Comprehensive Analytical Profiling of Soluble and Bound Phenolics of Beans from Selected Underutilised Legumes

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ABSTRACT

Beans are seeds of leguminous plants and are rich in macronutrients and micronutrients such as polyphenols predominantly phenolic acids and flavonoids that may have health benefits arising from their antioxidant and other properties. Beans, especially from underutilised legumes, may play an important role in future world food supply. However, there has been limited investigation into the nutritional composition of underutilised beans compared with commercial beans such as soya bean. Therefore, this study aimed to improve the extraction, identification and quantification methodologies for characterizing soluble free, conjugated and insoluble bound phenolics from underutilised beans using high performance liquid chromatography (HPLC) and liquid chromatography – mass spectrophotometry (LC-MS).

Six underutilised beans (adzuki bean, black eyed pea, bambara groundnut, lablab bean, mung bean, pigeon pea) were found to possess a comparable amount of antioxidant activity to commercial beans and this showed positive correlation with their phenolic compounds. Optimisation revealed that 80% methanol was most suitable for extracting soluble phenolics compared with 80% acetone and acetate buffer, although subsequent HPLC profiling showed strong similarities among all three solvents. Alkaline hydrolysis for 5 mins followed by SPE partitioning on soluble extracts and alkaline hydrolysis for 1 h followed by acetonitrile salting out liquid-liquid partitioning on residue were the optimum procedures for estimating the conjugated phenolics and releasing the bound phenolics. The methods showed better recovery, were more solvent friendly and had shorter drying times than ethylacetate liquid-liquid partitioning.

Using 20 phenolic standards, more phenolics were detected by LC-MS than HPLC. Black eyed pea had the most diverse soluble phenolics profile (n=13), followed by adzuki bean and bambara groundnut (n=11) whilst soya bean exhibited the most diverse bound phenolics profile (n=7), followed by pigeon pea and adzuki bean (n=5). Five phenolics were found at the highest concentration in bound extracts from adzuki bean (consist of gallic acid at least

5-fold higher than in soya bean), bambara groundnut (consist of protocatechuic acid at least 160-fold higher than in soya bean), lablab bean (consist of ρ-coumaric acid at least 3-fold higher than in soya bean), black eyed pea (consist of ferulic acid at least 0.5-fold higher than in soya bean), soya bean (sinapic acid) and others were found at highest concentrations in soluble extracts from different beans.

In conclusion, this study has achieved its objectives by developing the comprehensive profiling of phenolics for underutilised beans with optimised extraction methodologies and analysis techniques. The outcome was that underutilised beans are potential alternative resources to commercial beans since they possess higher concentrations of, and more diversified, phenolics than soya bean. This is the first report of optimised extraction methodologies and analysis techniques for the comprehensive analysis of phenolics in underutilised beans. It has generated phenolic profiles from the application of the optimised methodologies and produced useful reference databases for future studies and serves to create an awareness of the potential of underutilised beans as alternative food resources.

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DECLARATION

I hereby declare that the thesis is based on my original work except for the quotations and citations, which have been duly acknowledged. I also declare that it has not been previously submitted or concurrently submitted for any other degree at the University of Nottingham or other institutions.

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LIST OF ABBREVIATIONS

HPLC	High Performance Liquid Chromatography
LC-MS	Liquid Chromatography Mass Spectrophotometry
h	hour
min	minute
TPC	Total Phenolic Content
DPPH	2-Diphenyl-1-picrylhydrazyl
FRAP	Ferric Reducing Antioxidant Potential
M	Molar
SPE	Solid Phase Extraction
GAE	Gallic Acid Equivalent
DW	Dry Weight
w/v	weight per volume
LOQ	Limit of Quantification
LOD	Limit of Detection
ppm	Part per millions
m/z	ion mass over charge

CHAPTER 1
Introduction

Leguminous plants form the third largest plant family of the *Fabaceace* which consist of approximately 750 genera with more than 17,600 species (Du *et al.*, 2014; Lewis *et al.,* 2005). Beans are seeds of leguminous plants and found in pods of variable size, shape and colour. Varieties of beans like chickpea, dry bean, lentils, pigeon pea are classified as pulses that serve as dry grain for human consumption. Up to 2012, annual production of total pulses were ranked fifth among the major food commodities after maize, rice paddy, wheat and barley (FAOSTAT, 2015). Beans are listed in the same category as nuts, meat, poultry, fish and seeds in the US Department of Agriculture food guide pyramid (DOA, 1992) with the recommendation to consume ≥ 2 servings/ day. It reflects the importance of beans to the human diet.

There are a number of commercially significant legume crops like soya bean and chickpea with an average annual production of 245.2×10^6 and 10.0×10^6 tonnes, respectively between 2010 and 2015. However, annual production for beans like pigeon pea, black eyed pea and bambara groundnut were between 2 and 64 fold lower than chickpea within the same period (FAOSTAT, 2018). There are a large number of potential leguminous plants that are currently underutilised. Underutilised beans are those that have remained either unexplored or are localised in a particular region (Bhat & Karim, 2009). These underutilised beans are gaining attention as potential alternative food resources due to their nutritive content. The adoption of these underutilised crops for increased production could contribute to the problem of food security (FAO, 2009d) caused by the rapid growth of world population and reduction in food production (FAO, IFAD & WFP, 2013).

Beans in general are rich in macronutrients- proteins and carbohydrate and micronutrients like vitamins, such as folate and other B vitamins, and minerals such as calcium and zinc (Rebello, *et al.*, 2014; Zhao *et al*., 2014; Broughton *et al.*, 2003; Campos-Vega *et al.*, 2010; Olalekan *et al.*, 2010). On top of that, polyphenols predominantly phenolic acids and flavonoids have been reported

in beans (Rebello *et al.*, 2014). These phenolic compounds contribute to the antioxidant potential of the plant and may protect against oxidative stress (Chaurasia *et al.*, 2014; Zhao *et al.*, 2014; Cho *et al.*, 2013; Kim *et al.*, 2013; Talukdar 2013; Girish *et al.*, 2012; Sreerama *et al.*, 2012; Xu & Chang, 2012; Vadivel *et al.*, 2012; Marathe *et al.*, 2011; Wang *et al.*, 2011; Gutiérrez-Uribe *et al.*, 2011; Kanatt & Sharma, 2011; Vadivel *et al.*, 2011; Boateng *et al.*, 2008; López-Amorós *et al.*, 2006). Oxidative stress is a major cause of degenerative diseases such as coronary heart disease, diabetes and cancer.

Studies on the anti-oxidative properties of beans are limited to the commercial beans such as soya bean and chickpea. Similar research, especially information concerning the phenolic composition, from underutilised beans such as black eyed pea, adzuki bean, lablab bean, pigeon pea, mung bean and bambara groundnut are lacking. Past investigations of underutilised beans have been limited to identify some of the soluble conjugated and free phenolic acids such as gallic acid, cinnamic acid, ferulic acid and coumaric acid (Girish *et al.*, 2012; Gutiérrez-Uribe, *et al.*, 2011; Amarowicz & Pegg, 2008; Xu & Chang, 2007; Troszyńska *et al.*, 2006). Hence, the lack of more detailed phytochemical profiles is one of the hurdles in raising the awareness and promoting the benefits of underutilised beans. Phytochemical profiling becomes important because it provides a blueprint of compounds for individual plants and represents the hidden potential health benefits brought by the compounds. This database would be a useful tool for further development in agricultural research and development.

Most phenolics in plants exist as either free phenolics, often within the cell vacuole, soluble phenolic conjugates with other compounds such as sugars or as insoluble bound phenolics (Pandey and Rizvi, 2009). Insoluble phenolics can be covalently bound to cell wall components such as cellulose and are only released within the gastrointestinal tract by colonic microflora. The ability of these phenolics to survive through digestion in the stomach and intestinal tract and reach the colon may contribute to the prevention of colon cancer and other digestive tract cancers (Liu, 2007). About 85 %, 75 % and 62 % of the total phenolics in corn, wheat and rice, respectively have been reported as insoluble

bound phenolics while an average of 24 % of the total phenolics are bound phenolics present in other food matrices (Adom & Liu, 2002).

Free phenolics and soluble conjugates can be extracted directly from the bean using a range of solvents and without the need for any treatment other than raw material processing such as homogenisation and milling. Numerous efforts to investigate the soluble phenolics include optimisation of the extraction technique and determination of the health promoting potential of the crude extracts. There has been limited attention payed to analytical techniques like HPLC and LC-MS targeted at actual profiling of the phenolic compounds present (Pająk *et al.*, 2014; Ojwang *et al.*, 2013; Amarowicz *et al.*, 2008; Cai *et al.*, 2003; Arts & Hollman, 1998; Franke *et al.*, 1994). Similarly, most recent studies have mainly focussed on soluble phenolics whilst bound phenolics, especially in beans, are being neglected.

Therefore, this current study aimed to improve the extraction, identification and quantification methodologies for investigating the polyphenols in selected underutilised beans. These improved methodologies will enable the characterization of the soluble free, conjugated and bound phenolics found in beans by using high performance liquid chromatography (HPLC) and liquid chromatography mass spectrophotometry (LC-MS). This will be the first complete method set to investigate and profile the targeted soluble and bound phenolics for underutilised beans. Moreover, it is also the first report of profiling compounds from soluble and bound samples of selected underutilised beans.

The outcome of this study will be an improved methodology as initial guidelines for the future phytochemical study for legumes and potentially other food crops. On top of that, the study also generates a useful reference database for future research for tracking antioxidant contents of beans during the food supply chain, such as growth, postharvest handling, downstream processing and marketing, and future nutraceutical research in deciphering the role and mechanism of these phytochemicals in the human diet.

1.1 Aims and objectives

Hypothesis

Antioxidant potential and phenolic compounds are both variable in commercial and underutilised beans.

Aims

To investigate the antioxidant potential and profile the soluble and bound phenolics from selected commercial and underutilised beans.

Objective

To develop an improved extraction, identification and quantification methodology to profile phenolics in plant materials using high performance liquid chromatography (HPLC) and liquid chromatography mass spectrophotometry (LC-MS). Then to apply this methodology to selected commercial and underutilised beans.

Specific objectives

1. To screen the antioxidant activities of selected commercial and underutilised beans using three independent assays, namely total phenolic content assay (TPC), DPPH free radical scavenging potential assay (DPPH) and ferric reducing antioxidant potential assay (FRAP).

2. To develop optimised extraction techniques for both soluble and bound phenolics from beans.

3. To develop an optimised treatment for the analysis of soluble conjugated and bound phenolics.

4. To investigate the limitations of these three optimised techniques.

5. To apply the developed methodologies for the extraction and analysis of soluble phenolics, deconjugated free phenolics and bound phenolics to selected underutilised beans.

6. To determine the antioxidant activities of soluble, deconjugated free and bound phenolics using TPC, DPPH and FRAP assays.

7. To identify and quantify soluble phenolics, deconjugated free phenolics and bound phenolics from selected underutilised beans with phenolic standards using high performance liquid chromatography (HPLC) and liquid chromatography- mass spectrophotometer (LC-MS)

8. To analyse the HPLC and LC-MS profile of the unidentified peaks from selected underutilised beans.

CHAPTER 2

Literature review

2.1 Beans

2.1.1 Introduction to beans from underutilised legumes

Leguminous plants (Family: *Fabaceace*) consist of approximately 750 genera with more than 17,600 species, forming the third largest plant family (Du *et al.*, 2014; Lewis *et al.,* 2005). Seeds of leguminous plants - known as beans are found in pods of variable size, shapes and colour. They are grown in tropical and subtropical countries with warm temperature. Beans such as kidney bean, chickpea, mung bean, lentil, black eyed pea, adzuki bean are grown in Asia, America and Africa (Campos-Vega *et al.,* 2010).

It typically requires 55 to 60 days from planting to harvesting. When the pods mature, they turn yellow and dry up, the beans inside change from green to their mature colour (Food Outlook: FAO, 2014). Mature beans such as adzuki bean is in red colour, mung bean is in green colour and bambara groundnut in black to dark brown. They are consumed as whole beans (named as grains), split or dehusked (named as *dhals*), cooked, fermented, milled or ground into flour (Tharanathan and Mahadevamma, 2003). They are classified into commercial or underutilised beans, according to availability and popularity of consumption.

Commercial beans, such as soya bean and chickpea are widely consumed and the price is high. The range of annual producer price of soya bean and chick pea from the highest production region was higher than the underutilised beans between 2011 and 2015 (table 2.4). Consequently, consumer price also higher following the trend of producer price. As a result, beans are not affordable for every consumer despite their high nutrient value. Therefore, the world forum is focussing on underutilised beans as a sustainable food source with a more affordable price.

Apart from that, the range of producer price for underutilised beans were constant, in decreasing trend or huge fluctuation from 2011 to 2015 (table 2.4). For example, the range of producer price for bambara groundnut in Africa was

kept constant around 700 USD/ tonnes. And, the range of producer price for pulse (nes) showed a fluctuation between 1500 USD/ tonnes to 3000 USD/ tonnes within the range of year. It showed that farming the underutilised beans could not generate a constant and secure income to the farmers which subsequently lead to reduce in production.

Underutilised beans are those which have remained either unexplored or are localised in a particular region (Bhat & Karim, 2009). The Food and Agriculture Organization of the United Nation (FAO), the International Centre for Underutilised Crops (ICUC) and the Global Facilitation Unit for Underutilised Species (GFU) have established a reference list of proposed criteria for underutilised crops as shown in table 2.1 (GFU, 2013). In addition, beans such as *Vigna* spp - cowpea, mung bean, adzuki bean, lablab and *Cajanas cajan* have been mentioned as underutilised crops in some publications (GFU, 2013; Padulosi *et al.,* 2011; Williams & Haq, 2000). They are less popular for daily food consumption because their usage is restricted to specific regions and they are not commonly available.

Table 2.1 Reference list of proposed criteria for underutilised foods (GFU, 2013).

	Criteria
1.	For human consumption
2.	Have potential for contributing to food security and nutrition
3.	Are mainly local and traditional crops/animals and whose distribution, biology, cultivation and uses are poorly documented
4.	Limited attention from research, farmers, policy and decision makers and consumers
5.	Limited seed/animal germplasm supply systems
6.	Are farmed, reared, gathered and caught on a small scale
7.	Imported species are excluded as underutilised in that region

Pulses are defined as annual leguminous crops yielding one to twelve seeds of variable size, shape and colour within a pod (FAO, 1996). Pulses such as mung bean, chickpea, broad bean and pea are crops that are harvested solely for dry grain. Annual production of total pulses were constantly increased from 2004 to 2012 by at least 14%. Up to 2012, total pulses were ranked fifth after the major food commodities -maize, rice, wheat, barley (FAO, 2015). Hence, beans have been getting increased attention as food resources over the past 10 years. A number of the underutilised beans have been classified by the FAO as pulses (table 2.2) for human consumption.

Table 2.2 Pulses which are used mainly for human consumption.

Commodities	Latin Name
Bean, Dry (*Phaseolus spp*: include certain types of *Vigna* bean as highlighted which were classified as *Phaseolus* in the past)	
Kidney bean, haricot bean, adzuki bean*	*Phaseolus angularis*
Lima, butter bean *	*Phaseolus lunatus*
Mungo bean, golden, green gram*	*Phaseolus aureus*
Black gram, urd	*Phaseolus mungo*
Scarlet runner bean	*Phaseolus coccineus*
Rice bean*	*Phaseolus calcaratus*
Moth bean	*Phaseolus aconitifolius*
Tepary bean*	*Phaseolus acutifolius*
Broad bean, dry	
Horse bean	*Vicia faba var equine*
Broad bean	*Vicia faba var major*
Field bean	*Vicia faba var minor*
Peas, dry	
Garden pea	*Pisum sativum*
Field pea	*Pisum arvense*
Chick peas	
Chickpea, Bengal gram, garbanzos	*Cicer arietinum*
Cowpeas, dry*	
Cowpea, black eye pea/bean	*Vigna sinensis; Dolichos sinensis*
Pigeon peas*	
Pigeon pea, cajan pea, congo bean	*Cajanas cajan*
Lentils	*Lens esculenta*
Bambara beans*	
Bambara groundnut, earth pea	*Vigna subterranean*
Pulses Nes (Minor pulses)	
Lablab, hyacinth bean*	*Dolichos spp*
Jack or sword bean*	*Canavalia spp*
Winged bean*	*Psophocarpus tetragonolobus*
Guar bean*	*Cyamopsis tetragonoloba*
Yam bean*	*Pachyrrhizus erosus*

**classified as underutilised beans by GFU, 2013*

The majority of these pulses represent commercial beans and only a minority are underutilised beans. Detailed statistical reports revealed that the production of underutilised beans are far lower than commercial beans such as soya bean and chickpea. The annual production of underutilised beans ranged between 2 fold and 64 fold lower than soya bean from 2011 to 2015 (table 2.3). Lower demand for underutilised beans may be due to a number of reasons such as limited research studies and supporting data about underutilised beans globally. Consequently, greater understanding is needed to further develop these beans into greater production and to promote the underutilised beans worldwide.

Table 2.3 Production of selected beans from 2010 to 2013.

	Highest production region	production, x 10^6 tonnes				
		2011	2012	2013	2014	2015
Soya bean[++]	America	224.6	203.0	240.9	269.6	287.8
Chickpea[++]	Asia	10.1	9.5	11.1	11.4	9.1
Dry peas[+]	Europe	4.1	3.4	3.0	3.4	4.3
Pigeon pea	Asia	3.7	3.5	3.8	3.8	3.4
Broad peas[+]	Asia	1.8	1.8	1.7	1.5	1.6
Black eyed pea	Africa	4.5	8.0	5.9	5.4	5.6
Dry beans[+]	Asia	11.2	10.3	10.2	11.8	12.2
Bambara	Africa	0.15	0.22	0.24	0.16	0.16
Pulse (nes) [+]	Asia	1.6	1.9	2.1	2.1	2.0

+ consists of more than 1 species of legume

++ commercial beans

Table 2.4 Producer price of selected beans from 2011 to 2015.

	Highest production region	Producer price, USD per tonnes				
		2011	2012	2013	2014	2015
Soya bean[++]	America	308-694	363-867	321-3758	308-808	293-3316
Chickpea[++]	Asia	363-3910	520-3231	489-2669	479-2294	566-1979
Dry peas[+]	Europe	226-696	263-583	279-2404	213-861	182-1633
Pigeon pea	Asia	1081	1033	818	772	758
Broad peas[+]	Asia	633-1740	602-2151	739-1069	650-1563	751-1335
Black eyed pea	Africa	375-839	382-782	388-759	350-671	350-713
Dry beans[+]	Asia	587-4520	642-3909	769-1069	650-1563	751-1353
Bambara	Africa	636-683	658-758	721	726	654
Pulse (nes) [+]	Asia	463-1544	377-2867	771-2894	803-1507	474-1610

+ consists of more than 1 species of legume

++ commercial beans

2.1.2 Nutritional value of beans from underutilised legumes

Beans are a source of macronutrients such as proteins, micronutrients such as minerals, dietary fibre and phytochemicals (Zhao *et al*., 2014; Campos-Vega *et al.*, 2010; Olalekan *et al.*, 2010; Broughton *et al.*, 2003; Messina, 1999). A comprehensive datasheet about micronutrients and macronutrients of beans has been compiled by the United State Department of Agriculture. Some of the major nutrients in commercial and underutilised beans have been tabulated (table 2.5) from the datasheet for comparison purposes. Underutilised beans could potentially represent an alternative to current commercial beans without any significant loss of nutritional value.

The first criteria for any alternative food resource is to be able to provide sufficient energy – carbohydrate- for daily activities. We depend on the major food commodities such as rice, wheat and grain to provide energy all the time. Underutilised beans provide a carbohydrate content that is 2 fold higher than soya bean. The carbohydrate contents are about 15% less than the major food commodities such as white rice, wheat and corn grain with 79.15g, 75.36g and 74.26g per 100g dry material respectively (USDA Release 28, 2016). Therefore, they are potentially an alternative source of carbohydrate.

Secondly, beans are described as low cost protein sources. Protein plays an important role as a functional and structural component for every cell in the body. One notable study reported that the protein content of beans ranged from 17g to 40g/ 100g which is approximately three times higher than the protein content of cereal (Zhao *et al.,* 2014). This finding is supported by Du *et al.* (2014) who showed that the protein content of mung bean (27.1g/ 100g) is higher than in cereal (7.5 – 12g/ 100g), poultry (15 – 20g/ 100g), eggs (2.8g/ 100g) and meat (10-20g/ 100g). Despite the protein level from underutilised beans being lower than soya bean, they are compatible with other commercial beans such as chickpea (table 2.5).

Fat aids in the absorption of the fat soluble vitamins A, D, E, K and carotenoids. However, high fat intake is often correlated with weight gain and susceptibility to obesity and its complications such as high risk of coronary heart disease (Otten *et al.,* 2006). Hence, we should have a balance of fat intake among foods instead of consuming all foods that are high in fat. We have a choice to select foods that are lower in fat as alternatives such as from underutilised beans. Fat content of underutilised beans is far lower than soya bean (30.16 g/100g) and chickpea (6.04 g/100g).

Although micronutrients such as calcium, magnesium, potassium and iron in the underutilised beans are not as high as from soya bean, they are comparable with other commercial beans such as chickpea, lentil and kidney bean. In contrast, the sodium content from the listed underutilised beans are higher than the commercial beans (table 2.5).

In addition to the promising nutrient level, beans have been categorized as low glycaemic index (GI) foods especially underutilised beans (table 2.6). Their GI indexes are at least three fold lower than that of white rice. Low GI index foods reduce the risk of developing type 2 diabetes, cardiovascular disease and certain cancers such as colon cancer (Foster-Powell *et al.,* 2002). Jenkins *et al.* (2012) revealed that a low-GI beans diet reduced HbA1c values by 0.5% better than a wheat fibre diet. Hence, underutilised beans are a good alternative food especially for diabetic patients.

Table 2.5 National Nutrient Database for Standard Reference release 27 for legumes by United States Department of Agriculture (USDA).

		Chickpea	Kidney bean	Soya bean	lentil	Hyacinth bean^	Adzuki bean^	Pigeon pea^	Black eyed pea^	Mung bean^
Protein	g	20.47	22.53	36.49	24.63	23.9	19.87	21.7	23.52	23.86
Total lipid (fat)	g	6.04	1.06	19.94	1.06	1.69	0.53	1.49	1.26	1.15
Carbohydrate,	g	62.95	61.29	30.16	63.35	60.74	62.9	62.78	60.03	62.62
Calcium, Ca	mg	57	83	277	35	130	66	130	110	132
Iron, Fe	mg	4.31	6.69	15.7	6.51	5.1	4.98	5.23	8.27	6.74
Magnesium, Mg	mg	79	138	280	47	283	127	183	184	189
Potassium, K	mg	718	1359	1797	677	1235	1254	1392	1112	1246
Sodium, Na	mg	24	12	2	6	21	5	17	16	15

*value per 100g dry, raw material

^underutilised beans

Table 2.6 Glycaemic index for selected beans.

Beans	Glycaemic index, GI
Chickpea	33
Hyacinth bean*	na
Kidney bean	29
Adzuki bean*	na
Pinto bean	39
Mung bean*	31
Green pea	54
Soya bean	15
Black turtle bean	30
Black gram	43
Pigeon pea*	22
Black eyed pea*	33
Moth bean	36
Lima bean	32
25g portion of glucose, fed with oats	92
Jasmine rice, white long grain, cooked in rice cooker (Golden World Food, Thailand)	109

Notes:
na – not available
Adapted from (Foster-Powell *et al.*, 2002)
Selected GI are the closest description to the raw beans. GI value is differed by the source of sample and process involve while cooking
Glucose as reference standard, GI for glucose =100

2.1.3 Bio-protective potential of beans

Plant based diets, especially those containing fruits and vegetables, have been widely suggested to reduce the risk of developing diseases such as cardiovascular diseases, diabetes, cancer and hypertension (Deng *et al.*, 2012; Vadivel *et al.,* 2012; Naczk and Shahidi, 2006). The pathogenesis of these diseases are thought to involve oxidative stress (Mishra *et al.,* 2011). Hence, antioxidant capacity screening of food is a potentially useful indicator of health related benefits. Therefore, a quality daily food intake is recommended as a disease preventive measure.

Oxidative stress happens due to the accumulation of free radical species in the body. Free radicals are unstable molecules and occur in our body due to environmental factors or biological factors such as UV, pollution and smoking. They can also be naturally produced endogenously as a result of metabolic reactions such as lipid peroxidation where oxygen species are intermediate electron acceptors or donors (Wang *et al.*, 2011). There are two major types of free radicals, reactive oxygen species (ROS) such as superoxide anion, hydroxyl radical and reactive nitrogen species (RNS) such as nitric oxide.

Under normal conditions, biological systems will trigger several enzymatic activities involving superoxide dismutase, catalase and peroxidase to counter react the oxidative stress. However, the counter reaction becomes slower as we age, and if immunity is lowered due to illness and unhealthy lifestyle. Therefore, supplementary exogenous dietary antioxidants are highly recommended to stabilize the oxidants.

Antioxidants are compounds that are able to stabilise existing free radicals, to inhibit formation or to interrupt propagation of free radicals by different mechanisms such as by interrupting the auto oxidative chain reaction (Nawar, 1996). The most effective mechanism is the interruption of the free radical reaction by donating $H^{\bullet}$ to the free radicals formed during oxidation and becoming a radical themselves. This agent is known to be antioxidant and often contains aromatic or phenolic rings (Brewer, 2011).

Phytochemicals from plants are one source of dietary antioxidants due to their aromatic or phenolic rings that are able to stabilise the free radical (Nawar, 1996). Phenolic compounds from beans are generally correlated to the antioxidant activity and a wide spectrum of health related bioactivities (Dzialo, 2016; Chen *et al.,* 2015; Zhao *et al.*, 2014; Xu & Chang, 2012; Girish *et al.,* 2012; Yao *et al.,* 2011; Amarowicz and Pegg, 2008; Duenas *et al.*, 2006). Moreover, the antioxidant activity from beans has also been measured by cellular antioxidant activity assay instead of relying on the colorimetric assays (Xu and Chang, 2012).

A limited number of underutilised beans, such as adzuki bean, mung bean and black eyed pea, have been included in antioxidant activity studies (Luo *et al.,* 2016; Zhao *et al.,* 2014; Sreerama *et al.,* 2012; Mishra *et al.,* 2011; Kanatt *et al.,* 2011; Yao *et al.,* 2011; Amarowicz *et al.,* 2008; Siddhuraju & Becker, 2007;). There are many more underutilised beans that are awaiting to be investigated such as bambara groundnut, lablab bean and pigeon pea. A lack of such information pertaining to the health benefits is one reason that is inhibiting the promotion of underutilised beans.

Other health benefits from underutilised beans such as the potential presence of antidiabetic activity from adzuki bean and mung bean (Luo *et al.,* 2016; Itoh *et al.,* 2004), anti-proliferation of different types of cancer cells namely- human mammary hormone dependent cancer cell, colorectal adenocarcinoma cells, hepatocellular carcinoma cells, and gastric adenocarcinoma cells by black eyed pea (Xu & Chang, 2012; Gutiérrez-Uribe *et al.,* 2011), inhibition of pancreatic lipase activity from mung bean, red bean and moth bean(Sreerama *et al.,* 2012), antimicrobial activity from list of legumes such as pigeon pea (Devi *et al.,* 2016; Kanatt *et al.,* 2011), inhibition of tyrosinase activity, anti LDL-lipid peroxidation (Bazzano *et al*., 2011) and antihypertension from adzuki bean (Mukai & Sato, 2009; Xu *et al.,* 2007). These are believed to be related to antioxidant potential. Activity that may not be directly related to antioxidant potential include estrogen-like activities (Zhao *et al.*, 2005).

2.2 Plant polyphenols

Vascular plants synthesise a diverse range of organic molecules named as phytochemicals (Stalikas, 2007; Harborne, 1982). They are known as biologically-active, non-nutritive secondary metabolites that have many functions in plants ranging from structural to protection against insect, fungal, bacterial and viral infections (Wink, 2013; Johnson & Williamson, 2003). For example, seed coat tannin protect the cowpea from being eaten by insects (Lattanzio *et al.*, 2005). One class of phytochemical are the phenols which consist of approximately 8,000 naturally occurring compounds that play a protective function in the plant host and exhibit a series of potential health-related benefits (Dzialo, 2016; Stalikas, 2007).

In general, there are three main groups of polyphenolic compounds- phenolic compounds, glucosinolates and carotenoids (Johnson & Williamson, 2003) (figure 2.1). Each group has its own differential characteristics such as chemical structure, biosynthetic pathway, functionality and mode of action. Phenolic compounds from plants are of research interest due to their significant antioxidant activity (Xu & Chang, 2007). Apart from that, phenolic compounds are also known for their broad spectrum of biological properties due to their molecular structure. They possess one or more aromatic ring with one or more hydroxyl groups (Brandolini *et al.*, 2013).

Approximately 8000 different plant phenolics are known (Dzialo, 2016) and these are subdivided into flavonoids and non-flavonoids (Brewer, 2011) (figure 2.1) according to the number of phenol rings that they contain and the structural elements that bind rings to each another (Manach *et al.*, 2004). Flavonoids are further subdivided into six classes, flavonols, flavanones, flavones, isoflavones, flavanols and anthocyanidins. The subclasses for non-flavonoids are phenolic acids, lignans, stilbenes, tannins and lignins. Each category will express their individually varying reactions due to the variety of their functional groups.

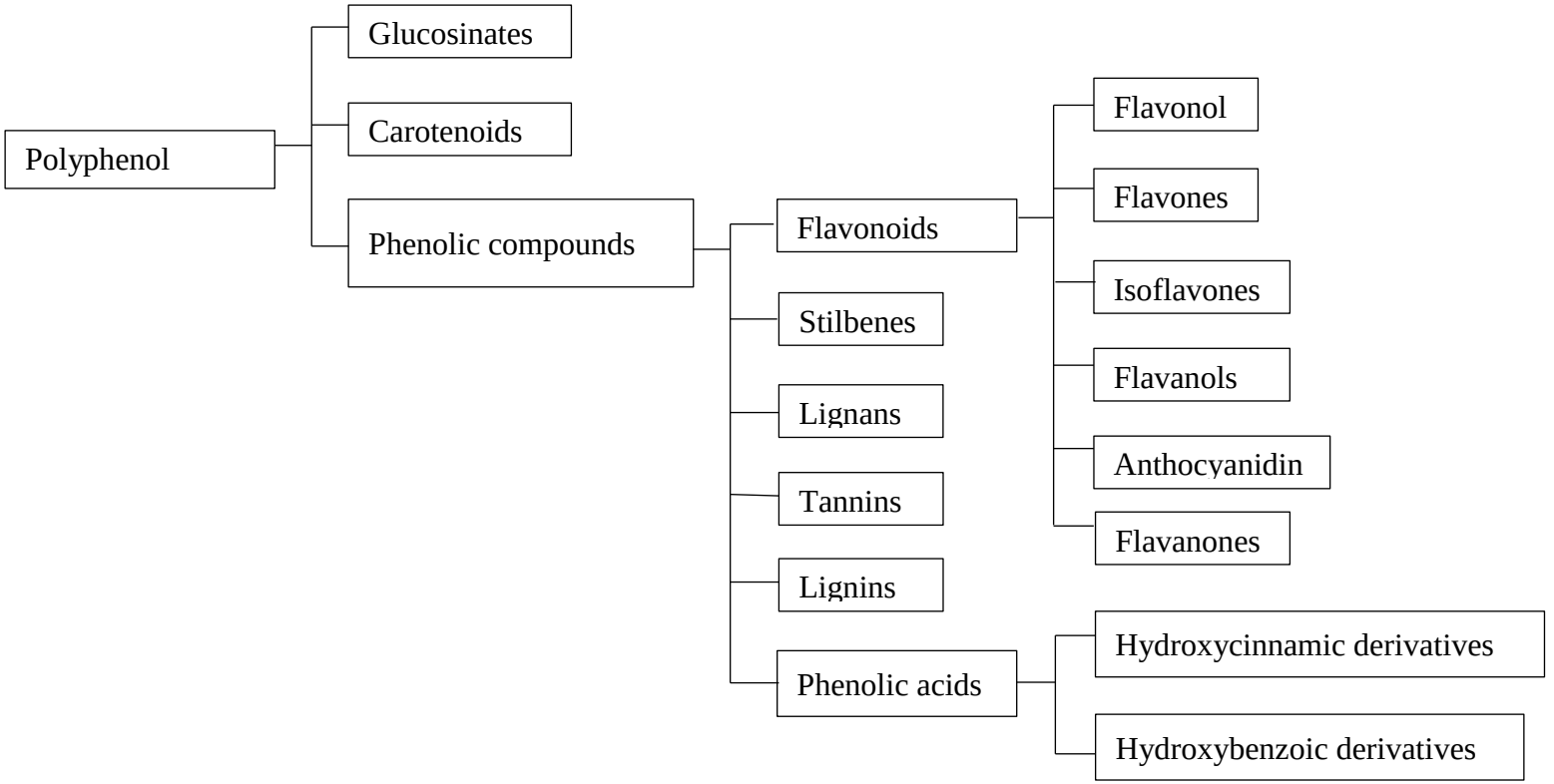


Figure 2.1 Classification of polyphenol.

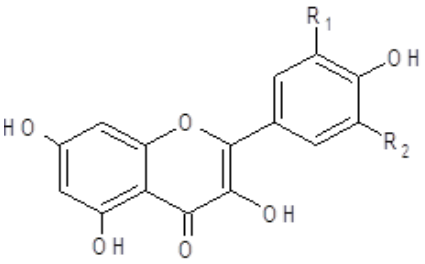
Plant phenolics are synthesized through either the shikimate (phenylpropanoid) or polyketide acetate (malonate) pathways, or both (Quidea, 2006; Quidea, 2004). Each class of phenolic compound can be detected at a distinctive absorption wavelength, thus phenols and simple phenolic acids show spectral maxima in the range 250 – 290 nm; hydroxycinnamic acid derivatives are detected in the range of 290 – 330 nm; flavones and flavonols exhibit absorption bands at 250 – 350 nm; anthocyanins exhibit absorption in the visible region 475 – 560 nm, 535 – 545 nm and have subsidiary peaks at 270 – 275 nm (Lattanzio *et al.*, 2008). This characteristic is useful to distinguish the compounds in biochemical assays and analytical studies.

Flavonoids are the largest and most diverse group of plant phenolics (figure 2.2). They share 2 aromatic rings that are linked by 3 carbon atoms that form an oxygenated heterocycle ring C (Manach *et al.*, 2004). Subclass - flavonols are most common in foods such as in broccoli, onion, leeks and tea but generally occur at relatively low concentrations. They exist as glycosylated forms, most often with glucose or rhamnose. They are found in the outer and aerial tissues (skin and leaves) because their synthesis requires light. Quercetin and kaempferol are the most commonly found examples. Another subclass - flavanones are found in high concentrations only in citrus fruit; but they exist in small amounts in tomatoes and some aromatic plants such as mint. For example, naringenin in grapefruit, hesperetin in oranges and eriodictyol in lemon (Manach *et al*, 2004).

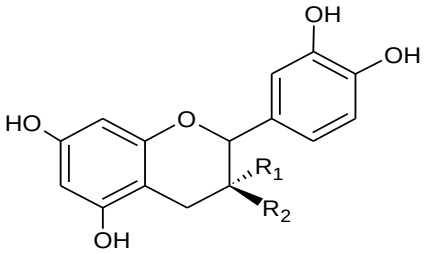
Isoflavones, as phytoestrogen, are structurally similar to estrogen and can elicite pseudohormonal effects. They are commonly found in leguminous plants. Genistein, daidzein and glycitein are the 3 major isoflavones in soya and its processed products are the main source of isoflavones in the human diet (Manach *et al*, 2004). However, flavones have been reported to be less common than flavonols and isoflavones in fruit and vegetables. They exist as C-glycosides flavones in millet and wheat and as tangeretin, nobiletin in the skin of citrus fruits (Manach *et al.*, 2004).

The molecular structure of flavanols (figure 2.2) shows that they can exist in monomer form, such as catechin, or polymer form, such as proanthocyanidins (Manach *et al.*, 2004). Catechin and epicatechin are mainly found in fruits such as apricot. However, gallocatechin, epigallocatechin and epigallocatechin gallate are found in certain leguminous plants, grapes and teas (Manach *et al.*, 2004). Proanthocyanidins are dimers oligomers and polymers of catechins that are bound together. They are responsible for the astringency of fruits such as grapes, peaches and berries and beverages such as wine, cider and beer and bitterness in chocolate. However, the astringency changes when the fruit reaches ripeness. It is hard to estimate the proanthocyanidin content due to its wide range of structures and molecular weights.

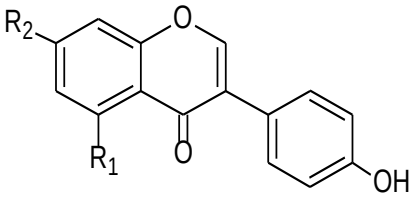
Lastly, the subclass- anthocyanins are pigments giving pink, red, blue or purple colours in fruits and flowers. They exist in the aglycone form (anthocyanidins), glycosylated, esterified with organic acids or phenolic acids in different chemical structures both coloured and uncoloured according to pH (Manach *et al.*, 2004). Some of the foods in our diet contain high amounts of anthocyanins e.g. red wine, cereals, leafy and root vegetables such as beans, onions, radishes and are abundant in fruit. Cyanidin is one of the most common anthocyanin in food.



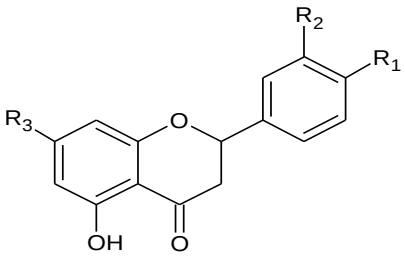
Flavonols	R1	R2
Kaempferol	H	H
Quercetin	OH	H
Myricetin	OH	OH



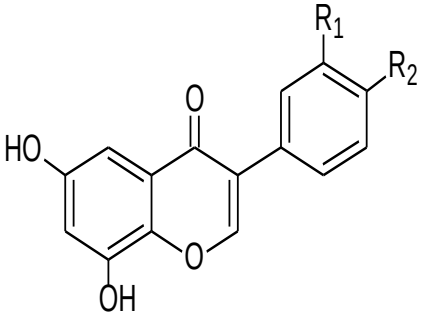
Flavanols	R1	R2
Epicatechin	OH	H
Catechin	H	OH



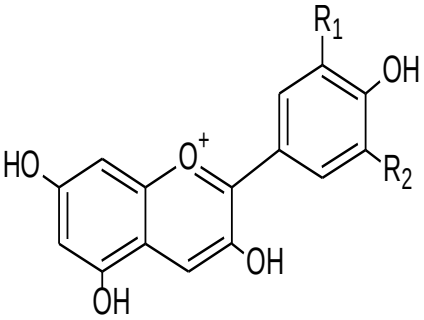
Isoflavones	R1	R2
Daidzein	H	OH
Genistein	OH	OH



Flavanones	R1	R2	R3
Naringenin	OH	H	OH



Flavones	R1	R2
Apigenin	H	OH
Luteolin	OH	OH



Anthocyanidins	R1	R2
Cyanidin	OH	H
Delphinidin	OH	OH

Figure 2.2 Chemical structure of flavonoids.

Phenolic acids are simple phenolic compounds and are divided into two subclasses, derivatives of benzoic acid and derivatives of cinnamic acid. Hydroxycinnamic acids such as caffeic acid, chlorogenic acid, ferulic acid, o-, ρ- coumaric acid and sinapic acid are the most common phenolic acids in plant tissues (Saxena *et al.,* 2012) (figure 2.3 to 2.4). They often exist as conjugated forms in plants forming ester or glycosides with carboxyl acids, such as quinic acid, shikimic acid and tartaric acid, or glucose (Manach *et al.*, 2004). However, they are rarely found in free form except in processed food. Chlorogenic acid (caffeic acid bound with quinic acid) is another example it is found in many types of fruit and has a high concentration in coffee (figure 2.3).

Derivatives of benzoic acid such as gallic acid, hydroxybenzoic acid and syringic acid are found in low concentrations in edible plants and occurs only in glycoside forms. They also form more complex structures such as hydrolysable tannins such as ellagitannins in red fruit such as strawberries (Manach *et al.*, 2004.). Both free and esterified forms are limited to certain plants, hence there is a lack of extensive study and nutritional interest (Manach *et al.*, 2004).

Other phenolic compounds include the tannins which are classified as either hydrolysable tannin (complex polymer structure consisting of phenolic acids and sugars) and condensed tannins (combination of flavonoids). Pomegranates are a rich source of hydrolysable tannins (Gil *et al.,* 2000). Other compounds include the lignans. Some of the main lignan compounds are secoisolariciresinol, lariciresinol, pinoresinol and matairesinol. Sources of lignan are oilseed, which is higher than sesame seed, flaxseed and buckwheat (Naczk & Shahidi, 2006; Thompson *et al.,* 1991). Resveratrol is an example of a stilbenes it is present in both grape leaves and berries (Versari *et al.*, 2001)

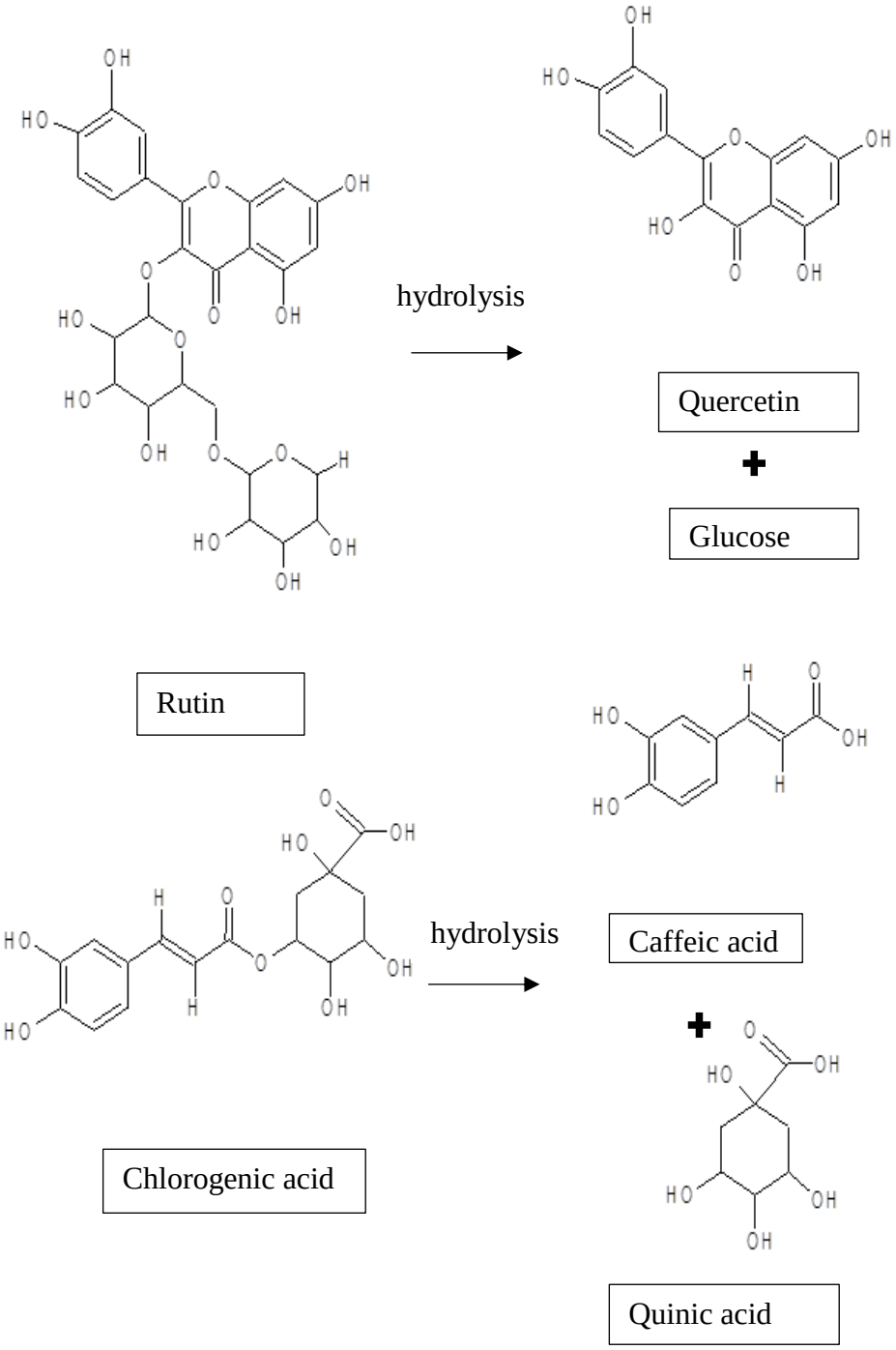
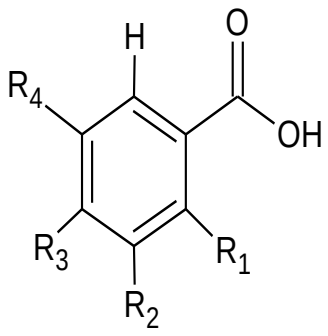
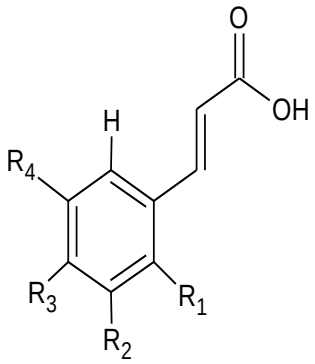


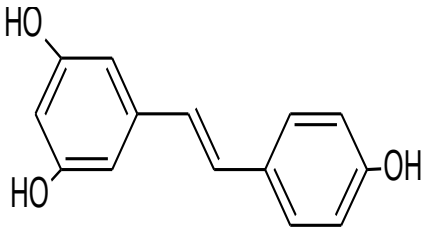
Figure 2.3 Breakdown of conjugated phenolics.

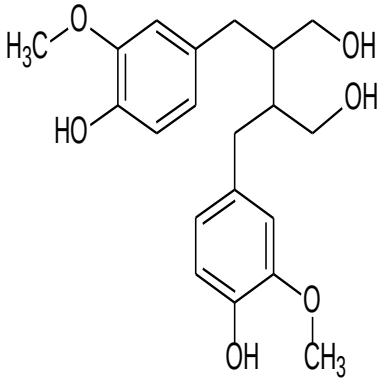




Derivatives of benzoic acid	R1	R2	R3	R4
Gallic acid	H	OH	OH	OH
Protocatechuic acid	H	OH	OH	H

Derivatives of cinnamic acid	R1	R2	R3	R4
ρ-coumaric acid	H	H	OH	H
Ferulic acid	H	OCH_3	OH	H





Stilbenes
Resveratrol

Lignans
Secoisolariciresinol

Figure 2.4 Chemical structure of phenolic acids, stilbenes and lignans.

2.3 Phenolic compounds

A diversity of phenolic compounds are found in beans such as flavonoids, phenolic acids, lignans and proanthocyanidin (Ramírez-Jiménez *et al.*, 2014; Yao *et al.*, 2011; Amarowicz & Pegg, 2008; Troszyńska *et al.*, 2006). These are found in the leaf, seed and seed coat at different concentration levels and categories (Cho *et al.*, 2013; Onyilagha *et al.*, 2009;). The seeds of leguminous beans consists of cotyledon (89%), seed coat (10%) and embryonic axe (1%) (Duenas *et al.*, 2006). The cotyledon is a protein and carbohydrate source while the seed coat contains the highest concentration of phenolic compounds because it acts as a protective barrier for the cotyledon (Duenas *et al.*, 2006; Lattanzio *et al.*, 2005). Whole bean has been the focus of previous investigations because it provides both nutrients and health related properties.

Phenolic compounds are present in either the soluble free, conjugated or insoluble forms (Acosta-Estrada *et al.*, 2014; Naczk and Shahidi, 2004; Krygier *et al.*, 1982). Research to estimate the levels of free, soluble conjugated and insoluble bound phenolics started in 1980s in different foods (Krygier *et al.*, 1982). Documented reports reveal that most phenolic compounds exist in either the soluble free or conjugated forms in fruit and vegetables (Acosta-Estrada *et al.*, 2014; Sun *et al.*, 2002; Chu *et al.*, 2002; Adom & Liu, 2002). An average of only about 24% of total phenolics are bound in food matrices (Adom and Liu, 2002). For example, carrots, banana and potato have 37.6%, 33.1% and 39.9% total phenolics as bound phenolics, respectively (Sun *et al.*, 2002; Chu *et al.*, 2002).

2.3.1 Soluble phenolics

Soluble phenolic compounds consist of free and conjugated phenolics that are extractable in solvents and found in plants at different concentration levels. Most of the soluble phenolic compounds exist in the conjugated form in the plant and they are linked with sugar residues through one or more hydroxyl groups or esterified (Ascensao and Dubery, 2003). Ferulic acid and caffeic acid are examples of free phenolic compounds whilst rutin is an esterified phenolic formed from the conjugation of quercetin and glucose.

The nature and characteristics of the soluble phenolic compounds determine the nature of the extraction process best used to recover or isolate these phytochemicals from plant materials (Stalikas, 2007). The solvent extraction system is different for hydrophilic and hydrophobic compounds. This means that there are no definitive solvents that can be used to extract soluble phenolics, the solvent of choice depending on the targetted compounds and their nature. Alcohols, acetone, hexane and ethylacetate are some examples of solvents that have been employed in the past.

Alcohols such as methanol, ethanol, acetone, either absolute or at varying ratios with water are commonly used extraction solvents. Thusfar, numerous reported solvents have been used on beans these include absolute methanol, 50%, 60% or 80% methanol (Pająk *et al.*, 2014; Durazzo *et al.*, 2013; Ortega *et al.*, 2013; Zhang *et al.*, 2013;Sreerama *et al.*, 2012; Nithiyanantham *et al.*, 2012; Marathe *et al.*, 2011; Vadivel *et al.*, 2011; Suneja *et al.*, 2011; Lin *et al.*, 2008; Hung & Morita, 2008; Duenas *et al.*, 2006), 70%, 80% acetone (Ojwang *et al.*, 2013; Nithiyanantham *et al.*, 2012; Siddhuraju & Becker, 2007; Xu & Chang, 2008; Amarowicz *et al.*, 2008) and 70% and 80% ethanol (Gutiérrez-Uribe *et al.*, 2011; Peng *et al.*, 2008).

The beneficial health properties of soluble phenolics from fruit and vegetables have been widely investigated. Some of the reported health benefits include antioxidant, antimicrobial activity, anticancer, anti-diabetic and estrogenic effects (Al-snafi, 2017; Tan *et al.*,2011; Dai & Mumper, 2010; Lim *et al.*, 2007; Bahorun *et al.*, 2004; Franke *et al.*, 1994). Some of the phenolic compounds such as daidzein, genestein, ferulic acid, catechin, caffeic acid and rutin have been directly implicated as being involved in promoting health (Sirotkin and Harrath, 2014; Silva *et al.*, 2013; Deng *et al.*, 2012; Röhrdanz *et al.*, 2002;Arts & Hollman, 1998; Mazur *et al.*, 1998)

2.3.2 Insoluble phenolics

Insoluble phenolics are also known as bound phenolics. They are covalently bound to cell wall material such as cellulose, lignin, hemicellulose and structural protein (Acosta-Estrada *et al.*, 2014). This can serve as a physical and chemical barrier for protection against pathogen invasion and can cause astringency that protects against insects or animals (Liu, 2007; Ascensao and Dubery, 2003). Phenolic acids such as hydroxycinammic acid are naturally found in the bound form (Acosta-Estrada *et al.*, 2014; Lozovaya *et al.*, 1999). They form ether linkages with lignin through their hydroxyl groups and ester linkages with structural carbohydrate and proteins through their carboxylic groups (Liu, 2007).

The interest in these bound phenolics is increasing due to the health related benefits. Adom and Liu (2002) stated that bound phenolics contributed to the total antioxidant activity and survive, by binding to the cell wall materials, transit through the stomach and intestinal digestion to reach the colon. Colonic microflora then digest the cell wall material to release the bulk phenolics. Hence, dietary intake of bound phenolics has been suggested to prevent colon cancer. On top of that, bound phenolics can contribute more than soluble phenolics to the total antioxidant potential as reported by Durazzo *et al.* (*2*013). The highest TPC and FRAP values from wheat, lentils, chickpea and sweet chestnut have been reported in insoluble phenolics.

The majority of phenolic compounds exist in the bound form in cereal and grains. For example, 85%, 75% and 62% of the total phenolics in corn, wheat and rice are insoluble bound phenolics (Adom and Liu, 2002). A significant amount of insoluble phenolics, particularly hydroxycinnamic acid, have been neglected because these compounds are concentrated in the bran layers and are lost with the separation of the seed coat during the milling process (Tian *et al.*, 2004). This has given a great impetus to look for the bound phenolics from potential staple food resources. Thus far, cowpea was the first underutilised bean to be investigated for bound phenolics. It was found that cowpea consisted of 29.6% bound phenolics (Gutiérrez-Uribe *et al.*, 2011) but others underutilised beans are lacking such information.

There are 3 common ways to release bound phenolics from the food matrix, alkaline hydrolysis, acid hydrolysis and enzyme treatment. Alkaline hydrolysis uses molarity ranging from 1 M to 4 M NaOH for hydrolysis between 1 to 24 hours and has been used to release the phenolic acids from the cell wall by breaking down the ester links between the phenolics and the cell wall (Sun *et al.,* 2012; Hung & Morita, 2008; Bonoli *et al.,* 2004; Tian *et al.,* 2004; Ascensao & Dubery, 2003; Adom & Liu, 2002). Acid hydrolysis uses molarity ranges 1 M to concentrated HCl for hydrolysis between 0.5 h to 1 h to release the ether linked phenolics (Bonoli *et al.,* 2004; Ascensao & Dubery, 2003).

The insoluble phenolics would be expected, when released, to have the same health benefits as soluble phenolics. For instance, gallic acid, protocatechuic acid, vanillic acid, syringic, caffeic and ferulic acids extracted from black gram (*Vigna mungo*) showed the same positive antioxidant effect as soluble phenolics (Girish *et al.,* 2012), and were expected to have the same benefits when bound. This depends on the mechanisms to release them as free phenolics and site of reaction. Hence, there is a great impetus to continue studying bound phenolics.

2.3.3 Extraction and analysis techniques for phenolic compounds

Extraction is the transfer or separation of material from the matrix or coexisting components (Anthemidis and Ioannou, 2009). Solid-liquid or liquid-liquid are common conventional extraction methods. These have been applied to plant material, biological samples such as human plasma and food samples. It is the main step required to recover or isolate phytochemicals from plant material. The efficiency of extraction is influenced by the chemical nature of the compounds, the method employed, sample particle size and interfering substances (Stalikas, 2007).

Solid-liquid extraction is capable of extracting soluble compounds such as soluble phenolics from solid materials. This method has been widely used with plants material such as medicinal herbs, fruits and vegetables in order to obtain valuable natural compounds. Application of solid-liquid extraction at elevated temperature such as boiling is common in our daily life. However, novel

extraction methods such as microwave-assisted extraction, supercritical fluid extraction and ultrasound-assisted extraction have been developed from these conventional methods. They are fast and efficient in extracting compounds from solid plant matrices (Wang and Weller, 2006).

Since the application of solid-liquid extraction is direct and simple, only 2 steps are involved in improving the efficiency. First is the pre-treatment of the samples for analysis by milling, grinding, freeze drying, homogenizing etc (Stalikas, 2007). This aims to reduce the particle size of the matrices thus increasing the surface area available for liquid extraction. Some of the frequently used traditional extraction techniques for soluble phenolic compounds from plants are shaking, stirring, blending and reflux (Sun *et al.,* 2012). Secondly is the optimisation of the extraction conditions, these include pH, temperature, sample to solvent ratio and extraction duration.

Liquid-liquid extraction is defined as the distribution of a solute or analyte between two immiscible solvents at different ratios. It is also known as a partition process whereby two visible layers of liquid are separated. The two immiscible solvents are usually a water or aqueous phase and an organic solvent or organic phase (Athemidis and Ioannou, 2009). This method has a secondary role since it can also be used after solid-liquid extraction. Samples extracted by this method are often clean and concentrated but the method is mostly inapplicable to hydrophilic compounds (Zhang *et al.*, 2009; Anthemidis and Ioannou, 2009) because of the nature of the liquid.

The disadvantage of liquid-liquid extraction is the need to use less polar solvents such as ethylacetate and hexane. The hydrophobic organic solvents are used to extract compounds from aqueous materials or non-polar extraneous compounds such as fat or chlorophyll (Stalikas, 2007; Yoshida *et al.,* 2004). The use of hazardous and excessive amounts of organic solvents is time consuming, expensive, environmentally unfriendly and tedious.

It is clear that this method is inapplicable to hydrophilic compounds which is another disadvantage of this method. This is because hydrophilic compounds

are immiscible in the organic solvent and prone to contamination due to the high volume of solvent used. More environmental friendly and less hazardous solvents such as acetonitrile, methanol and ethanol are omitted as extracting solvents because they are miscible with water (Anthemidis and Ioannou, 2009).

Therefore, a modern trend of liquid-liquid extraction has arisen to improve these shortcomings. It focusses on using less solvent and reagents which in turn reduces the laboratory waste. For example, cloud point extraction (CPE) and homogenous liquid-liquid extraction (HLLE). CPE is based on the phase separation procedure with addition of a small volume of surfactants (Anthemidis and Ioannou, 2009). Homogenous solution separates into a surfactant rich layer when heated above the cloud point temperature.

HLLE utilises the phase separation phenomena from a homogenous solution and extracts the target analyte into the separate phase at the same time. These methods are based on salting out, temperature, pH of perfluorinated surfactant systems and on ion-pair formation. Salting out HLLE is carried out by adding salt such as sodium chloride (NaCl) and magnesium sulphate. An even extraction is expected because the separation is from homogenous solutions without going through emulsion or suspension (Zhang *et al.*, 2009).

Three major factors that limit this separation are that the solubility of the salt in the organic solvent must be negligible, high solubility in water to allow maximum interaction with water molecules and ability of ions to precipitate hydrophilic substances according to the order $Mg^{2+} > Ca^{2+} > Sr^{2+} > Ba^{2+} > Li^{+} > Na^{+} > K^{+} > Rb^{+} > Cs^{+}$ (Anthemidis and Ioannou, 2009). Apart from that, the salt's anion also plays an important role to the phase separation following the order $SO_4^{2-} \approx CO_3^{2-} > CH_3COO^{-} \approx Cl^{-}$ (Zhang and Cremer, 2006).

Salting out liquid-liquid extraction has been proven to be comparable with conventional liquid-liquid extraction especially those using acetonitrile. Acetonitrile has been determined to be more promising than isopropyl alcohol and ethanol for use in salting out liquid-liquid extractions (Valente *et al.*,2013). It is less hazardous and thus a greener procedure and a reduced volume is

required (Zhang *et al.*, 2009). Indeed, acetonitrile is the favoured solvent to extract a wide range of compounds and is compatible with both gas and liquid chromatography (Valente *et al.*, 2013). The application of this method has mostly been on human biological samples such as serum (Zhao *et al.*, 2012; Zhang *et al.*, 2009; Anthemidis and Ioannou, 2009).

Solid phase extraction (SPE) uses solid absorbents to extract phytochemicals from liquid matrices. It is known to be a good tool for sample clean up especially from crude plant extracts and biological samples (Stalikas, 2007). It is often used in purification or pre-concentration because of the selectivity and saturation of absorbents (Tsao and Deng, 2004). SPE can be used for isolation of all acidic and basic analytes with high recoveries. Absorbent C_{18} bonded silica is the most frequently used matrix for isolating phenolics and flavonoids (Stalikas, 2007).

After extraction, the next major research challenge is the qualitative and quantitative analysis of the phenolics. There are several methods available for this and these include spectrophotometric and chromatographic techniques. There are several colorimetric assays either to determine total phenolic compounds such as Folin-Ciocalteu method (also known as total phenolic content assay- TPC) or those determine a specific class of phenolic compounds such as the ferric chloride assay that determines the presence of flavonoids (Devi *et al.*, 2016).

The principle of colorimetric assay built on the absorption maxima of the phenolic compounds which in turn is influenced by their characteristic behaviour at acidic, neutral or alkaline conditions (Naczk & Shahidi, 2006). For example, total monomeric anthocyanin assay is based on the compounds' characteristic behaviour at acidic condition whilst alkaline reagent test is determine the presence of flavonoids in response to alkaline condition.

The disadvantages of this technique include interference by other UV absorbing substances such as protein and nucleic acids, lack of suitable standards and uncertain reactivity of phenolics and other confounding

compounds (Naczk & Shahidi, 2006). As a consequence, this method often leads to overestimation of the phenolic content. However, these colorimetric assays, such as total phenolic content and monomeric anthocyanin assay, are relatively quick and easy to perform and may be useful indicators for phenolic compounds (Jonfia-Essien *et al.,* 2008; Klopotek *et al.,* 2005). Whilst an indication of the total phenolic content is useful, determination of the actual phenolic profile is more important.

Application of chromatographic techniques to address this began in the early 1960s when thin layer chromatography was applied to the analysis of phenolics (Stalikas, 2007). Paper, packed column and thin layer chromatography have since been widely used for the separation and purification of antioxidant phytochemicals (Tsao and Deng, 2004). Although these techniques are convenient and low cost, there are a few difficulties such as lack of separation efficiency and resolution, difficulties in detection, quantification and sensitivity. As a result, they are used less often nowadays.

Liquid chromatography and gas chromatography are commonly used as an alternative to the earlier methods. The separation, isolation and purification of phenolics are all improved using these approaches. The difference between these two techniques is that gas chromatography is meant for volatile compounds whilst liquid chromatography is suitable for non-volatile and soluble compounds. Thus far, high performance liquid chromatography (HPLC) has dominated the separation and identification of phenolic compounds (Stalikas, 2007).

The analysis of phenolic compounds in plants is challenging because of their chemical diversity, variability within the same species and the fact that their concentrations in the tissue can range from very low to high. The use of HPLC, as a preferred separation and analysis technique, is not capable of overcoming all of these challenges even though there are many types of detectors -UV-vis, photodiode array and fluorescence available. Mass-spectrophotometry (MS) has greatly improved the analytical ability especially in terms of identification

and quantification. Hence, the use of LC-MS for qualitative and quantitative analysis of phenolics has increased significantly.

Applications of LC-MS in identifying and quantifying secondary metabolites over the past ten years has included studies on alkaloids, coumarins, phenolic compounds, quinones and terpenes (Steinmann & Ganzera, 2011). However, despite the increased sensitivity and specificity of LC-MS, its use is still lower than HPLC because of the high instrument and experimental costs involved. However, chromatographic fingerprinting of plants can be achieved by LC-MS or LC-MS^n. Essential information is obtained such as retention time, UV-Vis spectra, ionization modes and characteristics ion were profiled. Such profiles or databases has been utilised for authentication, quality assurance, stability and similarity studies (Steinmann & Ganzera, 2011).

One of the example was the isoflavone profiling from soymilk (Zhang *et al.*, 2017). Total of 16 types of isoflavones include aglycones, glucosides, acetyl and malonyl isoflavone glucosides were identified by using 6 principal isoflavone standards - daidzein, glycitein, genistein, daidzin, glycitin and genistin. Derivatives from the principal isoflavones were estimated according to the molecular ions, specific fragment ions, UV information and published data. This result will be part of the profile for quality assurance in production. Another example was fingerprinting the phenolics from fruits tissues of Spanish lime (*Melicoccus bijugatus* Jacq). (Bystrom *et al.*, 2008) by LC-UV/vis, LC-MS-MS. The findings showed that total phenolics content from the tissues are in the order seed coat > embryo > pulp.

Comparatively, higher sensitivity, specificity and accuracy is achievable by chromatographic analysis. Whilst, an overall understanding of the contents and concentration is best achieved by spectrophotometric analysis. As a result, a number of current studies begin with spectrophotometric analysis followed by chromatographic analysis if conditions allow. Thus the majority of published studies on beans have focussed on spectrophotometric analysis more than chromatographic analysis. On top of that, there is a lack of profiling studies on beans especially from underutilised legumes.

CHAPTER 3

Determination of antioxidant activity of underutilised and commercial beans

3.1 Introduction

Three independent assays, total phenolic content assay (TPC), 2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging potential assay and ferric reducing antioxidant power assay (FRAP) were conducted to compare the antioxidant activity of underutilised and commercial beans. This preliminary screening was to select the underutilised beans with putative antioxidant potential for further study. In addition, the impact of two different solvents - sodium acetate buffer ($CH_3COONa.3H_2O$) and 80% methanol (CH_3OH) – on the extractable antioxidant activity from selected beans was investigated. Commercial beans have been included in this study as a comparator for the underutilised beans. Lastly, antioxidant activity of the beans in relation to phenolic content, anthocyanin content and tocopherol content was studied.

3.2 Methodology

3.2.1 Materials

The beans from a total of four commercial legumes- soya bean (*Glycine max*), chickpea (*Cicer arientinum*), kidney bean (*Phaseolus vulgaris*), lentil (*Lens culinaris*) and six underutilised beans - mung bean (*Vigna radiate*), black eyed pea (*Vigna unguiculata*), adzuki bean (*Vigna angularis*), lablab bean (*Lablab purpureus*) and pigeon pea (*Cajanus cajan*) were purchased from local hypermarkets in Malaysia, and bambara groundnut beans *(Vigna subterranean)* were sourced from the University farm, UK. A list of the beans used in this study is shown in table 3.1.

Table 3.1 Information for source of beans.

	Source	Country of origin	Brand
Soya bean	Giant Hypermarket	Canada	Che Ros
Mung bean		Australia	Che Ros
Chickpea		Mexico	Che Ros
Black eyed pea		USA	Giant
Lablab bean		China	Giant
Lentil		India	Giant
Pigeon pea		India	Giant
Adzuki bean	Jusco Hypermarket	unknown	Spring Food
Kidney bean	Jusco Hypermarket	unknown	Spring Food
Bambara groundnut	In house farm at Sutton Bonington, UK	unknown	-

3.2.2 Sample processing

Beans were pre-screened and any defective beans were discarded. After that, two processing methods were applied. Beans were either blended with a juice blender (Philips) until a homogenous powder was achieved or milled with a ball mill (Fritsch Pulverisette 5, Germany). To avoid overheating of the samples the ball mill was set at 250 rpm, with 2 min of milling followed by 2 min rest as one cycle. The cycle was repeated 9 times. Powders were passed through an 18 mesh sieve and kept at 4^{o}C for a maximum of 3 months. The study reported in section 3.3.1 used powder that had been processed using the juice blender and was carried out in Malaysia. Whilst, ball milled powder was used in the rest of the experiments across all chapters and was carried out in the UK.

3.2.3 Extraction procedure

Two solvents were compared for the extraction, 80% methanol (CH_3OH) 20% water (Fisher Scientific) and 300 mM sodium acetate buffer ($CH_3COONa.3H_2O$) (Sigma Aldrich) at pH 3.60. One gram of bean powder was extracted with 10 mL of the respective extraction solution. The mixture was homogenized with an ultra-Turrax T25 high speed homogenizer (IKA, USA) 13.5 L/min for 1 min and then stirred for 60 min at 23°C to 25°C. After that, the suspension was centrifuged (Thermo Scientific Heraeus Megafuge, USA) at 4696 *g*, 4°C for 5 min and filtered with Whatman paper No.4. The filtrate was immediately used for subsequent studies such as antioxidant assays (section 3.2.4), total monomeric anthocyanin assay (section 3.2.5), tocopherol analysis (section 3.2.6), other studies as in section 5.2.1, section 6.2.1, section 6.2.2, section 7.2.2 or dried completely for HPLC and LC-MS analysis.

3.2.4 Antioxidant assays

3.2.4.1 2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay

The DPPH free radical scavenging method was adopted from Wong *et al.* (2006). A standard calibration curve of trolox (Fluka) was constructed within the range 0 µg/mL to 320 µg/mL. A 0.1 mL of sample, or standard, was pipetted into a test tube followed by 3 mL of 0.1 mM DPPH (Sigma Aldrich). For the blank sample, the sample was replaced with extraction solvent. The mixture and the blank sample were then vortexed thoroughly and kept for 30 min in the dark. The absorbance for both sample and blank sample were measured at 515 nm with a spectrophotometer 7315 (Jenway, UK). Methanol was used to zero the spectrophotometer. The Δ absorbance (absorbance of sample – absorbance of blank) was calculated for standards and samples.
A typical standard calibration curve is presented in figure 3.1. Results were expressed as mg trolox equivalent (TE) per g dry weight (DW) bean powder.

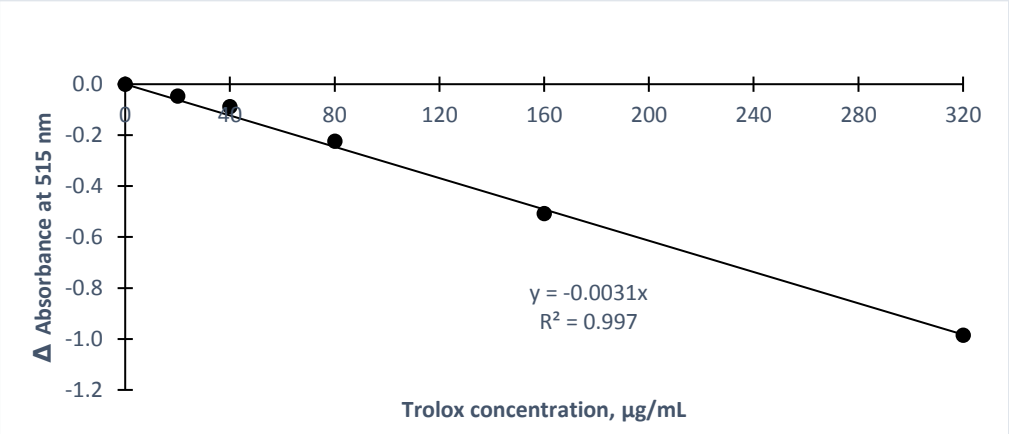


Figure 3.1 Standard calibration curve for the DPPH antioxidant assay.

3.2.4.2 Ferric reducing antioxidant potential (FRAP) assay

FRAP assay was adopted from Benzie and Strain (1996). FRAP reagent consisted of 10 mM 2,4,6-tripyridyl-s-triazine, TPTZ ($C_{18}H_{12}N_6$) (Sigma Aldrich), 20 mM ferric chloride ($FeCl_3$)(Sigma Aldrich) and 300 mM sodium acetate buffer ($CH_3COONa.3H_2O$) (Sigma Aldrich) at a ratio of 1:1:10 and was prepared fresh and only used if it gave a brown colour. Sample or standard, 0.1 mL, was pipetted into a test tube followed by 0.3 mL of distilled water. 3 mL of FRAP reagent was added at 1 min intervals for each sample. For the blank, the sample was replaced with extraction solvent and used to zero the spectrophotometer before taking measurements. The mixture was then vortexed thoroughly. The absorbance was measured after 4 min at a wavelength of 593 nm. Standard calibration curves using either 0 – 3.2 mM of ferrous sulphate heptahydrate ($FeSO_4.7H_2O$) (Fisher Scientific) or 0 – 400 µg/mL of trolox were constructed. Typical standard curves in each case are shown in figures 3.2 and 3.3, respectively. Results were expressed as mM ferrous sulphate ($FeSO_4$) equivalent per g dry weight (DW) or mg trolox equivalents (TE) per g dry weight (DW) bean powder.

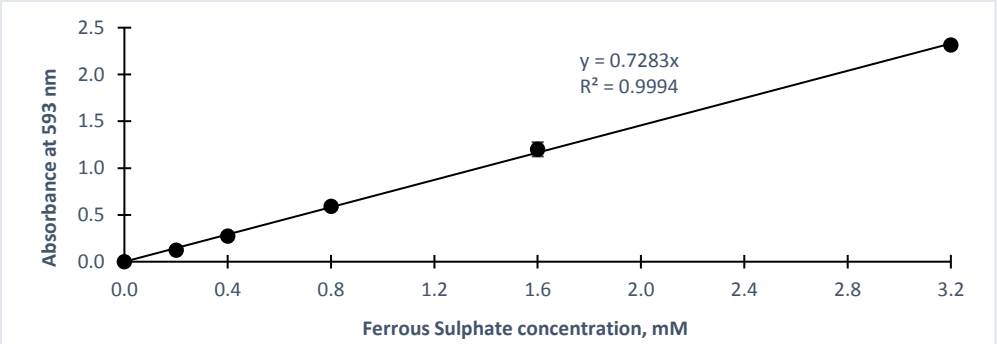


Figure 3.2 Standard calibration curve for the FRAP antioxidant assay using ferrous sulphate.

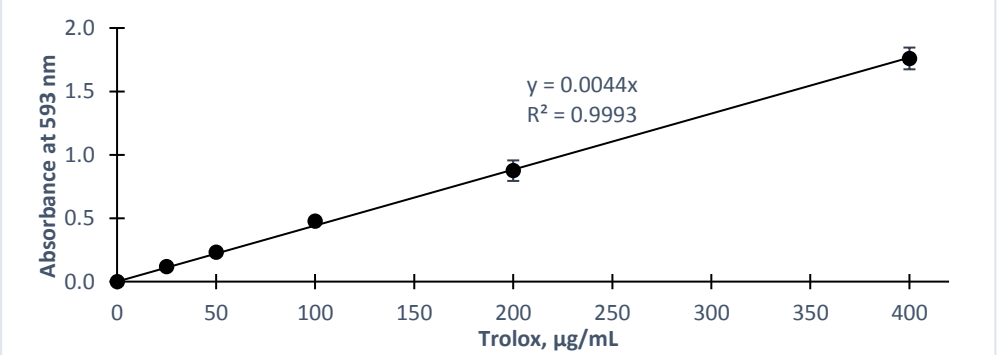


Figure 3.3 Standard calibration curve for the FRAP antioxidant assay using trolox.

3.2.4.3 Total phenolic content assay (TPC)

The total phenolic method was adopted from Lim *et al*. (2007). A standard calibration curve of gallic acid (Sigma Aldrich) was prepared in the range 0 µg/mL to 160 µg/mL. A 0.3 mL of sample, or standard, was pipetted into a test tube. Next, 1.5 mL of ten-fold diluted Folin Ciocalteu (Sigma Aldrich) and 1.2 mL of sodium carbonate (Na_2CO_3) (Merck) (7.5% w/v in water, freshly prepared) were pipetted into the test tube. For the blank, the sample was replaced with 80% methanol (CH_3OH) and used to zero the spectrophotometer before taking measurements. The mixture was then vortexed thoroughly and kept in the dark for 30 min. The absorbance was measured at a wavelength of 765 nm. A typical standard curve is presented in figure 3.4. Results were expressed in mg gallic acid equivalent (GAE) per g of dry weight of bean powder.

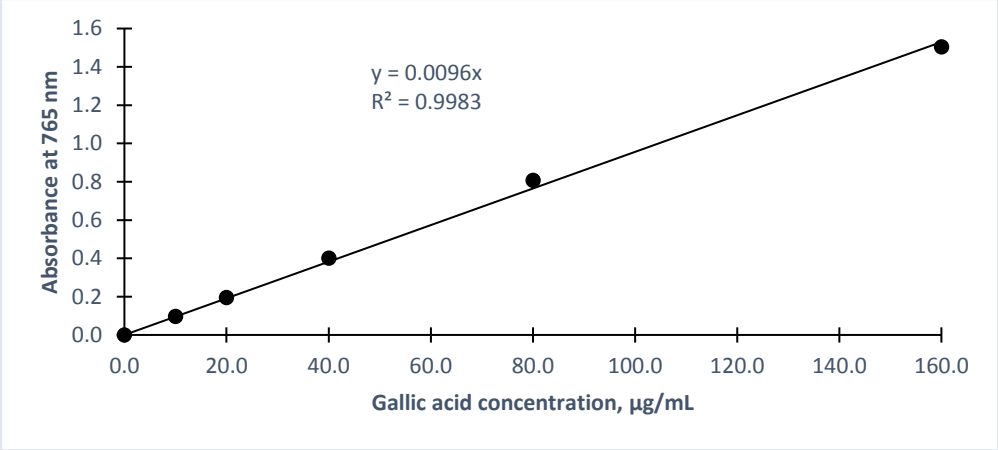


Figure 3.4 Standard calibration curve for the TPC assay of antioxidant activity.

3.2.5 Total monomeric anthocyanin content assay (TMA)

The total monomeric anthocyanin content was determined by the pH-differential method adopted from Giusti and Wrolstad (2001). Two solutions were prepared, one with 0.4 mL sample added to 3.6 mL of 0.025 M potassium chloride (KCl) buffer (Sigma Aldrich), pH 1.0 and the other one with 0.4 mL sample added to 3.6 mL of 0.4 M sodium acetate buffer ($CH_3COONa.3H_2O$) (Sigma Aldrich), pH 4.5. The solutions were left to equilibrate for 15 min. Next, the absorbance for each solution was measured at wavelengths of 510 nm and 700 nm, against the blank solution with a spectrophotometer 7315 (Jenway, UK). The sample was replaced with extraction solvent at pH 1.0 and pH 4.5 as blank solutions.

The absorbance of the sample (A) was calculated as follows:

$$A = (A_{510} - A_{700})_{pH\ 1.0} - (A_{510} - A_{700})_{pH\ 4.5}$$

The calculation of monomeric anthocyanin pigment concentration in the sample was as follows:

Monomeric anthocyanin pigment (mg/L) = (A x MW x DF x 1000)/ (ε x 1)

The concentration was converted to mg cyanidin-3-glucoside equivalent per 100 g sample considering the sample amount that has been used for testing. Results were expressed as cyanidin-3-glucoside equivalents because it is the most abundant anthocyanin in nature (Francis, 1989).

The lambda maximum to be used is 510 nm and the molecular weight (MW) is 449.2 g/mol, molar absorptivity (ε) is 26,900 and the calculated dilution factor (DF) is 10.

Fresh strawberry extract was used as a positive control to validate the experiment. 15 – 35 mg/ 100g fresh weight of monomeric anthocyanin content has been reported in strawberry (Timberlake, 1988).

3.2.6 Tocopherol analysis

3.2.6.1 Extraction

Tocopherol extraction method was adopted from Grela & Gunter (1995). This extraction is specifically targeting the tocopherol. Two grams of bean powder was added to extraction solution consisting of 24 mL of 96% ethanol (CH_3CH_2OH), 9 mL of 100% methanol (CH_3OH), 10 mL of 10% ascorbic acid in distilled water and 7 mL of 0.05 M potassium hydroxide (KOH). The mixture was homogenized by ultra-Turrax T25 high speed homogenizer (IKA, USA) 13.5 L/min for 1 min and then incubated for 20 min at 70°C. After cooling in cold water, 2 mL of this solution were transferred into a centrifuge tube and 0.5 mL of distilled water and 5 mL of heptane $CH_3(CH_2)_5CH_3$ / 2-propanol $(CH_3)_2CHOH$ (99.7 : 0.3 v/v) were added. The mixture was mixed vigorously and centrifuged at 2800 *g* for 7 min. The upper heptane layer was carefully removed and the residue was re-extracted with another 5 mL of heptane ($CH_3(CH_2)_5CH_3$) / 2-propanol ($(CH_3)_2CHOH$). The two fractions were combined and dried under nitrogen gas. Fully dried sample was then reconstituted in 2 mL of 100% methanol (CH_3OH) and filtered prior to HPLC analysis as described in section 3.2.6.2. 80% methanol extracts (section 3.2.3) without treatment were analysed simultaneously.

3.2.6.2 High Performance Liquid Chromatography (HPLC) analysis of Tocopherols

HPLC analysis was carried out to analyse the tocopherol content in the selected beans. This method was optimised from Pyka and Sliwiok (2001). A Discovery C18 (Sigma-Aldrich, USA) (150 mm x 4.6 mm, 5µm) column and Supelguard pre-column C18 (20 mm x 3 mm, 5µm) were used. HPLC grade methanol (CH_3OH) and purified water were used as mobile phases A and B. The sample was run isocratic with 90% methanol (CH_3OH) and 10% water for 60 min at 1.5 mL/min, 20 µL injection volume and the diode array detector set at 290 nm. Four types of tocopherol standards (α, β, γ, δ) each at 100 µg/mL were used to optimise the isocratic setting and as reference standards during analysis. Dried samples were reconstituted in 100% methanol and were filtered through a 0.45 micron nylon syringe filter prior to HPLC analysis.

3.2.7 Statistical analysis

All assays were carried out in triplicate. One-way ANOVA was used to identify differences between groups using Statgraphics Centurion version 16.1.11. When significant differences were detected ($p<0.05$), Fisher's least significant difference (LSD) was generated to determine the difference in mean. The Student's t tests have been used for small sample sizes. Correlation was analysed using Simple Regression Analysis. Statistical significance was declared as $p<0.05$.

3.3 Results

3.3.1 Preliminary screening for antioxidant activity and total phenolic content of beans

A total of six underutilised beans - adzuki bean (*Vigna angularis*), black eyed pea (*Vigna unguiculata*), bambara groundnut (*Vigna subterranean*), lablab bean (*Lablab purpureus*), mung bean (*Vigna radiate*), pigeon pea (*Cajanus cajan*) and four commercial beans – soya bean (*Glycine max*), chickpea (*Cicer arientinum*), kidney bean (*Phaseolus vulgaris*), lentil (*Lens culinaris*) (quoted scientific name as in GFU, 2013) were selected for this study based on their availability in Malaysia. The commercial beans were used as targeted comparisons for the screening of antioxidant potential of underutilised beans. A common blending process followed by 80% methanol extraction was applied prior to antioxidant assays. The antioxidant activity (DPPH, FRAP, and TPC) of commercial and underutilised beans are shown in figures 3.5 – 3.7 respectively.

