Phenolic compounds, functional and antioxidant properties of flours from microwave pre-treated Bambara groundnut seeds

By

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DEDICATION

This dissertation is dedicated to God and all my loved ones. To my mother and father, brothers, friends and my family.

I hope I made you all proud.

DECLARATION

I declare the dissertation which I hereby submit for the degree MSc (Food Science) at the University of Pretoria is my own work and has not previously been submitted by me for a degree at another university or institution of higher education.

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ABSTRACT

Phenolic compounds, functional and antioxidant properties of flours from microwave pre-treated Bambara groundnut seeds

by

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Degree: MSc (Food Science)

Bambara groundnut (*Vigna subterranea* L.) is an underutilised and under-researched legume crop which is an important source of protein and other nutrients, mainly in countries in sub-Saharan Africa. It is consumed in a wide variety of forms such as soups, stews and increasingly as a flour with various food applications. Bambara groundnut is also susceptible to the hard-to-cook (HTC) defect as in many other legumes. Microwave processing is increasingly being used as a pre-treatment method for legumes to alleviate the HTC defect and inactivate anti-nutritional factors in the production of legume flours. This presents an opportunity for application of microwave processing to Bambara groundnut seeds for preparation of flours. Bambara groundnut also contain bioactive phenolic compounds with potential health-promoting properties in terms of combating non-communicable diseases (NCDs) associated with oxidative stress. However, phenolic compounds can be affected by thermal processing treatments such as microwave processing. In this study, the effect of microwave pre-treatment of Bambara groundnut seeds on the HTC defect as well as functional and antioxidant properties and phenolic content of its flours were determined.

Seeds of two Bambara groundnut types (brown and red) were pre-soaked to a final moisture content of 40% and microwave treated at 900 W and 1200 W for 5 and 8 minutes with an air temperature of 130ºC. The effect of microwave pre-treatment on the cooking time (determined using a Mattson bean cooker) of the seeds were determined. The water solubility index (determined at 50ºC and 95ºC), nitrogen solubility index, pasting properties (determined using a rheometer) and thermal properties (determined using differential scanning calorimetry) of the resultant flours

were determined. The total phenolic content (Folin-Ciocalteu method), antioxidant activity [2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging], phenolic profile and concentration [Ultra Performance Liquid Chromatography-Quadropole Time-of-Flight Mass Spectrometry (UPLC-QToF-MS)] and inhibitory effects against peroxyl radical-induced DNA damage of the resultant flours were determined.

Microwave pre-treatment decreased cooking time of Bambara groundnut seeds possibly due to partial gelatinisation of starch, protein denaturation and depolymerisation of pectic substances in the middle lamella of parenchyma cells. The water solubility index, nitrogen solubility index and pasting viscosity of flours from microwave pre-treated Bambara groundnut seeds were significantly lower compared to the untreated flour. This could be due to the microwave-induced denaturation of proteins and exposure of hydrophobic sites as well as the formation of a hydrophobic protein-starch complex which decreased water uptake by starch and thus leading to a decrease in the afore-mentioned functional properties. The thermal properties (enthalpy) of the flours decreased with increase in microwave power and time, suggesting the melting of some crystalline regions of starch which was attributed to the partial gelatinisation of starch.

Gallic, protocatechuic and vanillic acid were the main hydroxybenzoic acids while coumaric acid isomer, caffeoyl glycerol and ferulic acid hexoside were the main hydroxycinnamic acid derivatives identified in the Bambara groundnut seeds. Flavonoids identified in Bambara groundnut seeds were flavan-3-ols (procyanidin B2 dimer, procyanidin C2 trimer, catechin and catehin glucoside), flavonols (kaempferol, quercetin-3-*O*-glucoside and myricetin), flavones (apigenin), flavanones (hesperidin, hesperetin, naringenin, eriodictyol and eriodictyol-7-*O*-β-D-glucoside) and flavononols (taxifolin). The predominant flavonoids identified in brown Bambara groundnut samples were catechin and hesperidin while quercetin-3-*O*-glucoside and hesperidin were the predominant flavonoids in the red variety.

Microwave pre-treatment of Bambara groundnut seeds led to both increases (greater extractability) and decreases (reduced extractability) in phenolic content (total phenolic content and concentration of phenolic compounds) and radical scavenging antioxidant properties of Bambara groundnut. Increases in phenolic content and antioxidant properties could be due to microwave-induced release of bound phenolic compounds while decreases in phenolic content and antioxidant properties may be due to microwave-induced oxidative degradation of phenolic compounds.

Flavonoids were generally more thermally stable in the red variety than the brown during microwave treatments, suggesting that the effect of microwave pre-treatment on flavonoids in Bambara groundnut seeds could be variety-dependent. Microwave pre-treated Bambara groundnut seeds extracts showed protective effects against 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) radical-induced DNA damage which was attributable to their content of antioxidant phenolics.

Overall, these results show the potential of microwave processing as a technique not only for reducing the cooking time of Bambara groundnut seeds but also for producing functional Bambara groundnut flours for different food applications. The retention of appreciable antioxidant properties supported by protective effects against AAPH radical-induced DNA damage by the flours indicates their potential to promote health in terms of offering protection from oxidative stress related non-communicable diseases. The outcomes and findings of this research open up further avenues and opportunities for increased utilisation of Bambara groundnuts which is an important crop for food security in sub-Saharan African countries.

Contents

DEDICATION i
DECLARATION ii
ACKNOWLEDGEMENTS iii
ABSTRACT iv
Chapter 1: Introduction 1
1.1) Introduction and problem statement 1
Chapter 2: Literature review 3
2.1) Bambara groundnut 3
2.1.1) Production and utilization 3
2.1.2) Nutritional composition 3
2.2) Phenolic compounds in Bambara groundnut 6
2.2.1) Chemistry of phenolic acids and flavonoids and their content in Bambara groundnut 6
2.3) Phenolics-related health-promoting properties of legumes 9
2.3.1) In vitro antioxidant activity 9
2.3.2) Antihypertensive properties 12
2.3.3) Anti-cancer properties 13
2.3.4) Anti-inflammatory properties 14
2.3.5) Anti-diabetic properties 14
2.4) Hard-to-cook (HTC) phenomenon in legumes and its alleviation 15
2.4.1) Phytase-phytate-pectin hypothesis 15
2.4.2) Lignification hypothesis 15
2.4.3) Protein functionality deterioration hypothesis 16
2.4.4) Alleviation of the HTC phenomenon in legumes 16
2.5) Applications of thermal treatments to legumes and effects on seed structure and flour functional properties 17
2.5.1) High pressure cooking 17
2.5.2) Roasting and boiling 17
2.5.3) Microwave treatment 18
2.5.4) Micronisation (infrared) treatment 19
2.6) Conclusion and gaps in knowledge 21
Chapter 3: Hypotheses and Objectives 22

3.1) Hypotheses 22
Hypothesis 1 22
Hypothesis 2 22
Hypothesis 3 23
3.2) Objectives 23
Objective 1 23
Objective 2 23
Objective 3 23
Chapter 4: Experimental design 24
Chapter 5: Research 25
5.1) Effects of microwave pre-treatment of pre-soaked Bambara groundnut seeds on the cooking time and functional properties of the resultant flours 26
Abstract 26
5.1.1) Introduction 27
5.1.2) Materials and methods 28
5.1.2.1) Materials 28
5.1.2.2) Proximate analysis 28
5.1.2.3) Hydration of Bambara groundnut seeds to 40% moisture content 28
5.1.2.4) Microwave treatment 29
5.1.3) Analytical methods: 29
5.1.3.1) Cooking time 29
5.1.3.2) Water solubility index (WSI) 29
5.1.3.3) Nitrogen solubility index (NSI) 30
5.1.3.4) Pasting properties 30
5.1.3.5) Thermal properties 31
5.1.3.6) Light Microscopy (LM) 31
5.1.3.7) Statistical analysis 31
5.1.4) Results and discussion 32
5.1.4.1) Proximate composition of Bambara groundnut seeds 32
5.1.4.2) Cooking time of microwave pre-treated Bambara groundnut seeds 33
5.1.4.3) Pasting properties of flours from microwave pre-treated Bambara groundnuts 34

5.1.4.4) Water solubility index (WSI) of flours from microwave pre-treated Bambara groundnut seeds 38

5.1.4.5) Nitrogen solubility index (NSI) of flours from microwave pre-treated Bambara groundnut seeds 40

5.1.4.6) Thermal properties of flours from microwave pre-treated bambara groundnut seeds 42

5.1.4.7) Light microscopy analysis of flours from microwave pre-treated Bambara groundnut seeds 45

5.1.5) Conclusion: 52

5.2) Effects of microwave pre-treatment of pre-soaked Bambara groundnut seeds on their phenolic composition and antioxidant properties 53

Abstract 53

5.2.1) Introduction 54

5.2.2) Materials and methods 55

5.2.2.1) Bambara groundnut samples 55

5.2.2.2) Chemicals 55

5.2.2.3) Hydration of Bambara groundnut seeds to 40% moisture content 55

5.2.2.4) Microwave treatment 55

5.2.2.5) Preparation of phenolic extracts 55

5.2.2.6) Preparation of phenolic extracts for Ultra-performance Liquid Chromatography-Mass Spectrometry (UPLC-MS) analysis 55

5.2.2.7) Folin-Ciocalteu method for total phenolic content 56

5.2.2.8) 2, 2'-azino-bis (3-ethyl) benzothiazoline-6-sulphonic acid (ABTS) radical scavenging assay 57

5.2.2.9) UPLC-MS analysis 57

5.2.2.10) Inhibition of oxidative DNA damage assay 58

5.2.2.11) Statistical analysis 58

5.2.3) Results and discussion 59

5.2.3.1) Total phenolic content (TPC) and ABTS radical scavenging activity of flours from microwave pre-treated Bambara groundnut seeds 59

5.2.3.2) Identification of phenolic compounds in flours from microwaved pre-soaked Bambara groundnut seeds. 62

5.2.3.3) Phenolic acids and derivatives identified 63

5.2.3.4) Flavonoids and derivatives identified 66

5.2.3.5) Quantification of phenolic compounds from flours of microwaved Bambara groundnut seeds 75

5.2.3.6) Inhibitory effects of extracts from raw and microwaved Bambara groundnut seeds against oxidative DNA damage 78

5.2.4) Conclusion 80

Chapter 6: General discussion 81

6.1. Discussion of research methodologies used 81

6.2. Discussion of key research findings 85

Chapter 7: Conclusions and recommendations 98

Chapter 8: References 100

Appendix A 121

Appendix B 141

List of tables:

Table 2.1.1 Proximate composition of Bambara groundnut, cowpea and maize (g/100 g sample dry basis) 4
Table 2.1.2 Essential (indispensible) amino acid content (g/ 100 g protein) of Bambara groundnut, cowpea and maize 5
Table 2.2.1 Backbone structures of different flavonoid groups and their examples 8
Table 2.3.1: Some *in vitro* antioxidant activities reported for different varieties of Bambara groundnut and cowpea 10

Table 5.1.4.1 Proximate composition (% db) of Bambara groundnut 32
Table 5.1.4.2 Effect of microwave time and power on the cooking time of pre-soaked Bambara groundnut seeds 34
Table 5.1.4.3: Effect of microwave time and power on water solubility index (WSI) of flours from pre-soaked Bambara groundnut seeds 39
Table 5.1.4.4: Effect of microwave time and power on nitrogen solubility index (NSI) of flours from pre-soaked Bambara groundnut seeds 41
Table 5.1.4.5: Pearson correlation coefficients (r) for Nitrogen solubility index (NSI) vs water solubility index (WSI) for flours from raw and microwaved brown and red Bambara groundnut seeds 41
Table 5.1.4.6: Effect of microwave time and power on the onset, peak, endset temperatures and transition enthalpy of flours from pre-soaked Bambara groundnut seeds……..42

Table 5.2.3.1: Effect of microwave pre-treatment of pre-soaked Bambara groundnut seeds on the total phenolic content (TPC) (mg CE/g db) of the resultant flours 59
Table 5.2.3.2: Effect of microwave pre-treatment of pre-soaked Bambara groundnut seeds on the ABTS radical scavenging activity (µmol TE/g db) of the resultant flours 60
Table 5.2.3.3: Pearson correlation coefficients (r) for total phenolic content (TPC) vs ABTS radical scavenging activity for flours from raw and microwaved brown and red Bambara groundnut seeds 61
Table 5.2.3.4: Retention time (tR), UV-visible absorption maxima and mass spectral characteristics of phenolic compounds identified in ethyl acetate fraction of extracts from raw and microwaved Bambara groundnut seeds 62
Table 5.2.3.5: Effects of microwave pre-treatment on the flavonoid content (µg/g sample dry basis) of extracts from flours of Bambara groundnut seeds 75

Table 5.2.3.6: Effects of microwave pre-treatment on the phenolic acid content (µg/g sample dry basis) of extracts from flours of Bambara groundnut seeds 76

Table 6.2.1: Summary of the effect of microwave pre-treatment of Bambara groundnut seeds on the functional properties of the resultant flours 87
Table 6.2.2: Effect of microwave pre-treatment on the presence of flavones and flavanones in Bambara groundnut seeds 90

Table 6.2.3: Effect of microwave pre-treatment on the presence of flavan-3-ols and flavonols in Bambara groundnut seeds 91
Table 6.2.4: Summary of the effect of microwave pre-treatment on the phenolic content and antioxidant properties of Bambara groundnut seeds 93

List of figures:

Figure 2.2.1: Two different phenolic acid groups (Awika & Duodu, 2017) 6
Figure 2.2.2: General flavan backbone of flavonoids (Awika 2017) 7
Figure 2.3.1: Quercetin structure showing structural features important for antioxidant activity. (Adapted from Williams, Spencer & Rice-Evans, 2004). 11
Figure 2.3.2: Structural difference between hydroxycinnamic (with the ethylenic group encircled) and hydroxybenzoic acids (Adapted from Cai et al. 2006) 12
Figure 2.5.2.1: Scanning electron micrograph of flour from boiled green lentil seeds (Adapted from Ma et al., 2011) 18
Figure 2.5.4.1: Light microscope image of toluidene stained whole micronised Bambara groundnut seed cotyledon (A) and scanning electron microscope image of whole micronised Bambara groundnut seed cotyledon (B). Cw, cell wall: Cs, Cell separation: S, Starch granules. (from Ogundele, 2016) 21

Figure 4.1: Experimental design used to determine the effect of microwave pre-treatment on cooking time of Bambara groundnut seeds and functional properties, phenolic content and antioxidant properties of their flours 24
Figure 5.1.4.1: Effect of microwave pre-treatment on the pasting properties of pre-soaked Brown (A) and Red (B) Bambara groundnuts 37
Figure 5.1.4.2 Effect of microwave pre-treatment on the thermal properties of flours from brown (A) and red (B) Bambara groundnut seeds. 44
Figure 5.1.4.3: Polarized light microscopy images of flour from raw and microwaved Bambara groundnut seeds. 48
Figure 5.1.4.4: Light microscope images of iodine stained flours from raw and microwaved Bambara groundnut seeds. 51
Figure 5.2.3.1: Proposed fragmentation patterns for phenolic acids and their derivatives identified in the raw and microwaved Bambara groundnut seeds 65
Figure 5.2.3.2: Proposed fragmentation patterns for procyanidin B 2 (epicatechin-(4-8)-epicatechin) 66
Figure 5.2.3.3: Proposed fragmentation patterns for procyanidin C 2 trimer 67
Figure 5.2.3.4: Proposed fragmentation pattern for catechin 68
Figure 5.2.3.5: Proposed fragmentation pattern for catechin glucoside 68
Figure 5.2.3.6*:* Proposed fragmentation pattern for kaempferol 69
Figure 5.2.3.7 Proposed fragmentation pattern for quercetin-3-*O*-glucoside 70
Figure 5.2.3.8 Proposed fragmentation pattern for taxifolin 70
Figure 5.2.3.9*:* Proposed fragmentation pattern for apigenin 71
Figure 5.2.3.10: Proposed fragmentation pattern for hesperidin 72
Figure 5.2.3.11*:* Proposed fragmentation for hesperetin (301 m/z) 73
Figure 5.2.3.12: Proposed fragmentation for naringenin 73
Figure 5.2.3.13*:* Proposed fragmentation for eriodictyol 74
Figure 5.2.3.14*:* Proposed fragmentation pattern for eriodictyol-7-*O*-D-glucoside 74
Figure 5.2.3.15: Agarose gel electrophoretogram showing the inhibitory effects of phenolic extracts from raw and microwaved Bambara groundnut seeds against AAPH radical-induced oxidative pBR322 vector DNA damage 79

Figure 6.2.1: β-elimination reaction in the depolymerization of pectin during microwave treatment 86
Figure 6.2.2: Basic representation of the formation of the protein-starch matrix (Adapted from Ogundele 2016) 88
Figure 6.2.3: Light microscope images of iodine stained flours from brown Bambara groundnut seeds microwaved for 5 minutes at 900 W (right) and 5 minutes at 1200 W (left). 89
Figure 6.2.1**:** An illustration of how quercetin-3-*O*-glucoside could be bound to proteins via hydrophobic interactions, hydrogen bonding and ionic bonds 94
Figure 6.2.5: Representation of microwave-induced hydrolysis of ether and ester bonds to release naringenin from hemicelluloses or pectic substances 95
Figure 6.2.6: Microwave - induced oxidation of kaempferol 96
Figure 6.2.7: An illustration of microwave-induced crosslinking of oxidized protocatechuic acid with proteins 96
Figure A 1: Mass spectrum of gallic acid (167 m/z) peak 1 121
Figure A 2: Mass spectrum of protocatechuic acid (153 m/z) peak 2 122
Figure A 3: Mass spectrum of vanillic acid (167 m/z) peak 3 123
Figure A 4: Mass spectrum of coumaric acid isomer (137 m/z) peak 4 124
Figure A 5: Mass spectrum of caffeoyl glycerol (253 m/z) peak 5 125
Figure A 6: Mass spectrum of ferulic acid hexoside (355 m/z) peak 6 126
Figure A 7: Mass spectrum of Procyanidin B 2 dimer (577 m/z) peak 7 127
Figure A 8: Mass spectrum of procyanidin C 2 trimer (865 m/z) peak 8 128
Figure A 9 : Mass spectrum of catechin (289 m/z) peak 9 129
Figure A 10:Mass spectrum of catechin-5-*O*- glucoside (451 m/z) peak 10 130
Figure A 11:Mass spectrum of kaempferol (285 m/z) peak 11 131
Figure A 12:Mass spectrum of quercetin-3-*O*-glucoside (463 m/z) peak 12 132
Figure A 13:Mass spectrum of myricetin (317 m/z) peak 13 133
Figure A 14:Mass spectrum of taxifolin (303 m/z) peak 14 134
Figure A 15:Mass spectrum of apigenin (269 m/z) peak 15 135
Figure A 16:Mass spectrum of hesperidin (609 m/z) peak 16 136
Figure A 17:Mass spectrum of hesperetin (301 m/z) peak 17 137
Figure A 18:Mass spectrum of naringenin (271 m/z) peak 18 138
Figure A 19:Mass spectrum of eriodictyol (287 m/z) peak 19 139
Figure A 20:Mass spectrum of eriodictyol -7-*O*-β-D- glucoside (449 m/z) peak 20 140
Figure B.1: UPLC-MS chromatograms (at 289 nm) of ethyl acetate extracts from raw untreated pre-soaked brown (A) and red (B) Bambara groundnut seeds. 141
Figure B. 2: UPLC-MS chromatograms (at 289 nm) of ethyl acetate extracts from pre-soaked red Bambara groundnut seeds microwaved for 5 minutes at 900 W. 142
Figure B. 3: UPLC-MS chromatograms (at 280 nm) of ethyl acetate extracts from pre-soaked brown (A) and red (B) Bambara groundnut seeds microwaved for 8 minutes at 900 W. 143
Figure B. 4: UPLC-MS chromatograms (at 280 nm) of ethyl acetate extracts from pre-soaked brown (A) and red (B) Bambara groundnut seeds microwaved for 5 minutes at 1200 W. 144
Figure B. 5: UPLC-MS chromatograms (at 280 nm) of ethyl acetate extracts from pre-soaked brown (A) and red (B) Bambara groundnut seeds microwaved for 8 minutes at 1200 W. 145

Figure B. 6: UPLC-MS chromatograms (at 280 nm) of formic acid-methanol extracts from pre-soaked raw untreated brown (A) and red (B) Bambara groundnut seeds. 146
Figure B. 7: UPLC-MS chromatograms (at 280 nm) of formic acid-methanol extracts from pre-soaked red Bambara groundnut seeds microwaved for 5 minutes at 900 W. 147
Figure B. 8*:* UPLC-MS chromatograms (at 280 nm) of formic acid-methanol extracts from pre-soaked brown (A) and red (B) Bambara groundnut seeds microwaved for 8 minutes at 900 W. 148
Figure B. 9: UPLC-MS chromatograms (at 280 nm) of formic acid-methanol extracts from pre-soaked brown (A) and red (B) Bambara groundnut seeds microwaved for 5 minutes at 1200 W. 149
Figure B. 10: UPLC-MS chromatograms (at 280 nm) of formic acid-methanol extracts from pre-soaked brown (A) and red (B) Bambara groundnut seeds microwaved for 8 minutes at 1200 W. 150

Chapter 1: Introduction

1.1) Introduction and problem statement

Bambara groundnut (*Vigna subterranea* L.) is an important grain legume consumed in many African communities (Phillips, McWatters & Enwere, 1998; Goudoum, Mgamo Tinkeu, Madou, Djakissam & Mbofung, 2016). Like other grain legumes such as cowpeas, Bambara groundnut is an important plant protein source and plays a role as a supplier of protein and essential amino acids in many rural African communities (Giami 2005). These legumes however have long cooking times due to the hard-to-cook (HTC) phenomenon which is a problem for rural communities in developing countries using conventional cooking as it impacts energy costs (Kayitesi, Duodu, Minnaar & De Kock, 2012). Some research studies aimed at allevaiting the HTC phenomenon in legumes have involved the application of pre-treatments such as hydration and micronization (Infrared treatment) as has been done with cowpea (Mwangwela, Waniska, McDonough & Minnaar 2007). Microwave treatment is a potential technology that could also be utilized in this way to reduce cooking time of legumes such as Bambara groundnut.

Non-communicable diseases (NCD's) such as cancer, cardiovascular diseases, diabetes and chronic respiratory diseases are said to be the leading cause of death in the world and these are increasing in Africa (Naik & Kaneda 2015). Growing urbanization in sub-Saharan African countries has contributed to change in dietary choices to less healthy and energy dense foods. This has brought about increase in obesity and susceptibility to oxidative stress and associated non-communicable diseases (Naik & Kaneda 2015). Legumes such as Bambara groundnuts are known to contain bioactive phenolic compounds which are hypothesized to have the ability to protect against oxidative stress and prevent NCD's (Ambriz-Pérez, Leyva-López, Gutierrez-Grijalva & Basilio Heredia, 2016), through their antioxidant properties (Jacob & Burri 1996).

Legume flours are extensively used in various food applications. For production of legume flours, legume seeds are usually taken through some thermal pre-treatments such as micronisation or microwaving. These thermal pre-treatments serve to reduce or eliminate anti-nutritional factors, reduce cooking time of the seeds and also have an effect on functional properties of the legume flours. Some examples of application of thermal pre-treatments to legumes include the pre-

treatment of Bambara groundnuts using micronisation (Ogundele, 2016) and pre-treatment of cowpeas with microwave radiation (Mokatso, 2017). Microwave treatment technology could therefore be potentially applied to Bambara groundnut to reduce its cooking time. Such microwave treatments can have an effect on functional properties of Bambara groundnut flours, their phenolic content and antioxidant properties.

The production of quick cooking Bambara groundnut seeds by application of microwave treatment and Bambara groundnut flours with good functional properties for different applications and important health-promoting properties will be beneficial for sub-Saharan African communities. In this research, the effect of microwave pre-treatments on cooking time of Bambara groundnut seeds, functional properties of resultant flours as well as their phenolic compounds and antioxidant properties will be studied.

Chapter 2: Literature review

2.1) Bambara groundnut

2.1.1) Production and utilization

Bambara groundnut is a grain legume grown mainly in sub-Saharan Africa, but also in other parts of the world such as Asia, Northern Australia, Central and South America (Goli, 1997; Eltayeb, Ali, Abou-Arab & Abu-Salem, 2011). The Bambara groundnut has been described as an easy-to-cultivate crop due to its high drought tolerance and ability to grow in poor soil conditions (Diedericks & Jideani, 2015). Burkina Faso is the world's largest producer of Bambara groundnut with an average production of 41 900 metric tons from 1993 to 2014 according to FAOSTAT, (2016). Bambara groundnut is consumed in a wide variety of ways including as a fried or boiled snack or milled into flour for use in soups or porridges in Nigeria (Adeleke, Adiamo & Fawale, 2018). Bambara groundnut is also processed into steamed foods named *akara, moin-moin* and *okpa* in Nigeria (Adeleke *et al*., 2018). It has also been used in making of bread in Zambia (Adebowale & Lawal, 2004).

2.1.2) Nutritional composition

The importance of Bambara groundnut as a food legume is demonstrated by its nutritional composition. Table 2.1.1 shows the proximate compositon of Bambara groundnut compared to cowpea (also an underutilized grain legume) and maize (as an example of a cereal). As expected, for legumes compared to cereals, the protein contents of Bambara groundnut and cowpea are approximately three times that of maize. Bambara groundnut protein consists mainly of albumins (73%), glutelins (8.6%), globulins (8.2%) and prolamins (0.8%) (Yagoub & Abdalla, 2007). The three grains have high carbohydrate content (mostly starch) and low fat content.

Maphosa and Jideani (2016) reported soluble dietary fibre and insoluble dietary fibre contents of Bambara groundnut to range from 15.4% to 17.1% and 12% to 15.6% respectively. These authors reported the composition of these dietary fibres to be comprised of galactomannans, arabinoxylans, arabinogalactans, rhamnogalacturans and pectic substances.

Table 2.1.1 Proximate composition of Bambara groundnut, cowpea and maize (g/100 g sample dry basis)

	Bambara groundnut[a]	Cowpea[b]	Maize[c]
Protein	21.9	26.7	7.7
Crude fat	6.4	1.4	4.4
Carbohydrates	64.3	68.1	86.3
Crude fibre	4.0	12.03	8.2
Ash	3.4	NA	1.6

[a]: (FAO, 2012)

[b]: (USDA, 2016)

[c]:(USDA, 2018)

NA: Not Available

Legume proteins are generally regarded to be of better quality than cereal proteins. This can be shown by a comparison of their indispensable (or essential) amino acid contents. Table 2.1.2 shows the indispensable amino acid contents for Bambara groundnut in comparison with cowpea and maize. Bambara groundnut and cowpea (the legumes) contain higher levels of the indispensable amino acids than maize (the cereal). This provides motivation for combining legumes with cereals in composite foods to achieve enhanced protein quality compared to the cereal on its own. Bambara groundnut seeds are superior to cowpea and maize in almost all of the indispensible amino acids including methionine and cystine with 2.9%, 2.5% and 1.87% respectively.

Table 2.1.2 Essential (indispensible) amino acid content (g/ 100 g protein) of Bambara groundnut, cowpea and maize

	Bambara groundnut[a]	Cowpea[b]	Maize[c]
Leucine	7.8	7.7	5.89
Isoleucine	4.3	4.1	1.73
Valine	5.3	4.8	2.43
Threonine	3.3	3.8	1.80
Phenylalanine + Tyrosine	9.3	9.1	2.15
Lysine	6.6	6.8	1.39
Histidine	3.2	3.1	1.46
Methionine + Cystine	2.9	2.5	1.87
Arginine	6.4	6.9	2.36

[a]: (Apata & Ologhobo, 1994)

[b]: (USDA, 2018)

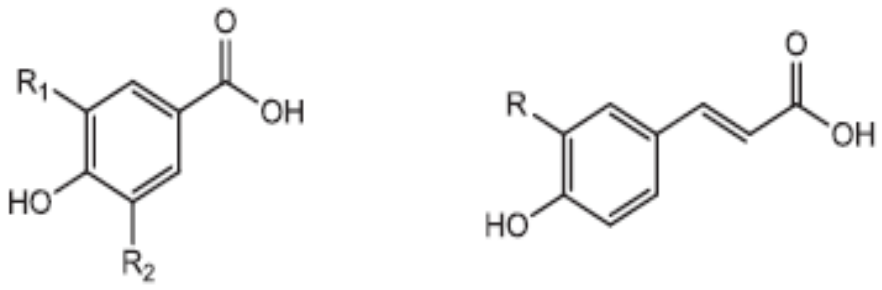
[c]: (USDA, 2018)

2.2) Phenolic compounds in Bambara groundnut

Phenolic compounds in Bambara groundnuts are mainly phenolic acids and flavonoids. The chemistry of phenolic acids and flavonoids and their contents reported in Bambara groundnut is discussed below.

2.2.1) Chemistry of phenolic acids and flavonoids and their content in Bambara groundnut

Phenolic acids are secondary metabolite compounds in plants involved in cell defense, structural support, cell signaling and biosynthetic intermediates (Awika & Duodu, 2017). Phenolic acids can be classified into hydroxycinnamic and hydroxybenzoic acids (Figure 2.2.1). Hydroxybenzoic acids contain a phenol ring with a carboxylic group attached forming a C6-C1 backbone (Teixeira, Gaspar, Manuela Garrido, Garrido & Borges, 2013) (Figure 2.2.1). Examples of hydroxybenzoic acids includes gallic, *p*-hydroxybenzoic, protocatechuic, syringic and vanillic acids. Hydroxycinnamic acids consist of a C6-C3 phenylpropanoid backbone structure (Teixeira *et al*., 2013) (Figure 2.2.1). Examples of hydroxycinnamic acids include caffeic, sinapic, *p*-coumaric and ferulic acids. Hydroxybenzoic acid and hydroxycinnamic acid derivatives are determined by the different substituents on the R, R1 and R2 position on the phenol ring (Figure 2.2.1). Phenolic acids are found in free or bound forms in grain legumes. The bound phenolic acids are esterified to cell wall components such as cellulose, lignins and proteins (Lui, 2007).



Hydroxybenzoic acids Hydroxycinnamic acids

Figure 2.2.1: Two different phenolic acid groups (Awika & Duodu, 2017)

Phenolic acids reported in Bambara groundnuts include caffeic acid and caffeic acid derivatives, *trans*-ferulic acid, *p*-coumaric acid and salicylic acid (Nyau *et al*., 2015). Tsamo, Ndibewu and Dakora (2018) reported that the main phenolic acids in Bambara groundnut are caffeic acids and its derivatives. These authors also reported the presence of syringic acid derivatives, gallic acid and its derivative pyrogallic acid, vanillic acid hexoside, isoferulic acid as well as chlorogenic acid.

Flavonoids are a group of plant secondary metabolite compounds containing a C6-C3-C6 flavan nucleus ring (Figure 2.2.2) (Awika & Duodu, 2017). Different types of flavonoids are classified according to substitutions around the C-ring. Flavonoids are concentrated in the seed coat of legumes and have an effect on the seed coat color of legume seeds (Ojwang, 2012). The different R-group constituents including hydroxyl, hydrogen, methoxyl and *O*-glucosides on the backbone determine their chemical activity such as antioxidant activity (Rice-Evans *et al*., 1996). Flavonoids can be classified into different groups depending on the level of oxidation or the pattern of substitution of the C-ring (Pietta, 2000). The main flavonoid groups include flavonols, flavan-3-ols, flavones and flavanones (Table 2.2.1).

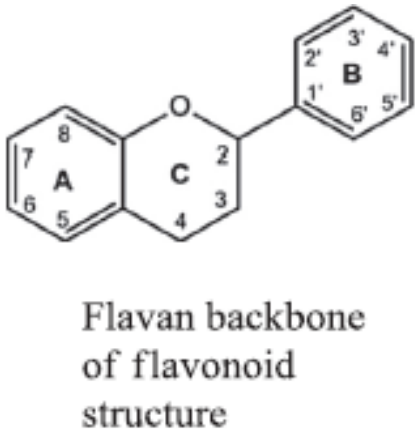
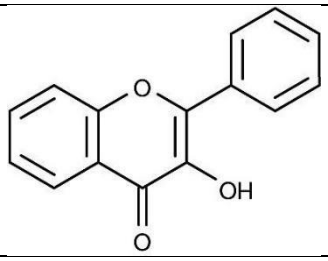
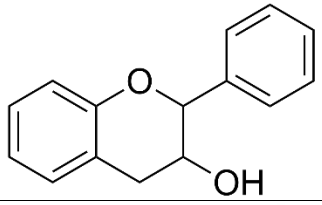
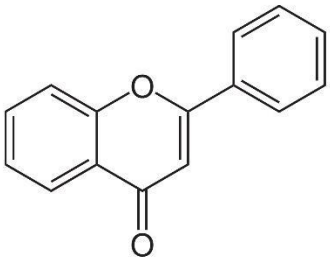
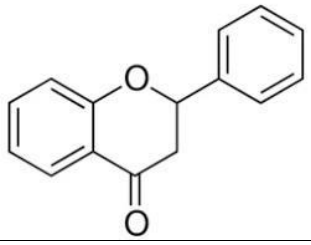


Figure 2.2. 2: General flavan backbone of flavonoids (Awika 2017)

Table 2.2.1 Backbone structures of different flavonoid groups and their examples

Flavonoid group and examples	Backbone structure
Flavonol (quercetin, myricetin, kaempferol)	
Flavan-3-ol (catechin, epicatechin)	
Flavone (apigenin, luteolin)	
Flavanone (naringenin, eriodictyol, hesperitin)	

Nyau *et al*. (2015) identified the flavonols myricetin hexoside, quercetin-3-*O*-rutinoside and quercetin-3-*O*-glucoside in red Bambara groundnut seeds. The authors also identified quinic acid, catechin glucoside, catechin, epicatechin and medioresinol (a lignan) in both brown and red Bambara groundnut seeds. Tsamo *et al*. (2018) identified free and glycoside derivatives of flavonoids such as kaempferol, quercetin and catechin in Bambara groundnut seeds.

2.3) Phenolics-related health-promoting properties of legumes

2.3.1) In vitro antioxidant activity

The health benefits associated with phenolic compounds in legumes such as the reduced risk of diet-related NCD's are attributed to their antioxidant activity. Phenolic compounds are known to be powerful antioxidants through their ability to exert radical scavenging activity by hydrogen donation (Silva, Borges, Guimarães, Lima, Matos & Reis, 2000). *In vitro* antioxidant activity for phenolic extracts for various legumes have been reported. Table 2.4.1 shows some *in vitro* antioxidant acitivity values for Bambara groundut and cowpea reported in literature. Generally for both Bambara groundnut seeds and cowpea, the *in vitro* antioxidant activity is higher for the red and reddish- brown varieties respectively than for the golden-yellow, cream colored and brown varieties. This could be due to the red varieties containing more flavonoids than the other colored varieties such as reported by Ojwang (2012) for cowpea.

Table 2.3.1: *In vitro* antioxidant activities reported for different varieties of Bambara groundnut and cowpea

Legume type	*In vitro* Antioxidant activity	Reference
Raw golden-yellow cowpea (Agrigold variety), aqueous acetone extract	38 ± 3 µmol Trolox Equivalents/g dry weight basis (ABTS radical scavenging)	Hachibamba *et al.* (2013)
Raw cream cowpea (Blackeye variety), acidified methanol extract	123.7 ± 6.0 µmol Trolox Equivalents/g dry weight basis (ABTS radical scavenging)	Kayitesi (2013)
Raw reddish-brown cowpea (Glenda variety), acidified methanol extract	157.7 ± 8.1 µmol Trolox Equivalents/g dry weight basis (ORAC-Oxygen radical absorbance capacity)	Kayitesi (2013)
Raw brown Bambara groundnut, aqueous methanol extract	477.5 ± 3.5 µg dried extract/ ml (EC50)* (DPPH radical scavenging)	Nyau *et al.* (2015a)
Raw red Bambara groundnut, aqueous methanol extract	495.5 ± 12.0 µg dried extract/ml (EC_{50}) (DPPH radical scavenging)	Nyau *et al.* (2015a)

*EC50 – Effective concentration of extract for inhibiting DPPH by 50%

Antioxidant activity of phenolic compounds is closely related to their structure. The structure-activity relationships of phenolic compounds has been described by Rice-Evans *et al.* (1996). The main structural features important for display of antioxidant activity by flavonoids are highlighted in the structure of quercetin shown in Figure 2.4.1. These structural features may be summarised as follows:

- The presence of a 3'4'-catechol group on the B-ring
- A 4-oxo functional group adjacent to a double bond between C2-C3
- OH groups on C5 and C7 on the A-ring

Hydroxylation of the B ring increases the ability of the flavonoid to donate hydrogen atoms for antioxidant activity. In addition, the catechol group on the B ring can engage in transition metal ion chelation which confers antioxidant effects. The 3-OH group in the C ring and the 2,3 double bond in conjugation with the 4-oxo carbonyl group in the C ring also makes it possible for transition metal chelation (Rice-Evans *et al*., 1996). The 2,3 double bond in conjugation with the 4-oxo carbonyl group such as in quercetin has been shown by Rice-Evans *et al*. (1996) to enhance the antioxidant activity compared to catechin which does not have the 2,3 double bond. The glycosylation of flavonoids has been shown by Shahidi and Wanasundara (1992) to decrease radical scavenging activity.The blocking or removal of the 3-OH group in the C ring of quercetin or its absence as in luteolin result in a decrease in antioxidant activity (Rice-Evans *et al*., 1996).

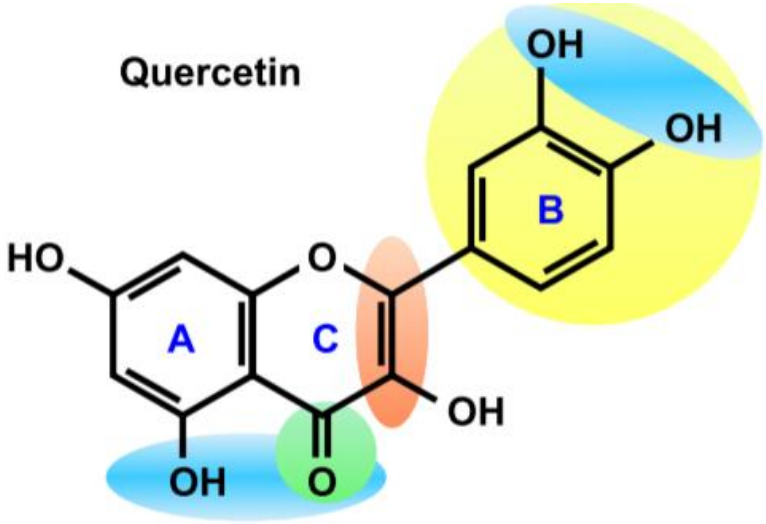


Figure 2.3.1: Quercetin structure showing structural features important for antioxidant activity. (Adapted from Williams, Spencer & Rice-Evans, 2004). The presence of a 3'4'-catechol group on the B-ring (shaded blue inside the yellow circle), a 4-oxo functional group (shaded in green) adjacent to a double bond between C2-C3 (shaded in red), and OH groups on C5 (shaded blue) and C7 (unshaded) on the A-ring are important structural features for antioxidant activity of flavonoids.

For phenolic acids, Cai, Sun, Xing, Luo and Corke (2006) showed that as a general principle, phenolic acids with the most hydroxyl groups possessed higher antioxidant activity. Hydroxycinnamic acids also generally show higher antioxidant activity than hydroxybenzoic acids. This is due to the presence of the ethylenic group in hydroxycinnamic acids (Figure 2.4.2)

which allows for enhanced resonance stabilization of the phenoxyl radical and therefore translates into greater antioxidant activity (Rice-Evans *et al*., 1996).

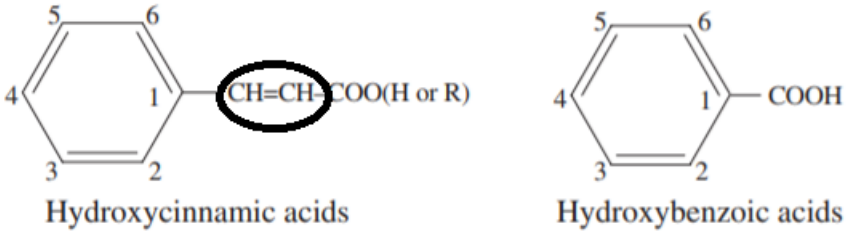


Figure 2.3.2: Structural difference between hydroxycinnamic (with the ethylenic group encircled) and hydroxybenzoic acids (Adapted from Cai *et al*. 2006)

2.3.2) Antihypertensive properties

An important step for the initiation of hypertention and artheroscrelosis is the oxidation of low density lipoprotein (LDL) and transport of the oxidized LDL across the epithelium into the artery wall (Madamanchi, Vendrov & Runge, 2005). The oxidized LDL loses the ability to regulate uptake of cholesterol and this results in the accumulation of cholesterol within the oxidized LDL (Jialal & Devaraj, 1996). Macrophages within the arteries take up the oxidized LDL and this results in the formation of foam cells (Diaz *et al*., 1997). The macrophages which contain oxidized LDL with increased cholesterol uptake then have reduced motility and so are retained and accumulate within the arterial walls which results in formation of a fatty streak within the artery (Jialal & Devaraj, 1996; Diaz *et al*., 1997). This increased cholesterol uptake by oxidized LDL and resultant formation of fatty streaks play a major role in hypertension and artherosclerosis. Therefore, prevention of LDL oxidation could be a way of combating hypertension and artherosclerosis.

Phenolic compounds can inhibit oxidation of lipid systems such as low density lipoproteins by chelation of pro-oxidant metal ions such as copper and iron (Rice-Evans *et al*., 1996). The ability of different types of extracts from legumes to inhibit LDL oxidation has been reported in the literature and this has been linked to their content of phenolic compounds. Hachibamba *et al*.

(2013) showed the potential of cowpea phenolic extracts (which contained a wide range of phenolic compounds including mono and diglucosides of quercetin) to inhibit LDL oxidation *in vitro* after simulated gastrointestinal digestion. Salawu, Bester & Duodu (2014) showed the potential of extracts from cell wall preparations and whole grain flours of cowpea (containing flavonoids such as catechin, quercetin, apigenin and naringenin and phenolic acids such as p-coumaric acid, p-hydroxy benzoic acid and cinnamic acid) to inhibit LDL oxidation. Phenolic extracts from other legumes such as red kidney beans, black beans and lentils have also proven to be effective in inhibition of LDL oxidation *in vitro* as shown in researh done by Xu, Yuan & Chang (2007).

2.3.3) Anti-cancer properties

Phenolic compounds can prevent cancer by inhibition of oxidative DNA damage. Various researchers have reported on the ability of phenolic extracts from legumes such as cowpeas to inhibit oxidative DNA damage (Ojwang, 2012; Hachibamba et al., 2013; Nderitu et al., 2013).

