Extraction, characterization and *in vitro* testing of flavonoids rich fractions obtained from *Actinidia macrosperma* fruit

Sujeewa Krishanthi Hettihewa

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ABSTRACT

In this PhD study, phenolic compounds extracted from *Actinidia macrosperma* L. fruit grown in New Zealand, were separated and isolated by series of chromatographic procedures followed by identification on high performance liquid chromatography-tandem mass spectrometry (HPLC-ESI-MS/MS).

Single factor experimental design was adopted to optimise the extraction conditions and their phenolic profiles, antioxidant and antihypertensive activities were compared to commercial kiwifruit varieties. Among the extracts tested, 70% aqueous acetone by steeping the *A. macrosperma* fruit variety showed the highest values of total phenolic (TP) content (823.1±14.4 mg gallic acid equivalent/100 g DW), total flavonoid (TFO) content (170.9±1.9 mg catechin equivalent/100 g DW), total flavanol (TFA) content (82.6±0.6 mg catechin equivalent/100 g DW) and antioxidant capacity (5.1±0.1 mmol Trolox equivalent (TE)/100 g DW and 8.3±0.1 mmol Fe (II) equivalent/100 g DW for DPPH and FRAP assays, respectively). The qualitative and quantitative analysis of each defatted crude extract on high pertformance liquid chromatography-diode array detection (HPLC-DAD) and high pertformance liquid chromatography-tandem mass spectrometry (HPLC-ESI-MS/MS) revealed that 70% aqueous acetone extract from the *A. macrosperma* fruit contains many potential antioxidant flavonoids compared to commercial kiwifruit varieties namely, *Actinidia deliciosa cv* Hayward, *Actinidia deliciosa cv* Sweetgreen, *Actinidia chinensis cv* Sungold and *Actinidia chinensis cv* Gold. Quercetin-3-*O*-galactoside was identified as the most abundant flavonoid among the other flavonoids present in *A. mac*rosperma fruit. Previously undiscovered eleven flavonoids from the *A. mac*rosperma fruit were tentatively identified by using LC-ESI/MS/MS after a series of purification steps including liquid-liquid partitioning, silica gel flash column chromatography, size exclusion chromatography on Sephadex LH-20, and semi-preparative HPLC.

It could be concluded that the optimized extraction conditions along with the HPLC-ESI-MS/MS technique were effectively performed to identify eleven flavonoid compounds, not previously reported from *Actinidia macrosperma* fruit. This is the first study regarding the phenolic composition and antioxidant properties of new commercial kiwifruit varieties, *Actinidia deliciosa cv* Sweetgreen, *Actinidia chinensis cv* Sungold and non-commercial variety, *Actinidia macrosperma* cultivated in New Zealand. Finally, the effect of *Actinidia macrosperma* fruit on inhibition of angiotensin converting enzyme (ACE) has not been previously reported and could therefore be recorded as a novel biological activity.

I dedicate this thesis

to

my dearest parents and my lovely husband

and kids (Sandeep, Dinuth) for their support, motivation & being

with me at every step of this journey.

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This thesis would have never been possible without the supervision and incredible guidance from my main supervisor; Associate professor, Yacine Hemar. Thank you so much for taking on this project and for your advice, assistance, encouragement, ideas, and many valuable comments from start to end and during the final preparation until the submission.

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I am indebted to the ZESPRI kiwifruit industry in New Zealand, in particular Morris Clare for donating kiwifruits to me. Thank you Dr. David Stevenson (Ruakura HortResearch, Hamilton) for donating non-commercial typed kiwifruit (*Actinidia macrosperma*) to me. There are many who helped me with some practical aspects of my research, for which I am very grateful, Dr. Don Otter (Palmerston HortResearch), Yusuf Odunbi (PhD student at Faculty of Engineering, University of Auckland).

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A	Aglycone
Aq.	Aqueous
AA	Antioxidant activity
AAE	Ascorbic acid equivalents
ABTS	Azino bis(3-ethylbenzothiozoline-6-sulfonic acid
ACE	Angiotensin converting enzyme
AH	Hydrogen donor
APCI	Atmospheric pressure chemical ionisation
BHA	*tert*-butylhydroxyanisole
BHT	*tert*- butylhydroxytoluene
C-18	HPLC- column coated with a carbon 18 reverse phase
CAE	Catechin equivalent
CID	Collision-induced dissociation
COSY	Correlated spectroscopy
CUPRAP	Copper reducing antioxidant power
d4-MeOH	Deuterated methanol
d_6- DMSO	Deuterated dimethyl sulfoxide
d	doublet
dd	doublet of doublet
Da	Daltons
DAD	Diode array detector
DMACA	Dimethylaminocinnamaldehyde

DPPH	2,2-diphenyl-2-picylhydrazyl
DW	Dry weight
EC_{50}	50% effective concentration
ESI-MS	Electrospray ionization mass spectroscopy
ESI-MS/MS	Electrospray ionization tandem mass spectroscopy
ET	Electron transfer
EtOAc	Ethyl acetate
FC	Folin-Ciocalteu phenol reagent
FRAP	Ferric reducing antioxidant power
g	grams
GAE	Gallic acid equivalent
GC	Gas chromatography
^{1}H	Proton nuclear resonance spectroscopy
h	hour
HAT	Hydrogen atom transfer
HL	Histidine-leucine
HHL	Histidine-$_{L}$-hippuryl-$_{L}$-leusine-chloride
HPLC	High performance liquid chromatography
HPLC-DAD	High performance liquid chromatography coupled with diode array detector
HPLC-ESI-MS/MS	High performance liquid chromatography electrospray ionization tandem mass spectroscopy
HSCCC	High speed counter current chromatography

HSQC	Heteronuclear single quantum correlation
J	Coupling constant in NMR spectroscopy
LC	Liquid chromatography
LC-DAD	Liquid chromatography coupled with diode array detector
LC-ESI-MS	Liquid chromatography coupled with electrospray ionization mass spectroscopy
LC-ESI-MS/MS	Liquid chromatography electrospray ionization tandem mass spectroscopy
LC-MS	Liquid chromatography coupled with mass chromatography
LC-MS/MS	Liquid chromatography tandem mass spectroscopy
LDL	Low density lipoprotein
m/*z*	Mass to charge ratio
MeOH	Methanol
Min	Minutes
mg	Milligrams
mL/min	Millilitre per minute
mmol	Millimoles
mol	Moles
MS	Mass spectrometry
MS^n	Tandem mass spectrometry
Mw	Molecular weight
ng	Nanograms
NMR	Nuclear resonance spectroscopy

ODS	Octadecyl silica
ORAC	Oxygen radical absorbance capacity
Prep HPLC	Preparative HPLC
pH	Power of hydrogen ions concentration
QE	Quercetin equivalent
s	Singlet
RT	Retention time
ROS	Reactive oxygen species
TE	Trolox equivalent
TFA	Total flavanol content
TFO	Total flavonoid content
TP	Total phenolic content
TPTZ	2,4,6-tripyridyl-s-triazine
U	Cushman and Cheng units
UV	Ultra violet
UHPLC	Ultra high performance liquid chromatography

Chapter 1

Introduction

Introduction

1.1. Research background

Epidemiological research studies suggest that the consumption of fruits and vegetables are precious sources of bioactive compounds and effective safeguards for human health. It is reported that the protection against chronic diseases such as cancer, arthritis, diabetes, atherosclerosis, and other aging related diseases has been attributed to various antioxidants contained in the diet (Ames et al., 1993; Ness and Powels, 1997; Prior et al., 2005; Wang et al., 2011). Since synthetic antioxidants such as *tert*-butylhydroxyanisole (BHA) and *tert*- butylhydroxytoluene (BHT) have been shown to have toxic and carcinogenic effects on human health (Branen, 1975; Shahidi and Wanasundara, 1992), different plant materials have recently become a major interest of scientific research as a natural source of phenolic antioxidants.

Phenolic compounds are commonly found in both edible (fruits, vegetables and grains) and non-edible plants, and they have been reported to have multiple biological effects, including antioxidant activity (Naczk and Shahidi, 2004). Crude extracts from fruits, herbs, vegetables, cereals, and other plant materials rich in phenolics are increased interest in the pharmaceutical industry for the maintenance of health and the protection from chronic diseases such as coronary heart disease and cancer (Kathirvel and Rupasinghe, 2012). Kiwifruit which belongs to the genus *Actinidia* and family *Actinidiace,* is one of the most popular fruits today. They have been widely used as food and medicinal materials such as antioxidants (Nishiyama et al., 2004; Ferguson, 2007; Nishiyama, 2007; Fiorentino et al., 2009; Bursal and Gulcin, 2011; Iwasawa et al., 2011; Park et al., 2011), antimicrobial agents (Nishiyama et al., 2004; Lu et al., 2007a; Nishiyama, 2007; Lu et al., 2011; Lu et al., 2012), anticancer agents (Motohashi et al., 2002; Lu et al., 2007b; Xu et al., 2009; Lu et al., 2011;

Lu et al., 2012), anti diabetic agents (Kurakane et al., 2011), immunomodulatory agents (Lu et al., 2007b) and anti-inflammatory agents (Kim et al., 2003). *Actinidia macrosperma* L. belongs to the family *Actinidiaceae* is a non-commercial type kiwifruit. There is a great interest in chemical composition and biological activities of this plant due to its bioactive compounds and their high potential to be nutraceutical and functional foods. Although a number of bioactive constituents present in stem and leaves of *A. macrosperma* have been reported (Lu et al., 2007b), phenolic compounds mainly flavonoids and flavonoid glycosides in this fruit have not been fully investigated yet.

1.2. Research objectives

The objectives of this study are to:

- Optimize the extraction conditions using single factor experimental design for extracting phenolic compounds from *Actinidia macrosperma* fruit.
- Compare the phenolic profiles and *in vitro* antioxidant activity of defatted crude extracts obtained from *A. macrosperma* fruit with the commercial kiwifruit varieties namely, *A.deliciosa cv* Sweetgreen, *A.deliciosa cv* Green (Hayward), *A.chinensis cv* Sungold (Gold3), *A.chinensis cv* Hort 16A (Gold) grown in New Zealand.
- Evaluate angiotensin converting enzyme inhibitory activity of flavonoid rich defatted crude extracts obtained from *Actinidia macrosperma* fruit *in vitro* by using fluorescence measurements based on biochemical reaction.
- Carry out bioassay guided extraction, separation and isolation followed by characterization of selected flavonoids on HPLC-ESI-MS/MS and NMR spectroscopy where required.

1.3. Thesis structure

The thesis is structured into two main parts: the first part contains an introduction and a review of the literature, and the second part is focused on the original contributions including experimental measurements, results and discussions.

Chapter 01 : contains a brief background of significance and the objectives of the thesis.

Chapter 02 : summarizes the kiwifruit industry in New Zealand, kiwifruit varieties, chemical composition of kiwifruits, health benefits of kiwifruits, Chemistry of phenolic compounds and their occurrence in kiwifruits, antioxidants, and methods for determination of antioxidant activity, extraction, separation and identification of phenolics.

Chapter 03 : reports on an experiment to optimize the extraction conditions for phenolics from *A. macrosperma* L. fruit and evaluating its phenolic profiles and *in vitro* antioxidant activity compared to commercial kiwifruit varieties.

Chapter 04 : describes the angiotensin converting enzyme inhibitory activity of flavonoid-rich extracts of *A. macrosperma* fruit and determination of kinetics of the inhibition.

Chapter 05 : deals with the determination of the structures of some selected flavonoids from *A. macrosperma* L. fruit by bioassay guided separation, HPLC-ESI-MS/MS and NMR spectroscopy .

Chapter 06 : is a general conclusion presenting the main findings, general discussion, and some suggestions for future work.

Chapter 2

Review of the literature

Review of the literature

This chapter reviews briefly about the kiwifruit industry in New Zealand including commercial and non-commercial varieties of kiwifruits and their health benefits reported in the literature. Also this reviews Chemistry of phenolic compounds as main bioactive components in kiwifruit, their diversity of occurrence in plants and importance of phenolics as antioxidants with free radical scavenging activity including commonly used antioxidant activity assays. This chapter closes with some of the methods reported in the literature for sampal preparation, extraction, separation, purification, identification and structure elucidation of phenolics. This does not describe application of nuclear magnetic resonance spectroscopy (NMR) in depth as it was minor application in my study. This literature review would also have some information written in the introductions in other chapters.

2.1. Kiwifruit industry in New Zealand

Kiwifruit originated in the Chang Kiang Valley of China which is the centre of diversity for the genus *Actinidia* belongs to the family Actinidiaceae which includes only two other genera (*Clematoclethra* and *Saurauia*) (Ferguson, 2007). The New Zealand kiwifruit industry began in the early twentieth with Isabel Fraser bringing kiwifruit seeds from China to New Zealand in 1904 for the first time (Ferguson, 2007). Hayward Wright, a New Zealand horticulturalist produced the green fleshed Hayward variety in 1925. In the early 1940s, the first commercial orchard was launched for the domestic market and began cultivating it in 1952 for commercial profit (Ferguson, 2007). The fruits were being marketed and commercial exporting was launched in 1953. Over 95 percent of world kiwifruit production is reported amongst a few countries. Italy (25% of world production), New Zealand (25-30% of world production) and Chile (7.5% of world production) have been the largest

exporters in terms of international trade (Ferguson and Seal, 2008; Bano and Scrimgeour, 2012). China joined these three countries as a major producer since 2005 (Bano and Scrimgeour, 2012).

2.2. Kiwifruit varieties in the family *Actinidiaceae*

The genus *Actinidia* is composed of over 76 species and about 120 taxa in all (Nishiyama et al., 2004). *Actinidia deliciosa, Actinidia chinensis* and *Actinidia arguta* are the three main commercial species and other *Actinidia* species also have commercial potential or are being investigated as useful genetic resources for cultivar development by interspecific hybridization techniques (Nishiyama et al., 2004; Ferguson and Seal, 2008). Some of the species of *Actinidia* are shown in **Figure 2.1**.

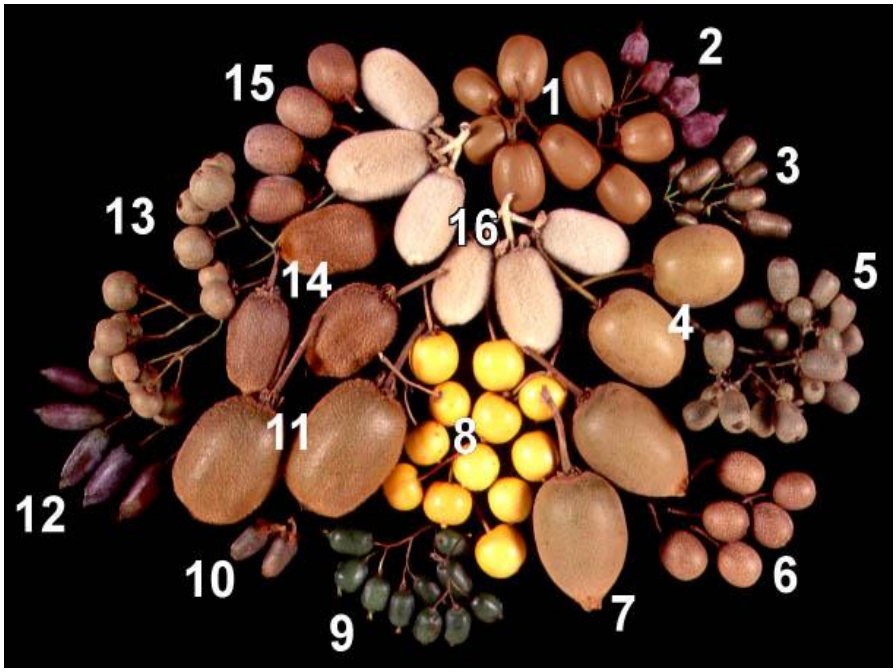


Figure. 2. 1. Fruit diversity in the genus *Actinidia* (adapted from Ferguson, 1999).

1 *A. rufa*	5 *A. latifolia*	9 *A. argute*	13 *A. guilinensis*
2 *A. melanandra*	6 *A. indochinensis*	10 *A. fulvicoma*	14 *A. setosa*
3 *A. glaucophylla*	7 *A. chinensis* 'Hort16A'	11 *A. deliciosa* 'Hayward'	15 *A. chrysantha*
4 *A. chinensis*	8 *A. macrosperma*	12 *A. arguta* var. *purpurea*	16 *A. eriantha*

Actinidia deliciosa is the most common type of kiwifruit with translucent and bright green flesh and dull-brown skin with dense hair (Nishiyama, 2007). Hayward is the most commercially available cultivar of this species and is considered as the standard cultivar against which the quality of a new cultivar is evaluated (Nishiyama, 2007). *Actinidia chinensis* is very similar to *A. deliciosa*, but it is a smooth-skinned almost hairless fruit usually with yellow flesh (Nishiyama, 2007). Hort 16A is a cultivar developed from *Actinidia chinensis* by HortResearch in New Zealand and marketed in 2000 under the commercial name ZESPRI GOLD (Ferguson and Seal, 2008). Sweetgreen and Sungold are new kiwifruit cultivars (not shown in **Figure 2.1**) marketed by ZESPRI, New Zealand in 2010 (Morris, ZESPRI, New Zealand, personal communication).

A. arguta is a species recently marketed with smooth and hairless grape-sized fruit with edible skin that is reported with shorter storage life and the shelf life than those of either *A. deliciosa* or *A. chinensis (*Ferguson and Seal, 2008).

2.3. Phenolic compounds and their occurrence in kiwifruits

2.3.1. Overview of phenolic compounds

Phenolic compounds are secondary metabolites which are derived from phenylalanine and tyrosine via the shikimic acid pathway (**Figure 2.2**) (Naczk and Shahidi, 2004). Phenolic compounds are synthesized by plants during development, in response to stress conditions such as infection, wounding and UV radiation (Harbone and Williams, 2000; Naczk and Shahidi, 2004). More than 8000 phenolic compounds have been reported, all of which share the common structural feature: an aromatic ring bearing at least one hydroxyl substituent (phenol) (Naczk and Shahidi, 2004). Since animals are unable to synthesize the flavone nucleus, flavonoids are found only in the plant kingdom (Baghel et al., 2012).

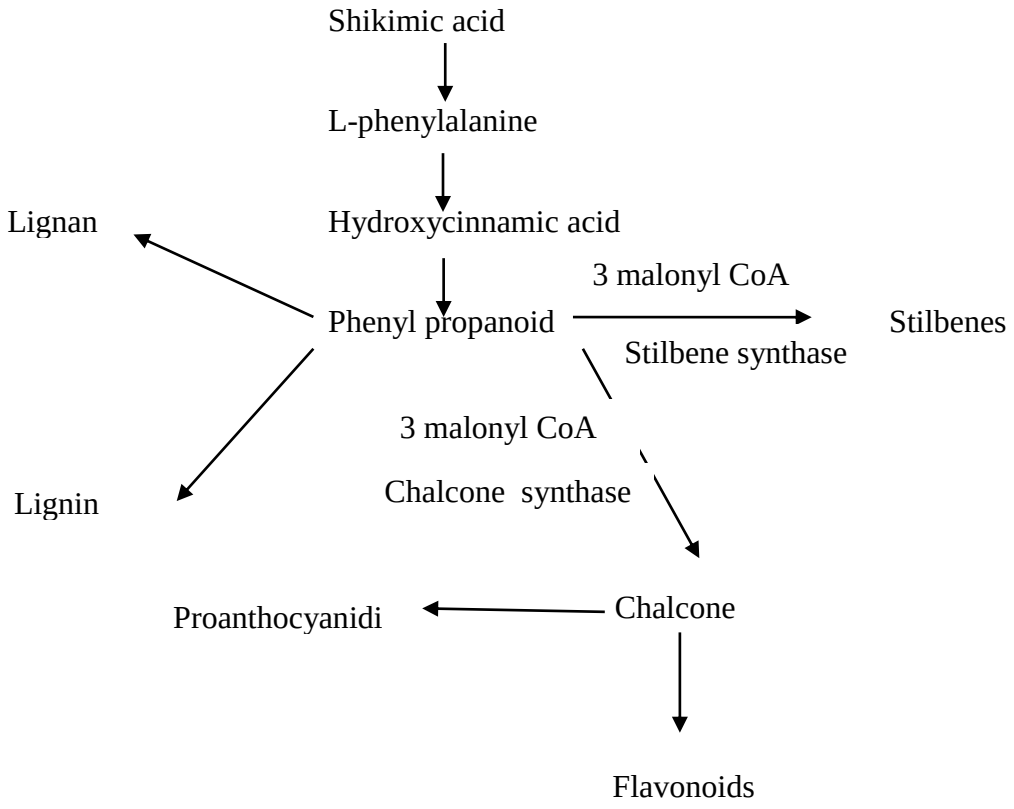


Figure 2.2. Schematic diagram of the production of phenyl propanoids from phenylalanine in the shikimic pathway (Naczk and Shahidi, 2004).

It has been reported that phenolics are distributed in plants at the tissue, cellular and subcellular levels. Insoluble phenolics (lignans and lignins) are the components of cell walls, while soluble phenolics occur within the plant cell vacuoles (Haslam, 1989). In plants, phenolics act as antifeedants, phytoalexins, and attractants for pollinators and contributors to the pigmentation, antioxidants and UV protection (Haslam, 1989; Naczk and Shahidi, 2004). Phenolics contribute to the colour, astringency, flavour, odour, and oxidative stability of products in foods (Haslam, 1989). Phenolics range from simple phenolics, phenolic acids, coumarins, flavonoids, stilbenes, up to hydrolysable and condensed tannins, lignans, and lignins (Manach et al., 2004; Naczk and Shahidi, 2004: Khoddami et al., 2013). They can be classified based on the number and arrangement of their carbon atoms (**Table 2.1**) and are commonly conjugated to sugars and organic acids.

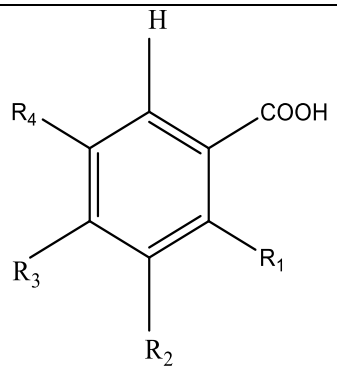
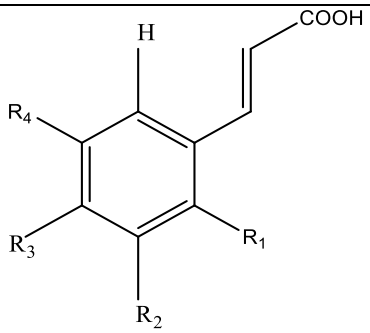
Table 2.1. Structural skeleton of simple phenolic and polyphenolic compounds (Garcia-Sales et al., 2010).

Number of Carbons	Skeeton	Classification	Example
7	C_6-C_1	Phenolic acids	Gallic acid
8	C_6-C_2	Acetophenones	Gallacetophenone
8	C_6-C_2	Phenylacetic acids	*p*-Hydroxyphenyl-acetic acid
9	C_6-C_3	Hydroxycinnamic acids	*p*-Coumaric acid
9	C_6-C_3	Coumarins	Esculetin
10	C_6-C_4	Napthoquinones	Juglone
13	C_6-C_1-C_6	Xanthones	Mangiferin
14	C_6-C_2-C_6	Stilbenes	Resveratol
15	C_6-C_3-C_6	Flavonoids	Quercetin

2.3.2. Phenolic acids and their derivatives

The term phenolic acids represent phenols with one carboxylic acid functionality. The phenolic acids include two types of carbon frameworks as shown in **Table 2.2**, the hydroxycinnamic and hydroxybenzoic structures (Manach et al., 2004; Khoddami et al., 2013).

Table 2.2. Structures of the prominent naturally occurring phenolic acids.

 Hydroxybenzoic acids	Benzoic acid $R_1= R_2= R_3 = R_4=H$ *p*-Hydroxybenzoic acid $R_1= R_2= R_4 = H, R_3=OH$ Vanillic acid $R_1= R_4 = H, R_2=OCH_3, R_3=OH$ Gallic acid $R_1= H, R_2= R_3 = R_4=OH$ Protocatehuic acid $R_1= R_4 =H, R_2=R_3= OH$ Syringic acid $R_1= H, R_2=R_3= R_4 =OCH_3$
 Hydroxycinnamic acids	Cinnamic acid $R_1= R_2= R_3 = R_4=H$ *O*- coumaric acid $R_1= OH, R_2= R_3 = R_4=H$ *m*- coumaric acid $R_1= R_3 = R_4=H, R_2=OH$ *p* - coumaric acid $R_1= R_2 = R_4=H, R_3=OH$ Ferulic acid $R_1= R_4=H, R_2=OCH_3, R_3 =OH$ Sinapic acid $R_1= H, R_2= R_4=OCH_3, R_3 =OH$ Caffeic acid $R_1= R_4 =H, R_2= R_3=OH$

Caffeic, *p*-coumaric, ferulic and sinapic acids are the major hydroxycinnamic acids and these are more common than the hydroxybenzoic acids in the plants. The bound forms of these acids such as glycosylated derivatives of esters of quinic acid, shikimic acid, and tartaric acid are common while free forms are rarely found in the nature. Chlorogenic acid which is found in high concentration in many types of fruits is a combination of caffeic and quinic acid (Manach et al., 2004). Caffeic acid represents between 75% to 100% of the total hydroxycinnamic acid content and is generally the most abundant phenolic acid in many fruits.

2.3.3. Flavonoids and their derivatives

Flavonoids are derived from the basic flavan nucleus, which consists of 15 carbon atoms arranged in the three rings (C_6-C_3-C_6) labelled as A, B, and C in **Figure 2.3**.

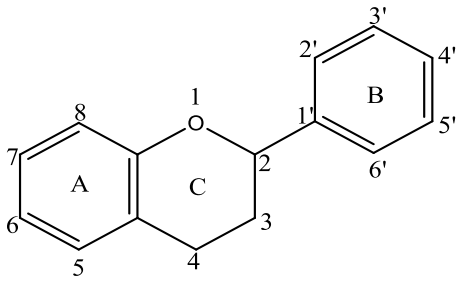
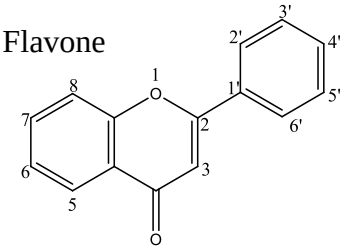


Figure 2. 3. Basic structure of flavonoids.

Flavonoids are derived from a chromane-type skeleton with various types of patterns of hydroxylation, methylation and methoxylation and having phenyl substituent in the C_2 or C_3 (isoflavone) positions (Manach et al., 2004). Variations such as the number and position of hydroxylation in the C ring also contribute to the diversity of subclasses. Therefore, according to their molecular structure, flavonoids are divided into several classes: flavone, flavanones, flavonol, flavan 3- ols and anthocyanidins (**Table 2.3**).

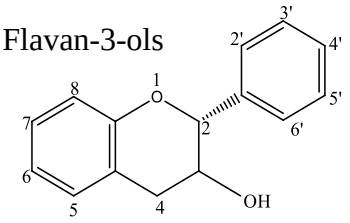
Table 2.3. Different classes of flavonoids and their substitution patterns.

Flavone



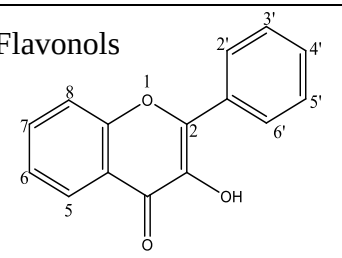
	5	7	3′	4′
Apigenin	OH	OH	-	OH
Luteolin	OH	OH	OH	OH
Chrysin	OH	OH	-	-

Flavan-3-ols



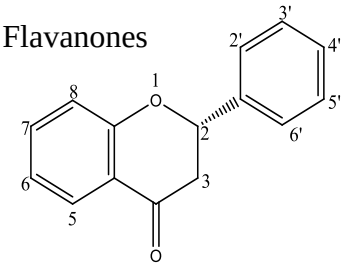
	3	5	7	3′	4′
(+) Catechin	βOH	OH	OH	OH	OH
(-) Epicatechin	αOH	OH	OH	OH	OH

Flavonols



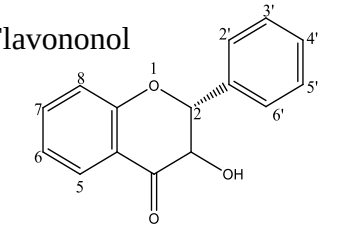
	5	7	3′	4′	5′
Quercetin	OH	OH	OH	OH	-
Kaempferol	OH	OH	OH	-	-
Myricetin	OH	OH	OH	OH	OH

Flavanones



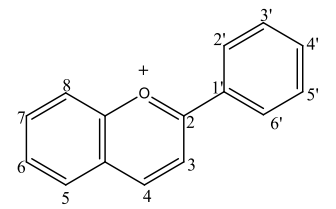
	5	7	3′	4′
Naringenin	OH	OH	-	OH
Hesperetin	OH	OH	OH	OCH_3

Flavononol



	5	7	3′	4′
Taxifolin	OH	OH	OH	OH

Anthocyanidins



	3	5	7	3′	4′
Cyanidin	OH	OH	OH	OH	OH
Peonidin	OH	OH	OH	OCH_3	OH

Flavonols

Flavonols which vary in colour from white to yellow, are the most widespread flavonoids in plants. They are mainly represented by quercetin, kaempherol, and myricetin and isorhamnetin (El Gharras, 2009). Quercetin is the most ubiquitous flavonol found in the diet, and is present in various fruits and vegetables (Manach et al., 2004; Jaganath and Crozier, 2010).

Flavones

The structure of flavones is very similar to flavonols and they differ only at the 3-position on the C-ring which shows the absence of hydroxylation. Apigenin and luteolin are two main flavones commonly found in the diet (**Table 2.3**). They are not widely distributed in plants with significant concentrations but reported in celery, parsely and artichoke (Manach et al., 2004; Jaganath and Crozier, 2010).

Flavan-3-ols

Flavan-3-ols are mainly found in the western diet and functional ingredients in various beverages (red wine, tea, coffee), herbal remedies, whole and processed foods (chocolates, beans, cherry, grapes), and supplements (Haslam, 1989). It is reported that the food quality parameters such as astringency, bitterness, sourness, sweetness, aroma and colour formation are affected by flavan-3-ols (Haslam, 1989). The flavan-3-ols are the most complex flavonoids ranging from simple monomers (catechin and epicatechin isomers) to oligomers and polymeric proanthocyanidins (Haslam, 1989; Jaganath and Crozier, 2010).

Flavanones

Naringenin, hesperitin, and eriodictyol are the main flavanones reported in plants such as citrus fruits in both the aglycone and their glycosidic forms (Manach et al., 2004;

Jaganath and Crozier, 2010). Hydroxylation, glycosylation, and *O*-methylation reactions are favoured for the flavanone structure due to its high reactivity (Manach et al., 2004).

Anthocyanidins

Anthocyanidins, which are responsible for a wide range of colours, are water-soluble plant pigments. They occur primarily as glycosides with the sugar moiety at the 3-position on the C-ring (Manach et al., 2004). They are involved in the defence mechanism of plants against pathogens and also have an important role to play in attracting pollinating insects (Jaganath and Crozier, 2010). A total of 539 anthocyanins are identified and they are derived from about 27 types of anthocyanidins in nature (Valls et al., 2009), but only delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin are commonly distributed in the plants (Manach et al., 2004). Anthocyanidin structures vary depending on the number and the position of hydroxyl and methoxyl groups attached and the positions at which sugars are attached (Manach et al., 2004; Jaganath and Crozier, 2010). Cyanidin-3-*O*-glucoside is the most widespread anthocyanin in fruits and malvidin-3-*O*-glucoside is the characteristic anthocyanin in red grapes (Jaganath and Crozier, 2010; Myjavcova et al., 2010).

2.3.4. Flavonoids in diet and plants

Flavonoids are an integral part of the daily diet (**Table 2.4)** and widely distributed in foods (vegetables, fruits, nuts, seeds, stem, and flowers) (Yan-Hwa et al., 2000) and beverages such as, tea and wine (Haslam, 1989). The daily intakes of flavonoids, anthocyanidins, flavonols and flavones were reported as 165.6, 27.6, 123.7 and 10.7 mg/day, respectively (Li et al., 2013a).

Table 2.4. Occurrence of flavonoids in foods (Manach et al., 2004).

Flavonoid subclass	Food source	Representative flavonoids
Flavonol	onion, kale, broccoli, red wine, apples, cherries, berries, tea	myricetin, quercetin, rutin, kaempherol
Flavones	parsley, thyme	apigenin, chrysin, luteolin
Flavonones	citrus	hesperitin, naringen
Flavan-3-ols	apple, tea	catechin, galocatechin
Anthocyanidins	cherry	malvidin, peteudin

In plants, flavonoids commonly occur as flavonoid *O*-glycosides even though they can occur as *O*-glycosides or *C*-glycosides. An acid-labile, acetal, glycosidic O-C bonds can be formed between a sugar and one or more hydroxyl groups on the aglycone (Cuyckens and Claeys, 2004). Glycosylation may also take place via an acid-resistant C-C bond, to form flavonoid *C*-glycosides at the C-6 and/or C-8 positions, by direct linkage of the sugar to the flavan nucleus, (Cuyckens and Claeys, 2004) (**Figure 2.4**). Certain positions of the hydroxyl groups are favoured for glycosylation, although many positions such as 3, 5, 7, 3′, 4′, and 5′ are available (Cuyckens and Claeys, 2004). Cuyckens and Claeys, (2004) reported that the 7-hydroxyl group in flavones, the 3-hydroxyl group in flavanones and isoflavones, and 7- hydroxyls in flavonols and flavan-3-ols are common glycosylation positions while the 3- and 5-hydroxyls in anthocyanins (**Figure 2.4**). Since the 5-hydroxyl group forms hydrogen bonds with the adjacent 4-carbonyl group, 5-*O*-glycosylations are rare for compounds with a carbonyl group on position 4 in the nature (Cuyckens and Claeys, 2004).

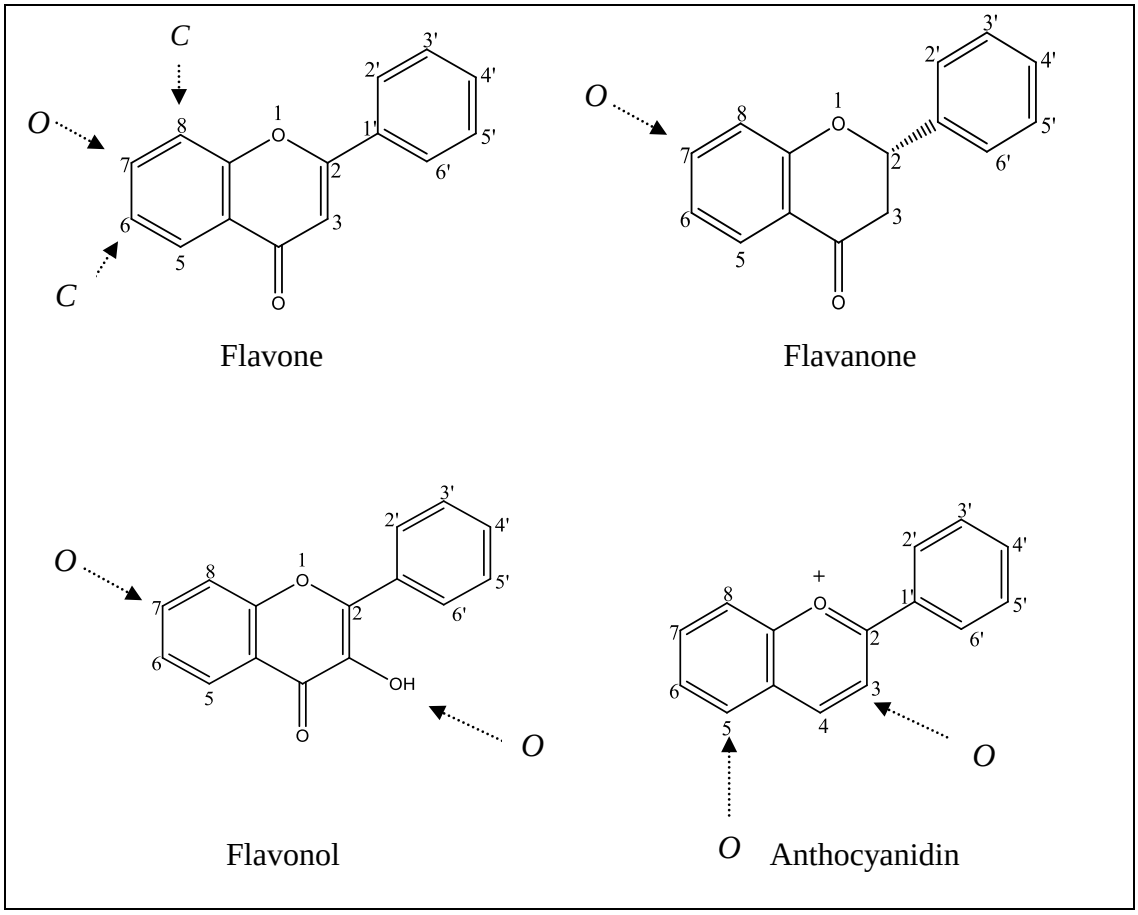


Figure 2.4. Common *O*- and *C*-glycosylation positions of flavonoids (Cuyckens and Claeys, 2004).

Glucose is the most commonly glycosylating sugar in flavonoids, while galactose, arabinose, rhamnose, and xylose are not uncommon. Mannose, fructose, glucuronic and galacturonic acids have been rarely reported (Cuyckens and Claeys, 2004; Ye et al., 2005; Inbaraj et al., 2010). Disaccharides such as rutinose and neohesperidose are also found in plants in association with flavonoids while tri- and tetra saccharides are occasionally reported (Leite et al., 2001; Tang et al., 2001; Mangaru et al., 2004; Ye et al. 2005; Inbaraj et al., 2010).

2.3.5. Occurrence of phenolic compounds and their derivatives in kiwifruits

Among many popular fruits, the kiwifruit is considered as abundant in different bioactive compounds such as phenolic compounds, pigments, organic acids, sugar and sugar alcohols and calcium oxalate (Nishiyama et al., 2007). Phenolic acids and flavonoids are the major two types of phenolic compounds which have been identified among phenolic compounds in *Actinidia* plants (Nishiyama et al., 2007). Phytochemical analysis carried out on kiwi (Hayward cultivar) peel extract and pulp extract had led to the isolation of several flavonoids, namely naringenin (only in peel matrix), quercetin, tricin, derivatives of kaempferol, rutin, epicatechin, catechin, gallocatechin and phenolic acids such as ursolic acid, caffeic acid glucosyl derivatives and chlorogenic acid using gravity column chromatography techniques (Fiorentino et al., 2009). Phenolic compounds had been previously separated from kiwi juice made up from the Hayward cultivar, and hydroxycinnamic acids, hydroxybenzoic acids such as protocatechuic acid, vanillic and syringic acids and flavonoids (epicatechin, catechin, procyanidin dimer, and tetramer) were identified (Dawes and Keene, 1999). Phenolic compounds extracted from six different *Actinidia* cultivars (*A. arguta, A. deliciosa cv* Hayward, *A. kolomikta*, *A. macrosperma, A. melanandra* and *A. purpurea)* were investigated by Latocha et al., (2010). In their study, quercetin (except in *A. melanandra*), epicatechin (in all) and catechin (except in Hayward, *A. melanandra* and *A. purpurea*) were found in different concentrations and tannic acid, 2,5-dihydroxybenzoic acid, hydroxybenzoic acid, chlorogenic acid, caffeic acid, and benzoic acid derivatives were identified in all those cultivars but *p*-coumaric acid was identified in *A. arguta, A. macrosperma, A. melanandra* and *A. purpurea* only (Latocha et al., 2010). Polymeric proanthocyanidins in *A. chinensis* fruit and leaves were identified by Foo and Porter, (1981).

2.4. Nonphenolic compounds and their presence in kiwifruits

Kiwifruit contains several types of pigments, including carotenoids, chlorophyll a, b and anthocyanins. Violaxanthin, neoxanthin, lutein, and β-carotene are the main carotenoids identified in kiwifruits (McGhie and Angie, 2002; Montefiori et al., 2005; Nishiyama et al., 2007; Latocha et al., 2010). Ascorbic, malic, citric and quinic acids are some of the organic acids reported in *Actinidia* fruits (Nishiyama et al., 2007). Of these, citric and quinic acid concentrations (0.99 - 1.29 and 0.74 - 1.18 g/100 g fresh weight) are higher than those of ascorbic and malic acid (0.08 - 0.19 g/100 g fresh weight) (Marsh et al., 2004). The organic acid composition in other kiwifruits (*A. deliciosa, A. chinensis* and *A. rufa*) is similar to those of Hayward (Nishiyama et al., 2007).

It is reported that *Actinidia* fruits are a rich dietary source of vitamin C and the values vary from less than 10 mg/100 g fresh weight to more than 2000 mg/100 g fresh weight (Huang et al., 2003). The vitamin C content reported in *A. deliciosa, A. chinensis* and *A. arguta* fruits are in the range of 50 - 250, 50 - 420 and 81 - 430 mg/100 g fresh weight, respectively (Huang et al., 2003). The highest vitamin C content is reported in *A. latifolia* fruit (671 - 2140 mg/100 g fresh weight), followed by *A. eriantha* fruit (500 - 1379 mg/100 g fresh weight) which are non- commercial crops (Huang et al., 2003).

The acidity and sugar content of the fruit are important parameters affecting the sensory characteristics of kiwifruit (Nishiyama et al., 2007). Glucose, fructose and sucrose are the major types of soluble sugars reported in *Actinidia* fruits (Nishiyama et al., 2007). The main soluble sugars in Hayward fruit are glucose and fructose which are present in equal amounts (3 - 5 g/100 g fresh weight) and a smaller amount of sucrose is present (0.7 - 1.5 g/100 g fresh weight) (Sanz et al., 2004). Hayward fruit also contains sugar alcohols such as *myo*-inositol and hexahydric alcohol (153 mg/100 g fresh weight) (Sanz et al., 2004). Glucose and fructose are the main sugars found in *A. chinensis* (Esti et al., 1998). The

composition of the soluble sugars reported in *A. arguta* fruit is different from those in *A. deliciosa* and *A. chinensis* fruit. This species contains sucrose (5.0 - 9.5 g/100 g fresh weight) as a predominant soluble sugar, and glucose and fructose at concentrations of 0.8 - 2.0 g/100 g fresh weight (Nishiyama et al., 2007). The amount of *myo*-inositol reported in *A. arguta* is 0.65 - 1.05 g/100 g fresh weight (Nishiyama et al., 2007).

Various concentrations of calcium oxalate are reported in *A. deliciosa, A. chinensis* and *A. arguta* fruits (37 - 65, 18 - 45, and 5.0 - 8.5 mg/100 g fresh weight, respectively) (Nishiyama et al., 2007).

2.5. Health benefits of Actinidia species

In terms of nutritional value, kiwifruits have a reputation as being particularly nutritious and excellent sources of vitamin C, carotenoids, polyphenols, flavonoids, folate, potassium and dietary fibre, although the levels vary among the different genotypes of *Actinidia* fruits (Nishiyama et al., 2004; Nishiyama, 2007; Hunter et al., 2011). Furthermore, they have been widely used as food (stewed and in jams, jellies and juices) (Bursal and Gulcin, 2011) and medicinal materials such as antioxidants (Nishiyama et al., 2004; Jung et al., 2005; Ferguson, 2007; Nishiyama, 2007; Fiorentino et al., 2009), antimicrobial agents (Nishiyama et al., 2004; Lu et al., 2007a; Nishiyama, 2007; Lu et al., 2012), anticancer agents (Lu et al., 2007b; Lu et al., 2011), anti-diabetic agents (Kurakane et al., 2011) and anti-inflammatory agents (Kim et al., 2003) because of the broad spectrum of pharmacological and biological properties of bioactive compounds in them (Nishiyama et al., 2004; Ferguson, 2007; Nishiyama, 2007; Fiorentino et al., 2009). It is also reported that polyphenolics among different substances contained in the kiwifruits are the major compounds which are responsible for most of the antioxidant capacity in kiwifruit (Hunter et al., 2011).

Some *Actinidia* species, high in vitamin C such as *A. kolomikta* and *A. latifolia* are reported to have antioxidant and immune-stimulation properties (Ferguson and Fergusan, 2003). The plant of the *Actinidia macrosperma* has a reputation as a treatment for various ailments like abscesses, leprosy, rheumatism, jaundice, arthritis inflammation and abnormal leucorrhoea in Chinese traditional medicine (Lu et al., 2007a; Lu et al., 2007b; Lu et al., 2011). The roots and stems of this plant have been extensively employed in some local cancer treatments (Lu et al., 2007; Lu et al., 2011).

Kiwifruit is used as an example of a food with putative antioxidant properties, and its effectiveness at decreasing oxidative DNA damage was assessed in *vivo* as well as *in vitro* tests (Collins et al., 2001). Collins and co-workers (2001) have reported that a simple extract of kiwifruit, buffered to pH 7, was more effective than a solution of vitamin C of equivalent concentration at protecting DNA from damage. The immunimodulary effects of *A. macrosperma* aqueous extract was examined by Lu et al., (2007b) who showed that an aqueous extract of *A. macrosperma* lacked significantly the inhibition of transplantable sarcomas 180, but significantly increased the overall immune functions. Jung et al., (2005) has investigated cardiovascular properties of green kiwifruit extracts by analysing the *in vitro* antioxidants and antihypertensive activities and reported that green kiwifruit has potential cardiovascular protective properties.

2.6. Antioxidants

2.6.1. Chemistry of antioxidants and the mechanism of their action

Antioxidants are compounds which are capable of delaying or inhibiting the oxidation processes which consist of the three major steps of initiation, propagation and termination under the influence of atmospheric oxygen or reactive oxygen species (ROS) (Shahidi and Wanasundara, 1992). In biological systems during respiration, a significant fraction of oxygen is incompletely reduced, leading to highly reactive and toxic reactive oxygen species. ROS include free radicals (superoxide anion, alkoyl, hydroxyl, and peroxyl) and non-radical derivatives of oxygen (singlet oxygen and hydrogen peroxide). They can cause functional damage to biological systems due to the unbalance between ROS and antioxidants, triggering a number of degenerative diseases, like carcinogenesis, mutagenesis, and aging (Singh and Singh, 2008).

Endogenous antioxidants, which occur in the human body such as enzymes (superoxide dismutase, catalase, glutathione peroxidase) or non enzymatic compounds (uric acid, bilrubin, albumin, metalothioneins), are associated with the attack of free radicals (Pisoschi, 2011). Exogenous antioxidants are needed when endogenous factors cannot control or provide complete protection against the ROS. Thus, nutritional supplements or pharmaceutical products with antioxidant compounds need to be taken (Pisoschi, 2011). Vitamin E, vitamin C, β-carotene and flavonoids are well known natural exogenous antioxidants (Shahidi and Wanasundara, 1992; Pisoschi, 2011). However, exogenous antioxidants can also be synthetic compounds such as butylhydroxyanisole (BHA) or butylhydroxytoluene (BHT) (Shahidi and Wanasundara, 1992; Pisoschi, 2011). Polyphenol based natural antioxidants have recently attracted considerable attention related to the radicals and oxidative stress, cancer and therapy (Pisoschi, 2011).

Antioxidants can be classified based on their mode of action, as primary and secondary antioxidants. Primary antioxidants are compounds that are able to donate a hydrogen atom rapidly to a lipid radical to form a more stable new radical. For example, vitamic C, flavonoids, and tocopherol can stop chain reactions by donating hydrogen to the peroxyl radical of the fatty acid which stops propagation in the oxidation process (Shahidi and Wanasundara, 1992). Secondary antioxidants are compounds which can retard the rate of radical initiation reaction by eliminating the initiators (or inhibit the initiating enzyme), or reduce the oxygen level (without generating reactive radical species).

2.6.2. Antioxidant capacity assays

On the basis of the chemical reaction, major antioxidant activity assays can be divided into two categories: (1) hydrogen atom transfer (HAT) reaction based assays and (2) single electron transfer (ET) based assays (**Table 2.5**). Regardless of the mechanism involved, the end result of antioxidant activity is the same, with different kinetics and potential for side reactions (Prior et al., 2005).

Table 2.5. *In vitro* antioxidant capacity assays (Huang et al., 2005).

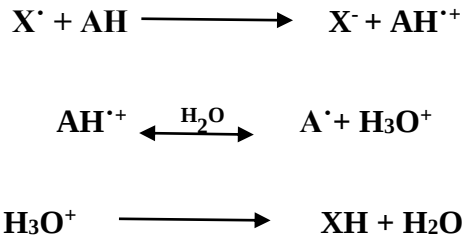
assays involving hydrogen atom transfer reactions	Oxygen radical absorbance capacity (ORAC) Total radical trapping antioxidant parameter (TRAP) Inhibition of low density lipoprotein (LDL) oxidation
assays by electron-transfer reaction	Ferric ion reducing antioxidant power (FRAP) Diphenyl-1-picrylhydrazyl (DPPH) Total phenol content assay by Folin-Ciocalteu Trolox equivalaent antioxidant capacity (TEAC) Copper (II) reduction capacity

Hydrogen atom transfer (HAT) methods measure the ability of an antioxidant to quench free radicals by hydrogen donation (Huang et al., 2005; Prior et al., 2005) as follows:

$$\mathbf{X^{\cdot} + AH \longrightarrow XH + A^{\cdot}}$$

$X^{\cdot}$ is a ROS radical, AH is hydrogen donor. Prior et al., (2005) reported that HAT reactions are solvent and pH independent and quite rapid, typically completed in seconds to minutes. Since the hydrogen atom transfer reaction is a key step in the radical chain reaction, Huang et al., (2005) reported that the HAT based method is more relevant to the radical chain-breaking antioxidant capacity.

The electron transfer (ET) based assays measure the ability of a potential antioxidant to transfer one electron to reduce any compound (antioxidant's reducing capacity) (Huang et al., 2005; Prior et al., 2005) as follows:



$X^{\cdot}$ is a ROS radical, AH is a hydrogen donor. ET reactions are solvent and pH dependent and usually slow (Prior et al., 2005). The electron transfer assays involve two components in the reaction mixture as shown above, hydrogen donor/antioxidants (AH) and oxidant (probe, $X^{\bullet}$). They are based on the following electron-transfer reaction (Huang et al., 2005):

Probe (oxidant) + electron from antioxidant ⟶ reduced probe + oxidized antioxidant

The probe itself is an oxidant and can abstract an electron from the antioxidant, which changes the colour of the probe. The degree of the colour change is proportional to the concentration of the antioxidant. The reaction end point is reached when colour change stops. The change of absorbance is plotted against the antioxidant concentration to give a linear curve. The slope of the curve which shows the antioxidant's reducing activity equals to antioxidant activity and is expressed as the Trolox equivalent (TE) or gallic acid equivalent (GAE) (Huang et al., 2005). It has been reported that ET and HAT reactions almost occur together in all samples, with the balance being determined by pH and antioxidant structure (Prior et al., 2005). A number of assays have been developed and reported in the literature for the detection of antioxidant capacity and **Table 2.6** shows the principles, advantages and limitations of commonly used antioxidant capacity assays.

Table 2.6. Advantages and limitations of commonly used methods for the evaluation of antioxidant (Singh and Singh, 2013).

Method	Principle	Advantages	Limitations
DPPH	Evaluation of scavenging activity of antioxidants by measurement of change in absorbance at 515 to 517 nm	Simple, accurate, sensitive, and inexpensive, highly reproducible	DPPH radicals interact with other radicals, also sensitive to some bases, compulsory to use organic solvents
FRAP	Measurement of blue colour of reduced complex at 593 nm	Simple, fast, inexpensive and reproducible	measured reducing capacity does not necessarily reflect antioxidant activity
TEAC	Measurement of inhibition of the absorbance at 415 nm	Useful for both hydrophilic and lipophilic compounds, rapid and easy	Time interval should be carefully monitored
ORAC	Calculating the net protection area under the time recorded fluorescence decay curve	Superior as it combines inhibition time and inhibition degree of free radical action	Primarily measures hydrophilic antioxidant activity against peroxyl radicals and not total antioxidant

2.6.3. Phenolics as antioxidants

The reported beneficial effects of phenolics are mainly based on their antioxidant properties. Depending on their chemical structures, phenolics can act as chain breakers or radical scavengers (Rice-Evans et al., 1996; Miller, 1996; Rice-Evans, 2001; Podsedek, 2007). It has been reported by Rice-Evans et al., (1996) and Kruzlicova et al., (2012) that the position of the hydroxyl groups and other features in the chemical structure of flavonoids are important determinants of their antioxidant and free radical scavenging activities. Fraga, (2007) reported that the presence of hydrogen on the phenoxyl groups, which can be donated to a radical, is important for antioxidant character. Bors, (1997) described three criteria that define the effective free radical scavenging activity for flavonoids; (1) the presence of two hydroxyl groups in the 3′, 4′ positions on the B ring (to stabilize the free radical formed mainly in the 3′ position) (**Figure 2.3**), (2) a double bond at the 2, 3 position (to provide higher conjugation with other double bonds), and (3) a 4-oxo function with 3- and 5-hydroxyl groups (**Figure 2.3**). Thus, the natural antioxidant quercetin shows all these structural features and is an effective antioxidant (Fraga, 2007; Kruzlicova et al., 2012). However, no definite structure-activity relationship can be established. In recent years, Yilmaz et al., (2006) reported applications of catechins as natural antioxidant in oils and fats to protect against lipid oxidation. However, anthocyanins have been reported to contribute more to the antioxidant activity of all fruits (≈90%) than phenolic acids, flavonols, and flavan-3-ols (≈10%) (Jakobek et al., 2009).

2.7. Methods for sample preparation, extraction, separation and identification of phenolic compounds

2.7.1. Sample preparation and extraction

Sample preparation and extraction of phenolic compounds from different types of samples such as fruits, cereals, juices, and vegetables, depend mostly on the chemical properties of the phenolics and the nature of the sample matrix (Khoddami et al., 2013). In general, plant material should be protected from sunlight to avoid photooxidation and also should be dried at temperatures below 30 °C to avoid decomposition of phenolics. The plant material should not be compacted to prevent the build-up of heat and moisture (Gabriela, 1999). It is advisable to extract phenolic compounds in organic solvents that can deactivate enzymes present in the plant, when fresh plant material is required for the study. If the plant material is to be ground to improve the efficacy of the extraction by increasing the surface area of the plant material, before the extraction, it may be necessary to grind in a buffered solvent (Khoddami et al., 2013). Since flavonoids can be degraded by enzyme action, it is advisable to use dry, lyophilized, or frozen samples for extracting flavonoids (Chan et al., 2009).

In the last several years, the extraction of phenolic compounds occurring in natural products has attracted much interest. Extraction is one of the most important steps in the isolation, identification and use of phenolic compounds. There is no standard extraction method for all types of polyphenols. Liquid-liquid extraction, solid-liquid extraction and supercritical fluid extraction are three typical common techniques used for the extraction process (Garcia-Sales et al., 2010; Ignat et al., 2011). The techniques are needed to be selected based on the original sample form, and the further analytical methods used after extraction. The solid-liquid extraction is a major extraction method used for plant-related compounds, in which a solid material comes in direct contact with a liquid solvent (Cacace

and Mazza, 2002). Extraction is typically performed with magnetic stirring, shaking or macerating but other methods such as supercritical fluid extraction, soxhlet extraction, microwave-assisted extraction, or ultrasound assisted extraction have recently been introduced to increase the efficacy and speed of the extraction procedure (Garcia-Sales et al., 2010**;** Khoddami et al., 2013).

Extraction is a function of process conditions, and several factors such as type of the solvent, number of steps, the volume of the solvent, pH, temperature, flow rate and the size of the particles in the sample, can affect the efficacy of extraction or concentration of the components to be analyzed in the extract (Nazck and Shahidi, 2004; Ignat et al., 2011). It is reported that the type of extraction solvent plays an important role (Marston and Hostettman, 2006) and the solvents are chosen based on the class of phenolic compounds being extracted (Nazck and Shahidi, 2004). Polyphenols are polar compounds and also are extracted using water, polar organic solvents such as acetonitrile, methanol, ethanol, and acetone, or a mixture of water (Nazck and Shahidi, 2004). Anthocyanins are extracted with acidified methanol at low temperatures to maintain the stable flavylium forms (Nazck and Shahidi, 2004; Comeskey et al., 2009).

Jones and Kinghorn, (2010) reported that the resulting solution from the extraction should be filtered, regardless of the extraction technique used, to remove any remaining particular matter. It is also advisable not to store the plant material in the solvent for long periods at room temperature, or in sunlight, to avoid formation of artifacts. Application of a stream of nitrogen or reduced pressure on a rotary evaporator is recommended to concentrate the filtrates prior to separation.

2.7.2. Separation and purification

The separation and purification stages include removing interfering compounds from the crude extract by separating using liquid-liquid extraction and/or using gravity and/or flash column chromatography (Nazck and Shahidi, 2004). Apart from the available chromatographic separation techniques, solvent partitioning has been successfully applied in the separation of an extremely complexed mixture of different classes of compounds present in a crude extract (Li et al., 2013b). In solvent partitioning, the compounds are distributed in two solvents according to their different partition coefficients (Jaganath and Crozier, 2010; Qiao et al., 2011). This step has been applied by several researchers to remove lipids, chlorophylls and unwanted polyphenols from the crude or partially purified extract (Nazck and Shahidi, 2004; Jaganath and Crozier, 2010; Li et al., 2013b).

