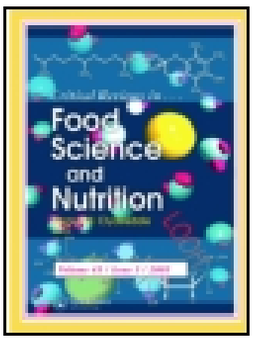

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Advances in techniques for reducing cholesterol in egg yolk: a review

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Abstract

Eggs are highly nutritious food whose high cholesterol content has been always an inconvenience due to concerns about the relationship between dietary cholesterol and atherosclerotic cardiovascular risk. As this remains uncertain, low cholesterol intake is recommended. This review deals with the techniques employed to reduce the cholesterol content in egg yolk once the egg is shelled. There are four main techniques: i) solvent extraction, ii) fractionation by centrifugation, iii) cholesterol chelates or adsorbents and iv) cholesterol biotransformation. Analyse of techniques, descriptions and recent advances are included in this review. Solvent extraction and cholesterol biotransformation allow to reduce up

to 94.7% and 93.4%, respectively. However, both methods have not been scaled up due to food safety and economic reasons. Nowadays, fractionation by centrifugation and cholesterol chelates are the only feasible methods with industrial applications, obtaining up to 82% and 99%, respectively. Fractionation method can be considered the best because no substances are added.

Keywords: cholesterol; extraction; egg; removal; yolk

Introduction

Cholesterol is a vital constituent of animal cell membranes and it serves as a precursor to various biomolecules such as steroids hormones, bile acids and vitamin D_3 (cholecalciferol). This is the reason for it presents in foods of animal origin such as meat, dairy product, poultry, shellfish and eggs. Moreover European Food Safety Authority (EFSA) says that traces amounts are present in plants membranes (EFSA Panel on Dietetic Products, Nutrition, 2010).

Cholesterol is a steroid. It is based on a tetracyclic ring system involving three six-membered rings and one five-membered (Figure 1). It contains a hydroxyl group that could be esterified or not given free cholesterol. Due to it lipophilic nature, it is transported by blood bound to lipoproteins such as very low-density lipoprotein (VLDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL).

Serum cholesterol was selected as one of the seven health metrics that characterised the cardiovascular health by the American Heart Association. It was considered a cardiovascular health factor with an ideal level lower than 200 mg/dL that clearly promote overall longevity (Lloyd-Jones et al., 2010). Better cardiovascular health is associated with less incident of heart failure, less subclinical vascular disease, better global cognitive performance and cognitive function, lower prevalence and incidence of depressive symptoms, and lower loss of physical

functional status (Benjamin et al., 2017).

Concerns about the relationship between dietary fat and atherosclerotic cardiovascular risk led to a number of reports encouraging changes in the human diet (Griffin, 1992; Lichtenstein et al., 2006). These studies included recommendations for the reduction in the total fat intake, in the ratio of saturated to unsaturated fatty acids and intake of total cholesterol to less than 300 mg/day (US Department of Agriculture & US Department of Health and Human Services, 2010).

However, this relationship has been always a controversy. There are some studies that reported a decreased risk or no change with higher cholesterol intake (Berger, Raman, Vishwanathan, Jacques, & Johnson, 2015). Studies have to be carefully fitted and well conducted. Dietary cholesterol is obtained from foods that are also significant sources of dietary saturated fatty acids. The positive relationship between consumption of saturated fat and the risk of cardiovascular disease has been already demonstrated (Berger et al., 2015; de Jesus, Kahan, & Eckel, 2016; EFSA Panel on Dietetic Products, Nutrition, 2010).

In 2010, EFSA decided not to propose a reference on cholesterol intake beside its conclusion on the intake of saturated fatty acids. However, EFSA recognised that there is a positive dose-dependent relationship between the intake of dietary cholesterol with blood LDL-cholesterol concentrations (EFSA Panel on Dietetic Products, Nutrition, 2010). The American Heart Association avoided the recommendation but it was due to the lack of enough evidence to determine whether lowering dietary cholesterol reduces LDL-cholesterol (Eckel et al., 2014).

Nowadays the *Dietary Guidelines for Americans 2015-2020* do not include the recommendation of "lower 300 mg/day" and claim for more research regarding the dose-

response relationship between dietary cholesterol and blood cholesterol levels to determine a quantitative limit for dietary cholesterol.

While this is unclear, they recommend to eat as little dietary cholesterol as possible, because humans are able to synthesize sufficient cholesterol to meet biologic requirements (U.S. Department of Health and Human Services & U.S. Department of Agriculture, 2015). Since the US regulatory authorities claimed, several studies have been published with controversy conclusions (Berger et al., 2015; Freeman et al., 2017; Grundy, 2016; Poggio et al., 2017; Rhee et al., 2017). Some of them were focus on how the egg intake modified the blood cholesterol levels (Clayton, Fusco, & Kern, 2017; Díez-Espino et al., 2017; Eckel, 2015; Kishimoto et al., 2017).

Eggs were always polemic due to their high cholesterol content. In 1968, the US authorities recommended not to eat more than 3 eggs per week. The egg industry responded it with research documenting the minimal effect of egg intake on plasma lipoprotein levels, as well as research verifying the importance of egg nutrients in a variety of issues related to health promotion (McNamara, 2015).

Cholesterol is contained in the yolk. The whole egg contains an average of 372 mg and the yolk egg around 1085 mg of cholesterol per 100 g of edible portion (US Department of Agriculture, Agricultural Research Service, & Nutrient Data Laboratory, 2016). If a large egg weighs around 50 g, more than one egg supposes an intake higher than 300 mg of cholesterol.

Egg yolk contains approximately 50% solids. The major constituents of the solid matter are lipids (65–70%) and proteins (30%), consisting of proteins in solution referred to as livetins, lipoprotein particles including HDLs and LDLs, and phosvitin (Amanda Laca, Paredes, & Díaz,

2010). Yolk cholesterol is mainly located in LDL particles (Lv et al., 2002). LDL are spherical nanoparticles (17 – 60 nm) with a lipid core of triglycerides and cholesterol esters in a liquid state surrounded by a monofilm of phospholipids, proteins and some cholesterol (Marc Anton, 2013).

Eggs are a highly nutritious food based on their high-quality proteins and compliment of vitamins and minerals. In addition, egg proteins, has a significantly greater satiety effect than other protein sources. Eggs provide highly bioavailable forms of the xanthophylls lutein and zeaxanthin and they are an excellent source of choline and phospholipids (Andersen, 2015; McNamara, 2015).

