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Research Article

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Free Amino Acids, Including Canavanine, in the Seeds from 24 Wild Mediterranean Legumes

Cristina Megias, Isabel Cortés-Giraldo, Julio Giron-Calle, Manuel Alaiz and Javier Vioque*

Food Phytochemistry Department, Instituto de la Grasa (C.S.I.C.), Campus Universidad Pablo de Olavide, Carretera de Utrera Km 1, 41013-Sevilla, Spain

***Correspondence to:**
Javier Vioque
Food Phytochemistry Department
Instituto de la Grasa (C.S.I.C.)
Campus Universidad Pablo de Olavide
Carretera de Utrera Km 1, 41013-Sevilla, Spain
Tel: +34 954611550
Fax: +34 954616790
E-mail: jvioque@cica.es

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Abstract

Seeds with a high concentration of specific free amino acids may represent a green alternative to industrial scale fermentation as sources of specific amino acids. Legume seeds are particularly rich in free amino acids. In order to explore this possibility, the free amino acid composition of the seeds from 24 wild Mediterranean legumes collected in southern Spain has been determined. These species represent fourteen genera belonging to tribes *Galegeae*, *Loteae* and *Trifolieae*. Free amino acid concentrations went from 0.21 g/100 g flour in *Scorpiurus sulcatus* to 1.89 g/100 g flour in *Anthyllis vulneraria* and *Medicago polymorpha*. The non proteinogenic amino acid canavanine, plus arginine, asparagine and aspartic acid were the most abundant amino acids. The highest content in canavanine, 1.26 g/100 g flour, equivalent to 66% (w/w) total free amino acids, was found in the seeds from *Anthyllis vulneraria*. Our results show that the seeds from several of the wild legumes included in this study represent a potential source of canavanine, arginine, asparagine and aspartic acid.

Keywords

Seed legumes, Free amino acids, Canavanine, Arginine

Introduction

Plants accumulate a variety of secondary compounds in their seeds that serve different functions, usually related with physical, chemical and biological stresses. The chemical nature of these compounds is very diverse. Secondary compounds include a great variety of polyphenols, alkaloids, and free amino acids. In legumes, free amino acids are, after alkaloids, the most abundant nitrogen containing secondary compounds [1]. Free amino acids and alkaloids may function as chemical defensive compounds, and also as sources of nitrogen for seedling growth until the symbiosis with nitrogen fixing bacteria is established [1]. Interestingly, it appears that free amino acids and alkaloids are exclusive in the sense that free amino acids are absent from legumes containing alkaloids and *vice versa* [1]. Legumes that store free amino acids in their seeds have been classified within the non-protein amino acids accumulating (NPAAA) clade [2], which includes around 9464 species and 305 genera. This clade includes 70% of the species and more than 60% of the genera in the subfamily *Papilionideae*, and almost 50% of the species and genera in the *Fabaceae* family [3].

Free amino acids include the 20 amino acids normally found in proteins, and a variety of other non proteinogenic amino acids such as 3-N-oxalyl-2,3-diaminopropionic acid (ODAP), homoarginine, canavanine, mimosine, and djenkolic acid [4]. Although better known for their presence at high concentrations in the legume family, free amino acids are also present in other nutritionally important plant families such as *Brassicaceae,* and also in algae and fungi [4].

Non protein free amino acids have been traditionally considered antinutritional compounds with undesirable functional properties. This is the case of 3-N-oxalyl-2,3-diaminopropionic acid (ODAP) and homoarginine from *Lathyrus* [5] and β-cyano-L-alanine and canavanine from *Vicia* [6, 7]. Nevertheless, some of these amino acids are now also been considered as potential health-promoting or therapeutic components in view of more recent discoveries. Legume seeds may also accumulate high amounts of proteinogenic amino acids that are also of interest because of their use in food supplements and pharmaceuticals. It is the case for instance of *Cicer arietinum* (chickpea) seeds, which accumulate more than 0.2% (w/w) arginine, accounting for more than half of total free amino acids [8].

