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Chemometric analysis on free amino acids and proximate compositional data for selecting cowpea (*Vigna unguiculata* L.) diversity

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ABSTRACT

Biodiversity of cowpea, along with the interesting agricultural and nutritional properties, has encouraged research with local landraces of Portugal. This work deals with the composition of dry beans of cowpea from 28 different Portuguese cultivars concerning dry matter (DM), crude proteins (CP), fat content (FC), ash, and concentration of free essential and non-essential amino acids. Data obtained on these traits showed contents of DM, FC, ash, and CP in the ranges of 95.7–98.9%, 1.0–1.6%, 2.7–4.7%, and 18.6–26.9%, respectively. The content in free amino acids was evaluated as an indicator of germinating potential of the diverse cultivars assessed. The amino acids content was the highest in the varieties ‘Vg50’ and ‘Vg58’. Cluster and principal component analyses of the data allowed the identification of the most promising Portuguese landraces of cowpea to be used for breeding new varieties with improved germination potential and nutritional traits.

1. Introduction

In the last decades, pulses have been stressed as an advantageous alternative to partially replace animal protein in human and animal diets (O’Neill et al., 2012; Day, 2013). One of the major advantages of increasing the proportion of grain legumes in foods is the reduction of the ingestion of saturated fats, which has been demonstrated to be deleterious for human health (Mitchell et al., 2009). Additionally, in regions where malnutrition with incidence of protein deficiency is prevalent, the introduction of new nutritive vegetable species, with high resistance to environmental stress factors, may be of great benefit (Henley et al., 2010).

In addition to human nutrition, with respect to livestock feedstuffs, to overcome the restrictions established by the European regulations on using meat and bone meal to accomplish food safety claims, soya has been used as the main substituent. However, the European industry is becoming increasingly reliant on imported soya bean meal. Its high price in global feed trade markets has encouraged research into alternative home-grown vegetables (O’Neill et al., 2012).

Consequently, in the last few years, special attention has been paid to pulses, which are robust crops with an interesting nutritional value, such as chickpea, faba bean, horse gram, and cowpea (Phillips et al., 2003). Among these crops, cowpea (*Vigna unguiculata* L.) is a legume species resistant to abiotic stress characteristic of semi-arid regions (Huynh et al., 2015), providing reasonable yields as well as a high content in protein, dietary fibre and amino acids (Henley et al., 2010; Anyango et al., 2011). Nevertheless, additional selection and breeding programs to obtain high yielding cultivars with desirable trait of resistance to biotic and abiotic stresses and valuable chemical composition (Huynh et al., 2015) is desirable. Regarding the selection of those varieties with high adaptative characteristics, it is crucial to take into account the wide range of internal mechanisms and

Abbreviations: Ala, L-alanine; Arg, L-arginine; Asn, L-asparagine; Asp, L-aspartic acid; DM, dry matter; CP, crude protein; FC, fat content; Gln, L-glutamine; Glu, L-glutamic acid; Gly, glycine; His, L-histidine; Ile, L-isoleucine; Leu, Leucine; MCE, 2-mercaptoethanol; Met, L-methionine; OPA, *o*-phthalaldehyde; PC, principal component; PCA, principal component analysis; Phe, L-phenylalanine; Ser, L-serine; Thr, L-threonine; Trp, L-tryptophan; Tyr, L-tyrosine; Val, L-valine (Val).

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physico-chemical factors that contribute to germination, amongst which the content in free amino acids is of critical relevance (Alhadi et al., 2012).

In this work, 28 accessions of cowpea representing the Portuguese variability were evaluated on their nutritional traits (content in dry matter (DM), crude protein (CP), crude fat (CF), and ash). Additionally, the content in free amino acids was assessed as a relevant property correlated with the germination capacity. This information will identify those varieties with the most promising characteristics to be included in further breeding programs or for production of innovative foods as edible sprouts. Amongst the evaluated varieties, 'Fradel', the most popular Portuguese cultivar commercially available, was included as a reference. The classification of the 28 varieties assessed was achieved using multivariate statistical methods (cluster and principal component analyses (PCA)).

2. Materials and methods

2.1. Chemicals

Cation exchange resin, Dowex (H^+) 50WX8-400, acetonitrile, methanol, *o*-phthalaldehyde (OPA)/2-mercaptoethanol, disodium hydrogen phosphate dihydrate, propionic acid, and standards of amino acids (L-alanine (Ala), L-arginine (Arg), L-asparagine (Asn), L-aspartic acid (Asp), glycine (Gly), L-glutamic acid (Glu), L-glutamine (Gln), L-histidine (His), L-isoleucine (Ile), leucine, (Leu), L-methionine (Met), L-phenylalanine, (Phe), L-serine (Ser), L-threonine (Thr), L-tryptophan, (Trp), L-tyrosine (Tyr), and L-valine (Val)) were purchased from Sigma Aldrich (Steinheim, Germany). Solid-phase extraction mini-columns were from Macherey-Nagel (Düren, Germany). Hydrochloric acid was obtained from Merck (Kenilworth, NJ).

2.2. Plant material

Seeds from distinct cowpea (*Vigna unguiculata* L.) cultivars, from 2013 season, were obtained from local producers in Portugal according to the information provided in Table 1, regarding harvest location with indication of longitude, latitude, and altitude. The selected locations corresponded to the main cowpea growing areas in Portugal, mainly from northern and central regions in the country, with the samples being collected from four distinct districts, namely, *Castelo Branco* (*Covilhã*, *Fundão*, and *Penamacor*), *Guarda* (*Sabugal*, *Celorico da Beira*, *Almeida*, *Trancoso*, *Pinhel*, *Figueira de Castelo Rodrigo*, and *Meda*), *Bragança* (*Torre de Moncorvo*, *Carrazeda de Ansiães*, *Vila Flor*, *Mogadouro*, *Miranda do Douro*, *Vimioso*, *Macedo de Cavaleiros*, *Mirandela*, *Valpaços*, and *Bragança*), and *Vila Real* (*Alijó*). The meteorological data corresponding to 2013 season is presented with indication of data for each district (Table 2). Cowpea seeds were ground to a fine powder required for chemical analysis and kept protected from light and humidity until analysis.

2.3. Basic chemical analysis

Samples were analyzed in triplicate ($n = 3$) using the procedures described by Association of Official Analytical Chemists (AOAC, 1995) to determine the content in ash and DM (AOAC protocol #942.05), CP (AOAC protocol #954.01), and CF (AOAC protocol #920.39).

