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Pasteurisation of liquid whole egg: optimal heat treatments in relation to its functional, nutritional and allergenic properties.

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Abstract

Pasteurised liquid egg products represent around 70% of total egg products. Pasteurisation, necessary for microbiological safety, is a unit operation undergone by the totality of liquid whole egg commercialized. The present work aims to investigate the impact of a wide range of pasteurisation rates (among those classically used in industry) on the interfacial properties, *in vitro* digestibility and antigenicity of whole egg proteins, in order to optimize the unit operation parameters to ensure all whole egg qualities. Pasteurisation from 4 to 10 min at 60°C improved protein interfacial properties, *in vitro* digestibility and decreased ovomucoid antigenicity but did not significantly change ovotransferrin and lysozyme antigenicity. On the opposite, pasteurisation from 4 to 10 min at 66°C decreased protein *in vitro* digestibility and increased drastically ovotransferrin antigenicity. Pasteurisation from 4 to 10 min at 60°C enabled to reach all whole egg properties, but for safety reasons, higher pasteurisation rates at 62 or 64°C are good compromises to ensure microbiological safety without excessively damaging the functional properties.

Keywords

Whole egg, pasteurisation, interfacial property, emulsion, *in vitro* digestion, antigenicity

1. Introduction Egg is a widely used ingredient in many food products. Especially, whole egg is indeed a major source of high quality proteins and essential nutrients and provides many desirable functional attributes such as foaming, emulsifying, gelling, colouring, flavouring (Yang & Baldwin, 1995). However, hen egg is also one of the leading causes of food allergy in childhood, affecting 1.6 to 3.2% of young children (Eggesbo et al., 2001).

In the egg product industry, microbiological safety of liquid products is mainly guaranteed by pasteurisation. The USDA requires that liquid whole egg is at least heated at 60°C for no less than 3.5 min, but in the United Kingdom the recommendations are to pasteurize at least at 64°C for 2.5 min (Cunningham, 1995). In France, there is no statutory heat treatment; only microbiological results are determined by regulations. To achieve this, the treatments classically used to pasteurize whole egg vary from 65 to 68°C for 2 to 5 min in order to ensure 5 to 6 decimal reductions of vegetative micro-organisms and especially *Salmonella* Enteritidis and *Listeria monocytogenes* (Baron et al., 2010). Pasteurisation temperatures used in the egg industry are limited by the sensitivity of egg proteins to heat treatment. Thus, pasteurisation for 2 to 10 minutes from 60 to 68°C modifies whole egg electrophoretic pattern by especially decreasing ovotransferrin, livetin, ovalbumin, apovitellenin, lysozyme and/or ovomucin band intensity (Dixon & Cotterill, 1981; Martinez et al., 1994; Mendes de Souza & Fernandez, 2013; Scholtyssek et al., 1981; Woodward & Cotterill, 1983). Moreover, an increase of whole egg viscosity is observed at temperature as low as 56°C (Cunningham, 1995), and whereas pasteurisation has little influence on liquid whole egg functionality when heated up to 60°C (Ball et al., 1987; Cunningham, 1995; Herald & Smith, 1988), heating above this temperature decreases foaming and emulsifying properties (Janssen, 1971; Montfort et al., 2012), custard height (Ball et al., 1987; Cunningham, 1995) or pie filling expansion (Herald & Smith, 1988).

The high content of highly digestible proteins in whole egg is of great benefit to human nutrition. However, the effects of industrial processing such as pasteurisation on the nutritional quality of whole egg proteins are poorly documented. Van der Plancken et al. (2002) and Jimenez-Saiz et al. (2011)

showed that ovalbumin susceptibility to hydrolysis was increased after heat treatment higher than 10 to 30 minutes at 60°C, whereas the ovomucoid susceptibility was not changed after 30 min at 60°C. Assuming that pasteurisation results in egg protein denaturation and/or aggregation, whole egg pasteurisation may hide, destroy or unmask protein allergenic epitopes. Moreover, the potential changes in protein digestion can also change the ability to sensitize and elicit the immune response (Jimenez-Saiz et al., 2011). The effect of heat treatment on the allergenic properties of egg proteins has been focused on cooking since some allergic children tolerate cooked eggs (Lemon-Mulé et al., 2008). Moreover, egg white proteins have been mainly studied since they are considered more allergenic than yolk proteins (Kosti et al., 2013), despite apovitellenins I & VI, α -livetine and phosvitin were reported as allergens by Mine & Yang (2008). In most cases, physicochemical changes caused by cooking either enhance a decrease or have no significant effect on allergenicity, depending on the susceptibility of the proteins to unfold and to lose their conformational epitopes. But in some cases, aggregates which are more allergenic and more resistant to digestion can be generated (Thomas et al., 2007). Up to the authors' knowledge, no study has been performed to test the effect of pasteurisation on whole egg antigenicity. Consequently, if the effects of pasteurisation on whole egg functional properties have been well documented, there is a lack of knowledge on the effect of this unit operation on whole egg protein digestibility and antigenicity. The present study aimed at investigating the effect of a wide range of pasteurisation rates on the interfacial properties, *in vitro* digestibility and antigenicity of whole egg proteins, and to determine the best conditions to optimize these different functionalities.

2. Material and methods

2.1. Experimental design

Raw liquid whole egg was supplied by Ovoteam (Plaintel, France). Dry matter was adjusted to 22% by adding raw egg white (Ovoteam, Plaintel, France). Homogenised whole egg was obtained from raw

liquid whole egg using a two-stage high-pressure homogeniser (Rannie LAB 16/15, APV France, Evreux, France) at 5 Mpa. Heat treatments of homogenised whole egg were performed with an indirect tubular processing system (Microthermics UHT/HTST Lab 25 EDH, Microthermics, Inc., Raleigh, NC 27615, USA) according to the following complete factorial design 4x3: whole egg was heated to 60, 62, 64 or 66°C and held at temperature for 4, 7 or 10 min. The pasteurisation temperature curves are given in Supplementary Data 1. After cooling to 25°C, glass bottles (volume 250 mL) were filled and then frozen. Homogenised raw whole egg was also frozen as a control. The time-temperature rates applied enables to reach from 23 to 1243 decimal reductions of *Salmonella enteritidis* according to D and z values of these bacteria in whole egg (Jin et al, 2008).

2.2. Interfacial properties

2.2.1. Langmuir isotherms at air-water interface

Measurements of the surface pressure (Π) - surface area (A) isotherms have been performed by compression – expansion cycles using the Wilhelmy plate method as described by Lechevalier et al. (submitted) except that egg white solutions was replaced by liquid whole egg diluted in 50 mM phosphate buffer pH 7, NaCl 300 mM to a final concentration of 1 mg.mL⁻¹ after measurement of protein content according to Markwell et al. (1978). The surface corresponding to an interfacial pressure of 10 mN.m⁻¹ (S_{10}) was selected as the indicator of the sample behaviour at the air-water interface.

2.2.2. Emulsifying properties

2.2.2.1. Preparation of oil-water emulsions

Emulsions were prepared as described by Rannou et al. (2015) with the aqueous phase being 1.5% (w/v) whole egg protein solutions in phosphate buffer pH7, NaCl 300 mM.

2.2.2.2. Droplet size distribution

The droplet size distribution (DSD) was determined as described by Lechevalier et al. (submitted). The volumetric mean diameter ($d_{4,3}$) was selected to measure droplet size distribution.

2.2.2.3. Emulsion stability

Emulsion stability towards creaming was determined as described by Lechevalier et al. (submitted). The creaming index (Cr_i) was calculated as follows:

$$Cr_i = \frac{V_a}{V_t} * \frac{100}{\phi} \quad \text{eq.1}$$

where V_a is the volume of separated aqueous phase, V_t is the total volume of sample in the tube after centrifugation, and ϕ is the volumic fraction of the aqueous phase used to prepare the emulsion (i.e. 0.7). The higher the creaming index, the lower the emulsion stability, since it assumes a high release of aqueous phase due to a high compaction of droplets with centrifugation.

2.3. Protein *in vitro* digestion and characterization of digested samples

2.3.1. *In vitro* digestion

Each pasteurised whole egg sample and the control sample were subjected to an *in vitro* digestion model which simulates successive gastric and intestinal stages of digestion in the adult human as proposed by Dupont et al. (2010) and detailed by Lechevalier et al. (2015). One ml of protein solution was aliquoted before digestion (gast ti), at the end of the gastric stage (gast tf) and at the end of the intestinal stage (int tf).

2.3.2. Degree of hydrolysis (DH)

The DH was calculated from the measurement of amino groups released during *in vitro* digestion using OPA (orthophtaldialdéhyde) according to Church et al. (1983), modified as described by Lechevalier et al. (submitted). The hydrolysis degree (DH) was calculated using the following formula:

$$\% \text{ DH} = (L_t - L_0) / L_{\text{tot}} \times 100 \quad \text{eq. 2}$$

where L_t is the amount of free NH_2 after t min hydrolysis, L_0 is the amount of initial free NH_2 (before digestion), and L_{tot} is the maximum amount of free NH_2 , i.e. after total acid hydrolysis. Free NH_2 was

expressed in mg per g of protein. The DH at the end of the gastric and the intestinal stages were kept as results.

2.3.3. Electrophoresis

The digested samples were analysed by SDS-PAGE on 12.5% polyacrylamide gels, according to Laemmli (1970), modified as explained by Lechevalier et al. (2015).

Samples were distributed onto 26 gels. For each pasteurised whole egg (one temperature, one duration) and the control, one gel was prepared with the samples of the gastric stage, and another gel was prepared with the samples of the intestinal stage (Figure 1).

The gels were Coomassie Blue stained, and digitised using the Image Scanner III device (GE Healthcare Europe GmbH). ImageQuant software (GE Healthcare) was used to quantify the band by Gaussian fitting, and to match the gels. All matches were checked manually and corrected if necessary. The band volume was used as the response variable in the statistical analysis. Up to 22 bands per sample could be identified (Figure 1).

