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Optimization of Egg White Removal from Waste Egg Shells and Membranes by Design of Experiments for their Refinement

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Production and processing of chicken eggs is a significant segment of the food industry, not only in the EU, but also globally, with a leading position of China reaching almost 40 % of global chicken eggs production. Egg processing industry generates a huge amount of waste in the form of eggshells and membranes that make up the share of about 11 % wt. of the egg. The utilization of this waste is low and often is only landfilled without further use or refinement to more valuable products.
The waste egg-shells and membranes can be used in their native or modified form. Among the main ways of usage belongs adsorbents (in particular for waste water), heterogeneous catalysts, fertilizers, additives for correction of the acidic soils pH, food supplements, bone implants components or cosmetics products components. Nevertheless, eggshells are recycled only rarely.
Utilization of waste consisting of eggshell in pharmacy is limited mainly by the presence of unwanted egg-white and other impurities. For pharmaceutical use, the feedstock must contain only minimal amount of contaminating proteins. Egg-white and other impurities must be removed e.g. by washing prior to further processing of shells and membranes.
For optimization of the parameters of the egg-white washing, the design of experiments (DOE) methodology in the form of a central compositional plan was chosen, to achieve rapid and effective washing out under optimal conditions. The protein concentration was measured photometrically by Biuret method. By iterative response surface analysis, statistically insignificant factors and their interactions were excluded. A model that is adequate and can be used to describe the process of removing undesirable substances from waste consisting of eggshell and membranes by washing was obtained.

1. Introduction

Egg shells and membranes are considered as waste material from chicken eggs processing. Most of this material is discarded, in spite of fact, it can be reutilized and refined to get valuable products (Oliveira et al., 2013). The amount of this waste material is quite extensive, so it is difficult to reutilize it all, but it should be reused of refined in greatest extent possible, because landfilling of this material causes environmental and health issues (Francis and Rahman, 2016).
One of the possible utilization is as a source for food supplements and pharmaceutical products. Egg shells and membranes obtained after chicken eggs processing contains a lot of contaminating substances as egg white, egg yolk, feather and another impurities, which must be removed e.g. by wet process as washing. Egg white forms the largest part of the presented impurities. Egg white is consisted mainly of water and proteins, therefore it is a breeding ground for propagation of microorganisms including pathogenic ones, despite its natural defence capabilities given by egg white proteins, such as lysozyme and ovotransferrin (Baron et al., 2016). Egg white total solids content is only 12 % wt. (i.e. 88 % wt. is water), so it can be considered to be aqueous solution of protein.
Natural properties of egg white proteins will influence the optimal conditions for washing. Important from the washing water pH adjustment viewpoint, is isoelectric point of each protein. Near the isoelectric point, the extraction of egg white proteins is impeded and can be considered as the limit value for extraction. Egg white of

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fresh chicken egg has a pH of 7.6, and after it is laid, the pH gradually increases to 9.4. This means that the extraction of egg white in the washing water will increase its pH.
For selecting the temperature of washing water, the temperature of the egg white denaturation is also important. Denaturation leads to changes in the spatial structure of biomolecules from the initial native state, wherein there is a loss of solubility (precipitation) (Akkouche and Madani, 2012), which negatively affects the extraction into the washing water. Denaturation temperature can be also like isoelectric point, for the purposes of the experiment, considered as a limit value. It is believed that increasing the temperature will gradually improve the extraction of the egg white in water up to the optimum point, beyond which the extraction already will deteriorate due to approach to the protein denaturation temperature. Selected physical properties of mainly represented proteins in egg white are shown in Table 1.
In the literature up to date, there is no evidence about measurement of optimal conditions of eggshells and membranes washing, so any obtained data (or model fitting the data) are valuable from the technological point of view for this process and are filling the current research gap in this field.