2.4. Free amino acids extraction and chromatographic determination

Samples (0.2 g) were extracted with 3.0 mL of methanol/Milli-Q water (70:30, v/v) at 70 °C for 10 min and centrifuged for 2 min at 5000 rpm. The supernatant was poured into a 10.0-mL volumetric flask. This step was repeated twice and the combined supernatants

Table 1
Geographical locations of collection of the *Vigna unguiculata* L. accessions used in the study.

Code	Harvest location (town (district)	Geographical location		
		Latitude	Longitude	Altitude (m)
Vg 47	S. Sebastião, Castelo Bom (Almeida)	40°35′595″N	6°53′595″W	794
Vg 48	Reigada (Figueira Castelo Rodrigo)	40°48′041″N	6°56′594″W	663
Vg 49	Quinta Nova (Pinhel)	40°47′549″N	7°03′282″W	573
Vg 50	Madalena (Pinhel)	40°51′154″N	7°08′224″W	523
Vg 51	Rabaçal (Meda)	40°50′585″N	7°15′163″W	580
Vg 52	Rio de Mel (Trancoso)	40°48′451″N	7°23′260″W	770
Vg 53	Franco (Mirandela)	41°43′458″N	7°39′609″W	593
Vg 54	Rio Torto (Valpaços)	41°44′383″N	7°38′575″W	673
Vg 55	Lamalonga (Macedo Cavaleiros)	–	–	–
Vg 56	Lamalonga (Macedo Cavaleiros)	–	–	–
Vg 57	Benhevai (Vila Flor)	41°37′874″N	7°09′291″W	600
Vg 58	Meimão (Penamacor)	40°17′070″N	7°07′020″W	607
Vg 59	Escarigo (Fundão)	40°14′572″N	7°17′227″W	507
Vg 60	Bendada (Sabugal)	40°22′009″N	7°15′325″W	1514
Vg 61	Cerdeira do Côa (Sabugal)	40°30′458″N	7°02′427″W	707
Vg 62	Caria (Covilhã)	40°17′521″N	7°22′016″W	511
Vg 63	Quinta das Neves/Cortes do Meio (Covilhã)	40°14′434″N	7°35′351″W	555
Vg 64	Casa Soeiro (Celorico da Beira)	40°37′343″N	7°24′296″W	519
Vg 65	Chã/Vila Chã (Alijó)	41°19′253″N	7°28′046″W	766
Vg 66	Pinhal do Norte (Carrazeda de Ansiães)	41°17′531″N	7°20′355″W	507
Vg 67	Sampaio (Vila Flor)	41°17′529″N	7°05′532″W	247
Vg 68	Larinho (Torre de Moncorvo)	41°12′192″N	7°00′481″W	494
Vg 69	Pinela (Bragança)	41°40′197″N	6°45′439″W	859
Vg 70	Carção (Vimioso)	41°35′321″N	6°35′168″W	679
Vg 71	Malhadas (Miranda do Douro)	41°32′367″N	6°19′354″W	770
Vg 72	Ventuzelo (Mogadouro)	41°16′571″N	6°35′060″W	726
Vg 73	Bornes (Macedo de Cavaleiros)	41°27′195″N	7°00′306″W	750
Vg 74 ('Fradel')	Local market (Vila Real)	–	–	–

Table 2
Climatic conditions for the districts of origin of the cowpea varieties collected for the study in 2013 in Portugal.

District[Z]	January			February			March			April			May			June		
	Tm[Y]	TM[X]	RR[W]	Tm	TM	RR	Tm	TM	RR	Tm	TM	RR	Tm	TM	RR	Tm	TM	RR
Bragança	2.3	9.7	142.6	0.9	10.3	69.0	3.9	11.6	168.0	4.5	16.2	64.1	6.4	18.9	35.8	9.9	25.4	3.6
Vila Real	3.9	10.7	194.2	2.9	11.3	71.5	5.4	12.4	247.1	6.3	16.9	80.6	7.5	18.8	29.1	11.2	24.5	3.5
Guarda	2.4	7.9	132.0	0.7	7.8	67.6	2.6	8.8	208.2	4.4	13.4	61.6	6.1	15.6	21.4	9.7	21.6	8.3
C. Branco	4.9	11.9	106.7	4.4	13.2	73.2	6.9	14.0	193.0	8.1	19.0	73.8	10.1	22.0	15.4	14.1	28.2	8.8

	July			August			September			October			November			December		
	Tm	TM	RR	Tm	TM	RR	Tm	TM	RR	Tm	TM	RR	Tm	TM	RR	Tm	TM	RR
Bragança	15.9	32.3	15.8	14.1	31.3	0.0	12.5	27.8	47.4	9.5	19.0	151.5	1.9	13.4	11.1	−1.2	9.1	161.6
Vila Real	16.2	31.6	4.8	15.2	31.2	0.0	14.6	28.3	54.5	11.0	19.5	160.0	4.8	13.4	14.9	1.8	9.5	214.6
Guarda	15.7	28.7	5.7	14.6	28.4	0.0	–	–	97.8	9.5	16.3	327.5	–	–	–	–	–	–
C. Branco	18.0	33.7	1.3	19.0	34.6	0.0	17.4	30.4	92.0	12.7	21.7	174.5	6.9	15.8	3.3	4.0	12.5	143.1

[Z] Geographical area of reference.
[Y] Mean daily minimum air temperature (°C).
[X] Mean daily maximum air temperature (°C).
[W] Total monthly rainfall (mm).

were made up to a final volume of 10.0 mL with methanol (70% in distilled water) and kept at −18 °C.

Sample purification was performed according to Cooper et al. (1984). Briefly, 2.0 mL of each extract were evaporated and resuspended in 2.0 mL of 0.1 M HCl. Mini-columns of 1.0 mL were set in a vacuum chamber (Varian Vac Elut SPS 24) and eluted with 0.5 mL of 0.1 M HCl before being filled up to 2 cm with a cation exchange resin, Dowex (H^+). The resuspended 2.0 mL of sample were passed through the column and washed with 5.0 mL of 0.1 M HCl. Free amino acids were eluted with 4 × 2.5 mL of 7.0 M NH_3. After evaporation by blowing air, the residue was dissolved in 0.3 mL of distilled water, filtered through 0.2-mm regenerated cellulose/polypropylene filters, and kept at −18 °C until analysis.