The goal of this work was to screen wild Mediterranean legume species for new taxa containing high concentrations of canavanine and other free amino acids that could be of interest as sources of these amino acids. Seeds were collected from 24 populations located in the Seville and Huelva provinces in southern Spain, and amino acids were determined by reversed-phase HPLC. These species are representative of widespread taxa, and their amino acid composition had not been previously reported, or had been reported based on outdated methods that had only provided qualitative or semi-quantitative information.

Material and Methods

Materials

D, L α-aminobutyric acid, amino acid standards, water (HPLC grade), and acetonitrile (HPLC grade) were purchased from Sigma-Aldrich. Diethyl ethoxymethylenemanolate was purchased from Fluka. All other chemicals were of analytical grade.

Plant material

Legume seeds were collected during May and June 2014 in Huelva and Seville provinces, Andalucía, Spain. Seeds at full maturity were collected from several plants in each population and completely dried in the laboratory at room temperature before storage in Falcon tubes at -20 °C. Voucher specimens of each population are deposited at the Instituto de la Grasa, Seville, Spain.

Determination of free amino acids

Legume seeds were ground using a domestic blender and the resulting flour was extracted (10%, w/v) by stirring in aqueous ethanol (60% ethanol) for 1 hour using a magnetic stirrer. The pellets resulting from centrifugation at 12000 g for 20 minutes were extracted twice more, and the combined

Table 1: Free amino acid composition (g/100 total free amino acids), and contents in total free amino acids and canavanine (g/100 g flour) in studied legumes belonging to tribes *Galegeae* and *Loteae*. Results are the average ± sd of two determinations.

Tribes	*Galegeae*			*Loteae*			
Species	*Astragalus cymbaecarpos*	*Astragalus hamosus*	*Astragalus pelecinus*	*Coronilla glauca*	*Hippocrepis ciliata*	*Scorpiurus sulcatus*	*Scorpiurus vermiculatus*
Aspartic acid	37.44 ± 0.74	19.57 ± 0.16	10.44 ± 0.30	15.93 ± 0.09	14.06 ± 0.78	15.04 ± 0.74	5.05 ± 0.13
Glutamic acid	4.80 ± 0.01	4.62 ± 0.04	3.97 ± 0.02	11.89 ± 0.11	10.89 ± 0.41	10.12 ± 0.14	4.18 ± 0.06
Asparagine	11.54 ± 0.25	12.91 ± 0.14	26.92 ± 0.25	14.28 ± 1.63	29.70 ± 0.75	20.76 ± 0.92	11.90 ± 0.05
Serine	0.62 ± 0.03	1.47 ± 0.01	2.11 ± 0.22	3.26 ± 0.29	2.82 ± 0.84	1.26 ± 0.13	0.61 ± 0.05
Glutamine	0.00 ± 0.00	0.43 ± 0.02	1.30 ± 0.04	1.10 ± 0.08	1.94 ± 0.31	0.59 ± 0.01	0.53 ± 0.07
Histidine	10.09 ± 0.26	12.39 ± 0.06	16.92 ± 0.24	14.33 ± 0.02	8.61 ± 0.18	20.55 ± 0.36	5.80 ± 0.07
Glycine	0.87 ± 0.01	1.10 ± 0.01	1.42 ± 0.02	0.97 ± 0.01	1.48 ± 0.14	1.87 ± 0.09	0.57 ± 0.06
Threonine	0.51 ± 0.09	0.57 ± 0.02	2.42 ± 0.15	1.58 ± 0.16	0.49 ± 0.09	2.91 ± 1.09	3.85 ± 0.08
Arginine	13.41 ± 0.22	27.09 ± 0.20	20.22 ± 0.17	10.79 ± 0.33	8.69 ± 0.13	15.13 ± 0.10	59.57 ± 0.62
Alanine	0.97 ± 0.05	1.34 ± 0.02	1.29 ± 0.07	4.43 ± 0.15	5.27 ± 0.34	1.28 ± 1.09	1.43 ± 0.01
Proline	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Tyrosine	0.61 ± 0.05	0.53 ± 0.03	0.97 ± 0.08	4.29 ± 0.34	2.37 ± 0.19	1.08 ± 0.35	0.80 ± 0.03
Valine	0.59 ± 0.05	2.73 ± 0.17	1.11 ± 0.05	4.48 ± 0.30	1.56 ± 0.55	2.07 ± 0.05	0.87 ± 0.06
Methionine	1.45 ± 0.04	1.07 ± 0.07	1.72 ± 0.00	2.00 ± 0.25	0.00 ± 0.00	1.78 ± 0.00	0.53 ± 0.08
Cysteine	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Isoleucine	0.57 ± 0.05	1.10 ± 0.03	3.50 ± 0.02	2.39 ± 0.03	1.83 ± 0.01	0.99 ± 0.03	0.45 ± 0.04
Tryptophan	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Leucine	1.39 ± 0.04	0.89 ± 0.18	4.34 ± 0.17	3.26 ± 0.77	4.06 ± 0.06	1.55 ± 0.30	0.82 ± 0.14
Phenylalanine	0.65 ± 0.08	0.58 ± 0.03	0.83 ± 0.06	1.30 ± 0.15	1.60 ± 0.43	1.31 ± 0.09	0.60 ± 0.13
Lysine	0.26 ± 0.06	0.56 ± 0.06	0.51 ± 0.04	1.67 ± 0.00	2.43 ± 0.07	1.61 ± 0.05	1.12 ± 0.03
Canavanine	14.24 ± 0.20	11.04 ± 0.20	0.00 ± 0.00	2.11 ± 0.21	2.19 ± 0.29	0.00 ± 0.00	1.33 ± 0.04
Total free amino acids (g/100 g flour)	1.19 ± 0.01	1.03 ± 0.00	1.18 ± 0.03	0.69 ± 0.02	0.55 ± 0.02	0.21 ± 0.01	0.99 ± 0.03
Canavanine (g/100 g flour)	0.17 ± 0.00	0.11 ± 0.00	0.00 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

