Purification of canavanine from the legume *Vicia disperma*

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ABSTRACT

Vicia disperma is an annual legume that accumulates high amounts of the non-protein amino acid canavanine in the seeds. The purification of this amino acid from the seeds of *V. disperma* has been investigated in order to assess the possibility of using this plant as a source of canavanine. Seeds had 3% (w/w) canavanine content, representing 84.3% (w/w) total free amino acids. Canavanine was extracted from seed flour extracts at pH 4 and purified by nanofiltration and ion-exchange chromatography, followed by either precipitation or crystallization, yielding canavanine 90% and 97% pure, respectively. This represents a simple and inexpensive procedure for production of canavanine, taking advantage of the high content of canavanine in *V. disperma*. This could have a very beneficial impact on the use of *V. disperma* and other *Vicia* rich in canavanine, some of which have good agronomic properties and were farmed in the past.

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1. Introduction

Vicia disperma DC. (*Fabaceae*), also known as two-seeded vetch, is a drought-resistant, annual climbing legume with stems up to one meter long. Fruits are very abundant and have one or two seeds, usually two. *V. disperma* is very common in the Mediterranean Region, especially in the Iberian Peninsula, Italy, south of France, and northern Africa. Nevertheless, and despite its high fruit yield and good agronomic properties, there are no reports on the use of this legume as food or animal feed. Previous studies in our laboratory showed that *V. disperma* seeds are rich in polyphenols with high antioxidant activity, and contain protein and fatty acids with good nutritional value (Pastor-Cavada et al., 2011a, 2011b, 2009).

Plants produce and accumulate different types of secondary compounds, including glucosinolates, alkaloids, polyphenols, essential oils, and free amino acids, which in many cases have a defensive role against predators. Many legumes, including legume crops, accumulate significant amounts of free amino acids in their seeds. Canavanine is a non-protein amino acid present in many legumes, such as *Canavalia, Dioclea*, *Colutea*, and *Robinia* (Rosenthal, 2001), and in several *Vicia* species including *V. disperma* (Bell and Tirimanna, 1965). Canavanine is an arginine analog with antinutritional activity as an antimetabolite that is incorporated into proteins of the predator substituting for arginine (Rosenthal, 1977a), leading to deficient protein conformations with loss of

functionality (Rosenthal, 2001). Nevertheless, canavanine also has some activities with potential health promoting and even therapeutic potential. Thus, canavanine inhibits the growth of colon tumors (Thomas et al., 1986), and in combination with low levels of arginine induces apoptosis in a number of cancerous cells (Vynnytska et al., 2011, 2012; Bence et al., 2002; Jang et al., 2002). The antiproliferative activity of the legume *Sutherlandia frutescens* has been attributed at least partially to its content in canavanine (Van Wyk and Albrecht, 2008). Canavanine also has antiviral activity as reviewed in Bell et al. (2008), and potential health promoting or therapeutic properties by inhibiting nitric oxide synthase (Teale and Atkinson, 1994).

Although there are studies on the free amino acid composition in species belonging to the *Fabaceae* family reporting the presence of canavanine (Bell and Tirimanna, 1965; Bell et al., 1978), the inter-population variability, and quantification of this amino acid in *V. disperma* seeds, as well as its purification from these seeds, have not been previously investigated. While large amounts of free amino acids are used by the pharmaceutical, cosmetic, agricultural, and feed and food industries, the nutrition and health products industries are the mayor consumers of free amino acids driving the search for new production and purification methods. Although fermentation, protein hydrolysis, chemical synthesis, and enzymatic catalysis are the traditional methods for production of amino acids (Leuchtenberger et al., 2005), plants represent "green sources" of amino acids with a great potential. The goal of the present research was to analyze canavanine in seeds collected from a number of *V. disperma* populations in southern Spain and to explore purification methods in order to determine whether

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V. disperma represents a potential source of this amino acid.

