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Cholesterol removal in liquid egg yolk using high methoxyl pectins

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Abstract

A process to reduce cholesterol in liquid *in natura* egg yolk using high methoxyl pectins was optimized by response surface methodology aiming to define the maximum level of cholesterol extraction at a minimum decrease of the yolk protein contend. The most important variables influencing the process were dilution level of egg yolk, ionic strength, and pH of yolk suspension, as well as the amount of pectin gel used in the extraction. The fractionary factorial 2^{4-1}_{III} was applied in the central composite design to determine the minimum number of experiments to be conducted. The yolk suspension conditions for the optimized process were 5 g of yolk, 31.23 g of water, ionic strength of 0.39 mol/L and pH equal to 9.2 using 5.78 g of pectin gel containing 3% of pectin. The egg yolk contends of cholesterol and protein decreased to 14.4% and 88.6%, respectively.

Keywords: Cholesterol; High methoxyl pectins; Egg yolk; Response surface methodology

1. Introduction

Cholesterol is present in the egg yolk in quantities varying from 180 to 250 mg, depending on hen genotype (Stadelman & Cotterill, 1995). However, when exposed to the air, at ambient temperature, it becomes susceptible to oxidation, generating over 60 different by-products, the so-called cholesterol oxides (Razzazi-Fazeli, Kleineisen, & Luf, 2000). Toxicological studies have shown that these oxides have mutagenic, cytogenic, and carcinogenic effects, being involved in the initiation and propagation of several diseases, such as cancer, atherosclerosis, and cardiovascular problems (Johnson, 1996; Missler, Wasilchuck, & Merrittt, 1985), inducing consumers to choose healthier food in the market, with lower cholesterol concentration or cholesterol-free. The demand for such products has prompted food and pharmaceutical industries to develop foods with desired characteristics, e.g., egg yolk with reduced content of fat and cholesterol (Smith, Awad, Bennink, & Gill, 1995). Thus, different processes to reduce egg yolk cholesterol were tested, such as extraction with organic solvents (Chung & Ferrier, 1991; Kwan, Helbig, & Nakai, 1991; Martucci & Borges, 1997); supercritical fluid (Froning et al., 1990; Ogasahara, Hariu, & Takahashi, 1992); vegetable oils (Conte, Jhonson, Hsieh, & Ko, 1992); cholesterol bio-transformation by enzyme action cholesterol reductase (Chenfeng et al., 2002; Watanabe et al., 1986); cholesterol complexing using β-cyclodextrins (BCD) (Shukla, 1991; Smith et al., 1995), *Quilaia saponaria* saponins (Sundfeld, Yun, Krochta, & Richardson, 1993) and gum arabic (Hsieh, Snyder, & Ford, 1994).

Gum arabic was used in the extraction of cholesterol based on properties of anionic polysaccharides with low pH values, which may be used as chelating agents forming insoluble complexes (chelating agent–lipoprotein) in water, which are easy to remove. However, a limitation of this process is high loss (over 66%) of proteins that, together with lipids, are responsible for the emulsifying properties of yolk. According to Lewis (1993), polysaccharides are hydrocolloid agents, which confer greater viscosity and

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gelatinous consistency to the foods they are added to, due to the hydrophilic and hydrophobic groups they contain, which favor their emulsifying properties. The major polysaccharides used in the food industry are alginate, xanthate, agar, carrageen, gum arabic, and pectin.

Pectin is a natural polysaccharide present in almost all the terrestrial plants and is responsible for structural properties of fruits and vegetables (Rollers & Jones, 1996). It is commercially extracted from citrus peels and apple pomace under mildly acidic conditions. Its major component is the galacturonic acid, partially esterified with a methoxyl group. Pectin is divided into two main categories, high methoxyl (HM) pectins and low methoxyl (LM) pectins, according to their degree of esterification (DE): any pectin of 50% DE or greater is a HM pectin and under a DE of 50% is a LM pectins (Wong, 1995).

An important functional property of this biopolymer is its ability to gel in aqueous solutions (Yokoi, Obita, Hirose, Hayashi, & Takasaki, 2002). HM pectin gels display a great amount of ester methyl groups ($COOCH_3$), whose hydrophobic interactions support the structure of the gel (Oakenfull & Scott, 1984). Pectin is widely used in the food industry in the production of jelly, fruit juices, confectionary products, and edible biodegradable films (Coffin & Fishman, 1997), separation of proteins (Yang, Chen, & Chang, 1998) and enantiomers (Phinney, Jinadu, & Sander, 1999) and in the reduction of cholesterol levels in human blood (Day & Kuhrts, 1989; Judd & Truswell, 1982).