Other mechanisms by which phenolic compounds are hypothesised to exert protective effects against cancer include inhibition of Phase I detoxifying enzymes which convert procarcinogens into reactive intermediate molecules (Benson, Batzinger, Ou, Bueding, Cha & Talalay, 1978) and induction of Phase II detoxifying enzymes (Awika et al., 2017). Phase II detoxifying enzymes protect cells by converting toxic substances introduced into the body to less reactive molecules which are easily excretable. Induction of these enzymes has been shown to occur at transcription level of genes (Hu, Shen, Yerramilli, Lin, Xu, Nair & Kong, 2006). Faris, Takruri, Shomaf and Bustanji (2009) showed the chemopreventative effect of lentils and soybeans against azoxymethane-induced carcenogenesis in rats. These authors reported an increase in glutathione-S-transferase (GST) (a Phase II enzyme) activity in rats fed a diet of lentils and soybeans as compared to the control group. The increases in GST activity was attributed to the detoxifying enzyme induction activities of phenolic compounds in lentils and soybeans. In a different study, Shomaf, Takruri and Faris (2012) found a reduction in azoxymethane-induced dysplastic lesions and neoplasms in colons of Fischer 344 (F 344) rats fed a diet of lentils as compared to the control group. These authors also found an increase in phase II GST activity in the F 344 rats and attributed the increase to bioactive phenolic compounds.

2.3.4) Anti-inflammatory properties

Inflammation is a cellular response to tissue injury and chronic inflammation is believed to be a precursor to the development of non-communicable diseases such as cardiovascular disease (CVD) and cancer (Santangelo, Scazzocchio, Di Benedetto, Filesi & Masella, 2007; García-Lafuente, Guillamón, Villares, Rostagno & Martínez, 2009). Phenolic compounds such as flavonoids inhibit inflammation through several mechanisms as described by García-Lafuente *et al*. (2009): i) Scavenging of highly reactive free radicals that would otherwise stimulate the action of various inflammatory mediators resulting in inflammatory response and tissue injury, ii) Inhibition of enzymes involved in arachidonic acid metabolism such as phospholipase A2, cyclooxygenase (COX), lipoxygenase (LOX) and nitric oxide synthase, iii) Modulation of proinflammatory molecules such as Nuclear factor-κB (NF-κB), a transcription factor involved in inflammation and stress and iv) Modulation of expression of genes involved in proinflammatory activities.

Boudjou, Oomah, Zaidi and Hosseinian (2013) reported high lipoxygenase (LOX) and cyclooxygenase (COX) inhibitory effects of tannins, flavonols and anthocyanins in lentil and faba bean cotyledon fractions and lentil hull extracts. The lentil hull extracts exhibited preferential inhibition of LOX (Boudjou *et al*., 2013). Ojwang (2012) studied anti-inflammatory properties of cowpeas and reported that a flavonol-rich red cowpea variety showed high inhibition of NF-κB by down-regulation of the NF-κB-mRNA. Ojwang (2012) also showed that cowpea phenolic compounds reduced vascular cell adhesion molecules (VCAM-1) gene expression in colon CCD18Co cells. Vascular cell adhesion molecules such as VCAM-1 are target genes for NF-κB production (Ojwang, 2012).

2.3.5) Anti-diabetic properties

The control of blood glucose levels is very important in the prevention of diabetes mellitus since in the case of type 2 diabetes, the cells fail to respond to insulin thus it is important to prevent the absorption of glucose (Orhan, Aslan, Şüküroğlu & Orhan, 2013). The inhibition of starch hydrolysing enzymes such as amylases and α-glucosidase is used to demonstrate potential anti-diabetic effects. α-Glucosidase inhibitors such as phenolic compounds are unique in that they do not target specific defects of type 2 diabetes (Inzucchi, 2002). Instead they bind to the α-glucosidase enzyme in the brush border cells of the small intestine (Inzucchi, 2002). These

enzymes, together with α-amylase, break down carbohydrates such as starch into smaller glucose units which can be absorbed into the body (Kang, Song & Gu, 2012).

Sreerama, Takahashi and Yamaki (2012) showed that phenolic methanol extracts from adzuki, moth bean and mung bean were all strong inhibitors of the α- glucosidase enzyme. They showed that the black adzuki bean had the most potent inhibitory affect. Carmona *et al*. (1996) showed that black bean tannins inhibited α-amylase activity of bovine pancreatin to the same extent as commercial tannic acid.

2.4) Hard-to-cook (HTC) phenomenon in legumes and its alleviation

Legumes are well known for having long cooking times. Three main theories have been proposed to explain the HTC phenomenon in legumes: the phytase-phytate-pectin hypothesis (Galiotau-Panayotau, Kyriakidis & Margaris, 2008), the lignification hypothesis (Hincks & Stanley, 1987) and the protein functionality deterioration hypothesis (Lui, MacWatters & Phillips, 1992b).

2.4.1) Phytase-phytate-pectin hypothesis

The phytase-phytate-pectin theory suggests that during storage of legumes at high temperature and high relative humidity, there is increased phytase enzyme activity which causes degradation of phytate (Galiotau-Panayotau *et al*., 2008). The phytate thereafter reacts with released divalent calcium cations to form crosslinked calcium-pectate bridges. During cooking, the calcium-pectates do not dissolve easily and therefore lead to long cooking time (Shomer, Paster, Lindner & Vasiliver, 1990) due to cell separation failure (Ndungu, Emmambux & Minnaar, 2012).

2.4.2) Lignification hypothesis

Varriano-Marston and Jackson (1981) reported lignification of the middle lamella as a possible reason for the decrease in bean cookability. This lignification proceeds by increased polymerisation of phenolic compounds as has been reported by Hincks and Stanley (1986) who found a decrease in extractable polyphenols in beans after storage. Although some researchers such as Srisuma *et al*. (1989) and Garcia *et al*. (1998) failed to find changes in lignin content during storage of beans, Srisuma *et al*. (1989) reported an increase in free hydroxycinnamic acids with increased hardening of dry beans. The authors suggested that the release or synthesis of phenolic

acids could lead to cross-linking reactions with proteins or pectin, thus resulting in increase seed toughness. Garcia *et al*. (1998) recorded a higher phenolic content for HTC beans than for the control through Fourier Transform Infrared (FTIR) analyses.

2.4.3) Protein functionality deterioration hypothesis

According to the protein functionality deterioration hypothesis, during storage of legumes the proteins may interact with the embedded starch which causes a decrease in the protein solubility and thermal stability (Lui *et al*., 1992a). The decrease in thermal stability of proteins means that the protein transition temperature is lower than that of starch. Due to this, protein coagulation occurs before starch gelatinization which allows the protein to form a water restricting barrier around the starch granules. This phenomenon restricts or retards water absorption by the starch granules leading to a longer cooking time.

2.4.4) Alleviation of the HTC phenomenon in legumes

Thermal treatments such as microwave processing and micronisation (infrared) have been successfully applied to legumes with the aim of reducing their cooking time and thus alleviate the effects of the HTC defect. Divekar, Karunakaran, Lahlali, Kumar, Chelladurai, Liu, Borondics, Shanmugasundaram & Jayas (2017) showed the reduction in cooking time of various pulses after microwave treatement including red lentil, chickpea, pigeon pea, mung bean, and pinto bean using microwave power of 400 to 600 W for 14 to 56 seconds. Kayitesi *et al*. (2013) reported a reduction in cooking time of four different cowpea types after micronisation treatment. Ogundele and Emmambux (2018) found a reduction in cooking time of micronised (infrared treated) Bambara groundnut seeds.

2.5) Applications of thermal treatments to legumes and effects on seed structure and flour functional properties

Various thermal treatments are applied to legume seeds mostly as pre-treatment processes for the production of legume flours. Apart from having effects on the legume seeds, such thermal treatments also have an effect on the functional properties of the resultant legume flours. Functional properties of flours such as nitrogen solubility index (NSI), water solubility index (WSI), pasting and thermal properties are important characteristics which determine the efficiency and applicability of the flours as functional ingredients for use in food. The effects of thermal treatment of legume seeds on the seed structure and functional properties of the resultant legume flours will now be discussed briefly.

2.5.1) High pressure cooking

Nagmani and Prakash (1997) showed a decrease in nitrogen solubility index in flours of black and green gram and lentils after pressure cooking for 10 minutes at 103.4 kPa. The authors suggested the decrease in protein solubility could be due to thermal denaturation of protein.

2.5.2) Roasting and boiling

Roasting and boiling have been applied to various pulses including lentils, chickpeas and peas by Ma *et al*. (2011). These authors found that both treatments reduced protein solubility as well as trypsin inhibitor activity of pulse flours. Reduction in protein solubility during roasting and boiling could be due to cross-linking reactions of protein and starch as well as formation of non-polar and intramolecular hydrogen bonds (Ma *et al*., 2011).

Boiling of Bambara groundnut seeds lowered the water absorption index (WAI) of the flour. The lowered WAI could be due to the exposure of hydrophobic sites upon thermal protein denaturation which could impede water absorption by starch granules.

Ma *et al*. (2011) found that boiling significantly changed the microstructure of pulse flours. However little difference was observed with flours from roasted pulse seeds. Boiling resulted in the absence of starch granules and the formation of amorphous flakes as shown in Figure 2.5.2.1.

Ma *et al*. (2011) suggested that the absence of starch granules may be due to the starch being involved in crosslinks with protein to form the amorphous flakes.

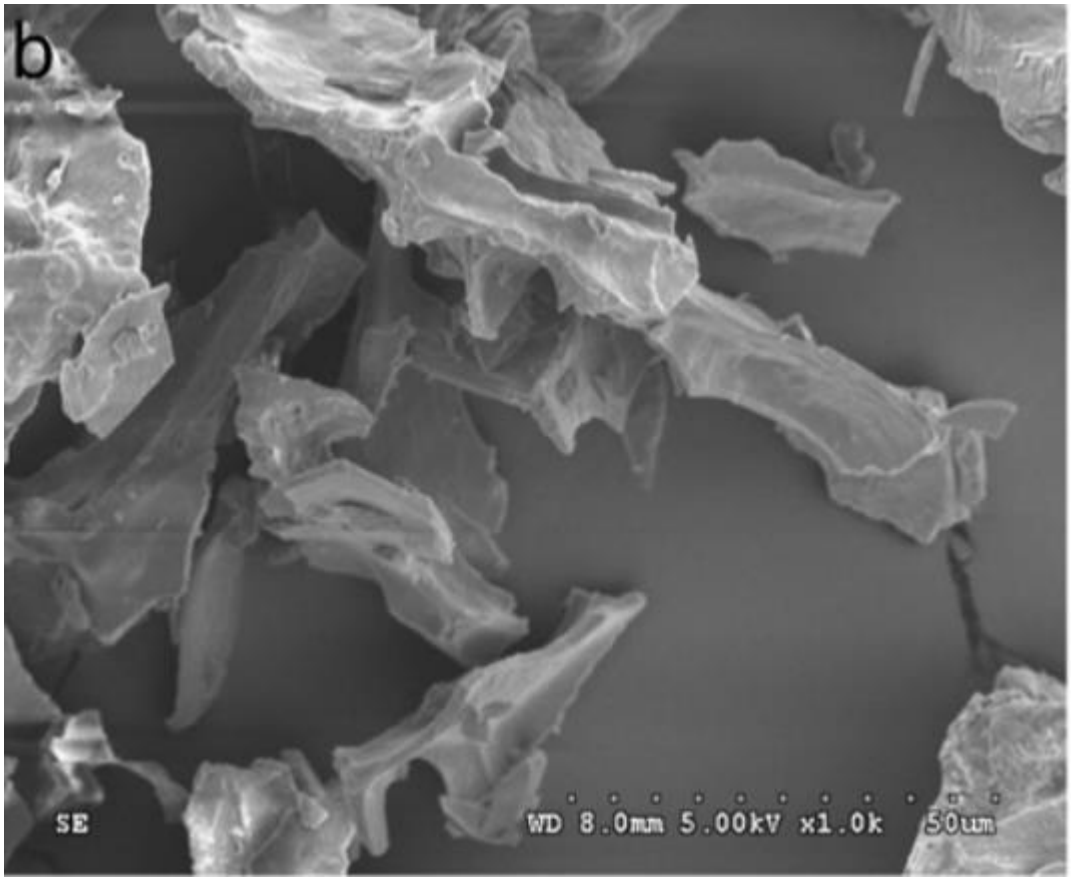


Figure 2.5.2.1: Scanning electron micrograph of flour from boiled green lentil seeds (Adapted from Ma *et al*., 2011)

2.5.3) Microwave treatment

Microwave heating uses electromagnetic waves with wavelengths in the range of 12.2-33.3 cm with frequencies in the range of 300 MHz to 300 GHz (Haque, 1999) but the frequencies often used in food applications range from 918 MHz to 2450 MHz (Galema, 1997). Microwaves have longer wavelengths and thus lower energy or temperature compared to infrared radiation (Haque, 1999). A very important factor for the use of microwave treatment is the ability of a food material to absorb microwaves and convert it into heat (Thostenson & Chou, 1999).

Mokatso (2017) showed that hot air-assisted microwave treatment of cowpea seeds also caused a decrease in protein solubility of the flour. The decrease in protein solubility was attributed to formation of cross-links between protein and starch which result in aggregation and renders the protein less soluble (Ma *et al*., 2011). Mokatso (2017) showed that flour from hot air-assisted microwaved cowpea seeds had a decreased WSI. The decrease in WSI of thermally treated flours was attributed to a decrease in protein solubility as suggested by Mwangwela and Waniska (2007b).

Pasting and thermal properties of legume flours are functional properties used to describe starch gelatinization and protein denaturation (Henshaw, McWatters, Akingbala & Chinnan, 2003). Mokatso (2017) reported a decrease in pasting viscosities of flours from hot-air assisted microwave treated cowpea seeds. The decrease in pasting viscosities could have been due to thermal degradation of protein and starch in the cowpea flour (Mokatso, 2017). The author also reported an absence of endotherms from flours from hot-air assisted microwaved cowpea seeds suggesting complete starch gelatinization.

Ashraf, Saeed, Sayeed and Ali (2012) investigated microwave heat treatment of legume flours. These authors reported that microwave heating caused starch gelatinization as well as protein denaturation of red bean flour which led to improvement of various functional properties such as an increased water absorption capacity, oil binding capacity, foaming capacity and viscosity. This was attributed to the rise in water vapour pressure due to internal heat causing seed volume increase. The cracking of legume seeds after microwave treatment have been reported to increase splitting during cooking that results in starch leaching which in turn causes a mushiness in texture (Mokatso, 2017). The mushy texture and splitting of seeds are considered undesirable according to research by Kayitesi *et al*. (2012).

2.5.4) Micronisation (infrared) treatment

Micronisation is a heat treatment using electromagnetic waves in the infrared region of the spectrum in the range of 1.8-3.4 µm (Zheng, Fasina, Sosulski & Tyler, 1998). Micronisation has been shown to reduce the cooking time of cowpeas by Kayitesi *et al*. (2012). Research by Bai, Stone and Nickerson (2018) showed that micronisation of desi chickpeas signifiantly affect the functional properties of their flours. They found that infrared treatment resulted in a reduction of

protein solubility of chickpea flour. Sibanda (2016) reported a decrease in WSI of flours from micronized cowpeas with increase in micronisation temperature and time. This was attributed to the exposure of hydrophobic sites on the proteins as it partially unflolds (Bai *et al*., 2018). Research by Mwangwela, Minnaar and Waniska (2007b) reported a decrease in NSI of cowpea flour after micronisation treatment.

Research by Cenkowski and Sosulski (1997) showed that micronisation treatment of lentils significantly decreased the transition enthalpy of endothermic peaks. They concluded that micronization gelatinized and solublized 45% to 65% of the starch in lentil seeds depending on moisture content.

Ogundele (2016) found that micronisation of Bambara groundnut seeds causes a reduction in protein solubility and results in the formation of a protein-starch complex. The protein-starch complex consists of partially gelatinised starch imbedded in hydrophobic protein matrix. This complex resulted in reduced pasting viscosity.

Ogundele (2016) observed with toluidene stained light microscopy and scanning electron microscopy that micronisation of Bambara groundnut seeds caused starch granule disruption and changes in the shape of the parenchyma cells and cell separation as seen in Figure 2.5.4.1A. Scanning electron microscope images revealed the disrupted starch granules coated with protein particles and other cell wall components (Figure 2.5.4.1B).

A **B**

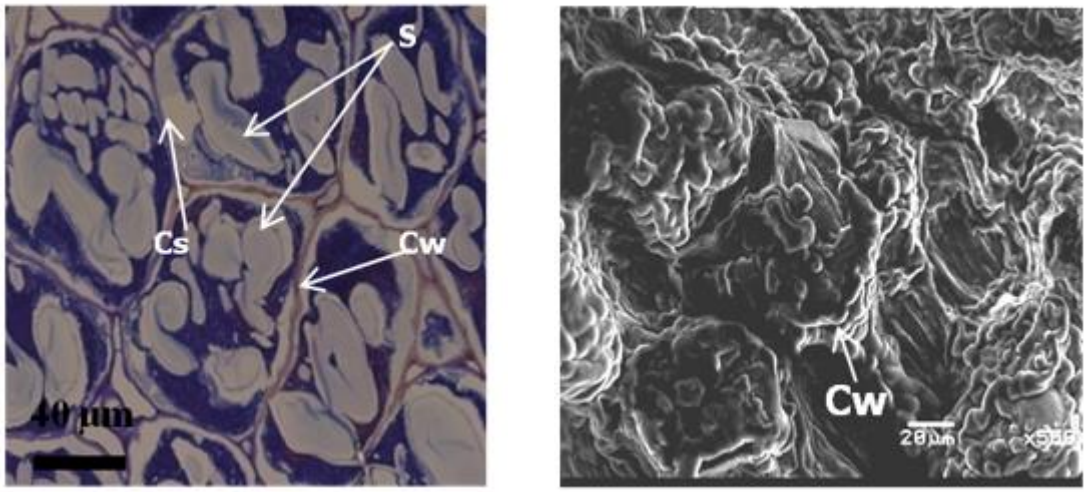


Figure 2.5.4.1: Light microscope image of toluidene stained whole micronised Bambara groundnut seed cotyledon (A) and scanning electron microscope image of whole micronised Bambara groundnut seed cotyledon (B). Cw, cell wall: Cs, Cell separation: S, Starch granules. (from Ogundele, 2016)

2.6) Conclusion and gaps in knowledge

Current research has focused on the use of thermal pre-treatments such as microwave and micronization on legumes to produce a flour with enhanced functional properties and health-promoting properties related to their phenolic compounds as well as reduced cooking time of the seeds. Most research has focused on cowpeas and other legumes but Bambara groundnuts are fairly unexplored or under-researched in terms of their functional properties and health benefits after microwave pretreatments. Thus studies are needed to assess the use of microwave pre-treatments to reduce the cooking time and enhance functional and health- promoting properties of Bambara groundnut seeds and flours.

Chapter 3: Hypotheses and Objectives

3.1) Hypotheses

Hypothesis 1

Pre-soaked microwaved Bambara groundnut seeds will have a reduced cooking time as compared to untreated samples. Pre-soaking increases the water content of the seeds which increases the efficiency of the microwaves as they generate heat through the induction of dipole rotation of water molecules (George, 1993). Thermal pre-treatments have been used to reduce cooking time of legumes such as micronisation of cowpeas (Kayitesi *et al*., 2012) and microwave treatment of various pulses (Divekar, Karunakaran, Lahlali, Kumar, Chelladurai, Liu, Borondics, Shanmugasundaram & Jayas, 2017). The reason for reduction in cooking time of legumes has been attributed to the thermal destruction of the middle lamella between the cotyledon parenchyma cells due to internal heat produced by ionic polarization and dipole rotation of water molecules (George, 1993) resulting in starch gelatinization and protein denaturation (Arntfield *et al*., 2001; Mwangwela, 2006).

Hypothesis 2

Flours produced from pre-soaked microwaved Bambara groundnut seeds will have lower nitrogen solubility index (NSI), water solubility index (WSI), pasting viscosities and enthalpy than that of untreated samples.

Heat treatments suchand microwaving have been reported to reduce protein solubility, NSI and WSI of various legumes (Cenkowski & Sosulski, 1998; Mwangwela *et al*., 2006a; Bellido, Arntfield, Cenkowski & Scanlon, 2006; Mokatso, 2017). The decrease in NSI and WSI, could be due to protein denaturation during heat treatment resulting in a decrease in solubility (Mahajan, Bhardwaj & Dua, 1999). It is also hypothesized that the denaturation of protein could cause exposure of hydrophobic sites in the protein due to unfolding which thus decreases its solubility. Microwave-induced thermal denaturation and degradation of proteins and depolymerization of

starch (Mahajan et al., 1999; Mokatso, 2017) could lead to reduced pasting viscosities and enthalpy of flours from microwave pre-treated Bambara groundnut seeds.

Hypothesis 3

Presoaked and microwaved pre-treated Bambara groundnut seeds will have higher phenolic content and radical scavenging antioxidant activity of phenolic compounds than the untreated Bambara groundnut seeds. Microwave pre-treatment could bring about heat-induced hydrolysis of bound phenolic compounds in Bambara groundnut seeds which will increase the extractability of phenolic compounds such as phenolic acids and flavonoids (Zilic *et al*., 2014; Mokatso 2017). The release of bound phenolic compounds will lead to increased phenolic content and antioxidant activity. The released phenolic compounds will then be more soluble compared to when they are in a bound form.

3.2) Objectives

Objective 1

To determine the effect of microwave pre-treatment on cooking time of Bambara groundnut seeds.

Objective 2

To determine the effect of microwave pre-treatment on the functional properties (NSI, WSI, pasting properties and enthalpy) of flours from Bambara groundnut seeds.

Objective 3

To determine the effect of microwave pre-treatment on the phenolic profile and antioxidant activity of Bambara groundnut seeds.

Chapter 4: Experimental design

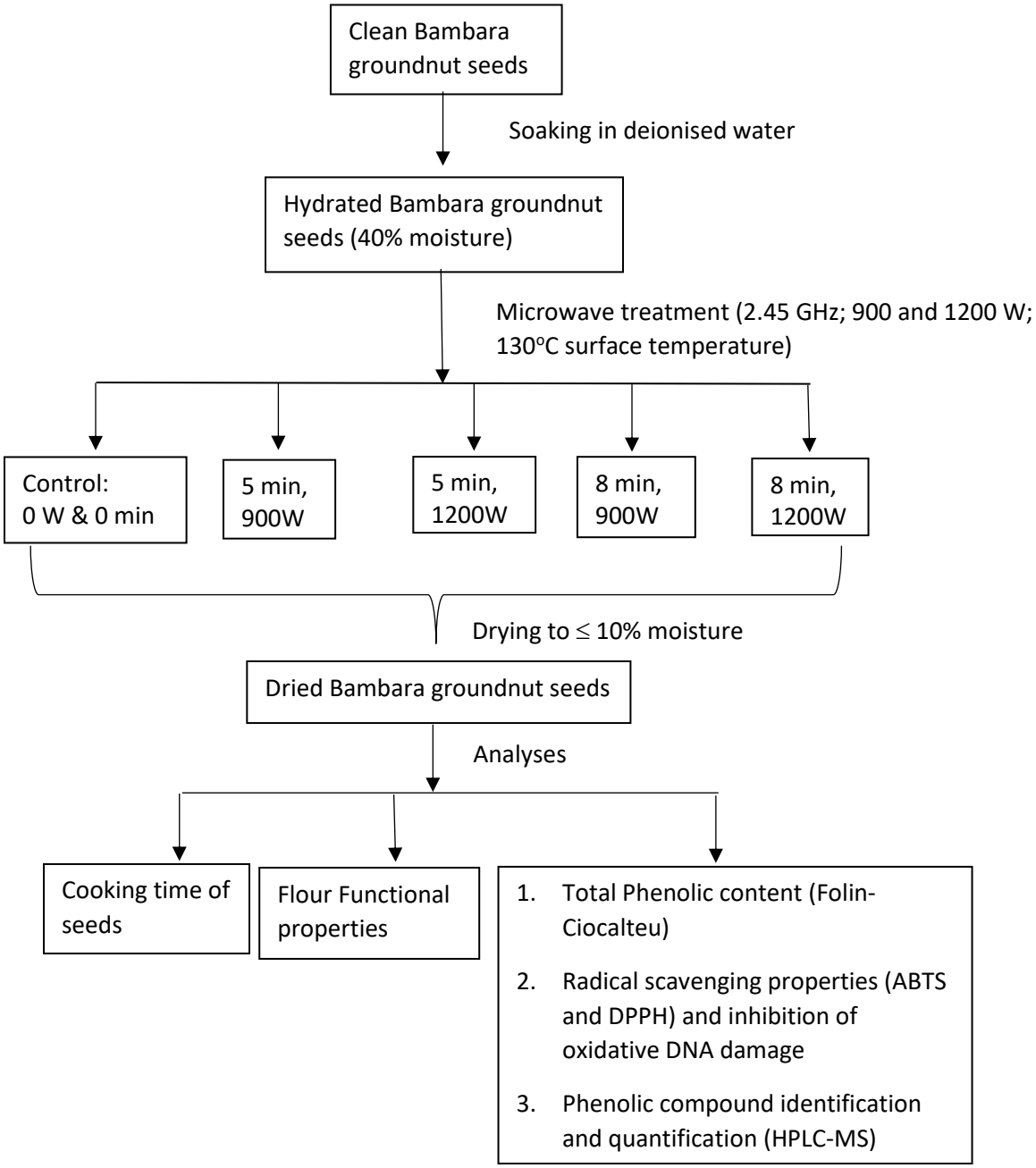


Figure 4.1: Experimental design used to determine the effect of microwave pre-treatment on cooking time of Bambara groundnut seeds and functional properties, phenolic content and antioxidant properties of their flours

Chapter 5: Research

The research conducted in this dissertation is organized into two chapters. The first chapter reports on the effect of microwave pre-treatment on the cooking time of pre-soaked Bambara groundnut seeds and the functional properties of the resultant flours. The second chapter reports on the effect of microwave pre-treatment on the phenolic composition and health promoting properties of pre-soaked Bambara groundnut seeds.

5.1) Effects of microwave pre-treatment of pre-soaked Bambara groundnut seeds on the cooking time and functional properties of the resultant flours

Abstract

The effect of microwave pre-treatment of pre-soaked brown and red Bambara groundnut seeds on the cooking time of the seeds and functional properties of the resultant flours were investigated. Bambara groundnut seeds were pre-soaked to a final moisture content of 40%. A fluidized bed microwave system was used to heat the pre-soaked Bambara groundnut seeds at 900 and 1200 W for 5 and 8 min. The cooking time of the seeds was determined using a Mattson bean cooker. Water solubility index (WSI) and nitrogen solubility index (NSI) were determined. The pasting properties of flours were determined using a rheometer. Thermal properties were determined using differential scanning calorimetry (DSC). Microwaving caused a significant ($p \leq 0.05$) decrease in the cooking time of the pre-soaked seeds. Microwave pre-treatment of pre-soaked Bambara groundnut seeds resulted in a decrease of WSI and NSI of the resultant flours as compared to the raw flour. Microwave pre-treatment of pre-soaked Bambara groundnut seeds resulted in the decrease of the pasting viscosity and enthalpy of the resultant flour as compared with the raw flour. The 1200 W treated Bambara groundnut seeds had higher pasting viscosity, WSI and NSI than the 900 W treatment. Results indicated that microwaving shows promise in alleviating the Hard to cook defect in pre-soaked Bambara groundnut seeds and in the production of a flour with the required functional properties for use in specific food applications.

Keywords: Bambara groundnut, nitrogen solubility index, water solubility index, pasting properties, thermal properties, microwave processing

5.1.1) Introduction

Bambara groundnut (*Vigna subterranea* L.) is an under researched and underutilized legume crop and has been called by some a "poor man's crop" (Muchuweti, Bhebhe, Chipurura, Kasiyamhuru & Chitindingu, 2013). Bambara groundnut is still a very important source of protein and other nutrients in rural Africa, but has problems regarding its long cooking times (Garcia, Filisetti, Udaeta & Lajolo, 1998). Bambara groundnut is consumed in a variety of ways in African countries (Adegunwa, Adebowale, Bakare & Kalejaiye, 2014) and therefore the need has arisen for scientists and food manufacturers to exploit different processing methods of Bambara groundnut to reduce the cooking times and for the use of the flour as a functional ingredient in foods.

Thermal treatments of Bambara groundnut affect the cooking time and functonal properties of the resultant flours. Yusuf, Ayedun and Sanni (2008) determined the effect of roasting on the functional properties of Bambara groundnut. They found that roasting decreases the nitrogen solubility index (NSI) of the Bambara groundnut flour at a wide pH range of 1-12. The use of γ-irradiation of Bambara groundnut seeds significantly reduced its cooking time in all cultivars as reported by Falade and Adebiyi (2015). The use of γ-irradiation also significantly decreased the pasting properties of Bambara groundnut flours but increased other functional properties such as water absorption capacity (Falade *et al*., 2015). Recently, Ogundele and Emmambux (2018) showed a decrease in functional properties such as nitrogen solubility, water solubility index and pasting viscosity after micronisation (infrared) treatment of Bambara groundnut seeds.

Although some research has been done on the application of thermal treatments such as roasting, γ-irradiation and micronisation to Bambara groundnut, limited research exists on the application of microwave treatment to Bambara groundnut seeds and its effect on the cooking time of the seeds and functional properties of resultant flours. Microwave processing is a thermal treatment that allows for decreased cooking time and energy costs compared to conventional cooking methods (Thostenson & Chou, 1999). Microwaves have wavelengths in the range of 1 mm to 1 m with frequencies of 300 MHz and 300 GHz. Microwaves have the advantage of penetrating food materials and thus deliver heat energy through molecular interaction with electromagnetic field. Microwaves penetrate the material accumulating energy which allows generation of heat throughout the volume of the material (Thostenson *et al*., 1999). Energy transfer in microwave

processing does not depend on diffusion but rather the conversion of electromagnetic energy to thermal energy in the material which allows for fast and uniform heating (Thostenson *et al*., 1999).

The objective of this study was to determine the effect of microwave pre-treatment of pre-soaked Bambara groundnut seeds on the cooking time of the seeds and functional properties of the resultant flours with the aim of enhancing its use as a functional ingredient in food applications.

5.1.2) Materials and methods

5.1.2.1) Materials

Bambara groundnut (brown- and red-coloured types) was procured from Triotrade CC (Pretoria, South Africa). Bambara groundnut seeds were sorted based on size for the red and brown varieties and all broken or damaged seeds were removed. The seeds were packed in airtight Ziploc bags and stored at 4 ºC.

5.1.2.2) Proximate analysis

Proximate composition (crude protein, crude fat, crude fibre and ash) of the Bambara groundnut seeds were determined using standard methods of the American Association of Cereal Chemists International. Moisture content was determined based on a method by Ajibola, Aviara and Ajetumobi (2003). Carbohydrates were determined by difference.

5.1.2.3) Hydration of Bambara groundnut seeds to 40% moisture content

Hydration of Bambara groundnuts to 40% moisture content was done as described by Sibanda, (2016) with modifications. The Bambara groundnuts (50 g) were placed in a polyethylene ziplock bag. The weight of water (16.5 g) required was calculated and put inside the ziplock bag. The bag was placed in an oven set to 37°C and left until all the water was soaked up by the Bambara groundnuts (24 hrs). After hydration the samples were processed.

5.1.2.4) Microwave treatment

The microwave treatments were done based on the method of Mokatso (2017) with some modifications using a fluidized bed microwave oven (Delphius, Centurion, South Africa). The microwave oven was first allowed to pre-heat to an air temperature of 130ºC with an electric heater set at 1.2 kW maximum. Approximately 50 g of presoaked and hydrated Bambara groundnut samples were treated with microwave power of 900 and 1200 W for 5 and 8 min. After allowing to cool the seeds were dried to a moisture content of ≤ 10% in an oven at 40ºC overnight and milled to a flour using a laboratory hammer mill (Falling Number 3100, Huddinge, Sweden) with a 500 µm sieve. After milling, the flours were stored in air tight Ziploc bags at 4ºC until analysis. Microwave treatments were done in triplicate.

5.1.3) Analytical methods:

5.1.3.1) Cooking time

The cooking time of untreated (raw pre-soaked) and treated pre-soaked Bambara groundnut seeds was determined using a Mattson bean cooker as described by Mwangwela et al. (2006) with some modifications. For treated and untreated samples, 25 Bambara groundnut seeds were placed in the perforations of the cooker with the pins placed on the surface of each seed. The cooker was placed in a stainless steel pot containing 1500 ml of deionized water and cooked. The cooking time was recorded as the moment when 80% of the pins had fallen through the seeds.

5.1.3.2) Water solubility index (WSI)

The WSI of flours from microwave pre-treated Bambara groundnut samples were determined using the method described by Adebowale, Adeniyi & Lawal (2002) with modification by Ocloo, Minnaar & Emmambux (2014). Approximately 0.125 g of treated and untreated flour samples were heated in 20 ml of distilled water at 50 and 95ºC for 30 min in a shaking waterbath at 100 rpm. The samples were allowed to cool and centrifuged at 30000 x g for 5 min at 25ºC. The supernatants were collected and evaporated in an air oven at 105ºC for 21 hr. The WSI (%) was calculated as the ratio of weight of dried supernatant to weight of flour using the following equation:

$$\%\, WSI = \frac{Weight\ of\ supernatant\ after\ drying}{Weight\ of\ flour} \times 100$$

5.1.3.3) Nitrogen solubility index (NSI)

The NSI of flours from the raw and microwaved pre-treated Bambara groundnut seeds were determined using AACC method 46-23 (AACC, 2000). Flour sample (1 g) was added to 50 ml of 0.1 M NaCl solution and stirred for 1 hr at 30ºC with pH kept at 7. Approximately 20 ml of the suspension was centrifuged at 1000 x g for 15 min at 4ºC. The supernatant was filtered through a Whatman No. 1 filter paper. The residue was washed twice with 10 ml of 0.1 M NaCl solution at pH of 7. The filtrate collected was frozen at -18ºC overnight in a freezer and freeze-dried (13KL Instruvac lyophilizer, Midrand, South Africa). The nitrogen content of the freeze-dried samples was determined using a Dumatherm nitrogen analyzer. A protein conversion factor of 6.25 was used for Bambara groundnuts (Aremu, Olaofe & Akintayo, 2006). The nitrogen solubility index was calculated using the following equation:

$$\%\, NSI = \frac{Protein\ content\ of\ supernatant}{Protein\ content\ of\ flour} \times 100$$

5.1.3.4) Pasting properties

The pasting properties of flours from raw and microwaved Bambara groundnut seeds were determined using a Physica MCR 101 Rheometer (Anton Paar, Ostfildern, Austria) based on a method by Wokadala, Ray and Emmambux (2012) with modifications. The pasting cycle began with an initial stirring speed of 960 rpm at 50ºC for 30 s and then stirred at 160 rpm for the remaining period. The temperature was increased at a rate of 5.5ºC/min to 91ºC, this temperature was held for 5 min for short pasting. The pastes were then cooled to 50ºC at a rate of 5.5ºC/min.

5.1.3.5) Thermal properties

The thermal properties of flours from the microwave pre-treated Bambara groundnut seed samples were determined as described by Wokadala, Ray and Emmambux (2012) with modifications using a high pressure DSC system with STARe ® software (HPDSC-827, Mettler Toledo, Greifensee, Switzerland) with modifications. Indium (Tp =156°C, heat endothermic flow = -28.6 J/g) was used to calibrate the instrument with regards to temperature and enthalpy. A weight of 10 mg (db) of treated and untreated flour samples was added to 30 ml of distilled water in a sealed aluminum pan. The samples were allowed to equilibrate at ambient temperature overnight. Scanning of samples was done at 40^{o}C-150^{o}C at a rate of 10^{o}C/min and at 40 ± 0.01 MPa with a flow rate of 60 ml/min. An empty pan was used as reference. Melting enthalpy (ΔH in J/g), onset (T_o), peak (T_p) and endset (T_c) temperatures of endothermic peaks were measured. Measurements were done in duplicate.

5.1.3.6) Light Microscopy (LM)

A small amout of flour was placed on a slide with the addition of glycerol. Iodine staining was done by placing a drop of iodine on the flour. The suspension was then mixed. The slides were viewed using a Nikon optiphot transmitted light microscope (Tokyo, Japan) with appropriate illumination sources and filters. Pictures was captured using a Nikon digital camera DXM1200 (Tokyo, Japan).

5.1.3.7) Statistical analysis

Experiments were conducted in triplicates. Multi-factor analysis of variance (MANOVA) was conducted as required using SPSS software. Significant differences between means was determined at $p \leq 0.05$. Significant means were seperated using the least significant difference (LSD) test at $p \leq 0.05$. Independent variables were microwave power (0, 900 W and 1200 W) and microwave time (0, 5 minutes and 8 minutes) and Bambara groundnut types. Dependent variables were the measured values of cooking time and the functional properties of the Bambara groundnut flour.

5.1.4) Results and discussion

5.1.4.1) Proximate composition of Bambara groundnut seeds

The proximate composition of the Bambara groundnut seeds is shown in Table 5.1.4.1. There was no significant ($p \leq 0.05$) difference between the proximate composition of brown and red Bambara groundnut seeds. The moisture contents of brown and red Bambara groundnuts were 8.78% and 8.44%, respectively. Protein content was 18.59% and 18.14%, respectively. Crude fat content of brown and red Bambara groundnut was 7.77% and 8.06%, respectively. Crude fibre content of brown and red Bambara groundnut seeds were 14.56% and 12.17%, respectively. The ash contents were 3.51% and 3.53% for brown and red Bambara groundnut seeds respectively. The % carbohydrates were calculated by difference as 46.79% and 49.66% for brown and red Bambara groundnut seeds respectively.

Table 5.1.4.1 Proximate composition (% db) of Bambara groundnut

Type	Moisture	Crude protein	Crude fat	Crude fibre	Ash	Carbohydrates (by difference)
Brown Bambara groundnuts	$8.78^{a} \pm 0.47$	$18.59^{a} \pm 0.11$	$7.77^{a} \pm 0.33$	$14.56^{a} \pm 1.41$	$3.51^{a} \pm 0.08$	46.79
Red Bambara groundnuts	$8.44^{a} \pm 0.23$	$18.14^{a} \pm 0.17$	$8.06^{a} \pm 0.78$	$12.17^{a} \pm 2.82$	$3.53^{a} \pm 0.06$	49.66

Values are means of triplicates ± standard deviations. Mean values with different superscript letters in columns differ significantly ($p \leq 0.05$) from each other.

a,b: different superscripts differ significantly ($p \leq 0.05$)

db: dry basis

5.1.4.2) Cooking time of microwave pre-treated Bambara groundnut seeds

The effects of microwave time and power on the cooking time of pre-soaked Bambara groundnut seeds is shown in Table 5.1.4.2. The microwave time and power interaction caused a significant reduction ($p \leq 0.05$) in the cooking time of Bambara groundnut seeds as compared with the raw seeds. The Bambara groundnut type (variety) had no significant effect ($p \leq 0.05$) on the cooking time of the seeds. The biggest decrease in cooking time for brown Bambara groundnut seeds was caused by the 1200 W microwave treatment for 5 minutes which had a cooking time of 45.33 minutes. The cooking time of untreated red Bambara groundnut seeds was 369 minutes. The red Bambara groundnut seeds microwaved at 900 W decreased cooking time up to 6 times whereas 1200 W treatment reduced the cooking time of red Bambara groundnut seeds up to 12 times. There were no significant differences ($p \leq 0.05$) between cooking time of Bambara groundnut seeds at 1200 W and 900 W treatment.

Reduction in cooking time of micronized cowpea seeds was also reported by Kayitesi et al. (2012) and Mwangwela *et al*. (2006a). Kayitesi *et al*. (2012) reported a reduction in cooking time by 34%, 49%, 35% and 28% for Black eye, Bechuana white, Glenda and Dr. Saunders cowpea varieties cowpea respectively after micronization treatment. Ogundele and Emmambux (2018) reported a reduction in cooking time from 162 minutes for raw seeds to 83, 75 and 62 minutes after micronisation at 5, 10 and 15 minutes respectively. The reduction in cooking time of legumes has been attributed to parenchyma cell separation along the middle lamella (Sefa-Dedeh & Stanley 1978) caused by β elimination reactions of pectic substances (Coultate, 2002) and gelatinization of starch in the parenchyma cells of the cotyledon.

Table 5.1.4.2 Effect of microwave time and power on the cooking time of pre-soaked Bambara groundnut seeds

Samples	Microwave power (Watt)	Microwave time (min)	Cooking time (min)
Brown Bambara groundnut*	**0**	**0**	$219.0^{a} \pm 8.6$
	900	**5**	$60.0^{b} \pm 2.9$
		8	$50.0^{b} \pm 3.6$
	1200	**5**	$45.3^{b} \pm 5.7$
		8	$55.7^{b} \pm 7.7$
Red Bambara groundnut*	**0**	**0**	$369.0^{a} \pm 4.0$
	900	**5**	$54.0^{b} \pm 5.3$
		8	$29.3^{b} \pm 6.4$
	1200	**5**	$31.5^{b} \pm 0.7$
		8	$29.3^{b} \pm 3.8$

Values are means of triplicates ± Standard deviations. For each Bambara groundnut type mean values with different superscript letters in columns are significantly different ($p \leq 0.05$) from each other

*: indicates no significant difference ($p \leq 0.05$) between Bambara groundnut type.

5.1.4.3) Pasting properties of flours from microwave pre-treated Bambara groundnuts

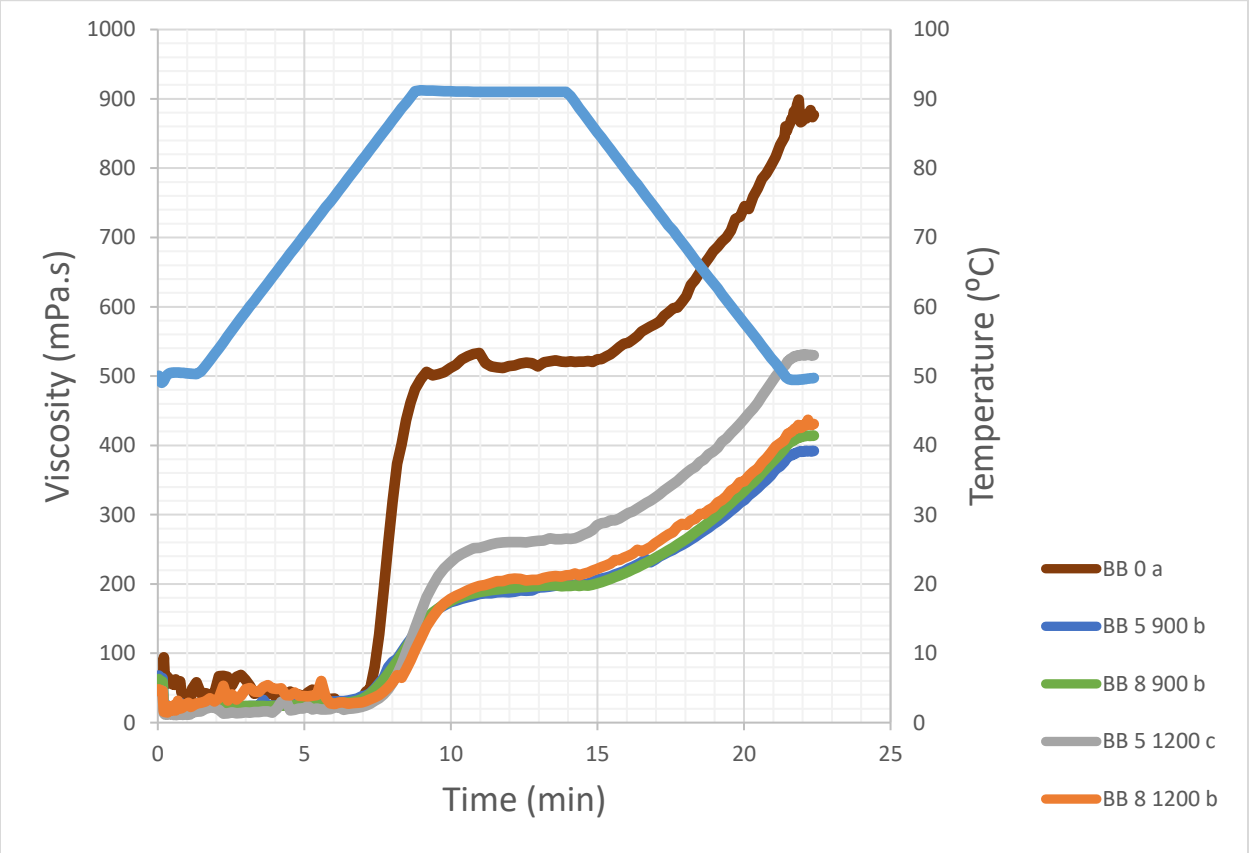
Figure 5.1.4.1 shows the effect of microwave pre-treatment on the pasting properties of flours from pre-soaked Bambara groundnut seeds. Bambara groundnut type had no significant ($p \leq 0.05$) effect on the pasting viscosity of the resulting flours. The pasting viscosity of flours from microwaved brown and red Bambara groundnut seeds were significantly lower ($p \leq 0.05$) than that of the raw untreated flour. The maximum, setback and final viscosity of flours from pre-soaked microwaved Bambara groundnut seeds at all processing parameters were significantly ($p \leq 0.05$) lower than that of the untreated flours.

