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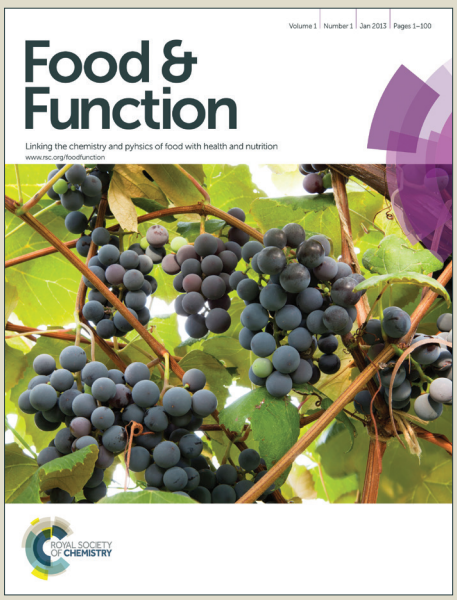

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Effects of dry heating on the progression of *in vitro* digestion of egg white protein: contribution of multifactorial data analysis.

Valerie Lechevalier*[a,b], Nuttinee Musikaphun[a,b], Angelique Gillard[a,b], Maryvonne Pasco[a,b], Catherine Guerin-Dubiard[a,b], Francois Husson[c], Francoise Nau[a,b]

[a]Agrocampus Ouest, UMR1253 STLO, F-35042 Rennes, France

[b]Institut National de la Recherche Agronomique (INRA), UMR1253 STLO, F-35042 Rennes, France

[c].Agrocampus Ouest, Applied Mathematics Department, F-35042 Rennes, France

*corresponding author : Valerie Lechevalier, Agrocampus Ouest UMR1253 STLO, 65 rue de Saint Brieuc, CS 84215, 35042 Rennes cedex, France

Tel : +33 (2) 23 48 58 95

Fax : +33 (2) 23 48 53 50

E-mail address :valerie.lechevalier@agrocampus-ouest.fr

maryvonne.pasco@agrocampus-ouest.fr

catherine.guerin@agrocampus-ouest.fr

francois.husson@agrocampus-ouest.fr

francoise.nau@agrocampus-ouest.fr

Abstract

The impact of dry heating on the progression of *in vitro* digestion of egg white proteins was investigated through application of multiple factor analysis (MFA) to electrophoresis data. Dry heating (from 1 to 10 days between 60 and 90°C) enhanced protein unfolding and aggregation, thus generating different SDS-PAGE patterns for each sample before digestion. The progression of *in vitro* digestion was then modified according to the degree of protein unfolding and/or aggregation.*In vitro* digestion tended to decrease the heterogeneity of sample electrophoretic patterns overall but it occurred either at the very beginning of the gastric stage or throughout the gastric stage or again during the duodenal stage, depending on the heat treatment to which the sample had been submitted. At the end of digestion, three groups of samples were obtained: all samples dry heated at 60°C and one sample dry heated for1 day at 70°C that were more hydrolysed than the control, samples dry heated for more than 2 days at 80°C or 90°C that were less hydrolysed than the control, and samples dry heated for more than 2 days at 70°C or 1 day at 80 or 90°C that were as hydrolysed as the control.

Keywords: egg white, dry heating, *in vitro* digestion, electrophoresis, principal component analysis, multiple factor analysis

1. Introduction

For centuries, and throughout the world, the egg has had an excellent reputation and has been used in many traditional dishes. It is still one of the most frequently consumed animal products, and is remarkable for its high functional and nutritional qualities. In particular, the protein content of eggs is high; 100 g of whole egg supplies about 30% of an adult man's protein requirements. Moreover, the quality of egg protein is high, because of its amino acid quality and its digestibility[1]. However, it has been established that protein quality cannot be evaluated solely on the ability to provide essential amino acids for protein synthesis. Several criteria such as physiological and metabolic responses to protein intake have also to be taken into account. For instance, digestion kinetics have been reported to play a major role in the nutritional quality of proteins[2, 3]. Then, any factor likely to modify digestion kinetics may have an impact on the nutritional quality of the proteins. In particular, although it is seldom studied, the consequences of food processing must be taken into consideration. For example, Lacroix *et* al[4] showed that spraying affects the protein quality of milk due to a high degree of protein lactosylation.

Egg white proteins are particularly sensitive to technological processes such as heating, freezing and drying[5]. The structural modifications thus induced may have both desirable and undesirable effects on protein susceptibility to digestive enzymes[6]. However, current results are not sufficient to prove or to disprove that such processing may affect the nutritional quality of egg proteins. In the study presented here, the consequences of dry-heating on the *in vitro* digestibility of egg white proteins were investigated. Dry heating, i.e. heating of a powder, is specifically and usually performed in the egg processing industry in order to ensure the microbiological safety of egg white powders. When long times and high temperatures are applied, dry heating also improves egg white foaming and gelling properties[7]. In the present study, several combinations of dry-heating temperature and time were tested in the ranges applied in the egg industry, and *in vitro* digestion of egg white proteins was followed by electrophoresis.

Electrophoresis is widely used to follow enzymatic protein digestion. However, the interpretation of electrophoresis gels is mainly qualitative, except when simplified systems such as purified proteins are studied; quantitative analysis is then sometimes performed[8]. However, when digestion is performed on real food, huge amounts of data are generated rapidly which make interpretation challenging. For example, although egg white is quite a simple food, it contains up to 158 proteins[9], 13 being easily detected by SDS-PAGE[10]. Including peptides generated by *in vitro* digestion, simultaneous analysis of around 20 electrophoresis bands is therefore required to follow egg white proteolysis by SDS-PAGE. Moreover, in order to follow the effects of an external factor (such as dry heating in the present study) on the progress of *in vitro* digestion, sampling has to be performed several times throughout digestion for each condition. A large data set is thus generated, which requires a global statistical analysis approach.

The classical data analysis is a univariate approach for which the protein bands are analysed one by one. However, this approach does not take into account all the information present in the experiment, and it may be negatively affected by the raw structure of electrophoretic data[11] (variability due to gel staining and image analysis, large number of missing data, few replications). The use of multivariate methods such as Principal Component Analysis (PCA) has spread in the past few years because it reduces the complexity of the data set and it is less affected by data distribution or variance heterogeneity[12]. However, PCA introduces only one set of variables as active, the others being illustrative. With this strategy, the only multidimensional structure really handled is that of the active variables, and the illustrative variables act independently. However, under specific circumstances, such as when following several samples at different times during a given process (digestion in the present study), it may be desirable to consider several sets of variables simultaneously as active elements, in order to take all of them into account in the definition of distance between individuals. Multiple Factor Analysis (MFA) makes this possible[13].

In the study presented here, first PCA, and then MFA were applied to follow the effects of dry-heating temperature and time on the changes in the electrophoretic

pattern of egg white protein during *in vitro* digestion. It is of note that a similar approach and strategy could be transferred to many measurements performed to study kinetic phenomena.

2. Materials and methods

2.1. Experimental design

2.1.1. Chemicals and materials

A premixed stock solution of acrylamide/bis-acrylamide 37.5:1 (2.6% C), ammonium persulfate, glycine, N,N,N',N'-tetra-methyl-ethylenediamine (TEMED), mini-PROTEAN® TGX™ Precast Gels (4-20 % polyacrylamide) and Bio-Safe Coomassie stain™ were purchased from Biorad (Marnes-la-Coquette, France). Tris, bis-tris, ammonium carbonate, glycerol, sodium dodecylsulfate (SDS), dithiothreitol (DTT), sodium carbonate, sodium taurocholate, sodium glycodeoxycholate, Bowman Birk (trypsin chymotrypsin inhibitor), bromophenol blue, glycin, porcine pepsin (EC 3.4.23.1, activity: 4223 U/mg), bovine chymotrypsin (EC 232-671-2, activity: 55 U/mg, porcine trypsin (EC 232-650-8, activity: 14230 U/mg) were purchased from Sigma (St Quentin Fallavier, France). Low Molecular Weight markers were purchased from GE Healthcare Europe GmbH (Velizy-Villacoublay, France).

2.1.2. Samples

Egg white protein powder was supplied by Igreca (Seiches sur le Loir, France). The powder was aliquoted into 17 samples of 100 g. Each sample was submitted to one dry heating treatment according to the following experimental design: complete factorial design 4^2 with 4 temperatures (60, 70, 80 and 90°C) and 4 durations (1, 2, 5 and 10 days). The control sample was maintained at 20°C.

2.1.3. *In vitro* digestion

Each dry-heated sample and the control sample were submitted 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*[14] and modified as follows:

Egg white powders were dissolved in simulated gastric fluid (SGF, 0.15 M NaCl pH 2.5) to a final protein concentration of 10 $mg.ml^{-1}$. One ml of each protein solution was

aliquoted (gast ti), and the remainder was used for digestion. *In vitro* digestion was performed under continuous stirring (80 rpm) in a KS 4000I incubator (IKA-Werke, Germany) at 37°C. Gastric digestion started when porcine pepsin, previously dissolved in SGF, was added at a final concentration of 182 U of pepsin per mg of protein. Aliquots (1 ml) were taken over the 60 min of gastric digestion, at 15 s after the addition of pepsin (gast t+), then at 1 min (gast t1), 2 min (gast t2), 5 min (gast t5), 10 min (gast t10), 20 min (gast t20), 40 min (gast t40), and 60 min (gast tf). After sampling, the reaction was immediately stopped by raising the pH to 7 by adding 0.5 M ammonium bicarbonate.

