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Characterization of *Vicia ervilia* (bitter vetch) seed proteins, free amino acids and polyphenols.

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29 **RUNNING TITLE:** *Vicia ervilia* seed proteins and functional components.

30 **ABSTRACT.**

31 *Vicia ervilia* is an ancient crop from the Mediterranean Region. It may
32 represent a useful source of proteins for food and animal feed, as well as bioactive
33 components. Seed samples from 39 populations of *V. ervilia* have been analyzed.
34 Polyphenol contents ranged from 0.09 to 0.19 %. Luteolin, kaempferol, apigenin, and
35 quercetin were the major aglycones. Total free amino acid content of the seeds was 0.05 to
36 0.19 % in which canavanine represented 9 to 22 %. Protein content was 24.1 %. The amino
37 acid composition indicated a high content in acidic amino acids and a deficit in sulphur
38 amino acids. *V. ervilia* seeds proved to be a good substrate for preparation of protein
39 isolates. The seed extracts inhibited proliferation of Caco-2 colon tumor cells,
40 simultaneously, exerting antioxidative effects. Hence, seeds of *V. ervilia* could
41 represent a source of high value food and feed components, as well as functional
42 components.

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45 **PRACTICAL APPLICATIONS.**

46 *Vicia ervilia* (bitter vetch) (*Leguminosae*) is an ancient crop from the
47 Mediterranean Region. Although it was still grown in many Mediterranean countries at
48 the beginning of the twentieth century, other crops that provide higher and more

consistent yield later replaced it. However, *V. ervilia* seeds may represent a useful source of proteins for human nutrition and animal feeding, and a source of bioactive components with health promoting properties. Our results show that the seeds of *V. ervilia* could indeed represent a source of high value food and feed components, as well as functional, health-promoting components. This may result in a revalorization of this neglected crop. The availability of numerous populations in seedbanks guarantees the preservation of a genetic diversity in *V. ervilia* that could be used for the production of new varieties with better nutritional and functional characteristics.

KEY WORDS: *Vicia ervilia*, bitter vetch, functional components, antioxidant activity, antiproliferative activity, proteins.

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79 **1. INTRODUCTION.**

80 *Vicia ervilia* (bitter vetch) (*Leguminosae*) is an ancient crop that was already
81 known in the Paleolithic (Mikic et al., 2015), and there are some evidences of it being
82 grown already about 46,000 years ago in Iraq (Henry et al., 2011). Some remains from
83 10,000 to 7,000 BC have been found in Spain (Aura et al., 2005). *Vicia ervilia* seeds
84 have been found in many archaeological places dating back to the Neolithic or later,
85 and *V. ervilia* is the most abundant pulse found in sites dating back to the Bronze Age.
86 *V. ervilia* is also associated with the start of the agricultural revolution in the Old World
87 (Erskine, 1998). The first farmers in Europe used *V. ervilia*, *Lens* sp., *Pisum sativum*,
88 *Lathyrus cicera* and *Lathyrus sativus*. At the beginning of the twentieth century *V.*
89 *ervilia* was still grown for production of grain and hay in many Mediterranean
90 countries including Spain, Italy, Greece, Turkey and Morocco. Nevertheless, *V. ervilia*
91 was later mostly replaced by other crops that provide higher and more consistent
92 yield.

93 Pulses have traditionally been recognized as a good source of major nutritional
94 components such as proteins and carbohydrates. *V. ervilia* can be used directly for
95 feeding ruminants, and after processing it also represents a good source of protein for
96 poultry (Farran et al., 2001; Sadeghi et al., 2004). In addition, pulses represent a source

of bioactive components with health promoting properties such as polyphenols and certain free amino acids. *V. ervilia* belongs to subgenus *Vicilla*, which includes many species that are characterized by the presence in their seeds of the non-protein amino acid canavanine, an analogous of arginine. *V. ervilia* is easy to grow and thrives in poor, alkaline and dry soils much better than others pulses do. It is also characterized by a high capacity for fixing nitrogen (López-Bellido, 1994). The goal of this work was to determine whether *V. ervilia* is a good source of bioactive components and protein for functional and nutritional applications. This could lead to a revalorization of this neglected, ancient crop. The samples that have been analyzed come from 39 populations of *V. ervilia* distributed throughout the Mediterranean Region.

2. MATERIAL AND METHODS.

2.1. Materials. Potassium ferricyanide, ferric chloride, trichloroacetic acid (TCA), trifluoroacetic acid (TFA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), 2,7-dichlorofluorescein diacetate (DCFH-DA), 2,2-azobis (2-amidinopropane) dihydrochloride (ABAP), pyrocatechol violet (PV) were provided by Sigma–Aldrich (St. Louis, MO, USA). Hanks’ Balanced Salt Solution (HBSS), fetal bovine serum (FBS) and Dulbecco’s modified Eagle’s medium (DMEM) were purchased from Gibco (Invitrogen, Barcelona, Spain). Ultrapure water was obtained using a Mili-Q system (Millipore, Bedford, MA, USA) and acetonitrile (UpS ultragradient) and methanol were from Teknokroma (Barcelona, Spain).

2.2. Plant Material. The following *V. ervilia* seeds were provided by the Centro de Recursos Fitogenéticos (CRF, INIA) at Madrid, Spain. Population number, location of origin and CRF reference number are as follows: 1, Spain, Almeria (29060); 2, Spain,

Cordoba (38405); 3, Spain, Granada (1115); 4, Spain, Jaen (41372); 5, Spain, Málaga (38406); 6, Spain, Teruel (1510); 7, Spain, Zaragoza (23586); 8, Spain, Ciudad Real (1533); 9, Spain, Cuenca (1469); 10, Spain, Guadalajara (1072); 11, Spain, Toledo (998); 12, Spain, Burgos (4270); 13, Spain, Leon (25615); 14, Spain, Palencia (7325); 15, Spain, Salamanca (1461); 16, Spain, Segovia (1138); 17, Spain, Valladolid (991); 18, Spain, Madrid (13842); 19, Greece, Ioanina (708); 20, Greece, Kozani (742); 21, Greece, Pela (701); 22, Greece, Arkadia (736); 23, Greece, Korinthia (719); 24, Greece, Lakonia (728); 25, Greece, Kriti, Hania (734); 26, Greece, Kriti, Lassithi (717); 27, Greece, Larissa (706); 28, Turkey, Anatolia (695); 29, Turkey, Antalya (759); 30, Turkey, Aydin (761); 31, Turkey, Denizli (722); 32, Turkey, Kayasik (694); 33, Iran, Isfahan (714); 34, Iran, Kahrise (749); 35, Albania, Korca (705); 36, Albania, Tirane (733); 37, Afghanistan, Kabul (730); 38, Cyprus (727); 39, Morocco, Chaouen (37450).

2.3 Extraction of polyphenols and free amino acids. Free amino acids and polyphenols were extracted by shacking a suspension of the seed flour or ground leaves (10 % w/v) in ethanol (70 % in water) at 4° for 30 min. The pellet resulting from centrifugation at 15,000 g for 15 min was extracted once more and supernatants were combined. These supernatants containing polyphenols and free amino acids were kept at -20 °C until further use.

