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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 have been suggested as possible mechanisms for 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, phosphotidylcholine, 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. D_9-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 D_9-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 D_9-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).

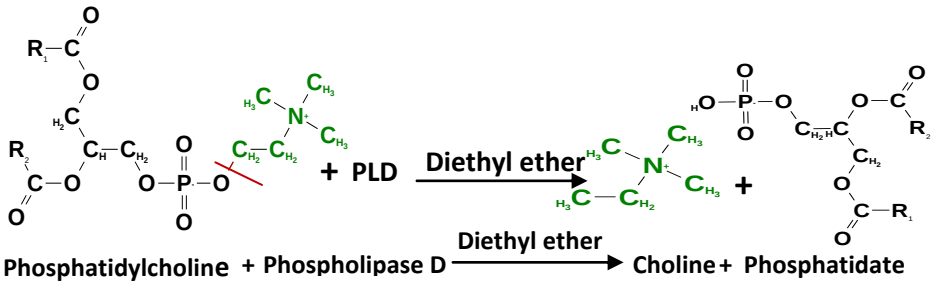

Figure 1: Enzymatic hydrolysis of phospholipids (phosphatidylcholine) using phospholipase D in the presence of diethyl ether as catalysing agent.

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 was 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 analysed 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

Food	Description	Choline content (mg/100g ± SD, n=3)		% Diff
		Acid hydrolysis	Enzymatic hydrolysis	
Cereal and cereal breakfast				
Quinoa	raw cereal	71 ± 9	85 ± 10	16
Quinoa, cooked	100g cereal + 250g water and boiling 15-20 min	20 ± 1	24 ± 2	16
Rye meal flour	whole meal flour	38 ± 1	42 ± 1	9
Breakfast cereal	containing 75% wheat bran	84 ± 1	97 ± 5	9
Breakfast cereal	containing 85% wheat bran	86 ± 5	103 ± 4	13
Breakfast cereal	whole wheat 53%, wheat bran 36%	77 ± 2	94 ± 4	18
Breakfast cereal	whole grain wheat 67, wheat bran 23%	77 ± 2	92 ± 2	16
Pearl barley	raw	33 ± 3	37 ± 2	10
Pearl barley, cooked	105g cereal + 250g water and boiling 25 min	22 ± 2	26 ± 1	15
Bread crumber whole meal	whole meal flour	58 ± 3	70 ± 2	17
Bread crumber superwhite	white flour	43 ± 7	50 ± 7	14
Crumpets Pams	wheat flour, water, raising agent	26 ± 1	32 ± 3	18
Crumpets Golden	wheat flour, water, raising agent	26 ± 1	29 ± 1	10
Couscous	raw	36 ± 1	42 ± 3	14
Couscous, cooked	200g + 250g boiling water and covered	28 ± 1	33 ± 1	15
Canned legume foods				
Chick peas canned	net weight 390 g, chickpeas 62.5%	40 ± 2	46 ± 4	13
Lentils canned	net weight 390 g, lentils 60%	34 ± 3	44 ± 1	22
Meat and meat products				
Lamb kidney, fresh	raw, not prepared	316 ± 10	na	
Lamb kidney, cooked	cooked 3min in 1 table spoon olive oil	278 ± 11	na	
Chicken liver, fresh	raw, not prepared	192 ± 16	172 ± 13	
Chicken liver, cooked	cooked 5min in 1 table spoon olive oil	206 ± 9	235 ± 33	
Lamb liver, fresh	raw, not prepared	330 ± 19	360 ± 19	
Lamb lever, cooked	cooked 5min in 1 table spoon olive oil	374 ± 8	397 ± 40	

% Diff = (enzymatic hydrolysis – acid hydrolysis)/enzymatic hydrolysis. na: not analyzed

