Folates in quinoa (*Chenopodium quinoa*), amaranth (*Amaranthus sp.*) and buckwheat (*Fagopyrum esculentum*): Influence of cooking and malting.

Carla Motta[1], Inês Delgado[1], Ana Sofia Matos[2], Gerard Bryan Gonzales[3], Duarte Torres[4,5], Mariana Santos[1], Maria V. Chandra-Hioe[6], Jayashree Arcot[6], Isabel Castanheira[1*]

[1] Departamento de Alimentação e Nutrição, Instituto Nacional de Saúde Doutor Ricardo Jorge, INSA. IP, Avenida Padre Cruz, 1649-016, Lisboa, Portugal

[2] Departamento de Engenharia Mecânica e Industrial, UNIDEMI, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal

[3] Laboratory of Food Analysis, Department of Bioanalysis, Faculty of Pharmaceutical Sciences, Ghent University, Ottergemsesteenweg 460, Ghent, Belgium

[4] Faculdade de Ciências da Nutrição e Alimentação da Universidade do Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

[5] Departamento de Bioquímica, Faculdade de Medicina da Universidade do Porto (U-38, FCT), Al. Prof. Hernâni Monteiro, 4200-319 Porto, Portugal

[6] Food Science and Technology, School of Chemical Engineering, University of New South Wales, High Street (Gate 2), Sydney NSW 2052, Australia

***Corresponding author:**

Isabel Castanheira

isabel.castanheira@insa.min-saude.pt

Departamento de Alimentação e Nutrição, Instituto Nacional de Saúde Doutor Ricardo Jorge, INSA. IP, Avenida Padre Cruz, 1649-016, Lisboa, Portugal

Abstract

Effects of processing on the contents of five folate vitamers in quinoa, amaranth and buckwheat were analyzed using a trienzymatic extraction method followed by LC-MS/MS. Total folate (TF) content, corresponding to the sum of folic acid (FA), 5-methyltetrahydrofolate (5-MTHF) and 10-formyltetrahydrofolate (10-CHOTHF) expressed as folic acid equivalent, in raw quinoa, amaranth and buckwheat were 309±8.07, 228±24.2 and 153±12.4 µg/100 g dw, respectively, being dominantly 5-MTHF. Boiling and steaming reduced the TF in amaranth by 58% and 22%, respectively, whereas up to a 10-15% increase was observed in quinoa. Boiling and steaming did not significantly alter the TF content in buckwheat although significant changes were observed in some individual folate vitamers. Malting, on the other hand significantly increased TF content in amaranth by 21% (276±14.2 µg/100 g dw) and buckwheat by 27% (193±20.0 µg/100 g dw), whereas no significant change in quinoa was observed. Based on the EFSA recommendations, a portion of amaranth and quinoa (either boiled, steamed or malted) may contribute up to more than 25% of the dietary reference value for folates, whereas buckwheat may contribute only 14% when cooked and 19% when malted. Results demonstrate that quinoa, amaranth and buckwheat are good sources of folates, regardless of processing.

Keywords: Dietary reference value; Total folates; Retention factor; 5-methyltetrahydrofolate; 10-formyltetrahydrofolate; Folic acid; Tetrahydrofolate; 5-formyltetrahydrofolate; Pseudocereals

1. Introduction

Folate is a generic term used for different water-soluble vitamins of the B-complex group. It is an essential micronutrient needed for optimal health, growth, and development. Dietary folate deficiency is common around the world, and deficiency of this vitamin is directly or indirectly associated with other metabolic disorders and pathophysiological conditions such as inflammatory bowel disease and celiac disease (Wani et al., 2008). Pregnancy and lactation also increase the risk of folate deficiency due to the high requirement to support optimal growth and development of the fetus (Stamm and Houghton, 2013). According to the European Food Safety Authority, the average requirement (AR) for healthy adult men and women is 250 µg of dietary folate equivalent (DFE) / day and the Population Reference Intake (PRI) is 330 µg DFE / day (European Food Safety Authority, 2014). Dietary reference values are in agreement with controlled studies showing that folate intake of around 200–300 µg/day may be sufficient to maintain folate concentration in serum and red blood cells (European Food Safety Authority, 2014).

Naturally occurring folates are present in a wide range of foods. However, some staple diets, especially those consisting of polished cereal grains and tubers, are very poor in folate but can be improved by the addition of legumes or green leafy vegetables (FAO and WHO, 2001). Thus, to achieve the recommended folate intake, efforts to fortify staple foods with folates have been explored. Alternatively, consumption of foods naturally high in folates could also serve as a good strategy to achieve recommended folate intake.

