Journal of Food Engineering 122 (2014) 62–67

Contents lists available at ScienceDirect

Journal of Food Engineering

journal homepage: www.elsevier.com/locate/jfoodeng

Total phenolic content and antioxidant activity of different streams resulting from pilot-plant processes to obtain *Amaranthus mantegazzianus* protein concentrates

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ARTICLE INFO

Article history:
Received 25 March 2013
Received in revised form 15 August 2013
Accepted 22 August 2013
Available online 28 August 2013

Keywords:
Amaranth
Phenolic compounds
Antioxidant activity
Protein concentration
Pilot-plant

ABSTRACT

Antioxidant properties of different pilot-plant process streams to obtain *Amaranthus mantegazzianus* protein concentrates (APC) were evaluated. Conventional process (CP) (alkaline extraction and isoelectric precipitation) and two alternative processes (APs): (1) acid pre-treatment stage combined with isoelectric precipitation and (2) acid pre-treatment stage combined with ultrafiltration were applied at pilot-plant scale to obtain APC. Methanol and water extracts of APC and other fractions obtained in the processes were evaluated by Folin–Ciocalteau method in order to determine total phenolic content and by DPPH radical scavenging activity method to determine antioxidant activity. Acid pre-treatment stage and ultrafiltration caused an effective removal of phenolic compounds yielding on the one hand APC with lower phenolic content than the ones obtained by CP. On the other hand, the acid extract and the whey obtained presented high phenolic content and antioxidant activity and could be used as additives to increased this parameters in food.

Finally, evaluated processes could be used to obtain several products (concentrates, whey, extracts) with different phenolic content and antioxidant activity suitable for different applications in food industry.

1. Introduction

Protein concentrates are widely used as ingredients in food industry because of their high nutritional quality, functional properties, high protein level and low content of antinutritional factors (Cordero-de-los-Santos et al., 2005). Several processes have been used to obtain protein concentrates, such as isoelectric precipitation (Paredes-López, 1991; Martínez and Añón, 1996; Salcedo-Chávez et al., 2002), dialysis (Fidantsi and Doxastakis, 2001) and micellisation (Cordero-de-los-Santos et al., 2005). Different processes and conditions used for concentrate preparation cause particular effects in their composition and functional properties, besides information about these effects is useful in order to control final product characteristics (Wagner, 2000). Isoelectric precipitation, for example, could cause some undesirable effects like loss of some functional properties, production of certain antinutritional factors (lysinoalanine) and reduction of protein nutritional quality due to the loss of some essential amino acids through the beta-elimination reaction (Sarwar et al., 1999). Therefore, it is important to have information about the effect of the different processes could produce.

The outstanding nutritional and physicochemical properties of amaranth proteins are well documented. The high nutritional quality of amaranth seed protein is one of the main factors that has attracted the attention of researchers. Amaranth seeds have a protein content of 14–18%, which is superior to that of most cereals (~10% dry basis). Its proteins have an exceptional essential amino acid pattern, being relatively rich in lysine, tryptophan, and methionine, which are found in low concentrations in cereals and leguminous grains of common usage. Moreover, amaranth seeds contain adequate levels of important micronutrients such as minerals and vitamins and significant amounts of other bioactive components such as saponins, squalene and phenolic compounds (Barba de la Rosa et al., 2008; Nsimba et al., 2008; Klimczak et al., 2002).

There has been increased interest in phenolic compounds and their antioxidant activity among consumers and the scientific community in the past decade because epidemiological studies linking the consumption of diets rich in natural antioxidants with decreased risk of diseases associated with oxidative stress, such as cancer and cardiovascular disease (Thaipong et al., 2006). Moreover, antioxidants also inhibit oxidation in food that causes many undesirable changes and leads both to the deterioration of sensory

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http://dx.doi.org/10.1016/j.jfoodeng.2013.08.032

characteristics and reduction of its nutritive value. Therefore, antioxidants have an important role inhibiting oxidative processes in the organism and in food processing and storage. Moreover, the fact that synthetic antioxidants are suspected to be carcinogenic promotes the research of natural antioxidants, mainly found in plants (Klimczak et al., 2002; Singh et al., 2002). Nevertheless, it has also been reported that proteins and peptides show antioxidant activity against the peroxidation of lipids and/or fatty acids, and some amino acids are known as effective primary antioxidants and synergists (Wang and Xiong, 2005; Sakanaka et al., 2004; Je et al., 2005; Mendis et al., 2005). Thereby, antioxidant activity of plant extracts is often the effect of two or more compounds acting according to different mechanisms (Klimczak et al., 2002).

