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TECNOLOGIAS INOVADORAS PARA O PROCESSAMENTO SEQUENCIAL COMBINADO VISANDO A MELHORIA DE PASTEURIZAÇÃO DE OVO-PRODUTOS

INNOVATIVE SEQUENTIALLY COMBINED PROCESSING TECNHOLOGIES TO IMPROVE THE QUALITY OF EGG PASTEURIZATION PRODUCTS

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Ciência e Tecnologia Alimentar e Nutrição, realizada sob a orientação científica do Professor Doutor Jorge Manuel Alexandre Saraiva, Professor Associado do Departamento de Química da Universidade de Aveiro e do Professor Doutor José António Teixeira Lopes da Silva, Professor Auxiliar do Departamento de Química da Universidade de Aveiro.

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Dedico este trabalho aos meus pais, ao Mário, às minhas madrinhas e tio Fernando pelo incansável apoio ao longo de todas as etapas da minha vida.

o júri

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palavras-chave

Clara de ovo, gema de ovo, ovo inteiro líquido, pressão moderada, ultrassom, pasteurização térmica, inativação de *Salmonella* Senftenberg 775/W, propriedades físico-químicas e funcionais.

resumo

Uma nova abordagem combinando pré-tratamentos não térmicos seguidos de uma pasteurização térmica (PT) menos intensa foi avaliada como uma alternativa à PT comercial de ovo-produtos. Assim, este trabalho teve como objetivo avaliar o efeito de pré-tratamentos de pressão (50 – 400 MPa/5 – 20 min) e ultrassom (US, 50% amplitude/1 – 3 min) (isoladamente e/ou combinados) antes de uma PT mais curta, na inativação de microrganismos (*Salmonella* Senftenberg 775/W e *Listeria innocua*) e nas propriedades de qualidade dos ovo-produtos, versus PT comercial.
Em clara de ovo (CO), a utilização de pressão moderada (PM) seguida de uma PT mais curta (PM-PT) inativou cerca de 4,4 ciclos log_{10} de *S.* Senftenberg 775/W e *L. innocua*, mas a PT comercial causou uma maior inativação. No geral, o tratamento combinado reteve melhor as propriedades de qualidade da CO do que a PT comercial. Porém, os merengues preparados com as amostras tratadas e não tratadas obtiveram a mesma aceitabilidade.
Para a gema de ovo (GO), obteve-se o mesmo nível de inativação utilizando PM-PT e TP comercial, mas em geral, o tratamento combinado permitiu obter uma GO com melhores propriedades de qualidade. No entanto, o PM-PT causou alterações mais pronunciadas no perfil volátil da GO, mas o doce de ovos preparado com esta amostra apresentou maior aceitabilidade sensorial.
Em ovo inteiro líquido (OIL), o tratamento combinado e a PT comercial alcançaram um efeito letal equivalente, todavia o tratamento de PM-PT manteve ou melhorou algumas das propriedades de qualidade do OIL pasteurizado. Além disso, as tartes de ovo preparadas com as amostras tratadas e não tratadas obtiveram aceitabilidade sensorial semelhante.
Posteriormente, a combinação de pré-tratamentos de PM e US com uma PT mais curta (PM-US-PT), aplicada em OIL, alcançou o mesmo nível de inativação que a PT comercial. Contudo, a PT comercial reteve melhor as propriedades de qualidade do OIL do que o tratamento de PM-US-PT.
Assim, os resultados indicaram que a redução da intensidade da PT quando aplicada em combinação com um pré-tratamento de PM pode ser uma alternativa à PT comercial, alcançando um nível de segurança semelhante, mas mantendo e/ou melhorando as propriedades de qualidade dos ovo-produtos.

keywords

Egg white, egg yolk, liquid whole egg, moderate pressure, ultrasound, thermal pasteurization, *Salmonella* Senftenberg 775/W inactivation, physicochemical and functional properties.

abstract

A new approach combining non-thermal pre-treatments before a less intense thermal pasteurization (TP) was evaluated as an alternative to commercial egg products TP. Thus, this work aimed to evaluate the performance of pressure (50 – 400 MPa/5 – 20 min) and ultrasound (US, 50% amplitude/1 – 3 min) pre-treatments (singly and/or combined) before a shorter TP on egg products, versus commercial TP, to assess the effect on microorganisms' inactivation (*Salmonella* Senftenberg 775/W and *Listeria innocua*) and egg quality properties.

Initially, in egg white (EW), using moderate pressure (MP) followed by a shorter TP (MP-TP) allowed to reduce *S.* Senftenberg 775/W and *Listeria innocua* population up to 4.4 $\log_{10}$ cycles, while a higher inactivation was obtained with commercial TP. Overall, the combined treatment resulted in better retention of EW properties than commercial TP. However, the meringues prepared with treated and non-treated EW showed similar global acceptability.

On the other hand, for egg yolk (EY), the same inactivation level was attained with MP-TP and commercial TP, yet, in general, the combined treatment allowed obtaining EY with better quality properties. However, MP-TP treatment caused more pronounced changes in EY volatile profile, but the "Doce de ovos" prepared with this sample had a higher sensory acceptability.

In liquid whole egg (LWE), the combined treatment and commercial TP achieved a similar lethal effect, with the MP-TP maintaining and/or improving some properties of pasteurized LWE. Still, the egg tart prepared based on the treated and non-treated LWEs obtained similar sensory acceptability.

Posteriorly, the combination of MP and US pre-treatments with a subsequent shorter TP (MP-US-TP), applied in LWE, reached a safety level against *S.* Senftenberg 775/W similar to that of commercial TP. Although, the latter retained better the quality properties of raw LWE.

Therefore, the results indicated that the reduction of TP intensity when applied in combination with a MP pre-treatment could be an alternative to commercial egg products TP, offering a similar safety level against *S.* Senftenberg 775/W, and also to maintain and/or improve egg quality properties.

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LIST OF ABBREVIATIONS

ANS	1-anilino-8-naphtalene-sulfonate
CFU	Colony Forming Unit
D	Decimal Reduction Time
DSC	Differential Scanning Calorimetry
EAI	Emulsifying Activity Index
EDTA	Ethylenediaminetetraacetic Acid
ESI	Emulsion Stability Index
EU	European Union
EW	Egg White
EY	Egg Yolk
FA	Fatty Acids
FAMEs	Fatty Acid Methyl Esters
FC	Foaming Capacity
FID	Flame Ionization Detection
FS	Foaming Stability
GC/MS	Gas Chromatography-Mass Spectrometry
HDLs	High-Density Lipoproteins
HP	High Pressure
HS-SPME	Headspace-Solid Phase Microextraction
IS	Internal Standard
LDLs	Low Density Lipoproteins
LWE	Liquid Whole Egg
MDA	Malondialdehyde
MP	Moderate Pressure
MP-TP	Moderate Pressure Followed by Thermal Pasteurization
MP-US	Moderate Pressure Followed by Ultrasound
MP-US-TP	Moderate Pressure Followed by Ultrasound Followed by Thermal Pasteurization
MUFA	Monounsaturated Fatty Acid
PCA	Principal Component Analysis
PEF	Pulsed Electric Fields
PUFA	Polyunsaturated Fatty Acid

SFA	Saturated Fatty Acid
SH	Sulfhydryl
TBARS	Thiobarbituric Acid-Reactive Substances
TC	Triethyl Citrate
TP	Thermal Pasteurization
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
US	Ultrasound
US-MP	Ultrasound Followed by Moderate Pressure
US-MP-TP	Ultrasound Followed by Moderate Pressure Followed by Thermal Pasteurization
US-TP	Ultrasound Followed by Thermal Pasteurization
WHC	Water Holding Capacity
z	Thermal Coefficient
ΔE^*	Total Colour Difference
ΔH	Enthalpy Variation

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Chapter I

General Introduction, Objectives and Thesis Structure

1.1.General introduction and objectives

Eggs and egg products are recognized as a basic foodstuff for humans, being consumed worldwide due to their high nutritional value, low cost, and low caloric content. However, they are a highly perishable product even stored under refrigeration and egg-associate salmonellosis is an important public health problem. Thus, to reduce consumer risks, egg products need to be thermally pasteurized, yet, such treatment has a detrimental effect on egg products characteristics, particularly on the functional and physicochemical properties. As an alternative to thermal pasteurization (TP), non-thermal technologies such as high pressure, pulsed electric fields and ultrasound (US) have been studied. Nevertheless, their use *per se* was shown to be not effective in reducing 5 log colony forming unit (CFU)/g of a foodborne pathogen, and also showed considerable detrimental effects on eggs quality when causing possible interesting microbial inactivation, being so no viable alternatives. Therefore, a new possibility to achieve eggs pasteurization with better quality is the combined use of pre-treatments, applied at sublethal intensities, with the aim to cause sublethal damages in microorganisms, thus reducing their resistance to temperature, allowing a further TP with lower thermal input.

The main objectives of this work were so to evaluate the effect on liquid whole egg (LWE), egg yolk (EY) and egg white (EW) of a pre-treatment by pressure and US (singly and/or combined) followed by a less intense TP on:

(i) The lethal efficacy on inoculated microorganisms after treatments and during storage;

(ii) Functional and physicochemical properties;

(iii) Sensorial characteristics of egg product-derived produced from treated LWE, EY and EW.

1.2. Thesis structure

In order to do that, the work performed during the PhD thesis was structured as follows (**Figure 1.1**). Initially, a general literature review on egg structure, composition and properties, egg-associated foodborne pathogens and the advantages/disadvantages of egg products TP was presented. In addition, a possible strategy to improve egg products TP was described, as well as an overview of the effect of combined treatments on microorganisms' inactivation in egg products and their effect on quality properties (**Chapter 2**). **Chapter 3** follows with a detailed description of the materials and methods used throughout this thesis. To optimize the combined treatments, the sequential combination of pressure and/or US pre-treatments followed by a shorter TP was evaluated in **Chapters 4**, **5**, **6** and **7**. The combination of pressure pre-treatment before a shorter TP applied to liquid EW was optimized taking into account the inactivation of *Salmonella* Senftenberg 775/W (inoculated study). For the most promising condition, a further evaluation of the physicochemical and functional properties was performed, as well as a post-TP refrigeration storage study (inoculated study). Furthermore, a sensory analysis of egg product-derived produced with treated EW was carried out. All results were compared with non-treated and commercial TP-treated samples (**Chapter 4**). **Chapters 5** and **6** were constructed along the same lines as **Chapter 4**, but studying liquid EY and LWE, respectively. In **Chapter 7**, a preliminary test was initially conducted in order to define the US conditions for further studies. After selecting the conditions, the sequentially combined effect of US and moderate pressure (MP) (singly and/or combined) before a shorter TP on the inactivation of *S*. Senftenberg 775/W inoculated in EW, EY and LWE was assessed. The most promising results were obtained for LWE, using MP followed by US pre-treatments before a shorter TP (MP-US-TP). Thus, the MP-US-TP conditions were optimized (*S*. Senftenberg 775/W inactivation), and for the combination that reached similar lethal effect to commercial TP, the LWE properties were evaluated. Finally, in **Chapter 8** the main conclusions obtained in this thesis are presented and some future work that can complement what was studied in this thesis is also suggested.

CHAPTER 2
LITERATURE REVIEW

CHAPTER 3
MATERIALS AND METHODS

CHAPTER 4
SEQUENTIAL COMBINED EFFECT OF MODERATE PRESSURE AND THERMAL PASTEURIZATION IN EGG WHITE

- ✓ *Salmonella* Senftenberg 775/W inactivation (conditions optimization)
- ✓ Post thermal pasteurization refrigeration study (inoculated studies)
- ✓ Physicochemical and functional properties
- ✓ Sensory analysis

CHAPTER 5
SEQUENTIAL COMBINED EFFECT OF MODERATE PRESSURE AND THERMAL PASTEURIZATION IN EGG YOLK

- ✓ *Salmonella* Senftenberg 775/W inactivation (conditions optimization)
- ✓ Post thermal pasteurization refrigeration study (inoculated studies)
- ✓ Physicochemical and functional properties
- ✓ Sensory analysis

CHAPTER 6
SEQUENTIAL COMBINED EFFECT OF MODERATE PRESSURE AND THERMAL PASTEURIZATION IN WHOLE EGG

- ✓ *Salmonella* Senftenberg 775/W inactivation (conditions optimization)
- ✓ Post thermal pasteurization refrigeration study (inoculated studies)
- ✓ Physicochemical and functional properties
- ✓ Sensory analysis

CHAPTER 7
EFFECT OF ULTRASOUND AND MODERATE PRESSURE (SINGLY AND/OR COMBINED) FOLLOWED BY THERMAL PASTEURIZATION IN LIQUID EGG PRODUCTS

- ✓ *Salmonella* Senftenberg 775/W inactivation (conditions optimization)
  - ❑ Egg White, Egg Yolk and Liquid Whole Egg
- ✓ Physicochemical and functional properties of liquid whole egg

CHAPTER 8
CONCLUDING REMARKS AND FUTURE WORK

Figure 1.1. Thesis structure overview.

CHAPTER II

LITERATURE REVIEW

2.1.Eggs and egg products

2.1.1. Eggs

Eggs for consumption are produced by “laying hens” this meaning adult hens of the species “*Gallus gallus*” [1]. Hens eggs have been used as a food by human since antiquity, and there is no other food of animal origin that is eaten by so many people all over the world [2]. They are recognized as a basic foodstuff for humans due to their high nutritional value (one egg contains ≈ 14 % of protein recommended daily intake), low cost, and low caloric content [3–5]. Therefore, eggs are considered by World Health Organization as a standard protein food of high biological value [6].

Eggs, in general, are considered as tasty, wholesome and a rich and well-balanced source of essential nutrients for human diet, naturally containing a couple of essential vitamins (vitamins A, B6, B12, D, E, and K), trace minerals, and represent an important source of phosphorus, iron and essential fatty acids (FA) [7,8]. In addition, hen egg has a versatility of functions such as: (1) coagulating; (2) foaming; (3) emulsifying; (4) flavouring; and (5) colouring [9]. They have also been classified as nature’s original functional food [10], being associated with some benefits that include anti-inflammatory, anticarcinogenic and antimicrobial activities [11]. Nevertheless, hen eggs are one of the most frequent causes of food allergy in infants and young children [12].

2.1.2. Egg structure and composition

Egg is a complex and highly differentiated reproductive cell. Egg structure moving from the outside to the inside (**Figure 2.1**) is composed by shell, shell membranes, egg white (EW) and egg yolk (EY).

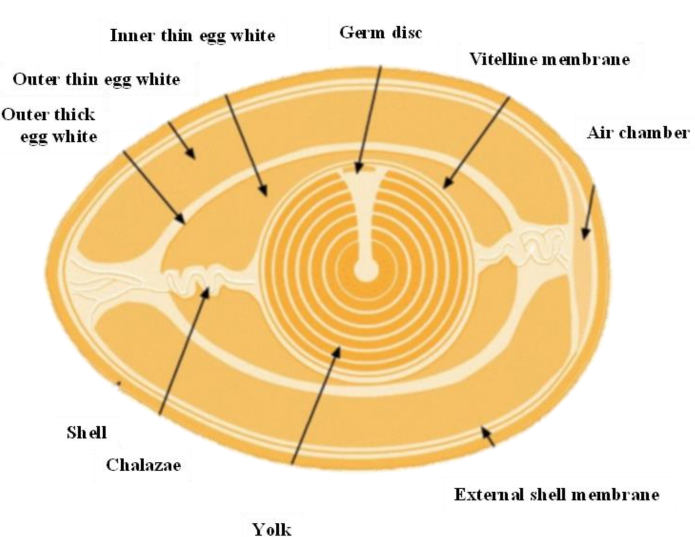

Figure 2.1. General egg structure. Adapted from [13].

Their composition and weight may vary due to factors such as the hen species, age and feeding [14]. **Table 2.1** shows the approximate composition (% w/w) of each egg component.

Table 2.1. Approximate composition (% w/w) of whole egg, eggshell, egg white, and egg yolk.

Egg Component	% of total	Approximate Composition (% w/w)				
		Moisture	Protein	Lipid	Carbohydrate	Ash (Minerals)
Whole egg	100	66.1	12.8 – 13.4	10.5 – 11.8	0.3 – 1.0	0.8 – 1.0
Eggshell	9 – 11	1.6	6.2 – 6.4	0.03	Trace	91 – 92
Egg white	60 – 63	87.6	9.7 – 10.6	0.03	0.4 – 0.9	0.5 – 0.6
Egg yolk	28 – 29	48.7	15.7 – 16.6	31.8 – 35.5	0.2 – 1.0	1.1

Adapted from [14–16].

The chemical composition of the edible portion of whole egg, EW and EY (per 100 g of edible portion) are shown in **Table 2.2**. Regarding egg lipid fraction, the FA present in highest amounts is the monounsaturated FA (MUFA) followed by saturated FA (SFA), and polyunsaturated FA (PUFA). The most abundant FA present are palmitic (C16:0), stearic (C18:0), palmitoleic (C16:1), oleic (C18:1), linoleic (C18:2) and eicosatetraenoic (C20:4) acids. Although, small amounts of α-linoleic (ALA, C18:3 ω-3), eicosapentaenoic (C20:5 ω-3), docosapentaenoic (DPA, C22:5 ω-3) and docosahexaenoic (DHA, C22:6 ω-3) acids may be present [5,11]. In addition, the amino acid composition of egg is very diverse with 18 different amino acids, containing the 9 essential amino acids with concentrations of 0.125 – 1.399 g/100 g [11]. Thus, one egg (56 g) contributes with 14 – 30 % of daily value of protein and minerals (selenium and iodine), and also with 10 – 40 % of vitamins daily value, namely vitamin A, niacin, vitamin B12, pantothenic acid, riboflavin, choline and biotin [5,17].

Table 2.2. Chemical composition of edible portion of whole egg, egg white and egg yolk[1,2].

Nutrient	Value per 100 g of edible portion		
	Whole egg	**Egg white**	**Egg yolk**
Energy (kcal/kJ)	131/547	43/184	347/1437
Water (g)	76.15	87.57	52.31
Protein (g)	12.56	10.9	15.86
Total nitrogen (g)	2.02	1.73	2.62
Carbohydrate, by difference (g)	0.72	0.73	3.59
Total sugars (g)	0.37	0.71	0.56
Total fat (g)	9.51	0.17	26.54
SFA (g)	3.13	< 0.2	9.55
MUFA (g)	3.66	< 0.2	11.74
PUFA (g)	1.91	< 0.2	4.20
Cholesterol (mg)	372	0	1085
Minerals			
Calcium (mg)	56	7	129
Iron (mg)	1.75	0.08	2.73
Magnesium (mg)	12	11	5
Phosphorus (mg)	198	15	390
Potassium (mg)	138	163	109
Sodium (mg)	142	166	48
Zinc (mg)	1.29	0.03	2.3
Copper (mg)	0.072	0.023	0.077
Manganese (mg)	0.03	< 0.01	0.11
Iodine (µg)	50	4	130
Selenium (µg)	30.7	20	56
Chloride (mg)	180	159	163
Vitamins			
Thiamine (mg)	0.040	0.004	0.176
Riboflavin (mg)	0.457	0.439	0.528
Niacin (mg)	0.075	0.105	0.024
Vitamin B6 (mg)	0.17	0.005	0.35
Folate (µg)	47	4	146
Vitamin B12 (µg)	0.89	0.09	1.95
Vitamin A (µg)	160	0	381

Vitamin E (mg)	1.05	0	2.58
Vitamin D (µg)	2.0	0	5.4
Vitamin K (µg)	0.3	0	0.7
Choline (mg)	293.8	1.1	820.2
Choline chloride (mg)	382	1.50	954
Biotin (µg)	19.5	5.6	63.6
Vitamin B5 (mg)	1.35	0.28	4.53
Carotenoids			
β-Carotene + α-Carotene (µg)	< 1	0	126
β-Cryptoxanthin (µg)	9	0	33
Lutein + Zeaxantin (µg)	503	0	1094

[1]SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid.

[2]Adapted from [5,18].

2.1.2.1. Eggshell

The eggshell represents about 9 – 11 % of total egg weight, consisting essentially of minerals, of which calcium represents the majority (**Table 2.1**). However, other minerals such as magnesium, phosphorus and iron may be present [14]. Eggshell is composed of a foamy layer of cuticle, a spongy calcium carbonate layer, a mamillary layer, and two shell membranes [19]. Numerous funnel-shaped pores canals (7 000 – 17 000 per egg) extend through the shell, forming passages between the shell membrane and cuticle, and the latter partially seals the pores, restricting microorganisms penetration but allowing water and gases exchange [11,20].

2.1.2.2. Egg white

EW represents about 60 – 63 % of fresh whole hen egg, consisting mainly of water (87.6 %), proteins (9.7 – 10.6 %), carbohydrates (0.4 – 0.9 %) and lipids (0.03 %) (**Table 2.1**) [14–16]. Its pH is around 8.0 after laying, gradually increasing to a maximum of 9.7 due to carbon dioxide loss during storage [16].

Structurally, EW is composed of four layers, namely an outer thin layer next to shell membrane; an outer thick layer; an inner thin and a thick (or chalaziferous) layer [15]. The thin layer is less viscous than the thick layer due to the high content of ovomucin in the latter,

and the chalaziferous layer consist of mucin fibres that cover the entire EY [21]. The latter twisted at both sides of the EY membranes, and formed the chalazae cord, which holds the yolk in the centre of the egg, allowing it to oscillate within a safe boundary [21].

Proteins are the main component of EW, representing more than 90 % of dry matter, among them, the major proteins are ovalbumin (54 %), ovotransferrin (12 %), ovomucoid (11 %), ovomucin (3.5 %), lysozyme (3.4 %) and ovoglobulin (4 % + 4 %) [16]. Ovalbumin is a globular protein and is primarily responsible for EW functionality [22]. This is a phosphoglycoprotein containing one disulphide bond and four free sulfhydryl (SH) groups buried within the protein core, with a denaturation temperature close to 84 ºC [22,23]. Ovotransferrin, also known as conalbumin, is a glycoprotein with the ability to binding Fe^{3+} ions, creating an iron-deficient environment with impact on bacteria [24,25]. Unlike ovalbumin, conalbumin is the most heat-sensitive EW protein with a denaturation temperature of about 61 ºC [16,23]. Instead, ovomucoid is a thermostable glycoprotein, composed of three distinct domains crosslinked only by intradomain disulphide bonds [26]. The ovomucin is a sulphated glycoprotein formed through flexible fibres, giving the jelly-like structure of the thick white layer [27]. Otherwise, lysozyme has a globular structure stabilised by four disulphide bonds, a denaturation temperature of 70 – 75 ºC, and antimicrobial activity against gram-positive bacteria [23,28]. Other proteins present in much minor quantities are: ovoinhibitor (1.5 %), ovoglycoprotein (1.0 %), ovoflavoprotein (0.8 %), ovostatin (0.5 %), cystatin (0.05 %) and avidin (0.05 %) [16]. Furthermore, the EW proteins are associated with diverse biological activities, including antimicrobial, immunomodulatory, anticancer, and antihypertensive activities, and protease inhibitory action [11]. Although, some proteins have been associated to egg allergenicity, but, in general, procedures such as boiling and baking seem to reduce allergenic activity [29].

2.1.2.3. Egg yolk

EY represents about 28 – 29 % of fresh whole hen egg, being composed of 48.7 % water, 31.8 – 35.5 % lipids, 15.7 – 16.6 % proteins, 1.1 % minerals, and 0.2 – 1.0 % carbohydrates (**Table 2.1**) [14–16]. After egg laying, the EY pH is about 6.0, yet, during storage gradually increases to a maximum of 6.9 due to the carbon dioxide loss [16].

Structurally, EY is encircled by the vitelline membrane and anchored by the chalazae cord in centre of the egg. In egg centre there is the labrum centre with connection to the

germinal disc, where the formation of the embryo occurs [14,30]. EY is distributed in concentric layers called by yellow yolk, consisting of deep yellow yolk (formed in daytime) and light yellow yolk (formed at night when the protein concentration in the bloodstream is lower than in the daytime) [31].

The EY consists of a non-soluble protein aggregate (granules) suspended in a clear yellow fluid (plasma). Plasma is composed of low-density lipoproteins (LDLs) and soluble proteins (livetins), containing about 90 % of yolk lipids and 50 % of yolk proteins [32]. Instead, the granules contains mainly high-density lipoproteins (HDLs) and phosvitin [33,34]. The lipids present are exclusively associated with lipoprotein assemblies, and in form of triglycerides, phospholipids and cholesterol [35]. In addition, carotenoids are responsible for egg colour and represent less than 1 % of yolk lipids. The main carotenoids are lutein and zeaxanthin, but other xanthophylls in minor amounts were found, including β-cryptoxanthin, β-carotene and α-carotene [5,18,35], however, its composition is highly variable, depending on the hen age, genotype and changes in diet [11]. On the other hand, the EY proteins are in the form of free proteins or apoproteins, comprising mainly 68 % of LDL, 16 % of HDL and 10 % of globular proteins [35]. They consists of apovitellenin I – VI, phosvitin, α- and β-lipovitellin apoproteins, α-livetin (serum albumin), β-livetin (α2-glycoprotein), γ-livetin (γ-globulin), and traces of biotin-binding protein [16,36]. Some biological activities have been attributed to EY, for instance antimicrobial, anti-inflammatory, anticancer, antiadhesive and antioxidant activities [11,37].

2.1.3. Egg properties

Foaming property is defined as the capacity to rapidly adsorb on the air-liquid interface during whipping or bubbling, with the formation of a cohesive viscoelastic film trough intermolecular interactions [38]. During the whipping process, air comes into the solution forming bubbles, and the protein hydrophobic regions facilitate the adsorption at the interface. Proteins undergo partial unfolding (increase surface hydrophobicity and flexibility), resulting in solubility losses or precipitation of some proteins, which accumulate at the liquid-air interface. Consequently, the surface tension decreases, facilitating the formation of new interfaces and more bubbles. The partially unfolded molecules associate, forming a stabilising film around the bubbles, which is crucial for the foam stability [39]. Nonetheless, the foams can be destabilized by three main mechanisms such as: coarsening

that is characterized by gas transfer from smaller to bigger bubbles, resulting in the disappearance of the smaller bubbles; coalescence is the fusion of two adjacent bubbles; and drainage consists of the movement of liquid out of the foam through the liquid channels, removing proteins from the film around the bubble [39,40]. In the case of egg, the foaming properties of EW are considered a reference when compared with other animal and plant protein ingredients [41], however, they can be affected by protein concentration and composition, pH, temperature and ionic strength [42].

Emulsifying activity is the capacity of surface-active molecules to cover the oil-water interface through mechanical homogenization, while the **emulsion stability** is related to the capacity to prevent flocculation, creaming and/or coalescence of oil droplets. The flocculation and creaming are reversible, but the coalescence is characterized by the irreversible fusion of oil droplets [43]. The emulsifying properties of EY are mainly attributed to LDL particles, due to their important role in the transport of surfactants in a soluble form, and their release at the interface [32]. When LDL come into contact with the interface, the apoproteins located on the LDL surface start the initial anchorage at the interface, leading to protein unfolding and destabilisation of LDL external layer. Then, LDL release neutral lipids, phospholipids and apoproteins from the lipoprotein core, allowing it to spread and to form an emulsion [44].

Another important property associated with egg components is the capacity of egg proteins clot and form gels (**gelling properties**). For heat-induced egg gels, firstly occurs the protein unfolding with the exposure of their hydrophobic internal structure. These unfolded proteins interact and form high molecular weight aggregates, and then, they interact with each other resulting in a three-dimensional gel [45]. The interactions involved in formation of gels are principally hydrophobic and electrostatic, but also disulphide links [43]. All EW proteins, with the exception of ovomucin and ovomucoid, coagulate when heated, being ovalbumin and ovotransferrin the main contributors [43,46], while in the case of EY, LDL is main contributor [32].

2.1.4. Egg industry

"Laying hens are the birds of the *Gallus gallus* species which have reached sexual maturity and been raised for egg production not intended for incubation" [47]. On the other hand, egg products means "processed products resulting from the processing of eggs, or of

various components or mixtures of eggs, or from the further processing of such processed products" [48].

Currently, egg products production takes place in specialist large plants [49]. When eggs arrive at the food processing industry, they must be inspected, washed and completely dried [50]. Then, the eggs are broken, separated using machines highly automatic, and the liquid products are filtered to eliminate any physical particles [43,50]. Ingredients such as salt, sugar, etc can be added, and subsequently homogenised to distribute uniformly throughout a batch [50,51]. The next step is pasteurization, which is carried out in accordance with the current legislation in each country [6]. If the product cannot be immediately pasteurized, it must be stored at ≤ 4 ºC, not exceeding 48 h [48,52]. Finally, the liquid product is aseptically packed, stored and delivered [53]. Additionally, the pasteurized liquid egg can be subjected to a transformation processes of freezing, concentration or dehydration [43,50].

The European Union (EU) legislation establishes the absence of *Salmonella* spp. in 25 g or 25 mL, and restricts the presence of *Enterobacteriaceae* to maximum 100 colony forming unit (CFU)/g. The legislation also limits the presence of wastes that should not exceed 100 mg/kg, and the chemical limits for hydroxybutyric acid that must be <10 mg/kg dry matter (to avoid the use of eggs rejected in incubators in industrial hatcheries to enter the commercial consumption circuit) [48,54]. The reference pH for the whole egg, EY and EW is about 7.1, 6.2 and 8.2, respectively, since the microorganisms remaining in the pasteurized egg can lead to acid production, with a consequent pH reduction [55].

The global egg production has grown due to a rapidly increasing demand for proteins in the developing world, having grown from 35 071 918 tonnes (in 1990) to 86 669 518 tonnes (in 2020). In the same year, 64.5 % of the total egg production in world are in Asia, 18.8 % in American continent, 11.7 % in Europe, 4.6 % in Africa and only 0.4 % in Oceania [56]. In the EU, the egg consumption was about 11.72 kg/capita/year in 2019, with Denmark, France and Italy being the largest consumers of egg products [56,57]. In Portugal, 80 % of annual egg production (2020) was eggs for consumption, with a consumption per capita of 11.0 kg/person [58]. Furthermore, the food industry consumed approximately 30 % of eggs produced annually, for processing into a wide range of egg products [6].

2.2. Microorganisms in eggs

The eggshell could be contaminated, after laying, with faeces (*Escherichia coli, Salmonella* spp.) and through its direct contact with the nest microorganisms, which can attach to the surface, such as *Staphylococcus aureus* [59]. Although, *Salmonella* can contaminate eggs before the shell formation (primary contamination) or after oviposition (secondary contamination), but cross-contamination between eggs and equipment along the food line can also occur [60,61]. Salmonellosis is the second most frequent zoonotic disease in the EU, and *Salmonella* is one of the main causes of foodborne outbreaks (22.5 %) [62]. One of the food vehicles most frequently associated to human infection are eggs and egg products, causing 44.0 % of strong-evidence outbreaks in 2020 [62]. The most common *Salmonella* serovars involved in human infections are *Salmonella* Enteritidis (48.7 %), *S.* Typhimurium (12.4 %) and monophasic *S.* Typhimurium (11.1 %) [62], yet, a very heat-resistant serovar, such as *S.* Senftenberg 775/W was also isolated from foods [63,64].

Otherwise, *Listeria monocytogenes*, a gram-positive bacterium, has been reported as another microorganism of public health concern in liquid egg [54]. The presence of *Listeria* spp. in raw liquid whole egg (LWE) can result in the most of cases of cross-contamination [53]. This microorganism was found in 8.5 % of raw egg products, while a high level of *L. innocua* contamination was observed, accounting for about 26.2 and 1.8 % of raw and pasteurized egg products, respectively [65].

2.3. Pasteurization

Thermal pasteurization (TP) of a certain food product is designed based on a target (such as a vegetative microbial cell or an enzyme), where the food product is heated to a target temperature (processing temperature) during a certain time (processing time) [66]. Pasteurization applies temperatures below 100 ºC, to inactivate vegetative microorganisms and undesirable enzymes, but does not remove all pathogenic microorganisms, neither spore-forming heat resistant bacteria [67]. This is a multitarget process that can act on the cell wall, cytoplasmatic membrane, nucleic acids, ribosomes, ribosomal RNA and enzymes of citric acid cycle [68,69]. Microbial inactivation is expressed by decimal reduction time (*D*, is the time (in minutes), at a given temperature, required to kill 90 % of the initial number of microorganisms) and thermal coefficient (*z*, is the change in temperature required to achieve

a tenfold reduction in *D*-value) [66,70]. The *D*- and *z*-values reported for different microorganisms in liquid egg products are available in literature [71–73].

On the other hand, TP requires high energy levels which could have a negative impact on the quality of final product such as: losses in nutritional content and sensory properties, and off-flavours development [74,75]. Despite the disadvantages, TP remains the most widely used food preservation technique, due to its high availability, low cost, and effectiveness [75]. Nevertheless, pasteurized products, usually, need storage at low temperatures, and have a shelf-life that may vary from few days (under refrigeration) up to months (high-acid products and/or with low water activity) [66].

2.3.1. Pasteurization of liquid egg products

Egg products pasteurization is limited by the sensitivity of egg proteins [76]. The most common pasteurization conditions used for LWE, EY and EW are 60 ºC/3.5 min, 60 ºC/6.2 min and 55.6 ºC/6.2 min, respectively. Other applied conditions can be consulted in the literature [51,77,78]. The shelf-life of these products depends on the initial microbiological quality, pasteurization conditions, product homogenization, packaging under aseptic conditions, and maintaining adequate refrigeration temperatures during storage, distribution and retail [79].

Although TP ensures egg products safety, it cause some undesirable effects on the overall product quality, as described below. The adverse effects are essentially due to the high content of heat-sensitive proteins, some soluble proteins began to precipitate at 57 ºC and coagulates at 73 ºC [80–82]. Initially, the heat can cause the unfolding of proteins, exposing hydrophobic amino acids buried inside, and promoting aggregation by hydrophobic interactions, and thus, reducing protein solubility [83]. Mild TP (56 – 61 ºC/3.5 min) induced a certain degree of protein aggregation [84], while at higher temperatures (73 – 85 ºC) occurred a strong decrease in protein solubility, probably due to the protein denaturation and aggregation [85]. In addition, at temperature below those that cause gelation, the unfold and aggregated proteins can lead to an increase in egg viscosity [86]. In fact, temperatures as low as 56, 60 and 61 ºC applied during 3.5 min enhanced viscosity of EW, LWE and EY, respectively [84]. Effects on foaming properties were observed when egg products were heated at and above 55 ºC, including foam volume and foaming capacity (FC) decrease, and longer whipping time [87–89]. Regarding to emulsifying properties, EY heated above 69 ºC

presented a reduced emulsifying activity, whereas the emulsion stability was not influenced up to 76 ºC [90]. Otherwise, heat triggers Maillard's reactions, accelerating the production of brown Maillard products (affects egg colour), and pointing out to the loss of essential amino acids [91,92]. Consequently, the undesirable effects can be reflected in egg-based products, such as emulsions, cakes and pies [82,93,94].

2.4. Non-thermal technologies

The undesirable impact of TP on food quality has driven consumer demand for microbiologically safe, convenient and high quality foods, with natural/fresh (fresh-like) flavour and appearance, minimal nutritional changes and chemicals and additives free [95,96]. In the recent years, as an attempt to response to the consumer's needs and to improve or replace TP, there has been increasing research/development of non-thermal food preservation technologies [97]. These technologies effectively inactivate microorganisms, extends the product shelf-life, and causes minimal or no changes in vitamins and nutrients content, since they are able to process food at temperatures lower than those used in TP [98,99]. Furthermore, these innovative techniques are environmentally friendly and sustainable, with reduced energy and water consumption [100].

2.4.1. High pressure technology

High pressure (HP) is a non-thermal technology that has attracted a lot of attention in several areas, including food and biosciences, among others [101]. This technology is based on the Le Châtelier principle, in which the application of pressure affects any phenomenon (for example, phase transition, chemical reactivity, chemical reactions), shifting equilibrium toward the state with the lowest volume; and the isostatic principle which states that pressure is transmitted quickly and uniformly in all directions [102]. Thus, the product is compressed regardless of its size, geometry and composition [103]. Although, it is also necessary to take into account the adiabatic heating occurred during compression, that generally, induces an increase of approximately 3 ºC for each increase of 100 MPa [102].

HP was first tested as a preservation technique by Hite [104] more than a century ago. This technology uses pressures between 300 and 600 MPa for short time periods [105], with the main objective of producing food with an extended shelf-life, without substantially

effects on nutritional, functional and organoleptic characteristics [106]. Commercially, a wide range of products are available, such as: fruit juices, jams, ham, seafood, fruit preparations, rice cakes, squid and guacamole [107,108].

HP has several effects on living organisms and the lethal effect results from simultaneous damage to different cellular structures and functions [109]. Pressure damages the cell membrane and its structure, affecting their mechanisms of nutrient absorption and metabolite release, and changing microorganisms metabolic pathways [110]. In biomolecules, such as proteins, HP can cause reversible (100 – 300 MPa) or irreversible (> 300 MPa) structural changes, resulting in denaturation, aggregation or gel formation [111,112]. These effects are associated with non-covalent bonds disruption (essential for the structure and function maintaining), mainly affecting tertiary and quaternary structures, but with little effect on the secondary structure [113]. Pressure also causes multimeric disintegration, promoting ribosomes dissociation [114]. As well, HP stabilizes hydrogen bonds of DNA molecules increasing the transition temperature from double strand to single strand, hindering the cellular processes [115]. Nonetheless, the extent of HP damages in microorganisms depends on the microorganism strain and growth cycle, treatment intensity, duration and temperature, pH, water activity and food composition [116]. In general, gram-positive bacteria are more resistant to pressure than gram-negative bacteria, moulds and yeasts, and the spores are the most resistant structures [105].

