Nutraceutical antioxidant potential and polyphenolic profiles of the Zambian market classes of bambara groundnuts (*Vigna subterranea* L. Verdc) and common beans (*Phaseolus vulgaris* L.)

Vincent Nyau (NYXVIN001)

Thesis Presented for the Degree of

DOCTOR OF PHILOSOPHY

in the Department of Molecular and Cellular Biology

UNIVERSITY OF CAPE TOWN

June 2013

Supervisor: Jill Farrant (University of Cape Town)

Co-supervisors: Jerry Rodriques (University of Cape Town)

Shiv Prakash (University of Zambia)

To refer to this thesis:

Nyau V. (2013), 'Nutraceutical antioxidant potential and polyphenolic profiles of the Zambian market classes of bambara groundnuts (*Vigna subterranea* L. Verdc) and common beans (*Phaseolus vulgaris* L.)', PhD Thesis, Faculty of Science, University of Cape Town.

Declaration

I declare that the work contained in this thesis has not been previously submitted for a degree at this or any other tertiary institution for examination, that it is my original work, and all materials previously published or unpublished contained herein have been appropriately acknowledged.

Vincent Nyau
June 2013, Cape Town

Dedication

This discourse is dedicated to the following extraordinary people:

My wife, Rosaline Mwape Nyau
My daughter Thulani and son Thembalethu
My late supervisor, Professor Wolf Brandt (MHSRIP)
My late brother, Patrick Akulowa Nyau (MHSRIP)

An individual succumbs, but he does not die if he has left something behind

- *WILL DURANT*

The final test of a leader is that he leaves behind in other people the convictions and the will to carry on

- *WALTER LIPPMAN*

Acknowledgements

First, I am thankful to God for the life and good health during this challenging adventure. I would like to acknowledge my first supervisor, late Professor Wolf Brandt for the early seed he sown in me on various assays. I would also like to acknowledge Olivier Kalenga, former assistant to Wolf for his technical assistance.

I wish to express my sincere personal thanks and gratitude to Professor Jill Farrant, Dr. Jerry Rodriques and Dr. Shiv Prakash for the valuable supervision of this thesis. Their support, guidance, confidence, helpful suggestions, goodwill and many fruitful discussions inspired a great deal on this accomplishment. Jill and Jerry, I am inspired and humbled by your receptiveness and aspiration to continue with the supervision of this thesis after the demise of my first supervisor Professor Wolf Brandt. Though I become hopeless and lost my confidence with the bereavement, my sanity was restored with your approachability and goodwill for continuance of this work. I shall forever be thankful.

I would also want to record my remarkable indebtedness to my lovely wife Rosaline Mwape Nyau, my two kids Thulani and Thembalethu, and nephew Akulowa Nyau for their love, encouragement, support and patience during my wondering days in the wilderness. You guys offered listening ears and hearts to my laughters and tears of failed assays after a long day in the laboratory. The support from my Dad, Mum and brothers cannot be overlooked; they have stood by me to this end.

I am greatly indebted to USHPiA for the financial assistance that facilitated my research and stay in Cape Town. The works and generosity of your organisation have immensely contributed to the career development of many scholars across the African continent. I am particularly thankful to Norma Derby, the Administrator of USHEPiA for logistical support, and to Nan Warner, former USHEPiA Manager for making earlier logistical arrangements in the planning phase of this research. I am also very grateful to Yolande Burrows, the Departmental Manager and Glynnis Spannenberg, the Purchasing Administrator in the Department of Molecular and Cell Biology for their passionate support and proficiency in handling my research funds.

I would like to extent my heartfelt thanks to all the member of the Plant Stress Research group, University of Cape Town, for their warm attitude and cooperation during my stay in the group. To the members of Lab 434, Keren, Claire, Millie, Ali, Ryman and Amelia among whom I have shared wonderful moments, I say thank you for the support, care and love. My special thanks should also go to the University of Zambia for granting me study leave which enabled me to pursue my research without any hindrance. I am grateful to all the staff in the School of Agricultural Sciences for the encouragement and moral support. To John Shindano and Himoonga Bernard Moonga, I say thank you for keeping the Department of Food Science and Technology alive and viable especially at a time when most members are on study leave.

I would like to acknowledge all the staff at ALL AFRICA HOUSE for the favourable environment during my entire stay in Cape Town. Professor Himoonga, Patrick, Cally and Nomonde, continue with your wonderful hospitality. I am also very thankful to Peggoty, Juliana, Deo and Rubby, my fellow USHEPiA warriors for the love, support and family care offered to me in this undertaking. I would also like to acknowledge Christian and Dennis for their wonderful friendship.

Thank you all – Naonga Zikomo

Vincent Nyau
June 2013, Cape Town

Doing nothing for others is the undoing of one's self. We must be purposely kind and generous, or we miss the best part of existence. The heart that goes out of self gets large and full of joy. This is the great secret of the inner life. We do ourselves the most good doing something for others.

- *HORACE MANN*

Abbreviations

Y-H	Antiradical
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
AOM	Azoxymethane
BAMnut	Bambara groundnuts crop simulation model
C-ring	Carbon ring
CVD	Cardiovascular disease
DNA	Deoxyribonucleic acid
DPPH	1,1-diphenyl-2- picrylhydrazyl
DW	Dry weight
EC_{50}	Half maximal effective concentration
EGC	(-)-epigallocatechin
EU	European Union
FAE	Ferulic Acid Equivalence
FAO	Food and Agriculture Organisation
FAOSTAT	Food and Agriculture Organisation Statistics
FRAP	Ferric Reducing Antioxidant Power
GAE	Gallic Acid Equivalence
GI	Glycemic Index
GIS	Geographical Information System
HPLC-PDA-ESI-MS	High-Performance Liquid Chromatography-Photo Diode Array-Electrospray Ionization- Mass spectrometry
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
K	Pseudo first-order rate constant
$[M - H]^-$	Negatively charged ion
ND	Not detected
Abs	Absorbance
ORAC	Oxygen Radical Absorbance Capacity
PVPP	Polyvinylpolypyrrolidone
TEAC	Trolox equivalent antioxidant capacity
TIC	Total Ion Chromatogram

TPTZ	2,4,6-tri-2-pyridyl-*s*-triazine
Trolox	6-hydroxy-2,5,7,8-tetra-methylchromane-2-carboxylic acid
UV-VIS	Ultraviolet-Visible
UAE	Ultrasound-Assisted Extraction
WHO	World Health Organisation

Abstract

There is a growing interest in legumes and legume based foods because of the health claims associated with their consumption. The aim of the current study was to explore the nutraceutical potential of bambara groundnuts (*Vigna subterranea* L. Verdc) and common beans (*Phaseolus vulgaris* L.) commonly grown in Zambia based on the antioxidant properties and phenolic phytochemical profiles. Two market classes of bambara groundnuts (red and brown) and four of common beans (red, grey mottled, brown and white) were screened in raw dry form. Effects of cooking and sprouting on the antioxidant activities and phenolic phytochemicals of the promising market classes were assessed. The study employed *in vitro* antioxidant assays (DPPH and FRAP) to screen for antioxidant properties, HPLC-PDA-ESI-MS and Folin Ciocalteu assay to screen for phenolic phytochemical profiles.

Bambara groundnuts were found to possess antioxidant activities. Brown bambara groundnuts exhibited the highest DPPH free radical scavenging activity with EC_{50} = 347 ± 4.2 µg dried extract / ml compared to 495 ± 12 µg dried extract / ml for the red bambara groundnuts. Again FRAP derived total antioxidant power was higher in the brown (6.00 ± 0.21 mmole Fe^{2+} / 100 g DW) compared to (5.00 ± 0.13 mmole Fe^{2+} / 100 g DW) in the red. Total polyphenol contents were 144.2 ± 1.7 and 117 ± 0.6 mg GAE / 100 g DW in the aqueous extracts of brown and red bambara groundnuts respectively. HPLC-PDA-ESI-MS-based identification revealed the presence of various phenolic compounds, mainly phenolic acids and flavonoids.

The various classes of common beans displayed varying antioxidant activities. The bean extracts exhibited DPPH free radical scavenging activities with EC_{50} ranging between 450.0 and 2534.5 µg dried extract / ml and FRAP derived antioxidant power between 1.69 and 6.88 Fe^{2+} / 100 g DW. The total polyphenol content ranged from 37.3 to 123.7 mg GAE / 100 g DW. The concentrations of *t*-ferrulic acid, gallic acid, salicylic acid, *p*-coumaric acid, epicatechin and catechin varied greatly. Ranking the common bean market classes based on the antioxidant activity and total polyphenol content revealed the following order: red beans > grey mottled beans > brown beans > white beans. HPLC-PDA-ESI-MS based identification revealed the presence of quinic acid, a syringic acid derivative, ferulic acid derivatives, medioresinol, *p*-coumaric acid and *t*-ferulic acid in all the market classes.

Phenolic compounds such as catechin, gallic acid, epicatechin, catechin glucoside, kaempferol glucoside and carnosol were variety-associated

Domestic cooking displayed positive effects on the antioxidant activity and phenolic phytochemical profiles of red beans and red bambara groundnuts that were investigated further. The free radical scavenging speed increased 10-fold in the methanolic extract from cooked red bambara groundnuts compared to uncooked. By contrast, the free radical scavenging speed increased 20-fold in the methanolic extract from cooked red beans compared to uncooked. Similarly, there were noticeable changes due to sprouting of the red bambara groundnuts and red beans. After 8 days of sprouting in red bambara groundnuts (at 98% germination capacity), the rate of free radical scavenging increased 1.3-fold. In red beans (at 100% germination capacity), the rate of free radical scavenging increased 2-fold. HPLC-PDA-ESI-MS profiles of the cooked as well as the sprouted red bambara groundnuts and red beans revealed a number of emergent phenolic compounds, mainly flavonoids. These data indicate that bambara groundnuts and common beans studied have the potential for use as nutraceuticals. Cooking and sprouting appear to enhance the nutraceutical profiles of these legumes.

Table of Content

Declaration.....ii
Dedication..... iii
Acknowledgements.....iv
Abbreviations.....vi
Abstract..... viii
Table of Content.....x
List of Figures.....xv
List of Tables..... xviii

Chapter 1.....1
Introduction.....1
1.1. Plant derived phenolic antioxidants.....3
1.2. Bambara groundnuts.....6
1.2.1. Description of bambara groundnuts.....6
1.2.2. History and origin of bambara groundnuts.....7
1.2.3. Distribution of bambara groundnuts.....7
1.2.4. Varieties of bambara groundnuts.....11
1.2.5. Global production of bambara groundnuts.....11
1.2.6. Nutritional value of bambara groundnuts.....13
1.2.7. Utilisation and consumption of bambara groundnuts.....13
1.2.8. Phenolic phytochemicals of bambara groundnuts.....14
1.2.9. Antioxidant properties of bambara groundnuts.....14
1.3. Common beans.....16
1.3.1. Varieties of common beans.....16
1.3.2. Production of common beans.....16
1.3.3. Nutritional value of common beans.....18
1.3.4. Anti-nutritional factors of common beans.....20
1.3.5. Processing and utilization of common beans.....20
1.3.6. Phytochemicals in common beans.....21
1.3.7. Biological activities of common beans.....21
1.4. Aim and objectives.....24

Chapter 2 25
Materials and Methods 25
2.1. Bambara groundnut and common bean varieties used, collection and storage 25
2.2. Characterisation of bambara groundnuts and common beans 28
2.2.1. Hydration, swelling capacity and indices 28
2.2.2. Chemical composition 28
2.2.3. Phenolic phytochemical investigations 29
2.2.3.1. Preparation and extraction of phenolic compounds 29
2.2.3.2. Cooking treatment 30
2.2.3.3. Preparation of extracts of cooked beans and bambara groundnuts 30
2.2.3.4. Sprouting of the common beans and bambara groundnuts 30
2.2.3.5. Preparation of extracts from the sprouted seeds 30
2.2.3.6. Determination of total polyphenols 31
2.2.3.7. Determination of tannins 31
2.2.3.8. Extraction and analysis of total flavonoids 32
2.2.3.9. HPLC –DAD-ESI-MS Instrumentation and chromatographic conditions 32
2.2.3.10. Preparation of the samples for HPLC -DAD-ESI-MS 33
2.2.3.11. HPLC-PDA-ESI-MS Identification and quantification of phenolic compounds 33
2.2.4. Antioxidant activity determination 34
2.2.4.1. Determination of free radical scavenging activity of the bambara groundnuts and common beans 34
2.2.4.2. Determination of Ferric Reducing Antioxidant Power (FRAP) 37

Chapter 3 39
Screening of the raw dry bambara groundnuts and common beans for phenolic phytochemicals and antioxidant activity 39
Introduction 39
3.1. Section A: Bambara groundnuts 40
Results 40
3.1.1. Nutritional and physicochemical properties of the Seeds 40
3.1.2. Phenolic phytochemical profiles of the Zambian bambara groundnuts 42
3.1.2.1. Water and methanol extractables 42
3.1.2.2. Total polyphenols and tannins 43

3.1.2.3. Total flavonoid concentration 44
3.1.2.4. Phenolic compounds identified by HPLC-PDA-ESI-MS in brown bambara groundnuts 45
3.1.2.5. Phenolic compounds identified by HPLC-PDA-ESI-MS in red bambara groundnuts 50
3.1.2.6. Concentration of some identified phenolic compounds in bambara ground nuts 53
3.1.3. Antioxidant activity of bambara groundnuts 53
3.1.3.1. Free radical scavenging activity of bambara groundnut antioxidants 54
3.1.3.2. Ferric Reducing Antioxidant Power of bambara groundnuts 58
Discussion 59
Conclusion 62
3.2. Section B: Common beans 63
Results 63
3.2.1. Nutritional and physicochemical properties of the seeds 63
3.2.2. Phenolic phytochemical profiles of the Zambian market classes of common beans 67
3.2.2.1. Water and methanol extractables 67
3.2.2.2. Total polyphenol and tannins 67
3.2.2.3. Total flavonoid concentration 70
3.2.2.4. Phenolic compounds identified by HPLC-PDA-ESI-MS in red beans 71
3.2.2.5. Phenolic compounds identified by HPLC-PDA-ESI-MS in grey mottled beans 75
3.2.2.6. Phenolic compounds identified by HPLC-PDA-ESI-MS in brown beans 78
3.2.2.7. Phenolic compounds identified by HPLC-PDA-ESI-MS in white beans 81
3.2.2.8. Concentration of some identified phenolic compounds in common beans 84
3.2.3. Antioxidant activity of the Zambia market classes of common beans 85
3.2.3.1. Free radical scavenging activity of antioxidants in common beans 85
3.2.3.2. Ferric Reducing Antioxidant Power of antioxidants in common beans 88
3.2.3.3. Correlation of antioxidant activities and phenolic contents of common beans 89
Discussion 92
Conclusion 94

Chapter 4 95
Domestic cooking effects of bambara groundnuts and common beans in the antioxidant properties and polyphenol profiles 95
Introduction 95
Results 96
4.1. Free radical scavenging activity of cooked red bambara groundnuts and red beans 96
4.2. Ferric Reducing Antioxidant Power of the cooked red bambara groundnuts and red beans 98
4.3. Polyphenol profiles of cooked red bambara groundnuts and red beans 99
4.3.1. Total polyphenol levels in cooked red bambara groundnuts and red beans 99
4.3.2. Levels of individual phenolic compounds in cooked red bambara groundnuts and red beans 100
4.3.3. HPLC-PDA-ESI-MS profiles of the cooked red bambara groundnuts and red beans 101
Discussion 107
Conclusion 110

Chapter 5 111
Antioxidant properties and polyphenol profiles of bambara groundnuts and common beans as affected by sprouting 111
Introduction 111
Results 111
5.1. Sprouts characteristics 111
5.2. Free radical scavenging activity of the sprouted red bambara groundnuts and red beans extracts 113
5.3. Ferric Reducing Antioxidant Power of the sprouted red bambara groundnuts and red beans extracts 115
5.4. Polyphenol profiles of sprouted red bambara groundnuts and red beans 116
5.4.2. HPLC-PDA-ESI-MS profiles of the sprouted red bambara groundnuts and red beans 117
Discussion 124
Conclusion 127

Chapter 6 128
Thesis summary and future perspectives 128
6.1. Thesis summary 128
6.2. Future perspectives 131

References 133

Appendices 154
Appendix A: UV-visible spectra of selected peaks 155
Appendix B: Selected MS and MS/MS spectra 160
Appendix C: Analytical scale chromatograms for external standards. 163
Appendix D: Total Ion Chromatograms (TIC) of the crude methanol extracts of bambara groundnuts and common beans 166

List of Figures

Figure 1-1 The structures of the six main classes of flavonoids.....5
Figure 1-2 Predicted pod yield of bambara groundnuts (kg / ha) across the world. 9
Figure 1-3 Predicted biomass of bambara groundnuts (kg / ha) across the world. 10
Figure 1-4 Africa and global production volumes for bambara groundnuts for the period 2002 to 2009. 12
Figure 1-5 Global production volumes for common beans for 2009. 18
Figure 1-6 Global production volumes for common beans for the period 2002 to 2009. 18
Figure 2-1 Map of Zambia showing the sample collection areas 26
Figure 3-1 Results of the spectrophotometric scanning at 200 nm to 400 nm for the 70% methanol extracts of bambara groundnuts. 44
Figure 3-2 HPLC-PDA-ESI-MS chromatogram of 70% methanol extract of brown bambara groundnuts.....49
Figure 3-3 HPLC-PDA-ESI-MS chromatogram of 70% methanol extract of red bambara groundnuts.....52
Figure 3-4 Disappearance pattern of DPPH free radicals with time in the presence of the aqueous and methanol extracts of bambara groundnuts 55
Figure 3-5 Dose response plot [concentration of aqueous extract of brown bambara groundnuts and DPPH scavenged (%)] fitted with linear regression line 57
Figure 3-6 Results of the spectrophotometric scanning at 200 nm to 400 nm for the common beans aqueous extracts.....69
Figure 3-7 HPLC-PDA-ESI-MS chromatogram of 70% methanol extract of red beans.....74
Figure 3-8 HPLC-PDA-ESI-MS chromatogram of 70% methanol extract of grey mottled beans.....77
Figure 3-9 HPLC-PDA-ESI-MS chromatogram of 70% methanol extract of brown beans.....80
Figure 3-10 HPLC-PDA-ESI-MS chromatogram of 70% methanol extract of white beans.....83
Figure 3-11 Disappearance pattern of DPPH free radicals with time in the presence of aqueous extracts of common beans.....86

Figure 3-12 Disappearance pattern of DPPH free radicals with time in the presence of the 70% methanol extracts of common beans 86
Figure 3-13 Correlation of antioxidant activities and phenolic compounds in the aqueous extracts of common beans.....90
Figure 3-14 Correlation of antioxidant activities and phenolic compounds in the 70% methanol extracts of common beans
..... 91
Figure 4-1 Disappearance pattern of DPPH free radicals with time in the presence of 70% methanol extracts of cooked and uncooked red bambara groundnuts. 97
Figure 4-2 Disappearance pattern of DPPH free radicals with time in the presence of 70% methanolic extracts of cooked and uncooked red beans 98
Figure 4-3 Concentration of individual phenolic compounds of 70% methanol extracts of cooked and uncooked red bambara groundnuts.....101
Figure 4-4 Concentration of individual phenolic compounds of 70% methanol extracts of cooked and uncooked red beans 101
Figure 4-5 HPLC-PDA-ESI-MS chromatogram of 70% methanol extract of cooked red bambara groundnuts.....103
Figure 4-6 HPLC-PDA-ESI-MS chromatogram of 70% methanol extract of the uncooked red bambara groundnuts.....104
Figure 4-7 HPLC-PDA-ESI-MS chromatogram of 70% methanol extract of cooked red beans. 105
Figure 4-8 HPLC-PDA-ESI-MS chromatogram of 70% methanol extract of the uncooked red beans. 106
Figure 5-1 Disappearance pattern of DPPH free radicals with time in the presence 70 % methanolic extracts of sprouted red bambara groundnuts. 113
Figure 5-2 Disappearance pattern of DPPH free radicals with time in the presence of 70% methanolic extracts of sprouted red beans 115
Figure 5-3 HPLC-PDA-ESI-MS chromatogram of 70% methanol extract of sprouted red bambara groundnuts.....119
Figure 5-4 HPLC-PDA-ESI-MS chromatogram of 70% methanol extract of the unsprouted red bambara groundnuts.....120
Figure 5-5 HPLC-PDA-ESI-MS chromatogram of 70% methanol extract of sprouted red beans. 121

Figure 5-6 HPLC-PDA-ESI-MS chromatogram of 70% methanol extract of the unsprouted red beans. 122
Figure A1 UV-visible spectrum of catechin 155
Figure A2 UV-visible spectrum of syringic acid derivative.....156
Figure A3 UV-visible spectrum of epicatechin.....157
Figure A4 UV-visible spectrum of catechin glucoside.....158
Figure A5 UV-visible spectrum of caffeic acid derivative.....159
Figure B1 MS and MS/MS spectrum showing a peak with molecular ion $[M - H]^-$ at m/z 259 in common beans.....160
Figure B2 MS and MS/MS spectrum showing a peak with molecular ion $[M - H]^-$ at m/z 205 in bambara groundnuts.....161
Figure B3 MS and MS/MS spectrum showing a peak with molecular ion $[M - H]^-$ at m/z 381 in bambara groundnuts.....162
Figure C1 Analytical scale chromatograms for external standards (catechin, epicatechin and salicylic acid 163
Figure C2 Analytical scale chromatograms for external standards (galic acid and ferulic acid).....164
Figure C3 Analytical scale chromatogram for external standard (*p* –coumaric acid).....165
Figure D1 TIC of 70% methanol extract of brown bambara groundnuts.....166
Figure D2 TIC of 70% methanol extract of red bambara groundnuts.....167
Figure D3 TIC of 70% methanol extract of red beans.....168
Figure D4 TIC of 70% methanol extract of grey mottled beans.....169
Figure D5 TIC of 70% methanol extract of brown beans.....170
Figure D6 TIC of 70% methanol extract of white beans.....171

List of Tables

Table 1-1 Flavonoids subclasses, their chemical structure and name of prominent food flavonoids.....4
Table 1-2 Proximate composition of common beans grown in Ethiopia.....19
Table 2-1 Market classes of bambara groundnuts investigated.....26
Table 2-2 Market classes of common beans investigated.....27
Table 2-3 Gradient solvent system for analysis of phenolic compounds by HPLC -PDA –ESI-MS.....33
Table 3-1 Physicochemical properties of the bambara groundnuts.....40
Table 3-2 Proximate composition (g / 100 g DW) of the whole seed sample.....41
Table 3-3 Mineral composition (mg / kg DW) of the whole seed sample.....42
Table 3-4 Yields of crude extracts of bambara groundnuts in ultrasonic-assisted extraction.43
Table 3-5 Total polyphenols and tannin concentration of bambara groundnuts.....44
Table 3-6 Flavonoids content of bambara groundnuts.....45
Table 3-7 HPLC-PDA-ESI-MS -based identification of phenolics in brown bambara groundnuts.....48
Table 3-8 HPLC-PDA-ESI-MS -based identification of phenolics in red bambara groundnuts.....51
Table 3-9 Individual phenolic compounds concentration (mg/ kg DW) in bambara groundnuts.....53
Table 3-10 Pseudo-first order rate constant of antiradical (A-H) in bambara groundnuts extracts and the amount of DPPH scavenged after 80 minutes of incubation.....56
Table 3-11 EC_{50} values for DPPH free radical scavenging by bambara groundnuts extracts after 30 minutes of incubation.....58
Table 3-12 Ferric Reducing Antioxidant Power (FRAP) values for bambara groundnuts.....59
Table 3-13 Ferric Reducing Antioxidant Power (FRAP) values for other legumes.....59
Table 3-14 Physico-chemical characteristics of the Zambian market classes of common beans.....63
Table 3-15 Proximate composition (g / 100g DW) of the whole seed sample.....64
Table 3-16 Mineral composition (mg / kg DW) of seed sample.....66
Table 3-17 Yields of crude extracts of common beans in ultrasonic- assisted extraction.....67
Table 3-18 Total polyphenol contents and tannin concentrations of common beans.....69

Table 3-19 Flavonoids content of common beans 70
Table 3-20 HPLC-PDA-ESI-MS -based identification of phenolics in red beans 73
Table 3-21 HPLC-PDA-ESI-MS -based identification of phenolics in grey mottled beans ... 76
Table 3-22 HPLC-PDA-ESI-MS -based identification of phenolics in brown beans 79
Table 3-23 HPLC-PDA-ESI-MS -based identification of phenolics in white beans..... 82
Table 3-24 Individual phenolic compounds concentration (mg/ kg DW) in common beans .. 84
Table 3-25 Pseudo-first order rate constant of antiradical (A-H) in common beans extracts and the amount of DPPH scavenged after 80 minutes of incubation 87
Table 3-26 EC_{50} values for DPPH scavenging by common beans extracts after 30 minutes of incubation 88
Table 3-27 Ferric Reducing Antioxidant Power (FRAP) values for common beans 89
Table 4-1 Pseudo-first order rate constant of antiradical (A-H) in cooked red bambara groundnuts and red beans and the amount of DPPH scavenged after 80 minutes of incubation 97
Table 4-2 FRAP values for the cooked red bambara groundnuts and red beans 99
Table 4-3 Total polyphenol concentration of cooked red bambara groundnuts and red beans 100
Table 4-4 Emerging phenolic compound tentatively identified in cooked red bambara groundnuts..... 107
Table 4-5 Emerging phenolic compound tentatively identified in cooked red beans..... 107
Table 5-1 Sprouted red beans and red bambara groundnuts 112
Table 5-2 Germination parameters of red bambara groundnuts and red beans 112
Table 5-3 Pseudo-first order rate constant of antiradical (A-H) in sprouted red bambara groundnuts and red beans and the amount of DPPH scavenged after 80 of minutes incubation 114
Table 5-4 FRAP values of sprouted red bambara groundnuts and red beans 116
Table 5-5 Polyphenol concentration of different day sprouts of red bambara groundnuts and red beans 117
Table 5-6 Concentration of individual phenolic compounds in sprouted red bambara groundnuts and red beans..... 123
Table 5-7 Emerging phenolic compound tentatively identified in sprouted red bambara groundnuts..... 124
Table 5-8 Emerging phenolic compound tentatively identified in sprouted red beans 124

Chapter 1

Introduction

Consumers are increasingly aware of the health benefits of foods and pay particular attention to the potential disease preventing and health promoting compounds that a given food contains. This awareness, coupled with a well-known understanding of how diet affects our health, is motivating a quest for natural health products. A healthy diet is an important element of disease prevention. There is a mounting market demand for natural products from traditional foods primarily due to the increasing consumer awareness of the role of food in health promotion and disease prevention (Liu 2009). Natural health food products provide an opportunity to improve people's health thereby reducing health care costs. Furthermore, they also provide prospects for farmers to diversify their agriculture in terms of the number of crops cultivated and in so doing support economic development in poverty stricken rural communities.

A diet based on plant products plays a very significant role in the prevention and fighting of degenerative diseases that are on the increase worldwide (Kushi et al. 1999). This is because plant food crops contain several bioactive compounds that have diverse disease fighting capabilities (Winter 2009). It is estimated that there are in excess of 200,000 chemical compounds that are synthesized by plants (Hartmann *et al.,* 2005). Given the essential biochemical functions that these chemicals play, it is not surprising that these compounds have been discovered to have medicinal applications (Thompson, 2009). In fact, plant-based preparations account for 70 percent of remedies used in traditional medicines around the world and are the basis of more than 50 percent of prescription and/or over the counter drugs used in the Western-type practice of medicine (Gad, 2005).

This thesis was undertaken to explore the nutraceutical antioxidant potential of the market classes of bambara groundnuts (*Vignea subterranean* L. verdc) and common beans (*Phaseoulus vulgaris* L.) grown in Zambia. Nutraceuticals are foodstuffs which provide health benefits in addition to their basic nutritional value (Merrian-Webster 2013). Whilst the nutritional value of bambara groundnuts is well known, their potential as nutraceuticals has not been exploited. Bambara groundnuts remain uncharacterised in many aspects and have

not been the subject of sustained research (Massawe *et al.,* 2005). Since under-utilised crops are grown for subsistence and contribute to the food security of many of the world's poorest people, attempts to improve them rarely attract interest from international agencies or commercial sponsors (FAO, 2001). Studies on the nutraceutical properties of bambara groundnuts are essential to establish their potential for use in the functional foods and nutraceutical industry. Additionally, this information is necessary for strategies aimed at promoting consumption of bambara groundnuts, which are slowly drifting into the category of "neglected species" or "forgotten crops" of Africa.

Common beans are an important food crop with an exceptional potential to deal with nutritional, health, income generation, and agricultural sustainability needs of developing countries in Sub-Saharan Africa and elsewhere in the world. They are a staple food crop for many regions of the world where they are eaten in large quantities on a daily basis as a rich source of protein, resistant starch, and dietary fibre (Geil and Anderson, 1994). They are also an excellent source of nutraceutical constituents such as fiber, protease inhibitors, phytic acid, and polyphenols such as tannins (Guzman and Lopez, 1999). A number of studies on common beans as a nutraceutical food have been conducted. Biological activities that have been described for fibre, polyphenolic compounds, lectins, trypsin inhibitors, and phytic acid from common beans include: enhancement of the bifidogenic effect (Queiroz-Monici *et al.,* 2005); antioxidant activity (Cardador-Martínez *et al.,* 2002); antimutagenic (Gonzalez *et al.,* 1999); anticarcinogenic effect (Kiss et al., 1997; Hangen and Bennink, 2002); and antiproliferative effect on transformed cells (Aparicio-Fernández *et al.,* 2006).

Whilst we appreciate and acknowledge the findings from previous workers on the nutraceutical perspectives of common beans, we were motivated to include the Zambian market classes in the current study with the intention of providing information to the stakeholders who are involved in the breeding of this crop in Zambia. The Department of Plant Science of the University of Zambia and other agricultural institutions have an ongoing programme that focuses on the breeding of improved varieties of common beans and other legumes. Initially, the breeding work was focussed on agronomical aspects such as pest resistance, drought resistance, and early maturation, but now there is a new approach to incorporate health attributes and nutrition. In any breeding programme, germplasm screening for traits of interest is an important first step to genetic improvement and it is imperative to evaluate locally available germplasm to have baseline information (Blair *et al.,* 2009).

In order to explore the nutraceutical antioxidant potential of the Zambian market classes of bambara groundnuts and common beans, their antioxidant activities and phenolic phytochemical profiles need to be investigated. Plant phenolics are important nutraceutical constituents and represent one of the major groups of compounds acting as primary antioxidants or free radical terminators (Miliauskas *et al.*, 2004; Saura-Calixto *et al.*, 2007). As domestic cooking and sprouting are the common ways by which legumes are prepared for consumption, their effects on the antioxidant properties and phenolic phytochemical profiles of the two legumes are addressed. Physicochemical characteristics and nutritional composition were also determined, though they are not the main focus of discussion in this thesis.

This chapter provides the background on a number of aspects of bambara groundnuts and common beans before briefly recognizing their biological activities or perceived health benefits. The chapter also gives a brief review on plant phenolic derived antioxidants.

1.1. Plant derived phenolic antioxidants

Phenolic compounds are the most widely distributed secondary metabolites, ubiquitously present in the plant kingdom (Mamoudou *et al.*, 2006). They have highly diverse chemical structures and more than 500 polyphenols have been described in common foods and beverages (Neveu *et al.*, 2010). The main classes of phenolic compounds are phenolic acids (hydroxybenzoic acids and hydroxycinnamic acids), flavonoids (flavanols, flavones, flavanones, isoflavones and anthocyanins), chalcones, aurones (hispidol), hydroxycoumarins, lignans, hydroxystilbenes and polyflavans (proanthocyanidins and prodeoxyanthocyanidins) (Chung *et al.*, 1998; Krueger *et al.*, 2003). Flavonoids constitute the largest class of phenolic compounds with more than 3000 structures, possessing in common a flavylium unit (C6-C3-C6) (Iacobucci and Sweeny, 1983). They may be divided into six different major classes (flavonols, flavanones, flavones, isoflavones, flavanols and anthocyanidins) based on differences in the structure of their molecular backbone (Beecher, 2003; Crozier *et al.*, 2000), (Table 1-1 and Figure 1-1).

Table 1-1 Flavonoids subclasses, their chemical structure and name of prominent food flavonoids.

Flavonoid subclass	C ring unsaturation	C ring function group	Prominent food flavonoids
Flavanols	none	3-hydroxy	(+)-Catechin
			(+)-Gallocatechin
		3-O-gallate	(-)-Epicatechin-3-gallate
Flavanones	none	4-Oxo	Eriodictyol
			Hesperetin
			Naringenin
Flavones	2-3 Double bond	4-Oxo	Apigenin
			Luteolin
Isoflavones	2-3 Double bond	4-Oxo	Daidzein
			Genistein
			Glycitein
Flavonols	2-3 Double bond	3-Hydroxy	Isorhamnetin
		4-Oxo	Kaemferol
			Myricetin
			Quercetin
Anthocyanidins	1-2,3-4 Double bonds	3-Hydroxy	Cyanidin
			Delphinidin
			Petunidin

Source: (Beecher 2003).

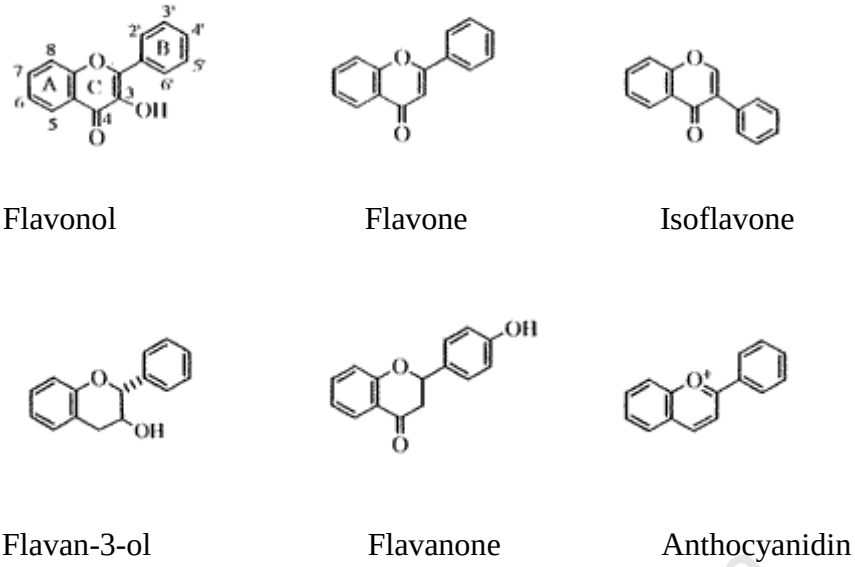


Figure 1-1 The structures of the six main classes of flavonoids (Crozier *et al.*, 2000)

Phenolic acids includes hydroxybenzoic acids (gallic and ellagic acids being major ones) and hydroxycinnamic acids (most common being coumaric, caffeic and ferulic acid). Added to this is quinic acid, a conjugate of caffeic acid and chlorogenic acid (Goldberg, 2003). Tannins are polyphenolic compounds that constitute hydrolysable and non hydrolysable tannins. They are complex polyphenols that can be degraded to sugars and phenolic acids by both enzymatic and non enzymatic hydrolytic processes (Wahle *et al.*, 2010).

Plant-derived phenolic compounds are important as nutraceutical constituents in our diet. They have antioxidant properties and may protect against major clinical conditions such as heart disease and cancer in which reactive oxygen species (i.e., superoxide anion, hydroxyl radicals and peroxy radicals) are involved (Rhodes and Price, 1997; Duthie and Crozier, 2000). Reactive oxygen species are generated through normal metabolism, environmental factors such as pollution, radiation, pesticides and cigarette smoke in which oxygen participates in the reactions. Reactive oxygen species attack cellular components such as DNA, lipids and proteins and are thought to be an initiating factor for several chronic diseases (Muller *et al.*, 2000). According to Yu *et al.*, (2002), dietary antioxidants may prevent these cellular components from oxidative damage and do this by undergoing

oxidation themselves. Antioxidant properties that have been described for plant-derived phenolic compounds include: stabilisation of unpaired electrons (Duthie *et al.*, 2003); scavenging of free radicals from lipid peroxidation (Nijverldt *et al.*, 2001); and the ability to chelate transition metal ions, which results in the inhibition of the reactive oxygen species production (Duthie and Crozier 2000). In view of the possibility that the antioxidant potential of plant-derived phenolic compounds may reduce the risk of developing chronic diseases, it is important to have a clear idea of the phenolic antioxidant compounds that plant foods contain. However, there is limited information on the antioxidant activities and phenolic phytochemical profiles of the market classes of bambara groundnuts and common beans grown in Zambia.

1.2. Bambara groundnuts

1.2.1. Description of bambara groundnuts

The bambara groundnuts are grown for their seeds which are used for dietary purposes. The pods develop underground and may attain a length of up to 3.7 cm, depending on the number of seeds they contain (Egoli 1995). The pods containing the seeds are round, wrinkled and may range from yellowish to reddish dark brown colour. Most varieties are characterized by pods that contain one seed, though varieties containing two or three seeds may rarely be encountered. The seed colour may vary from white to creamy, brown, red, or black (Egoli 1995). Bambara groundnuts have the ability to do well in poor soils and harsh climatic conditions. According to Doku and Karikari (1969), they are the most drought resistant pulse, producing a crop under conditions of high temperature and low rainfall, where other pulses fail to survive. They adapt to a wide range of soils and perform better on poor soils than common peanuts (Tweneboah, 2000).

Several researchers have reported that bambara groundnuts have high yield potentials. Yields of 300 kg / ha under marginal conditions and up to 4200 kg / ha with improved cultivars under optimum conditions are obtainable (Madamba 1995). According to Brink *et al.*, (2006), average yields are 300 – 800 kg / ha but yields of less than 100 kg / ha are not uncommon. Average yields may exceed 3000 kg/ha in intensive farming (Baudoin and Mergeai, 2001). Comparable ranges of 3000 kg / ha were reported for landraces in Tanzania (Collinson *et al.*, 2000) and South Africa (Swanevelder, 1998). Heller *et al.*, (1995) reported a yield of 400 –

1400 kg / ha unshelled pods in Zimbabwe and this range is similar to what is obtainable in Zambia. In experiments conducted at the University of Nottingham in the United Kingdom under controlled environments, the crop gave a yield of 4000 kg / ha (Collinson *et al.*, 1999).

1.2.2. History and origin of bambara groundnuts

Bambara groundnuts have a long history of cultivation and are predominantly grown in drier areas with short inconsistent rainfall in the Sub-Saharan Africa (FAO 2001). Several reports have been made regarding the origin of bambara groundnuts, with some claiming that they originated from Central Africa while others claim their centre of origin was West Africa. The crop is believed to have been named after the tribe, the Bambara who live in the Bambara district on the Upper Niger near Timbuktu in Mali (Holm & Marloth, 1940). However, wild relatives of cultivated bambara groundnuts have only been found in northeastern Nigeria and northern Cameroon (Egoli 1995).

According to Egoli (1995), this crop is first mentioned in the 17th century literature. Du Petit-Thouars (1806) found the crop in Madagascar, under the vernacular name "voanjo", subsequently written as "voandzou" in French. He then proposed the name *Voandzeia subterranean* (L) Thouars, which was broadly used by subsequent researchers for over a century (Egoli 1995). Verdcourt (1980) proposed *Vigna subterranean* (L) Verdc, as the correct name for bambara groundnuts and this has been accepted to date.

1.2.3. Distribution of bambara groundnuts

Bambara groundnuts have a long history of cultivation in tropical Africa and are often intercropped with cereal crops (Egoli 1995). They are reported to have been carried as far as India, Sri Lanka, Indonesia, the Philippines, Malaysia, New Caledonia and South America, particularly Brazil (Rassel 1960), but it seems that the present degree of cultivation outside Africa is negligible. Linneman and Azam-Ali, (1993) also reported that small quantities of bambara groundnuts were found in South and Central America, India, Indonesia, Malaysia, the Philippines, Sri Lanka and parts of Northern Australia.

At the moment, Sub-Saharan Africa still remains the dominant traditional constituency for bambara groundnuts. However, there may be locations outside its current distribution that offer conditions at least as conducive to their growth and yield as those that are currently

cultivated. For the first time in 1999, the Food and Agriculture Organization (FAO), in collaboration with the University of Nottingham, did a global mapping for bambara groundnut production to assess locations that have potential for their production across the world (FAO 2001). The methodology was applied both to regions such as Africa, where the crop is widely cultivated but where experimental evidence is limited, and to new regions that have not previously been associated with bambara groundnuts but where environmental factors are conducive for productive growth. A weather generator and a crop simulation model for bambara groundnuts (BAMnut) were incorporated into a Geographical Information Systems (GIS) model to predict, for the first time in 1999, bambara groundnut production for the world (FAO 2001).

A gridded mean monthly climate dataset at a resolution of 50 km x 50 km for global land areas (excluding Antarctica), for the period 1961-1990, was used as input to the weather data generator to generate daily weather data. Simulation results from BAMnut were used as input to the GIS model to provide the required maps and statistics. Given the time frame of the study, neither the model nor the overall methodology attempted to account for the specific effects of soil type, pests or diseases on the likely productivity of bambara groundnuts at any location. Similarly, the influence of daylength sensitivity for pod filling in many bambara groundnuts landraces was not assessed in relation to potential yield. Each site that was evaluated was approximately a 50 km x 50 km (at the equator) grid cell, of which 62,482 cells corresponded to global land areas.

Results showed that bambara groundnuts are likely to produce significant pod yields in many parts of the world beyond their current distribution with suitable areas of potential production in America, Australia, Europe and Asia as well as Africa (Figures 1-2 & 1-3). In fact, locations within the Mediterranean region showed the highest predicted biomass, often exceeding 8500 kg / ha.

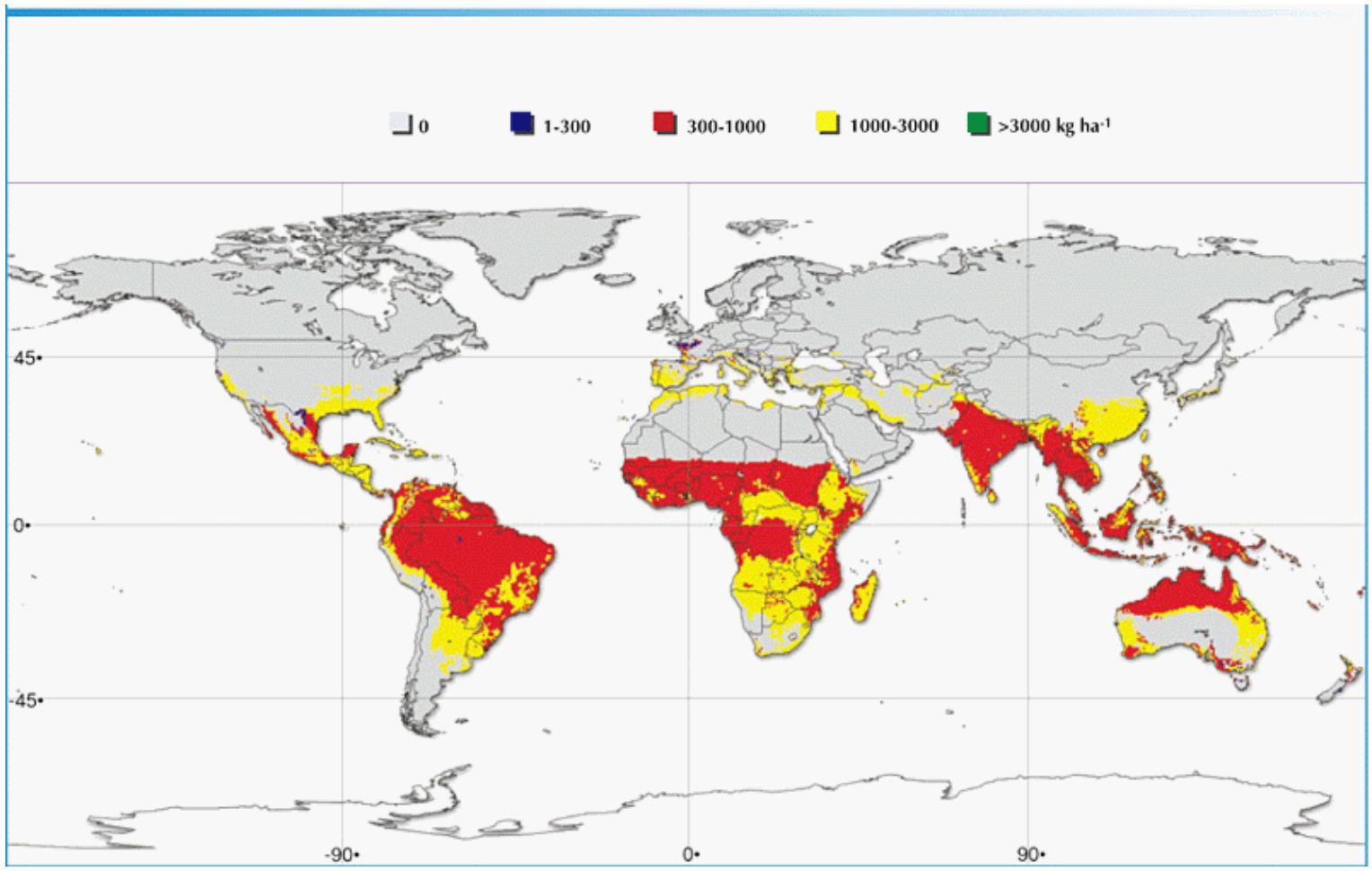


Figure 1-2 Predicted pod yield of bambara groundnuts (kg / ha) across the world. Source: FAO (2001)

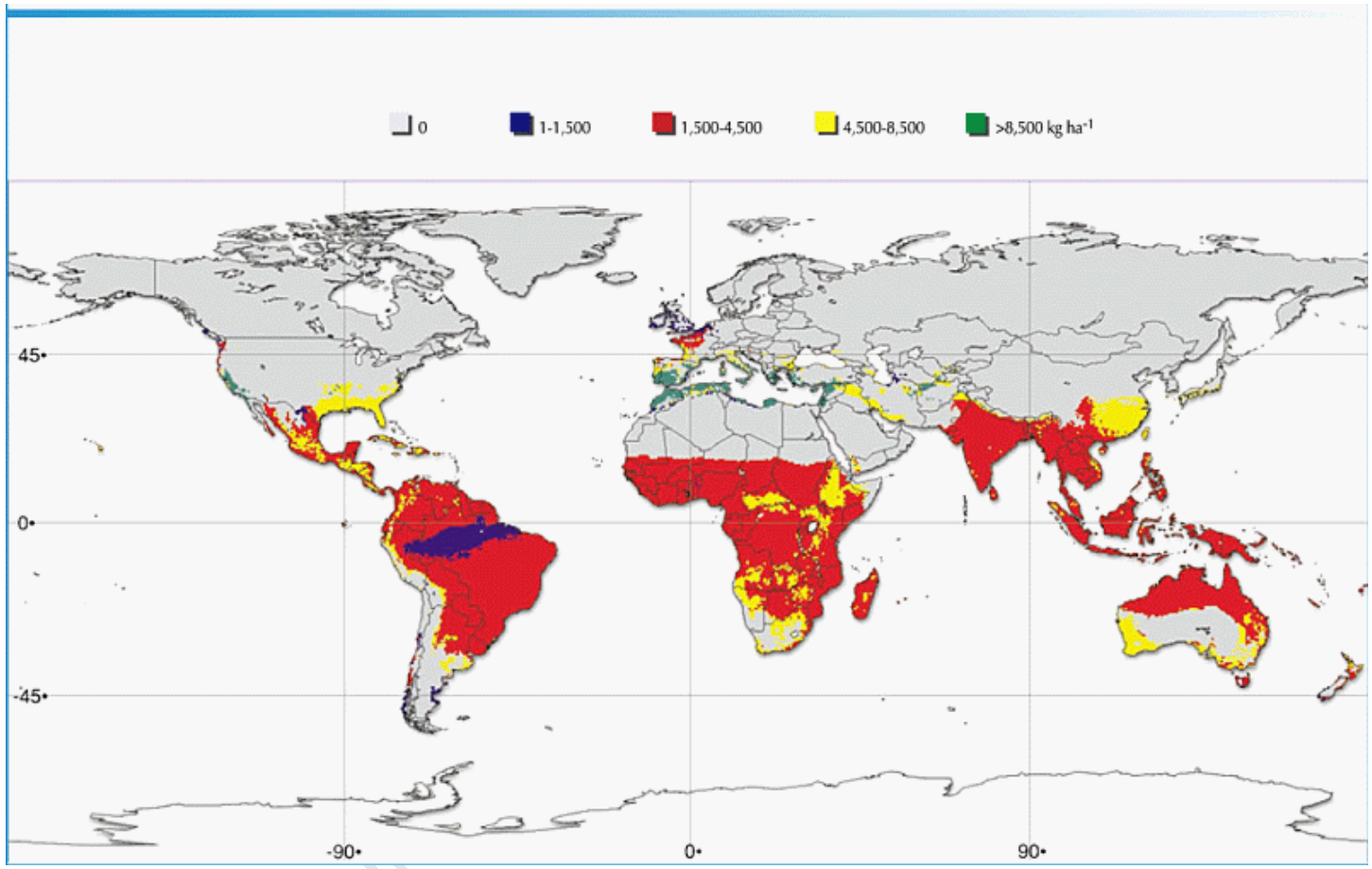


Figure 1-3 Predicted biomass of bambara groundnuts (kg / ha) across the world. Source: FAO (2001)

1.2.4. Varieties of bambara groundnuts

In most growing regions of Africa, bambara groundnuts are still cultivated from landraces and not necessarily from improved varieties that have been specifically bred to suit agro-ecological conditions in the growing areas. In Zambia for instance, the landraces of bambara groundnuts have been passed from one generation to the next and this system is well maintained in the traditional farming scheme. These landraces have become well adapted to the local climate and soils, and indigenous knowledge about the germplasm is well preserved in the communities. According to Azam-Ali *et al.*, (2001), the breeding system of bambara groundnuts is not well understood, and furthermore, local expertise and germplasm of bambara groundnuts have rarely been exchanged between growing regions in Africa.

In most farming communities, the landraces of bambara groundnuts are distinguished on the basis of shape and size of the leaves, and colour, size and hardness of the seed. Most often, landraces are called by local names and sometimes names are based on the location in which the seed was collected (Brink *et al.*, 2006). In Zambia for instance, varieties are distinguished mainly based on the colour. The red, brown and light cream varieties are grown. However, the red and the brown are the commonly found market classes. This thesis thus concentrated on the red and brown landraces.

1.2.5. Global production of bambara groundnuts

Global production of bambara groundnuts is generally lower as compared to other legumes like common beans, soya beans and common peanuts. The degree of negligence for this crop is reflected in the disparity of figures in terms of annual production volumes. According to the FAOSTAT (2011), global production of bambara groundnuts was barely 103,877 tonnes while that of common beans was over 20 million tonnes in 2009. In 2008, it was at 110, 614 tonnes while that of common beans stood at over 21 million tonnes. This is particularly so because the crop is cultivated by poor rural farmers in Sub-Saharan Africa and disappointingly, there seems to be no interest by commercial farmers to include it as a cash crop. In Zambia and other countries in the Sub-Saharan, it is slowly drifting into the category of “neglected” or “forgotten” crops. In most African countries, production data is not accurately collected and there are no accurate statistics on internal agricultural production or marketing of the crop.

In other regions of the world outside Sub-Saharan Africa, bambara groundnuts are barely known and their cultivation and consumption is negligible. According to the global production figures documented by FAOSTAT from 2002 to 2009, annual volumes remained stagnant below 100, 000 tonnes in the first five years and reached above 100, 000 tonnes in the last two years (2008 and 2009). It is however intriguing to note that the values indicated for the global annual volumes for the eight years period are the same values indicated for the total annual production for Africa alone; suggesting that bambara groundnuts cultivation outside Africa is negligible (Figure 1-4).

