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Analysis of Betaine and Choline Contents of Aleurone, Bran, and Flour Fractions of Wheat (*Triticum aestivum* L.) Using ^{1}H Nuclear Magnetic Resonance (NMR) Spectroscopy

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In conventional milling, the aleurone layer is combined with the bran fraction. Studies indicate that the bran fraction of wheat contains the majority of the phytonutrients betaine and choline, with relatively minor concentrations in the refined flour. This present study suggests that the wheat aleurone layer (*Triticum aestivum* L. cv. Tiger) contains the greatest concentration of both betaine and choline (1553.44 and 209.80 mg/100 g of sample, respectively). The bran fraction contained 866.94 and 101.95 mg/100 g of sample of betaine and choline, respectively, while the flour fraction contained 23.30 mg/100 g of sample (betaine) and 28.0 mg/100 g of sample (choline). The betaine content for the bran was lower, and the choline content was higher compared to previous studies, although it is known that there is large variation in betaine and choline contents between wheat cultivars. The ratio of betaine/choline in the aleurone fraction was approximately 7:1; in the bran, the ratio was approximately 8:1; and in the flour fraction, the ratio was approximately 1:1. The study further emphasizes the superior phytonutrient composition of the aleurone layer.

KEYWORDS: Aleurone layer; betaine; choline; wheat

INTRODUCTION

Wheat is a valuable source of betaine, choline (*1*, *2*), B vitamins, vitamin E, and a number of minerals, including iron, zinc, magnesium, and phosphorus (*3*). Epidemiological studies indicate that whole-grain consumption is protective against several chronic diseases (*4*−*12*). It has not been fully elucidated how whole-grain cereals or specific fractions (*13*) exert their protective effect, but it is thought to be due to their content of several nutrients associated with the reduced risk of disease.

Conventionally, whole grain is separated during milling into bran, germ, and flour (*14*). The nutrient composition of these fractions differ markedly; refined wheat flour contains approximately 50% less vitamins and minerals than whole-grain flour (*15*). Considerable amounts of bran are produced each year as a byproduct of refining flour, with the majority used as animal feed (*16*). Bran is the outermost portion of grain and, in wheat grain, constitutes approximately 15% (values may vary between cultivars) of its dry weight. The bran layers are composed of the pericarp, testa, and hyaline layer, while the inner layer is composed of aleurone cells constituting approximately 6.5% of the bran (*14*, *17*). In conventional milling, the aleurone layer is removed with the wheat bran. The aleurone layer is an excellent source of vitamins, minerals, and phenolic compounds (*18*), potentially useful for applications in the food industry. The flour makes up 82% of wheat grain and is composed mainly of starch (64% of dry matter of the flour), nonstarch polysaccharides, small amounts of protein, and lipids (*17*, *19*).

The concentration of betaine and choline varies depending upon the variety of wheat (*20*). A robust method for the quantification of betaine and choline in fractions and their products would be useful for breeding and marketing of wheat based on its potential to aid human health, in line with increasing consumer demands for healthier functional foods. In wheat bran, analyzed using liquid chromatography−electronspray ionization−isotope dilution mass spectrometry, 1505.60 mg/100 g of food of betaine and 74.39 mg of choline moiety/100 g of food for the total choline concentration were reported (*1*). In wheat bread, values of 226.5 mg/100 g of food and 26.53 mg of choline moiety/100 g of food were reported for betaine and choline, respectively (*1*). Measuring the total levels of betaine and choline

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Table 1. Total Betaine and Choline Concentrations of the Different Fractions ± Standard Deviation, $n = 12$ (mg/100 g Sample)

	aleurone	bran	flour
choline	209 ± 18	102 ± 10	28 ± 10
betaine	1553 ± 93	867 ± 74	23 ± 8

can be difficult and laborious. The total choline is particularly difficult to measure because of the various forms present in wheat. These include choline, glycerophosphocholine, phosphocholine, and phosphatidylcholine (*2*). ^{1}H nuclear magnetic resonance (NMR) represents the four choline compounds as one peak (δ 3.24) because it is measuring all of the hydrogen atoms of three methyl groups at the amino end of the molecule present on all forms of choline. The aim of this study was to develop a method that would quantify all of the free betaine and choline in the aleurone, bran, and flour fractions using NMR spectroscopy. This is economically relevant because substantial amounts of wheat bran are produced annually (of which 6.5% is made up of the aleurone layer), which is predominantly used in animal feed (*15*).