As eggs are higher in dietary cholesterol, but not in saturated fats, they are included in the protein foods group and considered nutrient dense foods (U.S. Department of Health and Human Services & U.S. Department of Agriculture, 2015). Eggs are included in a healthy eaten pattern and the recommendation for the meats, poultry, and eggs subgroup in the Healthy U.S. and Mediterranean-Style Eating Pattern at the 2.000-calorie level is 26 ounce-equivalents per week; considering that 1 large egg is equal to 1 ounce-equivalent protein foods.

Although it cannot be forgotten their high cholesterol content. Egg yolk should not be eaten indiscriminately by adults without regard to their global cardiovascular risk, genetic predisposition to heart attacks and overall food habits (Spence, Jenkins, & Davignon, 2010). Eggs should be eaten in a healthy diet. However, in order to avoid excessive intake of cholesterol from them and improve their nutritional value, the studies developed to reduce cholesterol levels in eggs are justified.

Some egg derived products labelled as “low cholesterol” are marketed. They contains only egg white, with the yolk fat portion being replaced by vegetable fats (phytosterols) or fish fats (omega-3 fatty acids) (Radlo & Delio, 2013).

This review deals with the available techniques employed to reduce the cholesterol of the egg yolk in the edible portion once the egg is shelled.

Techniques to reduce cholesterol

There are many patents and articles reporting techniques to reduce cholesterol content in egg yolk. They can be classified in two groups depending on the step where extraction is performed: a) In-shell egg: techniques that decrease the formation of cholesterol in the shell egg and b) Out-shell egg: techniques that reduce the cholesterol content once the egg is shelled. Figure 2 shows a classification scheme.

Inside the first group, there were described several strategies, such us breeding methods (Yadong et al., 2013) or feed control. The last one is the technique most described in patents (Guoqing, 2017; Jingshen, Dongxiang, & Xuezhi, 2017). However, it is difficult to decrease the cholesterol content of egg efficiently by alteration of the laying hens diet with various nutrients or genetic selection, non-nutritive factors, or pharmacological agents, as the vast majority of these experimental approaches elicited only minimal changes in the cholesterol level (<10%). This can be due to laying hens usually meet their bodies' needs for cholesterol entirely by *de novo* synthesis (Elkin, 2006). The best results were obtained when statins, garlic paste, or

pharmacological amounts of copper were orally administered to chickens. Yolk cholesterol levels were reduced up to 46%, 32%, or 34%, respectively (Elkin, 2007).

Within out-shell egg techniques, they can be classified in four groups: i) solvent extraction; ii) fractionation by centrifugation; iii) cholesterol chelates or adsorbents and iv) cholesterol biotransformation. These are the methods reviewed in this article. It is important to note that it is desirable to reduce the cholesterol content maintaining the egg-derived product edible with minimal changes in colour, flavour and functional properties of yolk.

Solvent extraction

Organic solvents

Due to its high lipophilicity, cholesterol determination was always laid to organic solvents (Sweeney & Weilhrauch, 1976). Consequently, several approaches were performed with organic solvents to remove cholesterol from eggs (Borges, 1997; Chung & Ferrier, 1991; Ezra, 1975; Melnick, 1971; Warren, Brown, & Davis, 1988; Yano, Fukinbara, Yoshida, & Wakiyama, 1980). Some of the solvents employed were: hexane, chloroform, acetone, petroleum ether, diethyl ether, dimethyl ether or heptane. However, solvents employed must be in the list of authorised extraction solvents in the production of foodstuff and food ingredients (European Parliament and Council, 2009). Within the organic solvents already studied, this list includes acetone, hexane, dimethyl ether (European Commission, 2010) and diethyl ether. But only acetone has not specific use.

The common steps in the extraction methods are: 1) mixing out-shell egg or yolk with solvent; 2) remove solvent, 3) dry the protein concentrate to remove the residual solvent. Mixing can be under continuous stirring (Paraskevopoulou & Kiosseoglou, 1994; Warren et al., 1988) or just by agitation for a few minutes (Borges, 1997). Separation of solvent is normally by filtration (Paraskevopoulou & Kiosseoglou, 1994; Warren et al., 1988) or centrifugation (Kijowski & Lombardo, 2000).

Table 1 summarizes the results where organic solvents were employed to reduce the cholesterol content in egg. It also includes some operational variables such as the dilution ratio employed with the solvent, temperatures for mixing and solvent evaporation, and mixing time. Only studies with vegetable oils employed liquid egg yolk. While the rest employed dried egg yolk.

In the first studies, it was detected that the amount of cholesterol removed was higher with a combination of polar and non-polar solvents. As a result of the disruption of lipid-protein bonds (Warren, Ball, Froning, & Davis, 1991). Moreover, the liquid egg (yolk and white) contains a lot of water. Therefore, an extraction with non-polar solvents will not be efficient due to the difference in polarities. The polar solvents, such as lower alcohols, denature yolk proteins destroying hydrogen bonds or electrostatic interaction in protein structure opening the way to the neutral lipids (LDL), what makes extraction with non-polar solvent possible (Kovalcuks & Duma, 2014).

However, whether proteins are denatured, functional properties are greatly impaired because they precipitate and emulsifying properties are closely related to protein solubility (Amanda Laca, Paredes, Rendueles, & Díaz, 2015). This explained why it was considered hexane as the best option for organic solvent extraction when compared with a mixture of polar and non-polar solvents. Hexane removed less cholesterol and more fats and phospholipids. Nevertheless, yolks defatted with hexane remained natured proteins and they were more easily dissolved in water and retained more pigments. Besides it was a solvent already used in commercial extraction of vegetable oils. These defatted yolks were incorporated into cakes and frozen and spray-dried scrambled egg products (Warren et al., 1991). Those cakes compared to full fat yolk cakes resulted in no difference in tenderness, flavour and volume; only a small colour difference was detected. In addition to the practical nutritional benefit of containing only

51 mg of cholesterol per egg (Warren et al., 1988). On the other side, different organic solvents (hexane, isopropanol, chloroform-methanol (2:1, v:v), hexane-isopropanol (77:23, w:w), hexane-ethanol (77:23, w:w) and 95% ethanol) were tested. It was detected that protein solubility and emulsifying activity of the extracted egg yolk powder decreased in all cases (Chung & Ferrier, 1991).

