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Diversity of Nutrient Content in Grains - A Pilot Metabolomics Analysis

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Abstract

Grains are a major food source providing many essential nutrients. The objective of this work was to determine the biochemical (<1,500 Da) composition of selected grains and grain fractions. We hypothesized that the nutrient composition of grains is not only dependent on the grain type, but also influenced by the milling process to generate individual fractions. Whole grain corn, oat, and wheat were milled and separated into bran and flour/meal fractions. The biochemical composition of the whole grains and their subcomponents were determined by untargeted metabolomic profiling on methanol extracts. This global analysis identified 325 biochemicals, belonging to diverse nutrient categories including carbohydrates, antioxidant, vitamins and amino acids. Many of the metabolites were significantly different between the grain types and grain fractions; statistical analysis showed clear differences in the biochemical composition of corn, oat, and wheat grains. Principle component analysis showed that whole wheat flour and whole oat flour were not distinguishable from their respective grains, while corn meal could be distinguished from the corn grain kernel. Many of these nutrients were decreased in the bran fractions upon processing. This preliminary study provides a glimpse into distinct profiles of different grains and their fractions, which can potentially have an impact on nutrition, health and other parameters. Further research is needed to better understand the health benefits of these compounds in individual grains and grain fractions. The use of metabolomics techniques to better understand the profiles of foods not only can help understand their role in improving their health, but also their impact on food product quality, food safety and other parameters.

Keywords: Metabolomics; Nutrient; Whole grain; Processed grain; Corn; Oat; Wheat; Bran

Abbreviations: Da: Daltons; YDC: Yellow Dent Corn; SOG: Stabilized Oat Groats; SWW: Soft White Wheat; UHPLC: Ultra High Performance Liquid Chromatography; GC/MS: Gas Chromatography-Mass Spectrometry; CV: Coefficient of Variance; FDR: False Discovery Rate Calculations

Introduction

Whole grains are defined by the American Association of Cereal Chemists International and Food and Drug Administration as consisting of the "intact, ground, cracked or flaked fruit of the grain whose principal components, the starchy endosperm, germ and bran, are present in the same relative proportions as they exist in the intact grain" [1,2]. That is, whole grain foods can contain the intact whole grain or they can be reconstituted from processed fractions, provided they have components of the whole grains recombined to the same relative proportions as naturally occurring in the grain kernel [1,2]. During the refining of whole grains into white flour the outer bran and inner germ layers are removed and the remaining endosperm is processed into white flour. Thus, compared to refined grains, whole grains are inherently richer in dietary fiber, containing approximately 80% more dietary fiber than refined grains [3,4]. Furthermore, as a consequence of this refining process, there is substantial loss of essential minerals, vitamins and phytonutrients [3,4].

Whole grains are rich sources of vitamins, minerals, dietary fiber, lignins, beta-glucan, inulin, numerous phytonutrients, including phytosterols, phytin, and sphingolipids [3,5]. The bran is the multi-layered outer skin of the grain that protects the germ and the endosperm from damage, such as sunlight, pests, water, and disease. The bran contains phenolic compounds, vitamins, minerals, and fiber. The endosperm is the largest component of the whole grain; it contains carbohydrates (mostly starch), protein, vitamins and minerals, and serves as the food supply for the seedling plant. The germ refers to the embryo, the part that forms the new plant, and contains vitamins, some protein, minerals, and fat. The relative proportion of these compounds varies by the species and by the whole grain fraction. For instance, corn has the highest phenolic content (265 mg gallic acid equivalent/100 g) followed by wheat, oats and rice, with, 136 mg, 111 mg, and 95 mg gallic acid equivalent/100 g, respectively [6]. As discussed above, whole grains have higher phytonutrient content and antioxidant activity than refined grains. Adom and Liu [7] reported that the majority of the beneficial phytonutrients are present in the bran and germ fractions of whole grains. For example, refined wheat flour looses 83% of total phenolics, 79% of total flavonoids, 93% of ferulic acid, 78% of total zeaxanthin, 51% of total lutein, and 42% of total β-cryptoxanthin when compared to whole wheat flour [7]. The additive and synergistic effects of these bioactive phytonutrients found in whole grains are purported to be responsible for the health benefits associated with whole grains [3,6,7]. Food processing followed by reconstitution, using techniques such as thermal processing and milling, can help release these phytonutrients, making them more bioaccessible, thus enhancing their health effects, both locally and systemically, upon absorption.

