A Modified Enzymatic Assay for Quantifying Choline in Fish Tissue and Common Feed Ingredients

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An enzymatic assay was developed to quantify total choline content in fish tissue and common feed ingredients. The sensitivity of the assay is within a range of 0.5−4 μg choline/mL, a level sensitive enough to analyze total choline for most nutritional studies. A coupled assay was performed linking acetylcholine esterase to choline kinase and pyruvate kinase. The production of ADP by choline kinase is equivalent to the total amount of choline present in the sample. ADP stoichiometrically drives the pyruvate kinase reaction, producing pyruvate. Pyruvate can then be quantified following conversion of a nitro-substituted phenylhydrazine (2,4-dinitrophenylhydrazine) into phenylhydrazone (2,4-dinitrophenylhydrazone), which can be monitored at 450 nm. This discontinuous assay is efficient, sensitive, and simple to perform.

Keywords: *Choline content; catfish tissue; choline kinase; feed ingredients; 2,4-dinitrophenylhydrazine*

INTRODUCTION

Choline is a precursor of several membrane phospholipids (e.g., phosphatidylcholine and sphingomyelin), the methyl donor betaine, and the neurotransmitter acetylcholine. Interest continues in analyzing choline in animal tissue and feed ingredients for various nutritional studies (Emmert and Baker, 1997; Hung, 1989; Kim et al., 1994). A number of chromatographic methods have been developed to quantify choline in animal tissue which can be divided into gas chromatographic (GC) methods (Hartmann and Kilbinger, 1974; Jenden and Hanin, 1974) and liquid chromatographic methods (Eksborg and Persson, 1974; Jones and Stutte, 1985; Pomfret et al., 1989; Ikarashi and Maruyama, 1993). Gas chromatography of choline esters requires an esterification of choline followed by a N-demethylation procedure before they can be volatilized for GC analysis. Demethylation is accomplished by a lengthy chemical demethylation procedure (2 working days) using sodium benzenedithiolate (Jenden and Hanin, 1974). This demethylation procedure has been reported to cause cleavage of the ester moiety from choline leading to potential erroneous results (Sheehan and Davis, 1964). Earlier application of high performance liquid chromatography (HPLC) analysis of choline and acetylcholine focused on ion-pair chromatography. Choline and acetylcholine were extracted as ion pairs with dipicrylamine to dichloromethane. The dipicrylamine was available only in an impure quality and had to be purified. The process requires extensive precautions to avoid contact with hands and skin. Recently improved HPLC methods for analyzing choline and acetylcholine use either a radioactivity detector (Pomfret et al., 1989) or an electrochemical detector (Ikarashi and Maruyama, 1993). An enzymatic method for determining choline is also available (Browning and Brostrom, 1974). This method utilizes the following series of enzymatic reactions coupled to the oxidation of NADH which is monitored spectrophotometrically at 340 nm.

$$\text{acetylcholine} \xrightarrow{\text{acetylcholinesterase}} \text{acetate} + \text{choline}$$

$$\text{choline} + \text{ATP} \xrightarrow[Mg^{2+}]{\text{choline kinase}} \text{phosphocholine} + \text{ADP}$$

$$\text{ADP} + \text{pyruvate} \xrightarrow[K^+,\, Mg^{2+}]{\text{pyruvate kinase}} \text{ATP} + \text{pyruvate}$$

$$\text{pyruvate} + \text{NADH} \xrightarrow{\text{lactate dehydrogenase}} \text{lactate} + \text{NAD}^+$$

This method has been used in nutritional studies (Emmert and Baker, 1997), because compared with the other available methods, it was less labor-intensive and requires no expensive instrumentation. In practice, the results from this method could also be erroneous because endogenous NADH-producing enzymes must first be depleted before choline kinase can be added to the assay solution, otherwise the spurious production of NADH could interfere with the final reading. For example, NADH can be produced through the glycolysis pathway by converting endogenous glucose to a series of pathway intermediates. If the amount of glucose in the sample exceeds the choline content by several 100-fold, the lactate dehydrogenase reaction may be significantly displaced. Adding excess hexokinase to remove endogenous glucose prior to the choline kinase addition has been suggested by the authors. However, possible interference from other intermediates which also produce NADH has not been addressed. Another shortcoming of the assay is that it requires continuous monitoring of the oxidation of NADH and is thus difficult to perform by normal laboratory staff in the nutrition, food, and feed industry.