MATERIALS AND METHODS

Milled fractions of aleurone, bran, and flour were derived from the hard winter wheat (*Triticum aestivum* L. cv. Tiger) and were a gift from Bühler AG, Uzwil, Switzerland. Six 50 and 500 mg replicates of aleurone, bran, and flour fractions were mixed with 3 mL of 40:60 (v/v) methanol/water. The samples were mixed for 10 min using a minimix standard shaker (Merris Enginering, Maidenhead, Berkshire, U.K.) at room temperature, sonicated for 15 min, and centrifuged (3500*g*) for 20 min, and the supernatant was decanted. The pellets were resuspended in 2 mL of 40:60 (v/v) methanol/water, and the procedure was repeated with six extractions undertaken in total. The supernatants were dried for 8 h in a vacuum concentrator at room temperature and lyophilized for 24 h in a freeze dryer. The samples were reconstituted in 650 μL of 0.1 M phosphate buffer (pH 7.0), in D_2O, containing 1 mM of the internal standard sodium trimethylsilyl-2,2,3,3-tetradeuteroproprionate (TSP, Sigma Aldrich, U.K.). Insoluble material was removed by centrifugation (16000*g* for 15 min), and 600 μL of the remaining supernatant was transferred to a 5 mm diameter NMR tube.

The spectra were recorded in D_2O on a Bruker AC 300 MHz spectrometer. The sample temperature was 303 K, and the samples were spun at 20 Hz. One-dimensional spectra were acquired across a spectral width of 7.5 kHz into 64 K data points: relaxation delay = 1 s, $t_1 = 6$ ms. A total of 32 transients were acquired. Spectral processing was carried out using ACDlabs NMR Processor, version 7.0 (ACD Laboratories, Toronto, Canada). The summed transients were multiplied by a 0.5 Hz apodization factor prior to Fourier transformation; chemical shifts (δ) are reported in parts per million (ppm) of the operating frequency and were referenced to the TSP resonance ($\delta = 0.0$). Baseline correction was performed manually. Data reduction was carried out by manually binning the peaks for betaine and choline and measuring the integral for each bin. The data sets for the 50 and 500 mg spectra were modeled together, and the data were normalized to the TSP peak.

RESULTS AND DISCUSSION

We found that the aleurone layer contains the greatest concentrations of both betaine and choline (1553 and 209 mg/100 g of sample, respectively), with approximately a 2-fold greater concentration for both betaine and choline relative to the bran fraction (**Table 1**). Previous work on wheat fractions looked at the bran and flour fractions but did not analyze the aleurone fraction separately (*1, 2*). In agreement, previous studies have reported that the bran contained more betaine and choline than any other fraction (*1, 2*). While the total concentrations of betaine and choline in each of the fractions were markedly different (highest in the aleurone layer), in this study, the ratio of betaine/choline in the aleurone and bran fractions were alike. Similar to previous studies, the flour had relatively low concentrations of both betaine and choline, which were in similar proportions to each other (*2*). A NMR spectrum of the aleurone layer extract (**Figure 1**) with prominent resonances at δ 3.27 [s, 9, $N(CH_3)_3^+$] (*22*) and δ 3.21 [s, 9, $N(CH_3)_3^+$] (*21*) corresponds to betaine and choline, respectively. These peaks can be used to calculate the concentration of betaine and choline as the internal standard (TSP) at δ 0.00 [s, 9, $N(CH_3)_3$], which