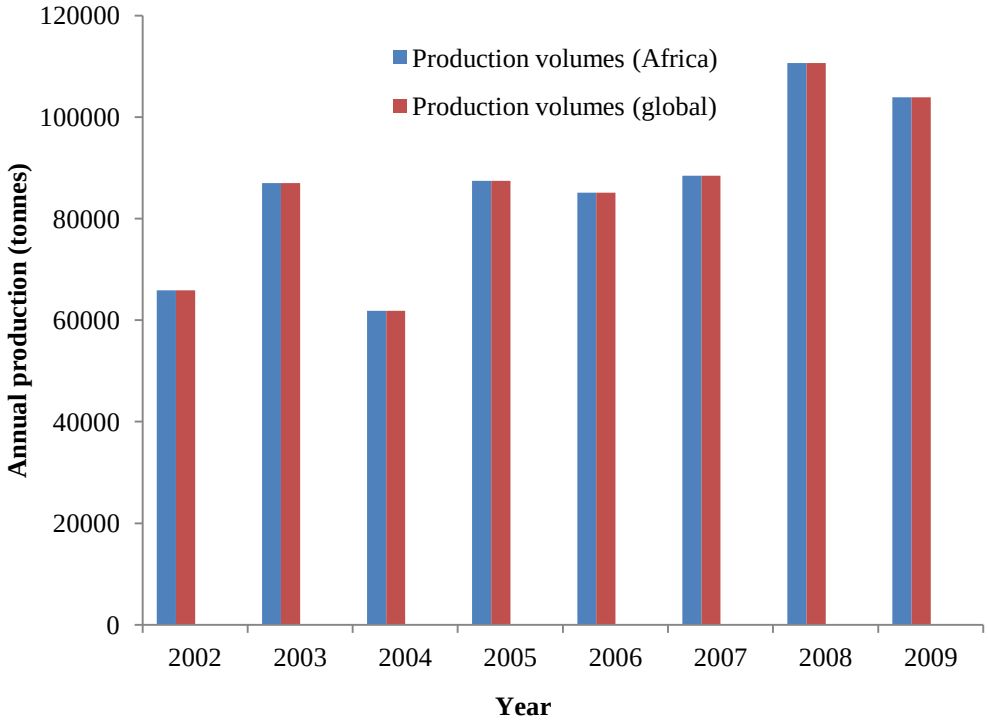


Figure 1-4 Africa and global production volumes for bambara groundnuts for the period 2002 to 2009. Source: FAOSTAT. (2011)

According to the report by Baudoin and Mergeai (2001), about 45 – 50% of the world production comes from West Africa. The major producers are Burkina Faso, Chad, Cote d'lvoire, Ghana, Mali, Niger and Nigeria, and that the main exporting countries are Burkina Faso, Chad, Mali, Niger and Senegal (Brinks *et al.*, 2006)

1.2.6. Nutritional value of bambara groundnuts

Work by Mahala and Mohammed (2010) demonstrated that bambara groundnuts are nutritionally rich containing 24.98% crude protein, 12.94% crude fibre, 1.6% fat and 3.6% ash. This report confirmed previous observation by Poulter and Caygill (1980) who described bambara groundnuts as nutritionally rich because they contain proteins, carbohydrates and fats in adequate proportions. According to the report by Purseglove (1992), ripe seeds contain 16 – 21% proteins, 4.5 - 6.5% fats, and 50 – 60% carbohydrates. Work at the University of Nottingham by Brough and Azam-Ali, 1992; Brough *et al.,* 1993 also demonstrated that bambara groundnuts are a rich source of proteins (16 – 25%), carbohydrates (43%) and lipid fraction (7.9%). They further revealed that the protein percentage was found to be superior to that reported for cowpeas, common peanuts and pigeon peas but that in terms of lipids, bambara groundnuts compares favourably with cowpeas and pigeon peas.

1.2.7. Utilisation and consumption of bambara groundnuts

Bambara groundnuts are primarily produced for household consumption by the rural population. The way they are prepared may differ from one regional grouping to the other. In Zambia, fresh seeds are boiled and eaten as snacks, while dry seeds are cooked and used as relish with nsima (maize meal cooked paste). Linnemann (1988) reported that in Nigeria, dry seeds are often crushed into flour to prepare a number of dishes: 'alele', 'alelen ganye', 'danwake', 'gauda', 'kosai', 'kunu', 'tuwo' and 'waina'. The fresh pods are boiled and eaten as a snack while the fresh immature seeds may be eaten raw. In South Africa, Swanevelder (1995) reported the following culinary uses:

- 'Sekome' (Sesotho), 'tihove' shangaan) or 'tshidzimba' (Venda) is prepared by adding bambara groundnuts and peanuts, or just one of the two, to maize or millet-meal and boiling the mixture until it forms a stiff dough. This is salted and pounded into a ball, and will often keep fresh for several days.
- Bambara groundnuts are boiled and then stirred, to make a thin porridge, which is known as 'tshipupu' (Venda). Like maize, they may also be added to 'lupida', a porridge made from peanuts.
- Fresh pods are often eaten when still immature, simply boiled until soft, and shelled. When quite dry and hard, they are generally shelled, and then boiled to make a stiff porridge.

- Dry seed can be cooked with maize and pounded into a thick, sticky dough known as 'dithaku' (in Sesotho).The only use of bambara groundnuts observed among the local white population was of the dried beans, to make a soup.

According to Nambou (1995), communities in Togo consume bambara groundnuts as boiled or fried, and fried or boiled puddings may be prepared with dough made from bambara groundnut flour, which may also be used as a base for many other dishes. In Zimbabwe, Mabika and Mofongoya (1995) reported that the dry seeds are eaten after boiling either on their own or mixed with maize grain and others may roast the seeds and consume as a snack. Another report by Doku (1995) indicated that in Northern Ghana, dry seeds are boiled, crushed and made into cakes or balls, which are then fried and used to prepare stews. In Southern Ghana, the dry seeds are usually soaked overnight, after which they are boiled until soft to produce a kind of porridge. Capsicum pepper and salt may be added during the boiling process and prepared with gari (roasted, grated cassava) or with mashed fried ripe plantain. Ngugi (1995) reported that in Kenya dry seeds are boiled, mixed with boiled sweet potatoes and mashed (a popular children's dish). The dry seed may also be ground into a meal that is made into a sauce and added to the traditionally prepared leafy vegetables. This is served with "ugali" or potatoes.

1.2.8. Phenolic phytochemicals of bambara groundnuts

To the author's knowledge, published reports on the phenolic phytochemical profiles of bambara groundnuts are limited. There are no reports on the identification of individual phenolic compounds in bambara groundnuts using authentic standards, UV-VIS and LCMS spectral data. This is the first time that identification of phenolic compounds in bambara groundnuts using these approaches has been attempted.

1.2.9. Antioxidant properties of bambara groundnuts

A number of food grains have been reported in literature to possess antioxidant activities that may be beneficial to health (Cardador-Martínez *et al.,* 2002; Winter 2009; Liu 2009). However, there are limited reports about the antioxidant activity or other nutraceutical properties of bambara groundnuts.

Traditional medicinal uses in some communities where bambara groundnuts are grown suggest, however, that they may possess some nutraceutical properties. Karikari *et al.,* (1995)

reported that the seeds of the mature black bambara groundnuts landrace are used in traditional medicine in Botswana. According to Swanevelder (1995), chewing and swallowing raw bambara groundnuts is said to check nausea and vomiting, a remedy that is often used to treat morning sickness in pregnant women. Ngugi (1995) reported the following medicinal use among the Luo tribal grouping of Kenya:

- Water from the boiled maize and bambara groundnuts seed mixture is drunk to treat diarrhoea.
- The leaves of bambara groundnuts are pounded with those of *Lantana trifolia* L. ('nyabend winyo','nyamrithi'), and then water is added to make a solution used to wash livestock as a preventation against ticks. This solution is used as a pesticide on vegetables too.
- The leaves of bambara groundnuts can be combined with those of 'nyajagra' (Mexican marigold) and *L. trifolia*, pounded, and water added. This mixture can also be used as an insecticide on vegetables.
- When dry, the leaves are pounded with traditional salt ('mbala', harvested at Sindo and Homalime), and fed to cattle infected with 'tuoolao' (a type of mouth disease).

Despite the reported medicinal uses of bambara groundnuts, there is limited experimental evidence to suggest that they possess nutraceutical properties or exhibit some form of bioactivity.

1.3. Common beans

1.3.1. Varieties of common beans

A high degree of diversity in terms of colour, size, growth habits and seed shape is present in *Phaseolus vulgaris*. Andean types of *Phaseolus vulgaris* tend to have larger seeds and leaves than the Central American, but both growth habits (erect bush and climbing) may exist in each gene pool (Wortmann 2006). Names like pinto beans, red kidney beans, and black beans are frequently encountered in the United States of America, Canada and other growing regions. According to Wortmann (2006), some genetic diversity found in tropical Africa is not found in the Americas.

The diversity of common bean seed types in Africa has been reported as massive but varies across the region (Wortmann *et al.*, 1998,). The most common varieties grown are of the bush type characterised by small to medium sized seeds. Bush type common beans are preferred to the climbing type because of their low cost of production requirements and convenience for the market (Katungi *et al.*, 2009). Climbing types are largely restricted to high altitude areas, especially in south-western Uganda, Rwanda, Burundi and eastern parts of Democratic Republic of Congo, but they are also grown in northern and western Malawi, northern and southern Tanzania and northern Zambia (Wortmann 2006). Red, brown, grey mottled, large-seeded cultivars and white, black, red small to medium-seeded cultivars are frequently found in tropical Africa.

In Zambia, the red large-seeded type, (non climber, locally called *Lundazi* beans), grey mottled large-seeded type, (climber, locally called *Kablangeti* beans), brown large-seeded type, (non climber, locally called *Chambishi* beans), white mediun-seeded variety, (non climber, locally called *Kalungu* beans), red speckled large-seeded type, (non climber, locally called *Lyambai* beans) and tan large-seeded type, (non climber, locally called *Lukupa* beans) are grown. This thesis concentrated on the red, grey mottled, brown and white beans as they are the widely consumed and commonly found varieties.

1.3.2. Production of common beans

Jones (1999) argued that statistics for global production of common beans are vague. According to his report, figures for the biggest producers in developing countries are often

underestimated because common beans are often intercropped and/ or grown in remote areas and as a result, data are often imprecise. Wortmann (2006) postulated a similar argument that reliable production statistics for common beans are difficult to obtain because their production is often lumped together with that of other *phaseolus* species.

According to the report given by Agriculture and Agric-Food Canada (2000), India, Brazil, United States of America, China, Mexico, Myanmar, Indonesia, Argentina, Uganda and Canada were ranked as the top ten world producers. Wortmann (2006) reported Brazil to be the world's largest producer. A report by Jones (1999) postulated a world-wide annual production of over 12 million tonnes. The continent of Africa, in addition makes a very significant contribution to the annual global volume of common beans. According to Wortmann (2006), Africa produces about 2 million tonnes annually on about 3.5 million ha, with large producers (100,000 – 600,000 ha annually) in tropical Africa being Democratic Republic of Congo, Rwanda, Burundi, Ethiopia, Kenya, Uganda, Tanzania, Malawi, Angola and Mozambique; and smaller producers (2,000 – 100,000 ha) being Cape Verde, Niger, Cameroon, Sudan, Zambia and Zimbabwe. Of the 2 million tonnes produced annually, Wortmann (2006) further reported that most of it is consumed by the producer, and that 40% may be marketed to supply urban areas and for export at a value of over USD 200 million per year. Africa's production volumes have not kept pace with the annual population growth rate which is estimated at 2% for some countries due to a number of abiotic, biotic and socio-economic constraints (Xavery *et al.,* 2007)

According to the FAOSTAT (2011), global production volume for common beans for 2009 was just above 20 million tonnes (Figure 1-5). Asia's contribution to the total was the largest, followed by South America, Africa and the least was Australia and New Zealand. The production volumes documented from 2002 to 2009 (Figure 1-6) indicate a sharp decline for the year 2004, followed by just a slight upswing in 2005. The year 2006 witnessed a sharp rise with over 20 million tonnes produced. Production was maintained fairly consistently above 20 million tonnes for the subsequent years up to 2009.

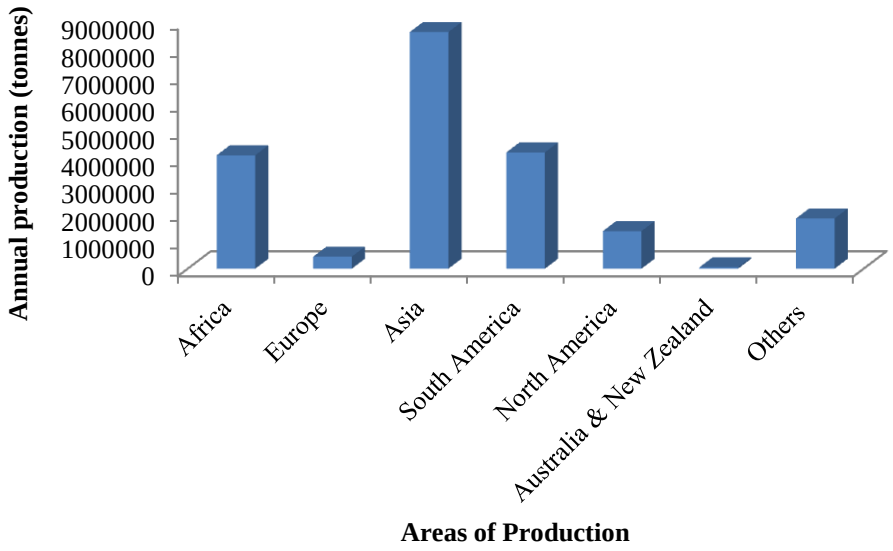


Figure 1-5 Global production volumes for common beans for 2009. Source: FAOSTAT (2011)

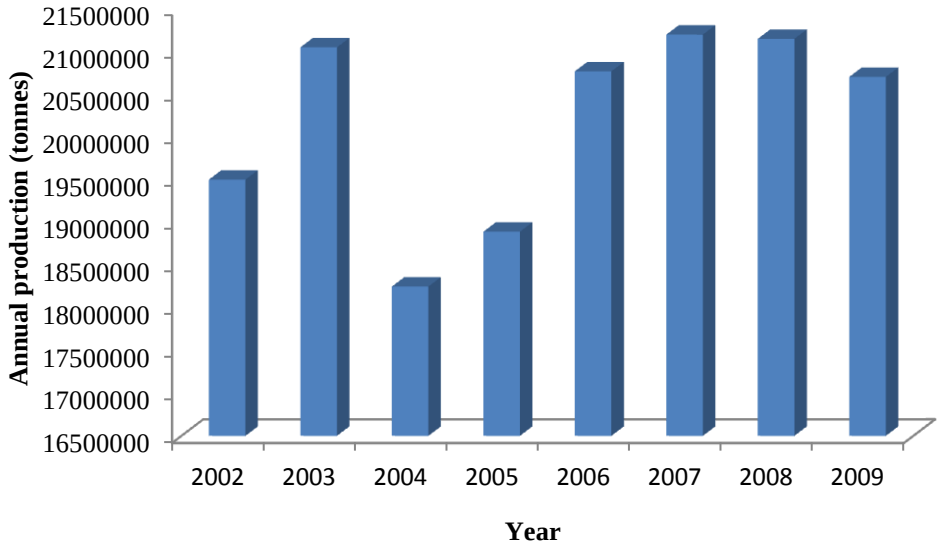


Figure 1-6 Global production volumes for common beans for the period 2002 to 2009. Source: FAOSTAT (2011)

1.3.3. Nutritional value of common beans

The contribution of common beans to world nutrition remains significant especially in developing countries where they are the main source of protein to the poor majority. They are

rich in protein, carbohydrate, fiber, minerals and vitamins. Protein has always been recognized as the most significant macronutrient in common beans. Crude protein content of common beans ranges from 18 – 22% and it is essentially among the highest vegetable sources; however, the amino acid profiles limit the quality of common bean protein (Uebersax 2006). Methionine, a sulphur containing amino acid is limited in common beans but it is often complemented by cereal sources such as maize and wheat which in turn are limited in lysine. It is not surprising therefore, that in developing regions, common beans and cereal grains such as maize and rice are eaten in blends. The essential amino acid composition per 100 g edible portion is: tryptophan 210 mg, lysine 1540 mg, methionine 240 mg, phenylalanine 1130 mg, threonine 860 mg, valine 990 mg, leucine 1640 mg and isoleucine 890 mg (Paul *et al.,*1980). Common beans are also one of the best non-meat sources of iron, providing 23 – 30% of daily recommended levels from a single serving (Pachico, 1993).

Samma *et al.,* (1999) observed that common beans contain 12 – 14 % moisture, 18 – 22% protein, 0.7 – 1.2% fat, 0.8 – 1.2 mg copper / 100g, 8 – 9 mg iron / 100g, 2.5 – 4 mg zinc/100g, and 295 – 542 mg phosphorus / 100g. Shimelis and Rakshit (2005) evaluated the approximate composition of the improved varieties of common beans grown in Ethiopia and the findings are presented in Table 1-2. Published reports on the nutritional aspects of the Zambian market classes of common beans are limited.

Table 1-2 Proximate composition of common beans grown in Ethiopia

	Proximate composition (g /100g)
Protein	17.96 – 22.07
Crude fat	1.27 – 3.02
Crude fibre	4.66 – 5.95
Ash	2.86 – 4.26
Carbohydrates	56.53 – 61.56
Moisture	9.08 – 11.00

Source: Shimelis and Rakshit (2005)

1.3.4. Anti-nutritional factors of common beans

Anti-nutritional factors (trypsin inhibitors, phytohemaglutinating compounds, commonly referred to as "lectins," and phytic acid) have long been recognized as concerns in common beans and require appropriate processing conditions to ameliorate adverse effects (Uebersax 2006). These compounds are intrinsic to the beans and serve vital physiological processes during growth and development. However, these components may affect the digestibility of common beans and therefore need to be inactivated by appropriate processing. Gupta (1987) reported saponins, protease inhibitors, lathyrogens, phytohaemagglutinins, favism, and cyanogenic glucosides as additional factors that may affect the digestibility of legumes. A report by Birk (1996) postulated protease inhibitors as widely distributed in legumes. Genovese and Lajolo (2002) reported that thermal inactivation of trypsin inhibitors is essential for use in animal and human food.

1.3.5. Processing and utilization of common beans

Consumption of common beans is high in the developing regions mostly among the rural populations with lower income levels. Uebersax (2006) reported that the regions of highest beans consumption include all of Latin America, Sub-Saharan Africa, which utilizes dry beans and other legumes (cowpea), and the subcontinent of India. Throughout Southeast Asia, consumption of legumes is moderate and a great variety of species are produced and used as mature seeds and immature vegetative pods. Uebersax further reported that the per capita consumption of legume-based food products in the United States, Europe (encompassing the EU) and other industrialized economies has generally and consistently been substantially lower than that observed in other regions of the world.

In Africa and Latin American countries where common beans are a staple food, they are prepared by traditional cooking which involves boiling followed by the addition of various seasonings. Traditionally cooked beans are often eaten in combination with a carbohydrate food such as maize, rice or potatoes. Other traditional methods of common bean preparation such as germination and fermentation in Africa and Asian countries have been widely accepted (Uebersax 2006). A report by Reddy *et al.,* (1982) documented that legume-based fermented foods are very popular in Southeast Asia, the Near East, and parts of Africa. According to Uebersax (2006), these methods produce highly specialized and culturally distinctive products and are recognized for improving beans digestibility and reducing anti-

nutritional factors. A review by Davila *et al.,* (2003) reported that germination and fermentation are presented as alternatives that are able to reduce or inactivate anti-nutritional factors in legumes.

There are also great opportunities to use common beans in the formulation of weaning foods. Rodriguez-Burger *et al.,* (1998) developed a nutritious weaning food using fermented black beans blended with rice. Mwikya *et al.,* (2000) assessed the inclusion into a weaning food formulation of kidney beans sprouted for 96 hours at 30°C. During the sprouting period, starch content decreased; reducing and non-reducing sugars increased; tannins, trypsin inhibitor and phytates decreased; and in-vitro digestibility increased.

1.3.6. Phytochemicals in common beans

Ocho-Anin *et al.,* (2010a) conducted a basic qualitative screening for common beans and reported that alkaloids, anthraquinone, catechictannins, flavonoids, gallotannins, glucosides, polyphenols, saponins, steroids and terpenoids were present. Phytochemical screening showed the presence of some bioactive components such as alkaloids, anthocyanin, carbohydrate, catechin, fibers, flavonoids, phasine, phytic acid, quercetin, saponins, steroids, tannins and terpenoids and a trypsin inhibitor. Further investigations by Ocho-Anin *et al.,* (2010b) identified compounds such as anthocyanins, flavanol monomers, and heterogeneous flavanol oligomers up to hexamers (also known as condensed tannins or proanthocyanidins). The presence of catechin, delphinidin, cyanidin, and phenolic acids such as vanillic, caffeic, coumaric and ferrulic acids in the seed coat of common beans have been reported by Madhujith *et al.,* 2004. Phenolic compounds such as anthocyanins, quercetin glycosides and proanthocyanidins were isolated and identified in dark red kidney beans by Beninger and Hosfield (2003). Espinosa- Alonso *et al.,* (2006) reported the presence of kaempferol and quercetin as the main flavonoids in different market classes of Mexican beans. To the author's knowledge, published reports on the phenolic phytochemicals of market classes of Zambian common beans are limited.

1.3.7. Biological activities of common beans

Common beans have biological activities that may be beneficial to health. Kushi *et al.,* (1999) reported that there is growing evidence that cereals and legumes play important roles in the prevention of chronic diseases and that overall, the substantial epidemiological evidence of legumes in the prevention of chronic diseases such as diabetes, cancer,

hypertension and obesity is very promising. The physiological effects of common beans may be due to the presence of abundant phytochemicals including polyphenolics, which possess both anticarcinogenic and antioxidant properties (Gonzalez *et al.,* 1999; Martínez *et al.,* 2002; Aparicio-Fernañdez *et al.,* 2006). The antioxidant activity of common beans has been investigated previously. Wu *et al.,* (2006) studied the antioxidant activity of more than 100 food items using the Oxygen Radical Absorbance Capacity (ORAC) assay and reported that common beans (navy, black, pinto, red kidney and small red) demonstrated the highest antioxidant activity among the food items evaluated. A study by Martínez *et al.,* (2002) on antioxidant potential of methanol extract, acetate/acetone and acetone fractions from common beans using the *β*-carotene-linoleate and the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assays demonstrated varying antioxidant activities which were concentration dependent. Madhujith and Shehini (2005) reported the DPPH free radical scavenging of 22% by the extract of black beans at 50 ppm. Beninger and Hosfield (2003) reported that pure flavonoid compounds such as anthocyanins, quercetin glycosides and protoanthocyanidins (condensed tannins), in the seed coat methanol extract and tannin fractions from 10 colored genotypes of common beans all demonstrated antioxidant activity, with the highest activity (> 50% inhibition of lipid oxidation) reported in extracts that were rich in condensed tannins. A study by Xu *et al.,* (2007) on the antioxidant activity of common beans grown in different regions of the United States of America using FRAP assay demonstrated varying antioxidant activities for the beans with FRAP values ranging from 1.27 to 9.70 mmol Fe^{2+} / 100 g DW. Information on the antioxidant activity of the common bean market classes grown in Zambia is limited.

There is substantial scientific evidence that regular consumption of common beans reduces the risks of colon cancer. Epidemiological studies show a low incidence of colon cancer in many Latin American countries where the consumption of common beans is high (Hughes *et al.,* 1997). Hangen and Bennink (2002) tested the effects of common beans on the inhibition of colon cancer induced by the carcinogen, azoxymethane (AOM) in rats. At 31 weeks after the second AOM injection, the incidence of colon adenocarcinomas was significantly lower in rats fed the black beans (9%) and navy beans (14%) than in rats fed the control diet (36%). Many theories have been offered to explain the protective effect of foods such as legumes and whole grains on the colon. These foods contain fermentable carbohydrates, including dietary fibre, resistant starch and oligosaccharides. Indigestible carbohydrates reach the colon and are fermented by intestine microflora to short chain fatty acids such as acetate, butyrate, propionate, which have been associated with lowered serum cholesterol and decreased risk of cancer (Cook *et al.,* 1998).

Legumes contain several antinutrients such as protease inhibitors, phytic acid, phenolics and saponins. These antinutrient compounds may act as cancer inhibitors by preventing the formation of carcinogens and by blocking the interaction of carcinogens with cells (Manson *et al.,* 2000).

Common beans may also play a role in maintaining stable blood glucose levels in the body. Low glycemic responses have been observed for common beans compared with other starchy foods such as bread, potato and certain breakfast cereals (Uebersax 2006). Food with low glycemic indices produce a small rise in blood sugar and may be beneficial to diabetic patients. Low-GI diets improved adipocyte insulin-mediated glucose uptake *in vitro* and was found to be useful in normalizing diet-insulin responses of hyperinsulinaemic subjects (Frost *et al.,* 1998). Wolever *et al.,* (1987) fed portions of five varieties of beans, both cooked and canned, to groups of diabetic patients, and found their mean glycemic index to be lower (GI = 47) than that of white bread (GI =100). On the other hand, Tovar *et al.,* (2003) demonstrated that the rate of hydrolysis of starch from black beans is markedly slower (41%) compared with white bread (50%). A study by Leathwood and Pollet (1988) on plasma glucose and satiety showed a sharp rise in plasma glucose levels after a meal containing potato, and in contrast, a slow sustained increase in blood glucose after consumption of bean puree. According to Ludwig (1999), a low glycemic index diet might also protect against the development of obesity.

Studies have also shown that consumption of common beans may reduce the risk of coronary heart disease. According to the findings in the Health Professional Follow-up Study by Hu *et al.,* (2000), men that adhered to a more prudent diet which included greater consumption of whole grains, legumes, fish, and poultry had a 30% lower risk of having heart disease. The amount of total cholesterol in the plasma may influence one's risk of developing heart disease. A 1% reduction in total cholesterol has been reported to correspond to about a 2% decrease in the risk of developing heart disease (Rifkind 1984). A regular consumption of common beans may contribute to lowering the plasma cholesterol levels (Anderson *et al.,* 1984; Kingman *et al.,* 1991and Shutler *et al.,* 1989). The inclusion of 450g baked beans into the daily diet of normocholesterolaemic men resulted in a significant reduction in the mean plasma cholesterol level from 5.1 to 4.5 mmol/l within 14 days. In contrast, no reduction in plasma cholesterol was observed when 440g spaghetti with tomato sauce was included in the daily diet of the same subjects for 2 more weeks (Shutler *et al.,* 1989). Beans are a good source of soluble dietary fibre, containing approximately 4 g per 1 cup cooked portion

(Anderson *et al.*, 1994). Brown *et al.*, (1999) concluded that soluble fibre from different fibre sources was associated with small but significant decrease in total cholesterol. The hypocholesterolemic effect of dietary fibre has been attributed to its ability to inhibit intestinal absorption of bile acids and neutral steroids, resulting in greater faecal bile acid and total steroids excretion (Moundras *et al.*, 1997). Resistant starch, which is part of the dietary fibre, has the property of gelling and binding bile acids, thus increasing viscosity of intestine contents and reducing absorption of bile acid from small intestine (Han *et al.*, 2004).

Some of the health benefits associated with common beans may come from fibre, unidentified phytochemicals, such as phytoestrogens, antioxidants, and phenols, which together with vitamins and minerals such as vitamin E and selenium may play important roles in disease prevention (Joanne *et al.*, 2001, Slavin *et al.*, 1999). These health benefits are achieved through multifactorial physiological mechanisms including antioxidant activity, mediation of hormones, enhancement of the immune system and facilitation of substance transit through the digestive tract, butyric acid production in the colon, and absorption of substances in the gut (Adom *et al.*, 2003).

1.4. Aim and objectives

Aim:

The study aimed to explore the nutraceutical antioxidant potential and polyphenolic profiles of the market classes of bambara groundnuts and common beans commonly grown in Zambia.

Objectives

1. To assess the *in vitro* antioxidant activities of the extractable phytochemicals in bambara groundnuts and common beans using chemical-based assays and to characterise the phytochemical nature, particulary their polyphenolic compound profiles.
2. To investigate the effects of domestic hydrothermal processing on antioxidant activities and phenolic phytochemical profiles of bambara groundnuts and common beans
3. To investigate the effects of sprouting on the antioxidant activities and phenolic phytochemical profiles of bambara groundnuts and common beans.

Chapter 2

Materials and Methods

2.1. Bambara groundnut and common bean varieties used, collection and storage

Two market classes of bambara groundnuts (red and brown, Table 2-1) were procured directly from the farmers in Chipata district of the Eastern region of Zambia during the 2010 crop harvesting season. Locally it is called *Nzyama* (in the Nyanja language), *Ntoyo* (in the Bemba language), and *Mbwila* (in the Tonga language). Both market classes are landraces that have become well adapted to the local climate and soils, and indigenous knowledge about the germplasm is well preserved in the communities. Four market classes of common beans (red, grey mottled, brown and white, Table 2-2) commonly grown were similarly obtained directly from the farmers in their traditional cultivating areas. Red beans (locally called *Lundazi*) was obtained from Lundazi district of the Eastern region, grey mottled (locally called *Kabulangeti*) from Nakonde district in the Northern region, brown beans (locally called *Chambeshi*) from Solwezi district in the North Western region and white beans (locally called *Kalungu*) from Lusaka region. The sample collection sites are shown in Figure 2-1 and a description of the characteristics of the bambara groundnuts and common bean market classes are shown in Tables 2-1 and 2-2 respectively. In order to make the samples representative, an attempt was made to collect the seeds of each market class from 15 farmers in each growing area with not less than 0.5 kg per farmer. Collected samples were stored in air and water tight containers and transported by air from Lusaka to Cape Town. Immediately after the arrival, samples were taken to the University of Cape Town and stored at 4^{o}C until they were used for biochemical and physiological assessments.

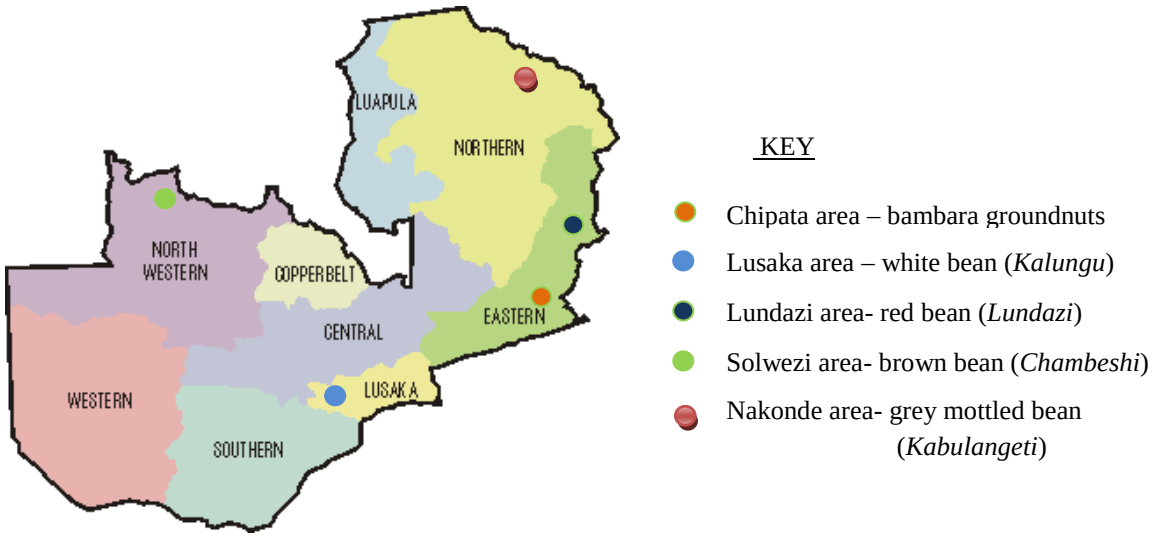


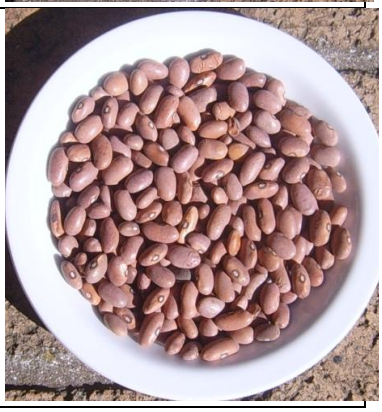
Figure 2-1 Map of Zambia showing the sample collection areas

Table 2-1 Market classes of bambara groundnuts investigated

Market class	Local name	*Descriptive characteristics	Appearance
Brown bambara groundnuts	*Nzyama* (Nyanja language *Ntoyo* (Bemba language) *Mbwiila* (Tonga language)	• Underground pod • 110-120 days to maturity • Potential yield: 400 – 1400 kg/ha	
Red bambara groundnuts	*Nzyama* (Nyanja language *Ntoyo* (Bemba language) *Mbwiila* (Tonga language)	• Underground pod • 110-120 days to maturity • Potential yield: 400 – 1400 kg/ha	

Market classes known by the same name in each language, they are only differentiated based on colour. *Information about the general attributes such as days to maturation, growth pattern and potential yields was obtained through informal interviews with the local people.

Table 2-2 Market classes of common beans investigated

Market class	Variety name	*Descriptive characteristics	Appearance
Red beans	*Lundazi*	• Non-climber • 78-80 days to maturity • Potential yield: 1500 kg/ha	
Brown beans	*Chambeshi*	• Non-climber • 78-80 days to maturity • Potential yield: 1500 kg/ha	
White beans	*Kalungu*	• Non-climber • 78-80 days to maturity • Potential yield: 1000-1500 kg/ha	
Grey mottled beans	*Kabulangeti*	• Climber • 80-100 days to maturity • Potential yield: 1000 kg/ha	

*Information about the general attributes such as days to maturation, growth pattern and potential yields was obtained through informal interviews with the local people.

2.2. Characterisation of bambara groundnuts and common beans

Bambara groundnuts and common beans were characterized on the basis of their phenolic phytochemical profiles, antioxidant activities, physicochemical and nutritional properties. Physicochemical properties assessed included water hydration and swelling capacities. The nutrition related parameters investigated were mineral content, moisture, ash, crude protein, crude fat, crude fibre and total carbohydrates.

2.2.1. Hydration, swelling capacity and indices

Hydration and swelling capacities were determined according to the procedure described by Bishnoi & Khetarpaul, (1993). One hundred seeds were counted, weighed and transferred to a measuring cylinder and 100 ml of water was added. The cylinder was covered with aluminium foil and left at room temperature for 24 hours. After 24 hours, all the seeds were drained, superfluous water removed with the filter paper and swollen seeds reweighed. Hydration capacity per seed was determined by dividing the mass gained by the seeds by the number of seeds present in a sample. Hydration index was then calculated as the ratio of average hydration capacity per seed and the mass of one seed. For swelling capacity, 100 seeds were counted, their volume noted and soaked for 24 hours as described above. The volume of the soaked seeds was noted in the graduated cylinder after 24 hours. Swelling capacity per seed was calculated as the volume gained by the seeds divided by the number of the seeds. The swelling index was then calculated as the ratio of the swelling capacity per seed to the volume of one seed. The experiment was conducted three times in triplicate.

2.2.2. Chemical composition

Chemical composition analysis of the seed flour involved analysis of crude moisture, crude fat, crude protein, total ash and crude fiber were determined using AOAC official methods of 934.01, 920.39 (A), 984 (A – D), 942.05 and 978.10 respectively (AOAC 2006). Total carbohydrate was calculated by difference. Calcium and magnesium were measured using the GBC Atomic Absorption Spectrophotometer (Sena *et al.,* 1998). Potassium and sodium were assayed using a Corning 410 flame photometer (Sena *et al.,* 1998). Phosphorus was determined using Technicon Auto-analyzer methodology (Lockett *et al.,* 2000). Iron, zinc, copper, manganese, lead, selenium and arsenic were measured using Perkin-Elmer 2001 Model Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Approximately 2 g of the

seed flour was used in each analysis and the experiment was performed three times in triplicate.

2.2.3. Phenolic phytochemical investigations

Total polyphenols, total flavonoids and tannin contents of different market classes of bambara groundnuts and common beans were assessed. Individual phenolic compounds were identified using HPLC-DAD-ESIMS and quantification was done where the standard was available.

2.2.3.1. Preparation and extraction of phenolic compounds

Raw dry bambara groundnuts and common beans were ground into powder of the same consistency using a coffee grinder (Braun, Mexico). Crude aqueous and 70% methanol extracts were obtained using Ultrasound-Assisted Extraction (UAE) from the seed flour (Dobiáš *et al.*, 2010). This method is an effective way to extract analytes from different matrices more rapidly than with other extraction techniques. The extraction process is faster and more complete in comparison to traditional methods such as maceration/stirring, because the surface area in contact with the solid and liquid phase is much larger due to particle disruption (Palma and Barroso, 2002). It is a simple method in which the crushed sample is mixed with a suitable solvent and placed into an ultrasonic bath, where the working temperature and extraction time are set (Klejdusa *et al.*, 2009). Extraction time varies, in some cases complete extractions are accomplished in as little as 30 min (Palma and Barroso, 2002).

Approximately 15 g of seed powder in 150 ml of either water or 70% methanol was sonicated for 30 minutes at 25^{o}C using the Eumax UD500SH 40 kHz ultrasonic bath. After extraction, the mixture was centrifuged at a speed of 10,000 rpm for 15 minute in Beckman Coulter JE centrifuge. The supernatant of the water extraction was frozen at -80 ^{o}C and freeze dried to get a powdered aqueous extract. The supernatant of the 70% methanol extraction, however, was first concentrated to 30 ml by evaporation under reduced pressure in a rotary evaporator (Buchi R-210 model, Switzerland) to remove methanol. The extract was then frozen at -80 ^{o}C and freeze dried to obtain a powdered methanolic extract using the Telstar LyoQuest -85 freeze dryer. The freeze dried aqueous and methanolic extracts were stored at -4 ^{o}C until further analysis.

2.2.3.2. Cooking treatment

Approximately 400 g of the seeds of each legume were cooked using the traditional cooking method commonly used in Zambia. Seeds were boiled in tap water on a hot plate at the temperature of 100 ± 5 ^{o}C, until they felt soft using the finger compression test. The finger compression test is a sensory based approach in which texture is treated as a perception or how a food material feels with the fingers. It is a very rapid and useful method employed to determine firmness or the degree of softness by consumers (Mitcham *et al.,* 1996). In this experiment, the beans were considered soft if they were able to deform under moderate pressure when compressed between the index finger and the thumb.

2.2.3.3. Preparation of extracts of cooked beans and bambara groundnuts

The cooked seeds together with the water that remained after cooking were immediately frozen at – 80 ^{o}C and freeze dried to obtain the dried material that was later ground to a powder. Approximately 15 g of the cooked seed powder in 150 ml of 70% methanol was extracted as previously described and the powdered extract was stored at - 4^{o}C (see section 2.2.3.1).

2.2.3.4. Sprouting of the common beans and bambara groundnuts

100 seeds of each legume were soaked in tap water for 12 hours until fully imbibed. The seeds were then spread on a moist muslin cloth and allowed to germinate at room temperature (25 ± 1^{o}C) in the dark. The seeds were kept moist by sprinkling the water on a muslin cloth at regular intervals. Germination capacity was expressed as the percentage of the seeds that germinated (Jimenez Martınez *et al.,* 2012). The sprouts were harvested on the 8th day of germination when 98 and 100% of the seeds had germinated in bambara groundnuts and common beans respectively. A seed was considered to have sprouted when the root growth of 2 mm had occurred. The experiment was conducted two times and all measurements were performed in duplicate.

2.2.3.5. Preparation of extracts from the sprouted seeds

Germination was stopped by putting the sprouted seeds into a – 80^{o} C freezer for 5 hours following which the seeds were freeze dried for 72 hours. The dried seeds were ground into powder using a coffee grinder (Braun, Mexico). The powdered sample (15 g) was extracted

in 150 ml of 70% methanol as previously described and the resultant powdered extract was stored at - 4^{o}C (see section 2.2.3.1.).

2.2.3.6. Determination of total polyphenols

In order to determine polyphenol intake in populations and study their association with health, it is essential to have detailed information on their content in foods (Neveu *et al.*, 2010). Powdered samples of bambara groundnuts or common beans (5 g) were placed in 50 ml of 70 % methanol or water and sonicated for 30 minutes at 25 ^{o}C followed by centrifugation at 10,000 rpm for 15 minute at 4 ^{o}C to obtain a clear supernatant. Total polyphenols were then determined by the Folin Ciocalteu assay according to the method of Makkar *et al.*, (2000). To 100 µl of sample extract, 400 µl of distilled water was added followed by the addition of 250 µl Folin Ciocalteu reagent. 20 % Sodium carbonate (1.25 ml) was then added and the mixture was incubated for 40 min. Absorbancies were read at 725 nm after 40 minutes using a spectrophotometer (Ultrospec 1000 model, England) against the blank (70% methanol or water) depending on whether it was the water or 70 % methanol extract. The amount of total polyphenols was calculated as gallic acid equilvalents from the calibration curve of gallic acid standard solution and expressed as mg gallic acid equivalents/ 100 g DW. The experiment was conducted three times and all measurements were performed in triplicate.

2.2.3.7. Determination of tannins

The determination of tannins was performed using the Polyvinylpolypyrrolidone (PVPP) tannin binding assay as described by Makkar *et al.*, (2000). Approximately 100 mg PVPP was placed in 100 x12 mm test tubes. One ml of distilled water was added to the test tubes containing PVPP followed by the addition of 1 ml of sample extract. The tube was vortexed, and kept at 4 ^{o}C for 15 minutes. After 15 minutes at 4 ^{o}C, the tube was vortexed again and then centrifuged for 10 minutes at 10,000 rpm. The supernatant has only simple phenolics other than tannins (the tannins would have been precipitated along with the PVPP). Total polyphenols of the supernatant was measured as described in section 2.2.3.6. and results of non tannin phenols were expressed as mg gallic acid equivalents / 100 g DW. The amount of polyphenols bound to PVPP as tannin was calculated by subtracting the total polyphenols in the supernatant after the PVPP assay from the amount of total polyphenols determined

without PVPP. The experiment was conducted three times and all measurements were performed in triplicate.

2.2.3.8. Extraction and analysis of total flavonoids

According to Amic *et al.*, (2003) many of the flavonoid phytochemicals have positive health benefits as antioxidants and their determination in foods is essential. Flavonoids were extracted from bambara groundnuts and common beans samples by the ultrasound-assisted system as described by Wang *et al* (2012). Powdered sample (5 g) was placed in 50 ml of 70% methanol or water and was sonicated for 30 minutes at 25 ^{o}C followed by centrifugation at 10,000 rpm for 15 minute at 4 ^{o}C to obtain a clear supernatant. Analysis of total flavonoids was done using the aluminium chloride colorimetric method according to Pourmorad et al. (2006). About 500 µl of 70% methanolic extract was mixed separately with 1.5 ml of methanol, 100 µl of 10% aluminium chloride, 100 µl of 1 M potassium acetate and 2.8 ml of distilled water. The absorbance of the solutions was measured after 30 minutes at 414 nm using a spectrophotometer (Ultrospec 1000 model, England). The amount of total flavonoids was calculated as quercetin equivalents (mg / 100g DW) using a calibration curve of quercetin standard solution. The experiment was conducted three times and all measurements were performed in triplicate.

2.2.3.9. HPLC –DAD-ESI-MS Instrumentation and chromatographic conditions

The freeze dried 70% methanolic extracts of bambara groundnuts and common beans were analysed using a Waters ZMD 4000 system that was equipped with a Waters 2690 HPLC, Waters 996 photodiode array, ZMD mass spectrophotometer, 717 Plus autosampler, and a quaternary pump (Waters Corp, Milford, MA, USA). Separations were carried out on a 300x3.9 mm, 4 µm reversed phase Nova-Pak C18 (Waters) column that was maintained at 40^{o}C. The photodiode array detector (PDA) was linked directly to a sprayer needle where ions were generated by electrospray ionisation (ESI) in a negative mode. The mobile phase A consisted of 5% (v/v) acetonitrile/water, containing 0.1% (v/v) formic acid and mobile phase B consisted of 100% acetonitrile containing 0.1% (v/v) formic acid. The sample was injected at a volume of 25 µl. The elution profile consisted of a stepwise linear gradient from 0% to 28% solvent B for 22 minutes with a flowrate of 0.3 ml/min. The PDA dectector was set to a scanning range of 200 to 700 nm and the UV-Vis absorption spectra were recorded online during the HPLC analysis. Phenolic acids and flavonols were detected at 280 and 360 nm

respectively. Continuous mass spectra data were recorded on a full scan negative ionisation mode for a mass range of m/z 85 to 1000. The capillary voltage was set at 2.5 kV, the cone at 20 V and the extractor at 5 V. Nitrogen gas was used for nebulising and drying at different fragmentation voltages. Data acquisition was controlled using MassLynx 4.1 (Micromass, Waters Corp., Beverly, MA, USA).

Table 2-3 Gradient solvent system for analysis of phenolic compounds by HPLC -PDA – ESI-MS

	Composition of the mobile phase (%)	
Time (minutes)	***Mobile phase (A)**	***Mobile phase (B)**
1	100	0
22	72	28
22.50	60	40
23	0	100
24.50	0	100
25	100	0
26	100	0

* Mobile phase A consisted of 5% (v/v) acetonitrile/water, containing 0.1% (v/v) formic; mobile phase B consisted of 100% acetonitrile containing 0.1% (v/v) formic acid.

2.2.3.10. Preparation of the samples for HPLC -DAD-ESI-MS

Preparation of the test solution for HPLC-DAD-ESI-MS was done according to the procedure by Gülçin *et al.,* (2010) with slight modifications. One hundred mg of the freeze dried 70% methanolic extract was dissolved in 5 ml of ethanol-water (50:50 v/v). 100 µl of the prepared extract was transferred into a 5 ml volumetric flask and diluted to the volume with ethanol-water (50:50). From the final solution, an aliquot of 1.5 ml was transferred into a capped autosampler vial and 25 µl of the sample was injected into the HPLC-DAD-ESI-MS system.

2.2.3.11. HPLC-PDA-ESI-MS Identification and quantification of phenolic compounds

Identification of phenolic compounds was accomplished using UV spectra and ESI-MS spectral data and by comparison with published data reported in the literature. Authentic

standards were also used where available by comparing their chromatograms with those of the samples. Quantification of individual phenolic compounds could only be done where authentic standards were available. The available standards were *t*-ferrulic acid, gallic acid, salicylic acid, *p*-coumaric acid, epicatechin and catechin. The concentrations of ferrulic acid, gallic acid, salicylic acid, *p*-coumaric acid, epicatechin and catechin were obtained from the linear regression equations of the standard curves (Gülçin *et al.*, 2010). The experiment was conducted three times and all measurements were done in duplicate.

2.2.4. Antioxidant activity determination

Antioxidant activity of both the aqueous and 70% methanolic extracts of bambara groundnuts and common beans was determined by two methods:

a) 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay
b) Ferric Reducing Antioxidant Potential (FRAP) assay

The two methods were used because they are rapid, accurate and inexpensive. The DPPH method is widely used for the measurement of free radical scavenging ability of antioxidants (Pérez-Jiménez *et al.*, 2008). The DPPH method is a rapid, simple and accurate assay for measuring the ability of different compounds to act as free radical scavengers or hydrogen donors, and to evaluate the antioxidant activity of foods and beverages (Prakash, 2001). According to Marxen *et al.,* (2007), the DPPH method is a convenient method which is independent of sample polarity and useful for screening of many samples for radical scavenging activity. Similarly, the FRAP assay is fast assay with little selectivity (Carlsen *et al.*, 2010). A study by Thaipong *et al*., (2006) on the comparison of ABTS, ORAC, FRAP and DPPH assays for estimating antioxidant activity demonstrated FRAP and DPPH to be highly reproducible as they showed no differences among determinations while ABTS and ORAC differed among the runs.

2.2.4.1. Determination of free radical scavenging activity of the bambara groundnuts and common beans

According to Oktay *et al.,* (2003), the free radical scavenging reaction between DPPH and an antioxidant (Y-H) can be written as:

(DPPH) + (Y-H) → DPPH-H + (Y)
(Purple) (Yellow)

DPPH stable free radicals are reduced to DPPH-H leading to discoloration from purple to yellow and consequently a decrease in absorbance. The degree of discoloration indicates the scavenging potential of the antioxidant compounds (Oktay *et al.*, 2003; Pal *et al.*, 2008). This assay therefore involves the measurement of hydrogen atom transfer or electron donation from a potential antioxidant to free radical molecules (Becker *et al.*, 2004).

First, it was important to study the kinetic behaviour of the extracts towards DPPH free radicals when the freeze dried extracts from each market class were added at the same concentration. The knowledge of the kinetics of atom transfer is important because free radicals in the organism are short-lived species, implying that the impact of a substance as an antioxidant depends on its fast reactivity towards free radicals (Villaño *et al.*, 2007). The free radical scavenging kinetic determinations were adapted from (Villaño *et al.*, 2007). Under the experimental conditions used, the DPPH concentration was in large excess with respect to that of the extracts in order to follow pseudo first-order kinetics. This was done to exhaust the hydrogen donating capacity of the extracts. The excess concentration of DPPH (200 mM) was determined to be the optimum concentration after performing a number of runs with the extracts. This was the only way the excess DPPH concentration could be determined since it was not posible to work it out based on the DPPH: antioxidant molar ratios as the antioxidants in the extracts were not pure compounds. In the assessement of the kinetic behaviour, 2 ml of the extracts were added at the same concentration (400 µg / ml) to 2 ml of DPPH radical solution (200 mM) prepared in 95% methanol. The reaction was run at room temperature within a time period of 80 minutes. The absorbances of the mixture were automatically measured every 10 seconds using the spectrophotometer at 517 nm connected to a computer and the output was displayed using SWIFT 1000 software (Ultraspec 1000 model, England). From the reaction between an antioxidant and DPPH;

(DPPH) + (Y-H) → DPPH-H + (Y), it can be deduced that:

$$-\frac{d[DPPH]}{dt} = k[DPPH][Y-H] \qquad (1)$$

Considering that DPPH was in excess and therefore the experiment was under pseudo first-order conditions, one can say:

$$InA = InA_o - kt \qquad (2)$$

Where A_0 is the absorbance of the reaction mixture (DPPH and the extract) at $t = 0$; A is the absorbance of the reaction mixture (DPPH and extract) at time t.
The pseudo first order rate constant 'k' for the reaction of the antioxidants in the extracts and DPPH in the first seconds of the reaction was calculated from the slopes of *InA* versus time plots.

According to Villaño *et al.*, (2007), the percentage of DPPH remaining at any time t can be determined as:

$$\%DPPH_{remaining} = \frac{A_t}{A_0} X100$$

Where A_0 is the initial absorbance and A_t is the absorbance at time = t, both measured at 517 nm respectively. Plots of percentage DPPH versus time were constructed to show the disappearance pattern of the DPPH with time in the presence of each extract.

After studying the kinetic behaviour of the extracts towards the DPPH free radicals, it was necessary to evaluate the Effective Concentration (EC_{50}) for each extract. The extracts were assayed with the DPPH and incubated in the dark at room temperature for 30 minutes. The degree of free radical scavenging activity in the presence of different concentration of extracts and their absorbance were measured using spectrophotometer at 517 nm (Ultrospec 1000 model, England). The degree of free radical scavenging activity was expressed as:

$$\text{DPPH free radical scavenging (\%)} = \frac{A_{Control} - A_{Sample}}{A_{Control}} X100$$

$A_{Control}$ = Absorbance of DPPH alone, A_{Sample} = Absorbance of DPPH in the presence of different concentrations of the extracts.

The EC_{50} value required for 50% of the DPPH free radicals scavenging by the extracts was obtained from a series of dose-response data (extract concentrations and DPPH free radical scavenging (%). Using an x-y plot, the data was fitted with a linear regression line (Iranshahi *et al.*, 2009), and the EC_{50} was estimated using the following relationship:

$$Y = a*X + b,$$

$$EC_{50} = (0.5 - b)/a$$

2.2.4.2. Determination of Ferric Reducing Antioxidant Power (FRAP)

The FRAP assay according to the procedure by Benzie (1996) was used to determine the ferric reducing antioxidant power of the bambara groundnuts and common beans. The method measures the ferric reducing ability of the antioxidants compounds in the extracts. At low pH, ferric-2,4,6-tri-2-pyridyl-*s*-triazine (TPTZ) complex (Fe^{3+} TPTZ) is reduced to the ferrous form Fe^{2+} in the presence of the antioxidant producing an intense blue colour with an absorption maximum at 593 nm. The reaction is non-specific and any half life reaction, which has a less positive redox potential, under reaction conditions, than the Fe^{3+}/ Fe^{2+}-TPTZ half-life reaction will drive the Fe^{3+}-TPTZ reduction. This method is based on electron transfer mechanism. Acording to Ou *et al*., (2002), the relevant chemical reaction of the FRAP method involves a single electron reaction between Fe^{3+}-TPTZ and a single electron donor ArOH. This reaction can be written as:

$$Fe^{3+}\text{-TPTZ} + ArOH \rightarrow Fe^{2+}\text{-TPTZ} + ArOH^{+\bullet}$$

The antioxidant (ArOH) is oxidized by an oxidant ($Fe^{3+)}$ and as a result, a single electron is transferred from (ArOH) to (Fe^{3+}).