supernatants were used for determination of free amino acids by HPLC.

Free amino acids including canavanine were analyzed by reverse phase HPLC after derivatization using diethyl ethoxymethylenemanolate according to the method described by Megias et al. [7]. D, L α-aminobutyric acid was used as internal standard, and the reversed phase column was a Novapack C18 (300 x 3.9 mm i.d., 4 mm) from Waters (Milford, Massachusetts, USA).

Results and Discussion

Seeds from species belonging to one genus in tribe *Galegeae*, 9 genera in tribe *Loteae,* and 4 genera in tribe *Trifolieae* have been analyzed (Tables 1-3). These tribes are included in the informal Hologalegina subclade, which is part of the non-protein amino acid accumulating (NPAAA) clade [9]. The Hologalegina subclade is divided into the Robinioids subclade, to which the *Loteae* tribe belongs, and the inverted repeat (IR)-lacking subclade, to which the *Galegeae* and *Trifolieae* tribes belong. The IR-lacking subclade is a monophyletic group characterized by the loss of one or two copies of a 25-kb inverted repeat in the plastid genome [3, 10] and contains most of the temperate herbaceous legumes.

The genus *Astragalus*, including 2300-2500 species, accounts for most of the species in the *Galegeae* tribe. Many *Astragalus* species are of importance in popular pharmacopeia [11]. The amino acid composition of the three *Astragalus* species that have been analyzed here revealed a total free amino acid content of 1.1 g/100 g flour. The most abundant amino acids in these three species were aspartic acid, asparagine, arginine, and histidine (Table 1). Nevertheless, the non-protein amino acid canavanine was also abundant in two of the species, *A. cymbaecarpos* and *A. hamosus*. A previous report based on reaction to the amino reagent ninhydrin after separation of free amino acids by paper chromatography described very variable contents of canavanine in 120 *Astragalus* species [12].