Solid-phase extraction (SPE) is a simple separation technique allowing the elimination of polar, non-phenolic impurities in one step (Park and Lee, 1996; Kraemer-Schafhalter et al. 1998; Dawes and Keene, 1999; Sun et al., 2006) SPE formats (cartridges to disks) are commercially available with a range of sorbents such as C_{18} and C_8 (Robards, 2003). With reversed-phase cartridges, interfering sugars can be eluted with acidified water (Dawes and Keene, 1999) or aqueous methanol prior to elution of phenols with methanol (Robards, 2003). Sun and co-workers (1999) have successfully fractionated proanthocyanidins according to their degree of polymerization from grapes using a wide range of solvent system namely, water, ethyl acetate and methanol, through two preconditioned neutral C_{18} Sep-Pak cartridges connected in series.

As reviewed by Naczk and Shahidi, (2004), the phenolic extracts can be partially purified using ion exchange resins such as Amberlite particles (XAD-2 and XAD-7), or Diaion HP-20. The isolation of flavan 3-ols such as catechins, epicatechins and proanthocyanidins is commonly carried out using Sephadex LH-20 size exclusion column

chromatography (Gabriela, 1999). There are different carbohydrate gels such as Sephadex G-10, G-15, G-25 and Sephadex G-100, rated depending on their swelling capacity. Sephadex LH-20 is a hydroxypropylated form of Sephadex G-25 which is one of the most frequently used gels in natural product isolation (Gabriela, 1999). As a result of the derivatization, LH-20 gel swells sufficiently in organic solvents and allows separation of natural products that are soluble in organic solvents. When a single eluent is used, the gel filtration mode is operational while the partition mechanism comes to play when the eluent is a mixture of solvents (polar and non polar solvents) (Robards et al. 1994).

Preparative high-performance/pressure liquid chromatography (Prep HPLC) has recently become a major technique for the isolation of natural products. Preparative high-performance liquid chromatography (prep HPLC) can purify compounds from complex mixtures and it is robust and versatile. The main difference between prep HPLC and other lower pressure column chromatographic systems is the consistency and size of the particles in the stationary phases. One of the following four chromatographic modes such as reversed-phase, normal-phase, ion exchange chromatography and gel permeation chromatography is used in prep HPLC purification of natural products. The modes are determined by the stationary phase and the preparative column used, and the solvents utilized for elution. Reversed-phase prep HPLC is commonly used to isolate phenolic compounds from crude mixtures or partially purified mixtures with the help of reversed phase non-polar octadecylsilyl bonded (ODS) C_{18} columns (Naczk and Shahidi, 2004). There is no single HPLC method established that can properly elute all flavonoids without difficulties related to co-elution or interference (Alonso-Salces et al., 2004; Aaby et al., 2007; Inbaraj et al., 2010; Latocha et al., 2010). It has been reported that flavonoid glycosides and flavonoids with higher numbers of hydroxyl groups elute before aglycones on C_{18} reversed-phase columns (Abad-Garcia et al., 2009). Flavanones elute before flavones due to the unsaturation

between carbon 2 and 3 on the C-ring of the flavone structure. Flavone *C*-glycosides generally elute at shorter retention times than their *O*-glycosides.

2.7.3. Identification and structure elucidation

The identification of phenolics often includes the combination of high performance liquid chromatography coupled to a diode array detector (HPLC-DAD) or with mass spectrometry (HPLC-MS). HPLC is typically based on measurements of ultra violet (UV) absorption, or visible absorption (for anthocyanins). Mobile phase compositions are typically altered during analysis in the form of gradient elution, which changes the solvation power or polarity to allow for better separation of analytes. This allows for detection and identification of different types of phenolic based compounds in a single run, such as hydrophobic flavonoid aglycones and hydrophilic glycosides.

In addition to HPLC-DAD techniques, one of the most common methods available for the identification of phenolic-based compounds is high performance liquid chromatography mass spectrometry (HPLC-MS). This technique consists of sample separation, followed by analysis of the mass of analytes in the sample mixture. In liquid chromatography mass spectroscopy (LC-MS) ions are generated from the eluting molecules of an HPLC device. They are subsequently separated by a mass-selective detector according to their mass-to-charge (*m*/*z*) ratio. The instrument generates a plot of intensity versus mass-to-charge (*m*/*z*) ratio, and the results are reported as a mass spectrum. The mass spectrum of a compound is based on the distribution of fragment ions generated from the dissociation of a given analyte (Cuyckens et al., 2000). The detection, interpretation and identification of phenolic compounds are based on the theory that different structures often have unique molecular weights but with similar dissociation fragmentation patterns for different sub classes of flavonoids either in negative or positive ionization mode. It has been reported that

the negative ionization mode provides the highest sensitivity and the results in limited fragmentation. It also has been reported that the peak at the highest *m*/*z* ratio is not always the molecular ion species ($[M+H]^+$) in the positive ionization mode, and ($[M-H]^-$) in the negative ionization mode, because adducts with solvent and/or acid molecules and also molecular complexes can be generated ($[2M+H]^+$) in the positive mode, and ($[2M-H]^-$) in the negative mode (Cuyckens et al., 2000).

The presence of hexose residue (glucose and galactose sugars) is characterized by neutral loss of 162 Da, while arabinose, a pentose sugar moiety results in a 132 Da loss. A 146 Da loss indicates the presence of rhamnose sugar moiety which is a methylpentose (deoxyhexose) (Cuychens and Claeys, 2004). Losses of small neutral molecules, such as CO (-28 u), CO_2 (-44 u), and C_2H_2O (-42 u) can be associated with flavonoid compounds via retro-Diels Alder (RDA) ring opening mechanism (Fabre et al., 2001; Dubber et al., 2005; Gates and Lopes, 2012). Methylated compounds are characterized by the loss of 15 amu resulting in an $[M-H-CH_3]^-$ (Cuyckens et al., 2000; Parejo et al., 2004; Rosch et al., 2004; Gates and Lopes, 2012).

For the analysis of phenolic-based compounds, various types of mass spectrometric techniques can be applied, with the most powerful technique reported being tandem mass spectrometry (MS/MS) or MS^n (Cuyckens et al., 2000; Parejo et al., 2004; Rosch et al., 2004; Ye et al., 2005). This is a multi-stage method which is combined with collision-induced dissociation (CID) (Vukics and Guttman, 2008; Appledorn et al., 2009). This method allows for more detailed structural information of the flavonoids such as the type of substituent attached to the flavonoid base-unit, the stereochemistry of terminal monosaccharide units, the aglycone base-unit, inter-glycosidic linkages, locations of substituent's on the aglycone, and the type of inter-flavonoid linkages or inter-flavonoid bonds (Cuychens and Claeys, 2004). In many cases, even the use of MS^n (MS/MS/MS)

cannot differentiate isomers such as glucose and galactose or catechin and epicatechin. Thus full stereochemistry cannot be determined by MS, but rather these require techniques such as nuclear magnetic resonance (NMR).

Both ^{1}H- and ^{13}C- NMR spectroscopy are useful tools for elucidating the structure of flavonoids (Batterham and Highet, 1963; Agrawel, 1992; Gohari et al., 2011). The chemical shifts of the protons of ring A and B (**Figure 2.3**) prove to be independent of each other, but are affected by the nature of the ring C (Batterham and Highet, 1963; Gohari et al., 2011). There are two characteristic regions that can be distinguished in the ^{1}H NMR spectra; the signals of the A and B rings at δ=6.0-7.8 and the presence of sugar moieties with characteristic signals at δ= 3.1- 5.2 (Batterham and Highet, 1963; Gohari et al., 2010; Gohari et al., 2011). Flavonoids are readily recognized by the peaks arising from ring A, which occur upfield from the other peaks (Batterham and Highet, 1963; Gohari et al., 2011). Thus, examination of an unfamiliar spectrum will commonly start by the recognition of these peaks, which will often allow the nature of rings A and C, and the class of compound in hand, to be inferred.

Chapter 3

Optimization of extraction conditions for phenolics

Optimization of extraction conditions for phenolics from *Actinidia macrosperma* L. fruit and evaluation of their antioxidant capacity and phenolic profiles compared to commercial kiwifruit varieties

3.1. Introduction

Many different plant sources have recently become of great interest to scientific research as a result of their naturally occurring antioxidants. As safe sources of phenolic antioxidants, edible fruits have been investigated for their antioxidant properties, for example pomelo (*Citrus grandis* (L) beck) (Toh et al., 2013), sour cherries (*Prunus cerasus* L.) (Piccolella et al., 2008), berries (Kahkonen et al., 2001; Nagai et al., 2011), mango (*Mangifera indica*, L.), (Riberio al., 2008), bitter gourd (*Memordica charantia* L.) (Kubola and Siriamornpun, 2008), guava fruit (Thaipong et al., 2006), snake fruit (Gorinstein et al., 2009), red fruit (*Pandanus conoideus* Lam) (Rohman et al., 2010), star fruit (Shui and Leong, 2006) and kiwifruits (Park et al., 2008; Du et al., 2009; Fiorentino et al., 2009; Bekhradnia et al., 2011; Park et al., 2011).

Kiwifruit, one of the most popular fruits today, is the common name of the edible berry of a cultivar group of the woody vine of several *Actinidia* species in the genus *Actinidia* (family *Actinidiacea*). Even though the kiwifruit is native to China, they are now well distributed throughout the world, especially in eastern Asia, Europe, United States and New Zealand (Kim et al., 2009; Bekhradnia et al., 2011). The most commonly consumed kiwifruits in the world are green-fleshed cultivar (*Actinidia deliciosa* (A. Chev.) C.F. Laing et A. R. Ferguson 'Hayward') and gold-fleshed cultivar (*A. chinensis* Planch. 'Hort16A') (Fiorentino et al., 2009) **(Figure 3.1a & b**). A few other varieties such as *A. arguta* marketed recently as baby kiwifruit (**Figure 3.1c**) are grown commercially (Kim et al., 2009; Kruppa

et al., 2011), and a number of other varieties are currently being assessed for future commercialization or are important as useful genetic resources for cultivar development by inter specific hybridization techniques (Ferguson, 2007). *A. chinensis cv* Gold3 kiwifruit is a new kiwifruit variety commercialized in 2010 by ZESPRI after 10 years of development and is marketed internationally under the name of Sungold (**Figure 3.1d**). This variety shows a greater level of tolerance to Psa (*Pseudomonas syringae pv actinidiae* bacteria) than the original ZESPRI gold kiwifruit variety. The second green variety which forms part of the ZESPRI suite of products is the sweet green (*sp. A. deliciosa*) which has a similar shape and visual appeal but offers a smoother, sweeter taste (**Figure 3.1e**) (Morris, ZESPRI, New Zealand, personal communication)**.**

Actinidia macrosperma L. belongs to the family *Actinidiaceae* and is a non-commercial fruit with orange coloured flesh, small size fruit with large seeds and relatively thick, hairless skin (**Figure 3.1f**). This plant is popularly called 'cat ginseng' due to its attractant effect on cats by giving off a specific odour and then cats preferred to eat fresh leaves of the plant or twigs to excite themselves and cure wounds (Lu et al., 2011b). This plant has a reputation to treat various diseases in Chinese traditional medicine (Lu et al., 2007a; Lu et al., 2007b; Lu et al., 2011). Recent studies have shown that the different parts of the *A. macrosperma* plant exhibit various biological activities including immunomodulary (Lu et al., 2007b), antioxidant, antibacterial and antifungal activities (Lu et al., 2007a; Lu et al., 2011; Lu et al., 2012). There is a great interest in evaluation of the chemical composition and biological activities of *A. macrosperma* plant attributed by bioactive compounds and their high potential as nutraceutical and functional foods. A number of bioactive constituents extracted from stem and leaves of *A. macrosperma* have been reported, including polysaccharides, alkaloids, saponinns and organic acids (Lu et al., 2011).

Figure 3.1. The fruit of (a) *A. deliciosa cv* Hayward, (b) *A. chinensis cv* Hort 16A, (c) *A. arguta,* (d) *A. chinensis cv* Sungold, (e) *A. deliciosa cv* Sweet green and (f) *A. macrosperma*.

Recent studies have reported that the fruit of *A. macrosperma* includes compounds such as lutein, β-carotenes, zeaxantin, violaxantin, chlorophyll a and b, catechin, epicatechin, quercetin, tannic acid, gentisic acid, hydroxy benzoic acid, chlorogenic acid, *p*-coumaric acid and caffeic acid (MacGie and Ainge, 2002; Latocha et al., 2010). However, phenolic compounds mainly flavonoids and flavonoid glycosides present in this fruit have not been fully characterized yet. Therefore, the extraction of antioxidant flavonoids and their glycosides from *A. macrosperma* can be considered to contribute to the value of these fruits.

Extraction is one of the most important steps in the isolation, identification and quantification of phenolic compounds from plant materials. However, there is no standardized extraction method for the extraction of phenolic compounds from different plants (Pinelo et al., 2004; Shahidi and Naczk, 2004; Chirinos et al., 2007). Since each plant material has its unique properties in terms of phenolic extraction, it is important to develop an optimal extraction method that allows the maximum recovery of phenolic compounds from the fruit. It is reported that the extraction of phytochemicals from solid plant materials using solvents is influenced by two fundamental concepts called equilibrium and mass transfer rate in the extraction process (Cacace and Mazza, 2002). Generally, it is a separation process where the distribution of the analyte (phenolic compounds) between two immiscible phases is made in order to derive the appropriate distribution coefficient (Cacace and Mazza, 2002). Solvent extraction which is a frequently used method, is a process designed to separate soluble phenolic compounds by diffusion (using shaking or sonicating or blending or steeping) from a solid matrix (plant tissue) using a liquid solvent matrix (Pinelo et al., 2004; Shahidi and Naczk, 2004; Chirinos et al., 2007). In this separation process, the efficacy of the extraction can be affected by several factors such as the extraction method employed, type of solvent, the chemical nature of the compounds (simple and complex phenolics), the storage or extraction time and the conditions (pH, temperature) (Pinelo et al.,

2004; Shahidi and Naczk, 2004; Chirinos et al., 2007). Among the above mentioned factors, polarity of the solvent plays an important role in the selective extraction of different flavonoids (Marston and Hostettmann, 2006).

Therefore, the objectives of this chapter were to: (1) optimize the conditions for extracting phenolic compounds using single factor experimental design applied to *A. macrosperma* fruit followed by evaluating contents of total phenolic, flavonoid, flavanol and the antioxidant potentials, (2) compare phenolic composition and antioxidant activity of the extract obtained under the optimized conditions of *A. macrosperma* fruit with those of commercial kiwifruit varieties namely, *A. deliciosa cv* Hayward (Green), *A. deliciosa cv* Sweetgreen, *A. chinensis cv* Hort16A (Gold), *A. chinensis cv* Gold3 (Sungold) grown in New Zealand and (3) analyze the phenolics present in various extracts using High performance liquid chromatography coupled with a ultra violet (UV)/visible spectroscopy to diode array detector (HPLC-UV-DAD) and electro spray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS). Findings from this study will help to evaluate the best among the five different varieties in terms of antioxidant capacity and phenolic composition and also help horticulturists to develop new kiwifruit cultivars with specific traits.

3.2. Plant materials and chemicals

A. deliciosa cv Hayward (Green kiwifruit), *A. deliciosa cv* Sweetgreen, *A. chinensis cv* Hort 16A (Gold kiwifruit) and *A. chinensis cv* Sungold were donated by ZESPRI-New Zealand. The fruits of *A. macrosperma* reaching physiological maturity were collected on 26th April 2010 at the Plant and Food research orchard in Te Puke Bay, New Zealand. Defective fruits were discarded and the remaining fruit were cut into small parts,

freeze dried and stored at -80 °C. The samples were prepared by grinding the lyophilized fruit samples using a pestle and mortar prior to the extraction.

Folin-Ciocalteu phenol reagent, iron(III) chloride 6-hydrate, hydrochloric acid, ferulic acid, caffeic acid, cholorogenic acid, *p*-coumaric acid, syringic acid, catechin, epicatechin, rutin, quercetin-3-*O*-glucoside, quercetin, myricetin, luteolin, 2,2-diphenyl-2-picylhydrazyl (DPPH), 2,4,6-tripyridyl-s-triazine (TPTZ), Trolox and *p*-dimethylaminocinnamaldehyde (DMACA) were purchased from Sigma, St Louis, USA. Sodium carbonate, sodium hydroxide, sodium nitrite, formic acid and aluminium hexahydrate, were from Scharlau, Spain. Gallic acid (ACROS, USA), iron (II) sulfate 7-hydrate (BDH Chemicals, England), HPLC grade methanol, HPLC grade acetonitrile, ethanol, methanol, hexane and all other chemicals were purchased from ECP Ltd, Auckland, New Zealand. HPLC grade methanol used for LC-ESI-MS/MS analysis was purchased from Sigma Aldrich, Oakville, ON, Canada.

3.3. Methodology

3.3.1. Single factor experiments for optimization of extraction conditions for phenolics from *A. macrosperma* fruit

Single factor experiments were performed to determine the optimal extraction conditions namely, extraction solvent and extraction technique by varying one independent variable at a time while keeping others constant. Therefore, the initial step of the preparation of defatted crude extracts was to select an appropriate extraction solvent by fixing extraction technique for phenolic compounds present in *A. macrosperma* fruit. Five different solvent systems, namely 70% aq. acetone, 80% aq. methanol, 80% aq. ethanol, 100% methanol and

100% water were selected based on their wide use in extraction of phenolics from plant material (Pinelo et al., 2004; Shahidi and Naczk, 2004; Chirinos et al., 2007). Extraction was carried out by steeping each lyophilized ground kiwifruit sample (3 g) in each solvent (50 mL) in a Scott Duran bottle for 6 h in the dark with nitrogen gas purging at room temperature (23±2 °C) to prevent phenolic oxidation during extraction. The extracts were filtered through a sintered glass filter and collected into a conical flask in an ice bath. The residue was subjected to re-extraction, and the filtrates were combined and concentrated on a rotary evaporator (Buchi, Switzerland) below 35 °C under vacuum.

Partitioning of phenolic compounds between water and organic (hexane) layers was carried out in a separating funnel to remove carotenoids and unwanted substances from the crude extract to get an accurate quantiy of phenolics. The partitioning step was carried out several times until the organic layer became colourless. The upper organic layer (yellow/orange colour) which was rich in carotenoids and other lipophilic compounds and the lower aqueous layer (red/orange colour) rich in phenolics were collected separately and then concentrated on a rotary evaporator below 35 °C under vacuum. Weight and volume of the concentrated filtrates were recorded and the defatted crude extract was subjected to freeze drying and stored at -80 °C. Total phenolic (TP), total flavonoid (TFO), total flavanol (TFA) contents, and antioxidant activity (AA) of each solvent extract were determined.

It was found that the 70% aq. acetone extraction allowed the recovery of polyphenols based on % yield, TP, TFO, TFA and AA values of each solvent extract tested. Thus, selection of the extraction technique was carried out by extracting phenolic compounds from lyophilized ground fruit samples into chosen solvent system (70% aq. acetone) by four different extraction techniques namely, steeping, shaking, sonicating and blending due to their wide use in conventional extractions.

Method A (Steeping): Briefly**,** the lyophilized ground fruit sample (3 g) was steeped in 70% aq. acetone (50 mL) in a Scott Duran bottle for 6 h in the dark with nitrogen gas purging at room temperature (23 ± 2 °C). Method B (Sonicating): The crude extract was prepared by homogenizing the lyophilized ground fruit sample (3 g) in 70% aq. acetone solvent (50 mL) in a Scott Duran bottle in an ultrasonic bath (40 kHz frequency and 150 W power) (Compact High-Performance Ultrasonic cleaner PC_3) for 2 h in the dark with nitrogen gas purging at room temperature (23 ± 2 °C). Method C (Shaking): The lyophilized ground fruit sample was homogenized (3 g) in 70% aq. acetone solvent (50 mL) in a Scott Duran bottle on the orbital shaker (KS 125 basic 1 KA LABORTECNIK) for 6 h at 250 rpm in the dark with nitrogen gas purging at room temperature (23 ± 2 °C). Method D (Blending): The lyophilized ground fruit sample was homogenized (3 g) in 70% aq. acetone solvent (50 mL) in a Waring blender for 5 minutes using high speed at room temperature (23 ± 2 °C).

Each extract was filtered through a sintered glass filter, followed by the partitioning step as described in Section 3.3.1. Weight and the volume of each concentrated filtrate were recorded and each defatted crude extract was subjected to freeze drying and stored in the freezer at -80 °C. Total phenolic (TP) content, total flavonoid (TFO) content, total flavanol (TFA) content and antioxidant activity of each solvent extract were determined.

3.3.2. Preparation of defatted crude extracts from commercial kiwifruit varieties

Phenolic compounds from commercial kiwifruit varieties were extracted by steeping each lyophilized ground fruit sample (3 g) in 70% aq. acetone (50 mL) in a Scott Duran bottle for 6 h in the dark with nitrogen gas purging at room temperature (23±2 °C) as described in Section 3.3.1. The extracts were filtered through a sintered glass filter and partitioned with hexane to get defatted crude extracts. Weight and volume of the

concentrated phenolics-rich filtrates obtained from commercial kiwifruits were recorded, subjected to freeze drying and stored at -80 °C. Total phenolic content (TP), total flavonoid content (TFO), total flavanol content (TFA), and antioxidant activity of each extract were determined.

3.3.3. Determination of total phenolic content

The Folin-Ciocalteu assay was performed to estimate the total phenol content (TP) of defatted crude mixtures as described by Singleton and Rossi, (1965), modified by Jayaprakasha et al., (2001). Briefly, 7.9 mL of distilled water, 0.1 mL of each defatted crude extract (50 mg/mL) (DW/volume) and 0.5 ml of 0.2 N Folin-Ciocalteu reagent (1:1 with water) were mixed in a 10 mL Eppendorf tube and left to stand. After one minute, 1.5 mL of sodium carbonate (20 g/100 mL) (DW/volume) was added and the mixture was vortexed and allowed to stand at room temperature in the dark for 2 h. The absorbance was measured at 765 nm with a UV-visible single beam spectrophotometer (UV mini-1240, Shimadzu**,** Japan). The total phenol content was calculated from a calibration curve using gallic acid standard (50-1000 mg/L) (**Appendix 1**) and expressed as mg gallic acid equivalents (GAE)/100 g dry weight (DW) of the fruit.

3.3.4. Determination of total flavonoid content

The aluminium chloride colorimetric method described by Marinova et al., (2005), slightly modified by Park et al., (2008) and Du et al., (2009) was used to estimate the total flavonoid content (TFO). In a 10 mL Eppendorf tube, 0.3 mL of each defatted crude extract (50 mg/mL) (DW/volume), 3.4 mL of 30% ethanol, 0.15 mL of 0.5 M $NaNO_2$ and 0.15 mL

of 0.3 M $AlCl_3.6H_2O$ were added and mixed. After 5 min, 1 mL of 1 M NaOH was added and the absorbance of the mixture was measured at 506 nm with a UV-visible single beam spectrophotometer (UV mini-1240, Shimadzu**,** Japan). The total flavonoid content was calculated from a calibration curve using catechin as a standard (6.25-300 mg/L) (**Appendix 2**) and expressed as mg catechin equivalents (CAE)/100 g DW of the fruit.

3.3.5. Determination of total flavanol content

The *p*-dimethylaminocinnamaldehyde (DMACA) method used by Du et al., (2009) was performed with slight modifications to determine the total flavanol (TFA) content. In a 10 mL Eppendorf tube, 0.2 mL of each defatted crude extract (10 mg/mL) (DW/volume) was mixed with 3.0 mL of DMACA solution (0.05% in 1 M HCl in MeOH). The mixture was vortexed and allowed to react at room temperature (23±2 °C) for 10 min. The absorbance of the mixture was measured against a blank prepared following the same procedure as above but with milli-Q-water replacing extract at 640 nm with a UV-visible single beam spectrophotometer (UV mini-1240, Shimadzu**,** Japan). The total flavanol content was calculated from a calibration curve using catechin as a standard (1.56-50 mg/L) (**Appendix 3**) and expressed as mg catechin equivalents (CAE)/100 g DW of the fruit.

3.3.6. Determination of ferric-reducing antioxidant power activity (FRAP assay)

The FRAP assay was used to determine the electron donating potential of the fruit extracts based on the assay described by Benzie and Strain, (1996) with slight modifications. The stock solutions included 300 mM acetate buffer (pH 3.6) (3.1 g $C_2H_3NaO_2.3H_2O$ and 16 mL $C_2H_4O_2$), 10 mM TPTZ (2,4,6-tripyridyl-*s*-triazine) solution in 40 mM HCl, and 20

mM $FeCl_3.6H_2O$ solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution, and 2.5 mL $FeCl_3.6H_2O$ solution and then warmed at 37 °C for 2 h before use. Each fruit extract was prepared in water with the concentration of 5 mg/mL (DW/volume) and 0.1 mL of each of them was allowed to react with 3 mL of the FRAP reagent in the reaction vial and incubated for 10 minutes in the dark. Absorbance of the coloured product (ferrous tripyridyltriazine complex) was recorded at 593 nm with a UV-visible single beam spectrophotometer (UV mini-1240, Shimadzu**,** Japan) after 10 minutes with reference to a reagent blank containing distilled water and FRAP reagent which was also incubated for 10 minutes. The standard curve was prepared by using dilution series of 0, 0.1, 0.2, 0.5, 1.0, 1.5 mM made up from the stock solution of aqueous $Fe(II)SO_4.7H_2O$ (5 mM) (**Appendix 4**) and the results were expressed in mmol Fe(II)equivalents/100 g DW of the fruit.

3.3.7. Determination of antioxidant capacity (DPPH assay)

The antioxidant capacity of all extracts was determined using the DPPH assay according to the method described by Brand-Williams et al., (1995) and modified by Du et al., (2009). The stock solution of DPPH (6.25×10^{-5} M) was prepared by dissolving 2.4 mg of DPPH in 100 mL methanol which was stored at -20 ^{o}C. Briefly, each fruit extract was prepared in water with the concentration of 5 mg/mL (DW/volume) concentration and 0.025 mL of each extract was added to 2 mL of 6.25×10^{-5} M solution of DPPH in the reaction vial and incubated for 30 minutes in the dark. A control sample containing the same volume of solvent (Mili-Q-water) in place of extract was used to measure the maximum DPPH absorbance. The absorbance at 517 nm was recorded with a UV-visible single beam spectrophotometer (UV mini-1240, Shimadzu**,** Japan) with reference to methanol to

determine the concentration of remaining DPPH. The standard curve was linear in the range of 0-1.0 mM Trolox (**Appendix 5**) and the results were expressed in mmol Trolox equivalents/100 g DW of the fruit.

The DPPH radical-scavenging activity (%) of 70% aq. acetone extract by steeping technique (under optimized conditions) obtained from all kiwifruit cultivars was studied with serial concentrations (0, 1, 2, 3, 4, and 5 mg/mL) (DW/volume), assayed according to the method described by Yan-Hwa and Hsu, (2000). The radical-scavenging activity (%) was calculated by following the equation:

$$\text{Scavenging activity (\%)} = \{1- (A_{\text{sample}} - A_{\text{control}})\} \times 100\ \% \quad \text{.....(1)}$$

A_{sample} is the absorbance of the solution with different concentrations of extract, and A_{control} is the absorbance of the DPPH solution without sample extract. The scavenging activity was expressed as 50% effective concentration (EC_{50}) which represented the concentration of sample having 50% of DPPH radical scavenging effect. EC_{50} was calculated using linear regression analysis of % scavenging activity vs. concentration of the extract/sample.

3.3.8. High performance liquid chromatography coupled to diode array detection (HPLC-DAD) fingerprint analysis

The phenolic profiles in each defatted crude extract were determined according to the procedure described by Gheldof et al., (2002) with slight modifications of gradient programme. Phenolics were separated using a reversed-phase HPLC column on an Agilent 1100 liquid chromatograph (Agilent 1100, New Zealand) equipped with an auto injector, column oven, auto sampler, and vacuum solvent degasser, a UV/vis photodiode array

detector (DAD), and the Empower soft-ware. A synergy fusion RP_{18} (150 × 4.6 mm, I.D. 4 µm particle size, 80 Å) column (Phenomenex, New Zealand) combined with a Phenomenex synergy fusion guard column (4.6 × 2 mm, 4 µm) was used for phenolic separation at 35 ^{o}C. A binary mobile phase system was employed consisting of solvent (A) 0.1 % formic acid in water (v/v) and (B) 100 % methanol. The gradient programme was used with the following proportions of solvent A applied at time *t* (min) (*t*, A%): (0, 95%), (10, 85%), (15, 70%), (20, 60%), (30, 55%), (40, 40%), (45, 50%), (55, 80%), (58, 95%). The flow rate of the mobile phase was 0.8 mL/min and 20 µL of each sample (50 mg/mL) was injected. Samples were filtered through a 0.45µm filter prior to HPLC injection. Spectral data and peaks were monitored at four different wavelengths: 280, 320, 360 and 520 nm.

3.3.9. Liquid chromatography coupled to mass spectrometry (LC-ESI-MS/MS) analysis

Analyses of major individual phenolic compounds present in all extracts obtained from *A. macrosperma* fruit were performed at the Department of Environmental Sciences, Faculty of Agriculture, Dalhousie University, Truro in Canada according to the procedure reported by Rupasinghe et al., (2008). All analyses were performed using a Waters Alliance 2695 separations module (Waters, Milford, MA) coupled with a Micromass Quattro *micro* API MS/MS system and controlled with Masslynx V4.0 data analysis system (Micromass, Cary, NC). The column used was a Phenomenex Luna C_{18} (150 mm x 2.1 mm, 5 µm) with a Waters X-Terra MS C_{18} guard column. For the separation of the flavonol, flavan-3-ol, phenolic acid and dihydrochalcone compounds, a gradient elution was carried out with 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) at a flow rate of 0.35 mL/min. A linear gradient profile was used with the following proportions of

solvent A applied at time *t* (min) (*t*, A %): (0, 94%), (9, 83.5%), (11.5, 83%), (14, 82.5%), (16, 82.5%), (18, 81.5%), (21, 80%), (29, 0%), (31, 94%), (40, 94%).

Electrospray ionization in negative ion mode (ESI-) was used for the analysis of the flavonol, flavan-3-ol, phenolic acid and dihydrochalcone compounds.The following conditions were used: capillary voltage -3000V, nebulizer gas (N_2) temperature 375 ^{0} C at a flow rate of 0.35 mL/min. The cone voltage (25 to 50 V) was optimized for each individual compound. Multiple reaction–monitoring (MRM) mode using specific precursor/ product ion transitions was employed for quantification in comparison with standards: *m/z* 301 105 for quercetin (Q), *m/z* 609 → 301 for Q-3-*O*-rutinoside, *m/z* 463→ 301 for Q-3-*O*-glucoside, and Q-3-*O*-galactoside, *m/z* 448 → 301 for Q-3-*O* -rhamnotoside, *m/z* 595 → 301 for Q-3-*O*-peltoside, *m/z* 273 → 167 for phloritin, *m/z* 435 → 273 for phloridzin, *m/z* 353 → 191 for chlorogenic acid, *m/z* 179 → 135 for caffeic acid, *m/z* 193 → 134 for ferulic acid and isoferulic acid, *m/z* 289 → 109 for catechin and epicatechin, and *m/z* 305→ 125 for epigallocatechin.

3.3.10. Statistical analysis

All experimental measurements were conducted in triplicate and the results are expressed as mean±SD. The effects of the extraction solvent, technique and the kiwifruit variety tested on the total phenol content, total flavonoid content, total flavanol content and antioxidant capacity values were analyzed by analysis of variance (ANOVA) using OriginPro8 software. Pairwise multiple comparisons were evaluated using Tukeyss' significance difference test used in Originpro8. Differences at p= 0.05 were considered significant.

3.4. Results and discussion

3.4.1. Extract yields, phenolic composition and antioxidant activities of different extraction solvents and techniques employed to *A. macrosperma* kiwifruit

3.4.1.1. Extract yields

Sample preparation, removal of unwanted substances and the extraction procedure are the primary determinants for the separation and recovery of phenolics. As mentioned earlier, extraction is one of the most important steps which are influenced by several factors including the extraction solvent and the technique employed (Pinelo et al., 2004; Shahidi and Naczk, 2004; Chirinos et al., 2007; Kohddami et al., 2013). The literature also indicates that extraction yield is dependent on the solvent and technique of the extraction, due to presence of different compounds with different polarity (Pinelo et al., 2004; Shahidi and Naczk, 2004; Goli et al., 2005; Chirinos et al., 2007; Mohdaly et al., 2010; Kohddami et al., 2013). For extraction, the solvent is chosen as a function of the type of required flavonoid. Less polar flavonoids such as isoflavones, flavanones, methylated flavone and flavonols are extracted with chloroform, dichloromethane, diethyl ether, or ethyl acetate while flavonoid glycosides and more polar aglycones are extracted with alcohols or alcohol-water mixtures. Since the attached sugar moieties in the glycosides increase solubility in water, aqueous alcoholic solutions are suitable for their extraction (Marston and Hostettmann, 2006). Therefore, it is worthwhile to determine their yields in the different extracts obtained from *A. macrosperma* fruit.

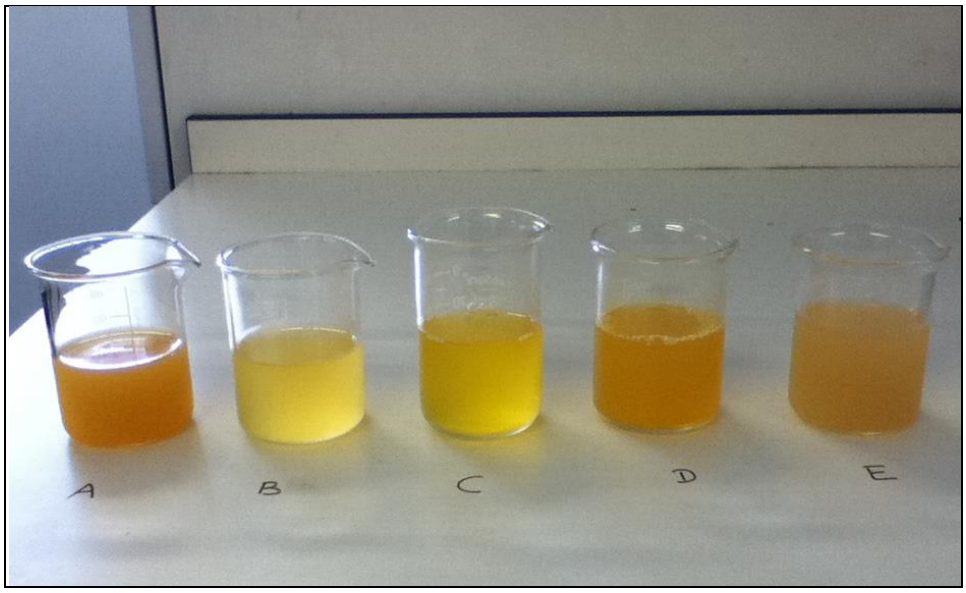


Figure 3.2. Physical appearance of each defatted solvent extracts (A; 70% aq. acetone, B; 80% aq. methanol, C; 80% aq. ethanol, D; 100% methanol and E; 100% water) obtained by steeping technique employed in *A.macrosperma* fruit.

Physical appearance of solvent extracts shown in **Figure 3.2**. shows that various solvent extracts obtained from *A. macrosperma* fruit have different colours. The observation could be due to the different compounds with different colour or/and different compounds with different concentration extracted. 80% aq. methanol and 80% aq. ethanol extracts had similar colours but less intense compared to 70% aq. acetone, 100% methanol and 100% water extracts.

The yields with different solvent extracts obtained from *A. macrosperma* fruit varied from 39.3±1.3 to 51.3±1.5 g/100 g DW of the fruit **(Table 3.1)**. Water as extraction solvent had the highest (51.3±1.5 g/100 g DW) in extraction percentage yield followed by 100% methanol (46.6±1.9 g/100 g DW), 70% aq. acetone (42.8±0.8 g/100 g DW), 80% aq. methanol (39.9±1.0 g/100 g DW), and 80% aq. ethanol (39.3±1.3 g/100 g DW). The yields from 80% aq. methanol and 80% aq. ethanol were not significantly different (p= 0.05) according to the Tukey test.

Table 3.1. Effect of extraction solvent on percentage of Yield, TP, TFO, and TFA values for *A. macorsperma* fruit.

Solvent	Yield (%)[a]	TP[b]	TFO[c]	TFA[d]
70% aq. acetone	42.8±0.8^{p}	823.1±14.4^{p}	170.9±1.9^{p}	82.6±0.6^{p}
80% aq. methanol	39.9±1.0^{q}	360.3±24.4^{q}	61.9±5.0^{q}	71.0±1.0^{q}
80% aq. ethanol	39.3±1.3^{q}	430.0±4.9^{r}	75.8±0.8^{r}	55.3±0.9^{r}
100% methanol	46.6±1.9^{r}	321.6±18.7^{q}	96.3±0.6^{s}	52.6±0.9^{s}
100% water	51.3±1.5^{s}	464.1±31.1^{r}	14.2±6.0^{t}	53.1±1.1^{r,s}

[a] Extraction yield is expressed as percentage g/100 g DW

[b] Total phenolic content is expressed as mg GAE/100 g DW

[c]Total flavonoid content is expressed as mg CAE/100 g DW

[d]Total flavanol content is expressed as mg CAE/100 g DW

Results are expressed as mean±standard error. Means followed by the same letter in a column are not significantly different at p= 0.05

It is reported that water and aqueous mixtures of ethanol, methanol, ethyl acetate and acetone have been commonly used for extracting phenolics and will influence the yields of phenolics extracted (Jayaprakasha et al., 2001; Pinelo et al., 2004; Shahidi and Naczk, 2004; Goli et al., 2005; Chirinos et al., 2007; Mohdaly et al., 2010; Kohddami et al., 2013). Variation in the yields of various solvent extracts obtained in this study could be attributed to differences in polarity of compounds present and these observations are in agreement with former studies reported in the literature (Pinelo et al., 2004; Goli et al., 2005; Chirinos et al., 2007; Mohdaly et al., 2010). The use of only water as solvent may yield an extract with a

high content of impurities such as organic acids, sugars, and soluble proteins, which could interfere in the phenolic identification and quantification. However, it is reported that the use of water in combination with other organic solvents contributes to the creation of a moderately polar medium that ensures the extraction and solubility of polyphenols (Jayaprakasha et al., 2001; Chirinos et al., 2007). It is also reported that the solubilisation of phenolic compounds is enhanced if the organic component in the extraction solvent is also a hydrogen–bond breaking agent such as acetone (Gabriela, 1999). Our research findings are in accordance with a previous study reporting that solvents with high polarity, such as water, or very low solvent strength, such as chloroform or hexane, do not give good extraction results for extracting phenolic compounds from *A.macrosperma* stems (Lu et al., 2011a), mashua tubers (Chirinos et al., 2007), and pine sawdust (Pinelo et al., 2004).

The yields with extracts obtained from *A. macrosperma* fruit using different techniques varied from 30.9±2.0 to 48.5±1.7 g/100 g of DW of the fruit with the descending order of shaking > steeping > sonicating > blending (**Table 3.2**). The population means of the yields from different techniques of steeping (42.8±0.8 g/100 g of DW) and sonicating (42.2±1.4 g/100 g of DW) showed no significant difference at p= 0.05. The extraction technique of shaking showed the highest yield (48.5±1.7 g/100 g of DW) while blending exhibited the lowest yield (30.9±2.0 g/100 g of DW).

Table 3.2. Effect of extraction technique employed on percentage of Yield, TP, TFO, and TFA values for *A. macorsperma* fruit.

Method	Yield (%)[a]	TP[b]	TFO[c]	TFA[d]
Steeping	42.8±0.8[p]	823.1±14.4[p]	170.7±1.9[p]	82.6±0.6[p]
Blending	30.9±2.0[q]	427.8±14.6[q]	99.7±4.3[q]	37.6±1.1[q]
Sonicating	42.2±1.4[p]	642.9±8.2[r]	170.7±3.4[p]	65.7±1.5[r]
Shaking	48.5±1.7[r]	781.4±13.5[p]	159.2±7.5[p]	69.6±1.1[s]

[a] Extraction yield is expressed as percentage g/100 g DW

[b] Total phenolic content is expressed as mg GAE/100 g DW

[c]Total flavonoid content is expressed as mg CAE/100 g DW

[d]Total flavanol content is expressed as mg CAE/100 g DW

Results are expressed as mean±standard error. Means followed by the same letter in a column are not significantly different at p= 0.05

3.4.1.2. Total phenolic content

The total phenolic contents of different extracts were measured by Folin-Ciocalteu method which is the most widely used procedure for quantification of phenolics in plant materials. This assay is based on the reduction of phosphomolybdic-phosphotungstic acid (FC) reagent to a blue complex in an alkaline solution occurs in the presence of phenolic compounds. The results of the total phenolic contents determined for the extracts from different extraction solvents and techniques were reported as gallic acid equivalents (mg GAE/100 g of DW of the fruit) with reference to the standard curve (y=0.001x, R^2=0.996) (**Appendix 1**). The concentration of phenolics in the extracts expressed was dependent on

the solvent (**Table 3.1)** and the technique employed (**Table 3.2**). This study revealed that different solvent extracts from *A. macrosperma* fruit had total phenolic contents ranging from 321.6±18.7 to 823.1±14.4 mg GAE/100 g DW of the fruit. It is interesting to notice that 70 % aq. acetone extract had the highest (823.1±14.4 mg GAE/100 g) amount of total phenols which is significantly different (p= 0.05) from other different extraction media tested. Research studies carried out by Xu and Chang, (2007) reported 70% aq. acetone was the best solvent for extracting phenolics from lentils, black soybean and common beans, while 50% aq. acetone was best for yellow pea, green pea, and chick pea. Research studies carried out by Pinelo et al., (2004); Liyanapathirana and Shahidi, (2005); Chirinos et al., (2007); Goli et al., (2007); Mohdaly et al., (2010) and Hismath et al., (2011) showed that total phenolic content varied according to the solvent employed for extracting phenolics. Furthermore, there was no significant difference of total phenolic content found between 100% methanol extract (321.6±18.7 mg GAE/100 g), and 80% aq. methanol extract (360.3±24.4 mg GAE/100 g) as well as between 80% aq. ethanol (430.0±4.9 mg GAE/100 g) and water extracts (464.1±31.1 mg GAE/100 g). It has been reported by several research studies that TP content increases with the methanol content in the solvent mixture upto 80% but started decreasing after that (Pinelo et al., 2004; Chirinos et al., 2007; Goli et al., 2007; Mohdaly et al., 2010).

However, all population mean values of TP obtained for different methods namely, steeping (823.1±14.4 mg GAE/100 g), blending (427.8±14.6 mg GAE/100 g), sonicating (642.9±8.2 mg GAE/100 g) and shaking (781.4±13.5 mg GAE/100 g) are significantly different at p= 0.05 level (**Table 3.2).** The technique, steeping showed the highest TP values while blending being the lowest. In this study, 70% aq. acetone and steeping technique were selected as the most efficient solvent and method for the extraction and the recovery of phenolics.

3.4.1.3. Total flavonoid content

Flavonoids are the most common and widely distributed group of plant phenolic compounds which are characterized by a benzo-γ-pyrone structure as described in Section 2. 3.1 in Chapter 2. They are likely to be the most important components of natural phenolics of human and animal diet. Therefore, the total flavonoid content of the different extracts obtained from *A. macrosperma* fruit was performed by the aluminium chloride colorimetric method. The content of flavonoids was expressed as catechin equivalents (CAE/100 g of DW of the fruit) with reference to the standard curve (y=0.0018x, R^2= 0.998) (**Appendix 2**). This study showed that the total flavonoid contents of the solvent extracts tested were significantly different (*p*= 0.05) from other solvent extracts tested and varied from 14.2±6.0 (100% water extract) to 170.9±1.9 mg CAE/100 g of DW of the fruit (70% aq. acetone extract) with the descending order of 70% aq. acetone > 100% methanol > 80% aq. ethanol > 80% aq. methanol > water (**Table 3.1**)**.** Interestingly, it is noticed that 70% aq. acetone extract had the highest and this observation was well supported by the research carried out by Xu and Chang, (2007) who revealed that 70% aq. acetone extract obtained from peas had the highest total flavonoid content (TFO). The total flavonoid content of the water extract was significantly lowest, although it showed the highest yield of the dry weight and moderate total phenolic content. This is in agreement with the published literature. The total flavonoid content extracted from *Teucrium montamum* (medicinal plant) was high in acetone and methanolic extracts while the lowest level was observed in water extract (Stankovic et al., 2011). Our data are in agreement with other studies reporting flavonoid concentration in various solvent extracts from different plant parts such as potato peel, sugar beet pulp, sesame cake, mashua and pistachio hull (Chirinos et al., 2007; Goli et al., 2007; Mohdaly et al., 2010).

It is well known that the total phenolic content measured by the Folin-Ciocalteu procedure does not give a full picture of the quantity or quality of the phenolic constituents in the extracts as there may be some interference rising from other chemical components present in the extract, such as ascorbic acid and sugars (Singleton and Rossi, 1965; Du et al., 2009). Therefore, total flavonoid and total flavanol contents were determined to get better picture of the phenolic contents of the different extracts.

The total flavonoid contents of the extracts with different extraction techniques obtained from *A. macrosperma* fruit varied from 99.7±4.3 to 170.9±1.9 mg CAE/100 g DW of the fruit (**Table 3.2**). Method of steeping as extraction method occupied the first place (170.9±1.9 mg CAE/100 g DW) followed by sonicating 170.7±3.4 mg CAE/100 g DW), shaking (159.2±7.5 mg CAE/100 g DW), and blending (99.7±4.3 mg CAE/100 g DW). However, there was no significant difference observed for total flavonoid contents among extracts obtained from different extraction techniques namely sonicating, shaking and steeping in the present study. This could be one of the reasons for these three extraction techniques to be highly employed in extraction of phenolic compounds from foods and medicinal plants (Aba-Garcia et al., 2007; Xu and Chang, 2007; Du et al., 2009; Bekhradnia et al., 2011; Al Juhaimi and Ghafoor, 2013).

3.4.1.4. Total flavanol content

Flavanols are a class of flavonoid compounds found in many plants where they are involved in defense mechanism as described in Section 2. 3.1 in Chapter 2. The total flavanols were estimated using the *p*-dimethylaminocinnamaldehyde (*p*-DMACA) reagent which is based on the reaction between the reagent and flavanol compounds to form a blue

complex **(Figure 3.3)** that can be measured at the maximum absorption wavelength of 640 nm. 0

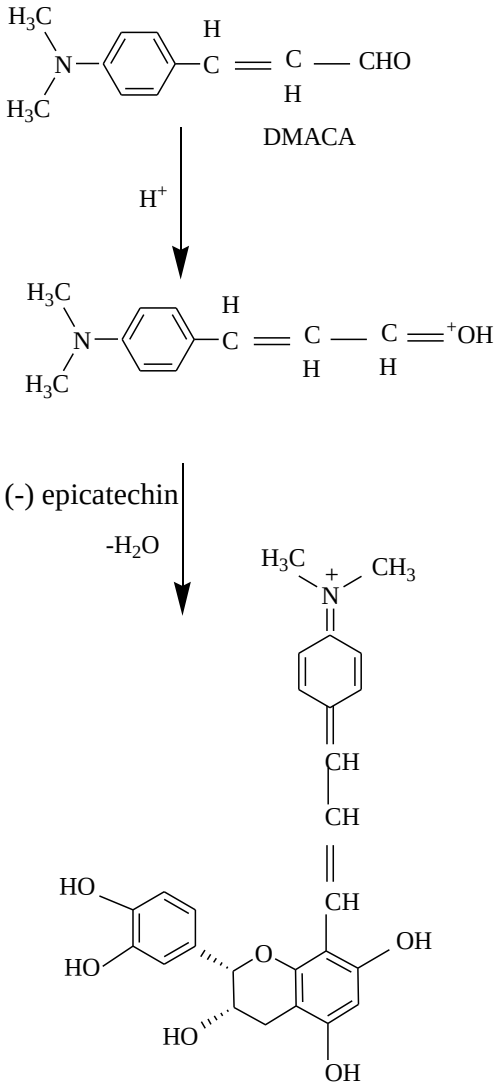


Figure 3.3. Condensation reaction of (-)-epicatechin with *p*-DMACA.

It reacts with the terminal groups of flavan-3-ols and it is sensitive to both monomeric and polymeric unit (**Figure 3.3**) (Gabriela, 1999). It was reported that other phenolic compounds, such as indoles and terpenes, react very weakly or give different reaction products with DMACA (Treutter, 1989). It is known that flavanols are very important phenolics known to influence the sensory properties of fruits by adversely affecting the

palatability by introducing astringency or bitterness in hardy kiwifruit (Kim et al., 2009) and provide a defense mechanism against plant pathogens (Haslam, 1989).

The total flavanol contents of the solvent extracts tested are shown in **Table 3.1** with reference to the standard curve (y=0.027x, R^2= 0.995) (**Appendix 3**). Those values varied from 52.6±0.9 to 82.6±0.6 mg CAE/100 g of DW of the fruit with the ranking order of 70% aq. acetone > 80% aq. methanol > 80% aq. ethanol > water > 100% methanol. It is interesting to notice that similarly to the total phenolic and flavonoid contents, 70% aq. acetone extract had the highest flavanol content (82.6±0.6 mg CAE/100 g of DW of the fruit) which was significantly different (*p*= 0.05) from the total flavanol contents (TFA) obtained from all other solvent extracts tested. Extract obtained from 100% methanol (52.6±0.9 mg CAE/100 g of DW of the fruit) exhibited the lowest but not significantly different from that of 100% water extract (53.1±1.1 mg CAE/100 g of DW of the fruit).

The flavanol contents of the extracts obtained from different techniques which were significantly different (*p*= 0.05) are shown in the **Table 3.2** and those values varied from 37.6±1.1 to 82.6±0.6 mg catechin equivalents (CAE)/100 g of DW of the fruit. The technique steeping showed the highest TFA (82.6±0.6 mg CAE/100 g of DW of the fruit) followed by shaking (69.6±1.1 mg CAE/100 g of DW of the fruit), sonicating (65.7±1.5 mg CAE/100 g of DW of the fruit), and the lowest was blending (37.6±1.1 mg CAE/100 g of DW of the fruit). There are no previous studies on the total flavanoid and total flavanol contents of *A. macrosperma* fruit reported in the literature for the comparison of our data obtained in this study. This is the first report on TFO and TFA of *A. macrosperma* fruit. However, similar as with the total phenolic and total flavonoid content, 70% aq. acetone extract from *A. macrosperma* fruit by technique of steeping had the highest total flavanol content.

3.4.1.5. Antioxidant activity

Reactive oxygen species (ROS) are constantly generated by biological processes occurring in the human body. To counteract ROS and to prevent their possible damage to biological molecules, especially to DNA, lipids and proteins, all oxygen-consuming organisms are endowed with well-integrated antioxidant systems (Wang et al., 1996; Prior et al., 2005). There are many different antioxidant components in animal and plant tissues, and it is relatively difficult to measure each antioxidant component separately. Therefore, several methods have been developed in recent years to evaluate the total antioxidant capacity of biological samples (Cao et al., 1993). Ferric reducing antioxidant power (FRAP), copper reducing antioxidant activity (CUPRAP), diphenyl picryl hydrazyl (DPPH), azinobis(3-ethylbenzothiozoline-6-sulfonic acid (ABTS), oxygen radical absorbance capacity (ORAC), total radical-trapping antioxidant parameter (TRAP) and low density lipoprotein (LDL) oxidation are some of the antioxidant capacity assays commonly used (Wang et al., 1996) as described in Section 2.6.2 in Chapter 2. Basic reactions occur in two different assays namely, FRAP and DPPH used for determination of antioxidant activities of different extracts in this study are shown in **Figure 3.4** and **Figure 3.5** respectively.

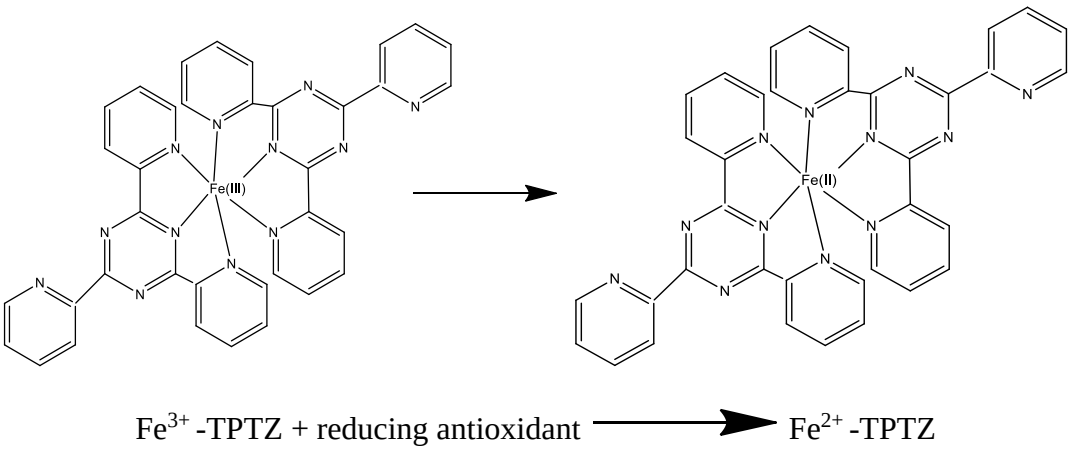


Figure 3.4. Reaction occurring in the FRAP assay.

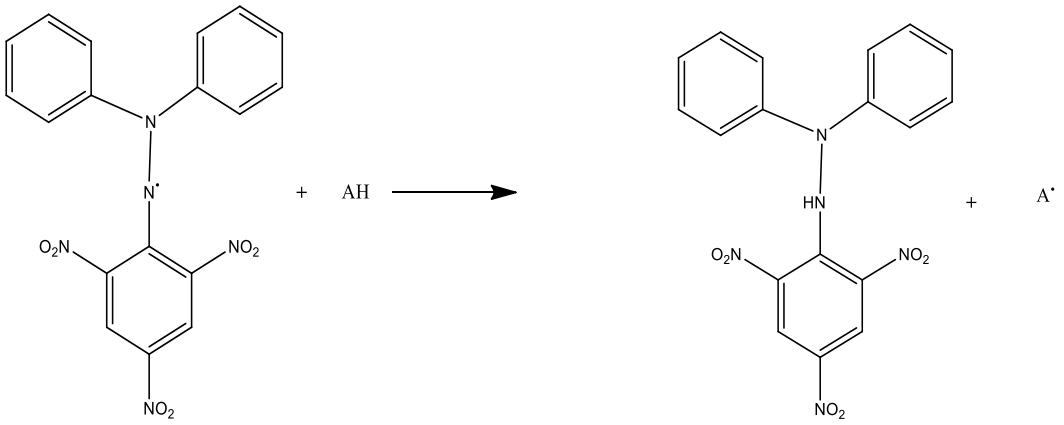


Figure 3.5. Reaction occuring in the DPPH assay (AH is Antioxidant).

This study showed that the FRAP values of the solvent extracts tested were significantly different (p= 0.05) and varied from 3.1±0.1 mmol Fe(II) equivalents/100 g DW (100% methanol extract) to 8.3±0.1 mmol Fe(II) equivalents/100 g DW of the fruit (70% aq. acetone extract) with reference to the standard curve (y=0.6225x, R^2= 0.998) (**Appendix 4**) with the descending order of 70% aq. acetone > 80% aq. methanol > water > 80% aq. ethanol > 100% methanol (**Figure 3.6A**). Antioxidant capacity measured by DPPH values for the different extraction solvents varied from 2.0±0.1 to 5.1±0.1 mmol Trolox equivalents/100 g DW of the fruit with reference to the standard curve (y=0.2635x, R^2=

0.997) (**Appendix 5**) with the order of 70% aq. acetone > 80% aq. methanol > water > 80% aq. ethanol = 100% methanol (**Figure 3.6B**). Interestingly, it is noticed that 70% aq. acetone extract exhibited significantly highest antioxidant capacity at p= 0.05 by both of assays performed in this study.

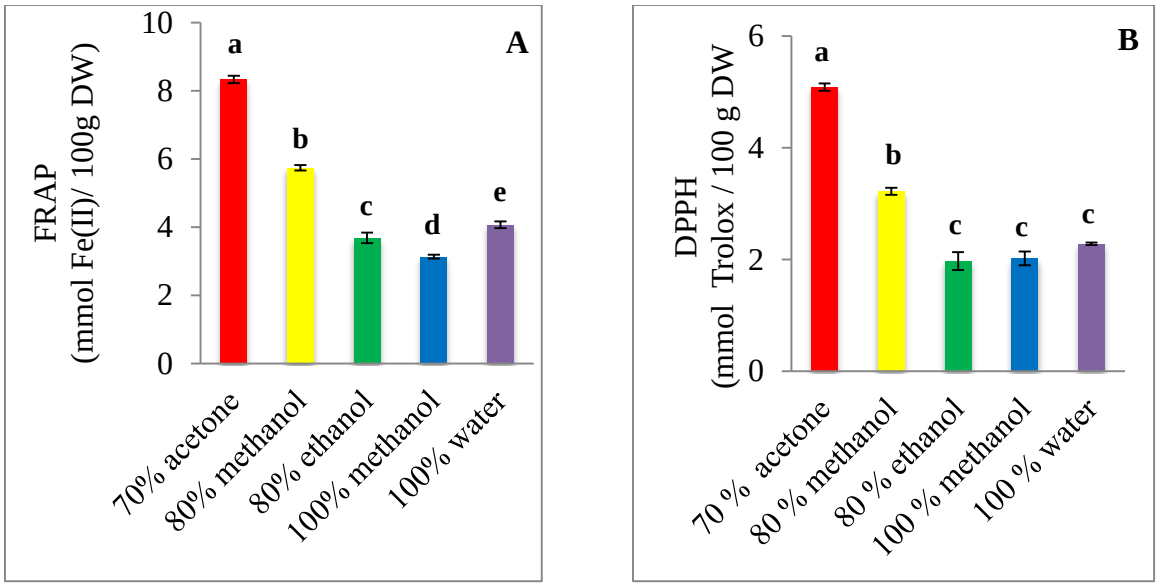


Figure 3.6. Effect of extraction solvent on (A) FRAP values and (B) DPPH values obtained from *A. macrosperma* fruit.