In addition to the type of grain and whether or not it is processed, other factors such as the genetic background, environmental factors, and agronomic practices can also impact the chemical composition of whole grains and their individual components [8,9]. Composition can

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also vary based on the stage of kernel development and on the specific types of processing used [8,9].

Metabolomics allows the non-targeted global profiling of structurally and functionally diverse metabolites. Metabolomics technology offers the capability to identify and measure hundreds of separate biochemical entities virtually in a simultaneous manner. Within the context of potentially selecting beneficial plant/seed traits, biochemical profiling of maize grain, using a GC-TOF-MS system, was recently reported [10-12]. Analyzing grain of different maize genotypes grown in different environments, 119 metabolites were identified, including free amino acids, free fatty acids, and carbohydrates. Dramatic variations in some metabolites were observed, varying ~1.5 to>150-fold [9].

The purpose of the present pilot study was to develop a better understanding of the nutritional and anti-nutritional components found in the whole grains of wheat, oats, and corn, and in their various processing fractions. Identifying the similarities and differences among the grains and their fractions could potentially help elucidate which grains and which of their components have the greatest potential for promoting health and reducing risk of chronic disease.

Methods

Grains and grain fractions

All samples of grains and their fractions, namely, yellow dent corn (YDC), stabilized oat groats (SOG) and soft white wheat (SWW), are used in regular food production and were provided by General Mills Inc (Minneapolis, MN). Briefly, the yellow dent corn, U.S. Grade #2 corn kernels, were heat treated to inactivate the enzymes present, and yellow whole grain corn flour was milled from the kernels. The corn meal is a finely ground product milled from the horny endosperm portion of dry, yellow, shelled, degermed dent corn. The corn bran, which is the coarse outer covering of the corn kernel, was separated from the cleaned yellow corn and was ground in the commercial milling process. The whole oat flour was produced from 100% de-hulled clean whole oat groats by steaming and grinding such that there was no significant loss of oat bran. The low bran oat flour, which is a by-product stream made from whole oat flour, was obtained by processing whole, clean, sound, de-hulled, steamed and ground oats, and by separating the appropriate proportion of bran. The oat bran was obtained by processing whole, clean, sound, de-hulled oats. Soft white wheat, U.S. #2 grade, which was suitably and adequately cleaned, was milled. The appropriate proportion of bran was separated to produce the soft white wheat bran. Whole grains and the grain fractions were analyzed by Metabolon's biochemical profiling platform (Metabolon Inc., Durham, NC).

Metabolomics analysis

Metabolomics analysis was conducted at Metabolon, Inc. (Durham, NC). Detailed methodology has been previously described [10-12]. Briefly, the small molecule metabolites were extracted from 100 mg of sample using a methanol based solvent, dried and reconstituted in chromatography solvent. The reconstituted extracts were divided into three portions and resolved using three separate chromatography platform systems coupled to mass spectrometry. The purpose of using multiple platforms was to provide broad coverage of the diverse small molecules present in biological samples. Two of the chromatography systems were UHPLC coupled to mass spectrometry, being separately optimized for the ionization of negative ions or positive ions. The third sample was derivatized using trimethylsilane prior to injection into the GC/MS instrument. The identity of metabolites was determined by matching the combination of chromatographic retention index and mass spectra signatures to reference library entries based on analysis of authentic chemical standards [12], the library contained 3066 named chemical entries. Performance standards spiked into each sample, as well as the analysis of a collection of sample technical replicates, allowed for estimations of overall process variation and data quality. The median coefficient of variance (CV) for the spiked standards (added after extraction, and a measurement of instrument reproducibility) was 5%. The median CV for all biochemical compounds measured in the sample technical replicates (a measurement of overall process variation) was 12%.