2.4. Preparation of protein extracts. For protein extraction a seed flour previously extracted as described in point 2.3 was used. Protein extracts were prepared by shaking a suspension of this seed flour or ground leaves (10 % w/v) in 0.1 N NaOH (pH 10) at 4° for 1 h. The pellet resulting from centrifugation at 15,000 g for 15 min was extracted once more and the supernatants were combined and acidified to the average isoelectric point of *V. ervilia* seed proteins, pH 4. Precipitated proteins

were recovered by centrifugation at 15,000 g for 15 min and freeze-dried after washing twice with water. The isoelectric pH of the different extracts had been previously determined by checking for precipitation in aliquots that were titrated to a range of pH values. Protein was determined according to Bradford (1976).

2.5. Polyphenols analysis. Total polyphenols were determined in ethanolic extracts using the Folin-Ciocalteou reagent and a catechin calibration curve (Singleton et al., 1999). Polyphenol aglycones were analyzed by RP-HPLC after hydrolysis by heating at 85° C for 2 h in 12 M HCl as described (Dinelli et al., 2006). Aglycones were extracted from this solution using ethyl acetate and suspended in 75 % ethanol in water. Analysis of aglycones was carried out by HPLC-RP and UV detection at 254 nm, using a 5 µm particle size, 25 x 4.6 mm Ultrasphere ODS C₁₈ column (Beckman-Coulter, CA, USA). Response factors were calculated from the corresponding calibration curves of the aglycone standards.

2.6. Amino acid analysis. Analysis of total amino acids was carried out by RP-HPLC after acid hydrolysis and precolumn derivatization with diethyl ethoxymethylenemalonate, using D,L-α-aminobutyric acid as internal standard, according to Alaiz et al. (1992). Tryptophan was determined by RP-HPLC after basic hydrolysis according to Yust et al. (2004). Amino acid score was calculated as % essential amino acids in sample / % FAO essential amino acid recommendations (FAO/WHO/UNU, 1985). Free amino acids in 70 % ethanol extracts were analyzed by RP-HPLC after derivatization with diethyl ethoxymethylenemalonate, using D,L-α-aminobutyric acid as internal standard, according to Megías et al. (2015).

2.7. SDS-PAGE electrophoresis. Protein samples were mixed (1:1) with solubilization buffer (80 mM Tris, 0.57 % EDTA, 0.26 % DTT, 3.3 % SDS, 0.008 %

bromophenol blue, 20 % sucrose, pH 6.8) and incubated at 100° C for 10 min. SDS-PAGE was carried out as described by Schagger & von Jagow (1987) with slight modifications. The separating gel consisted of 15 % T and 2.6 % C, where T is the total percentage concentration of acrylamide and bisacrylamide and C is the percentage concentration of bisacrylamide relative to T. The stacking gel consisted of 4 % T and 3 % C. Electrophoresis was performed at a voltage of 60 V for stacking and 120 V for separation. Proteins were stained with 0.25 % Coomassie Brilliant Blue G in 45 % methanol, 10 % acetic acid for 2 h at room temperature. Distaining of the gel was performed with 10 % acetic acid.

2.8. Analysis of polyphenols reducing power. Reducing power was analyzed according to Oyaizu (1986). Samples were incubated with potassium ferricyanide 1 % (w/v) in 0.2 M phosphate buffer pH 6.6 at 50° C for 20 min. Then, 2.5 % (w/v) TCA was added and the resulting solution was incubated with 0.01 % (w/v) ferric chloride at 50° C for 10 min. Finally, absorbance was read at 700 nm. Blank sample included neither sample nor ferric chloride.

2.9. Analysis of polyphenols chelating activity. Cu²⁺ chelating activity was determined using the pyrocatechol violet reagent according to Saiga, Tanabe, & Nishimura (2003). Polyphenols were added to 96-well plates containing 250 µL of 50 mM sodium acetate pH 6.0, 6.25 µL 4 mM pyrocatechol violet, and 1 µg, CuSO₄. Absorbance at 632 nm was determined after incubation at room temperature for 1 minute. Chelating activity (CA) was calculated according to the following formula:

$$\% \text{ CA} = [1 - \text{Abs}_{632 \text{ nm}} (\text{PV} + \text{Cu}^{++} + \text{sample}) / \text{Abs}_{632 \text{ nm}} (\text{PV} + \text{Cu}^{++})] \times 100$$

where Abs_{632 nm} (PV + Cu⁺⁺ + sample) is the absorbance of the PV + Cu⁺⁺ complex at 632 nm in the presence of samples, and Abs_{632 nm} (PV + Cu⁺⁺) is the absorbance of the PV + Cu⁺⁺ complex at 632 nm in the absence of samples.

2.10 Cell culture. Caco-2 cells were obtained from the European Collection of Authenticated Cell Cultures (Salisbury, UK, ECACC number 86010202) and cultured in low glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % heat inactivated fetal bovine serum (FBS), 1 % non-essential amino acids (NEAA), 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were grown in standard culture conditions (37° C and 5 % CO₂ in a humidified atmosphere).

2.11. Cellular antioxidant activity. Cellular antioxidant activity (CAA) was determined by monitoring the inhibition of the fluorescence emitted by cells loaded with (2',7'-dichlorofluorescein diacetate (DCFH-DA) in the presence of the free radical generator 2,2'-azobis (2-amidinopropane) dihydrochloride (ABAP) as previously described (Wolfe & Liu, 2007) with modifications. Caco-2 cells were seeded at a density of 2 x 10⁴ cells/well in 96-well microplates in DMEM and preincubated for 48 h under standard culture conditions. Cells were then washed twice with HBSS and incubated for 1 h in HBSS containing the samples and 25 µM DCFH-DA. Cells were washed again twice using HBSS and incubated for 1 h in HBSS containing 600 µM ABAP. Fluorescence (excitation at 485 nm and emission at 555 nm) was measured every 15 min for 1 h during incubation at 37° C in a Fluoroskan Ascent plate-reader (Thermo Fisher Scientific). Intracellular esterases hydrolyze the stable DCFH-DA to DCFH, which can then be easily oxidized by reactive oxygen species to fluorescent 2',7'-dichlorofluorescein (DCF). Negative controls (cells treated only with ABAP) and positive controls (cells untreated with ABAP or samples) were included.

2.12. *Cell proliferation assay.* Caco-2 cells were seeded in 96-well microplates (4×10^3 cells/well) in full medium containing the samples, and uptake of neutral red (Borenfreund & Puerner, 1985) was determined after incubation for 7 days under standard culture conditions as described above. Medium was removed by aspiration and cells were incubated in fresh culture medium containing neutral red (50 $\mu\text{g/ml}$) for 30 minutes. The stain was extracted using acetic acid (75 μl , 1 % v/v in ethanol 50 % v/v) after washing with HBSS (75 μl), and absorbance was measured at 550 nm using a plate reader.

2.13 *Statistical analysis.* Comparisons between treatment groups were carried by one-way ANOVA-Tukey test.

3. RESULTS AND DISCUSSION.

3.1. *Polyphenols in V. ervilia seeds.* Polyphenols are the most abundant bioactive components with health promoting properties in legumes. They are very well known for their antioxidant properties, but they also have antiproliferative, immunomodulatory, chelating and enzyme inhibitory activities that have been related with prevention of immune, neurological and cardiovascular diseases (Olivares-Vicente et al., 2018).