Table 1: Selected physical properties of mainly represented proteins in egg white (Alleoni, 2006)

Proportions of individual proteins in egg white proteins [%]	Protein	Isoelectric point – pH	Denaturation temperature [°C]
54.0	Ovalbumin	4.5	84.0
12.0	Ovotransferin / Conalbumin	6.1	61.0
11.0	Ovomucoid	4.1	77.0
4.0	Ovoglobulin G2	5.5	92.5
4.0	Ovoglobulin G3	5.8	-
3.5	Ovomucin	4.5-5.0	-

2. Materials and methods

For optimization, a methodology of design of experiments (DOE) was chosen. If the stable levels of selected input variables can be achieved, it is commonly used method for optimization, minimizing the required number of experiments (Belohradsky and Kermes, 2012), to acquire as much statistical data about impact of selected variables on the observed response as possible (Yusuf et al., 2015).
Time (t) in s, the initial pH of the washing water and the initial temperature of the washing water in °C were selected as factors of the model. To determine the levels of the pH factor, it was necessary to measure the native pH of the water eggshells suspension. The ratio of eggshells and water was set to 1:5. The suspension was stirred only briefly and the pH was measured (pH = 8.5). Selection of the level of factors was performed in the way to see the effect of the factor, but to avoid overstepping the denaturation temperature and isoelectric points of proteins. For initial pH adjustment, 2 % vol. H_2SO_4 and 20 g/L NaOH were used.
As a response from randomized trials, protein concentration in the extract suspension was chosen. To measure the protein concentration, Biuret method was chosen from various available methods mainly because of expected high concentration of protein in the extract. Biuret method enables measurement of the protein concentration in the order of g/L (thus has the highest detection limit of commonly available methods). Sample dilution may further increase the upper concentration limit. The method, however, had to be adjusted. Absorbance was measured at 340 nm because the protein after reaction with the reagent at 550 nm did not exhibit a sufficiently high absorbency. Measurement in UV region (310 - 340 nm), to increase the sensitivity (Nemcova et al., 1996) and reduce the interference (Koch and Putnam, 1971) is commonly used. As the standard, pure lyophilized egg white was utilized.
A form of a central compositional plan was chosen, in which star points are located in the middle of the wall of an imaginary cube. This means that the distance alpha = 1. Factors and their levels were selected according to Table 2.
Repetitions was carried out at the central point of the design. At other points of the design, only one trial was performed, however the samples taken from the resulting protein extract were determined by Biuret method in duplicate. For statistical evaluation, Minitab 15 software was used. Parameters of design of experiments was in accordance with Figure 1a).
On Figure 1b) an iterative process used to find a suitable model that describes well the measured data is also depicted. By performed analysis, statistically insignificant parameters (model factors and interactions) were identified. These parameters were excluded from the model and this procedure was repeated until all statistically insignificant factors were excluded from the model.

Table 2: Design of experiments – factors levels

Factor level / factor	-1	0	1
t [s]	30	150	270
pH [-]	7.0	8.5	10.0
T [°C]	22	34	46

a) Central Composite Design

Factors: 3 Replicates: 1
Base runs: 17 Total runs: 17
Base blocks: 1 Total blocks: 1

Two-level factorial: Full factorial

Cube points: 8
Centre points in cube: 3
Axial points: 6
Centre points in axial: 0

Alpha: 1

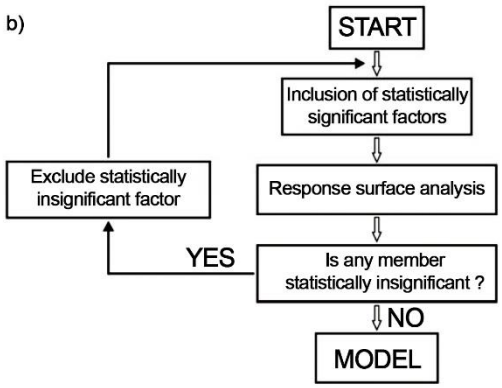

Figure 1: a) Definition of design of experiments in Minitab 15 and b) iterative process of finding an appropriate model

3. Results and discussion

Measured protein concentrations at each point of the design are summarized in Table 3.

