J Sci Food Agric 1998, **78**, 395–398

The Determination of the Choline Content of Feed Ingredients using Choline Kinase

José Fernando M Menten[1] and Gene M Pesti[2]*

[1] Dept Zootecnia, University of São Paulo, 13418-900 Piracicaba SP, Brazil
[2] Department of Poultry Science, University of Georgia, Athens, GA 30602-2772, USA

(Received 17 October 1997; revised version received 9 February 1998; accepted 11 March 1998)

Abstract: This study was conducted to establish a methodology for a more accurate and less laborious determination of the choline content of feed ingredients, compared to the available procedures. The methodology involved an extraction-hydrolysis step, in which the feed sample was heated in a methanolic KOH solution using a Goldfisch apparatus, followed by an enzymatic assay, involving a series of coupled reactions started by choline kinase, which allowed the quantitation of the extracted choline. The enzymatic assay was very accurate over the range of choline concentrations from 1 to 20 mg l^{-1}, with a lower detection limit in feeds of 50 mg kg^{-1}. The hot extraction procedure was more efficient than the conventional thimble cool extraction for maize, soybean meal and canola meal, especially for maize. Extraction for 2 h gave choline values similar to those obtained with 4 or 6 h extraction. The recovery of added choline to maize, soybean meal or canola meal samples averaged 97·4%, indicating very little loss of choline in the procedure. This methodology provided more realistic values of the choline content of feed ingredients than previous methods. © 1998 Society of Chemical Industry.

J Sci Food Agric **78**, 395–398 (1998)

Key words: choline; cholinekinase; feedstuffs

INTRODUCTION

Numerous enzyme-linked assays, gas and liquid chromatographic, radiochemical and fluorimetric procedures have been described for choline analysis (Hanin 1974). Schiefer and Beutler (1985) described an enzymatic method for the assay of phosphatidylcholine in amniotic fluid and in foods such as egg yolk and mayonnaise. This method was based on the quantitation of choline released from the phosphatidylcholine, and the conditions of the coupled reactions utilising choline kinase were described.

The knowledge of the choline concentration in feed ingredients is important because substantial amounts of choline chloride are often supplemented to animal diets to meet their needs. This study was conducted to establish a methodology for an accurate and less laborious determination of the choline content of feed ingredients applicable to most nutrition laboratories. The authors originally tried to use the Goldfisch apparatus without a thimble as a means of reducing costs, but they found that the new procedure resulted in the extraction of considerably higher amounts of choline from maize and canola meal (but not soybean meal).

EXPERIMENTAL

Reagents and solutions

The reagents and solutions needed were as follows. Extractant: 0·5 M KOH solution in methanol : EDTA solution; ethylene diamine tetracetic acid sodium salt, 0·03 M in water. Glycine buffer: glycine, 0·2 M, Mg^{+2}, 0·01 M (from $MgSO_4 . 7H_2O$), pH 8·0 in water; glycine buffer was stable for 3 months at 4°C. ATP/PEP solution: adenosine 5′-triphosphate disodium (bacterial source), 36 mM and phospho(enol)pyruvate tricyclohexylammonium salt, 20 mM in water; solution

* To whom correspondence should be addressed.

 J Sci Food Agric 0022–5142/98/$17.50. Printed in Great Britain

stable for 1 week at 4°C. NADH solution: reduced β nicotinamide-adenine dinucleotide disodium salt, 7 mM in water; solution prepared fresh each day, stable for a few hours at 4°C. PK/LDH suspension: Mix 650 U of pyruvate kinase from rabbit muscle (PK, ATP : pyruvate 2-0-phosphotransferase, EC 2.7.1.40) and 650 U of lactate dehydrogenase from rabbit muscle (LDH, L-lactate : NAD^+ oxidoreductatase, EC 1.1.1.27) in 1 ml of aqueous suspension: suspension stable for 1 year at 4°C. CK suspension: 1 U lyophilised choline kinase from yeast (CK, ATP : choline phosphotransferase EC 1.7.1.32) in 1 ml of glycine buffer; suspension stable for 1 week at 4°C. 6 M HCl solution; distilled–deionised water; choline stock solution; aqueous solution of choline chloride used to prepare different standards by dilutions; diluted standards stable for 1 week at 4°C.

