QUANTIFICATION OF TOTAL DIETARY CHOLINE CONTENT IN FOODS AFTER HYDROLYSIS WITH PHOSPHOLIPASE D IN COMPARISON WITH ACID HYDROLYSIS

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ABSTRACT

A previously we have developed a method for choline quantification that have been based on acid hydrolysis. In this study, a simple hydrolysis procedure for phospholipids in foods using only phospholipase D (PLD) was developed. Choline was extracted into a homogenized phase using chloroform/methanol/water and hydrolyzed using PLD (1:2:0.8). Choline was fully released from the phospholipids after incubation with PLD in the presence of diethyl ether as catalyzing agents. The established acid hydrolysis method and the developed PLD hydrolysis procedure was used for choline quantification in different food matrices and the results were compared. Quantitative results for foods analyzed using both methods were comparable ($R^2=0.9973$). The highest choline content was found in lamb liver (360 mg/100g fresh food) and lamb kidney (316 mg/100g fresh food) followed by chicken liver (172 mg/100g fresh food). Cereals and cereal breakfast contained moderate to little content of choline (20 – 86 to mg/100g fresh foods). In canned legumes the choline content was 45 mg/100 g fresh foods.

**Keywords:** Choline, Foods, Phospholipase D, Acid Hydrolysis, LC-MS.

INTRODUCTION

Choline is naturally found in plants and animals and is a significant component of many foods (Patterson et al. 2008, Zeisel et al., 2003), including, eggs, liver, shellfish, cereals and dairy products. The physiological functions of choline are as a component of the membrane phospholipids that are essential for cell integrity, a catabolic source of methyl-group metabolism, synthesis of the neurotransmitter acetylcholine, and in lipid transport in lipoproteins (Ueland 2011, Lever and Slow 2010, Penry and Manore, 2008). One important methylation reaction is that of the choline metabolite product betaine which acts as a methyl group donor for the remethylation of homocysteine to methionine (Ueland, 2011, Lever and Slow, 2010; Craig, 2004). Together with folate, good choline status is linked to decrease the risk of neural tube defects and other congenital defects (Zeisel, 2006, Kim, 2004). Choline deficiency is a well known cause of liver dysfunction, kidney failure, and may lead to increased free radical activity in...
the liver, which may lead to carcinogenesis (Zeisel and Da Costa, 2009). Choline can be endogenously synthesized in the human body, but dietary sources are necessary to maintain a good status (Blusztajn, 1998).

Choline is present in a wide range of foods as free choline (water-soluble), but the majority is found in esterified forms such as phosphocholine, phosphatidylcholine, glycerophosphocholine, and sphingomyelin (Patterson et al., 2008). A variety of hydrolysis producers have been reported for determination of total choline content in foods based on acid hydrolysis e.g., HCl (1 M) for 3 h at 70 °C (Fu et al., 2012; Andrieux et al., 2008; Laikhtman & Rohrer, 1999), or 45 min at 110 °C using a microwave-assisted hydrolysis procedure (Phillips & Sander, 2012). Phospholipase D is usually used after acid hydrolysis to fully release the choline that may be still bound to the phospholipids (Fu et al., 2012; Phillips & Sander, 2012; Andrieux et al., 2008; Laikhtman & Rohrer, 1999). In this study, a simple hydrolysis method was employed using only PLD, which eliminates the acid hydrolysis for measuring total dietary choline content.

**MATERIALS AND METHODS**

**Materials**

**Food samples**

Foods (Table 1) were purchased from a local supermarket in Christchurch, New Zealand.

**Chemicals and reagents**

Phospholipase D ≥ 50,000 units/mL (buffered aqueous glycerol solution) were purchased from Sigma-Aldrich (St. Louis, USA). A stock solution (400 U/mL) of phospholipase D was prepared using 50 mM Tris-HCL buffer (pH = 8) and stored portioned (0.5 mL) at −80 °C until use. D9-choline (HCl) was obtained from Isotec (Ohio, USA). Acetonitrile (HPLC grade) was obtained from Merck (Darmstadt, Germany). Methanol (HPLC grade) was obtained from Scharlau (Barcelona, Spain). A choline stock solution (10 mmol/L) was prepared using distilled water and stored at −20 °C until use. A set of choline calibration standards (10 – 1000 µmol/L), were prepared by serial dilution of the stock solution. All the stock and standard solutions were stored at −20°C.

