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Amaranth Seeds and Products – The Source of Bioactive Compounds

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In recent years, new products obtained from amaranth seeds have entered the food market including expanded "popping" seeds and flakes. Lipids and biologically-active substances dissolved in these products are susceptible to changes. Additionally, due to the fact that fat quality has high dietary importance, there is a need to conduct detailed quality and quantity studies on the lipid composition of *Amaranthus cruentus*.

For the samples under analysis, protein, fat, starch and ash content were determined. Fatty acids and sterols were analysed by gas chromatography. The analysis of tocopherols and squalene content was carried out with the application of high-performance liquid chromatography coupled with photodiode array and fluorescence detectors (HPLC-DAD-FLD).

Protein, fat and starch content did not change during seed processing. However in the case of tocopherols, the total tocopherol content was 10.6 mg/100 g for seeds, while in "popping" and in flakes it was reduced by approximately 35%. The squalene content ranged from 469.96 mg/100 g for seeds to 358.9 mg/100 g for flakes. No significant differences were observed in the fatty acid profile of seeds and products, but differences were observed in the sterol content.

INTRODUCTION

"Pseudocereals" is a term which has not been properly defined in the literature to date. This term covers plants such as amaranth, quinoa, tartary buckwheat, buckwheat and millet. Unlike plants that are recognised as true cereals, which are monocotyledonous plants, these are dicots. Owing to their chemical composition, seeds of these plants, just like cereal grains, are used for human nutrition [Ceglińska & Cacak-Pietrzak, 1999].

Amaranthus cruentus, a member of the *Amaranthaceae* family, is an unconventional plant that has gained popularity, both among consumers and farmers. The use of its seeds has also increased; it is now used as a source of lipids and a material for the production of flour, flakes, popped seeds, several sorts of bread [Januszewska-Jóźwiak & Synowiecki, 2008] and confectionery [Sindhuja *et al.,* 2005]. The amaranth-based products are a valuable source of nutrients and minerals. In addition, the amino acid profile of amaranth makes it an attractive source of protein. If consumed with other cereals, it is a balanced source of proteins; therefore, amaranth flour is often used in mixtures with maize or wheat [Alvarez-Jubete *et al.,* 2010; Escudero *et al.,* 2004]. It has also been reported that the addition of 10–20% expanded amaranth seeds instead of amaranth flour might be used to improve the nutritional value of bread [Bodroža-Solarov *et al.,* 2008]. Amaranth is rich in minerals including: magnesium, calcium, potassium and phosphorus, iron and sodium [Shukla *et al.,* 2006; Gajewska *et al.,* 2002]. Moreover, amaranth seeds and their products are a rich source of bioactive substances showing antioxidant properties [Worobiej *et al.,* 2009; Klimczak *et al.,* 2002]. The non-saponifiable substances of the grain lipid fraction include: squalene, tocopherols, sterols and others [Gamel *et al.,* 2007; Gorinstein *et al.,* 2007; León-Camacho *et al.,* 2001; Sun *et al.,* 1997].

The scope of this study was to examine the content of selected nutrients in the seeds, popped seeds and flakes of amaranth. Moreover, due to the importance of the fat quality on the products' dietary value and its influence on shelf life, a detailed examination of the lipid content was carried out to determine the fatty acid profile and the content of lipid-soluble bioactive substances.

MATERIALS AND METHODS

Materials

Amaranth (*Amaranthus cruentus)* seeds and commercially available products (expanded seeds - popping and flakes) were obtained from "Szarłat" company (Łomża, Poland). Seeds, popping and flakes were produced from the same lot of material.

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Proximate chemical composition

Assessment of the nutritional value of amaranth seeds included determination of starch content by the polarimetric method in a hydrochloric acid solution, in accordance to the procedure of the AOAC, following the guidelines provided by Bagdach, detailed in Rutkowska [1981]. Dry matter, protein and ash contents were determined following AACC methods 44–15, 46–10 and 08–03 [AACC, 1994], respectively. Protein content was calculated on the basis of total nitrogen using a 6.25 multiplication factor. Total fat was determined by the method described by Folch *et al.* [1957].