The final data set was composed of 39 different samples: 13 samples x 3 digestion times (gast ti, gast tf, int tf). The band volumes measured at gast ti were used to characterize sample protein composition after pasteurisation and before digestion; they were analysed separately by principal component analysis as described in section 2.5.2.

2.3.4. Pre-processing of the data

As explained by Lechevalier et al. (2015), raw electrophoresis data are not suitable for processing by statistics: pre-processing of the data was thus necessary. Firstly, the values that were missing in the final dataset, were manually assigned to zero after checking the absence of the corresponding band on the gel.

Then, since the 39 samples were not randomized on the 26 gels, band volumes were normalized using the 30 kDa band of the LMW kit to avoid any differences in staining from one gel to another. The volume of this band on the gel prepared with the digested gastric samples of the control sample (raw whole

egg) was taken as the reference value ($Gel_{ref}BV$). The volume of the 30 kDa band for each of the other 25 gels was called Gel_nBV ($1 < n < 25$). The correction coefficient (CC) for the gel n was then calculated as follows:

$$Gel_nCC = \frac{Gel_nBV}{Gel_{ref}BV} \quad \text{eq. 3}$$

All the band volumes of the Gel_n were then multiplied by the Gel_nCC coefficient. The band volumes at the three digestion stages characterized hydrolysates protein composition and were analysed by multiple factor analysis as described in section 2.5.4 to highlight pasteurisation effect on the progress of the *in vitro* digestion.

2.4. Protein antigenicity

Inhibition ELISA using ovomucoid, ovotransferrin or lysozyme-specific monoclonal antibodies was performed in order to determine the residual immunoreactivity of each protein after whole egg pasteurisation, according to Dupont et al. (2010) and as described by Lechevalier et al. (submitted). Results were expressed as ovomucoid, ovotransferrin or lysozyme concentration in samples using a four-parameter logistic (sigmoidal) function. The measurements were performed in duplicate.

2.5. Statistical analysis

2.5.1. ANOVA

ANOVA was used to test the effect of pasteurisation temperature and time on each measurement. The normal distribution of the data was first tested using a Shapiro Wilk normality test, and the homoscedasticity of the samples was tested using the Levene's test for homogeneity of variance of R language (R development core team, 2007). Since all variables fitted these ANOVA hypotheses, the following linear model procedure of R was applied:

$$Y_{ij} = T_i + t_j + T.t_{ij} \quad \text{eq.4}$$

where, T is the temperature i (20, 60, 62, 64, 66°C); t the treatment time j (0, 4, 7, 10 min); and $T.t$ the interaction. Differences were regarded significant at minimum level of 95% (p -value<0.05). The LS-Means were calculated and compared using multiple comparisons by means of LSD with the “agricolae” package of R software (R development core team, 2007).

2.5.2. Principal component analysis (PCA)

To analyse the effect of pasteurisation on all egg proteins at once, PCA was applied to the matrix with electrophoresis band volumes at each stage as active variables, and the 13 samples as individuals (Supplementary data 2). Pasteurisation temperature and time were added as supplementary variables. PCA transformed the observed variables into a new set of independent variables called principal components (PCs) which were uncorrelated linear combinations of the original variables. The first component was the one which explained most of the variance in the data. A large part of the information in the data could thus be plotted in 2-D or 3-D graphs defined by the PCs. PCA was performed using the FactoMineR package of the R software (Husson et al., 2008). The variables were automatically standardized (mean centred and scaled) by the software to give them all the same importance.

2.5.3. Multiple factor analysis (MFA)

MFA deals with a multiple table, composed of groups of either continuous, categorical variables or frequency tables (Escofier & Pagès, 1998). MFA balances the influence of the groups on the first principal dimension by dividing the weights of the variables (columns) of a group by the first eigenvalue of the separated PCA of this group. The highest axial inertia of each group is standardized at 1. MFA provides the classical results of principal component methods. PCA characteristics and interpretation rules are retained. MFA offers tools for comparing the different groups such as the partial representation of the individuals (rows). This representation allows comparison of the typologies provided by each group in a common space. In other words, it allows comparison of the PCA results in a common space. A graph of the groups allows global comparison of the groups and evaluation if the relative positions of the individuals are globally similar from one group to another. It also allows the comparison of partial

groups and assesses whether they provide the same information. Another graph gives the correlations between the dimensions of the MFA and each separate analysis (PCA dimensions).

In the present study, two MFA were performed. The first one ("digestion MFA") was performed on the table containing the quantified band volumes at three digestion sampling times (gast ti, gast tf and int tf), with the row representing the 13 samples (the control and the 12 pasteurised samples) (Supplementary data 3). It provided a representation of the effect of pasteurisation treatment by taking into account the digestion sampling time in a balance way. Moreover, it also provided a superimposed representation of pasteurised samples described by each of the groups of variables onto the subspace of the global analysis generated by PCs (Lechevalier et al., 2015).

The second MFA ("global MFA") was performed on the table containing all the previously described measurements dispatched in four groups (Supplementary data 4): the volumes of each SDS PAGE band before hydrolysis in "protein profiles" group; the surface pressure, the droplet size distribution and the emulsion stability in "protein interfacial properties" group; the degree of hydrolysis at the end of gastric and intestinal stages and the coordinates of the samples on the first 2 PCs of the digestion MFA in the "protein digestion" group; the ovomucoid, ovotransferrin and lysozyme concentrations determined by inhibition ELISA in the "protein antigenicity" group. The rows represented the control and the 12 pasteurised samples. Pasteurisation temperature and time were added as supplementary variables. The "global MFA" provided a representation of the diversity of pasteurisation treatment by taking into account the protein profile before digestion, the protein interfacial properties, their susceptibility to *in vitro* digestion and their antigenicity. The superimposed representation of pasteurised samples described by each one of the four groups of variables on the subspace of the global analysis generated by PCs highlighted the similarities and discrepancies between samples according to the properties considered.

3. Results and discussion

3.1. Pasteurisation under 66°C minimizes protein aggregation

Whole egg protein profile was analysed by SDS-PAGE for each pasteurised sample and the control. The volume of the protein bands measured on SDS-PAGE gels before digestion (gast ti samples) were submitted to PCA to have a global overview of the effect of pasteurisation on protein profile. The graph of the variables, i.e. of the electrophoretic band volumes, on the first two dimensions is given in Figure 1A. PC1 and PC2 explain 28% and 21.6% of the variability of the data set, respectively. PC1 is positively correlated to the volume of the band at 159, 138, 97, 94, 75 and 51 kDa, and negatively correlated to the volume of the band at 106 kDa (Figure 2A). PC2 is positively correlated to the volumes of the band at 198, 129, 118 and 14 kDa. Pasteurisation temperature and time are not well correlated with PC1 and PC2 assuming that pasteurisation treatment is not responsible for the heterogeneity of samples electrophoretic patterns. However, the samples pasteurised at 66°C, whatever the time, have especially low and high coordinates on PC1 and PC2, respectively (Figure 2B). These samples have thus more components of 198, 129, 118, 106 and 22 kDa and less components of 159, 138, 97, 94, 75 and 51 kDa than the other samples. The bands at 198, 118, 106 and 22 kDa are only present in pasteurised samples and specifically in samples pasteurised at 66°C (data not shown). On the opposite, the band at 51 kDa has completely disappeared in samples pasteurised at 66°C and the volume of the bands at 159 and 138 kDa decreases. The occurrence of high molecular weight components at the expense of lower molecular components suggests protein aggregation for the highest pasteurisation treatment, i.e. from a few minutes at 66°C. This is in agreement with Hamid-Samimi et al. (1984) who observed a rapid decrease of soluble protein in the pasteurisation temperature range 67.5-72.5°C. The analysis of whole egg electrophoretic pattern thus confirms that pasteurisation temperature should stay below 66°C to prevent protein aggregation.

3.2. Pasteurisation at 60°C maximizes whole egg interfacial properties

Interfacial properties were measured both on 2D through surface pressure-area isotherms and 3D through emulsifying properties. Surface pressure-area isotherms of films made with pasteurised whole egg proteins are shifted towards higher pressures and areas than the control, at least for the lowest pasteurisation temperatures (Figure 3). Low pasteurisation thus favours the spreading of whole egg proteins at the air-water interface, making pasteurised proteins more surface-active than control proteins. The best interfacial properties are obtained for whole egg pasteurised at 60°C, whatever the time. When pasteurisation temperature increases, surface pressure-area isotherms are shifted towards lower pressures and areas, so that the sample pasteurised 7 min at 66°C is not significantly different from the control one (Figure 3).

The interfacial properties of whole egg proteins were also investigated for emulsifying properties, as measured by droplet size ($d_{4,3}$) and creaming index (Cr_i). Samples pasteurised at 60°C have significantly lower $d_{4,3}$ and Cr_i than the control whatever pasteurisation time (Figures 4 & 5). When pasteurisation temperature increases, $d_{4,3}$ and Cr_i tend to increase to achieve the control values. A significant pasteurisation time effect is also observed for samples pasteurised at 66°C, $d_{4,3}$ and Cr_i increasing with pasteurisation time.

Then, whole egg pasteurisation at 60°C increases protein interfacial properties thus generating more stable emulsions with lower droplet size. Mc Clements (2004) already mentioned this relationship between protein surface activity and emulsion properties. But further increase in pasteurisation temperature decreases protein surface activity and emulsion properties getting back to the control values. The effect of pasteurisation temperature outweighs that of pasteurisation time as suggested by the analysis of variance (Table 1). Pasteurisation time is only significant for samples pasteurised at 66°C, emulsifying properties decreasing with pasteurisation time increase. Up to the authors' knowledge, the effect of pasteurisation rate has never been tested on whole egg interfacial properties. However, Le Denmat et al. (1999) highlighted that below 69°C pasteurisation had no effect on egg yolk emulsifying properties. The increase in protein interfacial properties after pasteurisation at 60°C may

thus be attributed to egg white proteins, consistently with Hagolle et al. (2000) who showed that pre-heating treatment of ovalbumin solution enhanced its interfacial properties for temperature as low as 62°C. On the contrary, Wang and Wang (2009) noticed that egg white foam expansion and foam stability decreased after pasteurisation at higher temperature (62 or 64°C for 4 min). The increase of whole egg interfacial properties after pasteurisation at 60°C is thus probably due to a moderate denaturation of the proteins that make them more surface active. And the decrease of protein interfacial properties with the increase of pasteurisation rate above 60°C suggests protein aggregation, consistently with the previous results of SDS-PAGE analysis and the literature (Campbell et al., 2005; Montfort et al., 2012; Niewiarowicz et al., 1980). A pasteurisation at 60°C maximum is thus advised to optimize the emulsifying properties of whole egg.