Microwave time of 8 minutes caused a significant ($p \leq 0.05$) decrease in pasting viscosities as compared to the 5 minutes treatment for both microwave powers for flour from red Bambara groundnut seeds. On the other hand, no significant difference ($p \leq 0.05$) in pasting viscosity of flours from pre-soaked red Bambara groundnut seeds was observed between microwave powers of 900 W and 1200 W. For the red Bambara groundnut flour, pasting viscosity decreased significantly ($p \leq 0.05$) with an increase in microwave power and time as compared to the untreated seeds.

The pasting viscosities of flours from microwaved brown Bambara groundnut seeds decreased with a decrease in microwave power. The pasting viscosity of flours from pre-soaked brown Bambara groundnut seeds microwaved at 1200 W for 5 minutes was significantly ($p \leq 0.05$) higher than that of seeds microwaved at 900 W for both microwave times. This is unusual as it is expected that a higher power would result in a bigger change in molecular structure of starch and protein leading to a decrease in pasting viscosity as seen in previous studies by Makatso (2017) with flour of microwaved cowpea seeds.

Ogundele (2016) and Mwangwela et al. (2007) reported reduction in pasting viscosities of flours of micronized Bambara groundnut and cowpea, respectively. Mokatso (2017) found that flours from microwaved cowpea seeds had reduced pasting viscosities compared to flours from tempered-only cowpea seeds. Mwangwela *et al*. (2007) hypothesised that the reduction in pasting viscosities of micronized cowpea flour could be due to starch depolymerization or starch cross-linking. Ogundele (2016) proposed that the decrease in pasting viscosities of thermally treated flours could be due to protein denaturation resulting in exposure of hydrophobic sites which could limit water access to starch granules surrounding protein matrix. According to Ogundele (2016), the hydrophobic protein surrounding the starch granules prevent starch from swelling and leaching out to form entangled networks called junction zones and thus resulting in reduced pasting viscosities.

A)


Viscosity (mPa.s)
Temperature (°C)
Time (min)
0
100
200
300
400
500
600
700
800
900
1000
10
20
30
40
50
60
70
80
90
5
15
25
BB 0 a
BB 5 900 b
BB 8 900 b
BB 5 1200 c
BB 8 1200 b

B)

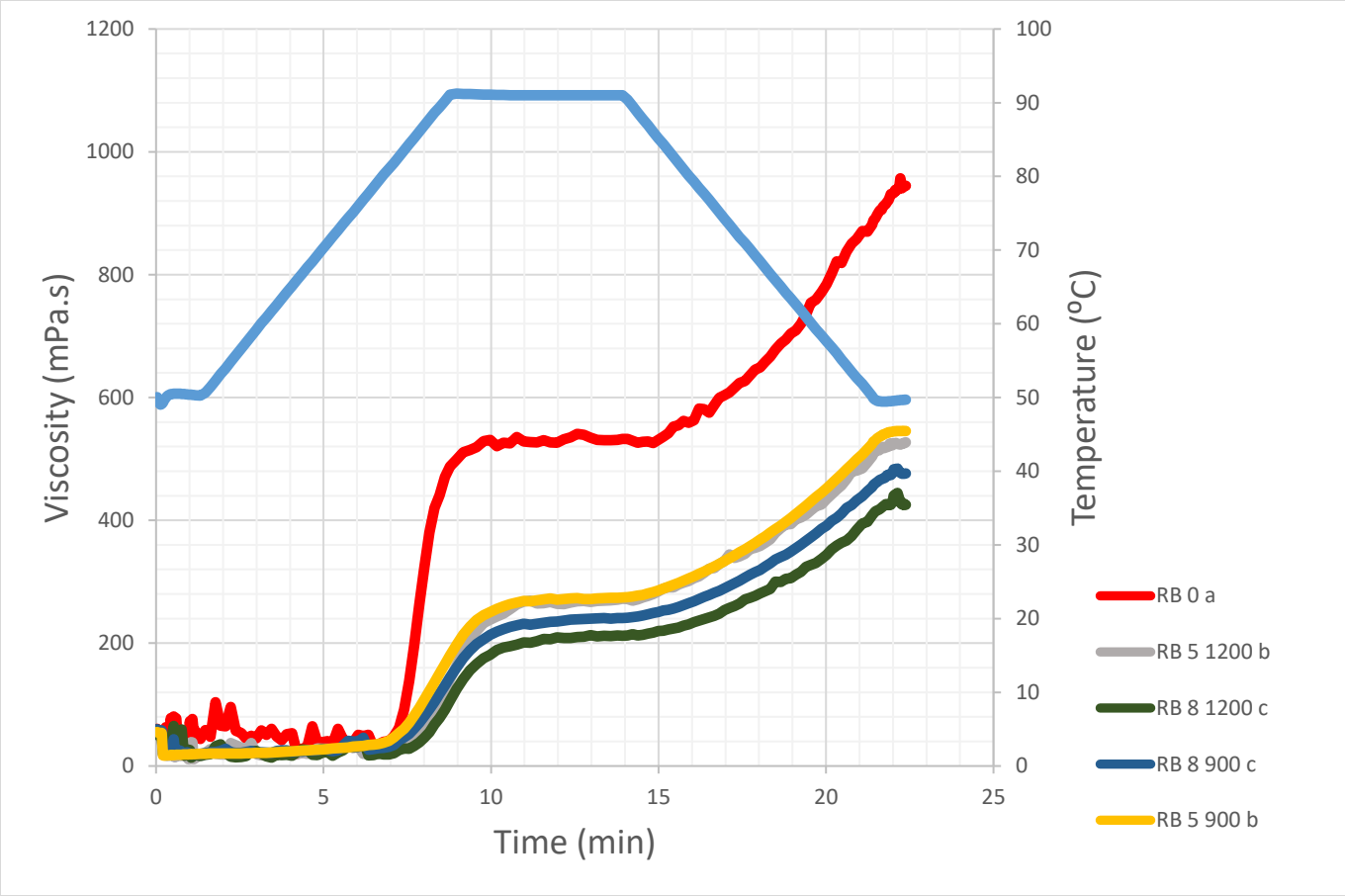


Figure 5.1.4.1: Effect of microwave pre-treatment on the pasting properties of pre-soaked Brown (A) and Red (B) Bambara groundnuts

BB 0: flour from untreated brown Bambara groundnut seeds (raw)
BB 5 900: flour from brown Bambara groundnut seeds microwaved at 900 W for 5 minutes
BB 8 900: flour from brown Bambara groundnut seeds microwaved at 900 W for 8 minutes
BB 5 1200 = flour from brown Bambara groundnut seeds microwaved at 1200 W for 5 minutes
BB 8 1200 = flour from brown Bambara groundnut seeds microwaved at 1200 W for 8 minutes
RB 0: flour from untreated red Bambara groundnut seeds (raw)
RB 5 900: flour from red Bambara groundnut seeds microwaved at 900 W for 5 minutes
RB 8 900: flour from red Bambara groundnut seeds microwaved at 900 W for 8 minutes
RB 5 1200: flour from red Bambara groundnut seeds microwaved at 1200 W for 5 minutes
RB 8 1200: flour from red Bambara groundnut seeds microwaved at 1200 W for 8 minutes
Pasting curves are means of triplicate treatments

5.1.4.4) Water solubility index (WSI) of flours from microwave pre-treated Bambara groundnut seeds

The effects of microwave time and power on water solubility index of flours from pre-soaked Bambara groundnut seeds are shown in Table 5.1.4.3. The microwave time and temperature interaction caused a significant ($p \leq 0.05$) reduction in WSI of Bambara groundnut flours.

The Bambara groundnut type and heating temperature had no significant ($p \leq 0.05$) effect on the WSI of the seed flours. The WSI of flours from both brown and red Bambara groundnut seeds microwaved at 900 W was significantly ($p \leq 0.05$) lower than that of seeds microwaved at 1200 W, respectively for 5 minutes microwave time. This corresponds with the lower pasting properties for flours from seeds microwaved at 900 W for 5 minutes compared to the 1200 W for 5 minutes treatment (Figure 5.1.4.1 A). The highest microwave time parameter of 8 minutes caused a significant ($p \leq 0.05$) reduction in WSI of both brown and red Bambara groundnut seed flour compared to the raw untreated flour. In contrast, the lowest microwave power parameter of 900 W significantly ($p \leq 0.05$) reduced the WSI of brown and red Bambara groundnut seed flours compared to the untreated samples.

Similar results in reduction in WSI with increase in microwave power and time were reported for cowpea flour by Mokatso (2017). Ogundele (2016) found a decrease in water solubility index with an increase in micronisation time and power in flours from whole and dehulled Bambara groundnut seeds. The reduction in WSI could be attribued to the denaturation of legume proteins during thermal treatment resulting in a decrease in protein solubility (Fasina *et al*., 2001; Mwangwela & Waniska, 2007). To confirm that the reduction in water solubility index was due to a reduction of protein solubilty, the nitrogen solubility index was determined.

Table 5.1.4.3: Effect of microwave time and power on water solubility index (WSI) of flours from pre-soaked Bambara groundnut seeds

Samples	Heating temperature (°C)	Microwave power (Watt)	Microwave time (min)	WSI (%)
Brown Bambara groundnut *	**50**	**0**	**0**	$51.30^{a} \pm 3.99$
		900	**5**	$18.93^{ab} \pm 1.01$
			8	$20.23^{ab} \pm 0.77$
		1200	**5**	$38.64^{ab} \pm 4.53$
			8	$17.48^{ab} \pm 0.58$
	95	**0**	**0**	$62.27^{a} \pm 3.41$
		900	**5**	$26.58^{ab} \pm 1.42$
			8	$25.90^{ab} \pm 0.93$
		1200	**5**	$31.03^{ab} \pm 2.94$
			8	$22.90^{ab} \pm 1.50$
Red Bambara groundnut *	**50**	**0**	**0**	$33.74^{a} \pm 2.18$
		900	**5**	$20.39^{ab} \pm 2.25$
			8	$19.35^{ab} \pm 0.49$
		1200	**5**	$16.89^{ab} \pm 1.00$
			8	$17.24^{ab} \pm 1.18$
	95	**0**	**0**	$73.71^{a} \pm 1.59$
		900	**5**	$26.04^{ab} \pm 0.24$
			8	$25.37^{ab} \pm 0.89$
		1200	**5**	$25.00^{ab} \pm 1.06$
			8	$25.09^{ab} \pm 0.88$

Values are means of triplicates ± standard deviations.

Mean values with different superscript letters in columns differ significantly ($p \leq 0.05$) from each other within heating temperatures.

*: indicates no significant difference ($p \leq 0.05$) between Bambara groundnut type and heating temperature.

5.1.4.5) Nitrogen solubility index (NSI) of flours from microwave pre-treated Bambara groundnut seeds

The effect of microwave power and time on the nitrogen solubility index (NSI) is shown in Table 5.1.4.4. The nitrogen solubility index of microwaved Bambara flours decreased significantly ($p \leq 0.05$) for both 900 and 1200 W treatment as compared to the raw flour for brown and red Bambara groundnut seed flour. The Bambara groundnut type had no significant ($p \leq 0.05$) effect on the NSI of the flours. NSI for the 1200 W treatments were higher than that of the 900 W treatments which corresponds to the results for WSI and which is confirmed by the strong positive correlation with WSI as shown in Table 5.1.4.5 with 0.87 and 0.97 correlation coefficients (r) for flours from brown and red Bambara groundnut seeds, respectively.

Bellido *et al*. (2006), reported a decrease in solubility of protein in navy and black beans after micronization treatment as compared to their non-processed counterparts. The NSI for the 1200 W treatments were higher than those of the 900 W treatments which corresponds to the results for WSI. Ogundele *et al*. (2017) reported the reduction of NSI of flours from micronised Bambara groundnnut seeds with increase in micronisation time of 5, 10 and 15 minutes. The decrease in protein solubility or NSI of flours after heat treatment was attributed to the denaturation of protein resulting in exposure of hydrophobic sites thus making the protein less soluble (Ogundele *et al*., 2017). Thus, the protein surrounding the starch granules became more hydrophobic and this influenced the water absorption by starch granule to paste for a higher viscosity.

Table 5.1.4.4: Effect of microwave time and power on nitrogen solubility index (NSI) of flours from pre-soaked Bambara groundnut seeds

Samples	Microwave power (Watt)	Microwave time (min)	NSI (%)
Brown Bambara groundnut *	**0**	**0**	$78.02^{a} \pm 1.20$
	900	**5**	$18.76^{b} \pm 0.27$
		8	$17.67^{b} \pm 0.37$
	1200	**5**	$25.96^{b} \pm 5.34$
		8	$21.59^{b} \pm 2.11$
Red Bambara groundnut *	**0**	**0**	$87.33^{a} \pm 4.16$
	900	**5**	$15.89^{b} \pm 0.40$
		8	$19.24^{b} \pm 5.36$
	1200	**5**	$18.65^{b} \pm 0.75$
		8	$19.30^{b} \pm 1.03$

Values are means of triplicates ± standard deviation. For each Bambara groundnut type mean values with different superscript letters in columns differ significantly ($p \leq 0.05$) from each other

*: indicates no significant difference ($p \leq 0.05$) between Bambara groundnut type.

Table 5.1.4.5: Pearson correlation coefficients (r) for Nitrogen solubility index (NSI) vs water solubility index (WSI) for flours from raw and microwaved brown and red Bambara groundnut seeds

	NSI vs WSI
Brown Bambara groundnut seeds	0.87
Red Bambara groundnut seeds	0.97

$p \leq 0.05$

5.1.4.6) Thermal properties of flours from microwave pre-treated bambara groundnut seeds

The effect of microwave time and power on the thermal properties of flours from Bambara groundnut seeds are shown in Figure 5.1.4.2. A single endothermic peak was observed in raw untreated flours from both brown and red Bambara groundnut seeds. Flour from raw brown Bambara groundnut had a thermal transition recorded at Onset (T_o) of 76.07°C, Peak (T_p) 81.33ºC and Endset (T_c) 87.11ºC temperatures with an enthalpy (Δ H) of 4.50 J/g. Flour from raw red Bambara groundnut had a thermal transition at Onset (T_o) of 71.86ºC, Peak (T_p) 81.27ºC and Endset (T_c) 90.96ºC temperatures with an enthalpy (ΔH) of 9.77 J/g (Table 5.1.4.6). These values are in agreement with values found for Bambara groundnut flour by Sirivongpaisal (2008) with Onset (T_o) of 76.84°C, Peak (T_p) 80.75ºC and Endset (T_c) 85.82ºC.

Microwave pre-treatment of Bambara groundnut seeds caused a significant ($p \leq 0.05$) reduction in endothermic peaks and transition enthalpy as compared to the flour from raw seeds. Very small endothermic peaks were observed for flours from all microwaved brown Bambara groundnut seeds as seen in Table 5.1.4.6. Similar endothermic peaks were observed for all flours from microwaved red Bambara groundnut seeds.

The endothermic peaks observed for raw Bambara groundnut flours may be due to starch gelatinization and protein denaturation (Henshaw *et al*., 2003). The small endothermic peaks observed for microwaved samples might explain the deformed but intact “maltese crosses” in the starch granules observed under light microscopy after microwave treatment indicating partially gelatinized starch (Figure 5.6.8). The low transition enthalpy values may indicate the presence of crystalline regions in the partially gelatinized starch granules after microwave pre-treatment. Sirivongpaisal (2008) showed that Bambara groundnut contain A-type crystalline starch through X-ray diffraction pattern which according to Gidley (1987) is the most thermodynamically stable form.

Mwangwela *et al*., (2007) also reported a decrease in endothermic peaks and enthalpy for flours from micronized cowpea seeds. Ogundele (2016) found a decrease in endothermic peaks and enthalpy of flours from dehulled and whole micronized Bambara groundnut seeds. Ogundele

(2016) attributed the reduction in endothermic peaks and enthalpy to the partial gelatinization of starch molecules. To confirm these results light microscopy was done.

Table 5.1.4.6: Effect of microwave time and power on the onset, peak, endset temperatures and transition enthalpy of flours from pre-soaked Bambar groundnut seeds

	Onset Temperature (To) ºC	**Peak Temperature (Tp) ºC**	**Endset Temperature (T_C) ºC**	**Transition Enthalpy (Δ H) J/g**
Brown 0min/0W	76.07	81.33	87.11	4.50[a]
Brown 5min/900W	81.94	85.67	89.72	0.14[b]
Brown 8min/900W	80.33	85.67	90.22	0.28[b]
Brown 5min/1200W	82.27	86.83	91.74	0.17[b]
Brown 8min/1200W	78.54	85.19	92.34	0.80[b]
Red 0min/0W	71.86	81.27	90.96	9.77[a]
Red 5min/900W	80.69	85.44	92.03	0.98[b]
Red 8min/900W	80.33	85.36	91.37	1.06[b]
Red 5min/1200W	76.08	83.45	100.88	0.33[b]
Red 8min/1200W	80.85	82.86	86.05	0.56[b]

$p \leq 0.05$

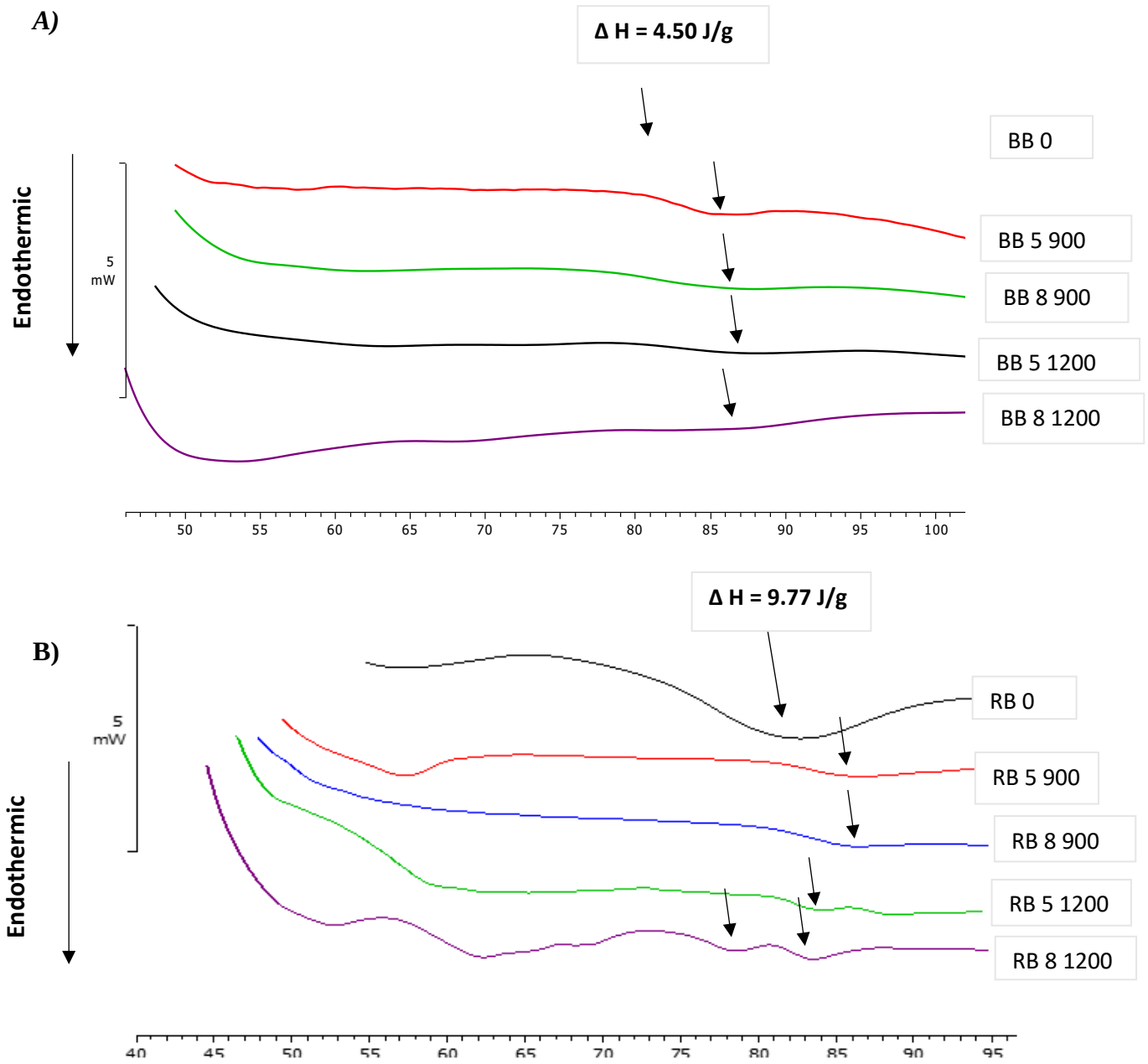


Figure 5.1.4.2 Effect of microwave pre-treatment on the thermal properties of flours from brown (A) and red (B) Bambara groundnut seeds

BB 0: flour from untreated brown Bambara groundnut seeds (raw)
BB 5 900: flour from brown Bambara groundnut seeds microwaved at 900 W for 5 minutes
BB 8 900: flour from brown Bambara groundnut seeds microwaved at 900 W for 8 minutes
BB 51200: flour from brown Bambara groundnut seeds microwaved at 1200 W for 5 minutes
BB 81200: flour from brown Bambara groundnut seeds microwaved at 1200 W for 8 minutes
RB 0: flour from untreated red Bambara groundnut seeds (raw)
RB 5 900: flour from red Bambara groundnut seeds microwaved at 900 W for 5 minutes
RB 8 900: flour from red Bambara groundnut seeds microwaved at 900 W for 8 minutes
RB 51200: flour from red Bambara groundnut seeds microwaved at 1200 W for 5 minutes
RB 81200: flour from red Bambara groundnut seeds microwaved at 1200 W for 8 minutes

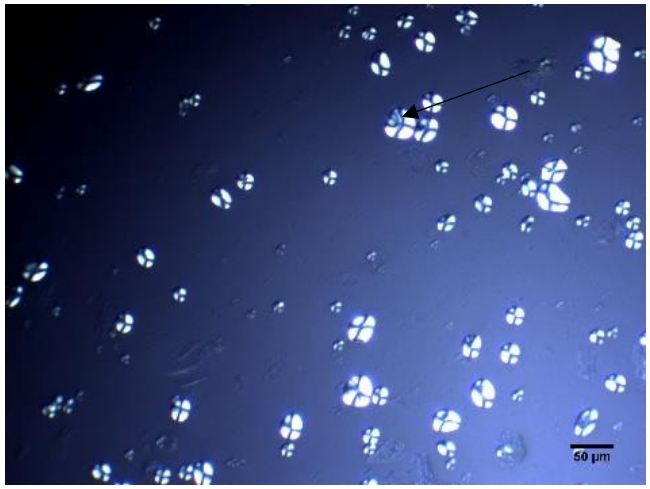
5.1.4.7) Light microscopy analysis of flours from microwave pre-treated Bambara groundnut seeds

Figures 5.1.4.3 and 5.1.4.4 show polarized light microscopy images and iodine stained light microscopy images respectively of flour from raw and microwaved Bambara groundnut seeds. In the raw untreated flour of both brown and red Bambara groundnut seeds, the polarized light microscopy images showed intact starch granules as evident by the "Maltese cross" birefringence (indicated with arrows). With increase in microwave power and time it could be seen that there was a decrease in "Maltese cross" birefringence which could explain the reduction in endothermic peak for microwaved flours as compared to raw samples.

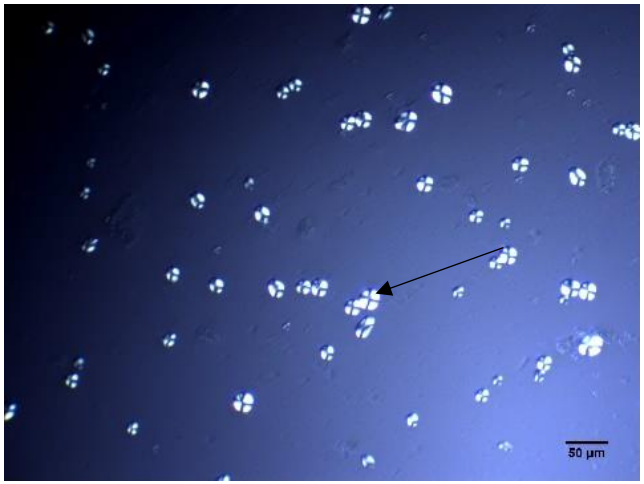
Iodine staining showed an increase in aggregation and swelling of black-blue starch (orange arrows) as microwave power and time increased which indicates partially gelatinized starch and cross-linking with protein. The starch granules (black) (blue arrow) embedded in a protein matrix (yellow) (black arrow) could be seen in the microscope image of flour from red Bambara groundnut treated for 5 minutes at 1200 W. The protein matrix surrounding the starch granules could be the reason for the lower WSI, NSI and pasting viscosities of the flour from microwave treated Bambara groundnut seeds. The hydrophobic protein matrix could restrict water access to starch granules which reduces starch swelling and leaching out.

The Iodine stained flours of both brown and red Bambara groundnut seeds treated for 8 minutes 1200 W showed a swelled up and slight disappearance of the black color of starch granules showing a distinct purple color (Figure 5.6.9) The intense blue color can be due to amylose that leached (red arrow) out while the purple color (amylopectin) (yellow arrow) remains as an insoluble irregular mass after heating. This is evidence of some regions of flour not having formed a protein matrix around partially gelatinized starch granules. This could be the reason for the increased pasting viscosities as compared to the 900 W treatments.

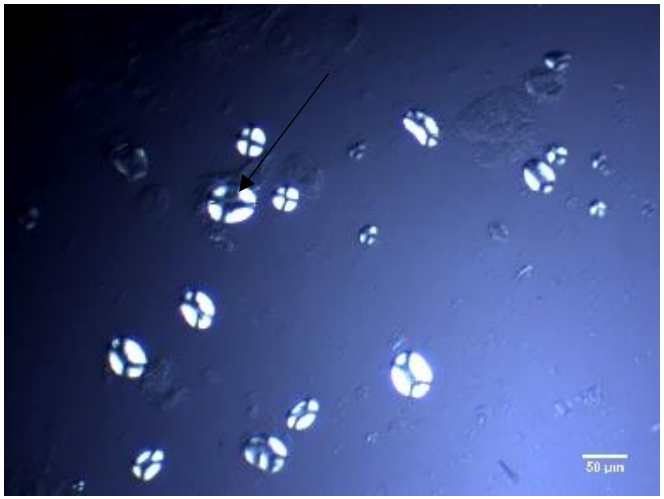
Brown Bambara groundnut flour (Raw)



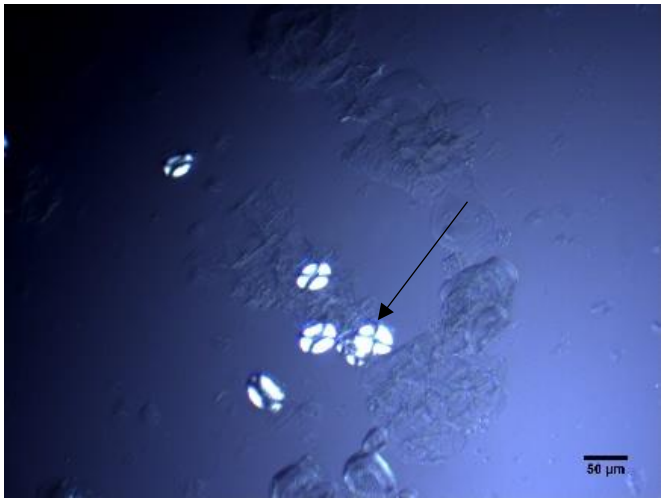
Red Bambara groundnut flour (Raw)



Brown Bambara groundnut flour 5min/ 900 W

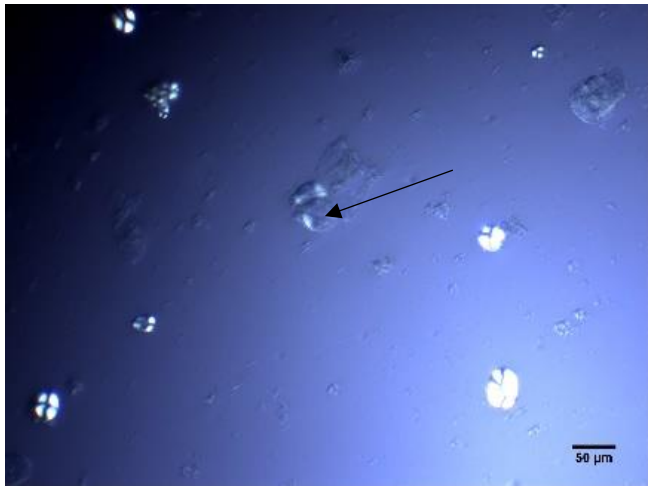


Red Bambara groundnut flour 5 min/ 900 W

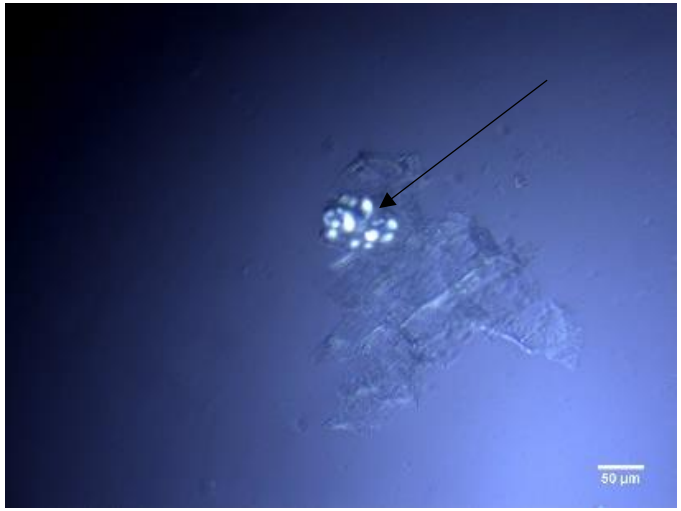


Brown Bambara groundnut flour 8 min/ 900 W

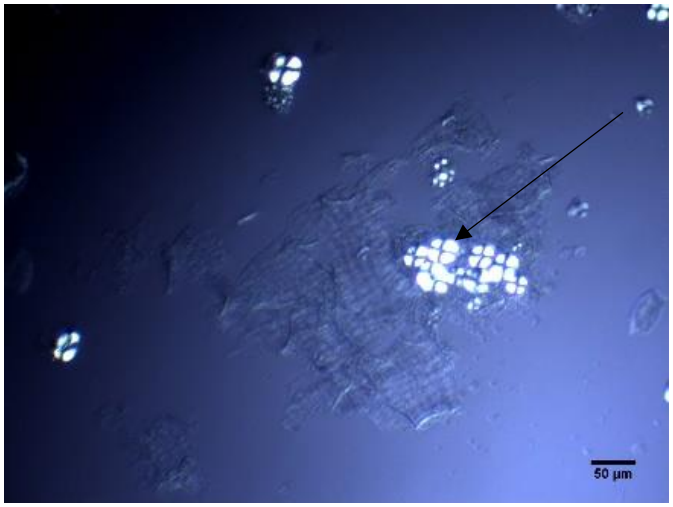
Red Bambara groundnut flour 8 min/ 900 W



Brown Bambara groundnut flour 5 min/ 1200 W



Red Bambara groundnut flour 5 min/ 1200 W



Brown Bambara
groundnut flour
8 min/ 1200 W

Red Bambara
groundnut flour
8 min/ 1200 W

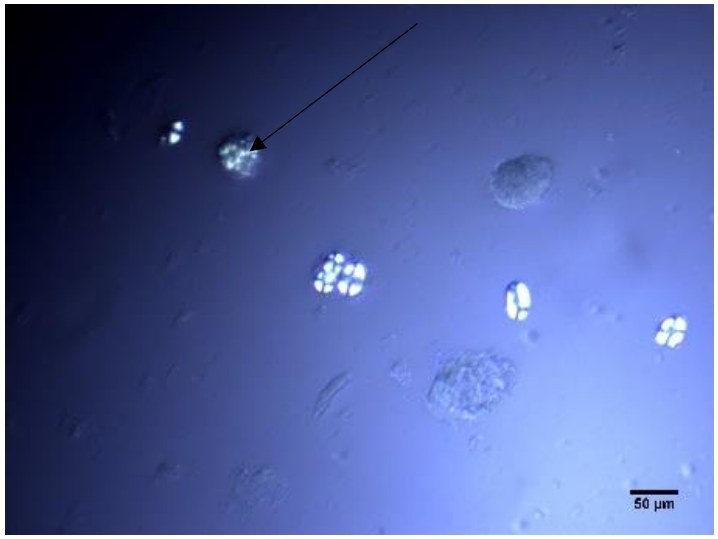
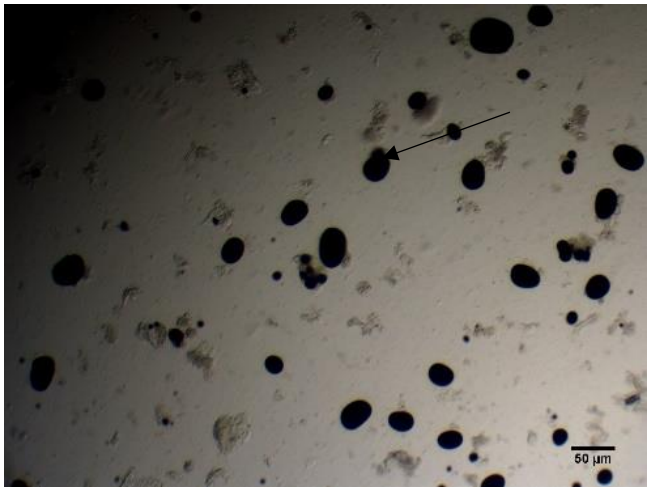
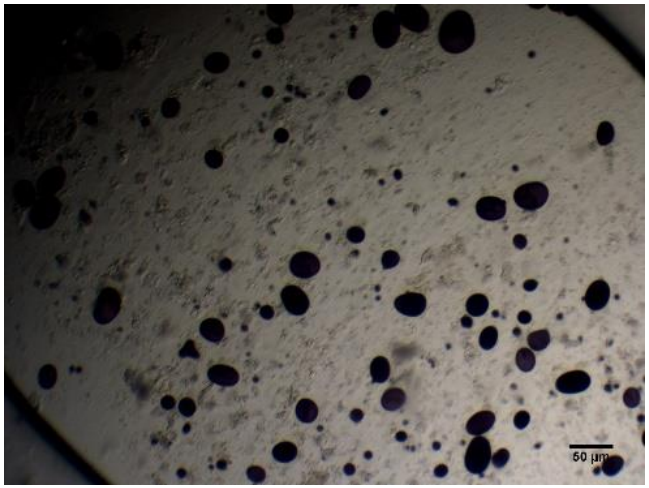


Figure 5.1.4.3: Polarized light microscopy images of flour from raw and microwaved Bambara groundnut seeds. Bambara groundnut flours from raw pre-soaked and microwaved (900W and 1200 W for 5 and 8 min) seeds were viewed under polarized light for loss of birefringence. Bar = 50 µm. Arrows indicate aggregates of partially gelatinized starch granules and birefringence of starch granules in flours from raw untreated and microwave treated samples.

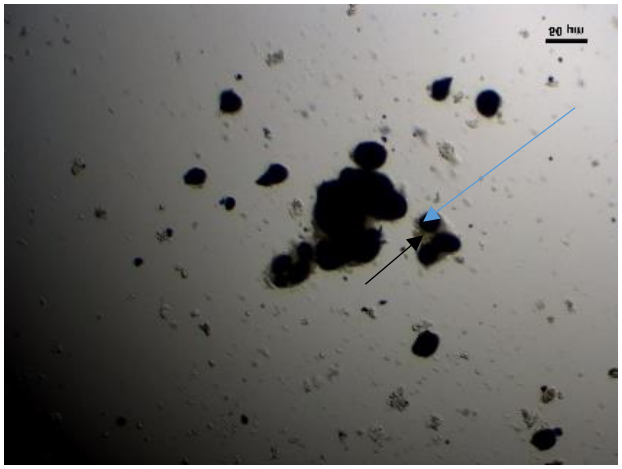
Brown Bambara groundnut flour (Raw)



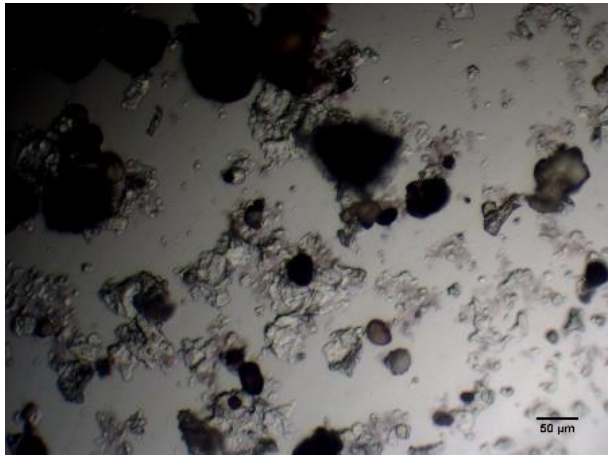
Red Bambara groundnut flour (Raw)



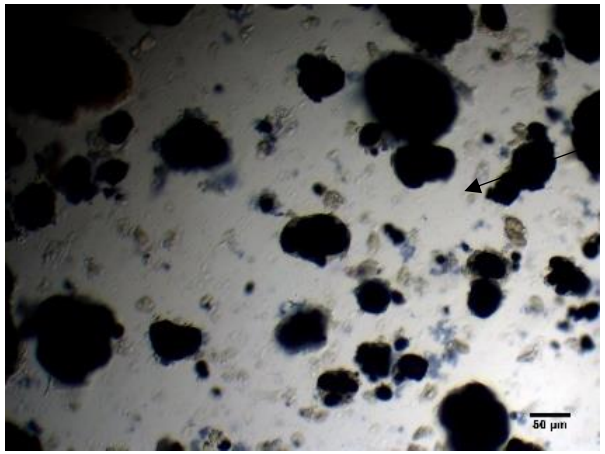
Brown Bambara groundnut flour 5 min/ 900 W



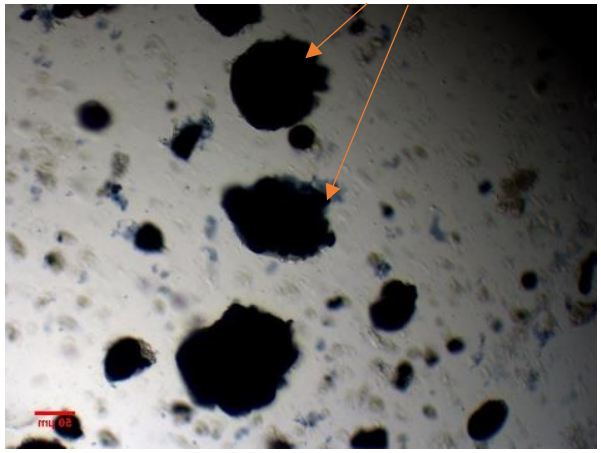
Red Bambara groundnut flour 5 min/ 900 W



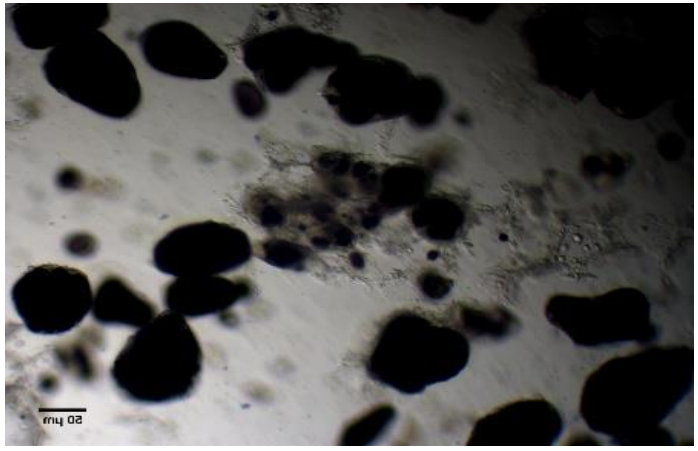
Brown Bambara
groundnut flour
8 min/ 900 W



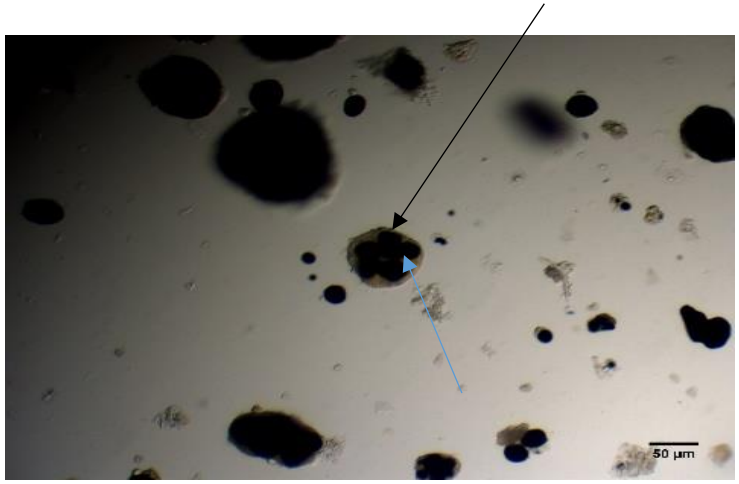
Red Bambara
groundnut flour
8 min/ 900 W



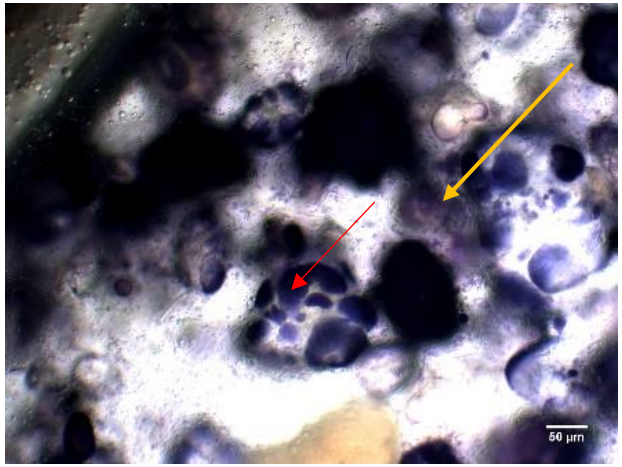
Brown Bambara
groundnut flour
5 min/ 1200 W



Red Bambara
groundnut flour
5 min/ 1200 W



Brown Bambara groundnut flour
8 min/ 1200 W



Red Bambara groundnut flour
8 min/ 1200 W

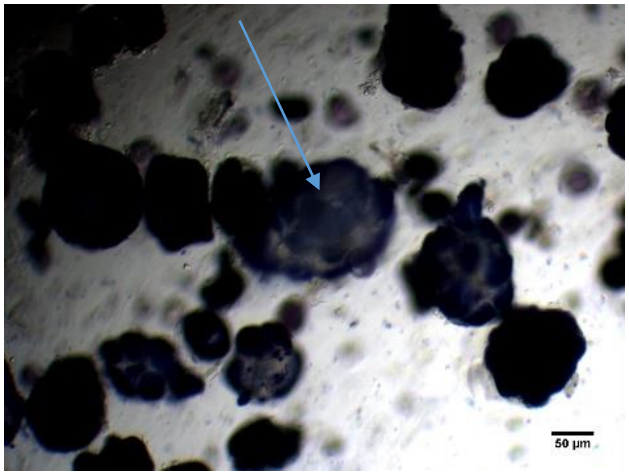


Figure 5.1.4.4: Light microscope images of iodine stained flours from raw and microwaved Bambara groundnut seeds. Flours from raw untreated Bambara groundnut seeds and microwaved (900 W and 1200 W for 5 and 8 min) were stained with iodine. Bar = 50 µm. Black arrows show iodine stained swelled starch granules and light blue arrows indicate hydrophobic protein layer surrounding starch granules. Orange arrows show aggregation and swelling of starch granules in microwaved and starch granules in raw untreated samples. Dark Blue arrows indicate the purple insoluble irregular mass of starch in microwaved samples.