On the other hand, phenolic compounds are the major cause of color in vegetable protein isolates because of the products of the protein–phenol reaction (Xu and Diosady, 2002). Protein–phenol interaction also has effects on the thermal denaturation, solubility and digestibility of the protein (Bejosano and Corke, 1998; Gonzáles-Pérez et al., 2002) and may interfere in the extraction of the protein and limit its use in food industry (Gamel et al., 2006). In some particular applications is necessary to avoid these disadvantages removing the phenolic compounds of the protein concentrate, with this aim several processes have been evaluated, such as diafiltration and treatment with NaCl (Xu and Diosady, 2002).

The conventional method used for amaranth proteins concentration has been the alkaline extraction followed by isoelectric precipitation (Paredes-López, 1991; Martínez and Añón, 1996; Fidantsi and Doxastakis, 2001; Salcedo-Chávez et al., 2002). In a previous work, we found that protein concentration in the final product increased using an acid pre-treatment stage previous to the isoelectric precipitation but protein yield of the entire process decreased. On the other hand, the acid pre-treatment stage combined with ultrafiltration improved protein concentration, decreased protein aggregation and yield a concentrate with better amino acid composition (Castel et al., 2012). Since, one important property of amaranth seeds is the phenolic compounds content because of its antioxidant activity, this is an important issue to take into account when evaluating the products of these processes. As far as we know, there is no previous study about *Amaranthus mantegazzianus* antioxidant properties neither of antioxidant properties of pilot plant process streams.

For all the mentioned, in this study, the objective was to evaluate the effects of the acid pre-treatment and the ultrafiltration pilot-plant processes on the total phenolic content and antioxidant activity of amaranth protein concentrates and others fractions obtained in the processes.

2. Materials and methods

2.1. Materials

Amaranth seeds (*A. mantegazzianus*) were obtained from an Argentinean grower (Reconquista, Santa Fe, Argentina). Whole seeds were ground in a Bühler Miag MLGV Variostuhl mill (Germany) and screened with 20 mesh. The flour was defatted three times with hexane in a 10% (w/v) suspension under continuous stirring for 24 h, air dried at room temperature, and then stored at 4 °C until use. Amaranth defatted flour (ADF) presented the following composition: 13.4% w/w moisture, 13.8% w/w protein (17% protein/dry material), 0.35% w/w fat and 2.74% w/w ash, determined according to the approved AOAC assays (1995).

2.2. Preparation of amaranth protein concentrates (APCs) at pilot scale

Processes were performed according to Castel et al. (2012). The conventional process (CP) is shown in Fig. 1. ADF was suspended in alkaline media (pH 9.0), stirred for protein solubilization and then centrifuged. This procedure was made twice and then the supernatants obtained were mixed and clarified by centrifugation in continuous disk centrifuge. The protein in the clarified solution was precipitated at pH 4.5, washed with tap water, centrifuged and finally spray-dried. This protein concentrate was named amaranth protein concentrate 1 (APC-1) and presented a protein content of 50.9 ± 0.1% w/w (Castel et al., 2012).

The alternative processes: (1) acid pre-treatment stage combined with isoelectric precipitation and (2) acid pre-treatment stage combined with ultrafiltration are shown in Fig. 2. In the acid pre-treatment stage, ADF was suspended in acid media (pH 4.5), stirred for 1 h at 25 °C. After centrifugation, an acid extract (AE) with 14.8 ± 0.8% w/w of protein (Castel et al., 2012) and a residue were separated. The residue was dispersed in water (1:10 w/v) and then two alkaline extraction steps at pH 9.0 were carried out. Supernatants were clarified and divided in two streams, one for (a) isoelectric precipitation, performed in the same conditions of CP result in an amaranth protein concentrate 2 (APC-2) with 73.1 ± 0.2% w/w of protein and a whey with 57.9 ± 0.1% w/w of protein; and the other stream for (b) ultrafiltration process, performed by 3 diafiltrations at 50 °C obtaining the ultrafiltration retentate (UR) with 52.5 ± 0.8% w/w of protein (Castel et al., 2012).