Pseudocereals, such as quinoa, amaranth and buckwheat, differ from true cereals (poaceae botanic family) like wheat and rice due to their differences in seed physiology and absence of gluten (Alvarez-Jubete et al., 2010; Saturni et al., 2010). Hence, amaranth, quinoa and buckwheat have been recommended by the World Gastroenterology Organization for celiac disease patients and as a base ingredient for baby foods due to their low allergenicity (WGO, 2012). However, although much has been said about the nutritive value of these pseudocereals, their folate content has not been elaborately reported in the literature. Since folates are unstable to heat, light and pH conditions, the content of folates in processed and stored food may be lower than in raw food. Several studies previously reported negative effects of cooking processes to folate in foods, especially in legumes and vegetables (Delchier et al., 2013, 2012; Stea et al., 2007). This effect is more pronounced with longer processing times and higher temperatures (Witthoft et al., 1999). On the contrary, other means of processing, like malting, have been reported to increase the physicochemical accessibility and compounds that intensify bioavailability of micronutrients (Platel and Srinivasan, 2016). Malting has also been shown to decrease the content of antinutrients such as phytates (Hotz and Gibson,

2007). The germination phase, which proceeds after the malting process in food products, has been reported as a way to increase the nutritional and bioactive profile of cereal grains (Hefni and Witthöft, 2011; Nelson et al., 2013; Shohag et al., 2012). Furthermore, malted grains are recommended as an ingredient for medicinal foods to increase their nutritional density without further processing (Kaur, 2009). In large-scale service systems, for example hospitals, knowledge of the content of folate in foods can be critical because of special or increased requirements for many patients. The content of folate after processing must be taken into consideration when calculating the real total folate intake from food. Thus, the effect of boiling, steaming and malting on the folate profile of these pseudocereals was also investigated. Lastly, the contribution of each portion of cooked or malted quinoa, amaranth and buckwheat to the recommended daily intake of folates was assessed.

2. Materials and methods

2.1. Reagents and chemical standards

Deionized water employed in all solutions was obtained by a Milli-Q purifier (Millipore, Eschborn, Germany). Folic acid (FA), 5-methyltetrahydrofolate (5-MTHF), tetrahydrofolate (THF), 5-formyltetrahydrofolate (5-CHOTHF) and 10-formyltetrahydrofolate (10-CHOTHF) standards were supplied by Schircks Laboratories (Jona, Switzerland). Stable isotope labelled ($^{13}C_5$) FA and ($^{13}C_5$) 5-MTHF, used as internal standard (I.S.), were purchased from Merck-Eprova (Schaffhausen, Switzerland) and stored at −80 °C. LC-MS grade acetonitrile and formic acid were from Merck Millipore (Germany). α-Amylase (Type I-A, from porcine pancreas, 23.5 units/μL, EC no. 232-565-6) and protease (Type XIV, from Streptomyces griseus, ≥ 3.5 units/mg, EC no. 232-909-5), ammonium bicarbonate, DL-Dithiothreitol (DTT) and L-ascorbic acid were purchased from Sigma (St. Louis, MO USA). Rat whole serum, lyophilized was purchased from Invitrogen Corporation (Waltham, MA USA).

2.2. Samples and Sample preparation

All pseudocereal samples white quinoa (*Chenopodium quinoa*), amaranth (*Amaranthus sp*.) and buckwheat (*Fagopyrum esculentum*)) seeds were purchased from local organic markets in Lisbon, Portugal, between March 2014 and September 2015. Sampling plan for primary samples (three with 0.5 to 1 kg each), laboratory samples and test portions was designed according to the protocol desbribed in Mota et al. (2016) to guarantee representativeness.

Sample preparation for cooking methods (boiled and steamed) were conducted three times according to Mota et al. (2016). Briefly, 50 g of raw pseudocereals were boiled for 15 min in 100°C using a Thermomix® TM31 food processor (Vorwerk, Germany) or steamed for 30 min using the same food processor.

For the malting process steeping phase, 50 g of each sample, weight in triplicate, was mixed with distilled water in a ratio of 1:10 and the samples were kept at 30 ºC for 48h until the moisture content is constant (with CV < 0.01 %). The germination process took place at 23 ºC for 48 hours until the germination rate (number of germinated seeds/number of non germinated seeds) was maximum. Steeping and germination were performed in dark conditions using a temperature controlled oven (Infors, Ecotron, Switzerland). The sprouts were dried in a food dehydrator (Excalibur®, California, USA) at 42 ºC for 10 h until moisture content were below 5%. Table 1 showed the malting optimization process conditions for all pseudocereals under study.

Raw, boiled, steamed and malted samples were milled in a GRINDOMIX GM 200, high speed grinder from Retsch (Germany) . After milling process samples were pooled and stored separately in aluminum foils in vacuum bags at -20 ºC until use. For each pooled sample four test portions were take to perform the assays (quadruplicate, n=4).

2.3. Moisture analysis

Moisture content was determined according to AOAC 952.08 (2000). The method was performed using a dry air oven (Heraeus Instruments, Hanau, Germany) at 102 Cº ± 2 Cº for 2 h, using test portions (3 g) of each sample, until constant weight.

