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A Comparison Study of Phenolic Contents and *in Vitro* Antioxidant Activities of Australian Grown Faba Beans (*Vicia faba* L.) Varying in Seed Coat Colours as Affected by Extraction Solvents

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Abstract

Twelve Australian grown faba bean genotypes with seed colour ranging from white, different shades of buff/beige, green, red and purple, were extracted using 80% methanol (v/v) or 70% acetone (v/v). The findings showed that commonly consumed buff-coloured genotypes have comparable phenolic contents and antioxidant activities to those with exotic seed coat colour (red, green and purple). In general, the extraction yield for methanol and acetone extracts of faba bean genotypes were similar. However, the acetone extracts of faba bean varieties with coloured seed coats exhibited higher antioxidant activities than their methanol extracts counterparts. The phenolic content and antioxidant activities of acetone extracts from white-genotypes were much lower than those of genotypes with coloured-seed coats. Phenolic compounds and antioxidant activities of the genotypes were compared in an array of chemical-based assays and profiled using an on-line high-performance liquid chromatography-post column derivatization (HPLC-PCD) system. The total phenolic content (TPC) and total flavonoid content (TFC) of acetone extracts were approximately twice those of methanol extracts. The acetone extracts contained six times higher levels of di(phenyl)-(2,4,6-trinitrophenyl) iminoazanium radical scavenging

[§]Deceased.

activity (DPPH), six times higher Total Equivalent Antioxidant Capacity (TEAC) and two times higher Ferric Reducing Antioxidant Power (FRAP) than those of methanol extracts. In general, the methanol extracts of white-coloured faba bean genotype exhibited comparable phenolic contents and antioxidant activities to varieties with coloured seed coats. However, the TPC, TFC, DPPH, TEAC and FRAP of acetone extracts from white-coloured genotype were 2 - 4, 1 - 2, 5 - 9, 2 - 3 and 1 - 2 times lower than those of faba bean varieties with coloured seed coats respectively. HPLC-PCD analyses showed substantial antioxidant responses, represented as a dense "hump" of peaks in the HPLC chromatograms of acetone extracts from coloured-genotypes. This "hump" was not detected in the chromatograms of white-genotype acetone extracts, or in chromatograms of methanol extracts regardless of genotype or seed coat colour. Hydroxybenzoic acids/flavanols, hydroxycinnamic acids and flavonols were dominant in coloured beans. The findings suggest that it is possible to select commonly consumed buff-coloured faba bean genotypes from breeding programs that have enhanced levels of phenolic compounds and antioxidant activities, potentially enhancing their health-promoting properties. Furthermore, an opportunity exists to develop faba bean extracts for pharmaceutical or natural medicines.

Keywords

Vicia faba, Extraction Solvent, Phenolic Compound, Antioxidant

1. Introduction

The faba beans (*Vicia faba* L.) typically grown in Australian broad acre agriculture and commonly marketed internationally are buff (light tan) in colour. Nevertheless, genotypes exist with other seed coat colours such as white, different shades of buff/beige, green, red and purple. The relationship between seed coat colour, phenolic content and antioxidant level of pulses is contentious. For example, darker-coloured cultivars are reported to have a higher phenolic content and antioxidant level than those of white-coloured cultivars [1], but this view is opposed by other reports [2] [3]. However, it is generally agreed that bean cultivars with different seed coat colours contain different dominant phenolic compounds and antioxidant levels [4] [5]. The phenolic contents and antioxidant levels also are affected by different cooking methods [6] [7] [8].

Various solvent systems have been applied in the extraction of phenolics from plant materials, with methanol and acetone-water mixtures being two of the most commonly used solvents. The type of solvent system used has a significant impact on the amount and type of phenolic compounds extracted from legume kernels [9]. For instance, it is advisable to extract anthocyanins using a slightly acidified methanol; while acetone is more efficient in extracting procyanidins from food products [10]. The combination of alcohols with water is more efficient than using a single solvent system for extracting phenolic compounds [9]

[11], possibly due to the fact that water-alcohol mixtures are capable of solubilizing a range of sugar attached glycosides as well as less polar aglycones [10].

In the present study, two extraction solvent systems: methanol-water (80:20; v/v) and acetone-water (70:30; v/v) were selected to extract phenolic compounds from 12 Australian grown faba bean genotypes. The faba bean genotypes chosen consisted of five different seed coat colour groups: white, buff, green, red and purple. A variety of reagent-based assays were applied to assess antioxidant activities of faba bean extracts. The composition of phenolic compounds were characterised by high performance liquid chromatography (HPLC) analyses. The results of this study may enable targeted breeding of faba bean genotypes with specific phenolic compositions and contents.

2. Materials and Methods

2.1. Sample Preparation and Storage

Twelve faba bean genotypes from five different seed coat colour groups were grown to maturity and mechanically harvested at Wagga Wagga Agricultural Institute, Australia in 2008. They included a white-coloured breeding line TF(Ic*As)*483/13, green-coloured cv. Icarus, red-coloured cv. Rossa, purple-coloured cv. Deep Purple and eight buff-coloured genotypes (breeding lines 974*(611*974)/42, 1269*483/6-1, 1323/3, S95007 and cultivars Doza, Farah, Fiord and Nura). Bean samples were cleaned by removing foreign materials, loosely packed and stored in the dark at room temperature for five months to naturally dry, then stored at −18˚C.

2.2. Preparation of Crude Phenolic Extract

Bean samples were ground into flour using an IKA-Universalmühle M20 Grinder (Janke and Kunkel, Staufen, Germany) and stored in screw cap plastic containers at −18˚C. Extraction of phenolic compounds was carried out by dispersing flour in aqueous acetone (acetone-water, 70:30, v/v) [12] or aqueous methanol (methanol-water, 80:20, v/v) [13] at a solid to solvent ratio of 1:10 and shaking (150 rpm, Orbital Mixer; Ratek OM11, Australia) for 2 hrs at room temperature. The supernatant was collected after centrifugation at 4000 *g* for 5 minutes at 5˚C. The extraction was repeated on the residue and the supernatant from subsequent extraction was pooled. These supernatants were then concentrated under reduced pressure at 40˚C using a rotary evaporator (Rotavapor R-205; Buchi, Switzerland) and freeze-dried to obtain a fine lyophilised powder using a Christ-Alpha 1-4 freeze dryer (Biotech International, Germany). This process was performed in duplicate to produce two independent extracts for each extraction method and genotype. The extracts were stored at −20˚C until required. Distilled water was used to dissolve the lyophilized extract and these reconstituted extracts were filtered through 0.45 μm Millipore filters before analysis.

