Folate distribution in barley (Hordeum vulgare L.), common wheat (Triticum aestivum L.) and durum wheat (Triticum turgidum durum Desf.) pearled fractions

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Running title: Folate distribution in barley and wheat pearling fractions

Authors:

Debora Giordano, Amedeo Reyneri, Massimo Blandino*

Affiliation:

University of Torino, Department of Agricultural, Forest and Food Sciences, Largo Paolo Braccini 2, 10095 Grugliasco (TO), Italy.

*Corresponding author: Massimo Blandino

Phone +39 011 6708895, massimo.blandino@unito.it

ABSTRACT

Background: Wholegrain cereals are an important source of folates. In this study, total folate was analyzed in different pearling fractions of hulled and hulless barley as well as in common and durum wheat in order to evaluate its distribution in the kernels.

Results: A noticeable variation in the folate content was observed between the barley and wheat varieties. The highest folate content was detected in the hulless barley variety. A significant reduction in total folate, from 63% to 86%, was observed in both barley and wheat varieties from the outermost to the innermost pearling fractions.

Conclusion: Results have proved that folates are mainly present in the germ and in the outer layers of the kernel. This is the first study reporting the folate distribution in both common and durum wheat and in hulless barley varieties. Results suggest that the folate content could be naturally enhanced by introducing grain pearling fractions into cereal food products.

KEYWORDS

Folates, Vitamin B9, Common wheat, Durum wheat, Barley

INTRODUCTION

Folates, also known as vitamin B9, are a group of water-soluble vitamins characterized by a similar biological activity to folic acid. Folates are naturally present in some food, are added to others, and are available as dietary supplements. This family of vitamins is currently one of the most actively studied, because of its pivotal role as an essential coenzyme to provide one-carbon units for nucleotide biosynthesis, amino acid metabolism and DNA methylation in organisms. [1] Unlike plants and microorganisms, animals cannot synthesize folates *ex novo* and depend entirely on their dietary supply. Although folates are omnipresent in a normal human diet, folate intake often falls below the recommended levels, even in highly-developed countries. [2,3] An insufficient folate intake is associated with a large number of health disorders, such as megaloblastic anemia, [4] and with an increased risk of Neural Tube Defects (NTDs) in the developing foetus. [5] In addition, there is scientific evidence of a relationship between folate deficiency and several other diseases, such as cardiovascular diseases, [6] Alzheimer's disease [7] and some forms of cancer. [8] The gap between the recommended and actual folate intake has led to mandatory folic acid food fortification in more than 60 countries around the world, but not in Europe, where only voluntary food fortification is practiced [Reg. (EC) No 1925/2006]. Although folic acid food fortification has been the main strategy undertaken over the years to increase folate diet levels, natural folate enhancement has recently also attracted attention in countries where mandatory folic acid food fortification is not practiced. Variations in the folate content of wheat, [9] barley, [10] oat [11] and rye [12] were examined in the comprehensive European HEALTHGRAIN project. Information is also currently available on folate levels in rice [13] and pseudocereals. [14] Even though cereal

grains and their derived products are important sources of natural folates, the folate content of cereal products depends on both the initial grain content and the severity of the grain milling process. In fact, bioactive compounds, such as folates, are unevenly distributed in cereal grains. As reported in previous studies, folates are mainly concentrated in the bran and in germ fractions. [15-17] These fractions are generally removed during the milling process and remain in the bran fraction, which is mainly used for feeding. Hegedüs and collaborators [18] showed that, with an extraction rate of 87% in the milling process, the wheat flour folate concentration is reduced to 79% of that of wholegrain flour. Similarly, with an extraction rate of 66%, the wheat flour folate concentration is reduced to 10% of that of wholegrain flour. These data are in agreement with other results [19] which show that the folate content is reduced remarkably in sifted wheat flours with extraction rates of 70-80%. Similarly, Arcot and collaborators [15] reported that wheat bran folate levels are 4-fold higher than those of flour and 2-fold higher than those of grain.

