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Lipidomic investigation of eggs' yolk: Changes in lipid profile of eggs from different conditions

Ana M. Campos [a], Fernando Ricardo [b], Eliana Alves [a], Ana Reis [a], Daniela Couto [a], Pedro Domingues [a], M. Rosário M. Domingues [a,*]

[a] Mass Spectrometry Centre, Department of Chemistry & QOPNA, University of Aveiro, 3810-193 Aveiro, Portugal
[b] Departamento de Biologia & CESAM, Universidade de Aveiro, 3810-193 Aveiro, Portugal

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ABSTRACT

Eggs are one of the main foods eaten worldwide. Nutritionally they are one of the main sources of dietary lipids, impacting human health. Egg yolk lipid composition changes depending on different conditions associated with hens raising. Therefore, the purpose of our work was to use a lipidomic approach as a tool to evaluate if different diets (vegetable *versus* animal) and raising environments (free range *versus* indoor) interfere in the triacylglycerol (TAG) and phospholipid (PL) profiles of eggs' yolks and to use such differences to differentiate eggs according to their origin. To achieve that goal, total lipid extracts were obtained and then fractionated by solid-phase chromatography. TAGs fraction was analysed by ESI-MS and PLs fraction by HILIC-LC-MS/MS. TAG and five PL classes were identified, namely PC, LPC, PE, LPE and SM. Fatty acids (FA) esterified to the glycerol backbone of PL ranged between C16:0 and C22:6. On the other hand, FA esterified to TAG ranged from C14:0 to C20:0. Major differences on the PL profile were observed regarding eggs from free-range hens and fed with vegetable origin food and eggs from the remaining conditions, once the former presented higher levels of PC (O-34:0), PC (34:1) and PE (34:1). Eggs from hens fed with animal origin food contained PL and TAG molecular species richer in *n*-6 FA, according to GC–MS and to LC-MS/MS data. The lipidomic approach used herein proved to be promising in differentiating eggs from hens with different raising conditions.

1. Introduction

Eggs have been collected and eaten for centuries worldwide, and are an important food due to their rich nutritional value. They are simultaneously an economical food and a food ingredient consumed in all cultures (Fredriksson, Elwinger, & Pickova, 2006). Because eggs are a system developed to nourish and protect the embryo, their shell and white have physical and biological defence mechanisms such as antimicrobial properties while egg yolk is a reservoir of nutrients (Nimalaratne & Wu, 2015). Nutritionally, eggs are very complete once they are rich in amino acids, lipids, vitamins and minerals, with some of these compounds possessing antioxidant properties (Fredriksson et al., 2006; Nimalaratne & Wu, 2015). Eggs' lipids are found mainly in the yolk, consisting in triacylglycerols (TAGs, 66%), phospholipids (PLs, 28%) and cholesterol (6%) (Belitz, Grosch, & Schieberle, 2009). Therefore eggs are one of the main sources of lipids in the human diet.

Some studies reported eggs' fatty acids (FAs) as beneficial to prevent some pathological conditions, such as coronary heart disease and some cancers (Simopoulos & Salem, 1992). Lipids from eggs are rich in monounsaturated fatty acids (MUFAs) and are being artificially enriched in omega-3 polyunsaturated fatty acids (*n*-3 PUFAs) through rations (Rodrigues & Arau, 2005). Since *n*-3 PUFAs have been reported as beneficial to human health, several studies addressed how the lipid profile of eggs from laying hens changes according to diets rich in such FAs. The FA profile of eggs changes according to the diet of laying hens, namely diets richer in *n*-3 PUFAs give rise to eggs richer in *n*-3 PUFAs (Fredriksson et al., 2006). These results indicate that PUFA profile of the yolk is highly dependent on the types of PUFAs present in the hens' diets.