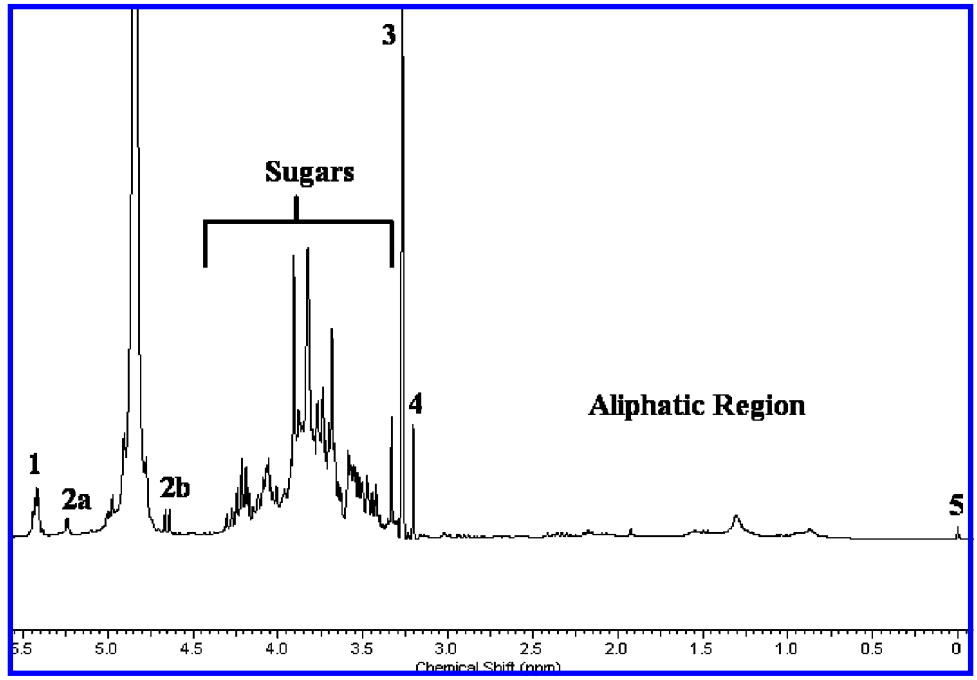

Figure 1. ^{1}H NMR spectra of the first extraction on the aleurone fraction. Peak assignments: 1, G1 proton of sucrose, δ 5.41−5.44 (m, 1, CH); 2a, α-glucose, δ 5.25 (d, 1, $J = 6$ Hz, CH); 2b, β-glucose, δ 4.66 (d, 1, $J = 9$ Hz; CH); 3, betaine, δ 3.27 [s, 9, $N(CH_3)_3^+$]; 4, choline, δ 3.21 [s, 9, $N(CH_3)_3^+$]; and 5, the internal standard (TSP), δ 0.00 [s, 9, $N(CH_3)_3$].

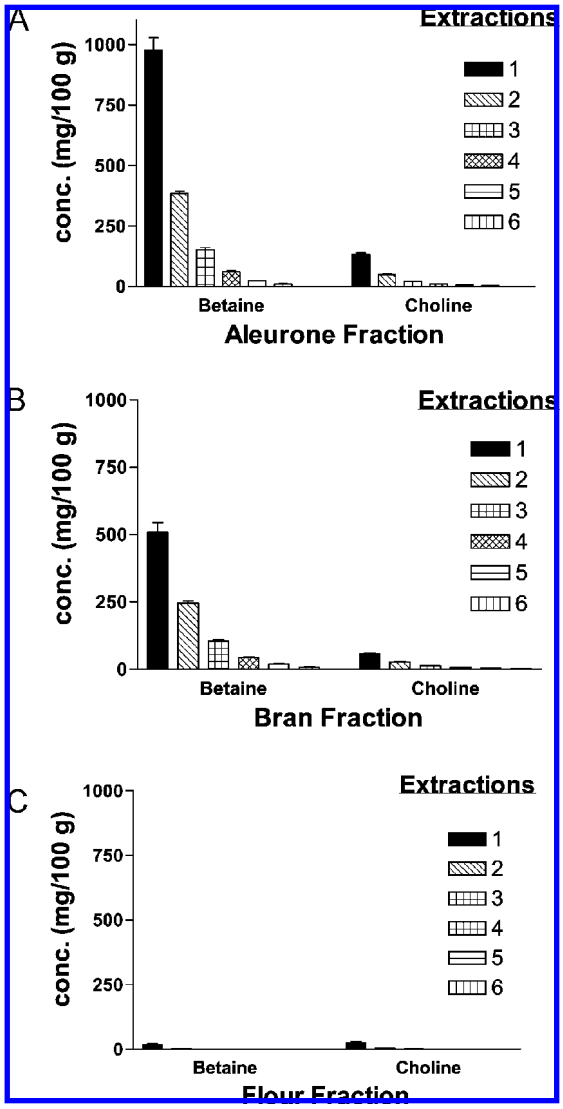

Figure 2. Betaine and choline content of (A) the aleurone fraction, (B) the wheat bran fraction, and (C) the flour fraction after each respective extraction ± standard deviation, $n = 12$.

is directly proportional to the peaks of betaine and choline because its signal corresponds to nine protons.