Data normalization and statistical analyses

Raw area counts for each metabolite in each sample were normalized to correct for variation resulting from instrument inter-day tuning differences. For each metabolite, the raw area counts were divided by its median value for each run-day, therefore setting the medians equal to 1 for each day's run. Missing values were assumed to result from areas falling below the limits of detection. For each metabolite, the missing values were imputed with the observed minimum after the normalization step. In order to compare nutrient content between grain fractions, values for grain fractions were normalized to the whole grain using the recovery rate.

Principal components analysis was performed using ArrayStudio (OmicSoft Corp., North Carolina, USA) on all the detected metabolites. Whole grains, meal/flour and bran from the corn, oat and wheat were compared to one another by performing Welch's *t*-test, using replicates (n=6) on log-transformed data for every metabolite (Figure 1). The full statistical table can be found in the Supplementary tables (available upon request from the authors). Multiple comparisons were compensated for using false discovery rate (FDR) calculations [13], and FDRs were estimated using the q-value method [14]. All T-tests were performed with R, which is an open-source software package (http://cran.r-project.org/).

Results

Cooking and heating deactivate grain metabolism

The corn, wheat, and oats grain samples could be completely separated by their biochemical profile using unsupervised classification (Figure 2). The separation indicated that the grains were distinctive in their biochemical composition and abundance. Principle component analysis showed that whole wheat flour and whole oat flour were not distinguishable from their respective grains, and in addition corn meal could be distinguished from corn grain. The greatest differences between corn grain and corn meal were identified by the *t*-test results ($p \leq 0.05$) and were related to stress responses and carbohydrate metabolism (Figure 3). The differences were not due to enrichment or dilution from the milling process since recovery of the corn meal was

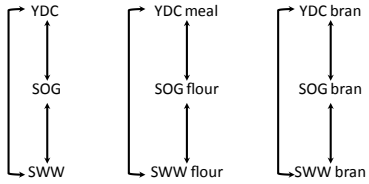

Figure 1: Statistical comparisons. Welch's T-tests were performed between the groups indicated by the arrows. YDC, yellow dent corn; SOG, stabilized oat groats; SWW, soft white wheat.

100%. These metabolic changes were not observed in oat or wheat grains (data not shown). Biochemicals including the antioxidant glutathione (reduced and oxidized) (Figure 3A) and several osmolytes (Figure 3B), are expected to accumulate normally during the desiccation of grains, and were abundant in the corn meal but were significantly depleted or in yellow dent corn. In corn grain, glucose oligosaccharides coming from starch catabolism, and glucose-1-phosphate from carbohydrate metabolism were all increased in corn grain (Figure 4). The depletion of anti-oxidants and osmolytes, and the production of carbohydrate in the whole grains were consistent with the presence of residual enzymatic activity that would be denatured with heating and boiling during the milling process.

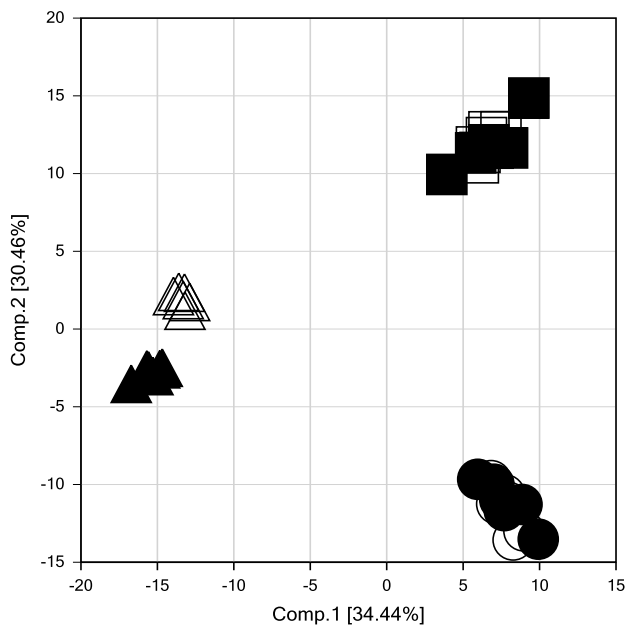

Figure 2: Principle component analysis plot showing separation of corn, wheat and oat grains. The biochemical composition between grains and between yellow dent corn and corn meal can be distinguished. In total, 64.9% of the variation of the data is represented by the first component (34.44%) and second component (30.46%). Filled Circles= soft white wheat; Filled squares= stabilized oat groats; Filled triangles= yellow dent corn; open circles= wheat flour; open squares= whole oat flour and open triangles= corn meal.