3.3. Low pasteurisation treatments increase the *in vitro* digestibility of whole egg proteins

Regarding the degree of hydrolysis at the end of both gastric and intestinal *in vitro* digestion, pasteurisation time and time x temperature interaction have a significant effect on the degree of hydrolysis at the end of both gastric and intestinal stages (Table 1). At the end of gastric stage, samples pasteurised 4 minutes at 60, 62 or 64°C and 7 minutes at 60°C have higher or equal degree of hydrolysis than the control whereas higher pasteurised samples have lower degree of hydrolysis than the control (Figure 6). At the end of intestinal stage, samples pasteurised at 60°C (4, 7 and 10min), 4 minutes at 62 and 64°C and 10 minutes at 66°C have significantly higher degree of hydrolysis than the control. On the opposite, samples pasteurised 10 minutes at 62°C, 7 and 10 minutes at 64°C and 4 and 7 minutes at 66°C have a significantly lower degree of hydrolysis than the control (Figure 6).

Protein and peptide profiles of whole egg digests were characterized by SDS-PAGE, and quantified by the band volumes at the three digestion stages (gast ti, gast tf and int tf). To highlight the effect of pasteurisation on the progress of protein *in vitro* digestion, these data were submitted to MFA according

to a method previously reported by Lechevalier et al. (2015). The coordinates of the samples on the first two PCs were used to test the effect of pasteurisation rate on the progress of protein digestion.

Pasteurisation time and time x temperature interaction have a significant effect on the coordinates of the samples along "digestion MFA" PC1 and only pasteurisation time x temperature interaction has a significant effect on the coordinates of the samples along "digestion MFA" PC2 (Table 1). These PCs explain 20% and 18% of the dataset variability, respectively (Figure 7A). PC1 positively correlated to the band volumes of the 51, 31 and 10 kDa bands of gast tf stage, and 40, 31, 23, 22, and 14.5 kDa bands of int tf stage ($p < 0.05$). It also significantly negatively correlated to the band volumes of the 129 kDa bands of gast ti stage (Figure 7A). PC2 significantly ($p < 0.05$) positively correlated to the band volumes of the 106, 86 and 22 kDa bands of gast ti stage, and 86, 68 and 55 kDa bands of gast tf stage. It also significantly negatively correlated to the band volumes of the 159, 138, 97, 75 and 44 kDa bands of gast ti stage and 40 kDa band of gast tf stage (Figure 7A). Pasteurisation time also positively correlated with PC2 (correlation coefficients equal to 0.41). Thus, when the intensity of pasteurisation increased, the band volumes of the proteins positively correlated to PC2 also tended to increase, whereas the band volumes of those negatively correlated rather decreased.

PC2 is correlated to the band volume of samples of the gastric stages only; it is thus representative of the effect of the gastric step of the *in vitro* digestion. Its correlation with pasteurisation time suggests that the progress of protein digestion during the gastric stage depends on pasteurisation time. The band volumes of the samples of the intestinal stage are correlated only to PC1, which is thus representative of the intestinal stage of the *in vitro* digestion.

Partial point positions on the PC1-PC2 plot give information on SDS-PAGE profiles before digestion (red points), at the end of gastric stage (green points), and at the end of intestinal stage (blue points). The way from one point to another is representative of what happens during gastric (red arrows) and intestinal (blue arrows) stages.

Before digestion (Figure 7B red points), control sample and mid pasteurised samples (i.e. 7 and 10 min at 60°C and 62°C and 4 and 10 min at 64°C) are close to the centre of the plot whereas low pasteurised samples (i.e. 4 min at 60°C and 62°C and 7 min at 64°C) have negative coordinates along PC2 and high pasteurised samples (i.e. 4, 7 and 10 min at 66°C) have positive coordinates along PC2. Low (respectively high) pasteurised samples have thus higher (respectively lower) concentration of 159, 138, 97, 75 and 44 kDa components than the control group in agreement with the results of the PCA given in the section 3.1.

For most samples (except samples pasteurised for 10 min at 60°C and for 7 and 10 min at 66°C), red arrows are mostly vertical suggesting that during the gastric stage, changes occurred according to PC2. For most samples, partial points at the end of gastric stage (Figure 7B, green points) are farer from the centre of the plot than the corresponding partial points before digestion. Gastric stage thus increases electrophoretic pattern heterogeneity. At the end of gastric stage (Figure 7B, green points), control and low pasteurised samples (i.e. 4 and 7 min at 60°C and 62°C and 7 min at 64°C) have negative coordinates along PC2 whereas mid and high pasteurised samples (4 min at 64°C, 7 min at 66°C and 10 min at 60, 62, 64 and 66°C) have positive coordinates along this PC. Control and low pasteurised samples have thus lower concentration of 86, 68 and 55 kDa components but higher concentration of 40 kDa component than the average whereas it is the opposite for mid and high pasteurised samples. Electrophoretic band at 68 and 55 kDa were attributed to apo-LDL + γ -livetin and phosvitin + α -livetin + apo-LDL, respectively, by Freschi et al. (2011) and Guilmineau et al. (2005). These proteins seemed thus more hydrolysed in control and low pasteurised samples than in mid and high pasteurised samples. The reduced hydrolysis observed for mid and high pasteurised samples during the gastric phase may be attributed to aggregation induced by heat treatment that can mask enzyme cleavage sites as suggested by Nyemb et al. (2014).

The effect of intestinal stage is given by blue arrows. For most samples (except control, 7 and 10 min at 60°C and 7 min at 64°C), the partial points at the end of intestinal stage (blue points) get closer to the centre of the plot i.e. the volumes of the bands correlated to PC1 and PC2 are closer to the mean. The intestinal stage thus reduces the electrophoretic pattern heterogeneity. At the end of digestion (Figure 7B, blue points), the 40, 31, 23, 22 and 14.5 kDa components are in higher quantity in low pasteurised samples (4, 7 and 10 min at 60°C, 10 min at 62°C, 4 min at 64 °C) than in the control one. Higher pasteurised samples are not significantly different from the control one. The 40, 23 and 22 kDa components appeared with hydrolysis, whereas the 44, 31 and 14.5kDa components corresponded to ovalbumin, apo-HDL and lysozyme, respectively (Freschi et al., 2011; Guérin-Dubiard et al., 2006). However, some peptides coming from the hydrolysis of higher molecular weight proteins or aggregates can also correspond to these molecular weights so that it is not possible to conclude to a lower degree of hydrolysis of ovalbumin, apo-HDL and lysozyme in low pasteurized samples. Nevertheless, Van der Plancken et al. (2002) showed that heat treatment below 78°C had negligible effects on ovalbumin susceptibility to enzymatic hydrolysis even if heat treatment had more effect when it was performed on egg white rather than on purified ovalbumin. Few data are available in the literature on the effect of heat treatment on egg protein susceptibility to *in vitro* digestion and most of them have been obtained on purified proteins and for much more drastic treatments (Jimenez-Saiz et al., 2011; Martos et al, 2011; Nyemb et al, 2014). Only, Schmidt et al. (2007) showed that whole egg spray drying increased its sensitivity to digestion (expressed as the fraction of peptide smaller than 12-14 kDa). However, spray drying conditions are hardly comparable to pasteurisation ones. Regarding digestion, rather low pasteurisation intensity (up to 10 min at 60°C, 10 min at 62°C and 4 min at 64°C) should finally be recommended to improve protein digestion.

3.4. Medium pasteurisation treatments prevent an increase of whole egg protein antigenicity

Pasteurisation temperature, time and their interaction have a significant effect on ovomucoid antigenicity tested using ELISA inhibition method (Table 1). Ovomucoid apparent concentration clearly decreased with pasteurisation time whatever the temperature (Figure 8A), such as it was lower than that of the control sample for all samples pasteurised for 10 minutes. Such a decrease in protein antigenicity after heat treatment has been described for many allergens since protein conformation and thus conformational epitopes are often modified by heating (Thomas et al., 2007). However, this result was more surprising in the case of ovomucoid since many studies reported the resistance of ovomucoid antigenicity to heat treatment in solution (Fu et al., 2010; Jimenez-Saiz et al., 2011; Kosti et al., 2013; Shin et al., 2013; Urisu et al., 1997). Only Mine & Zhang (2002) observed a decrease of ovomucoid antigenicity after heating 15 min at 95°C in solution. Such discrepancies between studies could be attributed to the different sources of antibodies and techniques used (Julia et al., 2007). Especially, the nature of monoclonal antibodies that are used is determining, since such antibodies recognize specific and small sequences of the proteins which can be, or not modified by heat treatments.

Pasteurisation time and temperature x time interaction also have a significant effect on lysozyme and ovotransferrin antigenicity (Table 1). Lysozyme apparent concentration appears higher in sample pasteurised at 60 and 62°C than in the control sample (Figure 8B). However, these concentrations were consistent with the theoretical concentration of lysozyme in whole egg (2.4 g.l⁻¹). For higher pasteurisation, ie at 64 and 66°C, lysozyme antigenicity decreased when temperature and/or time increased. This is consistent with the fact that egg cooking reduced egg allergenicity, even if pasteurisation treatment occurred at lower temperatures (Lemon-Mulé et al., 2008; Mine & Yang, 2008).

Ovotransferrin antigenicity clearly increased after pasteurisation at 66°C (Figure 8C), and longer the time at this temperature, higher the antigenicity, consistently with Tong et al. (2012) who showed that the potential antigenicity of ovotransferrin increased with the unfolding of its structure after heat treatment up to 1 hour at 55-60°C.