Thus, this work evaluated the use of HM pectin to reduce egg yolk cholesterol. Response surface methodology was applied to optimize the cholesterol extraction process. Protein loss during extraction was also minimized.

2. Materials and methods

2.1. Material

Fresh eggs were purchased from the local market in Viçosa-MG (Brazil). Egg yolks were manually separated from the albumen and placed on absorbing paper to remove adhering albumen and chalazas. GENU® 104 type – pectin (DE > 69%) was obtained from CP Kelco Brazil S.A. (São Paulo, Brazil). Pectin gel was prepared with 3% aqueous solution in pectin mass. Cholesterol and bovine serum albumin (BSA) standards were purchased from Sigma Chemicals (St. Louis, USA). All chemicals were of analytical grade and the water was deionized using a Milli-Q device (0.22 μm membrane, Millipore Inc., USA).

2.2. Extraction process

Cholesterol extraction involved the following stages: (1) 5 g of yolk (Denver Analytical Balance, M310, USA) were weighed and diluted with saline solution (NaCl) to a pre-determined yolk: saline solution ratio; (2) pH was adjusted to the desired value (Potentiometer DM20, Digimed, Brazil) with KOH 1 mol/L; (3) yolk suspension and pectin gel were mixed to a pre-established yolk suspension:pectin gel ratio and periodically stirred; (4) the mixture was cooled at 4 °C for 30 min, in a water bath (TE-184, Tecnal, Brazil); (5) the slurry was centrifuged at 5000*g* for 10 min, at 4 °C (Ultracentrifuge Sorvall RC-5B, USA); (6) the supernatant was decanted; and (7) the cholesterol content in the supernatant was quantified. The experiments were conducted in 50 mL centrifuge tubes.

2.3. Preliminary studies

Preliminary tests were carried out to evaluate the influence of five variables on cholesterol extraction:yolk:water dilution ratio (X_1), pectin gel amount (X_2), ionic strength of the yolk suspension (X_3), yolk suspension pH (X_4), and extraction time (X_5). The fractionary factorial 2_V^{5-1} of resolution 5 (Box, Hunter, & Hunter, 1978) was used for five independent variables at two levels each, as presented in Table 1. The experimental data were analyzed using the statistical package SAS®version 8.0, Stepwise procedure (SAS Institute Inc., 1999).

2.4. Experimental design

Response surface methodology (RSM) was applied to optimize the extraction process aiming to evaluate simultaneously the influence of four pre-selected variables on the extraction of yolk cholesterol using pectin. These independent variables were: water:yolk dilution ratio (X_1), pectin gel amount (X_2), yolk suspension ionic strength (X_3), and yolk suspension pH (X_4), as shown in Table 2. The depending variables were cholesterol reduction percentage (Y_1) and protein reduction percentage (Y_2). The fractionary factorial 2_{III}^{4-1} of resolution 3 (Box et al., 1978; Neto, Scarminio, & Bruns, 2001), was used to define the central composite design for four independent variables at five levels each, eight star points and four repetitions in the central

Table 1
Variables and respective levels in the preliminary process

Variable	Symbol	Levels	
		−1	+1
Yolk:water dilution (g:g)	X_1	0.17	0.27
Amount of gel (g)	X_2	5.00	7.00
Ionic strength (mol/L)	X_3	0.20	0.50
pH	X_4	7.50	10.50
Extraction time (min)	X_5	20	60

Table 2
Variables and respective levels in central composite design for yolk cholesterol reduction

Variable	Symbol	Codified variable levels				
		−1.68	−1	0	+1	+1.68
Yolk:water dilution (g:g)	X_1	0.13	0.17	0.22	0.27	0.30
Amount of gel (g)	X_2	4.32	5.00	6.00	7.00	7.68
Ionic strength (mol/L)	X_3	0.10	0.20	0.35	0.50	0.60
pH	X_4	6.50	7.50	9.00	10.50	11.50

point (Table 3). The optimum surface region was obtained by applying the maximum inclination path (Box et al., 1978; Myers & Montgomery, 1995). Such planning allowed reducing the number of experiments to 20. The optimum conditions of each variable in the process were obtained by applying the numeric optimization procedure (Myers & Montgomery, 1995) and response surface analysis.