Before the intestinal digestion stage, the pH of the gastric digest was adjusted to 6.5 by adding 0.5 M bis-tris pH 6.5, and 0.125 M bile salts mixture (0.0625 M sodium taurocholate and 0.0625 M sodium glycodeoxycholate). One ml of the digest solution was taken (int ti), and the remainder was used for intestinal digestion. Trypsin and α-chymotrypsin previously dissolved in simulated duodenal fluid (SDF, 0.15 M NaCl pH 6.5) were added to the protein solution (34.5 U trypsin per mg of protein and 0.4 U chymotrypsin per mg of protein). Aliquots (1 ml) were taken over the 30 min of intestinal digestion, at 15 s after the addition of enzyme (called int t+), then at 1 min (int t1), 2 min (int t2), 5 min (int t5), 10 min (int t10), 15 min (int t15), and 30 min (int tf). After sampling, the reaction was immediately stopped by adding 100 µl of 0.06 mM soybean Bowman-Birk trypsin-chymotrypsin inhibitor. Digested samples were frozen at -20°C until analysis.

2.1.4. Electrophoresis

Digested samples were analysed by SDS-PAGE on 12.5% polyacrylamide gels, according to Laemmli[15]. Samples were diluted with sample buffer (2% SDS, 20% glycerol, 1 M DTT, 0.5% bromophenol blue in 62.5 mM Tris-HCl buffer pH 6,8). To take into account the dilution due to the addition of the enzyme, the quantity of sample buffer solution was adjusted so that all samples had a final protein concentration of 5 $\mu g.\mu l^{-1}$. Samples were then heated for 3 min at 100°C. One hundred and fifty µg of protein were loaded onto each lane. Electrophoresis was carried out at a constant current (200 V) for 45 min. Gels were fixed in 20% v/v trichloroacetic acid for 90 min

and rinsed in distilled water before being stained with Bio-safe Coomassie G250 stain (Biorad).

Samples were distributed onto 34 gels. For each dry-heated egg white powder (one temperature, one duration), one gel was prepared with the samples of the gastric stage, and another gel was prepared with those of the intestinal stage. Similarly, two gels were prepared with the gastric and intestinal digests for the control powder, respectively.

The gels were 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. Up to 21 bands could be identified per sample (Figure 1). The band volume was used as the response variable in the statistical analysis.

Because of the high number of samples, only four lanes were analyzed quantitatively (gast ti, gast t+, gast t2 and gast tf and int ti, int t+, int t2 and int tf for gastric and intestinal stages, respectively). The final data set was thus composed of 17x8 (136) different digested samples.

2.2. Pre-processing of data

Raw electrophoresis data were not suitable for processing by either univariate or multivariate statistics[12]. Because of the high variability of staining between gels, the difference between variances from one band to another, and the potentially large number of missing values, pre-processing of the data was necessary.

2.2.1. Imputation of missing values

Few values were missing in the final data set, because not all the electrophoretic bands were systematically present and hence not systematically detected by ImageQuant software in all samples. In such cases, a zero value was assigned manually after checking the absence of the corresponding band on the gel.

2.2.2. Normalization of band volumes

Since the 136 samples were not randomized on the 34 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 powder was taken as the reference value ($Gel_{ref}BV$).The volume of the 30 kDa band for each of the other 33 gels was called Gel_nBV (1<n<33).The correction coefficient (CC) for the gel n was then calculated as follows:

$$Gel_nCC = \frac{Gel_nBV}{Gel_{ref}BV} \quad \text{eq. 1}$$

All the band volumes of Gel_n were then multiplied by the Gel_nCC coefficient.

2.3. Statistical methods

2.3.1. Principal Component Analysis (PCA)

The data set was a matrix consisting of the corrected quantified band volumes (21 columns) with a row representing the 17 samples (the control and the 16 dry-heated samples) at each digestion sampling time (i.e. 17x8=136 rows), as described in Supplementary Materials 1.

PCA transformed the variables observed into a new set of independent variables called Principal Components (PCs) which were uncorrelated linear combinations of the original variables. The first PC 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 on 2-D or 3-D graphs defined by the PCs.The matrix for PCA is described in Supplementary Materials 1. Dry-heating temperature, time and digestion sampling time were added as supplementary variables. PCA was performed using the FactoMineR package of the R software[16,17]. The variables were automatically standardized (mean centred and scaled) by the software to give them all the same importance.

2.3.2. Hierarchical Clustering Analysis (HCA)

HCA was performed in order to visualize and highlight the similarities between individuals. Briefly, HCA consists of calculating the dissimilarity, usually called the distance, between individuals[18]. In the present study, Ward's method was used for hierarchical clustering with the Euclidean distance as a similarity measure. The distances were calculated from the first 5 PCs only to suppress the noise in the data that occurred in the last PCs.

2.3.3. Multiple Factor Analysis (MFA)

MFA was performed on the table containing the quantified band values at each digestion sampling time, with the row representing the 17 samples (the control and the 16 dry-heated samples), as presented in Supplementary Materials 2. Dry-heating temperature and time were added as supplementary variables.

MFA deals with a multiple table, composed of groups of either continuous, categorical variables or frequency tables[19]. 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 separate 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 permits the comparison of partial groups and assesses whether they provide the same information. Another graph gives the correlations between the dimensions of the global analysis (MFA dimensions) and each separate analysis (PCA dimensions).

The aim of the present study was to determine the changes in electrophoretic pattern for each dry-heated sample during digestion. Each group of data was thus composed of the 21 band volume values at one sampling time (Supplementary Materials 2). Only four out of the eight sampling times performed were retained for MFA in order to clarify the results, as further explained in Section 3.2. The four sampling times retained (i.e. gast ti, gast t+, gast tf and int tf) were chosen according to the results of HCA performed after PCA, as described in Section 3.1. MFA provided a representation of the diversity of dry-heating treatments by taking into account the digestion sampling time in a balanced way. Representation of the variables made it possible to identify the main factors of variance, and to visualize the relationships between variables within a group (i.e. for one sampling time) and between groups (i.e.

between sampling times). Moreover, MFA provided a superimposed representation of dry-heated samples described by each of the groups of variables onto the subspace of the global analysis generated by PCs[20]. Thus, in our case, each dry-heated sample was represented by five spots: four partial points (i.e. each described by only one group) and a mean point (i.e. the centroid of the four previous points). The distance between partial points of a sample corresponded to the discrepancy between digestion sampling times.

3. Results

3.1. *In vitro* digestion progression as highlighted by PCA

Principal component analysis (PCA) was performed on the data set described in Supplementary Materials 1. The graph of the variables, i.e. of the electrophoretic band volumes, on the first two dimensions is given in Figure 2. PC1 and PC2 explain 32.8% and 19.6% of the variance of the data set, respectively. The correlations of the variables with these two PCs are indicated in Supplementary Materials 3. If only correlation coefficients over 0.5 are considered, PC1 is positively correlated with all the electrophoretic bands with a molecular weight equal to or higher than 42 kDa and with the band at 21 kDa, and negatively correlated with the bands at 18 and 41 kDa. From the highest to the lowest correlation, PC2 is positively correlated with the bands at 14, 15, 21, 93, 35 and 10 kDa, and negatively correlated with the bands at 71 and 174 kDa.

The graph of the individuals is given in Figure 3. Each point represents one dry-heated sample at one digestion time. The eight sampling times considered for the statistical analysis are identified by numbers, from 1 to 4 for gastric digestion stages, and from 5 to 8 for duodenal digestion stages (Figure 3A). PC1 and PC2 make it possible to split the samples according to the digestion time. Samples taken before addition of the enzyme (gast ti, numbered 1) have significantly higher coordinates on PC1 than all other samples (Figure 3A). Moreover, during digestion, the sample coordinates on PC1 and PC2 decrease while the digestion time increases (from number 2 to number 8). Considering the variables strongly correlated with PC1, both

positively and negatively (Supplementary Materials 3), indicates that as soon as the gastric stage starts and throughout the digestion process, high molecular weight components (over 42 kDa) are hydrolysed, whereas components of 41 and 18 kDa become more and more abundant. Similarly, when considering the variables strongly correlated with PC2, it can be concluded that the volume of the 10, 14, 15, 21, 35 and 93 kDa bands gradually decrease during digestion, assuming progressive hydrolysis of these proteins/peptides.

Moreover, it is noticeable that the dispersion of the individuals on the 2D plot defined by PCs 1 and 2 depends on the digestion time they belong to (Figure 3A). Samples from the gastric stage (numbered from 1 to 4) are much more scattered than samples from the intestinal stage (numbered from 5 to 8). This indicates that the differences in electrophoretic patterns between samples are greater before digestion and progressively decrease during digestion, especially during the intestinal stage. This decrease in variability occurs in such a way that all samples are quite close at the end of *in vitro* digestion according to PCs 1 and 2.

