Modelling *in vitro* gastrointestinal digestion of egg white gel matrix by laser-backscattering imaging

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Abstract

The objective of this work was to test the capability of laser-backscattering imaging technique to model changes produced throughout *in vitro* gastrointestinal digestion of chicken egg white gel as protein matrix model. Evolution of the matrix sample was monitored by analysing protein digestibility, zeta potential, particle size, and imaging every 20 min during the digestion process. Laser-backscattering was based on capturing the diffraction patterns generated by laser-light after being transmitted through matrix samples. Differences in imaging data were observed for the different zones of the studied diffraction patterns. This complexity was solved by a dimensional reduction using multivariate statistics, obtaining R^2 >0.90 in calibration studies. Results indicated that imaging device captured the variance produced due to changes in concentrations of protein compounds in despite of changes produced by pH and reagents addition. Therefore, laser backscattering imaging was capable of monitoring the digestion of egg white gel during gastric and intestinal phases.

Keywords: *protein matrix, in vitro digestion, imaging analysis, laser-backscattering, modelling*

1. Introduction

Gastrointestinal digestion is a complex process including mechanical, chemical and enzymatic processes which involve food breakdown and release of nutrients for subsequent absorption. Physical mechanisms, pH changes, and enzymes action as well as food material properties and structure influence the rate and extent of digestion. In the case of protein matrices, proteolysis begins in the gastric phase with the combined action of HCl and pepsin enzyme, which breaks down proteins generating a mixture of peptides and free amino acids. At the intestinal phase, the action of different enzymes, mainly trypsin and chymotrypsin, completes protein digestion by further hydrolysing peptide fractions for further intestinal absorption (Gropper and Smith, 2013).

In vitro digestion models, including static and dynamic methods, are simple and useful tools to evaluate structural and physicochemical changes occurring during the process as well as digestibility of protein compounds. In that regard, digested samples are analysed in order to measure concentration profiles of the released proteolytic products, generally by using traditional destructive approaches such as spectrophotometric, electrophoretic, chromatographic, or mass spectrometry methods (Gallego et al., 2021, 2020; Sánchez-Rivera et al., 2014). However, the use of non-destructive approaches to study profiles of food hydrolysis has generated a considerable interest over the last years. Examples include magnetic resonance techniques to evaluate the hydrolysis level in complex lipid mixtures (Nieva-Echevarría et al., 2014) and time-series micro-computed tomography to study structural breakdown during *in vitro* gastric digestion of apples (Olenskyj et al., 2020).

In the case of imaging, several techniques have been applied to analyse physicochemical properties and structure changes of foods during *in vitro* gastrointestinal digestion. For instance, Somaratne et al. (2019) mapped the distribution of digestive fluids in food

structures using hyperspectral imaging. Focusing on protein matrices, Deng et al. (2020) applied magnetic resonance imaging to explore *in vitro* gastric digestion of whey protein. Somaratne et al. (2020) reported a time-lapse imaging system to evaluate *in-situ* disintegration by pepsin of egg white gels with different microstructures and nutrient release kinetics. Pasquier et al. (2019) evaluated the effects of gastric and intestinal enzymes on disintegration kinetics of microstructured canola seed proteins by using small-angle scattering and imaging techniques.

Within imaging techniques, laser-backscattering imaging characterises the extinction of light transmittance across a changing magnitude measured in a given matrix. This principle is the basis of a wide range of analytical methods. However, in this case, imaging analysis and data mining techniques are followed to study the generated diffraction patterns because matrix-light interactions. In food-processing research, laser-backscattering imaging has been applied for modelling different food matrix transformations due to enzymatic action. Some examples are the characterisation of the tendering process of pork loin with papain enzyme addition (Grau et al., 2021), and of texture evolution in dairy matrices such as the milk curdling phase of cheese and yogurt fermentation (Verdú et al., 2021, 2019). These studies suggested the hypothesis of using this technique to study the physicochemical evolution of a given food matrix resulting from the action of enzymes throughout gastrointestinal digestion. There is little information about the capacities of imaging techniques combined with analytical methods to study digestion processes. This could improve existing methods and developing new applications in non-destructive analysis terms. The ultimate goal of the application would be the continuous monitoring of changes produced in a food matrix during *in vitro* digestion without sample modification/destruction. However, it was necessary to test

previously the capability of the technique to model sample changes measured by traditional analytical methods.