Comparison of grain composition

The biochemical profiling identified 325 distinct entities (Supplement for complete biochemical list available upon request from authors). To our best ability, a subset of the biochemicals from corn, wheat, and oat were categorized as shown in table 1. The classification scheme illustrates the distribution, number and diversity of compound classes detected in the various grains. A small portion of biochemicals (4-9.5%) were detected exclusively in one grain (Figure 5A); for example, ergothioneine and alpha tocotrienol were detected in oats; quercetin-3-galactoside, gamma tocopherol and campesterol were detected in yellow; choline phosphate and inositol 2-phosphate were detected in wheat. We also detected unique biochemicals in individual grain fractions (Figure 5B), but the majority of biochemicals were shared across at least two grain types. Statistical analysis showed that the levels of many metabolites were significantly different between the grain types and grain fractions, namely, the amino acids, flavonoids, choline and inositol metabolites, phytosterols, vitamins, and cofactors which are reported to be important or beneficial in human health. Table 2, shows the differences between corn, oat and wheat grains. The distribution of the nutrients was grain dependent and also varied within the same biochemical class (Table 2). With few exceptions, the majority of nutrient levels were significantly lower in bran fractions

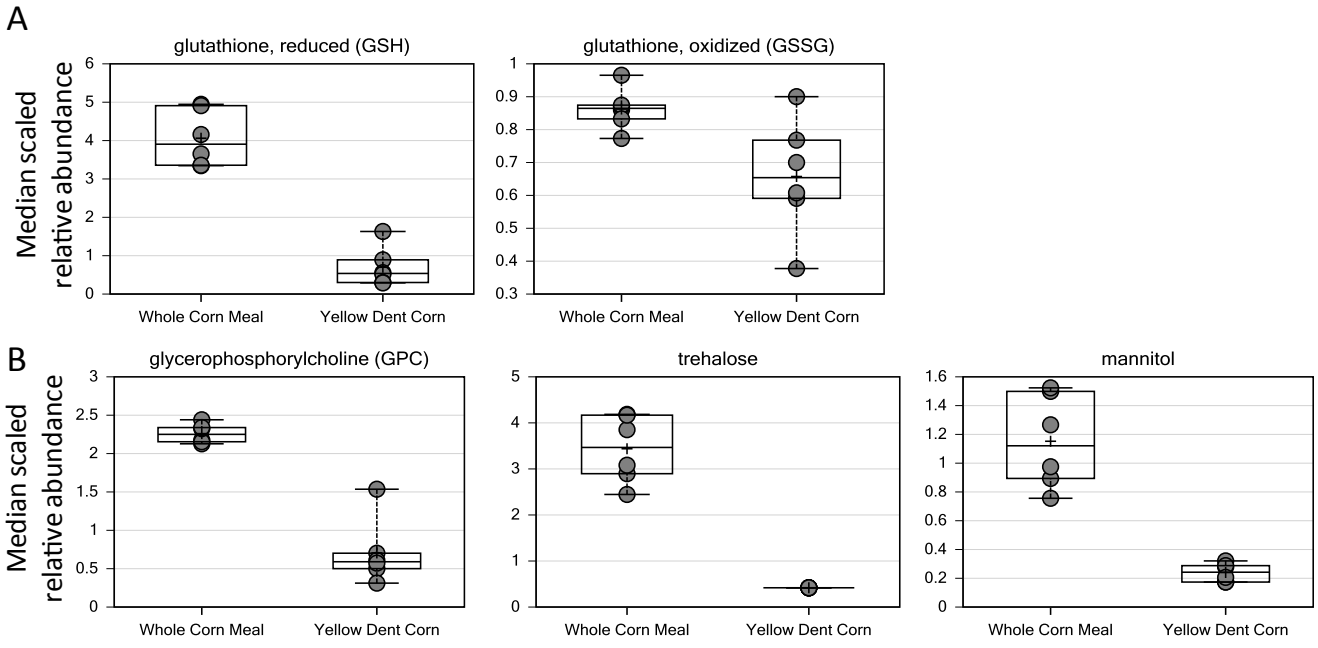

Figure 3: Boxplots showing metabolites elevated ($p \leq 0.05$) in whole corn meal A. glutathione metabolites; B. osmolytes. The boxed area represent the interquartile range; whiskers indicate maximum and minimum values; the horizontal line indicates median value; "+" indicates mean value; circles show biochemical level for individual samples.

