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

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28 **Abstract.**

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

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

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50 **Running title:** Purification of free arginine from chickpea.

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52 **1. Introduction.**

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59 times more abundant than polyphenols in lentil seeds (*Lens culinaris*) (unpublished  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

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

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

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

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

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100 **1. Material and methods.**

101 *1.1. Plant material.*

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

105 *2.2. Extraction of free amino acids.*

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

111 *2.3. Purification of arginine.*

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

121 incubated with 1 mL resin, in 10 mL water taken to pH 2, under continuous stirring at  
122 room temperature for 30 min, which led to binding of all free amino acids. The resin  
123 was then washed four times for 15 min with ten volumes of pH 2 water. Acidic amino  
124 acids were then released by washing with 10 volumes NH<sub>4</sub>OH 0.5 N for 30 min, and  
125 finally arginine was recovered by washing three times with 10 volumes NH<sub>4</sub>OH 7.5 N  
126 for 30 min. The combined washes were taken to dryness in a speed vacuum and  
127 redissolved in the minimum volume of water possible. Solid matter determinations  
128 were carried out after drying aliquots at 120° C overnight.

#### 129 *2.4. Precipitation and crystallization.*

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

#### 135 *2.5. Amino acids analysis.*

136 Amino acids were analyzed by RP-HPLC after derivatization with diethyl  
137 ethoxymethylenemanolate, and determined according to the method described by  
138 Alaiz, Navarro, Giron, & Vioque (1992), using D, L α-aminobutyric acid as internal  
139 standard and a Novapack C<sub>18</sub> column (300 x 3.9 mm i.d., 4 μm, Waters). Electro-spray-  
140 ionization high-resolution mass spectra were recorded with a micrOTOF-QII High  
141 Resolution-of-Flight mass spectrometer (UHR-TOF) with qQ-TOF geometry (Bruker  
142 Daltonic, Bremen, Germany).

#### 143 *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).

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## 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 isoelectric 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 isoelectric 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



192 80% of soluble sugars remained in the soluble phase (Figure 3, second group of bars).  
193 Washes using pH 2 water allowed for removal of the remaining sugars and unidentified  
194 components bound to the resin (Figure 3, third group of bars) without any losses of  
195 bound amino acids (Table 3). The resin was also washed using 0.5 N  $\text{NH}_4\text{OH}$  in order to  
196 remove poorly bound acidic amino acids (Figure 3, fourth group of bars). Aspartic and  
197 glutamic acid accounted for 85% of the eluted amino acids (Table 2). Finally, arginine  
198 was recovered from the resin by washing three times with 7.5 N  $\text{NH}_4\text{OH}$  (Figure 3, fifth  
199 group of bars, Tables 2 and 3).

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

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

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228

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289 **Table 1.** Total and free amino acid composition in chickpea seeds. Results,  
290 expressed as g / 100 g amino acids, are the average  $\pm$  sd of two determinations.

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

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293 <sup>a</sup>Asp + Asn. <sup>b</sup>Glu + Gln. <sup>c</sup>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<sub>4</sub> and concentrated NH<sub>4</sub> (7.5 N).

299 Results are the average ± sd of two determinations.

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	Permeate	0.5 N NH <sub>4</sub> OH wash	7.5 NH <sub>4</sub> OH first wash	7.5 NH <sub>4</sub> OH second wash	7.5 NH <sub>4</sub> OH third wash	Precipitated 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.

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302 \*n.d.: not detected.

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307 **Table 3.** Detailed total mass, soluble sugars, and amino acid contents, of a  
308 representative experiment, in the Amberlite washes using pH 2 water, 0.5 N NH<sub>4</sub>OH  
309 and 7.5 N NH<sub>4</sub>OH. Results are the average ± sd of three determinations.

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	Total mass (mg)	Sugars (mg)	Amino acids (mg)
First pH 2 water wash	141.9 ± 4.7	22.9 ± 2.0	tr <sup>1</sup>
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
<b>Total pH 2 water washes</b>	<b>198.9</b>	<b>51.6</b>	<b>tr</b>
0.5 N NH <sub>4</sub> OH wash	7.2 ± 0.0	tr	1.7 ± 0.1
First 7.5 N NH <sub>4</sub> OH wash	68.0 ± 0.0	tr	29.9 ± 0.6
Second 7.5 N NH <sub>4</sub> OH wash	33.0 ± 5.1	tr	6.9 ± 0.2
Third 7.5 N NH <sub>4</sub> OH wash	nd <sup>2</sup>	tr	2.5 ± 0.1
<b>Total 7.5 N NH<sub>4</sub>OH washes</b>	<b>101</b>	<b>tr</b>	<b>39.3</b>

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313 <sup>1</sup>traces, <sup>2</sup>not determined.