Analysis of the lipid fraction of amaranth seeds and amaranth products obtained as total lipids using the method described by Folch *et al.* [1957] involved characterisation of fatty acids, squalene, tocopherols and sterols.

Determination of fatty acids

Fatty acid methyl esters were prepared according to the method described by Zadernowski & Sosulski [1978]. The methyl esters were dried under a nitrogen stream, purified by hexane extraction and analysed by gas liquid chromatography (GLC) on a Fisons 8000 gas chromatograph equipped with a flame ionization detector (FID) (Carlo Erba, Italy). Separations were performed on a J&W Scientific DB-225 capillary column (30 m × 0.25 mm × 0.15 μm) using helium as a carrier gas. The GC oven was operated isothermally at 190°C. Peaks were identified on the basis of retention times determined for fatty acids standards mixture (Supelco® 37 Component FAME Mix) (Milwaukee, WI, USA).

Determination of squalene

The squalene content was determined with high performance liquid chromatography (HPLC), according to method described by Czaplicki *et al.* [2009] with modifications. Briefly, 0.01 g of oil (±0.0001 g) was transferred into a 10 mL measuring flask, and filled up with n-hexane. Next, 20 mL of diluted oil sample was injected into the chromatographic system. Analyses were conducted on a 1200 series high performance liquid chromatograph, manufactured by Agilent Technologies (Palo Alto, CA, USA) and equipped with a photodiode detector (PDA). Chromatographic separations were conducted on a LiChrospher RP-18 column (250 mm × 4.6 mm × 5 μm, Merck, Germany), at 30°C. The mobile phase contained acetonitrile, isopropyl alcohol and hexane at a flow rate of 1 mL/min. An analytical wavelength was set at 218 nm. An external calibration curve was used for quantitative analysis. The squalene analytical standard was supplied by Sigma-Aldrich (Milwaukee, WI, USA).

Determination of tocopherols

Analysis of tocopherols was carried out by high performance liquid chromatography (HPLC), according to the method described by Peterson & Qureshi [1993]. Briefly, 0.1 g of oil (±0.001 g) was diluted in n-hexane in a 10 mL measuring flask. After subsequent centrifugation (10 min at 16,000 rpm), the sample was transferred to a chromatographic vial. The analysis was carried out using a 1200 series liquid chromatograph manufactured by Agilent Technologies (Palo Alto, CA, USA), equipped with a fluorescent detector from the same manufacturer. Separations were performed on a LiChrospher Si60 column (250 mm × 4 mm × 5 μm, Merck). Next, 20 μL of sample was injected into the chromatographic column. A solution of 0.7% iso-propyl alcohol in hexane was used as a mobile phase at a flow rate of 1 mL/min. Excitation and emission wavelengths were set at 296 nm and 330 nm, respectively. Peaks were identified on the basis of retention times determined for α-, β-, γ- and δ-tocopherol standards (Calbiochem, UK) analysed separately, and their content was calculated using external calibration curves prepared for all analysed tocopherols.

Determination of phytosterols

Sterol content in oils was determined by gas chromatography coupled with mass spectrometry (GC-MS) according to the method described by Vlahakis & Hazebroek [2000]. The unsaponifiables were extracted with diethyl ether, and the extract was subsequently evaporated with rotary evaporator type R210 (Büchi Labortechnik AG, Postfach, Switzerland) under nitrogen. Dry residues were redissolved in 1.5 mL of n-hexane, and 0.2 mL (0.4 mg/mL) of 5α-cholestane internal standard solution was used. The extract was transferred into a vial and evaporated with a nitrogen stream. The residues were re-dissolved in 100 μL of pyridine and 100 μL of BSTFA (N,O-bis (trimethylsilyl) trifluoroacetamide) with 1% TMCS (trimethylchlorosilane) and left in the dark for 24 h to complete the derivatization reaction. This was followed by the addition of 1 mL of hexane, and 1 μL of obtained mixture was used for GC-MS analysis.

