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Processing Stability of Squalene in Amaranth and Antioxidant Potential of Amaranth Extract

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The processing stability of squalene in amaranth and the antioxidant capacity of the oil-rich fraction of amaranth were studied. The processes investigated were continuous puffing and roasting. Puffing was carried out using a single screw extruder, while roasting was carried out in a convection oven. High-performance liquid chromatography was used to quantify squalene content before and after processing. The L-ORAC method was used to study the antioxidant activity of pure squalene and lipophilic amaranth extract containing squalene. It was found that squalene was stable during all of the processing operations with a maximum loss of 12% during roasting (150 °C, 20 min) and no loss during puffing. The L-ORAC test showed pure squalene to be a weak antioxidant, whereas the lipophilic extract of amaranth showed higher antioxidant activity as compared to pure squalene at the same concentration, suggesting that tocotrienols and other minor ingredients also played a role as antioxidants.

KEYWORDS: Amaranth; squalene; L-ORAC; antioxidant

INTRODUCTION

Amaranth (*Amaranthus hypochondriacus*, *Amaranthus cruentus*, and *Amaranthus caudatus*) was cultivated by the Aztecs and Incas in ancient times. Today, it is grown in many tropical, subtropical, and temperate countries (*1*). It is a robust crop and can withstand a wide range of climatic conditions. Amaranth is rich in proteins, which have a balanced amino acid profile and are rich in lysine. Amaranth oil is highly unsaturated, containing about a 70% oleic acid fraction, and is a rich source of squalene and tocotrienols (*2*).

Squalene is an intermediate in cholesterol biosynthesis and is found in humans under the skin and inside the adipose tissue. It has significant use in the cosmetics and pharmaceutical industry as a solvent and as a moisturizer. The richest source of squalene is shark liver oil. Interest in more sustainable sources of squalene has increased recently due to a ban on shark hunting, and amaranth is the richest source of squalene among the plant sources (*2*). Squalene is a polyprenyl compound with six prenyl units (*3*). The molecular formula of squalene is $C_{30}H_{50}$, and the IUPAC nomenclature is (*all-E*)-2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene. The six double bonds in squalene are trans and are isolated from one another. Squalene at room temperature is a colorless viscous liquid with a bland taste and mild aroma. It is a nonvolatile compound with a high boiling point (284 °C). The double bonds impart considerable reactivity to squalene; however, it is generally stable to peroxidation, and many other reactions as double bonds are isolated from each other (*3*). **Figure 1** shows the molecular structure of squalene.

Squalene has been found to act as an anticancer agent (*4*−*7*) and has hypocholesterolemic effects (*8, 9*). Squalene acts as a hyocholesterolemic agent by inhibiting HMG-CoA reductase, a necessary enzyme in cholesterol biosynthesis. This also leads to reduction in the concentration of intermediates such as farnesyl pyrophosphate, a compound capable of farnesylating the Ras oncoprotein p21. Farnesylation of these proteins increases their carcinogenic activity. Thus, increasing squalene consumption leads to lowering the concentration of such oncoproteins, which is the reason for anticancer activity of squalene (*6*). Squalene can also act as a sink for xenobiotics, as it can dissolve most hydrophobic compounds (*10*). The very limited evidence showing the antioxidant activity of squalene (*3, 11, 12*) indicates that it may act as a quencher of photogenerated singlet oxygen, explaining its presence under the skin in large concentrations. No study has been reported showing the antioxidant activity in terms of widely accepted parameters such as the ORAC value. The only study pertaining to processing stability of amaranth has been on the frying stability of squalene in which it was found to be reasonably stable (*13*).

The overall objective of this research was (i) to study the stability of squalene during conventional processing methods like heat-induced puffing (popping) and roasting and (ii) to study the antioxidant activity of pure squalene and lipophilic extract of amaranth and to determine the effect of processing on their antioxidant activities.

MATERIALS AND METHODS

Materials. *Amaranth.* Amaranth seeds were procured from Barryfarm (Wapakoneta, OH). The seeds were a commercial blend of different varieties of amaranth, and the exact species of the seeds were not known.

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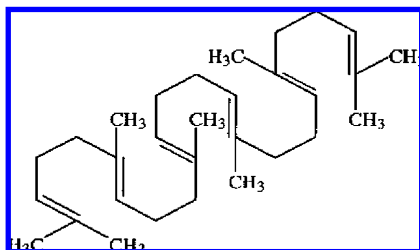

Figure 1. Structure of squalene.