5.1.5) Conclusion:

Microwave pre-treatment of pre-soaked Bambara grounndut seeds cause a reduction in coooking time and all functional properties such as pasting viscosity, thermal properties, WSI and NSI. The observed decreases can be attributed to microwave-induced changes in the microstructure of the flours. Microwave pre-treatment of Bambara groundnut seeds shows promise in the reduction in cooking time which aids in the alleviation of the hard-to-cook (HTC) defect as well as the modification of microstructural components which enhances its use in food application. The structural modification of the flours allows for its use in a variety of products such as porridges, soups and baked goods.

5.2) Effects of microwave pre-treatment of pre-soaked Bambara groundnut seeds on their phenolic composition and antioxidant properties

Abstract

The effect of microwave pre-treatment on the phenolic composition, total phenolic content (TPC) and antioxidant properties of pre-soaked brown and red Bambara groundnut seeds were investigated. Bambara groundnut seeds were pre-soaked to a final moisture content of 40%. A fluidized bed microwave system was used to heat the pre-soaked Bambara groundnut seeds at 900 and 1200 W for 5 and 8 min. Total phenolic content was determined using the Folin-Ciocalteu method and antioxidant capacity using the 2,2-azinobis-(3-ethyl-benzothiazoline-6-sulphonic acid (ABTS) radical scavenging activity. Quantification of flavonoids and phenolic acids in extracts of raw and microwaved Bambara groundnut flour were determined using Ultra-Performance Liquid Chromatography-Mass Spectrometry. Inhibitory effects of the extracts against AAPH radical-induced plasmid DNA damage was also determined. Microwaving led to both increases and decreases in TPC and radical scavenging activity of Bambara groundnut seeds. Hydroxybenzoic, hydroxycinnamic acid derivatives, flavan-3-ols, flavonols, flavones and flavanones were identified in extracts of Bambara groundnut flour. For the raw seeds, catechin and hesperidin were the major flavonoids in the brown type while quercetin-3-*O*-glucoside and hesperidin were the major flavonoids in the red type. There were varietal differences in the types and levels of flavonoids and phenolic acids in the two Bambara groundnut types with the red type containing higher levels of these phenolic compounds than the brown type. Microwaving at the higher power of 1200 W generally made flavonoids and phenolic acids more extractable especially in the red Bambara groundnuts. Extracts from microwaved samples exhibited protective effects against AAPH radical-induced DNA damage. Overall, flours from the microwave-treated Bambara groundnut seeds retained appreciable radical scavenging properties and this indicates the potential of microwave processing to be applied in the production of legume flours with enhanced health-promoting properties.

Keywords: Bambara groundnut, flavonoids, phenolic acids, total phenolic content, antioxidant activity, microwave processing

5.2.1) Introduction

Bambara groundnuts are an underutilized grain legume (Nyau, Prakash, Rodrigues & Farrant, 2015) and considered to be among some of the most significant African legumes (Duodu & Apea-Bah, 2017). Bambara groundnuts have a variety of food uses especially in sub-Saharan African countries and constitute an important source of protein, essential amino acids and other nutrients (Massawe, Mwale, Azam-Ali & Roberts, 2005; Mbata, Ikenebomeh & Ezeibe, 2009).

The increased consumption of plant foods in the form of grain legumes such as cowpeas and Bambara groundnuts has been accociated with a reduced risk of non-communicable diseases (NCD's) such as cardiovascular disease and cancer (Fraser, 1999; Velie, Schairer, Flood, He, Khattree & Schatzkin, 2005). It is hypothesised that this is due to the presence and activity of bioactive components such as phenolic compounds (Oomah, Tiger & Balasubramanian, 2006) mainly as a result of their antioxidant properties (Rice-Evans *et al*., 1996). There is therefore increasing interest in the bioactive phenolic composition of grain legumes such as Bambara groundnuts and how their health-promoting properties are affected during processing. For example, Muchuweti *et al*. (2013) reported that phenolic compounds in fresh and dried Bambara groundnut seeds exhibited free radical scavenging antioxidant activity. Nyau *et al*. (2015) also reported that Bambara groundnuts contain a wide variety of phenolic compounds consisting mainly of flavonoids.

An important form in which grain legumes such as Bambara groundnut is utilized is in the form of flours for various food applications (Duodu & Apea-Bah, 2017). For production of such flours, the legume seeds are usually subjected to thermal treatments for purposes such as reduction in cooking time of the legume seeds and enhancing functional properties of the resultant flours. Microwave processing is one of such thermal treatments used. For example, Mokatso (2017) used microwave processing for pre-treatment of cowpeas for processing into flour. However, as reported by Mokatso (2017) such microwave pre-treatment processes can affect the phenolic content and antioxidant properties of the legume. No research has been conducted on the phenolic composition and antioxidant activity of Bambara groundnut seeds after microwave pre-treatment.

Thus the objective of this study was to determine the effect of microwave pre-treatment on the phenolic composition and antioxidant health-promoting properties of pre-soaked Bambara groundnut seeds.

5.2.2) Materials and methods

5.2.2.1) Bambara groundnut samples

Bambara groundnut (brown and red types) was procured from Triotrade CC (Pretoria, South Africa).

5.2.2.2) Chemicals

Pure standard phenolic compounds: *p*-coumaric acid, ferulic acid, caffeic acid, (+)-catechin, quercetin, kaempferol, myricetin, rutin were all purchased from Sigma Aldrich. Folin-Ciocalteu's phenol reagent, methanol, hydrochloric acid and potassium persulphate were purchased from Merck. 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were purchased from Sigma-Aldrich. Formic acid and ethyl acetate solvents were purchased from Merck and Sigma-Aldrich.

5.2.2.3) Hydration of Bambara groundnut seeds to 40% moisture content

The hydration of Bambara groundnut seeds was conducted as described in section 5.1.

5.2.2.4) Microwave treatment

The microwave treatments of Bambara groundnut seeds were conducted as described in section 5.1.

5.2.2.5) Preparation of phenolic extracts

Phenolic extracts from the Bambara groundnut samples were prepared as described by Kayitesi (2013) with modifications. The extracts were prepared in three phases as follows: 10 ml 1% v/v HCl-methanol was added to 3 g and 1 g for extraction sample in an Erlenmyer flask, stirred for 3 h and centrifuged at 8217 x g for 10 min at ambient temperature (25^{o}C) and supernatant decanted. The pellet was collected and washed with 10 ml of solvent (1% v/v HCl-methanol), stirred for 20 min and centrifuged as above described. The washing step was repeated twice. The supernatants were pooled and placed in air-tight plastic tubes wrapped in aluminum foil and stored at -20^{o}C until analysis.

5.2.2.6) Preparation of phenolic extracts for Ultra-performance Liquid Chromatography-Mass Spectrometry (UPLC-MS) analysis

Extracts were prepared as described by Ojwang (2012) with some modifications. Approximately 5 g of raw and microwaved Bambara groundnut flour samples were weighed into a beaker and soaked in 15 ml 70% acetone acidified with 1% formic acid for 12 h at 4^{o}C. The mixtures were then stirred with a magnetic stirrer for 2 hrs at room temperature. The extracts were centrifuged at 135837 x g

for 10 min at room temperature. The pellets were collected and washed twice with 10 ml of extraction solvent for 30 min. The previous two steps were repeated. The supernatants were combined and concentrated under reduced pressure using a Buchmann R110 rotavapor (Westbury, NY, USA).

The extracts were purified and fractionated using Sep-Pak Solid Phase Octadecylsilane (C18) cartridges (Sigma, USA) as described by Prior, Lazarus, Cao, Muccitelli and Hammerstone (2001) and Monagas, Gómez-Cordovés, Bartolomé, Laureano and da Silva (2003) with some modifications. The C18 cartridges were briefly conditioned with 25 ml methanol:water (1:1) for 1 h and washed with 50 ml distilled water. At least 5 ml of the concentrated extracts were deposited into cartridges and washed with 5 ml distilled water to remove the sugars. Catechins, oligomeric proanthocyanins (PA) and other small phenolic compounds were eluted with 15 ml ethyl acetate. The ethyl acetate fraction was dried under vacuum and re-dissolved in 2 ml of methanol:water (50:50) containing 0.05% formic acid. Flavonols, isoflavonols and anthocyanin pigments were then eluted with 15 ml methanol acidified with 0.1% formic acid. The sequencial elution procedure was done to reduce the number of compounds in each fraction injected into the mass spectrometer to enable proper characterization. Before chromatographic analysis, the extract fractions were filtered through 0.45 µm PTFE membrane filters and deposited into amber vials.

5.2.2.7) Folin-Ciocalteu method for total phenolic content

The total phenolic content (TPC) was determined using the Folin-Ciocalteu assay modified for the use of a 96-well microplate as described by Ainsworth and Gillespie (2007). One hundred microliters of phenolic extracts and catechin standard solutions of different concentrations (0, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3 and 0.35 mg/ml) prepared in 1% HCl in methanol) were placed in Eppendorf tubes and 200 µl of 10% v/v Folin-Ciocalteu reagent was added to each tube. A volume of 800 µl of 250 mM Na_2CO_3 was added to each tube within 8 minutes after the addition of the Folin-Ciocalteu reagent. The solutions were vortexed and incubated for 2 h at room temperature. After incubation, 200 µl of the reaction mixture was placed into each well of a 96 well microplate and the absorbance read at 750 nm using a microplate reader (Multiskan FC, Thermo Fisher Scientific, Shanghai, China). The total phenolic content was expressed as milligram catechin equivalents (CE) per g sample on dry weight basis.

5.2.2.8) 2, 2'-azino-bis (3-ethyl) benzothiazoline-6-sulphonic acid (ABTS) radical scavenging assay

The ABTS radical scavenging activity assay was performed according to the method described by Awika, Rooney, Wu, Prior and Cisneros-Zevallos (2003) with some modifications for use in a 96-well microplate. The $ABTS^{\bullet+}$ radical mother solution was prepared by mixing equal volumes of 7 mM ABTS solution and 2.45 mM potassium peroxysulphate solutions prepared in distilled water and then allowing incubation of the solutions in the dark for 12-16 hrs. The mother solution was diluted by adding 29 parts phosphate buffer (pH = 7.4) to one part $ABTS^{\bullet+}$ radical cation mother solution to form an $ABTS^{\bullet+}$ radical working solution. A volume of 10 µl of phenolic extract and Trolox standard solutions of different concentrations (0, 50, 100, 200, 300, 400, 500 and 600 µM) prepared in 1% HCl in methanol was added to 190 µl of the $ABTS^{\bullet+}$ radical cation working solution in the microplate wells, stored in a dark area for 30 min and the absorbance then read at 750 nm using a microplate reader (Multiskan FC, Thermo Fisher Scientific, Shanghai, China). The results were expressed as µmol Trolox equivalents per gram sample on dry weight basis.

5.2.2.9) UPLC-MS analysis

The chromatographic analysis was preformed as described by Stander, Van Wyk, Taylor & Long, (2017). A Waters Synapt G2 Quadrupole Time-of-Flight (QToF) mass spectrometer (MS) connected to a Waters Acquity ultra-performance liquid chromatograph (Waters, Milford, MA, USA) was used for high-resolution UPLC-MS analysis. Electrospray ionization was used in negative mode with a cone voltage of 15 V, desolvation temperature of 275°C, desolvation gas at 650 L/h, and the rest of the MS settings optimized for best resolution and sensitivity. Data were acquired by scanning from m/z 150 to 1500 m/z in resolution mode as well as in MS^E mode. In MS^E mode two channels of MS data were acquired, one at a low collision energy (4 V) and the second using a collision energy ramp (40−100 V) to obtain fragmentation data as well. The instrument was calibrated with sodium formate. Separation was achieved on a Waters HSS T3, 2.1 × 100 mm, 1.7 µm column. An injection volume of 2 µL was used and the mobile phase consisted of 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid as solvent B.

Gradient elution was done according to the following program: 100% A from 0 to 0.5 min; 74% A and 26% B from 0.5 min to 6 min; 56% A and 44% B from 6 min to 15 min.; 0% A and 100% B from 15 min to 17.5 min. Ionisation was in negative mode with a capillary voltage of 3 kV and cone

voltage of 15 V. Identification was done by comparing chromatograms and retention times of phenolic constituents in the extracts with external phenolic acid and flavonoid standards as well as comparison of MS/MS fragmentation data and UV spectra with those of phenolic compounds reported in literature. Quantification was done by comparing integrated peak areas of phenolic compounds in extracts with that of standards and from generated calibration curves.

5.2.2.10) Inhibition of oxidative DNA damage assay

Inhibition of AAPH-induced oxidative DNA damage was determined by a method described by Apea-Bah *et al*. (2014) with some modifications. The phenolic extracts were diluted 10 times with 0.2 M sterile phosphate buffer (PH 7.4) solution. To test the inhibitory effect of the extracts against AAPH-induced oxidative damage of the DNA, 2.5 µl 10 times diluted phenolic extracts were reacted with 2.5 µl of 20 times diluted DNA and 2.5 µl of 3 mM AAPH. The reaction mixtures were incubated for 90 min at 37ºC. SYBR Green stain was used as the precasting DNA stain. After incubation, 2 µl of 6 times concentrated DNA loading dye was added to the reaction mixtures and 8 µl of the final reaction mixture were loaded into 1% (w/v) agarose gel wells. The gel was subjected to electrophoresis (60 V, 500mA, 150 W) for 60 min and the DNA bands were imaged (Gel Doc EZ imager, Bio-Rad Labs, Hercules, CA, USA) and the bands analyzed (Image Lab 5.1, Bio-Rad Labs, Hercules, CA, USA).

5.2.2.11) Statistical analysis

Experiments were conducted in triplicate. Multi-factorial analysis of variance (MANOVA) was conducted using SPSS software. Significant differences between means were determined at $p \leq 0.05$ using the least significant difference (LSD) test. Independent variables were microwave power (0, 900 W and 1200 W), microwave time (0, 5 and 8 min) and Bambara groundnut types. Dependent variables were the phenolic acid and flavonoid contents, total phenolic content and antioxidant activity.

5.2.3) Results and discussion

5.2.3.1) Total phenolic content (TPC) and ABTS radical scavenging activity of flours from microwave pre-treated Bambara groundnut seeds

The effect of microwave pre-treatment on the total phenolic content (TPC) and ABTS radical scavenging activity of flours from pre-soaked brown and red Bambara groundnut seeds are shown in Tables 5.2.3.1 and 5.2.3.2, respectively. The total phenolic content of flours from raw untreated brown and red Bambara groundnut seeds was 3.04 mg CE/g and 2.47 mg CE/g, respectively. These are similar to the TPC value of 2.54 mg CE/g reported by Ogundele (2016) for Bambara groundnut seeds.

Table 5.2.3.1: Effect of microwave pre-treatment of pre-soaked Bambara groundnut seeds on the total phenolic content (TPC) (mg CE/g db) of the resultant flours

	Microwave pre-treatment				
	Raw	5 min/ 900 W	8 min/ 900 W	5 min/1200 W	8 min/1200 W
Brown Bambara groundnut seeds	$3.04^{a} \pm 0.35$	$3.12^{a} \pm 0.11$	$3.20^{a} \pm 0.06$	$2.60^{a} \pm 0.20$	$2.64^{a} \pm 0.02$
Red Bambara groundnut seeds	$2.46^{a} \pm 0.38$	$3.19^{a} \pm 0.26$	$3.33^{a} \pm 0.39$	$3.14^{a} \pm 0.12$	$3.28^{a} \pm 0.01$

CE – catechin equivalents; db – dry basis
a,b,c,: numbers with different superscripts differ significantly ($p \leq 0.05$) within rows

Table 5.2.3.2: Effect of microwave pre-treatment of pre-soaked Bambara groundnut seeds on the ABTS radical scavenging activity (µmol TE/g db) of the resultant flours

	Microwave pre-treatment				
	Raw	5 min/ 900 W	8 min/ 900 W	5 min/ 1200 W	8 min/ 1200 W
Brown Bambara groundnut seeds	$47.77^{a} \pm 5.76$	$40.16^{a} \pm 0.42$	$38.69^{a} \pm 9.65$	$31.08^{a} \pm 2.95$	$31.43^{a} \pm 0.16$
Red Bambara groundnut seeds	$31.31^{b} \pm 9.65$	$43.55^{ab} \pm 1.34$	$41.85^{ab} \pm 0.30$	$38.14^{ab} \pm 1.58$	$42.88^{b} \pm 1.70$

ABTS – 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid
TE – Trolox equivalents; db – dry basis
a,b,c: numbers with different superscripts differ significantly ($p \leq 0.05$) within rows

For brown Bambara groundnut seeds, compared with the raw seeds, microwave treatment at 900 W increased TPC by up to 5% while microwave treatment at 1200 W decreased TPC by up to 5%. For red Bambara groundnut seeds, microwave treatment at both 900 W and 1200 W increased TPC by up to 26%. Unlike for TPC, microwave treatment at both 900 W and 1200 W decreased ABTS radical scavenging activity of brown Bambara groundnut seeds by up to 39%. The trends in ABTS radical scavenging activity of red Bambara groundnut seeds after microwave treatment were similar to the TPC with increases in radical scavenging activity by up to 26%. Overall, these results indicate that microwave treatment of Bambara groundnut seeds could lead to either increases or decreases in TPC and radical scavenging activity.

The observed decrease in TPC and radical scavenging activity of flour from Bambara groundnut seeds after microwave treatment could be due to thermally induced reaction of phenolic compounds with proteins (Bishnoi, Khetarpaul & Yadav, 1994) making them less extractable. Another possible reason could be due to thermal oxidation or degradation of phenolic compounds as reported by Luthria and Pastor-Corrales (2006).

On the other hand, observed increases in TPC and radical scavenging activity after microwave treatment may be attributed to the release of phenolic compounds bound to cell wall components as a result of thermal-induced changes in structural integrity to the seeds (Žilic, Mogol, Akillioğlu, Serpen, Delić & Gökmen, 2013). This makes the phenolic compounds more extractable and this is

reflected as increases in TPC and antioxidant activity. Maillard reaction products produced as a result of the thermal treatment are known to have reducing properties (Dittrich, El-massry, Kunz, Rinaldi, Peich, Beckmann & Pischetsrieder, 2003) and could also contribute to increases in TPC and antioxidant activity. Similar results have been reported by Nyembwe, Minnaar, Duodu and De Kock (2015) who observed an increase in total phenolic content of marama beans after roasting.

Table 5.2.3.3: Pearson correlation coefficients (r) for total phenolic content (TPC) vs ABTS radical scavenging activity for flours from raw and microwaved brown and red Bambara groundnut seeds

	TPC vs ABTS
Brown Bambara groundnut seeds	0.76
Red Bambara groundnut seeds	0.94

$p \leq 0.05$

For the two Bambara groundnut types studied, there were high and positive correlations between TPC and ABTS radical scavenging activity (Table 5.2.3.3). The Pearson correlation coefficient between TPC and ABTS radical scavenging activity (r) were 0.76 for brown Bambara groundnut seeds and 0.94 for red Bambara groundnut seeds. The high and positive correlations indicate that phenolic compounds were significant contributors to the observed radical scavenging activity. The relatively lower magnitude of the correlation for brown Bambara groundnut seeds is likely a reflection of the slightly different trends in TPC compared to ABTS radical scavenging activity due to microwave treatment. Similar positive correlations between TPC and ABTS radical scavenging antioxidant activity have been reported for various processed legumes including lentils, chickpeas, yellow and green peas and soybeans (Han & Baik, 2008), black beans, lentils and red kidney bean (Xu & Chang, 2007) and dry beans (*Phaseolus vulgaris* L.) (Oomah *et al*., 2014).

5.2.3.2) Identification of phenolic compounds in flours from microwaved pre-soaked Bambara groundnut seeds.

Table 5.2.3.4: Retention time (t_R), UV-visible absorption maxima and mass spectral characteristics of phenolic compounds identified in ethyl acetate fraction of extracts from raw and microwaved Bambara groundnut seeds

Peak number	*t_R(min)*	*λ_{max}(nm)*	*[M-H]⁻*	*MS/MS fragments*	*Proposed compound ID*
Hydroxybenzoic acid derivatives					
1	4.04	271, 289	169	125 (71)	Gallic acid
2	4.98	220, 266	153	109 (57)	Protocatechuic acid
3	4.51	220, 343	167	152 (20), 123 (20), 108 (26)	Vanillic acid
Hydroxycinnamic acid derivatives					
4	10.17	348, 311	163	119 (80)	Coumaric acid isomer
5	9.41	282	253	179 (18)	Caffeoyl glycerol
6	1.50	265	355	193 (23)	Ferulic acid hexoside
Flavan-3-ols					
7	5.26	276	577	407 (58), 289 (66), 125 (84)	Procyanidin B2 dimer
8	5.59	279	865	577 (13), 289 (53)	Procyanidin C2 trimer
9	5.71	280, 348	289	271 (5), 245 (24), 205 (21)	Catechin
10	4.77	277	451	289 (100)	Catechin glucoside
Flavonols					
11	9.42	341, 280	285	243 (9), 151 (17), 145 (25), 109 (11)	Kaempferol
12	7.24	353, 279	463	301 (75), 179 (5.4), 151 (20)	Quercetin-3-*O*-glucoside
13	10.33	281	317	317 (100)	Myricetin
Flavononol					
14	14.41	281	303	125 (5)	Taxifolin
Flavones					
15	11.38	348, 341	269	151 (46), 117 (8)	Apigenin
Flavanones					
16	6.46	348, 275	609	301 (75), 286 (6), 258 (2), 151 (14)	Hesperidin
17	9.88	368, 341	301	177 (5), 151 (54)	Hesperetin
18	11.21	348, 281	271	151 (20), 119 (50)	Naringenin
19	13.49	281	287	151 (2), 135 (10), 125 (10)	Eriodictyol
20	6.20	282	449	287 (35), 151 (29)	Eriodictyol-7-*O*-β-D- glucoside

5.2.3.3) Phenolic acids and derivatives identified

The compound labelled as peak 1 with retention time 4.04 min, molecular ion at m/z 169 and maximum UV-vis absorption wavelength 271 and 289 nm (Table 5.2.3.4) was identified as gallic acid. It produced a fragment at m/z 125 possibly due to loss of a CO_2 (-44 amu) from the carboxylate group as shown in Figure 5.2.3.1A (Fang, Yu & Prior, 2002). Its mass spectrum is shown in Appendix A: Figure A 1. Gallic acid has been previously reported in Bambara groundnut seeds by Tsamo, Ndibewu and Dakora (2018).

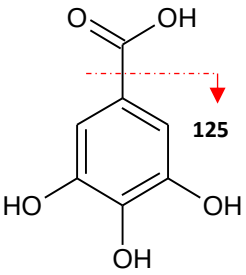
The compound labelled as peak 2 with retention time of 4.98 min, molecular ion at m/z 153 and UV-vis absorption wavelength 220 and 266 nm (Table 5.2.3.4) was identified as protocatechuic acid. The compound produced an ionic fragment at m/z 109 which corresponds to loss of a CO_2 (-44 amu) moiety from the carboxylate group as shown in Figure 5.2.3.1B (Fang *et. al*., 2002). Its mass spectrum is shown in Appendix A: Figure A 2.

The compound labelled as peak 3 (t_R = 4.51, λ_{max} = 220 and 343) with molecular ion at m/z 167 (Table 5.2.3.4) was tentatively identified as vanillic acid. It produced fragments at m/z 152 [due to loss of a methyl group (CH_3; –15 amu)], m/z 123 [due to loss of a carbon dioxide (CO_2; –44 amu)] and m/z 108 [due to loss of both CH_3 (–15 amu) and CO_2 (–44 amu)] (Griemann, Greaves & Saltzman, 2015). The fragmentation patterns are illustrated in Figure 5.2.3.1C and its mass spectrum is shown in Appendix A: A 3.

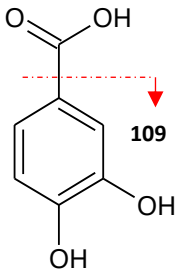
The compound labelled as peak 4 (t_R = 10.17, λ_{max} = 348, 311) with molecular ion at m/z 163 (Table 5.2.3.4) was tentatively identified as coumaric acid isomer. Fragmentation produced an ion at m/z 119 which corresponds to the loss of a CO_2 unit (-44 amu) (Fang *et al*., 2002). The fragmentation pattern is illustrated in Figure 5.2.3.1D and its mass spectrum is shown in Appendix A: A 4.

The compound labelled as peak 5 (t_R = 9.41, λ_{max} = 282) with molecular ion at m/z 253 (Table 5.2.3.4) was tentatively identified as caffeoyl glycerol. Fragmentation produced an ion at m/z 179 corresponding to the loss of a glycerol molecule (-74 amu) (Ma *et al*. (2007) as illustrated in Figure 5.2.3.1E. Its mass spectrum is shown in Appendix A: Figure A 5.

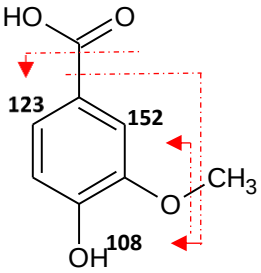
Peak 6 with retention time of 1.50 min, molecular ion at m/z 355 and UV-vis absorption maximum at 265 nm (Table 5.2.3.4) was tentatively identified as ferulic acid hexoside. Fragmentation produced an ionic fragment at m/z 193 corresponding to the loss of a hexose unit (-162 amu) (Aaby *et al*., 2007) as illustrated in Figure 5.2.3.1F. Its mass spectrum is shown in Appendix A: Figure A 6.



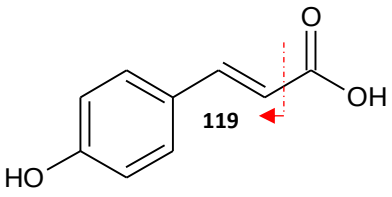
A) Gallic acid (m/z 169)



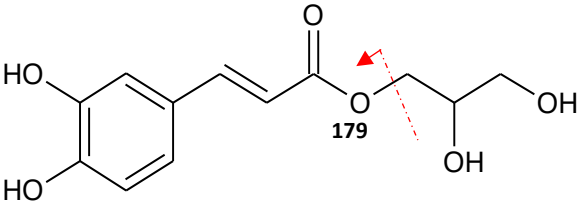
B) Protocatechuic acid (m/z 153)



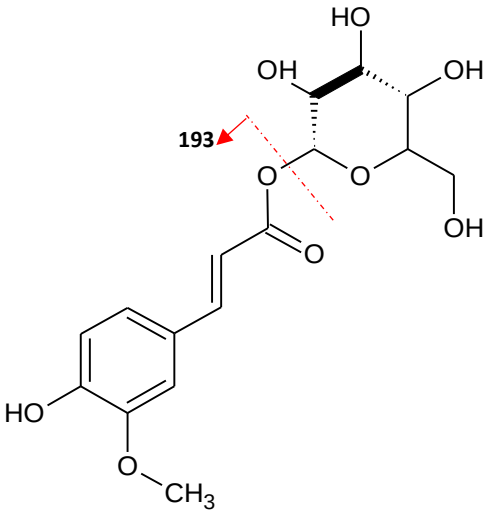
C) Vanillic acid (m/z 167)



D) Coumaric acid (m/z 163)



E) Caffeoyl glycerol (m/z 253)



F) Ferulic acid hexoside (m/z 355)

Figure 5.2.3.1: Proposed fragmentation patterns for phenolic acids and their derivatives identified in the raw and microwaved Bambara groundnut seeds

5.2.3.4) Flavonoids and derivatives identified

Peak 7 with retention time of 5.26 min, molecular ion at m/z 577 and UV-vis absorption at 276 nm (Table 5.2.3.4) was identified as procyanidin B 2 dimer. It produced fragments at m/z 407, 289 and 125. Retro-Diels-Alder (RDA) fragmentation of the C ring at positions 1 and 3 of one of the flavan-3-ol monomeric units produces an m/z 425 fragment (Figure 5.2.3.2) which can then undergo loss of a water (H_2O) molecule (-18 amu) to produce the m/z 407 fragment. Cleavage of the interflavan linkage produces the flavan-3-ol monomeric fragment at m/z 289 (Holt, Lazarus, Sullards, Zhu, Schramm, Hammerstone, Fraga, Schmitz & Keen, 2002). RDA cleavage of the C ring of one of the monomeric flavan-3-ol fragments at positions 1 and 3 produces a trihydroxy phenol moiety ($^{1,3}A^-$) at m/z 125 (Figure 5.2.3.2). Its mass spectrum is illustrated in Appendix A: Figure A 7.

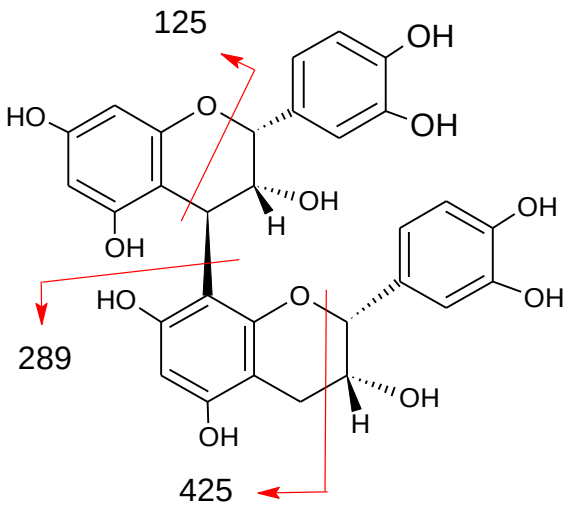


Figure 5.2.3.2: Proposed fragmentation patterns for procyanidin B 2 (epicatechin-(4-8)-epicatechin)

Peak 8 with retention time of 5.59 min, molecular ion at 865 m/z and UV-vis absorption at 279 nm (Table 5.2.3.4) was identified as procyanidin C 2 trimer. Its fragments are produced from cleavage of the interflavan bond to produce the dimeric (m/z 577) and monomeric (m/z 289) procyanidin species (Friedrich *et al.*, 2000; Tsang *et al.*, 2005) (Figure 5.2.3.3). Its mass spectrum is illustrated in Appendix A: Figure A 8.

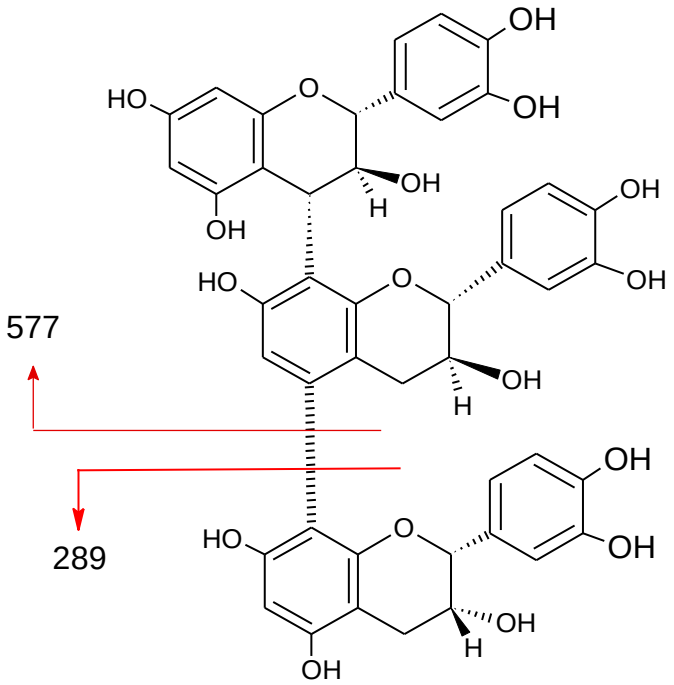


Figure 5.2.3.3: Proposed fragmentation patterns for procyanidin C 2 trimer

Peak 9 with retention time of 5.71 min, molecular ion at m/z 289 and UV-vis absorption at 280, 348 nm (Table 5.2.3.4) was identified as catechin. Fragmentation produced ionic fragements at m/z 271, 245 and 205. The m/z 271 fragment corresponds to the loss of a H_2O (- 18 amu) molecule. The m/z 245 fragment corresponds to either the loss of a CO_2 (-44 amu) residue (Ströggl, Huck & Bonn, 2004) or loss of a CH=C-OH group (– 42 amu) most likely from the A ring (Pérez-Magariño *et al*., 1999) together with hydrogen (H_2) molecule (– 2 amu). The m/z 205 fragment corresponds to the loss of the A ring (-84 amu) (Ströggl *et al*., 2004) (Figure 5.2.3.4). Its mass spectrum is illustrated in Appendix A: Figure A 9. Catechin has been previouly reported in Bambara groundnut seeds by Nyau *et al*. (2015) and Tsamo *et al*. (2018).

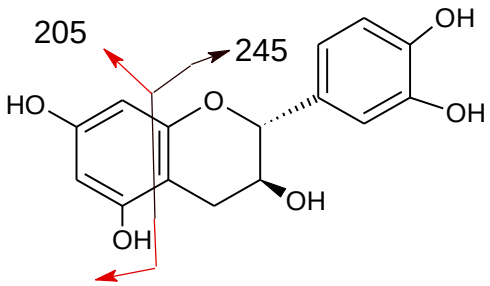


Figure 5.2.3.4*:* Proposed fragmentation pattern for catechin

Peak 10 with retention time of 4.77 min, molecular ion at m/z 451 and UV-vis absorption at 277 nm (Table 5.2.3.4) was tentatively identified as a glucoside derivative of catechin. Fragmentation produced an ionic fragment at m/z 289. The fragment at m/z 289 is the resulting catechin aglycone after loss of the glucose moiety (-162 amu) (Zerbib *et al*., 2018). The proposed fragmentation pattern is shown in Figure 5.2.3.5 using catechin-5-*O*-glucoside as an example. The mass spectrum of catechin glucoside is illustrated in Appendix A: Figure A 10.

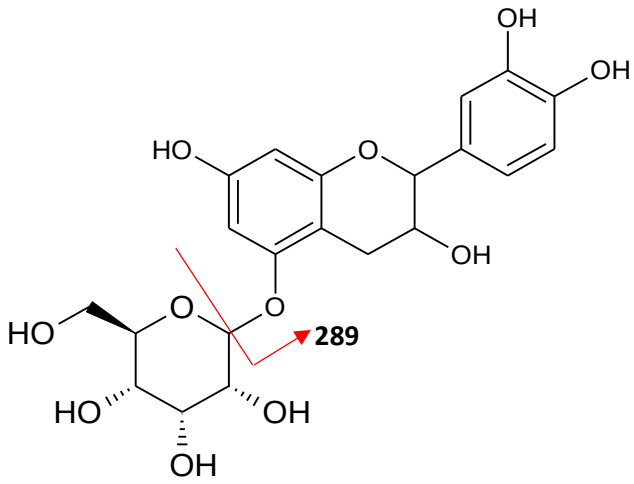


Figure 5.2.3.5: Proposed fragmentation pattern for catechin glucoside

The compound labelled as peak 11 (t_R = 9.42, λ_{max} = 341, 280) with molecular ion at m/z 285 was identified as kaempferol (Table 5.2.3.4). The compound produced ionic fragments at m/z 243, 151, 125 and 109. The m/z 243 fragment could be due to loss of a CH=C-OH group (– 42 amu) most likely from the A ring. RDA fragmentation of the C ring at positions 1 and 3 produces a $^{1,3}A^-$ fragment at m/z 151 (Fabre *et al*., 2001). Similarly, RDA fragmentation of the C ring at positions 1 and 4 produces a $^{1,4}A^-$ fragment (trihydroxy benzene molecule) at m/z 125 (Miketova *et al*., 2000). Loss of a CH=C-OH group (– 42 amu) from the A ring of the m/z 151 fragment produces the m/z 109 fragment (Ma *et al*., 1997). The proposed fragmentation pattern is shown in Figure 5.2.3.6. Kaempferol has been previously reported in Bambara groundnut seeds by Tsamo *et al*. (2018).

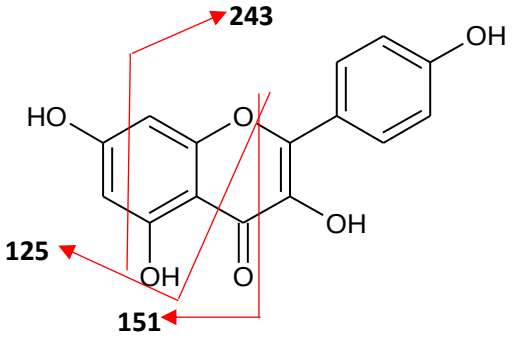


Figure 5.2.3.6*:* Proposed fragmentation pattern for kaempferol

The compound labelled peak 12 with retention time of 7.24 min, molecular ion at m/z 463 and UV-vis absorption at 353, 279 nm (Table 5.2.3.4) was identified as quercetin-3-*O*-glucoside. The compound produced fragments at m/z 301, 179 and 151. The m/z 301 fragment corresponds to quercetin aglycone after the loss of a glucose molecule (-162 amu) (Kajdžanoska, Gjamovski & Stefova, 2010). The m/z 179 fragment represents the $^{1,2}A^-$ ion resulting from fragmentation of the C ring of quercetin aglycone at positions 1 and 2 (Fabre *et al*., 2001). RDA fragmentation of the C ring of quercetin aglycone at positions 1 and 3 produces a $^{1,3}A^-$ fragment at m/z 151 (Fabre *et al*., 2001). The proposed fragmentation pattern is shown in Figure 5.2.3.7. Its mass spectrum is illustrated in Appendix A: Figure A 12. Quercetin-3-*O*-glucoside has been previously reported in Bambara groundnut seeds by Nyau *et al*. (2015).

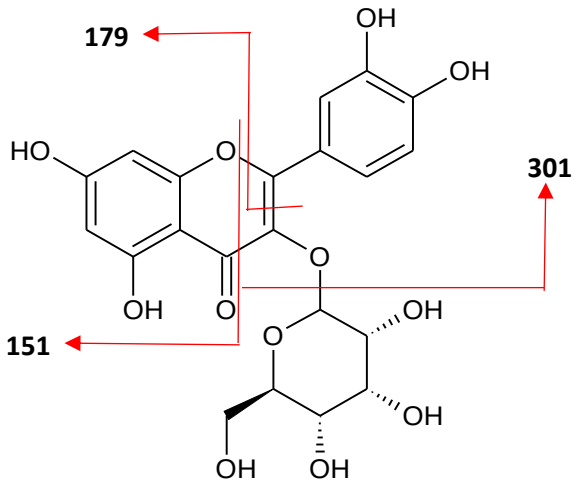


Figure 5.2.3.7 Proposed fragmentation pattern for quercetin-3-O-glucoside

Peak 13 with retention time of 10.33 min and UV-vis absorption at 281 nm (Table 5.2.3.4) was tentatively identified as myricetin based on the molecular ion at m/z 317. Its mass spectrum is illustrated in Appendix A: Figure A 13.

Peak 14 with retention time of 14.41 min, molecular ion at m/z 303 and UV-vis absorption at 281 nm (Table 5.2.3.4) was tentatively identified as taxifolin. RDA fragmentation of the C ring at positions 1 and 4 produces a $^{1,4}A^-$ fragment (trihydroxy benzene molecule) at m/z 125 (Miketova *et al*., 2000) as shown in Figure 5.2.3.8. Its mass spectrum is illustrated in Appendix A: Figure A 14.

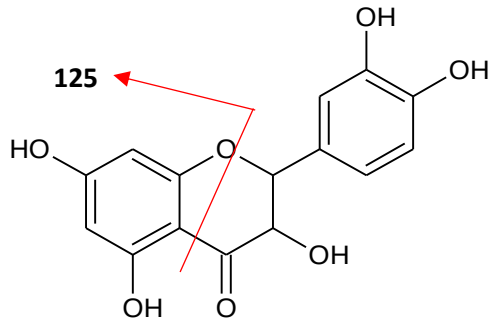


Figure 5.2.3.8 Proposed fragmentation pattern for taxifolin

Peak 15 with retention time of 11.38 min, molecular ion at m/z 269 and UV-vis absorption wavelength 348, 341 nm (Table 5.2.3.4 and Appendix A: Figure A 15, Appendix B) was identified as apigenin. The compound produced fragments at m/z 151 and 117. RDA fragmentation of the C ring at positions 1 and 3 produces a $^{1,3}A^-$ (m/z 151) and $^{1,3}B^-$ fragment (m/z 117) (Fabre *et al*., 2001) as shown in Figure 5.2.3.9. Its mass spectrum is illustrated in Appendix A: Figure A 15. Apigenin has been previously identified in Bambara groundnut seeds by Tsamo *et al*. (2018).