For these reasons, the “whole grain concept” promotes the consumption of all the grain components in the same proportion as in the native grain. [20] However, the outer layers of the grain may confer undesirable properties to bakery products, in terms of safety, processing, or acceptability by consumers. The outer layers of the wheat kernel are the most prone to contamination by mycotoxins, heavy metals and pesticides. [21] Moreover, wholegrain foods are not so attractive to consumers, because of the high bran content in wholegrain flour, which reduces the sensory value of the end-use products. [22] Therefore, the cereal grain fractionation technology, which could also be applied easily with a selective dry pearling process, is receiving a great deal of attention because of its capacity to efficiently separate the negative and positive aspects, in order to

produce new ingredients and flour mixes with technologically optimized functional and nutritional attributes.[23] This will allow to produce new flour mixes and ingredients with technologically optimized functional and nutritional attributes. The pearling (debranning) of wheat, before roller milling, is going to be increasingly accepted by wheat millers to improve milling performance, since it sequentially removes the outer kernel bran layers through an abrasive scouring and increases the efficiency of the milling process.[24] The average concentrations of mycotoxins and heavy metals are more effectively reduced by pearling than milling.[21] Moreover, the degree of pearling could be carefully modulated to separate the external bran fractions, which are characterized by a higher toxicity risk and coarse fiber, from the cereal fractions which offer potentially high health benefits. In this way, it would therefore be possible to enrich conventional flour with cereal bran fractions, obtained from sequential pearling and characterized by higher antioxidant activity and bioactives content, but also by a lower risk of contamination.[25] For this reason, the distribution of the different nutrients in the cereal grain should be evaluated.

Although the distribution of folates in hulled barley fractions was already investigated,[26] to the best of the authors' knowledge no information is available, in the scientific literature, on the distribution folates in pearling fractions of either common or durum wheat or hulless barley. The aim of this study was to determine the folate distribution in the kernels of two wheat and three barley cultivars, in order to enhance the achievement of grain fractions rich in these compounds through the pearling process.

MATERIALS AND METHODS

Plant material

Three commercial barley varieties (*Hordeum vulgare* L.) and two wheat varieties, namely, one common wheat (*Triticum aestivum* L.), and one durum wheat (*Triticum turgidum durum* Desf.), were cultivated side by side on the same field, in a medium-texture fertile soil in Carignano, Piedmont, NW Italy (44°53'8.69"N, 7°41'16.75"E, 232 m a.s.l.), during the 2011-12 growing season, according to the ordinary crop management program applied to these crops in the growing area.

The compared barley varieties were:

- Mona (S.I.S, San Lazzaro di Savena, BO, Italy): spring, hulless and two-row variety, used for food purposes;
- Trasimeno (Geo Seed, Grinzano di Cervere, CN, Italy): winter, hulled and two-row variety, used both for food and feed purposes;
- Ketos (Limagrain Italia Spa, Busseto, PR, Italy): winter, hulled and six-row variety, mainly used for feed purposes.

The compared wheat varieties were:

- Generale (Consorzio Nazionale Sementi, Conselice, RA, Italy): common wheat variety classified, according to the Italian bread-making quality grade, [27] as superior bread making-quality wheat;
- Colombo (Apsovsementi S.p.A., Voghera, PV, Italy): durum wheat variety classified as high quality wheat.

Planting was conducted in 12 cm wide rows at a seeding rate of 450 seeds m^{-2} on October 24, 2011 for the winter barley and wheat, while cv. Mona was planted on March 1, 2012. The previous crop was maize for grain. Fungicide treatments were only performed on the common and durum wheat to avoid the

development of foliar and head fungal diseases at stem elongation (GS-35, a.i. azoxystrobin and cyproconazole applied at 0.2 kg ha^{-1} and 0.08 kg ha^{-1}, respectively) and at heading (GS-58, a.i. prothioconazole applied at 0.250 kg ha^{-1}).