Powdered sample of bambara groundnuts or common beans (5 g) in 50 ml of 70 % methanol or water was sonicated for 30 minutes at 25 ^{o}C followed by centrifugation at 10,000 rpm for 15 minutes at 4 ^{o}C to obtain a clear supernatant. Working FRAP reagent was prepared by mixing 25 ml of acetate buffer (300 mM, pH 3.6); 2.5 ml ferric chloride solution (prepared by dissolving 54 mg ferric chloride in 10 ml distilled water) and 2.5 ml TPTZ solution (prepared by dissolving 31 mg TPTZ in 40 mM HCl at 50 ^{o}C). The mixture was placed in a water bath at 37 ^{o}C for 10 minutes. The assay was performed as follows: 1 ml of water and 80 µl of the test sample were pipetted into a cuvette. About 600 µl of the incubated FRAP reagent was added to the cuvette and mixed by inversion. A reagent blank was prepared as above with 80 µl water added instead of the test sample. The change in absorbance was recorded at 593 nm using a spectrophotometer after exactly 4 minutes (Ultrospec 1000 model, England). The amount of Fe^{2+} produced from the reduction of Fe^{3+} by the extract was calculated from the standard curve prepared from ferrous sulphate solution and results were expressed as mg Fe^{2+}

/ 100 g dry sample. The experiment was conducted three times and all measurements were performed in triplicate.

2.3. Statistical analysis

Statistical analysis was performed using S-PLUS 6 Windows Professional 2001. Experimental results were expressed as mean values ± standard error. Data was analysed using either a t-test or one way and two way analysis of variance (ANOVA) models. Values at $p < 0.05$ were considered statistically significant. Correlation coefficients of variable parameters were analysed by Pearson correlation test.

Chapter 3

Screening of the raw dry bambara groundnuts and common beans for phenolic phytochemicals and antioxidant activity

Introduction

The focus in this chapter was to screen the market classes of raw dry bambara groundnuts and common beans commonly grown in Zambia for their nutraceutical potential based on the antioxidant activities and polyphenolic phytochemical profiles. Physicochemical properties and nutritional composition of both legumes are also reported. Physical properties that included water hydration capacity, swelling capacity, hydration index, and swelling index were explored. The nutritional parameters studied were mineral content, moisture, ash, crude protein, crude lipid, crude fibre and total carbohydrates. This information is important when it comes to the exploitation of these landraces by breeders, food processors, nutritionists, farmers and policy makers. Water hydration capacity, swelling capacity, hydration index, and swelling index are also very useful parameters in grain processing as they are correlated with cooking time (Shimelis and Rakshit 2005).

This Chapter is divided into sections A and B. Section A gives the report on bambara groundnuts, whereas the section B reports on common beans.

3.1. Section A: Bambara groundnuts

Results

3.1.1. Nutritional and physicochemical properties of the Seeds

Physicochemical properties of the red and brown bambara groundnuts are summarized in Table 3-1. Generally, there were no significant differences ($p > 0.05$) in the swelling capacity, swelling index, hydration capacity, and hydration index of the two market classes of bambara groundnuts. Higher values for these parameters are an indication of shorter cooking time. Hence, the red and brown bambara groundnuts would require similar cooking times.

Table 3-1 Physicochemical properties of the bambara groundnuts

Property	Market classes of bambara groundnuts	
	Red	Brown
Hydration capacity (g / seed)	0.173 ± 0.014	0.176 ± 0.006
Hydration index	0.125 ± 0.004	0.119 ± 0.008
Swelling capacity (ml / seed)	0.180 ± 0.036	0.184 ± 0.031
Swelling index	0.118 ± 0.005	0.122 ± 0.002

Concentrations of moisture, crude protein, total ash, fat, crude fibre and carbohydrates of the red and brown bambara groundnuts market classes are presented in Table 3-2. The protein content of the red market class was within the range (16 – 21 %) reported by Brough and Azam-Ali, 1992; Brough *et al.,* 1993 and Purseglove 1992. The brown market class had the protein content of 14.62 % which was lower than the average reported literature values. The observed lower protein value in this market class may be due to the genotype (Salunkhe *et al.,* 1985). The concentrations of carbohydrate for both market classes were higher than 43 % reported by Azam-Ali, 1992, but consistently within the range (50 – 60 %) reported by Purseglove (1992). Ash contents for both market classes were higher than 3.6 % reported previously by Mahala and Mohammed (2010). The differences in ash may be attributed to

soil types (Mesquita *et al.*, 2007). The fat concentrations for both market classes were within the range (4.5 – 6.5 %) previously reported by Purseglove (1992).

Table 3-2 Proximate composition (g / 100 g DW) of the whole seed sample

Property	Market classes of bambara groundnuts	
	Red	Brown
Moisture	8.95 ± 1.26	9.13 ± 0.71
Crude protein	18.55 ± 1.55	14.62 ± 0.01
Total ash	4.21 ± 0.68	4.29 ± 0.51
Fat	6.54 ± 0.38	6.28 ± 0.77
Crude fibre	2.79 ± 0.01	5.33 ± 0.37
Carbohydrates	59.23 ± 3.11	60.34 ± 0.81

The mineral contents (mg / kg DW) of the seed sample are presented in Table 3-3. Potassium was found to be the most abundant mineral in both market classes, followed by phosphorous magnesium and calcium respectively. Sodium, iron, manganese and copper were generally very low while lead, mercury and arsenic were not detected in both samples. The amount of potassium was within the range (15780 – 17420 mg / kg DW) previously reported by Amarteifio *et al.*, (2002) for Botswanan bambara groundnut landraces. Concentrations of the rest of the minerals investigated were different from those reported by Amarteifio *et al.*, (2006); Amarteifio *et al.*, (2002) and Kemo (2000). Mineral content of agricultural products vary with geographical location and agricultural practices (John 1992; Amarteifio *et al.*, 2006). According to the information from the local people in the area where sampling was done, bambara groundnut landraces are normally intercropped with maize and no chemicals or fertilizers are used. Most growers in the area prefer growing the crop in sandy loam soil that is well drained. Agricultural practices may differ from one growing region of Africa to the other and this may affect the mineral content of bambara groundnuts.

Table 3-3 Mineral composition (mg / kg DW) of the whole seed sample

Minerals	Market classes of bambara groundnuts	
	Red	Brown
Calcium	516.6 ± 0.59	593.7 ± 0.67
Magnesium	2024.4 ± 0.20	2054 ± 0.21
Potassium	16670.1 ± 1.08	16282.2 ± 1.56
Phosphorous	3398.5 ± 0.03	3221.1 ± 0.02
Sodium	26.2 ± 1.97	38.4 ± 2.89
Iron	27.25 ± 1.3	20.97 ± 1.9
Arsenic	ND	ND
Zinc	19.02 ± 1.4	18.45 ± 1.0
Lead	ND	ND
Copper	5.51 ± 2.4	5.12 ± 1.0
Manganese	13.87 ± 2.3	19.62 ± 1.9
Mercury	ND	ND

ND = Not detected

3.1.2. Phenolic phytochemical profiles of the Zambian bambara groundnuts

The identification of phenolic compounds in bambara groundnuts using HPLC-PDA-ESI-MS is reported below. To the authors' knowledge, this is the first time that phenolic compounds in bambara groundnuts have been identified using this approach. Bambara groundnut characterisation based on the tannin concentrations, total polyphenols and flavonoids determined using non chromatographic approaches is also reported.

3.1.2.1. Water and methanol extractables

Extraction with different solvent systems have been reported in the research of phytochemicals and antioxidant activity of plant foods (Adon and Lui 2002; Yu *et al.,* 2002; Liu 2009). In this study, either water or 70% methanol were used in ultrasonic-assisted extraction of bambara groundnuts. The yields obtained are presented in Table 3-4. The red market class had significantly lower yield for the 70% methanol extraction. This could probably be due to the differences in the nature of compounds in this market class compared to the brown market class. Extraction yields obtained using water were significantly higher

than those obtained using 70% methanol. Water has been reported to give higher yields than ethanol and hexane using ultrasonic-assisted extraction in wheat bran (Liu 2009).

Table 3-4 Yields of crude extracts of bambara groundnuts in ultrasonic-assisted extraction

Market classes of bambara groundnuts	Yield (mg / g DW)	
	70 % Methanol extract	Aquoeus Extract
Brown	76.4 ± 2.1	192 ± 6.3
Red	30.3 ± 3.5	190.2 ± 2.1

3.1.2.2. Total polyphenols and tannins

Determination of total polyphenols in plant foods is important because plant phenolics represent one of the major groups of compounds acting as primary antioxidants or free radical terminators (Miliauskas *et al.,* 2004). Total polyphenols and tannin concentrations of the brown and red market classes of bambara groundnuts are presented in Table 3-5. Spectrophotometric scanning of the aqueous and 70% methanol extracts between 200 to 400 nm showed a stronger absorbance at 280 nm for the brown compared to the red market class, indicating the presence of more polyphenol compounds (see Figure 3-1). Total polyphenol concentration of the brown market class was higher than the red for both the aqueous and 70% methanol extracts. The aqueous extracts recorded the highest amount of extractable polyphenols compared to the 70% methanol extract in both market classes. Most likely, the aqueous extract contained mainly hydrophilic components while the 70% methanol contained both hydrophilic and the hydrophobic components. The effect of the solvent type is significant to the extraction of the phenolic phytochemicals in the complex seed flour matrix, in addition to the varietal effects. These results are within the range (117 – 440 mg GAE / 100 g DW) reported by Heimler *et al.,* (2005) for other legumes.

The results for tannin concentrations followed a similar trend as that found for total polyphenols. The brown bambara market class recorded the highest tannin concentration in both the aqueous and 70% methanol extracts (Table 3-5).

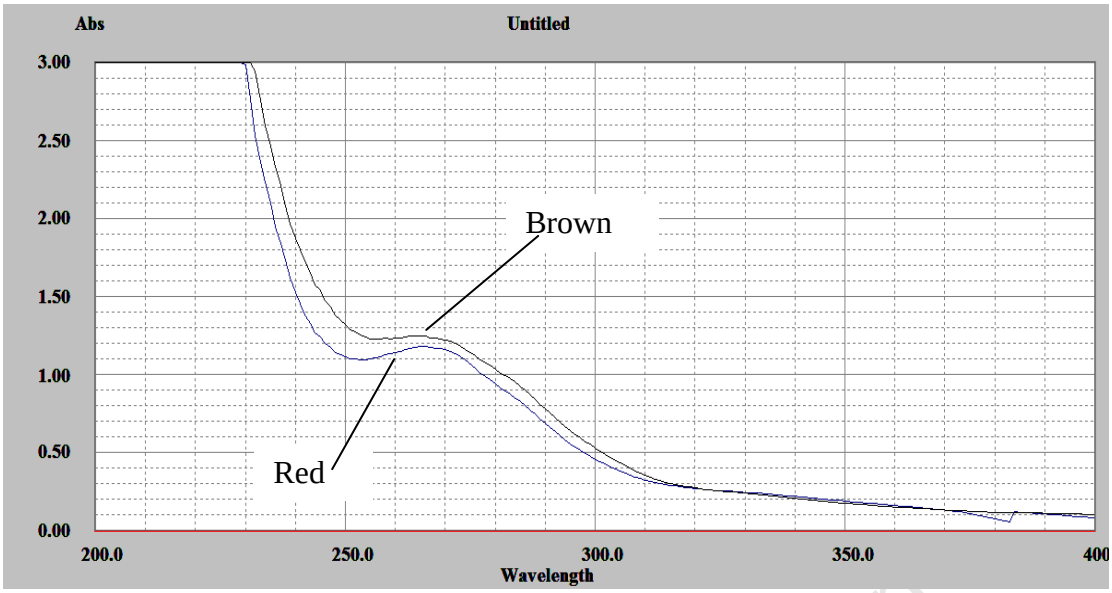


Figure 3-1 Results of the spectrophotometric scanning at 200 nm to 400 nm for the 70% methanol extracts of bambara groundnuts.

Table 3-5 Total polyphenols and tannin concentration of bambara groundnuts

Market classes of bambara groundnuts		Total polyphenols (mg GAE / 100 g DW)	Tannins (mg GAE / 100 g DW)
Aqueous			
	Brown	144.2 ± 1.7	112.8 ± 1.0
	Red	117.4 ± 0.6	95.5 ± 2.2
70% methanol			
	Brown	138.6 ± 2	82.3 ± 3.2
	Red	109.3 ± 1.2	69.1 ± 0.6

3.1.2.3. Total flavonoid concentration

Flavonoids constitute the largest class of phenolic compounds and the most diverse in plants (Miliauskas *et al.*, 2004). They possess a broad spectrum of chemical and biological activities including radical scavenging properties (Miliauskas *et al.*, 2004). Table 3-6 presents the results of the total flavonoid concentrations of the two market classes of bambara groundnuts.

It is intriguing that the total flavonoids, unlike the total polyphenols and tannins were higher in the red than the brown market class. The effect of the solvent on the extraction efficiency of the flavonoids was significant. In both market classes, the aqueous extract had the higher flavonoid contents compared to the 70% methanol extracts. The aqueous extracts for the two market classes were not significantly different ($p > 0.05$). The concentrations of total flavonoids in the present study are within the range (4.1 – 133 mg quercetin equivalents / 100 g DW) reported by Lin and Tang (2007) for different fruits and vegetables. Fruits and vegetables have health benefits and are good sources of flavonoids (Qian *et al.,* 2004; Cieslik *et al.,* 2006). Bambara groundnuts can therefore be used as an alternative source of flavonoids in the diet.

Table 3-6 Flavonoid content of bambara groundnuts

Market classes of bambara groundnuts		Flavonoid content expressed as quercetin equivalents (mg / 100 g DW)
Aqueous		
	Brown	127.4 ± 2.7
	Red	130.1 ± 1.2
70% methanol		
	Brown	71.6 ± 2.0
	Red	104.3 ± 1.2

3.1.2.4. Phenolic compounds identified by HPLC-PDA-ESI-MS in brown bambara groundnuts

Ten phenolic compound were identified based on co-chromatography with available authentic standards and mass spectra obtained in the negative mode by using their fragmentation pattern and data from published literature (Wu and Prior 2005; Seeram *et al.,* 2006). The chromatogram of the 70% methanol extract of brown bambara groundnuts is presented in Figure 3-2 and the identified compounds are summarised in Table 3-7. Selected UV-visible spectra for selected peaks are presented in Appendix A, fragment ion peaks in negative ion mode in Appendix B and Total Ion Chromatograms (TIC) in Appendix D. The mass spectrum in full scan negative ionisation mode showed the deprotonated molecule [M –

H] ⁻ of quinic acid at m/z 191 (peak 1) that fragmented to yield MS/MS spectrum with an ion at m/z 127, characteristic of quinic acid (Gouveia and Castilho 2011).

Peak 2 was provisionally identified as (E) GC-hexoside, a flavan-3-ol monomer with $[M - H]^-$ ion at m/z 467, a double molecular ion $[2M - H]^-$ at m/z 935, and a fragment ion at m/z 305 indicative of the hexosyl residual. These fragmentation patterns are in agreement with the previously published data (Yang *et al.*, 2011), where this phenolic compound was detected in bayberry. Dinelli *et al.*, (2006) reported the presence of flavanol compounds in free and conjugated forms as one of the principal phenolics in common legumes.

Peak 3 was tentatively identified as catechin glucoside with $[M - H]^-$ at m/z 451 that fragmented to yield a MS/MS spectrum with an ion at m/z 289 indicative of the catechin fragment and a loss of the hexose moiety (162 amu). This compound has been reported previously in pinto beans with similar fragmentation pattern by Estrella *et al.*, (2011).

Peak 4 had a deprotonaed molecular ion $[M - H]^-$ with m/z 205. MS/MS fragmentation yielded fragments at m/z (179, 143, 129) indicating the presence of caffeic acid. The fragmentation pattern is characterised by the loss of a hydrocarbon moiety (amu 26). This molecule was be tentatively assigned as caffeic acid derivative.

Peak 5 showed a deprotonated molecule $[M - H]^-$ of catechin at m/z 289, a fragment at m/z 245, and HPLC retention time of 6.08 min. Co-chromatography of authentic standard verified the identification of this peak as catechin.

Peak 6 revealed a negatively charged $[M - H]^-$ ion at m/z 289, MS/MS spectrum with m/z at 245, and HPLC retention time of 8.12 min . The spectra and the UV data gave a suggestive indication that compounds represented in peaks 5 and 6 are isomers. Co-chromatography of authentic standard verified the identification of peak 6 as epicatechin.

Peak 7 had $[M - H]^-$ ion at m/z 387 with MS/MS fragmentation at m/z 207 was identified at medioresinol, a phenolic lignin. The compound has been reported previously in lamiaceae species with similar fragmentation pattern (Hassain *et al.*, 2010).

Peaks 8 and 9 revealed negatively charged ions $[M- H]^-$ at m/z 163 and 193, and HPLC retention times at 8.92 and 11.1 respectively. Co-chromatography with authentic standards verified the identification of peak 8 as *p*-coumaric acid and peak 9 as *t*-ferulic acid.

Peaks 10 revealed negatively charged ions $[M- H]^-$ at m/z 137, and HPLC retention time at 12.37 min. Co-chromatography with authentic standard verified peak 10 as salicylic acid.

Peak 11 had $[M – H]^-$ ion at m/z 529 with MS/MS fragmentation at m/z 368, 367 and 179 (indicating the presence of caffeic acid fragment). Compounds with similar fragmentation characteristics have been reported previously in plants (Gouveia and Castilho 2011). Based on the fragmentation pattern and published data, this compound was tentatively identified as a caffeic acid derivative.

Peak 12 revealed a negatively charged ion ions $[M- H]^-$ at m/z at 582 with MS/MS fragmentation at 245, 205 and 289 (indicative of the presence of catechin fragment). The compound was provisionally assigned to catechin dimer.

Table 3-7 HPLC-PDA-ESI-MS -based identification of phenolics in brown bambara groundnuts

Peak	t_R(min)	Molecular weight	[M-H]- (m/z)	[M-H]- fragments (m/z)	Tentative identification
1	1.36	192	191	127	Quinic acid
2	4.31	468	467	935, 467	(E)GC-hexoside
3	4.58	452	451	289	Catechin glucoside
4	5.27	206	205	179,143,129	Caffeic acid derivative
5	6.08	290	289	245	Catechin
6	8.12	290	289	245	Epicatechin
7	8.62	388	387	207.1	Medioresinol
8	8.92	164	163	119	*p*-coumaric acid
9	11.1	193	194	149,134	*t*-ferulic acid
10	12.37	138	137		Salicylic acid
11	15.7	530	529	368, 367, 179	Caffeic acid derivative
12	16.71	583	582	289, 245, 205	Catechin dimer

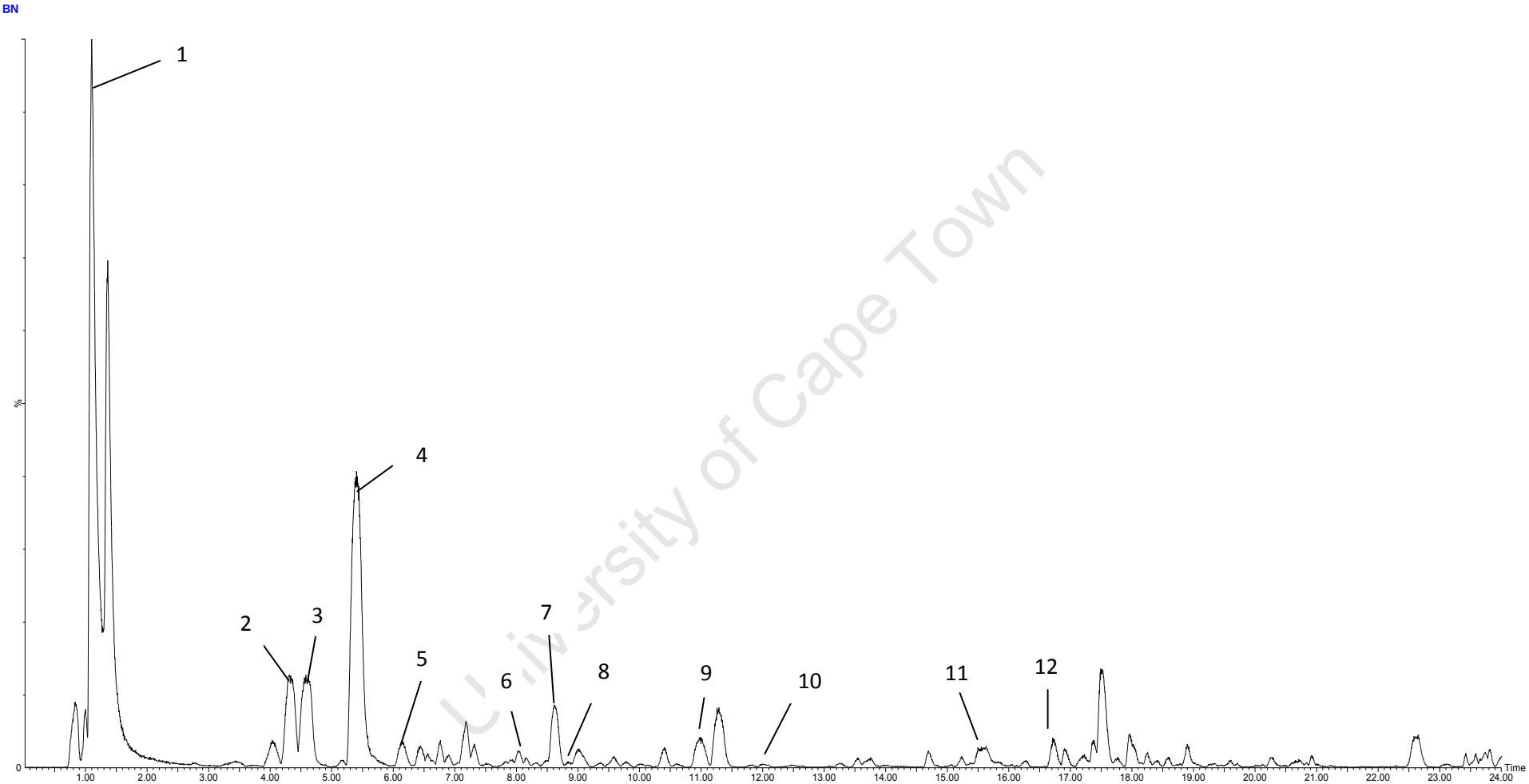


Figure 3-2 HPLC-PDA-ESI-MS chromatogram of 70% methanol extract of brown bambara groundnuts.

3.1.2.5. Phenolic compounds identified by HPLC-PDA-ESI-MS in red bambara groundnuts

The chromatogram of the 70% methanol extract of red bambara groundnuts is presented in Figure 3-3. Fifteen phenolic compounds were provisionally identified and are summarised in Table 3-8. The following phenolic compounds that were identified in the brown market class of bambara groundnuts were also found in the red one: Quinic acid (peak 1), (E) GC – hexoside (peak 2), catechin glucoside (peak 3), caffeic acid derivative (peak 4), catechin (peak 5), epicatechin (peak 6), medioresinol (peak 7), *p*- coumaric acid (peak 8), *t*-ferulic acid (peak 11) salicylic acid (peak 13), caffeic acid derivative (peak 14) and catechin dimer (peak 15).

The red bambara groundnuts contained the following phenolic compounds that were not detected in the brown market class:

Peak 9 revealed a negatively charged [M – H]- ion at m/z 479 and MS/MS spectrum with m/z at 317, corresponding to a loss of 162 amu: hexose moiety, probably a galactoside or glucoside leaving the myricetin fragment. This compound was tentatively identified as myricetin hexoside. This compound has been reported in other legumes by Lin *et al.,* (2008) with similar fragmentation pattern.

Peak 10 revealed a negatively charged ions [M – H]- at m/z 609 with MS/MS fragmentation at 301 (indicative of the presence of quercetin fragment). Tentatively, this compound is a quercetin derivative. Lin *et al.,* (2008) reported a compound in common beans with similar fragmentation pattern that was identified as quercetin-3-O-rutinoside by confirmation with a standard. In this research, the standard was not available to do the confirmation. Based on the literature data and the fragmentation pattern, this compound can provisionally be identified as quercetin-3-0-rutinoside.

Peak 12 had a negatively charged [M – H]- ion at m/z 463. MS/MS fragmentation yielded a fragment with m/z 301 (quercetin), after losing a hexose moiety (amu 162). This compound was identified as quercetin-3-O-glucoside. The presence of this flavonoid in legumes has been reported previously by Lin *et al.,* (2008). Kajdžanoska *et al.,* (2010) identified a compound with similar fragmentation pattern in strawberries.

Table 3-8 HPLC-PDA-ESI-MS -based identification of phenolics in red bambara groundnuts

Peak	t_R(min)	Molecular weight	[M-H]- (m/z)	[M-H]- fragments (m/z)	Tentative identification
1	1.36	192	191	127	Quinic acid
2	4.31	468	467	935,467	(E)GC-hexoside
3	4.58	452	451	289	Catechin glucoside
4	5.27	206	205	178,143,129	Caffeic acid derivative
5	6.08	290	289	245	Catechin
6	8.12	290	289	245	Epicatechin
7	8.62	388	387	207.1	Medioresinol
8	8.92	164	163	119	*p*-coumaric acid
9	9.81	480	479	317	Myricetin hexoside
10	10.11	610	609	301	Quercetin-3-O-rutinoside
11	11.1	194	193	149, 134	*t*-ferulic acid
12	11.59	464	463	301	Quercetin-3-O-glucoside
13	12.37	138	137	-	Salicylic acid
14	15.7	530	529	368, 367, 179	Caffeic acid derivative
15	16.71	583	582	289, 245, 205	Catechin dimer

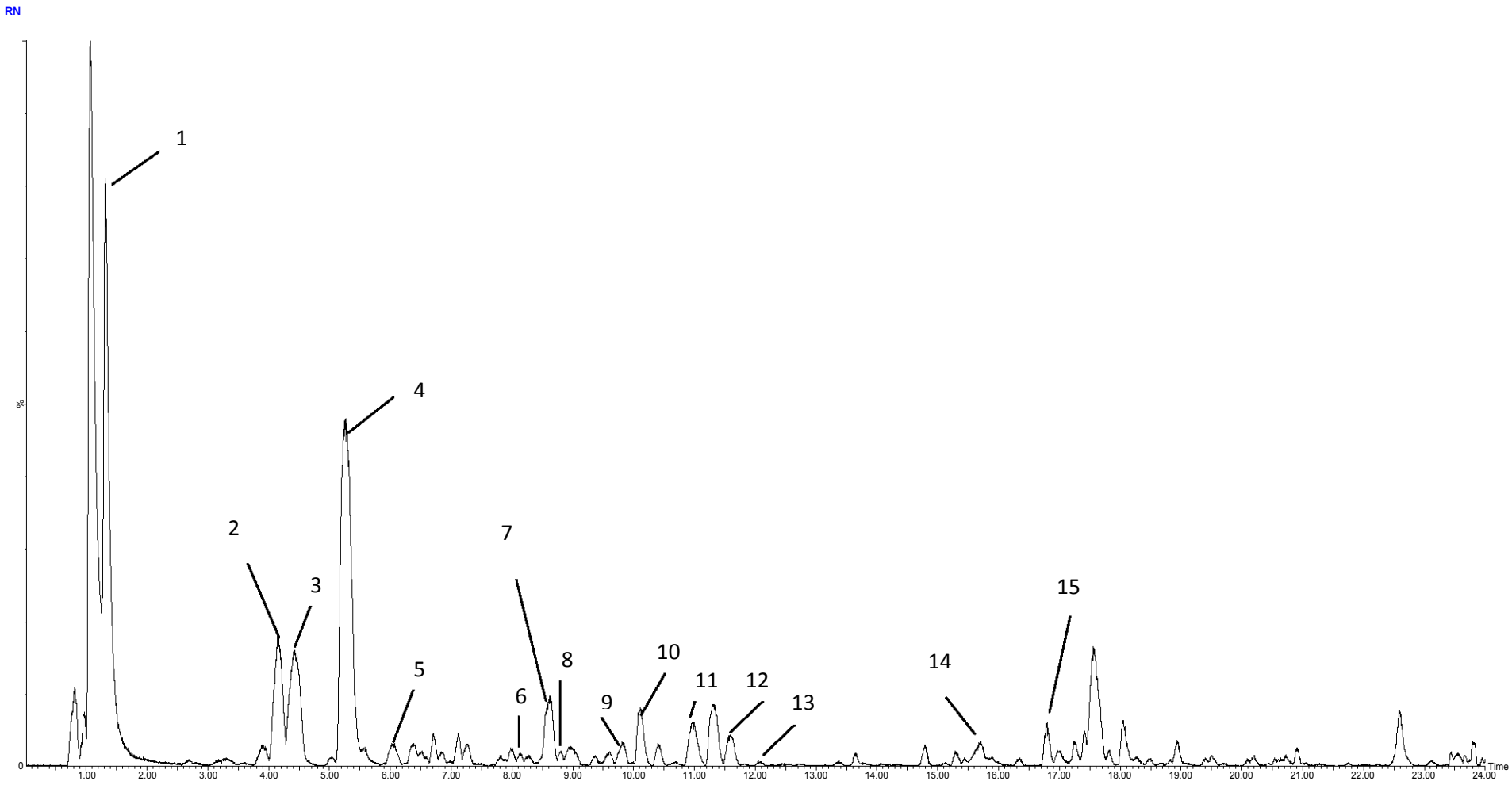


Figure 3-3 HPLC-PDA-ESI-MS chromatogram of 70% methanol extract of red bambara groundnuts.

3.1.2.6. Concentration of some identified phenolic compounds in bambara ground nuts

Concentrations of epicatechin, catechin, *p*-coumaric acid, *t*-ferulic acid, salicylic acid in bambara groundnuts were determined by the HPLC-PDA method and results are presented in Table 3-9. Among the phenolic compounds investigated, catechin was observed in higher concentrations than the others. There was a significant difference in catechin concentrations in the two bambara groundnut market classes. The concentrations of catechin and epicatechin were found to be higher in the brown than in the red market class. The red market class however had higher phenolic acids (*p*-coumaric, *t*-ferulic and salicylic acids) than the brown one. Salicylic acid had the highest concentration among the phenolic acids investigated in both bambara groundnuts market classes. The amount of phenolic acids t can be attributed to various factors such as the variety, growing and storage conditions (Hakkinen and Torronen, 2000; Ninfali and Bacchiocca, 2003). Generally the concentrations of phenolic compounds in foods is influenced by genotype, agronomical practices (irrigation, fertilization, pest management), maturity at harvest, post-harvest storage and climatic conditions (Hakkinen and Torronen, 2000; Ninfali and Bacchiocca, 2003).

Table 3-9 Individual phenolic compound concentration (mg/ kg DW) in bambara groundnuts

Phenolic compound concentration (mg / kg)	Market classes of bambara groundnuts	
	Red	**Brown**
Epicatechin	17.30 ± 0.26	26.32 ± 0.03
Catechin	266.41 ± 1.12	337.10 ± 8.7
p-coumaric acid	5.32 ± 0.22	4.29 ± 0.13
t-ferulic acid	3.29 ± 0.13	2.79 ± 0.39
Salicylic acid	33.57 ± 1.72	27.61 ± 0.48

3.1.3. Antioxidant activity of bambara groundnuts

Antioxidant properties of the brown and the red Zambian market classes of bambara groundnuts based on the free radical scavenging activity and ferric reducing power are reported here.

3.1.3.1. Free radical scavenging activity of bambara groundnut antioxidants

The free radical scavenging ability of the extracts is reported on the basis the kinetics of the DPPH radical-bambara extracts reaction and the effective concentration of the extract required to scavenge 50% of the DPPH radicals.

Kinetics of the DPPH free radical reaction with antioxidants in the bambara groundnuts

Figure 3-4 presents the disappearance pattern of DPPH free radicals with time in the presence of the aqueous and methanol extracts within a time period of 80 minute. The free radical scavenging pattern is biphasic, characterised by the fast initial decay, followed by the subsquent slower step in which degradation of by products may be involved. According to Villaño *et al.,* (2007), the fast initial decay is attributed to reactions (i) and (ii)

$$DPPH + (Y\text{-}H) \rightarrow DPPH\text{-}H + Y \qquad (i)$$

$$Y + DPPH \rightarrow products \qquad (ii),$$

while the subsequent decay is attributed to secondary slow reactions from the products of dimerization (or disproportionation) of A or from the products of reaction (ii). From the decrease in absorbance versus time in the first few seconds of the reaction, information about the pseudo first-order rate constant for reaction (i) can be acquired (Villaño *et al.,* 2007). Employing equations (1) and (2) in section 2.2.4.1 (chapter 2), and the plot of InA versus time, the pseudo first-order rate constant K of the aqueous and methanol extracts of bambara groundnuts were obtained. The pseudo first-order rate constants (K) for the bambara groundnuts extracts in a DPPH reaction are presented in Table 3-10. The K values for the brown bambara groundnuts were higher than the red one in both the aqueous and methanol extracts. The results would indicate that the antioxidants from the brown market class have faster reaction kinetics than the antioxidants from the red one. The slower kinetics for the red bambara groundnuts can also be observed by looking at the amount of DPPH scavenged after 80 minutes incubation time (Table 3-10). By the end of 80 minutes, over half of the DPPH was scavenged by the brown bambara groundnuts antioxidants in both the aqueous and methanol extracts, whereas 39 and 43 % DPPH was scavenged by the red bambara antioxidants in the aqueous and methanol extracts respectively. Trolox showed a very fast

initial decay with the higest K value 1.55 (min^{-1}) and the amount of DPPH scavenged (85.5%). The free radical scavenging ability of bambara groundnuts antioxidants was found to be moderate as compared to Trolox. This, however, may not be very surprising because Trolox is a pure compound and is considered to be a powerful antioxidant (Madhavi *et al.*, 1996). These observations have positive implications on the potential of bambara groundnuts as a dietary source of antioxidants.

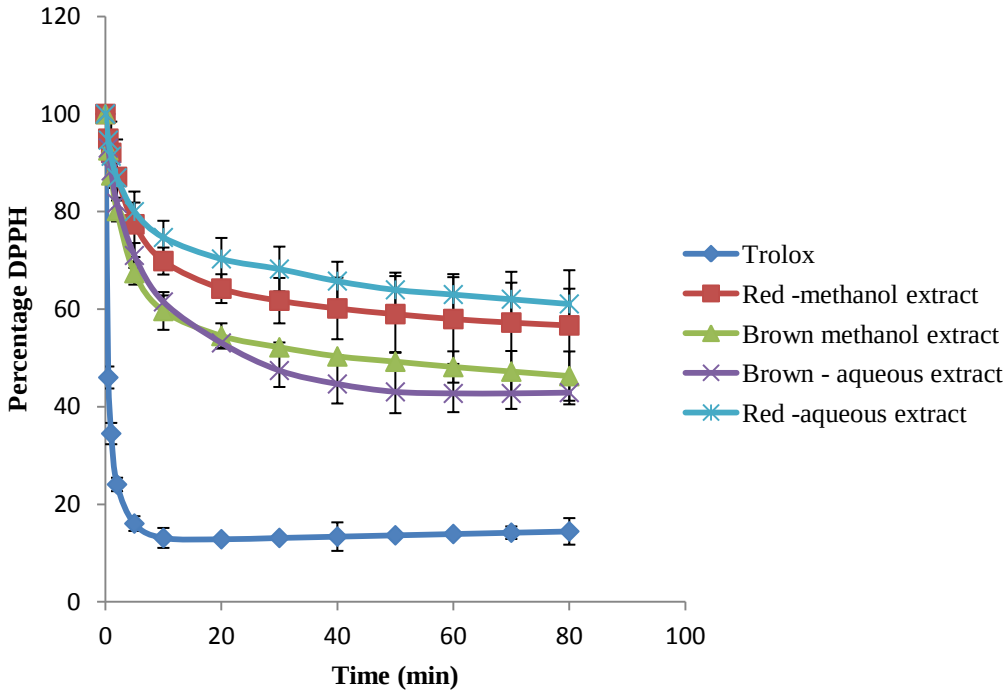


Figure 3-4 Disappearance pattern of DPPH free radicals with time in the presence of the aqueous and methanol extracts of bambara groundnuts

Table 3-10 Pseudo-first order rate constant of antiradical (Y-H) in bambara groundnuts extracts and the amount of DPPH scavenged after 80 minutes of incubation

	Pseudo-first order rate constant (K) [min^{-1}]		Amount $DPPH^{\bullet}$ quenched [%] after 80 minutes incubation	
Market class	Aqueous extract	70% Methanol extract	Aqueous extract	70% Methanol extract
Brown	0.056	0.053	57.1 ± 3.1	53.7 ± 2.6
Red	0.034	0.041	38.9 ± 1.8	43.3 ± 4.6

Effective concentration (EC_{50})

The EC_{50} values were obtained from the full dose-response curves fitted with the regression line (see example in Figure 3-5). The EC_{50} is inversely related to the antioxidant capacity of the compound. The lower the EC_{50}, the higher the antioxidant activity of the compound is (Villaño *et al.,* 2007). The EC_{50} of bambara groundnuts extracts and Trolox, which was used as a positive reference standard, are presented in Table 3-11. Generally, in comparison with the positive reference standard, all extracts displayed moderate scavenging activity. Among the extracts, the aqueous extract from the brown bambara groundnuts was the most efficient scavenger with the lowest EC_{50} value (347 µg dried extract / ml). The lowest scavenging effect was displayed by the methanol extract of the red bambara groundnuts (EC_{50} = 525.5 µg dried extract / ml). Both the aqueous and the methanol extracts of the brown bambara groundnuts showed higher scavenging activity than both extracts from the red one.

Zia-Ul-Haq *et al.,* (2012) studied the DPPH free radical scavenging ability of five legumes that included chickpea, lentil, mung beans, mash beans and peas extracted in aqueous methanolic mixture (80:20 v/v). The following EC_{50} values were reported: Desi chickpea (367.2 µg dried extract / ml), Kabuli chickpea (432.1 µg dried extract / ml), lentil (465.5 µg dried extract / ml), mung beans (389.2 µg dried extract / ml), mash beans (401.4 µg dried extract / ml) and peas (457.1 µg dried extract / ml) respectively. The DPPH free radical scavenging of the four legumes was found to be moderate compared to ascorbic acid (192 µg / ml) used as a positive reference standard. The results on bambara groundnuts in the present study are somewhat comparable to these findings. Spanou *et al.,* (2007) investigated the DPPH radical scavenging capacity of the *Lupinus albus*, *Lens culinaris* and *Phaseolus*

vulgaris seeds and reported the EC_{50} values ranging from 2300 to 7600 µg dried extract / ml. Comparing these findings to the present study, bambara groundnuts with EC_{50} ranging from 347 to 525 µg dried extract / ml can be said to be more potent scavengers than the leguminous seeds studied by Spanou *et al.*, (2007). The DPPH free radical scavenging of bambara groundnuts indicates that they contain compounds capable of scavenging free radicals.

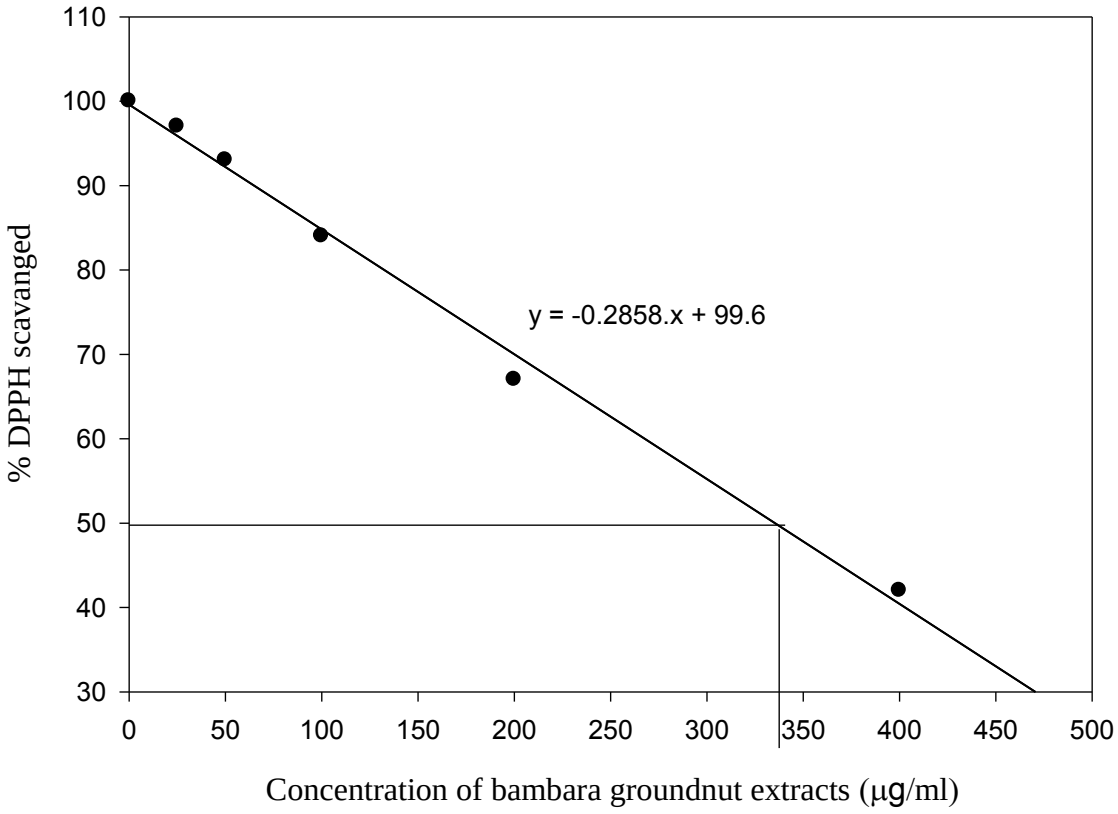


Figure 3-5 Dose-response plot [concentration of aqueous extract of brown bambara groundnuts and DPPH scavenged (%)] fitted with linear regression line

Note: y = a*x + b, and y = 0.5,
EC_{50} = (0.5 - b)/a = (0.5 - 99.6)/-0.2858 = 347.0

Table 3-11 EC_{50} values for DPPH free radical scavenging by bambara groundnut extracts after 30 minutes of incubation

Market classes of bambara groundnuts	DPPH radical scavenging EC_{50} (µg dried extract / ml)	
	70 % Methanol extract	Aqueous Extract
Brown	477.5 ± 3.5	347.0 ± 4.2
Red	525.5 ± 7.8	495.5 ± 12.0

Trolox EC_{50} = 21.0 ± 1.4

3.1.3.2. Ferric Reducing Antioxidant Power of bambara groundnuts

The reducing power of the antioxidants in the bambara groundnuts was assessed using the FRAP assay. This assay has been used frequently in the assessement of various foods and biological samples. The FRAP assay treats the antioxidants contained in the sample as reductants in a redox-linked colorimetric reaction and the value reflects the reducing power of the antioxidants (Hajimahmoodi *et al.,* 2008). The FRAP values of the aqueous and methanol extracts of the brown and the red market classes of bambara groundnuts are presented in Table 3-12. As can be seen, the methanol extract of both market classes had higher FRAP values than the aqueous extract. The results showed that the brown groundnuts had higher ferric reducing ability than the red bambara groundnuts and the difference was significant ($p < 0.05$). Xu *et al.,* (2007) evaluated the antioxidant reducing power of different market classes of yellow peas, green peas, chickpea, lentils and common beans grown in North Dakota, Idaho and Washington regions of the United States of America, and their FRAP values are presented in Table 3-13. In comparison to the present study, bambara groundnuts can be considered to have stronger antioxidant reducing power than peas, but comparable to ranges reported for lentils and common beans.

Table 3-12 Ferric Reducing Antioxidant Power (FRAP) values for bambara groundnuts

Market classes of bambara groundnuts	FRAP value (mmole Fe^{2+} / 100 g DW)	
	70 % Methanol extract	Aqueous Extract
Brown	9.70 ± 0.07	5.65 ± 0.21
Red	8.01 ± 0.13	5.00 ± 0.13

Table 3-13 Ferric Reducing Antioxidant Power (FRAP) values for other legumes

Legume type	Range of FRAP value (mmole Fe^{2+} / 100 g DW)
Yellow peas	0.62 – 0.82
Green peas	0.43 – 0.86
Lentils	8.75 – 13.92
Common beans	1.27 – 9.70

Source: Xu *et al.,* (2007)

Discussion

Both the red and brown bambara groundnuts had high concentrations of total polyphenols that were within the range (117 – 440 mg GAE / 100g DW) reported by Heimler *et al.,* (2005) for other legumes. However, total polyphenol content for the brown bambara groundnuts was significantly higher ($p < 0.05$) compared to that observed in the red market class. Tannin concentration also followed the similar trend. The total flavonoid contents in the aqueous extract of both market classes were not significantly different ($p > 0.05$). Differences in the total polyphenol and tannin concentration may be attributed to the varietal effect. The metabolism of secondary metabolites in plants is affected by various factors that are complex and each market class respond differently to these factors. Solvent interaction with the food matrix during the extraction of polyphenolic compounds also has some influence.

Where authentic standards were available individual phenolic compounds were investigated. Catechin was observed in higher concentrations than the others in both bambara groundnuts market classes. The difference in catechin concentrations of the two market classes of bambara groundnuts was significant. Catechin and epicatechin concentrations were found to be higher in the brown compared to the red market class. Salicylic, *t*-ferulic, and *p*-coumaric acids were higher in the red compared to the brown market class. Concentrations of salicylic acid were highest among the phenolic acids investigated in both bambara groundnut market classes. Difference in the concentrations of individual phenolic compounds can be attributed to factors such as the variety, growing and storage conditions (Hakkinen and Torronen, 2000; Ninfali and Bacchiocca, 2003).

The identification of phenolic compounds by HPLC-PDA-ESI-MS revealed various compounds in bambara groundnuts. This is the first time that identification of phenolic compounds using HPLC-PDA-ESI-MS is being reported for bambara groundnuts. In both the red and brown bambara groundnuts, the following phenolic compounds were tentatively identified: Quinic acid, (E) GC-hexoside, catechin glucoside, catechin, epicatechin, medioresinol, *p*-coumaric acid, salicylic acid, caffeic acid derivative and catechin dimer. The red bambara groundnuts revealed the following phenolic compounds that were absent in the brown: myricetin hexoside, quercetin-3-O-rutinoside and quercetin-3-O-glucoside. Myricetin hexoside, quercetin-3-O-rutinoside and quercetin-3-O-glucoside have been reported in other legumes by Lin *et al.,* (2008).

The majority of the phenolic compounds identified in bambara groundnuts were flavonoids. Various health benefits have been described previously for flavonoids. An inverse correlation between flavonoid intake and total plasma cholesterol concentrations has been shown (Patel 2008). Flavonoid intake has also been reported to have a protective effect against coronary heart disease (Hertog *et al.,* 1995; Middleton *et al.,* 2000). Cocoa beans have historically been used as a treatment for diarrhoea (Schuier *et al.,* 2005). The nature of the active ingredient, nor the exact mechanism of action was not known but the recent research attributes the antidiarrhoea effect to the flavonoids present in cocoa (Schuier *et al.,* 2005). Similarly, bambara groundnuts have been reported to be used as an ingredient in the preparation of traditional medicine for diarrhoea among the Luo tribal grouping in Kenya (Ngugi 1995). Flavonoids like quercetin have been shown to have anti-inflammatory properties and do so by inhibiting the cyclooxygenase pathway (Nijveldt *et al.,* 2001).

Quercetin has also been shown to inhibit the growth of *helicobacter pylori* bacteria in *in-vitro* studies (Middleton *et al.,* 2000). Methyl-3-(+)-catechin interferes with the formation of histamine in gastric mucosa and hence produces the protective effect (Farkas *et al.,* 1981). Most flavonoids have anti-viral effects against *Herpes* simplex virus, respiratory syncytial virus, parainfluenza virus, and adenovirus (Nijveldt *et al.,* 2001). Flavonoids have also been shown to have free radical scavenging properties (Pietta 2000). In a summary, one may speculate that bambara groundnuts may be useful as nutraceuticals due to various phenolic compounds from the flavonoids class that they contain.

The market classes of bambara groundnuts in this study also demonstrated antioxidant activities comparable to the report of Zia-Ul-Haq *et al.,* (2012) on other legumes. The brown market class had higher antioxidant activities compared to the red. This may be due to the differences in the concentrations, type, hydrogen or electron donating capacities of the polyphenolic compounds present. The antioxidant activities of phenolic compounds are closely related to their structures (Mat Ali, 2008). It seems reasonable to assume that the contribution of catechin, epicatechin, catechin glucoside and other flavonoids to the total antioxidant activity of the Zambian market classes of bambara groundnuts studied was substantial. According to Jovanovic *et al.,* (1994), flavonoids are thermodynamically able to reduce most oxidizing free radicals relevant to the biological systems due to their low redox potentials ($0.2 < E < 0.8$). Han *et al.,* (2012) pointed out three structural requirements that seem important to the efficiency of flavonoids as antioxidants:

(i) The *ortho*-dihydroxy (catechol) structure in the B-ring, increasing the stability of oxidized flavonoid radicals through H – bonding or electron delocalization;

(ii) The 2,3 – double bond, in conjugation with 4-oxo function, enhancing electron transfer and radical scavenging through electron delocalization;

(iii) The presence of both 3- and 5-OH groups, enabling the formation of stable quinonic structures upon flavonoid oxidation.

According to Han *et al*., (2012), a typical flavonoid which meets the above three structural criteria is quercetin and has the highest antioxidant activity. This flavonoid was idenfied in red bambara groundnuts in the form of quercetin glucoside and quercetin rutinoside but was absent in the brown variety. Despite red bambara groundnuts having quercetin, it recorded lower antioxidant activity than the brown variety possibly due to the interfearance by the sugar moiety. Any sugar substituent is capable of diminishing complarity of the B-ring relative to the rest of the flavonoid by exerting steric effects through blocking of the B-ring

catechol (Helm *et al*., 2002). The catechol structure in the B-ring is a salient feature of most potent scavengers of peroxyl, superoxide and peroxynitrite radicals (Dagas *et al*., 2000; Haenen *et al*., 1997; Hu *et al*., 1995). Peroxynitrite scavenging by catechin is mainly attributed to its B-ring catechol (Kerry and Rice-Evance, 1999). Catechin was detected in both market classes of bambara groundnuts. Besides structural requirements, the number and position of hydroxyl substituents on the flavonoid molecule, the presence of glucosides, and overall degree of conjugation are important in determining their activities (Han *et al*., 2012). Glucosides in the form of catechin glucoside and quercetin glucoside were identified in bambara groundnuts. Phenolic acids, most likely, also contributed greatly to the total antioxidant capacity of bambara groundnuts. Salicylic acid, *t*- ferulic acid, *p*-coumaric acid, quinic acid and caffeic acid were the hydroxycinnamic acids identified in bambara groundnuts while their hydroxybenzoic acid counterparts were absent. Hydroxycinnamic acids are more effective as antioxidants than hydroxybenzoic acid possibly due to the aryloxy-radical stabilizing effect of the – CH=CH – COOH linked to the phenyl ring (Rice-Evance *et al*., 1996).

Antioxidant activities demonstrated by bambara groundnuts suggest that it could be very useful as a source of antioxidants besides the commonly consumed legumes. This in itself presents an opportunity for diversification of food resources considering that bambara groundnuts are currently very much under utilized.

Conclusion

The study has shown that bambara groundnuts have antioxidant activities that are comparable to commonly consumed legumes such as lentils, common beans and chickpeas. The nuts contain various polyphenolic compounds, mainly from the class of flavonoids that have been reported to have a variety of medicinal properties. The brown market class variety has higher antioxidant activities and phenolic contents compared to the red variety. These findings have demonstrated that bambara groundnuts have the potential for use in the nutraceutical industry. Based on this, it is suggested that consumption of bambara groundnuts could possibly offer some health benefits since they contain phytochemical constituents that have been reported to possess protective functions. Consumption of bambara groundnuts can be as good as other commonly consumed legumes and this is an opportunity for dietary and crop diversification on the part of the consumers and farmers respectively.

3.2. Section B: Common beans

Results

3.2.1. Nutritional and physicochemical properties of the seeds

Physicochemical characteristics that include hydration capacity, hydration index, swelling capacity and swelling index of the four market classes of common beans commonly grown in Zambia are presented in Table 3-14. Hydration capacity (g / seed) ranged from 0.285 to 0.416 among the different market classes. Brown beans had the minimum whereas white had the maximum hydration capacity, but the difference between the white and the red was not significant ($p > 0.05$). The hydration index, swelling capacity and the swelling index followed a similar trend as the hydration capacity among the market classes. The brown market class, having the lowest hydration capacity, hydration index, swelling capacity and swelling index would require longer cooking times than the rest. These parameters have been shown to be inversely related to the cooking time (Bishnoi and Khetarpaul, 1993; Wang *et al.*, 2003; Shimelis and Rakshit 2005).