Twelve species belonging to nine genera in tribe *Loteae* have been analyzed (Tables 2 and 3). This tribe includes 22 genera and around 282 species. Total contents in free amino acids were quite variable in this tribe, ranging from 0.21 to 1.89 g/100 g flour in *Scorpiurus sulcatus* and *Anthyllis vulneraria*, respectively. The most abundant amino acids were again histidine and the metabolically related aspartic acid,

Table 2: Free amino acid composition (g/100 total free amino acids), and contents in total free amino acids and canavanine (g/100 g flour) in studied legumes belonging to tribe *Loteae*. Results are the average ± sd of two determinations.

Tribe	*Loteae*							
Species	*Hymenocarpos cornicina*	*Hymenocarpos hamosus*	*Hymenocarpos lotoides*	*Tripodium tetraphillum*	*Anthyllis vulneraria*	*Dorycnopsis gerardi*	*Ornithopus compressus*	*Lotus creticus*
Aspartic acid	2.95 ± 0.00	7.44 ± 0.13	9.21 ± 0.12	10.52 ± 2.57	16.15 ± 0.08	3.41 ± 0.11	18.05 ± 0.19	4.26 ± 0.12
Glutamic acid	3.73 ± 0.19	7.98 ± 0.03	3.23 ± 0.12	9.41 ± 0.10	1.68 ± 0.18	1.61 ± 0.15	6.67 ± 0.06	8.49 ± 0.08
Asparagine	12.07 ± 0.49	29.87 ± 0.41	4.82 ± 0.03	5.36 ± 0.16	5.09 ± 0.52	40.86 ± 0.79	16.36 ± 0.06	6.26 ± 0.05
Serine	0.43 ± 0.21	1.26 ± 0.17	0.78 ± 0.00	1.34 ± 0.21	0.58 ± 0.03	2.09 ± 0.05	1.21 ± 0.09	0.83 ± 0.02
Glutamine	0.43 ± 0.05	0.25 ± 0.06	0.28 ± 0.01	0.65 ± 0.22	0.71 ± 0.01	0.88 ± 0.16	0.93 ± 0.07	0.78 ± 0.02
Histidine	5.50 ± 0.15	17.43 ± 0.26	5.43 ± 0.01	12.10 ± 0.03	2.00 ± 0.02	3.10 ± 0.31	13.15 ± 0.02	5.86 ± 0.09
Glycine	0.88 ± 0.06	2.02 ± 0.01	0.87 ± 0.11	1.74 ± 0.02	0.17 ± 0.00	0.81 ± 0.00	1.03 ± 0.00	1.09 ± 0.21
Threonine	0.49 ± 0.07	1.69 ± 0.02	0.33 ± 0.00	2.04 ± 0.07	0.24 ± 0.01	1.95 ± 0.75	0.89 ± 0.02	1.29 ± 0.07
Arginine	2.72 ± 0.39	25.72 ± 0.31	3.16 ± 0.19	47.73 ± 0.95	1.10 ± 0.06	33.92 ± 0.81	25.68 ± 0.21	16.33 ± 0.49
Alanine	0.91 ± 0.23	2.80 ± 0.01	1.78 ± 0.01	1.94 ± 0.04	0.86 ± 0.04	1.25 ± 0.22	0.86 ± 0.09	2.46 ± 0.12
Proline	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Tyrosine	0.67 ± 0.01	0.41 ± 0.04	0.63 ± 0.02	0.93 ± 0.07	0.55 ± 0.05	0.76 ± 0.08	1.81 ± 0.13	4.04 ± 0.13
Valine	0.41 ± 0.01	0.72 ± 0.06	0.78 ± 0.10	0.56 ± 0.04	0.56 ± 0.13	1.54 ± 0.30	0.84 ± 0.01	5.43 ± 0.17
Methionine	0.82 ± 0.01	0.00 ± 0.00	1.92 ± 0.00	0.00 ± 0.00	2.18 ± 0.00	0.00 ± 0.00	0.52 ± 0.05	0.45 ± 0.01
Cysteine	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Isoleucine	2.03 ± 0.10	0.30 ± 0.01	0.20 ± 0.01	0.58 ± 0.06	0.30 ± 0.00	0.73 ± 0.00	0.49 ± 0.03	0.62 ± 0.03
Tryptophan	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Leucine	0.52 ± 0.03	0.56 ± 0.02	0.38 ± 0.03	1.82 ± 0.12	0.55 ± 0.01	5.22 ± 0.17	3.50 ± 0.04	0.63 ± 0.00
Phenylalanine	0.34 ± 0.02	1.02 ± 0.00	0.60 ± 0.03	0.79 ± 0.05	0.48 ± 0.34	1.14 ± 0.02	0.82 ± 0.19	1.20 ± 0.01
Lysine	0.46 ± 0.01	0.55 ± 0.03	0.37 ± 0.01	0.96 ± 0.00	0.34 ± 0.03	0.76 ± 0.02	0.66 ± 0.00	1.19 ± 0.02
Canavanine	64.62 ± 0.34	0.00 ± 0.00	65.25 ± 0.06	1.49 ± 0.53	66.43 ± 0.08	0.00 ± 0.00	6.52 ± 0.00	39.15 ± 0.14
Total amino acids (g/100 g flour)	1.78 ± 0.03	0.50 ± 0.00	1.75 ± 0.06	0.49 ± 0.01	1.89 ± 0.01	0.53 ± 0.02	0.67 ± 0.01	1.62 ± 0.02
Canavanine (g/100 g flour)	1.15 ± 0.01	0.00 ± 0.00	1.14 ± 0.03	0.01 ± 0.00	1.26 ± 0.00	0.00 ± 0.00	0.04 ± 0.00	0.62 ± 0.01

