Saponins, phytic acid, tannins and protease inhibitors in quinoa (*Chenopodium quinoa*, Willd) seeds

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The seeds of quinoa (*Chenopodium quinoa*, Willd), a food crop of the Andean region of Latin America, contain protein of good quality and high amounts of carbohydrates, fat, vitamins, and minerals. An industrial process for manufacturing infant food using quinoa as a basic raw material is being developed. The presence of antinutrients are of importance in this context, and this paper deals with saponins, phytic acid, tannins and protease inhibitors in quinoa seeds.

The samples of quinoa analysed in this experiment contained two main types of saponins. The amount of saponin A (β-D-glucopyranosyl-[β-D-glucopyranosyl-(1 → 3)-α-L-*arabino*-pyranosyl-(1 → 3)]-3-β-23-dihydroxy-12-en-28-oate methyl ester) was 0·7% of the dry weight and that of the saponin B (β-D-glucopyranosyl-[β-D-glucopyranosyl-(1 → 3)-α-L-*arabino*-pyranosyl-(1 → 3)]-3-β-23-dihydroxyolean-12-en-28-oate was 0·2% of the dry weight. These were the major saponins found in the quinoa bran collected while polishing the seeds.

After scrubbing and washing, the level of saponin-A remaining in the seeds decreased to 0·31% of the dry weight, and saponin-B was completely removed by this process.

The content of phytic acid in the quinoa seeds was about 1% of the dry matter, and scrubbing and washing reduced the phytic acid content of the seeds by about 30%. Neither protease inhibitor nor tannins were detected in the quinoa seeds.

INTRODUCTION

Quinoa (*Chenopodium quinoa*, Willd) has been an indigenous crop of the Andean region of South America since ancient times. Quinoa can be used as flour for making bread or as whole grain in gruels, porridge or soups. The protein content in quinoa (15% dry basis) is much higher than that found in cereals such as wheat, barley, oats, rice and sorghum (Ruales & Nair, 1992*a*; Kent, 1984). The results of animal feeding experiments showed high net protein utilization for raw whole (76) and polished and washed quinoa (74) (Ruales & Nair, 1992*a*). Quinoa also contains more carotene, riboflavin, tocopherols and folic acid than wheat, rice, oats and maize, and can supply the daily requirements of certain vitamins and several minerals for children between 1 and 3 years (Ruales & Nair, 1992*b*).

One of the factors which limit the widespread utilization of quinoa is its bitter taste caused by the presence of saponins. Saponins are triterpene glucosides that consist of a linear arrangement of one to six hexose or

pentose glycoside units joined to the sapogenin aglycone, which can be a steroidal or a triterpenoid aglycone. Quinoa saponins are soluble in methanol or water. They produce stable foams in aqueous solutions, and haemolyse red blood cells (Ruiz & Amaya, 1979; Birk & Peri, 1980; Oakenfull, 1981; Price *et al.*, 1987, 1989). Saponins are reported to be toxic for cold-blooded animals and they were used as fish poison by the inhabitants of South America. Some saponins form chemical complexes with iron and reduce its absorption (West & Greger, 1978; West *et al.*, 1978; Southon *et al.*, 1988; Price *et al.*, 1989). However, no evidence of formation of complexes of saponin with vitamin A, E or D_3 was found by West & Greger (1978). Saponins are located on the outer layers of the seeds, and can be removed by polishing and washing with water.

Phytic acid (myoinositol hexaphosphoric acid) reduces the availability of many minerals like iron, zinc, calcium and magnesium. The formation of iron–phytate complexes of low solubility, in the small intestine is considered to be the basis for the interference of phytate with iron absorption (Davies & Reid, 1979; Hallberg, 1984; Hallberg & Rossander, 1984; Hallberg *et al.*, 1987; Brune, 1989). Although the iron content in

cereals is usually high, the iron absorption from them is often poor due to the presence of high amounts of phytates (Brune, 1989; Sandberg & Svanberg, 1991). Adverse effects on zinc utilization when the diet contains large amounts of phytates were also reported by Sandberg (1990). They are also found to inhibit the proteases and amylases (Vaintraub & Bulmaga, 1991) of the intestinal tract. Quinoa contains many essential minerals like calcium (874 mg/kg), phosphorus (5·3 g/kg), iron (5·3 g/kg) and zinc (36 mg/kg) and their availability is of importance in relation to its use as a basic source of nutrients in an infant food.