In the DPPH assay, there was a significant difference in free radical scavenging activity shown between the tested beans. Statistical analysis showed the ranking of free radical scavenging activity of beans in graph (*).The underutilised adzuki bean had the highest free radical scavenging activity and this was similar to that from commercial soya bean (figure 3.5). While, lablab bean, chickpea and pigeon pea exhibited the lowest free radical scavenging activities. Further ranking of kidney bean, black eyed pea, mung bean, lentil, pigeon pea, chickpea and lablab bean was difficult because the statistical analysis showed no significant differences when comparing the mean value by pairs. However, adzuki bean, soya bean and bambara groundnut are the top three highest in sequence on absolute values whilst pigeon pea, chickpea and lablab bean were the last three lowest in value.

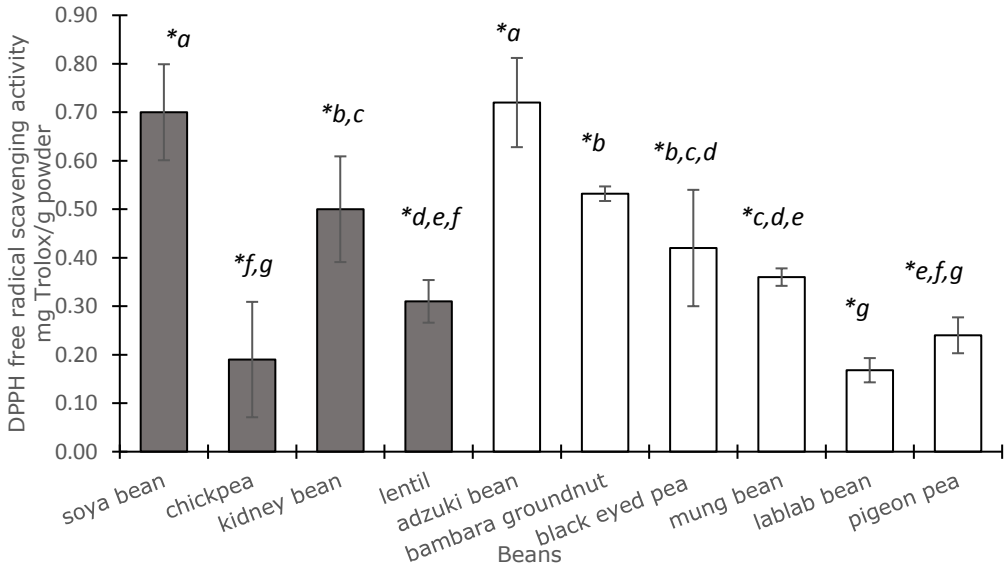


Figure 3.5 DPPH free radical scavenging activity of beans. Bars highlighted in black represent the commercial beans while in white represent the underutilised beans. Results are shown as the mean ± SD (n=9). Different letters represent significant differences at $p<0.05$.

Results from the FRAP assay, revealed statistically significant differences in antioxidant activity between the beans. Statistical analysis showed the ranking of the FRAP antioxidant activity of beans in graph (*). The result showed that adzuki bean again exhibited the highest FRAP antioxidant activity at 0.31 mM ferrous sulphate equivalent / g DW powder. While chickpea and lablab bean had the lowest reducing power (figure 3.6). In addition, bambara groundnut, kidney bean and soya bean had the second highest ferric reducing antioxidant value.

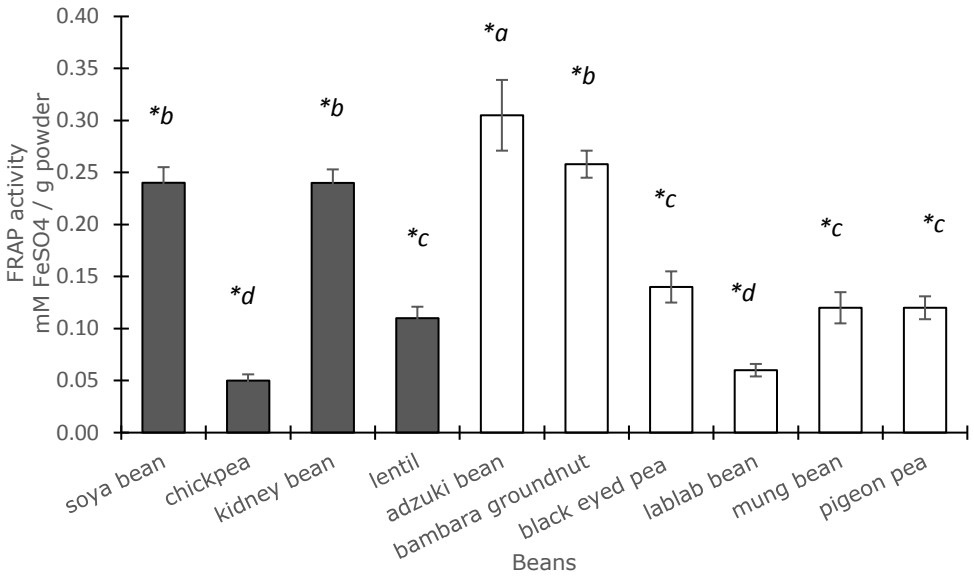


Figure 3.6 Ferric reducing antioxidant power (FRAP) of beans. Bars highlighted in black represent the commercial beans while in white represent the underutilised beans. Results are shown as the mean ± SD (n=9). Different letters represent significant differences at $p<0.05$.

The results for the TPC assay are shown in figure 3.7. Statistical analysis showed the ranking of the phenolic content for beans in graph (*). In this assay the soya bean exhibited the highest TPC (1.46 mg GAE / g DW), while lablab bean had the lowest TPC (0.27 mg GAE / g DW). All tested beans were at least 43% lower than soya bean in TPC. It was again hard to further rank amongst black eyed pea, mung bean, lentil, pigeon pea and chickpea because the statistical analysis showed no statistically significant differences when comparing mean value by pairs. However, kidney bean and adzuki bean were the second highest after soya bean based on the absolute values.

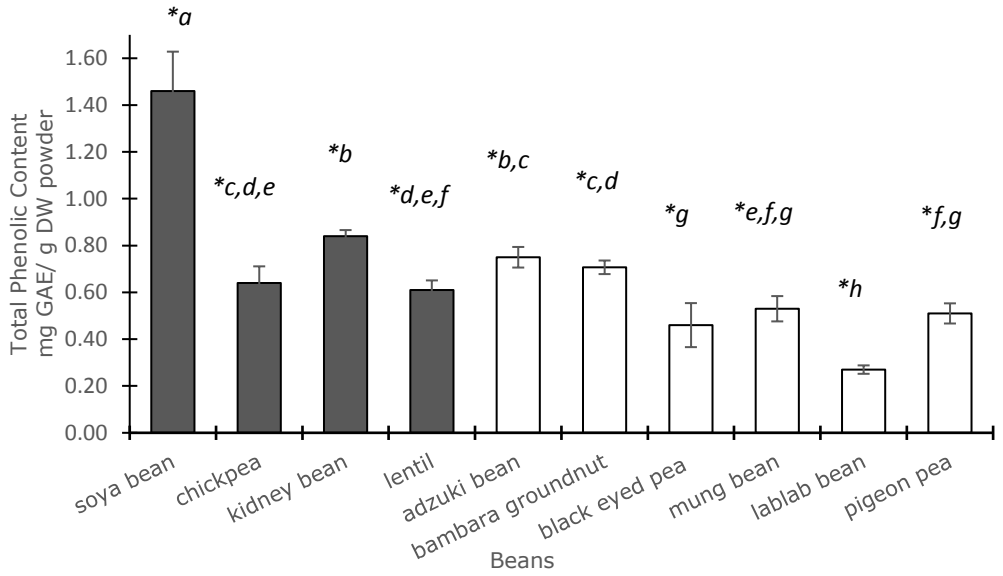


Figure 3.7 Total phenolic content of selected beans. Bars in black represent the commercial beans while those in white represent the underutilised beans. Results are shown as the mean ± SD (n=9). Different letters represent significant differences at $p<0.05$.

Correlations between these three assays for antioxidant potential have been determined (table 3.2). Moderately positive correlations were found between TPC and DPPH ($r^2=0.699$) and between TPC and FRAP ($r^2=0.599$). Whilst, there was a strong positive relationship between the DPPH and FRAP assays ($r^2=0.890$).

Table 3.2 Correlation coefficient, r^2 between assays, significantly correlated at $p<0.05$

	TPC	DPPH assay	FRAP assay
TPC	-	0.699	0.599
DPPH assay	0.699	-	0.890
FRAP assay	0.599	0.890	-

The strong correlation between the DPPH and FRAP results is perhaps to be expected since these assays are both based on the general reducing potential of the extracts. This involves the electron transfer mechanism between an oxidant and reductant (Vadivel *et al.,* 2011; Xu *et al.,* 2007; Benzie & Strain, 1996; Cuvelier & Berset, 1995). The TPC, as its name implies, is biased towards

antioxidant activity associated with phenolic compounds. As such this assay can also be used to gain an estimate of the phenolic content of the various beans but might not represent the specific reducing power of the antioxidants. Thus, this screening has demonstrated the variation in antioxidant potential amongst the beans.

In this study, adzuki bean showed high DPPH and FRAP antioxidant activity when compared to soya bean. Whereas the remaining five underutilised beans also contained promising antioxidant activities that are comparable to commercial beans. As a result, adzuki bean, bambara groundnut, black eyed pea, mung bean, lablab bean and pigeon pea were selected to investigate the types of phenolic compounds present.

3.3.2 Total monomeric anthocyanin content (TMA)

Previous findings suggested that underutilised beans possessed potential antioxidants and that this may be associated with phenolic compounds. A further investigation of respective antioxidant activity associated with anthocyanin was conducted. These beans have coloured seed coats which might indicate the present of anthocyanin. Anthocyanins are water-soluble plant pigments providing blue, purple and red colours of plant tissue (Prior, 2012). They are known to contribute to the antioxidant activity of many foods such as brown and black rice, red cabbage, grapes, kiwi and strawberry (Giusti and Wrolstad, 2001; Timberlake, 1988).

In this study, the level of anthocyanin in the beans was determined using the pH differential method and the results are shown in figure 3.8. Fresh strawberry, which was used as a control, was found to contain 30.0 mg cyanidin-3-glucoside equivalent/ 100 g fresh weight which is comparable to values in the literature (Timberlake, 1988). However, a similar anthocyanin content to strawberry was detected from soya bean. And, positive results ranging from 2.9 to 22.0 mg cyanidin-3-glucoside equivalent/ 100 g FW were found from other beans (figure 3.8).

It was thought that it would be unlikely for soya bean to contain levels of anthocyanin similar to that found in strawberry. Soya bean as well as fava bean, chickpea, lima bean and broad bean have not been reported to be rich sources of anthocyanin (Bhagwat *et al.*, 2011). Therefore, the current observation suggested that further validation was needed for the efficiency of the pH differential method in determining anthocyanin content in beans.

The pH differential method used is based on the reversible structural transformation of anthocyanin pigment into oxonium and hemiketal forms in response to a change in pH and measured using optical spectroscopy. The coloured oxonium forms at pH 1.0 and the colourless hemiketal forms at pH 4.5. Hence, a significant peak which represents the coloured oxonium should be observed between the visible wavelengths of 490 nm to 550 nm in the samples at pH 1. However, no similar peak should be observed for the colourless hemiketal forms at pH 4.5. Thus, a further validation step was carried out by measuring the absorbance spectra between 240 to 700 nm for each type of extract.

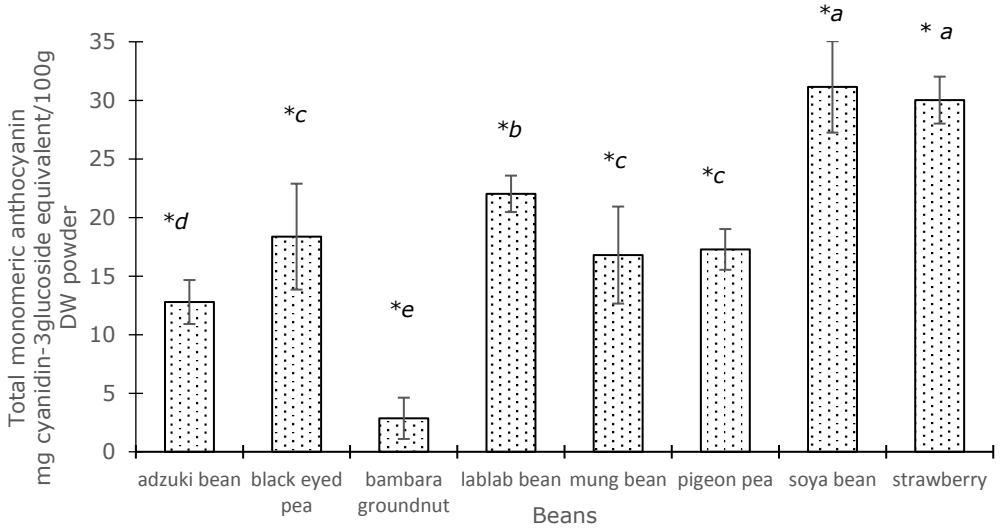


Figure 3.8 Total monomeric anthocyanin content (TMA) of methanol bean extract. Results are shown as the mean ± SD (n=9). Different letters represent significant differences at $p<0.05$. Strawberry was used as positive control.

The spectra for strawberry and soya bean are shown in figure 3.9 and for the rest of the extracts in appendix 3.1. The three lines on the graphs represents the absorbance spectrum of the soluble crude extract (labelled as TSP); the sample at pH 1.0 (labelled as pH 1.0) and the sample at pH 4.5 (labelled as pH 4.5). The spectra for the strawberry extract showed the expected peak between 480 nm and 540 nm at pH 1.0 that represents the coloured oxonium (figure 3.9). There was no corresponding peak associated with the colourless hemiketal (labelled as pH 4.5) at pH 4.5. Similarly no peaks could be detected for anthocyanin in the crude extract (labelled as TSP). These results serve to verify that the pH differential method can successfully detect anthocyanin in the strawberry control.

On the other hand, no equivalent peak was detected at pH 1.0 for any of the bean extracts (figure 3.9 and appendix 3.1). Instead it was found that absorbance of the pH 1.0 sample in these cases was generally higher across most wavelengths and this has contributed to an anomalous measurement of anthocyanin content. Hence, it led to a false positive value calculated according to the formula. The increased absorbance may have been due to turbidity in the samples.

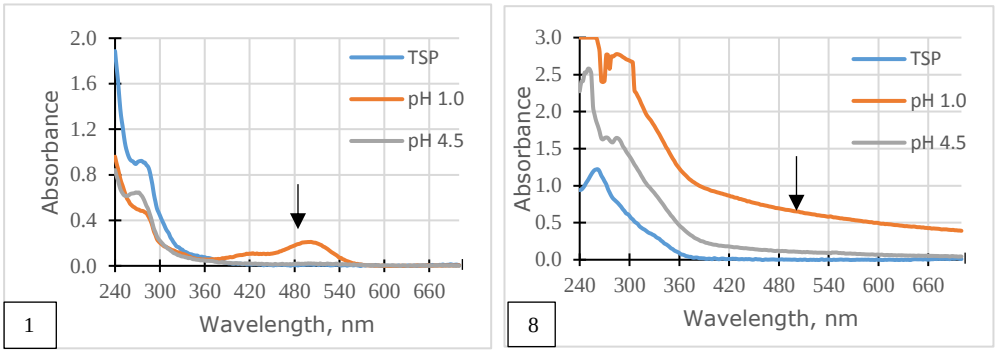


Figure 3.9 Absorbance spectra in the range of 240 nm to 700 nm for total soluble phenolics (TSP) from methanol extract, after reaction at pH 1.0 and pH 4.5. Graph 1 represents strawberry sample and 8 represents soya bean sample.

Therefore, this method may not be suitable for beans due to the turbidity formed. As a result, a modified protocol was carried out. Solutions at pH 1.0 and pH 4.5 were filtered before measuring at the designated wavelengths. The results (figure 3.10) showed that anthocyanin did not exist in the selected underutilised beans except perhaps for a small amount in bambara groundnut (0.92 mg cyanidin-3-glucoside equivalent per 100 g DW). This result was the total opposite to the previous result (figure 3.8) that suggested that all underutilised beans showed positive value and bambara groundnut actually had the lowest value. The anthocyanin content for strawberry was reduced from 30.0 mg cyanidin-3-glucoside equivalent/ 100 g fresh weight to 25.6 mg cyanidin-3-glucoside equivalent/ 100 g fresh weight.

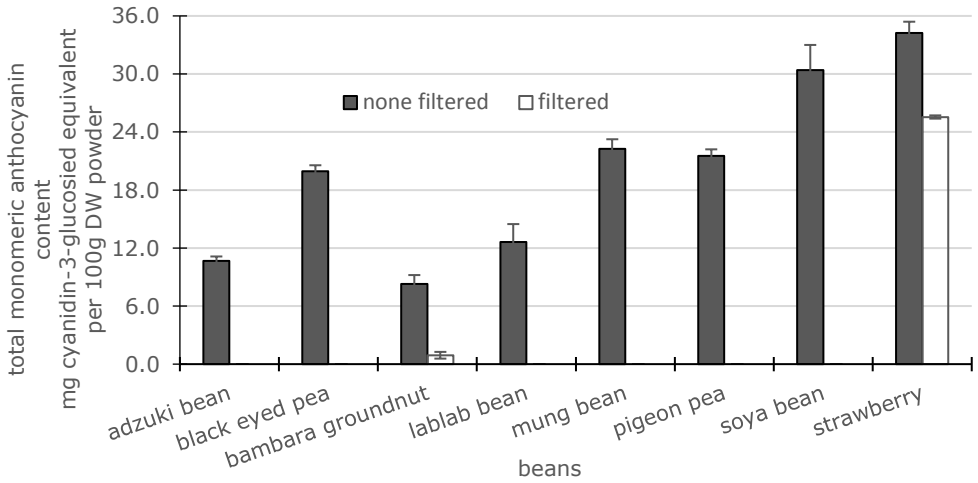


Figure 3.10 Total monomeric anthocyanin content (TMA) of filtered and unfiltered methanol bean extract. Strawberry was used as positive control. Results are shown as the mean ± SD (n=6).

Further observation of the absorbance spectra showed that there was a small peak between 480 nm to 540 nm at pH 1.0 for bambara groundnut but not for any other beans extract (figure 3.11 and appendix 3.2). There were major differences between these spectra and those obtained previously (figure 3.9). Background noise that may due to turbidity, has been avoided by filtration. This confirmed that a false positive result was obtained in the previous experiment and that anthocyanin content in these beans was negligible. This study confirmed that an additional filtration step could avoid false positive results using this assay.

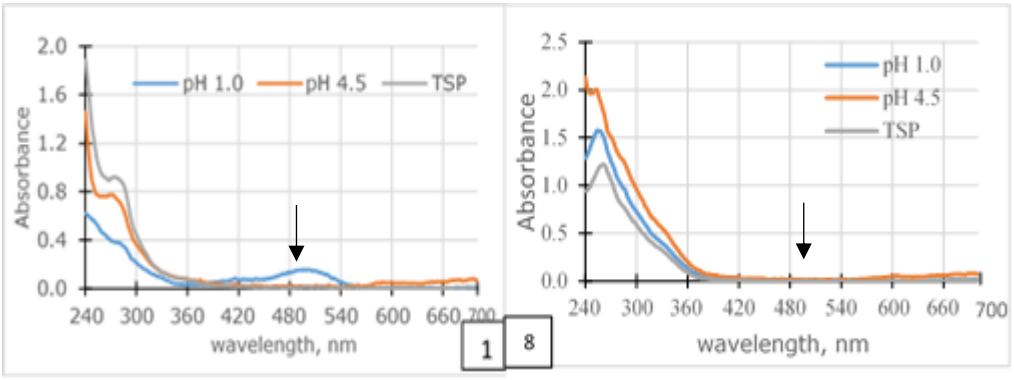


Figure 3.11 Absorbance spectra in the range of 240 nm to 700nm for total soluble phenolics (TSP) from CH_3OH extract, after reaction at pH 1.0 and pH 4.5 followed by filtration. Graph 1 represents strawberry sample and 8 represents soya bean sample.

3.3.3 Tocopherol content

Beans have been reported to have high fat (0.53- 19.94 g / 100 g dry weight) (USDA, 2015) and as such are likely to have fat soluble antioxidants such as Vitamin E. Hence, a current study was carried out to investigate the tocopherol content in underutilised beans.

Vitamin E consists of eight different vitamers (α-,β-, γ-. δ-tocopherols and (α-,β-, γ-. δ-tocotrienols) with varying biological activities. These are known to be lipid soluble antioxidants occurring ubiquitously in plants (Abidi and Mounts, 1997). Tocopherol, the major vitamers of vitamin E, has been reported as being higher in seeds and beans than in cereals (Ryan *et al.*, 2007). However, very limited studies have been conducted on tocopherol levels in the selected underutilised beans.

Standards for 4 different vitamers of tocopherol (100 µg/mL) namely α-,β-, γ-. δ-tocopherol were obtained. The retention time for all four vitamers of tocopherols from HPLC analysis were – tocopherol α - 43.9 min, tocopherol β+γ – 31.0 min and tocopherol δ – 27.9 min. Preliminary HPLC result showed that none of these vitamers of tocopherol were detectable in the 80% methanol bean extracts.

Another extraction methodology that included a saponification treatment was thus applied (section 3.2.6.1) and result are shown in table 3.3. Tocopherol-δ was detected in adzuki bean, black eyed pea, bambara groundnut and soya bean only. However, tocopherol-β+γ was found in all underutilised beans except adzuki bean and black eyed pea. On top of that, tocopherol has not been detected from 80% methanol extracts and thus the antioxidant activity from 80% methanol is not related to tocopherol.

Table 3.3 Identification of vitamers for tocopherol from different types of beans.

Tocopherol	Type of beans						
	Adzuki bean	Black eyed pea	Bambara groundnut	Lablab bean	Mung bean	Pigeon pea	Soya bean
Vitamer - δ	√	√	√				√
Vitamer - β+γ			√	√	√	√	√

3.3.4 Comparison of extraction methods on the antioxidant activity and total phenolic content of underutilised beans

Previous section has shown the relative correlation of antioxidant activity to phenolic content, anthocyanin and tocopherol. In this section, a further investigation on the efficiency of the aqueous and solvent extraction methods on the antioxidant activity of underutilised beans and soya bean was carried out. Beans were processed using the ball mill to obtain a finer form of powder to achieve a more efficient extraction process by increasing the surface area. Next, the beans were extracted with either 80% methanol or sodium acetate buffer at pH 3.6.

Figure 3.12 shows the comparison of solvent and aqueous extracts in terms of the DPPH free radical scavenging activity of beans. Results showed a significant difference of activity between the two extracts across all beans except adzuki bean. There was no clear effect of solvent identified in this case. Methanol extracts gave the highest activity in three of the remaining beans and acetate buffer the highest activity in the other three. Adzuki bean possessed the highest free radical scavenging ability in both extracts. While, lablab bean exhibited the lowest activity in 80% methanol and soya bean showed the lowest activity in acetate buffer extract. Adzuki bean and bambara groundnut were the two underutilised beans that showed higher antioxidant potential compared with soya bean in both solvents. The ranking of the free radical scavenging activity for 80% methanol extract from beans was adzuki bean> bambara groundnut> soya bean> black eyed pea = mung bean; mung bean = pigeon pea; pigeon pea = lablab bean.

When comparing the ranking of the activity from 80% methanol extract with the preliminary study (section 3.3.1), a consistent result has been observed for adzuki bean, black eyed pea, mung bean, pigeon pea and lablab bean (previous result was adzuki bean = soya bean > bambara groundnut = black eyed pea; black eyed pea = mung bean; mung bean = pigeon pea; pigeon pea = lablab bean). DPPH activity were generally increased across all beans after changing the raw material processing from blending to milling. Among all beans, the free radical scavenging activity from adzuki bean and bambara groundnut

increased by the greatest amount, 2 times when using the milling process as compared with the blending process.

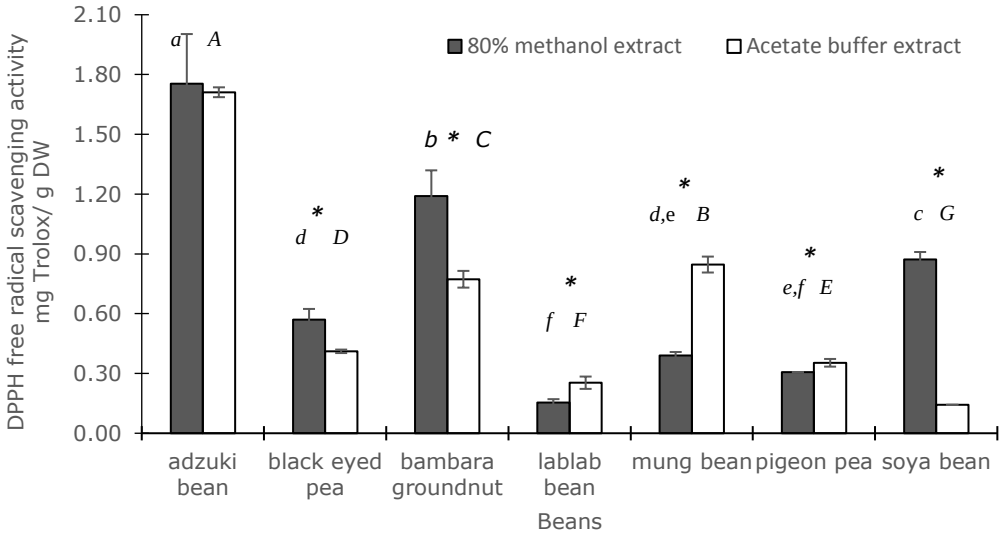


Figure 3.12 DPPH free radical scavenging activity of methanol and sodium acetate buffer extracts. Results are shown as the mean ± SD (n=3). * represents a significant difference between the values obtained for the two solvents at $p<0.05$. Different letters represent significant differences among beans for methanol extract at $p<0.05$.

The FRAP assay results also revealed significant differences of activity between the two solvents across all beans (figure 3.13). Adzuki bean exhibited the highest antioxidant power. While lablab bean had the lowest reducing power for both types of solvent among all underutilised beans. Adzuki bean and bambara groundnut were the two underutilised beans that showed better reducing power as compared with soya bean in both type of extracts. The ranking of the FRAP antioxidant reducing activity of beans was adzuki bean > bambara groundnut = soya bean > black eyed pea > mung bean = pigeon pea > lablab bean. These result showed a consistent ranking when compared with the result from the preliminary study (section 3.3.1) but, the absolute values had increased at least 2 times when changing the raw material processing to milling.

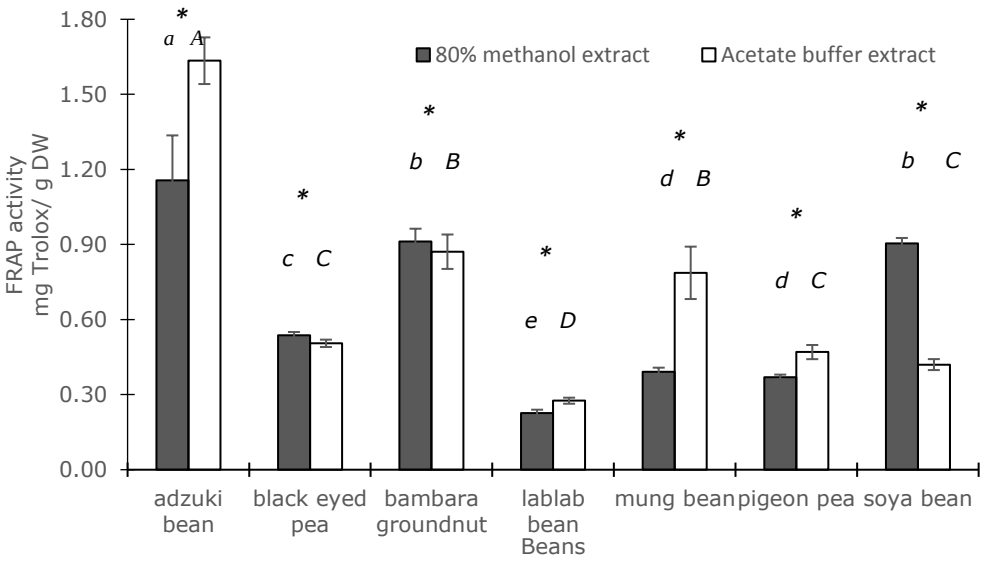


Figure 3.13 Ferric reducing antioxidant power (FRAP) of methanol and sodium acetate buffer extracts. Results are shown as the mean ± SD (n=3). * represents a significant difference between the values obtained for the two solvents at $p<0.05$. Different letters represent significant differences among beans for methanol extract at $p<0.05$.

The results for the TPC assay are shown in figure 3.14. Results showed that sodium acetate buffer extracts exhibited significantly higher TPC compared with 80% methanol across all beans except soya bean. Adzuki bean exhibited the highest TPC content amongst the underutilised beans. While lablab bean had the lowest TPC content for both types of extract among all underutilised beans. The ranking of phenolic content for methanol extract was soya bean > adzuki bean > bambara groundnut > mung bean > pigeon pea = black eyed pea > lablab bean. This result was consistent with the result obtained from the preliminary study in section 3.3.1. Again, the absolute values were generally increased when applying the milling process to the raw material.

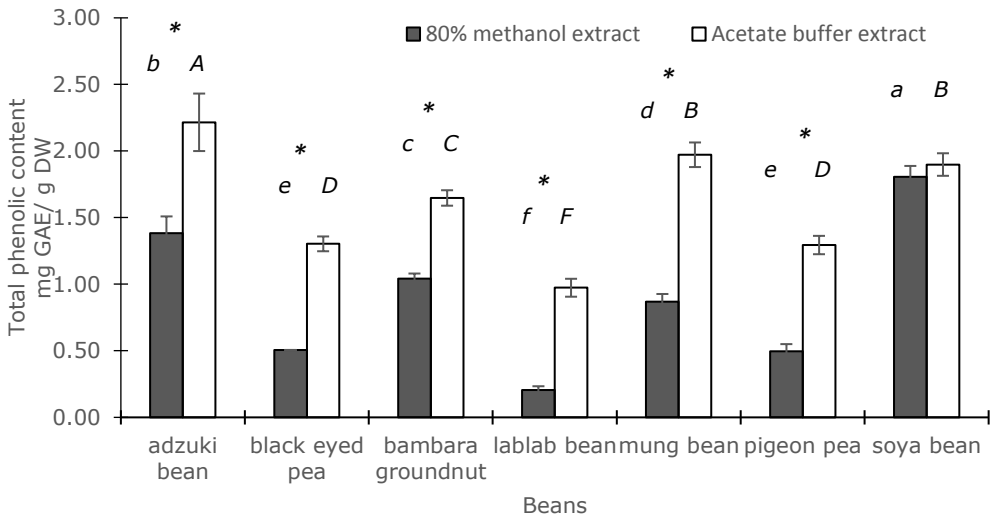


Figure 3.14 Total phenolic content of methanol and sodium acetate buffer extracts. Results are shown as the mean ± SD (n=3). * represents a significant difference between the values obtained for the two solvents at $p<0.05$. Different letters represent significant differences among beans for methanol extract at $p<0.05$.

A statistical correlation has been applied to compare the relationship of both types of solvents from each of the three antioxidant assays (table 3.4). The analysis showed a moderately positive relationship between the two solvents for all three assays. This suggests that whilst the individual beans may have differing profiles of antioxidant compounds, these antioxidant assays may be nonetheless able to rank them effectively.

Table 3.4 Correlation coefficient, r^2 between the activities extracted by the two solvents for antioxidant assays.

		Methanol extract		
		TPC	DPPH	FRAP
Acetate	TPC	0.813	-	-
buffer	DPPH	-	0.750	-
extract	FRAP	-	-	0.702

'-' represents no information

3.4 Discussion

3.4.1 Antioxidant potential of commercial and underutilised beans

The antioxidant activity of six underutilised beans - adzuki bean, black eyed pea, bambara groundnut, lablab bean, mung bean, pigeon pea, and four commercial beans – soya bean, chickpea, kidney bean, lentil were determined via three independent colorimetry assays - DPPH free radical scavenging assay, FRAP assay and TPC assay. These assay are widely used due to the availability of the reagents and ease of handling.

The general mechanism of both the DPPH free radical scavenging and FRAP reducing assays involve an electron transfer mechanism between an oxidant and reductant (Vadivel *et al.,* 2011; Xu *et al.,* 2007; Benzie & Strain, 1996; Brand-Williams *et al.,* 1995). Different reagents are used and the changes of colour after reaction are measured by spectrophotometry in order to determine the antioxidant activity. Lastly, the result is expressed with reference to a standard.

DPPH assay is commonly used to determine antioxidant activity *in vitro* (Al-Temimi and Choudhary, 2013). DPPH is one of the few stable and commercially available organic nitrogen radicals (Dejian *et al.,* 2005) exhibiting a dark purple colour at 515 nm. In the process of being scavenged by the compounds in the sample extract, DPPH will be reduced, producing a colour change from purple to yellow. Whilst, FRAP assay measures the ability of sample to reduce a ferric-tripyridyl- triazine complex (Fe3+-TPTZ) to the ferrous form, Fe^{2+} producing a blue colour with absorption at 593 nm.

The tested underutilised and commercial beans demonstrated different levels of antioxidant activity from both DPPH and FRAP assays. The results for underutilised beans were within the range of the tested commercial beans or higher except for lablab bean. This showed that the antioxidant potential of underutilised beans are comparable with commercial beans. Positive findings of antioxidant potential from DPPH and FRAP in this study are in agreement with published reports (Durazzo *et al.*, 2013; Xu and Chang, 2012; Kanatt *et al.,* 2011; Marathe *et al.,* 2011; Xu and Chang, 2007; Duenas *et al.,* 2006).

Marathe *et al* (2011) showed that 80% methanol extracts of soya bean exhibited higher DPPH (expressed in units/ g) and FRAP (expressed in µmol FRAP/ g) antioxidant values than chickpea, pigeon pea, lablab bean, lentil and black eyed pea. Next, Xu and Chang (2012) reported that adzuki bean, mung bean, kidney bean and lentil, extracted with acidic 70% acetone, exhibited higher DPPH values than the 50% acetone extracted chickpea and soya bean when expressed in µmole TE/g.

Adzuki bean exhibited the highest DPPH and FRAP activity among all the underutilised and commercial beans tested. This is in line with the published report from Xu and Chang (2012). Their findings indicated that adzuki bean possessed higher DPPH free radical scavenging activity than mung bean, kidney bean, black eyed pea, chickpea and soya bean. Thus, adzuki bean is potentially an alternative antioxidant source to soya bean.

Lablab bean exhibited the lowest activity in this study and this has also been reported by Marathe *et al* (2011). They showed that lablab bean exhibited the lowest antioxidant value among over 30 tested beans when measured by DPPH, ABTS, FRAP, metal ion chelating and TPC. In another words, there are clear differences between lablab bean and adzuki bean. A lower amount of antioxidants, or less diversification of antioxidant compounds, might exist in lablab bean.

A direct comparison of antioxidant activity results from this study with those from published reports could not be performed. This is because of differences in the extraction conditions e.g. solvent, unit of measurement and source of selected bean. One unanimous finding from both this study and previous reports is that the antioxidant activity can be correlated with TPC. The positive correlation between the DPPH, FRAP and TPC suggest that phenolics may play a significant role in determining antioxidant activity.

Xu *et al.* (2007a) reported that antioxidant activities from beans (peas, lentils, beans, soybeans and chickpea) measured by FRAP, DPPH, and ORAC were all correlated with TPC. Similarly, Marathe *et al.* (2011) reported that the

antioxidant activity from more than 30 beans measured by DPPH, ABTS and FRAP could be correlated with TPC. There are thus several published studies imply that phenolic compounds may predominantly contribute to antioxidant activity (Xu and Chang, 2012; Marathe *et al.*, 2011; Kanatt *et al.*, 2011).

TPC is one of the most commonly used tools to estimate the phenolic content. The TPC assay involves a complex redox reaction between compounds in the sample and phosphotungstic and phosphomolybdic acids present in the TPC reagent (Wong *et al.*, 2006). Although the principle is the same as for the DPPH and FRAP assays, it is biased towards antioxidant activity associated with phenolic compounds. As such this assay has been widely employed to estimate the phenolic content from various beans but might not represent the specific total reducing power of the antioxidants.

In this study, soya bean had 1.5 times higher TPC than all the tested underutilised beans. However, the TPC values obtained for soya bean, chickpea, pigeon pea, lentil, black eyed pea and lablab bean from this study were all lower than those reported by Marathe *et al*. (2011). For example, reported values were 2.17, 1.05 and 0.33 mg GAE / g of TPC were found in soya bean, pigeon pea and lablab bean as compared with 1.46, 0.51 and 0.27 mg GAE/ g in this study. The reported values were 20% to 100% higher than the values in this study. Although a similar extraction solvent was used, Marathe *et al* (2011) employed a longer extraction time (2h) and repeated the extraction step twice. This may account for the higher TPC values compared to the current study.

The ranking of beans' TPC, as reported in section 3.3.1, suggested that those beans with coloured seed coats and higher colour intensity (darker) tended to have higher TPC values than those that were lighter coloured seed coat and with lighter colour intensity. This was agreed by Kanatt *et al.* (2011) and Barampama and Simard (1995).TPC value as in ranking soya bean (yellow) > kidney bean (red) > adzuki bean (red) > bambara groundnut (black) > chickpea (creamy brown) > lentil (dark orange) > mung bean (green) > pigeon pea (light orange) > black eyed pea (creamy brown) > lablab bean (creamy white).

The colour could possibly arise through the present of anthocyanin. Thus the content of this compound in underutilised beans was also examined. These compounds are also known to contribute antioxidant activity and might correlate to the positive antioxidant activity as reported previously. In this study anthocyanin was only detectable in bambara groundnut which showed a small amount of anthocyanin (0.92 mg cyanidin-3-glucoside equivalent per 100 g DW) using the modified methodology (section 3.3.2).

Some previous investigations have reported finding anthocyanin in underutilised beans. A total of 97 mg/g dry weight of anthocyanin, consisting of peonidin-3-rutinoside, pelargonidin-3-O-glucoside and malvidin-3-O-glucoside, was reported from adzuki bean paste (Han *et al.,* 2015). Anthocyanin at 3.14 mg cyanidin-3-glucoside equivalents/ g was detected in flour from the same bean (Sreerama *et al.,* 2012). Delphidin 3-glucoside, cyanidin 3-glucoside, delphinidin 3-(ρ-coumaryl) glucoside and delphinidin 3,5-diglucoside have all been found in mung bean (Mazza and Miniati, 1996).

In contrast, other reports support the findings in this study. Cho *et al.,* (2013). failed to find any appreciable anthocyanin in yellow, brown or green soya bean but stated that anthocyanins such as cyanidin- 3-O- glucoside and delphinidin – 3-O- glucoside could be detected in black soya bean. Similarly, Giusti *et al.,* (2017)'s reported that cyanidin-3-glucoside and delphinidin 3,5 diglucoside were not detectable by HPLC in extracts from adzuki bean.

Bambara groundnut was the only bean found to contain detectable anthocyanin in this study. Bambara groundnut has been shown to possess delphinidin 3-O-β-glucoside, petunidin 3-O-β-glucoside, malvidin 3-O-glucoside based on chromatographic data, UV-VIS, ^{1}H-NMR and mass spectrophotometry (Pale *et al.,* 1997). Mbaiogaou *et al.(*2014) reported a range of 1- 3 mg cyanidin 3 – glucoside / 100g DW from 17 varieties of bambara groundnut. The current study found 0.92 mg cyanidin-3-glucoside equivalent per 100 g DW, which is at the lower end of the range reported previously. However, the previous report extracted with acidic 70% acetone at 4°C for more than 24 hours, which is far more rigorous than the method used in this study.

Tocopherols are insoluble in water but soluble in organic solvents such as ethanol, acetone and vegetable oils (Pyka and Sliwiok, 2001). They play an important role as fat soluble antioxidants that scavenge the lipid peroxyl radicals (Ryan *et al.*, 2007). The same study also showed that tocopherols content was higher in seeds and beans than in cereals. The antioxidant potential of the different tocopherol vitamers are not identical. The most common tocopherols in the human diet are α- and γ-. Thus, the tocopherol levels in the beans was investigated.

Tocopherols were not detected in any of the beans' using direct extraction into 80% methanol. This might be due to the extraction methodology itself failing to solubilise the tocopherol. It is recommended that the sample is treated with organic solvent before, or simultaneously, with saponification, so as to disrupt the association of tocopherol with lipoproteins and membranes (Ruperez *et al.*, 2001). A modified extraction (as shown in section 3.2.6.1) from beans showed that tocopherol-δ could be detected only in adzuki bean, black eyed pea, bambara groundnut and soya bean. Tocopherol-β+γ was found in all underutilised beans except adzuki bean and black eyed pea. Some previous studies have also shown that tocopherols can be found in adzuki bean (β-, γ-, δ-), pigeon pea (α-, γ-, δ-), red seed lablab bean (β-, γ-, δ-) and black eyed pea (α-, β+γ, δ-) (Kalogeropoulos *et al.,* 2010; Takanori *et al.*, 1993).

More investigations on tocopherol content have been carried out on commercial beans than on underutilised beans (Kalogeropoulos *et al.,* 2010; Ryan *et al.*, 2007; Grela and Gunter, 1995; Takanori *et al.,* 1993). Tocopherols in soya bean found in this thesis were similar to those reported by Grela and Gunter (1995) except for α-tocopherol. They reported concentrations in soya bean of γ-tocopherol (237.8 mg/ kg), α-tocopherol (65.5 mg/ kg) and δ-tocopherol (62.4 mg/ kg). Ryan *et al* (2007) reported that α-tocopherol and β+γ-tocopherol could be found in chickpea, kidney beans and lentil. However, only γ- and δ-tocopherol have been found in kidney bean whilst α- and γ-tocopherol were found in lentil (Grela and Gunter, 1995).

The HPLC setup could affect the analysis of tocopherols and that could lead to any variation of results in this study and those in published reports. C_{18} RP-HPTLC, NP-HPLC and GC are the recommended techniques for the separation of tocopherols (Pyka and Sliwiok, 2001). However, a drawback is that β- and γ- tocopherols can hardly be distinguished via C_{18} RP-HPLC (Gornas *et al.,* 2014; Ryan *et al.,* 2007; Pyka and Sliwiok, 2001; Kamal-Eldin *et al.,* 2000; Kramer *et al.,* 1997). And, destruction of heat sensitive tocopherols happens during sample treatment prior to GC analysis (Kua *et al.,* 2016).

Therefore, NP-HPLC with fluorescence detection is commonly used. The fluorescence detector possesses higher sensitivity, lower detection limits, in the range of 0.1 – 7.2 ng/L, and increased specificity compared with UV (Cunha *et al.,* 2006; Kamal-Eldin *et al.,* 2000). Some reports mentioned that approximately 20% of tocopherol content was degraded during the extensive sample treatment steps such as saponification and extraction (Gornas *et al.,* 2014; Bonvehi *et al.,* 2000). The use of C_{18}RP-HPLC with photo diode array used in this study may thus have contributed to a lower detection of the tocopherols from beans.

3.4.2 Comparison of solvent and aqueous extracts on the antioxidant activity and total phenolic content of underutilised beans

Numerous investigation on the effect of different solvents on extractable antioxidant activity from have been conducted (Bai *et al.,* 2017; Hi *et al.,* 2016; Deng *et al.,* 2012; Wang & Weller, 2006). This reflect the fact that extraction conditions contribute to the efficiency of extraction. Selection of a suitable analysis technique is also important in order to obtain rapid and easily handle results.

Methanol was selected because it is commonly used to extract phenolic compounds from plants and for its efficient (Al-Temimi and Choudhary, 2013; Sreerama *et al.,* 2012; Marathe *et al.,* 2011; Stalikas, 2007; Escarpa & Gonzalez, 2001; Arts and Hollman, 1998). However, it has been suggested that a mixture of alcohol and water is more efficient at extracting phenolic

compounds than the mono component solvent systems (Pinelo *et al.*, 2005). Up to 80% of antioxidant activity can be extracted using 80% methanol (Ortega *et al.*, 2013). Additionally, 80% methanol has been shown to be better than 30%, 50% or pure methanol for extracting isoflavones, a type of flavonoid (Vila-Donat *et al.*, 2015; Chen *et al.*, 2009). Therefore, 80% methanol was chosen as the organic solvent to be used in this study.

A buffered aqueous solution- sodium acetate buffer at pH 3.6 was also selected for this study. This buffer system is used in the FRAP antioxidant assay to increase the stability of antioxidants during the extraction. Hence, the efficiency of a buffered solution is expected to be better than pure water. Apart from having a different polarity index than the organic solvent, the extracts obtained from this system are free from alcohol soluble compounds (Jones, 1968). Hence, it might be expected to find differences in the types of compounds that are being extracted by the two solvent systems.

Colorimetric antioxidant assays are widely used to monitor the efficiency of different solvents in extracting antioxidant compounds (Lv *et al.*, 2012; Ikram *et al.*, 2009; Chu *et al.*, 2002). They are fast, simple and easy to handle. Therefore, three colorimetric antioxidant assays, -DPPH, FRAP and TPC - were used to compare the efficiency of 80% methanol and acetate buffer extract in extracting antioxidant compounds and phenolics.

The outcome from this study revealed that both solvents are suitable, for either the DPPH or FRAP assays, but that the most effective for any particular bean was dependent on the bean. Aqueous being best for some beans and methanol for others. Hence, there was no solvent that was better across all beans and this may reflect a different profile of compounds in each case. This is supported by Krygier *et al.* (1982) who showed that no single solvent was able to perform a complete extraction of soluble phenolic compounds.

The TPC results, in contrast, suggested that sodium acetate buffer was better at extracting antioxidants. Since this assay tends to favour phenolic compounds this could suggest that these are also being more efficiently extracted.

However, phenolics tend to favour solubilisation into organic solvents and it is possible that in this case the aqueous solvent is extracting a greater amount of non phenolic confounding compounds than with the 80% methanol. However, a good outcome from these investigation were 80% methanol and acetate buffer extracts were positively related.

An indirect observation showed that the processing of the raw material has affecting the extraction efficiency. Milling the raw material (section 3.3.4) effectively increased the overall antioxidant potential as compared with the blended raw material (section 3.3.1). The reason was most likely due to the reduction of particle size, in milled versus blended material, increasing the surface area available for mass transfer and thus increasing extraction yield (Spigno *et al.,* 2007). This is also in line with other published statements (Sun *et al.,* 2012).

Apart from that, variation of results have been observed between this study and others published reports (Zhao *et al.,* 2014; Xu and Chang, 2012; Sreerama *et al.,* 2012) that differed in solvent, pH condition and extraction conditions such as ultrasonication. For example, TPC values from Zhao *et al* (2014) showed that 15.2 and 26.7 mg GAE/ g of phenolics in black eyed pea and mung bean whilst only 0.46 and 0.53 mg GAE/ g DW in this report. Those were extracted three times using 60% ethanol with 2% HCl and ultrasonication for 30 mins at 60°C. Next, Sreerama *et al* (2012)'s report showed that TPC values of 2.35 and 4.89 mg GAE/ g for mung bean and adzuki bean. This was at least four times higher than the values in this study. Beans were extracted three times by 80% methanol in 1% HCl and refluxed in water bath at 55°C for 1 h. As a result, there is no standard extraction procedure for the highest yield of extract and types of compounds when comparing more than one type of bean.

3.5 Conclusion

The underutilised beans and commercial beans demonstrated a large variation in levels of antioxidant activity. Among the underutilised beans, adzuki bean showed the highest antioxidant activity, as measured by DPPH and FRAP, and this was comparable to soya bean. However, soya bean gave the highest TPC value. Of the known antioxidants only bambara groundnut contained traces of anthocyanin. While four beans (adzuki bean, black eyed pea, bambara groundnut and soya bean) had low levels of tocopherol-δ. Interestingly, tocopherol-β+γ was detected in all tested underutilised beans except adzuki bean and black eyed pea.

The type of solvent used for the extraction had a significant effect on recoverable antioxidant activity. The relative efficiency of the buffered aqueous and 80% methanol solvents varied with bean type and neither was universally more effective than the other. This may reflect differences in the antioxidant compounds present in each bean. Significant correlations were found between the antioxidant assays- DPPH and FRAP- and the TPC values suggesting that phenolics may play a significant role in the determination of the antioxidant capacity.

In the following chapters, more precise HPLC, LC-MS methods will be developed to determine the soluble (free and conjugated) and insoluble bound phenolics from selected underutilised beans.

CHAPTER 4

Analysis of soluble phenolics from selected beans

4.1 Introduction

Folin Ciocalteu assay is used to determine phenolic content. Phenolic compounds act as antioxidants and undergo a complex redox reaction with phosphotungstic and phosphomolybdic acids that act as oxidants in the reagent. The colour of the reagent is then changed from yellow to blue. Due to its fast reaction and ease of handling, this assay is commonly used in determine antioxidant activity associated with phenolic compounds from fruits and vegetables (Ti *et al.,* 2014; Thaipong *et al.,* 2006; Sun *et al.,* 2002).

Results from total phenolic content (TPC) measurements in chapter 3 could suggest that sodium acetate ($CH_3COONa.3H_2O$) buffer was better than 80% methanol for extracting phenolic compounds. This assay gives an indication of total phenolic content but does not provide detailed identification of specific phenolics. Furthermore the reagent used can also react with non-phenolic compounds with reducing power, and the number of hydroxyl groups in the compound will also influence the reaction (Singleton & Rossi, 1965). Hence, due to the limitations of the TPC assay, high performance liquid chromatography (HPLC) is recommended for further confirmation and analysis. HPLC is a more advanced analytical technique that could fractionate compounds according to their polarity which in turn provides more information in terms of numbers and types of phenolics present in the sample.

In this chapter, both TPC assay and HPLC were used to compare their efficiency in determining the total soluble phenolic compounds extracted from selected beans using three solvents (sodium acetate, 80% methanol and 80% acetone). Subsequently, the HPLC method was optimised to identify and quantify the specific phenolic compounds with the aid of known reference standards.

4.2 Methodology

4.2.1 Sample preparation

Beans were extracted, as per section 3.2.3, with either 80% methanol/20% water, 80% acetone /20% water or sodium acetate buffer. Two mL of the extract was fully dried under nitrogen gas and kept at -20^{o}C for further analysis. The dried extract was reconstituted in one mL of 50% methanol in 0.02 M phosphate buffer, pH 2.4. It was then filtered through a 0.45 micron nylon syringe filter ready for HPLC and TPC analysis.

4.2.2 Total phenolic content assay (TPC)

TPC assay was carried as per section 3.2.4.3 with 80% methanol, 80% acetone and sodium acetate extracts.

4.2.3 Preparation of phenolic standards

Naringenin, ρ-coumaric acid, sinapic acid, caffeic acid, genistein, epigallocatechin gallate, epicatechin gallate, kaempferol, rutin, trans-3-hyroxycinnamic acid (Sigma Aldrich), quercetin, (Tocris Bioscience), ferulic acid, gallic acid, epicatechin (MP Biomedicals), 3-hydroxybenzoic acid, protocatechuic acid (Ark Pharmaceutical), daidzein (Acros), myricetin (Alfa ceasar), luteolin (Cayman Chemical) and chlorogenic acid (Fluorochem) were used. All standards were supplied in powder form. An appropriate amount was weighed into a microcentrifuge tube and dissolved in 100% methanol (CH_3OH) (Fisher Scientific) to make 1 mg/mL stock solutions. These solutions were stored in aliquots at -20°C.

Stock solutions were thawed and further diluted to 100 µg/mL (with 50% methanol) as a working solution. A serial two-fold dilution was carried out to build a linear standard curve within the range of 3.13 µg/mL to 50 µg/mL.

4.2.4 High performance liquid chromatography (HPLC)

High performance liquid chromatography was carried out using a Waters, 2695 fitted with a Discovery C18 (150 mm x 4.6 mm, 5 µm) column, Superguard pre-column C18 (20 mm x 3 mm, 5 µm) and photodiode array detector. The method was adapted from that described by Jonfia-Essien *et al.,* 2008 with the optimisation of the gradient setting for mobile phase. Phosphate buffer (0.02 M pH 2.4) and HPLC grade methanol (CH_3OH) were used as mobile phases A and B. The sample was run with a gradient starting at 10% B, increasing to 70% B over 30 min, and then increasing to 90% at 32 min. A 90% methanol wash to remove the retained compounds in the column was carried out for 5 min before the column was equilibrated with 10% B for 5 min between each sample. The detection wavelength range was set at 210 nm to 700 nm for section 4.3.1.1 and at 210 nm to 400 nm for section 4.3.1.2.

The maxplot tool was used to plot the maximum spectral absorbance measured at each time point. This includes all chromatographic peaks in the sample regardless of lambda max. This was applied to analyse the results for section 4.3.1.1. After that, the comparisons were made at 280 nm. Total area of those peaks with > 1% of total peak area were integrated and used in section 4.3.1.1. In addition, the ratio of integrated area between solvents was also calculated in order to compare with the ratio of TPC activity between solvents.

4.2.5 Statistical analysis

All assays were carried out in triplicate. One-way ANOVA was used to identify differences between groups using Statgraphics Centurion version 16.1.11. When significant differences were detected ($p<0.05$), Fisher's least significant difference (LSD) was generated to determine the difference in mean. The Student's t test was used to compare solvents and correlation was analysed using simple regression analysis. Statistical significance was declared as $p<0.05$.

4.3 Results

4.3.1 Extraction efficiency of solvents for soluble phenolics in beans

Seven beans – namely adzuki bean, black eyed pea, bambara groundnut, lablab bean, mung bean, pigeon pea and soya bean were used in this study. This study was divided into two parts, part 1 compared the extraction efficiency of 80% methanol and sodium acetate buffer that have different polarities. While part 2 compared the extraction efficiency of 80% methanol and 80% acetone that have the same polarity index of 5.1. The extracts were then subjected to TPC assay and HPLC analysis to determine the total soluble phenolic in tested beans.

4.3.1.1 80% methanol (CH_3OH) versus sodium acetate buffer ($CH_3COONa.3H_2O$)

The initial experiment was to compare the effect of methanol and sodium acetate buffer, two solvents with varied polarity. Methanol was expected to extract more non polar compounds than the sodium acetate buffer.

After the HPLC analysis, the 3D maxplots were first generated to compare the extracts for each type of bean and obtain an overview of the distribution pattern of compounds at different time points and wavelengths (210 nm to 700 nm) concurrently. The typical 3D maxplots for methanol and sodium acetate extracts of soya bean are shown in figure 4.1. The corresponding 3D maxplots for the other beans are shown in appendix 4.1 to 4.6. These 3D maxplots showed that the majority of the compounds were eluted within 30 min and detected within the range of 210 to 400 nm for both extracts.

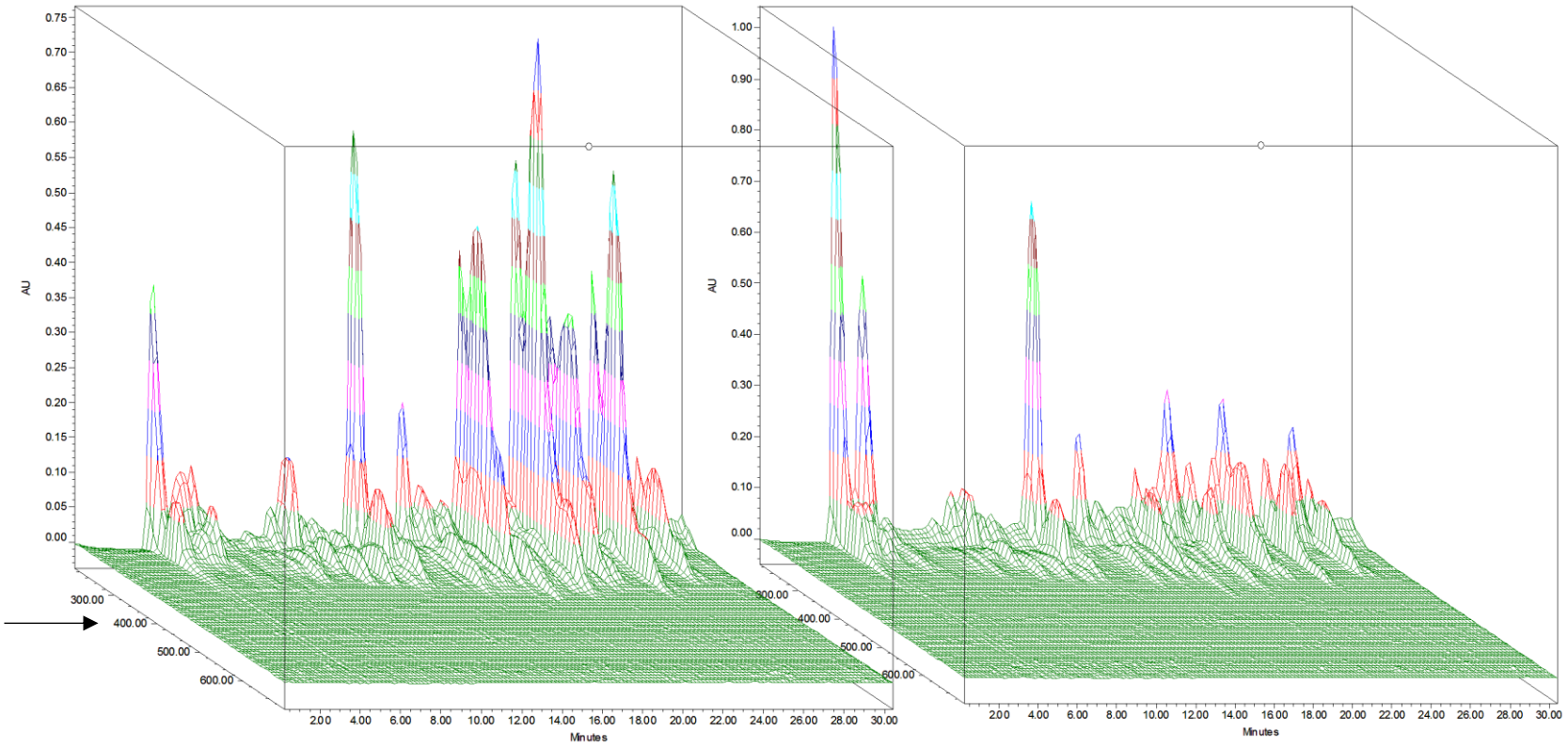


Figure 4.1 3D Maxplot for soya bean extracts. Chromatogram (Left) the 80% methanol extract; (Right) the sodium acetate buffer extract.

A direct comparison of both extracts was then carried out by overlaying the 2D maxplot chromatograms for all the beans tested (figures 4.2 and 4.3). Overall, both extracts showed good match in peak profiles. The resolved peaks in most cases have similar retention times, absorbance values and peak numbers. The exceptions are lablab bean, pigeon pea and soya bean. From the overlay chromatograms (figures 4.2 and 4.3), the sodium acetate extracts of lablab bean and pigeon pea had an additional peak at retention time of 5.53 min and 6.06 min, respectively. While, the sodium acetate extract of soya bean had 2 additional peaks at the retention times of 21.23 min and 27.03 min. Furthermore, the methanol extract of soya bean had 3 peaks (19.46, 22.22 and 26.09 min) that have higher absorbance than the corresponding peaks in the sodium acetate extract.

Overlay maxplot chromatogram were not sufficient to show the commonality or variation between the extracts. A comparison of the relative peak sizes from the maxplots was carried out to further investigate the extraction efficiency of both methanol and sodium acetate extracts (table 4.1 to table 4.3). The analysis in general showed a high degree of similarity between the compounds that have been extracted by both solvents, although there were some minor variations in relative peak sizes.

A total of 63 peaks have been integrated from both extracts for all beans tested. However, 80% methanol extracts had more unique peaks which were not found in acetate extracts. There were 13 unique peaks in the 80% methanol extracts (6.53 min, 8.84 min, 9.97 min, 10.42 min, 11.98 min, 13.10 min, 15.77 min, 20.55 min, 21.46 min, 24.20 min, 24.20 min, 24.72 min, 25.97 min and 29.81 min) while only 3 unique peaks were found in the acetate extract (4.67 min, 7.67 min and 14.61 min) when comparing all tested beans at the same retention time. The remaining 46 peaks were shared at least once between both extracts but may and may not be from the same beans.

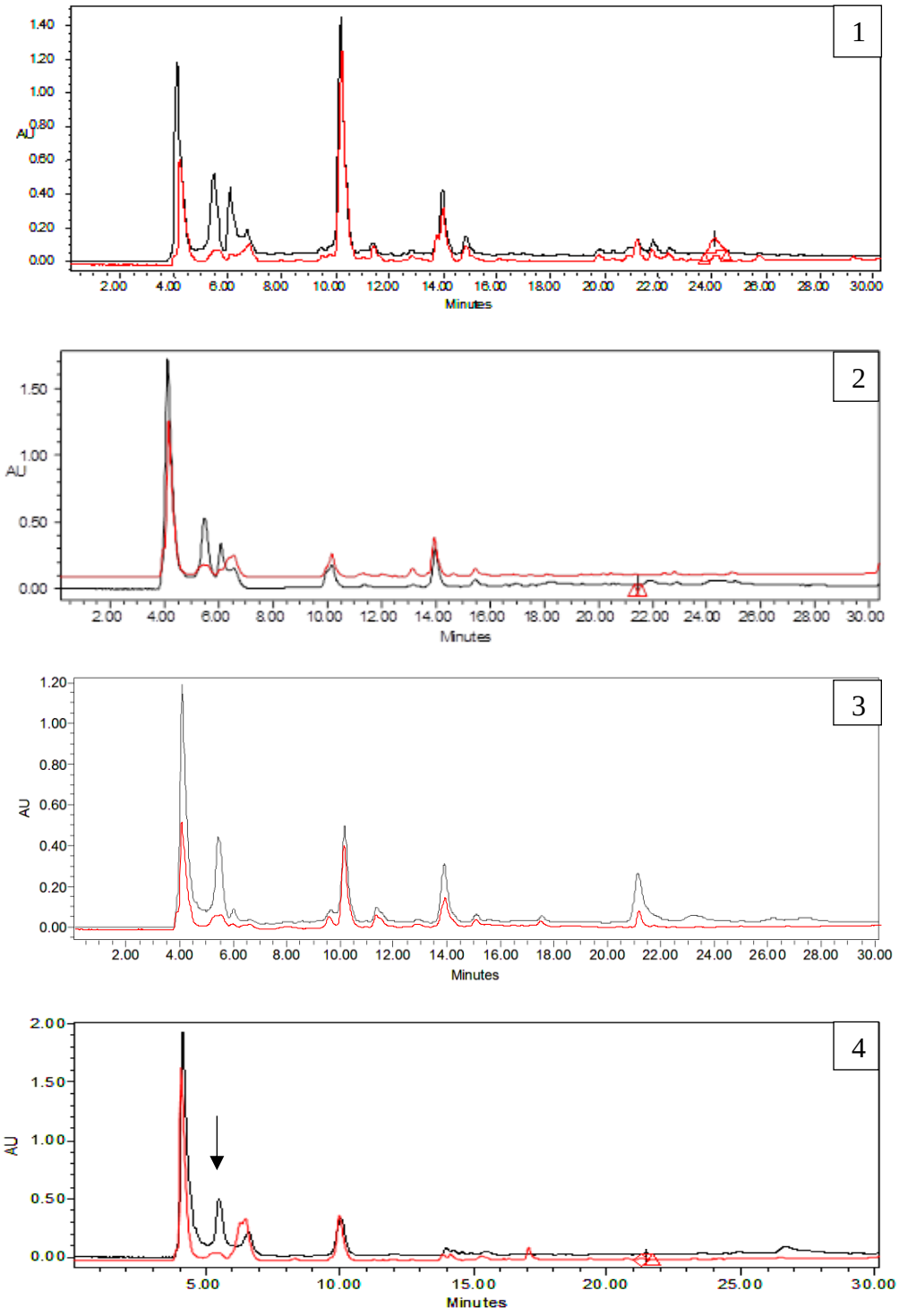


Figure 4.2 Overlay of 2D Maxplot. Red line represents the 80% methanol extract while black line represents the sodium acetate buffer extract. 1- adzuki bean, 2- black eyed pea, 3-bambara groundnut and 4-lablab bean.

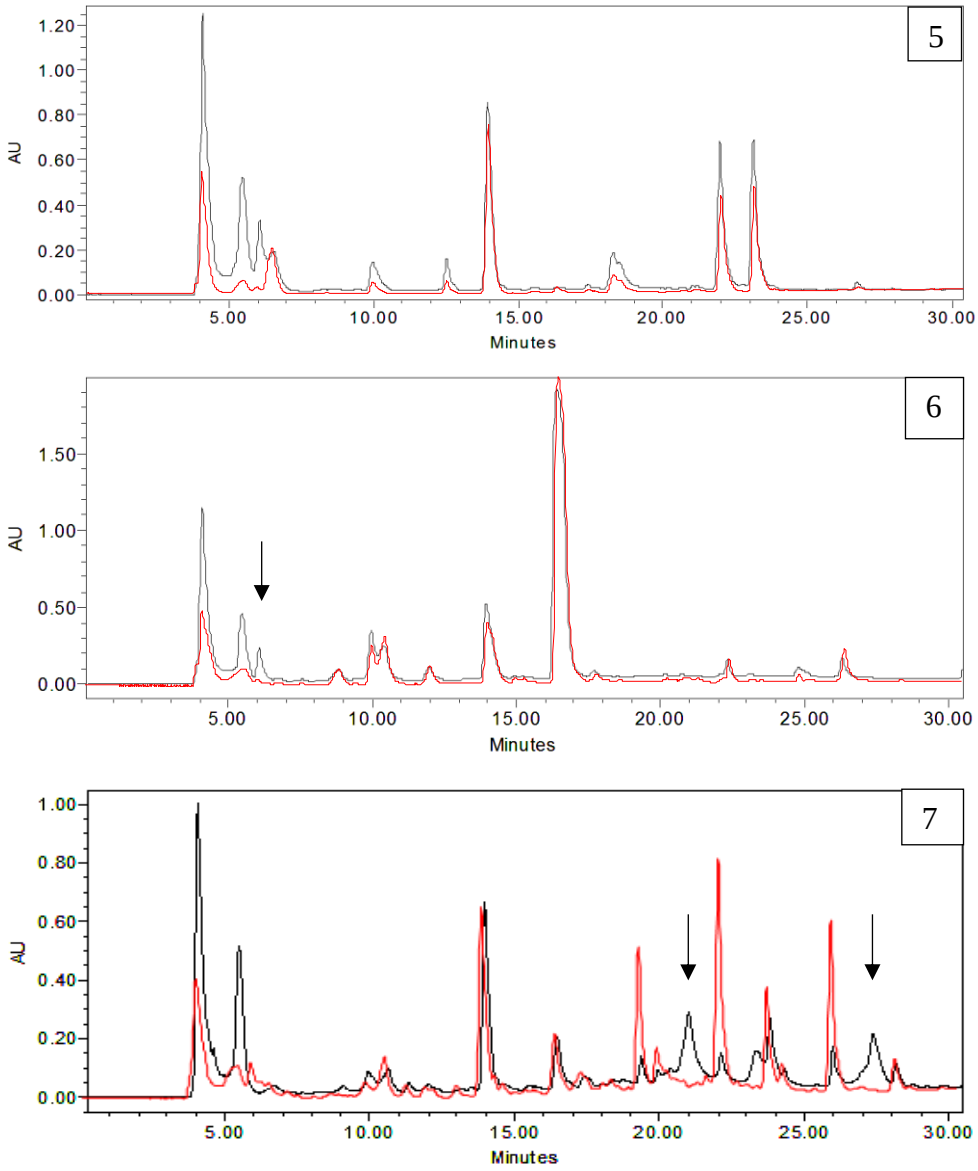


Figure 4.3 Overlay of 2D Maxplot. Red line represents the 80% methanol extract while black line represents the sodium acetate buffer extract. 5-mung bean, 6-pigeon pea and 7-soya bean.

Table 4.1 Tabulation of relative size for integrated peaks by retention time at maxplot for 80% methanol and acetate buffer extract.

Retention time, min	80 % methanol extract (µV*sec)							Acetate buffer extract (µV*sec)						
	Adzuki bean	Black eyed pea	Bambara groundnut	Lablab bean	Mung bean	Pigeon pea	Soya bean	Adzuki bean	Black eyed pea	Bambara groundnut	Lablab bean	Mung bean	Pigeon pea	Soya bean
4.13	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
4.67	-	-	-	-	-	-	-	-	-	-	++++	-	+++	++++
5.43	++++	++++	++++	++++	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++
5.58	-	-	+++	-	-	-	-	-	-	++++	-	-	-	-
6.06	++	++	+	-	+	+	++++	++++	++++	++	-	++++	++++	-
6.35	-	-	+	++++	-	-	++	-	++++	+	++++	++++	-	++
6.53	-	++++	-	++++	++++	-	++	-	-	-	-	-	-	-
6.72	++++	-	-	-	-	-	-	++++	-	+	-	-	-	-
7.28	-	-	-	-	-	+	+	+	-	-	-	-	+	-
7.57	-	-	-	-	-	-	-	+	-	+	-	-	+	-
8.15	-	-	+	-	-	-	-	-	-	+	+	-	-	-
8.59	+	-	-	+	-	-	-	+	-	-	-	+	+++	+
8.84	-	-	-	-	-	++++	+	-	-	-	-	-	-	-
9.46	+	-	-	-	-	+	+	++	-	-	-	+	+	-
9.76	+	-	+++	-	-	-	-	++	-	++	-	++++	++++	+++
9.97	-	-	-	-	++	++++	++	-	-	-	-	-	-	-
10.18	++++	++++	++++	++++	-	-	-	++++	++++	++++	++++	-	++++	++++
10.42	-	-	-	-	-	++++	++++	-	-	-	-	-	-	-
11.01	+	-	-	-	-	-	-	+	-	-	-	-	-	-
11.35	+++	++	+++	+	-	+	++	+++	+	+++	-	-	+	+
11.98	-	-	-	-	-	++++	-	-	-	-	-	-	-	-

‘-’ indicated not detectable; ‘+’ indicated the relative peak size < 500,000 µV*sec; ‘++’ indicated the relative peak size from 500,000 to 1,000,000 µV*sec; ‘+++’ indicated the relative peak size between 1,000,000 and 1,500,000 µV*sec; ‘++++’ indicated the relative peak size > 1,500,000 µV*sec.

Table 4.2 Tabulation of relative size for integrated peaks by retention time at maxplot for 80% methanol and acetate buffer extract (contd).

Retention time, min	80 % methanol extract (µV*sec)							Acetate buffer extract (µV*sec)						
	Adzuki bean	Black eyed pea	Bambara groundnut	Lablab bean	Mung bean	Pigeon pea	Soya bean	Adzuki bean	Black eyed pea	Bambara groundnut	Lablab bean	Mung bean	Pigeon pea	Soya bean
12.04	+	+	-	+	-	-	+	+	-	-	+	-	++++	+
12.53	+	-	-	-	++	-	-	+	-	-	-	++++	-	-
12.79	+	-	+	+	-	-	-	+	+	+	-	-	+	+
13.10	+	++	-	-	-	+	++	-	-	-	-	-	-	-
13.76	+++	-	-	-	-	-	-	+	-	-	-	-	+	+
13.96	++++	++++	++++	++	++++	++++	++++	++++	++++	++++	++	++++	++++	++++
14.28	-	-	-	++	-	-	++	-	-	-	++	-	-	-
14.61	-	-	-	-	-	-	-	-	-	-	+	-	-	-
14.84	+++	+	+	+	-	+	++	++++	-	+	+	-	+	-
15.14	-	-	+	+	-	+	+	-	+	+	-	-	+	-
15.51	-	++	-	++	+	-	+	-	++	-	++	+	-	+
15.77	-	-	-	-	-	-	+	-	-	-	-	-	-	-
16.08	+	-	-	+	+	-	-	+	-	-	-	-	-	-
16.54	+	+	-	-	-	++++	++++	+	+	-	+	+	++++	++++
16.90	-	+	-	-	-	-	-	+	+	-	-	+	-	-
17.54	-	+	+	+++	+	++	++++	+	+	+	-	+	-	+
18.17	-	+	-	-	+++	-	+	+	+	-	-	++++	-	+
18.50	-	-	-	-	++	+	++	-	-	-	-	++++	+	+
18.66	-	-	-	-	-	+	+	-	-	-	-	-	+	+
19.00	-	-	-	-	-	-	+	-	-	-	-	-	+	+++
19.44	-	+	-	+	-	+	++++	-	+	-	+	-	+	-
19.81	++	+	-	+	-	+	++++	++	+	-	-	-	+	++

'-' indicated not detectable; '+' indicated the relative peak size < 500,000 µV*sec; '++' indicated the relative peak size from 500,000 to 1,000,000 µV*sec; '+++' indicated the relative peak size between 1,000,000 and 1,500,000 µV*sec; '++++' indicated the relative peak size > 1,500,000 µV*sec.

Table 4.3 Tabulation of relative size for integrated peaks by retention time at maxplot for 80% methanol and acetate buffer extract.

Retention time, min	80 % methanol extract (µV*sec)							Acetate buffer extract (µV*sec)						
	Adzuki bean	Black eyed pea	Bambara groundnut	Lablab bean	Mung bean	Pigeon pea	Soya bean	Adzuki bean	Black eyed pea	Bambara groundnut	Lablab bean	Mung bean	Pigeon pea	Soya bean
20.18	-	+	+	-	-	+	++	++	+	+	-	+	-	++
20.55	-	+	-	-	+	-	++	-	-	-	-	-	-	-
20.74	-	+	-	+	+	+	+	++	+	-	-	-	+	-
21.23	-	+	+++	+	-	+	++	++++	+	++++	+	+	+	++++
21.46	-	+	-	-	-	-	++	-	-	-	-	-	-	-
21.81	-	-	+	-	++++	-	++++	+++	++	+	+	++++	+	++++
22.53	-	+	-	-	-	++++	+	++	-	-	-	+	+++	-
22.90	-	+	-	-	++++	+	+	+	+	-	+	++++	+	++++
23.48	-	-	-	+	-	-	+	+	-	-	-	-	+	++++
24.10	-	+	-	-	-	-	++++	++++	+	-	-	-	+	++
24.20	-	+	-	-	-	+	+++	-	-	-	-	-	-	-
24.72	-	-	-	-	-	+	-	-	-	-	-	-	-	-
25.05	-	+	-	+	-	+	-	-	+	-	+	-	+++	+
25.40	-	-	-	-	-	-	+	+	-	-	-	-	++++	++++
25.97	-	-	-	-	-	-	++++	-	-	-	-	-	-	-
26.67	-	-	+	+	+	++++	-	-	-	+	+++	+	-	-
27.03	-	-	-	-	-	-	+	-	-	-	-	+	-	++++
28.18	-	-	-	-	-	+	++++	-	-	-	-	+	+	+++
28.70	-	-	-	-	-	-	+	-	-	-	-	-	+	+
29.81	-	-	-	-	-	-	+	-	-	-	-	-	-	-

‘-’ indicated not detectable; ‘+’ indicated the relative peak size < 500,000 µV*sec; ‘++’ indicated the relative peak size from 500,000 to 1,000,000 µV*sec; ‘+++’ indicated the relative peak size between 1,000,000 and 1,500,000 µV*sec ; ‘++++’ indicated the relative peak size > 1,500,000 µV*sec.

Further analysis involved comparing the total integrated areas at 280 nm for both 80% methanol and sodium acetate buffer extracts. Statistical analysis showed a significant difference in total integrated area at 280 nm between the tested beans ($p<0.05$) for both extracts. The ranking of relative total area at 280 nm for 80% methanol extracts was soya bean > mung bean > pigeon pea > lablab bean = adzuki bean = black eyed pea, black eyed pea = bambara groundnut. The ranking of relative total area at 280 nm for acetate buffer extracts was mung bean > soya bean > pigeon pea = adzuki bean, adzuki bean = lablab bean > black eyed pea > bambara groundnut. Although slight variations were observed in these rankings, both soya bean and mung bean ranked the highest, whereas black eyed pea and bambara groundnut ranked the lowest. Next, the ratio of the total peak areas for 80% methanol to sodium acetate extracts (table 4.4) were compared with the corresponding TPC ratios for the same extracts.

The extracts were also subjected to TPC assay. Statistical analysis showed a significant difference between the tested beans ($p<0.05$) for both extracts (figure 4.4). The ranking of the TPC for the 80% methanol extracts was soya bean > adzuki bean > bambara groundnut > mung bean > black eyed pea = pigeon pea > lablab bean. And, the ranking for the bean extracts from sodium acetate buffer was adzuki bean > mung bean = soya bean > bambara groundnut > black eyed pea = pigeon pea > lablab bean. These results were in accordance to TPC result reported in section 3.3.4, figure 3.14. At the same time, the TPC results were calculated as the ratio of the total peak areas for 80% methanol to sodium acetate extracts (table 4.4).

From table 4.4, the higher total peak area in sodium acetate extracts suggest that these extracts potentially contain more phenolics than the methanol extracts. This was found for all beans except for soya bean where the total absorbance at 280 nm showed a higher phenolic content in the methanol extract. Similarly, the ratio of the TPC values also suggest that all sodium acetate bean extracts demonstrated higher phenolic content than methanol extracts.

However, comparing the ranking of the result for total integrated area at 280 nm and TPC values for each extract did not show a correlation. A high 280 nm absorbance was not reflected as a high TPC ranking. This was true for both extracts. Further study using phenolic standards is essential to identify these resolved peaks. The only conclusion from this study was the high similarity of peaks between 80% methanol and acetate buffer extract but 80% methanol contained more unique peaks (table 4.1 to 4.3).

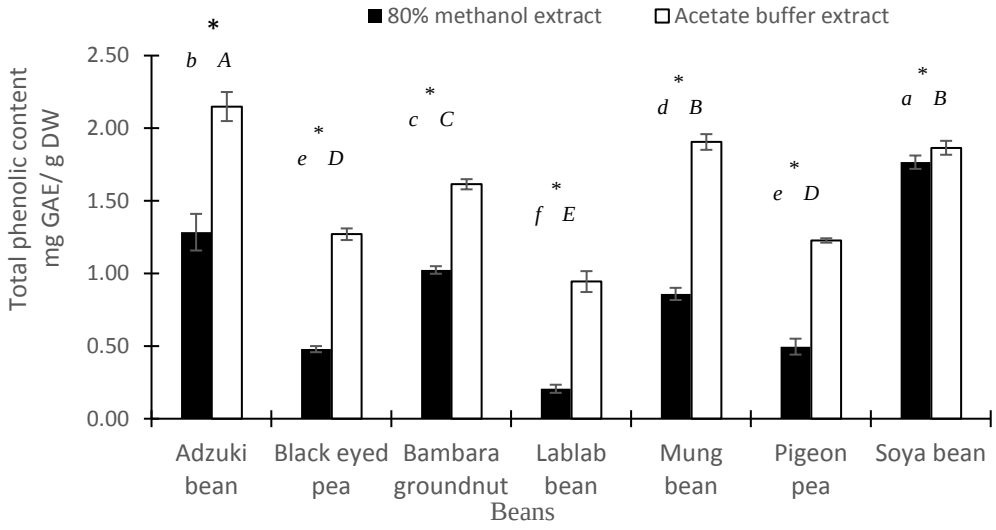


Figure 4.4 Total phenolic content of methanol and sodium acetate buffer extracts. Results are shown as the mean ± SD (n=3). * represents a significant difference between the values obtained for the two solvents at $p<0.05$. Different letters represent significant differences among beans for methanol extract at $p<0.05$.

Table 4.4 Ratio of integrated area at 280 nm and TPC value for 80% methanol to sodium acetate buffer extract for different beans.

	Ratio of integrated area at 280 nm	Ratio of TPC value
Adzuki bean	1 : 1.7	1 : 1.6
Black eyed pea	1 : 1.5	1 : 2.6
Bambara groundnut	1 : 1.8	1 : 1.6
Lablab bean	1 : 1.5	1 : 4.7
Mung bean	1 : 0.8	1 : 2.3
Pigeon pea	1 : 1.2	1 : 2.6
Soya bean	1 : 0.5	1 : 1.1

4.3.1.2 80% methanol (CH_3OH) versus 80% acetone $(CH_3)_2CO$

In Part 2, the efficiency of methanol and acetone in extracting soluble phenolics was compared. The main reason for selecting these solvents even though both had the same polarity index of 5.1 was that the acetone bean extracts had a greater colour intensity when compared to methanol extracts. Hence, both TPC and HPLC was carried out to investigate the possibility of different soluble phenolics being present in these extracts.

A comparison of physical characteristics was made between extracts in 80% methanol and 80% acetone. It was noted that the colour of the extracts varied among the beans tested. Observation found that 80% acetone extracts in general showed darker colour intensity than 80% methanol extracts. Mung bean extract showed more greenish colour as compared with bambara groundnut extract. Adzuki bean extract showed reddish to purplish colour while black eyed pea, lablab bean and soya bean extracts gave light yellow colours. However, this variation was not thought to impact directly on the subsequent TPC analysis due to the dilution factor involved in the assay.

The findings from HPLC analysis were first analysed as 2D maxplots and subsequently at 280 nm. Figures 4.5 – 4.6 show the over-layered 2D maxplot

of the 2 types of extract for all tested bean extracts. Over-layered chromatograms showed that there were no differences in terms of the number of major peaks and peak heights. The comparison of relative peak sizes from maxplot for bean extracts are shown in table 4.5 to 4.8. The analysis showed a high similarity of compounds whereby a total of 83 peaks have been integrated from both extracts for all beans tested. 80% methanol extract has 7 unique peaks which were not found in acetone extracts while 9 unique peaks were observed in acetone extracts when comparing all tested beans at the same retention time.