Table 3-14 Physico-chemical characteristics of the Zambian market classes of common beans

Property	Market classes of Common beans			
	Red	**Grey mottled**	**Brown**	**White**
Hydration capacity (ml / seed)	0.407 ± 0.005	0.363 ± 0.006	0.285 ± 0.024	0.416 ± 0.001
Hydration index	0.503 ± 0.028	0.360 ± 0.016	0.255 ± 0.046	0.594 ± 0.009
Swelling capacity (ml / seed)	0.425 ± 0.049	0.365 ± 0.042	0.380 ± 0.049	0.455 ± 0.014
Swelling index	0.283 ± 0.032	0.197 ± 0.030	0.185 ± 0.018	0.227 ± 0.007

Table 3-15 presents the proximate composition of the four market classes of common beans. The proximate composition concentrations varied from 10.09 to 11.47 g / 100g DW (moisture), from 16.78 to 20.47 g / 100 g DW (crude protein), from 4.73 to 5.15 g / 100 g DW (ash), from 1.34 to 3.04 g / 100 g DW (fat), from 0.91 to 6.57 g / 100 g DW (fibre) and from 54.89 g / 100 g DW (carbohydrate) respectively. The crude protein concentrations of red, brown and white market classes were within the range (17 – 22 g / 100 g DW) previously reported by Shimelis and Rakshit (2005) for eight different market classes of common bean grown in Ethiopia, whereas the grey mottled was below this range. For all the market classes, ash content was higher than what has been previously reported (Samma *et al.*, 1999; Shimelis and Rakshit 2005). Moisture and fat contents for all the market classes were comparable with some reports in the literature (Samma *et al.*, 1999). The concentrations of carbohydrate observed for red, grey mottled and brown were lower than (66.39 to 76.79 g / 100 g DW) reported in common bean market classes grown in Burundi (Barampama and Simard, 1993). Most likely, the differences in the concentration of carbohydrate may be due to the varietal effect.

Table 3-15 Proximate composition (g / 100g DW) of the whole seed sample

	Market classes of Common beans			
Property	**Red**	**Grey mottled**	**Brown**	**White**
Moisture	10.84 ± 0.23	11.42 ± 0.55	11.47 ± 0.11	10.09 ± 1.05
Crude protein	17.57 ± 0.18	16.78 ± 0.12	20.47 ± 0.04	18.29 ± 1.35
Total ash	4.73 ± 0.67	4.74 ± 0.79	5.13 ± 0.74	5.15 ± 0.42
Fat	3.04 ± 0.93	1.66 ± 0.06	1.46 ± 0.06	1.34 ± 0.03
Crude fibre	5.93 ± 0.09	4.92 ± 0.21	6.57 ± 0.28	0.91 ± 0.01
Carbohydrates	57.88± 0.76	60.48 ± 1.38	54.89 ± 1.14	67.57 ± 2.29

Table 3-16 presents the mineral composition (mg / kg DW) of the four market classes of common beans. Iron varied from 60.85 to 72.42 mg / kg DW and was comparable to the concentrations given by many researchers (Meiners *et al.*, 1976; Barampama and Simard, 1993; Shimelis and Rakshit 2005). Potassium varied depending on the market class and was found to be the most abundant mineral, followed by phosphorous, magnesium and calcium respectively. Zinc concentration varied from 22.35 to 35.49 mg / kg DW. These values are

similar or slightly greater than concentrations reported by Shimelis and Rakshit (2005). Zinc, an important trace element for growth, development and reproduction, can be cheaply obtained from common beans. The concentration of calcium was similar for red and grey mottled beans, whereas the white beans recorded the highest value. Lead, mercury and arsenic were not detected in any of the market classes. It is generally accepted that mineral content of agricultural products vary with the environment in which the crop is grown (Amarteifio *et al.*, 2006).

Table 3-16 Mineral composition (mg / kg DW) of seed sample

Property	Market classes of Common beans			
	Red	**Grey mottled**	**Brown**	**White**
Calcium	1479.6 ± 16.9	1486.2 ± 17.0	1672.5 ± 19.1	1961.9 ± 22.4
Magnesium	1869.8 ± 19.0	2213.3 ± 22.5	2121.3 ± 21.6	2096.3 ± 21.4
Potassium	15157.7 ± 98.3	16647.1 ± 108.0	17852.1 ± 115.5	16790.5 ± 108.9
Phosphorous	4884.7 ± 36.6	4723.8 ± 35.2	5344.8 ± 40	4782.4 ± 35.8
Sodium	46.8 ± 3.52	16.4 ± 1.2	108.9 ± 8.2	14.7± 1.1
Iron	72.21 ± 2.27	60.85 ± 1.9	75.88 ± 2.3	72.42 ± 2.3
Zinc	29.58 ± 1.5	22.35 ± 1.1	22.57 ± 1.14	35.49 ± 1.8
Copper	5.07 ± 0.1	5.58 ± 0.2	6.88 ± 0.19	8.35 ± 0.2
Manganese	21.56 ± 1.1	15.69 ± 0.8	13.47 ± 0.67	21.66 ± 1.1
Mercury	ND	ND	ND	ND
Lead	ND	ND	ND	ND
Arsenic	ND	ND	ND	ND

ND = Not detected

3.2.2. Phenolic phytochemical profiles of the Zambian market classes of common beans

The HPLC-PDA-ESI-MS based identification of phenolic compounds in the Zambian market classes of common beans is reported below. Characterisation of market classes based on the tannin concentrations, total polyphenols and flavonoids determined using non chromatographic approaches is also reported.

3.2.2.1. Water and methanol extractables

The yields obtained in the extraction of the four market classes of common beans using water and 70% methanol solvent systems are presented in Table 3-17. Higher extraction yields were obtained with water compared to the 70% methanol solvent system. The brown and the white market classes gave slightly similar yields for the aqueous extracts but were significantly different from the red and grey mottled. The red market class gave the highest yield for the 70% methanol solvent system, whereas the white gave the lowest. The solvent effect for the 70% methanol system was significant on the extraction yields ($p > 0.05$). The differences in the yields of extractable material are expected because the seed properties such as the hydration capacity, hydration index, swelling capacity and swelling index are different. Each seed material interacted differently with the solvent system.

Table 3-17 Yields of crude extracts of common beans in ultrasonic- assisted extraction

Market classes of common beans	Yield (mg / g DW)	
	70 % Methanol extract	Aqueous extract
Red	73.7 ± 14.1	121.4 ± 7.7
Grey mottled	51.4 ± 2.8	132.2 ± 4.9
Brown	48.0 ± 4.2	154.8 ± 0.7
White	41.5 ± 2.1	152.6 ± 1.4

3.2.2.2. Total polyphenol and tannins

Table 3-18 presents the results of the total polyphenol and tannins concentrations of the four market classes of common beans. Total polyphenol concentrations for the four market classes in both extracts were significantly different ($p > 0.05$). The red market class had the highest

total polyphenol contents in the aqueous and 70% methanol extracts, followed by the grey mottled, brown and last, the white. The red market class showed stronger absorption than the rest at 280 nm when the aqueous was scanned spectrophotometrically between 200 and 400 nm (see Figure 3-6). The results of the present study are close to the range (64 – 95 mg GAE / 100 g DW) reported by Deshpande *et al.,* (1987) and less than the range (117 to 427 mg GAE / 100 g DW) reported by Heimler *et al.,* (2005) for the twelve different market classes of common beans from Italy. Wu *et al.,* (2006) reported values from 223 to 1247 mg GAE / 100 g DW of total polyphenols for various common beans cultivars, which is very different from what has been observed in this research. In polyphenol research, several authors have reported different findings on the total polyphenol concentration of common beans. This may be attributed, in part, to the fact that the samples are from diverse growing areas with different environmental conditions, and furthermore, the methods and the solvents used in the extraction of polyphenol compounds differ. The varietal effect may also play a significant role. Previous authors have reported that due to the lack of standardization of the analytical methods, concentrations for polyphenols in a given food are often not easily comparable (Merken and Beecher, 2000; Santos-Buelga and Williamson 2003; Harnly *et al.,* 2010; Amarowicz *et al.,* 2009.

The results for tannin concentration ranged from 10.2 to 55.4 mg GAE / 100 g DW for the aqueous and from 3.1 to 53 mg GAE / 100 g DW for the 70% methanol extract. The grey mottled and white beans consistently demonstrated the highest and the lowest tannin concentration respectively.

Table 3-18 Total polyphenol contents and tannin concentrations of common beans

Market classes of common beans		Total polyphenols (mg GAE / 100 g DW)	Tannins (mg GAE / 100 g DW)
Aqueous			
	Brown	61.6 ± 1.1	24.7 ± 0.7
	Red	105.4 ± 0.5	51.3 ± 2.0
	Grey mottled	90.3 ± 3.8	55.4 ± 1.3
	White	45.2 ± 1.4	10.2 ± 0.2
70% methanol			
	Brown	60.2 ± 3.8	19.6 ± 1.7
	Red	123.7 ± 4.3	49.4 ± 2.7
	Grey mottled	85.4 ± 2.9	53.1 ± 1.6
	White	37.3 ± 2.6	3.1 ± 0.2

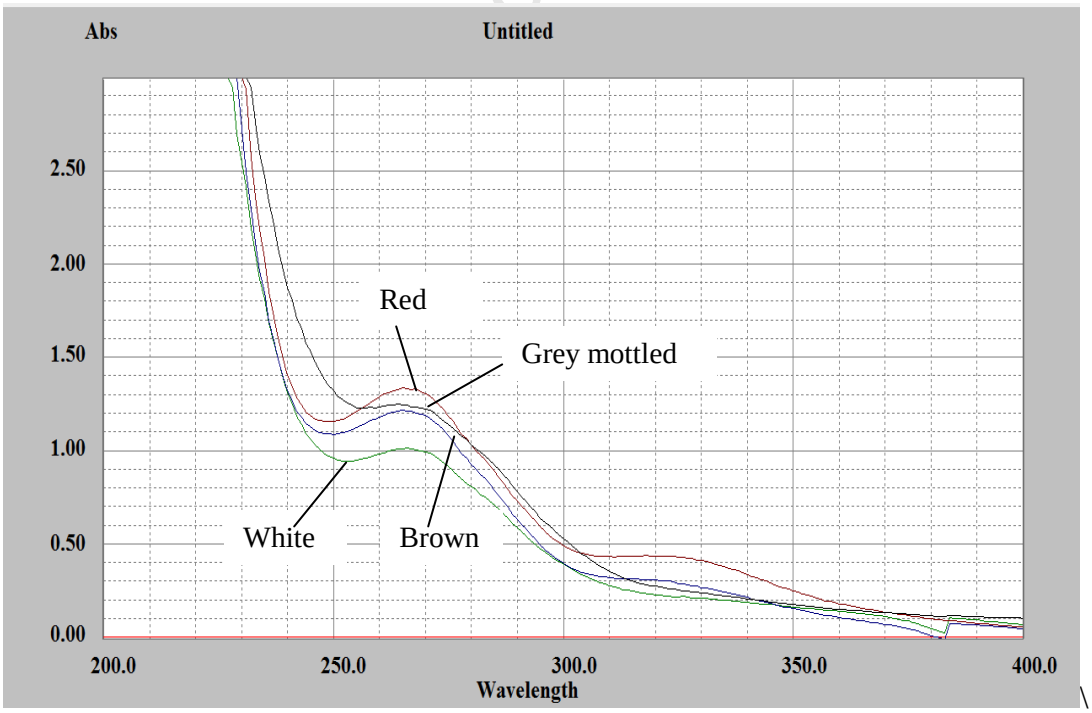


Figure 3-6 Results of the spectrophotometric scanning at 200 nm to 400 nm for the aqueous extract of common beans.

3.2.2.3. Total flavonoid concentration

Flavonoids are pigments responsible for seed coat colour in common beans (Beninger *et al.*, 1998). The flavonoid concentrations of the four market classes of common beans grown in Zambia are shown in Table 3-19. The concentrations of flavonoids in the aqueous extract were generally lower than in the 70% methanol extract. Flavonoid concentration ranged from 42.1 to 62.6 mg quercetin equivalents / 100 g DW (aqueous extraction) and 95.2 to 123.5 mg quercetin equivalents / 100 g DW (70% methanol extraction). The concentrations obtained in the present study are in agreement and slightly greater than the range (19 – 84 mg quercetin equivalents / 100 g DW) obtained by Dinelli *et al.*, (2006) for twenty three Italian varieties of common beans. Mishra *et al.*, (2012) reported a total quercetin ranging from 5 to 41 mg / 100g DW for twenty landraces of common beans grown in different regions of Uttarakhand, which is lower than in our study. As mentioned earlier, concentration of phytochemicals may vary depending on a number of factors.

Table 3-19 Flavonoid content of common beans

Market classes of common beans		Flavonoids content expressed as quercetin equivalents (mg / 100 g dry mass)
Aqueous		
	Brown	56.3 ± 2.0
	Red	42.1 ± 1.2
	Grey mottled	55.8 ± 0.9
	White	62.6 ± 3.1
70% methanol		
	Brown	101.4 ± 2.8
	Red	123.5 ± 1.9
	Grey mottled	95.2 ± 1.1
	White	117.7 ± 2.2

3.2.2.4. Phenolic compounds identified by HPLC-PDA-ESI-MS in red beans

Thirteen phenolic compounds were identified by HPLC-PDA-ESI-MS in red beans. The results of the identified compounds are presented in Table 3-20, and the numbered peaks in the chromatogram (Figure 3-7). A compound with a negatively charged $[M - H]^-$ ion at m/z 191 (peak 1), with a fragment ion at m/z 127 was was identified as quinic acid. Peak 2 was identified as gallic acid with $[M - H]^-$ at m/z 169 that fragmented to yield a MS/MS spectrum at m/z 125, an indicator of trihydroxy phenol moiety (Mammela *et al.*, 2000). Furthermore, co-chromatography of the authentic standard verified the identification of this peak as gallic acid.

Peaks 3, 6, 7 and 8 were identified as isomers of ferulic acid derivatives. These compounds were eluted at different retention times but showed similar depronated molecules $[M - H]^-$ at m/z 385 that fragmented to yield MS/MS spectra with ions at m/z 193, indicative of ferulic acid fragment. Four isomers of ferulic acid derivatives have been reported previously in common beans by Lin *et al.*, (2008).

Peak 4 showed a deprotonated molecule $[M - H]^-$ at m/z 259 and its MS/MS fragmentation yielded fragment ions at m/z (241, 223, 197), indicating the presence of a syringic acid fragment (a phenolic acid with a depronated molecule at m/z 197, and with a molecular weight of 198). The fragmentation pattern is characterised by the loss of two water moieties (18 amu, each subsequently) and a hydrocarbon moiety (26 amu), which probably could be the acetylene (C_2H_2). This compound was tentatively identified as a syringic acid derivative.

Peaks 5 and 9 were isomers that eluted at 6.08 and 8.12 minutes respectively. The two compounds showed similar deprotonated molecules $[M - H]^-$ at m/z 289 that fragmented to yield MS/MS spectras with ions at 245. Co-chromatography with authentic standards verified peak 5 as catechin and peak 9 as epicatechin. Peak 10 revealed a negatively charged $[M - H]^-$ ion at m/z 387 with MS/MS fragmentation at m/z 207. The compound was identified as medioresinol, a phenolic lignin. Hassain *et al.*, (2010) reported a compound in lamiaceae with similar fragmentation pattern that was tetantively identified as medioresinol.

Peaks 11 and 12 revealed negatively charged ions $[M- H]^-$ at m/z 163 and 193, and HPLC retention times at 8.92 and 11.1 minutes respectively. Co-chromatography with authentic

standards verified the identification of peak 11 as *p*-coumaric acid and peak 12 as *t*-ferulic acid. These compounds have been reported in common beans by Lin *et al.*, (2008)

Peak 13 showed a deprotonated molecule $[M - H]^-$ ion at m/z 567 and its MS/MS fragmentation yielded a fragment ion at m/z 341 with absorption maxima at 217 and 287 nm, suggesting the presence of a flavanone skeleton as proposed by Portet *et al.*, (2008). The fragmentation observed for this depronated molecule has been previously described in the literature for a flavanone derivative (Gouveia and Castilho 2011).

Table 3-20 HPLC-PDA-ESI-MS -based identification of phenolics in red beans

Peak	t_R(min)	Molecular weight	[M-H]- (m/z)	[M-H]- fragments (m/z)	Tentative identification
1	1.36	192	191	127	Quinic acid
2	1.99	170	169	125	Gallic acid
3	4.95	386	385	193	Ferulic acid derivatives
4	5.57	260	259	241,233,197	Syringic acid derivative
5	6.08	290	289	245	Catechin
6	6.45	386	385	193	Ferulic acid derivates
7	7.42	386	385	193	Ferulic acid derivatives
8	8.04	386	385	193	Ferulic acid derivatives
9	8.12	290	289	245	Epicatechin
10	8.62	388	387	207	Medioresinol
11	8.92	164	163	119	*p*-coumaric acid
12	11.1	194	193	149, 134	*t*-ferulic acid
13	19.38	568	567	342, 341, 330	Flavanone derivative

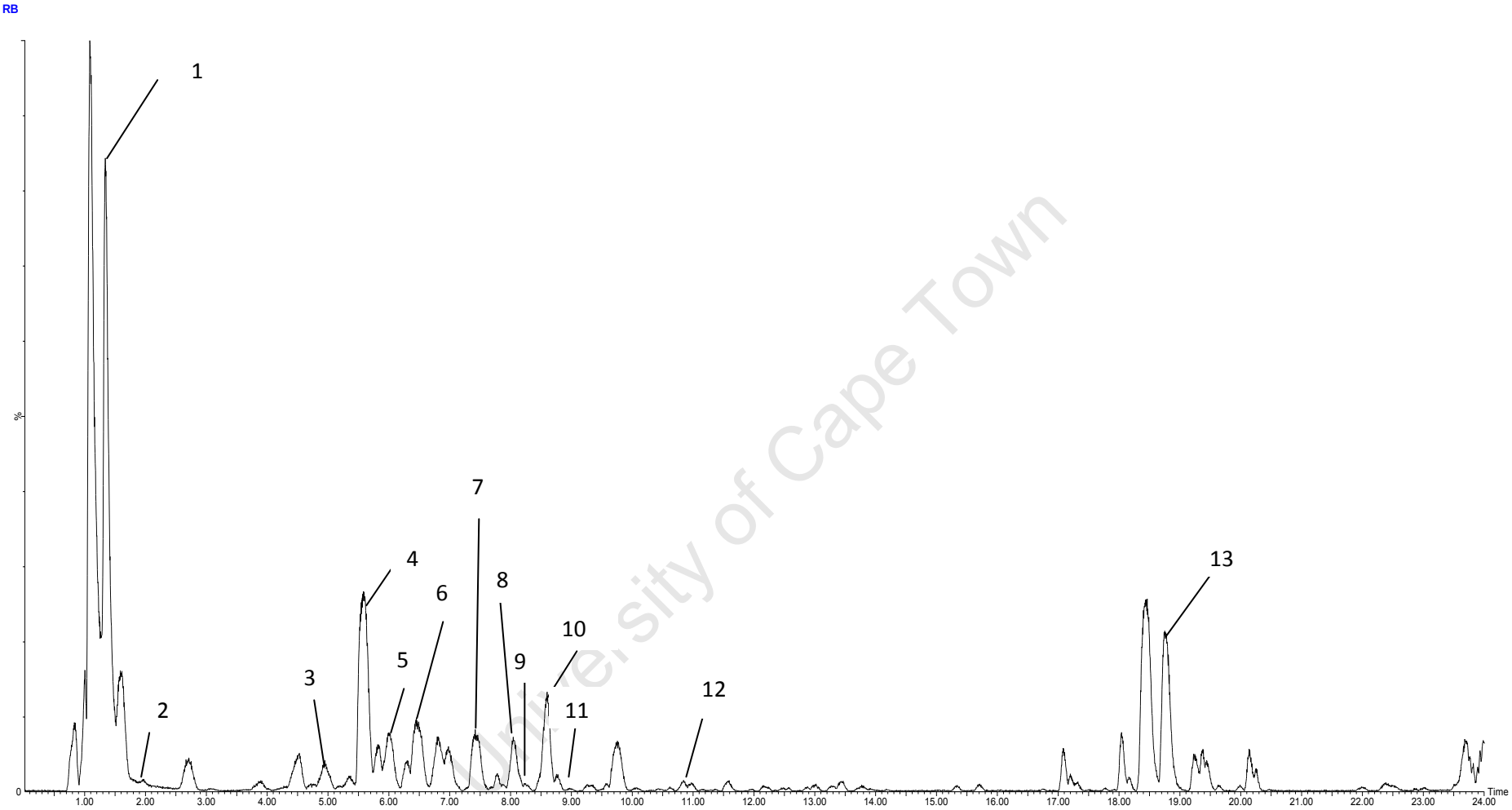


Figure 3-7 HPLC-PDA-ESI-MS chromatogram of 70% methanol extract of red beans.

3.2.2.5. Phenolic compounds identified by HPLC-PDA-ESI-MS in grey mottled beans

The chromatogram of methanolic extract of grey mottled beans is presented in Figure 3-8. Eleven phenolic compounds were identified and are summarised in Table 3-21. The following phenolic compounds that were identified in the red beans were also found in the grey mottled one: quinic acid (peak 1), gallic acid (peak 2), a syringic acid derivative (peak 4), catechin (peak 5), ferulic acid derivatives (peak 6), epicatechin (peak 7), medioresinol (peak 8), *p*- coumaric acid (peak 9) and *t*-ferulic acid (peak 10). For the peaks in the brackets, refer to the chromatogram (Figure A4 in Appendix A). Only one ferulic acid derivative was reported in grey mottled beans compared to the four that were detected in red beans.

Peak 11 with the retention time of 12.37 minutes was identified as salicylic acid based on the co-chromatography with the authentic standard. Peak 3 showed a deprotonated molecule $[M - H]^-$ ion with m/z at 451 that fragmented to yield a fragment ion at m/z 289 indicating the presence of catechin residual and a loss of a hexose moiety (162 amu). This compound was tentatively identified as catechin glucoside and has been reported by Estrella *et al.*, (2011) previously in pinto beans.

A flavone derivative with $[M - H]^-$ on at m/z 567 that was observed in red beans was not detected in grey mottled beans. Similarly, catechin glucoside observed in grey mottled beans was not detected in red beans.

Table 3-21 HPLC-PDA-ESI-MS -based identification of phenolics in grey mottled beans

Peak	t_R(min)	Molecular weight	[M-H]- (m/z)	[M-H]- fragments (m/z)	Tentative identification
1	1.36	192	191	127	Quinic acid
2	1.98	170	169	125	Gallic acid
3	4.50	452	451	289	Catechin glucoside
4	5.57	260	259	241,233,197	Syringic acid derivative
5	6.08	290	289	245	Catechin
6	7.42	386	385	193	Ferulic acid derivates
7	8.12	290	289	245	Epicatechin
8	8.62	388	387	207	Medioresinol
9	8.92	164	163	119	*p*-coumaric acid
10	11.1	194	193	149, 134	*t*-ferulic acid
11	12.37	138	137		Salicylic acid

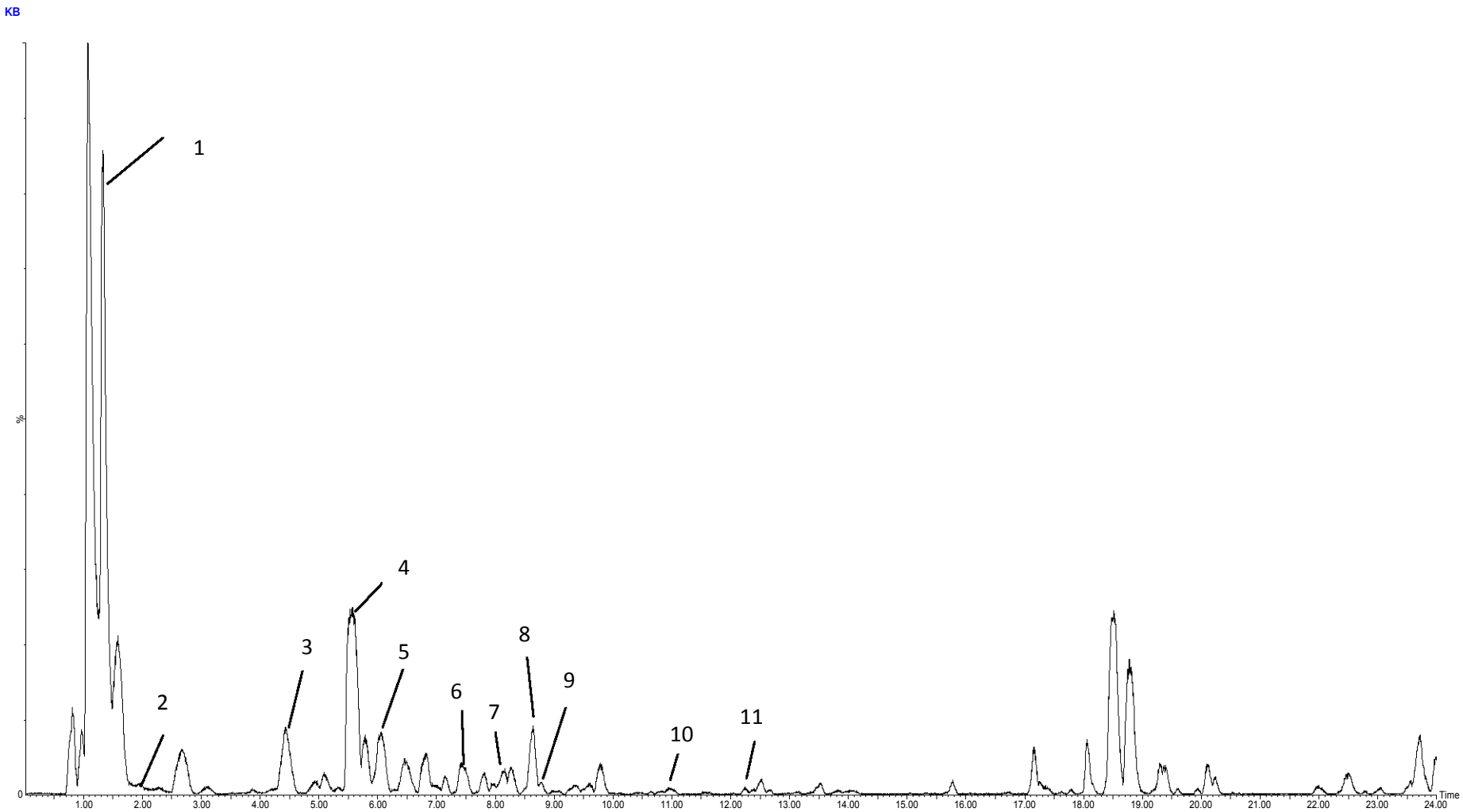


Figure 3-8 HPLC-PDA-ESI-MS chromatogram of 70% methanol extract of grey mottled beans.

3.2.2.6. Phenolic compounds identified by HPLC-PDA-ESI-MS in brown beans

Phenolic compounds identified by HPLC-PDA-ESI-MS in brown beans are presented in Table 3-22 and the numbered peaks in the chromatogram (Figure 3-9). Quinic acid, gallic acid, a syringic acid derivative, catechin, ferulic acid derivatives, medioresinol, *p*-coumaric acid and *t*-ferulic acid detected in both the red and the grey mottled beans were also observed in the brown beans. (For peaks numbers for these compounds refer to Table 3-22 and Figure A5 in Appendix A). Three isomers of ferulic acid derivaties were observed as opposed to one detected in grey mottled and four in red beans. Epicatechin, observed in both the red and grey mottled, was not detected in the brown beans. However, catechin glucoside and salicylic acid which were absent in the red beans but present in the grey mottled were detected in the brown beans. A flavanone derivative observed in red beans was not detected in brown beans.

Peak 14 revealed a negatively charged ion [M – H]- at m/z 447 with MS/MS fragment ion at m/z 285 indicating the presence of kaempferol fragment and a loss of hexose moiety (162 amu). Based on the similar fragmentation pattern reported previously for this ion by Kajdžanoska *et al.,* 2010, this compound was identified as kaempferol glucoside. The presence of kaempferol glucoside in common beans has been reported previously by Lin *et al.,* (2008).

Peak 15 had [M – H]- ion at m/z 329 with MS/MS fragmentation yielding a major fragment ion at m/z 285, indicating the loss of carbon dioxide (44 amu). The fragmentation pattern observed for this ion after MS/MS experiment has been previously described in literature for carnosol (Hassain *et al.,* 2010). Carnosol, a phenolic antioxidant has been reported by Herrero et al (2009; Hassain *et al.,* (2010) in rosemary extracts. To the authors' knowledge, this is the first time carnosol has been reported to be present in common beans.

Kaempferol glucoside and carnosol detected in this market class were not observed in the red and grey mottled beans.

Table 3-22 HPLC-PDA-ESI-MS -based identification of phenolics in brown beans

Peak	t_R(min)	Molecular weight	[M-H]- (m/z)	[M-H]- fragments (m/z)	Tentative identification
1	1.36	192	191	127	Quinic acid
2	1.98	170	169	125	Gallic acid
3	4.50	452	451	289	Catechin glucoside
4	5.57	260	259	241,233,197	Syringic acid derivative
5	6.08	290	289	245	Catechin
6	6.43	386	385	193	Ferulic acid derivates
7	7.42	386	385	193	Ferulic acid derivates
8	8.10	386	385	193	Ferulic acid derivates
9	8.62	388	387	207	Medioresinol
10	8.92	164	163	119	*p*-coumaric acid
11	9.79	388	387	207	Medioresinol
12	11.1	194	193	149, 134	*t*-ferulic acid
13	12.37	138	137		Salicylic acid
14	13.13	148	447	287	Kaempferol glucoside
15	23.55	330	329	285	Carnosol

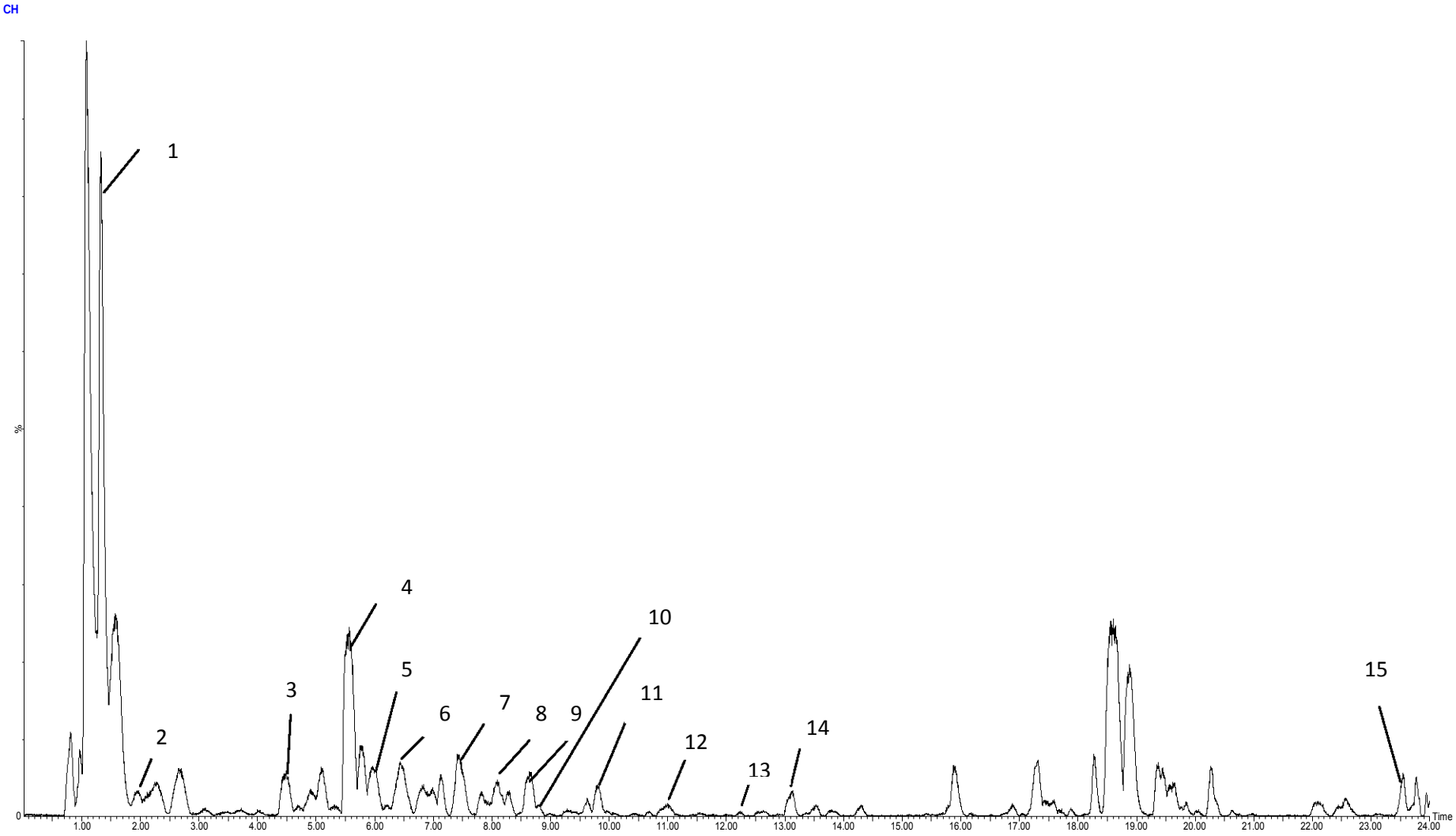


Figure 3-9 HPLC-PDA-ESI-MS chromatogram of 70% methanol extract of brown beans.

3.2.2.7. Phenolic compounds identified by HPLC-PDA-ESI-MS in white beans

Table 3-23 presents the compound identified in white beans. The chromatogram is presented in Figure 3-10. Quinic acid, 3 ferulic acid derivatives, medioresinol, *p*-coumaric acid and *t*-ferulic acid were the only compounds identified in white beans. The majority of the compounds detected in the red, grey mottled and brown were not observed in white beans.

Table 3-23 HPLC-PDA-ESI-MS -based identification of phenolics in white beans

Peak	t_R(min)	Molecular weight	[M-H]- (m/z)	[M-H]- fragments (m/z)	Tentative identification
1	1.36	192	191	127	Quinic acid
2	5.57	260	259	241,233,197	Syringic acid derivative
3	6	386	385	193	Ferulic acid derivates
4	6.52	386	385	193	Ferulic acid derivates
5	7.52	386	385	193	Ferulic acid derivates
6	8.62	388	387	207	Medioresinol
7	8.92	164	163	119	*p*-coumaric acid
8	9.79	388	387	207	Medioresinol
9	11.1	194	193	149, 134	*t*-ferulic acid

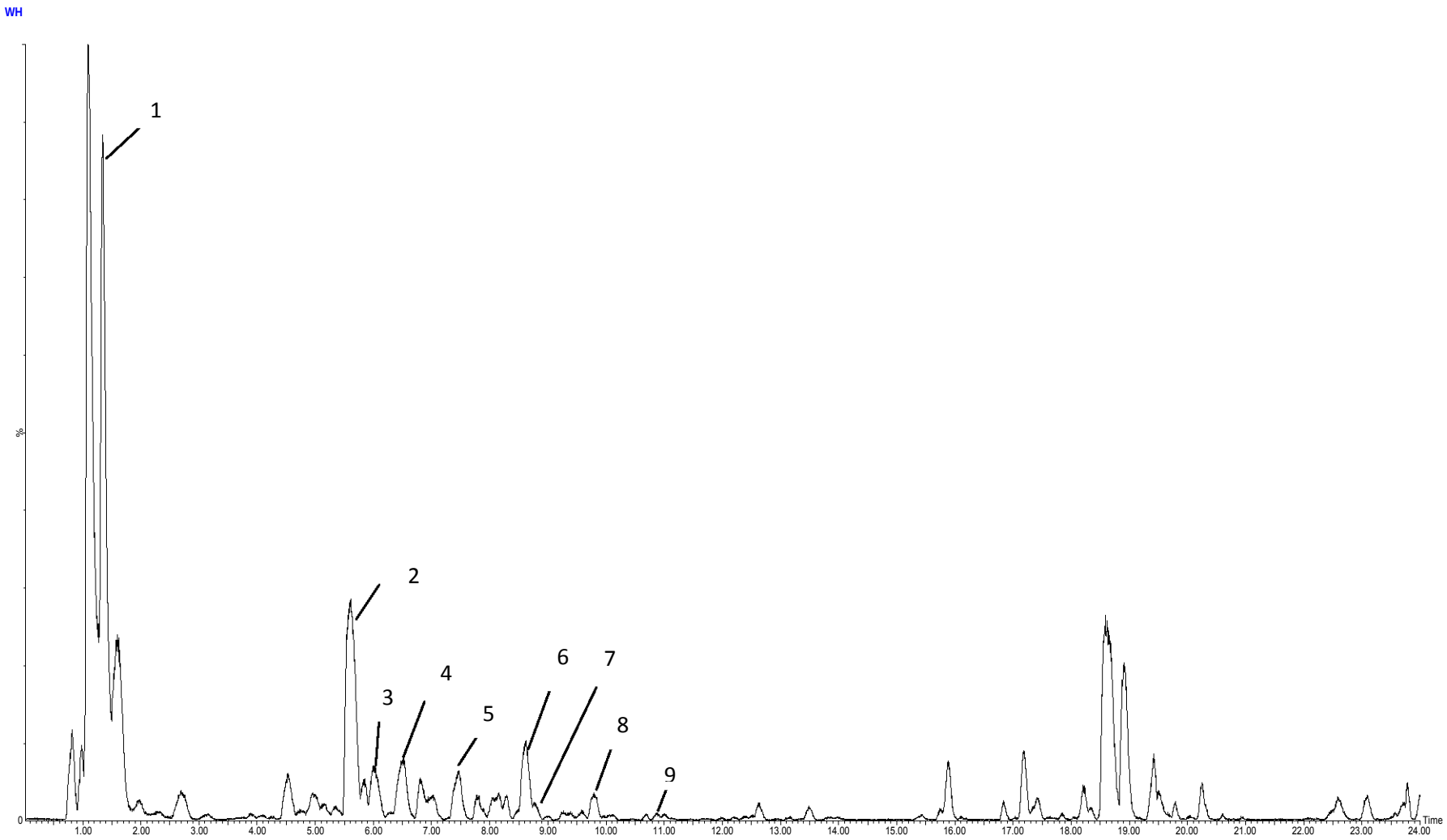


Figure 3-10 HPLC-PDA-ESI-MS chromatogram of 70% methanol extract of white beans.

3.2.2.8. Concentration of some identified phenolic compounds in common beans

Table 3-24 presents the concentrations of epicatechin, catechin, *p*-coumaric acid, *t*-ferulic acid, gallic acid and salicylic acid in the four market classes of common beans. The concentration of catechin ranged from 54.30 to 435.10 mg / kg DW and was the most abundant among the phenolic compounds investigated. Catechin was not detected in white beans, whereas epicatechin was not observed in white and the brown market classes. Grey mottled beans had the highest concentrations of both catechin and and epicatechin. Salicylic acid was not observed in the white market class. Among the phenolic acids investigated, the concentration of *t*-ferulic acid was higher than *p*-coumaric, gallic and salicylic acid and there was a significant difference in the *t*-ferulic acid content among the market classes. As mentioned earlier, concentration of individual phenolic compounds in food crops is affected by a number of factors (pre-harvest and post-harvest). Data from the literature (Luthria *et al.*, 2006; Romani *et al.*, 2004; Macz-pop 2006) on the concentration of individual phenolic compounds in common beans show wide variations.

Table 3-24 Individual phenolic compound concentration (mg/ kg DW) in common beans

Phenolic compound concentration (mg /kg DW)	Market classes of Common beans			
	Red	**Grey mottled**	**Brown**	**White**
Epicatechin	7.51 ± 0.22	23.13 ± 0.68	-	-
Catechin	108.6 ± 10.3	435.10 ± 25.4	54.30 ± 0.57	-
p-Coumaric acid	2.73 ± 0.02	5.40 ± 0.06	5.45 ± 0.25	3.24 ± 0.02
t-Ferulic acid	20.46 ± 3.18	37.87 ± 2.46	45.06 ± 0.6	27.64 ± 0.01
Gallic acid	3.04 ±0.03	6.35 ± 0.17	3.72 ± 0.31	-
Salicylic acid	-	9.47 ± 0.31	3.19 ± 0.10	-

3.2.3. Antioxidant activity of the Zambia market classes of common beans

Antioxidant properties of common beans based on the free radical scavenging activity and ferric reducing power are reported in this part of the thesis. DPPH assay was used to evaluate the free radical scavenging activity and the FRAP assay to assess the ferric reducing antioxidant power.

3.2.3.1. Free radical scavenging activity of antioxidants in common beans

The DPPH free radical scavenging ability of common beans was explored by studying the kinetics of the DPPH radical-common beans extracts reaction and the effective concentration (EC_{50}) of the common beans extract required to scavenge 50% of the DPPH free radicals.

Kinetics of the DPPH free radical reaction with antioxidants in common beans

Figures 3-11 and 3-12 present the disappearance pattern of DPPH free radicals with time in the presence of the aqueous and methanol extracts within the time period of 80 minutes. The reaction was performed under pseudo first-order condition that was achieved by making the concentration of DPPH in large excess. The free radical scavenging pattern was characterised by the fast initial decay, followed by the subsquent slower step. The pseudo first-order rate constants (K) of the four market classes of common beans were obtained using equations (1) and (2) in section 2.2.4.1 (chapter 2), and the plot of InA versus time, whose slope was equal to K. Ranking the free radical scavenging capacities of the common bean market classes based on K, the order would be as follows: red beans > grey mottled beans > brown beans > white beans (Table 3-25). The same order is obtained by ranking the market classes based on the amount of DPPH radicals scavenged after 80 minutes incubation (Table 3-25). White beans showed far lower antiradical capacity than the other three. Compared to Trolox which was used as a positive reference standard, the free radical scavenging ability of the red, grey mottled and the brown beans can be considered moderate.

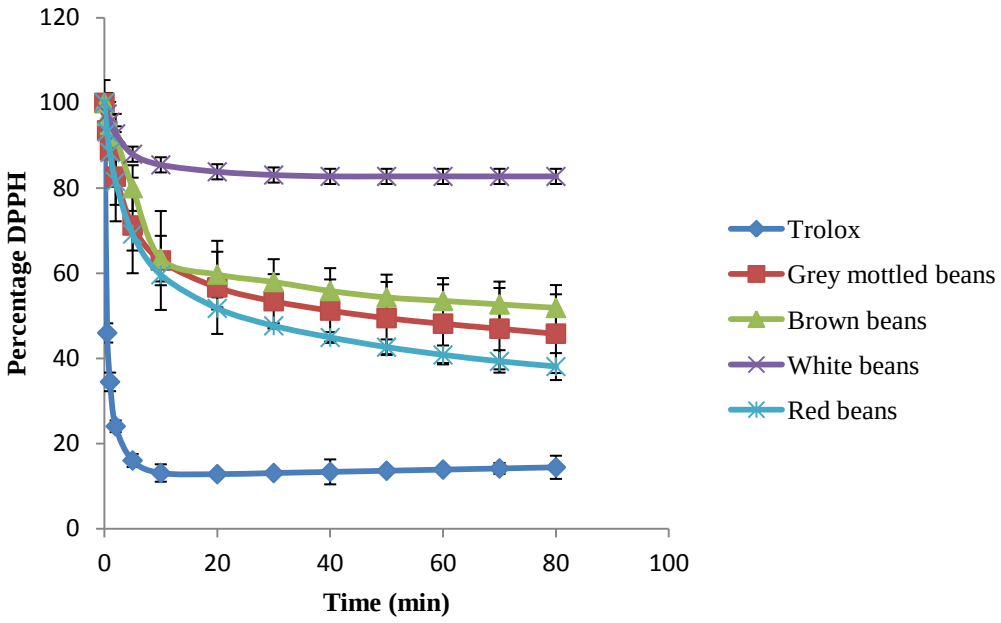


Figure 3-11 Disappearance pattern of DPPH free radicals with time in the presence of aqueous extracts of common beans

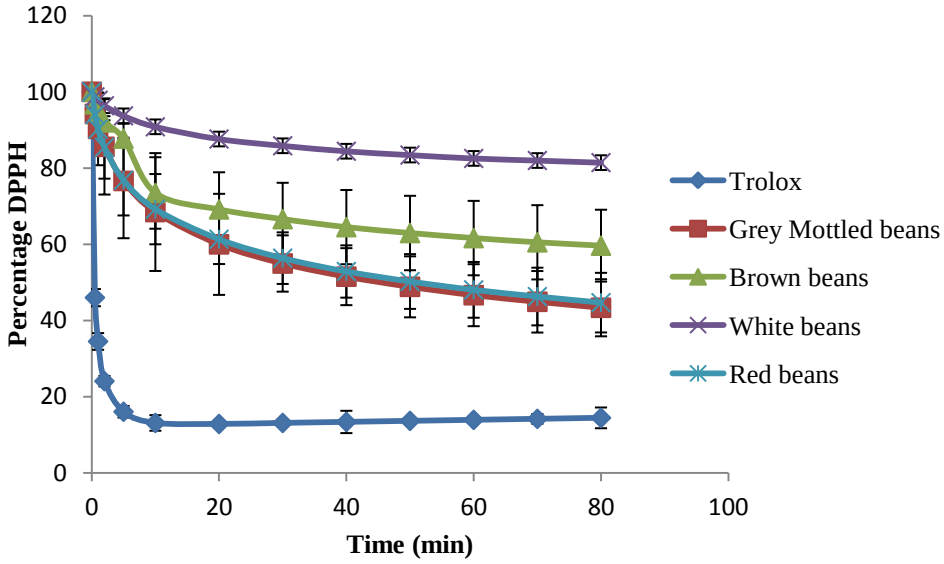


Figure 3-12 Disappearance pattern of DPPH free radicals with time in the presence of the 70% methanol extracts of common beans

Table 3-25 Pseudo-first order rate constant of antiradical (Y-H) in common beans extracts and the amount of DPPH scavenged after 80 minutes of incubation

Market class of common beans	Pseudo-first order rate constant (K) [min^{-1}]		Amount DPPH quenched [%] after 80 minutes incubation	
	Aqueous extract	70% Methanol extract	Aqueous extract	70% Methanol extract
Red	0.043	0.053	55.3 ± 2.0	61.9 ± 3.1
Grey mottled	0.038	0.047	56.6 ± 0.9	54.2 ± 1.4
Brown	0.019	0.034	40.3 ± 1.1	48.1 ± 5.2
White	0.006	0.013	18.5 ± 0.9	17.3 ± 1.0

Trolox K = 1.55, amount quenched in 80 minutes = 85.5%

Effective concentration (EC_{50})

The EC_{50} values for common beans extracts and Trolox in a DPPH free radical scavenging reaction are presented in Table 3-26. The calculation was done as in Figure 3-5 (Section A). In both the aqueous and methanol extracts, the following order of free radical scavenging capacity based on effective concentrations can be observed: red beans > grey mottled beans > brown beans > white beans. Generally, the red and grey mottled beans demonstrated very good DPPH free radical scavenging capacities. The white beans market class was very poor in stabilizing the DPPH free radicals.

The findings on red beans in the present study are comparable to the range (367.2 to 465.5 µg dried extract / ml) reported for five legumes (chickpea, lentil, mung beans, mash beans and peas) previously by Zia-Ul-Haq *et al.,* (2012). Spanou *et al.,* (2007) investigated *Lupinus albus, Lens culinaris* and *Phaseolus vulgaris* seeds for DPPH free radical scavenging ability and reported EC_{50} values ranging from 2300 to 7600 µg dried extract / ml. Red, grey mottled, and brown beans appear to be more potent scavengers than the leguminous seeds studied by Spanou *et al.,* (2007).

Table 3-26 EC_{50} values for DPPH scavenging by common beans extracts after 30 minutes of incubation

Market classes of common beans	DPPH radical scavenging EC_{50} (µg dried extract / ml)	
	70 % Methanol extract	Aqueous extract
Red	450.0 ± 14.1	489.5 ± 6.36
Grey mottled	507.5 ± 5.5	525 ± 4.8
Brown	989.0 ± 12.7	1020.1 ± 4.1
White	2425.0 ± 35.4	2534.5 ± 7.8

3.2.3.2. Ferric Reducing Antioxidant Power of antioxidants in common beans

The FRAP values of the aqueous and methanol extracts of the four market classes of common beans are presented in Table 3-27. The methanol extracts demonstrated higher FRAP values than the aqueous extracts in all the common bean market classes. FRAP values ranged from 1.69 to 6.88 mmol Fe^{2+} / 100 g DW. The four market classes of common beans can be ranked as follows based on the antioxidant reduction power: red beans > grey mottled beans > brown beans > white beans. Our results are within the range (1.27 to 9.70 mmol Fe^{2+} / 100 g DW) reported previously by Xu *et al.,* (2007) for different market classes of common beans grown in North Dakota, Idaho and Washington regions of the United States of America. In comparison to other legumes, all four market classes had higher FRAP values than peas (0.62 to 0.82 mmol Fe2+ / 100 g DW), but lower than lentils (8.75 to 13.92 mmol Fe^{2+} / 100 g DW) reported previously by Xu *et al.,* (2007). Similar comparison can be made with the findings of Halvorsen *et al.,* (2002) on the FRAP values of lentils (11.37 to 13.92 mmol Fe^{2+} / 100 g DW), peas (0.43 to 0.86 mmol Fe^{2+} / 100g DW), soyabeans (1.09 to 1.49 mmol Fe^{2+} / 100 g DW) and chickpeas (0.8 mmol Fe^{2+} / 100 g DW).

Table 3-27 Ferric Reducing Antioxidant Power (FRAP) values for common beans

Market classes of common beans	FRAP value (mmole Fe^{2+} / 100 g DW)	
	70 % Methanol extract	Aqueous extract
Red	6.88 ± 0.04	4.86 ± 0.05
Grey mottled	6.74 ± 0.08	4.70 ± 0.03
Brown	4.46 ± 0.14	2.25 ± 0.02
White	2.96 ± 0.04	1.69 ± 0.03

3.2.3.3. Correlation of antioxidant activities and phenolic contents of common beans

The correlations between the antioxidant activity and total polyphenols, tannins and flavonoids of common beans were established using regression analysis. Correlations of antioxidant activities and phenolic compound in the aqueous extracts of common beans are presented in Figure 3-13. Correlations between the ferric reducing antioxidant power and phenolic compounds were as follows: total polyphenols (0.9684), tannins (0.9549), flavonoids (0.0988). The correlations between the DPPH free radical scavenging and phenolic compounds were: total polyphenols (0.8642), tannins (0.9462) and flavonoids (0.0127). Correlations of antioxidant activities and phenolic compound in methanol extracts are presented in Figure 3-14. With the ferric reducing antioxidant power, the following correlations were observed: total polyphenols (0.8841), tannins (0.9724) and flavonoids (0.6898), and with the DPPH free radical scavenging: total polyphenols (0.7826), tannins (0.7850) and flavonoids (0.6887). In both extracts, total polyphenols and tannins showed a positive linear relationship with the antioxidant activities. This suggests that their contribution to the antioxidant activities of the investigated market classes of common beans is considerable. These results are consistent with the findings of previous researchers, who reported such positive correlations for total polyphenols and tannins with antioxidant activity (Hajimahmoodi *et al.,* 2008; Xu *et al.,* 2007; Miliauskas *et al.,* 2004, Silva *et al.,* 2007; Pourmorad *et al.,* 2006). Surprisingly, there was no correlation between antioxidant activity and flavonoids content of the aqueous extracts, whereas negative correlations were observed in the methanol extracts. Low correlations between flavonoids and antoxidant activity have been reported previously in other studies (Luís *et al.,* 2009; Tawaha *et al.,* 2007, Silva *et al.,* 2007; Miliauskas *et al.,* 2004). It is known that only flavonoids of certain structure and

containing certain groups, particularly hydroxyl groups in certain positions in the molecule determine antioxidant properties (Luís *et al.*, 2009); and that in general, these properties depend on the ability to donate hydrogens or electrons to a free radical (Miliauskas *et al.*, 2004).