Seeds of 39 populations of *V. ervilia* distributed throughout the Mediterranean Region were analyzed for their polyphenol contents and biological activities. Polyphenol content in *V. ervilia* seeds ranged from 0.09 to 0.19 g / 100 g flour, with an average content of 0.13 % (Table 1). These are lower than those reported for other *Vicias* belonging to subgenus *Vicilla* such as *V. benghalensis*, *V. cracca* and *V. tenuifolia* (Megías et al., 2016). Curiously, the lowest and highest contents were found in *V.*

ervilia populations from the same country, Albania, and no correlation between content in polyphenols and geographical origin was found. No significant differences in the average content in polyphenols in different countries and regions were observed (Figure 1).

Luteolin, kaempferol, apigenin and quercetin were the major aglycones in the extracts from two of the populations (Table 2). It has been described that these polyphenols have antioxidant and antiproliferative activities (Kashyap et al., 2017; Chen et al., 2017; Madunic et al., 2018; Ali et al., 2017). As shown by determination of reducing power, the *V. ervilia* polyphenol extracts have high antioxidant activity. Thus, reducing power ranged from 0.17 to 0.35 absorbance units, with an average at 0.22 (Table 1). Copper chelating activity ranged from 29.8 % to 57.5 % with an average chelating activity of 43.9 % (Table 1). The chelating activity of polyphenols may prevent the formation of damaging reactive oxygen species in the presence of transition metals, which have been related with different diseases (Gaetke & Chow, 2003).

3.2. Free amino acids in *V. ervilia* seeds. It is usual to find high concentrations of a specific non-protein free amino acid in leguminous seeds. For example, 3-N-oxalyl-2,3-diaminopropionic acid and homoarginine are present in *Lathyrus* species (Megías, Cortés-Giraldo, Alaiz, Girón-Calle, Vioque, Santana-Meridas, Herraiz-Peñalver & Sanchez-Vioque, 2015), and β -cyano-L-alanine (Megías, Cortés-Giraldo, Girón-Calle, Vioque & Alaiz, 2014) and canavanine (Megías, Cortés-Giraldo, Girón-Calle, Alaiz & Vioque, 2016) are present in different *Vicia* species. *Vicia* species belonging to subgenus *Vicilla*, the same subgenus to which *V. ervilia* belongs, are especially rich in the non-protein amino acid canavanine (Megías et al., 2016). This amino acid is an analogue of arginine and may represent more than 2.5 % of the seed dry weight in *V.*

incana, *V. disperma*, *V. tenuifolia* and *V. cretica* (Megías et al., 2016). Canavanine is incorporated by *Vicia* predators in their proteins leading to synthesis of non-functional, aberrant proteins (Rosenthal, 2001). Canavanine also exhibits antiproliferative (Vynnytska-Mynorovska, Bobak, Garbe, Dittfeld, Stasyk & Kunz-Schughart, 2012) and immunomodulatory properties (Akaogi, Barker, Kuroda, Nacionales, Yamasaki, Stevens, Reeves & Satoh, 2006). Although the content of canavanine in the seeds from *V. ervilia* is low, the antinutritional properties of this amino acid have been considered a limiting factor for a more extended use of this legume in human nutrition and for feeding animals.

The seeds from four *V. ervilia* populations from distant locations were selected for analysis of the free amino acid composition. Total free amino acids in these seeds ranged from 0.05 to 0.19 % dry weight, and canavanine represents between 9 % and 22 % of these free amino acids, corresponding to 0.005 % - 0.018 % dry weight (Table 3). The composition in total and free amino acids in *V. ervilia* seeds are compared in Figure 2; significant differences in the contents of Thr, Ala, Pro, Tyr, Ile, Trp, Phe, and canavanine were observed.

The composition in free amino acids was also determined in the leaves from population 35 (Table 3). The total free amino acid content in leaves was higher than in the seeds, 1.7 % versus 0.12 %, but no canavanine was found in the former. Asparagine was the most abundant free amino acid in the leaves, representing around 50 % of the total. This is rather surprising because unlike seeds, leaves would not be protected from herbivores by the presence of canavanine. This would probably highlight the fact that seeds are more important than leaves for survival of the population. Alternatively, it could be considered that canavanine is selectively accumulated in the seeds as a

form of nitrogen storage. In fact, canavanine has a carbon to nitrogen molar ratio higher than any of the amino acids present in proteins. These ratios are 4/5 and 1/2 for canavanine and asparagine, respectively, being the ratio for asparagine the highest among the amino acids commonly found in proteins. In addition, mobilization of nitrogen during seed germination from a free amino acid such as canavanine is faster than mobilization from storage proteins.

3.3. Cellular antioxidant activity (CAA) of V. ervilia seed extracts. The seed extracts from populations 11 and 29, which have both a high content in polyphenols and a high reducing activity, were assayed for their cellular antioxidant activity using Caco-2 cells. These cells are originally derived from a colon tumor and represent a model of exposure of cancerous cells to components in the diet. The assay for cellular antioxidant activity determines the inhibition of the intracellular oxidative activation of a fluorescent probe, so that it is a measure of the antioxidant potential of components in the samples that have an effect inside cells (Kellet, Greenspan, & Pegg, 2018). As shown in figure 3, *V. ervilia* extracts exhibited a concentration dependent antioxidant effect ($P < 0.001$) although the differences between populations were not significant.

3.4. Antiproliferative activity of V. ervilia seed extracts. Many polyphenols, including polyphenols present in leguminous seeds, inhibit the growth of cancerous cells (Duthie, Duthie & Kyle, 2000). The Caco-2 cell line, originally derived from a colon tumor, has been used to determine the effect of two of the *V. ervilia* seed extract on the proliferation of transformed, cancerous cells. As shown in figure 4, both extracts inhibited cell proliferation ($P < 0.001$). Population 29 showed a significant increase in the antiproliferative activity ($P < 0.005$) with increased amount of extract assayed. As previously reported, polyphenols in *V. ervilia* have antiproliferative activity (Tsai et al.,

2018; Li, Zhang, Chen, & Li, 2018). Functional components other than polyphenols that are also present in the extracts may also inhibit cell proliferation, namely canavanine (Staszek, Weston, Ciacka, Krasuska, & Gniazdowska, 2017) and lectins (Islam et al., 2018). *V. ervilia* population 29 possess both higher antioxidant and antiproliferative activities than the others do. Considering that transformed cells often develop a microenvironment characterized by a higher concentration of free radicals, it might be possible that both antioxidant and antiproliferative properties in this population were related.