Free amino acids were quantified by HPLC (Gilson system) using a 150 mm C18 column (Waters Spherisorb S3 ODS2, i.d. 4.6 mm) and a UV–vis detector set at 340 nm, after pre-column derivatisation with OPA/MCE, following a combination of procedures previously described (Sørensen et al., 1999; Carvalho et al., 2012). The mobile phase was made of two solvents: $Na_2HPO_4{\cdot}2H_2O$ 350.0 mM/propionic acid (1:1, v/v)/acetonitrile/Milli-Q water (40:8:52, v/v/v) (Solvent **A**), and acetonitrile/methanol/water (30:30:40, v/v/v) (Solvent **B**). The chromatographic analysis was performed using the linear gradient scheme (*t* in min; flow rate; %**B**): (0.0; 1.3; 0%), (9.5; 1.3; 11%), (11.0; 1.3; 12%), (13.6; 1.3; 20%), (20.4; 1.3; 45%), (23.4; 1.3; 50%), (25.4; 0.8; 60%), (32.0; 0.8; 100%), and (34.0; 1.3; 0%). Identification and quantification of the detected amino acids were done against external standards after adjustment through regression lines and expressed as mmol 100 g^{-1} dw.

2.5. Statistical analysis

Data were processed using OriginPro 9.1 (OriginLab, Northampton, MA). Analysis of variance (ANOVA) and Tukey's multiple range test were carried out. Spearman's correlation analysis was performed to corroborate relationships between selected parameters. Principal components analysis (PCA) was carried out using the analytical data as variables, resorting to triplicate values for each parameter, and without solution rotation. Cluster analysis was applied to the means of the analytical values, which were used to obtain hierarchical associations, as standardised data, employing Euclidean distance and Ward's method as dissimilarity measure and amalgamation rule, respectively.

3. Results and discussion

3.1. Basic chemical determinations

The mean values of proximate nutritional composition performed on 28 cowpea accessions are presented in Table 3. The

Table 3
Content in dry matter, ash, crude protein, and crude fat of different Portuguese varieties of cowpea (*Vigna unguiculata* L.).

Variety	Dry matter (%)	Ash (%)	Crude Protein (%)	Fat content (%)
Vg47	98.3 ijklm[Z]	4.4 ef	23.1 ghi	1.1 bcde
Vg48	95.7 a	3.6 cd	22.3 efgh	1.0 ab
Vg49	97.9 efghijkl	3.3 abcd	23.1 hij	1.1 abc
Vg50	97.3 cdefgh	3.9 ed	21.9 cdefgh	1.2 bcdef
Vg51	97.9 efghijkl	3.3 abcd	21.1 bcdefg	1.1 bcde
Vg52	97.0 bcde	3.5 bcd	21.0 bcdef	1.2 bcdefg
Vg53	97.7 efghijk	3.2 abcd	26.9 e	1.2 bcdefg
Vg54	98.5 klm	3.1 abc	21.7 bcdefgh	1.0 ab
Vg55	98.3 jklm	3.1 abc	21.7 bcdefgh	1.2 bcdefg
Vg56	98.3 jklm	3.8 cde	19.9 ab	1.1 bcd
Vg57	98.6 lm	3.5 bcd	20.7 bcde	1.3 defgh
Vg58	98.0 efghijkl	3.4 abcd	18.6 a	1.3 cdefgh
Vg59	97.5 defghij	2.8 ab	21.9 cdefgh	1.4 fgh
Vg60	98.1 ghijklm	3.3 abcd	20.0 abc	1.2 bcdefg
Vg61	97.3 cdefg	4.7 f	25.1 jke	1.2 bcdef
Vg62	97.3 cdefgh	3.1 abc	24.6 ijk	1.2 bcdef
Vg63	96.4 ab	3.8 cde	19.8 ab	1.2 bcd
Vg64	97.4 cdefghi	3.9 de	21.3 bcdefgh	1.1 bcde
Vg65	98.1 hijklm	3.8 cde	24.7 ab	1.6 i
Vg66	96.6 bc	3.8 cde	22.1 efgh	1.2 bcd
Vg67	98.9 m	3.7 cde	25.5 kl	1.1 bcd
Vg68	96.7 bcd	3.7 cde	21.7 bcdefgh	0.8 a
Vg69	97.5 defghij	3.9 de	21.6 bcdefgh	1.1 ab
Vg70	98.4 klm	2.7 a	23.0 fghi	1.5 hi
Vg71	97.2 bcdef	3.6 cd	21.6 bcdefgh	1.4 fgh
Vg72	98.1 ghijklm	3.3 abcd	20.1 abcd	1.1 bcd
Vg73	97.1 bcdef	3.9 de	22.9 fghi	1.2 bcdefgh
Vg74 ('Fradel')	97.7 efghijkl	3.7 cde	22.0 defgh	1.4 ghi
p-value	*	*	***	***
LSD ($P < 0.001$)	0.4	0.4	1.0	0.1

[Z] Means ($n = 3$) within a column followed by different letters are significantly different at $p < 0.05$ (*) and $p < 0.001$ (***) according to Tukey's multiple range test.

content in CP recorded in the 28 cultivars of cowpea evaluated showed values ranging from 18.6 to 26.9% (for 'Vg58' and 'Vg53', respectively), the highest corresponding to the varieties 'Vg61', 'Vg62', 'Vg65', and 'Vg67', which presented higher values than the average (22.1%). These levels were in agreement with contents described in the literature concerning high yielding cowpea cultivars, which reported values from 22.5 to 26.0% (Cheng and Hardy, 2003; Ragab et al., 2004; Giami, 2005; Vasconcelos et al., 2010; Carvalho et al., 2012; Antova et al., 2014) and further support the high nutritional value of cowpea seeds. This interesting nutritional composition provide added value to a legume crop perfectly adapted to the growing conditions in southern Europe even in the most demanding areas.

Regarding the FC, differences were observed between cultivars, with values ranging from 0.8% for 'Vg68' to 1.6% for 'Vg65', with an average of 1.2%. The contents in FC observed in the present work are in good agreement with those previously reported in the literature, which range between 1.3 and 3.9% (Zia-Ul-Haq et al., 2010; Carvalho et al., 2012; Antova et al., 2014; Ojiako and Kayode, 2014). In this concern, the minor variations observed on FC in varieties from very different locations and agro-climatic conditions suggest the partial dependence of the fat composition on these factors. However, this content decreases as a consequence of drying procedure employed to obtain marketable seeds and on the treatment with natural and synthetic insecticides (Ojiako and Kayode, 2014).