2.4. Calibration standards

Folates stock standard solutions for FA, 5-MTHF, THF, 5-CHOTHF and 10-CHOTHF (100 µg/mL) were prepared under subdued light in ammonium bicarbonate buffer 0.5 mM, pH 7.2 with 0.5% of DTT and 1% of ascorbic acid as antioxidants. Working solutions were prepared by diluting the stock solution (25 µg/mL, pH 7.2) with water and reading in a UV-spectrometer (Thermo Scientific™ Evolution 300, England) in a wave length of 200-400 nm. The concentration of each folate was calculated at a maximum absorvance of 307 nm, and a molar absorptivity of 250 M, according to the equation (1).

$$A\lambda = \varepsilon \times c \times L \qquad (1)$$

where Aλ is absorbance in a specific wavelength, ε is molar absorptivity for the dissolved substance, c is the molar concentration and L is light path length in centimeters. An acceptance criteria of 80% to 120% was established for the standard solutions.

2.5. Folate Extration

The extraction of folates was performed with a tri-enzyme treatment (α-amylase, protease and rat serum). One gram of each pseudocereal was diluted in ten milliliters of ammonium bicarbonate buffer (0.5 mM, pH 7.2 with 0.5% of DTT and 1% of ascorbic acid, containing all I.S. (40 µg/mL of stable isotope labelled ($^{13}C_5$) FA to quantify FA and ($^{13}C_5$) 5-MTHF to quantify 5-MTHF, THF, 5-CHOTHF and 10-CHOTHF). After homogenisation in a vortex, the mixtures were boiled for 10min and cooled down on ice. Subsequently, an aliquot of 1.5mL from each sample was taken and mixed with 10µL of α-amylase. After 10 min at room temperature, 150µL of protease (4 mg/ml) was added and the tubes were incubated for 1h at 37◦C. The capped tubes were boiled for 10min to stop the enzymatic reaction followed by cooling on ice. For deconjugation of the polyglutamylated folates, 100µL of rat serum was added to the solution followed by incubation at 37 ◦C for 2h. Again, the enzyme was inactivated by boiling for 10min followed by cooling on ice. After centrifugation of the resulting solution for 30 min at 11500×g, the supernatant was ultrafiltrated at 13000 ×g for 60min on a 15 mL 5kDa Amicom filter (Merck Millipore, USA). The above steps were carried out under subdued light (Motta, 2016).

2.6. Folate LC-MS/MS analysis

The determination of FA, 5-MTHF, THF, 5-CHOTHF and 10-CHOTHF was performed using a ultrahigh pressure liquid chromatographer with a triple quadrupole mass spectrometric detection (UPLC-MS/MS) (Waters ACQUITY® TQD, Waters Co., Milford, USA). The chromatographic separation was achieved using an HSS T3 1.8 µm 2.1 x 150 mm column (Waters Co.) at 45˚C, 0.35 ml/min flow rate of 0.1% formic acid solution in water (A) and 0.1% formic acid solution in acetonitrile (B) in gradient elution. The proportion of B was increased to 25% in 4min, and finally increased to 70% and held for 1min. Subsequently, the mobile phase was adjusted to its initial composition (0.5% B) and held for 2min (Ramos et al., 2016).

The ion source voltage was 3kV, source and desolvation temperature were 150ºC and 350ºC, respectively. The fragments used for MRM were determined via an infusion experiment. Estimation was performed in

166 negative ion mode by multiple reaction monitoring (MRM) with a scan time of 7 min. First transition was
167 used for the quantification and the other was used for the confirmation (Table 2).
168 Analyses were carried out by a laboratory accreditated for flexible scope within food analysis in accordance
169 with EN ISO/IEC 17025. All test portions were analysed in quadruplicate. Quantification of FA, 5-MTHF,
170 THF, 5-CHOTHF and 10-CHOTHF was performed using a 6-point calibration curve between 7 ng/ml and
171 100 ng/ml. The correlation coefficients of all endogenous folates were ≥ 0.9985. The limit of detection
172 (LoD) in µg/100 g was 1.02 to 5-CHOTHF; 0.97 to 10-CHOTHF; 0.73 to THF; 0.68 to FA and 0.56 to 5-
173 MTHF. The limit of quantification (LoQ) in µg/100 g was 3.09 to 5-CHOTHF; 2.93 to 10-CHOTHF; 2.22
174 to THF; 2.06 and 1.70 to 5-MTHF. In all runs the medium value of the calibration curve (30 ng/ ml) was
175 injected six times (quality control) to monitor repeatability under acceptance criteria which is below 10 %
176 for quality control and for samples.
177 Laboratory competence was demonstrated through satisfactory participation in proficiency testing schemes
178 (FAPAS Proficiency testing, Fera Science, UK). Also a Standard Reference Material from National
179 Institute of Standards and Technology (NIST SRM® 1849a Infant/Adult Nutritional Formula)
180 (Gaithersburg, MD, USA) was used to assess the accuracy of method.
181
182 2.7. Determination of folate retention
183 The true retention (%TR) of folates in the processed samples was calculated according to US Department
184 of Agriculture (2007) definition, using the following equation:
185 $\%TR = (A \times B \div C \times D) \times 100$ (2)
186 where A - nutrient content (g) of cooked sample; B - sample weight (g) after cooking; C - nutrient content
187 (g) of raw sample and D - weight (g) of raw sample.
188
189 2.8. Calculation of the equivalent portion of raw food
190 Equivalent portion corresponds to a standard amount used to give advice about how much food an
191 individual should eat according to the food plate model (Direção Geral da Saúde, 2012). The Direção Geral
192 da Saúde (2012) table of equivalence was used to calculate the equivalent portion (EP) for each cooked
193 pseudocereal. In this table, each portion of raw cereals and derivatives corresponds to two tablespoons or
194 35 g. Initially, the weight gain or loss during processing, or cooking yield (Yc), was calculated according