One-way ANOVA performed on the coordinates of the samples on both PC1 and PC2 showed a significant effect of the digestion time categorical variable (student t-test, $p<10^{-4}$). This confirmed that the digestion time had a significant effect on electrophoretic patterns, in keeping with the individual map (Figure 3A).

3.2. Key digestion times as determined by HCA

Hierarchical clustering analysis (HCA) performed on the first five PCs defined four clusters (Figure 4). Cluster A included all the gast ti samples, cluster B 53% of the gast t+ samples and 65% of the gast t2 samples, cluster C 88% of the gast tf samples and all the int ti samples, and cluster D all the int t+, t2 and tf samples. The definition of these clusters thus revealed three key times for the digestion of egg white proteins: the addition of gastric enzyme (between clusters A and B), the gastric stage (between clusters B and C), and the addition of intestinal enzymes (between clusters C and D).

3.3. Impact of dry-heating conditions tested by PCA

Unlike the significant effect of digestion time on electrophoretic patterns mentioned above, one-way ANOVA performed on the coordinates of the samples on both PC1 and PC2 did not reveal any significant effect of the dry heating treatment categorical variable. This is consistent with the 2D-graph shown on figure 3B, on which the barycentres for each dry-heating treatment are very close to the centre of the graph, although the coordinates on PCs 1 and 2 increase with dry-heating treatment intensity when the centre of the graph is enlarged.

This low effect of dry heating treatment was a consequence of the strong effect of digestion time, which explains at least 52.4% of the variance of the data set (variance explained on PCs 1 and 2). The digestion time effect thus concealed the dry heating treatment effect that was significant only on PCs 4 and 5 (data not shown), explaining 14% of the variance of the data set. To investigate the impact of dry heating conditions in detail, the digestion time effect had therefore to be cancelled out. Multiple factor analysis was implemented to achieve this.

3.4. Global dry heating treatment effect as highlighted by MFA

Multiple factor analysis (MFA) was performed on the data set described in Supplementary Materials 2. To make the analysis easier to read, and because only four critical digestion times were previously defined by HCA, four groups were considered for MFA, i.e. gast ti, gast t+, gast tf, and int tf.

The plane defined by PC1 and PC2 represents 43.8% of the variance of the data set (Figure 5). Only variables with a correlation coefficient over 50% are presented. PC1 correlated with variables of the four groups (Table 1). Moreover, PC1 also correlated positively with dry heating temperature and to a lesser extent dry heating time, which were considered as supplementary variables in the data set (correlation coefficients equal to 0.61 and 0.41, respectively). As shown in Figure 6A, PC1 contrasted (left side) control and slightly to moderately dry-heated samples (all durations at 60°C and 70°C, 1 or 2 days at 80°C, and 1 day at 90°C), with (right side) strongly dry-heated samples (more than 5 days at 80°C and more than 2 days at 90°C). PC1 was thus representative of the dry-heating effect. This discrepancy between samples according

to the intensity of the dry-heating treatment was also highlighted by HCA performed on the MFA data set (Figure 7). Of the six clusters thus defined, three clusters corresponded to low or medium dry-heating treatments (clusters 1 to 3) and three clusters corresponded to the most severe dry-heating treatments (clusters 4 to 6). PC2 was correlated with variables of the three gastric stage groups only (Table 2). As shown in Figure 6A, PC2 contrasted (upper part) samples dry-heated for 5 or 10 days at 80°C, with (lower part) both slightly dry-heated samples (control, 1 day at 60°C or 80°C) and strongly dry-heated samples (2, 5 or 10 days at 90°C). PC2 could thus represent a quadratic effect of dry heating treatment.

3.5. Effects of dry-heating treatment on the progress of digestion as evidenced by MFA

MFA complemented the representation of the barycentre of dry-heated samples with four partial points for each dry-heated sample (Figure 6B). Each partial point corresponds to one dry-heated sample described by a single group of variables, i.e. electrophoresis band volumes of gast ti, gast t+, gast tf, or int tf times, respectively. First, all the partial points corresponding to a given digestion time should be considered. Then, the distances between partial points of a given sample should be considered; these distances represent the change in electrophoresis band volumes that occurred during digestion for the sample in question.

3.5.1. Electrophoretic patterns of samples before digestion

In order to compare the egg white samples before digestion, the gast ti partial points (red points) had to be considered (Figures 6B and 6C). Their dispersion on the PC1-PC2 plane emphasized the discrepancies between samples before enzymatic digestion, as a consequence of the different dry-heating treatments. The coordinates of the gast ti partial points on PC1 increased when dry-heating treatment intensity increased. The volume of the bands positively correlated with PC1, i.e. 164, 152, 93, 30, and 10 kDa (Table 1), was then higher in strongly dry-heated samples whereas that of the bands negatively correlated with PC1, i.e. 130, 40 and 32 kDa (Table 1),was then lower. On PC2, the coordinates of the gast ti partial points from the

control sample first increased up to the dry-heating treatment of 10 days at 80°C, and then decreased for dry-heating treatments at 90°C for 2 days or longer. The volume of the bands positively correlated with PC2, i.e. 130, 121, 112, 55, 42, 35 and14 kDa (Table 2) first increased with dry-heating treatment for up to 10 days at 80°C, but then decreased in samples dry-heated over 2 days at 90°C.

3.5.2. Changes in electrophoretic patterns during the gastric stage

As in Section 3.5.1. for comparison of samples before digestion, analyses of the PC1-PC2 plane were undertaken for each digestion time. It is noticeable that for most samples, the partial points at the end of the gastric stage (blue spots) are closer to the centre of the graph than that before digestion (red spots) (Figure 6D). Since the centre of the graph represents mean values for the band volumes correlated with PC1 and PC2, this indicates that the gastric digestion stage decreased the initial electrophoretic pattern discrepancy due to dry- heating. Only samples dry-heated for 2 days at 70°C, or for 5 days at 80°C had coordinates on PC1 and/or 2 further from the mean at the end of the gastric stage than at the beginning. These two samples thus had high band volumes for the bands at 130, 93, 42 and 14 kDa, suggesting their low hydrolysis during the gastric stage. For some other samples (60°C-5d, 70°C-1 and 10d, 80°C-2d and 90°C-10 days), partial points corresponding to gast ti and gast tf were close, which suggests hardly any effect of the gastric stage on SDS PAGE pattern.

Considering the distances between partial points of a given sample, which represent changes in electrophoresis band volumes that occur during digestion for the sample in question, Figure 6D highlights the effects of dry heating on the progression of the gastric stage. Red, green and blue spots represent the electrophoresis band volumes before, a few seconds after the addition of the gastric enzyme and at the end of the gastric stage, respectively. Red arrows thus illustrate the effects of the addition of the gastric enzyme on the electrophoretic pattern in the very first few seconds, while green arrows represent the effects of the following 60 min of the gastric stage. It is noticeable that most of the arrows are vertical, indicating that the gastric stage mainly affects the volume of the bands correlated with PC2 (Table 2). However, the relative importance of the very few seconds and of the following 60 minutes differs since the

sizes of red and green arrows vary from one sample to another. For example, for samples 80°C-1d and 90°C-2d (Figure 6D), the red arrow is smaller than the green arrow . This indicates hardly any difference between gast ti and gast t+ electrophoretic patterns and that hydrolysis mainly occurs during the 60 minutes of the gastric stage. In contrast, for samples 60°C-1d and 70°C-5d, the red arrow is longer and the green arrow shorter, indicating that most of the gastric stage effects occur during the first few seconds after addition of the enzyme. The progression of the gastric stage thus depends on the initial state of the sample.

Although the gastric stage tended to standardize the electrophoretic patterns of the samples, there were still significant differences between samples at the end of this digestion stage. In particular, strongly dry-heated samples (i.e. more than 5 days at 80°C or 2 days at 90°C) still had high coordinates on both PC1 and PC2. These samples thus had high band volumes for the bands at 130, 121, 93, 55, 42, 35, 30, 18 and 14 kDa (Tables 1 and 2). Most of these bands (130, 121, 93, 55, 42, 18, 14 kDa) were present in samples before digestion: their high volume at the end of the gastric stage therefore suggests their low proteolysis by pepsin. However, the bands at 35 and 30 kDa appeared only in gastric digests, suggesting that they corresponded to peptides that were released in higher quantities in the strongly dry-heated samples.

3.5.3. Changes in electrophoretic patterns during the intestinal stage

The partial points at the end of the intestinal stage (black spots) were closer to the centre of the graph than those before digestion (red spots), and even than those at the end of the gastric stage (blue spots) (Figure 6E). In continuation of the gastric stage, the intestinal stage thus decreased the initial dispersion of the electrophoretic pattern of samples which became confined along PC1. This is consistent with the correlation of int tf variables only with PC1 (Table 1).

However, for samples dry-heated from 2 to 10 days at 60°C and 1 day at 70°C, and for 2 and 10 days at 80°C and 2 and 5 days at 90°C, the coordinates at the end of the intestinal stage were further from the centre of the graph than those at the end of the gastric stage (Figure 6E). At the end of the intestinal stage, samples dry-heated from 2 to 10 days at 60°C and 1 day at 70°C thus had lower band volumes than the mean

for the bands at 121, 93, 42, 55, 14 and 10 kDa (Table 1), whereas samples dry-heated for 2 and 10 days at 80°C and 2 and 5 days at 90°C had higher band volumes than the mean for the same bands. This was also the case for the sample dry-heated for 10 days at 90°C since it still had high coordinates along PC1 at the end of intestinal step.