The FRAP values of the extracts from different techniques tested varied from 4.5±0.2 mmol Fe(II) equivalent/100 g DW to 8.3±0.1 mmol Fe(II) equivalents/100 g DW of the fruit with reference to the standard curve (y=0.6225x, R^2= 0.998) (**Appendix 4**) with the descending order of steeping > shaking > sonicating > blending (**Figure 3.7A**).

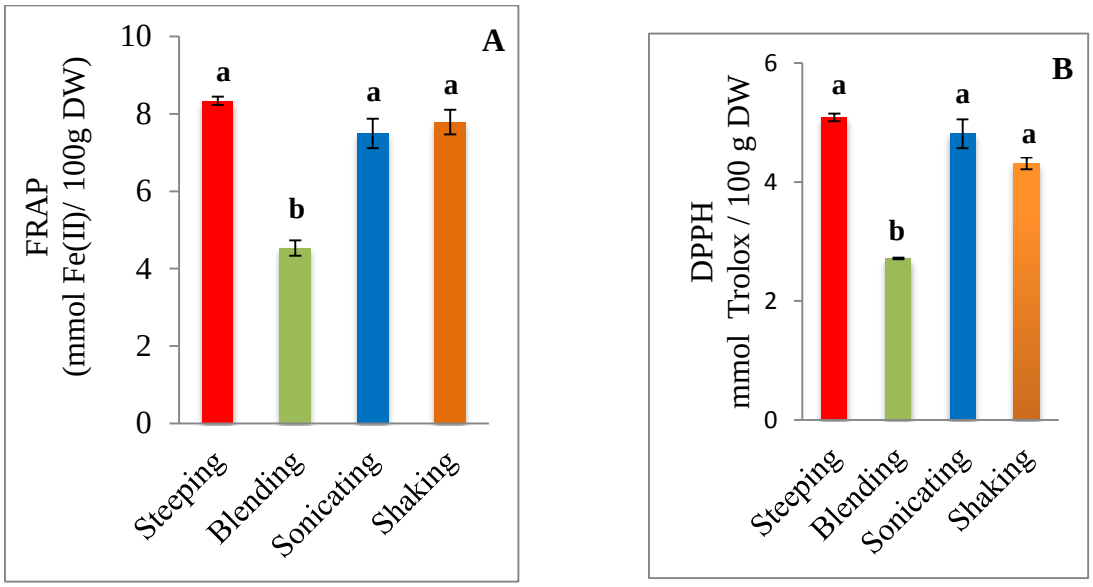


Figure 3.7. Effect of extraction technique on (A) FRAP and (B) DPPH values for *A. macrosperma* fruit.

Antioxidant capacity measured by DPPH values of the extracts from different techniques tested varied from 2.7±0.1 mmol Trolox equivalents/100 g DW to 5.1±0.1 mmol Trolox equivalents/100 g DW of the fruit with reference to the standard curve (y=0.2635x, R^2= 0.997) (**Appendix 5**) with the ranking order of steeping > sonicating > shaking > blending (**Figure 3.7B**). The extraction technique named blending showed the lowest antioxidant activity measured by both of the assays and they were significant different (*p*= 0.05) from all other techniques employed. There was no significantly difference among the techniques used namely, steeping, shaking, and sonicating in terms of antioxidant activity measured. Interestingly, it is noticed that 70% aq. acetone extract by using steeping extraction technique exhibited significantly highest antioxidant capacity at *p*= 0.05, according to the assays performed in this study.

There are a few previous studies reported on the antioxidant capacity of *Actinidia* fruits but only one report on *A. macrosperma* fruit. As mentioned earlier, the antioxidant activity of kiwifruit measured *in vitro* is dependent on several factors; extraction medium,

plant matrix and the laboratory assay employed (Hunter et al., 2011). However, the antioxidant activity previously reported of *A. macrosperma* fruit assessed by DPPH assay was 27.1 mg Ascorbic acid Equivalent (AAE)/100 g fresh weight of the fruit (Latocha et al., 2010). The observed differences in antioxidant activity could be related to the result of different methods of extraction, solvents, analysis and the origin of the kiwifruit used for the analysis.

3.4.1.6. Correlations of phenolic profile with antioxidant capacity

It is important to examine the correlation between phenolic profiles and the antioxidant potential as well as inter-correlated coefficients amongst phenolic profiles in different solvent extracts and techniques to optimize the extraction conditions for phenolics. The correlations between composition and antioxidant capacities amongst the different solvent extracts employed in *A.macrosperma* fruit analysed are shown in **Table 3.3.**

In this study, the results showed that the total phenolic content was not strongly correlated with the total flavonoid (R^2= 0.489) (**Table 3.3**) and flavanol (R^2 = 0.557) (**Table 3.3**) contents of the extracts obtained from the different extraction solvents. Antioxidant capacities by FRAP and DPPH assays from solvent extracts were moderately correlated with total phenolic (R^2= 0.722 and 0.746 respectively) but not well correlated with total flavonoid contents (R^2= 0.456 and 0.548 respectively) (**Table 3.3**).

Table 3.3. Correlation coefficients (R^2) between composition and antioxidant capacities amongst the different solvent extracts employed in *A.macrosperma* fruit.

	TFO	TFA	FRAP	DPPH
TP	0.489	0.557	0.722	0.746
TFO	-	0.495	0.456	0.548
TFA	-	-	0.956	0.935
FRAP	-	-	-	0.982

Total phenolic (TP) content is expressed as mg GAE/100 g DW

Total flavonoid (TFO) content is expressed as mg CAE/100 g DW

Total flavanol (TFA) content is expressed as mg CAE/100 g DW

FRAP is expressed as mmol Fe(II) equivalents/100 g DW

DPPH is expressed as mmol Trolox equivalents/100 g DW

The correlation between phenolic profiles and the antioxidant activity has been widely studied in different foodstuffs such as fruits and vegetables (Jayaprakasha et al., 2001; Klimczak et al., 2007; Kumaran and Karunakaran, 2007**;** Olajire, and Azeez, 2011). Positive and negative correlations were reported in the literature. Therefore, this lack of relationship is in agreement with the former studies reported (Heinonen et al., 1998; Ghaseme et al., 2009; Olajire, and Azeez, 2011). It could be due to the fact that only phenolics/flavonoids with a certain structure and particularly hydroxyl position (3-OH group attached to the 2, 3- double bond and adjacent to the 4-carbonyl in the C ring) in the molecule can act as proton donating and show radical scavenging activity effectively (Ghaseme et al., 2009).

The results obtained in this study support that high total flavanol content increases the antioxidant activity measured by DPPH and FRAP assays applied to solvent extracts and there is a strong linear correlation (R^2) between total flavanol content and antioxidant activity (**Figure 3.8A and Figure 3.8B**). Flavan-3-ols are the most common among the flavanols which belongs to flavonoids class. Catechin, epicatechin, epicatechin gallate, epigallocatechin, epigallocatechingallate, proanthocyanidins, theaflavins, and thearubgins which are well known antioxidants are the most common compounds of this flavanols (Haslam, 1989; Heinonen et al., 1998).

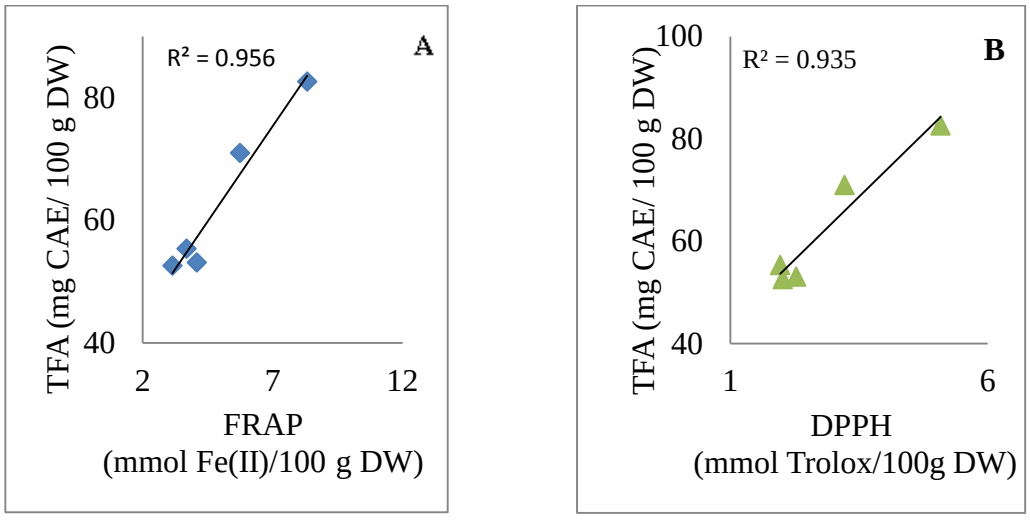


Figure 3.8. Correlations between total flavanol and (A) FRAP value and (B) DPPH value obtained from different extraction solvents employed in *A. macrosperma* fruit.

Similar trend was observed regarding the extraction techniques as well (**Table. 3.4)**. The results showed that the total phenolic content was not strongly correlated with the total flavonoid (R^2= 0.064), flavanol (R^2 = 0.056) contents, FRAP (R^2 = 0.022) and DPPH (R^2 = 0.145) of the extracts obtained from the different extraction techniques (**Table 3.4)**.

Table 3.4. Correlation coefficients (R^2) between composition and antioxidant capacities amongst the extracts obtained from different extraction techniques employed in *A.macrosperma* fruit.

	TFO	TFA	FRAP	DPPH
TP	0.064	0.056	0.022	0.145
TFO	-	0.867	0.945	0.973
TFA	-	-	0.964	0.906
FRAP	-	-	-	0.930

Total phenolic (TP) content is expressed as mg GAE/100 g DW

Total flavonoid (TFO) content is expressed as mg CAE/100 g DW

Total flavanol (TFA) content is expressed as mg CAE/100 g DW

FRAP is expressed as mmol Fe(II) equivalents/100 g DW

DPPH is expressed as mmol Trolox equivalents/100 g DW

This observation could be due to the various non phenolic compounds like proteins, vitamin C, sugars extracted which might interfere with FC reagent which was used to quantify total phenolic content (Singleton and Rossi, 1965). There is a strong linear correlation (R^2) between total flavanol content and antioxidant activity was obsrved (**Figure 3.9A** and **Figure 3.9B**).

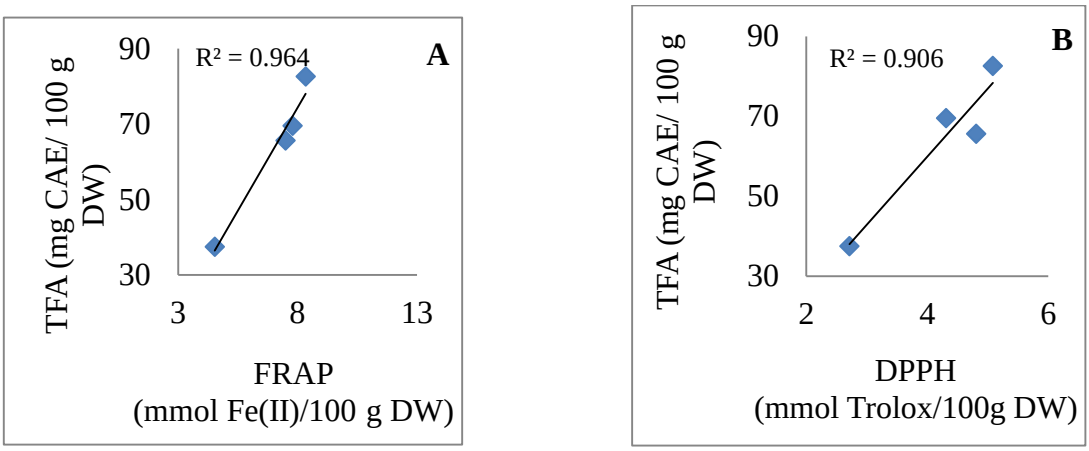


Figure 3.9. Correlations between total flavanol and (A) FRAP value and (B) DPPH value obtained from different extraction techniques employed in *A. macrosperma* fruit.

The antioxidant activities obtained from solvent extracts and extraction techniques employed by using FRAP and DPPH assays were well correlated with R^2= 0.982 and R^2= 0.930 as shown in **Figure 3.10A** and **Figure 3.10B** which implied that antioxidants in these fruit extracts were capable of scavenging free radicals (DPPH) and reducing oxidants.

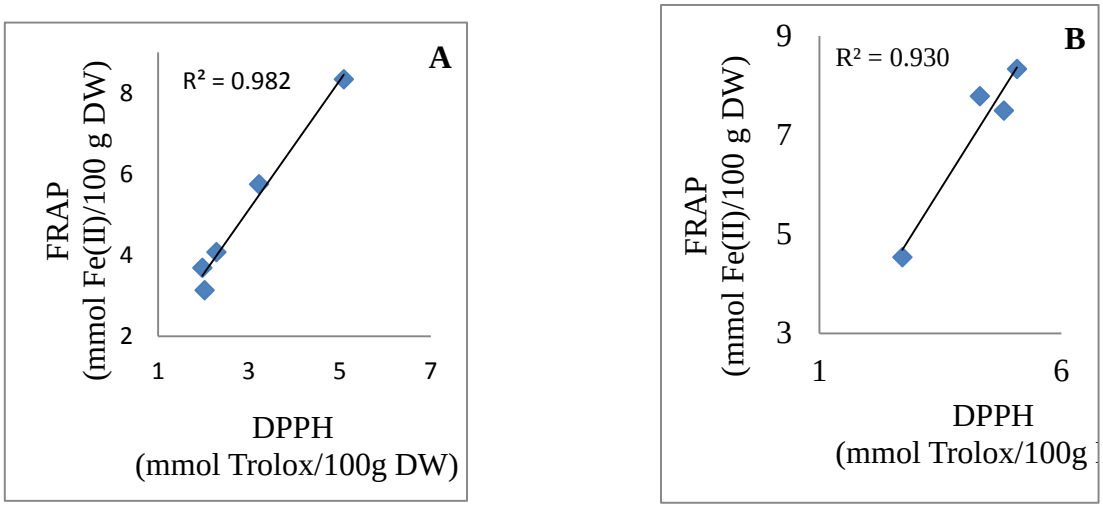


Figure 3.10. Correlation between the antioxidant activities measured by DPPH and FRAP assays in (A) different extraction solvents (B) extraction techniques obtained from *A. macrosperma* fruit.

3.4.1.7. Liquid chromatography coupled to diode array detector (LC-DAD) and mass spectrometry (LC-ESI-MS/MS) analysis of extracts obtained from *A. macrosperma* fruit

All extracts were qualitatively (on LC-DAD) and quantitatively (on LC-ESI-MS/MS) analyzed for most abundant phenolic compounds common to many fruits. The five different solvent extracts showed approximately the similar phenolic profile but with differences in the proportions of various compounds extracted (**Figure 3.11, Figure 3.12** and **Figure 3.13**). Liquid chromatography (LC) coupled to mass spectrometry (MS) has been demonstrated to be a powerful tool for the identification of natural products in crude plant extracts owing to their soft ionization which favours the analysis of flavonoids. The LC-ESI-MS/MS analysis further confirmed that all extracts obtained from *A. macrosperma* fruit are rich in mainly flavonoids (**Table 3.5** and **Table 3.6**). Flavonoids: quercetin, and its glycosides namely, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-arabinoglucoside and phenolic acids namely, chlorogenic acid and caffeic acid were identified in all extracts obtained from *A. macrosperma* fruit (**Table 3.5** and **Table 3.6**). Recent studies have reported that the fruit of *A. macrosperma* contains lutein, β-carotenes, zeaxanthin, violaxanthin, chlorophyll a and b, catechin, epicatechin, quercetin, tannic acid, gentisic acid, hydroxy benzoic acid, chlorogenic acid, *p*-coumaric acid and caffeic acid (Latocha et al., 2010). Quercetin-3-*O*-galactoside was identified as the most abundant among other phenolic compounds tested in all extracts in this study (**Table 3.5** and **Table 3.6**).

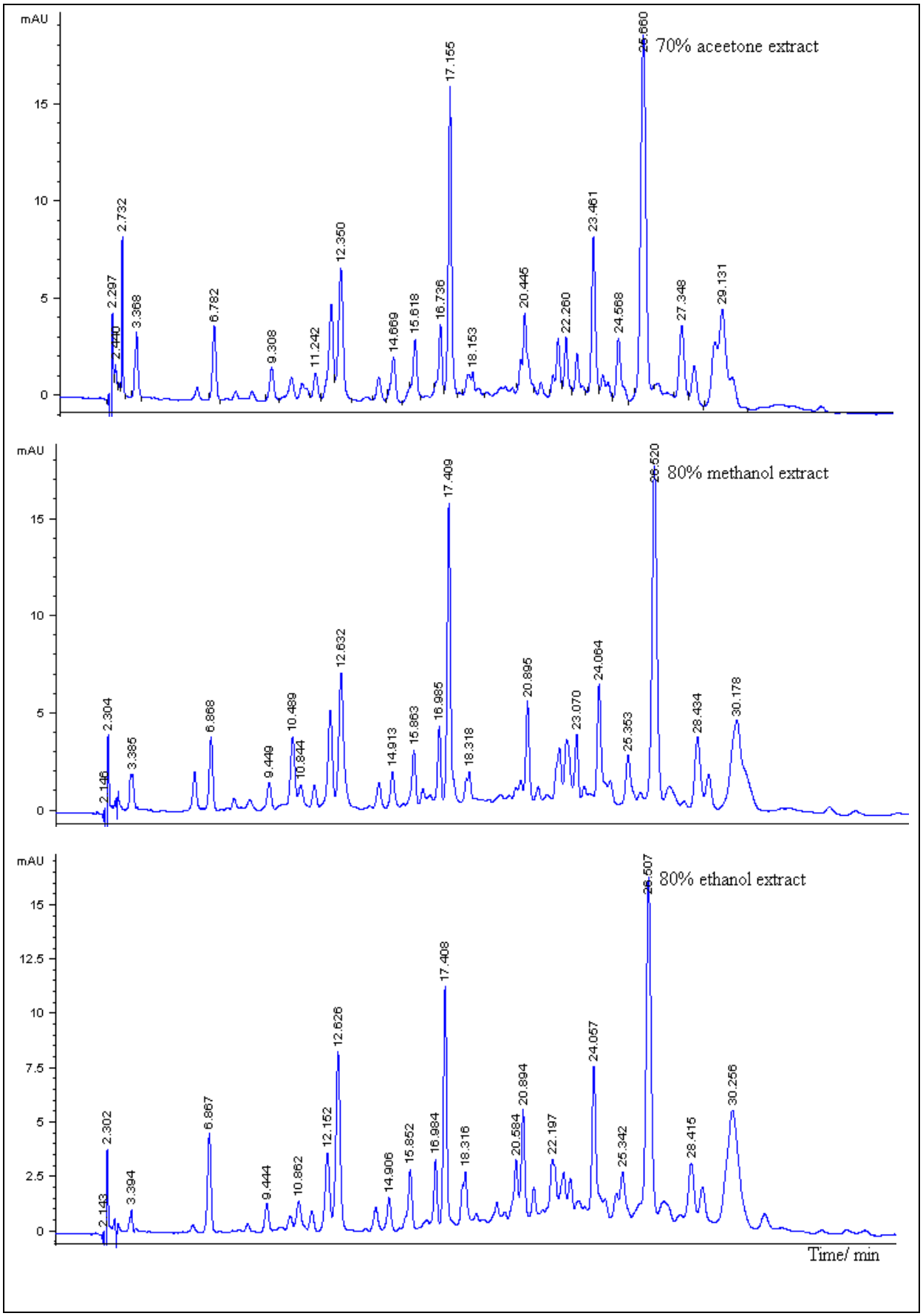


Figure 3.11. HPLC-DAD chromatograms of flavonoid patterns of 70% acetone, 80% methanol and 80% ethanol extracts (by steeping technique) at 360 nm.

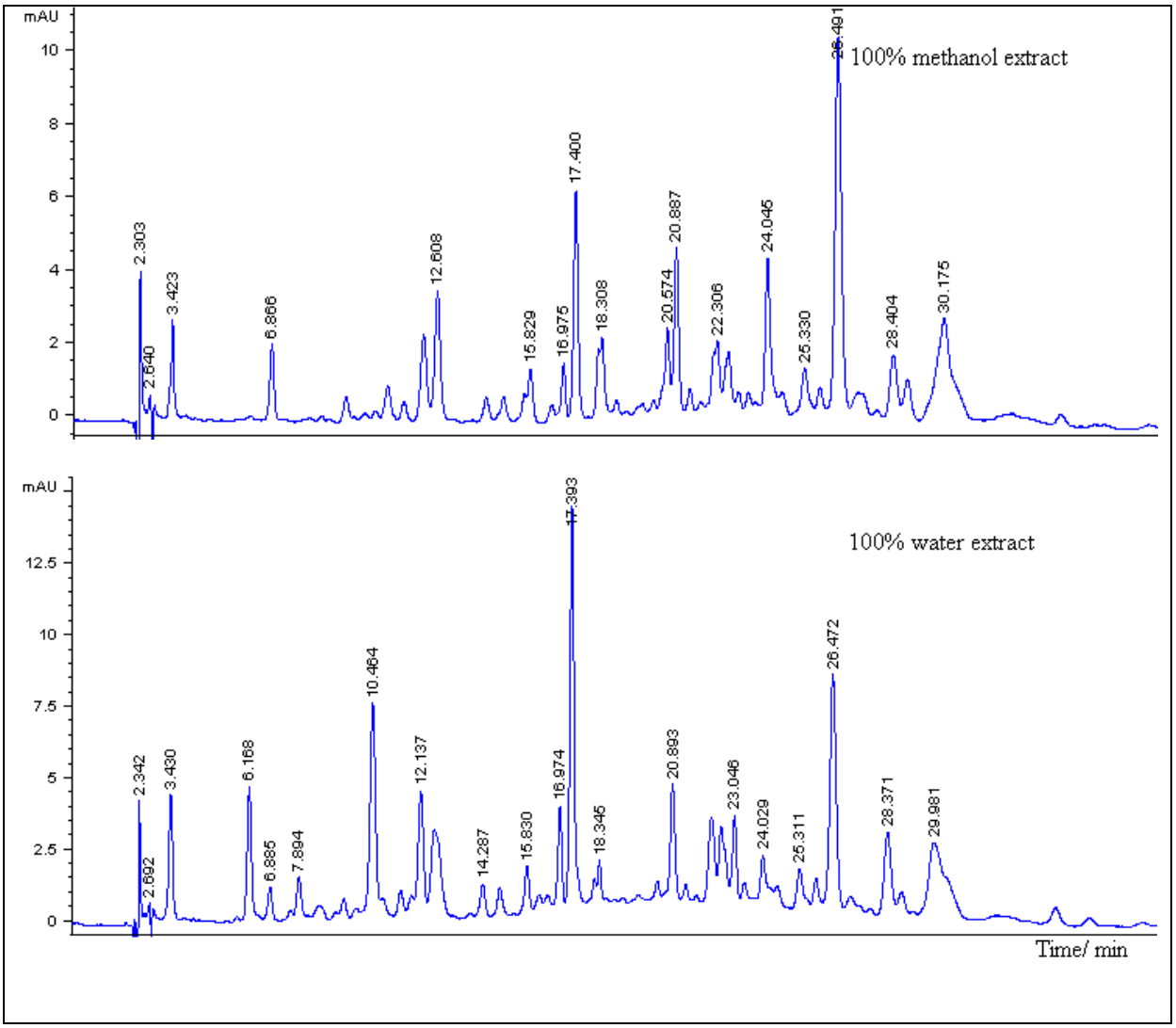


Figure 3.12. HPLC-DAD chromatograms of flavonoid patterns of 100% methanol and 100% water extracts (by steeping technique) at 360 nm.

Although 70% aq. acetone showed the presence of less quantities of flavonoid glycosides compared to that of 80% aq. methanol on LC-MS/MS analysis, it showed high antioxidant activity and total flavonoid content among others tested. It may be because either presence of other flavonoids which could be highly potential antioxidants which were not detected in this LC-MS/MS analysis due to the lack of flavonoid standards to identify or presence of higher quantities of quercetin, catechin, epicatechin gallate which are considered to be strong antioxidants due to its ability to scavenge free radicals and bind transition metal ions.

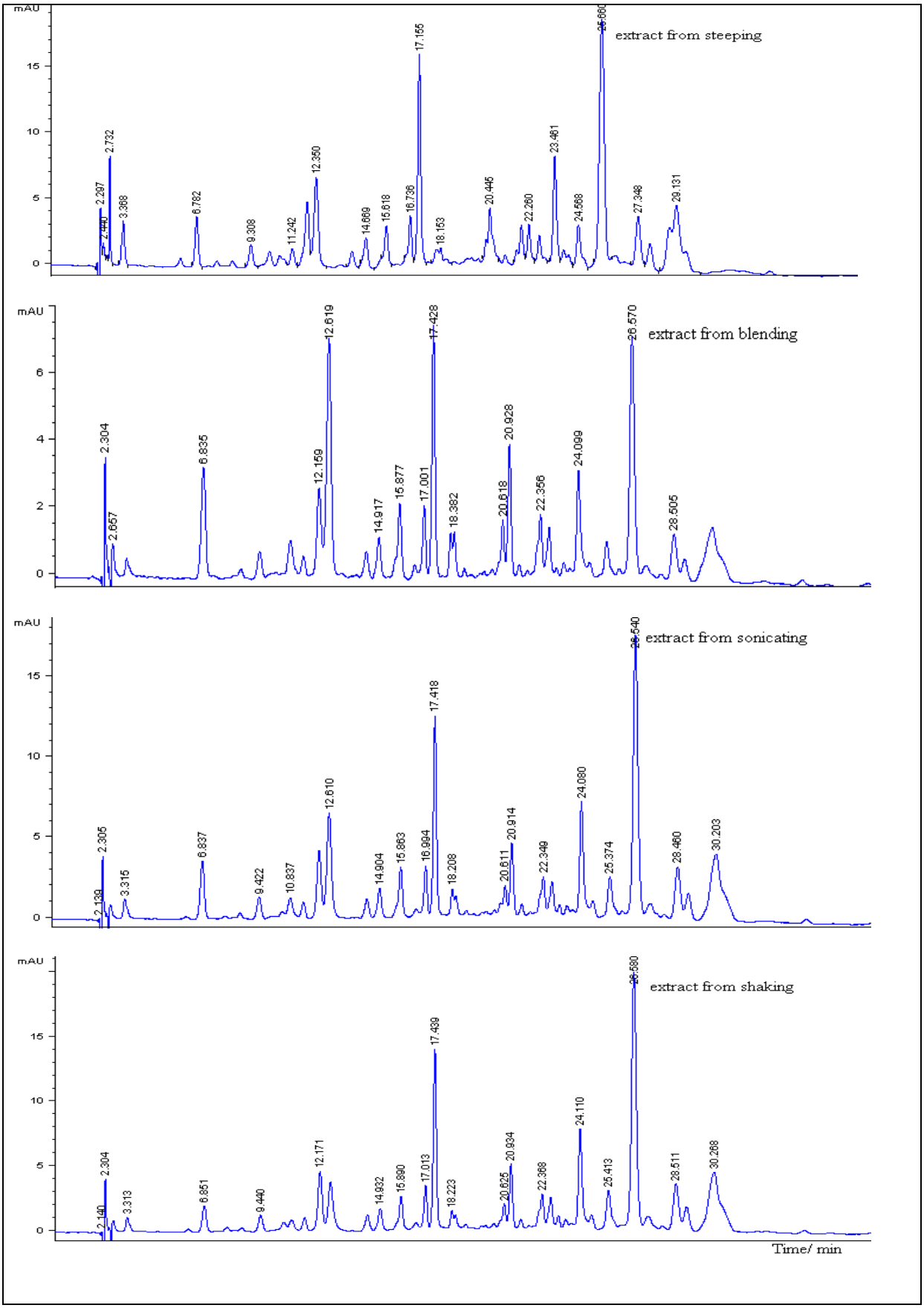


Figure 3.13. HPLC-DAD chromatograms of flavonoid patterns obtained for various techniques (steeping, blending, sonicating and shaking) into 70% acetone at 360 nm.

Table 3.5. HPLC-MS/MS analysis data of phenolics in different extraction solvents from *A. macorsperma* fruit.

Group/ Name of the Flavonoid	Concentration of phenolics (µg/g DW)				
	70% acetone	80% methanol	80% ethanol	100% methanol	100% water
Flavonol					
Quercetin-3-*O*-galactoside	470.90	851.31	456.63	525.38	282.58
Quercetin-3-*O*-glucoside	4.16	2.64	3.21	2.68	1.31
Quercetin arabinoglucoside	2.53	1.43	3.08	2.26	1.45
Quercetin-3-*O*- rhamnoside	2.99	6.89	1.72	1.64	nd*
Quercetin	2.56	1.46	0.74	0.52	0.26
Quercetin -3-*O*-rutinoside	1.96	nd	0.84	2.33	0.24
Flavanol					
Epigallocatechin	1.55	nd	1.60	2.17	2.66
Catechin	54.31	19.02	34.63	75.64	7.0
Epicatechin	0.91	1.01	0.30	nd	0.50
Epigallocatechingallate	0.75	1.85	0.71	1.71	0.77
Dihydrochalcones					
Phloridzin	3.12	4.69	0.61	3.58	1.80
Phloritin	0.14	0.11	0.38	0.30	0.26
Phenolic acids					
Chlorogenic acid	1.97	5.24	1.71	4.74	2.81
Caffeic acid	1.65	1.39	0.32	1.28	0.27
Ferulic acid	4.70	1.42	0.78	4.52	nd
Isoferulic acid	32.71	45.48	nd	37.96	78.99

DW: dry weight of the fruit, nd*: not detected

Table. 3.6. HPLC-MS/MS analysis data of phenolics in different extraction techniques from *A. macorsperma* fruit.

Group/ Name of the Flavonoid	Concentration of phenolics (µg/g DW)			
	steeping	sonicating	shaking	blending
Flavonol				
Quercetin-3-*0*-galactoside	470.90	630.09	1051.31	507.38
Quercetin-3-*0*-glucoside	4.16	3.07	4.80	1.54
Quercetin arabinoglucoside	2.53	0.77	1.81	0.96
Quercetin-3-*0*- rhamnoside	2.99	0.62	3.82	0.69
Quercetin	2.56	1.17	0.34	0.34
Quercetin -3-*0*-rutinoside	1.96	nd*	5.33	1.46
Flavanol				
Epigallocatechin	1.55	1.35	1.92	0.83
Catechin	54.31	68.34	79.86	35.58
Epicatechin	0.91	1.89	1.80	1.47
Epigallocatechingallate	0.75	nd	1.10	1.07
Dihydrochalcones				
Phloridzin	3.12	1.83	0.71	0.28
Phloritin	0.14	0.09	0.16	0.10
Phenolic acids				
Chlorogenic acid	1.97	1.47	2.05	3.22
Caffeic acid	1.65	1.04	0.41	0.58
Ferulic acid	4.70	2.86	6.04	nd
Isoferulic acid	32.71	nd	14.73	47.56

DW: dry weight of the fruit, nd*: not detected

3.4.2. Phenolic profiles and antioxidant activity of 70% aq. acetone extract by steeping technique employed in *A. macrosperma* compared to those of commercial kiwifruit varieties

3.4.2.1. Extract yields

The yields of the extracts obtained from commercial kiwifruit varieties and *A. macrosperma* fruit under the same conditions (steeping in 70% aq. acetone solvent as desccribed in Section 3.3.2) ranged from 16.0±1.1 to 58.4±1.4 g/100 g DW of the fruit (**Table 3.7**).

Table 3.7. Effect of kiwifruit cultivar on TP, TFO, and TFA values.

Sample type	Yield[a] (%)	TP[b]	TFO[c]	TFA[d]
A. deliciosa (green)	16.0±1.1^{p}	112.0±0.5^{p}	12.9±0.7^{p}	1.2±0.1^{p}
A. chinensis (gold)	20.8±0.9^{q}	243.2±8.1^{q}	31.2±0.8^{q}	8.1±0.4^{q}
A. deliciosa (sweetgreen)	58.4±1.4^{r}	561.5±5.9^{r}	118.7±3.7^{r}	40.4±1.4^{r}
A. chinensis (sungold)	57.7±1.8^{r}	616.5±8.3^{s}	116.8±6.8^{r}	51.8±0.2^{s}
A. macrosperma	42.8±0.8^{s}	823.1±14.4^{t}	170.9±1.9^{s}	82.6±0.6^{t}

[a]Extraction yield is expressed as percentage g/100 g DW

[b]Total phenolic content is expressed as mg GAE/100 g DW

[c]Total flavonoid content is expressed as mg CAE/100 g DW

[d]Total flavanol content is expressed as mg CAE/100 g DW

Results are expressed as mean±standard error. Means followed by the same letter in a column are not significantly different at p= 0.05.

Sweetgreen kiwifruit had the highest in extraction percentage yield (58.4±1.4 g/100 g DW) followed by sungold (57.7±1.8 g/100 g DW), *A. macrosperma* (42.8±0.8 g/100 g DW), gold (20.8±0.9 g/100 g DW) and green (16.0±1.1 g/100 g DW). The yields from sweetgreen and sungold were similar and significantly higher than from the others (p= 0.05) according to the Tukey test. Variation in the yields of the extracts obtained in this study could be resulted due to the polarity of various compounds present in different kiwifruit cultivars tested (**Figure 3.14**) and these observations are in agreement with the former studies reported in the literature on other plants such as potato peel, sugar beet pulp, sesame cake, mashua and pistachio hull (Pinelo et al., 2004; Goli et al., 2005; Chirinos et al., 2007; Mohdaly et al., 2010).

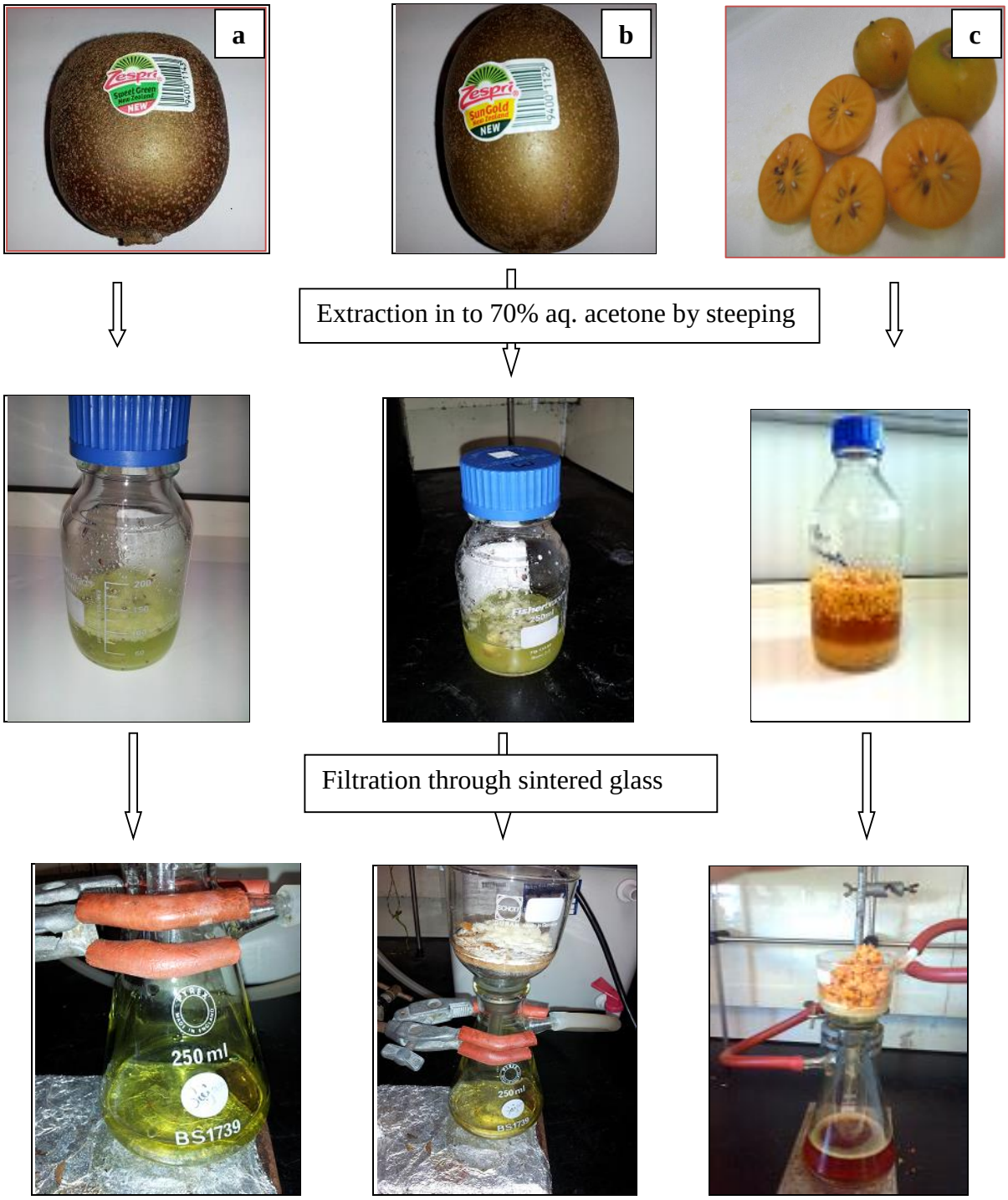


Figure 3.14. Various colours of crude extracts due to the different compounds extracted from (a) *A. deliciosa cv* Sweetgreen, (b) *A. chinensis cv* Sungold and (c) *A. macrosperma* kiwifruits.

3.4.2.2. The total phenolic contents

The total phenolic contents of different kiwifruit varieties were measured by the Folin-Ciocalteu method as described in Section 3.3.3 and the results were reported as gallic acid equivalents (mg GAE/100g of DW of the fruit) (**Table 3.7**) with reference to the standard curve (y=0.001x, R^2=0.996) given in **Appendix 1**. The total content of phenolics in the extracts was dependent on the variety of kiwifruit as shown in **Table 3.7.** This study showed that total phenolic contents ranged from 112.0±0.5 to 823.1±14.4 mg GAE/100 g DW of the fruit with a descending order of *A.macrosperma* > sungold > sweetgreen > gold > green. Interestingly, it is noticed that the *A.macrosperma* fruit extract had the highest amount of total phenols (823.1±14.4 mg GAE/100 g of DW of the fruit) which is significantly higher (p= 0.05) than from all other varieties tested. The TP of the extracts obtained from *A. deliciosa cv* Hayward (Green kiwifruit) and *A. chinensis cv* Hort 16A (Gold kiwifruit) (112.0 ±0.5 and 243.2±8.1 mg GAE/100 g of DW of the fruit respectively) were significantly lower than the new commercial kiwifruits namely, sweetgreen (561.5±5.9 mg GAE/100 g of DW of the fruit) and sungold (616.5±8.3 mg GAE/100 g of DW of the fruit).

Total phenolic contents of different kiwifruit cultivars previously reported by Latocha et al., (2010) using the Folin-Ciocalteu method was cultivar dependent. Their results showed that TP of *A. deliciosa cv* Hayward and *A. macrosperma* fruits were 128.5 mg GAE/100 g fresh weight and 164.6 mg GAE/100 g fresh weight of the fruit, respectively (Latocha et al., 2010). Research studies carried out on determination of total phenols extracted into ethanol:acetone (70:30) of different kiwifruits by Du et al., (2009) reported that the TP values varied from 41.7 to 710 mg/100 g fresh weight. Bursal and Gulcin, (2011) reported that the total phenolic content determined in water extract from *A. deliciosa* (Green kiwifruit) in 1 mg of lyophilized kiwifruit extract was 16.67±2.83 µg GAE. The total

phenolic content determined for *A. chinensis* kiwifruit under slightly different conditions of the Folin-Ciocalteu method showed 9.3±0.4 µg GAE/mL (Bekhradnia et al., 2011). The total phenolic contents evaluated for *A. chinensis cv* Gold, *A. deliciosa cv* Hayward and *A. macrosperma* fruits in this study were different from the reported values. The observed differences in total phenolic content could be related to the result of different methods of extraction, solvents, analysis and the origin of the fruit used.

3.4.2.3. Total flavonoid and total flavanol content

The colorimetric method based on aluminium chloride ($AlCl_3$) was performed as described in Section 3.3.4 to determine the total flavonoid content of the different kiwifruit varieties. The total flavonoid contents of the extracts tested are shown in **Table 3.7**. Those values varied from 12.9±0.7 to 170.9±1.9 mg catechin equivalents (CAE)/100 g of DW of the fruit (**Table 3.7**)**,** with reference to a standard curve (y=0.0018x, R^2= 0.998) (**Appendix 2**). Similarly to the total phenolic content measured by the Folin-Ciocalteu method, *A. macrosperma* fruit extract had the highest total flavonoid content (170.9±1.9 mg CAE/100 g of DW of the fruit) which was significantly different (p= 0.05) from the total flavonoid content (TFO) values obtained from all the commercial cultivars tested. The new commercial kiwifruits namely, sweetgreen (118.7±3.7 mg CAE/100 g of DW of the fruit) and sungold (116.8±6.8 mg CAE/100 g of DW of the fruit) had the second and third highest amount in the total flavonoid content determined. Extracts obtained from green and gold kiwifruit exhibited the lowest total flavonoid contents (12.9±0.7 and 31.2±0.8 mg CAE/100 g of DW of the fruit respectively).

By comparing with the literature, the flavonoid content of *A. deliciosa cv* Hayward (Green kiwifruit) was reported as 6.69±0.08 mg rutin/100 g fresh weight of the fruit among

different kiwifruit cultivars tested using the same method (Du et al., 2009). Bursal and Gulcin, (2011) reported that the total flavonoid content detected in water extract from *A. deliciosa* (Green kiwifruit) in 1 mg of lyophilized kiwifruit extract was 12.95±0.52 µg quercetin equivalent (QE). Research studies carried out by Bekhradnia et al., (2011) reported a flavonoid content of *A. chinensis* kiwifruit of 7.9±0.63 µg QE/mL. The total flavonoid contents evaluated for *A. chinensis cv* Gold and *A. deliciosa cv* Hayward fruits in this study are different from the reported values, which may again be related to the different extraction conditions, analysis and the origin of the fruit used.

The total flavanol contents of the different kiwifruit varieties obtained as described in Section 3.3.5 with reference to a standard curve (y=0.027x, R^2= 0.995) (**Appendix 3**) are reported in **Table 3.7**. Those values varied from 1.2±0.1 to 82.6±0.6 mg CAE/100 g of DW of the fruit with the ranking order of *A.macrosperma* > sungold > sweetgreen > gold > green. It is interesting to notice that similarly to the total phenolic and flavonoid contents, *A.macrosperma* kiwifruit extract had the highest TFA (82.6±0.6 mg CAE/100 g of DW of the fruit) which was significantly different (p= 0.05) from the total flavanol content (TFA) values obtained from all commercial cultivars tested. The new commercial kiwifruits, sungold (51.8±0.2 mg CAE/100 g of DW of the fruit) and sweetgreen (40.4±1.4 mg CAE/100 g of DW of the fruit) were second and third to *A.macrosperma* in terms of TFA values. Extracts obtained from green and gold kiwifruit (8.1±0.4 and 1.2±0.8 mg CAE/100 g of DW of the fruit, respectively) exhibited the lowest TFA contents.

Only a few studies on the total flavanol content of kiwifruits are reported in the literature. The total flavanol content value reported by Du et al., (2009) for *A. deliciosa cv* Hayward (Green kiwifruit) originated from China was 4.34±0.25 mg CAE/100 g fresh weight of the fruit. TFA value reported by Du et al., (2009) for *A. deliciosa cv* Hayward

(Green kiwifruit) was slightly different from the TFA value found in this study. This could be due to the different origin of the fruit, method and solvent used for the extraction as well as analysis. Therefore, our experimental observations related to total phenolic, total flavonoid and flavanol contents of different kiwifruit cultivars are also in agreement with other reports in the literature which showed that total phenolic, flavonoid, and flavanol contents are cultivar-dependent parameters (Du et al., 2009).

3.4.2.4. Antioxidant capacity

Antioxidant capacities of all *Actinidia* extracts were evaluated with the most commonly used *in vitro* assays namely the FRAP and DPPH assays as described in Section 3.3.6 and 3.3.7. The FRAP values varied from 1.3±0.1 to 8.3±0.1 mmol Fe(II)/100 g DW of the fruit (**Table 3.8**) with the order of *A.macrosperma* > sungold > sweetgreen > gold > green. Antioxidant capacity measured by DPPH values varied from 0.6±0.1 to 5.1±0.1 mmol Trolox equivalents/100 g DW of the fruit. In the FRAP and DPPH assays, the antioxidant capacity of *A. deliciosa cv* Sweetgreen (5.6±0.2 mmol Fe(II) equivalents/100 g DW and 5.0±0.1 mmol Trolox equivalent/100 g DW of the fruit respectively), *A. chinensis cv* Sungold (6.7±0.4 mmol Fe (II) equivalents/100 g DW and 4.4±0.3 mmol Trolox equivalents/100 g DW of the fruit) was stronger than those of other commercial varieties namely, *A. chinensis cv* Hort 16A (3.0±0.1 mmol Fe (II) equivalents/100 g DW and 2.0±0.1 mmol Trolox equivalents/100 g DW of the fruit respectively) and *A. deliciosa cv* Hayward (green) (1.3±0.1 mmol Fe (II) equivalents/100 g DW and 0.6±0.1 mmol Trolox equivalents/100 g DW respectively). It is interesting to observe that *A.macrosperma* had the highest FRAP and DPPH values which were significantly different (p= 0.05) among the five different cultivars compared, (**Table 3.8**).

Table 3.8. Effect of kiwifruit cultivar on FRAP, DPPH and EC_{50} values.

Sample type	FRAP[a]	DPPH[b]	EC_{50}[c]
A.deliciosa (green)	1.3±0.1[p]	0.6±0.1[p]	40.96
A.chinensis (gold)	3.0±0.1[q]	2.0±0.1[q]	17.48
A.deliciosa (sweetgreen)	5.6±0.2[r]	5.0±0.1[r]	14.82
A.chinensis (sungold)	6.7±0.4[s]	4.4±0.3[s]	14.75
A.macrosperma	8.3±0.1[t]	5.1±0.1[t]	12.98

[a]Antioxidant activity assessed by FRAP is expressed as mmol Fe(II) equivalents/100 g DW

[b]Antioxidant activity assessed by DPPH is expressed as mmol Trolox equivalents/100 g DW

[c]Radical scavenging activity (EC_{50}) by DPPH assay is expressed as mg/mL DW

Results are expressed as mean±standard error. Means followed by the same letter in a column are not significantly different at p= 0.05.

Antioxidant activity has been expressed in various ways including the percentage of the reagent used in reference to a common standard, such as Trolox (Du et al. 2009), or the oxidation incubation rate (Wang et al. 1996). Therefore, the effective concentration of scavenging anti-radicals (EC_{50}) was also determined with the DPPH assay for the different kiwifruit varieties. The DPPH radical scavenging capacity was dose-dependent in the concentration range used in this study (1-5 mg/mL^{-1}) as presented in the **Figure 3.15**. The EC_{50} values for *A.macrosperma*, *A. chinensis cv* Sungold, *A. deliciosa cv* Sweetgreen, *A. chinensis cv* Hort 16A and *A. deliciosa cv* Hayward were 12.98, 14.75, 14.82, 17.48 and 40.96 mg/mL respectively.

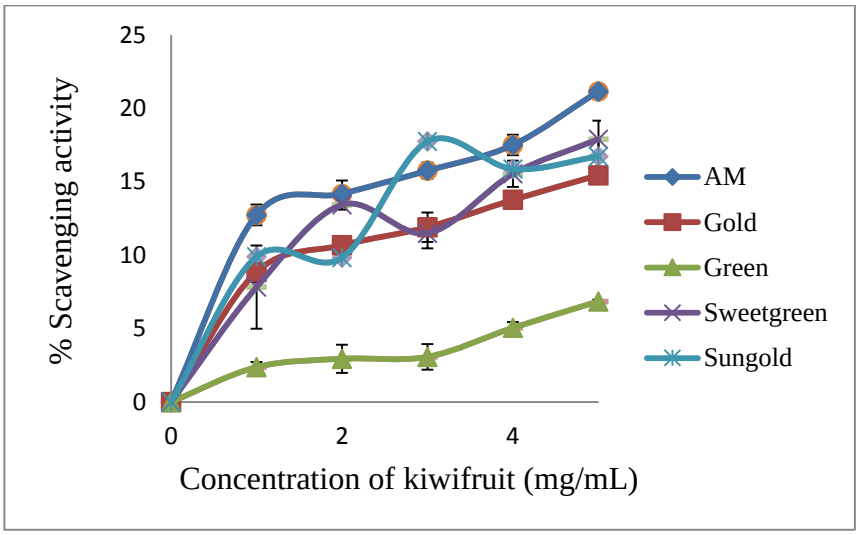


Figure 3.15. Antiradical-scavenging activity (%) of 70% aq. acetone extracts obtained from five different kiwifruit varieties assayed by the DPPH.

There are a few previous studies on the antioxidant capacity of kiwifruits in the literature. The antioxidant potential of commonly consumed fruits has been rated in the order of plum > kiwi > apple > pear and the kiwifruit have demonstrated significant antioxidant capacity *in vitro* and in *vivo* (Wang et al., 1996). The chemical antioxidant activity of kiwifruit measured *in vitro* is dependent on several factors such as extraction medium, plant matrix and the laboratory assay employed. The antioxidant activity of different kiwifruit cultivars previously reported in the literature using the DPPH assay was cultivar dependent (Latocha et al., 2010). The DPPH values of *A. deliciosa cv* Hayward and *A. macrosperma* fruits were 116.6 mg ascorbic acid equivalents (AAE)/100 g fresh weight and 27.1 mg AAE/100 g fresh weight of the fruit respectively (Latocha et al., 2010). The results for the antioxidant activity of ethanol:acetone (70:30) extracts from different kiwifruit cultivars reported by Du and co-workers (2009), were cultivar and assay dependent (Du et al., 2009). Bursal and Gulcin, (2011) showed that the antioxidant activity detected in water extract from *A. deliciosa* (Green kiwifruit) was dependent on the laboratory assay used (Hunter et al., 2011). The reported antioxidant capacities of 'Hayward' and 'Hort 16A' kiwifruit ranged

from 6.0 to 9.2 and 12.1 µmol Trolox equivalent (TE)/g fresh weight (FW), when assayed by the ORAC method (Hunter et al., 2011). In a study of six *Actinidia* species, the higher levels of both vitamin C and phenolic compounds found in *A. kolomikta* cv 'Dr Szymanowski' compared with those in the remaining examined species showed high antioxidant activity of these species, as measured by the DPPH assay (Latocha et al., 2010). The observed differences in antioxidant activity could be related to the result of different methods of extraction, solvents, analysis and the origin of the kiwifruit used for the analysis.

3.4.2.5. Correlation between antioxidant capacity and phenolic composition

To explore the influence of the phytochemical constituents among TP, TFO, and TFA as well as on antioxidant capacity in different kiwifruit varieties, correlations were analyzed in the present study (**Table 3. 9**). The total phenol, flavonoid, flavanol contents and antioxidant capacity of the different kiwifruit varieties were strongly correlated. The total flavonoid content of five different varieties of kiwifruits was in good correlation with the total phenolic contents of those extracts (R^2= 0.977) (**Table 3.9**) which suggested that TFO was the main component of the total phenolic content of different kiwifruit varieties tested. TFA was well correlated with TP (R^2=0.973) and TFO (R^2=0.942) (**Table 3.9**). Antioxidant activities assayed with FRAP and DPPH are strongly correlated with TP (R^2= 0.992, 0.927), TFO (R^2= 0.974, 0.956) and TFA (R^2= 0.960, 0.834) (**Table 3.9**) respectively.

Table 3.9. Correlation coefficients (R^2) between composition and antioxidant capacities amongst the different kiwifruit varieties.

	TFO	TFA	FRAP	DPPH
TP	0.977	0.973	0.992	0.927
TFO	-	0.942	0.974	0.956
TFA	-	-	0.960	0.834
FRAP	-	-	-	0.942

Total phenolic (TP) content is expressed as mg GAE/100 g DW

Total flavonoid (TFO) content is expressed as mg CAE/100 g DW

Total flavanol (TFA) content is expressed as mg CAE/100 g DW

FRAP is expressed as mmol Fe(II) equivalents/100 g DW

DPPH is expressed as mmol Trolox equivalents/100 g DW

Therefore, our research study reveals that high phenolic, flavonoid and flavanol contents of different kiwifruit varieties tested are important factors in determining the antioxidant activities of the kiwifruits studied. This observation is in good agreement with the data generated by Park et al., (2011) on comparison of bioactive compounds and antioxidants in different kiwifruit cultivars grown in South Korea. Well correlated relationships between phenolic composition and antioxidant activity of different kiwifruit cultivars were also identified by Du et al., (2009) and other studies reported in the literature (Nickavar et al., 2007; Olajire and Azeez, 2011**;** Tian et al., 2013). The antioxidant activities obtained from the FRAP and those obtained from the DPPH assay were well correlated (R^2= 0.942) as shown in **Table 3.9** which implies that antioxidants in these different kiwifruit extracts were capable of scavenging free radicals (DPPH) and reducing oxidants (ferric ions). It has also been reported that phenolic acids and flavonoid compounds are the main

phytochemicals responsible for the antioxidant capacity of fruits and radical scavenging capacity might be mostly related to the concentration of phenolic hydroxyl groups, the molecular structure, the availability of phenolic hydrogens and the possibility for stabilization of the resulting phenoxyl radicals formed by hydrogen donation (Wang et al., 1996).

3.4.2.6. High performance liquid chromatography coupled to diode array spectrometry (HPLC-DAD) fingerprint analysis of kiwifruit varieties

Since there are a large number of different types of antioxidants and phenolic compounds that might contribute to the total antioxidant activity, extracts from all kiwifruit varieties with concentration of 50 mg/mL (DW/volume) are analyzed using HPLC-DAD and the chromatograms of all defatted crude extracts were presented in **Figures 3.16-3.20**.

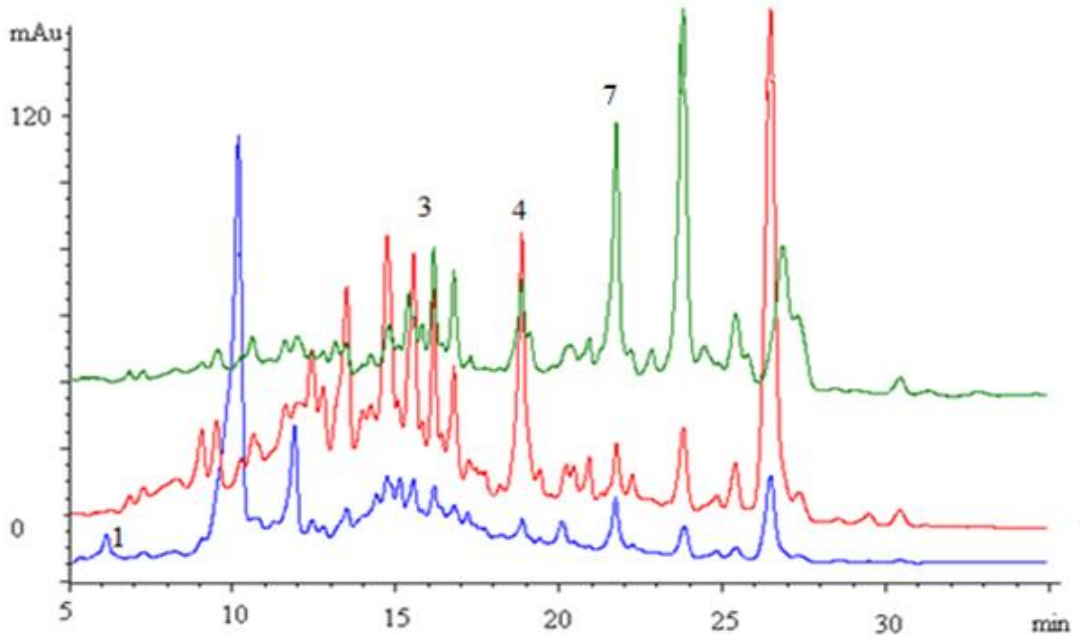


Figure 3.16. HPLC-DAD chromatograms of defatted crude extract of *A. macrosperma* fruit recorded at 360 (—), 320 (—) and 280 nm (—).

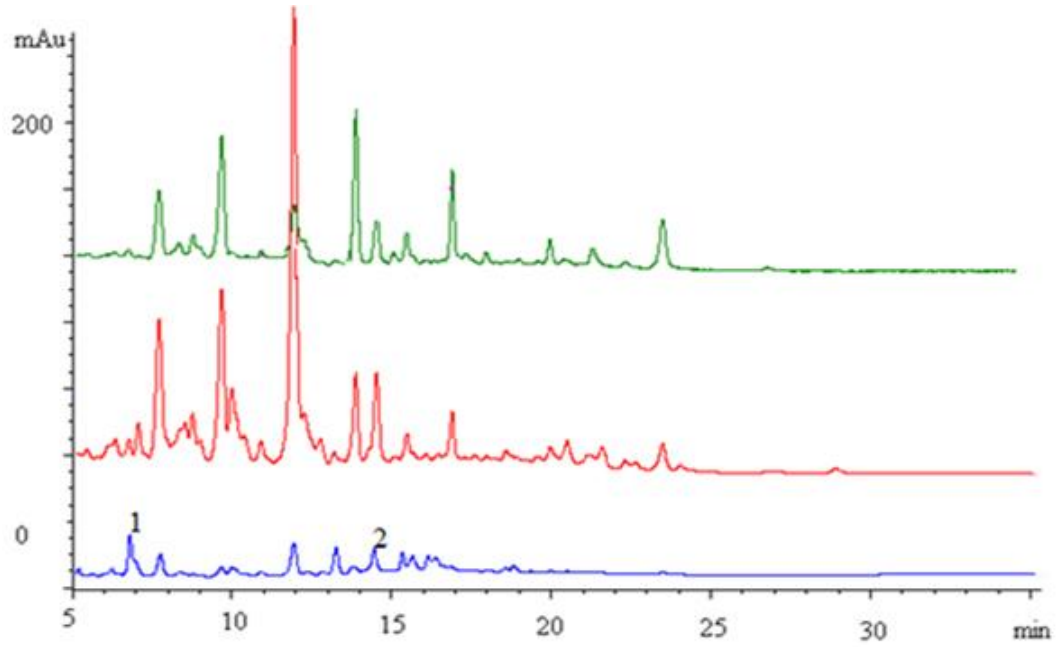


Figure 3.17. HPLC-DAD chromatograms of defatted crude extract of *A. deliciosa cv* Sweet green kiwifruit recorded at 360 (—), 320 (—) and 280 nm (—).