Thus, this work focused on testing the capability of laser-backscattering imaging technique to model protein changes, occurring during gastric and intestinal digestion, of egg white as model of protein gel matrix. For that, digested samples were evaluated in terms of protein digestibility, by both analytical and *in silico* assays, and of zeta potential and particle size distributions in order to improve understanding of information captured in the image data.

2. Materials and methods

2.1 Chemicals and reagents

Enzymes pepsin from porcine gastric mucosa and pancreatin from porcine pancreas as well as bile extract porcine, trinitrobenzenesulfonic acid (TNBS), Coomassie Brilliant Blue G-250, bovine serum albumin (BSA), L-tyrosine, and L-leucine were purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA). Trichloroacetic acid (TCA), ethanol and phosphoric acid were from Scharlau Chemie, S.A. (Sentmenat, Barcelona, Spain). All other reagents and chemicals were of analytical grade.

2.2 Sample preparation

Pasteurised liquid egg white from chicken eggs was obtained from a local supermarket and subjected to heat-induced gelation. For that, the sample was heated at 100 °C under agitation for 12 min in order to form a homogeneous gel protein matrix. The sample was kept at 4 °C before further analysis. This homogeneous and highly reproducible sample was used with the aim of ensuring the capacity of the technique to capture the variance

generated by changes produced by the enzymatic activity, therefore reducing within-sample variability before digestion.

2.3 *In vitro* gastrointestinal digestion

The simulated gastrointestinal digestion was carried out following the standardised INFOGEST method (Minekus et al., 2014; Brodkorb et al., 2019). In duplicate, 1 g of each sample was diluted with simulated salivary fluid (SSF) in a ratio 1:1 (w/v), and mixed at 40 rpm for 2 min at 37 °C using an Intell-Mixer™ RM-2 (ELMI Ltd., Riga, Latvia) within an incubator chamber (JP Selecta, S.A., Barcelona, Spain). In the gastric phase (*G*), the oral bolus was diluted 1:1 (v/v) with simulated gastric fluid (SGF), $CaCl_2$ (0.15 mM) and pepsin enzyme (2000 U/mL). The pH was adjusted to 3.0 with HCl (1 M) and the mixture was then incubated under agitation at 37 °C for 2 h as previously described. In the intestinal phase (*I*), the gastric chyme was diluted (1:1, v/v) with simulated intestinal fluid (SIF), $CaCl_2$ (0.6 mM), pancreatin (trypsin activity of 100 U/mL) and bile salts (10 mM). The pH was adjusted to 7.0 with NaOH (1 M), and the sample was mixed at 37 °C for 2 h. After digestion, samples were immediately placed in ice, centrifuged (8000 *g*, 4°C, 10 min), and the resultant supernatants were taken for following analyses. Samples were taken every 20 min during the process, using separate tubes for each sampling time point. The number of experimental data points was established based on preliminary assays considering possible data-loss for the imaging technique. Preliminary kinetics obtained for free amino groups showed non-loss of information using that sampling frequency. Control samples containing digestive fluids, enzymes, and bile (without sample) were run in parallel.

147 **2.4 Protein digestibility**

148 The protein digestibility of the control and egg white gel samples during *in vitro*
149 gastrointestinal digestion was evaluated, in duplicate, by determining the content of free
150 amino groups, soluble proteins, and TCA-soluble peptides.

151 The content of free amino groups was determined using the TNBS method (Adler-Nissen,
152 1979). For that, 25 µL of sample was mixed with 200 µL of sodium phosphate buffer (0.2
153 M, pH 8.2) and 200 µL of TNBS (0.1 %), and incubated at 50 °C for 1 h. After adding
154 640 µL of HCl (0.1 N), the mixture was incubated for 30 min and then the absorbance
155 was measured at 340 nm using a UV-Visible spectrophotometer (Helios Zeta, Thermo
156 Scientific, UK). Results were expressed as mg of leucine per mL of sample.

157 Total soluble proteins were evaluated according to the Bradford assay (Bradford, 1976).
158 Briefly, 40 µL of sample was mixed with 2 mL of Bradford reagent, which was comprised
159 of Coomassie Brilliant Blue G-250 (0.01 %), ethanol (4.7 %), and phosphoric acid (8.5 %).
160 The mixture was incubated for 5 min at room temperature and the absorbance measured
161 at 595 nm. Results were expressed as mg of BSA protein per mL of sample.