2. Material and methods

2.1. Plant materials

Nine populations of *V. disperma* were collected during May and June 2013 in the Sierra de Aracena y Picos de Aroche Natural Park (Huelva province, Andalucía, Spain). GPS locations of the nine populations were: (1) N 37.905335, W 6.495641; (2) N 37.888402, W 6.502365; (3) N 37.902965, W 6.667023; (4) N 37.869679, W 6.708495; (5) N 37.880731, W 6.642170; (6) N 37. 912344, W 6.551116; (7) N 37.878791, W 6.530140; (8) N 37.844395, W 6.709989; (9) N 37.882119, W 6.741575. Seeds at full maturity were collected from several plants in each population and were allowed to completely dry at room temperature before storage at -20 °C. Soybeans (*Glycine max*), chickpeas (*Cicer arietinum*), and lentils (*Lens culinaris*) were purchased in a local market. Seeds from population n° 9 were used for the canavanine purification assays.

2.2. Determination of free amino acids

V. disperma seeds were ground using a blender (Moulinex, France) and the resulting flour was extracted by stirring in aqueous ethanol [10% (w/v) in 60% (v/v) ethanol] for 1 h. The pellets resulting from centrifugation at 12,000g for 20 min were extracted twice more, and the combined supernatants were used for determination of canavanine by HPLC.

Free amino acids including canavanine were analyzed by reverse phase HPLC after derivatization using diethyl ethoxymethylenemanolate and D, L α-aminobutyric acid as internal standard according to the method described (Megias et al., 2015). A Novapack C_{18} column (300×3.9 mm^2 i.d., 4 µm, Waters) was used. Electro-spray-ionization high-resolution mass spectra were recorded with a micrOTOF-QII High Resolution-of-Flight mass spectrometer (UHR-TOF) with qQ-TOF geometry (Bruker Daltonic, Bremen, Germany).

2.3. Purification of canavanine from V. disperma seed flour extracts

A suspension of flour in water 20% (w/v) was taken to pH 4, stirred for 1 h at room temperature, and centrifuged at 12,000g for 15 min. The pellets were extracted twice more as described above, and the three resulting supernatants were combined and stored at -20 °C. Purification of canavanine was carried out as described by Bass et al. (1995) for *Canavalia ensiformis* seeds with modifications. Seed extracts were reduced to half their volume using an Amicon cell filtration unit (Millipore, MA, USA) equipped with a nanofiltration membrane (Koch Membrane Systems, MA, USA). Amberlite IR-120 (Fluka, MO, USA) resin was used to further purify canavanine from the permeates. The resin was pre-conditioned, washed and loaded using a batch procedure. Pre-conditioning involved treatment with 10% (w/v) of 2 N HCl for two hours, 10% (w/v) of 2 N NaOH for two hours, 10% (w/v) of 2 N HCl for two hours more, and finally washing several times using water. Free amino acid permeates were incubated with the resin (1 mg free amino acids/mL resin in water pH 2) with shacking at room temperature for 30 min, which led to binding of all free amino acids. The resin was then washed four times for 15 min with water in a 1/10 (v/v) proportion. Acidic amino acids were then released by washing with 0.5 N NH$_4$OH for 30 min, and finally canavanine was recovered by washing three times with 7.5 N NH$_4$OH for 30 min. The combined washes were taken to dryness and redissolved in the minimum volume of water. Solid matter determination was carried out by weighing after drying aliquots at 120 °C overnight.

2.4. Precipitation and crystallization

Precipitation was carried out by adding excess ethanol (four times the volume of the canavanine extract), and the precipitates were recovered after centrifugation at 12,000g for 15 min. Crystallization was carried out by allowing a 1:1 (v/v) mixture of the canavanine extract and ethanol to rest for 24 h at 4 °C. The crystallized amino acids were recovered by centrifugation at 12,000g for 15 min.

3. Results and discussion

3.1. Free amino acids and canavanine in V. disperma seeds

Free amino acids were extracted from the seeds taken from nine populations of *V. disperma* using aqueous ethanol. Total free amino acids ranged from 2.55% to 3.55% (w/w), with an average content of $2.99 \pm 0.31\%$ (w/w). These values are much higher than those found in soy (0.42%), chickpea (0.45%), and lentil (0.67%), which were also analyzed as reference materials, and represent contents in total free amino acids that are ten times higher than the content in polyphenols as reported, 0.28% (w/w) (Pastor-Cavada et al., 2011a).