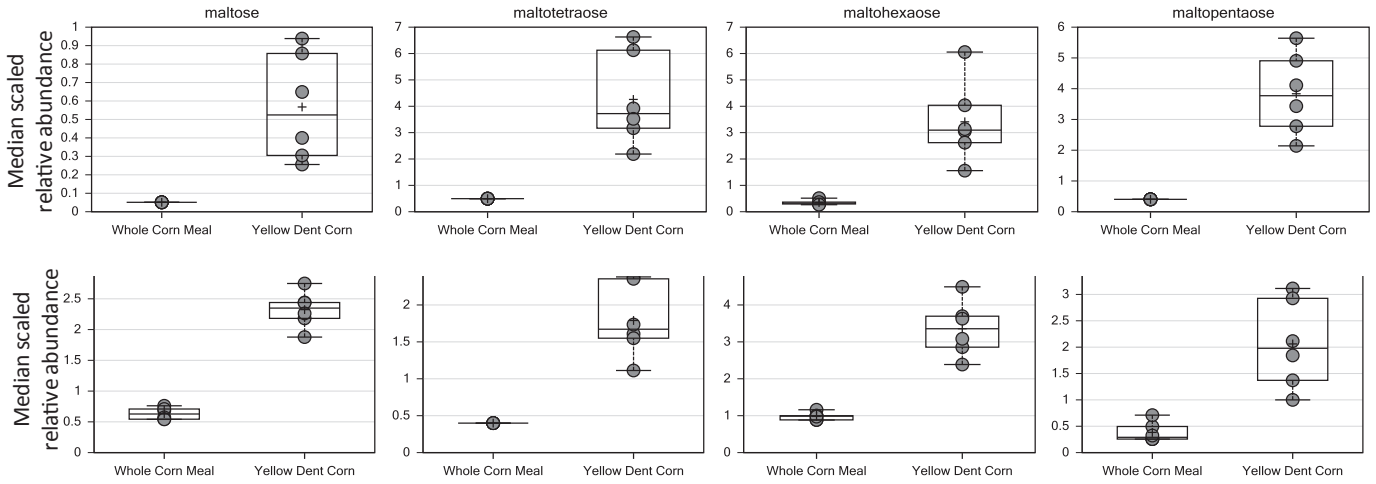

Figure 4: Boxplots showing carbohydrate metabolites ($p \leq 0.05$) elevated in corn grain but not corn meal. The boxed area represent the interquartile range; whiskers indicate maximum and minimum values; the horizontal line indicates median value; "+" indicates mean value; circles show biochemical level for individual samples.

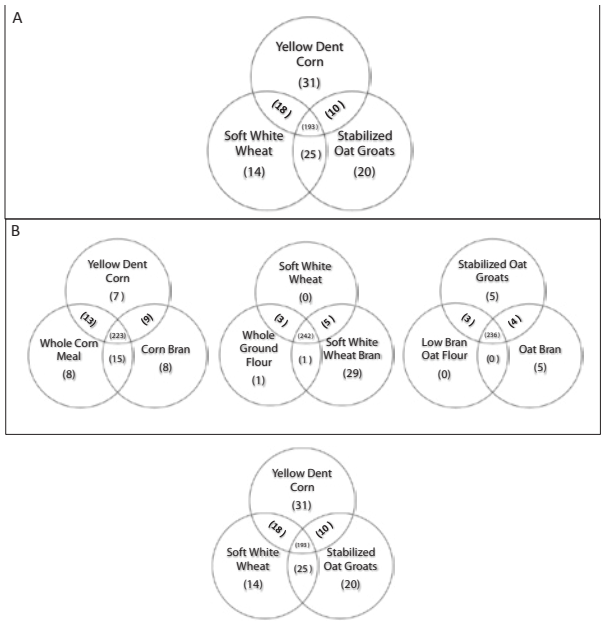

Figure 5: Venn diagram showing number of detected biochemicals in the intact grain (A); Venn diagrams showing number of detected metabolites in the intact grain, milled whole grain flour/meal and bran fractions for yellow dent corn, stabilized oat groats and soft white wheat (B).