2.3. Seed Coat Colour Determination

Colour determination was conducted using a Minolta Chronometer CR-310

(Minolta Co., Osaka, Japan) with a CR-A33E class light projection tube and a white calibration tile. A white container was used to hold whole bean samples and at least two layers of the bean samples were placed in the containers for each measurement to avoid background interference. Six independent measurements were taken for each genotype and averaged. Colour was defined by the L*, a* and b* tristimulus system (CIELAB), where L* was a measure of lightness (0 = black; 100 = white); a* was a measure of red/green (+60 = red; −60 = green); and b* was a measure of blue/yellow (+60 = yellow; −60 = blue).

2.4. Chemical Assays

2.4.1. Total Phenolic Content (TPC) Assay

The total phenolic content (TPC) was measured [14]. Gallic acid (Sigma) was used as a standard and the TPC was expressed as mg of gallic acid equivalent per gram of dry bean (mg GAE/g DW).

2.4.2. Total Flavonoid Content (TFC) Assay

The total flavonoid content (TFC) assay was determined according to Michalska *et al.* (2007) [13]. (+)-Catechin gallate (Sigma) was included as a standard and the TFC was expressed as mg of catechin equivalent per gram of dry bean (mg CE/g DW).

2.4.3. Trolox Equivalent Antioxidant Capacity (TEAC) Assay

The Trolox equivalent antioxidant capacity (TEAC) assay measures the relative ability of extracts to react with the radical cation of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{+}$). The assay was conducted using 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Sigma-Aldrich) as the standard and the results were expressed as μM of Trolox equivalent per gram of dry bean (μmol TE/g DW) [15].

2.4.4. 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) Radical Scavenging Assay

The 1,1-diphenyl-2-picrylhydrazyl (DPPH•) radical scavenging assay determines the relative ability of extracts to scavenge 1,1-diphenyl-2-picrylhydrazyl radicals (DPPH). The assay was carried out and the total volume was reduced to fit in microcuvettes [13]. Trolox was used as a standard and the results were expressed as μM of Trolox equivalent per gram of dry bean (μmol TE/g DW).

2.4.5. Ferric Reducing Antioxidant Power (FRAP)

The ferric reducing antioxidant power (FRAP) assay was used to measure ferric reducing ability of faba bean extracts. The assay was performed according to Benzie and Strain [16].

2.5. Separation Using Column Chromatography

2.5.1. Preparation of 2,2'-Azino-Bis(3-Ethylbenzthiazoline-6-Sulphonic Acid) (ABTS) Free Radical for Post Column Derivatisation Assay (PCD)

The 7 mM $ABTS^{\cdot+}$ solution was prepared by dissolving the ABTS in deionised

water and mixed with 2.45 mM of potassium persulfate overnight to allow a complete reaction. The solution was diluted using distilled water to obtain an absorbance of 0.70 ± 0.02 at 734 nm and filtered through a 0.45 µm polypropylene membrane (Millipore).

2.5.2. On-Line PCD with High Performance Liquid Chromatography (PCD-HPLC) for Phenolic Compound Profiling

Analysis of the antioxidant activity was carried out on-line using $ABTS^{\cdot+}$ cation radicals [17]. The HPLC system (ProStar model 410) consisted of a Phenomenex Luna 5U C18 column (100 A pore size; 150 × 3 mm) preceded by a guard column (Phenomenex, 4 × 3 mm), a Varian 240I pump and a Varian 335 PDA Detector. The mobile phase A was water-acetic acid (99:1, v/v) and phase B was methanol-acetonitrile (50:50, v/v). An aliquot (8 µL) of sample extract (50 mg/mL) dissolved in solvent A was injected in a gradient of 0% - 48% phase B over 40 min at a flow-rate of 0.4 mL/min. UV spectra were recorded at 280 nm. PCD on-line antioxidant activity was determined on the HPLC eluent which arrived at a "T" piece and reacted with $ABTS^{\cdot+}$ being added at a flow rate of 0.4 mL/min. The absorbance of the reaction products was measured by a UV-Vis detector at 414 nm.

2.5.3. Quantification of Phenolic Classes Using High-Performance Liquid Chromatography (HPLC)

The HPLC system (Shimadzu, Japan) consisted of an auto injector (SIL-10AD VP), a degasser (DGU-20A, two pumps (LC-10AD), a diode array detector (SPD-M10A), a column oven (CTO-10AS) and a system controller (SCL-10A). The solvents A and B used were 0.5% trifluoroacetic acid (TFA, Sigma-Aldrich) (v/v) in distilled water and acetonitrile-TFA-water (95:0.5:4.5, v/v/v) respectively. The column temperature was maintained at 25˚C and the solvent elution rate was set at 1 mL/min. The gradient elution method was used to separate phenolic compounds with modifications [14], where solvent B was eluted from 0% - 10% over first 10 min, 10% - 50% for 45 min, 50% - 80% for 15 min, 80% - 100% for 15 min and lastly an isocratic elution with 100% solvent B over 10 min, for an overall 95 min HPLC run. The HPLC eluents were monitored at four different wavelengths: 280 nm for determination of hydroxybenzoic acids/flavanols; 326 nm for determination of hydroxycinnamic acids; 370 nm for determination of flavonols and 520 nm for determination of anthocyanins. Amounts of respective phenolic classes were expressed as GAE for hydroxybenzoic acids/flavanols; chlorogenic acid equivalents (CHAeq) for hydroxycinnamic acids; rutin equivalents (REeq) for flavonols and cyanidin 3-glucoside equivalents (C3Geq) for anthocyanins.