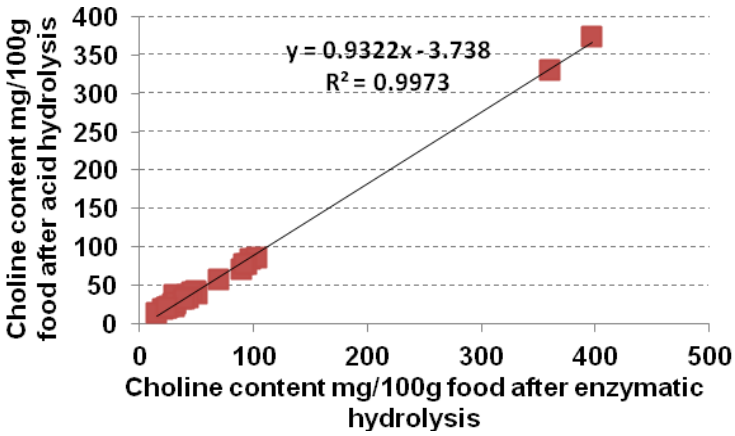

Fig. 2. Correlation between quantification after acid hydrolysis vs. enzymatic hydrolysis of choline content (mg/100g) in analysed foods

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 (R^2 = 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

Food	Choline content (mg/100g)	
	Current data	USDA data base
Quinoa	85 ± 10	70
Quinoa, cooked	24 ± 2	23
Rye meal flour	42 ± 1	31
Pearl barley	37 ± 2	38
Chicken liver, fresh	172 ± 13	194

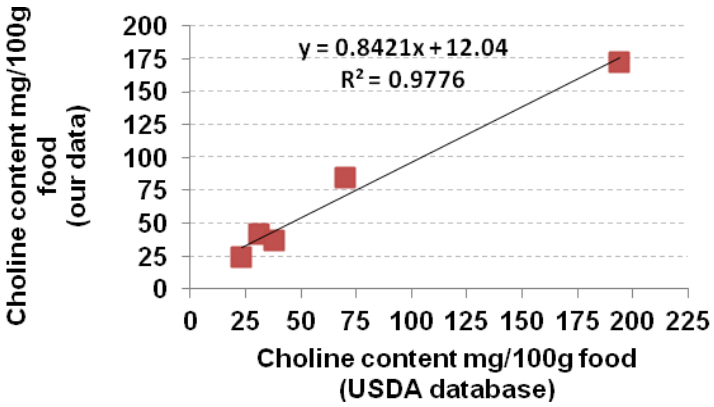

Fig. 3. Correlation between current choline data quantified after enzymatic hydrolysis vs. USDA database

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.

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تقدير الكولين في الأغذية بعد التحلل الأنزيمي مقارنة بالتحلل الحامضي

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في دراسة سابقة تم تطوير طريقة لتقدير الكولين قي الأغذية تعتمد علي التحلل الحامضي للفوسفولبيدات. في هذ البحث قمنا بتطوير طريقة أخري لتقدير الكولين في الأغذية باستخدام انزيم الفوسفوليبيز D. تم استخلاص الكولين في مزيج من الكلولورفورم والميثانول والماء ثم استخدام انزيم الفوسفوليبيز D في تحليل الفوسفولبيدات. تم استخلاص الكولين كلية من الفسفولبيدات بعد التحضين مع انزيم الفوسفوليبيز D في وجود اثير ثنائي الأثيل كعامل مساعد لمدة ٩٠ دقيقة. تم استخدام هذه الطريقة لتقدير الكولين في بعض الأغذية ومقارنة النتائج المتحصل عليها مع التحلل الحامضي. اظهرت النتائج ان كلا الطريقتين يعطي نتائج متقاربه ومعامل ارتباط قوي (R^2=0.9973). كان اعلي محتوي للكولين في الكبد البقري بتركيز يصل إلي ٣٦٠ مجم/١٠٠جم يليه كبد الدجاج بتركيز ١٧٢ مجم/١٠٠جم. الحبوب وأغذية الإفطار المصنعة من الحبوب كانت ذات محتوي متوسط من الكولين يتراوح من ٢٠-٨٦ مجم/١٠٠جم. أما البقوليات فكانت ذات تركيز منخفض من الكولين حوالي ٤٥ مجم/١٠٠جم.