PC1 contrasted (left side) low to medium dry-heated samples (clusters 1 and 3), with (right side) high dry-heated samples (clusters 4, 5 and 6); the control and low-treated samples (cluster 2) being in the middle. From the variable correlation with PC1 (Table 1), and cluster distribution along PC1 (Figure 7), it can be concluded that slightly to moderately dry-heated samples had lower band volumes than the control sample for the bands at 10, 14, 42, 55, 93 and 121 kDa at the end of the intestinal stage, i.e. at the end of *in vitro* digestion. In contrast, strongly dry-heated samples had higher band volumes for these components at the end of *in vitro* digestion.

4. Discussion

4.1. The digestion time effect accounted for more than half of the data variability and concealed the dry-heating effect on the progression of *in vitro* protein digestion

PCA is a method that projects the data in a new space spanned by principal components. These components reduce the number of dimensions in a data set, and can be used to summarize the structure of the data. This is achieved by transforming the variables observed into a new set of independent variables called principal components (PCs) which are linear combinations of the original variables, and whose correspondence to phenomena such as biological processes can be assumed. PCs are not correlated with each other, and are extracted from the sample one by one, the first PC being the one which explains most of the systematic variation in the data[12].

In the present study, the first two PCs explain 52.4% of the data set variability (Figure 3) and appear correlated with digestion time (Supplementary Materials 3), which makes sense considering the data set. Indeed, enzymatic digestion of protein is a progressive process that aims at hydrolysing proteins and then extensively changes

electrophoretic patterns. PCA thus makes it possible to highlight the effects of enzymatic digestion on sample electrophoretic patterns, with an individual distribution on the PCA plane mainly governed by digestion time (Figure 3A). In addition, HCA analysis (Figure 4) makes it possible to determine the key digestion times to describe the progression of digestion in a relevant way: before and after addition of pepsin, beginning and end of the gastric stage, before and after addition of trypsin and chymotrypsin.

In contrast, the information about the progression of digestion depending on the dry-heating treatment previously applied to the samples remains difficult to extract from the PCA analysis, although a tendency can be seen (Figure 3B). This is a consequence of the strong effect of digestion time described above (52.4% of the variability explained on PCs 1 and 2), meaning that the dry-heating treatment effect appeared significant only on PCs 4 and 5; the dry-heating treatment effect explains only 14% of the variability of the data set. In other words, the digestion time effect conceals the dry-heating treatment effect. Thus the digestion time effect had to be cancelled out to investigate the impact of dry-heating conditions specifically. Multiple Factor Analysis was implemented for that purpose.

4.2. Dry-heating induced protein aggregation

MFA made it possible to take into account several sets of variables as active elements simultaneously in the definition of distances between individuals. It brought out main factors of data variability, individuals being described by several groups of variables in a balanced manner. In the present study, MFA highlighted the dry-heating effect according to PC1 and PC2 (Figure 6A), the variability induced by digestion time being controlled by the groups defined. The map of the partial individuals before digestion showed high degrees of difference in electrophoretic patterns according to the dry-heating treatment (figure 6C). The coordinates of the samples increased along PC1 when dry-heating intensity increased, whereas along PC2, the coordinates first increased from the control sample up to 10 days' treatment at 80°C, before decreasing for stronger treatments (from 2 to 10 days at 90°C). Since high molecular

weight bands (164, 152, 93 kDa) were positively correlated with PC1, this suggests that dry-heating induces egg white protein aggregation. When samples were dry-heated for more than 1 day at 90°C, the coordinates along PC2 were low, assuming low band volumes for the bands at 130, 121, 112, 42, ,and 35 kDa. The 42 kDa band was attributed to ovalbumin. The intensity of this band decreased for the strongest dry-heating treatments, suggesting that ovalbumin is involved in aggregation due to heat treatment. In particular, the 93 kDa band, the volume of which increased with dry-heating intensity, could correspond to the ovalbumin dimer [21, 22]. In contrast, the low volumes for the bands at 130, 121 and 112 kDa in these strongly dry-heated samples was more surprising. It can be assumed that these high molecular weight components were integrated into the larger aggregates, such as those at 152, 164 and 174 kDa, in higher quantities in these samples.

Several studies have previously reported such aggregation resulting from dry-heating of egg white[7, 21-27] and purified egg proteins[29, 30]. The aggregation is usually assigned to disulfide bonds, hydrophobic interactions, and/or non disulfide covalent bonds[7, 21, 24, 27, 28, 29]. In the present study, the electrophoresis performed in denaturing conditions confirmed the involvement of covalent bonds. In particular, the free SH group of the ovalbumin molecules might explain why this protein is involved in such covalent aggregates through SS bonds when dry-heating intensity increases.

4.3. Dry-heating impacts on the progress of *in vitro* digestion of egg white proteins

The PC1-PC2 plane (figure 5) represents the variables, i.e. the electrophoresis bands, which contribute most to the variability of the data set. They correspond to the constituents whose *in vitro* digestion progression is the most affected by dry-heating treatment. Many bands do not appear because the progression of *in vitro* digestion of the corresponding constituent does not depend on dry-heating conditions. This is, for example, the case for the ovotransferrin band that disappeared in all cases as soon as gastric enzymes were added (data not shown).

The partial points describing each sample became closer to the centre of the MFA map as digestion progressed (Figure 6B), indicating that *in vitro* digestion reduces the

differences in the electrophoretic pattern due to dry-heating. However, digestion progression differed according to the samples considered.

In most samples, the gastric stage was responsible for most of the proteolysis, as also suggested by PCA analysis (Figure 3A). Similar results were reported by Nyemb et al[30] for *in vitro* digestion of different types of ovalbumin aggregates. For the moderately to high dry-heated samples (1 to 5 days at 60°C, 5 days at 70°C and 80°C, and 1 and 5 days at 90°C), most of the proteolysis even occurred during the first few seconds of the gastric stage (long red arrows on Figure 6D). Digestion of globular proteins in SGF by pepsin has been reported to be a two-step mechanism[31]: protein unfolding due to the acid conditions, followed by pepsinolysis, the first step being the slowest and therefore the rate limiting step. The rapid hydrolysis of the moderately dry-heated samples may be due to the unfolded state of some of the proteins after dry-heating treatment.

The relationship between the progression *of in vitro* digestion and dry-heating treatment was not easily determined. Nevertheless, at the end of digestion, digest composition differed according to heat treatment intensity. Slightly to moderately dry-heated samples (2 to 10 days at 60°C and 1 day at 70°C) were more extensively hydrolysed than the control sample, as indicated by the lower band volumes of most of the proteins originally present in egg white (int tf partial points mainly on the left of the MFA map, Figure 6E). This sensitivity to *in vitro* digestion was specifically due to the intestinal stage since these samples had electrophoretic patterns closer to that of the control sample at the end of the gastric stage. The bands mainly affected by hydrolysis were those at 121, 93, 55, 42 (ovalbumin), 14 (lysozyme) and 10 kDa (Table 1). Although ovalbumin and lysozyme have low susceptibility to digestion[32], this can be modified by processing. Thus, heat treatment of ovalbumin in solution over 15 min at 90°C or 6h at 80°C increases its susceptibility to *in vitro* digestion[33, 30]. Ovalbumin unfolding or even aggregation (according to the size and the shape of aggregates)[30] might make it easier for the enzyme to access proteolytic sites. However, in the present study, the most strongly dry-heated samples (over 2 days at 80°C or 90°C) were less hydrolysed than the control or the least dry-heated samples,

as suggested by their position on the right of the MFA map (Figure 6E). Such intense dry-heating treatments are believed to lead to the formation of around 40% of soluble aggregates[22] and also unfolded proteins, that are more flexible and prone to aggregation[28]. It can then be assumed that, after the strongest dry-heating treatments, protein aggregation is thus extended or the shape of aggregates is such that it prevents the enzyme from accessing cleavage sites. The overall extent of pepsinolysis has indeed been shown to be mainly governed by the degree of protein unfolding in the material prior to ingestion[34]. Furthermore, Nyemb et al[30] showed that the surface area to volume ratio and the degree of unfolding of proteins inside aggregates are the major contributing factors governing the extent and nature of digestion. Jimenez Saiz et al[35] also showed that a high proportion of intact lysozyme may reach the duodenum in an *in vivo* situation. Intact lysozyme then precipitates under duodenal conditions, probably due to electrostatic interaction with negatively charged bile salts at pH 6.5, and this helps it to resist digestion by pancreatic enzymes. Moreover, dry-heating at 80°C increases the lysozyme net charge due to isoaspartate formation[36]. This increase in protein net charge could thus enhance its reactivity towards bile salts, increasing its resistance to digestion.