162 The content of TCA-soluble peptides was determined based on the methodology
163 described by Ketnawa and Ogawa (2019). For that, 50 µL of sample was mixed with 450
164 µL of TCA (5 %), vortexed and kept at 4 ºC for 1 h. The mixture was centrifuged at 8000
165 *g* for 10 min, and the absorbance of the supernatant was measured at 280 nm. Results
166 were expressed as mg of tyrosine per mL of sample.

167

168 **2.5 *In silico* analysis**

169 The UniProt database (https://www.uniprot.org/) was used to obtain the sequence data in
170 FASTA format of ovalbumin (accession number P01012), which is the major protein in
171 egg white. Then, the gastrointestinal digestion was simulated by using the "enzyme(s)

action" tool of BIOPEP-UWM database (http://www.uwm.edu.pl/biochemia/index.php/en/biopep) (Minkiewicz et al., 2019). Pepsin (EC 3.4.23.14) was used to predict the proteolysis occurring during the gastric phase, whereas trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) were used subsequently to pepsin in order to simulate the protein hydrolysis during the complete digestion process. The ToxinPred software (http://crdd.osdd.net/raghava/toxinpred/) was used to predict the molecular weight (MW) and isoelectric point (pI) of the peptides obtained after *in silico* digestion.

2.6 Particle size and zeta potential

The supernatants extracted from digestion matrix were analysed in particle size (Ps) and zeta potential (ζ-potential) terms. These parameters were measured to achieve a better understanding of imaging information since laser-backscattering imaging is based on capturing the alterations in diffraction patterns produced by light-particle interaction. Measurements were carried out in a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). The instrument was set up for proteins as material and water as dispersion medium**.** Ps was expressed as the average particle size (nm). The Smoluchowski mathematical model was used to convert the particle mobility measurements into ζ-potential values (mV) (Fuentes et al., 2020). All the determinations were performed in triplicate at each sampling time.

2.7 Imaging device and capture

A low-cost imaging device was designed based on Verdú et al. (2020) in order to collect information from each sampling time generated by light-matrix interactions. The objective was to use images to collect the variance generated in the matrix during

digestion by capturing the diffraction patterns transmitted due to laser-matrix interaction at each sampling time. For that purpose, samples were prepared placing 3 mL supernatant (obtained at each sampling time during *in vitro* digestion) on a transparent well plate (12-well plate, 21.2 mm ø, 6.9 mL, Corning Costar®). Figure 1-A shows a scheme of the device setup. The elements were a digital capture system (digital camera), a laser light, and a computer. The setup was installed inside a dark chamber away from light. All the camera's light controls were set to the manual mode, avoiding uncontrolled alterations to the work regime (gain, shutter speed, white balance, etc.). A digital Logitech C920 camera (maximum resolution: 1080p/30 fps - 720p/30 fps) was the capture system. Images were captured in the RGB (red, green and blue) format and were saved as .JPEG (1980x1080). The camera was vertically placed 15 cm over the sample, which lays in the middle of the vision field. The laser beam was used in line mode (650 nm, 50 mW), and it was perpendicularly placed 20 cm under the zone where the wells plate placed. This configuration allowed projecting laser-line on the diameter of each well bottom surface. The laser properties and placement distances in the device were selected after previous studies in food applications (Verdú et al., 2021, 2020). The main objective of this configuration was to transmit enough light to capture changes in the studied matrix without saturating the camera sensor. Both the camera and the laser line were connected to a computer. The capture procedure was static for all sampling time (well). It means that images were manually captured by placing the well plates into imaging device. Captures were taken every 20 min during the digestive process for both egg white and control samples following the above-mentioned procedure. Five replicates of images per well (at each sampling time) were taken from each of the two digestion replicates.