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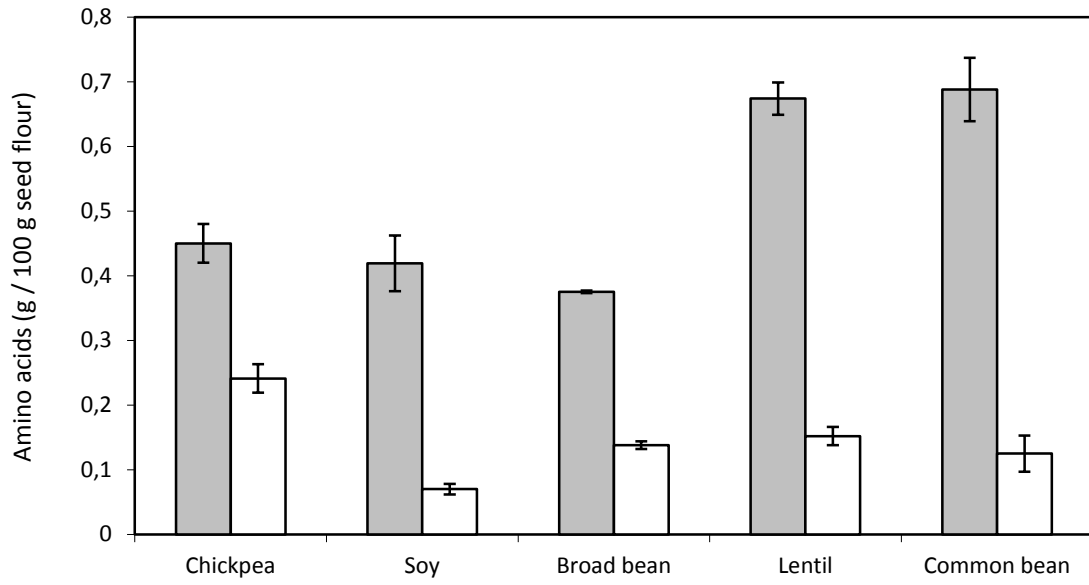
**Figure legends.**

**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  $\pm$  sd of three determinations.

**Figure 2.** FPLC gel filtration profile of **A)** chickpea extracts, **B)** nanofiltration retentate, **C)** nanofiltration permeate. Dashed line in Figure 1A represents the cut-off molecular weight (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  $\pm$  sd of three determinations.