In this paper, we present a modified assay based on the original principle from Browning and Brostrom (1974) to quantify total choline in fish tissue and

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common feed ingredients. The modified procedure is more efficient and easier to perform compared to the original NADH method.

MATERIALS AND METHODS

Principles and Reactions of the Assay. The assay utilizes 2,4-dinitrohydrazone as the colorimetric indicator for pyruvate which is generated from a series of coupled reactions. The reactions are as follows:

$$\text{acetylcholine} \xrightarrow{\text{acetylcholinesterase}} \text{acetate} + \text{choline}$$

$$\text{choline} + \text{ATP} \xrightarrow[Mg^{2+}]{\text{choline kinase}} \text{phosphocholine} + \text{ADP}$$

$$\text{ADP} + \text{phosphoenolpyruvate} \xrightarrow[Mg^{2+}]{\text{pyruvate kinase}} \text{ATP} + \text{pyruvate}$$

$$\text{pyruvate} + \text{dinitrophenylhydrazine} \xrightarrow{\text{NaOH}} \text{pyruvate-dinitrophenylhydrazone}$$

The conditions for the above enzymatic reactions are compatible, and the equilibrium of each reaction lies to the right at pH 8.5 (Browning and Brostrom, 1974) with the exception of the last colorimetric reaction which requires a pH >10 (Neish, 1957) to ensure a decent sensitivity.

Chemicals and Reagents. All chemicals and reagents were purchased from Sigma Co., St. Louis, MO, unless otherwise indicated. The necessary assay solutions were prepared as follows: (1) Extractant: 0.5 M methanolic KOH: dissolve 14.025 g of KOH in 500 mL of methanol. (2) 50 mM glycylglycine buffer: dissolve 0.66 g of glycylglycine (free base) in approximately 80 mL of double distilled water, adjust pH to 8.5 with KOH, add 1.5 mL of 1 M $MgCl_2$ and 0.4 mL 0.25 M EDTA; readjust pH with KOH before making the volume up to 100 mL and store frozen. (3) Phosphoenolpyruvate (PEP), tricyclohexylammonium salt, 25 mg/mL of double distilled water and store frozen. (4) 0.1 M ATP: dissolve 0.623 g of ATP disodium salt in 10 mL of double distilled water and store frozen. (5) Rabbit muscle pyruvate kinase (EC 2.7.1.40): 1000 units/0.6 mL vial was diluted to 1000 units/1.333 mL with double distilled water and stored frozen. (6) Choline kinase from *Saccharomyces cerevisiae* (EC 2.7.1.32): 5 units/vial was dissolved to 10 units/mL with double distilled water and stored frozen. (7) Acetylcholinesterase from electrical eel (EC 3.1.1.7): 500 units/vial was dissolved in 0.25 mL of double distilled water to generate a solution of 2000 units/mL and stored frozen. (8) Standard choline chloride: 10 mM choline chloride stock solution was diluted to provide 0.1, 0.2, 0.3, and 0.4 mM concentrations for preparation of a standard curve. Stock solution of choline chloride can be made monthly, but the standard solutions should be prepared daily. (9) 2,4-Dinitrophenylhdrazine (DNPH) was purchased from Eastman Kodak Co., New York, and used as a 0.05% solution (0.05 g/100 mL of 2 M HCl).

Instrument. Samples were clarified by using a clinical centrifuge, and absorbance was monitored at 450 nm with a Hitachi 100-80A spectrophotometer.

Fish Samples. Juvenile channel catfish were obtained from our departmental fish nutrition laboratory. Fish tissue was chopped to a slurry, and 1 g of sample was weighed, using an analytical balance, into a 100 mL round-bottom flask.

Feed Ingredient Samples. Cooked corn meal, corn meal, wheat middlings, cottonseed meal, soybean meal, fish meal, and meat and bone/blood meal were obtained from the Delta Research and Extension Center, Stoneville, MS. The ingredients were ground to pass through a #40 mesh sieve, and then 1 g was weighed into a 100 mL round-bottom flask.

Extraction Procedures. The following extraction procedure was adapted from Schneider (1987). Twenty milliliters of 0.5 M methanolic KOH was added to the sample in the round-bottom flask fitted with a reflux condenser. The flask was placed in a water bath for 2 or 3 h reflux with periodical shaking. At the end of extraction, the flask was removed from the water bath, 30 mL of distilled water was added, and the content allowed to cool to room temperature. The content of the flask was then filtered with a Whatman 4 filter paper into a 100 mL volumetric flask along with several rinsings of the solid residue with distilled water. Five milliliters of 1% EDTA was added, and then the filtrate was diluted to 100 mL with distilled water.