to equation 3 (Charrondiere, 2014). In this work it was assumed that the variations in food weights were due to losses or gains in water content. Then, the EP was calculated using equation 4.

$$Yc = \frac{Weight\ of\ cooked\ sample\ (g)}{Weight\ of\ raw\ sample\ (g)} \quad (3)$$

$$EP\ raw\ food\ (g) = 35\ g\ \times Yc \quad (4)$$

2.9. Statistical Analysis

Data were reported as mean ± standard deviation (SD). Analysis of variance (ANOVA) was used to determine difference among groups. Tukey-Kramer multiple comparison test was performed to identify each of the significantly different cooking processes when the overall result was statistically significant. Homogeneity of variances was tested by Cochran's and Levene's test and the results were undertaken using Kruskal-Wallis non-parametric test for heterogeneous variances. Differences were considered significant at a *p*-value below 0.05. All data were standardized to zero mean and unit standard deviation before HCA analysis. All statistical tests were conducted by Statistica v. 8 software (Statsoft Ibérica, Lisboa, Portugal).

3. Results and Discussion

3.1 Quality control

In SRM® 1849a, values from 2.25 mg/kg to 2.35 mg/kg for FA and from 0.044 mg/kg to 0.058 mg/kg for 5-MTHF was quantified. The certified values in SRM® 1849a for FA is 2.293 ± 0.062 mg/kg and for 5-MTHF is 0.0482 ± 0.0085 mg/kg. In the proficiency testing schemes FAPAS 2186, 2191 and 2199 (breakfast cereal test material), the quantified values for FA, in each participation were 181 ± 15 µg/100g, 127 ± 10 µg/100g and 137 ± 12 µg/100g , with a Z-Score of 1.6, -1.9 and -0.2, respectively.

3.2 Folate content in raw, cooked and malted pseudocereals

The results of total folate content in raw, cooked and malted pseudocereals, are presented in Table 3. Five folate vitamers were analysed. The concentations for FA, 5-MTHF and 10-CHOTHF were above the LoD, whereas for the vitamers 5-CHOTHF and THF were below the LoD.

The moisture content increased significantly in all cooked samples due to water addition, and decreased significantly in the malted samples ($p < 0.001$), because those were dried after germination to preserve the grains. Total folates were presented as the sum of FA, 5-MTHF and 10-CHOTHF expressed as folic acid

equivalents, using for conversion the molecular weights respectively 441.4 for folic acid, 459.5 for 5-MTHF and 469.4 for 10-CHOTHF. The results of all vitamers were expressed in µg / 100 g dry matter basis. In amaranth, total folate content ranged from 95.3 µg / 100 g in boiled samples to 276 µg / 100 g in malted samples. Raw amaranth showed a significant decreased in the total folate amount when compared to boiled samples. On the contrary, malting significantly increased the total folate content when compared to raw amaranth samples ($p < 0.001$). For quinoa, folate contents ranged from 285 µg / 100 g to 354 µg / 100 g for malted and boiled, respectively. Steamed and malted quinoa did not show significant differences when compared with raw. Boiled samples however showed significantly higher total folates than raw and malted ($p < 0.01$). The increase of total folates, which occur after cooking in quinoa, was also verified by Stea, Johansson, Jägerstad, & Frølich (2007) when analyze the content of different forms of folates in broccoli after different cooking methods. Delchier, Reich, & Renard (2012) also reach to the same conclusions after analysed spinach and green beans. Low cooking temperatures can be used to maximize folate retention in whole grain seeds, as quinoa, which present higher content of folate due to their composition of bran and germ. This improvement would be advantageous especially in the development of medical foods (Witthoft et al., 1999). The reason for these increases, verified in quinoa as well as in the previous studies, remain unclear and need further studies. For buckwheat, folate content ranged from 146 µg / 100 g to 193 µg / 100 g for boiled and malted, respectively. Only malted samples showed significantly higher total folate values than raw and cooked samples ($p < 0.01$). The increase in folate content observed in amaranth and buckwheat after malting process, was also previously reported by several authors in legume seeds and wheat after germination (Hefni and Witthöft, 2011; Koehler et al., 2007; Shohag et al., 2012). As shown in Table 3, all pseudocereals contained considerable amount of total folates, sorted in decreasing order as follows: quinoa, amaranth and buckwheat.