Chloroform [high-performance liquid chromatography (HPLC) grade], hexane (HPLC grade, 99% pure), acetone, α-amylase, and docosane were procured from Fischer Scientific (St. Louis, MO). The L-ORAC reagents were as follows: AAPH [2,2′-azobis (2-amidinopropane) dihydrochloride] was purchased from Wako Chemicals (Richmond, VA). Trolox and disodium fluorescein (FL) were obtained from Sigma-Aldrich (Milwaukee, WI). Randomly methylated β-cyclodextrin (RMCD) (Trappsol) (pharmacy grade) was procured from Cyclodextrin Technologies Development Inc. (High Springs, FL).

Puffing of Amaranth. We developed a new technique of using a single screw extruder with a tapered screw and without a die to puff the amaranth seeds. This processing technique enabled us to have accurate control over the temperature and the residence time of the seeds.

A C. W. Brabender (Hackensack, NJ) (model, Plasti-Corder) single screw extruder without a die was used for puffing of amaranth seeds. A tapered screw with a 3:1 compression ratio and having a L/D (length/diameter) ratio of 20:1 was used to convey the seeds. The dimensions of the screw were as follows: length, $L = 380$ mm; maximum diameter, $D = 19$ mm; and pitch $P = 19$ mm. The maximum channel depth was 3.72 mm near the hopper end, and the minimum channel depth at the die end was 1.23 mm. The extruder barrel could be heated to a preset temperature, and the screw speed could be varied from 0 to 232 rpm.

The extruder operating conditions were obtained from the preliminary experiments carried out by varying the screw speed and barrel temperature. The seeds were fed into the extruder through the hopper at the rate of 16−20 g/min manually. As seeds passed though the screw channel and the barrel wall, they came in contact with the heated barrel, and the temperature started to increase. As the seeds progressed further downstream where the channel depth of the screw was more shallow, the seeds came in constant contact with the wall, leading to a further increase in temperature. Toward the end of the barrel, the seeds essentially formed a monolayer in the screw channel and the temperature was high enough for the seeds to puff. The mass flow rate was measured at the exit of the extruder, and it was controlled by feeding a known and fixed amount of grain in a definite time period, as uniformly as possible. The final operating window for the extruder was as follows: screw speed in the range 75−175 rpm and barrel temperature in the range 250−290 °C.

Roasting of Amaranth Flour. A natural convection oven (Fisher Scientific Isotemp vacuum oven model 282A) was used for roasting the amaranth flour. The oven was set to the desired temperature, and 50 g of amaranth flour was spread in a thin layer in a metallic tray (760 mm × 240 mm) and placed in the oven for a specific duration. After it was roasted, the flour was allowed to cool for 2 min in open air, filled in poly ethylene zip-lock bags, and immediately stored at −4 °C till further use.

Quantification of Squalene. The squalene content was measured in amaranth samples processed at the following conditions of puffing and roasting. Puffing: (i) 250 °C, 75 rpm; (ii) 270 °C, 100 rpm; (iii) 290 °C, 125 rpm; and (iv) 290 °C, 150 rpm. Those processing conditions were chosen that gave a puffing efficiency of more than 50%. Roasting: (i) 125 °C, 5 min; (ii) 125 °C, 20 min; (iii) 150 °C, 5 min; and (iv) 150 °C, 20 min. The rationale behind choosing these processing conditions was that (i) the moisture content of the final product was low enough to make the flour shelf stable, (ii) the roasted flour gave a characteristic pleasant aroma, and (iii) the experimental design would help to examine the effect of time and or temperature on stability.

After the moisture content was measured, the raw and the processed amaranth flour samples were weighed and dispersed in 50 mL of solution containing 0.6% (w/v) α-amylase. The solution was kept at 25 °C for 24 h. The rationale behind this step was to hydrolyze the starch that would help in releasing lipids that are physically entrapped in the starch or starch−protein matrix (*14*).