Analyses were conducted on a GCMS-QP2010S gas chromatograph coupled with mass spectrometer manufactured by Shimadzu Corporation (Japan). A DB-5ms (30 m × 0.25 mm × 0.25 μm film thickness) Agilent J&W Scientific (Perlan Technologies, Poland) capillary column was used for separation of phytosterols with helium as a carrier gas at a flow rate of 0.9 mL/min. The injector temperature was 230°C, and the column temperature was programmed as follows: 50°C for 2 min, a subsequent increase to 230°C at the rate of 15°C/min, to 310°C at the rate of 3°C/min, 10 min hold. The interface temperature of GC-MS was 240°C. Temperature of the ion source was 220°C and the electron energy was 70 eV. The total ion current (TIC) mode was used for quantification (100–600 m/z range).

Statistical analysis

All analyses were performed in three replications. The significance of differences between the mean values was estimated by Duncan's test. Statistical analysis was performed at a significance level of $*p<0.05$. The statistical analysis was conducted using Statistica v. 10.0 software (StatSoft, Kraków, Poland).

RESULTS AND DISCUSSION

What distinguishes amaranth seeds from other cereals is its high protein and valuable lipid contents and an abundance of diverse minerals [Grajeta 1997].

Protein content in amaranth seeds is 16.1% and is similar in popped seeds and flakes (Table 1). These results have

been confirmed by other researchers. Alvarez-Jubete *et al.* [2010] studied the chemical composition of amaranth seeds and found a protein content of 16.5%. Similar, but slightly higher protein levels, were determined by Bodroža-Solarov *et al.* [2008]: 16.96% in amaranth seeds and 16.89% in popped seeds. A protein content of 10.50% in popped seeds was found by Guerra-Matias & Arêas [2005]. There are few reports on the chemical composition of amaranth flakes. Piecyk *et al.* [2009] reported the protein content in flakes obtained from *Amaranthus cruentus* to be 16.25%. The same authors also analysed the fat content in seeds of *Amaranthus cruentus* and products obtained from them, with the following results: 7.91%, 6.35%, 6.88% for seeds, popped seeds and flakes, respectively. These results are lower than those reported in this study, where the total fat content determined by extraction with chloroform-methanol mixture was 8.3% (seeds), 8.03% (popped seeds), 7.15% (flakes) (Table 1). Similar results for *Amaranthus cruentus* have been reported by Ratusz & Wirkowska [2006], Becker *et al.* [1981] and Gamel *et al.* [2007]. Piecyk *et al.* [2009] analysed amaranth products and found that the fat content was 6.35% in popped seeds and 6.88% in flakes. The fat content determined in amaranth seeds is higher than that in cereal grains. For example, wheat contains 2.1% fat and maize contains 4.5% fat [Barba de la Rosa *et al.,* 2009].

Starch is the main component of amaranth seeds [Qian & Kuhn, 1999; Hoover *et al.,* 1998]. In the present study the starch content was 49.5% in amaranth seeds, 55.53% in popped seeds, and 48.9% in flakes (Table 1). These values are in accordance with those reported by other authors. Gajewska *et al.* [2002] determined the starch content at 54.3% in seeds and at 56.6% in popped seeds. A higher starch content in amaranth seeds, at 59.9–60.52 %, was found by Alvarez-Jubete *et al.* [2010], Bodroža-Solarov *et al.* [2008] and by Choi *et al.* [2004]. Bodroža-Solarov *et al.* [2008] examined popped seeds and found them to contain 61.25% of starch, whereas Piecyk *et al.* [2009] obtained the value of 61.73%. According to these authors, the starch content in amaranth flakes is 65.64%.

Water content in amaranth products ranged from 2.27% to 9.66% (Table 1). The lowest values were found in popped seeds, whereas the highest in the seeds. The popping process causes the product to dry out and therefore results in the consequent water loss [Gajewska *et al.,* 2002]. The ash content in seeds and products was alike; at 3.2%, 3.3%, 3.28% in seeds, popped seeds and flakes, respectively (Table 1). Similar results were obtained by Piecyk *et al.* [2009] and Gajewska *et al.* [2002]. Bodroža-Solarov *et al.* [2008] determined ash content in popped seeds to be 3.67%.