2.6. Statistical Analysis

The analysis of variance (ANOVA) on data was carried out using GenStat 13th edition (VSN International Ltd, UK). Colour (L*, a* and b* values) was analysed by ANOVA with genotype as the only term in the model. The means for each

genotype with least Significant Difference (LSD) was calculated at the 5% level.

There were two independent extractions for each genotype and solvent extraction system combination. Depending on the variable, there was one (material yield, TPC and FRAP) or three (TFC, DPPH and TEAC) measurements per extraction. These data were analysed by ANOVA with genotype, extraction replicate and their interaction as terms in the model. Where there were three measurements (TFC, DPPH and TEAC), the extraction replicate was included in the model as a blocking factor. Examination of the residuals from the models indicated that data for some variables needed to be log or square root transformed to ensure that the residuals were normally distributed. The LSD was calculated at the 5% level to enable comparisons across genotypes and solvent extraction systems. Means for each genotype and solvent extraction system combination were also used to calculate Pearson correlations.

Student *t*-tests were performed to generate the significant differences ($p < 0.05$) using Prism 5 (Graphpad Software, CA, USA).

3. Results

3.1. Colour Determination

The image of 12 faba bean genotypes with different seed coat colours is illustrated in Figure 1. Seed coat colours as measured by the CIELAB colour system, seed weight and description of 12 faba bean genotypes are shown in Table 1. The violet-coloured Deep Purple and red-coloured Rossa had the lowest L* value, followed by green-coloured Icarus and other buff-coloured genotypes. As expected, TF(Ic*As)*483/13 had the highest L* value, due to its white-coloured

Figure 1. Image of faba bean genotypes with different seed coat colours (for illustration purposes only).

Table 1. Seed coat colours as measured by the CIELAB colour system, seed weight and description of the 12 faba bean genotypes.

Faba bean genotype	Seed coat colour	Seed weight (g/100 seeds)	Description	L[†,‡]	a[†,‡]	b[†,‡]
TF(Ic*As)*483/13	White	60	Tannin free, white hilum breeding line (combination of white flower, white testa and white hilum)	56.0[a]	1.4[g]	17.3[g]
974*(611*974)/42	Buff[§]	85	Breeding line, low discolouration[ʃ] at Tamworth	49.0[c]	7.1[b]	17.7[def]
1269*483/6-1	Buff[§]	83	Breeding line, good disease resistance, white hilum, low discolouration[ʃ] at Tamworth	48.7[cd]	7.9[a]	18.8[a]
1323/3	Buff	60	Germplasm from ICARDA[≠]	46.5[g]	3.6[e]	17.9[cde]
Doza	Buff	45	Variety, from Northern New South Wales	48.0[de]	4.5[d]	17.4[fg]
Farah	Buff	67	Variety, selected from Fiesta, based on germplasm from Spain	47.7[ef]	7.0[b]	18.4[abc]
Fiord	Buff	46	Variety, based on germplasm from Greece	47.0[fg]	5.9[c]	17.6[defg]
Nura	Buff	53	Variety, progeny of Icarus*Ascot	49.1[c]	5.9[c]	18.5[ab]
S95007/1	Buff	67	Germplasm from ICARDA	50.0[b]	2.3[f]	18.0[bcd]
Icarus	Green	80	Variety, based on germplasm from Ecuador	45.3[h]	−2.2[h]	17.5[efg]
Rossa	Red	53	Variety, based on germplasm from Ecuador	28.3[i]	5.0[d]	0.9[h]
Deep Purple	Dark Purple	41	Single plant selection from Fiord, made in Western Australia	29.0[i]	4.4[d]	−1.1[i]
			5% LSD	0.9	0.6	0.5

[†]Values were based on six measurements. The data marked by the same superscripts in each column are not significantly different ($p < 0.05$). [≠]ICARDA—International Center for Agricultural Research in the Dry Areas; [‡]L*—lightness (0 = black; 100 = white); a*—red/green (+60 = red; −60 = green); b*—blue/yellow (+60 = yellow; −60 = blue); [§]Seed coat colour was between buff and green, buff in colour but with modifier genes that result in a green tinge; [ʃ]Discolouration refers to the darkening rate of the seed coat after harvest.

seed coat. Some of the buff-coloured genotypes, such as 1323/3, Fiord and Farah were darker than the other buff-coloured genotypes including 974*(611*974)/42, 1269*483/6-1 and S95007/1. Only green-coloured Icarus showed a negative a* value, while the rest of the genotypes showed positive a* values. The Deep Purple was the only genotype that exhibited a negative b* value, which appeared in the blue hue region. On the other hand, Rossa had a very low but positive b* value. The rest of the white-, buff- and green-coloured genotypes shared similar b* values indicating a yellowish hue.

3.2. Material Yield

Material yield of the crude phenolic extracts from all of the genotypes comprised about 7% - 9% of the total bean weight (dry basis) regardless of choices of solvent system (Table 2). The material yield from TF(Ic*As)*483/13 was lower when extracted using 80% methanol (v/v), however, the inverse was true for 974*(611*974)/42 and Fiord. For all the other faba bean genotypes, material yields from the methanol and acetone extract pairs for each genotype were not significantly different.

3.3. Effect of Solvent Extraction Systems on Phenolic Content of 12 Faba Bean Genotypes

The effect of solvent extraction system on TPC and TFC of the 12 faba bean

Table 2. Comparison of material yield, total phenolic content (TPC) and total flavonoid content (TFC) of 12 Australian grown faba bean genotypes extracted using different solvent extraction systems: 80% methanol (v/v) and 70% acetone (v/v).