2.8 Image processing and data collection

The first step was to transform images from RGB to 8 bits (grayscale) format with the aim of simplifying intensity information. Next operation was to create an image stack from the images captured at each sampling time of the digestion process (Figure 1-B). The second step was to select five regions of interest (ROIs) and extract five orthogonal slices from each stack corresponding to each of them. Those slices formed images with 150 pixels in Y-axis, maintaining the laser-line in the middle of them (Figure 1-B, white dotted lines delimited by *a* and *b*). Five replicates from each stack were parallelly extracted, leaving 50 pixels between them across X-axis. That extraction was carried out by slicing the stack across the time axis (Z-axis). Finally, five replicates of images crossing the central zone of the captured diffraction pattern (Figure 1-C, example of orthogonal image) was obtained from each sampling time of the digestion process. In these new images (orthogonal images), the X-axis corresponded to time while Y-axis corresponded to pixel position. Information across pixel position described the diffraction pattern. The intensity data from each pixel across all positions were taken as single variables to collect variance during the digestion process. Thus, any change in light intensity at each sampling point was captured in these orthogonal images to simplify the image-stack from the entire process to a single image. The obtained images contained the intensity profile of the diffraction pattern along the selected pixels in the two studied phases of the digestion. Each image represented a data matrix containing the changes in the intensity profiles with time (Figure 1-E). The extracted matrix from each replicate was organised and labelled in a common data matrix to study the evolution of the information captured during digestion process, as well as the relationship to the physicochemical properties in composition and particle properties terms. Thus, the data matrix provided spectra of the intensities across pixel positions, which were used as a

246 basis for numerical analytics. These procedures were run with the ImageJ software
247 (http://rsb.info.nih.gov/ij/) (Schindelin et al., 2012).
248
249 **2.9 Statistical analysis**
250 The evolution of physicochemical data of the protein gel matrix (obtained by
251 determinations of free amino groups, soluble proteins, TCA-soluble peptides, *in silico*
252 analysis, Ps and ζ-potential) was analysed by studying time series. Moreover, the imaging
253 data were explored after applying multivariable statistical procedures to reduce data
254 dimensionality. For this purpose, a principal component analysis (PCA, a multivariate
255 unsupervised statistical method) was employed. This method allowed a simplification of
256 the analysis of variance collected by the image data matrix across digestion process.
257 Calibration studies were carried out by applying PLS-R (Partial Least Square Regression).
258 This method allowed to evaluate the dependence between the image information
259 (multivariate data matrix) and analytical data (contents of free amino groups, soluble
260 proteins, and TCA-soluble peptides) generating linear regressions. The calibrations were
261 evaluated based on the R^2 and root mean square error (RMSE). These procedures were
262 run with the PLS Toolbox, 6.3 (Eigenvector Research Inc., Wenatchee, Washington,
263 USA), a toolbox extension in the Matlab 7.6 computational environment (The Mathworks,
264 Natick, Massachusetts, USA).
265
266 **3. Results and discussion**
267 **3.1 Digestibility of the protein gel matrix**
268 Heating of proteins matrices, such as chicken egg white, leads to conformational changes
269 that affect the folded structure of proteins, producing aggregates that join together to form
270 gels (Ma and Holme, 1982). Physicochemical properties and gel structure influence the

extent of digestion, amount and nature of generated peptides (Nyemb et al., 2016). The proteolysis occurring in the egg white gel matrix during *in vitro* gastrointestinal digestion was evaluated by determining the content of total free amino groups, which include the available amino groups of proteins, peptides, and amino acids (Adler-Nissen, 1979). The content of soluble proteins and TCA-soluble peptides were also measured to obtain information about the solubility of the digestive products. Results of protein digestibility in the controls (without sample) and samples obtained before digestion (t0) and during the gastric phase (*G*, 0-120 min) and intestinal phase (*I*, 120-240 min) were presented in Figure 2. Results were expressed as mg/mL of sample in the digestive medium for a better comparison with the data subsequently obtained by using laser-backscattering imaging to model the digestion process. Controls presented values below 0.2 mg/mL in the content of free amino groups, soluble proteins and peptides during *G*, whereas an increased proteolysis and solubility was observed during *I* (Figure 2-A). Regarding the samples, a gradual increment in the content of free amino groups was observed as the digestion advanced, with values increasing from 0.27 to 1.63 mg/mL in *G* and reaching up to 14.52 mg/mL at the end of *I* (Figure 2-B). These results evidence the action of digestive enzymes, mainly those of the intestinal phase, hydrolysing the protein gel matrix. Moreover, the solubility of proteins increased during the *in vitro* digestion, with values ranging from 1.68 to 2.19 mg/mL, whereas the content of TCA-soluble peptides varies throughout the process with values between 7.44 and 13.62 mg/mL (Figure 2-B).