Finally, medium pasteurisation intensity (64°C) should be recommended to keep ovomucoid, lysozyme and ovotransferrin antigenicity as low as possible.

3.5. Which conditions of pasteurisation to simultaneously optimize protein interfacial properties, antigenicity and *in vitro* digestibility ?

To consider all together the results of protein interfacial properties, antigenicity and *in vitro* digestibility, MFA was performed on the dataset that contained all the previously described measurements, with the row representing the 12 pasteurised samples and the control. Pasteurisation temperature and time were added as supplementary variables. Four groups of measurements were constituted: “protein profiles”, “protein interfacial properties”, “protein digestion” and “protein antigenicity” as described in section 2.5.3. PC1 and PC2 respectively explain 32% and 18% of the dataset variability. PC1 was significantly positively correlated to ovotransferrin antigenicity, electrophoresis band volume of the 129 and 106 kDa components before digestion, digestion MFA-PC2, d_{4.3} and creaming index (Cri) (Figure 9A). It was also negatively correlated to lysozyme antigenicity, S₁₀, digestion MFA-PC1, DH gast and DH int and electrophoresis band volume of the 51 and 138 kDa components before digestion. PC1 thus contrasted on the right side samples with poor interfacial properties, weak lysozyme antigenicity but strong ovotransferrin antigenicity and poorly hydrolysed during both gastric and intestinal stages of *in vitro* digestion, with on the left side samples with good interfacial properties, strong lysozyme antigenicity but weak ovotransferrin antigenicity and highly hydrolysed during both gastric and intestinal stages of *in vitro* digestion.

PC2 was significantly positively correlated to the electrophoresis band volume of the 175, 159, 138, 97, 94 and 75 kDa components before digestion and d_{4.3} and negatively correlated to the electrophoresis band volume of the 198 and 22 kDa components before digestion and digestion MFA-PC2. PC2 thus contrasted on the upper side samples with aggregates after pasteurisation, poor emulsifying properties and highly hydrolysed during the gastric stage of *in vitro* digestion, with on the lower side samples with

less aggregates after pasteurisation, good emulsifying properties and poorly hydrolysed during the gastric stage of *in vitro* digestion.

As shown in Figure 9B, PC1 contrasted the samples pasteurised at 66°C (right side) with the samples pasteurised at 60°C (left side). The control and the samples pasteurised at 62 and 64°C were in the middle. PC2 contrasted the samples pasteurised 7 min at 64°C (upper side), with the samples pasteurised 7 and 10 minutes at 60°C and all the samples pasteurised at 66°C (lower side), other samples being in the middle (Figure 9B).

Samples pasteurised at 60°C had thus the best interfacial properties since they were more surface-active (S_{10} increases) and generated more stable emulsions (Cr_i decreased) with smaller droplets ($d_{4,3}$ decreased). They were also the more sensitive to protein *in vitro* digestion since they had the highest DH_{gast} and DH_{int} and the highest concentration in low molecular weight components at the end of intestinal stage as suggested by the positive correlation with digestion MFA-PC1 (Figure 9A). At least, pasteurisation at 60°C increased slightly lysozyme antigenicity but kept ovotransferrin antigenicity to a low level compared to the other samples. An increase in pasteurisation temperature then increased samples coordinates on global MFA-PC1 (Figure 9B). Increasing pasteurisation temperature thus decreased interfacial and emulsifying properties, protein digestibility, lysozyme antigenicity but increased ovotransferrin antigenicity.

Finally, thanks to multiple factorial analysis that enabled to consider all the dimensions of the study at once, a good compromise to improve the interfacial properties of whole egg, without enhancing too much protein resistance to digestion and/or protein antigenicity could be proposed: low pasteurisation treatment of 4 to 10 min at 60°C. However, for hygienic reasons, pasteurisation can also be performed during 4 to 10 min at 62 and 64°C without excessively damaging functional properties or enhancing protein resistance to digestion and/or protein antigenicity. On the contrary, pasteurisation at 66°C should be avoided to prevent resistance to digestion and ovotransferrin antigenicity increase.

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ACCEPTED MANUSCRIPT

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Figure Captions

Figure 1: *In vitro* digestion of whole egg followed by SDS PAGE analysis for gastric (A) and intestinal (B) stages. Lane frames (gast ti, gast tf and int tf) correspond to digestion times used for quantification by Imagequant software. Bands marked with spots correspond to the quantified bands. Ovotransferrin (ovt), ovalbumin (ova) and lysozyme (lyso) bands are identified. The electrophoresis gel presented here as example show the digestion of whole egg pasteurised 4 min at 62°C.

Figure 2 :

A) Projection of variables (electrophoresis band volumes identified by their molecular weight) onto the plane defined by the first two principal components of Principal Component Analysis (PCA). The coordinates of each variable are the correlation coefficients with the two first principal components. The variable significantly correlated with PC1 and/or PC2 are written in bold and underlined, respectively. B) PCA map of the individuals projected on the 2D plane defined by PC1 and PC2.

Figure 3: Surface pressure – surface area isotherms obtained by the compression of the film after adsorption during 30 min of 15 µg whole egg protein: comparison of the 12 pasteurisation treatments and the control sample.

Figure 4: Droplet size distribution of emulsions (O:W / 30:70) prepared with 1.5% whole egg proteins as a function of pasteurisation temperature and time. Samples with different letters are significantly different (LSD test, $p < 0.05$).

Figure 5: Index of creaming of emulsions (O:W / 30:70) prepared with 1.5% whole egg proteins as a function of pasteurisation temperature and time. Samples with different letters are significantly different (LSD test, $p < 0.05$).

Figure 6: Degree of hydrolysis at the end of gastric stage (plained bars) and intestinal stage (hatched bars) of whole egg proteins *in vitro* digestion, as a function of pasteurisation temperature and time. Samples with different letters are significantly different (lower case and upper case letters are used for DH gast and DH int, respectively) (LSD test, $p < 0.05$).

Figure 7: A) Projection of variables (electrophoresis band volumes before digestion (red), at the end of gastric stage (green), and at the end of intestinal stage (blue)) onto the plane defined by the first two principal components of Multiple Factor Analysis (MFA). The coordinates of each variable are the correlation coefficients with the two first principal components. The closest the arrow is to the circle, the best the variable is represented. Considering the variables correctly represented, the correlations between two variables are all the most high given that the angle between their directions is small. B) MFA map of the individuals projected on the 2D plane defined by PC1 and PC2. For reason of clarity, MFA map of the individuals was split into 4 graphs: control and samples pasteurised at 60°C (B1), 62°C (B2), 64°C (B3), 66°C (B4). Mean points (in black) are the centroid of the three partial points, each representing the individual described by only one group: gast ti (red), gast tf (green), int tf (blue). Arrows between partial points represent electrophoresis profile changes during gastric stage (red arrows) and intestinal stage (blue arrows).

Figure 8: Ovomucoïd (A), lysozyme (B) and ovotransferrin (C) apparent concentration determined by ELISA inhibition as a function of dry heating treatment intensity. Samples with different letters are significantly different (LSD test, $p < 0.05$).

Figure 9: A) Projection of variables onto the plane defined by the first two principal components of the global Multiple Factor Analysis (MFA). The coordinates of each variable are the correlation coefficients

with the two first principal components. The closest the arrow is to the circle, the best the variable is represented. Considering the variables correctly represented, the correlations between two variables are all the most high given that the angle between their directions is small. B) MFA plot of the individuals projected on the 2D plane defined by PC1 and PC2. Mean points (in black) are the centroid of the four partial points, each representing the individual described by only one group (data not shown).

Table 1: Significant levels of variables and their interaction for the different analytical parameters, and model R² and p-value.

	Temperature	Time	Temperature x Time	R ²	p-value
PCA PC1	NS	NS	NA	0.73	0.12
PCA PC2	NS	NS	NA	0.22	0.92
S 10	***	NS	NS	0.77	0.0088
d4/3	**	***	**	0.91	7 10⁻⁵
Cr _i	***	NS	NS	0.92	4 10⁻⁵
Lysozyme immunoreactivity	***	**	NS	0.94	3 10⁻¹³
Ovomucoid immunoreactivity	***	***	NS	0.62	3 10⁻⁵
Ovotransferrin immunoreactivity	***	*	***	0.92	2 10⁻¹¹
DH gast	***	***	***	0.99	< 2 10⁻¹⁶
DH duo	***	***	***	0.99	< 2 10⁻¹⁶
Digestion MFA PC1	NS	*	*	0.49	0.09
Digestion MFA PC2	NS	NS	*	0.48	0.10

* significant effect at P < 0.05; ** significant effect at P < 0.01; *** significant effect at P < 0.001; NS, no significant effect; NA, non available