3.2. Content in free essential and non-essential amino acids

Generally, free amino acids content of legume seeds is neglected when considering its nutritional value as they tend to be lost during cooking. However, their concentration in seeds needs to be considered when selecting varieties with agronomical purposes because of their critical role on the germination process (Alhadi et al., 2012). Moreover, current trends in consumption habits enhance the possibility for the development of new improved legume seed products, such as sprouts. In this regard, the evaluation of the content in free amino acids of cowpea seeds would constitute a valuable indicator for identifying those varieties with the highest potential to be used for new envisaged applications.

The scored amino acids of the cowpea varieties assessed in the present work are the essential amino acids Ala, Arg, His, Met, Phe, Thr, Val, and Trp; and the non-essential amino acids Asn, Asp, Glu, Gln, Gly, Ile, Ser, and Tyr. When evaluating the content in free essential and non-essential amino acids, the concentrations observed with respect to essential amino acids showed differences amongst the separate cultivars evaluated, except for Arg and Ala (Table 4). Thus, Arg and Ala were identified in limited amounts (0.003 and 0.001 mmol 100 g^{-1} dw, on average, respectively) that were consistent with previous reports pointing out these two free amino acids as minor in cowpea seeds (Alhadi et al., 2012). Similarly, in agreement with data reported in the literature, free lysine and leucine were not found in cowpea seeds. The majority of the cultivars presented similar quantities of His, which ranged from 0.022 to 0.023 mmol 100 g^{-1} dw. However, regarding this essential amino acid, three varieties ('Vg48', 'Vg49', and 'Vg59') showed a much higher concentration (0.043 mmol 100 g^{-1} dw, on average). Concerning the content in free Phe, Thr, and Trp, the cultivar 'Vg50' displayed the highest values (0.078, 0.067, and 0.059 mmol 100 g^{-1} dw, on average, respectively), whilst 'Vg58' cv. showed the highest concentration of co-eluting free essential amino acids Met/Val (0.143 mmol 100 g^{-1} dw, on average) (Table 4). With respect to non-essential amino acids, only Tyr presented

Table 4
Content in free essential amino acids (mmol 100 g^{-1} dw) of different Portuguese varieties of cowpea (*Vigna unguiculata* L.).

variety	Essential amino acids						
	arginine	histidine	phenylalanine	methionine/valine	threonine	alanine	tryptophan
Vg47	0.003 a[Z]	0.023 a	0.059 fghi	0.042 a	0.039 hi	0.001 a	0.033 abcde
Vg48	0.003 a	0.040 ab	0.025 abc	0.025 a	0.029 defghi	0.001 a	0.039 abcdefg
Vg49	0.003 a	0.046 b	0.040 cdefg	0.036 a	0.029 cdefghi	0.001 a	0.028 abcde
Vg50	0.003 a	0.022 a	0.078 i	0.074 ab	0.067 j	0.001 a	0.059 g
Vg51	0.003 a	0.023 a	0.067 hi	0.070 ab	0.052 ij	0.001 a	0.044 cdefg
Vg52	0.003 a	0.023 a	0.028 abc	0.038 a	0.035 fghi	0.001 a	0.036 abcdef
Vg53	0.003 a	0.022 a	0.038 bcdef	0.056 a	0.010 abcde	0.001 a	0.022 abc
Vg54	0.003 a	0.023 a	0.027 abc	0.039 a	0.033 efghi	0.001 a	0.025 abcd
Vg55	0.003 a	0.023 a	0.035 bcde	0.040 a	0.020 abcdefgh	0.001 a	0.034 abcdef
Vg56	0.003 a	0.023 a	0.056 efghi	0.064 ab	0.036 ghi	0.001 a	0.041 bcdefg
Vg57	0.003 a	0.023 a	0.042 cdefg	0.070 ab	0.033 efghi	0.001 a	0.043 cdefg
Vg58	0.003 a	0.022 a	0.051 defgh	0.143 b	0.007 abcd	0.001 a	0.028 abcde
Vg59	0.003 a	0.044 b	0.041 cdefg	0.072 ab	0.024 bcdefgh	0.001 a	0.043 cdefg
Vg60	0.003 a	0.023 a	0.035 bcde	0.045 a	0.036 ghi	0.001 a	0.036 abcdefg
Vg61	0.003 a	0.023 a	0.032 abcd	0.031 a	0.033 efghi	0.001 a	0.032 abcde
Vg62	0.003 a	0.023 a	0.041 cdefg	0.075 ab	0.034 fghi	0.001 a	0.049 efg
Vg63	0.003 a	0.023 a	0.039 cdefg	0.049 a	n.d.[Y]	0.001 a	0.039 abcdefg
Vg64	0.003 a	0.023 a	0.022 abc	0.036 a	0.005 abc	0.001 a	0.028 abcde
Vg65	0.003 a	0.023 a	0.022 abc	0.055 a	0.003 ab	0.001 a	0.031 abcde
Vg66	0.003 a	0.023 a	0.011 a	0.023 a	0.000 a	0.001 a	0.019 ab
Vg67	0.003 a	0.023 a	0.019 abc	0.043 a	0.012 abcdef	0.001 a	0.028 abcde
Vg68	0.003 a	0.023 a	0.061 ghi	0.063 ab	0.050 ij	0.001 a	0.056 hg
Vg69	0.003 a	0.023 a	0.015 ab	0.032 a	0.023 abcdefgh	0.001 a	0.018 a
Vg70	0.003 a	0.023 a	0.021 abc	0.033 a	0.017 abcdefgh	0.001 a	0.022 abc
Vg71	0.003 a	0.023 a	0.028 abc	0.081 ab	0.029 defghi	0.001 a	0.035 abcdef
Vg72	0.003 a	0.023 a	0.039 cdefg	0.026 a	0.017 abcdefgh	0.001 a	0.036 abcdef
Vg73	0.003 a	0.023 a	0.035 bcde	0.044 a	0.047 ij	0.001 a	0.028 abcde
Vg74 (Fradel)	0.003 a	0.023 a	0.052 defgh	0.042 a	0.014 abcdefg	0.001 a	0.047 defg
p-value	n.s.[X]	***	***	***	***	n.s	***
LSD ($p < 0.05$)	0.000	0.014	0.012	0.052	0.000	0.000	0.012

[Z] Means ($n = 3$) within a column followed by different letters are significantly different at $p < 0.05$ (*) and $p < 0.001$ (***) according to Tukey's multiple range test. [Y] n.d.: not detected. [X] n.s.: not significant.

Table 5
Content in free non-essential amino acids (mmol 100 g^{-1} dw) of different Portuguese varieties of cowpea (*Vigna unguiculata* L.).