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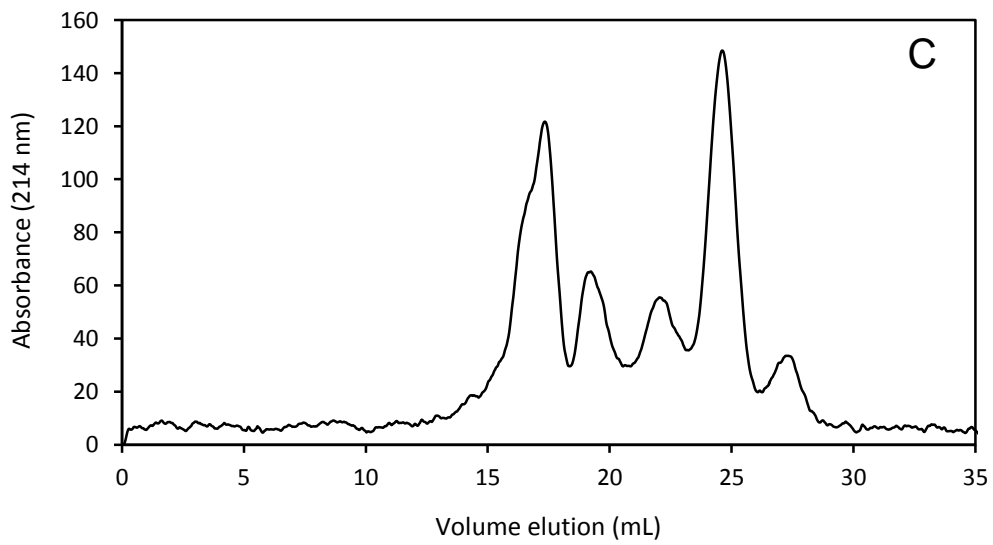
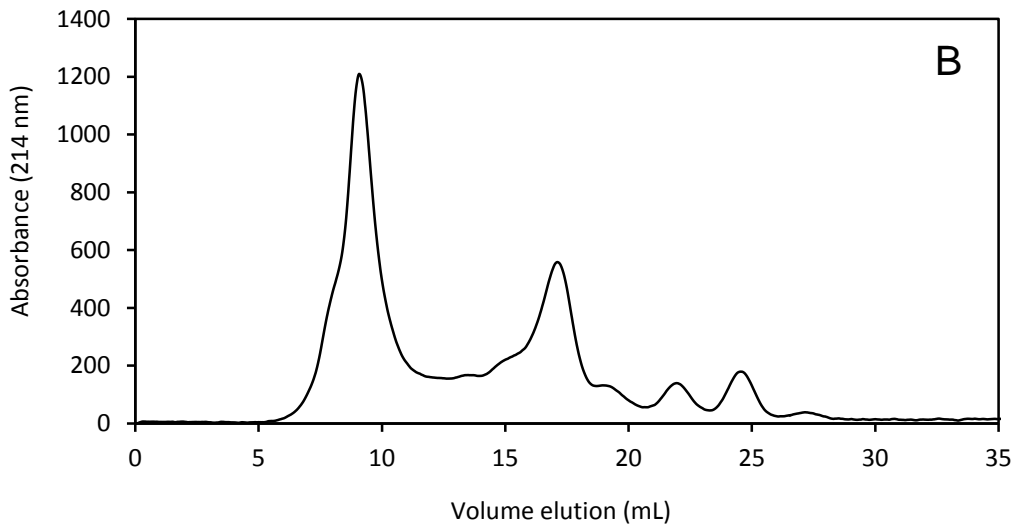
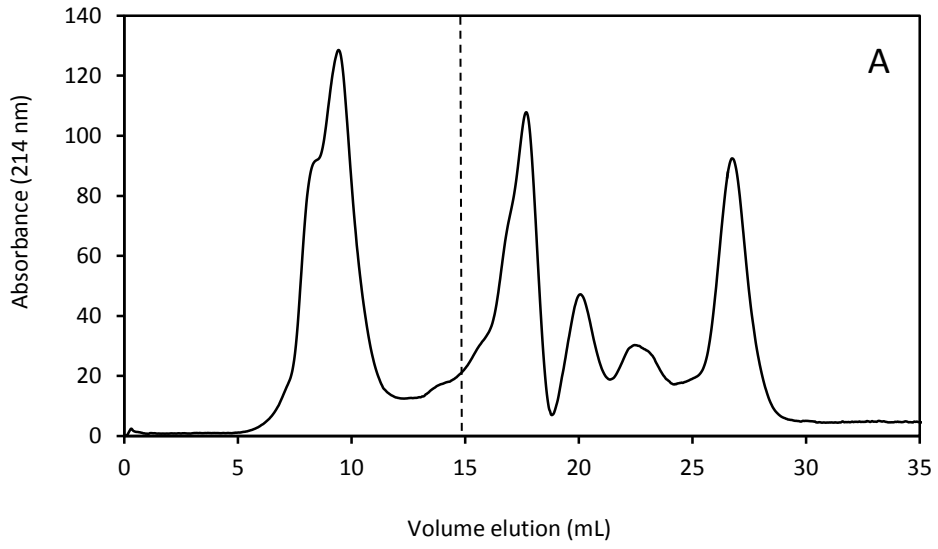
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**FIGURE 1**

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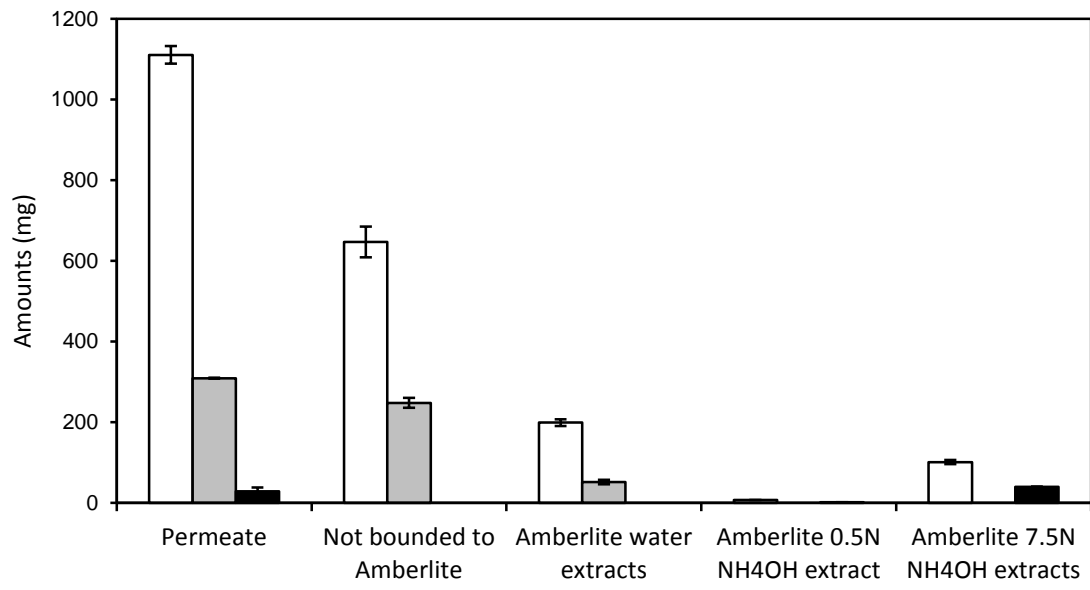
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**FIGURE 2**

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**FIGURE 3**