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Quantitative Measurement of Betaine and Free Choline in Plasma, Cereals and Cereal Products by Isotope Dilution LC-MS/MS

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Betaine and choline are important components of the one-carbon metabolism cycle, linked with the amino acid homocysteine and lipid metabolism. Analyses of broad ranges of foods point to cereal based foods being important sources of betaine and choline, however to date there has been no detailed analysis of these compounds in cereal flours or cereal products. An analytical method based on optimization of an existing extraction followed by LC-MS/MS analysis was used to analyze 47 plasma samples, 32 cereal flours and cereal fractions, and 51 cereal products. For the method validation LLOQ, recovery, inter- and intraday repeatability were all performed. Whole-grain wheat and rye flours, and products based on these were the best whole cereal sources of betaine (747−1508 μg/g) and to a lesser extent choline (76−159 μg/g), while the bran fraction contained the highest concentrations of betaine and free-choline (2350−2899 μg/g and 366−384 μg/g respectively). Refined wheat flour and products contained lower concentrations, while rice and maize contained only very low and no detectable amounts of betaine respectively (0−10 μg/g), and low amounts of free-choline (<31 μg/g). These results were mirrored in cereal products analyzed, with whole-grain wheat or rye-based cereal products having the highest concentrations of the two metabolites. Plasma concentrations for betaine and free-choline in a group of 47 subjects ranged from 15.2−66.3 and 9.8−18.5 μmol/L respectively, within the range of previous reports. This LC-MS/MS method can be used to rapidly and sensitively quantify betaine and free-choline in plasma and cereal products. Whole-grain cereal products and products containing cereal bran appear to be excellent dietary sources of betaine and free-choline.

KEYWORDS: Betaine; choline; LC-MS; cereals; isotope dilution; HILIC

INTRODUCTION

Methylation via the one-carbon/methionine metabolic cycle is an important area of metabolism with known implications in the development of cardiovascular disease, folate and vitamin B_{12} status, hepatic lipid metabolism, renal function (*1*), and DNA methylation (*2*). Two of the critical compounds in the process of remethylation of homocysteine are betaine (glycine-betaine; trimethylglycine) and free-choline. Free choline is irreversibly converted to betaine, which in turn donates a methyl group for the betaine homocysteine methyltransferase catalyzed reaction converting homocysteine to methionine (*2*). Betaine is known to be involved in other biochemical reactions (see refs *2* and *3* for reviews). Several studies have linked intake of betaine to increased plasma concentrations of betaine and lower concentrations of homocysteine (*4*, *5*). In addition, considerable work has been undertaken which is suggestive that betaine may be useful in treating fatty liver disease (*6*, *7*). As oral free-choline is rapidly converted to betaine *in vivo*, while choline esters such as phosphatidylcholine can remain circulating intact for over 24 h post dose (*8*), both dietary betaine and free-choline are potential determinants of plasma betaine concentrations. Lipophilic choline esters are less likely to have a rapid impact on plasma betaine concentrations (*8*). At present, there is still relatively little data on the amount of betaine and free-choline in food.

Zeisel et al. (*9*) reported on the analysis of a wide variety of U.S. foods for betaine and free-choline (including esterified forms of choline), while De Zwart et al. (*10*) and Slow et al. (*11*) reported on the analysis of different forms of betaine in New Zealand foods. Both analytical food surveys point to cereal foods being important sources of betaine (and, to a lesser extent, free-choline). With the exception of the analysis of wheat milling streams for free-choline and betaine (*12*) and total choline and betaine (*13*), no detailed analysis of betaine and free-choline in cereals and cereal foods has been reported.

Betaine and its analogues can be analyzed using HPLC-UV after chemical derivatization by 2-naphthacyl trifluoromethanesulphonate or bromophenacyl trifluoromethanesulfonate (*10*, *11*, *14*), betaine, and diverse forms of choline by isotope dilution LC-MS (*15*), while betaine and free-choline in plasma have been analyzed using LC-MS/MS (*16*). An LC-MS/MS based method provides advantages in terms of selectivity and sensitivity of

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analysis, but as yet, no such method has been reported for the analysis of betaine and free-choline in food samples.

Here we present an isotope dilution LC-MS/MS method for the rapid quantification of betaine and free-choline that can be applied to plasma samples and cereal foods. Additionally, different methods for extracting betaine and free-choline from cereal foods have been compared. Such a method will help in establishing if there is any link between betaine and free-choline intake in food and concentrations in plasma.