Variety	non-essential amino acids							
	aspartic acid	asparagine	glutamic acid	glutamine	glycine	isoleucine	serine	tyrosine
Vg47	0.98 hijk[Z]	0.37 cde	0.59 efg	1.017 cdef	0.994 klm	0.014 abcd	0.154 bcde	0.014 a
Vg48	0.51 bcdefg	0.22 abcde	0.42 abcdef	0.712 abcde	0.573 defghij	0.010 ab	0.080 abcd	0.014 a
Vg49	1.13 kl	0.18 abcde	0.32 abcdef	0.822 abcdef	1.411 n	0.013 abcd	0.093 abcd	0.014 a
Vg50	1.38 e	0.46 e	0.90 g	1.400 f	1.387 mn	0.033 cde	0.199 de	0.014 a
Vg51	1.05 jkl	0.39 de	0.65 fg	1.285 ef	1.235 lmn	0.027 bcde	0.127 abcde	0.014 a
Vg52	0.53 bcdefg	0.28 adcde	0.54 cdefg	0.767 abcdef	0.754 hijk	0.014 abcd	0.113 abcd	0.014 a
Vg53	0.64 efgh	0.06 ab	0.19 abcdef	0.703 abcde	0.587 efghij	0.016 abcd	0.068 ab	0.014 a
Vg54	0.83 ghijk	0.17 abcde	0.37 abcdef	0.755 abcdef	0.592 efghij	0.019 abcd	0.092 abcd	0.014 a
Vg55	0.74 fghij	0.13 abcd	0.34 abcdef	0.864 bcdef	1.183 lmn	0.011 ab	0.114 abcd	0.014 a
Vg56	0.84 ghijkl	0.25 abcde	0.48 abcdefg	0.871 bcdef	0.667 ghijk	0.024 abcde	0.117 abcd	0.014 a
Vg57	0.64 efgh	0.14 abcd	0.28 abcdef	0.726 abcdef	0.593 efghij	0.023 abcde	0.098 abcd	0.014 a
Vg58	0.15 a	0.01 ab	0.04 ab	0.288 ab	0.077 ab	0.044 e	0.022 a	0.014 a
Vg59	0.28 abcd	0.06 ab	0.13 abcde	0.461 abc	0.362 abcdefgh	0.034 de	0.036 ab	0.014 a
Vg60	0.60 cdefg	0.09 abcd	0.23 abcdef	0.587 abcd	0.837 ijkl	0.019 abcd	0.078 abc	0.014 a
Vg61	0.62 defg	0.09 abcd	0.21 abcdef	0.727 abcdef	0.876 jkl	0.009 ab	0.083 abcd	0.014 a
Vg62	0.51 bcdefg	0.11 abcd	0.22 abcdef	0.496 abc	0.402 abcdefgh	0.022 abcde	0.079 abc	0.014 a
Vg63	0.55 cdefg	0.04 ab	0.14 abcde	0.457 abc	0.466 bcdefghi	0.008 ab	0.060 ab	0.014 a
Vg64	0.16 a	0.01 ab	0.07 ab	0.281 ab	0.063 a	0.007 ab	0.021 a	0.014 a
Vg65	0.27 abc	0.01 ab	0.04 ab	0.231 ab	0.226 abcde	0.012 abc	0.034 a	0.014 a
Vg66	0.16 a	0.01 a	0.02 a	0.161 a	0.118 abc	0.007 ab	0.020 a	0.014 a
Vg67	0.66 efghi	0.18 abcde	0.31 abcdef	0.810 abcdef	0.644 fghijk	0.009 ab	0.100 abcd	0.014 a
Vg68	0.85 ghijk	0.38 de	0.569 defg	1.261 def	0.876 jkl	0.013 abcd	0.244 e	0.014 a
Vg69	0.35 abcde	0.05 ab	0.111 abcd	0.338 abc	0.328 abcdefg	0.009 ab	0.052 ab	0.014 a
Vg70	0.38 abcde	0.02 ab	0.080 abc	0.406 abc	0.278 abcdefg	0.006 ab	0.050 ab	0.014 a
Vg71	0.44 abcdef	0.06 abc	0.110 abcd	0.436 abc	0.479 cdefghij	0.015 abcd	0.069 ab	0.014 a
Vg72	0.48 abcdef	0.25 abcde	0.402 abcdef	0.864 cdef	0.247 abcdef	0.004 a	0.058 ab	0.014 a
Vg73	1.00 ijk	0.31 bcde	0.491 bcdefg	1.187 def	1.201 lmn	0.010 ab	0.197 cde	0.014 a
Vg74 ('Fradel')	0.19 ab	0.02 ab	0.075 abc	0.381 abc	0.181 abcd	0.005 ab	0.038 ab	0.014 a
p-value	***	***	***	***	***	***	***	n.s.[Y]
LSD ($p < 0.05$)	0.179	0.155	0.237	0.338	0.213	0.010	0.052	0.000

[Z] Means ($n = 3$) within a column followed by different letters are significantly different at $p < 0.05$ (*) and $p < 0.001$ (***) according to Tukey's multiple range test.
[Y] n.s.: not significant.

similar values for all varieties (0.014 mmol 100 g^{-1} dw, on average). Variety 'Vg50' displayed the highest values for Asn, Asp, Glu, Gln and Ser (0.464, 1.379, 0.900, 1.400, and 0.199 mmol 100 g^{-1} dw, respectively), whilst the highest value of Gly and Ile corresponded to 'Vg49' and 'Vg58' (1.411 and 0.044 mmol 100 g^{-1} dw, respectively) (Table 5).

Free amino acids have a critical role in dormancy and germination in plants. In this regard, Phe has been related with the synthesis of abscisic acid, which is partially responsible for seeds dormancy, whilst Arg, Glu, and Met have been reported to enhance germination, suggesting that this essential process is regulated, even partially, by these amino acids. Thus, amino acid reserves in dry seeds have been emphasised as the major determinant for germination capacity (Canton et al., 2005; Alhadi et al., 2012). In this regard, from the batch of cowpea varieties evaluated, those containing the highest amount of Arg, Glu, and Met ('Vg50' and 'Vg58') might be promising cultivars to be used for agronomical purposes.