The results presented in this paper were found to be higher when compared to the folate composition, expressed in DFE, reported in the USDA National Nutrient Database for Standard Reference (US Department of Agriculture and Agricultural Research, 2016) for pseudocereals raw and cooked or by Schoenlechner, Wendner, Siebenhandl-Ehn, & Berghofer, (2010) for total folate content in pseudocereals wholemeal flour expressed in µg/100g dw. After conversion, when we compared values of total folates in quinoa and amaranth raw and cooked, presented in our study, these are around 3 times higher than those reported in USDA National Nutrient Database for Standard Reference (US Department of Agriculture and Agricultural Research, 2016) or in Schoenlechner et al. (2010). The values for buckwheat are around 4

times higher, when compared with the same references.The difference in their results are however expected since the method employed in this study is more sensitive. LC-MS/MS method provides a more sensitive, specificity and accurate separation of several folate forms (Arcot and Shrestha, 2005) when compared to the microbiological method (Ringling and Rychlik, 2017) or with high performance liquid chromatography (HPLC). The microbilogical method usually applied used *Lactobacillus rhamnosus*, that could respond differently to different tipes of glutamates and could also may be stimulated or inhibited by non-folate substances (Arcot and Shrestha, 2005; Ringling and Rychlik, 2017). The HPLC methods present low sensitivity due to incomplete separation of all types of glutamates (Arcot and Shrestha, 2005; Mönch and Rychlik, 2012). Further, the differences in extraction methods between laboratories, can also explain some of the variations (Stea et al., 2007). Also the use of the tri-enzymatic extraction, which generally provides the highest detectable value of food folate concentration, could explain the differences found (Johnston et al., 2002). Furthermore, as the plants were cultivated under different climatic conditions, and on different soil types, it is also expected that the total content of folate is different (Miranda et al., 2012).

In our studie the predominant vitamer was 5-MTHF. This vitamer was also the predominant in white rice (De Brouwer et al., 2010; Pfeiffer, 1997) in cooked semolina (Vishnumohan, Arcot, & Pickford (2011), in mongbean seeads raw and germinated (Shohag et al., 2012) and in wheat bran and oat (Patring, Wandel, Jägerstad, & Frølich 2009). On the contrary, 5-HCO-THF was reported to be the dominant form in several cereal grain products, such as wheat and rye flour, and in buckwheat (Gujska & Kuncewicz, 2005). These different findings could be explained by the different extraction procedure used by the latter, as previously explained. Enzyme parameters (reaction time and amount), especially of deconjugase, could lead to incomplete desconjugation of all glutamates (De Brouwer et al., 2007; Ringling and Rychlik, 2017). Also, the extraction buffer pH could lead to the same phenomenon, as well as to the oxidation or interconversion of the folate forms, as already described by Mönch & Rychlik (2012), Ringling & Rychlik (2013, 2017) and Stea et al. (2007). The behaviour of folates under different experimental conditions could explain the different results among the studies.

The content of 5 – MTHF is significantly lower in boiled (59.3%) and steamed (24.4%) amaranth than in raw amaranth. However, 5 – MTHF is significantly higher in malted amaranth (27.0%) and buckwheat (26.4%), as well as in boiled (20.8%) and steamed (16.4%) quinoa when compared with raw amaranth, buckwheat and quinoa. The amount of 10 – CHOTHF in buckwheat increased in malting, when compared to raw (47.0 %). As folates occur intracellularly, they are released from the matrix when the cell/seed

structure is destroyed. Based on our observations, amaranth seeds are smaller and have higher surface area than the other pseudocereals. This may explain how boiling and steaming increase the extracted folates from amaranth more than in quinoa and buckwheat. The higher surface area of amaranth, may have led to a more efficient release of the folates from the seeds. This, however, needs further investigation.

3.3 Nutrient retention values

Retention values (%TR) of vitamers were calculated in order to measure the proportion of folate vitamers remaining after the cooking and malting processes in relation to the nutrient originally present in raw seeds. As presented in Figure 1a, boiled amaranth presented the lowest %TR, below 60% for all vitamers. Furthermore, during malting a significant increase in the content of 5-MTHF and consequently in total folates were observed (%TR > 120%). Malted amaranth presented the highest %TR, followed by steamed and finally by boiled ($p < 0.05$). Contrary to amaranth, in malted quinoa %TR of 5 – MTHF was below 100%, whereas in boiled and steamed the %TR was significantly higher exceeding 110% (Figure 1b). In malted quinoa, only 10 – CHOTHF presented a significantly higher %TR above 100%, but with no effect on the sum of folates. FA in buckwheat showed the lowest values in %TR, near 20 % in steamed and below 60 % in boiled and malted (Figure 1c). For 10 – CHOTHF, malted buckwheat presented %TR above 120 %, significantly higher than boiled and steamed, both below 100 %. In 5 – MTHF, as in amaranth, malted buckwheat presented %TR above 120 %. The findings in amaranth and buckwheat could be attributed to *de novo* synthesis of folate vitamers, specially 5 – MTHF, caused by an increased demand for methyl groups (one carbon unit) during germination in malting process, as described by Hefni & Witthöft (2011, 2012) for wheat and rye cultivars. Hübner & Arendt (2013) also suggested that germination may be used to increase the level of folate in wheat, barley, oats and rye.