2.5. Cholesterol quantification

The cholesterol present in the previously saponified yolk (Bragagnolo & Rodriguez-Amaya, 2003; Zhang, Li, Liu, Chen, & Rao, 1999) was quantified by high performance liquid chromatography (HPLC). The equipment used was a Shimadzu (LC-10ADVP, Tokio, Japan) consisting of a pump (LC-10ADVP), auto-injector (SIL-10ADVP), and a diode photo-detector (SPD-M10AVP) fixed at 208 nm. Separation was performed at 35 °C, on a reverse phase column, C_{18} Shim-pack CLC-ODS (M)® (250 mm × 4.6 mm, 5 μm of particle diameter and 100 Å diameter pore, Shimadzu, Tokio) preceded by a guard column of the same material (10 mm × 3.2 mm). The injection volume of the sample was of 10 μL, with the mobile phase (isocratic) being composed by a mixture of isopropanol and acetonitrile (1:1) and a flow rate of 1.2 mL/min. The external standard method was used in the analysis, at a cholesterol concentration range from 0.02 to 1.0 mg/mL. The experimental data were correlated by linear regression analysis.

2.6. Protein quantification

Protein content was determined by the Bradford method (1976), with 5 mL of Bradford reagent being added to 0.1 mL of diluted yolk. The Bradford reagent forms a blue color complex when added to the proteins. After 2 min of incubation, the absorbance was recorded at 595 nm. The analytical curve was prepared with BSA in an aqueous solution, containing 0.15 M of NaCl, and at a concentration range from 10 to 100 μg of BSA/0.1 mL of solution. The Bradford reagent was prepared with 200 mg of Cromassie Brilliant blue G-250 dissolved in 100 mL of 95% ethanol and 200 mL of 85% phosphoric acid.

Table 3
Experimental data for the optimization process

Run	Variables				Response*	
	X_1	X_2	X_3	X_4	Y_1	Y_2
1	−1	−1	−1	+1	75.61	24.49
2	+1	−1	−1	−1	83.45	75.44
3	−1	+1	−1	−1	87.96	71.94
4	+1	+1	−1	+1	96.36	72.22
5	−1	−1	+1	+1	77.20	26.61
6	+1	−1	+1	−1	75.23	42.13
7	−1	+1	+1	−1	81.46	28.23
8	+1	+1	+1	+1	74.46	33.80
9	0	0	0	0	73.09	53.06
10	0	0	0	0	72.73	55.69
11	0	0	0	0	72.11	54.47
12	0	0	0	0	74.43	52.17
13	−1.68	0	0	0	82.31	15.37
14	1.68	0	0	0	59.19	64.42
15	0	−1.68	0	0	76.96	47.18
16	0	1.68	0	0	75.18	67.27
17	0	0	−1.68	0	90.17	77.73
18	0	0	1.68	0	77.44	33.70
19	0	0	0	−1.68	86.07	60.52
20	0	0	0	1.68	82.89	37.57

* Mean value.

2.7. Statistical analysis

A software package (SAS Institute Inc., 1999) was used for experimental data analysis. The coefficient of determination R^2, the analysis of variance (ANOVA) result, and the level of statistical significance by the Fisher (F) test were used to evaluate the reliability of the polynomial model equation obtained. The level of significance of the regression coefficients was obtained by the Student's t test.

3. Results and discussion

3.1. Preliminary study

The preliminary tests for yolk cholesterol reduction defined the variables that most affected cholesterol extraction, i.e., X_1, X_2, X_3, X_4. The variable X_5 was not considered due to the lack of significance in the F test (>0.1). Extraction time has likely little influence on the stabilization of the polymer hydrophobic interactions (Oakenfull & Scott, 1984). A similar behavior was reported by Smith et al. (1995), after evaluating the impact of extraction time on yolk cholesterol reduction using β-cyclodextrin. Extraction time was fixed in 30 min in the posterior experiments.

3.2. Cholesterol reduction

The result obtained from the analysis of variance of the regression of the variable cholesterol reduction response shown in Table 4, indicates that the second order model is significant, with the F value equal to 19.71 and a low probability value [$(P_{\text{model}} > F) = 0.0001$]. The good fit of the model to the experimental data can also be verified by the average absolute deviation

Table 4
Analysis of Variance of the variable cholesterol reduction response (quadratic model)

Source of variation	Quadratic sum	Degrees of freedom	Quadratic mean	F value	Pr > F
Model	2275.66	14	162.50	19.71	<0.0001
Residue (error)	173.19	21	8.24		
Lack of adjustment	2.75	2	1.37	0.15	0.8588
Pure error	170.44	19	8.97		
Total	2448.85	35			

(AAD) under 2.5% (Eq. (1)) and the behavior of residual analysis with $R^2 = 0.93$. The low value of the coefficient of variation (CV = 3.62%) also indicated a greater experiment reliability and precision (Box et al., 1978).

$$\mathrm{AAD} = \left[\sum_{i=1}^{m}\left(\frac{|\delta_{\exp,i} - \delta_{\mathrm{cal},i}|}{\delta_{\exp,i}}\right)\right] \times \frac{100}{m} \quad (1)$$

where $\delta_{\exp,i}$ and $\delta_{\mathrm{cal},i}$ are the experimental and calculated values of cholesterol reduction, respectively, and m is the number of experimental points.