3.5. Nutritional characteristics of V. ervilia seed and leaf protein. Production of protein isolates. The average protein content in *V. ervilia* seeds is 24.1 g / 100 g flour (Table 4), which is consistent with previous reports of protein content in other *Vicia* species (Pastor-Cavada et al., 2011; Aletor et al. 1994; Farran et al. 2001a). Thus, the possibility of using these seeds for production of protein isolates has been explored. The amino acid composition of the seed proteins from five *V. ervilia* populations is shown in Table 4. This composition is characteristic of the storage proteins in leguminous seeds, the globulins. Hence, the amino acid composition indicated high acidic and low sulphur amino acid levels and a content in lysine above FAO recommendations. Considering this composition, these seeds may represent a good source of proteins for human nutrition and feeding animals. However, the presence of undesirable secondary compounds such as canavanine in these seeds has so far prevented a more extensive utilization of these seeds. Flour can be further processed and their proteins extracted for production of protein concentrates and protein isolates by alkaline extraction followed by precipitation of protein at the isoelectric pH. Alkaline extraction yields an amino acid composition similar to the proteins in the

flour, with all amino acids above FAO requirements except for methionine and cysteine (Table 5). Precipitation at the isoelectric point, which was previously calculated as shown in figure 5, yielded a 91.9 % protein isolate free of canavanine, which was also deficient in methionine and cysteine but had a content in lysine above FAO recommendations. While globulins are purified in this isolate, the pH 4 soluble albumin fraction includes functional proteins such as enzymes, lectins, and protease inhibitors (Duranti, 2006) (Figure 6).

Considering that *V. ervilia* has also been used as forage in animal feeding (Mohammadi & Sadeghi, 2009), the protein content in the leaves of one of the populations was also determined. As shown in table 5, the nutritional quality of the protein in leaves was lower than the quality of protein in seeds. Thus, protein in leaves was deficient in methionine, cysteine, histidine and lysine. This is further exemplified by the amino acid score (AAS) and % essential amino acids (% EAA), that are lower in leaf proteins than in seed proteins.

V. ervilia is an ancient crop that was widely grown in the Meditarrean Region in the past but has been mostly abandoned because of the implantation of uniform commercial varieties, and also because of the presence of antinutritional components in the seed. In this work we have shown that *V. ervilia* seeds can be processed and represent a good source of high quality protein and functional components. These functional properties include antioxidant and antiproliferative effects with potential health promoting effects. This may result in a revalorization of this neglected crop. The availability of numerous populations in seedbanks guarantees the preservation of a genetic diversity in *V. ervilia* that could be used for the production of new varieties with better nutritional and functional characteristics.

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REFERENCES.

Akaogi, J., Barker, T., Kuroda, Y., Nacionales, D.C., Yamasaki, Y., Stevens, B.R., Reeves, W.H., & Satoh, M., 2006. Role of non-protein amino acid L-canavanine in autoimmunity. *Autoimmunity Review*, 5, 429-435.

Alaiz, M., Navarro, J.L., Giron, J., & Vioque, E., 1992. Amino acid analysis by high-performance liquid chromatography after derivatization with diethylethoxymethylenemalonate. *Journal Chromatography*, 591, 181-186.

Aletor, V.A., Goodchild, A.V., & Abd El Moneim, A.M., 1994. Nutritional and antinutritional characteristics of selected *Vicia* genotypes. *Animal Feed Science Technology*, 47, 125-139.

Ali, F., Rahul, Naz, F., Jyoti, S., & Siddique, Y.H., 2017. Health functionality of apigenin: a review. *Int. Jour. Food Properties*, 20, 1197- 1238.

Aura, J.E., Carrión, Y., Estrelles, E., & Jordà, G.P., 2005. Plant economy of hunter-gatherer groups at the end of the last Ice Age: Plant macroremains from the cave of Santa Maira (Alacant, Spain) ca. 12000 – 9000 B.P. *Vegetal History Archaeobotany*, 14, 542-550.

Borenfreund, E., & Puerner, J.A., 1985. Toxicity determined in vitro by

383 morphological alterations and neutral red absorption. *Toxicology Letters*, 24, 119-124.

384 Bradford, M., 1976. A Rapid and Sensitive Method for the Quantification of
385 Microgram Quantities of Protein Utilizing the Principle of Protein-Dye
386 Binding. *Analytical Biochemistry*, 72, 248–254.

387 Chen, A.Y., & Chen, Y.C., 2017. A review of the dietary flavonoid, kaempferol on
388 human health and cancer chemoprevention. *Food Chemistry*, 138, 2099-2107.

389 Dinelli, G., Bonetti, A., Minelli, M., Marotti, I., Catizone, P., & Mazzanti, A.,
390 2006. Content of flavonols in Italian bean (*Phaseolus vulgaris* L.) ecotypes. *Food*
391 *Chemistry*, 99, 105-114.

392 Duranti, M. (2006). Grain legume proteins and nutraceutical properties.
393 *Fitoterapia*, 77, 67-82.

394 Duthie, G.G., Duthie, S.J., & Kyle, J.A.M., 2000. Plant polyphenols in cancer and
395 heart disease: implications as nutritional antioxidants. *Nutrition Research Review*, 13,
396 79-106.

397 Erskine, W., 1998. Use of historical and archaeological information in lentil
398 improvement today. In: Damania A B, Valkoun J, Willcox G, Qualset CO (eds) *Origins of*
399 *Agricultural and Crop Domestication*. ICARDA, Aleppo, Syria, 191-198.

400 FAO/WHO/UNU, 1985. Energy and protein requirements. Report of the joint
401 FAO/WHO/UNU expert consultation. Technical report series No. 724. FAO. WHO and
402 the United Nations University. Geneva. Switzerland.

403 Farran, M.T., Barbour, G.W., Uwayjan, M.G., & Ashkarian, V.M., 2001.
404 Metabolizable energy values and amino acids availability of vetch (*Vicia sativa*) and
405 ervil (*Vicia ervilia*) seeds soaked in water and acetic acid. *Poultry Science*, 80, 931-936.

406 Gaetke, L.M., & Chow, C.K., 2003. Copper toxicity, oxidative stress, and
 407 antioxidant nutrients. *Toxicology*, 189, 147–163.

408 Henry, A.G., Brooks, A.S., & Piperno, D.R., 2011. Microfossils in calculus
 409 demonstrate consumption of plants and cooked foods in Neanderthal diets (Shanidar
 410 III, Iraq; Spy I and II, Belgium). *Proceedings Natural Academic of Science USA*, 108, 486-
 411 491.

412 Islam, F., Gopalan, V., Lam, A.K.Y., & Kabir, S.R., 2018. Pea lectin inhibits cell
 413 growth by inducing apoptosis in SW480 and SW48 cell lines. *International Journal*
 414 *Biological Macromolecules*, 117, 1050-1057.

415 Kashyap, D., Sharma, A., Tuli, H.S., Sak, K., Punia, S., & Mukherjee, T.K., 2017.
 416 Kaempferol – A dietary anticancer molecule with multiple mechanisms of action:
 417 recent trends and advancements. *Journal Functional Foods*, 30, 203-219.

418 Kellett, M.E., Greenspan, P., & Pegg, R.B., 2018. Modification of the cellular
 419 antioioxidant activty (CAA) assay to study phenolic antioxidants in a Caco-2 cell line. *Food*
 420 *Chemistry*, 244, 359-363.

421 López-Bellido, L. Grain legumes for animal feed, 1994. In *Neglected Crops*. 1492
 422 from a different perspective. Eds. Hernández-Bermejo, J.E. & León, J. FAO, Rome; pp.