Moreover, not only FAs can bring positive outcomes to human health but also dietary PLs. Despite the relationship between dietary lipids and some pathological conditions, several studies discovered beneficial health effects associated with dietary PLs, namely PLs from eggs. One of PLs main roles is to provide unsaturated FAs, such as *n*-3 FAs

Abbreviations: TAG, triacylglycerol; PL, phospholipid; *n*-3, omega-3; *n*-6, omega-6; FA, fatty acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; SM, sphingomyelin; dMPC, 1;2-dimyristoyl-*sn*-glycero-3-phosphocholine; dMPE, 1;2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine; MeOH, methanol; $CHCl_3$, chloroform; ANOVA, analysis of variance; ANOSIM, analysis of similarity; CAP, canonical analysis of principal coordinates; LA, linoleic acid; AA, arachidonic acid; OA, oleic acid; CVD, cardiovascular disease; DPA, docosapentaenoic acid.

* Corresponding author.
E-mail address: mrd@ua.pt (M.R.M. Domingues).

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which can generate anti-inflammatory eicosanoids (Küllenberg, Taylor, Schneider, & Massing, 2012). Some studies have contributed to the growing interest in using dietary PLs for the treatment of inflammatory diseases. In fact, soybean phosphatidylcholine (PC), major component of foodstuff and eggs, was proved to be able of limiting the inflammatory process in animal models (Erős, Ibrahim, Siebert, Boros, & Vollmar, 2009), to protect gastrointestinal tract (Dial, Zayat, Lopez-Storey, Tran, & Lichtenberger, 2008), as well as lowering total cholesterol levels (Simons, Hickie, & Ruys, 1977). Marine PLs have also been proved as capable of reducing inflammatory reactions (Deutsch, 2007) and of inhibiting the growth of chemically induced colon cancer *in vitro* (Hossain, Hosokawa, & Takahashi, 2009).

Studies conducted around eggs' PLs have also reported these PLs as advantageous to human health, being capable of reducing cholesterol and FA absorption (Jiang, Noh, & Koo, 2001; Noh & Koo, 2004), lowering lipid levels in liver and simultaneously increasing fecal sterol excretion (Chung et al., 2013; Yang, Ma, Xu, Yu, & Qiu, 2012). Other studies reported eggs' PL as capable of reducing both C-reactive protein and tumor necrosis factor alpha in plasma (Blesso et al., 2013; Ratliff, Mutungi, Puglisi, Volek, & Fernandez, 2008).

Despite the wide diversity of studies concerning the alterations in the FA profiles of eggs, no study has ever addressed the differences in the total lipid profile between eggs obtained from laying hens raised in open air and fed with food from biological agriculture and eggs obtained from laying hens raised in different environments but fed with animal origin food. Also no previous studies aimed at developing a lipidomic approach capable of differentiating eggs proceeding from hens raised in different environments and fed with different feeds. However, this approach has been recently shown to be capable of differentiating the growing phase of organisms based on its lipid profile (Rey et al., 2016). In this work, a sophisticated lipidomic approach based on solid phase extraction (SPE) and liquid chromatography coupled to mass spectrometry and tandem mass spectrometry (LC-MS and MS/MS) was used to evaluate if the raising environment and the feeding of laying hens affects TAGs and PLs molecular profile of eggs' yolk, as well as the FAs esterified to them. It was also studied if these differences could predict the eggs' origin.

2. Materials and methods

2.1. Material

PL internal standards 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (dMPC) (ref. 850345C), 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (dMPE) (ref. 850745P) and 1-nonadecanoyl-2-hydroxy-sn-glycero-3-phosphocholine (LysoPC) (ref. 855476P) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Chloroform ($CHCl_3$), methanol (MeOH) and hexane of HPLC grade were used (Fisher Scientific, Leicestershire, UK), All other reagents and chemicals used were of the highest grade of purity commercially available. The water was ultra-pure water obtained using a milli-Q® direct water purification system (Millipore, USA).

2.2. Sampling and sample preparation

Eggs from laying hens raised in free-range conditions and fed exclusively with vegetal origin and organic food (food cultivated using environmentally friendly agricultural practices, including no use of pesticides) (eggs_org) were obtained from a local farm. The parent hens were maintained outdoors under free-range conditions with free access to grazing on grasses and were also provided exclusively with vegetables, fruit and corn. Despite the proportion of grasses, vegetables, fruit and corn is not constant, hens were fed everyday with the same diet.

