

Influence of heating on chemical composition, antioxidant activity and protein quality of an advanced line *Amaranthus cruentus* L. seed flour

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ABSTRACT

Amaranth is a pseudocereal of Andean origin, and compared to other crops, its seeds have a higher content of proteins, lipids and bioactive compounds of nutraceutical relevance.

The goal of the present work is to study the chemical composition, antioxidant activity and biological value of the protein of an advanced line *Amaranthus cruentus* L. seed flour (ACRU), compared with the same flour subjected to thermal treatment (90 °C, 1 h). Regarding the proximal chemical composition, the protein and lipid contents stand out, reaching values of 19.59 g % and 7.47 g %, reflecting an increase of 17% and 50% in the treated sample, respectively. A significant increase ($p < 0.05$) is observed in the ash content, as well as in the content of the main elements of nutritional interest, of the treated samples. The anti-nutrients values are within the acceptable limits in all samples, and present an adequate content of total phenols, with an antioxidant activity highlighted by its free-radical scavenging capacity. In the biological tests, the Net Protein Utilization (NPU) presents lower values for the treated samples, the True Digestibility (tD) does not show significant differences, and the Biological Value (BV) turns out to be lower in the treated sample ($p < 0.05$). A significant hypotriglyceridemic effect is observed. The applied thermal treatment, even though increases the nutrients concentration and the total phenols, according to the biological tests, it decreases the protein quality. These are aspects that should be contemplated in the food technology to optimize the nutritional quality of this amaranth.

Keywords: Amaranth, Antioxidant capacity, Biological value, Chemical composition, Thermal treatment

Introduction

Amaranth is an indigenous plant from America that has been used for more than 4000 years. In the last decades, it has regained popularity due to beneficial nutritional aspects, its agronomic advantages, such as its wide adaptability, and the possibility of its use as horticultural, graniferous and fodder, as well as for being one of the most promising resources to contribute towards mitigating food deficit (Dodok, 1997).

The amaranth nutritional composition is distinguished by the protein and lipid contribution. The protein content is between 12 to 22% (Tosi et al. 2001; Escudero et al. 2004; Barba de la Rosa et al. 2009). The proteins quality depends on the composition of essential amino acids and digestibility. Protein digestibility, lysine availability and net protein utilization of the amaranth proteins are higher than of cereals, and similar to those of casein (Salcedo-Chávez et al. 2002). The constituent amino acids of food proteins are not always fully available, due to that the protein digestibility and amino acid adsorption can be incomplete. Thus, the thermal treatment and milling applied to the seeds for the production of flour can raise the foods nutritional quality by the denaturalization of its proteins and the digestibility increase (Giami et al. 2001; Sun et al. 2014).

In order to expand the biodiversity and increase the agronomical efficiency of crops in the Central - West region of Argentina, new improved varieties have been obtained, such as the Acru-G10/13II, the advanced line belonging to the *Amaranthus cruentus* L. species. The latter is characterized for expressing good yields and excellent adaptation; it presents dark green foliage, semi-compact to compact dark red panicle, and a height between 1.40 and 1.70 m. It has a total cycle of 115 days and an acceptable behavior against "stem borer" (*Conotrachelus spp.*). The goal of this study is to evaluate the effect of thermal treatment on the chemical composition, antioxidant activity and biological quality of a new amaranth variety seed flour.

Materials and Methods

Sample

Seeds of a new variety of *Amaranthus* (Acru-G10/13 II) were supplied by the Faculty of Agronomy and Veterinary, National University of Río Cuarto, Córdoba, Argentina (experimental crop from 2016 vintage).

Sample Treatment

Dried seeds were ground in a grain mill and sieved through a 200 µm diameter mesh. A flour portion was used untreated (ACRU) and another was exposed to 90°C for 1 hour in an air-current oven (Dalvo HER/F/I, Argentina) (ACRU treated). The flours obtained were kept in closed containers

protected from light, in a cool and dry environment until analysis.