Canavanine was the major free amino acid in *V. disperma* samples, ranging from 80.4% to 88.6%, and with an average content of $84.3 \pm 2.72\%$ as referred to total free amino acids. Other free amino acids were present at much lower concentrations, including threonine, arginine, aspartic acid, asparagine, glutamic acid, and histidine, all of them at concentrations between 0.5% and 9% (w/w) in all populations (Table 1). Only traces of the remaining free amino acids were present. The predominance of a single free amino acid in *V. disperma* seeds, in this case canavanine, was less apparent in crop legumes. Thus, asparagine, arginine and threonine accounted for 24.8%, 52.2%, and 22.7% (w/w) total amino acids in soybean, chickpea, and lentil, respectively.

The content in canavanine as referred to the whole seeds was between 2.2% and 3.0% (w/w) with an average content of $2.54 \pm 0.25\%$ (w/w) as referred to the flour, which is similar to the content in the most popular source of canavanine, the legume *C. ensiformis* (Rosenthal, 1977b) with 2.6% (w/w). The inter-population variability of the content in free amino acids and canavanine for the nine populations was low, suggesting that it is well fixed genetically and quite independent of environmental conditions.

3.2. Purification of canavanine from V. disperma seed flour

Canavanine was extracted using water instead of aqueous ethanol in order to limit extraction of polyphenols and other ethanol soluble components. Adjusting to pH 4.0 greatly reduces solubilization of storage proteins that have their isoelectric point at this pH (Vioque et al., 2012). The extraction was repeated three times in order to assure extraction of free amino acids as shown in Fig. 1. Table 2 shows the free amino acid composition of these extracts. Free amino acids represent 6.6% (w/w) total matter in the combined extracts, the remaining 93.4% (w/w) corresponding to albumins, polyphenols, sugars, soluble fiber and organic acids. Nanofiltration of the combined extracts using a 200 Da cut-off membrane increased free amino acid content from 6.6% (w/w) in the original extract to 45.5% (w/w) in the permeate.

Ion exchange resins are frequently used for the purification of amino acids in industrial settings (Leuchtenberger et al., 2005). Strong cationic resins are especially indicated for the purification of basic amino acids such as arginine, which has a positively charged side chain with a pK of 12.5 (Utagawa, 2004). Homoarginine and canavanine are structurally similar to arginine and

Table 1
Free amino acids in *V. disperma* seeds. Data represent g free amino acids/100 g total free amino acids for the nine populations that were sampled, average ± standard deviation of three determinations.