Apparatus

The apparatus needed was as follows: Goldfisch apparatus; filtering equipment including vacuum pump, filtering funnel with tubes and glass microfiber filters (Whatman 934-AH).

Procedure

Treatment of the feed sample with the methanolic KOH solution at elevated temperatures results in the extraction of the choline-containing phospholipids from the feed matrix followed by hydrolysis of choline yielding choline hydroxide. The choline is assayed enzymatically by the coupled reactions described below:

$$\text{Choline} + \text{ATP} \xrightarrow[\text{kinase, } Mg^{2+}]{\text{choline}} \text{phosphorylcholine} + \text{ADP} \quad (1)$$

$$\text{ADP} + \text{PEP} \xrightarrow[\text{kinase, } Mg^{2+}]{\text{pyruvate}} \text{ATP} + \text{pyruvate} \quad (2)$$

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow[\text{dehydrogenase}]{\text{lactate}} \text{L-lactate} + NAD^+ \quad (3)$$

The choline is phosphorylated in the presence of ATP to phosphorylcholine by the enzyme choline kinase. The ADP formed in ration (**1**) is reconverted to ATP by PEP with the formation of pyruvate in the presence of pyruvate kinase. In the presence of lactate, dehydrogenase pyruvate is reduced to L-lactate by NADH with the oxidation of NADH to NAD. The amount of NADH oxidised in reaction (**3**) is stoichiometric with the amount of choline. NADH is determined by means of its absorbance at 340 nm.

Extraction and preparation of the extract

Accurately weigh approximately 2 g of the feed ingredient sample into Goldfisch beakers, in duplicate. Add 25 ml of the extractant and some boiling beads and heat on the Goldfisch apparatus under reflux. Allow to simmer, and avoiding boiling and splashing of the beaker contents. Extract for 2 h. Cool, add 30 ml water and adjust pH to 6·0–6·5 with HCl. Transfer the liquid fraction to a 100-ml volumetric flask, washing the beaker and feed several times with water. Adjust volume, stopper and mix well. Dilute the extract with water using dilution factors (F) 5, 10 or 20, and add 5% of the volume as the EDTA solution. The dilution factor is chosen according to the choline concentration of the sample and the presence of pigments in the extract which have high absorbance at 340 nm. Mix again and filter under vacuum. Discard the initial portion of the filtrate and store an aliquot in capped vials under refrigeration.

Enzymatic assay

Bring the sample solution and the reagents to room temperature. Pipette successively into cuvettes: 1 ml glycine buffer, 50 µl ATP/PEP solution, 50 µl NADH solution and 20 µl PK/LDH suspension. Then add 1 ml of sample (or water for blank or 1 ml choline chloride standard). Cover and mix by gentle inversion. Allow to stand for 10 min at room temperature and read absorbance (A_1) against air at 340 nm. Pipette 50 µl CK suspension into each cuvette, cover and mix by gentle inversion. Allow to stand for 30 min at room temperature and read absorbance (A_2).

Calculation

$$\Delta A = (A_1 - A_2) \text{ sample or standard} - (A_1 - A_2) \text{ blank} \quad (4)$$

The concentration of choline in the feed sample is given by

$$c = \frac{4{\cdot}175 \times F + \Delta A}{w} \text{ (g kg}^{-1}\text{)} \quad (5)$$

where F is the dilution factor, ΔA is the difference in absorbance as shown in (**1**) and w is the feed sample weight (g).

The concentration of choline in the standard solution is given by the following general equation:

$$c = \frac{V \times MW \times \Delta A}{\varepsilon \times d \times v \times 1000} \text{ (g litre}^{-1}\text{)} \quad (6)$$

where V is the final volume (ml), v is the sample solution volume (ml), MW is the molecular weight of choline (121·2); ΔA is the difference in absorbance as shown in (**4**); ε is the absorbance coefficient for NADH at 340 nm (6·3 litres mmol^{-1} cm^{-1}) and d is the light path (cm).