2.4.1.1. High pressure effects on egg products

In literature, there is an extensive review on the effects of HP on liquid egg (see **Annex A**, **Table A1** and **Table A2**). Overall, HP treatments (300 – 450 MPa for 10 – 30 min) inactivated more than 4 $\log_{10}$ cycles of *Salmonella* Enteritidis. Instead, the use of pulsed HP reduced *S.* Enteritidis, *L. innocua* CECT 910 and *E. coli* CECT 405 counts by at least 6.63 $\log_{10}$ cycles [117–121]. Though, at these higher pressures, the soluble protein of egg products decreases with increasing pressure, and it was accompanied by a decreasing trend in surface hydrophobicity and SH groups content [3,122]. These results suggest the occurrence of protein denaturation and aggregation [80,86], and consequently the viscosity increment (with treatment time and pressure rise) [122,123]. In addition, the foaming properties of HP-treated EW improved with pressure increasing (up to 450 MPa), while the EY emulsifying properties were gradually deteriorated [3,122,124]. The water holding

capacity (WHC) of heat-induced egg gels enhanced with increasing pressure, while reduced with treatment time increase [124].

2.4.2. Ultrasound technology

Ultrasound (US) technology began to be used at an industrial level in food processing (cleaning and homogenization) in the 50's [125]. US is an acoustic wave with a frequency above the human hearing (> 20 kHz), which needs a medium to propagate [126]. This can be applied at low intensity and frequencies higher than 100 kHz (non-destructive) or at frequencies between 18 – 100 kHz and high intensities (destructive) [127,128].

The waves propagate through the liquid via a series of compressions (positive pressure) and rarefactions (negative pressure) [129]. When the power applied is sufficiently high, the rarefaction cycle exceeds the attractive forces between liquid molecules, forming cavitation bubbles which can continue to grow during each cycle due to the net flow of gas from the liquid to the bubble [130,131]. One the one hand, the cavitation probably induced the formation of large bubble clouds with equilibrium size, causing microstreaming and high shear, and resulting in the thinning of cell membranes and their disruption [126,132–134]. On the other hand, if the bubbles continue to grow until to a critical size, they become unstable and end up collapsing violently [130,131]. This causes severe turbulence in the surrounding liquid, the generation of high temperature (hotspots) and high pressure zones, and form highly reactive free radicals (by water hydrolyses within the bubble) [135–137]. These free radicals can act on the cell envelope, disrupt the cell walls, and then, the cell dies [137,138]. In biomolecules, the US can induce changes in the protein structure (secondary and tertiary) due to the breakdown of hydrogen or Van der Walls bindings. Besides, the free radicals can also react with amino acids residues involved in structure stability, substrate binding, or catalytic functions, and consequently, change their biological activity [139].

The main advantages of US are low cost, ease of operation, minimal loss of flavour and nutrients, increased product homogeneity, and another advantage over other non-thermal technologies, is that cells ruptured and disintegrated by US cannot be revived [140–142]. On the contrary, the weak lethal action when used alone, high energy consumption, long treatment times, product heating and industrial-scale equipment with limited intensity are the limiting factors of this technology [143,144].

2.4.2.1. Ultrasound effects on egg products

Data on the application of US technology to ensure the safety of liquid egg products are very limited, and when applied alone, this technology is not very effective in inactivating microorganisms (≤ 2.3 log_{10} cycles) [145,146]. Concerning egg properties, at first, the US increased the solubility, surface hydrophobicity, exposed and total SH groups of treated egg products, and then declined, as the treatment power increased [42,147]. In addition, US-treated samples were less viscous, showed improved FC and emulsifying properties, and reduced foaming stability (FS) compared to non-treated samples [42,147–150]. More detailed information on the effect of this technology on egg products can be consulted in **Annex A** (**Table A3** and **Table A4**).

2.5. Hurdle concept

A strategy based on the “hurdle” concept has been developed by Leistner, combining several factors to preserve foods [151]. The concept advocates the combination of existing and novel preservation techniques, that can be applied simultaneously or sequentially, depending on the type of hurdle. Each factor is a hurdle that any microorganisms should not be able to overcome, and the 'higher" the hurdle, the greater the effort [151]. The advantages are (1) avoid the severity of one hurdle alone, (2) can act synergistically, and (3) many of the hurdles come from past experience [152]. Thereby, when different processes are combined, the intensity of individual preservation techniques can be kept comparatively low, to minimise product quality loss, while overall there is a high impact on controlling microbial growth [153].

The preservation factors disturb the microorganism’s **homeostasis**, and if they cannot repair the internal homeostasis (re-established), they will not multiply remaining in the lag-phase or even die [154]. Moreover, when a microorganism is under stress (such as heat, pH, water activity, ethanol and starvation) could become more resistant or more virulent, due to the stress shock proteins production (**stress reaction**). If different stresses are applied at the same time, the microorganism will require more energy for protective proteins synthesis, and when they exhaust completely their energy become metabolically exhausted and die (**metabolic exhaustion**) [154]. Thus, in a **multitarget** approach, the microorganisms are exposed to various sublethal stresses (simultaneous or sequential) that act on different cell

targets (cell membrane, DNA, enzyme systems, pH, water activity) disturbing the microorganisms homeostasis [143,154]. The techniques are effective when they overcome, temporarily or permanently, one or more of the homoeostasis mechanisms of microorganisms. Although, the changes in surrounding determine whether the microorganism loses viability, becomes injured (surviving cells that have been injured by sublethal stresses and with increased sensitivity to adverse environmental conditions), or express adaptive mechanisms [155].

As the use of non-thermal technologies *per se*, such as pulsed electric fields (PEF)/HP/US, have not been shown to be effective in reducing 5 log_{10} cycles of a foodborne pathogen (require to achieve pasteurization) or shown considerable detrimental effects on eggs quality when causing possible interesting microbial inactivation, they are not a viable alternative to pasteurize egg products [119,120,122,146]. Consequently, the hurdle concept has gained greater interest as a possible approach to new alternatives to egg products thermal pasteurization (**Table 2.3** – **Table 2.6**).

2.5.1. Sequentially combined processing of egg products

2.5.1.1. Microbial inactivation

Several studies confirmed that sequentially combined treatments can be an alternative to commercial TP, and a compilation of the results concerning microbial inactivation (inoculated studies) obtained so far is presented in **Table 2.3**. Inactivation studies with *Salmonella* spp. were conducted in LWE, supplemented with additives, applying PEF (25 kV/cm, 18 – 48 μs, 0.5 Hz, <35 ºC) followed by a TP (52 – 60 ºC/1 – 3.5 min) (PEF-Additives-TP) [156–159], obtaining an inactivation of 1.5 to 8.3 log_{10} cycles. In the absence of additives (PEF-TP), only 0.6 log_{10} cycles of *S.* Enteritidis population reduction was achieved, but the D_T values (at 52 and 55 ºC) were slightly reduced and the z value increased (from 4.2 to 4.5 ºC) [156,158]. Other authors using the same approach (PEF-TP, 25 kV/cm/250 μs and 55 ºC/3.5 min) attained a higher inactivation for *S.* Enteritidis ATCC 13076 and *S.* Typhimurium DT104 (up to 4.29 log_{10} cycles), but when they reversed the order of treatments (TP followed by PEF) observed no improvement on microorganisms inactivation [161,163]. The inactivation of *L. monocytogenes* STCC 5672 was also studied by Monfort et al. [157,159], using PEF before a TP (with additives), achieving reductions a TP (with additives), achieving reductions up to 4.8 log_{10} cycles. However, using PEF

Table 2.3. Effects of sequentially combined treatments on microorganisms' inactivation in liquid egg.

Microorganism	Egg product	Process Conditions	Treatment Combination	Log $_{10}$ reductions	References
Salmonella Enteritidis PT4 E10	Liquid whole egg	250 – 450 MPa, 5 – 15 min, 20 ºC 0.1 – 1.0 %, 1h, 20 ºC	HP-H_2O_2	2.74 – 7.95	[160]
Salmonella Enteritidis ATCC 13076	Liquid whole egg	25 kV/cm, 250 µs, 200 Hz, < 38 ºC 55 ºC/3.5 min	PEF-TP TP-PEF	4.29 2.26	[161]
Salmonella Enteritidis STCC 7300	Liquid whole egg	25 kV/cm, 48 µs, 0.5 Hz, < 35 ºC 55 ºC/2 min 2 % Triethyl citrate 10 mM EDTA	PEF-Additives PEF-TP PEF-Additives-TP	0.6 – 8.3	[156]
Salmonella Enteritidis STCC 4300	Liquid whole egg	25 kV/cm, 18 – 48 µs, 0.5 Hz, < 35 ºC 52 ºC/3.5 min, 55 ºC/2 min, 60 ºC/1 min 2 % Triethyl citrate	PEF-Additives-TP	> 5	[158]
Salmonella Enteritidis STCC 4396	Liquid whole egg	25 kV/cm, 18 – 48 µs, 0.5 Hz, < 35 ºC 52 ºC/3.5 min, 55 ºC/2 min, 60 ºC/1 min 2 % Triethyl citrate	PEF-Additives-TP	> 6.92	[158]
Salmonella Senftenberg 775W STCC 4565	Liquid whole egg	25 kV/cm, 48 µs, 0.5 Hz, < 35 ºC 52 ºC/3.5 min, 55 ºC/2 min, 60 ºC/1 min, 60 ºC/3.5 min 1 – 2 % Triethyl citrate 10 mM EDTA	PEF-Additives-TP	2.4 – 5.6	[157]
Salmonella Senftenberg 775W STCC 4565	Liquid whole egg	25 kV/cm, 24 µs, 0.5 Hz, < 35 ºC 60 ºC/3.5 min 200 µl/L Carvacrol, (+)-limonene, Citral, Lemon, Mandarin, Rosemary EO	PEF-Additives-TP	Up to 4	[159]

Salmonella Senftenberg STCC 4384	Liquid whole egg	25 kV/cm, 18 – 48 µs, 0.5 Hz, < 35 ºC 52 ºC/3.5 min, 55 ºC/2 min, 60 ºC/1 min 2 % Triethyl citrate	PEF-Additives-TP	> 4.97	[158]
Salmonella Enteritidis CB 919 Lux AB	Liquid whole egg	5.67 kV/cm, 60 µs, 0.5 Hz, 55 ºC 138 MPa, 2-2-4 min, 20 ºC 40 W, 5 min, 55 ºC, 12.5 and 25 mL	PEF-US US-PEF HP-PEF PEF-HP US-HP HP-US	2.30 2.25 < 3 < 2.5 < 3 3.23	[162]
Salmonella Typhimurium DT104	Liquid whole egg	25 kV/cm, 250 µs, 20 ºC 55 ºC/3.5 min	PEF-TP	Up to 3.5	[163]
Salmonella Typhimurium STCC 878	Liquid whole egg	25 kV/cm, 18 – 48 µs, 0.5 Hz, < 35 ºC 52 ºC/3.5 min, 55 ºC/2 min, 60 ºC/1 min 2 % Triethyl citrate	PEF-Additives-TP	> 6.44	[158]
Salmonella Typhi STCC 409	Liquid whole egg	25 kV/cm, 18 – 48 µs, 0.5 Hz, < 35 ºC 52 ºC/3.5 min, 55 ºC/2 min, 60 ºC/1 min 2 % Triethyl citrate	PEF-Additives-TP	> 6.73	[158]
Salmonella Dublin STCC 4152	Liquid whole egg	25 kV/cm, 18 – 48 µs, 0.5 Hz, < 35 ºC 52 ºC/3.5 min, 55 ºC/2 min, 60 ºC/1 min 2 % Triethyl citrate	PEF-Additives-TP	> 6.60	[158]
Salmonella Virchow STCC 4154	Liquid whole egg	25 kV/cm, 18 – 48 µs, 0.5 Hz, < 35 ºC 52 ºC/3.5 min, 55 ºC/2 min, 60 ºC/1 min 2 % Triethyl citrate	PEF-Additives-TP	> 5	[158]

Listeria monocytogenes STCC 5672	Liquid whole egg	25 kV/cm, 24 – 48 µs, 0.5 Hz, < 35 ºC 52 ºC/3.5 min, 55 ºC/2 min, 60 ºC/1 min, 60 ºC/3.5 min 1 – 2 % Triethyl citrate 10 mM EDTA 200 µl/L Carvacrol, (+)-limonene, Citral, Lemon, Mandarin, Rosemary EO	PEF-Additives-TP	1.6 – 4.8	[157,159]
Listeria innocua ATCC 51742	Liquid whole egg	30 – 50 kV/cm, 21.2 – 64 µs, 3.5 Hz, ≤ 36 ºC 0 – 100 IU/mL Nisin	PEF-Nisin	0.6 – 5.5	[164]
Listeria innocua BGA 3532	Liquid whole egg	200 – 300 MPa, 3 – 30 min, 20 ºC 52 ºC/3.5 min, 55 ºC/2 min 2 % Triethyl citrate	HP-TP HP-Additives-TP	1.6 – 2.3 2.6 – 5.5	[123]
Listeria seeligeri NCTC 11289	Liquid whole egg	250 – 300 MPa, 3.33 – 14.76 min, 5 ºC 24.6 – 42.0 W, 5 min, < 30 ºC, 10 mL 0 – 20 mg/L Nisin	Nisin-HP US-HP	Up to 5 < 0.5	[146]
Escherichia coli K12 DH 5α	Liquid whole egg	250 – 300 MPa, 3.33 – 14.76 min, 5 ºC 24.6 – 42.0 W, 5 min, < 30 ºC, 10 mL 0 – 20 mg/L Nisin	Nisin-HP US-HP	> 2 ~ 2 – 3	[146]
Escherichia coli K12 DH 5α	Liquid whole egg	200 – 300 MPa, 3 – 30 min, 20 ºC 52 ºC/3.5 min, 55 ºC/2 min 2 % Triethyl citrate	HP-TP HP-Additives-TP	1.5 – 3.1 At least 6	[123]

followed by nisin obtained higher bactericidal effect against *L. innocua* ATCC 5174 (up to 5.5 log_{10} cycles) [164].

In addition, the potential application of HP (200 – 300 MPa, 3 – 30 min) before a TP (52 – 55 ºC/2 – 3.5 min) in effectively inactivating *L. innocua* BGA 3532 and *E. coli* K12 DH 5α (*S.* Enteritidis surrogate), inoculated into LWE, was investigated by Monfort et al. [123]. A maximum reduction of 3.1 log_{10} cycles was reported for these microorganisms, however, the inactivation improved (at least 6 log_{10} cycles) when the treatments were performed in presence of 2 % triethyl citrate (TC) [123]. The combination of HP (250 – 450 MPa/ 3.33 – 15 min) with other compounds, such as nisin and hydrogen peroxide was explored, and all combinations resulted in 2 to 5 log_{10} cycles reductions of *S.* Enteritidis PT4 E10, *L. seeligeri* NCTC 11289 and *E. coli* K12 DH 5α [146,160].

Otherwise, the application of irradiation treatments prior TP decreased the thermal resistance of *Salmonella* serovars. For *S.* Senftenberg, the thermal D_T-values were reduced between 1.9 – 3.7-fold (at 55, 57, and 60 ºC), while for *S.* Typhimurium and *S.* Enteritidis a smaller effect was observed [165,166]. Other treatments combining US, HP and/or PEF, for LWE pasteurization were also studied. A 3 log_{10} cycles reductions was attained for *L. seeligeri*, *E. coli* and *S.* Enteritidis CB 919 Lux AB, when undergoing a treatment of US before HP (US-HP), yet, HP-US combination was reported to be more effective against *S.* Enteritidis CB 919 Lux AB [146,162]. Additionally, other combinations, such as HP-PEF, PEF-HP, PEF-US or US-PEF did not show an advantage compared to HP-US combination [162].

2.5.1.2. Physicochemical and functional properties

The few existing studies confirmed that the use sequentially combined treatments managed to maintain or even improve the egg products properties. A compilation of these studies in what concerns physicochemical and functional properties is presented in **Table 2.4**. Parameters such as pH, electrical conductivity and ºBrix did not undergo significant changes after sequential combined treatments [87,123,161]. Regarding to LWE colour, the results showed that the colour variation ($\Delta E^*<3$) caused by HP-Additives-TP or PEF-Additives-TP combinations were not detectable to the naked eye [87,123,157,161]. Although, other combinations such as US-HP and HP-US had a more pronounced effect in this parameter ($\Delta E^*>3$)[167,168].

From the studies performed so far, it can be inferred that the sequential combination of PEF or HP before a TP, in LWE supplemented with additives, leads to a loss of soluble protein [87,123,157,158]. The decrement was about 0.2 – 5.0 % for treatments using PEF-Additives-TP, but a higher reduction (about 12 %) was obtained with HP-Additives-TP. The same treatments, in presence of TC, increased viscosity of non-treated LWE, while ethylenediaminetetraacetic acid (EDTA) addition had no effect on this parameter [87,123,157]. Contrarily to what was observed for the previous treatments, no significant changes in LWE viscosity was reported by Hermawan et al. when used a PEF-TP treatment (PEF: 25 kV/cm/250 μs/< 38 ºC; TP: 55 ºC/3.5 min) [161].

Concerning foaming properties, Monfort et al. [87,123,157] studied the impact of PEF-TC-TP and HP-TC-TP treatments in LWE, and showed improved FC and FS, up to 26.5 % and 148 %, respectively, probably due to the presence of TC. Differently, when TC was replaced by EDTA, the foaming properties reduced, especially FS [157]. In another study, using US-HP combination, it was possible to enhance LWE FC as compared to non-treated samples, whereas US-HP and Nisin-US treatments did not change FS [167].

Furthermore, the HP-TC-TP treatment seems to slightly reduced LWE emulsifying properties compared to non-treated LWE [123], whereas the treatments combining PEF with 2 % TC and TP maintained or improved (up to 10 %) these properties [87]. By replacing TC with EDTA, it was observed a 10 % improvement in the emulsifying capacity of LWE [157]. Lee (2002) [167] reported that emulsions produced with US-HP-treated LWE had higher droplet sizes than that of non-treated samples, contrarily to what was observed for emulsions made with nisin-HP-treated LWE. This effect can be explained by the low viscosity of US-HP-treated samples, since the emulsion droplet diameters increase with a viscosity decrease [169].

Finally, the impact of these treatments on texture profile of heat-induced LWE gels has been studied by some authors. Gels prepared with HP-TC-TP-treated LWE, at a lower temperature (52 ºC/3.5 min), were harder than gels produced with non-treated LWE and LWE treated at a higher temperature (55 ºC/2 min) [123]. In the case of LWE treated by PEF-TC-TP, the heat-induced gels exhibited higher cohesiveness, gumminess, chewiness and resilience, and a lower hardness and springiness than those obtained with non-treated LWE [87,157]. Concerning WHC, the treatments (PEF-TC-TP; PEF-EDTA-TP; HP-TC-TP) had no significant effect on this property [87,123,157].

Table 2.4. Main effects of sequentially combined treatments on physicochemical and functional properties of egg products.

Egg product	Process Conditions	Treatment Combination	Effects	References
Liquid whole egg	300 MPa, 3.33 min, 5 °C 34.6 W, 0.5 min, 5 °C	US-HP Nisin-HP	No significant change in FS Increased in FC, emulsions droplet sizes, L* and a* Decreased in b*	[167]
Liquid whole egg	12.50 W, 10 – 60 min, room temperature 300 MPa, 3 – 10 min	US-HP	No significant change in a* Increased in L, b* and ΔE	[168]
Liquid whole egg	300 MPa, 3 min, 20 ºC 52 ºC/3.5 min, 55 ºC/2 min 2 % Triethyl citrate	HP-Additives-TP	No significant change in pH and WHC Colour variation not detected by naked eyes Reduction of soluble protein and emulsifying properties Increased in viscosity, FC and FS Harder gels	[123]
Liquid whole egg	25 kV/cm, 250 µs, 200 Hz, < 38 ºC 55 ºC/3.5 min	PEF-TP	No significant change in viscosity, electrical conductivity, colour, pH and ºBrix	[161]
Liquid whole egg	25 kV/cm, 18 µs, 38 ºC 52 °C/3.5 min, 55 °C/2 min, 60 °C/1 min 2 % triethyl citrate	PEF-Additives-TP	No significant change in pH, WHC and ES Similar colour and gelling properties Increased in viscosity, FC, FS and EC Decreased in soluble protein and electrical conductivity	[87]
Liquid whole egg	25 kV/cm, 48 µs, 0.5 Hz, < 35 ºC 60 ºC/3.5 min 1 % Triethyl citrate 10 mM EDTA	PEF-Additives-TP	Colour variation not detected by naked eyes Increased in viscosity, FC and FS with TC Reduction of foaming properties with EDTA Improved EC with EDTA No significant change in WHC and gel hardness	[157]

(1)EC: Emulsifying capacity; ES: Emulsifying stability; FC: Foaming capacity; FS: Foaming stability; HP: High pressure; PEF: Pulsed electric fields; TC: Triethyl citrate; US: Ultrasound; WHC: Water holding capacity.

2.5.2. Simultaneously combined processing of egg products

2.5.2.1. Microbial inactivation

The simultaneous combination of different technologies for microorganisms' inactivation has also been assessed as a possible alternative to commercial egg products TP (**Table 2.5**). Inactivation studies with *S.* Typhimurium and *S.* Enteritidis were conducted applying US at higher temperatures (40 – 55 ºC), and obtaining no more than 2.30 $\log_{10}$ cycles reductions [162,170]. Instead, the manosonification (117 mm, 200 kPa, 40 – 60 ºC) inactivated about 3 $\log_{10}$ cycles of *S.* Senftenberg 775/W counts [171]. On the other hand, simultaneously combining HP (350 MPa/10 – 40 min) and higher temperature (40 and 50 ºC) were enough to reduce about 6 $\log_{10}$ cycles of *S.* Enteritidis population, however, pulsed HP (pulses of 2, 4 and 5 min) completely inactivated this microorganism [121]. At lower temperatures (-15, 2, <15 ºC), HP treatments (200 – 450 MPa, 3 – 17 min) has been reported by some authors to reduce 0.90 – 6.41 $\log_{10}$ cycles of *S.* Enteritidis, *L. monocytogenes* and *Staphylococcus aureus* [119,172]. Although, similar conditions only eliminated 0.1 – 0.4 $\log_{10}$ cycles of *L. seeligeri* NCTC 11289 [173]. A higher inactivation of *L. innocua* (6.63 $\log_{10}$ cycles) was obtained, combining simultaneously, HP (200 – 450 MPa/3 – 30 min), temperature (4, 20 and 50 ºC) and/or additives (nisin or TC) [118,120,123]. Moreover, the inactivation of *E. coli* was studied by Lee et al. [173], that found a reduction up to 3.0 $\log_{10}$ cycles performing an HP treatment (250 – 400 MPa/0.38 – 14.8 min) at 5 ºC, and improved efficiency with the additives addition and temperature increasing [118,123]. As observed for *Salmonella*, pulsed HP (at -15, 2 and 50 ºC) yielded more promising results against *E. coli* CECT 405 (up to ~ 7 $\log_{10}$ cycles) [117].

Additionally, the lethal effect of HP (250 – 400 MPa/0.38 – 14.8 min) at lower temperatures (5 ºC) against *Pseudomonas fluorescens* and *Paenibacillus polymyxa* was studied by other authors, showing reductions < 5 $\log_{10}$ cycles in their counts [173], whereas, the use of weaker conditions (133.33 – 266.6 MPa/5 min) combined with additives, improved *Pseudomonas fluorescens* lethality [174].

Table 2.5. Effects of simultaneously combined treatments on microorganisms' inactivation in liquid egg.

Microorganism	Egg product	Process Conditions	Log 10 reductions	References
Salmonella Enteritidis SE-4	Liquid whole egg	350 MPa, 2+2+2+2, 5+5+5+5, 10+10+10+10 min, 50ºC	> 2 – at least 8	[121]
Salmonella Enteritidis CB 919 Lux AB	Liquid whole egg	40 W, 3 – 5 min, 50, 55 ºC, 12.5 and 25 mL	0.71 – 2.30	[162]
Salmonella Enteritidis	Liquid whole egg	300 – 450 MPa, 5, 5+5, 5+5+5, 10, 15 min, -15, 2, 50 ºC	1.48 – 6.86	[119]
Salmonella Enteritidis	Liquid whole egg	200 – 400 MPa, 3 – 17 min, < 15 ºC	4.89 – 6.41	[172]
Salmonella Senftenberg 775W ATCC 43845	Liquid whole egg	Manosonication: 117 mm, 200 kPa, 40, 60ºC	3	[171]
Salmonella Typhimurium ATCC 14028	Liquid whole egg	Ultrasonic cleaning bath, 15 – 30 min, 40, 50 ºC	< 1	[170]
Listeria monocytogenes	Liquid whole egg	200 – 400 MPa, 3 – 17 min, < 15 ºC	0.90 – 1.98	[172]
Listeria innocua CECT 910	Liquid whole egg	300 – 450 MPa, 5 –15 min, -15, 2, 20 ºC 1.25 – 5 mg/L Nisin	0.5 – up to 6.63	[118,120]
Listeria innocua BGA 3532	Liquid whole egg	200 – 300 MPa, 3 – 30 min, 4, 50 ºC 2 % Triethyl citrate	< 0.5 – 6	[123]
Listeria seeligeri NCTC 11289	Liquid whole egg	250 – 400 MPa, 0.38 –14.8 min, 5 ºC	0.1 – 0.4	[173]
Escherichia coli K12 DH 5α	Liquid whole egg	200 – 300 MPa, 3 – 30 min, 4, 50 ºC 2 % Triethyl citrate	~ 2– 6	[123]
Escherichia coli K12 DH 5α	Liquid whole egg	250 – 400 MPa, 0.38 – 14.8 min, 5ºC	2.1 – 3.0	[173]

Escherichia coli CECT 405	Liquid whole egg	300 – 450, 5, 5+5, 10, 5+5+5, 15 min, -15, 2, 20, 50 ºC 1.25 – 5 mg/L Nisin	~ 1 – ~7	[117,118]
Staphylococcus aureus	Liquid whole egg	200 – 400 MPa, 3 – 17 min, < 15 ºC	0.92 – 2.63	[172]
Pseudomonas fluorescens	Liquid whole egg	133.33 – 266.6 MPa, 5 min, ~ 22ºC 0.05 – 0.1 % Methyl:propyl (3:1) 0 – 0.1 % Parabens 0.15 – 0.5 % Citric acid 5 mM EDTA 200 UI Nisin 0 – 1000 UI Lysozyme	**≤ 7**	[174]
Pseudomonas fluorescens DSM 50090	Liquid whole egg	250 – 400 MPa, 0.38 – 14.8 min, 5ºC	< 5	[173]
Paenibacillus polymyxa DSM 36	Liquid whole egg	250 – 400 MPa, 0.38 – 14.8 min, 5ºC	< 5	[173]

2.5.2.2. Physicochemical and functional properties

An overview of how simultaneous combined treatments affect the physicochemical and functional properties of egg products is presented in **Table 2.6**. The colour of LWE was affected by treatments combining HP (130 – 270 MPa/5 min) and additives, presenting less yellowness and greater lightness and whiteness. The presence of citric acid significantly reduced the product redness, but had no effect on the other parameters [174]. However, the use of more intense processing conditions (300 MPa/3.33 min/5 ºC), in the presence of nisin, increased redness and decreased yellowness of LWE [167].

On the other hand, the impact of HP treatments (100 – 700 MPa) in combination with temperature (10 – 60 ºC) on turbidity, protein solubility, surface hydrophobicity and SH groups of EW solutions was studied by Van der Plancken et al. [175–177]. These authors reported that the EW solutions turbidity (pH 7.6) showed a strong increase with pressure and temperature increasing, while almost no increase occurred at pH 8.8 (except for pressure treatments at 60 ºC) [177]. For protein solubility, pressure increase caused a protein solubility reduction, but at pH 8.8 there was only a decrease when HP treatments were performed at 60 ºC (denaturing temperature) [177]. Regarding surface hydrophobicity, treated EW solutions showed a pressure-dependent increase in this property, with a strong rise at pressures above 400 MPa. With temperature increasing (10 – 40 ºC), at 550 MPa, also occurred a surface hydrophobicity increase [175,177]. Moreover, the exposure of buried SH groups of treated EW solution showed a pressure-dependent behaviour, with a greater increase above 500 MPa. This was accompanied by a decrease in buried SH groups, and at 700 MPa almost all SH groups were exposed [175,176]. Following the same trend, total SH groups content decreased with increasing pressure, probably due to the oxidation and formation of disulphide bonds, particularly at pH 8.8. Otherwise, when the temperature rise (10 – 40 ºC) there was a decrease in exposed SH groups, followed by an increase in buried SH groups [175,176].

Differential scanning calorimetry (DSC) thermogram of EW solutions showed that the peaks remained visible even after treatment at 400 MPa/10 ºC, although, they disappeared at 60 ºC and at pressures above 100 MPa. The residual denaturation enthalpy (ΔH) tended to decrease with increasing pressure, with a more pronounced effect at 10 ºC, while at 60 ºC a more gradual change was observed [177]. Additionally, depending on the processing conditions used, egg coagulation can occur, for instance, at 100 and 150 MPa/5 ºC up to 60

min coagulation did not occur, while it was observed at pressures above 200 MPa. Although, at 45 ºC, coagulation was detected at 100 and 150 MPa, and after 1 sec at pressures above 250 MPa [178].

Concerning rheological properties of egg products, the effect of HP (281.8 – 618.2 MPa during 1.6 –18.4 min) and temperature (8.2 – 41.8 ºC) were also assessed. All egg components (EW, EY and LWE) showed a thixotropic fluid behaviour, and their rheological properties were most significantly affected by pressure followed by holding time and temperature. In case of EY, there was a progressive change from a thin liquid to a thicker liquid, increasing viscosity and restricting the free flow of the product at pressures above 550 MPa [179]. For LWE, the apparent viscosity remained almost constant after treatments at 100 – 150 MPa/0 – 60 min/5 ºC, while stronger treatments (200 – 400, 3 – 17 min, < 15º C) increased this property [172,178].

Furthermore, the impact of simultaneously combined treatments on egg foaming properties was evaluated by Lee (2002) [167], that reported no effect on LWE foaming power and stability as a result of HP (300 MPa during 3.33 min at 5 ºC) combined with nisin. Otherwise, an improvement of EW solutions FC (pH 7.6) was found in the range of 100 – 500 MPa/10 – 40 ºC, and at 600 – 700 MPa occurred a reduction to even lower values than that of non-treated solution. At pH 8.8, FC improved with increasing pressure level, presenting a more pronounced effect at 60 ºC [88]. Although, these treatments had no detrimental effect on FS, producing more stable foams with EW solutions treated above 500 MPa/10 – 40 ºC [88].

Table 2.6. Main effects of simultaneously combined treatments on physicochemical and functional properties of egg products and egg derivatives.

Egg product	Process Conditions	Effects	References
Liquid whole egg	300 MPa, 3.33 min, 5 °C 10 mg/L Nisin	No significant change in foaming power, FS and droplet sizes Similar L* Increased a* Decreased b*	[167]
Liquid whole egg	100 – 400 MPa, 0 – 60 min, 5, 45 ºC	Coagulation at pressures > 200 MPa/5 ºC Apparent viscosity remained almost constant at 100 – 150 MPa/5 ºC Coagulation at 100 and 150 MPa/45 ºC Almost instantaneously coagulation > 250 MPa/45 ºC	[178]
Liquid whole egg	281.8 – 618.2 MPa, 1.6 – 18.4 min, 8.2 – 41.8 ºC	Pressure followed by holding time and temperature was the most significant variables Thixotropic fluid behaviour HP affected the protein structure	[179]
Liquid whole egg	200 – 400, 3 – 17 min, < 15º C	Colour change with pressure Increased in viscosity	[172]
Liquid whole egg	Ultrasonic cleaning bath: 15 – 30 min, 40 – 50 ºC	No significant change in soluble protein	[170]
Liquid whole egg	133.33 – 266.6 MPa, 5 min, ~22ºC 0.05 – 0.1 % Methyl:propyl (3:1) 0 – 0.1 % Parabens 0.15 – 0.5 % Citric acid 5 mM EDTA 200 UI Nisin 0 – 1000 UI Lysozyme	No significant change in redness Decreased in yellowness Increased in lightness and whiteness Citric acid significantly reduced redness and did not affect whiteness, lightness, or yellowness	[174]

Liquid egg yolk	281.8 – 618.2 MPa, 1.6 – 18.4 min, 8.2 – 41.8 ºC	Pressure followed by holding time and temperature was the most significant variables Thixotropic fluid behaviour Progressively changed from thin liquid to thicker liquid Increased in viscosity	[179]
Liquid egg white	281.8 – 618.2 MPa, 1.6 – 18.4 min, 8.2 – 41.8 ºC	Pressure followed by holding time and temperature was the most significant variables Thixotropic fluid behaviour HP affected the protein structure	[179]
Liquid egg white	400 – 800 MPa, 5 min, 9 ºC	Egg remained liquid at 400 MPa Formed a very soft gel Greater pepsin digestibility with increasing pressure Extensive protein hydrolysis and release of various peptides	[180]
Egg white solutions (10 % v/v, pH 7.6)	100 – 700 MPa, 0 – 50 min, 10 – 40 ºC	Increased in residual ΔH, solubility and buried SH groups with temperature Decreased in turbidity, surface hydrophobicity, exposed SH groups with temperature Decreased in residual ΔH, solubility, buried and total SH groups with pressure Increased in turbidity and surface hydrophobicity with pressure	[175]
Egg white solutions (10 % v/v)	100 – 700 MPa, 20 min, 10 – 60 ºC, pH 7.6 and 8.8	Antagonistic effect between pressure and temperature Increased in turbidity and exposed SH groups Increased in surface hydrophobicity with pressure increasing Decreased in surface hydrophobicity with temperature increasing Increased in enzymatic susceptibility > 400 MPa Decreased in buried and total SH groups, protein solubility and trypsin inhibitory activity Decreased in ΔH with increasing pressure SH groups were more sensitive to oxidation at higher pressure Faster denaturation at lower temperatures (up to 40 ºC) Peaks visible at 400 MPa/10 and 25 ºC	[88,176,177]

		Peaks disappeared above 100 MPa/60 ºC Increased in FC with increasing pressure, and higher at 60 ºC (pH 8.8) Increased in FC until 500 MPa, followed by a decreased (< 60 ºC) (pH 7.6) Had no effect in FS Foams had a moist and creamy appearance	
Egg white solution (10 % w/w)	21.3 – 65.7 W, 15 – 60 min, 25 – 55 ºC, 35 – 40 kHz, ≈ 360 mL, pH 7.00 – 10.0 (Ultrasonic water bath)	Protein aggregation caused by excess of ultrasound treatment and heating Increased alkalase hydrolysis Decreased in hydrolysis rate with acoustic power, treatment time and temperature increase Produced hydrolysate with higher antioxidant activity	[181]

[(1)] ΔH: Enthalpy of denaturation; FC: Foaming capacity; FS: Foaming stability; HP: High Pressure; SH groups: Sulfuryl groups.

2.6. References

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Chapter III

Materials and Methods

3.1.Sample preparation

Fresh shell eggs were purchased from a local supermarket the day before they were used and held overnight under refrigeration. The egg shells were thoroughly washed with 70 % ethanol, allowed to dry at room temperature, then broken aseptically and the egg white (EW) and egg yolk (EY) was manually separated, and the chalazae removed. The EW was transferred to a sterile cup and gently homogenized for 20 min at 1250 rpm using a magnetic stirrer (MS-3000, Biosan, Riga, Latvia). Each yolk was passed through a colander to remove remains of chalazae and albumen, then, the vitellin membrane was disrupted with a scalpel blade and yolk was collected in a sterile cup. The separated EY was then manually homogenized by gentle stirring. For whole egg, after breaking, the egg was carefully passed through a colander and the vitellin membrane disrupted with a scalpel blade, then, it was collected in a sterile cup and gently homogenized for 20 min at 1250 rpm using a magnetic stirrer (MS-3000, Biosan, Riga, Latvia).

3.2.Microorganisms

The strains used in this study were *Salmonella enterica* serovar Senftenberg (ATCC 43845) (throughout this thesis it will be mentioned as *S.* Senftenberg 775/W) and *Listeria innocua* (ATCC 33090). This *Salmonella* serovar was selected because it is widely used as a microorganism to study eggs pasteurization, due to its higher thermal resistance compared to the serovars associated with most human infections (*S.* Typhimurium and *S.* Enteritidis) [1,2]. On the other hand, *L. innocua* was used because it is a gram-positive bacteria and a *L. monocytogenes* surrogate, which has been reported as a microorganism of public health concern in liquid eggs [3].

One glass bead from a deep-frozen culture was transferred to 250 mL of sterile Tryptic Soy Broth (TSB, VWR Chemicals, Carnaxide, Portugal) and incubated overnight at 37 ºC, under rotational stirring (150 rpm) (Incubating orbital shaker, VWR, Portugal). Then, 1 mL from this culture was transferred to 250 mL of sterile TSB and incubated again at 37 ºC under rotational stirring (150 rpm) for 24 h. During this incubation period and at every hour, an aliquot of 1 mL of the culture was removed to count microorganisms in Tryptic Soy Agar (TSA, VWR Chemicals, Carnaxide, Portugal), and to measure the optical density (600 nm), until the turbidity values did not vary significantly. The TSA plates were incubated at 37 °C

during 24 h for *S.* Senftenberg 775/W and 48 h for *L. innocua*. Growth curves were constructed for each microorganism (**see Annex B**) when cells reached the stationary growth phase. The liquid culture was kept at rest at room temperature during 15 min, and glycerol was added to a final 10 % (v/v) concentration. Then, the culture was manually homogenized, transferred to Eppendorf tubes, rapidly frozen under liquid nitrogen and stored at – 80 °C for later use.

3.3.Sample inoculation and packaging

The microorganism suspension prepared above (**section 3.2**) was thawed at room temperature and used immediately afterwards. This suspension was inoculated into liquid egg (liquid whole egg (LWE), EW, or EY) and mixed under magnetic stirring for 5 min at 1250 rpm (MS-3000, Biosan, Riga, Latvia). Then, non-inoculated and inoculated samples were packed under aseptic conditions in polyamide-polyethylene bags (Plásticos Macar Lda., Santo Tirso, Portugal), previously sterilized with ultraviolet radiation, being manually heat sealed to minimize the amount of air inside the bags (**Figure 3.1**). The initial concentration of *S.* Senftenberg 775/W and *L. innocua* was approximately $10^6 – 10^7$ colony forming unit (CFU)/mL.