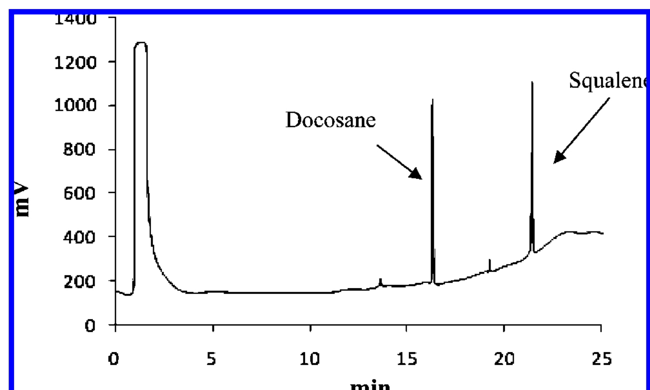

Figure 2. Chromatogram for docosane and squalene in extract.

The slurry was extracted after 24 h using 250 mL of chloroform in two stages followed by washing with chloroform to ensure complete extraction. The chloroform extract was then concentrated using a Buchi vacuum evaporator. The concentrated extract was then dissolved in hexane, injected with 1 mL of docosane (20 mg/mL in hexane) as an internal standard (docosane is a C:22 aliphatic hydrocarbon with bp close to that of squalene, i.e., 224 °C), and made up to 10 mL. A 0.2 mL aliquot of this stock was then taken and diluted up to 10 mL with hexane. One microliter of this solution was injected into a gas chromatography instrument (HP 5890 Series II, FID detector). A DB-5MS (60m) column was used, and splitless injections were done. The gas flow rates were as follows: air, 300 mL/in.; hydrogen, 30 mL/min; and helium (carrier gas), 30 mL/min. The settings for GC were as follows: injector temperature, 300 °C; detector temperature, 320 °C; initial oven temperature, 80 °C; and final oven temperature, 310 °C at a ramp of 10 °C/min. PeakSimple (model:203) software was used for the data acquisition. **Figure 2** shows a chromatogram for squalene and docosane in amaranth extract. Squalene was quantified by comparing the area under the curve (AUC) for squalene to that of a known concentration of docosane incorporated in each sample.

L-ORAC for Pure Squalene and Amaranth Extract. The modified L-ORAC method was used to study the antioxidant capacity of pure squalene and amaranth extracts (*15, 16*). A 0.045 g FL sample was dissolved in 100 mL of 0.075 M phosphate buffer (pH 7). One-half milliliter of this stock was further diluted to 100 mL with phosphate buffer. A working FL solution was made by further diluting 0.8 mL of the above solution to 50 mL with phosphate buffer.

A 17.2 mg/mL solution of AAPH was made in phosphate buffer. A 5% solution of RMCD was made using phosphate buffer. Trolox solution was made by dissolving 0.375 g of trolox into 50 mL of phosphate buffer; 0.1 mL of this solution was further diluted to 10 mL by using 3.5 mL of acetone and 6.5 mL of 5% RMCD solution.

Approximately 0.5 g of pure squalene was first dissolved in 3.5 mL of acetone and then made up to 10 mL by adding 5% RMCD solution. Four milliliters of this solution was diluted to 10 mL using phosphate buffer and used as a working solution. Four grams of amaranth flour (from raw or processed samples) was dispersed in 50 mL of 0.6% (by weight) α-amylase solution made in distilled water and incubated for 24 h at 25 °C. The lipids were then extracted using 250 mL of chloroform. Chloroform was then evaporated, and the extract was concentrated using a Buchi vacuum evaporator. The extract was then made to 10 mL using 6.5 mL of 5% RMCD solution and 3.5 mL of acetone. One-half milliliter of this solution was then diluted to 10 mL with 5% RMCD solution and was used as a working solution.

In a clean quartz cuvette, 2.7 mL of FL solution was added, followed by the addition of 0.25 mL of varying concentrations of trolox/amaranth extract/squalene. One milliliter of AAPH was injected into the cuvette and stoppered. The reaction mixture was mixed for 10−15 s using a Fischer Vortex Genie 2 shaker and then placed in a Varian Cary Eclipse fluorescence spectrophotometer (excitation wavelength, 485 nm; emis-

Table 1. Stability of Squalene during Various Puffing Conditions[a]

puffing parameter	squalene content (mg/100 g dw of flour) (SD)
raw	386.00 (14.1) a
250 °C, 75 rpm	409.8 (18.6) a
270 °C, 100 rpm	392.6 (23. 3) a
290 °C, 125 rpm	378.6 (19.3) a
290 °C, 150 rpm	385.3 (17.3) a

[a] Values in parentheses are SDs. Each number is an average of six measurements. The same letter (a) in the column indicates that the values are not significantly different from each other.

