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ORIGINAL PAPER

Increasing the Antioxidant Activity, Total Phenolic and Flavonoid Contents by Optimizing the Germination Conditions of Amaranth Seeds

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Abstract The aim of this study was to optimize the germination conditions of amaranth seeds that would maximize the antioxidant activity (AoxA), total phenolic (TPC), and flavonoid (TFC) contents. To optimize the germination bioprocess, response surface methodology was applied over three response variables (AoxA, TPC, TFC). A central composite rotable experimental design with two factors [germination temperature (GT), 20–45ºC; germination time (Gt), 14–120 h] in five levels was used; 13 treatments were generated. The amaranth seeds were soaked in distilled water (25 °C/6 h) before germination. The sprouts from each treatment were dried (50 °C/8 h), cooled, and ground to obtain germinated amaranth flours (GAF). The best combination of germination bioprocess variables for producing optimized GAF with the highest AoxA [21.56 mmol trolox equivalent (TE)/100 g sample, dw], TPC [247.63 mg gallic acid equivalent (GAE)/100 g sample, dw], and TFC [81.39 mg catechin equivalent (CAE)/100 g sample, dw] was GT=30ºC/Gt=78 h. The germination bioprocess increased AoxA, TPC, and TFC in 300–470, 829, and 213 %, respectively. The germination is an effective strategy to increase the TPC and TFC of amaranth seeds for enhancing functionality with improved antioxidant activity.

Keywords Amaranth · Antioxidant activity · Germination bioprocess · Optimization · Response surface methodology

Introduction

Amaranth is a pseudo-cereal that has been widely grown by the Aztecs, Incas and Mayas in Latin America since pre-Columbian times for millennia; this grain belongs to dicotyledonous class in *Amaranthaceae* family. Amaranth grains have an excellent nutritional quality; they contain approximately 15 % protein with an adequate balance of aminoacids, high lysine content, 60 % starch and 8 % fat [1,2]. The amaranth grain has higher concentration of soluble fiber than others cereals, such as wheat, corn or oats. Its lipid composition presents polyunsaturated fatty acids and squalene [3]. Polyphenolic compounds, such as phenolic acids and flavonoids, have been characterized in amaranth grains [2,4]. In addition to its promising nutritional qualities, amaranth grains are considered to be an important source of food for patients with celiac, diabetic and hypercholestrolemic problems [2,5].

In recent years, a new way in nutrition, is the consumption of sprouts, which have received attention as functional foods, because of their nutritive value including amino acids, fibre, trace elements and vitamins as well as flavonoids and phenolic acids [6]. It is believed that sprouts are rich in phytochemicals for health-promoting compared with their mature counterparts. Germination (sprouting) has been suggested as an inexpensive and effective way to improve the nutraceutical

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quality of cereals, pseudocereals, and legumes. During this bioprocess, some compounds with antioxidant activity increase, mainly polyphenols and flavonoids, which provide protection against oxidative damage [6–11]. Antioxidants are one of the most common active ingredients of nutritional and functional foods, which can play an important role in the prevention of oxidation and cellular damage by inhibiting or delaying the oxidative process. These are one of the reasons why the consumption of sprouts can be very important in reducing human diseases associated with oxidative stress [10,12]. There are some researches where it has been reported that germination is an efficient process to increase the antioxidant activity and phenolics content of amaranth and other pseudocereals [8–11].

In most of the previous studies, it has been reported antioxidant activity and phenolics contents of raw and germinated grains using various aqueous solutions of methanol, ethanol, and acetone to extract soluble (free) phenolics. Adom and Liu [13] reported the importance of the determination of free and bound phenolic compounds. Bound phytochemicals, mostly in cell wall materials, are difficult to digest in the upper gastrointestinal tract and may be digested by bacteria inside the colon to provide health benefits and reduce the risk of colon cancer. There has been limited research that has tried to explain the complete profile (free and insoluble bound) of phenolic compounds and total antioxidant activity of grains. Therefore, in this study, the free and bound phenolic fractions were considered to determine total antioxidant activity and total phenolics contents in raw and germinated amaranth.

Response surface methodology (RSM) has been considered as an effective mathematical statistics method for establishing models to evaluate the relative significance of variables and determine optimal conditions of desirable responses. The utility of RSM for bioprocesses optimization has been previously demonstrated [14–16]. Between these studies, only the research by Mora-Escobedo et al. [16] was carried out to optimize the germination bioprocess of amaranth. In that work, they studied the effect of process variables over the attributes of germination quality protein content, percentage germination, and total color difference.