**Methods**

**Choline extraction and quantification**

Before extraction, samples were minced using a household food processor (Braun, Germany). Choline extraction was based on the method of Bligh & Dyer (1959). Briefly, ~0.5 g of the food samples were homogenized in 2 mL extraction solvent (chloroform/methanol/water, 1:2:0.8) for 5 min then centrifuged at 3000 rpm for 5 min. The supernatant was collected and the extraction procedure repeated a further two times. Extracts were combined and stored at −20 °C until use.
Acid hydrolysis of choline-phospholipids

A 1 M solution of HCl was freshly prepared in acetonitrile-water (9:1) and used for acid hydrolysis (Hefni et al., 2015) as follow: In a 30 mL tube 3 mL HCl- acetonitrile was added to 1 mL of solvent extract. The mixture was heated at 115 °C for 30 min in a block heater. The caps were occasionally loosened during the first 2 min of heating to avoid excessive pressure build-up. Thereafter, samples were cooled to room temperature and pH was neutralized using NaOH (10 M).

Enzymatic hydrolysis of choline-phospholipids

Briefly: in a 1.5 mL microcentrifuge tube 50 μL of the PLD (400U/mL) was added to 50 μL of the solvent extract and 50 μL diethyl ether, was added and vortex mixed. The tubes were incubated for 90 min at 37 °C. The choice of optimum conditions for the enzymatic reaction were based on the results of preliminary experiments using phosphatidylcholine as a substrate (unpublished data).

Choline quantification by LC-MS

Choline was measured by LC-Ms as previously described (Hefni et al., 2015). Briefly, in a 1.5 mL microcentrifuge tube, 50 μL from the sample or the standard was added to 1 mL of extraction solvent (90% acetonitrile and 10% methanol) containing D₉-choline. 200 μL of the mixture was transferred to HPLC vials and capped for analysis. Separation of choline was performed on LC-MS (Agilent 1100, Agilent Technologies, USA) using A Cogent Diamond Hydride silica column (100 x 2.1 mm, 4 μm, MicroSolv Technology, Eatontown, NJ, USA) (Lenky et al., 2012). The injection volume was 5 μL and the oven temperature was set at 40 °C. A total run time was 11 min, and there was a pre-run equilibration time of 3 min, giving a run sample time of 8 min. A gradient system was used with solvent A containing 10 mM ammonium formate, 10 mM formic acid, 50% water, and 50% acetonitrile, and solvent B containing 90% acetonitrile and 10% water. The gradient used for the analysis was as follows: 0 min of 50% A and 50% B, 7 min of 100% A and 0% B, and 7.1 min of 50% A and 50% B. The mobile phase was delivered linearly at a flow rate of 0.3 mL/min. An Agilent 6120 quadrupole mass spectrometer (Agilent 1100, Agilent Technologies, USA) was used for detection using an electrospray ion source (ESI) and selected ion monitoring (SIM). Choline was measured in positive ion mode using m/z =104, and D₉-choline was used as the internal standard using m/z =113.

RESULTS AND DISCUSSION

A growing realization of the importance of adequate choline nutrition has led to determine the distribution of this compound in different foods. Choline is an essential nutrient, found in a wide variety of foods but is also formed by de novo synthesis. The free choline is highly soluble in water and are therefore easily to extract from foods. But the majority of choline in foods is esterified (phospholipids) (Zeisel et al., 2003) and therefore needs to be
hydrolysed for complete extraction. Initially, various conditions, with respect to incubation times and the concentration of catalysing agent (diethyl ether), were tested in order to define the optimum conditions for phospholipids hydrolysis using PLD (unpublished data). The optimum of conditions for the enzymatic reaction was selected, based on the results of preliminary experiments using phosphatidylcholine as substrate. The ability of PLD to be used to fully hydrolyse the choline-phospholipids compounds in foods was confirmed through this study. Usually, PLD is used to achieve a complete hydrolysis of choline phospholipids compounds after acid hydrolysis. In this study, a comparable extraction to acid hydrolysis was achieved using only PLD in the presence of diethyl ether which has been reported to increase the velocity of PLD (Fig. 1) (Yang & Roberts, 2004; El Kirat et al., 2002; Imamura & Horiuti, 1979), and the results were compared with acid hydrolysis (Hefni et al., 2015).

Figure 1: Enzymatic hydrolysis of phospholipids (phosphatidylcholine) using phospholipase D in the presence of diethyl ether as catalysing agent.