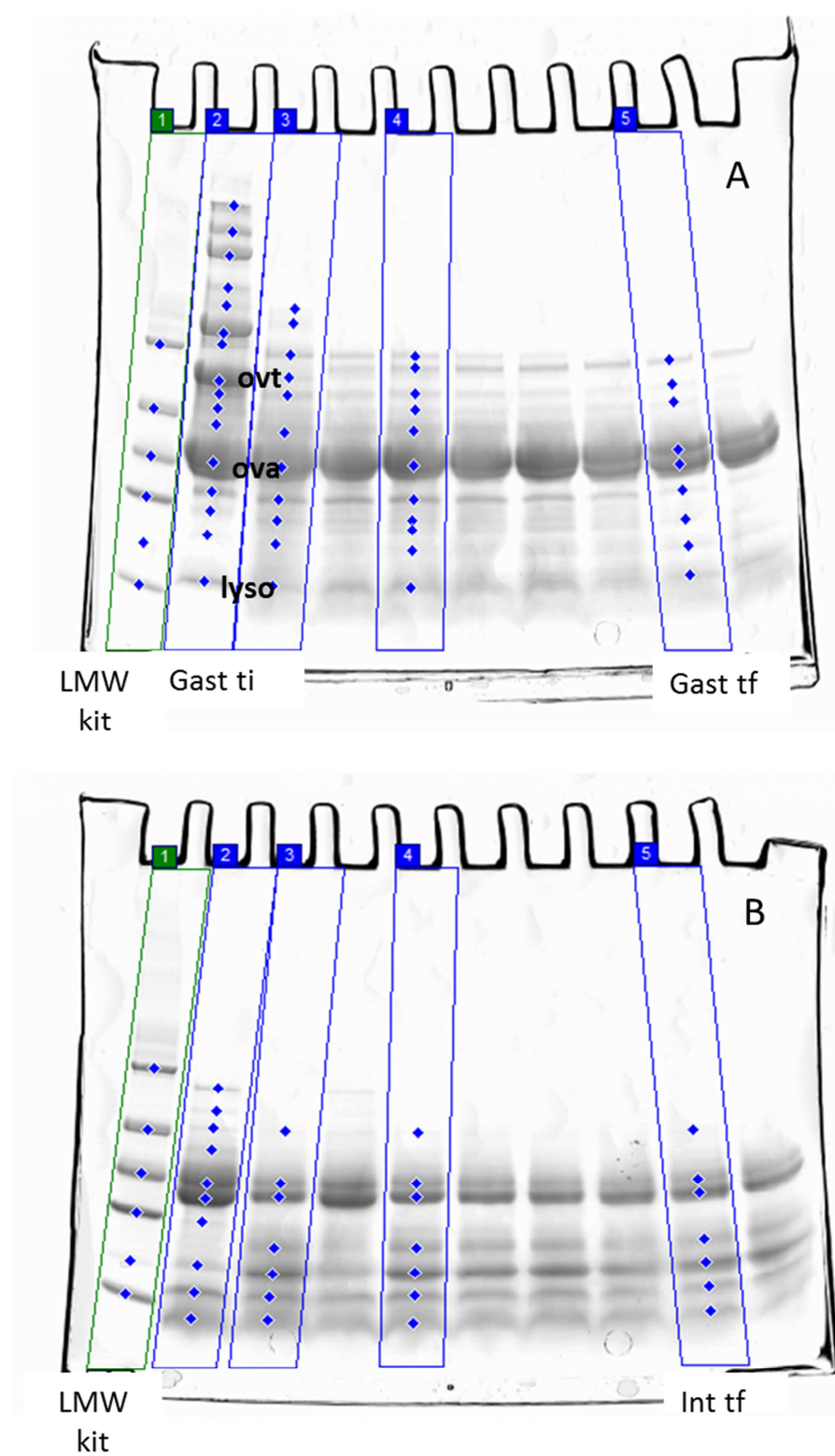


Figure 1

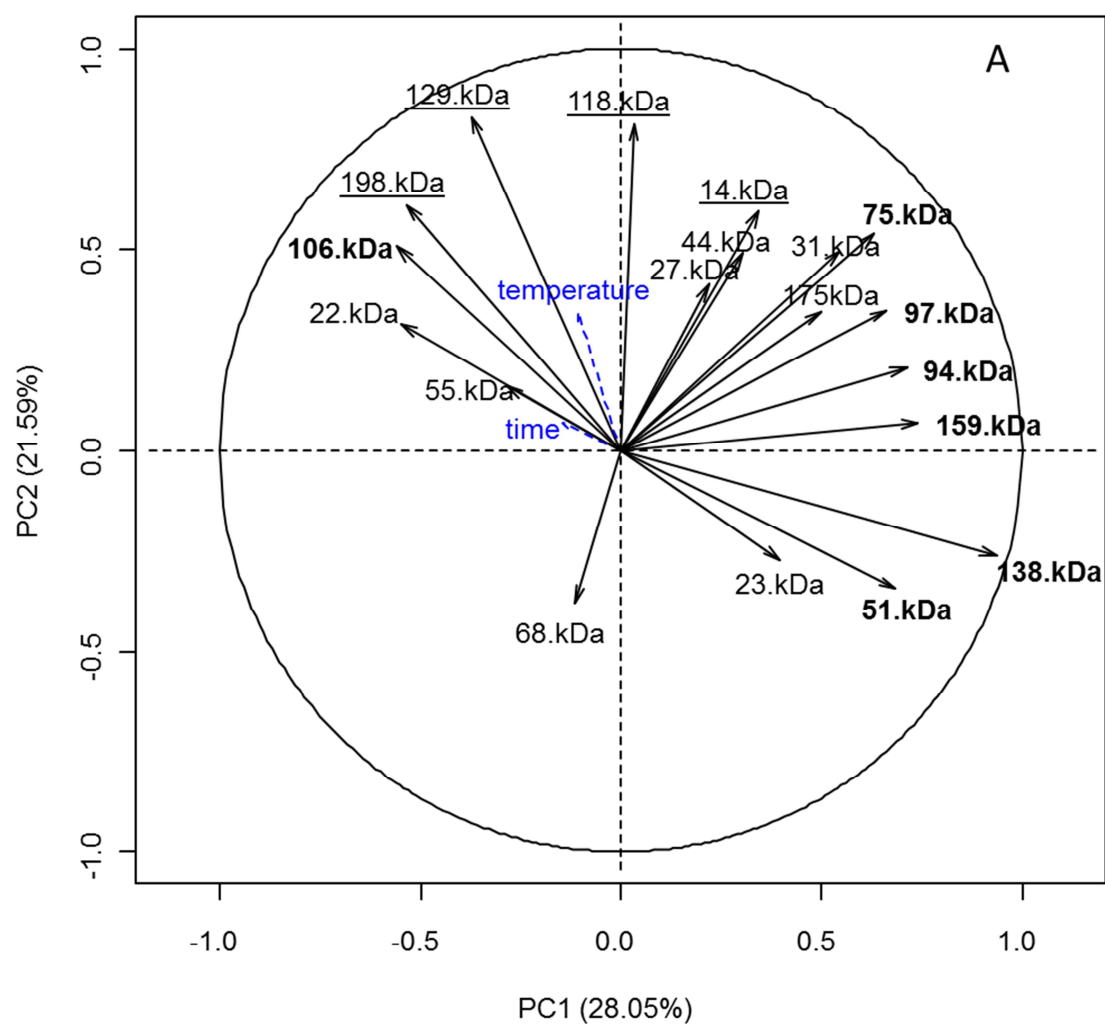


Figure 2A

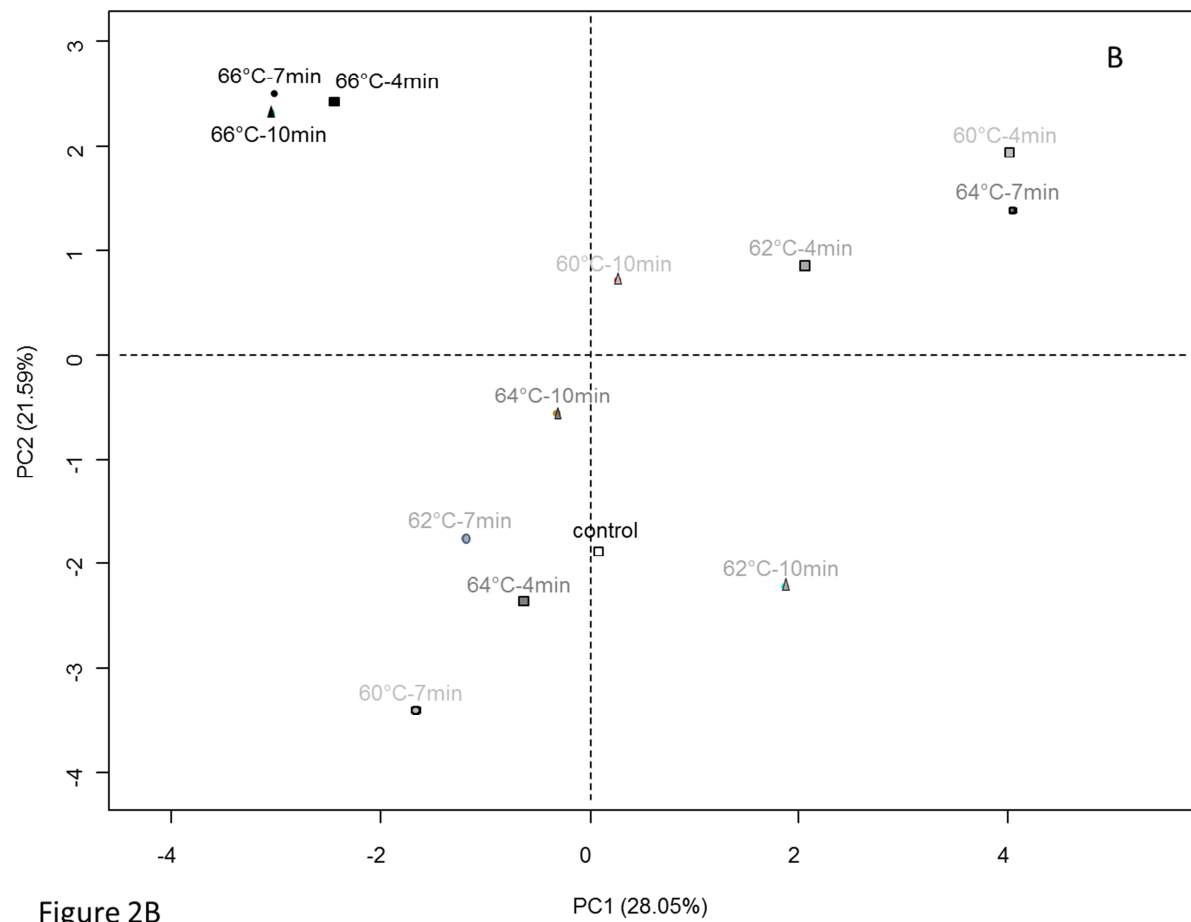


Figure 2B