Even when there are diverse studies about the effect of germination on the nutraceutical value of pseudocereals such as amaranth [8–11], there is not any research about the optimization of germination bioprocess to increase the antioxidant value of this grain. Therefore, this study was conducted to find the best germination conditions of amaranth seeds that would maximize the antioxidant activity, total phenolic and flavonoid contents, and obtain bioprocessed amaranth flour with added value that could be used by the food industry as functional ingredient.

Materials and Methods

Materials

Amaranth (*Amaranthus hypochondriacus*) grains were purchased at a local market in Temoac, Morelos, Mexico.

Methods

Production of Germinated Amaranth Flours (GAF)

A portion of 200 g of amaranth seeds was soaked in 1,000 mL of 0.1 % sodium hypochlorite for 10 min. Then, these seeds were washed with distilled water until reaching neutral pH. Afterwards, seeds were soaked with 1,000 mL of distilled water at 25ºC for 6 h. The hydrated seeds were placed in germination trays on wet laboratory paper. The trays were introduced in the germination chamber (manufactured by Centro de Instrumentos, Universidad Autónoma de Sinaloa). A relative humidity of 80–90 % within the chamber was maintained using trays with water. The germination bioprocess was achieved by applying combinations of temperature/time in the intervals of 20–45ºC and 14–120 h, respectively (Table 1). The conditions of temperature/time germination were based on previous and preliminary studies. In all cases, the seeds were germinated under light/darkness in periods of 50/50 % of the germination time daily [light source: fluorencente tubes (light white, 16 W/2,700 K, Tecno Lite, China)]. The resulting bioprocessed amaranth seeds were dried (50ºC/8 h), cooled (25ºC) and ground (80-US mesh=0.180 mm) to obtain germinated amaranth flours (GAF). Additionally, amaranth seeds were ground (80-US mesh=0.180 mm) to obtain unprocessed amaranth flours (UAF), which was used as control. GAF and UAF were packed and kept at 4ºC in tightly sealed containers until further analysis.

Extraction of Free and Bound Phenolic Compounds

Free phenolic compounds were extracted as reported by Dewanto *et al*. [17]. Briefly, 1 g of ground sample was blended with 10 mL of 80 % chilled ethanol for 10 min and then centrifuged (2,500 g/10 min); the supernatant was concentrated under vacuum at 45ºC and stored at −20ºC until its evaluation. Bound phenolic compounds were extracted from the residue according to Adom and Liu [13]. The residue was hydrolyzed with 10 mL of 2 M NaOH at 95ºC for 30 min with previous removal of O_2 using N_2, followed by 1 h at 25ºC. The mixture was acidified (pH<2.0) with 2 mL of 2 M HCl and extracted with hexane to remove lipids. The final solution was extracted five times with 10 mL of ethyl acetate; the fractions were pooled and dried under vacuum at 35 °C. Bound phenolic compounds were reconstituted in 2 mL of

Table 1 Experimental design[a] used to obtain different combinations of germination temperature/germination time for producing germinated amaranth flours, and experimental results for response variables

Assay[b]	Process variables		Response variables		
	Germination temperature (ºC)	Germination time (h)	Antioxidant activity[c]	Total phenolic content[d]	Total flavonoid content[e]
1	23.6	29.5	9.6	94.3	29.0
2	41.3	29.5	7.6	42.5	23.4
3	23.6	104.5	16.2	187.4	55.4
4	41.3	104.5	10.5	66.7	40.0
5	20.0	67.0	19.8	214.2	69.3
6	45.0	37.0	9.6	80.3	41.0
7	32.5	14.0	8.0	45.9	25.8
8	32.5	120	19.5	158.4	70.6
9	32.5	67	23.4	225.2	90.2
10	32.5	67	22.0	217.6	75.3
11	32.5	67	20.8	245.2	84.4
12	32.5	67	19.2	230.5	80.5
13	32.5	67	21.0	255.2	88.1

[a] Central composite rotatable design with two factors and five levels; 13 assays

[b] Does not correspond to order of processing

[c] mmol Trolox equivalents (TE)/100 g (dw)

[d] mg gallic acid equivalents (GAE)/100 g sample (dw)

[e] mg catechin equivalents (CAE)/100 g sample (dw)

methanol–water (50:50, *v*/*v*). The extracts were frozen and stored at −20ºC until evaluation. All extractions were made by quadruplicated.