One hundred and thirty and 170 kg N ha^{-1} were applied as granular ammonium nitrate fertilizers for the barley and wheat cultivars, respectively. Harvesting was conducted with a combine-harvester on June 23 and July 10, 2012 for the barley and wheat cultivars, respectively. Kernel samples of each cultivar were stored at 4℃ until the beginning of the tests.

Barley and wheat grain pearling

From 6 to 9 pearling fractions of the kernels were obtained through the incremental pearling of the barley and wheat varieties, according to the approach described by Beta and collaborators. [28] The pearling consisted of consecutive passages of kernels and pearled kernels in an abrasive-type grain testing mill (Model TM-05C, Satake, Tokyo, Japan) at a constant speed of 55 Hz. The pearling process was monitored by means of a time control. After each assay, the laboratory pearler was cleaned thoroughly to minimize equipment contamination. Initially, a 500-g portion of each unprocessed sample was sub-sampled from a 5-kg sample, and the remaining 4.5 kg was pearled.

A different number of bran fractions was obtained for the barley, according to its hulled or hulless nature, in order to obtain a similar kernel pearling degree. Starting from unprocessed hulless barley grain (cv. Mona), the kernels were initially pearled to remove 5% of the original grain weight, and this resulted in a first fraction (0-5%). Then, the remaining kernels were pearled to remove a second fraction of 5% (5-10%). The pearling process was repeated to remove a

214 third, fourth and fifth fraction (designed fractions of 10-15%, 15-20%, 20-25%).
215 A residual 75% of the kernel (25-100%) was also collected.
216 Kernel fractions of 5% in weight were obtained in the hulled barley varieties.
217 Nevertheless, in this case, the first two passages mainly removed the hull
218 fractions. The corresponding fractions of the hulless barley were obtained from
219 the third pearling passage. Thus, these pearled fractions were called hull1, hull2,
220 0-5%, 5-10%, 10-15%, 15-20%, 20-25%, 25-30% and 30-100%.
221 The pearling process for the wheat varieties was the same described for the
222 hulless barley variety. The kernels were pearled in order to obtain 6 pearling
223 fractions (see above).
224 The whole unprocessed grain samples and the residual fractions were milled
225 using a laboratory centrifugal mill (Model ZM-100, Retsch, Haan, Germany) with
226 a 1-mm opening. All the samples were stored at -25°C before the chemical
227 analyses were performed.
228
229 **Chemical analyses**
230 The moisture and the total folate content were determined on ground whole
231 kernels and their pearled fractions. The moisture content was obtained, using a
232 Sartorius MA30 thermo-balance (Sartorius AG, Goettingen, Germany), in order
233 to express the results on a dry matter (dm) basis.
234 The total folate content was determined using a microbiological assay,
235 according to the AOAC Official Method 2004.05, with a few modifications. This
236 method is applied to cereal grains and cereal grain foods containing added
237 folates (folic acid) or natural occurring folates with levels of 7.6 μg 100 g^{-1} to
238 100% folates.

Briefly, a 1.0 g sample was mixed thoroughly with a phosphate buffer, pH 7.8 (0.1 M sodium phosphate dibasic, 1% ascorbic acid) and then diluted with water to a 50 ml volume. Samples were autoclaved for 15 min at 121-123℃ and treated with Termamyl®Ultra 300 L (Novozymes, Denmark). The homogeneate was then treated with human plasma conjugase (Sigma-aldrich, Saint Luis, Missouri) and Creon 10000 [8000 EP-e amylase, 10000 EP-e lipase and 600 EP-e protease (Abbott Healthcare Products, Maidenhead, United Kingdom)]. The samples were treated for 3 min at 100℃ and then cooled. The pH was adjusted to 4.5 and the samples were then diluted to a volume of 100 ml and filtered through a 2V Whatman filter paper.