Chromatograms at 280 nm were first over layed (appendix 4.7 and 4.8) and the total integrated area of the peaks for both 80% methanol and acetone bean extracts was calculated, except for bambara groundnut and adzuki bean. This was due to the fact that the chromatogram for the acetone extracts from adzuki bean and bambara groundnut gave an irregular 'hump' peak shape whereby the integration could not be carried out accurately.

The ranking of relative total area at 280 nm for 80% methanol extracts was soya bean > mung bean > pigeon pea = lablab bean, lablab bean = black eyed pea. The ranking of relative total area at 280 nm for acetone extracts was soya bean > mung bean > pigeon pea > lablab bean = black eyed pea. Both extracts gave the same ranking for all the beans. Adzuki bean and bambara groundnut have not been included in this ranking.

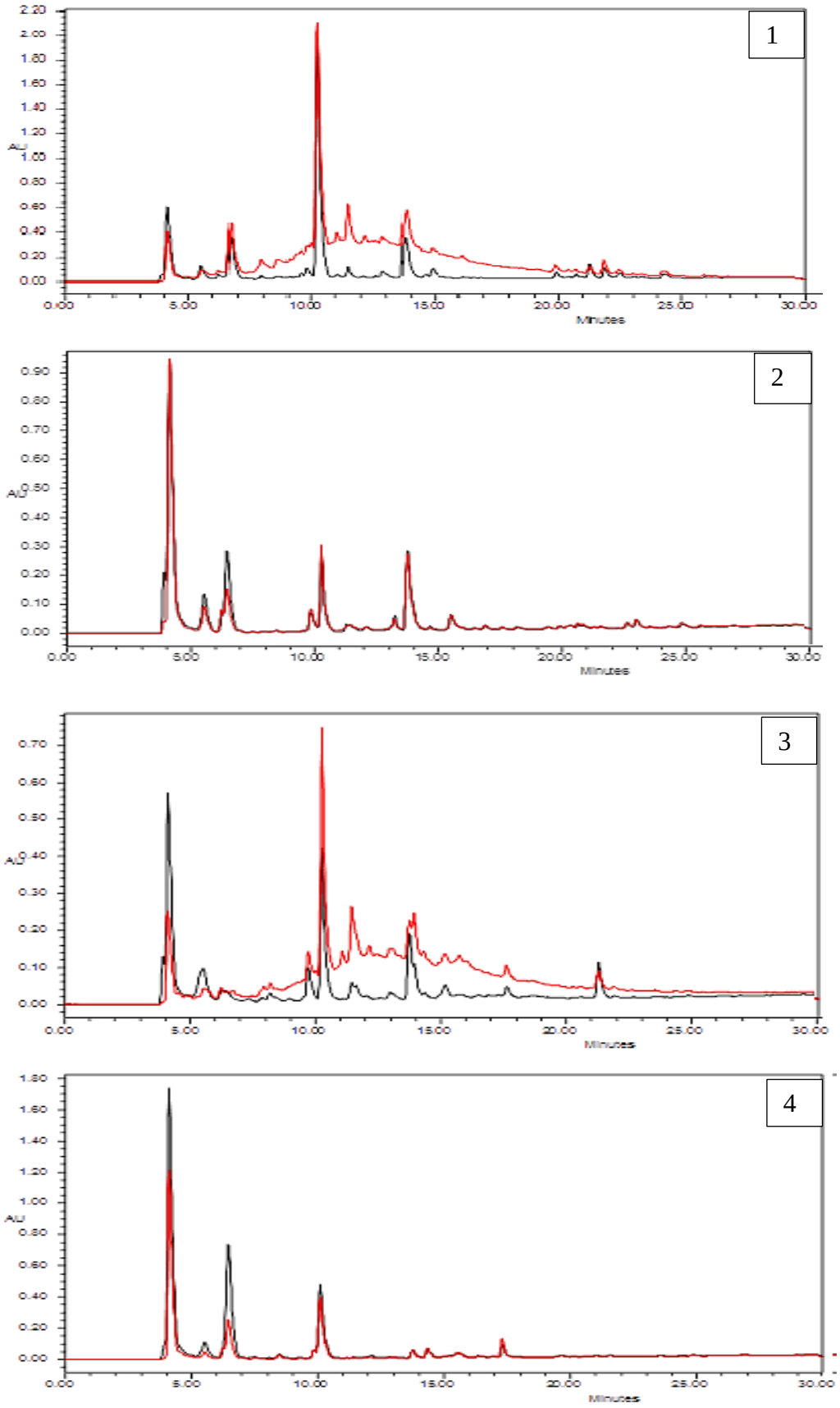


Figure 4.5 Overlay of HPLC 2D maxplot. Red line represents the 80% acetone extract while black line represents 80% methanol extract. 1-adzuki bean, 2-black eyed pea, 3-bambara groundnut and 4-lablab bean.

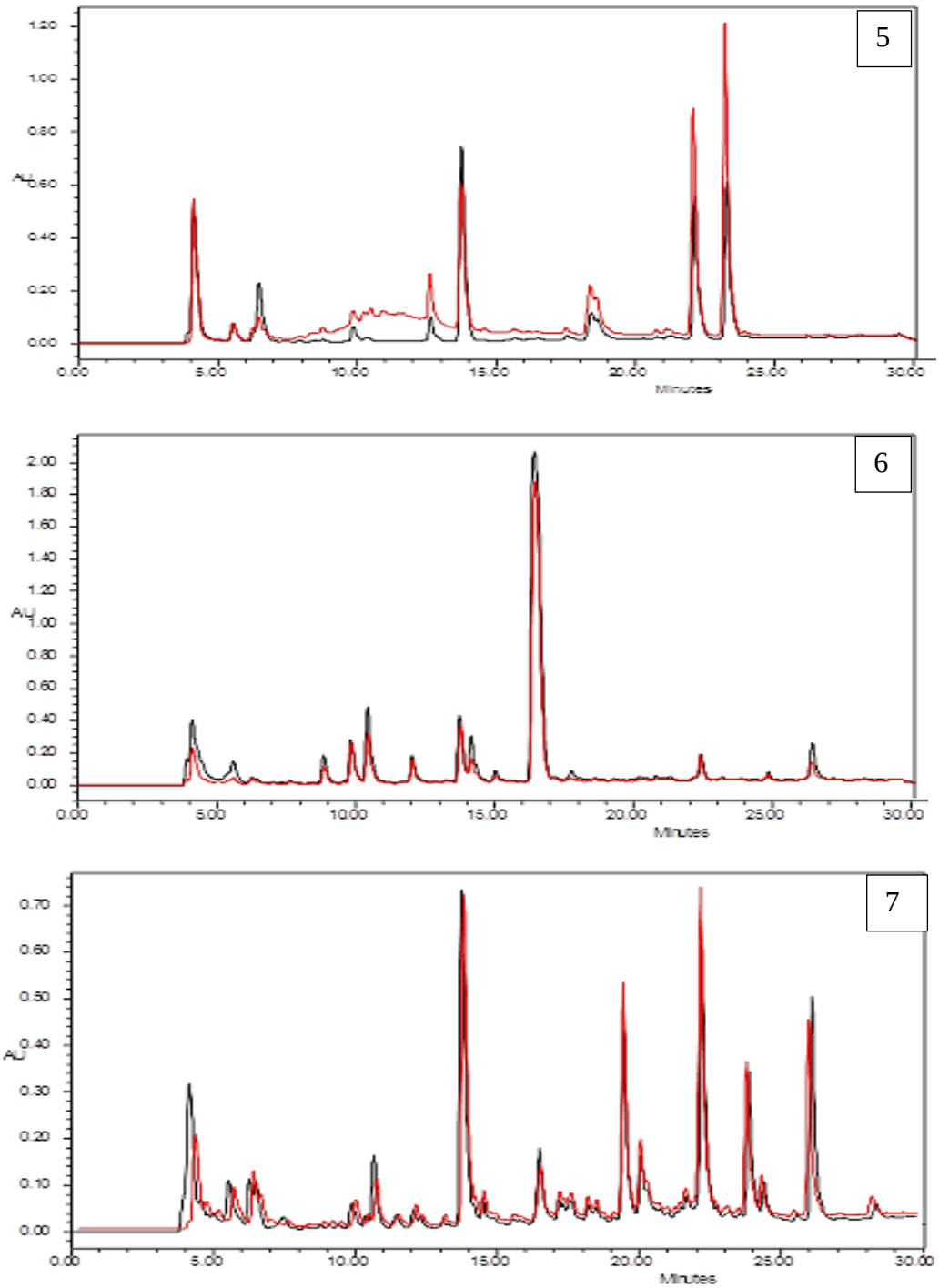


Figure 4.6 Overlay of HPLC 2D maxplot. Red line represented the 80% acetone extract while black line represented 80% methanol extract. 5-mung bean, 6-pigeon pea and 7-soya bean.

Table 4.5 Tabulation of relative size for integrated peaks by retention time at maxplot for 80% methanol and acetone extract.

Retention time, min	80 % methanol extract (µV*sec)							Acetone extract (µV*sec)						
	Adzuki bean	Black eyed pea	Bambara groundnut	Lablab bean	Mung bean	Pigeon pea	Soya bean	Adzuki bean	Black eyed pea	Bambara groundnut	Lablab bean	Mung bean	Pigeon pea	Soya bean
4.13	++++	++++	++++	++++	++++	++++	++++	-	++++	-	++++	++++	++++	++++
4.56	-	-	-	-	-	-	+++	-	-	-	-	-	-	++
4.87	-	-	-	-	-	-	-	-	-	-	+	-	-	+
5.02	+	-	-	-	+	+	++	-	+	-	+	+	+	++
5.53	++++	++++	++++	++++	+++	++++	++++	-	++++	-	++	+++	++	++++
6.25	++	-	+	-	-	+++	+++	-	++	-	++	++	++	+++
6.45	+	++++	++	++++	++++	-	++++	-	++++	-	++++	++++	+	++
6.72	++++	-	-	-	-	-	-	-	+	-	+	-	+	+
7.00	-	+	-	-	-	+	-	-	-	-	-	+	-	+
7.31	+	-	+	-	+	+	-	-	-	-	-	-	-	-
7.46	+	+	-	+	-	+	+	-	+	-	+	+	+	+
7.96	++	+	+	-	-	+	+	-	+	-	-	++	-	+
8.20	-	+	+	+	+	-	+	-	-	-	-	-	-	+
8.46	-	+	-	+	+	+	+	-	+	-	+	++	+	+
8.61	++	+	+	+	+	++++	+	-	+	-	-	+++	+++	+
9.06	+	-	-	-	-	-	+	-	-	-	-	-	-	-
9.30	+	+	-	+	-	-	+	-	+	-	+	+	-	+
9.53	++	+	-	-	-	+	-	-	+	-	-	-	+	+
9.84	++++	++	+++	++	++	++++	++	-	+++	-	++	++++	++++	++
10.27	++++	++++	++++	++++	+	++++	+	-	++++	-	++++	++++	-	+
10.49	-	-	-	-	-	-	-	-	-	-	-	++++	++++	+++

'-' indicated not detectable; '+' indicated the relative peak size < 500,000 µV*sec; '++' indicated the relative peak size from 500,000 to 1,000,000 µV*sec; '+++' indicated the relative peak size between 1,000,000 and 1,500,000 µV*sec ; '++++' indicated the relative peak size > 1,500,000 µV*sec.

Table 4.6 Tabulation of relative size for integrated peaks by retention time at maxplot for 80% methanol and acetone extract (contd).

Retention time, min	80 % methanol extract (µV*sec)							Acetone extract (µV*sec)						
	Adzuki bean	Black eyed pea	Bambara groundnut	Lablab bean	Mung bean	Pigeon pea	Soya bean	Adzuki bean	Black eyed pea	Bambara groundnut	Lablab bean	Mung bean	Pigeon pea	Soya bean
10.77	-	-	-	+	+	-	++++	-	-	-	+	++++	+	-
11.10	++	+	+	+	+	+	-	-	-	-	+	+++	+	-
11.46	++++	+	++	+	+	+	+	-	++	-	+	+++	+	+
11.63	-	-	-	-	-	-	-	-	-	-	-	++++	+	-
11.84	-	-	++	+	+	-	-	-	-	-	+	-	+	-
12.08	++	+	+	+	+	++++	+	-	+	-	+	++	++++	+
12.55	+	-	+	+	+	-	+	-	+	-	+	+++	+	+
12.87	+++	+	++	+	+++	+	-	-	+	-	+	++++	+	+
13.13	-	++	-	+	-	+	+	-	++	-	+	-	+	-
13.75	++++	++++	++++	++	++++	++++	++++	-	++++	-	++	++++	++++	++++
13.97	-	-	++	-	-	++++	+	-	-	-	-	-	-	-
14.35	-	-	+	++	+	-	-	-	-	-	++	-	++++	++
14.59	+	+	-	-	+	-	++	-	-	-	-	+++	-	++
14.67	-	-	-	-	-	-	-	-	+	-	-	-	-	+
14.89	+++	-	-	+	-	+++	+	-	+	-	+	++	++	+
15.34	+	-	++	+	-	-	+	-	-	-	+	-	-	+
15.50	-	++	-	+	-	+	+	-	++	-	++	+++	+	+
15.67	-	-	-	+	+	-	-	-	-	-	-	-	+	+
16.12	+	+	-	-	-	+	+	-	+	-	+	++	+	-
16.33	+	+	-	+	+	-	-	-	-	-	-	-	-	-
16.59	+	-	-	+	+	++++	++++	-	+	-	+	++	++++	++++

'-' indicated not detectable; '+' indicated the relative peak size < 500,000 µV*sec; '++' indicated the relative peak size from 500,000 to 1,000,000 µV*sec; '+++' indicated the relative peak size between 1,000,000 and 1,500,000 µV*sec ; '++++' indicated the relative peak size > 1,500,000 µV*sec.

Table 4.7 Tabulation of relative size for integrated peaks by retention time at maxplot for 80% methanol and acetone extract (contd).

Retention time, min	80 % methanol extract (µV*sec)							Acetone extract (µV*sec)						
	Adzuki bean	Black eyed pea	Bambara groundnut	Lablab bean	Mung bean	Pigeon pea	Soya bean	Adzuki bean	Black eyed pea	Bambara groundnut	Lablab bean	Mung bean	Pigeon pea	Soya bean
16.86	+	+	-	+	+	-	+	-	+	-	+	-	-	+
17.08	-	-	-	-	-	-	-	-	-	-	-	+	+	++
17.28	-	+	-	+++	+	-	+	-	+	-	+++	+	-	+
17.59	-	+	++	-	+	+++	+++	-	+	-	-	+++	++	+++
18.17	-	+	-	+	-	+		-	+	-	+	-	+	++
18.45	-	-	-	+	++++	+	++	-	-	-	+	++++	+	++
18.66	+	-	-	-	+++	+	++	-	-	-	-	++++	+	+
18.95	-	-	-	+	-	+	+	-	-	-	-	-	+	-
19.16	+	-	-	-	-	+	+	-	-	-	-	-	+	+
19.44	-	+	-	+	+	+	++++	-	+	-	+	+	+	++++
19.90	++	+	-	-	+	+	-	-	+	-	-	+	+	-
20.09	-	-	-	-	-	-	-	-	-	-	+	+	-	++++
20.26	-	+	-	+	+	+	++++	-	+	-	-	+	+	++++
20.41	+	-	-	-	-	+	++	-	-	-	-	-	+	+++
20.72	+	+	-	+	+	+	+	-	+	-	+	++	+	+
20.97	+	+	-	+	+	+	+	-	+	-	-	-	-	-
21.00	-	-	-	-	-	-	-	-	-	-	+	+	+	+
21.30	++++	+	+++	-	+	+	+	-	+	-	-	+	+	+
21.48	-	-	-	-	-	-	+	-		-	-	-	-	+
21.57	-	+	-	+	+	+	++	-	+	-	+	-	-	-
21.90	+++	-	+	+	-	-	-	-		-	-	-	-	-

'–' indicated not detectable;'+' indicated the relative peak size < 500,000 µV*sec; '++' indicated the relative peak size from 500,000 to 1,000,000 µV*sec; '+++' indicated the relative peak size between 1,000,000 and 1,500,000 µV*sec ; '++++' indicated the relative peak size > 1,500,000 µV*sec.

Table 4.8 Tabulation of relative size for integrated peaks by retention time at maxplot for 80% methanol and acetone extract.

Retention time, min	80 % methanol extract (µV*sec)							Acetone extract (µV*sec)						
	Adzuki bean	Black eyed pea	Bambara groundnut	Lablab bean	Mung bean	Pigeon pea	Soya bean	Adzuki bean	Black eyed pea	Bambara groundnut	Lablab bean	Mung bean	Pigeon pea	Soya bean
22.17	-	+	-	+	++++	+	++++	-	+	-	+	++++	+	++++
22.53	++	+	-	+	-	++++	+	-	+	-	+	-	++++	+
23.03	+	+	-	-	-	+	-	-	+	-	+	-	+	-
23.27	+	+	-	-	++++	+	+	-	-	-	-	++++	+	+
23.55	-	-	-	+	-	+	+	-	+	-	+	-	+	+
23.88	-	-	-	-	+	+	++++	-	-	-	-	-	+	++++
23.92	-	-	-	-	-	-	-	-	-	-	-	+	+	-
24.25	+	-	-	+	+	+	+++	-	+	-	+	+	+	+++
24.40	+	+	-	-	-	-	-	-	-	-	-	-	-	-
24.88	+	+	+	+	+	++	+	-	+	-	+	+	++	+
25.90	+	+	+	+	+	+	+	-	+	-	+	+	+	+
26.22	-	+	-	+	+	-	++++	-	+	-	+	+	+	++++
26.45	+	+	-	-	+	++++	-	-	-	-	-	+	++++	-
26.84	-	-	-	+	+	+	+	-	+	-	+	+	+	-
26.95	+	+	-	-	-	-	-	-	+	-	+	+	+	+
27.22	-	-	-	-	-	-	+	-	-	-	-	-	-	-
27.94	-	-	-	-	-	-	-	-	+	-	+	+	+	+
28.55	-	+	-	+	-	+	+	-	+	-	+	+	+	++
29.01	-	+	-	+	-	+	+	-	+	-	+	-	-	+
29.46	-	+	-	+	+	+	+	-	+	-	+	+	+	+

'-' indicated not detectable; '+' indicated the relative peak size < 500,000 µV*sec; '++' indicated the relative peak size from 500,000 to 1,000,000 µV*sec; '+++' indicated the relative peak size between 1,000,000 and 1,500,000 µV*sec ; '++++' indicated the relative peak size > 1,500,000 µV*sec.

The findings from TPC assay showed that the phenolic content was highest in both methanol and acetone extracts of adzuki bean and lowest in lablab bean (figure 4.7). The ranking of phenolic content for 80% methanol extracts was adzuki bean > soya bean > mung bean > black eyed pea = bambara groundnut > pigeon pea > lablab bean. The ranking for acetone extracts was adzuki bean > mung bean > bambara groundnut > black eyed pea = soya bean > lablab bean = pigeon pea.

A follow up analysis using the ratios of the total peak areas for 80% methanol to 80% acetone is shown in table 4.9 along with the corresponding TPC ratios for the same extracts. The result showed that only black-eyed pea had similar ratios in total peak area for both extracts. The ratios of total peak area in acetone extract of mung bean and soya bean were larger than 1.0. Whereas the ratios for lablab bean and pigeon pea acetone extracts were lower than 1.0.

The ratio of the TPC values showed that acetone extract might have higher phenolic content than the methanol extract, in which all beans had a ratio of more than 2 and bambara groundnut has the highest ratio of 5.3. This again highlights the difficulty in using either of these approaches to estimate phenolic content.

The current study suggested that all three solvents are suitable for extraction of phenolic contents and for analysis via TPC assay and HPLC technique. The 80% methanol was selected for further HPLC analysis coupled with phenolic standards. This is because overall methanol extract generated more unique peaks when compare to the other solvents. Furthermore, methanol extract has a shorter drying time when compared with sodium acetate buffer extract, and the chromatogram shows greater resolution with better peak separation than 80% acetone extract.

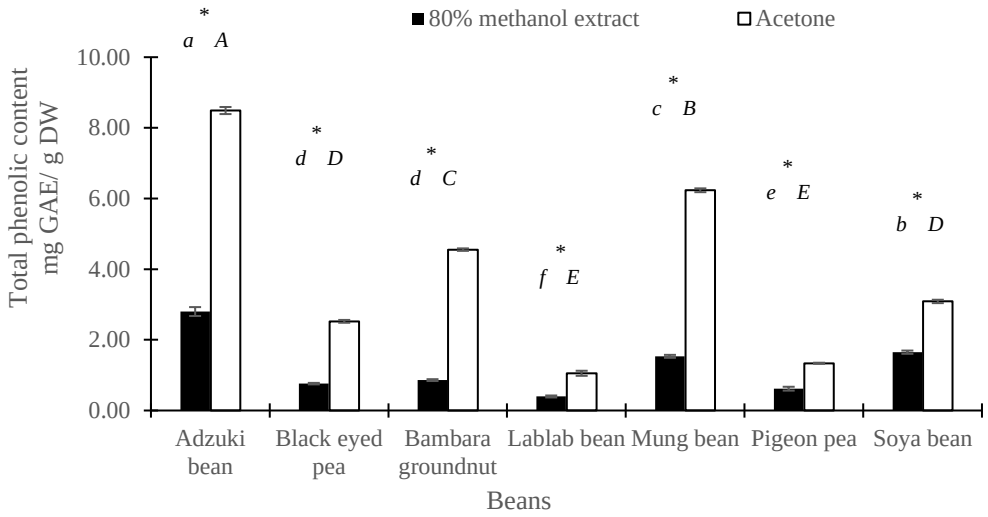


Figure 4.7 Total phenolic content of methanol and acetone extracts. Results are shown as the mean ± SD (n=3). * represents a significant difference between the values obtained for the two solvents at $p<0.05$. Different letters represent significant differences among beans for methanol extract at $p<0.05$.

Table 4.9 Ratio of integrated area at 280 nm and TPC value for 80% (CH_3OH) to 80% acetone $(CH_3)_2CO$ extract for different underutilised beans.

	Ratio of integrated area at 280 nm	Ratio of TPC value
Adzuki bean	-	1 : 3.0
Black eyed pea	1.0 : 1.0	1 : 3.3
Bambara groundnut	-	1 : 5.3
Lablab bean	1.0 : 0.9	1 : 2.6
Mung bean	1.0 : 1.6	1 : 4.0
Pigeon pea	1.0 : 0.8	1 : 2.2
Soya bean	1.0 : 1.1	1 : 1.9

4.3.2 Method development for identification and quantification using HPLC

After solvent selection, the optimisation of the HPLC separation method was then carried out using 20 phenolic standards. The retention time and spectra of each standard are shown in table 4.10. The wavelength of 280 nm was generally used in identification and quantification of phenolic compounds. However in this study, the optimised wavelength designated for each phenolic standard (appendix 4.9 to 4.10) was adopted as shown in table 4.10. Next, a standard curve has been built, as shown in appendix 4.11, within the range of 3.13 µg/mL to 50 µg/mL for each standard tested. The formula generated was applied for the quantification of the phenolics in chapter 5 and 6.

Apart from understanding the basic characteristics of the standard reference compounds when run on the HPLC method, a check for recovery and stability was also carried out. A 25 µg/mL of standard was spiked into 80% methanol solvent and underwent the same extraction protocol as the bean extract before being analysed by HPLC. Results showed that all standards were stable in 80% methanol with a recovery of >70% (figure 4.8). Hence, 80% methanol is a suitable extraction solvent with minimal compound degradation.

Table 4.10 Tabulation of data for retention time, spectral and lambda max for phenolic standards.

	Compounds	RT, minute	spectral	λ_{max} , nm	Standard Curve, $n=3^2$	R^2
1.	Gallic acid	7.8	216.9, 272.2	220	y=150132x	0.9996
2.	Protocatechuic acid	11.57	260.4, 294.7	260	y=67743x	0.9992
3.	Chlorogenic acid	15.03	219.2, 241.6, 328.0	320	y=46091x	0.9968
4.	Epigallocatechin gallate	15.91	276.9	280	y=23423x	0.9964
5.	Epicatechin	17.13	279.3	280	y=11435x	0.9844
6.	Caffiec acid	17.63	218.1, 240.4, 325.6	320	y=92358x	0.9998
7.	3-hydroxybenzoic acid	17.98	236.8, 298.3	220	y=56133x	0.9985
8.	Epicatechin gallate	19.46	279.3	280	y=29955x	0.9980
9.	p-coumaric acid	20.98	227.4, 310.1	320	y=121060x	0.9991
10.	Sinapic acid	21.25	236.8, 322.0	320	y=105097x	0.9960
11.	Ferulic acid	21.44	218.1, 235.7, 324.4	320	y=79093x	0.9998
12.	3-hydroxycinnamic acid	23.18	214.5, 279.3	280	y=109256x	0.9998
13.	Rutin	24.08	256.9, 358.8	260	y=28966x	0.9939
14.	Myricetin	25.92	254.5, 371.8	250	y=53951x	0.9982
15.	Daidzein	28.15	249.8, 303.0	250	y=105097x	0.9997
16.	Quercetin	29.27	255.7, 370.6	250	y=46527x	0.9948
17.	Naringenin	29.76	213, 290	220	y=88544x	0.9994
18.	Luteolin	30.32	254.5, 350.6	360	y=19310x	0.9981
19.	Genistein	30.71	261.6	260	y=120434x	0.9983
20.	Kaempferol	32.13	266.3, 369.4	360	y=51739x	0.9983

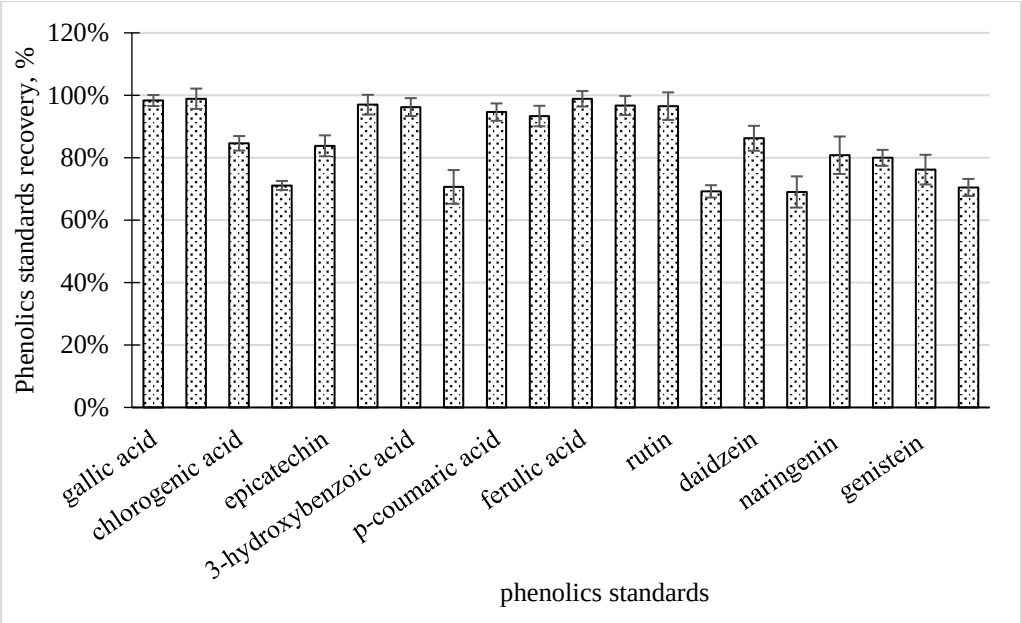


Figure 4.8 Stability of 20 phenolic standards in 80% methanol extraction. 1 mL of 25 µg/mL standard mixtures was added to 9 mL of 80% methanol. This was then subjected to extraction (section 4.2.1) and the recovery of the standard was assessed. Results are shown as the mean ± SD (n=3).

4.4 Discussion

Current findings (chapter 3) suggested that colorimetric antioxidant assays may not be sufficient in differentiating the efficiency of different solvents in extracting phenolic compounds. An additional analytical tool, HPLC technique was applied in this further study that involved sodium acetate buffer, 80% methanol and 80% acetone extracts.

The 3D maxplots are useful overviews of the compounds that exist in each bean and their absorption maxima. This can be used to inform the range of wavelengths to be employed and the run time for the analysis. For example, 30 mins of HPLC run time and range of 210 to 400 nm are sufficient to separate and detect the compounds in the bean samples in this study. On top of that, this is also a preliminary indication that the profile of compounds obtained from individual beans differ between beans.

Different compounds have different absorption spectra. For example, simple phenolic acids show spectral maxima in the range 250 – 290 nm whilst anthocyanins exhibit absorption in the visible region 475 – 560 nm, 535 – 545 nm and subsidiary peaks at 270 – 275 nm (Lattanzio *et al.*, 2008). The lack of peaks corresponding to anthocyanin (figure 4.1, appendix 4.1 to 4.6) is the confirmation of the previous result from the total monomeric anthocyanin assay in chapter 3. Results (section 3.3.2) showed that among all the beans tested only bambara groundnut contained traces of anthocyanin.

Next, 2D plots at 280 nm were then used as this is a general absorption for compounds associated with phenolics as mentioned by Lattanzio *et al* (2008). However, this does not mean that all peaks at 280 nm are going to be associated with phenolics. The HPLC technique does however, increase the chance of detecting phenolic compounds by eliminating other compounds that do not absorb at 280 nm. Having said that, there are still going to be confounding compounds that absorb at 280 nm but are not phenolics. However, comparisons of these 2D plots does providing an indication of the differences in compounds being extracted from the individual beans.

Two approaches – total integrated area at 280 nm and TPC- have been utilised in an attempt to compare the differences in phenolic content among the acetate buffer, methanol and acetone extracts. The ratio for the integrated area at 280 nm showed that both 80% methanol and 80% acetone potentially contain similar compounds but acetate buffer extract was better than methanol extract giving a higher ratio value. This could be taken to indirectly show that both methanol and acetone were extracting similar soluble compounds. However, acetate buffer was extracting more non solvent soluble compounds.

Next, the ratios for TPC values showed that methanol was perhaps weaker than acetate buffer and acetone in extracting the compounds associated with phenolics. This is the opposite conclusion to that from comparing the ratios for integrated area at 280 nm. Further analysis showed that there was no correlation between the ranking obtained from the total integrated area at 280 nm and the TPC values. A high TPC value was not associated with high 280 nm absorbance using any of the three solvents. For example, 1: 4.7 ratio of TPC value but only 1:1.5 ratio of integrated area at 280 nm for methanol and acetate extracts of lablab bean.

This might indicate that TPC is less capable in differentiating phenolic associated compounds as compared to HPLC techniques. This may be because the TPC assay has been used as a tool to estimate the phenolic content but is not entirely suitable since it can react with non-phenolic compounds and can thus over estimate their content (Singleton and Rossi, 1965). And, previous results have also shown that this assay was unable to detect any variation in extracts using different solvents (Xu and Chang, 2007). As a result, Bahorun *et al.* (2004) suggested that this assay be renamed the 'Folin-Ciocalteu index' rather than total phenol.

The HPLC technique generated more detailed information such as peak number, height and integrated peak area that could be used to quantify phenolic compounds. On top of that, HPLC analysis gave a more rational outcome in that there was a higher ratio value for methanol and acetate buffer extracts (different polarity) than for methanol and acetone extracts (same polarity). This

might be expected as the phenolics are more likely to be soluble in the organic solvents.

Although application of HPLC may start to overcome some of the limitations of the biochemical assay, it is also true that neither method can be used to measure the phenolic content with total accuracy without referring to phenolic standards. As a result, application of integrated area at 280 nm will be more complete and better than TPC if using actual phenolic standards.

The current study suggested that all three solvents are suitable for extracting phenolic associated compounds. The 80% methanol was selected for further HPLC analysis coupled with phenolic standards. This is because overall methanol extract generated more unique peaks when compared to the other two solvents. Furthermore, methanol extract has a shorter drying time when compared with sodium acetate buffer extract, and the chromatogram shows greater resolution with better peak separation than 80% acetone extract.

This finding is supported by a published report that showed that 80% methanol was recommended as the best solvent system (Zielinski and Kozlowska, 2000). Variation in water and solvent ratio did not help in diversifying the type of compounds but did effect the concentration (Spigno *et al.*, 2007). Thus, variation in concentration may not be as important as identifying the diversity of the extracted compounds at the initial stage of compound profiling. Therefore, no further analysis on the efficiency of extraction at different ratios of methanol to water were carried out in this study. However, the stability of a range of standard phenolics in 80% methanol was assessed. This is because stability of the target compound is a key determinant when designing an extraction technique.

Since HPLC technique gives a better comparison than colorimetric assay, HPLC analysis coupled with phenolic standards is important in identifying and quantifying soluble phenolics in bean samples. Therefore, a range of standards was obtained and their characteristics in terms of retention time and absorbance

spectra were determined. These data can also be applied to identify and quantify bound phenolics.

4.5 Conclusion

Both TPC and HPLC analysis showed that all three solvents are suitable for extracting the phenolic associated compounds. But, HPLC technique provides more detailed information when compared with TPC. It showed that a similar range of compounds appear to have been extracted with 80% methanol, sodium acetate buffer and 80% acetone solvent even though the TPC assay in each case exhibited a significant variation.

Although HPLC analysis showed the similarities among the solvents, 80% methanol was more suitable than either sodium acetate buffer or 80% acetone. This is because the resultant extract did not give any irregular peak shapes and had a short drying time. On top of that, this solvent provides more than 70% stability to the standard phenolics used in this study.

Characteristics of a range of phenolic standards in terms of retention time and absorbance spectra were determined using HPLC technique. This data will be used in the following chapters to identify and quantify the phenolics that exist in the beans.

CHAPTER 5

Development of methodologies for the analysis of conjugated and bound phenolics from selected beans

5.1 Introduction

Soluble free, conjugated or insoluble bound phenolics are commonly found in plants (Acosta-Estrada *et al.*, 2014). Soluble free phenolics are phenolic acids that are not conjugated. Carboxylic acid and hydroxyl groups in the structure of phenolic acids can easily form ester and ether bonds with other compounds, often with sugar moieties, forming soluble conjugated phenolics. The carboxylic acid and hydroxyl groups are also able to bind with cell wall polysaccharides, cellulose or hemicellulose to become insoluble bound phenolics (Yu *et al*., 2001).

Phenolics are known for promoting antioxidant activity and health benefits (Xu & Chang, 2007). Hence, soluble phenolics are reeasonably well understood. However, investigations of bound phenolics in beans are still lacking and are mostly focussed in cereal and grains that are major staple food commodities (Miller *et al.*, 2000). The analysis of deconjugated phenolics is an indirect methodology to estimate the possibility of phenolic acids that exist in conjugated phenolics since standard references for conjugated phenolics are costly and not always available. Thus, a developed methodology targetting deconjugated phenolics will be useful especially in determining phenolic profiles. It will also help in the analysis by tandem mass spectrophotometry.

The previous chapter developed an optimised extraction method for soluble phenolics from beans. Hence, this chapter will focus on developing optimised extraction and analysis methods for the extraction and analysis of conjugated and bound phenolics from beans. It will begin with the investigation of hydrolysis treatment. Acid and alkaline hydrolysis are the two most common methods (Stalikas, 2007) to break the ester and ether bonds because they are not as costly compared with enzymatic hydrolysis. Hence, these methods will be used in analysing conjugated and bound phenolics from selected beans followed by ethyl acetate ($CH_3COOC_2H_5$) liquid/liquid partitioning.

Lablab bean and mung bean were selected for this preliminary investigation in order to determine the type of hydrolysis that would be most suitable for the analysis of conjugated and bound phenolics. Subsequently, optimisation of the hydrolysis treatment was carried out using phenolic standards. Firstly to compare the efficiency of different post hydrolysis partition methods. Then the selected hydrolysis treatment, in combination with the optimum partition method, was applied to phenolic standards in order to finalise the procedure for both conjugated and bound phenolics. All comparisons were carried out using the HPLC technique.

5.2 Methodology

5.2.1 Hydrolysis treatments

5.2.1.1 Acid hydrolysis of conjugated phenolics

An 80% methanol (CH_3OH) extract was prepared according to chapter 3, section 3.2.3. Four mL of 80% methanol (CH_3OH) extract was added to an equal volume of 5 M hydrochloric acid (HCl) and stirred for either 2 or 24 h followed by extraction through ethyl acetate partitioning (section 5.2.2) to obtain phenolic compounds.

5.2.1.2 Acid hydrolysis for bound phenolics

Residue after the 80% solvent extraction was used for bound phenolics study. 40 mL of 5 M hydrochloric acid (HCl) was added to the residue from 1 g of methanol extracted bean and homogenized for 1 s. Next, the mixture was stirred for 2 or 24 h. After the treatment, the mixture was then centrifuged at 4696 *g*, 4 °C for 15 min. Supernatant was then transferred into a new tube. Hexane ($CH_3(CH_2)_4CH_3$) was added to the supernatant at volume ratio of 1:1 (v/v) to remove the fat which will reduce the extraction efficiency. The defatting treatment was repeated twice. Next, the aqueous layer was extracted through liquid-liquid partitioning (section 5.2.2).

5.2.1.3 Alkaline hydrolysis treatment of conjugated phenolics

An 80% methanol (CH_3OH) extract was prepared according to chapter 3, section 3.2.3. Four mL of 80% methanol (CH_3OH) extract was added to an equal volume of 2 M sodium hydroxide (NaOH) and stirred for a range of times between 5 min and 24 h. Next, the pH was adjusted to pH 2.0 with 5 M hydrochloric acid (HCl) followed by extraction though partition to obtain phenolic compounds (section 5.2.2).

5.2.1.4 Alkaline hydrolysis treatment of bound phenolics

Residue after 80% solvent extraction was used for bound phenolics study. 40 mL of 2 M sodium hydroxide (NaOH) was added to the residue from 1 g of bean after methanol extraction and homogenized for 1 s. Next, the mixture was stirred for a range of times between 5 min and 24 h. After the treatment, the pH was adjusted to pH 2.0 with 5 M hydrochloric acid (HCl). The mixture was then centrifuged at 4696 *g*, 4°C for 15 min. Supernatant was then transferred into a new tube. Hexane ($CH_3(CH_2)_4CH_3$) was added to the supernatant at a volume ratio of 1:1 (v/v) to remove the fat content which will reduce the extraction efficiency. The defatting treatment was repeated twice. Next, the aqueous layer was extracted through partitioning (section 5.2.2).

5.2.2 Partitioning methods

5.2.2.1 Ethyl acetate ($CH_3COOC_2H_5$) liquid/liquid partitioning

Ethyl acetate ($CH_3COOC_2H_5$) was added to the hydrolysed sample at a volume ratio of 1:1 (v/v) and vortexed vigorously. The mixture was then left for 10 min for partitioning to occur. The solvent layer was then transferred to a new tube. This step was repeated 5 times and the pooled solvent layers were then dried under nitrogen gas. Dried extract was then kept in 4°C before further analysis.

5.2.2.2 Acetonitrile (CH_3CN) salting out liquid/liquid partitioning

Acetonitrile (CH_3CN) was added to the hydrolysed sample at a volume ratio of 1:1 (v/v) and vortexed vigorously. The mixture was then left for 10 min for partitioning to occur. The solvent layer was then transferred to a new tube. This step was repeated 3 times and the pooled solvent layers were then dried under nitrogen gas. Dried extract was then kept in 4°C before further analysis.

5.2.2.3 Solid phase extraction (SPE) partitioning

A SPE column (Oasis HLB, 3 cc, 60 mg) was preconditioned with 4 mL of 100% methanol (CH_3OH). Next, 4 mL of water was loaded to flush the solvent out from the column. A maximum of 10 mL of sample was loaded onto the SPE column. The elute was collected as fraction 1, consisting of non-retained compounds. After that, 4 mL of water was run through the column to wash out the very polar compounds as fraction 2. Next, 4 mL of 100% methanol was run through the column to elute the less polar compounds as fraction 3.

5.2.3 Evaluation of extraction efficiency using phenolic standards through different partitioning methods

5.2.3.1 Ethyl acetate ($CH_3COOC_2H_5$) liquid-liquid partitioning

7 mL of 2 M sodium hydroxide (NaOH) was adjusted with 5 M hydrochloric acid (HCl) to pH 2.0 to give a final volume between 9 and 9.5 mL. Then, 1 mL of 25 µg/mL of the phenolic standard mixture (prepared as in chapter 4, section 4.2.3) was added to the solution and vortexed vigorously. Next, 10 mL of ethyl acetate ($CH_3COOC_2H_5$) was added and the procedure in section 5.2.2.1 was followed. Dried samples were then kept at 4°C ready to be analysed by HPLC.

5.2.3.2 Acetonitrile (CH_3CN) salting out liquid-liquid partitioning

7 mL of 2 M sodium hydroxide (NaOH) was adjusted with 5 M hydrochloric acid (HCl) to pH 2.0 with the final volume between 9 to 9.5 mL. Then, 1 mL of 25 µg/mL of phenolic standard mixture (prepared as in chapter 4, section 4.2.3) was added to the solution and vortexed vigorously. Next, 10 mL of acetonitrile (CH_3CN) was added and the procedure in section 5.2.2.2 was followed. But, in this case the three individual partitioning were dried separately without being pooled. Dried samples were then kept at 4°C ready to be analysed by HPLC.

5.2.3.3 Solid phase extraction (SPE) partitioning

7 mL of 2 M sodium hydroxide (NaOH) was adjusted with 5 M hydrochloric acid (HCl) to pH 2.0 with the final volume being between 9 to 9.5 mL. Then, 1 mL of 25 µg/mL phenolic standard mixture (prepared as in chapter 4, section 4.2.3) was added to the solution and vortexed vigorously. Next, the SPE column was precondition and partitioning performed as described in section 5.2.2.3. An additional 4 mL of 100% methanol (CH_3OH) was added to elute any remaining compounds as fraction 4. All eluted fractions were dried under nitrogen gas. Dried samples were then kept at 4°C to be analysed by HPLC.

5.2.4 Optimisation of hydrolysis treatment in combination with partitioning method

The study started with the 25 ug/mL mixed standards being investigated in 2 groups. Group 1 consisted of gallic acid, naringenin, myricetin, daidzein, quercetin, epigallocatechin gallate, epicatechin gallate, trans-hyroxycinnamic acid, caffeic acid, ferulic acid. Group 2 consisted of hydroxybenzoic acid, protocatechuic acid, rutin, genistein, epicatechin, chlorogenic acid, p-coumaric acid, sinapic acid, luteolin and kaempferol. One mL of each mixed standard was added to 0.5 mL 80% methanol (CH_3OH) and hydrolysed with 1.5 mL of 2 M sodium hydroxide (NaOH) for 5 min, 30 min, 1 h, 2 h, 4 h and 24 h. The samples were then adjusted to pH 2.0 and extracted with the selected optimised partition method and dried under nitrogen gas. Dried samples (n=3) were then analysed with HPLC according to section 5.2.5

5.2.5 Preparation for HPLC analysis

Dried sample was dissolved in 1 mL of 50% methanol. Mixture was vortexed vigorously and filtered into an HPLC vial. HPLC setup was as described in chapter 4, section 4.2.4. All chromatograms were analysed at 280 nm for section 5.3.1. Results for sections 5.3.2 to 5.3.4 were obtained at optimal spectrum (table 4.11) for each individual phenolic standards.

The integrated areas obtained from the untreated samples (total soluble phenolics) need to be increased by two when comparing with the areas from the chromatograms obtained from deconjugated phenolic samples. This consideration only applies to section 5.3.1.

5.3 Results

5.3.1 Hydrolysis treatment

The study began by determining whether either acid or alkaline hydrolysis were best suited to be applied to the bean samples prior to further investigation of the factors in detail. There are many factors that contribute to the suitability of the hydrolysis method such as sample type, hydrolysis duration and extraction method after hydrolysis.

Previous published literature reported that hydrolysis duration of 1 – 4 h is needed for deconjugating soluble conjugated phenolics (Madhujith & Shahidi, 2009; Ascensao & Dubery, 2003; Adom & Liu, 2002). In the case of bound phenolics, the hydrolysis duration can be prolonged to 24 h (Ross *et al.,* 2009). Based on these published reports, the hydrolysis duration for this preliminary study was set at 2 and 24 h for both soluble conjugated and bound phenolics. Also for this preliminary study only 2 selected beans, lablab bean and mung bean were investigated. Following hydrolysis, the ethyl acetate liquid-liquid extraction technique, which has been widely used in the past, was used to extract compounds from the hydrolysed sample. All samples were injected into the HPLC for analysis.

5.3.1.1 Acid hydrolysis of conjugated and bound phenolics

The HPLC chromatogram of the untreated lablab bean extract showed eleven peaks (labelled 1-11 on figure 5.1 (A), appendix 5.1). It would be expected that if the acid hydrolysis was successful, then some of these peaks (conjugated phenolics) would be seen to decline, whilst other peaks (free phenolics) would appear or increase.

The HPLC chromatograms for samples after 2 or 24 h of acid hydrolysis are shown in figures 5.1 (B) and (C), respectively. A comparison of the profiles with that from the untreated sample showed that some peaks have been totally lost (e.g. retention at 4.09 min in figure 5.1) whilst new peaks have appeared. A total of 10 new peaks were formed. These peaks can be seen in both the 2 h and 24 h hydrolysis samples - peaks 12 (14.16 min), 13 (15.33 min) and 14 (17.58 min). While, peak 15 (29.16 min) can only be seen in the 2 h hydrolysis

sample. The other six peaks were only detected in the 24 h hydrolysis sample - 16 (19.48 min), 17 (20.70 min), 18 (22.90 min), 19 (26.12 min), 20 (28.15 min) and 21 (29.15 min). Peaks 4 and 9 in the untreated sample were reduced to at least half of the original concentration during the hydrolysis whilst other peaks present in the untreated sample may have increased in 2 h and 24 h such as peak 11. Peak 3 and 10 in the untreated sample showed minor reductions after 2 h hydrolysis and were totally lost in the 24 h hydrolysis sample.

The HPLC chromatogram of the untreated mung bean extract showed seven peaks (labelled 1-7 on figure 5.2 (A), appendix 5.2) whilst figure 5.2 (B) and (C) represented the chromatograms for samples after 2 or 24 h hydrolysis. There were two peaks totally lost after hydrolysis, peak 1 (4.09 min) and 5 (17.74 min) while several peaks in the untreated sample such as peak 3 (15.31), 4 (16.90 min), 6 (21.49 min) and 7 (22.62 min) showed reductions in both 2 and 24 h hydrolysis samples. Five new peaks, peaks 8 to 12 appeared in the 2 h hydrolysis sample only.

The residue from the methanol extraction was subjected to acid hydrolysis in order to obtain the bound phenolic samples. The HPLC chromatogram for 2 h (figure 5.3-1A) and 24 h (figure 5.3-1B) (appendix 5.3) acid hydrolysis of lablab bean showed a reduction of peak numbers from nine to eight. If the acid hydrolysis is effective in cleaving the bond between the compounds and the cell wall then during prolonged hydrolysis it might be expected that the numbers of peaks and their intensities would increase unless the compounds were unstable in acid conditions. Four peaks, peaks 1 (11.10 min), 5 (17.65 min), 6 (20.80 min) and 9 (26.19 min) showed an increase of concentration from 2 h to 24 h hydrolysis duration whilst others showed a reduction in concentration (appendix 5.3). Peak 8 (23.01 min) was totally lost in the 24 h hydrolysis sample.

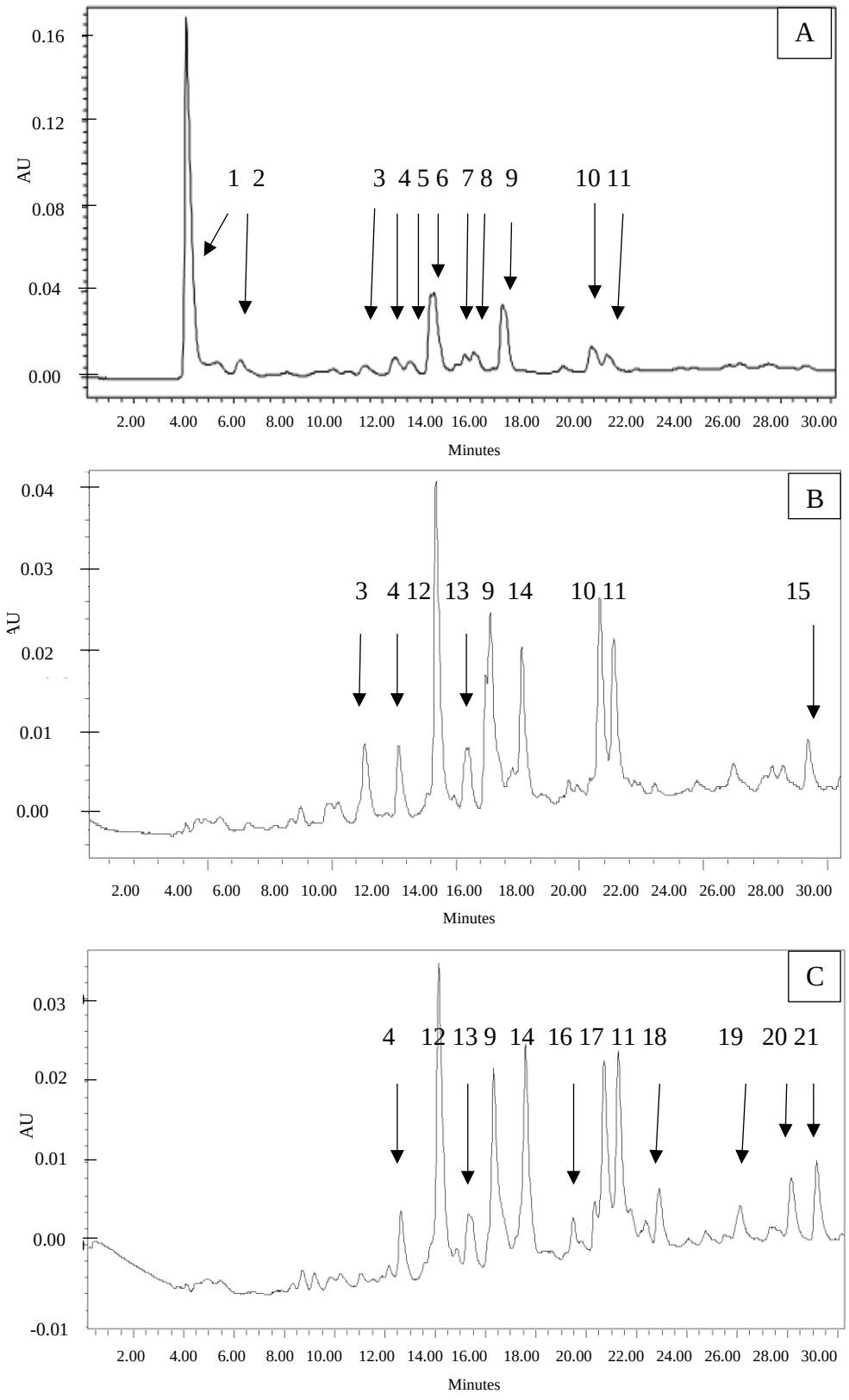


Figure 5.1 Effect of acid hydrolysis on soluble conjugated compounds from lablab beans. A methanolic extract from lablab beans was subjected to acid hydrolysis for 2 h and 24 h. Then analysed by HPLC. The HPLC chromatograms at 280 nm are presented for (A) untreated sample, (B) sample after 2 h hydrolysis and (C) sample after 24 h hydrolysis.

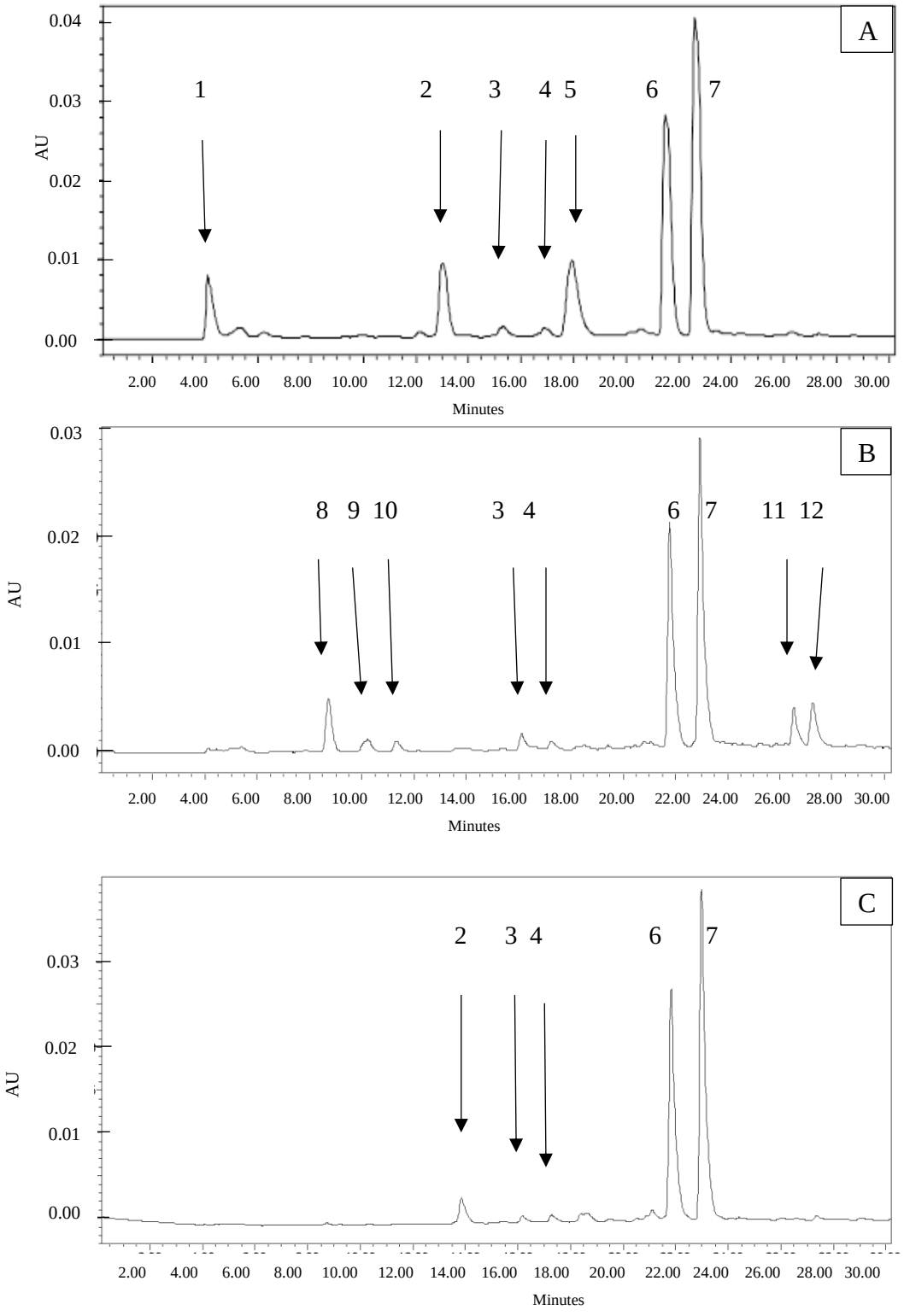


Figure 5.2 Effect of acid hydrolysis on soluble conjugated compounds from mung beans. A methanolic extract from mung beans was subjected to acid hydrolysis for 2 h and 24 h. Then analysed by HPLC. The HPLC chromatograms at 280nm are presented for (A) untreated sample, (B) sample after 2 h hydrolysis and (C) sample after 24 h hydrolysis.

A different observation has been seen for bound compounds from mung bean (figure 5.3-2). A total of seven peaks have been detected for both 2 h (figure 5.3-2A) and 24 h (figure 5.3-2B) acid hydrolysis samples. All peaks showed an increased concentration from 2 h to 24 h of hydrolysis (appendix 5.4). Peaks other than 6 and 7 were not shown in the chromatogram due to the huge peak intensity variation among the peaks. All peaks showed more than 100% increase in concentration between 2 h and 24 h acid hydrolysis except minor changes for peaks 1 and 2. Therefore, this treatment appears to be suitable to release bound compounds from mung beans.

In conclusion, from the number of peaks and peaks' intensity showed that majority of peaks in conjugated sample of lablab bean and bound sample of mung bean showed no further or slow degradation with prolonged acid hydrolysis of 24 h. This suggests that these compounds are relatively stable under acid condition. In comparison, a majority of peaks were further degraded in bound sample of lablab bean and conjugated samples of mung bean in 24 h which suggests that they are sensitive to acid conditions. This suggests that acid hydrolysis may be selectively effective depending on the type of bean and compounds. Due to the limitations above, further investigation on alternative hydrolysis methods such as alkaline hydrolysis is suggested in order to obtain a common methods for all beans. On top of that, reference standards are required to confirm that the compounds of interest in both conjugated and bound samples are actually phenolics.

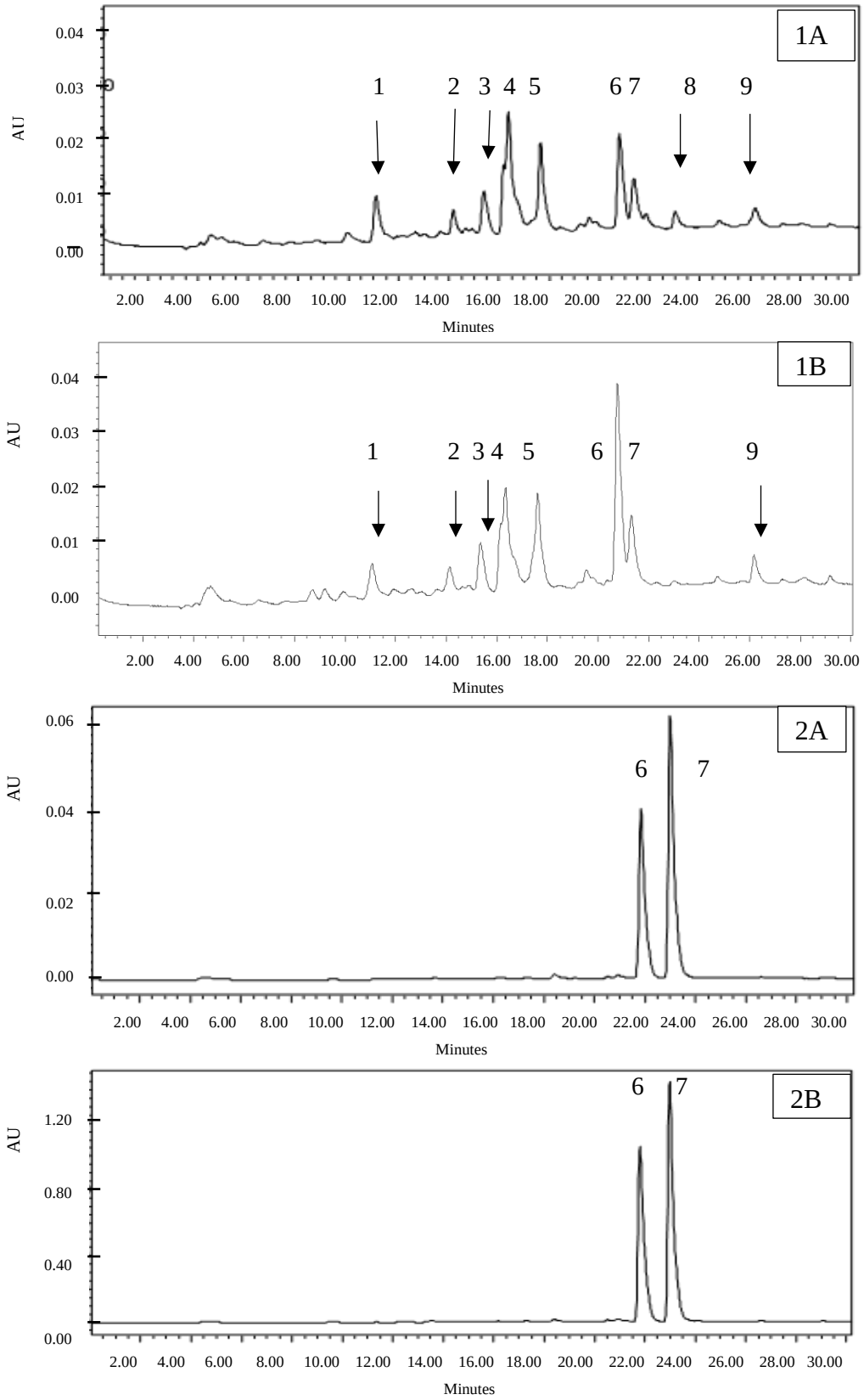


Figure 5.3 Effect of acid hydrolysis on bound compounds from lablab beans and mung beans. Residue after methanol extraction from both beans were subjected to acid hydrolysis for 2 h and 24 h. Then analysed by HPLC. The HPLC chromatograms at 280 nm are presented for lablab beans (1) and mung beans (2)-(A) sample after 2 h hydrolysis and (B) sample after 24 h hydrolysis.

5.3.1.2 Alkaline hydrolysis of conjugated and bound phenolics

The HPLC chromatogram for 2 h and 24 h alkaline hydrolysis of the methanol extracts from lablab bean are shown in figure 5.4-B and figure 5.4-C. The results show a change of 11 peaks (untreated sample- figure 5.4-A) to eight peaks after 2 h and 24 h hydrolysis. Four peaks were totally lost after 2 h of alkaline hydrolysis - peaks 1 (4.09 min), 2 (6.26 min), 5 (13.17 min) and 9 (16.82 min) whilst 1 new peak 12 (25.94 min) was formed. The remaining seven peaks were detected in both 2 and 24 h alkaline hydrolysis samples. Four peaks - peak 3 (11.23 min), 4 (12.53 min), 8 (15.66 min) and 10 (20.38 min) showed an increase in concentration after 2 h hydrolysis followed by a reduction after 24 h of hydrolysis (appendix 5.5). However, peak 6 (14.06 min) showed a continuous reduction in peak size whilst peaks 7 (15.26 min) and 11 (21.05 min) showed a continuous increase from 2 h to 24 h of hydrolysis (appendix 5.5).

The HPLC chromatogram for the equivalent mung bean samples showed a change of seven peaks in the untreated extract (figure 5.5-A) to nine peaks in 2 h hydrolysis samples (figure 5.5-B) and ten peaks in 24 h hydrolysis samples (figure 5.5-C) (appendix 5.6). Two peaks were totally lost (peaks 1 and 3) whilst four and two new peaks were formed in the 2 h and 24 h hydrolysis samples, respectively. A comparison of profiles from untreated with 24 h hydrolysis samples showed that some peaks are continuously reduced in size - peaks 2 (13.01 min), 5 (17.94 min) and 7 (22.62 min). This indirectly indicated the success of alkaline hydrolysis in breaking the conjugated compounds.

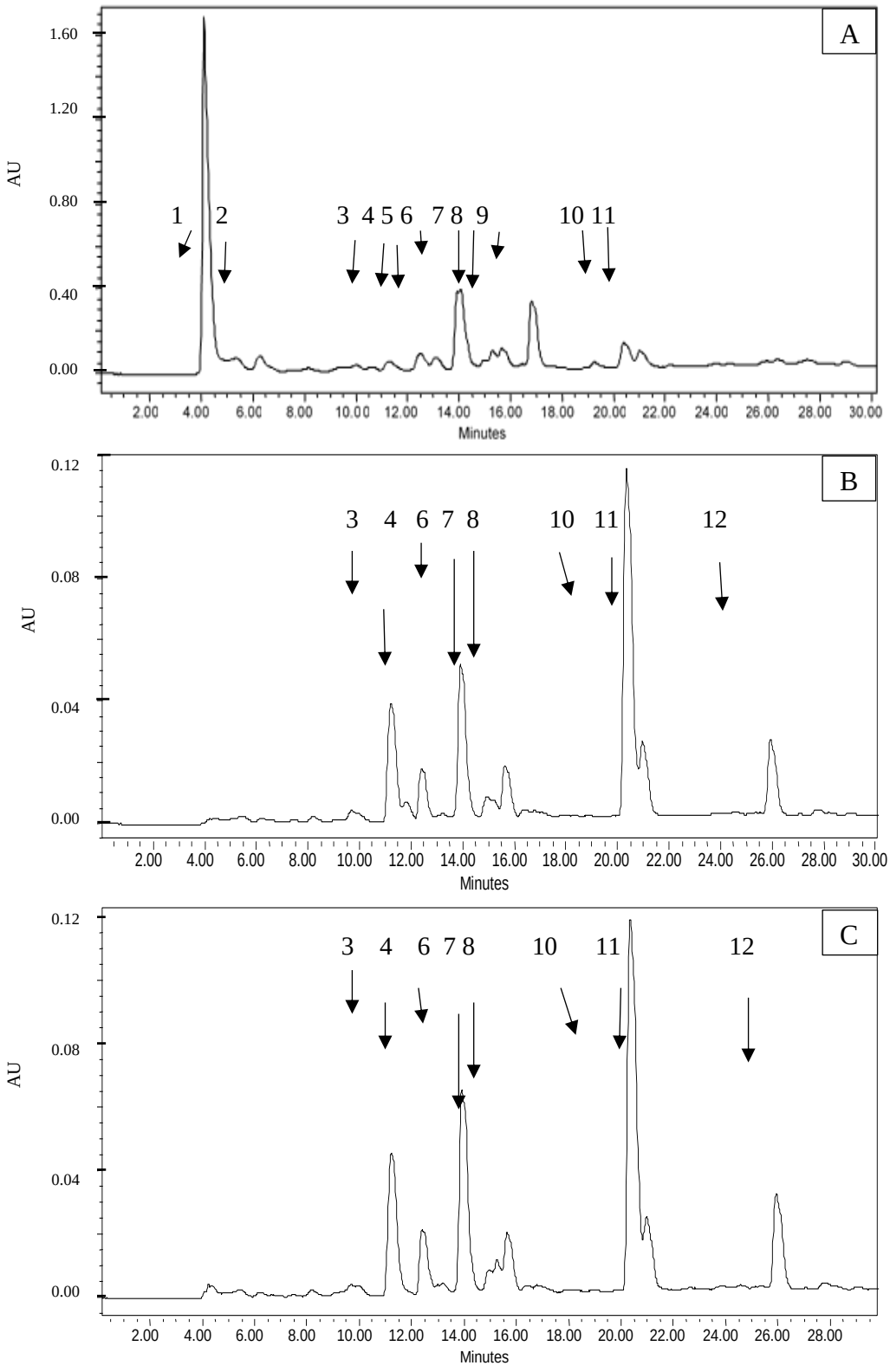


Figure 5.4 Effect of alkaline hydrolysis on soluble conjugated compounds from lablab beans. A methanolic extract from lablab beans was subjected to alkaline hydrolysis for 2 h and 24 h. Then analysed by HPLC. The HPLC chromatograms at 280 nm are presented for (A) untreated sample, (B) sample after 2 h hydrolysis and (C) sample after 24 h hydrolysis.

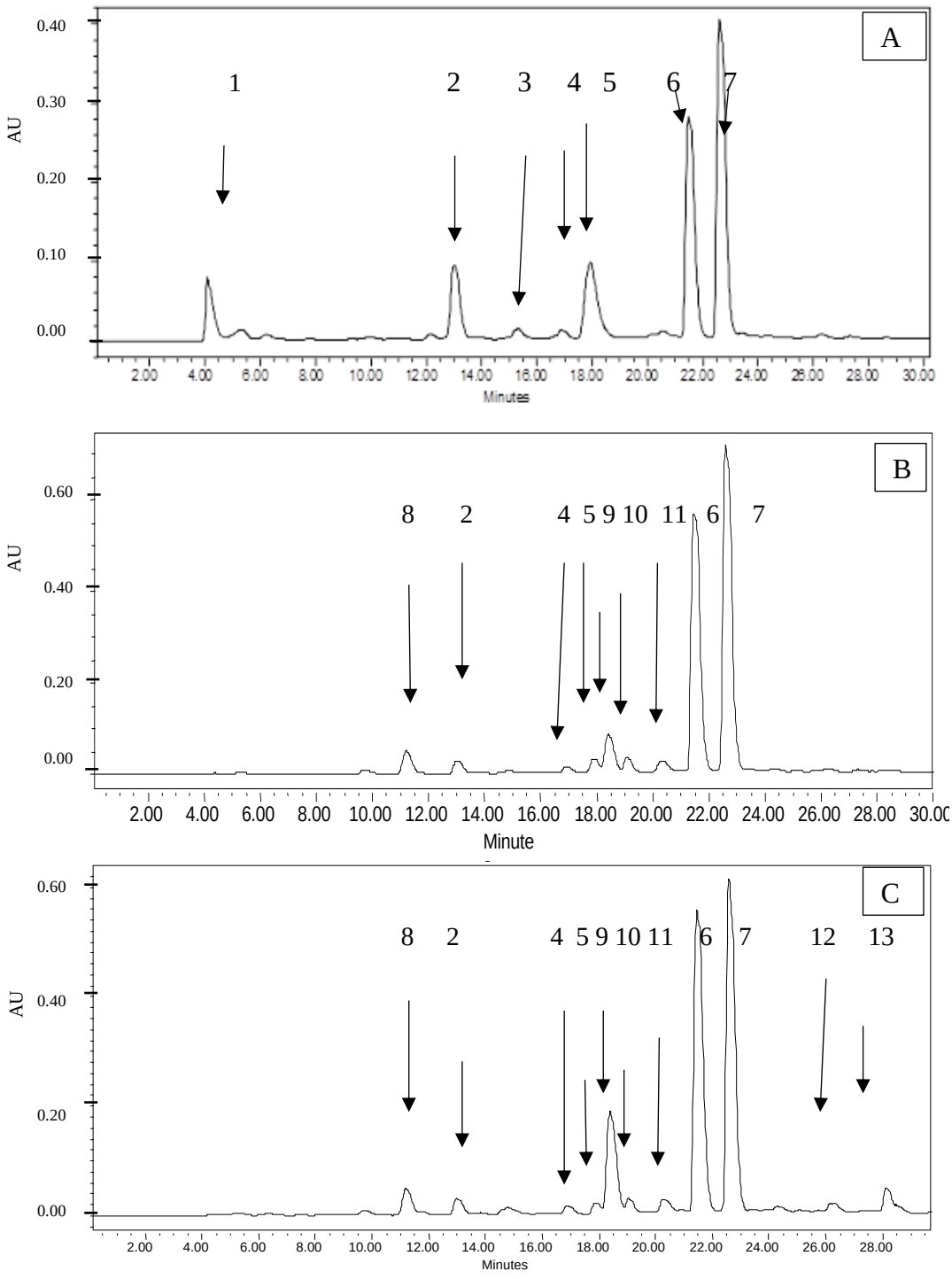


Figure 5.5 Effect of alkaline hydrolysis on soluble conjugated compounds from mung beans. A methanolic extract from mung beans was subjected to alkaline hydrolysis for 2 h and 24 h. Then analysed by HPLC. The HPLC chromatograms at 280 nm are presented for (A) untreated sample, (B) sample after 2 h hydrolysis and (C) sample after 24 h hydrolysis.

The residues after the methanolic extraction were subjected to alkaline hydrolysis to release bound phenolics. The changes in profile for alkaline hydrolysis between 2 h and 24 h for lablab bean samples are shown in figure 5.6-1A and 5.6-1B. A total of 7 peaks were seen in 2 h hydrolysis samples but three peaks - peak 1 (12.54 min), 2 (14.04 min) and 4 (15.82 min) were totally lost after 24 h of alkaline hydrolysis. Other peaks showed an increased in size with prolonged alkaline hydrolysis – e.g. peak 6 (20.53 min) (appendix 5.7).

In comparison a total of 8 and 9 peaks were observed from the 2 h and 24 h hydrolysis samples from mung bean without any peak losses but with one new peak, 9 (24.11 min), found in 24 h hydrolysis sample. The majority of the peaks increased in size (appendix 5.8) (e.g. peak 5- 18.51 min) but three peaks showed a slight decrease, such as peak 1 (9.41 min), after 24 h hydrolysis.

In conclusion, both acid and alkaline hydrolysis were seen to result in releasing deconjugated and bound compounds. Acid hydrolysis causes at least 50% of the soluble conjugated compounds to degrade completely and formed new peaks within 2 h of hydrolysis duration. The, results were varied between soluble conjugated and bound samples, between the two tested beans. In contrast, a more observable, instead of rapid degradation of conjugated compounds appears to occur during alkaline hydrolysis. It helps to estimate the relative free phenolics for conjugated phenolics. Hence, alkaline hydrolysis appears to be better because the compounds appear to be more stable under alkaline conditions and it may be less effected in terms of type of bean or compounds. As a result, alkaline hydrolysis was selected for the next study.

However, it was noted that even with alkaline hydrolysis some of the peaks observed were transient suggesting instability of the underlying compounds. Some of these may be the phenolics of interest. Thus duration of hydrolysis has to be optimised by using phenolic standards prior to application to bean samples. The next section will focus on the effect of alkaline hydrolysis on both conjugated and free phenolic compounds as well as optimising the extraction after hydrolysis using phenolic standards.

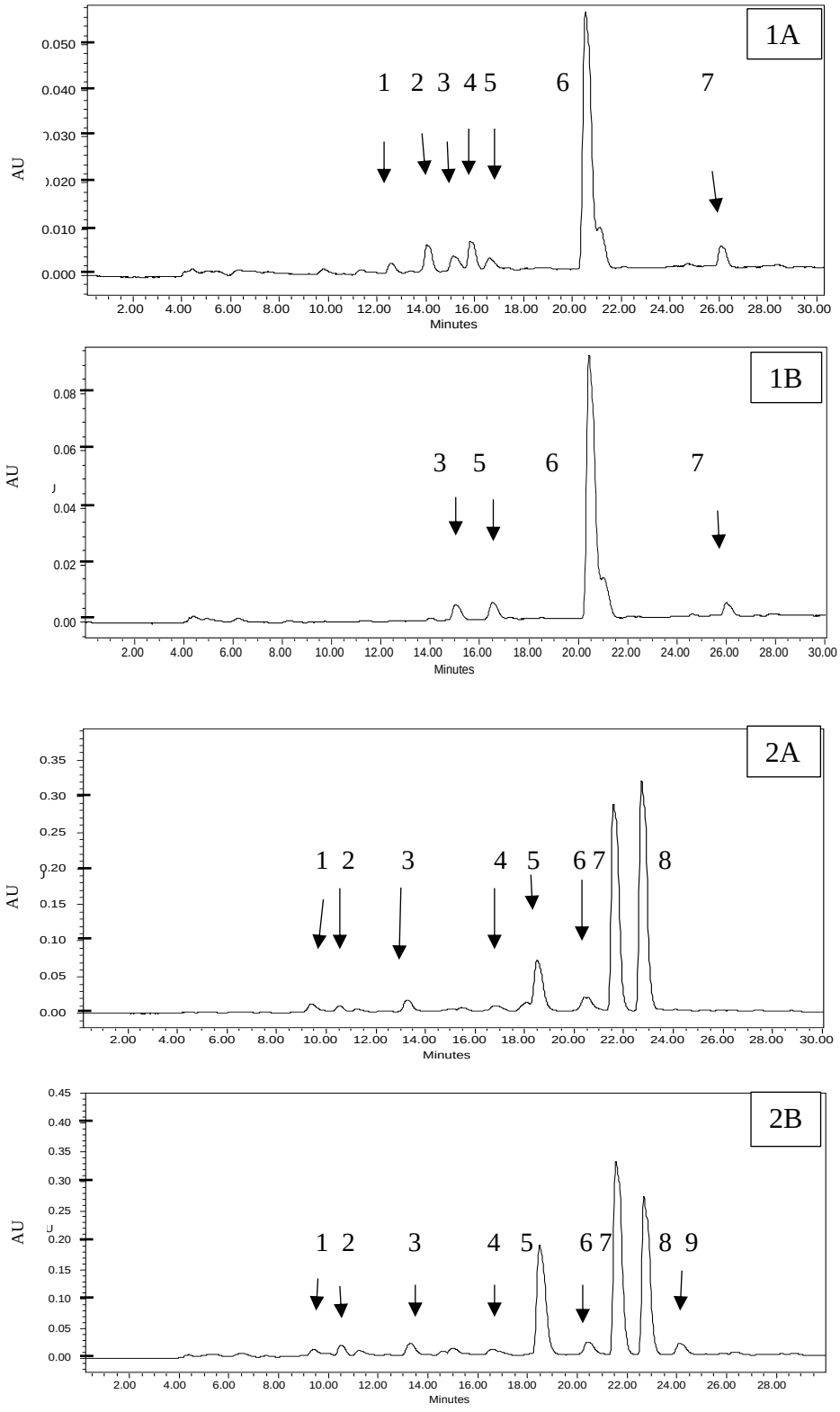


Figure 5.6 Effect of alkaline hydrolysis on bound compounds from lablab beans and mung beans. A methanolic extract from both beans were subjected to alkaline hydrolysis for 2 h and 24 h. Then analysed by HPLC. The HPLC chromatograms at 280 nm are presented for lablab beans (1) and mung beans (2)-(A) sample after 2 h hydrolysis and (B) sample after 24 h hydrolysis.

5.3.2 Effect of alkaline hydrolysis on known phenolic standards

The previous section justified the used of alkaline hydrolysis in obtaining deconjugated and bound phenolics. However, it also suggested that compounds may be unstable and as such the actual effect of the hydrolysis on phenolics can only be confirmed when applying this treatment to phenolic standards. Hence, an initial study was conducted using 20 known phenolic standards (refer to section 4.2.2) in order to assess stability under alkaline conditions. The standards were divided into 3 groups. Group 1 (epicatechin gallate, rutin, luteolin, daidzein, naringenin, sinapic acid and epigallocatechin gallate), group 2 (chlorogenic acid, ρ-coumaric acid, ferulic acid, genistein, myricetin, protocatechuic acid and caffeic acid) and group 3 (gallic acid, epicatechin, genestein, hydroxybenzoic acid, trans-3 hydroxycinammic acid and kaempferol). Each group was prepared and analysed by HPLC prior to any treatment. The composite HPLC profile is shown in figure 5.7.

Each group was then treated with alkaline hydrolysis for 1, 3 and 6 h (as described in section 5.2.1.3) followed by ethyl acetate liquid/liquid partitioning (section 5.2.2.1) and analysis by HPLC. A maximum 4 h of alkaline hydrolysis is commonly applied however, this experiment was carried out with an additional duration to 6 h in order to examine the suitability of a hydrolysis of more than 4 h.

The HPLC profiles from the hydrolysed samples are shown in figure 5.8. The results demonstrate that 12 out of 20 standards were not detected by HPLC after a 1 h alkaline hydrolysis. Among these were four conjugated phenolic standards – rutin, epigallocatechin gallate, epicatechin gallate and chlorogenic acid. These would not be expected to be detectable and serve to demonstrate the effective deconjugation process. The fact that 1h of treatment has successfully deconjugated these compounds suggests that a shorter treatment may be more applicable. The disappearance of eight phenolic standards (luteolin, epicatechin, myricetin, protocatechuic acid, genestein, caffeic acid, quercetin and kaempferol), which are free phenolics, showed the instability of these compounds in alkaline hydrolysis.

The remaining standards all showed a reduction in concentration of between 10% and 100%. The most stable being naringenin, daidzein, sinapic acid, ρ-coumaric, ferulic acid, trans- hydroxycinnamic acid and hydroxybenzoic acid. As a result, the hydrolysis duration needs to be redesigned with a shorter duration since some phenolic standards were unstable under alkaline conditions. Partition efficiency is also one of the factors determining the recovery of the standards after hydrolysis and this may add to the reason for the low detection.

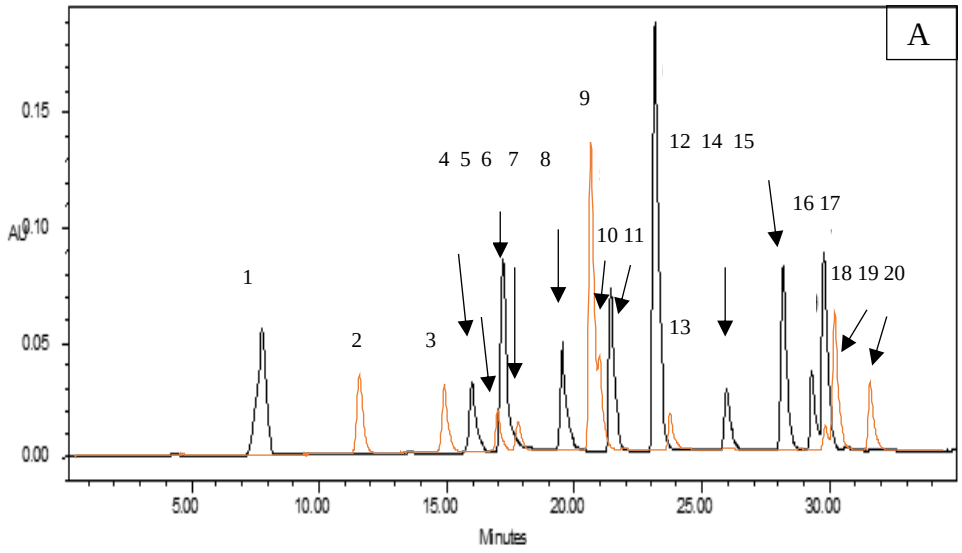


Figure 5.7 Untreated phenolic standards (25 µg/mL).1- gallic acid, 2-protocatechuic acid, 3-chlorogenic acid, 4- epigallocatechin gallate, 5-epicatechin , 6- caffeic acid, 7- hydroxybenzoic acid, 8- epicatechin gallate, 9-p-coumaric acid, 10- sinapic acid, 11- ferulic acid, 12- trans-hydroxycinnamic acid, 13- rutin, 14-myricetin, 15- daidzein, 16-quercetin, 17-naringenin, 18-luteolin, 19-genistein, 20-kaempferol. The figure show the result HPLC chomatogram at 280 nm.