Antioxidant Activity (AoxA)

Free and bound hydrophilic antioxidant capacities were determined using the oxygen radical absorbance capacity (ORAC) assay and the ABTS radical cationdecolorization assay, using a Microplate Reader (Synergy™ HT Multi-Detection, BioTek, Inc., Winooski, VT, USA). For ORAC assay the extracts were evaluated against a standard of Trolox with Fluorescein as a probe as described by Ou *et al.* [18]. For ABTS assay the extracts were evaluated against a standard of Trolox as a probe as it was described by Re *et al.* [19]. The results of ORAC and ABTS assays were expressed as mmol of Trolox equivalents (TE)/100 g of dry weight (dw) sample. All measurements were made by triplicated.

Total Phenolic and Flavonoid Contents (TPC, TFC)

The total phenolic and flavonoid contents of free and bound extracts from ground samples was determined using the colorimetric methods of Singleton *et al.* [20] and Heimler *et al.* [21], respectively. The absorbance was measured using a Microplate Reader (Synergy™ HT Multi-Detection, BioTekInc, Winooski, VT, USA). Total phenolics were expressed as milligrams of gallic acid equivalents (mg GAE)/100 g sample (dw), while total flavonoids were expressed as milligrams catechin equivalents (mg CAE)/100 g sample (dw). All measurements were made by triplicated.

Proximate Composition

The official AOAC [22] methods 925.09B, 920.39C and 960.52 were used to determine moisture, lipids and protein contents, respectively.

Response Surface Methodology (RSM) Experimental Design, Statistical Analysis and Optimization

A central composite experimental design was chosen for RSM, with two factors [germination temperature (GT), germination time (Gt)] and five variation levels (GT= 20,23.6,32.5,41.3,45ºC; Gt=14,29.5,67,104.5,120 h) (Table 1). Applying the stepwise regression procedure, non-significant terms (p >0.1) were deleted from a second order polynomial and a new polynomial was used to obtain a predictive model for each response variable. The conventional graphical method was applied as optimization technique to obtain maximum AoxA, TPC, and TFC. Predictive models were used to graphically represent the system. Contour plots of each of the response variables were superimposed to obtain

a contour plot for observation and selection of the best (optimum) combination of GT and Gt for the production of optimized germinated amaranth flour (OGAF) through the germination bioprocess. The statistical software Design Expert version 7.0.0 (Stat-Ease, Minneapolis, MN, USA) was used for the RSM analyses.

Results of chemical composition and nutraceutical properties of OGAF and unprocessed amaranth flour were subjected to one-way analysis of variance (ANOVA) followed by Duncan's multiple range test comparison among means with 5 % of significance level.

Results and Discussion

Predictive Models for Antioxidant Activity (AoxA) and Total Phenolic and Flavonoid Contents (TPC, TFC)

The *AoxA*, *TPC* and *TFC* experimental values of the germinated amaranth flours varied from 7.6 to 23.4 mmol TE/100 g sample (dw), 42.5 to 255.2 mg GAE/100 g sample (dw), and 23.4 to 90.2 mg CAE/100 g sample (dw), respectively (Table 1). Analysis of variance showed that *AoxA*, *TPC* and *TFC* were significantly ($p<0.1$) dependent on linear and quadratic terms of GT and Gt. Predictive models using uncoded variables for the response variables (*AoxA*, TPC, TFC) were:

$$AoxA = 21.27 - 2.76GT + 3.22Gt - 4.09GT^2 - 4.56Gt^2$$

$$TPC = 234.77 - 45.24GT + 34.55Gt - 50.51GT^2 - 73.07Gt^2$$

$$TFC = 83.71 - 7.62GT + 13.31Gt - 17.97GT^2 - 21.43Gt^2$$

The regression models explained 90, 96 and 90 % of the total variability ($p<0.0006$) in *AoxA*, *TPC* and *TFC*, respectively. The lack of fit was not significant ($p>0.05$) and the relative dispersion of the experimental points from the predictions of the models (CV) was found to be < 10 %. These values indicated that the experimental models were adequate and reproducible. In general, AoxA, TPC and TFC of bioprocessed amaranth flours increased with GT and Gt, until reach maximum values around 30 °C and 70–80 h (data not shown); after, these responses decreased with the highest values of GT and Gt. Pasko *et al.* [9,10] reported similar results. They found that AoxA and TPC of germinated amaranth increased with Gt until fourth day (96 h) of germination, and after this time, AoxA and TPC decreased significantly. There are not any reports in literature about the effect of GT over AoxA, TPC and TFC of germinated amaranth.