5. Conclusion

The nutritional quality of proteins depends on both their amino acid composition and their digestibility. Industrial processes applied to food stuffs to ensure their safety, especially heat treatments, are known to denature proteins. In the present study, we investigated the effects of dry-heating on the progression of *in vitro* digestion of egg white proteins. To do so, the use of multiple factor analysis (MFA) was extended to electrophoretic follow up of *in vitro* digestion kinetics. Classically used in the field of sensory analysis, MFA is designed to analyse data tables in which individuals are described by different groups of variables that could be the same variables observed in different conditions or times. This method can thus be applied to every case where variables can be gathered according to their nature (sensorial, physicochemical variables,etc), time or space (same variables measured at different times or in

different spaces). In the present study, MFA made it possible to determine which electrophoresis bands were affected by dry-heating, considering their *in vitro* digestion.

Dry-heating of egg white powder enhances protein aggregation: high molecular weight bands became more intense (higher band volumes) in dry-heated samples before digestion, as compared to control sample. This caused different courses of *in vitro* digestion, depending on the dry-heating treatment applied: slightly to moderately dry-heated samples were more prone to *in vitro* digestion than the non-treated sample, whereas strongly dry-heated samples were the most resistant. It can therefore be assumed that, after low to medium dry-heating, protein unfolding or increased flexibility make *in vitro* digestion easier, whereas after high dry-heating treatment (over 80°C for 2 days), protein aggregation slows down *in vitro* digestion. By taking into consideration the extent of digestion, such a statistical approach might help to determine optimal treatment.

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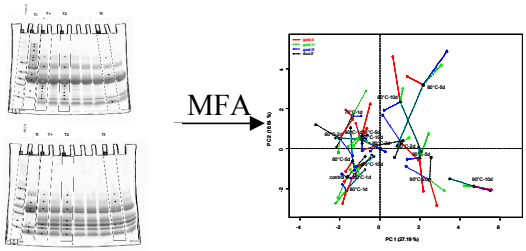


17 SDS PAGE gels 1 graphical display

In vitro digestion of egg white powders: Multiple Factor Analysis to follow dry-heating effect

Table 1: MFA : significant correlation coefficients ($p<0.05$) between band volumes at a given sampling time and PC1. NS = non-significant. Band molecular weights in italics = bands that appeared during digestion.

	Gast ti	Gast t+	Gast tf	Int tf
174 kDa	*NS*	*NS*	*NS*	*NS*
164 kDa	**+0.78**	**+0.78**	*NS*	*NS*
152 kDa	**+0.92**	**+0.50**	*NS*	*NS*
130 kDa	**-0.55**	*NS*	*NS*	*NS*
121 kDa	*NS*	**+0.67**	**+0.67**	**+0.64**
112 kDa	*NS*	*NS*	*NS*	*NS*
93 kDa	**+0.70**	**+0.82**	**+0.75**	**+0.88**
71 kDa (ovotransferrin)	*NS*	*NS*	*NS*	*NS*
55 kDa	*NS*	**+0.52**	**+0.68**	**+0.77**
42 kDa (ovalbumin)	*NS*	*NS*	*NS*	**+0.65**
41 kDa	*NS*	**+0.55**	**-0.54**	*NS*
40 kDa	***-0.64***	***+0.64***	*NS*	*NS*
35 kDa	*NS*	*NS*	**+0.69**	*NS*
32 kDa	***-0.66***	*NS*	*NS*	*NS*
30 kDa	***+0.66***	***+0.62***	***+0.71***	*NS*
21 kDa	*NS*	*NS*	*NS*	*NS*
18 kDa	*NS*	*NS*	**+0.65**	*NS*
15 kDa	*NS*	*NS*	*NS*	*NS*
14 kDa (lysozyme)	*NS*	*NS*	*NS*	**+0.58**
10 kDa	**+0.91**	*NS*	*NS*	**+0.63**
9 kDa	*NS*	***+0.87***	*NS*	*NS*

Table 2: MFA: significant correlation coefficients ($p<0.05$) between band volumes at a given sampling time and PC2. NS = non-significant. Band molecular weights in italics = bands that appeared during digestion.

	Gast ti	Gast t+	Gast tf	Int tf
174 kDa	*NS*	*NS*	*NS*	*NS*
164 kDa	*NS*	*NS*	*NS*	*NS*
152 kDa	*NS*	*NS*	*NS*	*NS*
130 kDa	**+0.5**	**+0.70**	**+0.60**	*NS*
121 kDa	**+0.55**	*NS*	*NS*	*NS*
112 kDa	**+0.77**	*NS*	*NS*	*NS*
93 kDa	*NS*	*NS*	**+0.56**	*NS*
71 kDa (ovotransferrin)	*NS*	*NS*	*NS*	*NS*
55 kDa	**+0.73**	**+0.74**	*NS*	*NS*
42 kDa (ovalbumin)	**+0.71**	**+0.76**	**+0.78**	*NS*
41 kDa	*NS*	*NS*	*NS*	*NS*
40 kDa	*NS*	*NS*	*NS*	*NS*
35 kDa	**+0.53**	*NS*	*NS*	*NS*
32 kDa	*NS*	*NS*	*NS*	*NS*
30 kDa	*NS*	*NS*	*NS*	*NS*
21 kDa	*NS*	**+0.65**	*NS*	*NS*
15 kDa	*NS*	*NS*	*NS*	*NS*
14 kDa (lysozyme)	**+0.59**	**+0.70**	**+0.70**	*NS*
10 kDa	*NS*	*NS*	*NS*	*NS*
9 kDa	*NS*	*NS*	*NS*	*NS*

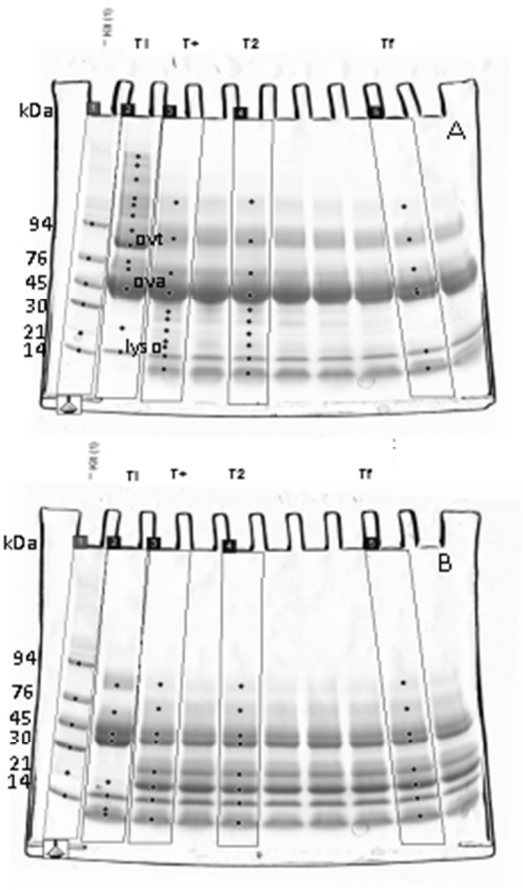


Figure 1: In vitro digestion of egg white followed by SDS PAGE analysis for gastric (A) and intestinal (B) stages. Lane frames (gast ti, gast t+; gast t2, gast tf; int ti, int t+, int t2 and int tf) correspond to digestion times used for quantification by ImageQuant software. Bands marked with spots correspond to the quantified bands. Ovotransferrin (ovt), ovalbumin (ova) and lysozyme (lyso) bands are identified (A). The two electrophoresis gels presented here as examples show the digestion progression of egg white dry-heated at 80°C for 2 days.
89x117mm (96 x 96 DPI)