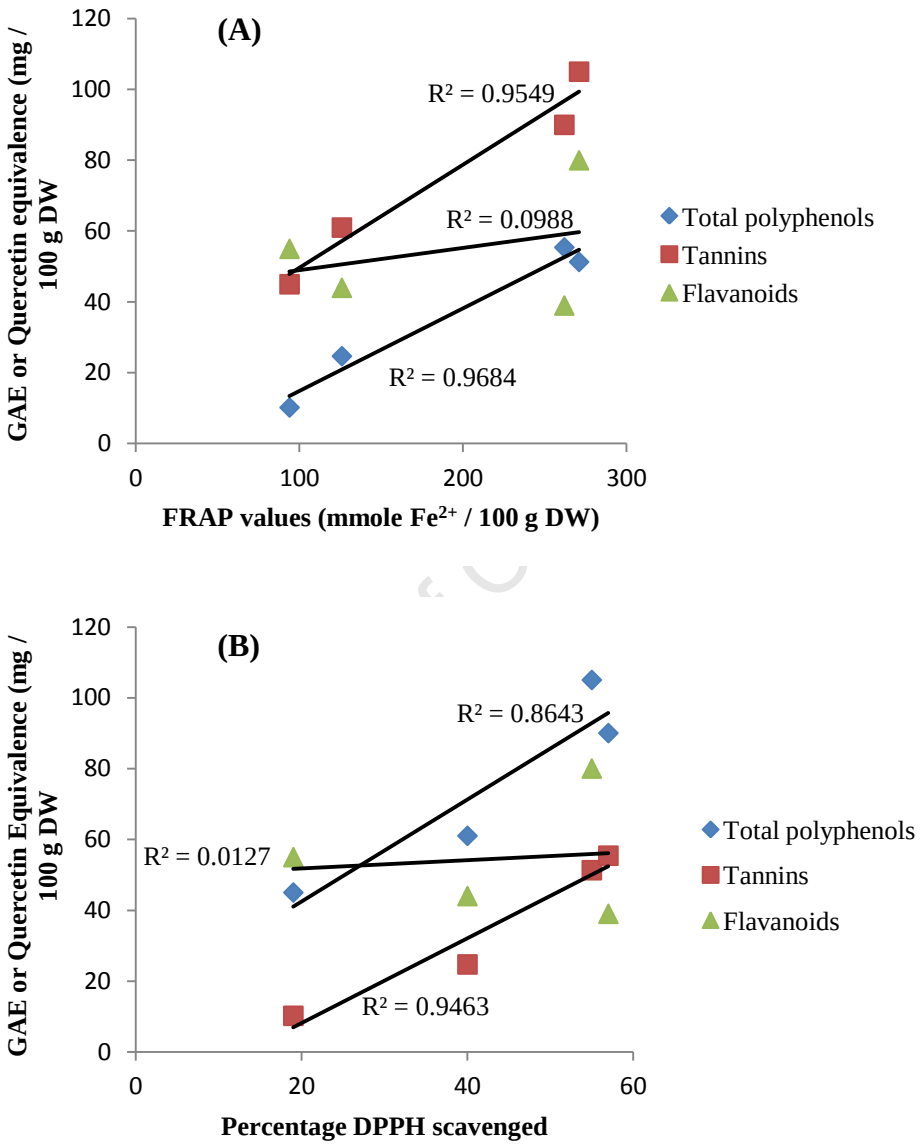


Figure 3-13 Correlation of antioxidant activities and phenolic compounds in the aqueous extracts of common beans: (A) Ferric Reducing Antioxidant Power, (B) DPPH free radical scavenging

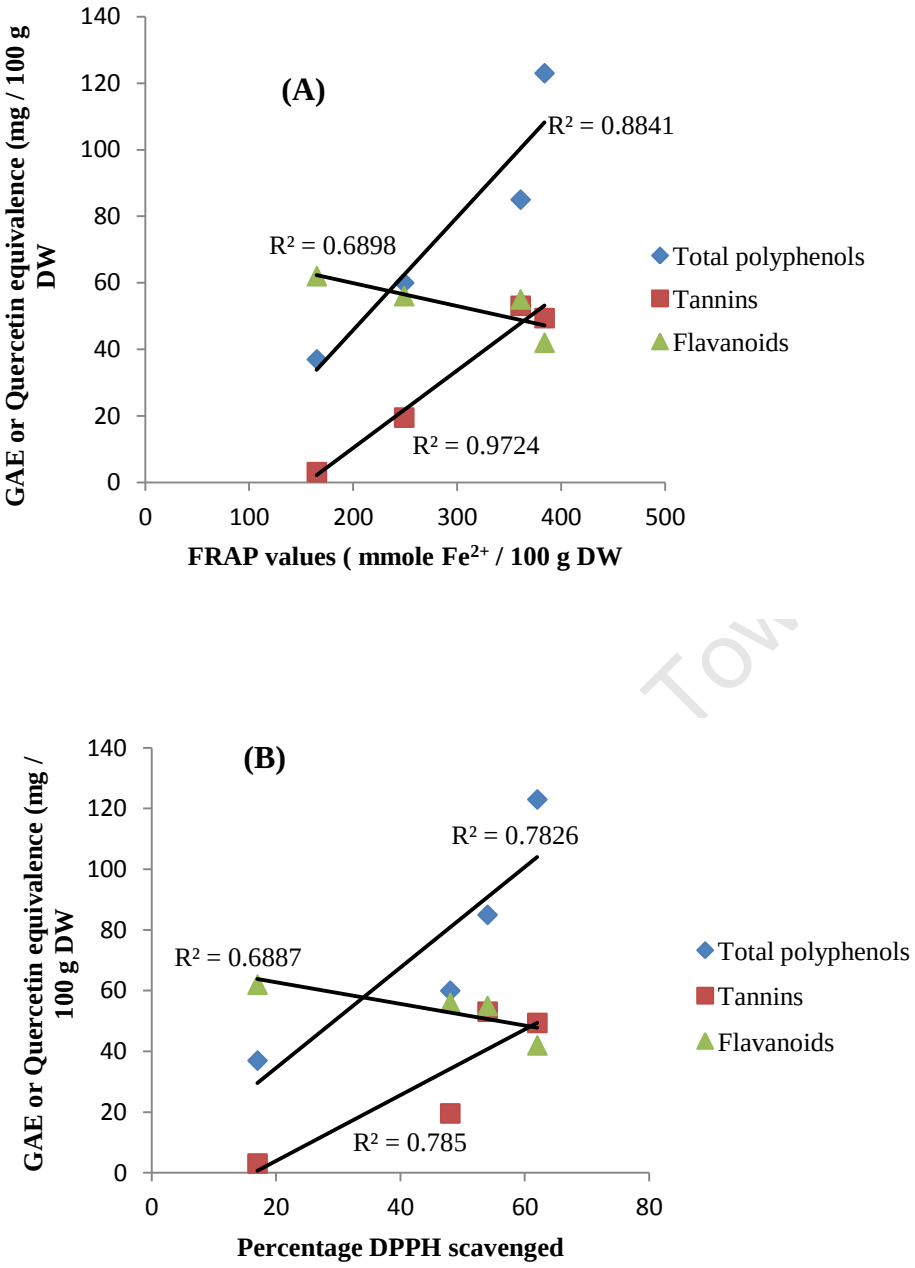


Figure 3-14 Correlation of antioxidant activities and phenolic compounds in the 70% methanol extracts of common beans: (A) Ferric Reducing Antioxidant Power, (B) DPPH free radical scavenging

Discussion

Upon reviewing the literature on the biological activity of common beans, there is enough evidence to conclude that common beans have the potential for use as nutraceuticals. Nutraceutical foods are thought to play an important role in the prevention and management of cardiovascular disease, diabetes, obesity and some cancers. The main interest in this section, however, was to screen the market classes of common beans grown in Zambia to provide information for a preliminary database that may be useful to researchers and other stakeholders who are involved in the breeding of this crop with the view of improving its nutraceutical value.

The four market classes of common beans displayed differences in phenolic phytochemical profile. Red beans consistently displayed the highest total polyphenol contents in both the methanolic and aqueous extracts, followed by grey mottled, brown and white. Differences in total polyphenol contents were significant. The polyphenol contents of the red and grey mottled beans were within the range (64 – 95 mg GAE / 100g DW) reported by Deshpande *et al.,* (1987). The tannin concentrations of the red and grey mottled were not significantly different. White beans had the lowest tannin concentration. The concentration of total flavonoids varied greatly and was influenced by the solvent used for extraction. In the methanolic extract, red beans displayed the highest total flavonoids content, followed by the white, brown, and grey mottled. In the aqueous extract, the white beans displayed the highest total flavonoid concentration, followed by brown, grey mottled and red. However, total flavonoid contents for all the market classes were in agreement and slightly greater than that reported by Dinelli *et al.*, 2006 (19 – 84 mg quercetin equilvalets / 100 g DW) for the twenty Italian varieties of common beans. Concentration of phenolic compounds may vary depending on a number of factors such as variety, agricultural conditions, storage and processing conditions (Amarowicz *et al.,* 2009).

Concentrations of individual phenolic compounds also varied greatly. Among the investigated compounds, catechin was found to be the abundant compound in the grey mottled, red and brown beans, with the grey mottled recording the highest concentration. Catechin and epicatechin were found to be absent in white beans and this may be responsible for the low antioxidant activity observed. Salicylic acid was absent in red and white beans.

The concentrations of *p*-coumaric and ferulic acid varied greatly among the market classes and the differences were significant.

The use of HPLC-PDA-ESI-MS led to the identification of various phenolic compounds in the Zambian market classes of common beans. Even though total polyphenol contents varied greatly between the common beans market classes, quinic acid, a syringic acid derivative, ferulic acid derivatives, medioresinol, *p*-coumaric acid and ferulic acid were identified in all the market classes. However, the isomers of ferulic acid derivatives observed were 4 in red, 3 in brown, 2 in white and 1 in grey mottled beans. Catechin and gallic acid were only identified in the red, grey mottled and brown beans. Epicatechin was only identified in the red and grey mottled beans. A compound with the molecular ion at m/z 567, tentatively identified as a flavonone derivative was only observed in red beans. Catechin glucoside was only identified in grey mottled and brown beans. Compounds tentatively identified as kaempferol glucoside and carnosol were only observed in brown beans and not in the other market classes.

Though some phenolic compounds were common in all the market classes of common beans, there was variation in the types of other phenolic compounds identified. It is possible to discover variation in almost every conceivable trait, including agronomical and nutritional qualities. And if a trait cannot be found in the crop itself, it can often be found in a wild relative of the crop; a plant that has similar species that have not been farmed or used in agriculture, but exist in the wild (Biodiversity International 2005). Diversity in the phenolic phytochemical profile offers great opportunities for the improvement of common beans by plant breeders.

The antioxidant activities of the market classes of common beans showed a similar trend as the total polyphenol contents. The red market displayed the highest antioxidant activity in the aqueous extract, but showed little difference with the grey mottled beans in the methanolic extract. Ranking the market classes based on the free radical scavenging capacities and the FRAP-derived total antioxidant power, the following order was observed: red beans > grey mottled beans > brown beans > white beans. On a comparative basis, white beans displayed far lower antioxidant activities compared to the others. Variations in the antioxidant behaviour can be attributed partly to the differences in the polyphenolic compound concentrations and the types of phenolic compounds in the market classes of common beans.

There were positive correlations between total phenolic content and the antioxidant activities in these beans. White beans had the lowest total polyphenol content and was found not to contain such important polyphenols as catechin, epicatechin and catechin glucoside from the flavonol sub group. This may suggest that polyphenols from this sub group could be among important phenolic antioxidants in common beans from Zambia. For example, brown beans, despite not having epicatechin displayed moderate antioxidant activities because it at least had catechin. Red and grey mottled beans which both contained catechin and epicatechin displayed higher antioxidant activities compared to white and brown beans. There are also many environmental and other factors that could influence the different antioxidant activity levels.

Conclusion

This study found that market classes of common beans grown in Zambia possess antioxidant properties that may be valuable for human health. The red beans have the highest free radical scavenging activity and FRAP – derived total antioxidant power, followed by the grey mottled, brown and white beans. Similarly, red beans is the richest in total polyphenol concentration, followed by grey mottled, brown and white beans, and there is a strong positive correlation between antioxidant activity and total polyphenol content. Some identified phenolic compounds are common in all the market classes while others are variety associated. The study assumes that the diversity in the phenolic phytochemical profile is an excellent opportunity for genetic improvement in the nutraceutical attributes of these common bean market classes by crop breeders.

Chapter 4

Domestic cooking effects of bambara groundnuts and common beans in the antioxidant properties and polyphenol profiles

Introduction

Generally most food legumes have to be processed before consumption. Different processing methods are applied depending on the intended use of the final product and the availability of the processing facilities. The most common method of processing is domestic cooking and involves boiling the seed legumes until soft using fire wood or electricity as heating sources.

Concentration of plant secondary metabolites having antioxidant activity is affected by a number of factors, including genetics and growing conditions (Kalt 2005). Processing is another important factor that can impact total antioxidant activity (Papas 1996). Knowledge about the fate of total antioxidant activity as a result of home processing may have a significant impact on consumers' food selection and processing (Danesi 2009). Few studies have been done on seed legumes to investigate the effect of domestic processing on the total antioxidant activity and the phytochemical profiles. Although common beans are widely consumed all over the world, very little information is available in the literature regarding the changes in total phenols, total flavonoids and antioxidant activities following food preparation methods (Akillioglu and Karakaya 2010).

In this section of the thesis, the effect of domestic cooking on the antioxidant activities and phenolic phytochemical profiles of the red Zambian market classes of common beans and bambara groundnuts was investigated. The red beans were chosen for this investigation because they demonstrated excellent antioxidant activities in the raw form compared to the other types investigated here (Section B of Chapter 3). The red bambara groundnuts, though demonstrating slightly lower antioxidant activity in raw form than the brown nuts (Section A of Chapter 3), were chosen for further investigations in this section partly because their HPLC-PDA-ESI-MS profile revealed more phenolic compounds than the brown bambara groundnuts. The methanolic extract was used because most likely it contained both

hydrophilic and hydrophobic compounds. Antioxidant properties of cooked seeds were measured based on their free radical scavenging activity and ferric reducing power (see Chapter 2, section 2.2.4.1. and 2.2.4.2.). The free radical scavenging ability of the methanolic extracts is reported on the basis of kinetic behaviour of the DPPH free radicals with antioxidants in the two legumes. The ferric reducing power is reported on the basis of the number of mmoles Fe^{2+} produced in the reduction of Fe^{3+} by the antioxidants in the extracts.

Results

4.1. Free radical scavenging activity of cooked red bambara groundnuts and red beans

Figure 4-1 presents the disapearance of the DPPH free radicals in the presence of methanolic extract of cooked and uncooked red bambara groundnuts. The initial decay of DPPH was faster in the presence of methanolic extracts from the cooked red bambara groundnuts than in the uncooked. The pseudo first-order rate constant was 0.43 min^{-1} in the cooked and 0.042 min^{-1} in the uncooked bambara groundnuts (Table 4-1). This is a very desirable property for the antioxidants because free radicals are very fast reactive species that equally need fast-acting scavengers. There was a significant difference in the amount of DPPH free radicals scavenged at the end of the incubation time by the extracts from the uncooked and cooked red bambara groundnuts. It was intriguing to note that the amount of the DPPH free radicals scavenged by the extracts from the cooked red bambara groundnuts was similar to that scavenged by the positive reference standard trolox towards the end of the assay period chosen (Table 4-1). This observation implies that the cooked nuts most likely, contained more antioxidant compounds than the uncooked nuts.

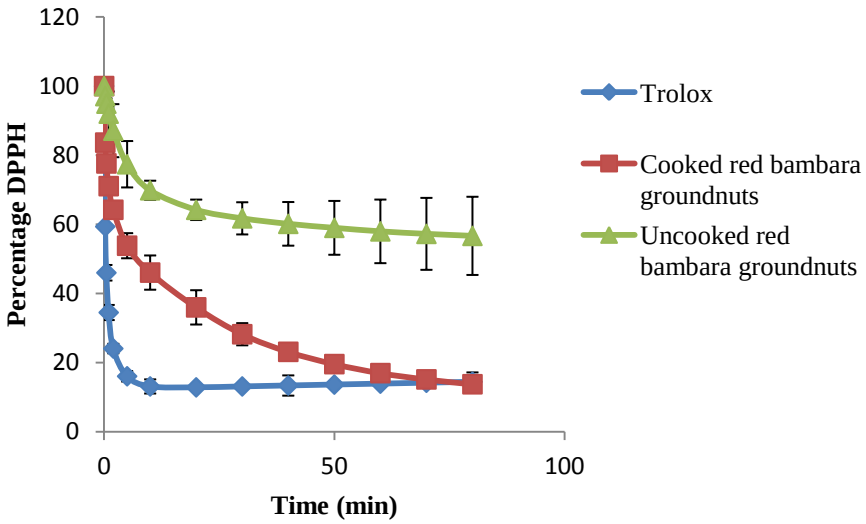


Figure 4-1 Disappearance pattern of DPPH free radicals with time in the presence of 70% methanol extracts of cooked and uncooked red bambara groundnuts.

Table 4-1 Pseudo-first order rate constant of antiradical (Y-H) in cooked red bambara groundnuts and red beans and the amount of DPPH scavenged after 80 minutes of incubation

Market classes of bambara groundnuts and common beans		Pseudo-first order rate constant (K) [min^{-1}]	Amount DPPH quenched [%] after 80 minutes incubation
Red bambara groundnuts	Control (uncooked)	0.042	44.01 ± 2.96
	Cooked	0.43	86.38 ± 1.36
Red beans			
	Control (uncooked)	0.050	62.01 ± 2.21
	Cooked	1.12	65.22 ± 2.36

Trolox K = 1.55, amount quenched in 80 minutes = 85.5%

The free radical scavenging pattern by antioxidants in the cooked and uncooked red beans is presented in Figure 4-2. The dispearance pattern of the DPPH in the presence of the extract from the cooked red beans was much faster than that of the uncooked. There was a very sharp

initial decay with a pseudo first-order rate constant (K) of 1.12 (min^{-1}) for the cooked red beans compared to 0.05 (min^{-1}) for the uncooked beans (see Table 4-1 above). The cooked extract had a better pseudo first-order rate kinetic than the extract from the uncooked beans. As discussed above with respect to red bambara groundnuts, this is a very desirable attribute for the antioxidants because free radicals react extremely quickly and require fast-acting antioxidants.

The results from our study are in agreement with Akillioglu and Karakaya (2009) who reported the increase in the amount of DPPH free radicals scavenged when common beans were cooked by first soaking in water for 3 hours prior to cooking. However, the study by Akillioglu and Karakaya did not report the free radical scavenging pattern by elaborating on the kinetics of the DPPH free radicals and the antioxidants in common beans in the course of the reaction.

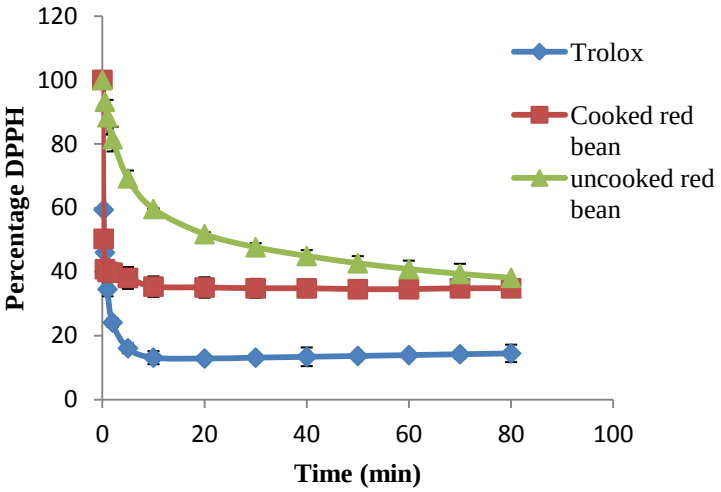


Figure 4-2 Disappearance pattern of DPPH free radicals with time in the presence of 70% methanolic extracts of cooked and uncooked red beans

4.2. Ferric Reducing Antioxidant Power of the cooked red bambara groundnuts and red beans

The FRAP values of the cooked red bambara groundnuts and red beans are presented in Table 4-2. The FRAP values of the uncooked and cooked red bambara groundnuts were not significantly different. However, the extract from the cooked red beans had greater reducing

power than the extract from the uncooked material, suggesting that the cooked sample contained more antioxidant compounds or more reactive antioxidants than the uncooked.

Table 4-2 FRAP values for the cooked red bambara groundnuts and red beans

Market classes of bambara groundnuts and common beans		FRAP values (mmole Fe^{2+}/ 100g DW)
Red Bambara groundnuts	Control (uncooked)	8.01 ± 0.41
	Cooked	8.55 ± 0.02
Red beans		
	Control (uncooked	4.81 ± 0.01
	Cooked	5.05 ± 0.04

4.3. Polyphenol profiles of cooked red bambara groundnuts and red beans

Cooked samples showed higher antioxidant activities than uncooked. This observation prompted the investigation into the effect of domestic cooking on total polyphenols contents and the concentrations of selected individual phenolic compound, where standards were available. The effect of cooking on the HPLC-PDA-ESI-MS profiles of the two legumes was thus investigated.

4.3.1. Total polyphenol levels in cooked red bambara groundnuts and red beans

Total polyphenol levels in cooked red bambara groundnuts and red beans are presented in Table 4-3. There was an increase in total polyphenol content in both the red bambara groundnuts and red beans after cooking. Total polyphenol content increased by 6% in red bambara groundnuts and 41% in red beans respectively.

Table 4-3 Total polyphenol concentration of cooked red bambara groundnuts and red beans

Market classes of bambara groundnuts and common beans		Total polyphenols (mg GAE / 100 g DW)
Red Bambara groundnuts	Control (uncooked)	109.12 ± 2.35
	Cooked	116.03 ± 3.27
Red beans		
	Control (uncooked)	84.50 ± 2.55
	Cooked	119.31 ± 3.04

4.3.2. Levels of individual phenolic compounds in cooked red bambara groundnuts and red beans

Quantitative contents of *t*-ferrulic acid, *p*-coumaric acid, catechin, epicatechin and salicylic acid of cooked red bambara groundnuts and red beans are presented in Figures 4-3 and 4-4. Generally, there was an increase in all phenolic compounds investigated in red bambara groundnuts, except for salicylic acid which showed a 59% decrease after cooking. Epicatechin increased by 92%, followed by catechin (54%), *t*-ferrulic acid (39%) and *p*-coumaric acid (30%) respectively. Similarly, there was an increase in the concentration of all the phenolic compounds investigated in red beans after cooking, except for salicylic acid which was completely missing. Phenolics in the flavonol category recorded higher increase than phenolic acids. Epicatechin increased by 96%, followed by catechin (80%), *p*-coumaric (41%) and *t*-ferrulic acid (40%) respectively.

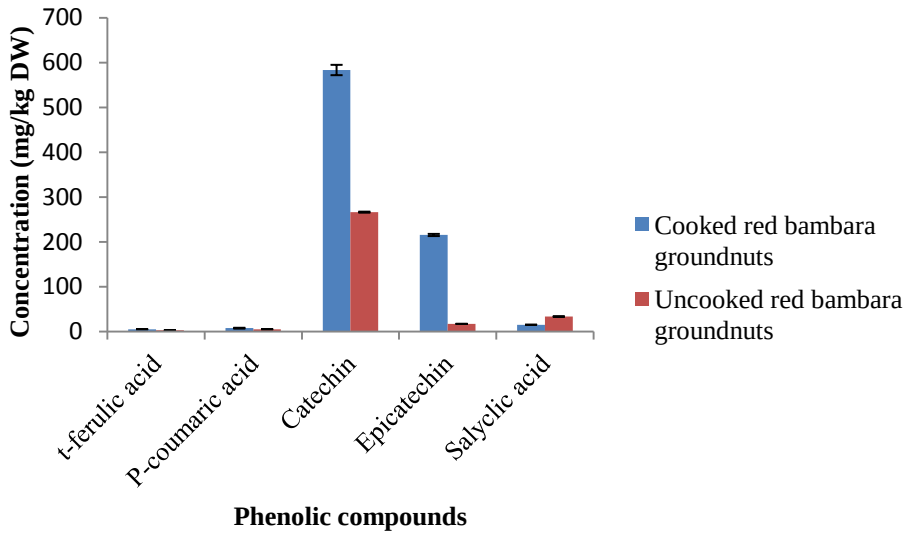


Figure 4-3 Concentration of individual phenolic compounds of 70% methanol extracts of cooked and uncooked red bambara groundnuts

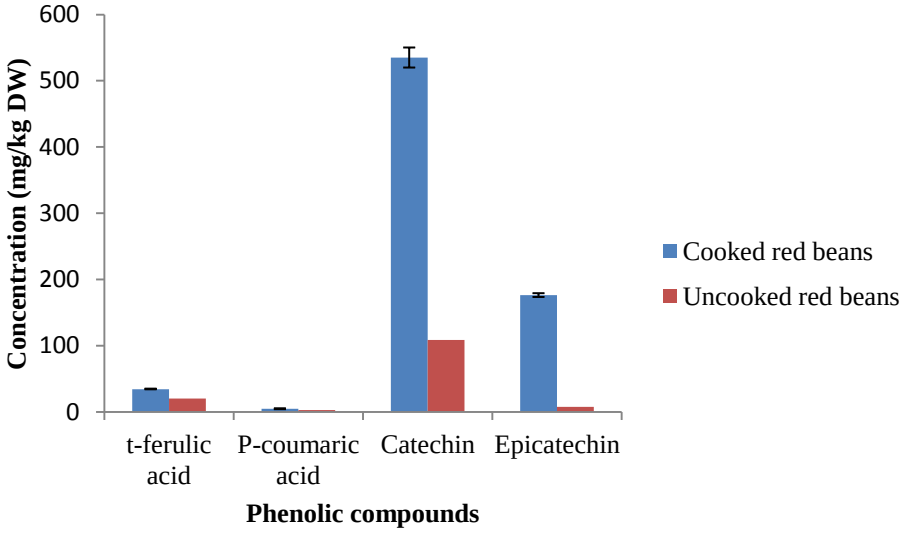


Figure 4-4 Concentration of individual phenolic compounds of 70% methanol extracts of cooked and uncooked red beans

4.3.3. HPLC-PDA-ESI-MS profiles of the cooked red bambara groundnuts and red beans

The HPLC-PDA-ESI-MS chromatograms of methanolic extracts from the uncooked and cooked red bambara groundnuts and red beans are compared in Figures 4-5 & 4-6 (for red bambara groundnuts) and 4-7 & 4-8 (for red beans) respectively. A slight drift in the retention

times of peaks was observed in these runs. This may be attributed to minor temperature changes or the increase in the back pressure in the column. Shifts in laboratory temperature and a slight increase of the back pressure in the column may cause drifting retention times in long automated operations (Waters Corporation, 2002).

Significant differences were observed in the HPLC-PDA-ESI-MS profiles of uncooked and cooked seeds for both legumes. In the profile of cooked red bambara groundnuts (Figure 4-5), there were ten emergent deprotonated molecules [M – H^-] of m/z 341, 639, 305, 495, 577, 451, 323, 389, 625 and 463 respectively. In the profile of the cooked red beans (Figure 4-7), there were five new deprotonated molecules [M – H^-] of m/z 164, 451, 608, 463 and 447 that emerged respectively. Differences in the number of emergent compounds between bambara groundnuts and common beans confirm that there are variations in the amount, types and distribution of phytochemicals in legumes.

For both red bambara groundnuts and red beans, there were variations in the peak heights (concentrations) for the compounds that were detected in the uncooked and cooked seeds. Peak heights were higher in the extracts of the cooked than the uncooked seeds, confirming that more phytochemicals are extracted when the seeds are cooked. These results suggested that there are changes in the phytochemical profiles of bambara groundnuts and common beans after the cooking process and validates our earlier observation on the changes in total polyphenol contents and concentrations of individual phenolic compounds. The observed changes are positive and dispel the generally held concerns that nutrients and phytonutrients are significantly lost during the cooking process.

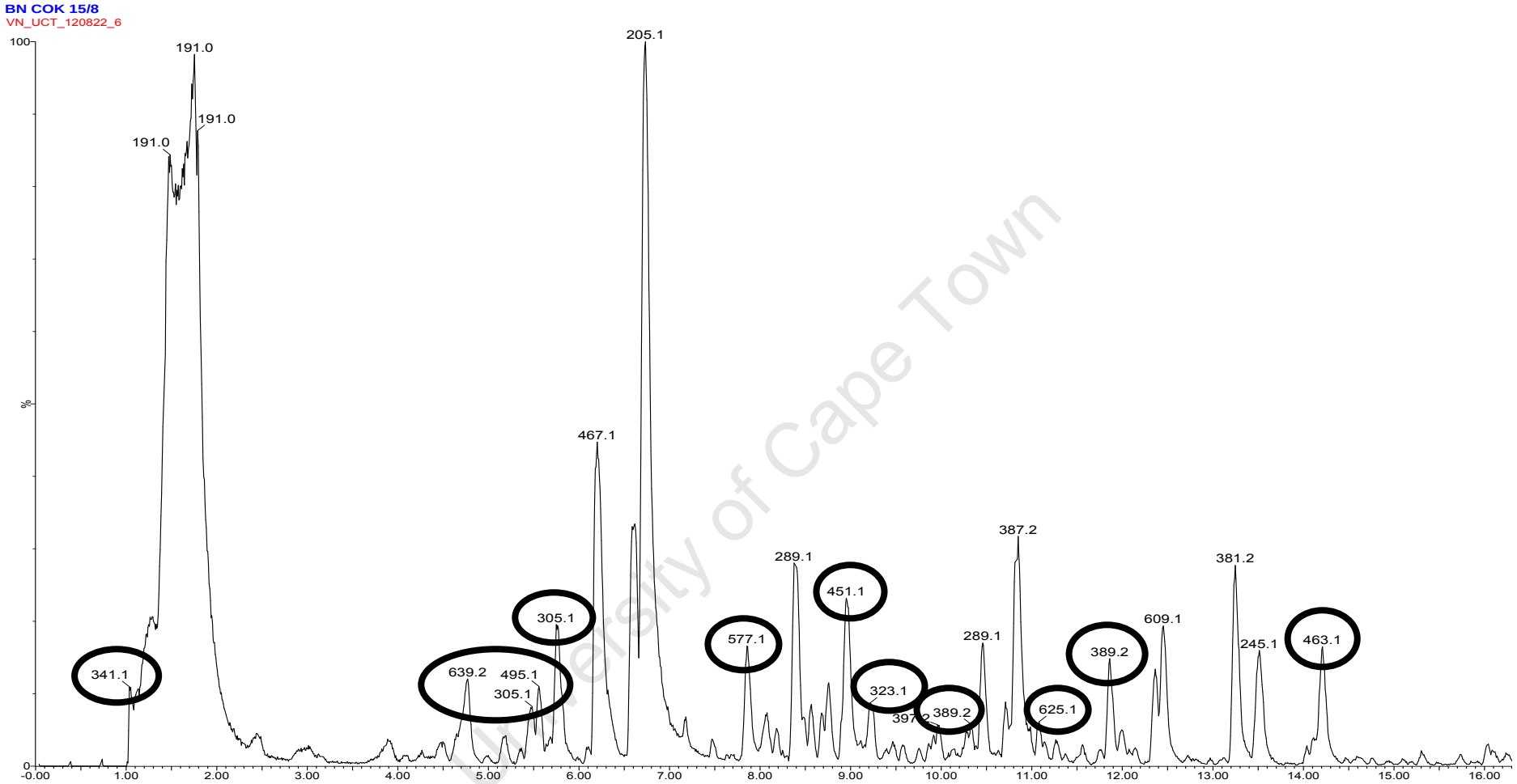


Figure 4-5 HPLC-PDA-ESI-MS chromatogram of 70% methanol extract of cooked red bambara groundnuts. O represents emergent compounds after processing. Number on top of each peak is the m/z of the deprotonated molecule M – H] ⁻ of each compound

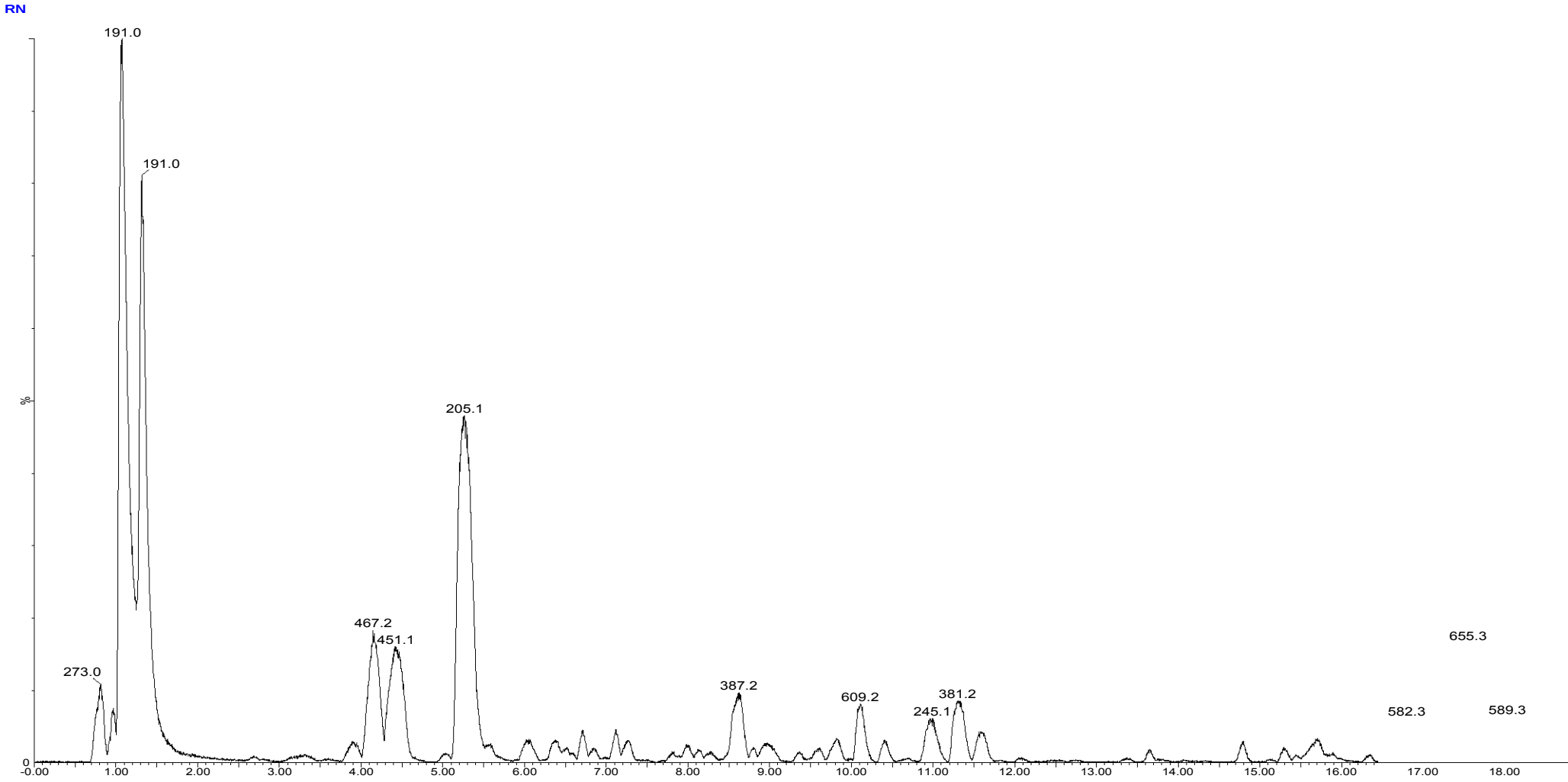


Figure 4-6 HPLC-PDA-ESI-MS chromatogram of 70% methanol extract of the uncooked red bambara groundnuts.

Number on top of each peak is the m/z of the deprotonated molecule $[M - H]^-$ of each compound.

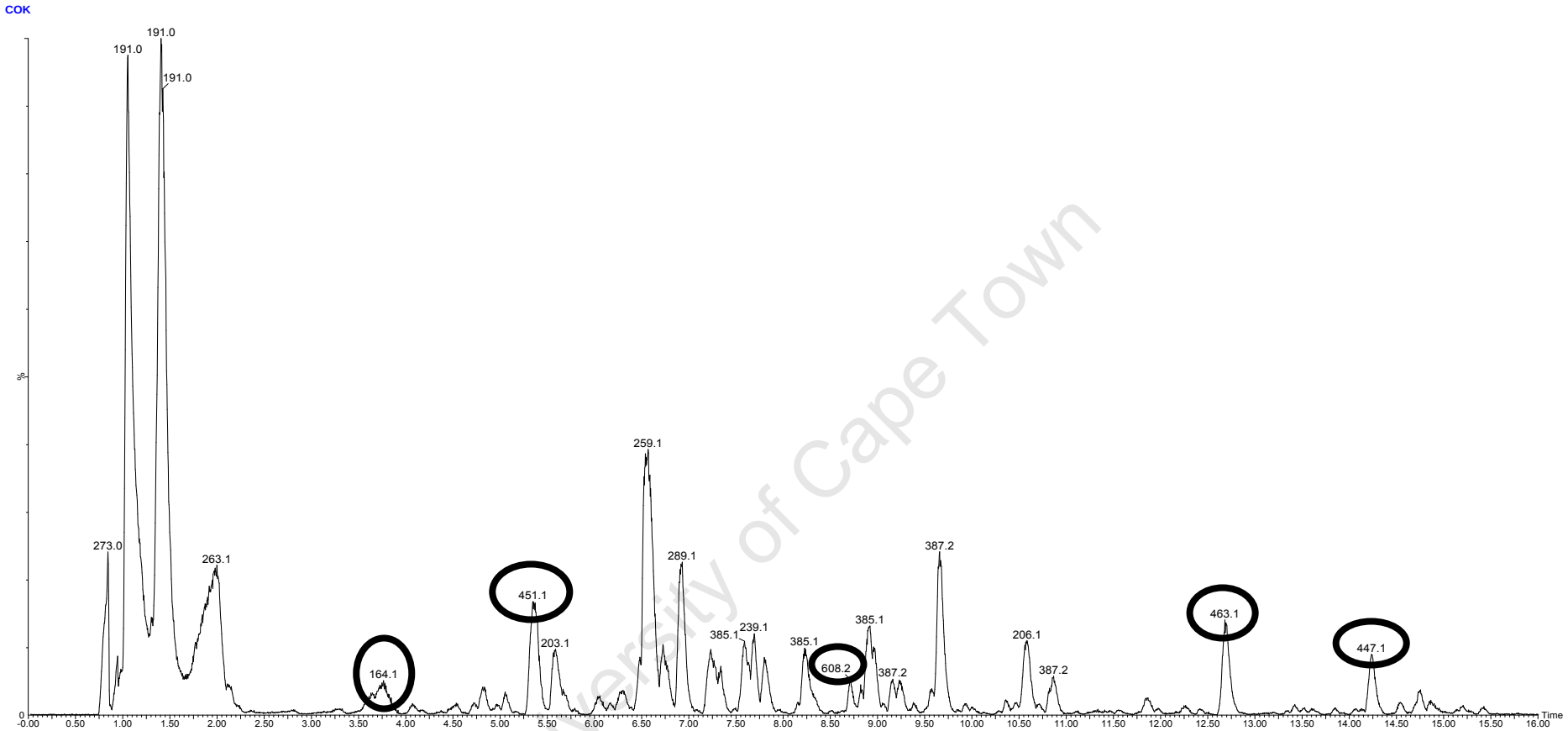


Figure 4-7 HPLC-PDA-ESI-MS chromatogram of 70% methanol extract of cooked red beans.

O represents emergent compounds after processing. Number on top of each peak is the m/z of the deprotonated molecule $[M - H]^-$ of each compound.

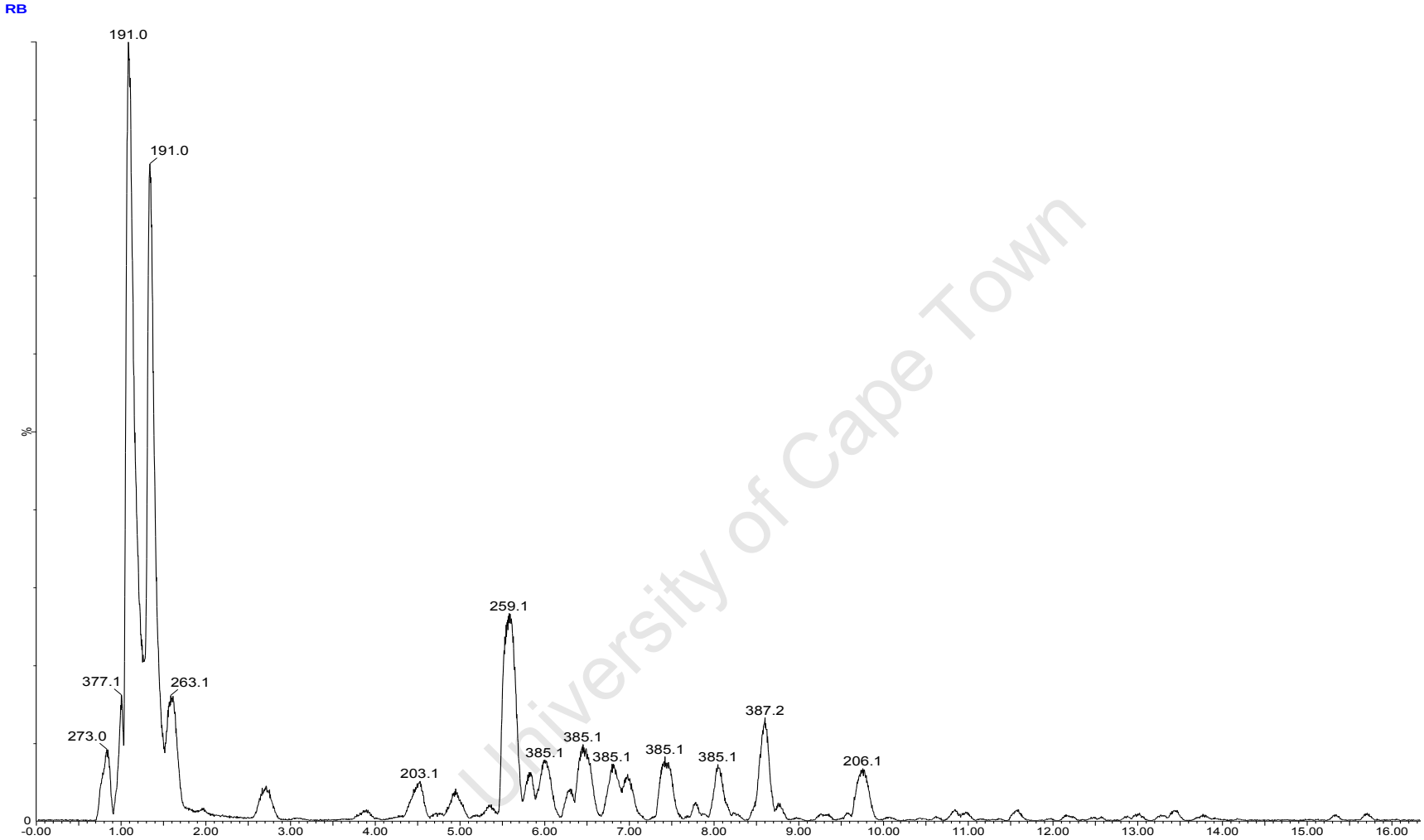


Figure 4-8 HPLC-PDA-ESI-MS chromatogram of 70% methanol extract of the uncooked red beans.

Number on top of each peak is the m/z of the deprotonated molecule $[M - H]^-$ of each compound.

Based on the literature data and fragmentation pattern, attempts were made to identify the new emerging compounds in both the cooked red beans and red bambara groundnuts. Some emergent compounds that were tentatively identified are presented in Tables 4-4 and 4-5 respectively.

Table 4-4 Emerging phenolic compound tentatively identified in cooked red bambara groundnuts

Parent ion [m/z]	Fragment ions [m/z]	Tentative identification
341	179, 161	Caffeic acid hexoside [v]
305	289,263, 247,219	Gallocatechin [w]
389	185,157,143	resveratrol glucoside [w]
625	239,179, 164	caffeic acid derivative [x]

[v] Hassain *et al.,* 2010, [w] Amandeep *et al.,* 2010, [x] Rabaneda *et al.,* 2003, [x] Gouveia and Castilho 2011

Table 4-5 Emerging phenolic compound tentatively identified in cooked red beans

Parent ion [m/z]	Fragment ions [m/z]	Tentative identification
451	289	Catechin glucoside [a]
608	301	Quercetin conjugate [b]
463	301	Quercetin-3-O-glucoside [b]
447	287	Kaempferol glucoside [b]

[a]Estrella *et al.,* 2011, [b] Lin *et al.,* 2008

Discussion

Cooking has displayed positive changes in the antioxidant activity and the polyphenol phytochemical profiles of both red beans and red bambara groundnuts. The increase in polyphenol concentration may be attributed to the release of bound polyphenols as a result of cooking process. Generally, complex polypenols have the ability to bind with other natural compounds within the cell. According to Harbaum (2007), cell wall bound phenolics are present as monomeric, dimeric, or oligomeric compounds which are esterified to the cell components (carbohydrates, lignin, pectin and proteins). These cell components may undergo

various changes during the cooking process. For instance, proteins may undergo thermal denaturation in which a number of bonds are weakened and broken, resulting in the loss of the tertiary structure. The loss of protein structure affects the polyphenol-protein interaction and bound phenolic compounds may be released. On the other hand, long chain carbohydrates tend to break down into simple sugars when cooked while simple sugars may form syrups. These changes may affect carbohydrate-polyphenol interaction resulting in the release of bound phenolic compounds. The study by Akillioglu and Karakaya (2010) on the total polyphenol content of cooked common beans that were first soaked in water for 3 hours reported a 78% increase in total polyphenol content, which was higher compared to the increase that has been observed in the present study. This could be due to the differences in the methodology and the samples used. In our study, pretreatment by soaking the samples prior to cooking was not done. According to Akillioglu and Karakaya (2009), differences between total polyphenol content of the beans cooked without soaking and cooked after soaking in water may be due to the increase in the efficacy of the heat process to extract phenolic compounds from the food matrix.

Changes in the concentration of invidual phenolic compounds after cooking can be explained in two ways: firstly, the bound phenolics present are released during the heating process due to changes that take place to the components to which they bind. Secondly, phenolic compounds which exist as oligomers and polymers (condensed tannins or proanthocyanidins) may disintegrate to release different constitutive units when heated. According to Cheynier (2005), phenolic compounds are highly unstable and are rapidly transformed into various products when the plant cells are damaged (for instance, during processing). The large increase in the concentration of flavanols (catechin and epicatechin) after cooking as observed in this study supports the second assumption. Catechin, epicatechin, epigallocatechin and gallocatechin are the main monomeric units present in condensed tannins (Shadkami *et al.,* 2009). Proanthocyanidins may occur as polymers containing as much as 50 catechin units (Wahle *et al.,* 2010). HPLC-PDA-ESI-MS profiling of the extracts from the cooked seeds revealed ten and five new compounds in the cooked red bambara groundnuts and red beans respectively. In cooked red bambara groundnuts, new compounds tentatively identified include caffeic acid hexoside, gallocatechin, resveratrol glucoside and a caffeic acid derivative. New compounds tentatively identified in red beans include catechin glucoside, quercetin-3-O-glucoside, kaemferol glucoside and quercetin conjugate.

Thermal processes have a large influence on the availability of phenolic compounds in food regardless of the method used. Changes in the polyphenol content have been reported in other foods as a result of heating using other methods other than domestic boiling. Roasting of peanuts at 130 ^{o}C for 33min caused an increase in the total polyphenol content (Yu *et al*., 2005); similar results are observed for cashew nuts when they were roasted using the same processing conditions (Chandrasekara and Shahidi, 2011). In apple juice processing, an increase in temperature from 40 ^{o}C to 70 ^{o}C caused increase of flavonoid content by 50% (Gerard and Roberts, 2004). According to the study by Fuleki and Ricardo-DaSilva (2003), pasteurization of grape juice increased the concentration of catechin and procyanidins in cold pressed juices. In these situations, an increase of temperature improves the extraction of phenolic compounds from foods. In other foods, thermal processes have been reported to decrease the content of phenolic compounds. Significant losses are noticed in tomato sauce pasteurized at 115C for 5 min (Valverdú-Queralt *et al*., 2011). A loss of 40% in total phenolic compounds was observed in strawberries pasteurized at 85C for 5 min (Hartman *et al*., 2008). It seems reasonable to assume that each type of food responds differently with regards to polyphenol stability when subjected to different thermal processes. This may be ascribed to the difference in the food matrix. The food matrix can act as a barrier to heat effect or induce the degradation polyphenols that exist as polymers and oligomers. It is very difficult to dissociate the thermal processing effect from the food matrix effects when discussing changes that occur due to thermal processing (Irina and Mohamed, 2012).

The antioxidant activities of both the cooked red beans and red bambara groundnuts were higher compared to the uncooked. The free radical scavenging speed increased 10-fold in the presence of methanolic extract from cooked red bambara groundnuts compared to uncooked. By contrast, there was a 20-fold increase in the presence of the methanolic extract from cooked red beans compared to uncooked. This finding is of great significance because free radicals react very quickly and require equally fast-acting scavengers. Yet again, extracts from the cooked red beans had greater FRAP derived total antioxidant power compared to the uncooked. However, the FRAP values for the uncooked and cooked red bambara groundnuts were not significantly different. Changes in the antioxidant activities are positive and may be attributed to the increase in the concentrations of total polyphenols and individual phenolic compounds. Further, the increase in the speed of DPPH free radical scavenging reaction by the antioxidants in the cooked legumes may be attributed to the new emerging compounds. There is a possibility that the emergent compounds have good free radical scavenging

abilities. An increase in the antioxidant activity of various foods subjected to thermal processes has been reported by many workers (Chandrasekara and Shahidi, 2011; Freeman *et al.*, 2010; Hartman *et al*., 2008; Sharma and Gujral 2011). Emergent compounds can have antioxidant activity sometimes higher than the initial phenolic compounds (Buchner *et al*., 2006). Due to thermal processing, synergies between antioxidant compounds and the food matrix can occur resulting into enhanced antioxidant activity of polyphenolic compounds (Wang *et al*., 2011). Increase in the antioxidant acitivity of flavonoids has been reported in thermally treated food matrix (Freeman *et al*., 2010). It seems that products of degradation, which are assumed to be more reactive than the intial phenolic compound and the synergistic interaction between the antioxidant compounds and the food matrix are key factors that may be ascribed to the enhanced antioxidant activity of thermally processed foods in some cases.

Conclusion

The study has demonstrated that cooking has positive effects on the antioxidant activities and phenolic phytochemical profiles of common beans and bambara groundnuts. In both common beans and bambara groundnuts, antioxidant activities are higher in the cooked samples compared to the uncooked. Cooking favoured the release of phenolic compounds and subsequently resulted in high concentrations of total polyphenols and individual phenolic compounds. Additionally, HPLC-PDA-ESI-MS advanced analytical technique revealed that new phenolic compounds emerge after cooking, and these may have an additive effect on the antioxidant activities. New compounds tentatively identified in cooked red bambara groundnuts include caffeic acid hexoside, gallocatechin, resveratrol glucoside and a caffeic acid derivative. In cooked red beans, new compounds tentatively identified include catechin glucoside, quercetin-3-O-glucoside, kaempferol glucoside and a quercetin conjugate. Cooking therefore enhances the nutraceutical profiles in both common beans and bambara groundnuts.

Chapter 5

Antioxidant properties and polyphenol profiles of bambara groundnuts and common beans as affected by sprouting

Introduction

Another processing method which is becoming popular at household level in Africa is sprouting of the seed legumes. Sprouting is the way of germinating seeds to be eaten either raw or cooked and is an opportune way to have fresh vegetables at any time of the year. Seeds can be sprouted at home or industrially. In this section, sprouting effects of bambara groundnuts and common beans on the antioxidant properties and polyphenolic profiles are reported. Red beans and red bambara groundnuts market classes studied in Chapter 4 were investigated. As was the case in Chapter 4, the effects of sprouting on the antioxidant properties of red bambara groundnuts and red beans are reported on the basis of free radical scavenging activity and ferric reducing power of the methanolic extracts from the sprouted samples. The free radical scavenging potential of the methanolic extracts is reported based on the kinetic behaviour of the DPPH free radicals with antioxidants in the two legumes. The ferric reducing power is reported on the basis of the number of mmoles Fe^{2+} produced in the reduction of Fe^{3+} by the antioxidants in the extracts.

Results

5.1. Sprouts characteristics

Sprouted red bambara groundnuts and red beans are presented in Tables 5-1. For the experiments outlined below, seeds were harvested on the 8^{th} day of germination when 100% and 98% germination capacities were recorded in red beans and red bambara groundnuts respectively. It is noticeable that red bambara groundnuts showed a slightly lower germination capacity than red beans (Tables 5-2).

Table 5-1 Sprouted red beans and red bambara groundnuts

Red beans	
Red bambara groundnuts	

Table 5-2 Germination capacity of red bambara groundnuts and red beans

	Number of seeds germinated (out of 100)
Red beans	100 (0)
Red bambara groundnuts	98 (1.5)

Numbers in parentheses are standard deviations of duplicate determinations

5.2. Free radical scavenging activity of the sprouted red bambara groundnuts and red beans extracts

Figure 5-1 gives the disappearance pattern of the DPPH free radicals in the present of the extract from sprouted red bambara groundnuts. The disappearance pattern of the DPPH free radicals was biphasic, characterised by the fast initial decay, followed by the slow step. The amount of the DPPH free radicals scavenged and the scavenging speed for the unsprouted and sprouted seeds were significantly different (Figure 5-1 and Table 5-3). By the end of the assay period, 58% of the DPPH free radicals were scavenged by the extract from the sprouted seeds compared to 32.0% scavenged by the extracts from the unsprouted seeds. In comparison to the positive reference standard trolox, the radical scavenging capacity of the extracts from the sprouted seeds can be considered moderate. Considering that trolox is a pure compound and a very strong antioxidant, this observation reveals a good perspective for the sprouted bambara groundnuts with regards to antiradical activity.