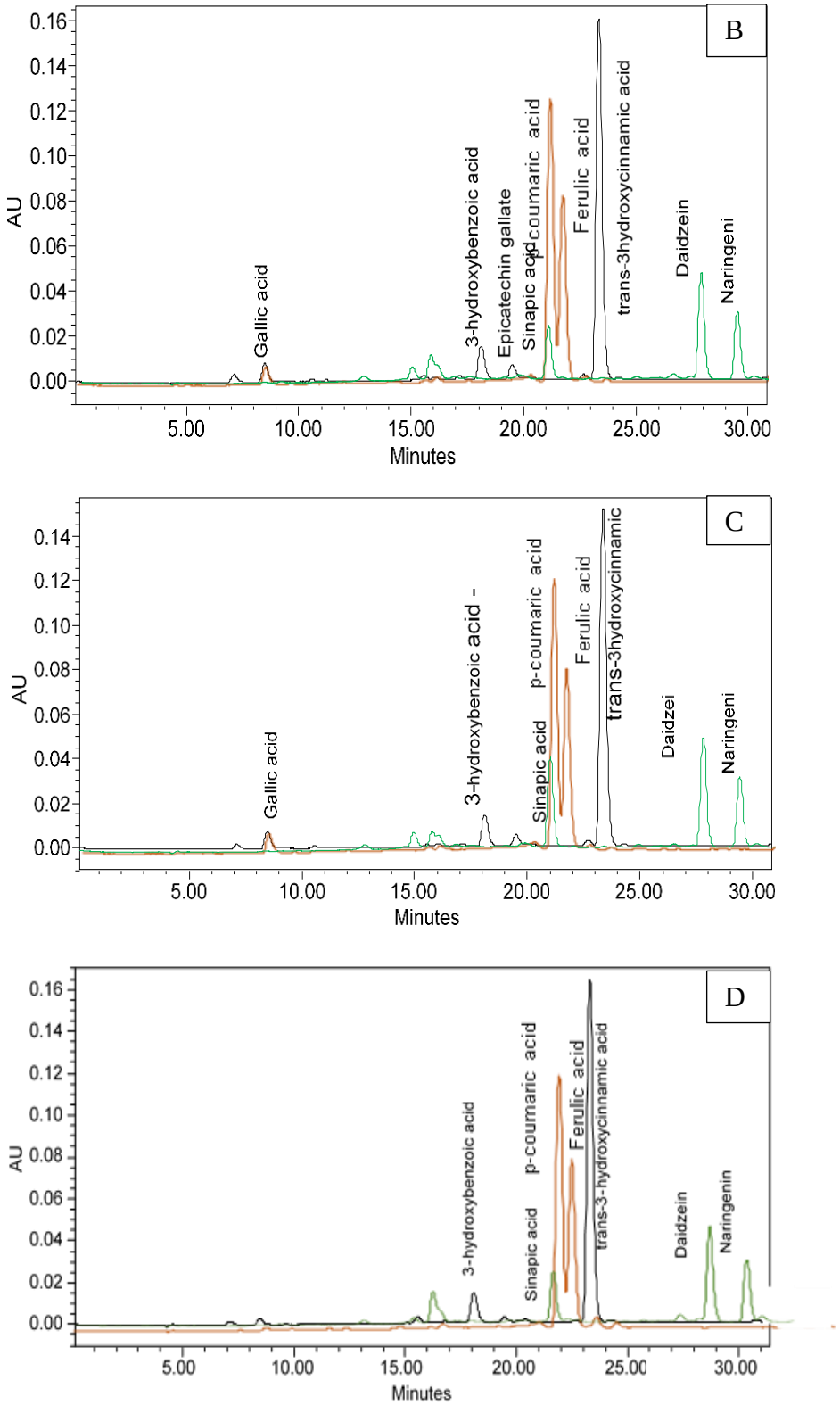


Figure 5.8 Effect of alkaline hydrolysis on stability of phenolic standards. Phenolic standards were subjected to alkaline hydrolysis for between 1 and 6 h. The figure shows result and HPLC chromatograms. For (B) 1 h, (C) 3 h and (D) 6 h hydrolysis samples.

5.3.3 Effect of partitioning method on extraction efficiency of hydrolysed samples

The previous investigation concluded that a short hydrolysis duration is favoured due to degradation of certain phenolics. The extraction method employed after hydrolysis may also play an important role in the recovery of phenolics after hydrolysis. Therefore, this section will focus on the study of extraction efficiency for hydrolysed samples using different partitioning methods. Three partitioning methods were used - ethyl acetate, acetonitrile salting out and SPE partitioning.

The adjustment of samples from alkaline (pH 13.0), as used in the hydrolysis, to acidic (pH 2.0) as required for the partitioning, leads to the formation of a high salt content. This could result in system blockage in HPLC and LC-MS if mishandled and, it may also affect liquid-liquid partitioning efficiency. Hence, an extraction method that can reduce the salt content with maximum recovery of phenolic compounds is required. In order to determine the partition efficiency, the starting conditions such as initial volume for sample after hydrolysis has to be similar to actual sample conditions in order to get the same amount of salt content. All solutions for each liquid-liquid partition were prepared as in section 5.2.3 and applied to phenolic standards.

An initial trial of ethyl acetate and acetonitrile salting out liquid/liquid partitioning for both hydrolysed deconjugated and bound compounds showed that acetonitrile salting out liquid-liquid extraction failed to partition the hydrolysed deconjugated samples into 2 layers. Subsequent experiments suggested that this was due to the methanol content of the sample. The partitioning will only be effective if the solutions contain less than 20% methanol (appendix 5.9 – 5.10). Therefore, acetonitrile liquid-liquid partitioning failed to separate the deconjugated sample after hydrolysis because it contained around 40% methanol. However, this method was suitable to be applied to bound phenolic samples because no methanol existed in these samples. In addition, acetonitrile liquid-liquid partitioning may be more suitable than SPE partitioning because it is cheaper. In conclusion, ethyl acetate and acetonitrile salting out liquid-liquid partition were selected for

further studies into the recovery of the bound phenolics. Ethyl acetate and SPE partitioning were selected for studies into the recovery of deconjugated phenolics.

The recoveries of phenolic standards by ethyl acetate liquid-liquid partitioning, acetonitrile salting out liquid-liquid partitioning and SPE partitioning are shown in figure 5.9. Ethyl acetate liquid-liquid partitioning failed to recover epigallocatechin gallate and epicatechin whilst the other methods managed to recover all the phenolics standards albeit at different percentage efficiencies. SPE partitioning worked best as a method to recover phenolic standards from hydrolysed samples as compared to ethyl acetate. It was able to recover all phenolics at similar or higher percentage efficiencies than ethyl acetate. On top of that, the ethyl acetate liquid-liquid partitioning method required five repetitive extractions and a longer drying time due to the high volume of pooled sample (250 mL).

The requirement for any further elution from the SPE column, in addition to the 4 mL fraction 3, was tested by including a further 4 mL elution with 100% methanol (fraction 4). The result, as shown in table 5.1, showed that compounds were fully eluted at fraction 3. Hence, only 4 mL of eluted sample was sufficient to obtain a recovery better than with ethyl acetate liquid-liquid partitioning. On top of that, SPE partitioning also had the advantage of desalting the sample which is good for subsequent HPLC and LC-MS analysis. To conclude, SPE partitioning was selected as the best method for the recovery of deconjugated phenolics from hydrolysed samples.

Acetonitrile salting out liquid-liquid partition was able to recover all the phenolic standards and 16 out of 20 showed a recovery higher than or close to that for the ethyl acetate liquid/liquid partitioning (table 5.2). Less solvent is used in this method as compared with ethyl acetate liquid-liquid partitioning. The partitioning was repeated 3 times and each extract was analysed separately for the recovery for each phenolic standard. Result showed that 3 repetitions were sufficient to achieve the maximum recovery as compared with ethyl acetate liquid-liquid partitioning. This method is also able to more effectively

desalt the samples as the salt is partitioned into the aqueous layer. And, it is cheaper than SPE partitioning especially for high volume samples. Thus, acetonitrile salting out liquid-liquid partition is recommended to extract bound phenolics.

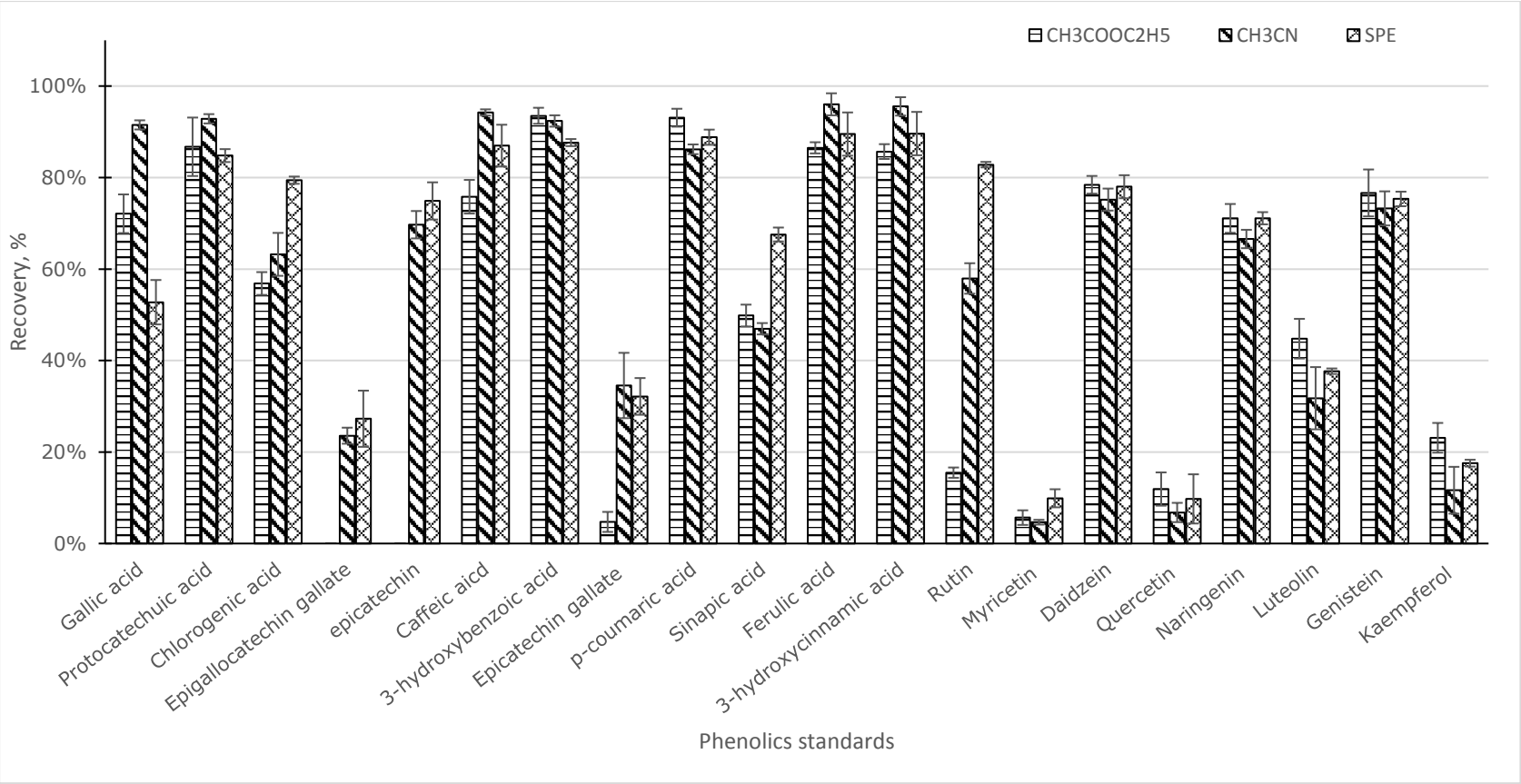


Figure 5.9 Recovery of phenolic standards from different liquid-liquid partitioning labelled as ethyl acetate liquid-liquid partitioning ($CH_3COOC_2H_5$), acetonitrile salting out liquid-liquid partitioning (CH_3CN) and solid phase partitioning (SPE). Results are shown as the mean ± SD (n=3).

Table 5.1 Recovery of phenolic standards from SPE partitioning specially in 3rd and 4th elutions.

	Compounds	Concentration of fraction, µg/mL			Recovery, %
		3	4	Total	
1.	Gallic acid	13.2	0	13.2 ± 1.2	53 ± 5%
2.	Protocatechuic acid	21.2	0	21.2 ± 0.4	85 ± 1%
3.	Chlorogenic acid	19.9	0	19.9 ± 0.2	79 ± 1%
4.	Epigallocatechin gallate	4.4	2.0	6.8 ± 1.5	27 ± 6%
5.	Epicatechin	18.7	0	18.7 ± 1.0	75 ± 4%
6.	Caffiec acid	21.7	0	21.7± 1.1	87 ± 5%
7.	3-hydroxybenzoic acid	21.9	0	21.9 ± 0.2	88 ± 1%
8.	Epicatechin gallate	5.9	2.0	8.0 ± 1.0	32 ± 4%
9.	p-coumaric acid	22.2	0	22.2± 0.4	89 ± 2%
10.	Sinapic acid	16.9	0	16.9 ± 0.4	68 ± 2%
11.	Ferulic acid	22.4	0	22.4 ± 1.2	90 ± 5%
12.	Trans-3-hydroxycinnamic acid	22.4	0	22.4 ± 1.2	90 ± 5%
13.	Rutin	20.7	0	20.7 ± 0.1	83 ± 1%
14.	Myricetin	2.5	0	2.5 ± 0.5	10 ± 2%
15.	Daidzein	19.5	0	19.5 ± 0.6	78 ± 3%
16.	Quercetin	3.4	0	2.4 ± 1.3	10 ± 5%
17.	Naringenin	17.8	0	17.8 ± 0.3	71 ± 1%
18.	Luteolin	9.4	0	9.4 ± 0.2	38 ± 1%
19.	Genistein	18.8	0	18.8 ± 0.4	75 ± 2%
20.	Kaempferol	4.4	0	4.4 ± 0.2	18 ± 1%

Table 5.2 Recovery of phenolic standards from different fractions of acetonitrile salting out liquid-liquid partitioning.

	Compounds	Concentration of fraction, µg/mL				Recovery, %
		1	2	3	Total	
1.	Gallic acid	5.8	13.0	4.1	22.9 ± 0.2	92 ± 1%
2.	Protocatechuic acid	8.2	11.8	3.2	23.2 ± 0.3	93 ± 1%
3.	Chlorogenic acid	4.4	8.4	3.0	15.8 ± 1.2	63 ± 5%
4.	Epigallocatechin gallate	1.9	4.0	0	5.9 ± 0.4	24 ± 2%
5.	Epicatechin	7.6	8.8	1.0	17.4 ± 0.7	70 ± 3%
6.	Caffeic acid	8.0	13.1	2.4	23.6 ± 0.2	94 ± 1%
7.	3-hydroxybenzoic acid	10.3	10.9	1.9	23.1 ± 0.3	92 ± 1%
8.	Epicatechin gallate	3.1	5.2	0.3	8.6 ± 1.8	35 ± 7%
9.	p-coumaric acid	11.5	8.3	1.7	21.5 ± 0.3	86 ± 1%
10.	Sinapic acid	6.1	5.7	0	11.7 ± 0.3	47 ± 1%
11.	Ferulic acid	9.4	12.7	1.9	24.0 ± 0.6	96 ± 2%
12.	Trans-3-hydroxycinnamic acid	9.6	12.5	1.8	23.9 ± 0.5	96 ± 2%
13.	Rutin	3.0	6.8	4.7	14.5 ± 0.8	58 ± 3%
14.	Myricetin	0.6	0.6	0	1.2 ± 0.1	5 ± 1%
15.	Daidzein	8.5	9.4	0.9	18.8 ± 0.6	75 ± 2%
16.	Quercetin	0.7	1.0	0	1.7 ± 0.5	7 ± 2%
17.	Naringenin	8.9	7.3	0.5	16.6 ± 0.5	67 ± 2%
18.	Luteolin	5.3	2.6	0	17.9 ± 1.7	32 ± 7%
19.	Genistein	12.0	6.4	0	18.3 ± 0.9	73 ± 4%
20.	Kaempferol	2.2	0.7	0	2.9 ± 1.3	12 ± 5%

5.3.4 Optimisation of duration of alkaline hydrolysis

The previous results suggest that alkaline hydrolysis followed by either SPE or acetonitrile salting out liquid-liquid partitioning may be the best approaches to obtain deconjugated and bound phenolics. However, the duration of the alkaline hydrolysis still required optimisation. This was explored using the standard phenolic mixtures as above. SPE partition has been used here, instead of acetonitrile salting out liquid-liquid partitioning to avoid failure of partitioning because methanol was the solvent used for the mixture of standards. The hydrolysis duration was set at 5 min, 30 min, 60 min, 2 h, 4 h and 24 h and the outcome analysed by HPLC. The results are shown in figures 5.10 and 5.11.

Results revealed that phenolics such as gallic acid, epicatechin, caffeic acid, myricetin, quercetin, luteolin and kaempferol were totally degraded within 5 min of alkaline hydrolysis. However, protocatechuic acid was found to be stable over 5 min hydrolysis but not after 30 min. Meanwhile, conjugated phenolics such as chlorogenic acid (esterified of caffeic acid with quinic acid), epigallocatechin gallate, epicatechin gallate were completely degraded within 5 min of hydrolysis. Rutin also known as quercetin-3-O-rutinoside showed a complete deconjugation after 30 min of alkaline hydrolysis. The most stable phenolic standards were hydroxybenzoic acid and hydroxycinnamic acid where no significant degradation was observed.

On the other hand, p-coumaric acid, sinapic acid, ferulic acid, daidzein, naringenin and genistein were stable for up to 4 h of hydrolysis. But, a significant degradation of 10 % to 60% was then observed after 24 h. As a result, a 5 min hydrolysis was deemed optimal, followed by 30 min and 1 h. The optimum depends on the types of phenolic compounds that are to be analysed, for example, releasing bound phenolics may need a hydrolysis duration of longer than 5 minutes. In conclusion, 5 min and 1 h hydrolysis were applied for subsequent studies on conjugated and bound phenolic samples.

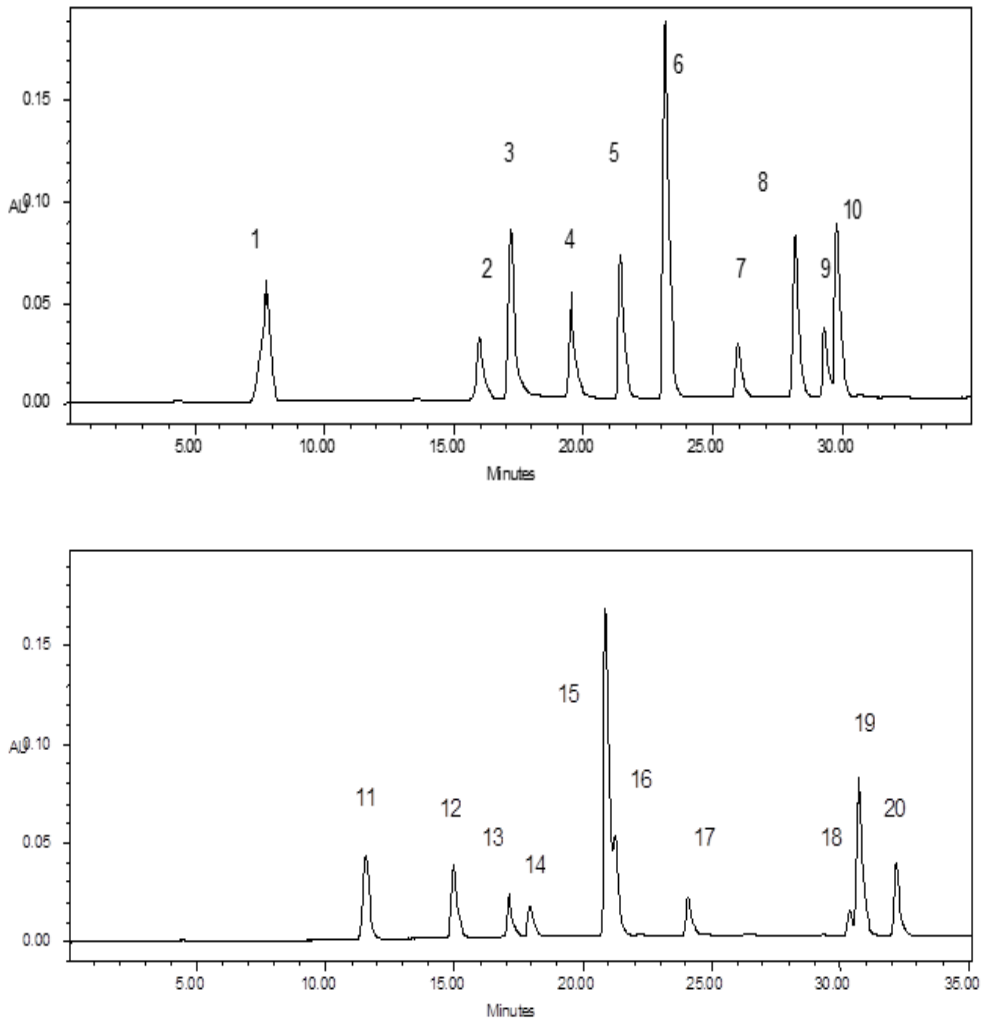


Figure 5.10 HPLC chomatogram for phenolic standards (25 µg/mL).1- gallic acid, 2-epigallocatechin gallate, 3-caffeic acid, 4-epicatechin gallate, 5-ferulic acid, 6-trans-hydroxycinnamic acid, 7-myricetin, 8-daidzein, 9-quercetin, 10-naringenin, 11-protocatechuic acid, 12-chlorogenic acid, 13-epicatechin, 14-hydroxybenzoic acid, 15-p-coumaric acid, 16-sinapic acid, 17-rutin, 18-luteolin, 19-genistein, 20-kaempferol.

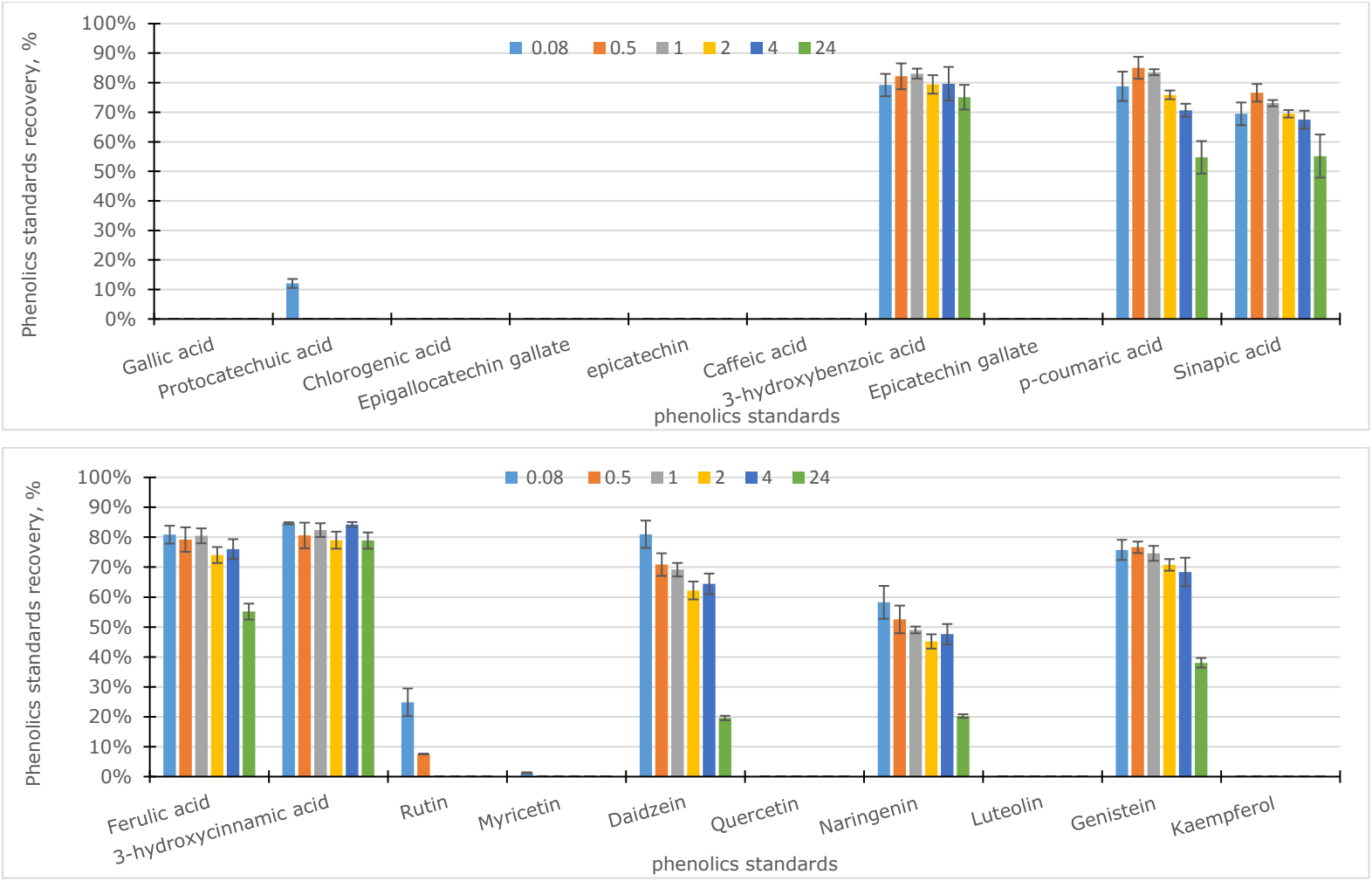


Figure 5.11 Stability of phenolic standards at different time interval (h) of alkaline hydrolysis. Results are shown as the mean ± SD (n=3).

5.4 Discussion

Selection of the most appropriate type of hydrolysis is essential in obtaining deconjugated and bound phenolics samples. Out of the three types of most commonly used hydrolysis methods, only acid and alkaline hydrolysis were investigated in this study. Enzyme treatment was not considered since it is more costly than the others. Alkaline hydrolysis is commonly used to extract esterified bound phenolics whilst acid hydrolysis is used to extract ether bonded phenolics (Madhujith and Shahidi, 2009; Ascensao and Dubery, 2003; Lozovaya *et al.,* 1999). Despite some reports that mention that alkaline hydrolysis failed to cleavage ether bond, alkaline hydrolysis has the advantage of releasing ferulic acid ether-linked to the cell wall by solubilising the wall polymers that are linked to the ferulic acid (Lozovaya *et al.,* 1999).

Comparison between acid and alkaline hydrolysis treatments revealed two noticeable outcomes with respect to the expected changes to conjugated and bound compounds. Firstly, alkaline hydrolysis appears to be better because the compounds are more stable under alkaline conditions. Alkaline hydrolysis caused less than 36% loss of total peak area from soluble conjugated samples but acid hydrolysis resulted in up to 55% loss of total peak area. Alkaline hydrolysis also resulted in higher increases of peak intensity within 2 h and 24 h hydrolysis for bound phenolics than acid hydrolysis. This is important and may suggest that more bound phenolics were released. The advantage of alkaline hydrolysis has been reported by Krygier *et al* (1982) who showed that hydroxycinnamic acids such as ferulic acid, coumaric acid, caffeic acid and sinapic acid were degraded within the range of 15% to 92% under acid hydrolysis, but that the same group of phenolics were relatively stable under alkaline hydrolysis for 4 h and the losses were < 10%.

Secondly, acid hydrolysis may be more selectively effective than alkaline hydrolysis and as such its relative efficiency may depend on the type of bean and compounds to be extracted. This potential disadvantage can be demonstrated by comparing the effect of acid hydrolysis on mung bean and lablab bean extracts. The peaks obtained from the hydrolysis of conjugated lablab bean extract and bound mung bean extracts showed no further, or slow

degradation, with prolonged acid hydrolysis of 24 h. In contrast, the majority of peaks in the bound sample of lablab bean and conjugated sample of mung bean were further degraded after 24 h. This could indirectly suggest that ether bonding is not found in all the test beans. Hence, there is no common effect of hydrolysis across all beans because acid hydrolysis treatment is meant to cleave ether bonds (Ascensao and Dubery, 2003). Alkaline hydrolysis is potentially better than acid hydrolysis since it has the ability to cleave the ester- and ether linked phenolics as mentioned above. Alkaline hydrolysis was thus selected for this preliminary study.

The follow up investigation involved investigating two factors- partition efficiency and hydrolysis duration- that could affect the analysis. Partition efficiency reflects the percentage recovery of hydrolysed compounds and plays an important role in getting the highest signal. The selection of the most appropriate partition method is determined by the criteria of the method and the efficiency of recovery. Three partition methods- two liquid-liquid partition methods and one solid-liquid partition method, were tested. They are each designed to perform sample clean up and to enrich and enhance signal by increasing the concentration of the target compounds (Anthemidis and Ioannou, 2009; Zhang *et al.,* 2013).

Conventional liquid-liquid partitions use an organic solvent, such as ethyl acetate or hexane, to create a phase separation with an aqueous solution so that analytes move to the organic layer. Hydrophilic compounds are not extracted by liquid-liquid partition (Zhang *et al.,* 2009). Acetonitrile salting out liquid-liquid partition is a type of homogenous liquid-liquid extraction and is best suited in this study because its criteria fits to the sample condition.

Acetonitrile salting out liquid-liquid partition has been applied to biological samples such as serum, plants, foods and environmental specimens (Park and Jung, 2017; Zhao *et al.,* 2012; Zhang *et al*., 2010; Chen *et al.*, 2009;Yoshida *et al.,* 2004; Rustum, 1989). Due to the fact that acetonitrile is miscible with the aqueous phase it forms a homogenous liquid, and as such it is suitable for extracting hydrophilic compounds. The phase separation can be achieved by

adding salt or applying sub-zero temperatures. The sub-zero temperature method has not been applied in this case. Acetonitrile is also a useful solvent because it is the diluent used to solubilise the dried extract prior to HPLC and LC-MS analysis.

A high concentration of salt (NaCl) is formed when the pH of the alkaline hydrolysis is decreased to pH 2.0. A low pH has been shown to increase the extraction efficiency of flavonoids targeting to isoflavone (Park and Jung, 2017). The high salt could be an advantage if applying subsequent acetonitrile salting out partitioning. However, anions show decreasing efficiencies in phase separation according to the Hofmeister series, $CO_3^{2-} > SO_4^{2-} > Cl^- > NO_3^-$ (Zhang and Cremer, 2006). Noubigh *et al.* (2007) demonstrated that the partition efficiency increased relative to the salt content. Solubility of ferulic acid and syringic acid in the organic solvent increased when the salt concentration increased. However, this may be a slight disadvantage since unknown salt concentrations, following the neutralisation of the sample, could limit studies to compare phenolic variability and concentrations between beans.

Another disadvantage of this method is that high salt is incompatible with LC-MS (Yoshida, 2004). Therefore, the post-column cut technique has been used to remove the salt content as suggested by Zhao *et al.* (2012). Direct injection of the organic layer was not practical in this study because of the low concentrations of analytes resulting in low or no signal in LC-MS. Increasing the injection volume does not increase the signal but broadens the peaks resulting in shortened retention times (Yoshida, 2004). As a result, sample drying was necessary to increase the concentration instead of increasing the injection volume. However, under some conditions direct injection could be feasible. Zhang *et al.,* (2010) found that acetonitrile salting out partitioning was compatible with reverse phase LC-MS and that solvent evaporation was not required.

During this investigation, a new limitation of acetonitrile salting out liquid-liquid partitioning has been found. Despite published reports showing that given a sufficient amount of salt and centrifugation separation will always be

facilitated (Zhang, 2009), in this study this method failed to partition the hydrolysed deconjugated samples. This could possibly be because of the methanol content of the sample. Further investigation found that acetonitrile salting out partitioning is workable only if the methanol content of the sample is less than 20% and with at least 6% salt weight to volume (appendix 5.9 – 5.10). In another words, two miscible solvents, acetonitrile and methanol co-exist at > 20% of methanol and this reduces the partition efficiency of acetonitrile.

Recovery and type of solvent were the two parameter used to compare the partition efficiencies of the three methods using 20 phenolic standards. In terms of recovery ethyl acetate liquid-liquid partition was weaker than the other two methods. Ethyl acetate liquid-liquid partitioning failed to recover epigallocatechin gallate and epicatechin whilst the other methods managed to recover all the phenolic standards albeit at different percentage efficiencies. SPE partitioning was able to recover all phenolics at similar or higher percentage efficiencies than ethyl acetate. On the other hand, acetonitrile salting out liquid-liquid partitioning was able to recover all the phenolic standards and 16 out of 20 showed a recovery higher than, or close to, that for the ethyl acetate liquid-liquid partitioning.

Solvents that promote cost saving and are environmental friendly should be considered as being the most appropriate for use in such studies. Acetonitrile salting out liquid-liquid extraction is a greener method that requires less solvent (150 mL) than ethyl acetate liquid-liquid partitioning (250 mL). It is supported by Zhang *et al.* (2009). It costs less than SPE partitioning when extracting high volume samples. But, SPE is recommended to be applied to hydrolysed deconjugated samples in order to overcome the limitations of the acetonitrile salting out liquid-liquid partition as mentioned earlier. Meanwhile, although SPE is more expensive (Zhang *et al.*, 2010; Zhang *et al.*, 2009) than ethyl acetate liquid-liquid partitioning it requires a much smaller sample volume (4 mL) and is more efficient at recovering phenolics.

To date, the stability of phenolic during the hydrolysis treatment has not been investigated in detail. As a result, the stability findings from HPLC analysis for a list of 20 phenolic standards were first reported here. This helped to understand the need to optimise hydrolysis treatment which has tended to be neglected in the past. The hydrolysis duration was set at 5 min, 30 min, 60 min, 2 hr, 4 hr and 24 hr and the samples were subjected to the optimised extraction protocol and analysed by HPLC.

The results revealed clearly that different phenolic compounds are being hydrolysed at various efficiencies (figures 5.10 and 5.11). Phenolics such as gallic acid, epicatechin, caffeic acid, myricetin, quercetin, luteolin and kaempferol were totally degraded within 5 min of alkaline hydrolysis. However, protocatechuic acid was found to be stable over 5 min hydrolysis but not after 30 min. This revealed that a hydrolysis duration of less than 5 min is the optimal.

On the other hand, p-coumaric acid, sinapic acid, ferulic acid, daidzein, naringenin and genistein were stable for up to 4 hr of hydrolysis with a significant degradation of between 10 % and 60% after 24 hr. The stability of p-coumaric acid, sinapic acid and ferulic acid in alkaline hydrolysis treatment up to 4 h has also been reported by Krygier *et al*. (1982). The only difference between their results and those of this study was in the percentage losses which might be due to the different extraction protocols used in each case. The most stable phenolic standards were hydroxybenzoic acid and hydroxycinnamic acid where no significant degradation was observed.

As a result, a 5 min hydrolysis was deemed optimal, followed by 30 min and 1 h. The optimum depends on the types of phenolic compounds that are to be analysed, for example, releasing bound phenolics may need a hydrolysis duration of longer than 5 minutes. In conclusion, 5 min and 1 h hydrolysis were selected for subsequent studies on conjugated phenolics and bound phenolics from the beans. The results from this thesis may provide useful information for further research involving any form of material not just beans. The findings might also impact on the results from previous studies that supported the use of

prolonged alkaline hydrolysis digestion times in order to increase the extraction yield (Bonoli *et al.*, 2004) since the majority of the phenolics were lost when subjected to prolonged hydrolysis.

5.5 Conclusion

In conclusion, alkaline hydrolysis was found to be most suitable in breaking down the conjugated compounds and releasing the bound compounds from bean samples. Acetonitrile salting out liquid-liquid partitioning and SPE partitioning are better methods than ethyl acetate liquid-liquid partitioning, to partially purify and concentrate the phenolics after hydrolysis for either deconjugated and bound phenolics. These methods use less solvent, have shorter drying times, due to the smaller volume of sample, and were able to recover all tested phenolic standards. On top of that, SPE has the advantage of desalting the sample. Examination of the limitations of these methodologies concluded that the optimum approach for deconjugation of the soluble phenolics was a hydrolysis of 5min followed by SPE partitioning and for bound samples a hydrolysis of 1 h followed by acetonitrile salting out liquid-liquid partitioning. These approaches will be employed in the next chapter to analyse conjugated and bound phenolics in the underutilised and commercial beans.

CHAPTER 6

Profiling of soluble, conjugated and bound phenolics from underutilised beans using optimised methodology and HPLC

6.1 Introduction

After optimisation of extraction methodologies including type of solvent used for extraction of phenolics (chapters 3 and 4), treatment conditions and limitations (chapter 5), a method was developed to profile the soluble, conjugated and bound phenolics from beans. The objective of this study was to profile the soluble, conjugated and bound phenolics of six underutilised beans (adzuki bean, black eyed pea, bambara groundnut, lablab bean, mung bean and pigeon pea) and commercial soya bean by using the optimised extraction methods followed by detection via HPLC.

This optimised method will use 80% methanol extraction to obtain soluble phenolic compounds. Next, alkaline hydrolysis of soluble phenolic sample for 5 min followed by SPE partitioning to profile the phenolic component of conjugated compounds (deconjugated phenolic sample). While, an hour of alkaline hydrolysis of the residue after 80% methanol extraction followed by acetonitrile salting out liquid -liquid partitioning was used to profile the bound phenolics. All extracts were further analysed using HPLC and individual phenolics will be identified by comparing with phenolic standards. Meanwhile, the antioxidant activities from soluble, deconjugated free and bound phenolics samples were determined using TPC, DPPH and FRAP.

6.2 Methodology

The flowchart (figure 6.1) represents the preparation of the soluble, deconjugated and bound phenolic samples from adzuki bean, black eyed pea, bambara groundnut, lablab bean, mung bean, pigeon pea and one commercial bean – soya bean.

6.2.1 Soluble phenolic sample preparation

One gram of bean powder was extracted with 10 mL of 80% methanol (CH_3OH) (Fisher Scientific) (see section 3.2.3). The mixture was homogenized using an ultra-Turrax T25 high speed homogenizer (IKA, USA) 13.5 L/min for 1 min and then stirred for 60 min at 23°C to 25°C. After that, the suspension was centrifuged (Thermo Scientific Heraeus Megafuge, USA) at 4696 *g*, 4°C for 5 min and filtered through Whatman paper No.4. The liquid extract was immediately used for antioxidant assays (section 6.2.5) and also as a substrate to obtain deconjugated phenolic samples according to section 6.2.2. Finally 2 mL of liquid extract was completely dried under nitrogen gas for HPLC analysis.

6.2.2 Deconjugated phenolic sample preparation

Four mL of 80% methanol (CH_3OH) extract (see section 6.2.1) was added to 2 M sodium hydroxide (NaOH) at a volume ratio of 1:1 (v/v) and stirred at 23°C to 25°C for 5 min. Next, the pH was adjusted to pH 2.0 with 5 M acid hydrochloride (HCl). A maximum of 10 mL of sample was filtered in order to remove colloidal material after hydrolysis which would otherwise block and reduced the partition efficiency of SPE. The sample was loaded onto a pre-conditioned SPE column (see section 5.2.2.3). The flow through volume was collected as fraction 1, this consisted of non-retained compounds. After that, 4 mL of water was passed through the column to wash out the very polar compounds as fraction 2. Next, 4 mL of 100% methanol was passed through the column to elute the compounds as fraction 3. This final fraction 3 was dried completely under nitrogen gas then kept in 4°C before further analysis.

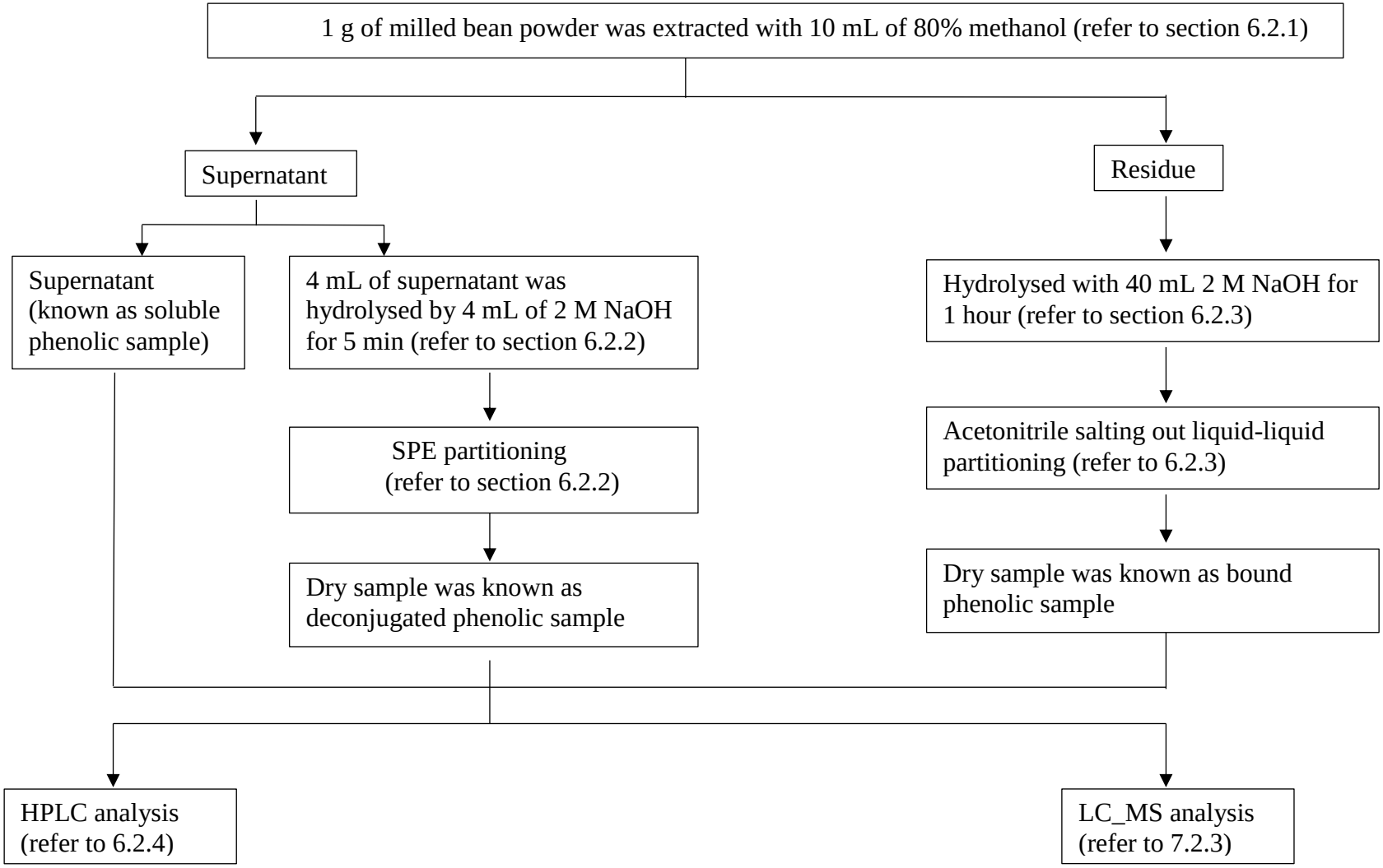


Figure 6.1 Flowchart of sample preparation for soluble, deconjugated and bound phenolic samples.

6.2.3 Bound phenolic sample preparation

The residue after 80% methanol extraction (see section 6.2.1) was used for the bound phenolic study. 40 mL of 2 M sodium hydroxide (NaOH) was added to the residue from the 1 g of bean and homogenized for 1 sec. Next, the mixture was stirred at 23°C to 25°C for 1 hour. After treatment, the pH was adjusted to pH 2.0 with 5 M hydrochloride acid (HCl). The mixture was then centrifuged at 4696 *g*, 4°C for 15 min. The supernatant was then transferred into a new tube. Hexane ($CH_3(CH_2)_4CH_3$) was added to the supernatant at a volume ratio of 1:1 (v/v) to remove the fat content which would reduce the extraction efficiency. The defatting treatment was repeated twice (see 5.2.1.4). Next, the aqueous layer was extracted through acetonitrile salting out liquid-liquid partitioning (see section 5.2.2). Acetonitrile (CH_3CN) was added to the hydrolysed sample at a volume ratio of 1:1 (v/v) and vortexed vigorously. The mixture was then left for 10 min to partition. The solvent layer was then transferred to a new tube. This step was repeated 3 times and the pooled solvent layers were then dried under nitrogen gas. Dried extract was then kept in 4°C before further analysis.

6.2.4 HPLC procedure

Dried soluble and deconjugated phenolic samples were dissolved in 1 mL of 50% methanol (CH_3OH) and 3 mL of 50% methanol was used to dissolve bound phenolic samples. Mixtures were vortexed vigorously and filtered into HPLC vials. The HPLC setup was as described in section 4.2.4. A Discovery C18 (150 mm x 4.6 mm, 5 µm particle diameter) column and Supelguard pre-column C18 (20 mm x 3 mm, 5 µm particle diameter) and photodiode array detector were used. The method was adapted from that described for profiling phenolics from cocoa bean (Jonfia-Essien *et al.,* 2008) with optimisation of the gradient settings for the mobile phase. Phosphate buffer (0.02 M pH 2.4) and HPLC grade (methanol) CH_3OH were used as mobile phases A and B. The sample was run with a gradient starting at 10% B, increasing to 70% B over 30 min, and then increasing to 90% at 32 min. A 90 % methanol wash to remove the retained compounds in the column was carried out for 5 min. The column was re-equilibrated with 10% B for 5 min between each sample. The detection wavelength was set at 210 nm to 700 nm.

A panel of 20 phenolic standards consisting of naringenin, ρ-coumaric acid, sinapic acid, caffeic acid, genistein, epigallocatechin gallate, epicatechin gallate, kaempferol, rutin, trans-3-hyroxycinnamic acid, quercetin, ferulic acid, gallic acid, epicatechin, 3-hydroxybenzoic acid, protocatechuic acid, daidzein, myricetin, luteolin and chlorogenic acid were used to identify potential phenolics. After that, there were used to build standard calibration curves in order to quantify the identified phenolics at optimal wavelength as listed in table 4.11. Integrated area was used for the calculation and the final results expressed as µg/g DW powder after correction for recovery from 80% methanol, acetonitrile liquid-liquid partitioning and SPE partitioning.

Those peaks that could not be assigned to a standard phenolic were profiled by using the integrated peak area between 20,000 to 5,500,000 µV*sec at 280 nm. Detection wavelength 280 nm was chosen since it is known to be the recommended wavelength for the detection of phenolic compounds.

6.2.5 Antioxidant assays

Soluble phenolic, deconjugated and bound phenolic samples were assayed for antioxidant activity as per chapter 3, section 3.2.4.1 and section 3.2.4.2 followed by total phenolic content assay as per chapter 3, section 3.2.4.3. This was to determine the antioxidant potential and correlation among the samples.

6.2.6 Statistical analysis

All assays were carried out in triplicate. One way ANOVA was used to identify differences between groups using Statgraphics Centurion version 16.1.11. When significant differences were detected ($p<0.05$), Fisher's least significant difference (LSD) was generated to determine the difference in mean. Correlation was analysed using Simple Regression Analysis. Statistical significance was declared as $p<0.05$.

6.3 Results

6.3.1 Determination of antioxidant activity and phenolic content of soluble, deconjugated and bound samples

Three antioxidant assays (TPC, DPPH and FRAP) were conducted to investigate the antioxidant activity of the soluble, deconjugated and bound phenolics extracted from beans (figures 6.2 to 6.4). The DPPH free radical scavenging assay revealed significant differences between the antioxidant activity of the extracts for each soluble, deconjugated and bound phenolic samples (figure 6.2). The soluble phenolic samples displayed the highest free radical scavenging activity across all beans. Adzuki bean exhibited the highest DPPH free radical scavenging activity (1.75 mg trolox/ g DW). The deconjugated phenolic sample showed the lowest activity across all beans with the lablab bean showing the lowest activity (0.034 mg trolox/ g DW). The ranking of the activity for soluble phenolic samples was adzuki bean > bambara groundnut > soya bean > black eyed pea = mung bean, mung bean = pigeon pea, pigeon pea = lablab bean. This is consistent with the result obtained from chapter 3.3.1. The ranking was different in deconjugated phenolic samples (soya bean > bambara groundnut > adzuki bean = black eyed pea = mung bean > pigeon pea > lablab bean) and bound phenolic samples (adzuki bean = bambara groundnut = soya bean, soya bean = mung bean, mung bean = black eyed pea, black eyed pea = pigeon pea = lablab bean).

The FRAP assay revealed significant differences in antioxidant activity between the extracts in each of the soluble, deconjugated and bound phenolic samples (figure 6.3). Soluble phenolic samples again showed the highest antioxidant activity across all beans. However, bound phenolic samples exhibited the lowest activity across all beans except for adzuki bean and bambara groundnut. Soluble phenolic sample of adzuki bean exhibited the highest antioxidant power and bound phenolic samples of black eyed pea, lablab bean, mung bean and pigeon pea showed the lowest antioxidant power. The ranking of the antioxidant activity for soluble phenolic samples was adzuki bean > bambara groundnut = soya bean > black eyed pea > mung bean = pigeon pea > lablab bean. Again, the result obtained is consistent with the

outcome from chapter 3.3.1. A different ranking was observed in deconjugated phenolic and bound phenolic samples.

The TPC assay showed that soluble phenolic samples had the highest antioxidant activity and the deconjugated phenolic samples had the lowest activity. This was the same finding as in the DPPH free radical scavenging assay. Result revealed a significant difference in antioxidant activity between the extracts in each of the soluble, deconjugated and bound phenolic samples. Soluble phenolic sample of adzuki bean exhibited the highest TPC content amongst the underutilised beans. Whilst, deconjugated phenolic sample of pigeon pea and lablab bean had the lowest TPC content. The ranking of phenolic content was soya bean > adzuki bean > bambara groundnut > mung bean > pigeon pea = black eyed pea > lablab bean. This result was again consistent with the result obtained from the preliminary study from section 3.3.1. Again, a different ranking of the antioxidant level for deconjugated and bound phenolic samples was observed.

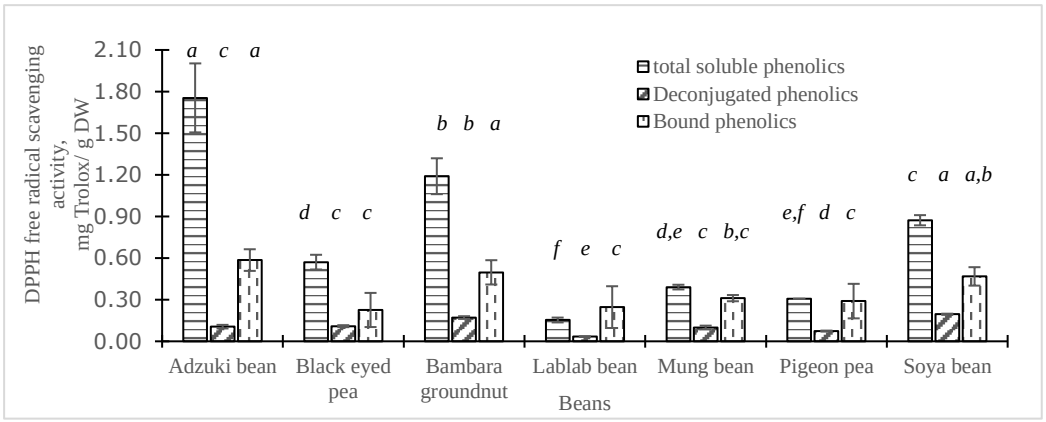


Figure 6.2 DPPH free radical scavenging activity of total soluble, deconjugated and bound phenolic samples. Results are shown as the mean ± SD (n=3). Different letters represent significant differences at $p<0.05$ for soluble, deconjugated and bound phenolic samples.

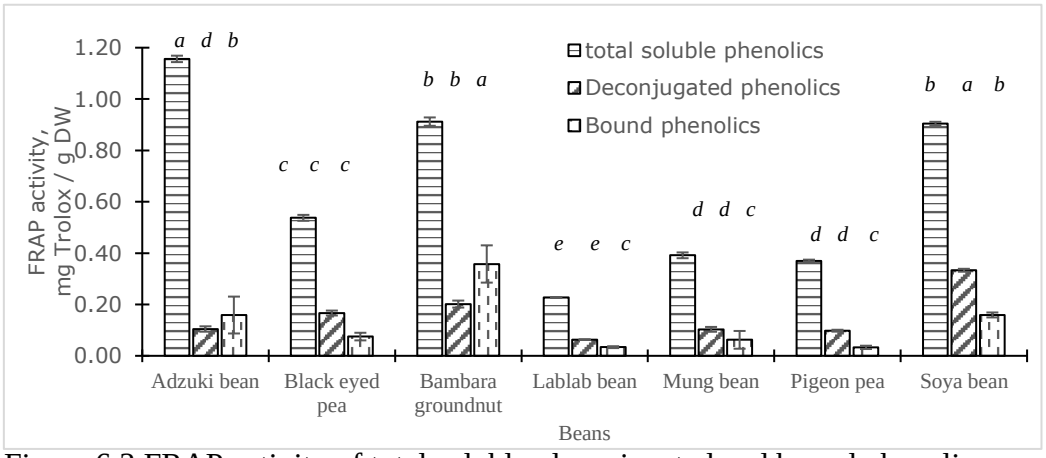


Figure 6.3 FRAP activity of total soluble, deconjugated and bound phenolic samples. Results are shown as the mean ± SD (n=3). Different letters represent significant differences at $p<0.05$ for soluble, deconjugated and bound phenolic samples.

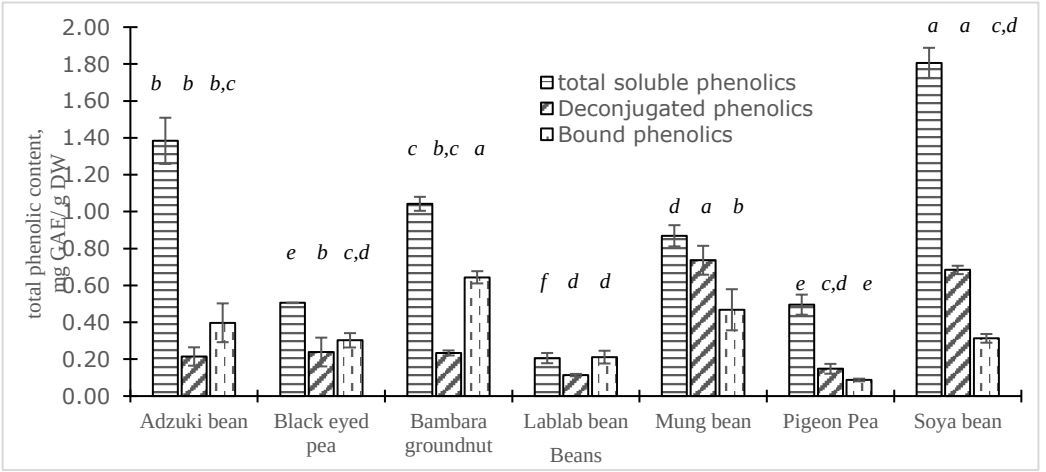


Figure 6.4 Total phenolic content of total soluble, deconjugated and bound phenolic samples. Results are shown as the mean ± SD (n=3). Different letters represent significant differences at $p<0.05$ for soluble, deconjugated and bound phenolic samples.

The correlations between the three antioxidant assays for each of the soluble, deconjugated and bound phenolic samples were determined (tables 6.1, 6.2 and 6.3). Results showed a positive relationship between all three assays for each fraction ($0.497 < r^2 < 0.965$). Strong correlations were shown between DPPH and FRAP for soluble ($r^2=0.965$) and deconjugated ($r^2=0.915$) phenolics.

On top of that, the correlations between the soluble, deconjugated and bound phenolic samples for each of the three assays-DPPH, FRAP and TPC - were determined (tables 6.4, 6.5 and 6.6). A positive correlation ($p<0.05$) was observed between soluble and deconjugated phenolic samples in all three antioxidant assays. This finding is to be expected since the deconjugated phenolic samples were prepared from the total soluble phenolic samples. On the other hand, correlations between total soluble phenolics and bound phenolics, deconjugated phenolics and bound phenolics samples were not consistently shown (table 6.6). It is because the amount of extractable bound phenolics are affected by hydrolysis conditions and extraction techniques but the soluble phenolics are limited by the extraction techniques only.

In conclusion, different form of compounds (soluble free, conjugated and bound) from all beans exhibited antioxidant activities and correlate with phenolics compounds. A further study in profiling the phenolic compounds with HPLC is highly recommended to investigate the differences in amount and variety of phenolic compounds.

Table 6.1 Correlation coefficients, r^2 between assays for total soluble phenolic samples, significantly correlated at $p<0.05$.

	TPC	DPPH assay	FRAP assay
TPC	-	0.699	0.829
DPPH assay	0.699	-	0.965
FRAP assay	0.829	0.965	-

Table 6.2 Correlation coefficients, r^2 between assays for deconjugated phenolic samples, significantly correlated at $p<0.05$.

	TPC	DPPH assay	FRAP assay
TPC	-	0.502	0.497
DPPH assay	0.502	-	0.915
FRAP assay	0.497	0.915	-

Table 6.3 Correlation coefficients, r^2 between assays for bound phenolic samples, significantly correlated at $p<0.05$.

	TPC	DPPH assay	FRAP assay
TPC	-	0.507	0.785
DPPH assay	0.507	-	0.652
FRAP assay	0.785	0.652	-

Table 6.4 Correlation coefficients, r^2 between total soluble, deconjugated and bound phenolics for DPPH assay, significantly correlated at $p<0.05$.

	Total soluble phenolics	Deconjugated phenolics	Bound phenolics
Total soluble phenolics	-	0.507	0.749
Deconjugated phenolics	0.507	-	0.488
Bound phenolics	0.749	0.488	-

Table 6.5 Correlation coefficients, r^2 between total soluble, deconjugated and bound phenolics for FRAP assay, significantly correlated at $p<0.05$.

	Total soluble phenolics	Deconjugated phenolics	Bound phenolics
Total soluble phenolics	-	0.498	0.637
Deconjugated phenolics	0.498	-	0.459
Bound phenolics	0.637	0.459	-

Table 6.6 Correlation coefficients, r^2 between total soluble, deconjugated and bound phenolics for TPC assay, significantly correlated at $p<0.05$ except *

	Total soluble phenolics	Deconjugated phenolics	Bound phenolics
Total soluble phenolics	-	0.586	0.379 *p=0.090
Deconjugated phenolics	0.586	-	0.323 *p=0.153
Bound phenolics	0.379 *p=0.090	0.323 *p=0.153	-

6.3.2 Identification and quantification of soluble, deconjugated and bound phenolics

The total soluble, deconjugated and bound extracts, described above, were all subjected to profiling by HPLC. Samples were first analysed chromatographically at wavelength of 280 nm as shown in figures 6.5 and 6.6 for adzuki bean and black eye pea samples, respectively, and in appendices 6.1 to 6.5 for the remaining beans. The results showed clear differences in the profiles of the peaks among the soluble, deconjugated and bound samples.

Next, the retention times and spectra of the peaks obtained were compared to those for the panel of 20 phenolic standards. A limited number of putative identifications were obtained- four phenolic acids (ρ- coumaric acid, ferulic acid, protocatechuic acid and sinapic acid), one isoflavone (daidzein) and one conjugated phenolic (rutin). These putative phenolics were then quantified in the soluble, deconjugated and bound phenolic samples of selected beans (tables 6.7 to 6.9).

Daidzein was found in soluble and deconjugated samples only while protocatechuic acid and sinapic acid were only found in bound phenolic samples. No phenolics compounds were identified in soluble and deconjugated samples of bambara groundnut, mung bean and all extracts from pigeon pea. Bound phenolic samples contained more variety of phenolic acids as compared with the conjugated phenolic samples. Additionally bound phenolics samples had the highest concentration of phenolic acids while soluble conjugated samples had the highest concentration of daidzein and rutin.

Soluble phenolic sample of adzuki bean had the highest concentration of rutin (37 µg/g DW bean powder) and soluble phenolic sample of soya bean had the highest daidzein (101.9 µg/g DW bean powder) (table 6.7). Whilst, the highest amount of ρ- coumaric acid, ferulic acid, protocatechuic acid and sinapic acid were found in the bound phenolic samples of different beans (table 6.9). ρ-coumaric acid was highest in the bound phenolic sample of lablab bean (48 µg/g DW bean powder), ferulic acid was highest in black eyed pea (30.5 µg/g

DW bean powder), protocatechuic acid was highest in bambara groundnut (24.2 µg/g DW bean powder) and sinapic acid was found in soya bean (19.1 µg/g DW bean powder) only.

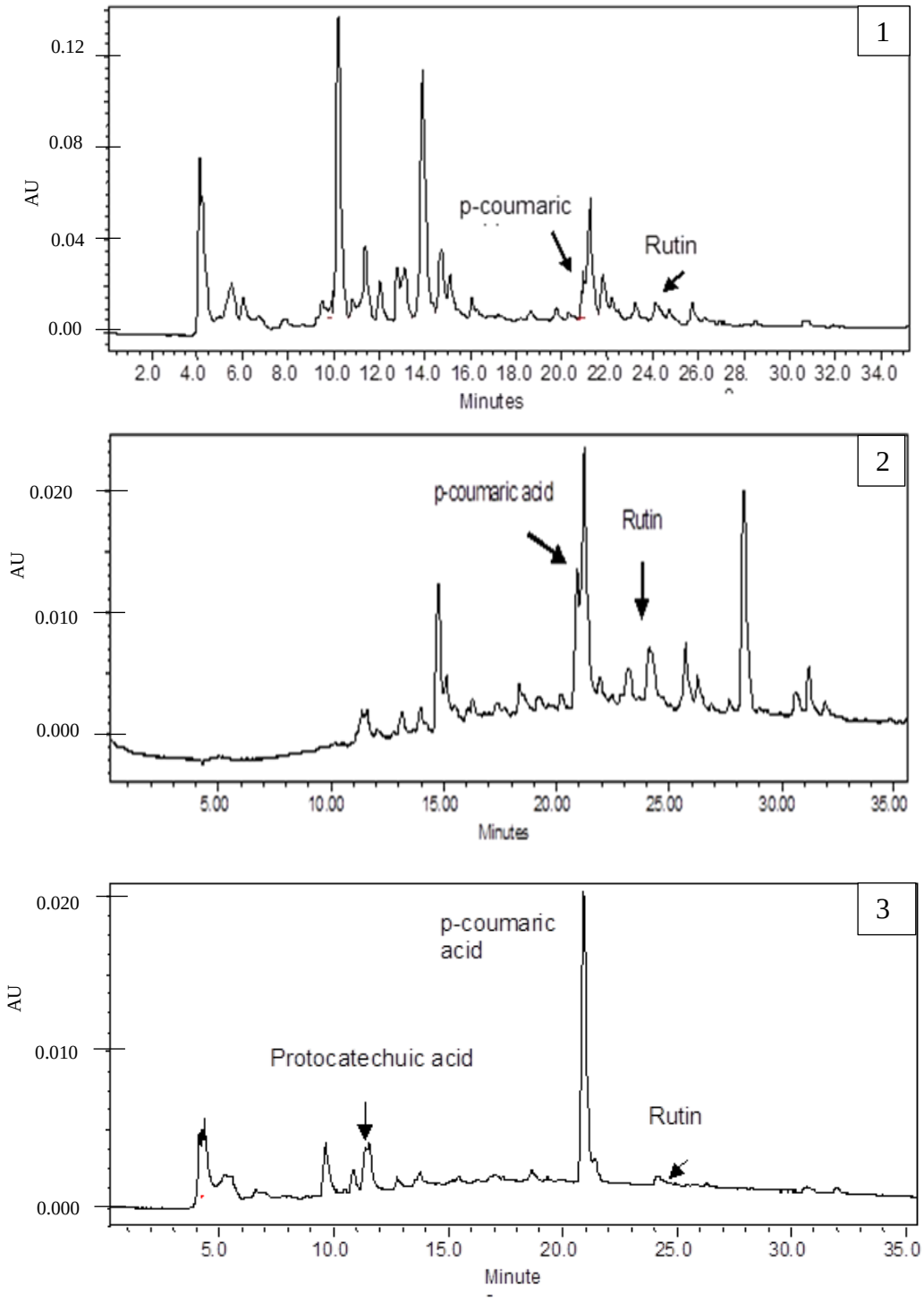


Figure 6.5 HPLC chromatogram for adzuki bean extracts detected at 280 nm. 1- soluble phenolic sample, 2- deconjugated phenolic sample, 3- bound phenolic sample.

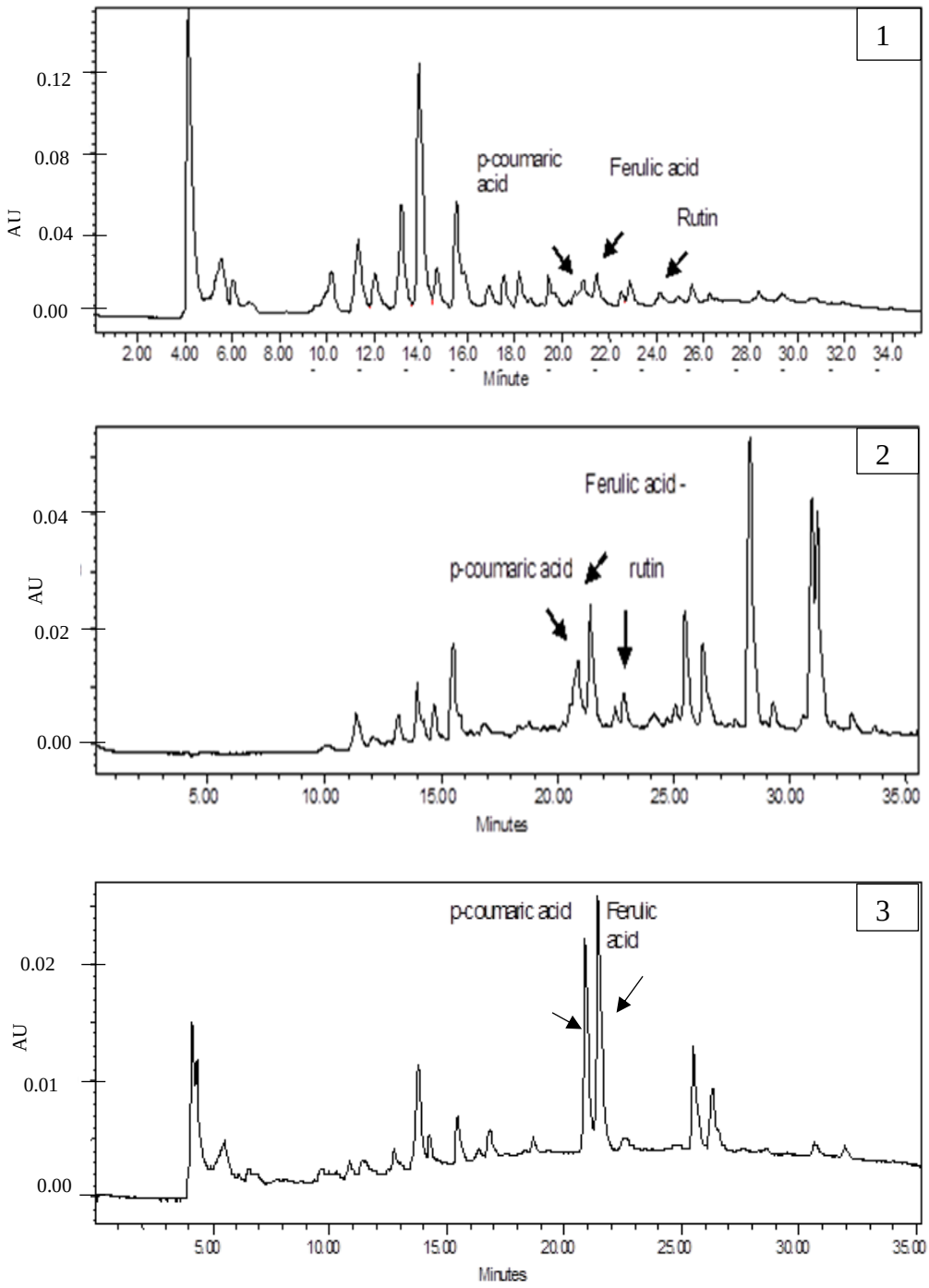


Figure 6.6 HPLC chromatogram for black eyed pea extracts detected at 280 nm. 1- soluble phenolic sample, 2- deconjugated phenolic sample, 3- bound phenolic sample.

Table 6.7 Quantification of identified phenolics from soluble phenolic samples. Results are shown as the mean ± SD (n=3).

Compound	Soluble phenolic content, µg/g DW bean powder						
	Adzuki bean	Black eyed pea	Bambara groundnut	Lablab bean	Mung bean	Pigeon pea	Soya bean
ρ-coumaric acid	10.6 ± 0.70	6.3 ± 0.35	ND	13.2 ± 1.51	ND	ND	ND
Rutin	37.0 ± 0.13	10.0 ± 0.43	ND	ND	ND	ND	ND
Ferulic acid	ND	9.6 ± 0.84	ND	17.1 ± 1.46	ND	ND	ND
Daidzein	ND	ND	ND	ND	ND	ND	101.9± 5.41

Table 6.8 Quantification of identified phenolics from deconjugated phenolic samples. Results are shown as the mean ± SD (n=3).

Compound	Deconjugated phenolic content, µg/ g DW bean powder						
	Adzuki bean	Black eyed pea	Bambara groundnut	Lablab bean	Mung bean	Pigeon pea	Soya bean
ρ-coumaric acid	6.9 ± 0.95	6.6 ± 0.67	ND	17.8 ± 1.09	ND	ND	ND
Rutin	29.4 ± 2.11	18.4 ± 1.00	ND	ND	ND	ND	ND
Ferulic acid	ND	17.2 ± 1.05	ND	15.9 ± 0.27	ND	ND	ND
Daidzein	ND	ND	ND	ND	ND	ND	73.3 ± 5.28

Table 6.9 Quantification of identified phenolics from bound phenolic samples. Results are shown as the mean ± SD (n=3).

Compound	Bound phenolic content, µg/ g DW bean powder						
	Adzuki bean	Black eyed pea	Bambara groundnut	Lablab bean	Mung bean	Pigeon pea	Soya bean
ρ-coumaric acid	40.2 ± 4.14	11.2 ± 1.35	2.9 ± 0.39	48.0 ± 5.62	4.7 ± 1.53	ND	15.1 ± 1.37
Rutin	25.3 ± 2.81	ND	ND	ND	ND	ND	ND
Ferulic acid	ND	30.5 ± 6.26	4.4 ± 0.85	9.2 ± 1.39	ND	ND	26.3 ± 2.53
Protocatechuic acid	18.1 ± 0.6	ND	24.2 ± 1.86	ND	ND	ND	ND
Sinapic acid	ND	ND	ND	ND	ND	ND	19.1 ± 1.87

6.3.3 Analysis of HPLC profile from selected beans

Only a minority of the peaks observed in the HPLC profiles could be putatively assigned to individual phenolics using the panel of standards. This implies that the remaining peaks are either phenolics for which standards were not applied, or represent other types of compounds. In either case, further analysis of these unidentified peaks in terms of their variation between beans was carried out. This was conducted by obtaining the integrated peak area at 280 nm as mentioned in the methodology. Integrated area (x) for each individual untargeted peak is categorized as x <100,000 µV*sec, 100,000 < x < 500,000 µV*sec, 500,000 < x < 1000,000 µV*sec and x> 1,000,000 µV*sec.

6.3.3.1 Profiling the soluble phenolic extracts

The total number of peaks (in the soluble sample from each bean) is shown in table 6.10 along with the number of those peaks that were unique to that individual bean. This varied from 10 peaks in lablab bean, of which 3 were unique, to 24 in peaks in soya bean, of which 10 were unique. Table 6.11 correlates the number of common peaks between the bean samples. The highest commonality of 13 peaks have been observed between soya bean and adzuki bean and the lowest commonality of 3 peaks were found between soya bean and mung bean. A total of 67 individual peaks with integrated areas between 20,000 to 5,500,000 µV*sec were identified across all the soluble extracts examined. The absorbance spectra for these peaks are shown in appendices 6.8 to 6.19. The retention times, spectral maxima and relative abundance of the individual peaks are summarised in appendices 6.26 to 6.30. Only two out of 67 peaks (4.10 min and 13.85 min) were found across all beans. The peak areas of both these showed a marked difference in concentration between the beans but the relative ranking varied. For example the peak intensity at 4.10 min was similar in both lablab and mung bean but that at 13.85 min was much lower in lablab than mung bean.

Table 6.10 Total number of individual HPLC peaks detected at 280 nm (soluble phenolic samples) and the number of those peaks that were unique to the specific bean.

	Adzuki bean	Black eyed pea	Bambara groundnut	Lablab bean	Mung bean	Pigeon pea	Soya bean
Total number of peaks detected	20	17	12	10	12	19	24
Number of unique peaks	6	8	4	3	7	11	10

Table 6.11 Total number of peaks common between bean samples (soluble phenolic samples).

	Adzuki bean	Black eyed pea	Bambara groundnut	Lablab bean	Mung bean	Pigeon pea	Soya bean
Adzuki bean	-	9	7	4	3	4	13
Black eyed pea	-	-	5	3	3	4	9
Bambara groundnut	-	-	-	4	3	5	7
Lablab bean	-	-	-	-	3	5	4
Mung bean	-	-	-	-	-	3	3
Pigeon pea	-	-	-	-	-	-	4

6.3.3.2 Profiling the bound phenolic extracts

The total number of peaks (in the bound sample from each bean) is shown in table 6.12 along with the number of those peaks that were unique to that individual bean. This varied from 6 peaks in adzuki bean, of which no unique peak was found, to 10 peaks in bambara groundnut, of which 5 were unique. Table 6.13 correlates the number of common peaks between the bean samples. The highest commonality of 5 peaks was observed between soya bean and pigeon pea and the lowest commonality of 1 peak, between adzuki bean and lablab bean, lablab bean and pigeon pea have been found. A total of 32 individual peaks with integrated areas between 24,000 and 3,989,000 µV*sec were identified across all the bean extracts examined. The absorbance spectra for these peaks are shown in appendices 6.20 to 6.25. The retention times, spectral maxima and relative abundance of the individual peaks are summarised in appendices 6.31 to 6.32. Only one out of 32 peaks (4.15 min) was found across all beans. The peak area was similar across all beans.

In general, soluble extracts showed 35 peaks more than bound samples and only a limited number peaks were found in both soluble and bound samples. Peaks at 4.10 min and 13.85 min were found in all beans of both soluble and bound samples except bambara groundnut and lablab bean bound samples. On top of that, the following peaks were uniquely found in both soluble and bound samples of selective beans, black eyed pea (5.53 min), bambara groundnut (13.03 min), mung bean (17.40 min, 21.95 min, 23.10 min), pigeon pea (7.58 min, 24.73 min) and soya bean (19.35 min). Besides, these are the peaks that were found in both soluble and bound samples although not unique to these beans, adzuki bean (9.63 min, 11.36 min), bambara groundnut (17.55 min), lablab bean (12.77 min), pigeon pea (14.12 min and 22.32 min) and soya bean (11.36 min).

Table 6.12 Total number of individual HPLC peaks detected at 280 nm (bound phenolic samples) and the number of those peaks that were unique to the specific bean.

	Adzuki bean	Black eyed pea	Bambara groundnut	Lablab bean	Mung bean	Pigeon pea	Soya bean
Total number of peaks detected	6	10	10	6	8	9	9
Number of unique peaks	0	4	5	2	4	4	1

Table 6.13 Total number of peaks common between bean samples (bound phenolic samples).

	Adzuki bean	Black eyed pea	Bambara groundnut	Lablab bean	Mung bean	Pigeon pea	Soya bean
Adzuki bean	-	3	3	1	3	2	3
Black eyed pea	-	-	2	4	3	2	4
Bambara groundnut	-	-	-	2	3	2	2
Lablab bean	-	-	-	-	2	1	2
Mung bean	-	-	-	-	-	2	2
Pigeon pea	-	-	-	-	-	-	5

6.4 Discussion

6.4.1 Antioxidant activity of soluble, deconjugated and bound phenolic extracts

The optimised methodology was applied to identify and quantify the soluble, deconjugated and bound phenolics from selected underutilised beans with reference to phenolic standards using HPLC. At the same time, three independent assays – DPPH, FRAP and TPC assays were used concurrently to compare the antioxidant activities of the different extracts from selected beans.

The soluble phenolic samples displayed the highest free radical scavenging activity (DPPH), FRAP reducing potential and TPC across all beans. Whilst, the deconjugated phenolic sample showed the lowest activity with the DPPH and TPC assays but bound phenolic samples exhibited the lowest FRAP reducing activity across all beans except for adzuki bean and bambara groundnut. This outcome showed that the soluble phenolic extracts demonstrated the highest level of antioxidant potential as compared with the deconjugated and bound phenolic samples.

This result is in line with published studies that have shown antioxidant potential of soluble phenolics was highest from whole buckwheat grains, peels of rambutan and black eyed pea (Sun *et al.,* 2012; Gutiérrez-Uribe *et al.*, 2011; Hung & Morita, 2008). This fraction either having a greater concentration of, or a more diverse profile of, antioxidant compounds because soluble phenolic samples consist of both free phenolic acids and conjugated phenolics that will give higher antioxidant potential activity. This rationale is not applicable to cereals and grains because in these commodities' antioxidant potential is always higher in the bound phenolic than the soluble phenolic extracts (Adom & Liu, 2002)

Deconjugated phenolic samples showed much lower antioxidant activity than conjugated samples possibly because they contain deconjugated free phenolic acids and a lower concentration of conjugated phenolics. On top of that, free phenolics acids might be at lower concentrations because of their sensitivity to alkaline hydrolysis. This finding is in agreement with that of Meyer and

Andreasen (1999) who found that antioxidant activities from deconjugated samples were expected to be lower than the soluble sample because soluble samples consisted of both conjugated and free phenolics. And, the conjugated phenolic have been shown to exhibit higher antioxidant activity than the free forms.

To the author's knowledge, this is the first report of significant antioxidant potential from bound compounds that correlate with phenolics from underutilised beans. Thus far, only bound phenolics from cereal and grains have received similar attention due to their role as major staple foods (Miller *et al.,* 2000). Durazzo *et al* .(*2*013) have reported that bound phenolics possess higher antioxidant potential than soluble phenolics and that was reflected in higher TPC and FRAP values from wheat, lentils, chickpea and sweet chestnut.

The health promoting benefits of bound phenolics are thought to be due to their ability to survive stomach and intestinal digestion to reach the colon by binding to the cell wall material. Hence, their natural characteristics such as antioxidant potential are freely effective at the point of release (Adom & Liu, 2002). These findings thus add to our understanding of the nutritional, and potentially also the pharmaceutical value of the beans.

On top of that this is probably the first report of the application of acetonitrile salting out partitioning targeting bound phenolics from plant material. This technique has been mostly applied to human biological samples such as serum (Zhao *et al.*, 2012; Zhang *et al.,* 2009; Anthemidis and Ioannou, 2009). In addition, this technique has proven its advantage by being less hazardous and able to extract a wide range of compounds and is compatible with gas and liquid chromatography (Valente *et al.,* 2013).

6.4.2 Preliminary profiling of soluble, deconjugated and bound phenolics from underutilised beans by HPLC

In the HPLC analysis, A limited number of putative phenolics were identified in the soluble, deconjugated and bound phenolic extracts from the six underutilised beans – three phenolic acids (ρ- coumaric acid, ferulic acid and protocatechuic acid) and one isoflavone (daidzein) and one conjugated phenolic (rutin). Whilst, three phenolic acids (ρ- coumaric acid, ferulic acid and sinapic acid) and one isoflavone (daidzein) were identified in soya bean. Soluble phenolic sample of adzuki bean had the highest concentration of rutin and soya bean had the highest daidzein (table 6.7).

There have been a limited number of publications that identify individual phenolics from beans. Thus far, only one validated HPLC-DAD investigation targeting phenolics has been reported (Giusti *et al.,* 2017). This group attempted to identify phenolics in mung bean, adzuki bean, black eyed pea and soya bean and some of the phenolics' selection (gallic acid, caffeic acid, rutin, ρ- coumaric acid, chlorogenic acid, ferulic acid, quercetin and epicatechin) are the same as in this study. From their findings, a concentration of 9.6 µg/ g DW ferulic acid was identified in black eyed pea and 41.2 µg/ g DW rutin in adzuki bean. These are similar to the current study which also found 9.6 µg/ g DW of ferulic acid in black eyed pea and 37.0 µg/ g DW of rutin in adzuki bean. Other phenolics were not detected and again this is consistent with the current study but with minor exceptions such as chlorogenic acid which was found in mung bean (10.3 µg/ g DW) and black eyed pea (53.3 µg/ g DW).

Soya bean is the major source of daidzein, a type of isoflavones that has distinct health benefits (Vila-Donat *et al.,* 2015; Manach *et al.,* 2004; Messina, 1999). Soluble phenolics like isoflavone- daidzein, genestein, glycitein and phenolic acids such as caffeic acid, ρ- coumaric acid, ferulic acid, sinapic acid, gallic acid, epicatechin have been reported in soya bean (Bai *et al.,* 2017; Giusti *et al.,* 2017; Cho *et al.,* 2013;Yao *et al.,* 2011). However, only daidzein (101.9 µg/ g DW) has been found in this study as compared to 300 µg/ g DW in Bai *et al.* (2017) and 2.65 µg /g DW in Cho *et al.* (2013).

The fact that the majority of the phenolics previously reported from beans were not found in this study may due to differences in extraction solvent (80% methanol), shorter extraction time (1 h) and extraction condition (room temperature). The published studies applied different solvents -70% or 95% ethanol, longer extraction duration (up to 2 h) and extraction conditions (sonication, acidic condition and higher temperature up to 70°C).