423 Madunic, J., Madunic, I.V., Gajski, G., Popic, J., & Garaj-Urhovac, U., 2018.
 424 Apigenin: a dietary flavonoid with diverse anticancer properties. *Cancer Letteres*, 413,
 425 11-22.

426 Megías, C., Cortés-Giraldo, I., Alaiz, M., Girón-Calle, J., Vioque, J., Santana-
 427 Meridas, O., Herraiz-Peñalver, D., & Sanchez-Vioque, R., 2015. Determination of the
 428 neurotoxin 3-N-oxalyl-2,3-diaminopropionic acid and other free amino acids in

429 Lathyrus cicera and L. sativus seeds by reversed-phase high-performance liquid
 430 chromatography. *Food Analytical Methods*, 8, 1953-1961.

431 Megías, C., Cortés-Giraldo, I., Girón-Calle, J., Vioque, J., & Alaiz, M., 2015.
 432 Determination of l-canavanine and other free amino acids in Vicia disperma (Fabaceae)
 433 seeds by precolumn derivatization using diethyl ethoxymethylenemalonate and
 434 reversed-phase high-performance liquid chromatography. *Talanta*, 131, 95–98.

435 Megías, C., Cortés-Giraldo, I., Girón-Calle, J., Alaiz, M., & Vioque, J., 2016. Free
 436 amino acids, including canavanine, in the seeds from 32 Vicia species belonging to
 437 subgenus Vicilla. *Biocatalysis Agricultural Biotechnology*, 8, 126-129.

438 Megías, C., Cortés-Giraldo, I., Girón-Calle, J., Vioque, J., & Alaiz, M., 2014.
 439 Determination of beta-cyano-L-alanine, gamma-glutamyl-beta-cyano-L-alanine, and
 440 common free amino acids in Vicia sativa (Fabaceae) seeds by reversed-phase high-
 441 performance liquid chromatography. *Journal Analytical Methods Chemistry*, doi.
 442 10.1155/2014/409089.

443 Mikic, A., Medovic, A., Jovanovic, Z., & Stanisavljevic, N., 2015. A note on the
 444 earliest distribution, cultivation and genetic changes in bitter vetch (Vicia ervilia) in
 445 ancient Europe. *Genetika*, 47, 1-11.

446 Mohammadi, L., & Sadeghi, Gh. (2009). Using different ratios of bitter vetch
 447 (Vicia ervilia) seed for moult inductino and post-moult performance in commercial
 448 laying hens. *British Poultry Science*, 50, 207-212.

449 Olivares-Vicente, M., Barrajon-Catalan, E., Herranz-Lopez, M., Segura-Carretero,
 450 A., Jove, J., Encinar, J.A., & Micol, V., 2018. Plant-derived polyphenols in human health:
 451 biological activity, metabolites and putative molecular targets. *Current Drugs*
 452 *Metabolism*, 19, 351-369.

453 Oyaizu, M., 1986. Studies of products of browning reaction: antioxidative
 454 activity of products of browning reaction prepared from glucosamine. *Japan Journal*
 455 *Nutrition*, 44, 307-315.

456 Pastor-Cavada, E., Juan, R., Pastor, J.E., Alaiz, M., & Vioque, J., 2011. Nutritional
 457 characteristics of seed proteins in 28 *Vicia* species (Fabaceae) from southern Spain.
 458 *Journal Food Science*, 76, C1118-C1124.

459 Rosenthal, G.A., 2001. L-Canavanine: a higher plant insecticidal allelochemical.
 460 *Amino Acids*, 21, 319-330.

461 Sadeghi, G., Samie, A., Pourreza, J., & Rahmani, H.R., 2004. Canavanine content
 462 and toxicity of raw and treated bitter vetch (*Vicia ervilia*) seeds for broiler chicken.
 463 *International Journal Poultry Science*, 3, 522-529.

464 Saiga, A., Tanabe, S., Nishimura, T., 2003. Antioxidant activity of peptides
 465 obtained from porcine myofibrillar proteins by protease treatment. *Journal*
 466 *Agricultural Food Chemistry*, 51, 3661–3667.

467 Schägger, H., & von Jagow, G., 1987. Tricine-sodium dodecyl sulfate
 468 polyacrylamide gel electrophoresis for the separation of proteins in the range from 1
 469 to 100 kDa. *Analytical Biochemistry*, 166, 368-379.

470 Singleton, V.L., Orthofer, R., & Lamuela-Raventos, R.M., 1999. Análisis of total
 471 phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu
 472 reagent. *Methods Enzymology*, 299, 152-178.

473 Staszek, P., Weston, L.A., Ciacka, K., Krasuska, U., & Gniazdowska, A., 2017. L-
 474 Canavanine: How does a simple non-protein amino acid inhibit cellular function in a
 475 diverse living system. *Phytochemistry Review*, 16, 1269-1282.

Vynnytska-Mynorovska, B., Bobak, Y., Garbe, Y., Dittfeld, C., Stasyk, O., & Kunz-Schughart, L.A., 2012. Single amino acid arginine starvation efficiently sensitizes cancer cells to canavanine treatment and irradiation. *International Journal Cancer*, 130, 2164-2175.

Wolfe, K.L., & Liu, H.L., 2007. Cellular antioxidant activity (CAA) assay for assessing antioxidants, foods, and dietary supplements. *Journal Agricultural Food Chemistry*, 55, 8896–8907.

Yust, M.M., Pedroche, J., Girón-Calle, J., Vioque, J., Millán, F., & Alaiz, M., 2004. Determination of tryptophan by high-performance liquid chromatography of alkaline hydrolysates with spectrophotometric detection. *Food Chemistry*, 85, 317-320.

FIGURE LEGENDS.

Figure 1. Polyphenol content in *V. ervilia* seeds classified according to their countries or Spanish region of origin. Data represent the average and standard deviation of populations corresponding to a specific geographical area.

Figure 2. Comparison of average seed total amino acid composition (open bars) and free amino acid composition (close bars) in *V. ervilia* populations 5, 18, 25 and 35. Asterisks indicate significantly different contents ($P < 0,05$) in total and free amino acids.

Figure 3. Cellular antioxidant activity of *V. ervilia* seed polyphenols. Polyphenols were assayed at two concentrations, 0.5 μg (white bars) and 1 μg (grey bars) /microplate well. Antioxidant activity was shown as the percent increase in the fluorescence with respect to a positive control (without ABAP) and compared with a negative control that contains ABAP and no extract (black bar). Results are average and standard deviation of six determinations.

Figure 4. Effect of *V. ervilia* seed ethanolic extracts on proliferation of Caco-2 cells. Cells were incubated in the presence of 0.5 (white bars) or 1 (black bars) μg / well ethanolic extracts from populations 11 and 29 for one week. Results are average and

standard deviation of six determinations and are plotted as percentage of neutral red uptake by control cells (no extract added).

Figure 5. Isoelectric point of seed proteins of five populations of *V. ervilia*.

Results are the average of three determinations.

Figure 6. SDS-PAGE of the seed proteins from *V. ervilia* population 35. Total

seed proteins (lane 1), pH 4 insoluble proteins (lane 2) and pH 4 soluble proteins (lane 3) are shown. Molecular weight markers (kDa) are shown on the left.