Eggs from laying hens fed with animal origin food and raised either in free-range conditions (eggs_fr) or indoors (eggs_ind) were purchased from a local supermarket, from the same supplier, using the same feed conditions. Parent hens were maintained outdoors under free-range conditions or in pavilions, respectively. In both conditions, according to the information provided by the company in eggs box, laying hens were provided with animal origin food, also designated as commercial feed, meaning that commercial feed was made of <60% cereals, according to the Portuguese legislation (Regulamento (CE) no 589/2008, de 23 de Junho, artigo 150, alínea a). The amount of food provided to the hens is controlled by the company that raises the hens. All eggs were laid in late August. Four eggs from all different conditions were randomly selected. Egg yolks were separated manually from the albumen.

2.3. Phospholipid extraction

PLs were extracted from the yolks by the Bligh and Dyer method (Bligh & Dyer, 1959). Briefly, 2.50 mL of MeOH were added to the egg yolks (≈200 mg) and well homogenized in a vortex. Subsequently, 1.25 mL of $CHCl_3$ were added to the previous mix, well homogenized in a vortex and incubated in ice for 30 min. Then, 1.25 mL of $CHCl_3$ and 1.25 mL of H_2O were added and well mixed. The mix was centrifuged at 1000 ×*g* for 5 min, at room temperature (Mixtasel Centrifuge, JP Selecta, Spain) and the aqueous phase was separated from the organic phase, from where lipids were retrieved. The extracts were dried under a nitrogen stream and stored at −20 °C.

2.4. Solid phase extraction

The total lipid extract was fractionated into TAG and PL using solid phase extraction (SPE) with 500 mg aminopropyl cartridges (Supelco, 52,637-U). The cartridge was activated with 7.5 mL of hexane and loaded with 10 mg of total lipid extract which was previously resuspended in 100 μL of hexane:$CHCl_3$:MeOH (95:3:2, v/v/v). TAGs were eluted with 5 mL of $CHCl_3$. Then, 2.5 mL of diethylether:acetic acid (98:2, v/v) were applied to the cartridge and the fraction with free FA was retrieved. The PL fraction was collected after eluting with 5 mL of MeOH:$CHCl_3$ (6:1, v/v) (Archer et al., 2013; Ruiz, Antequera, Andres, Petron, & Muriel, 2004). Using this protocol, it was possible to retrieve around 80–90% of TAG and of PL.

2.5. Quantification of phospholipids

The amount of total PL in each extract was estimated by phosphorus quantification (Bartlett & Lewis, 1970). Briefly, 125 μL of perchloric acid (70%, w/v) was added to the dried samples and incubated 60 min at 200 °C in a heating block (Block Heater, SBH200D/3, Stuart®, Bibby Scientific Ltd., Stone, UK). Milli-Q H_2O (825 μL) and 125 μL of aqueous ammonium molybdate (2.5%, w/v) and 125 μL of ascorbic acid (10%, w/v) were added to all samples and vortexed after addition of each solution. The samples were further incubated in a bath at 100 °C for 10 min. Simultaneously, standards were prepared using 0.1 to 2 μg of phosphate (100 μg mL^{-1}), which underwent the same treatment as the samples. The absorbance of samples and standards were measured at 797 nm, in a microplate reader (Multiskan GO, Thermo Scientific, Hudson, NH, USA).

2.6. HILIC-ESI-iontrap-MS and MS/MS conditions

To identify the molecular PL species and their changes in eggs from different conditions, PL classes were separated by HILIC-LC-MS, using an HPLC system (Waters Alliance 2690) coupled to an electrospray (ESI) linear ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA). The mobile phase A was composed of 25% water, 50% acetonitrile, 35% MeOH with 1 mM ammonium acetate. The mobile phase B was composed of acetonitrile 60%, MeOH 40% with 1 mM ammonium acetate. Sample (25 μg) was dissolved in the mobile phase B and