|	1	2	3	4	5	6	7	8	9
Asp	1.48 ± 0.07	1.95 ± 0.16	1.97 ± 0.04	2.21 ± 0.07	1.76 ± 0.05	2.24 ± 0.08	3.32 ± 0.09	3.02 ± 0.11	2.09 ± 0.06
Glu	1.23 ± 0.04	1.41 ± 0.01	1.98 ± 0.07	1.48 ± 0.11	1.70 ± 0.08	1.76 ± 0.04	1.62 ± 0.12	1.63 ± 0.05	2.10 ± 0.18
Asn	1.34 ± 0.00	2.46 ± 0.01	1.03 ± 0.03	1.85 ± 0.05	2.04 ± 0.05	1.88 ± 0.09	2.88 ± 0.17	1.28 ± 0.03	1.67 ± 0.06
Ser	0.18 ± 0.01	0.20 ± 0.00	0.24 ± 0.03	0.15 ± 0.04	0.16 ± 0.02	0.15 ± 0.02	0.24 ± 0.05	0.26 ± 0.00	0.19 ± 0.01
Gln	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.07 ± 0.00	0.06 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
His	0.94 ± 0.05	0.88 ± 0.01	1.49 ± 0.07	0.71 ± 0.05	0.83 ± 0.10	0.68 ± 0.01	1.50 ± 0.08	0.58 ± 0.02	0.94 ± 0.04
Gly	0.22 ± 0.01	0.20 ± 0.00	0.33 ± 0.01	0.22 ± 0.00	0.18 ± 0.02	0.21 ± 0.03	0.20 ± 0.01	0.29 ± 0.00	0.22 ± 0.02
Thr	4.31 ± 0.11	3.60 ± 0.13	8.53 ± 0.32	5.88 ± 0.04	4.31 ± 0.27	6.22 ± 0.19	6.73 ± 0.12	7.08 ± 0.32	4.48 ± 0.18
Arg	1.42 ± 0.15	0.49 ± 0.03	1.25 ± 0.23	1.47 ± 0.22	1.75 ± 0.79	1.53 ± 0.19	1.61 ± 0.07	1.03 ± 0.28	4.17 ± 0.18
Ala	0.00 ± 0.00	0.32 ± 0.09	0.35 ± 0.03	0.12 ± 0.01	0.27 ± 0.07	0.31 ± 0.28	0.23 ± 0.02	0.26 ± 0.08	0.54 ± 0.01
Pro	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
Tyr	0.00 ± 0.00	0.00 ± 0.00	0.16 ± 0.02	0.09 ± 0.01	0.08 ± 0.00	0.07 ± 0.03	0.23 ± 0.03	0.11 ± 0.03	0.09 ± 0.01
Val	0.05 ± 0.02	0.10 ± 0.01	0.10 ± 0.02	0.10 ± 0.03	0.15 ± 0.07	0.09 ± 0.06	0.19 ± 0.02	0.14 ± 0.01	0.11 ± 0.01
Met	0.11 ± 0.02	0.13 ± 0.01	1.03 ± 0.09	0.12 ± 0.01	0.14 ± 0.02	0.11 ± 0.01	0.00 ± 0.00	0.17 ± 0.00	0.10 ± 0.09
Cys	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
Ile	0.00 ± 0.00	0.08 ± 0.00	0.11 ± 0.04	0.06 ± 0.02	0.08 ± 0.00	0.05 ± 0.01	0.07 ± 0.02	0.09 ± 0.01	0.06 ± 0.01
Leu+Trp	0.00 ± 0.00	0.10 ± 0.00	0.58 ± 0.00	0.12 ± 0.07	0.32 ± 0.20	0.08 ± 0.03	0.19 ± 0.01	0.24 ± 0.02	0.19 ± 0.03
Phe	0.00 ± 0.00	0.14 ± 0.00	0.22 ± 0.01	0.15 ± 0.00	0.24 ± 0.01	0.14 ± 0.01	0.20 ± 0.01	0.20 ± 0.01	0.13 ± 0.00
Lys	0.16 ± 0.00	0.14 ± 0.00	0.26 ± 0.02	0.14 ± 0.01	0.22 ± 0.15	0.13 ± 0.02	0.11 ± 0.00	0.15 ± 0.00	0.16 ± 0.01
Canavanine	88.55 ± 0.00	87.81 ± 0.06	80.37 ± 0.09	85.17 ± 0.32	85.70 ± 0.54	84.27 ± 0.26	80.67 ± 0.64	83.45 ± 0.53	82.76 ± 0.16
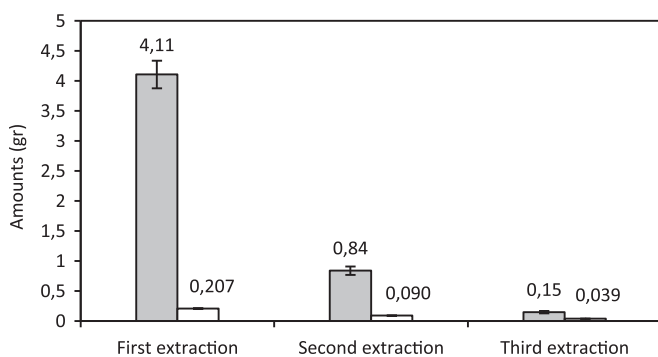
Fig. 1. Content in total matter (gray bars) and free amino acids (open bars) resulting from extraction using water pH 4 ($x \pm$ sd, $n=3$).

Table 2
Free amino acid composition of the extracts resulting from extraction of *V. disperma* seed flour using water pH 4. Data represent % (w/w) free amino acids, average ± standard deviation of three determinations.

