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PURIFICATION OF FREE ARGININE FROM CHICKPEA (CICER ARIETINUM) SEEDS.

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Abstract.

Chickpea is a grain legume widely consumed in the Mediterranean Region and other parts of the world. Chickpea seeds are rich in proteins but they also contain a substantial amount of free amino acids, especially arginine. Hence chickpea may represent a useful source of free amino acids for nutritional or pharmaceutical purposes. Arginine is receiving great attention in recent years because it is the substrate for the synthesis of nitric oxide, an important signalling molecule involved in numerous physiological and pathological processes in mammals. In this work we describe a simple procedure for the purification of arginine from chickpea seeds using nanofiltration technology and an ion exchange resin, Amberlite IR-120. Arginine was finally purified through precipitation or cristalization yielding preparations with purities of 91 and 100%, respectively. Chickpea may represent an affordable green source of arginine, and a useful alternative to production through fermentation or protein hydrolysis.

Key words: Chickpea, seed flour, free amino acids, arginine.

Running title: Purification of free arginine from chickpea.

1. Introduction.

Chickpea (*Cicer arietinum* L.) is a staple food in large areas of the world, including the Mediterranean Region, the Middle East, India, and South and Central America. It is a grain legume rich in proteins and carbohydrates (Sánchez-Vioque, Clemente, Vioque, Bautista, & Millán, 1999), and also in secondary components such as polyphenols, alkaloids and free amino acids. Free amino acids are among the most abundant secondary compounds in some legume seeds. For example, they are 3.5 times more abundant than polyphenols in lentil seeds (*Lens culinaris*) (unpublished results). Free amino acids may function in the seeds as phytoalexins, storage of nitrogen, and in metabolic signalling (Bell, 2003). Their abundance in legume seeds may be influenced by environmental factors such as temperature, hydric stress, salt stress, availability of nitrogen, and light (Reggiani, Cantu, & Brambilla, 1988; Wallace, Secor, & Schrader, 1984; Roosens, Thu, Iskandar, & Jacobs, 1998; Colling, Stander, & Makunga, 2010).

Free amino acids in legume seeds include both proteic and nonproteic amino acids (Bell, 2003). Chickpea seeds are particularly rich in free arginine (unpublished results). This amino acid is considered semi essential or conditionally essential because, although animals can synthesize it, exclusion of arginine from the diet results in a sub-optimal weight gain (Tapiero, Mathé, Couvreur, & Tew, 2002). In animals, arginine is an intermediate in the urea cycle and is also the substrate for the synthesis of many nitrogen-containing compounds including nitric oxide, ornithine and

polyamines (Morris, 2006). Arginine is directly implicated in ATP synthesis, cellular proliferation, vasodilatation, neurotransmission, calcium release, and immunity (Nieves, & Langkamp-Henken, 2002). In particular, the role of arginine in the synthesis of nitric oxide has stimulated research in this amino acid because nitric oxide is involved in many physiological and pathological processes. The therapeutic properties of arginine in the treatment of diseases related with shortage of NO, including nervous, muscular, circulatory, respiratory, digestive, urinary, reproductive, endocrine, and immune systems have been studied as reported by Ignarro (1989). Enteral and parenteral administration of arginine decreases the probabilities of suffering cardiovascular diseases (Flynn, Meininger, Haynes, & Wu, 2002), lower glucose concentration in blood, and improves reproductive, pulmonary, renal, gastrointestinal, hepatic, and immune functions (Tapiero, Mathé, Couvreur, & Tew, 2002)

Although free arginine, as most amino acids, is industrially produced by fermentation using microorganisms (Utagawa, 2004), plants rich in free arginine such as chickpea seeds may represent a very afordable “green alternative”. This is especially true for inexpensive, readily available sources of material, such as chickpeas that are damaged during harvesting and processing. These chickpeas, representing about 20 % of chickpea production, are considered as a by-product and are sold at low prices for feeding livestock, (Ulloa, Valencia, & Garcia, 1988).

The objective of this work was to design an improved purification method to efficiently produce arginine rich extracts that could be used as ingredients for foods, nutritional supplements, and pharmaceuticals.