Table 5 shows the estimates of each parameter of the second order model, as well as their values of the Student's t test and the probability (Pr). According to Box et al. (1978), high t and low Pr values lead to higher significance of the studied parameters.

The highly significant variables ($Pr < 0.0001$) in the cholesterol reduction process were yolk:water dilution and suspension ionic strength. Thus, a greater cholesterol reduction will occur with decreased ionic strength and with increased yolk dilution (Fig. 1). A greater dilution of yolk in water decreases viscosity and favors the formation of the pectin–cholesterol complexes, leading to a greater cholesterol reduction. Hsieh et al. (1994) and Smith et al. (1995) reported a similar behavior using, respectively, β-cyclodextrin and gum arabic to extract cholesterol. However, higher dilution values may decrease yield leading to economic losses.

Increased ionic strength in yolk suspension leads to an exposure of the hydrophobic amino acids of protein (Janson & Rydén, 1998), favoring the hydrophobic interaction of the yolk proteins with pectin methyl ester groups, likely decreasing the formation of the pectin–cholesterol complexes. However, low ionic strengths favor the solubility of these proteins (Causaret, Matringe, & Loriet, 1991).

Fig. 2 shows a greater cholesterol reduction with increased pectin gel and lower ionic strength. Since HM pectin gel is characterized by the presence of methyl ester hydrophobic groups (Wong, 1995), the increased number of these hydrophobic groups will favor the formation of

Table 5
Parameters estimated for the second order model

Parameter	Estimate	t Value	$Pr > \|t\|$
Intercept	72.945	56.37	<0.0001
X_1	−6.878	−8.05	<0.0001
X_2	−0.529	−0.62	0.5420
X_3	−4.136	−7.52	<0.0001
X_4	−0.948	−1.11	0.2799
$X_1\ X_1$	−0.737	−1.24	0.2272
$X_2\ X_1$	0.392	0.35	0.7292
$X_2\ X_2$	1.151	1.94	0.0657
$X_3\ X_1$	−3.150	−4.39	0.0003
$X_3\ X_2$	−2.720	−379	0.0011
$X_3\ X_3$	3.891	6.56	<0.0001
$X_4\ X_1$	4.121	3.69	0.0014
$X_4\ X_2$	7.784	6.97	<0.0001
$X_4\ X_3$	−0.702	−0.98	0.3390
$X_4\ X_4$	4.129	6.97	<0.0001

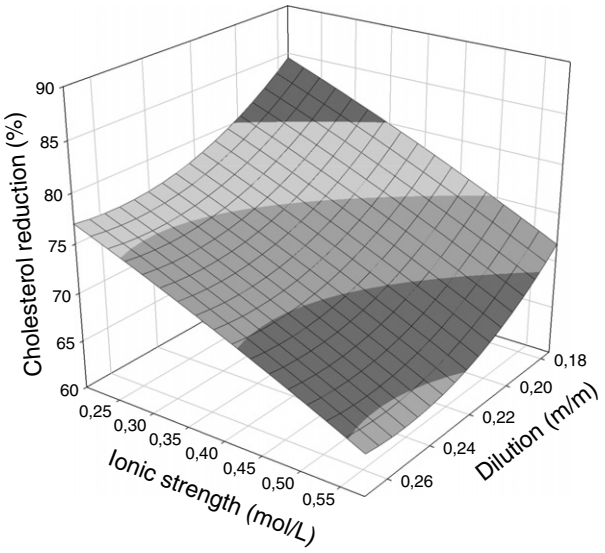

Fig. 1. Influence of dilution level and ionic strength on cholesterol reduction.

the pectin–cholesterol complex. Although pectin may be utilized as a substitute of fats in the food industry, as in the case of mayonnaise, with substitution of up to 1.2% of the total content of the product, the physical–chemical characteristics of the yolk must not be altered (Rollers & Jones, 1996).