Polyphenolic compounds like tannins are known to interfere with digestion and absorption in monogastric animals (Eggum *et al.*, 1983; Bach Knudsen *et al.*, 1988). Polyphenols bind proteins, and those which precipitate protein from aqueous solutions are usually called tannins (Makkar *et al.*, 1988). They form complexes, not only with dietary proteins, but also with digestive enzymes, thus reducing the digestibility of protein in foods (Singh & Eggum, 1984).

Many plant foods contain substances which inhibit the activity of certain proteolytic enzymes, reducing, in this way, the digestibility of dietary proteins (Liener & Kakade, 1980). Some protease inhibitors like the heat-labile trypsin inhibitor found in raw soybeans are, however, inactivated by heat treatment. In infant foods the factors which affect the digestibility of the protein are especially important.

The present paper deals with the determination of saponins, phytic acid, tannins and protease inhibitors present in quinoa seeds.

MATERIALS AND METHODS

Materials

Seeds of quinoa (*Chenopodium quinoa*, Willd) variety Latinreco-40057, were from the experimental farm of Latinreco, Nestlé Research Centre in Quito, Ecuador, where quinoa was grown under controlled conditions of agriculture, harvesting and storage. The saponin standards used in high-performance liquid chromatography (HPLC) analysis, saponin A and saponin B, were isolated from quinoa seeds by Dr C. Borel, Nestlé Research Centre, Vevey, Switzerland.

All other reagents were of analytical grade.

Methods

Sample preparation

Quinoa seeds were scrubbed and polished using a pulper (model 5707, Langsenkamp, Indianapolis, IN, USA) and, after removing the dust, they were washed with running tap water for 20 min and finally dried (Ruales & Nair, 1992*a*).

The material separated with a grain separator (model 100, Hance Corp., Westville, OH, USA) from the seeds, after scrubbing and polishing the quinoa seeds, was collected as quinoa bran.

The samples of clean dry quinoa were milled to a flour in a sample mill (Cyclotec 1093, Tecator AB, Höganäs, Sweden) equipped with a sieve of 0·25 mm pore size.

Saponins

A modified HPLC method described by Kesselmeir *et al.* (1981) was used for the determination of saponins. The saponins were extracted from 15 g of flour with 150 ml of methanol for 24 h, using a Soxhlet apparatus, after the seeds were defatted with 150 ml of petroleum ether for 16 h. After the extraction, the methanol was evaporated at 35°C using vacuum, and the residue was dissolved in 5 ml of methanol. Separation of the saponins was performed by injecting 20 μl of the sample into a high-performance liquid chromatograph (Varian Model 5000 Liquid Chromatograph, Varian Associates, Sunnyvale, CA, USA) equipped with a column (4 mm $\times$ 250 mm) packed with LiChrospher 100 CH-8/2 (5 μm) a UV detector and an integrator (Shimadzu C-R3A Chromatopac, Kyoto, Japan). The detection wavelength was 200 nm. A gradient elution was performed with 25 to 40% acetonitrile in water during 15 min. The flow rate was 2·0 ml/min. Saponins A and B isolated from quinoa seeds were used as standards.

Phytic acid

Phytate was quantified as hexaphosphate equivalents, using the method described by Harland & Oberleas (1986). The sample (2 g of quinoa flour) was mixed with 40 ml of HCl (2·4%) by shaking vigorously for 3 h at 20°C. The suspension was filtered with vacuum through a Whatman No. 1 paper. An aliquot (1 ml) of the filtrate was mixed with 1 ml of EDTA/NaOH solution (0·11M Na_2EDTA and 0·75M NaOH in H_2O), made up to 25 ml with water and placed on the ion-exchange (anion-exchange resin AG1-X4, 100–200 mesh chloride form, Bio-Rad Laboratories, Richmond, CA, USA) column (0·7 cm $\times$ 15 cm). The column was washed first with 15 ml of H_2O and then with 15 ml of 0·1M NaCl before it was eluted with 15 ml of 0·7M NaCl. The eluate was collected in a digestion vessel. A blank was prepared by mixing 1 ml of 2·4% HCl with 1 ml of Na_2EDTA–NaOH reagent and diluting with water to 25 ml. A mixture of concentrated H_2SO_4 (0·5 ml) and HNO_3 (3·0 ml) was added to the eluate to release the phosphorus by wet digestion in a Kjeldahl rack over medium heat (about 150°C), until active boiling ceased, and a cloud of thick yellow vapour filled the neck of the flask. The content was then heated for 5 min at about 150°C, followed by heating for 5 min at about 80°C. The samples were cooled to room temperature and then H_2O (10 ml) was added to dissolve the salts. They were again heated for 10 min under low heat (about 80°C). After cooling, the solutions were transferred to a 50 ml volumetric flask. An aliquot (2 ml) of 2·5% ammonium molybdate solution in 1N H_2SO_4 was added and mixed well, and then 1 ml of sulphonic acid reagent (0·16 g of 1-amino-2-naphthol-4-sulphonic acid, 1·92 g of Na_2SO_3 and 9·6 g