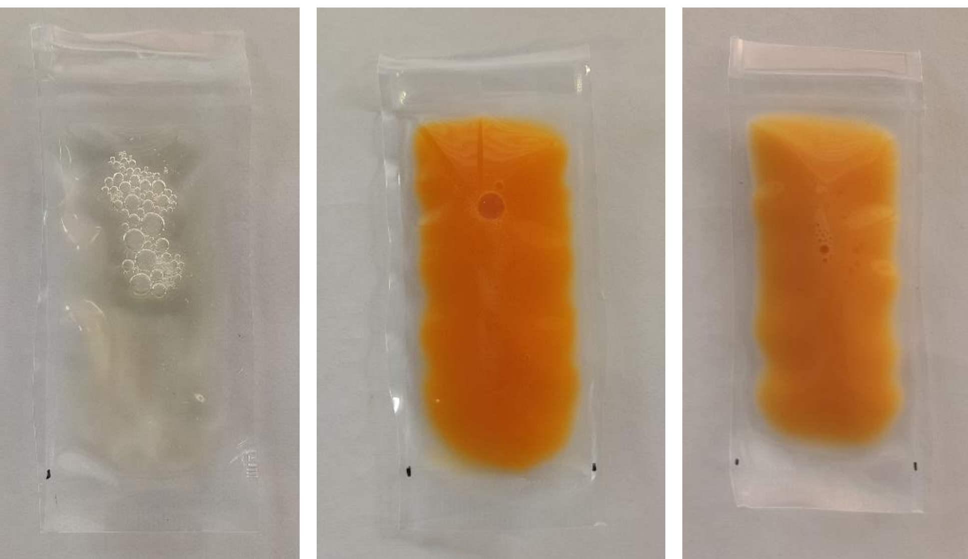

Figure 3.1. Pictures of the packaged egg samples. From left to right: egg white, egg yolk and whole egg.

3.4.Microbiological analyses

The samples were evaluated in triplicated for microorganisms counts, and the appropriate serial dilutions were prepared in Ringer's solution. The *S.* Senftenberg 775/W and *L. innocua* were quantified by the spread plate method, by plating 100 µL of the appropriate dilution, in duplicate, onto Xylose Lysine Deoxycholate Agar (Himedia, Mumbai, India) [4,5] and Listeria Palcam Agar (Liofilchem, Roseto degli Abruzzi, Italy) [6], respectively (Chapters IV, V and VI). In Chapter VII, after the experiments, 1.0 mL of each sample was obtained aseptically and thoroughly mixed with 9.0 mL of Ringer's solution. Further, decimal dilutions were made with the same diluent and duplicates of dilutions were plated on TSA. In this chapter (Chapter VII) only *S.* Senftenberg 775/W was evaluated and therefore TSA (non-selective medium) was used, since no other microorganisms were present in egg samples. The plates were incubated at 37 ºC for 24 h and 48 h for *S.* Senftenberg 775/W and *L. innocua*, respectively. Plates containing 10 to 300 colonies were selected for counting and the results were expressed as log CFU/mL (microbial load). The results are shown as microbial log load variation (log (N/N_0)) calculated by the log load difference between the microbial load at each treatment (N) and the initial microbial load (without any treatment) (N_0).

3.5.Treatment conditions

3.5.1. High pressure

The pressure experiments were performed at room temperature, approximately at 20 ºC, using a High-Pressure industrial equipment (Modelo 55, Hyperbaric, Burgos, Spain), with a 55 L pressure vessel using water as the pressure-transmitting medium.

3.5.2. Ultrasound

The ultrasound (US) experiments were carried out using a lab-scale equipment UP400St (Hielscher Ultrasound Technology GmbH, Chamerau, Germany) with a working frequency of 24 kHz and equipped with a 3 mm diameter probe (amplitude at 100 % of 166 µm). The probe was used to sonicate 50 mL of egg product in a 100 mL glass beaker and was maintained at 1.5 cm under the surface of egg product, and the beaker was kept in an ice water bath. The sample temperature was monitored by using a *K*-type thermocouple, with

an initial temperature of 15 °C, and achieving a maximum of 29 ºC. The US treatments were performed without any packaging (in a beaker), in a biosafety cabinet (Telstar, Bio II Advanced, Lisboa, Portugal). After US treatments, the samples were packaged in polyethylene plastic bags (Plásticos Macar Lda., Santo Tirso, Portugal) for further treatments.

3.5.3. Thermal pasteurization

The TP was performed using a circulating water bath (Circulator Bath, FALC, Treviglio, Italy), with the temperature monitored by using a *K*-type thermocouple (Thermometer 305, Roline, Bassersdorf, Switzerland). The time started to count after a come-up time of 40 sec (previously verified at the geometrical centre of the bags (4x3x0.5 cm)).

3.5.4. Sequentially combined treatments

The sequentially combined treatments were performed following the procedures described above, and the maximum time between treatments was approximately 20 min. Thereafter, all samples bags were immediately placed on ice and kept at 4 °C until analysis. The conditions used in each chapter are shown in **Erro! A origem da referência não foi encontrada.**.

Table 3.1. Pressure, ultrasound and thermal pasteurization conditions used.

Egg Product	Chapter	Conditions		
		Pressure	Ultrasound	Thermal Pasteurization
Egg white	Chapter IV	50 – 200 MPa/5 – 20 min	-	55 – 55.6 ºC/3 – 6.2 min
Egg yolk	Chapter V	50 – 400 MPa/5 min	-	60 ºC/3 – 6.2 min
Whole egg	Chapter VI	50 – 250 MPa/5 min	-	60 ºC/1.75 – 3.5 min
Egg white Egg yolk Whole egg	Chapter VII	50 – 160 MPa/5 min	50 % amplitude/1 – 3 min	55 – 60 ºC/1.75 – 6.2 min

3.6.Post thermal pasteurization refrigeration storage

Egg products pasteurized by the optimized combined treatment were stored for 61 days at 4 ºC and compared with commercial thermal pasteurization (TP) to study microbial development of *S.* Senftenberg 775/W and *L. innocua*. For this, microbiological analysis was performed before and immediately after treatments, and on different storage days. The experiment was carried out in three replicates at each time point.

3.7.Physicochemical analyses

3.7.1. pH and total soluble solids

The pH was measured at 20 ºC with a properly calibrated glass electrode (pH electrode 50 14, Crison Instruments, S. A., Barcelona, Spain), by directly submerging it into the homogenized egg samples.

The total soluble solids content was determined by measuring °Brix using a handheld refractometer (Handheld Refractometer Atago ATC-IE, Tokyo, Japan) at 20 °C.

3.7.2. Turbidity

Turbidity was determined as the absorbance at 650 nm and expressed as a percentage (%), with a 0 % turbidity corresponding to a totally clear solution. The absorbance was measured using a Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific Inc., Massachusetts, USA), according to the method of Van der Plancken et al. [7].

3.7.3. Colour

Colour of egg samples was assessed using a Konica Minolta CM 2300d spectrophotometer (Konica Minolta, Osaka, Japan). The colour parameters L* - lightness (0, dark; 100, light), a* – redness (+, red; – green) and b* – yellowness (+, yellow; – blue) were obtaining at room temperature with the CIELab system. The CIELab parameters were determined using the original SpectraMagicTM NX Software (Konica Minolta, Osaka, Japan), according to the International Commission on Illumination regulations. For measurements, 6 mL of sample were homogenized and placed in a sample holder. The total colour difference (ΔE^*) was calculated by **Equation 1** [8]:

$$\Delta E^* = [(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2]^{1/2} \quad (1)$$

where ΔE^* is the total colour difference between the sample and the control (raw sample), L^* and L_0^* are the lightness of treated and raw samples, respectively; a^* and a_0^* are the redness of treated and raw samples, respectively; and b^* and b_0^* are the yellowness of treated and raw samples, respectively.

3.7.4. Soluble protein

The egg samples were diluted at 10 % (m/v) in distilled water and then centrifuged at 10,000 g during 15 min at 4 °C (Heraeus Biofuge Stratos, Thermo Electron Corporation, Massachusetts, USA) [9]. Soluble protein content of the supernatant was quantified using the microassay Bradford method [10,11]. Two hundred µL of Bradford reagent was added to 40 µL of sample and shaked for 40 sec. Absorbance at 595 nm (Multiskan GO Microplate Spectrophotometer, Thermo Fisher Scientific Inc., Massachusetts, USA) was measured 20 min after Bradford reagent addition. A standard curve was built using bovine serum albumin (Sigma-Aldrich, Lisboa, Portugal) in the range of 0.02 – 0.40 g/L (**Annex C – Table C1**). Measurements were performed in triplicate and the results were expressed in wt.% (g/100 g egg product) or in percentage (%) with respect to values of non-treated samples.

3.7.5. Surface hydrophobicity

The surface hydrophobicity (H_0) was determined with the fluorescence probe 1-anilino-8-naphtalene-sulfonate (ANS, Cayman Chemical, Michigan, USA) as described by Sheng et al. [9]. The soluble fraction (see section 3.7.4) was diluted to 0.10 – 0.50 mg/mL with 0.010 M phosphate buffer (pH 7.2). To 4 mL of EW diluted solution, 20 µL of ANS solution (8 mM in 10 mM phosphate buffer, pH 7.2) was added, and the fluorescence intensity was measured at 390 nm (excitation wavelength) and 470 nm (emission wavelength), using an F-2000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). Surface hydrophobicity (expressed in arbitrary units, a.u.) was calculated using the initial slope of fluorescence intensity versus soluble protein concentration plot (calculated by linear regression analysis). Samples were measured in triplicate of sample and quadruplicate of analysis.

3.7.6. Viscosity

Viscosity was measured in a rotational rheometer (Kinexus PRO, Malvern Panalytical, Malvern, United Kingdom) with an attached cone-and-plate geometry (stainless steel cone, 4º and diameter of 40 mm). The experimental temperature was controlled at 20.0 ± 0.1 ºC by a Peltier system at the bottom plate. After transferring the sample to the rheometer bottom plate and after a 5 min equilibration time, the sample was subjected to a 3 min increasing shear rate ramp, from 0.1 to 100 s^{-1}, followed by a 3 min decreasing ramp, from 100 to 0.1 s^{-1}. Determination of the apparent viscosity and analysis of the flow curves (**Annex D – Figure D2**, **Annex E – Figure E2, Annex F – Figure F2, Annex G –Figure G2**) were performed using the rSpace software (Malvern Panalytical version 1.76s). Apparent viscosity measured at an intermediate shear rate of 50 s^{-1} was used for comparison among samples. Measurements were made in triplicate of sample and duplicate of analysis.

3.8.Thermal properties

The thermal properties were determined by a differential scanning calorimeter (DSC). Egg sample was accurately weighed (7 – 15 ± 2.1 mg) in a stainless-steel pan and then sealed hermetically. A sealed empty pan was used as a reference and the heating rate of the DSC scan was 10 °C/min over a range of 5 to 110 °C for EW, and over a range of 30 to 120 °C for LWE. The obtained thermograms were used to determine both enthalpy variation (ΔH) and peak denaturation temperatures (T_{peak}) of each sample. The DSC measurements were done in duplicate and the data was analysed with the Pyris Series – Diamond DSC Software (PerkinElmer, Massachusetts, USA).

3.9.Functional analyses

3.9.1. Foaming properties

Foaming properties were measured according to the method used by Sheng et al. [9]. Foams were produced using 7 mL of egg sample by whipping for 2.5 min with a laboratory homogenizer (Ultra-Turrax, T25 basic, IKA®-Werke, Staufen, Germany) at a speed of 9500 rpm and room temperature, in a 20 mL graduated cylinder. Foaming capacity (FC) was calculated as percentage of increase in foam volume using the **Equation 2**:

$$FC\ (\%) = \frac{V_1}{V_0} \times 100 \quad (2)$$

Foaming stability (FS) was determined by loss of foam volume after 30 min of standing, at room temperature. Foaming stability was calculated by **Equation 3**:

$$FS\ (\%) = \frac{V_2}{V_1} \times 100 \quad (3)$$

where V_0 is the initial volume of egg (mL); V_1 is the foam volume after whipping (mL); V_2 is the foam volume after 30 min (mL). Foaming properties was measured in triplicate of sample and duplicate of analysis.

3.9.2. Emulsifying properties

Emulsifying activity index (EAI) and emulsion stability index (ESI) were measured at room temperature according to the previous researches [12,13]. The EY was diluted in distilled water (1:1 (v/v)) and stirring at 250 rpm for 20 min (Orbital shaker PSU-10i, Biosan, Riga, Latvia) and collected for further use (kept for 1 h). To prepare emulsions, 3 mL of egg sample was stirred by a homogenizer (Ultra-Turrax, T25 basic, IKA®-Werke, Staufen, Germany) with 2 mL of sunflower oil, at 9500 rpm during 90 sec. An aliquot of emulsion was taken from the bottom of the homogenized emulsion immediately (0 min) or 10 min after homogenization and diluted 300 times in a 0.1 % (w/v) SDS solution. After dilution, the emulsion was shaken and the absorbance was read at 500 nm using a UV-Vis spectrophotometer (UVmini-1240, Shimadzu, Kyoto, Japan). Measurements were performed in triplicate of sample and duplicate of analyse, and the EAI and ESI were calculated by **Equation 4** and **5** [13]:

$$EAI\ (m^2/g) = \frac{2 \times 2.303 \times A_0 \times DF}{c \times \Phi \times 10^4} \quad (4)$$

$$ESI\ (min) = \frac{A_0}{(A_0 - A_{10}) \times \Delta t} \quad (5)$$

where A_0 and A_{10} are the absorbance of the diluted emulsions at 0 and 10 min, respectively, DF is the dilution factor, *c* is the initial concentration of protein (g/mL); Φ is the fraction of oil used to form the emulsion (0.4), Δt is the time interval between taking the first and second aliquots of emulsion.

3.9.3. Gelling properties

3.9.3.1. Gel's texture

Heating set gels were prepared by pouring each egg sample into a plastic casing, closed at the ends and heating at 85 ºC for 35 min in a programmable circulating water bath (FALC, Faenza RA, Italy). Then, the gels were quickly cooled in a water-ice bath and stored at 4 ºC for 18 h. Before texture analysis the gels were brought to room temperature, removed from the plastic and cut in cylinders of 22 mm diameter × 19 mm height with a wire knife.

Textural analysis was carried out with a TA.XT.plusC Texture Analyzer (Stable Micro Systems, Godalming, United Kingdom) equipped with a 30 kg load cell and a 50 mm diameter cylinder aluminium probe (P/50). The uniaxial compression tests up to rupture were performed at 2.00 mm/s. The Exponent Connect software (version 8.0.3.0, Stable Micro Systems, Godalming, United Kingdom) was used to construct stress-strain and force-time curves, respectively, for EW and LWE heat-induced gels, and to obtain the mechanical parameters of interest. The mechanical parameters determined were: stress at rupture (kPa), strain at rupture (%), Young modulus (kPa) and distance at first break (mm) for EW heat-induced gels; and maximum force (N), area under the curve (N.sec) and slope at 10 % height for LWE heat-induced gels. At least six cylindrical gels of each treatment were used for the analysis.

3.9.3.2. Water holding capacity

The water holding capacity (WHC) of egg heat-induced gels was measured as described by Marco-Molés et al. [14], with some modifications. Centrifuge tubes filled with liquid egg (2.0 mL) were placed in a water bath (Circulator Bath, FALC, Faenza RA, Italy) at 85 ºC for 35 min for gel formation. After heating, the samples were immediately placed on ice for 15 min and then, left at room temperature (20 ± 1 ºC) for 1 h. The tubes were then centrifuged

at 740 *g* for 10 min at 20 ºC (Centurion Scientific Ltd, Scansci, Vila Nova de Gaia, Portugal). The WHC was given by **Equation 6**:

$$\text{WHC (\%)} = \frac{\text{WAC}}{\text{WBC}} \times 100 \quad (6)$$

where WAC is the weight of gel after centrifugation and WBC is the weight of gel before centrifugation.

3.10. Fatty acids profile

Total lipids were extracted according to Cruz et al. [15], with minor adjustments. In brief, 1 mg of the internal standard triundecanoin (C11:0 triglyceride; 10 mg/mL in hexane) were added to EY samples (100 – 150 mg), mixed with 1.5 mL of 2-propanol (to precipitate proteins) followed by 2 mL of cyclohexane and the mixture was vigorously vortexed for 30 sec. Then, 2.25 mL of NaCl solution (1 % (m/v)) was added to allow the separation of the upper organic phase containing the lipids and centrifuged at 5000 rpm for 5 min. Afterwards, the upper layer was transferred to a 4 mL vial and evaporated to dryness under a gentle nitrogen stream at 40 ºC.

The fatty acids (FA) profile was determined by gas chromatography (GC) as fatty acid methyl esters (FAMEs) converted by cold methylation according to ISO 12966-2:2017 [16]. Briefly, extracted fat was dissolved in 2 mL of n-heptane and anhydrous sodium sulphate, mixed with 200 µL of potassium hydroxide (KOH, 2 M in methanol), followed by the addition of sodium hydrogen sulphate (500 mg) to guarantee samples dehumidification. Finally, 50 µL of injection standard (21.46 mg/mL of methyl tridecanoate (C13:0 methyl ester) in hexane) were added and centrifuged at 3500 rpm for 5 min. The FAMEs were determined by GC in the upper layer (Chrompack CP-9001 model, Apeldoorn, Netherlands) with flame ionization detection (FID). FAMEs separation was carried out on a Select FAME (50 m × 0.25 mm x 0.25 µm) column (Agilent, California, USA) using helium as carrier gas (pressure of 190 kPa), and temperature of injector and detector set at 250 °C and 270 °C, respectively. The oven temperature was set at 140 °C for 0 min, then raised to 210 °C at a rate of 2 °C/min and raised again at a rate of 20 °C/min to 250 °C, in a total run of 37 min. The FA identification and FID calibration was accomplished with a certified reference

mixture of FAME (TraceCert – Supelco 37 component FAME mix, Supelco). Data were processed by the CP Maitre chromatography data system program (version 2.5, Chrompack International B. V., Middelburg, Netherlands). The relative proportion of each FA was obtained by normalization of the peak areas, after calibration of the individual FA FID responses. The results are expressed in relative percentage of each FA (% fatty acid).

3.11. Secondary lipid oxidation: Thiobarbituric acid-reactive substances

Lipid oxidation was evaluated by quantification of secondary lipid oxidation products using the thiobarbituric acid-reactive substances (TBARS) method, performed as described by Vyncke [17], with slight modifications. Two grams of egg product were homogenized with 2 mL of 7.5 % trichloroacetic acid and the obtained suspension was centrifuged at 6000 rpm during 15 min at 4 ºC (Centurion Scientific Ltd, Scansci, Vila Nova de Gaia, Portugal). 1 mL of the supernatant was added to 1 mL of 2-thiobarbituric acid (20 mM TBA in 99 % acetic acid glacial) and slightly shaken. The mixture was placed in a water bath at 100 ºC for 40 min and then cooled on ice. The absorbance was measured at 538 nm (Multiskan GO Microplate Spectrophotometer, Thermo Fisher Scientific Inc., Massachusetts, USA). A standard curve of malondialdehyde (MDA) was obtained with 1,1,3,3-tetramethoxypropane, in the range of 0.5 – 10 µM MDA (**Annex C – Table C2**). Measurements were performed in triplicate and the results were expressed as µg MDA/100 g of egg product.

3.12. Total carotenoids

Total carotenoids were quantified based on the method described by Nagata and Yamashita [18]. Briefly, 20 mL of acetone/hexane solution (4:6 (v/v)) was added to one gram of egg sample, and homogenized during 40 sec at 10,000 rpm (MICCRA D-9 Homogenizer, MICCRA GmbH, Heitersheim, Germany). Then, the absorbance was measured at 663, 645, 505 and 453 nm (UVmini-1240, Shimadzu, Kyoto, Japan), following the calculations given by the cited authors [18]. Measurements were performed in triplicate of sample and duplicate of analysis and the results were expressed in mg/100 g of egg product.

3.13. Volatile profile

The volatile compounds of egg samples were collected through headspace solid-phase microextraction (HS-SPME) using a DVB/CAR/PDMS fiber (50/30 mm; Supelco, Lisboa, Portugal), and analysed by gas chromatography-mass spectrometry (GC/MS). To a 15 mL glass vial, containing a stirring bar and 2.0 g of EW, 0.20 g of sodium chloride and 25 µL of the internal standard (IS; cyclohexanone, 25 mg/L in ultrapure water) were added and immediately sealed with a screw cap with a silicone septum. For EY samples, to a 15 mL glass vial containing a stirring bar and 2.0 g of EY, 0.40 g of sodium chloride, 2.0 g of ultrapure water and 50 µL of the IS (cyclohexanone, 25 mg/L in ultrapure water) were added and immediately sealed. In the case of LWE, to a glass vial containing a stirring bar and 2.0 g of LWE, 0.60 g of sodium chloride and 50 µL of the IS (cyclohexanone, 25 mg/L in ultrapure water) was added and immediately sealed. Then, samples were equilibrated for 10 min at 40 °C (EW) or 15 min at 45 °C (EY and LWE) followed by HS-SPME exposure at the same temperature under moderate stirring (100 rpm) for 20 min [19,20]. Finally, the retained compounds were thermally desorbed from the fibre for 5 min in the injection port, with simultaneous cleaning and conditioning for subsequent analyses. Triplicate egg samples were analysed for each processing condition.

Chromatographic analysis was performed using an Agilent 7890N (Agilent Technologies, California, USA) coupled to an Agilent 5977B single quadrupole inert mass selective detector (Agilent Technologies, California, USA) operating in positive electron ionization mode. Injections were performed in splitless mode (250 ºC) and separation was performed in a 60 m × 0.25 mm × 0.25 µm DB-5MS capillary column (Agilent Technologies, California, USA), using helium as a carrier gas at a constant flow of 1 mL/min. The oven temperatures were progressively increased with the following ramp: 35 °C (5 min), 4 °C per min until 100 °C and 10 °C per min until 220 °C, with a total run of 33.5 min. The MS transfer line and ion source were at 250 °C and 230 °C, respectively, and the MS quadrupole temperature was 150 °C, with electron ionization of 70 eV; set in full scan mode (m/z 25 to 550 at 2.8 scan/s).

MassHunter quantitative analysis software (v. B.07.06.2704) was used for data processing, and the identification of the volatile compounds was based on computer matching with the reference mass spectra of the MS library of NIST 2.2, retention index and

available standards. Semi-quantification was performed using cyclohexanone as IS and results were expressed in µg IS equivalents/100 g of egg product.

3.14. Sensory analyses

3.14.1.Meringue

Sensory analysis was carried out for meringue batters prepared using a reference recipe: 40.0 mL of EW and 100.0 g powder sugar [21]. The EW was whipped for 2 min using an Electric-Hand-Mixer at a constant speed of 4 (the equipment maximum was 5). The sugar was gradually added over an additional 4 min of whipping (speed 5), and after all sugar had been added, the batter was whipped for another 3 min. Meringues were shaped in balls of about 3 cm diameter using a manual pastry bag and cooked at 140 ºC during 20 min (Flama, Cesar, Portugal). The cooked meringues were stored at room temperature for cooling before the sensory analysis.

A panel of 13 untrained volunteers from the Chemistry Department at University of Aveiro, Aveiro, Portugal (4 females and 9 males aged between 24 and 54 years) evaluated the sensory characteristics of meringues. Each sample composed of 2 merengues was randomly presented to the panellists in plastic dishes labelled with randomized three-digit codes. Water and crackers were provided to the panellists and they asked to cleanse their palate between tastings. The acceptability of meringues was evaluated based on their visual appearance, aroma, surface colour, interior colour, texture when breaking, crunchiness, flavour and global acceptability using a 9-point hedonic scale (1 = ‘dislike extremely’, 9 = ‘like extremely’). In addition, panellists were asked to order the samples according to their preference, from the one they liked least to the one they liked the most.

3.14.2.“Doce de ovos”

Sensory analysis was carried out for a traditional dessert (“Doce de Ovos”) prepared with the untreated and treated EYs. “Doce de Ovos” was prepared based on the following recipe: 40 g of EY, 190 g of sugar and 100 mL of water. The sugar was dissolved in water under stirring and then placed on an induction heating plate until it reached the thread stage. Next, the mixture was slowly added to the yolks (under stirring), and finally, this mixture was placed on the induction heating plate until reaching the desired consistency. The hot "Doce

de Ovos" was placed in plastic cups and stored at room temperature for cooling before the sensory analysis.

A panel of 13 untrained volunteers from the Chemistry Department at University of Aveiro, Aveiro, Portugal (6 females and 7 males aged between 24 and 54 years) evaluated the sensory characteristics of “Doce de Ovos”. Each sample composed of approximately 15 g of “Doce de Ovos” was randomly presented to the panellists in plastic cups labelled with randomized three-digit codes. Water and crackers were provided to the panellists and they were asked to cleanse their palate between tastings. Sensory characteristics such as visual appearance, colour, consistency, aroma, flavour, consistency in the mouth, mouthfeel and global acceptability were evaluated by the panel on a 9-point hedonic scale (1 = ‘dislike extremely’, 9 = ‘like extremely’) in part I of the questionnaire. Part II of the questionnaire assessed the granularity, viscosity, colour intensity and uniformity of the product using a 5-point scale (1 = ‘lowest’, 5 = ‘highest’) [22]. In addition, panellists were asked to order the samples according to their preference, from the one they liked least to the one they liked the most.

3.14.3.Egg tart

Sensory analysis was carried out for egg tarts prepared with non-treated and treated LWE. The ingredients (LWE and salt) were mixed with a hand mixer for 30 sec, and then, they were poured into 8-mold silicone pie tray (each one with 7 cm in diameter at the top and 5 cm in diameter at the bottom). The samples were baked in a pre-heated oven at 190 ºC for 12 min (Flama, Cesar, Portugal). The egg tarts were stored at ambient temperature for cooling before the sensory analysis.

A panel of 13 untrained volunteers from the Chemistry Department at University of Aveiro, Aveiro, Portugal (5 females and 8 males aged between 24 and 54 years) evaluated the sensory characteristics of egg tart. Each egg tart sample was randomly presented to the panellists in plastic dishes labelled with randomized three-digit codes. Water and crackers were provided to the panellists and they were asked to cleanse their palate between tastings. Egg tart prepared with LWE samples were considered to test for differences in which panellists were asked to evaluate each sample regarding acceptability based on 8 criteria (visual appearance, surface colour, interior colour, aroma, texture when cutting, texture when cracking, overall sensation when chewing, flavour) using a 9-point hedonic scale (1 =

‘dislike extremely’, 9 = ‘like extremely’). In addition, panellists were asked to order the samples according to their preference, from the one they liked least to the one they liked the most.

3.15. Statistical analysis

3.15.1. Analysis of variance

All data were tested at a 0.05 probability level ($p<0.05$) and the effect of each condition was tested with a one-way analysis of variance (ANOVA), followed by a multiple comparisons test (Tukey’s Honestly Significant Difference, HSD) to identify statistically significant differences between treatments (STATISTICA v.10).

3.15.2. Principal component analysis

Principal components analysis (PCA) was applied to identify the variables responsible for most of the data variability caused by the different treatments studied, grouping the samples according to their similarity. PCA was performed with the data from the volatile profile and quality parameters, using STATISTICA software, version 10.

3.16. References

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CHAPTER IV

RESULTS AND DISCUSSION | EGG WHITE

This chapter is adapted from the published work: Ana C. Ribeiro, Susana Casal, Francisco J. Barba, José A. Lopes-da-Silva and Jorge A. Saraiva. *Sublethal moderate pressure pre-treatments for subsequent shorter and improved egg white thermal pasteurization. Applied Food Research. 2022;2(2):100200.* DOI: 10.1016/j.afres.2022.100200.

4.1. Overview

This chapter describes the effect of pressure pre-treatments followed by a shorter thermal pasteurization (TP), compared to commercial TP, against *Salmonella* Senftenberg 775/W, and on the quality of liquid egg white (EW). Firstly, the processing conditions were optimized using *S.* Senftenberg 775/W inactivation as a reference (inoculated studies). Then, the development of *S.* Senftenberg 775/W and *Listeria innocua* (inoculated pathogenic surrogate) was evaluated during a post-TP refrigeration storage. Additionally, the EW treated under these conditions was also evaluated for physicochemical parameters such as pH, colour and soluble protein content, thermal properties, foaming and gelling properties, volatile profile and sensory analyses of an EW derived product (meringue), and the results were compared with non-treated and commercial TP-treated samples.

4.2. Microbial analyses

4.2.1. Microbial inactivation by moderate pressure treatments

The results (**Figure 4.1**) showed a general tendency to increase *S.* Senftenberg 775/W inactivation as pressure and holding time increased (the initial load of *S*. Senftenberg 775/W was 6.58 ± 0.22 log colony forming unit (CFU)/mL), which is in agreement with published works [1–3]. Moderate pressure (MP) treatments at 50 – 90 MPa/5 – 20 min reduced *S.* Senftenberg 775/W population by 0.21 – 0.38 log_{10} cycles ($p \geq 0.05$), while a higher inactivation was achieved (0.70 – 2.22 log_{10} cycles) in the range of 125 – 200 MPa/5 – 20 min. Other studies, despite applying higher pressure treatments (200 – 300 MPa/3 – 30 min), also obtained a similar or slightly higher inactivation (0.7 – 3.66 log_{10} cycles) for *S.* Enteritidis PT4 E10 and *Escherichia coli* K12 DH 5α (*S.* Enteritidis surrogate in liquid egg) in LWE, compared to our study [3,4].

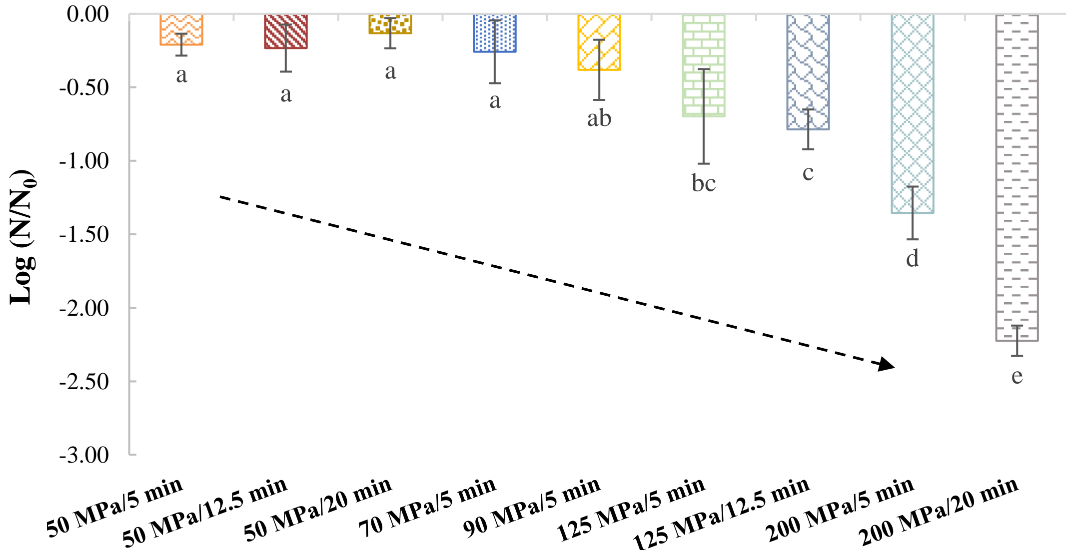


Figure 4.1. Log_{10} cycles reductions of *Salmonella* Senftenberg 775/W (ATCC 43845) in egg white treated by moderate pressure at 50 – 200 MPa/5 – 20 min. N denotes the microbial load measured for each treatment and N_0 the initial microbial load (6.49 – 7.12 log CFU/mL). Different letters indicate significant differences ($p < 0.05$) between treatments.

4.2.2. Microbial inactivation by moderate pressure followed by thermal pasteurization

Currently, the European Regulation CEE 1441/2007 [5] requires the absence of *Salmonella* spp. in 25 mL of liquid egg after pasteurization. One possible useful strategy to increase *Salmonella* spp. inactivation may be to apply MP pre-treatments at sublethal conditions to cause sublethal damages in microorganisms, possibly reducing their thermal resistance, allowing gentler TP conditions. In this study, the lethal effectiveness of a MP pre-treatment (50 – 200 MPa/5 – 20 min) followed by a shorter TP (55 ºC/3 min) (MP-TP) was evaluated against *S.* Senftenberg 775/W and compared with commercial TP (55.6 ºC/6.2 min). As shown in **Figure 4.2**, commercial TP achieved at least 5.49 log_{10} cycles of *S.* Senftenberg 775/W inactivation, while the shorter TP inactivated only 3.01 log_{10} cycles. MP at lower pressures (50 – 70 MPa/ 5 – 20 min) followed by TP, allowed to inactivate 2.44 to 3.10 log_{10} cycles ($p<0.05$). *S.* Senftenberg 775/W inactivation improved significantly ($p<0.05$) with MP-TP (90 MPa) (at least 4.4 log_{10} cycles reductions), but for MP-TP (125 – 200 MPa) the lethality increased even more, to at least 5.49 log_{10} cycles (counts below the detection limit, ≤ 1.00 log CFU/mL). A lower inactivation (1.5 – 3.1 log_{10} cycles) of *E. coli* K12 DH 5α (*S.* Enteritidis surrogate in liquid egg) was obtained by Monfort et al. [3] for LWE treated by pressure (200 – 300 MPa/3 – 30 min) combined with TP (52 – 55 ºC/2 – 3.5 min). For MP-TP at lower pressures (≤ 70 MPa), the combined treatments originated a lower or similar inactivation than the additive sum of the two individual treatments alone, meaning that there was not any synergistic effect. For MP-TP at pressures ≥ 90 MPa a synergistic effect was observed, indicating possible sublethal injuries in *S.* Senftenberg 775/W caused by the MP pre-treatments.

According to the results discussed above, two combined treatments were chosen for further analysis: 90 MPa /5 min – 55 ºC/3 min (MP-TP) as it was the combination that inactivated at least 4.4 log_{10} cycles of S. Senftenberg 775/W (close to 5 log_{10} cycles) and did not cause a visible increase in EW viscosity to the naked eye (contrarily the treatments at higher pressures), and 50 MPa/5 min – 55 ºC/3 min (MP-TP, that reduced about 2.5 log_{10} cycles of *S.* Senftenberg 775/W), in order to determine the effect of treatments on the physicochemical and functional properties of EW. The latter case (50 MPa/5 min – 55 ºC/3 min) was studied due to the intention of the authors to carry out further studies involving two nonthermal pre-treatments (pressure and ultrasound) for EW improved pasteurization

(see Chapter VII). For post-TP refrigeration storage evolution of *S.* Senftenberg 775/W and *L. innocua*, only the 90 MPa/5 min – 55 ºC/3 min treatment was studied due to the higher microbial inactivation achieved.

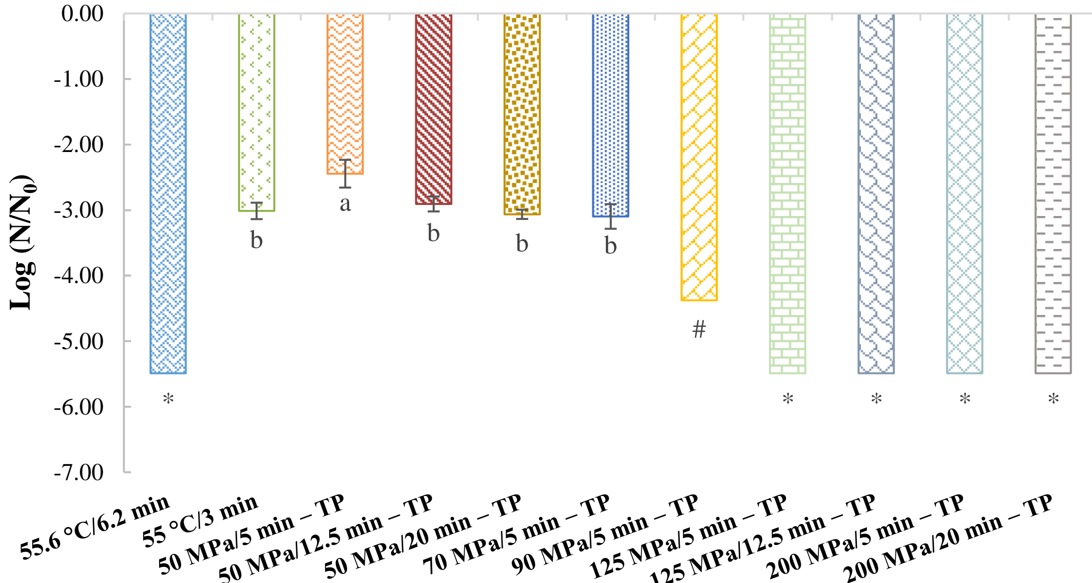
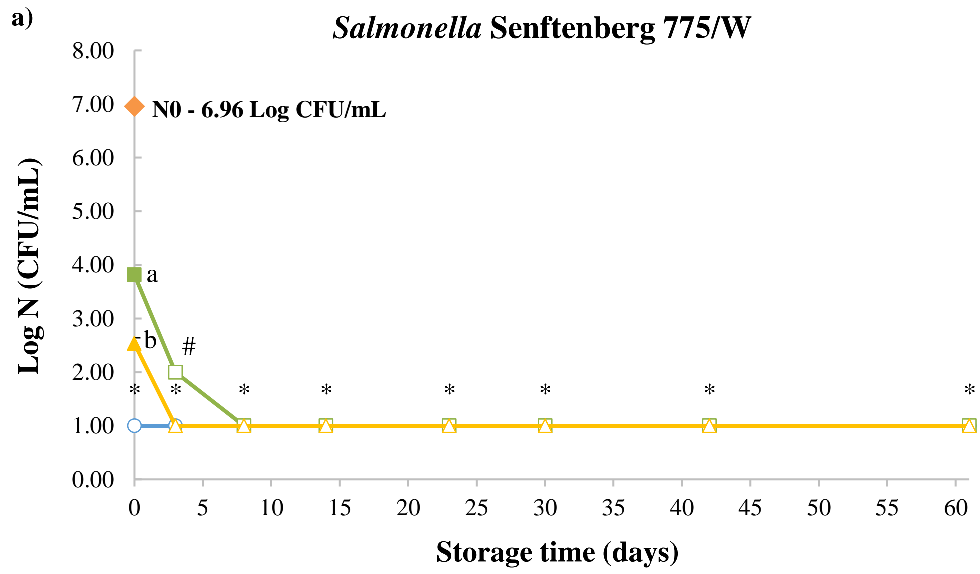


Figure 4.2. Log_{10} cycles reductions of *Salmonella* Senftenberg 775/W (ATCC 43845) in egg white treated by the designed combined treatments (moderate pressure followed by a shorter thermal pasteurization (TP) at 55 ºC/3 min) or TP only (55 ºC/3 min and 55.6 ºC/6.2 min). N denotes the microbial load measured for each treatment and N_0 the initial microbial load (6.49 – 7.12 log CFU/mL, without any treatment). The symbol # or * means that microbial counts were below the quantification limit (≤ 2.00 log CFU/mL) or detection limit (≤1.00 log CFU/mL), respectively. Different letters indicate significant differences ($p < 0.05$) between treatments.