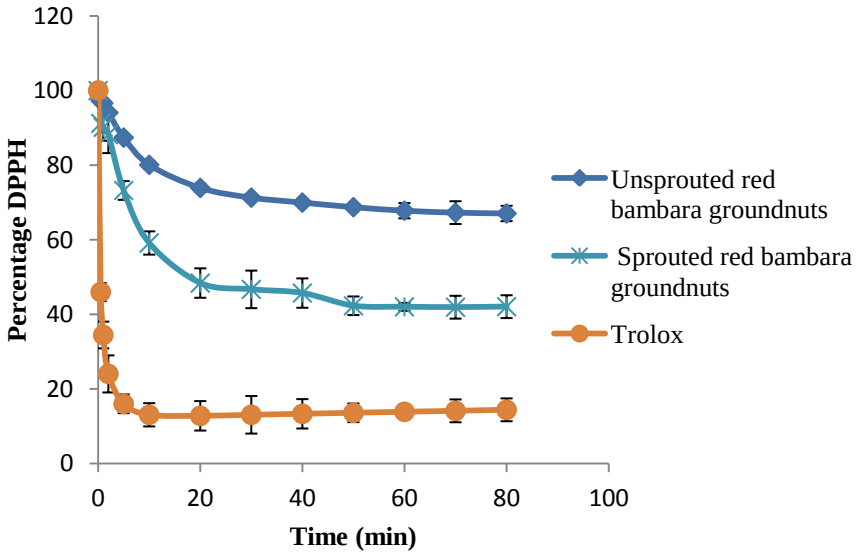


Figure 5-1 Disappearance pattern of DPPH free radicals with time in the presence 70 % methanolic extracts of sprouted red bambara groundnuts.

Table 5-3 Pseudo-first order rate constant of antiradical (Y-H) in sprouted red bambara groundnuts and red beans and the amount of DPPH scavenged after 80 of minutes incubation

Market classes of bambara groundnuts and common beans		Pseudo-first order rate constant (K) [min^{-1}]	Amount DPPH quenched [%] after 80 minutes incubation
Red bambara groundnuts	Control (unsprouted)	0.042	32.03 ± 2.30
	Sprouted	0.056	57.91 ± 3.96
Red beans			
	Control (unsprouted)	0.049	61.91 ± 2.81
	Sprouted	0.095	77.1 ± 4.26

Trolox K = 1.55, amount quenched in 80 minutes = 85.5%

The disappearance pattern of the DPPH free radicals in the present of the extract from sprouted red beans is presented in Figure 5-2. As was the case for red bambara groundnuts, the disappearance pattern of the DPPH free radicals was characterised by the fast initial decay, followed by the subsequent slower step. The amount of the DPPH free radicals scavenged and the scavenging speed for the unsprouted and sprouted seeds were significantly different (Table 5-3 above). The sprouted seeds displayed better kinetics than the unsprouted seeds. By the end of the assay period, the amount of DPPH free radicals scavenged by the extract from the sprouted beans (77%) was a little closer to that scavenged by the positive reference standard trolox (86%). As discussed above with respect to bambara groundnuts, this observation reveals a good perspective for the sprouted beans regarding antiradical activity.

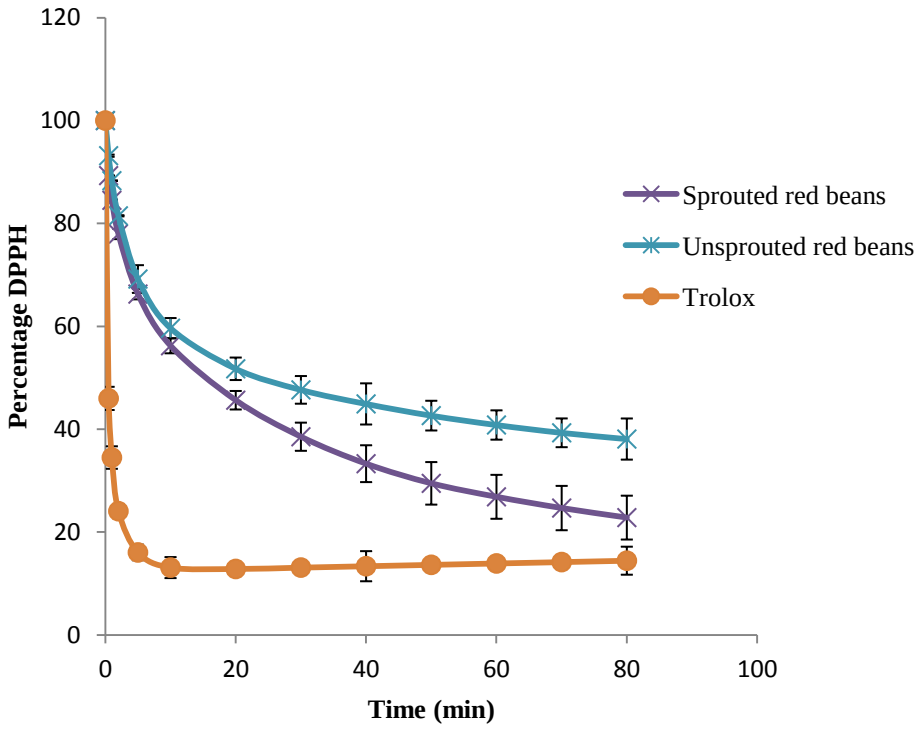


Figure 5-2 Disappearance pattern of DPPH free radicals with time in the presence of 70% methanolic extracts of sprouted red beans

5.3. Ferric Reducing Antioxidant Power of the sprouted red bambara groundnuts and red beans extracts

The influence of sprouting on the ferric reducing power of red bambara groundnuts and red beans is shown in Table 5-4. The FRAP value of the sprouted red bambara groundnuts was higher compared to that of unsprouted groundnuts. The difference in FRAP values was significant. As observed for red bambara groundnuts, the FRAP value of the sprouted red beans was higher compared to the unsprouted beans and the difference was significant. Most likely, sprouted seeds contained more antioxidant compounds with ferric reducing properties than the unsprouted seeds.

.

Table 5-4 FRAP values of sprouted red bambara groundnuts and red beans

Market classes of bambara groundnuts and common beans		FRAP values (mmole Fe^{2+}/ 100g DW)
Red bambara groundnuts	Control (unsprouted)	8.60 ± 0.09
	Sprouted	10.75 ± 0.12
Red beans		
	Control (unsprouted)	4.62 ± 0.12
	Sprouted	6.51 ± 0.18

5.4. Polyphenol profiles of sprouted red bambara groundnuts and red beans

Sprouting showed some effects on the antioxidant activities of red bambara groundnuts and red beans and thus the effect of sprouting on the total polyphenol contents of red bambara groundnuts and red beans was investigated. We also investigated the effects of sprouting on the concentration of individual phenolic compounds and the HPLC-PDA-ESI-MS profiles of the sprouted seeds.

5.4.1. Total polyphenol levels in sprouted red bambara groundnuts and red beans

The total polyphenol concentrations of the sprouts of red bambara groundnuts and red beans are presented in Table 5-5. Sprouting displayed a positive effect on the total polyphenol concentration of both the red bambara groundnuts and red beans. In red bambara groundnuts, the total polyphenol content increased by 1.3-fold after sprouting. The difference in the total polyphenol concentration of the sprouted and unsprouted seeds was significant.

In red beans, total polyphenol concentration increased by 3-fold after sprouting. As observed above with respect to red bambara groundnuts, the difference in the total polyphenol concentration of the sprouted and unsprouted seeds was significant. Increase in the concentration of total polyphenols after sprouting has been reported in other leguminous seeds. Oloyo (2004) reported a 5-fold increase in total polyphenol content of *Cajanus* seeds after 5 days of germination. The increase in the free radical scavenging activity and the ferric reducing power of the extracts from both the sprouted red bambara groundnuts and red beans

as observed above may partly be due to the increase in the concentrations of total polyphenols.

Table 5-5 Polyphenol concentration of different day sprouts of red bambara groundnuts and red beans

Market classes of bambara groundnuts and common beans		Total polyphenols (mg GAE / 100 g DW)
Red bambara groundnuts	Control (unsprouted)	135.9 ± 2.0
	Sprouted	174.2 ± 4.8
Red beans		
	Control (unsprouted)	84.5 ± 4.8
	Sprouted	253.1 ± 5.7

5.4.2. HPLC-PDA-ESI-MS profiles of the sprouted red bambara groundnuts and red beans

Figures 5-3 and 5-4 present the HPLC-PDA-ESI-MS chromatograms of methanolic extract from the sprouted and unsprouted red bambara groundnuts. There were eleven emergent deprotonated molecules [M – H-] of m/z 341, 391, 205, 405, 189, 389, 463, 271, 543, 529 and 447 that emerged in the HPLC-PDA-ESI-MS profile as a result of sprouting (Figure 5-3).

The HPLC-PDA-ESI-MS chromatograms of the methanolic extract from the sprouted and unsprouted red beans are presented in Figures 5-5 and 5-6. There were eight new deprotonated molecules [M – H^-] of m/z 297, 587, 451, 429, 609, 463, 505 and 489 that emerged in the HPLC-PDA-ESI-MS profile as a result of sprouting (Figure 5-5).

For both red bambara groundnuts and red beans, there were variations in the peak heights (concentrations) for the compounds that were detected in the unsprouted and sprouted seeds. Peak heights were higher in the extracts of the sprouted than the unsprouted seeds. Higher concentrations for these compounds may also be responsible for the increase in the free

radical scavenging activity and ferric reducing power of the sprouted samples. Furthermore, the emergent compounds may also have an effect on the antioxidant activities of the sprouts.

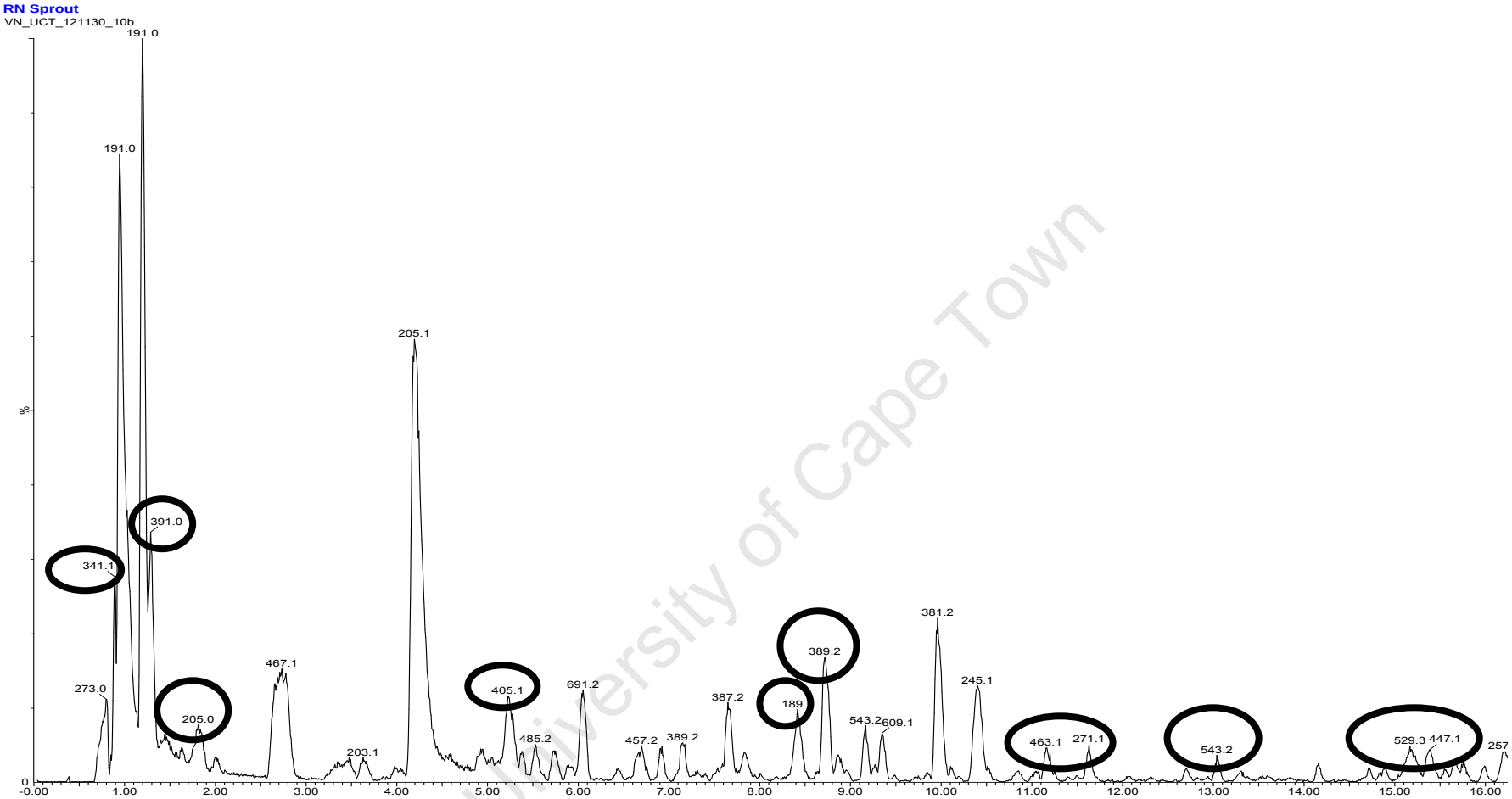


Figure 5-3 HPLC-PDA-ESI-MS chromatogram of 70% methanol extract of sprouted red bambara groundnuts.

O represents emergent compounds after processing. Number on top of each peak is the m/z of the deprotonated molecule $[M - H]^-$ of each compound.

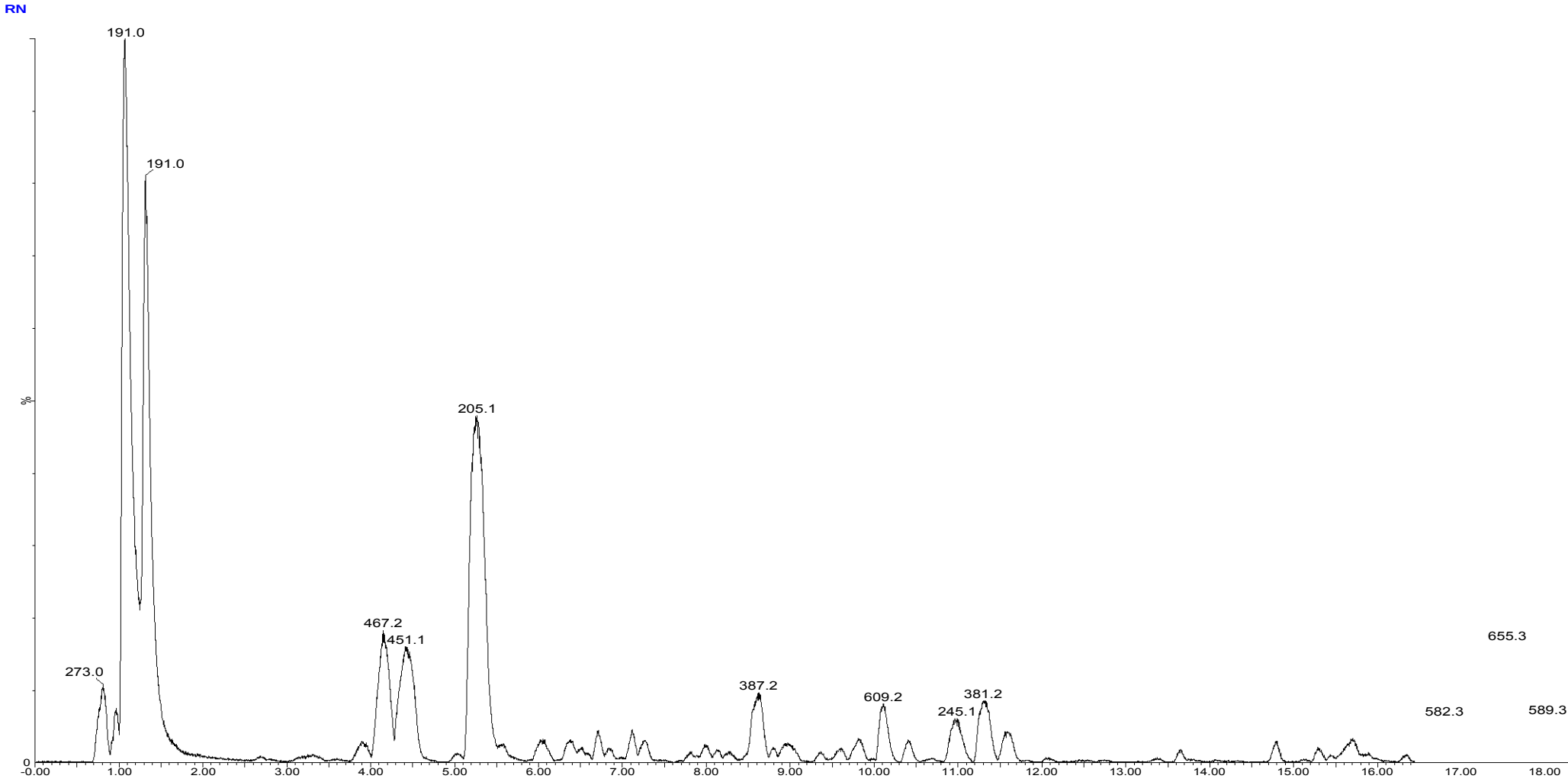


Figure 5-4 HPLC-PDA-ESI-MS chromatogram of 70% methanol extract of the unsprouted red bambara groundnuts.

Number on top of each peak is the m/z of the deprotonated molecule $[M - H]^-$ of each compound.

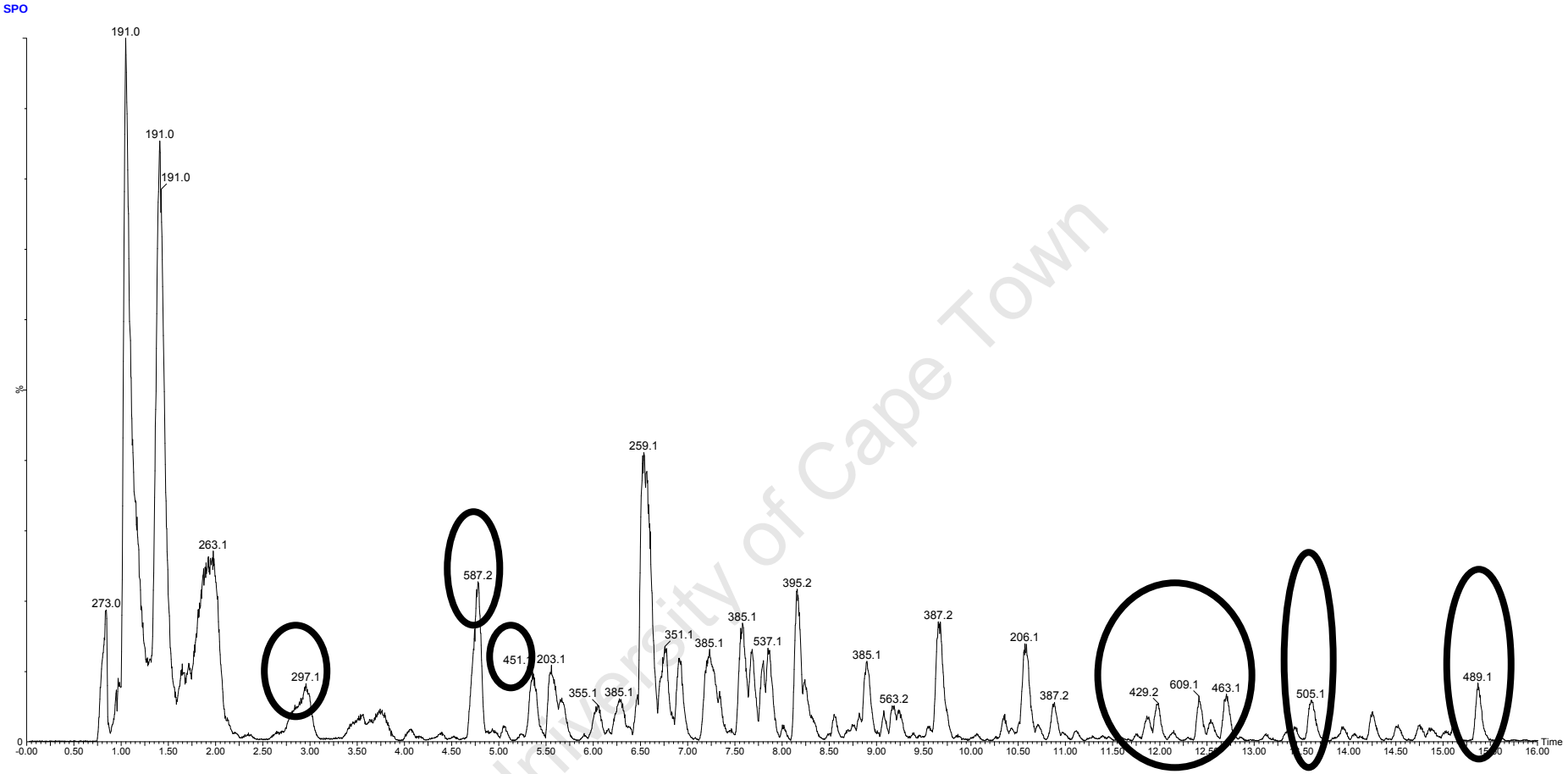


Figure 5-5 HPLC-PDA-ESI-MS chromatogram of 70% methanol extract of sprouted red beans.

O represents emergent compounds after processing. Number on top of each peak is the m/z of the deprotonated molecule $[M - H]^-$ of each compound.

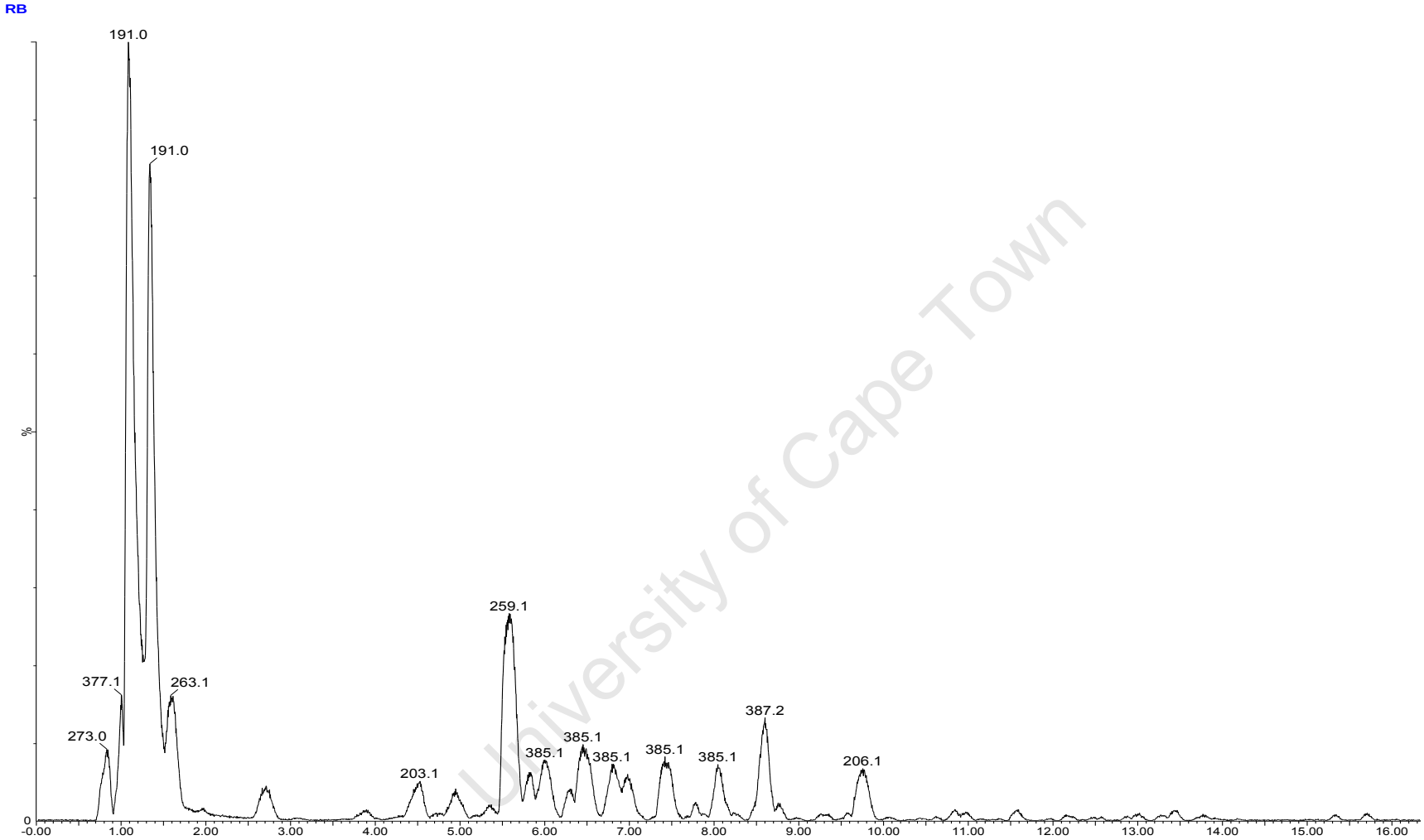


Figure 5-6 HPLC-PDA-ESI-MS chromatogram of 70% methanol extract of the unsprouted red beans.

Number on top of each peak is the m/z of the deprotonated molecule $[M - H]^-$ of each compound.

Quantification of individual phenolics using available standards was also done for the extracts from both the unsprouted and sprouted seeds. The results are presented in Table 5-6. In red bambara groundnuts, catechin increased by 5%, epicatechin by 12%, *t*-ferrulic acid by 17% and contrary, *p*-coumaric decreased by 2%. In red beans, catechin and epicatechin increased by 62 and 61% respectively. However, there was a decrease in the concentration of *t*-ferrulic acid and *p* – coumaric acid by 73 and 22% respectively.

Table 5-6 Concentration of individual phenolic compounds in sprouted red bambara groundnuts and red beans

Market classes of bambara groundnuts and common beans		Individual phenolic compounds (mg / kg DW)			
		Catechin	**Epicatechin**	***p*-coumaric acid**	***t*-ferrulic acid**
Red bambara groundnuts					
	Unsprouted	266.4±1.1	17.3 ± 0.3	5.22 ± 0.2	3.29 ± 0.1
	Sprouted	280.8±5.8	19.4 ± 1.5	5.10 ± 0.1	3.85 ± 0.2
Red beans	Unsprouted	108.6 ± 4.9	7.5 ± 0.2	2.73 ± 0.1	20.5 ± 3.2
	Sprouted	284.6 ±3.2	19.3 ± 0.7	2.23 ± 0.5	11.8 ± 1.1

Based on the literature data and fragmentation pattern, attempts were made to identify the new emerging coumpounds in both the spouted red beans and red bambara groundnuts. Some emergent compounds that were tentatively identified in red bambara groundnuts and red beans are presented in Tables 5-7 and 5-8 respectively.

Table 5-7 Emerging phenolic compound tentatively identified in sprouted red bambara groundnuts

Parent ion [m/z]	Fragment ions [m/z]	Tentative identification
341	179, 161	Caffeic acid hexoside [v]
389	185,157,143	resveratrol glucoside [w]
271	151,119	Naringenin [x]
529	368,367, 179	caffeic acid derivative [y]
447	285	kaemferol glucoside [z]

[v] Hassain *et al.,* 2010, [w] Amandeep *et al.,* 2010, [x] Rabaneda *et al.,* 2003, [y] Gouveia and Castilho 2011, [z] Lin *et al.,* 2008

Table 5-8 Emerging phenolic compound tentatively identified in sprouted red beans

Parent ion [m/z]	Fragment ions [m/z]	Tentative identification
451	289	Catechin glucoside [a]
429	217, 285	Luteolin hexoside [b]
609	301	Quercetin -3-rutinoside [c]
463	301	Quercetin -3-O-lucoside [c]
505	301	Quercetin glucoside acylated [a]
489	155, 163, 327	*p*- coumaric acid hexoside [b]

[a]Estrella *et al.,* 2011, [b]Palafox-Carlos *et al.,* 2012, [c]Lin *et al.,* 2008,

Discussion

The red beans and red bambara groundnuts used in the sprouting experiment were suitable for sprouting, though the germination capacity in red beans was higher compared to red bambara groundnuts. Changes that occurred as a result of sprouting were positive and beneficial in both common beans and bambara groundnuts. According to Brajdes and Vizireanu (2012), germination is the only process of agro-food processing which provides significant increase of the nutritional value by increasing the bioavailability of vitamins, bioelements and other biologically active compounds. Sprouted samples displayed positive changes in antioxidant activity in both legumes. After 8 days of sprouting of red bambara groundnuts, the free radical scavenging speed and FRAP derived antioxidant power increased 1.3-fold. After 8

days of sprouting in red beans, free radical scavenging speed increased by 2-fold and FRAP derived antioxidant power increased 1.4-fold respectively. The increase in the antioxidant activity as a result of sprouting has been reported previously in other seeds. Jimenez Martınez *et al.,* (2012) observed a 10% increase in the amount of DPPH free radicals scavenged by the extract from the *Campestris* L. Seeds that were sprouted for two days. After 9 days of sprouting in lupin seeds, the antioxidant capacity determined as TEAC of the hydrophilic extract, increased by 46% (Frias *et al.,* 2005). Kim *et al*., (2012) reported higher DPPH free radical scavenging activity of the methanol extracts of sprouted mungbeans than the dry seeds. At 2,000 mg / kg methanol extracts, DPPH activities of the seeds and the sprouts were 24.9% and 74.2 % respectively, showing higher activity in the sprouts. DPPH activities from all fractions from young sprouts and seeds at 2,000 mg / kg ranged from 18.5 to 90.9% and 13.5 to 24.9% respectively. Similarly, results from the investigation by Pasko *et al*., (2009) on various seeds showed that sprouts have a significantly higher antioxidant activity than dry seeds. The increase in the antioxidant activity of the sprouts can be attributed to the increase in the concentrations of total polyphenols and some individual phenolic compounds. Additionally, new compounds emerging after sprouting may have positive effects on the antioxidant activity.

Likewise, sprouting displayed a positive effect on the total polyphenol levels of the red bambara groundnuts and red beans. After 8 days of sprouting in red bambara groundnuts, total polyphenol levels increased 1.3-fold, whereas in red beans, there was a 3-fold increase. The HPLC-PDA-ESI-MS chromatograms of the methanolic extracts of the sprouted red bambara groundnuts and red beans revealed new emerging compounds. In red bambara groundnuts, eleven new compounds emerged. The new compounds tentatively identified include caffeic acid hexoside, resveratrol glucoside, a caffeic acid derivative, naringenin and kaempferol glucoside. In red beans, eight new compounds emerged. Catechin glucoside, quercetin-3-O-glucoside, quercetin-3-rutinoside, luteolin hexoside, quercetin glucoside acylated and *p*-coumaric acid hexoside were new compounds tentatively identified.

The increase in the total polyphenol levels as a result of sprouting has been reported previously in other seeds. Nwanguma *et al.,* (1996) found an increase of several fold in total phenolic content in all the four sorghum varieties after germination. According to the findings of Jimenez Martınez *et al.,* (2012), total polyphenol concentration increases by 2-fold in *Campestris* L. seeds after 8 days of germination. Oloyo (2004) reported a 5-fold increase in

total polyphenol content of *Cajanus* seeds after 5 days of germination. Methanolic extract of mungbeans that was sprouted for 7 days had total phenolics ranging from 166.5 to 191.7 mg ferulic acid equivalents (FAE) kg /DW whereas that for dry seeds ranged from 97.8 to 101 (FAE) kg/DW (Kim *et al*., 2012). A study by Brajdes and Vizireanu (2012) reported a number of important changes in the amount of biologically active compounds when buckwheat was germinated for 7 days. The amount of polyphenols increased from 50.36 to 298.3 mg GA / 100g DW, the amount of rutin increased from 13.66 to 283.42 mg / 100g DW, the amount of quercetin increased from 4.77 to 223.76 mg / 100g DW, whereas the amount of ascorbic acid increased from 0 to 1.09 mg / 100g DW

Changes in the total polyphenol concentration during sprouting may be attributed to the enzymatic activies in the seed during germination process. As mentioned earlier, polyphenols in the cell are bound to the cellular components (carbohydrates, pectin, lignin and proteins). During the enzymatic degradation of the said cellular components, bound phenolics may be released and made available for quantification. According to Brajdes and Vizireanu (2012), the increase of phenolic compounds in the germinated seeds can be explained by an increase in the amount of free forms occurring as a consequence of hydrolytic enzyme activity, due to the breakdown of the cell wall during germination. It can be assumed that conjugated phenolic acids are released from the breakdown of cell walls, maybe to protect the inner parts of the caryopsis which is still needed to support the developing germ (Engert *et al*., 2011).

Studies on the enzymatic release of bound phenolics from celluar components have been reported previously. Sinapic acid and *p*-coumaric acid from wheat bran were released by human colonic cinnamoyl esterase (Andreasen *et al.*, 2001). The release of the ferrulic acid of soluble feruloylated oligosaccharides by microbial esterase in the human colony was reported (Kroon *et al.*, 1997). Arnous (2009) reported the release of phenolic acid (both hydroxycinnamic and hydroxybenzoic), anthocyanins and quercetin during enzymatic (pectinolytic and cellulolytic) degradation of the cell wall polysaccharides of grape skin. Condensed tannins or proanthocyanidins that exist as oligomer and polymers may disintegrate into simple phenolics due to the activities of the hydrolytic enzymes. A study by Cheng et al., (2006) suggests that polyphenolic compounds such as tannins are broken and simple phenolics are released.

The reduction in the concentration of *t*-ferrulic and *p*-coumaric acids after sprouting in red beans did not have a significant effect on the total polyphenol content or the antioxidant activities because the two compounds were at much lower concentration in the unsprouted samples compared to others like catechin and epicatechin. Furthermore, the corresponding increase of catechin and epicatechin by over 50% coupled with the emergence of new compounds after sprouting might have contributed significantly to the total polyphenol content and antioxidant activities. The overall effect therefore, is that the nutraceutical profile was enhanced after sprouting.

Conclusion

The study has revealed that sprouting enhances the nutraceutical profiles of common beans and bambara groundnuts. After 8 days of sprouting of the red bambara groundnuts (at 98% germination capacity), and red beans (at 100% germination capacity), the antioxidant activities and the concentration of total polyphenols were higher compared to the unsprouted seeds. HPLC-PDA-ESI-MS advanced analytical technique revealed eleven new compounds in red bambara groundnuts and eight in red beans. Caffeic acid hexoside, resveratrol glucoside, a caffeic acid derivative, naringenin and kaempferol glucoside were new compounds tentatively identified in sprouted red bambara groundnuts. In red beans, new compounds tentatively identified include catechin glucoside, quercetin-3-O-glucoside, quercetin-3-rutinoside, luteolin hexoside, quercetin glucoside acylated and *p*-coumaric acid hexoside. The increase in the concentration of some phenolic compounds and the emergence of new ones after sprouting may be responsible for the enhanced nutraceutical profile.

Chapter 6

Thesis summary and future perspectives

6.1. Thesis summary

There is a growing interest in legumes and legume based foods because of the health claims associated with their consumption. With the high rates of chronic diseases such as cancer, diabetes, cardiovascular diseases and obesity, foods with health promoting properties are becoming popular. The aim of the current study was to explore the nutraceutical potential of the market classes of bambara groundnuts and common beans grown in Zambia based on the antioxidant activity and phenolic phytochemical profile. Because cooking and sprouting are popular methods of preparing legumes for consumption, their effects on the antioxidant activities and phenolic phytochemicals of the two food crops were examined. This study employed *in vitro* antioxidant assays (DPPH and FRAP) to screen for antioxidant properties and the HPLC-PDA-ESI-MS advanced analytical technique to identify phenolic phytochemicals in the Zambian market classes of bambara groundnuts and common beans. The DPPH is rapid, accurate and independent of sample polarity, whereas the FRAP is a rapid assay with little selectivity.

Like many neglected and under-utilized food crops, bambara groundnuts remain largely uncharacterised. This is the first detailed report on the nutraceutical aspects of bambara groundnuts. Bambara groundnuts in raw dry form demonstrated free radical scavenging capacities and FRAP derived total antioxidant power that are comparable to commonly consumed legumes such as lentils, common beans and chick peas. The brown market class had higher antioxidant activities and phenolic contents compared to the red one. HPLC-PDA-ESI-MS based identification of phenolic phytochemicals revealed that both the brown and red bambara groundnuts market classes contain various phenolic compounds that include Quinic acid, (E) GC –hexoside, catechin glucoside, a caffeic acid derivative, catechin, epicatechin, medioresinol, *p*-coumaric acid, salicylic acid and a catechin dimer. Besides the above mentioned phenolic compounds, myricetin hexoside, quercetin-3-O-rutinoside and quercetin-3-O-glucoside were identified in the red bambara groundnuts only. These compounds, which are mainly from the phenolic acid and flavonoids subclasses, have been reported previously to have various medicinal properties such as antioxidant and anti-inflamatory properties,

cholesterol lowering, anti-diarrhoea, antibacterial, antiviral and protective effects against coronary heart diseases. Bambara groundnuts therefore have the potential for use as nutraceuticals and their consumption could possibly offer some health benefits. The results from this research have provided valuable knowledge that may lead to the development of new natural products based on bambara groundnuts.

As mentioned above, the nutraceutical potential of common beans is well known based on the previous studies. The interest on common beans in this study, however, was to screen the market classes that are commonly grown in Zambia with the view of providing information for a preliminary database that may be useful to researchers and other stakeholders involved in its breeding. The four market classes of common beans in raw dry form demonstrated differences in phenolic phytochemical profile. Red beans showed the highest total polyphenol contents followed by grey mottled, brown and white respectively. Differences in total polyphenol contents were significant. The tannin concentrations of the red and grey mottled were not significantly different. White beans had the lowest tannin concentration. The concentration of total flavonoids varied greatly and showed different trends depending on the solvent used. In the methanolic extract, red beans displayed the highest total flavonoid content, followed by the white, brown, and grey mottled respectively. In the aqueous extract, the white beans displayed the highest total flavonoid concentration, followed by brown, grey mottled and red in that order.

HPLC-PDA-ESI-MS based identification revealed various phenolic compounds, some of which were common in all the market classes, whereas others were variety associated. Quinic acid, syringic acid derivative, ferulic acid derivatives, medioresinol, *p*-coumaric acid and ferulic acid were present in all the market classes. Four isomers of ferulic acid derivatives were observed in red, three in brown, two in white and one in grey mottled beans. Catechin and gallic acid were only identified in the red, grey mottled and brown beans. Epicatechin was only identified in the red and grey mottled beans. A compound with a molecular ion at m/z 567, tentatively identified as a flavonone derivative was only observed in the red beans. Catechin glucoside was only identified in grey mottled and brown beans. Compounds tentatively identified as kaempferol glucoside and carnosol were only observed in brown beans.

Screening of the market classes of common beans based on antioxidant activity revealed the following order of ranking: red beans > grey mottled beans > brown beans > white beans. White beans displayed far lower antioxidant activities compared to the other varieties. Variations in the antioxidant behaviour were attributed partly to the differences in the polyphenolic compound concentrations and the types of phenolic compounds in the market classes of common beans. White beans had the lowest total polyphenol content and were found not to contain important polyphenols such as catechin, epicatechin and catechin glucoside from the flavonol sub group. Positive correlations between total phenolic content and the antioxidant activities were observed. Diversity in the phenolic phytochemical profile and antioxidant properties of the four market classes of common beans investigated offers an excellent opportunity for genetic improvement in the nutraceutical attributes of these market classes by crop breeders.

Domestic cooking demonstrated positive effects on the antioxidant activity and phenolic phytochemical profile of both bambara groundnuts and common beans. The free radical scavenging speed increased 20-fold in the presence of methanolic extract from the cooked common beans compared to the uncooked. By contrast, there was a 10-fold increase in the presence of the methanolic extract from the cooked bambara groundnuts compared to uncooked. This observation in both bambara groundnuts and common beans is of great significance because the action of free radicals is very rapid and requires equally fast reactions from the antioxidants in order to prevent damage. Again, extracts from the cooked bambara groundnuts and common beans had greater FRAP derived total antioxidant power compared to the uncooked. The total polyphenol content increased by 6 and 41 % in the cooked red bambara groundnuts and red beans compared to the uncooked respectively. The concentration of catechin, epicatechin, *p*-coumaric and *t*-ferulic acid were greater in the cooked red bambara groundnuts and red beans compared to the uncooked. In bambara groundnuts, epicatechin increased by 92%, catechin (54%), *t*-ferrulic acid (39%), *p*-coumaric acid (30%) and by contrast, salicylic acid decreased by 59%. In red beans, epicatechin increased by 96%, catechin (80%), *p*-coumaric (41%) and *t*-ferrulic acid (40%) respectively. However, salicylic acid was completely missing in red beans after cooking. HPLC-PDA-ESI-MS profiles revealed ten and five new compounds in the cooked red bambara groundnuts and red beans respectively. In cooked red bambara groundnuts, new compounds tentatively identified include caffeic acid hexoside, gallocatechin, resveratrol glucoside and a caffeic acid derivative. New compounds tentatively identified in cooked red beans include catechin

glucoside, quercetin-3-O-glucoside, kaempferol glucoside and a quercetin conjugate. Cooking therefore was found to have positive effects as it enhances the nutraceutical profiles in both bambara groundnuts and common beans.

Sprouting similarly displayed positive effects on the antioxidant activity and phenolic phytochemical profiles of red beans and red bambara groundnuts. After 8 days of sprouting of red bambara groundnuts (at 98% germination capacity), the free radical scavenging speed, FRAP derived antioxidant power increased and total polyphenol levels increased by 1.3-fold. After 8 days of sprouting of red beans (at 100% germination capacity), free radical scavenging speed increased by 2-fold, FRAP derived antioxidant power by 1.4-fold and total polyphenol levels by 3-fold respectively. Changes in the concentrations of individual phenolic compounds were observed after sprouting. In red bambara groundnuts, catechin increased by 5%, epicatechin (12%), *t*-ferrulic acid (17%) and by contrast, *p*-coumaric decreased by 2%. In red beans, catechin and epicatechin increased by 62 and 61% respectively. However, there was a decrease in the concentration of *t*-ferrulic acid and *p* – coumaric acid by 73 and 22% respectively. The reduction in the concentration of *t*-ferrulic and *p*-coumaric acids after sprouting in red beans did not have an effect on the overall total polyphenol content or the antioxidant activities because the two compounds were at much lower concentrations compared to others like catechin and epicatechin, which subsequently increased by over 50% after sprouting. The HPLC-PDA-ESI-MS chromatograms of the methanolic extracts of sprouted red bambara groundnuts and red beans revealed new emerging compounds. In red bambara groundnuts, eleven new compounds emerged. The new compounds tentatively identified include caffeic acid hexoside, resveratrol glucoside, a caffeic acid derivative, naringenin and kaempferol glucoside. In red beans, eight new compounds emerged. Catechin glucoside, quercetin-3-O-glucoside, quercetin-3-rutinoside, luteolin hexoside, quercetin glucoside acylated and *p*-coumaric acid hexoside were new compounds tentatively identified. Sprouting therefore enhances the nutraceutical profiles of the two legumes.

6.2. Future perspectives

This study has provided valuable information for a preliminary database to stimulate further interest among researchers on bambara groundnuts in the area of nutraceuticals. As this was a screening project, nutraceutical potential of bambara groundnuts was assessed based on their antioxidant properties and phenolic phytochemicals. However, this is just the beginning of a

journey in the nutraceutical pipeline of bambara groundnuts. Other studies to evaluate them for compounds such as α-glucosidase, α-amylase and lipase inhibitors may be undertaken in view of escalating diabetes and obesity, and since bambara groundnuts are natural products, most likely there may be no adverse effects compared to synthetic drugs. It is also important to further screen them for other compounds such as phytosterols and fatty acid antioxidants that are also claimed to have health benefits. To fully acknowledge bambara groundnuts as nutraceutical targets, further studies looking at the bioavailabity and bioactivity of isolated compounds in animal models would be required followed by *in vivo* clinical trials.

References

1. Adom, K.K., Sorrells, M.E., & Liu, R.H. (2003), 'Phytochemical profiles and antioxidant activity of wheat varieties', J. *Agric. Food Chem.* **51**, 7825 – 7834.
2. Adon, K.K. & Liu, R.H. (2002), 'Antioxidant activity of grains', *J.Agric. Food Chem.* **50 (21),** 6182-6187.
3. Agriculture and Agric – Food Canada (2000), 'Dry Beans: Situation and Outlook', *Bi- weekly bulletin* (16) No 13.
4. Akıllıoğlu, G. H. & Karakaya, S. (2009), 'Effect of Some Domestic Cooking Methods on Antioxidant Activity, Total Phenols and Total Flavonoid Content of Common Beans', *Akademil Gida* **7(6),** 6-12.
5. Akillioglu, G.H. & Karakaya, S. (2010), 'Changes in Total Phenols, Total Flavonoids, and Antioxidant Activities of Common Beans and Pinto Beans after Soaking, Cooking, and in vitro Digestion Process', *Food Sci. Biotechnol.* **19(3),** 633-639.
6. Amandeep, K. sandhu & Liwei G. (2010), 'Antioxidant Capacity, Phenolic Content, and Profiling of Phenolic Compounds in the Seeds, Skin, and Pulp of *Vitis rotundifolia* (Muscadine Grapes) As Determined by HPLC-DAD-ESI-MSn', *J.Agric. Food Chem.* **58**, 4681–4692 4681.
7. Amarowicz, A., Carle, R., Dongowski, G. et al. (2009), 'Influence of postharvest processing and storage on the content of flavonoids and phenolic acids in foods', *Mol. Nutr. Food Res*., **53,** S151–S183.
8. Amarteifio, J.O., Karikari, S.K. & Modise, O.J. (2002), 'The proximate and mineral composition of six landraces of Bambara groundnuts', *Tropical Science* **42,** 188-191.
9. Amarteifio, J.O., Tibe, O. & Njogu, R.M. (2006), 'The mineral composition of Bambara groundnuts (*Vigna subterranean* (L) Verdc) grown in Southern Africa', *Afr. J. Biotechnol*.**5 (23),** 2408-2411.
10. Amic, D., Beslo, D. & Trinajstic N. (2003), 'Structure-radical scavenging activity relationships of flavonoids', *Croatica Chemica Acta* **76,** 55-61.
11. Anderson, J. W., Smith, B. M., & Gustafson, N. J. (1994), 'Health benefits and practical aspects of high-fiber diets', *Am J Clin Nutr.* **59**, S1242-S1247
12. Anderson, J.W., Story, L., Sieling, B., Chen, W.J.L., Petro, M.S. & Story, J. (1984), 'Hypocholesterolaemic effects of oat-bran or beans intake for hypercholesterolaemic men', *Am. J. Clin. Nutr*. **40,** 1146-1155.

13. Andreasen, M. F., Kroon, P. A., Williamson, G., & Garcia-Conesa, M.T. (2001), 'Esterase activity able to hydrolyze dietary antioxidant hydroxycinnamates is distributed along the intestine of mammals', *J.Agric. Food Chem.* **49,** 5679-5684.
14. Arnous, A. (2009), 'Enzymatic release of phenolics from fruit skin – with grape as the main model', PhD thesis, Technical University of Denmark.
15. AOAC. (2006), 'Methods of Analysis - Official methods 923.03, 923.05, 925.09, 962.09, and 979.09.', Association of Official Analytical Chemists, (Vol. II 17th edition) of AOAC International, Washington, DC, USA.
16. Aparicio-Fernández X.,Yousef G., Loarca-Pina G., De Mejia E. & Ann-Lila M. (2005), 'Characterization of polyphenolics in the seed coat of Black Jamapa beans (*Phaseolus vulgaris* L.)', *J.Agric. Food Chem, Volume*: 53, Issue: **11**, 4615-4622
17. Aparicio-Fernández, X., García-Gasca, T., Yousef, G.G., Lila, M. A., González de Mejía, E. & Loarca-Piña G. (2006), 'Chemopreventive activity of polyphenolics from black Jamapa Beans (*Phaseolus vulgaris* L.) on HeLa and HaCaT cells', *J. Agric. Food Chem*. **54,** 2116-2122.
18. Azam-Ali, S. N, Aguilar-Manjarrez, J. Karikari S.K & Massawe F.J. (2001), 'Assessing the Potential of an Underutilized Crop: A Case study using Bambara Groundnuts', *Expl Agric*. **(37),** 433 – 472.
19. Azam–Ali, S. N. (1992), 'Evaluating the potential of bambara groundnuts (*Vigna subterranean L. Verdc)* as a food crop for semi-arid Africa', In: a paper given at the proceedings of the third SADC Regional Conference on land and water Management, Harare, October, 1997.
20. Barampana, Z. & Simard, E.R. (1993), 'Nutrient composition, protein quality and antinutritional factors of some varieties of dry beans (*Phaseolus vulgaris*, L) grown in Burundi', *Food Chemistry* **47,** 159-167.
21. Baudoin, J. P. & Mergeai, G. (2001), 'Grain Legumes', In: Crop production in Tropical Africa, 313 – 317.
22. Becker, E.M., Nissen, L.R. & Skibsted, L.H. (2004), 'Antioxidant evaluation protocols: Food quality or health effects', *Eur. Food Res. Technol*. **19,** 561-571.
23. Beecher, G.R. (2003). 'Overview of Dietary Flavonoids: Nomenclature, Occurrence and Intake', *J Nutr*. **133,**3248-3254.
24. Beninger, C. W. & Hosfield, G. L. (2003), 'Antioxidant activity of extracts, condensed tannins fractions, and pure flavonoids from *Phaseolus* V*ulgaris* L. Seed coat color genotypes', *J. Agric. Food Chem.* **51,** 7879-7883.

25. Beninger, C.W., Hosfield, G.L. & Nair M.G. (1998), ' Glycosides from the seed coat of a new Manteca-type dry beans (phaseolus vulgaris L.)', *J. Agric. Food Chem*. **46**, 2906 – 2910.
26. Benzie, I. F. F. (1996). *Clin. Biochem.* **29**, 111–116.
27. Bioversity International (2005), 'Crop Wild Relatives - Global Portal', Via dei Tre Denari 472/a 00057 Maccarese (Fiumicino) Rome Italy.
28. Birk, Y. (1996), 'Protein proteinase inhibitors in legume seeds – overview', *Arch Latinoam Nutr*. **44(4 Suppl 1),** 26S-30S
29. Bishnoi, S. & Khetarpaul, N. (1993), 'Variability in physico-chemical properties and nutrient composition of different pea cultivars', *Food Chemistry,* **47,** 371– 373.
30. Blair, M.W., Astudillo, C., Beebe, S.E., Roa, I., Kimani, P. & Chirwa R. (2009), 'Biofortification breeding of common beans (Phaseolus vulgaris L.)', *Biozoom* **1,** 25-8.
31. Brajdes C. & Vizireanu C. (2012), 'Sprouted buchwheat, an important vegetable source of antioxidants', Food Technology, **36**, 53 – 60.
32. Brink, M., Ramolemana, G.M & Sibuga, K.P. (2006), '*Vigna subterranean* (L.) Verdc', PROTA (Plant Resources of Tropical Africa), Wageningen, Netherlands.
33. Brough, S. H. & Azam-Ali, S. N. (1992). The effect of soil moisture on the proximate composition of bambara groundnuts (Vigna subterranea L. Verdc). *Journal of the Science of Food and Agriculture* **60,** 197 - 203.
34. Brough, S. H., Taylor, A. J. & Azam-Ali, S. N. (1993), 'The potential of bambara groundnuts (Vigna subterranea) in vegetable milk production and basic protein functionality systems', *Food Chemistry* **47,** 277 - 283.
35. Brown, L., Rosner, B., Willett, W.W. & Sacks, F.M. (1999), 'Cholesterol lowering effects of dietary fibre: A meta-analysis', *Am J Clin Nutr.* **69**, 30 – 42.
36. Buchner, N., Krumbein, A., Rhon, S. & Kroth, L.W. (2006), ' Effect of thermal processing on the flavonol rutin and quercetin', *Rapid Communication in Mass Spectrometry*, **20**, 3229 – 3235.
37. Cardador-Martínez, A., Loarca-Piñan, G. & Oomahn, B. D. (2002), 'Antioxidant activity in common beans (*Phaseolus Vulgaris* L.)', *J.Agric. Food Chem*. **50,** 6975-6980.
38. Carlsen, H., Bente, L., Holte1 K., Siv, K. Steinar B., *et al.*, (2010), 'The total antioxidant content of more than 3100 foods, beverages, spices, herbs and supplements used worldwide', *Nutrition Journal* **9,** 3.