Optimization

Figure 1, which was obtained of the superimposition of contour plots, was utilized to determine the best combination of process variables for production of germinated amaranth flour (GAF). The central point of the optimization region in Fig. 1 corresponds to the optimum combination of process variables (GT=30 °C/Gt=78 h) for GAF production with the highest *AoxA*, *TPC*, and *TFC* values. The predicted values of *AoxA*, *TPC* and *TFC*, using the predictive models of each response variable and the optimal conditions of germination, were 22.3 mmol ET/100 sample (dw), 247 mg GAE/100 sample (dw), and 86.3 mg CAE/100 sample (dw), respectively (Fig. 1). Optimized germinated amaranth flour (OGAF) was produced applying the best combination of germination bioprocess variables. The experimental values of *AoxA*, *TPC* and *TFC* of OGAF (Table 2) were similar to the predicted values, above mentioned, indicating that the optimal conditions of germination bioprocess were appropriated and reproducible.

Chemical Composition and Nutraceutical Properties of Optimized Germinated Amaranth Flour (OGAF)

Germination affects protein, lipid and dietary fibre contents of amaranth seeds (Table 2). The protein content of OGAF after 78 h had a trend toward a significant ($p<0.05$) increase (40 %) compared to that in the raw seeds. This increased protein content may be attributed to losses in dry weight, particularly in carbohydrates, through respiration during germination. The losses in dry weight can be accounted mainly as loss in sugars during respiration due to production of carbon dioxide and water, which escape from the seeds [23]. During germination process, soluble sugars are produced due to the needs of

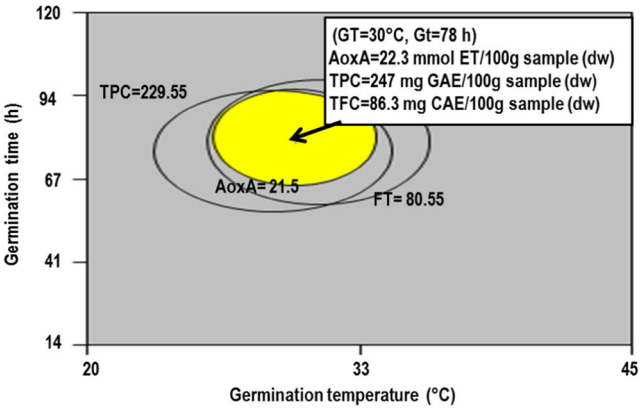

Fig. 1 Region of best combination of process variables (GT=germination temperature/Gt=germination time) for production of optimized germinated amaranth flour through germination bioprocess

Table 2 Proximate composition, physicochemical properties, antioxidant activity, and phytochemicals content of amaranth flours[a]

Property	Amaranth flour[b]	
	Unprocessed	Optimized germinated
Chemical composition (%, dw)		
Proteins	13.00±0.22^{B}	18.21±0.19^{A}
Lipids	7.68±0.05^{A}	5.36±0.03^{B}
Total dietary fibre	10.67±0.45^{B}	23.86±0.74^{A}
Soluble fibre	0.47±0.01^{B}	3.55±0.04^{A}
Insoluble fibre	10.20±0.42^{B}	20.31±0.79^{A}
Antioxidant activity ORAC[c]		
Free phytochemicals	2.32±0.12^{B}	11.26±0.20^{A}
Bound phytochemicals	3.08±0.12^{B}	10.30±0.21^{A}
Total phytochemicals	5.40±0.13^{B}	21.56±0.30^{A}
Antioxidant activity ABTS[c]		
Free phytochemicals	0.90±0.06^{B}	7.71±0.13^{A}
Bound phytochemicals	1.23±0.09^{B}	4.44±0.11^{A}
Total phytochemicals	2.13±0.12^{B}	12.16±0.23^{A}
Phenolic content[d]		
Free phenolics	12.14±0.05^{B}	146.04±1.50^{A}
Bound phenolics	14.51±0.08^{B}	101.59±2.10^{A}
Total phenolics	26.65±1.10^{B}	247.63±2.20^{A}
Flavonoid content[e]		
Free flavonoids	16.83±0.19^{B}	51.46±1.12^{A}
Bound flavonoids	9.18±0.07^{B}	29.93±1.22^{A}
Total flavonoids	26.01±0.14^{B}	81.39±1.17^{A}

[a] Data are expressed as means±standard deviation

[b] Means with different superscripts (A-B) in the same row are significantly different (Duncan, $p \leq 0.05$)

[c] mmol Trolox equivalents (TE)/100 g (dw)

[d] mg gallic acid equivalents (GAE)/100 g sample (dw)

[e] mg catechin equivalents (CAE)/100 g sample (dw)

growing plants [8]. The increase in protein content was similar as that found by Paredes-López and Mora-Escobedo [24] who reported that germination for 72 h of *A. hypochondriacus* increased the protein content by about 41 %. The increase in protein content after germination was also found in other seeds such as soybean [25] and mungbean [26].