3.2 *In silico* analysis

In silico analyses were performed to predict the peptide sequences released during a simplified gastrointestinal digestion of ovalbumin, which is the major protein present in egg white. The theoretical hydrolysis of the protein with enzymes found in the

296 gastrointestinal tract, including pepsin (gastric phase) and trypsin and chymotrypsin
297 (intestinal phase), is shown in Figure 3-A. The MW and pI of the generated sequences
298 was also predicted *in silico* with the aim of knowing the properties of produced peptides
299 and thereby improving the physicochemical characterisation at the end of each digestion
300 phase. Figures 3-B and 3-C show the MW of the generated peptides expressed in
301 percentiles (*P*). The peptides generated during *G* showed higher average weight than *I*.
302 Compounds within P_{65} of *G* had the highest MW obtained in *I* (~2 g/mol) while the rest
303 of compounds in *G* rose to 5 g/mol. During gastrointestinal digestion, protein chains were
304 cleaved by peptidase enzymes during the gastric and intestinal phases, releasing smaller
305 peptides as the digestion advances. Moreover, in the same way of MW, peptides obtained
306 from each phase presented differences in pI. Figures 3-D and 3-E show pI of each block
307 of peptides expressed in percentiles. In the case of *G*, 20% of peptides had a difference
308 of one pH unit between pI and the pH used in this phase (pH=3), whereas 50% of peptides
309 presented pI more than threefold (pI~6) (Figure 3-D). More differences between pI and
310 pH would mean more stability for the generated dispersion. In *I* (pH=7), 65% of peptides
311 presented differences of less than one unit between pI and pH, suggesting a low stability
312 of the dispersion. The observed theoretical effect of each digestion phase on both MW
313 and pI of the generated compounds should impact on the properties of the generated
314 particles (such as ζ-potential and particle size). That impact should modify matrix
315 properties not only at the end of the phase, but also at each sampling point. Since these
316 modifications were directly related to the matrix structure, the generated changes in the
317 laser-matrix interaction should correlate with information captured by the imaging
318 technique.
319

3.3 Particle size and zeta potential distributions

After observing the results from computational analysis of egg white digestion, the extracts of the digested matrix were analysed in Ps and ζ-potential terms for evidencing matrix structure modifications at each sampling point. The aim was to get a better understanding of produced changes and thus improve the interpretation of laser-matrix interaction captured in imaging data. Figures 4-A and 4-B show the time series of the measurements, from which kinetics of both parameters during the digestion process were studied. Ps of egg white gel was maintained nearly constant during *G*, whereas the addition of reagents (digestive fluids, enzymes and bile) and the pH change from 3 to 7 in *I* drastically increased Ps (Figure 4-B). This effect was also observed in the control samples (Figure 4-A), suggesting that it could be mainly due to the formation of large flocs in presence of bile and pancreatin enzyme (Liu and Kong, 2019; Sharkar et al., 2018). Accordingly, *in silico* results indicated that 50% of the peptides generated at the end of *G* presented pI close to the pH of *I* (pH=7) (Figure 3-D). Thus, when matrix was modified to that pH, stability of peptides would decrease due to reduction of solubility and aggregation of particles (Nyemb et al., 2016), which would increase Ps.

These phenomena matched with ζ-potential evolution. In egg white gel samples, values reduced magnitude of negative charge (from -15 to -10 mV) during the first 40 min of *G*, from which it was constant up to the end of this phase. At the beginning of *I*, ζ-potential drastically changed and then decreased up to values between 0 and -5 mV (Figure 4-B). Similar to that suggested from the *in silico* results, ζ-potential values indicated lower stability of the compounds in *I* than *G*, which also matched with the observed increase of Ps. So, results evidenced the progressive physical changes in the studied matrix due to the digestion process. Changes in chemical composition of samples produced by enzymes and pH during digestion produced alterations in particles and matrix structure that should

affect the interaction with laser and therefore the information captured in diffraction patterns. Thus, after characterising these modifications, the objective was evaluating the capacity of the imaging technique to detect these changes and relate them to the released protein compounds.

3.4 Image analysis

3.4.1. Imaging data exploration

Image data were explored in raw form in order to observe if there was evolution of intensity data during the digestion process at different pixel positions into the diffraction patterns. In this regard, Figure 5 shows the results obtained for the control sample (Figure 5-left) and egg white gel sample (Figure 5-rigth). A real sequence of images corresponding to half capture (from central peak of diffraction pattern, pixel position 75, to an extreme) were included. Three pixels positions from the central peak (A, B and C) were selected to explore whether differences in data were produced depending on the pattern zone. That study was necessary to evaluate if pixel positions collected redundant data or, by contrast, different information was collected from different pixel positions and therefore no positions should be obviated.