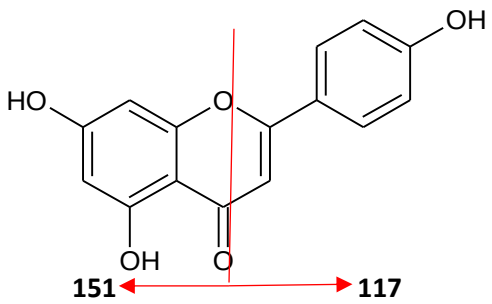


Figure 5.2.3.9*:* Proposed fragmentation pattern for apigenin

Peak 16 with retention time of 6.46 min, molecular ion at m/z 609 and UV-vis absorption wavelength at 348 and 275 nm (Table 5.2.3.4) was identified as hesperidin. It produced fragments at m/z 301, 286, 258 and 151. Loss of the diglucoside moiety (-308 amu) produces the m/z 301 fragment (Xu *et al*., 2009) which is hesperitin (the aglycone form of hesperidin) (Figure 5.2.3.10). Loss of a methyl group (CH_3, -15 amu) from the hesperitin aglycone produces the fragment at m/z 286, which can further lose a carbonyl group (CO, -28 amu) to produce the m/z 258 fragment. RDA cleavage of the C ring of the hesperitin aglycone at positions 1 and 3 produces a $^{1,3}A^-$ fragment at m/z 151. Its mass spectrum is illustrated in Appendix A: Figure A 16.

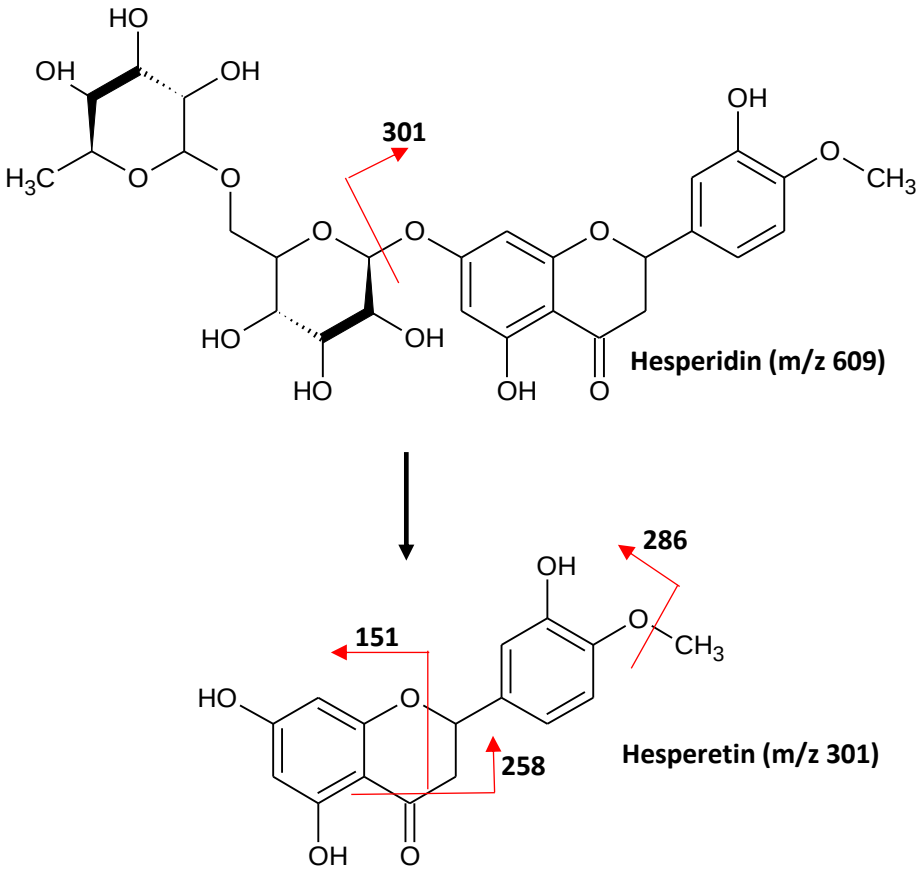


Figure 5.2.3.10: Proposed fragmentation pattern for hesperidin

The compound labelled peak 17 (t_R = 9.88, λ_{max} = 368, 341) with molecular ion at m/z 301 was identified as hesperetin (Table 5.2.3.4). Fragmentation produced ions at m/z 177 and 151. Cleavage of the bond joining the B ring and carbon 2 of the C ring leads to loss of the B ring moiety and produces the fragment at m/z 177. The RDA cleavage of the C ring of hesperitin at positions 1 and 3 produces the $^{1,3}A^-$ fragment at m/z 151 (Figure 5.2.3.11). Its mass spectrum is illustrated in Appendix A: Figure A 17.

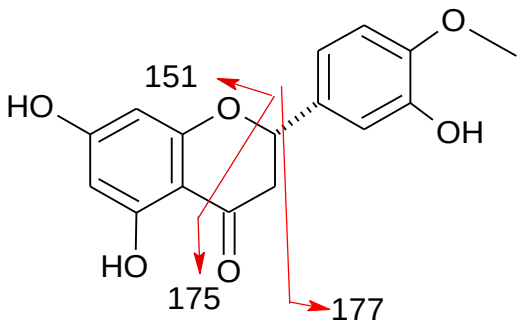


Figure 5.2.3.11*:* Proposed fragmentation for hesperetin (301 m/z)

Peak 18 (t_R = 11.21 min, λ_{max} = 348 and 281) with molecular ion at m/z 271 was identified as naringenin (Table 5.2.3.4). The compound produced fragments at m/z 151 and 119 which respectively correspond to the ^{1,3}A- and ^{1,3}B- fragments after RDA cleavage of the C ring at positions 1 and 3 (Fabre *et al*., 2000) as shown in Figure 5.2.3.12. Its mass spectrum is illustrated in Appendix A: Figure A 18.

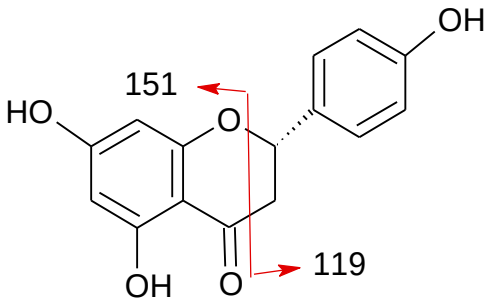


Figure 5.2.3.12: Proposed fragmentation for naringenin

Peak 19 with retention time of 13.49 min, molecular ion at m/z 287 and UV-vis absorption wavelength 281 nm (Table 5.2.3.4) was tentatively identified as eriodictyol. Fragmentation produced ionic fragments at m/z 151, 135 and 125. RDA cleavage of the C ring at positions 1 and 3 produces the ^{1,3}A- fragment (m/z 151) and the ^{1,3}B- fragment (m/z 135) (Miketova *et al*., 2000; Fabre *et al*., 2001) (Figure 5.2.3.13). RDA cleavage of the C ring at positions 1 and 4 produces a trihydroxy benzene moiety which is the ^{1,4}A- fragment at m/z 125 (Miketova *et al*., 2000). Its mass spectrum is illustrated in Appendix A: Figure A 19.

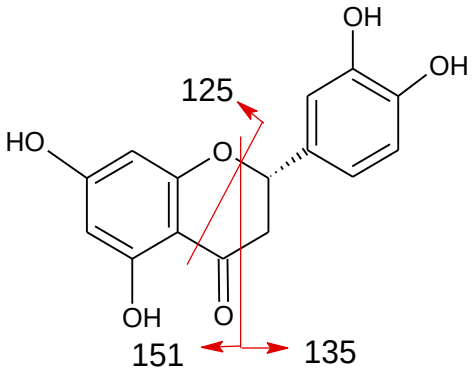


Figure 5.2.3.13*:* Proposed fragmentation for eriodictyol

Peak 20 with retention time of 6.20 min, molecular ion at m/z 449 and UV-vis absorption wavelength 282 nm (Table 5.2.3.4) was tentatively identified as eriodictyol-7-*O*-β-D-glucoside. Fragmentation produced ions at m/z 287 and 151. The m/z 287 fragement corresponds to the eriodictyol aglycone after loss of the glucose moiety (-162 amu) (Pereira *et al*., 2013). The ion at m/z 151 is the ^{1,3}A- fragment produced through the RDA cleavage of the C ring of the eriodictyol aglycone at positions 1 and 3 as shown in Figure 5.2.3.14 (Fabre *et al*., 2001). Its mass spectrum is illustrated in Appendix A: Figure A 20.

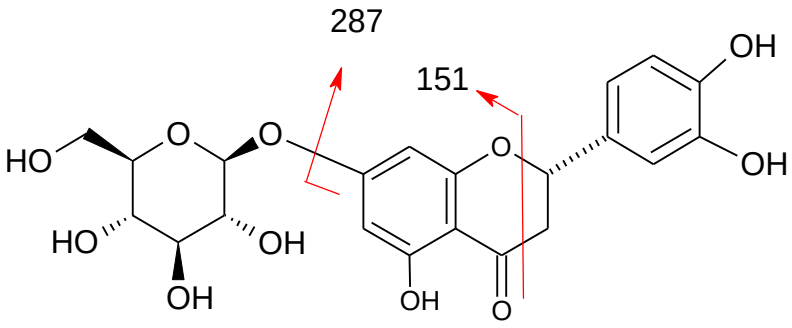


Figure 5.2.3.14*:* Proposed fragmentation pattern for eriodictyol-7-*O*-D-glucoside

5.2.3.5) Quantification of phenolic compounds from flours of microwaved Bambara groundnut seeds

Table 5.2.3.5: Effects of microwave pre-treatment on the flavonoid content (µg/g sample dry basis) of extracts from flours of Bambara groundnut seeds

	Raw Bambara groundnut seeds		Microwaved Bambara groundnut seeds							
	Brown	Red	Brown				Red			
			5 min/900 W	8 min/900 W	5 min/1200 W	8 min/1200 W	5 min/900 W	8 min/900 W	5 min/1200 W	8 min/1200 W
Catechin	$47.8^{b} \pm 4.6$	$29.3^{b} \pm 1.5$	$163.8^{ab} \pm 8.2$	$55.4^{ab} \pm 19.4$	$27.7^{a} \pm 10.0$	$324.5^{a} \pm 35.1$	$115.7^{ab} \pm 10.5$	$102.4^{ab} \pm 19.3$	$206.4^{a} \pm 38.2$	$218.2^{a} \pm 0.07$
Kaempferol	$10.3^{a} \pm 1.2$	$11.2^{a} \pm 4.0$	ND	$2.8^{ab} \pm 0.1$	$10.5^{ab} \pm 0.4$	$19.4^{b} \pm 2.4$	$17.6^{a} \pm 0.4$	$7.8^{ab} \pm 0.02$	$10.8^{ab} \pm 5.0$	$19.6^{b} \pm 2.9$
Quercetin-3-*O*-glucoside	$17.2^{a} \pm 1.6$	$117.3^{a} \pm 36.3$	$3.4^{a} \pm 0.1$	$9.3^{a} \pm 5.0$	$23.2^{a} \pm 2.6$	$15.7^{a} \pm 5.3$	$127.7^{a} \pm 14.2$	$127.8^{a} \pm 14.3$	$187.5^{a} \pm 7.0$	$178.6^{a} \pm 8.3$
Naringenin	ND	$3.9^{b} \pm 0.3$	ND	ND	ND	32.7 ± 0.6	$16.8^{b} \pm 0.4$	$7.8^{ab} \pm 0.5$	ND	$59.8^{a} \pm 1.8$
Hesperidin	$71.4^{a} \pm 1.6$	$364.5^{a} \pm 87.8$	ND	$33.2^{a} \pm 1.7$	$53.3^{a} \pm 9.0$	$41.9^{a} \pm 25.2$	$351.3^{a} \pm 11.1$	$217.3^{a} \pm 61.2$	$676.9^{a} \pm 92.9$	$750.1^{a} \pm 57.4$
Apigenin	$15.9^{a} \pm 6.7$	$22.9^{a} \pm 0.4$	ND	ND	$24.4^{ab} \pm 15.1$	$36.8^{ab} \pm 12.9$	$8.1^{a} \pm 0.5$	$9.6^{a} \pm 0.6$	$33.8^{ab} \pm 0.8$	$13.5^{ab} \pm 8.4$
Hesperetin	ND	$7.2^{a} \pm 0.1$	ND	ND	ND	ND	$39.2^{a} \pm 1.6$	$56.0^{a} \pm 6.4$	$181.4^{a} \pm 67.9$	$100.2^{a} \pm 47.0$
Total flavonoids	162.6	556.3	167.2	100.7	139.1	507.8	676.4	528.7	1296.8	1340.0

Values = mean ± SD

nd = not detected

a,b: values in a row with different superscripts differ significantly ($p \leq 0.05$) from each other

Table 5.2.3.6: Effects of microwave pre-treatment on the phenolic acid content (µg/g sample dry basis) of extracts from flours of Bambara groundnut seeds

	Raw Bambara groundnut seeds		Microwaved Bambara groundnut seeds							
	Brown	Red	Brown				Red			
			5 min/900 W	8 min/900 W	5 min/1200 W	8 min/1200 W	5 min/900 W	8 min/900 W	5 min/1200 W	8 min/1200 W
Gallic acid	ND	$3.6^{a} \pm 0.6$	ND	ND	ND	ND	$9.8^{a} \pm 0.6$	$2.4^{a} \pm 0.3$	$8.6^{a} \pm 2.3$	$14.6^{a} \pm 6.8$
Protocatechuic acid	$3.9^{a} \pm 0.4$	$24.5^{a} \pm 3.1$	ND	ND	$17.7^{a} \pm 6.5$	ND	$25.2^{a} \pm 2.3$	$19.8^{a} \pm 13.2$	$63.4^{a} \pm 13.4$	$83.5^{a} \pm 10.6$
Total phenolic acids	3.9	28.1	ND	ND	17.7	ND	35.0	22.2	72.0	98.1

Values = mean ± SD

ND = not detected

a,b: values in a row with different superscripts differ significantly ($p \leq 0.05$) from each other

Table 5.2.3.5 shows the effect of microwave pre-treatment on the flavonoid content of phenolic extracts from flours of Bambara groundnut seeds. There were varietal differences in the types and levels of flavonoids in the two Bambara groundnut types. For the raw seeds, catechin and hesperidin were the major flavonoids in the brown type while quercetin-3-*O*-glucoside and hesperidin were the major flavonoids in the red type. Naringenin and hesperetin were not detected in the brown type. Overall, the raw red Bambara groundnut type contained a higher level of total flavonoids than the brown type.

For the two Bambara groundnut types, seeds microwaved at the higher power of 1200 W generally had higher levels of flavonoids than seeds microwaved at 900 W. This means that the flavonoids (e.g. catechin, quercetin-3-*O*-glucoside and hesperidin) were rendered more extractable when microwaved at a higher power of 1200 W. The higher microwave power may facilitate the thermal hydrolysis of these compounds from cell wall components of Bambara groundnut seeds (Žilic *et al*., 2013) and thus increase their extractability. This observation also suggests that these compounds are relatively thermally stable at the high microwave power of 1200 W.

For the brown Bambara groundnut seeds, microwaving time generally increased extractability of flavonoids. This is evident from the observation that microwaving for 8 min at either 900 W or 1200 W mostly led to increases in levels of specific flavonoids and total flavonoids than for the shorter duration of 5 min. However, the effect of microwaving time seemed to be different for the red Bambara groundnut seeds where there were mostly decreases in levels of flavonoids after microwaving for 8 min compared to 5 min or the flavonoid levels did not change significantly. This suggests that variety may play a role in determining the effect of microwaving time on flavonoids in Bambara groundnut. This hypothesis needs to be tested using a larger number of Bambara groundnut varieties.

Table 5.2.3.6 show the effect of microwave pre-treatment on the phenolic acid content of phenolic extracts from flours of Bambara groundnut seeds. It is important to note that in this work, only a small number of phenolic acids were detected in the Bambara groundnut samples among which only two could be quantified. This is because the phenolic extraction methodology applied is specifically designed for optimum extraction of flavonoids. Therefore, conclusions about the effect of microwave pre-treatment on phenolic acids in this research can only be made to a limited extent. In agreement with the results on flavonoid levels, varietal differences were also found in the levels

of phenolic acids in the two Bambara groundnut types. Gallic acid and protocatechuic acid could be detected and quantified in all the red Bambara groundnut samples. In contrast, apart from protocatechuic acid in the 5 min/1200 W treated seeds, no phenolic acids could be identified and quantified in any of the brown Bambara groundnut seeds. The results also appear to indicate that overall, microwave processing made phenolic acids more extractable. As can be seen in Table 5.2.3.3, total phenolic acids in the microwave treated brown Bambara groundnut seeds were much higher than in the raw. This was also predominantly the case with the red Bambara groundnut seeds, the exception being the 8 min/900 W treated sample. The increased extractability may be due to release of phenolic acids from bound forms as a result of the hydrothermal microwave treatment.

5.2.3.6) Inhibitory effects of extracts from raw and microwaved Bambara groundnut seeds against oxidative DNA damage

Figure 5.2.3.15 shows the effect of extracts from raw and microwaved brown and red Bambara groundnut seeds on the inhibition of peroxyl radical- induced pBR322 vector DNA damage. AAPH radicals react with nucleotide bases in DNA molecules which leads to damage of the DNA (Madhujith, Amarowicz & Shahidi, 2004). The oxidative reaction with AAPH radicals leads to single strand (circular) or double strand (linear) breaks in the pBR322 DNA vector (Wei, Zhou, Cai, Yang & Liu, 2006). The undamaged supercoiled form of DNA and the damaged circular or linear forms have differing mobility during electrophoresis (Damiani, Kalinska, Canapa, Canestrari, Wozniak, Olmo & Greci, 2000).

The negative control in Lane 1 (DNA + H_2O), showed the DNA predominantly in the supercoiled form (Figure 5.2.3.15). Lane 1 also showed a faint DNA band in the circular form. This may suggest that either the DNA may have been slightly damaged by the hydrochloric acid (Sasaki, Adachi, Yamamoto, Murakami, Tanaka & Takahashi, 1988) used in the solvent or some of the DNA as supplied by the manufacturer was already in the damaged circular form. According to Wei *et al*. (2006) and Adarkwah-Yiadom and Duodu (2017), depending on the batch, some plasmid DNA as supplied by the manufacturer may have some damaged forms. The positive control in Lane 2 (DNA + H_2O + AAPH) showed the DNA predominantly in the circular (single strand break) form as indicated by the high intensity band. A faint band of supercoiled DNA could

still be seen in Lane 2 which suggests that the DNA was not completely damaged by the AAPH radicals (Adarkwah-Yiadom *et al*., 2017).

Lanes 3 to 12 which contained plasmid DNA, AAPH and phenolic extracts from the raw and microwave-treated seeds showed the DNA predominantly in the supercoiled form with high band intensity. This is an indication that the phenolic extracts exerted protective effects against damage of the DNA by the AAPH radicals. Phenolic compounds in the extracts could scavenge the AAPH radicals and thus prevent them from reacting with the DNA. Similar protective effects of phenolic extracts from whole red, brown, black and white beans and hulls (Madhujith *et al*., 2004) and from raw and microwaved cowpeas (Mokatso, 2017) against AAPH radical-induced oxidative DNA damage have been reported.

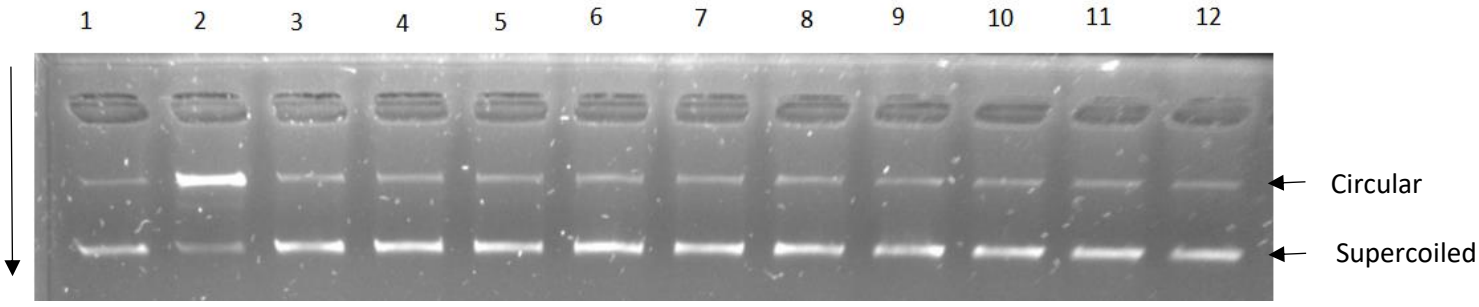


Figure 5.2.3.15: Agarose gel electrophoretogram showing the inhibitory effects of phenolic extracts from raw and microwaved Bambara groundnut seeds against AAPH radical-induced oxidative pBR322 vector DNA damage

Lane 1: negative control (DNA + H_2O)
Lane 2: positive control (DNA + H_2O + AAPH)
Lane 3: DNA + extract from raw brown Bambara groundnut seeds + AAPH
Lane 4: DNA + extract from raw red Bambara groundnut seeds + AAPH
Lane 5: DNA + extract from brown Bambara groundnut seeds microwaved at 900 W for 5 minutes + AAPH
Lane 6: DNA + extract from brown Bambara groundnut seeds microwaved at 900 W for 8 minutes + AAPH
Lane 7: DNA + extract from brown Bambara groundnut seeds microwaved at 1200 W for 5 minutes + AAPH
Lane 8: DNA + extract from brown Bambara groundnut seeds microwaved at 1200 W for 8 minutes + AAPH
Lane 9: DNA + extract from red Bambara groundnut seeds microwaved at 900 W for 5 minutes + AAPH
Lane 10: DNA + extract from red Bambara groundnut seeds microwaved at 900 W for 8 minutes + AAPH
Lane 11: DNA + extract from red Bambara groundnut seeds microwaved at 1200 W for 5 minutes + AAPH
Lane 12: DNA + extract from red Bambara groundnut seeds microwaved at 1200 W for 8 minutes + AAPH. Downward arrow shows direction of DNA electrophoretic migration.

5.2.4) Conclusion

Microwave pre-treatment of Bambara groundnut seeds can lead to increases or decreases in extractability of phenolic compounds and influence antioxidant activity depending on Bambara groundnut type, microwave power and time. The phenolic compounds seem to have thermal stability during the microwave treatment under the conditions used. Thus, microwave pre-treatment holds promise for the production of Bambara groundnut flours with enhanced health promoting properties.

Chapter 6: General discussion

6.1. Discussion of research methodologies used

In this research study, pigmented (brown and red) Bambara groundnut seeds were used due to expected higher content of phenolic compounds. Seed coat colour is said to be one of the factors that affects the phenolic content of legumes. Generally, it is considered that darker colored legumes contain higher levels of phenolic compounds than lighter colored ones (Ojwang, 2012).

The processing parameters used in this study namely, microwave frequency of 2.45 MHz, microwave times of 5 and 8 min and microwave powers of 900 W and 1200 W were chosen based on the use of similar parameters by Yoshida and Kajimoto (1988) for soybean and Mokatso (2017) for cowpea. Yoshida and Kajimoto (1988) found that a soybean internal temperature of 135ºC with a 6 min and 500 W microwave treatment with microwave frequency of 2.45 MHz caused complete trypsin inhibitor inactivation. Thus to ensure complete trypsin inhibitor inactivation Bambara groundnut samples were microwaved at an air temperature of 130ºC as measured by the internal thermometer of the microwave system used in this study.

A fluidized bed microwave system was used in this study due to it offering several advantages including high heat and mass transfer, good temperature control, uniform temperature and drying capacity (Cil & Topuz, 2010). However, the disadvantage with the use of the closed fluidized bed microwave system in this study was the difficulty in obtaining an accurate measurement of the internal temperature of the Bambara groundnut seeds during microwaving without thermocouples. Thermocouples allow for accurate measurement of the internal temperature of seeds. Notwithstanding this limitation, there is a fair degree of confidence that a high enough internal temperature of the Bambara groundnut seeds was attained. This is due to the fact that Mokatso (2017) showed by using a non-fluidized bed microwave system that an internal temperature and maximum surface temperature of 140ºC and higher in cowpeas could be achieved with wattages ranging from 800 to 1200 W for 5 min.

After microwave treatment, Bambara groundnut seeds required an additional drying step at 40ºC for 24 hours to decrease the moisture content to below 10%. The microwave treatment was unable to decrease the moisture content below the required 10% possibly due to the relatively high water holding properties of the Bambara groundnut seeds perhaps due to their large size as well as their dietary fibre content as has been reported by Tosh and Yada (2010). Maphosa and Jideani (2016) showed that dietary fibre components from brown and red Bambara groundnut seeds had notable water holding capacities of 2.6 g water /g sample and 2.41 g water/g sample respectively.

The cooking time of Bambara groundnut seeds was determined using a manually operated Mattson bean cooker. The major problem with this version of the Mattson bean cooker was the need for constant and continuous observation for the recording of the dropping of the plungers which may take up to 6 hrs depending on the sample under study. In this case, a more modern version in the form of an automated Mattson bean cooker would have been advantageous whereby the plunger drops are recorded via a computer or plotter (Wood, 2017). In the absence of such an automated version of the cooker, simple and practical steps such as careful scheduling of cooking time experiments were taken to enable the critical observation required for accurate recording of the cooking time.

As indicated by Proctor and Watts (1987), another problem with the manually operated Mattson bean cooker is the difficulty in obtaining consistency in orientation of the beans under the plunger for determination of the cooking time as well as the force and weight of the plunger which inevitably introduces variability. In relation to this, it was observed in this research that the plungers (49.8 g each) were sometimes unable to pierce the seedcoat of the pre-soaked Bambara groundnut seeds possibly due to the hard-to-cook defect as evidenced by the long cooking time of 6 hours in some instances. Texture analysis could be used to support the cooking time determined by the Mattson bean cooker by confirming the softness of the Bambara groundnut seeds.

A Physica MCR 101 Rheometer was used to determine the effect of microwave treatment on the flour components such as starch and protein and its effect on viscosity. The preparation of sample is very important in order to obtain accurate results. The formation of clumps may result in variablity in the results. For this reason, during sample preparation, the flour and water was carefully stirred to ensure a uniform distribution and to prevent or reduce clump formation.

The effect of microwave treatment on the thermal properties of flours from pre-soaked Bambara groundnut seeds were determined using Differential scanning calorimetry (DSC). DSC has the disadvantage of being very sensitive to enviromental factors such as temperature of surroundings and cleanliness of pans among other factors such as the operator's experience with the instrument which may result in variation in data (Kolbe, Wilson & Hartel, 1999). To ensure accurate results, clean pans were used during each run and environmental temperature in the laboratory was maintained constant at ambient.

To observe the structural organelles and their interaction in flours, a polarized light microscope was used as well as iodine staining to observe starch granules. Polarized light microscopy provides a way of visualizing the "maltese cross" birefringence pattern of intact starch granules. The iodine staining of starch allows for the visualization of individual swollen starch granules (Flint, 1982). Staining with iodine leads to formation of a starch-iodine complex which is black-blue in colour. It would have been beneficial to use protein staining with Safarin-O with confocal laser scanning microscopy (CLSM) in this study. CLSM is a useful tool for the observation of the microstructure of samples and allows for the detailed observation of the interaction between proteins and starch (Jekle & Becker, 2011).

The total phenolic content (TPC) was determined using the Folin-Ciocalteu method. This method was originally designed for protein analysis but was later adapted to measure phenolic content in wine by Singleton, Orthofer and Lamuela-Ravenos (1999). The method measures the reducing power of phenolic hydroxyl groups (reducing agent) in a sample in a reduction-oxidation reaction with the Folin-Ciocalteu phenol reagent (oxidising agent) (Macdonald-Wicks *et al*., 2006). In effect, the Folin-Ciocalteu reagent can react with any reducing substances in the sample be they phenolic or not. The implication of this is that the Folin-Ciocalteu method is not specific for phenolic compounds and suffers from interference from non-phenolic reducing substances such as peptides, reducing sugars, ascorbate and some metal ions (Macdonald-Wicks *et al*., 2006). Notwithstanding this demerit, the Folin-Ciocalteu assay is convenient, reproducible and simple (Macdonald-Wicks *et al*., 2006) and finds wide application particularly in screening experiments to obtain an indication of the gross phenolic content of a sample.

An extraction regime for phenolic compounds described by Ojwang (2012) involving purification using C18 solid phase extraction cartridges was used in this research for LC-MS analysis. This extraction method allowed for the use of different solvent systems for separation and purification of different fractions of phenolic compounds during solid phase extraction. However, a major limitation with this extraction method is that it is designed for flavonoids and therefore was limited in its ability to extract phenolic acids. This was clearly apparent in this research as an overwhelming majority of the phenolic compounds identified and quantified in the extracts were flavonoids. For future research, a separate extraction regime for phenolic acids involving extraction of free, soluble-esterified and insoluble-bound phenolic acids as described by Krygier, Sosulski and Hogge, (1982) and Moore, Hao, Zhou, Luther, Costa and Yu (2005) could be used in addition to the solid phase extraction methods for flavonoids used in this research.

Due to the non-specificity of the Folin-Ciocalteu method and its inability to identify phenolic compounds, it was necessary to use a chromatographic method for the characterisation and quantification of phenolic compounds in the Bambara groundnut samples. The LC-MS system consisting of an ultra performance liquid chromatograph (UPLC) coupled with a Quadrupole Time of-Flight (QToF) mass spectrometer with electrospray ionisation in negative mode proved invaluable in this regard. The negative mode electrospray ionisation method used for LC-MS provides efficient ionization and has high sensitivity and selectivity towards biologically significant compounds (Challamalla, Ghosh, Parthiban, Rao & Banji, 2012). Overall, the LC-MS system enables identification and quantification of phenolic compounds and gives information about the compound molecular structure from the molecular mass obtained from the mass-to-charge (m/z) ratio (Mojzer, Hrncic, Skerget, Knez & Bren, 2016).

With regard to indication of health-promoting properties, a disadvantage of the ABTS radical scavenging assay is that the ABTS radical is unknown to biological systems and therefore has limited biological relevance (Shalaby & Shanab, 2013). Therefore in this research, inhibition of oxidative DNA damage was included as a more biologically relevant assay to indicate health-promoting properties. Oxidative damage to DNA is considered to be a key feature of the development of non-communicable diseases such as cancer and conditions such as chronic inflammation and ageing (Harsha & Anilakumar, 2014). DNA damage occurs as a result of reaction between AAPH radicals and DNA nucleotide bases such as guanine to produce 8-

hydroxyguanine or dehydroxy guanosine and sugar residues (Valavanidis, Vlachogianni & Fiotakis, 2009). Such a reaction converts the undamaged supercoiled DNA into damaged forms that may be open-circular, linear strands or DNA fragments. The undamaged supercoiled DNA and the damaged forms have different electrophoretic mobility and can therefore be separated by gel elctrophoresis. The inhibition of oxidative DNA damage assay tests the ability of phenolic extracts to inhibit the AAPH radical-induced DNA damage. For future research, assays that measure the ability of phenolic extracts to inhibit or scavenge more biologically relevant radical species such as nitric oxide and hydroxyl radicals (Valavanidis, et al., 2009; Harsha & Anilakumar, 2014) could be included.

6.2. Discussion of key research findings

In this study it was shown that microwave pre-treatment of pre-soaked Bambara groundnut seeds caused a significant decrease in cooking time. Generally, the cooking time decreased with an increase in microwave power and time. The decrease in cooking time could be due to the microwave-induced partial starch gelatinization as confirmed by the deformation and aggregation of intact starch granules as seen under light microscope imaging and iodine stained microscope images and the decrease in enthalpy. Although pectic substances were not measured during this study, decreases in cooking time could also be due to depolymerization reactions such as β-elimination of pectic substances (Van Buggenhout, Sila, Duvetter, Van Loey & Hendrickx, 2009) (Figure 6.2.1) in the paranchyma cells of the cotyledon of the Bambara groundnut seeds leading to solubilization of pectin and thus decrease in cooking time.

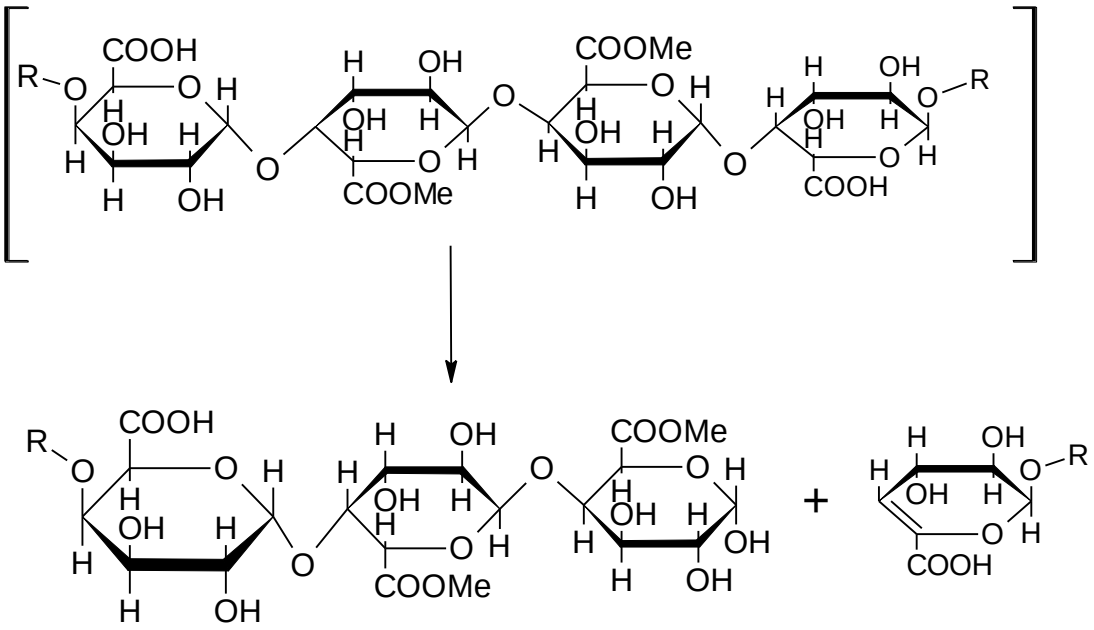


Figure 6.2.1: β-elimination reaction in the depolymerization of pectin during microwave treatment

Table 6.2.1: Summary of the effect of microwave pre-treatment of Bambara groundnut seeds on the functional properties of the resultant flours

	Brown 5 min/ 900 W	**Brown 8 min/ 900 W**	**Brown 5 min/ 1200 W**	**Brown 8 min/ 1200 W**
Pasting viscosity	Decrease	Decrease	Decrease	Decrease
Nitrogen solubility index	Decrease	Decrease	Decrease	Decrease
Water solubilty index	Decrease	Decrease	Decrease	Decrease
Enthalpy (Δ H)	Decrease	Decrease	Decrease	Decrease
	Red 5 min/ 900 W	**Red 8 min/ 900 W**	**Red 5 min/ 1200 W**	**Red 8 min/ 1200 W**
Pasting viscosity	Decrease	Decrease	Decrease	Decrease
Nitrogen solubility index	Decrease	Decrease	Decrease	Decrease
Water solubility index	Decrease	Decrease	Decrease	Decrease
Enthalpy (Δ H)	Decrease	Decrease	Decrease	Decrease

Table 6.2.1 shows a summary of the effects of microwave pre-treatment on the functional properties of flour from Bambara groundnut seeds. Microwave pre-treatment caused a decrease in all of the functional properties of the flour as compared to the raw untreated flour. A hypothesis about formation of a hydrophobic protein layer around starch granules as shown in Figure 6.2.2, could be proposed to account for the observed decreases in functional properties of the flours after microwave pre-treatment of the Bambara groundnut seeds. The raw Bambara groundnut seeds contain intact raw starch granules as seen by the "maltese crosses" as well as individual protein bodies. After microwave pre-treatment, the pre-gelatinized starch granules, with deformed "maltese crosses", appear to be embedded in a protein matrix. This protein matrix is actually a hydrophobic protein layer formed by the thermal denaturation of the protein by microwave treatment resulting in unfolding and exposure of hydrophobic sites of the protein and reaction with starch. The hydrophobic protein layer surrounds the partially gelatinized starch granules and this arrangement has increased hydrophobicity, decreased protein solubility and decreased water absorption by the partially gelatinized starch granules. Overall, this results in a decrease in WSI and NSI and lowered pasting viscosity.

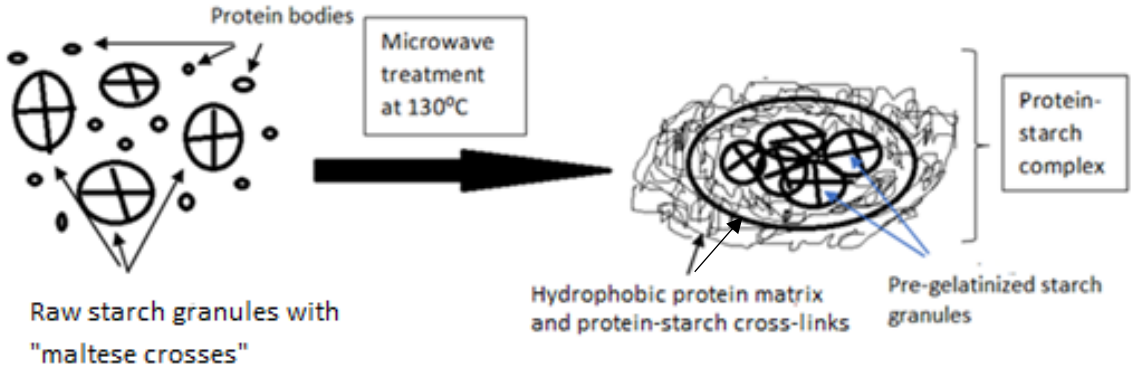


Figure 6.2.2: Basic representation of the formation of the protein-starch matrix (Adapted from Ogundele, 2016)

However, an interesting finding was that the highest microwave power (1200 W) treatments for brown Bambara groundnuts seeds resulted in a higher pasting viscosity, WSI and NSI for the resulting flours as compared to the 900 W treatments. The higher pasting viscosities of the 1200 W treatments could be explained by looking at the structure of the flour under light microscope. Iodine stained light microscopy images such as in Figure 6.2.3 which show areas of the flour that did not form the starch-embedded protein matrix. The light purple areas show the amylopectin (yellow arrow) while the blue show leaching of amylose from the starch granules (red arrow). The leaching of amylose indicates the gelatinization of starch. The higher WSI and NSI as compared to the 900 W treatment also indicates that a hydrophobic protein layer did not form which allows water inside the granules and this may be the reason for the higher pasting viscosity of the flour from the 1200 W treatment as compared to the 900 W treatment of brown Bambara groundnut seeds.

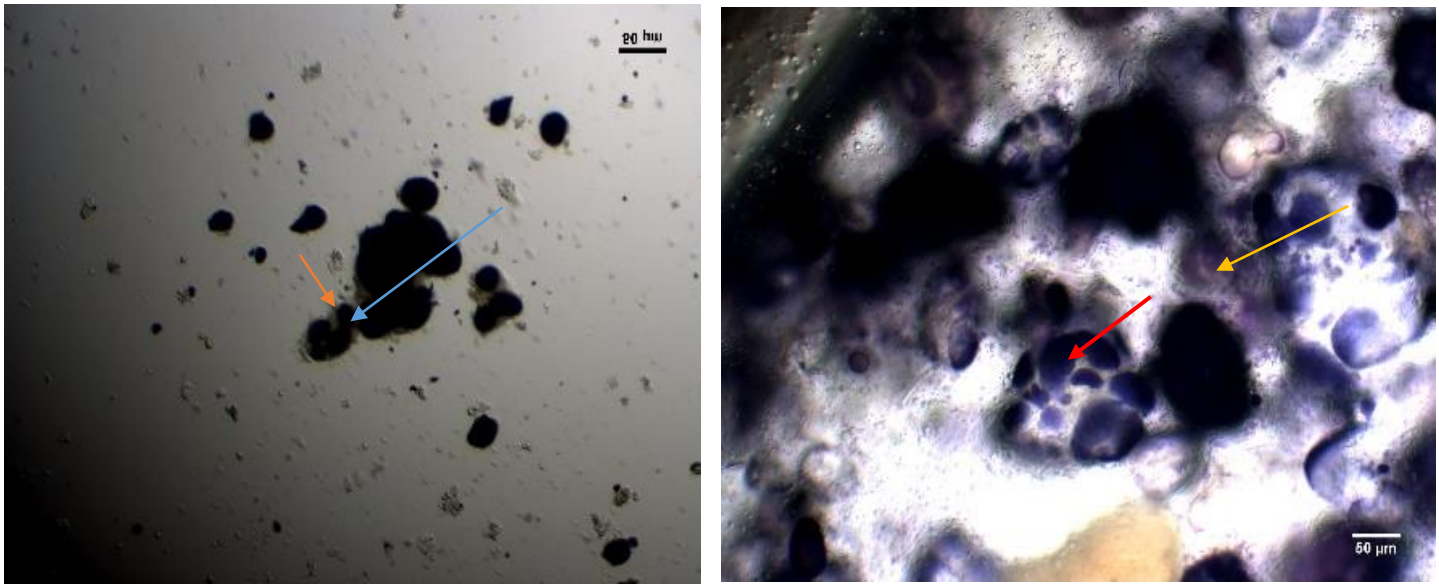


Figure 6.2.3: Light microscope images of iodine stained flours from brown Bambara groundnut seeds microwaved for 5 minutes at 900 W (right) and 5 minutes at 1200 W (left). Bar = 50 µm. Orange arrow indicate the protein layer around aggregated partially gelatinized starch (blue arrow). The yellow arrow indicates amylopectin while the red arrow indicates the leaching of amylose.

Table 6.2.2: Effect of microwave pre-treatment on the presence of flavones and flavanones in Bambara groundnut seeds

	Raw samples		Microwaved samples							
	Brown	**Red**	**Brown 5 min/900 W**	**Brown 8 min/900 W**	**Brown 5 min/1200 W**	**Brown 8 min/1200 W**	**Red 5 min/900 W**	**Red 8 min/900 W**	**Red 5 min/1200 W**	**Red 8 min/1200 W**
Flavones										
Apigenin	+	+	–	–	+	+	+	+	+	+
Flavanones										
Naringenin	–	+	–	–	–	+	+	+	–	+
Hesperidin	+	+	–	+	+	+	+	+	+	+
Hesperetin	–	+	–	–	–	–	+	+	+	+
Eriodictyol	+	+	–	–	–	–	–	–	–	–
Eriodictyol-7-*O*-β-D-glucoside	+	+	–	+	+	–	+	+	+	+

Table 6.2.3: Effect of microwave pre-treatment on the presence of flavan-3-ols and flavonols in Bambara groundnut seeds

	Raw samples		Microwaved samples							
	Brown	**Red**	**Brown 5 min/900 W**	**Brown 8 min/900 W**	**Brown 5 min/1200 W**	**Brown 8 min/1200 W**	**Red 5 min/900 W**	**Red 8 min/900 W**	**Red 5 min/1200 W**	**Red 8 min/1200 W**
Flavan-3-ols										
Catechin	+	+	–	+	+	+	+	+	+	+
Catechin-5-*O*-glucoside	+	+	–	+	+	+	+	+	+	+
Procyanidin B2 dimer	+	+	–	+	+	+	+	+	+	+
Procyanidin C2 trimer	+	+	–	+	+	+	+	+	+	–
Flavonols										
Kaempferol	+	+	–	+	+	+	+	+	+	+
Quercetin-3-*O*-glucoside	+	+		+	+	+	+	+	+	+
Myricetin	+	+	–	–	+	+	+	–	+	+
Flavononol										
Taxifolin	+	+	–	+	+	+	+	+	+	+

Tables 6.2.2 and 6.2.3 show an overview of the effects of microwave pre-treatment on the presence of flavonoid compounds in the Bambara groundnut seeds. The presence or absence of any flavonoid compound in the Bambara groundnut seeds after microwave pre-treatment could potentially provide an indication of its thermal stability. Two main trends can be observed in Tables 6.2.2 and 6.2.3. Firstly, for brown Bambara groundnut seeds, while flavones and flavanones were absent in most of the microwaved samples (Table 6.2.2), flavan-3-ols, flavonols and a flavononol were present in majority of the microwaved samples (Table 6.2.3). This suggests that flavan-3-ols, flavonols and the flavononol were more thermally stable in brown Bambara groundnut seeds than flavones and flavanones during microwave pre-treatment. The second observable trend is that the presence of flavonoids could be detected in a much greater number of microwaved red Bambara groundnut seeds compared to the microwaved brown Bambara groundnut seeds. This suggests a potential varietal effect with flavonoids in red Bambara groundnut seeds being relatively more thermally stable during microwave pre-treatment than in brown Bambara groundnut seeds. This potential varietal effect also seems evident in the effect of microwave pre-treatment on changes in phenolic content (i.e. concentration) and antioxidant activity of the Bambara groundnut seeds.

