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Moisture impact on extractability of phospholipids from leftover egg yolk after enzymatic treatment using supercritical carbon dioxide

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A B S T R A C T

The leftover egg yolk after immunoglobulin Y (IgY) extraction was subjected to enzymatic hydrolysis with Protease P and Lipase AY30, which resulted in the formation of cream. The extractability and purity of phosphatidylcholine (PC) and total phospholipids (PL) at three levels of moisture content in cream (freeze dried cream, 20% and 45%, w/w) were studied using supercritical carbon dioxide (SC-CO_2) extraction with ethanol addition (8%, mole%) as co-solvent at 48.3 MPa and 70 °C. The results were compared to those obtained from fresh yolk and dry yolk. The highest recoveries of PC and total PL were for freeze-dried cream and cream with 20% moisture in which the recovery of PC was about 85% and 65%, respectively, based on the initial mass of yolk used for cream formation and 94% and 106% recovery, respectively, based on the amount of cream used as extraction feed. The highest purity of PC was about 87% obtained from fresh yolk and 84% from cream with 20% moisture content. This study showed that enzymatic treatment of the pellet can improve the recovery of PC and PL from egg yolk at moisture contents of up to 20%.

Keywords: Enzymatic hydrolysis; Moisture content; Phosphatidylcholine; Supercritical CO_2; Egg yolk; Egg phospholipids; Extraction

1. Introduction

Egg yolk is rich in high-value proteins and phospholipids (PL). About one-third of egg yolk lipid composition is PL with a high content of about 70% phosphatidylcholine (PC) (Kovacs-Nolan et al., 2005; Rossi, 2007), which have broad applications in pharmaceutical and nutraceutical products and are routinely added into infant formula (Rader et al., 2004; Zeisel, 1992).

Moisture content of the feed material used for supercritical carbon dioxide (SC-CO_2) extraction has a significant effect on the extractability of solutes present in the matrix. The presence of a high amount of moisture can hinder the extraction kinetics by acting as a barrier for SC-CO_2 to diffuse into the matrix and can also result in possible alteration of the pH of the extraction environment due to CO_2 solubilisation and formation of carbonic acid. On the other hand, the presence of a small amount of moisture in biological matrices may decrease the affinity of oil for the matrix, perform as a co-solvent and induce selective extraction of certain compounds (Dunford and Temelli, 1997). Therefore, before their use as feed for SC-CO_2 extraction fresh biological materials are usually dried to certain levels of moisture content to enhance extraction efficiency. Egg yolk is an emulsion with about 50% moisture (Anton, 2007; Hatta et al., 1990).

Due to its high moisture content, strong emulsion structure and the position of PL in its structure, i.e. their non-covalent bonding to apoproteins, the diffusivity of CO_2 into the fresh egg yolk and extractability of PC is very low (Burley and Vadehra, 1989). Therefore, similar to other biological materials, egg yolk dehydration is a prerequisite to de-oiling and PL extraction using SC-CO_2. However, despite all the achievements in terms of the know-how and development of SC-CO_2 extraction technology, the low recovery of PC, even in the presence of ethanol as the co-solvent, has been a great barrier for scale up of SC-CO_2 extraction of PL from egg yolk (Aro et al., 2009; Shah et al., 2004). On the other hand, the extraction and fractionation of PL from egg yolk using conventional organic solvents is a complicated process, which involves the use of a large amount of hazardous organic solvents, specifically during neutral lipids removal (de-oiling process) (Gadkowski et al., 2012; Palacios

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and Wang, 2005a, 2005b; Schneider, 1989). Therefore, a new processing approach that could improve the recovery of high valued PC at a satisfactory level using a "green" technology like SC-CO_2 extraction would be beneficial.

Egg yolk is also an abundant source of immunoglobulin Y (IgY), which can find applications as an oral vaccination agent for the treatment of viral and bacterial diseases. IgY can be easily extracted from egg yolk using 10-fold water dilution, leaving an abundant amount of leftover egg yolk, referred to as pellet. Pellet contains over 90% of lipids and phospholipids of egg yolk (Kwan et al., 1991). The pretreatment of the pellet by enzymatic hydrolysis prior to SC-CO_2 extraction could be a worthwhile approach to increase the extractability of PC from yolk. In our previous study, we showed that enzymatic treatment could improve PL extraction from dried egg yolk samples (Navidghasemizad et al., 2014). However, enzymatic hydrolysis followed by a dehydration step prior to SC-CO_2 extraction of PC can be very costly for scale up purposes. Therefore, it is important to establish the extent of drying necessary to achieve efficient extraction of PC from fresh hydrolysate.

The objectives of this study were: (a) to investigate the extractability of PC and total PL from fresh egg yolk with intact structure and high moisture content compared to fresh, intermediate moisture and dry pellet hydrolysates, as well as dry yolk; and (b) to determine the effect of moisture content on the recovery and selective extraction of the two major PL classes in egg yolk i.e. PC and phosphatidylethanolamine (PE) using SC-CO_2 + ethanol.