Reagents

All reagents used were of analytical grade and acquired from Sigma (St. Louis, MO). All standard solutions were prepared using reagents of spectroscopic grade supplied by Merck (Darmstadt, Germany). Ultrapure water (18.2 MΩ cm) was used to prepare all standard and sample solutions.

Chemical Composition

The determination of moisture, ash, protein, total lipids and crude fiber was performed according to the methodology proposed by the AOAC (2012). Carbohydrates are determined by the following calculation:

$$\text{Carbohydrates} = 100 - (\% \text{ Ash}) - (\% \text{ Total Fat}) - (\% \text{ Moisture}) - (\% \text{ Protein})$$

The quantification of mineral elements was performed by Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-OES). The procedure was performed following the methodology used by Aguilar et al. (2011).

The antinutrients determined were: nitrates (Cataldo et al. 1975), hemagglutinin activity (lectins) (Das Gupta and Boroff 1968; Do Prado et al. 1980), saponins (WHO/PHARM/92559 1992; Duarte Correa and Carlsson, 1986), antitrypsin activity (Kakade et al. 1974), oxalic acid (AOAC 1995) and phytic acid (Rucci and Bertoni, 1974).

The extraction of total phenols was performed from a defatted sample with 1.2 M HCl in 50% methanol/water. The sample was heated at 90 °C for 3 h, cooled, and then diluted with methanol. The supernatant was used for the determination of total phenols and antioxidant activity (Vinson et al. 2001). The concentration of the obtained extract was 5 mg/mL. The determination of total phenols was performed using Folin Ciocalteu reagent with gallic acid as a standard. The absorbance was measured at 750 nm (UV-vis BeckmanDK-2^a). Results were expressed as mg/100 g of dry weight of gallic acid equivalent (Emmons et al. 2001).

Antioxidant Activity

DPPH Free Radical-Scavenging Assay. This spectrophotometric assay uses the stable radical DPPH (1,1-diphenyl-2-picrylhydrazyl) as a reagent (Burits and Bucar, 2000). Various concentrations of the extract in methanol were added to a 40 mg/L methanol solution of DPPH. The absorbance was read at 517 nm (UV-Vis Beckman DK-2a). Results were expressed as percentage (%) of radical scavenging activity (RSA).

Scavenging Activity against Nitric Oxide (NO Test). Nitric oxide (NO) was generated from sodium nitroprusside and measured by Griess reaction (Marcocci et al. 1994; Saija et al. 1999). Nitrite concentration was calculated by referring to the absorbance of standard solutions of sodium nitrite. Absorbance was measured in a spectrophotometer (UV-Vis Beckman DK-2a) at 542 nm. Results were expressed as percentage (%) of RSA with respect to blank.

β-Carotene–Linoleic Acid Assay

This assay involves measuring β -carotene bleaching, at 470 nm, resulting from the β -carotene oxidation by linoleic acid degradation products at 50 °C (Koleva et al., 2002). The absorbance at 470 nm was taken at time zero ($t = 0$), and measured every 15 min until the color of β -carotene disappeared in the control tubes ($t = 60$ min). A mixture prepared as above but without β -carotene served as blank. BHT (butylated hydroxytoluene) was included in the experiments as a positive control. Results were expressed as percentage (%) of RSA.

All determinations above were performed in triplicate.

Biological Assay

The Pprotein quality of the amaranths flour was measured by three different indices: Net Protein Utilization (NPU), true digestibility (tD), and Biological Value (BV) (Miller and Bender, 1955; Pellet and Young, 1980). Four groups of 30-day-old *Wistar* rats weighing 30–40 g (± 2 g weight difference) were used (six animals per group). One group received a protein-free diet, another received a control diet (casein), and the remaining groups received a diet with protein provided by the material under study. The preparation and composition of the diets were carried out according to AIN 93G (Table 1) and the NPU method at 10% of proteins and 7% of lipids (Reeves et al. 1993). The amount of flour incorporated in the diets to achieve 10 % of protein, was defined taking into account the protein content of the sources, ACRU: 19.59 % and ACRU treated: 23.54 %. Casein: 80 % purity. The amount of oil added to achieve 7 % of lipids, was defined taking into account the fat content obtained for the sources, ACRU: 7.47 % and ACRU treated: 14.70 %.