2.3. Extracts preparation

Methanol-soluble components were extracted from the samples using the method described by Tsaliki et al. (1999) with some modifications. The samples were mixed with methanol (HPLC, code 9093-68, lot C52E02-JTBaker, Mallinckrodt Baker, Inc. Phillisburg, NJ, USA) in tubes with screw caps in a 1:5 (w/v) proportion and stirred in a mixer for 10 min. The tubes were placed in a water bath at 50 °C for 1 h, then the temperature was raised to 65 °C for 5 min. Tubes were left to cool down to room temperature at 25 °C and the supernatant was subsequently filtered through a 0.45 μm Millipore filter.

Water extracts of the samples were obtained according to the method developed by Eberhardt et al. (2005) with some modifications. The samples were suspended in distilled water in a concentration of 50 mg mL^{-1} and mixed with a magnetic stirrer for

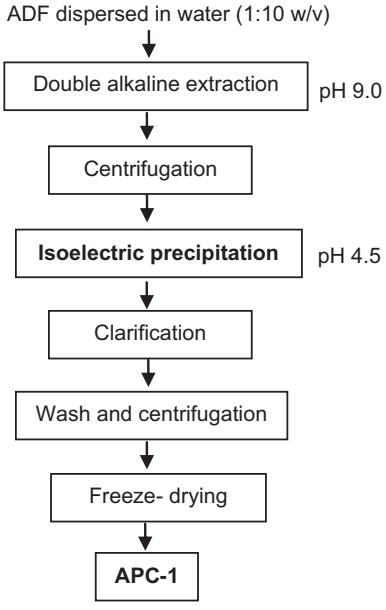

Fig. 1. Conventional process to obtain amaranth protein concentrates. ADF: amaranth defatted flour from *A. mantegazzianus* seeds. APC-1: amaranth protein concentrate obtained by conventional process.

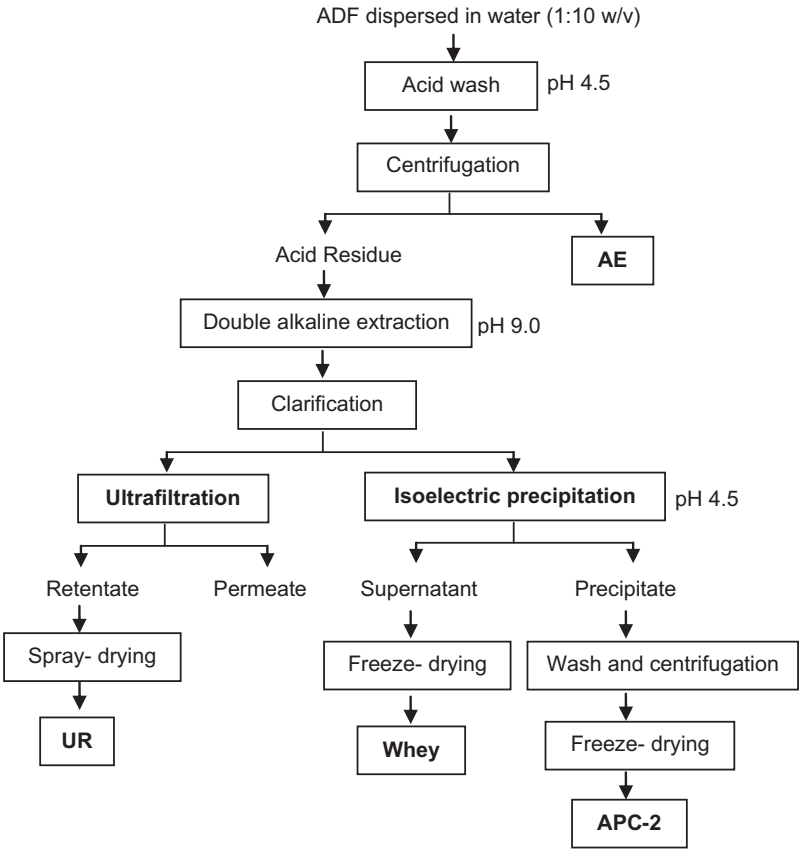

Fig. 2. Alternative processes to obtain amaranth protein concentrates. ADF: amaranth defatted flour from *A. mantegazzianus* seeds; APC-2: amaranth protein concentrate obtained by alternative process (1): acid pre-treatment process combined with isoelectric precipitation; UR: ultrafiltration retentate obtained by alternative process (2): acid pre-treatment process combined with ultrafiltration; whey: isoelectric precipitation supernatant of alternative process (1); AE: acid extract.