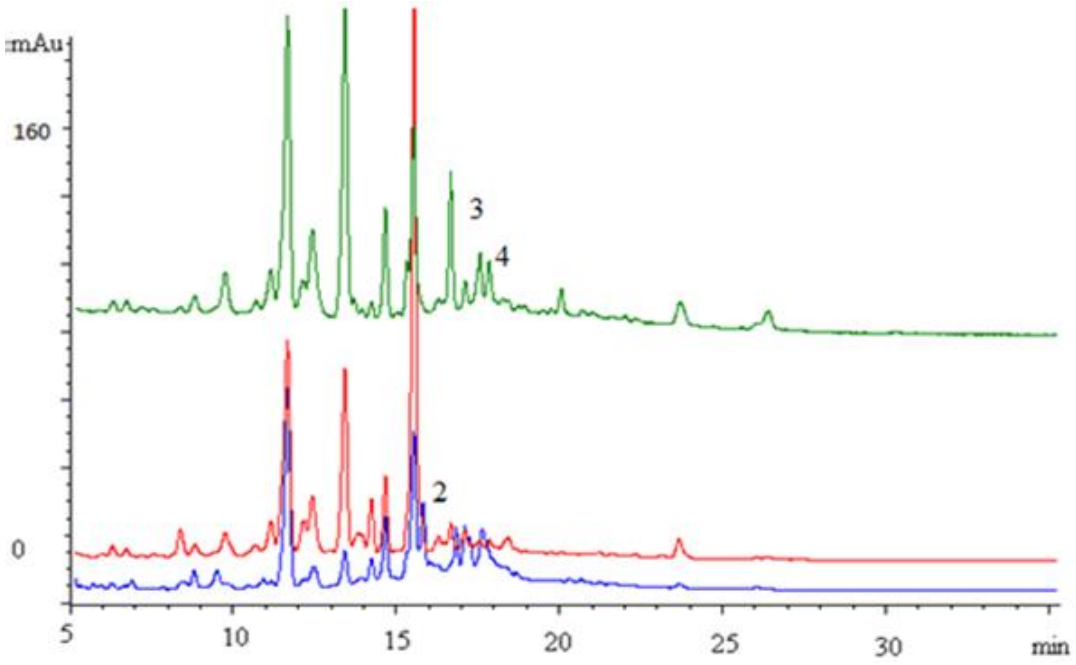


Figure 3.18. HPLC-DAD chromatograms of defatted crude extract of *A. chinensis cv* Sungold kiwifruit recorded at 360 (—), 320 (—) and 280 nm (—).

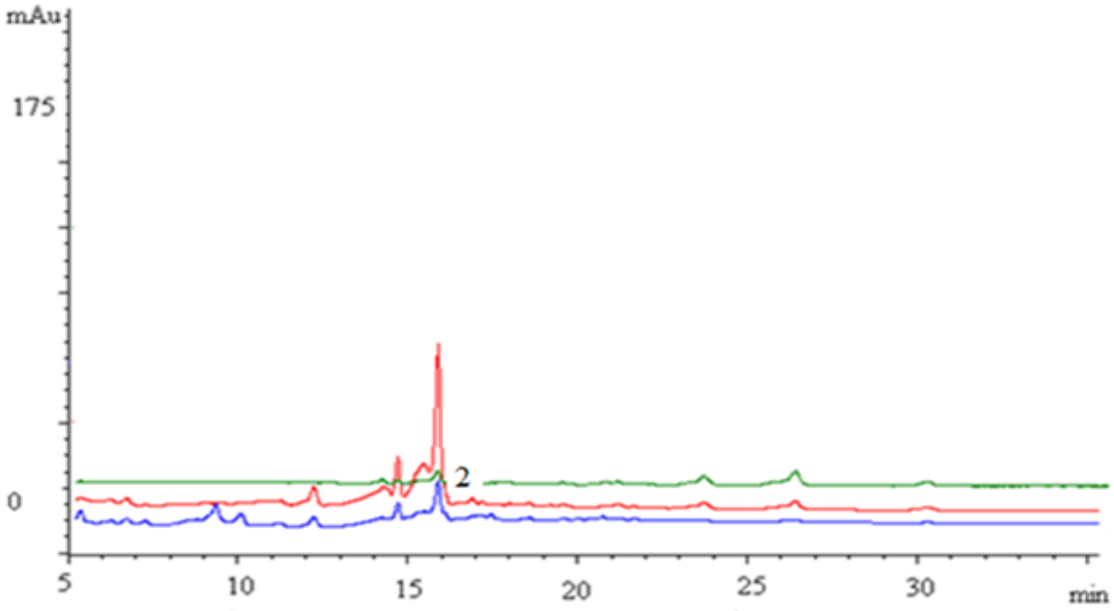


Figure 3.19. HPLC-DAD chromatograms of defatted crude extract of *A. deliciosa cv* Hayward (Green) kiwifruit recorded at 360 (—), 320 (—) and 280 nm (—).

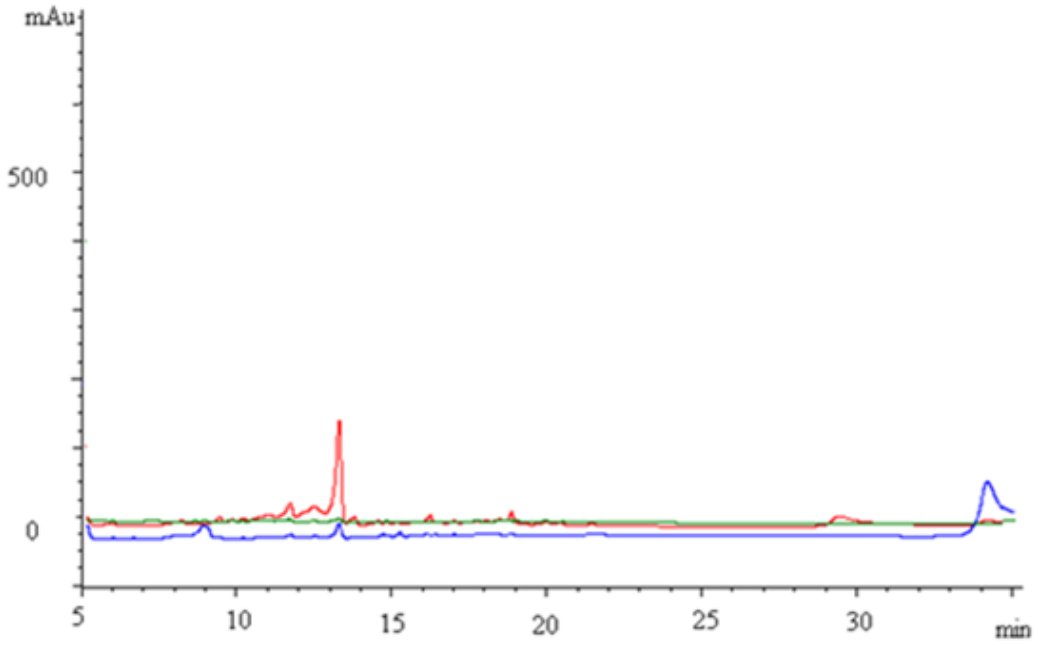


Figure 3.20. HPLC-DAD chromatograms of defatted crude extract of *A. chinensis cv* Hort 16A (Gold) kiwifruit recorded at 360 (—), 320 (—) and 280 nm (—).

In addition, a mixture of the most abundant standard phenolic compounds namely gallic acid, ferulic acid, caffeic acid, chlorogenic acid, *p*-coumaric acid, syringic acid,

catechin, epicatechin, rutin, quercetin-3-*O*-glucoside, quercetin, and luteolin which are common to many fruits, was also separated under the same conditions **(Figure 3.21)**.

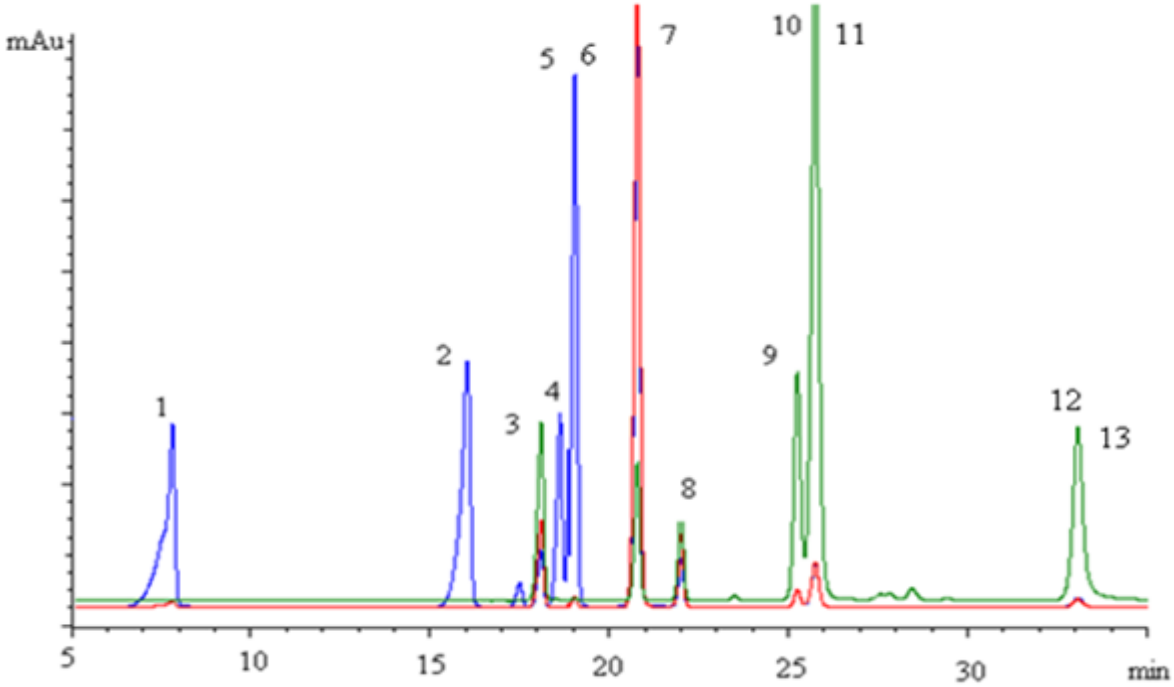


Figure 3.21. HPLC-DAD chromatograms of the standard mixture of phenolic compounds (1) gallic acid, (2) catechin, (3) caffeic acid, (4) chlorogenic acid, (5) epicatechin, (6) syringic acid (7) *p*-coumaric acid, (8) ferulic acid, (9) rutin, (10) quercetin-3-*O*-glucoside, (11) myricetin (12) quercetin, and (13) luteolin recorded at 360 (—), 320 (—) and 280 nm (—).

Simultaneous monitoring was carried out at four different wavelengths: 280 nm (flavan-3-ols, proanthocyanidins and benzoic acids), 320 nm (hydroxycinnamic acids), 360 nm (flavonols) and 520 nm (anthocyanidins). The UV/vis spectra were recorded between 200-550 nm. By comparing the retention times of standard compounds and the absorption spectra of the peaks (**Table 3.10**) with that of each component in the defatted crude extracts of kiwifruits, it could be preliminarily concluded that there was a correspondence to some of standard compounds in the extracts analysed.

Table 3.10. Chromatographic parameters employed in the identification of standard phenolics.

Peak number	Phenolic compound	RT*	Phenolic structure	Maximum wavelengths (λ_{max}) (nm)
1	Gallic acid	6.323	Hydroxybenzoic acid	270
2	(+)-Catechin	15.620	Flavan-3-ol	279
3	Caffeic acid	17.241	Hydroxycinnamic acid	244, 323
4	Chlorogenic acid	18.154	Hydroxycinnamic acid	244, 324
5	(-)-Epicatechin	18.547	Flavan-3-ol	279
6	Syringic acid	18.818	Hydroxycinnamic acid	242, 314
7	*p*-Coumaric acid	20.058	Hydroxycinnamic acid	242, 314
8	Ferulic acid	22.315	Hydroxycinnamic acid	244, 323
9	Rutin	25.993	Flavonol	259, 355
10	Quercetin-3-*O*-glucoside	26.573	Flavonol	257, 353
11	Myricetin	26.812	Flavonol	256, 370
12	Quercetin	34.820	Flavonol	256, 368
13	Luteolin	34.820	Flavonol	255, 365

*Retention times are given in minutes

The phenolic constituents of *A. macrosperma* cultivar were found to be much more complex than other cultivars tested, and the phenolic patterns were remarkably different (**Figure 3.16**). Gallic acid, caffeic acid, chlorogenic acid, *p*-coumaric acid and ferulic acid were detected in the defatted extract of *A. macrosperma* fruit (**Figure 3.16**). The HPLC-DAD

chromatograms at different wavelengths of *A. macrosperma* cultivar showed unidentified peaks (in the retention time range 15-34 minutes) representing high peak areas that might contribute to the antioxidant activity. Gallic acid and catechin were identified in sweetgreen cultivar (**Figure 3.17**) while catechin, caffeic acid and chlorogenic acid were identified in sungold cultivar (**Figure 3.18**). Several peaks detected at 360 nm in the defatted extracts from *A.chinensis cv* Sungold and *A.deliciosa cv* Sweetgreen kiwifruits could not be identified. There was no correspondence to any of the standard phenolics detected in *A.chinensis cv* Hort 16A (Gold) (**Figure 3.20**) while a very weak peak corresponding to the catechin in *A.deliciosa cv* Hayward (Green) kiwifruits (**Figure 3.19**) was identified at the concentration of 50 mg/mL (DW/volume). This observation further supported and confirmed the presence of poor antioxidant capacity and the phenolic profiles found in gold and green kiwifruits in this study could be due to the lack of phenolics in them. The defatted crude extracts from *A. macrosperma* fruit **(Figure 3.16)** analyzed on an analytical HPLC-DAD without further purification, allowed us to identify only a few phenolic compounds based on the peak maxima at 280, 320 and 360 nm. This is due to the other compounds with associated spectra could elute in this region and thus interfere preventing identification of the compounds present in the chromatogram. This observation corresponds well to the recent studies reported by Latocha et al., (2010) showed that the fruit of *A. macrosperma* contains mainly phenolic acids namely tannic acid, gentisic acid, hydroxy benzoic acid, chlorogenic acid, *p*-coumaric acid and caffeic acid. Only a few flavonoids such as, catechin, epicatechin and quercetin, were identified in *A. macrosperma* fruit due to co-elution of many phenolic compounds (Latocha et al., 2010).

Research study carried out on phenolic composition of kiwifrui juice extracted from *A. deliciosa cv*. Hayward by Dawes and Keene, (1999) characterized some of the derivatives of coumaric acid, caffiec acid, catechin, epicatecin, procyanidins, dimeric and tetrameric

flavanols, quercetin glucoside, quercetin rutinoside, quercetin rhamnoside, rhamnoside and rutinoside of kaempferol in very low concentrations in this fruit. There are no reports on the identification of phenolics in the new commercial kiwifruit varieties, sungold and sweetgreen. The HPLC-DAD analysis further revealed that the extract obtained from *A. macrosperma* fruit is richer in flavonoids than the other commercial kiwifruit varieties tested. This is based on the high number of peaks in the retention time region of 20-40 minutes and the spectra in the chromatogram at 360 nm **(Figure 3.16**).

3.5. Conclusion

In this study, extraction conditions were optimized by using single factor experimental design for extracting phenolic compounds from *Actinidia macrosperma* kiwifruit. Initially, phenolics were extracted from each lyophilized ground fruit sample obtained from *Actinidia macrosperma* fruit into different extraction solvents (70% aq. acetone, 80% aq, methanol, 80% aq. ethanol, 100% methanol and water) followed by evaluating yield, total phenolic, total flavonoid, total flavanol contents and antioxidant capacities. The antioxidant capacity was determined by two different assays, namely, the ferric-reducing antioxidant power (FRAP) and 2,2-diphenyl-2-picylhydrazyl (DPPH) radical scavenging assays. After the quantification of phenolic profiles and antioxidant capacities in each solvent extract, 70% aq. acetone was chosen as the optimum solvent for the extraction in this study. Second step was to select the optimum extraction technique out of some selected conventional techniques (steeping, shaking, sonicating and blending) by keeping the solvent type constant. Among the solvents and extraction techniques employed, 70% aq. acetone by steeping resulted in the highest values of total phenolic (823.1±14.4 mg GAE/100 g DW), total flavonoid (170.9±1.9 mg CAE/100 g DW), total flavanol (82.6±0.6

mg CAE/100 g DW) contents and the antioxidant activity (5.1±0.1 mmol Trolox equivalents/100 g DW and 8.3±0.1 mmol Fe (II) equivalents/100 g DW for DPPH and FRAP assays, respectively. Analysis of each extract on LC-DAD and LC-ESI-MS revealed that 70% aq. acetone extract from *A. macrosperma* fruit contains many potential antioxidant flavonoids and quercetin-3-*O*-galactoside was the most abundant among them.

Phenolic profiles and *in vitro* antioxidant activity of 70% aq. acetone extract by steeping technique employed in *A. macrosperma* fruit was compared with those of different commercial kiwifruit varieties grown in New Zealand. Among the different kiwifruit varieties tested, the *A. macrosperma* fruit showed the highest values of total phenolic (TP) content (823.1±14.4 mg gallic acid equivalent/100 g DW), total flavonoid (TFO) content (170.9±1.9 mg catechin equivalent/100 g DW), total flavanol (TFA) content (82.6±0.6 mg catechin equivalent/100 g DW) and antioxidant activity (5.1±0.1 mmol Trolox equivalent/100 g DW and 8.3±0.1 mmol Fe (II) equivalent/100 g DW for DPPH and FRAP assays, respectively).

To explore the influence of the phenolic composition on antioxidant capacity in different kiwifruit varieties, the correlations were analyzed in the present study. The total phenol, flavonoid and flavanol contents and antioxidant capacity obtained from different kiwifruit varieties tested showed strong correlation**.** The total flavonoid content of five different varieties of kiwifruits was in good correlation with the total phenolic content of those extracts (R^2= 0.977) which suggests that TFO was the main component of TP in these different varieties. TFA was well correlated with TP (R^2=0.973) and TFO (R^2=0.942). High correlation (R^2= 0.942) observed between FRAP and DPPH antioxidant capacities implied that the antioxidants in these kiwifruit extracts were capable of scavenging free radicals (DPPH) and reducing oxidants.

Since there are a large number of different types of antioxidants and phenolic compounds that might contribute to the total antioxidant capacity, all defatted extracts from different varieties were analyzed using HPLC-DAD and compared to a mixture of standard compounds. The data suggests that the *A. macrosperma* fruit could be a rich source of phenolic antioxidants.

In conclusion, the antioxidant activities and phenolic composition of *A. macrosperma* fruit extracts were well evaluated in this study and the 70% aq. acetone by steeping technique was selected as the optimized extraction method to isolate and to distinguish structural features most important for scavenging and antioxidant activities. It was noticed that total phenolic content, total flavonoid content, total flavanol content and antioxidant capacity determined in *A. macrosperma* fruit were affected by the extraction solvent and the extraction technique. According to this work, *A. macrosperma* fruit is a natural source of potential antioxidants of polyphenols compared to commercially available kiwifruits. To the best of our knowledge, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, and quercetin-3-*O*-arabinoglucoside were identified in *A. macrosperma* fruit for the first time. The investigation of five different varieties has demonstrated that the antioxidant activities and the phenolic composition showed great variation among *Actinidia* varieties and high correlation between them. To the best of our knowledge, this is the first study to investigate the antioxidant activity and the phenolic profiles of *A. deliciosa cv* Sweetgreen, and *A. chinensis cv* Sungold kiwifruits. The results from these *in vitro* assays provide data as a preliminary step in assessing commercialization prospects for *A. macrosperma* fruit. Therefore, further studies are needed to start searching for any other potential health benefits, isolation, purification and identification of individual antioxidant components which could be an economically interesting phytochemical source for the nutraceutical and functional food market.

Chapter 4

Angiotensin converting enzyme (ACE) inhibition

Angiotensin converting enzyme (ACE) inhibition by flavonoid-rich defatted crude extracts of *A.macrosperma* fruit and determination of kinetics of the inhibition of ACE

4.1. Introduction

Hypertension is a common cardiovascular condition which has become a worldwide problem of epidemic proportions affecting 15 to 20% of all adults, leading to chronic diseases such as arteriosclerosis, stroke, renal disease and diabetes (Murray and FitzGerald, 2007). Hypertension can affect people of all ages from children through to the elderly with the incidence increasing with age especially in people 50 years or older (Murray and FitzGerald, 2007). One quarter of the world's adult population is already afflicted with hypertension and the numbers are likely to increase up to 29 % by the year 2025 (Mittal and Singh, 2010). As this chronic disorder elevates the blood pressure in the arteries and is asymptomatic in nature, unawareness could lead to development of fatal conditions such as stroke or heart failure. Frequent measurement of blood pressure is the most convenient way to detect hypertension.

Many pathophysiological factors have been implicated in the genesis of hypertension: increased activity of the renin angiotensin aldosterone system (RAAS) (**Figure 4.1**) and the sympathetic nervous system (SNS) activity, endothelial dysfunction and genetic influence (Oparil et al., 2003). Increased activity of the sympathetic nervous system increases high blood pressure and contributes to the development and maintenance of hypertension through stimulation of the heart by causing rapid contraction of the heart muscle to increase cardiac output, increasing the vascular resistance in the peripheral vasculature, and increasing fluid retention in the kidneys (Oparil et al., 2003).

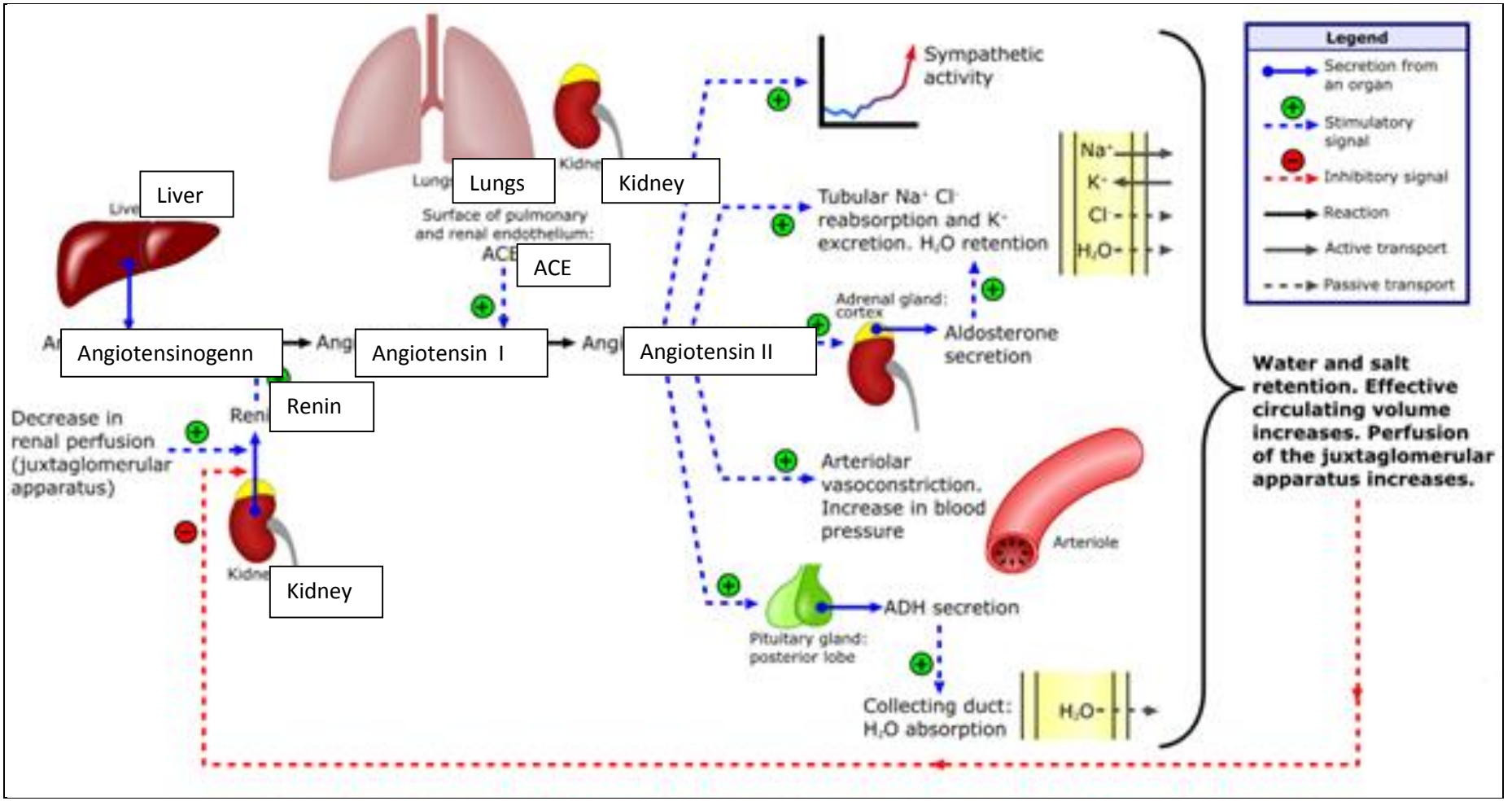


Figure 4.1. Schematic diagram showing the sequential process of the circulatory Renin-angiotensin aldosterone system (RAAS)

(http://en.wikipedia.org/wiki/Macula_densa).

Overactivation of the renin angiotensin aldosterone system (RAAS) is a prominent factor which is caused by the pathogenesis of hypertension (Atlas, 2007). The RAAS begins with the biosynthesis of renin which is a circulating enzyme secreted by juxtaglomerular cells (JG cells or granular cells) in the kidney via three responses: (1) a decrease in arterial blood pressure as detected by pressure sensitive cells called baroreceptors (2) a decrease in sodium chloride levels in the ultra-filtrate of the nephron (3) sympathetic nervous system activity which controls blood pressure through the $beta_1$ adrenergic receptors. Renin participates in maintaining extracellular volume, and arterial vasoconstriction by contributing to regulation of the blood pressure through breaking down the globular protein called angiotensinogen secreted from the liver into Angiotensin-I (**Figure 4.1**) (Atlas, 2007). The conversion of angiotensinogen to Angiotensin I by renin is identified as the rate limiting factor of RAAS where control of renin activity plays a significant role in controlling RAAS (Atlas, 2007). Angiotensin-I is subsequently converted to angiotensin II by angiotensin converting enzyme (ACE) which is secreted in the lungs (**Figure 4.1**.) (Atlas, 2007; Balasuriya and Rupasinghe, 2012). ACE is a zinc-dipeptidylcarboxypeptidase (EC 3.4.15.1) which is a glycoprotein and multifunctional enzyme in RAAS that plays a key role in regulating blood pressure, fluid and electrolyte balance. The overactivation of renin-angiotension system, leads to vasoconstriction and retention of sodium and water, and these effects lead to hypertension (**Figure 4.2**).

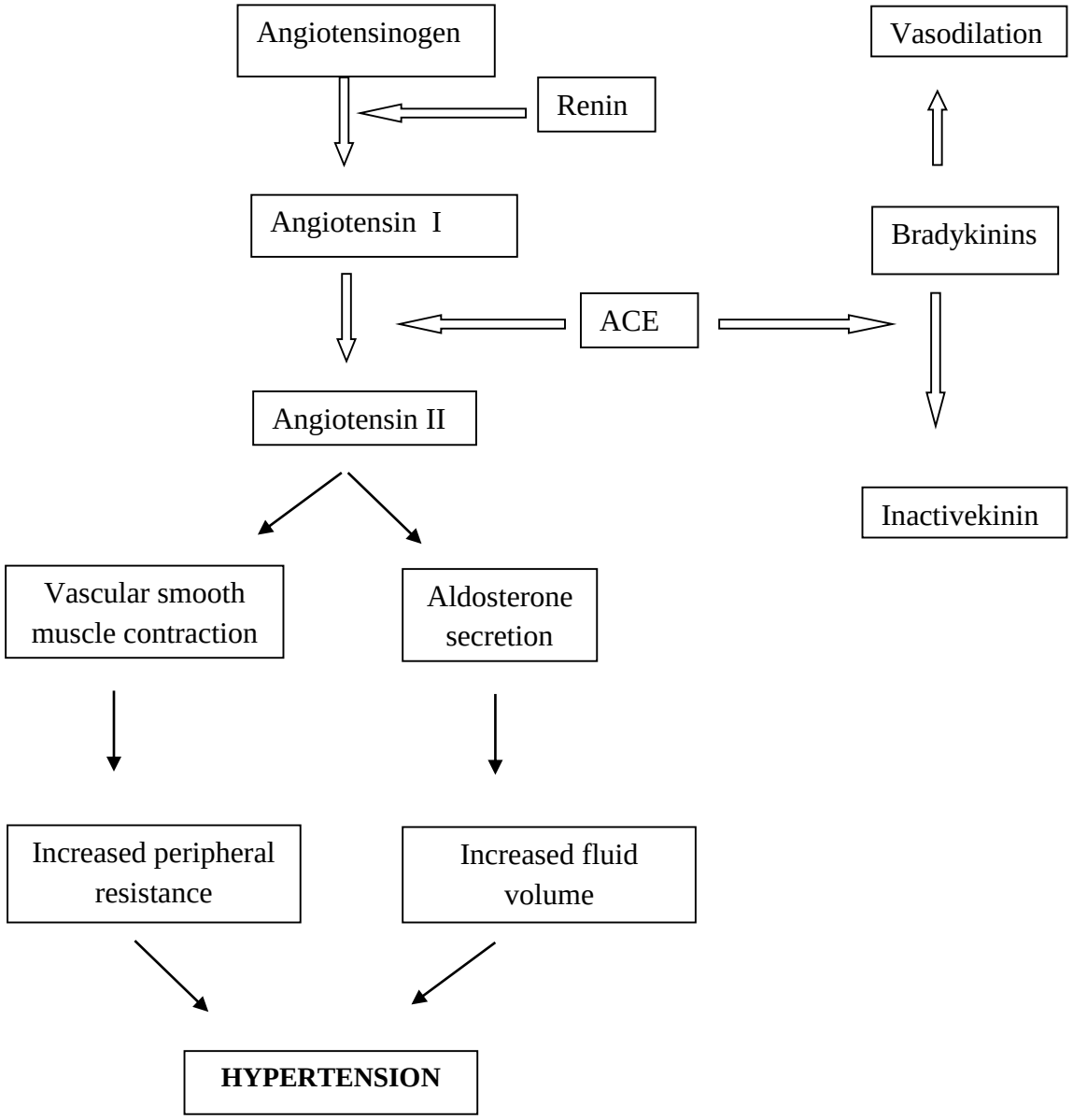


Figure 4.2. The suggested hypertensive mechanism of angiotensin.

Therefore, control of circulatory RAAS had been identified as a major therapeutic target. Several classes of pharmacological agents have been used in the treatment of hypertension. One class of antihypertensive drugs, known as renin inhibitors, act on the rate limiting step of the RAAS system which is conversion of angiotensinogen into angiotensin I. Aliskiren (TekTurna) was the first renin inhibitor to be approved by the US Food and Drug

Administration (Vijayaraghavan and Deedwania, 2011). In current medical practice, the renin-angiotensin-aldosteron-system's overactivity is more commonly reduced using ACE inhibitors or angiotensin II receptor blockers as they are associated with low rates of adverse side-effects as compared to direct oral renin inhibitors (Fotherby and Panayiotou, 1999; Loizzo et al., 2007). Briefly, this group of drugs causes dilation of blood vessels which results in lower blood pressure by the suppression of ACE activity and decreases the formation of angiotensin II and inactivation of bradykinin. Therefore, ACE inhibition has been considered as one of the effective and key therapeutic approaches for the treatment of hypertension and cardiovascular diseases (Hoe et al., 2007).

ACE inhibitors can be divided into several groups based on their molecular structure:

1. Sulfhydryl-containing agents (Captopril, Zofenopril)
2. Dicarboxylate-containing agents (Enalapril, Rampiril, Quinapril, Lisinopril, Benezepril, Imidapril)
3. Phosphate-containing agents (Fosinopril)
4. Naturally occurring agents (peptides, flavonoids)

Although synthetic ACE-inhibitor drugs such as Captopril, Rampiril, Lisinopril, and Enalapril are currently in use and are effective at treating hypertension (Quan, 2006), they are often accompanied by undesirable side effects, including a persistent non-productive dry cough, allergic reactions and difficulty swallowing or breathing due to angioedema (Braga et al., 2007; Guang and Philips, 2009). In contrast to the synthetic ACE inhibitor drugs, no such side effects have been observed for ACE inhibitors derived from natural sources (Braga et al., 2007; Guang and Philips, 2009). Different types of naturally derived compounds have

been investigated for their ACE inhibitory properties. Many studies have shown that plant extracts rich in phytochemicals including protein derivatives (Cinq-Mars and Li-Chan, 2007; Hong et al., 2008; Farzamirad and Aluko, 2008; Guan and Phillips, 2009; Udenigwe et al., 2009), hydrolysable tannins, phenylpropanes, fatty acids, xanthones, terpenoids (Braga et al., 2000; Braga et al., 2007), alkaloids, oligosaccharaides, and polyphenolic compounds like proanthocyanidins (Actis-Goretta et al., 2003) and flavonoids, caffeolyquinic acid derivatives (Braga et al., 2007; Loizzo et al., 2007; Balasooriya and Rupasinghe, 2012; Umamaheshwari et al., 2012) can act as effective natural ACE inhibitors.

Many recent studies have investigated flavonoid rich food constituents isolated from fruits, vegetables and their by-products, including the capability of these constituents to prevent and/or limit increase in blood pressure (Actis-Goretta et al., 2006; Hodgson and Croft, 2006; Ottaviani et al., 2006; Ahmed et al., 2010; Da Silva Pinto et al., 2008; Da Silva Pinto et al., 2010; Lecour and Lamont, 2011; Balasooriya and Rupasinghe, 2012).

Kiwifruits namely *A. deliciosa cv* Hayward, *A. chinensis cv* Hort 16A) and *A. macrosperma* have been evaluated for their pharmacological applications including antimicrobial activity (Lu et al., 2007a; Lu et al., 2011a), antioxidant activity (Fergusan, 2007; Nishiyama, 2007; Latocha et al., 2010; Hunter et al., 2011), immune modulatory activity (Collin et al., 2002; Lu, Y., 2011b) and anticancer activities (Motahashi et al., 2002; Lu et al., 2011b). The *A. macrosperma* fruit have been used on the management and treatment of human diseases such as leprosy, abscess, rheumatism, arthritis inflammation, jaundice, abnormal leucorrhoea and various types of cancers (Lu et al., 2007a; Lu et al., 2007b; Lu et al., 2011b). These findings open up the possibility of finding newer plants or plant derived compounds which mimic synthetic ACE inhibitors from *A. macrosperma* fruit and provide health benefits.

To date, only one research study on the antihypertensive activity of kiwifruits has been reported in the literature (Jung et al., 2005) and there do not appear to be any reports on the ACE inhibitory activity of *A. macrosperma* fruit. Therefore, the potential ACE inhibitory activity of extracts obtained from *A. macrosperma* fruit was investigated as this fruit was found to be a rich source of antioxidants, particularly flavonoids, in Chapters 3, compared to the commercially available kiwifruits namely, *A. deliciosa cv* Hayward and *A. chinensis cv* Hort 16A.

4.2. Plant materials and chemicals

The fruits of *A. macrosperma* reaching physiological maturity were collected on 26th April 2010 at the Plant and Food research orchard in Te Puke Bay, New Zealand. *A. deliciosa cv* Hayward (Green kiwifruit), and *A. chinensis cv* Hort 16A (Gold kiwifruit) were donated by ZESPRI, New Zealand. Defective fruits were discarded and the remaining fruit were cut into small parts, freeze dried and stored at -80 °C. The samples were prepared by grinding the lyophilized fruit samples with a mortar and pestle prior to the extraction.

The ACE extracted from rabbit lung, histidine-$_L$-hippuryl-$_L$-leucine-chloride (HHL), histidineleucine (His-Leu), sodium hydroxide (NaOH), hydrochloric acid (HCl), ethanol anhydrous, Captopril, *O*-phthaldialdehyde (OPT), dimethyl sulfoxide (DMSO) and HPLC grade methanol were purchased from Sigma Aldrich Canada Ltd, Oakville, ON, Canada. Borate saline buffer (100 mM boric acid, 1.5 M sodium chloride, sterile, pH adjusted to 8.3) was purchased from Teknova, Hollister, CA, USA.

4.3. Methodology

4.3.1. Preparation of flavonoid rich defatted crude fruit extracts

The flavonoid rich defatted crude extracts from *A. macrosperma* fruit were prepared according to the procedure as described in Section 3.3.1 in Chapter 3. Briefly, the extracts were prepared using different extraction solvents namely 70% aq. acetone, 80% aq. methanol, 80% aq. ethanol, 100% methanol, and 100% water and four different extraction techniques namely, steeping, shaking, sonicating and blending from *A. macrosperma* fruit analysis. The flavonoids rich defatted crude extracts from *A. deliciosa* and *A. chinensis* kiwifruits were prepared in 70% aq. acetone by steeping technique as described in Section 3.3.2 in Chapter 3. The composition of the flavonoid rich defatted crude kiwifruit extracts (FKE) obtained from these commercial varieties was analysed using HPLC-MS/MS according to the procedure given in the Section 3.3.9 in Chapter 3.

4.3.2. ACE inhibitory assay

The ACE inhibitory assay was performed according to the method described by Balasooriya and Rupasinghe, (2012) with slight modifications. This inhibitory assay consists of two reactions: a bioassay based on cleavage of Histidine-Leucine (His-Leu) from histidine-$_{L}$-hippuryl-$_{L}$-leucine-chloride (HHL) substrate in the presence of ACE followed by quantification of His-Leu using fluorescence detection.

A preliminary experiment was carried out to select the optimum composition of DMSO in borate saline buffer to dissolve the samples using the ratios given in **Table 4.1**. The experimental testing units (1-5) showed in **Table 4.1** represent the sample units in the

presence of ACE activity while 6-10 represent blank units for the relevant concentrations without ACE activity.

The experimental testing units were prepared by mixing sodium borate buffer (pH 8.3) (9 µL), 0.78 mM HHL in buffer (150 µL), and different concentrations of DMSO in buffer (21 µL) followed by addition of 2.5 mU ACE in buffer (30 µL) in an eppendorf tube (**Table 4.1**). All the experimental units (testing and blank units) were incubated at 37 °C using a shaker oven (Model: HP 50, Appolo Instrumentation for Molecular Biology, CA, USA) for 1 h followed by adding 0.35 M NaOH (150 µL) to stop the enzyme activity in the experimental unit. The formation of His-Leu by the cleavage from HHL in the presence of ACE was quantified by measuring fluorescence after formation of *O*-phthaldialdehyde adduct.

Table 4.1. Experimental design for the optimization of DMSO:Buffer on the ACE assay.

Experimental Unit No	No of Replicates	Volume of Buffer (µL)	Volume of HHL (µL)	Volume of DMSO (µL)	[DMSO] used (%)	Volume of ACE (µL)	Volume of NaOH (µL)	Volume of OPT (µL)	Volume of HCl (µL)	Total Volume (µL)
1	3	9	150	21	3.75	30	150	100	100	560
2	3	9	150	21	1	30	150	100	100	560
3	3	9	150	21	0.38	30	150	100	100	560
4	3	9	150	21	0.04	30	150	100	100	560
5	3	9	150	21	0.0	30	150	100	100	560
6	3	39	150	21	3.75	0	150	100	100	560
7	3	39	150	21	1	0	150	100	100	560
8	3	39	150	21	0.38	0	150	100	100	560
9	3	39	150	21	0.04	0	150	100	100	560
10	3	39	150	21	0.0	0	150	100	100	560

4.3.3. Fluorescence measurements

100 µL of *O*-phthaldialdehyde (20 mg/mL) (DW/volume) prepared in absolute ethanol was added to each experimental unit to make the fluorescent adduct and the solution was left for 10 min before hydrochloric acid (3 M, 100 µL) was added to stop the reaction (Balasooriya and Rupasinghe, 2012). 100 µL of the experimental reaction mixture was transferred into a *96*–well polystyrene plate. Fluorescence was measured using a FLUOstar OPTIMA plate reader (BMG LabtechInc, Offenburg, Germany) at excitation and emission wavelengths of 360 nm and 500 nm, respectively.

4.3.4. ACE inhibitory activity of various extracts from solvents, techniques and cultivars

The ACE inhibitory assay was carried out according to the procedure described by Balasooriya and Rupasinghe, (2012) with slight modifications. Briefly, sodium borate buffer (pH 8.3) (9 µL) and 0.78 mM HHL in buffer (150 µL) were mixed in Eppendorf tubes followed by addition of the fruit extract (FKE) reconstituted in 0.38% DMSO (21 µL) at different concentrations. 2.5 mU ACE in buffer (30 µL) was added into each testing experimental unit to produce His-Leu before fluorescence detection. All experimental units including testing units, negative control/blank (without ACE and inhibitors), positive control (with ACE but no inhibitors) and the standard, Captopril (10 mg/L) were run in triplicates. All the experimental units were incubated and followed by formation of *O*-phthaldialdehyde adduct as described in Sections 4.3.2 and 4.3.3 respectively.

The percentage inhibition of the ACE enzyme was calculated according to the equation (1) (Balasooriya and Rupasinghe, 2012):

Percent enzyme inhibition (%) $=\{1-(F_{sample}-F_{sample\ blank})/F_{positive\ control}\}x100$.....(1)

F_{sample}; fluorescence of the sample extract, $F_{sample\ blank}$; fluorescence of the blank prepared for the sample extract, $F_{positive\ control}$; fluorescence of the positive control

Dose responsive enzyme inhibition was determined using different concentrations of each test extract/sample. The concentration of a test extract/sample which could inhibit 50 % of enzyme activity (IC_{50}) was calculated using linear regression analysis of % ACE inhibition vs. concentration of the extract/sample.

4.3.5. Determination of kinetic parameters of ACE activity without inhibitors

In order to study the type of inhibition of the ACE activity observed in this study, kinetic studies were performed with and without inhibitors. Enzyme kinetic analysis for ACE activity without inhibitors was performed by preparing a serial dilution (0.125, 0.25, 0.5, 1, 2, 4, 8 mM) of HHL standard substrate in buffer solution according to the experimental design given in **Table 4.2**. His-Leu formed from HHL substrate in the presence of ACE and without inhibitor was referred to as the maximum enzyme activity**.** His-Leu formed was calculated by using His-Leu standard curve. Five different concentrations (0.125, 0.25, 0.5, 1, 2 mM) of His-Leu were prepared in buffer using standard His-Leu compound followed by formation of the fluorescent adduct as described in Section 4.3.3. The fluorescence values obtained were plotted against the concentration of His-Leu to obtain the standard curve (**Appendix 6**).

Table 4.2. Experimental design for the determination of kinetic parameters without inhibitors.

Experimental Unit No	No of Replicates	Volume of Buffer (µL)	Volume of HHL (µL)	[HHL] used (mM)	Volume of ACE (µL)	Volume of NaOH (µL)	Volume of OPT (µL)	Volume of HCl (µL)	Total Volume (µL)
1	3	30	150	8	30	150	100	100	560
2	3	30	150	4	30	150	100	100	560
3	3	30	150	2	30	150	100	100	560
4	3	30	150	1	30	150	100	100	560
5	3	30	150	0.5	30	150	100	100	560
6	3	30	150	0.25	30	150	100	100	560
7	3	30	150	0.125	30	150	100	100	560
8	3	60	150	8	0	150	100	100	560
9	3	60	150	4	0	150	100	100	560
10	3	60	150	2	0	150	100	100	560
11	3	60	150	1	0	150	100	100	560
12	3	60	150	0.5	0	150	100	100	560
13	3	60	150	0.25	0	150	100	100	560
14	3	60	150	0.125	0	150	100	100	560

4.3.6. Determination of kinetic parameters of ACE activity with inhibitors

The kinetics of the ACE activity in the presence of known concentrations of 70 % aq. acetone extracts obtained from *A.macrosperma* (2.64 mg/mL), *A.chinensis cv* Hort 16 A (13 mg/mL) and *A.deliciosa cv* Hayward (31 mg/mL) kiwifruits were determined with different concentrations of HHL (0.125, 0.25, 0.5, 1, 2, 4, 8 mM) (**Table 4.3**), then followed by fluorescence measurements as described in Section 4.3.3. These fluorescence measurements were used to calculate the liberated concentration of His-Leu in each experimental unit in the presence of inhibitors using the His-Leu standard curve as described in Section 4.3.5.

4.3.7. Kinetic calculations

The kinetic parameters were calculated by fitting the data to the curves to the Lineweaver-Burk linerised form of the Michaelis-Menten kinetic equation (Balasooriya and Rupasinghe, 2012):

$$V=V_{max}[S] / (Km+[S]) \quad (2)$$

where V is the reaction rate, V_{max} is the maximum reaction rate, K_m is the Michaelis-Menten constant, and S is the substrate concentration.

Lineweaver-Burk double reciprocal plots using equation (3) were used to determine the type of the inhibition (Balasooriya and Rupasinghe, 2012).

$$1/V = (Km/V_{max}) (1/[S]) + 1/V_{max} \quad (3)$$

where the symbols are as defined for eqn (2).

Table 4.3. Experimental design for the determination of kinetic parameters in the presence of inhibitors.

Experimental Unit No	No of Replicates	Volume of Buffer (µL)	Volume of HHL (µL)	[HHL] used in the Unit (mM)	Volume of fruit extract (µL)	Volume of ACE (µL)	Volume of NaOH (µL)	Volume of OPT (µL)	Volume of HCl (µL)	Total Volume (µL)
1	3	9	150	8	21	30	150	100	100	560
2	3	9	150	4	21	30	150	100	100	560
3	3	9	150	2	21	30	150	100	100	560
4	3	9	150	1	21	30	150	100	100	560
5	3	9	150	0.5	21	30	150	100	100	560
6	3	9	150	0.25	21	30	150	100	100	560
7	3	9	150	0.125	21	30	150	100	100	560
8	3	39	150	8	21	0	150	100	100	560
9	3	39	150	4	21	0	150	100	100	560
10	3	39	150	2	21	0	150	100	100	560
11	3	39	150	1	21	0	150	100	100	560
12	3	39	150	0.5	21	0	150	100	100	560
13	3	39	150	0.25	21	0	150	100	100	560
14	3	39	150	0.125	21	0	150	100	100	560

The kinetic parameters {V_{max}, Km, and K_i (dissociation constant)} were determined using equations (3) and (4).

$$m_i = m\,(1+[I]/K_i) \quad \text{.....(4)}$$

where, m_i is the slope from Lineweaver-Burk plot for the inhibited reaction, m is the slope from Lineweaver-Burk plot for the non-inhibited reaction, [I] is the concentration of inhibitor, and K_i: dissociation constant of the inhibitor-enzyme complex (inhibitory constant)

4.3.8. Statistical analysis

All experimental measurements were conducted in triplicate and the results are expressed as mean±SD. Selected inhibition percentage values obtained from three different kiwifruit cultivars were analyzed by analysis of variance (ANOVA) using OriginPro8 software. Pairwise multiple comparisons were evaluated using Tukeyss' significance difference test in OriginPro8. Differences at p= 0.05 were considered significant.

4.4. Results and Discussion

4.4.1. ACE assay sensitivity on the composition of DMSO

The reaction of *O*-phthalaldehyde (OPT) with Histidine-Leucine (His-Leu) affords a highly fluorescent product which enables the quantification of trace amounts of His-Leu cleaved from the HHL substrate in the presence of ACE (Balasooriya and Rupasinghe, 2012). However, attention should be paid to influences on the assay by the solvents and their composition in the buffer used, as it has been reported that the presence of DMSO even in

concentrations as low as 1% decreases the observed yield of fluorescence (Moldt et al., 1988). In this work, DMSO was selected as the solvent since it is the most commonly used solvent for water insoluble materials (Moldt et al., 1988). The effects of various DMSO-to-buffer ratios (as given in **Table 4.1**) on the fluorescence in the present assay were investigated with the working concentration of HHL solution (7.8 mM). As can be seen from **Figure 4.3**, DMSO was found to severely affect the fluorescence in this assay. As shown by the red line on the graph (b), increasing the concentration of DMSO in the experimental unit caused a decrease in the fluorescence yield in the absence of ACE (**Figure 4.3**). When the molar ratios was less than 1% of DMSO in buffer in the experimental unit, it caused a dose-dependent response on the fluorescence. But over that ratio, the intensity tended to level off. Also increasing concentrations of DMSO in the medium caused a decrease in the fluorescence yield. The line (a) represents the fluorescence yield under the same conditions but in the presence of ACE. This resulted in the production of the fluorescent adduct (OPT-His-Leu). This result is in agreement with the literature which reported that DMSO can reduce the fluorescence yield due to quenching (Moldt et al., 1988; Cho and Mattice, 1990; Ottaviani et al., 2006). Therefore, 0.38% DMSO in the experimental unit was selected as the optimum ratio of DMSO to buffer in this study.

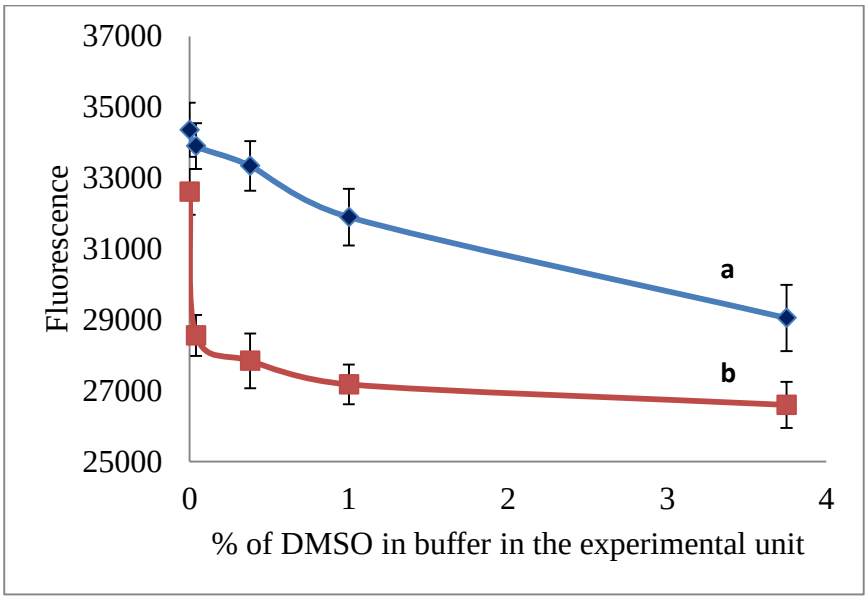


Figure 4.3. Effect of DMSO: buffer ratio on fluorescence on ACE inhibitory assay

(a) The reactions were performed in the presence of ACE, (b) the reactions were performed in the absence of ACE. All values are mean±SD of three experiments, with bars corresponding to standard deviations.

The biochemical assay used in the present study consists of enzymatic cleavage followed by detection of the florescent adduct formed by the reaction of *O*-pthaldialdehyde with the cleavage product (Balasooriya and Rupasinghe, 2012). To ensure accurate function of the slightly modified microplate fluorometric assay, the IC_{50} for a standard compound called Captopril, a known ACE inhibitor was determined to be 19 ng/L (**Table 4.4**). Captopril is a common orally administered ACE inhibitor used to treat hypertension and congestive heart failure. Various values for the IC_{50} of Captopril have been reported; 0.02 µM (4340 ng/L) was reported for Captopril by measuring histidine-Leucine (HL) cleaved from the reaction between HHL and rabbit ACE with fluorometry (Balasooriya and Rupasinghe, 2012) while 1.9 ng/mL (1900 ng/L) was reported by measuring hippuric acid liberated from the reaction of HHL and rabbit ACE with HPLC (Tsai et al., 2012). Hansen and co-workers (1995) have reported 11 nM (2387 ng/L), which was comparable to 14 nM (3038 ng/L) reported by Elbi and Wagner, (1991). The differences in the observed values

for IC_{50} determined for Captopril could be due to the influences of several factors, including the conditions of the assay, the enzyme source, and the calculation method (Neels et al., 1982).

Table 4.4. Concentration responsive ACE inhibition by Captopril *in vitro*.

Concentration of Captopril (µg/mL)	% inhibition of ACE
100	99.88±0.36
10	100.79±0.89
0.1	91.07±2.41
0.001	73.50±0.38
0.00001	36.55±1.34
0.0000001	31.61±1.60

Values are expressed as mean±SD of three measurements.

4.4.2. ACE inhibition affected by extraction conditions employed

The potential antihypertensive activity of *A. macrosperma* fruit extracts obtained from different solvents and techniques employed was evaluated by the inhibition of the angiotensin converting enzyme (ACE), using a fluorometric method (Balasooriya and Rupasinghe, 2012). All the solvent extracts tested inhibited the ACE activity in a dose-dependent manner and big variation of inhibition was observed among the different solvent extracts (**Table 4.5**).

Table 4.5. Concentration responsive ACE inhibition by different extraction solvents employed in *A. macrosperma* fruit.

Concentration of FKE (mg/mL)	% Inhibition of ACE by the extract				
	100% water	100% methanol	80% Methanol	80% Ethanol	70% Acetone
100	83.0±0.7	nd	nd	nd	Nd
80	68.6±5.6	nd	nd	nd	nd
70	nd	78.3±0.6	nd	nd	nd
60	52.9±1.3	71.0±0.8	nd	nd	nd
50	nd	65.5±2.4	25.2±2.7	91.7±2.0	nd
40	4.6±3.8	49.8±2.9	nd	nd	nd
30	nd	11.4±3.7	nd	nd	nd
25	nd	nd	nd	82.5±3.0	nd
20	0.0	0.0	nd	nd	nd
12.5	nd	nd	69.1±4.5	69.2±2.0	nd
10	0.0	0.0	nd	nd	49.4±3.9
6.25	nd	nd	35.5±3.4	77.7±3.0	nd
5	0.0	0.0	nd	nd	83.0±3.3
3.125	nd	nd	nd	37.2±2.0	nd
2.5	0.0±0.0	nd	nd	nd	78.4±5.1
1.563	nd	nd	5.4±0.1	nd	nd
1.25	nd	nd	nd	nd	64.3±4.2
0.786	nd	nd	nd	9.6±0.1	nd
0.625	nd	nd	nd	nd	51.8±4.0
IC_{50} (mg/mL)	**67.10**	**47.48**	**9.09**	**4.10**	**0.38**

FKE: Flavonoids rich defatted crude kiwifruit extracts; nd: not detected; Values are expressed as mean±SD of three measurements

The 100% water extract had an inhibition range from 4.6±3.8% to 83.0±0.7% in the concentration range of 40-100 mg/mL of flavonoids rich kiwifruit extracts (FKE) while the 100% methanol extract showed inhibition of 11.4±3.7% to 78.3±0.6% in the concentration range of 30-70 mg/mL (DW/volume) of FKE. From 5.4±0.1% to 69.1±4.5% inhibition in the range of 7.56-12.5 mg/mL (DW/volume) was exhibited by 80% aq. methanol extract while the 70% aq. acetone extract showed the highest inhibition within the lowest concentration range from 51.8±4.0% to 83.0±3.3% in the range of 5-0.625 mg/mL of FKE (**Table 4.5**). The concentration which could inhibit 50 % of enzyme activity (IC_{50}) of all extracts tested were calculated using linear regression analysis plot of % inhibition of ACE vs. concentrations. It has been reported that IC_{50} can be determined by several methods such as by regression analysis of ACE inhibition versus analyte concentration, ACE inhibition versus logarithmic analyte concentration and fitting inhibitor concentration ACE activity data to a four parametric logistic model (Guang and Philips, 2009). IC_{50} calculated for solvent extraction in this study, ranged from 0.38 to 69.1 mg/mL of flavonoid rich extract (**Table 4.5**). By comparing the IC_{50} determined, 70% aq. acetone extract showed the greatest ACE inhibitory activity with the lowest IC_{50} value of 0.38 mg/mL (DW/volume) among the other solvent extracts tested.

The present data are consistent with former studies reported in the literature. Studies published by Siddesha et al., (2011) reported that the effect of solvent extracts obtained from the leaves of *Artocarpus altilis* on ACE inhibitory activity (IC_{50}) varied with the order of ethyl acetate (85.4±0.9 µg/mL), *n*-butanol (106.6±0.6 µg/mL), methanol (125.0±0.4 µg/mL), water (210.3±5.4 µg/mL), ethanol (400.2±3.2 µg/mL) and acetone (441.7±6.2 µg/mL) depending on the type of the bioactive compounds extracted into each solvent due to the polarity of the functional groups present. Tsai et al., (2012) have reported that a 50% aq. ethanol solvent extract obtained from flavonoids rich buckwheat exhibited the greatest

(30±2 µg/mL) ACE inhibitory activity among water, 20% ethanol and 50% ethanol extracts tested.