Different results were obtained when petroleum-ether alone or with ethanol (35:65) was used. It was observed that both egg yolk protein concentrates showed better foaming activity and foam stabilizing ability than dried yolk. But yolk extracted with petroleum-ether gave emulsions of similar rates of coalescence to those prepared with dried yolk and mayonnaise-like emulsions of higher rheological properties (Paraskevopoulou & Kiosseoglou, 1994).

Good results were also obtained with acetone. An egg-derived product with 30% less of total lipids, 81% less of cholesterol and increased 42% the phospholipid content was produced. As ingredient in mayonnaise preparation, significant difference was not detected in emulsion stability compared to that obtained with the egg yolk control. However, the sensory tests showed that the product was considered acceptable by only 55% of the judges (Borges, 1997). A computer simulation suggested an extraction process with up to 91% cholesterol removal yield (Martucci & Borges, 1997).

Due to the low lipid selectivity of hexane solvent extraction, it has been employed to obtain egg yolk oil. It is considered a very good additive for infant and adult nutrition that contains vitamin, lecithin and very high nutritional value lipids (Kovalcuks & Duma, 2014; Tokarska & Clandinin, 1985). Organic solvents extraction has been also applied to phospholipids isolation from egg yolk (Gładkowski, Chojnacka, Kiełbowicz, Trziszka, & Wawrzeńczyk, 2012). When using these solvents it must be consider that only residues or unavoidable quantities presenting no danger to human health can be left in the egg-derived

product (European Parliament and Council, 2009). This risk disappears if edible oils are used. Some of the oils described were from safflower, corn, cottonseed, sunflower, soybean, olive or peanut.

In the early 1970s, it was patented a method where vegetable oils were combined with wet egg yolk by high shear mixing. The inventors sustained that a water barrier that surrounded the yolk fat globules prevented the contact between them and the oil solvent. They thought that it could be broken with a mixing device, as it would apply energy to disrupt particles and the water barrier, allowing the contact between the yolk fat globules and the oil droplets. Then, it was employed a centrifugal separator to separate the oil yolk mixture. Other advantages of this method are: a) the possibility of increasing the yolk polyunsaturated/saturated ratio when the edible oil was high in polyunsaturated; b) a last dry step was not needed, thus the end product was liquid egg yolk in which the natural water content has been substantially retained (Fioreti, Stahl, Sims, & Spotholz, 1973).

In the 1990s, the technique was improved by the addition of monoglyceride to the oil. Instead of an energetic mixing, the egg yolk lipoproteins were destabilized previously in water preferably with salts as they aided in destabilizing the lipoprotein complexes and granules, decreased the viscosity of the solution, and increased the solubility of some egg proteins. At this step, monoglyceride was added with oil facilitating oil-lipoprotein interaction in that aqueous phase (Conte, Jr., Johnson, Hsieh, & Ko, 1992). All process was performed under temperature control (50-60 ºC) to facilitate emulsion formation without protein denaturation.

Moreover, it was patented a method that substituted monoglyceride by food grade acid such as acetic acid to destabilize egg yolk emulsion at low pH and avoid the emulsion formation. Once the oil-in-water emulsion is formed, it was extremely difficult to separate. Therefore, the extraction of cholesterol is inhibited. While the acid and/or salt facilitated the rapid coalescence of oil droplets as they were sheared into smaller size particles by the mixer. This method required less oil and reduced production cost (Lombardo & Kijowski, 1994). The highest cholesterol removal obtained was 81.4 %. This percentage was increased later to 84.6 % by the same inventors who authored a patent were no acid or salt was employed. However, this second patent used nearly twice more soybean oil to obtained almost the same removal (Kijowski & Lombardo, 2000).

Supercritical fluids

In 1990, it was published the first study where it was removed selectively the cholesterol from egg products without removing the polar lipids with supercritical carbon dioxide (CO_2) extraction. Previous studies with ethylene and carbon dioxide were published but their extraction was not such selective (Froning et al., 1990). This method exploits the dissolving power of supercritical CO_2 under conditions above its critical temperature and pressure. In these conditions of high pressure and moderate temperature, cholesterol has higher fluid density and greater volatility and supercritical fluid has high diffusivity and low viscosity. Then it is able to dissolve and sweep selectively the cholesterol and triacylglicerols, the non-polar lipids (Del Valle & Aguilera, 1999).

Table 2 included results published where supercritical fluids were employed to removed cholesterol in egg. Different pressure and temperature conditions were tested (163 atm/40 °C; 238 atm/45 °C; 306 atm/45 °C; 374 atm/55 °C). The increase in temperature and pressure raised the yield of extracted lipids and cholesterol. The best low-fat and low-cholesterol (LFLC) egg product was obtained using 306 atm at 45 °C where the improved protein concentration did not

impaired functional properties of the egg yolk (even ameliorated cake volume) and approximately 66% of the cholesterol was removed.

Several studies were performed to evaluate the properties of LFLC egg yolk produced with this method. It was proved that emulsifying properties do not change because the glycoproteins are not removed (Bringe, Howard, & Clark, 1996). When it was increased the amount of CO_2 employed, higher amounts of cholesterol were removed (75%). They tested a mayonnaise-like emulsion and a cake prepared with the LFLC egg yolk. The LFLC mayonnaise had lower values for consistency index and yield stress, and less pronounced pseudoplasticity. However, it had greater foaming activity and foam liquid stability. Assessed by sensory tests, the LFLC cake had a texture different to the cake prepared with dried egg yolk (Paraskevopoulou, Panayiotou, & Kiosseoglou, 1997).

There are some patents that described methods using supercritical fluids, carbon dioxide alone (Ogasahara, Hariu, & Takahashi, 1991) or in combination with other solvents such as propane (Heidlas, Vollbrecht, & Cully, 1997) or ethanol (Zeidler, Pasin, & King, 1996). This last one also combined edible oils with supercritical fluid extraction. The advantages of this technique are the selective extraction of cholesterol from egg yolk while the phospholipids and proteins are retained. Therefore, nutrient composition is not adversely affected, and the egg product saves most of its functional and sensory properties. Nevertheless, due to its high costs, it is not widely spread in food industry (Miranda et al., 2015).

Supercritical fluid extraction has been also employed to isolate the egg yolk phospholipids in a two steps process: first neutral lipids were removed using neat CO_2 and then phospholipids were extracted using CO_2 and ethanol, a polar co-solvent. This extraction method was improved with a previous enzymatic treatment that destabilized the lipoproteins and then increased accessibility of phospholipids to polar co-solvent, leading to shorter extraction time (Navidghasemizad, Temelli, & Wu, 2014).