The animals were kept in individual suspended cages with screen bottoms. Temperature and relative humidity were held at 21 ± 1 °C and 60%, respectively. Lighting was controlled by alternating 12-h periods of light and darkness. All animals received potable water and food *ad libitum* for 14 days. Ingestion was recorded on days 3, 6, and 10; weight gain was

recorded at the end of the experiment. Feces were collected and weighed. After the experiment, the euthanasia of the animals was performed through a carbon dioxide chamber. Subsequently, a thoracic and abdominal incision was performed, and the rats were weighed and placed in a forced air oven at 100 – 105 °C for 48 hours to determine the body water by weight difference. We followed the general guidelines for the care and use of laboratory animals recommended by the Animal Care Committee of the National University of San Luis.

The NPU is defined as the portion of nitrogen intake that is retained. The formula used was

$$NPU = \frac{B - (BK - IK)}{I} \times 100$$

where B is the corporal nitrogen of the experimental group; BK is the corporal nitrogen of the group on the protein-free diet; IK is the nitrogen intake of the group on the protein-free diet; and I is the nitrogen intake in the experimental group. Corporal nitrogen (N) was calculated by using the following equation:

$$Y = 2.92 + 0.02X \quad (1)$$

where X is the rats age in days, and Y is calculated as

$$Y = \frac{N(g) \times 100}{H_2O(g)} \quad (2)$$

By equating Eqs. (1) and (2), N is calculated as

$$N(g) = \frac{H_2O(2.92 + 0.02X)}{100}$$

tD was determined along with NPU, and was considered as the absorbed nitrogen with respect to the N intake. Unabsorbed nitrogen was calculated by quantification of the fecal nitrogen in the group fed with the protein-free diet. The formula used was

$$tD = \frac{I - (F - FK)}{I} \times 100$$

where I is the ingested nitrogen; F is the fecal nitrogen in the group that received the experimental diet; and FK is the fecal nitrogen of the group consuming the protein-free diet.

The biological value (BV) was calculated as the NPU/tD ratio.

Table 1. Composition of diets AIN 93-G

Nutrients (g/kg)	Protein free	Casein	ACRU	ACRU Treated
Cornstarch	397.48	397.48	85.16	164.16
Protein ^a	0	125.00	510.50	424.71
Soybean oil ^b	70.00	70.00	31.86	7.56
Fiber	50.00	50.00	20.08	29.23
Sucrose	100.00	100.00	100.00	100.00
Mineral mix	35.00	35.00	35.00	35.00
Vitamin mix	10.00	10.00	10.00	10.00
Choline bitartrate	2.50	2.50	2.50	2.50
L-Cystine	0	3.00	0	0
Dextrinized cornstarch	335.00	207.00	284.87	226.80
Tert-butylhydroquinone	0.014	0.014	0.014	0.014

^a The amount of flour incorporated in the diets to achieve 10 % of protein was defined taking into account the protein content of the sources, ACRU: 19.59 % and ACRU treated: 23.54 %. Casein: 80 % purity.

^b The amount of oil added to achieve 7 % of lipids, was defined taking into account the fat content obtained for the sources, ACRU: 7.47 % and ACRU treated: 14.70 %.

Blood Analysis

Glucose, total cholesterol and triglycerides, were determined by enzymatic methods using commercial kits.

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Statistical Analysis

Results are expressed as mean \pm standard deviation. Statistical differences were tested by the Student's *t*-test and ANOVA. Probabilities of 0.05 or less indicate significant difference (Snedecor and Cochran 1991).

Results and Discussion

The present work studied the effect of thermal treatment on the proximal chemical composition, elemental profile, antioxidant activity and biological value of the protein of a new *Amaranthus cruentus* L. variety seed flour, as well as the effect of its intake on some serum parameters.

Table 2 shows the proximal chemical composition, where the protein and lipid contents stand out, reaching values of 19.59 and 7.47 g/100 g, respectively, in ACRU. These values are higher than the informed by Bressani (2003) for proteins, and similar in the case of lipids. In ACRU treated, these results increase significantly in 17% and 50%, respectively.