4.2.3. Assessment of post-thermal pasteurization refrigeration storage

Salmonella spp. is the most frequent cause of egg foodborne outbreaks, but *L. monocytogenes* is another microorganism of public health concern in egg products [5,6] and so, *L. innocua* was used as a surrogate for *L. monocytogenes*. **Figure 4.3** shows the effect of treatments under study on the counts of *S.* Senftenberg 775/W and *L. innocua,* during storage at 4 ºC. Liquid EW samples were inoculated with an initial load of 6.96 ± 0.07 and 6.83 ± 0.08 log CFU/mL of *S.* Senftenberg 775/W and *L. innocua*, respectively. For day 0, immediately after the treatments, regarding *S.* Senftenberg 775/W (**Figure 4.3a**), the



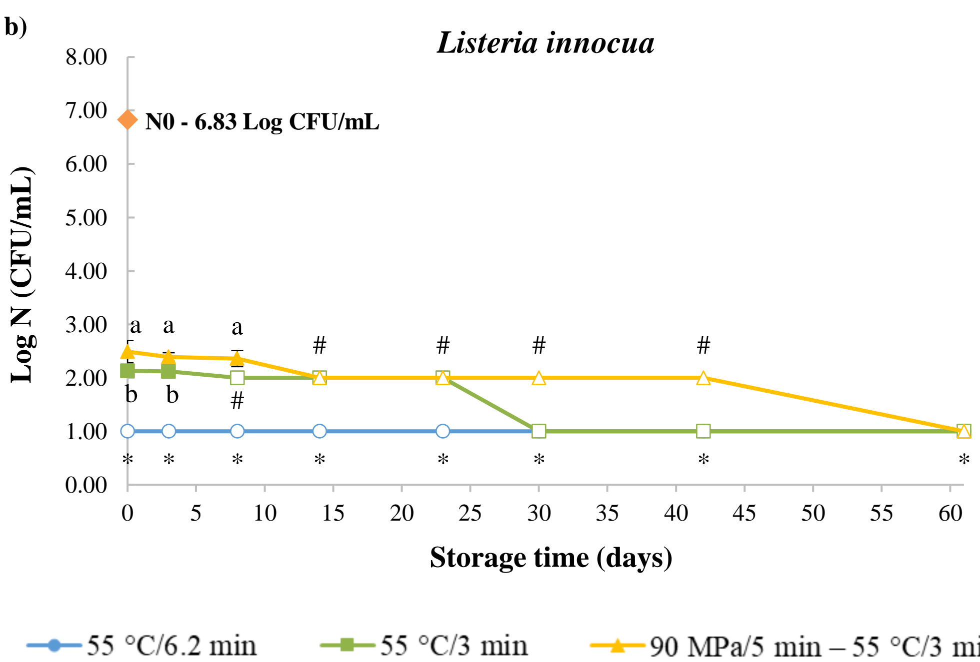


Figure 4.3. Evolution of a) *Salmonella* Senftenberg 775/W (ATCC 43845) and b) *Listeria innocua* (ATCC 33090) up to 61 days of refrigeration storage after moderate pressure followed by a shorter thermal pasteurization (TP) (90 MPa/5 min – 55 ºC/3 min) or TP only (55 ºC/3 min and 55.6 ºC/6.2 min). Unfilled symbols on graphics and # or * mean that microbial counts were below the quantification limit (≤ 2.00 log CFU/mL) or detection limit (≤1.00 log CFU/mL), respectively, and N_0 corresponds to the initial load before each treatment. Different letters denote significant differences ($p<0.05$) between each processing condition and storage days.

MP-TP (90 MPa/5 min – 55 ºC/3 min) originated a reduction of 4.43 $\log_{10}$ cycles, whereas the shorter TP and commercial TP alone reduced 3.15 and at least 5.96 $\log_{10}$ cycles, respectively (these results are in agreement with those shown above (section 4.2.2) for the same treatments). During 61 days of storage, *S.* Senftenberg 775/W counts remained below the detection limit (≤ 1.00 log CFU/mL) in commercial TP-treated samples. On the other hand, in EW treated by the MP-TP, a reduction in counts to a value ≤ 1.00 log CFU/mL occurred after 3 days, which remained until the end of storage. For the shorter TP only, *S.* Senftenberg 775/W counts decreased to below the quantification limit after 3 days (≤ 2.00 log CFU/mL), and below detection limit (≤ 1.00 log CFU/mL) after 8 days, and then remained without significant variation until the 61st day.

Concerning *L. innocua* (**Figure 4.3b**), the initial counts decreased by 4.34, 4.70 and at least 5.83 $\log_{10}$ cycles, for the MP-TP, the shorter TP and the commercial TP, respectively. The counts of this microorganism in commercial TP-treated samples showed a behaviour similar to *S.* Senftenberg 775/W, keeping counts below the detection limit for 61 days. For shorter TP- and MP-TP-treated samples, *L. innocua* seemed to be unaffected during the first days, although, a further decrease in counts to ≤ 2.00 log CFU/mL and ≤ 1.00 log CFU/mL occurred, a trend alike for *S.* Senftenberg 775/W, but at a slower rate.

4.3. Physicochemical properties

4.3.1. pH, total soluble solids and colour

The effects of treatments on physicochemical properties of EW are shown in **Table 4.1**. The pH is an essential attribute to achieve adequate EW quality properties, and the values ranged from 9.33 (raw EW) to 9.31 for the treated EW samples. Total soluble solids content was not significantly affected by any of the treatments, with measured values in the range 14.9 – 15.0 (**Table 4.1**).

In general, all treated samples showed lower L* (lightness), higher b* (yellowness) and similar a* (redness) when compared to raw EW (**Table 4.1**). The colour variation (ΔE^*) produced by the four treatments would be not detected by the naked eye ($\Delta E^*<3$) [7], with similar results being observed by other authors in LWE submitted to combined sequential treatments (pressure (300 MPa/5 min) or pulsed electric fields (25 kV/cm) followed by TP (52 – 55 ºC/2 - 3.5 min), with 2 % triethyl citrate (TC)) [3,8].

Table 4.1. Physicochemical properties of raw and treated egg white (mean ± standard deviation)). Different letters indicate significant differences ($p<0.05$) between processing conditions.

Properties	Raw	Thermal Pasteurization		Combined Treatments	
		55.6 ºC/6.2 min	55 ºC/3 min	50 MPa/5 min – 55 ºC/3 min	90 MPa/5 min – 55 ºC/3 min
pH	9.33 ± 0.01 ^{a}	9.32 ± 0.01 ^{b}	9.31 ± 0.01 ^{b}	9.32 ± 0.01 ^{ab}	9.32 ± 0.01 ^{b}
Total Soluble Solids (°Brix)	14.9 ± 0.08 ^{a}	15.0 ± 0.05 ^{a}	15.0 ± 0.05 ^{a}	15.0 ± 0.07 ^{a}	15.0 ± 0.09 ^{a}
L*	55.66 ± 1.00 ^{a}	54.72 ± 0.15 ^{b}	54.61 ± 0.04 ^{b}	55.86 ± 0.09 ^{a}	54.51 ± 0.16 ^{b}
a*	-0.42 ± 0.04 ^{ab}	-0.39 ± 0.06 ^{a}	-0.43 ± 0.01 ^{ab}	-0.38 ± 0.01 ^{a}	-0.44 ± 0.01 ^{b}
b*	2.37 ± 0.08 ^{bc}	2.46 ± 0.19 ^{ab}	2.56 ± 0.05 ^{a}	2.55 ± 0.03 ^{a}	2.32 ± 0.02 ^{c}
ΔE^*	-	0.99 ± 0.06 ^{b}	1.06 ±0.04 ^{ab}	0.28 ± 0.07 ^{c}	1.15 ± 0.16 ^{a}
Turbidity (%)	2.37 ± 0.29 ^{c}	4.39 ± 0.67 ^{a}	3.51 ± 0.96 ^{b}	3.33 ± 0.48 ^{b}	3.24 ± 0.59 ^{b}
Soluble Protein (g/100 g egg white)	2.96 ± 0.51 ^{a}	1.59 ± 0.09 ^{c}	2.57 ± 0.05 ^{b}	2.43 ± 0.17 ^{b}	2.42 ± 0.18 ^{b}
Surface Hydrophobicity (a.u.)	639 ± 59 ^{a}	643 ± 40 ^{a}	616 ± 23 ^{a}	601 ± 34 ^{a}	724 ± 24 ^{a}
Viscosity (mPa.s, at shear rate of 50 s^{-1})	31.8 ± 1.1 ^{c}	70.8 ± 7.9 ^{a}	40.1 ± 2.3 ^{b}	37.2 ± 1.0 ^{bc}	39.9 ± 1.4 ^{b}

4.3.2. Turbidity, soluble protein and surface hydrophobicity

Turbidity is an useful indicator of protein aggregation in EW [9], and increased from 2.4 ± 0.3 % for raw EW to 4.4 ± 0.7 % (about 85 %) for the EW treated by commercial TP ($p<0.05$) (**Table 4.1**). For the shorter TP, the turbidity increase was less pronounced, just about 48 %, when compared to the non-treated EW, being like the increment verified for MP-TP-treated EW (around 37 – 40 %, ($p<0.05$)). The increase in turbidity observed for all treated samples is probably related to increased protein aggregation (aggregates not visible to the naked eye, which is in agreement with ΔE results), although, as will be discussed below, there were no significant differences in protein denaturation state as measured by differential scanning calorimetry (DSC).

The soluble protein content of EW has been correlated with egg functionality and its reduction may indicate a lower functionality of egg proteins [3,10,11]. For all treatments, a significant reduction in soluble protein was observed ($p<0.05$), compared to non-treated EW (**Table 4.1**). The commercial TP caused a more significant loss of protein solubility (46 %, $p<0.05$), a value in agreement with Van Der Plancken et al. [12]. The heat probably led to the unfolding of EW proteins, exposing hydrophobic amino acids buried inside the protein, promoting aggregation by hydrophobic interactions, and thus reducing protein solubility [13]. Shorter TP and MP-TP led to 13 % and 18 % reduction ($p\geq0.05$), respectively, indicating a possible less pronounced unfolding and aggregation, which in agreement with turbidity results presented above (the soluble protein content of MP-TP-treated EW was 1.5-fold higher than commercial TP-treated samples).

Furthermore, surface hydrophobicity is an index of the number of hydrophobic groups on the surface of a protein molecule and is used to evaluate the change in protein conformation [14]. As can be seen in **Table 4.1**, surface hydrophobicity did not change significantly ($p\geq0.05$) regardless of the treatments applied, although an increment of about 13 % (85 a.u.) was observed for the MP-TP (90 MPa). Considering the effects of the different treatments on protein solubility and dispersion turbidity, we can suggest that any possible increase in surface hydrophobicity due to conformational changes, namely due to heat treatments, has been masked due to protein aggregation.

4.3.3. Apparent viscosity

As can be observed in **Table 4.1**, the apparent viscosity of raw EW (31.8 ± 1.1 mPa.s) was lower than the viscosity measured for treated samples. The more pronounced viscosity increment, up to 123 % (70.8 ± 7.9 mPa.s, $p<0.05$), was observed for the samples subjected to the commercial TP, probably due to aggregation of proteins, despite that the thermal conditions used were below those that cause denaturation (as can be confirmed in section 4.3.2) [10]. On the other hand, the viscosity increase observed for the shorter TP-treated samples were about 23 % ($p<0.05$), an increment similar to that observed for both MP-TP treatments (apparent viscosities about 17 – 25 % higher than raw EW), suggesting that the effect observed in MP-TP-treated EW was mainly caused by heat. The study of Monfort et al. [3] showed that the viscosity of LWE treated at 100 MPa/40 min did no change compared with a non-treated sample, thus supporting our interpretation that the effect of the combined MP-TP treatment is mainly due to the heat treatment and not to the MP treatment step. In addition, the MP-TP-treated samples revealed to be 1.8-fold less viscous than commercial TP-treated samples, in agreement with the turbidity and soluble protein results (section 4.3.2), suggesting that greater aggregation caused a greater increment in viscosity.

4.4. Thermal properties

Thermal properties of EW scanned in the temperature range from 5 to 110 °C at the rate of 10 °C/min, are summarized in **Table 4.2**. The two major endotherms of raw EW were found at temperatures of 68.5 ºC (conalbumin) and 82.3 ºC (ovalbumin) (see **Annex D – Figure D3**), which agrees with previous studies [15]. The denaturation temperature and enthalpy variation (*ΔH*) of the first peak ranged between 68.2 – 68.9 ºC and from 0.21 to 0.28 J/g, respectively, and were not significantly ($p\geq0.05$) modified by the treatments applied, likely indicating a similar denaturation process for the conalbumin and, therefore, no significant changes in structural integrity of this protein fraction caused by the studied treatments. For the ovalbumin peak, the denaturation temperature (81.9 – 82.5 ºC) also did not change significantly ($p\geq0.05$) with treatments. Otherwise, the *ΔH* of this second peak was significantly ($p<0.05$) higher in commercial TP-treated samples than in raw EW, while the other treatments did not show significant differences ($p\geq0.05$). Although the treatments temperature was much lower than the typical denaturation temperature observed for the

Table 4.2. Thermal properties of raw and treated egg white (mean ± standard deviation). Different letters indicate significant differences (p <0.05) between processing conditions.

Thermal Properties	**Raw**	**Thermal Pasteurization**		**Combined Treatments**	
		55.6 ºC/6.2 min	**55 ºC/3 min**	**50 MPa/5 min – 55 ºC/3 min**	**90 MPa/5 min – 55 ºC/3 min**
$T_{onset\ (peak\ 1)}$ (ºC)	64.8 ± 0.0	64.6 ± 0.1	64.5 ± 0.2	64.4 ± 0.1	64.9 ± 0.4
$T_{max\ (peak\ 1)}$ (ºC)	68.5 ± 0.6	68.2 ± 0.2	68.5 ± 0.4	68.7 ± 0.1	68.9 ± 0.2
ΔH_1 (J/g)	0.27 ± 0.03	0.24 ± 0.01	0.24 ± 0.02	0.28 ± 0.04	0.21 ± 0.13
$T_{onset\ (peak\ 2)}$ (ºC)	77.4 ± 0.00	76.3 ± 0.4	77.1 ± 0.2	77.2 ± 0.1	76.5 ± 0.7
$T_{max\ (peak\ 2)}$ (ºC)	82.3 ± 0.4	81.9 ± 0.2	82.4 ± 0.2	82.3 ± 0.1	82.5 ± 0.4
ΔH_2 (J/g) [(1)]	1.22 ± 0.06	1.63 ± 0.10	1.49 ± 0.11	1.26 ± 0.05	1.43 ± 0.08

[(1)]Only ΔH_2 showed significant differences between treatments and different letters (with: *b*, *a*, *ab*, *b* and *ab*, respectively for raw, 55.6 ºC/6.2 min, 55.6 ºC/6.2 min, 50 MPa/5 min – 55 ºC/3 min and 90 MPa/5 min – 55 ºC/3 min).

ovalbumin, an increasing trend was observed for treated EW compared to raw samples. According to Chandrapala et al. [16], the treatments could disrupt some intramolecular forces, namely that at higher temperature, thus allowing later the occurrence of protein aggregation in some extension, causing slightly higher ΔH.

4.5.Functional properties

4.5.1. Foaming properties

Foaming properties of EW are related to the ability of proteins that make up EW to rapidly adsorb on the air-liquid interface during whipping or bubbling, with the formation of a cohesive viscoelastic film through intermolecular interactions [17]. The influence of the studied treatments on foaming properties of EW are summarized in **Table 4.3**. In relation to raw EW, the application of both TP significantly reduced EW foaming capacity (FC, 65 – 74 %, $p<0.05$), otherwise, the foaming stability (FS) was improved. Moreover, MP followed by TP also reduced FC, but less (43 %, $p<0.05$), while no significant changes were observed for FS. These results show that the combination of MP and TP results in a less pronounced effect on FC than when only the shorter TP was applied, suggesting that MP pre-treatment counteracts the TP effects. The negative effect on FC, which coincides with a turbidity increase and a soluble protein decrease, could probably be due to the formation of more stable aggregates than their corresponding native protein. These aggregates are likely to have a greater difficulty in diffusing and adsorbing at the air-liquid interface and as a result, the FC was reduced [18]. Instead, the FS improvement may be due to the presence of aggregates that did not adsorb at the interface, and remained in liquid film, which could slow down the drainage and limit coalescence [19]. Comparatively to commercial TP, the results show that MP-TP-treated EW presented a FC up to 2.6-fold higher ($p<0.05$), while the FS of the first was 1.3-fold higher ($p<0.05$). In addition, although there were no significant structural changes, observable at a macroscopic/thermal level, according to the results obtained regarding DSC measurements and surface hydrophobicity, we hypothesize that some molecular changes may occur leading to aggregation and changes on foaming properties.

Table 4.3. Foaming properties of EW and gelling properties of heat-induced gels produced from raw and treated egg white (mean ± standard deviation). Different letters indicate significant differences ($p<0.05$) between processing conditions.

Properties	**Raw**	**Thermal Pasteurization**		**Combined Treatments**	
		55.6 ºC/6.2 min	**55 ºC/3 min**	**50 MPa/5 min – 55 ºC/3 min**	**90 MPa/5 min – 55 ºC/3 min**
Foaming Capacity (%)	69.6 ± 9.0 ^{a}	18.3 ± 3.1 ^{c}	24.6 ± 4.8 ^{c}	48.3 ± 6.91 ^{b}	40.0 ± 6.4 ^{b}
Foaming Stability (%)	66.1 ± 5.0 ^{b}	90.7 ± 8.6 ^{a}	86.9 ± 10.3 ^{a}	71.0 ± 4.2 ^{b}	69.0 ± 3.7 ^{b}
Stress at Rupture (kPa)	68.8 ± 3.4 ^{d}	73.8 ± 5.3 ^{cd}	77.2 ± 2.0 ^{bc}	89.5 ± 5.1 ^{a}	80.1 ± 4.4 ^{b}
Strain at Rupture (%)	59.0 ± 1.5 ^{b}	58.3 ± 1.0 ^{b}	60.4 ± 0.8 ^{ab}	62.4 ± 1.6 ^{a}	60.4 ± 1.4 ^{ab}
Young Modulus (kPa)	29.5 ± 1.0 ^{b}	30.8 ± 1.2 ^{b}	29.4 ± 1.0 ^{b}	36.1 ± 1.7 ^{a}	35.9 ± 2.6 ^{a}
Water Holding Capacity (%)	98.2 ± 1.0 ^{a}	97.6 ± 0.7 ^{a}	97.9 ± 1.0 ^{a}	98.5 ± 0.9 ^{a}	98.6 ± 0.9 ^{a}

4.5.2. Gelling properties

Mechanical properties (stress and strain at rupture, and Young`s modulus) of thermal-induced EW gels were evaluated (**Table 4.3**) by uniaxial compression tests. Typical stress-strain curves were characterized by an increase in stress and strain up to the breaking point of gel, at which there was a sudden drop in stress, followed by a further increase until the end of the test (examples of these curves are shown in **Annex D – Figure D4**). Young`s modulus was defined as the initial curve slope, within the linear elastic behaviour, and stress and strain at rupture were defined as the stress and strain measured for the breaking point. The gels from the MP-TP-treated EW samples showed a significant ($p<0.05$) higher stress at rupture, a measure of gels' hardness, compared to the other gels, mainly those gels obtained from MP-TP (50 MPa), which reflects the mechanical properties of the gels in the non-linear zone, under high deformations. In addition, also considering the difference in the surface hydrophobicity observed for the MP-TP treated samples, the explanation considered most likely is that the molecular changes that occurred, despite not being detected at a macroscopic/thermal level, were sufficient to allow the establishment of S-S bonds to a greater extent, leading to thermally induced gels with greater hardness. Regarding the commercial TP, non-significant differences ($p\geq0.05$) were found for stress at rupture compared with gels from raw EW. The strain at rupture (%) corresponds to the percentage of gel height that had to be compressed/deformed until it breaks. Non-significant differences ($p\geq0.05$) were observed among samples subjected to the different treatments regarding this parameter. Additionally, samples treated by MP-TP also showed a significant increase ($p<0.05$) in Young`s modulus (**Table 4.3**), a measure of the stiffness of a material, with increments of about 18 and 15 % compared to the gels produced from raw EW and commercial TP, respectively. No significant changes ($p\geq0.05$) were obtained for the TP treated samples compared to the raw EW.

The water holding capacity (WHC) is an important property to establish the strength of the water binding to a solid matrix [20]. As observed in **Table 4.3**, non-significant differences ($p\geq0.05$) were found between the WHC of raw and treated EW. Previous studies also showed that different combined treatments (pressure (300 MPa/5 min) or pulsed electric fields (25 kV/cm) followed by TP (52 – 55 ºC/2 - 3.5 min), with 2 % TC) had no significant effects on the WHC of LWE heat-induced gels [3,8].

4.6. Volatile profile

To avoid the formation of new volatiles induced by the temperature used in the extraction procedure, the volatiles were extracted at 40 ºC. The composition of EW samples regarding identified volatile compounds is shown in **Table 4.4**. A total of 16 volatiles were observed using headspace analysis of which 8 were identified, and they were grouped into 4 classes.

All treatments increased the volatiles against raw EW, more significantly with commercial TP (+ 129 %) than the combined MP-TP treatments under testing (+ 57 %). The hydrocarbons were the major components present in EW samples, with heptane being the main hydrocarbon found in raw EW, followed by hexane. Compared to raw EW, hexane content only reduced in the MP-TP samples (65 – 70 %, $p<0.05$), while for heptane content an opposite tendency was noticed in the MP-TP- and commercial TP-treated samples (22 – 167 %, $p<0.05$). Nonane was not detected in raw EW, but it was noticed after treatments. These hydrocarbons were also identified by other authors, suggesting decarboxylation of fatty acids from glycerides as a possible production pathway [21–23]. Furthermore, the sum of hydrocarbons detected in commercial TP- and MP-TP-treated samples was 138 % and 38 – 63 % higher, respectively, than in raw EW, showing that a greater increase in oxidative process occurred after the application of commercial TP. However, hydrocarbons are reported in literature to have negligible effect on aroma perception due to high odour thresholds [24].

The two esters identified were ethyl acetate and butyl acetate, accordingly to previously published data, being derived from esterification of free fatty acids and alcohols [21,23,25]. Ethyl acetate exhibited a decreasing trend, while butyl acetate showed an increase after treatments, with a more pronounced effect observed in TP-treated samples ($p<0.05$).

The aromatic compounds found in EW samples were toluene, followed by *o*-xylene (1,2-dimethylbenze), also previously identified by other authors [21,23], being typical volatile organic compounds released during food decaying processes. A significantly higher content of toluene was detected in commercial TP- and MP-TP (50 MPa)-treated EW (118 %, $p<0.05$) compared to raw EW, likely due to thermal degradation of glucose [22], while the *o*-xylene content decreased only in samples treated by MP-TP (50 MPa). Eucalyptol was the compound found in the lowest amount in raw EW samples, presenting a tendency to increase after treatments, mainly after commercial TP.

Table 4.4. Volatile profile of raw and treated egg white, expressed in µg internal standard equivalents/100 g egg white (mean ± standard deviation). Different letters indicate significant differences ($p < 0.05$) between processing conditions.

Volatile compounds	RT [1] (min)	KI [2]	Egg White (µg/100 g) [3,4]				
			Raw	55.6 ºC/6.2 min	55 ºC/3 min	50 MPa/ 5 min – 55 ºC/3 min	90 MPa/ 5 min – 55 ºC/3 min
Hexane	3.31	603	6.96 ± 1.62 [a]	5.47 ± 0.96 [a]	6.24 ± 0.29 [a]	2.06 ± 0.04 [b]	2.45 ± 0.12 [b]
Heptane	5.16	689	72.04 ± 6.54 [c]	166.28 ± 21.47 [a]	28.86 ± 4.13 [d]	105.34 ± 7.89 [b]	88.23 ± 0.95 [bc]
Nonane	13.18	900	n.d.	0.57 ± 0.05 [b]	0.41 ± 0.11 [b]	1.65 ± 0.20 [a]	0.39 ± 0.07 [b]
∑ Hydrocarbons			87.76 ± 5.76 [c]	208.72 ± 23.85 [a]	82.73 ± 5.78 [c]	120.92 ± 8.39 [b]	143.19 ± 1.82 [b]
Ethyl Acetate	3.59	616	1.28 ± 0.20 [a]	0.58 ± 0.11 [b]	0.51 ± 0.10 [b]	1.08 ± 0.25 [a]	0.94 ± 0.32 [a]
Butyl Acetate	9.65	820	1.85 ± 0.10 [b]	4.15 ± 0.96 [a]	2.59 ± 0.24 [b]	2.91 ± 0.49 [ab]	3.09 ± 0.35 [ab]
∑ Esters			3.13 ± 0.23 [b]	4.73 ± 0.85 [a]	3.10 ± 0.30 [b]	3.99 ± 0.39 [ab]	4.03 ± 0.23 [ab]
Toluene	7.60	766	9.24 ± 0.61 [b]	20.11 ± 3.43 [a]	7.55 ± 0.50 [b]	18.60 ± 1.44 [a]	11.41 ± 0.17 [b]
o-xylene	13.09	897	1.06 ± 0.11 [a]	0.82 ± 0.04 [ab]	0.88 ± 0.11 [a]	0.55 ± 0.03 [b]	0.81 ± 0.17 [ab]
∑ Aromatic			10.30 ± 0.72 [b]	20.94 ± 3.40 [a]	8.43 ± 0.58 [b]	19.16 ± 1.46 [a]	12.21 ± 0.16 [b]
Eucalyptol			0.78 ± 0.21 [c]	1.60 ± 0.18 [a]	1.10 ± 0.17 [bc]	0.86 ± 0.08 [bc]	1.24 ± 0.16 [ab]
∑ Total			103.29 ± 6.29 [c]	237.01 ± 26.71 [a]	96.70 ± 5.51 [c]	146.41 ± 9.03 [b]	162.47 ± 2.27 [b]

[1]RT – Retention time; [2] KI – Experiment value of Kovats Index; [3] Values are from semi-quantification using cyclohexanone as internal standard; [4] Detection limit is 0.14 µg/100 g egg white; n.d. – not detected.

Overall, a more significant effect on EW volatile profile was observed with commercial TP than shorter TP, as the samples were exposed to a longer TP. However, in the MP-TP samples, the MP pre-treatment seems to potentiate the thermal effect of the shorter TP, with the pre-treatment at 50 MPa causing a greater impact than at 90 MPa on heptane, nonane and aromatic compounds content. Otherwise, concerning hexane and ethyl acetate content, the results suggest that MP pre-treatment attenuated the effect caused by shorter TP.

Compared with non-treated EW, no compounds such as aldehydes, alcohols and ketones, mainly found in cooked eggs [26] and egg samples submitted to extractions at higher temperatures and longer times [23,25,27], were detected in treated EW, since the TP and MP-TP treatments applied were not strong enough to induce its production. According to published literature, these classes of compounds could be originated from lipid oxidation, Strecker and Maillard reactions, which are favored during the heating process [22], and in matrices such as egg yolk and whole egg [23,25] with a high content of fatty acids.

In order to simplify the interpretation of the correlation between treatments and their effects, principal component analysis (PCA) was carried out on the volatile compounds of EW samples. The first two principal components accounted for 49.41 % and 21.96 % of the total variance, respectively, as can be seen in **Figure 4.4**. The distribution of the scores on the first two PCs showed four separate groups of points corresponding to: (1) raw EW and shorter TP (2) MP-TP at 90 MPa; (3) MP-TP at 50 MPa; and (4) commercial TP. The group 1 was mainly influenced by nonane, toluene and aromatic compounds (**Table 4.5**). Otherwise, the MP-TP (50 MPa)-treated EW were characterized principally by the lowest content of hexane and 1,2-dimethylbenze, whereas the group of commercial TP-treated EW were clearly differentiated by the highest content of heptane, butyl acetate, esters, eucalyptol and hydrocarbons compounds. Therefore, the results clearly show that shorter TP is more related to raw EW than the other treatments, suggesting a change in the volatile profile of raw EW to a higher extent.

4.7.Sensory analysis

Sensory analysis of egg-derived product prepared with MP-TP-treated EW (90 MPa/5 min followed by 55 ºC/3 min) was studied, and compared with those of raw and commercial TP. The sensory evaluation scores of meringues are shown in **Figure 4.5**.

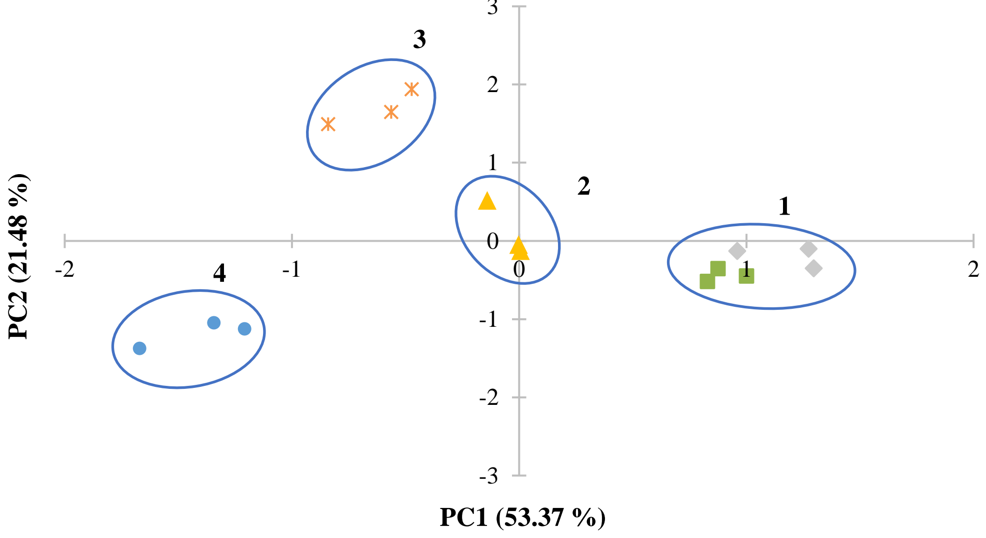


Figure 4.4. Principal component analysis (PCA) score plot of volatile compounds of raw and treated egg white. The principal components (PC) explain 74.85 % of the total variance of the data.

Table 4.5. Loadings of the variables in the first two principal component (PC) analysis of volatile compounds in egg white samples.

Compounds	Principal Components	
	PC 1	**PC 2**
Hexane	0.468	-0.669
Heptane	-0.866	-0.252
Nonane	-0.560	0.729
Ethyl acetate	0.270	0.533
Butyl acetate	-0.812	-0.302
Toluene	-0.907	0.168
o-xylene	0.576	-0.638
Eucalyptol	-0.627	-0.602
Esters	-0.842	-0.107
Aromatic compounds	-0.904	0.148
Hydrocarbons compounds	-0.885	-0.338

The attributes score ranged from 6.3 to 8.2, which is equivalent to "like slightly" and "like very much" respectively on 9-point hedonic scale. The meringues produced from non-treated EW presented the highest score for visual appearance, being significantly different from meringues produced with thermally treated EW ($p<0.05$). Despite this, for the other attributes evaluated, no significant differences ($p\geq 0.05$) were observed among samples. The meringues from non-treated EW have a round and pointed shape (**Annex D – Figure D**7) characteristic of this product, a morphological attribute that decreased in the meringues produced with thermally treated EW, supporting the visual appearance score reduction. Besides, during the meringues production, it was possible to see that the meringue batter produced from thermally treated EW was the most fluid (**Annex D – Figure D6**), with this having a possible contributing for meringues with a flatter and more cylindrical-like shape. Although, the three meringues produced showed similar global acceptability by the panellists, however, the most preferred meringue was the produced with non-treated EW.

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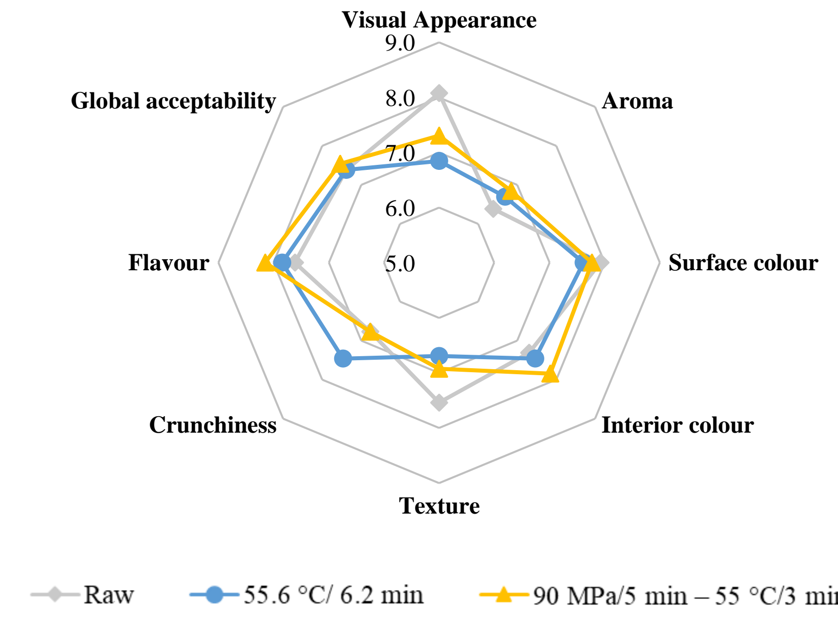

Figure 4.5. Sensory evaluation scores of meringues produced from raw and treated egg white, using a 9-point hedonic scale.

4.8. Conclusions

Sublethal MP followed by a shorter TP (90 MPa/5 min followed by 55 ºC/3 min), inactivated at least 4.4 $\log_{10}$ cycles of *S.* Senftenberg 775/W and *L. innocua* in EW. EW treated by MP-TP presented lower turbidity and viscosity, and a higher soluble protein content than commercial TP-treated EW, suggesting a higher protein aggregation in the latter. The aggregates formed were likely to be more stable than the corresponding native protein, leading to a decreasing in FC, while maintaining or improving FS. Despite, the heat-induced gels produced with MP-TP-treated EW were harder and stiffer than those produced with other EW samples. In addition, the volatile profile of EW was affected by all treatments, with MP-TP EW volatile profile being closer to that of raw EW (and so less affected) than commercial TP EW. Sensory analysis of EW prepared meringues revealed no differences in the product global acceptability independently of treatment (commercial TP and MP-TP (90 MPa)). Therefore, the results of the present work indicate that a shorter TP (55 ºC/3 min) than commercial TP (55.6 ºC/6.2 min), when applied in combination with a MP pre-treatment (90 MPa/5 min), allowed to achieve *S.* Senftenberg 775/W inactivation over at least 4.4 $\log_{10}$ cycles, and maintaining and/or improving the physicochemical and functional properties of commercial TP-treated EW.

This new approach avoids the severity of one technique alone, uses an environmentally friendly technology (high pressure technology, HP), and has a relatively easy industrial implementation as it only needs to add the pre-treatment technology upstream (no scale-up required). However, a possible limitation is associated with the large initial investment in HP equipment. On the other hand, further research is needed to clarify some important aspects related to chemical and structural changes affecting EW proteins, namely related to protein aggregation, and also about the effectiveness of combined treatments on the inactivation of other pathogenic microorganisms.

4.9. References

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CHAPTER V

RESULTS AND DISCUSSION | EGG YOLK

This chapter is adapted from the submitted manuscript: Ana C. Ribeiro, Susana Casal, José A. Lopes-da-Silva and Jorge A. Saraiva. *Improved egg yolk pasteurization using sublethal moderate pressure pre-treatments.* *Food Chemistry Advances*.

5.1.Overview

This chapter describes the possibility to use a moderate pressure (MP) pre-treatment before a shorter thermal pasteurization (TP) (compared to commercial TP) for liquid egg yolk (EY) pasteurization. The sequential combined treatment (MP-TP) was optimized (testing different pressure levels) taking into account the inactivation of *Salmonella* Senftenberg 775/W (inoculated studies). For the optimized combination, a post-TP refrigeration study was performed, evaluating the evolution of *S.* Senftenberg 775/W and *Listeria innocua* (inoculated pathogenic surrogate) after pasteurization, and comparing to the behaviour observed after commercial TP. Then, pH, colour, soluble protein content, viscosity, volatile profile, lipid oxidation, fatty acid profile, total carotenoids, emulsifying properties and sensory properties of an EY derived product ("Doce de Ovos") were also evaluated. These results were compared to those obtained for non-treated and commercial TP-treated samples.

