Quantitative Determination of Total Choline in Grains and Feed by High Performance Liquid Chromatography-Tandem Mass Spectrometry

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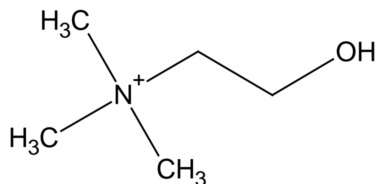
Abstract: Choline has been an established dietary essential since 1932 and is required in relatively large quantities in humans and animals when compared to other vitamins *(1)*. In addition to free choline, other choline-contributing compounds in plant-derived materials include glycerophosphocholine, phosphocholine, phosphatidylcholine and sphingomyelin. The most commonly reported procedure for the determination of total choline in grains and feeds involves soxhlet extraction with methanol, hydrolysis and saponification of the lipid fraction with barium hydroxide, complexation and precipitation with a reinecke salt followed by dissolution of the reinecke salt-choline complex and spectrophotometric analysis. A new procedure for analysis of corn grain, soybean seed, canola seed and a formulated rodent feed utilizing high performance liquid chromatography with tandem mass spectrometry detection (HPLC-MS/MS) was developed and validated. The HPLC column selected for the assay contained a pentalflurophenylpropyl (PFP) stationary phase which provided symmetric peak shape and narrow chromatographic band widths without derivatization or complexation of the cationic choline. MS/MS detection provided excellent sensitivity and selectivity. The new procedure replaces the labor-intensive steps required for reaction and precipitation of the reinecke salt complex and spectrophotometric measurement. The HPLC-MS/MS assay precision was 7% (relative standard deviation) and the overall mean spiking recovery was 100% across four sample matrices.

Chemicals and Reagents: The chemical structure of choline:



Choline chloride analytical standard was obtained from Sigma-Aldrich, St. Louis, MO. A stock solution was prepared at a concentration of 4,000 µg/mL choline (corrected for chloride content) in deionized (DI) water. A working standard solution was prepared in DI water by dilution of the stock standard to achieve a concentration of 1 µg/mL choline. Serial dilutions of the 1 µg/mL working standard solution in 50:50 (v/v) acetonitrile:DI water were prepared to produce instrument calibration standards ranging in concentration from 1 to 50 ng/mL. All choline standard solutions were stored in a refrigerator (approximately 4ºC). DI water was obtained from a Barnstead NANOPure water system. Additional reagents used included:

Acetic acid, glacial, ACS, Fisher Scientific, St. Louis, MO
Acetonitrile, HPLC grade, Fisher Scientific, St. Louis, MO

Barium hydroxide octahydrate, ACS, Fisher Scientific, St. Louis, MO
Ethyl alcohol, anhydrous, denatured, EMD, Billerica, MA
Formic acid, 88%, ACS, Fisher Scientific, St. Louis, MO
Methanol, gas chromatography grade, Omnisolv®, EMD, Billerica, MA
Thymolphthalein, ACS, VWR, Radnor, PA

A saturated barium hydroxide solution was prepared by weighing 18 g of barium hydroxide octahydrate into a 1 L bottle and adding 500 mL of warm DI water. Thymolphthalein indicator solution was prepared by weighing 0.5 g of thymophthalein into a 125 mL bottle and dissolving in 50 mL of ethyl alcohol. An acetonitrile:water solution (50:50 v/v) was prepared by mixing equal volumes of acetonitrile and DI water. Mobile phase solutions containing 0.1% formic acid in either DI water or acetonitrle were prepared by adding 1 mL of 88% formic acid to 1 L of solvent.