Regarding the elemental profile (Table 3), in general, the results obtained are increased in the treated sample, in accordance with the ash value informed. Among the main elements of nutritional interest (expressed in $\mu\text{g/g}$ of flour), it is highlighted the presence of Ca: 1592.15 and 1974.25, Fe: 85.50 and 106.02, Na: 49.00 and 60.76, S: 7.71 and 9.56, and Cu: 6.49 and 8.03, for ACRU and ACRU treated, respectively, and a high content of P and K in both samples. The concentrations of these minerals is similar to the informed by Nascimento et al. (2014), and do not exceed the maximum tolerable levels. The Ca contribution of the studied flours could participate in the prevention of osteopenia and osteoporosis, which frequently affect coeliacs patients. No presence of toxic mineral elements, such as As and Cd, is detected, while Pb and Cr slightly exceed the limit allowed by the FAO/WHO (2015) (0.20 ppm for Pb and 0.10 ppm for Cr).

Form the investigated anti-nutrients (Table 4), it is observed that nitrates increase significantly (35%) in the treated sample; however, both values obtained (206.44 and 316.27 mg/100 g) are within the acceptable range. The Joint FAO/WHO Expert Committee has determined as Acceptable Daily Intake (ADI) of nitrates a value of 0-3.7 mg/kg of body weight (FAO/WHO 2002). Regarding the hemagglutinin activity, it is observed that they are concordant with the obtained by Escudero et al. (2004) for grain amaranths, and do not affect health. No presence of saponins is observed. A significant decrease is observed in the antitrypsin activity in

ACRU treated, due to that these proteases inhibitors are thermolabile. The results obtained for ACRU and ACRU treated, 3.49 and 2.86 TIU/mg, respectively, are similar to the reported for seeds of other amaranth species (Bressani, 1994). The values informed are close to the levels considered as safe (5 TIU/mg of sample). The concentration of oxalic acid in ACRU is 156.00 mg/100 g, decreasing in 8% for ACRU treated, value that is similar to regular consumption cereals. Considering that the Hendek Ertop and Bektaş (2015) recommends patients with kidney stones an oxalate-restricted diet with values below 40 to 50 mg per day, the daily intake of ACRU should be reduced. The phytic acid content was of 0.36 and 1.38 mg P/100g in ACRU and ACRU treated, respectively, lower than the informed for amaranth (82 mg/100 g) by Ferreira and Arêas (2010).

The amaranth grain contributes with natural antioxidants that play an import role in the inhibition of free radicals, preventing oxidative deterioration. The values obtained for

total phenols were 16.23-39.23 mg gallic acid / 100 g for ACRU and ACRU treated, respectively (Table 5), similar to the informed by Repo de Carrasco and Encina Zelada (2008) for six varieties of *Amaranthus caudatus*. The significant increase of phenols in the treated sample would be a consequence of the release of these compounds by the action of the thermal treatment. The evaluated antioxidant activity does not present significant differences between both samples, with varying ranges for % Inhibition for DPPH: 88.84-89.51; NO: 65.73-72.21; and β -carotene: 25.03-26.65. It is noted the

capacity of free radical scavenging given by the DPPH inhibition percentage. It is interesting the high percentage obtained for the NO Test, considering that NO produced in excess interacts with oxygen forming nitrites that transform into harmful peroxides; Czerwinski et al. (2004) inform lower values that vary between 23.00 and 25.10 % NO inhibition. The bleaching percentage of β -carotene, as a measurement of the lipid peroxidation inhibition, is relatively low and in agreement with the informed for *Amaranthus hypochondriacus* by the same author.

Regarding the evaluation of the biological quality of the studied samples (Table 6), it is observed that the diets consumption does not present significant differences between the experimental diets, and neither with respect to casein taken as reference. However, the weight gain decreases significantly (87%) with respect to the animal protein; these results can be attributed to the characteristics that distinguish a vegetable protein. The feces weight presents a significant increase with respect to casein, probably due to the fiber present in the vegetable diet. In the biological tests, NPU presents lower values for the studied samples with respect to casein, being lower for the treated sample. The tD does not show significant differences between the experimental samples, so it can be inferred that it is not affected by the thermal treatment. The BV results are lower for the treated sample, indicating that this treatment decreases the protein quality, and consequently, its utilization for protein synthesis.