1. Material and methods.

1.1. Plant material.

Chickpea (*Cicer arietinum*), soy (*Glycine max*), broad bean (*Vicia faba*), lentil (*Lens culinaris*) and common bean (*Phaseolus vulgaris*) seeds were purchased in a local market.

2.2. Extraction of free amino acids.

Seeds were milled using a domestic blender. Flour was suspended in water and taken to pH 4.3 with 1 N HCl for extraction under continuous stirring for 1 hour. Extracts were centrifuged at 15.000 g for 20 min, and pellets were extracted two more times as described above. The three resulting supernatants were pooled and used for the purification of arginine.

2.3. Purification of arginine.

Aliquots of the extracts prepared as described above were concentrated to half of their volume using an Amicon cell filtration unit (Millipore, MA, USA) equipped with a 200 Da nanofiltration membrane (Koch Membrane Systems, MA, USA). The permeate, containing free amino acids and other low molecular weight molecules, was incubated with Amberlite IR-120 (Fluka, MO, USA) to further purify arginine. Amberlite IR-120 is a strong cationic resin that binds arginine at acidic pH. The resin was pre-conditioned by treatment with ten volumes of HCl 2 N for two hours, ten volumes of NaOH 2 N for two hours, ten volumes of HCl 2 N for two additional hours, and it was finally washed four times with water. The free amino acid permeate (1 mL) was

incubated with 1 mL resin, in 10 mL water taken to pH 2, under continuous stirring 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 ten volumes of pH 2 water. Acidic amino acids were then released by washing with 10 volumes NH_4OH 0.5 N for 30 min, and finally arginine was recovered by washing three times with 10 volumes NH_4OH 7.5 N for 30 min. The combined washes were taken to dryness in a speed vacuum and redissolved in the minimum volume of water possible. Solid matter determinations were carried out after drying aliquots at 120° C overnight.

2.4. Precipitation and crystallization.

Precipitation was carried out by addition of excess ethanol, at least four times the volume of extract, and the precipitate was recovered by centrifugation at 15.000 g for 15 min. Crystallization was carried out by addition of the same volume of ethanol and letting the resulting mixture rest for 24 hours at 4º C. Crystals were recovered by centrifugation at 15.000 g for 15 min.

2.5. Amino acids analysis.

Amino acids were analyzed by RP-HPLC after derivatization with diethyl ethoxymethylenemanolate, and determined according to the method described by Alaiz, Navarro, Giron, & Vioque (1992), using D, L α–aminobutyric acid as internal standard and a Novapack C_{18} column (300 x 3.9 mm i.d., 4 μm, Waters). 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).

2.6. Soluble sugars determination.

144 Soluble sugars were measured according to Dubois, Gilles, Hamilton, Reber, &
145 Smith (1956), using a standard curve of glucose.
146 *2.7. FPLC gel filtration chromatography.*
147 Samples were analyzed by gel filtration chromatography using a Superdex-
148 peptide column coupled to a FPLC AKTA-purifier system. The eluent used was 0.75 M
149 ammonium bicarbonate at a flow rate of 0.5 mL/min. Elution was monitored at 214 nm
150 and the molecular masses of eluted compounds were determined by comparison with
151 the following molecular weights standards from Pharmacia: blue dextran (2000 kDa),
152 cytochrome C (12500 Da), aprotinin (6512 Da), bacitracin (1450 Da), cytidine (246 Da)
153 and glycine (75 Da).
154
155 **2. Results and discussion.**
156 Many legumes store free amino acids in their seeds. As an example, figure 1
157 shows total free amino acids and free arginine contents in the seeds of five of the most
158 popular grain legumes, which accumulate in particular large amounts of arginine. This
159 is especially the case of chickpea, with arginine representing 53 % of total free amino
160 acids. This is in contrast to total arginine content in chickpea, which represents 10 % of
161 all proteic and non-proteic amino acids. Thus, chickpea could be a good source of
162 arginine provided that an affordable purification protocol could be implemented to
163 purify arginine from the free amino acids pool. Other free amino acids at substantial
164 levels in chickpea seeds are glutamic acid, aspartic acid, leucine and tryptophan (Table
165 1). Extraction of free amino acids was carried out at pH 4.3 in order to minimize
166 protein solubilization, since this pH corresponds to the isolectric point of storage
167 proteins in chickpea seeds (Sanchez-Vioque et al., 1999). Figure 2A shows the FPLC gel