Fractionation by centrifugation

When viewed under the light microscope, yolk appears as a suspension of several small drops, called granules, dispersed in a clear yellow fluid, called plasma, which contains the LDL micelles and the water-soluble livetins (Paraskevopoulou & Kiosseoglou, 1995b). Since the 1970s, it is well known that egg yolk can be easily separated into two fractions by centrifugation. The major fraction is plasma (supernatant), whereas granules (pellet) represent the smallest part of the yolk dry matter. Egg yolk granules are mainly composed of 70% HDLs, 16% phosvitin and 12% LDL. This fraction is high in protein content, low in lipids with approximately 25% of the total cholesterol found in egg yolk (Laca, Paredes, Rendueles, & Díaz, 2014). As a result, this fractionation method has been employed to produce low-cholesterol egg-derived products such as mayonnaise (Laca, Sáenz, Paredes, & Díaz, 2010; Motta-Romero, Zhang, Tien Nguyen, Schlegel, & Zhang, 2017), muffins (Marcet, Paredes, & Díaz, 2015), snacks (Valverde et al., 2016) or desserts (García et al., 2015).

The common steps in the fractionation methods are an egg yolk dilution and a centrifugation. Table 3 shows the cholesterol reduction percentages from egg yolk fractionation studies where cholesterol was measured. The Table also shows the main parameters defined in the process: dilution ratio, dilution solvent, middle step (between dilution and centrifugation) and centrifugation parameters. The percentage of cholesterol reduced was calculated as follow:

$$Cholesterol\ reduced = \frac{Cholesterol_{yolk} - Cholesterol_{granule}}{Cholesterol_{yolk}}\ x\ 100$$

Table 3 reflects the variability between methods. The highest cholesterol reduction was obtained by methods that obtained granules with around 20 % of the total cholesterol found in egg yolk while the simplified method that employed less time and lower centrifugation force got granules with approximately 70 % of cholesterol.

In the early 80s, a fractionation process combining centrifugation with organic solvents such as ethanol, isopropanol (IPA), hexane-ethanol or hexane-IPA was developed. The ratio

was 1:1:1 (egg yolk, organic solvent and water). After centrifugation, three fractions were obtained: lipid layer, middle aqueous layer and protein precipitate. The highest yield was obtained with hexane-IPA 77:23 (w/w). The top layer contained 80 to 95% lipids. However, the mayonnaise tests showed that none of the fractions, alone or in combination were as stable as emulsions made with native yolk (Larsen & Froning, 1981). As mentioned above, organic solvents result in a significant impairing of the emulsifying properties.

Another approach failure was obtained when spray-dried egg yolk was fractionated. Only minor reduction in cholesterol and lipid was observed. However, with non spray-dried egg yolk granules, 31.7 % cholesterol was reduced. Good emulsion and foam stability values were detected and it was suggested as stabilizers of dispersion systems (Dyer-Hurdon & Nnanna, 1993).

There are two main dilution solvents: water and NaCl solutions. Recently, a study compared both solvents and the cholesterol content did not differ significantly between the granules obtained. The increase in the ionic strength with the salt supposed lower granule yield, because it increased solubility of granules, and it is linked with better emulsion stability. It was due to the disruption of the electrostatic repulsion forces between proteins (Motta-Romero et al., 2017). As a result, instead of native granules, it is more effective to use disrupted granules with NaCl to form and stabilise oil-in-water emulsions.

Egg yolk and granules have shown similar emulsifying activities, but since granules have demonstrated better emulsion stabilization properties and less lipid and cholesterol content, they are a promising alternative as emulsifier for food applications (Motta-Romero et al., 2017). Moreover, granules are thermal resistance and can be heated to pasteurization temperature without losing their emulsifying properties (Anton, Le Denmat, & Gandemer,

2000). Nevertheless, granules generated an heterogeneous gel during the gelation process and show partial thermoreversible properties (Laca et al., 2014).

Two modified fractionation methods were developed to scale-up the process. First method was a continuous centrifugal separation of liquid egg yolk into plasma and granules (40,000 g and 100 mL/min) where cholesterol was reduced 68 % (Naderi, House, & Pouliot, 2016). Recently, it was tested a simplified method with lower speed (8000 g) and shorter time (10 min) obtaining 30 % cholesterol reduction in a batch centrifugal separation (Motta-Romero et al., 2017). When it was compared laboratory and pilot-scale centrifuge methods, good agreement was achieved between them in terms of chemical composition and recovery of egg yolk components (Naderi et al., 2016). The simplified method obtained granule yields similar to those achieved by applying higher centrifuge forces with longer times, however the percentage of cholesterol reduced is the lowest in Table 3.

Cholesterol chelates or adsorbents

There are molecules that are able to establish hydrophobic interactions with cholesterol and isolate it from its surroundings. They are β-cyclodextrin (β-CD), polysorbate 80 or polysaccharides such as chitosan, gum arabic or high methoxyl (HM) pectines (Figure 3). They are called adsorbents. Studies with direct addition of cholesterol chelates or adsorbents have been developed to remove cholesterol from egg yolk. Table 4 shown results for these adsorbents. Nearly all of them started the isolation with liquid egg yolk.

β-CD is an authorised food additive (E-459). It is a cyclic oligosaccharide consisting of seven α-1,4-linked D-glucopyranosyl units (Mortensen et al., 2016). Due to the slightly hydrophobic inner surface of its ring-shaped, it is able to form stable inclusion complex with cholesterol. When the number of glucopyranose units increased, six for α-CD and eight for γ-CD, there is practically no interaction as a result of inadequate steric fit into the cavity. β-CD and its derivatives form complex with cholesterol. While cholesterol is chelated with β-CD, the

complexes with β-CD derivatives are soluble in water. The chelated cholesterol effect of β-CD has been utilized in the production of various food products with reduced cholesterol content, such as dairy products or eggs (Fenyvesi, Vikmon, & Szente, 2016). In fact, the most prevalent use of ciclodextrins in food industry is as complexing agents for the elimination of cholesterol from animal foods (Iacovino et al., 2017).

The chelating agent is added to the egg yolk and stirred for the reaction time and temperature as reported in Table 4. Then the mixture is centrifuged, filtered or decanted (when it is an adsorbent) to separate the isolating agent linked to cholesterol from egg yolk. The egg yolk is normally diluted in aqueous solution because it reduces the viscosity of the reaction system and facilitates subsequent contact and interaction between lipoprotein complex and isolating agent (Hsieh, Snyder, & Ford, 1994). In addition, working in aqueous solution has the advantage that physical, chemical, and organoleptic characteristics of the final product are not altered (Garcia Rojas, Dos Reis Coimbra, & Minim, 2006).