Enzymatic Assay. The assay includes three steps: blank checking, basal α-keto acid determination, and the stoichiometric conversion of choline to pyruvate for final color development. Both the standard solutions and the samples were taken through the three steps so that they were comparable.

A. Blank Checking. Assay mixtures were prepared to contain 2 mL of glycylglycine buffer and 0.21 mL of water in a test tube. Fifty microliters of PEP, 10 μL of ATP, 20 μL of pyruvate kinase, and 10 μL of acetylcholinesterase were added. Assay mixtures were allowed to stand 10 min at room temperature. The total volume of the reaction solution was 3 mL. Then 0.1 mL of DNPH was added to the tube and allowed to stand for 10 min. One milliliter of 2 N NaOH was added and mixed. After 10 min, the tube was centrifuged for 5 min. One milliliter of the supernatant was pipetted into a disposable plastic cuvette, and the absorbance read at 450 nm. This reading was a *Blank* for both standard and sample solutions.

B. Basal α-Keto Acid Determination. This step is used to determine the concentration of endogenous α-keto acids in the sample solution. The basal determination step was the same as in the blank checking procedure except that the 0.21 mL of water was replaced with 0.2 mL of sample extract and 0.01 mL of water. This absorbance reading was called *Basal*.

C. Stoichiometric Conversion of Choline to Pyruvate. The choline conversion step was the same as in the basal α-keto acid determination step except that the 0.01 mL of water was replaced with 0.01 mL of choline kinase, which was added following the acetylcholinesterase. This reading was called *Final*.

Calculation. For the standard solutions, no other α-keto acids should be present, and thus the absorbance reading for the *Blank* should be the same as that for the *Basal*. Thus

$$\Delta A_{\text{Standard}} = (A_{Final} - A_{Blank}) - (A_{Basal} - A_{Blank}) = (A_{Final} - A_{Basal}) = (A_{Final} - A_{Blank}) \quad (1)$$

This procedure was used to calculate absorbance for the choline chloride standards. For a sample solution, the difference in absorbance due to the basal α-keto acids and the choline converted to pyruvate was calculated as

$$\Delta A_{\text{Sample}} = (A_{Final} - A_{Blank}) - (A_{Basal} - A_{Blank}) = (A_{Final} - A_{Basal}) \quad (2)$$

The molar concentration of choline = 0.868 × molar concentration of choline chloride.

RESULTS AND DISCUSSION

Standard Curve Equation. A set of standard solutions was prepared daily to contain 0, 0.1, 0.15, and 0.2 mM of choline chloride. A reference standard curve based on a 10 day consecutive analysis is presented in Figure 1. The linear equation is given

$$Y = -0.0042 + 0.5283X \quad (3)$$

where Y is the absorbance at 450 nm, X is the concentration of choline chloride (mM), and −0.0042 and 0.5283 are the intercept and slope, respectively. The coefficient of determination (R^2) of this equation was 0.9964.

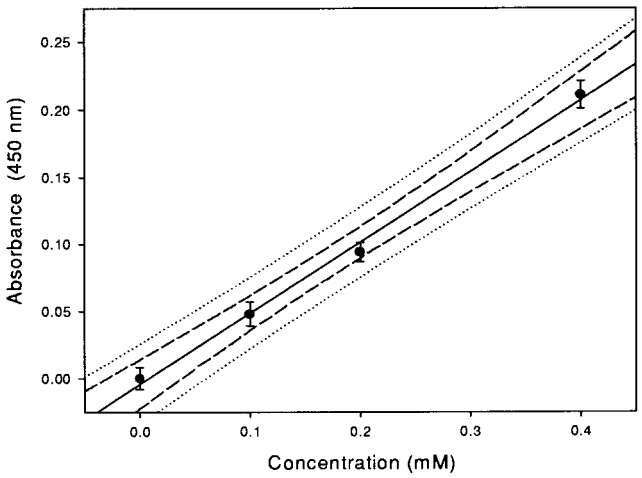

Figure 1. Plot of the standard curve of the choline chloride concentration (mM) versus absorbance (450 nm). The standard deviations, along with 95% confidence intervals (– –) and prediction intervals (· · ·) for the standard curve ($n = 10$) are shown.