168 filtration profile of these extracts. Although globulins are insoluble at this pH, other
169 components including albumins, polyphenols and sugars are solubilized in addition to
170 free amino acids. Similarly, during the process of production of chickpea protein
171 concentrates and isolates, the aqueous fraction generated after isolectric precipitation
172 of proteins at pH 4.3 (Sanchez-Vioque et al., 1999) may be used for the production of
173 an arginine rich fraction.

174 Nanofiltration using a 200 Da membrane allows for separation of free amino
175 acids from higher molecular weight components such as proteins, polysaccharides,
176 polyphenols and fibre. Figures 2B and 2C show the FPLC gel filtration profile of the
177 nanofiltration retentate and permeate, respectively.

178 Ion exchange resins are frequently used in the purification of amino acids in
179 industrial settings (Leuchtenberger, Hutmacher, & Drauz, 2005). Strong cationic resins
180 are especially useful in the purification of amino acids with basic R-functional groups
181 such as the guanidinium group in arginine (Utagawa, 2004). These resins have been
182 used in the past for the purification of free amino acids from legume seeds, including
183 homoarginine from *Lathyrus sativus* (Rao, Ramachandran, & Adiga, 1963) and *L. cicera*
184 (Bell, 1962), and canavanine from *Canavalia ensiformis* (Bass, Harper, Rosenthal,
185 Phuket, & Crooks, 1995). Specifically Amberlite IR-120 resin has been used for the
186 determination of arginine in grape juice (Li, Liang, Feng, Liu, & Wang, 2008). Figure 3
187 shows the total mass, soluble sugars and free amino acids contents in a representative
188 experiment of purification arginine from chickpea seeds using Amberlite IR-120 resin.
189 Free amino acids and soluble sugars represented 2.6 % and 27.8 % of total mass in the
190 permeate from nanofiltration, respectively (Figure 3 first group of bars). All free amino
191 acids were bound to the resin after incubation for 30 min, while 58% of total mass and

80% of soluble sugars remained in the soluble phase (Figure 3, second group of bars). Washes using pH 2 water allowed for removal of the remaining sugars and unidentified components bound to the resin (Figure 3, third group of bars) without any losses of bound amino acids (Table 3). The resin was also washed using 0.5 N NH_4OH in order to remove poorly bound acidic amino acids (Figure 3, fourth group of bars). Aspartic and glutamic acid accounted for 85% of the eluted amino acids (Table 2). Finally, arginine was recovered from the resin by washing three times with 7.5 N NH_4OH (Figure 3, fifth group of bars, Tables 2 and 3).

The final washes were pooled and taken to dryness to yield a brownish syrup that was used for precipitation or crystallization in order to further purify arginine. Precipitation yielded a white pellet containing 90.7 % arginine (Table 2). The exact mass of this precipitate was 175.1191 (M^+) similar to 175.1190 (M^+) of the pure arginine. Crystallization yielded white crystals containing pure arginine (Table 2). The exact mass of this pellet was 175.1190 (M^+) identical to the theoretic mass expected for pure arginine. While crystallization would be the method of choice to produce arginine of the highest purity, precipitation might be preferred when a higher yield is required. Thus, although the precipitated preparation is only 90.7 % arginine, the yield of precipitation is 38% vs. 22% for crystallization.

In conclusion, although the presence of free amino acids in seed legumes has been known for a long time the contents and potential interest of free amino acids in pulses such as chickpea have not received much attention. We show that chickpea is a potential source of free amino acids, especially arginine, that can be easily purified, as a green alternative to production by fermentation.

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Table 1. Total and free amino acid composition in chickpea seeds. Results, expressed as g / 100 g amino acids, are the average ± sd of two determinations.