Table 2. Chemical composition of the new variety in the dry weight of Acru-G10/13 II and Acru-G10/13 II treated seed flours

Determination (g/100g)	ACRU	ACRU treated
Moisture	7.28 ± 0.15 ^a	1.13 ± 0,01 ^b
Ash	4.46 ± 0,16 ^a	5.53 ± 0,04 ^b
Protein (N x 6.25)	19.59 ± 0,13 ^a	23.54 ± 0,15 ^b
Total lipids	7.47 ± 0.18 ^a	14.70 ± 0.12 ^b
Carbohydrates*	61.20 ± 0.43 ^a	55.10 ± 0.30 ^b
Crude fiber	5.86 ± 0.14 ^a	5.91 ± 0.06 ^a

Values are mean ± standard deviation of three measurements. Different letters indicate significant differences (p<0.05).

*Carbohydrates are determined by the following calculation:

$$\text{Carbohydrates} = 100 - (\% \text{ Ash}) - (\% \text{ Total Fat}) - (\% \text{ Moisture}) - (\% \text{ Protein})$$

Table 3. Concentration of 21 elements analyzed in the dry weight of Acru-G10/13 II and Acru-G10/13 II treated seed flours ($\mu\text{g/g}$)

Element	ACRU	ACRU treated
As	ND	ND
Ca	1592.15 \pm 75 ^a	1974.25 \pm 83 ^b
Cd	ND	ND
Co	0.11 \pm 0.005 ^a	0.14 \pm 0.008 ^b
Cu	6.49 \pm 0.03 ^a	8.03 \pm 0.06 ^b
Cr	0.16 \pm 0.007 ^a	0.18 \pm 0.009 ^b
Fe	85.50 \pm 5.70 ^a	106.02 \pm 7.50 ^b
K*	>10	>10
Hg	ND	ND
Li	0.98 \pm 0.06 ^a	1.21 \pm 0.04 ^b
Mg*	>10	>10
Mn	37.47 \pm 2.40 ^a	46.46 \pm 3.35 ^b
Mo	0.53 \pm 0.06 ^a	0.64 \pm 0.07 ^a
Na	49.00 \pm 2.78 ^a	60.76 \pm 4.25 ^b
Ni	0.37 \pm 0.001 ^a	0.46 \pm 0.002 ^b
P*	>10000	>10000
S	7.71 \pm 0.50 ^a	9.56 \pm 0.45 ^b
I	ND	ND
Pb	1.15 \pm 0.009 ^a	1.43 \pm 0.01 ^b
Se	ND	ND
Zn	32.33 \pm 1.87 ^a	40.09 \pm 2.25 ^b

Values are mean \pm standard deviation of three measurements. Different letters indicate significant differences ($p < 0.05$).

*Exceeds the upper quantification limit. ND: not detected

Table 4. Anti-nutrient factors in the dry weight of Acru-G10/13 II y Acru-G10/13 II treated seed flour

Anti-nutrient factors	ACRU	ACRU treated
Nitrates (mg/100 g)	206.44 \pm 17.67 ^a	316.27 \pm 32.19 ^b
Hemagglutinin activity	1/64	1/16
Hemolytic activity	ND	ND
Foam index*	<100	<100
Antitrypsin activity (TIU/mg sample) [#]	3.49 \pm 0.30 ^a	2.86 \pm 0.10 ^b
Oxalic acid (mg/100 g)	156.00 \pm 4.04 ^a	144.32 \pm 3.90 ^b
Phytic acid (mg P/100 g)	0.36 \pm 0.01 ^a	1.38 \pm 0.09 ^b

Values are mean \pm standard deviation of three measurements. Different letters indicate significant differences ($p < 0.05$)

ND: Not detected.

