Detection of Choline in Food and Feed Matrices by HPLC-Fluorescence through Chemical Derivatization

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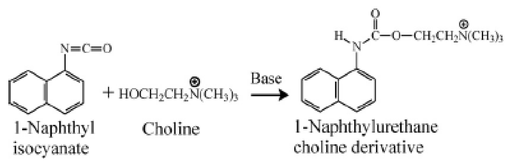
Abstract

Choline is an important component of membrane phospholipids and plays an essential role in the nervous system. A continuous supply of choline from diet and supplements provides great benefits for both humans and animals.

A chemical derivatization method with HPLC-Fluorescence detection is presented here to quantify the amount of total choline in various matrices including food, feed, commodities, supplements and infant formula. Different from most HPLC methods using post-column reaction, this approach selects 1-naphthyl isocyanate for derivatization. 1-naphthyl isocyanate reacts with choline quickly in non-aqueous condition and forms a sensitive fluorescent compound. The excessive 1-naphthyl isocyanate is then removed by the addition of water and the product of derivatization is separated by cation exchange column.



This method was originally designed for plasma samples. It was modified to apply to food, feed, commodities, and infant formula matrices. Compared to the official AOAC method 999.14 (an enzyme method), this method provides a much higher sensitivity while maintaining a fast and low cost procedure. The data shows high reproducibility (RSD ≤5%) and precision (spike recoveries are between 95% - 105%) for all matrices tested. The range of the standard curve is set at 10 -200 µg/mL and the linearity of the curve (R^2) is above 0.995. For samples with very low amounts of choline, the standard curve can be adjusted to go as low as 0.5 µg/mL and the LOQ can go as low as 5 mg/100g.

Background

Choline is a water-soluble essential nutrient and it is usually grouped within the B-complex vitamins. Choline has important roles in many physiological activities, such as cell signaling. It serves as constructional component of cell membranes and precursor molecule of neurotransmitter acetylcholine, which is directly related to many nervous functions such as memory and muscle control. Choline is also a major source for methyl groups through the S-adenosylme-thionine (SAMe) synthesis pathways. Deficiency of choline may result fatty liver, hemorrhagic kidney necrosis, fish odor syndrome, etc.

Choline is commonly fortified in infant formula, supplements, food, and pet food. Although choline naturally presents in many food matrices such as soybean, vegetables, meat, fish, etc., (Fig 1), sufficient amount of choline needs to be consumed and absorbed through diet in order to remain healthy status.

Choline can exist in different forms. Free choline normally refers to inorganic chemicals containing the choline ion such as choline chloride, choline bitartrate, choline hydroxide, etc. Choline can also exist in the forms of phosphatidylcholine, sphingomyelin, acetylcholine, etc., which can be hydrolyzed to liberate choline component.

The quantification of choline is always challenging. Choline is difficult to measure because it doesn't have native chromophore or fluorphore. The official AOAC (999.14) method was developed about 15 years ago. It is based on the coupled enzymes reaction (choline oxidase & peroxidase). It is widely used but the lack of selectivity of peroxidase in some matrices may restrict its application in food analysis.

Background (Continue)

LC-MS/MS method was also developed and it provides superior selectivity and sensitivity. However, MS method may face challenges when food or feed matrices are assayed. Choline is a small molecule; therefore the choline signal may experience interference from high background or non-specific peaks when complicated matrices are analyzed. Further investigation is needed to apply MS on choline analysis in food and feed matrices.

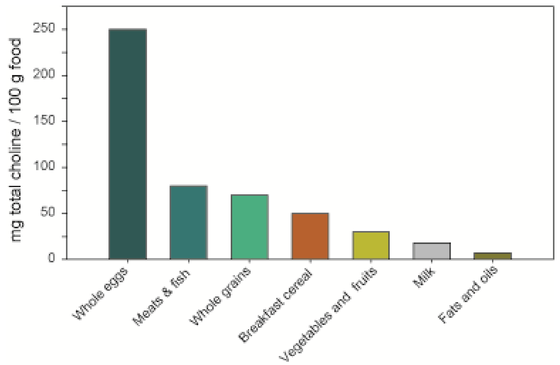


Fig 1: Total Choline in select food (from USDA)

Eurofins developed a chemical derivatization method, which provides a fast and inexpensive solution for high throughput in choline analysis. Compared to AOAC method, it has a better sensitivity. Compared to existing the LC-MS/MS method, our method provides an alternative way to quantify choline in certain complicated matrices with lower cost.

Procedure

- **Sample extraction:**

Weigh proper amount of samples and extract with 1 N HCl solution. Incubate the tube in an oven at 60 ± 5 ºC overnight (15 - 18 hours).

- **Chemical Derivatization**

Cool down samples to room temperature, adjust pH to 3.5– 4.0. Bring volume to 50 mL with distilled water and filter all samples with filter paper.

Perform further dilutions based on the expected level so the concentration of diluted sample falls in the range of standard curve 10 – 200 µg/mL.