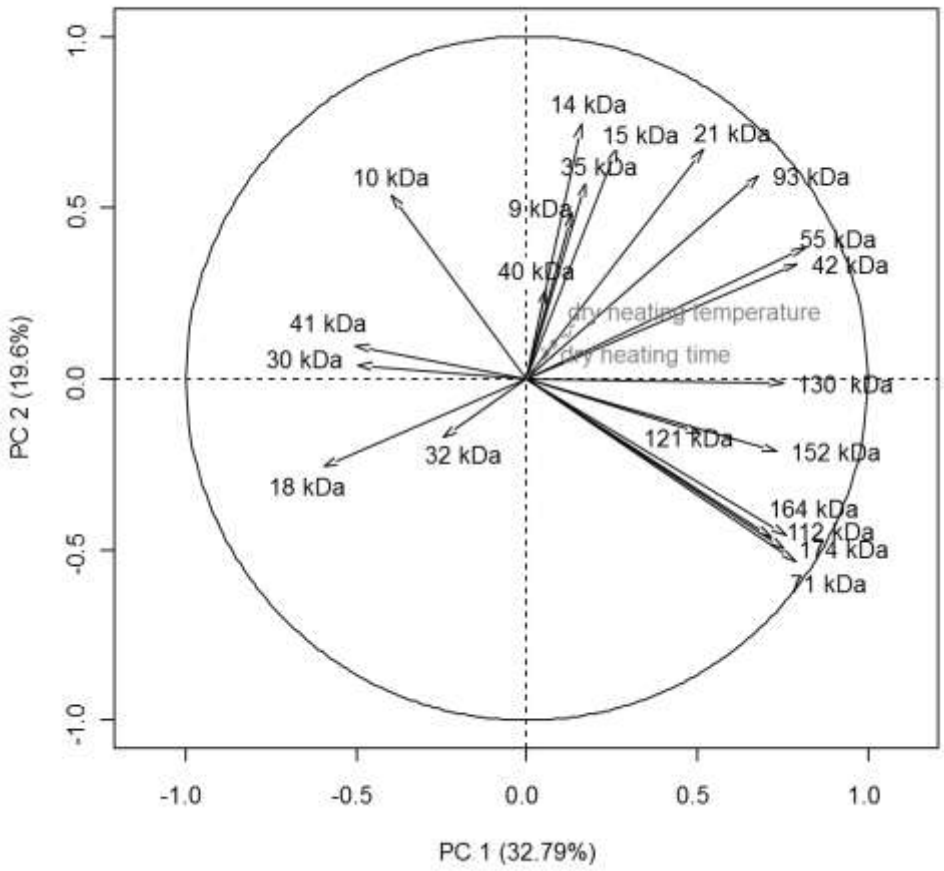
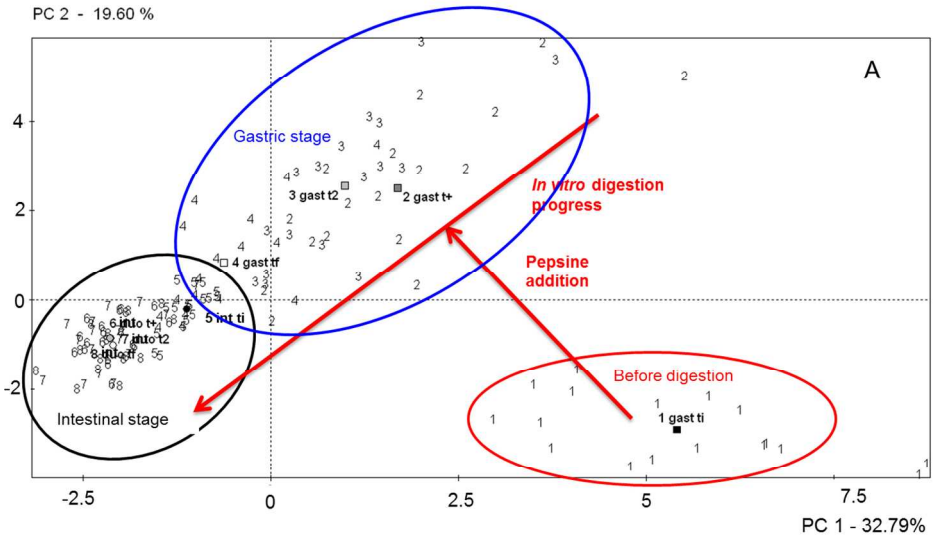


Figure 2: Projection of variables (electrophoresis bands) onto the plane defined by the first two principal components (PCs) of Principal Component Analysis (PCA). The coordinates of each variable are the correlation coefficients with the two first PCs: the closer the arrow to the circle, the better the representation of the variable. Of the variables significantly correlated with PC1 and 2, the correlations between two variables were all the highest, given that the angle between their directions was small.
192x191mm (150 x 150 DPI)



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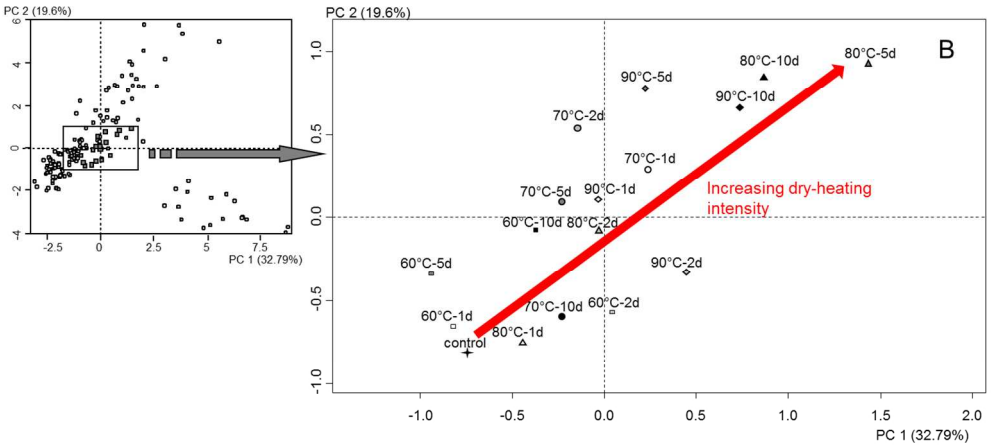

Figure 3: PCA map of individuals projected on the 2D plane defined by PC1 and PC2. A) Individuals identified according to digestion time: gast ti (1), gast t+ (2), gast t2 (3), gast tf (4), int ti (5), into t+ (6), int t2 (7) and int tf (8). B) Individuals identified by empty circles and representation of the "dry-heating treatment" qualitative variable by grey squares. In the zoom, the position of the barycentres for the different dry-heating treatments.
254x115mm (150 x 150 DPI)

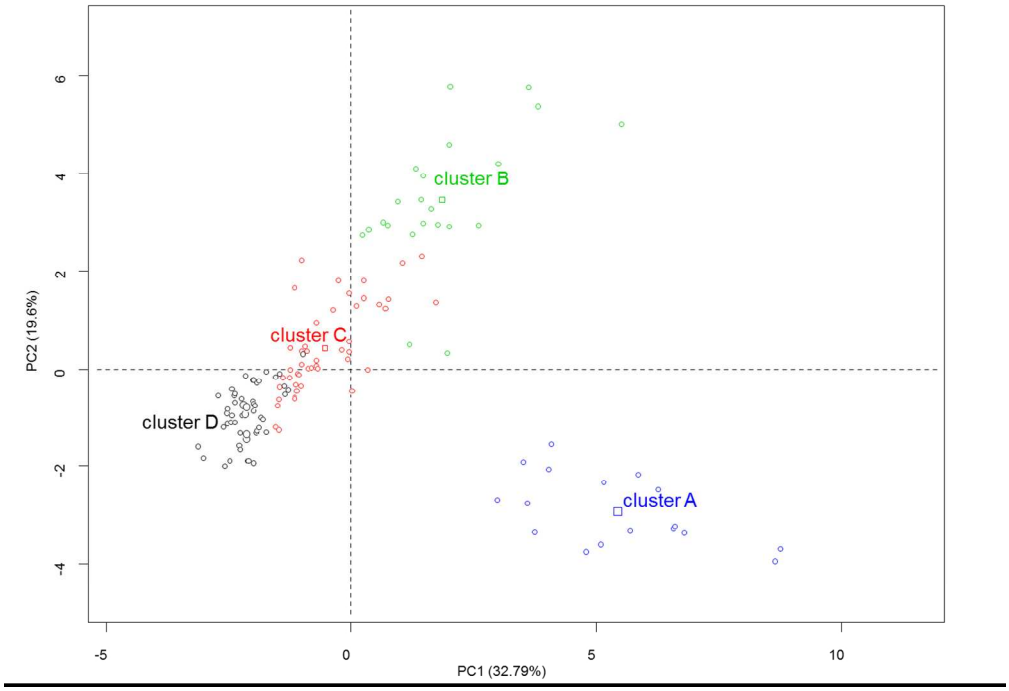

Figure 4: Hierarchical Clustering Analysis (HCA) applied to the first two principal components of PCA. Ward's method was used for hierarchical clustering, with the Euclidean distance as a similarity measurement.
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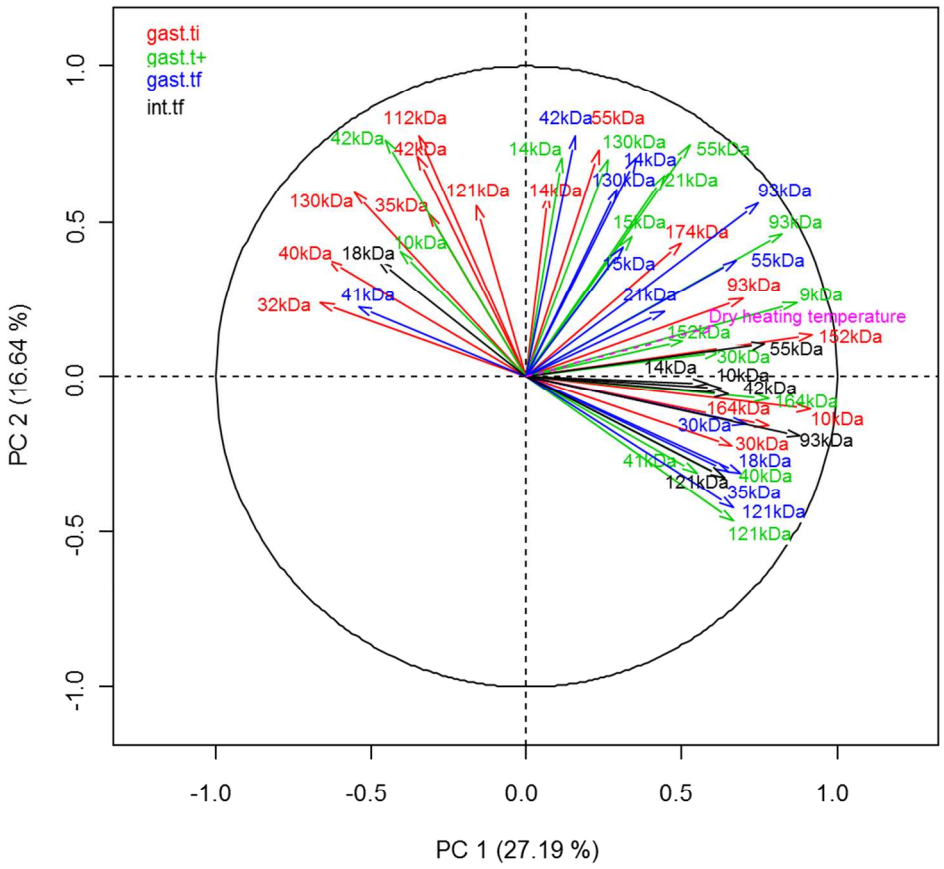