All the extracts obtained from different techniques employed inhibited the ACE activity in a dose-dependent manner and variation of inhibition was observed among the different extracts (**Table 4.6**). Among the flavonoids rich extracts obtained from the different extraction techniques from *A. macrosperma* kiwifruit, steeping had the highest inhibition which ranged from 49.4±3.9% to 83.0±3.3% in the lowest concentration of 5-0.625 mg/mL (DW/volume) of FKE with the lowest IC_{50}=0.38 mg/mL (DW/volume). All other extraction techniques namely, shaking, sonicating and blending had similar IC_{50} values as shown in **Table 4.6**. Higher the IC_{50} lower the ACE inhibitory activity. Therefore, the 70% aq. acetone extract obtained by steeping technique employed in *A. macrosperma* fruit showed prominent ACE inhibitory activity (IC_{50}=0.38 mg/mL) compared to the other solvents and techniques tested.

Table 4.6. Concentration responsive ACE inhibition by different extraction techniques employed on *A. macrosperma* fruit.

Concentration (mg/mL)	% Inhibition of ACE by the extract			
	Shaking	Sonicating	Blending	Steeping
100	nd	nd	96.7±0.9	Nd
80	nd	nd	95.1±1.1	nd
70	88.4±1.7	78.3±0.6	nd	nd
60	82.2±1.4	71.0±0.8	86.6±2.2	nd
50	65.7±0.1	65.5±2.4	nd	nd
40	49.7±0.1	49.8±2.9	64.6±2.4	nd
30	13.8±0.1	11.4±3.7	nd	nd
20	0.0	0.0	nd	nd
10	0.0	0.0	18.7±0.6	49.4±3.9
5	0.0	0.0	nd	83.0±3.3
2.5	nd	nd	nd	78.4±5.1
1.563	nd	nd	0±0	nd
1.25	nd	nd	nd	64.3±4.2
0.625	nd	nd	nd	51.8±4.0
IC_{50}(mg/mL)	**43.5**	**37.0**	**32.9**	**0.38**

nd: not detected; Values are expressed as mean±SD of three measurements.

4.4.3. ACE inhibition affected by kiwifruit cultivar

All the extracts obtained from different kiwifruit cultivars tested inhibited the ACE activity in a dose-dependent manner (**Figure 4.4**) with different extent of inhibition being observed among the different cultivars (**Table 4.7**).

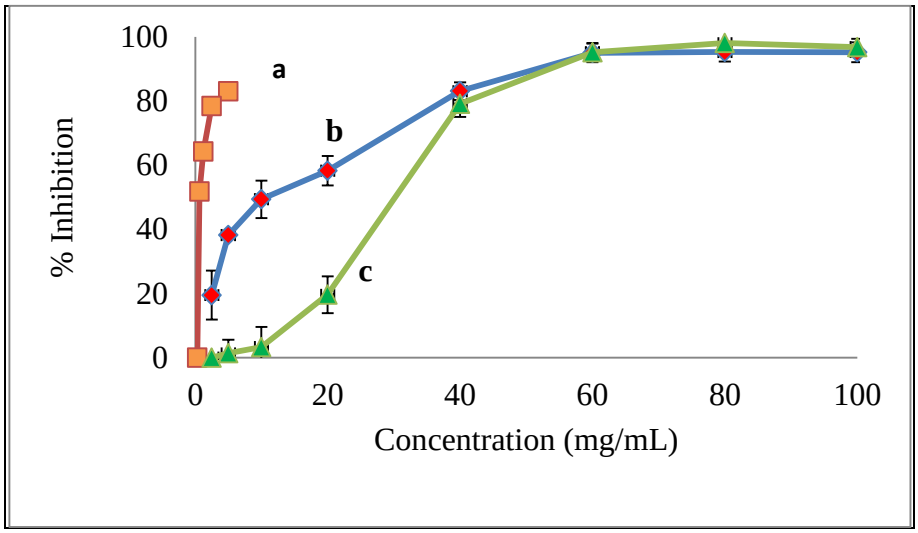


Figure 4.4. The dose response curves for the extracts from different cultivars on ACE assay (a) *A. macrosperma* (b) *A. chinensis cv* Hort 16A (c) *A. deliciosa cv* Hayward.

Flavonoids rich extracts obtained under the same extraction conditions for *A. chinensis cv* Hort 16A and *A. deliciosa cv* Hayward had inhibition ranges from 19.5±7.6% to 95.3±2.9% in the concentration range of 2.5-80 mg/mL (DW/volume) and 0.00±0.0% to 98.1±2.6% in the range of 2.5-80 mg/mL (DW/volume) of FKE respectively (**Table 4.7**). The concentration which could inhibit 50% of enzyme activity (IC_{50}) of all three extracts were calculated as 0.38, 12.6 and 32.1 mg/mL (DW/volume) for *A. macrosperma*, *A. chinensis cv* Hort 16A and *A. deliciosa cv* Hayward, respectively. Among the cultivars, the *A. macrosperma* fruit extract possessed the lowest IC_{50} compared to other commercial cultivars tested. Pairwise multiple comparison evaluated by Tukeys's test using Originpro8

showed that the percentage inhibitions determined at 5 and 2.5 mg/ml concentrations for *A. macrosperma*, *A. chinensis cv* Hort 16A and *A. deliciosa cv* Hayward were significantly different (p= 0.05).

Table 4.7. Concentration responsive ACE inhibition by different kiwifruit cultivars.

Concentration (mg/mL)	% Inhibition of ACE by the extract		
	A.deliciosa cv Hayward	*A.chinesis cv* Hort 16A	*A.macrosperma*
100	96.8±2.6	95.2±31	nd
80	98.1±2.6	95.3±2.9	nd
60	95.2±2.9	95.0±2.9	nd
40	79.2±4.2	83.1±2.8	nd
20	19.6±5.7	95.0±2.9	nd
10	3.3±6.3	49.4±5.8	49.4±3.9
5	1.3±4.3	38.2±1.4	83.0±3.3
2.5	0.0	19.5±7.6	78.4±5.1
1.25	nd	nd	64.3±4.2
0.625	nd	nd	51.8±4.0
IC_{50}(mg/mL)	**32.1**	**12.6**	**0.38**

nd: not detected; Values are expressed as mean±SD of three measurements

This indicates that the flavonoids rich extract obtained from *A. macrosperma* contains potential anti-hypertensive agents. A number of different classes of compounds with ACE-inhibitory activity have been isolated from plants in previous studies including

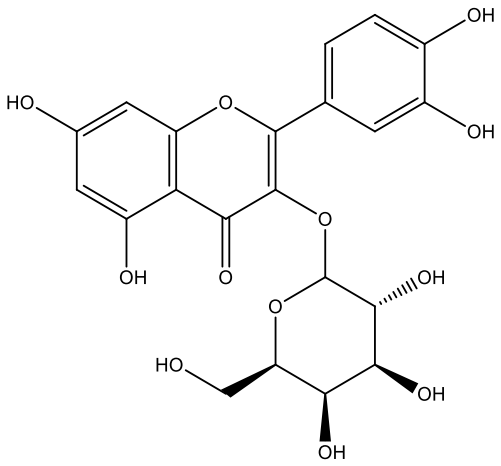
phenolics, glycosides, tannins, flavonoids, alkaloids, xanthones, terpenes and peptides as mentioned in Section 4.1. Therefore, LC-ESI-MS/MS was performed to identify the possible classes of compounds accounting for the ACE-inhibitory activity found in this study.

The major phenolic compounds detected in each kiwifruit cultivar namely, *A. macrosperma*, *A. deliciosa* and *A. chinensis* are compared in **Table 4.8**. The sub-class, flavonol was the most abundant group of flavonoids detected in all the extracts tested from three different kiwifruit cultivars. Quercetin, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-rutinoside, quercetin-arabinoglucoside, catechin, epigallocatechingallate, epigallocatechin, chlorogenic, ferulic, isoferulic and caffeic acids were found in *A. macrosperma* fruit. Some of these phenolics shown in **Figure 4.5** and **Figure 4.6** could be responsible for the strong ACE inhibitory activity determined in the extract of *A. macrosperma* fruit. Among the flavanols detected, catechin, which is a well known ACE inhibitor, is the most abundant in *A. macrosperma* fruit cultivar. The literature indicates that quercetin, quercetin sugar derivatives, and flavan-3-ols such as catechins, epigallocatechingallate, and epigallocatechin are also well known ACE inhibitors (Actis-Goretta et al., 2003; Braga et al., 2007; Loizzo et al., 2007; Balasooriya and Rupasinghe, 2012; Umamaheshwari et al., 2012).

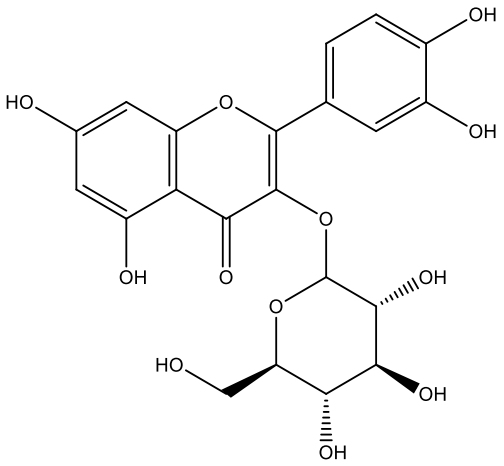
Table 4.8. LC-MS/MS analysis data of phenolics in three different kiwifruitcultivars.

Group/ Name of the Flavonoid	Concentration of phenolics (µg/g DW)		
	A.macrosperma	*A chinensis cv* Hort 16A	*A.deliciosa* Hayward
Flavonol			
Quercetin-3-*O*-Galactoside	470.9	205.19	441.39
Quercetin-3-*O*-Glucoside	4.16	0.45	0.14
QuercetinArabinoglucoside	2.53	0.06	0.21
Quercetin-3-*O*- Rhamnoside	2.99	0.61	0.31
Quercetin	2.56	nd*	0.17
Quercetin -3-*O*-Rutinoside	1.96	0.29	0.55
Flavanol			
Epigallocatechin	1.55	0.61	0.46
Catechin	54.31	0.75	0.3
Epicatechin	0.91	5.15	0.74
Epigallocatechingallate	0.75	nd	0.4
Dihydrochalcones			
Phloridzin	3.12	2.03	5.08
Phloritin	0.14	0.21	0.14
Phenolic acids			
Chlorogenic acid	1.97	0.39	0.28
Caffeic acid	1.64	0.04	0.08
Ferulic acid	4.7	0.42	0.6
Isoferulic acid	32.71	15.12	28.17

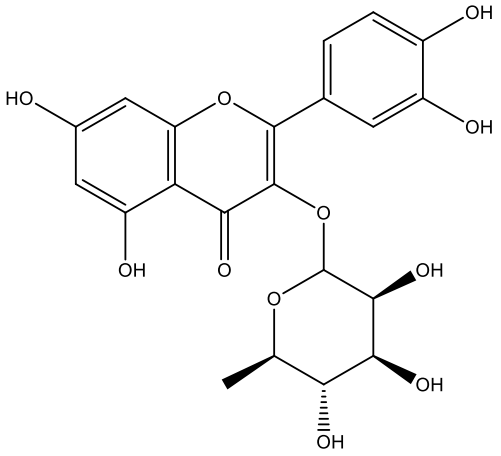
DW: dry weight of the fruit, nd*: not detected



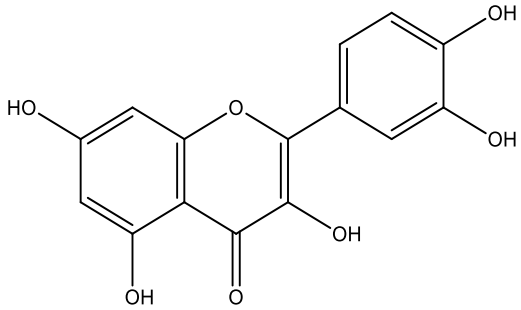
Quercetin-3-*O*-galactoside



Quercetin-3-*O*-glucoside

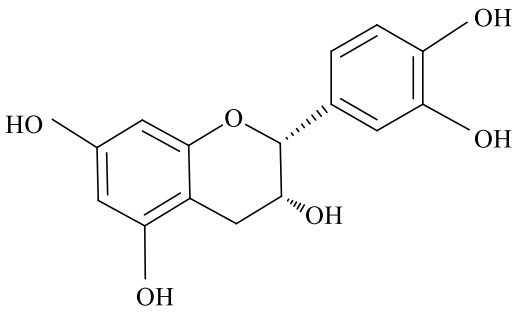


Quercetin-3-*O*- rhamnoside

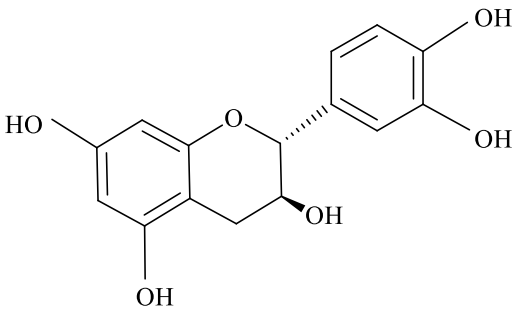


Quercetin

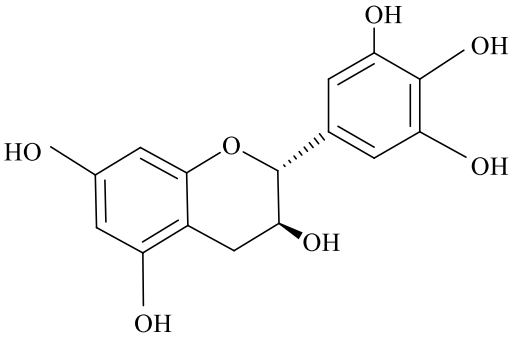
Figure 4.5. Most common types of flavonols identified by LC-MS/MS analysis in three different *Actinidia* genotypes.



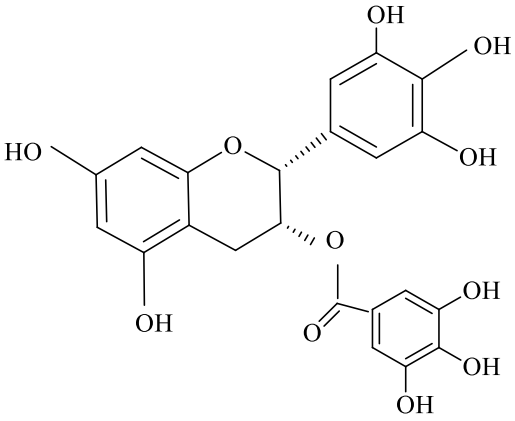
Catechin



Epicatechin (EC)



Epigallocatechin



Epigallocatechingallate

Figure 4.6. Most common types of flavan-3-ols identified by LC-MS/MS analysis in three different *Actinidia* genotypes.

There are various reports that demonstrated flavonoids and flavonoids rich plant extracts inhibit the ACE activity. The *in vitro* hypertensive activities of the methanol (MeOH) extracts and some flavonoids namely apigenin, luteolin, kaempferol-3-*O*-α-arabinopyranoside, kaempferol-3-*O*-β-galactopyranoside, quercetin-3-*O*-α-arabinopyranoside isolated from *Ailanthus excelsa* (Roxb) were reported by Loizzo et al., (2007). The ACE inhibitory properties of flavonoid rich apple peel extracts and selected

quercetin derivatives were assayed by Balasooriya and Rupasinghe, (2012) and they reported that apple extracts are potential ACE inhibitors. The major flavan-3-ols namely, catechins, (-)-epicatechins, (-)-epigallocatechins, (-)-epicatechingallate, and (-)-epigallocatechingallate isolated from green and black tea are reported to show dose dependent ACE inhibition in a human umbilical vein endothelial cells (HUVEC) culture model (Persson et al., 2006). Inhibition of Angiotensin converting enzyme has been evaluated in the presence of flavanol-rich foods like wines, chocolates, and teas and of purified flavonoids in the literature. The results have indicated a significant correlation between ACE inhibition and the concentration of proanthocyanidin and epicatechin (Actis-Goretta et al., 2006). The relationship between the structure of flavanols and *in vitro* ACE inhibitory properties has shown that the enzyme inhibition increased with the number of catechin units in the proanthocyanidin (Ottaviani et al., 2006). It has also been reported that aqueous extracts of *Ginkgo biloba*, rich in quercetin derivatives as the major flavonoids, have higher ACE inhibitory activity than ethanol extracts (Pinto et al., 2009). Oh and co-workers (2004) reported that fractions of stonecrop (*Sedum sarmentosum*) and five purified flavonols had ACE inhibitory activities (Oh et al., 2004). Kaempferol-rich stem bark extracts of *Ficusracemosa* have shown a dose dependent ACE inhibition property *in vitro* (Ahmed et al., 2010). The aqueous extracts of red currents (*Ribesnigrum* L.) exhibited ACE inhibition *in vitro* but not the extracts of red and green gooseberries (Da Silva Pinto et al., 2010).

There is less information about ACE inhibitory properties of flavones compared to other types of flavonoids. Apigenin and luteolin the two major flavones found in Roxb (*Ailanthus excelsa*), have demonstrated a dose dependent enzyme inhibition. The results showed that luteolin-7-*O*-glucoside has reduced enzyme activity comprising to higher IC_{50} value than luteolin aglycone, (Loizzo et al., 2007). Chalcones are another type of molecules which are precursors of the biosynthetic pathways of flavonoids. Chalcones and their

pyrazole derivatives inhibited ACE in a concentration-dependent manner *in vitro* (Bonsei et al., 2010). Anthocyanins have shown a competitive inhibition of ACE. Delphinidin-3-*O*-sambubiosides, and cyanidin-3-*O*-sambubiosides isolated from hibiscus (*Hibiscus sabdariffa* L.) extracts showed a dose dependent ACE inhibition with the IC_{50} values between 100-150 µM (Ojeda et al., 2010).

4.4.4. Determination of kinetic parameters of ACE inhibition

In order to study the type of inhibition of the ACE inhibitory activity, kinetic studies were performed using known concentrations of the extracts of these kiwifruit cultivars tested. ACE showed a Michaelis-Menten behaviour which has been used to describe the kinetic properties of many enzymes. In this model, an enzyme combines with a substrate to form an enzyme-substrate complex which proceeds to form the product.

The maximum rate of substrate hydrolysis when the enzyme is completely saturated with substrate (*Vmax*) and Michaelis constant (*Km*), which is the substrate concentration at half of the *Vmax*, were determined to characterize the type of inhibition exerted by the extracts using Lineweaver-Burk plots shown in **Figures 4.7A**, **B** and **C**.

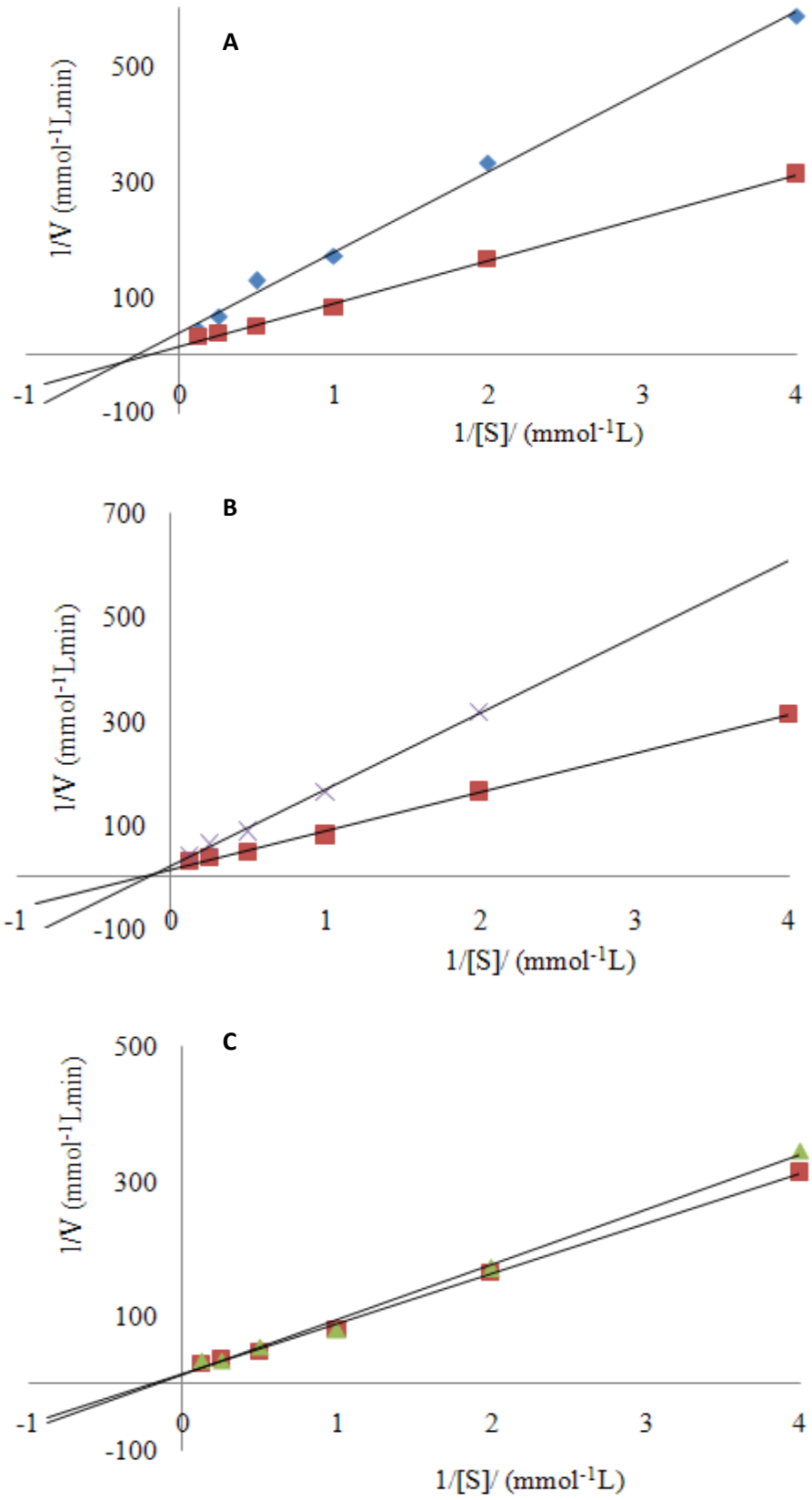


Figure 4.7. Lineweaver-Burk plots of (A) *A.macrosperma* fruit extract (B) Green kiwifruit extract and (C) Gold kiwifruit extracts compared to control (■).

As shown in **Figures 4.7A**, **B** and **C**, these plots have coinciding intercept on the 1/S axis indicating that the enzymatic inhibition of ACE was non-competitive (Liu et al., 2003). The *V*max values were markedly reduced while *Km* remained unchanged by each inhibitor which suggests that act as a non-competetive inhibitor for ACE (**Table 4.9)**.

Table 4.9. Kinetic prameters of the ACE inhibitory activity of extracts from different kiwifruit cultivars.

Extract/Compound	Concentration tested (mg/mL)	*K*m (mM)	V_{max} (mM/min)	*K*i (mg/mL)
No inhibitor	0	7.258	0.069	
A.chinesis cv Hort 16A	13.00	6.614	0.081	44.516
A.deliciosa cv Hayward	31.00	7.258	0.049	64.041
A. macrosperma	2.60	3.559	0.026	78.312

V_{max} is the maximum reaction rate, K_m is the Michaelis-Menten constant, and K_i is the dissociation constant

This means that the bioactives from the flavonoids rich extracts did not bind to the catalytic site of ACE but may have bound to other sites on the ACE molecules to produce an inactive complex (enzyme-substrate-inhibitor), irrespective of substrate binding. These findings are in agreement with the studies reported by Liu et al., (2003) on tannins and flavan-3-ols isolated from traditional Chinese herbs with non-competitive inhibition of ACE. The inhibition mechanism investigated using Lineweaver-Burk plots on lentil proteins by Barbana and Bye, (2011) also revealed a non-competitive inhibition on ACE.

ACE is a zinc-containing peptidyldipeptide hydrolase where the active site is known to have three parts; a carboxylate binding functionality such as the guanidinium group of arginine, a pocket that accommodates a hydrophobic side chain of C-terminal amino acid residues, and a zinc ion which coordinates to the carbonyl of the penultimate peptide bond of the substrate, whereby the carbonyl group becomes polarized and is subjected to a nucleophilic attack (Loizzo et al., 2007). It has been proposed that the *in vitro* ACE inhibitory activity of flavonoids may be due to the formation of chelate complexes with the zinc atom within the active centre of zinc-dependent mettalopeptidases or possibly by the formation of hydrogen bridges between the inhibitor/ phenolics and the active site (Wagner et al., 1991; Loisso et al., 2007; Ojeda et al., 2010). Furthermore, the ability of flavonoids to form chelates with the active centre of ACE depends on the substitution pattern of the hydroxy groups in the flavonoids (Elbi and Wagner, 1991; Guerrero et al., 2012). Thus, the presence of high phenolic and flavonoid content in the extract obtained from *A. macrosperma* fruit could have contributed towards ACE inhibition.

4.5. Conclusion

Several biological activities such as antioxidant, anticancer, anti-inflammatory, antimicrobial activities have already been reported for kiwifruit cultivars. Inhibition of angiotensin converting enzyme (ACE) is identified as a main therapeutic target to control high blood pressure. As far as we know, only one study supporting the use of kiwifruit species as antihypertensive has been published to date (Jung et al., 2005). The present study investigated the ACE inhibitory property of different flavonoid rich kiwifruit extracts (FKE) *in vitro* by using a fluorescence based biochemical assay. Based on our results, kiwifruit has potential use as a cardiovascular protective agent against high blood pressure. The kiwifruit

extracts obtained using different extraction solvents, techniques and cultivars were examined for their ACE inhibitory activity and results showed that they are moderately effective ACE inhibitors in *in vitro* models when compared with other plant extracts reported in the literature (Balasooriya and Rupasinghe, 2012). Among the results obtained in the present study, it should be highlighted that the activity observed for the extract from steeping with 70% aq. acetone exhibited quite promising ACE inhibitory activity (lowest IC_{50} values), as compared to the other extracts tested. Kinetic determinations suggested that flavonoids rich extracts from *A. macrosperma* fruit inhibit the enzyme activity by non-competitive binding with the substrate for the enzyme active site. Investigating the ACE enzyme inhibition by kiwifruits along with their kinetic parameters generated valuable information supporting the concept of health effects of flavonoids rich kiwifruits in general. Further studies are needed to isolate and to identify these bioactive compounds with active ACE inhibition from the most active extract from *A.macrosperma*.

Chapter 5

Extraction, separation, and identification

Extraction, bioassay guided separation, and identification of flavonoids from *A. macrosperma* L fruit using HPLC-ESI-MS/MS

5.1. Introduction

Many plants and herbs are considered to have antioxidant properties and are extensively studied for their beneficial effects on the health of animals and humans, based on their scavenging ability of free radicals generated by environmental and metabolic factors (Shahidi and Wanasundara, 1992; Kathirvel and Rupasinghe, 2012). Many of these antioxidant compounds, such as polyphenols, vitamin C, E and A, are present in fruits and vegetables that contribute most significantly to their total antioxidant capacity (Shahidi and Wanasundara, 1992; Matthaus, 2002).

The phenolic phytochemicals include simple phenols, benzoic and cinnamic acid derivatives, coumarins, tannins, lignins and flavonoids as described in Chapter 2 (Section 2.3.1) (Haslam, 1989; Khoddami et al., 2013). Flavonoids are derived from the aromatic amino acids, phenylalanine and tyrosine, and have three rings in the structures. They comprise one of the most diverse and wide groups of secondary metabolites occurring among natural products (Haslam, 1989). Flavonoids share a C6-C3-C6 backbone with two benzene rings (A and B) linked through a heterocyclic ring (C) (**Figure 5.1**) which is commonly glycosylated (at one or more sites with a variety of sugars) and they may also be alkoxylated or esterified. Flavonoids can be subdivided into six structural categories: flavones, flavonols, flavononol, flavanones, flavanols, and anthocyanidins as described in Chapter 2 (Section 2.3.1) (**Figure 5.1)**.

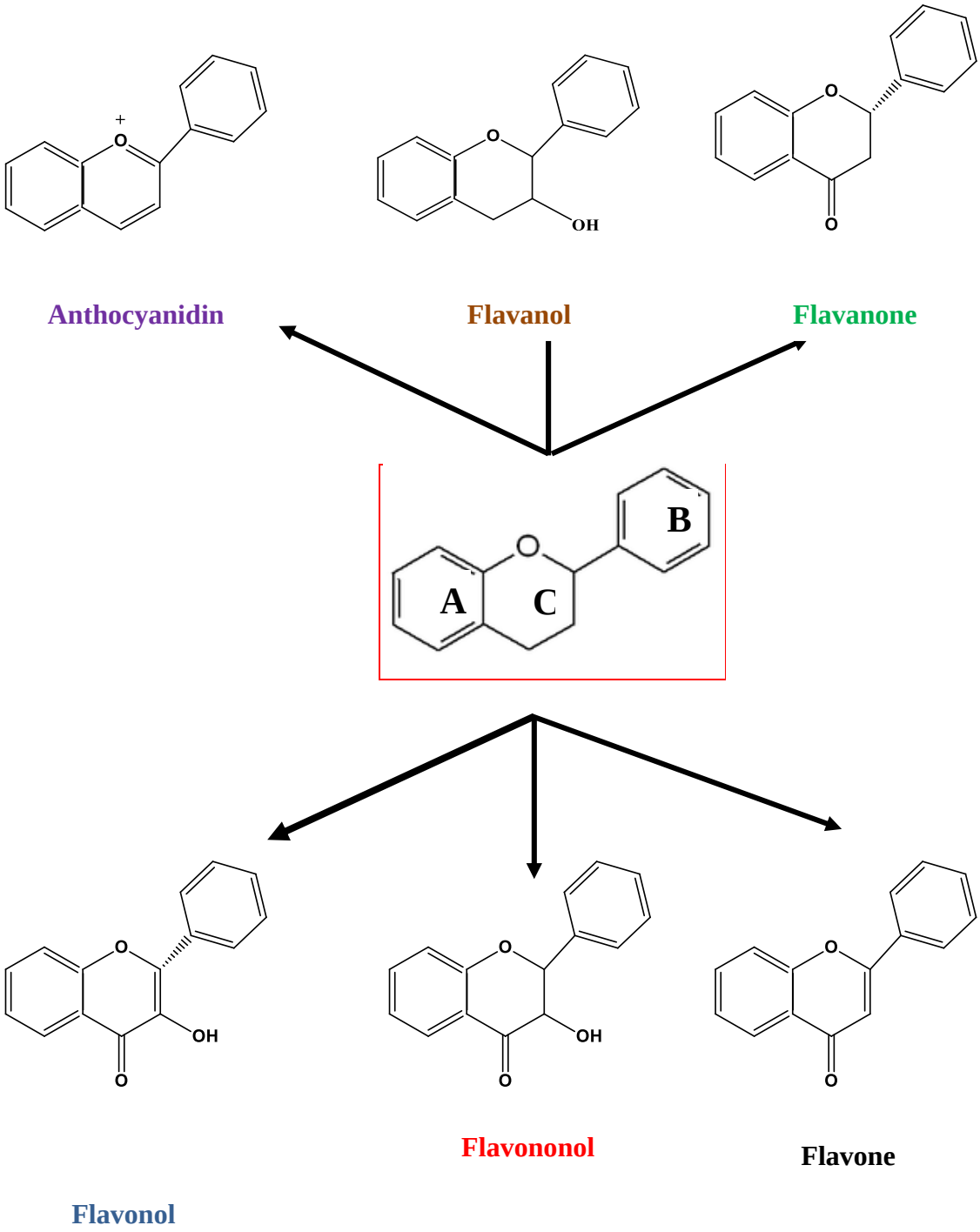


Figure 5.1. Flavan skeleton and subclasses of flavonoids.

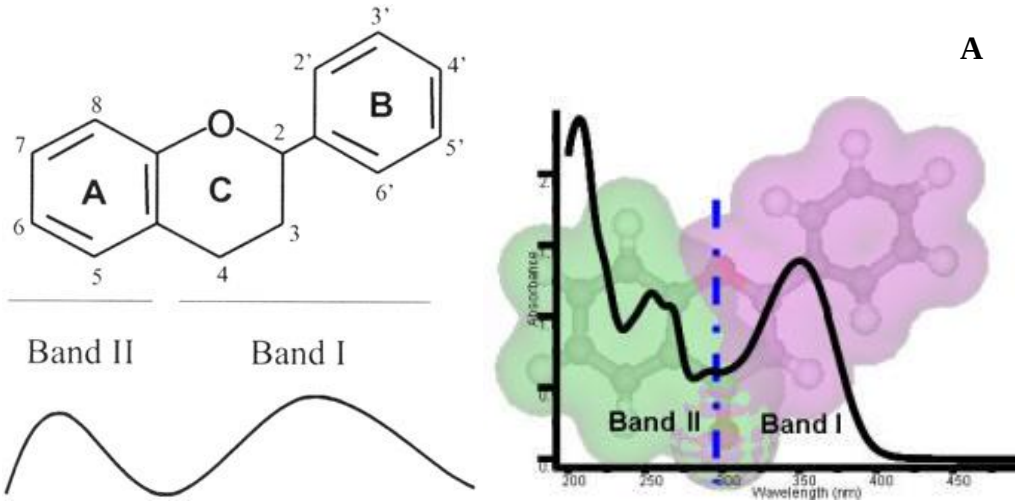
Research on the therapeutic potential of medicinal plants validates ethnopharmacological usage of plant materials, but also isolates and focuses on the characterization of the active components (Owen et al., 2003; Yu et al., 2006; Meena and Patni, 2008; Abadio Finco et al., 2012; Sampath and Vasanthi, 2013). Therefore, interest in

the role of phenolic antioxidants in human health has prompted studies on the separation, isolation and characterization of active phenolic components in various plant-derived foods (Yu et al., 2006; Gavrilova et al., 2011; Abadio Finco et al., 2012). Various chromatographic methods available for separation (liquid-liquid chromatography, solid phase chromatography), isolation {semi preparative high-performance liquid chromatography, open column chromatography, flash column chromatography, high speed counter current chromatography (HSCCC)}, and identification {high-performance liquid chromatography coupled with diode array detection (HPLC-DAD), gas chromatography (GC), mass spectroscopy, nuclear magnetic resonance spectroscopy (NMR)} are reported in the literature (Zhang et al., 2001; Owen et al., 2003; Sun et al., 2006; Liu and Zhu, 2007; Wu et al., 2007; Jeffery et al., 2008; Ana et al., 2009; Sun et al., 2009; Latocha et al., 2010; Abadio Finco et al., 2012; Dipjyoti, 2013).

The most frequently used analytical technique for the separation of phenolic compounds is high-performance liquid chromatography (HPLC), with diode array detection (DAD) for routine analysis and mass spectroscopy (MS) as a more sophisticated method especially for identification of phenolic compounds (Khoddami et al., 2013). High performance liquid chromatography coupled with a UV-visible photodiode-array detector (HPLC-DAD) provides characteristic UV-visible spectra which can characterize flavonoid compounds in crude or semi-purified plant extracts (Alonso-Salces et al., 2004; Latocha et al., 2010).

The UV-visible spectra of phenolic compounds are mainly composed of two bands named Band I (300-380 nm) and Band II (240-280 nm) (**Figure 5.2A**) (Alonso-Salces et al., 2004; Seijas and Vzquez-Tato, 2012). Band I is considered to be associated with the absorption due to the structure of B-ring cinnamoyl system, and Band II with the absorption

involving the A-ring benzoyl system in their flavan skeleton (**Figure 5.2B**) (Alonso-Salces et al., 2004). Thus, hydroxybenzoic acids are detected at 280 nm, hydroxycinnamic acids are at 300-320 nm, flavonols and ellagic acid at 330-365 nm, anthocyanidins at 500-520 nm and flavan-3-ols such as catechin, epicatechin and proanthocyanidins at 280 nm (**Figure 5.3**).



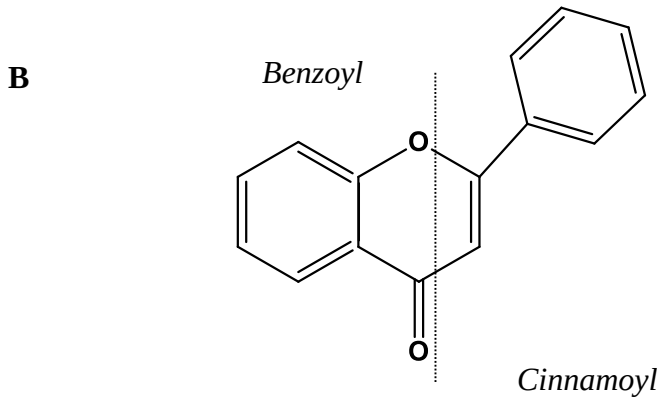


Figure 5.2. The spectra formed by (A) Band I and Band II (adapted from Seijas and Vzquez-Tato, 2012) related to the (B) structure of the flavan skeleton.

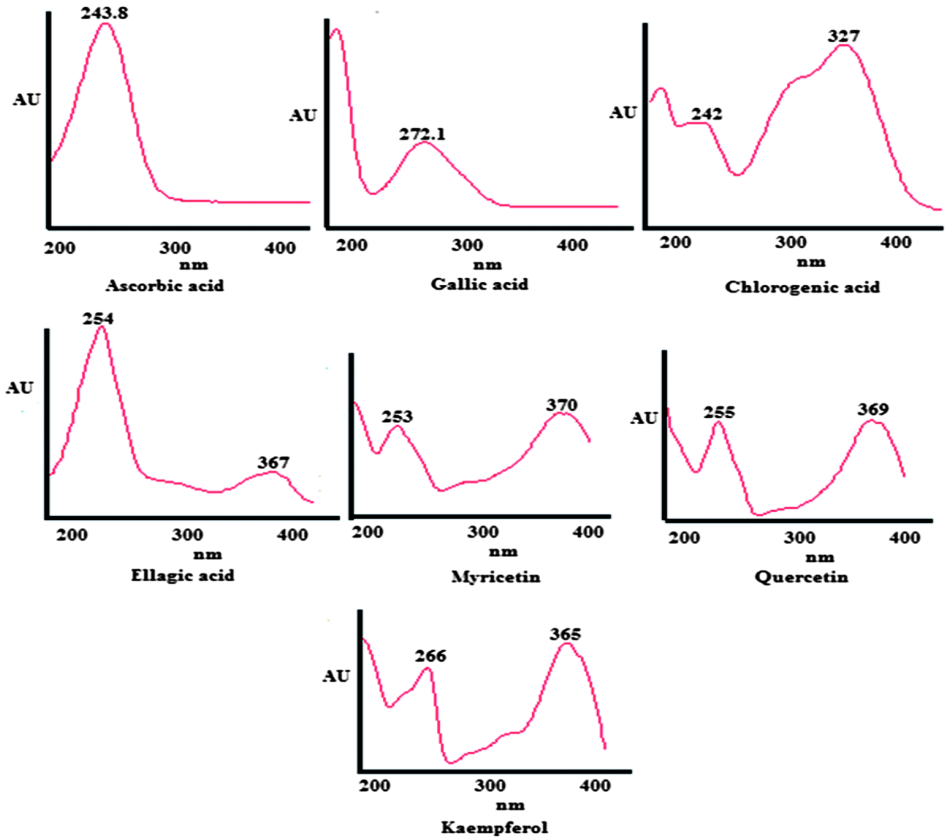


Figure 5.3. Characteristic UV-visible spectra representing different phenolic compounds and ascorbic acid (adapted from Bansal et al., 2013).

However, the identification of non-phenolic moieties in phenolic compounds, such as sugars or aliphatic acyl groups which do not have strong UV chromophores is difficult. Therefore, while UV-visible spectra of phenolic compounds are valuable for preliminary identification and for quantification using the characteristic absorption maxima (Dawes and Keen, 1999; Latocha et al., 2010), combining these data with mass spectral data and information from the literature can contribute greatly to structural characterization of such phenolic compounds (Zhang et al., 2001; Owen et al., 2003; Sun et al., 2006; Yu et al., 2006; Jeffery et al., 2008; Ana et al., 2009; Sun et al., 2009; Abadio Finco et al., 2012; Dipjyoti, 2013).

Electrospray ionization (ESI) is a commonly used soft ionization technique for mass spectrometry that provides the pseudomolecular masses of analytes in the positive and/or negative mode (Zhang et al., 2001; Owen et al., 2003; Sun et al., 2006; Yu et al., 2006; Liu and Zhu, 2007; Jeffery et al., 2008; Ana et al., 2009). Ionization in the positive ion mode is used mainly for the detection of anthocyanins in their native forms whereas hydroxybenzoic and hydroxycinnamic acids, flavonol glycosides and condensed tannins show good response in the negative ionization mode (Inbaraj et al., 2010; Kajdzanoska et al., 2010; Gouveia and Castilo, 2011).

As reported in the literature, the large number of compounds typically present in plant extracts often result in the co-elution of multiple compounds during HPLC separation, making identification impossible with single-step mass spectrometers (Alonso-Salces et al., 2004; Aaby et al., 2007; Inbaraj et al., 2010; Latocha et al., 2010). This can be circumvented by the use of more advanced methods such as tandem mass spectrometry (MS^n) which provides structural details of the phenolic compounds, since the mass spectrometer is tuned to simultaneously identify multiple compounds in different molecular weight ranges with various fragmentation patterns (Zhang et al., 2001; Owen et al., 2003; Sun et al., 2006; Yu et al., 2006; Jeffery et al., 2008; Ana et al., 2009).

There is only one reported research study in the literature on the identification of the phenolics in the fruit of *A. macrosperma,* and it identified some phenolics such as catechin, epicatechin, quercetin, tannic acid, gentisic acid, hydroxy benzoic acid, chlorogenic acid, *p*-coumaric acid and caffeic acid by using HPLC-DAD analysis (Latocha et al., 2010). However, phenolic compounds such as the flavonoids and their glycosides present in this fruit were not characterized due to the co-elution of multiple compounds (Latocha et al., 2010). Therefore, the objective of this chapter was to use HPLC-ESI-MS^n for the possible

identification of potent antioxidant, undiscovered phenolic compounds in the previous study carried out by Latocha et al., (2010) in *A.macrosperma* fruit. Isolation of some selected flavonoid compounds was carried out by flash chromatography and size exclusion chromatography followed by semi-preparative HPLC. Structural information on isolated compounds was obtained using nuclear magnetic resonance spectroscopy where required.

5.2. Plant materials and chemicals

The fruits of *A. macrosperma* were collected at the Plant and Food research orchard in Te Puke Bay, New Zealand as mentioned in the Section 3.2. in Chapter 3. The samples were prepared by grinding the lyophilized fruit samples with a mortar and pestle prior to the extraction.

Folin-Ciocalteu phenol reagent, iron (III) chloride 6-hydrate, hydrochloric acid, catechin, 2,2-diphenyl-2-picylhydrazyl (DPPH), 2,4,6-tripyridyl-s-triazine (TPTZ), Trolox, and *p*-dimethylaminocinnamaldehyde (DMACA) were purchased from Sigma, St Louis, USA. Sodium carbonate, sodium hydroxide, sodium nitrite, formic acid and aluminium hexahydrate, were from Scharlau, Spain. Gallic acid (ACROS, USA), iron (II) sulphate-7-hydrate (BDH Chemicals, England), HPLC grade methanol, HPLC grade acetonitrile, ethanol, methanol, acetone, ethyl acetate, chloroform, and hexane and all other chemicals were purchased from ECP Ltd, Auckland, New Zealand. Deuterated methanol (MeOH-d_4) and dimethylsulfoxide (DMSO-d_6) were purchased from Cambridge Isotope Laboratories, USA.

5.3. Methodology

Schematic diagram of the experimental design used for the extraction and separation was presented in **Figure 5.4**.

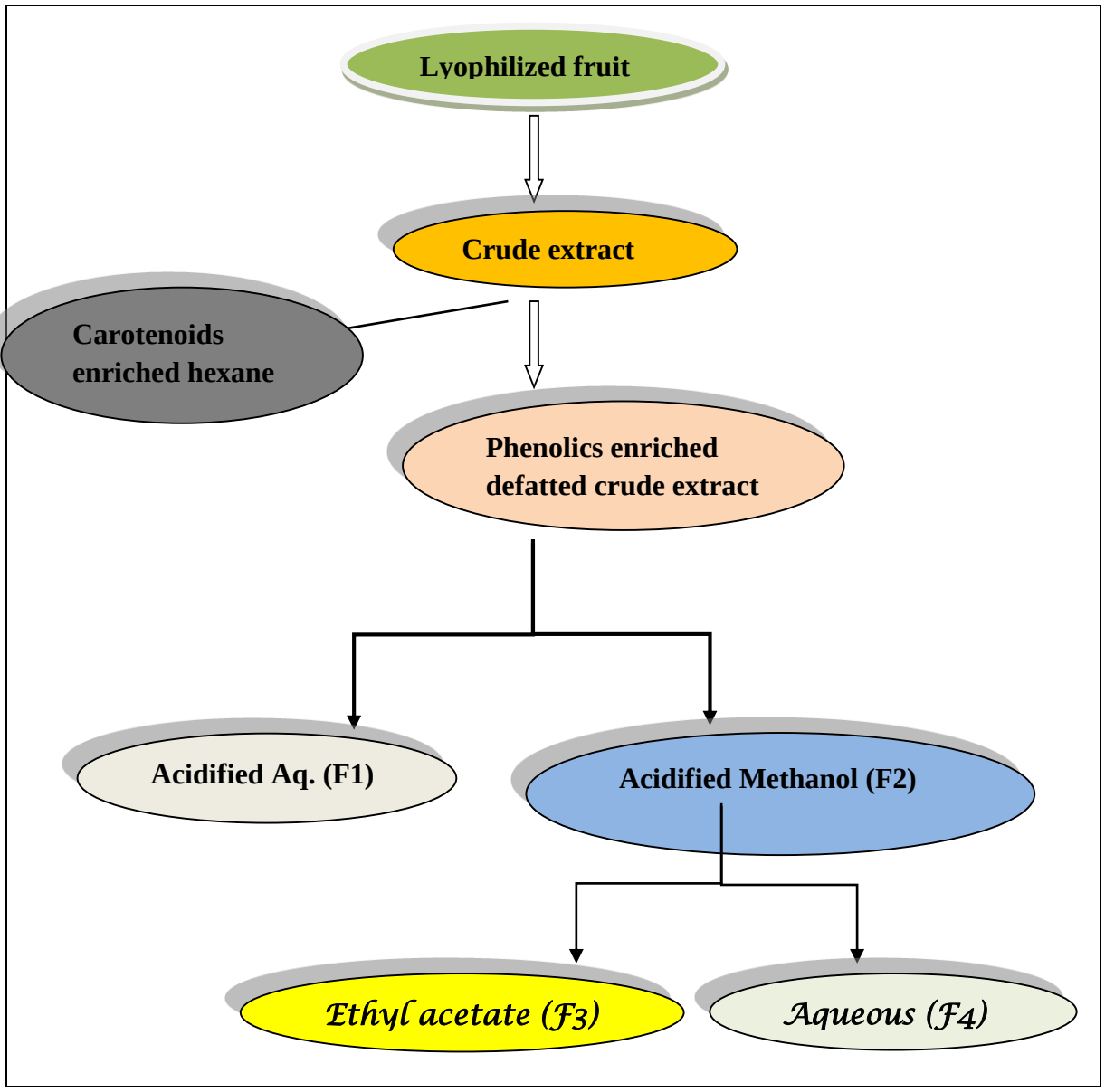


Figure 5.4. Experimental design for the extraction, and separation of the *A. macrosperma* fruit extract.

5.3.1. Extraction of phenolics

Phenolic compounds were extracted by steeping lyophilized ground fruit sample (500 g) in optimized solvent (70% aq. acetone) (500 mL x 5) in a Scott Duran bottle for 6 h in the dark with nitrogen gas purging at room temperature (23±2 °C) to prevent phenolic oxidation during extraction as described in Chapter 3. The extracts were then filtered through a sintered glass filter and collected into a conical flask in an ice bath. The residue was subjected to re-extraction, and the filtrates were combined together and concentrated on a rotary evaporator (Buchi, Switzerland) below 35 °C under vacuum. Partitioning of phenolic compounds between water and organic (hexane) layers was carried out using a separating funnel to remove carotenoids and unwanted substances from the crude extract which might interfere with the identification of phenolic compounds. The UV-visible spectra of the carotenoids-enriched hexane phase and phenolics-enriched aqueous phase were scanned in the range of 250 nm-700 nm (UV-160 Shimadzu, double beam spectrophotometer, Japan) to ensure the presence of phenolics in the aqueous layer and carotenoids in the hexane layer. The mass and volume of the concentrated filtrate (aqueous crude extract) were recorded and freeze dried then stored at -80 °C for subsequent steps.

5.3.2. Bioassay guided fractionation by vacuum manifold solid phase extraction (VSPE)–Strata® SPE C_{18}

The defatted crude extract could contain sugars, sugar derivatives, phenolic compounds and any other hydrophilic compounds extracted by the optimized extraction solvent (70% aq. acetone) which was described in Chapter 3 (Section 3.4). These compounds were separated into different fractions depending on their polarity and the acidity using Strata® C_{18} SPE cartridge (12 mL, SPE; Phenomenex, New Zealand) on a vacuum manifold

according to the method described by Sun et al., (2006) with some modifications of the solvents selected for the separation. The C_{18} SPE cartridges were preconditioned with methanol (20 mL) and conditioned with 2% formic acid in Milli-Q-water (100 mL). The freeze dried defatted crude extract (2.220 g in 3 ml of 2% formic acid Milli-Q-water) was applied to the SPE cartridge and 2% formic acid in Milli-Q-water (100 mL) was passed through the SPE cartridge to elute sugars and highly polar compounds such as phenolic acids into fraction F1 (**Figure 5.4**). The rest of the compounds remaining on the cartridge were eluted with 2% formic acid in methanol (100 mL), giving fraction F2. The solvents from the fractions F1 and F2 were removed on a rotary evaporator below 35 °C under vacuum and subjected to freeze drying and then were stored at -80 °C. This process was repeated several times on two SPE cartridges to attain enough material for the liquid-liquid extraction.

5.3.3. Liquid- liquid extraction

The phenolic profiles and antioxidant capacity determinations described in Section 5.3.11 showed that the acidified methanol fraction (F2) was rich in flavonoids and had stronger antioxidant activity than the acidified aqueous fraction (F1). Therefore, the acidified methanol fraction (F2) was selected for further fractionation using liquid-liquid extraction (**Figure 5.4**) as described by Li et al., (2013b). The sample of F2 for the liquid-liquid extraction was prepared by reconstituting the combined freeze dried fractions (F2) (3 g) in a minimum volume of methanol (approximately 5 mL) as the F2 fraction was partially soluble either in water or ethyl acetate, and the solvent was removed on a rotary evaporator below 35 °C under vacuum. Then liquid-liquid extraction of the sample was carried out between ethyl acetate and water in a separatory funnel several times until the ethyl acetate upper layer became colourless. The combined upper ethyl acetate layers (yellow colour)

which was rich in moderately polar compounds and the lower aqueous layer (red) which was rich in more polar compounds were collected separately (F3 and F4 respectively) then concentrated using a rotary evaporator below 35 °C under vacuum. Fractions F3 and F4 were subjected to freeze drying and stored at -80 °C. This process was repeated several times to get enough material for the sub-fractionation step described in Section 5.3.4.

5.3.4. Sub-fractionation of the ethyl acetate fraction (F3) using silica gel column chromatography

The ethyl acetate fraction (F3) (211 mg) was subjected to column chromatography on silica gel 922 (200-325 mesh) (ECP, Auckland, New Zealand) according to the method described by Li et al., (2013b) with some modifications of elution gradient system used. A silica gel slurry prepared by mixing 25 g of silica gel 922 adsorbent (200-300 mesh) in chloroform ($CHCl_3$, 50 mL) was packed in a glass column (29 cm x 3 cm, i.d) using nitrogen under the wet loading method. The column was then well conditioned with chloroform (250 mL) before applying the sample. The sample (F3) to be analysed was prepared by the dry loading method by mixing, F3 (211 mg) in methanol (5 ml) with silica gel 922 (3 g) followed by concentrating on rotary evaporator below 35 °C under vacuum. The dried sample was applied to the top of the column and a small amount of sand was added to the top of the column to prevent it from being disturbed when fresh solvent was added. After elution with chloroform, a gradient of chloroform/acetone (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 0:10) was applied with an increasing amount of acetone to collect 11 fractions (fractions F3a to F3k). All the compounds remaining in the column were eluted with 100% methanol (100 x 3 mL) and three fractions were collected ($F3L_I$-$F3L_{III}$). The fractions F3a-F3k and $F3L_I$-$F3L_{III}$ collected were concentrated and subjected for HPLC-DAD analysis as described in

Section 5.3.6. Fractions ($F3L_I$-$F3L_{III}$), which showed similar phenolic profiles on HPLC-DAD analysis were pooled together to get the fraction F3L. This fraction (F3L) revealed on the HPLC-DAD chromatogram at 360 nm a pool of flavonoids which had not been reported in the previous study on this fruit carried out by Latocha et al., (2010). Therefore, this fraction (F3L) was selected for further purification with semi-preparative HPLC to obtain flavonoids from selected peaks (P1, P2, P3 and P4) prior to mass spectroscopic analysis.

5.3.5. Sub-fractionation of the aqueous fraction (F4) using Size exclusion chromatography

The lyophilized aqueous fraction (F4) (800 mg) was dissolved in water (3 mL) and chromatographed on Sephadex LH-20 (29 cm x 3 cm i.d.) (Amersham Biosciences, Uppsala, Sewden) which was conditioned with water (500 mL), as described by Li et al., (2013b) with some modifications of elution gradient system used. The compounds were serially eluted with water (100%, 100 mL), water:methanol (80:20%, 100 mL), water:methanol (50:50, 200mL), water:methanol (20:80%, 100 mL), methanol (100%, 200 mL) and water:acetone (30:70%, 100 mL) to derive fractions FW1, FW2, FW3, FW4, FW5 and FW6 respectively. All the fractions FW1-FW6 were concentrated and subjected to HPLC-DAD analysis as described in Section 5.3.6. The fraction collected with water: methanol (5:5) (FW3) which showed characteristic UV-visible spectra of flavonols on a HPLC-DAD chromatogram at 360 nm was selected for further purification on semi-preparative HPLC prior to mass spectroscopic analysis.

5.3.6. Analytical high performance liquid chromatography coupled to diode array spectrophotometry (HPLC-DAD) analysis

The phenolic profiles in all fractions were qualitatively determined according to the procedure described by Gheldof et al., (2002) with slight modifications as described in Section 3.3.6 in Chapter 3.

5.3.7. Semi-preparative HPLC analysis

Semi-preparative HPLC was conducted on an Agilent 1100 liquid chromatograph with a Synergy fusion-RP (reversed-phase) C_{18} column (150 x 10 mm, I.D. 4 µm particle size, 80 Å) (Phenomenex, Auckland, New Zealand). The HPLC system consits of a column oven (35 °C), auto sampler, vacuum solvent degasser and diode-array detector (DAD) set at 280, 320 and 360 nm. A binary mobile phase system similar to that used for analytical HPLC (Section 3.3.6 in Chapter 3) consisting of solvent (A) 0.1 % formic acid in water (v/v) and (B) 100 % methanol was employed. The gradient programme was used with the following proportions of solvent A applied at time *t* (min) (*t*, A%): (0, 95%), (10, 85%), (15, 70%), (20, 60%), (30, 55%), (40, 40%), (45, 50%), (55, 80%), (58, 95%). The flow rate of the mobile phase was scaled up to 3.8 mL/min and the volume of the injected sample was 80 µL (maximum 100 µL) of each fraction which was filtered through a 0.45 µm filter prior to HPLC injection. Peaks eluting from the column were collected on an Agilent Micro plate sampler. Similar fractions obtained after several runs on semi preparative HPLC were pooled and solvents were evaporated prior to freeze drying before mass spectral analysis.

5.3.8. Liquid chromatography coupled to mass spectrometry (LC-ESI-MS) analysis

LC-ESI–MS analysis of ethyl acetate (F3) and aqueous (F4) fractions was performed on a Shimadzu series LC-MS 2020 system equipped with a degasser, a binary pump, an auto sampler, and a column oven. The detector of this system was an ESI-quadruple mass spectrometer. The analysis was carried out on Synergy fusion RP_{18} (150 x 4.6 mm, I.D. 4 µm particle size, 80 Å) column (Phenomenex, New Zealand) combined with a Phenomenex synergy fusion guard column (4.6 x 2 mm, 4 µm). The column oven temperature was set to 30 °C. A binary mobile system was employed consisting of solvent (A) 0.1 % formic acid in water (v/v) and (B) 100 % methanol. The gradient programme was used with the following proportions of solvent A applied at time *t* (min) (*t*, A%): (0, 95%), (10, 85%), (15, 70%), (20, 60%), (30, 55%), (40, 40%), (45, 50%), (55, 80%), (58, 95%). The flow rate of the mobile phase was 0.2 mL/min and 20 µL of each sample prepared in methanol with a concentration of 5 mg/mL (DW/volume) was injected. Samples were analysed by MS under full scan mode (*m/z* 50-1000) in the positive and negative ionization modes.

5.3.9. Liquid chromatography coupled to mass spectrometry (LC-ESI-MS/MS) analysis

High resolution mass spectrometric analysis of flavonoid fractions isolated on semi-preparative HPLC were performed at AgResearch, Palmerston North, New Zealand according to the procedure reported by Fraser et al., (2013). All analyses were performed using the Thermo LC-MS system (Thermo Fisher Scientific, Waltham, MA, USA) consisted of an Accela 1250 quaternary UHPLC pump, a PAL auto sampler fitted with a 15 000 psi injection valve (CTC Analytics AG, Zwingen, Switzerland), 20 µL injection loop, and an

Exactive Orbitrap mass spectrometer with electrospray ionization. An aliquot of 2 µL of each sample was injected on an Agilent RRHD SB-C_{18} column (150 mm x 2.1 mm, 1.8 µm) at 25 °C with a gradient elution programme and a flow rate of 400 µL/min. A binary mobile system was employed consisting of solvent (A) water-formic acid (99.9:0.1, v/v) and solvent (B) acetonitrile-formic acid (99.9:0.1, v/v). The gradient elution programme was as follows: held at 5% B (0-0.5 min), 5-99% B (0.5-13 min), held at 99% B (13-15 min), returned to 5% B (15-16 min) and allowed to equilibrate for further 4 min prior to the next injection. Mass spectral data were collected in profile mode over the mass range *m/z* 60-1200, at a mass resolution setting of 25 000 with a maximum trap fill time of 100 ms using the Xcalibur software. Samples were run using electrospray ionization in negative ion mode (ESI-) with the following conditions (spray voltage -3500 V, capillary voltage -90 V, and nebulizer gas (N_2).