Table 6.2.4 shows a summary of the effect of microwave pre-treatment on the phenolic content and antioxidant properties of Bambara groundnut seeds. The table shows that microwave pre-treatment can lead to either increases or decreases in phenolic content and antioxidant activity. These increases or decreases do not appear to follow a consistent trend in response to microwave time and power as independent variables. It is important to provide some clarification about the use of the terms “increase” and “decrease” to describe how phenolic content and antioxidant activity are affected by microwave pre-treatment. As explained by Duodu (2014), the terms “increase” and “decrease” could be misleading. An “increase” in phenolics may suggest that matter is being created while a “decrease” may suggest that matter is being destroyed. These are contrary to the law of conservation of mass (Duodu, 2014) which states that matter can neither be created nor destroyed. Although the terms “increases” and “decreases” in phenolics may still be used, it is important to note that these are actually referring to phenolics becoming either more extractable (in the case of “increases”) or less extractable (in the case of “decreases”)

Table 6.2.4: Summary of the effect of microwave pre-treatment on the phenolic content and antioxidant properties of Bambara groundnut seeds

Analyses	Effect of microwave pre-treatment							
	Brown 5 min/ 900 W	**% Effect**	**Brown 8 min/ 900 W**	**% Effect**	**Brown 5 min/ 1200 W**	**% Effect**	**Brown 8 min/ 1200 W**	**% Effect**
Total phenolic acid content	Decrease	100	Decrease	32.9	Increase	112	Decrease	100
Total flavonoid content	Increase	2.82	Decrease	26.5	Decrease	0.08	Increase	207
Total phenolic content (TPC)	Increase	2.63	Increase	5.26	Decrease	14.5	Decrease	13.2
ABTS radical scavenging	Decrease	15.9	Decrease	19.0	Decrease	34.9	Decrease	34.2
	Red 5 min/ 900 W	**% Effect**	**Red 8 min/ 900 W**	**% Effect**	**Red 5 min/ 1200 W**	**% Effect**	**Red 8 min/ 1200 W**	**% Effect**
Total phenolic acids	Increase	101	Decrease	21.8	Increase	235	Increase	252
Total flavonoids content	Increase	21.3	Decrease	12.1	Increase	133	Increase	141
Total phenolic content (TPC)	Increase	29.7	Increase	35.4	Increase	27.6	Increase	33.3
ABTS radical scavenging	Increase	39.1	Increase	33.7	Increase	21.8	Increase	37.0

In summary, any increases in phenolic content and antioxidant activity may be due to the release of phenolic compounds from substances they may be bound to as a result of the microwave pre-treatment. Quercetin-3-*O*-glucoside and naringenin are used here as examples to demonstrate this. Figure 6.2.5 shows the different ways in which quercetin-3-*O*-glucoside could be bound to proteins in the Bambara groundnut seeds through either hydrophobic interactions, hydrogen bonding or ionic bonding. Figure 6.2.6 shows how naringenin could be bound to hemicellulose or pectic substances through ether and ester bonds. The protein, hemicellulose or pectic substances may protect the phenolic compounds from thermal degradation, thus conferring thermal stability (Baxter, Lilley, Haslam & Williamson, 1997; He, Chen & Moser, 2015). The hydrothermal microwave pre-treatment could however bring about hydrolysis and breakage of the hydrophobic interactions, hydrogen, ionic, ether and ester bonds which then releases the phenolic compounds, thus making them more extractable.

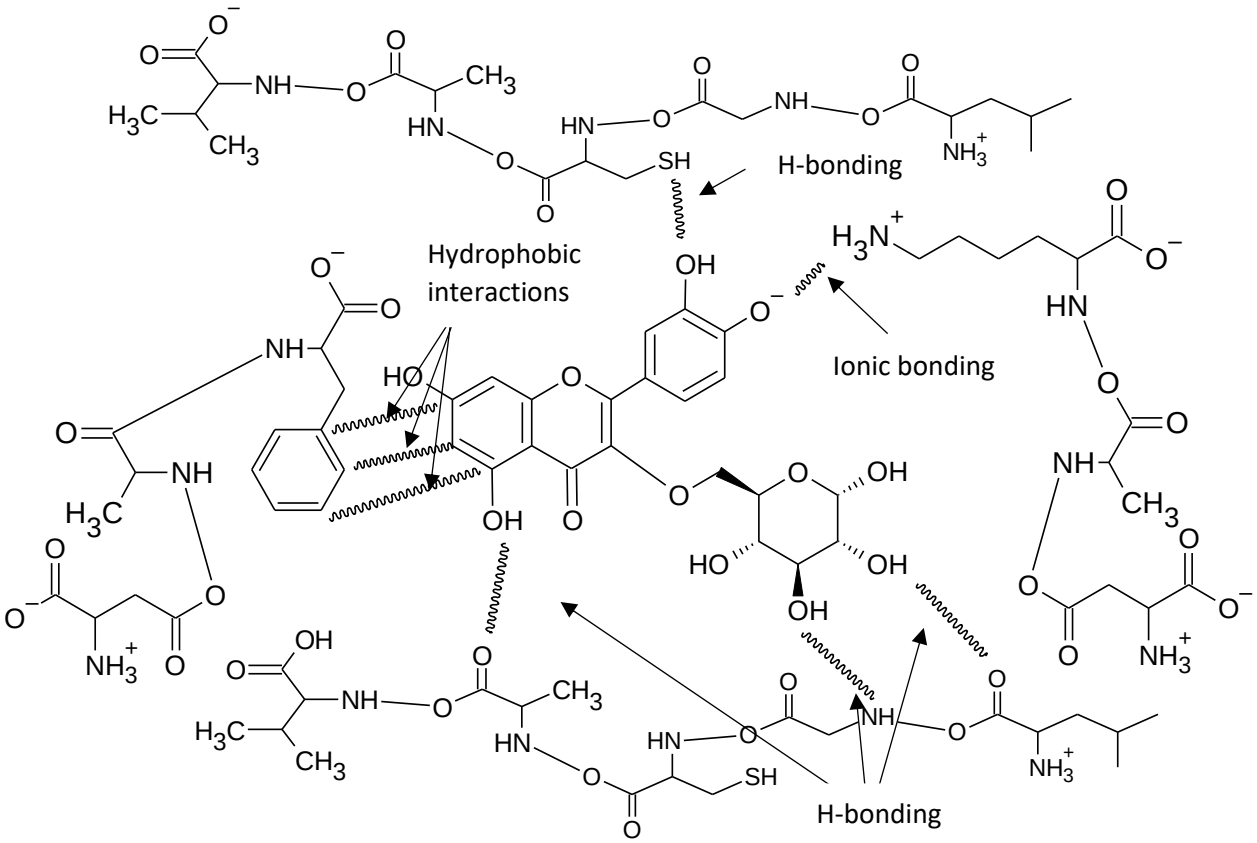


Figure 6.2.4*:* An illustration of how quercetin-3-*O*-glucoside could be bound to proteins via hydrophobic interactions, hydrogen bonding and ionic bonds

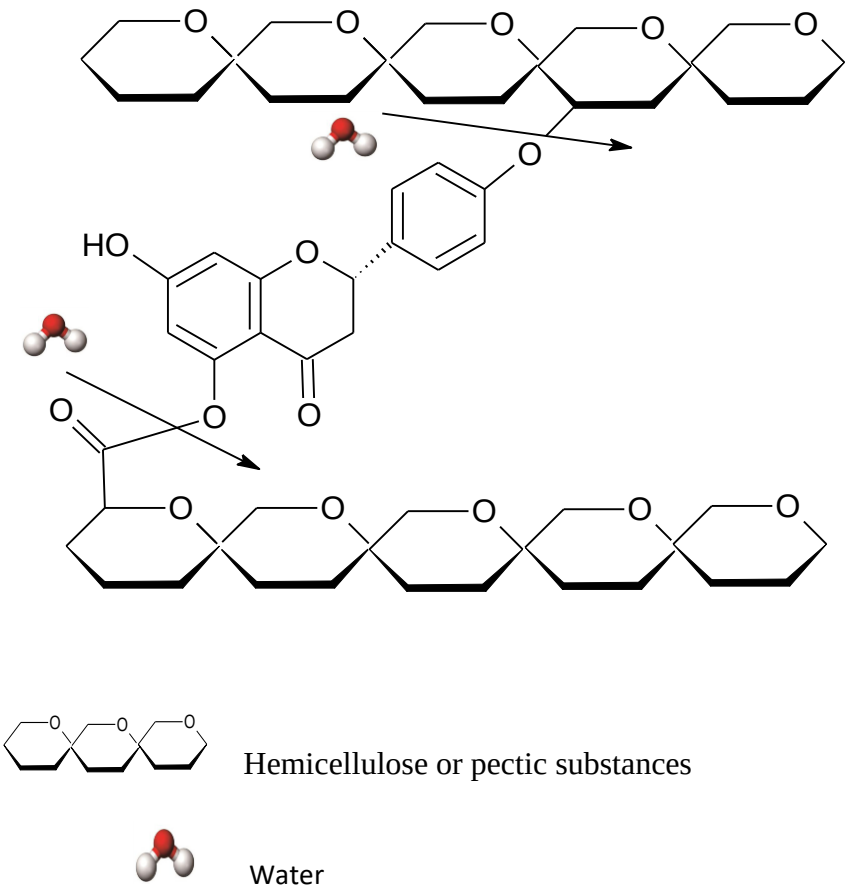


Figure 6.2.5: Representation of microwave-induced hydrolysis of ether and ester bonds to release naringenin from hemicelluloses or pectic substances

On the other hand, decreases in phenolic content and antioxidant activity may be attributed to either microwave-induced degradation of phenolic compounds or promotion of their crosslinking with other macromolecules such as proteins which then makes them less extractable. Phenolic compounds can be degraded in the presence of heat and oxygen via oxidation reactions. This is illustrated in Figure 6.2.7 using kaempferol as an example. Oxidative degradation of kaempferol during microwave pre-treatment can lead to formation of various quinone-like intermediates which can be subsequently degraded into various non-phenolic reaction products or form polymerisation products and melanins (Rohn, 2014). Phenolic compounds such as protocatechuic acid, after oxidation, may cross-link with proteins via the ε-amino group from lysine or thiol groups (Rohn, 2014) as shown in Figure 6.2.8 and this then decreases extractability of the phenolic compound.

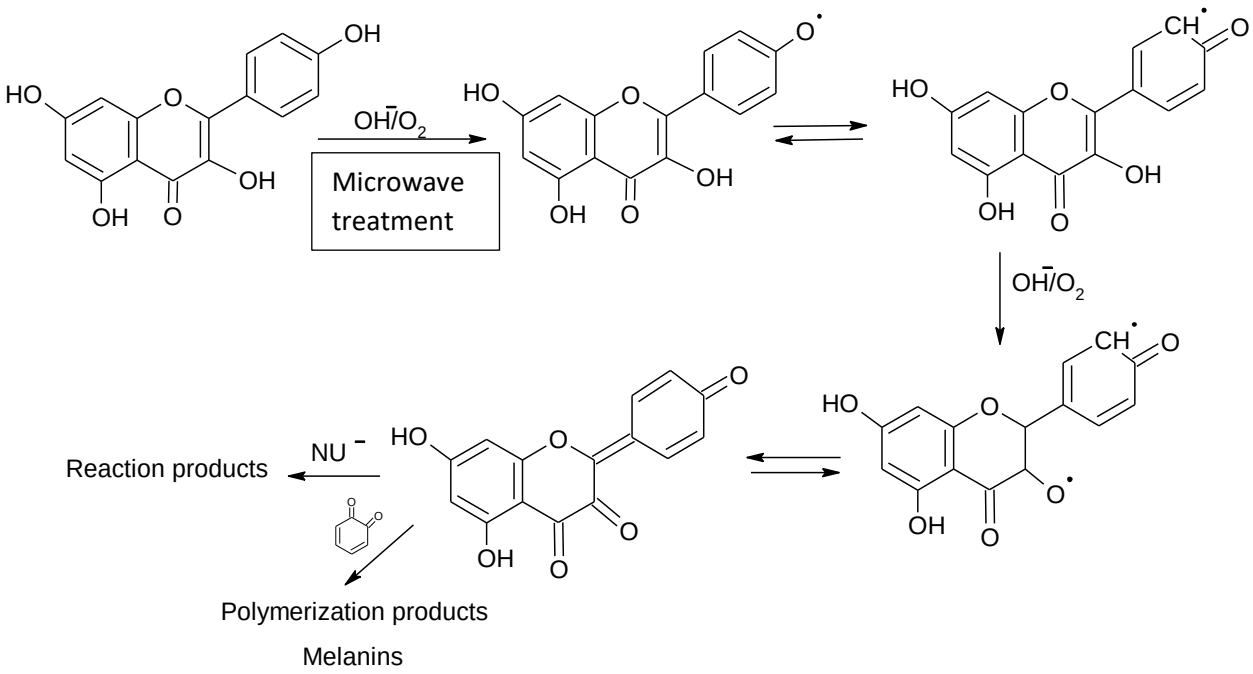


Figure 6.2.6: Microwave-induced oxidation of kaempferol

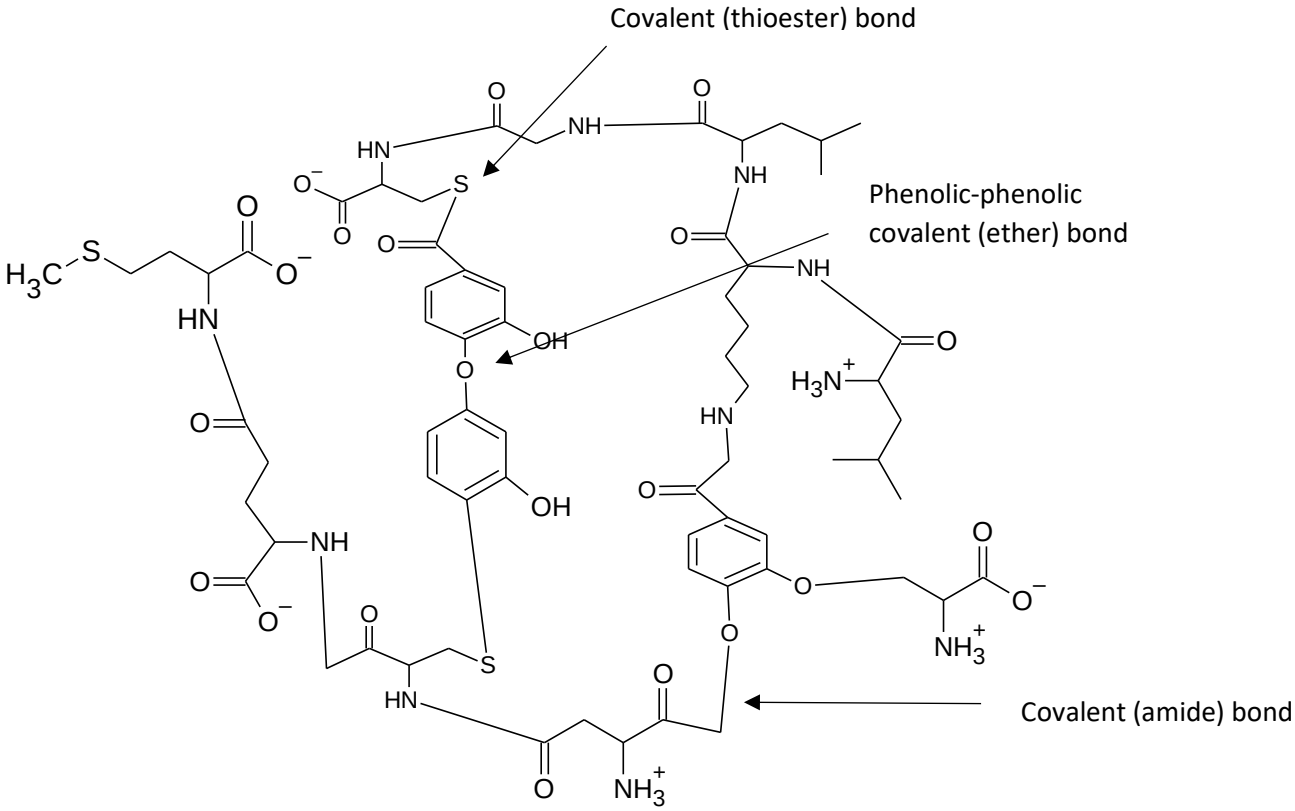


Figure 6.2.7: An illustration of microwave-induced crosslinking of oxidized protocatechuic acid with proteins

Table 6.2.4 shows that there was a potential varietal effect with regard to the observed increases and decreases in phenolic content and antioxidant activity of Bambara groundnut seeds after microwave pre-treatment. Predominantly, increases in phenolic content and antioxidant activity were observed for red Bambara groundnut seeds while mainly decreases were observed for brown Bambara groundnut seeds. This suggests that phenolic compounds in red Bambara groundnut seeds were relatively more thermally stable and became more extractable after microwave pre-treatment. An overall hypothesis that emerges from these observations is that phenolic compounds in different Bambara groundnut varieties may be bound to other components to different extents. A study of free and bound phenolic compounds in a wider range of Bambara groundnut varieties could provide some more insights regarding this varietal effect.

Chapter 7: Conclusions and recommendations

This study has shown that microwave pre-treatment reduces the cooking time of Bambara groundnut seeds significantly. The reduction in cooking time is due to partial starch gelatinization and protein denaturation as well as possible breakdown of pectic substances in the middle lamella of the parenchyma cells through β-elimination reactions.

Microwave pre-treatment of Bambara groundnut seeds reduces the functional properties (pasting properties, WSI, NSI and thermal properties) of the resultant flour. The high temperature conditions during microwave pre-treatment leads to a series of events such as partial gelatinisation of starch, protein denaturation and exposure of hydrophobic sites of the protein and possible formation of a hydrophobic protein matrix layer surrounding the partially gelatinised starch granules. Overall, these events bring about reduction in water uptake and subsequent reduction in functional properties of the Bambara groundnut flours.

The presence of flavonoids is more evident in microwave pre-treated red Bambara groundnut samples than the brown variety. This indicates that flavonoids are more thermally stable in the red variety than the brown during microwave treatments and suggests that the effect of microwave pre-treatment on flavonoids in Bambara groundnut seeds could be variety-dependent. Thermal stability could be conferred by interactions of flavonoids with macromolecules such as proteins through hydrogen, ionic and hydrophobic interactions.

Microwave pre-treatment leads to both increases (greater extractability) and decreases (reduced extractability) in phenolic content and antioxidant properties of Bambara groundnut. Increases in phenolic content and antioxidant properties could be due to microwave-induced release of bound phenolic compounds. On the other hand, decreases in phenolic content and antioxidant properties may be due to microwave-induced oxidative degradation of phenolic compounds.

Overall, this research shows that microwave heating is a promising processing technique for pre-treatment of Bambara groundnut seeds for production of flours which could have various food applications. Although the flours had reduced functional properties, they could still find application for use as ingredients in food products such as baked goods, porridges, soups and snacks. The fact that the flours retained appreciable antioxidant properties shows their potential to promote health through combating NCDs especially in sub-Saharan Africa where Bambara groundnut is widely consumed.

It is recomended that future research could test the hypothesis of a link between depolymerisation of pectin by β-elimination and reduction in cooking time by measuring the changes in pectic substances and their degree of polymerisation during microwave pre-treatment of Bambara groundnut seeds. A more accurate and reliable method than the Mattson bean cooker for determination of the cooking time of Bambara groundnut seeds needs to be investigated. A possibility could be the use of a texture analyzer whereby changes in textural properties of the seeds such as force of deformation, hardness or softness could be related to cooking time.

Future research can also make use of confocal laser scanning microscopy (CLSM) with the use of Safarin O dye to observe interations between starch and protein together with scanning electron microscopy (SEM). These powerful microscopic techniques have the potential to enhance understanding of starch-protein interactions during microwave pre-treatment of Bambara groundnut seeds and how this influences functional properties of resultant flours.

In terms of phenolic content and antioxidant activity, further research needs to be done on *in vitro* gastrointestinal digestion of Bambara groundnut flour to better understand the bioaccessibility of phenolic compounds. The bioaccessible phenolic compounds can also be tested using cell culture models such as Caco 2 cells to establish their role in the induction of cancer cellular death and the viability of cancer cells.