Like other vegetable originating lipids (those obtained from sunflower, maize, soya), the lipid fraction of amaranth seeds contains mostly unsaturated fatty acids [León-Camacho *et al.,* 2001; Dodok *et al.,* 1997] – ranging from 67 to 80% [Grajeta, 1997]. The saturated to unsaturated fatty acids ratio ranges from 0.2 to 0.5 [Grajeta, 1997].

Among the fatty acids present in amaranth seeds and products, the dominant one was linoleic acid ($C_{18:2}$), whose content was about 47%. No significant differences were observed for seeds, popped seeds and flakes (Table 2). León-Camacho *et al.* [2001] analysed seeds of *Amaranthus cruentus* and found linoleic acid at a concentration of 38.2%, similar values have been obtained by Gamel *et al*. [2007] in seeds and popped seeds. A much lower content of linolenic acid in popped seeds at 27.04% was determined by Singhal & Kulkarni [1990], but their result for seeds (46.79%) was similar. Palmitic ($C_{16:0}$) and oleic ($C_{18:1}$) acids were found in amaranth seeds and products at similar amounts from 23.45% to 24.37% of palmitic acid and about 24% of oleic acid. A lower content of palmitic acid and higher of oleic acid was determined by Gamel *et al.* [2007]. In our study, the content of stearic acid ($C_{18:0}$) was 4.16% in seeds, 3.9% in popped seeds and 3.32% in flakes. The lowest content was determined for α-linolenic acid: 0.69%, 0.81% and 0.77%, respectively. The results of this study confirm data reported by Berganza *et al.* [2003], who determined the stearic acid content ranging from 3.05% to 3.80%. They also analysed the content of squalene in seeds, which they determined in the range of 3.20% to 5.80%, depending on the species and place of cultivation.

TABLE 1. Proximate composition of amaranth seeds and its products (%).

Nutritional components	Seeds	Expanded seeds	Flakes
Protein	16.1 ± 1.3^a	15.8 ± 0.5^a	16.0 ± 1.0^a
Fat	8.30 ± 0.28^a	8.03 ± 0.22^a	7.15 ± 0.28^b
Starch	49.50 ± 0.32^a	55.53 ± 0.59^b	48.90 ± 0.48^a
Ash	3.20 ± 0.11^a	3.30 ± 0.22^a	3.28 ± 0.24^a
Water	9.66 ± 0.98^a	2.27 ± 0.07^b	9.22 ± 0.42^a

Results are the mean value of at least three replications, ± standard deviation. Different letters in the same row indicate significant differences, $p<0.05$.

TABLE 2. Fatty acids compositions of amaranth seeds and its products lipids (% of total fatty acids).

Fatty acid	Seeds	Expanded seeds	Flakes
$C_{16:0}$	23.45 ± 1.57^a	23.52 ± 0.37^a	24.37 ± 1.26^a
$C_{18:0}$	4.16 ± 0.37^a	3.90 ± 0.16^{ab}	3.32 ± 0.53^b
$C_{18:1}$	24.66 ± 1.76^a	24.74 ± 0.85^a	24.22 ± 1.86^a
$C_{18:2}$	47.05 ± 1.30^a	47.04 ± 0.76^a	46.72 ± 0.92^a
$C_{18:3}\alpha$	0.69 ± 0.14^a	0.81 ± 0.02^a	0.77 ± 0.80^a

Results are the mean value of at least three replications, ± standard deviation. Different letters in the same row indicate significant differences, $p<0.05$.

TABLE 3. Tocopherols content of amaranth seeds and its products (mg/100 g).

Tocopherol	Seeds	Expanded seeds	Flakes
α-Tocopherol	1.91 ± 0.33^a	0.49 ± 0.02^b	1.07 ± 0.08^c
β-Tocopherol	4.07 ± 0.44^a	1.08 ± 0.08^b	2.19 ± 0.13^c
γ-Tocopherol	1.29 ± 0.24^a	0.60 ± 0.02^b	0.57 ± 0.02^b
δ-Tocopherol	3.34 ± 0.38^a	2.35 ± 0.19^b	2.12 ± 0.01^b
Total tocopherols	10.61 ± 1.22^a	4.52 ± 0.31^b	5.95 ± 0.27^b

Results are the mean value of at least three replications, ± standard deviation. Different letters in the same row indicate significant differences, $p<0.05$.