The concentration of daidzein from soya reported by Bai *et al.* (2017) was higher than found in this study and this may be because they used a more exhaustive extraction method (50 g extracted with 500 mL of 95% ethanol with three repeated extractions and then concentrated at 45°C under reduced pressure). However, a lower concentration of daidzein, than found in this study, was reported by Cho *et al.* (2013) despite both studies using 80% methanol and the other group applying a longer (12 h) extraction. The reason maybe due to the type of soya beans, which in the case of Cho *et al* (2013) were grown internally at an experimental field. They also demonstrated observable differences in soluble phenolics between cultivars, crop years and seed colour, again factors that could account for the discrepencies between their values and those in this study.

In terms of previous studies into phenolics from underutilised beans these are again limited and are mostly from adzuki and mung bean. Soluble phenolics that have been previously found in adzuki bean, but not in the current study, were caffeic acid (11.2 µg/ g DW), chlorogenic acid (2.0 µg/ g DW-14.0 mg/ g DW), ρ-coumaric acid (26.8 µg/ g DW), ferulic acid (154.1 µg/ g DW) and sinapic acid (68.0 µg/ g DW) (Bai *et al.,* 2017; Yao *et al.,* 2011). The rutin level from this study (0.04 mg/ g DW) was far below the reported value by Gan *et al.,*(2016) and Bai *et al.,* (2017) (0.42 - 4.1 mg/ g DW). Negative results for daidzein and genestein in the current study were supported by the above mentioned publications.

The phenolics previously reported in mung bean but which were not detected in this study include gallic acid (3.0 µg/ g DW), caffeic acid (0.3 µg/ g DW), ρ-coumaric acid (0.2 µg/ g DW), ferulic acid (2.2 µg/ g DW), sinapic acid (1.5

µg/ g DW), quercetin (0.2 µg/ g DW) and kaempferol (0.1 µg/ g DW) (Pająk *et al.,* 2014). Another report by Yao *et al.* (2011) showed that ρ- coumaric acid (57.6 µg/ g DW), ferulic acid (198.6 µg/ g DW) and sinapic acid (78.5 µg/ g DW) exist in mung bean. The negative results for soluble protocatechuic acid, chlorogenic acid, caffeic acid and luteolin in this study were in line with the above two reports. In contrast, Giusti *et al.,* (2017) reported chlorogenic acid (10.3 µg/ g DW) and rutin (5.4 µg/ g DW) which were not found in either this study or the above published studies.

Cai *et al.* (2003) conducted phenolic analysis from 17 varieties of black eyed pea using HPLC. The results reported in this study were within the range reported by this group. Firstly, ρ- coumaric acid (6.3 µg/ g DW in this study) was within the range 3 to 42 µg/ g DW. Secondly, ferulic acid (9.6 µg/ g DW in this study) was within the range 6 to 62 µg/ g DW (Cai *et al.,* 2003). However, protocatechuic acid, hydroxybenzoic acid, caffeic acid and hydroxycinnamic acid were positively reported by Cai *et al.* (2003) but were not found in this study. Whilst, the presence of rutin (10.0 µg/ g DW) reported in this study appears to be a novel finding.

Other results from Gutiérrez-Uribe *et al.* (2011) using black coloured black eyed pea extracted with 80% ethanol showed results similar to, or lower than, those in this study such as a 80% lower concentration of ρ-coumaric acid (1.25 µg/ g DW) and negative detection for myricetin, kaempferol, gallic acid and protocatechuic acid have been demonstrated. In contrast the reported results for ferulic acid (26.25 µg/ g DW), quercetin (1.26 µg/ g DW) and hydroxybenzoic acid (0.95 µg/ g DW) were all higher than in this study. The major reason for this variation may have been in the different types of black eyed pea used in each case and the extraction method.

Phenolics such as caffeic acid (9.6 µg/ g DW), ρ- coumaric acid (46.3 µg/ g DW), ferulic acid (190.5 µg/ g DW), sinapic acid (32.5 µg/ g DW) have been detected in lablab bean but chlorogenic acid was not detectable (Yao *et al.,* 2011). The concentration of these phenolics detected in this study were far

below the result above. Yao *et al.* (2011) conducted an extraction for 2 hours at room temperature with 2 repetition. The current study used only 1 hour at room temperature without repetition and that might be part of the reason for the variation in results.

In terms of the comparison between the published reports and this study, a range of similar soluble phenolic compounds have been observed despite variations in extraction methods and conditions. The previous report utilising a validated HPLC technique has shown to have results that are closest to this study. Therefore, a further investigation with LC-MS is suggested to confirm and quantify the identified soluble phenolics instead of relying on HPLC only.

The application of optimised methodologies to actual beans samples and subsequent analysis by HPLC appeared to show a different result to that obtained from a similar hydrolysis of phenolic standards. It was estimated that there was a 20% reduction of the phenolic standards -ρ- coumaric acid, ferulic acid and daidzein (figure 5.11). However, analysis of the deconjugated bean samples suggests a mixture of reduction and increase in concentration is occurring for these phenolics. For instance ρ- coumaric acid from adzuki bean appears to decrease by 39.2%, ferulic acid from lablab bean to decrease by 7.1%, daidzein from soya bean to decrease by 28.6%, ρ- coumaric acid to increase by 4.8% from black eyed pea and by 35.0% from lablab bean and lastly ferulic acid increased by 79.0% from black eyed pea.

This inconsistency between the phenolic standards and actual samples are probably to be expected and indeed indirectly serve to demonstrate the success of the optimised methodologies. Reductions less than expected (< 20%) or an increase in concentration after hydrolysis may be expected because the phenolics probably exist in both conjugated and free forms. Hydrolysis being designed to deconjugate the phenolic compounds leads to increased concentrations of the free forms.

A comparison of phenolic acids before and after hydrolysis for deconjugated samples indicated that rutin and ferulic acid for black eyed pea existed mostly in the esterified form. Conjugated ferulic acid was converted to free ferulic acid after hydrolysis and this was supported by Cai *et al*.(2003). Whilst, rutin may exist in more complex conjugated forms that gave higher concentrations after hydrolysis. Another phenomena that has been observed was the slight increase of concentration after hydrolysis which may suggest low amounts of esterified forms exist. This was true for ρ- coumaric acid for lablab bean. Conversely for those phenolic acids that showed a reduction in concentration after hyrolysis, such as ρ- coumaric acid and rutin for adzuki bean, ferulic acid for lablab bean and daidzein in soya bean may indicate that esterified forms of these compounds do not exist and the reduction is due to their sensitivity to alkaline conditions.

Comparison between the soluble and bound phenolics showed that a more diversified and higher concentration of phenolics were found in the bound form. Among all those phenolics examined, ρ- coumaric acid, ferulic acid and protocatechuic acid were found to be the major phenolic acid and had their highest concentrations in bound phenolic samples (table 6.9). ρ- coumaric acid was highest in the bound phenolic sample of lablab bean, ferulic acid was highest in black eyed pea, protocatechuic acid was highest in bambara groundnut and sinapic acid was found in soya bean only.

Bound phenolic from black eyed pea have been reported in the literature. Gallic acid, protocatechuic acid, hydroxybenzoic acid, ρ-coumaric acid, myricetin, quercetin, kaempferol and ferulic acid were not detected from black coloured black eyed pea (Gutiérrez-Uribe *et al*, 2011). This finding is contradictory to the result obtained in this study since both ρ-coumaric acid and ferulic acid were detected. On top of that, ferulic acid was found to have the highest concentration in the bound extract for all beans tested in this current study. This variation of results may be due to different varieties of bean, a ratio of sample to volume of 2M NaOH is 1: 20 (w/v) was used in the published study but a ratio of 1: 40 (w/v) was applied in this study. And, ethyl acetate

liquid-liquid partitioning was used in the published study instead of acetonitrile salting out liquid/liquid partitioning as used in this study. It is thus a novel finding for black eyed pea.

Bound phenolics from mung bean have been reported by Pająk *et al.* (2014) using HPLC. Among their findings, only ρ- coumaric acid at 10.6 µg/ g DW was similar to the results in this study. Other phenolics such as protocatechuic acid, ferulic acid, chlorogenic acid and kaempferol were not detected, again a similar result to that in this thesis. They also showed that gallic acid, caffeic acid, sinapic acid, quercetin and luteolin were detectable in the bound form but all of these were undetectable in this study. The differences between the reported data and the current study are probably due to the relative hydrolysis methods used. The published method applied the methanol, alkaline hydrolysis and post hydrolysis extraction sequentially to the raw material. Hence, their results probably consisted of a mixture of free, deconjugated and bound phenolics. The current study in contrast extracted the soluble phenolics first followed by direct alkaline hydrolysis of the residue after extraction in order to obtain bound phenolics only.

Despite the limited number of putative phenolics identified by this preliminary profiling, HPLC analysis and comparison with published reports has clearly resulted in 4 important findings. Firstly, that the levels of conjugated phenolic compounds might be quite low and this is in line with the publications mentioned above. Secondly, bound phenolic samples contained more variety of phenolic acids and higher concentrations as compared with the soluble phenolic samples. Thirdly, the concentrations of these phenolics are very different in the different varieties of beans. Fourthly, due to the limitation of HPLC, some phenolic compounds may not be being detected. Hence, further analysis with LC-MS is crucial. LC-MS is known to have higher selectivity and sensitivity in identification and quantification (Brooke and Huggett, 2012; Gross, 2011; Korfmacher, 2005)

6.4.3 Profiling of unknown compounds

Only a minority of the peaks observed by HPLC profiling could be putatively assigned to individual phenolics using the panel of standards. This implies that the remaining peaks are either phenolics for which standards were not applied, or represent other types of compound. These results provide markers for these compound in terms of their retention times and optimal wavelengths. This database for each individual bean for compounds that exist in soluble and bound forms may provide a useful reference tool for future study.

6.5 Conclusion

In conclusion, soluble, deconjugated and bound phenolic samples showed significant differences in antioxidant activities and correlations. The soluble phenolic samples displayed the highest free radical scavenging activity (DPPH), FRAP reducing potential and TPC across all beans. Soluble extracts from adzuki bean exhibited the highest DPPH and FRAP antioxidant activity among all beans. Soluble extract from soya bean exhibited the highest TPC value.

In the HPLC analysis, ρ- coumaric acid, ferulic acid, rutin and daidzein were found in soluble phenolic samples as either soluble free or conjugated compounds. And, ρ- coumaric acid, rutin, ferulic acid, protocatechuic acid and sinapic acid were found in bound phenolic samples. Highest content of phenolics were obtained from adzuki bean (rutin), lablab bean (ρ- coumaric acid), black eyed pea (ferulic acid), bambara groundnut (protocatechuic acid) and soya bean (sinapic acid and daidzein). On top of that, ρ- coumaric acid, ferulic acid and protocatechuic acid were found to be the major phenolic acids with the highest concentrations found in bound phenolic samples.

Apart from that, profiling the data of unidentified phytochemicals according to retention time and optimal detection wavelength may provide a database of compound markers for each individual bean. This also indicated the peaks commonality among the beans. This is a useful reference tool for future study.

CHAPTER 7

Profiling of soluble, conjugated and bound phenolics from underutilised beans using optimised methodology and liquid chromatography coupled with mass spectrophotometry

7.1 Introduction

Liquid chromatography coupled with mass spectrophotometry (LC-MS) separates compounds by retention time according to their polarity followed by an ionization process which gives different mass-to-charge ratio (*m/z*) of charged particles (ions). These *m/z* values of particles will then allow the elucidation of the elemental composition and chemical structures of the sample tested (Kruve *et al.*, 2015). This LC-MS analytical approach has high selectivity and sensitivity in confirmation of identity in accordance to the mass-to-charge ratio, and has proven to be immensely useful in a wide range of fields such as environmental, food and new drug discovery (Brooke and Huggett, 2012; Gross, 2011; Korfmacher, 2005)

A limited number of putative phenolics were detected in bean samples using the developed methods by HPLC in the previous chapter. A further analysis with LC-MS is crucial to reconfirm the phenolic contents. Moreover, a validation of the result obtained from HPLC using LC-MS analysis is essential for future elucidation of the elemental composition and chemical structures of these phenolics. Hence, the aim for the current chapter was to profile the soluble, conjugated and bound phenolics in six underutilised beans by LC-MS adapting the method first developed for HPLC. The LC-MS method used was also optimised to enhance the detection efficiency of the tested samples.

On top of that, a validation procedure for soluble phenolics was undertaken using the final method. There are few, if any, reports in the scientific literature of fully validated LC-MS methods for the analysis of bean samples. This is because the task is complex and time consuming. The validation procedure imparts a way to confirm whether a developed method is suitable for practical application for the analysis of phenolics in bean samples. As a result, the validation was applied to pooled bean samples as preliminary data.

7.2 Methodology

7.2.1 Preparation of phenolic standard solutions

Phenolic standards were weighed into microcentrifuge tubes separately and dissolved in 100% methanol to make 1 mg/mL stock solutions. These solutions were stored at -20°C. For analytical purposes the mixture of stock solutions were prepared with water as diluent to a final 50% methanol and the final concentration as shown in table 7.1. A serial two fold dilution with 50% methanol was carried out to build a linearity curve within the concentration ranges shown in table 7.2.

Table 7.1 Concentration of stock solutions for phenolic standards.

	Compounds	Molecular Weight (MW)	Concentration (mM)
1	Gallic acid	170.12	11.76
2	Protocatechuic acid	154.12	12.98
3	Chlorogenic acid	354.31	5.64
4	Caffeic acid	180.16	11.10
5	Epicatechin	290.27	3.45
6	Epigallocatechin gallate	458.37	2.18
7	Hydroxybenzoic acid	138.12	14.48
8	ρ-Coumaric acid	164.16	12.18
9	Rutin	610.52	3.28
10	Epicatechin gallate	442.37	2.26
11	Ferulic acid	194.18	10.30
12	Sinapic acid	224.21	8.92
13	3-Hydroxycinnamic acid	164.16	12.18
14	Myricetin	318.24	3.14
15	Daidzein	254.24	3.93
16	Luteolin	286.24	3.49
17	Quercetin	302.24	3.31
18	Naringenin	272.25	7.35
19	Genestein	270.24	3.70
20	Kaempferol	286.24	3.49
21	Phloretin	274.27	3.49

Table 7.2 Concentration range for calibration curve and validation protocol.

Phenolic standards	Concentration range (µM)	Spiked concentration, µM		
		Low	Medium	High
Gallic acid	0.094 - 3.000	0.19	0.75	3.00
Protocatechuic acid	0.375 - 12.000	0.75	3.00	12.00
Chlorogenic acid	0.200 - 6.400	0.40	1.60	6.40
Caffeic acid	0.141 - 4.500	0.28	1.13	4.50
Epicatechin	0.063 - 2.000	0.13	0.25	1.00
Epigallocatechin gallate	0.188 – 6.000	0.38	1.50	6.00
3-Hydroxybenzoic acid	0.188 – 6.000	0.38	1.50	6.00
ρ-Coumaric acid	0.356 – 11.400	0.71	2.85	11.40
Rutin	0.375 – 12.000	0.75	3.00	12.00
Epicatechin gallate	0.055 – 1.750	0.11	0.44	1.75
Sinapic acid	0.125 – 4.000	0.25	1.00	4.00
Ferulic acid	0.547 – 17.500	1.10	4.38	17.50
3-Hydroxycinnamic acid	0.075 – 2.400	0.15	0.60	2.40
Myricetin	0.219 – 7.000	0.44	1.75	7.00
Daidzein	0.038 – 1.200	0.08	0.30	1.20
Luteolin	0.031 – 1.000	0.06	0.25	1.00
Quercetin	0.094 – 3.000	0.19	0.75	3.00
Genistein	0.038 – 1.200	0.08	0.30	1.20
Naringenin	0.009 – 0.300	0.02	0.08	0.30
Kaempferol	0.024 – 0.760	0.05	0.19	0.76
Phloretin (internal standard)	0.156 – 5.000	1.00	1.00	1.00

7.2.2 Soluble, deconjugated and bound phenolic sample preparation

Preparation of the soluble, deconjugated and bound phenolic samples from adzuki bean, black eyed pea, bambara groundnut, lablab bean, mung bean, pigeon pea and one commercial bean – soya bean were as shown in figure 6.1. The samples were prepared according to the developed methodologies (section 6.2.1, 6.2.2 and 6.2.3). A slight change to the protocol for soluble phenolic samples was that 1 g of bean powder was extracted with 9 mL of 80% methanol and spiked with 1 mL of phloretin (1 µM). Other methods remain the same as previously described. Dried samples (n=3) were kept at 4°C until ready to be used.

7.2.3 LC-MS procedure

Dried soluble and deconjugated phenolic samples were dissolved in 1 mL of 50% methanol and 3 mL of 50% methanol was used to dissolve bound phenolic samples. Mixtures were vortexed vigorously and filtered into HPLC vials. The samples were then analysed with a Thermo Scientific Accela U-HPLC system coupled with high resolution Exactive benchtop Orbitrap mass spectrometer. The setup was divided into the LC and MS separately.

For the LC setup, an Agilent Zorbax eclipse plus C18 (Rapid resolution HD, 100 mm x 2.1 mm, 1.8 µm) column and guard column C18 (2.1 mm x 5 mm, 1.8 µm) were used. Mobile phase A consisted of 0.01% formic acid in filtered water and mobile phase B consisted of HPLC grade acetonitrile with 0.01% formic acid. The samples were run using a gradient setting: starting with 10% B, increasing to 70% B within 20 min duration, increasing to 99% at 23 min and an organic solvent wash for 2 min was performed to remove the retained compounds in the column. Finally, the gradient was decreased to 10% B at 27 min and back to the equilibrium status of 10% B for 3 min. The flow rate was set at 250 µL/min and 5 µL of sample volume per injection. The system was setup such that initial flow out of samples from LC will be directed to waste for 2 min prior to mass spectrophotometer to minimize the contamination of salt to the MS system.

The MS setup had to be optimised according to the reference phenolic standards. The outcomes are presented in the results section.

7.2.4 Validation procedure for soluble phenolic samples

This experiment was carried out to validate the developed methodologies for identifying and quantifying the soluble phenolic compounds using LC-MS. Validation parameters include recovery and the matrix effect on extraction and MS response efficiency. 1 mL of internal standard phloretin (1 µM) was used throughout the validation procedure. All samples (n=6) were analysed by using the developed LC-MS setup (section 7.2.3). Varied types of sample preparation were involved in the validation procedure as shown in figure 7.1.

A mixture of phenolic standards (table 7.2) at different concentration ranges were prepared. A set of six calibration points obtained from two fold serial diluted mixtures of phenolic standards were prepared on the same day as the analysis. Peak areas for each phenolic standard were plotted against concentration to give calibration graphs, a value for linearity could then be determined. Next, 2^{nd}, 4^{th} and 6^{th} concentration level from low to high along the calibration curve were selected and labeled as low, medium and high concentrations (table 7.2) for the validation experiments (figure 7.1).

A solvent blank (80% methanol) was 'spiked' separately with low, medium and high concentrations of mixed phenolic standards along the calibration graphs (table 7.2). The solvent blank 'spiked' samples (n=6) were extracted (section 6.2.1) and analysed by LC-MS and named as sample A. A solvent blank sample (E) without 'spike' was prepared at the same time as a negative control. Recovery values, as percentage for each phenolics standard, were calculated as concentration obtained from the 'spiked' sample divided by actual 'spiked' concentration multiplied by 100.

Three types of sample extract were involved in investigating the matrix effect on the recovery, extraction and MS response efficiency (figure 7.1). All samples were extracted according to the optimised soluble phenolic extraction procedure (section 6.2.1). Firstly, bean powders were pooled at an equal ratio

of weight and extracted without 'spiked' with phenolic standards, sample extract was named as sample B. Next, another set of pooled bean powders were spiked separately with low, medium and high concentrations of mixed phenolic standards before extraction and was named as sample C. Third sample was named as sample D, whereby the pooled bean samples were extracted first, centrifuged and filtered followed by spiking separately with low, medium and high concentrations of mixed phenolic standards prior to the drying process. All dried extracts were injected to LC-MS for further analysis (section 7.2.3).

Recovery value (as a percentage) for phenolic standards extracted from pooled beans was defined as the difference in concentration obtained from sample C and B divided by actual spiked concentration, multiplied by 100. Matrix effect on extraction efficiency was determined as the ratio of peak area for each individual phenolic from sample C to sample B. If the ratio >1, matrix has enhanced the extraction efficiency whilst a ratio < 1 suggests the matrix has suppressed the extraction efficiency. Another approach of the analysis was to determine the matrix effect on MS response. The ionization effect is either enhanced or suppressed. This was calculated as the variation of peak area for individual phenolics for sample D and B divided by peak area for the same phenolic from sample A. If the ratio >1, matrix has enhanced the ionization efficiency whilst a ratio < 1 represents the ionization has been suppressed.

7.2.5 Statistical analysis

All investigations for section 7.2.4 were carried out in six replicates. The Student's t test was used to compare recovery of individual phenolic standards with and without matrix. Statistical significance was declared as $p<0.05$.

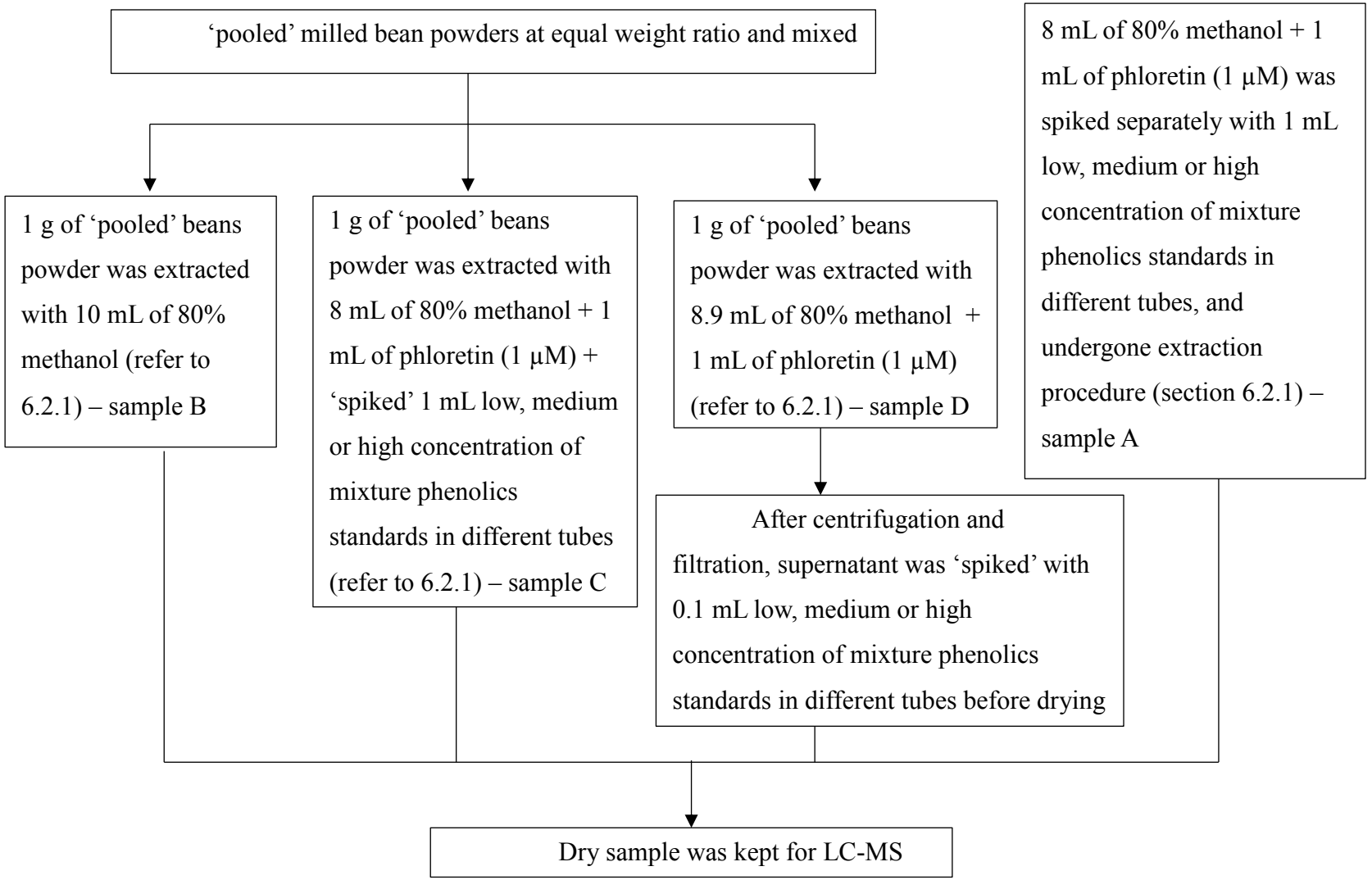


Figure 7.1 Flowchart of sample preparation for soluble phenolic sample method validation. A sample consisting of blank solvent only was used as negative control.

7.3 Results

7.3.1 Method development for identification and quantification of soluble, conjugated and bound phenolics using LC-MS

Table 7.3 shows the optimised MS parameters for electrospray ionisation in positive mode (ESI+) and negative mode (ESI-) when analysing by direct infusion of individual phenolic standards. The elution times and ionization intensities in both ESI+ and ESI- were also investigated for individual standards (1 mg/mL) as shown in tables 7.4 and 7.5. Interpretation of these results showed that positive ionization intensity ranged between 10^2 and 10^5 whilst negative ionization intensity ranged between 10^4 and 10^6. Therefore, negative ionization mode was more suitable than positive mode since it gave higher ionisation intensity. Hence, electrospray ionization in negative mode was selected for further study. Tables 7.4 and 7.5 also showed that identification by mass to charge is selective even though the elution times can be as close as 0.04 min. This is because formula structure of individual phenolics defines the ratio mass to charge (*m/z*) characteristic and the retention time (appendices 7.1 to 7.5). This key factor makes LC-MS more sensitive and selective than HPLC. Formic acid was used to reduce the peak tailing (Prokudina *et al.*, 2012).

Table 7.3 Optimised Exactive Orbitrap MS parameters.

Parameters	Value and units	
	ESI -	ESI +
Mass scan range	70 – 1200	
Automatic gain control (AGC) target value	Balanced	
Resolution	Enhanced	
Heated Electrospray Ionization (HESI) Source condition :		
Sheath gas	40 au	
Auxiliary gas	20 au	
Sweep gas	5 au	
Spray voltage	4.0 kV	
Capillary temperature	250°C	
Heater temperature	250°C	
Capillary voltage	-32.5 V	25 V
Tube lens voltage	-100 V	90 V
Skimmer voltage	-24 V	16 V

au= arbitrary unit

ESI – represents electrospray ionization in negative mode

ESI + represents electrospray ionization in positive mode

Table 7.4 Ionization characterization for phenolic standards in electrospray ionisation in negative and positive modes.

Standards		Molecular Formula	Molecular Weight (g/mol)	*m/z*		Elution time, min	Ionization intensity	
				ESI-	ESI+		ESI-	ESI+
1	Gallic acid	$C_7H_6O_5$	170.120	169.013	171.029	1.61	6.56E+04	7.50E+02
2	Protocatechuic acid	$C_7H_6O_4$	154.120	153.018	155.034	2.45	9.81E+04	1.56E+03
3	Chlorogenic acid	$C_{16}H_{18}O_9$	354.309	353.087	355.102	3.11	2.56E+04	2.94E+03
4	Caffeic acid	$C_9H_8O_4$	180.157	179.033	181.050	4.22	4.69E+04	2.24E+03
5	Epicatechin	$C_{15}H_{14}O_6$	290.268	289.071	291.086	4.40	1.61E+05	1.56E+04
6	Epigallocatechin gallate	$C_{22}H_{18}O_{11}$	458.372	457.077	459.092	4.52	2.02E+04	1.99E+03
7	3-Hydroxybenzoic acid	$C_7H_6O_3$	138.121	137.023	139.039	4.56	7.14E+04	4.10E+04
8	ρ-Coumaric acid	$C_9H_8O_3$	164.158	163.039	165.055	5.68	5.16E+05	6.70E+04
9	Rutin	$C_{27}H_{20}O_{16}$	610.518	609.145	611.161	5.90	7.20E+05	6.96E+04
10	Epicatechin gallate	$C_{22}H_{18}O_{10}$	442.372	441.081	443.097	6.23	2.28E+04	1.50E+03

Table 7.5 Ionization characterization for phenolic standards in electrospray ionisation in negative and positive modes (Contd.)

Standards		Molecular Formula	Molecular Weight (g/mol)	*m/z*		Elution time, min	Ionization intensity	
				ESI-	ESI+		ESI-	ESI+
11	Sinapic acid	$C_{11}H_{12}O_5$	224.210	223.060	225.076	6.34	7.45E+04	1.36E+04
12	Ferulic acid	$C_{10}H_{10}O_4$	194.184	193.050	195.065	6.34	4.39E+05	1.20E+05
13	3-Hydroxycinnamic acid	$C_9H_8O_3$	164.158	163.039	165.055	6.67	5.16E+05	6.70E+04
14	Myricetin	$C_{15}H_{10}O_8$	318.235	317.029	319.045	7.93	1.61E+04	1.48E+03
15	Daidzein	$C_{15}H_{10}O_4$	254.238	253.049	255.065	9.04	4.86E+04	1.67E+04
16	Luteolin	$C_{15}H_{10}O_6$	286.236	285.039	287.055	9.66	3.09E+04	9.64E+03
17	Quercetin	$C_{15}H_{10}O_7$	302.236	301.034	303.050	9.77	5.62E+04	8.30E+03
18	Genistein	$C_{15}H_{10}O_5$	270.237	269.044	271.060	11.14	1.04E+04	1.81E+03
19	Naringenin	$C_{15}H_{12}O_5$	272.253	271.060	273.076	11.14	3.20E+04	4.99E+03
20	Kaempferol	$C_{15}H_{10}O_6$	286.236	285.039	287.055	11.46	5.38E+04	7.69E+03
21	Phloretin	$C_{15}H_{14}O_5$	274.268	273.076	275.091	11.10	1.86E+06	2.79E+05

7.3.2 Identification and quantification of soluble, conjugated and bound phenolics

The six underutilised beans, namely adzuki bean, black eyed pea, bambara groundnut, lablab bean, mung bean, pigeon pea and soya bean were extracted using optimised procedures as reported in previous chapters and analysed using the optimised MS setup. By comparison to the 20 known phenolic standards, a total of 13, 10 and 8 phenolics from soluble, deconjugated and bound phenolic samples have been identified and quantified, respectively (tables 7.6 to 7.8). A total of eight phenolics, namely gallic acid, protocatechuic acid, caffeic acid, ρ-coumaric acid, rutin, sinapic acid, ferulic acid and daidzein were found in all soluble, deconjugated and bound phenolic samples at different concentrations, ranging from 0.5 to 9100 µg/100 g DW bean powder (tables 7.6 to 7.8). Five out of eight phenolics were found in bound samples at their highest concentration, they were gallic acid, protocatechuic acid, ρ-coumaric acid, sinapic acid and ferulic acid.

Table 7.6 shows the 13 phenolic compounds identified from soluble phenolic extracts of the seven bean samples. Among all tested beans, only black eyed pea contained all 13 known phenolic compounds, while both soya bean and mung bean had the least number of known phenolic compounds (9 phenolics). The four phenolic compounds that were not detected in soya bean were chlorogenic acid, epicatechin, rutin and quercetin. Whereas, protocatechuic acid, chlorogenic acid, sinapic acid and quercetin were not found in mung bean.

When the soluble extracts from the beans tested were compared (table 7.6), adzuki bean was found to be high in quercetin and rutin. Bambara groundnut was high in gallic acid, protocatechuic acid and chlorogenic acid. Lablab bean was high in caffeic acid, ρ-coumaric acid and ferulic acid. Pigeon pea was high in epicatechin. Soya bean was high in sinapic acid, daidzein, geneistein and naringenin. While black-eyed pea and mung bean had lower phenolic content than the remaining beans. Black eyed pea has the greatest variety of soluble phenolics but lower content than the other beans.

Table 7.7 shows the 10 phenolic compounds identified from deconjugated phenolic extracts of the seven bean samples. Among all tested beans, only adzuki bean and soya bean contained all 10 known phenolic compounds, while pigeon pea had the least number of known phenolic compounds (8 phenolics). The two phenolic compounds that were not detected in pigeon pea were protocatechuic acid and caffeic acid.

When the deconjugated beans were compared (table 7.7), adzuki bean was found to be high in rutin, while black eyed pea was high in ferulic acid. Bambara groundnut was high in gallic acid and protocatechuic acid. Lablab bean was high in caffeic acid and ρ-coumaric acid. Soya bean was high in sinapic acid, daidzein, naringenin and genestein. Whereas mung bean and pigeon pea had lower phenolic contents than the remaining beans.

Table 7.8 shows the 8 phenolic compounds identified from bound phenolic extracts of the seven bean samples. Among all tested beans, only soya bean contained 7 of the 8 known phenolic compounds. All six tested beans contained gallic acid, protocatechuic acid, ρ-coumaric acid and ferulic acid. Only soya bean had caffeic acid and daidzein while only adzuki bean had rutin. When bound phenolic extracts from beans tested were compared (table 7.8), adzuki bean had the highest gallic acid and rutin. Black eyed pea was high in ferulic acid, bambara groundnut was high in protocatechuic acid and lablab bean was high in ρ-coumaric acid.

Analysis of deconjugated phenolics was used to estimate the type of free phenolics hydrolysed from conjugated compounds. A majority of the phenolics showed an increased in concentration after hydrolysis (tables 7.6 and 7.7). Protocatehuic acid in adzuki bean and rutin in soya bean exist only after hydrolysis. This reveals that protocatechuic acid (adzuki bean) and ruitn (soya bean) exist in esterified form only but not in free form.

Table 7.9 revealed the possibility of the phenolics existing in esterified forms by calculating the increase of concentration in percentage for each phenolic

from the soluble phenolics extract to that after hydrolysis. Referring to the percentage increase, high amounts of ρ-coumaric acid, sinapic acid, daidzein and naringenin in bambara groundnut appear to exist mostly in the esterified or conjugated form. They showed the highest increase in concentration after hydrolysis. Pigeon pea has the highest esterifed rutin and ferulic acid while esterified caffeic acid was highest in mung bean.

However, chlorogenic acid, epicatechin and quercetin were not found in deconjugated samples. Most likely chlorogenic acid had been fully hydrolysed to caffeic acid and quinic acid even after a 5 min hydrolysis treatment. Increased concentration of caffeic acid has been observed in black eyed pea and lablab bean extracts. Others were degraded during hydrolysis.
The increased concentration for selected phenolics in deconjugated extracts indicated that these originate from conjugated phenolics that consist of this free phenolic. Therefore, the concentration increased after hydrolysis. In contrast, decreased concentration may suggest that free phenolics are being degraded during hydrolysis.

When comparing known phenolics detected from soluble and bound phenolic bean extracts (tables 7.6 and 7.8), these 5 phenolic compounds, gallic acid, protocatechuic acid, ρ-coumaric acid, sinapic acid and ferulic acid were higher in bound phenolic extracts than in soluble phenolic extracts. Whereas the remaining phenolic compounds, chlorogenic acid, caffeic acid, epicatechin, rutin, daidzein, naringenin, genistein and quercetin were higher in soluble phenolic extracts.

Table 7.6 Identified and quantified phenolic compounds from soluble phenolic samples detected using LC-MS.

Phenolic compounds		Concentration, µg/100g DW bean powder						
		Adzuki bean	Black eyed pea	Bambara groundnut	Lablab bean	Mung bean	Pigeon pea	Soya bean
1	Gallic acid	26.5 ± 2.1	5.2 ± 0.6	30.3 ± 2.7	3.6 ± 0.6	3.6 ± 0.5	8.6 ± 0.7	5.4 ± 0.8
2	Protocatechuic acid	nd	109.8 ± 7.8	658.4 ± 25.4	24.0 ± 3.0	nd	49.5 ± 4.1	276.2 ± 32.2
3	Chlorogenic acid	nd	11.4 ± 1.1	16.2 ± 1.5	14.7 ± 1.9	nd	nd	nd
4	Caffeic acid	10.0 ± 1.1	16.7 ± 0.5	3.6 ± 0.2	21.1 ± 1.0	7.0 ± 1.0	nd	14.9 ± 1.8
5	Epicatechin	30.4 ± 2.3	51.9 ± 4.0	33.2 ± 3.0	nd	4.7 ± 0.3	73.4 ± 9.1	nd
6	ρ-Coumaric acid	387.0 ± 9.3	374.1 ± 21.8	60.5 ± 3.9	1600.0 ± 0.1	43.7 ± 1.7	14.9 ± 0.7	745.2 ± 26.7
7	Rutin	3500.0 ± 0.3	105.6 ± 10.5	nd	10.3 ± 1.2	68.3 ± 5.2	9.3 ± 1.2	nd
8	Sinapic acid	77.7 ± 2.0	35.5 ± 3.5	38.8 ± 2.0	41.2 ± 1.3	nd	10.0 ± 0.9	144.3 ± 22.0
9	Ferulic acid	64.2 ± 1.0	722.1 ± 22.7	54.6 ± 3.4	1100.0 ± 0.0	21.9 ± 1.1	8.7 ± 0.8	320.4 ± 17.2
10	Daidzein	16.1 ± 2.1	16.2 ± 1.1	36.2 ± 4.3	2.5 ± 0.4	1.7 ± 0.1	1.5 ± 0.3	9100.0 ± 0.6
11	Naringenin	7.6 ± 0.4	5.2 ± 0.1	11.3 ± 1.3	nd	12.5 ± 0.4	5.0 ± 0.3	488.2 ± 34.7
12	Genistein	5.2 ± 0.7	14.4 ± 1.4	17.9 ± 2.3	1.3 ± 0.1	1.4 ± 0.2	23.4 ± 2.2	10500.0 ± 0.6
13	Quercetin	43.1 ± 7.1	19.7 ± 3.1	nd	nd	nd	nd	nd

Notes: nd – not detected; Epicatechin gallate, hydroxycinnamic acid, epigallocatechin gallate, myricetin, luteolin, kaempferol and hydroxybenzoic acid were not detected across all tested beans

Table 7.7 Identified and quantified phenolic compounds from deconjugated phenolic samples detected using LC-MS.

Phenolic compounds		Concentration, µg/100g DW bean powder						
		Adzuki bean	Black eyed pea	Bambara groundnut	Lablab bean	Mung bean	Pigeon pea	Soya bean
1	Gallic acid	9.8 ± 1.7	6.4 ± 1.8	22.9 ± 7.3	2.4 ± 0.6	2.6 ± 0.2	11.1 ± 1.4	6.8 ± 2.5
2	Protocatechuic acid	115.0 ± 7.8	115.1 ± 11.3	585.3 ± 88.1	18.4 ± 2.9	nd	nd	80.8 ± 17.3
3	Caffeic acid	2.1 ± 0.3	25.0 ± 4.4	3.1 ± 0.8	24.9 ± 0.6	15.8 ±1.0	nd	10.8 ±0.2
4	ρ-Coumaric acid	898.6 ± 71.1	731.8 ± 24.5	228.7 ± 5.9	2400.0 ± 0.2	190.2 ± 27.5	55.3 ±10.9	1500.0 ± 0.2
5	Rutin	5100.0 ± 0.1	nd	nd	52.7 ± 8.0	232.9 ± 5.2	63.0 ± 5.9	274.6 ±10.3
6	Ferulic acid	90.6 ± 7.4	1200.0 ± 0.1	157.9 ±6.1	1100.0 ± 0.0	40.8 ± 2.1	25.4 ± 7.4	408.3 ±98.0
7	Sinapic acid	88.6 ± 5.9	61.9 ± 1.5	79.0 ± 1.4	29.5 ± 7.1	1.2 ± 0.2	19.6 ± 1.6	223.7 ± 51.2
8	Daidzein	7.8 ± 1.0	11.2 ± 0.3	72.4 ± 4.5	1.0 ± 0.0	1.3 ± 0.1	0.5 ± 0.2	1600.0 ±0.2
9	Naringenin	3.4 ± 0.6	4.2 ± 0.1	17.9 ± 1.3	nd	14.9 ±0.4	4.5 ± 0.3	83.7 ± 13.5
10	Genistein	1.0 ± 0.4	5.5 ± 0.1	24.5 ± 2.3	0.3 ± 0.0	1.2 ± 0.1	15.2 ± 1.1	1100.0 ± 0.1

Notes: nd – not detected; Epicatechin gallate, hydroxycinnamic acid, epigallocatechin gallate, myricetin, luteolin, kaempferol and hydroxybenzoic acid were not detected across all tested beans

Table 7.8 Identified and quantified phenolic compounds from bound phenolic samples detected using LC-MS.

Phenolic compounds		Concentration, µg / 100g DW bean						
		Adzuki bean	Black eyed pea	Bambara groundnut	Lablab bean	Mung bean	Pigeon pea	Soya bean
1	Gallic acid	40.1 ± 13.2	17.0 ± 0.8	38.9 ± 20.0	13.8 ± 1.5	13.6 ± 2.3	7.0 ± 2.0	6.2 ± 1.2
2	Protocatechuic acid	900.0 ± 0.2	122.9 ± 47.0	1500.0 ± 0.1	4.5 ± 1.0	132.6 ± 10.1	7.0 ± 1.1	8.9 ± 2.2
3	Caffeic acid	nd	nd	nd	nd	nd	nd	10.0 ± 1.7
4	ρ-Coumaric acid	3900.0 ± 0.8	1300.0 ± 0.1	400.0 ± 0.1	6000.0 ± 1.3	700.0 ± 0.1	55.6 ± 5.9	2000.0 ± 0.1
5	Rutin	1500.0 ± 2.0	nd	nd	nd	nd	nd	nd
6	Sinapic acid	nd	nd	nd	nd	nd	14.3 ± 1.3	700.0 ± 0.1
7	Ferulic acid	101.0 ± 6.5	1700.0 ± 0.3	300.0 ± 0.1	400.0 ± 0.0	42.5 ± 1.6	20.7 ± 5.5	1300.0 ± 0.0
8	Daidzein	nd	nd	nd	nd	nd	nd	72.2 ± 24.4

Notes: nd – not detected; Epicatechin gallate, hydroxycinnamic acid, epigallocatechin gallate, myricetin, luteolin, kaempferol and hydroxybenzoic acid were not detected across all tested beans

Table 7.9 Increase of phenolics concentration (%) after alkaline hydrolysis.

Phenolics	Increase in concentration, x % after hydrolysis		
	< 100%	100% < x < 200%	> 200%
Gallic acid	Black eyed pea (19%) Pigeon pea (29%) Soya bean (26%)		
Caffeic acid	Black eyed pea (50%) Lablab bean (18%)	Mung bean (125%)	nr
ρ-coumaric acid	Black eyed pea (95%) Lablab bean (50%)	Adzuki bean (132%) Soya bean (100%)	Bambara groundnut (278%) Mung bean (235%) Pigeon pea (271%)
Rutin	Adzuki bean (46%)	nr	Lablab bean (411%) Mung bean (241%) Pigeon pea (577%)
Ferulic acid	Adzuki bean (41%) Black eyed pea (66%) Mung bean (86%) Soya bean (27%)	Bambara groundnut (189%)	Pigeon pea (200%)
Sinapic acid	Adzuki bean (14%) Black eyed pea (74%) Pigeon pea (96%) Soya bean (55%)	Bambara groundnut (104%)	nr
Daidzein	nr	Bambara groundnut (100%)	nr
Naringenin	Bambara groundnut (58%)	nr	nr

*protocatechuic acid for adzuki bean and rutin for soya bean exist after hydrolysis

**nr- not relevant

7.3.3 Validation of extraction procedure for soluble phenolics using LC-MS

Validation of the optimised extraction method targeting soluble phenolics using LC-MS has been carried out. This included the linearity, recovery, precision, limit of detection (LOD) and limit of quantification (LOQ) of each individual phenolic and investigation of matrix effect towards extraction efficiency and ionization effect. This was carried out on pooled bean samples (figure 7.1) instead of one single bean in order to obtain an overview of the methodology prior to a potentially more in depth study of each bean in the future.

A total of 7 concentrations were used in building the linear curve in accordance to the recommendation of a minimum of 5 concentrations (ICH, 2005). The linearity results, as shown in tables 7.10 to 7.12, indicated the response of the MS for the studied concentrations were on the whole proportional to the concentration of the analyte in the samples with only a few exceptions when the phenolic concentration in the samples were extremely low. Values for the linearity (coefficient of regression, r^2) of the MS response to the panel of phenolic standards in the blank solvent (no matrix) were all above 0.99. It followed the criteria of linearity, $r^2 > 0.99$ (ICH, 2005). However, there are a few exceptional cases such as epigallocatechin gallate, epicatechin gallate, luteolin, myricetin, quercetin, kaempferol, where the r^2 for the said phenolics are generally $0.90 < r^2 < 0.98$.

Recovery at three concentrations (low, medium and high) represents the accuracy of the experimental value to true value. Relative standard deviation (RSD- also known as coefficient of variation) represents the precision of repeatability of six repetitions under the same analytical conditions within a day (intraday) in the same laboratory and equipment.

A total of 16 out of the 20 phenolic standards were successfully studied for their recovery in blank solvent, the exceptions being luteolin, myricetin, quercetin and kaempferol (tables 7.10 to 7.12). The recovery values showed a high level of variation at different concentrations. The value varied between

2.7% < x < 82%, 25% < x < 115%, 26% < x < 99% among the phenolic compounds at low, medium and high spiked concentration ranges, respectively. The majority of the phenolics such as hydroxybenzoic acid, ρ-coumaric acid, rutin, hydroxycinnamic acid and daidzein showed a closely related value of recovery at low, medium and high concentration within the same sample. Whilst, phenolics such as gallic acid, caffeic acid and sinapic acid had different values of recovery at the low concentration (> 50%) compared with higher concentrations.

The recovery of phenolics from actual beans samples showed changes of recovery value from non-matrix to matrix conditions (tables 7.10 to 7.12). It is expected that sample matrix will influence the recovery value. Statistical analysis revealed that significant changes of recovery value at $p < 0.05$ due to matrix were found except in five cases. There were no significant changes of recovery value due to matrix from 'spiked' low concentration of rutin, 'spiked' medium concentration of protocatechuic acid, rutin, epigallocatechin gallate and 'spiked' high concentration of naringenin.

Although there were significant changes of recovery value, there was no definite trend in the value of recovery extracted from blank solvent (no matrix) to pooled beans (matrix) neither among the concentrations nor the bean samples. Some of the significant changes were that the recovery value of ρ-coumaric acid decreased from 94.8% (no matrix) to 83.6%, (matrix), 93.5% (no matrix) to 48.0% (matrix) for protocatechuic acid and 58.9% (no matrix) to 19.7% (matrix) for rutin at high concentration levels. The changes of recovery value extracted from blank solvent and beans when spiked at low and medium concentrations were as low as 63.7% to 62.2% for rutin to 62.1 % to 114.8 % for daidzein.

The validation procedure also took into account the effect of sample matrix on extraction and MS response efficiency during analysis. The enhancing or suppressing effect is related to the recovery value of phenolics extracted from pooled beans samples at different spiked concentration level. In general, results

in tables 7.10 to 7.12 showed that matrix affected the recovery value for all phenolic compounds at all tested concentrations.

There was a total of 15 responses showing decreased recovery value at 'spiked' medium concentration such as from gallic acid, chlorogenic acid, hydroxybenzoic acid and 'spiked' high concentration such as from epicatechin, caffeic acid, epigallocatechin gallate, hydroxybenzoic acid, ρ-coumaric acid, rutin, sinapic acid, hydroxycinnamic acid and naringenin showed suppression of MS response only due to matrix. Whilst, only 7 responses showed a reduction in recovery value due to the suppression in both extraction efficiency and MS response.

A total of 10 responses showed that increase of recovery value was due to matrix enhancement of the extraction efficiency only. These can be seen from 'spiked' low concentrations of gallic acid, protocatechuic acid, epicatechin, hydroxybenzoic acid, ρ-coumaric acid, rutin and sinapic acid and medium concentrations of epicatechin, ρ-coumaric acid and sinapic acid. Whilst, another 4 cases showed that increased recovery value was due to matrix enhanced MS response only such as from epigallocatechin gallate, luteolin and quercetin.

In table 7.13, the LODs and LOQs were tabulated. LOD is the lowest concentration of analyte in a sample that can be detected but not necessarily quantified. Whilst, LOQ is the lowest concentration of analyte to be quantified. The LODs and LOQs for each phenolic compound were obtained by the analysis of standard solutions at known concentrations of analyte. Accepted signal to noise ratios were as suggested as 3:1 for estimation of the LODs and 10:1 for the LOQs (ICH, 2005). LODs ranged from 0.001 to 0.036 µM and LOQs ranged from 0.007 to 0.120 µM. All identified and quantified phenolics in section 7.3.2 were higher than the LODs and LOQs.

Table 7.10 Validation parameters for the analysis of phenolic standards when spiked into solvent blank and 'pooled' bean samples.

Compounds	Conc, µM	Linearity equation	R^2	'Spiked' blank solvent n=6	'Spiked' pooled beans samples, n=6				
				Recovery,% (RSD)	Recovery (%)	RSD (%)	SD	Extraction efficiency	Ionization efficiency
Gallic acid	0.19	$y = 747{,}466x$	0.9966	17.5 (14.1)	47.0	19.2	9.0	≥1 (1.5)	<1 (-1.1)
	0.75			115.0 (6.3)	30.7	8.2	2.5	≥1 (1.0)	<1 (0.7)
	3.00			72.1 (3.6)	10.2	4.9	0.5	<1 (0.9)	<1 (0.1)
Protocatechuic acid	0.75	$y = 1{,}072{,}500x$	0.9989	76.1 (10.2)	114.1	12.6	14.4	≥1 (1.2)	<1 (-1.0)
	3.0			99.0 (2.5)	113.3	16.0	18.1	≥1 (1.0)	<1 (0.3)
	12.0			93.5 (6.0)	48.0	6.7	3.2	<1 (0.9)	<1 (0.4)
Chlorogenic acid	0.40	$y = 1{,}084{,}637x$	0.9992	52.5 (15.4)	nc	nc	nc	nc	nc
	1.60			112.7 (4.5)	31.1	7.8	2.4	≥1 (1.0)	<1 (0.5)
	6.40			91.2 (5.1)	27.1	1.2	0.3	<1 (0.9)	<1 (0.3)
Caffeic acid	0.28	$y = 2{,}214{,}253x$	0.9992	6.3 (18.0)	51.0	11.9	6.1	≥1 (1.3)	≥1 (4.3)
	1.13			89.4 (1.9)	67.6	2.2	1.5	<1 (0.9)	<1 (0.6)
	4.50			80.1 (2.7)	59.4	1.7	1.0	≥1 (1.0)	<1 (0.8)
Epicatechin	0.13	$y = 2{,}084{,}870x$	0.9988	29.2 (17.2)	82.5	16.3	13.4	≥1 (1.3)	<1 (-4.1)
	0.25			75.7 (17.9)	89.0	3.7	3.3	≥1 (1.1)	<1 (0.4)
	1.0			62.2 (10.5)	32.2	8.1	2.6	≥1 (1.0)	<1 (0.3)
Epigallocatechin gallate	0.38	$y = 962{,}575x$	0.9784	2.7 (18.0)	20.6	11.6	2.4	<1 (0.6)	≥1 (11.1)
	1.50			25.2 (19.2)	21.8	16.3	3.6	<1 (0.7)	<1 (0.9)
	6.0			26.0 (12.0)	18.4	12.7	2.3	≥1 (1.0)	<1 (0.3)
Hydroxybenzoic acid	0.38	$y = 1{,}246{,}102x$	0.9969	81.6 (2.7)	35.3	11.0	3.9	≥1 (1.3)	<1 (0.3)
	1.50			94.6 (1.1)	56.3	6.3	3.5	≥1 (1.0)	<1 (0.3)
	6.0			95.7 (5.8)	50.4	3.5	1.8	≥1 (1.0)	<1 (0.4)

Note: nc – not countable

Table 7.11 Validation parameters for the analysis of phenolic standards when spiked into solvent blank and 'pooled' bean samples. (contd).

Compounds	Conc, µM	Linearity equation	R^2	'Spiked' blank solvent n=6	'Spiked' pooled beans samples, n=6				
				Recovery,% (RSD)	Recovery (%)	RSD (%)	SD	Extraction efficiency	Ionization efficiency
ρ-Coumaric acid	0.71	$y = 2{,}053{,}503x$	0.9996	82.4 (2.8)	191.9	12.7	24.3	≥1 (1.4)	<1 (-2.0)
	2.85			89.9 (1.3)	186.1	4.7	8.8	≥1 (1.1)	<1 (0.5)
	11.4			94.8 (3.0)	83.6	4.6	3.8	≥1 (1.0)	<1 (0.6)
Rutin	0.75	$y = 2{,}896{,}919x$	0.9947	60.4 (7.1)	67.2	9.4	6.3	≥1 (1.2)	<1 (0.3)
	3.0			63.7 (3.5)	62.2	5.4	3.3	≥1 (1.0)	<1 (0.4)
	12.0			58.9 (9.1)	19.7	6.5	1.3	≥1 (1.0)	<1 (0.3)
Epicatechin gallate	0.11	$y = 1{,}365{,}578x$	0.9803	33.3 (10.0)	nc	nc	nc	nc	nc
	0.44			52.3 (21.3)	14.6	19.7	2.9	<1 (0.9)	<1 (0.3)
	1.75			35.0 (14.7)	16.0	7.9	1.3	<1 (0.9)	<1 (0.5)
Ferulic acid	1.10	$y = 783{,}265x$	0.9991	134.7 (9.6)	nc	nc	nc	nc	nc
	4.38			163.1 (0.8)	206.0	5.6	11.6	≥1 (1.0)	<1 (0.5)
	17.5			160.4 (4.5)	103.3	3.8	3.9	≥1 (1.0)	<1 (0.5)
Sinapic acid	0.25	$y = 1{,}739{,}936x$	0.9966	7.2 (14.4)	68.5	11.4	7.8	≥1 (1.3)	<1 (-4.1)
	1.0			66.4 (3.5)	85.5	6.7	5.7	≥1 (1.1)	<1 (0.5)
	4.0			75.5 (5.9)	40.3	4.9	2.0	≥1 (1.0)	<1 (0.4)
Hydroxycinnamic acid	0.15	$y = 2{,}306{,}972x$	0.9992	80.4 (1.9)	49.7	5.5	2.8	≥1 (1.3)	<1 (0.4)
	0.60			91.9 (1.5)	63.4	6.4	4.1	≥1 (1.0)	<1 (0.5)
	2.40			87.4 (5.2)	49.9	6.8	3.4	≥1 (1.0)	<1 (0.6)
Daidzein	0.08	$y = 8{,}636{,}817x$	0.9995	56.6 (3.3)	nc	nc	nc	nc	nc
	0.30			64.3 (3.7)	nc	nc	nc	nc	nc
	1.20			62.1 (6.9)	114.8	7.9	9.1	≥1 (1.0)	<1 (-2.0)

Note: nc – not countable

Table 7.12 Validation parameters for the analysis of phenolic standards when spiked into solvent blank and 'pooled' bean samples. (contd).

Compounds	Concent-ration, µM	Linearity equation	R^2	'Spiked' blank solvent n=6	'Spiked' pooled beans samples, n=6				
				Recovery,% (RSD)	Recovery (%)	RSD (%)	SD	Extraction efficiency	Ionization efficiency
Naringenin	0.02	$y = 5{,}232{,}869x$	0.9967	40.1 (24.1)	nc	nc	nc	nc	nc
	0.08			66.4 (10.8)	nc	nc	nc	nc	nc
	0.30			63.9 (7.2)	59.4	16.1	9.6	≥1 (1.0)	<1 (-0.1)
Genestein	0.08	$y = 4{,}399{,}689x$	0.9920	38.6 (14.7)	nc	nc	nc	nc	nc
	0.30			51.5 (10.3)	nc	nc	nc	nc	nc
	1.20			49.0 (9.3)	nc	nc	nc	nc	nc
Luteolin	0.06	$y = 509{,}677x$	0.9416	nc	nc	nc	nc	nc	nc
	0.25			nc	nc	nc	nc	nc	nc
	1.0			4.8 (11.7)	25.8	16.7	4.3	<1 (0.8)	≥1 (10.5)
Myricetin	0.44	$y = 122{,}419x$	0.9441	nc	nc	nc	nc	nc	nc
	1.75			nc	nc	nc	nc	nc	nc
	7.0			nc	nc	nc	nc	nc	nc
Quercetin	0.19	$y = 393{,}601x$	0.9618	nc	nc	nc	nc	nc	nc
	0.75			0.2 (28.2)	8.1	12.9	1.1	<1 (0.5)	nc
	3.0			2.4 (18.3)	17.9	19.5	3.5	<1 (0.5)	≥1 (11.2)
Kaempferol	0.05	$y = 438{,}832x$	0.9701	nc	nc	nc	nc	nc	nc
	0.19			nc	nc	nc	nc	nc	nc
	0.76			nc	nc	nc	nc	nc	nc

Note: nc- not countable

Table 7.13 Tabulation of concentration for limit of detection (LOD) and limit of quantification (LOQ).

Compounds	LOD (μM)	LOQ (μM)
Gallic acid	0.010	0.033
Protocatehuic acid	0.010	0.033
Chlorogenic acid	0.025	0.082
Caffeic acid	0.005	0.015
Epicatechin	0.002	0.007
Epigallocatechin gallate	0.036	0.120
Hydroxybenzoic acid	0.006	0.019
ρ-coumaric acid	0.004	0.012
Rutin	0.003	0.011
Epicatechin gallate	0.011	0.036
Ferulic acid	0.006	0.021
Sinapic acid	0.004	0.012
Hydroxycinnamic acid	0.003	0.011
Daidzein	0.001	0.003
Naringenin	0.001	0.004
Genestein	0.002	0.007
Luteolin	nc	nc
Myricetin	nc	nc
Quercetin	nc	nc
Kaempferol	nc	nc

7.3.4 Analysis of LC-MS profile from selected beans

In the bean extracts, only a minority of the peaks observed in the LC-MS profiles could be matched to the respective phenolic standards (tables 7.6 to 7.8). The majority of the peaks could not be identified using the current set of standards. This implies that the remaining peaks are either phenolics for which standards were not applied, or represent other types of compounds. A further analysis of these unidentified peaks, in terms of their variation between beans was carried out. These ions may or may not be phenolic compounds. The *m/z* species that showed a relative abundance area > 100,000 ppm have been shortlisted for further comparison. These ions were further categorized according to their relative abundance 100,000 < x < 500,000 ppm, 500,000 < x < 1,000,000 ppm and x> 1,000,000 ppm. Ions with relative abundance lower than 100,000 ppm were not shortlisted for comparison.

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7.3.4.1 Profiling the soluble phenolic extracts by LC-MS

The total number of unidentified ions and ions that were unique to respective soluble phenolic bean extracts are shown in table 7.14. This varied from 215 ions in soya bean, of which 139 were unique to 91 ions in mung bean, of which 36 were unique.

Table 7.15 shows the tabulation of ions which were commonly found in the soluble bean extracts. The highest commonality was observed between soya bean and pigeon pea, black eyed pea and bambara groundnut. Both pairs shared 11 ions, respectively. Whereas no common ions were found between pigeon pea and adzuki bean, black eyed pea or bambara groundnut.

A total of 583 ions with relative abundance > 100,000 ppm were profiled across all the soluble phenolic bean extracts examined. The relative abundance, retention time and individual ion (*m/z*) are summarised in appendices 7.6 to 7.29. The appendices also revealed the ions that uniquely found in specific beans. Only 15 ions were found in all soluble phenolics bean extracts (table 7.16).

Table 7.14 Total number of unidentified and unique ions in soluble phenolic bean extracts.

	Adzuki bean	Black eyed pea	Bambara groundnut	Lablab bean	Mung bean	Pigeon pea	Soya bean
Total number of ion (*m/z*) detected	117	110	123	118	91	127	215
Number of unique ion(*m/z*)	50	37	52	51	36	74	139

Table 7.15 Total number of ions which are common between soluble phenolic bean extracts.

	Adzuki bean	Black eyed pea	Bambara groundnut	Lablab bean	Mung bean	Pigeon pea	Soya bean
Adzuki bean	-	4	10	2	3	-	5
Black eyed pea	-	-	11	2	3	-	6
Bambara groundnut	-	-	-	1	2	-	5
Lablab bean	-	-	-	-	2	6	3
Mung bean	-	-	-	-	-	3	2
Pigeon pea	-	-	-	-	-	-	11

Table 7.16 List of *m/z* that found in all tested beans either in soluble phenolics or bound phenolics beans extracts.

Retention time, min	*m/z*	Soluble phenolics beans extracts	Bound phenolics beans extracts
1.15	562.588	-	+
1.17	94.925	-	+
	266.804	-	+
	560.591	-	+
	270.798	-	+
	268.801	-	+
	92.928	-	+
1.48	191.019	-	+
	111.009	-	+
7.35	713.473	+	+
7.37	723.502	+	+
11.81	329.233	+	-
15.69	311.222	+	-
17.85	476.277	+	+
18.44	595.288	+	-
18.62	564.330	+	-
19.03	452.278	+	-
19.49	571.288	+	-
20.41	478.293	+	-
20.43	311.168	+	+
20.48	295.227	+	-
22.43	325.184	+	+
24.49	339.199	+	+
25.42	112.985	-	+
26.47	106.401	+	+

‘+’ represents detected in all seven beans extracts

‘-‘ represents not detected in all seven beans extracts

7.3.4.2 Profiling the bound phenolic extracts by LC-MS

A total of 160 ions with integrated relative abundance areas (> 100,000 ppm) were identified across all the beans extracts. The ion (*m/z*), retention time and relative abundance of individual ions are summarised in appendices 7.30 to 7.36. Altogether 17 out of 160 ions were found across all beans (table 7.14). The total number of ion (in the bound sample from each bean) is shown in table 7.17 along with the number of ion unique to the individual beans. This varied from 57 ion in soya bean, of which 32 ion were unique, to 32 ion in black eyed pea, of which 2 were unique.

Table 7.18 shows the tabulation of ions which are commonly found in the bound phenolics bean extracts. The highest commonality was observed between lablab bean and mung bean. Both pairs shared 4 ions, respectively. Whereas the lowest commonality was observed between adzuki bean and mung bean, black eyed pea and soya bean. They shared 1 ion respectively.

In general, soluble extracts showed 423 ions more than bound extracts and only a limited number of ions (64 ions) were found in both soluble and bound samples. The ion can be found in soluble and bound sample of the same bean only, different beans or all beans. A total of seven ions were found in both soluble and bound samples for all beans tested (table 7.16).

Table 7.17 Total number of unidentified and unique ions in bound phenolic bean extracts.

	Adzuki bean	Black eyed pea	Bambara groundnut	Lablab bean	Mung bean	Pigeon pea	Soya bean
Total number of ion m/z detected	45	32	35	48	53	50	57
Number of unique ion m/z	17	2	5	18	13	20	32

Table 7.18 Total number of ions which are common between bound phenolic bean extracts.

	Adzuki bean	Black eyed pea	Bambara groundnut	Lablab bean	Mung bean	Pigeon pea	Soya bean
Adzuki bean	-	2	-	-	1	-	1
Black eyed pea	-	-	2	-	-	-	1
Bambara groundnut	-	-	-	-	-	-	-
Lablab bean	-	-	-	-	4	-	-
Mung bean	-	-	-	-	-	2	-
Pigeon pea	-	-	-	-	-	-	2

7.4 Discussion

7.4.1 Profiling of soluble, conjugated and bound phenolics from underutilised beans using LC-MS

LC-MS results showed that a total of 6 flavonoids and 7 phenolic acids have been identified from soluble, deconjugated and bound phenolic extracts of beans at different concentrations. A total of eight phenolics, namely gallic acid, protocatechuic acid, caffeic acid, ρ-coumaric acid, rutin, sinapic acid, ferulic acid and daidzein were found in soluble, deconjugated and bound phenolic samples from at least one bean but at different concentrations. Naringenin and genistein were found in both soluble and deconjugated phenolics of bean extracts. Whilst, quercetin, chlorogenic acid and epicatechin were only found as soluble phenolics in bean extracts.

Five out of eight of these phenolics were found across all soluble, deconjugated and bound phenolic samples but were at their highest concentration in bound samples, they were gallic acid, protocatechuic acid, ρ-coumaric acid, sinapic acid and ferulic acid. This positive finding of higher concentrations in bound than soluble phenolic extracts is similar to that reported for white rice. White rice has been reported to contain higher levels of protocatehuic acid (0.17 mg/100g flour), ferulic acid (5.26 mg/100g flour) and ρ-coumaric acid (0.34 mg/100g flour) as bound phenolics than as soluble phenolics (Tian *et al.*, 2004). Whilst, daidzein, caffeic acid and rutin have higher levels in soluble bean samples.

Ranking of the beans in terms of which have the most diversified soluble phenolics were black eyed pea> adzuki bean = bambara groundnut > lablab bean = pigeon pea > mung bean =soya bean. From here we can see that underutilised beans appear to exhibit more diversification of their phenolics than soya bean. Hence, consumption of underutilised beans may be recommended because the greater mixture of phytochemicals may provide better protective health benefits than single compounds through additive or synergistic effects (Eberhardt *et al.*, 2000).

Apart from that, underutilised beans appear to be better than soya bean in that they may have a higher concentration of soluble phenolics. Adzuki bean was high in quercetin (43.1 µg/100g DW powder) and rutin (3.5 mg/100g DW powder). This result is supported by Han *et al.,* (2015) who showed that a total of 20.7 mg/g of rutin could be obtained from the bean paste, and, 36.2 µg/g of quercetin has been reported from adzuki bean crude extract (Amarowicz *et al.,* 2008). The value from this study is lower than the reported values since different forms of raw material and extraction protocols were used. Han *et al.* (2015) used bean paste and a boiled water extraction. Amarowicz *et al.* (2008) defatted the blended bean powder with hexane prior to extraction with 80% acetone at 50°C with a repeated extraction. Whilst, the current study applied 80% methanol extraction to milled bean flour at room temperature without repetition.

The results in chapter 6 showed that only a limited number of phenolics from beans could be identified and quantified by HPLC. Similar study to those in this thesis, using LC-MS or LC-MS-MS, appear to be limited even though the MS system is more efficient. Adzuki bean is perhaps one of the best studied beans and a list of previously reported soluble phenolics from this bean, that were included in the range of standards in the current study, are protocatechuic acid (67.6 µg/ g DW), ρ - coumaric acid (31.3 µg/ g DW), epicatechin (4.57 µg/ g DW), epigallocatechin gallate (0.14 µg/ g DW), quercetin (36.2 µg/ g DW) and rutin (38.2 µg/ g DW) (Amarowicz *et al.,* 2008). The quantified amounts for the phenolics in this study were either lower than the above report or were not detected, except for rutin (35.0 µg/ g DW) which had the closest value with the previous report.

Up to date, there has been only one report concerning soluble phenolics of bambara groundnut that has been conducted using an MS system. It showed a list of identified phenolics that is in line with the current study such as epicatechin, ferulic acid, ρ-coumaric acid, derivatives of caffeic acid and rutin which were identified from red and brown bambara groundnut by LC-MS-MS (Nyau *et al,* 2015). Hence, the current phenolic study on black bambara

groundnut, a different variety of bean, is the first to report on identified and quantified phenolics by LC-MS.

The increase in concentration of some compounds in deconjugated samples suggested that they exist as both conjugated and free phenolics. The outcome from the deconjugated phenolics analysis clearly showed the presence of esterified or conjugated forms in the beans (table 7.9). This gives a hint to the expected fragmentation pattern when analysed with tandem mass spectrophotometry by showing *m/z* and the increase of fragmented ion concentration.

This is the first report of bound phenolics identified from beans and within this fraction a total of eight individual phenolics were identified. In terms of the number of phenolics identified the ranking was soya bean> adzuki bean = pigeon pea > black eyed pea= bambara groundnut= lablab bean= mung bean. Although underutilised beans had a smaller number of phenolics when compared with soya bean, they had the highest concentration for most of the detected compounds (5 out of 8). Adzuki bean had the highest gallic acid and rutin, black-eyed pea was high in ferulic acid, bambara groundnut was high in protocatechuic acid and lablab bean was high in ρ-coumaric acid.

Among all the beans, adzuki bean (0.9 mg/ 100g powder) and bambara groundnut (1.5 mg/ 100g poweder) showed higher protocatechuic acid in the bound form and this was higher than that found in white rice (0.17 mg/ 100g flour). All beans, except pigeon pea, had higher bound ρ-coumaric acid than white rice (0.34 mg/100g flour). However, ferulic acid from white rice (5.25 mg/ 100g flour) is 2 to 25 fold higher than in the tested beans (Tian *et al.*, 2004).

Protocatehuic acid (bambara groundnut), ρ-coumaric acid (lablab bean), sinapic acid (soya bean) and ferulic acid (black eyed pea) were all highest in the bound forms with bound samples providing at least 100% more concentration than soluble samples. Gallic acid also showed a higher content in

the bound extract than in the soluble form but in this case the percentage was only 30% more in the bound form. Other phenolic compounds- caffeic acid, rutin and daidzein- were highest in the soluble extracts from lablab bean, adzuki bean and soya bean, at more than 100% concentration.

Bound phenolics are thought to play an important role in the prevention of intestinal and colonic diseases. These phenolic acids bind to cell wall materials as insoluble phenolics and hydrolysable conjugated phenolics. These bound phenolic acids are expecting to promote the same health benefits as soluble phenolics but at more targeted locations such as the colon. Soluble phenolics are likely to be beneficial before reaching the colon because they will be available in the gastrointestinal tract. The natural characteristics of those phenolics that are associated with cell wall materials lead to difficult in their release by gastrointestinal enzymes, hence they pass the upper intestinal tract intact to the colon (Madhujith and Shahidi, 2009).

Previous studies have also shown that hydroxycinnamic acid, ferulic acid, ρ-coumaric acid and 5-5-dehydrodiferulic acid derived from the cell wall had antimutagenic properties and could provide preventitive activity against cancer (Ferguson *et al.,* 2003). Hydroxycinamic acids have strong antioxidant activity but their effect in the body may be relative to their bioavailability in the gut. Zhao, Egashira and Sanada (2003) reported that free ferulic acid has a higher bioavailability than the conjugated form, feruloylarabinose and thus can be completely absorbed in the gastrointestinal tract.

This novel finding of bound phenolics in beans, especially those that have higher content than in the soluble phenolic fraction such as protocatehuic acid (bambara groundnut), ρ-coumaric acid (lablab bean), sinapic acid (soya bean) and ferulic acid (black eyed pea) may have implications for the beneficial impact of bean consumption on the gastrointestinal tract. Increased intake of beans rich in bound or free forms of ferulic acid may help to reduce dietary carcinogen-induced mutations and other mutations associated with reactive oxygen species especially because of the high absorption in the gastrointestinal tract of the free form.

7.4.2 **Validation of soluble phenolics extraction procedure**

The validation of an optimised extraction method and analysis by LC-MS for soluble phenolics from beans was first reported here. Linearity, accuracy, precision, matrix effect, LOD and LOQ of the optimised methodology were evaluated. These provide an indication that the current reported phenolic concentration values from plants need to be reconfirmed since matrix was found to affect the recovery value. There is very little previous information about procedures or analytical methods used in plants that have been similarly validated (Alonso-Salces *et al.*, 2005)

Validation procedure with the pooled beans powder indicated that matrix affects the recovery value for phenolic compounds by enhancing or suppressing the extraction and MS response efficiency. For example, changes of recovery from non matrix to matrix for 'spiked' 4.5 µM of caffeic acid (high concentration) was 80.1% to 59.4% (table 7.10). This represents a significant difference of 20% ($p<0.05$) and is sufficient to show that the reported values without a validated protocol are unconvincing. Therefore, previous reported studies quantifying phenolics that did not include the matrix effect on the recovery may not be sufficient to represent the actual value in plants.

There was no definitive pattern discernible for changes in the recovery of the phenolics from the blank solvent (no matrix) to pooled beans (matrix). No definite correlations with any specific phenolic category or bean type were observed. This may be because a pooled bean powder was used instead of individual beans. Physical and chemical components vary among beans which may result in different matrix effects that lead to the changes in recovery value. For example, the ability to trap moisture in the vacuole and the moisture content for pigeon pea, black eyed pea and mung bean are 11.07%, 10.39% and 8.3% (Butt and Batool, 2010). This indirectly showed that the pooled bean powders may not be proportionally equal during sample preparation and the affinity of solvent and materials interaction may be different as well.

The recovery was also shown to change with increasing concentration. The recovery values showed a high level of variation at different concentrations.

The values for recovery were generally better from high concentrations as compared with low concentrations. But, this would seem to be against the principle of successful validation for salting out liquid-liquid partitioning. The extraction recovery and matrix effect at different concentrations should be constant with good precision and high sensitivity along the calibration curve (Zhao *et al.*, 2012). This observation might be simply due to the fact that the preparation of solutions at higher concentration was more accurate due to unavoidable error after each dilution step (Kua *et al.*, 2016).

In addition, simple phenolic acids (hydroxycinnamic acid and hydroxybenzoic acid derivatives) such as caffeic acid and ρ-coumaric acid showed better recovery (>70%) than flavonoid compounds like daidzein, naringeinin and epicatechin (<70%) at medium and high concentrations. Therefore, the findings indicated that one single extraction method may not be suitable across all types of phenolic compound. Some phenolics might be increased while others might be lost during extraction.

This preliminary validation has built up a concrete understanding that further method validation for individual beans is necessary because each individual bean is likely to affect the analysis differently through matrix effects. Besides, the validation experiment for the procedure to obtain bound phenolics from bean extracts are expected to be more complicated. A more comprehensive validation procedure may be required since there may be many other limiting factors or uncertainties in addition to the matrix effect.

One of the additional factors may be the high concentration of salt that could cause lower ion suppression (Zhao *et al.*, 2012). Secondly, the extraction recovery for bound phenolics may be directly affected by the amount of acetonitrile organic layer to be transferred out for analysis. Zhang *et al.* (2009) has shown from a validation experiment that half of the acetonitrile layer being transferred out during the post hydrolysis extraction led to 31% to 42% of extraction recovery.

7.4.3 Profiling of unknown compounds

Only a minority of the peaks observed by LC-MS profiles could be putatively assigned to individual phenolics using the panel of standards. This implies that the remaining peaks are either phenolics for which standards were not applied, or represent other types of compounds. Profiling the unknown compounds by LC-MS provide a number of databases to show interesting compound markers for each individual bean and the number of peaks that are common among the beans. These LC-MS profiles for unknown compounds consist of retention time and ion (*m/z*) for those compounds that exist in either the soluble or bound forms.

The methods and database will be a good reference for further study of phenolics profile by liquid chromatography tandem mass spectrophotometry in order to confirm the identity of these unknown compounds in the database.

7.5 Conclusion

In conclusion, the soluble, deconjugated and bound phenolic bean samples showed varied types of phenolics at different concentrations. All the phenolics identified in the bound phenolic samples were also found in soluble phenolic samples as well but at varied concentrations except protocatechuic acid for adzuki bean and mung bean where the phenolic was only found in bound phenolic samples.

Quantification results from both soluble and bound phenolics showed that phenolics were found at the highest concentration in the bound phenolic extracts in different beans: gallic acid (adzuki bean), protocatechuic acid (bambara groundnut), ρ-coumaric acid (lablab bean), sinapic acid (soya bean), ferulic acid (black eyed pea). Others were highest in the soluble phenolic extracts in different beans: chlorogenic acid (bambara groundnut), caffeic acid (lablab bean), epicatechin (pigeon pea), rutin (adzuki bean), daidzein (soya bean), naringenin (soya bean), genistein (soya bean) and quercetin (adzuki bean).

An initial validation of the procedure, with pooled bean powders, for soluble phenolic analysis by LC-MS was carried out. This gave an indication that matrix affects the recovery value for phenolic compounds by enhancing or suppressing the extraction and MS response efficiency. However, definite correlations with any specific phenolic category or bean type were not shown. Hence, further validation of the analytical procedure applied to individual bean is necessary to make the quantification results obtained more reliable and convincing.

Apart from that, profiling of the unidentified compounds according to their ratio mass-to-charge has provided a database to show interesting ions for each individual bean and identifying the number of ions commonly found among the beans. This database could be used as a reference tool for future study of legume' phenolic, or other, compounds.

CHAPTER 8
General discussion

This study aimed to improve the extraction, identification and quantification methodologies for characterizing soluble free, conjugated and insoluble bound phenolics from selected underutilised beans via HPLC and LC-MS. Firstly, a preliminary screening to compare the antioxidant potential from commercial and underutilised beans has been conducted via DPPH, FRAP and TPC assays. Secondly, an optimised extraction methodology for soluble phenolics from selected underutilised beans was designed. This involved solvent selection by comparing 80% methanol, 80% acetone and sodium acetate buffer. At the same time, colorimetric assays and HPLC techniques were applied to detect the extractable phenolics. Thirdly, the optimisation of extractions targeting conjugated and bound phenolics were conducted. Upon completion of the method optimisation, these were applied to selected underutilised beans in order to profile the soluble and bound phenolics as well as estimating the conjugated phenolics by HPLC and LC-MS with 20 phenolic standards. On top of that, the untargeted compounds were profiled concurrently. Lastly, a validation procedure for soluble phenolics was undertaken using the final method.

8.1 Preliminary antioxidant screening

A total of six underutilised beans - adzuki bean, black eyed pea, bambara groundnut, lablab bean, mung bean, pigeon pea and four commercial beans – soya bean, chickpea, kidney bean, lentil were selected for this study based on their availability in Malaysia. This study involved three independent colorimetry assays - DPPH free radical scavenging assay, FRAP assay and TPC assay aimed to compare the antioxidant activity between underutilised and commercial beans.

The selected underutilised beans are different in size and color. The sequence of the length is bambara groundnut > lablab bean> black eyed pea> pigeon pea> adzuki bean> mung bean. On top of that, a diversify of seed coat colors have been observed, such as creamy white for black eyed pea, green color for

mung bean, red color for adzuki bean, white color for lablab bean, orange yellow for pigeon pea and black color for bambara groundnut.

They are difference in the main region of producers for the selected beans such as Asia, America and Africa (table 2.3) and the physical characteristics indicated a vast growing conditions for this crop. Thus, different in phytochemical profiles are expected. The similarities for the selected beans apart from being classified as underutilised, they are cooked as a side dish or main dish for a meal, a dessert, or processed into flour for pastry making as mentioned in the literature review. Hence the understanding of the phytochemical profile is essential.

Current findings in section 3.3.1 revealed significant differences in antioxidant activity and relative phenolic contents between the commercial and underutilised beans' methanolic extracts. In DPPH assay, adzuki bean exhibited the same scavenging activity as soya bean and was the highest among all tested beans (0.7 mg Trolox/ g powder). Moreover, adzuki bean also possessed the highest FRAP antioxidant activity (0.31 mM ferrous sulphate / g powder) followed by the group of soya bean = bambara groundnut = kidney bean. However, when examined by TPC assay, that could be considered as monitoring both antioxidant capacity and providing an estimation of phenolic content, soya bean was shown be the highest in TPC (1.45 mg GAE/ g powder) among all tested beans.

The correlation analysis showed that antioxidant potential from DPPH (r^2 =0.699) and FRAP (r^2 =0.599) were associated to phenolic compounds (TPC assay) although the correlation was not as strong as between the DPPH and FRAP assays (r^2=0.890). TPC assay is used to estimate the phenolic content but might not represent the specific reducing power of the antioxidants. Therefore, soya bean has been observed to give the highest TPC value but not the highest DPPH or FRAP value. Some other none phenolic compounds in soya bean may thus be reacting in the TPC assay.

In addition to the antioxidant potential associated with the beans' phenolic content, a further study on total monomeric anthocyanin content (TMA) and tocopherol content was also conducted. TMA was carried out using a modified pH differential method and results showed that only bambara groundnut has any detectable monomeric anthocyanin content (0.92 mg cyanidin-3-glucoside/ 100g DW), strawberry was used as a positive control in this investigation. Next, tocopherol-δ was detected in adzuki bean, black eyed pea, bambara groundnut and soya bean only. However, tocopherol-β+γ was found in all underutilised beans except adzuki bean and black eyed pea. The concentrations of these detected vitamers were lower than in published reports although most of the tocopherol investigations in the past have been conducted on commercial beans.

8.2 Optimisation of extraction method for soluble phenolics from underutilised beans

After determination of beans' antioxidant potential, a study was carried out to establish an optimised method to profile the phenolics from underutilised beans. Two types of phenolics are found in beans, soluble and insoluble phenolics. Soluble phenolics are further divided into conjugated and free phenolics, whilst insoluble phenolics are known as bound phenolics.

Optimisation of solvent to extract the soluble phenolics is important in the profiling process. In this study, 80% methanol, 80% acetone and sodium acetate were compared for their ability to extract soluble phenolics (section 3.3.4 and section 4.3.1). Methanol has been selected in this study due to its efficiency in extracting polyphenolic compounds and the fact that it is commonly used for extracting phenolic compounds from plants (Al-Temimi and Choudhary, 2013; Sreerama *et al.*, 2012; Marathe *et al*., 2011; Stalikas, 2007; Escarpa & Gonzalez, 2001; Arts and Hollman, 1998).

Selection of acetate buffer was due to its role in stabilizing antioxidants in the FRAP assay. Hence, the efficiency of buffer solution is expected to be better than water and it has a different polarity as compared with organic solvent. Those compounds that are soluble in alcohol will not be extracted. Thus, a variation of compounds was expected.

Acetone has also been commonly used as a solvent for extracting compounds from fruits and vegetables such as cinnamon and peppermint (Lv *et al.*, 2012; Naczk & Shahidi, 2006; Seeram *et al.*, 2006). 80% acetone was thus included even though it has the same index polarity as methanol and it was expected that a similar range of compound might be extracted between both solvents. But, it was of interesting to investigate potential differences since acetone extracts were observed to have a greater colour intensity that may bring variation in the concentration of important compounds when compared to methanol extracts.