The crowded carbohydrate region of the spectrum consists of overlapping peaks corresponding to fructose, glucose, and sucrose. In all of the fractions, an unassigned peak was recognized that overlapped with the anomeric proton of sucrose at δ 5.41−5.44 (m, 1, CH) (*22*). However, unlike the other fractions, β-maltose was identified in the flour extraction, with a doublet occurring at δ 4.66 (d,1, $J = 9$ Hz, CH), characteristic of the sugar (*23*). This was verified through spiking of the sample. Although some overlap between the doublet of β-glucose at δ 4.65 (d, 1, $J = 9$ Hz, CH) (*24*) and maltose was absent. In smaller concentrations, the amino acids alanine, aspartate, glutamine, glutamate, isoleucine, leucine, and valine were identified in the aliphatic region of the spectrum. The results of each extraction for the separate fractions are presented in parts **A**−**C** of **Figure 2**. The first wash for each fraction removes between 58 and 62% betaine and between 55 and 85% choline, demonstrating the importance of undertaking more than one wash for quantification purposes. In all of the fractions, <3% of the final concentrations recorded for betaine and choline are recovered in wash number 6.

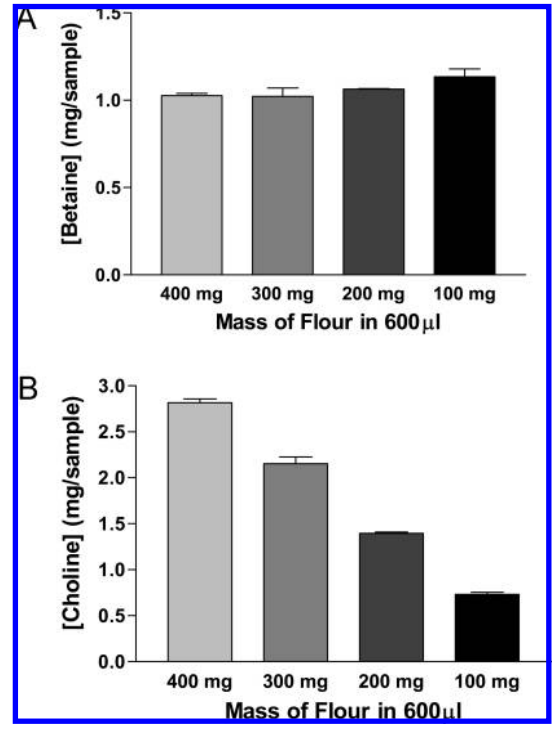

Figure 3. (A) Betaine and (B) choline content of four different masses of flour ± standard deviation, $n = 3$.

Two separate extraction masses were used in this investigation to determine whether mass had any bearing on the final concentration recorded. We found that the results remained consistent across both masses for both betaine and choline. However, the values of betaine varied between the different masses of flour used in the extractions, whereas choline concentrations for the same extractions remained consistent for this fraction. Because this was the only fraction where this was evident, a secondary experiment was undertaken to investigate the varying levels of betaine in the flour samples. The experiment was run using four different masses of flour and the same protocol carried out previously. The data (**Figure 3**) show that the levels of betaine remain almost consistent across all of the masses, whereas the concentration of choline decreases as the mass of flour used decreases. From the results, we determined that the concentrations of betaine are affected by the signal-to-noise ratio in the spectra because it is at such a low concentration. Betaine in the spectrum (relating to the flour fraction) is surrounded by peaks on either side (**Figure 1**); these peaks at such low concentrations make the accurate measurement of betaine difficult because they subsequently decrease the signal-to-noise ratio and effectively minimize the sensitivity and the signal that can be detected. This however is not the case for choline because it is not engulfed by surrounding peaks, and therefore, we are able to maximize the signal-to-noise ratio and effectively quantify the metabolite at lower concentrations.

In the current study, the value of betaine in the bran was 867 mg/100 g of sample (minus the aleurone), which differs from that of the published values of 1505 and 1293 mg/100 g of sample for betaine in wheat bran (Kansas Hard Red Winter Wheat) (*1*, *2*). However, it is known that there are substantial differences in betaine concentrations between genotypes (*19*). This study was carried out on one cultivar, Tiger; further research should be undertaken over a wider range of wheat genotypes, to determine which cultivars contain the greatest concentrations of betaine and choline. In the studies by Zeisel et al. (*1*) and Likes et al., (*2*) values of 74 and 88 mg/100 g of

bran are reported for choline, which are lower than values reported here (102 mg/100 g of bran). The aleurone layer is a valuable source of vitamins and minerals in wheat (*17*), which can be additionally considered to have higher concentrations of the phytonutrients betaine and choline.

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