A decrease in the pH value and increase in dilution favor cholesterol reduction, as shown in Fig. 3. Decreased pH results in apparent increase of the hydrophobic interactions (Janson & Rydén, 1998; Pharmacia Biotech, 1993). However, with lower pH values (5.0–7.0), pectin behaves as an anionic polysaccharide that produces complexes insoluble in water with other molecules positively charged, such as

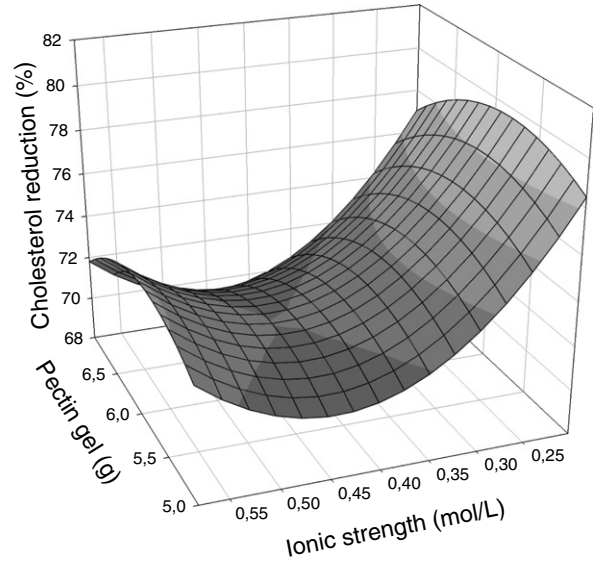

Fig. 2. Influence of the amount of pectin gel and ionic strength on cholesterol reduction.

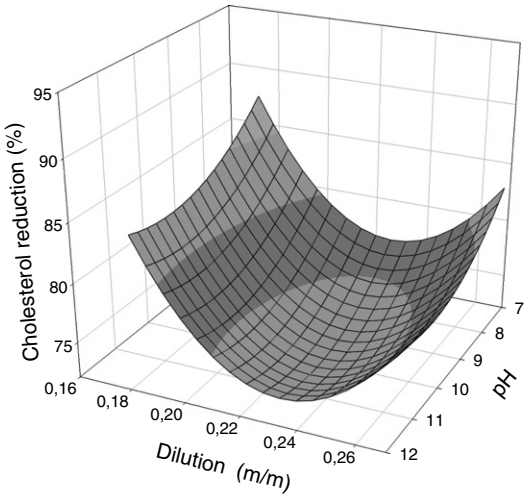

Fig. 3. Influence of the dilution level and pH ratios on cholesterol reduction.

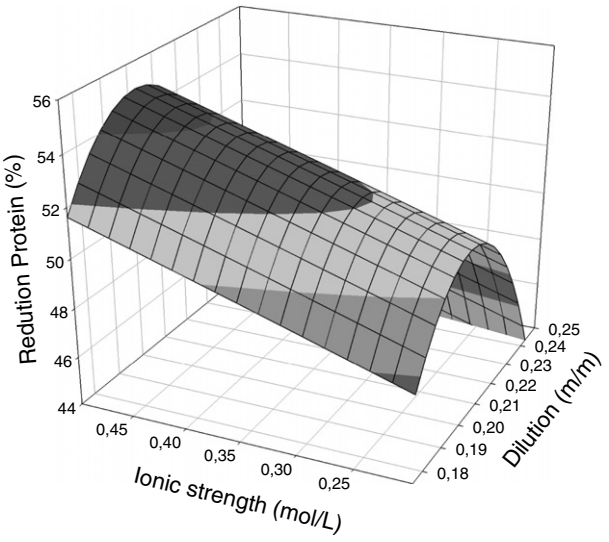

Fig. 4. Influence of the dilution and ionic strength ratio on protein reduction.

metals, proteins, and lipoproteins (Hsieh et al., 1994; Lewis, 1993). Such behavior does not favor the process studied.

3.3. Protein reduction

The results of the analysis of variance of the regression of the variable yolk protein reduction response are listed on Table 6, showing that the second order model is highly significant, with an F value equal to 43.77, and a low probability value $[(P_{\text{model}} > F) = 0.0001]$. The good fit of the model to the experimental data can be evaluated by the AAD, under 7.5%, and the behavior of residual analysis with $R^2 = 0.97$. The coefficient of variation was 9.45%.