Both antioxidant assays and HPLC techniques were applied in an attempt to determine the relative phenolic content followed by comparing the contents

from three different solvents (section 4.3.1). The study started by comparing sodium acetate buffer and 80% methanol using DPPH, FRAP and TPC. These solvents have a distinctive variation of polarity- one is an organic solvent (polarity index = 5.1) whilst the other is aqueous based and were expected to give different results from the colorimetric antioxidant assays.

In the DPPH assay, there were no clear differences in the antioxidant activity between the two solvents used. Adzuki bean possessed the highest antioxidant activity from both extracts. Three methanol extracts (from black eyed pea, bambara groundnut and soya bean) showed higher activity than the equivalent acetate buffer extract. But, another 3 acetate buffer extracts (from lablab bean, mung bean and pigeon pea) showed higher values than the equivalent methanol extracts. FRAP assay results showed that methanol extracts (from black eyed pea, bambara groundnut, soya bean) had higher antioxidant activity than acetate buffer extracts. Whilst, the acetate buffer extract for the remaining beans (adzuki bean, lablab bean, mung bean and pigeon pea) contained higher values than the corresponding methanol extracts.

In contrast, TPC assay revealed that acetate buffer extracts for all beans contained higher TPC value than the methanol extracts except soya bean where there was no difference between both types of extract. Both methanol and acetate buffer are thus suitable in extracting the antioxidants although TPC showed that acetate buffer is better than methanol in extracting putative phenolic compounds. In short, there was no solvent that was best across all beans as reflected from the colorimetric assays. However, a good outcome from these investigation were 80% methanol and acetate buffer extract were positively correlated.

Colorimetric assays are commonly used to assess antioxidant activity mainly due to their simplicity and speed of analysis (Blekas *et al.,* 2002). There are a few disadvantages that have been reported such as restriction of maximum colour intensity for each assay. Thus, there is no optimum recording when large quantities of samples are to be analysed (Porretta & Sandei, 1991). A disadvantage of the TPC assay is its low specificity, because some non

phenolic compounds can react with the reagents giving a false positive result (Singleton & Rossi, 1965). Furthermore, the molar mass and structure (namely number of hydroxyl groups) of individual compounds cannot be distinguished using this method (Hrncirik & Fritsche, 2004).

Therefore, HPLC analysis is recommended, in addition to, colorimetric assays to determine the phenolic compounds. HPLC analysis coupled with TPC assay showed that all three solvents were suitable for extracting the phenolic compounds (section 4.3.1). However, it was difficult to justify whether HPLC was better than the colorimetric assay from this investigation. Hrncirik & Fritsche (2004) showed a strong correlation between HPLC and colorimetric assay and that TPC was a reasonable method for prediction of total phenolic content. But, the current study managed to show the advantages of HPLC in providing more information than the colorimetric assay. Also, HPLC is able to overcome the disadvantages of the colorimetric assay as mentioned above.

HPLC analysis showed that similar compounds were extracted in both methanol and acetate extracts (table 4.1 to 4.3), and between methanol and acetone extracts (table 4.5 to 4.8). The similarity of compounds extracted by different solvents has been investigated by Jyothiprabha & Venkatachalam (2016). Qualitative analysis in their report clearly showed that methanol, acetone and distilled water extracted the same compound types such as phenolics, alkaloids and saponins from spices. Hrncirik & Fritsche (2004) also commented that this analytical procedure is capable of quantitative isolation of the phenolic fraction from olive oil.

In short, application of HPLC technique is recommended for the analysis and quanification of phenolics. Therefore, the next step involved the use of reference standards in the development of a qualitative and quantitative HPLC method.

8.3 Optimisation of extraction methods for conjugated and bound phenolics from underutilised beans

Following the success in establishing the extraction methodology targeting the soluble phenolic compounds from beans, the next stage was to develop optimised extraction methods to obtain soluble conjugated and bound phenolics. It consisted of optimisation of hydrolysis conditions and extraction/partitioning after hydrolysis.

Optimisation of hydrolysis conditions began with a comparison between acid and alkaline hydrolysis using lablab bean and mung bean and a hydrolysis duration of 2 hours and 24 hours to obtain deconjugated and bound compounds. Lablab bean and mung bean were used for this initial stage in order to overview the effect of hydrolysis on actual samples. They were selected because of the relatively few significant major peaks that were observed from chromatogram at 280 nm (chapter 4, appendix 4.7 and appendix 4.8). Changes of these major peaks after hydrolysis were used to justify the suitability of the type of hydrolysis used.

Current findings showed that both acid and alkaline hydrolysis were effective in deconjugating the conjugated phenolic compounds and releasing the bound phenolic compounds. However, alkaline hydrolysis was considered better because the changes from conjugated to deconjugated free phenolics appeared to be accompanied by the appearance of new peaks and the reduction of conjugated phenolics instead of what appeared to be complete degradation of conjugated compounds. Also, the compounds appeared to be relatively more stable in alkaline conditions and it was less selective in terms of type of bean or compound.

Optimisation of hydrolysis using phenolic reference standards showed that 12 out of 20 standards were totally degraded after 1 hour of alkaline hydrolysis while the remaining standards showed a reduction in concentration of between 10% and 100%. This suggested the high possibility of deconjugated free phenolics and bound phenolics being degraded completely after 1 hour of hydrolysis. Upon completion of this optimisation a hydrolysis durations of 5

min and 1 hour were applied on conjugated phenolic and bound phenolic samples, respectively.

The recommended hydrolysis duration above targeting conjugated and bound phenolics could provide useful information for further research on plant materials since the stability of the reference standards have been tested and considered. The finding could have a negative impact on previous studies that obtained the deconjugated and bound phenolics without considering the degradation of standards under the hydrolysis conditions. A range of 1 h to 24 h hydrolysis duration have been employed in previous studies (Chen *et al.,* 2015; Wang *et al.,* 2015; Pająk *et al.,* 2014; Silva *et al.,* 2013; Chandrasekara & Shahidi, 2011; Madhujith & Shahidi, 2009; Verma *et al.,* 2009; Cai *et al.,* 2003; Adom & Liu, 2002). It is not clear whether or not reference standards have been investigated in these reports. Moreover, Bonoli *et al.* (2004) suggested prolonging alkaline hydrolysis to 20 h to increase the extraction yield. But, in fact the majority of the phenolics may be lost during prolonged hydrolysis as shown in this current study.

Next, optimisation of the post hydrolysis separation techniques was carried out. Again this was with reference to a range of phenolic standards. The conclusion (section 5.3.3) was that acetonitrile salting out liquid-liquid partitioning is recommended during the extraction of bound phenolics whilst SPE partitioning is the best method for the recovery of deconjugated phenolics from hydrolysed samples. The criteria for these selections were based on the recovery of reference standards, ease of handling and cost compared with the more conventional ethyl acetate liquid-liquid partitioning.

Application of acetonitrile salting out liquid-liquid partitioning has been applied to biological samples such as serum since 1989 and recently extended to plants, foods and environmental specimens but thus far not to beans (Park and Jung, 2017; Zhao *et al.*, 2012; Zhang *et al*., 2010; Chen *et al.*, 2009;Yoshida *et al.,* 2004; Rustum, 1989). The outcome from optimisation showed its ability to recover all phenolic standards and that 16 out of 20 showed a recovery higher than, or close to, that for ethyl acetate liquid-liquid

partitioning. This technique uses less solvent and was able to perform sample cleaning prior to HPLC and LC-MS analysis. The only disadvantage was no optimal yield could be reached. It is subjected to the step of transferring the organic solvent layer after partitioning.

The advantage of SPE partitioning was its ability to recover all phenolics at similar to, or higher percentage, efficiencies than ethyl acetate liquid-liquid partitioning. This technique is commonly used to concentrate samples or to clean-up samples prior to HPLC or LC-MS analysis. It is also user friendly and requires shorter drying times. The only disadvantage is that the cost is higher than other types of liquid-liquid partitioning.

8.4 Identification and quantification of phenolics by HPLC and LC-MS

The total soluble, deconjugated and bound extracts, prepared as described (chapter 4 and 5), were all subjected to profiling by HPLC and LC-MS with reference to 20 phenolic standards. These extracts were also subjected to antioxidant assays- TPC, DPPH and FRAP assays. Results showed that soluble phenolic samples exhibited the highest DPPH, FRAP and TPC antioxidant activities across all beans. Whilst, deconjugated phenolic samples showed the lowest DPPH and TPC activities, bound phenolics samples exhibited the lowest FRAP activities across all beans except adzuki bean and bambara groundnut.

Soluble phenolic extract of adzuki bean revealed the highest DPPH free radical scavenging activity (1.75 mg Trolox/ g DW) and FRAP activity (1.16 mg Trolox/ g DW), across all beans inclusive of soya bean. However, TPC value from soluble phenolic extract of soya bean was still the highest. Deconjugated extract of lablab bean showed the lowest DPPH and TPC values whilst bound extract of lablab bean was the lowest in FRAP activity. In conclusion, different forms of compound (soluble free, conjugated and bound) from all beans exhibited antioxidant activities and in general these appear to correlate with phenolic compounds.

This is in line with published studies that show antioxidant potential of soluble phenolic extracts was the highest and deconjugated phenolic extracts were much lower than soluble phenolic extracts (Sun *et al.,* 2012; Gutiérrez-Uribe *et al.,* 2011; Hung & Morita, 2008). Soluble phenolic extracts consist of conjugated and free phenolics as well as several non-phenolic compounds that are soluble in the extraction solvent and may also possess antioxidant activity. This is probably the reason why it has higher antioxidant activity. Deconjugated phenolic samples probably show much lower activity because the hydrolysis conditions may degrade some phenolic and non-phenolic compounds that have antioxidant activity.

Identification and quantification of the compounds existing in all three forms of sample extracts rely on HPLC and LC-MS techniques. A total of 13, 10 and 8 soluble, deconjugated and bound phenolics have been identified across all beans by LC-MS. However, only 4, 4 and 5 phenolics were identified in the corresponding samples by HPLC. All the phenolics identified using HPLC were also found using LC-MS. For example, rutin, ferulic acid and ρ-coumaric acid were found in soluble and deconjugated samples from the majority of beans by LC-MS (table 7.6 to 7.8) but not by HPLC (table 6.7 to 6.9). This indicated that LC-MS was more efficient than HPLC at identifying the diversity of phenolics.

The concentration of phenolics identified by LC-MS were as low as < 0.1 µg/g DW powder but the detection was only as low as > 5 µg/g DW powder by HPLC. Moreover, the identification by LC-MS using the mass to charge ratio was more selective and could separately identify compounds even though the elution times could be as close as 0.04 min. This is because formula structure of individual phenolics defines the mass to charge ratio and the retention time. Therefore, LC-MS is more sensitive and selective than HPLC.

The advantage of high sensitivity increased the detection of deconjugated phenolics. The findings showed that many of the identified phenolics existed more as conjugated than as free phenolics (table 7.9). This provides a clue on the expected fragmentation pattern when being analysed with tandem mass

spectrophotometry by showing *m/z* and the increase of fragmented ion concentration. As a result, application of HPLC analysis during the method optimisation stage and pre-screening for profiling in order to obtain an overview result is highly recommended. Whilst, LC-MS is recommended in profiling and quantifying the phenolics compounds.

The overview from the HPLC analysis was that, soluble phenolic sample of adzuki bean had the highest concentration of rutin (37 µg/g DW bean powder) and soluble phenolic sample of soya bean had the highest daidzein (101.9 µg/g DW bean powder) (table 6.7). Whilst, the highest amount of ρ- coumaric acid, ferulic acid, protocatechuic acid and sinapic acid were found in the bound phenolic samples of different beans (table 6.9). ρ- coumaric acid was highest in the bound phenolic sample of lablab bean (48 µg/g DW bean powder), ferulic acid was highest in black eyed pea (30.5 µg/g DW bean powder), protocatechuic acid was highest in bambara groundnut (24.2 µg/g DW bean powder) and sinapic acid was found in soya bean (19.1 µg/g DW bean powder) only.

As discussed in chapters 6 and 7, to the author's knowledge, there are only five publications concerning the anlysis of soluble and bound phenolics by HPLC-DAD from mung bean, adzuki bean, black eyed pea and lablab bean that can be found. (Giusti *et al.,* 2017; Bai *et al.,* 2017; Gan *et al.,* 2016; Pająk *et al.,* 2014; Yao *et al.,* 2011; Gutierrez-Uribe *et al.,* 2011; Cai *et al.*, 2003). Similar studies with LC-MS or LC-MS-MS are even more limited and only one for adzuki bean could be found. As a result, comprehensive profiling of soluble and bound phenolics by LC-MS for six underutilised beans – adzuki bean, black eyed pea, bambara groundnut, lablab bean, mung bean and pigeon pea were first reported in this study.

Two important outcomes resulted from the LC-MS analysis. Firstly, underutilised beans appear to possess more diversity of soluble phenolics than soya bean. Among all the beans tested, black eyed pea contained all 13 identified phenolic compounds whilst both soya bean and mung bean had the least number of identified phenolic compounds (9 phenolics). The

concentrations of these phenolics were higher in underutilised beans than in soya bean. For example, adzuki bean was found to be high in quercetin and rutin, bambara groundnut was high in gallic acid, protocatechuic acid and chlorogenic acid.

Secondly, although underutilised beans seem to show less variety in their bound phenolics when compared with soya bean, they had the highest concentrations for most of the detected compounds (5 out of 8). Adzuki bean had the highest gallic acid and rutin, black-eyed pea was high in ferulic acid, bambara groundnut was high in protocatechuic acid and lablab bean was high in ρ-coumaric acid. In many cases the bound samples showed at least 100% higher concentrations than the equivalent soluble samples. Others phenolic compounds, caffeic acid, rutin, daidzein had highest concentrations in soluble extracts from lablab bean, adzuki bean and soya bean again often 100% more.

Profiling the phenolics in individual beans by LC-MS has added to our knowledge of the potential nutraceutical and pharmaceutical value for each bean, especially in terms of the bound phenolics. The health promoting benefits of bound phenolics are thought to be due to their ability to survive through the stomach and intestinal digestion to reach the colon. Thence, its natural characteristics such as antioxidant potential are freely effective at the point of release (Adom & Liu, 2002). Those beans that possess significant amounts of bound phenolics may thus have the potential to serve as functional foods.

Among all the tested beans, adzuki bean, black eyed pea, bambara groundnut and lablab bean appear to show the most potential as functional foods. This is because these beans contain a significant amount of phenolics such as protocatehuic acid, ferulic acid, rutin and ρ-coumaric acid. These groups of phenolics are gaining interest not only because of their antioxidant effect but also other physiological functions and potential application as preservatives in the food industry. Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) are widely used as food preservatives. However, weak carcinogenic effects have been proven in some animals at high levels (Velasco & Pamela, 2011). Therefore, using phenolic

compounds as an alternative is a trend due to their antioxidant ability. They are believed to not only act as antioxidants but also as antimicrobial agents that prolong the shelf life of food products (Stojkovic, 2013)

Many phenolic compounds possess antioxidant activity but not all can be used in food products. Several criteria are important for use in food products such as cost, nontoxicity, capable of surviving processing, stable in the finished products and do not cause undesirable colour, flavour or odour effects (Shahidi & Zhong, 2010; Djilas & Jasna, 1998). Among those phenolics examined in this study, caffeic acid, ρ-coumaric acid and rutin are of particular interest as food preservatives (Stojkovic, 2013).

A level of 1.87 mg/ mL of rutin or ρ-coumaric acid were reported sufficient to inhibit the growth of *S. aureus* in chicken soup at 25°C over 24 h (Stojkovic, 2013). The current study showed that soluble extract of adzuki bean was able to provide 3.5 mg rutin and bound extract of lablab bean was able to provide 6 mg of ρ-coumaric acid from 100g of dry powder. Hence, adzuki bean and lablab bean are the best sources for rutin and ρ-coumaric acid that are suitable as food preservatives.

Protocatehuic acid has elicited the interest of researchers because of its antioxidant activity, antibacterial activity, anti-cancer and ability to protect against the oxidative stress that leads to neuronal cell death, as neuroprotective and anti-inflammatory agent (Winter *et al.*, 2017; Semaming *et al.*, 2015; Kakkar & Bais, 2014). Moreover, it is also able to exert biological functions – such as its ability to activate insulin signalling pathway by mimicking insulin activity (Scazzocchio *et al.*, 2015).

Various concentrations of protocatechuic acid have been reported to be involved in the activities above. For example, protocatechuic acid isolated from the dried fruits of Alpinia was found to possess an anti-ageing effect at doses of 5 – 10 mg/kg for 7 days in young and old rats (Kakkar & Bais, 2014). Therefore, bambara groundnut (the highest concentration from bound extract –

1.5 mg/ 100g DW) could be a good source since it provides the highest levels of both soluble and bound protocatehuic acid.

Ferulic acid is a phenolic acid with low toxicity, it can be easily absorbed and metabolized in the human body. Some of the reported physiological functions, include antioxidant, antimicrobial, anti-inflammatory, anti-thrombosis and anti-cancer activities and this compound is widely used in the food and cosmetic industries (Ghosh *et al.,* 2017; Mancuso & Santangelo, 2014; Yan *et al.,* 2013; Ou & Kwok, 2004). Soluble ferulic acid from lablab bean and bound ferulic acid from black eyed pea could strongly recommend these as food sources. Additionally, bound ferulic acid concentration is highest in black eyed pea and might be expected to promote health benefits to the colon.

8.5 Profiling the unknown compounds

Only a minority of the peaks observed by HPLC and LC-MS profiles could be putatively assigned to individual phenolics using the panel of standards. This implies that the remaining peaks are either phenolics for which standards were not applied, or represent other types of compounds. Profiling the unknown compounds by HPLC and LC-MS provides a database of markers for potentially interesting compound for each individual bean. HPLC profile for unknown compounds provides a database based on the retention time and optimal wavelength for compounds that exist in soluble and bound forms. Whilst, the LC-MS profiles for unknown compounds provide markers based on retention time and ions for compounds that exist in soluble and bound forms.

There are 3 major pieces of information that can be obtained from the HPLC database. Firstly, it provides an overview of the number of peaks that were unique to individual beans such as 6 out of 20 peaks are unique to the soluble sample of adzuki bean but no unique peaks were observed in the bound sample of the same bean. Secondly, it shows the commonality of peaks between beans. For example, the highest commonality of 13 peaks have been observed between soya bean and adzuki bean and the lowest commonality of 3 peaks was found between soya bean and mung bean. Lastly, commonality of peaks between soluble and bound extracts can be obtained as well. The same

information can be obtained from the LC-MS database. The differences are that this compilation is based on both the retention time and ion (*m/z*).

8.6 Validation of extraction procedure for soluble phenolics using LC-MS

Validation of the LC-MS procedure has been carried out based on the linearity, recovery, precision, limit of detection (LOD) and limit of quantification (LOQ) of each individual phenolic and investigation of matrix effect towards extraction efficiency and ionization effect. This was carried out on pooled bean samples (figure 7.1) instead of one single bean in order to obtain an overview of the methodology.

The linearity results indicated that the response of the MS over the concentrations used were on the whole proportional to the concentration of the analyte in the samples. Whilst other parameters as mentioned above have been justified as well. The major outcomes were that matrix affects the recovery value for phenolic compounds by either enhancing or suppressing the extraction and MS response efficiency. However, no definitive correlations with any specific phenolic category or bean were found. Hence, validation of the analytical procedure for each individual bean is necessary to make the quantification results obtained more reliable and convincing.

There are four areas suggested for future studies. Firstly, identification of unknown compounds. The optimised methodologies could be used to extract the soluble and bound phenolics followed by identification using LC-MS-MS. This system possesses a quadrupole (Q1) that is capable of defining the *m/z* value (parent ion or precursor ion) and fragmented ion (as product ion). Thus a range of data such as retention time, spectra, precursor ion and its relationship with fragmented ion can be collected for each unknown peak. This data set could be used to estimate the type of compounds by referring to published reports and international database such as the dictionary of natural products and mass bank. Therefore, some of the untargeted peaks could be identified. Unfortunately time did not allow such detailed interrogation of the data in this thesis.

Secondly, the validation of methodologies to extract, identify and quantify soluble and bound phenolics is recommended to be conducted for individual beans. This is because the natural characteristic of beans means they may behave differently. Hence, the outcome from the individual validation investigation would provide more reliable results for the specific bean.

Thirdly, the current study focussed on optimising the extraction and analysis methodologies for beans samples follow by compound profiling. A single batch of samples from retail suppliers in this project failed to reflect the actual variation in phytochemical profiles. There are many factors that cause the variation in phytochemical compounds in a plant such as ecological factors, choice of cultivars, storage condition and production practices.

Liu et al. (2015) have mentioned that annual average precipitation, temperature, sunshine duration, soil pH and soil organic matter affect the production of active substances in *Sinopodophyllum hexandrum* T.S. Ying, a type of Chinese traditional medicinal herb. And, higher temperature and high rainfall in a year increased the phytochemical composition and antioxidant activity of Ontario vegetable crops (Hu, 2012).

Hence, comparisons of phenolic profiles from different batches of beans that grow under the controlled condition and storage time are recommended. This will minimize the variation due to the genetic and environmental factors. And, this will provide a reliable and accurate comparison of information specifically for bean selection, potential environmental impacts and preservation. Next, the complete improved method is convinceable to be applied in further study how the ecological factors affect the production of phytochemicals. Through this, the optimum ecological conditions can be concluded for each bean. This will help the producers to increase the global yield and minimize the losses due to changing of climates.

Lastly, it would be interesting to further investigate the phenolic profiles specifically from the seed coat in order to understand its proportional contribution to the total phenolics in beans. The seed coat is quite likely to

contain higher amounts of phenolics than the cotyledon. Therefore, the outcome from this study could help in the formulation of diets with higher consumption of phenolics if the total bean, including the seed coat, could be utilised as a food source.

The success in improving the extraction, identification and quantification methodologies for investigating the profile of soluble and bound phenolics in selected underutilised beans has played two important role in competing the underutilised beans with soya bean. Firstly, the databases of phenolic profiles managed to show that the phytochemicals from some of the selected underutilized beans are having higher concentration and more diversified compounds. In another words, it contains more health related properties than the soya bean.

As a result, it has filled up the gap of lacking the scientific data to understand the nutritious properties of underutilised beans which lead to lack of awareness and popularity of the underutilised beans. With these profiles, it will bring up the awareness of underutilised beans easier therough global policies without hesitation. Next, it encourages more studies to be carried out on underutilised beans.

Secondly, the improved method has made the utilization and analysis of underutilized beans easier and approachoable as compared with previously whereby lack of analysis method. Thus, the will aids the downstream study and quality control of cultivars more efficient by looking at the phytochemical contents. Genetic cultivar of the beans are often to be an issued when comes to the beans selection. Once, this has been solved by referring to the phenolic profiles, the quality of beans are under controlled and it reduces losses.

Besides that, this method can also help the manufacturers to produce more nutritious food by referring to the profile when applying different processes without affecting the active susbtances after the process. Hence, we no longer require to relay on commercial beans but more affordable and cheaper price and yet quality beans in the future.

CHAPTER 9
Conclusions

A total of six underutilised beans - adzuki bean, black eyed pea, bambara groundnut, lablab bean, mung bean, pigeon pea and four commercial beans – soya bean, chickpea, kidney bean, lentil were screened for antioxidant potential using DPPH, FRAP and TPC assays. All beans showed substantial antioxidant potential and the amount was variable in both commercial and underutilised beans. The antioxidant potential was found to be positively correlated with phenolic compounds. Adzuki bean had the highest DPPH and FRAP antioxidant activities whilst soya bean possessed the highest TPC value.

Optimised extraction methodologies and analysis techniques targeted to soluble and bound phenolics from underutilised beans have been developed. This included solvent selection, hydrolysis conditions and the use of LC-MS. This has been successfully applied to profile the soluble and bound phenolics from underutilised beans. In this method, 80% methanol was used to extract soluble phenolics and resulted in >70% recovery of standard phenolics.

Alkaline hydrolysis was used to obtain deconjugated free and bound phenolics. It is more capable than acid hydrolysis in breaking down the soluble conjugated compounds within 5 min and released bound phenolics within 1 h. Moreover, phenolics compounds are found to be more stable under alkaline than acidic conditions. Next investigation showed that acetonitrile salting out liquid-liquid partitioning and SPE partitioning were better than ethyl acetate liquid-liquid partitioning in recovering the phenolics from hydrolysed samples. These methods use less solvent, have shorter drying times, due to smaller volume of sample, and were able to recover all tested phenolic standards.

The application of alkaline hydrolysis for 5 mins followed by SPE partitioning on soluble extracts and alkaline hydrolysis for 1 h followed by acetonitrile salting out liquid-liquid partitioning on residue were the optimum procedures for estimating conjugated phenolics and releasing bound phenolics, repectively. Soluble phenolic samples exhibited the high antioxidant activities than

deconjugated and bound phenolic samples across all beans as determined by all three assays (DPPH, FRAP and TPC). Follow up analysis showed that more phenolics were detected via LC-MS than HPLC using 20 phenolics standards.

Using LC-MS, a total of 13, 10 and 8 phenolics from soluble, deconjugated and bound phenolic samples have been identified and quantified, respectively. Black eyed pea has the most diverse soluble phenolics profile (n=13), followed by adzuki bean and bambara groundnut (n=11), lablab bean and pigeon pea (n=10), lastly mung bean and soya bean (n=9). Soya bean contained the most diversified bound phenolics (n=7), followed by pigeon pea and adzuki bean (n=5), and lastly the black eyed pea, bambara groundnut, lablab bean and mung bean (n=4).

Comparison of the results by LC-MS from both soluble and bound extracts showed that phenolics were found at the highest concentrations in bound extracts from different beans: gallic acid (adzuki bean), protocatechuic acid (bambara groundnut), ρ-coumaric acid (lablab bean), sinapic acid (soya bean), ferulic acid (black eyed pea). Others were highest in soluble extracts from different beans: chlorogenic acid (bambara groundnut), caffeic acid (lablab bean), epicatechin (pigeon pea), rutin (adzuki bean), daidzein (soya bean), naringenin (soya bean), genistein (soya bean) and quercetin (adzuki bean).

An initial study, carried out to validate the procedure with pooled bean powder for the soluble phenolics analysis, revealed that matrix affects the recovery via enhancing or suppressing the extraction and MS response efficiency. However, definite correlations with any specific phenolic category or bean type were not shown. Hence, validation of soluble phenolics analysis for each individual bean is highly recommended in order to obtain more reliable and convincing results.

Apart from that, profiling the unidentified phytochemicals according to their spectrum by both HPLC and LC-MS may provide markers for interesting compound for each individual bean and shows the number of peaks either

unique or common among the beans, and this may be useful for future research.

Overall, a total of five key findings were identified from this study. Firstly, a complete methodology to profile the soluble and bound phenolics in underutilised beans has been established. Secondly, a breakthrough methodology to extract bound phenolics from beans has been developed. Thirdly, individual profiles of targeted soluble and bound phenolics by HPLC and LC-MS have been obtained. This is the first report on the analysis of bound phenolics from underutilised beans and soya bean. Fourthly, database profiles of unknown compounds have been prepared by both HPLC and LC-MS for individual beans. Fifthly, a preliminary validation protocol for the extraction and analysis of soluble phenolics has been carried out with pooled bean samples. As a result, the objective of obtaining a methodology for the comprehensive analysis to profile soluble and bound phenolics from beans has been achieved.

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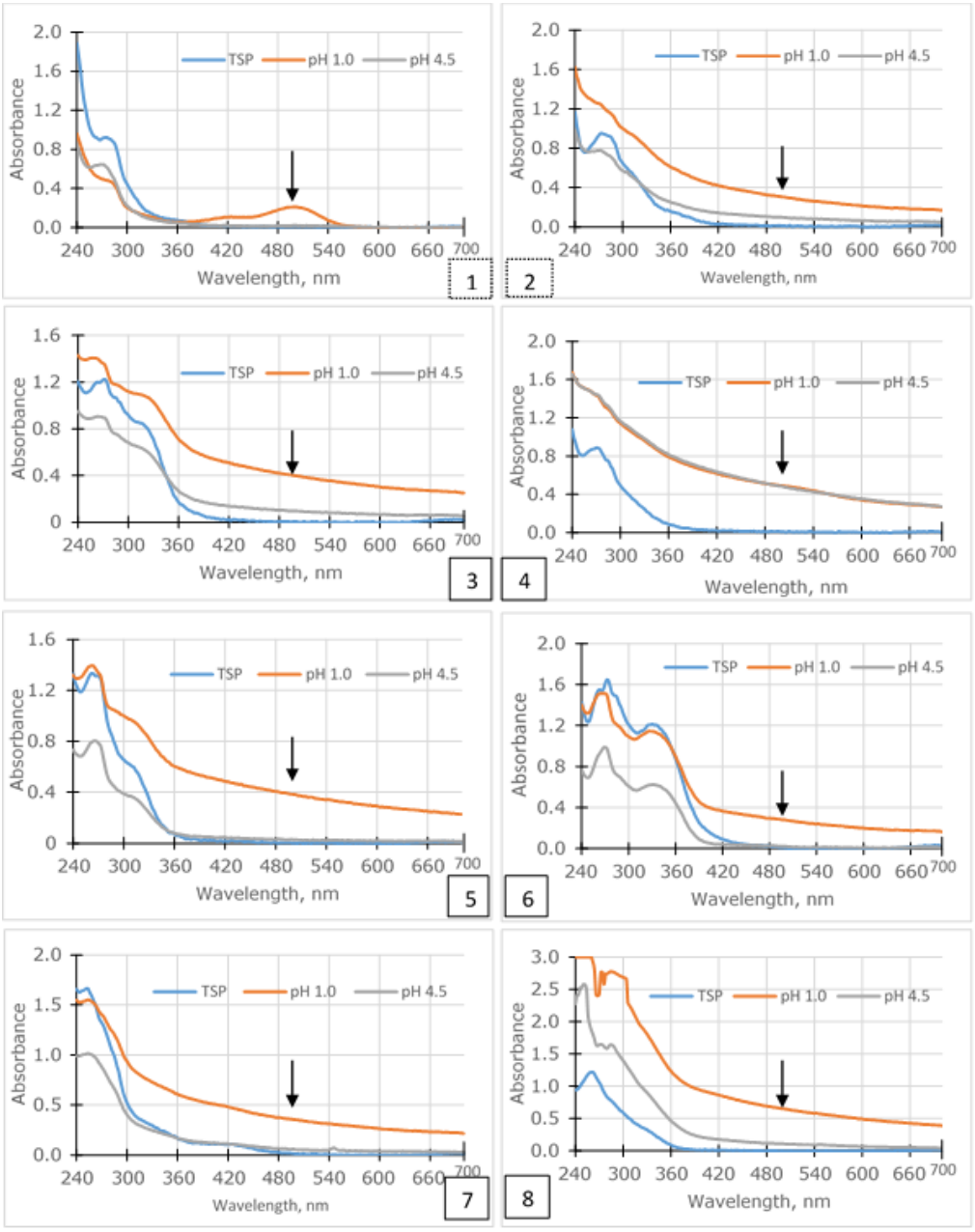
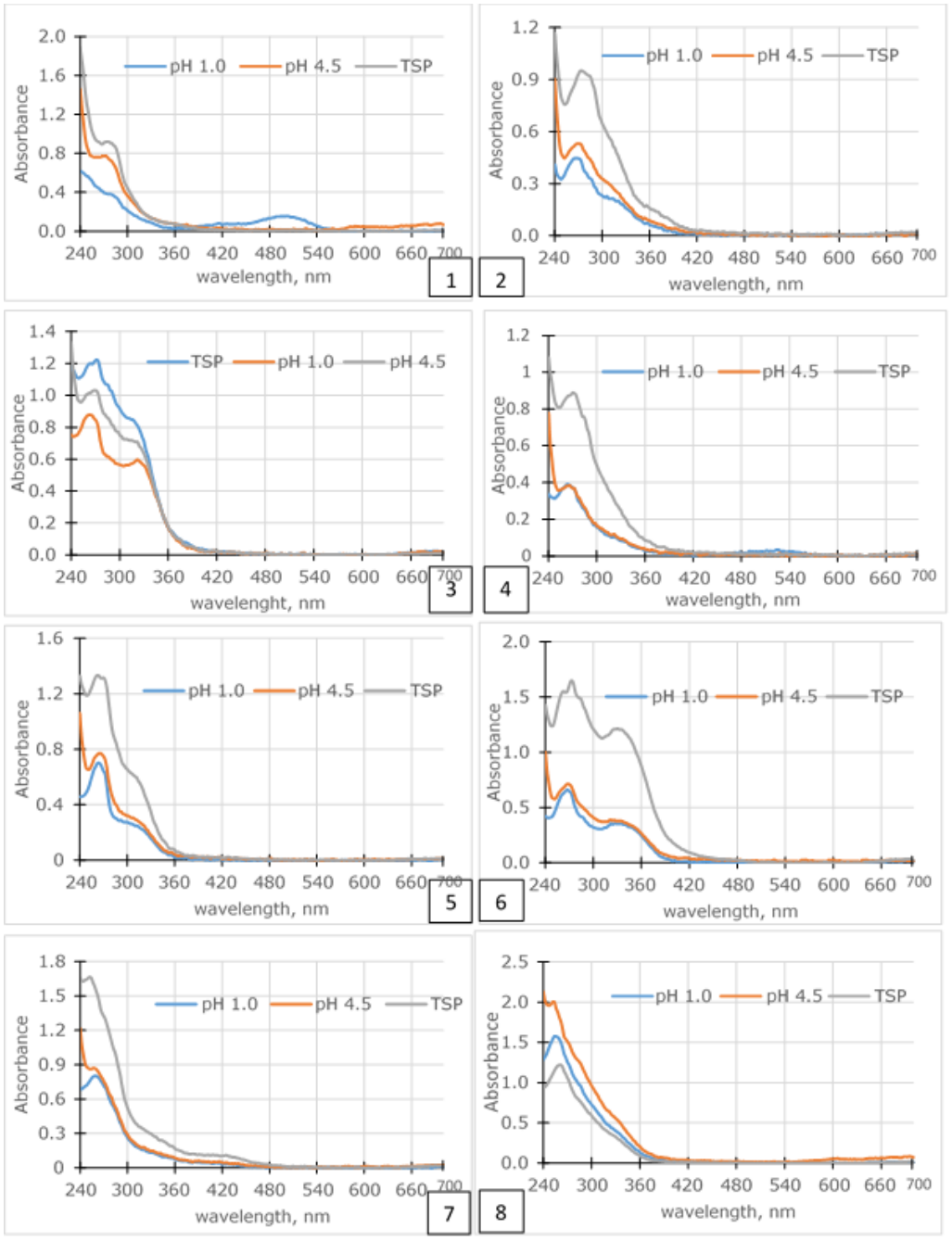
LIST OF APPENDICES

Appendices

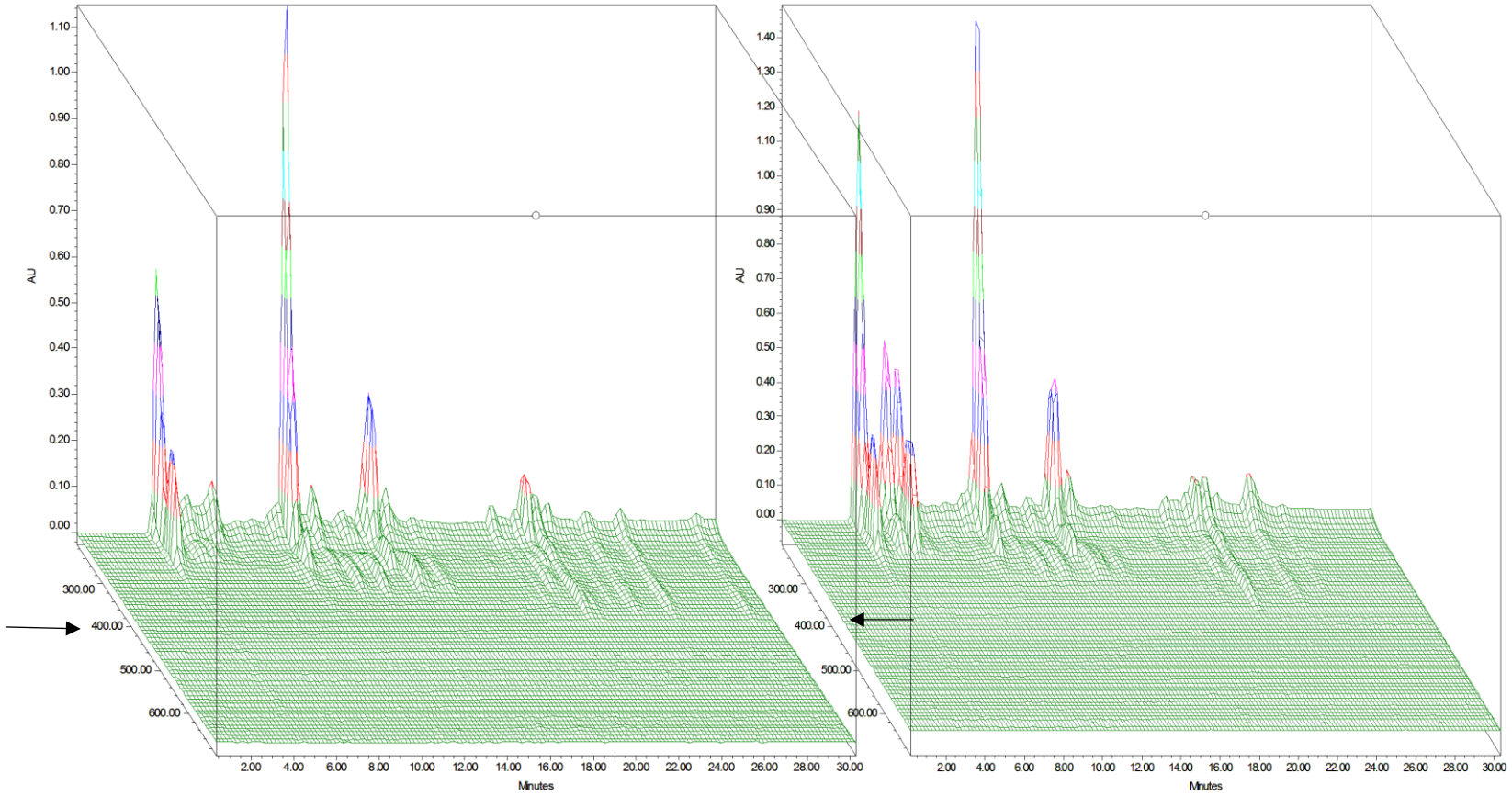
Appendix 3.1 Absorbance spectra in the range of 240 nm to 700 nm for total soluble phenolics (TSP) from methanol extract, after reaction at pH 1.0 and pH 4.5. TSP for all samples were diluted five times however, 25 times of dilution is required for soya bean extract. 1- strawberry, 2- adzuki bean, 3- black eyed pea, 4- bambara groundnut, 5- lablab bean, 6- mung bean, 7- pigeon pea, 8- soya bean



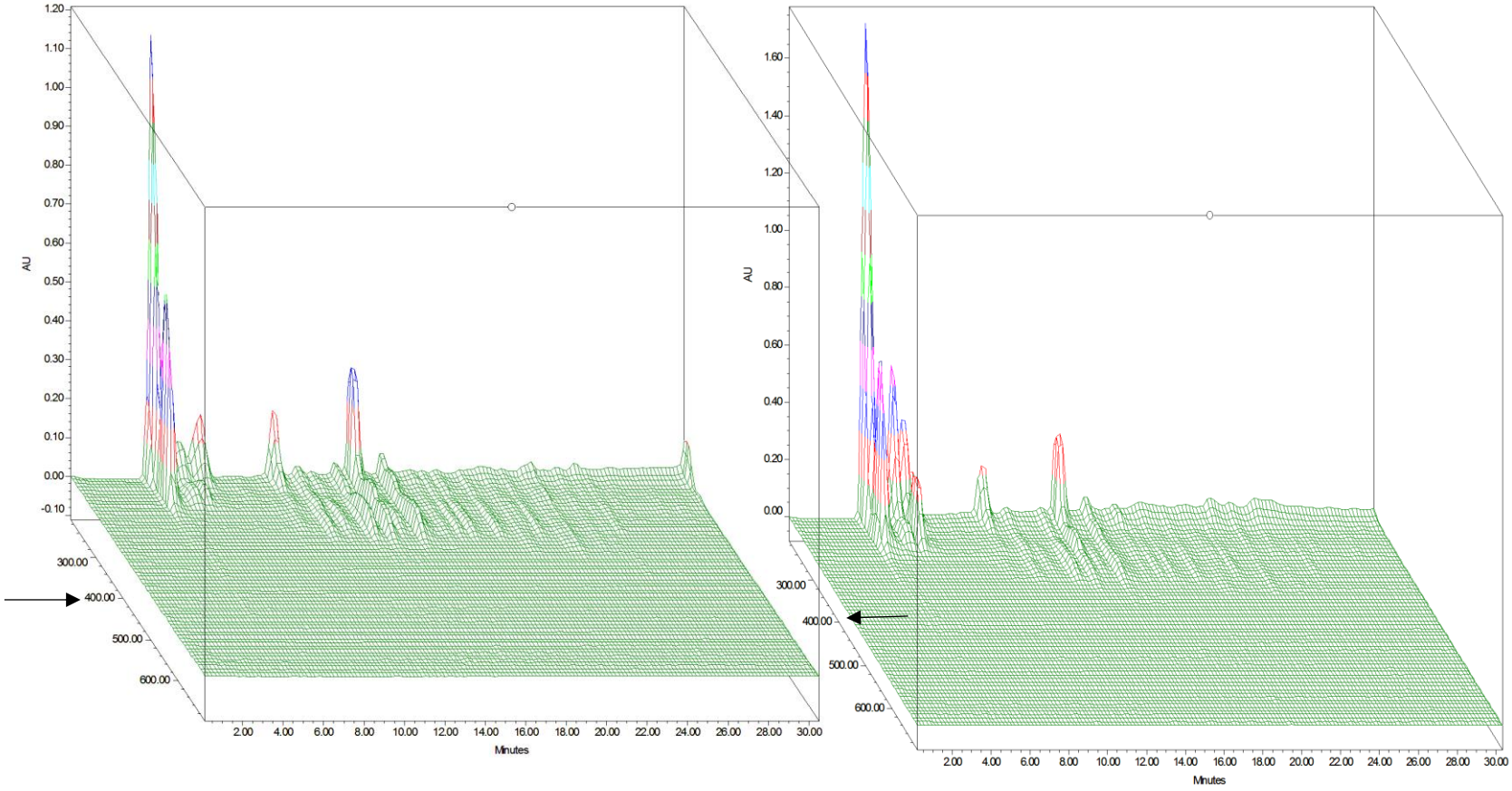
Appendix 3.2 Absorbance spectra in the range of 240 nm to 700 nm for total soluble phenolics (TSP) from CH_3OH extract, after reaction at pH 1.0 and pH 4.5 followed by filtration. TSP for all samples were diluted five times however, 25 times of dilution is required for soya bean extract. 1- strawberry, 2- adzuki bean, 3- black eyed pea, 4- bambara groundnut, 5- lablab bean, 6- mung bean, 7- pigeon pea, 8- soya bean



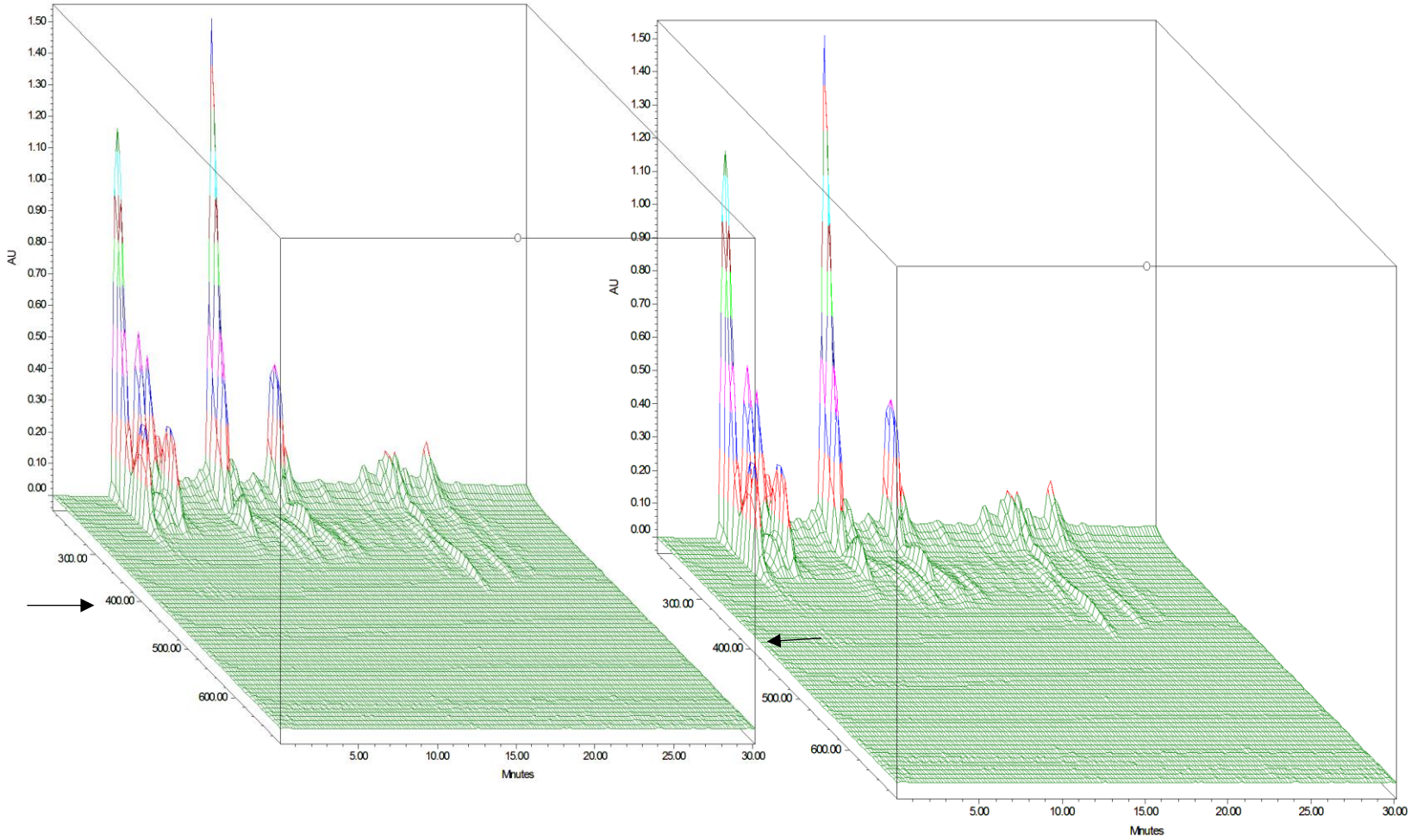
Appendix 4.1 3D maxplot chromatogram for adzuki bean. Chromatogram (Left) represented the 80% CH_3OH extract while (Right) represented the $CH_3COONa.3H_2O$ extract



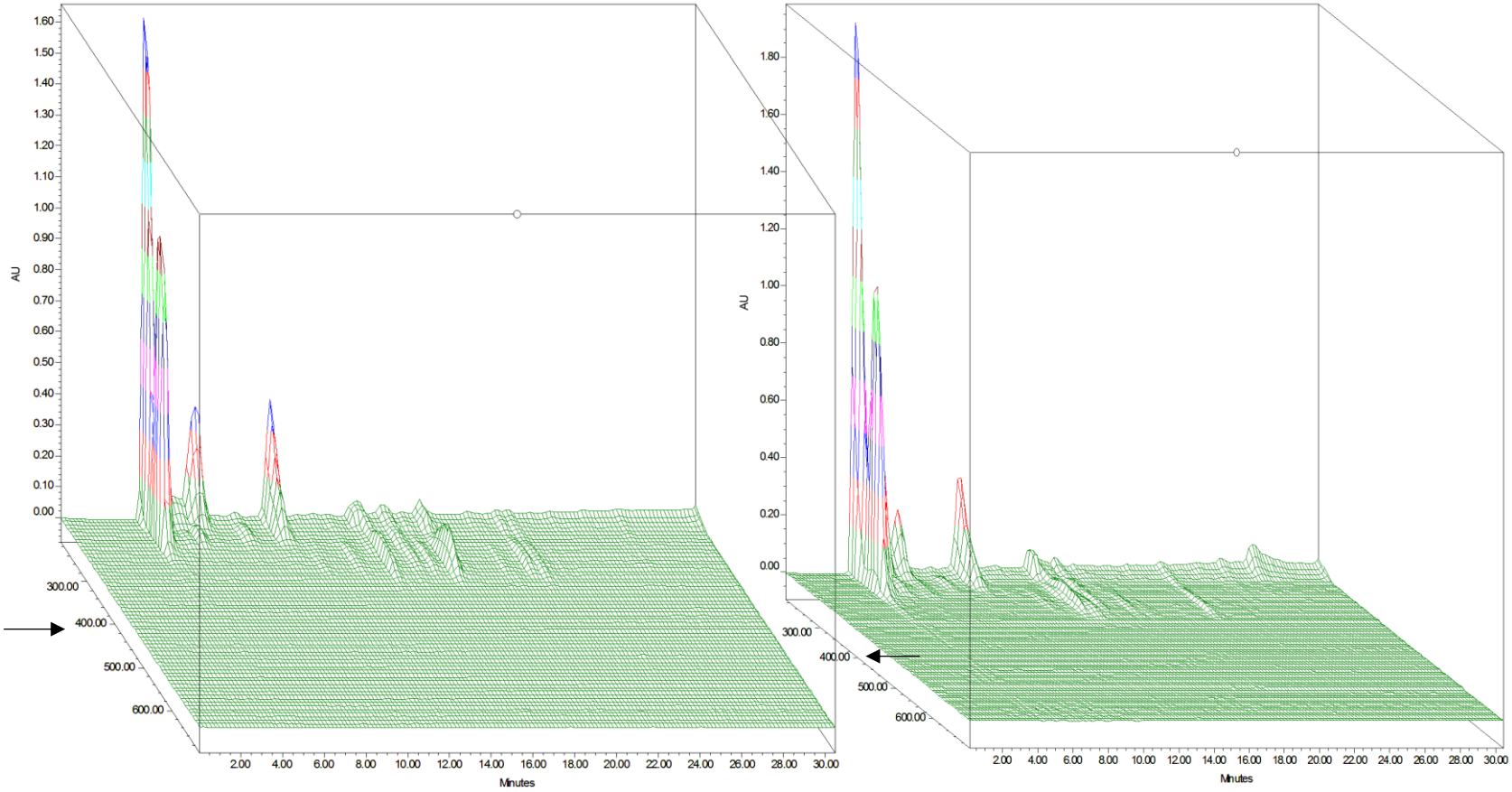
Appendix 4.2 3D maxplot chromatogram for black eyed pea. Chromatogram (Left) represented the 80% CH_3OH extract while (Right) represented the $CH_3COONa.3H_2O$ extract



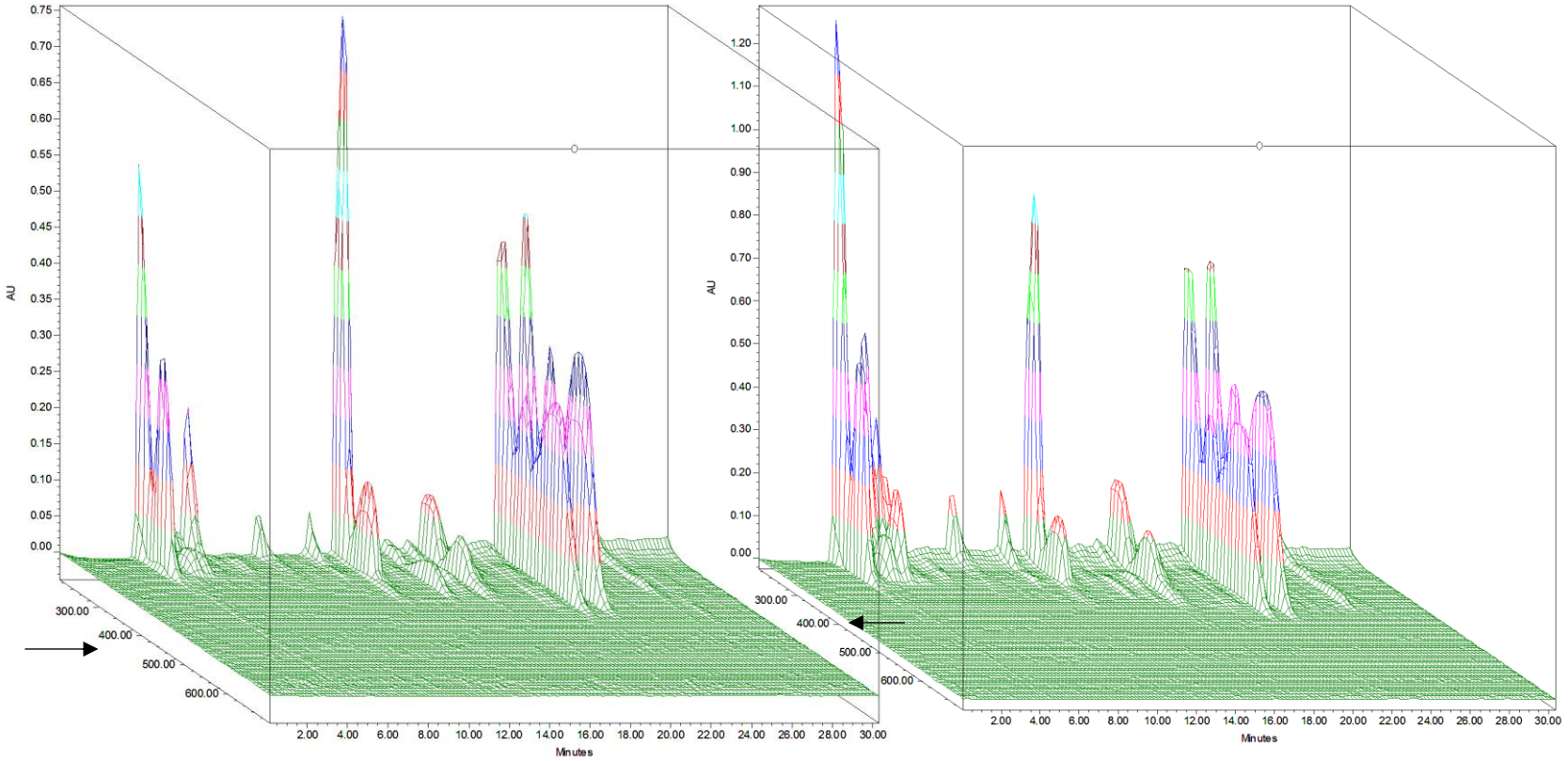
Appendix 4.3 3D maxplot chromatogram for bambara groundnut. Chromatogram (Left) represented the 80% CH_3OH extract while (Right) represented the $CH_3COONa.3H_2O$ buffer extract



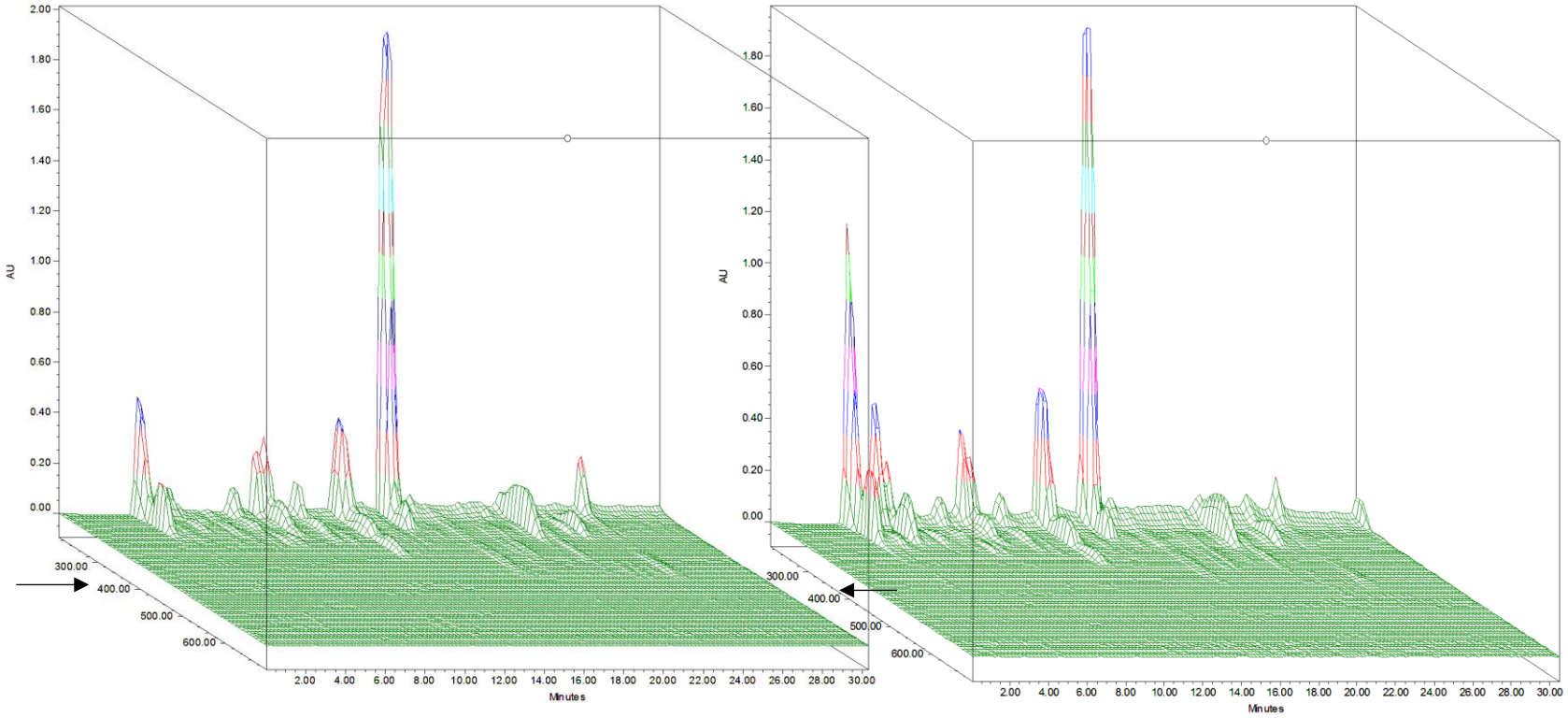
Appendix 4.4 3D maxplot chromatogram for lablab bean. Chromatogram (Left) represented the 80% CH_3OH extract while (Right) represented the $CH_3COONa.3H_2O$ buffer extract



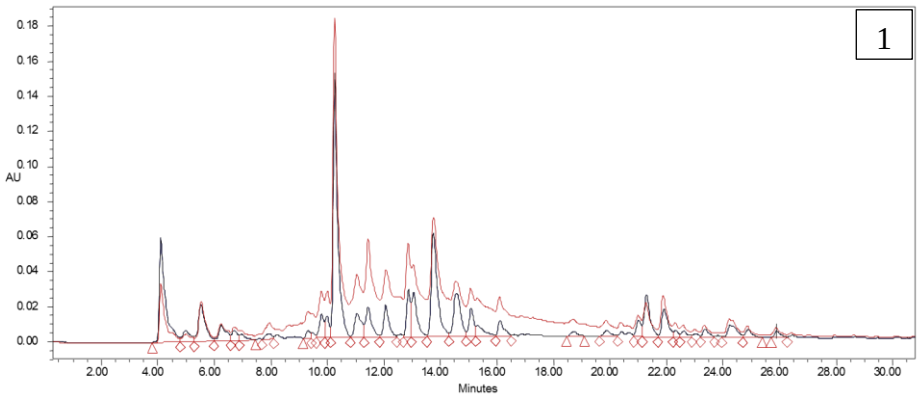
Appendix 4.5 3D maxplot chromatogram for mungbean. Chromatogram (Left) represented the 80% CH_3OH extract while (Right) represented the $CH_3COONa.3H_2O$ buffer extract

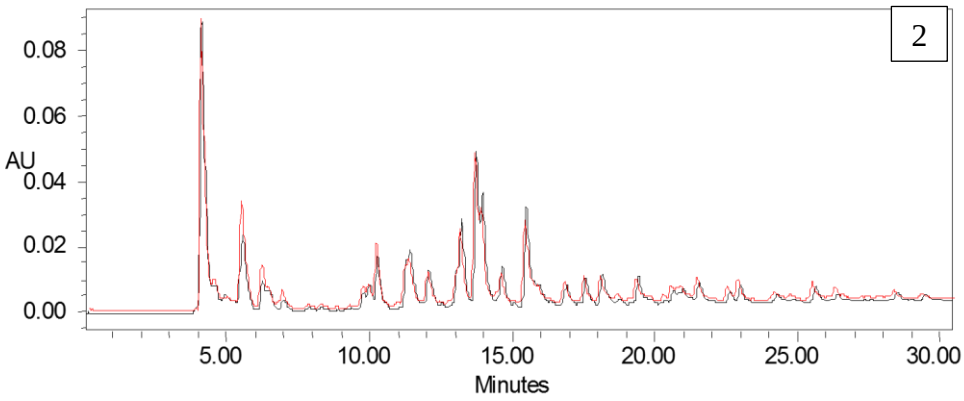


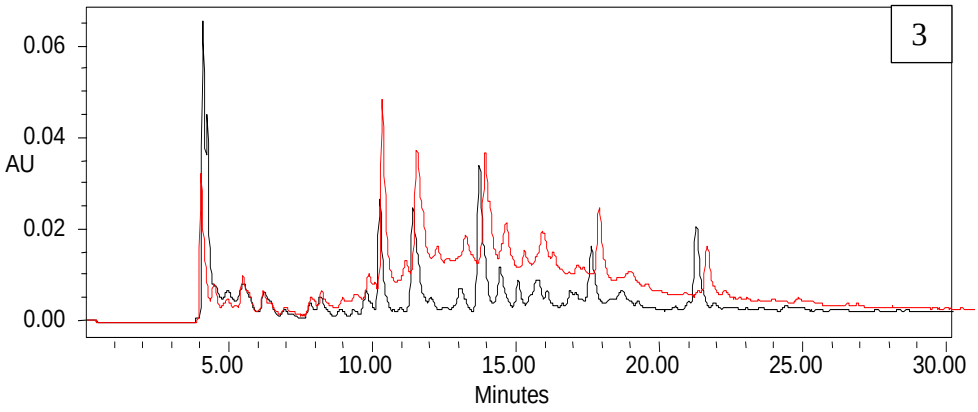
Appendix 4.6 3D maxplot chromatogram for pigeon pea. Chromatogram (Left) represented the 80% CH_3OH extract while (Right) represented the $CH_3COONa.3H_2O$ buffer extract

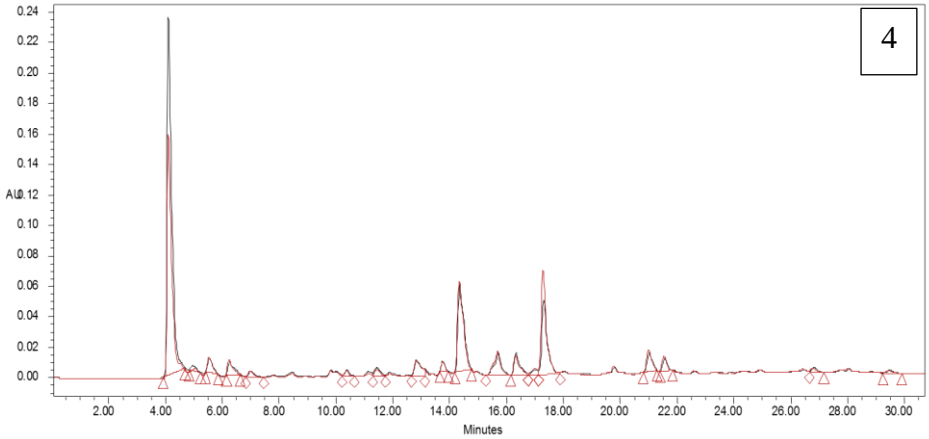


Appendix 4.7 Overlay HPLC chromatogram at 280nm. Red line represents the 80% acetone extract and the black line represents the 80% methanol extract. 1- adzuki bean, 2-black eyed pea, 3- bambara groundnut and 4-lablab bean.

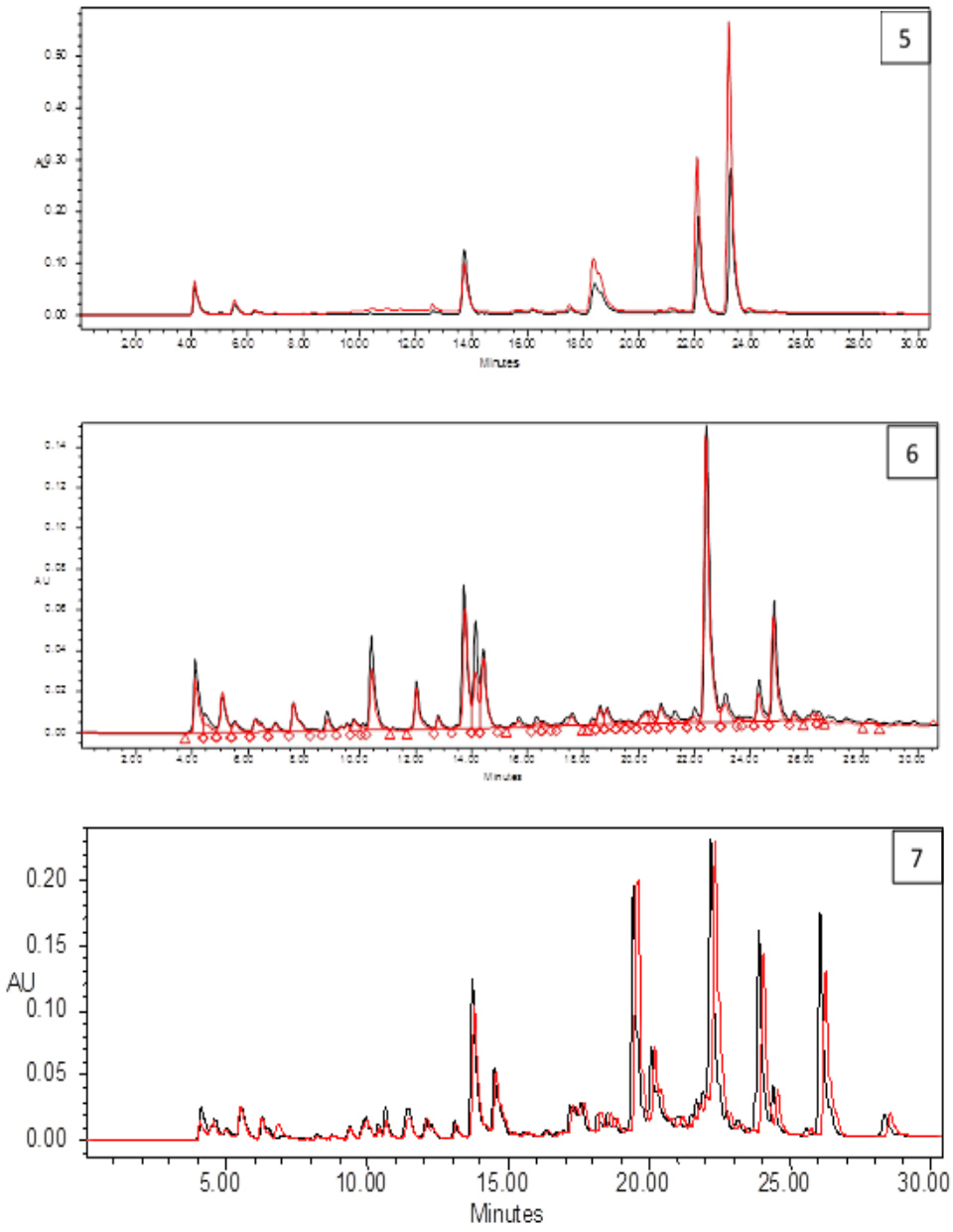




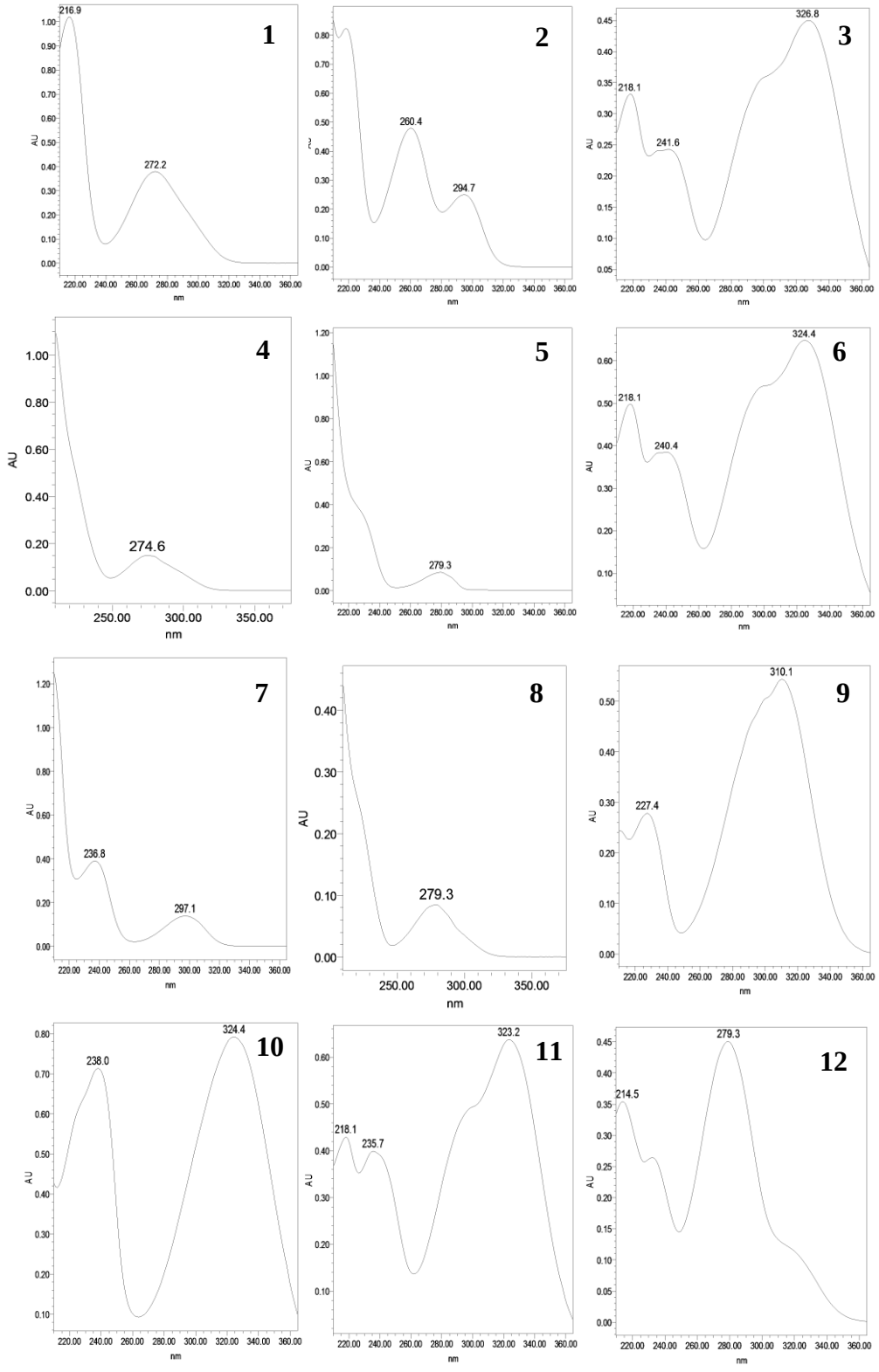




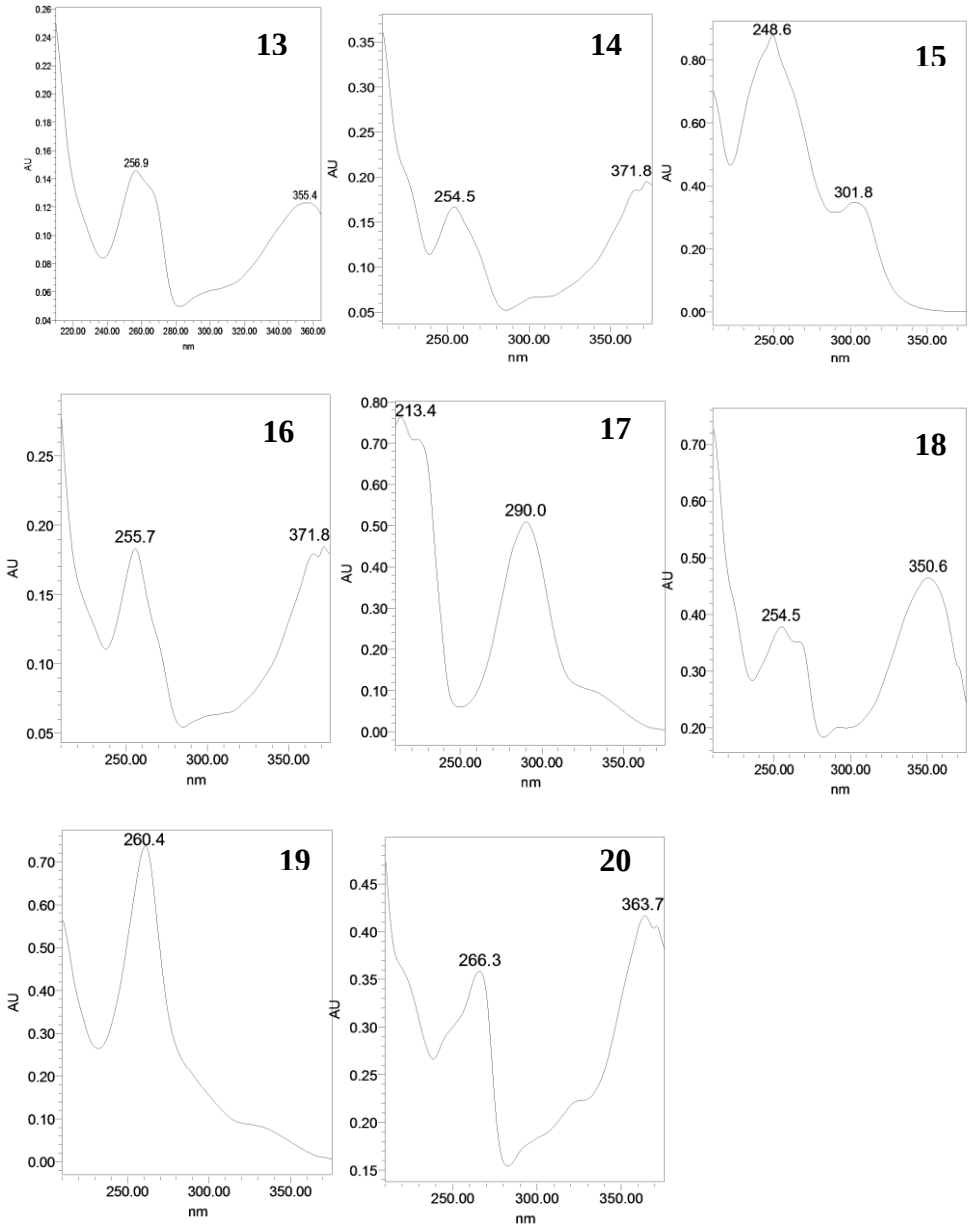
Appendix 4.8 Overlay HPLC chromatogram at 280 nm. Red line represented the 80% acetone extract and the black line represented the 80% methanol extract. 5-mung bean, 6-pigeon pea and 7-soya bean.



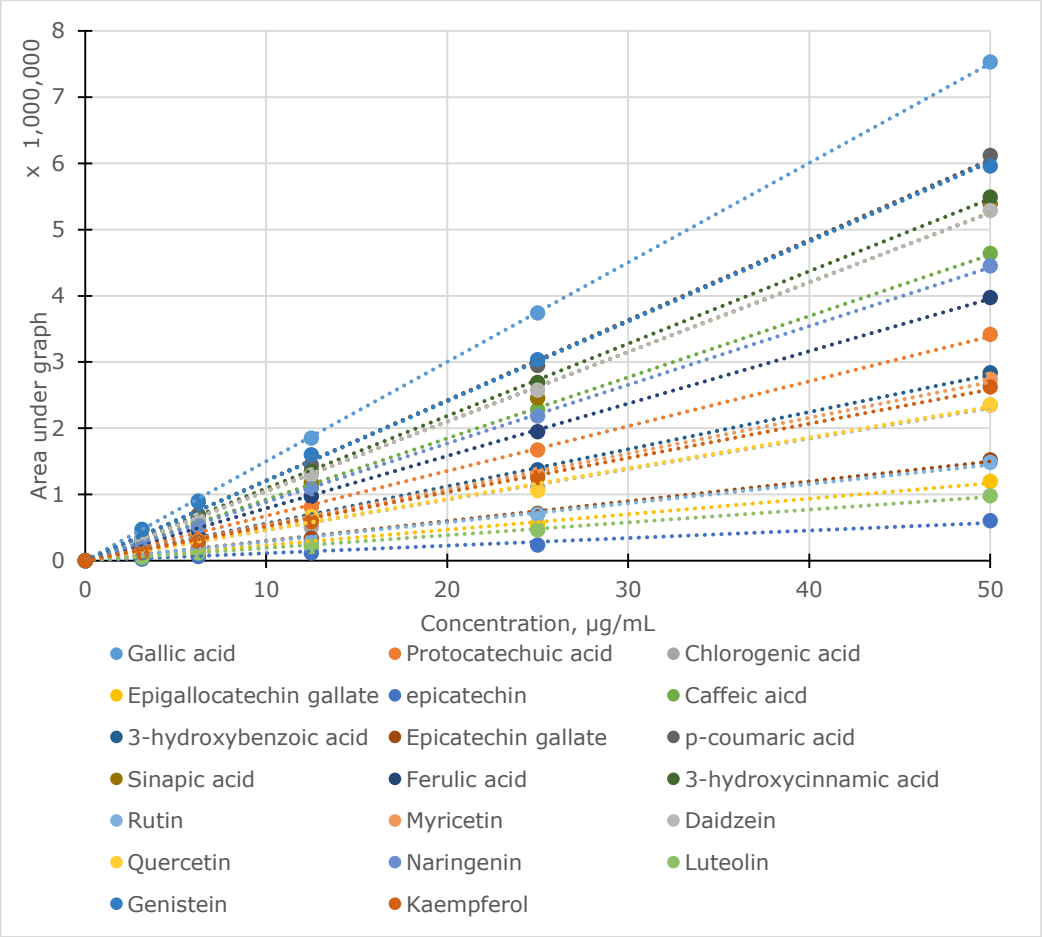
Appendix 4.9 Spectral for phenolics standards.