Equipment:

Analytical balance, capable of weighing to the nearest 0.01 mg (stock standard preparation)
Top-loading balance, capable of weighing to the nearest 1 mg (sample weighing)
Boiling chips, Teflon
Class A volumetric glassware
Flasks, 250 mL, flat bottom, round
Graduated glass tubes, 15 mL, conical
HPLC autosampler vials, 1.5 mL with screw caps, Waters Corporation, Milford, MA
HPLC column, Pentafluorophenylpropyl (PFP), 100 x 2.1 x 3 µm, Ultra PFP, Restek, Bellefonte, PA
HPLC, 2695 Separations Module, Waters Corporation, Milford, MA
Mass spectrometer detector, Quattro micro™ API, Waters Corporation, Milford, MA
Pipetters, adjustable volume
Soxhlet extraction apparatus
Soxhlet extraction thimbles, cellulose
Syringe filters, 0.45 µm, Teflon
Syringes, 20 cc, plastic

Procedures: The extraction and saponification procedures described herein are similar to those appearing in AACC method 86-45 *(2)*. Grain samples were ground with dry ice using a Retsch centrifugal grinder equipped with a 0.75 mm screen. The rodent diet was already in a ground, homogeneous state, so no further processing was required. Samples were stored frozen at -20ºC. Sub-samples (1.0 g) of feed, grain and seed were weighed into cellulose soxhlet extraction thimbles. The thimbles were placed in a soxhlet extraction tube which was connected to a water cooled condenser. Methanol, 150 mL, was transferred to a 250 mL flat bottom, round flask along with a few Teflon boiling chips. The flask was connected to the soxhlet extraction tube and placed on a hot plate. The hot plate setting was adjusted to bring the methanol to a steady boil. Once reflux of the solvent was observed, the extraction was allowed to proceed for 24 hours. After cooling, methanol extracts were quantitatively transferred to 200 mL volumetric flasks and brought to volume with methanol. Approximately 10 mL aliquots of each extract were transferred to glass vials. A 0.25 mL aliquot of each extract was transferred to a 15 mL

graduated glass conical tube, and 3 mL of saturated barium hydroxide were added. The tubes were heated at 55ºC for 90 minutes on a heating block. After cooling, 1 mL of thymolphthalein indicator was added producing a deep blue color. Glacial acetic acid was added drop wise to each tube while swirling to mix until the solutions became colorless. Each tube was diluted to the 10 mL mark with DI water. Sample solutions were filtered through 0.45 µm Teflon syringe filters into glass vials. Sample solutions were diluted in 50:50 (v/v) acetonitrile:DI water in HPLC autosampler vials according to the scheme in Table 1 using adjustable pipetters. Diluted sample solutions were analyzed by HPLC-MS/MS. A linear mobile phase gradient program and flow rate of 0.3 mL/minute was used to elute choline from the PFP column (Table 2). The run time was 10 minutes per injection. The expected retention time for choline was 1.9 minutes. The column was held at 40˚C. The injection volume for sample and calibration standard solutions was 1 µL. Multiple reaction monitoring (MRM) was used with positive ion polarity to detect choline (Table 3). The mass spectrometer was tuned periodically to maintain optimal sensitivity and mass accuracy by infusing choline standard solutions and adjusting detector settings accordingly. The daughter ion spectrum for choline displaying the molecular ion $[M]^+$ at a mass to charge ratio (m/z) of 104 and the daughter ion used for quantitation at m/z 60 appears in Figure 1. The daughter ion at m/z 60 represents the trimethyl amine cation $(CH_3)_3NH^+$. Representative HPLC-MS/MS chromatograms appear in Figures 2a-e.

Table 1. Sample Dilution Scheme

Matrix	Sample Volume (mL)	Diluting Solvent (mL)	Dilution Factor
Maize Grain	0.1	0.9	10
Soybean Seed	0.05	0.95	20
Canola Seed	0.01	0.99	100
Rodent Feed	0.05	0.95	20

Table 2. Mobile Phase Gradient Program

Time (min.)	%A	%B
0.00	75	25
0.20	75	25
4.00	100	0
6.00	100	0
6.01	75	25
9.00	75	25
9.01	75	25
10.00	75	25

A: 0.1% Formic Acid in DI Water. B: 0.1% Formic Acid in Acetonitrile

Table 3. MRM Program for the Detection of Choline

Ion (m/z)		Dwell Time (s)	Cone Voltage	Collision Energy
Parent	Daughter			
104	60	1.0	35	16

The HPLC-MS/MS system was calibrated by analysis of external calibration standard solutions ranging from 1 to 50 ng/mL choline. Typically, six calibration standards were injected with each set of sample solutions. A linear regression plot of the peak area (y-axis) and the standard solution concentration in units of ng/mL (x-axis) was constructed. An inverse (1/x) weighting function was used. Sample solution concentrations were calculated using the linear regression equation $y = mx + b$; where y is the sample solution peak area, m is the slope of the linear regression plot, x is the sample solution concentration, and b is the y-intercept. Masslynx version 4.1 was used to collect the chromatographic data, construct calibration curves and calculate sample solution concentrations.