Our results indicate that cooking methods cause a major effect on amaranth seeds with a total folates %TR below 80%, but not on quinoa and buckwheat (%TR around 100%). These findings were in accordance with retention values obtained by Stea, Johansson, Jägerstad, & Frølich (2007) for green peas and potatoes boiled and steamed.

3.4 Dietary Reference Intake

To calculate the equivalent portion of each cooked food, the weight gain after cooking was monitored and expressed as Yc (equation 3). The Yc of amaranth boiled seeds was 2.87 and 1.60 for steamed seeds. For

boiled quinoa Yc was 2.38 and for steamed quinoa it was 2.30. Buckwheat presented 2.89 of Yc after boiling and 1.89 after steaming. Based on the 35g recommendation of Direção Geral da Saúde (2012), the EP for amaranth, quinoa and buckwheat were therefore 100 g, 83 g and 101 g for boiled and 56 g, 81 g and 66 g for steamed, respectively. For raw and malted samples the EP was 35 g.

According to European Food Safety Authority (2014), the dietary reference values (DRVs) for adults considering PRI was established at 330 µg of DFE / day. Figure 2 shows the recommendation, in percentage, provided by the consumption of one EP of each pseudocereal.

Results indicate that over 30% and 25% of the DRV for folate/day is met by the consumption of a portion of quinoa and amaranth, respectively. On the other hand, a portion of buckwheat only contributes from 14% to 19% of the DRVs, representing the lowest values. For amaranth and quinoa, the processing methods did not cause significant differences in DRV´s whereas malting of buckwheat showed significantly higher contribution to the DRVs than when raw and cooked (boiled and steamed). In general, the results suggested that the studied pseudocereals, with or without processing, are important sources of folates.

4. Conclusions

Results from this study showed that quinoa, amaranth and buckwheat are good sources of folates, especially of 5-MTHF. The retention of folates were dependent on both the food matrix and the method of processing (such as boiling, steaming and malting). According to the DRV for folate as per EFSA recommendations, a portion, corresponding to two tablespoons of raw pseudocereals, amaranth and quinoa (35g) contributes at least 25% of the DRV for folates. Malting increased the contribution of a portion of buckweat to the DRV for folates from 14% to 19%.

Conflict of interest

The authors declare to have no potential sources of conflict of interest

Acknowledgements

The scientific work was funded by the Portuguese Fundação para a Ciência e a Tecnologia (FCT) under the scope of the strategic project UID/EMS/00667/2013. The analytical work has been financially supported by Project ELEMENTARIA funded by the Instituto Nacional de Saúde Doutor Ricardo Jorge, I.P. Lisbon,

Portugal (2013DAN850) and PRO-METROFOOD project, funded by European Union's Horizon 2020 research and innovation programme under grant agreement No 739568.

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Table 1 - Conditions in malting process.

	Steeping		Germination		Kilning	
Pseudocereals	Time (Hours)	Temperature (ºC)	Time (Hours)	Temperature (ºC)	Time (Hours)	Temperature (ºC)
Amaranth	8	30	24	23	4	42
Quinoa	8	30	24	23	6	42
Buckwheat	10	30	40	23	8	42

Table 2 - Multiple reaction monitoring (MRM) transitions and compound parameters of folates and internal standards.

	Retention time (min)	Precursor ion (m/z)	Product ions (m/z)	Cone Voltage (V)	Collision energy (eV)
THF	3.63	444.4	315.5	45	26

			128.2		27
5-MTHF	3.76	458.4	329.4	45	27
			128.3		27
^{13}C-5-MTHF	3.76	463.4	329.3	40	27
			133.3		30
10-CHOTHF	3.88	468.3	339.4	48	22
			249.3		28
5-CHOTHF	3.98	472.4	315.3	48	28
			128.3		36
FA	4.17	440.3	311.3	42	22
			175.2		28
^{13}C-FA	4.17	445.3	311.3	53	20
			175.2		34

446
447
448

Table 3 – Effect of cooking methods and malting on folate composition (µg / 100 g dry weight basis)