Table 3: Measured response - protein concentrations at each point of the design

Run No.	Factor 1: t	Factor 2: pH	Factor 3: T	$c_{protein1}$	$c_{protein2}$	$\mathbf{c}_{protein,average}$	RES**
[-]	[s]	[-]	[°C]	[g/L]	[g/L]	[g/L]	[-]
1	30	7.0	22	3.461	3.681	3.571	-0.94
2	270	7.0	22	5.745	6.862	6.304	-0.76
3	30	10.0	22	8.077	8.827	8.452	0.73
4	270	10.0	22	17.212	17.741	17.477	-0.95
5	30	7.0	46	8.033	8.474	8.253	1.07
6	270	7.0	46	8.920	9.920	9.420	-0.32
7	30	10.0	46	10.003	10.370	10.186	-0.20
8	270	10.0	46	21.387	21.358	21.373	0.28
9	30	8.5	34	8.841	9.047	8.944	-0.99
10	270	8.5	34	16.977	18.976	17.977	1.42
11	150	7.0	34	8.568	10.391	9.479	-0.12
12	150	10.0	34	15.771	16.124	15.948	-0.94
13	150	8.5	22	11.361	11.332	11.346	1.92
14	150	8.5	46	10.949	11.596	11.273	-0.83
15	150	8.5	34	10.773	12.008	11.390	-1.85
16	150	8.5	34	12.831	12.802	12.816	-0.43
17*	150	8.5	34	15.830	16.477	16.154	2.91

* the value measured at this point is outlier and therefore this measurement was excluded
** RES = residuals. Residuals are deviations of the measured values from the values predicted by the model

The effect of selected factors (a,b,c) and their interactions (d) on the egg white concentration in the extract solution is shown in Figure 2. It is clear, that optimal temperature for washing lies within the scope of experiment (see a and b) and that raise in pH also improved washing of proteins (c). Interactions plot (d) indicated pH-t interaction, which was later proved to be significant.