Test Matrices: The method was validated using Purina Certified Rodent Diet 5002 (feed), dent corn (*Zea mays*), soybean seed (*Glycine max*) and canola seed (*Brassica napus*).

Results/Discussion: Seven replicate sub-samples of each matrix were analyzed to determine the precision of the developed method via calculation of the relative standard deviation (RSD). Choline concentrations were calculated on a dry weigh basis (DB) in units of mg choline/100 g of sample. Mean (n=7) choline results ranged from 59 to 610 mg/100 g DB for corn grain and canola seed, respectively (Table 4). RSD values ranged from 4.98 to 11.5% for rodent feed and canola seed, respectively. The mean RSD over all four matrices was 7%.

The choline concentrations reported herein were compared to literature derived concentrations for the grain/seed matrices. Good agreement between the literature values and the validation data were observed (Table 4).

Table 4. Summary of Unfortified Sample Analysis Results (Precision Assessment)

Matrix	Mean (n=7) (mg/100 g DB)	Std. Dev. (n=7)	RSD (%)	Literature Mean (mg/100 g DB)
Corn Grain	59	3.48	5.89	57 *(3)*
Soybean Seed	245	12.4	5.06	222 *(4)*
Canola Seed	610	70.0	11.5	619 *(4)*
Rodent Feed	194	9.65	4.98	174 *(5)*

For accuracy validation, three replicate sub-samples of each matrix were fortified with choline at a level approximately equal to the expected native choline content. These laboratory fortified sub-samples were used to assess method accuracy through calculation of percent recovery. Mean (n=3) recovery values ranged from 87 to 112% for soybean seed and corn grain, respectively (Table 5). The mean (n=12) recovery over all four matrices was 100%.

Table 5. Summary of Choline Recoveries

Matrix	Fortification Level (mg/100 g DB)	Mean (n=3) Recovery (%)
Corn Grain	57	112
Soybean Seed	226	87
Canola Seed	753	108
Rodent Feed	226	103

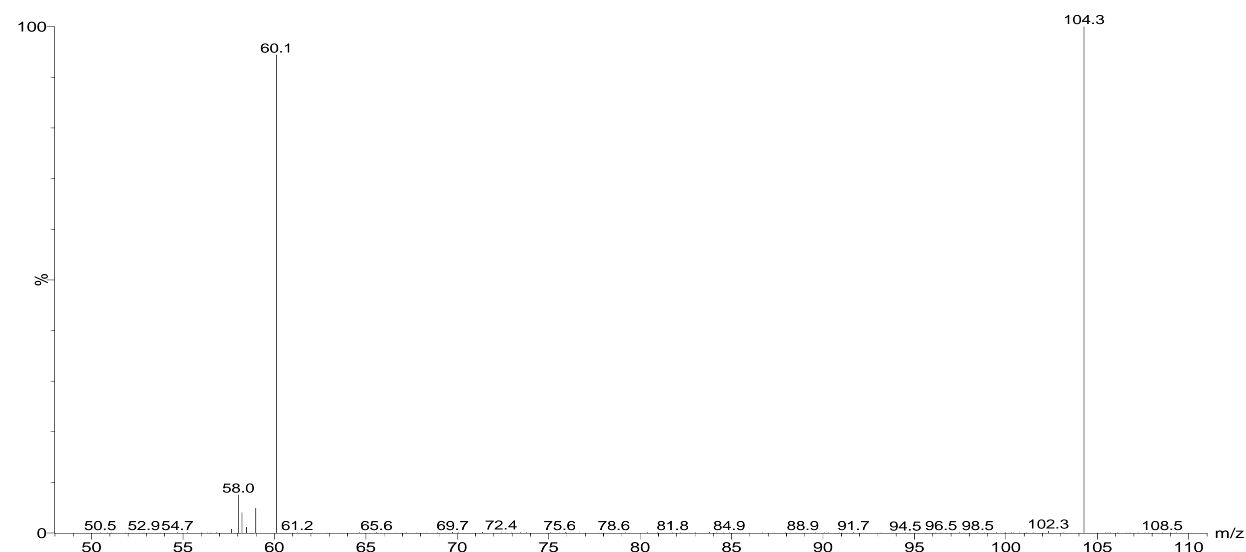
The linear working range for choline was established by the analysis of external standard solutions. An optimum linear range of 1 to 50 ng/mL was established (Figure 3). Linear regression calibration plots of the peak area (y-axis) and the standard concentration in units of ng/mL (x-axis) consistently yielded a coefficient of determination (r^2) value of 0.995 or greater.