During the desiccation stage concentrations of free amino acids increase, being available to be involved as physiological factors and/or chemical promoters via various internal germination mechanisms (Footitt et al., 2002; Angelovici et al., 2010; Lau and Deng, 2010). This fact is reinforced by the description of the critical role of the free amino acids Asp, Asn, Arg and Glu in relation with germination/dormancy capabilities (Kuo et al., 2004), these being mainly located in the seed coat, which constitutes the most important reservoir (Górecki et al., 2001).

3.3. Cluster and principal component analysis

In order to identify the most valuable cultivars concerning the basic chemical composition of cowpea seeds and free amino acids content, multivariate approaches were applied to gain conclusions from the plethora of data recorded in this work. Hence, from PCA, sixteen components, representing the total variance (100.0%) were extracted, with the first nine explaining 96.4% of variance, whilst the first three components explained 70.0% (Table 6). Concomitantly, the weight of each variable (chemical parameter) for the separate principal components (PC) was determined, while the set of these loadings constitutes an Eigenvector (Wold et al., 1987). The Eigenvectors obtained for the first three components are represented in Fig. 1; 44.4% of variance is explained by PC1 (Table 6) and is mostly attributable to Asp, Glu, and Gln, whilst additional amino acids, such as Asn, Ser, and Thr, also displayed a lesser contribution to this component (Fig. 1). With respect to PC2, the contents in the free essential amino acids Val, Leu, and Phe were the most relevant contributors, although contrasting with PC1, CP and ash contents also provided a significant input to PC2 by displaying negative loadings (Fig. 1). DM constituted the most important variable for PC3, along with contents in CP and CF, which also displayed high scores for this component. Furthermore, the

Table 6
Variance explained by the first 9 principal components, accounting for 96.4% of the total variance.

PC	Eigenvalues	percentage of variance	cumulative variance
1	7.10	44.4%	44.4%
2	2.64	16.5%	60.9%
3	1.46	9.1%	70.0%
4	1.34	8.4%	78.4%
5	0.99	6.2%	84.6%
6	0.70	4.4%	88.9%
7	0.49	3.1%	92.0%
8	0.42	2.6%	94.6%
9	0.28	1.7%	96.4%

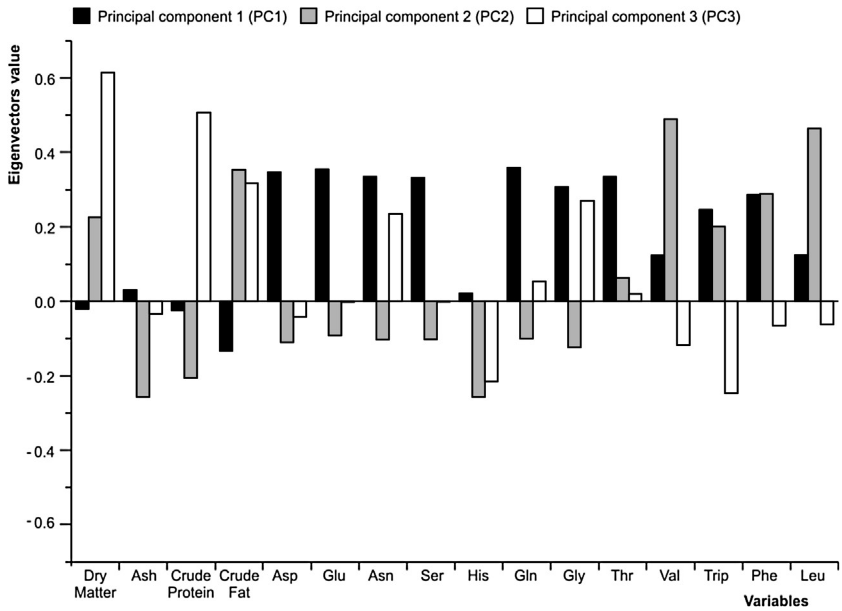

Fig. 1. Eigenvectors of the first three Principal components (PCs). Accounting for 70.0% of the total variance.

content of free amino acids displayed no significant contributions, with the exceptions of Gly and Trp. Trp was negatively correlated with this component, in line with the trend observed for the essential amino acids (Fig. 1). The percentage of variance explained by PC's 2 and 3 was 16.5 and 9.1%, respectively (Table 6).

The cluster analysis undertaken with all the analytical parameters revealed five different clusters (Fig. 2) that can be further understood alongside the PCA, by marking the different samples according to their clusters in the plot of PC1 *vs* PC2, which together represented 57.4% of the variance (Fig. 3A). In this plot, two distinct clusters (1 and 2) presented positive scores for PC1, whilst 'Vg60' from Cluster 2, represented the only exception, with all the replicates displaying negative scores for this PC. Additionally, both clusters (1 and 2) are composed of samples from either the districts of *Bragança* or *Guarda*. On the other hand, clusters 3, 4, and 5 presented negative scores for PC1. With respect to PC2, clusters 1, 2, and 3, displayed the same behavior, presenting samples scattered around this axis, while clusters 4 and 5 displayed negative and positive scores for this component, respectively. The latter cluster (5) comprised only two samples,

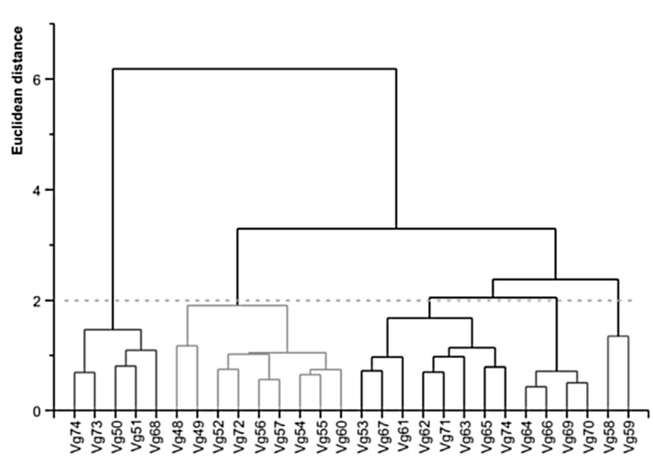

Fig. 2. Dendrogram showing the correalation between the 28 Portuguese cultivars of cowpea.

'Vg58' and 'Vg59', which displayed the highest scores for PC2, both from the district of *Castelo Branco*.