asparagine, arginine, and canavanine. The highest contents of canavanine, more than 1 g/100 g flour, were found in *Anthyllis vulneraria*, *Hymenocarpos cornicina*, and *H. lotoides*, formerly in genus *Anthyllis* [13]. However, canavanine contents in *Dorycnopsis gerardi* and *Tripodium tetraphyllum*, that were also classified within genus *Anthyllis* in the past, were 0.00 and 0.02 g/100 g flour, respectively. The most abundant amino acids in *Dorycnopsis gerardi* and *Tripodium tetraphillum* were asparagine and arginine, respectively. Our results are consistent with most of previous reports on the presence of canavanine in some species within tribe *Loteae*. Thus, our results confirm previous reports on the concentration of canavanine in *Ornithopus compresus* [14] in *Lotus creticus* [15], and in genus *Hymenocarpos* [14, 16]. Although canavanine was previously found in *Coronilla* species [16], it was not found in *Coronilla glauca* (Table 1). Consistent with previous reports showing that canavanine is absent from genus *Hippocrepis* [17], no canavanine was found in *H. ciliata*. However, the absence of canavanine in genus *Scorpiurus* does not agree with a previous report [14].

Nine species belonging to four of the six genera in tribe *Trifoliae* were analyzed (Table 3). This tribe includes species of great economic importance as forage such as *Medicago sativa* and *Trifolium repens*. More than half of the 485 species belonging to *Trifoliae* are included in genus *Trifolium*. The average free amino acids content in the *Trifolium* species was around 1 g/100 g flour (Table 3). In two of these species, namely *T. cherleri* and *T. scabrum*, the content in arginine was very high, representing around half of the total content in free amino acids. *T. angustifolium* was characterized by a very low content in arginine but a very high content in canavanine, while *T. repens* had relatively high levels of both arginine and canavanine. The metabolically related aspartic acid and asparagine were also abundant in *Trifolium* seeds (Table 3). These results are consistent with previous reports showing the presence of canavanine in genus *Trifolium* [16].

Table 3: Free amino acid composition (g/100 total free amino acids), and contents in total free amino acids and canavanine (g/100 g flour) in studied legumes belonging to tribe *Trifolieae*. Results are the average ± sd of two determinations.