MATERIALS AND METHODS

Chemicals and Samples. Cereal flour and food samples were commercially available samples from the European Union, Switzerland, and the United States, or from Nestec SA. Fasting lithium heparin plasma samples from 47 healthy volunteers were from a food frequency questionnaire validation study described previously (*17*). Betaine and choline were purchased from Fluka (Steinheim, Germany), and D_{11}-betaine and D_9-choline were purchased from Eurisotop (Saint-Aubin Cedex, France). High purity HPLC grade water, methanol, ammonium acetate, and acetic acid were purchased from Merck (Darmstadt, Germany). High purity HPLC grade acetonitrile was purchased from J. T. Baker (Deventer, The Netherlands). The plasma sample used for the method validation (QC plasma) was purchased from the CHUV (University Hospital, Lausanne, Switzerland), based on freshly pooled samples from healthy volunteers. The plasma was delivered in a sealed bag on dry ice, thawed on delivery to create smaller aliquots that were stored at −80 °C.

Method Optimization. Method development was based on three previously published methods for analysis of betaine in food by HPLC (*10*), betaine and choline derivatives in food by LC-MS (*15*), and betaine and free choline in plasma by LC-MS/MS (*16*).

The extraction method based on the protocol of de Zwart et al. involved wetting 1 g of sample with 1 mL of water and 100 μL of 10 ng/μL D_{11}-betaine and D_9-choline in methanol (internal standard solution). This slurry was vortexed for 5 min and centrifuged at 2000*g* for 5 min. The aqueous supernatant was extracted with 1 mL of dichloromethane, and following a second centrifugation, the aqueous layer was removed for analysis by LC-MS/MS (see below).

The extraction method based on that of Koc et al. used 100 mg of sample, to which 100 μL of internal standard solution was added. This was followed by the addition of 400 μL of a methanol/chloroform (2:1 v/v) solution; the sample was then vortexed for 20 s and kept at −20 °C for 16 h. Following centrifugation, the supernatant was removed into a new glass tube. To the sample residue was added 250 μL of methanol/chloroform/water (2:1:0.8, v/v/v), and the sample was vortexed again for 20 s. Following centrifugation (1500*g*, 5 min), the two supernatants were combined and 100 μL each of chloroform and water was added. The upper aqueous layer was removed into a 2 mL microcentrifuge tube and dried. To the dried extract, 20 μL of water and then 800 μL of methanol were added, and the extract was vortexed for 20 s. This was centrifuged at 3000*g* for 1 min and analyzed by LC-MS/MS as described below. Note: the complexity of this extraction procedure is in part due to its development for analysis of a wide range of choline derivatives that were not analyzed in this study.

The analysis of betaine and free choline in plasma by Holm et al. was achieved by simple deproteinization using acetonitrile spiked with internal standards followed by centrifugation and injection of the supernatant. Early trials with the Holm et al. method found that deproteinization with methanol led to a better peak shape and greater column lifespan than with acetonitrile. For the application of the Holm et al. method to cereal samples, 100 mg of sample was weighed and 100 μL of internal standard solution added. Following the addition of 1.5 mL of a 50% methanol/water solution, samples were vortexed for 2 min and centrifuged (10 min, 4 °C, 5350*g*) prior to LC-MS/MS analysis.

All three extraction methods were tested on five samples, which would cover a likely range expected in food, as well as different processing methods: white wheat flour, whole-grain wheat flour, whole-grain wheat bread, whole-grain wheat pasta, and rye bran. Initially for the modified Holm et al. method, samples were extracted with 800 μL of a 50% methanol/water solution. Due to the high concentration of betaine and free-choline in most samples, this was adjusted to 1.5 mL of a 50% methanol/water solution, without any observed differences in results.

As betaine and choline may not be fully released from the sample matrix (*18*), samples of refined wheat flour, wheat bran, and rye bran were extracted sequentially six times, and extracts were pooled and compared to samples extracted only once.

Inter- and intraday repeatability were determined on samples of white wheat flour and brown wheat flour. Note: brown wheat flour is wheat flour that has an extraction rate of 85−90% (10−15% of the kernel removed by milling).

Final Extraction Protocols. Plasma samples were thawed on ice and centrifuged (5 min, 4 °C, 5350*g*). Aliquots (30 μL) were taken and placed into 2 mL microcentrifuge tubes. To the aliquots of plasma (30 μL) was added 90 μL of internal standard solution (10 μM of D_{11}-betaine and D_9-choline in methanol) to each microcentrifuge tube, followed by vortexing for 5 min. The tubes then underwent centrifugation (10 min, 4 °C, 5350*g*), and the supernatant (60 μL) was placed in an LC-MS vial with a space saver fitting for LC-MS/MS analysis.

All food samples were milled to a uniform powder using a coffee grinder. Samples with a high water content (bread, fresh pasta) were dried in a freeze-dryer for >48 h prior to milling. Approximately 100 mg of a flour or milled food sample was weighed into 2 mL microcentrifuge tubes. Then 100 μL of methanol containing the labeled internal standards D_{11}-betaine and D_9-choline (both at 10 ng/μL) was added to the sample followed by 1500 μL of a 50% methanol/water solution. Samples were then vortexed for 2 min in a multivortex mixer and then centrifuged (10 min, 4 °C, 5350*g*). The supernatant was then transferred to LC-MS vials for analysis.