The total folate content was determined through a microbiological assay with *Lactobacillus casei* subspecies *rhamnosus* (ATCC 7469). A folic acid-free double strength basal medium was added to each tube and then autoclaved for 6 min at 121-123℃. The tubes were rapidly cooled to minimize browning reactions between the amino acids and sugars, and then aseptically inoculated with a 50 µl drop working inoculum. After incubation at 37℃ for 22 h, bacteria growth was measured as optical density at 595 nm using inoculated blanks as the reference blank. The amount of folates in each sample was determined through a comparison with calibration solutions of known concentrations.

Statistical analyses

All the analyses were performed in triplicate. One-way analysis of variance (ANOVA) was applied separately for each cultivar in order to compare the folate content among the different wheat and barley varieties and among the pearling fractions. The residual normal distribution was verified using the Shapiro-Wilk test, while variance homogeneity was verified by performing the Levene test.

The REGW-Q test was performed for multiple comparison purposes. When the ANOVA assumptions were not verified, rank transformation of the data was performed. [29] SPSS for Windows statistical package, Version 20.0 (SPSS Inc., Chicago, Illinois) was used for the statistical analyses. A 0.05 threshold was used as the cut-off value for significance in all the tests.

RESULTS and DISCUSSION

Total folate content in barley and wheat cultivars

The comparison of folate content of the different barley and wheat varieties showed significant differences (Table 1).

Three barley varieties were selected for the study, considering spring and winter types, as well as two-row and six-row types. The total folate content of the three genotypes was, on average, 806 ng g^{-1} dm, and ranged between 653 and 1033 ng g^{-1} dm. The mean folate level observed in this study fell within the range reported in previous studies for this crop (518-918 ng g^{-1} dm [10,26]). The lowest folate content was observed in the hulled winter barley cv. Ketos (653 ng g^{-1} dm), which is normally used for animal feeds. A similar folate level was observed in cv. Trasimeno (732 ng g^{-1} dm), which is also intended for the food chain. Conversely, a significantly higher folate content was observed in the hulless barley cv. Mona (1033 ng g^{-1} dm). Andersson and collaborators, [10] comparing 10 barley varieties, including 2 hulless and 8 hulled, observed that there is no clear trends in the folate content, when either winter or spring varieties or hulled and hulless ones are considered. Thus, other genetic components would seem to play a more important role. Recently, hulless barley has gained particular attention for food purposes because of the content of several other nutritional compounds, in particular β-glucans, which is higher than in hulled barley varieties. [30] Thus, the identification of hulless varieties rich in both folates and β-glucans could increase the nutritional value of barley food products.

The total folate content in the common and durum wheat cultivars was, on average, 1072 ng g^{-1} dm. Both cultivars were characterized by a similar folate content (1024 ng g^{-1} dm and 1119 ng g^{-1} dm, respectively). The total folate

content of both common and durum wheat cultivars fell within the wide range reported in previous studies. [15] However, the results were over the range reported by Piironen and collaborators [9] for 130 winter wheat cultivars (mean 561 ng g^{-1} dm, range 364-774 ng g^{-1} dm) and for 10 durum wheat cultivars (mean 741 ng g^{-1} dm, range 637-891 ng g^{-1} dm) cultivated in Hungary. The folate level in the wheat grains was shown to vary over a wide range. The variation in folate content that we observed could partly be explained by the different genetic backgrounds or growing conditions. [9] The folate content of another common wheat cultivar grown in climatic and agronomic conditions similar to the ones described above was shown to be lower (659 ng g^{-1} – data not showed) than those observed for cvs. Generale and Colombo.

Total folate content in barley pearling fractions

The folate content in the fractions obtained from the sequential barley pearling is reported in Figure 1. Folate-rich fractions were achieved for both the hulled and hulless cultivars by pearling.