Add acetonitrile into centrifuge tube to provide a non-aqueous reaction condition. Add sample or standard, and MgO powder into centrifuge tubes. Cap and briefly vortex so MgO can remove the water from sample. In each centrifuge tube, add 1-naphthyl isocyanate to derivatize choline.

Centrifuge the tubes and transfer supernatant to a second labeled centrifuge tube. Add distilled water and incubate 2 hours at room temperature to remove the excessive 1-naphthyl isocyanate.

Centrifuge all tubes at ~14,000 rpm for 5 minutes on an ultracentrifuge. Transfer supernatant and filter through Mini-UniPrep Syringeless Filter. Load vials into HPLC.

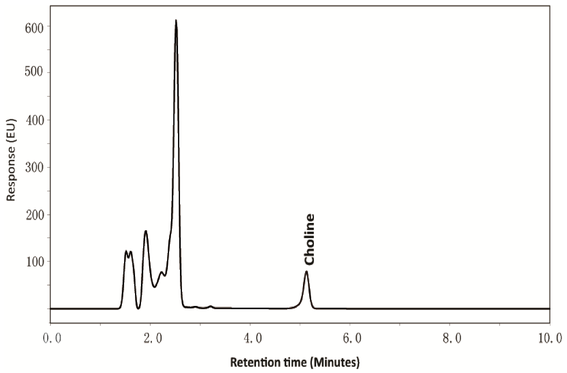
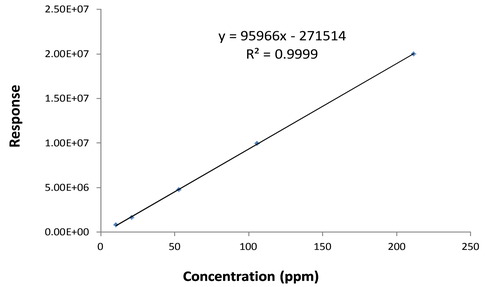


Fig 2: Chromatogram of choline in canola

Fig 3: Choline Standard Curve



Quality Control Plan

System Suitability: Perform six replicate injections of a standard. The results must meet the following specifications: %RSD of the peak area and retention times for the standard peaks must be ± 5 %

Linearity: Inject all standards and calculate the correlation coefficient (R^2) ≥0.995 on a minimum of four points.

Standard Checks: Every ten injections and at the end of the run must be bracketed by an injection of a standard. The area of the standard check must be within ± 10% of the corresponding standard from the standard curve.

Table I: Certified Reference Material - NIST 1849a

Date	Choline (ppm)
Average	1163
Std Dev	62
% RSD	5.31%
NIST Range	980 - 1200

Table II: Accuracy - Spike study

Sample Matrices	Level Spiked	Recovery	Reproducibility (RSD)
Infant Formula	600 µg	100%	0.88 %
	1200 µg	100%	0.42 %
	2400 µg	99%	3.24 %
Dry Pet Food	1200 µg	99%	2.63 %
	2400 µg	99%	2.06 %
	4800 µg	99%	2.31 %
Canola	1700 µg	99%	1.40 %
	3400 µg	100%	4.18 %
	6800 µg	102%	2.41 %

Table III: Reproducibility

Matrices	Average (mg/kg)	Standard Deviation	Range ± 2 STDEV (mg/kg)	Reproducibility (% RSD)
Infant Formula	1236	59	1118 to 1354	4.77
Dry Pet Food	2468	64	2340 to 2596	2.59
Wet Pet Food	1439	61	1317 to 1561	4.21
Canola	3459	142	3175 to 3743	4.11
Supplement	31.72%	1.48%	28.77% to 34.67%	4.65

Discussion:

Magnesium Oxide (MgO) is an essential factor in this method. The role of MgO is to remove the water from the sample solution thus preventing undesired reaction during derivatization. It was found that the excessive MgO may be carried over and precipitated out in the following cleanup step thus affecting the assay. To ensure sufficient but not excessive amount of MgO is added, a range needs to be defined. MgO can absorb moisture from the air very easily. The storage time and condition of MgO may affect the results. When needed, the MgO needs to be dried down to remove the moisture, or the amount of MgO added needs to be adjusted.

For samples with very low amount of choline (less than 25 mg/100g), the standard curve can be adjusted to go lower. The least standard can go as low as 0.5 µg/mL.

Conclusions:

1) This method provides a fast, inexpensive, accurate and precise way to measure the amount of choline in various food and feed samples.
2) This method is easy to set up, and can be used in food, feed and clinical analysis. It can be also used in academic labs.
3) This method provides an alternative to the traditional enzymatic method with superior sensitivity and versatility.

References:

1). *AOAC 999.14* – Choline in Infant Formula and Milk. (Modified)

2). *Analytica Chimica Acta* 664 (2009) 90-94 – Measurement of plasma free choline by high performance liquid chromatography with fluorescence detection following derivatization with 1-naphthyl isocyanate.

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