Concentrations in cereal flours and foods are reported on a dry matter (DM) basis. Dry matter was determined by weighing approximately 50 mg of sample, drying for 105 °C for 16 h, and then reweighing the cooled sample. All samples were analyzed in triplicate and reanalyzed if the coefficient of variation for the three replicates was above 10%.

LC-MS/MS Analysis. The analysis was performed using a Transcend TLX1 high pressure LC system (ThermoFisher Scientific) coupled to a TSQ Quantum Ultra AM triple quadrupole MS/MS system (ThermoFisher Scientific). The TLX1 system was equipped with a UHPLC HILIC column (2.1 mm × 150 mm, 1.7 μm) (Waters), kept at 30 °C. The mobile phase consisted of 10 mM ammonium acetate in water containing 0.005% acetic acid (A) and 100% acetonitrile (B). The flow rate was 600 μL/min, and the injection volume was 3 μL. Analytes were eluted using the following linear gradient conditions: 20% (A) from 0 to 0.12 min, ramped to 100% (A) at 2.45 min, maintained at 100% (A) to 11.28 min, stepped back to the initial condition 20% (A) at 11.29 min until 14.95 min. Data was acquired using positive electrospray ionization in the SRM mode by monitoring two transition reactions per analyte [betaine: 118 → 58 (collision energy (CE): 25 eV), 118 → 59 (CE: 29 eV); choline: 104 → 60 (CE: 17 eV), 104 → 58 (CE: 33 eV)] and their corresponding internal standards [D_{11}-betaine: 129 → 66 (CE: 31 eV), 129 → 68 (CE: 20 eV); D_9-choline: 113 → 69 (CE: 19 eV), 113 → 66 (CE: 32 eV)], with a total cycle time of 200 ms (scan width: 0.6 *m*/*z*). The MS data acquisition window was set from 1.0 to 3.65 min, where the flow was diverted to the MS probe and to waste between 0 and 1.0 min and 3.65 and 14.95 min. The spray voltage was adjusted to 4000 V (and to 300 V while the flow was diverted to waste). The vaporizer and capillary temperatures were both set to 325 °C. Nitrogen was used for the sheath and auxiliary gases set at arbitrary values of 60 and 45, respectively. The tube lens voltage was optimized for each analyte (betaine, 58 V; choline, 39 V) (Supporting Information Table 1). Aria (version 4) and Xcalibur (version 1.2) software were used for the data acquisition. Xcalibur was also used for all peak integration, while an in-house macro was used for quantification. Quantification results were validated using the qualifier ratio approach, following EU guidelines (*19*).

Statistical Analyses. Differences between different types of cereal flours were determined using ANOVA with the Tukey-Kramer Multiple Comparison test. Relationships between plasma betaine and free-choline concentrations, with age, height, weight, and BMI, were tested using linear regression, while differences between genders were tested using a *t* test. Results were considered significant at $P < 0.05$. All statistical analyses were performed using NCSS for Windows 2007 (Kaysville, UT).

Table 1. Results from the Comparison of Different Extraction Methods, All Using the Same LC-MS/MS Method for Quantification[a]

		Koc (*15*)		De Zwart (*10*)		modified Holm (*16*)	
		mean μg/g DM	% CV	mean μg/g DM	% CV	mean μg/g DM	% CV
refined wheat flour	betaine	141.2	5	176.8	9	179.0	9
	choline	38.9	1	48.0	2	47.4	5
whole grain wheat flour	betaine	604.0	7	767.8	4	717.0	13
	choline	37.3	7	60.5	3	54.2	9
whole grain wheat bread	betaine	579.1	9	781.4	9	834.2	2
	choline	151.6	5	200.2	4	204.6	3
whole grain wheat pasta	betaine	374.7	4	420.1	6	457.2	9
	choline	60.8	7	65.7	5	68.8	8
rye bran	betaine	1651.5	9	2134.7	9	2122.4	5
	choline	142.6	8	217.3	13	176.2	3

[a] The modified Holm method was based on direct extraction with 1500 μL of 50% methanol. All samples were analyzed in triplicate for each extraction method.