of $NaHSO_3$ in 100 ml of H_2O) was added and mixed well; thereafter, the volume of the solution was made up with water to 50 ml. After mixing well, the solution was allowed to stand 15 min before the absorbance at 640 nm was measured in a spectrophotometer. The amount of phytate (phytate = 28·2% P) in the sample was calculated as hexaphosphate equivalents. Sodium phytate (Sigma P 5756) solutions were used as standards.

Tannins
Tannins were determined as flavanols as described by Truelsen (1984). The flavanols from 0·3 g of quinoa flour were extracted with 5 ml of 70% acetone in 10 mM HCl, at room temperature (20°C) during 2 h. The suspension was centrifuged at 3000g for 5 min. The supernatant was collected in a beaker and the residue was treated again with 5 ml of 70% acetone in 10 mM HCl, under the same conditions as before. The filtrates from the two extractions were pooled together and made up to 10 ml with the extraction solution. An aliquot of 200 μl was mixed with 3 ml of 4-dimethyl-aminocinnamaldehyde solution (140 ml of methanol and 50 ml of conc. HCl were mixed at 20°C and 200 mg of 4-dimethylaminocinnamaldehyde (Sigma, D 4506) was added and made up to 200 ml with methanol). The absorbance was measured at 640 nm, 3 min after ixing the reagents. Catechin (Sigma, C1788) was used as standard.

Protease inhibitors
The presence of protease inhibitors was determined by a method described by Tovar (1983). Quinoa flour (2 g) was treated with distilled water (10 ml) with continuous stirring for 16 h at 4°C. The insoluble material was separated by centrifugation at 10000g for 20 min and discarded. A 0·8% agarose solution was prepared in 0·1 M Tris–HCl buffer of pH 8·6. This solution was used to cast 2 nm thick gels on standard microscopy slides. After the gel was solidified, wells were punched in the gels. After filling the wells with 5 μl of test solution the slides were incubated in a chamber for 20 min at 37°C to allow inhibitor diffusion. The gels were immersed in 0·1 M Tris–HCl buffer of pH 8·6 containing trypsin (Sigma T 8253) (100 μg/litre). The gels were then washed three times with distilled water, and were incubated for 5 min at 37°C with excess of a substrate solution of 3 mg of N-acetyl-DL-phenylalanine 2-naphthyl ester (Sigma A 7512) dissolved in 1·25 ml of N,N-dimethylformamide (Sigma D 8654) and mixed with 11 ml of 0·1 M Tris–HCl buffer of pH 8·6 containing 6 mg of tetrazotized o-dianisidine (Sigma D 3502). Finally, the gels were transferred to 7·5% acetic acid to fix the colour.

Statistical analysis
The statistical evaluation of the results was done by one-way ANOVA using Statgraphics (Statistical Graphics Corp., MD, USA) software. Multiple comparison tests were performed adopting Tukey's test at the 95% confidence level.

RESULTS AND DISCUSSION

Saponins

The analysis of saponins has been done by different methods. One method deals with the measurement of the amount of foam produced after shaking the sample with water. This afrosimetric method does not take into account the presence of any other surfactants. Moreover, it is not particularly sensitive (Oakenfull, 1981). The saponins present in quinoa differ in proportions with the variety. Some saponins may form a stable and compact foam. Koziol (1991) found, when using the afrosimetric method, that the variety Porotok produced an unstable foam described as larger air bubbles which were easily collapsed making the determination of saponins difficult. The accuracy and precision of the afrosimetric method (Koziol, 1991) depends on the type of saponins present in the seeds. However, this method is satisfactory for screening different varieties and for obtaining a rapid knowledge about the efficiency of the process of removing saponins from the quinoa seeds.