	Total amino acids	Free amino acids
Asp	13.5[a] ± 0.1	5.7 ± 0.5
Glu	18.7[b] ± 0.1	20.3 ± 1.6
Asn	---	3.9 ± 0.5
Ser	5.9 ± 0.1	0.9 ± 0.2
Gln	---	0.0 ± 0.0
His	2.3 ± 0.1	0.5 ± 0.0
Gly	4.3 ± 0.1	1.6 ± 0.0
Thr	4.1 ± 0.0	0.7 ± 0.0
Arg	10.4 ± 0.1	53.5 ± 5.0
Ala	4.6 ± 0.0	0.9 ± 0.0
Pro	1.8 ± 0.1	0.0 ± 0.0
Tyr	2.3 ± 0.0	0.5 ± 0.0
Val	4.1 ± 0.0	0.5 ± 0.0
Met	0.8 ± 0.0	0.5 ± 0.0
Cys	1.1 ± 0.1	0.0 ± 0.0
Ile	3.6 ± 0.1	0.2 ± 0.0
Trp	0.8 ± 0.0	---
Leu	8.0 ± 0.1	7.3[c] ± 0.7
Phe	6.0 ± 0.1	1.4 ± 0.0
Lys	7.2 ± 0.1	1.8 ± 0.2

293 [a]Asp + Asn. [b]Glu + Gln. [c]Leu + Trp.

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297 **Table 2.** Amino acid composition (g / 100 g amino acids) of chickpea permeate

298 and washes of the Amberlite resin using 0.5 N NH_4 and concentrated NH_4 (7.5 N).

299 Results are the average ± sd of two determinations.

300

	Permeate	0.5 N NH_4OH wash	7.5 NH_4OH first wash	7.5 NH_4OH second wash	7.5 NH_4OH third wash	Precipited Arg	Crystalized Arg
Asp	5.29 ± 0.16	27.51 ± 0.09	4.19 ± 0.02	3.23 ± 0.03	1.66 ± 0.00	2.91 ± 0.02	n.d.
Glu	29.40 ± 0.53	57.31 ± 0.09	31.01 ± 0.20	23.69 ± 0.20	15.50 ± 0.10	6.37 ± 0.08	n.d.
Asn	3.45 ± 0.08	4.01 ± 0.03	3.64 ± 0.07	2.61 ± 0.10	1.72 ± 0.02	n.d.	n.d.
Ser	1.36 ± 0.07	1.75 ± 0.00	1.69 ± 0.01	1.14 ± 0.10	0.71 ± 0.01	n.d.	n.d.
Gln	0.26 ± 0.04	0.22 ± 0.00	0.14 ± 0.00	0.12 ± 0.00	0.08 ± 0.01	n.d.	n.d.
His	0.43 ± 0.05	0.38 ± 0.03	0.15 ± 0.04	0.09 ± 0.01	0.06 ± 0.00	n.d.	n.d.
Gly	1.89 ± 0.04	1.24 ± 0.02	2.11 ± 0.00	1.51 ± 0.00	0.93 ± 0.01	n.d.	n.d.
Thr	1.92 ± 0.15	2.43 ± 0.01	1.69 ± 0.01	1.27 ± 0.01	0.76 ± 0.00	n.d.	n.d.
Arg	30.05 ± 0.89	n.d.	25.37 ± 0.28	38.89 ± 0.26	55.99 ± 0.37	90.73 ± 0.07	102.87 ± 0.66
Ala	3.35 ± 0.19	1.23 ± 0.02	2.86 ± 0.10	1.74 ± 0.08	0.94 ± 0.13	n.d.	n.d.
Pro	n.d.*	1.60 ± 0.13	2.17 ± 0.14	1.42 ± 0.07	0.32 ± 0.06	n.d.	n.d.
Tyr	1.77 ± 0.04	0.29 ± 0.01	1.94 ± 0.02	1.81 ± 0.00	1.58 ± 0.02	n.d.	n.d.
Val	0.95 ± 0.12	0.74 ± 0.06	1.94 ± 0.23	1.24 ± 0.15	0.71 ± 0.24	n.d.	n.d.
Met	0.05 ± 0.07	n.d.	0.47 ± 0.04	0.29 ± 0.01	n.d.	n.d.	n.d.
Cys	0.04 ± 0.05	n.d.	0.25 ± 0.01	0.14 ± 0.00	n.d.	n.d.	n.d.
Ile	0.92 ± 0.02	0.20 ± 0.00	1.11 ± 0.01	0.86 ± 0.01	0.54 ± 0.02	n.d.	n.d.
Leu + Trp	14.69 ± 0.29	0.78 ± 0.00	14.69 ± 0.13	15.97 ± 0.08	15.90 ± 0.07	n.d.	n.d.
Phe	2.68 ± 0.07	0.31 ± 0.01	3.53 ± 0.03	3.19 ± 0.00	2.19 ± 0.12	n.d.	n.d.
Lys	0.71 ± 0.04	n.d.	1.05 ± 0.01	0.78 ± 0.00	0.42 ± 0.00	n.d.	n.d.