The Trasimeno and Ketos hulled cultivars were characterized by covered kernels, and the hulls were mainly removed in the first two pearling passages. Thus, the first two fractions (hull1 and hull2) mainly consisted of lignocellulosic material characterized by a high mineral content. [31] The folate concentration in the first two pearling fractions for both cv. Ketos and cv. Trasimeno was not significantly different from that of the whole grain. The authors hypothesized that total folates in the first two fractions were mainly derived from the germ and pericarp tissues. Since the germ is located at the proximal end of the barley kernel, as reported by Edelmann and collaborators, [26] tiny fragments were apparently removed within the first two fractions, as demonstrated by the

343 presence of white germ particles in the fibrous hull. Furthermore, in the second
344 pearling fraction, a part of the folates might have come from the seed coats
345 (pericarp and testa). Thus, all these facts could be responsible for the high total
346 folate content observed in the hull fractions. After hull removal, a characteristic
347 folate distribution was observed in both hulled barley cultivars and a progressive
348 reduction was observed from the outermost to the innermost layers. In fact, in
349 both cultivars, the highest folate concentration was observed in the 0-5%
350 pearling fraction, which corresponds to the outermost layers of the kernel. In
351 particular, the 0-5% fraction of cv. Trasimeno was characterized by a folate
352 concentration of 1038 ng g^{-1} dm (1.4-fold higher than the whole grain), while the
353 Ketos one was characterized by 2393 ng g^{-1} dm (about 4-fold higher than the
354 whole grain). Moreover, a reduction in the folate level was observed after each
355 progressive pearling passage towards the innermost layers. In particular, a
356 significant reduction in the folate level was observed for the two-row barley cv.
357 Trasimeno after the removal of 30% of the kernel weight. The mean folate level
358 observed from the 0-5% to the 25-30% fraction was 903 ng g^{-1} dm. The residual
359 30-100% fraction, which mainly corresponds to the endosperm, was
360 characterized by a folate concentration of 385 ng g^{-1} dm. Even thought the
361 reduction in folate concentration was not significant, a gradual reduction in
362 folate concentration was observed from the 15-20% fraction to the 25-30%
363 fraction. This was probably due to an increase in endosperm content.
364 Conversely, a gradual significant reduction was observed in the six-row cv.
365 Ketos from the 0-5% fraction to the innermost one. In particular, as reported
366 above, the 0-5% fraction, corresponding to the outer layers of the kernel, was
367 the one that was characterized by the highest folate level. A not significant
368 reduction of 25% was observed in the subsequent 5-10% fraction. After the

removal of the first two fractions, a higher reduction was observed, ranging from 58% to 86%, in comparison to the 0-5% fraction. The folate distribution observed in the hulled barley varieties analyzed in this study has confirmed data reported for other hulled two-row and six-row varieties. [26]

To the best of the authors' knowledge no other work has described the folate distribution in hulless barley kernels. As shown for the hulled barley cultivars, a significant reduction in the folate content was also observed in the hulless two-row cv. Mona from the outermost to the innermost kernel layers. In particular, the highest folate concentration was found in the outermost 0-5% fraction, which is characterized by a concentration of 4647 ng g^{-1} dm. In this fraction, the folate content was about 4.5-fold higher than in the whole grain. A high folate level (3970 ng g^{-1} dm) was also observed in the second 5-10% fraction. This content was about 4-fold higher than that of the whole grain. The remaining parts of the pericarp and testa, but also a part of the aleurone layer and of the germ were probably collected in this fraction. [26,32] Thus, on the basis of previous observations on barley fractionation, [26,32,33] it was assumed that the high level of folate observed in the first fractions mainly came from the germ, and from the outer layers of the kernel. The folate content in the next three fractions (10-15%, 15-20% and 20-25%) was still fairly high, compared to the whole grain (3350 ng g^{-1} dm, 2652 ng g^{-1} dm and 2009 ng g^{-1} dm, respectively). The folates in these fractions probably mainly came from the aleurone layer, because most of the germ had been eliminated earlier. After removing 25% of the kernel weight by pearling, the germ and the outer layers of the kernel were removed, and the rest of the grain probably mainly consisted of endosperm tissues, with small amounts of aleurone and subaleurone. In fact, a significantly lower folate concentration was observed in the innermost fraction (620 ng g^{-1} dm). The folate

content of the innermost residual fraction was significantly lower than that of the whole grain.