Table 2. Stability of Squalene during Various Roasting Conditions[a]

processing parameters	mg/100 g flour (dw) (SD)	% loss of squalene
raw	386.0 (10.0) a	
125 °C, 5 min	382.2 (11.1) a	0.98
150 °C, 5 min	347.7 (33.5) a	9.92
125 °C, 20min	337.5 (14.1) a	12.7
150 °C, 20min	339.2 (28.3) a	12.12

[a] Values in parentheses are SD. Each number is an average of six measurements. The same letter (a) in the column indicates that the values are not significantly different from each other.

sion wavelength, 510 nm). The fluorescence intensity was measured immediately ($t = 0$) and then measured every 2 min until the fluorescence dropped to less than 2% of the starting intensity.

The percentage relative fluorescence was plotted against time. The AUC for each concentration was plotted against the corresponding concentration of the antioxidant in the reaction mixture. The relative ORAC value was calculated from the following formula (eq 1) (*15*):

$$\text{relative ORAC} = \frac{[(\text{AUC}_{\text{sample}} - \text{AUC}_{\text{blank}})/(\text{AUC}_{\text{trolox}} - \text{AUC}_{\text{blank}})]}{(\text{molarity of sample}/\text{molarity of trolox})} \quad (1)$$

RESULTS AND DISCUSSION

Stability of Squalene during Puffing. Samples obtained from puffing conditions that gave a puffing efficiency greater than 50% were tested for the stability of squalene. The results for average squalene content are shown in **Table 1**. An *F* test showed that the mean values of squalene content for raw and puffed amaranth samples were not statistically significantly different from each other (as indicated by same letter in **Table 1**) ($\alpha = 0.05$). The excellent processing stability of squalene could be attributed to the HTST (high temperature−short time) nature of the process with the residence time at high temperature being in the range of 9−15 s.

Stability of Squalene during Roasting. Table 2 shows the squalene content of samples roasted at different conditions. Although an *F* test showed that there is no statistically significant difference between raw (the control sample) and any of the samples processed at different temperature−time combinations ($\alpha = 0.05$), an average maximum measured loss of approximately 12% could be seen for sample processed at 150 °C, 20 min.

Antioxidant Activity of Pure Squalene and Lipophilic Amaranth Extract. Figure 3a shows the standard fluorescence decay curves obtained at different concentrations of trolox. From **Figure 3b**, it can be seen that trolox shows a linear dose-dependent response. **Figure 4** shows a plot of AUC vs concentration of pure squalene. A straight line was fitted into the data for squalene to compare with the trolox curve. The relative ORAC value of squalene was found to be 0.00062, which means that squalene has an extremely low antioxidant activity as compared to trolox.

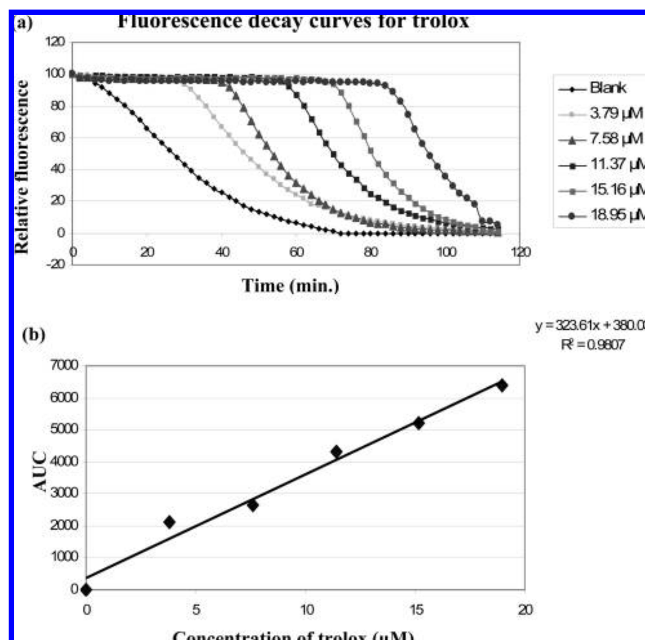

Figure 3. (**a**) Fluorescence decay curve using trolox; (**b**) AUC vs concentration for trolox.