Faba bean genotype	Material yield (g/100gDW)*†		TPC (mgGAE/gDW)*‡^		TFC (mgCE/gDW)*$^	
	80% Methanol	70% Acetone	80% Methanol	70% Acetone	80% Methanol	70% Acetone
TF(Ic*As)*483/13	8.8^{abc}	7.9^{fghijk}	1.5^{efgh} (2.3)	1.7^{e} (2.8)	0.31^{gh} (1.4)	0.02^{lm} (1.0)
974*(611*974)/42	7.8^{ghijk}	8.6^{abcde}	1.3^{i} (1.6)	2.8^{bc} (8.0)	0.13^{jk} (1.1)	1.05^{ab} (2.8)
1269*483/6-1	7.5^{jk}	7.3^{k}	1.3^{i} (1.7)	2.3^{d} (5.4)	0.08^{kl} (1.1)	0.62^{f} (1.9)
1323/3	8.5^{abcdef}	8.8^{abc}	1.5^{efgh} (2.3)	3.0^{b} (8.9)	0.01^{lm} (1.0)	0.86^{de} (2.3)
Doza	8.2^{cdefghi}	8.5^{abcdef}	1.4^{ghi} (2.0)	2.9^{bc} (8.6)	−0.03^{m} (1.0)	0.10^{jk} (1.1)
Farah	8.2^{cdefgh}	8.6^{abcde}	1.4^{hi} (1.8)	2.9^{bc} (8.5)	−0.11^{n} (0.9)	0.80^{e} (2.2)
Fiord	8.4^{bcdefg}	9.2^{a}	1.5^{efgh} (2.3)	3.5^{a} (12.0)	0.09^{kl} (1.1)	0.93^{cd} (2.5)
Nura	7.5^{ijk}	7.7^{hijk}	1.4^{ghi} (2.0)	3.3^{a} (10.9)	0.24^{hi} (1.3)	1.09^{a} (3.0)
S95007/1	8.0^{efghij}	8.1^{defghij}	1.6^{efg} (2.5)	2.7^{c} (7.5)	0.09^{kl} (1.1)	1.00^{bc} (2.7)
Icarus	8.6^{abcde}	8.7^{abcd}	1.5^{efgh} (2.3)	3.3^{a} (10.8)	0.29^{gh} (1.3)	1.11^{a} (3.0)
Rossa	8.7^{abcd}	8.3^{bcdefgh}	1.6^{ef} (2.6)	3.4^{a} (11.2)	0.33^{g} (1.4)	1.08^{a} (2.9)
Deep Purple	8.3^{bcdefgh}	8.9^{ab}	1.5^{fghi} (2.1)	3.3^{a} (10.8)	0.18^{ij} (1.2)	0.96^{c} (2.6)
5% LSD (genotype × solvent extraction system)	0.7		0.2		0.08	

*The data marked by the same superscripts within two types of solvent extraction systems in the respective assay are not significantly different ($p < 0.05$); †g/100gDW—gram of extract per 100 gram of dry bean; ‡TPC—Total phenolic content; mgGAE/gDW—mg gallic acid equivalent per gram of dry bean; Square root transformed to achieve equal variances in the population for obtaining 5% LSD (the original data are in brackets); $TFC—Total flavonoid content; mgCE/gDW—mg of catechin equivalent per gram of dry bean; $\text{Log}_{(2.7)}$transformed to achieve equal variances in the population for obtaining 5% LSD (the original data are in brackets).

genotypes is shown in Table 2. The TPC of acetone extracts from all genotypes were 2 - 3 times greater than those of methanol extracts, except for TF(Ic*As)*483/13. Among the acetone extracts, TF(Ic*As)*483/13 exhibited the lowest TPC, which was 2 - 4 times lower than those of coloured-genotypes. The highest TPC levels were found in Fiord, Nura, Icarus, Rossa and Deep Purple.

Similarly, the TFC in acetone extracts from all genotypes were 2 - 3 times higher than those of methanol extracts, except for TF(Ic*As)*483/13. The acetone extracts from Nura, Icarus, 974*(611*974)/42 and Rossa similarly exhibited the highest TFC among all genotypes.

3.4. Effect of Solvent Extraction Systems on Antioxidant Levels of 12 Faba Bean Genotypes

The effect of solvent extraction system on the antioxidant levels of 12 faba bean genotypes is shown in Table 3. The acetone extracts from all genotypes exhibited greater levels of DPPH radical scavenging activity than those of methanol extracts by 4 - 8 times, except for TF(Ic*As)*483/13. The methanol extract from TF(Ic*As)*483/13 had a similar level of DPPH radical scavenging activity to other genotypes. Inversely, the acetone extract of TF(Ic*As)*483/13 had a much lower DPPH activity compared to the other genotypes. The highest level of DPPH radical scavenging activity was found in the acetone extracts from Fiord, Icarus and Deep Purple.

The TEAC of the acetone extracts from all genotypes were about 2 - 7 times

Table 3. Effect of different solvent extraction systems, 80% methanol (v/v) and 70% acetone (v/v) on the level of antioxidant activities of 12 Australian grown faba bean genotypes with different seed coat colours.

Faba bean genotype	DPPH (μmolTE/gDW)*†		TEAC (μmolTE/gDW)*‡		FRAP (μmolFe^{2+}eq/gDW)*§	
	80% Methanol	70% Acetone	80% Methanol	70% Acetone	80% Methanol	70% Acetone
TF(Ic*As)*483/13	2.2^{gh} (8.9)	2.0^{lm} (7.3)	2.5^{l} (12.0)	3.2^{i} (24.0)	8.2^{ghij}	10.9^{f}
974*(611*974)/42	2.1^{ij} (8.1)	3.9^{bc} (48.1)	2.6^{l} (13.2)	4.3^{c} (71.6)	7.7^{hij}	19.1^{cd}
1269*483/6-1	2.0^{klm} (7.3)	3.4^{f} (29.3)	2.5^{mn} (12.0)	3.9^{h} (48.1)	6.8^{j}	14.9^{e}
1323/3	1.9^{n} (6.6)	3.7^{de} (39.4)	2.4^{op} (10.8)	4.1^{g} (58.7)	8.6^{ghi}	19.1^{cd}
Doza	2.0^{m} (7.3)	3.8^{d} (43.6)	2.4^{op} (10.8)	4.1^{f} (58.7)	7.3^{ij}	19.7^{cd}
Farah	1.9^{n} (6.6)	3.8^{d} (43.6)	2.4^{p} (10.8)	4.2^{ef} (64.8)	6.7^{j}	19.9^{c}
Fiord	2.1^{jkl} (8.1)	4.2^{a} (64.8)	2.4^{no} (10.8)	4.4^{a} (79.1)	6.7^{j}	22.2^{a}
Nura	2.1^{jk} (8.1)	4.0^{b} (53.1)	2.6^{k} (13.2)	4.3^{c} (71.6)	7.9^{hij}	18.2^{d}
S95007/1	2.1^{ij} (8.1)	3.7^{e} (39.4)	2.6^{k} (13.2)	4.2^{de} (64.8)	8.7^{ghi}	16.5^{e}
Icarus	2.2^{gh} (8.9)	4.1^{a} (58.7)	2.5^{lm} (12.0)	4.4^{a} (79.1)	8.2^{ghij}	20.4^{bc}
Rossa	2.3^{g} (9.8)	3.9^{c} (48.1)	2.7^{j} (14.6)	4.3^{cd} (71.6)	9.6^{fg}	20.1^{bc}
Deep Purple	2.2^{hi} (8.9)	4.1^{a} (58.7)	2.5^{l} (12.0)	4.4^{b} (79.1)	9.2^{gh}	21.7^{ab}
5% LSD (genotype × solvent extraction system)	0.1		0.1		1.62	