Table 1. Choline Content in Channel Catfish Liver and Muscle

tissue	choline content[a] (mg/kg)	SD[b]	RSD[c]
liver	3595	198	4.2
muscle	3768	159	4.5

[a] Means of 10 samples of each tissue on dry matter basis. [b] Standard deviation. [c] Relative standard deviation.

Sensitivity. The enzymatic assay was sensitive within a range of 0.5−4 μg choline/mL of sample extract. A choline concentration greater than this range may give incorrect readings because ATP acts as both a substrate and an inhibitor of pyruvate kinase. ATP concentration in the 3 mL of enzymatic reaction solution should be kept within a range from 0.0025 to 0.005 M. For a sample containing higher choline content, more water can be added after extraction so that the required ATP concentration remains within this range. PEP was added in excess to the assay to remove endogenous pyruvate by reactions taking place prior to adding choline kinase. Pyruvate kinase was also added in sufficient quantity to guarantee depletion of endogenous pyruvate prior to adding choline kinase. Therefore, PEP and pyruvate kinase concentrations should be proportionally increased if a sample contains a high level of glucose or other pyruvate-generating intermediates. The colorimetric part of this assay was originally developed for determination of α-keto acids in animal and plant tissues by nitro-substituted phenylhydrazines (Dakin, 1908; Lu, 1939; Neish, 1957). The absorbance at 450 nm was stable for more than 30 min. Compared with the continuous enzymatic assay of Browning and Brostrom (1974), the presented discontinuous assay is easier to perform by an average laboratory technician in a nutrition, food, or feed industry.

Reproducibility and Recovery Rate. The reproducibility of the standard assay was checked by 10 measures of the standard solutions. The relative standard deviations (RSD) were in the ±2.3−4.1% range. The reproducibility of the assay for the liver and muscle sample was checked by 10 measures, and the RSDs of the fish tissues were in the ±4.2−5.5% range (Table 1). The choline content of the various feed ingredients obtained by our new method was compared with those reported by the National Research Council (NRC) which are considered to be obtained by using classical methods (Table 2). The choline values obtained by our method are within the same range as the NRC values. The reproducibility of the assay for the seven feed ingredients was measured in three replicate samples, and the RSDs for the seven feed ingredients were in the ±1.7−5.3% range (Table 2). To estimate the recovery of the extraction method, 1 mL of 10 mM choline chloride was added to a round-bottom flask before the extraction, and the final absorbance reading was compared with a normal sample. The spiking was repeated three times for each of the seven feed ingredients and two types of tissue. A range of recovery values of 86−94% was observed (Table 2).

Table 2. Choline Content in Seven Common Feed Ingredients

ingredient	choline content[a] (mg/kg)	av recov[b] (%)	RSD[c] (%)	NRC value
cooked corn meal	396	86	5.3	N/A
corn meal	682	89	4.9	504
cottonseed meal	2625	91	5.1	2764
wheat middlings	1221	90	4.6	1247
soybean meal	2647	94	1.7	2421
menhaden fish meal	2614	93	4.2	3112
meat & bone/blood meal	1178	88	4.8	N/A

[a] Means of three samples from each of the feed ingredients on dry matter basis. [b] Average recovery rate. [c] Relative standard deviation.

Comparison of Choline Content in Catfish Liver and Muscle. Table 1 shows that the choline content in channel catfish liver and muscle are similar based on 10 analyses of each tissue. There was no significant difference ($p < 0.05$) between the choline content in channel catfish liver and muscle tissue based on a t-test. No previous studies have reported the choline content of fish tissues.

Choline Content in the Seven Feed Ingredients. Table 2 lists the choline content in seven feed ingredients compared with data published by the National Research Council (1993). Although NRC values for cooked corn and meat and bone/blood meal are not available, there was reasonable agreement between the values obtained by our new method and those reported by the NRC which had been obtained by using classical methods. Variable processing time and procedures in feed mills might cause differences between the results from the above two sources. In our experiment, plant materials, corn meal, cooked corn meal, wheat middlings, and cottonseed meal required 2 h of extraction. Animal materials, fish meal, and meat and bone/blood meal required 3 h of extraction. This might be due to the different forms and structural location of choline in plant and animal tissues.

Conclusions. The method reported herein is simple, suitable, and sensitive enough to quantitatively analyze total choline content in fish tissue and feed ingredients used in nutritional studies. This method can also be easily adapted to routine choline analysis by the food industry.

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