RESULTS AND DISCUSSION

Accuracy, precision, sensitivity

The enzymatic determination of choline proved to be very accurate and the determined values were very close to the actual concentrations (determined = 1·0041 × actual − 0·0083). The differences between the actual and the analysed choline concentrations were not greater than 0·07 mg litre^{-1}; the proportional difference was less than 5% even for the lowest concentration used in the assay. The response of the method was linear over the range 1–20 mg choline per litre of assay solution ($r = 0{\cdot}999$). The detection limit of 1·2 mg litre^{-1} corresponds to a choline concentration in feeds of 50 mg kg^{-1}. The coefficient of variation of a series of enzymatic determinations on a standard solution of choline was 0·2%, indicating the excellent precision of the assay.

Extraction procedure

In the conventional procedure (Lim and Schall 1964), a porous thimble containing the feed ingredient is suspended in a beaker containing the extractant ('cool' method). The methanol in the beaker is boiled. Methanol condenses above the thimble and drips through the feed and thimble, bringing methanol and soluble choline and choline-containing compounds into the beaker. Hydrolysis during this reflux period liberates free choline in the beaker. The modification found to work better was to remove the thimble and place the ingredients directly in the beaker with the methanolic KOH ('hot' method). Duplicate samples of maize, soybean meal and canola meal were extracted for 2, 4 or 6 h using the conventional technique or for 2 h heating the sample in the boiling extractant (Table 1). Heating the sample immersed in the boiling extractant resulted in a more thorough extraction of choline than dripping the cooled extractant onto the sample for the three feed ingredients tested. Although the 6 h extraction in the thimble appeared to result in a better extraction for soybean meal and canola meal than 2 or 4 h, the analysed choline values were still lower than those obtained with 2 h in the boiling extractant. The choline content of these samples of soybean meal and canola meal extracted for 2 h in the beaker were similar to values appearing in widely accepted tables of feed composition (NRC 1988, 1994).

The most marked differences between the cool and the hot extractions occurred for maize; in this case, the 2 h beaker values for choline were more than double those of the thimble extraction. Increasing the time of cool extraction for maize up to 6 h did not increase the determined choline values, which were close to table values (NRC 1988, 1994). The higher value obtained with the 2 h hot extraction was comparable to the 1·30 g kg^{-1} choline reported for maize by Lim and Schall (1964), but nearly double those reported by other workers (Almquist and Maurer 1951; Fritz *et al* 1967). The differences in choline extraction between the cool and the hot techniques were also very substantial for canola meal but not for soybean meal.

Samples of maize, soybean meal and canola meal were also used to study the time effect of the new extraction procedure. Duplicate samples were heated for 2, 4 or 6 h in beakers containing the extractant and assayed for choline (Table 2). For all three feed ingredients, the 2 h heating proved to be sufficient for adequate extraction of choline, and this length of time was established as the standard for the procedure.

Recovery assays

Recovery assays were carried out using samples of the three feed ingredients previously tested. For each ingredient a known amount of choline was added to approximately 2 g of feed sample, as an aqueous solution of choline chloride. The determined choline values of these preparations and those of the non-supplemented

TABLE 1
Analysed values of choline (g kg^{-1})[a] in feed ingredients subjected to the conventional extraction (cool) or the new procedure (hot)

Extraction	*Ingredient*		
	Maize	*Soybean meal*	*Canola meal*
2 h cool	0·53 ± 0·07	2·08 ± 0·17	3·87 ± 0·25
4 h cool	0·53 ± 0·11	2·04 ± 0·26	3·81 ± 0·02
6 h cool	0·50 ± 0·01	2·35 ± 0·08	4·18 ± 0·27
2 h hot	1·12 ± 0·06	2·58 ± 0·09	6·90 ± 0·05

[a] Mean ± standard deviation.