High resolution mass spectra of compound 11 were recorded on a Bruker MicrOTOF-QII mass spectrometer over the mass range *m*/*z* 50-2900. Samples were run using electrospray ionization in negative ion mode (ESI-) with the following conditions (capillary voltage 3200 V, dry heater 180 °C, nebulizer gas (N_2, 0.4 Bar).

5.3.10. NMR spectroscopy

NMR spectra were recorded on a Bruker DRX-400 spectrometer operating at 400 MHz for ^{1}H nuclei and ^{13}C nuclei. All chemical shifts are reported in parts per million (ppm) relative to tetramethyl silane. ^{1}H NMR data are reported as chemical shift, multiplicity and coupling constant (*J* Hz). Assignments were made with the aid of one dimensional (1D), two dimensional (2D) ^{1}H NMR and ^{13}C NMR where required. The 2D ^{1}H NMR experiments

were correlated spectroscopy (COSY) and heteronuclear single quantum correlation (HMQC).

5.3.11. Determination of phenolic profile and antioxidant capacity

The Folin-Ciocalteu assay was performed to estimate the total phenol content (TP) of all fractionated samples (**Table 5.1**) as described by Singleton and Rossi, (1965), modified by Jayaprakasha et al., (2001), as mentioned in Section 3.3.3 in Chapter 3.

Table 5.1 Preparation of samples for bioassays.

Sample type	Concentration (in water) (mg/mL) (DW/volume)			
	TP	TFO	DPPH	EC_{50}
Crude extract	50	50	5	1-5
Defatted crude	50	50	5	1-5
F1	50	50	5	1-5
F2	5	5	1	0.06-1
F3	7.5	7.5	1	0.06-1
F4	15	7.5	1	0.06-1

TP: total phenolic content, TFO: total flavonoid content, DPPH: 2,2-diphenyl-2-picrylhydrazyl and EC_{50}: efficient radical scavenging concentration

The total flavonoid content (TFO) was estimated by the aluminium chloride colorimetric method described by Marinova et al., (2005), slightly modified by Park et al., (2008) and Du et al., (2009) as described in Section 3.3.4 in Chapter 3. The total flavanol (TFA) contents of fractions ethyl acetate (F3) and aqueous (F4) with the concentrations of

10 and 5 mg/mL (DW/volume) were determined according to the *p*-dimethylaminocinnamaldehyde (DMACA) method used by Du et al., (2009) with slight modifications as described in the Section 3.3.5 in Chapter 3. The antioxidant capacity of all extracts was determined using DPPH assay according to the method described by Brand-Williams et al., (1995) modified by Du et al., (2009), as described in Section 3.3.7 in Chapter 3.

5.3.14. Statistical analysis

All measurements obtained for phenolic profiles and antioxidant capacities were conducted in triplicate and the results are expressed as mean±SD. The effect of the fractionation and purification steps on the total phenol content, total flavonoid content and antioxidant capacity values were analyzed by analysis of variance (ANOVA) using OriginPro8 software. Pairwise multiple comparisons were evaluated by Tukeyss' significance difference test in OriginPro8. Differences at $p = 0.05$ were considered significant.

5.4. Results and discussion

5.4.1. Sample preparation and extraction

Phenolic compounds from lyophilized *A. macrosperma* fruit (500 g) were extracted into 70% acetone by steeping the lyophilized fruit sample according to the scaled up optimized extraction method described in Chapter 3. The preparation of an extract consisted of disintegrating and homogenizing the sample followed by transferring the compounds of interest to a suitable solvent. The extraction step was especially delicate when dealing with reactive compounds like phenols. To overcome the detrimental effect of air causing oxidation, the homogenization was carried out by steeping the lyophilized fruit sample of *A. macrosperma* in the extraction solvent under a nitrogen gas purge. Polar compounds such as phenolic compounds are vacuole–bound in living plant cells (Haslam, 1989). During freeze drying and homogenization, the vacuoles distort or disrupt, allowing the formation of strong hydrogen bonds between phenolic compounds and other molecules such as proteins, polysaccharides and nucleic acids. Therefore, phenolic extracts of plant materials are always a mixture of different classes of chemicals that are soluble in the solvent system used (Gabriela, 1999). Thus, an additional step of partitioning the crude extract (292 g) with hexane was carried out to remove carotenoids and other lipophilic compounds that could be present in the fruit matrix. The two immiscible phases of phenolic-enriched defatted lower aqueous phase and carotenoids–enriched upper hexane phase were collected separately. The mass of the lyophilized phenolic-enriched defatted crude extract was recorded (222.0 g). The UV-visible spectra scanned in the range of 250 nm-700 nm showed a strong peak at 280 nm for the phenolic rich phase and several peaks between 400-600 nm were observed for the carotenoid–enriched upper phase which is consistent with a high carotenoid content (**Appendix 7).** Some of the carotenoids were identified by comparison with a xanthophyll

(mixture of lutein and β-carotene) standard, ran under the same conditions on a UV-vis spectrophotometer. Recent studies have reported that the fruit of *A. macrosperma* is rich in carotenoids (lutein, β-carotenes, zeaxanthin, violaxanthin) and chlorophylls (chlorophyll a and b) (MacGie and Ainge, 2002; Latocha et al., 2010).

5.4.2. Fractionation by solid phase extraction technique on C18 Strata cartridges

The phenolics–enriched defatted crude extract (2.22 g) was fractionated by solid phase extraction technique on reverse phase silica bonded C18 Strata cartridges on a vacuum manifold. The colourless acidified aqueous (F1, 2.01 g) and red coloured acidified methanol (F2, 0.21 g) fractions were obtained from a single cartridge in a single run. This process was repeated on two cartridges several times (**Appendix 8**)**.** The phenolic profiles and antioxidant capacities were determined for lyophilized crude extract, defatted crude extract, F1 and F2 fractions followed by HPLC-DAD analysis. Total phenolic content, total flavonoid content and antioxidant activity determined for the acidified methanol fraction (F2) (476.3±12.7 mg GAE/100 g DW, 106.7±7.5 mg CAE/100 g DW and 2.9±0.1 mmol Trolox equivalents/100 g DW respectively) were significantly (p=0.05) higher than those for the acidified aqueous fraction (F1) (363.2±18.9 mg GAE/100 g DW, 46.2±0.7 mg CAE/100 g DW and 2.0±1.0 mmol Trolox equivalents/100 g DW respectively) (**Table 5.2**).

Table 5.2. Phenolic profiles and antioxidant capacity of crudes and fractions obtained from *A. macrosperma* fruit.

Sample type	TP[a]	TFO[b]	DPPH[c]	EC_{50}[d]
Crude	695.9±24.0[p]	58.2±8.6[p]	6.0±0.1[p]	11.67
Defatted crude	823.1±14.4[q]	170.9±1.9[q]	5.1±0.1[q]	12.98
F1	363.2±18.9[r]	46.2±0.7[r]	2.0±1.0[r]	49.99
F2	476.3±12.7[s]	106.7±7.5[s]	2.9±0.1[s]	1.95
F3	84.1±5.0[t]	41.2±1.2[t]	0.5±0.1[t]	2.40
F4	189.9±10.7	58.1±3.8	1.9±0.1	1.91

[a]Total phenolic content is expressed as mg GAE/100 g DW

[b]Total flavonoid content is expressed as mg CAE/100 g DW

[c]Antioxidant activity assessed by DPPH is expressed as mmol Trolox equivalents/100 g DW

[d]Radical scavenging activity (EC_{50}) by DPPH assay is expressed as mg/mL DW

Results are expressed as mean±standard error. Means followed by the same letter in a column are not significantly different at p= 0.05.

HPLC-DAD chromatograms along with the UV-vis spectra obtained at 360 nm showed that the phenolic compounds present in defatted crude extract were successfully fractionated on C_{18} cartridges (**Figures 5.5 A, B & C**). Compounds with shorter retention times such as phenolic acids (maximum absorption wavelength between 300-320 nm), were eluted with acidified water (fraction F1) (**Figure 5.5B**) while compounds with longer retention times such as flavonols and their glycosides (maximum absorption wavelength between 330-360 nm) were eluted with acidified methanol (fraction F2) as shown in **Figure 5.5C.**

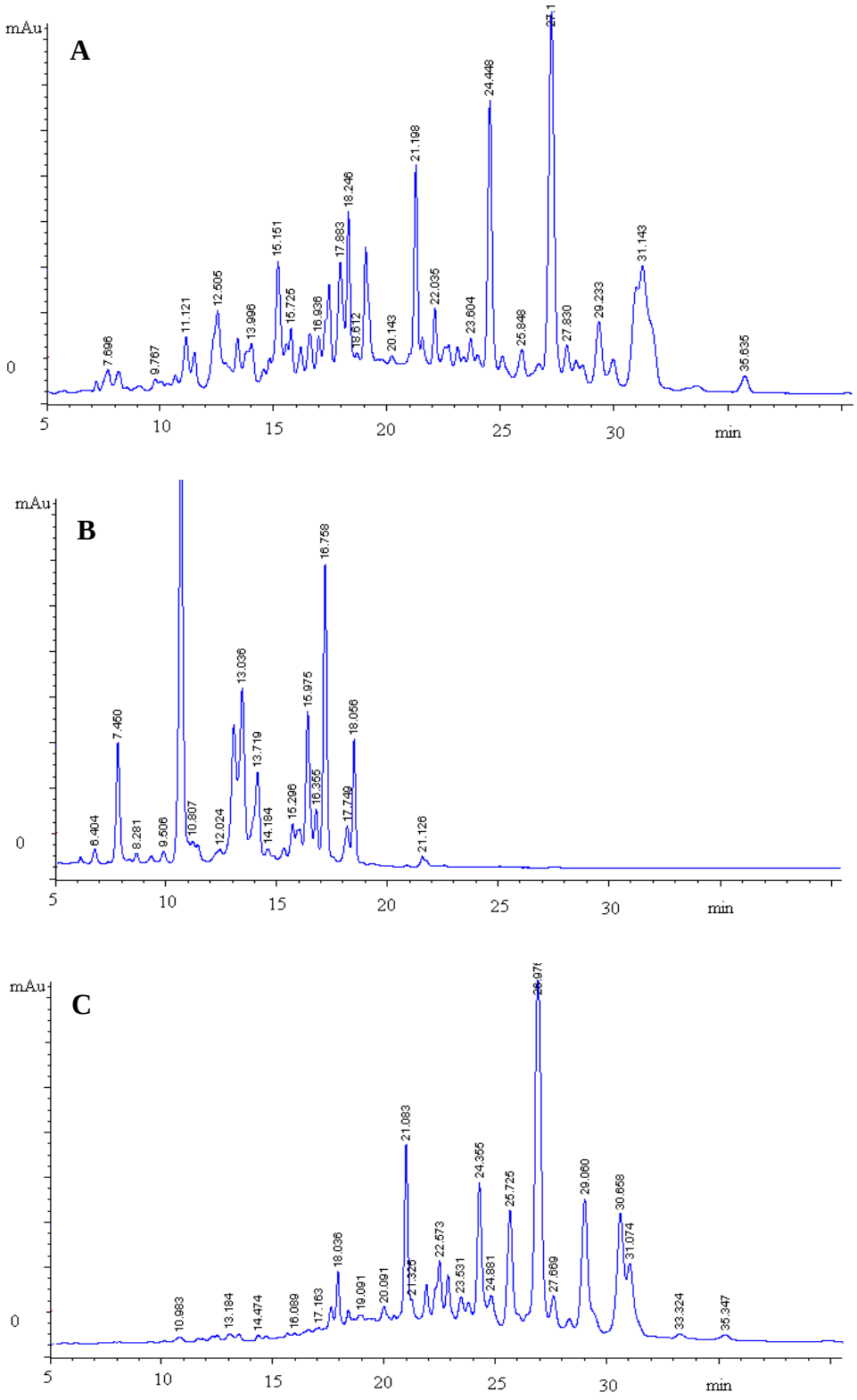


Figure 5.5. HPLC-DAD chromatograms of (A) defatted crude, (B) acidified aqueous fraction-F1 and (C) acidified methanol fraction-F2 recorded at 360 nm.

The elution order of phenolic compounds on reverse phase mode has been described previously (Abad-Garcia et al., 2009). More polar phenolics elute at the beginning of the chromatogram, with the following order: hydroxybenzoic acids, flavan-3-ols, hydroxycinnamic acids, coumarins, flavanones, dihydrochalcones, flavonols and flavones. Within the same polyphenol class (a) the retention time decreased as more hydroxyl groups were present in the polyphenol, (b) if the polyphenol contained less polar substituents, such as methoxy groups, the retention time increased, (c) if the chemical structures of polyphenolic compounds include sugars, the polyphenols eluted before their aglycones (Abad-Garcia et al., 2009). Our observations are in agreement with research on the phenolic composition of kiwifruit juice extracted from *A. deliciosa cv*. Hayward using a similar fractionation process on SPE cartridges as Dawes and Keene, (1999). Using HPLC-DAD analysis, they characterized strongly acidic phenolics such as *p*-coumaric acid, protocatechuic acid, syringic aldehyde, and derivatives of coumaric acid were eluted into acidified water, and weakly acidic phenolic compounds such as catechin, epicatechin, procyanidins, dimeric and tetrameric flavanols, quercetin glucoside, quercetin rutinoside, quercetin rhamnoside, kaempferol rhamnoside and kaempferol rutinoside were eluted with acidified methanol (Dawes and Keene, 1999).

5.4.3. Application of column chromatography, size exclusion and semi-preparative HPLC

The most active fraction F2 obtained after repeated runs on two SPE cartridges was selected for further studies on the purification and identification of flavonoids in *A. macrosperma* fruit. Fraction F2 (3 g) was partitioned between ethyl acetate and water to obtain fractions F3 (615 mg in a single run) which showed strong yellow colour representing

flavonols and F4 (2.31 g in a single run) which showed red colour representing flavan-3-ols (**Appendix 9**). The total phenolic content, total flavonoid content, total flavanol content and antioxidant activity (189.9±10.7 mg GAE/100 g DW, 58.1±3.8 mg CAE/100 g DW, 140.2±3.5 mg CAE/100 g DW, 1.9±0.1 mmol Trolox equivalents/100 g DW respectively) showed by the aqueous fraction (F4) were significantly (p=0.05) higher than those of the ethyl acetate fraction (F3) (84.1±5.0 mg GAE/100 g DW, 41.2±1.2 mg CAE/100 g DW, 16.1±1.9 mg CAE/100 g DW, 0.5±0.1 mmol Trolox equivalents/100 g DW respectively). The significantly high antioxidant activity determined for the aqueous fraction (F4) could be due to the higher total flavanol content in F4. Monomeric (catechin, epicatechin), dimeric, trimeric and polymeric (proanthocyanidins) flavanols are all well known antioxidants (Haslam, 1989; Gu et al., 2006).

HPLC-DAD chromatograms obtained from F3 and F4 are shown in **Figures 5.6** and **5.7**. The labelled flavonoid peaks on the HPLC-DAD chromatograms obtained from F3 (P1, P2, P3 and P4) in **Figure 5.6** and F4 (P5) in **Figure 5.7** do not correspond with the previously reported flavonoids in this fruit such as quercetin (maximum absorption wavelength, λ_{max} 256, 368 with retention time of 34.8 min), catechin (λ_{max}, 279 with retention time of 15.6 min) or epicatechin (λ_{max}, 279 with retention time of 18.5 min) (**Table 3.10** given in Chapter 3).

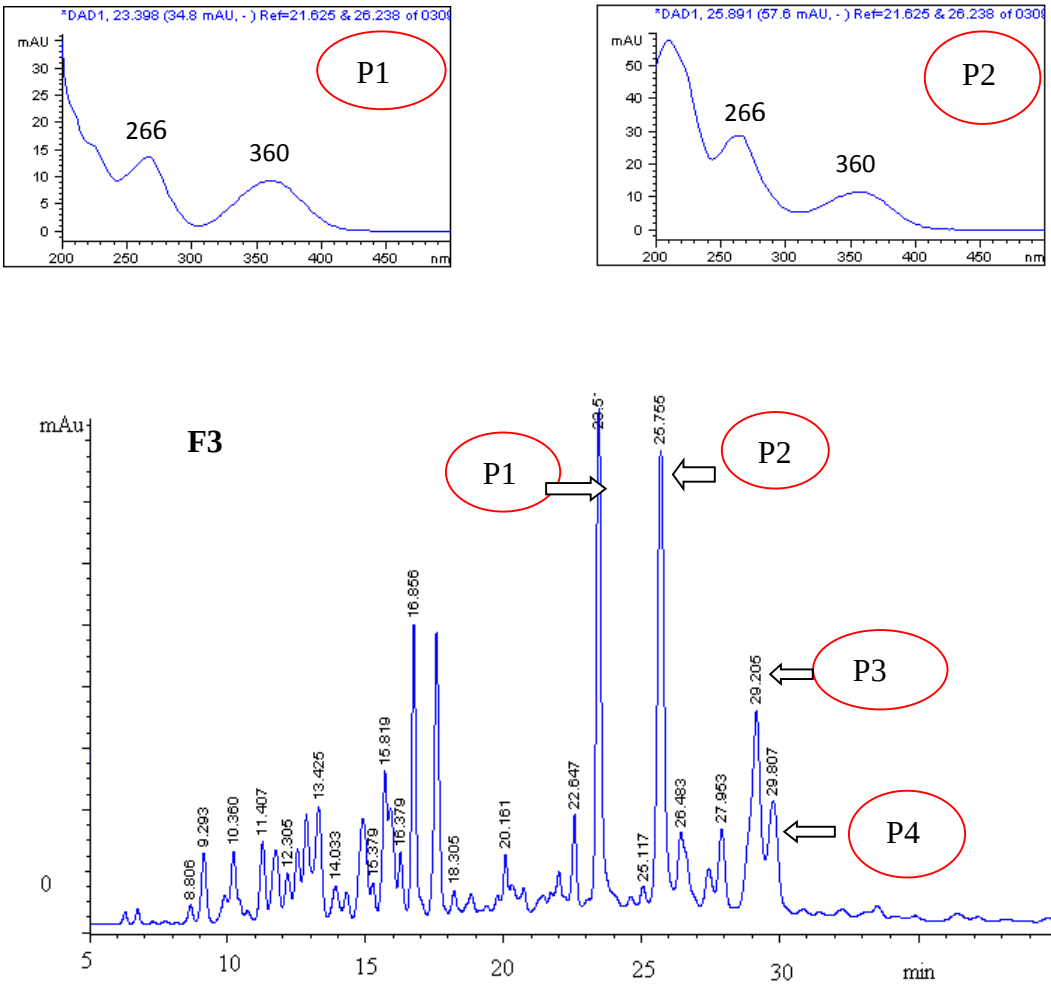


Figure 5.6. HPLC-DAD chromatogram of the ethyl acetate fraction (F3) at 360 nm and spectra of peaks P1 and P2.

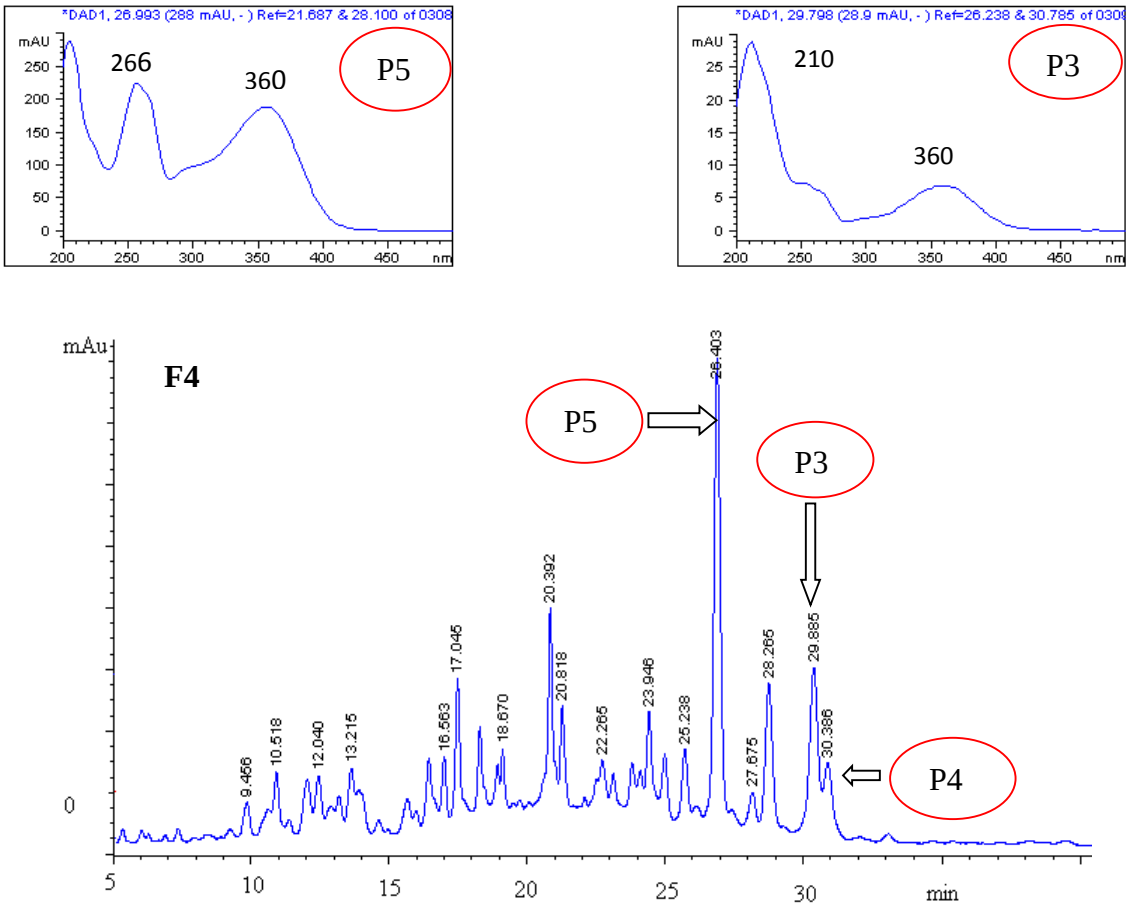


Figure 5.7. HPLC-DAD chromatogram of the aqueous fraction (F4) at 360 nm and spectra of P3 and P5 peaks.

Therefore, liquid chromatography coupled with electrospray ionization mass spectrometry (LC-ESI-MS) was performed on the ethyl acetate fraction (F3) and the aqueous fraction (F4) in an attempt to identify these previously unidentified flavonoids. Mass spectra obtained for the peaks labelled in the LC-ESI-MS chromatograms (**Appendix 10** and **Appendix 11**) are shown in **Table 5.3** and did not correspond to any common flavonoids such as catechin, epicatechin, quercetin and rutin, thus confirming that the peaks in **Figure 5.6** (peaks P1, P2, P3, and P4) and **Figure 5.7** peaks P3, P4, and P5 were not flavonoids previously reported in *A. macrosperma* fruit.

Table 5.3. LC-ESI-MS mass data obtained for fractions F3 and F4.

Fraction	Peak No	Retention Time (min)	MS in negative mode	MS in positive mode
Ethyl	1	**23.62**	199, 399, 589,779	232, 351, 413, 503
acetate (F3)	2	**25.60**	369, 781	416, 517, 807
	3	31.78	197, 377, 529, 677	315, 369, 667
	4	34.55	215, 345, 559, 677	365, 667
	5	37.68	313, 345, 691	369, 715
	6	41.50	337, 347, 695	371, 719
	7	44.00	349, 543, 677	369, 701
	8	46.50	197, 337, 405, 711	383, 701
Aqueous	1	**26.18**	361, 723	385, 747
(F4)	2	31.53	355, 391, 783	214, 415,485, 807
	3	33.17	377, 519, 663	355, 485, 687
	4	39.00	345	369, 519, 729
	5	40.93	347, 505, 695	371, 491, 719

Therefore, sub-fractionation of fractions F3 and F4 using column chromatography and size exclusion chromatography and isolation on semi-preparative HPLC was conducted in an attempt to furnish the structural information relevant to peaks P1 (retention time, (RT) at 23.59 min), P2 (RT, 25.76 min), P3 (RT, 29.20 min), P4 (RT, 29.81 min) and P5 (RT, 26.43 min) using ESI-MS/MS and NMR spectroscopy.

The ethyl acetate fraction (F3) (211 mg) was rechromatographed on a silica gel 922 column and this was repeated several times to get enough material for the subsequent separation steps. Eleven fractions from F3a to F3L were obtained from a single chromatographic run. After repetition of this silica gel subfractionation, F3L (~540 mg) was subjected to semi-preparative HPLC according to the method described in Section 5.3.7. The aqueous fraction (F4) (800 mg) was rechromatographed on a Sephadex LH-20 and six fractions {FW1 (15.4 mg), FW2 (654.7 mg), FW3 (49.9 mg), FW4 (13.9 mg), FW5 (22.8 mg) and FW6 (36.3 mg)} were collected and this was repeated several times. The fraction collected with water:methanol (5:5) (FW3) which showed characteristic UV-visible spectra of flavonols obtained from the HPLC-DAD chromatogram was selected for further purification with semi-preparative HPLC to isolate the flavonoids in P3, P4 and P5. Based on the similar behaviour on the HPLC-DAD analysis, the fractions (P3, P4) obtained from the ethyl acetate fraction (F3) were combined with the similar fractions of P3, P4 from the aqueous fraction F4 (**Figure 5.8**).

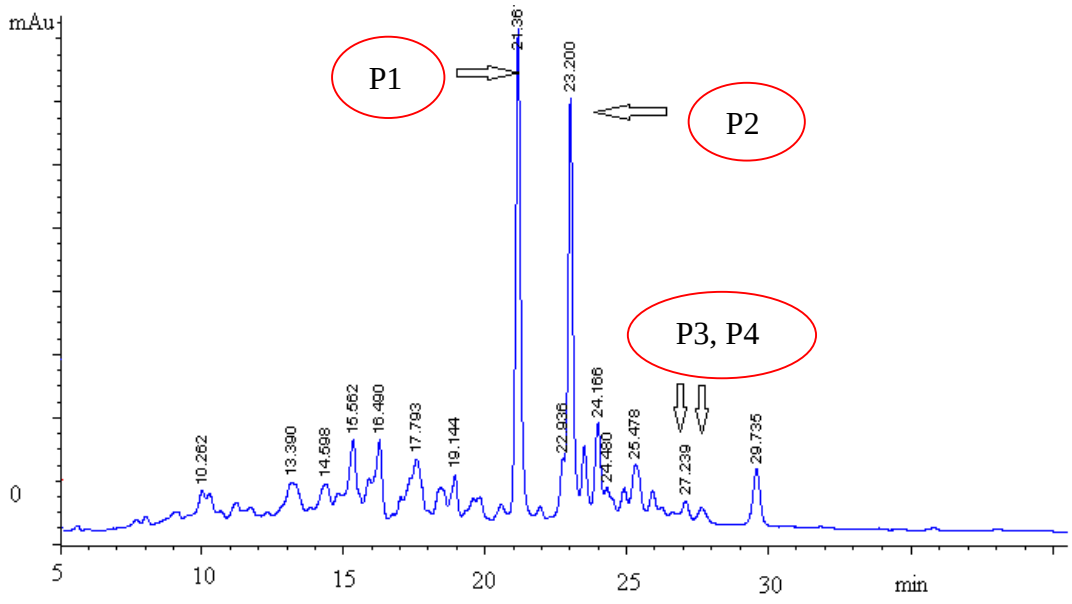


Figure 5.8. HPLC-DAD chromatogram of fraction F3L at 360 nm.

5.4.4. ESI-MS/MS analysis

HPLC-ESI-MS/MS analyses of the flavonoids isolated from peaks P1, P3, P4, and P5 with semi preparative HPLC were performed according to the procedure described in Section 5.3.9. The tentative structures of the flavonoids present in these fractions isolated on semi-preparative HPLC were proposed based on their HPLC-DAD patterns and mass fragmentation behaviour along with their corresponding reported values for comparison. In this study, most of the flavonoids were identified as glycosides containing one, two or three sugar moieties. Under the applied conditions in the mass spectrometry, the pseudomolecular $[M-H]^-$ ions readily eliminate the sugar moieties to produce $[Aglycone-H]^-$ ions. The presence of hexose residues was characterized by a neutral loss of 162 Da, typically glucose and galactose sugars. Arabinose is one of the common pentose sugar moieties reported in flavonoid glycosides which could be characterized by a 132 Da loss in the plants. A 146 Da loss indicates the presence of a methylpentose (deoxyhexose) such as a rhamnose sugar moiety (Ye et al., 2005).

5.4.4.1. Identification of flavonoids present in peak P1

Three major flavonoid compounds (1-3) in the yellow fraction isolated from peak P1 with retention times of 5.13, 5.34 and 5.46 min respectively were tentatively identified based on LC-ESI-MS/MS data. Compound 1 showed a pseudomolecular ion peak $[M-H]^-$ at *m*/*z* 761.33 with fragment ions at 629.23 (-132 Da, $[M-H\text{-pentose}]^-$), 300.00, 179.08 and 150.75 in the full mass spectrum (**Appendix 12**). The MS^2 of the 761.33 ion gave several fragment ions at 629.23 (-132 Da, $[M-H\text{-pentose}]^-$), 497.25 (-132 Da, $[M-H\text{-2xpentose}]^-$), and 335.08 (-162 Da, [M-H-2xpentose-hexose] (**Appendix 13**). The aglycone having (*m/z*) at 335 is not reported in the literature. The ions at *m*/*z* 179 and 151 in the negative ionization mode are

typical for flavonols and their glycosides (Abadio Finco et al., 2012). Therefore, compound 1 was tentatively identified as flavonol-di-(pentose)-hexoside based on the mass fragmentation patterns.

Compound 2 showed $[M-H]^-$ of *m*/*z* 767.08 with ion at 617.25, 479.08, 316.00 in the full mass spectrum (**Appendix 14**). The MS^2 of the 316 ion gave several fragment ions at 287.00 (-28 Da, -CO), 271.00 (-16 Da, O), 178.92 and 150.92 (Retro Diels Alder arrangement, RDA products) which are typical for flavonols and their glycosides (Abadio Finco et al., 2012) (**Appendix 15**). The ion at *m*/*z* 316 in the MS/MS spectrum accorded with the myricetin aglycone ion. In a former study, myricetin–malonylglucoside (molecular weight, MW=596 $gmol^{-1}$) was identified with fragments at *m*/*z* 521, 479, 316 in the negative ionization mode (Sojka et al., 2009). Therefore, compound 2 was tentatively identified as a derivative of myricetin glucoside based on its mass fragmentation patterns along with comparison with corresponding literature (Abadio Finco et al., 2012).

The mass spectrum of compound 3 in the negative ionization mode produced a $[M-H]^-$ ion at *m*/*z* 477.17 and a fragment ion at *m*/*z* 345.17 (-132 Da, $[M-H\text{-pentose}]^-$) in the full mass spectrum (**Appendix 16**). MS^2 of *m*/*z* 345.08 gave several fragment ions at *m*/*z* 301.08 (-44 Da, CO_2), 179 and 161 (RDA) (**Appendix 17**). Since *m*/*z* 347.00 is characteristic of the syringetin aglycone in the positive ionization mode, compound 3 was tentatively assigned as syringetin pentoside (Na et al., 2009; Guo et al., 2010; Yang et al; 2013).

5.4.4.2. Identification of flavonoids in peak P3

The total ion chromatogram of peak P3 showed the presence of at least two flavonoids (compounds 4 and 5). Compound 4 with a retention time of 5.39 min displayed

a pseudomolecular ion $[M-H]^-$ at *m*/*z* 739.25 and fragment ions at *m*/*z* 677.33 (-162 Da, [M-H-hexose]$^-$) and 285.00 in the full mass spectrum (**Appendix 18**). The MS^2 of the *m*/*z* 739.25 ion gave fragment ions at *m*/*z* 593.25 (-146 Da, [M-H-rhamnose]$^-$) and 285.00 (-308 Da, [M-H-rhamnose-rutinose]$^-$) (**Appendix 19**). It has been reported that kaempferol and luteolin produce the aglycone at *m*/*z* 285 as a result of the loss of sugar moieties, in the negative ionization mode in ESI experiments (Sanchez-Rabaneda et al., 2004; Schmidt et al., 2010; Abadio-Finco et al., 2012). Studies by Ye et al., (2005) on the characterization of phenolic compounds in the Chinese herbal drug, Tu-Si-Zi reported kaempferol-*O*-rhamnosylhexoside ([M-H]$^-$, *m*/*z* 593) with fragment ions at *m*/*z* 327 (3%) and 285 (100%) in the MS/MS spectrum. Since kaempferol produces a high relative abundance (100%) for the aglycon at *m*/*z* 285 in MS^2, compound 4 in peak P3 was tentatively assigned as kaempferol-*O*-di-(rhamnosyl) hexoside which is a trisaccharide with two rhamnose sugar moieties and one hexose sugar moiety, which is identified as based on mass fragmentation patterns (Tian et al., 2002; Sanchez-Rabaneda et al., 2004; Ye et al., 2005; Inbaraj et al., 2010; Schmidt et al., 2010).

Compound 5 in peak 3 (RT, 5.98 min) showed a pseudomolecular ion $[M-H]^-$ of *m*/*z* 675.17 with fragment ions at *m*/*z* 643.17, 599.08 and 319.00 in the full mass spectrum (**Appendix 20**). The MS^2 of the 675.17 ion gave fragment ions at *m*/*z* 643.08 and 599.57 (**Appendix 21**). The structure of compound 5 could not be assigned.

5.4.4.3. Identification of flavonoids present in peak P4

Three flavonoid compounds (6-8) with retention times of 5.48, 5.64 and 5.98 min were tentatively identified in the yellow fraction isolated from peak P4 based on LC-ESI-MS/MS data. Compound 6 (RT, 5.48 min) showed a pseudo molecular ion $[M-H]^-$ at *m*/*z*

769.17 with fragments at *m*/*z* 623.17 (-146 Da, [M-H-rhamnose]$^-$), 503.25 (-120 Da) and 315.00 (-308 Da, [M-H-rhamnose-rutinose]$^-$) in the full scan mass spectrum (**Appendix 22**). MS^2 of *m*/*z* 769.25 ion gave fragment ions at *m*/*z* 623.17 (-146 Da, [M-H-rhamnose]$^-$), 315.00 (-146-308 Da, [M-H-2x rhamnose-hexose]$^-$), 300.00 (-15 Da, [M-H-2x rhamnose-hexose-CH_3]$^-$) and 271.00 (-29 Da, CH_2-CH_3) (**Appendix 23**). The aglycon ion at (*m/z*) 315 is characterestic of rhamnetin and isorhamnetin as reported in the literature (Abadio Finco et al., 2012). It has been reported that rhamnetin and isorhamnetin are isomers with mostly similar mass fragments in ESI-MS/MS analysis (Ye et al., 2005) but with different fragmentation patterns such as *m*/*z* 165 fragment for rhamnetin while *m*/*z* 151 for isorhamnetin in negative ionization mode on atmospheric pressure chemical ionization mass spectrometry (APCI-MS) (Justeen, 2000; Schieber et al., 2002). Therefore, compound 6 was identified as isorhamnetin-*O*-di-(rhamnosyl)hexoside based on the mass fragmentation patterns observed in this study together with comparison to the literature (Parejo et al., 2004; Rosch et al., 2004; Ye et al., 2005; Schmidt et al., 2010; Abadio Finco et al., 2012).

Compound 7 (RT, 5.64 min) showed an peak [M-H]$^-$ at *m*/*z* 477.08 with fragments at *m*/*z* 314.00, 271.00, 243.00, 150.92 in the full mass spectrum (**Appendix 24**). MS^2 of the *m*/*z* 477.08 gave several fragment ions at *m*/*z* 449.00 (-28 Da, -CO), 356.92 (-92 Da), 314.00 (-43 Da), 285.00 (-29 Da), 178.92 and 150.92 (RDA products) (**Appendix 25**). MS^2 of *m*/*z* 314.00 spectrum exihibited three major peaks at *m*/*z* 284.92 (-29 Da, CH_2-CH_3), 270.92 (-14 Da, CH_2) and 243.00 (-29 Da, CH_2-CH_3) (**Appendix 26**). Since it has been observed that the retention time decreases with the number of sugar moieties or hydroxyl groups attached to the flavonoid aglycone, in the reverse phase liquid chromatography (Abad-Garcia et al., 2009), compound 7, which showed similar fragments to compound 6, was tentatively identified as isorhamnetin-3-*O*-glucoside (Parejo et al., 2004; Rosch et al., 2004; Ye et al., 2005). Parejo et al., (2004) reported the pseudomolecular ion [M-H]$^-$ at *m*/*z* 477 with MS^2

fragments at *m*/*z* 449, 357, 315 and 314.00 while *m*/*z* 315, 314, 300, 285, 271.00 and 243.00 appear in the MS^3 spectrum.

The mass spectrometric experiments of compound 8 (RT, 5.98) in peak P4 in the negative ionization mode produced as $[M-H]^-$ ion at *m*/*z* 505.00 and fragment ions at *m*/*z* 314.00 (-191 Da), 271.00 (-43 Da), 243 (29-Da, CH_2-CH_2) and 152.92 (-90 Da) in the full mass spectrum (**Appendix 27**). MS^2 of *m*/*z* 314.00 ion gave several fragment ions at *m*/*z* 284.92 (-29 Da, CH_2-CH_3), 271 (-14 Da, CH_2) and 150.83 (-120 Da, RDA) (**Appendix 28**). The fragment of 191 Da has been reported for quinic acid (Saldanha et al., 2013). Since this compound showed the similar fragmentation patterns of the aglycone to compounds 6 and 7, compound 8 was tentatively assigned as a quinic acid derivative of isorhamnetin.

5.4.4.4. Identification of flavonoids present in peak P5

The MS data of two flavonoid glycosides (compounds 9 and 10) identified in the yellow fraction P5 revealed that they were quercetin glycosides. The compound 9 with a retention time of 5.36 min displayed a pseudomolecular ion $[M-H]^-$ at *m*/*z* 755.25 with fragments at *m*/*z* 609.08 (-146 Da, $[M-H\text{-rhamnose}]^-$) and 300.00 in the full mass spectrum (**Appendix 29**). MS^2 of *m*/*z* 609.08 ion gave two fragment ions at 301.08 (-308 Da, [M-H-rutinose] and 178.83 (RDA) (**Appendix 30**). The aglycon ion at *m/z* 301 in the negative ionization mode is characteristic of quercetin, morin (flavonol), hesperitin (flavanone) or elagic acid (Justeen, 2000). According to the maxima in the UV absorption spectrum (λ_{max}, 266, 300, 360 nm) of peak 5 shown in **Figure 5.7** and the MS^2 fragmentation pattern compared to the reported values (Justeen, 2000; Parejo et al., 2004; Rosch et al., 2004), the aglycone could be assigned as quercetin. It has been reported that quercetin mono and diglycosides produce the quercetin aglycon at *m*/*z* 301 as a result of the loss of sugar moieties

in the negative ionization mode in ESI experiments (Parejo et al., 2004; Rosch et al., 2004; Ye et al., 2005; Abadio-Finco et al., 2012). Therefore, compound 9 in P5 was identified as quercetin-di-(rhamnosylhexoside) based on comparison to the MS/MS data with the corresponding literature reports (Parejo et al., 2004; Rosch et al., 2004; Ye et al., 2005; Inbaraj et al., 2010; Abadio-Finco et al., 2012).

Compound 10 with a retention time of 5.52 min showed a $[M-H]^-$ ion at *m*/*z* 463.00, with fragment ions at *m*/*z* 300.00, 270.92, 178.92 and 150.92 in the full mass spectrum (**Appendix 31**). MS^2 of *m*/*z* 300.00 ion showed several fragment ions at 271 (-29 Da), 255 (-46 Da), 178.92 and 150.83 (RDA) (**Appendix 32**). The characteristic pattern of $[M-H]^-$ at *m*/*z* 463 with fragments 301, 271, 179 and 151 identified the compound as belonging to the quercetin-3-*O*-galactoside or quercetin-3-*O*-glucoside flavonoids (Parejo et al., 2004; Rosch et al., 2004; Ye et al., 2005). A similar fragmentation pattern has been reported in the negative ionization mode for quercetin-3-*O*-coumaroylgalactoside (*m*/*z,* 610) (Ye et al. 2005). Therefore, compound 10 was identified as quercetin-3-*O*-galactoside (Parejo et al., 2004; Rosch et al., 2004; Ye et al., 2005).

5.4.4.5. Structural charaterization of flavonoid in peak P2

Compound (11), which was isolated from peak P2 (yellow, 3.2 mg) after several runs on semi-preparative HPLC, showed sufficient purity (>98%) based on HPLC-DAD analysis. The time of flight-mass spectroscopy (micrOTOF-ESI-MS) performed on a Bruker micrOTOF-Q-II mass spectrometer further confirmed the presence of the single compound with a molecular ion at *m/z* 447.0953 ($[M-H]^-$) (**Appendix 33**), which corresponds to the molecular formula of $C_{21}H_{19}O_{11}$. The UV-visible spectrum bands (λ_{max} 266, 360 nm) (**Figure 5.6**) indicated the presence of a phenolic functionality, and together with the

micrOTOF-ESI-MS data and anomeric proton signals (in the 3-5.2 ppm range) in the NMR spectrum (**Figure 5.9**), suggested this compound was a mono-substituted flavonoid glycoside.

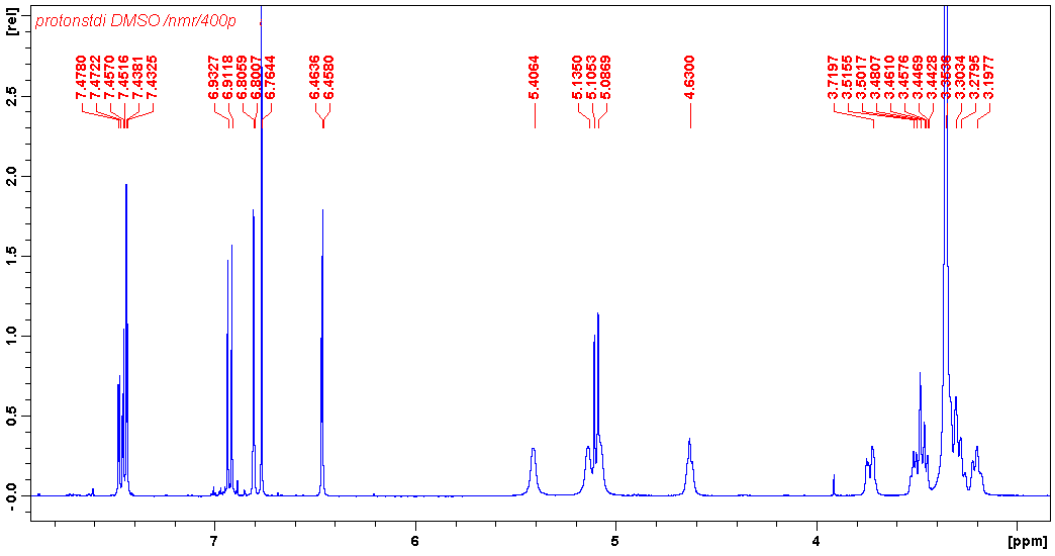


Figure 5.9. Expanded region of the ^{1}H NMR spectrum of compound 11 in d_6-DMSO showing the presence of anomeric proton signals at 3-6 ppm.

The presence of a sharp peak in the ^{1}H NMR spectrum (**Figure 5.10**) at 12.99 ppm indicated (Loo and Bruyn, 1986; Messens and Montagu, 1989) a hydrogen bond between HO-5 and the carbonyl function on C-4, which demonstrates that HO-5 is not substituted. Two broad signals at 10.00 and 9.40 ppm were identified as OH-3´, OH-4´ with comparison to the literature (Lee et al., 2008).

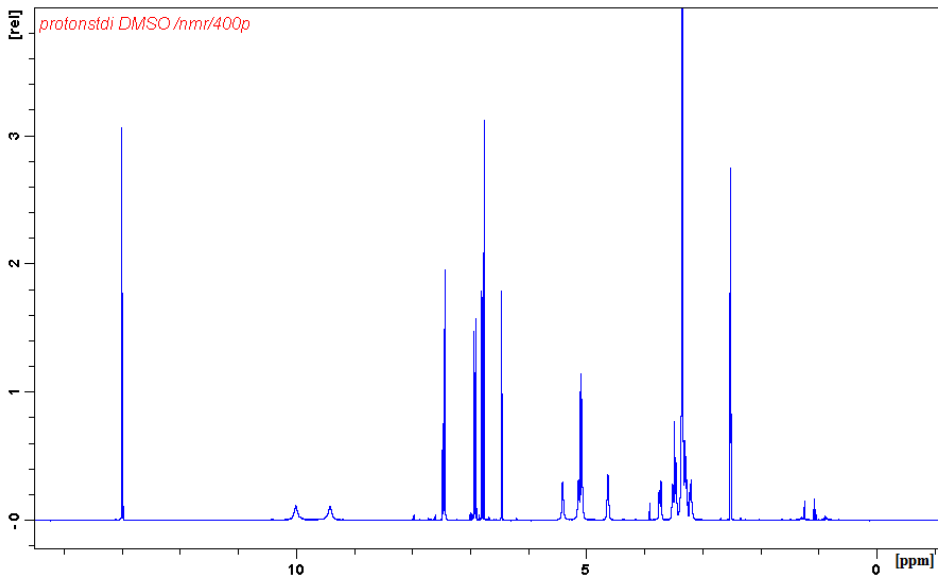


Figure 5.10. The ^{1}H NMR spectrum of compound 11 in d_6-DMSO.

The aromatic region of signals in the ^{1}H NMR spectrum showed a singlet at δ 6.76 ppm (H-3), four doublets (*d*) at δ 6.46 (*J*=2.0 Hz, H-6), 6.80 (*J*=2.0 Hz, H-8), 7.43 (*J*=2.5 Hz, H-2′), 6.92 (*J*=8.5 Hz ,H-5′) and a doublet of doublets (*dd*) at δ 7.47 (*J*=2.5 Hz, 8.5 Hz, H-6′) (**Table 5.4**), Therefore, the resonances in this region accord with the luteolin nucleus (**Figure 5.11**) for compound 11.

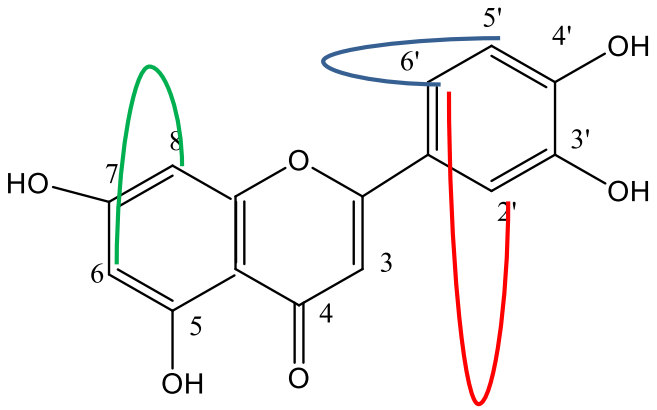


Figure 5.11. The structure of luteolin nucleus related to the ^{1}H NMR resonance.

Table 5. 4. ^{1}H and ^{13}C NMR spectroscopy data for the compound 11 in d_6 DMSO.

Carbon number	Chemical shift (ppm)	
	^{1}H	^{13}C
1	-	-
2	-	164.4
3	6.76 (*s*)	99.8
4	-	181.9
5	-	161.1
6	6.46 (*d*, *J*=2.0 Hz)	95.0
7	-	162.9
8	6.80 (*d*, *J*=2.0 Hz)	99.5
9	-	156.9
10	-	103.1
1′	-	121.3
2′	7.43 (*d*, *J*=2.5 Hz)	113.5
3′	-	145.8
4′	-	149.9
5′	6.92 (*d*, *J*=8.5 Hz)	116.0
6′	7.47 (*dd*, *J*=2.48 Hz, 8.48 Hz)	119.0
1′′	5.1 (*d*)	99.5
2′′	3.17- 3.5 (*m*)	73.0
3′′		76.2
4′′		69.5
5′′		77.0
6′′		60.5
5-OH	12.99 (*s*)	-
3′-OH	10.00 (*s*)	-
4′-OH	9.40 (*s*)	-

The presence of one glucosyl sugar moiety with a characteristic signals at δ 5.1 ppm for the anomeric proton and 3.17- 3.49 was identified as given in **Table 5.4.** Comparison of the ^{1}H and ^{13}C data shown in **Table 5.4** with literature values led to the conclusion that a glucose moiety was substituted at the 7-*O*-position of the luteolin (Hartwig et al., 1990; Gohari et al., 2011). Although, COSY and HSQC spectral data were attempted on compound 11, due to the very low concentration of compound 11 in DMSO-d_6 these were unsuccessful. Therefore, the structure of compound 11 was tentatively assigned as luteolin-7-*O*-glucoside (**Figure 5.12**) on the basis of 1 D NMR data along with previous literature report (Gohari et al., 2011).

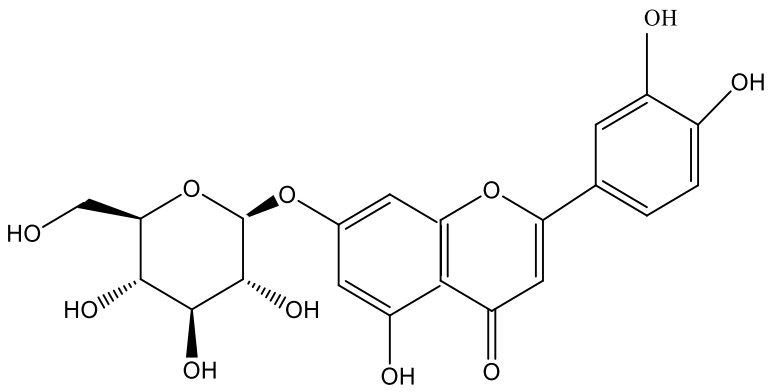


Figure 5.12. The structure of luteolin-7-*O*-glucoside.

5.5. Conclusion

This study was designed to use HPLC-ESI-MS^n to identify previously undiscovered phenolic antioxidant compounds in *A. macrosperma* fruit. Vacuum manifold solid phase extraction (SPE) using Strata C18 cartridges was used for the preliminary purification to remove more polar compounds such as sugars and proteins from the defatted crude extract resulting in an acidified aqueous fraction (F1), while moderately polar compounds were obtained in an acidified methanol fraction (F2). The phenolic profile, antioxidant capacity

and the HPLC-DAD fingerprints of the SPE–purified extracts showed that the acidified methanol fraction (F2) was rich in flavonoids and was the most active fraction. So it was subjected to further purification. The acidified methanol fraction (F2) was subjected to a series of separation procedures, including liquid-liquid partitioning, silica gel flash column chromatograghy, size exclusion chrmatograghy on sephadex LH-20 and semi-preparative HPLC, resulting in flavonoid rich fractions (P1-P5) prior to LC-ESI-MS/MS analysis of the individual fractions.

For the sensitive detection of flavonoid glycosides, LC-ESI-MS was operated in negative ionization mode resulting in abundant $[M-H]^-$ ions. Further MS^2 experiments showed intense fragment ions for the flavonoid glycones. The observed molecular weight differences provide information about the sugar type of the glycones. The neutral loss of a glucose or galactose (162 Da), rhamnose (146 Da), or pentose (132 Da) were observed, if mono, di or trisaccharide units were present in the structure. LC-ESI-MS/MS revealed that several compounds which were hidden by compounds with higher absorbance co-eluted in the peaks of P1, P3, P4 and P5 of the HPLC-DAD chromatograms. Therefore, ten compounds were tentatively identified (**Table 5.5**) in these peaks by using ESI-MS/MS analysis and HPLC-DAD analysis in combination with the reported data in the literature. All these compounds identified in this chapter were not previously reported in the *A. mac*rosperma fruit. Only a flavonoid in peak P5 obtained from the semi-preparative HPLC showed sufficient purity (>98%) for NMR spectroscopy analysis. ^1H and ^{13}C NMR data along with the reported data in the literature allowed identification of this compound as luteolin-7-*O*-glucoside.

Table. 5.5. Mass spectral characterestics and identity of phenolics in *A. macrosperma* fruit.

Compound No	Peak No	Retention time (min)	$[M-H]^-$ (*m/z*)	Other ions in full mass (*m/z*)	MS^2 fragment ions (*m/z*)	Tentative assignment
1	P1	5.13	761	629, 300, 179, 151	(761) 629, 497, 335	flavonol-di-(pentose)-hexoside
2	P1	5.34	767	617, 479, 316	(316); 287, 271, 179, 151	derivative of myricetin glucoside
3	P1	5.46	477	345	(345); 301, 179, 161	syringetin pentoside
4	P3	5.39	739	677, 285	(739); 593, 285	kaempferol-*O*-di-(rhamnosyl) hexoside
5	P3	5.98	675	643, 599, 319	(675); 643, 599	Unknown
6	P4	5.48	769	623, 503, 315	(769); 623, 315, 300, 271	isorhamnetin-*O*-di-(rhamnosyl) hexoside
7	P4	5.64	477	314, 271, 243, 151	(477); 449, 357, 314, (314); 285, 179, 151	isorhamnetin-3-*O*-glucoside
8	P4	5.98	505	314, 271, 243, 153	(314); 285, 271, 151	quinic acid derivative of isorhamnetin
9	P5	5.36	755	609, 300	(609); 301, 179	quercetin-di-(rhamnosylhexoside)
10	P5	5.52	463	300, 271, 179, 151	271, 255, 179, 151	quercetin-3-*O*-galactoside

Chapter 6

General conclusion and future works

6.1 General conclusions

This thesis reports experimental findings of optimization of extraction conditions for phenolics from *A. macrosperma* fruit, evaluation of the phenolic composition, antioxidant and antihypertensive properties compared to commercial kiwifruit varieties, and identification of flavonoids and their derivatives isolated from *A. macrosperma* kiwifruit with bioassay guided extraction and fractionation using chromatographic procedures.

Considering the objectives of the research and the results obtained by different experimental investigations as described in Chapters 3 to 5, the main achievements could be summarized as follows:

The optimised extraction conditions for phenolic compounds from *A. macrosperma* kiwifruit, using the single factor experimental design was obtained. Phenolics were initially extracted from *A. macrosperma* fruit in to different extraction solvents (70% aq. acetone, 80% aq. methanol, 80% aq. ethanol, 100% methanol and water). After quantification of yield, total phenolic, total flavonoid, total flavanol contents and antioxidant activities of the different extracts, 70% aq. acetone were found to be the optimum extraction solvent. Secondly, the optimum extraction technique was optimized out of some selected conventional techniques (steeping, shaking, sonicating and blending) by keeping the solvent type constant. Among the solvents and extraction techniques employed, 70% aq. acetone by steeping resulted in the highest values of total phenolic (823.1±14.4 mg GAE/100 g DW), total flavonoid (170.9±2.0 mg CAE/100 g DW), total flavanol (82.6±0.6 mg CAE/100 g DW) contents and the antioxidant activity (5.1±0.1 mmolTrolox equivalents/100 g DW and 8.3±0.1 mmol Fe (II) equivalents/100 g DW for DPPH and FRAP assays, respectively. Analysis of each extract on HPLC-DAD and HPLC-ESI-MS/MS revealed that 70% aq.

acetone extract from *A. macrosperma* fruit contains many potential antioxidant flavonoids and quercetin-3-*O*-galactoside was the most abundant among them.

The optimised conditions (70% aq. acetone employing the steeping technique) used for the extraction of phenolics from non-commercial fruit, *A. macrosperma*, were applied to extract phenolics from commercial kiwifruit varieties grown in New Zealand. A comparative evaluation of phenolic profiles, antioxidant activity of defatted crude extracts obtained from these kiwifruit were compared with the of *A. macrosperma* fruit. Among the extracts tested, the *A. macrosperma* fruit cultivar showed the highest values of total phenolic (TP) content (823.1±14.4 mg gallic acid equivalent (GAE)/100 g DW), total flavonoid (TFO) content (170.9±1.9 mg catechin equivalent (CAE)/100 g DW), total flavanol (TFA) content (82.6±0.6 mg catechin equivalent (CAE)/100 g DW) and antioxidant capacity (5.1±0.1 mmol Trolox equivalent (TE)/100 g DW and 8.3±0.1 mmol Fe (II) equivalent/100 g DW for DPPH and FRAP assays, respectively). This study showed that the FRAP values of the kiwifruit cultivars tested varied with the descending order of *A. macrosperma* > sungold> sweetgreen > gold > green while DPPH values varied with the ranking order of *A. macrosperma* > sweet green > sungold > gold > green. High correlation (R^2= 0.942) observed between FRAP and DPPH antioxidant capacities implied that the antioxidants in these kiwifruit extracts were capable of scavenging free radicals (DPPH) and reducing oxidants. The data obtained from HPLC-DAD and HPLC-MS/MS analysis of defatted crude extracts obtained from the kiwifruit suggest that the *A. macrosperma* fruit is a very good source of phenolic antioxidants.