*1000/a; a = mL of filtrate in the tube that reached, when no tube exhibited 1 cm of foam, foam index <100.

[#]Trypsin inhibited units per mg of flour.

Table 5. Total phenols content and antioxidant activity in the dry weight of Acru-G10/13 II and Acru-G10/13 II treated seed flours

	ACRU	ACRU treated
Total phenols (mg gallic acid /100 g)	16.23 ± 0.10 ^a	39.23 ± 0.99 ^b
DPPH inhibition (%)	89.51 ± 1.66 ^a	88.84 ± 2.14 ^a
NO inhibition (%)	65.73 ± 1.63 ^a	72.21 ± 2.58 ^a
β-carotene inhibition (%)	25.03 ± 1.89 ^a	26.65 ± 0.97 ^a

Values are mean ± standard deviation of three measurements.

Different letters indicate significant differences (p<0.05)

Table 6. Biological quality from seed flour of Acru-G10/13 II y Acru-G10/13 II treated

	Casein	ACRU	ACRU treated
Intake (g)	74.08±10.94 ^a	60.70±7.17 ^a	66.90±7.37 ^a
Weight gain (g)	17.70±2.38 ^a	2.75±0.75 ^b	1.83±0.15 ^b
Feces weight (g)	5.51±0.46 ^a	8.44±1.96 ^b	7.44±0.85 ^b
NPU	63.21±6.85 ^a	45.39±5.23 ^b	33.99±3.22 ^c
tD	96.23±2.19 ^a	83.90±4.32 ^b	86.33±4.60 ^b
BV	65.69±5.69 ^a	54.10±4.53 ^a	39.37±3.20 ^b

The results are expressed as mean ± standard deviation

Different letters indicate significant differences (p<0.05)

NPU: Net protein utilization, tD: true digestibility, BV: Biological value

Table 7. Effect of ACRU-G10/13 II and ACRU-G10/13 II treated seed flours on biochemical variables

Biochemical variables (mg/dL)	Casein	ACRU	ACRU treated
Glucose	53.25 ± 11.70 ^a	62.40 ± 8.88 ^a	77.66 ± 21.08 ^a
Total Cholesterol	53.40 ± 11.33 ^a	39.60 ± 12.34 ^a	38.00 ± 5.05 ^a
Triglycerides	131.40 ± 33.28 ^a	89.40 ± 6.58 ^b	70.20 ± 18.40 ^b

The results are expressed as mean ± standard deviation

Different letters indicate significant differences (p<0.05)

The evaluated serum parameters (Table 7) indicate that the triglycerides level decreases significantly in the experimental samples, with a tendency in the decrease of total cholesterol with respect to casein, values similar to the reported by Escudero et al. (2006). This could be explained by the presence of dietary fiber and the prevalence of unsaturated fatty acids in the vegetal diet.

Conclusions

The obtained results allow to conclude that the thermal treatment used (90°C for 1h) affects the content of nutrients. It improves the protein and lipid contents, however, the biological study indicates that the thermal treatment decreases the protein quality when its biological value and net protein utilization are affected. The content of minerals of nutritional importance increases in the treated sample, highlighting the presence of Ca, Fe and P, without observing toxic elements.

The antinutrients studied are within ranges that do not affect health, suggesting the potential consumption of this variety without apparent toxicity risk. The concentration of total phenols indicates that the amaranth grain contributes with antioxidants that play an important role in free-radical scavenging, preventing oxidative deterioration; the sample subjected to thermal treatment increases its phenol content. On the other hand, a beneficial effect of the amaranths grains is observed by producing a hypotriglyceridemic effect. However, and despite that, in general, the thermal treatment improves some of the evaluated parameters, it decreases the protein quality. This is an aspect that should be contemplated for performing future studies, subjecting that sample to different types of thermal treatments that do not affect the biological quality of the protein. This also becomes a challenge for the food technology, with the goal of optimizing the nutritional quality of this amaranth.

Compliance with Ethical Standard

Conflict of interests: The authors declare that for this article they have no actual, potential or perceived the conflict of interests.

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