	First extraction	Second extraction	Third extraction
Asp	2.13 ± 0.01	1.88 ± 0.01	0.93 ± 0.00
Glu	2.06 ± 0.02	2.03 ± 0.02	1.67 ± 0.01
Asn	1.94 ± 0.03	1.68 ± 0.03	1.23 ± 0.03
Ser	0.21 ± 0.03	0.31 ± 0.03	0.31 ± 0.02
Gln	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
His	0.70 ± 0.01	0.69 ± 0.01	0.50 ± 0.10
Gly	0.30 ± 0.00	0.29 ± 0.00	0.28 ± 0.06
Thr	6.13 ± 0.01	5.30 ± 0.12	4.38 ± 0.01
Arg	2.60 ± 0.25	2.93 ± 0.01	2.94 ± 0.13
Ala	0.86 ± 0.01	0.76 ± 0.05	0.81 ± 0.28
Pro	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Tyr	0.23 ± 0.01	0.45 ± 0.01	0.59 ± 0.15
Val	0.19 ± 0.01	0.14 ± 0.08	0.32 ± 0.00
Met	0.12 ± 0.00	0.14 ± 0.00	0.04 ± 0.06
Cys	0.13 ± 0.00	0.14 ± 0.00	0.14 ± 0.00
Ile	0.10 ± 0.00	0.12 ± 0.00	0.19 ± 0.01
Leu+Trp	0.33 ± 0.00	0.59 ± 0.03	0.92 ± 0.01
Phe	0.15 ± 0.01	0.29 ± 0.01	0.49 ± 0.00
Lys	0.33 ± 0.00	0.42 ± 0.00	0.56 ± 0.00
Canavanine	81.30 ± 0.06	83.07 ± 0.07	83.68 ± 0.00

Fig. 2. Content in total matter (gray bars) and free amino acids (open bars) resulting from purification using Amberlite IR-120. All the fractions related with the procedure are shown in order to show the specificity and yield of the different steps ($x \pm$ sd, $n=3$).

Amberlite IR-120 was complete, and washes using water pH 2 allowed for removal of other components. Acidic amino acids were successfully removed from the resin by washing with 0.5 N NH$_4$OH as shown in Fig. 2 and Table 3. Canavanine was finally recovered from the resin by carrying out three extractions using 7.5 N NH$_4$OH. These three extracts, which have very similar amino acid compositions as shown in Table 3, were combined and analyzed by mass spectrometry, yielding a major fragment with a mass identical to that of the theoretic fragment expected for canavanine, 177.098 Da (M$^+$).

Precipitation using excess ethanol yielded a solid containing 90% and 96% (w/w) canavanine and total amino acids, respectively. Alternatively, crystallization using a smaller amount of ethanol yielded white crystals containing 97% and 100% (w/w) canavanine and total amino acids, respectively, the remaining 3% corresponding to glycine. Table 4 and Fig. 3 show the amino acid composition and the reverse phase HPLC profile of these two canavanine preparations.

In conclusion, *V. disperma* could represent a very good source of

have also been purified using cationic resins (Bell, 1962; Rao et al., 1963; Bass et al., 1995). Amberlite IR-120 is an example of such cationic resins. As shown in Fig. 2, binding of free amino acids to
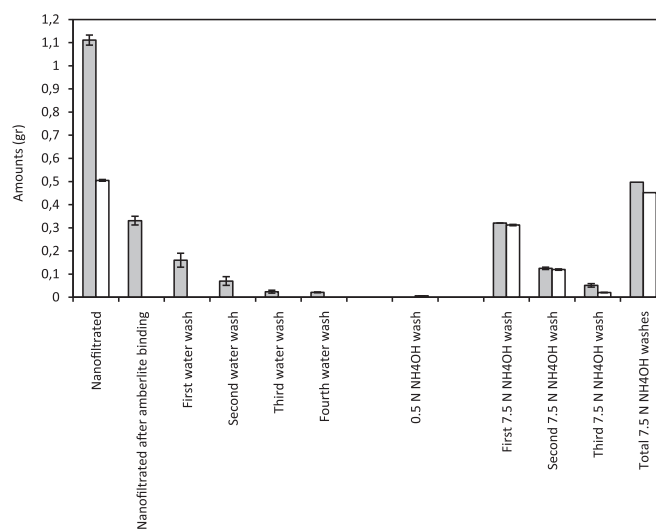
Table 3
Free amino acid composition of the fractions resulting from washing the resin using 0.5 N NH_4OH and 7.5 N NH_4OH. Data represent g free amino acid/100 g total free amino acids, average $\pm$ standard deviation of three determinations.