It was detected that low cholesterol yolk obtained by treatment with β-CD contained less lipid and protein and more carbohydrate and ash than the original egg yolk. Both free and esterified cholesterol in cholesterol-reduced yolk were reduced by above 90%. Triglycerides became the major lipid class in the product, which contained more oleic acid and less linoleic acid than the control. The low cholesterol yolk was less yellow than the original egg because β-CD also removed a considerable fraction of carotenoids (18,3 %). The increased carbohydrate content was due to residual β-CD in the product (Awad, Bennink, & Smith, 1997). The cholesterol reduced egg-derived products have been used to make foods such as egg-derived sticks (Lamas et al., 2016) or mayonnaise where no adverse effect on rheological properties was detected (Yüceer, İlyasoğlu, & Özçelik, 2016).

Some studies developed a method to recover and recycle the complexing agent (Chiu, Chung, Giridhar, & Wu, 2004; Jeong, Sun, Chogsom, & Kwak, 2014; Jung, Park, & Kwak, 2005; Su et al., 2015). It was tried with crosslinked β-CD, a product of a crosslinking reaction between C-2 position of β-CD and one of the carboxyl groups of the crosslinking agent. Nevertheless, they detected that cholesterol removal ability was reduced when using whole egg. This suggested that other white egg components, such as proteins, were binding the complexing agent. Then, the crosslinked β-CD could not be applied into whole egg with an effective recycling (Jeong et al., 2014).

A promising support for β-CD was developed and tested with milk: colloidal magnetic mesoporous silica (MMS) (Sinha, Basiruddin, Chakraborty, & Jana, 2015). Another approach studied allowed reusing and reducing residues of β-CD in the final product by immobilizing the complexing agent in agarose (Lamas et al., 2016) or chitosan (Chiu et al., 2004). Agarose results were not as good as β-CD added directly. This lower cholesterol reduction with immobilized β-CD might be because the position of some molecules in the support prevented them from being correctly oriented to chelate cholesterol. On the other side, when β-CD were immobilized in chitosan beads the amount of adsorbent needed was decreased. They explained that it might be due to the chelation of more cholesterol molecules in the gap between chitosan and β-CD thereby causing an increased cholesterol adsorption (Chiu et al., 2004). But it has been demonstrated that chitosan is also a cholesterol adsorbent itself.

Chitosan is a linear polysaccharide consisting of (1,4)-linked, 2-amino- deoxy-β-D-glucan units derived from the alkaline deacetylation of chitin, which cannot be digested by human digestive enzymes. Since it is recognized as being able to bind neutral lipids and have lipid-lowering effects, it was tested as a chelating agent to reduce cholesterol content in egg. Unlike other methods, the cholesterol remained in the final product after using chitosan. With the aim to confirm that the complex chitosan-cholesterol was not affected during human digestion, in vitro simulation of human gastrointestinal conditions was performed. It confirmed that the complex remained stable. Then this complexing agent has multiple advantages. Because

even that the amount of cholesterol removed was lower than other techniques, it is enough to be labelled as egg with reduced cholesterol content. It is an easy technique to scale-up, as it only requires stirring at room temperature for 15 min and the structure of the egg-derived yolk is unchanged. In addition, there is not limit for chitosan addition as there is not maximum intake limit. Indeed, there is a 3 g/day recommendation (Lamas et al., 2016). While for β-CD there is a maximum recommended amount in food of 5 mg/kg per day (Iacovino et al., 2017).

Other polysaccharides tested for cholesterol removal in egg yolk were gum arabic (Garcia Rojas, dos Reis Coimbra, Minim, & Freitas, 2007) and high methoxyl (HM) pectins (Garcia Rojas et al., 2007). However, both techniques reduce the amount of proteins in the low-cholesterol egg-derived more than β-CD.

An alternative water-soluble food additive was employed to remove cholesterol in egg yolk. It was polyoxyethylene (20) sorbitan monooleate (polysorbate 80), an emulsifying food additive (E-333). It was tested in an ethanol/water (20/80) mixture containing 1.5 % (w/v). Dried egg yolk was mixed with the aqueous solution (1:10) and homogenized. Likely this additive acted in a way similar to sodium dodecyl sulphate, an anionic surfactant which breaks hydrophobic bonds between protein molecules that contributed to the stabilization of LDL micelle and granule lipoprotein structures and aids in the release of yolk lipids. The homogenized suspension was centrifuged, and the yolk precipitate was washed with ethanol/water (20/80) and recentrifuged. This yolk precipitate contained around 60 % less cholesterol than the initial dried egg and around 50 % less total lipids. While the lipid phosphorus content was not lowered to the same extent, around 37 %. The yolk protein concentrate gave mayonnaise-like emulsions of higher shearing stress - rate of shear values when compared to mayonnaises prepared with the dried yolks. Foam liquid stability was similar to the dried yolks but foaming activity differed. Spray-dried yolk decholesterolized gave an

emulsion which showed the same stability as the emulsion prepared with the respective yolk. This was attributed to the existence of egg white proteins in the latter (Paraskevopoulou & Kiosseoglou, 1995b). Other food decholesterolized with an emulsifying agent was butteroil with quillaja saponin (Sundfeld, Yun, Krochta, & Richardson, 1993).

Another cholesterol adsorption was tested with a resin (Streamline Phenyl®) and egg yolk plasma. The adsorbent was added to egg yolk plasma diluted in phosphate buffer (Garcia Rojas et al., 2006). Cholesterol was reduced in 70 % but tests were not done with the decholesterolized product and its final components were not described. Recently, it was tested pure cholesterol with another adsorbent (Silicalite-1). It was obtained a higher maximum adsorption: 70 mg of cholesterol /g adsorbent versus 12.65 mg/g in the previous study. However, when egg lecithin was also in the solution, they detected that cholesterol adsorption decreased due to preferential adsorption. Cholesterol was adsorbed only on the remaining free outer surface of the adsorbent (Atyaksheva, Ivanova, Ivanova, Tarasevich, & Fedosov, 2017).