Total folate content in wheat pearling fractions

The folate content in the fractions obtained from the sequential wheat pearling is reported in Figure 2. As previously reported for barley, folate-rich fractions were achieved from both the common and durum wheat cultivars by pearling.

The folate content in the first three fractions of the common wheat was significantly higher than that of both the whole grain and of all the other pearling fractions. The folate content in these three fractions (0-5%, 5-10% and 10-15%) was 2050 ng g^{-1} dm, 1895 ng g^{-1} dm and 1801 ng g^{-1} dm, respectively. The folate concentration in the first three pearling fractions was about 2-fold higher than that of the whole grain (1024 ng g^{-1} dm). After the removal of the external fractions, a significant reduction in the folate concentration was observed; the folate concentration of the intermediate pearling fractions (15-20% and 20-25%) and of the residual fraction (25-100%) was 883 ng g^{-1} dm, 727 ng g^{-1} dm and 726 ng g^{-1} dm, respectively. Although no significant differences were observed between the last three fractions, a decrease in folate content was observed from the outermost fraction to the innermost one. The folate content observed in these pearling fractions was not significantly different from that of the whole grain.

A similar trend was also observed in the durum wheat pearling fractions. The folate content in the first three fractions (0-5%, 5-10% and 10-15%) was 2670 ng g^{-1} dm, 2651 ng g^{-1} dm and 2765 ng g^{-1} dm, respectively. The folate content in these three fractions was significantly higher than that of both the whole grain and of all the other fractions. This content was about 2.5-fold higher than that

observed in the whole grain (1119 ng g^{-1} dm). A significant reduction in the folate level was observed after the removal of the external fractions. In fact, the intermediate pearling fractions (15-20% and 20-25%) were characterized by a folate content of 1890 ng g^{-1} dm and 2095 ng g^{-1} dm, respectively. However, the folate content in these two fractions was about 2-fold higher than that of the whole grain. The lowest folate content was observed in the 25-100% residual fraction (784 ng g^{-1} dm). No significant difference was observed between the innermost fraction and the whole grain.

Shetlar and collaborators [34] reported that the outer pericarp, the inner pericarp, the testa and the aleurone layer, represent 3.9%, 0.9%, 0.7% and 9% of the kernel weight, respectively. Therefore, according to data reported in other studies, [35-37] pearling up to the 5% level on average removed most of the outer pericarp, while the aleurone layer was removed at the 5-10% and 10-15% level. Furthermore, as reported for barley, part of the folates observed in the first pearling fractions might have originated from the germ. The results have confirmed that the folates were mainly concentrated in the outer layers and in the germ of wheat kernels. Similarly, Hemery and collaborators [17] showed that the electrostatic wheat bran separation process produced a fraction that was rich in aleurone cells and which was therefore characterized by a large amount of folates (1188 ng g^{-1} dm). Moreover, Fenech and collaborators [16] reported a folate concentration of between 4000 and 6000 ng g^{-1} in wheat aleurone flour containing aleurone and germ particles, and the isolated wheat germ contained 2000 ng g^{-1} dm folates. [38]

To our knowledge, this is the first study that describes the folate distribution in pearling fractions of common wheat, durum wheat and hulless barley kernels. Even though the first pearling fractions were characterized by the highest folate