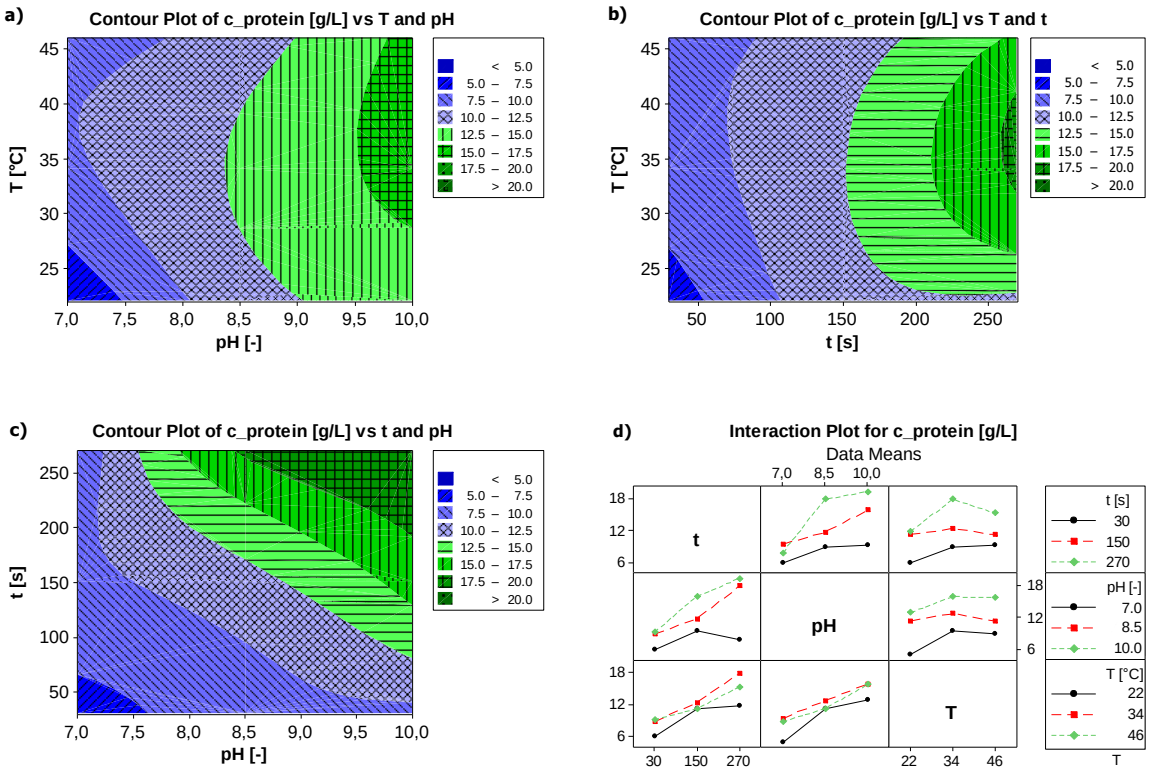

Figure 2: Influence of factors (a,b,c) and their interactions (d) on final concentration of egg white in extract solution

Factors optimization was done in Minitab. Results of factors levels optimization are shown together with main factor effects on Figure 3. The optimum for temperature was found and it was 38 °C. The pH and of course also time optima was out of the scope of selected factors levels, however it indicated that the alkaline washing regime can be recommended to improve proteins removal.

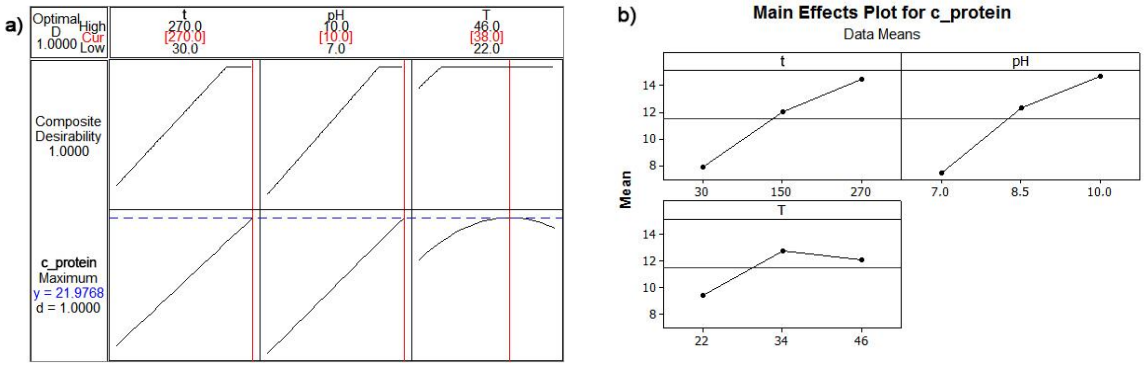

Figure 3: a) Optimization of factors and b) main factor effects on response

3.1 Search for a suitable model of egg white washing

The resulting model from the iteration response analysis after exclusion of statistically insignificant members is summarized in Figure 4. P value for the "Lack of fit," indicated that the model is adequate and can be used for the description of experimental data. P > α (95 % reliability α = 0.05), i.e. 0.565 > 0.05.
The value of R-Sq = 95.45 % is high and is approaching 100 %. The model regression curve therefore very well fit the measured data.

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Response Surface Regression: c_protein versus t; pH; T

The analysis was done using coded units.

Estimated Regression Coefficients for c_protein

Term        Coef  SE Coef       T      P
Constant  12.759   0.4951  25.773  0.000
t          3.314   0.3835   8.643  0.000
pH         3.641   0.3835   9.494  0.000
T          1.336   0.3835   3.483  0.006
T*T       -1.994   0.6262  -3.184  0.010
t*pH       2.039   0.4287   4.756  0.001


S = 1.21265    PRESS = 36.2290
R-Sq = 95.45%  R-Sq(pred) = 88.79%  R-Sq(adj) = 93.17%