30 min. Then, they were centrifuged (35,735*g*/15 min/25 °C) and filtered through a Whatman n. 1 paper. The obtained volume was completed up to the initial volume of extraction. All the extracts were stored at −20 °C until used.

2.4. Total phenolic content determination

Total phenolic content of methanol and water extracts was determined colorimetrically using Folin–Ciocalteu reagent (Sigma Chemical Co, St. Louis, MO, USA) as described by Gamel et al. (2006) with some modifications. The extracts (100 μL) were added to 2 mL aqueous sodium carbonate (Na_2CO_3) solution at 2% (w/v). After 2 min, 100 μL of Folin–Ciocalteu reagent aqueous solution (1:1) was added. The mixture was vigorously shaken and allowed to stand for 30 min. Then, absorbance was measured at 750 nm against a test blank using a Beckman Coulter spectrophotometer (DU 640, Beckman Instrument Inc., Fullerton, CA, USA). A calibration curve of gallic acid (3,4,5-trihydroxybenzoic acid, Sigma Chemical Co, St. Louis, MO, USA) in a range of 0.002–0.12 mg mL^{-1} was prepared and the concentration of phenolic compounds was expressed as mg of gallic acid equivalents per gram of dry weight (mg GAE/g dw).

2.5. Protein content in water extracts

Protein concentration of water extracts were determined by Biuret method (Layne, 1957) using bovine serum albumin (Sigma Chemical Co, St. Louis, MO, USA) as a standard. The results were expressed as mg of protein per mL of extract.

2.6. Determination of antioxidant activity in methanol and water extracts

In vitro antioxidant activity of methanol and water extracts of the samples was evaluated using the DPPH radical scavenging activity assay as described by Thaipong et al. (2006). The stock solution was prepared by dissolving 24 mg DPPH (2,2-diphenyl-1-picrilhidrazyl, Sigma Chemical Co, St. Loius, Mo, USA) in 100 mL methanol solution and then stored at −20 °C until needed. The working solution was obtained by mixing 8.6 mL of stock solution with 50 mL of methanol. The extracts (150 μL) were left to react with 2.9 mL of the DPPH working solution for 24 h in a dark place. Then, absorbance was measured at 515 nm using a spectrophotometer (Beckman Coulter, DU 640, Beckman Instrument Inc., Fullerton, CA, USA). The calibration curve was done using Trolox ((R)-(+)-6-hidroxy-2,5,7,8-tetramethylcroman-2-carboxylic acid, 98%, Sigma Chemical Co, St. Louis, MO, USA) dissolved in methanol in a range of 25–1558 μM. Test were performed by triplicate and results were expressed as mg of Trolox equivalents per gram of dry weight (mg TE/g dw). Additional dilution was needed when the DPPH value measured was over the linear range of the standard curve.

2.7. Statistical analysis

The results were evaluated statistically by analysis of variance (ANOVA) and LSD test at 5% level of significance ($p < 0.05$) using STATGRAPHIC Centurion XV software. Linear regression analysis was performed in order to obtain correlation coefficients (R) between antioxidant activity, total phenolic content and protein concentration.

3. Results and discussion

3.1. Total phenolic content

The total phenolic content in methanol and water extracts of the samples are shown in Table 1. Water and methanol extracts of ADF of *A. mantegazzianus* presented higher phenolic content than similar extracts reported in the literature. Carrasco and Encina-Zelada (2008) reported total phenolic content for methanol extracts from six varieties of *Amaranthus caudatus* ranging from 0.19 to 0.30 mg GAE/g dw. Czerwinski et al. (2004) reported values of 0.149 and 0.147 mg GAE/g of flours from two varieties of *Amaranthus hypochondriacus*. Gorinstein et al. (2007) found much lower values (from 0.011 to 0.034 μg GAE/g dw) in water and acetone extracts of *Amaranthus cruentus*, *Amaranthus hybridum* and *A. hypochondriacus*. However, using methanol–HCl as extraction solvent, higher phenolic content was reported for *A. cruentus* (3.0 mg GAE/g dw) (Paśko et al., 2009). The methanol/acid extraction produces an acid hydrolysis that releases phenolic compounds linked to proteins increasing the extraction yield (Gorinstein et al., 2007). In this regard, several authors have remarked the importance of the extraction method and the solvent used (Gorinstein et al., 2007; Nsimba et al., 2008; Ozsoy et al., 2009). The use of solvents with different polarities leads to differences in phenolic content and antioxidant capacity (Matthäus, 2002; Gorinstein et al., 2007).