TABLE 2
Analysed values of choline (g kg^{-1})[a] in feed ingredients submitted to increasing extraction times under the new procedure (hot extraction)

Extraction time	*Ingredient*		
	Maize	*Soybean meal*	*Canola meal*
2 h	1·41 ± 0·01	2·56 ± 0·09	7·58 ± 0·25
4 h	1·56 ± 0·20	2·62 ± 0·09	7·61 ± 0·24
6 h	1·49 ± 0·11	2·57 ± 0·03	7·68 ± 0·08

[a] Mean ± standard deviation.

TABLE 3
Recovery assays of choline added to different feed ingredients

Ingredient	*Added* ($g\ kg^{-1}$)	*Analysed choline*[a] ($g\ kg^{-1}$)	*Recovery* (%)
Maize	0	1·21 ± 0·12	—
	1·988	3·16 ± 0·01	98·8
Soybean meal	0	2·80 ± 0·08	—
	1·836	4·51 ± 0·09	97·3
Canola meal	0	6·63 ± 0·05	—
	4·026	10·24 ± 0·29	96·1

[a] Mean ± standard deviation.

counterparts are shown in Table 3. Duplicate samples were used for the extraction and enzymatic procedures. The recovery values obtained were more than 96% for the three ingredients, being similar or better than previously reported by Betz *et al* (1991). The results of the recovery assays demonstrated that there was no appreciable loss of choline in the extraction procedure. The results also demonstrated that the method is equally suitable for determining choline concentration in feed ingredients or in feeds to which choline chloride has been added.

The greatly increased extraction of choline for maize and canola meal samples by the hot method was entirely unexpected; the authors only hoped to lower expenses by altering the extraction procedure. Apparently there are choline-containing compounds that are not soluble in the cooled and condensed methanol but are soluble in boiling methanol. Further there appears to be a higher proportion of these compounds in maize, than canola meal, as well as soybean meal. The data in Table 1 show that a clear plateau has been reached for maize, but not clearly for the soybean or canola meal samples. In other trials with extraction times greater than 4 h, no increase was found in apparent choline content, but the values with the cool extraction method never approached those from the hot extraction method. Lim and Schall (1964) also showed a plateau for soybean meal, but this is dependent on the drip rate which is difficult to control with the Goldfisch apparatus.

CONCLUSIONS

The methodology for the determination of the choline content of feed ingredients presented in this paper combined an extraction procedure, which was more efficient and less time-consuming than the conventional thimble extraction, and an enzymatic assay of the extracted choline, which was less laborious and more specific and accurate compared to the commonly used reineckate procedure. This methodology can be conducted using standard equipment in a nutrition laboratory.

ACKNOWLEDGEMENTS

This paper was supported by State and Hatch funds allocated to the Georgia Agricultural Stations of The University of Georgia, an FAPESP Scholarship to the senior author, and a gift from DuCoa Inc, Highland, IL, USA.

REFERENCES

Almquist H J, Maurer S 1951 Choline content of certain feedstuffs. *Poultry Sci* **30** 789–790.

Betz R, Schneider J, Chen J 1991 Enzymatic determination of choline. In: *NFIA Laboratory Methods Compendium* (Vol 1 *Vitamins and Minerals*). National Feed Ingredients Association, West Des Moines, IA, USA, pp 77–81.

Fritz J C, Roberts T, Boehne J W 1967 The chick's response to choline and its application to an assay for choline in feedstuffs. *Poultry Sci* **46** 1447–1453.

Hanin I 1974 Choline and acetylcholine. In: *Handbook of Chemical Assay Methods*. Raven Press, New York, USA, pp 1–162.

Lim F, Schall E D 1964 Determination of choline in feeds. *J Assoc Off Agric Chem* **47** 501–503.

NRC 1988 *Nutrient Requirements of Swine* (9th edn). National Research Council, National Academy Press, Washington, DC, USA, pp 56–57.

NRC 1994 *Nutrient Requirements of Poultry* (9th edn). National Research Council, National Academy Press, Washington, DC, USA, pp 62–65.

Schiefer S, Beutler H S 1985 Phosphatidylcholine (Lecithin). In: *Methods of Enzymatic Analysis* (Vol VIII *Metabolites* 3 *Lipids, Amino Acids and Related Compounds*), ed Bergmeyer H U. VCH Publishers, Weinheim, Germany, pp 87–104.