Figure 5 shows the intensity evolution observed at the selected pixel positions, presenting different kinetics for each one. The digestion phases also presented differences within the same position due to the changes in composition and pH. The same alteration in the time series observed in the physicochemical study due to the changes produced at gastric-intestinal intersection (pH and reagents addition) (Figures 2 and 4) was reproduced in the image data. On the other hand, intensity captured within each phase presented a different evolution depending on the position. The digestion of egg white gel was also differenced from the control sample. In addition to the observed differences in kinetics, the digestion

of egg white gel generated higher intensity changes compared to control in A and B, while less at C position (Figure 5).

Thus, the explored raw data evidenced the capacity of the imaging technique to capture the variance generated from different factors such as the enzymatic action on both egg white gel and themselves, and the changes produced because the digestive phases. However, results suggest that different fractions of total variance collected by images were contained in each pixel position. It meant that pixel positions provided different information of the digestion process and therefore all of them (150 variables) had to be considered from data matrix to attempt process modelling.

Since entire data matrix from imaging was considered, it was processed by a PCA with the aim of analysing imaging data in a reduced dimensionality. PCA reduced 150 variables to one synthetic variable (linear combination from the original ones) called PC1, which collects 72.2% and 75.1% total variance for control and digested sample, respectively. Kinetics of PC1 scores are included in Figure 6. The differences between control and egg white gel samples were better observed after this data transformation. Control showed fluctuant evolution within each digestion phase while egg white gel presented different descendent tendencies for each phase. The range of scores for *G* was 20-15, from t=0 to t=120 min, while *I* showed 55-21 from t=120 to t=240 min. The observed kinetics for PC1 scores were in accordance with that observed in Ps and ζ-potential results. Figure 6-A and 6-B include ζ-potential evolution as example. Those similar evolutions evidenced the sensibility of the imaging technique to capture changes in the matrix due to physicochemical modifications of the protein compounds during digestion. The alterations in Ps and ζ-potential produced changes in the interaction patterns of light-matrix because modifications in particle properties, enabling to be modelled by the imaging technique. In the same way of the rest of analysis, this effect

was maximum at the beginning of *I*. Although changes in matrix did not impede the capture of differences during *I*, the impact of pH changes and adding intestinal enzymes and bile produced a new matrix that could produce important differences in the diffraction pattern basis.

This effect was confirmed studying the patterns from all samples at t=120 min for both phases. In this point, since enzymes in *G* were blocked and no time had passed for action of intestinal enzymes in *I*, the observed differences in the diffraction patterns were exclusively produced due to pH changes and reagents addition. Figure 6-A and 6-B shows the diffraction patterns at t=120 min for both phases. Differences in the control were visually limited because the absence of sample, while the digestion of egg white gel generated strong differences in the intensity distribution. The central peak lost intensity while the extremes increased. This phenomenon could be explained according to the Ps modification; however, results did not match to the principle that is used by particle size analysers. These analytical devices are based on the fact that the angle of light diffraction is inversely proportional to the particle size (Emmerich et al., 2019). It matched with an increase of diffraction angle, contrary to what was expected for larger particles. Therefore, this effect evidenced the result of the combined effect of Ps modifications and addition of new compounds like bile, which increased opacity of matrix and therefore led to a more diffuse intensity pattern.

These interactions difficulted visualising a global evolution of the imaging signal for the entire digestion process. For this reason, PC1 scores were normalised transforming data to score increases (Δ). It was expressed in absolute value and assumed t=120 min as equal for both phases. The transformation made it visualisable the changes captured by the imaging technique, achieving a better comparison with physicochemical results. Figure 6-A and 6-B shows the evolution of transformed scores data (black continuous lines),

which was in accordance with free amino groups results (Figure 2). This relation could be due to free amino groups were the unique analysed compounds accumulated over time, unlike soluble protein and peptides that fluctuated depending on sampling point. In this sense, the normalised scores represented the accumulation of changes during the entire digestion process, whereby this data expression could be largely affected by the evolution of this fraction of compounds.