PLD are enzymes that catalyze the hydrolysis of phospholipids to phosphatidic acid and choline (Buxmann et al., 2010) (Fig. 1). Moreover, the optimum conditions of PLD hydrolysis were applied to quantify the choline content in selected foods and results were compared to the choline levels measured after acid hydrolysis (Table 1). The quantified choline content after acid hydrolysis was linearly related to the acid hydrolysis results ($R^2 = 0.996$, Fig. 2). The results of both methods were nearly identical and therefore comparable. Confirming a full hydrolysis of choline phospholipids after incubation with PLD in the presence of diethyl ether as catalysing agent. The most obvious strength of this method is the simplicity of the hydrolysis procedure that eliminated the acid hydrolysis and the capacity to analyse many samples at the same time.

A variety of analytical approaches have also been reported for choline quantification e.g., NMR spectroscopy (Graham et al., 2009), mass spectrometry (LC-MS/MS) (Xiong et al., 2012; Zhao et al., 2011; Bruce et al., 2010), or isotope dilution LC-MS (Koc et al., 2002). However, previously we have shown that choline could be separated on strong cation exchange and detected using fluorescence detection after simple derivatization procedure using (1-naphthyl isocyanate), which provides comparable results to LC-MS (Hefni et al., 2015).
The quantitative results for the choline content in the analyzed foods were expressed as mg/100 g food (Table 1). Among the analyzed foods, the highest sources of dietary choline are lamb liver (360 mg/100g fresh food) and lamb kidney (316 mg/100g fresh food) followed by chicken liver (172 mg/100g fresh food). The high choline content in meat products made them an important choline sources in the diet.

Table 1: Total dietary choline content in selected foods quantified using LCMS after acid and enzymatic hydrolysis

<table>
<thead>
<tr>
<th>Food</th>
<th>Description</th>
<th>Choline content (mg/100g ± SD, n=3)</th>
<th>% Diff</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Acid hydrolysis</td>
<td>Enzymatic hydrolysis</td>
</tr>
<tr>
<td>Cereal and cereal breakfast</td>
<td>Quinoa</td>
<td>71 ± 9</td>
<td>85 ± 10</td>
</tr>
<tr>
<td></td>
<td>Quinoa, cooked</td>
<td>20 ± 1</td>
<td>24 ± 2</td>
</tr>
<tr>
<td></td>
<td>Rye meal flour</td>
<td>38 ± 1</td>
<td>42 ± 1</td>
</tr>
<tr>
<td></td>
<td>Breakfast cereal containing 75% wheat bran</td>
<td>84 ± 1</td>
<td>97 ± 5</td>
</tr>
<tr>
<td></td>
<td>Breakfast cereal containing 85% wheat bran</td>
<td>86 ± 5</td>
<td>103 ± 4</td>
</tr>
<tr>
<td></td>
<td>Breakfast cereal whole wheat 53%, wheat bran 36%</td>
<td>77 ± 2</td>
<td>94 ± 4</td>
</tr>
<tr>
<td></td>
<td>Breakfast cereal whole grain wheat 67, wheat bran 23%</td>
<td>77 ± 2</td>
<td>92 ± 2</td>
</tr>
<tr>
<td></td>
<td>Pearl barley</td>
<td>33 ± 3</td>
<td>37 ± 2</td>
</tr>
<tr>
<td></td>
<td>Pearl barley, cooked</td>
<td>22 ± 2</td>
<td>26 ± 1</td>
</tr>
<tr>
<td></td>
<td>Bread crumber whole meal</td>
<td>58 ± 3</td>
<td>70 ± 2</td>
</tr>
<tr>
<td></td>
<td>Bread crumber superwhite</td>
<td>43 ± 7</td>
<td>50 ± 7</td>
</tr>
<tr>
<td></td>
<td>Crumpets Pams</td>
<td>26 ± 1</td>
<td>32 ± 3</td>
</tr>
<tr>
<td></td>
<td>Crumpets Golden</td>
<td>26 ± 1</td>
<td>29 ± 1</td>
</tr>
<tr>
<td></td>
<td>Couscous</td>
<td>36 ± 1</td>
<td>42 ± 3</td>
</tr>
<tr>
<td></td>
<td>Couscous, cooked</td>
<td>28 ± 1</td>
<td>33 ± 1</td>
</tr>
<tr>
<td>Canned legume foods</td>
<td>Chick peas canned</td>
<td>40 ± 2</td>
<td>46 ± 4</td>
</tr>
<tr>
<td></td>
<td>Lentils canned</td>
<td>34 ± 3</td>
<td>44 ± 1</td>
</tr>
<tr>
<td>Meat and meat products</td>
<td>Lamb kidney, fresh</td>
<td>316 ± 10</td>
<td>na</td>
</tr>
<tr>
<td></td>
<td>Lamb kidney, cooked</td>
<td>278 ± 11</td>
<td>na</td>
</tr>
<tr>
<td></td>
<td>Chicken liver, fresh</td>
<td>192 ± 16</td>
<td>172 ± 13</td>
</tr>
<tr>
<td></td>
<td>Chicken liver, cooked</td>
<td>206 ± 9</td>
<td>235 ± 33</td>
</tr>
<tr>
<td></td>
<td>Lamb liver, fresh</td>
<td>330 ± 19</td>
<td>360 ± 19</td>
</tr>
<tr>
<td></td>
<td>Lamb liver, cooked</td>
<td>374 ± 8</td>
<td>397 ± 40</td>
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</tbody>
</table>