This study also revealed the potential application of antihypertensive activity of flavonoid rich defatted crude extracts obtained from *Actinidia macrosperma* fruit. Based on the experimental results by *in vitro* assay using fluorescence based biochemical reaction,

kiwifruit has potential use as a cardiovascular protective agent against high blood pressure. The kiwifruit extracts obtained from different extraction solvents, techniques and cultivars were performed for the ACE inhibitory activity and results showed that they are moderately effective ACE inhibitors. Among the results obtained in the present study, it should be highlighted that the activity observed for the extract from 70 % acetone by steeping method exhibited quite promising ACE inhibitory activity (lowest IC_{50} values), as compared to the other solvent extracts tested. Kinetic determinations suggested that flavonoids rich extracts from *A. macrosperma* kiwifruit inhibit the enzyme activity by non-competitive binding activity with the substrate for the active site. Investigating the ACE enzyme inhibition by kiwifruits along with their kinetic parameters generated valuable information for supporting the general concept that flavonoids rich kiwifruits have health effects.

Therefore, it is of interest to isolate and further characterize these potential antioxidants from the *A. macrosperma* kiwifruit using bioassay guided extraction, separation and isolation steps. The different chromatographic procedures, including liquid-liquid partitioning, silica gel flash column chromatography, size exclusion chromatography, and semi-preparative HPLC were performed to yield five flavonoids rich fractions prior to LC-ESI-MS/MS analysis to identify some flavonoids present in *A. macrosperma* fruit. For the sensitive detection of flavonoid glycosides, HPLC-ESI-MS was operated in negative ionization mode resulting in abundant $[M-H]^-$ ions. Further MS^2 experiments showed intense fragment ions for the flavonoid glycones. The observed molecular weight differences provide information about the sugar type of the glycones such as glucose or galactose (162 Da), rhamnose (146 Da), or pentose (132 Da). Ten chemical structures for flavonoids were tentatively identified by ESI/MS/MS analysis, HPLC-DAD analysis, in combination with the published literature. To the best of our knowledge, all those compounds identified in this thesis were not previously reported in the case of *A. mac*rosperma fruit. ^1H and ^{13}C nuclear

magnetic resonance (NMR) data of one highly purified compound allowed the identification of luteolin-7-*O*-glucoside.

Considering the main results of the research, presented in this thesis, it could be concluded that the optimized extraction protocol and the HPLC-ESI-MS/MS technique allowed the identification of eleven flavonoid compounds, from *Actinidia macrosperma* fruit. Further, the effects of *Actinidia macrosperma* fruits on inhibition of angiotensin converting enzyme (ACE) has not been previously reported and could therefore be potentially used as a novel biological activity. It is believed that the experimental results presented in this thesis, contribute to the research areas related to chemical and biological properties of phenolics present in kiwifruits. The thesis also allowed demonstrating the potential of flavonoids from *Actinidia macrosperma* kiwifruit as antioxidants and antihypertensive agents.

6.2. Future research work

As previously discussed, the flavonoids rich defatted crude extracts from *Actinidia macrosperma* exhibited significantly higher antioxidant and antihypertensive activities compared to commercial kiwifruit varieties. Consequently, there are several lines of research arising from this work which could be considered in the future:

6.2.1. *Extraction of phenolics from other non-commercial Actinidia species*

Further studies should be conducted on the extraction of phenolics from other non-commercial species in the genus *Actinidia* and comparing the antioxidant activities with the

antioxidant activity of *A. macrosperma* fruit on searching for health benefits. As described in Section 2.2 in Chapter 2, there are more than 76 known *Actinidia* species (Ferguson, 2007) but only three species have been marketed (Nishiyama et al., 2004; Ferguson and Seal, 2008).

6.2.2. Quantification *of phenolics and their derivatives of new commercial kiwifruits*

As described in Section 2.2 in Chapter 2, the new commercial kiwifruits namely "Sungold" and "Sweetgreen" which were marketed in 2010, were found to be richer in antioxidants than old commercial kiwifruit varieties (gold and green kiwifruits). Therefore it is worth quantifying and identifying the phenolic compounds present in them to correlate with their defence mechanism against *Pseudomonas syringae pv actinidiae* bacteria (PSA) attack and develop more resistant kiwifruit cultivars.

6.2.3. Application *of hyphenated techniques for structure elucidation*

The present study indicates that the *A. macrosperma* kiwifruit is rich in many phenolic compounds; only eleven compounds were identified although it showed many peaks on HPLC-DAD chromatograms. Further experiments should be carried out to identify other phenolic compounds present in the *Actinidia macrosperma* kiwifruit, not identified in this thesis. Since it is understood that the abundance of these individual phenolic compounds is very low in the fruit, it is recommended to use hyphenated techniques such as liquid chromatography coupled with nuclear magnetic resonance (LC-NMR) for the direct identification of phenolics.

6.2.4. *Screening for biological activities of isolated fractions and compounds isolated from A. macrosperma kiwifruit*

The results of the Chapter 3 and 4 revealed that the flavonoids rich defatted crude extracts exhibited significantly higher antioxidant and antihypertensive activities compared to commercial kiwifruit varieties. Therefore, studies should be focused on the isolation individual compounds with active ACE inhibition and antioxidant capacity from the most active extract from *A.macrosperma*, followed by *in vitro* testing.

6.2.5. *Screening for anticancer/ antitumor activities of A. macrosperma kiwifruit*

Although anticancer activities of extracts obtained from stem of *A. macrosperma* were reported in the literature (Lu et al., 2007; Luet al., 2012), and highly employed in Chinese traditional medicine to cure several tumours (Lu et al., 2007; Luet al., 2012), there are no reports found on the anticancer activity of the fruit of *A. macrosperma*. Therefore, preliminary studies should be launched to search for anticancer agents from the *A. macrosperma* kiwifruit as it was found to be rich in antioxidants.

6.2.6. *Performance of A. macrosperma kiwifruit as antioxidants rich pharmaceutical products*

It has been proved that the *A. macrosperma* contains many antioxidants such as carotenoids, and many phenolics (Montefiori and McGhie, 2005; Nishiyama et al., 2005; Nishiyama et al., 2007; Latocha et al., 2010), it should be considered to introduce *A.macrosperma* kiwifruit as a natural antioxidants and antihypertensive agents rich nuetraceutical health products (NHP) to the market in the near future.

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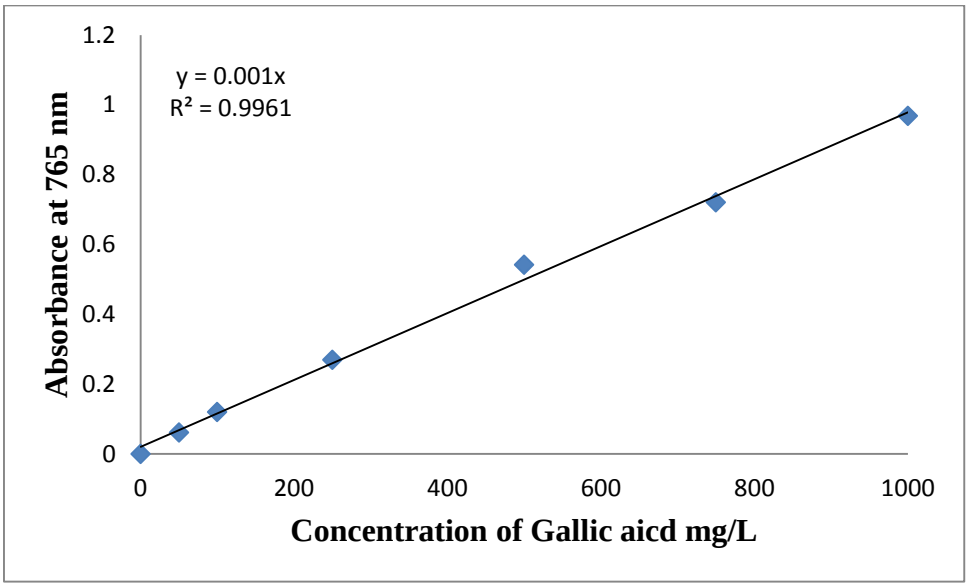
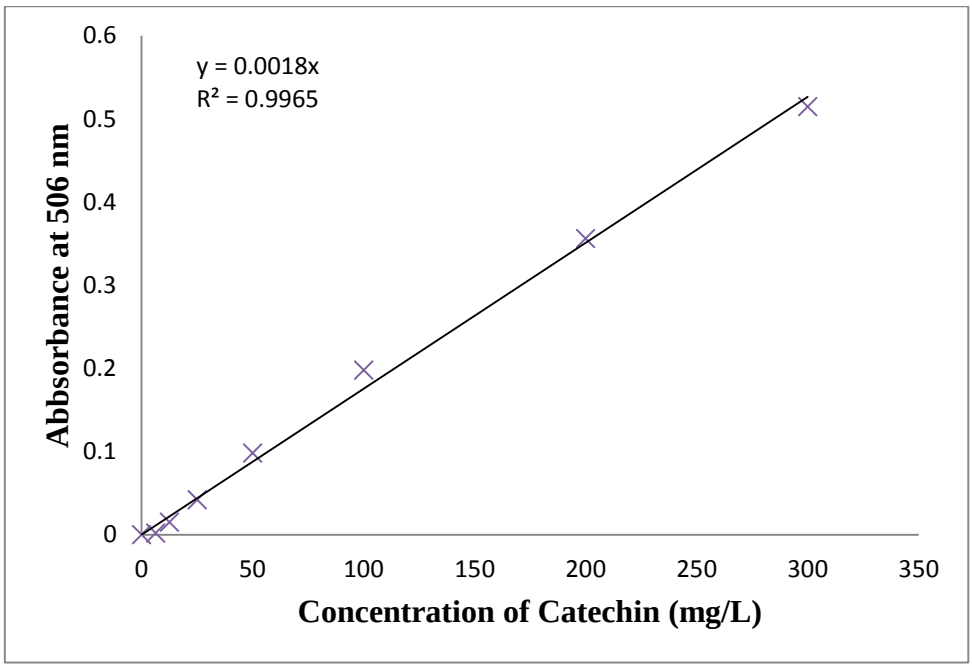
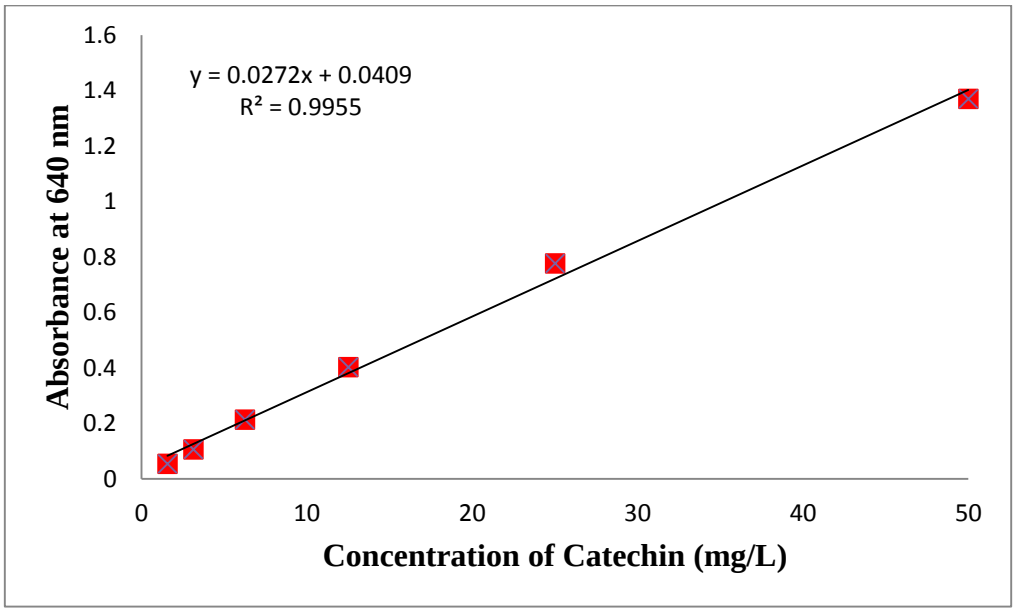
Appendices



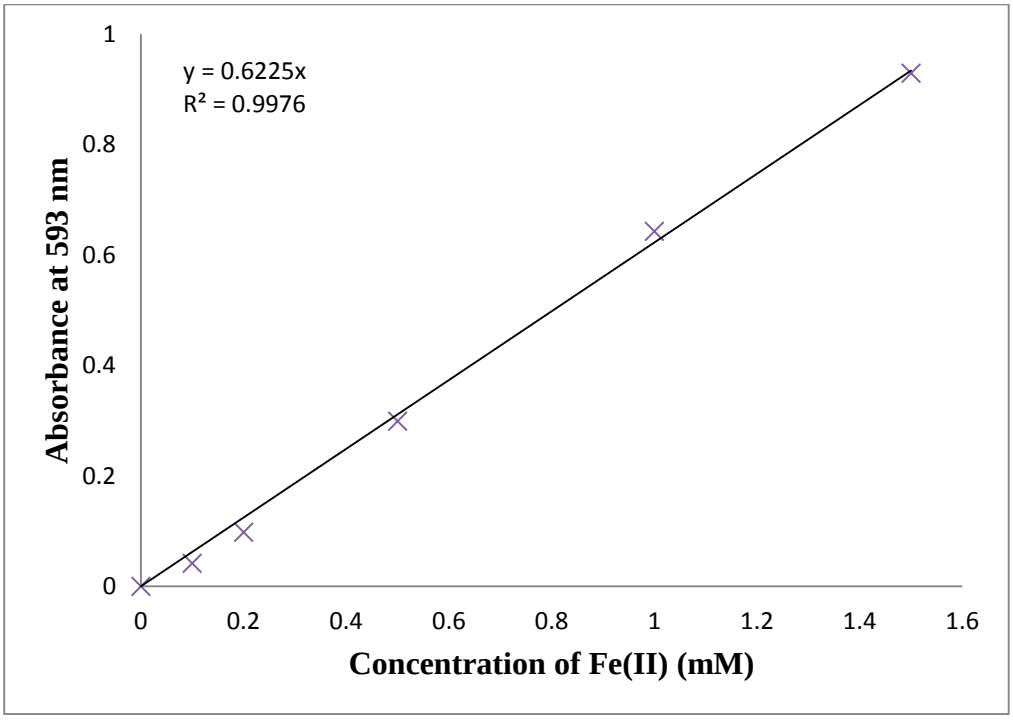
Appendix 1. Standard curve for Total Phenol Content.



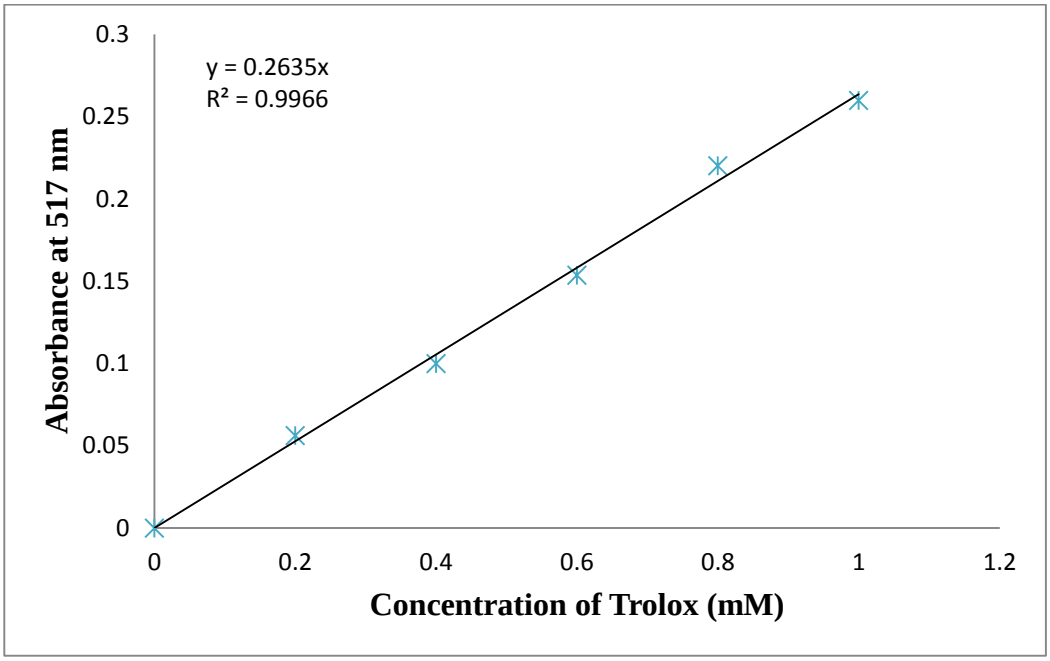
Appendix 2. Standard curve for Total Flavonoid Content.



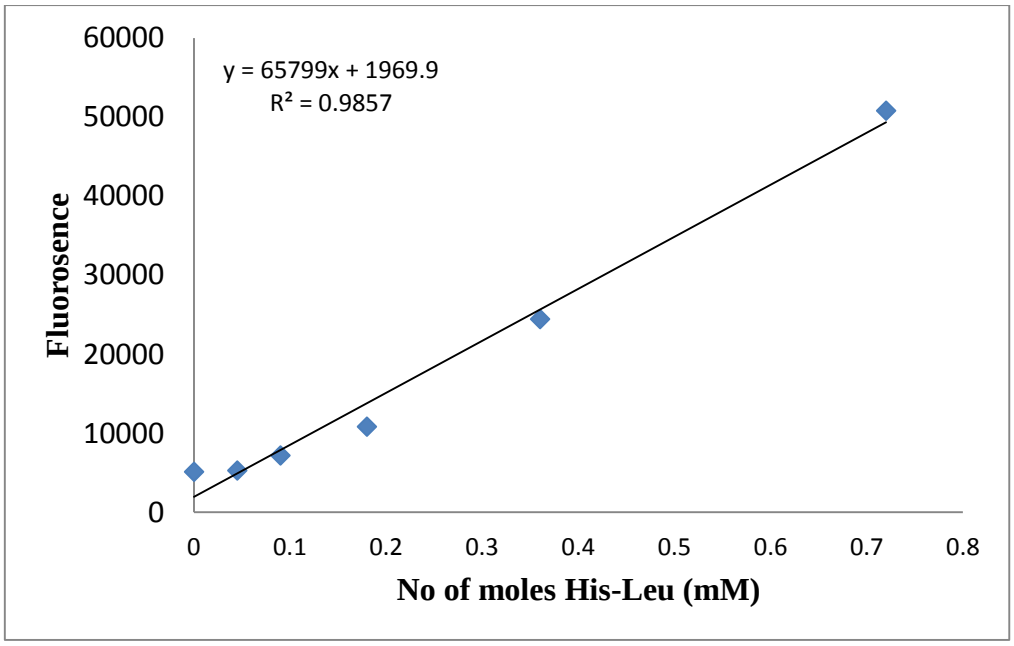
Appendix 3. Standard curve for Total Flavanol Content.



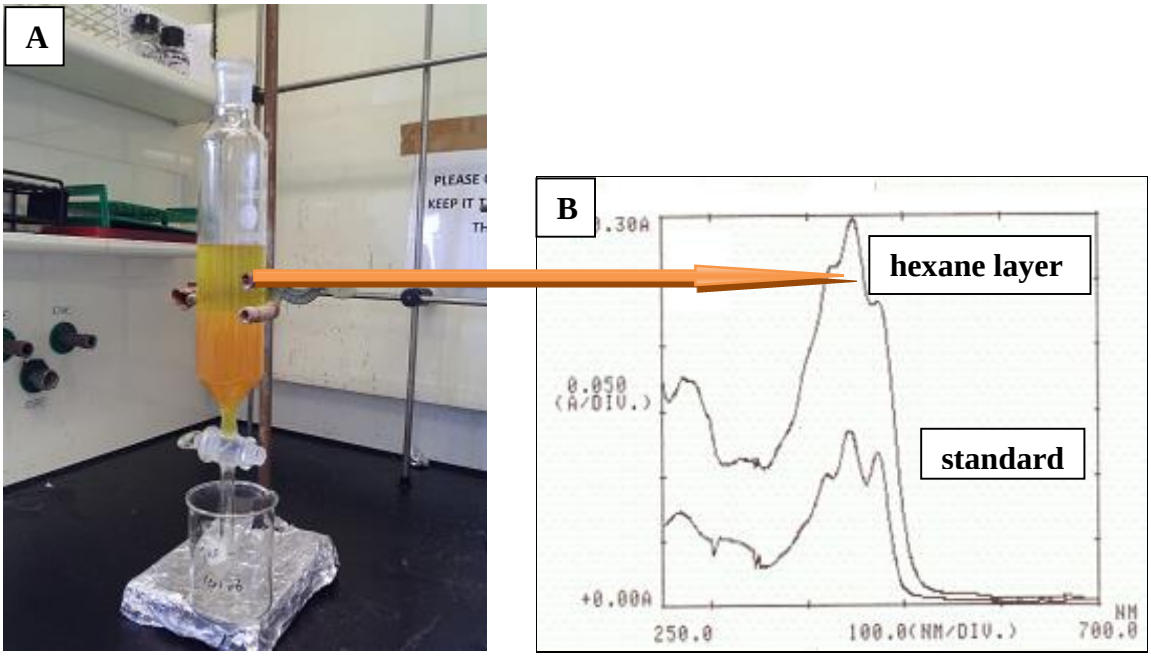
Appendix 4. Standard curve for FRAP.



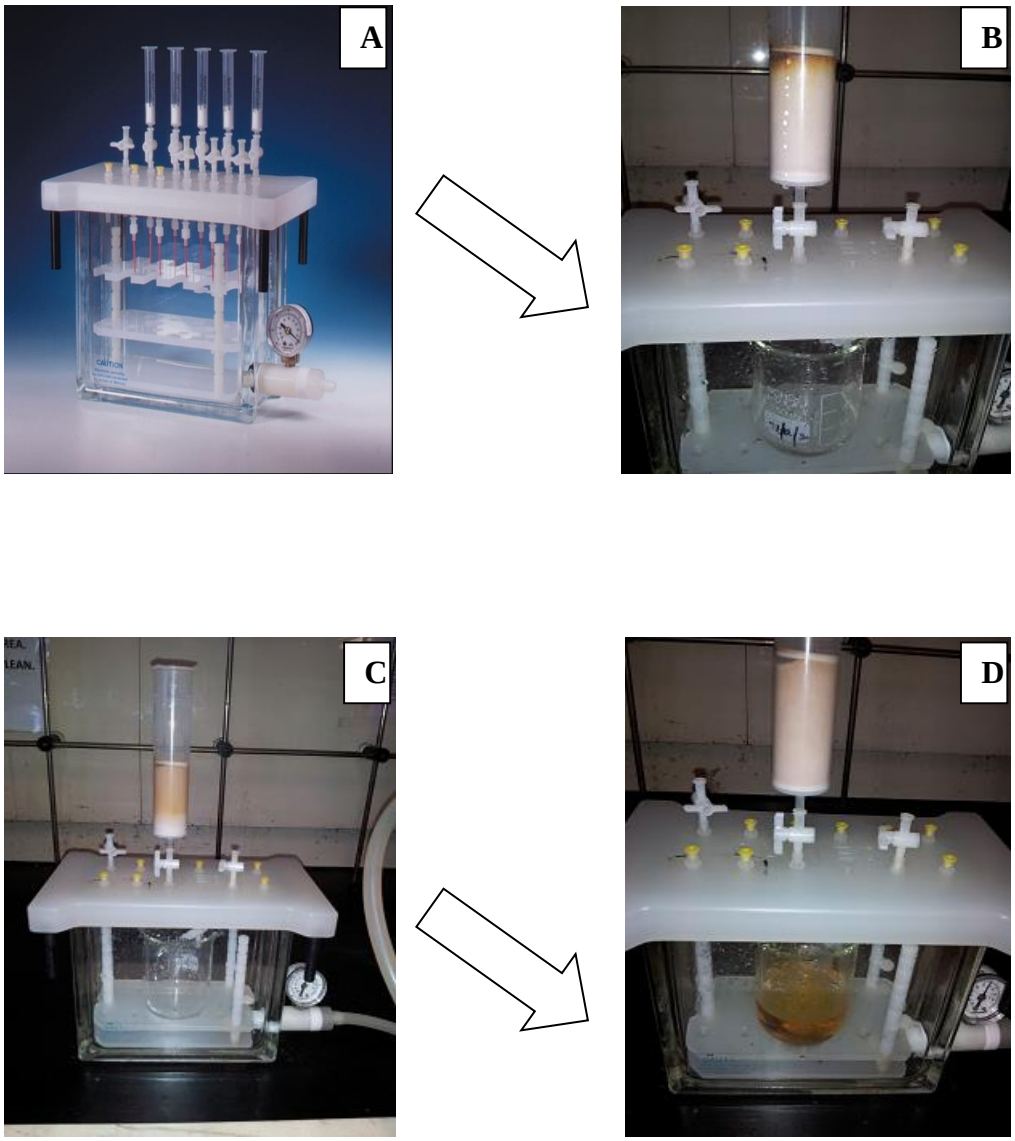
Appendix 5. Standard curve for DPPH.



Appendix 6. Standard curve for His-Leu.

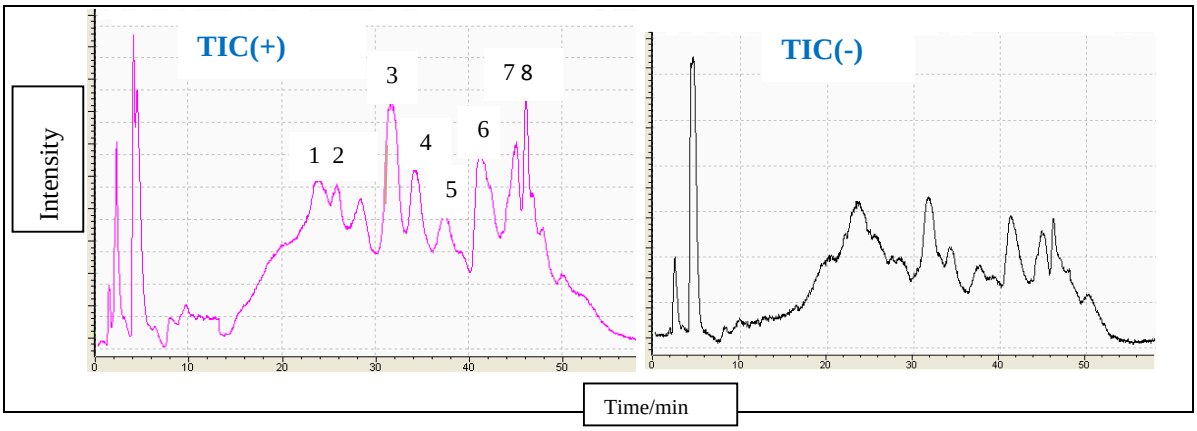


Appendix 7. Partitioning of crude extract in a separating funnel (A) and the UV-vis spectrum of hexane layer obtained on the double beam spectrophotometer compared to standard xanthophyl (B).

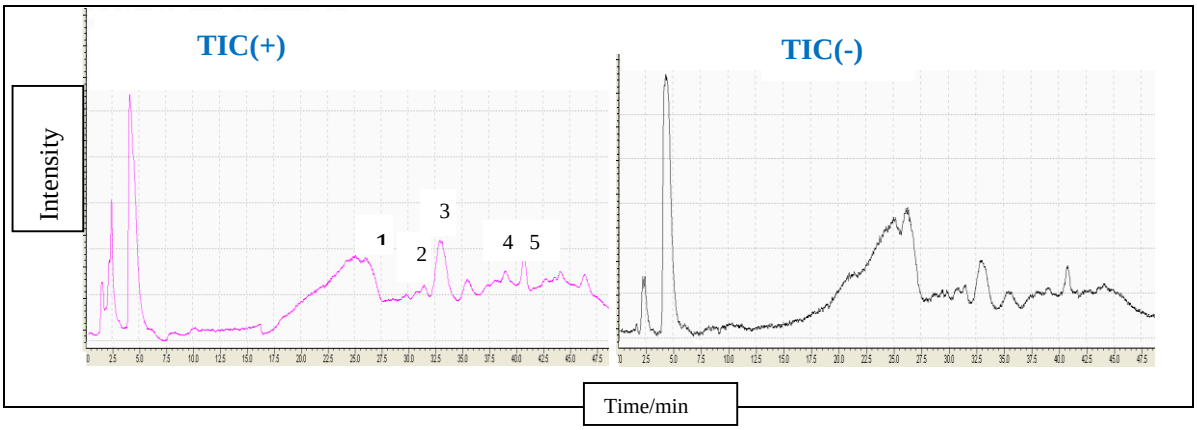


Appendix 8. Fractionation process of defatted crude extract on vacuum manifold strata solid phase C18 (A) multiple cartidges (B) collection of F1 fraction (C) collection of F2 fraction (D) after collection of F2 fraction.

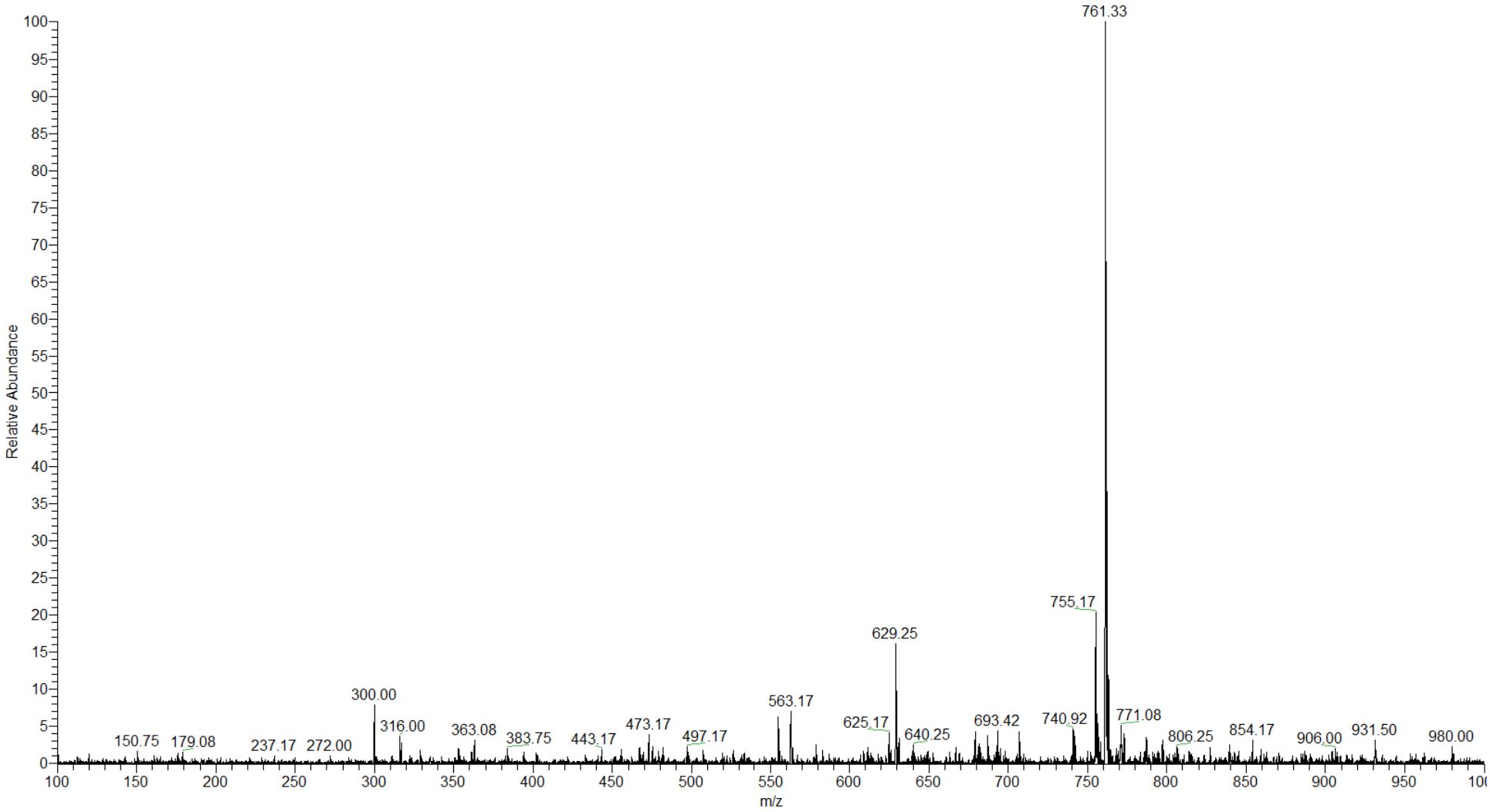
Appendix 9. Colour appearence of F3 and F4 fractions.



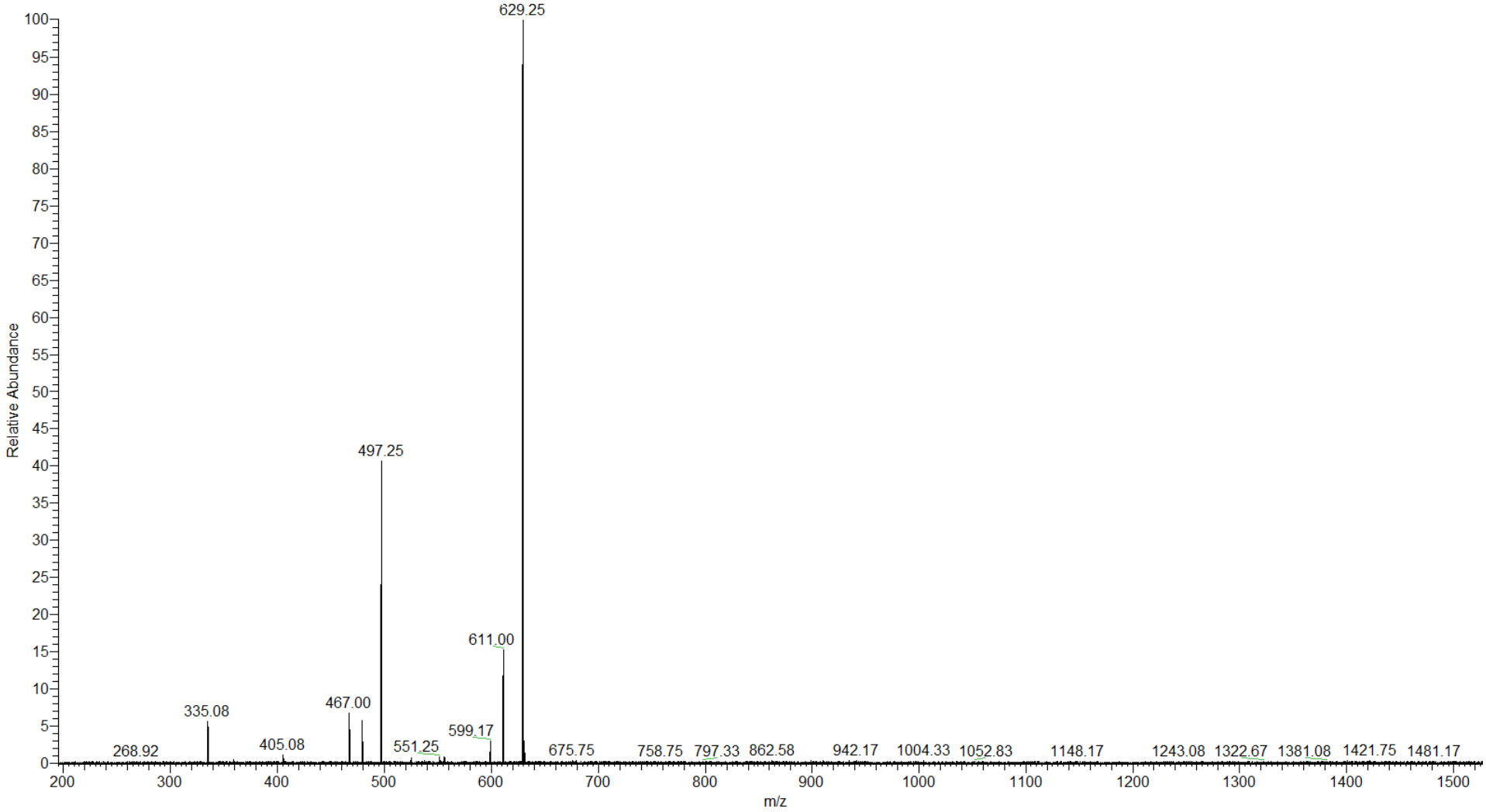
Appendix 10. HPLC-ESI-MS extended ion chromatograms in positive and negative modes obtained for the ethyl acetate fraction (F3).



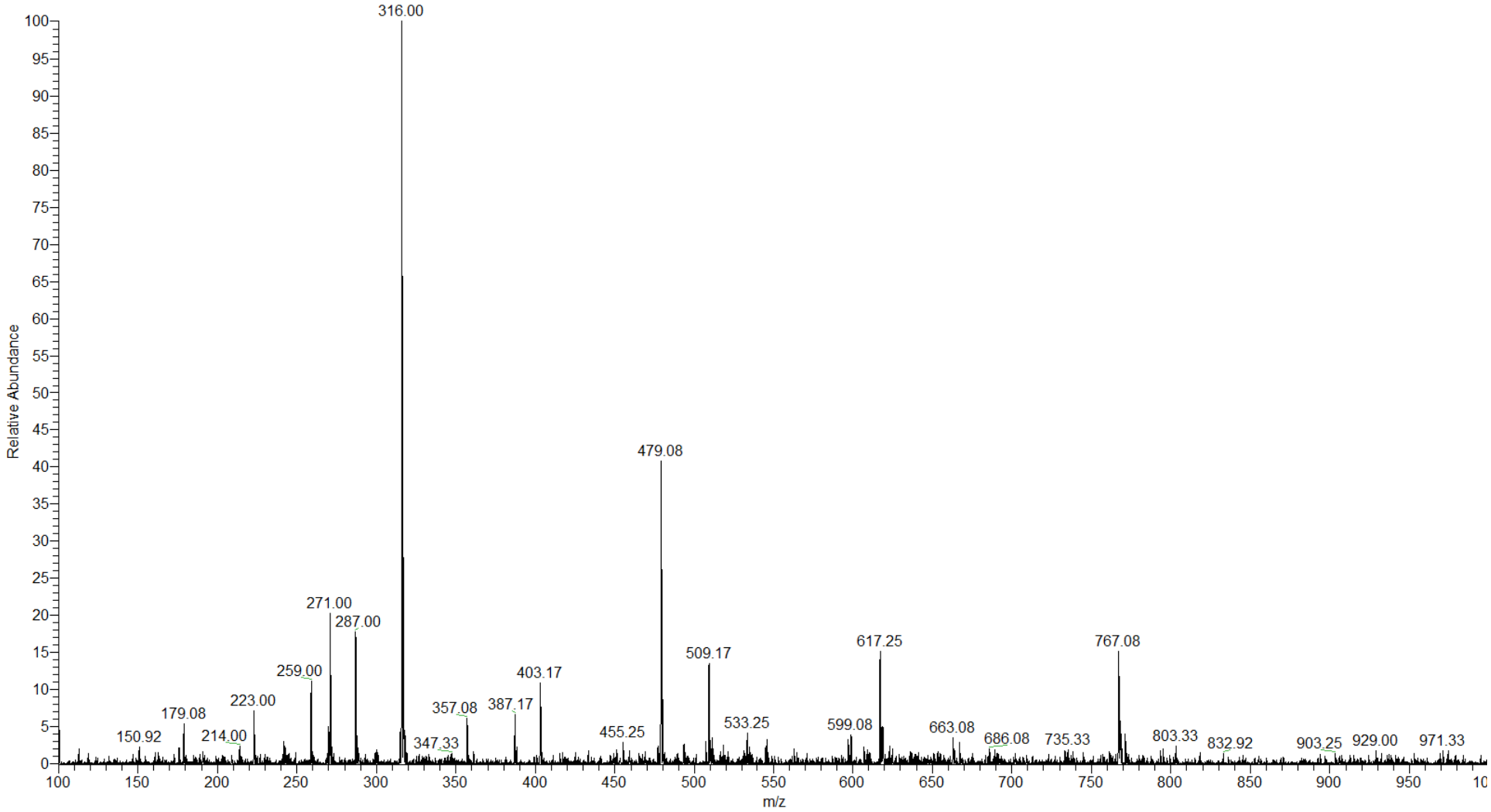
Appendix 11. HPLC-ESI-MS extended ion chromatograms in positive and negative modes obtained for the aqueous fraction (F4).



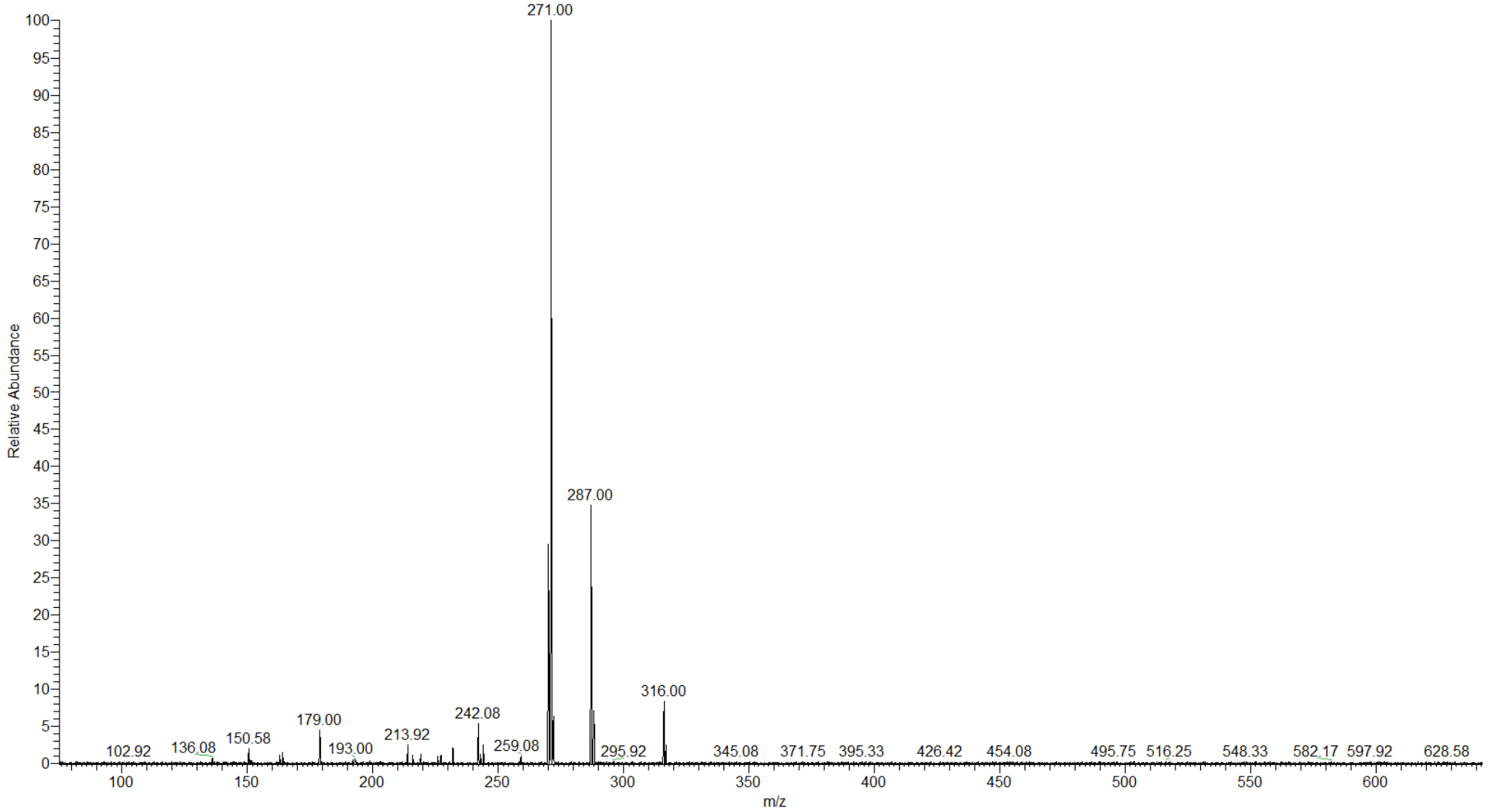
Appendix 12. Full mass spectrum obtained on HPLC-MS for compound 1, isolated from peak 1.



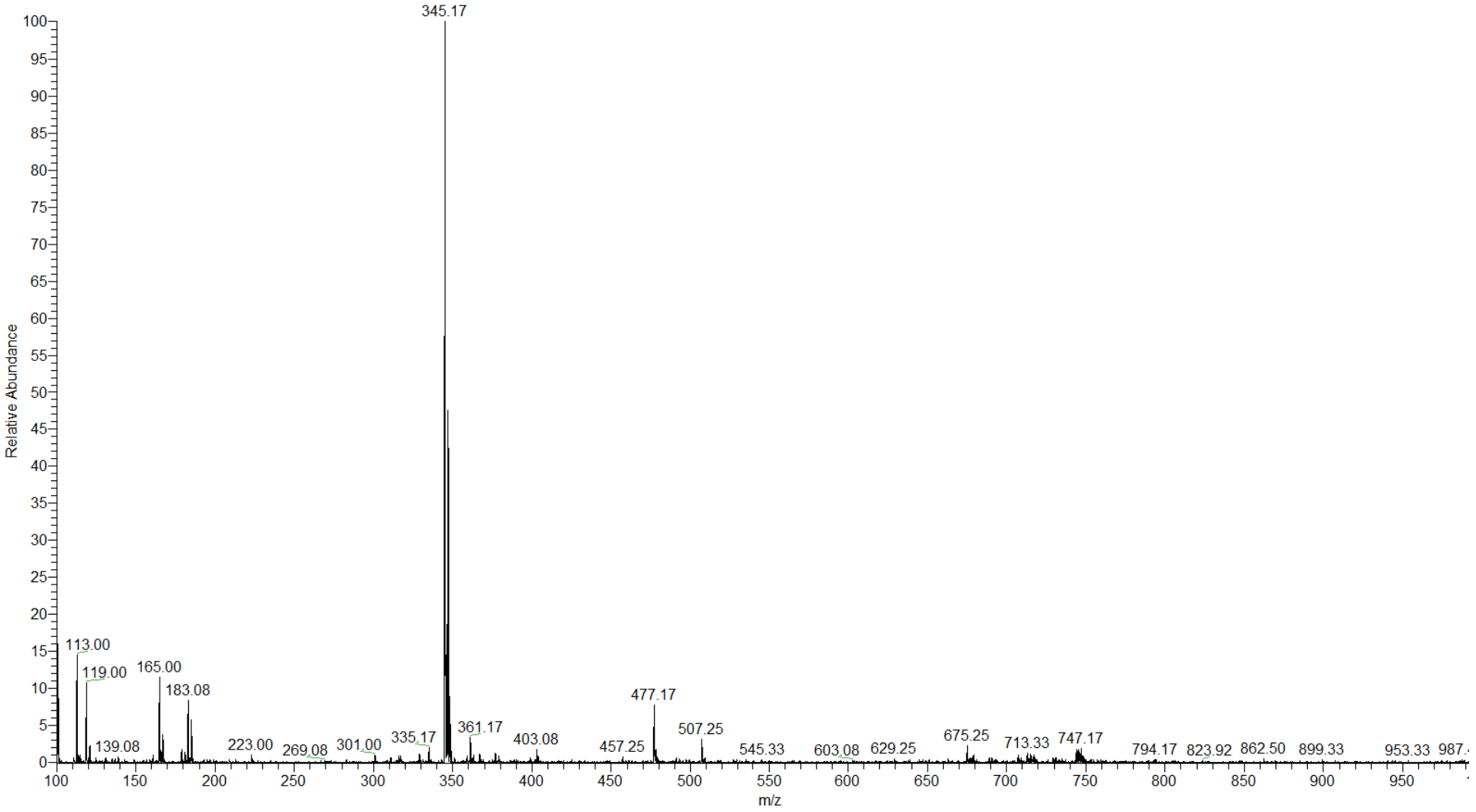
Appendix 13. MS/MS spectrum of the *m/z* 761.33 obtained for compound 1, isolated from peak P1.



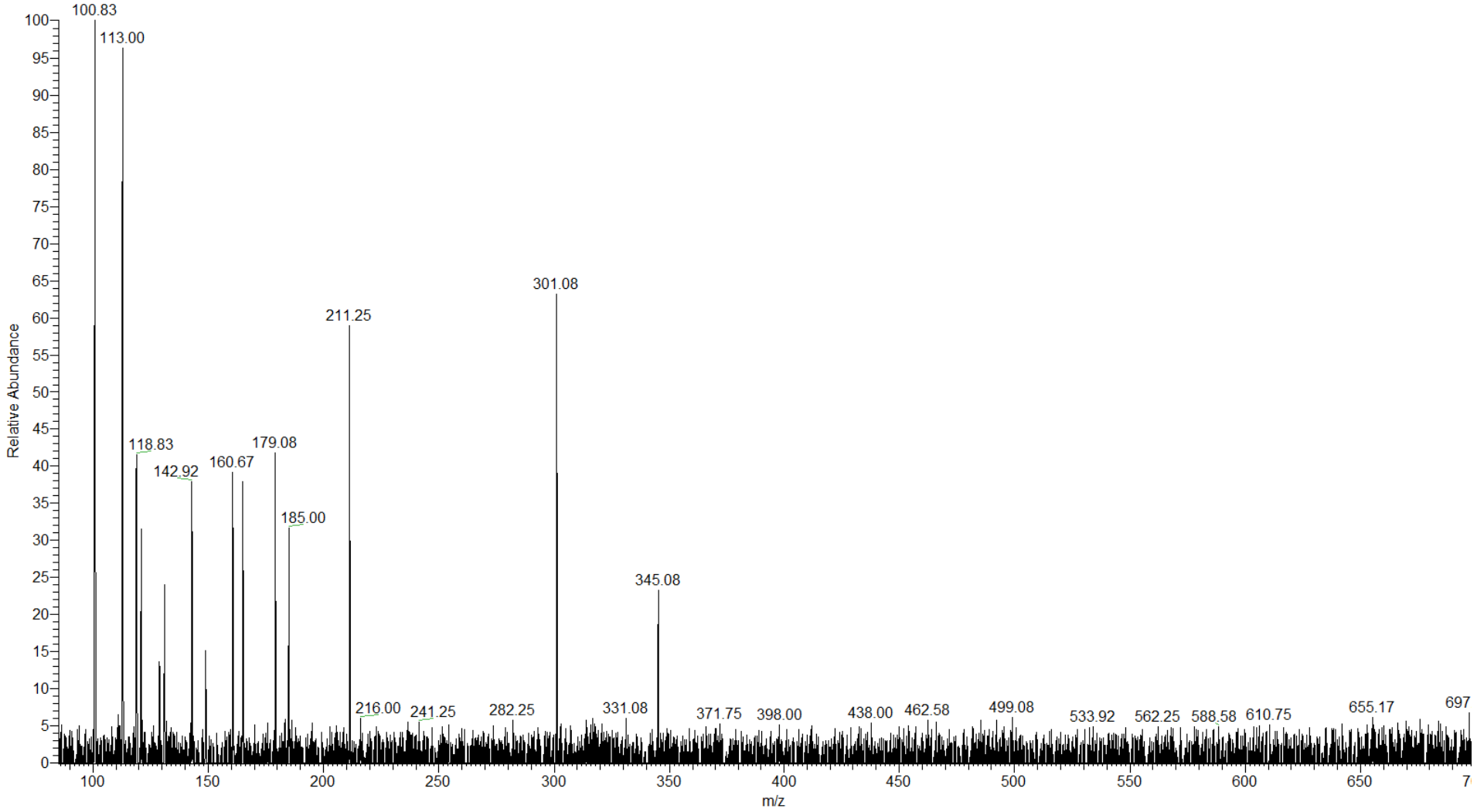
Appendix 14. Full mass spectrum obtained on HPLC-MS for compound 2, isolated from peak 1.



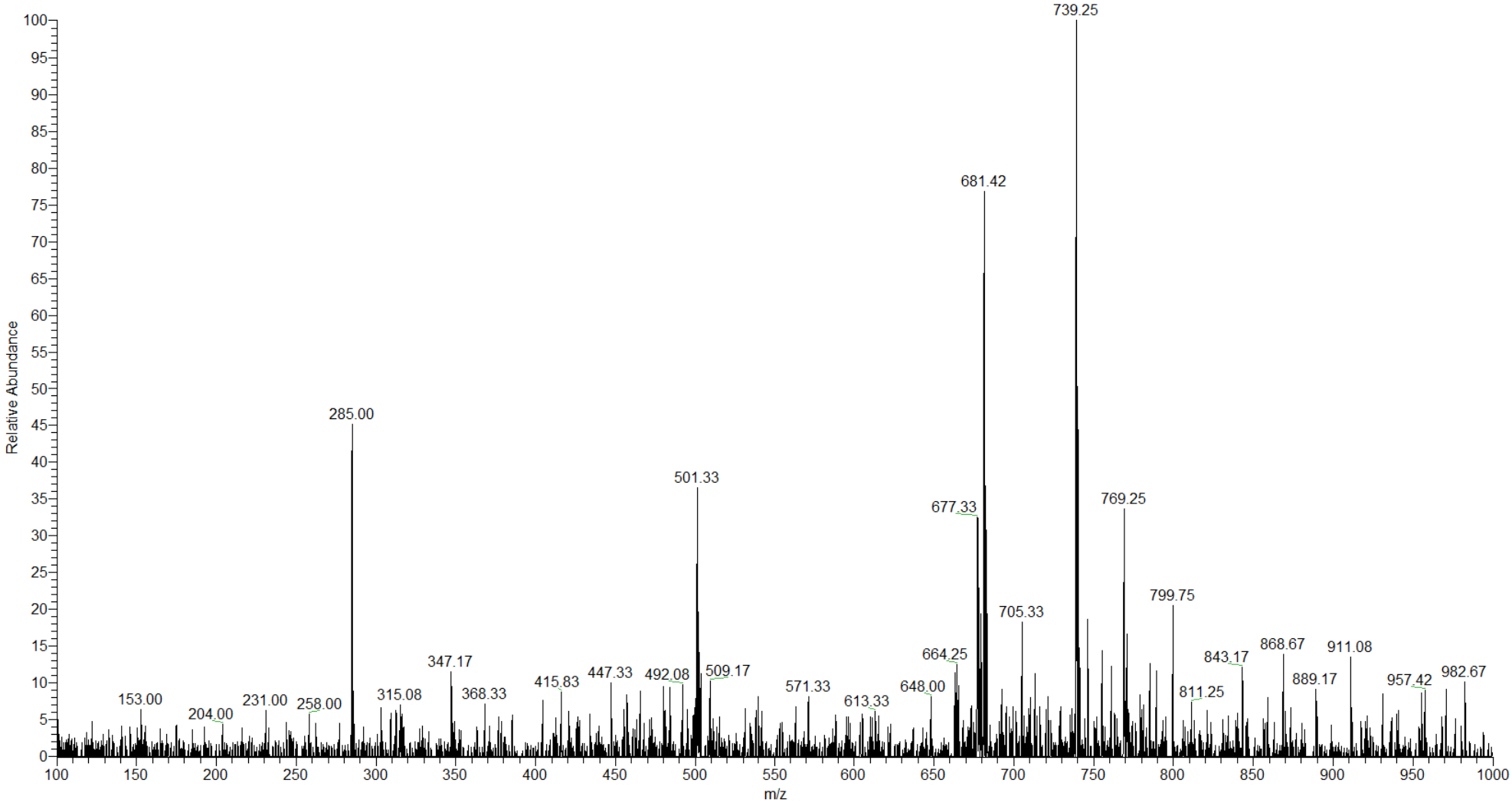
Appendix 15. MS/MS spectrum of the *m/z* 316 obtained for compound 2, isolated from peak P1.



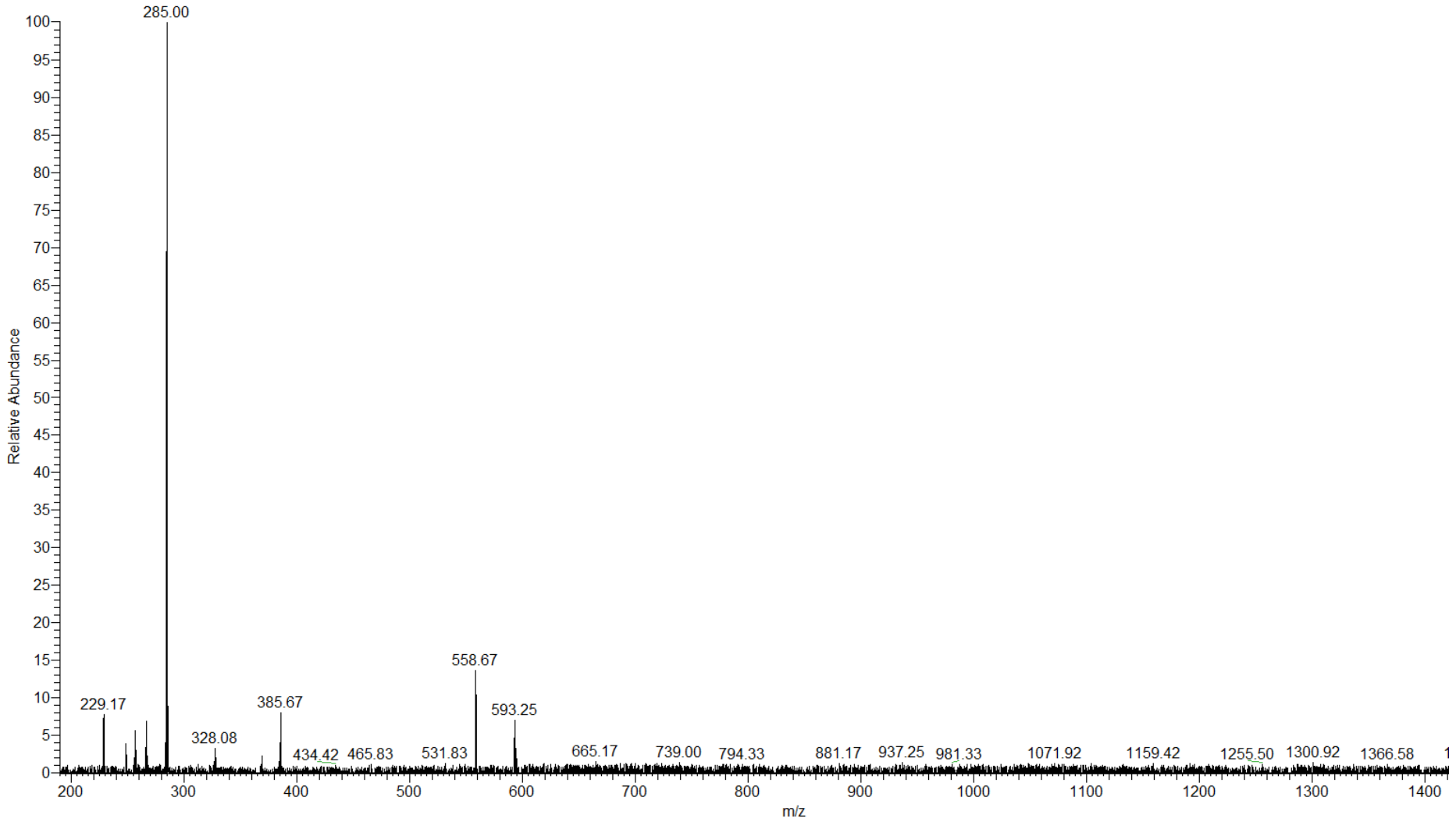
Appendix 16. Full mass spectrum obtained on HPLC-MS for compound 3, isolated from peak 1.