Appendix 4.10 Spectral for phenolics standards



Appendix 4.11 Standard calibration curve for phenolics standards between 3.13 µg/mL to 50 µg/mL



Appendix 5.1 Profiling of the peaks from untreated soluble sample, 2 and 24 hours acid hydrolysis lablab bean sample

No	Retention time	Spectra, nm	Integrated area at 280 nm, µV*sec		
			Untreated sample	2 hours hydrolysis sample	24 hours hydrolysis sample
1	4.09	265	5,611,674	-	-
2	6.26	258	307,414	-	-
3	11.23	213, 275	249,494	210,857	-
4	12.53	230, 298	261,362	133,182	113,075
5	13.17	219, 279	152,586	-	-
6	14.06	217,288	1,888,366	-	-
7	15.26	260, 299	120,106	-	-
8	15.66	222, 309	134,118	-	-
9	16.82	229, 313	1,216,694	457,627	235,141
10	20.38	227, 310	368,662	324,085	-
11	21.05	220,236, 327	140,972	217,181	281,104
12	14.16	217, 232, 291	-	544,063	476,542
13	15.33	256	-	132,893	104,167
14	17.58	313	-	198,448	360,707
15	29.16	243, 270	-	110,870	-
16	19.48	223, 309	-	-	65,736
17	20.70	211, 310	-	-	327,594
18	22.90	211, 273, 315	-	-	74,357
19	26.12	219, 257, 315	-	-	67,453
20	28.15	230, 315	-	-	65,560
21	29.15	222, 262	-	-	84,201

‘-‘ Not detected

Appendix 5.2 Profiling of the peaks from untreated soluble sample, 2 and 24 hours acid hydrolysis mung bean sample

No	Retention time	Spectra, nm	Integrated area at 280 nm, µV*sec		
			Untreated sample	2 hours hydrolysis sample	24 hours hydrolysis sample
1	4.09	265	2,813,450	-	-
2	13.61	219, 279	4,105,938	-	476,855
3	15.31	226, 314	432,840	164,187	108,606
4	16.90	295	334,324	110,329	112,451
5	17.94	217, 292	5,703,850	-	-
6	21.49	215, 269, 338	12,215,574	3,076,802	4,131,655
7	22.62	213, 270, 338	17,094,018	4,175,308	5,616,799
8	8.72	231, 285	-	781,698	-
9	10.24	252	-	247,244	-
10	11.32	213, 277	-	130,974	-
11	26.54	216, 270, 339	-	424,340	-
12	27.27	213, 273, 339	-	664,426	-

‘-‘ Not detected

Appendix 5.3 Profiling of the peaks from 2 and 24 hours acid hydrolysis bound sample of lablab bean

No	Retention time	Spectra, nm	Integrated area at 280 nm, µV*sec	
			2 hours hydrolysis sample	24 hours hydrolysis sample
1	11.10	229, 314	89,023	93,714
2	14.18	218, 288	75,125	62,636
3	15.39	256	116,534	91,014
4	16.21	227, 309	473,587	438,173
5	17.65	229, 313	192,370	233,787
6	20.80	227, 311	270,951	553,817
7	21.37	217, 321	92,946	145,179
8	23.01	215, 271, 312	42,891	-
9	26.19	269	39,649	71,119

Appendix 5.4 Profiling of the peaks from 2 and 24 hours acid hydrolysis bound sample of mung bean

No	Retention time	Spectra, nm	Integrated area at 280 nm, µV*sec	
			2 hours hydrolysis sample	24 hours hydrolysis sample
1	16.20	229, 314	87,948	102,423
2	17.30	211, 295	68,831	129,350
3	18.42	294	99,300	364,260
4	20.53	212, 257, 349	60,314	155,557
5	20.92	212, 271, 349	155,858	465,478
6	21.84	216, 270, 339	6,321,421	15,293,095
7	23.01	215, 271, 338	9,452,183	21,583,465

‘-‘ Not detected

Appendix 5.5 Profiling of the peaks from untreated soluble sample, 2 and 24 hours alkaline hydrolysis lablab bean sample

No	Retention time	Spectra, nm	Integrated area at 280 nm, µV*sec		
			Untreated sample	2 hours hydrolysis sample	24 hours hydrolysis sample
1	4.09	265	5,611,674	-	-
2	6.26	258	307,414	-	-
3	11.23	213, 275	243,494	973,981	766,196
4	12.53	230, 298	261,362	376,640	310,349
5	13.17	219, 279	152,586	-	-
6	14.06	217,288	1,888,366	1,373,603	1,075,147
7	15.26	260, 299	120,106	163,932	186,808
8	15.66	222, 309	134,118	379,105	326,484
9	16.82	229, 313	1,216,694	-	-
10	20.38	227, 310	368,662	2,711,911	2,600,549
11	21.05	220,236, 327	140,972	478,110	491,252
12	25.94	267	-	646,220	509,123

Appendix 5.6 Profiling of the peaks from untreated soluble sample, 2 and 24 hours alkaline hydrolysis mung bean sample

No	Retention time	Spectra, nm	Integrated area at 280 nm, µV*sec		
			Untreated sample	2 hours hydrolysis sample	24 hours hydrolysis sample
1	4.09	265	2,813,450	-	-
2	13.01	219, 279	4,105,938	628,699	619,581
3	15.31	226, 314	432,840	-	-
4	16.90	295	334,324	279,971	316,749
5	17.94	217, 292	5,703,850	627,765	-
6	21.49	215, 269, 338	12,215,574	12,673,542	11,977,829
7	22.62	213, 270, 338	17,094,018	15,508,748	12,585,101
8	11.21	213, 275	-	1,064,056	948,537
9	18.40	220, 276	-	1,961,691	4,342,154
10	19.05	272, 305	-	603,300	485,405
11	20.28	227, 310	-	522,021	536,618
12	26.32	215, 271, 336	-	-	380,672
13	28.13	276, 332	-	-	650,359

Appendix 5.7 Profiling of the peaks from 2 and 24 hours alkaline hydrolysis bound sample of lablab bean

No	Retention time	Spectra, nm	Integrated area at 280 nm, µV*sec	
			2 hours hydrolysis sample	24 hours hydrolysis sample
1	12.54	297.0	57,039	-
2	14.04	215, 290	121,767	-
3	15.12	257	65,116	117,957
4	15.82	220, 310	100,375	-
5	16.42	220, 268, 288	68,127	133,670
6	20.53	227, 310	1,118,387	1,814,134
7	26.07	269	88,940	85,347

Appendix 5.8 Profiling of the peaks from 2 and 24 hours alkaline hydrolysis bound sample of mung bean sample

No	Retention time	Spectra, nm	Integrated area at 280 nm, µV*sec	
			2 hours hydrolysis sample	24 hours hydrolysis sample
1	9.41	219, 305	153,186	139,762
2	10.53	291	135,929	351,908
3	13.26	231, 281, 310	314,912	437,368
4	16.94	217, 291	173,481	137,453
5	18.51	220, 275	1,298,479	4,421,164
6	20.41	227,312	458,204	492,012
7	21.59	215, 269, 338	6,135,235	7,375,520
8	22.72	215, 269, 338	6,609,498	5,790,948
9	24.11	242	-	429,136

‘-‘ Not detected

Appendix 5.9 Initial percentage of methanol before acetonitrile liquid/liquid partitioning

	Concentration (%)				
	1	2	3	4	5
Water	100	90	80	70	-
CH_3OH	-	10	20	30	100

*sample 1 represented the positive control

Appendix 5.10 Observation result of partitioning formation at different combination of solvents

Sodium chloride	Sample (n=5)				
(w/v), %	1	2	3	4	5
0	_	_	_	_	_
1	_	_	_	_	_
2	_	_	_	_	_
4	+	+	_	_	_
6	+	+	+	_	_
8	+	+	+	_	_
10	+	NA	+	_	_
20	NA	NA	NA	_	_
30	NA	NA	NA	_ (cloudy)	NA
40	NA	NA	NA	NA	_ (cloudy)
50	NA	NA	NA	_ (cloudy)	NA
60	NA	NA	NA	NA	_ (cloudy)
70	NA	NA	NA	_ (cloudy)	NA

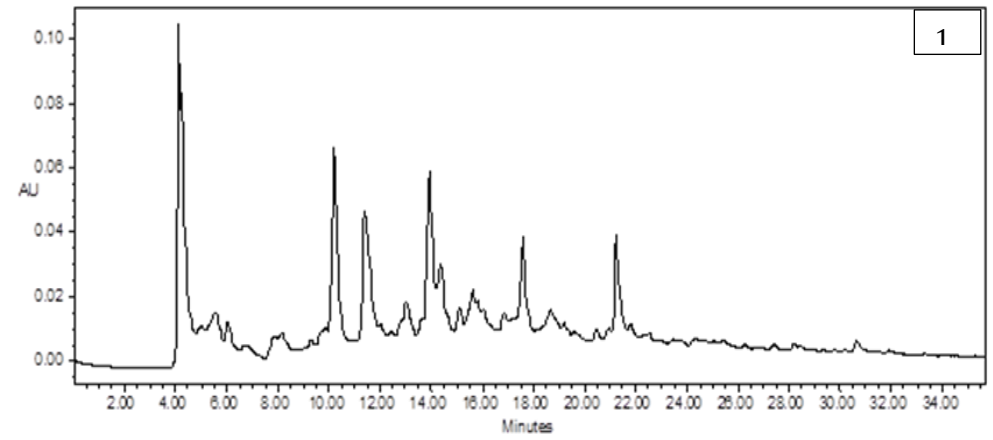
* '+' represented formation of partition layer

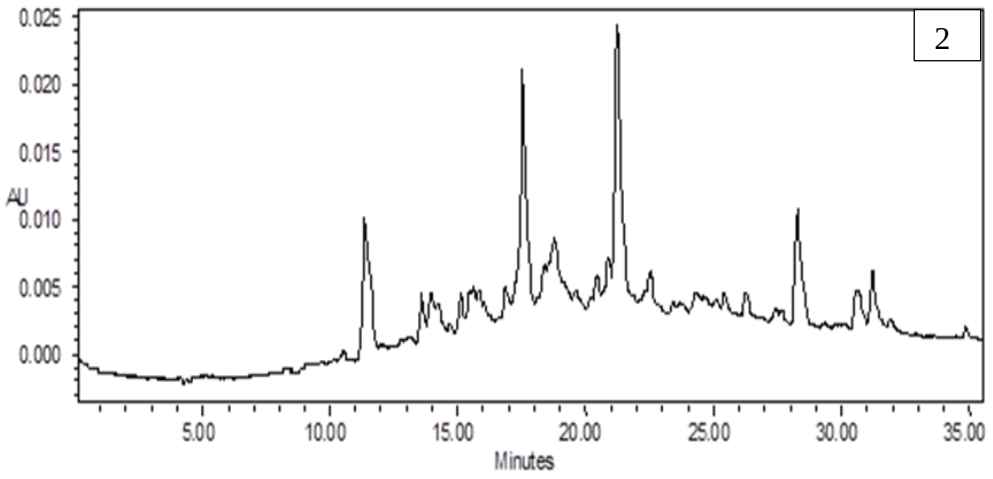
* '_'represented no formation of partition layer

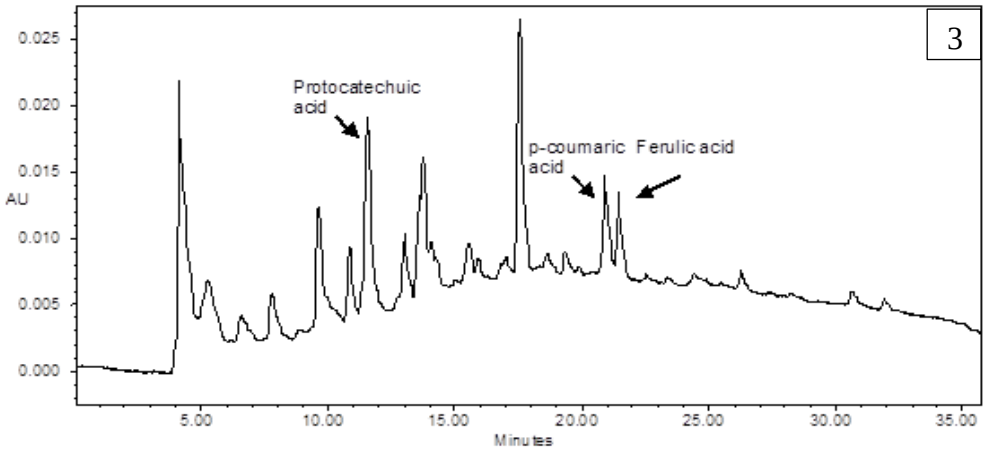
* NA represented no data available due to no experiment has been carried out

* sample 1 represented the positive control

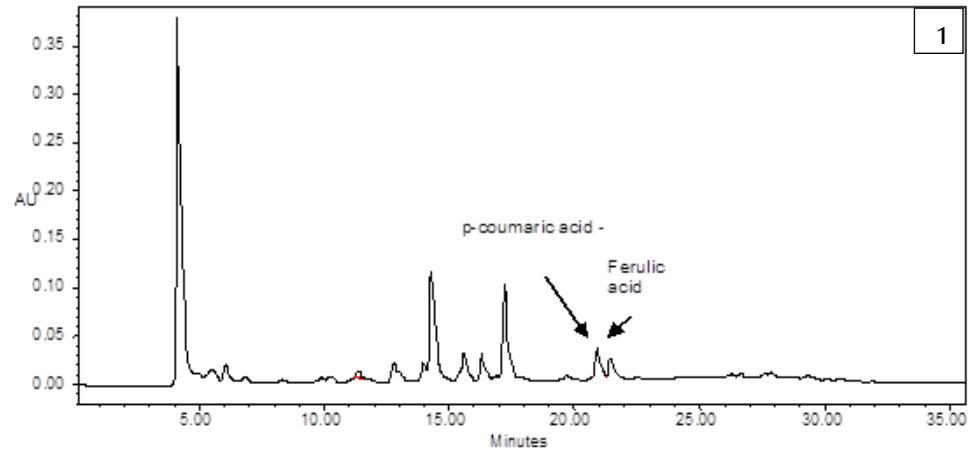
Appendix 6.1 HPLC chromatogram for bambara groundnut extracts detected at 280 nm. 1- soluble phenolic sample, 2- deconjugated phenolic sample, 3- bound phenolic sample

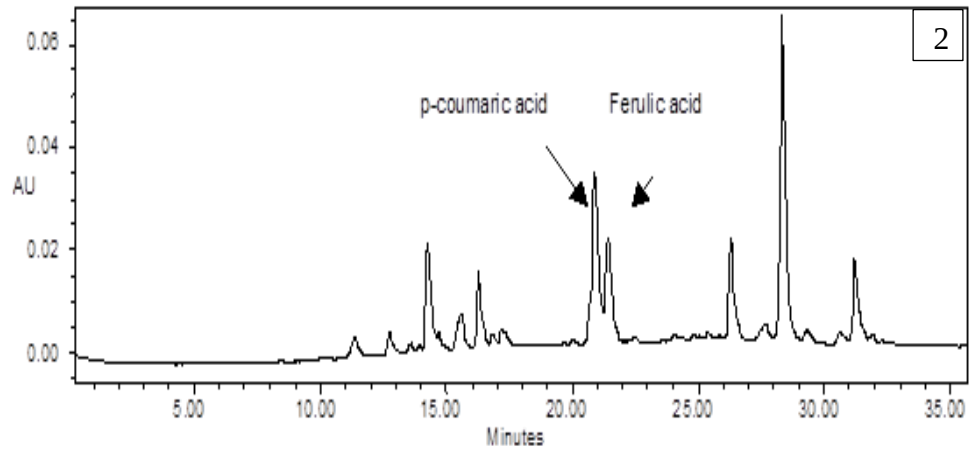


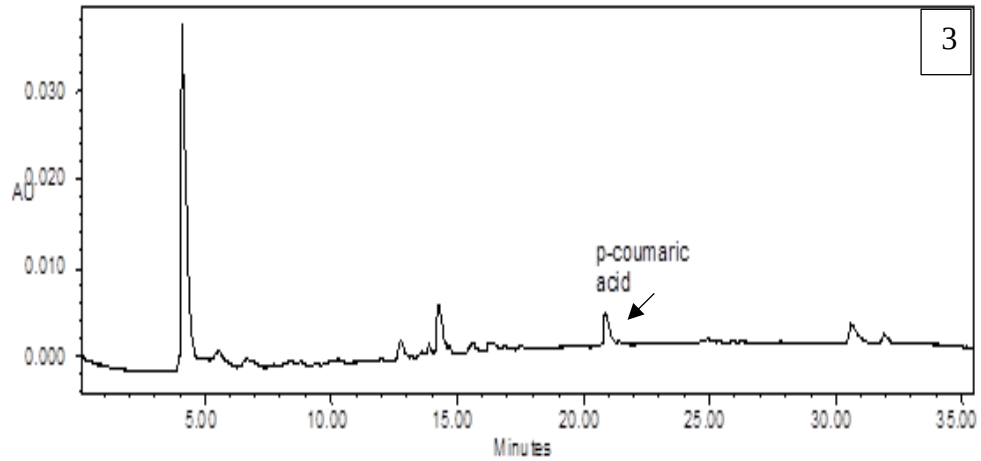




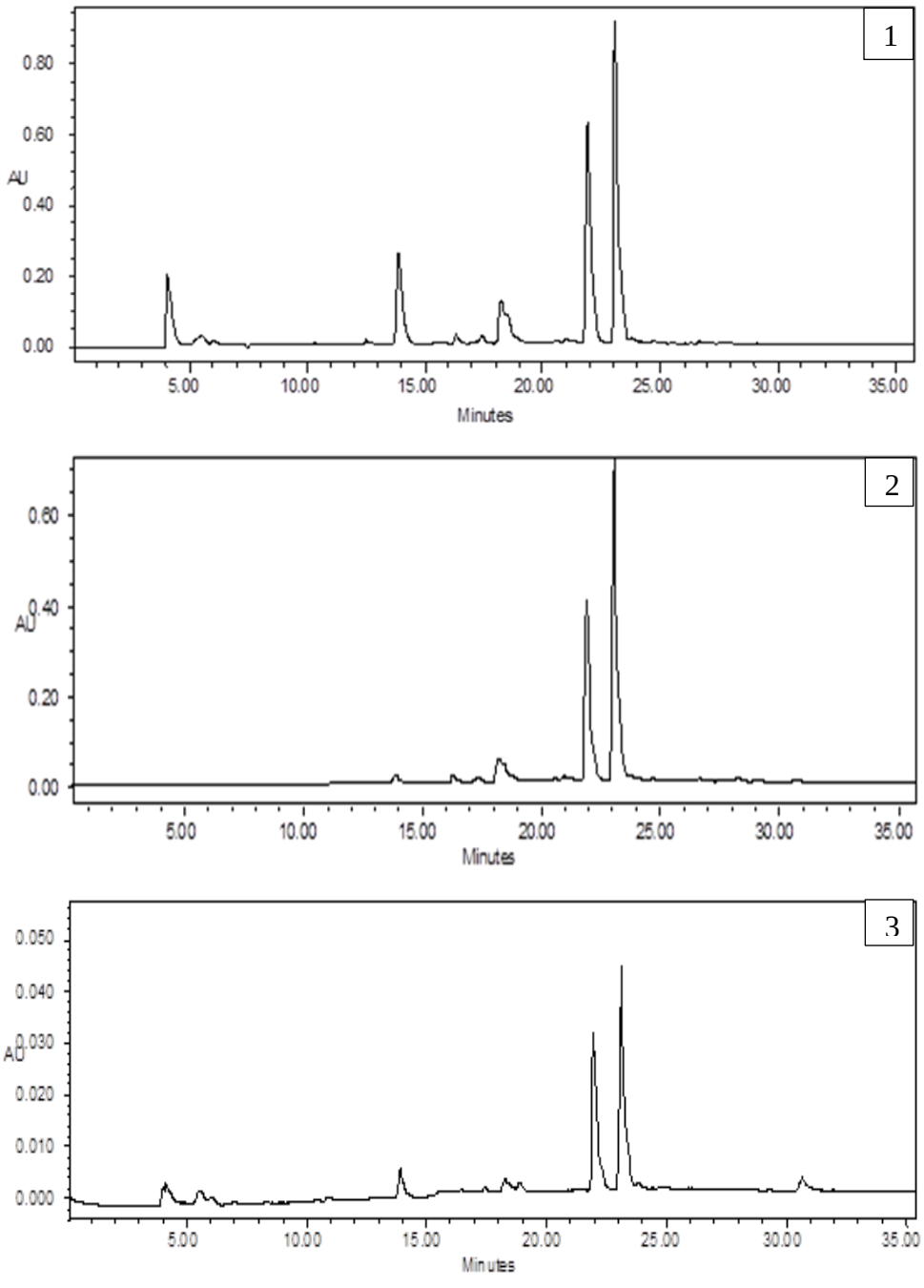
Appendix 6.2 HPLC chromatogram for lablab bean extracts detected at 280 nm. 1- soluble phenolic sample, 2- deconjugated phenolic sample, 3- bound phenolic sample



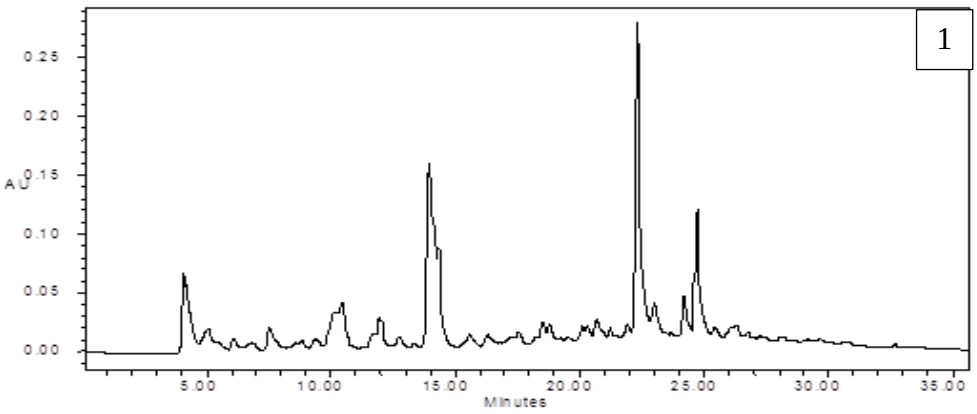


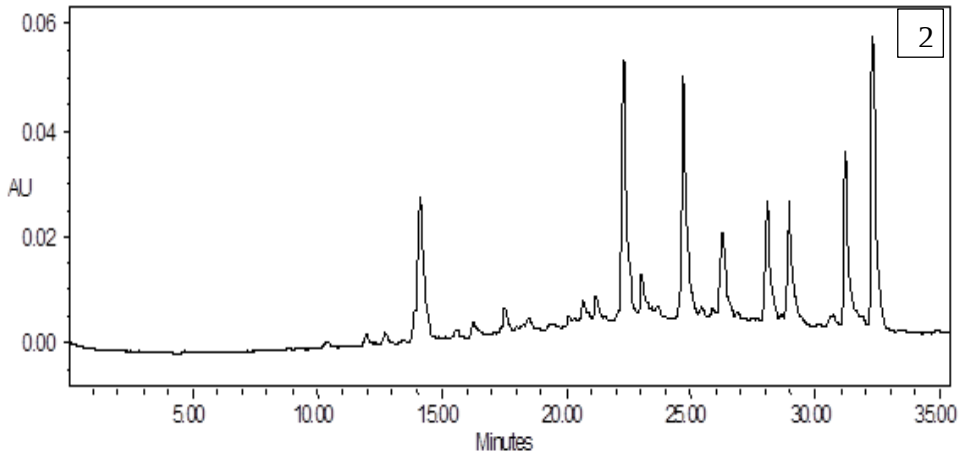


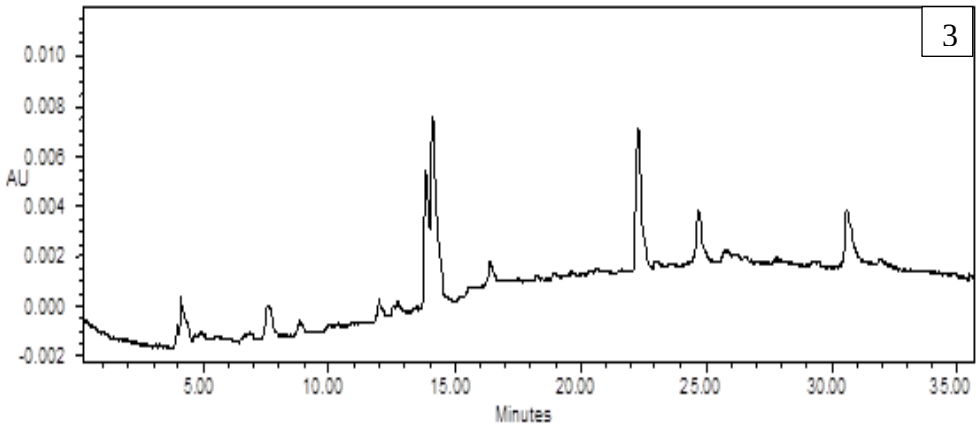
Appendix 6.3 HPLC chromatogram for mung bean extracts detected at 280 nm. 1- soluble phenolic sample, 2- deconjugated phenolic sample, 3- bound phenolic sample



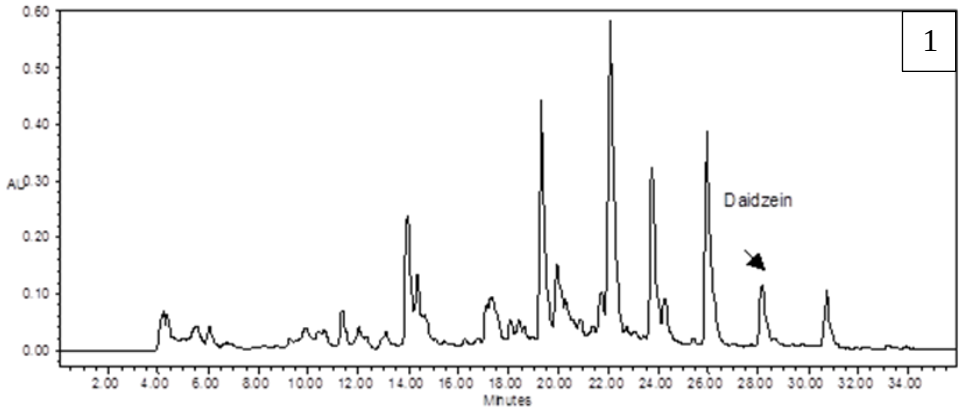
Appendix 6.4 HPLC chromatogram for pigeon pea extract detected at 280 nm. 1- soluble phenolic sample, 2- deconjugated phenolic sample, 3- bound phenolic sample

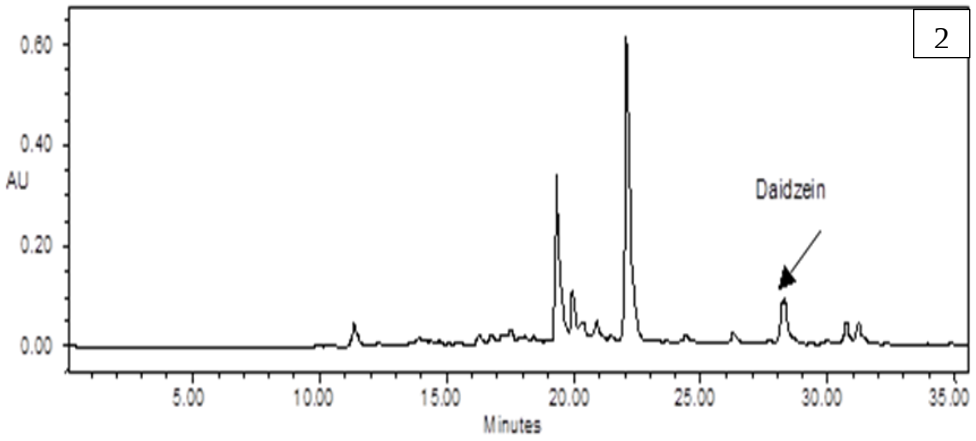


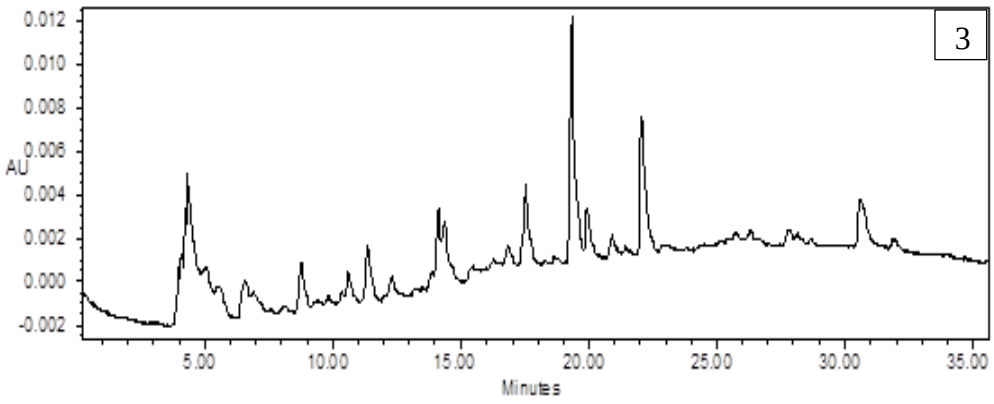




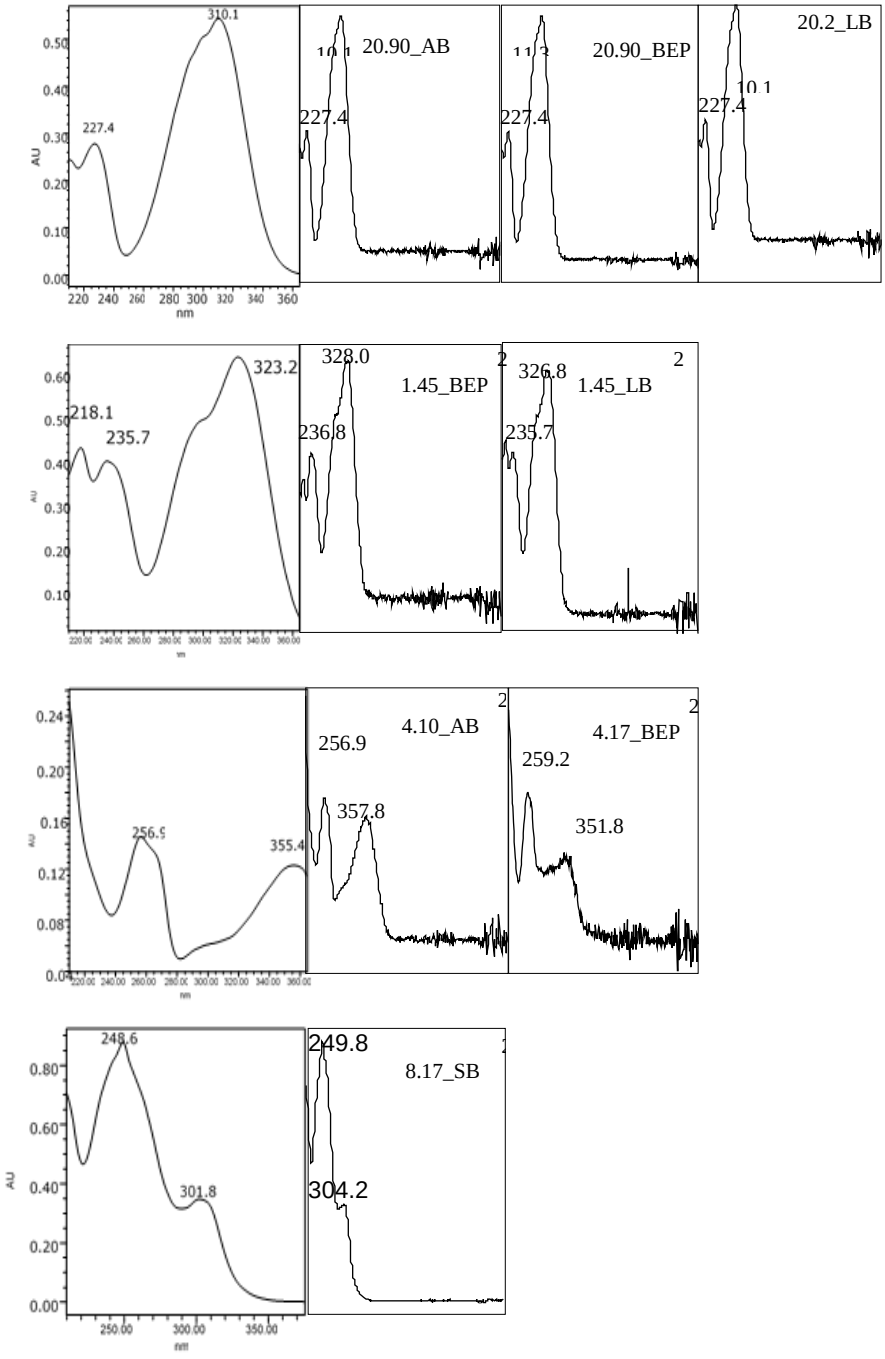
Appendix 6.5 HPLC chromatogram for soya bean extracts detected at 280 nm. 1- soluble phenolic sample, 2- deconjugated phenolic sample, 3- bound phenolic sample



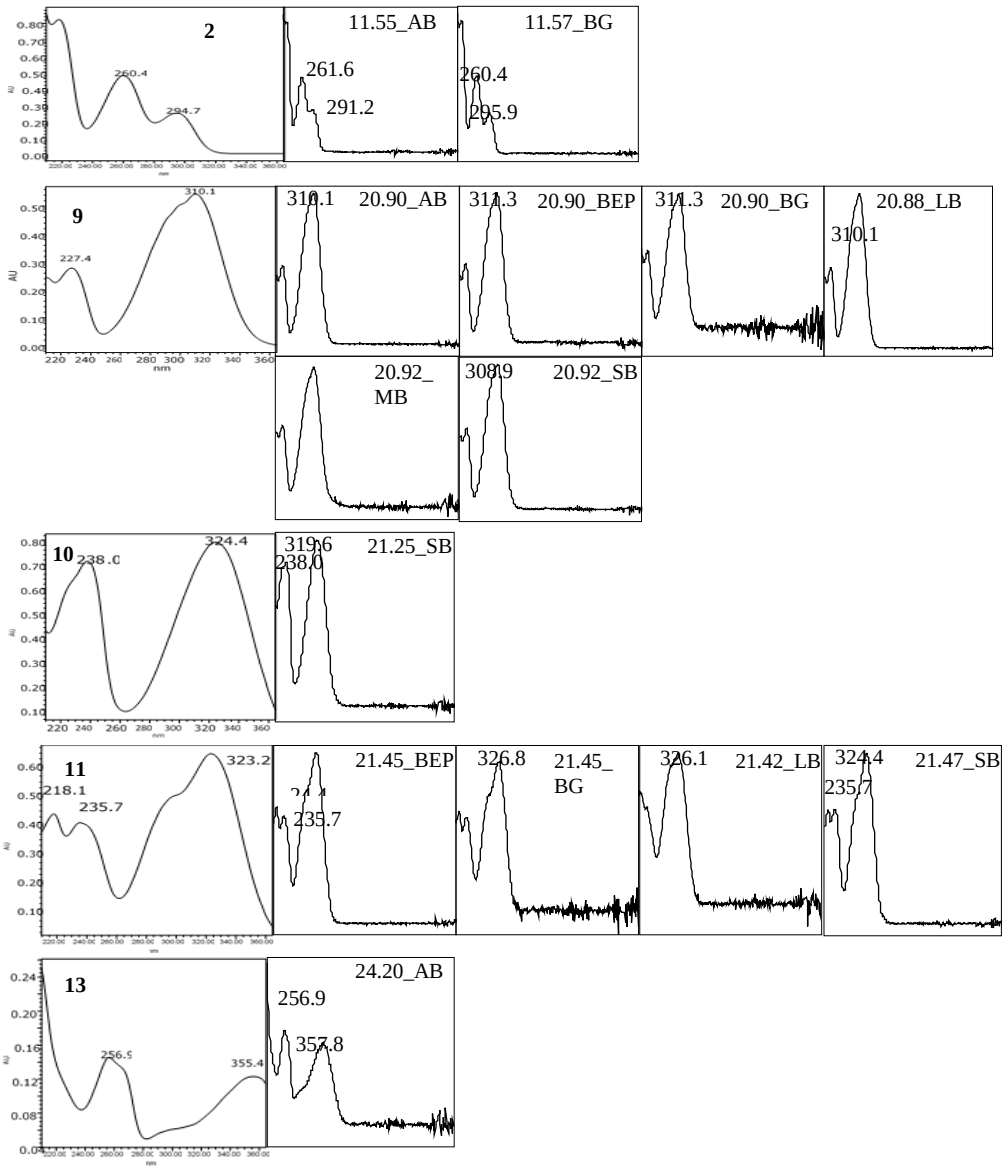




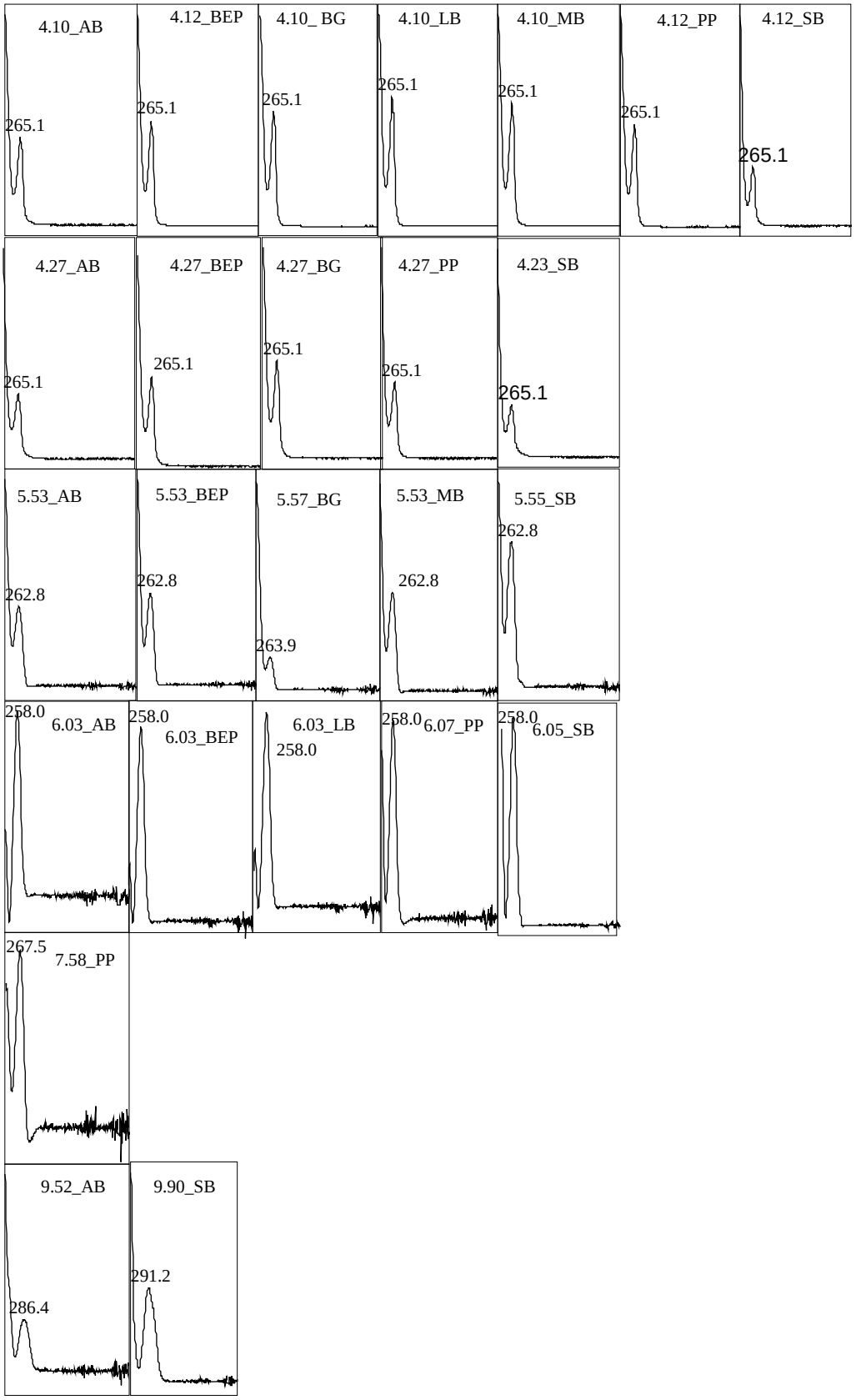
Appendix 6.6 Spectra for the identified targeted soluble phenolics that matched with phenolic standards. Label 9 - ρ-coumaric acid, 11 – ferulic acid, 13 – rutin, 15 –daidzein, AB - adzuki bean, BEP – black eyed pea, LB – lablab bean, SB – soya bean



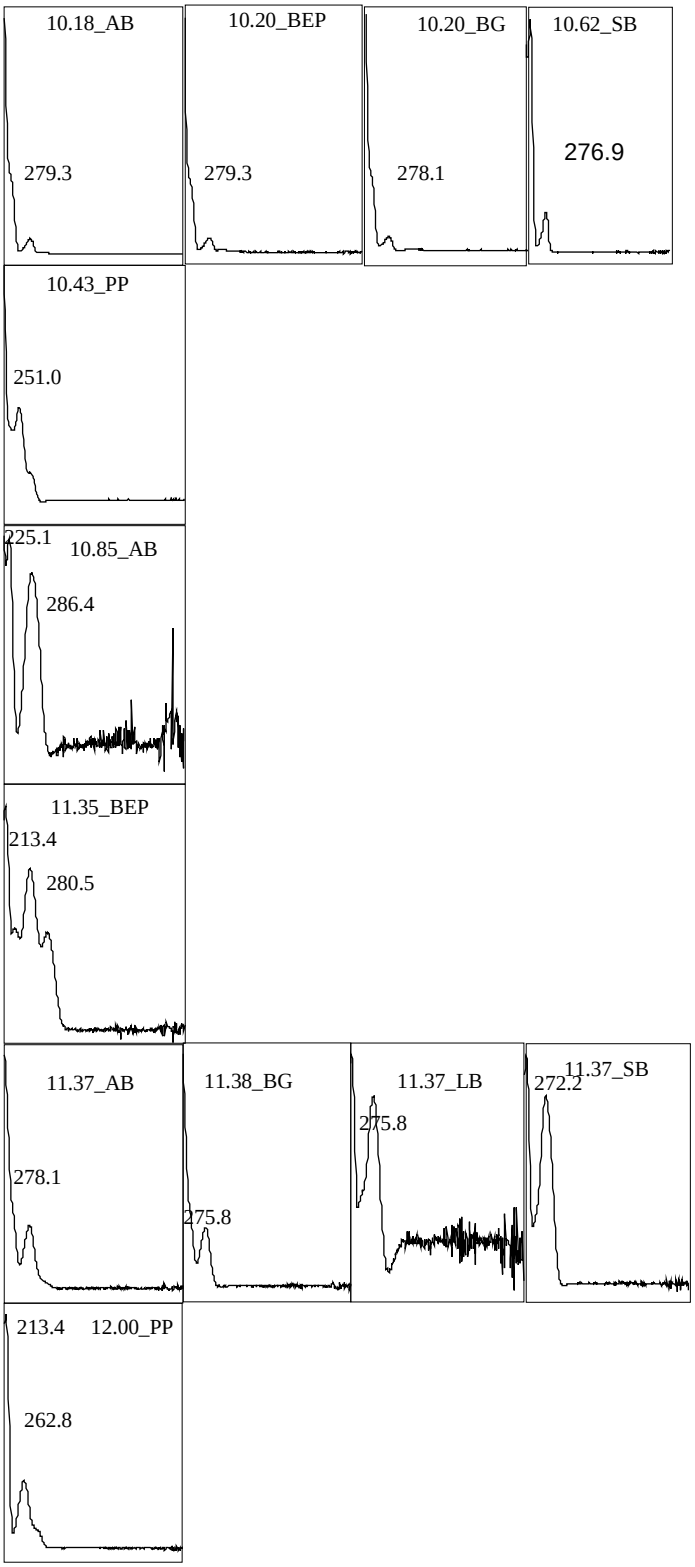
Appendix 6.7 Spectra for the identified targeted bound phenolics that matched with phenolic standards. Label 9 - ρ-coumaric acid, 11 – ferulic acid, 13 – rutin, 15 –daidzein, AB - adzuki bean, BEP – black eyed pea, LB – lablab bean, SB – soya bean



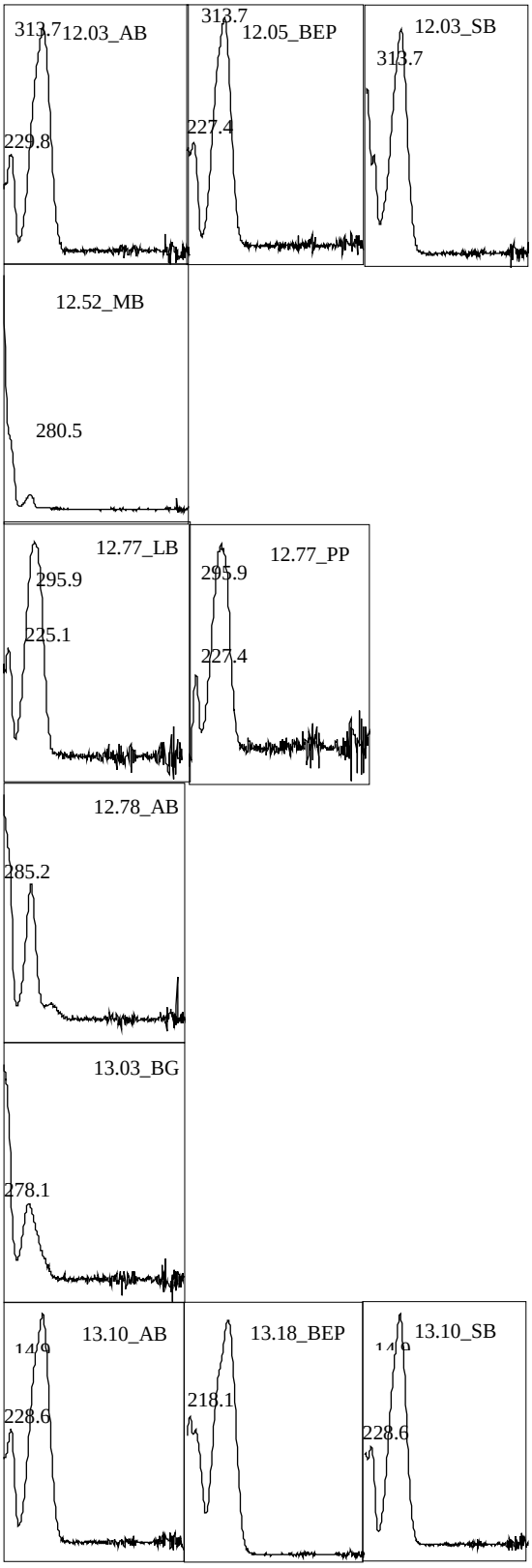
Appendix 6.8 Spectra for each unidentified soluble compound detected at different time interval and at 280 nm for adzuki bean (AB), black eyed pea (BEP), bambara groundnut (BG), lablab bean (LB), mung bean (MB) and pigeon pea (PP)



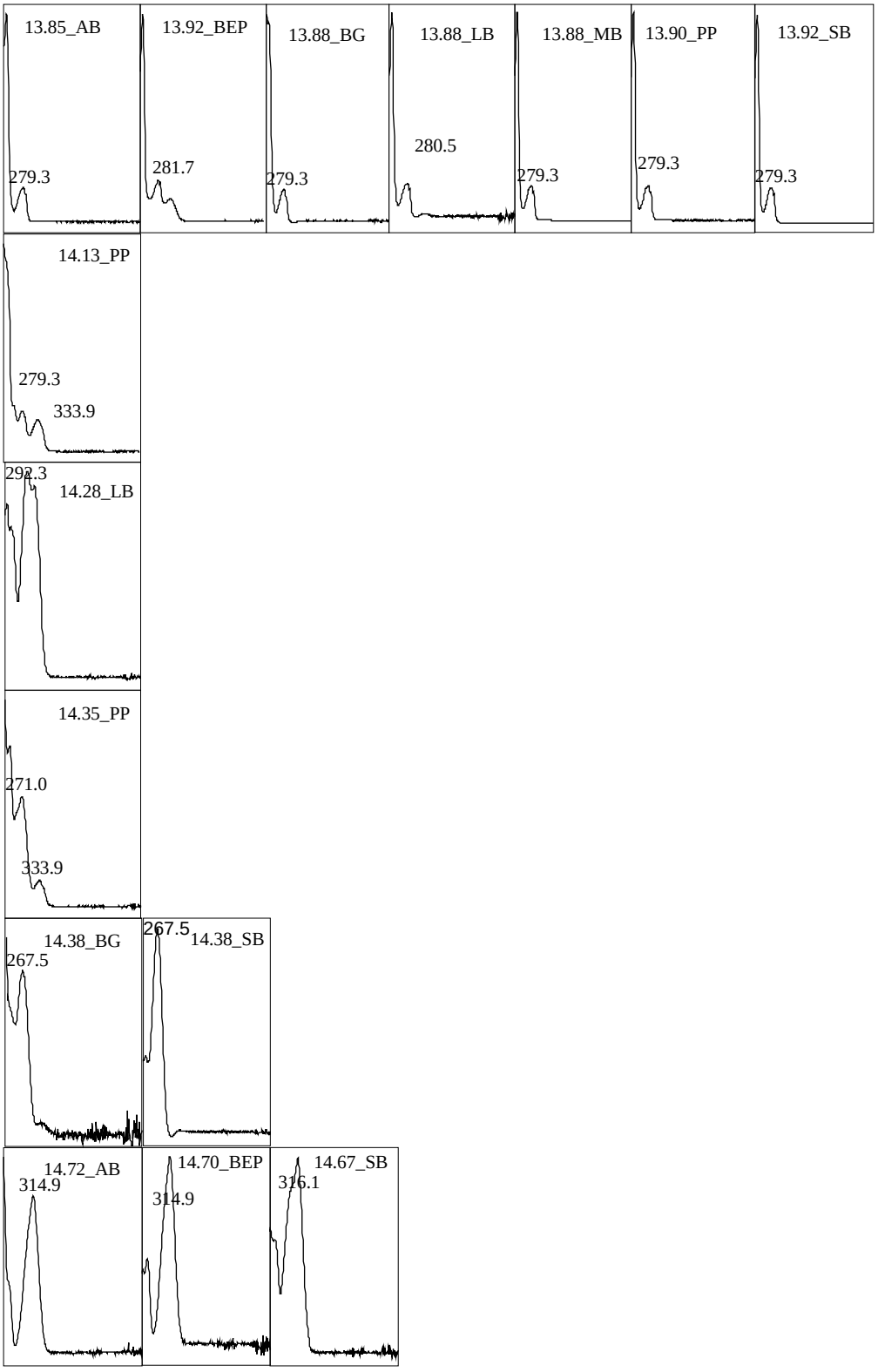
Appendix 6.9 Spectral for each unidentified soluble compound detected at different time interval and at 280 nm for adzuki bean (AB), black eyed pea (BEP), bambara groundnut (BG), lablab bean (LB), mung bean (MB) and pigeon pea (PP) (contd).



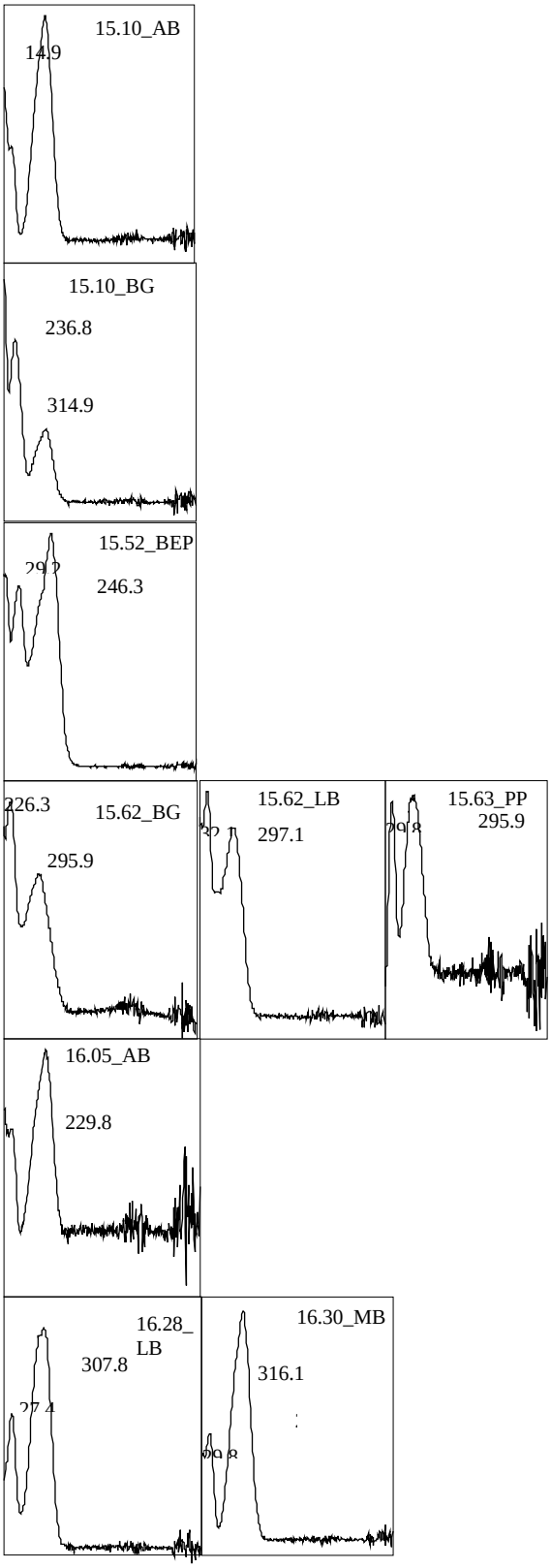
Appendix 6.10 Spectral for each unidentified soluble compound detected at different time interval and at 280 nm for adzuki bean (AB), black eyed pea (BEP), bambara groundnut (BG), lablab bean (LB), mung bean (MB) and pigeon pea (PP) (contd).



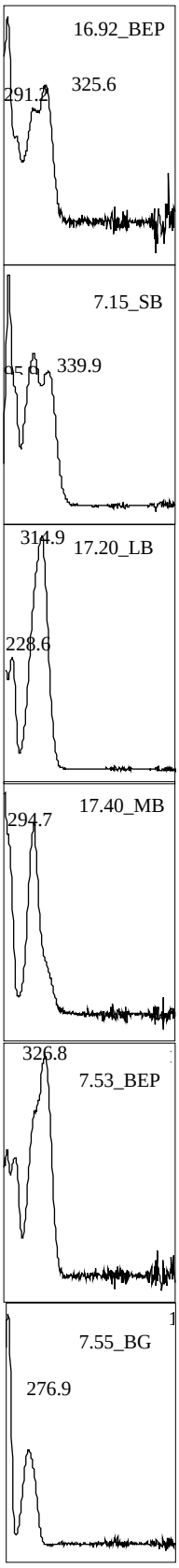
Appendix 6.11 Spectral for each unidentified soluble compounds detected at different time interval and at 280 nm for adzuki bean (AB), black eyed pea (BEP), bambara groundnut (BG), lablab bean (LB), mung bean (MB) and pigeon pea (PP) (contd.)



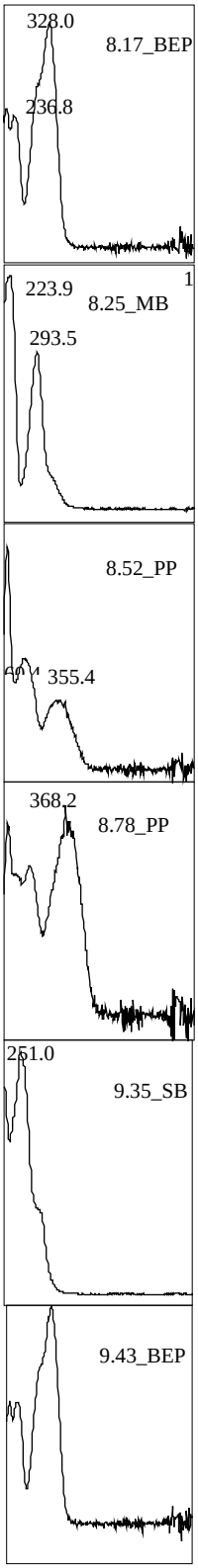
Appendix 6.12 Spectral for each unidentified soluble compounds detected at different time interval and at 280 nm for adzuki bean (AB), black eyed pea (BEP), bambara groundnut (BG), lablab bean (LB), mung bean (MB) and pigeon pea (PP) (contd.)



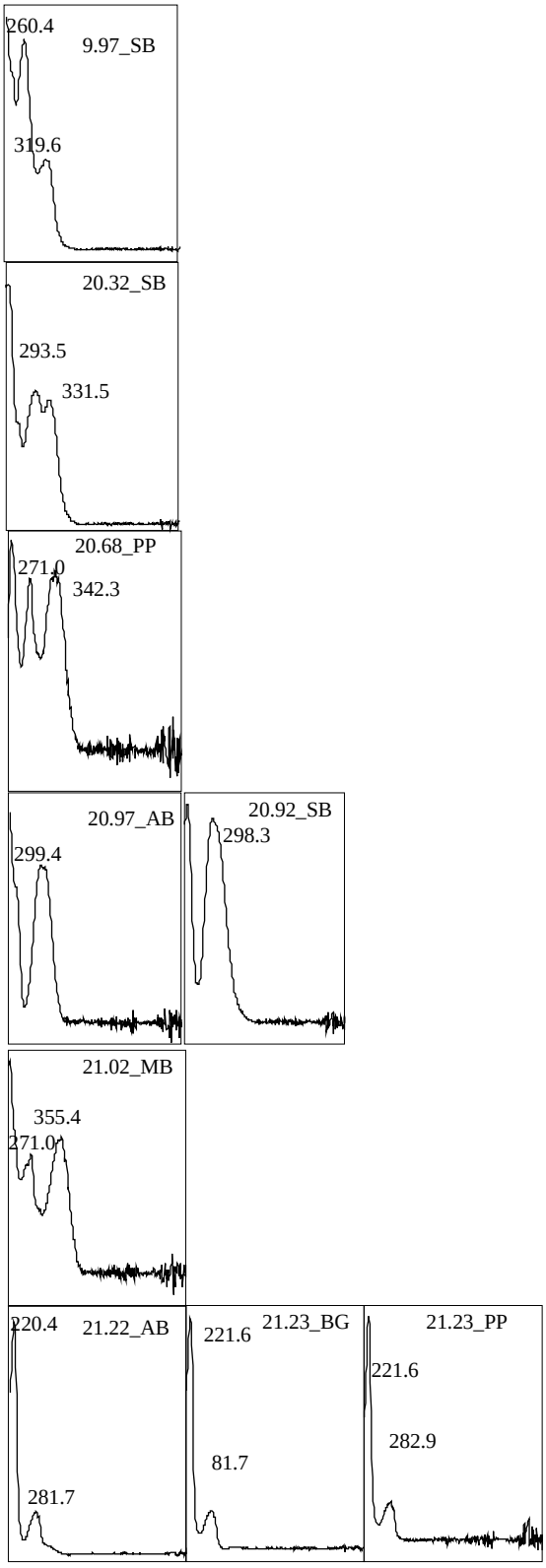
Appendix 6.13 Spectral for each unidentified soluble compounds detected at different time interval and at 280 nm for adzuki bean (AB), black eyed pea (BEP), bambara groundnut (BG), lablab bean (LB), mung bean (MB) and pigeon pea (PP) (contd.)



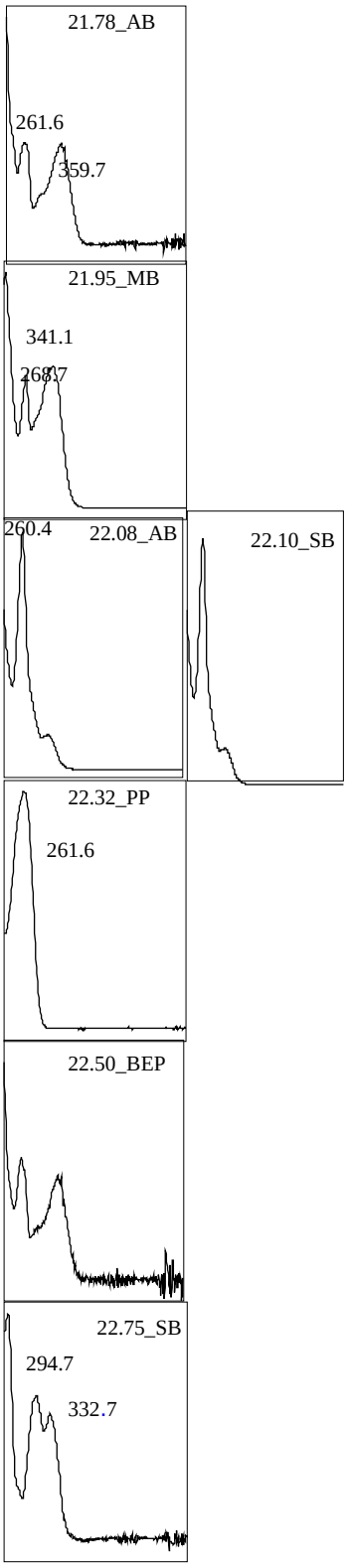
Appendix 6.14 Spectral for each unidentified soluble compounds detected at different time interval and at 280 nm for adzuki bean (AB), black eyed pea (BEP), bambara groundnut (BG), lablab bean (LB), mung bean (MB) and pigeon pea (PP) (contd.)



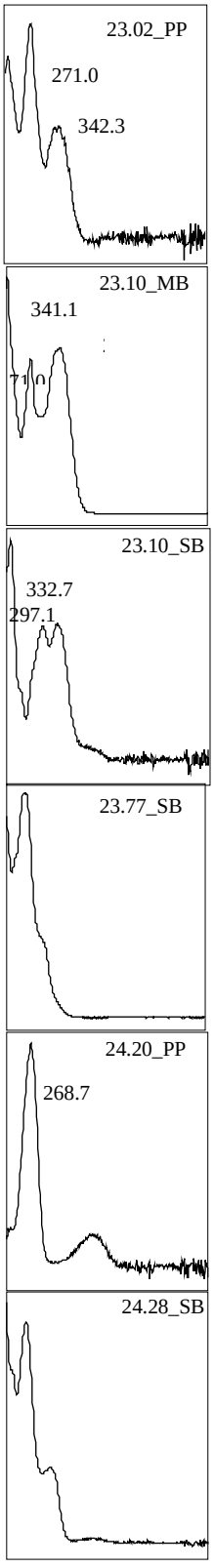
Appendix 6.15 Spectral for each unidentified soluble compounds detected at different time interval and at 280 nm for adzuki bean (AB), black eyed pea (BEP), bambara groundnut (BG), lablab bean (LB), mung bean (MB) and pigeon pea (PP) (contd.)



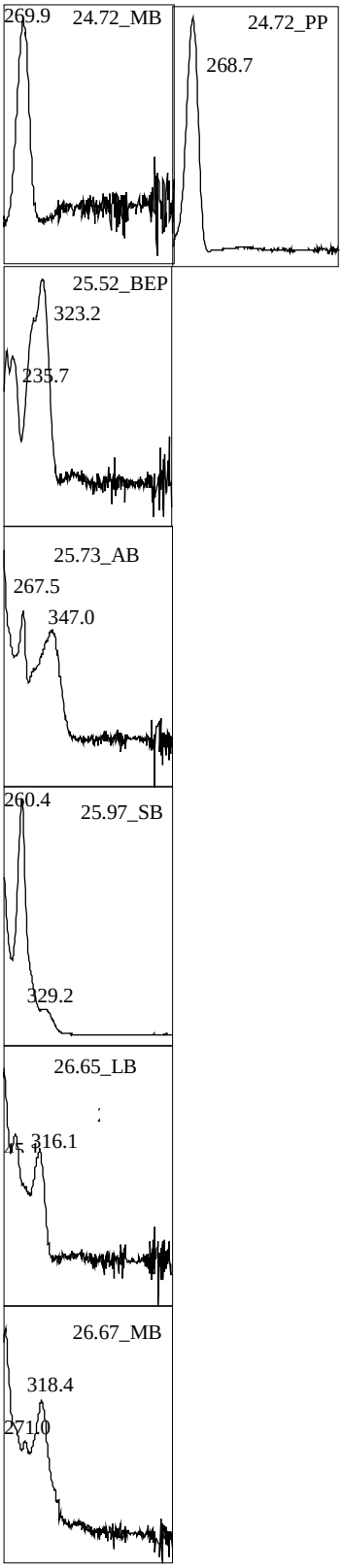
Appendix 6.16 Spectral for each unidentified soluble compounds detected at different time interval and at 280 nm for adzuki bean (AB), black eyed pea (BEP), bambara groundnut (BG), lablab bean (LB), mung bean (MB) and pigeon pea (PP) (contd).



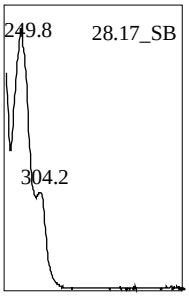
Appendix 6.17 Spectral for each unidentified soluble compounds detected at different time interval and at 280 nm for adzuki bean (AB), black eyed pea (BEP), bambara groundnut (BG), lablab bean (LB), mung bean (MB) and pigeon pea (PP) (contd.)



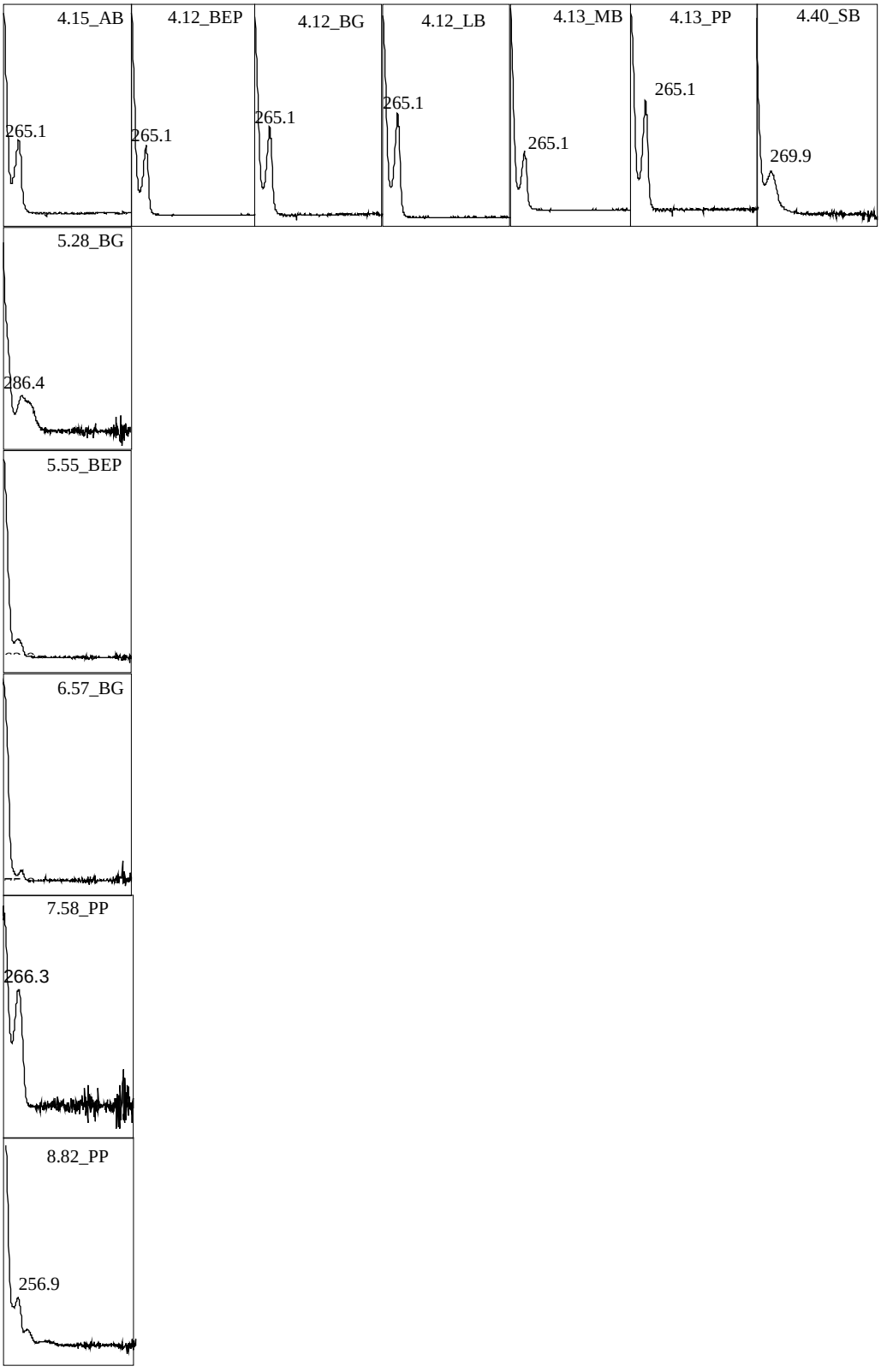
Appendix 6.18 Spectral for each unidentified soluble compounds detected at different time interval and at 280 nm for adzuki bean (AB), black eyed pea (BEP), bambara groundnut (BG), lablab bean (LB), mung bean (MB) and pigeon pea (PP) (contd.)



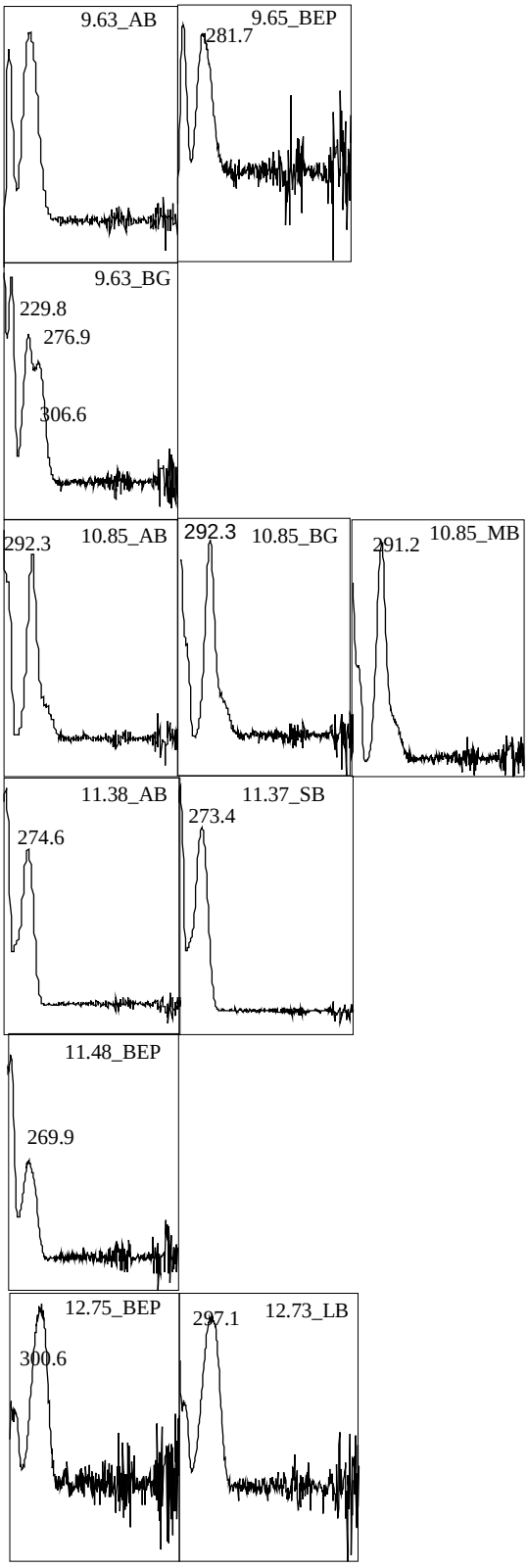
Appendix 6.19 Spectral for each unidentified soluble compounds detected at different time interval and at 280 nm for adzuki bean (AB), black eyed pea (BEP), bambara groundnut (BG), lablab bean (LB), mung bean (MB) and pigeon pea (PP)



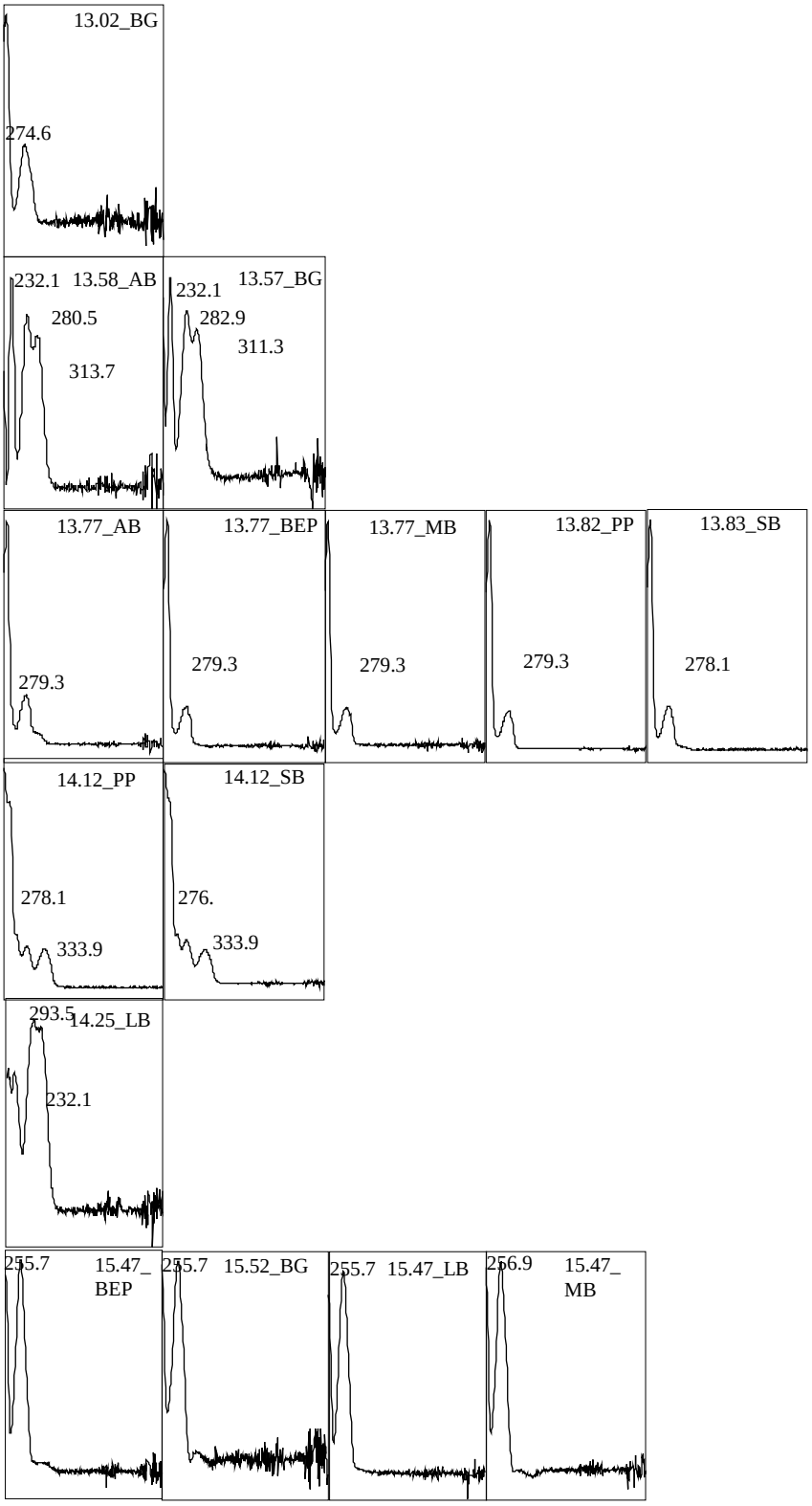
Appendix 6.20 Spectral for each unidentified bound compounds detected at different time interval and at 280 nm for adzuki bean (AB), black eyed pea (BEP), bambara groundnut (BG), lablab bean (LB), mung bean (MB) and pigeon pea (PP)



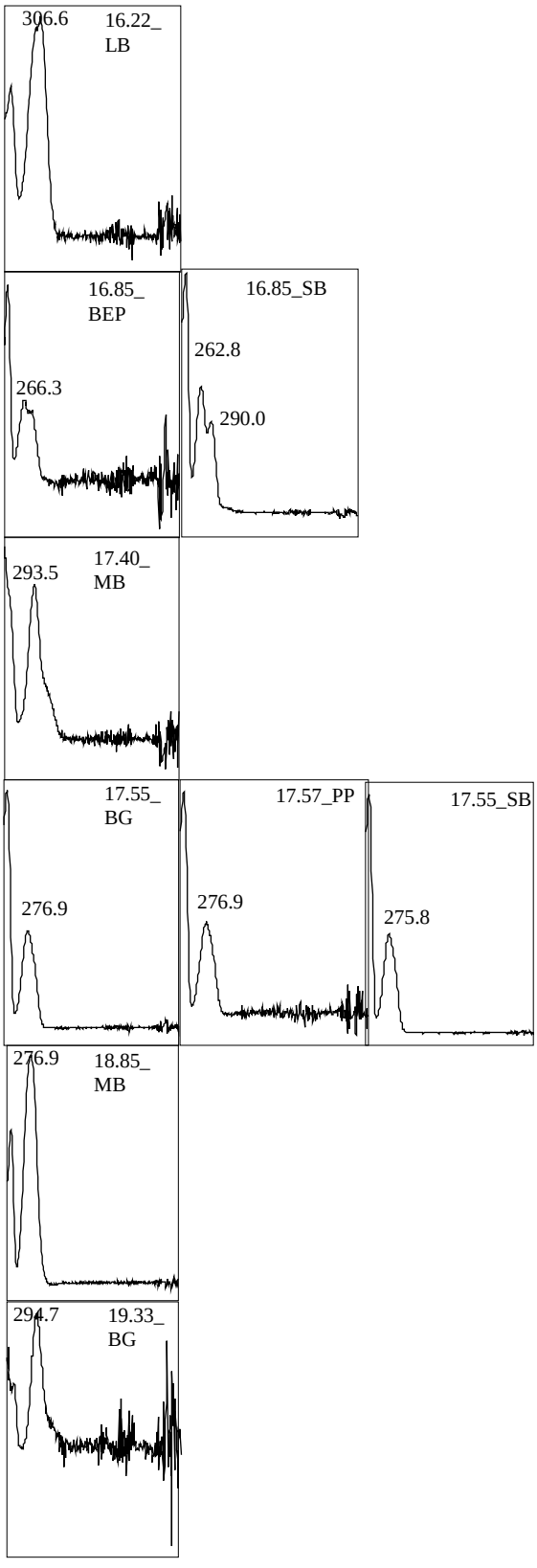
Appendix 6.21 Spectral for each unidentified bound compounds detected at different time interval and at 280 nm for adzuki bean (AB), black eyed pea (BEP), bambara groundnut (BG), lablab bean (LB), mung bean (MB) and pigeon pea (PP) (contd).



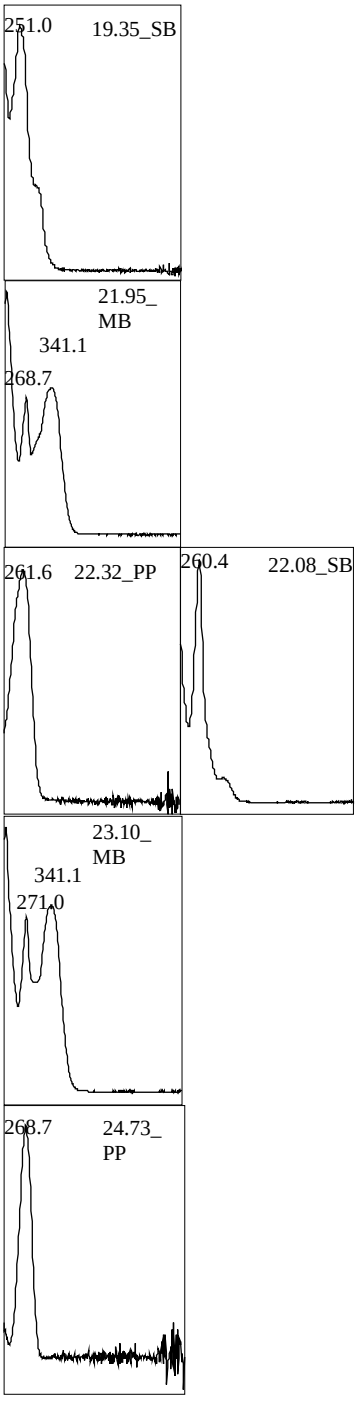
Appendix 6.22 Spectral for each unidentified bound compounds detected at different time interval and at 280 nm for adzuki bean (AB), black eyed pea (BEP), bambara groundnut (BG), lablab bean (LB), mung bean (MB) and pigeon pea (PP) (contd).



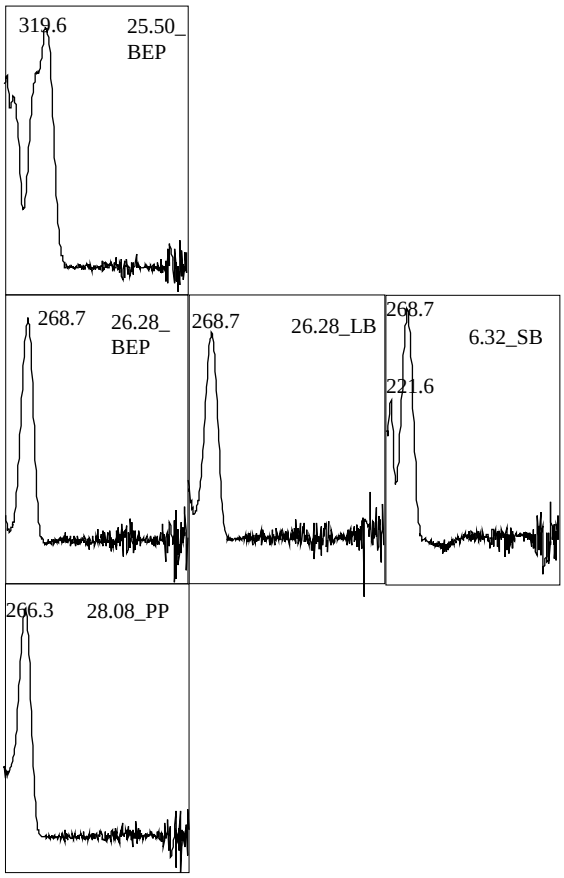
Appendix 6.23 Spectral for each unidentified bound compounds detected at different time interval and at 280 nm for adzuki bean (AB), black eyed pea (BEP), bambara groundnut (BG), lablab bean (LB), mung bean (MB) and pigeon pea (PP) (contd).



Appendix 6.24 Spectral for each unidentified bound compounds detected at different time interval and at 280 nm for adzuki bean (AB), black eyed pea (BEP), bambara groundnut (BG), lablab bean (LB), mung bean (MB) and pigeon pea (PP) (contd).



Appendix 6.25 Spectral for each unidentified bound compounds detected at different time interval and at 280 nm for adzuki bean (AB), black eyed pea (BEP), bambara groundnut (BG), lablab bean (LB), mung bean (MB) and pigeon pea (PP) (contd).



Appendix 6.26 Tabulation of detected unidentified soluble compounds from selected beans at different time interval and at 280 nm

Retention time	Spectral	Integrated area at 280 nm, x 10^3 µV*sec						
		Adzuki bean	Black eyed pea	Bambara groundnut	Lablab bean	Mung bean	Pigeon pea	Soya bean
4.10	265	++	++++	+++	++++	++++	++	++
4.27	265	++	++	++	-	-	++	++
5.53	263	++	++	+	-	++	-	++
6.03	258	+	+	-	+	-	+	++
7.58	268	-	-	-	-	-	+	-
9.52	286	+	-	-	-	-	-	++
10.18	279	+++	++	++	-	-	-	++
10.43	251	-	-	-	-	-	+++	-
10.85	225, 286	+	-	-	-	-	-	-
11.35	281	-	++	-	-	-	-	-
11.37	278	++	-	++	++	-	-	++
12.00	263	-	-	-	-	-	++	-
12.03	223, 314	+	++	-	-	-	-	++
12.52	281	-	-	-	-	+	-	-
12.77	225,296	-	-	-	+	-	+	-

Appendix 6.27 Tabulation of detected unidentified soluble compounds from selected beans at different time interval and at 280 nm (contd).