compared to whole grain flour/meal (Figure 6). A graphical example of this distribution is reflected by selected amino acids (Figure 7).

Discussion

The nutritional benefits of whole grain are well recognized. Techniques to study grain composition are labor intensive. Specifically, optimized chromatographic methods for each class of biochemical are used to identify and quantitate metabolites including amino acids, flavonoids, lipids and vitamins [15].

Metabolomics technology offers the capability to identify and measure hundreds of separate biochemical entities simultaneously. For this reason, the adoption of metabolomics in the study of nutrient content in grains has been recently gaining popularity [8,16-18]. The influence of organic farming on the nutrient profile of wheat [19], and the effect of drought and genetic engineering on maize [20] have been explored by metabolomics. Metabolomic approaches have also been recognized as an emerging tool in determining the nutrient equivalence of the genetically engineered crop compared to its non-transgenic counterpart [21-23]. Novel insights into plant physiology have been advanced by metabolomics [24-26]. For example, mechanistic insight on how grasses tolerate dehydration was reported by Oliver et al. [26]. High throughput applications are also possible, as recently demonstrated in a human genome wide association study [27] that metabolomics technology can process thousands of samples. This study describes the detection and "relative" quantitation of over 300 metabolites within two days of analytical time on platforms consisting of GC and UHPLC in tandem with MS/MS.

In the current pilot study, our comparisons of the biochemical nutrients in yellow dent corn, soft white wheat and stabilized oat groats and their related fractions reveal that the grains contain diverse compositional makeup, and the nutrient distribution after fractionation is altered. The milling process was preceded by heating and boiling, which softens the grains and inactivates residual metabolic activity in corn. The heating and cooking did not appear to increase the release or degradation of nutrients as others have reported with more aggressive methods including lime and enzymatic treatments [28,29].

Our findings show good agreement, among several compounds including flavonoids, choline, riboflavin, phytic acid and campesterol, with the relative levels reported in literature. The flavonoids detected in the grain samples showed wide distribution, up to 10-fold difference among the different grain types (Table 2) using our extraction method which is limited to the soluble form. The vast majority of phenolics and flavonoids however actually exist in non-soluble forms [6,7]. The relative distribution between grains is surprisingly consistent with other determinations of total flavonoid levels showing higher levels in corn than in wheat or oat [7], suggesting an association between the soluble and insoluble content in grains.

After whole eggs, meats and fish, whole grains are an important

Super Pathway	Corn	Oat	Wheat
Amino acid	41	40	39
Carbohydrate	39	33	36
Cofactors, Prosthetic Groups, Electron Carriers	13	11	12
Lipids	36	37	38
Nucleotide	18	16	14
Peptide	4	2	4
Secondary metabolism	10	7	4
Unnamed	125	107	116
Total	286	253	263

Table 1: Classification of grain metabolites- Number of biochemicals. Secondary metabolism includes flavanoids, phytosterols, phenylpropanoids and terpenoids. Unnamed metabolites represent biochemicals whose identities have not yet been confirmed with a reference standard.