	0.5 N NH_4OH	7.5 N NH_4OH First wash	7.5 N NH_4OH Second wash	7.5 N NH_4OH Third wash
Asp	22.99 $\pm$ 0.10	2.13 $\pm$ 0.02	2.44 $\pm$ 0.11	2.42 $\pm$ 0.01
Glu	13.99 $\pm$ 0.13	2.76 $\pm$ 0.04	2.85 $\pm$ 0.16	2.88 $\pm$ 0.01
Asn	22.95 $\pm$ 0.00	2.96 $\pm$ 0.31	3.06 $\pm$ 0.23	3.02 $\pm$ 0.06
Ser	3.62 $\pm$ 0.10	0.45 $\pm$ 0.03	0.35 $\pm$ 0.06	0.32 $\pm$ 0.03
Gln	0.20 $\pm$ 0.00	0.08 $\pm$ 0.00	0.07 $\pm$ 0.01	0.07 $\pm$ 0.02
His	3.35 $\pm$ 0.04	0.88 $\pm$ 0.00	1.07 $\pm$ 0.12	1.13 $\pm$ 0.03
Gly	2.78 $\pm$ 0.18	0.77 $\pm$ 0.01	0.57 $\pm$ 0.01	0.63 $\pm$ 0.07
Thr	14.47 $\pm$ 0.09	12.07 $\pm$ 0.02	10.88 $\pm$ 0.88	10.86 $\pm$ 0.18
Arg	0.00 $\pm$ 0.00	1.25 $\pm$ 0.06	2.03 $\pm$ 0.27	4.16 $\pm$ 0.44
Ala	8.63 $\pm$ 0.02	2.71 $\pm$ 0.07	2.76 $\pm$ 0.44	2.80 $\pm$ 0.15
Pro	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
Tyr	0.35 $\pm$ 0.02	0.43 $\pm$ 0.01	0.52 $\pm$ 0.03	0.56 $\pm$ 0.07
Val	0.91 $\pm$ 0.07	0.28 $\pm$ 0.03	0.35 $\pm$ 0.04	0.20 $\pm$ 0.02
Met	0.00 $\pm$ 0.00	0.11 $\pm$ 0.01	0.20 $\pm$ 0.07	0.16 $\pm$ 0.01
Cys	0.88 $\pm$ 0.01	0.24 $\pm$ 1.31	0.26 $\pm$ 0.02	0.23 $\pm$ 0.00
Ile	0.23 $\pm$ 0.33	0.14 $\pm$ 0.01	0.13 $\pm$ 0.01	0.13 $\pm$ 0.00
Leu+Trp	0.41 $\pm$ 0.38	0.95 $\pm$ 0.00	0.96 $\pm$ 0.02	0.87 $\pm$ 0.01
Phe	0.18 $\pm$ 0.13	0.44 $\pm$ 0.00	0.49 $\pm$ 0.02	0.57 $\pm$ 0.01
Lys	0.00 $\pm$ 0.00	0.11 $\pm$ 0.00	0.10 $\pm$ 0.00	0.11 $\pm$ 0.01
Canavanine	0.00 $\pm$ 0.00	72.12 $\pm$ 1.30	70.73 $\pm$ 4.09	68.90 $\pm$ 0.54

Table 4
Free amino acid compositions of the fractions resulting from canavanine purification. Data represent g free amino acids/100 g of total free amino acids, average $\pm$ standard deviation of three determinations.

Amino acids	Nanofiltrate	Amberlite bound	Precipite	Cristals
Asp	2.32 $\pm$ 0.13	2.36 $\pm$ 0.03	1.27 $\pm$ 0.04	0.00 $\pm$ 0.00
Glu	3.49 $\pm$ 0.14	3.13 $\pm$ 0.01	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
Asn	3.51 $\pm$ 0.30	2.99 $\pm$ 0.02	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
Ser	0.46 $\pm$ 0.03	0.52 $\pm$ 0.01	0.18 $\pm$ 0.02	0.00 $\pm$ 0.00
Gln	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
His	1.02 $\pm$ 0.07	1.03 $\pm$ 0.01	0.45 $\pm$ 0.01	0.00 $\pm$ 0.00
Gly	0.70 $\pm$ 0.06	0.83 $\pm$ 0.06	0.30 $\pm$ 0.01	3.03 $\pm$ 0.01
Thr	12.76 $\pm$ 0.30	11.60 $\pm$ 0.16	3.77 $\pm$ 0.13	0.00 $\pm$ 0.00
Arg	1.87 $\pm$ 0.23	1.32 $\pm$ 0.08	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
Ala	2.33 $\pm$ 0.10	2.11 $\pm$ 0.15	0.57 $\pm$ 0.06	0.00 $\pm$ 0.00
Pro	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
Tyr	0.57 $\pm$ 0.00	0.63 $\pm$ 0.45	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
Val	0.35 $\pm$ 0.04	0.32 $\pm$ 0.01	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
Met	0.17 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
Cys	0.28 $\pm$ 0.00	0.15 $\pm$ 0.01	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
Ile	0.15 $\pm$ 0.04	0.18 $\pm$ 0.01	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
Leu+Trp	0.72 $\pm$ 0.01	0.64 $\pm$ 0.10	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
Phe	0.45 $\pm$ 0.00	0.46 $\pm$ 0.01	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
Lys	0.12 $\pm$ 0.01	0.18 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
Canavanine	69.68 $\pm$ 0.51	71.51 $\pm$ 0.65	93.26 $\pm$ 0.32	96.94 $\pm$ 0.21