Table 1. Polyphenols content, reducing power and chelating activity in the seed extracts from the 39 populations of *V. ervilia*. Results are the average $\pm$ sd of three determinations.

<i>V. ervilia</i> populations	Location	Polyphenols (g/100 g flour)	Reducing power (Abs 700 nm)	Chelating activity (%)
1	Spain	0.14 $\pm$ 0.002	0.20 $\pm$ 0.015	39.1 $\pm$ 2.6
2	Spain	0.14 $\pm$ 0.007	0.19 $\pm$ 0.004	50.8 $\pm$ 5.5
3	Spain	0.12 $\pm$ 0.001	0.18 $\pm$ 0.008	48.6 $\pm$ 2.2
4	Spain	0.12 $\pm$ 0.002	0.19 $\pm$ 0.005	54.9 $\pm$ 2.0
5	Spain	0.12 $\pm$ 0.003	0.19 $\pm$ 0.002	54.0 $\pm$ 1.1
6	Spain	0.10 $\pm$ 0.002	0.19 $\pm$ 0.012	54.9 $\pm$ 1.1
7	Spain	0.15 $\pm$ 0.004	0.20 $\pm$ 0.009	53.8 $\pm$ 1.5
8	Spain	0.11 $\pm$ 0.001	0.18 $\pm$ 0.007	44.5 $\pm$ 1.2
9	Spain	0.16 $\pm$ 0.003	0.31 $\pm$ 0.005	37.8 $\pm$ 2.4
10	Spain	0.15 $\pm$ 0.002	0.25 $\pm$ 0.016	37.5 $\pm$ 0.4
11	Spain	0.18 $\pm$ 0.004	0.32 $\pm$ 0.015	32.9 $\pm$ 4.1
12	Spain	0.15 $\pm$ 0.007	0.25 $\pm$ 0.004	36.0 $\pm$ 4.0
13	Spain	0.18 $\pm$ 0.004	0.26 $\pm$ 0.007	39.1 $\pm$ 1.1
14	Spain	0.17 $\pm$ 0.009	0.24 $\pm$ 0.008	49.0 $\pm$ 3.1
15	Spain	0.11 $\pm$ 0.003	0.20 $\pm$ 0.002	57.5 $\pm$ 2.7
16	Spain	0.11 $\pm$ 0.014	0.20 $\pm$ 0.006	52.7 $\pm$ 0.4
17	Spain	0.13 $\pm$ 0.002	0.21 $\pm$ 0.003	50.7 $\pm$ 4.4
18	Spain	0.13 $\pm$ 0.001	0.19 $\pm$ 0.002	43.3 $\pm$ 1.8
19	Greece	0.12 $\pm$ 0.003	0.20 $\pm$ 0.005	45.8 $\pm$ 4.9
20	Greece	0.14 $\pm$ 0.011	0.25 $\pm$ 0.006	43.6 $\pm$ 3.2
21	Greece	0.12 $\pm$ 0.006	0.23 $\pm$ 0.006	45.5 $\pm$ 4.5
22	Greece	0.12 $\pm$ 0.005	0.20 $\pm$ 0.004	43.5 $\pm$ 1.0
23	Greece	0.12 $\pm$ 0.002	0.20 $\pm$ 0.008	40.7 $\pm$ 1.6
24	Greece	0.18 $\pm$ 0.005	0.27 $\pm$ 0.005	29.8 $\pm$ 2.0
25	Greece	0.11 $\pm$ 0.006	0.18 $\pm$ 0.008	45.4 $\pm$ 3.3
26	Greece	0.16 $\pm$ 0.008	0.30 $\pm$ 0.005	35.3 $\pm$ 1.6
27	Greece	0.16 $\pm$ 0.011	0.22 $\pm$ 0.003	33.0 $\pm$ 1.0
28	Turkey	0.13 $\pm$ 0.002	0.19 $\pm$ 0.006	44.2 $\pm$ 2.6
29	Turkey	0.18 $\pm$ 0.004	0.30 $\pm$ 0.006	40.8 $\pm$ 2.0
30	Turkey	0.11 $\pm$ 0.003	0.17 $\pm$ 0.005	40.5 $\pm$ 2.1
31	Turkey	0.15 $\pm$ 0.004	0.26 $\pm$ 0.003	41.5 $\pm$ 1.5
32	Turkey	0.12 $\pm$ 0.005	0.20 $\pm$ 0.010	40.8 $\pm$ 2.6

33	Iran	0.12 ± 0.003	0.21 ± 0.013	46.1 ± 1.1
34	Iran	0.10 ± 0.003	0.18 ± 0.006	43.8 ± 2.2
35	Albanie	0.09 ± 0.005	0.18 ± 0.005	39.0 ± 0.6
36	Albanie	0.19 ± 0.001	0.35 ± 0.011	39.2 ± 2.0
37	Afghanistan	0.10 ± 0.002	0.17 ± 0.006	48.1 ± 1.9
38	Cyprus	0.12 ± 0.001	0.19 ± 0.002	44.3 ± 1.0
39	Maroc	0.15 ± 0.010	0.20 ± 0.007	44.5 ± 2.1
Average		0.13 ± 0.026	0.22 ± 0.05	43.9 ± 6.6
Range		0.09 – 0.19	0.17 – 0.35	29.8 – 57.5

Table 2. Polyphenol composition of *V. ervilia* seeds from populations 11 and 29 (µg/g flour). Results are the average ± sd of two determinations.

<i>V. ervilia</i> populations	ERV-11	ERV-29
Myricetin	1.5 ± 0.083	4.5 ± 0.25
Daidzein	4 ± 0.17	1 ± 0.24
Quercetin	25.5 ± 1.2	0.0 ± 0.0
Genistein	7.0 ± 0.34	0.0 ± 0.0
Luteolin	551 ± 3.5	316 ± 2.1
Kaempferol	5.4 ± 1.7	152 ± 2.0
Apigenin	19.5 ± 2.5	4 ± 0.098
Formononetin	1 ± 0.15	5.5 ± 0.34
Diosmetin	2 ± 0.17	6.5 ± 0.43
Biochanin A	7 ± 0.34	1.5 ± 0.25

Table 3. Free amino acid composition of *V. ervilia* seeds (populations 5, 18, 25, and 35) and leaves (population 35). Data is shown as g/100 g free amino acids, and represent the average $\pm$ sd of two determinations.