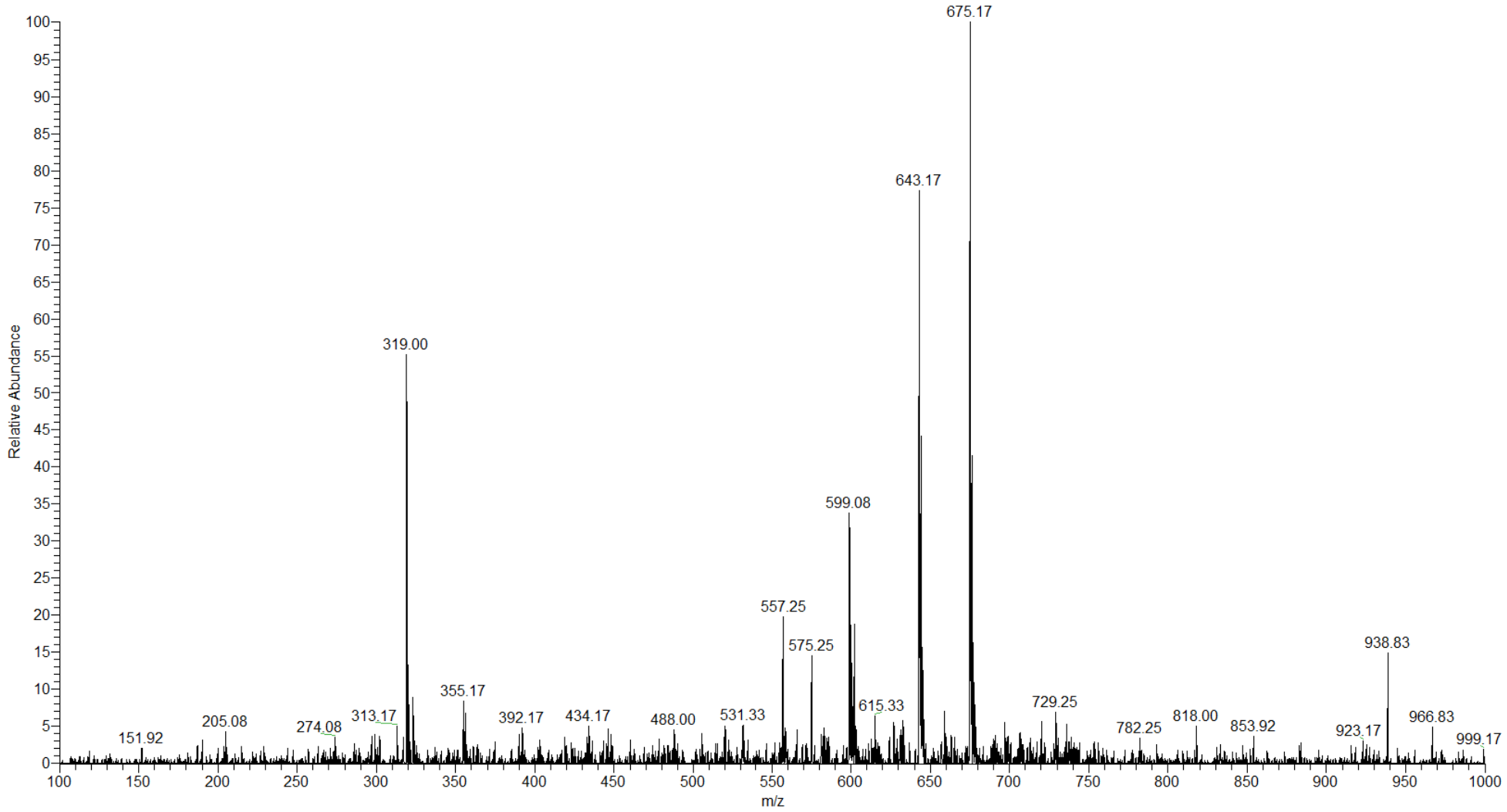
Appendix 17. MS/MS spectrum of the *m/z* 345.17 obtained for compound 3, isolated from peak P1.



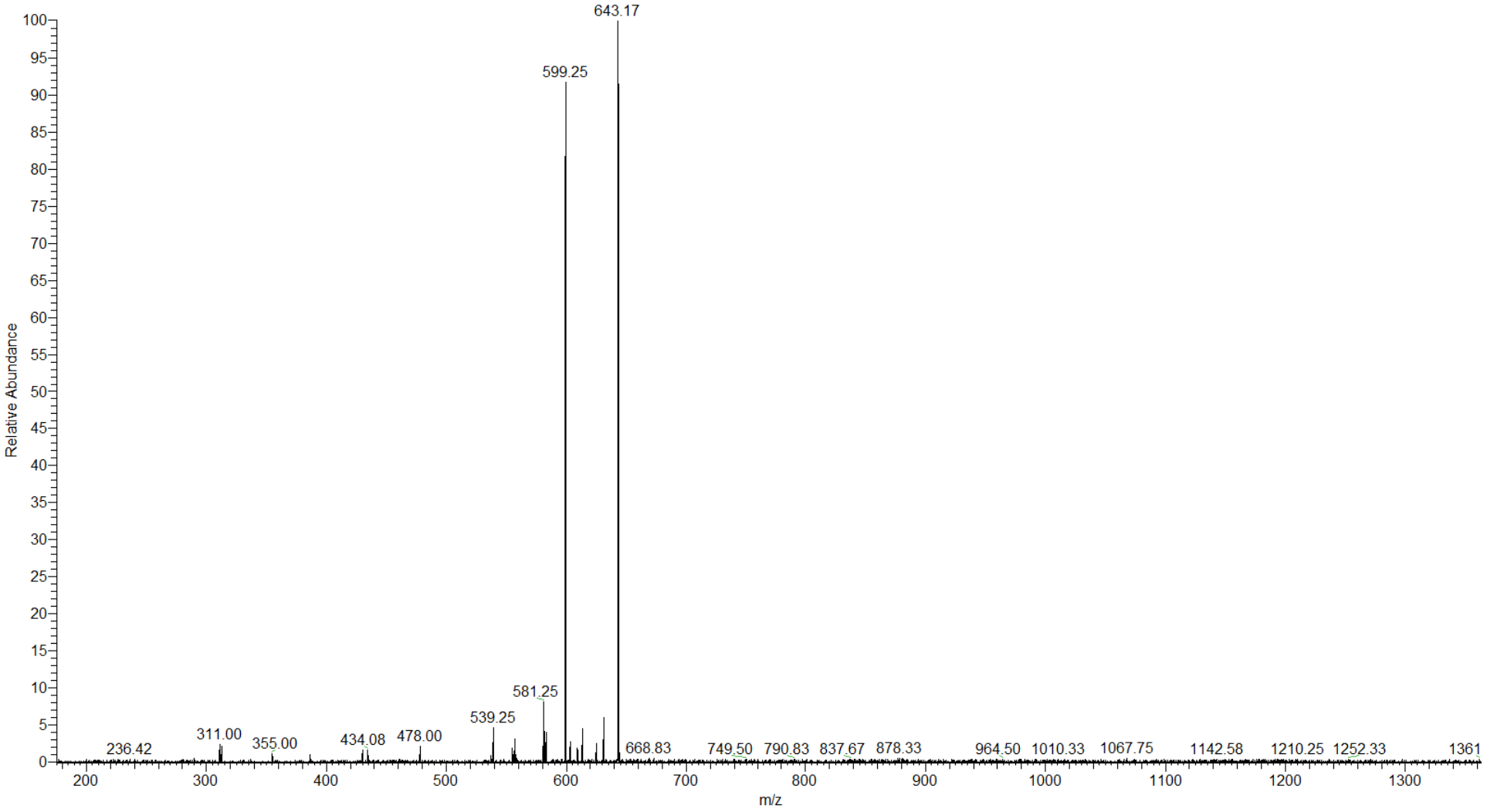
Appendix 18. Full mass spectrum obtained on HPLC-MS for compound 4, isolated from peak 3.



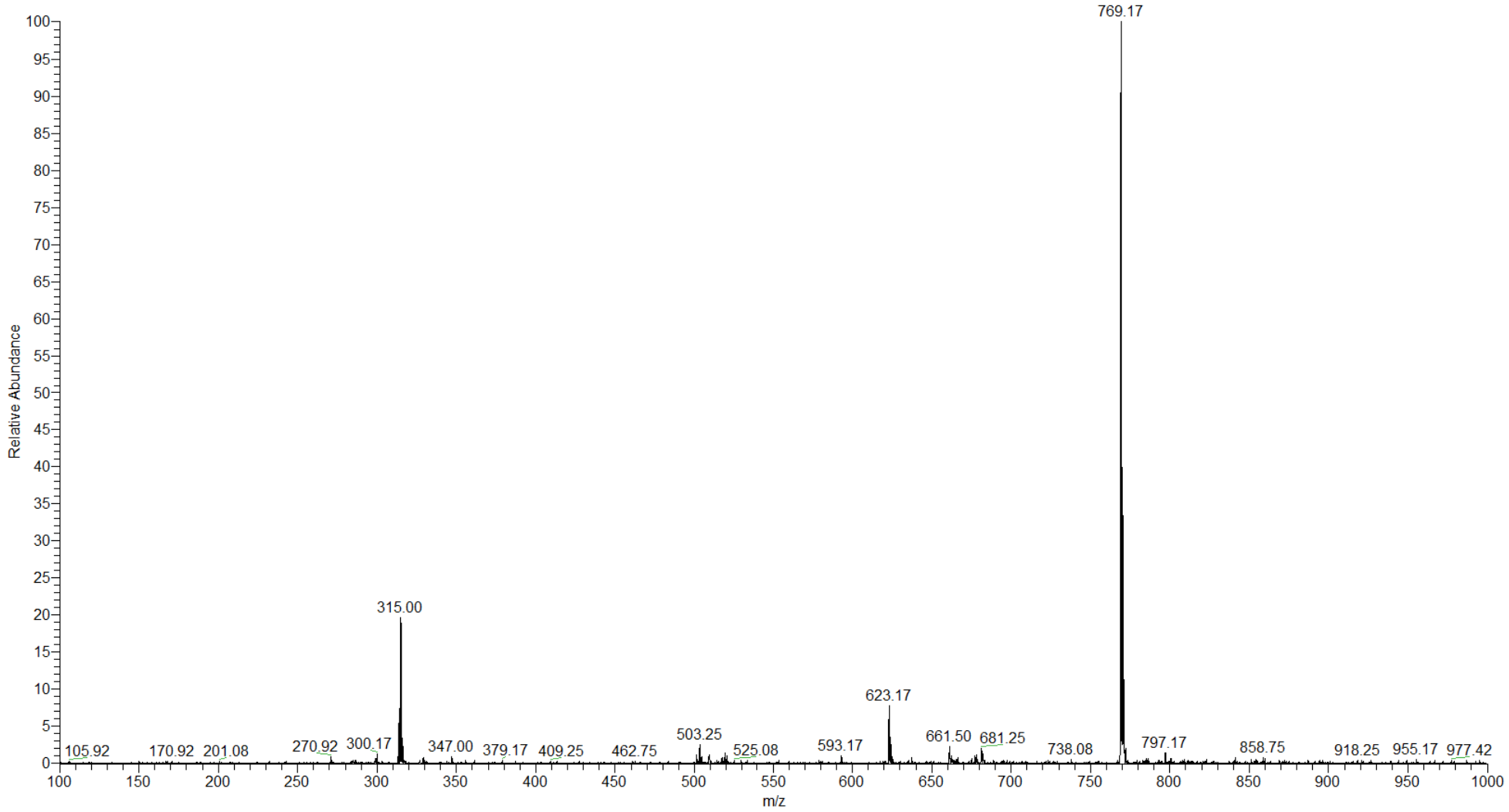
Appendix 19. MS/MS spectrum of the *m/z* 739.25 obtained for compound 4, isolated from peak P3.



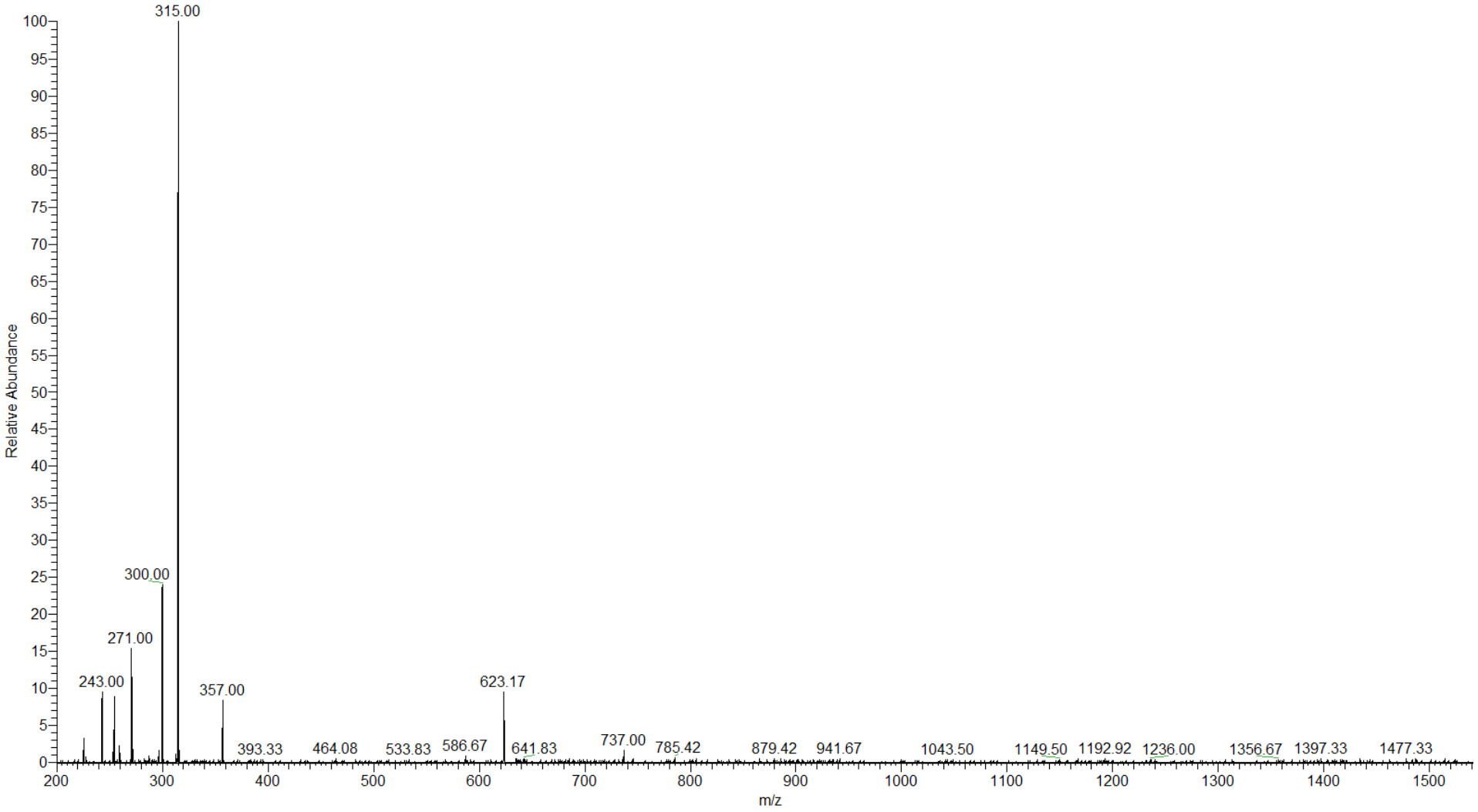
Appendix 20. Full mass spectrum obtained on HPLC-MS for compound 5, isolated from peak 3.



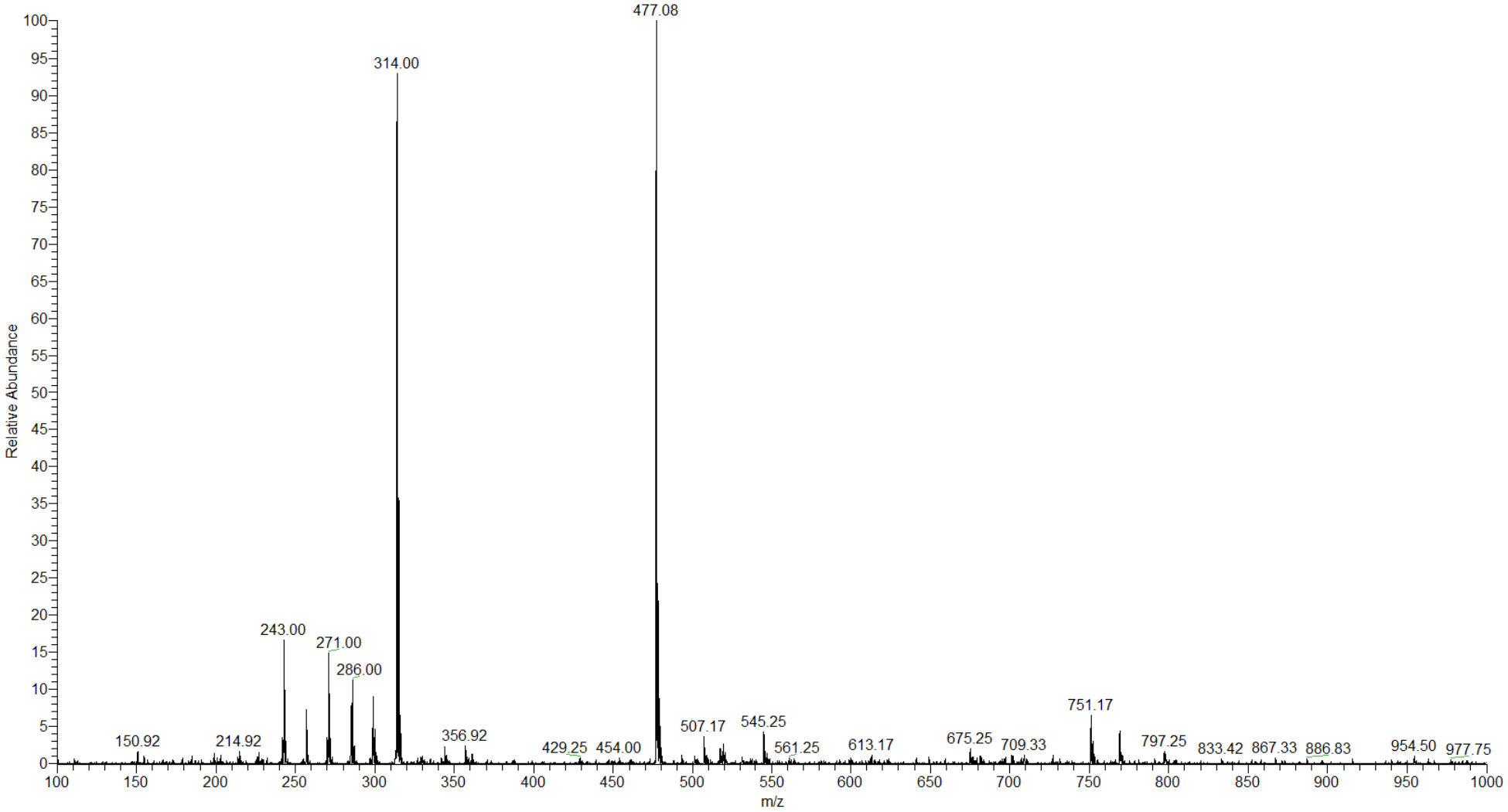
Appendix 21. MS/MS spectrum of the *m/z* 675.17 obtained for compound 5, isolated from peak P3.



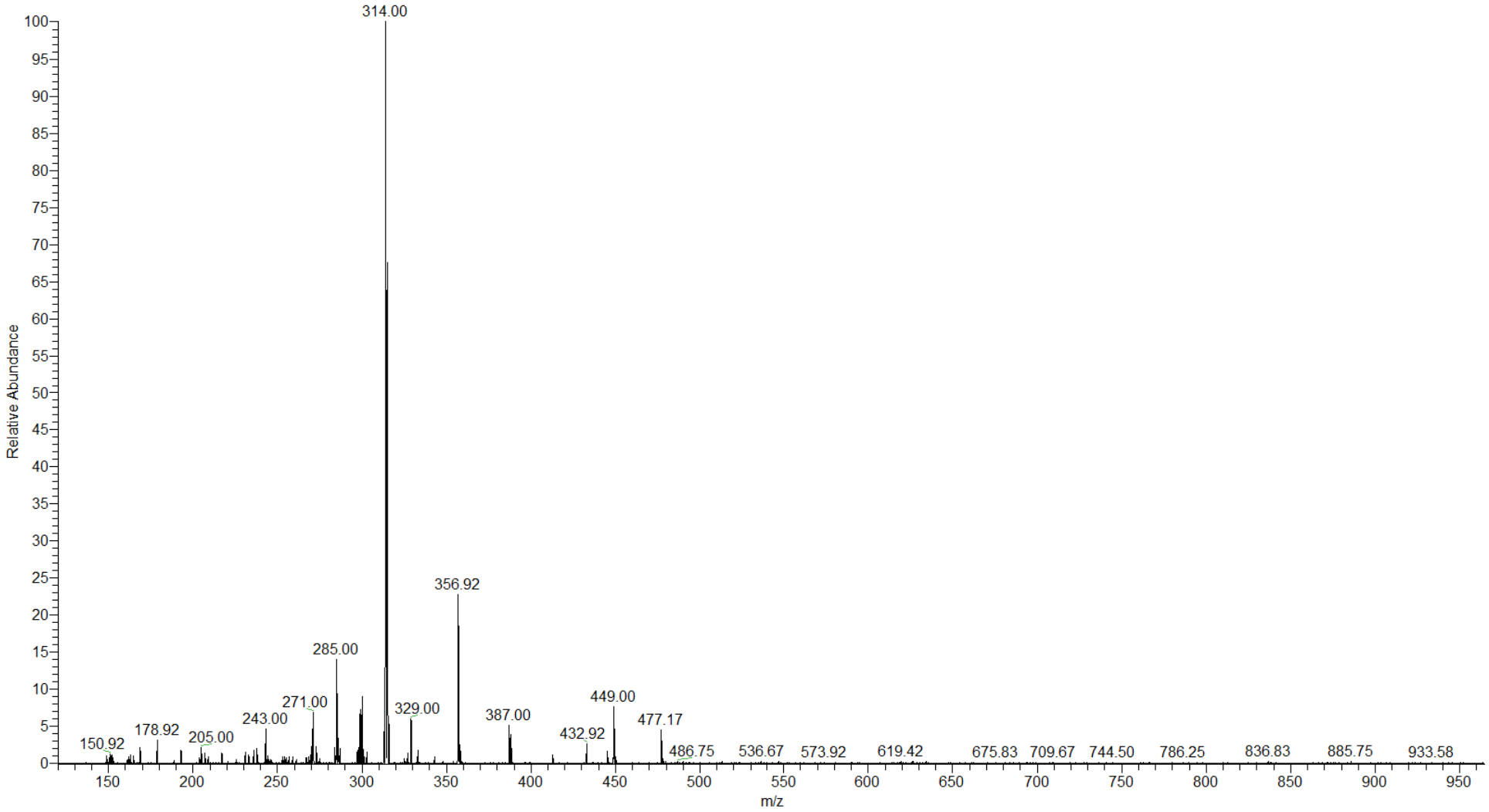
Appendix 22. Full mass spectrum obtained on HPLC-MS for compound 6, isolated from peak 4.



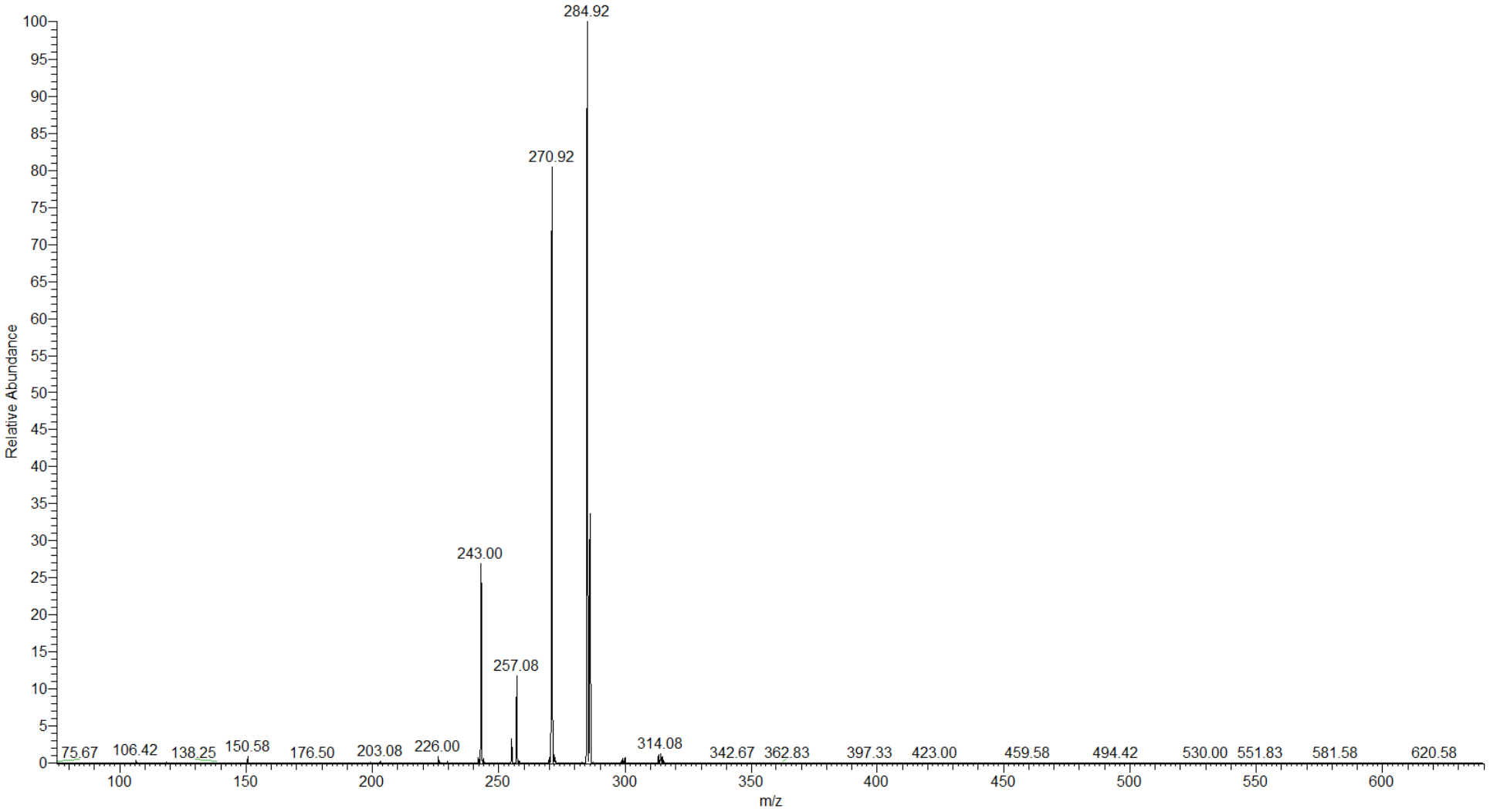
Appendix 23. MS/MS spectrum of the *m/z* 769.25 obtained for compound 6, isolated from peak P4.



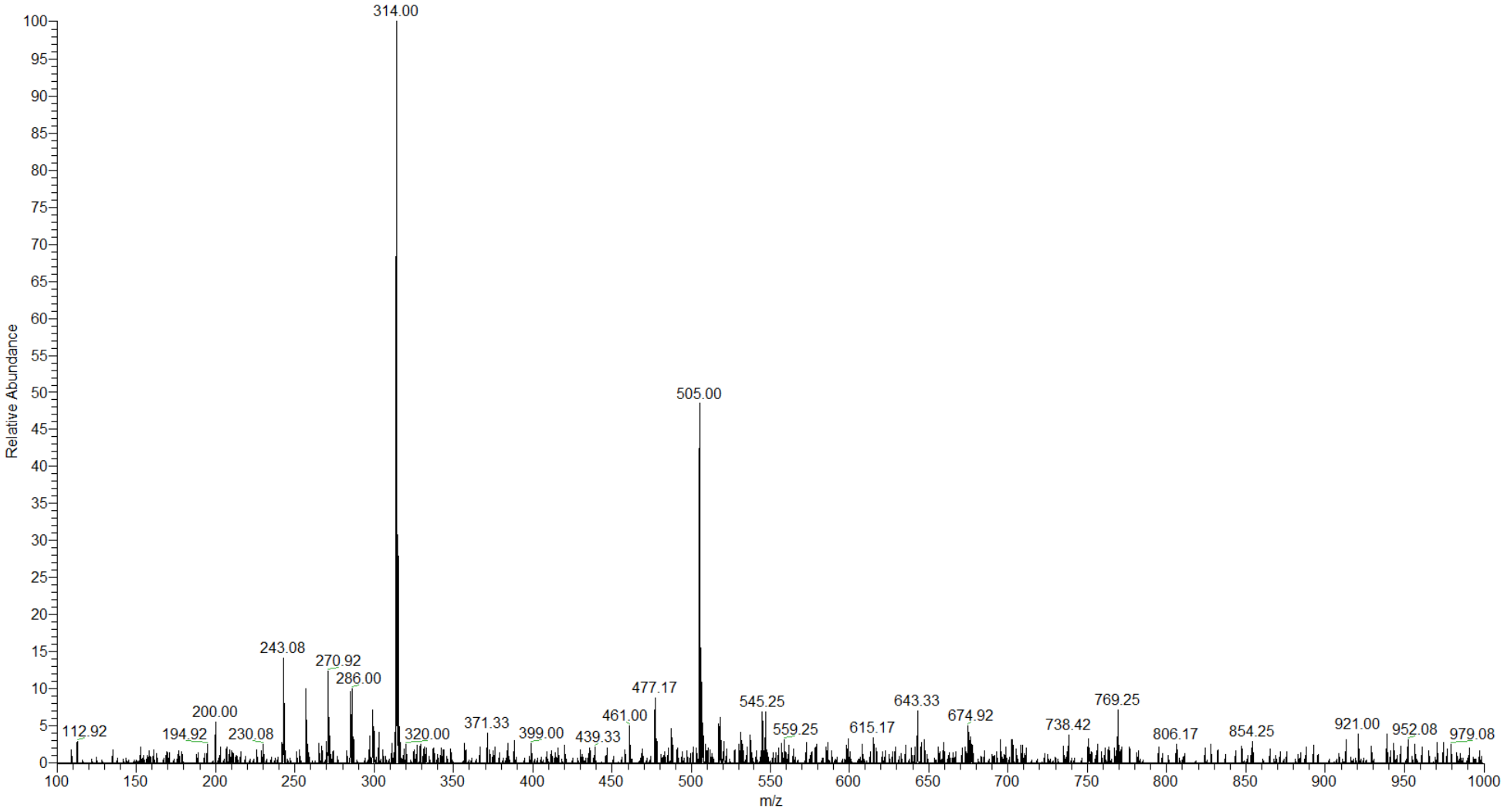
Appendix 24. Full mass spectrum obtained on HPLC-MS for compound 7, isolated from peak 4.



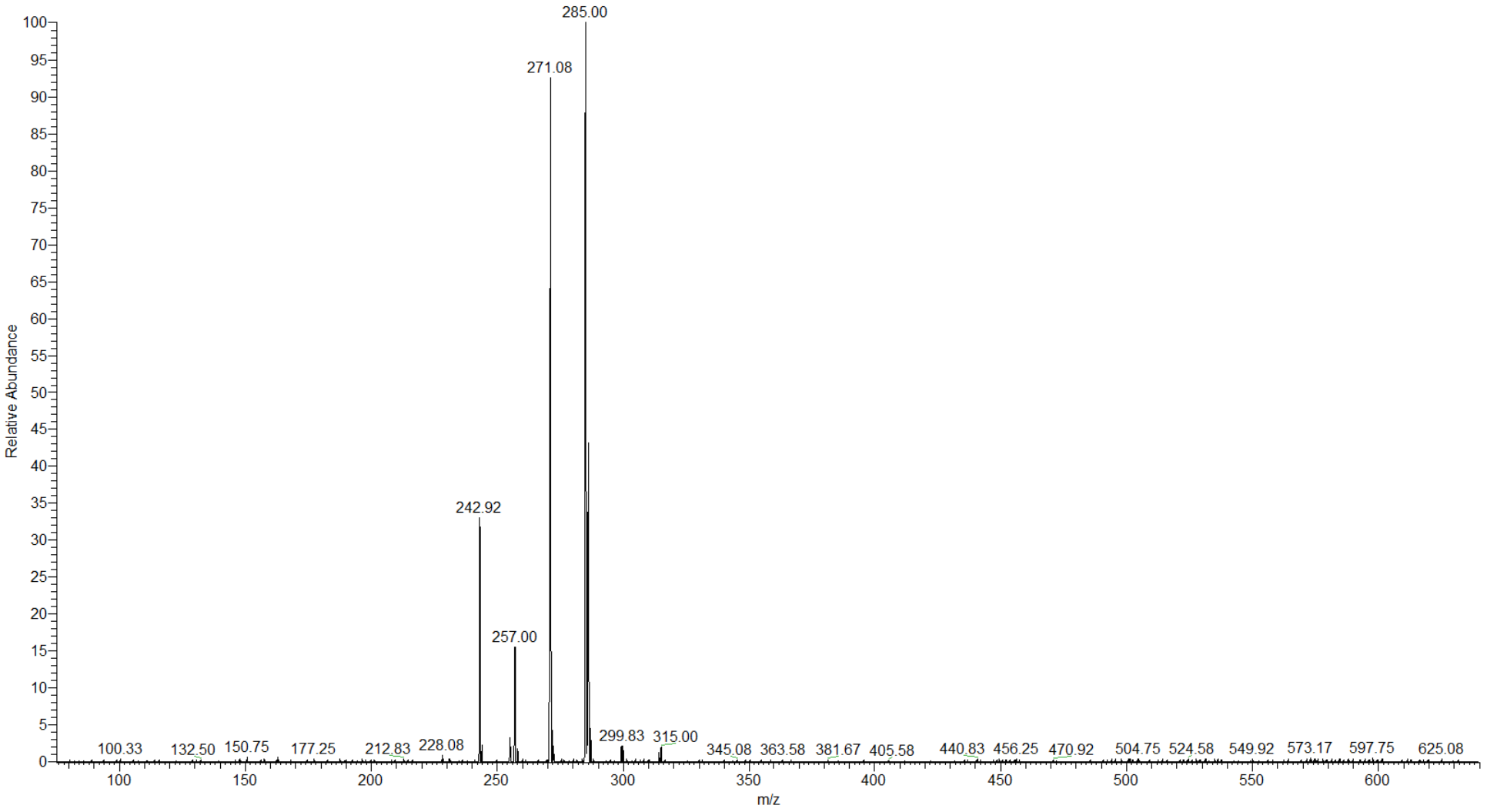
Appendix 25. MS/MS spectrum of the *m/z* 477.08 obtained for compound 7, isolated from peak P4



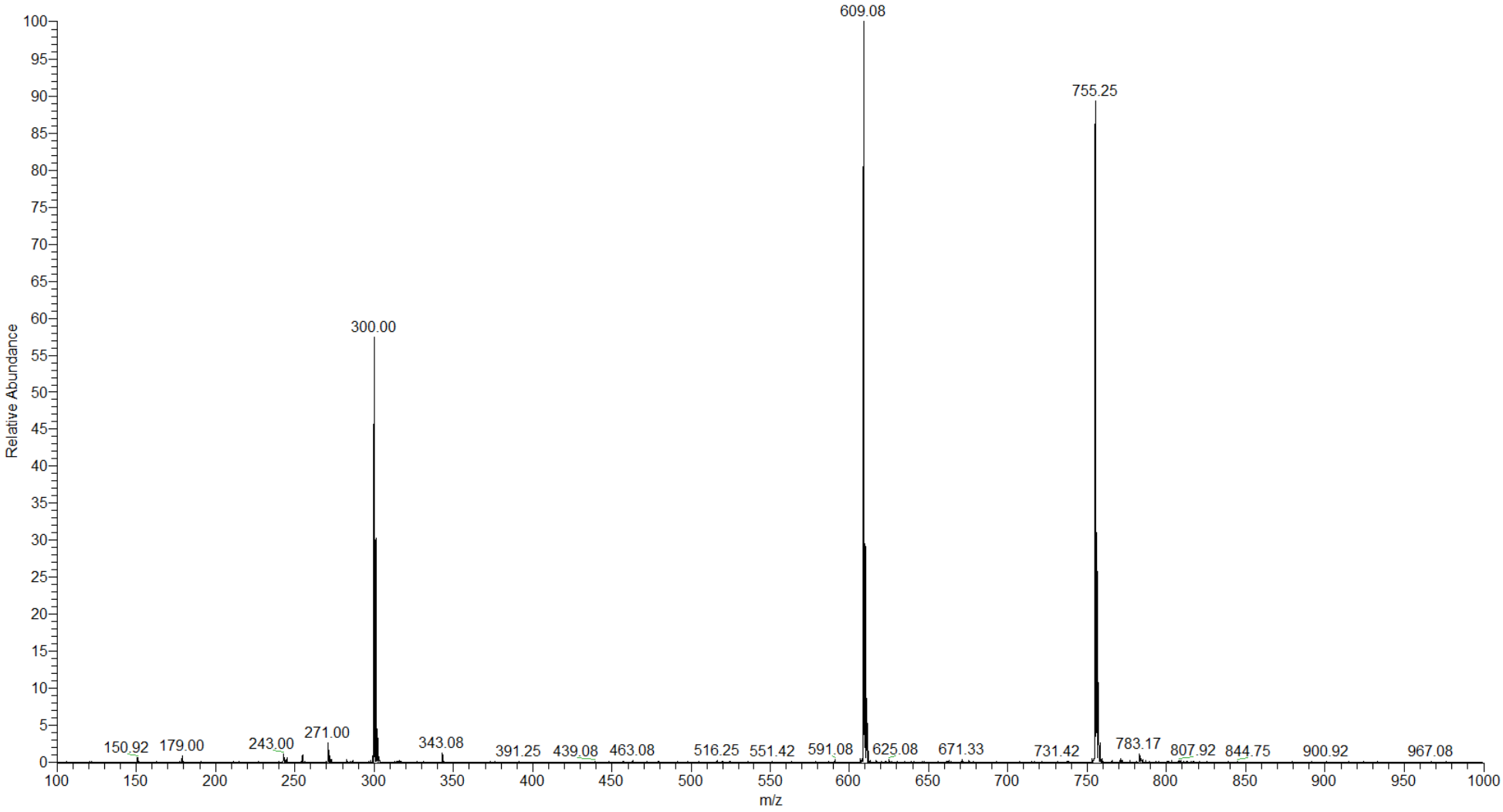
Appendix 26. MS/MS spectrum of the *m/z* 314.00 obtained for compound 7, isolated from peak P4.



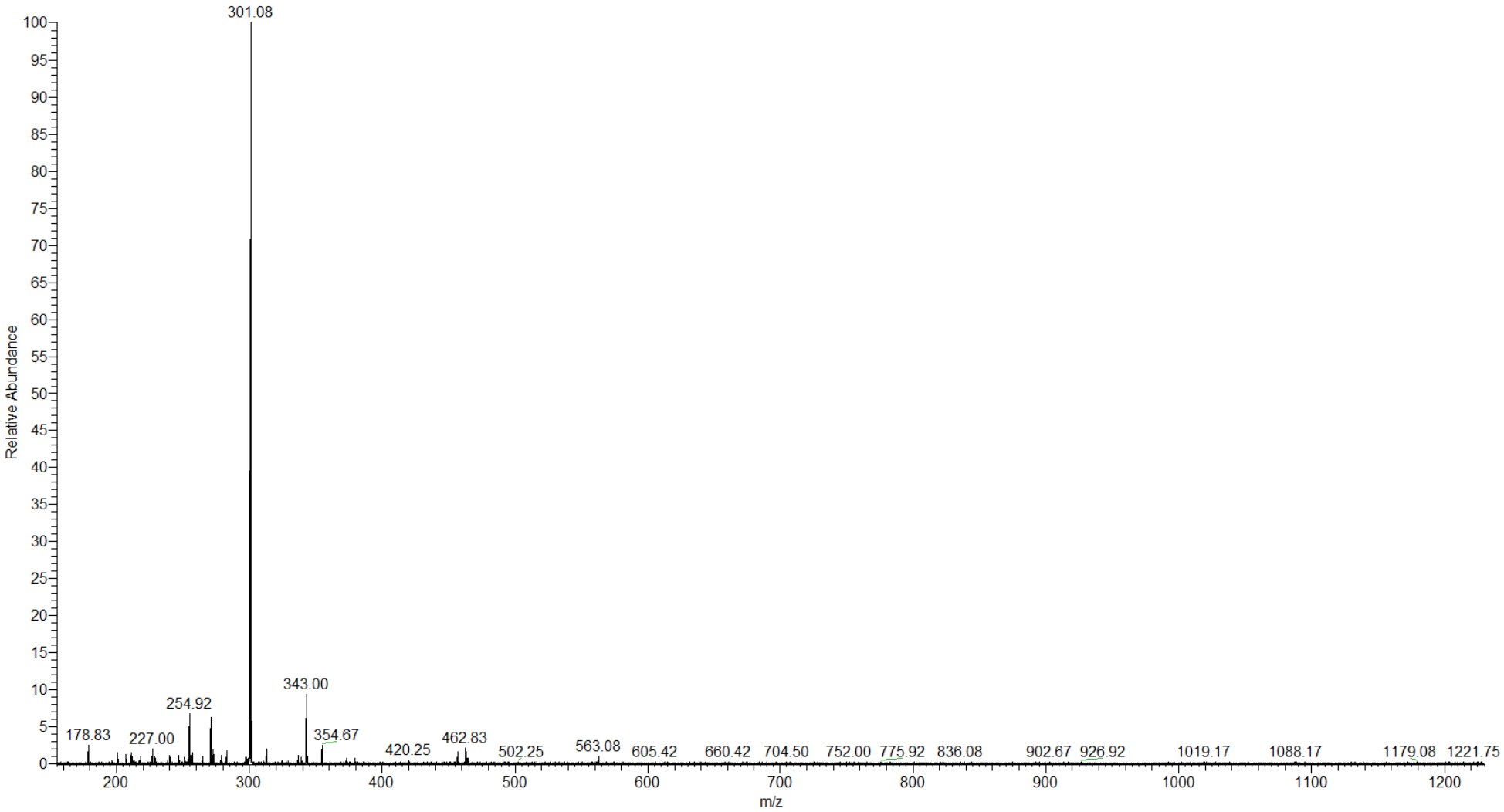
Appendix 27. Full mass spectrum obtained on HPLC-MS for compound 8, isolated from peak 4.



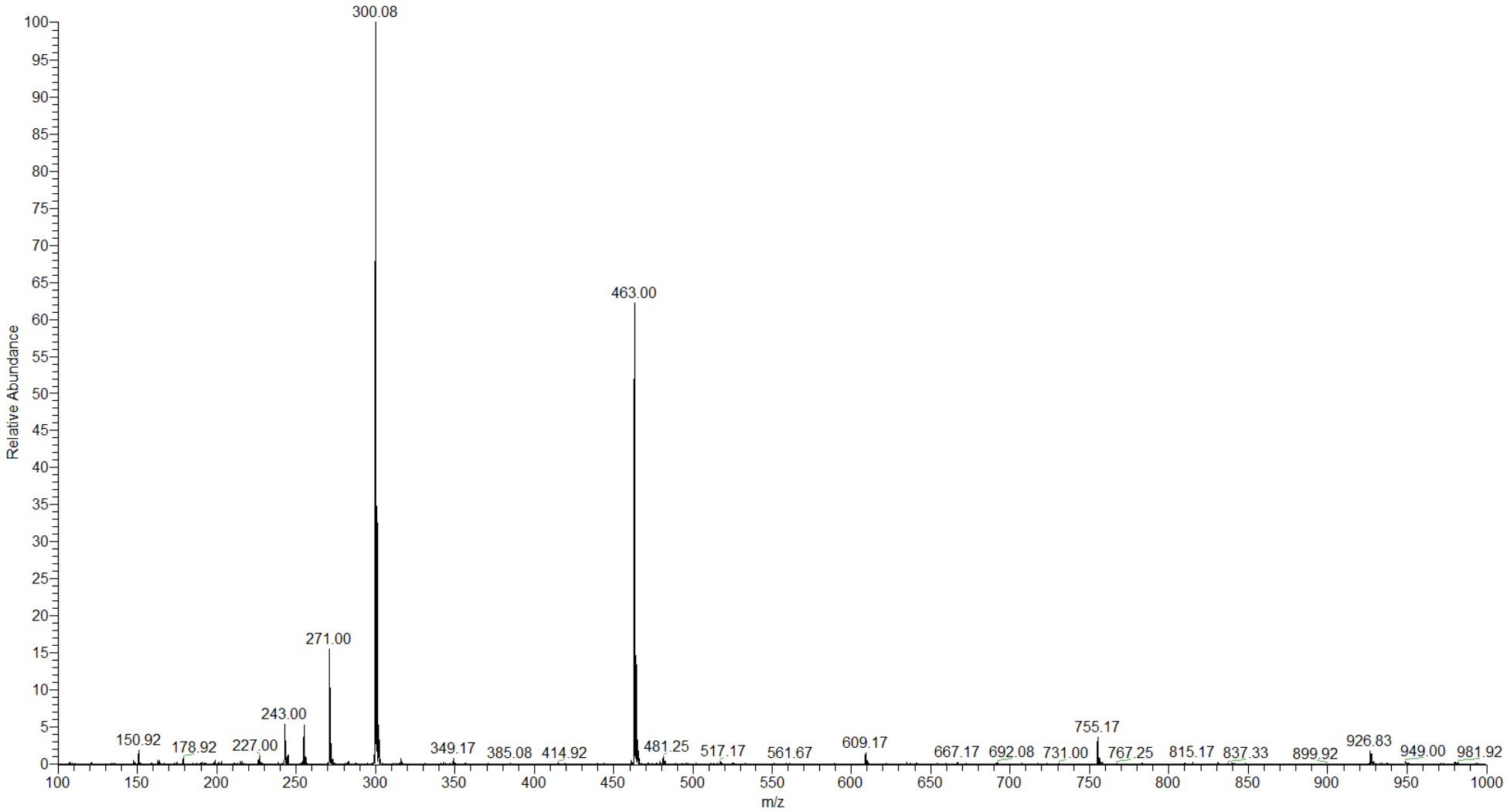
Appendix 28. MS/MS spectrum of the *m/z* 314.00 obtained for compound 8, isolated from peak P4.



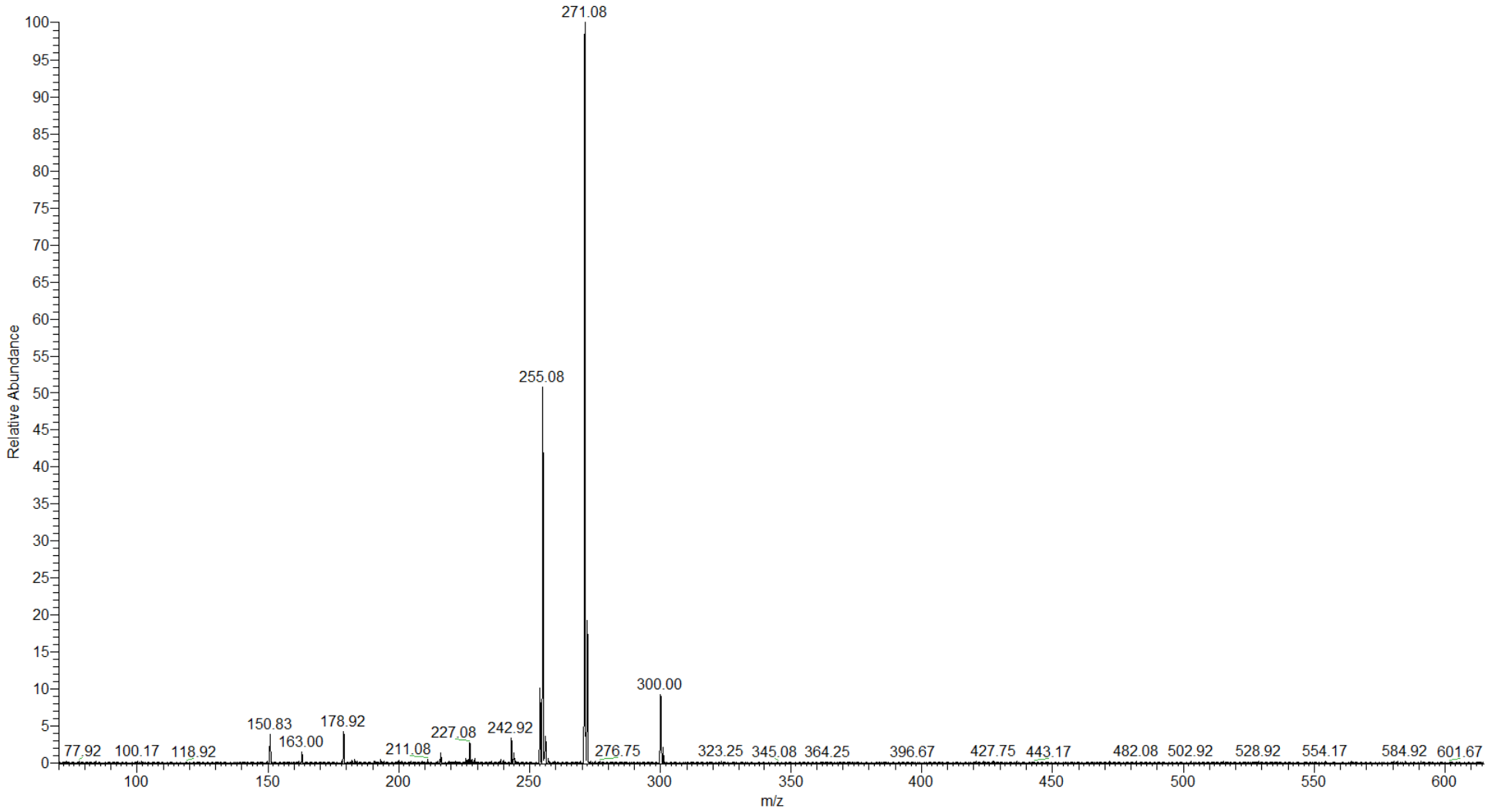
Appendix 29. Full mass spectrum obtained on HPLC-MS for compound 9, isolated from peak 5.



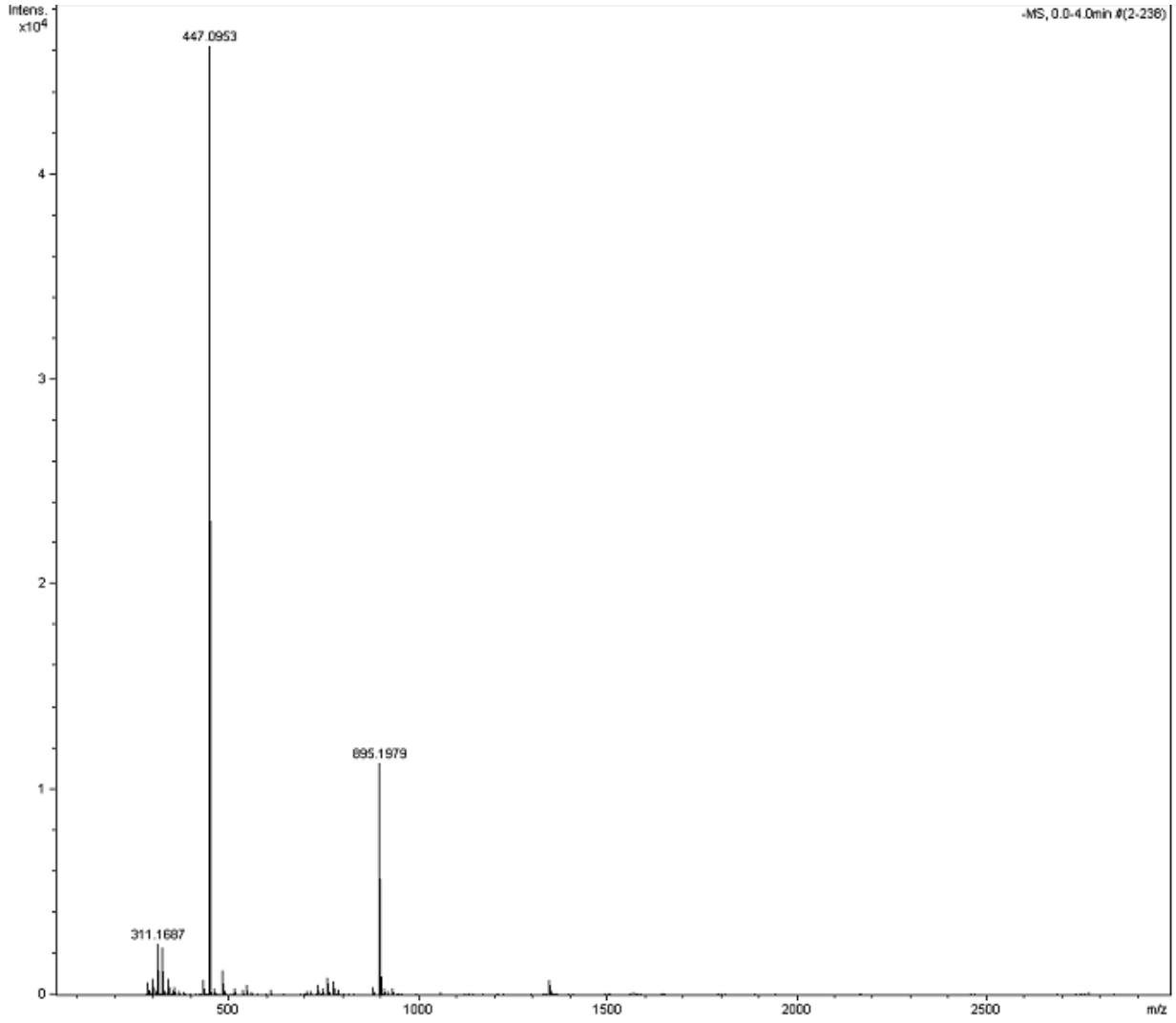
Appendix 30. MS/MS spectrum of the *m/z* 609.08 obtained for compound 9, isolated from peak P5.



Appendix 31. Full mass spectrum obtained on HPLC-MS for compound 10, isolated from peak 5.



Appendix 32. MS/MS spectrum of the *m/z* 300.00 obtained for compound 10, isolated from peak P5.



Appendix 33. MicrOTOF-ESI-MS chromatogram of compound 11, isolated from peak P2.

Appendix 34. Poster presentations

1. S.K. Hettihewa, S.Y.Quek, D.E. Stevenson, Fractionation of Polyphenols in *Actinidia macrosperma* genotype by Solid-Phase Extraction and High-Performance Liquid Chromatography, International conference at New Zealand Institute of Food Science and Technology (NZIFST) held in **2011** in Rotuora, New Zealand.

2. S.K. Hettihewa, S.Y.Quek, D.E. Stevenson, Isolation of flavonoids from *Actinidia macrosperma* fruit Solid-Phase Extraction and High-Performance Liquid Chromatography, International conference at New Zealand Institute of Chemistry (NZIC) held in **2011** in Hamilton, New Zealand.

3. S.K. Hettihewa, S.Y.Quek, D.E. Stevenson, Total Phenol Content and characterization of flavonoid glycosides from *Actinidia macrosperma*. Functional food symposium held in **2011**, University of Auckland, New Zealand.

4. S.K. Hettihewa, D.E. Stevenson, Y. Hemar, C. Hartinger, and P. Kilmartin. Flavonoid glycosides in the extract of kiwifruit (*Actinidia macrosperma*) by HPLC-DAD, ESI-MS and NMR, Research show Case held in **2012**, School of Chemical Sciences University of Auckland, New Zealand.

5. S.K. Hettihewa, Y. Hemar, and C. Hartinger**.** Polyphenol contents and *in vitro* antioxidant activities of different extracts from kiwifruit (*Actinidia macrosperma*). International conference & Exhibition-Functional Foods, Nutraceuticals, Natural Health Products and Dietary Supplements held in December, **2012,** Hawaii, USA.

6. S.K. Hettihewa, Y. Hemar, C. Hartinger, and H.P.Vasantha Rupasinghe. Phenolic composition and antioxidant activities of different extracts from *Actinidia macrosperma,* Research show Case held in **2013**, School of Chemical Sciences University of Auckland, New Zealand.