Cholesterol biotransformation

Another method studied to reduce the amount of cholesterol in eggs is its conversion into an oxidised product (cholest-4-en-3-one) by a cholesterol oxidase enzyme. Figure 4 shows the reaction. The enzyme is obtained from bacteria. First studies were done with *Rhodococcus equi* Nº 23 (Aihara, Watanabe, & Nakamura, 1988). However, the direct addition of a strain plus the incubation temperature made the eggs highly sensitive to microbial spoilage. In addition, the egg-yolk product was altered considerably because *Rhodococcus equi* belongs to risk group 2, being then an organism that can cause disease in humans or animals (Lamas et al., 2016).

Following studies employed the isolated enzyme instead of the bacterium. In table 5 are shown the results where the cholesterol biotransformation was studied to reduce cholesterol content in egg. Each study employed different enzyme sources, enzyme activities and incubation conditions. There was a study where the employed cholesterol oxidases were from different bacteria such as *Pseudomonas fluorescens*, *Nocardia erythropolis*, *Streptomyces* species and *Brevibacterium* species (Christodoulou, Hung, Trewhelv, & Blackz, 1994). The enzymes from the three first bacteria reduced more than 50 % of egg cholesterol within 24 h. The cholesterol oxidase from *Pseudomonas fluorescens* had the advantages of being effective over a wide temperature range and it was required in low concentrations to achieve significant reduction in yolk cholesterol.

Later studies used a mutant of *Brevibacterium* to produce the enzyme (Lv et al., 2002; Sun, Yang, Zhong, Zhang, & Wang, 2011). This enzyme had extracellular and intracellular cholesterol oxidase activities. This mutant produced a cholesterol oxidase that degraded more cholesterol than the *Brevibacterium* species previously mentioned. Incubation time was signed as a positive factor to raise cholesterol degradation. However, it was reduced because after 15 h only limited increased in degradation was detected while the risk of bacterial invasion was there. It was observed that this enzyme activity and the yolk cholesterol bioconversion had a broad temperature. Cholesterol oxidase activity remained relatively high at 20 ºC but cholesterol conversion became low. While enzyme lost activity at 50 ºC and cholesterol conversion remained high. It was explained to the better solubility of granule at a higher temperature, thus affecting contact between yolk cholesterol and enzyme (Lv et al., 2002). Higher amount of cholesterol was removed (91.7 %) when ultrasonic-assisted extraction was performed. Because it helped the yolk granules solubilisation, facilitated the enzyme action and allowed to reduce units of enzyme added, incubation temperature and time (Sun et al., 2011). It was also studied the proteins and phospholipids composition in treated egg yolk and there were not significant differences due to the high enzyme specificity (Sun et al., 2011). Other benefits of this biotransformation are that no other substances are added except for enzymes and that the

remaining cholestenone is an effective antiobesity medicine in the product, that may raise its commercial value (Lv et al., 2002). Nevertheless, the enzyme has to be subject to safety evaluation by the EFSA and subsequently approved by the European Commission (European Parliament and Council, 2008).

A study was recently developed using a *Rhodococcus* strain isolated from wastewater, which has not been previously used to reduce cholesterol in egg yolk. As it was not from mutant strains, it had lower ability to produce cholesterol oxidase. Only intracellular enzyme was employed and it resulted in low enzymatic activity (0.13 U/ml) (Lamas et al., 2016). The cholesterol reduction (26.8%) was not as good as preceding studies.

Conclusions

All methods described employed mild conditions. However, since cholesterol is presented combined with high weight molecules with functional properties in egg yolk, it is difficult to isolate it alone and maintain appearance and functional properties. Supercritical fluids extraction has shown the highest selectivity, but it is too expensive for industrial application. Organic solvents extraction and cholesterol biotransformation could be economically viable; nevertheless, more research is needed to comply with food safety regulation while substantial amounts of cholesterol are removed. Nowadays, the fractionation by centrifugation and cholesterol chelates are the only feasible methods with industrial applications, obtaining up to 82% and 99% of cholesterol reduction, respectively. Cholesterol chelates technique has the highest cholesterol reduction but added compounds should be removed. Fractionation method can be considered the best because no substances are added to product.

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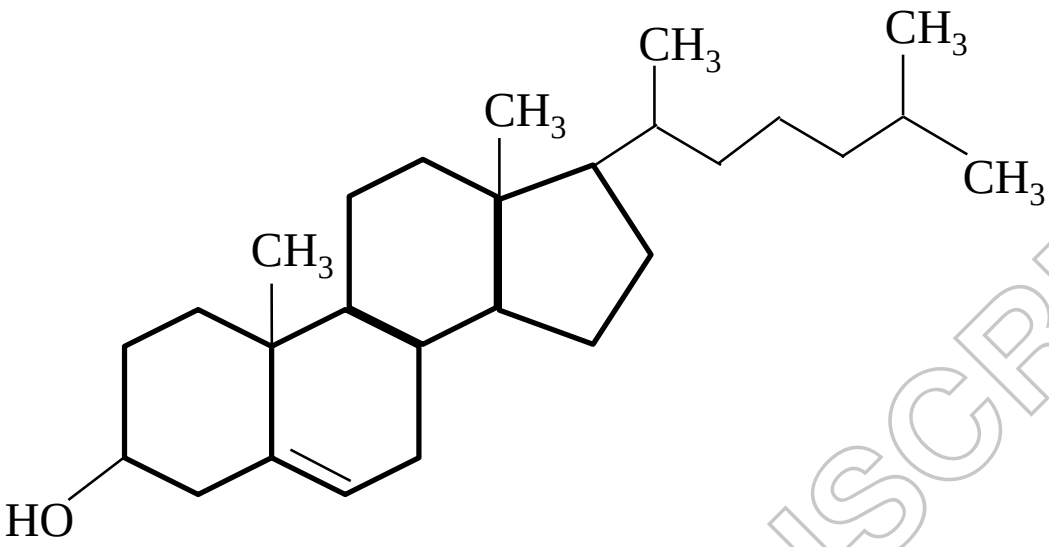