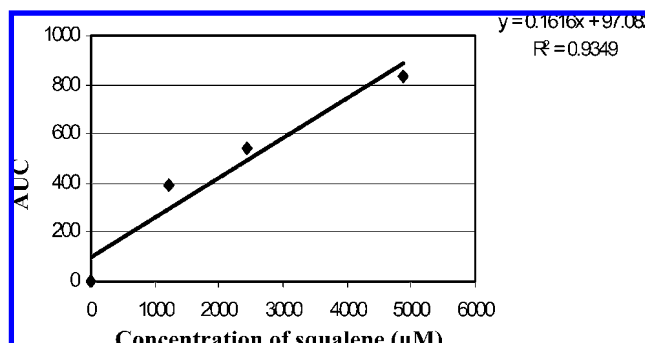

Figure 4. AUC vs concentration for squalene.

Table 3. Antioxidant Activity of Pure Squalene and Raw and Processed Amaranth Flour Expressed as μmol Trolox Equiv/g[a]

sample	μmol trolox equiv/g (SD)
pure squalene	0.74 (0.31) (/g squalene)
raw amaranth	0.14 (0.03) (/g flour) a
puffed amaranth: 250 °C, 75 rpm	0.71 (0.65) (/g flour) a
puffed amaranth: 270 °C, 100 rpm	0.62 (0.30) (/g flour) a
puffed amaranth: 290 °C, 125 rpm	0.29 (0.41) (/g flour) a
roasted amaranth: 150 °C, 20 min	0.39 (0.55) (/g flour) a

[a] Values with the same letter (a) are not significantly different from each other statistically.

An antioxidant capacity assay for raw amaranth, puffed amaranth (250 °C, 75 rpm; 270 °C, 100 rpm; and 290 °C, 125 rpm) and roasted amaranth (150 °C, 20 min.) was performed. The above-mentioned puffing conditions that gave maximum puffing efficiency and the roasting condition that led to maximum squalene loss were chosen for the antioxidant assay. The antioxidant activity can also be reported in terms of μmol trolox equiv/g sample by comparing the AUC for the extract with that of trolox and then by multiplying the corresponding concentration of trolox by the dilution factor. **Table 3** shows these values for pure squalene and raw and processed amaranth flour. From the data in **Table 3**, it can be seen that pure squalene and amaranth flour (raw and processed) have comparable

antioxidant values on a per gram basis. However, 1 g of flour contained only 3.37−3.86 mg of squalene (depending upon whether it was raw or processed), which is extremely low as compared to 1 g of pure squalene. However, because the relative ORAC value for squalene was extremely low (∼0), it can be concluded that the contribution of squalene to antioxidant activity of amaranth flour is negligible. In spite of negligible activity of squalene, the amaranth extract showed considerable antioxidant activity, which could be due to tocotrienols and other minor components that are present in amaranth oil. The antioxidant activity of amaranth extract is comparable with other cereal extracts, almonds, and many fruit extracts (*17*).

Statistically, the values for raw and processed amaranth samples were not found to be significantly different ($\alpha = 0.05$) (shown by same letter in the column in **Table 3**), although the trend of higher trolox equivalent for processed samples as compared to raw was consistent and discernible. This could be due to change in extractability of minor components after processing (as the extraction was standardized only for complete squalene extraction and not for other minor components like tocotrienols). Another reason could be the generation of compounds having antioxidant properties during processing. The Maillard reaction can play a significant role as both lysine (amaranth proteins are rich in lysine) and sugars (from starch) are present in flour at significant proportions. Several prior studies have found that Maillard reactions lead to the synthesis of various compounds having antioxidant properties (*18*−*20*). This needs to be further researched.

In summary, it was found that squalene was stable during all of the puffing conditions studied, with no significant loss during any puffing condition. Squalene showed good stability after roasting, and the maximum loss was found to be 12.7% under the conditions tested. L-ORAC antioxidant studies showed that squalene had very low antioxidant activity. However, the lipophilic amaranth extract showed significant antioxidant activity that was comparable with nuts and cereal grains, etc. The antioxidant activity of puffed and roasted samples was slightly higher as compared to raw amaranth seeds, which could be due to enhanced extractability of other minor ingredients like tocotrienols after processing and/or due to generation of Maillard reaction products (MRPs) having antioxidant activity.

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