5.2. Microbial analyses

5.2.1. Microbial inactivation by pressure, thermal pasteurization and pressure followed by thermal pasteurization

The European Regulation CEE 1441/2007 requires the absence of any *Salmonella* species in 25 mL of liquid egg after pasteurization [1]. In this study, the resistance of *S.* Senftenberg 775/W in liquid EY to moderate pressure (MP, 50 – 200 MPa/5 min) and high pressure (HP, 225 – 400 MPa/5 min) treatments was assessed. As observed in **Figure 5.1**, in general, increasing pressure led to an increasing trend in *S.* Senftenberg 775/W inactivation (the initial load *S.* Senftenberg 775/W was 7.06 ± 0.09 log colony forming unit (CFU)/mL). Pressure treatments, in range of 50 – 125 MPa/5 min, only reduced the initial population of *S.* Senftenberg 775/W up to 0.19 log_{10} cycles ($p \geq 0.05$), but rising pressure up to 300 MPa significantly increased inactivation (0.46 – 2.91 log_{10} cycles, $p < 0.05$). In addition, when the treatment was carried out at 400 MPa/5 min, the *S.* Senftenberg 775/W counts were reduced to below the quantification limit (≤ 2.00 log CFU/mL), inactivating at least 5.09 log_{10} cycles (**Figure 5.1**). The results are in accordance with what was observed by Ponce et al. [2], reducing by 1.96 – 5.39 log_{10} cycles of *S.* Enteritidis population using pressure treatments at 350 – 450 MPa/5 min.

The results obtained confirmed the expectable effect of pressure treatments upon *S.* Senftenberg 775/W inactivation. Following the main objective of this study, the combination of a pressure pre-treatment before a shorter TP (60 ºC/3 min) on the inactivation of *S.* Senftenberg 775/W was then tested, and the results are shown in **Figure 5.2**. The treatments at 50 – 200 MPa/5 min followed by shorter a TP resulted in a microbial reduction of 1.82 to 3.09 log_{10} cycles ($p < 0.05$), but a similar effectiveness ($p \geq 0.05$) was observed among treatments in range of 70 – 200 MPa/5 min. The combined treatments performed at pressures ≥ 225 MPa significantly enhanced *S.* Senftenberg 775/W inactivation ($p < 0.05$), being the microbial loads measured for the treatments in the range of 250 – 400 MPa/5 min even below the detection limit (≤ 1.00 log CFU/mL), with reductions of at least 6.09 log_{10} cycles. More promising results were obtained in our work than in the Monfort et al. study [3], who obtained up to 3.1 log_{10} cycles reductions of *Escherichia coli* K12 DH 5α (*S.* Enteritidis surrogate) applying pressure treatments (200 – 300 MPa/3 – 30 min) followed by TP (52 ºC/3.5 min and 55 ºC/2 min). The same authors obtained results equivalent to those of the present study only by adding triethyl citrate (TC).

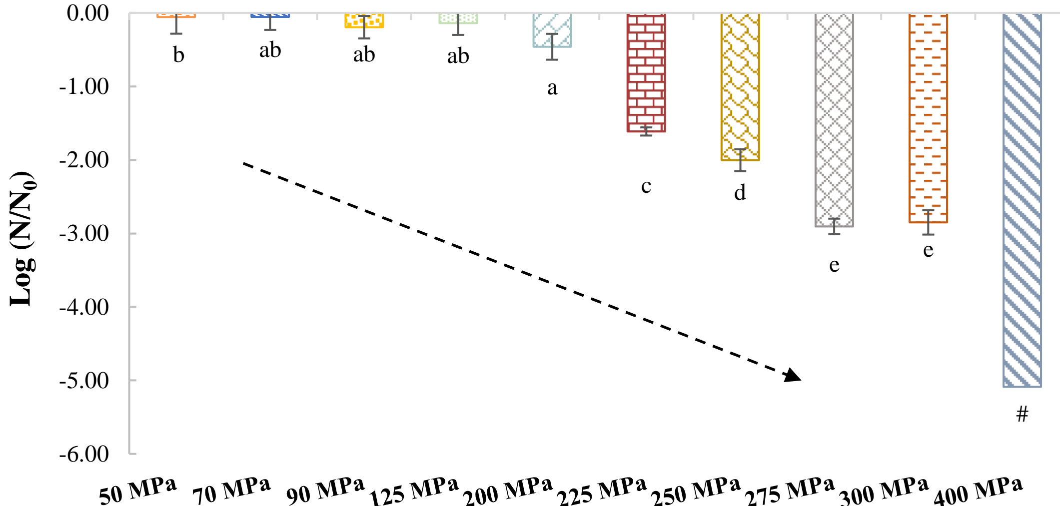


Figure 5.1. Log_{10} cycles reductions of *Salmonella* Senftenberg 775/W (ATCC 43845) in egg yolk treated by pressure treatments at 50 – 400 MPa during 5 min. N denotes the microbial load measured for each treatment and N_0 the initial microbial load (6.97 – 7.12 log CFU/mL, without any treatment). The symbol # means that microbial counts were below the quantification limit (≤ 2.00 log CFU/mL). Different letters indicate significant differences ($p < 0.05$) between treatments.

As observed in **Figure 5.2**, the lethal efficacy of the shorter TP against this *Salmonella* serovar was about 2 log_{10} cycles, while the commercial TP (60 ºC/6.2 min) achieved a reduction of about 3.25 ± 0.14 log_{10} cycles, which is in line with the 1 – 4 log_{10} cycles reduction of *S.* Senftenberg 775/W caused by commercial pasteurization [4,5]. Compared to commercial TP, the combined treatments showed similar (at pressures of 90 – 200 MPa, $p \geq 0.05$) to higher (at pressures ≥ 225 MPa, $p < 0.05$) inactivation of *S.* Senftenberg 775/W. In addition, a synergistic effect was found for combined treatments carried out at pressures ≥ 70 MPa, in which the combined effect was greater than the additive sum of each individual treatment. This suggest that pressure pre-treatments might have caused sublethal injuries in *S.* Senftenberg 775/W cells, reducing their thermal resistance, which led to the subsequent TP being more effective than when applied alone.

According to the results presented above, two combined treatments were selected for a further analysis: 90 MPa /5 min – 60 ºC/3 min (MP-TP), corresponding to processing condition that caused similar inactivation level to that caused by commercial TP, and 50 MPa/5 min – 60 ºC/3 min (MP-TP), in order to assess the effect of treatments on the

physicochemical and functional properties of EY. The latter case (50 MPa/5 min – 55 ºC/3 min) was studied to assess if it could result in lower quality parameters changes, although it resulted in lower microbial reduction, due to the intention of the authors to carry out further studies involving the sequential combination of two nonthermal pre-treatments (pressure and ultrasound), as an attempt to increase microbial inactivation (see Chapter VII). Additionally, post-TP refrigeration storage evolution of *S.* Senftenberg 775/W and *L. innocua* was studied only for the 90 MPa /5 min – 60 ºC/3 min treatment, since this was the case where microbial inactivation was equivalent to commercial TP.

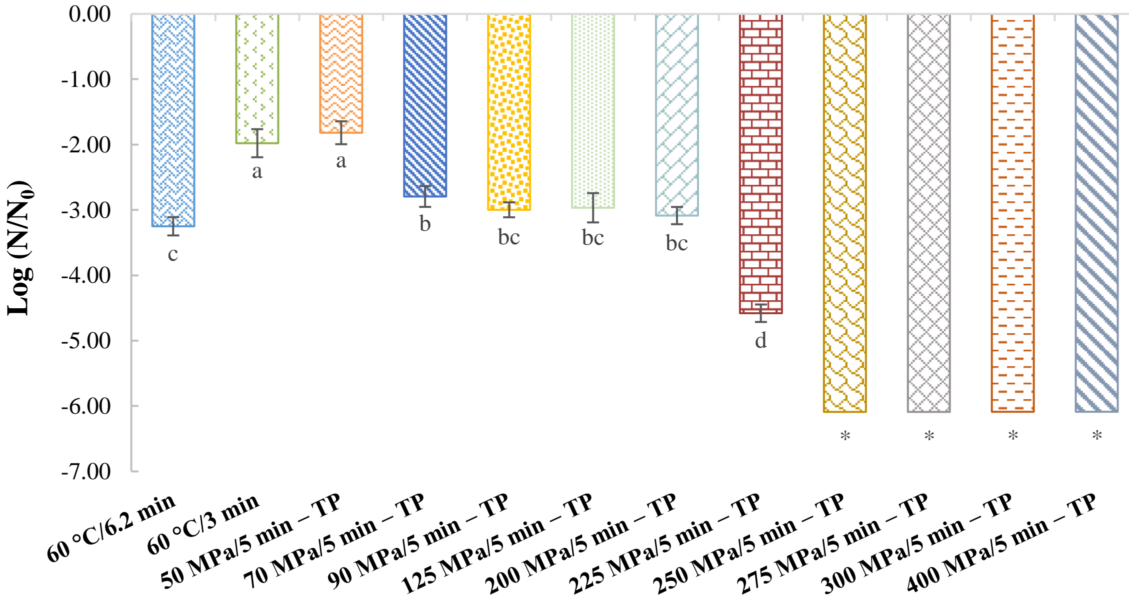


Figure 5.2. Log_{10} cycles reductions of *Salmonella* Senftenberg 775/W (ATCC 43845) in egg yolk treated by pressure pre-treatment (50 – 400 MPa/5 min) before a shorter thermal pasteurization (TP, at 60 ºC/3 min) or TP only (60 ºC/3 min and 60 ºC/6.2 min). N denotes the microbial load measured for each treatment and N_0 the initial microbial load (6.97 – 7.12 log CFU/mL, without any treatment). The symbol * means that microbial counts were below the detection limit (≤1.00 log CFU/mL). Different letters indicate significant differences ($p < 0.05$) between treatments.

5.2.2. Assessment of post-thermal pasteurization refrigeration storage

The effect of MP-TP (90 MPa/5 min followed by 60 ºC/3 min), shorter TP and commercial TP on *S.* Senftenberg 775/W and *L. innocua* (used as a surrogate for *L.*

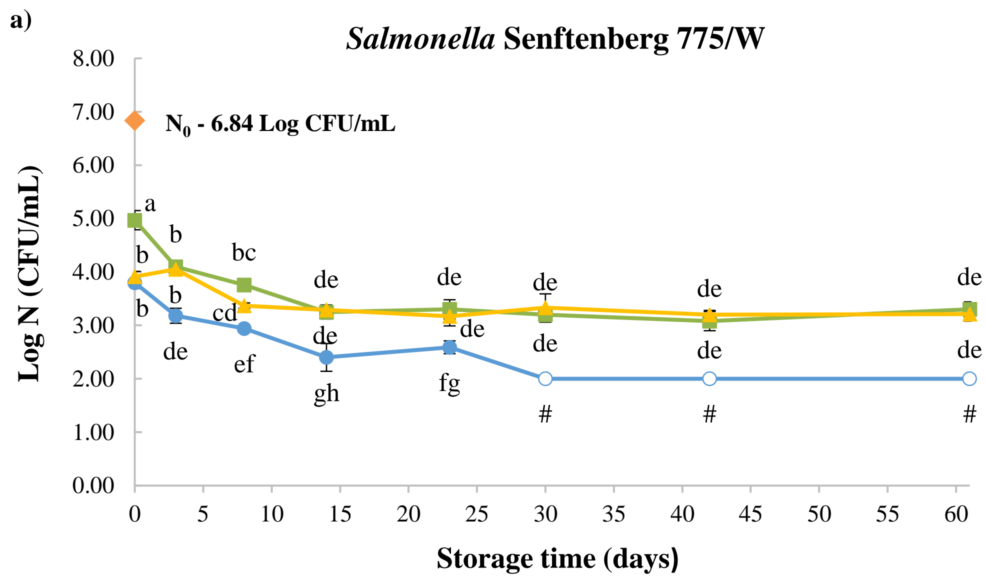
monocytogenes) was evaluated. **Figure 5.3** shows the evolution of these microorganisms counts during storage at 4 ºC. The EY was inoculated with 6.84 ± 0.06 log CFU/mL of *S.* Senftenberg 775/W, and a reduction of 1.87, 2.93 and 3.04 log_{10} cycles ($p<0.05$) was obtained right after each treatment (time 0) with the shorter TP, MP-TP and commercial TP, respectively. During the storage, for commercial TP-treated EY, *S.* Senftenberg 775/W counts diminished during the first 30 days ($p<0.05$), attaining counts below the quantification limit (≤ 2.00 log CFU/mL, day 30), that remained until the end of storage (**Figure 5.3a**). Otherwise, a reduction of 0.68 log_{10} cycles was observed for MP-TP-treated EY right after the first 8 days ($p<0.05$), with no further significant variation until the 61st day ($p\geq0.05$). Furthermore, for shorter TP-treated EY, the *S.* Senftenberg 775/W population decrease about 1.72 log_{10} cycles ($p<0.05$) during the first 14 days of storage, with the counts remaining between 3.25 and 3.30 log CFU/mL ($p\geq0.05$) until the end of storage. Though, from day 14 onwards, the MP-TP- and the shorter TP-treated EY showed similar *S.* Senftenberg 775/W counts ($p\geq0.05$), while the commercial TP-treated EY presented significantly lower counts along storage.

Concerning *L. innocua*, fresh EY was inoculated with an initial load of 7.18 ± 0.07 log CFU/mL, and the treatments reduced counts by at least 6.18 log_{10} cycles (counts below the detection limit, ≤ 1.00 log CFU/mL), with no changes during the 61 days of storage under refrigeration (**Figure 5.3b**). Contrary to what is reported in the literature [2,6], in this study, a greater resistance to inactivation was observed for *S.* Senftenberg 775/W, for both thermal and combined treatments, compared to *L. innocua*. This difference may be related to the *L. innocua* strain used, which may be less resistant, the age of the culture used for inoculation or inoculation together rather than an individual culture only [7].

5.3. Physicochemical properties

5.3.1. pH and colour

The pH of EY measured in the present study ranged from 6.19 (raw EY) to 6.23 for the treated EY samples (**Table 5.1**). The colour of raw EY colour was assessed and compared with the different treatments, showing a similar ($p\geq0.05$) L* (lightness), a* (redness), and b* (yellowness) values among them. Furthermore, the colour variation (ΔE^*) produced by the four treatments revealed no significant differences among treatments ($p\geq0.05$) (**Table 5.1**), being in the level of variation considered not detected by the naked eye ($\Delta E^*<3$) [8].



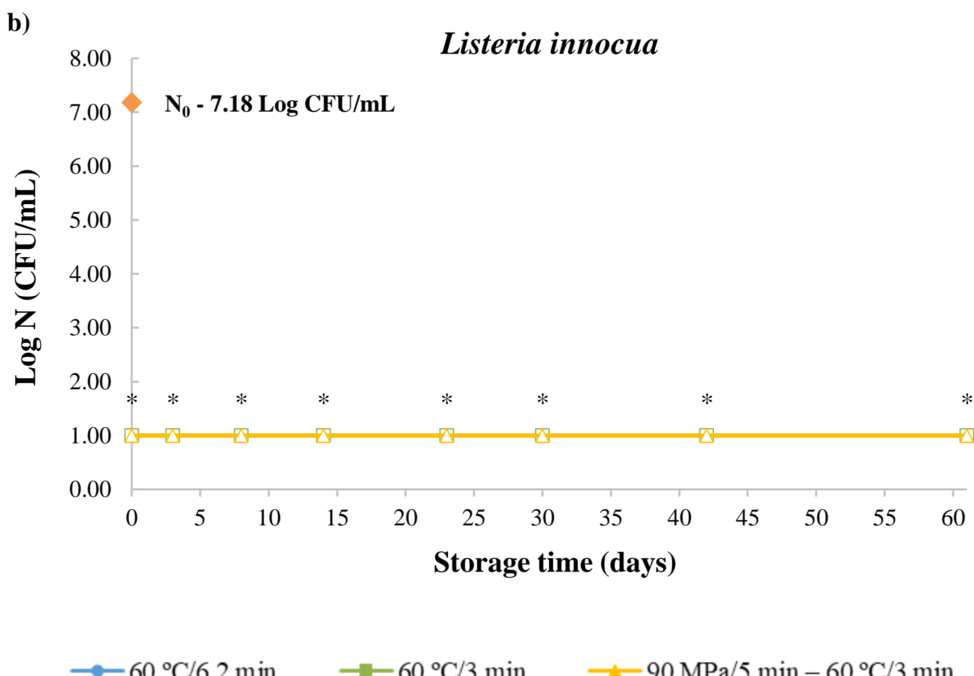


Figure 5.3. Evolution of a) *Salmonella* Senftenberg 775/W (ATCC 43845) and b) *Listeria innocua* (ATCC 33090) up to 61 days of storage after moderate pressure before a shorter thermal pasteurization (TP) (90 MPa/5 min – 60 ºC/3 min) or TP only (60 ºC/3 min and 60 ºC/6.2 min). Unfilled symbols on graphics and # or * mean that microbial counts were below the quantification limit (≤ 2.00 log CFU/mL) or detection limit (≤1.00 log CFU/mL), respectively, and N_0 corresponds to the initial load before each treatment. Different letters denote significant differences ($p<0.05$) between each processing condition and storage days.

Similar results were also reported for treated liquid whole egg (LWE) after a combined treatment of pulsed electric fields (25 kV/cm, 75 kJ/kg) followed by 60 ºC/1 min with 2 % TC [9].

5.3.2. Soluble protein

Concerning soluble protein, the content of non-treated and treated EY was also evaluated and as can be seen in **Table 5.1**, the raw EY contain 1.67 g of soluble protein/100 g of yolk. All treatments significantly ($p<0.05$) decreased the soluble protein content compared to raw EY, with a maximum decrement of 17 % for commercial TP-treated samples. Moreover, non-significant differences were found among treated samples, suggesting that the reduction observed in MP-TP-treated EY could be attributed to the thermal effects, although, a downward trend with increasing pressure pre-treatment was observed. This reduction may be associated with the exposure of hydrophobic groups buried within proteins, promoting aggregation by hydrophobic interactions, and consequently reducing soluble protein [10]. In fact, previous studies reported that a mild heat pasteurization (61.1 ºC/3.5 min) and a moderate pressure treatment (100 MPa/10 min) caused unfolding or aggregation of EY proteins, possibly affecting livetins and some low density lipoproteins (LDL) apoproteins [11,12]. Otherwise, a behaviour similar to that observed in MP-TP-treated samples was reported for LWE (300 MPa/5 min followed by 55 ºC/2 min with TC) [3].

5.3.3. Apparent viscosity

In this investigation, the raw EY exhibited an apparent viscosity of 0.60 ± 0.01 Pa.s, and, in general, all studied treatments led to a viscosity increment (**Table 5.1**). The shorter TP- and MP-TP-treated EY showed an apparent viscosity 51 – 57 % higher than non-treated sample, however, this increment was significantly lower than the caused by the commercial TP (66 %, $p<0.05$). Consequently, the results suggest that the TP was the main responsible for the viscosity increase in MP-TP-treated EY. Indeed, published studies indicated that the MP treatments (100 MPa/5 – 40 min) did not change the viscosity of egg products compared with non-treated samples [3,12], supporting this hypothesis. The thermal effects may be related to thermally unfolded livetins and some LDL apoproteins, that lead to protein aggregation and, consequently, to an increase in apparent viscosity [13], as it has been

previously proposed to explain the soluble protein reduction (**Table 5.1**). The more pronounced impact caused by commercial TP, indicates more marked protein aggregation under these conditions. Although the soluble protein data did not show a significant effect between TP-treated samples, a decreasing trend was observed with increasing pasteurization time. As for MP-TP-treated EY, an increase in LWE viscosity was also found after a TP (52 ºC/3.5 min) preceded by a pressure treatment (300 MPa/5 min) in the presence of TC [3].

5.4. Functional properties

The emulsifying properties of eggs are principally related to EY compounds, and the parameters used to characterize them were: emulsifying activity index (EAI) and emulsion stability index (ESI). In general, the commercial TP and MP-TP treatments improved EAI (35 – 52 %) and ESI (41 – 66 %) ($p<0.05$) compared to raw EY, although the shorter TP did not significantly change the ESI ($p\geq0.05$) (**Table 5.1**). The emulsifying properties enhancement caused by heat could be a consequence of LDL apoproteins unfolding, which may lead to an increase of surface hydrophobicity and molecular flexibility. This allows a faster and a more effective adsorption of LDL apoproteins at the oil/water interface [14]. As observed in **Table 5.1**, a higher emulsifying activity and emulsion stability were obtained for TP-treated EY with increasing pasteurization time, but also for MP-TP-treated EY compared to shorter TP. This is probably related to a more protein unfolding and higher adsorption of EY lipoproteins at interfaces, as emulsion stability is related to the interfacial area that can be coated by proteins [15]. The results are consistent with data from previous studies [12], who reported EY proteins unfolded at MP (100 MPa), indicating that pressure pre-treatment may influence this property, and therefore an intermediate behaviour between the two TP was observed for MP-TP-treated EY.

5.5. Lipid compounds analysis

5.5.1. Fatty acids profile

The results for the amount of fatty acids (FA) quantified from EY samples treated under different conditions is given in **Table 5.2**. A total of 21 FA was identified, revealing a moderated proportion of polyunsaturated FA (PUFAs, 22.12 – 22.45 %), and a high prevalence of monounsaturated FA (MUFAs, 44.32 – 45.20 %) followed by saturated FA

Table 5.1. Physicochemical and functional properties, lipid oxidation and total carotenoids content of raw and treated egg yolk (mean ± standard deviation). Different letters along each row denote significant differences ($p<0.05$) between processing conditions.

Properties	Raw	Thermal Pasteurization		Combined Processes	
		60 ºC/6.2 min	60 ºC/3 min	50 MPa/5 min – 60 ºC/3 min	90 MPa/5 min – 60 ºC/3 min
pH	6.19 ± 0.01 [d]	6.23 ± 0.01 [a]	6.19 ± 0.01 [d]	6.21 ± 0.01 [c]	6.22 ± 0.01 [b]
L*	49.57 ± 0.51 [a]	49.63 ± 0.28 [a]	49.76 ± 0.16 [a]	49.27 ± 0.40 [a]	49.67 ± 0.09 [a]
a*	9.96 ± 0.66 [a]	9.76 ± 0.31 [a]	9.84 ± 0.09 [a]	9.42 ± 0.51 [a]	9.86 ± 0.19 [a]
b*	10.73 ± 0.78 [a]	10.89 ± 0.47 [a]	11.01 ± 0.16 [a]	10.16 ± 0.75 [a]	10.92 ± 0.13 [a]
ΔE^*	-	1.38 ± 0.10 [a]	1.40 ± 0.08 [a]	1.28 ± 0.05 [a]	1.31 ± 0.21 [a]
Soluble Protein (g/100 g egg yolk)	1.67 ± 0.08 [a]	1.38 ± 0.02 [b]	1.48 ± 0.15 [b]	1.45 ± 0.09 [b]	1.41 ± 0.10 [b]
Viscosity (Pa.s, at shear rate of 50 s^{-1})	0.60 ± 0.01 [c]	0.99 ± 0.03 [a]	0.93 ± 0.04 [b]	0.92 ± 0.01 [b]	0.90 ± 0.02 [b]
Emulsifying Activity Index (m^2/g)	57.97 ± 3.12 [c]	87.94 ± 6.53 [a]	73.21 ± 3.07 [b]	81.08 ± 7.05 [ab]	78.26 ± 7.81 [ab]
Emulsifying Stability Index (min)	0.50 ± 0.02 [c]	0.82 ± 0.07 [a]	0.53 ± 0.03 [c]	0.70 ± 0.05 [b]	0.71 ± 0.06 [b]
Lipid Oxidation (TBARS, µg/100 g egg yolk)	40.68 ± 3.24 [c]	60.78 ± 6.72 [a]	49.93 ± 3.12 [b]	48.85 ± 5.39 [b]	49.52 ± 2.40 [b]
Total Carotenoids (mg/100 g egg yolk)	1.88 ± 0.03 [a]	1.51 ± 0.21 [b]	1.87 ± 0.08 [a]	1.79 ± 0.17 [ab]	1.73 ± 0.21 [ab]

(SFAs, 31.86 – 32.47 %). SFAs detected in EY were mainly palmitic (C16:0, 23.03 – 23.39 %) followed by stearic (C18:0, 8.23 – 8.55 %) acids, being in agreement with other published studies [16]. Regarding to MUFAs, oleic acid (C18:1ω-9, 38.31 – 39.09 %) was the most abundant FA, followed by palmitoleic (C16:1ω-7) and vaccenic (C18:1ω-7) acids. Relatively to PUFAs, the majority were ω-6 FAs (20.23 – 20.45 %), mainly linoleic (C18:2ω-6) and arachidonic (C20:4ω-6) acids, while only three ω-3 PUFA acids were detected in the EY samples: α-linoleic (ALA, C18:3ω-3), docosapentaenoic (DPA, C22:5ω-3) and docosahexaenoic (DHA, C22:6ω-3) acids, consistent with the results provide in literature [16]. In general, the FA profile of treated EY did not change ($p \geq 0.05$) compared to raw EY (**Table 5.2**). These observations are consistent with previous investigations for pasteurized milk (72 ºC/30 sec) and pressure treated turbot fillets (≤ 200 MPa/15 – 30 min), pointing out that even stronger treatments had no significant impact on FAs content [17,18]. In line with these data, the combination of pressure and TP, using milder conditions (50 and 90 MPa/5 min and 60 ºC/3 min), does not affect the FA content. A similar behaviour was also observed for the ω-6/ω-3, MUFA/SFA and PUFA/SFA ratios ($p \geq 0.05$) (**Table 5.2**). Worth to mention that the ω-6/ω-3 and PUFA/SFA ratios were higher than those recommended [16]. Thus, no consistent pattern relating treatments and FA composition arise from results, and no particular detrimental effect were caused by the treatments studied, maintaining the nutritional value of treated EY.

5.5.2. Secondary lipid oxidation

Lipid oxidation is one of the main causes of food deterioration, and thiobarbituric acid-reactive substances (TBARS), an index of secondary lipid oxidation (expressed as malondialdehyde (MDA) content), was determined (**Table 5.1**). The MDA content of raw EY was 40.68 ± 3.24 µg/100 g, comparatively lower than values reported in the literature [8]. Analysing the results, it is clear that occurred an increase (20 – 49 %) of MDA content in treated EY compared to raw samples ($p < 0.05$), clearly higher for commercial TP, possibly causing a negative impact on the organoleptic characteristics of the final product. The commercial TP-treated EY exhibited an MDA content about 1.4- and 1.2-fold higher compared to raw and MP-TP-treated samples, respectively. Moreover, the increase caused by the shorter TP was lower than that of commercial TP ($p < 0.05$), but similar to that of MP-TP. This suggests that the effect on the latter was probably related with the thermal effects,

Table 5.2. Fatty acids profile (% of total fatty acids) of raw and treated egg yolk [1,2]. Different letters along each row denote significant differences ($p<0.05$) between processing conditions.

Fatty Acids	Raw	Thermal Pasteurization		Combined Processes	
		60 ºC/6.2 min	60 ºC/3 min	50 MPa/5 min – 60 ºC/3 min	90 MPa/5 min – 60 ºC/3 min
C14:0	0.31 ± 0.01 [a]	0.31 ± 0.01 [a]	0.30 ± 0.01 [a]	0.30 ± 0.01 [a]	0.31 ± 0.01 [a]
C16:0	23.26 ± 0.34 [a]	23.03 ± 0.25 [a]	23.22 ± 0.21 [a]	23.30 ± 0.24 [a]	23.39 ± 0.19 [a]
C17:0	0.17± 0.00 [ab]	0.17± 0.00 [b]	0.17 ± 0.00 [ab]	0.17 ± 0.00 [a]	0.17 ± 0.00 [a]
C18:0	8.35 ± 0.13 [b]	8.23 ± 0.10 [b]	8.29 ± 0.09 [b]	8.55 ± 0.17 [a]	8.35 ± 0.06 [b]
∑ SFA	32.20 ± 0.43 [ab]	31.86 ± 0.18 [b]	32.11 ± 0.18 [ab]	32.47 ± 0.34 [a]	32.36 ± 0.20 [a]
C16:1ω9	0.81 ± 0.01 [ab]	0.80 ± 0.01 [ab]	0.81 ± 0.01 [ab]	0.79 ± 0.01 [b]	0.81 ± 0.01 [a]
C16:1ω7	2.62 ± 0.04 [ab]	2.61 ± 0.05 [ab]	2.62 ± 0.03 [ab]	2.56 ± 0.05 [b]	2.63 ± 0.02 [a]
C17:1	0.10 ± 0.00 [a]	0.11 ± 0.00 [a]	0.09 ± 0.03 [a]	0.10 ± 0.00 [a]	0.10 ± 0.00 [a]
C18:1ω9	38.79 ± 0.42 [ab]	39.09 ± 0.12 [a]	38.64 ± 0.15 [ab]	38.31 ± 0.42 [b]	38.63 ± 0.16 [ab]
C18:1ω7	1.89 ± 0.02 [ab]	1.90 ± 0.02 [a]	1.89 ± 0.02 [a]	1.86 ± 0.02 [b]	1.88 ± 0.01 [ab]
C20:1	0.29 ± 0.01 [a]	0.29 ± 0.02 [a]	0.28 ± 0.01 [a]	0.28 ± 0.01 [a]	0.29 ± 0.01 [a]
∑ MUFA	44.90 ± 0.45 [a]	45.20 ± 0.12 [a]	44.73 ± 0.21 [ab]	44.32 ± 0.51 [b]	44.76 ± 0.16 [ab]
C18:3ω3	0.56 ± 0.01 [ab]	0.57 ± 0.00 [a]	0.58 ± 0.01 [a]	0.55 ± 0.01 [b]	0.57 ± 0.01 [ab]
C22:5ω3	0.19 ± 0.01 [bc]	0.18 ± 0.01 [c]	0.23 ± 0.03 [a]	0.22 ± 0.01 [ab]	0.20 ± 0.01 [bc]
C22:6ω3	1.03 ± 0.03 [a]	1.01 ± 0.06 [a]	1.03 ± 0.04 [a]	1.09 ± 0.06 [a]	1.01 ± 0.04 [a]
∑ ω3	1.80 ± 0.03 [a]	1.79 ± 0.07 [a]	1.86 ± 0.06 [a]	1.88 ± 0.06 [a]	1.79 ± 0.04 [a]
C18:2ω6	17.38 ± 0.06 [c]	17.51 ± 0.08 [ab]	17.60 ± 0.03 [a]	17.43 ± 0.05 [bc]	17.39 ± 0.07 [c]
C18:3ω6	0.12 ± 0.01 [a]	0.13 ± 0.01 [a]	0.13 ± 0.00 [a]	0.13 ± 0.00 [a]	0.13 ± 0.00 [a]
C20:2ω6	0.18 ± 0.01 [a]	0.18 ± 0.01 [a]	0.18 ± 0.00 [a]	0.18 ± 0.00 [a]	0.17 ± 0.00 [a]
C20:3ω6	0.16 ± 0.00 [a]	0.16 ± 0.01 [a]	0.16 ± 0.00 [a]	0.17 ± 0.00 [a]	0.16 ± 0.00 [a]
C20:4ω6	2.22 ± 0.09 [ab]	2.11 ± 0.08 [b]	2.20 ± 0.06 [ab]	2.33 ± 0.11 [a]	2.21 ± 0.05 [ab]
C22:4ω6	0.18 ± 0.01 [ab]	0.18 ± 0.01 [ab]	0.17 ± 0.01 [ab]	0.19 ± 0.01 [a]	0.17 ± 0.01 [b]
∑ ω6	20.24 ± 0.08 [b]	20.32 ± 0.12 [ab]	20.45 ± 0.10 [a]	20.42 ± 0.16 [ab]	20.23 ± 0.10 [b]
∑ PUFA	22.14± 0.12 [ab]	22.21 ± 0.18 [ab]	22.41 ± 0.15 [a]	22.41 ± 0.21 [a]	22.12 ± 0.14 [b]
Ratio ω6/ ω3	11.24 ± 0.16 [ab]	11.38 ± 0.38 [a]	11.00 ± 0.32 [ab]	10.89 ± 0.25 [b]	11.30 ± 0.23 [ab]
Ratio MUFA/SFA	1.39± 0.03 [ab]	1.42 ± 0.01 [a]	1.39 ± 0.01 [ab]	1.37 ± 0.03 [b]	1.38 ± 0.01 [ab]
Ratio PUFA/SFA	0.69 ± 0.01 [ab]	0.70 ± 0.01 [ab]	0.70 ± 0.01 [a]	0.69 ± 0.01 [ab]	0.68 ± 0.01 [b]
∑ *Trans* FA	0.26 ± 0.02 [a]	0.26 ± 0.02 [a]	0.27 ± 0.03 [a]	0.29± 0.03 [a]	0.27 ± 0.01 [a]

[1]Data are means of six replicates (each treatment) (mean ± standard deviation); [2]SFA: Saturated fatty acid; MUFA: Monounsaturated fatty acid; PUFA: Polyunsaturated fatty acid; FA: Fatty acid.

which occur through the formation of free radicals and the consequent production of secondary oxidation products [19]. These results are consistent with data from previous studies [20,21], who showed no significant effect of MP (up to 200 MPa) on the TBARS values of other food products, whereas an increase was found in poultry muscles heated at 50 and 60 ºC (30 min). Furthermore, similarly to what was noted for MP-TP treatments, higher lipid oxidation was found in pressure treated poultry (200 MPa/50 and 60 ºC/20 min) than in non-treated samples [21]. Thus, although a higher level of secondary lipid oxidation was obtained in treated EY, this did not lead to significant changes in FA content, as mentioned above (section 5.5.1).

5.5.3. Total carotenoids

The carotenoids content of EY from different sources changes considerably, with the raw EY presenting a content of 1.88 ± 0.03 mg/100 g yolk (**Table 5.1**). For all treatments, no significant differences ($p<0.05$) in total carotenoids content were observed compared to non-treated EY, with exception of commercial TP. In the latter occurred a loss of about 20 % ($p<0.05$), likely due to a greater increase in free radical formation during this longer TP [19], consistent with what was noted above for TBARS.

5.6. Volatile compounds analysis

The volatile profile in the headspace over EY samples using solid phase microextraction-gas chromatography-mass spectrometry (SPME-GC/MS) analysis is listed in **Table 5.3**. To avoid additional thermal effects, the volatiles were extracted at 45 ºC, at a lower temperature than that used in EY TP (60 ºC). A total of 12 volatiles were quantitated, of which 5 were identified.

In general, the treatments increased the volatiles content of raw EY, with MP-TP treatment causing an increment of 7.8-fold ($p<0.05$) compared to other samples ($p<0.05$). The hexane and heptane were the main hydrocarbons present in EY samples, compounds that were also identified in egg products by other authors, and will likely originate from the lipid oxidation (free radicals) [22,23]. The sum of hydrocarbons followed the same trend as heptane, with a content 5.4- and 7.8-fold higher detected in MP-TP-treated EY than in TP-treated and raw EY, respectively ($p<0.05$) (**Table 5.3**). Thus, the results suggests that MP

Table 5.3. Volatile profile of raw and treated egg yolk (expressed in µg internal standard equivalents/100 g egg yolk) (mean ± standard deviation). Different letters indicate significant differences ($p<0.05$) between processing conditions.

Volatile compounds	RT [1] (min)	KI [2]	Egg Yolk (µg/100 g) [3,4]				
			Raw	**60 ºC/6.2 min**	**60 ºC/3 min**	**50 MPa/5 min – 60 ºC/3 min**	**90 MPa/5 min – 60 ºC/3 min**
Hexane	3.35	605	5.67 ± 0.12 ^{b}	5.07 ± 1.28 ^{b}	6.04 ± 0.25 ^{ab}	6.97 ± 0.16 ^{ab}	7.88 ± 0.94 ^{a}
Heptane	5.15	686	0.45 ± 0.07 ^{b}	4.25 ± 0.03 ^{b}	3.94 ± 0.10 ^{b}	70.32 ± 10.17 ^{a}	59.24 ± 15.92 ^{a}
∑ Hydrocarbons			11.08 ± 1.67 ^{b}	15.90 ± 1.03 ^{b}	16.96 ± 0.25 ^{b}	85.96 ± 10.53 ^{a}	73.29 ± 15.93 ^{a}
Toluene	7.64	766	0.89 ± 0.26 ^{c}	1.52 ± 0.03 ^{c}	2.82 ± 0.05 ^{c}	6.32 ± 1.38 ^{b}	13.58 ± 1.67 ^{a}
o-xylene	13.16	899	0.29 ± 0.14 ^{b}	0.46 ± 0.06 ^{b}	0.48 ± 0.29 ^{b}	0.64 ± 0.10 ^{ab}	1.17 ± 0.40 ^{a}
∑ Aromatic			1.18 ± 0.25 ^{c}	1.98 ± 0.07 ^{c}	3.30 ± 0.34 ^{c}	6.95 ± 1.43 ^{b}	14.75 ± 1.27 ^{a}
Ethyl Acetate	3.64	618	0.82 ± 0.00 ^{b}	7.72 ± 1.41 ^{a}	2.19 ± 0.59 ^{b}	10.94 ± 2.53 ^{a}	7.60 ± 1.93 ^{a}
∑ Total			13.36 ± 1.72 ^{b}	25.84 ± 2.10 ^{b}	22.76 ± 0.85 ^{b}	104.26 ± 14.24 ^{a}	96.00 ± 15.85 ^{a}

[1]RT –Retention time; [2] KI – Experiment value of Kovats Index; [3] Values are from semi-quantification using cyclohexanone as internal standard; [4] Detection limit is 0.09 µg/100 g egg yolk.

pre-treatment appears to have initiated lipid oxidation, and subsequently intensified by TP. One of the suggested mechanisms is that oxygen is more soluble under pressure, increasing the formation of hydroperoxides (lipid oxidation), and then more hydrocarbons [24] (and not compounds quantified by the TBARS method, see section 5.5.2). However, the hydrocarbons probably have negligible effect on aroma perception due to high odour thresholds [25].