Figure 1. Cholesterol molecule

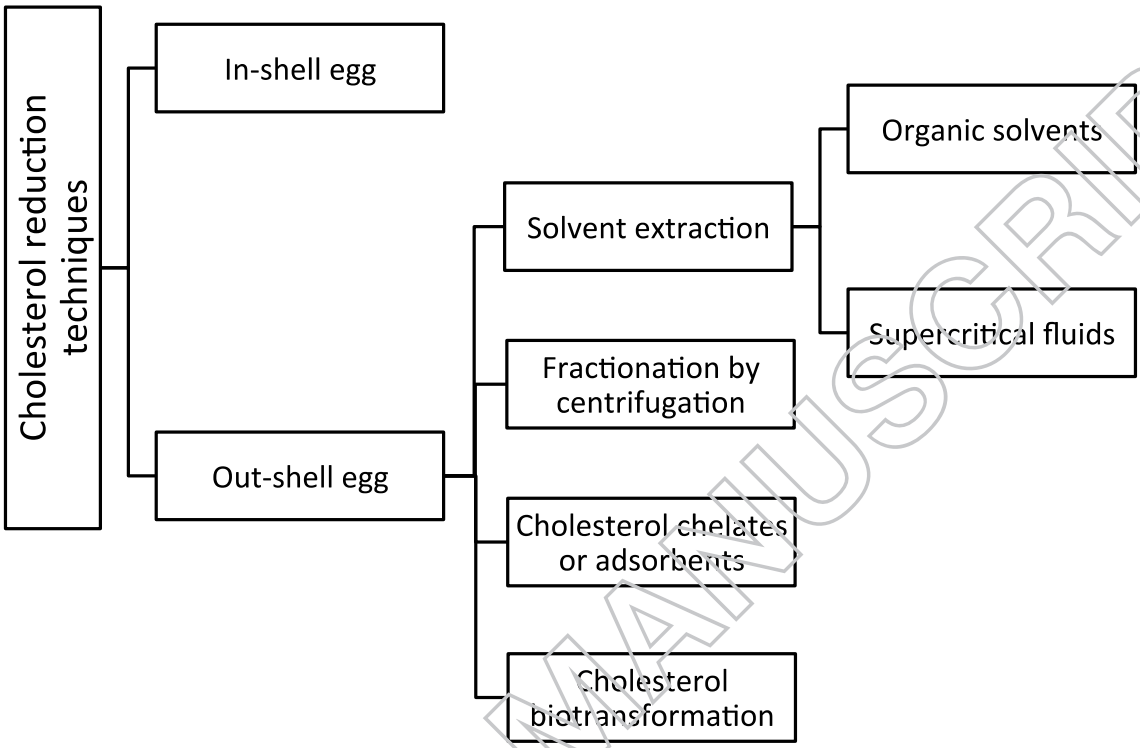

Figure 2. Classification of techniques to reduce cholesterol content in egg yolk

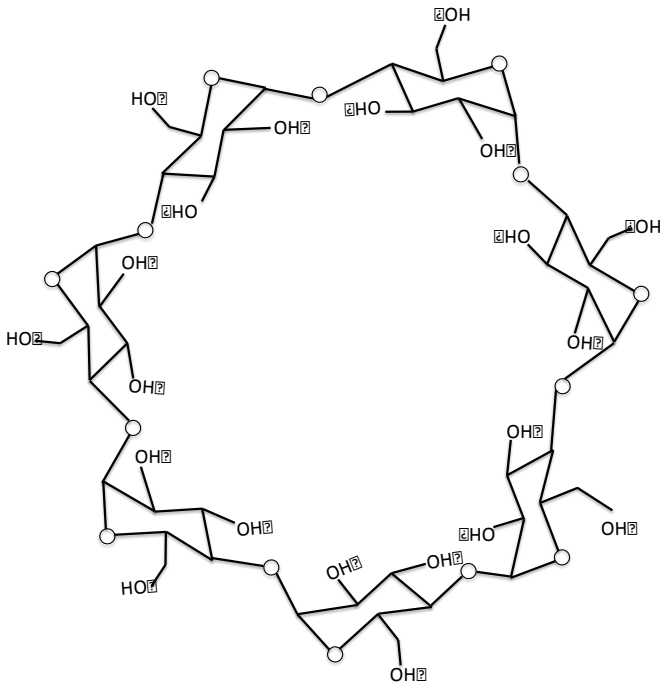

Figure 3. Chemical structure of β-CD

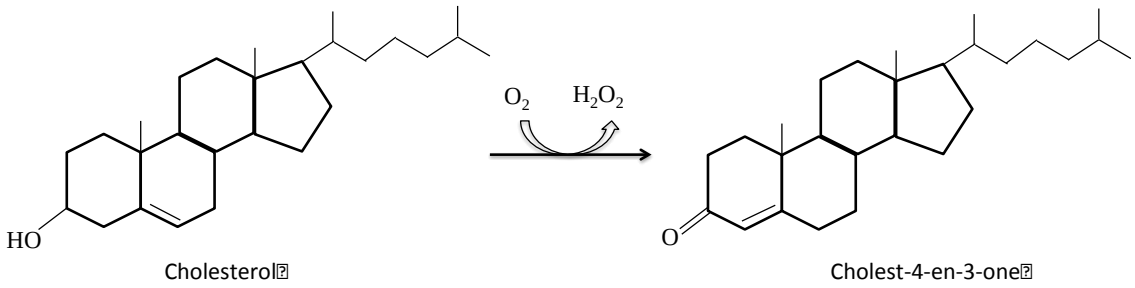

Figure 4. Cholesterol oxidase reaction

Table 1. Egg cholesterol reduction results with organic solvent extraction methods.

Cholesterol removed	**Organic solvent**	**Dilution ratio (OS:yolk)**	**Mixing**	**Solvent evaporation**	**Mixing time**	**Reference**
			Temperature			
>94.7%	Petroleum ether-ethanol (35:65)	10:1 (v:w)	NS	55 ºC	2.5 h	(Paraskevopoulou & Kiosseoglou, 1994)
91 %	Acetone	3.5:1 (w:w)	25 ºC	60 ºC	15 min	(Martucci & Borges, 1997)
81 %	Acetone	12:1 (w:w)	25 ºC	60 ºC	2 min	(Borges, 1997)
74.7 %	Chroloform-methanol (2:1)	3:1 (v:w)	23 ºC*	RT	30 min	(Warren et al., 1988)
73 %	Hexane-isopropanol (2:1)	3:1 (v:w)	23 ºC*	RT	30 min	(Warren et al., 1988)
70 %	Petroleum ether	10:1 (v:w)	NS	55 ºC	2.5 h	(Paraskevopoulou & Kiosseoglou, 1994)
66.3 %	Hexane	3:1 (v:w)	23 ºC*	RT	30 min	(Warren et al., 1988)
99.8 % (after 3 extractions)	Safflower oil	9:1 (v:w)**	55 ºC	NP	5 min	(Fioreti et al., 1973)

87-91 %	Sunflower oil + 8 % monoglyceride**	2:1 (w:w)	57-60 ºC	NP	30 min	(Conte, Jr. et al., 1992)
84.6 %	Soybean oil - water (3:0.4)**	1:1 (w:w)	49-54 ºC	NP	90 min	(Kijowski & Lombardo, 2000)
81.4 %	Soybean oil – water – salt - vinegar (1.67:0.37:0.29:0.037)**	1:1 (w:w)	48-65 ºC	NP	18 min	(Lombardo & Kijowski, 1994)

OS: organic solvent. RT: room temperature. NS: not specified. NP: not performed.