Among the methanol extracts, AE presented the highest phenolic content (7.09 mg GAE/g dw) followed by the whey (6.89 mg GAE/g dw). This would indicate that most phenolic compounds remain soluble in the initial acid extraction and in the isoelectric precipitation. As a result of this, the phenolic content of APC-2 was lower than that of APC-1. On the other hand, UR methanol extract presented lower phenolic content than APC-1 and APC-2 indicating that most of the phenolic compounds permeated by the ultrafiltration membrane.

Water extracts of ADF, APC-1, APC-2 and UR showed higher phenolic content than their corresponding methanol extracts. This finding could be the result of non-specific reactions of Folin–Ciocalteu reagent with other components of the water extract which could overestimate the phenolic content in these extracts (Galher et al., 2003). Folin–Ciocalteu reagent is not specific for phenolic compounds and can be reduced by non-phenolic compounds such as tertiary aliphatic amines, hydroxylamine, vitamin C, Cu (I), sugars, aromatic amino acids (tryptophan, phenylalanine and tyrosine), and other reducing agents.

In order to evaluate the possible reaction between Folin–Ciocalteu reagent and free amino acids, peptides and proteins present in water extracts, total phenolic content and protein concentration in water extracts were correlated in Fig. 3. The correlation coefficient was not low (R^2 = 0.7799) showing that there might be a relationship between total phenolic contents measured by Folin–Ciocalteu method and protein concentrations in water extracts. Therefore, results obtained in water extracts are difficult to analyze because the measurement might not show the real concentration of phenolic compounds.

Table 1
Total phenolic content of methanol and water extracts of the samples. Protein concentration in water extracts.

Samples	Total phenolic compounds (mg GAE/g dw)		Protein (mg/mL)
	Methanol extracts	Water extracts	Water extracts
ADF	$0.98 \pm 0.07^{a,A}$	$1.12 \pm 0.02^{a,B}$	0.75 ± 0.09^{a}
APC-1	$2.51 \pm 0.06^{c,A}$	$3.85 \pm 0.07^{d,A}$	5.83 ± 0.10^{d}
APC-2	$1.16 \pm 0.04^{b,A}$	$5.50 \pm 0.08^{f,B}$	7.07 ± 0.14^{f}
Whey	$6.89 \pm 0.12^{e,B}$	$6.47 \pm 0.05^{g,A}$	14.54 ± 0.04^{h}
UR	$0.88 \pm 0.05^{a,A}$	$3.95 \pm 0.07^{d,B}$	9.39 ± 0.12^{g}
AE	$7.09 \pm 0.10^{f,B}$	$4.36 \pm 0.02^{e,A}$	6.85 ± 0.09^{e}

Data expressed as average ± SD (n = 3). Different lowercase letters in the same column and different capital letters in the same line indicate statistical difference (p < 0.05).
GAE: gallic acid equivalent, ADF: amaranth defatted flour, APC-1: amaranth protein concentrate obtained by conventional process, APC-2: amaranth protein concentrate obtained by alternative process (1), UR: ultrafiltration retentate obtained by alternative process (2), whey: isoelectric precipitation supernatant of alternative process (1), AE: acid extract.

3.2. Antioxidant activity

Antioxidant activities evaluated by the DPPH radical scavenging capacity of methanol and water extracts of the samples are presented in Table 2. The ADF of *A. mantegazzianus* showed higher antioxidant activity in methanol extracts (TE 0.71 mg/g dw) than in the water extracts (0.16 mg TE/g dw). Paśko et al. (2009) obtained similar results in methanol acid extracts (HCl–methanol–water) from *A. cruentus* (varieties Aztek and Rawa) (1.1 mg TE/g dw and 0.7 mg TE/g dw, respectively). Carrasco and Encina-Zelada (2008) reported antioxidant activity values in the range of 0.56–0.66 mg TE/g dw for hydrophilic extracts (acetone: water: acetic acid) of six varieties of *A. caudatus*, which are higher than those obtained in this study for the water extracts of the ADF.