content in both the wheat and the hulless barley, these fractions could result in a higher sanitary risk as a consequence of the high concentrations of mycotoxins, heavy metals and pesticides. [39] In order to obtain a functional ingredient through wheat pearling, Sovrani and collaborators, [39] suggested the 10-15% intermediate fraction for common wheat as the best compromise between high nutritional value and low mycotoxin contamination risks. Moreover, it was observed that the addition of this intermediate fraction with a 10% substitution level [25] to refined flour could increase the content of bioactives with limited effects on the technological properties. Considering our results, the addition of cvs. Colombo and Mona 10-15% fraction with a 10%-substitution level [25] to the refined flour could increase the folate level up to 15% of the Nutrient Reference Value [NRV – Reg. (EU) No 1169/2011]. The addition of cv. Generale 10-15% fraction under the same conditions could improve the folate level up to 9% of the NRV. Instead, the addition of barley cvs. Ketos and Trasimeno 10-15% fraction could only increase the folate level up to 5% of NRV. Further studies are necessary in order to identify the functional ingredients that would be able to enrich the folate contents in bakery products, while considering that these compounds are unstable at high temperatures and as a result their levels could decrease in the final food products. [40] In conclusion, our results suggest that the pearling process could be a useful and practical tool in order to select intermediate bran fractions from small cereals, as a natural source of folates, separated from detrimental components, in order to develop nutritionally enhanced ingredients and products.