Concerning Cluster 3, some local correlations were observed, with three sub-clusters, presenting Euclidean distances greater than 1. Thus, the first was composed of two samples from the district of *Bragança*, 'Vg53' and 'Vg67', and one from *Guarda*, 'Vg61', the second included two samples from *Castelo Branco*, 'Vg62' and 'Vg63', and one from *Bragança* 'Vg71', whereas the third sub-cluster corresponded exclusively to samples from *Vila Real*, 'Vg65' and 'Vg74' (Fig. 2). Cluster 4 was formed of four samples close together, presenting short Euclidean distances between them. Three arise from *Bragança* and one from the district of *Guarda*. Finally, Cluster 5 was formed exclusively of samples from a single district, *Castelo Branco*, the most southern district amongst those considered for sampling with somewhat warmer weather (Table 2). *Bragança* region is surrounded by *Guarda* to the south, and *Vila Real* to the west. The two first regions have similar weather conditions, which might be reflected in the proximity between the samples from these two origins, which are often placed in common clusters. Concerning *Vila Real*, which is the district nearest to the Atlantic seashore, the rainfall in this area is slightly higher, and the temperature range lower. Hence, the two samples from this district, 'Vg65' and 'Vg74', isolated in a sub-cluster within cluster 3, did not represent a significant sampling, suggesting that more cultivars from this origin should be evaluated to ascertain any influence from either regional or climatic factors (Tables 1 and 2).

Since PC1 was mainly related with amino acids such as Glu, Gln, and Asp, as well as others (including essential amino acids), a positive score along this axis provides interesting information respecting the nutritional value. The fact that some samples displayed a reduced content in Asp and Glu increases the relevance of the positive scores of PC1. In addition, positive scores for PC2 are meaningful as well, although being negatively related with the content in His (Fig. 1), they are associated with the essential amino acids Met and Leu. Two pairs of cultivars ('Vg50'/'Vg51' and 'Vg56'/'Vg57') belonging to different clusters, are emphasised, since they are placed in the top right quadrant of the plot PC1 vs PC2, presenting positive scores for both components (Fig. 3A).

PC3 split the aforementioned pairs, with 'Vg51' and 'Vg57' remaining on the upper part, whilst their counterparts, 'Vg50' and 'Vg56' displayed negative scores for this component. Thus, PC3

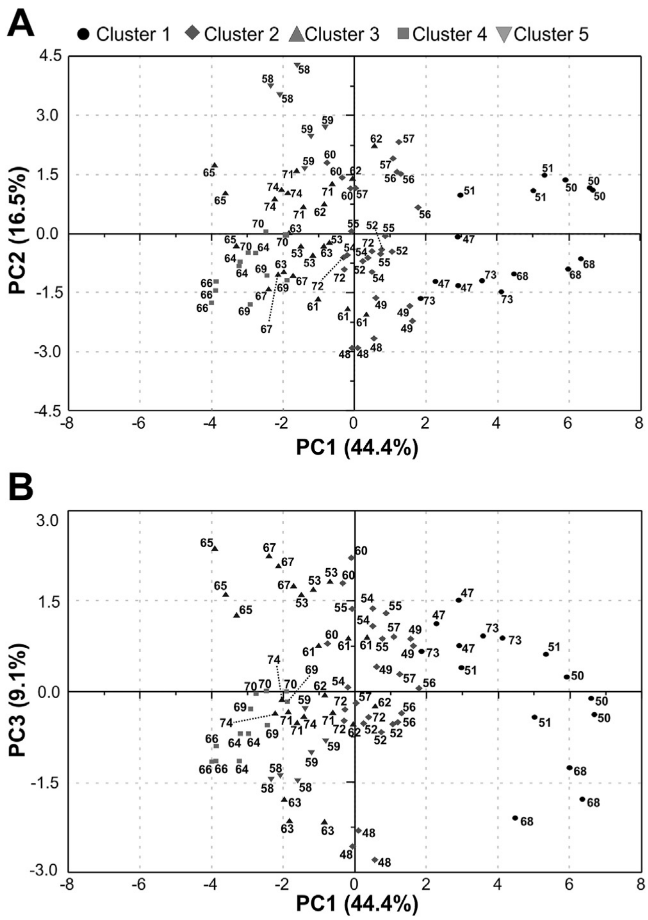

Fig. 3. Principal Component Analysis (PCA) developed resorting to the values retrieved on proximate and free amino acid composition of 28 cultivars of cowpea. PC1 vs PC2 (A) and PC1 vs PC3 (B).

separated samples with high content in fat and protein to positive scores, whilst was negatively related with the content in free essential amino acids, and the content in DM. Consequently, the varieties 'Vg50' and 'Vg56', placed in the negative half of PC3 due to their balanced contents of free essential amino acids, arise amongst the richest varieties to be considered (Table 3, Fig. 3B).

Additionally, the varieties 'Vg58' and 'Vg59', which were found detached in Fig. 3A due to the high scores for PC2, constituted Cluster 5 and were also identified as valuable cultivars. Though presenting negative scores for PC1 (due to the low quantity of non-essential amino acids displayed), this pair of samples has to be taken into account regarding the richest varieties, as they presented the highest scores for PC2, as well as negative scores for PC3 (Tables 3 and 5).

4. Conclusions

From the comparison of dry seeds of Portuguese cultivars of cowpea regarding their nutritional composition and the evaluation of their content in free amino acids, significant differences between the cultivars analyzed were emphasized highlighting the utility of multivariate statistical methods for the discrimination of the origin and the quality of agro food samples. This information provided valuable data for the selection of those cowpea varieties suitable to be included in informed breeding programs as donors of agronomical and nutritional quality. Varieties 'Vg50' and 'Vg58' showed the most promise, due to their appreciable nutritional content and the level of free amino acids. Additional cultivars, namely 'Vg51', 'Vg56', 'Vg57', and 'Vg59', should be considered in further studies focused on the resistance to biotic and abiotic stresses based on their nutritional composition and positive germinating properties. The most promising samples arise from distinct regions, namely, 'Vg50' and 'Vg51' from *Guarda*, "Vg56" and 'Vg57' from *Bragança*, and finally 'Vg58' and "Vg59" from *Castelo Branco*. The regional factor should be taken into account, when selecting cultivars, due to the specific agro-climatic conditions in these geographical areas.