Among the samples, the whey presented the highest antioxidant activity in methanol and water extracts (3.32 and 5.54 mg TE/g dw, respectively). On the one hand, the high antioxidant activity in methanol extracts of the whey could be related to the high phenolic compounds content showed by this extract. On the other hand, the high antioxidant activity in the water extract of the whey could be probably related to the high protein content of this extract. Several authors, like Wang and Xiong (2005) and Je et al. (2005) observed high antioxidant power in low molecular weight peptides (<6 kDa and <1 kDa, respectively). Then, there is a possibility that peptides and low molecular weight proteins that not precipitate at isoelectric pH could be contributing in the antioxidant activity of this extract. In a previous study (Castel et al., 2012), it was found that the proteins present in the whey were rich in amino acids known as primary antioxidants and synergists: cysteine and methionine. The presence of these amino acids in the water extract might be contributing to the high antioxidant activity.

The APC-2 showed the lowest antioxidant activity in the methanol extract (0.08 mg TE/g dw), indicating that most of the antioxidant compounds were separated in the soluble fraction at

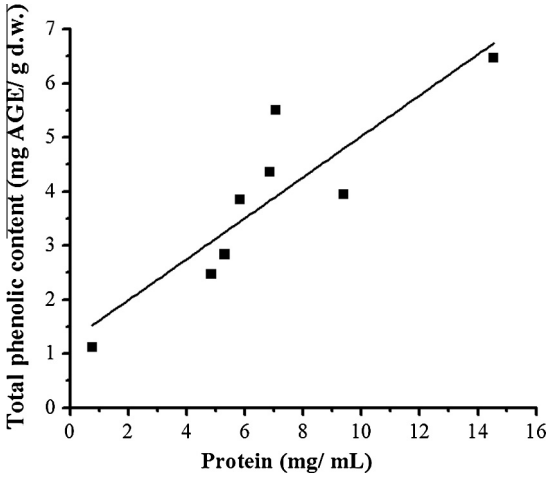

Fig. 3. Correlation between total phenolic content and protein concentration in water extracts, R^2 = 0.7799, y = 0.3785x + 1.2369.

Table 2
In vitro antioxidant activity of methanol and water extracts of the samples.

Samples	Antioxidant activity (mg TE/g dw)	
	Methanol extracts	Water extracts
ADF	$0.71 \pm 0.01^{b,B}$	$0.16 \pm 0.06^{a,A}$
APC-1	$1.69 \pm 0.05^{c,B}$	$1.44 \pm 0.01^{d,A}$
APC-2	$0.08 \pm 0.02^{a,A}$	$0.89 \pm 0.01^{b,B}$
Whey	$3.32 \pm 0.04^{e,A}$	$3.46 \pm 0.05^{g,B}$
UR	$0.83 \pm 0.03^{b,A}$	$1.82 \pm 0.02^{e,B}$
AE	$2.72 \pm 0.05^{d,B}$	$1.90 \pm 0.02^{f,A}$

Data expressed as average ± SD ($n = 3$) standard deviations. Different lowercase letters in the same column and different capital letters in the same line indicate statistical difference ($p < 0.05$). TE: Trolox equivalent, ADF: amaranth defatted flour, APC-1: amaranth protein concentrate obtained by conventional process, APC-2: amaranth protein concentrate obtained by alternative process (1), UR: ultrafiltration retentate obtained by alternative process (2), whey: isoelectric precipitation supernatant of alternative process (1), AE: acid extract.

isoelectric pH. The methanol extract of UR presented a ten times higher antioxidant activity than the APC-2. On the other hand, the APC-1 obtained by CP showed higher antioxidant activity and phenolic content than the UR and APC-2 obtained by the AP. Then, it could be said that the initial acid extraction was able to separate compounds with antioxidant activity, mainly phenols, which were soluble at acid pH. As a result of the AP, AE with relatively high antioxidant activity and phenolic compounds concentration was obtained, and therefore, phenolic content and antioxidant activity of UR and APC-2 were diminished in contrast to APC-1.