*The data marked by the same superscripts within two types of solvent extraction systems in the respective assay are not significantly different ($p < 0.05$); †DPPH—2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity; μmolTE/gDW—μmol Trolox equivalent per gram of dry bean; $Log_{(2.7)}$transformed to achieve equal variances in the population for obtaining 5% LSD (the original data are in brackets); ‡TEAC—Trolox equivalent antioxidant capacity; μmolTE/gDW—μmol Trolox equivalent per gram of dry bean; $Log_{(2.7)}$transformed to achieve equal variances in the population for obtaining 5% LSD (the original data are in brackets); §FRAP—Ferric Reducing Antioxidant Power; μmolFe^{2+}eq/gDW—μmol Fe^{2+} equivalent per gram of dry bean.

greater than those of methanol extracts. The genotype Rossa exhibited the highest TEAC among the methanol extracts, while Fiord and Icarus exhibited the highest TEAC among the acetone extracts. The genotype TF(Ic*As)*483/13 had the lowest TEAC among the acetone extracts, but its methanol extract had a TEAC close to the median of all other faba bean genotypes.

The FRAP of the acetone extracts from faba bean genotypes were about 2 - 3 times greater than those of methanol extracts, except the FRAP of acetone extract from TF(Ic*As)*483/13 was only 1.3 times higher than its methanol extract counterpart. The TF(Ic*As)*483/13 exhibited the lowest FRAP among the acetone extracts, while Fiord and Deep Purple were the highest.

3.5. Ranking of Phenolic Extraction Yields, TPC, TFC, FRAP, DPPH Radical Scavenging Activity and TEAC and Correlation between the Levels of Phenolic Compounds and Antioxidant Activities in Methanol and Acetone Extracts of Faba Bean Cultivars

The ranking of phenolic extraction yields, TPC, TFC, FRAP, DPPH radical scavenging activity and TEAC of 12 faba bean cultivars extracted by 80% methanol and 70% acetone respectively is shown in **Figure 2**. There is little consistency in rankings of genotypes from both extraction solvent systems of all variables.

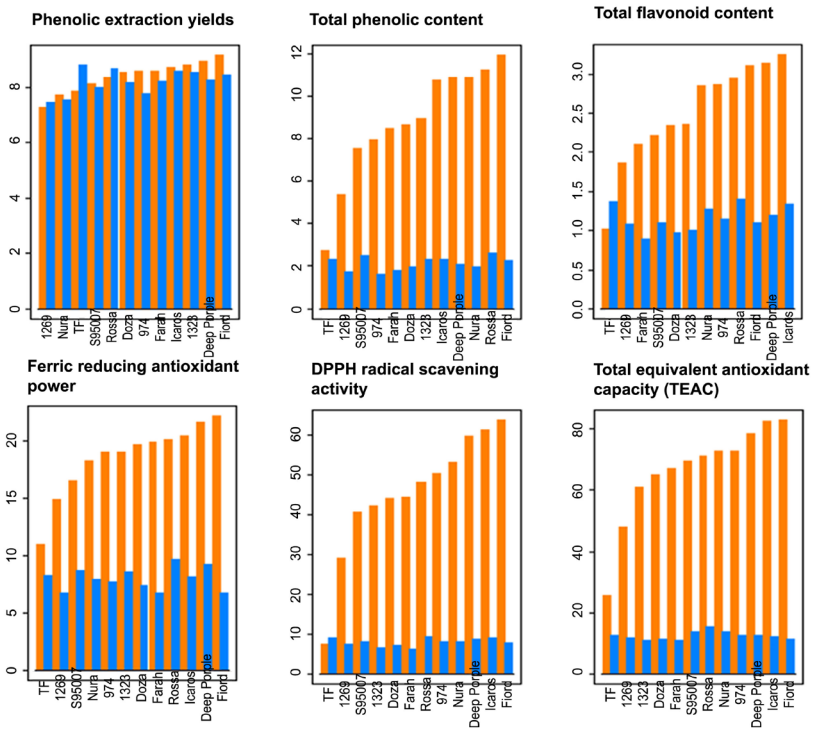

Figure 2. Ranking of phenolic extraction yields, total phenolic content, total flavonoid content, Ferric reducing antioxidant power, DPPH radical scavenging activity and total equivalent antioxidant capacity of 12 faba bean cultivars extracted by 80% methanol (blue bar) and 70% acetone (orange bar).

The correlations between data from the variables: TPC, TFC, DPPH, TEAC and FRAP for faba bean methanol and acetone extracts from 12 faba bean genotypes are shown in **Table 4**. The values of DPPH, TEAC and FRAP were positively correlated with each other for both methanol and acetone extractions ($r^2 > 0.62$ and $r^2 > 0.91$ respectively). There was a positive correlation between the values of TPC and TFC ($r^2 > 0.93$) of acetone-based but not methanol-based extracts.