Tribe	***Trifolieae***								
Species	***Trifolium angustifolium***	***Trifolium cherleri***	***Trifolium repens***	***Trifolium scabrum***	***Trifolium stellatum***	***Medicago minima***	***Medicago polymorpha***	***Melilotus elegans***	***Ononis natrix***
Aspartic acid	20.61 ± 1.03	23.74 ± 0.01	16.16 ± 0.48	23.72 ± 0.92	8.53 ± 0.29	9.51 ± 0.38	5.73 ± 0.02	43.92 ± 0.07	8.41 ± 0.60
Glutamic acid	2.64 ± 0.06	6.27 ± 0.09	5.59 ± 0.03	6.40 ± 0.02	6.78 ± 0.27	3.55 ± 0.09	2.31 ± 0.00	7.35 ± 0.64	10.53 ± 0.12
Asparagine	3.64 ± 0.05	7.92 ± 0.84	11.54 ± 0.37	10.09 ± 0.87	14.90 ± 0.38	17.29 ± 0.18	19.56 ± 0.42	11.33 ± 0.33	6.17 ± 0.25
Serine	0.43 ± 0.05	0.97 ± 0.09	7.24 ± 0.10	0.81 ± 0.29	0.89 ± 0.22	1.13 ± 0.27	0.49 ± 0.04	1.12 ± 0.02	1.47 ± 0.03
Glutamine	0.07 ± 0.03	0.47 ± 0.04	0.68 ± 0.23	0.81 ± 0.06	1.16 ± 0.13	0.73 ± 0.19	0.00 ± 0.00	0.79 ± 0.06	1.31 ± 0.02
Histidine	1.47 ± 0.02	2.46 ± 0.17	6.56 ± 0.14	1.06 ± 0.26	4.37 ± 0.10	3.02 ± 0.08	0.33 ± 0.07	14.06 ± 0.26	8.79 ± 0.37
Glycine	0.43 ± 0.03	1.53 ± 0.04	0.61 ± 0.05	0.98 ± 0.13	0.54 ± 0.01	0.66 ± 0.06	0.35 ± 0.01	0.96 ± 0.01	1.53 ± 0.02
Threonine	0.80 ± 0.02	1.84 ± 0.03	1.88 ± 0.04	1.85 ± 0.32	2.35 ± 0.02	0.97 ± 0.04	0.70 ± 0.05	2.86 ± 0.06	1.79 ± 0.07
Arginine	3.60 ± 0.08	42.40 ± 0.71	25.48 ± 0.21	42.40 ± 2.21	51.76 ± 0.33	11.87 ± 0.27	6.19 ± 0.24	6.17 ± 0.06	47.57 ± 0.09
Alanine	1.58 ± 0.02	2.37 ± 0.06	2.11 ± 0.02	1.42 ± 0.20	1.37 ± 0.06	1.82 ± 0.04	0.41 ± 0.00	1.78 ± 0.00	2.42 ± 0.03
Proline	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Tyrosine	0.47 ± 0.09	0.69 ± 0.02	1.58 ± 0.03	0.37 ± 0.04	0.87 ± 0.02	0.70 ± 0.08	0.21 ± 0.03	2.15 ± 0.02	0.95 ± 0.01
Valine	0.42 ± 0.01	1.01 ± 0.14	2.09 ± 0.05	1.15 ± 0.21	0.97 ± 0.15	0.83 ± 0.18	0.48 ± 0.03	1.03 ± 0.06	1.71 ± 0.97
Methionine	2.06 ± 0.01	1.13 ± 0.05	0.66 ± 0.01	0.73 ± 0.14	0.57 ± 0.05	0.96 ± 0.04	0.73 ± 0.06	0.00 ± 0.00	0.00 ± 0.00
Cysteine	0.00 ±0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Isoleucine	0.19 ± 0.13	1.24 ± 0.05	0.80 ± 0.02	1.07 ± 0.07	0.27 ± 0.04	0.70 ± 0.01	0.13 ± 0.03	0.77 ± 0.09	1.97 ± 0.06
Tryptophan	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Leucine	0.85 ± 0.14	4.25 ± 0.09	4.70 ± 0.24	3.19 ± 0.21	1.09 ± 0.30	1.08 ± 0.12	1.02 ± 0.06	3.78 ± 0.02	1.72 ± 0.07
Phenylalanine	0.84 ± 0.02	0.91 ± 0.11	0.97 ± 0.09	1.33 ± 0.49	0.35 ± 0.05	0.88 ± 0.10	0.19 ± 0.27	1.01 ± 0.55	1.07 ± 0.09
Lysine	0.36 ± 0.16	0.80 ± 0.02	0.70 ± 0.02	1.19 ± 0.00	0.81 ± 0.03	1.01 ± 0.02	0.43 ± 0.02	0.88 ± 0.07	1.67 ± 0.02
Canavanine	59.54 ± 0.46	0.00 ± 0.00	10.63 ± 0.02	1.40 ± 0.03	2.42 ± 0.32	43.29 ± 0.28	60.73 ± 0.64	0.00 ± 0.00	0.92 ± 0.42
Total amino acids (g/100 g flour)	1.22 ± 0.01	0.89 ± 0.04	0.99 ± 0.02	1.15 ± 0.02	1.16 ± 0.03	1.82 ± 0.05	1.89 ± 0.03	0.50 ± 0.02	0.79 ± 0.01
Canavanine (g/100 g flour)	0.73 ± 0.01	0.00 ± 0.00	0.11 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.79 ± 0.02	1.15 ± 0.02	0.00 ± 0.00	0.01 ± 0.00