RESULTS AND DISCUSSION

Method Optimization and Validation. Due to the endogenous levels of betaine and free-choline present in our samples, the recovery results were determined in plasma, white wheat flour, and brown wheat flour by pre- and postextraction spiking with D_{11}-betaine and D_9-choline at two different spiking levels (50 and 100 μL of a 10 ng/μL solution of both internal standards into a constant final volume). The normal preparation procedure was initially followed, but with all the collected supernatants dried under N_2 and resuspended in either 50 or 100 μL of methanol (prespiked) or 50 or 100 μL of internal standards (10 ng/μL) in methanol (postspike). Moreover, extraction efficiency was also determined using a repeated extraction approach due to the findings of Graham et al. (*18*), who found that, for wheat bran and aleurone layer samples, extraction efficiency was below 100%. Sequential extraction experiments were also performed and results compared to single extractions. For all the tested samples (white wheat flour, rye bran, and wheat bran), pooled extracts had the same betaine and free-choline concentrations as single extractions. Graham et al. (*18*), using NMR, found that sequential extraction extracted more analyte from wheat bran and aleurone. In this work, the use of isotopically labeled internal standards corrected for any apparent loss of analyte during the extraction, so single extractions were used for the final method to enable higher throughput. For the food samples, interday repeatability was determined using two samples (white and brown wheat flour) that were analyzed along with all other samples, over a period of two months ($n = 3$). Intraday repeatability was determined by extracting and analyzing the same white wheat flour sample on the same day ($n = 3$). For plasma, inter- and intraday repeatability was determined using the QC plasma, interday ($n = 6$) and intraday ($n = 4$) (Supporting Information, Table 2).

Extraction Methods. Of the three different extraction methods tested for food samples (Koc et al. (*15*), De Zwart et al. (*10*), and Holm et al. (*16*)), similar results were obtained for the modified Holm et al. and the De Zwart et al. methods, while results were 20−30% lower for the method of Koc et al. (**Table 1**). For the methods of De Zwart et al. and Holm et al., the repeatability based on the coefficient of variation for independent triplicate analysis was < 10% for every extract with the exception of two (whole-grain wheat flour betaine, Holm et al. method (13.3%); rye bran choline, de Zwart et al. extraction (13.0%)), though this improved to < 10% with later analyses of the same samples. The lower concentrations obtained using the extraction method of Koc et al. (*15*) may be due to a more complex extraction method, which is designed to extract not only betaine and free-choline but also a wide range of esterified forms of choline. The Koc et al. extraction method was repeated, and results were again 20−30% lower than those for the other two methods. Due to the greater ease of extraction and similar results to those for the method of de Zwart et al., the modified Holm et al. method was used for all subsequent analyses after confirming that single extraction gave the same results as multiple extractions (see previous section).

The recoveries of D_{11}-betaine and D_9-free-choline for plasma, refined white flour, and brown flour extracts were calculated ranging from 102 to 119% and from 82 to 105%, respectively. The interday repeatabilities for white and brown wheat flour samples were calculated with a coefficient of variation (CV) of 5 and 11% for betaine and 9 and 14% for free-choline, respectively (inter- and intraday $n = 3$ for each sample). The same parameters calculated for plasma found a CV of 5% for both betaine and choline (interday $n = 6$ and intraday $n = 4$ for both). Intraday repeatability ($n = 3$) for white wheat flour had a CV of 1−8% for betaine and 3−5% for free-choline. For brown wheat flour the same values for betaine and free-choline were 3−9% and 1−5%, respectively (Supporting Information, Table 2). Koc et al. (*15*) found lower inter- and intraday repeatability for their method but did not state what matrix this was tested in. Similar to the results in this work, they found a better repeatability for betaine compared to free-choline.

For plasma, CV calculated from the intraday repeatability ranged from 4 to 9% (for betaine) and from 3 to 9% (choline) ($n = 4$). Lever et al. (*14*) reported that the intraday precision of the HPLC method was slightly lower than reported here (between 2.6 and 3.3% for urine and plasma samples); however, their method of extraction/preparation was relatively long and complex, including the use of drying reagents and forming 4-bromophenacyl ester derivatives.

The LLOQ values (determined via estimation to get a S/N = 10) in white flour extract for betaine and free-choline were 21 ng/g and 57 ng/g, respectively. Similarly, the LLOQ in the QC plasma for betaine and free-choline were calculated at 60 nmol/L and 100 nmol/L, respectively.

Analysis of Plasma Samples. Analysis of plasma betaine and free-choline in 47 subjects (27 females, 20 males) found an average concentration (range) of 40.1 (15.2−66.3) and 13.6 (9.8−18.5) μmol/L, respectively. **Figure 1** depicts the chromatograms obtained from a typical plasma extract. The betaine results are in the same range as those of previous studies (*20*, *21*), though the choline concentrations are slightly higher than those reported previously (*16*). This slight difference is probably due to a previous freeze−thaw cycle for these samples, which has previously been shown to lead to elevated free-choline, especially for heparin plasma (*22*). Males had higher average betaine concentrations compared to females (mean ± SEM; 46.1 ± 2.5 μmol/L vs 35.7 ± 2.8 μmol/L; $P = 0.01$), similar to what has been found previously by Lever et al. (*21*). Possibly related, weight and height