Antioxidant activities of water extracts were different from those of methanolic extracts. APC-2, UR and whey show higher antioxidant activity in the water extracts than in methanol extracts, and the rest of the samples otherwise. These differences might be explained by different interactions between antioxidant compounds and solvents, which could determine the solubility and degree of extraction of the compounds. In water extracts is expected the main presence of peptides and amino acids, which have high solubility in water. While, in methanol extracts, is mainly expected the presence of hydrophobic compounds such as phenolic compounds and possibly hydrophobic amino acids or peptides. Also, it must be taken into account the possible interference of the solvent in the antioxidant activity determination. In polar solvents, hydrogen bonds of the medium results in changes in the ability of phenolic antioxidants to donate hydrogen atoms, decreasing their antioxidant activity. According to Pérez-Jiménez and Saura-Calixto (2006), among the most used methods, DPPH radical scavenging assay is the least affected by the solvent, even so, they found a 20% higher antioxidant activity in a methanol solution than in an aqueous solution. Moreover, it has been shown that certain non-antioxidant compounds could interfere in antioxidant capacity assay (Pérez-Jiménez and Saura-Calixto, 2006). When measuring antioxidant capacity with DPPH method a clear interaction between polyphenols and common components in foods was observed; these components have no effect by themselves but altered the original capacity of the polyphenols. Glycosidic compounds, such as glucose, galacturonic acid and pectins, have been shown to increase the value of antioxidant activity, while other compounds diminish it.

Total phenolic contents of water and methanol extracts were correlated with the DPPH scavenging activities. The correlation obtained in methanol extracts was higher than the one obtained in water extracts ($y = 0.3868x - 0.2338$, $R^2 = 0.8875$ and $y = 0.4484x - 0.2325$, $R^2 = 0.6057$, respectively). On the other hand, both correlations were lower than others found in the literature where the antioxidant activity was determined by other methods (Gorinstein et al., 2007; Paśko et al., 2009; Carrasco and Encina-Zelada, 2008; Czerwinski et al., 2004). The low correlations (<0.9) obtained in this work could suggest that the antioxidant activities of these extracts are not only the results of phenolic compounds effects, others non-phenolic compounds might be contributing to the DPPH scavenging activity. These data is consistent with the finding of Nsimba et al. (2008).

A significant correlation ($y = 0.2318x - 0.0997$, $R^2 = 0.8783$) was obtained between protein concentration and antioxidant activity in water extracts. This correlation was higher than the one obtained to the phenolic compounds and antioxidant activity in water extracts, this might indicate that proteins, peptides or free amino acid present in water extracts have high contribution into the antioxidant activity of these extracts.

Finally, results suggest that both, phenolic compounds and proteins, might be contributing to the antioxidant activity of amaranth extracts evaluated by DPPH radical scavenging activity method.

4. Conclusions

The ADF obtained from *A. mantegazzianus* seeds showed higher phenolic content than other varieties of amaranth found in literature. All samples obtained showed antioxidant activity evaluated by the DPPH radical scavenging capacity.

Acid pre-treatment stage separated phenolic compounds in the soluble fraction AE which results in high concentration of phenolic compounds and antioxidant activity. The acid pre-treatment stage combined with isoelectric precipitation produced APC-2 with lower phenolic content than APC-1 obtained by the conventional process. The whey obtained in this process presented the highest phenolic compound content and antioxidant activity. Both, AE and whey were particularly suitable to be used as food additives to enhance nutritional and health-related values. Here, for the first time, process streams, which in general are discharged as waste in the environment, were identify as potential antioxidant compound sources. These *A. mantegazzianus* extracts might be of interest to food technologists as they could be incorporated into the diet, either forming part of the food, replacing the used artificial antioxidants or acting together therewith, reducing their concentration.

Ultrafiltration process also separated phenolic compounds that cross the membrane obtaining a protein concentrate (UR) with lower content of phenolics than the APC-2.

Finally, the proposed processes could be used to obtain several products (concentrates, whey, extracts) with different total phenolic content and antioxidant activity suitable for different applications in food industry.

Acknowledgements

The authors are thankful to the Projects: CAI + D type II, PI 57-283 (UNL), PICTO 35831 (ANCYPT – UNL) and PICTO 36237 (ANCYPT – UNL).

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