Table 6
Analysis of variance of the variable yolk protein reduction response (quadratic model)

Source of variation	Quadratic sum	Degrees of freedom	Quadratic average	F value	Pr > F
Model	13278	14	948.42	43.77	<0.0001
Residue (error)	455.008	21	21.66		
Lack of fit	28.032	2	14.01	0.62	0.54
Pure error	426.97	19	22.47		
Total	13733.08	35			

Table 7
Parameter estimates of the second order model

Parameter	Estimate	t Value	Pr > $\lvert t \rvert$
Intercept	54.836	26.15	<0.0001
X_1	14.597	10.54	<0.0001
X_2	5.979	4.32	0.0003
X_3	−13.733	−15.41	<0.0001
X_4	−6.830	−4.93	<0.0001
X_1 X_1	−5.592	−5.82	<0.0001
X_2 X_1	−0.748	−0.41	0.6836
X_2 X_2	0.549	0.57	0.5736
X_3 X_1	−3.768	−3.24	0.0039
X_3 X_2	−6.369	−5.47	<0.0001
X_3 X_3	0.005	0.01	0.9956
X_4 X_1	−1.289	−0.71	0.4838
X_4 X_2	−5.556	−3.07	0.0058
X_4 X_3	5.091	4.37	0.0003
X_4 X_4	−2.351	−2.45	0.0233

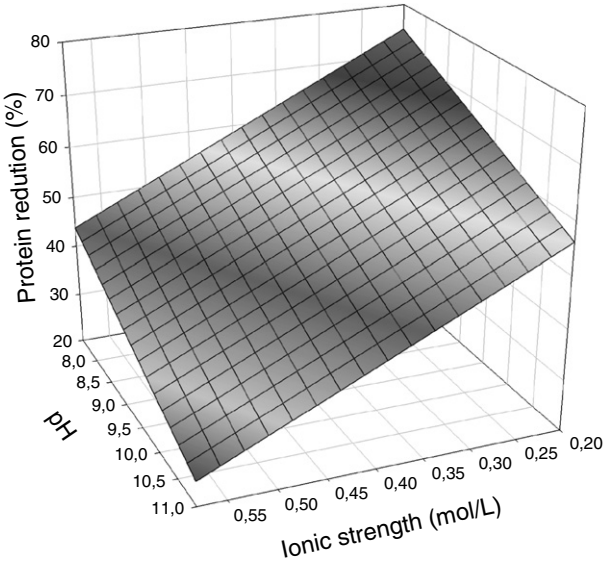

Fig. 5. Influence of pH and ionic strength on protein reduction.

Table 8
Optimum conditions for the codified and real variables

Variable	X_1	X_2	X_3	X_4	Y_1 (Eq. (1))	Y_2 (Eq. (2))
Codified	−0.727	−0.798	0.144	−1.279	98.94	32.52
Real	5:22.2	5.200	0.372	7.0		

Table 9
Optimum conditions for the codified and real variables

Variables	X_1	X_2	X_3	X_4	Y_1 (Eq. (2))	Y_2 (Eq. (3))
Codified	−1.635	−0.227	0.278	0.139	83.37	12.37
Real	5: 31.23	5.773	0.392	9.2		

Table 10
Comparison of the efficiency in removing cholesterol from egg yolk using different extracting agents

Extractive agent	Temperature (°C)	Protein reduction (%)	Cholesterol removed (%)	References
Gum arabic	21	75.0	94.0	Hsieh et al. (1994)**
β-Cyclodextrin	50	9.2	89.2	Smith et al. (1995)
β-Cyclodextrin*	N.R.	N.R.	90.0	Vollbrecht et al. (1991)**
Oil (peanut)	50	N.R.	89.3	Braco and Viret (1982)**
Acetone	25	N.R.	91.0	Martucci and Borges (1997)
HM pectins	25	11.3	85.7	This study

* Using yolk plasma.
** Patents; N.R. not reported.

Table 7 shows the estimates of each parameter of the second order model, besides the value Pr and t of each parameter.

In Fig. 4, it is observed that a greater dilution and lower ionic strength reduce protein loss, likely due to increased protein solubility. According to Wong (1995), low salt concentrations tend to increase the solubility of most proteins. Thus, in an aqueous medium, the protein hydrophobic groups tend to repulse the water molecules, grouping themselves in the regions of the protein molecule farthest from their surface.

Another characteristic is the favoring of protein protonation at low pH values (Wong, 1995), leading to electrostatic interactions between the protein and other component in the medium, e.g., a polymer (Doublier, Garnier, Renard, & Sanchez, 2000). Maroziene and Kruif (2000) carried out casein adsorption using HM pectin at pH 5.3, desorbing it with pH increase. In Fig. 5, a lower protein reduction is verified with increased pH, at a fixed ionic strength value.

3.4. Extraction process optimization

The maximum or minimum point method, as described by Myers and Montgomery (1995), was used to optimize the extraction process. Maximization of the variable cholesterol reduction response led to the following second order equation, considering the most significant variables (<0.0001), as shown in Table 5:

$$Y_1 = 72,945 - 6878X_1 - 4136X_3 + 3891X_3^2 + 7784X_4X_2 + 4129X_4^2 \quad (2)$$

The minimization of the variable yolk protein reduction response led to the following second order equation, considering the most significant variables (<0.0001), as shown in Table 7:

$$Y_2 = 54,836 + 14,597X_1 - 13,733X_3 - 6830X_4 - 5592X_1^2 - 6369X_3X_2 \quad (3)$$

Table 8 lists the optimum conditions for maximizing cholesterol reduction. In this work, a high cholesterol reduction value (98.94%) was observed, although the concomitant increase in the yolk protein reduction obtained (32.52%) may not favor this process.