Retention time	Spectral	Integrated area at 280 nm, x 10^3 µV*sec						
		Adzuki bean	Black eyed pea	Bambara groundnut	Lablab bean	Mung bean	Pigeon pea	Soya bean
12.78	285	++	-	-	-	-	-	-
13.03	278	-	-	+	-	-	-	-
13.10	229, 315	++	++	-	-	-	-	++
13.85	279	+++	++++	++	++	++++	+++	++++
14.13	279, 334	-	-	-	-	-	+++	-
14.28	292	-	-	-	+++	-	-	-
14.35	271, 334	-	-	-	-	-	+++	-
14.38	268	-	-	+	-	-	-	+++
14.72	315	++	++	-	-	-	-	++
15.10	314	+	-	-	-	-	-	-
15.10	237, 315	-	-	+	-	-	-	-
15.52	246, 329	-	++	-	-	-	-	-
15.62	226, 296	-	-	++	++	-	+	-
16.05	230, 314	+	-	-	-	-	-	-
16.28	227, 309	-	-	-	++	++	-	-

Appendix 6.28 Tabulation of detected unidentified soluble compounds from selected beans at different time interval and at 280 nm (contd).

Retention time	Spectral	Integrated area at 280 nm, x 10^3 µV*sec						
		Adzuki bean	Black eyed pea	Bambara groundnut	Lablab bean	Mung bean	Pigeon pea	Soya bean
16.92	291, 326	-	+	-	-	-	-	-
17.15	296, 340	-	-	-	-	-	-	++
17.20	229, 315	-	-	-	+++	-	-	-
17.40	295	-	-	-	-	+	-	-
17.53	327	-	+	-	-	-	-	-
17.55	277	-	-	++	-	-	-	-
18.17	237, 328	-	+	-	-	-	-	-
18.25	224, 294	-	-	-	-	++++	-	-
18.52	260, 355	-	-	-	-	-	++	-
18.78	275, 368	-	-	-	-	-	+	-
19.35	251	-	-	-	-	-	-	++++
19.43	329	-	+	-	-	-	-	-
19.97	260, 320	-	-	-	-	-	-	+++
20.32	294, 332	-	-	-	-	-	-	+++
20.68	271, 342	-	-	-	-	-	+	-

Appendix 6.29 Tabulation of detected unidentified soluble compounds from selected beans at different time interval and at 280 nm (contd).

Retention time	Spectral	Integrated area at 280 nm, x 10^3 µV*sec						
		Adzuki bean	Black eyed pea	Bambara groundnut	Lablab bean	Mung bean	Pigeon pea	Soya bean
20.97	299	+	-	-	-	-	-	++
21.02	271, 355	-	-	-	-	++	-	-
21.22	282	++	-	++	-	-	+	-
21.78	262, 360	+	-	-	-	-	-	-
21.95	269, 341	-	-	-	-	++++	-	-
22.08	260	+	-	-	-	-	-	++++
22.32	262	-	-	-	-	-	++++	-
22.50	258, 358	-	+	-	-	-	-	-
22.75	295, 333	-	-	-	-	-	-	++++
23.02	271, 342	-	-	-	-	-	++	-
23.10	271, 341	-	-	-	-	++++	-	-
23.10	297, 333	-	-	-	-	-	-	++
23.77	253	-	-	-	-	-	-	++++
24.20	269	-	-	-	-	-	++	-
24.28	260, 318	-	-	-	-	-	-	++

Appendix 6.30 Tabulation of detected unidentified soluble compounds from selected beans at different time interval and at 280 nm

Retention time	Spectral	Integrated area at 280 nm, x 10^3 µV*sec						
		Adzuki bean	Black eyed pea	Bambara groundnut	Lablab bean	Mung bean	Pigeon pea	Soya bean
24.72	270	-	-	-	-	+	+++	-
25.52	236, 323	-	+	-	-	-	-	-
25.73	268, 347	+	-	-	-	-	-	-
25.97	260, 329	-	-	-	-	-	-	++++
26.65	245, 316	-	-	-	+	-	-	-
26.67	271, 318	-	-	-	-	+	-	-
28.17	250, 304	-	-	-	-	-	-	+++

‘+’ represents the integrated area, x< 100,000 µV*sec

‘++’ represents the integrated area 100,000 < x < 500,000 µV*sec

‘+++’ represents the integrated area 500,000 < x < 1000,000 µV*sec

‘++++’ represents the integrated area x > 1000,000 µV*sec

‘-‘ represents no detection

Appendix 6.31 Tabulation of integrated area from unidentified bound compounds detected at different time interval and at 280 nm

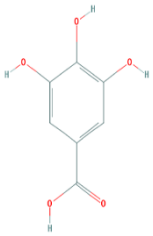
Retention time	Spectral	Integrated area at 280 nm, x 10^3						
		Adzuki bean	Black eyed pea	Bambara groundnut	Lablab bean	Mung bean	Pigeon pea	Soya bean
4.15	265	++	++	++	++	++	++	++
5.28	286	-	-	+	-	-	-	-
5.55	264	-	+	-	-	-	-	-
6.57	276	-	-	+	-	-	-	-
7.58	266	-	-	-	-	-	+	-
8.82	257	-	-	-	-	-	+	-
9.63	285	++	+	-	-	-	-	-
9.63	230,277,307	-	-	+	-	-	-	-
10.85	292	+	-	+	-	+	-	-
11.38	275	++	-	-	-	-	-	+
11.48	270	-	+	-	-	-	-	-
12.75	301	-	+	-	+	-	-	-
13.02	275	-	-	+	-	-	-	-
13.58	232,281,311	+	-	++	-	-	-	-
13.77	279	++	+	-	-	++	++	++

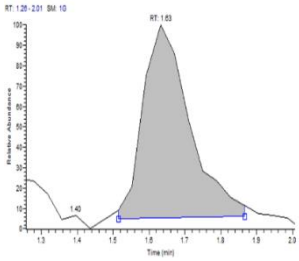
Appendix 6.32 Tabulation of integrated area from unidentified bound compounds detected at different time interval and at 280 nm (contd).

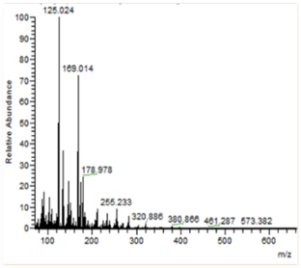
Retention time	Spectral	Integrated area at 280 nm, x 10^3						
		Adzuki bean	Black eyed pea	Bambara groundnut	Lablab bean	Mung bean	Pigeon pea	Soya bean
14.12	278,334	-	-	-	-	-	+++	++
14.25	232,294	-	-	-	+	-	-	-
15.47	256	-	+	+	+	+	-	-
16.22	307	-	-	-	+	-	-	-
16.85	266	-	+	-	-	-	-	++
17.40	294	-	-	-	-	+	-	-
17.55	277	-	-	++	-	-	+	+++
18.85	277	-	-	-	-	+++	-	-
19.33	295	-	-	+	-	-	-	-
19.35	251	-	-	-	-	-	-	++
21.95	269,341	-	-	-	-	++++	-	-
22.32	262	-	-	-	-	-	++	+++
23.10	271,341	-	-	-	-	++++	-	-
24.73	269	-	-	-	-	-	++	-
25.50	311	-	++	-	-	-	-	-
26.28	269	-	+	-	+	-	-	+
28.08	266	-	-	-	-	-	+	-

Appendix 7.1 Ionization characteristics and formula structure for the phenolic standards

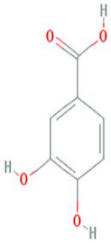
Gallic acid

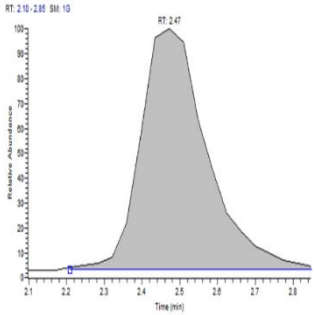


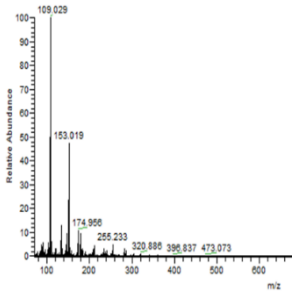




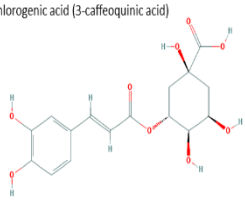
Protocatechuic acid

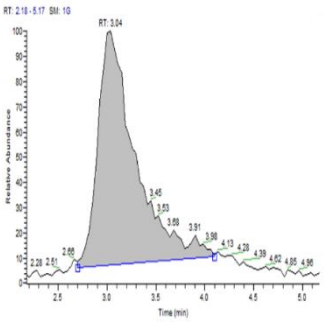


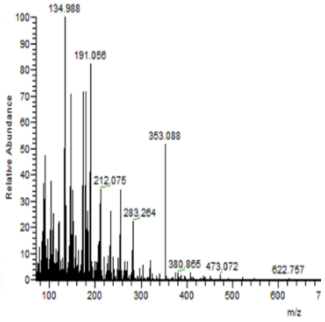




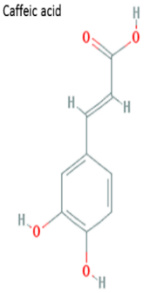
Chlorogenic acid (3-caffeoquinic acid)

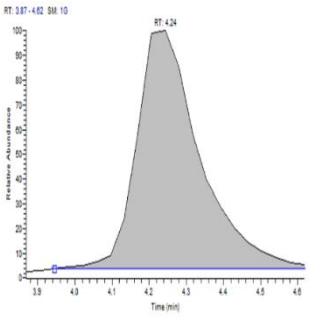


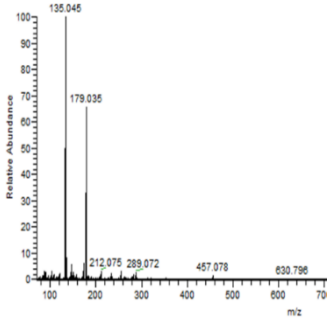




Caffeic acid

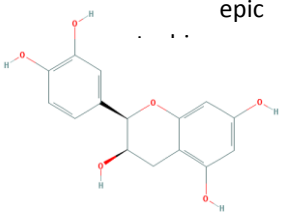


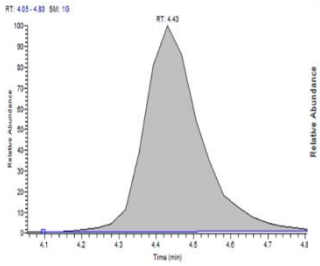


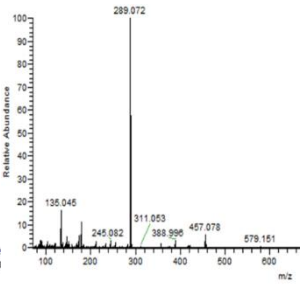


Appendix 7.2 Ionization characteristics and formula structure for the phenolic standards (contd)

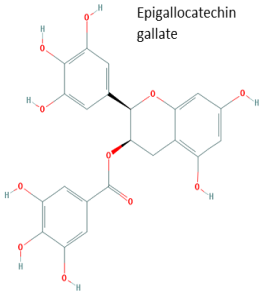
epic

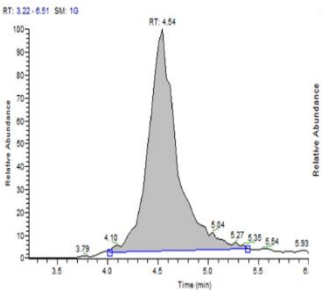


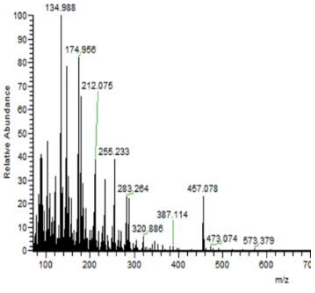




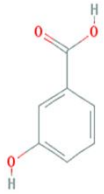
Epigallocatechin gallate

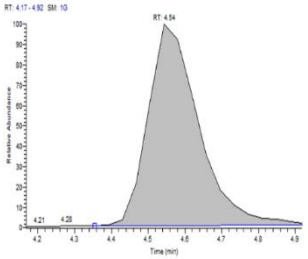


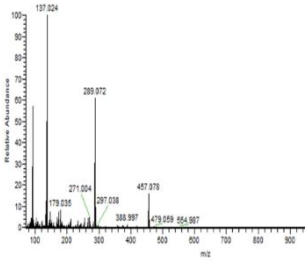




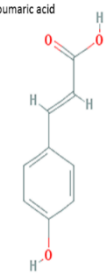
3-hydroxybenzoic acid

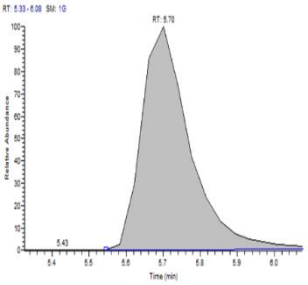


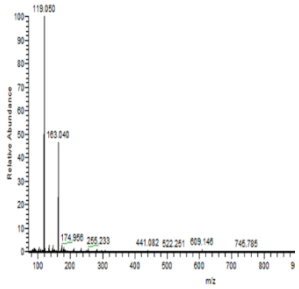




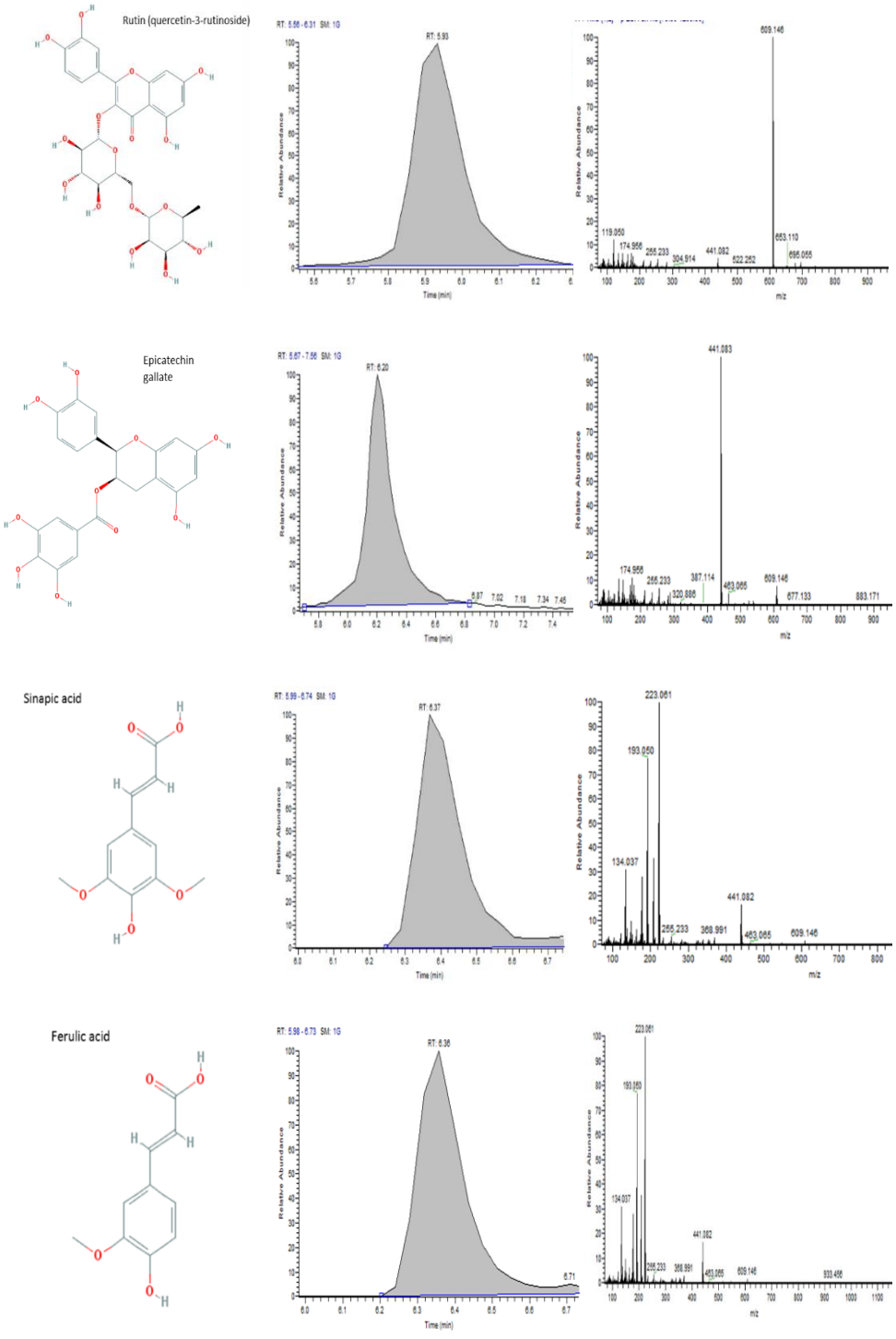
p-coumaric acid





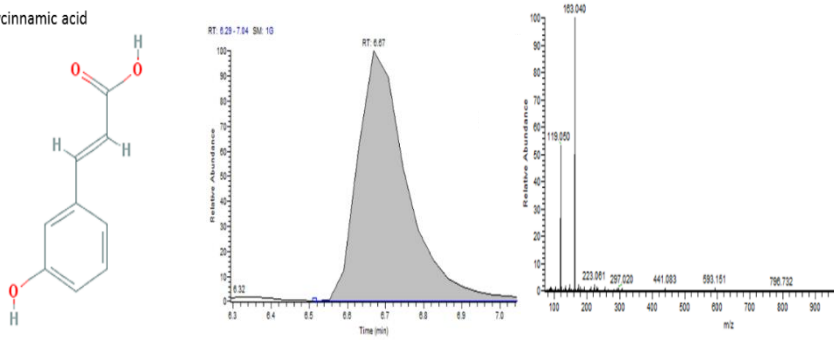


Appendix7.3 Ionization characteristics and formula structure for the phenolic standards (contd)

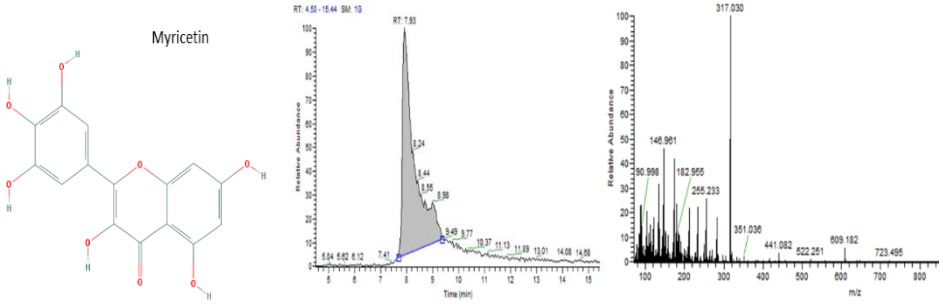


Appendix 7.4 Ionization characteristics and formula structure for the phenolic standards (contd)

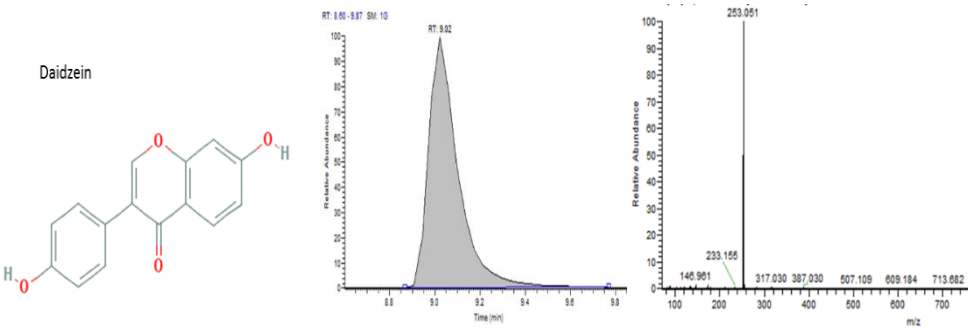
3-hydroxycinnamic acid



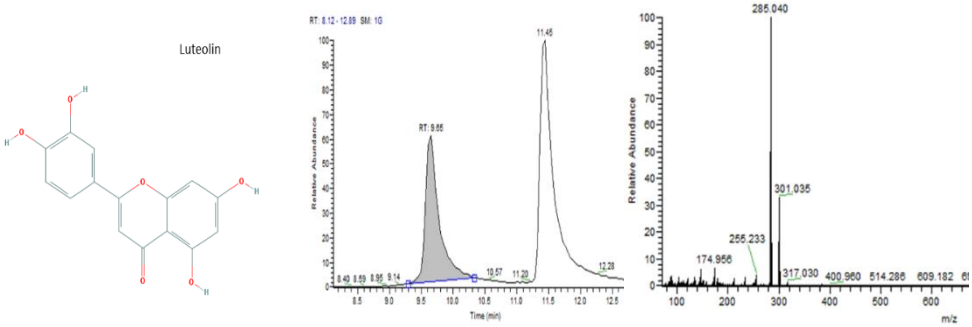
Myricetin



Daidzein

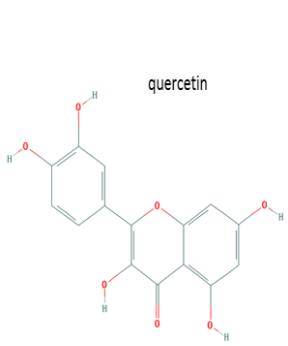


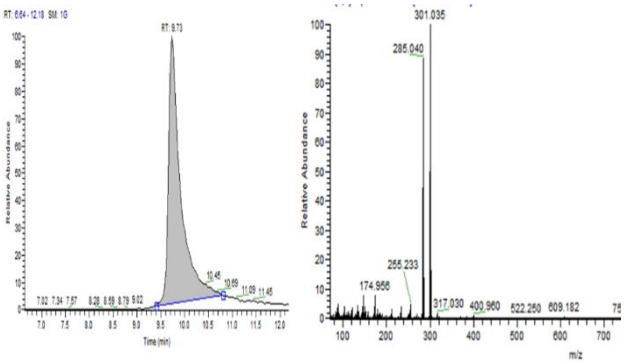
Luteolin



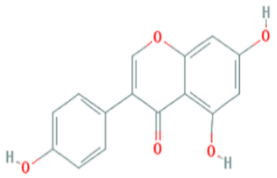
Appendix 7.5 Ionization characteristics and formula structure for the phenolic standards (contd)

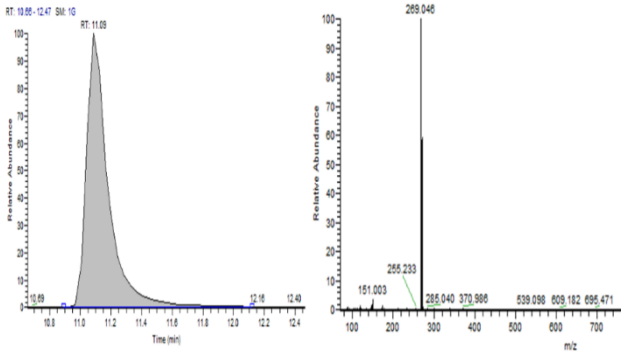
quercetin



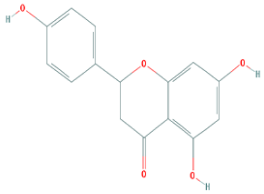


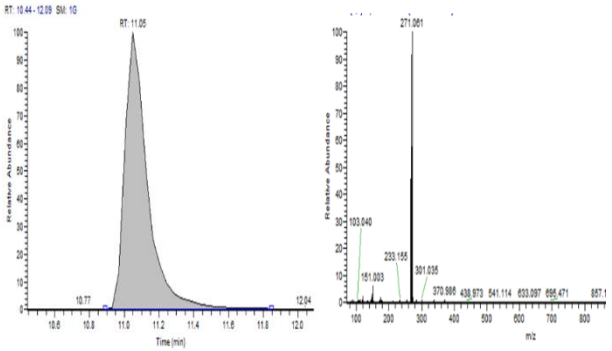
Genistein



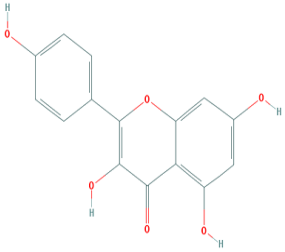


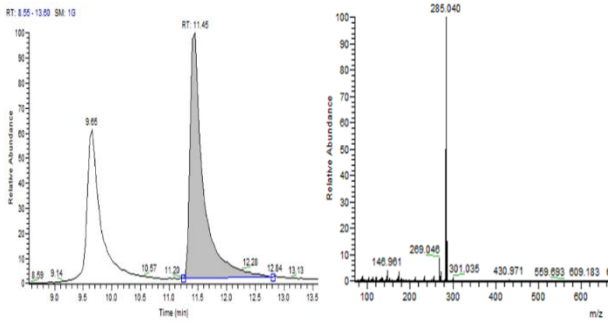
Naringenin





kaempferol





Appendix 7.6 Tabulation of relative abundance of unidentified ions (*m/z*) from soluble phenolic beans extracts

Retention time, min	*m/z*	Relative abundance, ppm						
		AB	BEP	BG	LB	MB	PP	SB
1.71	321.072	-	-	-	-	-	++	-
1.92	389.072	-	-	-	-	-	-	++
1.95	226.035	-	-	-	+++	-	-	-
2.14	417.103	-	-	-	-	-	++	-
2.15	203.082	-	-	-	-	++	-	++
2.37	581.244	-	-	-	-	-	++	-
2.41	303.061	-	-	-	-	-	++++	++
2.41	607.130	-	-	-	-	-	++	-
2.43	493.193	-	-	-	-	-	++	-
2.43	443.191	-	-	-	-	-	-	++
2.45	295.045	++	-	-	-	-	-	-
2.53	205.071	-	-	++	-	-	-	-
2.59	355.066	++	++	-	++	-	-	++
2.82	569.113	-	-	-	-	-	-	++
2.84	369.082	++	-	-	-	-	-	-
2.85	293.114	-	-	-	-	-	++++	+++
2.85	587.235	-	-	-	-	-	++++	++
2.87	128.035	-	-	-	-	-	++	-
2.87	164.071	-	-	-	-	-	++	-
2.87	275.103	-	-	-	-	-	+++	-
2.93	433.113	-	-	-	-	-	-	++
3.06	391.090	-	-	-	-	-	++	-
3.06	385.077	-	++++	-	-	-	-	-
3.20	278.066	-	-	-	++	-	-	-
3.23	509.129	-	-	++	-	-	-	-

AB – adzuki bean, BEP- black eyed pea, BG- bambara groundnut, LB- lablab bean, MB- mung bean, PP- pigeon pea, SB- soya bean

'-' represents the ion either not being detected or < 100,000 ppm

'++' represents the detected ion 100,000 ppm < x < 500,000 ppm

'+++' represents the detected ion 500,000 ppm < x < 1,000,000 ppm

'++++' represents the detected ion x > 1,000,000 ppm

Appendix 7.7 Tabulation of relative abundance of unidentified ions (*m/z*) from soluble phenolic beans extracts (contd)

Retention time, min	*m/z*	Relative abundance, ppm						
		AB	BEP	BG	LB	MB	PP	SB
3.30	422.156	-	-	-	-	-	+++	-
3.32	177.055	-	-	-	-	++	-	-
3.34	399.092	-	++	++	-	-	-	-
3.38	433.113	-	-	-	-	-	-	++
3.38	531.089	-	-	-	-	-	-	++
3.38	507.208	-	-	-	-	-	+++	-
3.42	563.233	-	-	-	-	-	++	-
3.50	485.140	-	-	-	-	-	++	-
3.61	487.145	-	-	-	++	-	-	-
3.66	305.069	-	-	-	-	-	-	++
3.70	543.244	-	-	++	-	-	-	-
3.89	472.102	-	-	-	++	-	-	-
3.90	369.082	++	-	-	-	-	-	-
3.93	387.165	-	++	-	-	+++	+++	++
3.93	485.142	-	-	-	-	++	++	-
3.93	775.338	-	-	-	-	++	++	-
3.95	463.124	-	-	-	-	-	-	++
3.97	507.208	-	-	-	-	-	++	-
3.99	292.082	-	-	-	-	-	++	-
4.01	387.165	-	-	-	++	-	-	-
4.08	595.129	-	-	-	-	-	-	++++
4.26	757.182	-	++	-	-	-	-	-
4.29	381.191	-	-	++++	-	-	-	-
4.29	763.390	-	-	++	-	-	-	-
4.31	250.071	-	-	-	-	-	++	++

AB – adzuki bean, BEP- black eyed pea, BG- bambara groundnut, LB- lablab bean, MB- mung bean, PP- pigeon pea, SB- soya bean

‘-' represents the ion either not being detected or < 100,000 ppm

‘++’ represents the detected ion 100,000 ppm < x < 500,000 ppm

‘+++’ represents the detected ion 500,000 ppm < x < 1,000,000 ppm

‘++++’ represents the detected ion x > 1,000,000 ppm

Appendix 7.8 Tabulation of relative abundance of unidentified ions (*m/z*) from soluble phenolic beans extracts (contd)

Retention time, min	*m/z*	Relative abundance, ppm						
		AB	BEP	BG	LB	MB	PP	SB
4.32	565.118	-	-	-	-	-	-	++++
4.36	465.103	-	-	-	-	++	-	-
4.36	389.181	-	-	-	-	-	++	-
4.38	915.218	-	-	-	-	++	-	-
4.38	517.228	-	-	-	-	-	-	++
4.43	755.203	++	-	-	-	-	-	-
4.43	329.066	-	-	-	-	++	-	-
4.43	449.108	-	-	-	-	++++	++	+++
4.43	899.223	-	-	-	-	+++	-	-
4.44	399.093	-	++	++	-	-	-	-
4.47	547.085	-	-	-	-	++	-	-
4.48	431.097	-	-	-	-	-	-	++
4.50	569.113	-	-	-	-	-	-	++
4.53	467.129	-	-	-	-	-	++++	-
4.54	625.140	-	++	-	-	-	-	-
4.68	563.139	-	-	-	-	-	++	-
4.70	509.092	-	-	-	-	-	-	++
4.70	513.079	-	-	-	-	-	-	++
4.70	535.108	-	-	-	-	-	-	+++
4.70	539.103	-	-	-	-	-	-	++
4.76	447.093	-	-	-	-	++	-	-
4.77	392.182	-	-	-	-	-	++	-
4.77	723.117	-	++	-	-	-	-	-
4.79	253.050	-	-	-	-	-	-	++
4.79	415.103	-	-	-	-	-	-	++

AB – adzuki bean, BEP- black eyed pea, BG- bambara groundnut, LB- lablab bean, MB- mung bean, PP- pigeon pea, SB- soya bean

‘-' represents the ion either not being detected or < 100,000 ppm

‘++’ represents the detected ion 100,000 ppm < x < 500,000 ppm

‘+++’ represents the detected ion 500,000 ppm < x < 1,000,000 ppm

‘++++’ represents the detected ion x > 1,000,000 ppm

Appendix 7.9 Tabulation of relative abundance of unidentified ions (*m/z*) from soluble phenolic beans extracts (contd)

Retention time, min	*m/z*	Relative abundance, ppm						
		AB	BEP	BG	LB	MB	PP	SB
4.79	461.108	-	-	-	-	-	-	++++
4.79	669.160	-	-	-	-	-	-	++
4.79	831.212	-	-	-	-	-	-	+++
4.79	877.217	-	-	-	-	-	-	++
4.80	625.140	++	++++	-	-	-	-	-
4.81	451.079	-	-	-	-	-	-	++
4.81	294.098	-	-	-	-	-	++	++
4.91	335.124	-	-	-	-	-	-	++
4.93	739.208	++	-	-	-	-	-	-
4.94	289.082	-	-	-	-	-	++	-
5.02	206.082	-	++	-	-	-	-	-
5.04	447.092	-	-	-	-	++	-	-
5.04	623.210	++	-	-	-	-	-	-
5.04	283.060	-	-	-	-	-	-	++
5.04	445.113	-	-	-	-	-	-	++
5.04	491.118	-	-	-	-	-	-	++++
5.04	671.233	-	-	-	-	-	-	++
5.04	891.233	-	-	-	-	-	-	++
5.06	1051.473	++	-	-	-	-	-	-
5.08	543.090	-	-	-	-	-	-	++
5.08	595.129	-	-	-	-	-	-	++++
5.09	481.243	+++	-	-	-	-	-	-
5.09	525.233	++++	-	-	-	-	-	-
5.11	399.093	-	++	-	-	-	-	-
5.12	547.238	-	-	-	-	-	++	-

AB – adzuki bean, BEP- black eyed pea, BG- bambara groundnut, LB- lablab bean, MB- mung bean, PP- pigeon pea, SB- soya bean

‘-' represents the ion either not being detected or < 100,000 ppm

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‘+++’ represents the detected ion 500,000 ppm < x < 1,000,000 ppm

‘++++’ represents the detected ion x > 1,000,000 ppm

Appendix 7.10 Tabulation of relative abundance of unidentified ions (*m/z*) from soluble phenolic beans extracts (contd)

Retention time, min	*m/z*	Relative abundance, ppm						
		AB	BEP	BG	LB	MB	PP	SB
5.13	613.285	+++	-	-	-	-	-	-
5.13	657.275	++++	-	-	-	-	-	-
5.13	1183.515	++	-	-	-	-	-	-
5.23	707.121	-	-	-	-	-	-	++
5.24	565.118	-	-	-	-	-	-	++++
5.27	250.108	-	-	-	-	-	++	-
5.30	593.150	-	-	-	-	++	-	-
5.34	569.113	-	-	-	-	-	-	++
5.38	459.129	-	-	-	-	-	-	++
5.42	163.039	-	-	-	++	-	-	++
5.42	509.092	-	-	-	-	-	-	++
5.42	535.108	-	-	-	-	-	-	+++
5.42	539.103	-	-	-	-	-	-	++
5.53	609.145	++++	-	-	-	-	-	++++
5.57	533.129	-	-	-	-	-	++	-
5.61	415.102	-	-	-	-	-	-	++
5.63	706.270	-	-	-	-	-	++	-
5.65	441.176	-	-	-	-	-	++++	-
5.67	539.153	-	-	-	-	-	++	++
5.67	883.358	-	-	-	-	-	++++	-
5.68	431.098	-	-	-	-	++++	-	-
5.68	863.202	-	-	-	-	++++	-	-
5.75	237.040	-	-	-	-	++	-	-
5.77	669.145	-	-	-	-	+++	-	-
5.78	731.265	-	-	-	-	-	++	-

AB – adzuki bean, BEP- black eyed pea, BG- bambara groundnut, LB- lablab bean, MB- mung bean, PP- pigeon pea, SB- soya bean

‘-' represents the ion either not being detected or < 100,000 ppm

‘++’ represents the detected ion 100,000 ppm < x < 500,000 ppm

‘+++’ represents the detected ion 500,000 ppm < x < 1,000,000 ppm

‘++++’ represents the detected ion x > 1,000,000 ppm

Appendix 7.11 Tabulation of relative abundance of unidentified ions (*m/z*) from soluble phenolic beans extracts (contd)

Retent ion time, min	*m/z*	Relative abundance, ppm						
		AB	BEP	BG	LB	MB	PP	SB
5.79	387.059	++	-	++	-	-	-	-
5.81	245.093	++++	-	++++	-	-	++	++
5.81	289.082	++	-	++	-	-	-	-
5.81	579.172	++++	-	++++	-	-	-	-
5.84	577.155	-	-	-	-	++	-	-
5.86	721.187	-	-	-	-	++	-	-
5.86	693.249	-	-	-	-	-	++	-
5.90	807.327	-	-	-	-	-	+++	-
5.90	463.088	-	++	-	-	++	+++	-
5.91	403.160	-	-	-	-	++	-	-
5.91	529.074	-	-	-	-	++	-	-
5.91	835.265	-	-	-	-	-	-	++++
5.92	371.061	-	-	-	-	-	-	++
5.92	595.129	-	-	-	-	-	-	++
5.92	1191.264	-	-	-	-	-	++	-
5.93	652.260	-	-	-	-	-	++	-
5.97	1161.254	-	-	-	-	-	-	++
5.99	649.250	-	-	-	-	-	++	++
6.02	193.050	-	++	-	-	-	-	-
6.02	593.150	+++	-	-	-	++	-	-
6.03	447.092	-	-	-	-	++	-	-
6.07	565.118	-	-	-	-	-	-	++++
6.08	415.124	-	++	-	-	-	-	-
6.10	597.265	-	++	-	-	-	-	-
6.10	1149.531	-	++	-	-	-	-	-

AB – adzuki bean, BEP- black eyed pea, BG- bambara groundnut, LB- lablab bean, MB- mung bean, PP- pigeon pea, SB- soya bean

‘-' represents the ion either not being detected or < 100,000 ppm

‘++’ represents the detected ion 100,000 ppm < x < 500,000 ppm

‘+++’ represents the detected ion 500,000 ppm < x < 1,000,000 ppm

‘++++’ represents the detected ion x > 1,000,000 ppm

Appendix 7.12 Tabulation of relative abundance of unidentified ions (*m/z*) from soluble phenolic beans extracts (contd)

Retent ion time, min	*m/z*	Relative abundance, ppm						
		AB	BEP	BG	LB	MB	PP	SB
6.15	535.108	-	-	-	-	-	-	++++
6.19	547.145	-	-	-	-	-	++	-
6.22	698.266	-	-	-	-	-	++	-
6.23	689.298	-	-	++	-	-	-	-
6.24	701.149	-	-	-	-	-	-	++
6.25	269.045	-	-	-	-	-	-	++
6.25	431.097	-	-	-	-	-	-	++++
6.25	467.074	-	-	-	-	-	-	++
6.25	477.102	-	-	-	-	-	-	++++
6.25	863.201	-	-	-	-	-	-	++++
6.25	909.207	-	-	-	-	-	-	++
6.25	640.301	-	-	++	-	-	-	-
6.38	248.092	-	-	-	-	-	++	-
6.40	529.074	-	-	-	-	-	-	++
6.40	641.290	-	-	-	-	-	-	++
6.40	664.293	-	-	-	-	-	-	++
6.45	361.222	-	-	-	-	-	-	++
6.47	579.171	-	-	-	-	++	-	-
6.49	435.129	-	-	-	-	++	-	-
6.51	1027.232	-	-	-	-	-	-	++
6.58	500.249	-	-	-	-	++	++	-
6.62	569.224	-	-	-	-	-	++++	++
6.62	1139.455	-	-	-	-	-	++	-
6.65	440.182	-	-	-	-	-	++	++
6.69	595.129	-	-	-	-	-	-	++++

AB – adzuki bean, BEP- black eyed pea, BG- bambara groundnut, LB- lablab bean, MB- mung bean, PP- pigeon pea, SB- soya bean

‘-' represents the ion either not being detected or < 100,000 ppm

‘++’ represents the detected ion 100,000 ppm < x < 500,000 ppm

‘+++’ represents the detected ion 500,000 ppm < x < 1,000,000 ppm

‘++++’ represents the detected ion x > 1,000,000 ppm

Appendix 7.13 Tabulation of relative abundance of unidentified ions (*m/z*) from soluble phenolic beans extracts (contd)

Retent ion time, min	*m/z*	Relative abundance, ppm						
		AB	BEP	BG	LB	MB	PP	SB
6.79	565.118	-	-	-	-	-	-	++++
6.83	656.296	-	++	-	-	-	-	++++
6.85	535.108	-	-	-	-	-	-	+++
6.89	597.265	-	++	-	-	-	-	-
6.89	597.767	-	++	-	-	-	-	-
6.89	527.249	-	-	-	-	++	-	-
6.89	563.225	-	-	-	-	++	-	-
6.89	573.254	-	-	-	-	++++	-	-
6.89	625.225	-	-	-	-	++	-	++
6.89	1055.504	-	-	-	-	++	-	-
6.95	487.181	-	-	-	-	-	++	-
6.95	543.244	-	-	-	-	-	++	-
6.96	633.293	-	-	-	-	-	-	++
6.96	633.795	-	-	-	-	-	-	++
6.96	241.107	-	-	-	-	-	++	-
6.98	502.265	-	-	-	-	-	++	-
7.04	534.233	-	-	-	-	-	++	-
7.05	241.108	-	++	-	++	-	-	++
7.06	440.182	-	-	-	-	-	+++	-
7.16	433.113	-	-	-	-	-	-	++
7.16	648.298	-	-	-	-	-	-	+++
7.16	590.275	-	+++	-	-	-	-	++
7.16	590.777	-	++	-	-	-	-	-
7.19	647.291	-	-	++	-	-	-	++
7.19	670.293	-	-	++	-	-	-	-

AB – adzuki bean, BEP- black eyed pea, BG- bambara groundnut, LB- lablab bean, MB- mung bean, PP- pigeon pea, SB- soya bean

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‘++++’ represents the detected ion x > 1,000,000 ppm

Appendix 7.14 Tabulation of relative abundance of unidentified ions (*m/z*) from soluble phenolic beans extracts (contd)

Retention time, min	*m/z*	Relative abundance, ppm						
		AB	BEP	BG	LB	MB	PP	SB
7.19	670.795	-	-	++	-	-	-	-
7.26	582.278	-	-	+++	-	-	-	-
7.26	1119.557	-	-	++	-	-	-	-
7.26	825.426	-	++	-	-	++	-	-
7.28	429.176	-	-	-	-	++	-	-
7.28	618.288	-	-	-	-	-	-	++
7.28	641.290	-	-	-	-	-	-	++
7.28	641.792	-	-	-	-	-	-	++
7.31	673.305	-	-	++	-	-	-	-
7.34	479.192	-	-	-	-	-	++	-
7.35	713.473	+++	++	++	++	++	++	++
7.35	723.502	++++	++++	++++	++++	++++	+++	++++
7.37	775.472	++	++	-	++	++	-	++
7.37	354.192	-	++	-	++	-	-	-
7.38	661.250	-	-	-	++	++	-	-
7.40	529.265	-	-	++	-	-	-	-
7.40	654.801	-	-	++	-	-	-	-
7.40	633.293	-	-	-	-	-	-	++
7.40	633.795	-	-	-	-	-	-	++
7.41	663.304	-	++	++	-	-	-	-
7.42	662.296	-	-	++	-	-	-	-
7.42	662.798	-	-	++	-	-	-	-
7.42	865.600	++	-	-	-	-	-	++
7.43	663.806	-	++	++	-	-	-	-
7.53	632.304	-	-	+++	-	-	-	-

AB – adzuki bean, BEP- black eyed pea, BG- bambara groundnut, LB- lablab bean, MB- mung bean, PP- pigeon pea, SB- soya bean

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‘++++’ represents the detected ion x > 1,000,000 ppm

Appendix 7.15 Tabulation of relative abundance of unidentified ions (*m/z*) from soluble phenolic beans extracts (contd)

Retention time, min	*m/z*	Relative abundance, ppm						
		AB	BEP	BG	LB	MB	PP	SB
7.53	632.806	-	-	+++	-	-	-	-
7.53	655.307	-	++	++++	-	-	-	-
7.53	655.808	-	++	++++	-	-	-	-
7.53	678.309	-	-	++	-	-	-	-
7.53	678.811	-	-	++	-	-	-	-
7.55	618.789	-	-	-	-	-	-	++
7.55	641.291	-	-	-	-	-	-	++++
7.55	641.792	-	-	-	-	-	-	+++
7.56	552.267	-	-	-	-	-	-	++
7.56	552.768	-	-	-	-	-	-	++
7.56	575.269	-	-	++	-	-	-	+++
7.56	575.771	-	-	-	-	-	-	++
7.56	618.288	-	-	-	-	-	-	++
7.63	582.278	++	-	++	-	-	-	-
7.64	373.186	-	-	-	-	-	-	++
7.64	747.379	-	-	-	-	-	-	++
7.66	553.192	-	++	-	-	-	-	-
7.66	839.405	-	++	-	-	++	-	-
7.68	419.199	-	++	-	-	-	-	-
7.69	529.265	-	-	++	-	-	-	-
7.73	525.233	-	-	++	-	-	-	-
7.73	589.267	-	-	+++	-	-	-	-
7.74	537.262	-	-	-	-	-	-	++
7.74	560.264	-	-	-	-	-	-	++
7.74	560.766	-	-	-	-	-	-	++

AB – adzuki bean, BEP- black eyed pea, BG- bambara groundnut, LB- lablab bean, MB- mung bean, PP- pigeon pea, SB- soya bean

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‘++++’ represents the detected ion x > 1,000,000 ppm

Appendix 7.16 Tabulation of relative abundance of unidentified ions (*m/z*) from soluble phenolic beans extracts (contd)

Retention time, min	*m/z*	Relative abundance, ppm						
		AB	BEP	BG	LB	MB	PP	SB
7.74	633.289	-	-	-	-	-	-	++
7.74	633.795	-	-	-	-	-	-	++
7.75	566.265	-	-	++	-	-	-	-
7.75	566.766	-	-	++	-	-	-	-
7.75	589.769	-	-	++	-	-	-	-
7.77	516.238	-	-	++	-	-	-	-
7.77	516.740	-	-	++	-	-	-	-
7.77	987.478	-	-	++	-	-	-	-
7.79	624.307	-	-	++	-	-	-	-
7.79	647.309	-	-	++	-	-	-	-
7.79	647.811	-	-	++	-	-	-	-
7.84	526.277	-	-	-	-	-	-	++
7.84	626.285	-	-	-	-	-	-	++
7.87	582.780	++	-	++	-	-	-	-
7.91	491.213	-	-	-	++	-	-	-
7.91	354.191	-	-	-	++	-	++	-
7.91	559.275	++	-	-	-	-	-	-
7.98	657.311	-	-	-	-	++	-	-
7.98	703.317	-	-	-	-	++	-	-
7.99	433.113	-	-	-	-	-	-	++
8.01	525.196	-	-	-	-	-	-	++
8.01	618.287	-	-	-	-	-	-	++
8.04	561.254	-	-	-	-	-	+++	-
8.07	421.207	-	-	-	-	-	-	++
8.07	467.212	-	-	-	-	-	-	++++

AB – adzuki bean, BEP- black eyed pea, BG- bambara groundnut, LB- lablab bean, MB- mung bean, PP- pigeon pea, SB- soya bean

'-' represents the ion either not being detected or < 100,000 ppm

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'++++' represents the detected ion x > 1,000,000 ppm

Appendix 7.17 Tabulation of relative abundance of unidentified ions (*m/z*) from soluble phenolic beans extracts (contd)

Retent ion time, min	*m/z*	Relative abundance, ppm						
		AB	BEP	BG	LB	MB	PP	SB
8.08	809.431	-	-	-	++	-	-	-
8.12	537.261	-	-	-	-	-	-	++
8.12	537.763	-	-	-	-	-	-	++
8.12	560.264	-	-	-	-	-	-	+++
8.12	560.766	-	-	-	-	-	-	++
8.12	1075.529	-	-	-	-	-	-	++
8.12	776.359	-	-	-	-	-	++	-
8.15	352.176	-	-	-	-	-	++	-
8.16	579.207	-	++	-	-	-	-	-
8.17	640.301	-	-	++	-	-	-	-
8.17	640.803	-	-	++	-	-	-	-
8.19	539.212	-	-	-	-	-	++	-
8.20	949.668	++	-	-	-	++	-	-
8.20	352.176	-	++	-	++	-	-	-
8.24	819.328	++	-	-	-	-	-	-
8.24	343.212	++	++	-	++	-	-	++
8.24	581.150	-	-	-	-	-	++	-
8.26	417.176	-	-	-	-	-	+++	-
8.28	522.256	-	-	-	-	-	-	++
8.28	545.259	-	-	-	-	-	-	++
8.28	545.761	-	-	-	-	-	-	++
8.28	835.358	-	-	-	-	-	++	-
8.31	663.304	-	++	-	-	-	-	++
8.31	663.806	-	++	-	-	-	-	-
8.36	511.253	-	-	++	-	++	-	-

AB – adzuki bean, BEP- black eyed pea, BG- bambara groundnut, LB- lablab bean, MB- mung bean, PP- pigeon pea, SB- soya bean

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‘++’ represents the detected ion 100,000 ppm < x < 500,000 ppm

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‘++++’ represents the detected ion x > 1,000,000 ppm

Appendix 7.18 Tabulation of relative abundance of unidentified ions (*m/z*) from soluble phenolic beans extracts (contd)

Retention time, min	*m/z*	Relative abundance, ppm						
		AB	BEP	BG	LB	MB	PP	SB
8.36	557.259	-	-	++	-	++	-	-
8.36	262.108	++	-	-	-	-	-	-
8.42	655.296	-	++	-	-	-	-	-
8.42	655.798	-	++	-	-	-	-	-
8.42	845.334	-	-	-	-	-	++	-
8.44	632.286	-	++	-	-	-	-	-
8.44	632.788	-	++	-	-	-	-	-
8.45	481.220	-	-	-	-	++	-	-
8.48	207.066	-	++	-	-	-	-	-
8.49	573.254	-	-	++	-	-	-	-
8.56	478.249	-	-	++	-	-	-	-
8.59	501.252	-	-	++	-	-	-	-
8.60	803.333	++	-	-	-	-	-	-
8.69	751.320	++	-	-	-	-	-	-
8.69	751.821	++	-	-	-	-	-	-
8.73	589.268	-	++	-	-	-	-	-
8.73	648.299	-	+++	-	-	-	-	-
8.73	648.801	-	++	-	-	-	-	++
8.73	1133.536	-	++	-	-	-	-	-
8.73	743.347	-	-	-	-	-	-	++
8.75	253.050	-	-	-	-	-	-	++++
8.75	507.107	-	-	-	-	-	-	++
8.76	1119.557	++	-	-	-	-	-	-
8.84	640.803	-	++	++	-	-	-	-
8.84	609.145	-	-	-	-	-	-	++

AB – adzuki bean, BEP- black eyed pea, BG- bambara groundnut, LB- lablab bean, MB- mung bean, PP- pigeon pea, SB- soya bean

‘-' represents the ion either not being detected or < 100,000 ppm

‘++’ represents the detected ion 100,000 ppm < x < 500,000 ppm

‘+++’ represents the detected ion 500,000 ppm < x < 1,000,000 ppm

‘++++’ represents the detected ion x > 1,000,000 ppm

Appendix 7.19 Tabulation of relative abundance of unidentified ions (*m/z*) from soluble phenolic beans extracts (contd)

Retention time, min	*m/z*	Relative abundance, ppm						
		AB	BEP	BG	LB	MB	PP	SB
8.84	640.301	-	++	++	-	-	-	-
8.90	741.332	-	-	-	-	-	-	++
8.92	483.235	-	-	-	-	-	-	++
8.93	262.108	-	-	-	-	-	++	-
8.96	596.257	++	-	-	-	-	-	-
9.03	409.199	-	-	-	-	-	-	++
9.03	582.278	-	++	-	-	-	-	-
9.07	624.305	-	-	-	-	-	-	++
9.07	647.308	-	-	-	-	-	-	+++
9.07	647.810	-	-	-	-	-	-	++
9.10	1104.063	-	-	++	-	-	-	-
9.13	574.280	-	-	+++	-	-	-	-
9.13	574.782	-	-	++	-	-	-	-
9.13	1103.562	-	-	++	-	-	-	-
9.13	1149.567	-	-	++	-	-	-	-
9.20	283.060	-	-	-	-	-	-	++
9.26	389.172	-	-	-	-	-	-	++
9.26	971.483	++ ++	-	++	-	-	-	-
9.29	735.324	-	-	-	++	-	-	-
9.33	632.303	-	-	-	-	-	-	++
9.33	632.805	-	-	-	-	-	-	++
9.35	651.322	-	-	-	-	-	-	++
9.38	508.743	++	-	-	-	-	-	-
9.40	971.985	++	-	-	-	-	-	-
9.50	704.325	-	-	-	++	-	-	-

AB – adzuki bean, BEP- black eyed pea, BG- bambara groundnut, LB- lablab bean, MB- mung bean, PP- pigeon pea, SB- soya bean

‘-' represents the ion either not being detected or < 100,000 ppm

‘++’ represents the detected ion 100,000 ppm < x < 500,000 ppm

‘+++’ represents the detected ion 500,000 ppm < x < 1,000,000 ppm

‘++++’ represents the detected ion x > 1,000,000 ppm

Appendix 7.20 Tabulation of relative abundance of unidentified ions (*m/z*) from soluble phenolic beans extracts (contd)

Retention time, min	*m/z*	Relative abundance, ppm						
		AB	BEP	BG	LB	MB	PP	SB
9.50	727.328	-	-	-	++++	-	-	-
9.50	727.829	-	-	-	+++	-	-	-
9.50	750.330	-	-	-	++	-	-	-
9.50	750.832	-	-	-	++	-	-	-
9.60	670.294	++	-	-	-	-	-	-
9.60	670.795	++	-	-	-	-	-	-
9.64	613.285	++	-	-	-	-	-	-
9.64	657.276	+++	-	-	-	-	-	-
9.65	719.330	-	-	-	++++	-	-	-
9.65	719.831	-	-	-	+++	-	-	-
9.72	653.337	-	-	-	-	-	-	++
9.77	957.503	-	-	++	-	-	-	++
9.77	1003.508	-	-	++	-	-	-	++
9.78	655.295	-	-	-	-	-	++	-
9.79	486.270	-	-	-	++	-	++	-
9.88	1089.545	-	-	-	-	-	-	++
9.89	625.322	++	-	-	-	-	-	-
9.90	565.257	-	-	++	-	-	-	-
9.90	565.759	-	-	++	-	-	-	-
9.90	1131.520	-	-	++	-	-	-	-
9.93	391.188	-	-	-	-	-	-	+++
9.94	654.299	-	-	-	+++	-	-	-
9.94	654.801	-	-	-	++	-	-	-
9.97	736.333	-	-	-	-	++	-	-
9.97	736.834	-	-	-	-	++	-	-

AB – adzuki bean, BEP- black eyed pea, BG- bambara groundnut, LB- lablab bean, MB- mung bean, PP- pigeon pea, SB- soya bean

‘-' represents the ion either not being detected or < 100,000 ppm

‘++’ represents the detected ion 100,000 ppm < x < 500,000 ppm

‘+++’ represents the detected ion 500,000 ppm < x < 1,000,000 ppm

‘++++’ represents the detected ion x > 1,000,000 ppm

Appendix 7.21 Tabulation of relative abundance of unidentified ions (*m/z*) from soluble phenolic beans extracts (contd)

Retent ion time, min	*m/z*	Relative abundance, ppm						
		AB	BEP	BG	LB	MB	PP	SB
9.98	177.055	++	++	-	++++	++	-	+++
9.98	811.447	+++	-	-	-	-	-	-
10.01	368.183	-	-	-	-	-	+++	-
10.03	655.260	-	-	-	-	-	++	-
10.05	1119.556	-	-	++	-	-	-	-
10.07	663.806	-	-	-	-	++	-	-
10.09	663.304	-	-	-	-	++	-	-
10.10	927.493	-	-	-	-	-	-	++
10.12	646.301	-	-	-	++	-	-	-
10.12	646.803	-	-	-	++	-	-	-
10.13	971.483	-	-	++	++	++	-	-
10.14	597.290	++	-	-	-	-	-	-
10.14	641.281	++++	-	-	-	-	-	-
10.19	573.252	-	-	-	++	-	-	-
10.22	795.452	-	-	++	-	-	-	-
10.30	825.426	++	-	-	-	-	-	-
10.30	1147.515	++	-	++	-	-	-	-
10.39	523.217	-	-	-	-	-	++	-
10.43	497.238	-	-	-	-	-	+++	++
10.46	593.223	-	++	-	-	-	-	-
10.47	581.270	-	-	-	+++	-	-	-
10.47	581.772	-	-	-	++	-	-	-
10.47	1117.541	-	-	-	++	-	-	-
10.47	255.123	-	+++	-	++	-	+++	++++
10.47	511.254	-	++	-	-	-	++	++

AB – adzuki bean, BEP- black eyed pea, BG- bambara groundnut, LB- lablab bean, MB- mung bean, PP- pigeon pea, SB- soya bean

‘-' represents the ion either not being detected or < 100,000 ppm

- ‘++’ represents the detected ion 100,000 ppm < x < 500,000 ppm

‘+++’ represents the detected ion 500,000 ppm < x < 1,000,000 ppm

‘++++’ represents the detected ion x > 1,000,000 ppm

Appendix 7.22 Tabulation of relative abundance of unidentified ions (*m/z*) from soluble phenolic beans extracts (contd)

Retention time, min	*m/z*	Relative abundance, ppm						
		AB	BEP	BG	LB	MB	PP	SB
10.61	207.066	-	++	-	-	-	-	-
10.65	1133.536	+++	-	++	-	-	-	-
10.71	955.488	-	-	-	++	-	-	+++
10.77	809.431	-	-	-	+++	-	-	-
10.77	1101.546	-	-	-	++	-	-	-
10.78	651.410	-	-	-	-	-	-	++
10.88	269.045	-	-	-	-	-	-	+++
10.89	508.243	+++	-	++	++	-	-	-
10.93	434.206	-	-	-	++	-	-	-
10.93	823.411	-	-	-	++++	-	-	-
10.93	823.912	-	-	-	++	-	-	-
10.93	897.447	-	-	-	++	-	-	-
10.93	796.456	++	-	-	-	-	-	-
11.05	985.462	-	-	-	++	-	-	-
11.07	573.254	++	-	-	++	-	-	-
11.07	573.756	++	-	-	++	-	-	-
11.07	1131.520	++	-	-	-	-	-	-
11.07	1147.515	+++	-	-	-	-	-	-
11.08	925.477	-	-	-	-	-	-	++
11.10	955.489	-	-	++	++++	-	-	-
11.10	971.482	-	-	-	-	-	-	++
11.15	327.217	++	-	+++	++	++++	-	++++
11.20	643.332	-	-	+++	-	-	-	-
11.21	655.442	-	-	-	-	++	-	-
11.23	809.432	+++	-	-	-	-	-	-

AB – adzuki bean, BEP- black eyed pea, BG- bambara groundnut, LB- lablab bean, MB- mung bean, PP- pigeon pea, SB- soya bean

'-' represents the ion either not being detected or < 100,000 ppm

'++' represents the detected ion 100,000 ppm < x < 500,000 ppm

'+++' represents the detected ion 500,000 ppm < x < 1,000,000 ppm

'++++' represents the detected ion x > 1,000,000 ppm

Appendix 7.23 Tabulation of relative abundance of unidentified ions (*m/z*) from soluble phenolic beans extracts (contd)

Retention time, min	*m/z*	Relative abundance, ppm						
		AB	BEP	BG	LB	MB	PP	SB
11.28	1119.556	++	-	-	-	+++	-	-
11.32	991.465	-	-	-	++	-	-	-
11.32	1001.494	-	-	-	+++	-	-	++
11.35	1053.465	-	-	-	++	-	-	-
11.37	500.244	-	-	-	++	-	-	-
11.38	1133.536	++++	-	-	-	-	-	-
11.43	881.954	-	-	-	++	-	-	-
11.47	426.207	-	-	-	++	-	-	-
11.47	807.416	-	-	-	++++	-	-	-
11.47	807.918	-	-	-	++	-	-	-
11.47	881.452	-	-	-	++	-	-	-
11.51	969.468	-	-	-	++	-	-	-
11.67	135.081	-	-	-	-	-	++	-
11.67	211.097	-	-	-	-	-	++	-
11.75	969.468	-	-	-	++	-	-	-
11.80	645.348	-	-	++	-	-	-	-
11.80	659.473	-	-	++	-	-	-	++++
11.81	329.233	++	++	+++	+++	++	+++	++++
11.89	793.437	-	-	-	++	++	-	-
11.96	427.209	-	-	-	-	-	-	++
11.98	681.454	-	-	-	-	-	-	++
12.08	809.431	-	-	-	++	-	-	-
12.08	939.494	-	-	-	++	-	-	-
12.20	327.217	++	++	-	++	-	-	+++
12.38	1117.541	++	-	-	-	-	-	-

AB – adzuki bean, BEP- black eyed pea, BG- bambara groundnut, LB- lablab bean, MB- mung bean, PP- pigeon pea, SB- soya bean

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‘+++’ represents the detected ion 500,000 ppm < x < 1,000,000 ppm

‘++++’ represents the detected ion x > 1,000,000 ppm

Appendix 7.24 Tabulation of relative abundance of unidentified ions (*m/z*) from soluble phenolic beans extracts (contd)

Retent ion time, min	*m/z*	Relative abundance, ppm						
		AB	BEP	BG	LB	MB	PP	SB
12.52	405.204	-	-	-	-	-	-	++
12.54	281.139	-	-	-	-	-	-	++
12.54	419.217	-	-	-	+++	-	-	-
12.54	419.719	-	-	-	++	-	-	-
12.54	793.437	-	-	-	++++	-	++++	-
12.54	793.938	-	-	-	++++	-	-	-
12.54	1190.658	-	-	-	++	-	-	-
12.54	1191.160	-	-	-	++	-	-	-
12.63	971.483	++++	+++	++	++	-	-	-
12.85	396.215	-	-	-	-	-	++++	-
12.85	815.419	-	-	-	-	-	++	-
12.87	955.489	++	++	++	-	++	-	-
12.88	957.504	-	++++	-	-	++	-	-
12.91	939.494	-	+++	++	++	++	-	++
12.93	823.445	++	-	-	-	-	-	-
12.93	941.472	-	-	-	-	+++	-	-
13.09	957.503	++	-	-	-	-	-	++
13.21	993.477	-	++	-	-	-	-	-
13.21	1003.510	-	++	-	-	-	-	-
13.31	492.272	-	++	-	++	-	-	++
13.32	795.452	+++	-	-	-	-	-	-
13.32	977.483	-	++	++	++	++	-	++
13.34	941.509	+++	++++	++++	+++	++++	-	+++ +
13.34	987.515	++	++++	++++	++	++++	-	+++ +
13.35	1039.486	-	++	++	-	-	-	-

AB – adzuki bean, BEP- black eyed pea, BG- bambara groundnut, LB- lablab bean, MB- mung bean, PP- pigeon pea, SB- soya bean

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'++++' represents the detected ion x > 1,000,000 ppm

Appendix 7.25 Tabulation of relative abundance of unidentified ions (*m/z*) from soluble phenolic beans extracts (contd)

Retention time, min	*m/z*	Relative abundance, ppm						
		AB	BEP	BG	LB	MB	PP	SB
13.45	955.489	++	++	-	++	-	-	-
13.47	329.233	-	-	++	-	-	-	-
13.59	793.437	++	-	-	-	-	-	-
13.65	647.379	-	-	-	++	-	-	-
13.65	693.384	-	-	-	++	-	-	-
13.67	809.431	+++	-	+++	-	-	-	-
13.85	795.452	++++	+++	++	++	++	-	++
13.87	831.428	++	-	-	-	-	-	-
13.87	893.429	++	-	-	-	-	-	-
13.91	957.503	-	-	-	-	-	-	+++
13.93	911.498	-	-	-	-	-	-	++++
13.93	947.474	-	-	-	-	-	-	++
13.93	925.515	-	++++	+++	++	-	-	-
14.03	307.191	-	-	-	-	-	-	++
14.07	961.491	-	++	-	-	-	-	-
14.07	971.517	-	++	-	-	-	-	-
14.31	955.487	-	++	-	-	-	-	++
14.42	528.248	-	-	-	-	-	++	-
14.45	343.248	-	-	-	-	-	-	++
14.46	387.238	-	-	-	-	++	-	-
14.55	939.494	-	+++	++	-	-	-	++++
14.55	985.499	-	++	++	-	-	-	+++
14.61	975.469	-	-	-	-	-	-	++
14.68	1083.535	-	++	-	-	-	-	-
14.74	661.358	-	-	-	++	-	-	-

AB – adzuki bean, BEP- black eyed pea, BG- bambara groundnut, LB- lablab bean, MB- mung bean, PP- pigeon pea, SB- soya bean

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‘++’ represents the detected ion 100,000 ppm < x < 500,000 ppm

‘+++’ represents the detected ion 500,000 ppm < x < 1,000,000 ppm

‘++++’ represents the detected ion x > 1,000,000 ppm

Appendix 7.26 Tabulation of relative abundance of unidentified ions (*m/z*) from soluble phenolic beans extracts (contd)

Retent ion time, min	*m/z*	Relative abundance, ppm						
		AB	BEP	BG	LB	MB	PP	SB
14.83	343.248	-	-	-	-	-	-	++
14.83	779.456	++	-	-	-	-	-	-
14.87	528.249	-	-	-	-	-	++	-
14.90	1067.540	-	++	++	-	-	-	-
14.90	1113.546	-	++	++	-	-	-	-
15.02	807.416	++	-	-	-	-	-	-
15.08	793.437	++	-	-	-	-	-	++
15.19	909.483	-	-	-	-	-	-	+++
15.19	955.488	-	-	-	-	-	-	++
15.22	823.411	++	-	-	-	-	-	-
15.24	333.206	-	-	-	-	-	++	-
15.28	389.253	-	-	-	-	-	-	++
15.44	969.503	-	-	-	-	-	-	++
15.46	309.206	-	-	-	-	-	-	++
15.48	809.432	++++	-	-	-	-	-	
15.61	1121.661	++	-	-	-	-	-	-
15.69	311.222	++++	++++	++	++	++	++	++++
15.80	623.452	++	++	-	-	-	-	-
15.81	645.434	++	-	-	-	-	-	-
15.83	309.206	-	-	-	-	-	-	++
15.84	849.499	-	-	-	-	-	++	-
15.86	447.249	-	-	-	-	-	++	-
15.90	424.246	-	-	-	-	-	++++	-
16.32	593.272	++	++	-	-	-	-	-
16.38	474.262	++++	++++	-	++++	++++	++++	++++

AB – adzuki bean, BEP- black eyed pea, BG- bambara groundnut, LB- lablab bean, MB- mung bean, PP- pigeon pea, SB- soya bean

‘-' represents the ion either not being detected or < 100,000 ppm

‘++’ represents the detected ion 100,000 ppm < x < 500,000 ppm

‘+++’ represents the detected ion 500,000 ppm < x < 1,000,000 ppm

‘++++’ represents the detected ion x > 1,000,000 ppm

Appendix 7.27 Tabulation of relative abundance of unidentified ions (*m/z*) from soluble phenolic beans extracts (contd)

Retent ion time, min	*m/z*	Relative abundance, ppm						
		AB	BEP	BG	LB	MB	PP	SB
16.38	949.530	++	++	-	++	-	-	++
16.89	593.272	+++	++++	-	++	++	-	-
17.00	313.238	++	++	++	++	-	+++	+++
17.19	562.314	++	+++	-	-	++	-	-
17.32	313.238	+++	++	+++	++	-	++	+++
17.32	627.483	++	-	++	-	-	-	-
17.69	595.288	-	++	++	++	-	-	-
17.76	953.561	+++	++	-	++++	+++	++++	++++
17.85	476.277	+++ +	++++	++++	++++	++++	++++	++++
17.85	576.202	-	-	-	++	-	++	-
17.85	975.543	-	-	-	++	-	++	-
17.89	297.152	-	++	-	++	++	++	++
17.89	311.222	-	-	-	-	-	++	-
18.04	544.265	++	++	-	++	++	++	++
18.22	723.380	-	-	++	-	-	-	-
18.25	564.330	-	-	-	++	++	++	-
18.31	821.431	++	-	-	-	-	-	-
18.36	564.330	-	++	-	-	-	-	-
18.44	595.288	+++ +	++++	++++	++++	++++	+++	++
18.62	564.330	++	++++	+++	++++	+++	+++	++
18.69	475.280	-	-	-	-	-	+++	-
18.69	994.611	-	-	-	-	-	++	-
18.75	1083.661	-	++	-	-	-	-	-
18.79	554.301	-	++	-	-	-	-	-
18.85	571.288	-	++	++	-	++	-	-

AB – adzuki bean, BEP- black eyed pea, BG- bambara groundnut, LB- lablab bean, MB- mung bean, PP- pigeon pea, SB- soya bean

‘-' represents the ion either not being detected or < 100,000 ppm

‘++’ represents the detected ion 100,000 ppm < x < 500,000 ppm

‘+++’ represents the detected ion 500,000 ppm < x < 1,000,000 ppm

‘++++’ represents the detected ion x > 1,000,000 ppm

Appendix 7.28 Tabulation of relative abundance of unidentified ions (*m/z*) from soluble phenolic beans extracts (contd)

Retent ion time, min	*m/z*	Relative abundance, ppm						
		AB	BEP	BG	LB	MB	PP	SB
18.86	293.212	++	-	-	++	++	-	-
18.86	823.447	++	-	-	-	-	-	-
18.92	315.253	-	-	++	-	-	++	-
19.03	452.278	++++	++++	+++	++++	++++	++++	++++
19.11	905.562	-	-	-	++	-	++	-
19.20	540.330	-	++	++	++	++	++	-
19.49	571.288	++	++++	++++	+++	+++	++	++
19.70	540.330	-	+++	-	-	-	-	-
20.41	478.293	++	++	+++	++++	++	++++	+++
20.43	311.168	++	++	++	++	++	+++	+++
20.46	566.345	-	++	++	++	-	-	-
20.48	295.227	++	++	+++	+++	++	+++	+++
20.68	597.304	-	++	+++	++	-	-	-
20.95	297.243	-	-	-	++	-	-	-
21.37	293.211	-	-	-	-	-	++	++
21.57	503.311	-	-	-	-	-	++	-
22.01	243.196	-	-	-	-	-	++	-
22.42	199.170	-	-	-	-	-	++	-
22.43	325.184	++	+++	++	+++	+++	++++	+++
22.67	295.227	-	-	-	-	-	++	-
23.03	555.343	-	-	-	-	-	++	-
24.35	277.217	-	-	-	-	++	-	-
24.49	339.199	++	++	++	++	++	++	++
25.26	116.928	++	++	++	++	-	++	++
25.26	279.232	-	-	++	++	++	++	

AB – adzuki bean, BEP- black eyed pea, BG- bambara groundnut, LB- lablab bean, MB- mung bean, PP- pigeon pea, SB- soya bean

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‘++++’ represents the detected ion x > 1,000,000 ppm

Appendix 7.29 Tabulation of relative abundance of unidentified ions (*m/z*) from soluble phenolic beans extracts (contd)

Retention time, min	*m/z*	Relative abundance, ppm						
		AB	BEP	BG	LB	MB	PP	SB
25.26	339.199	-	-	++	-	-	-	-
25.26	379.157	++	-	++	-	-	++	++
25.39	112.985	++	++	++	++	++	-	-
25.39	333.228	++	-	-	-	-	-	++
25.39	667.464	++	-	-	-	-	-	++
26.47	106.041	++	++	++	++	++	++	++
27.04	1000.735	++	++	-	-	-	-	-
27.06	974.719	++	++	++	-	-	-	-

AB – adzuki bean, BEP- black eyed pea, BG- bambara groundnut, LB- lablab bean, MB- mung bean, PP- pigeon pea, SB- soya bean

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Appendix 7.30 Tabulation of relative abundance of unidentified ions (*m/z*) from bound phenolic beans extracts (*m/z*)

Retention time, min	*m/z*	Relative abundance, ppm						
		AB	BEP	BG	LB	MB	PP	SB
1.14	326.759	-	-	-	-	++	-	-
1.14	442.676	-	-	-	-	++	-	-
1.14	504.629	-	-	-	-	++	-	-
1.15	562.588	++	++	++	++	++	++	++
1.15	502.632	++	-	++	++	++	++	-
1.15	794.422	++	-	++	-	++	++	-
1.15	558.593	++	-	++	-	++	++	-
1.16	792.424	-	-	-	-	++	-	-
1.18	796.418	-	-	-	-	++	-	-
1.18	500.635	-	-	-	-	++	-	-
1.17	92.928	++	++	++	++	++	++	++
1.17	268.801	++	++	++	++	++	++	++
1.17	270.798	++	++	++	++	++	++	++
1.17	560.591	++	++	++	++	++	++	++
1.17	266.804	++	++	++	++	++	++	++
1.17	94.925	++	++	++	++	++	++	++
1.17	270.960	-	++	++	-	-	-	++
1.20	191.019	-	-	-	++	++	++	++
1.21	444.673	++	-	-	-	++	-	-
1.46	427.010	-	++	-	-	-	-	++
1.48	191.019	+++	+++	+++	+++	++	+++	+++
1.48	111.009	++	++	++	++	++	++	++
1.48	405.028	-	++	-	-	-	++	++
1.50	619.037	-	-	-	-	-	-	++
1.95	226.035	-	-	-	+++	-	-	-

AB – adzuki bean, BEP- black eyed pea, BG- bambara groundnut, LB- lablab bean, MB- mung bean, PP- pigeon pea, SB- soya bean

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Appendix 7.31 Tabulation of relative abundance of unidentified ions (*m/z*) from bound phenolic beans extracts (contd)

Retention time, min	*m/z*	Relative abundance, ppm						
		AB	BEP	BG	LB	MB	PP	SB
1.95	475.060	-	-	-	++	-	-	-
1.97	182.046	-	-	-	++	-	-	-
2.53	259.130	-	++	-	-	-	-	-
2.56	205.071	-	-	+++	-	-	-	-
2.56	433.132	-	-	++	-	-	-	-
2.56	661.193	-	-	++	-	-	-	-
2.41	303.062	-	-	-	-	-	+++	++
2.86	128.035	-	-	-	-	-	++	-
2.86	164.071	-	-	-	-	-	++	-
2.89	293.114	++	-	-	-	-	++++	++
2.88	587.235	-	-	-	-	-	++++	-
2.88	275.103	-	-	-	-	-	+++	-
3.03	609.217	-	-	-	-	-	++	-
3.27	422.156	-	-	-	-	-	++	-
3.29	433.113	-	-	-	-	-	-	++
3.94	387.166	-	-	-	-	++	++	-
4.30	381.192	-	-	++	-	-	-	-
4.23	449.108	-	-	-	-	++	-	-
4.14	197.045	-	-	-	-	-	-	++
4.19	467.130	-	-	-	-	-	++	-
4.35	389.181	-	-	-	-	-	++	-
4.44	409.125	-	-	-	-	-	++	-
4.48	467.130	-	-	-	-	-	+++	-
4.48	449.120	-	-	-	-	-	++	-
4.67	451.079	-	-	-	-	-	-	++

AB – adzuki bean, BEP- black eyed pea, BG- bambara groundnut, LB- lablab bean, MB- mung bean, PP- pigeon pea, SB- soya bean

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Appendix 7.32 Tabulation of relative abundance of unidentified ions (*m/z*) from bound phenolic beans extracts (contd)

Retention time, min	*m/z*	Relative abundance, ppm						
		AB	BEP	BG	LB	MB	PP	SB
4.67	461.108	-	-	-	-	-	-	++
4.67	867.189	-	-	-	-	-	-	++
5.09	239.092	++	-	-	-	-	-	-
5.10	307.129	-	-	-	-	-	++	-
5.20	525.234	+++	-	-	-	-	-	-
5.20	481.244	++	-	-	-	-	-	-
5.25	657.276	++	-	-	-	-	-	-
5.41	119.050	-	-	-	++	-	-	-
5.47	135.045	-	-	-	-	++	-	-
5.64	441.176	-	-	-	-	-	+++	-
5.64	883.359	-	-	-	-	-	++	-
5.68	609.146	++	-	-	-	-	-	-
5.69	431.098	-	-	-	-	++++	-	-
5.69	863.203	-	-	-	-	+++	-	-
5.78	899.179	-	-	-	-	+++	-	-
5.78	467.074	-	-	-	-	++	-	-
5.83	529.074	-	-	-	-	++	-	-
5.88	403.160	-	-	-	-	-	++	-
5.89	489.056	-	-	-	-	++	-	-
6.11	467.074	-	-	-	-	-	-	+++
6.11	477.103	-	-	-	-	-	-	+++
6.11	899.179	-	-	-	-	-	-	++
6.11	431.098	-	-	-	-	-	-	++
6.17	361.223	-	-	-	-	-	-	++
6.50	830.404	-	-	-	-	-	-	+++

AB – adzuki bean, BEP- black eyed pea, BG- bambara groundnut, LB- lablab bean, MB- mung bean, PP- pigeon pea, SB- soya bean

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Appendix 7.33 Tabulation of relative abundance of unidentified ions (*m/z*) from bound phenolic beans extracts (contd)

Retention time, min	*m/z*	Relative abundance, ppm						
		AB	BEP	BG	LB	MB	PP	SB
6.58	569.224	-	-	-	-	-	++++	-
6.58	591.206	-	-	-	-	-	++	-
6.63	773.314	-	-	-	++	-	-	-
6.83	685.299	++	-	++	++++	+++	-	-
6.67	721.275	-	-	-	++	-	-	-
6.67	743.257	-	-	-	++	-	-	-
6.91	864.388	-	-	-	-	-	++	++
7.12	812.393	-	-	-	-	-	-	++
7.22	686.287	-	-	++	++	++	-	-
7.51	723.502	+++	+++	+++	+++	++++	++++	++++
7.53	713.473	++++	++++	++++	++++	+++	+++	+++
7.38	846.379	-	-	-	-	-	-	++
7.37	865.600	-	-	-	-	++	-	-
7.42	187.097	-	-	-	++	++	-	-
7.45	703.264	-	-	-	++	++	-	-
7.45	667.288	-	-	-	++	-	-	-
7.52	465.059	-	-	-	-	++	-	-
7.71	685.298	-	-	-	++	-	-	-
8.05	685.298	-	-	-	++	++	-	-
8.09	343.212	-	-	-	-	-	-	++
8.32	407.183	-	-	-	-	-	-	++
8.69	483.236	-	-	-	-	-	-	++
8.76	407.183	-	-	-	-	-	-	++
8.82	409.199	-	-	-	-	-	-	+++
8.99	343.212	-	-	-	-	-	-	++

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Appendix 7.34 Tabulation of relative abundance of unidentified ions (*m/z*) from bound phenolic beans extracts (contd)

Retention time, min	*m/z*	Relative abundance, ppm						
		AB	BEP	BG	LB	MB	PP	SB
9.19	409.199	-	-	-	-	-	-	+++
9.60	653.338	-	-	-	-	-	-	++
10.13	491.285	-	-	-	-	-	-	++
10.93	497.251	-	-	-	-	-	-	++
10.76	809.432	-	-	-	++	-	-	-
10.91	823.411	-	-	-	++	-	-	-
11.05	421.199	-	-	-	-	-	-	++
11.15	327.217	-	++	-	++	+++	-	++++
11.15	423.215	-	-	-	-	-	-	++
11.32	991.465	-	-	-	++	-	-	-
11.32	955.489	-	-	-	++	-	-	-
11.79	659.473	-	-	-	-	-	-	++++
11.79	365.209	-	-	-	-	-	-	++
11.79	753.455	-	-	-	-	-	-	++
11.82	329.233	-	-	++	++	-	++	++++
11.82	681.455	-	-	-	-	-	-	++
11.82	427.209	-	-	-	-	-	-	++
12.48	793.437	-	-	-	++++	-	-	-
12.48	793.938	-	-	-	++	-	-	-
12.48	829.413	-	-	-	++	-	-	-
12.50	327.217	-	-	-	++	-	-	-
12.81	971.484	++	-	-	-	-	-	-
12.82	396.215	-	-	-	-	-	+++	-
13.10	957.505	-	++	-	-	-	-	-
13.36	941.510	-	++	+++	-	++	-	-

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Appendix 7.35 Tabulation of relative abundance of unidentified ions (*m/z*) from bound phenolic beans extracts (contd)

Retention time, min	*m/z*	Relative abundance, ppm						
		AB	BEP	BG	LB	MB	PP	SB
13.36	977.487	-	++	+++	-	++	-	-
13.36	987.516	-	++	++	-	-	-	-
13.76	809.432	-	-	++	-	-	-	-
14.02	795.453	++++	-	-	-	-	-	-
14.05	831.429	++	-	-	-	-	-	-
14.67	661.358	-	-	-	++	-	-	-
15.02	779.457	++	-	-	-	-	-	-
15.02	815.434	++	-	-	-	-	-	-
15.37	823.411	++	-	-	-	-	-	-
15.67	809.432	++++	-	-	-	-	-	-
15.67	845.407	++	-	-	-	-	-	-
15.76	424.246	-	-	-	-	-	++	-
15.82	311.222	++	++	-	-	-	-	-
16.28	474.262	+++	++	-	++	-	++	-
17.02	793.437	++	-	-	-	-	-	-
17.75	476.278	++++	++	++	+++	-	+++	++
17.80	297.153	++	-	-	-	-	-	-
18.80	452.277	-	-	-	-	-	-	++
19.65	291.197	-	-	-	-	++	-	-
19.57	311.168	++	++	++	++	++	++	++
21.13	293.212	-	-	-	-	++	-	-
21.39	325.184	+++	++	++	++	++	+++	+++
23.17	339.199	++	++	++	++	++	++	+++
24.38	277.217	++	-	-	-	-	-	-
25.40	116.928	-	++	++	-	-	-	-

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Appendix 7.36 Tabulation of relative abundance of unidentified ions (*m/z*) from bound phenolic beans extracts (contd)

Retention time, min	*m/z*	Relative abundance, ppm						
		AB	BEP	BG	LB	MB	PP	SB
25.42	112.985	++	++	++	++	++	++	++
25.43	116.928	-	-	-	-	-	++	-
25.44	333.228	++	-	-	-	-	-	-
26.52	106.041	++	++	++	++	++	++	++
27.03	1000.735	++	-	-	-	++	++	-
27.03	976.734	-	-	-	-	++	-	-
27.03	974.721	++	++	-	-	++	-	-
27.04	116.928	-	-	-	++	++	-	++
27.02	896.674	-	++	++	++	-	-	-
27.05	671.465	++	++	++	++	++	++	-

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