% Diff = (enzymatic hydrolysis – acid hydrolysis)/enzymatic hydrolysis. na: not analyzed
Hence, information with respect to the effects of food processing and household preparation could be helpful to improve dietary intake. This study confirmed that cooked meat products (frying 3-5 min) rich source of choline (>200 mg/100 g cooked food). Confirming that frying had no major effect on choline content in meat products.

Cereals are a moderate source of choline (< 100 mg/100g fresh food) (Table 1). These results are in agreement with those of Zeisel et al. (2003), who reported that richest sources of dietary choline are eggs, liver, soybean, and wheat germ, whereas betaine is obtained from wheat bran, wheat germ, and spinach (Slow et al. 2005; Sakamoto et al. 2002). In conclusion, the main source of choline in the diet is from meat products, but cereals are a good source as well with respect to consumption.

Most of traditional foods, has no choline data in the USDA database, and therefore, each country needs to quantify the choline content of the foods consumed locally. Our data on choline content in some foods (Table 2, which have a choline data in the USDA database) are well in line with the USDA database (Table 2). The choline data from our analysis was linearly related to the USDA database (R² = 0.9776, Fig. 3). Confirming the adequacy of both hydrolysis (acid and enzymatic hydrolysis) procedure for choline extraction in foods.

Table 2: Comparison of total dietary choline data (mg/100 g fresh weight) quantified after enzymatic hydrolysis to USDA data base

<table>
<thead>
<tr>
<th>Food</th>
<th>Choline content (mg/100g)</th>
<th>Current data</th>
<th>USDA data base</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quinoa</td>
<td>85 ± 10</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>Quinoa, cooked</td>
<td>24 ± 2</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Rye meal flour</td>
<td>42 ± 1</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>Pearl barley</td>
<td>37 ± 2</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>Chicken liver, fresh</td>
<td>172 ± 13</td>
<td>194</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 3. Correlation between current choline data quantified after enzymatic hydrolysis vs. USDA database

CONCLUSION

Phospholipase D was used to fully release the bound choline in foods. A comparison of acid hydrolysis with the enzymatic hydrolysis procedure showed a similar choline content in selected foods.

ACKNOWLEDGMENTS

Scholarship funding from the Partner Ownership Initiative Program (ParOwn, grant number 20150), the Ministry of Higher Education, Egypt, is gratefully acknowledged. Assistance from the National Heart Foundation of New Zealand and from the Maurice & Phyllis Paykel Trust is also acknowledged.

REFERENCES


Hefni, M. *et al.*

**Predicting Choline in Foods After Enzymatic Hydrolysis Compared to Acid Hydrolysis**

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In a previous study, we developed a method for determining choline in foods that relies on acid hydrolysis of phospholipids. In this study, we developed another method for determining choline in foods using phospholipase D. Choline was extracted from a mixture of chloroform and methanol and water, then phospholipase D was added to the phospholipids. Choline was isolated from the phospholipids after hydrolysis with phospholipase D in the presence of ether as an auxiliary agent for 90 minutes. This method was used to determine the choline in some foods and compare the results obtained with acid hydrolysis. The results showed that both methods gave similar results with a high correlation coefficient ($R^2 = 0.9973$). The highest choline content in the bovine liver was 259 mg/100 g, followed by chicken liver with 71 mg/100 g. Wheat and breakfast cereals of wheat were found to be average in choline content with a range of 19 to 65 mg/100 g. Beans and legumes had a low choline content with a range of 3.4 to 1.9 mg/100 g.

460