Results reported the capability of the imaging technique to capture changes produced during the digestion process of egg white gel, and its differences with control samples. The information obtained by imaging captured variance along time following the evolution of the physicochemical changes generated because enzyme action and reagents addition. Therefore, after that data exploration in non-supervised way, studies of dependence between concentration of released protein compounds and imaging data were carried out to determine the possibility of calibration between both data blocks.

3.4.2. Calibration studies

After evidencing the capacity of the imaging technique to capture the variance generated because digestion of egg white gel, calibration studies were carried out between the concentration of released protein compounds and the data from diffraction patterns. *PLS-R* was the method used to done it. Figure 7 shows the calibration plots reported by *PLS-R* after reducing the dimensionality of imaging data to one latent variable (*LV1*). This variable also represented a lineal combination from original variables (pixel positions) of diffraction pattern, in this case, optimised to obtain the maximum correlation with each measured protein parameter. The R^2 of calibration for all parameters were higher than 0.90, and *RMSE* also showed reduced values, which indicated strong relationship between both data sets with independence of digestion phase. Note that soluble protein contents

445 (Figure 7-C) showed overlapping between phases around 1.75 mg/mL because
446 concentrations around those values were registered at both phases. This overlapping was
447 not observed across calibration model for free amino groups (Figure 7-A) and soluble
448 peptides (Figure 7-B) since their concentration ranges were different at each phase,
449 principally for free amino groups. In such a case, correlation was principally produced
450 with data from *I* because a reduced variation was observed during *G*. This fact decreased
451 the correlation coefficient comparing to the rest of parameters.
452 The weight of each pixel position on the explanation of variance retained in *LV1* were
453 explored by loadings. Figures 7-D, -E and -F show loading data of *LV1* for each regression
454 study overlapped on an example of diffraction pattern. The distribution of weights was
455 similar for all analytes. The central zone was maintained around zero (dotted line), which
456 meant reduced weight for those pixel positions in the explanation of digestion evolution.
457 Moreover, external zones presented higher weight towards negative zone of *LV1* axis.
458 This distribution of weights indicated that the pixel positions placed in the extremes of
459 the pattern were the main zones collecting information from physicochemical changes
460 occurring during digestion. It followed the observed in the Figure 6-D, where external
461 zones collected the effect of pH changes and reagents addition at the beginning of *I*. The
462 explanation could be attributed to the fact that central zones represented pixels with higher
463 level of light saturation than the external ones. This saturation could reduce the sensitivity
464 of the central zone of sensor, needing intense changes in light-matrix interaction to detect
465 significant changes. However, external zones, which collect the most diffracted light
466 fraction, kept away from saturation, maximising the capacity to detect soft changes due
467 to the moderate amount of arrived light.
468 Calibration results proved the linear relationship between captured imaging data and the
469 evolution of released protein compounds throughout gastrointestinal digestion of egg

white gel. These results suggested that the used imaging data captured information dependent from protein compounds but independent from digestion phase. This concept was important since clashed with the observed impact of phase change on diffraction pattern showed in Figure 6-D. This meant that the used imaging technique captured digestion process-dependent information in images; however, it was necessary supervising the reduction of dimensionality to calibrate both datasets. Therefore, after a supervised data-transformation, this information correlated linearly with the concentration of analysed protein compounds but was not affected by changes in pH and intestinal enzymes addition, obtaining R^2 >0.90 for all of them. The calibrations were obtained from a specific type of protein sample processed under certain conditions, which was used as protein matrix model. The observed capacity of the technique to register digestion changes should be maintained if different protein matrix were tested. However, modifications in matrix samples resulting from egg specie, age, presence of colouring agents or particles as well as from other type and origin of protein (e.g, casein or whey protein) require a new calibration study to explore the effect on the observed models.

4. Conclusions

Gastrointestinal digestion of egg white gel showed different profiles of protein products depending on the sampling time and phase (*G* and *I*). The evolution of compounds concentration, pH changes, and reagents addition produced physicochemical changes in matrix that induced modifications in Ps and ζ-potential. Those changes led to alterations in the laser-matrix interaction that were registered by the imaging technique in form of diffraction patterns. The captured diffraction patterns showed different evolution depending on the pixel position and the digestion phase. After a dimensionality reduction of image data, capability to capture variance generated because phase change and

enzymatic action was evidenced. Moreover, high calibration level (R^2 >0.90) was obtained between imaging data and analytical data (content of free amino groups, soluble protein, and soluble peptides). This indicated that, although pH and added reagents produced non-desirable variance in imaging data with phase changes, the variance produced because changes on concentrations of protein compounds was successfully registered. Therefore, results suggested that laser backscattering imaging was capable of monitoring the gastrointestinal digestion of egg white gel as protein matrix model. This technology could improve existing analytical methods for developing non-destructive approaches within food digestibility research area. The next step should focus on monitoring *in situ* the food matrix digestion, without sample modification/destruction, with the aim of obtaining continuous image data during the entire process in a non-destructive way. Moreover, new factors such as different protein origin, protein mixes and sample pre-treatments need to be included in future studies to test the capability of this technique to model sample variability sources.