39. Chandrasekara, N. & Shahidi, F. (2011). Effect of roasting on phenolic content and antioxidant activities of whole cashew nuts, kernels and testa', *J.Agric. Food Chem.* **59,** 5006 – 5014.
40. Cheynier, V. (2005), 'Polyphenols in foods are more complex than often thought', *American Journal of Clinical Nutrition* **81**(suppl), 223S-9S.
41. Cheng, Z., Su, L., Moore, J., Zhou, K., Luther, M. & Yin J.J. (2006), 'Effects of post harvest treatment and heat stress on availability of wheat antioxidants', *J.Agric. Food Chem.*, **54**, 5623 – 5629.
42. Chung, T., Wong, T.Y., Wei, C.I, Huang, Y.W. & Lin Y. (1998), 'Tannins and human health: A review', *Crit. Rev. Food Sci. Nutr*. **38**, 421–464.
43. Cieslik, E., Greda, A. & Adams, W. (2006), 'Contents of polyphenols in fruits and vegetables', *Food Chemistry* **94,** 135-142.
44. Collinson, S.T., J. Berchie & S.N. Azam-Ali, (1999). The effect of soil moisture on light interception and the conversion coefficient for three landraces of bambara groundnuts (*Vigna subterranea*). *Journal of Agricultural Science* **133,** 151-157
45. Collinson, S.T., Sibuga, K.P., Tarimo, A.J.P. & Azam-Ali, S.N. (2000), 'Influence of sowing date on the growth and yield of bambara groundnuts landraces in Tanzania', *Experimental Agriculture* **36**, 1-13
46. Cook, S.I. & Sellin, J.I. (1998), 'Review article: Short chain fatty acids in health and disease', Aliment *Pharm Ther*. **12**, 499 – 507.
47. Crozier, A., Burns, J., Aziz, A.A., Stewart A.J, Rabiasz H.S., *et al.*, (2000), 'Antioxidant flavonols from fruits, vegetables and beverages: measurements and bioavailability', *Bio. Res.* **33(2),** 79 – 88.
48. Danesi (2009), 'PhD thesis Biological Effects of Bioactive Components and Extracts derived from Edible plants Commonly used in Human Nutrition', PhD thesis, University of Bologna
49. Davila, M.A., Sangronis, E. & Granito, M. (2003), 'Germinated or fermented legumes: food or ingredients of functional food', *Arch Latinoam Nutr*. **53(4)** 348-54.
50. Deshpande, S.S. & Cheryan, M. (1987), 'Determination of phenolic compounds of dry beans using vanillin, redox and precipitation assays', *Journal of Food Science* ***52,*** 332-334.
51. Dinelli, G., Bonetti, A., Minelli, M., Marotti I., Catizone P. & Mazzanti A. (2006), 'Content of flavonols in Italian beans (*Phaseolus vulgaris*, L.) ecotypes', *Food Chemistry* **99,** 105-114.

52. Dobiáš, P., Pavlíková, P., Adam, M., Eisner, A., Beňová, B. & Ventura, K. (2010), 'Comparison of pressurised fluid and ultrasonic extraction methods for analysis of plant antioxidants and their antioxidant capacity', *Cent. Eur. J. Chem.* ***8,*** 87-95.
53. Doku, E.V. & Karikari, S.K. (1969), 'Growth habit and pod production in Bambara groundnuts (*Voandzeia subterranea)*', *Ghana J. Agric. Sci.* **2,** 91-95.
54. Doku, E.V. (1995), 'Ghana country report on Bambara groundnuts', Proceedings of the workshop on Conservation and Improvement of Bambara Groundnuts (*Vigna subterranea* (L.) Verdc.) 14–16 November, Harare, Zimbabwe, in: Heller, J. Begemann, F. and Mushonga, J. (Eds.), 128-134.
55. Dugas Jr, A.J., Castaneda-Acosta, G.C., Bonin, K.L., Price, N.H. & Fischer G.W. (2000), 'Evaluation of the total peroxyl radical scavenging capacity of flavonoids: structure activity relationships', *J. Nat. Products*, **63**, 327 – 331.
56. Duthie G. & Crozier A. (2000), 'plant-derived phenolic antioxidants', Curr *Opin Clin Nutr Metab Care*, **3(6)**, 447-451.
57. Duthie, G., Gardner, P.T. & Kyle J. (2003), 'Plant polyphenols: are the new magic bullet', *Proc. Nutr. Soc.* **62**, 599-603.
58. Du Petit-Thouars, L.M.A. (1806), 'Genera nova Madagascariensis', Paris, 23.
59. Dziedzic, S.Z. & Hudson B.J.F. (1983), 'Polyhydroxy chalcones and flavanones as antioxidants for edible oils', *Food Chem.* **12**, 205 – 212.
60. Egoli, A. (1995), 'Bibliographical Review', Proceedings of the workshop on Conservation and Improvement of Bambara Groundnuts (*Vigna subterranean* (L.) Verdc.) 14–16 November, Harare, Zimbabwe, in: Heller, J. Begemann, F. and Mushonga, J. (Eds.), 128-134.
61. Engert, N., John A., Henning W. & Honermeier, B. (2011), 'Effect of sprouting on the concentration of phenolic acids and antioxidative capacity in wheat cultivas (Triticum aestivum ssp. Aestivum L.) in dependency of nitrogent fertlization', *Journal of Applied Botany and Food Quality*, **84**, 111 – 118.
62. Espinosa-Alonso G.L., Lygin A., Jack M., Widholm, Maria, E., Valverde, & Paredes-Lopez O. (2006), 'Polyphenols in Wild and Weedy Mexican Common Beans (*Phaseolus vulgaris* L.)', *J. Agric. Food Chem.* **54**, 4436-4444.
63. Estrella, I., Aguilera**,** Y. Benitez, V., Rosa, M. Esteban,. Martín-Cabrejas (2011), 'Bioactive phenolic compounds and functional properties of dehydrated beans flours', *Food Research International* **44**, 774–780.

64. FAO (2001), 'A Global Mapping System for Bambara Groundnuts Production', FAO Agricultural Information Management Series. No. 1, Viale delle Terme di Caracalla, 00100 Rome, Italy.
65. FAOSTAT. (2011), 'Food and Agricultural Organization Statistical Databases', URL: http//www.faostat.fao.org/
66. Farkas, L., Gabor, M., Wagner, H., et al, (1981), '*Flavonoids and Bioflavonoids*', Amsterdam, Elsevier.
67. Freeman, B.L., Eggett, D.L. & Parker, T.L. (2010), 'Synergistic and antagonistic interactions of phenolic compounds foung in navel oranges', *J. Food Science*, **75(6)**, C570 – C576.
68. Frias, J., Miranda, M.L., Doblado, R. and C. Vidal-Valverde, C. (2005), 'Effect of germination and fermentation on the antioxidant vitamin content and antioxidant capacity of *Lupinus albus* L.', *Food Chemistry*, **92(2)**, 211–220.
69. Frost, G., Leeds, A., Trew, G., Margara, R. & Dornhorst, A. (1998), 'Insulin sensitivity in women at risk of coronary heart disease and the effect of a low glycemic diet', *Metabolism*, **47,** 1245-51.
70. Fuleki, T. & Ricardo-Da-Silva, J.M. (2003), 'Effect of cultival and processing methods on the contents of catechin and procyanidins in grape juice', *J.Agric. Food Chem.* **51,** 640 -648.
71. Gad, S.C. (2005), 'Drug Discovery Handbook', Wiley, Hoboken.
72. Geil, P.B. & Anderson, J.W. (1994), 'Nutrition and health implications of dry beans: a review' *Am J Clin Nutr*. 13.
73. Gerard, K.A. & Roberts, J.S. (2004), 'Microwave heating of apple mash to improve juice yield and quality', *Food Science and Technology*, **37**, 551 – 557.
74. Goldberg, G. (2003), 'Plants: Diet and health', British nutrition foundation. Edit. Blackwell publ.
75. Gonzalez, M. E., Castano-Tostado, E. & Loarca-Pina, G. (1999), 'Antimutagenic effects of natural phenolic compounds in beans', *Mutat. Res. Genet. Toxicol. En*V*iron. Mutagen*. ***44,*** 1-9.
76. Gouveia S. & Castilho P.C. (2011), 'Characterisation of phenolic acid derivatives and flavonoids from different morphological parts of *Helichrysum obconicum* by a RP-HPLC–DAD–ESI-MS^n method', *Food Chemistry* **129**, 333–344.

77. Gülçin, E., Bursal, H.M., Sehitoglu, M., Bilsel, & Gören, A.C. (2010), 'Polyphenol contents and antioxidant activity of lyophilized aqueous extract of propolis from Erzurum, Turkey', *Food Chem. Toxicol.* **48**, 2227-2238.
78. Gupta, Y.P. (1987), 'Anti-nutritional and toxic factors in food legumes: a review', *Plant Foods Hum Nutr.* **37 (3),** 201-8.
79. Guzmañ-Maldonado, S. H. & Paredes-Lo´pez, O. (1999), 'Biotechnology for the improvement of nutritional quality of food crop plants', In *Molecular Biotechnology for Plant Food Production*; Paredes- Lo´pez, O., Ed.; Technomic Publishing Company, Inc.: Lancaster, PA, 590-591.
80. Haenen, G.R.., Paquay, J.B., Korthuwer, R.E. & Bast, A. (1997), 'Peroxynitrite scavenging by flavonoids', *Biochem. Biophys. Res. Commin*., **236**, 591 – 593.
81. Hajimahmoodi, M., Sadeghi, N., Jannat, B., Oveisi, M.R., Madani, S., Kiayi, M., Akrami, M.R. & Ranjbar, A.M. (2008), 'Antioxidant Activity, Reducing Power and total Phenolic Content of Iranian Olive Cultivar', *Journal of Biological Sciences* 8**(4),** 779-783.
82. Hakkinen, S. & Torronen, R. (2000), 'Content of flavonols and selected phenolic acids in strawbewrriesVaccinium species: Influence of cultivar, cultivation site and technique', *Food Reviews International* **33,** 517–524.
83. Halvorsen, B.L, Holte, K., Myhrstad, M.C.W., Barikmo, I. & Blomhoff, R. (2002), 'A systematic screening of total antioxidants in dietary plants', *J Nutr.* **132,** 461–71.
84. Harnly, J.M, Bhagwat, S., Lin L.Z. (2007), 'Profiling methods for the determination of phenolic compounds in foods and dietary supplements', *Anal. Bioanal.* Chem. **389,** 47-61.
85. Han, K.H., Sekikawa, M., Shimada, K., Sasaki, K., Ohba, K. & Fukushima, M. (2004), 'Resistant starch fraction prepared from kintoki beans affects gene expression of genes associated with cholesterol metabolism in rats', *Exp. Biol. Med.* **229,** 787-92.
86. Hangen, L. & Bennink, M.R. (2002), 'Consumption of black beans and navy beans (*Phaseolus vulgaris*) reduced azoxymethane-induced colon cancer in rats', *Nutrition and Cancer* **44(1),** 60-65.
87. Harbaum, B. (2007), 'Characterization of Free and Cell-Wall-Bound Phenolic Compounds in Chinese *Brassica* Vegetables', PhD thesis, Christian-Albrechts-Universität zu Kiel.
88. Hartmann,T., Kutchan, T.M., & Strack, D. (2005), 'Evolution of metabolic diversity', *Phytochemistry* **66,** 1198-1199.

89. Hartmann, A., Patz, C.D. Andlauer, W., Dietrich, H. & Ludwig, M. (2008), ' Influence of processing on quality parameters of strawberries', *J.Agric. Food Chem.* **56(20),** 9484 – 9489.
90. Hassain, M., Dilip K., Brunton, Nigel, Martin-Diana, A. & Barry-Ryan, C. (2010), 'Characterization of Phenolic Composition in Lamiaceae Spices by LC-ESI-MS/MS', *J. Agric. Food Chem.* **58 (19),** pp 10576–10581.
91. Heimler, D., Vignolini, P., Dini, M.G. & Romani, A. (2005), 'Rapid tests to assess the antioxidant activity of *Phaseolus vulgalis* L dry beans', *J.Agric. Food Chem.* **53,** 3053-3056.
92. Heller, J., Begemann F. & J. Mushonga, (1995), **'**Bambara groundnuts. *Vigna subterranea* (L.) Verdc)', Promoting the conservation and use of underutilised and neglected crops', Proceedings of the Workshop on Conservation and Improvement of Bambara Groundnuts (*Vigna subterranea* (L.) Verdc.), 14-16 November, Harare, Zimbabwe, in: Heller, J. Begemann, F. and Mushonga, J. (Eds.), 128-134.
93. Helm K.E., Tagliaferro A.R. & Bobilya D.J. (2002), 'Flavoniods antioxidants: chemistry, metabolism and structure activity relationship', *Journal of Nutritional Biochemistry*, 13, 572 – 584.
94. Herrero, M., Plaza, M., Cifuentes, A. &. Ibáñez, E. (2009), 'Green processes for the extraction of bioactives from Rosemary: Chemical and functional characterization via ultra performance liquid chromatography-tandem mass spectrometry and in-vitro assays', *J.Chromatogr. A* **2010**, *1217*, 2512-2520.
95. Hertog, M.G., Kromhout, D., Aravanis, C., et al (1995), 'Flavonoid intake and long term risk of coronary heart disease and cancer in the seven countries study', *Arch Intern Med.* **155,** 381-386.
96. Holm, J.M. & Marloth, B.W. (1940), **'**The Bambara groundnuts or njugo beans', *Farming in South Africa* **15,** 195-200.
97. Hassain, M., Dilip, K., Brunton, Nigel, Martin-Diana, A. & Barry-Ryan, C. (2010), 'Characterization of Phenolic Composition in Lamiaceae Spices by LC-ESI-MS/MS', *J.Agric. Food Chem.* **58 (19)**, 10576–10581.
98. Hu, F. B., Rimm, E. B., Stampfer, M. J., Ascherio, A., Spiegelman, D., and Willett, W. C. (2000), 'Prospective study of major dietary patterns and risk of coronary heart disease in men', *American Journal of Clinical Nutrition* **72**, 912-921

99. Hu, J.P., Calomme M., Lasure, A., DeBruyne, T., Pieters, A., Vlietinick, A. & Vanden Berghe, D.A. (1995), 'Structure-activity relationship of flavonoids with superoxide scavenging activity', *Bio. Trace Elem. Res*. **47**, 327 – 331.

100. Hughes, J.S., Ganthavorn, C. & Wilson-Sanders, S. (1997), 'Dry beans inhibit azoxymethane-induced colon carcinogenesis in F344 rats', *Journal of Nutrition* **127**, 2328-2333.

101. Iacobucci, G.A. & Sweeny, J.G. (1983), 'The chemistry of anthocyanins, anthocyanidins and related flavylium salts', *Tetrahedron* **39**, 3005- 3015.

102. Iranshahi, M., Askari, M., Sahebkar, A. & Hadgipavlou-Litina, D. (2009), 'Evaluation of antioxidant, anti-inflamatory, and lipoxygenase inhibitory activities of prenlated coumarin umelliprenin', *DARU J. Pharmaceut. Sci*., **17**, 99-103.

103. Irina, I. & Mohamed, G. (2012), ' Biological activitities and effects of food processing on flavonoids as phenolic antioxidants', Advances in Applied Biotechnology, ISBN: 978-953-307-820-5, In Tech.

104. Jimenez Martınez, C., Cardador Martınez, A., Martinez Ayala, A.L., Muzquiz, M. Martin Pedrosa, M. and Davila-Ortiz G. (2012), 'Changes in Protein, Nonnutritional Factors, and AntioxidantCapacity during Germination of L. *campestris* Seeds', *Int. J. Agronomy*, doi:10.1155/2012/387407

105. Joanne, L., Slavin, J.L., Jacobs, D., Marquart, L., & Weimer, K. (2001), 'The role of whole grains in disease prevention', *J. Am Dietetic Assoc.* **101,** 780 – 785.

106. John M.K. (1992), 'Uptake of soil applied Cadmium and its distribution in Redisties', *Can. J. Plant Sci*. **52,** 715-719.

107. Jones, A.L. (1999), 'Phaseolus Beans: Post-harvest Operations', International Centre for Tropical Agriculture (CIAT), Cali, Colombia.

108. Jovanovic, S.V, Steenken, S., Tosic, M., Marjanovic B. & Simic, M.G. (1994), 'Flavonoids as antioxidants', *J. Am. Chem. Soc*., **116**, 4846 – 4851.

109. Kajdžanoska M., Gjamovski V. & Stefova M. (2010), 'HPLC-DAD-ESI-MSn Identification of Phenolic compounds in cultivated Straberries from Macedonia', *Macedonian Journal of Chemistry and Chemiscal Engineering* **29 (2),** 181-194.

110. Kalt, W. (2005), 'Effects of Production and Processing Factors on Major Fruit and Vegetable Antioxidants', *J Food Sci* **70(1)**, R11-R19.

111. Karikari, S.K., Wigglesworth, D.J, Kwerepe, B.C., Balole, T.V., Sebolai, B. & Munthali, D.C. (1995), 'Botswana country report on Bambara groundnuts', Proceedings of the workshop on Conservation and Improvement of Bambara

Groundnuts (*Vigna subterranea* (L.) Verdc.) 14–16 November, Harare, Zimbabwe, in: Heller, J. Begemann, F. and Mushonga, J.(Eds.), 128-134.

112. Katungi, E., Farrow, A., Chianu, J., Sperling, L., & Beebe, S. (2009), 'Common beans in Eastern and Southern Africa: a situation and outlook analysis', International Centre for Tropical Agriculture (CIAT).
113. Kemo, M.S. (2000), 'Chemical composition of four landraces of Bambara groundnuts grown in Botswana', B.Sc thesis, Botswana College of Agriculture.
114. Kerry, N. & Rice-Evance, C. (1999), 'Inhibition of peroxynitrite-mediated oxidation of dopamine by flavonoid and phenolic antioxidants and their structural relationship', *J. Neurochem*. **73**, 247 – 253.
115. Kim, D.K., Jeong, S.C., Gorinstein, S. & Chon, S.U. (2012), 'Total polyphenols, antioxidant and antiproliferative activities of different extracts in Mungbeans seeds and sprouts', *Plant Foods Hum. Nutr*., **67**, 71 – 75.
116. Kingman, S. (1991), 'The influence of legume seeds on human plasma lipid concentrations', *Nutr. Res*. **4,** 97–123.
117. Kiss, R., Camby, I., Duckworth, C., Salmon, I. et al. (1997) 'In vitro influence of Phaseolus vulgaris, Griffonia simplicifolia, concanavalin A, wheat germ, and peanut agglutinins on HCT- 15, LoVo, and SW837 human colorectal cancer cell growth', *Gut*. **40,** 253 - 261.
118. Klejdusa, B., Kopecký, J., Benesˇová, L. & Vaceka, J. (2009), 'Solid phase/supercritical-fluid extraction for liquid chromatography of phenolic compounds in freshwater microalgae and selected cyanobacterial species', *Journal of Chromatography A* ***1216,*** 763-771.
119. Kroon, P. A., Faulds, C. B., Ryden, P., Robertson, J. A., Williamson, G. (1997), 'Release of covalently bound ferulic acid from fiber in the human colon', *J.Agric. Food Chem*. **45**, 661-667.
120. Krueger, C.G., Vestling, M.A. & Reed, J.D. (2003), 'Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of heteropolyflavan-3-ols and glucosylated heteropolyflavans in sorghum (Sorghum bicolor (L.) Moench', *J. Agric. Food Chem*. **51**,538–543.
121. Kushi, L.H, Meyer, K.A. & Jacobs, D.R. (1999), 'Cereals, legumes, and chronic disease risk reduction: evidence from epidemiologic studies', *Am J Clin Nutr***. 70(suppl),** 451S-458S.

122. Genovese, M.I & Lajolo, F.M. (2002), 'Nutritional significance of lectins and enzyme inhibitors from legumes', *J Agric Food Chem*. **50(22),** 6592-8.
123. Leathwood, P. & Pollet, P. (1988), 'Effects of slow release carbohydrates in the form of beans flakes on the evolution of hunger and saiety in man', *Appetite*. **10**, 1-11.
124. Lin L.Z., James M. Harnly, Matcial S. Pastor-Corrales & Devanand L. (2008), 'The Polyphenolic profiles of common beans (*Phaseolus vulgaris* L.)', *Food Chemistry* **107,** 399-410.
125. Lin, J.Y. & Tang, C.Y. (2007), 'Determination of total phenolic and flavonoids contents in selected fruits and vegetables, as well as their stimulatory effects on mouse splenocyte proliferation', *Food Chemistry* **101,** 140-147.
126. Linnemann, A. R. (1988), 'Cultivation of bambara groundnuts in Northern Nigeria', *Trop. Crops. Comm*. **15**, 1-13
127. Linnemann, A. R. & Azam-Ali, S. N. (1993), 'Bambara groundnuts (Vigna subterranea)', In Underutilized Crops: Pulses and Vegetables, 13 -57 (Ed. J. T. Williams). London: Chapman & Hall.
128. Lockett, C.T., Calvet, C.C., & Grivetti, L.E. (2000), 'Energy and micronutrient composition of dietary and medicinal wild plants consumed during drought. Study of rural Fulani, Norheastern Nigeria', *Int. J. Food Sci. Nutr.* **51,** 195-208.
129. Liu L. (2009), 'Phytochemical and Pharmacological perspectives of Wheat grain and Lupin seed', PhD thesis, Southern Cross University.
130. Ludwig D.S., Majzoub, J.A., Al-Zahrani, A., Dallal GE, Blanco I. and Roberts S.B. (1999), 'High glycemic index foods, overeating, and obesity', *Pediatrics* **103**, E26.
131. Luís, Â. Domingues F., Gil C., & Duarte A.P. (2009), 'Antioxidant activity of extracts of Portuguese shrubs: *Pterospartum tridentatum, Cytisus scoparius* and *Erica', Journal of Medicinal Plants Research* **3(11),** 886-893.
132. Luthria, A. & Pastor-Corrales Devanand, L., (2006), 'Phenolic acids content of fifteen dry edible beans (*Phaseolus vulgaris* L.) varieties', *Journal of Food Composition and Analysis* **19,** 205-211.
133. Mabika, V. & Mofongoya, P. (1995), 'Zimbabwe country report on Bambara groundnuts', Proceedings of the workshop on Conservation and Improvement of Bambara Groundnuts (*Vigna subterranea* (L.) Verdc.) 14–16 November

Harare, Zimbabwe, in: Heller, J. Begemann, F. and Mushonga, J. (Eds.), 128-134.

134. Macz-Pop, G.A., Rivas-Gonzalo, J.C., Perez-Alonso, J.J. & Gonzalez-Paramas A.M. (2006), 'Natural occurrence of free anthocyanin aglycones in beans (*Phaseolus vulgaris* L.)', *Food Chemistry* **94,** 448-456.

135. Madamba, R. (1995), 'Breeding bambara groundnuts varieties suitable for Zimbabwean conditions', Proceedings of the workshop on Conservation and Improvement of Bambara Groundnuts (*Vigna subterranea* (L.) Verdc.) 14–16 , Harare, Zimbabwe, in: Heller, J. Begemann, F. and Mushonga, J.(Eds.), 128-134.

136. Madhavi, D. L., Singhal, R. S., Kulkarni, P. R. (1996), 'Technological aspects of food antioxidants', In *Food Antioxidants. Technological, Toxicological, and Health Perspectives*; Madhavi, D. L., Deshpande, S. S., Salunkhe, D. K., Eds.; Marcel Dekker: New York, pp 194-197.

137. Madhujith, T., Amarowicz, R., & Shahidi, F. (2004), 'Phenolic antioxidants in beans and their effects on inhibition of radical-induced DNA damage', *Journal of the American Oil Chemists' Society* **81,** 691–696.

138. Madhujith, T. and Shehini, F. (2005), 'Antioxidant Potential of Pea Beans (*Phaseolus vulgaris L.*)', *Journal of Food Science*, **70**, 85.

139. Mahala, A.G. & Mohammed, A.A.A. (2010), 'Nutritive Evaluation of Bambara Groundnuts (*Vigna subterranean*) Pods, Seeds and Hull as Animal Feeds', *Journal of Applied Sciences Research*, **6(5),** 383-386.

140. Makkar, H.P.S., Hagerman, A. & Mueller-Harvey, I. (2000), 'Quantification of tannins in tree foliage – a laboratory manual', FAO/IAEA Working Document. IAEA, Vienna, 23–24.

141. Mammela, P., Savolainen H., Lindroos L., Kangas, J. & Vartiainen, T. (2000), 'Analysis of Oak tannins by Liquid Chromatograpghy – Electrospray Ionisation Mass Spectrometry', *Journal of Chromatograpghy A* 891, 75-83.

142. Mamoudou, H. D, Gruppen, H., Alfred, S., Traoré Alphons, G. J., Voragen,& Willem, J. H. (2006), 'Phenolic compounds and related enzymes as determinants of sorghum for food use', *Biotechnology and Molecular Biology*, **1 (1),** pp. 21-38.

143. Manson, M.M., Gescher, A., Hudson, E.A., Plummer, S.M., Squires, M.S. & Prigent S.A. (2000), 'Blocking and Suppressing Mechanism of Chemoprevention by Dietary Constituents', *Toxicology Lett*. **112 – 113**, 499 – 505.

144. Martinez, A.C., Loarca-Pin˜a, G. & Oomah, B. D. (2002), 'Antioxidant activity in common beans (*Phaseolus* V*ulgaris* L.)', *J. Agric. Food Chem*. ***50***, 6975-6980.

145. Marxen, K., K. H., Vanselow, S., Lippemeier, R., Hintze, A. Ruser, & Hansen U.P. (2007), 'Determination of DPPH radical oxidation caused by methanolic extracts of some Microalgal Species by linear regression analysis of spectrophotometric measurements', *Sensors* **7,** 2080-2095.

146. Massawe, F.J., S.S. Mwale, S.S., Azam-Ali, S.N & Roberts, J.A. (2005), 'Breeding in Bambara groundnuts (Vigna subterranea (L.) Verdc.): strategic considerations', *Afr. J. Biotechnol.* **4 (6),** 463–471.

147. Mat Ali, M.S. (2008), 'Analysis of Phenolics and Other Phytochemicals in Selected Malaysian Traditional Vegetables and Their Activities *In Vitro'*, PhD Thesis, University of Glasgow.

148. Meiners, C.R., Derise, N.L., Lau, H.C., Crews, M.G., Ritchey, S.J. & Maurphy E.W. (1976), 'The contents of nine mineral elements in raw and cooked mature dry legumes', *J.Agric. Food Chem.* **24,** 1126-1130.

149. Merken, H.M. & Beecher, G.R. (2000), 'Measurement of food flavonoids by High Performance Liqiud Chromatograph: A review', *J.Agric. Food Chem.* **48,** 577-599.

150. Merrian-Webster dictionary (2013), 'Nutraceutical', In *Merriam-Webster.com*, retrieved February 25, 2013, from http://www.merriam-webster.com/dictionary/nutraceuticals

151. Mesquita, F. R., Corrêa, A. D., Abreu, C. M. P., Lima, R. A. Z. & Abreu, A. F. B. (2007), 'Linhagens de feijão (*Phaseolus vulgaris* L.): composição química e digestibilidade protéica', *Ciênc. Agrotec. Lavras* **31(4)**, 1114-1121.

152. Middleton, E., Kandaswami, C. & Theoharides T.C. (2000), 'The Effects of Plant Flavonoids on Mammalian Cells: Implications for Inflammation, Heart Disease, and Cancer', *Pharmacol. Rev* **52,** 673-751.

153. Miliauskas, G., Venskutonis, P. & van Beek, T., (2004), 'Screening of radical scavenging activity of some medicinal and aromatic plant extracts', *Food Chemistry* **85,** 231-237.

154. Muller, H. E., Rigelhof, F., Marquart, L., Prakash, A. & Kanter, M. (2000), 'Antioxidant content of whole grain breakfast cereals, fruits and vegetables', *Journal of the American College of Nutritio* **19, (3 Suppl),** 312S-319S.

155. Mishra, J., Tattan, T.D., Jadon, V.S. Manju P. & Gusain, Aradhana. (2012), 'HPTLC Profile of Quercetin Content of Common Beans (Uttarakhand)

Landraces growing in Uttarakhand', *American Journal of Food Technolgy* **7(2),** 96-100.

156. Mitcham, B., Cantwell, M and Kader A. (1996), 'Methods for Determining Quality of Fresh Commoditites', *Perishables Handling Newsletter,* February Issue ,**85,** 1.
157. Moundras, C., Behr, S.R., Remesy, C. & Demigne, C. (1997), 'Fecal losses of sterols and bile acids induced by feeding rats guar gum are due to greater pool size and liver bile acid secretion', *J. Nutr.* **127,** 1068-76.
158. Mwikya, S., Ooghe, W., Camp J-van, Ngundi, D. & Huyghebaert, A. (2000), 'Amino acid profiles after sprouting, autoclaving, and lactic acid fermentation of finger millet (*Eleusine coracan*) and kidney beans (*Phaseolus vulgaris* L.)', *J.Agric. Food Chem.* **48(8),** 3081-3085.
159. Nambou, B. (1995), 'Togo country report on Bambara groundnuts', Proceedings of the workshop on Conservation and Improvement of Bambara Groundnuts (*Vigna subterranea* (L.) Verdc.) 14–16 November, Harare, Zimbabwe, in: Heller, J. Begemann, F. and Mushonga, J.(Eds.), 128-134.
160. Neveu, V., Perez-Jime'nez, J., Vos, F., Crespy, V., du Chaffaut, L., Mennen, L., Knox, C., Eisner, R., Cruz, J., Wishart, D. & Scalbert A. (2010), 'Phenol Explorer: an online comprehensive database on polyphenol contents in foods', *Database, Vol. 2010, Article ID bap024, doi:10.1093/database/bap024.*
161. Ngugi, G.W. (1995), 'Kenya country report on Bambara groundnuts', Proceedings of the workshop on Conservation and Improvement of Bambara Groundnuts (*Vigna subterranea* (L.) Verdc.) 14–16 November, Harare, Zimbabwe, in: Heller, J. Begemann, F. and Mushonga, J.(Eds.), 128-134.
162. Nijveldt, R.J., Nood, E., Hoorn, D., et al, (2001), 'Flavonoids: a review of probable mechanisms of action and potential applications' *Am J Clin Nutr.* **74,** 418-425.
163. Ninfali, P. & Bacchiocca, M. (2003), 'Polyphenols and antioxidant capacity of vegetables under fresh and frozen conditions', *J.Agric. Food Chem.* **51,** 2222–2226.
164. Nwanguma, B. C. & Eze, M. O. (1996), 'Changes in the concentrations of the phenolic constituents of sorghum during malting and mashing', *J. Sci. Food Agric.* ***70***, 162-166.
165. Ocho-Anin, A.A.L., Brou1, K.D, Kouakou, T.H., Kouadio, Y.J. & Gnakri, D. (2010a), 'Screening for antidiabetic activity and phytochemical constituents of

common beans (*Phaseolus vulgaris* L.) seeds',. *Journal of Medicinal Plants Research Vol.* **4(17)**, 1757-1761

166. Ocho-Anin, A.A.L., Brou1, K.D., Kouakou, T.H., Kouadio, Y.J. & Gnakri, D. (2010b), 'Evaluation of bioactive components in seeds of *Phaseolus vulgaris* L. (fabaceae) cultivated in Côte d'Ivoire', *Journal of Applied Biosciences* **31,** 1928 – 1934.
167. Oktay, M., Gulein, I. & Kufreviolglu, I. (2003), 'Determination of in vitro antioxidant activity of fennel (*Foeniculum vulgare*) seed extracts', *Labenson-Wiss U. Technol.* **36**, 263-71.
168. Oloyo, R.A. (2004), 'Chemical and nutritional quality changes in germinating seeds of *Cajanus cajan* L.', *Food Chemistry*, **85(4)**, 497–502, 2004.
169. Ou, B., Huang, D., Woodill, M.H., Flanagan, J. & Deemer, E.K. (2002), ' Analysis of Antioxidant Activities of Common Vegetables Employing Oxygen Radical Absorbance Capacity (ORAC) and Ferric Reducing Antioxidant Power (FRAP) assays: A Comparative Study', *J.Agric. Food Chem.* **50,** 3122 – 3128.
170. Pachico, D. (1993), 'The demand for beans technology. In G. Henry (Ed.). Trends in CIAT commodities', Cali, Colombia: Centro International de Agricultura Tropical (CIAT).
171. Pal, D.K., Kumar, S., Chakraborty, P. & Kumar, M.A. (2008), 'A study on the antioxidant activity of *Semecarpus anacardium* L. f. Nuts', *Journal of Natural Remeady* **8**, 160–163.
172. Palafox-Carlos, H., Yahia, E.M. & González-Aguilar, G.A. (2012), 'Identification and quantification of major phenolic compounds from mango (Mangifera indica, cv. Ataulfo) fruit by HPLC–DAD–MS/MS-ESI and their individual contribution to the antioxidant activity during ripening', Food *Chemistry* **135**, 105–111.
173. Palma, M. & C.G Barrosa, C. (2002), 'Ultrasound-Assisted Leaching', *Anal. Chim. Acta,* **458**.
174. Papas, A. (1996), 'Determinants of antioxidant status in humans', *Lipids,* **31**(1)' S77-S82.
175. Paskoa, P., Barton H., Zagrodzki P., Gorinstein S., Folta, M. & Zachwieja Z. (2009), ' Anthocyanins, total polyphenols and antioxidant activity in amaranth and quinoa seeds and sprouts during their growth', *Food chem.*,**115**, 994 – 998.

176. Patel, J.M. (2008), 'A Review of Potential Health Benefits of Flavonoids', *Lethbridge Undergraduate Research Journal.* **3 (2).**

177. Paul, A.A., Southgate, D.A.T. & Russell, J. (1980), 'First supplement to McCance and Widdowson's The composition of foods: amino acids (mg per 100 g food), fatty acids (g per 100 g food)', Elsevier, Amsterdam, Netherlands 112.

178. Pérez-Jiménez, J., S. Arranz., M. Tabernero, M. E. Diaz-Rubio, J., Serrano, I. Goñi & Saura-Calixto, F. (2008), 'Updated methodology to determine antioxidant capacity in plant foods, oils and beverages: extraction, measurement and expression of results', *Food ResearchInternational* **41 (3),** 274-285.

179. Pietta, P.G. (2000), 'Flavonoids as Antioxidants', *J Nat. Prod*. **63,** 1035-1042.

180. Portet, B., Fabre, N., Rozenberg, R., Habib-Jiwan, J.L., Moulis, C. & Quetin-Leclercq, J. (2008), 'Analysis of minor flavonoids in Piper hostmannianum var. Berbicense using liquid chromatography coupled with atmospheric pressure chemical ionization mass spectrometry', *Journal of Chromatography A* **1210,** 45–54.

181. Poulter, N.H & Caygill, J. C. (1980), 'Vegetable milk processing and rehydration characteristic of Bambara groundnuts *(Voandzeia subterranea (L) Thouars)*', *Journal of the Science of Food and Agriculture,* **31,** 1158-1163.

182. Pourmorad, F., Hosseinimehr. S. & Shahabimajd N. (2006), 'Antioxidant activity, phenol and flavonoid contents of some selected Iranian medicinal plants', *Afr. J. Biotechnol.* **5,** 1142-1145.

183. Prakash, A. (2001), 'Antioxidant Activity', *Medallion Laboratories Analytical Progress* **19 (2)**.

184. Purseglove, J. W. (1992), 'Tropical Crops (Dicotyledons)', Longman House Burnt Mill, Harlow, Essex CM20 2JE England.

185. Qian, J.Y., Lui, D. & Huang, A.G. (2004), 'The efficiency of flavonoids in polar extracts of Lycium Chinese Mill fruit as free radical scavengers', *Food Chemisrty* **87,** 283-288.

186. Queiroz-Monici, K. S., Costa, G.E.A., da Silva N., Reis S.M.P.M. & de Oliveira A.C.(2005), 'Bifidogenic effect of dietary fiber and resistant starch from leguminous on the intestinal microbiota of rats', *Nutrition,* **21,** 602-609.

187. Rabaneda, F.S., Olga Ja´uregui, Rosa Maria Lamuela-Ravento Jaume Bastida, Francesc Viladomat & Carles Codina (2003), 'Identification of phenolic

compounds in artichoke waste by high performance liquid chromatography–tandem mass spectrometry', *Journal of Chromatography A* **1008**, 57–72.

188. Rassel, A. (1960), 'Le voandzou *Voandzeia subterranea* Thou. et sa culture au Kwango', *Bull. agric. du Congo Belge et du Ruanda-Urundi* **51**,1-26.
189. Reddy, N.R., Pierson, M.D., Sathe, SK. & Salunkhe, DK. (1982), 'Legume-based fermented foods: their preparation and nutritional quality', *Crit Rev Food Sci Nutr*. **17(4),** 335-70.
190. Rhodes M.J.C & Price K.R. (1997), 'Identification and analysis of plant phenolic antioxidants', *Eur. J. Cancer prev*. **6**, 5188.
191. Rice-Evance, C.A., Miller, N.J. & Paganga, G. (1996), ' Structure-antioxidant activity relationships of flavonoids and phenolic acids', *Free Radic. Biol. Med*. **20**, 933 – 956.
192. Rifkind, B. M. (1984), 'The Lipid Research Clinics coronary primary prevention trial results .2. The relationship of reduction in incidence of coronary heart disease to cholesterol lowering', *Jama-Journal of the American Medical Association* **251**, 365-374.
193. Rodriguez-Burger, A.P., Mason, A. & Nielsen, S. (1998), 'Use of fermented black beans combined with rice to develop a nutritious weaning food', *J.Agric. Food Chem.* **46(12),** 4806-4813.
194. Romani, A., Vignolini, P., Galardi, C., Mulinacci, N., Benedettelli, S. & Heimler, D. (2004), 'Germplasm characterization of Zolfino landraces (*Phaseolus vulgaris L*.) by flavonoid content', J. *Agric. Food Chem.* **52**, 3838-3842.
195. Rui-Min Han, I., Jian-Ping Zhang, I. & Skibsted L.H. (2012), 'Reaction Dynamic of Flavonoids and Carotenoids as Antioxidants', *Molecules*,**17**, 2140 – 2160; doi:10.3390/molecules17022140.
196. Salunkhe, D.K., Kadon, S.S. & Charan, J.K. (1985), 'Postharvest Biotechnology of food legumes', Bocaranton F.L. CRC press.
197. Samma, N., Maldonado, S., Alfaro, M.E., Farfa´n, N. & Gutierrez, J. (1999), 'Composition of Different Beans Varieties (*Phaseolus vulgaris*) of Northwestern Argentina (Region NOA): Cultivation Zone Influence', *J. Agric. Food Chem.* **47**, 2685-2689
198. Santos-Buelga, C. & Williamson, G. (2003), 'Methods in Polyphenol Analysis', Royal Society of Chemistry, Cambridge.

199. Saura-Calixto, F., Serrano, J. & Goñi, I. (2007), 'Intake and bioaccessibility of total phenols in a whole diet', *Food Chemistry*. **101**, 492-501.

200. Schuier, M., Sies, H., Illek, B., et al, (2005), 'Cocoa-Related Flavonoids Inhibit CFTR-Mediated Chloride Transport across T84 Human Colon Epithelia', *J Nutr*. **135,** 2320-2325.

201. Seeram, P. N., Lee, R., Scheuller, S. H. & Heber, D. (2006), 'Identification of phenolic compounds in strawberries by liquid chromatography electrospray ionizationmass spectroscopy', *Food Chemistry.,* **97**, 1–11.

202. Sena, L.P., Van der jagt, D.J., Rivera, C., Tsin, I., Muhamadu, A.T.C., Mahamadou, O., Millson, M., Pastuszyn, A. & Glew, R.H. (1998), 'Analysis of nutritional components of eigt famine foods of the republic of Niger', *Plant Foods Hum. Nutr*. **52,** 17-30.

203. Shadkami F., Estevez S., & Helleur R. (2009), 'Analysis of catechins and condensed tannins by thermally assisted hydrolysis/methylation-GC/MS and by a novel two step methylation', Journal *of Analytical and Applied Pyrolysis* **85**, 54-65.

204. Sharma, P. & Gujral, H.S. (2011), 'Effect of sand roasting and microwave cooking on antioxidant activity of barley', *Food Research International*, **44**, 235-240

205. Shimelis, E.A & Rakshit, S.K. (2005), 'Proximate composition and physico-chemical properties of improved dry beans (Phaseolus vulgaris L.) varieties grown in Ethiopia', *LWT* **38**, 331–338

206. Shutler, S.M., Bircher, G.M., Tredger, J.A., Morgan, L.M. & Walker, A.F. (1989), 'The effect of daily baked beans (*Phaseolus vulgaris*) consumption on the plasma lipid levels of young, normo-cholesterolaemic men', *Br. J. Nutr*. **61,** 257-265.

207. Silva, E., Souza, J., Rogez, H., Rees, J. & Larondelle, Y. (2007), 'Antioxidant activities and polyphenolic contents of fifteen selected plant species from the Amazonian region', *Food Chemistry* **101,** 1012-1018.

208. Slavin, J.L, Martin, P.C, Jacob, D., & Maquart, L. (1999), 'Plausible mechanism for protectiveness of whole grains', *Am J Clin Nutr.* 459S – 463S.

209. Spanou, C., Stagos, D., Tousias, I., Angelis, A., Aligiannis, N., Skaltsounis, I. & Kouretas, D. (2007), 'Assessment of Antioxidant Activity of Extracts from Unique Greek Varieties of *Leguminosae* Plants Using *In vitro* Assays', *Anticancer research **27,*** 3403-3410.

210. Swanevelder, C.J. (1995), 'South Africa country report on Bambara groundnuts', Proceedings of the workshop on Conservation and Improvement of Bambara Groundnuts (*Vigna subterranea* (L.) Verdc.) 14–16 November, Harare, Zimbabwe, in: Heller, J. Begemann, F. and Mushonga, J.(Eds.), 128-134.

211. Swanevelder, C.J. (1998), 'Bambara – food for Africa', National Department of Agriculture, Government Printer, Republic of South Africa

212. Tawaha, K., Alali, F., Gharaibeh, M., Mohammad, M. & El-Elimat T. (2007), 'Antioxidant activity and total phenolic content of selected Jordanian species', *Food Chemistry* **104,** 1372-1378.

213. Thaipong k., Boonprakob, U., Crosby, K., Zevallos, L.C. & Byrne, D.H. (2006), 'Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts', *Journal of Food Composition Analysis*, **19,** 669 – 675.

214. Thompson H.J. 14th Australasian Plant Breeding and 11th SABRAO Conference, Cairns, Queensland, Australia, 10-14 August 2009.

215. Tovar, J., Sayago-Ayerdi, S.G., Penalver, C., Paredes-Lopez, O. & Bello-Perez, L.A. (2003), '*In vitro* starch hydrolysis index and predicted glycemic index of corn tortilla, black beans (*Phaseolus vulgaris* L.), and Mexican taco', *Cereal Chem*. **80,** 533-535.

216. Tweneboah, C. K. (2000). Modern Agriculture in the Tropics, Food crops. Co-wood Publishers.

217. Uebersax (2006), 'Dry Edible Beans: Indigenous Staple and Healthy Cuisine', *Forum on Public Policy*: *Journal of the Oxford Round Table,* September issue.

218. Valverdú-Queralt, A., Medina-Remón, A., Andres-Lacueva, C. & Lamuela-Raventos, R.M.(2011), 'Changes in phenolic profile and antioxidant activity during production of diced tomatoes', *Food Chem.,* **126,** 1700-1707.

219. Verdcourt, B. (1980), 'The correct name for the bambara groundnuts', *Kew Bull.* **35(3),** 474.

220. Villaño, D., M.S. Fern´andez-Pach´on, M.S., M.L. Moy´a, M.L., Troncoso, A.M. & Garc´ıa-Parrilla, M.C. (2007), 'Radical scavenging ability of polyphenolic compounds towards free radicals', *Talanta* **71,** 230–235.

221. Wahle, K.W., Brown, I., Rotondo, D. & Heys, S.D. (2010), 'Plant Phenolics in the Prevention and Treatment of Cancers', *Adv. Exptl. Med. Biol*. **698**, 36 – 51

222. Wang, J., Zhao, Y.M., Guo, C.Y., Zhang, S.M, Liu, C.L., Zhang, D.S. & Bai, X.M. (2012), 'Ultrasound-Assisted ectraction of total flavonoids from Inula Helenium', *Pharmacogn Mag.* April-Jun; **8(30),** 166 – 170.

223. Wang, N., Daun, K.J. & Malcomson, J.L. (2003), 'Relationship between physicochemical and cooking properties, and effects of cooking on antnutrients of yellow field peas (*Pisum sativum*)', *Journal of the Science of Food and Agriculture* **83,** 1228-1237.

224. Wang, S., Meckling, K.A., Marcone, M.F., Kakuda, Y. & Tsao, R. (2011), 'Synergistic, additive, and antagonistic effects of food mixtures on total antioxidant capacities', *J.Agric. Food Chem.*, **59**, 960 - 968.

225. Waters Corporation (2002), 'HPLC troubleshooting guide', American Laboratory and Waters Corporation, 720000181EN, 08/02.

226. Winter K.M. (2009), 'Identification of Bioactive Compounds in Wheat', PhD thesis, Southern Cross University.

227. Wolever, T.M.S., Jenkins, D.J.A., Thompson, L., Wong, G. & Josse, R.G. (1987), 'Effect of canning on the blood glucose response beans in patients with type 2 diabetes', *Human Nutrition: Clinical Nutrition.* **41C**, 135-140.

228. Wortmann, C.S. (2006), '*Phaseolus vulgaris* L. (common beans)', PROTA (Plant Resources of Tropical Africa), Wageningen, Netherlands.

229. Wortmann, C.S., Kirby, R.A., Eledu, C.A. & Allen, D.J. (1998), 'Atlas of common', International Centre for Tropical Agriculture (CIAT), Cali, Colombia.

230. Wu, X. L., Beecher, G. R., Holden, J. M., Haytowitz, D. B., Gebhardt, S. E. & Prior, R. L. (2004), 'Lipophilic and hydrophilic antioxidant capacities of common foods in the United States', *J.Agric. Food Chem.* **52,** 4026-4037.

231. Wu, X.L, & Prior, L. R. (2005), 'Systematic identification and characterization of anthocyanins by HPLC-ESI-MS/ MS in common foods in the United States: Fruits and Berries', *J. Agric. Food Chem.* **53**, 2589– 2599.

232. Wu, X., Beecher, G.R., Holden J.M., Haytowitz, D.B., Gebhardt S.E. & Prior R.L. (2006), 'Concentrations of anthocyanins in common foods in the United States and Estimation of normal consumption', *J. Agric. Food Chem.* **54**, 4069-4075.

233. Xavery, P., Ngulu, F., Kasambala, S. & Muthoni, R. (2007), 'Factors affecting Uptake of beans based technologies in Northern Tanzania', Unpublished report.

234. Xu, B.J., Yuan, S.H. & Chang, S.K.C (2007), 'Comparative Analyses of Phenolic Composition, Antioxidant Capacity, and Color of Cool Season Legumes and other selected food legumes', *Journal of Food Science* **72**, 2.

235. Yang, H., Ge, Y., Sun, Y., Liu, D., Xingqian Ye, X. & Wu, D. (2011), 'Identification and characterisation of low-molecular-weight phenolic compounds in bayberry (*Myrica rubra Sieb. et Zucc*.) leaves by HPLC-DAD and HPLC-UV-ESIMS', *Food Chemistry* **128**, 1128–1135

236. Yu, J., Ahmedna, M. & Goktepe, I. (2005), ' Effects of processing methods and extraction solvents on concentration and antioxidant activity of peanut skin phenolic', *Food Chem*., **90**, 199 – 206.

237. Yu, L., Haley, S., Perret, J., Harris, M., Wilson, J. & Qian, M.(2002), 'Free radical scavenging properties of wheat extracts', *J.Agric. Food Chem.* **50 (6),** 1619-1624.

238. Zia-Ul-Haq, M., Shahid, S.A, Ahmad, S., Qayum, M. & Rasool, N. (2012), 'Mineral contents and antioxidant potential of selected legumes of Pakistan', *Journal of Medicinal Plants Research* **6(32),** 4735-4740.