2. Materials and methods

2.1. Egg yolk pellet preparation

White shell eggs (grade A), produced by Lucerne Foods Ltd. (Calgary, AB, Canada), were obtained from Safeway (Edmonton, AB, Canada). Egg yolks were separated from the albumen manually and gently rolled on Whatman paper to remove albumen from yolk. The vitelline membrane was punctured with a sharp blade and egg yolk content was collected in a beaker placed in an ice bath. Egg yolk pellet was prepared according to Kwan et al. (1991) with some modifications. Egg yolk was 10-fold diluted with MilliQ water and pH was adjusted to 6 using 1 N HCl. The yolk slurry was mixed for 1 h at 4 °C and centrifuged at 10,000 × *g* for 15 min to separate water-soluble proteins. Then, the supernatant was separated from the pellet and the pellet was collected for enzymatic hydrolysis.

2.2. Enzymatic treatment

For enzymatic treatments, the fresh pellet was diluted 6-fold with distilled water and mixed for 1 h using a magnetic stirrer to obtain a homogenous slurry, and then hydrolysed by a combination of Lipase AY 30 (EC 3.1.1.3 from *Candida rugosa*, a powder triacylglycerol lipase, active on short, medium and long fatty acid chains on sn-1, 2 and 3 positions of triacylgylcerol, Amano Enzyme Inc., Elgin, IL, USA) and Protease P (EC 3.4.x.x from *Aspergillus melleus*, a powder enzyme complex of proteinases and peptidases, minimum proteinase activity 60,000 units/g, Amano Enzyme Inc., Elgin, IL USA) at pH 6 and 40 °C. Both protease and lipase enzymes were added at a level of 2% (w/w), based on the pellet dry matter weight. Hydrolysis reaction was performed for 3 h in a 300 mL jacketed vessel to keep the temperature constant, while stirring at 300 rpm using a magnetic stirrer. The pH of slurry was maintained constant using 0.5 M NaOH or HCl manually. After 3 h, hydrolysis was terminated by increasing the temperature to 90 °C and holding the samples for 10 min at this temperature to inactivate the enzymes. Then, hydrolysates were immediately chilled by placing them in an ice bath. Later, hydrolysates were transferred to 50 mL tubes and centrifuged at 6000 × *g* for 30 min to separate cream phase from liquid phase. Cream phase was separated from the subnatant liquid by puncturing the bottom of the tubes and removing the liquid part. Cream part was collected, divided into 50 mL tubes and stored at −70 °C.

2.3. Proximate analyses

Samples were freeze dried and then their lipid content was determined according to Hara and Radin (1978) using hexane and isopropanol and the results are expressed as g/100 g on dry weight basis. Moisture content was determined gravimetrically after drying in an oven at 105 °C with measurements taken for 16 h, until constant weight was reached.

2.4. SC-CO_2 extraction process

For SC-CO_2 extraction, the following treatments were used as feed material: fresh egg yolk (48% w/w moisture), freeze-dried yolk, and creams with different moisture levels: fresh (45% w/w moisture), partially dried (20% w/w moisture) and freeze-dried cream. To obtain samples with an intermediate moisture (20%) content, samples were frozen at −70 °C and then freeze dried to the targeted level of moisture content (Labconoco, model 7806020, Kansas, MO, USA). The laboratory scale supercritical fluid extraction system described previously by Dunford and Temelli (1995) was used for the extraction process. Details of the operation of the system were similar to those reported earlier (Navidghasemizad et al., 2014). Schematic presentation of the whole experimental protocol for different treatments and SC-CO_2 extraction is demonstrated in Fig. 1.

About 3–4 g of sample was mixed with 150 g of glass beads (3 mm, Fisher Scientific, Fair Lawn, NJ, USA) and filled into a stainless steel basket to be placed into the 300 mL extraction vessel. Freeze-dried samples were crushed into powder using mortar and pestle. Lipids from the yolk and cream samples were extracted in two steps at 48.3 MPa, 70 °C using CO_2 (99.9% (wt%) pure, bone dry, Praxair Inc., Edmonton, AB, Canada) at a flow rate of 1 L/min (measured at ambient conditions, 20 °C and 101.1 kPa), corresponding to a mass flow rate of 1.83 g/min. The extraction condition was selected based on the available lipid solubility data and previous reports (Guclu-Ustundag and Temelli, 2005; Aro et al., 2009) in an effort to maximise lipid solubility and the CO_2 flow rate of 1 L/min was selected to allow sufficient residence time to approach the saturation limit (Sun and Temelli, 2006). In the first step, neutral lipids were extracted using neat CO_2 and extract fractions were collected at each 20 L of CO_2 consumption, except for the pellet where fractions were collected every 40 L of CO_2 consumption until no more extract was collected. When the extraction curve reached a plateau, 100% ethanol was injected into the CO_2 line prior to entry into the extraction vessel using a piston pump at a flow rate of 0.2 mL/min in order to get a molar concentration of 8% ethanol in CO_2. For the second part of extraction with ethanol injection, extract fractions were collected at every 20 L of CO_2 consumption for the first six collections. After that, extracts were collected every 40–60 L of CO_2 consumption. Extract fractions from CO_2 + ethanol step were dried under a gentle stream of nitrogen at 50 °C until they were completely dry and reached constant weight. Polar lipids were extracted in the second step for about 6 h, except for fresh yolk, which