Toluene and 1,2-dimethylbenzene (*o*-xylene) were the two aromatic compounds identified, what is in agreement with other authors [22,26], being typically released during food decaying processes. They showed an increasing trend with the treatments, probably due to the thermal degradation of glucose [23], but only in MP-TP-treated EY was found a significant higher content (up to 12.5-fold higher, $p<0.05$). Furthermore, as previously reported [16,22,26], the ethyl acetate was the only ester in headspace volatiles (**Table 5.3**). This compound could be formed by the esterification of free fatty acids [22], presenting a content up to 13-fold higher in commercial TP- and MP-TP-treated EY than in raw EY ($p<0.05$).

Otherwise, compounds such as aldehydes, alcohols and ketones were not detected in treated EY, but are often found in cooked eggs, and egg samples subjected to high temperatures and long times of extraction [16,22]. Despite the EY being a matrix with a high FA content, the treatments studied were not strong enough to cause a large lipid oxidation, and consequently, to produce these compounds [23].

Thereby, the results clearly show that the EY volatile profile was significantly more affected by the MP-TP treatments than for TP (**Figure 5.4**), characterized by the highest content of hydrocarbons compounds and toluene, proposing that MP pre-treatment seems to potentiate the thermal effect of the shorter TP.

5.7.Sensory analysis

Sensory analysis of egg-derived product prepared with MP-TP-treated EY (90 MPa/5 min followed by 60 ºC/3 min) was evaluated, and compared with those of raw and commercial TP. The sensory evaluation scores of “Doce de Ovos” produced from EY previously treated with different conditions are shown in **Figure 5.5a** (see **Annex E –Figure E4**). The attributes score ranged from 6.3 to 8.2, which is equivalent to “like slightly” and

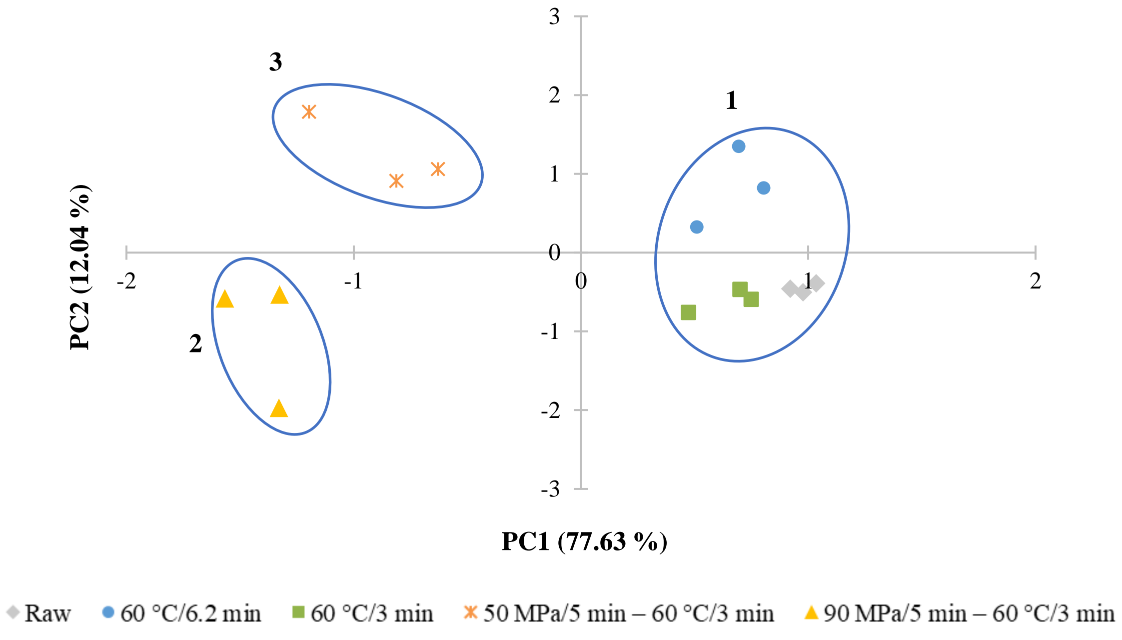


Figure 5.4. Principal component analysis (PCA) score plot of volatile compounds of raw and treated egg yolk. The principal components (PC) explain 89.67 % of the total variance of the data.

"like very much" respectively on 9-point hedonic scale. The scores given for all sensory characteristics, except aroma, of "Doce de Ovos" produced from non-treated EY (6.4 – 7.1) were similar to the thermally treated EY (6.3 – 7.5, $p \geq 0.05$), but significantly lower than those of the MP-TP-treated EY (scores above 7.7, $p < 0.05$). Besides, the "Doce de Ovos" produced with MP-TP-treated EY presented a significantly higher score for consistency, flavour and global acceptability (7.7 – 8.2, $p < 0.05$) than TP-treated EY (6.3 – 7.5). Despite the differences observed in the volatile profile of EY, these did not change the aroma of "Doce de Ovos", since hydrocarbons compounds (higher content in MP-TP-treated EY), due to high odour thresholds, probably had little effect on aroma perception [25], however, seem to have resulted in a high score for flavour. Otherwise, the specific sensory attributes examined (part II – **Figure 5.5b**) were not significantly different ($p \geq 0.05$) among samples, except the colour intensity. This attribute exhibited a significantly higher score ($p < 0.05$) in "Doce de Ovos" prepared from MP-TP-treated EY than the other samples, showing a more orange and luminous colour. This is probably due to greater carotenoids stability after the MP-TP treatment (as seen in the carotenoid section) and therefore, a final product with a more appealing colour was obtained, although, this colour difference was not noticeable in

EY samples after treatments (see colour section). Furthermore, the results clearly show that the preferred sample was the "Doce de ovos" prepared with MP-TP-treated EY.

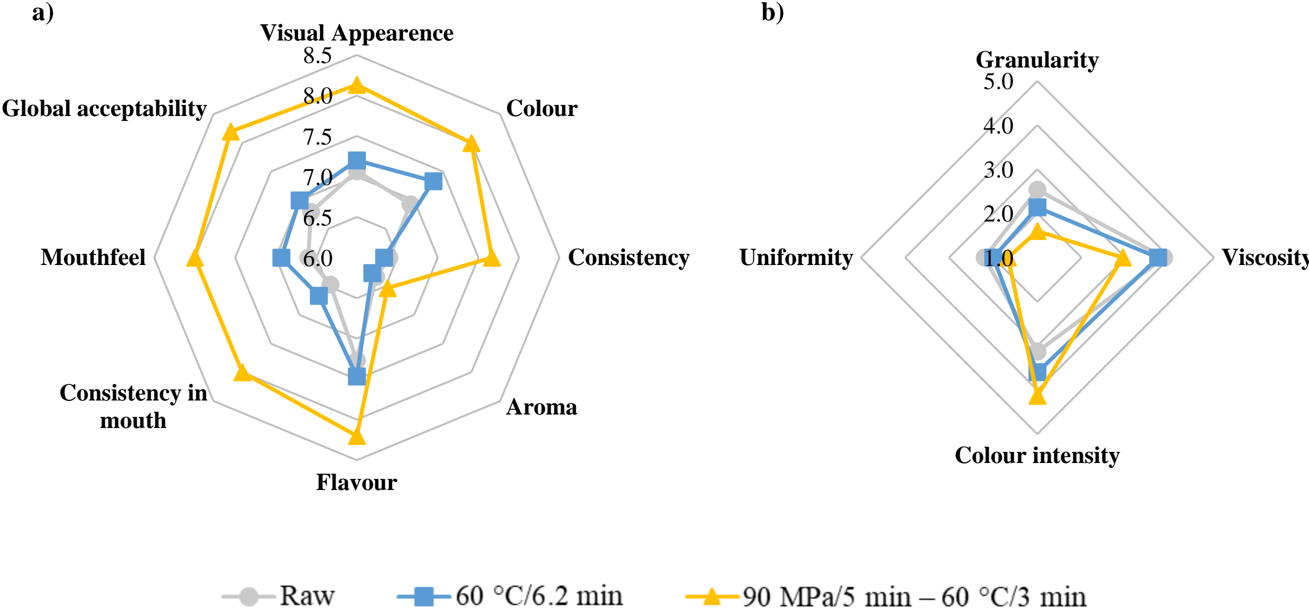


Figure 5.5. Sensory evaluation scores of "Doce de Ovos" produced from raw and treated egg yolk, on 9-point hedonic scale (1 = 'dislike extremely', 9 = 'like extremely') (a) and on 5-point scale (1 = 'lowest', 5 = 'highest') (b).

5.8.Conclusions

The results of the present work provided new insights of the effect of a shorter TP preceded by a sublethal pressure treatment on inoculated microorganisms and quality properties of EY. The use of pressure pre-treatment before a shorter TP reduced the thermal resistance of *S.* Senftenberg 775/W (synergistic lethal effect), achieving similar or even higher inactivation than that of commercial TP. Concerning egg quality, reduced soluble protein and increased viscosity was found in treated EY, probably due to the unfolding and aggregation of proteins, while heat-triggered lipid oxidation resulted in secondary oxidation products increase and carotenoids reduction, with the greatest impact being caused by commercial TP. However, despite the treatments impairing some EY physicochemical properties, the unfolded proteins might be adsorbing more at the interfaces, improving the emulsifying properties of raw EY. In addition, a higher content of volatile compounds was

found in MP-TP-treated EY, indicating that MP pre-treatment seems to enhance the thermal effect of the shorter TP. Regarding sensory analysis, the "Doce de Ovos" prepared with EY treated by MP-TP presented the highest global acceptability, with a higher score for colour and flavour, possibly due to the high content of volatile compounds and higher stability of carotenoids in these samples. Overall, the successive application of a shorter TP preceded by MP pre-treatments could be an alternative to commercial TP, offering a similar safety level against *S.* Senftenberg 775/W and *L. innocua,* and with lower impact on global EY quality.

5.9.References

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CHAPTER VI

RESULTS AND DISCUSSION | WHOLE EGG

This chapter is adapted from the submitted manuscript: Ana C. Ribeiro, Francisco J. Barba, Xavier Barber, José A. Lopes-da-Silva and Jorge A. Saraiva. *Influence of pressure pre-treatments on liquid whole egg thermal pasteurization – microbiological, physicochemical and functional properties*. Food Chemistry Advances.

6.1. Overview

This chapter describes the effect of a shorter thermal pasteurization (TP), compared to commercial TP, preceded by pressure pre-treatments on *Salmonella* Senftenberg 775/W inactivation (inoculated studies), in liquid whole egg (LWE), also as a possible alternative to commercial TP. A post-TP refrigeration storage was assessed to study the development of *S.* Senftenberg 775/W and *Listeria innocua* (inoculated pathogenic surrogate) after pasteurization. In addition, the physicochemical parameters, functional properties (foaming, emulsifying and gelling properties), and sensory analyses of an LWE derived product (egg tart) were also evaluated. The results were compared to those obtained for non-treated and commercial TP-treated samples.

6.2. Microbial analyses

6.2.1. Microbial inactivation by pressure treatments

The inactivation of *S.* Senftenberg 775/W using moderate pressure (MP, 50 – 250 MPa/5 min) and high pressure (225 – 250 MPa/5 min) treatments was evaluated, as can be seen in **Figure 6.1**. In general, as expected, an improvement in *S.* Senftenberg 775/W inactivation was obtained (the initial load was 6.94 ± 0.08 log colony forming unit (CFU)/mL) with increasing pressure, which is in agreement with what has been reported by other authors [1,2]. MP treatments in the range of 50 – 90 MPa caused a reduction from 0.17 to 0.39 $\log_{10}$ cycles ($p \geq 0.05$), while at pressure ≥ 125 MPa a significantly higher inactivation was achieved (0.53 – 2.46 log CFU/mL, $p < 0.05$). Nevertheless, other studies, despite applying similar or stronger conditions (200 – 250 MPa/5 – 30 min), obtained a lower inactivation (about 1 $\log_{10}$ cycles) for *Escherichia coli* K12 (*S.* Enteritidis surrogate in liquid egg) and *S.* Enteritidis PT4 E10 compared to our study, suggesting a greater resistance to pressure of these two microorganisms [2,3]. Therefore, the pressure conditions tested were not sufficient to guarantee the LWE safety, although, higher pressures were not considered, since treatments over 300 MPa may have a drastic effect on the physical properties of LWE [2].

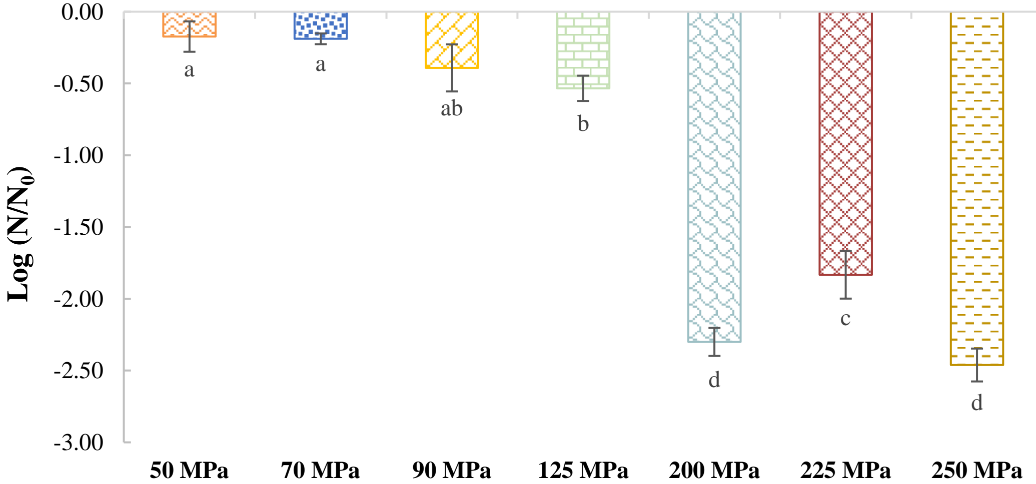


Figure 6.1. $\log_{10}$ cycles reductions of *Salmonella* Senftenberg 775/W (ATCC 43845) in liquid whole egg treated by pressure treatments at 50 – 250 MPa during 5 min. N denotes the microbial load measured for each treatment and N_0 the initial microbial load (6.89 – 7.01 log CFU/mL, without any treatment). Different letters indicate significant differences ($p < 0.05$) between treatments.

6.2.2. Microbial inactivation by pressure followed by thermal pasteurization

In this study, the use of pressure pre-treatments (50 – 250 MPa/5 min, at room temperature), aiming to cause sublethal damages in microorganisms and reduce their resistance to heat, before a shorter TP (60 ºC/1.75 min), was then tested to evaluate the lethal effect on *S.* Senftenberg 775/W, and compared with commercial TP (60 ºC/3.5 min) (**Figure 6.2**). When the MP pre-treatments were performed at 50 and 70 MPa, about 3.4 log_{10} cycles of *S.* Senftenberg 775/W population were inactivated ($p \geq 0.05$), whereas at 90 MPa lethality improved slightly (3.8 log_{10} cycles, $p < 0.05$) and then a similar lethal effect ($p \geq 0.05$) was obtained with increasing pressure up to 125 MPa. Moreover, the treatments carried out at pressures ≥ 200 MPa caused even greater inactivation ($p < 0.05$), resulting in reductions of at least 4.95 log_{10} cycles, reaching counts below the quantification limit (≤ 2.00 log CFU/mL) for 200 MPa and below detection limit (≤ 1.00 log CFU/mL) for 225 and 250 MPa. These findings showed to be more promising than published works, who reported reductions of 1.5 to 3.1 log_{10} cycles for *E. coli* K12 DH 5α (*S.* Enteritidis surrogate in liquid egg) in LWE treated at 200 – 300 MPa/3 – 30 min followed by TP at 52 – 55 ºC/2 – 3.5 min.

Otherwise, the shorter TP alone diminished *S.* Senftenberg 775/W counts by 3.01 log_{10} cycles, while a higher reduction of at least 4.95 log_{10} cycles (counts below the quantification limit, ≤ 2.00 log CFU/mL) was obtained with commercial TP. Compared to the latter, the combined treatments caused a similar (at 200 MPa, $p \geq 0.05$) or higher (at pressures ≥ 225 MPa, $p < 0.05$) inactivation, and at higher pressures (≥ 225 MPa) a synergistic effect was noticed.

According to the results discussed above, two combined treatments were selected for further studies: 200 MPa/5 min – 60 ºC/1.75 min (MP-TP), corresponding to a processing condition that accomplished similar inactivation level to commercial TP, and did not cause a large increase in viscosity (verified at a first glance by a simple test of flow time along a glass surface at a 45 º angle), and 50 MPa/5 min – 60 ºC/1.75 min (MP-TP). The latter case was studied to evaluate whether it would result in lower quality changes, despite having caused a lower microbial reduction, due to the intention of the authors to perform further studies involving the sequential combination of two nonthermal pre-treatments (pressure and ultrasound), in order to enhance microbial inactivation (see Chapter VII). Furthermore, a post-TP refrigeration storage development of *S.* Senftenberg 775/W and *L. innocua* was

studied, only for the 200 MPa /5 min – 60 ºC/1.75 min treatment, condition that obtained microbial inactivation like to commercial TP.

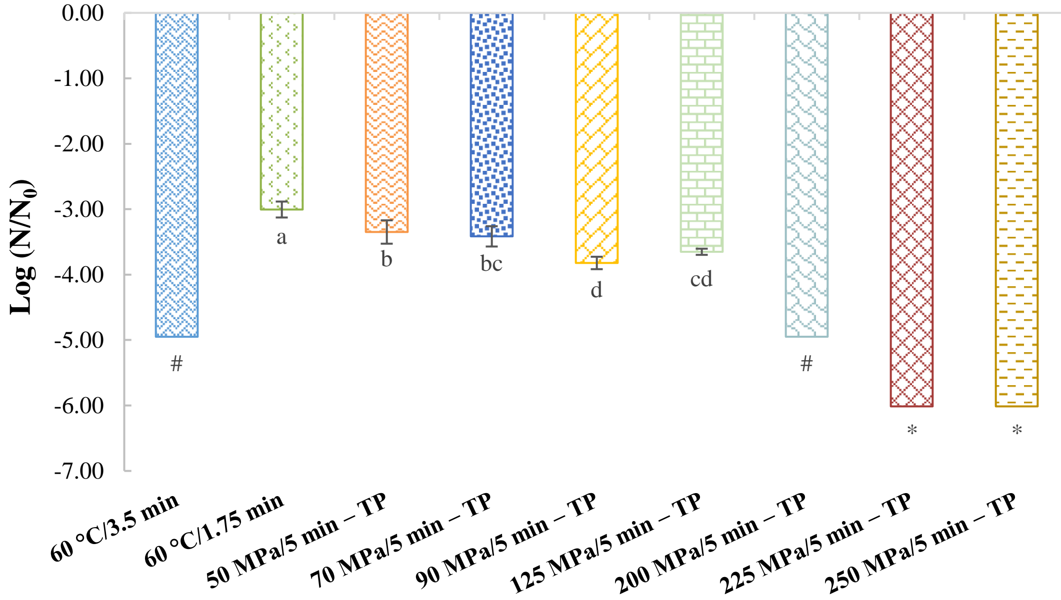


Figure 6.2. Log_{10} cycles reductions of *Salmonella* Senftenberg 775/W (ATCC 43845) in liquid whole egg treated by pressure pre-treatments (50 – 250 MPa/5 min) before a shorter thermal pasteurization (TP, at 60 ºC/1.75 min) or TP only (60 ºC/1.75 min and 60 ºC/3.5 min). N denotes the microbial load measured for each treatment and N_0 the initial microbial load (6.89 – 7.01 log CFU/mL, without any treatment). The symbols # and * means that microbial counts were below the quantification limit (≤ 2.00 log CFU/mL) or detection limit (≤1.00 log CFU/mL), respectively. Different letters indicate significant differences ($p<0.05$) between treatments.

6.2.3. Assessment of post-thermal pasteurization refrigeration storage

The effect of MP before a shorter TP (MP-TP at 200 MPa/5 min followed by 60 ºC/1.75 min), shorter TP and commercial TP on *S.* Senftenberg 775/W and *L. innocua* (*L. monocytogenes* surrogate) counts during storage at 4 ºC, was evaluated (**Figure 6.3**). LWE was inoculated with an initial load of 6.62 ± 0.11 log CFU/mL of *S.* Senftenberg 775/W (**Figure 6.3a**), and immediately after treatments (day 0), a reduction of 4.15 log_{10} cycles was obtained for shorter TP and at least 4.62 log_{10} cycles for MP-TP and commercial TP ($p<0.05$). During storage, *S.* Senftenberg 775/W counts decreased in all samples, with a faster decrease in commercial TP- followed by MP-TP- and shorter TP-treated samples,

reaching counts below the detection limit (≤ 1.00 log CFU/mL) after 3, 8 and 42 days of storage, respectively. No further variation until the end of storage was observed.

Concerning *L. innocua* (**Figure 6.3b**), the initial load was 6.99 ± 0.03 log CFU/mL, and the three treatments (day 0) decreased the counts by at least 4.99 log_{10} cycles (below the quantification limit, ≤ 2.00 log CFU/mL). Regarding to commercial TP- and MP-TP- treated LWE, the loads did not change during 23 and 14 days (≤ 2.00 log CFU/mL), respectively, but then increased until day 42, slightly more pronounced in MP-TP-treated samples (up to 4.92 log CFU/mL), maintaining similar counts until the end of experiment. A greater and faster *L. innocua* growth was observed in shorter TP-treated LWE, with values of 5.5 log CFU/mL after 30 days of storage, without significant variations until day 61. In addition, the prevalence and growth of *L. innocua*, during storage, suggests that, despite this microorganism counts being below the quantification limit after treatments, the remaining cells were able to multiply under refrigerated conditions, although to a less extent and later for commercial TP, followed by MP-TP and shorter TP.

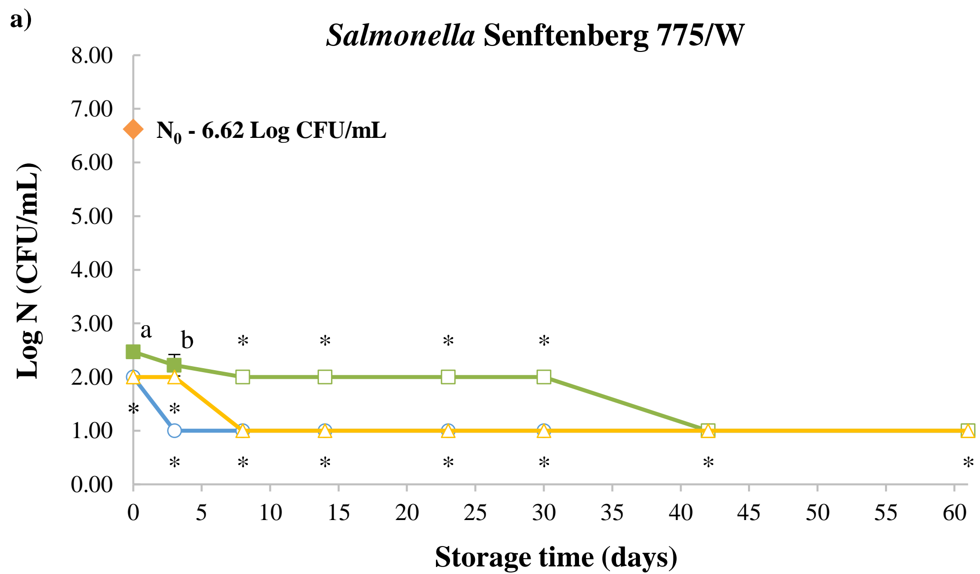
6.3.Physicochemical properties

6.3.1. pH and colour

The pH values measured in this study range from 7.72 (raw LWE) to 7.68 for treated samples (**Table 6.1**). Concerning LWE colour, in general, treated samples showed similar lightness (L*), redness (a*) and yellowness (b*) ($p \geq 0.05$) to non-treated sample. However, the MP-TP (200 MPa)-treated LWE showed a decrease of yellow colour (lower b* value, $p < 0.05$), resulting in a significantly higher colour variation (ΔE^*), which could indicate a lower presence of carotenoids [4]. Nonetheless, the colour variation produced by the four treatments would be not detected by the naked eye ($\Delta E^* < 3$) [5]. Similar results were reported by Monfort et al. [4] for LWE treated with a pulsed electric fields pre-treatment (25 kV/cm, 75 kJ/kg) followed by a TP (60 ºC/1 min) in the presence of triethyl citrate (TC).

6.3.2. Soluble protein

The soluble protein content represents the most practical criterion to predict the overall functionality of LWE, and a reduction usually indicates a lower functionality of egg proteins



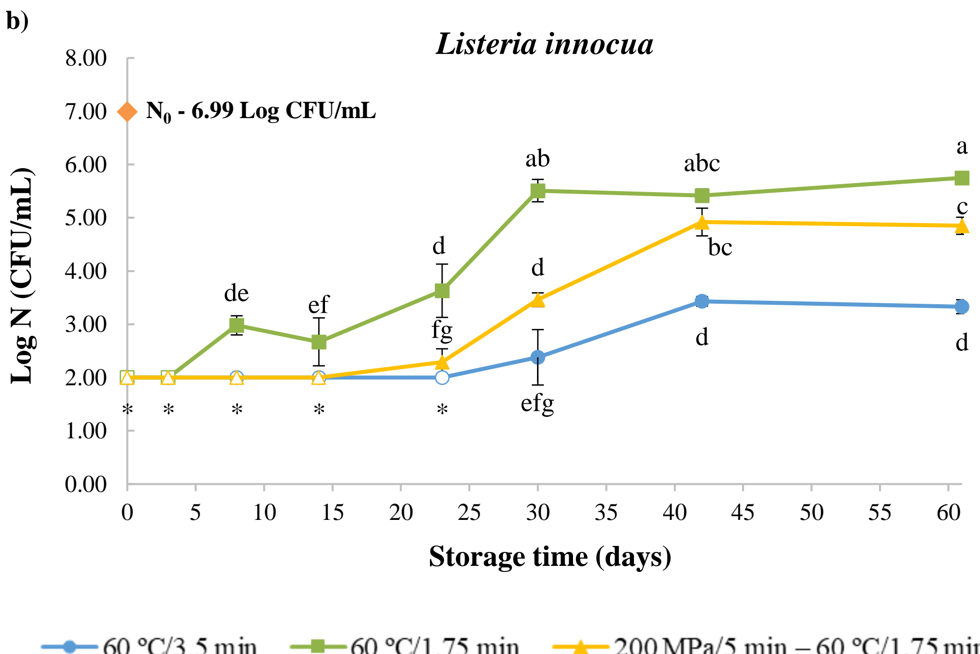


Figure 6.3. Development of a) *Salmonella* Senftenberg 775/W (ATCC 43845) and b) *Listeria innocua* (ATCC 33090) up to 61 days of storage after moderate pressure followed by a shorter thermal pasteurization (TP) (200 MPa/5 min – 60 ºC/1.75 min) or TP only (60 ºC/1.75 min and 60 ºC/3.5 min). Unfilled symbols on graphics and # or * mean that microbial counts were below the quantification limit (≤ 2.00 log CFU/mL) or detection limit (≤1.00 log CFU/mL), respectively, and N_0 corresponds to the initial load before each treatment. Different letters denote significant differences (p<0.05) between each processing condition and storage days.

[2,6]. As can be seen in **Table 6.1**, all treatments reduced significantly the soluble protein content of non-treated LWE (3.16 ± 0.11 to 2.78 ± 0.10 g/100 g, $p<0.05$), showing a decrement of about 7 % for MP-TP (50 and 200 MPa)- and shorter TP-treated LWE, while a slightly higher loss (12 %, $p<0.05$) was caused by commercial TP. Heat can cause the unfolding of egg proteins, by exposing the hydrophobic groups buried inside, promoting aggregation by hydrophobic interactions, and thus reducing soluble protein content [7]. Moreover, the negative effect on MP-TP-treated samples could be attributed to the themal effects leading to a less pronounced unfolding than commercial TP. Our results are supported by the findings of Souza and Fernández [8], who observed that a mild LWE pasteurization (60 ºC/3.5 min), can cause proteins unfolding and aggregation, possibly affecting ovomucoid, livetins, and some LDL apoproteins. As for MP-TP-treated, previous works in literature for LWE reported a soluble protein decreased (about 12 %) after TP (52 ºC/3.5 min) preceded by a pressure treatment (300 MPa/5 min) in presence of TC [2].

6.3.3. Apparent viscosity

The apparent viscosity of raw LWE was 13.0 ± 1.0 mPa.s (**Table 6.1**), what is in agreement with other authors [2], and all treated LWE samples showed higher apparent viscosity (20.53 – 30.64 mPa.s, $p<0.05$). The increment found for TP-treated LWE (about 59 %) is in line with the soluble protein decrement (**Table 6.1**), probably due to protein unfolding and consequent aggregation of unfolded proteins [9]. A more pronounced increase was observed in MP-TP (50 and 200 MPa)-treated LWE, being up to 49 % and 136 % higher ($p<0.05$) than TP- and non-treated LWE, respectively. However, despite the soluble protein data did not show a significant difference between shorter TP- and MP-TP-treated samples, the higher impact caused by MP-TP on viscosity, may indicate more marked protein aggregation under these conditions. Consequently, the results suggest that, in addition to thermal effects, the MP pre-treatment might also have an impact on viscosity *per se*. In fact, previous studies reported that increasing pressure and treatment time resulted in a LWE viscosity increment, possibly due to a greater protein-protein interaction under pressure [2,10].

Table 6.1. Physicochemical properties, lipid oxidation and total carotenoids content of raw and treated liquid whole egg (mean ± standard deviation). Different letters along each row denote significant differences ($p<0.05$) between processing conditions.

Properties	Raw	Thermal Pasteurization		Combined Treatments	
		60 ºC/3.5 min	60 ºC/1.75 min	50 MPa/5 min – 60 ºC/1.75 min	200 MPa/5 min – 60 ºC/1.75 min
pH	7.72 ± 0.01 ^{a}	7.69 ± 0.01 ^{bc}	7.70 ± 0.01 ^{b}	7.68 ± 0.01 ^{c}	7.69 ± 0.01 ^{c}
L*	47.16 ± 0.16 ^{ab}	47.24 ± 0.07 ^{ab}	46.89 ± 0.67 ^{bc}	46.65 ± 0.72 ^{c}	47.35 ± 0.13 ^{a}
a*	7.37 ± 0.12 ^{ab}	7.40 ± 0.06 ^{a}	7.13 ± 0.39 ^{b}	7.19 ± 0.45 ^{ab}	7.41 ± 0.09 ^{a}
b*	11.30 ± 0.16 ^{a}	10.96 ± 0.22 ^{ab}	10.57 ± 0.73 ^{b}	10.55 ± 0.74 ^{b}	10.62 ± 0.16 ^{b}
ΔE^*	-	0.46 ± 0.16 ^{b}	0.38 ± 0.09 ^{b}	0.40 ± 0.11 ^{b}	0.64 ± 0.04 ^{a}
Soluble Protein (g/100 g whole egg)	3.16 ± 0.11 ^{a}	2.78 ± 0.09 ^{c}	2.93 ± 0.10 ^{b}	2.94 ± 0.17 ^{b}	2.95 ± 0.19 ^{b}
Viscosity (mPa.s, at shear rate of 50 s^{-1})	12.98 ± 1.03 ^{c}	20.62 ± 1.33 ^{b}	20.53 ± 1.41 ^{b}	28.64 ± 2.26 ^{a}	30.64 ± 4.17 ^{a}
Lipid Oxidation (TBARS, µg/100 g whole egg)	13.29 ± 0.56 ^{a}	13.11 ± 0.51 ^{a}	12.92 ± 0.58 ^{a}	13.15 ± 0.73 ^{a}	13.36 ± 0.57 ^{a}
Total Carotenoids (mg/100 g whole egg)	0.92 ± 0.02 ^{a}	0.90 ± 0.03 ^{a}	0.94 ± 0.01 ^{a}	0.93 ± 0.02 ^{a}	0.81 ± 0.04 ^{b}

6.4. Functional properties

6.4.1. Foaming properties

The foaming properties of LWE is attributed to the egg white (EW) proteins, while egg yolk (EY) is considered as an inhibitor due to the competition by yolk compounds (proteins and lipids) [11]. **Table 6.2** summarizes the influence of the studied treatments on foaming properties of LWE. All treatments improved the foaming capacity (FC) of non-treated LWE (47 – 67 %, $p<0.05$), while they had no impact on foaming stability (FS). Additionally, the treated LWE samples exhibited similar foaming properties to each other ($p\geq0.05$), and so, suggesting that the effect on MP-TP-treated LWE was related with the thermal effects. The improvement of FC could probably be associated with the conditions used for foam production. As reported above, the treatments led to the formation of aggregates by hydrophobic interactions, which are quite weak and can probably be dissociated during the homogenization used for foaming (9500 rpm/2.5 min), thus forming smaller aggregates. Consequently, these aggregates can likely move faster to the foam interface, than their corresponding native proteins, improving FC [12,13]. Similar findings were described by Van der Plancken et al. [14], when heated EW solutions (pH 7.6) at 60 ºC. Actually, other works also reported no significant changes on FC of EW treated at 200 MPa [15] or EW solutions (pH 7.6) pressure-treated (0.1 – 700 MPa) at 60 ºC [14].

6.4.2. Emulsifying properties

Egg emulsifying properties are mainly associated to yolk compounds, but whole egg is also considered a good emulsifier [16]. The parameters used to describe emulsifying properties of LWE were emulsifying activity index (EAI) and emulsion stability index (ESI), as shown in **Table 6.2**. For all treatments, in general, it was observed a significant reduction ($p<0.05$) of EAI (11 – 38 %) and ESI (22 – 53 %) compared to non-treated LWE, however, a less pronounced effect was found for MP-TP-treated samples. This decrease could be attributed to the proteins unfolding, that promotes aggregation by hydrophobic interactions, and consequently, these aggregated proteins could not adsorb at the interface [7,17]. In opposition to what was suggested for foaming properties, the homogenization conditions used for emulsions production were less intense (9500 rpm/1.5 min) and were probably not sufficient to allow the dissociation of aggregates formed with the treatments. Furthermore,

Table 6.2. Functional properties of raw and treated liquid whole egg (mean ± standard deviation). Different letters along each row denote significant differences ($p<0.05$) between processing conditions.

Properties	Raw	Thermal Pasteurization		Combined Treatments	
		60 ºC/3.5 min	60 ºC/1.75 min	50 MPa/5 min – 60 ºC/1.75 min	200 MPa/5 min – 60 ºC/1.75 min
Foaming Capacity (%)	39.3 ± 7.3 ^{b}	60.5 ± 7.9 ^{a}	58.6 ± 6.5 ^{a}	65.5 ± 2.8 ^{a}	57.6 ± 9.5 ^{a}
Foaming Stability (%)	82.9 ± 8.1 ^{a}	70.3 ± 6.2 ^{a}	72.1 ± 5.7 ^{a}	73.7 ± 10.6 ^{a}	72.0 ± 9.6 ^{a}
Emulsifying Activity Index (m^2/g)	65.8 ± 4.2 ^{a}	44.2 ± 4.8 ^{c}	40.8 ± 1.0 ^{c}	45.1 ± 1.6 ^{c}	58.5 ± 1.9 ^{b}
Emulsifying Stability Index (min)	1.3 ± 0.3 ^{b}	0.6 ± 0.0 ^{d}	1.0 ± 0.2 ^{c}	1.5 ± 0.2 ^{a}	1.0 ± 0.0 ^{bc}
Maximum Force (N)	79.0 ± 11.2 ^{b}	98.6 ± 9.7 ^{a}	79.1 ± 7.9 ^{b}	80.9 ± 12.1 ^{b}	113.2 ± 9.7 ^{a}
Area Under the Curve (N.sec)	639 ± 31 ^{b}	689 ± 51 ^{b}	631 ± 32 ^{b}	688 ± 51 ^{b}	800 ± 37 ^{a}
Slope at 10 % Height	0.62 ± 0.01 ^{bc}	0.63 ± 0.04 ^{bc}	0.61 ± 0.01 ^{c}	0.66 ± 0.02 ^{b}	0.70 ± 0.03 ^{a}
Water Holding Capacity (%)	99.0 ± 0.6 ^{a}	99.0 ± 0.9 ^{a}	99.1 ± 0.7 ^{a}	99.4 ± 0.4 ^{a}	98.6 ± 0.7 ^{a}

as less aggregates adsorb at the interface, the stability of the formed emulsions reduce, as this property is related to the interfacial area that can be coated by proteins [18].

Further, LWE pasteurized at commercial conditions presented an ESI 1.6-fold lower than shorter TP-treated LWE ($p<0.05$), showing that the pasteurization time had a significant effect on this parameter. On the contrary, Lechevalier et al. [19] highlighted that pasteurization at 60 ºC (maximum) optimized the emulsifying properties of LWE, and only at 66 ºC the pasteurization time has a significant effect. Regarding MP-TP-treated samples, similar or higher emulsifying properties than shorter-TP-treated LWE was detected. This is consistent with Chen et. al (2019) [20] work, who noticed an increase in EAI and ESI when using pressures up to 200 MPa, possibly indicating that in our work the thermal effects counteract the positive effect of MP pre-treatments. Additionally, improved emulsifying properties (1.3 – 1.7-fold higher) was found for MP-TP (200 MPa)-treated LWE compared to commercial TP-treated LWE.