For acetone-based extracts, a significant correlation between the TPC/TFC values and the results of all the antioxidant activities assays was found (r2 > 0.88). For methanol-based extracts, the values of TFC positively correlated with DPPH (r2 = 0.92), TEAC (r2 = 0.74) and FRAP (r2 = 0.59) antioxidant capacities, but the TPC values correlated only with the FRAP values (r2 = 0.66).

3.6. High Performance Liquid Chromatography-Post Column Derivatisation (HPLC-PCD) Profiles

Figure 3 illustrates the HPLC-post column derivatisation (PCD) profiles of crude phenolic extracts (70% acetone versus 80% methanol) from four faba bean genotypes representing different seed coat colour groups; white (TF(Ic*As)*483/13), buff (Nura), green (Icarus) and red (Rossa). The faba bean phenolic compounds from both solvent extraction systems eluted in two separate regions of the HPLC chromatogram, which are arbitrarily classified as polar (retention time: 0 - 15 min) and less-polar (retention time: 15 - 40 min) regions.

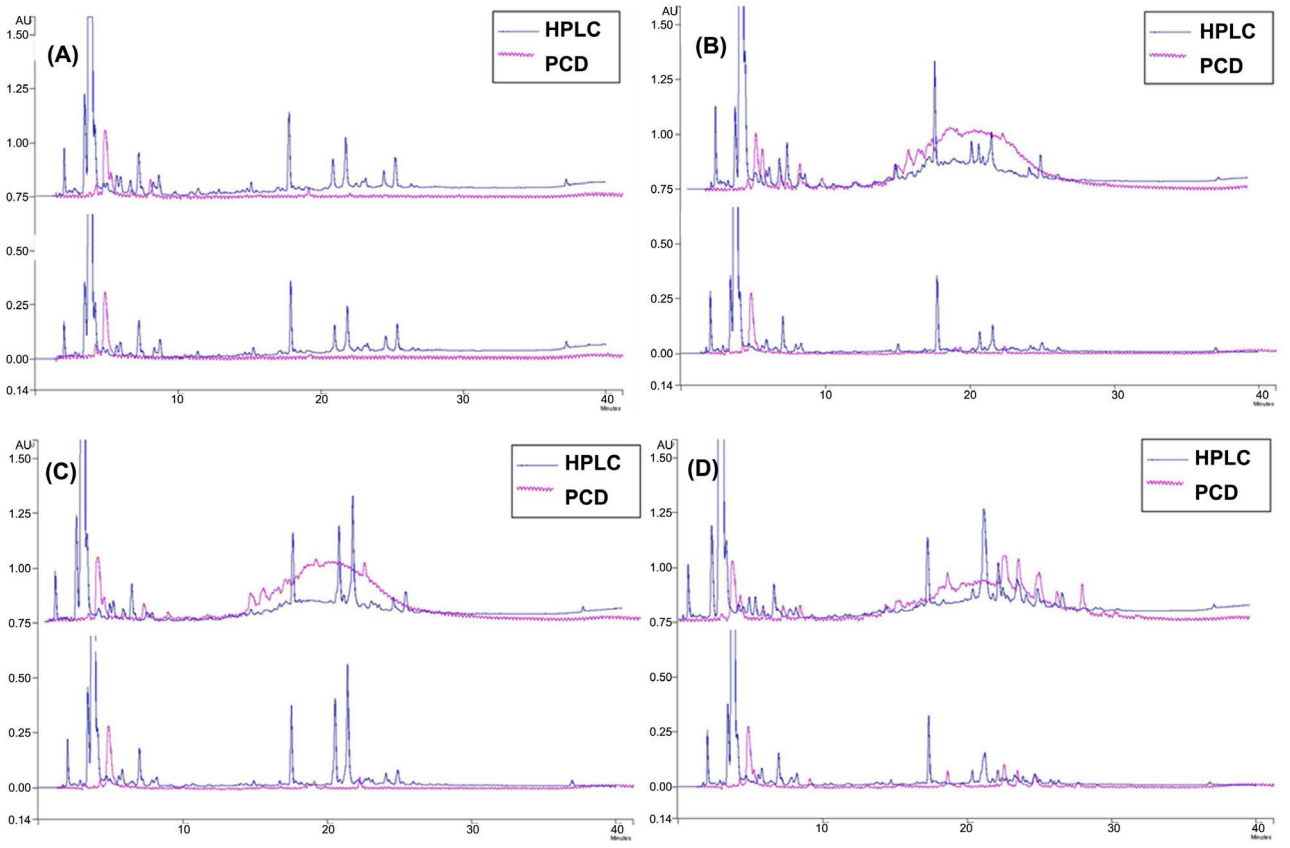

Figure 3. On-line high-performance liquid chromatography-post column derivatization (HPLC-PCD) profiles (280 nm) of faba bean genotypes extracted using 70% acetone (top) and 80% methanol (bottom); (A) white-coloured TF(Ic*As)*483/13; (B) green-coloured Icarus; (C) buff-coloured Nura and (D) red-coloured Rossa.

Table 4. Correlations of total phenolic content (TPC), total flavonoid content (TFC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, total equivalents antioxidant capacity (TEAC) and ferric reducing antioxidant power (FRAP) of methanol (white background) and acetone extracts (shaded background) from 12 faba bean genotypes (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

	TPC	TFC	DPPH	TEAC	FRAP
TPC	1.00	0.48	0.51	0.48	0.66*
TFC	0.93***	1.00	0.92***	0.74*	0.59*
DPPH	0.94***	0.97***	1.00	0.75*	0.62*
TEAC	0.92***	0.94***	0.98***	1.00	0.68*
FRAP	0.92***	0.88***	0.94***	0.91***	1.00

The HPLC profiles of both methanol and acetone extracts from all of the faba bean genotypes contained similar major peaks eluted at retention times: 4, 18, 21 and 22 min. Nevertheless, the chromatograms of acetone extracts from all genotypes showed a few additional small peaks in the polar region that showed an active PCD response but were absent in those of methanol extracts. On the other hand, a noticeable "hump" of densely co-eluted peaks appeared at the less-polar region in chromatograms of acetone extracts from the three coloured-genotypes (**Figures 3B, 3C and 3D**; retention time: 15 - 25 min). Moreover, these HPLC

"humps" directly corresponded to a substantive PCD response indicating a potent antioxidant activity, which was not prominent in the methanol extracts. In contrast, the HPLC-PCD profiles of both acetone and methanol extracts from TF(Ic*As)*483/13 (Figure 3A) appeared to be similar, where only the polar region in their chromatograms had a small active PCD response while the less-polar region lacked active compounds.