TABLE 4. Squalene and phytosterols content of amaranth seeds and its products (mg/100 g).

Compound	Seeds	Expanded seeds	Flakes
Squalene	469.96±104.23 [a]	428.64±25.08 [a]	358.90±16.02 [a]
Campesterol	3.49±0.00 [a]	4.88±0.18 [b]	4.36±0.07 [c]
24-Methylenecholesterol	2.51±0.01 [a]	1.91±0.31 [b]	1.70±0.22 [b]
Δ7-Stigmastenol	20.85±1.29 [a]	20.43±0.63 [a]	18.45±1.93 [a]
α-Spinasterol plus sitosterol	100.03±1.84 [a]	124.22±5.63 [b]	112.39±0.09 [c]
Stigmasterol	2.64±0.41 [a]	1.24±0.50 [b]	1.20±0.05 [b]
Sitostanol	1.94±0.04 [a]	1.01±0.01 [b]	1.13±0.13 [b]
Δ5-Avenasterol	0.61±0.02 [a]	0.63±0.02 [a]	0.63±0.16 [a]
Δ7-Ergosterol	28.64±1.05 [a]	24.07±0.57 [b]	24.82±2.57 [b]
Δ7-Avenasterol	14.37±0.67 [a]	10.86±0.46 [b]	11.44±1.23 [b]
Cycloartenol	n. d.	0.31±0.08 [a]	0.35±0.05 [a]
Citrostadienol	n. d.	0.69±0.08 [a]	0.63±0.02 [a]
5,24-Stigmastadienol	2.58±0.15 [a]	n. d.	5.89±0.48 [b]
Total sterols	177.66±5.48 [a]	190.25±8.47 [a]	182.99±7.00 [a]

Results are the mean value of at least three replications, ± standard deviation. Different letters in the same row indicate significant differences, $p<0.05$. n. d. – not determined.

Vegetable oils provide the richest source of tocopherols in the diet. The *Amaranthus cruentus* seeds and the products analysed in this study were rich in tocopherols (Table 3). The highest tocopherols content was found in seeds, followed by flakes. A slightly lower tocopherols content, with no significant difference, was found in popped seeds than in flakes. β-Tocopherol is the dominant homologue found in amaranth seeds, with determined contents at 4.07 mg/100 g, which contributes to approximately 38% of the total tocopherols. A contribution of 32% and 18% was found for δ-tocopherol and α-tocopherol, respectively. The lowest content was determined for γ-tocopherol – 1.29 mg/100 g, which accounted for 12% of all tocopherols. The dominant homologue in popped seeds was δ-tocopherol, with approximate contents at 2.35 mg/100 g of the product (52% of all tocopherols). Contribution of β-tocopherol was much lower – 24% of total tocopherols. On the other hand, similar contents were found of γ-tocopherol and α-tocopherol. The content of β-tocopherol and δ-tocopherol in amaranth flakes was also similar, 2.19 mg/100 g and 2.12 mg/100 g of the product, respectively. α-Tocopherol content was determined at 1.07 mg/100 g, which accounted for 18% of the total tocopherols in the flakes. The content of γ-tocopherol was the lowest. There are no reports in the literature about the content of different tocopherols in amaranth products. According to results of this study, the content of tocopherols in amaranth products decreases when compared to the raw seeds. According to a study by Réblová [2006], considerable decreases in the activity of α- and δ-tocopherols can be observed by heating oils at temperatures above 100°C. Table 4 shows the content of squalene and phytosterols in seeds of *Amaranthus cruentus* and products based on them. The highest squalene content was found in seeds, followed by popped seeds and flakes. However, mentioned results were not significantly different at the level of $p<0.05$. The results are consistent with the findings of other authors. For example, León-Camacho *et al.* [2001] analysed seeds of *Amaranthus cruentus* and found contents of squalene at 416 mg/100 g. Similar values presented in the paper by Bodroža-Solarov *et al.* [2008] were – 517.1 mg/100 g in seeds and 485.5 mg/100 g in popped seeds. Singhal & Kulkarni [1990] examined seeds of *Amaranthus cruentus* and determined the squalene content in seeds to be at 4884 mg/100 g of fat, whereas in popped seeds the squalene content was 5642 mg/100 g of fat. These results are in concordance with the current findings. In our research the squalene content was 5662 mg/100 g of fat in seeds and 5338 mg/100 g of fat in popped seeds. As amaranth oil many other vegetable oils contain squalene. Some of them are not as rich as amaranth oil but their consumption is higher. As reported by Owen *et al.* [2000], virgin olive oil contains 424 mg/kg of this compound, whereas squalene in argan oil is reported to reach 314 mg/100 g, in pumpkin seeds oil 89 mg/100 g, in quinoa oil 58.4 mg/100 g and in sunflower oil 6 mg/100 g [Khallouki *et al.,* 2003; Ryan *et al.,* 2007].