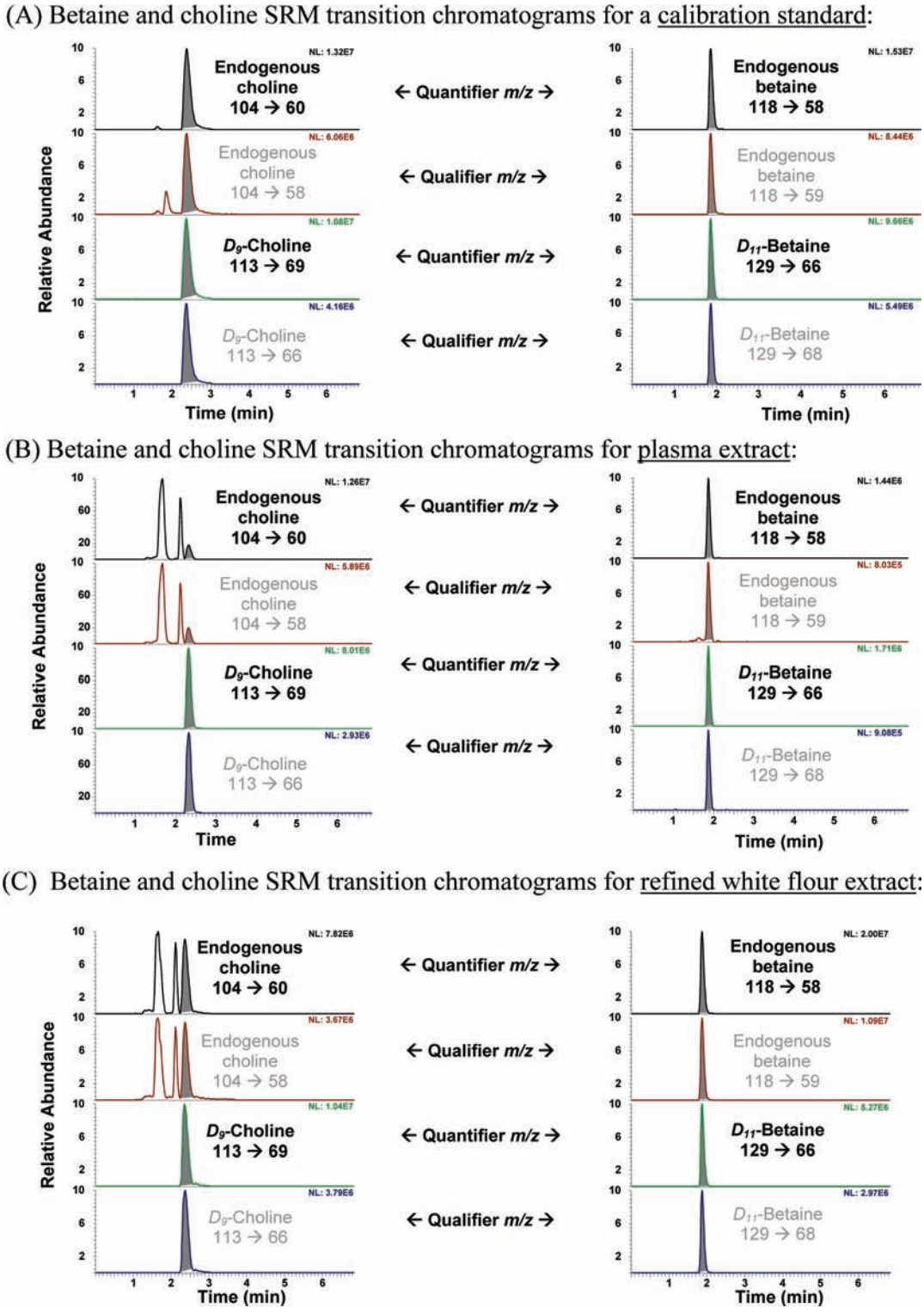

Figure 1. Betaine and choline SRM transitions for (**A**) a calibration standard (250 μM) spiked with a 10 ng/μL internal standard solution (IS), (**B**) plasma spiked with a final concentration of 7.5 and 5.7 μM IS for betaine and choline, respectively, and (**C**) refined white flour extracts spiked with a 10 ng/μL IS solution.

were correlated with plasma betaine concentration (r = 0.31, P = 0.04; r = 0.39, P = 0.01, respectively). There was no gender difference for free choline concentrations, though they were correlated with age (r = 0.33, P = 0.026). There was a weak trend (P = 0.092) for a relationship between plasma betaine and free choline; Holm et al. (*16*) found a correlation of 0.54 for the two metabolites in fasted and nonfasted plasma samples from 120 healthy subjects.