6.4.3. Gelling properties

The texture properties (maximum force, area under the curve, and slope at 10 % of height) of thermal-induced LWE gels were evaluated by uniaxial compresion tests. Typical force-time curves were characterized by an increase in force until the end of the test (examples of these curves are shown in **Annex F – Figure F3**), and the obtained results are shown in **Table 6.2**. The gels from raw LWE showed a significantly lower maximum force ($p<0.05$) than those obtained from commercial TP- and MP-TP (200 MPa)-treated LWE (25 – 43 % higher), and an increment of about 20 % was found with the treatment time increase (1.75 to 3.5 min). As noticed in **Table 6.2**, the area under the curve calculated for gels from raw LWE showed non-significant differences among samples ($p\geq0.05$), with exception of gels produced with MP-TP (200 MPa)-treated LWE that presented a higher value ($p<0.05$). Moreover, these last gels also showed a significant increment ($p<0.05$) in slope value compared to the other gels, and no significant changes ($p\geq0.05$) were found between TP-treated samples and raw LWE. Furthermore, the higher hardness and rigidity (15 – 43 %) of gels from MP-TP (200 MPa)-treated LWE, compared to the gels treated by only temperature, suggest that the pre-treatment at the highest pressure was responsible for the increment observed. In addition, for MP-TP (50 MPa)-treated gels, although there were no significant differences compared to shorter TP and non-treated gels, an increasing trend was observed.

Therefore, the results indicate that gels produced from commercial TP presented, overall, texture characteristics more similar to gels from raw LWE, than those produced from MP-TP (200 MPa)-treated LWE.

Concerning water holding capacity (WHC), as can be seen in **Table 6.2**, non-significant differences ($p \geq 0.05$) were found among the WHC of gels produced from raw and treated LWE, as also observed by Monfort et al. [2,4] in LWE supplemented with 2 % TC and treated by pressure (300 MPa/5 min) or pulsed electric fields (25 kV/cm) followed by TP (52 – 55 ºC/2 - 3.5 min).

6.5. Secondary lipid oxidation

Secondary lipid oxidation (thiobarbituric acid-reactive substances (TBARS), expressed as malondialdehyde (MDA) content) was assessed (**Table 6.1**), with the MDA content of raw LWE (13.29 ± 0.56 µg /100 g) being comparable to values reported by other authors [21]. No significant differences were noticed among raw and treated LWE, ranging between 12.92 and 13.36 µg MDA/100 g ($p \geq 0.05$). These findings are consistent with what was reported in a previous study [5], with no significant effect on TBARS values being observed for pasteurized LWE (60 ºC/3.5 min).

6.6. Total carotenoids

The non-treated LWE presented a total carotenoids content of 0.92 ± 0.02 mg/100 g (**Table 6.1**), which agrees with values reported by Fredriksson et al. [22]. As observed in **Table 6.1**, for all treatments, no significant differences ($p \geq 0.05$) were observed compared to raw LWE, with exception of MP-TP (200 MPa) (a reduction of about 12 %). The lack of effect noted for the TP is corroborated by published data when EY was heated at 61.5 °C for 3.5 min [23]. On the other hand, the significant loss observed for MP-TP-treated LWE at the highest pressure (~12 %), could be attributed to degradation reactions that take place during processing [24], suggesting that the reduction was probably related with the pressure pre-treatment. Indeed, published studies reported that MP treatment (200 MPa/6 min) reduced the total carotenoid content of persimmon fruit [24]. In addition, the carotenoids reduction resulted in a less yellow colour and a greater colour variation compared to the

other treated LWE, however, these differences are not consideraded noticeable by the consumer as $\Delta E^*<3$ [5].

6.7.Sensory analysis

Sensory analysis of an egg-derived product (egg tart) prepared with non-treated and treated LWE (200 MPa/5 min followed by 60 ºC/1.75 min and 60 ºC/3.5 min) was assessed, and the results are shown in **Figure 6.4** (see **Annex F – Figure F4**). The attributes score ranged from 5.2 to 7.3, which is equivalent to "neither like nor dislike slightly" and "like moderately" respectively on 9-point hedonic scale. The sensory characteristics scores (interior colour, texture when cracking, overall feeling when chewing, flavour and global acceptability) were highest in the non-treated samples, while the scores of visual appearance, surface colour, aroma and texture when cutting were higher in the LWE thermally treated. Besides, the egg tarts prepared with MP-TP-treated LWE obtained the lowest scores for all sensory characteristics. For this sample, the lower score for texture when cracking is in line with the highest texture properties observed for thermal-induced gels (see section 6.4.3). Nevertheless, no signifcant differences were found between the three egg tarts ($p\geq0.05$). They obtained a similar global acceptability by the panellists, although they were ordered by preference as follows: egg tart produced from MP-TP-treated LWE, raw LWE and commercial TP-treated LWE (from the one panellist liked least to the one they liked the most).

6.8.Conclusions

The application of pressure treatments alone (50 – 250 MPa/5 min) was not effective in controlling *S.* Senftenberg 775/W, but when followed by a shorter TP significantly increased inactivation (at least 6.09 $\log_{10}$ cycles), obtaining similar or even higher lethality than commercial TP, using a pressure pre-treatment ≥ 200 MPa. On the other hand, all the treatments caused protein unfolding and aggregation, leading to a decrease in soluble protein content and an increase in viscosity. Consequently, these changes in treated LWE resulted in improved foaming capacity (47 – 88 %), higher texture properties (13 – 43 %) and reduced emulsifying properties (18 – 53 %) compared to non-treated LWE. Compared to commercial TP, the MP-TP treatment presented higher emulsifying and gelling properties and viscosity,

similar foaming properties and lower total carotenoids content. Additionally, egg tarts produced with non-treated and treated LWE had a similar global acceptability. Thus, the results hint the possibility to use sequentially combined treatments to enhance inactivation of *S.* Senftenberg 775/W, using a shorter TP and maintaining/improving some properties of pasteurized LWE.

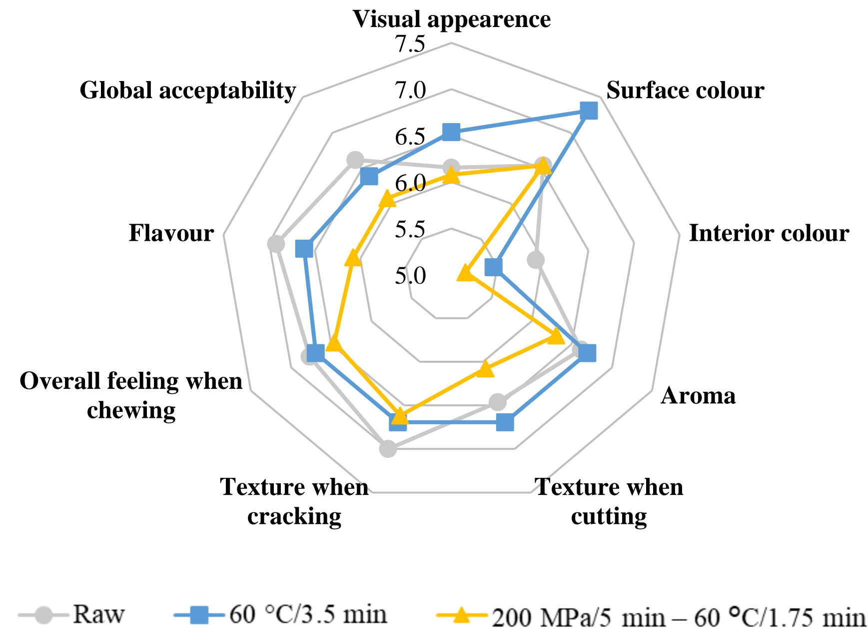


Figure 6.4. Comparative diagram of sensory attributes of the egg tart prepared from raw and treated liquid whole egg (9-point hedonic scale: 1-disliked extremely, 9-liked extremely).

6.9.References

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CHAPTER VII

RESULTS AND DISCUSSION | EGG PRODUCTS

This chapter is adapted from the manuscript under preparation: Ana C. Ribeiro, Susana Casal, José A. Lopes-da-Silva and Jorge A. Saraiva. *Effects of sequential combination of moderate pressure and ultrasounds on subsequent thermal pasteurization of liquid whole egg.*

7.1.Overview

This chapter describes the effect of moderate pressure (MP) and ultrasound (US) pre-treatments, singly and/or combined, before a shorter thermal pasteurization (TP), compared to commercial TP, on the inactivation of *Salmonella* Senftenberg 775/W inoculated in liquid whole egg (LWE), egg white (EW) and egg yolk (EY), as a possible substitute for commercial TP. Initially, the processing conditions were optimized, testing different pressure levels and US treatment time levels (inoculated studies). Then, the MP-US-TP combination was the one that attained a food safety level similar to commercial TP, in LWE, and thus it was evaluated for the effect on physicochemical parameters such as pH, colour, protein solubility and viscosity, emulsifying properties, thermal properties, lipid oxidation and volatile profile. The results were compared with non-treated and commercial TP-treated samples.

7.2. Pre-test studies and conditions selection

There are few data available about the effect of US treatments on the inactivation of pathogenic microorganisms in egg products. For instance, US application at temperatures below 30 ºC during long periods (up to 30 min) and with a working volume of 10 – 25 mL achieved only a maximum reduction of 2.3 log_{10} cycles of *Salmonella* spp., *Listeria* spp., and *E. coli* [1–3]. Therefore, based on these results and aiming to sequentially combine US technology with TP and/or MP, a maximum treatment time of 5 min and a working volume of 50 mL was defined. Indeed, increasing the treatment volume decreased the energy applied per mL of liquid and consequently, reduce the inactivation effect [1]. Despite this, in our study, we choose to work with a higher working volume compared to the studies cited above, as the US technology will be applied in combination with other technologies.

The first US pre-tests carried out at 100 % amplitude during 5 min produced a large amount of foam in LWE and EW samples and caused EY gelation after 3 min of treatment (**Figure 7.1**), probably leading, at least, to partial protein denaturation. The following pre-tests revealed that using amplitudes above 65 % caused some EY gelation in the sonotrode proximity, but at 50 % amplitude EY appeared more fluid than the non-treated sample. So, according to these pre-tests results, an amplitude of 50 % was selected for further studies, corresponding to the first amplitude level with lower foam production and no EY gelation. Though, under the defined conditions (50 % amplitude/5 min) a "burning smell" was noticed in all egg samples (aroma linked with sonication treatment also reported by some researchers [4]). To avoid this, in the present work the treatment time was reduced to 1 min and the samples so processed showed no signs of burned smell.

Concerning MP pre-treatment, a treatment of 50 MPa during 5 min was previously studied in Chapter IV, V and VI, showing no major changes in quality, but with reduced effect on microbial inactivation, revealing so it could be a good condition to combine with other technologies.

In summary, it was selected an US pre-treatment of 50 % amplitude during 1 min with a working volume of 50 mL, a MP pre-treatment at 50 MPa for 5 min, and a TP at commercial pasteurization temperature during a shorter time (55 ºC/3 min (EW), 60 ºC/3 min (EY) and 60 ºC/1.75 min (LWE)). The following combinations were so studied: US-TP, MP-TP, US-MP, MP-US, US-MP-TP, MP-US-TP and compared with the respective commercial TP (55.6 ºC/6.2 min (EW), 60 ºC/6.2 min (EY) and 60 ºC/3.5 min (LWE)) [5,6].

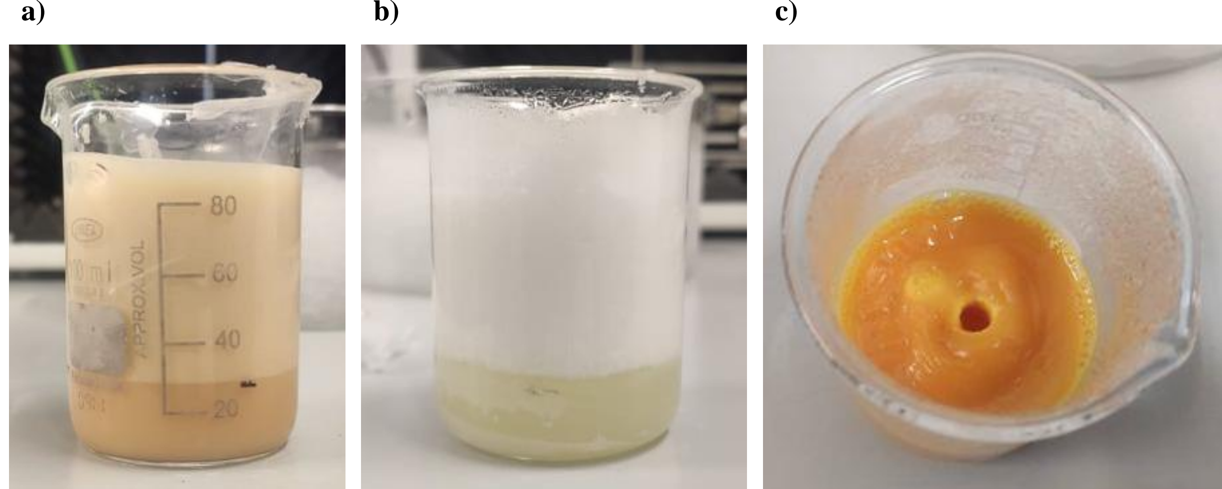

Figure 7.1. Samples of (a) whole egg, (b) egg white and (c) egg yolk treated by ultrasound at 100 % amplitude during 5 min.

7.3. Microbial analyses

7.3.1. Liquid egg white

The lethal effect of US, MP and TP, individually and sequentially combined, on *S.* Senftenberg 775/W inoculated in EW was assessed and is shown in **Figure 7.2**. The lethal efficacy of the commercial TP (55.6 ºC/6.2 min) against this *Salmonella* serovar was at least 5.63 log_{10} cycles, while the shorter TP (55 ºC/3 min) inactivated only 2.55 log_{10} cycles. For US, a single treatment of 1 min at 50 % amplitude resulted in 0.26 log_{10} cycles reductions in the viable cell counts of *S.* Senftenberg 775/W, with no significant differences compared to MP-only treatment (0.20 log_{10} cycles, $p \geq 0.05$). The consecutive combination involving US or MP pre-treatments before a shorter TP (US-TP and MP-TP) improved significantly the lethal effect against this microorganism (up to 3.15 log_{10} cycles, $p < 0.05$), compared to individual treatments. In fact, there are no data on sequential combination of US and temperature in egg products, however, the use of US at 40 – 55 ºC reduced up to 2.30 log_{10} cycles of *S.* Enteritidis and *S.* Typhimurium [1,3], showing a slightly lower inactivation than that obtained in our study.

Besides, the combination of US-MP or MP-US did not show a significantly greater effect on *S.* Senftenberg 775/W inactivation ($p \geq 0.05$) compared to each treatment individually (0.19 – 0.28 log_{10} cycles reductions). Yet, a higher inactivation (about 3.23 log_{10} cycles) was

reported by Huang et al. [1], when sequentially combined treatments of US (40 W/ 55 ºC/5 min) and MP (138 MPa/2-2-4 min/20 ºC) were used.

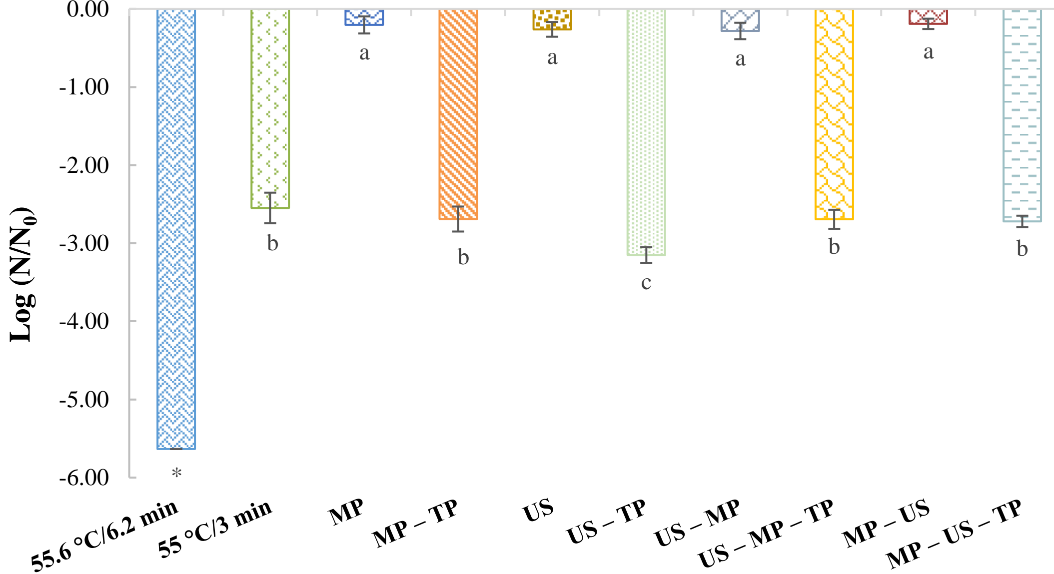

Figure 7.2. Log_{10} cycles reductions of *Salmonella* Senftenberg 775/W (ATCC 43845) in liquid egg white (EW) treated by moderate pressure (MP, 50 MPa/5 min,), ultrasound (US, 50 % amplitude/1 min,), shorter thermal pasteurization (TP, 55 ºC/3 min,), designed combined treatments (MP and/or US before a shorter TP) and commercial TP (55.6 ºC/6.2 min,). N denotes the microbial load measured for each treatment and N_0 the initial microbial load (6.55 – 6.98 log CFU/mL, without any treatment). The symbol * mean that microbial counts were below the detection limit (≤ 1.00 log CFU/mL). Different letters indicate significant differences ($p<0.05$) between treatments.

On the other hand, the use of a shorter TP preceded by US-MP or MP-US pre-treatments (US-MP-TP or MP-US-TP) attained similar reductions for both combinations (2.72 log_{10} cycles, $p\geq0.05$), but significantly improved the lethal effect compared to US-MP and MP-US ($p<0.05$). Compared to both TP, MP-US-TP or US-MP-TP combinations reached a similar inactivation level ($p\geq0.05$) to the shorter TP alone, yet, they were far from those reached with commercial TP (5.63 log_{10} cycles) ($p<0.05$) (**Figure 7.2**).

For the combinations studied in the present work, additive effects were found for MP-TP, US-MP, US-MP-TP and MP-US-TP, while for US-TP a synergic effect was obtained. Therefore, the combination of the three technologies (MP-US-TP or US-MP-TP) did not

prove to be advantageous compared to MP-TP or US-TP combinations. The optimization of MP-TP combination was already studied and is described in Chapter IV, while US-TP combination seems not to be a viable better alternative, taking into account the microbiological results (inactivated 3.15 log_{10} cycles which was far from 5.63 log_{10} cycles of commercial TP) and the large increase in US treatment time required to achieve satisfactory possible microbial inactivation (which could result in quality deterioration, as observed in pre-tests section).

7.3.2. Liquid egg yolk

Figure 7.3 shows the loads variation (log (N/N_0)) of *S.* Senftenberg 775/W in liquid EY submitted to MP, US and TP, individually and/or in combination. The shorter TP alone (60 ºC/3 min) diminished *S.* Senftenberg 775/W counts by 1.94 log_{10} cycles, while a higher reduction of 3.72 log_{10} cycles was obtained with commercial TP (60 ºC/6.2 min) ($p<0.05$). The MP-only treatment caused almost negligible inactivation of *S.* Senftenberg 775/W, while the US-only treatment decreased their counts by about 0.29 log_{10} cycles, and as expected the combination with a shorter TP (MP-TP and US-TP) increased lethality ($p<0.05$) up to 2.86 log_{10} cycles.

The consecutive combination of US and MP (US-MP and MP-US) was explored with the aim to achieve enhanced microbial inactivation, but no significant differences were obtained compared to each treatment individually ($p\geq0.05$), while the subsequent combination with a shorter TP (US-MP-TP and MP-US-TP) enhanced inactivation, from 0.36 to 2.05 log_{10} cycles ($p<0.05$). Nevertheless, regardless of the combination order (US-MP or MP-US, US-MP-TP or MP-US-TP) there were no significant differences ($p\geq0.05$). Compared to the inactivation obtained with US-MP and MP-US combinations, more promising results were found by other authors, after treating *S.* Enteritidis CB 919 Lux AB and *E. coli* K12 DH 5α with MP-US or US-MP combinations (US: 34.6 – 40 W/0.5 – 5 min/55 ºC, MP: 138 – 300 MPa/3 – 15 min) [1,2]. Furthermore, none of the studied combinations attained an inactivation level equivalent to that of commercial TP.

A synergy was observed for US-TP or MP-TP combinations, in which the combined effect was greater than the additive sum of each individual treatment, while for the other combinations only additive effects were observed. Consequently, the combination of the three technologies was not more effective than MP-TP or US-TP combinations. The former

was already optimized (Chapter V), while US-TP combination appears not to be a feasible alternative, taking into consideration the reasons mentioned above for EW optimization.

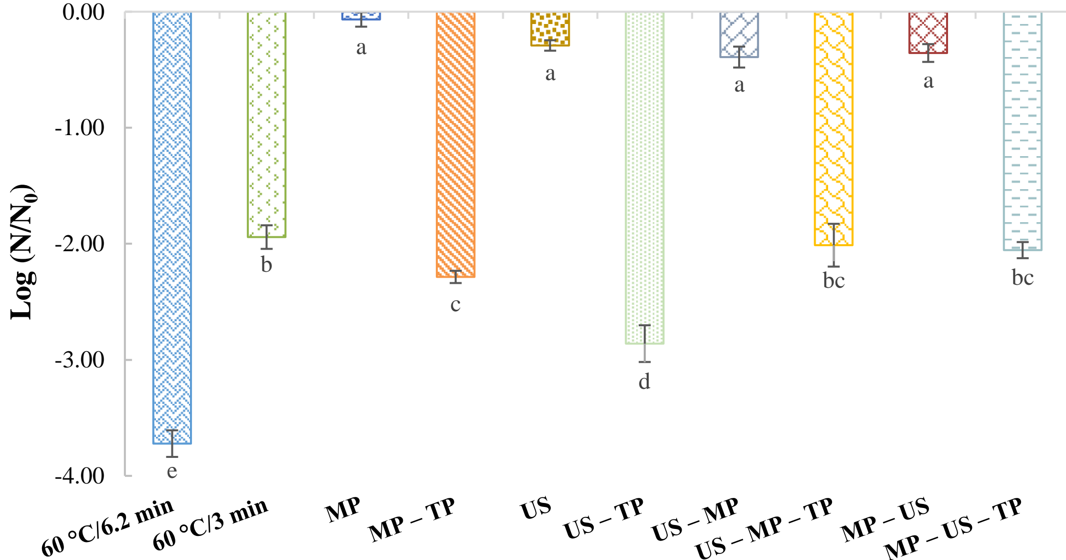

Figure 7.3. Log_{10} cycles reductions of *Salmonella* Senftenberg 775/W (ATCC 43845) in liquid egg yolk (EY) treated by moderate pressure (MP, 50 MPa/5 min, ▨), ultrasound (US, 50 % amplitude/1 min, ▨), shorter thermal pasteurization (TP, 55 ºC/3 min, ▨), designed combined treatments (MP and/or US before a shorter TP) and commercial TP (55.6 ºC/6.2 min, ▨). N denotes the microbial load measured for each treatment and N_0 the initial microbial load (6.55 – 6.98 log CFU/mL, without any treatment). Different letters indicate significant differences ($p<0.05$) between treatments.

7.3.3. Liquid whole egg

The inactivation of *S.* Senftenberg 775/W using MP, US and TP (shorter TP: 60 ºC/1.75 min and commercial TP: 60 ºC/3.5 min), individually or sequentially combined, was assessed as shown in **Table 7.1** and **Figure 7.4**. As can be seen in **Figure 7.4**, commercial TP inactivated 5.25 log_{10} cycles of *S.* Senftenberg 775/W, while the shorter TP reduced 3.34 log_{10} cycles. The MP (50 MPa/ 5 min) and US treatments (50 % amplitude/1 min), individually, decreased their counts up to 0.26 log_{10} cycles, in good agreement with previously reported data for other *Salmonella* spp. [1,3] (**Table 7.1**). No significant differences were found when these two techniques were combined (US-MP or MP-US, $p\geq0.05$), but an improved microbial inactivation (up to 3.90 log_{10} cycles) was observed when

US or MP was applied before a shorter TP (US-TP and MP-TP), as observed previously for EW and EY samples. In fact, a slightly higher inactivation was obtained in our work than in the study of Monfort et al. [7], who inactivated up to 3.1 log_{10} cycles reductions of *E. coli* K12 DH 5α (*S.* Enteritidis surrogate) applying a TP (52 ºC/3.5 min and 55 ºC/2 min) preceded by pressure (200 – 300 MPa/3 – 30 min) pre-treatments.

Table 7.1. Log_{10} cycles reductions in the population of *Salmonella* Senftenberg 775/W (ATCC 43845) in liquid whole egg (with a initial microbial load (N_0) of 6.55 – 6.98 log CFU/mL) treated by moderate pressure (MP, 50 – 160 MPa/5 min), ultrasound (US, 50 % amplitude/1 – 3 min), MP followed by a shorter thermal pasteurization (TP, 60 ºC/1.75 min) (MP-TP), US followed by a shorter TP (US-TP), US followed by MP and/or followed by a shorter TP (US-MP and US-MP-TP) or MP followed by US (MP-US). Different letters indicate significant differences ($p<0.05$) between treatments.

Treatments	*Salmonella* Senftenberg 775/W	
	MP	**MP-TP**
50 MPa/5 min	0.05 ± 0.06 [a]	3.90 ± 0.17 [i]
90 MPa/5 min	0.11 ± 0.11 [ab]	4.06 ± 0.08 [ij]
125 MPa/5 min	0.16 ± 0.09 [abc]	3.38 ± 0.16 [h]
160 MPa/5 min	0.67 ± 0.07 [fg]	4.45 ± 0.16 [lm]
	US	**US-TP**
50 % amplitude/1 min	0.26 ± 0.17 [abcd]	3.54 ± 0.15 [h]
50 % amplitude/2 min	0.25 ± 0.05 [abcd]	3.40 ± 0.05 [h]
50 % amplitude/3 min	0.38 ± 0.02 [de]	4.15 ± 0.06 [jk]
	US-MP	**US-MP-TP**
50 % amplitude/1 min – 50 MPa/5 min	0.31 ± 0.15 [bcde]	3.53 ± 0.15 [h]
	MP-US	
50 MPa/5 min – 50 % amplitude/1 min	0.37 ± 0.14 [cde]	
50 MPa/5 min – 50 % amplitude/2 min	0.35 ± 0.03 [cde]	
50 MPa/5 min – 50 % amplitude/3 min	0.50 ± 0.02 [ef]	
90 MPa/5 min – 50 % amplitude/1 min	0.20 ± 0.06 [abcd]	
125 MPa/5 min – 50 % amplitude/1 min	0.30 ± 0.07 [bcde]	
160 MPa/5 min – 50 % amplitude/1 min	0.87 ± 0.08 [g]	

In addition, when US-MP and MP-US were also combined with a shorter TP (US-MP-TP and MP-US-TP), a significant improvement in *S.* Senftenberg 775/W reduction was showed (up to 4.60 log_{10} cycles, $p<0.05$) (**Table 7.1** and **Figure 7.4**). Indeed, as stated by

other authors, using US as a second pre-treatment (MP-US-TP) reached higher inactivation, suggesting that a synergistic effect of US and TP acted on the injured survivors cells from the MP treatment [1]. Compared to commercial TP, the MP-TP, US-TP, US-MP-TP and MP-US-TP combinations achieved a lower reduction (3.53 – 4.60 $\log_{10}$ cycles, $p<0.05$), however, the MP-US-TP combination resulted in a closer inactivation level.

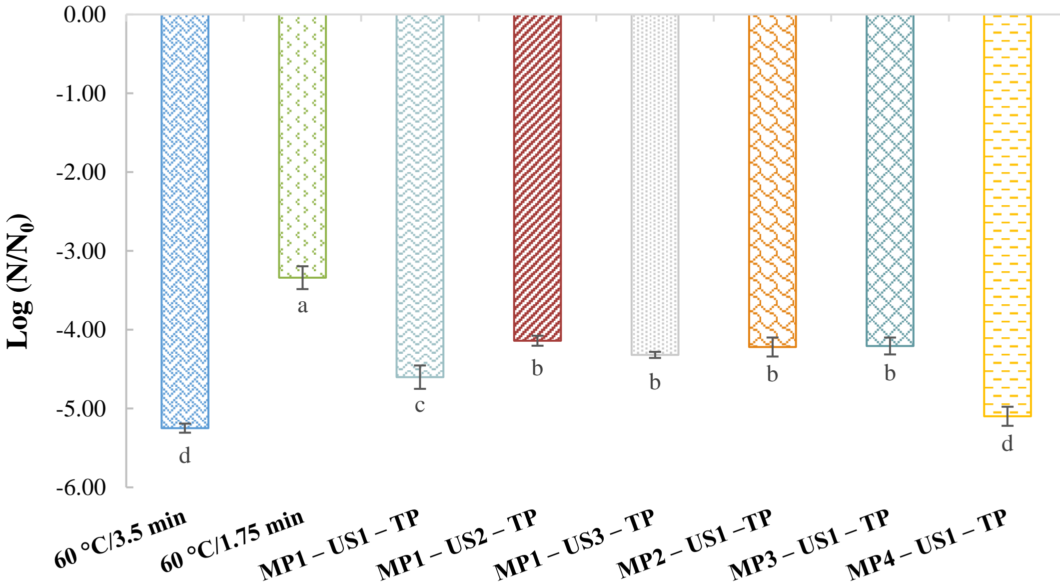

Figure 7.4. Inactivation of *Salmonella* Senftenberg 775/W (ATCC 43845), inoculated in liquid whole egg, by commercial thermal pasteurization (TP, 60 ºC/3.5 min,); shorter TP (60 ºC/1.75 min), and moderate pressure (MP) and ultrasound (US) followed by a shorter TP: ( ) 50 MPa/5 min – 50 % amplitude/1 min – TP (MP1 – US1 –TP); ( ) 50 MPa/5 min – 50 % amplitude/2 min – TP (MP1 – US2 – TP); ( ) 50 MPa/5 min – 50 % amplitude/3 min – TP (MP1 –US3 – TP); ( ) 90 MPa/5 min – 50 % amplitude/1 min – TP (MP2 – US1 – TP); ( ) 125 MPa/5 min – 50 % amplitude/1 min – TP (MP3 – US1 – TP); ( ) 160 MPa/5 min – 50 % amplitude/1 min – TP (MP4 – US1 –TP). N denotes the microbial load measured for each treatment and N_0 the initial microbial load (6.56 – 6.88 log CFU/mL, without any treatment). Different letters indicate significant differences ($p < 0.05$) between treatments.

For LWE were observed additive effects with US-TP and US-MP-TP combinations and a synergy for MP-TP and MP-US-TP combinations. The US-TP combination was not optimized, in line with the reasons given above for EW and EY samples, and the MP-TP combination was already optimized (see Chapter VI). Therefore, only the optimization of

MP-US-TP combination was investigated, increasing the US treatment time (1 – 3 min, without signs of burned smell) and the MP intensity (50 – 160 MPa), as an attempt to replace commercial TP in achievement of satisfactory microbial inactivation.

First, the US treatment time was increased (from 1 to 2 and 3 min), maintaining the MP pre-treatment (50 MPa/5 min). As observed in **Table 7.1** and **Figure 7.4**, for US, individually or in combination with MP and/or shorter TP, in general, increasing treatment time did not increase the lethal effect on *S.* Senftenberg 775/W. Then, the US treatment was maintained at 50 % amplitude for 1 min, and the pressure intensity raised (50 – 160 MPa/5 min) (**Table 7.1**). The progressive increase of MP intensity, applied individually or in combination with US and/or shorter TP, showed a *S.* Senftenberg 775/W inactivation increasing trend, mainly at pressures ≥ 125 MPa. In addition, for MP-US-TP combination, the pressure increments had only a significant effect at 160 MPa (4.60 to 5.10 log_{10} cycles reductions, $p<0.05$), obtaining a similar lethal effect ($p≥0.05$) to that of commercial TP with the MP-US-TP combination at 160 MPa/5 min – 50 % amplitude/1 min – 60 ºC/1.75 min. Thereby, considering the results obtained in this section, the *S.* Senftenberg 775/W seems to be less resistant to combined treatments when inoculated in LWE, than in EW or EY (section 7.3.1 and 7.3.2). In summary, from a food safety point of view, the results indicate that the following combination: 160 MPa/5 min – 50 % amplitude/1 min – 60 ºC/1.75 min could be an alternative to commercial TP, and so, main LWE quality properties were further assessed using this processing combination, and compared to commercial TP and each treatment individually.

7.4. Liquid whole egg quality properties

7.4.1. Physicochemical properties

7.4.1.1. pH and colour

Table 7.2 shows the physicochemical properties of LWE treated by MP followed by US before a shorter TP (MP-US-TP), compared with each treatment individually and commercial TP (60 °C/3.5 min). The pH values ranged from 7.9 (raw LWE) to 7.8 for the treated LWE samples, being the results for US-treated LWE (pH 7.8) in good agreement with those of O'Sullivan et al. [8]. On the contrary, the pH decrease observed for MP-treated samples was not supported by other published studies, which did not reported changes in pH with pressure [9].

Colour of non-treated LWE was assessed and compared with the different treatments studied (**Table 7.2**), and overall, the treatments had a tendency to increase the product lightness (L*) and maintain redness (a*), while a yellowness (b*) decrement was found for MP-only and MP-US-TP treatments ($p<0.05$). The colour variations produced by the treatments (ΔE = 0.4 – 1.0) were in the range considered not detected by the naked eye ($\Delta E^*<3$) [10], with TP (shorter and commercial) presenting a less pronounced effect (0.4 – 0.5). In fact, similar results ($\Delta E^*<3$) were reported by other authors for EW and LWE submitted to sequential combined treatments [7,11].

7.4.1.2. Protein solubility

Concerning protein solubility (**Table 7.2**), a reduction of about 7 – 12 % was found for LWE after shorter TP, commercial TP and MP-only treatments compared to non-treated LWE ($p<0.05$), with a more significant loss caused by commercial TP, as reported in our previous study (Chapter VI). Heat and MP can disrupt non-covalent bonds, leading to protein unfolding by exposing the hydrophobic groups buried inside, promoting aggregation by hydrophobic interactions, and thus reducing protein solubility [12]. Our results are consistent with the observations of De Souza and Fernández and Yang et al. [13,14]. However, the treatments using US showed a tendency to improve protein solubility (compared to raw sample). Additionally, the MP-US-TP-treated LWE presented the highest protein solubility increment, up to 1.2-fold higher than the other samples ($p<0.05$), pointing to the possibility that the negative effect of a MP pre-treatment (alone) may have been counteracted by the subsequent US treatment, since within the studied pressure range the changes that can occur in protein structure are expected to be reversible [15]. The sonication could destroy the aggregated state of proteins, possibly exposing hydrophilic regions toward water molecules, resulting in protein solubility increase [16,17]. Therefore, the subsequent shorter TP had no impact on this property, as opposed to when it was applied alone.

7.4.1.3. Apparent viscosity

As can be seen in **Table 7.2**, the apparent viscosity of raw LWE was 12.98 ± 1.03 mPa.s, which is in agreement with other authors [7]. Compared to raw LWE, a viscosity increment was found after both TP (shorter and commercial), as reported in our previous study (Chapter VI), about 59 % ($p<0.05$), and to a lesser extent with MP-only treatment (41 %, $p<0.05$).

Table 7.2. Physicochemical, thermal and functional properties and lipid oxidation of raw and treated liquid whole egg (mean ± standard deviation). Different letters along each row denote significant differences ($p<0.05$) between processing conditions.

Properties	Raw	Thermal Pasteurization		Ultrasound	Moderate Pressure	Combined Process (MP-US-TP)[1]
		60 ºC/3.5 min	60 ºC/1.75 min	50 %/1 min	160 MPa/5 min	160 MPa/5 min – 50 %/1 min – 60 ºC/1.75 min
pH	7.9 ± 0.0 ^{a}	7.9 ± 0.0 ^{ab}	7.9 ± 0.00 ^{b}	7.8 ± 0.0 ^{c}	7.8 ± 0.0 ^{d}	7.8 ± 0.0 ^{c}
L*	44.0 ± 0.7 ^{bc}	44.7 ± 0.12 ^{a}	44.3 ± 0.7 ^{ab}	44.7 ± 0.2 ^{a}	44.1 ± 0.2 ^{b}	43.5 ± 0.7 ^{c}
a*	9.8 ± 0.5 ^{a}	10.0 ± 0.1 ^{a}	9.7 ± 0.4 ^{a}	9.9 ± 0.0 ^{a}	10.0 ± 0.0 ^{a}	9.8 ± 0.4 ^{a}
b*	11.1 ± 0.9 ^{ab}	11.3 ± 0.3 ^{ab}	11.0 ± 0.7 ^{bc}	11.6 ± 0.1 ^{a}	10.5 ± 0.2 ^{c}	9.9 ± 0.7 ^{d}
ΔE	-	0.5 ± 0.2 ^{c}	0.4 ± 0.1 ^{c}	1.0 ± 0.1 ^{a}	0.8 ± 0.1 ^{b}	0.8 ± 0.1 ^{b}
Protein solubility (%)	100.0 ± 3.4 ^{b}	87.9 ± 2.9 ^{d}	92.6 ± 3.2 ^{c}	103.2 ± 2.6 ^{ab}	90.8 ± 2.0 ^{cd}	104.4 ± 3.9 ^{a}
Viscosity (mPa.s, at shear rate of 50 s^{-1})	13.0 ± 1.0 ^{c}	20.6 ± 1.3 ^{a}	20.5 ± 1.4 ^{a}	8.6 ± 0.1 ^{d}	18.3 ± 0.5 ^{b}	9.1 ± 0.1 ^{d}
T_{onset} (ºC)	75.9 ± 0.1 ^{a}	77.9 ± 0.1 ^{a}	77.6 ± 2.2 ^{a}	78.3 ± 0.5 ^{a}	80.0 ± 1.6 ^{a}	77.8 ± 1.2 ^{a}
T_{peak} (ºC)	84.2 ± 0.6 ^{b}	83.8 ± 1.0 ^{b}	84.2 ± 0.1 ^{b}	84.9 ± 0.1 ^{b}	87.5 ± 0.1 ^{a}	85.2 ± 0.4 ^{b}
ΔH (J/g)	6.7 ± 0.8 ^{a}	4.4 ± 0.4 ^{ab}	4.7 ± 1.0 ^{ab}	4.3 ± 0.3 ^{ab}	3.94 ± 0.9 ^{b}	6.1 ± 0.4 ^{ab}
Emulsifying Activity Index (m^2/g)	65.8 ± 4.2 ^{a}	44.2 ± 4.8 ^{b}	40.8 ± 1.0 ^{b}	23.4 ± 0.6 ^{c}	67.1 ± 0.8 ^{a}	24.8 ± 0.6 ^{c}
Emulsifying Stability Index (min)	1.3 ± 0.3 ^{b}	0.6 ± 0.0 ^{d}	1.0 ± 0.2 ^{c}	0.6 ± 0.1 ^{d}	1.7 ± 0.1 ^{a}	0.7 ± 0.1 ^{d}
Lipid Oxidation (TBARS, µg/100 g whole egg)	13.3 ± 0.6 ^{ac}	13.1 ± 0.5 ^{a}	12.9 ± 0.6 ^{a}	13.3 ± 0.4 ^{ac}	10.7 ± 0.3 ^{b}	13.8 ± 0.3 ^{c}

[1]MP: Moderate pressure; US: Ultrasound; TP: Thermal pasteurization.