301

302 *n.d.: not detected.

Table 3. Detailed total mass, soluble sugars, and amino acid contents, of a representative experiment, in the Amberlite washes using pH 2 water, 0.5 N NH_4OH and 7.5 N NH_4OH. Results are the average ± sd of three determinations.

	Total mass (mg)	Sugars (mg)	Amino acids (mg)
First pH 2 water wash	141.9 ± 4.7	22.9 ± 2.0	tr[1]
Second pH 2 water wash	40.8 ± 2.9	18.4 ± 1.7	tr
Third pH 2 water wash	11.0 ± 1.0	7.2 ± 0.3	tr
Fourth pH 2 water wash	5.2 ± 0.0	3.1 ± 1.4	tr
Total pH 2 water washes	**198.9**	**51.6**	**tr**
0.5 N NH_4OH wash	7.2 ± 0.0	tr	1.7 ± 0.1
First 7.5 N NH_4OH wash	68.0 ± 0.0	tr	29.9 ± 0.6
Second 7.5 N NH_4OH wash	33.0 ± 5.1	tr	6.9 ± 0.2
Third 7.5 N NH_4OH wash	nd[2]	tr	2.5 ± 0.1
Total 7.5 N NH_4OH washes	**101**	**tr**	**39.3**

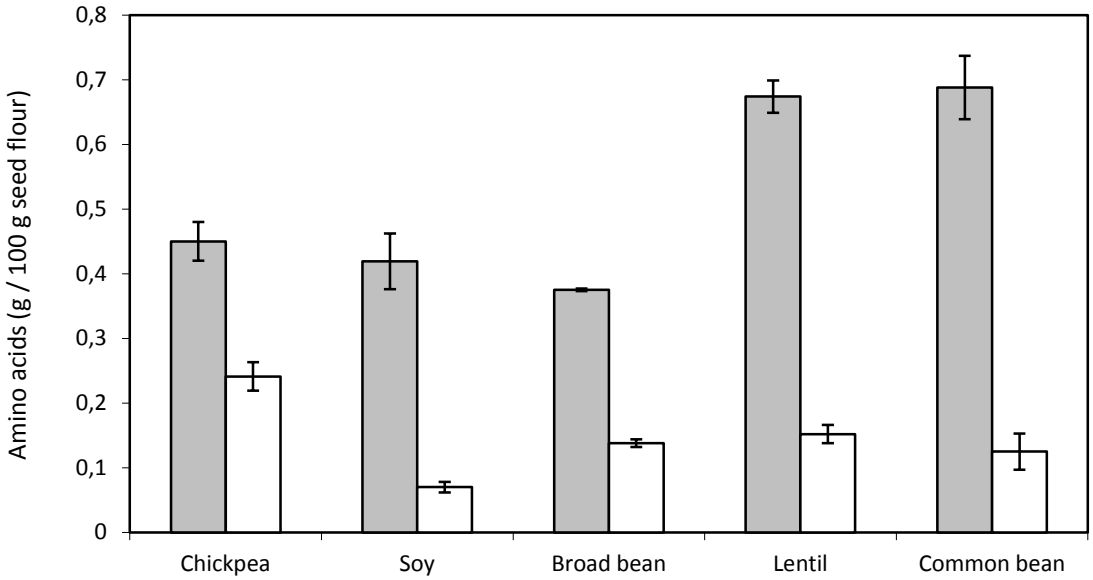
[1]traces, [2]not determined.