<i>V. ervilia</i> populations	5 seeds	18 seeds	25 seeds	35 seeds	Seeds x $\pm$ sd	35 leaves
Aspartic acid	2.3 $\pm$ 0.01	5.9 $\pm$ 0.04	7.2 $\pm$ 0.65	9.5 $\pm$ 0.38	6.2 $\pm$ 3.0	4.2 $\pm$ 0.05
Glutamic acid	16.5 $\pm$ 0.59	22.1 $\pm$ 0.17	14.6 $\pm$ 0.38	17.8 $\pm$ 0.61	17.8 $\pm$ 3.2	2.6 $\pm$ 0.01
Asparagine	9.3 $\pm$ 0.19	6.9 $\pm$ 0.00	2.4 $\pm$ 0.04	1.0 $\pm$ 0.04	4.9 $\pm$ 3.9	46.7 $\pm$ 0.66
Serine	1.4 $\pm$ 0.01	2.9 $\pm$ 0.13	2.7 $\pm$ 0.00	8.7 $\pm$ 0.01	3.9 $\pm$ 3.2	4.7 $\pm$ 0.18
Glutamine	2.1 $\pm$ 0.03	1.6 $\pm$ 0.07	2.1 $\pm$ 0.03	4.5 $\pm$ 0.05	2.6 $\pm$ 1.3	3.2 $\pm$ 0.09
Histidine	2.6 $\pm$ 0.10	1.6 $\pm$ 0.06	1.9 $\pm$ 0.04	2.4 $\pm$ 0.05	2.1 $\pm$ 0.5	2.8 $\pm$ 0.06
Glycine	5.8 $\pm$ 0.08	5.7 $\pm$ 0.05	6.1 $\pm$ 0.13	9.0 $\pm$ 0.01	6.7 $\pm$ 1.6	1.0 $\pm$ 0.02
Threonine	2.7 $\pm$ 0.15	2.5 $\pm$ 0.00	0.8 $\pm$ 0.02	1.7 $\pm$ 0.18	1.9 $\pm$ 0.8	3.3 $\pm$ 0.03
Arginine	7.3 $\pm$ 0.40	6.9 $\pm$ 0.22	16.2 $\pm$ 1.26	6.8 $\pm$ 0.32	9.3 $\pm$ 4.6	1.0 $\pm$ 0.03
Alanine	25.0 $\pm$ 0.74	15.2 $\pm$ 0.02	12.7 $\pm$ 0.27	9.9 $\pm$ 0.08	15.7 $\pm$ 6.6	3.9 $\pm$ 0.04
Proline	0.0 $\pm$ 0.00	0.0 $\pm$ 0.00	0.0 $\pm$ 0.00	0.0 $\pm$ 0.00	0.0 $\pm$ 0.0	3.5 $\pm$ 0.10
Tyrosine	1.8 $\pm$ 0.02	2.2 $\pm$ 0.05	1.4 $\pm$ 0.03	2.1 $\pm$ 0.34	1.9 $\pm$ 0.4	0.7 $\pm$ 0.04
Valine	3.0 $\pm$ 0.03	2.1 $\pm$ 0.04	4.1 $\pm$ 0.07	5.4 $\pm$ 0.21	3.6 $\pm$ 1.4	8.2 $\pm$ 0.04
Methionine	0.0 $\pm$ 0.00	0.0 $\pm$ 0.00	0.0 $\pm$ 0.00	0.0 $\pm$ 0.00	0.0 $\pm$ 0.0	0.6 $\pm$ 0.05
Cysteine	0.0 $\pm$ 0.00	0.0 $\pm$ 0.00	0.0 $\pm$ 0.00	0.0 $\pm$ 0.00	0.0 $\pm$ 0.0	1.4 $\pm$ 0.07
Isoleucine	1.2 $\pm$ 0.13	0.6 $\pm$ 0.04	0.0 $\pm$ 0.00	1.3 $\pm$ 0.04	0.8 $\pm$ 0.6	3.5 $\pm$ 0.02
Tryptophan	0.0 $\pm$ 0.00	0.0 $\pm$ 0.00	0.0 $\pm$ 0.00	1.9 $\pm$ 0.05	0.5 $\pm$ 1.0	0.0 $\pm$ 0.00
Leucine	5.4 $\pm$ 0.14	9.8 $\pm$ 0.15	2.1 $\pm$ 0.03	2.3 $\pm$ 0.05	4.9 $\pm$ 3.6	3.6 $\pm$ 0.04
Phenylalanine	1.7 $\pm$ 0.13	1.7 $\pm$ 0.04	1.7 $\pm$ 0.06	2.7 $\pm$ 0.08	2.0 $\pm$ 0.5	4.8 $\pm$ 0.00
Lysine	2.8 $\pm$ 0.18	2.1 $\pm$ 0.02	2.3 $\pm$ 0.23	2.8 $\pm$ 0.08	2.5 $\pm$ 0.4	0.4 $\pm$ 0.05
Canavanine	9.0 $\pm$ 0.18	9.9 $\pm$ 0.20	21.9 $\pm$ 1.59	10.2 $\pm$ 0.22	12.8 $\pm$ 6.1	0.0 $\pm$ 0.00
Total FAA ^a	0.19 $\pm$ 0.00	0.18 $\pm$ 0.00	0.06 $\pm$ 0.00	0.05 $\pm$ 0.00	0.12 $\pm$ 0.08	1.7 $\pm$ 0.01
Total canavanine ^a	0.018 $\pm$ 0.001	0.018 $\pm$ 0.001	0.014 $\pm$ 0.001	0.005 $\pm$ 0.00	0.013 $\pm$ 0.006	0.0 $\pm$ 0.00

^ag/100 g flour.

Table 4. Total amino acids composition and seed protein content in selected *V. ervilia* populations. Data is shown as g/100 g free amino acids, and represent the average $\pm$ sd of two determinations.

<i>V. ervilia</i> populations	5	18	25	35	x $\pm$ sd	FAO ^a
Aspartic a. ^b	11.5 $\pm$ 0.02	11.4 $\pm$ 0.01	12.2 $\pm$ 0.03	11.4 $\pm$ 0.12	11.7 $\pm$ 0.35	
Glutamic a. ^c	16.3 $\pm$ 0.01	17.1 $\pm$ 0.02	16.5 $\pm$ 0.04	16.6 $\pm$ 0.06	16.6 $\pm$ 0.34	
Serine	5.9 $\pm$ 0.01	5.9 $\pm$ 0.01	5.5 $\pm$ 0.02	6.0 $\pm$ 0.02	5.8 $\pm$ 0.21	
Histidine	2.6 $\pm$ 0.01	2.7 $\pm$ 0.04	2.5 $\pm$ 0.01	2.7 $\pm$ 0.02	2.6 $\pm$ 0.07	1.9
Glycine	4.7 $\pm$ 0.00	5.1 $\pm$ 0.00	4.8 $\pm$ 0.01	4.8 $\pm$ 0.00	4.9 $\pm$ 0.18	
Threonine	4.1 $\pm$ 0.03	4.1 $\pm$ 0.02	4.1 $\pm$ 0.01	4.0 $\pm$ 0.00	4.1 $\pm$ 0.06	3.4
Arginine	7.4 $\pm$ 0.01	7.9 $\pm$ 0.08	7.9 $\pm$ 0.01	8.0 $\pm$ 0.02	7.8 $\pm$ 0.30	
Alanine	5.7 $\pm$ 0.06	5.3 $\pm$ 0.01	5.1 $\pm$ 0.01	5.2 $\pm$ 0.00	5.4 $\pm$ 0.26	
Proline	4.4 $\pm$ 0.01	4.5 $\pm$ 0.02	4.5 $\pm$ 0.02	4.8 $\pm$ 0.06	4.6 $\pm$ 0.19	
Tyrosine	2.4 $\pm$ 0.04	2.5 $\pm$ 0.01	2.6 $\pm$ 0.02	2.4 $\pm$ 0.01	2.5 $\pm$ 0.07	6.3 ^d
Valine	6.2 $\pm$ 0.01	4.9 $\pm$ 0.05	4.9 $\pm$ 0.01	4.9 $\pm$ 0.02	5.2 $\pm$ 0.65	3.5
Methionine	0.1 $\pm$ 0.01	traces	0.3 $\pm$ 0.00	0.1 $\pm$ 0.00	0.1 $\pm$ 0.13	2.5 ^e
Cysteine	0.2 $\pm$ 0.01	traces	0.5 $\pm$ 0.02	0.1 $\pm$ 0.01	0.2 $\pm$ 0.19	
Isoleucine	4.7 $\pm$ 0.02	4.6 $\pm$ 0.03	4.7 $\pm$ 0.00	4.7 $\pm$ 0.04	4.7 $\pm$ 0.07	2.8
Tryptophan	2.3 $\pm$ 0.00	2.5 $\pm$ 0.04	2.3 $\pm$ 0.01	2.6 $\pm$ 0.04	2.4 $\pm$ 0.12	1.1
Leucine	8.1 $\pm$ 0.05	8.2 $\pm$ 0.05	8.3 $\pm$ 0.03	8.4 $\pm$ 0.00	8.3 $\pm$ 0.15	6.6
Phenylalanine	5.6 $\pm$ 0.03	5.5 $\pm$ 0.04	5.4 $\pm$ 0.01	5.7 $\pm$ 0.05	5.6 $\pm$ 0.11	
Lysine	7.8 $\pm$ 0.01	7.7 $\pm$ 0.01	7.6 $\pm$ 0.03	7.5 $\pm$ 0.02	7.7 $\pm$ 0.10	5.8
Canavanine	traces	traces	traces	traces	traces	
Protein	21.5 $\pm$ 0.01	23.7 $\pm$ 0.04	25.8 $\pm$ 0.09	25.5 $\pm$ 0.16	24.1 $\pm$ 1.98	