During the development phase of the research, several HPLC columns were evaluated. The Restek PFP column was chosen because it provided the best retention and peak symmetry.

The limit of quantitation (LOQ) was determined by signal to noise measurements of calibration standards analyzed over the course of the research. A signal to noise ratio of five was used as a benchmark for the LOQ. The method LOQ was estimated at 1 ng/mL for a 1 µL HPLC injection.

Conclusions: An analytical method for the determination of choline in grains and feed was successfully validated. The method demonstrated acceptable accuracy and precision, and provided good agreement with literature values for four matrices. A method LOQ of 1 ng/mL was estimated based on instrument signal to noise measurements. A 50x linear range was established and found to be reproducible from run to run.

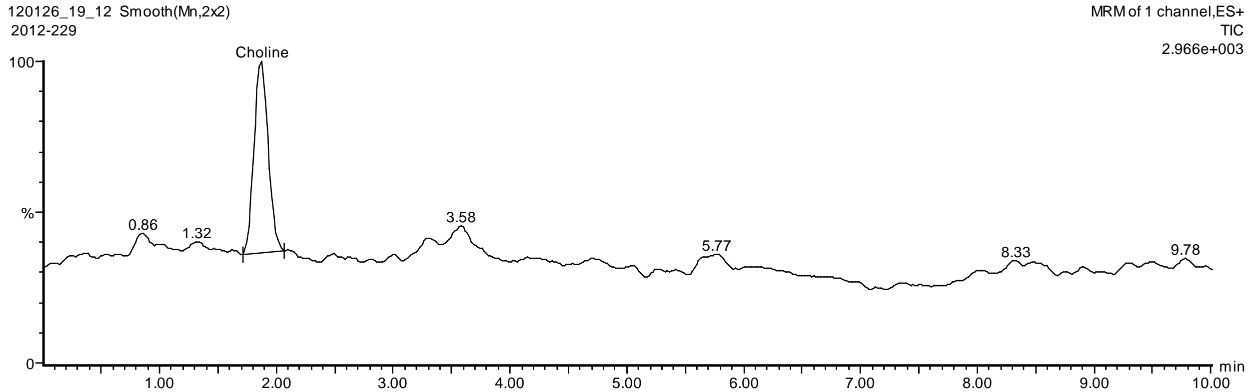
Figure 1. Daughter Ion Spectrum of Choline