Figure legends.

Figure 1. Free amino acids (full bars) and arginine (open bars) contents (g / 100 g seed flour) in five commercial grain legumes. Results are the average ± sd of three determinations.

Figure 2. FPLC gel filtration profile of **A)** chickpea extracts, **B)** nanofiltration **r**etentate, **C)** nanofiltration permeate. Dashed line in Figure 1A represents the cut-off molecular weigth (200 Da) of the nanofiltration membrane.

Figure 3. Balance of total mass (open bars), soluble sugars (grey bars) and free amino acids (black bars) in a representative experiment of arginine purification using Amberlite IR-120 resin. Results are the average ± sd of three determinations.



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355 **FIGURE 1**

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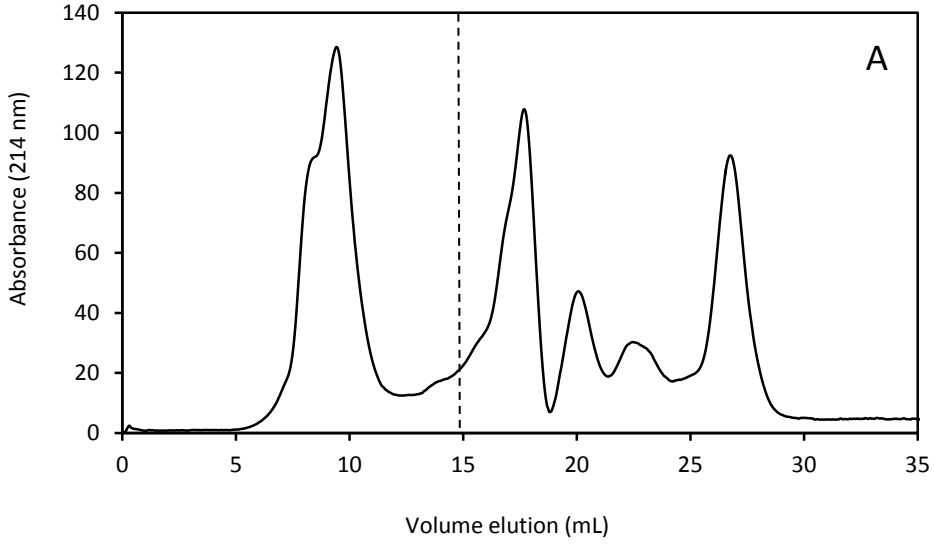

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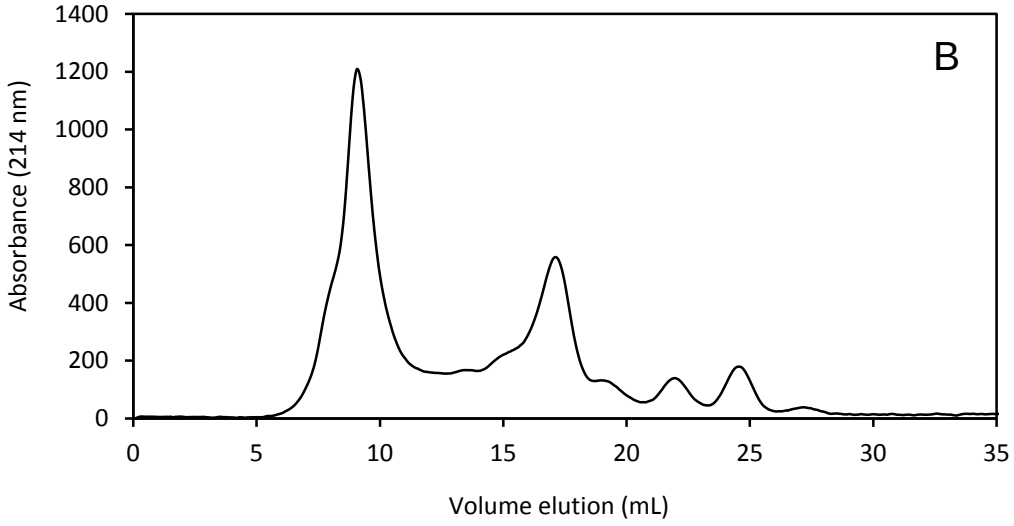