3.7. Analysis of Phenolic Classes in Faba Bean Extract

The HPLC results in Table 5 showed that both of the crude acetone extracts from Nura and Rossa contained three major phenolic classes: hydroxybenzoic acids/flavanols, hydroxycinnamic acids and flavonols, which were detectable at 280 nm, 326 nm and 370 nm, respectively. However, no anthocyanins were detectable at 520 nm in the extracts of either genotype. The extract of Rossa contained a significantly higher amount of all three phenolic classes than Nura.

4. Discussion

4.1. Efficiency of Extraction Solvents

This study demonstrates that 70% acetone (v/v) is generally more efficient than 80% methanol (v/v) in extracting phenolic compounds with a high level of antioxidant activity from faba beans. This result is likely attributed to the ability of acetone to extract the less polar active compounds, represented by the "humps" as indicated in HPLC chromatograms of the acetone extracts from coloured-genotypes. Our results are in agreement with other reports [9] [11] [18], where acetone was proven to be more efficient than methanol in extracting phenolic compounds in a variety of legume seeds.

4.2. Solvent Extraction System × Genotype and Their Interaction

The ANOVA analysis indicates that main factors affecting phenolic contents of extracts (TPC and TFC) and their antioxidant capacities (DPPH radical scavenging activity, TEAC and FRAP) are the type of solvent extraction system used and genotypes of faba bean, both being significant. Conversely, the effect of solvent extraction system is not significant for material yields, while the effect of genotypes is significant.

Table 5. Quantification of phenolic classes in faba bean genotypes crude acetone extracts using HPLC.

Detection wavelength (nm)	Phenolic class*	Faba bean genotype*	
		Nura	*Rossa*
280	Hydroxybenzoic acids/flavanols (mgGAE/gDW)†	2.94 ± 0.04^{a}	3.18 ± 0.09^{b}
326	Hydroxycinnamic acids (mgCHAeq/gDW)‡	0.16 ± 0.00^{a}	1.86 ± 0.11^{b}
370	Flavonols (mgRE/gDW)§	0.18 ± 0.01^{a}	2.42 ± 0.09^{b}
520	Anthocyanins	n/d	n/d

*Results were based on two independent extractions and single measurement taken per extraction (mean ± SD); n/d = not detected; the data marked by different superscripts across columns in the respective measurement wavelengths were significantly different ($p < 0.05$); †mgGAE/gDW—mg of gallic acid equivalent per gram of dry bean; ‡mgCHAeq/gDW—mg of chlorogenic acid equivalent per gram of dry bean; §mgRE/gDW—mg of rutin equivalent per gram of dry bean.

The solvent extraction system × genotype interaction is also significant for all of the mentioned variables (phenolic and antioxidant), including material yield. While the material yields from both solvent extraction systems are generally similar, those from acetone extracts of 974*(611*974)/42 and Fiord are higher than those of their methanol extracts. On the other hand, the extraction yield of the acetone extract is lower than that of methanol extract for TF(Ic*As)*483/13. Furthermore, there is little consistency in genotype rankings of phenolic (TPC and TFC) and antioxidant (DPPH, TEAC and FARP) variables between acetone and methanol extracts. These inconsistent effects from both factors reflect on the significant genotype × solvent extraction system interaction for material yields. It is also apparent that the acetone extracts produce a much greater spread of TPC, TFC, DPPH, TEAC and FRAP results amongst the genotypes than the methanol extracts. Of interest, Fiord tends to be the highest in TPC, FRAP, DPPH radical scavenging activity and TEAC for acetone extracts but it tends to fall below the median for methanol extracts. The opposite is true for TF(Ic*As)*483/13.

4.3. Role of Genotype (and Colour) in the Nature of Phenolic Content and Antioxidant Capacity

Genotype contributes to significant differences in phenolic contents and antioxidant levels in faba beans as reported in other studies [19] [20]. The differences in phenolic contents and antioxidant capacities among faba bean genotypes are likely to be related to seed coat colours. The material yields of acetone and methanol extracts are shown to be similar for most of the tested genotypes, but the acetone extracts exhibit a noticeably higher TPC, TFC and antioxidant levels for all genotypes except the white-coloured TF(Ic*As)*483/13. In addition, the majority of the antioxidant activity in faba bean acetone extracts are contributed by compounds represented by the HPLC "humps" observed in the chromatograph of genotypes with coloured seed coats, but not that of white-coloured geneotype. These compounds appear to be tightly grouped on the chromatograph and cannot be separated through varying polarities and elution flow rates. Similar HPLC "humps" in the chromatographic profiles of cider apples and faba beans detectable at 280 nm were also reported elsewhere [12] [21]. The HPLC "humps" are thought to be oligomeric and highly polymerised procyanidins and are reportedly too complex to be easily separated for chromatography analysis. In support of this, most compounds in the faba bean extracts are visible at 280 nm as observed using the HPLC system in this study, suggesting the compounds could be flavan-3-ols or catechins, the building block of procyanidins. Furthermore, acid hydrolysis of faba bean acetone extract generates anthocyanins detectable at 520 nm, indicating the presence of high molecular weight proanthocyanidins (results not shown).