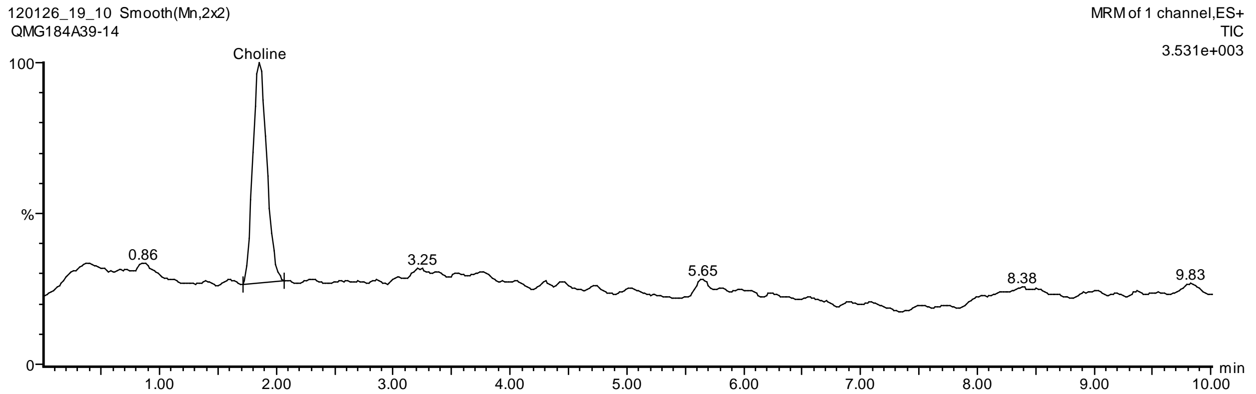
Molecular Ion at m/z 104. Daughter ion at m/z 60.

Figure 2. HPLC-MS/MS Chromatograms

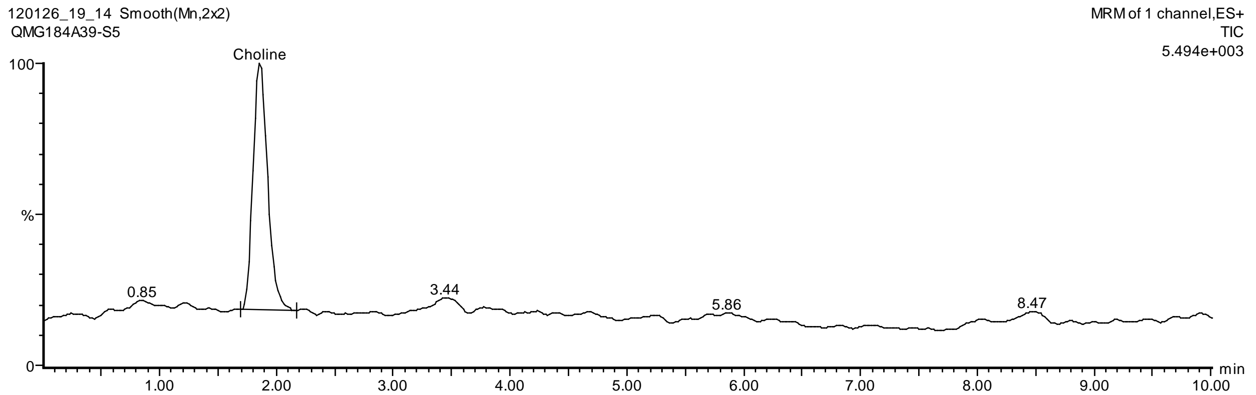
2a. Choline Calibration Standard (5 ng/mL)



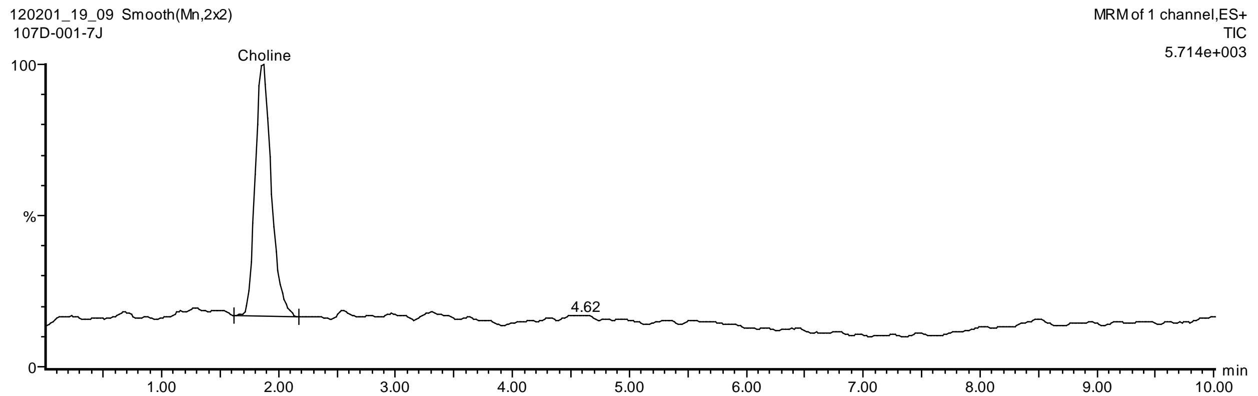
2b. Unfortified Corn Grain Sample (64 mg/100 g DB)