Amaranth					
	Treatments				
	Raw	Boiled	Steamed	Malted	p^A
Moisture g/100g (fresh)	$12.2^a \pm 0.09$	$73.6^b \pm 0.84$	$52.4^c \pm 0.13$	$5.50^d \pm 0.04$	***
FA	$4.88^a \pm 0.63$	$1.79^b \pm 0.33$	$3.42^c \pm 0.72$	$4.87^a \pm 0.66$	***
5 – MTHF	$211^a \pm 23.8$	$85.9^b \pm 6.69$	$160^c \pm 11.5$	$268^d \pm 13.9$	***
10 - CHOTHF	$21.2^a \pm 2.21$	$11.7^b \pm 1.44$	$20.8^a \pm 2.42$	$14.6^b \pm 1.74$	***
Sum of vitamers as folic acid equiv	$228^a \pm 24.2$	$95.3^b \pm 7.64$	$176^c \pm 11.8$	$276^d \pm 14.2$	***
Quinoa					
	Treatments				
	Raw	Boiled	Steamed	Malted	P^A
Moisture g/100g (fresh)	$11.7^a \pm 0.17$	$66.6^b \pm 0.21$	$62.8^c \pm 0.02$	$5.90^d \pm 0.03$	***
FA	$9.35^a \pm 1.15$	$7.57^{ab} \pm 1.10$	$4.81^b \pm 1.01$	$6.41^b \pm 1.59$	*
5 – MTHF	$259^a \pm 13.4$	$313^b \pm 11.4$	$302^b \pm 16.7$	$233^a \pm 29.5$	**
10 - CHOTHF	$53.4^a \pm 8.35$	$47.9^a \pm 9.86$	$47.9^a \pm 17.1$	$57.8^a \pm 8.22$	ns
Sum of vitamers as folic acid equiv	$309^{ac} \pm 8.07$	$354^b \pm 18.1$	$340^{ab} \pm 20.5$	$285^c \pm 30.0$	**
Buckwheat					
	Treatments				
	Raw	Boiled	Steamed	Malted	P^A
Moisture g/100g (fresh)	$13.3^a \pm 0.12$	$68.5^b \pm 0.21$	$52.7^c \pm 0.09$	$6.70^d \pm 0.02$	***
FA	$1.75^a \pm 0.38$	$0.55^b \pm 0.04$	$0.33^b \pm 0.09$	$0.94^c \pm 0.30$	***
5 – MTHF	$148^a \pm 14.4$	$144^a \pm 10.8$	$154^a \pm 15.4$	$187^b \pm 22.1$	**
10 - CHOTHF	$9.10^a \pm 2.72$	$7.78^a \pm 0.85$	$5.04^b \pm 0.58$	$13.4^c \pm 1.70$	***
Sum of vitamers as folic acid equiv	$153^a \pm 12.4$	$146^a \pm 9.58$	$153^a \pm 15.3$	$193^b \pm 20.0$	**

Mean ± sd (standard deviation); dw – dry weight; [A] statistical probability of treatment: ns (not significant), $P > 0.05$; *, $P < 0.05$; **, $p < 0.01$; ***, $P < 0.001$; means in the same row with different superscripts are significantly different ($P < 0.05$) ($n = 4$).

[B]Sum of vitamers after conversion using factors based on the molecular weights: 0.9607 for 5-MTHF and 0.9403 for 10 - CHOTHF.