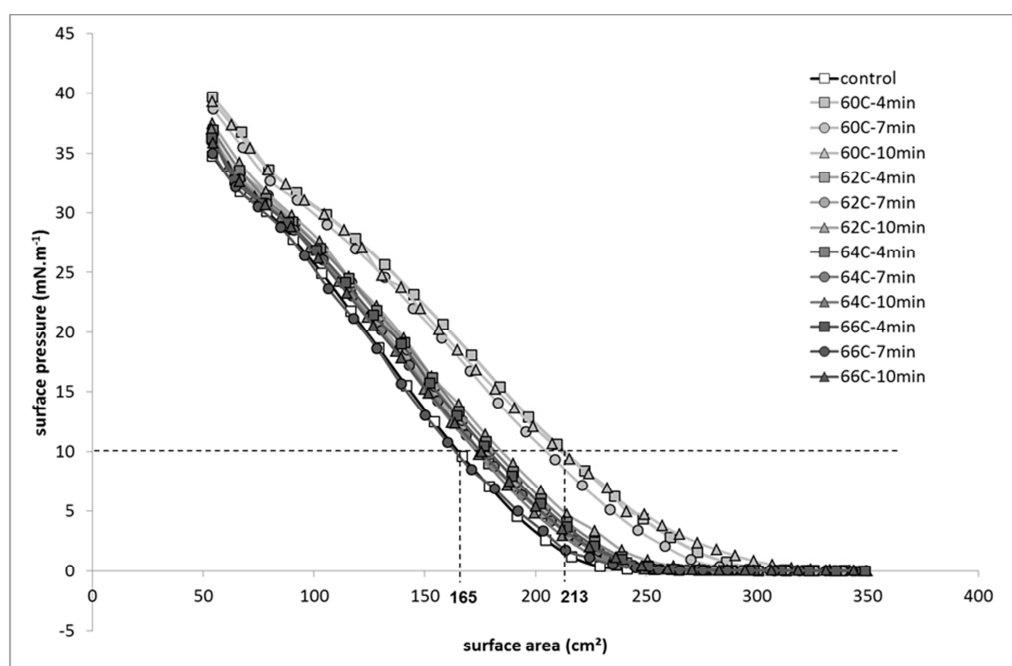


Figure 3

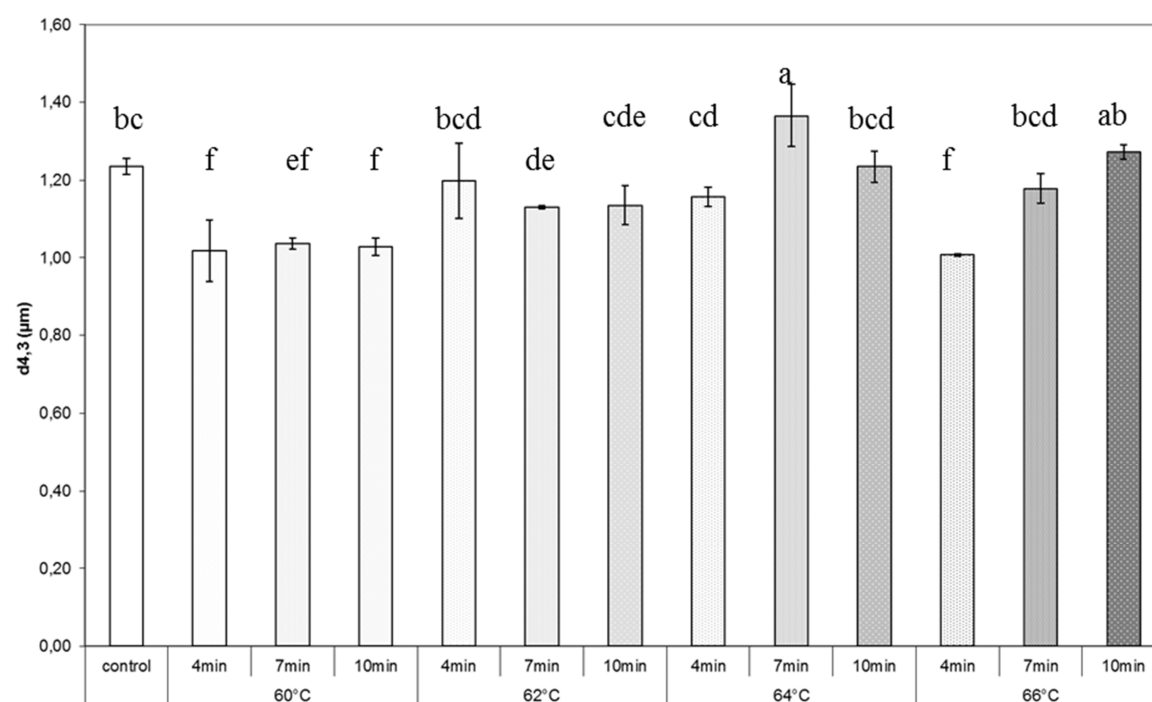


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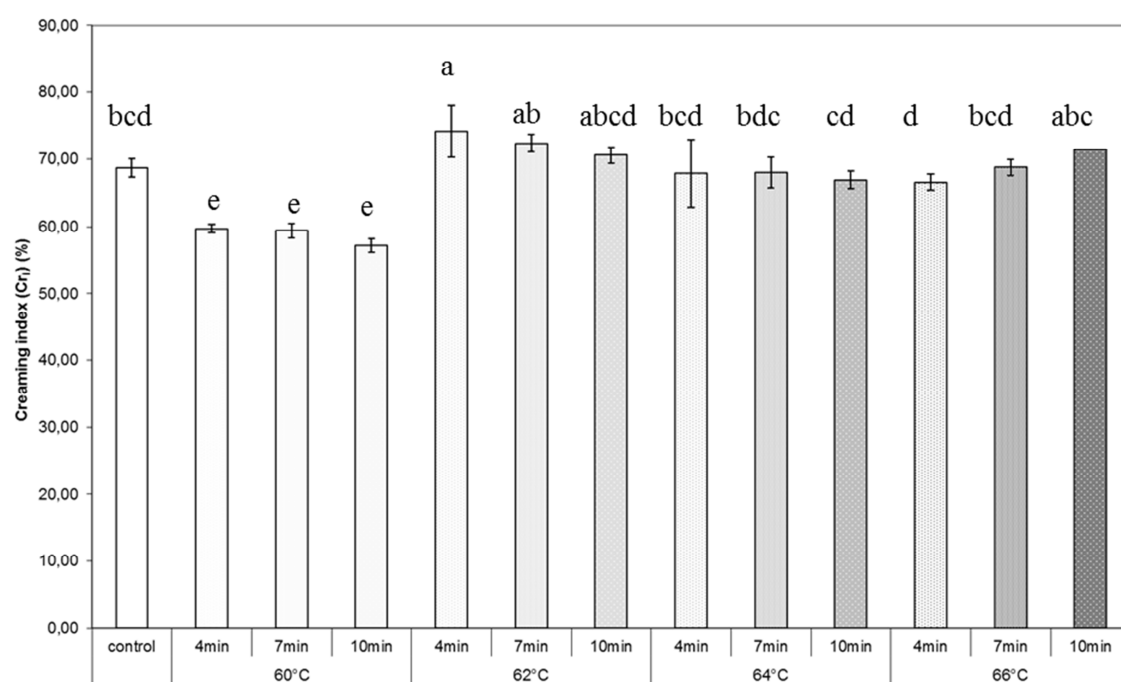