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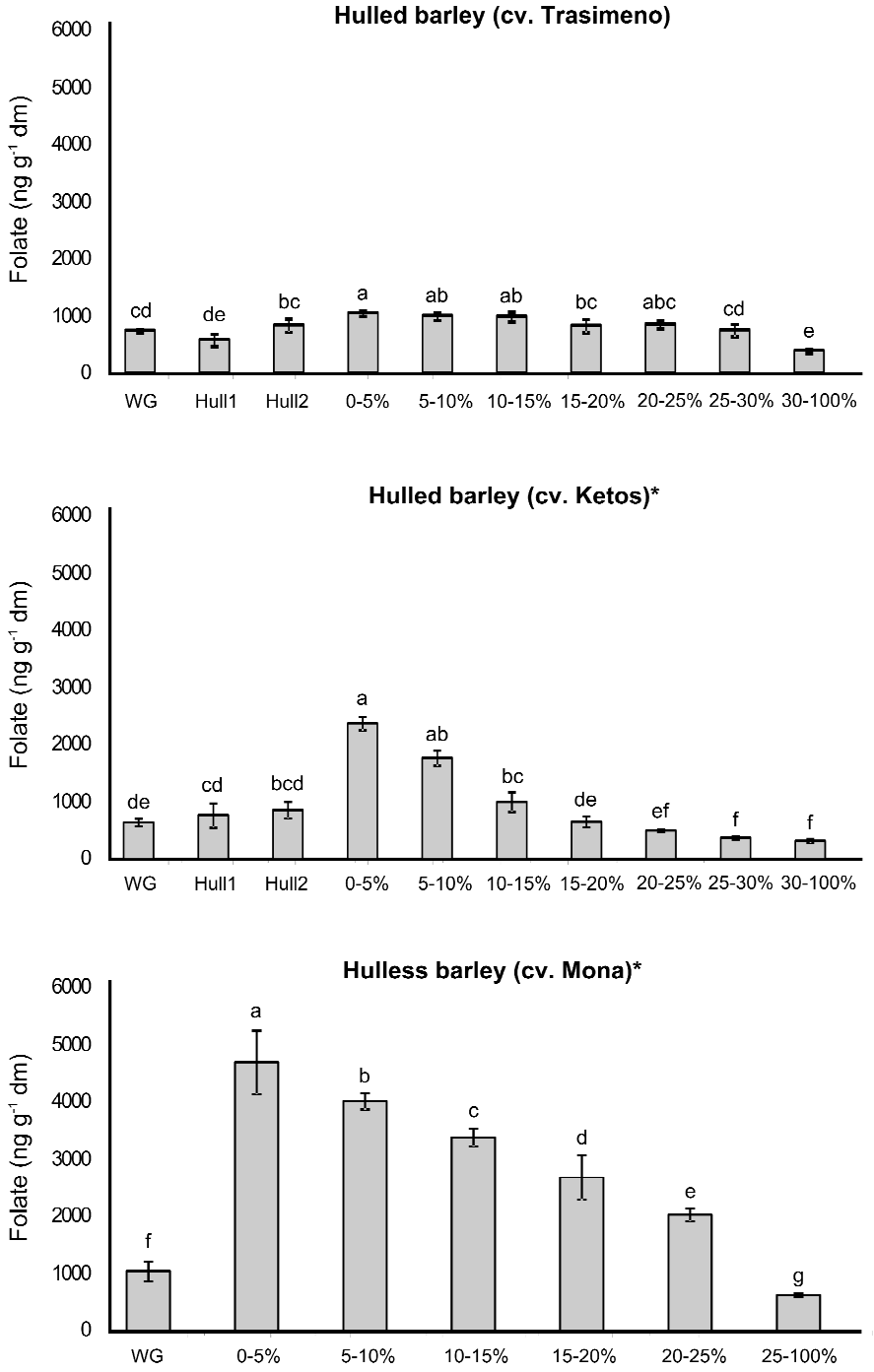
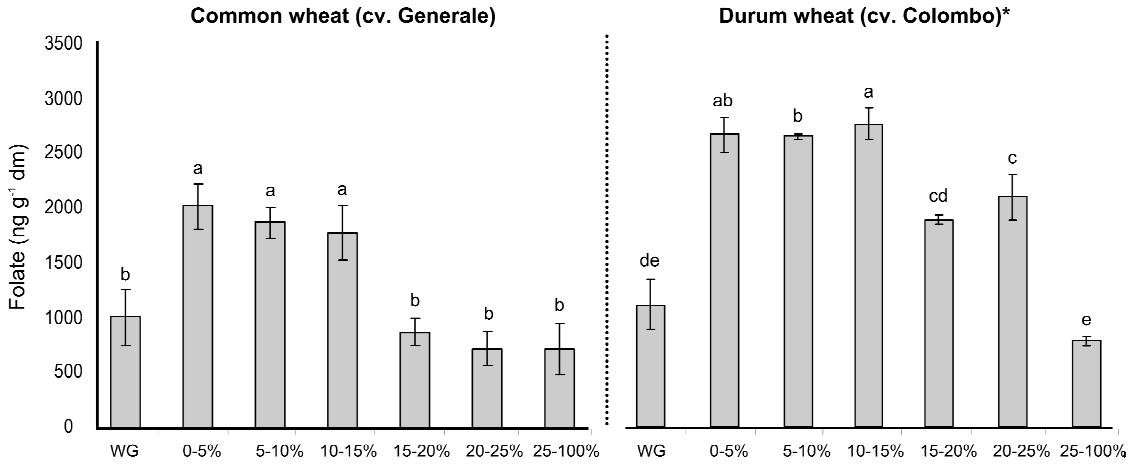
FIGURES

Figure 1. Folate content in the barley pearling fractions.



The reported data are the means of three values; values with different letters differ significantly ($P<0.05$). The error bars indicate the standard deviation. (WG: Whole Grain, *data analyzed after rank transformation).

Figure 2. Folate content in the wheat pearling fractions.



The reported data are the means of three values; values with different letters differ significantly ($P<0.05$). The error bars indicate the standard deviation. (WG: Whole Grain, *data analyzed after rank transformation).

TABLE

Table 1. Folate content in the whole grain of the compared barley and wheat varieties.

Species	Cultivar	Type	Folate content (ng g^{-1} dm)
H. vulgare	Mona	Hulless, spring, two-row barley	1033 ± 165 a
H. vulgare	Ketos	Hulled, winter, six-row barley	653 ± 65 c
H. vulgare	Trasimeno	Hulled, winter, two-row barley	732 ± 25 bc
T. aestivum	Generale	Winter common wheat	1024 ± 261 ab
T. turgidum durum	Colombo	Winter durum wheat	1119 ± 219 a

Data were analyzed after rank transformation. Values with different letters differ significantly ($P<0.05$)

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