The content in total free amino acids was higher in *Medicago* than in *Trifolium*, and canavanine represented about half of this content (Table 3). These results agree with previous reports of canavanine in *Medicago* [14]. In contrast, canavanine was not found at significant levels in *Melilotus elegans* and *Ononis natrix*, which also exhibited a relatively high content in total free amino acids. Aspartic acid and arginine represented almost half of the free amino acids in *Melilotus elegans* and *Ononis natrix*, respectively.

Arginine and canavanine are the most abundant amino acids in most of the leguminous seeds that have been analyzed. Canavanine is an arginine analogue with an important role in defense against predation because it can be incorporated into the newly synthesized proteins of plant predators, resulting in aberrant non-functional proteins [18, 19]. Nevertheless, it has also been found more recently that canavanine could have important therapeutic properties. Thus, canavanine inhibits cell proliferation [20] and induces apoptosis [21, 22] in cell cultures, and it also increases susceptibility to radiation in cancer cells [23]. In addition, it inhibits some of the deleterious effects of endotoxic shock in rat models [24, 25] and has potential as an antidiabetic agent because it has been found to increase uptake of glucose [26] and release of insulin [27] in cell cultures.

Considering our data, the legumes that have been analyzed can be classified into two groups according to their content in canavanine. Those with high content in canavanine are characterized by an inverse correlation between the contents in canavanine and arginine, as illustrated in Figure 1 (R^2 = 0.81). Those species with no canavanine or a low content in canavanine are characterized by having arginine, followed by asparagine and aspartic acid, as the most abundant free amino acid. The limit between these two groups could be set at about 2.5% total free amino acids. It has been partially demonstrated that arginine and canavanine share a similar biosynthetic pathway through the ornithine-urea cycle, where the precursors ornithine and canaline are precursors for the synthesis of arginine and canavanine, respectively [28, 29]. Same or very similar enzymes are involved in the synthesis of both amino acids. Also, in some reactions of the ornithine-urea cycle, arginine and canavanine share identical substrates such as carbamyl phosphate or aspartic acid. However, the genetic and biochemical differences that diverge to the synthesis of arginine or canavanine remain to be defined.