Figure 5

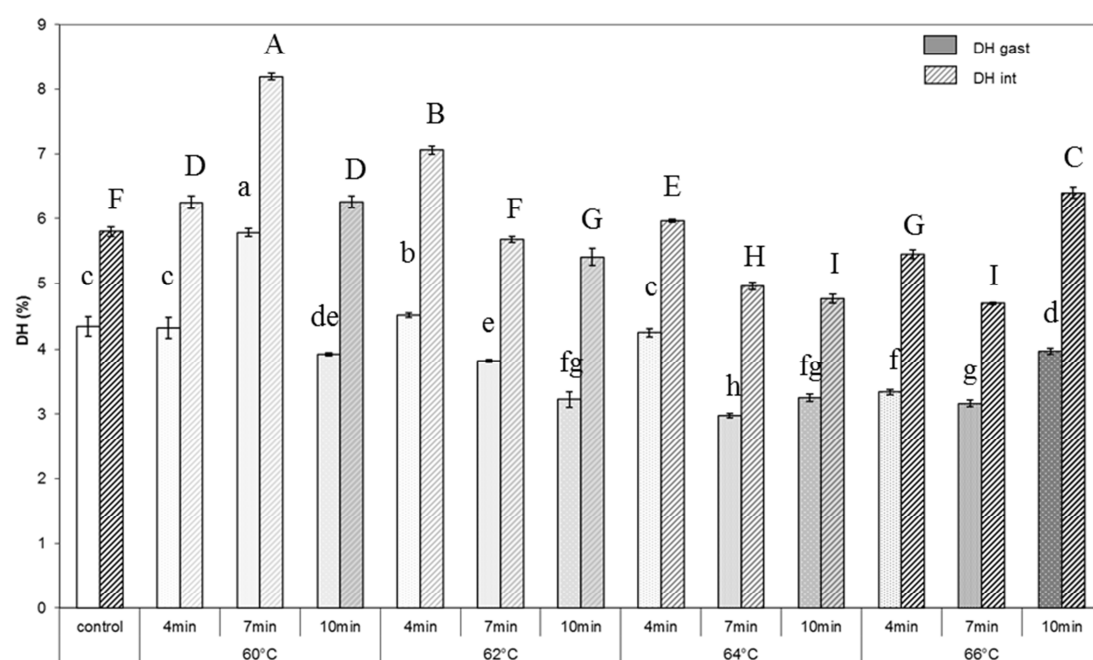


Figure 6

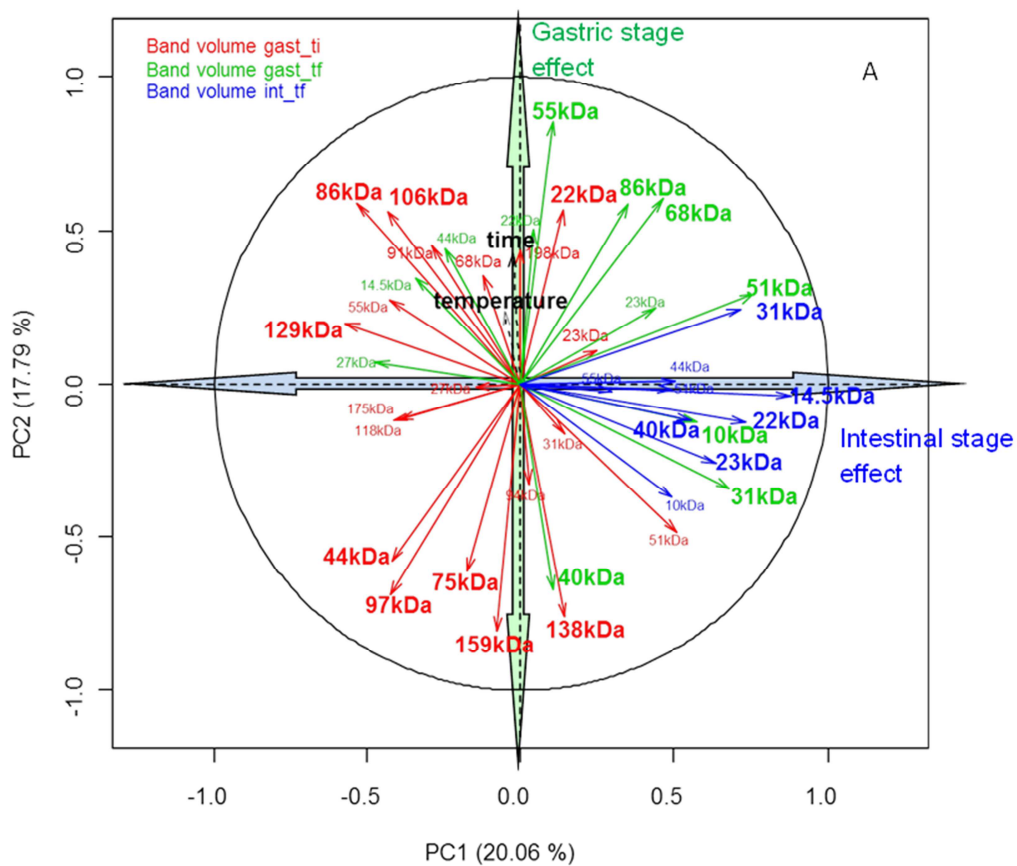


Figure 7A

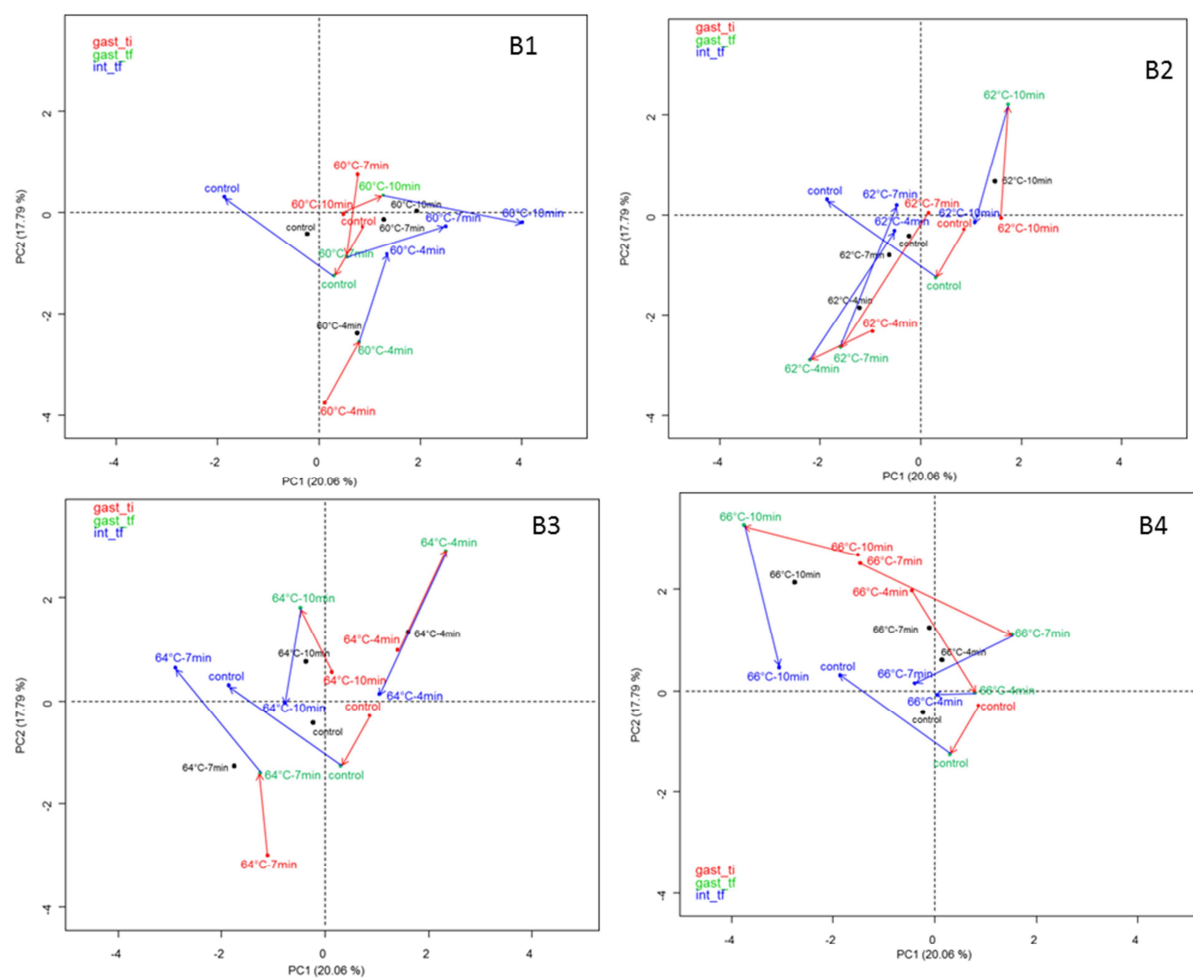


Figure 7B

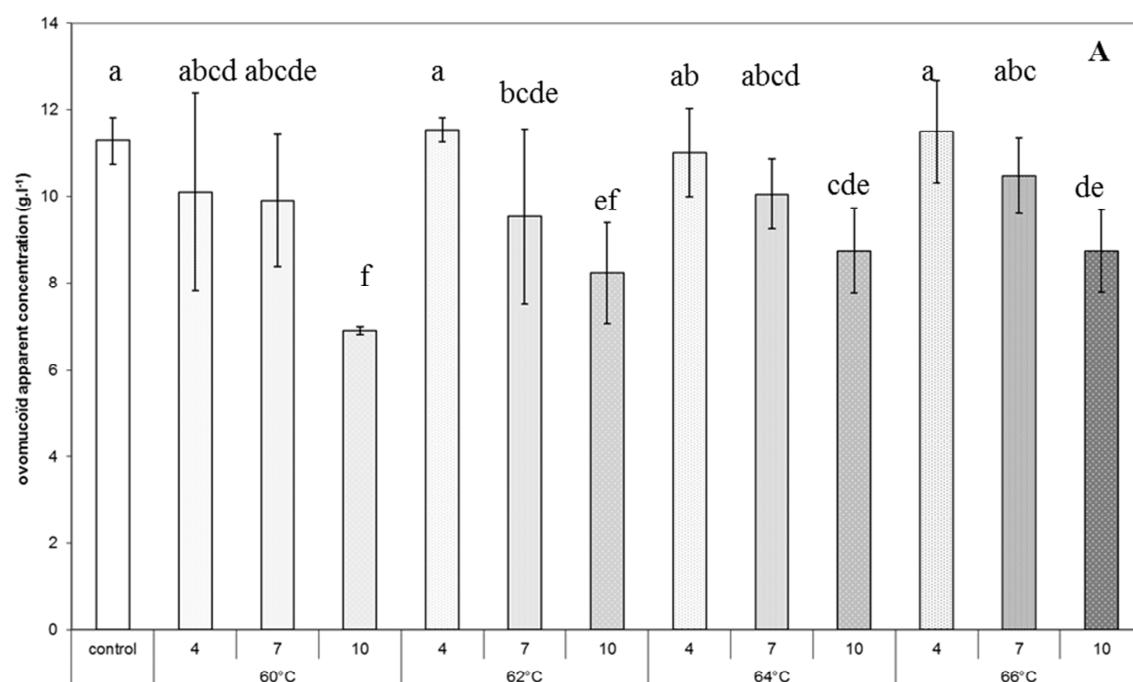


Figure 8A

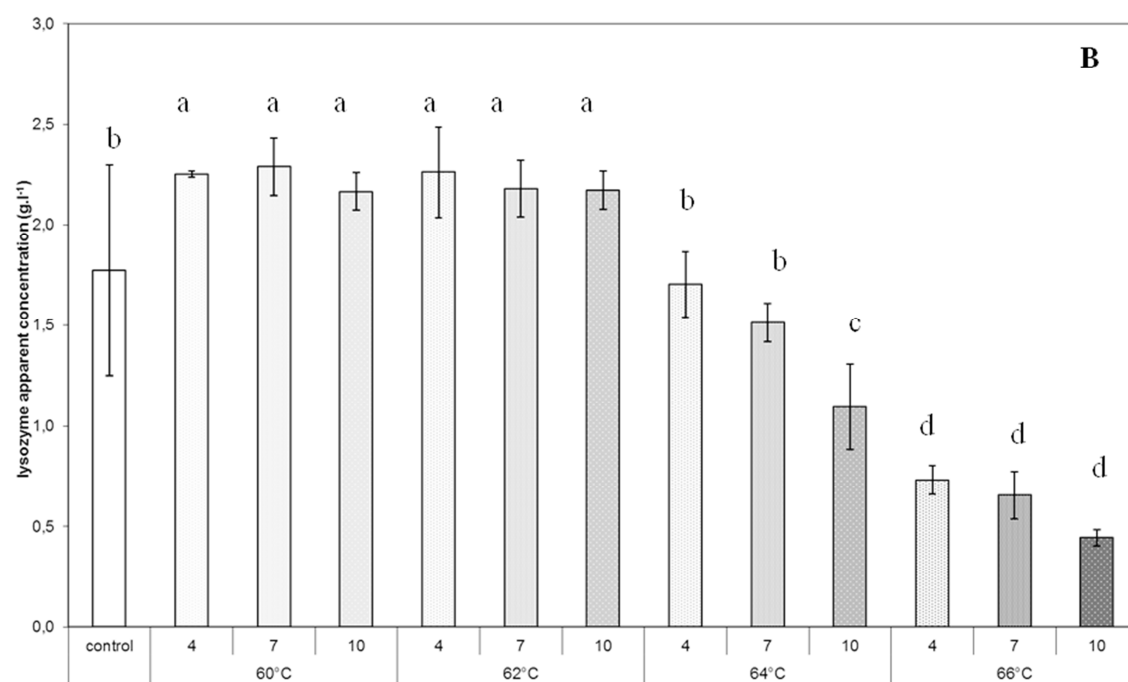


Figure 8B

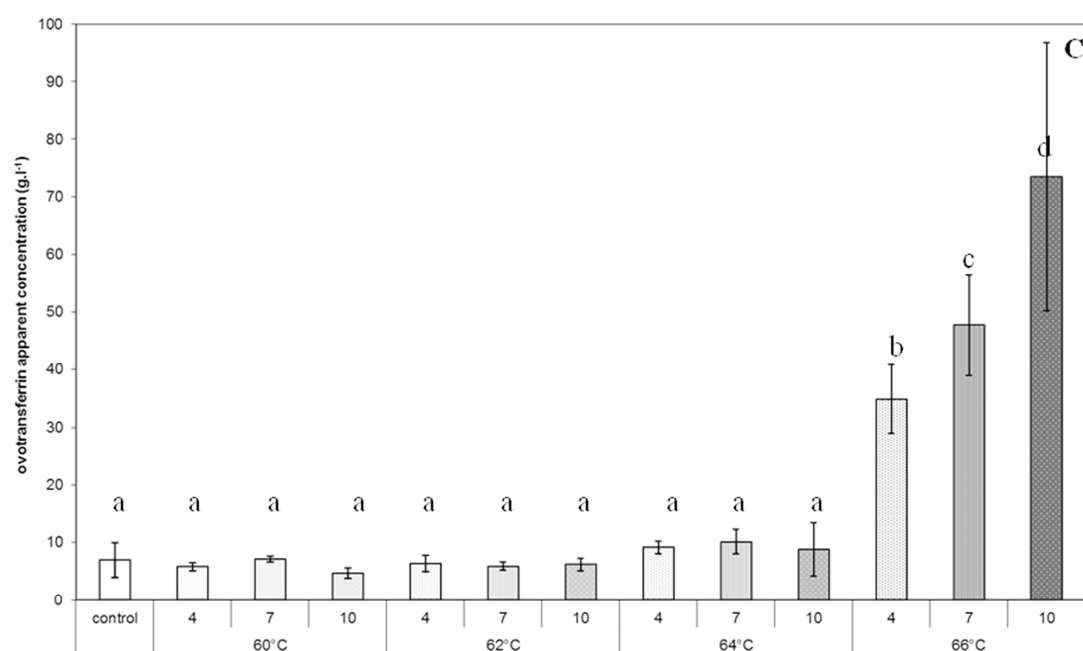


Figure 8C