It is often thought that darker-coloured beans exhibit a higher level of phenolic content than white-coloured beans and our findings support this concept [1]. However, the current study reveals that some lighter-coloured genotypes, such as the buff-coloured genotypes 974*(611*974)/42, Fiord and Nura, demon-

strated a comparable level of antioxidant activity to the darker-coloured genotypes Rossa, Deep Purple and Icarus. These findings will be received with interest by the food industry since buff-coloured faba beans are the only colour that is commercially acceptable for the export human consumption market.

The variation in phenolic contents of faba beans might be more strongly related to flower colour than testa colours where varieties with solid pigmented flowers tended to have higher tannin content in their beans than those with spotted flowers and white flowers [22]. This is particularly true for the tannin-free genotypes, which are known to have white-flowers with no spots [23]. Low tannin faba beans have white flowers with yellow spots [24], whereas the regular buff-coloured faba bean varieties have white flowers with black spots. In addition, increased variation in phenolic contents could also be caused by unintentional hybridisation between genotypes with different polyphenol contents in the field, since faba beans are outcrossing in nature. In support of this, crossing different soybean genotypes is known to result in offspring with kernels that contain high polyphenol contents and high antioxidant activities [25].

The TEAC and FRAP of faba bean methanol extracts obtained in the present study are comparable to what has been reported in other studies [26]. The TPC, TFC, DPPH radical scavenging activity and TEAC of the acetone extracts in the present study are also comparable or higher than those of acetone (80% v/v) extracts from eight different types of coloured peas and beans [9]. However, the crude ethanolic extracts of 13 faba beans grown in Tunisia [19] contain higher TPC, TFC and FRAP compared to our methanol extracts (by approximately 15-, 5- and 50-times) and our acetone extracts (by about 4-, 3- and 20-times respectively). The markedly higher levels of phenolic contents and antioxidant levels in the faba beans cultivated in Tunisia might be attributed to genetic and/or environmental differences in comparison to the present study [20].

In general, the white-coloured TF(Ic*As)*483/13 exhibits a lower phenolic content and antioxidant capacity in comparison to the coloured-genotypes. This was expected as this genotype is the only tannin free genotype with a white hilum (combination of white flower, white testa and white hilum) among the faba bean genotypes researched in the present study. Unlike the coloured-genotypes, both solvent extraction systems produce similar levels of TPC and DPPH radical scavenging activity for TF(Ic*As)*483/13. The acetone extract of TF(Ic*As)*483/13 contains a significantly lower level of TFC than its methanol extract (the inverse trend to coloured-genotypes). It is known that proanthocyanidins are not detectable in white-coloured faba beans [27]. Also, tannins (polymeric proanthocyanidins) in the coloured-genotypes are likely to contribute to the majority of antioxidant activity.

There have been on-going efforts in breeding faba bean cultivars with low tannin contents for feeding monogastric animals [28]. Tannins in faba beans are reported to lower animal weight and sizes due to its tendency to form indigestible complexes with proteins [29]. In addition, the tannin-free beans tend to have a lower proportion of seed coat [22] but are also more susceptible to seed coat

cracking and plant diseases [30], which makes their adaptation to farming situations difficult.

4.4. Nature of Antioxidant Capacities in Faba Beans in Relation to Extraction Solvent Systems

The FRAP values of the acetone extracts are 2-times higher than those of methanol extracts. In comparison, the values of DPPH radical scavenging activity and TEAC of acetone extracts are higher than their methanol counterparts by 6- and 5-times respectively. This suggests that acetone is vastly more efficient at extracting antioxidants from faba bean with free radical scavenging activities, and to a lesser extent reducing powers, than methanol. In support of this, it is reported that the antioxidant activity of faba beans is mainly based on chain-breaking ability rather than chelating activity with transition metals [31].

4.5. Correlation between Phenolic Compounds and Antioxidant Activities of Faba Bean Extracts

The correlations between phenolic contents and antioxidant capacities of acetone extracts are much stronger than those of methanol extracts. This might suggest that aqueous acetone is more efficient than methanol in extracting phenolic compounds that exhibit a high level of antioxidant activity. The antioxidant activity is likely to be contributed by compounds represented by the "hump" in the HPLC chromatograms of acetone extracts.

5. Conclusions

This study shows that 70% acetone is more efficient than 80% methanol in extracting phenolic compounds with a higher antioxidant activity from faba beans. The TPC and TFC of faba bean acetone extracts are twice as high as methanol extracts counterparts. The acetone extracts also contain six times higher levels of DPPH radical scavenging activity, six times higher TEAC and two times higher FRAP than those of methanol extracts. The antioxidant activities of faba bean acetone extracts are mainly based on radical scavenging activity rather than reducing ability. Among the faba bean genotypes, white-coloured genotype contains the lowest phenolic content and antioxidant level in the acetone extracts. Conversely, methanol extract of the white-coloured genotype exhibits an average phenolic content and antioxidant capacity to other coloured-genotypes in the methanol extracts. The antioxidant capacities of acetone extracts from coloured-beans are shown to be largely contributed by compounds represented by the "hump" which appears at the relatively less-polar region of the HPLC chromatograms. However, no active HPLC "hump" is observed in either the methanol, or acetone extract from white-coloured faba bean genotype. This study justifies the suitability of using aqueous acetone for analyses of phenolic compounds from faba beans to optimize extract abilities and antioxidant capacities of these extracts. Furthermore, the current study demonstrates the variability in phenolic

contents and antioxidant activities among faba bean genotypes with different seed coat colours. Our findings suggest potentials to select desirable buff-coloured faba bean genotypes within the breeding program to enhance the antioxidant activity of new varieties since buff-coloured varieties are the only commercially desirable colour currently. This finding may be useful if faba bean extracts are used in the pharmaceutical or natural medicine disciplines, as yield of bioactive compounds is an important factor in the economic viability of producing such products.

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Highlights

- Antioxidant activities in some buff- and darker-coloured beans were comparable
- Coloured-seed exhibits higher phenolic and antioxidant levels than white-seed
- Phenolic contents of faba bean acetone extracts were 2-fold those of methanol extracts
- Antioxidant activities of faba bean acetone extracts were 2 - 6 folds those of methanol extracts
- High antioxidant activity "hump" presents in HPLC chromatography of acetone extract