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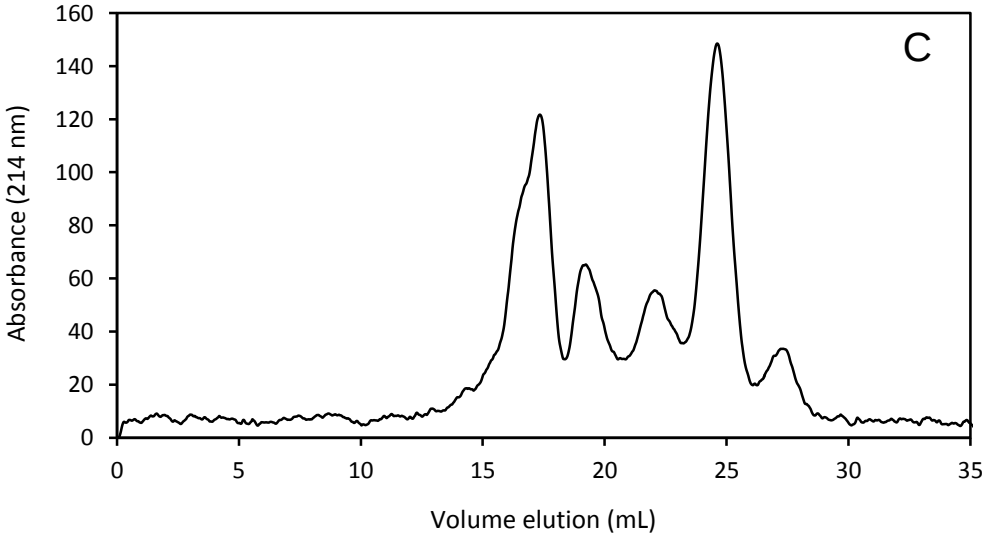

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362 **FIGURE 2**

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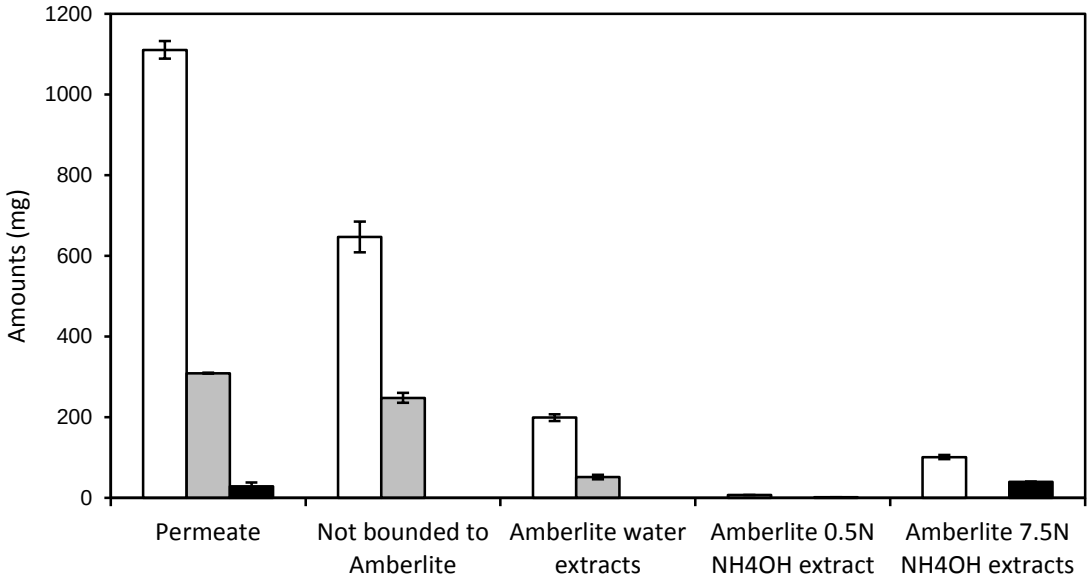

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366 **FIGURE 3**