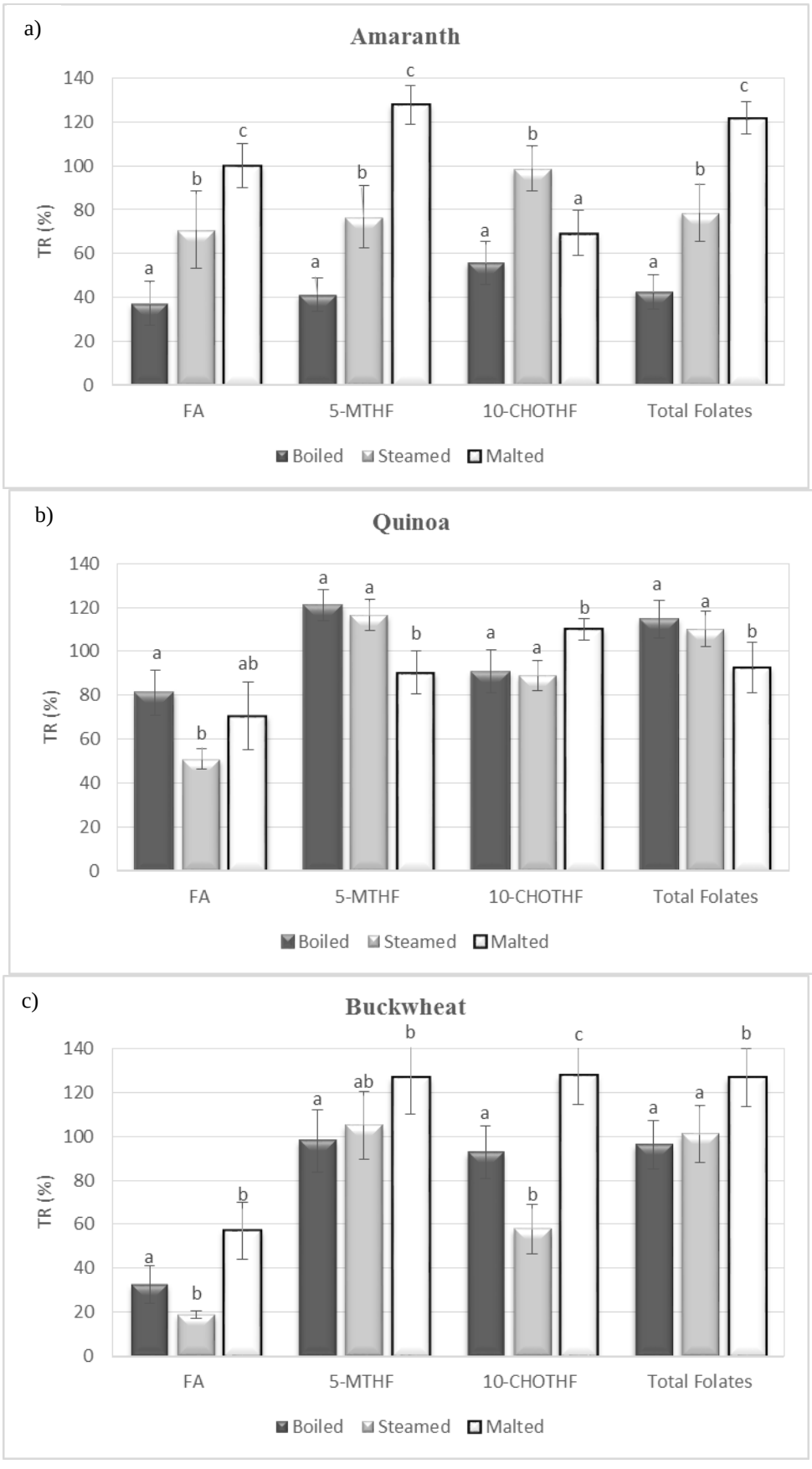

Figure 1 – Folate vitamers for True Retention (%TR) in boiled, steamed and malted pseudocereals (amaranth a), quinoa b) and buckwheat c)). Total folates represent the sum of vitamers after conversion using factors based on the molecular weights: 0.9607 for 5-MTHF and 0.9403 for 10 – CHOTHF.

Different letters show statistically significantly ($p < 0.05$) differences in folate content after different processes compared with raw.

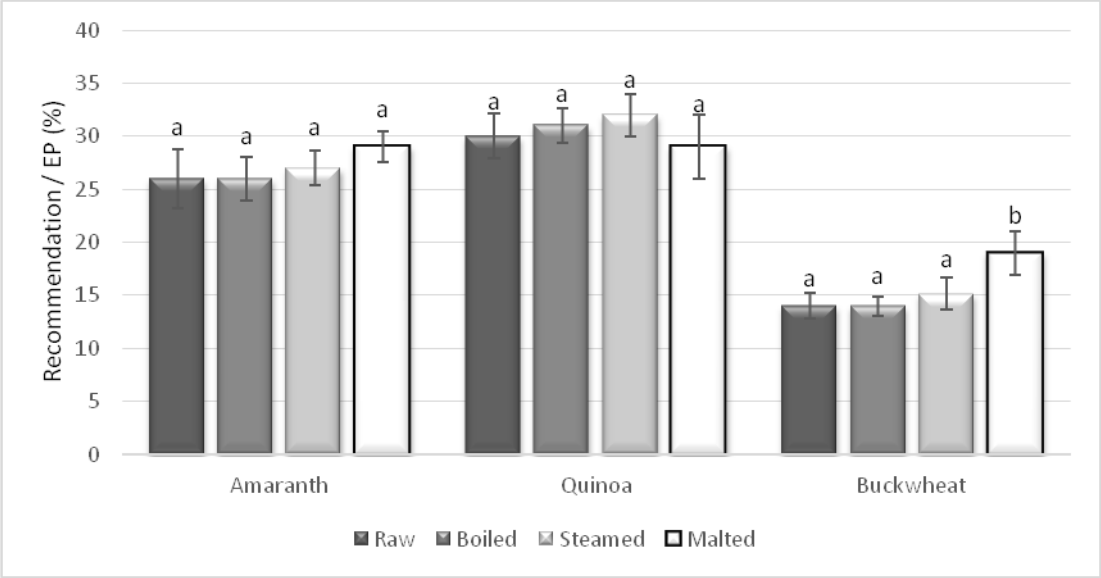

Figure 2 – Dietary Reference Values for folate (DFE % / Day) by equivalent portion (35 g for raw and malted pseudocereals; in amaranth 100 g for boiled and 56 g for steamed; in quinoa 83 g for boiled and 81 g for steamed; in buckwheat 101 g for boiled and 66 g for steamed). Calculation of DFE % / Day based on the sum of vitamers after conversion using factors based on the molecular weights: 0.9607 for 5-MTHF and 0.9403 for 10 – CHOTHF. Different letters within each group represent significant difference at $p < 0.05$.