Metabolite	Fold Difference SOG/ YDC	p value	Fold Difference SWW/ YDC	p value	Fold Difference SWW/ SOG	p value
Amino Acid related						
threonine	**3.07**	< 0.001	**2.11**	< 0.001	0.69	0.0644
lysine	**0.37**	< 0.001	**0.33**	< 0.001	0.87	0.5742
phenylalanine	**1.79**	0.0012	**1.94**	< 0.001	1.08	0.4092
tryptophan	**5.85**	< 0.001	**19.50**	< 0.001	**3.33**	< 0.001
isoleucine	**1.54**	0.0092	**2.43**	< 0.001	**1.58**	0.0043
leucine	1.26	0.0669	**2.05**	< 0.001	**1.62**	0.0016
valine	**2.69**	< 0.001	**2.82**	< 0.001	1.05	0.5950
ergothioneine	**4.07**	< 0.001	1.00	-	**0.25**	< 0.001
Flavanols and Phenolics						
4-hydroxycinnamate	**0.12**	0.0014	**0.18**	0.0035	**1.55**	0.0141
sinapate	**0.60**	0.0095	**0.08**	< 0.001	**0.14**	< 0.001
quinate	2.06	0.1232	**4.62**	0.0240	**2.24**	< 0.001
caffeate	**4.90**	0.0051	**2.25**	0.0319	0.46	0.0811
ferulate	**0.20**	< 0.001	**0.47**	< 0.001	**2.32**	< 0.001
quercetin-3-galactoside	0.54	0.0981	0.54	0.0981	1.00	-
vanillate	**0.16**	< 0.001	**0.39**	0.0108	2.38	0.0879
vanillin	**3.64**	< 0.001	1.00	-	**0.27**	< 0.001
Choline related						
betaine	**13.79**	< 0.001	**40.99**	< 0.001	**2.97**	< 0.001
choline	**0.36**	< 0.001	**0.71**	0.0031	**1.98**	< 0.001
choline phosphate	1.00	-	**4.32**	< 0.001	**4.32**	< 0.001
glycerophosphorylcholine	**3.28**	0.0012	0.65	0.2054	**0.20**	< 0.001
Phytic Acid related						
inositol 1-phosphate (I1P)	**0.31**	0.0458	**3.88**	< 0.001	**12.35**	0.0057
myo-inositol-1,4,5-triphosphate	**0.07**	< 0.001	**7.43**	< 0.001	**110.65**	< 0.001
inositol 2-phosphate (I2P)	1.00		**2.45**	< 0.001	**2.45**	< 0.001
myo-inositol hexakisphosphate	**0.00**	< 0.001	**0.00**	< 0.001	0.05	0.1762
Phytosterols						
beta-sitosterol	**0.59**	< 0.001	**2.58**	< 0.001	**4.38**	< 0.001
campesterol	**0.09**	< 0.001	**0.09**	< 0.001	1.00	-
fucosterol	**9.52**	0.0181	0.18	0.1757	**0.02**	0.0043
Vitamins and Cofactors						
pantothenate	1.07	0.3669	**2.37**	< 0.001	**2.22**	< 0.001
riboflavin (Vitamin B2)	0.39	0.0781	0.62	0.2963	1.61	0.3632
thiamin (Vitamin B1)	**0.49**	< 0.001	**0.36**	< 0.001	**0.73**	0.0038
gamma-tocopherol	0.81	0.1095	0.81	0.1095	1.00	-
alpha-tocotrienol	**4.55**	< 0.001	1.00	-	**0.22**	< 0.001
pyridoxate	1.03	0.6208	**0.10**	< 0.001	**0.10**	< 0.001

Table 2: Comparison of amino acid, antioxidant, choline, phytic acid, phytosterol and vitamin/cofactors between stabilized oat groats (SOG), yellow dent corn (YDC) and soft white wheat (SWW).

dietary source of choline [30]. Choline and betaine were higher in wheat (Table 2), consistent with a previous report [31]. The USDA database of choline and choline containing metabolites in various foods is also consistent with our results [30]. Free choline, betaine, and phosphocholine were highest in wheat compared to whole-grain corn meal and whole grain oat flour. Our results are consistent with the USDA report except that we saw 30% more phosphocholine in corn compared to wheat. A diet rich in choline is important because under certain circumstances, such as pregnancy or breastfeeding, dietary choline may be limited [11]. Wheat is a good source of betaine, an intermediate in choline metabolism, and both choline and betaine are important nutrients that are recognized to provide health benefits [32].