Analysis of Cereal Samples. The average (range) betaine and free-choline concentration within the white wheat flour products (including the half-white wheat flour) analyzed was 229 (165.9−325.8) μg/g and 55 (53.6−64.6) μg/g, respectively (n = 8), while for whole-grain wheat flour (including white wheat cultivars and spelt) it was 1030 (747.0−1502.7) μg/g for betaine and 106 (75.9−134.9) μg/g for choline (n = 6) (**Figure 2**; **Table 2**). While whole-grain wheat had much greater concentrations of

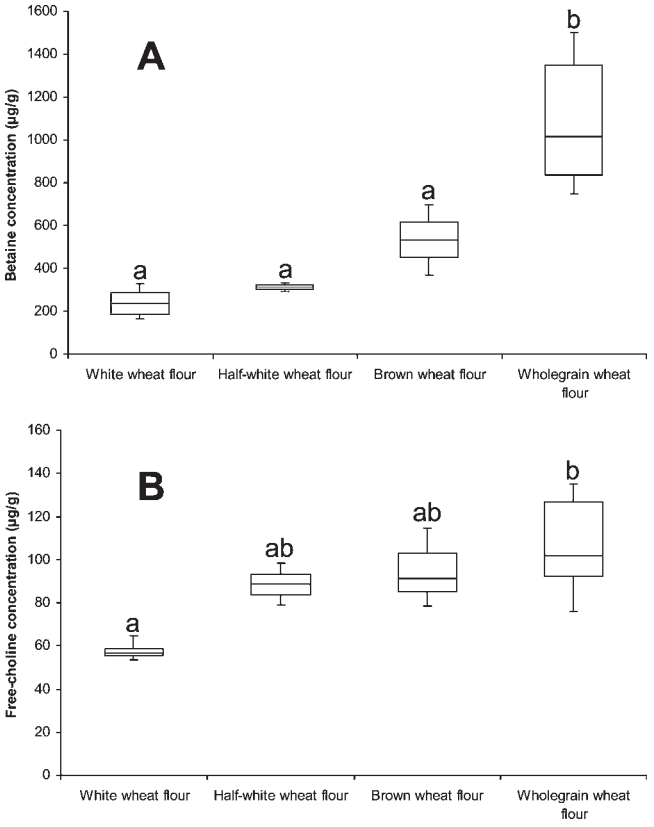

Figure 2. Distribution of betaine and free-choline in commercially available flours with different extraction rates. The box plot represents the range and 25%, 50%, and 75% quartiles. White wheat flour has an extraction rate of <75% ($n = 6$); half-white wheat flour 70−80% ($n = 2$), brown wheat flour 85−90% ($n = 3$), and whole-grain wheat flour 100% ($n = 6$). Samples not sharing a common lowercase letter are different ($P < 0.01$).

betaine than white and half-white wheat flours and to some extent brown wheat flour, the difference for choline was less marked, particularly between half-white, brown, and whole-grain wheat flours (**Figure 2**). Non-whole-grain wheat flour with higher extraction rates than white wheat flour ("half-white" flour 70−80% extraction rate) and brown wheat flour (extraction rate 85−90%) contained higher concentrations of betaine compared to white wheat flours. Rice and corn only contained low or no measurable concentrations of betaine (0−8.9 μg/g), while whole-grain oats and barley contained intermediate amounts (113.7−759.5 μg/g). (**Table 2**). Cereal products made from whole-grain wheat or rye had the highest concentrations of betaine and free-choline, with whole-grain breads and pasta being the best cereal product sources of betaine (**Table 3**). A whole-grain gluten free bread, made predominantly from rice and maize flours contained comparatively little betaine but had a high concentration of free-choline (35.8 and 338.2 μg/g, respectively). While this is only one sample, it and the fact that the major gluten free cereal grains measured in this study (corn and rice, as well as the pseudocereal buckwheat) also contained negligible betaine do suggest that people following a gluten free diet, such as those with celiac disease, may need to compensate for this by eating other foods that are rich in betaine.

The concentrations of betaine found in cereal samples in this study were in the same range as those found in New Zealand foods (*9*, *10*) and in the 2008 USDA database (*23*) but were a factor of around 4 times lower than those reported in U.S. foods and wheat milling streams (*9*, *12*). Free-choline concentrations were similar to those reported in U.S. foods (*9*, *23*) and wheat

Table 2. Betaine and Free-Choline Content in Cereal Flours (All Numbered Entries Refer to Different Items/Products Tested, Not Replicates)[a]

	betaine (μg/g)	free choline (μg/g)
white wheat flour 1 (CH)	194.7	53.6
white wheat flour 2 (CH)	325.8	64.6
white wheat flour 3 (CH)	277.9	55.6
white wheat flour 4 (CH)	182.2	55.7
white wheat flour 5 (CH)	165.9	57.0
white wheat flour 6 (SE)	287.8	58.8
half-white flour 1 (CH)	298.9	98.2
half-white flour 2 (CH)	332.0	79.0
brown wheat flour 1 (CH)	699.7	91.4
brown wheat flour 2 (CH)	533.8	78.5
brown wheat flour 3 (CH)	368.7	114.5
refined wheat/rye flour mix (CH)	516.9	83.3
wheat semolina (CH)	856.6	48.5
whole-grain wheat flour 1 (CH)	747.0	96.4
whole-grain wheat flour 2 (CH)	1157.2	75.9
whole-grain wheat flour 3 (CH)	876.1	133.3
whole-grain white wheat flour 1 (CH)	819.1	107.8
whole-grain white wheat flour 2 (PH)	1502.7	134.9
whole-grain spelt flour (SE)	1411.2	90.6
wheat bran (SE)	2899.4	366.2
white rice (IT)	5.0	23.2
brown rice (IT)	8.9	30.3
maize semolina (CH)	0.0	24.52
whole-grain oats 1 (E.U.)	155.0	25.6
whole-grain oats 2 (U.K.)	113.7	19.7
oat bran (E.U.)	187.5	39.5
whole-grain barley 1 (CH)	398.1	109.7
whole-grain barley 2 (HU)	759.5	68.7
whole-grain rye (SE)	1507.7	159.3
rye bran (SE)	2349.5	384.3
whole-grain buckwheat 1 (E.U.)	9.3	324.2
whole-grain buckwheat 2 (CN)	5.5	423.2