*It was also tested 55 ºC and determined that temperature did not affect the extraction of components.

**Results described in the best conditions.

Table 2. Egg cholesterol reduction results with supercritical fluid extraction methods.

Cholesterol removed	**Supercritical fluid**	**Temperature and pressure conditions**	**Amount employed (g SCF/ g of sample)**	**Target**	**Reference**
90 %	CO_2	NS	NS	Dried egg yolk	(Bringe et al., 1996)
78 %	CO_2	310 atm/35 ºC	50 g CO_2/g egg yolk	Spray-dried egg yolk	(Paraskevopoulou et al., 1997)
70-75 %	CO_2	193.6 atm/ 35 ºC	NS	Liquid egg yolk	(Ogasahara et al., 1991)
66 %	CO_2	306 atm/ 45 °C	45 ± 1 g CO_2/ g egg yolk	Spray dried egg yolk	(Froning et al., 1990)

SCF: Supercritical fluid. NS: not specified.

Table 3. Egg cholesterol reduction results with fractionation by centrifugation methods.

Cholesterol reduced	**Dilution ratio**	**Dilution solvent**	**Middle step**	**Centrifugation conditions**	**Reference**
82 %	1:1	NaCl 0.17 M	Mix for 1 h	10 ºC/ 10000 g/ 45 min	(Jin, Huang, Ding, Ma, & Ch, 2013)
50 %	1:1	NaCl 0.16 M		10 ºC/ 10000 g/45 min	(Marc Anton & Gandemer, 1997)
31.7 %[1]	1:1	NaCl 0.16 M	Mix for 5 min and dialyzed at 4 ºC for 12 h	10 ºC/ 13000 g/ 1 h	(Dyer-Hurdon & Nnanna, 1993)
30 %[2]	1:1	NaCl 0.17 M or water	Settle 30 min at RT	<10 ºC/ 8000 g/ 10 min	(Motta-Romero et al., 2017)
76.9 %	1:1,5	deionized water	pH 7 adjustment + settle overnight 4 ºC	4 ºC/ 10000 g/ 45 min	(A. Laca, Paredes, & Díaz, 2010)
69.6 %	1:1,5	deionized water	pH 7 adjustment + settle overnight 4 ºC	4 ºC/ 6000 rpm/ 45 min	(Lamas et al., 2016)
68 %	1:1	Milli-Q water	Cold mix	maximun 40000 g/ continously	(Naderi et al., 2016)
51.1 %[3]	1:1:1	hexane-IPA:water	pH 7 adjustment + settle 1h RT	10-15 ºC/ 6500 g/15 min	(Larsen & Froning, 1981)

RT: room temperature

[1] Results from nonspray-dried egg yolk.

[2] Average from the three isolations methods, because there was not significance difference in cholesterol content.

[3] Calculated from the 3 fractions obtained.

Table 4. Egg cholesterol reduction results with methods employing cholesterol chelates or adsorbents.

Cholesterol reduced	**Isolating agent**	**Temperature**	**Reaction time**	**Reference**
99 %	β-CD [1]	50 ºC	30 min	(Su et al., 2015)
95.4 %	β-CD	50 ºC	6.5 min	(Awad et al., 1997)
92.7 %	β-CD [2]	50 ºC	45 min	(Mine & Bergougnoux, 1998)
89.2 %	β-CD	50 ºC	6.5 min	(Smith, Awad, Bennink, & Gill, 1995)
87.5 %	β-CD	RT	30 min	(Lamas et al., 2016)
83 %	β-CD	35 ºC	15 min	(Yüceer et al., 2016)
37.7 %	β-CD immobilizated in agarose beads	4 ºC	24 h	(Lamas et al., 2016)
28.9 %	β-CD agarose film	4 ºC	24 h	(Lamas et al., 2016)
95 %	crosslinked β-CD	40 ºC	30 min	(Jung et al., 2005)
92.8 %	crosslinked β-CD [3]	40 ºC	30 min	(Jeong et al., 2014)
92 %	cross-link β-CD to chitosan beads	25 ºC	50 min	(Chiu et al., 2004)
94 %	Gum arabic	21 ºC	10 min	(Hsieh et al., 1994)
85.7 %	HM pectines	4 ºC	30 min	(Garcia Rojas et al., 2007)
49.8 %	Chitosan	RT	15 min	(Lamas et al., 2016)
~60 %	Polysorbate 80[4]	NS	10 min	(Paraskevopoulou & Kiosseoglou, 1995a)

70 %	Streamline Phenyl® resin [5]	25 ºC	120 min	(Garcia Rojas et al., 2006)

RT: room temperature. NS: not specified.

[1] Chelating reaction performed after ethanol extraction.

The starting material was: [2]LDL from egg yolk, [3]whole egg,[4] dried egg yolk, [5] egg yolk plasma.

Table 5. Egg cholesterol reduction results with cholesterol biotransformation methods.

Cholesterol removed	**Cholesterol oxidase source**	**Enzyme activity/ egg yolk**	**Incubation time**	**Incubation temperature**	**Reference**
93.4 %	*Pseudomonas fluorescens* and *Streptomyces* sp.	39 U /g	72 h	37 °C	(Christodoulou et al., 1994)
91.7 %	*Brevibacterium* sp. DGCDC-82	0.6 U /g	10 h	37 °C	(Sun et al., 2011)
90 %	NS	NS	24 h	55 °C	(Jones & Losso, 2005)
86.5 %	*Brevibacterium* sp. ODG-007	5.4 U /g	13.75 h	39 °C	(Lv et al., 2002)
64.9 %	*Pseudomonas fluorescens*	39 U /g	48 h	4 °C	(Christodoulou et al., 1994)
60 %	*Rhodococcus equi* Nº 23*	NS	72 h	37 °C	(Aihara et al., 1988)
26.8 %	*Rhodococcus* strain CECT 3014	0.13 U /ml	48 h	30 °C	(Lamas et al., 2016)

NS: not specified.

* Study performed with the bacteria directly added to egg yolk.