In Table 9, the optimum conditions for minimization of protein loss are listed, and the increase in the dilution and pH values is verified to favor yolk solubility in the water phase. The pH and ionic strength values obtained are close to those reported by Causeret, Matringre and Loriet (1991) for maximum solubility of yolk protein.

The use of these conditions led to Y_1 and Y_2 values of 85.65 and 11.38, respectively, thus, validating the use of the model (Eq. (3)) for yolk cholesterol extraction process.

In this work, the yolk protein reduction values, around 12%, were under those obtained in the process using gum arabic and higher to those obtained with β-cyclodextrin by Smith et al. (1995), as shown in Table 10. Smith et al. (1995) obtained smaller protein loss, around 9%, using higher temperature.

4. Conclusions

Response surface methodology was shown to be an efficient technique in the optimization of the process of liquid egg yolk cholesterol reduction, using HM pectin. The fractionary factorial 2^{4-1}_{III} used in the central composite design was found to be adequate to explain the effects of the four most significant variables (yolk dilution level, ionic strength, yolk suspension pH, and pectin gel amount used for extraction) in the process with a minimum number of experiments. The optimum conditions of the process were 5 g of yolk for 31.23 g of water, ionic strength of 0.39 mol/L and pH equal to 9.2 for yolk suspension and 5.77 g of pectin gel with 3% (m/m) pectin. A reduction of 83.4% in yolk cholesterol content and a decrease of 12.4% in yolk protein content were observed.

Acknowledgments

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References

Braco, H., & Viret J. -L. (1982). Decholesterization of egg yolk. US Patent 4,333,959.

Bragagnolo, N., & Rodriguez-Amaya, D. B. (2003). Comparison of the cholesterol content of Brazilian chicken and quail eggs. *Journal of Food Composition and Analysis, 16*(2), 147–153.

Box, G. E. P., Hunter, W. G., & Hunter, J. S. (1978). *Statistics for experimenters*. New York: John Wiley & Sons.

Causaret, D., Matringe, E., & Loriet, D. (1991). Ionic strength and pH effects on composition and microstructure of yolk granules. *Journal of Food Science, 56*, 1532–1536.

Chenfeng, L. V., Tang, Y., Wang, L., Wenming, J. L., Chen, Y., Yang, S., et al. (2002). Bioconversion of yolk cholesterol by extra-cellular cholesterol oxidase from *Brevibacterium* sp. *Food Chemistry, 77*, 457–463.

Chung, S. L., & Ferrier, L. K. (1991). Partial lipid extraction of egg yolk powder: effects on emulsifying properties and soluble protein fraction. *Journal of Food Science, 56*(5), 1255–1258.

Coffin, D. R., & Fishman, M. L. (1997). Films fabricated from mixtures of pectin and Poly(vynyl alcohol). US Patent 5,646,206.

Conte, Jr., Jhonson, B. R., Hsieh, R. J., & Ko, S. S. (1992). Method for removing cholesterol from eggs. US Patent 5,091,203.

Day, C. E., & Kuhrts, E. H. (1989). Method and composition for reducing serum cholesterol. US Patent 4,824,672.

Doublier, J.-L., Garnier, C., Renard, D., & Sanchez, C. (2000). Protein–polysaccharide interactions. *Current Opinion in Colloid and Interface Science, 5*, 202–214.

Froning, G. W., Wehling, R. L., Cuppett, S. L., Pierce, M. M., Niemann, L., & Siekmank, D. K. (1990). Extraction of cholesterol and other lipids from dried egg yolk using supercritical carbon dioxide. *Journal of Food Science, 55*(1), 95–98.

Hsieh, R. J., Snyder, D. P., & Ford, E. W. (1994). Method for removing cholesterol and fat from egg yolk by chelation and reduced-cholesterol egg product. US Patent. 5,302,405.

Janson, J.-C., & Rydén, L. (1998). *Protein purification: principles, high resolution methods and applications*. New York: John Wiley & Sons.

Johnson, C. B. (1996). Isolation of cholesterol oxidation products from animal fat using aminopropyl solid-phase extraction. *Journal of Chromatography A, 736*, 205–210.