[a] The country of origin is specified in parentheses after the sample: China (CN), European Union (E.U.), Hungary (HU), Italy (IT), Philippines (PH), Sweden (SE), Switzerland (CH), United Kingdom (U.K.). Concentrations reported are means of triplicate analyses with a coefficient of variation < 10%.

milling streams (*12*). There is no obvious reason for the difference between results obtained by the two LC-MS methods for betaine, and our use of the same sample preparation as reported in Koc et al. (*14*) found only slightly (20%) lower concentrations of betaine and free-choline. Graham et al. (*18*) used nuclear magnetic resonance (NMR) to quantify total choline and betaine in wheat bran, aleurone, and endosperm after 40% methanol extraction, and they found considerably higher amounts of total choline (280 and 1020 μg/g for endosperm and bran, respectively), indicating that most choline present in wheat is conjugated. Betaine concentrations determined by NMR were comparable for white flour but much higher for wheat bran (8670 μg/g). Results for betaine in rye and wheat bran from our study were similar to those found by Kamal-Eldin et al. (*24*) using the HPLC method of De Zwart et al., where rye bran was found to range between 1940 and 2780 μg/g and wheat bran between 4310 and 4410 μg/g. An earlier study (*13*) using a titration method for betaine analysis found among nine different cultivars of wheat a range of <220−1442 μg/g, which is similar to those found for whole-grain wheat flours in this study. While it is known that there can be considerable variation in betaine concentration due to agricultural conditions (*13*), this is unlikely to explain the apparently large differences seen between the published studies. Finding the reason for this apparent difference is important, as it may lead to an over- or underestimation of betaine and free-choline intake. Likes et al. (*12*) estimated daily betaine intake in the USA to be from 163 to 699 mg/d depending on if one was to eat only refined

Table 3. Betaine and Free-Choline in Cereal Products (All Numbered Entries Refer to Different Items/Products Tested, Not Replicates)[a]

	betaine (μg/g)	free choline (μg/g)
Breakfast Cereals		
breakfast cereal 1 (0% WG; CH)[b]	10.1	25.8
breakfast cereal 2 (0% WG; CH)[b]	13.6	25.1
breakfast cereal 3 (0% WG; CH)[c]	75.3	30.9
breakfast cereal 4 (27% WG; U.K.)[d]	180.6	61.0
breakfast cereal 5 (23% WG; U.K.)[d]	279.7	48.8
breakfast cereal 6 (45% WG; CH)[d]	482.2	42.4
breakfast cereal 7 (57% WG; U.K.)[f]	674.5	63.4
breakfast cereal 8 (64% WG; CH)[f]	789.6	59.7
breakfast cereal 9 (67% WG; CH)[f]	807.3	63.4
breakfast cereal 10 (74% WG; U.K.)[g]	235.9	56.7
breakfast cereal 11 (82% WG; U.K.)[f]	810.8	98.0
breakfast cereal 12 (93% WG; U.K.)[f]	1041.2	80.4
breakfast cereal 13 (100% WG; U.K.)[f]	885.5	84.1
Bread		
white wheat bread 1 (CH)	433.0	138.5
white wheat bread 2 (CH)	407.4	116.6
white wheat bread 3 (CH)	461.6	113.9
white wheat bread 4 (CH)	314.4	86.3
white wheat bread 5 (CH)	421.7	124.4
white wheat bread 6 (CH)	423.8	131.8
brown wheat bread 1 (CH)	596.5	183.5
brown wheat bread 2 (CH)	619.3	132.0
whole-grain wheat bread 1 (CH)	706.2	124.4
whole-grain wheat bread 2 (CH)	921.4	206.9
whole-grain wheat bread 3 (CH)	842.5	215.0
whole-grain wheat bread 4 (CH)	517.4	126.2
whole-grain wheat bread 5 (CH)	672.7	132.0
whole-grain wheat/rye bread 1 (DK)	1258.2	191.3
whole-grain wheat/rye bread 2 (SE)	1347.6	132.4
whole-grain rye bread 1 (SE)	1092.9	152.7
whole-grain rye bread 2 (SE)	1666.5	162.9
gluten-free whole-grain bread (DK)[h]	35.8	338.2
Pasta/Couscous/Noodles		
refined wheat pasta 1 (U.S.)	225.9	92.6
refined wheat pasta 2 (IT)	773.2	73.3
refined wheat pasta 3 (U.S.)	249.9	41.2
refined wheat pasta 4 (U.S.)	222.5	104.8
refined wheat pasta 5 (IT)	611.2	56.2
white rice noodles (E.U.)	3.2	6.9
refined couscous (IS)	384.1	36.9
WG wheat pasta 1 (IT)	1068.2	191.9
WG wheat pasta 2 (U.S.)	419.1	67.3
WG wheat pasta 3 (IT)	1326.6	196.8
WG wheat pasta 4 (E.U.)	798.4	104.0
WG couscous 1 (SE)	1299.0	107.7
WG couscous 2 (IS)	544.1	48.1
brown rice noodles (E.U.)	5.6	22.3
Crackers		
refined grain crackers 1 (IT)[c]	460.3	67.8
refined grain crackers 2 (IS)[c]	432.9	88.6
refined grain crackers 3 (IS)[c]	400.9	98.7
whole-grain crackers 1 (IS)[e]	919.7	247.6
whole-grain crackers 2 (IT)[e]	693.8	169.0
whole-grain crackers 3 (CH)[e]	823.2	88.1