Acknowledgements

Postdoctoral grant (PAID-10-19) from the Universitat Politècnica de València to M.G. is acknowledged. Authors also thank the Ministerio de Ciencia, Innovación y Universidades, Agencia Estatal de Investigación and Fondo Europeo de Desarrollo Regional for the financial support provided through the RTI2018-098842-B-I00 and RTI2018-101599-B-C21-AR projects.

Author contributions

Conceptualization, S.V., M.G., P.T. and R.G.; Methodology, S.V. and M.G.; Formal Analysis, S.V. and M.G.; Investigation, S.V. and M.G.; Writing – Original Draft

Preparation, S.V. and M.G; Writing – Review & Editing, S.V., M.G., JM.B., P.T. and R.G.; Visualization, S.V. and M.G; Project Administration, JM.B., P.T. and R.G.; Funding Acquisition, JM.B., P.T. and R.G.

Conflicts of interest

None

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FIGURE CAPTIONS

Figure 1. Capture device and image processing schemes. A: Capture system configuration. B: Images from each sampling time from complete digestion process (white dotted lines: the five ROIs for data extracting, *a*: pixel position 1; *b*: pixel position 150). C: Example of ROI from original stack. D: Visualising intensity (grayscale) differences captured at each pixel position of selected zone. E: Data matrix structure after extraction. *G*-red series: gastric phase; *I*-blue series: intestinal phase. Red and blue dashed lines mark specific sampling times as example (t=60 and t=180 min, respectively).

Figure 2. Evolution of the content of free amino groups (●), soluble proteins (■), and TCA-soluble peptides (▲) during *in vitro* gastrointestinal digestion. A: control; B: egg white gel. *G*-red series: gastric phase; *I*-blue series: intestinal phase. Green dashed line marks change of phase.

Figure 3. Results of *in silico* studies. A: amino acid sequence of ovoalbumin protein before digestion (up), after the gastric phase (middle), and after the intestinal phase (bottom). B and C: MW of the peptides obtained after each phase expressed in percentile profile. D and E: pI of the peptides obtained after each phase expressed in percentile profile. *G*-red series: gastric phase; *I*-blue series: intestinal phase. Dashed lines indicate pH at *G* (red) and *I* (blue). Watermarks indicate specific percentiles of pI near to pH in each phase.

Figure 4. Evolution of particle size (Ps) (■) and zeta potential (●) during digestion. A: control; B: egg white gel. *G*-red series: gastric phase; *I*-blue series: intestinal phase. Green dashed line marks change of phase.

Figure 5. Evolution of intensity data from captured image sequences at different pixel position. Left: raw image sequences of half pattern. Right: time series of intensity along phases corresponding to A, B and C positions. A, B and C: pixel positions along pattern.

G-red series: gastric phase; *I*-blue series: intestinal phase. Green dashed line marks change of phase.

Figure 6. Evolution of processed imaging data with PCA. A: control. B: egg white gel. C: diffraction patterns of control from both phases at t=120 min. D: diffraction patterns of egg white gel from both phases at t=120 min. Arrows mark t=120 min. *G*-red series: gastric phase; *I*-blue series: intestinal phase. Grey dotted lines mark ζ-potential evolution.

Figure 7. Results of calibration studies between protein compounds and imaging data. Regression studies (up) between *LV1* from *PLS-R* and free amino groups (A), soluble peptides (B) and soluble protein (C). Green series: loadings from *LV1* (down) for free amino groups (D), soluble peptides (E) and soluble proteins (F). Dotted black lines mark no charge for variables (pixel position). Black series: example of captured diffraction pattern. *RMSEC*: root mean square error calibration. R^2: calibration coefficient. *G*-red series: gastric phase; *I*-blue series: intestinal phase. Bars indicate standard deviation.