Fig. 3. HPLC-C_{18} profile of the solids that resulted from precipitation (A) and crystalization (B) using ethanol.

Fig. 4. Free amino acids (gray bars) and canavanine (black bars) in the fractions produced during the canavanine purification process. ($x \pm$ sd, $n=3$).

canavanine. Its seeds have as much canavanine as *C. ensiformis*. The procedure described in this article allows for purification of this amino acid from *V. disperma* seeds to different degrees of purity without requiring specialized equipment or reagents, resulting in several fractions with potential nutritional and pharmaceutical applications (Fig. 4). Some of these fractions are rich in components in addition to canavanine, that could be of interest for particular applications. Thus, the solid residue resulting from acid amino acid extraction is rich in storage proteins, the retentate resulting from nanofiltration is rich in functional polyphenols and albumins including lectins and protease inhibitors, and the fractions resulting from washing the resin with water are rich in soluble monosaccharides. This procedure might also facilitate the promotion of other related *Vicia* species that are also rich in canavanine, including old crops that were farmed in the past such as *Vicia cracca* and *Vicia benghalensis*. This could have a very favorable impact in biodiversity and protection of phytogenetic resources.
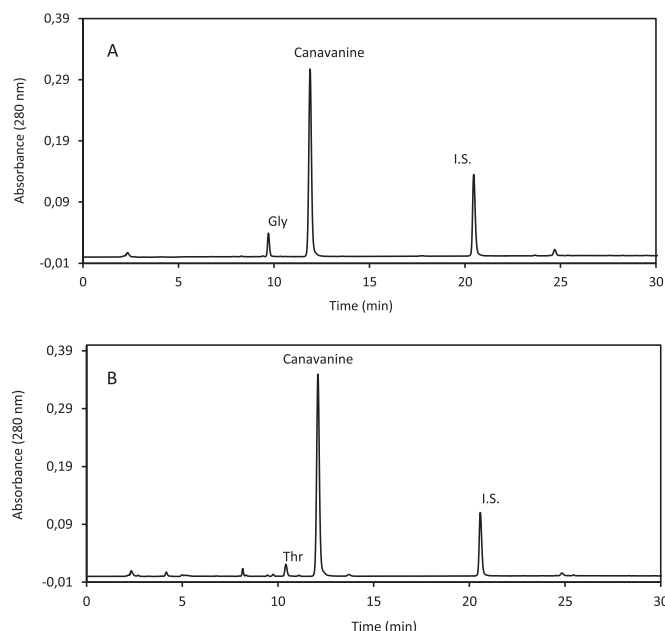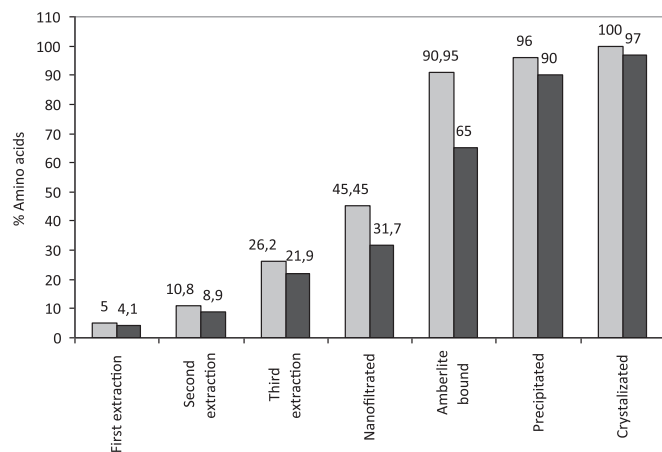
Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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