Using a thin-layer chromatography technique, a water extract of the sample was separated on a silica gel plate using chloroform–acetic acid–methanol–water or n-butanol–acetic acid–water, and saponins were developed with sulphuric acid (Ballon *et al.*, 1976; Romero, 1981). Saponins have also been determined by spectrophotometric methods, using oleanolic acid as reference (Elías & Diaz, 1988). The spectrophotometric methods utilize the colour produced by the reaction of saponins with vanillin or anisaldehyde. These methods are not suitable for estimating saponins in plant extracts due to the fact that the reactions are not specific, and coloured products can be produced from other compounds such as flavonoids (Oakenfull, 1981). Further, the capacity of saponins to inhibit growth of the fungus *Trichoderma viride* on potato dextrose agar was used to quantify saponins (Birk & Peri, 1980). Good correlation between the values from bioassay using red flour beetle larve and a HPLC method were obtained by Bercker & Hanners (1990). Quinoa saponins were isolated by monitoring the fractions with brine shrimp lethality and a taste for bitterness (Ma *et al.*, 1989). The same authors identified some quinoa saponins, using chemical spectral and enzymatic methods. Quinoa saponins have also been characterized, after acid hydrolysis producing oleanic acid and hederagenin, and the saponins were detected by using gas chromatography and by mass spectrometry (Meyer *et al.*, 1990). In addition, a gravimetric method (Junac, 1983), a method based on the ability of saponins to cause haemolysis (Jones & Elliott, 1969; Reichert *et al.*, 1986) and a gas chromatographic method have also been developed for analysis of saponins in quinoa (Ridout *et al.*, 1991).

The HPLC method is suitable for the analysis of saponins in crude extracts of plant tissues. It has the advantage over other methods used in this study in that it has high accuracy and precision. However, suitable standards of saponins are necessary for correct
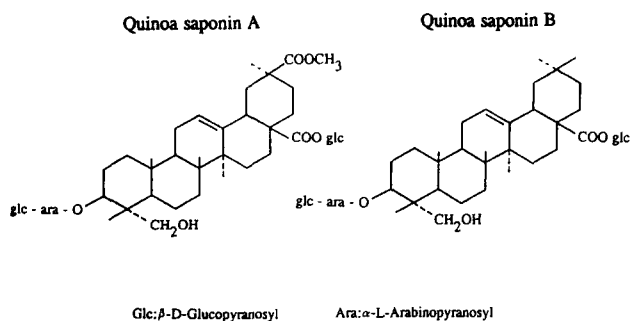
Quinoa saponin A   Quinoa saponin B

Glc:β-D-Glucopyranosyl   Ara:α-L-Arabinopyranosyl

Fig. 1. Proposed structure of quinoa saponins.

Table 1. Content of saponins and phytic acid in quinoa seeds (g/100 g dry basis)[a]

	Raw whole quinoa	Polished and washed quinoa	Bran
Saponin A	0.7 ± 0.05^b	0.3 ± 0.02^c	1.7 ± 0.08^d
Saponin B	0.2 ± 0.02^b	0.0 ± 0.00^c	0.6 ± 0.03^d
Phytic acid	1.0 ± 0.08^c	0.8 ± 0.01^b	—

[a] Means $(n = 3) \pm$ SD.
[b,c,d] Means in the same row followed by the same letter are not significantly different by Tukey's test at 95% confidence limits.

identification of the components and their quantitative determination (Kesselmeir & Stuck, 1981; Ireland & Dziedzic, 1987).

In the present study an HPLC method was used for the determination of the saponin content in quinoa seeds used for making an infant food. Two major saponins were identified in quinoa seeds and also in quinoa bran. These are present in relatively high amounts. Known amounts of both standards (saponins A and B) were separately spiked and coeluted with the sample in HPLC.

Saponin A, with a retention time of 7.04 min, corresponds to β-D-glucopyranosyl-[β-D-glucopyranosyl-(1 → 3)-α-L-*arabino*-pyranosyl-(1 → 3)]-3-β-23-dihydroxy-12-en-28-oate-30 methyl ester, and saponin-B, with a retention time of 12.53 min, corresponds to β-D-gluco-pyranosyl-[β-D-glucopyranosyl-(1 → 3)-α-L-*arabino*-pyranosyl-(1 → 3)]-3-β-23 dihydroxyolean-12-en-28-oate (Fig. 1). Thirteen saponins from quinoa bran, have been isolated using preparative methods and their structures were identified. Seven of them were oleanane (Mizui *et al.*, 1988, 1990). The chromatogram of the quinoa bran showed a pattern of saponin content similar to that of raw whole quinoa seeds.