Sterols are the major constituents of the non-saponifiable fraction of most edible oils [León-Camacho *et al.,* 2001]. Consumption of phytosterols is believed to positively affect the human blood cholesterol level. According to numerous reports, if phytosterols are consumed regularly at 1–3 mg/day dose, phytosterols and/or phytostanols may reduce blood cholesterol levels by 10 to 15% [EFSA Report, 2008]. Other studies have shown that phytosterols may prevent cancer, atherosclerosis and inflammations [Ryan *et al.,* 2005].

As it is presented in Table 4, the dominant sterols in amaranth seeds and products were α-spinasterol and sitosterol. Although, the content of these dominant sterols was significantly different in seeds and products, the total sterols content did not differ in the analysed samples. The results are consistent with those reported by Grajeta [1997], according to which the spinasterol content in amaranth seeds ranges from 46 to 54% of total sterol content. Both amaranth seeds and products contained considerable amounts of Δ7-sterols *i.e.*: Δ7-ergosterol, Δ7--stigmastenol and Δ7-avenasterol. The other sterols determined in amaranth include: 24-methylenecholesterol, Δ5-avenasterol,

citrostadienol, cycloartenol, campesterol, sitostanol and stigmasterol as well as 5,24-stigmastadienol. Amaranth seeds were not found to contain cycloartenol or citrostadienol, but these compounds were present in popped seeds and in flakes. The results are in accordance with previously published data. In the study conducted by Berger *et al.* [2003], the total phytosterol content in crude oil was 2.73 g/100 g.

A comparison of the results of studies concerning the presence of phytosterols in amaranth seeds, conducted by the methods applied so far indicates that the identification of sterols is problematic. For example, in the study conducted in 2001, León-Camacho *et al.* [2001] found that the main phytosterol present in amaranth oil was clerosterol; however the authors did not find α-spinasterol in the analysed samples. In an experiment conducted by Chernenko *et al.* [1999], the non-saponifiable fraction of amaranth oil, analysed by mass spectrometry, contained high levels of α-spinasterol and β-sitosterol, stigmasterol, campesterol and cholesterol, but no Δ7-sterols. On the other hand, Marcone *et al.* [2003] found only β-sitosterol, stigmasterol and campesterol in different kinds of amaranth seeds. To avoid such diversity in results obtained in different studies, sterol identification should be performed by chromatographic techniques coupled with a mass spectrometer, which could aid the unambiguous identification of individual sterols.

CONCLUSIONS

1. The protein content in amaranth seeds, popped seeds and flakes is up to 16%. No effect of technological treatment on protein content in the material was observed.
2. The main component of amaranth seeds and products is starch, which accounts for about 50% of dry matter.
3. Technological treatment of amaranth to produce popped seed and flakes causes a decrease in tocopherol by about 50%.
4. Amaranth seeds are a source of vegetable oil with a high squalene content.

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