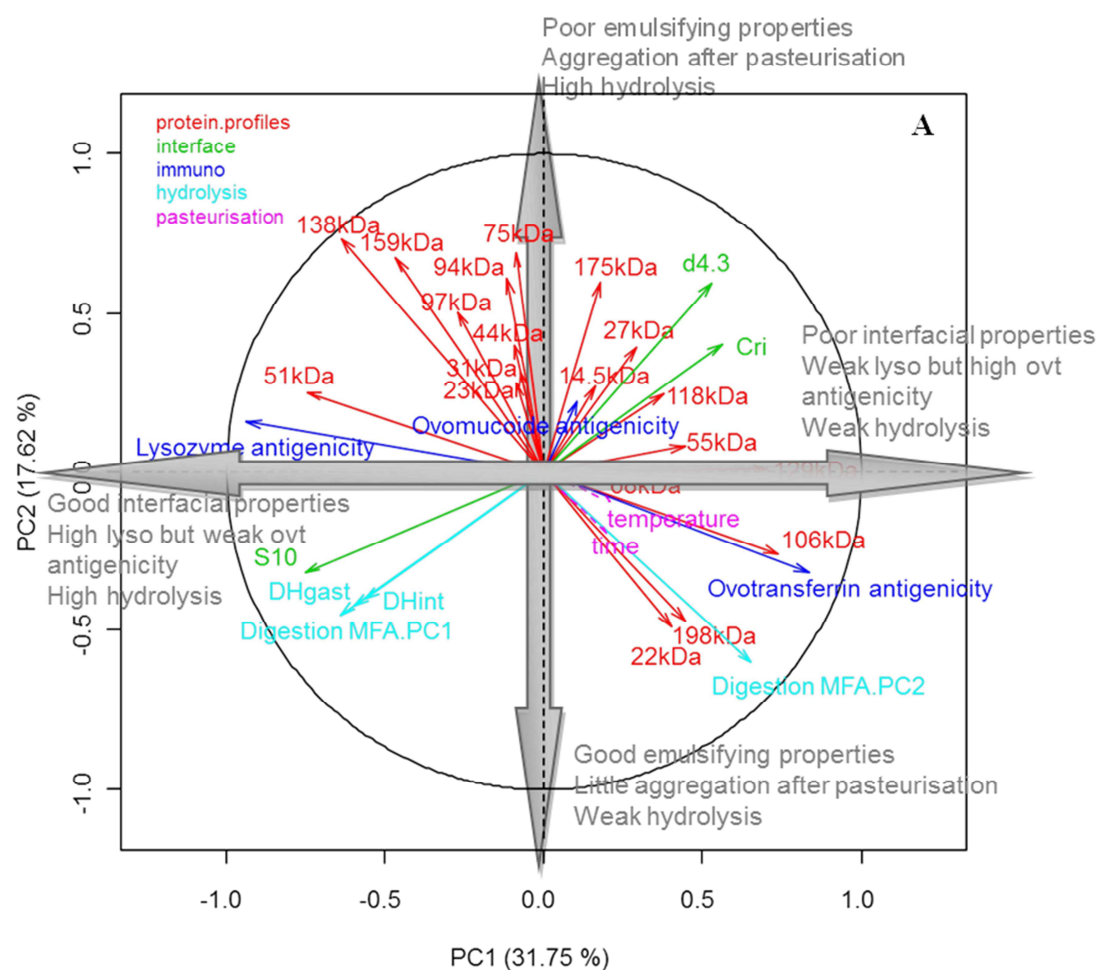


Figure 9A

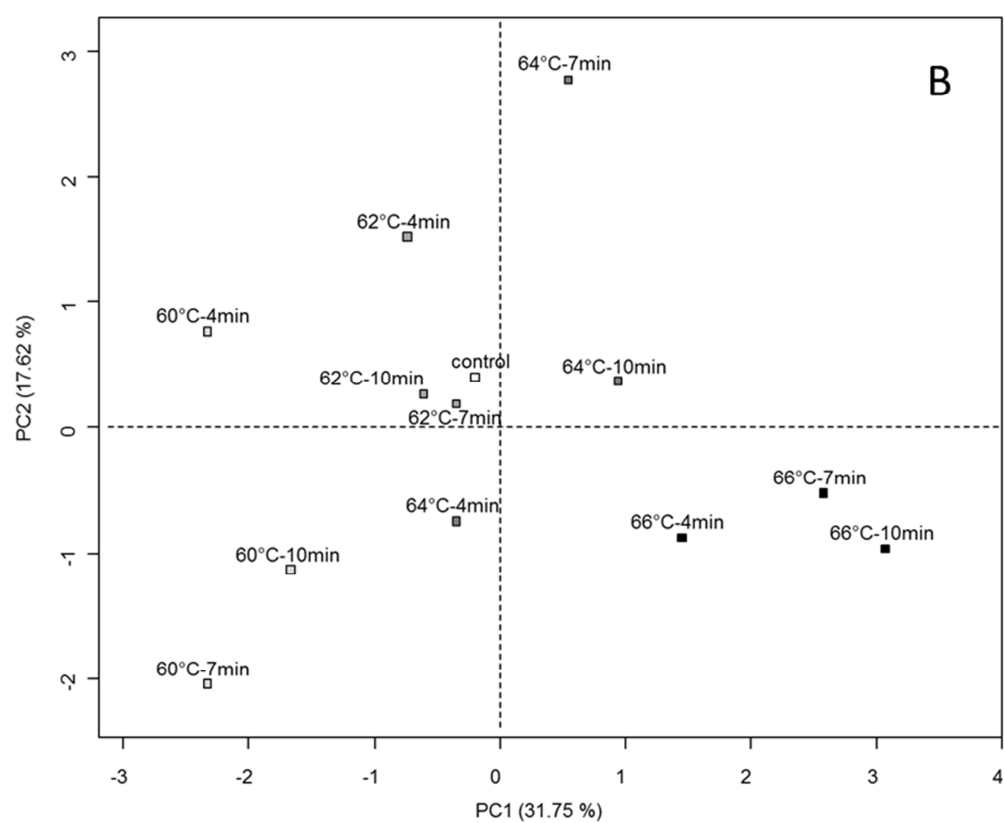


Figure 9B

Highlights:

Pasteurisation up to 10 min at 60°C improves whole egg functionalities

Pasteurisation between 62 and 64°C (up to 10min) preserves whole egg functionalities

Pasteurisation over 64°C decreases whole egg functionalities