[a] The country of origin is specified in parentheses after the sample: Denmark (DK), European Union (E.U.), Israel (IS), Italy (IT), Philippines (PN), Sweden (SE), Switzerland (CH), United Kingdom (U.K.), United States (U.S.). Concentrations reported are means of triplicate analysis with a coefficient of variation < 10%. [b] Principle ingredient is refined corn. [c] Principle ingredient is white rice. [d] Principle ingredient is refined wheat flour. [e] Principle ingredients are whole-grain wheat flour and refined wheat flour. [f] Principle ingredient is whole-grain wheat. [g] Principle ingredients are whole-grain corn and oats. [h] Principle ingredients are whole-grain rice and corn.

or only whole-grain wheat products. Based on the same premises, but using the concentrations found in our study, intake of betaine from wheat in the USA would be 54−233 mg/day, or a factor of 3 lower than that estimated by Likes et al. (*12*). There is a need for more data on the effect of cultivar and growing conditions on the concentration of betaine and choline in cereal grains, as all of the recent studies have focused either on single cultivars (*12*, *18*) or on food (refs 9−11 and this study).

There was a good correlation between betaine and free-choline in cereal flours and fractions ($r = 0.89$), while for all cereal flours and products analyzed the correlation was 0.78. This was probably lower due to the effect of adding noncereal ingredients. It was notable that the pseudocereal buckwheat had very low betaine concentrations (< 10 μg/g) but among the highest of the free-choline concentrations measured (up to 423 μg/g). While whole-grain cereals do have higher concentrations of free-choline compared to white wheat flour-based products, it would appear that the difference is less marked than that for betaine (**Figure 2**). Some of the white wheat bread samples analyzed did have higher concentrations of free-choline than found in white wheat flour, which is probably due to the addition of fats during the baking process and conversion of conjugated choline forms into free-choline. It would be useful to know the composition of different choline-conjugated compounds in order to confirm possible cleavage into free-choline during processing, though present methods are complex and relatively time-consuming (*15*) and were out of the scope of the objectives of this work.

This LC-MS/MS method was able to be used to analyze betaine and free-choline in both cereals and plasma. With a relatively rapid and simple sample preparation and short run time, this method is faster and more efficient than most existing methods used for food analysis. In a wide-ranging analysis of different cereal-based foods, it is confirmed that wheat- and rye-based whole-grain cereal products are an important food source of betaine in the diet, which may be one of the components contributing to the health benefits of a diet rich in whole-grain cereal foods (*25*). This method, validated for both cereal foods and plasma, should aid in the investigations of links between betaine and free-choline intake and corresponding concentrations in plasma.

ABBREVIATIONS

HILIC, hydrophilic interaction liquid chromatography; HPLC-UV, high performance liquid chromatography with ultraviolet light detection; LC-MS, liquid chromatography−mass spectrometry; LC-MS/MS, liquid chromatography−tandem mass spectrometry; LLOQ, lower limit of quantification; S/N, signal to noise ratio; UHPLC, ultra high pressure liquid chromatography; SRM, single reaction monitoring; CE, collision energy; CV, coefficient of variation.

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Supporting Information Available: MS/MS acquisition parameters (quan = quantifier ion, qual = qualifier ion) and inter- and intraday repeatability of betaine and free choline analyses in white wheat flour (intraday $n = 3$), brown wheat flour (intraday $n = 3$), and QC plasma (intraday $n = 4$). This material is available free of charge via the Internet at http://pubs.acs.org.

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