Appendices

Appendix A: UV-visible spectra of selected peaks

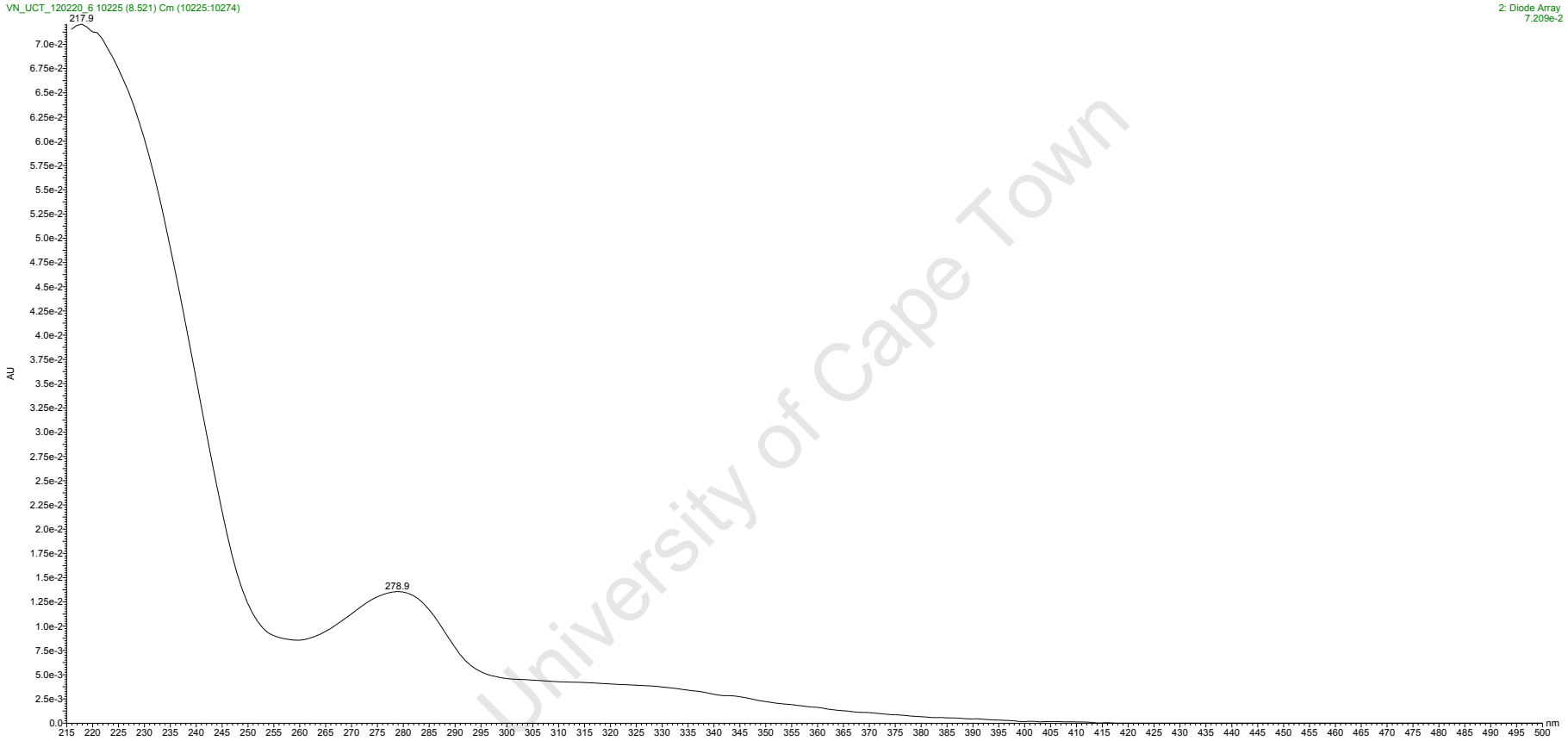


Figure A1: UV-visible spectrum of catechin

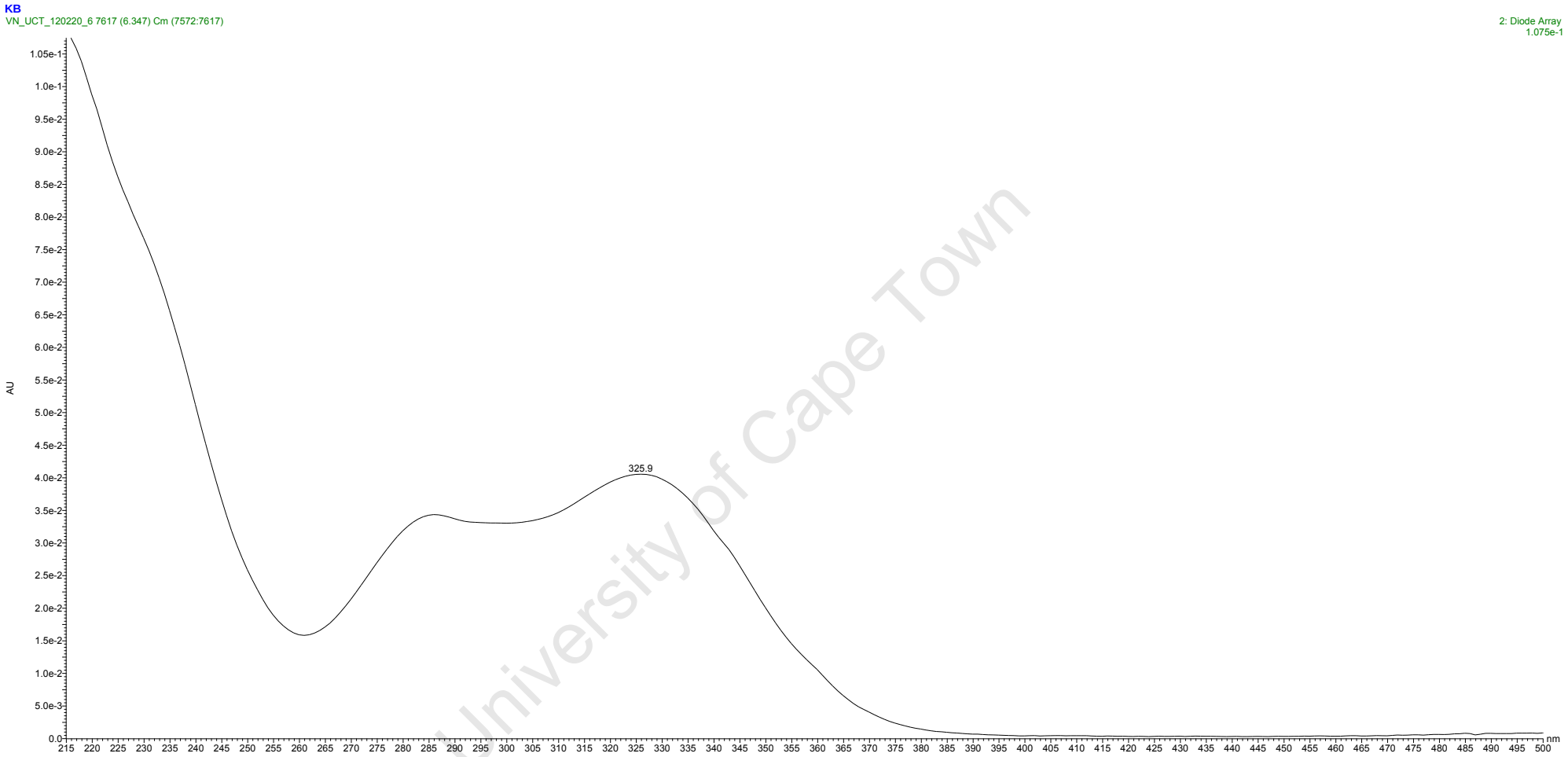


Figure A2: UV-visible spectrum of Syringic acid derivative

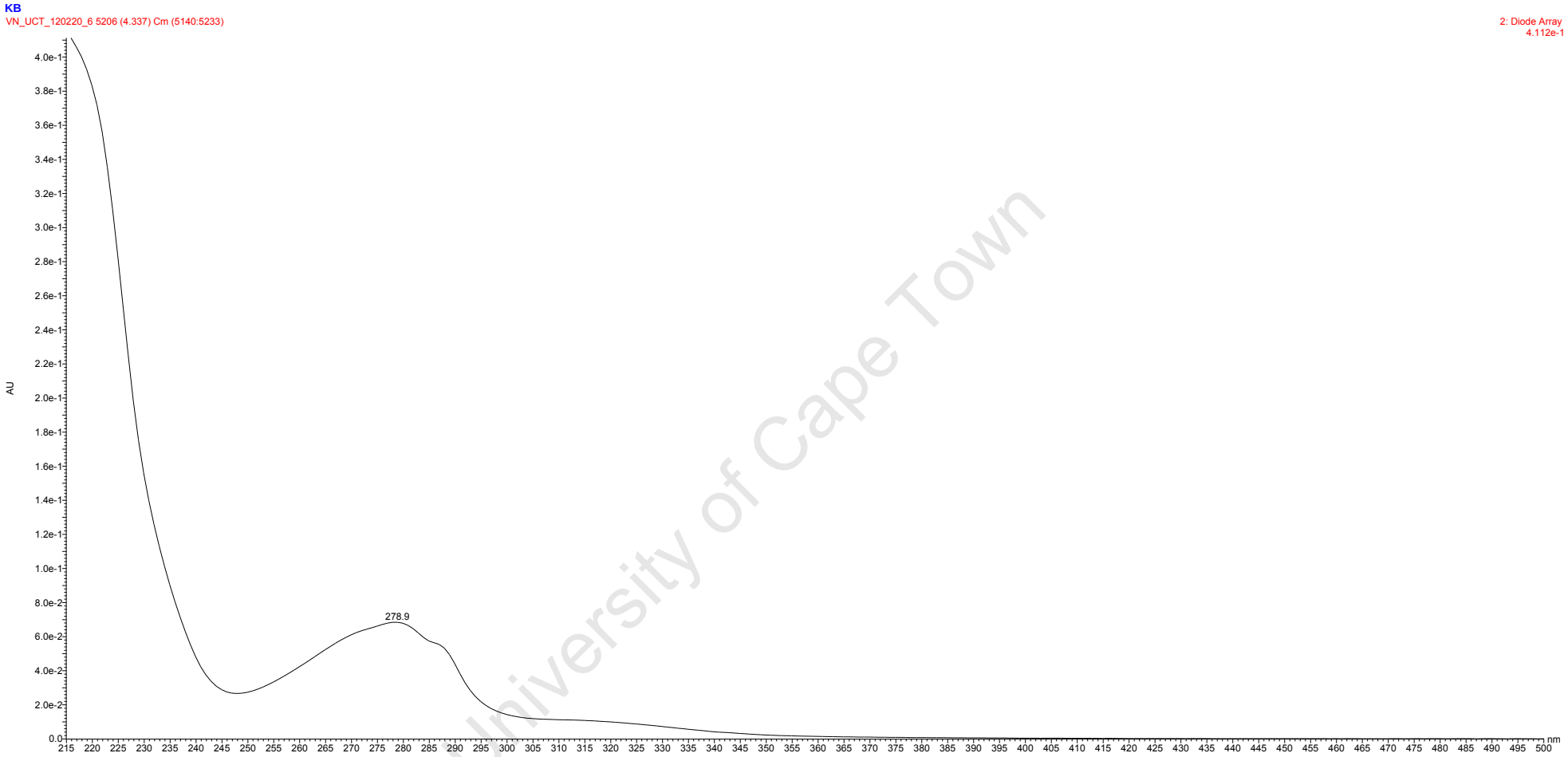


Figure A3 UV-visible spectrum of epicatechin

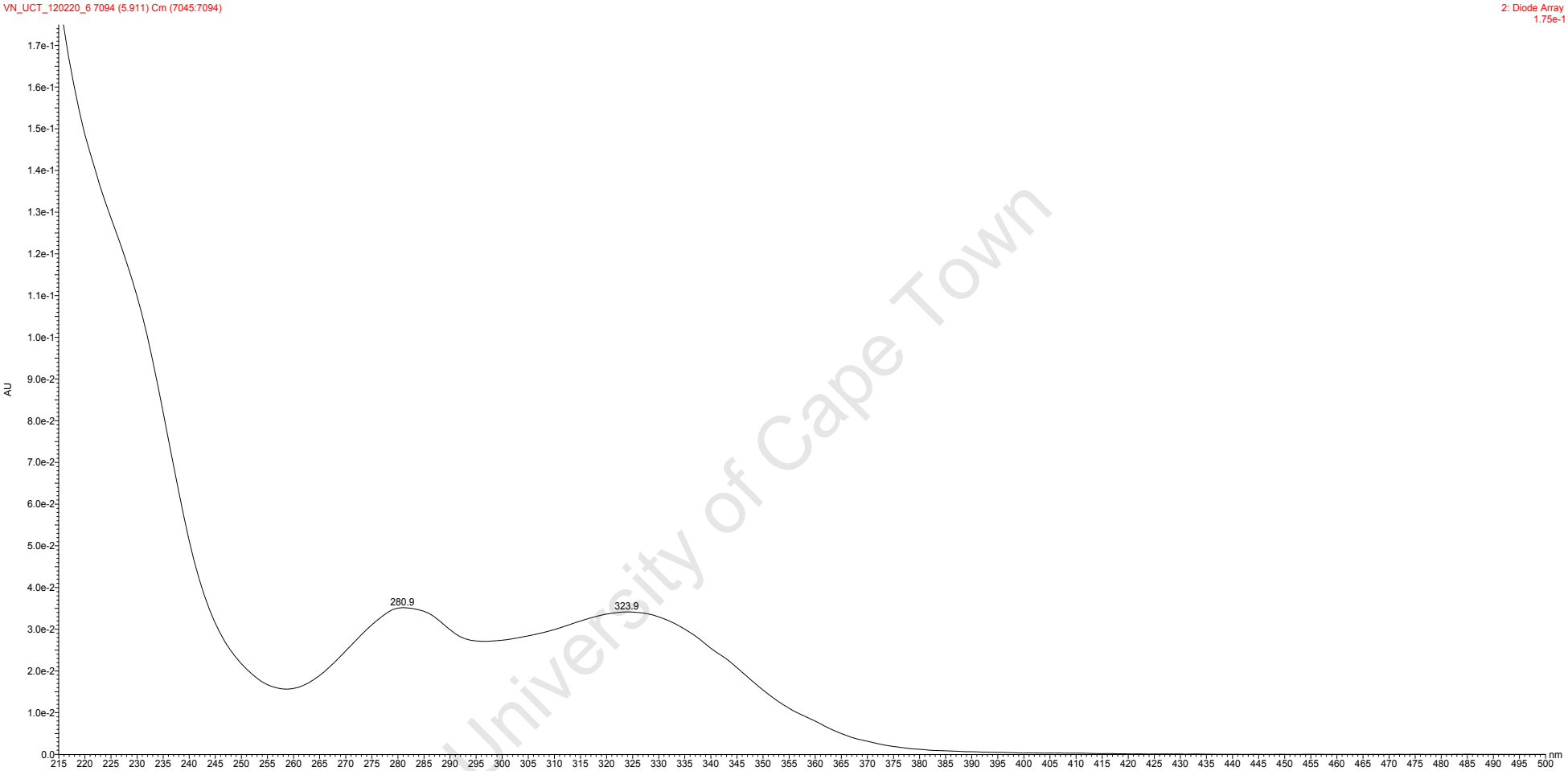


Figure A4 UV-visible spectrum of Catechin glucoside

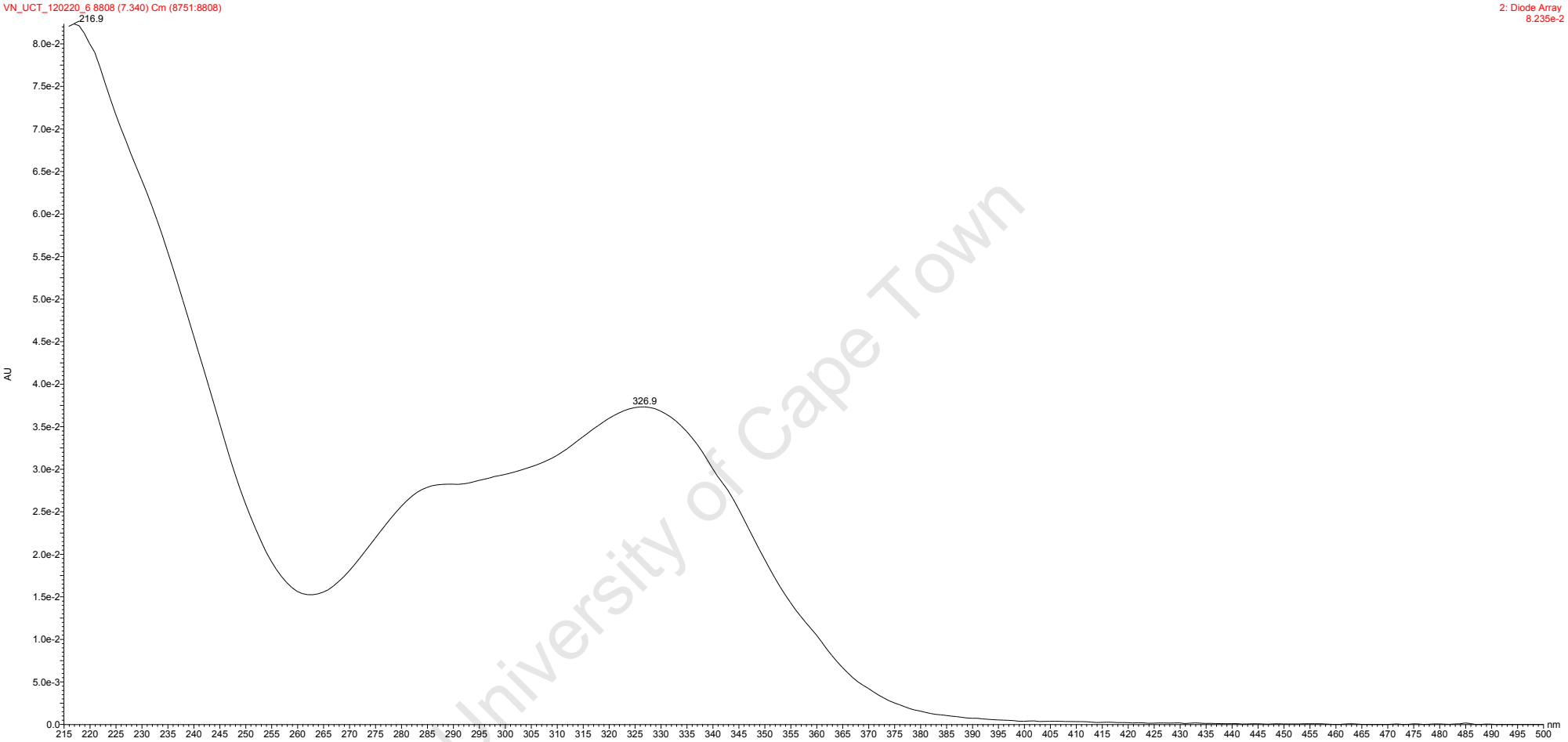


Figure A5 UV-visible spectrum of Caffeic acid derivative

Appendix B: Selected MS and MS/MS spectra

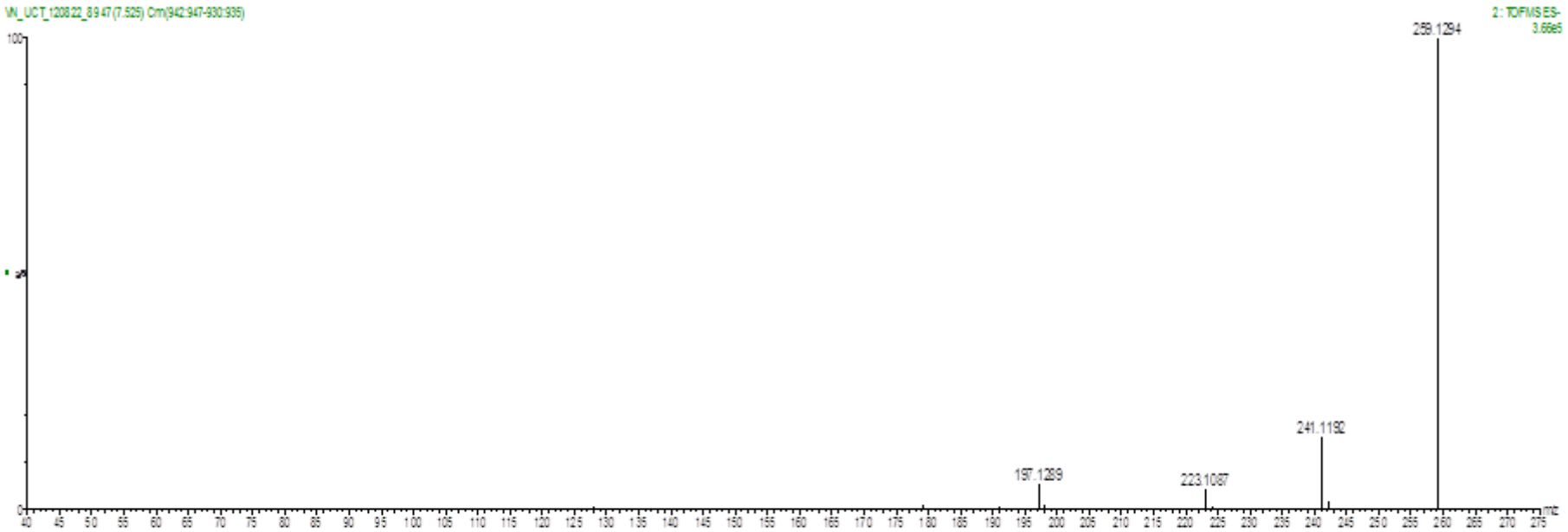


Figure B1 MS and MS/MS spectrum showing a peak with molecular ion [M – H]- at m/z 259 in common beans. This peak was common in all the market class common beans.

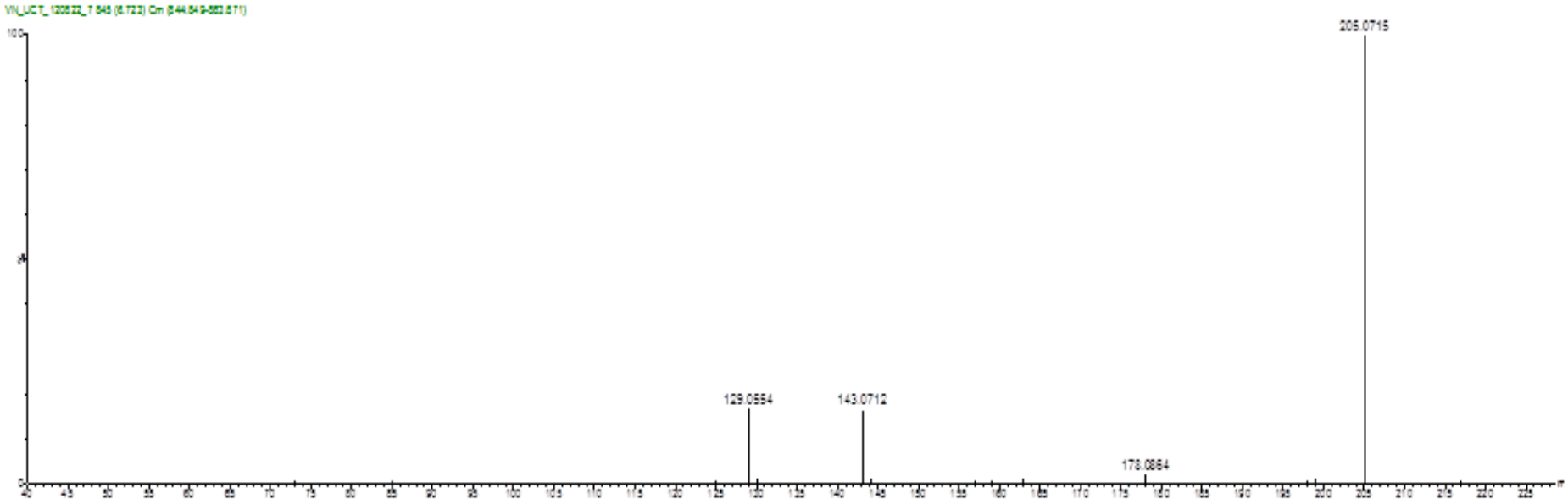


Figure B2 MS and MS/MS spectrum showing a peak with molecular ion [M – H]- at m/z 205 in bambara groundnuts. This peak was common in both the red and brown bambara groundnuts.

Figure B3 MS and MS/MS spectrum showing a peak with molecular ion [M – H]- at m/z 381 in bambara groundnuts.

Appendix C: Analytical scale chromatograms for external standards.

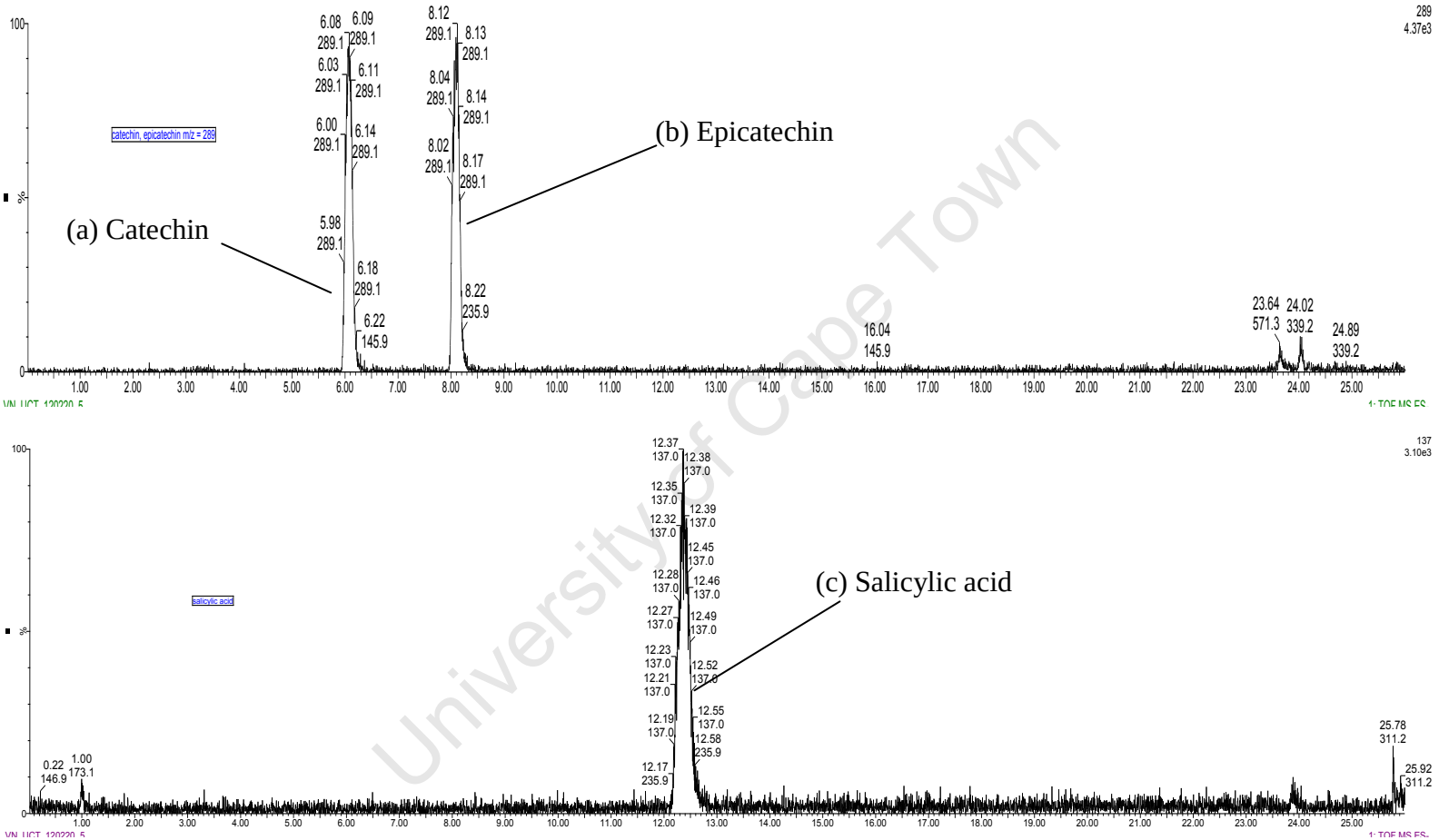


Figure C1 Analytical scale chromatograms for external standards: (a) Catechin, (b) Epicatechin and (c) Salicylic acid

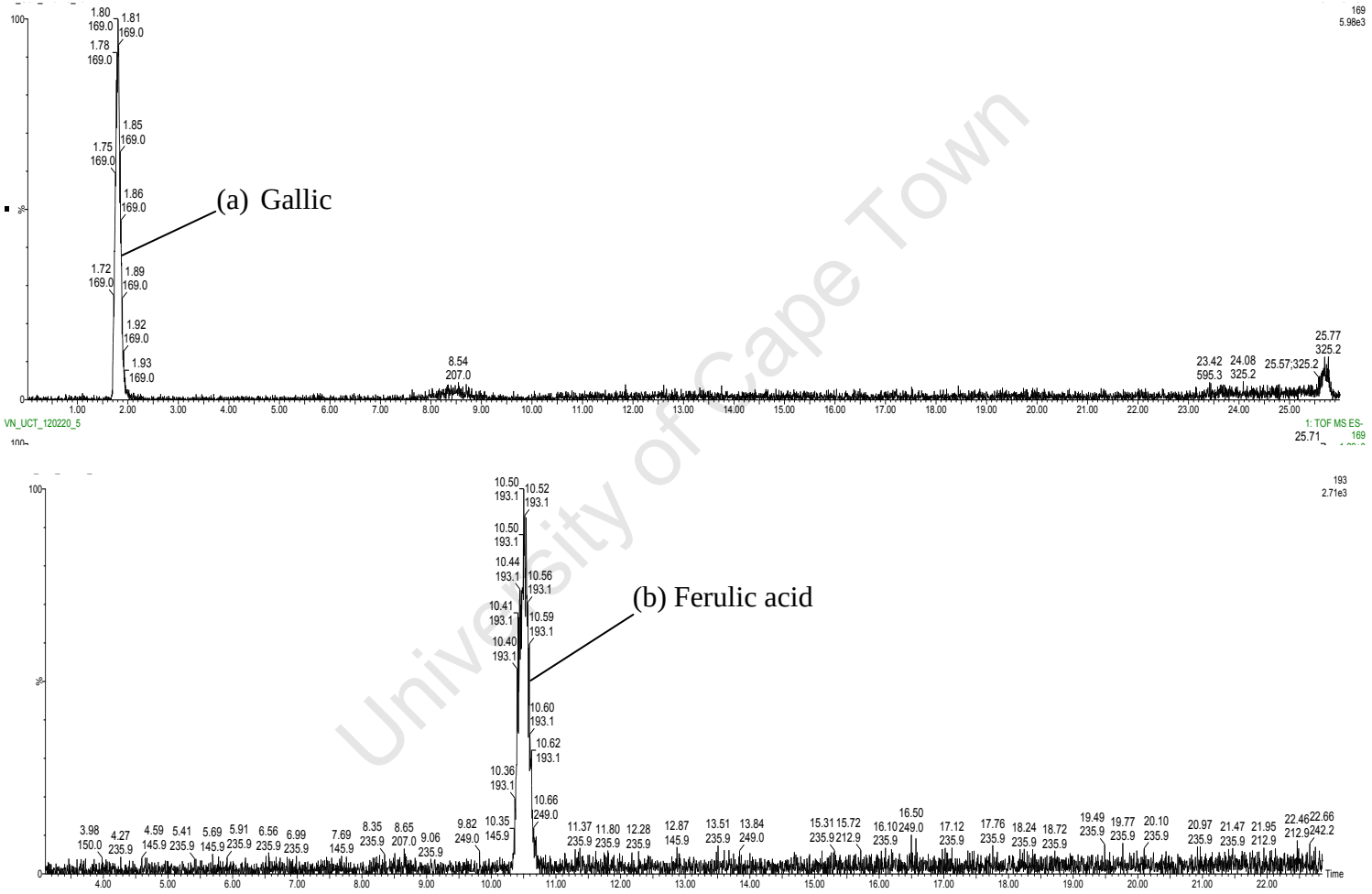


Figure C2 Analytical scale chromatograms for external standards: (a) Galic acid, (b) Ferulic acid

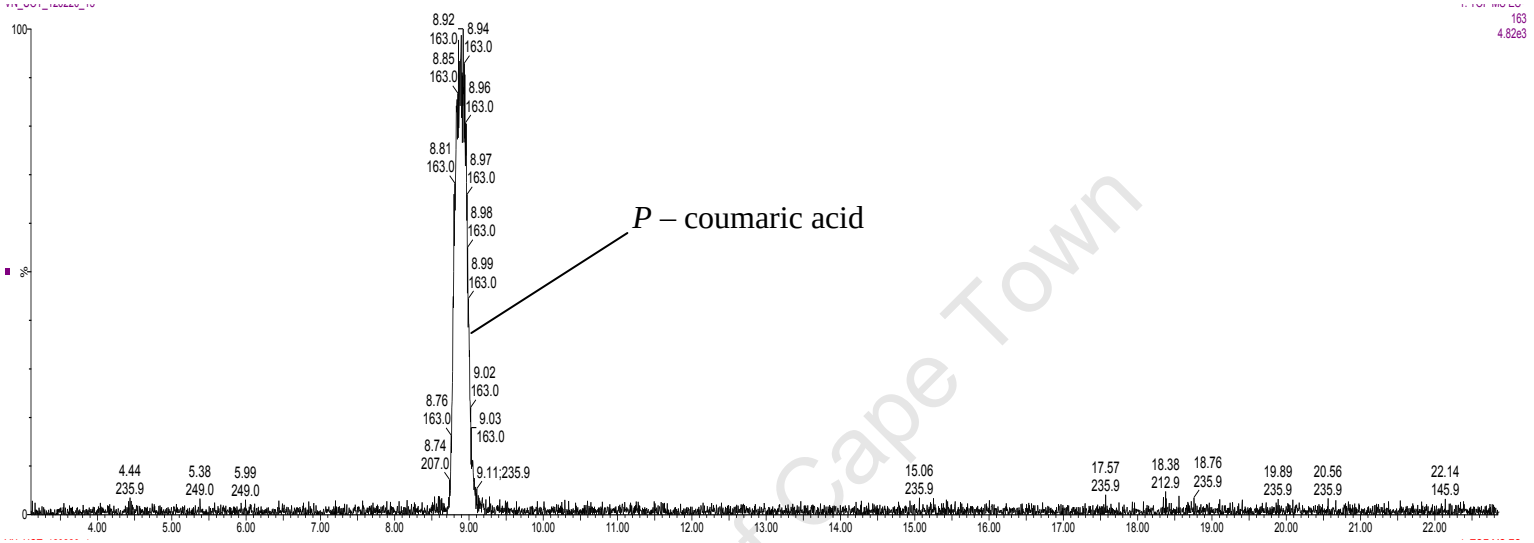


Figure C3 Analytical scale chromatogram for external standard (*p* –coumaric acid)

Appendix D: Total Ion Chromatograms (TIC) of the crude methanol extracts of bambara groundnuts and common beans

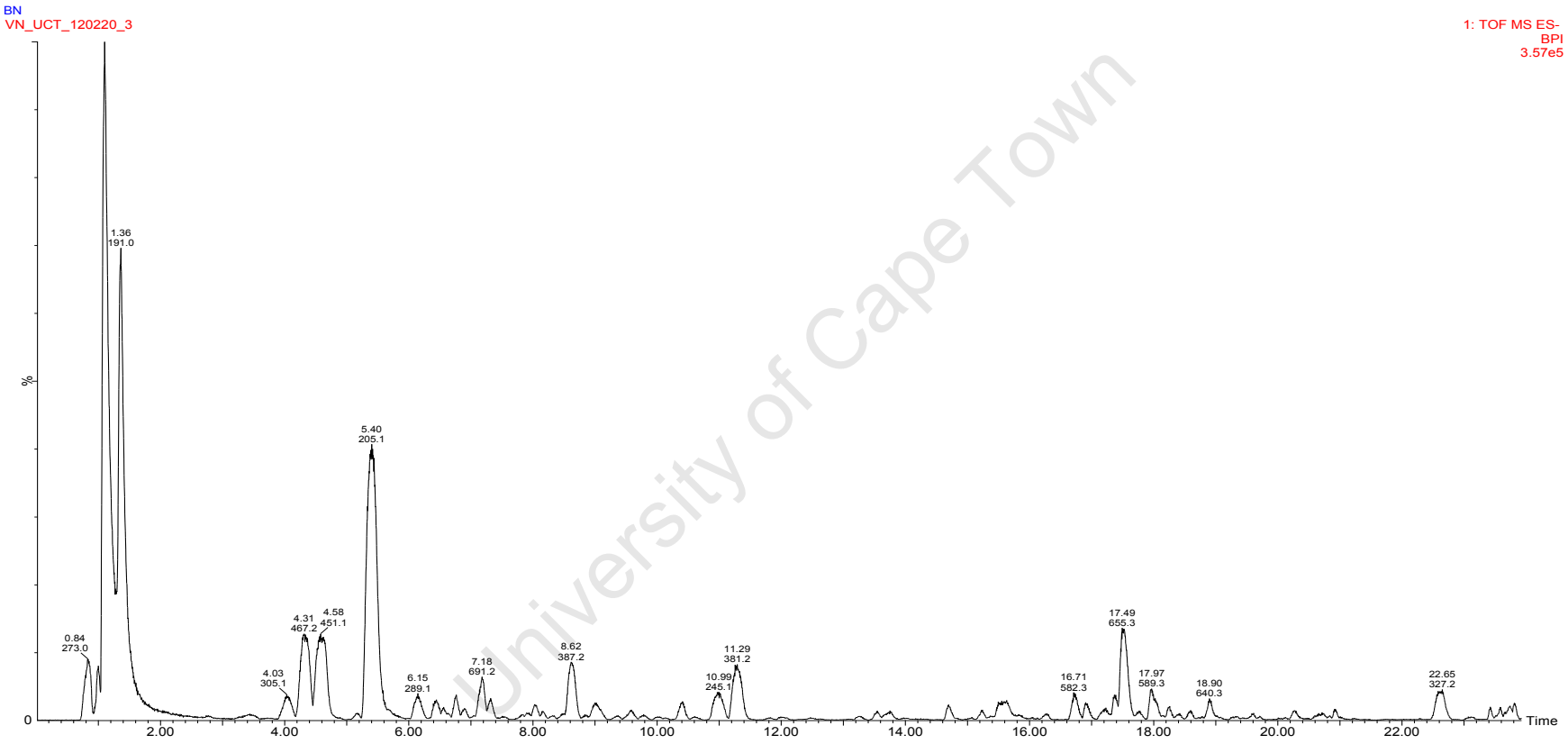


Figure D1 TIC of 70% methanol extract of brown bambara groundnuts. Numbers on top of each curve are m/z values of the deprotonated molecule [M – H] – of each compound and their retention times

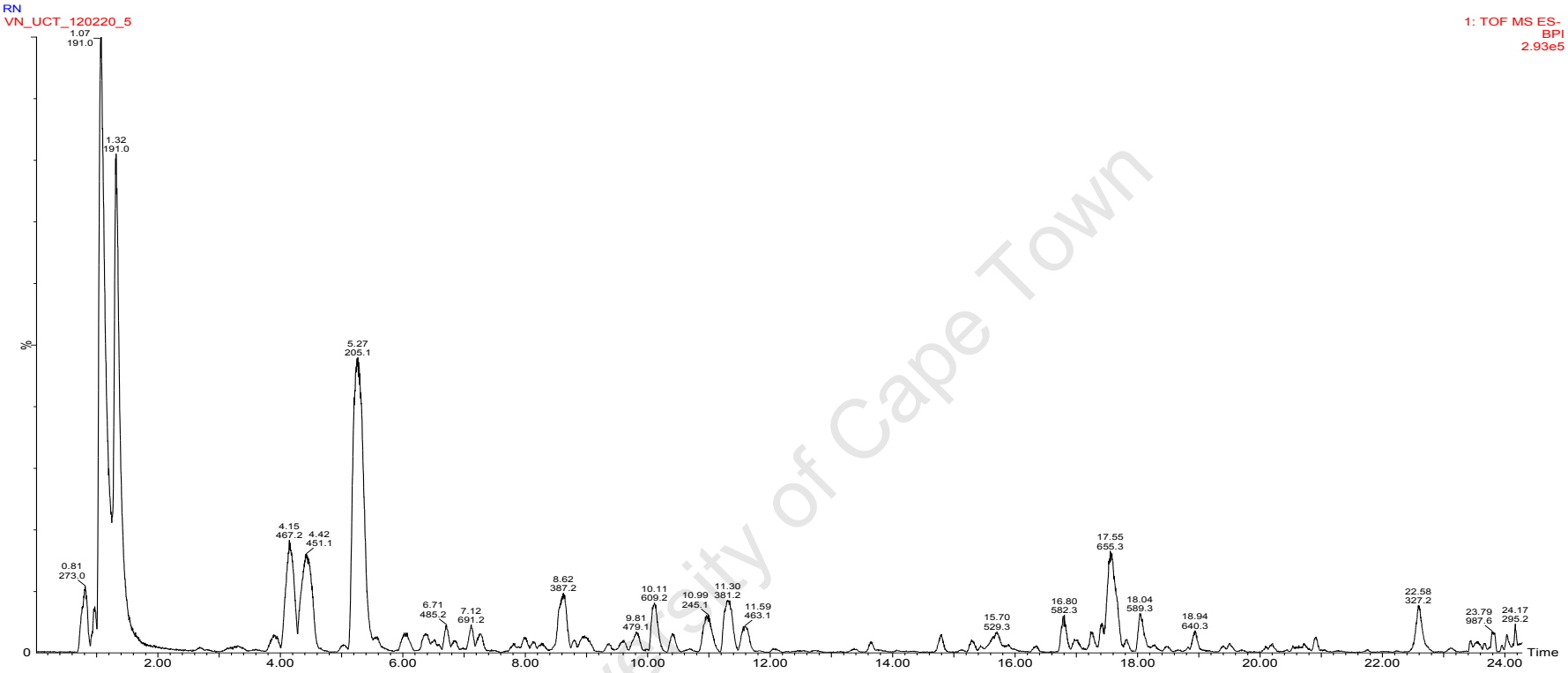


Figure D2 TIC of 70% methanol extract of red bambara groundnuts. Numbers on top of each curve are m/z values of the deprotonated molecule [M – H] – of each compound and their retention times.

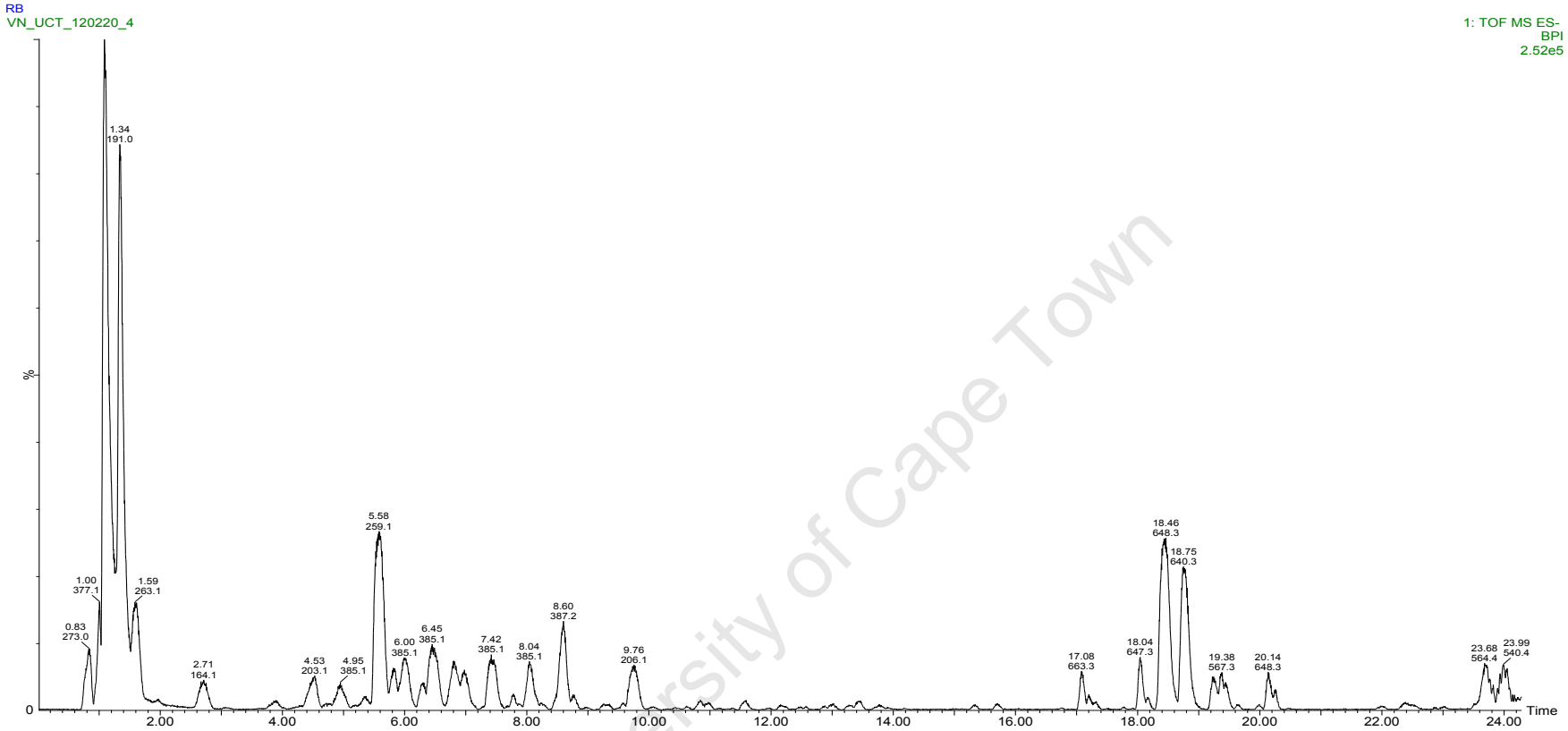


Figure D3 TIC of 70% methanol extract of red beans. Numbers on top of each curve are m/z values of the deprotonated molecule [M – H]$^-$ of each compound and their retention times.

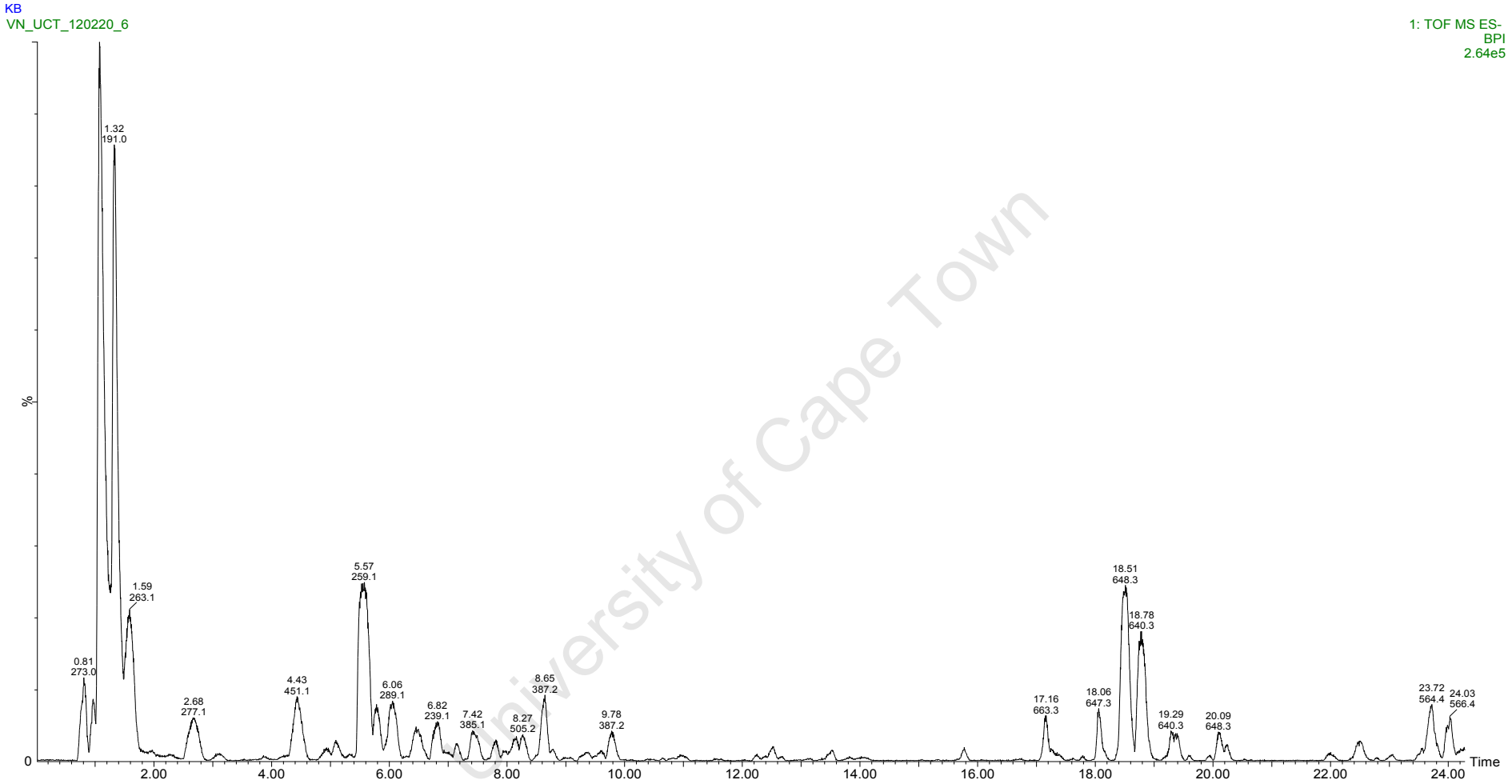


Figure D4 TIC of 70% methanol extract of grey mottled beans. Numbers on top of each curve are m/z values of the deprotonated molecule $[M - H]^-$ of each compound and their retention times.

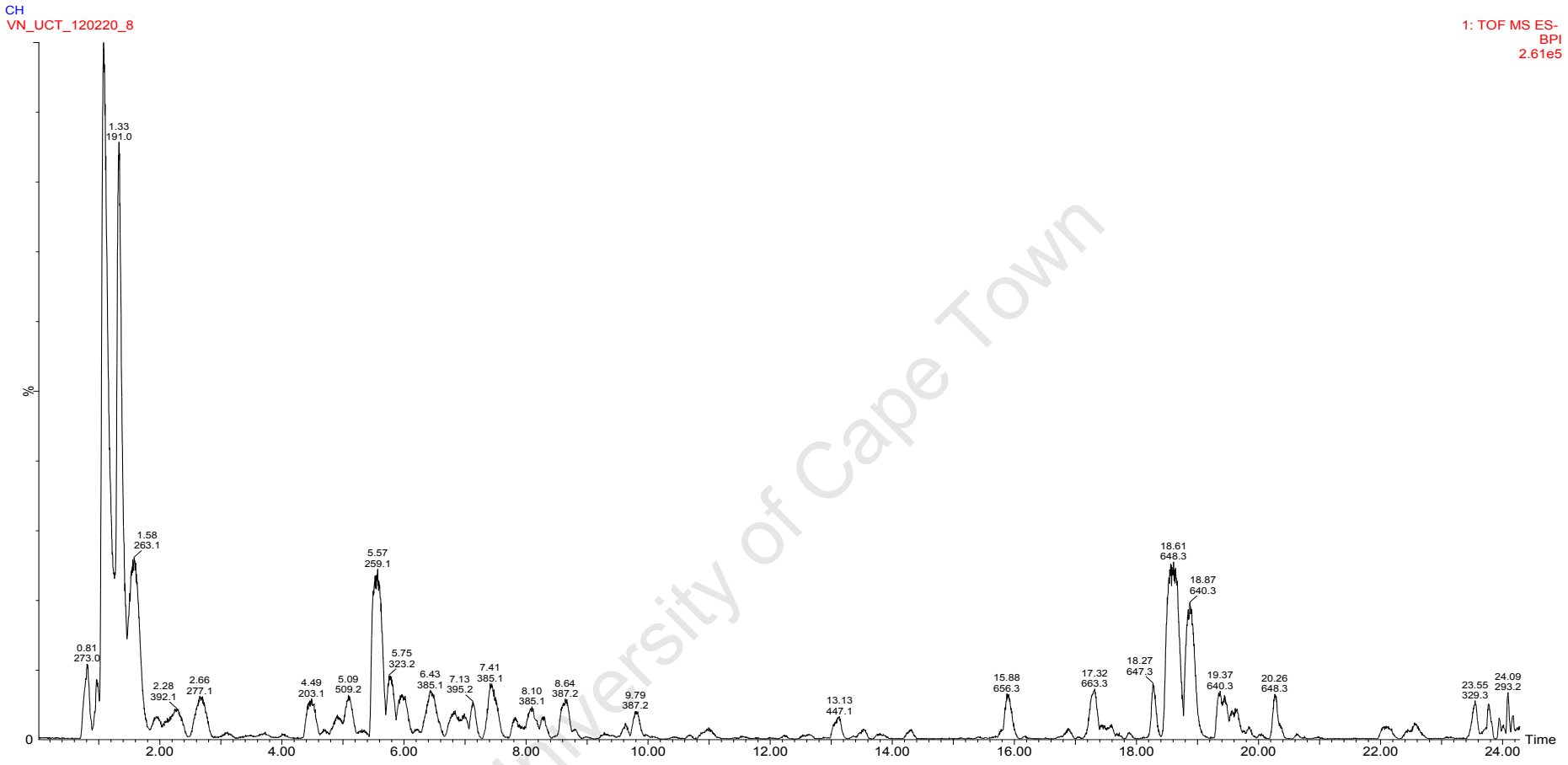


Figure D5 TIC of 70% methanol extract of brown beans. Numbers on top of each curve are m/z values of the deprotonated molecule $[M - H]^-$ of each compound and their retention times.

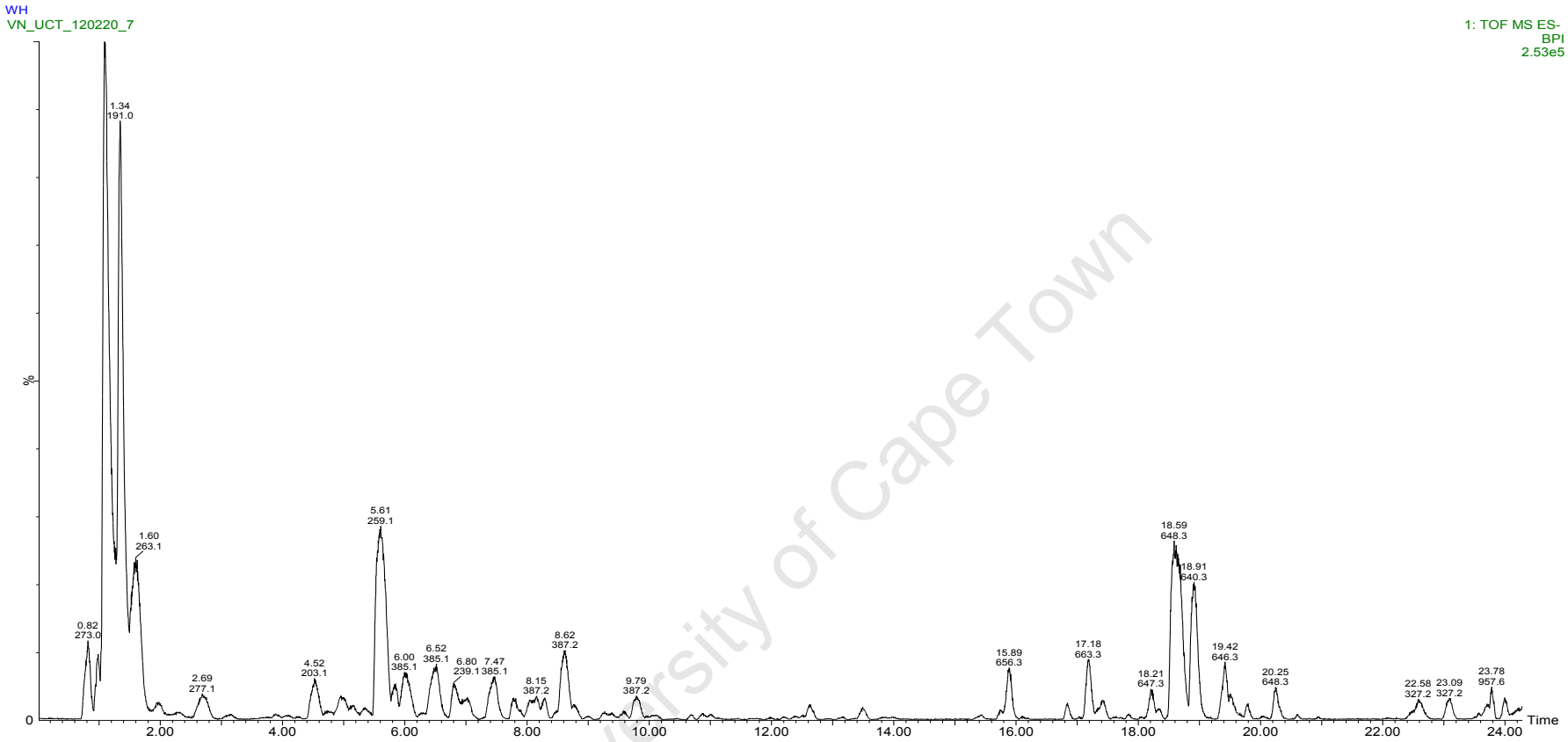


Figure D6 TIC of 70% methanol extract of white beans. Numbers on top of each curve are m/z values of the deprotonated molecule [M – H] ⁻ of each compound and their retention times.