^aFAO/WHO/UNU Energy and Protein Requirements, 1985. ^bAspartic acid + Asparagine. ^cGlutamic acid + Glutamine. ^dTyrosine + Phenylalanine. ^eMethionine + Cysteine. ^fLower than 0.05 %.

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Table 5. Total amino acid composition of seed flour, seed fractions, and leaves from population 35. Data (g/100 g protein) are the average $\pm$ sd of two determinations.

	Flour	Alkaline extract	pH 4 soluble proteins	pH 4 insoluble proteins	Leaf proteins	FAO ^a
Aspartic a. ^b	11.4 $\pm$ 0.12	10.0 $\pm$ 0.02	12.1 $\pm$ 0.06	11.5 $\pm$ 0.14	22.1 $\pm$ 0.01	
Glutamic a. ^c	16.6 $\pm$ 0.06	16.8 $\pm$ 0.06	16.3 $\pm$ 0.18	17.6 $\pm$ 0.20	10.2 $\pm$ 0.01	
Serine	6.0 $\pm$ 0.02	5.7 $\pm$ 0.05	4.9 $\pm$ 0.02	5.4 $\pm$ 0.05	4.7 $\pm$ 0.08	
Histidine	2.7 $\pm$ 0.02	4.4 $\pm$ 0.10	3.3 $\pm$ 0.01	4.4 $\pm$ 0.05	1.2 $\pm$ 0.02	1.9
Glycine	4.8 $\pm$ 0.00	5.1 $\pm$ 0.07	7.2 $\pm$ 0.02	4.1 $\pm$ 0.07	5.5 $\pm$ 0.00	
Threonine	4.0 $\pm$ 0.00	4.2 $\pm$ 0.03	6.4 $\pm$ 0.10	3.5 $\pm$ 0.01	4.9 $\pm$ 0.04	3.4
Arginine	8.0 $\pm$ 0.02	8.8 $\pm$ 0.06	6.1 $\pm$ 0.02	8.9 $\pm$ 0.15	4.4 $\pm$ 0.04	
Alanine	5.2 $\pm$ 0.00	5.6 $\pm$ 0.28	9.3 $\pm$ 0.11	4.2 $\pm$ 0.05	7.1 $\pm$ 0.03	
Proline	4.8 $\pm$ 0.06	1.6 $\pm$ 0.02	1.4 $\pm$ 0.00	3.3 $\pm$ 0.15	5.2 $\pm$ 0.01	
Tyrosine	2.4 $\pm$ 0.01	2.6 $\pm$ 0.01	3.1 $\pm$ 0.06	2.3 $\pm$ 0.02	2.2 $\pm$ 0.02	6.3 ^d
Valine	4.9 $\pm$ 0.02	5.8 $\pm$ 0.03	5.2 $\pm$ 0.03	5.6 $\pm$ 0.03	6.5 $\pm$ 0.04	3.5
Methionine	0.1 $\pm$ 0.00	0.2 $\pm$ 0.00	0.3 $\pm$ 0.00	0.3 $\pm$ 0.00	0.8 $\pm$ 0.02	2.5 ^e
Cysteine	0.1 $\pm$ 0.01	0.1 $\pm$ 0.00	1.2 $\pm$ 0.09	0.1 $\pm$ 0.00	0.1 $\pm$ 0.01	
Isoleucine	4.7 $\pm$ 0.04	5.2 $\pm$ 0.06	3.6 $\pm$ 0.05	5.0 $\pm$ 0.04	4.9 $\pm$ 0.00	2.8
Tryptophan	2.6 $\pm$ 0.04	1.9 $\pm$ 0.05	2.3 $\pm$ 0.01	2.3 $\pm$ 0.01	2.0 $\pm$ 0.07	1.1
Leucine	8.4 $\pm$ 0.00	8.5 $\pm$ 0.09	5.0 $\pm$ 0.05	8.4 $\pm$ 0.04	7.8 $\pm$ 0.07	6.6
Phenylalanine	5.7 $\pm$ 0.05	6.0 $\pm$ 0.07	3.7 $\pm$ 0.01	5.9 $\pm$ 0.04	5.8 $\pm$ 0.08	
Lysine	7.5 $\pm$ 0.02	7.4 $\pm$ 0.06	8.5 $\pm$ 0.10	7.0 $\pm$ 0.04	4.7 $\pm$ 0.03	5.8
Canavanine	0.005 $\pm$ 0.00	0.0 $\pm$ 0.00	0.0 $\pm$ 0.00	0.0 $\pm$ 0.00	0.0 $\pm$ 0.00	
Protein	25.5 $\pm$ 0.16			91.9 $\pm$ 2.03	15.3 $\pm$ 0.18	
% EAA ^f	43.2	46.3	38.9	44.8	40.6	
AAS ^g	127.5	136.6	87.2	132.2	119.9	

^aFAO/WHO/UNU Energy and Protein Requirements, 1985. ^bAspartic acid + Asparagine. ^cGlutamic acid + Glutamine. ^dTyrosine + Phenylalanine. ^eMethionine + Cysteine. ^f% essential amino acids of sample (g / 100 g protein). ^gAmino acid score (% essential amino acids of sample / % essential amino acids suggested by FAO, 1985).

FIGURE 1

FIGURE 2

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FIGURE 3

FIGURE 4

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