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Appendix A

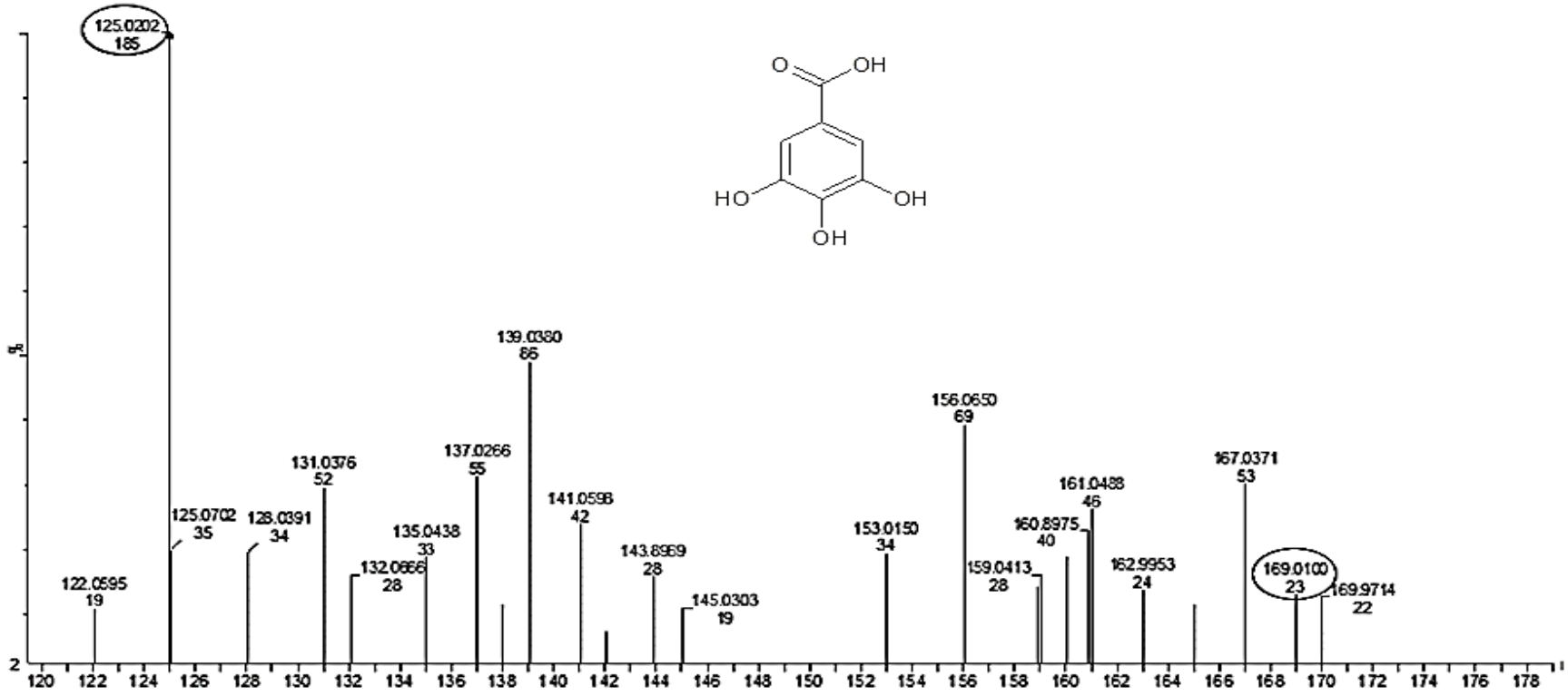


Figure A 1: Mass spectrum of gallic acid (167 m/z) peak 1

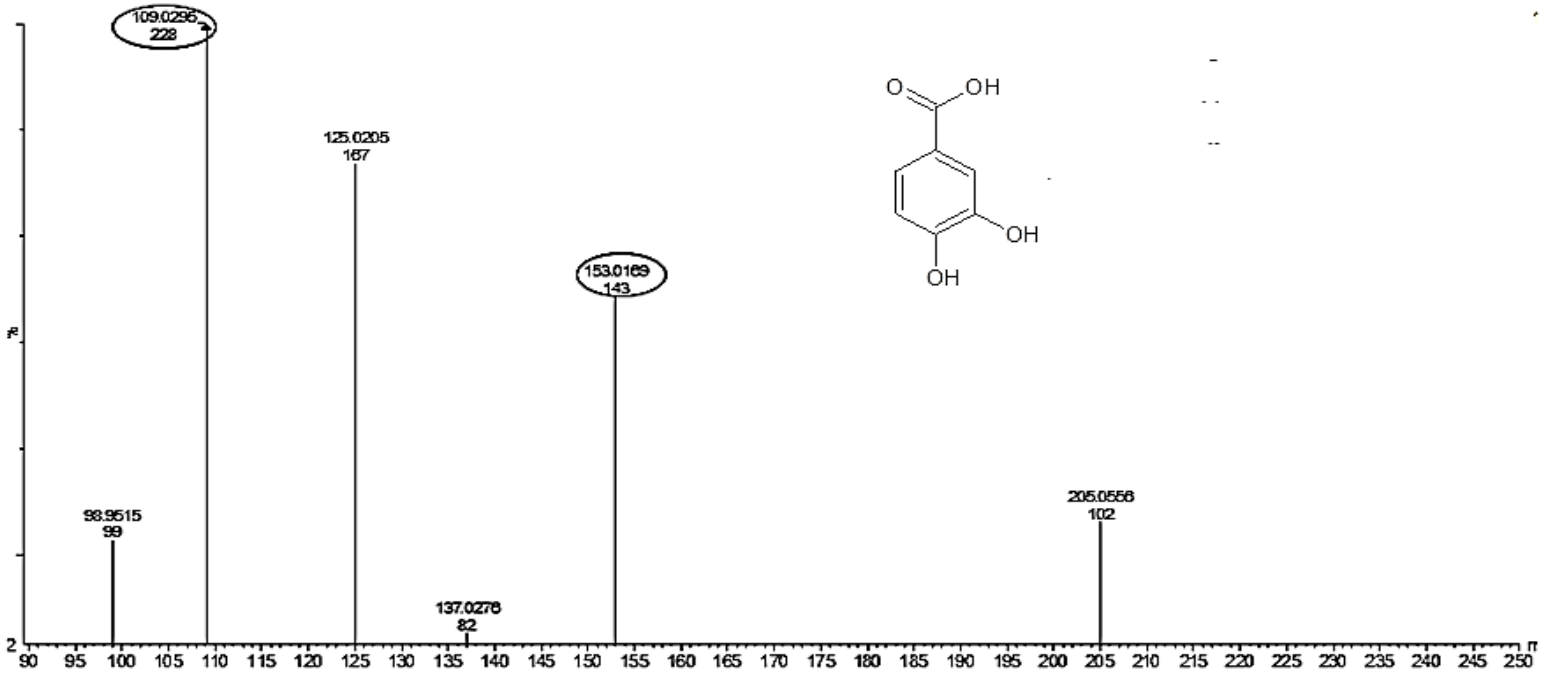


Figure A 2: **Mass spectrum of protocatechuic acid (153 m/z) peak 2**

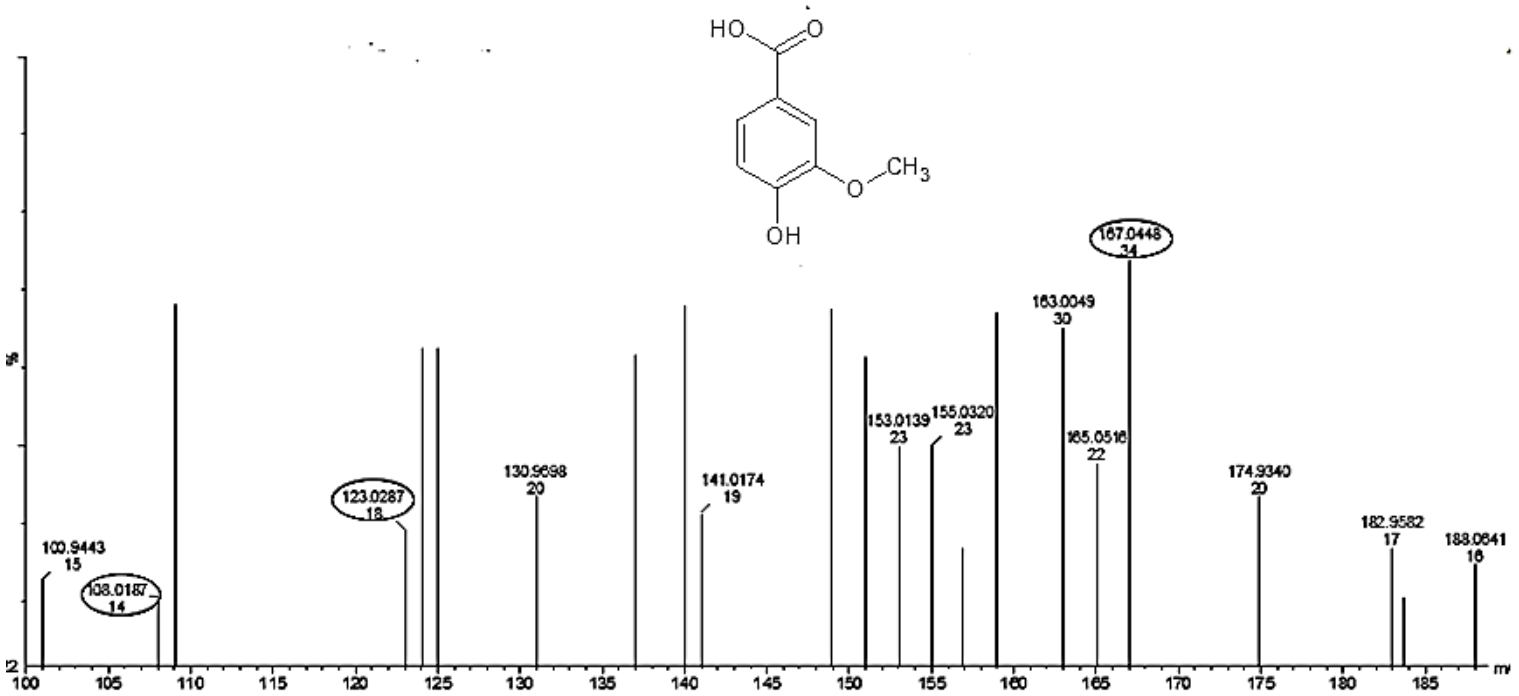


Figure A 3: Mass spectrum of vanillic acid (167 m/z) peak 3

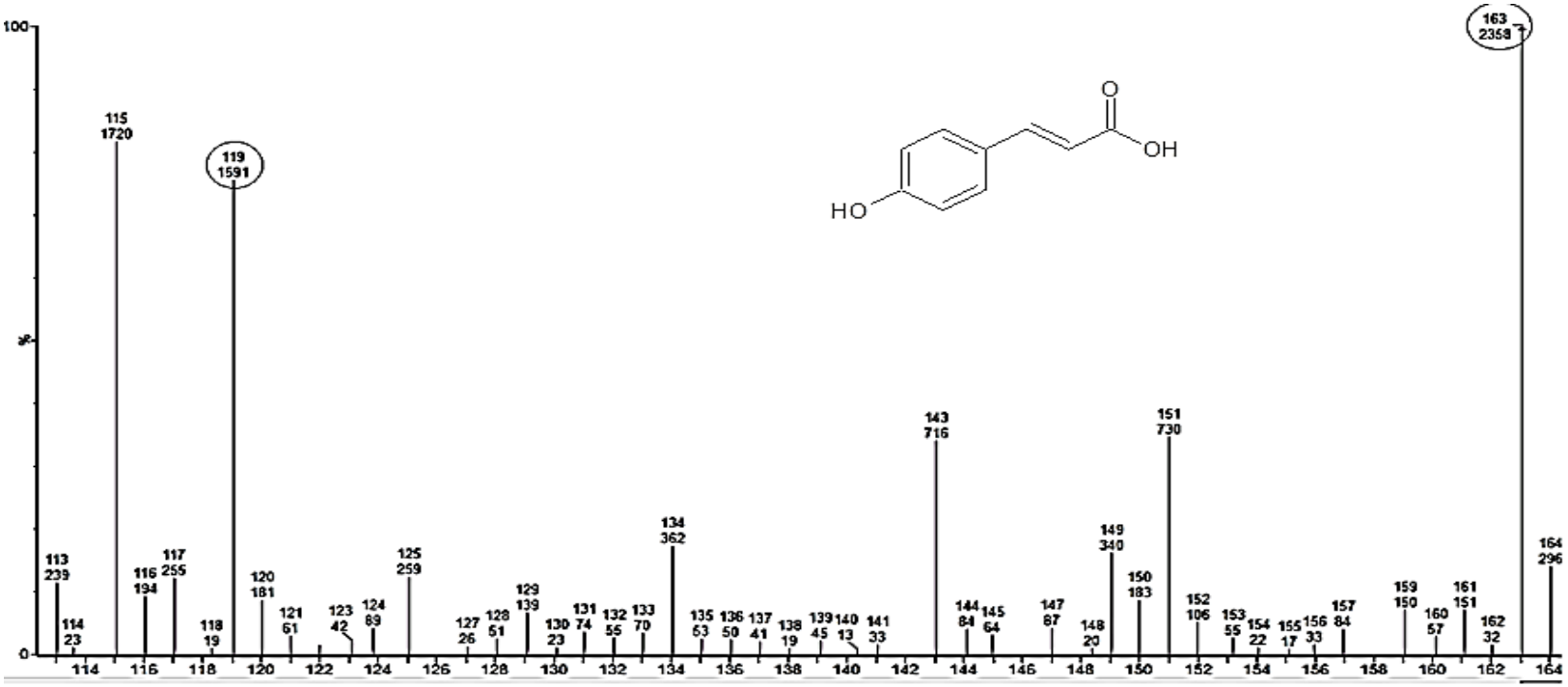


Figure A 4: Mass spectrum of coumaric acid isomer (137 m/z) peak 4

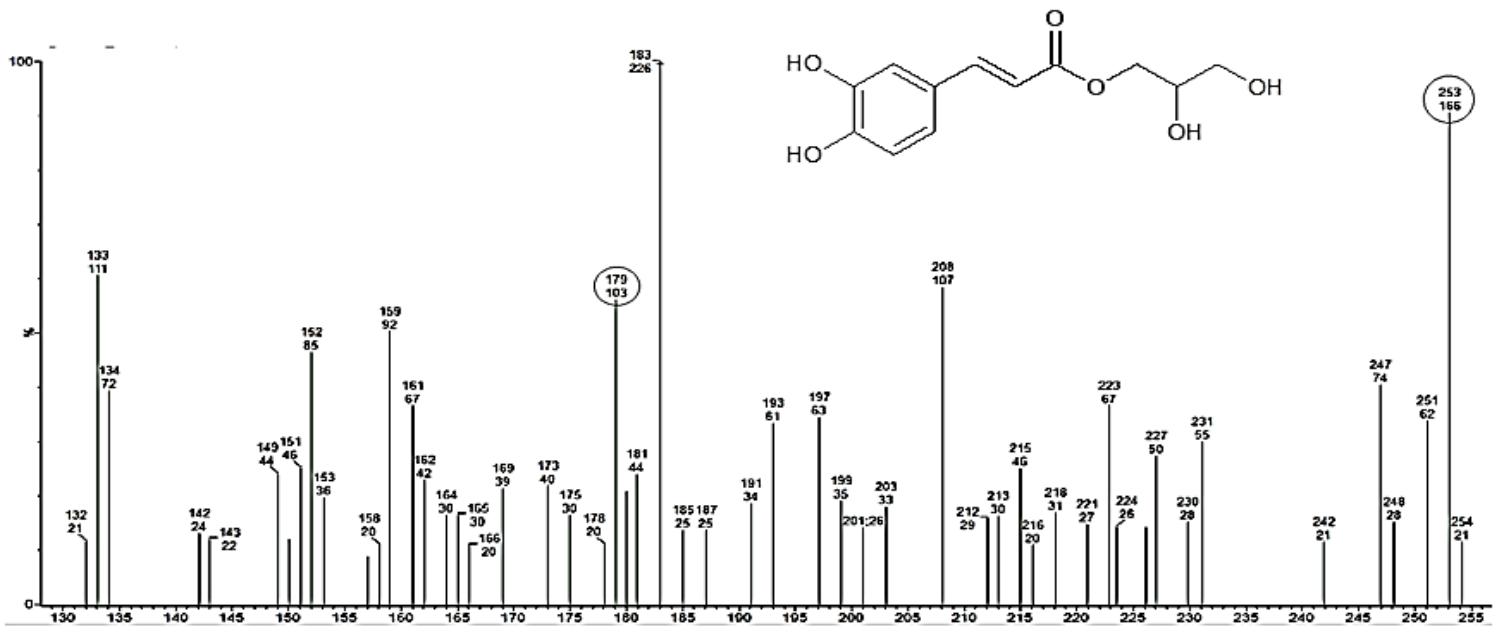


Figure A 5: Mass spectrum of caffeoyl glycerol (253 m/z) peak 5

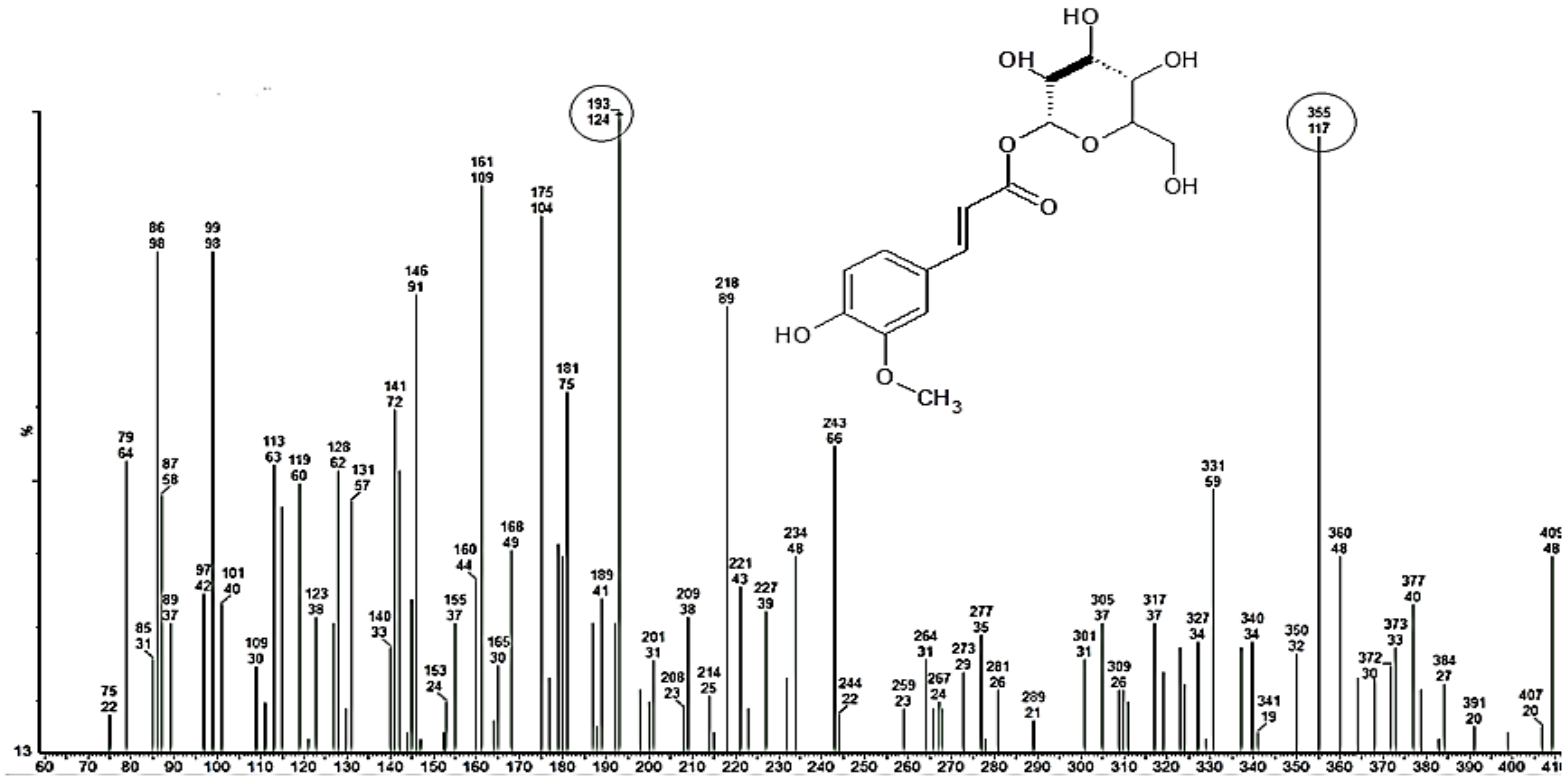


Figure A 6: Mass spectrum of ferulic acid hexoside (355 m/z) peak 6

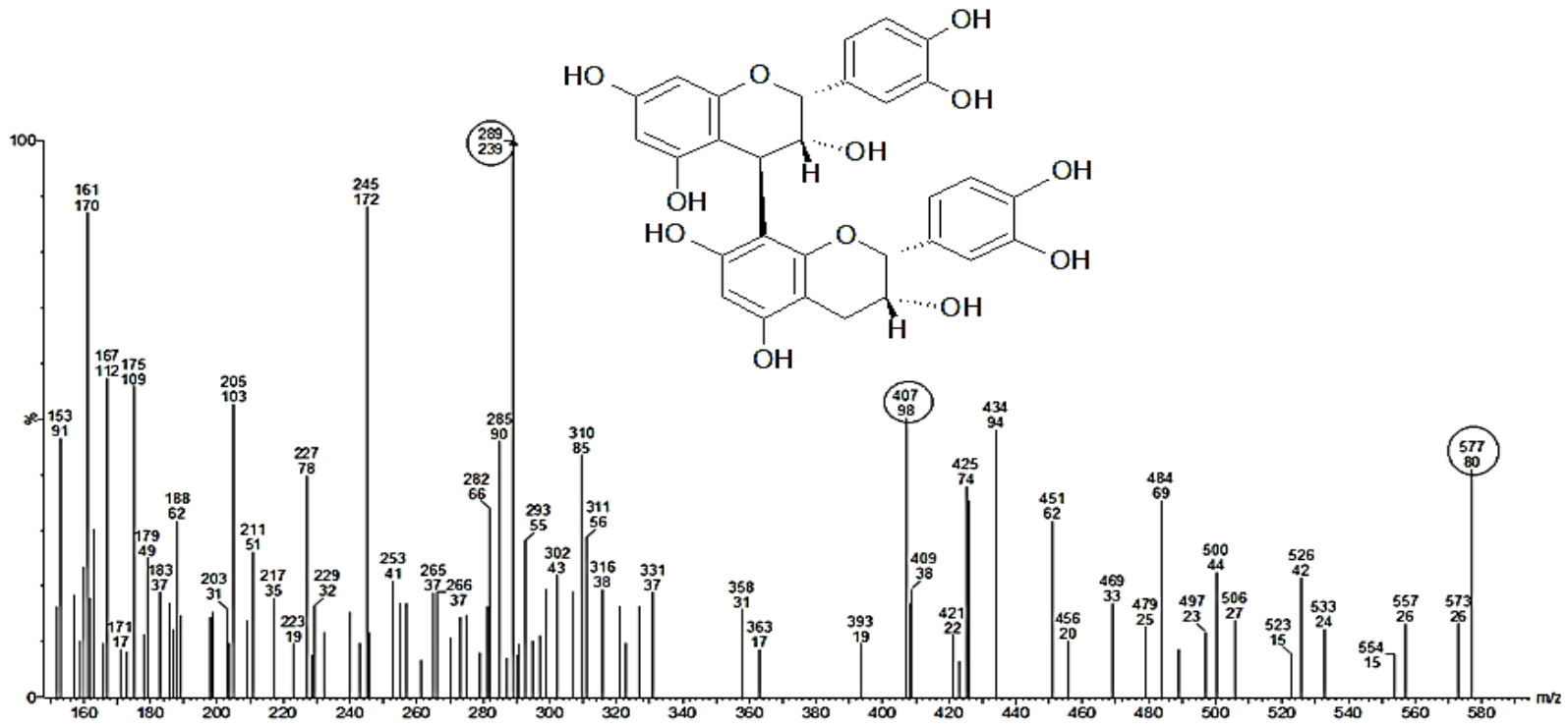


Figure A 7: Mass spectrum of Procyanidin B 2 dimer (577 m/z) peak 7

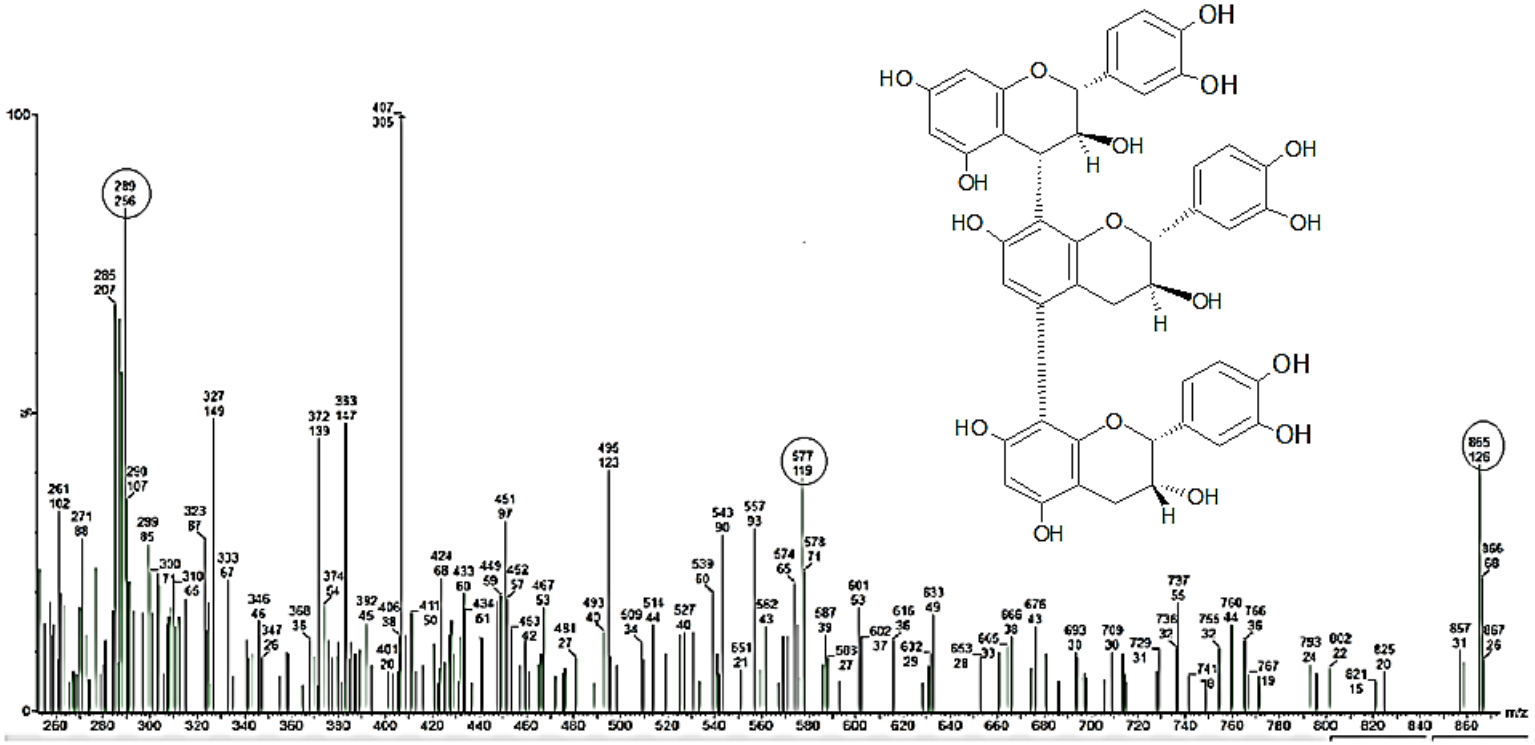


Figure A 8: Mass spectrum of procyanidin C 2 trimer (865 m/z) peak 8

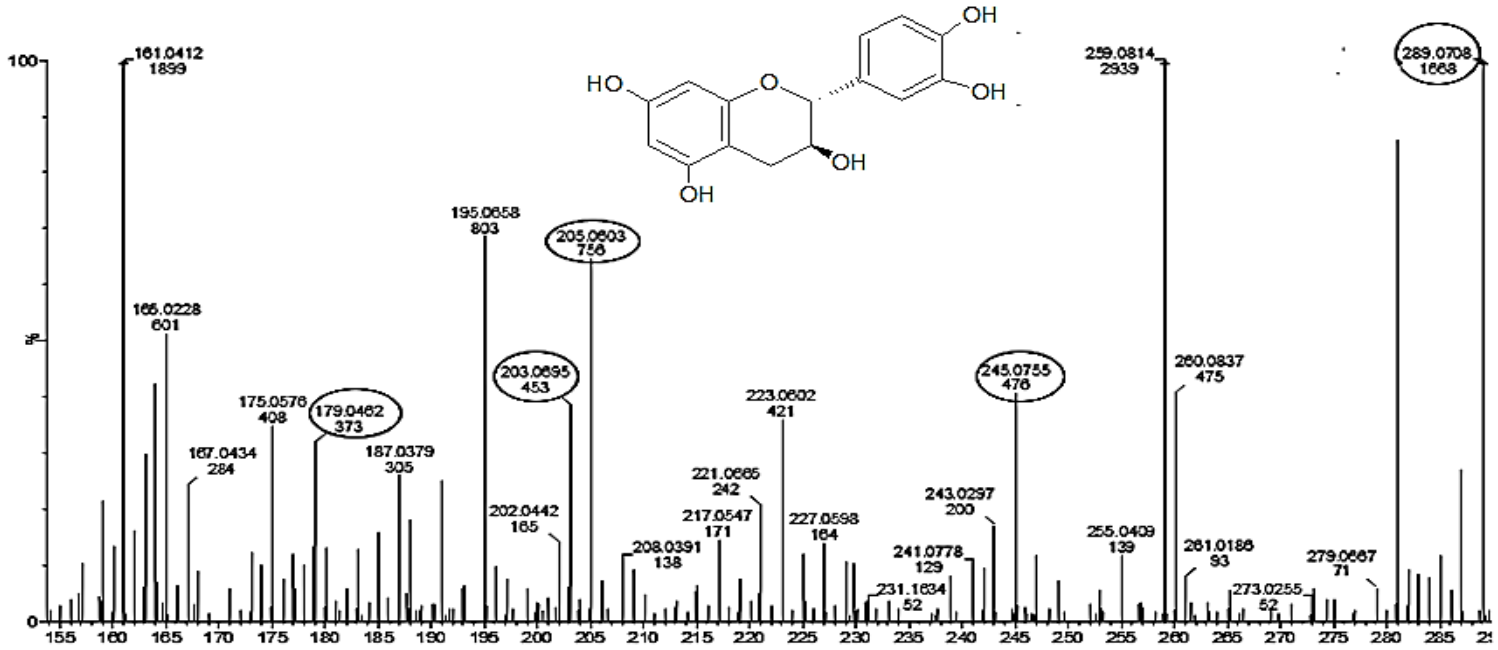


Figure A 9 : Mass spectrum of catechin (289 m/z) peak 9

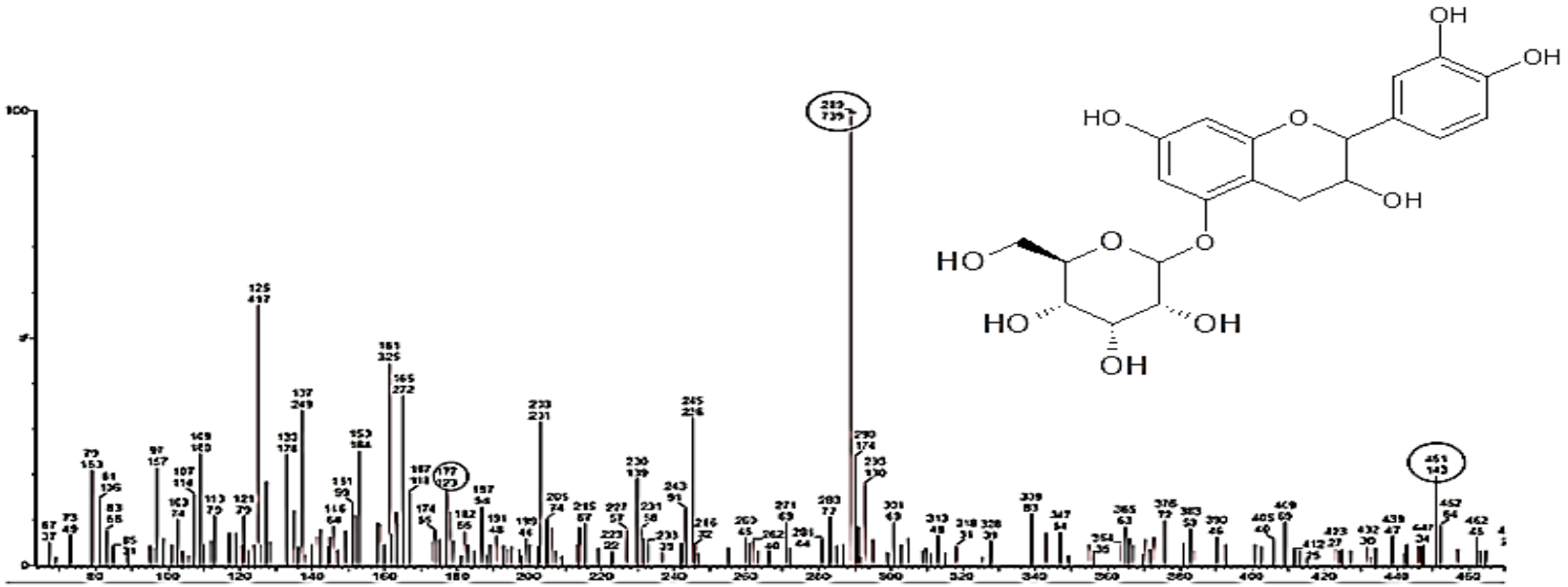


Figure A 10: Mass spectrum of catechin-5-O- glucoside (451 m/z) peak 10

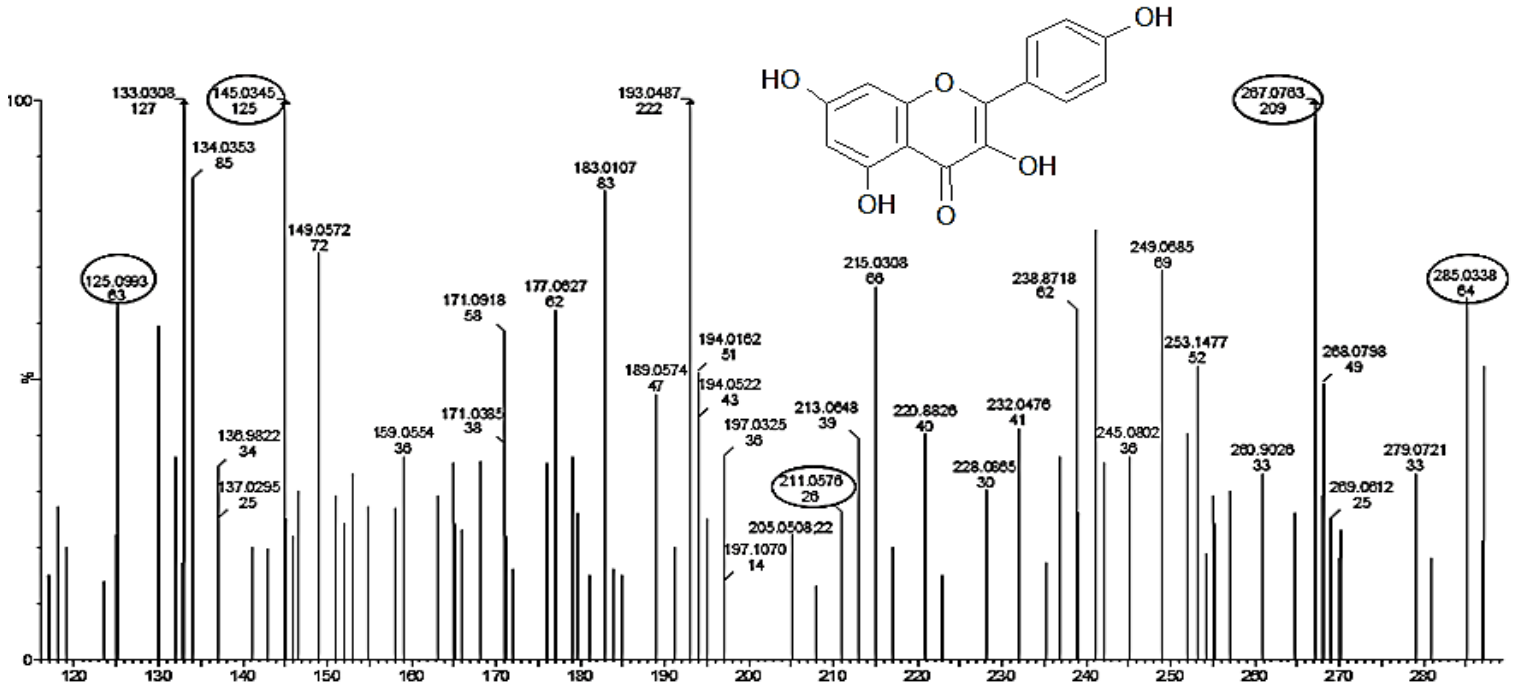


Figure A 11: Mass spectrum of kaempferol (285 m/z) peak 11

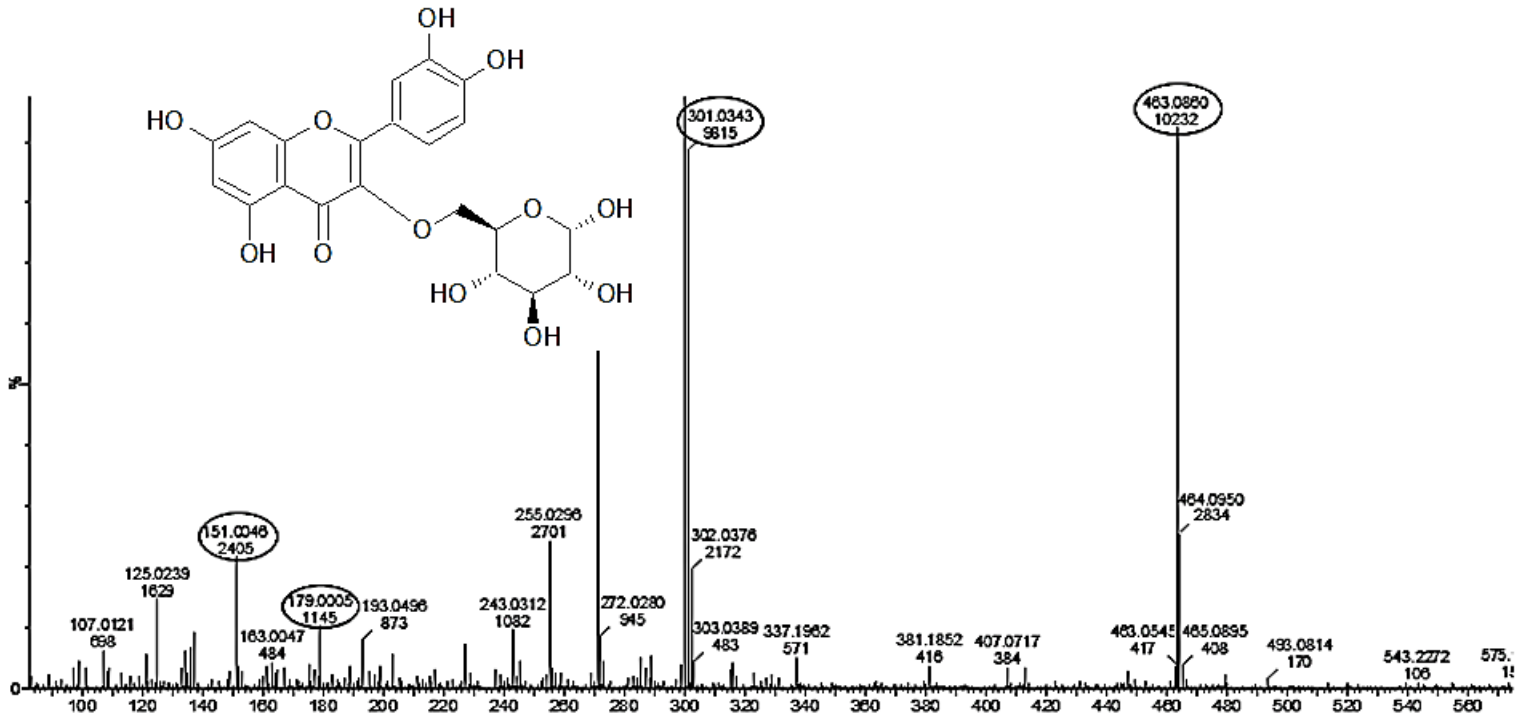


Figure A 12: Mass spectrum of quercetin-3-O-glucoside (463 m/z) peak 12

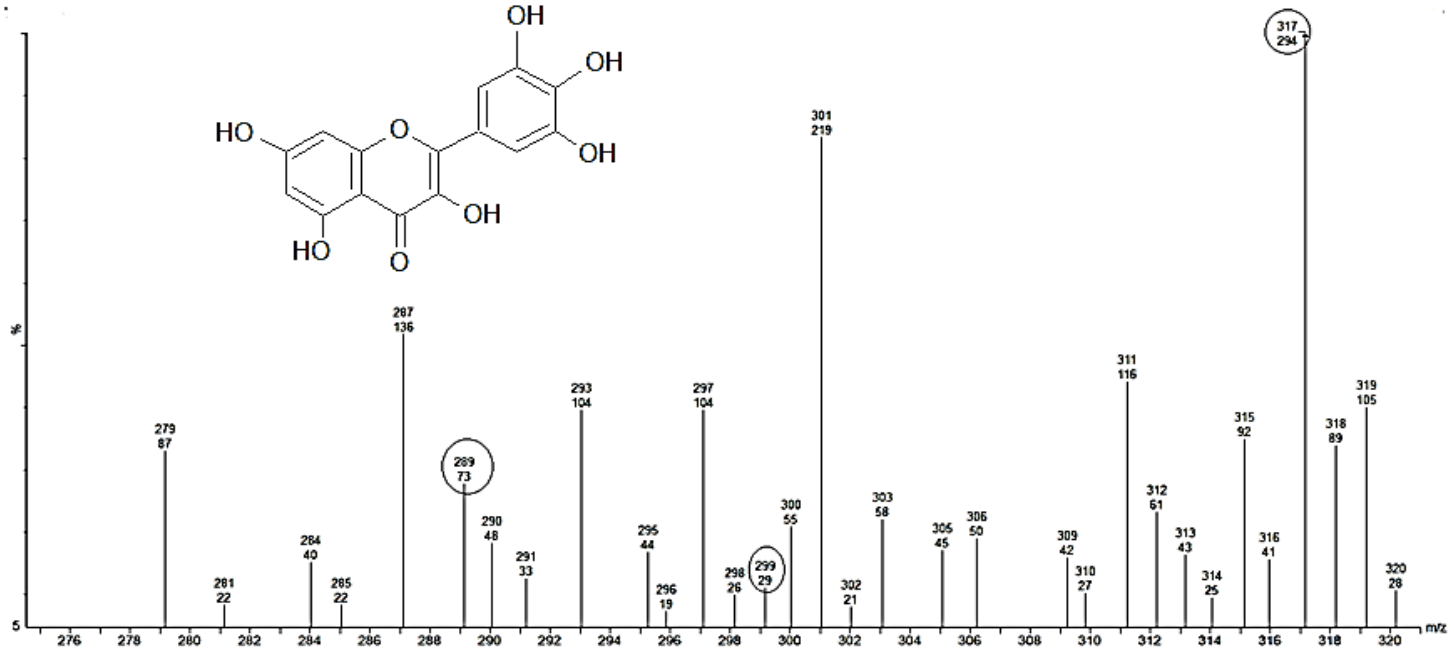


Figure A 13: Mass spectrum of myricetin (317 m/z) peak 13

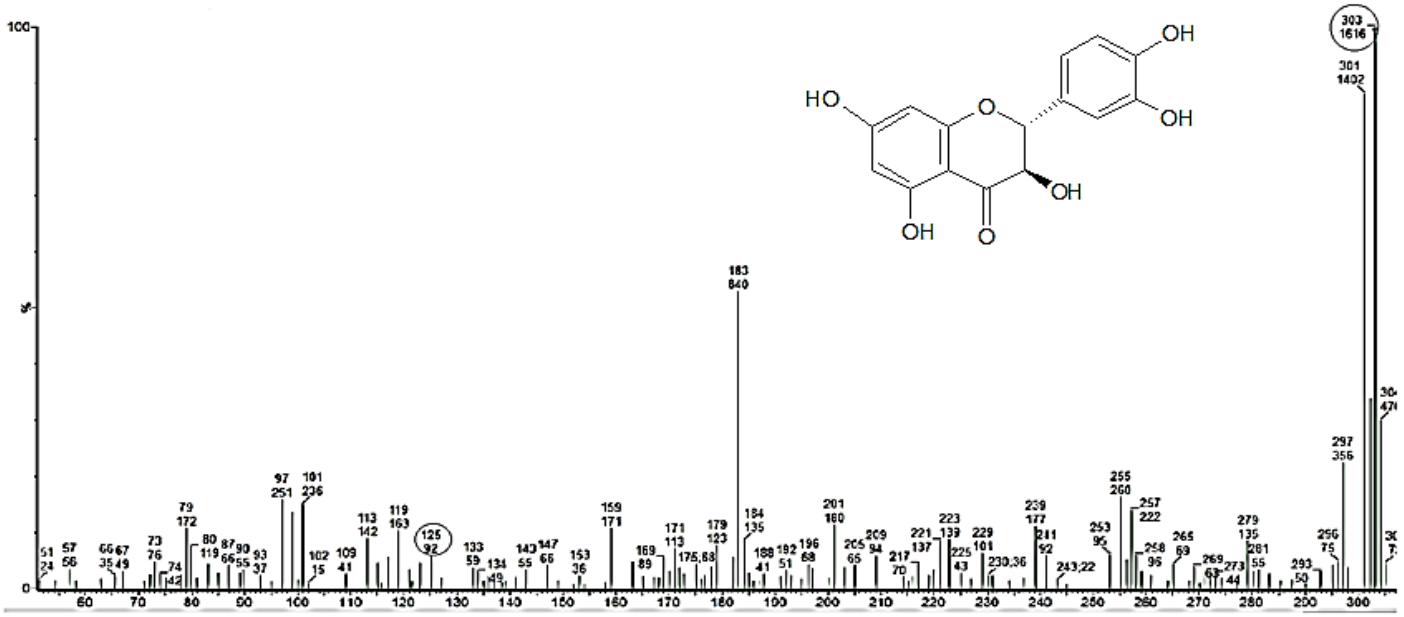


Figure A 14: Mass spectrum of taxifolin (303 m/z) peak 14

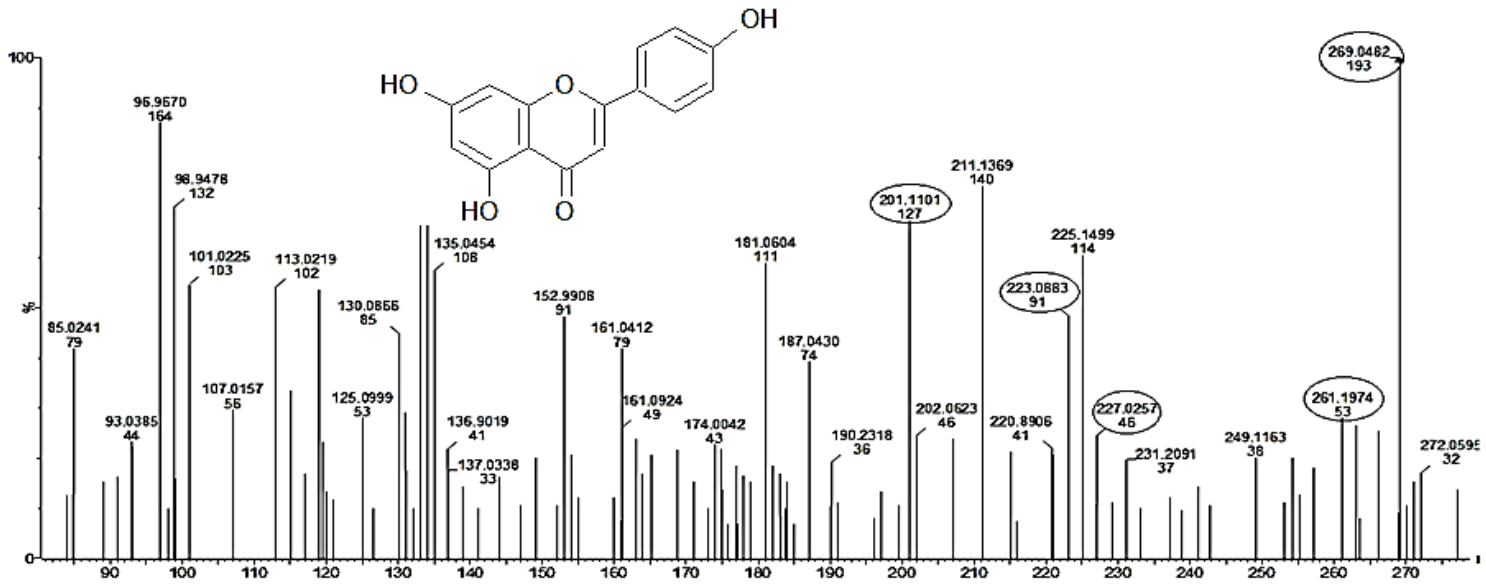


Figure A 15: Mass spectrum of apigenin (269 m/z) peak 15

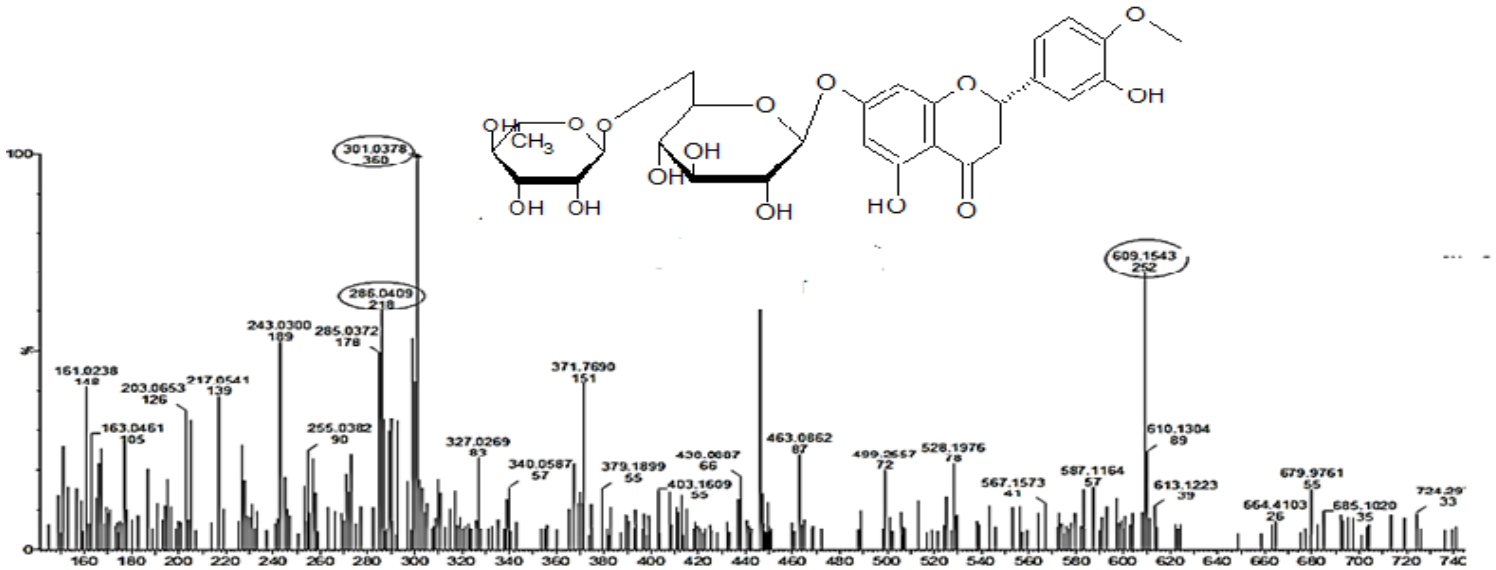


Figure A 16: Mass spectrum of hesperidin (609 m/z) peak 16

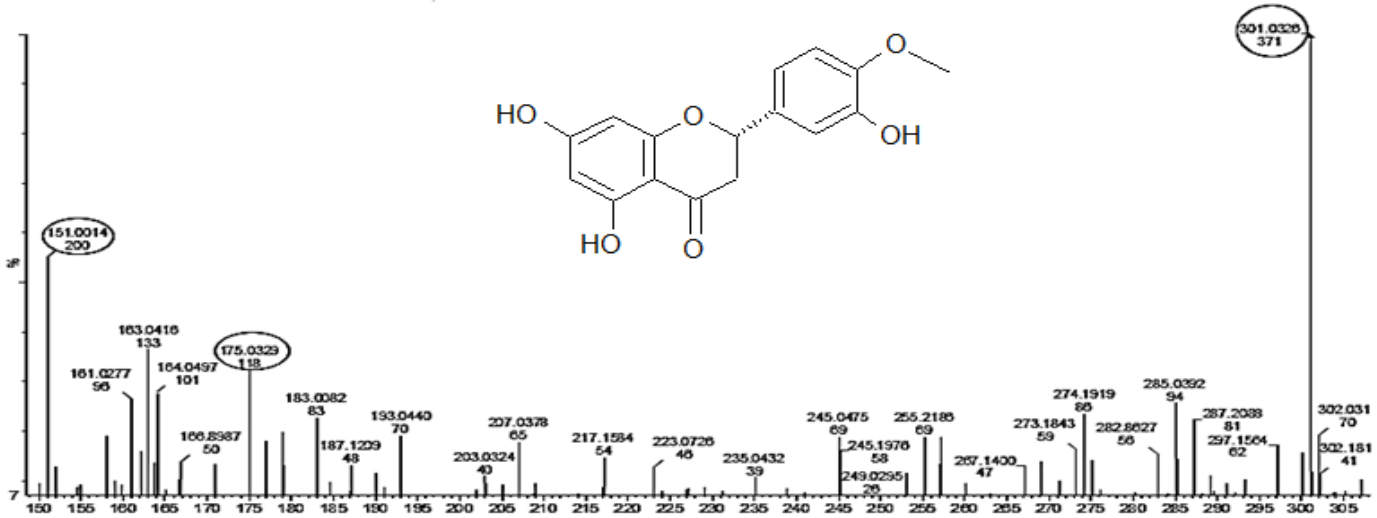


Figure A 17: Mass spectrum of hesperetin (301 m/z) peak 17

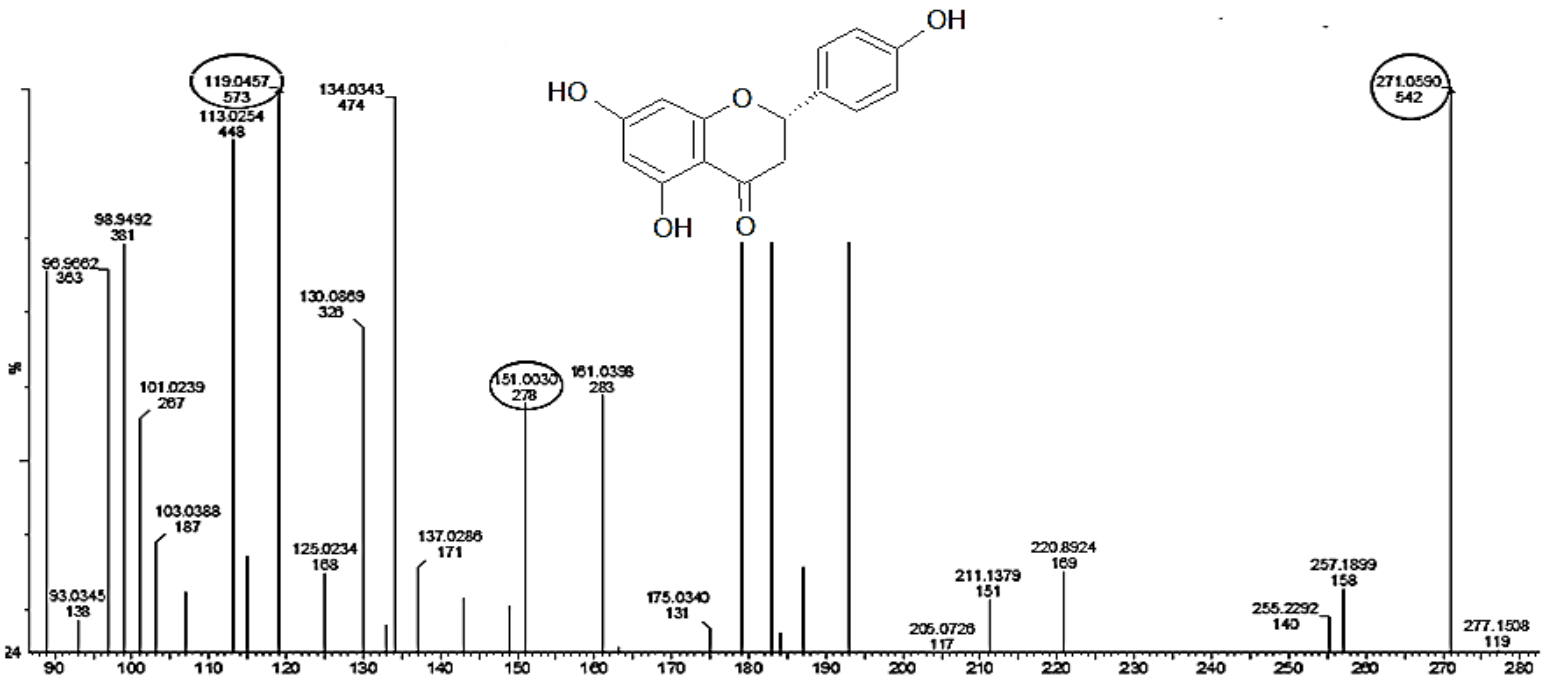


Figure A 18: Mass spectrum of naringenin (271 m/z) peak 18

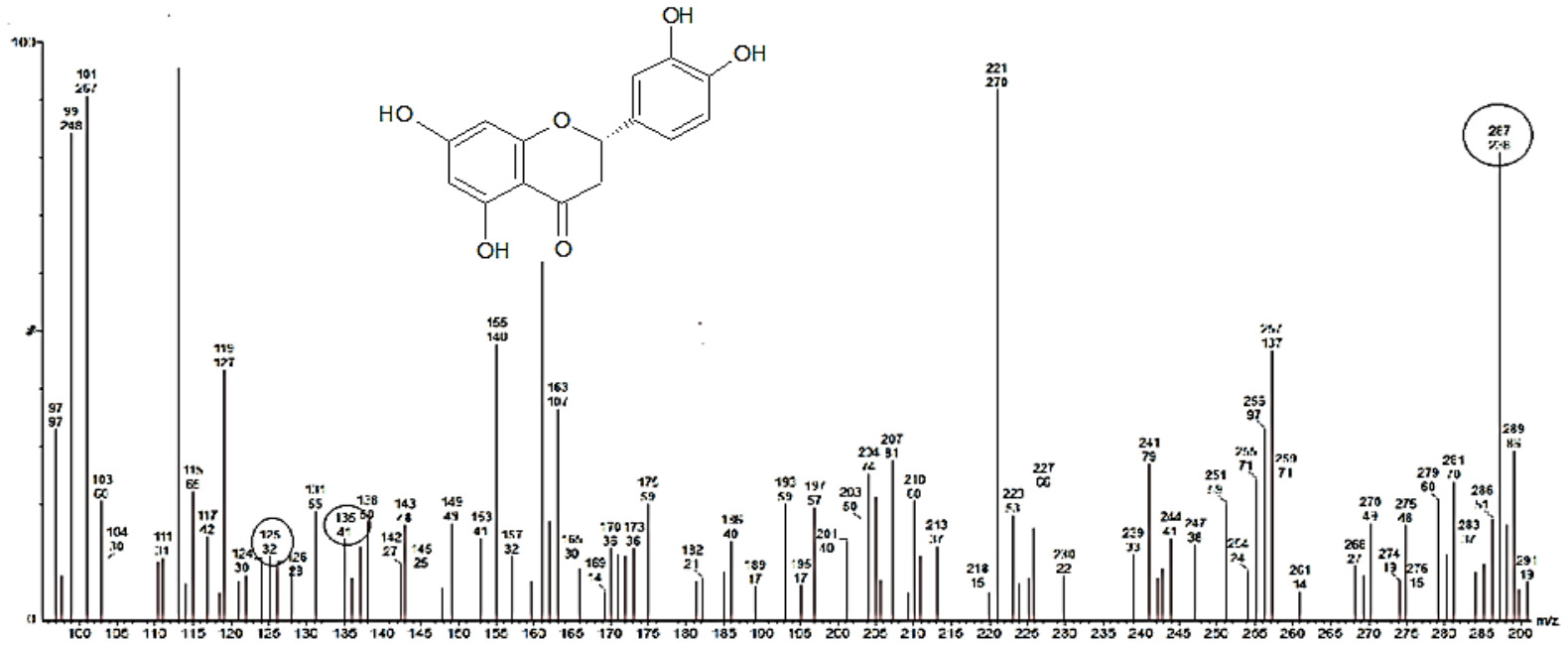


Figure A 19: Mass spectrum of eriodictyol (287 m/z) peak 19

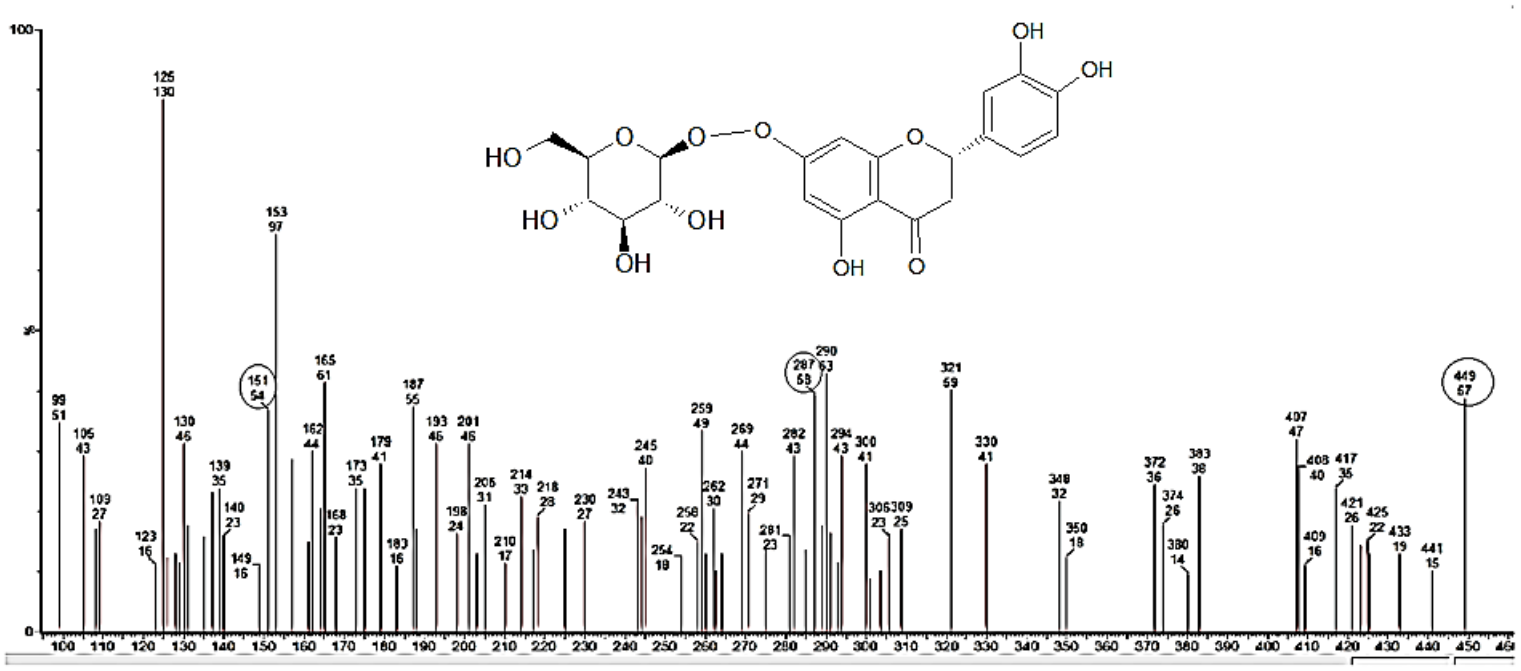


Figure A 20: Mass spectrum of eriodictyol -7-O-β-D- glucoside (449 m/z) peak 20

Appendix B

A

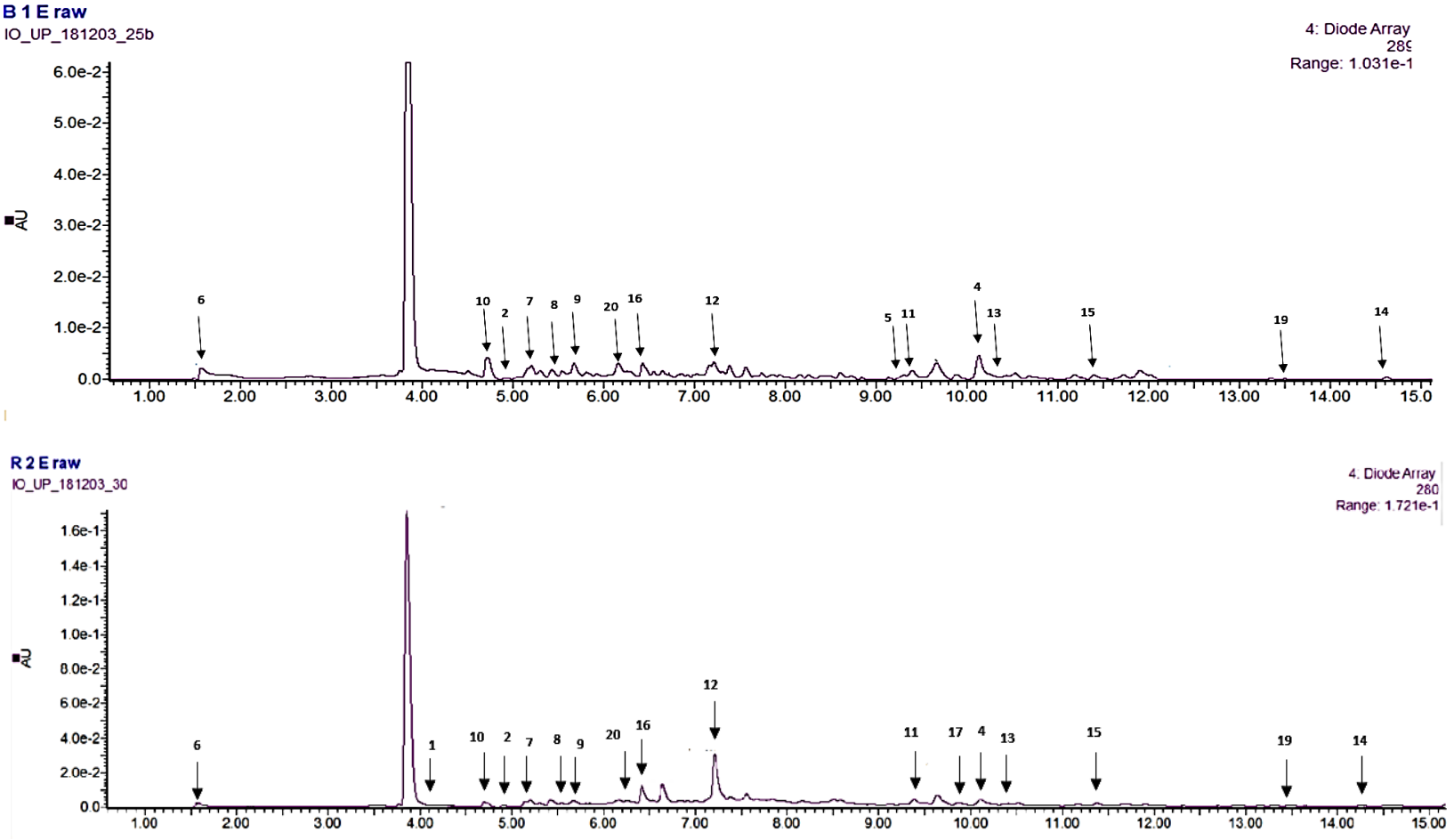


Figure B. 1: UPLC-MS chromatograms (at 289 nm) of ethyl acetate extracts from raw untreated pre-soaked brown (A) and red (B) Bambara groundnut seeds. 1 = Gallic acid, 2 = Protocatechuic acid, 4 = Coumaric acid isomer, 5 = Caffeoyl glycerol, 6 = Ferulic acid hexoside, 7 = Procyanidin B 2 dimer, 8 = Procyanidin C 2 trimer, 9 = Catechin, 10 = Catechin glucoside, 11 = Kaempferol, 12 = Quercetin-3-O-glucoside, 13 = Myricetin, 14 = Taxifolin, 15 = Apigenin, 16 = Hesperidin, 17 = Hesperetin, 19 = Eriodictyol, 20 = Eriodictyol -7-O-β-D- glucoside.