Figure 5: Projection of variables (electrophoresis bands) onto the plane defined by the first two principal components (PCs) of Multiple Factor Analysis (MFA). Only variables with a correlation coefficient >0.5 are presented.
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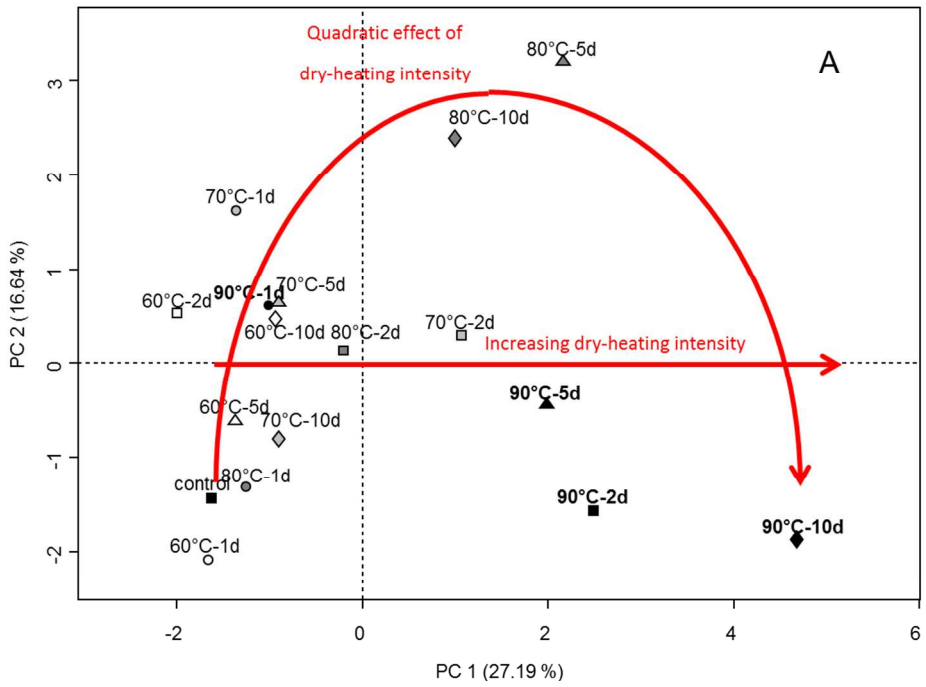

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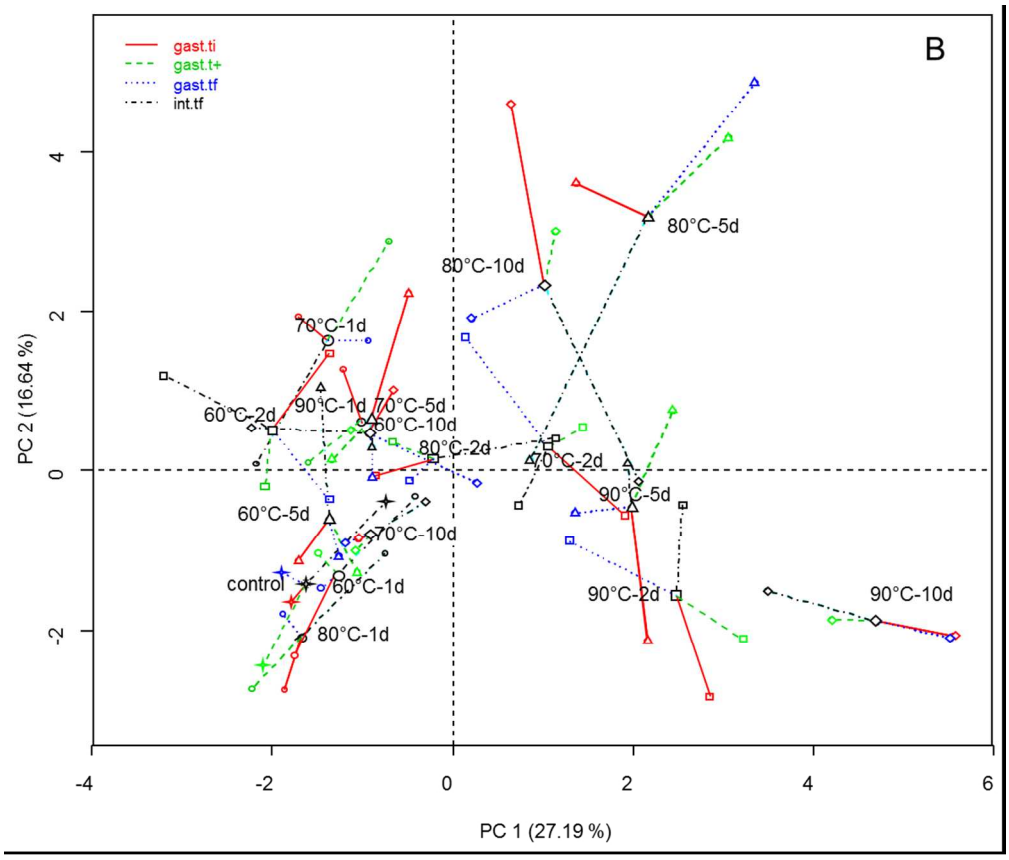

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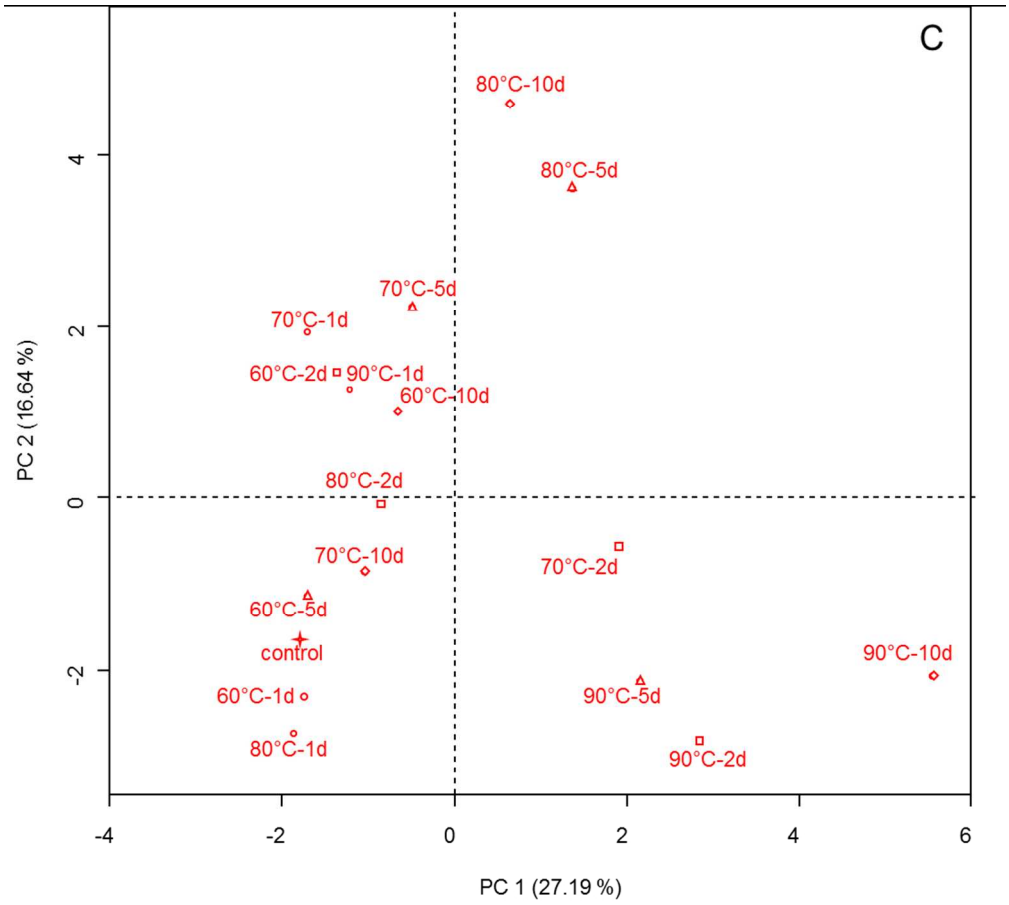