Analysis of Variance for c_protein

Source          DF   Seq SS   Adj SS  Adj MS      F      P
 Regression      5  308.402  308.402  61.680  41.94  0.000
  Linear         3  260.241  260.241  86.747  58.99  0.000
  Square         1   14.904   14.904  14.904  10.14  0.010
  Interaction    1   33.257   33.257  33.257  22.62  0.001
Residual Error  10   14.705   14.705   1.471
  Lack-of-Fit    9   13.688   13.688   1.521   1.50  0.565
  Pure Error     1    1.017    1.017   1.017
Total           15  323.108
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Figure 4: Definition of the final model in Minitab 15

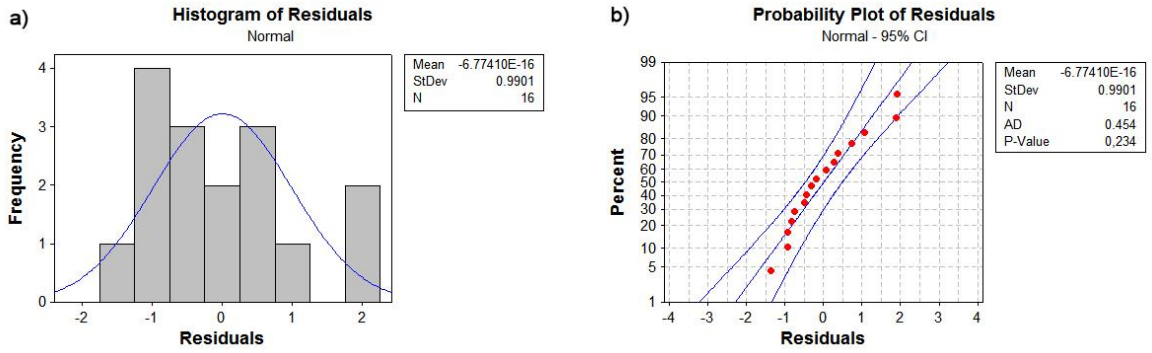

Figure 5: a) Histogram of residues frequency with normal distribution curve and b) probability graph of normal distribution of residues with a 95 % confidence bands

As shown in Figure 5a, a histogram of residuals frequencies did not fit optimally the curve of normal distribution. This is probably given by a low number of measurement points. Nevertheless, a histogram is not the best choice for judging the distribution of residuals for small sample sizes of residuals, a more sensitive approach is to use normal probability plot (Nist and Sematech, 2013) (Figure 5b). As can be seen in Figure 5b, that the P-value is larger than the chosen significance level α = 0.05, so it can be concluded that the residuals had normal distribution. The resulting regression equation Eq(1) is shown below:

$$c_{proteins}\left[g \cdot L^{-1}\right] = -17.3614 - 0.06866 \cdot t\,[s] + 0.72812 \cdot pH\,[-] + 1.05273 \cdot T\,[°C] - 0.01384 \cdot T^2\left[°C^2\right] + 0.01133 \cdot t \cdot pH\,[s] \quad (1)$$

4. Conclusions

In this study, the optimal parameters (pH, temperature and time) of the egg-white washing were searched. The optimum for the initial temperature of water for the extraction was found, namely 38 °C. The pH optimum is out the range of the selected pH factor levels of the experiment, but it is clear that it lies in the alkaline range, in particular pH > 10. From the perspective of the increasing of egg white washing speed, any addition of acid into the wash liquor therefore cannot be recommended. Usage of higher extraction water pH than 10 is not recommended, because it could cause increased material corrosion of the process equipment. The addition of NaOH can shorten the time needed for washing, however it also increases the process operating costs. On the other side, it can suppress proliferation and activity of certain bacteria, e.g. Salmonella already at pH > 9.5 do not multiply.

To maintain the pH of the extraction water at 10, approximately 125 g NaOH/$m^3_{washing\ water}$ has to be added. Considering the price of 0.8 €/kg_{NaOH}, the addition of NaOH brings additional operation cost 0.1 €/$m^3_{washing\ water}$. Time factor response showed relatively quick washing of proteins into water also with significant time-pH interaction.

The regression equation for adequate model for the process of removing eggwhite and other impurities from waste consisting of eggshell and membranes by washing was obtained and is stated in Eq(1). This model can be used to design a new washing process or to optimize an existing one.

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