After quinoa had been polished and washed with water, saponin A was decreased by 56% of the original content, while saponin B was not detectable in the processed samples (Table 1), i.e. <50 ng/100 g. A linearity response was seen up to 10 μg (Fig. 2). As quinoa had no bitter taste, after final washing, assayed by an informal panel composed of researchers who were taking

part in the processing, it can be assumed that the bitter taste most probably is the contribution of saponin B.

The amount of remaining saponins in the seeds after scrubbing and washing was much lower than the lethal doses of saponins by oral intake reported by George (1965), which were 3 to 1000 times higher than that by intravenous injections. The lethal dose by oral digestion in rodents varies from 1.9 to 6000 mg saponins/kg body weight. On the other hand, the bran obtained from quinoa seeds could be a source of saponins. Its potential toxicity to various organisms makes it an attractive insecticide (Price *et al.*, 1987). Saponins are used, as natural detergents, for flavouring, in health foods, in tonics, etc. (Birk & Peri, 1980; Oakenfull, 1981; Merk, 1983; Price *et al.*, 1987). Saponins are also of interest from the pharmacological point of view as they have been shown to modify the permeability of the small intestine, which may help the absorption of specific drugs (Oakenfull & Sidhu, 1990). The addition of saponins is found to lower the level of cholesterol in the plasma by increasing the faecal bile excretion (Oakenfull & Fenwich, 1978; Topping *et al.*, 1980; Southon *et al.*, 1988).

Phytic acid

The content of phytates present in the unpolished seeds was 1.04 ± 0.08 g/100 g and for polished and washed seeds it was 0.78 ± 0.01 g of dry matter. Relative standard deviations for repeatability ranging from 2.5 to 10%, and for reproducibility from 4.5 to 11.0%, have
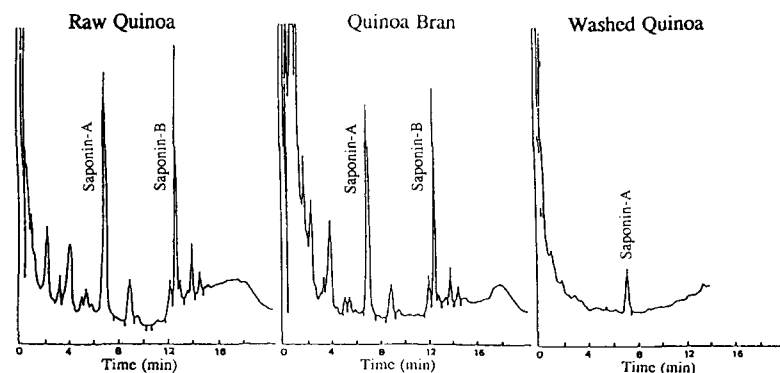

Fig. 2. Separation of saponins from quinoa seeds after methanol extraction. The separation was done on a LiChrosorb 100 CH-8/2 (5 μm) column (125 m × 4 mm) by 15 min gradient elution from 25 to 40% acetonitrile in water; flow rate, 2 ml/min; injection, 20 μl; detection (λ = 200 nm).

been reported for the contents of phytic acid in food samples (Harland & Oberleas, 1986). The process of removing the bitter taste from quinoa seeds was carried out by first scrubbing and then washing, leading to a significant decrease (about 30%) in the recovery of phytic acid. The phytic acid concentration in processed quinoa seeds was comparable with those values for whole grain rye flour (7·7 mg/g), whole grain wheat flour (8·7 mg/g), lentils (8·4 mg/g) and faba bean (8·0 mg/g) (Torelm & Bruce, 1982; Lombardi-Boccia *et al.*, 1991).

However, this shows that phytic acid is present not only in the outer layers of quinoa seeds as is the case in rye and wheat (Kent, 1984; Hallberg *et al.*, 1987) but also evenly distributed in the quinoa endosperm. The amounts of phytic acid obtained in this study are in concordance with the values reported by Koziol (1992) for five different varieties of quinoa, which ranged from 10·5 to 13·1 mg/g. However, the content of phytic acid obtained in this study are higher than the values reported by Chauhan *et al.* (1992).