Judd, P. A., & Truswell, A. S. (1982). Comparison of the effects of high and low methoxyl pectins on blood and faecal lipids in man. *Brazilian Journal Nutrition, 48*, 451–458.

Kwan, L. E., Helbig, L. N., & Nakai, S. (1991). Fractionation of water-soluble and insoluble components from egg yolk with minimum use of organic solvents. *Journal of Food Science, 56*(6), 1537–1541.

Lewis, M. J. (1993). *Physical properties of foods and food processing systems*. Chichester: Ellis Horwood Ltd.

Maroziene, A., & Kruif, C. G. (2000). Interaction of pectin and casein micelles. *Food Hydrocolloids, 14*, 391–394.

Martucci, E. T., & Borges, S. V. (1997). Extraction of cholesterol from dehydrated egg yolk with acetone: determination of the practical phase equilibrium and simulation of the extraction process. *Journal Food Engineering, 32*, 365–373.

Missler, S. R., Wasilchuck, B. A., & Merrittt, J. R. (1985). Separation and identification of cholesterol oxidation products in dried egg preparations. *Journal of Food Science, 50*, 595–598.

Myers, R. H., & Montgomery, D. C. (1995). *Response surface methodology: process and product optimization using designed experiments*. New York: John Wiley & Sons.

Neto, B. B., Scarminio, I. S., & Bruns, R. E. (2001). *Como fazer experimentos: pesquisa e desenvolvimento na ciência e industria*. Campinas: Editorial Unicamp.

Oakenfull, D., & Scott, A. (1984). Hydrophobic interaction in the gelation of high methoxyl pectins. *Journal of Food Science, 49*, 1093–1098.

Ogasahara, J., Hariu, H., & Takahashi, M. (1992). Process for producing liquid having cholesterol content. US Patent 5,116,628.

Pharmacia Biotech (1993). *Hydrophobic interaction chromatography principles and methods*. Uppsala, Sweden.

Phinney, K. W., Jinadu, L. A., & Sander, L. C. (1999). Chiral selectors from fruit: application of citrus pectins to enantiomer separations in capillary electrophoresis. *Journal of Chromatography A, 857*, 285–293.

Razzazi-Fazeli, E., Kleineisen, S., & Luf, W. (2000). Determination of cholesterol oxides in processed food using high performance liquid chromatography–mass spectrometry with atmospheric pressure chemical ionisation. *Journal of Chromatography A, 896*, 321–334.

Rollers, S., & Jones, S. A. (1996). *Handbook of fat replacers*. New York: CRC Press Inc.

SAS Institute Inc. (1999). *SAS user's guide: Statistics*, version 8 edition; Cary, North Carolina.

Shukla, T. P. (1991). Industrial research on cyclodextrins. *Cereal Foods World, 36*, 347–356.

Smith, D. M., Awad, A. C., Bennink, M. R., & Gill, J. L. (1995). Cholesterol reduction in liquid egg yolk using β-cyclodextrin. *Journal of Food Science, 60*(4), 691–694.

Stadelman, W. J., & Cotterill, O. J. (1995). *Egg science and technology* (3rd ed.). Wesport: AVI Publischer Company.

Sundfeld, E., Yun, S., Krochta, J. M., & Richardson, T. (1993). Separation of cholesterol from butter oil using Quillaja Saponins: 1. effects of pH, contact time and adsorbent. *Journal of Food Process Engineering, 16*, 191–205.

Vollbrecht, H. R., Cully, J., & Wiesmuller, J. (1991). Process for the removal of cholesterol esters from egg yolk. US Patent 5,063,077.

Watanabe, A., Shimizu, K., Aihara, H., Nakamura, H., Suzuki, R., & Komagata, K. (1986). Isolation and identification of cholesterol-degrating Rhodococcus strains from food of animal origin and their cholesterol oxidase activities. *Journal General Applied Microbiology, 32*, 137–147.

Wong, D. W. S. (1995). *Mechanism and theory in food chemistry*. New York: Van Nostrand Reinhold.

Yang, C. C., Chen, C. C., & Chang, H. M. (1998). Separation of egg white lysosyme by anionic polysaccharides. *Journal of Food Science, 63*, 962–965.

Yokoi, H., Obita, T., Hirose, J., Hayashi, S., & Takasaki, Y. (2002). Flocculation properties of pectin in various suspensions. *Bioresource Technology, 84*, 287–290.

Zhang, R.-Z., Li, L., Liu, S.-T., Chen, R.-M., & Rao, P.-F. (1999). An improved method of cholesterol determination in egg yolk by HPLC. *Journal of Food Biochemistry, 23*, 351–361.