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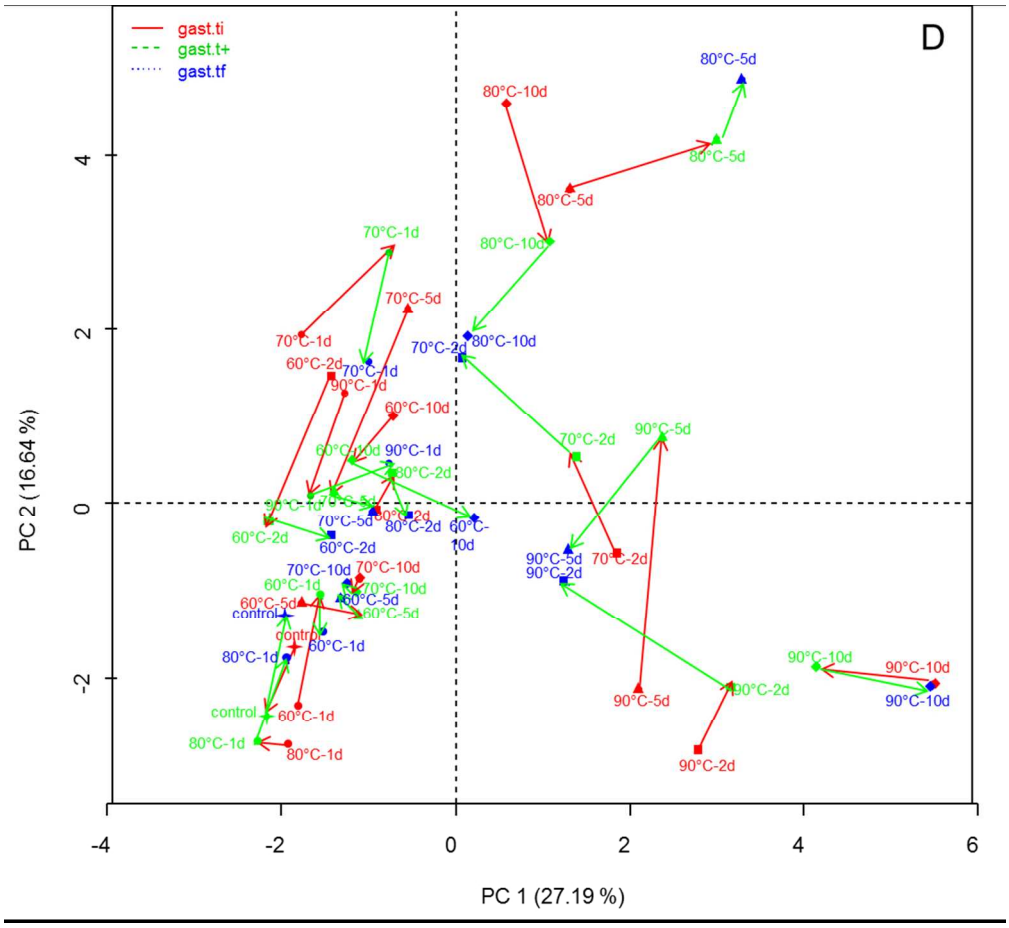

184x168mm (150 x 150 DPI)

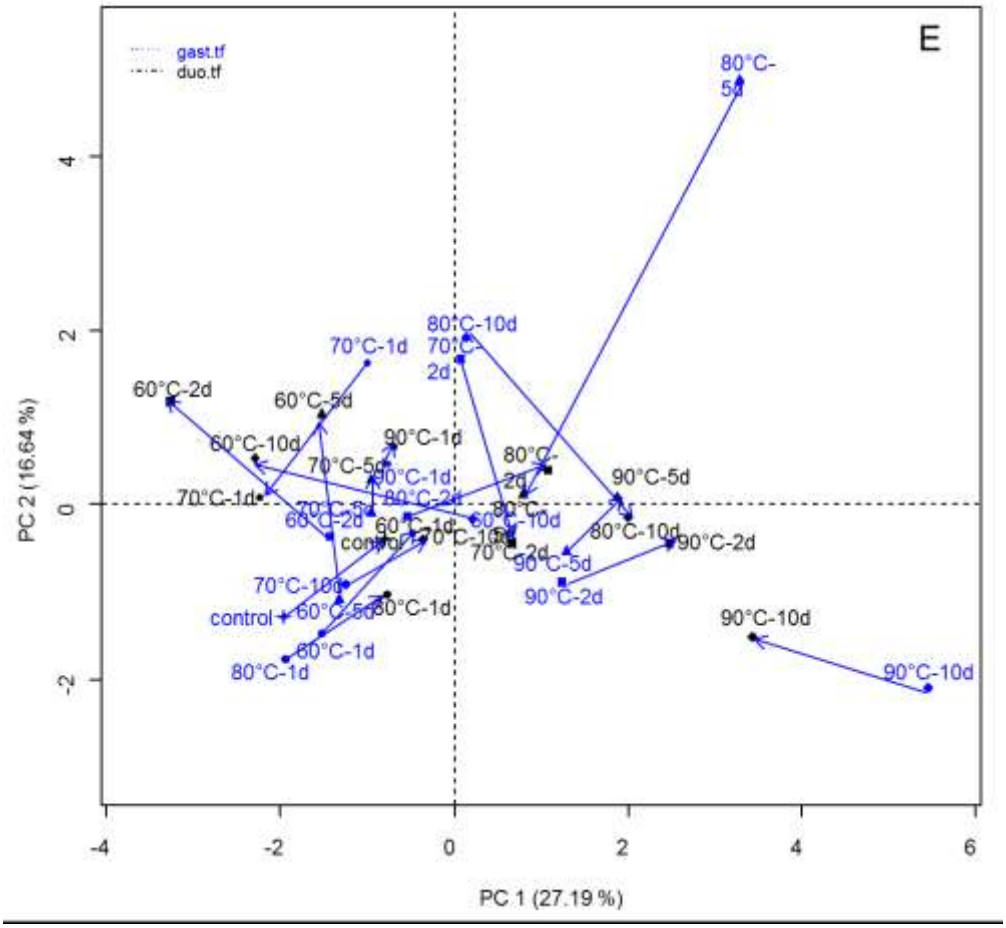

Figure 6: MFA maps of the individuals projected on the 2D plane defined by PC1 and PC2. Each dry-heated sample is represented by five points: the middle point (Figure 6A) corresponds to characterization of variables by the four groups; each extremity (three full symbols) corresponds to characterization by one group of variables: gast ti (red), gast t+ (green), gast tf (blue) and int tf (black), respectively (Figure 6B). Figures 6C to 6E represent MFA maps of the individuals before digestion, during the gastric stage, and during the intestinal stage, respectively; arrows illustrate the effects of digestion stages on the coordinates of the individuals, i.e. on their electrophoretic pattern.
184x170mm (150 x 150 DPI)

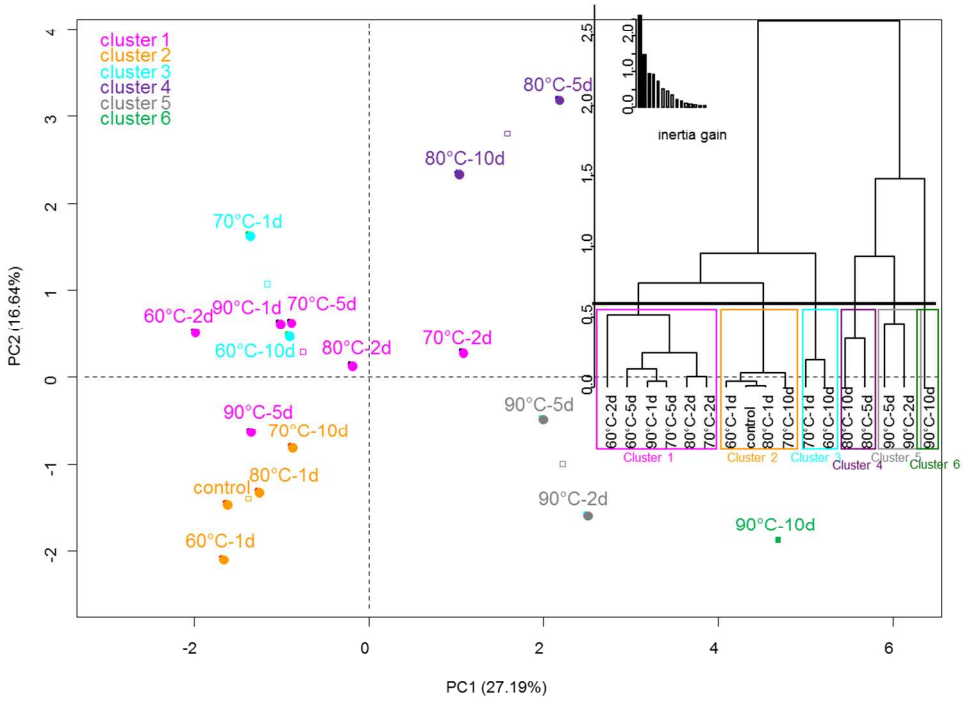

Figure 7: Hierarchical Clustering Analysis (HCA) applied to the first two principal components of MFA; this HCA takes into account the balance of the groups of variables considered with MFA. Ward's method was used for hierarchical clustering, with the Euclidean distance as a similarity measurement.
224x177mm (150 x 150 DPI)