Several compounds reflecting anti-nutritive effects were also apparent. Phytic acid is found in many foods, but cereal bran is a rich source [33]. Its levels can be dependent on the extrusion process [33] and can be reduced by phytase activity or cooking [34]. We found high phytic acid levels in wheat compared to corn and oat, consistent with previous reports [35]. It plays an important role in human health by functioning as an antioxidant [33] and by chelating various metals, which suppresses damaging iron-catalyzed redox reactions. Because

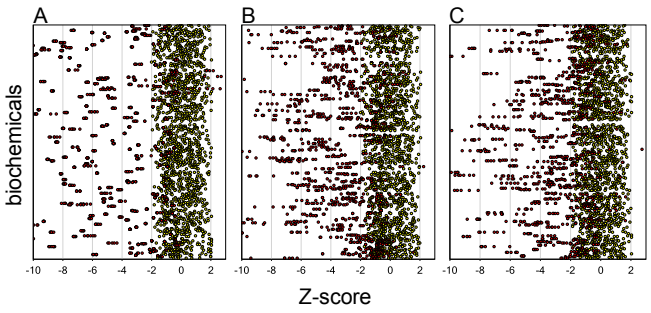

Figure 6: Depletion of compounds in bran fraction relative to whole grain. Z-score plots of whole grain flour/meal (yellow) and bran (red) for yellow dent corn (A), stabilized oat groats (B) and soft white wheat (C). Each row along the y-axis represents a different biochemical. Z-scores were normalized against whole grain flour/meal.

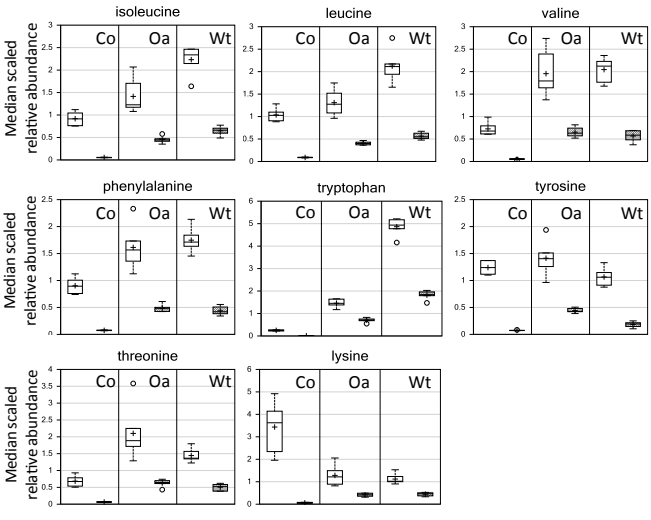

Figure 7: Boxplots of selected amino acids. Co, corn; Oa, oat; Wt, wheat. Open box, whole grain; shaded box, bran fraction. The boxed area represent the interquartile range; whiskers indicate maximum and minimum values; the horizontal line indicates median value; "+" indicates mean value.

of its chelating properties, phytic acid over-consumption may have unintended anti-nutritive effects by binding essential minerals. Wheat bran was also relatively enriched in riboflavin and phytosterols (Table 2). Sequestration of riboflavin [36] by wheat bran can potentially reduce riboflavin bioavailability.

Wheat bran is a rich source of phytosterols [37] and consumption of plant sterols has been demonstrated to reduce cholesterol [38]. Several essential amino acids including tryptophan, isoleucine, leucine were in greater abundance in soft white wheat than stabilized oat groats and yellow dent corn. Corn was high in free lysine, and threonine was high in oats. While corn is considered low in lysine, this refers to the composition of the main storage proteins, and not the free amino acid fraction. Free amino acid levels can be dependent on the extent of protein degradation, cultivar strain, geography and stage of plant development. Ergothioneine is derived from histidine and also has anti-oxidant properties [39]. Our detection of ergothionine only in oats is consistent with previous literature showing that oat grains are a rich source of this compound [40].

In conclusion, this study demonstrates that grain components can be usefully evaluated using a non-targeted metabolomics approach. The analysis also reflects how milling and fractionation can alter the biochemical content of refined grains. Furthermore, because different grains have different and complementary biochemical compositions, the analysis supports the notion that ingestion of multiple grains is nutritionally beneficial.

Acknowledgements

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