A study (Allred *et al.*, 1976) carried out in rats showed that the availability of iron from polished and washed quinoa was at least equal to the availability of iron from $FeSO_4$. When the diets of the rates were supplemented with 30% of quinoa, the iron gained as haemoglobin per mg of iron intake was 0·74. When diets had 50% of quinoa, the efficiency diminished to 0·51, compared to an efficiency of 0·55 for $FeSO_4$ added to the wheat based diet. Moreover, Allred *et al.* (1976) reported quinoa as a better source of iron for haemoglobin regeneration than wheat flour in anaemic rats. Thus, animal experiments indicate a good bio-availability of iron in spite of the rather high phytate content. Studies on human beings are needed to explore this further.

The ability of the phytates to form complexes with minerals like iron, zinc, calcium and magnesium can make the mineral content of a food inadequate especially for children. The Food and Nutrition Board (National Academy of Science, Washington, DC (NRC, 1989) recommends 10 mg of iron per day for children between 1 to 10 years. Sandberg (1990) reported that the minimum amounts of phytic acid to avoid negative effects on iron and zinc absorption were 10 and 50 mg per meal, respectively. The reduction of phytic acid in foods by processes like fermentation, sprouting and scalding has been reported. The hydrolysis of phytate by endogenous and exogenous phytases increased the availability of iron in rye, oats and wheat bran (Sandberg & Svanberg, 1991; Snider & Liebman, 1992). Sandberg *et al.* (1987) found degradation of inositol hexaphosphate to inositol penta- and tetra-phosphates during extrusion of wheat bran, and the phytase activity was lost. Reductions of phytic acid content in doughs made from coarse meals are reported to be small, but with increasing temperature up to 55°C the phytic acid concentrations were less than 8 and 4% of original concentrations for wheat and rye, respectively (Fretzdorff & Brümmer, 1992).

Tannins

Tannins measured as flavonols were not present at detectable levels in raw whole quinoa or raw polished and washed quinoa. Chauhan *et al.* (1992), on the other hand, found 0·53 g of tannins in whole quinoa seeds, 0·28 g in manually dehulled (flour) and 0·23 g in water dehulled (flour) per 100 g on a dry matter basis. The compositions of nutrients and antinutrients in food plants may vary, depending on the variety and growing conditions. This may partly explain the difference between our report and that of Chauhan *et al.* (1992). Nevertheless, the values reported by Chauhan *et al.* are appreciably lower than those of rice beans (1·3%), green gram (1·1%) and black gram 1·1% (Kaur & Kapoor, 1992), but higher than that of barley 0·12% (Kent, 1984). The method applied in this study presented a standard deviation of 0·0012% and a coefficient of variation of 1·1% between 0·078 and 0·131% of the content of tannins in 80 barley samples (Truelsen, 1984).

Protease inhibitors

Using the qualitative method for determination of trypsin inhibitor, no protease inhibitor was detected even when a concentrated extract from quinoa flour was analysed (2 g sample in 10 ml of water). In any case, protease inhibitors in quinoa seeds are below 50 ppm (0·97 TUI/100 g where TUI is Trypsin units inhibited; Kakade *et al.*, 1969), which is the detection limit of the method. Romero (1981) analysed trypsin inhibitors in eight varieties of quinoa and found values ranging from 1·36 to 5·04 TUI/mg. These values are much lower than those reported for soybean (*Glycine max*) 24·5–41·5 TUI/mg, kidney beans (*Phaseolus vulgaris*) 12·9–42·8 TUI/mg, and lentils (*Lens esculenta*) 17·8 TUI/mg (Romero, 1981). In addition, Romero (1981) reported that trypsin inhibitors present in quinoa are thermolabile, which means that cooking, auto-laving and extrusion will inactivate these antinutrients.

Quinoa seeds were reported (Ruales & Nair, 1992*a*) to have high digestibility and high biological value as assayed by animal feeding experiments. This further supports the conclusion that any protease inhibitor activity in quinoa is too low to reduce the bioavailability of quinoa protein.

CONCLUSIONS

Saponins and phytic acid are the two main antinutrients present in quinoa seeds. The process of scrubbing and washing quinoa seeds to remove the bitter taste reduced the saponin A content by 56% and the phytate content by 30%. Saponin B was completely removed. The saponins appeared to be mainly, but not entirely, in the outer layer of the seeds, whereas phytates seemed more evenly distributed. No tannins or protease inhibitors were detected in raw whole or polished and washed quinoa seeds.

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