Another major chemical component of amaranth seeds is lipid, which is a source of nutritional components and bioactive compounds such as mono and polyunsaturated fatty acids, tocopherols, and phytosterols. As shown in Table 2, following germination for 78 h, a significant (p <0.05) decline in amaranth total lipid content (30 % decrease) was observed. This result was lower than the reported by Paredes-López and Mora-Escobedo [24] who observed that germination of *A. hypochondriacus* for 72 h decreased its lipid content by 41 %. The decline is likely to be due to the use of the lipid as energy source during germination. Lipid content has also been reported to decrease during germination in soybean [25] and flax seeds [27].

The soluble, insoluble and total dietary fibre or fiber contents in amaranth seeds increased in 655, 99 and 124 %, respectively, after germination bioprocess (Table 2). The effect of germination on dietary fibre has been studied in peas where it was found that the total dietary fibre increased substantially during germination for about 100 % [28]. The germination process tends to modify the structure of cell wall polysaccharides of the seeds, possibly by affecting the intactness of tissue histology and disrupting the protein-carbohydrate interaction. This will involve extensive cell wall biosynthesis and therefore the production of new dietary fiber. Germination produces an increase of cellulose, hemicellulose, accompanied by an increase of pectic polysaccharides [28]. Therefore, germination of amaranth seeds in the present study appears to be an effective way to improve dietary fibre content in amaranth. The presence of dietary fiber in foods is important in health because they have been considered as functional ingredients to reduce colon cancer [29] and other diseases.

The germination bioprocess increased (p <0.05) free, bound and total phenolic contents of amaranth seeds 1,103, 600, and 829 %, respectively (Table 2). Some researchers [9–11] have suggested that the germination is an adequate and effective bioprocess for increasing the concentration of phenolic compounds in amaranth seeds, and hence their nutraceutical quality. This could be due to the release and biosynthesis of phenolic compounds. Cell wall-degrading enzymes are active during germination, and they contribute to modification of the cell wall structure of the grain. The significance of this lies in the fact that phenolic compounds such as hydroxycinnamates (*e.g.*, ferulic and *p*-coumaric acids) are bound to nonstarch polysaccharides in grain cell walls through associations such as ester and ether bonds. The action of cell wall-degrading enzymes (mainly esterases) on these bonds contributes to the release of bound phenolic compounds. On the other hand, activation of phenylalanine ammonia lyase (key enzyme in phenolic biosynthesis) during germination of seeds also has been previously reported [30].

Bioprocessing of the whole amaranth seeds throughout germination increased (p <0.05) free, bound and total flavonoid contents in 206, 226, and 213 %, respectively, when were compared with the unprocessed material (Table 2). Flavonoids are common constituents of pseudocereals and can provide health-promoting functions.

The AoxA, evaluated by ORAC assay, for free, bound and total phytochemicals increased in 386, 235, and 300 %, respectively, after germination (Table 2), while the AoxA, evaluated by ABTS method, for free, bound and total phytochemicals increased in 756, 261, and 470 %, respectively. Both, ORAC and ABTS methods, showed similar tendency. In general, our results show that bound phytochemicals, which commonly were underestimated in the literature,

contributed significantly to the total AoxA. Some researchers also reported that the germination increased the antioxidant activity of amaranth [8–11]; however, they did not evaluate AoxA in bound phenolic extracts. The increase in antioxidant activity with the germination bioprocess is one of the many metabolic changes that take place upon germination of seeds, mainly due to an increase in the content of phenolic compounds by the action of the endogenous hydrolytic enzymes, as discussed above [11]. Pasko et al. [10] have reported, in germinated amaranth, a highly significant correlation between TPC and AoxA.

The increase of antioxidant activity, phenolic and flavonoid contents in amaranth seeds shows a potentially important role of phenolics during seed germination, as well as the potential enhancement of the nutraceutical value of seeds by the germination process [31].

Conclusions

The optimum combination of bioprocess variables for the production of amaranth germinated flour with the highest antioxidant activity and total phenolic and flavonoid values was: germination temperature=30 °C/germination time=78 h. The optimized germination process resulted to be an effective strategy to increase the antioxidant activity, total phenolic and flavonoid, protein, and dietary fibre contents of amaranth seeds. Therefore, the optimized germinated amaranth flour could be used as a source of natural antioxidants, protein, and dietary fibre in the formulation of functional foods.

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