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References

AOAC, 1995. Official Methods of Analysis, 16th ed. Association of Official Analytical Chemists, Washington, DC.
Alhadi, F.A., A.L-Asbahi, A.A.S., Alhammadi, A.S.A., Abdullah, Q.A.A., 2012. The effects of free amino acids profiles on seeds germination/dormancy and seedlings development of two genetically different cultivars of Yemeni Pomegranates. J. Stress Physiol. Biochem. 8, 114–137.
Angelovici, R., Galili, G., Fernie, A.R., Fait, A., 2010. Seed desiccation: a bridge between maturation and germination. Trends Plant Sci. 15, 211–218.
Antova, G.A., Stoilova, T.D., Ivanova, M.M., 2014. Proximate and lipid composition of cowpea (*Vigna unguiculata* L.) cultivated in Bulgaria. J. Food Compos. Anal. 33, 146–152.
Anyango, J.O., de Kock, H.L., Taylor, J.R.N., 2011. Impact of cowpea addition on the protein digestibility corrected amino acid score and other protein quality parameters of traditional African foods made from non-tannin and tannin sorghum. Food Chem. 124, 775–780.
Canton, F.R., Suárez, M.F., Canovas, F.M., 2005. Molecular aspects of nitrogen mobilization and recycling in trees. Photosynth. Res. 83, 265–278.
Carvalho, A.F.U., de Sousa, N.M., Farias, D.F., da Rocha-Bezerra, L.C.B., da Silva, R.M.P., Viana, M.P., Gouveia, S.T., Sampaio, S.S., de Sousa, M.B., de Lima, G.P.G., de Morais, S.M., Barros, C.C., Filho, F.R.F., 2012. Nutritional ranking of 30 Brazilian genotypes of cowpeas including determination of antioxidant capacity and vitamins. J. Food Compos. Anal. 26 (1–2), 81–88.
Cheng, Z.J., Hardy, R.W., 2003. Effects of extrusion processing of feed ingredients on apparent digestibility coefficients of nutrients for rainbow trout (*Oncorhynchus mykiss*). Aquacult. Nutr. 9, 77–83.
Cooper, J.D.H., Ogden, G., McIntosh, J., Turnell, D.C., 1984. The stability of the *o*-phthalaldehyde/2-mercaptoethanol derivatives of amino acids: an investigation using high-pressure liquid chromatography with a precolumn derivatization technique. Anal. Biochem. 142, 98–102.
Day, L., 2013. Proteins from land plants—potential resources for human nutrition and food security. Trends Food Sci. Technol. 32, 25–42.
Footitt, S., Slocombe, S.P., Larner, V., Kurup, S., Wu, Y., Larson, T., Graham, L., Baker, A., Holdsworth, M., 2002. Control of germination and lipid mobilization by COMATOSE, the Arabidopsis homologue of human ALDP. EMBO J. 21, 2912–2922.
Górecki, R.J., Fordoñski, G., Halmajan, H., Horbowicz, M., Jones, R.G., Lahuta, L.B., 2001. Seed physiology and biochemistry. In: Hedley, C.L. (Ed.), Carbohydrates in Grain Legume Seeds. Improving Nutritional Quality and Agronomic Characteristics. CAB International, Wallingford, Oxon, UK, pp. 117–143 ISBN 0851994679.
Giami, S.Y., 2005. Compositional and nutritional properties of selected newly developed lines of cowpea (*Vigna unguiculata* L. Walp). J. Food Compos. Anal. 18, 665–673.
Henley, E.C., Taylor, J.R.N., Obukosia, S.D., 2010. Chapter 2–The importance of dietary protein in human health: combating protein deficiency in Sub-Saharan Africa through transgenic biofortified sorghum. In: Taylor, S. (ed.), Advances in Food and Nutrition Research 60 21–52.
Huynh, B.-L., Ehlers, J.D., Ndeve, A., Wanamaker, S., Lucas, M.R., Close, T.J., Roberts, P. A., 2015. Genetic mapping and legume synteny of aphid resistance in African cowpea (Vigna unguiculata L. Walp.) grown in California. Mol. Breed. 35 (36) .

Kuo, Y.H., Rozan, P., Lambein, F., Frias, J., Vidal-Valverde, C., 2004. Effects of different germination conditions on the contents of free protein and non-protein amino acids of commercial legumes. Food Chem. 86, 537–545.

Lau, O.S., Deng, X.W., 2010. Plant hormone signaling lightens up: integrators of light and hormones. Curr. Opin. Plant Biol. 13, 571–577.

Mitchell, D.C., Lawrence, F.R., Hartman, T.J., Curran, J.M., 2009. Consumption of dry beans, peas, and lentils could improve diet quality in the US population. J. Am. Diet Assoc. 109, 909–913.

O'Neill, H.V.M., Rademacher, M., Mueller-Harvey, I., Stringano, E., Kightley, S., Wiseman, J., 2012. Standardised ileal digestibility of crude protein and amino acids of UK-grown peas and faba beans by broilers. Anim. Feed Sci. Technol. 175, 158–167.

Ojiako, F.O., Kayode, R.M.O., 2014. Nutritional quality and consumer acceptability of cowpea seeds (*Vigna unguiculata* L. Walp) treated with natural and synthetic insecticides against bruchid infestation. J. Stored Prod. Res. 56, 9–15.

Phillips, D.R., McWatters, K.H., Chinnan, M.S., Hung, Y.C., Beuchat, L.R., Sefa-Dedeh, S., Sakyi-Dawson, E., Ngoddy, P., Nnanyelugo, D., Enwere, J., Komey, N.S., Liu, K., Mensa-Wilmot, Y., Nnanna, I.A., Okeke, C., Prinyawiwatkul, W., Saalia, F.K., 2003. Utilization of cowpeas for human food. Field Crops Res. 82, 193–213.

Ragab, D.M., Babiker, E.E., Eltinay, A.H., 2004. Fractionation, solubility and functional properties of cowpea (*Vigna unguiculata*) proteins as affected by pH and/or salt concentration. Food Chem. 84, 207–212.

Sørensen, H., Sørensen, S., Bjergegaard, C., Michaelsen, S., 1999. Chromatography and Capillary Electrophoresis in Food Analysis. The Royal Society of Chemistry, Cambridge, UK.

Vasconcelos, I.M., Maia, F.M.M., Farias, D.F., Campello, C.C., Carvalho, A.F.U., Moreira, R.A., Abreu de Oliveira, J.T., 2010. Protein fractions, amino acid composition and antinutritional constituents of high-yielding cowpea cultivars. J. Food Compos. Anal. 23, 54–60.

Wold, S., Esbensen, K., Geladi, P., 1987. Principal component analysis. Chemom. Intell. Lab. Syst. 2, 37–52.

Zia-Ul-Haq, M., Ahmad, S., Chiavaro, E., Mehjabeen Sagheer, A., 2010. Studies of oil from cowpea (*Vigna unguiculata* (L.) Walp) cultivars commonly grown in Pakistan. Pak. J. Bot. 42 (2), 1333–1341.