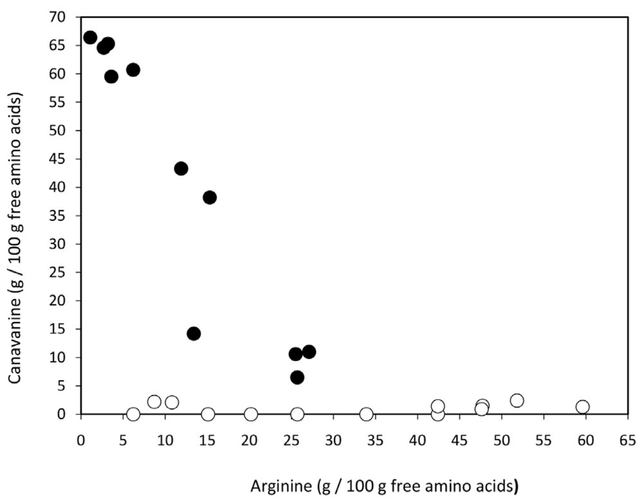

Figure 1: Correlation between contents in canavanine and arginine (g/100 g free amino acids). Open and full circles represent seeds with canavanine content lower or higher than 2.5% total free amino acids, respectively.

Interestingly, there is a positive correlation between the contents in canavanine and total free amino acids (Figure 2, R^2 = 0.67), indicating that accumulation of free amino acids in the seeds is associated with accumulation of canavanine specifically, and to a lesser degree with accumulation of those amino acids metabolically related with canavanine. This is further illustrated in figure 3 that shows the average free amino acid composition of the 24 species that have been analyzed. Canavanine and the metabolically related aspartic acid, asparagine, and arginine are the most abundant amino acids, although there is a high variability in their concentrations. This is especially true for canavanine, which is found at concentrations that go from 0.0 to 66.4% total free amino acids.

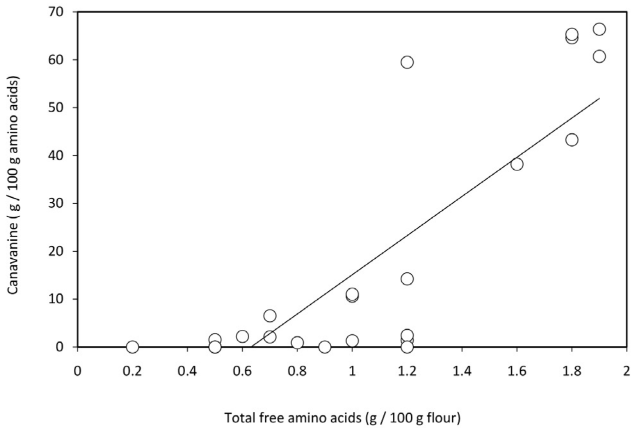

Figure 2: Correlation between contents in canavanine (g canavanine/100 g free amino acids) and free amino acids (g free amino acids/100 g flour).

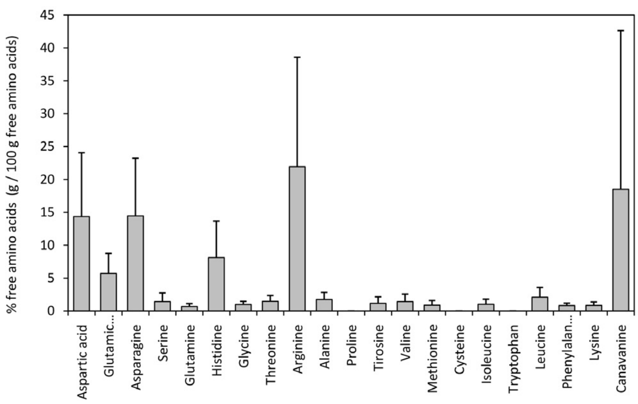

Figure 3: Average free amino acids composition (g free amino acid/100 g free amino acids) of the 24 species that were analyzed.

We have shown recently that other annual mediterranean legumes belonging to genus *Vicia* are also rich in free amino acids, including canavanine [31]. However, the highest contents in canavanine have been found in perennial herbs, shrubs and trees from tropical and subtropical regions. This is the case of *Canavalia ensiformis*, with canavanine contents higher than 2.5% [31], *Robinia pseudoacacia* (9.8% dry matter), *Wisteria floribunda* (12.3%), and *Dioclea megacarpa* (12.7%) [32].

In conclusion, results show that several Mediterranean wild legumes represent a potential source of free amino acids with favorable bioactive properties, especially canavanine. Some of these legumes are forage species that could be harvested and exploited for feeding and the seeds for canavanine purification.

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