2c. Fortified Corn Grain Sample (109% Recovery)



2d. Unfortified Rodent Feed Sample (208 mg/100 g DB)



2e. Fortified Rodent Feed Sample (104% Recovery)

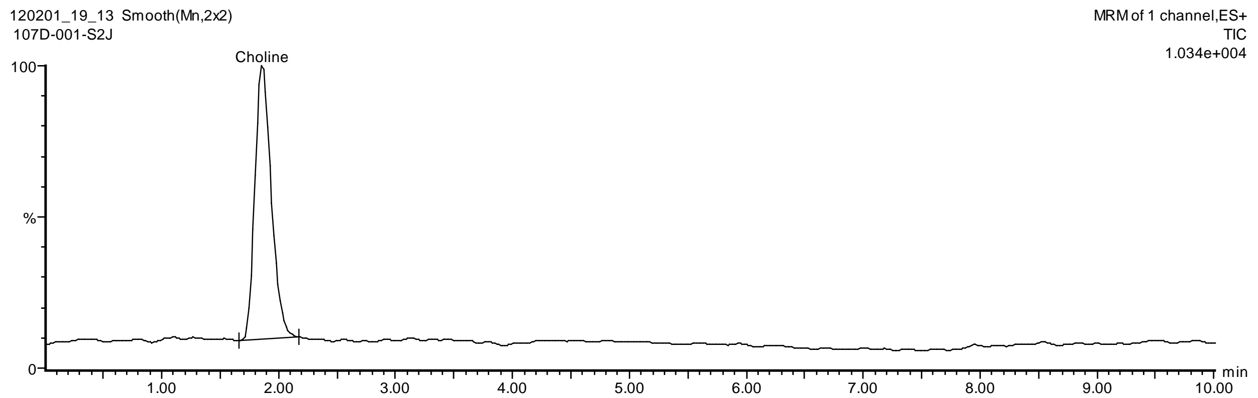
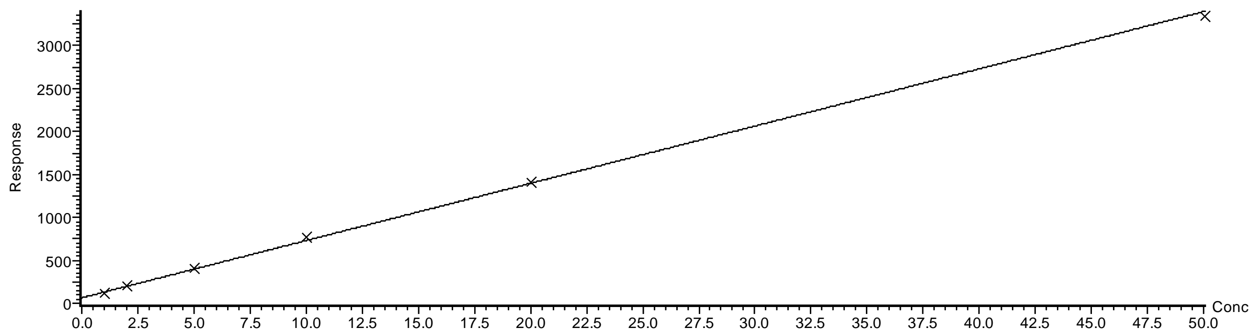


Figure 3. Choline Linear Range

Compound name: Choline
Correlation coefficient: r = 0.999309, r^2 = 0.998618
Calibration curve: 66.6213 * x + 63.9839
Response type: External Std, Area
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None



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(5) (2012) Purina Certified Rodent Diet 5002. http://www.labdiet.com/pdf/5002.pdf