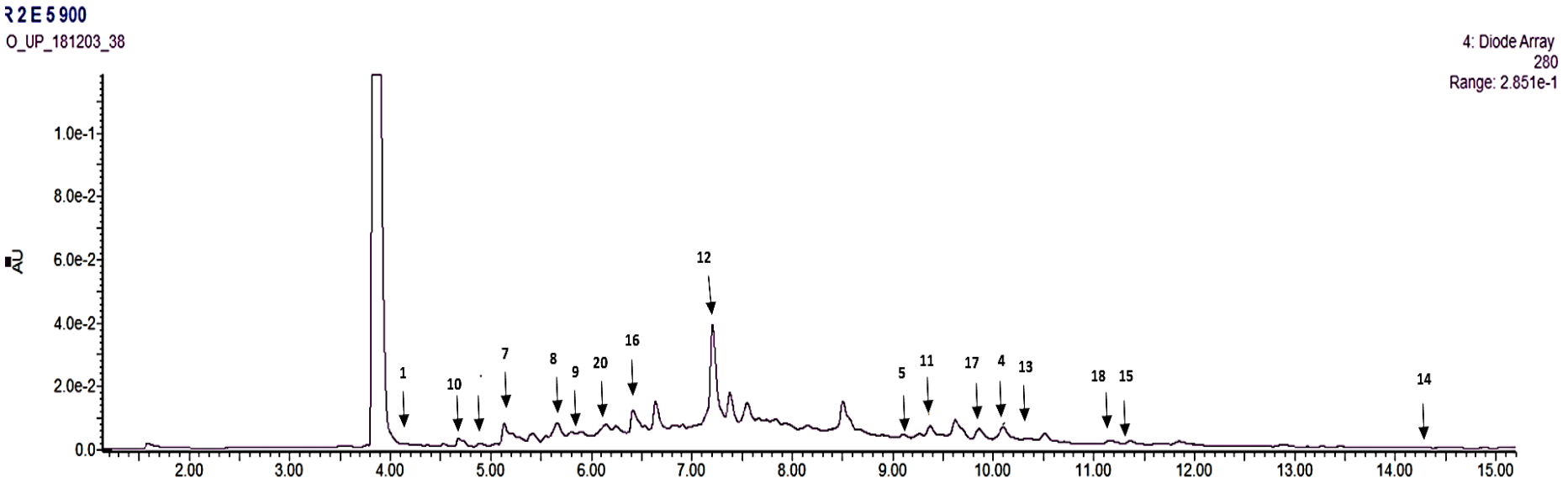


Figure B. 2: UPLC-MS chromatograms (at 289 nm) of ethyl acetate extracts from pre-soaked red Bambara groundnut seeds microwaved for 5 minutes at 900 W. 1 = Gallic acid, 2 = Protocatechuic acid, 4 = Coumaric acid isomer, 5 = Caffeoyl glycerol, 6 = Ferulic acid hexoside, 7 = Procyanidin B 2 dimer, 8 = Procyanidin C 2 trimer, 9 = Catechin, 10 = Catechin glucoside, 11 = Kaempferol, 12 = Quercetin-3-O-glucoside, 13 = Myricetin, 14 = Taxifolin, 15 = Apigenin, 16 = Hesperidin, 17 = Hesperetin, 18 = Naringenin, 19 = Eriodictyol, 20 = Eriodictyol -7-O-β-D- glucoside.

A)

B)

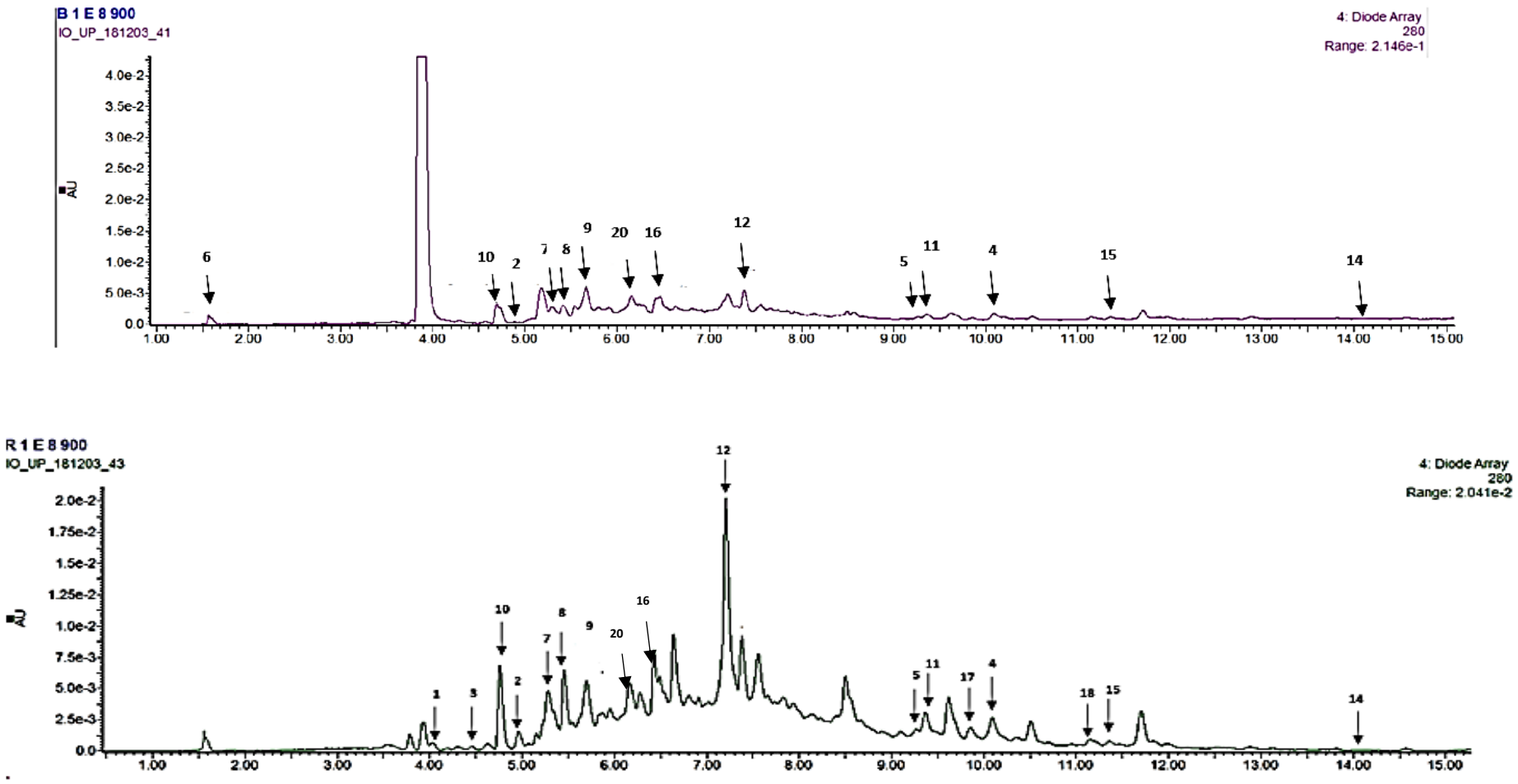
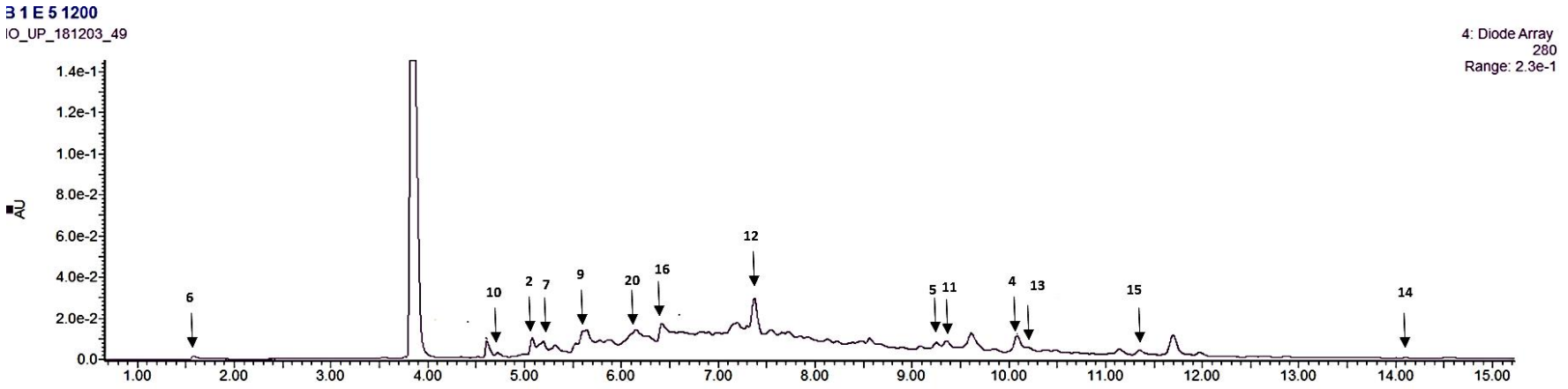


Figure B. 3: UPLC-MS chromatograms (at 280 nm) of ethyl acetate extracts from pre-soaked brown (A) and red (B) Bambara groundnut seeds microwaved for 8 minutes at 900 W. 1 = Gallic acid, 2 = Protocatechuic acid, 3 = Vanillic acid, 4 = Coumaric acid isomer, 5 = Caffeoyl glycerol, 6 = Ferulic acid hexoside, 7 = Procyanidin B 2 dimer, 8 = Procyanidin C 2 trimer, 9 = Catechin, 10 = Catechin glucoside, 11 = Kaempferol, 12 = Quercetin-3-O-glucoside, 14 = Taxifolin, 15 = Apigenin, 16 = Hesperidin, 17 = Hesperetin, 20 = Eriodictyol -7-O-β-D- glucoside.

A)



B)

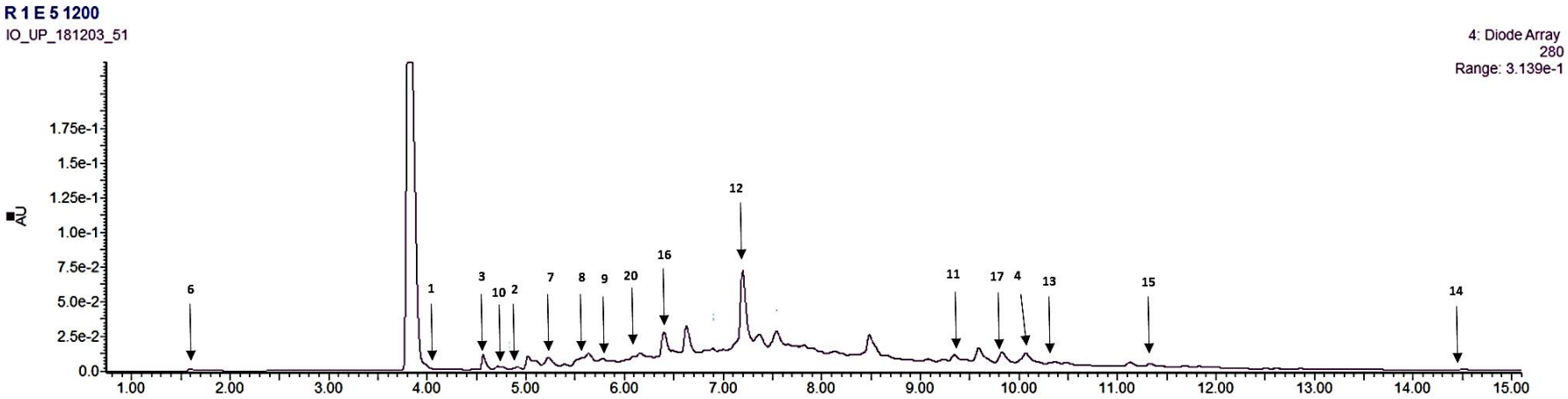


Figure B. 4: UPLC-MS chromatograms (at 280 nm) of ethyl acetate extracts from pre-soaked brown (A) and red (B) Bambara groundnut seeds microwaved for 5 minutes at 1200 W. 1 = Gallic acid, 2 = Protocatechuic acid, 3 = Vanillic acid, 4 = Coumaric acid isomer, 5 = Caffeoyl glycerol, 6 = Ferulic acid hexoside, 7 = Procyanidin B 2 dimer, 8 = Procyanidin C 2 trimer, 9 = Catechin, 10 = Catechin glucoside, 11 = Kaempferol, 12 = Quercetin-3-O-glucoside, 14 = Taxifolin, 15 = Apigenin, 16 = Hesperidin, 17 = Hesperetin, 20 = Eriodictyol -7-O-β-D- glucoside.

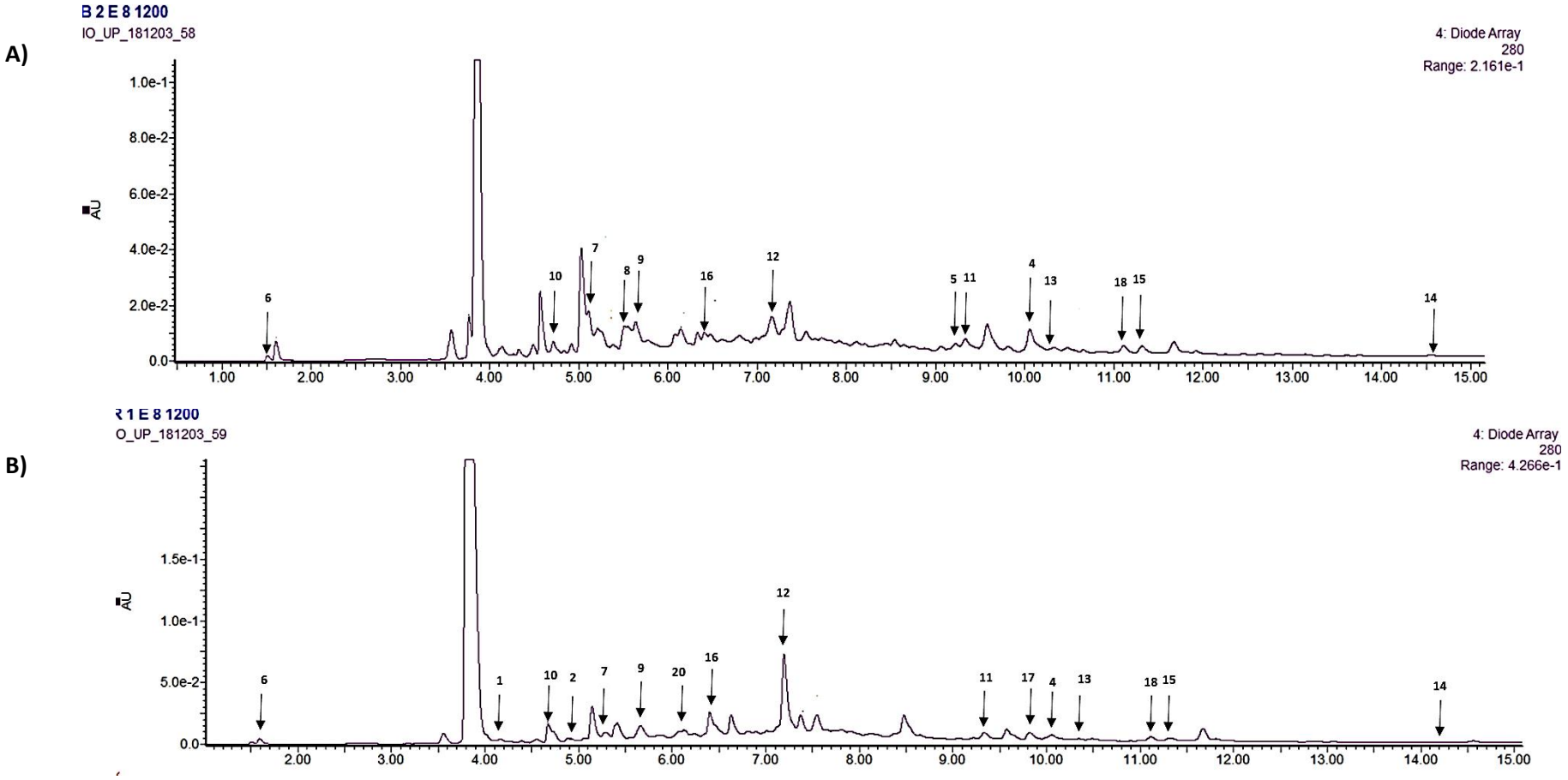
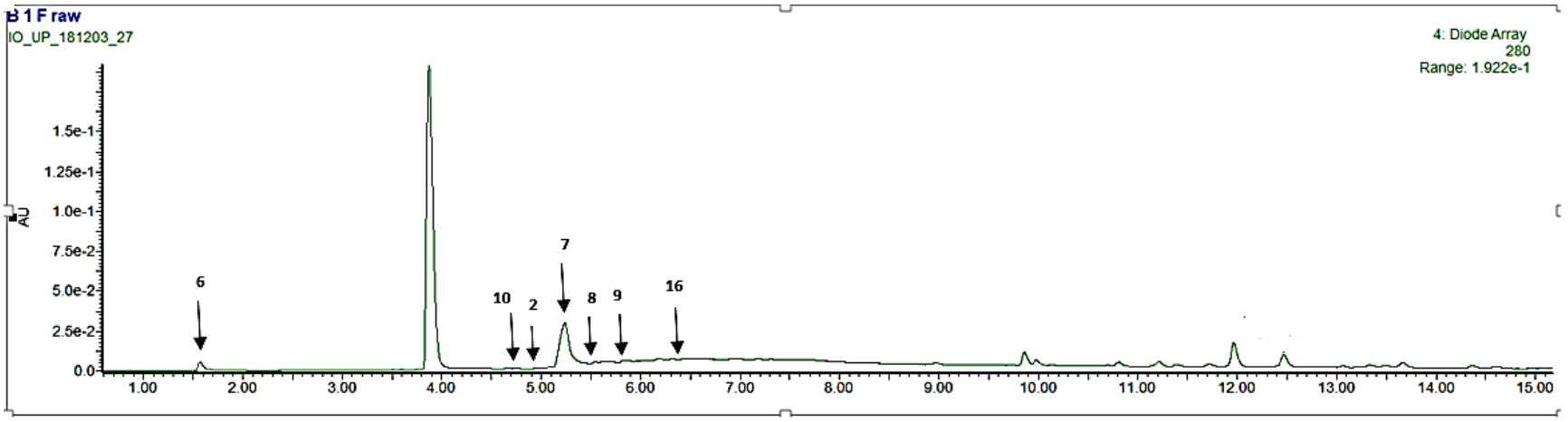


Figure B. 5: UPLC-MS chromatograms (at 280 nm) of ethyl acetate extracts from pre-soaked brown (A) and red (B) Bambara groundnut seeds microwaved for 8 minutes at 1200 W. 1 = Gallic acid, 2 = Protocatechuic acid, 3 = Vanillic acid, 4 = Coumaric acid isomer, 6 = Ferulic acid hexoside, 7 = Procyanidin B 2 dimer, 9 = Catechin, 10 = Catechin glucoside, 11 = Kaempferol, 12 = Quercetin-3-O-glucoside, 13 = Myricetin, 14 = Taxifolin, 15 = Apigenin, 16 = Hesperidin, 17 = Hesperetin, 18 = Naringenin, 20 = Eriodictyol-7-O-β-D- glucoside.

A)



B)

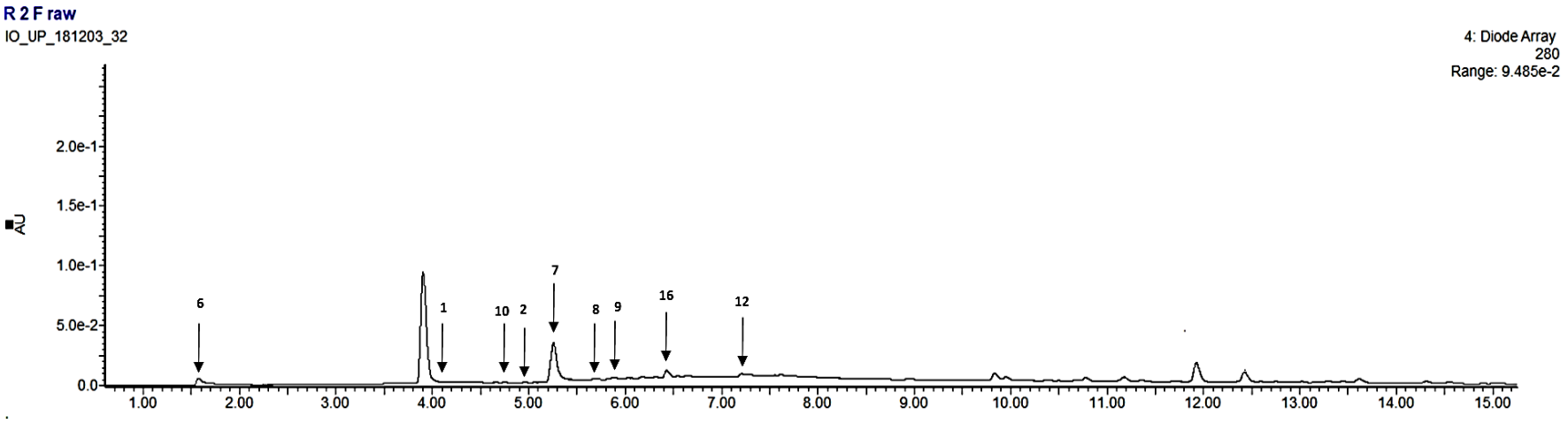


Figure B. 6: UPLC-MS chromatograms (at 280 nm) of formic acid-methanol extracts from pre-soaked raw untreated brown (A) and red (B) Bambara groundnut seeds. 1 = Gallic acid, 2 = Protocatechuic acid, 6 = Ferulic acid hexoside, 7 = Procyanidin B 2 dimer, 9 = Catechin, 10 = Catechin glucoside, 12 = Quercetin-3-O-glucoside, 16 = Hesperidin

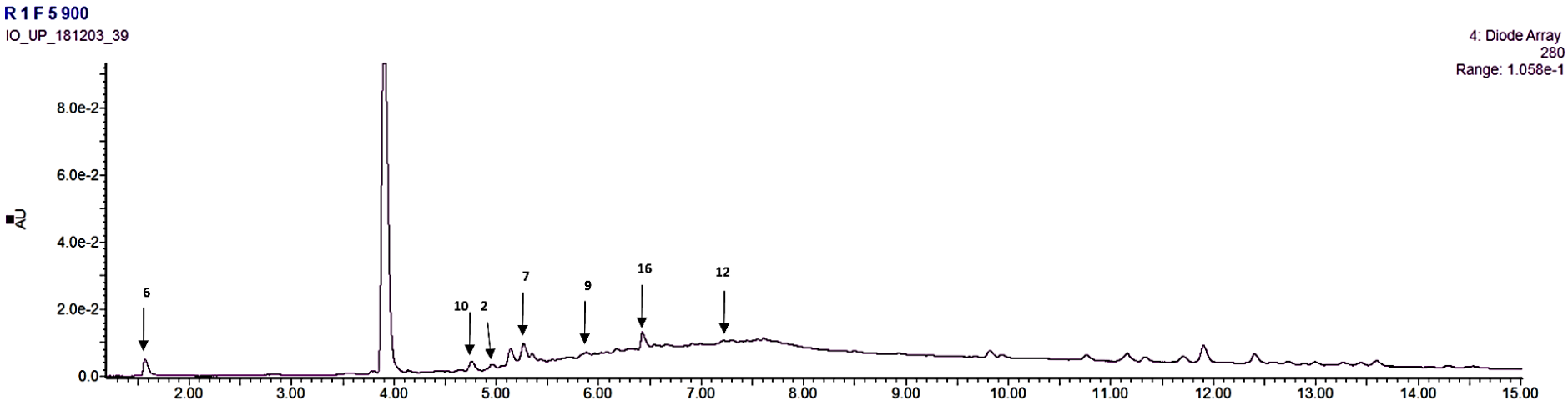
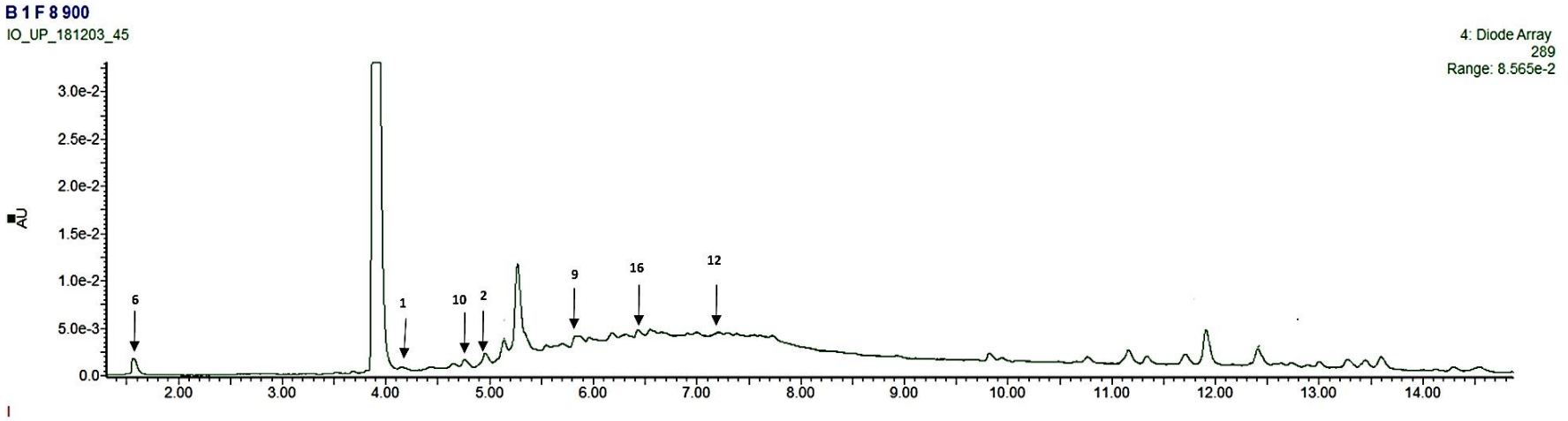


Figure B. 7: UPLC-MS chromatograms (at 280 nm) of formic acid-methanol extracts from pre-soaked red Bambara groundnut seeds microwaved for 5 minutes at 900 W. 1 = Gallic acid, 2 = Protocatechuic acid, 6 = Ferulic acid hexoside, 7 = Procyanidin B 2 dimer, 9 = Catechin, 10 = Catechin glucoside, 12 = Quercetin-3-O-glucoside, 16 = Hesperidin

A)



B)

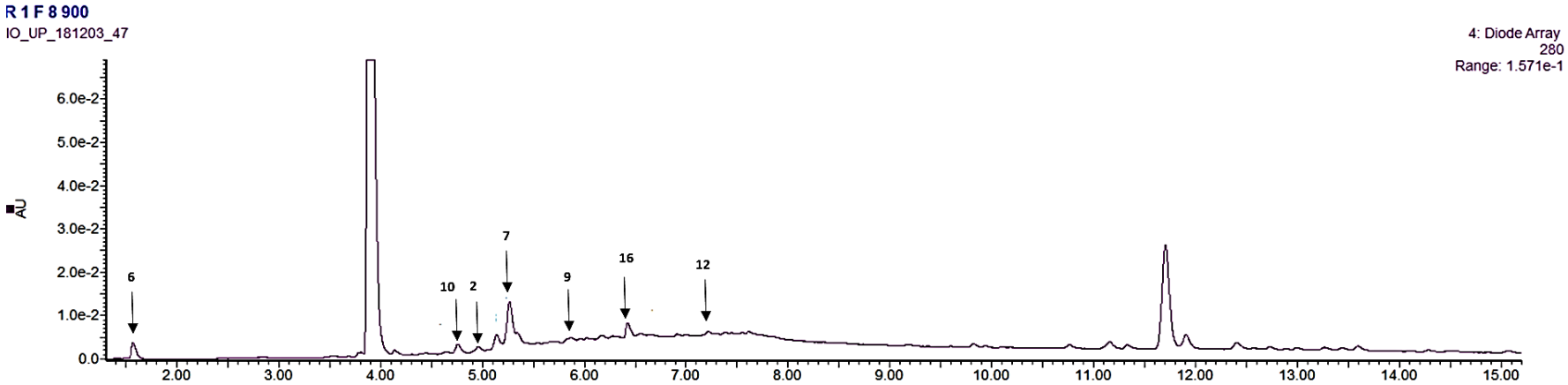
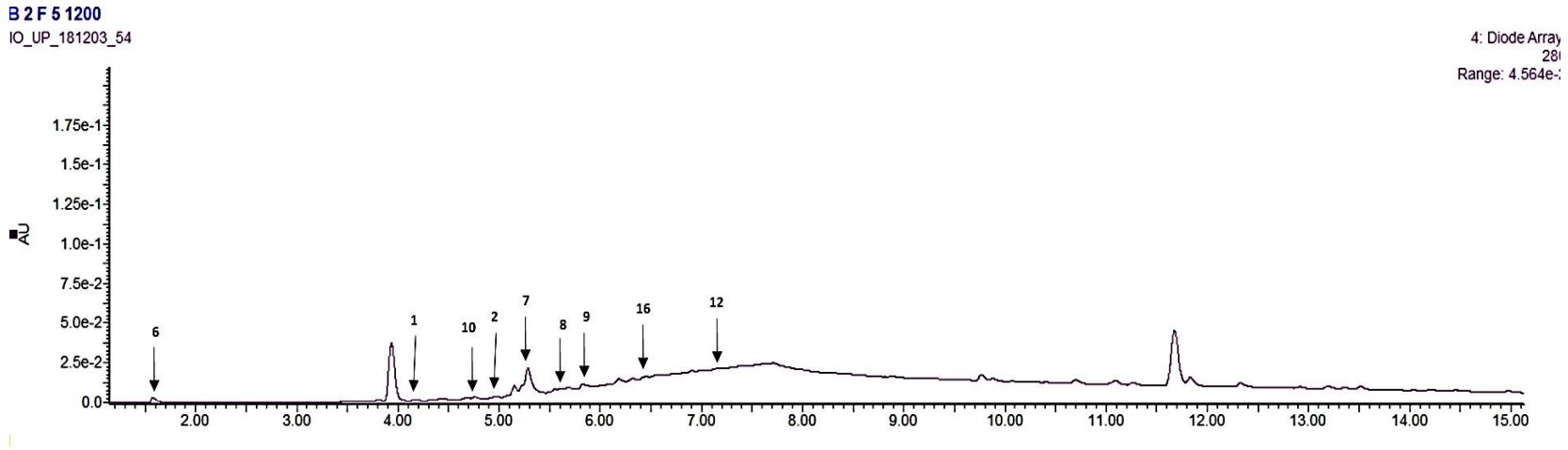


Figure B. 8*:* UPLC-MS chromatograms (at 280 nm) of formic acid-methanol extracts from pre-soaked brown (A) and red (B) Bambara groundnut seeds microwaved for 8 minutes at 900 W. 1 = Gallic acid, 2 = Protocatechuic acid, 6 = Ferulic acid hexoside, 7 = Procyanidin B 2 dimer, 9 = Catechin, 10 = Catechin glucoside, 12 = Quercetin-3-O-glucoside, 16 = Hesperidin

A)



B)

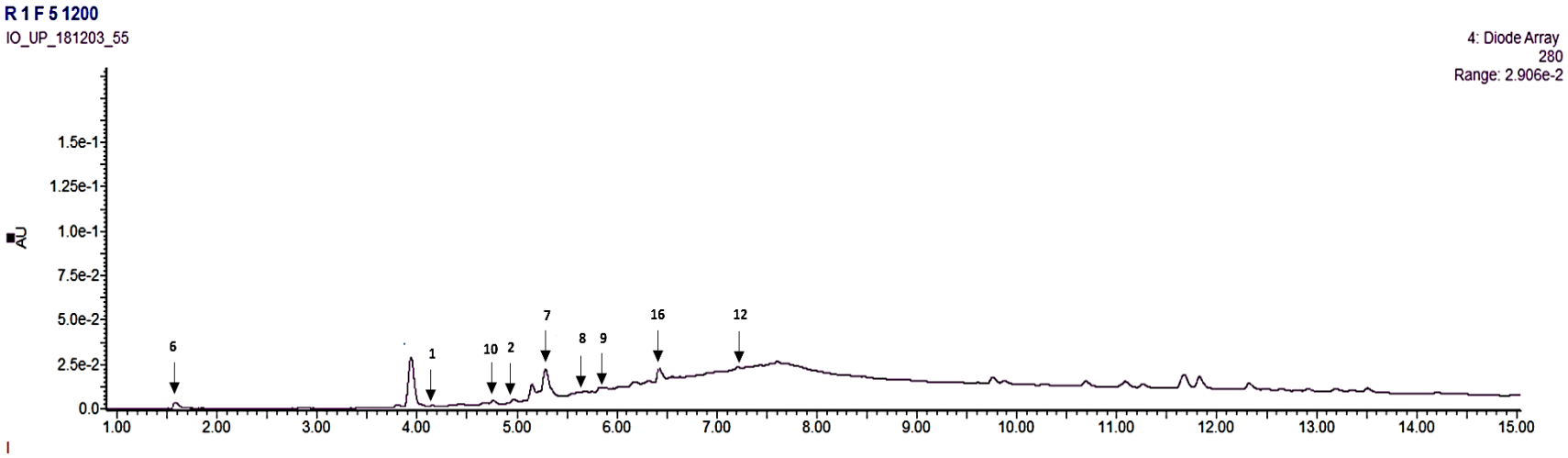
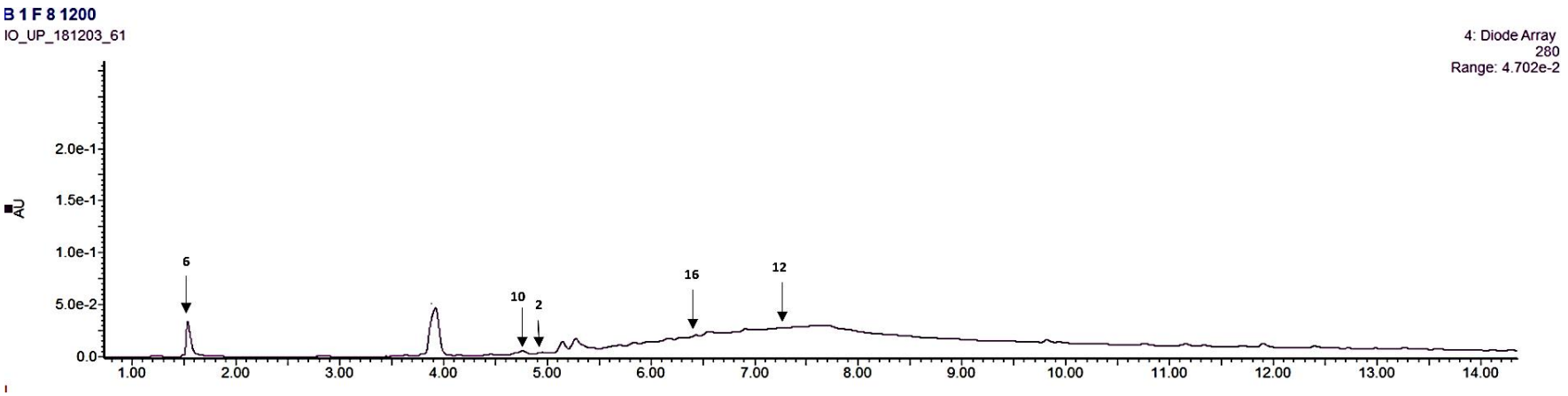


Figure B. 9: UPLC-MS chromatograms (at 280 nm) of formic acid-methanol extracts from pre-soaked brown (A) and red (B) Bambara groundnut seeds microwaved for 5 minutes at 1200 W. 1 = Gallic acid, 2 = Protocatechuic acid, 6 = Ferulic acid hexoside, 7 = Procyanidin B 2 dimer, 9 = Catechin, 10 = Catechin glucoside, 12 = Quercetin-3-O-glucoside, 16 = Hesperidin

A)



B)

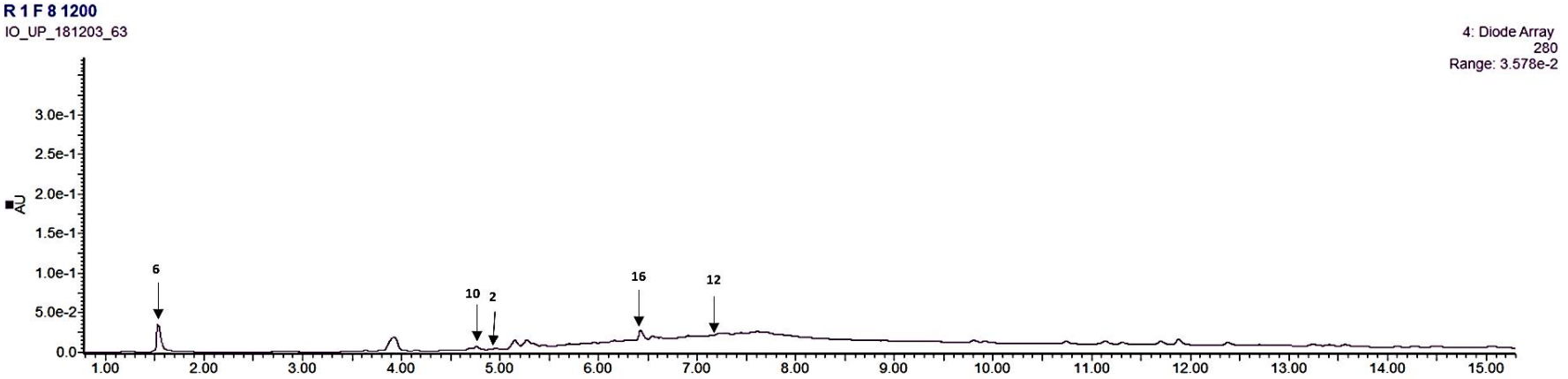


Figure B. 10: UPLC-MS chromatograms (at 280 nm) of formic acid-methanol extracts from pre-soaked brown (A) and red (B) Bambara groundnut seeds microwaved for 8 minutes at 1200 W. 2 = Protocatechuic acid, 6 = Ferulic acid hexoside, 10 = Catechin glucoside, 12 = Quercetin-3-O-glucoside, 16 = Hesperidin