These results are in line with the soluble protein decrement (**Table 7.2**), and probably occurred due to protein unfolding and aggregation [18], being supported by the findings of Monfort et al. and Souza and Fernández [7,13].

On the contrary, the US-only and MP-US-TP-treated LWE exhibited a lower viscosity compared to non-treated LWE (30 – 34 %, $p<0.05$), which was accompanied by a protein solubility increase. In fact, a previous study of Sheng et al. [17] suggested that mechanical energy and cavitation effect reduced the aggregation degree between the proteins molecules, decreasing viscosity of sonicated EW [17]. Moreover, the samples treated by MP-US-TP had a viscosity 2.3-fold lower than MP- and shorter TP-treated LWE ($p<0.05$), and no significant difference was found compared to US-treated LWE ($p\geq0.05$), suggesting that the effect observed for MP-US-TP can probably be attributed to the sonication effects. In this case, the initial viscosity increase induced by MP treatment appears to have been reversed (at pressures below 300 MPa, changes on protein structure caused by pressure are expected to be reversible) during sonication. Thus, further protein aggregation caused by thermal treatments occurred to a much lesser extent than when shorter TP was applied alone [15,17,19]. Analogous results were reported by Ashokkumar et al. [19], who found a reduction in viscosity of whey protein concentrate submitted to a pre-heat treatment (80°C/1 min) followed by sonication (for less than 5 min) and followed by a post-heat treatment (85°C/20 min). Furthermore, compared with commercial TP, a 2.3-fold less viscous LWE was obtained with the combined treatment, which could be an advantage in terms of industrial LWE processing (pumping, mixing, and so on).

7.4.2. Thermal properties

The thermal properties of LWE were studied in the temperature range from 30 to 120 °C at the rate of 10 °C/min (**Table 7.2**). As noted by other authors [20] only one major endotherm was found in LWE samples (see **Annex G – Figure G3**), and the peak was visible for all the treatments. The peak denaturation temperature of raw LWE did not change significantly after treatments, except for MP-only treatment (83.83 – 85.2 ºC, $p\geq0.05$), likely indicating a similar denaturation process for this protein fraction, and thus no significant changes in structural integrity caused by the treatments. The enthalpy variation (ΔH) of both TP-, US- and MP-US-TP-treated LWE did not show significant differences compared to raw LWE ($p\geq0.05$), but a decreasing trend was observed for TP and US treatments. However,

for MP-US-TP-treated LWE, it appears that US and shorter TP reversed the effects of MP, as reported above. Additionally, for the MP-only treatment was noticed a higher peak denaturation temperature and a lower *ΔH* compared to raw LWE ($p<0.05$), possibly suggesting a partial loss of protein structure in this sample [21]. Indeed, these observations were consistent with previous investigations, who stated a decrease in these parameters at pressures as low as 100 MPa [20,22].

7.4.3. Functional properties

In general, all treatments reduced the emulsifying properties of raw LWE ($p<0.05$), with exception of MP-only treatment that did not change the EAI ($p\geq0.05$), but increased the ESI ($p<0.05$) (**Table 7.2**). For MP-treated LWE, the observed increase in ESI could be due to the moderate protein unfolding [22], consistent with the protein solubility results. The lack of effect caused by MP on EAI was corroborated by the study of Khan et al. [23], when subjecting sweet potato protein-guar gum model to a MP treatment (200 MPa for 20 min at 25 ºC).

In addition, as previously reported in Chapter VI, the TP (shorter and commercial) significantly reduced both EAI and ESI ($p<0.05$), possibly as a result of proteins unfolding and aggregation, consequently hindering protein adsorption at interfaces [24]. As less aggregates adsorb at the interface, the emulsion stability reduces, since this property is related to the interfacial area that can be coated by proteins [25]. Dissimilar to our observations, Le Denmat et al. [24], highlighted that EY emulsifying properties was not affected at temperatures below 69 ºC.

An even higher decrement in emulsifying properties was found for LWE treated by US-only and MP-US-TP treatments (43 – 65 %), which could be a result of protein molecular flexibility changes [26]. Our findings were consistent with Zhou et al. [26], who also reported that a sonication treatment decreased the EAI of glycinin, even though the treatment improved the protein solubility. In opposition, several authors found a positive effect of sonication on emulsifying properties of EW and EY solutions [16,27]. Concerning the combined treatment, LWE presented similar emulsifying properties to US-treated LWE ($p\geq0.05$), suggesting that the effect of MP pre-treatment was countered by the subsequent sonication, as mentioned above, and the following treatment at 60 °C did not produced a

significant effect. Compared to commercial TP, lower emulsifying properties were obtained for MP-US-TP-treated LWE.

7.4.4. Secondary lipid oxidation

As an estimation of lipid oxidation extent caused by processing, the secondary lipid oxidation (TBARS, expressed as MDA content) was evaluated. As reported in Chapter VI, the measured TBARS values for raw LWE was 13.3 ± 0.6 μg MDA/100 g whole egg, similar to what was described by other authors [28]. TP and US treatments alone did not cause any significant effect on MDA content ($p>0.05$) in comparison to non-treated LWE (**Table 7.2**), what is corroborated by published data, using similar or stronger treatment conditions [10,29]. Compared to the non-treated LWE samples or those treated by commercial TP, the MP treatment alone caused a significant decrease in MDA content (reduction about 20 %, $p<0.05$) what may be due to the degradation or additional reactions among TBA-reactive substances, producing tertiary lipid oxidation products that are not detected by the TBARs method [30,31], whereas the combined treatment caused a slight increase ($p<0.05$) (**Table 7.2**), probably associated to a more pronounced contribution of the US treatment, as previously verified for protein solubility and viscosity. Literature data on the effect of pressure treatments on lipid oxidation can be considered somehow controversial. Some researchers have shown no significant effect on the TBARS values of pork as a result of MP treatment (up to 200 MPa) [32], although, other authors reported a secondary lipid oxidation increase in pressure treated coho salmon (200 MPa/30 sec) [33] or even a decreased in sliced cooked ham treated at 100 MPa during 8 h (at 30 ºC) [31].

7.4.5. Volatile profile

The composition of LWE samples regarding identified volatile compounds is listed in **Table 7.3**. To avoid the eventual formation of new volatiles induced by the temperature used in the extraction procedure, the volatiles were extracted at 45 ºC, at a lower temperature than that used for LWE TP (60 ºC). A total of 14 volatiles were found using headspace analysis of which 6 were identified.

In general, the total volatiles content of raw LWE decreased with US-only, MP-only and MP-US-TP treatments ($p<0.05$), with a more pronounced impact of the MP-only treatment

Table 7.3. Volatile profile of raw and treated whole egg, expressed in µg internal standard equivalents/100 g whole egg (mean ± standard deviation). Different letters along the same line indicate significant differences ($p<0.05$) between conditions.

Volatile compounds	RT (min)	Liquid whole egg (µg/100 g whole egg) [1,2]					
		Raw	Thermal Pasteurization		Ultrasound	Moderate Pressure	Combined Process (MP-US-TP) [3]
			60 ºC/3.5 min	60 ºC/1.75 min	50 %/1 min	160 MPa/ 5 min	160 MPa/ 5 min – 50 %/1 min – 60 ºC/1.75 min
2-methyl pentane	2.962	0.20 ± 0.06 [abc]	0.18 ± 0.04 [bc]	0.16 ± 0.02 [c]	0.23 ± 0.09 [abc]	0.32 ± 0.05 [ab]	0.33 ± 0.04 [a]
3-methyl pentane	3.088	0.24 ± 0.02 [b]	0.47 ± 0.06 [a]	0.27 ± 0.01 [b]	0.30 ± 0.06 [b]	0.36 ± 0.04 [ab]	0.32 ± 0.04 [b]
Hexane	3.242	9.52 ± 1.45 [a]	9.38 ± 2.85 [a]	12.32 ± 0.61 [a]	2.90 ± 0.33 [b]	2.72 ± 0.65 [b]	2.52 ± 0.25 [b]
Heptane	5.002	18.50 ± 8.80 [a]	24.26 ± 3.28 [a]	22.82 ± 0.54 [a]	2.13 ± 0.44 [b]	4.24 ± 1.11 [b]	12.91 ± 4.51 [ab]
Decahydro-2-methylnaphthalene	22.273	0.11 ± 0.01 [b]	0.19 ± 0.05 [a]	0.09 ± 0.02 [b]	0.08 ± 0.00 [b]	0.09 ± 0.02 [b]	0.10 ± 0.02 [b]
∑ Hydrocarbons	-	35.51 ± 9.78 [a]	41.06 ± 4.27 [a]	42.63 ± 0.59 [a]	8.42 ± 0.66 [b]	9.83 ± 1.66 [b]	19.47 ± 4.31 [b]
Toluene	7.498	133.22 ± 7.28 [a]	125.31 ± 10.51 [a]	113.08 ± 27.91 [a]	29.71 ± 1.15 [b]	3.60 ± 0.11 [b]	4.33 ± 0.22 [b]
∑ Total	-	168.73 ± 8.89 [a]	166.38 ± 6.34 [a]	155.71 ± 28.34 [a]	38.13 ± 1.53 [b]	13.43 ± 1.77 [b]	23.80 ± 4.50 [b]

[1]Values are from semi-quantification using cyclohexanone as internal standard; [2] Detection limit is 0.01 µg/100 g whole egg; [3] MP: Moderate pressure; US: Ultrasound; TP: Thermal pasteurization.

(– 92 %), while both TP (shorter and commercial) did not change the total volatiles content ($p \geq 0.05$). The volatile profile of LWE was essentially composed by hydrocarbons, being heptane and hexane the main ones, as also identified in egg by other authors [34]. A significantly lower content of these compounds (3.5 – 8.7-fold) was found in MP- and US-treated LWE compared to raw and both TP-treated LWE ($p<0.05$), whereas in MP-US-TP-treated samples only hexane content significantly decreased. The other identified hydrocarbons compounds were 2-methyl pentane, 3-methyl pentane and decahydro-2-methylnaphthalene, the latter being already reported in a previous study [35]. In general, for these compounds, no significant differences were found between raw and treated samples ($p \geq 0.05$). In addition, a 1.9-fold increase in 2-methyl pentane was observed in LWE treated by MP-US-TP in comparison to the commercial TP-treated LWE, while the first presented a lower content of 3-methyl pentane and decahydro-2-methylnaphthalene (1.5 – 1.9-fold lower) ($p<0.05$). Thus, the sum of hydrocarbons was up to 80 % lower ($p<0.05$) in US-, MP- and MP-US-TP-treated LWE than in the other samples. Yet, due to high odour threshold values of hydrocarbons, significant differences in their contents probably do not originate important changes in aroma perception [36]. Toluene was the compound found in highest amount in raw and both TP-treated LWE ($p \geq 0.05$), being previously identified by other authors [34,37]. A decrease of 97 % was found in MP- and MP-US-TP-treated LWE compared to raw and both TP-treated samples ($p<0.05$), however, this decrement was much higher than the produced by US-only treatment (about 78 %, $p<0.05$). The results obtained for MP-treated samples were consistent with Contador et al. [38] work, who noticed a reduction of aliphatic hydrocarbons and toluene after application of a pressure treatment during 6 min. Otherwise, US treatment increased hydrocarbons content in spiced beef using more severe treatment conditions [36], and the occurrence of pyrolysis when cavitation bubbles collapse is a possible pathway to generation and degradation of volatile compounds [39].

In comparison with non-treated LWE, compounds such as aldehydes, alcohols and ketones, mainly found in cooked eggs and egg samples submitted to extractions at higher temperatures and longer times [32,40], were not found in treated samples, since the treatments applied were not strong enough to induce its production. Thus, the results clearly show that the LWE volatile profile was significantly more affected by MP-US-TP treatment than commercial TP, the former being characterized by the low compounds content.

7.4.6. Principal component analysis

The measured parameters for the treated LWE samples were submitted to a principal component analysis (PCA) in order to obtain complementary information and to identify variables responsible for most of the data variability caused by the different treatments. Regarding volatile compounds composition, as shown in **Figure 7.5**, the first two principal components (PC1 and PC2) accounted for 65.29 and 20.63 % of the total variance, respectively. The results showed three well-defined groups, corresponding to: (1) commercial TP-treated LWE, (2) raw and shorter TP-treated LWE and (3) US-, MP- and MP-US-TP-treated samples. The group 1 is mainly influenced by 3-methyl pentane and decahydro-2-methylnaphthalene content, while the group 2 was differentiated by the highest amount of hexane, toluene and hydrocarbons compounds (**Table 7.4**). Moreover, the group 3 was characterized principally by the lowest compounds content.

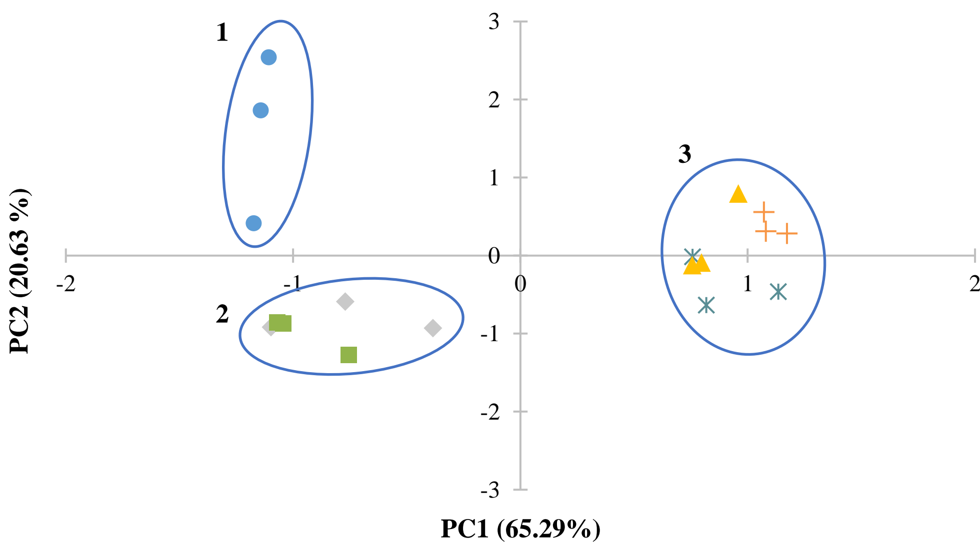

Figure 7.5. Principal component analysis (PCA) score plot of volatile compounds of raw and treated whole egg. The principal components (PC) explain 85.92 % of the total variance of the data.

Table 7.4. Loadings of the variables in the first two principal component (PC) analysis of volatile compounds in liquid whole egg samples.

Compounds	Principal Components	
	PC 1	**PC 2**
2-methyl pentane	0.732	0.211
3-methyl pentane	-0.092	0.935
Hexane	-0.929	-0.246
Heptane	-0.898	0.056
Decahydro-2-methylnaphthalene	-0.491	0.812
Toluene	-0.949	-0.054
Hydrocarbons compounds	-0.959	-0.049

PCA was also performed taking into consideration the other measured properties. **Figure 7.6** shows the factorial coordinate diagram representing the score plots of pH, colour, protein solubility, viscosity, thermal properties, emulsifying properties, secondary lipid oxidation plus volatile compounds. As shown, the first two principal components explained 38.55 and 23.12 % of the total variance, respectively, observed for the raw and treated LWE samples, discriminating three separate groups of points corresponding to: (1) raw, shorter TP- and commercial TP-treated LWE (2) MP-treated LWE; and (3) US- and MP-US-TP-treated LWE. The raw and both TP-treated samples were discriminated from the other samples by changes in pH, ΔH, TBARS and protein solubility (**Table 7.5**), while the group 2 was clearly differentiated by the lowest pH, TBARS and ΔH. In addition, the samples submitted to US-only and MP-US-TP treatments were discriminated from the others mainly by the highest protein solubility and 2-methyl pentane content.

Therefore, the results show that the TP-treated LWE caused less changes (negative) in LWE properties than the other treatments evaluated, whereas the samples treated by MP-only treatment were the most distinct. Consequently, these results indicate that US-only, MP-only or MP-US-TP treatment induced overall more pronounced changes (negative) in the properties of raw LWE than thermal treatments.

7.5. Conclusions

Considering raw LWE as a reference, treatments such as TP and MP decreased protein solubility with a consequent increase in viscosity, while the US treatment and the MP-US-

TP combination improved protein solubility and reduced viscosity of LWE. However, the US-only and MP-US-TP treatments also negatively affected the LWE emulsifying properties, whereas the MP treatment alone slightly improved them. Considering that any selected treatment should not significantly influence the volatile profile of the LWE, none of the studied treatments fulfilled this requirement, with exception of shorter TP. Clearly, based on the studied parameters, it is difficult to select a particular treatment that allows beneficial effects without adversely affecting certain LWE properties. However, the MP-US-TP combined treatment could be an effective means to pasteurize LWE, and the application of MP (160 MPa/5 min) followed by US (50 % amplitude/1 min) before a shorter TP (60 °C/1.75 min) permitted to obtain a similar safety level to the commercial TP (60 °C/3.5 min).

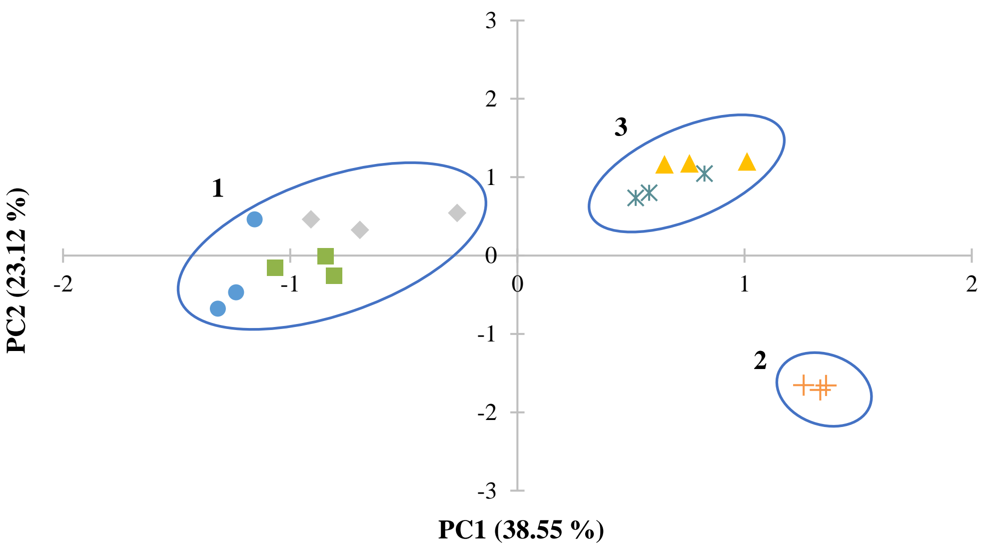

Figure 7.6. Principal component analysis (PCA) score plot of thermal, physicochemical and functional properties and lipid oxidation plus volatile compounds of raw and treated whole egg. The principal components (PC) explain 61.67 % of the total variance of the data.

Table 7.5. Loadings of the variables in the first two principal component (PC) analysis of thermal, physicochemical and functional properties and lipid oxidation plus volatile compounds in liquid whole egg samples.

Compounds	Principal Components	
	PC 1	PC 2
pH	-0.809	0.558
L*	-0.326	-0.156
a*	-0.008	-0.207
b*	-0.446	-0.029
Protein solubility	0.398	0.868
Lipid oxidation	-0.238	0.931
Emulsifying activity index	-0.083	-0.684
Emulsifying stability index	0.371	-0.606
Viscosity	-0.518	-0.794
T_{peak}	0.807	-0.459
ΔH	-0.144	0.621
2-methyl pentane	0.748	0.011
3-methyl pentane	-0.116	-0.481
Hexane	-0.902	-0.114
Heptane	-0.876	-0.029
Decahydro-2-methylnaphthalene	-0.489	-0.281
Toluene	-0.936	-0.056
Hydrocarbons compounds	-0.936	-0.047

7.6.References

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Chapter VIII

Concluding Remarks and Future Work

8.1.Conclusions

As a possible alternative to commercial egg products thermal pasteurization (TP), the sequential combination of pressure and/or ultrasound (US) pre-treatments, singly or combined, before a shorter TP (compared to commercial TP) was studied, for three different egg products: egg white (EW), egg yolk (EY) and liquid whole egg (LWE). The observed effects on the inactivation of *Salmonella* Senftenberg 775/W and *Listeria innocua,* and on selected quality properties were dependent on the type of egg product, thus pointing out the importance of the food matrix.

Regarding the EW samples, the application of moderate pressure (MP) pre-treatments before a shorter TP (MP-TP) decreased *S.* Senftenberg 775/W thermal resistance, and a similar behaviour was observed when inoculated in EY and LWE samples. Although, for both MP-TP and commercial TP treatments, *S.* Senftenberg 775/W showed a higher resistance to inactivation in EY followed by LWE and EW. On the contrary, for *L. innocua*, a lower resistance to inactivation was found when inoculated in EY compared to EW or LWE. In addition, during post-TP storage, *S.* Senftenberg 775/W counts decrease in all egg product samples, while an increase of *L. innocua* population was observed in LWE samples, independently of the treatment, showing that the remaining cells were able to grow under refrigerated conditions. Thus, despite the significant effect of the food matrix on microorganisms' inactivation, it was possible to achieve, for the three egg products, a MP-TP combination that originated a lethal effect, at least, similar to commercial TP.

On the other hand, for the three egg products, the MP-TP and commercial TP did not cause significant changes in the pH and colour, compared to the unprocessed samples, but induced a decrease in soluble protein which was accompanied by a viscosity increase. The effects on MP-TP-treated samples are mainly due to the heat treatment, probably due to protein unfolding and consequent aggregation of unfolded proteins, although, in LWE samples, the MP pre-treatment (applied at higher pressure than that used in EW and EY) might also have an impact on viscosity.

Concerning foaming and emulsifying properties, the treatments (MP-TP and commercial TP) reduced EW foaming capacity and improved EY emulsifying properties (compared to non-treated samples), while for LWE a contrary behaviour was showed. These differences are possibly due to the different treatment conditions used for each egg product, and also to the type of protein aggregates formed. In the whole egg, yolk and white proteins are present

and different aggregates can be formed, compared to what can happen for yolk or white proteins alone. Heat-induced gels prepared with MP-TP-treated samples (EW and LWE) showed higher hardness and rigidity compared to the gels produced from raw samples, which may be related the pressure effects (MP pre-treatment) on protein structure. The effects of treatment were more pronounced for LWE heat-induced gels, and the same was verified for the gels produced with commercial TP-treated LWE.

The volatile profile of egg products (EW and EY) was mainly composed of hydrocarbons followed by aromatic compounds, with the EW samples exhibiting the highest content. The commercial TP and MP-TP treatments increased the total volatile content (compared to non-treated samples), with the former showing a greater effect on EW samples, while the MP-TP caused a more pronounced increment in EY samples.

EY fatty acid profile and total carotenoids remained stable after MP-TP treatments, but these samples presented a higher secondary lipid oxidation than non-treated samples, possibly due to the thermal effects. For LWE, the MP-TP treatment decreased total carotenoids content, probably related to the MP pre-treatment, without inducing secondary lipid oxidation. The differences may be due to the different treatment conditions used for each egg product, for instance, the use of a stronger MP pre-treatment and a less intense TP (200 MPa/5 min – 60 °C/1.75 min) to treat LWE compared to the conditions used for EY (90 MPa/5 min – 60 °C/3 min). However, the matrix is another important factor, also taking into account the effects observed for egg products treated by commercial TP, it was shown that EY was more susceptible to oxidation than LWE.

Furthermore, the sensory analysis of an egg-derived product was assessed for the three egg products, showing that the “Doce de ovos” produced with EY stood out from all the other samples, presenting the highest global acceptability (MP-TP-treated EY), followed by meringues (EW) and egg tart (LWE).

Overall, the results of MP-TP treatments suggest that in addition to the thermal effects, the MP pre-treatment was also responsible for some of the observed changes (viscosity, functional properties, volatile profile and total carotenoids). However, despite the combined treatments impairing some egg properties (compared to non-treated samples), samples treated by MP-TP showed similar and/or improved physicochemical and functional properties when compared to the commercial TP-treated samples.

Considering the use of US pre-treatment before a shorter TP (US-TP) to pasteurize egg product, a decreased in *S.* Senftenberg 775/W thermal resistance were observed only in EW and EY samples. For LWE, only the combination of MP followed by US pre-treatments before a shorter TP (MP-US-TP) showed a synergy, pointing out that the order of pre-treatments (US-MP-TP or MP-US-TP) had a significant effect on *S.* Senftenberg 775/W inactivation. Regarding MP-US-TP-treated LWE properties, opposite effects were observed for soluble protein and viscosity when compared to commercial TP- and MP-TP-treated LWE, with a less pronounced reduction in the emulsifying properties of the latter samples. The LWE volatile profile differs from the EW and EY profiles by the higher content of total volatile compounds, being the toluene the major compound present. However, MP-US-TP combination decreased the content of LWE total volatile compounds, while MP-TP increased the total volatile content of EW and EY samples. In addition, as observed for EY samples, the commercial TP had no effect on LWE volatile profile. So, the MP-US-TP effects are probably associated with a more pronounced contribution of the US treatment, suggesting that US countered the MP effects, and the subsequent shorter TP had no significant effect.

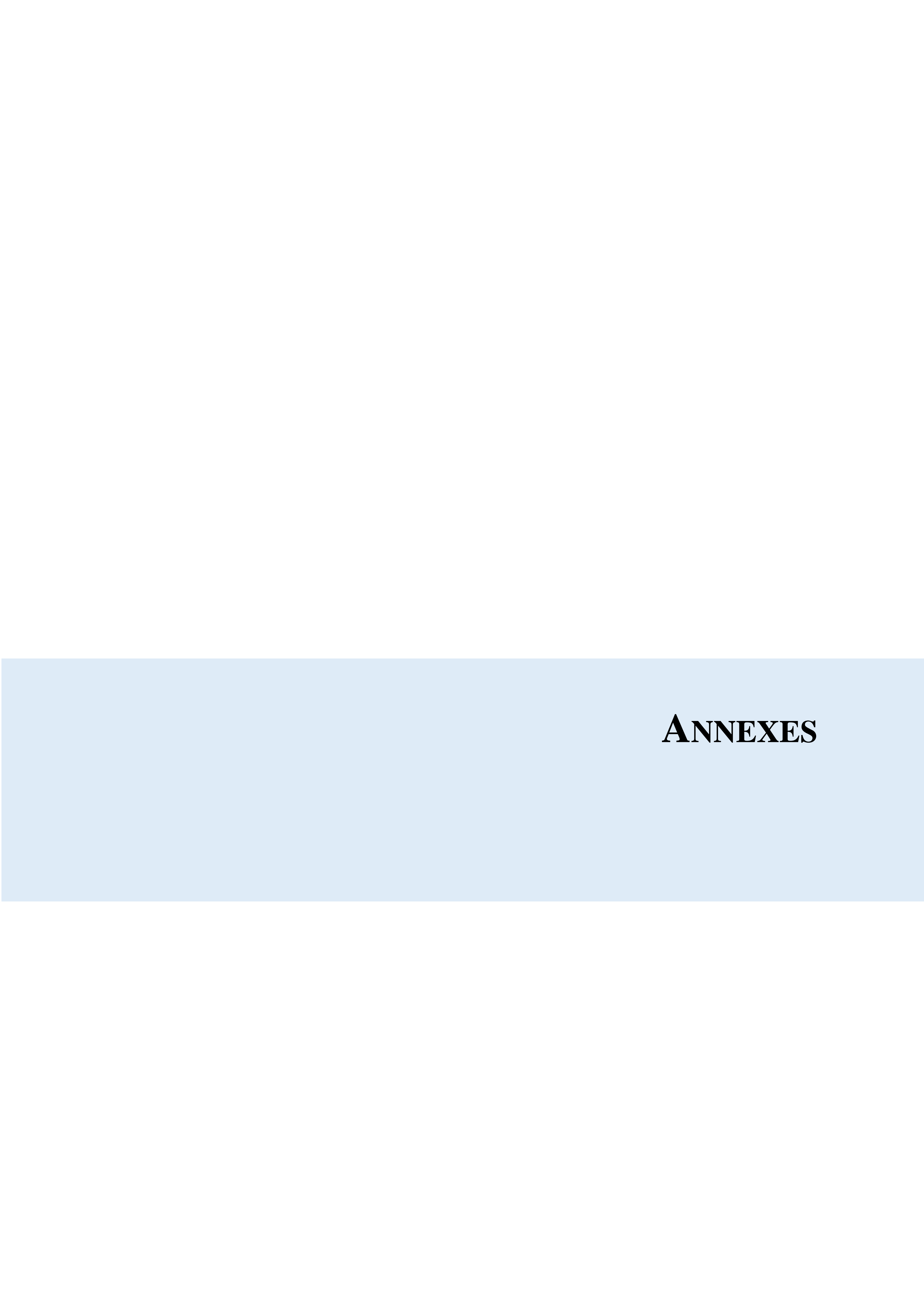
Therefore, the heat sensitization effect induced by MP pre-treatments on *S.* Senftenberg 775/W population allows the reduction of TP time and could be an alternative to commercial egg products TP, offering a similar safety level against *S.* Senftenberg 775/W and also maintaining or improving egg quality properties. This strategy avoids the severity of one technique alone, and industrial implementation would only need to add the pre-treatment technology upstream pasteurization, without a need to scale up.

8.2.Future work

The promising results obtained in this work indicate that the sequential combination of moderate pressure and/or ultrasound followed by a shorter thermal pasteurization may be a possible alternative to commercial egg products thermal pasteurization. However, further research is needed to improve our understanding of the effect of these treatments on liquid egg products such as:

- ✓ Evaluation of the effect of treatments (immediately after treatment and during storage) on other foodborne egg-associated microorganisms, such as *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, among others.

- ✓ Testing the combination with other processing technologies, such as pulsed electric fields technology, namely combined with the shorter thermal pasteurization.
- ✓ Conducting a shelf-life study of liquid egg products after combined treatments, as well as, the evaluation of their properties during storage.
- ✓ Deeper evaluation of the effect of treatments on the characteristics of egg proteins, namely changes in their structure, and on the structural characteristics of the possible formed aggregates.
- ✓ Better assessment of other parameters such as primary and tertiary lipid oxidation, triglycerides, phospholipids and cholesterol composition, and carotenoids content (lutein and zeaxanthin).
- ✓ Study the impact of treatments on nutritional value, for example, on the content of vitamins and amino acids and evaluation of digestibility in vitro.
- ✓ Assessment of possible changes in egg proteins allergenicity.
- ✓ Conducting a sensory analysis with a trained panel, and other products of interest obtained from liquid egg products.



ANNEXES

Annex A

Table A1. Effect of high pressure treatments on microorganisms' inactivation in liquid whole egg.

Microorganism	Egg product	Pressure (MPa)	Time (min)	Temperature (ºC)	Log $_{10}$ reductions	References
Salmonella Enteritidis SE-4	Liquid whole egg	300, 350, 400	10 – 30	25	4.06 – 5.96	[1]
Salmonella Enteritidis PT4 E10	Liquid whole egg	250	5 – 15	20	1.47 – 3.66	[2]
Salmonella Enteritidis CB 919 Lux AB	Liquid whole egg	138	8, 4+4, 2+2+4, 1+1+2	20	0.20 – 0.59	[3]
Salmonella Enteritidis	Liquid whole egg	350, 400, 450	5, 10, 15, 5+5, 5+5+5	20	1.96 – 7.96	[4]
Listeria innocua CECT 910	Liquid whole egg	300, 350, 400, 450	5 – 15	20	0 – 6.63	[5,6]
Listeria innocua BGA 3532	Liquid whole egg	200, 250, 300	3 – 30	20	0.2 – 0.6	[7]
Listeria seeligeri NCTC 11289	Liquid whole egg	250, 300, 350, 400	0.38 – 14.8	25	0.0 (1) $IC_{400\ MPa}$	[8]
Escherichia coli K12 DH 5α	Liquid whole egg	250, 300, 350, 400	0.38 – 14.8	25	0.2 – 0.6 $IC_{400\ MPa}$	[8]
Escherichia coli K12 DH 5α	Liquid whole egg	200, 250, 300	3 – 30	20	0.7 – 1.5	[7]
Escherichia coli CECT 405	Liquid whole egg	300, 350, 400, 450	5, 10, 15, 5+5, 5+5+5	20	0.39 – 7	[5,9]
Escherichia coli ATCC 25922	Liquid whole egg with 10 % of NaCl	400, 600, 800	5	25	> 5	[10]

Escherichia coli	Liquid egg white	300	2+2+2, > 10	20	Up to 7	[11]
Staphylococcus aureus A110	Liquid whole egg with 10 % of NaCl	400, 600, 800	5	25	0.2 – 2.7	[10]
Pseudomonas fluorescens DSM 50090	Liquid whole egg	250, 300, 350, 400	0.38 – 14.8	25	< 5.0 $IC_{400\ MPa}$	[8]
Paenibacillus polymyxa DSM 36	Liquid whole egg	250, 300, 350, 400	0.38 – 14.8	25	< 5.0 $IC_{400\ MPa}$	[8]
Streptococcus faecalis QM-2	Liquid whole egg with 10 % of NaCl	400, 600, 800	5	25	1.0 – 2.5	[10]

(1)$IC_{400\ MPa}$: Instantaneously coagulation at 400 MPa.

Table A2. Effect of high pressure treatments on physiochemical and functional properties of egg products and egg derivatives.

Egg product	Conditions	Effects/Results	References
Liquid whole egg	0 – 551.58 MPa, 0 – 9 min, 22 ºC	LWE appearance began to change at 344.74 MPa Electrophoretic protein pattern changed at ≥ 275.79 MPa Protein coagulation or denaturation occurred at ≥ 413.69 MPa Ovomacroglobulin faded	[12]
Liquid whole egg	350 – 550 MPa, 5 – 15 min, ~ 20 ºC	Increase in viscosity, L*, a*, b*, foam density, WHC, *G'* and *G"* with pressure increase FS increase until 450 MPa Decrease in foam density, FS and WHC with increasing time Reduction of ΔH value and flow behaviour with pressure and time increase Crossover of *G'* and *G"* (gel point) at 550 MPa/10 and 15 min Increase inconsistency coefficient with pressure and time	[13,14]
Liquid whole egg	100 – 400 MPa, 30 min, 20 ºC	Coagulation started at 250 MPa Irreversible protein coagulation Thixotropic fluid behaviour No effect on native protein components	[15]
Liquid whole egg	100 – 400 MPa, 0 – 60 min, 25 ºC	No coagulation at 100 and 150 MPa until 3600 sec Almost instantaneously coagulation at 400 MPa	[16]
Liquid egg yolk	100 – 500 MPa, 10 min, 25 ºC	Decrease in solubility, surface hydrophobicity, SH groups content, ESI, and ΔH Increase in apparent viscosity Increase in ESI at 100 MPa, followed by a decrease Gel was formed at 500 MPa Gradual increase in peak temperature up to 400 MPa	[17]

Liquid egg yolk	100 – 400 MPa, 30 min, 20 ºC	Thixotropic fluid behaviour No effect on native protein components Increase in yield stress and consistency coefficient Decrease in flow behaviour	[15]
Liquid egg white	100 – 400 MPa, 10 min, 18 ºC	Improved FC and FS (maximum at 350 MPa) Decrease in protein solubility and surface hydrophobicity Increase in exposed SH groups content (maximum at 350 MPa)	[18]
Liquid egg white	200 – 400 MPa, 5 – 15 min, 24 ºC	Remained in a liquid state up to 400 MPa No substantial effect on foam volume and amino acids content Decrease in FS with increasing pressure No important change in gel strength Decrease in elasticity modulus at 400 MPa	[19]
Liquid egg white	100 – 400 MPa, 30 min, 20 ºC	No effect on native protein components Thixotropic fluid behaviour Protein unfolding start above 200 MPa Complete aggregation and precipitation at 300 MPa Irreversible protein coagulation Decrease in yield stress and consistency coefficient	[15]
Liquid egg white	350 – 550 MPa, 5 – 15 min, ~ 20 ºC	Increase in *G'*, *G"* and consistency coefficient with pressure and time increase Increase in turbidity, L*, b*, viscosity, foam density and WHC with pressure increase FS increased up to 450 MPa Decrease in foam density, FS and WHC with increasing time Reduction of ΔH value and flow behaviour with pressure and time increase Decrease in a* with increasing pressure EW became a self-holding translucent gel at 550 MPa/15 min EW coagulated and changed from clear liquid to opaque First peak disappeared at 550 MPa/10 and 15 min	[13,14]

Egg white solution with 10 % NaCl or sucrose (w/w)	400 – 600 MPa, 5 – 15 min, 25 ºC	Sucrose and NaCl addition prior to HP treatments prevented gel formation Increase in viscosity Foam density increase with NaCl and decrease with sucrose Foam consistency decrease with NaCl and increase with sucrose Decrease in gel strength with pressure and additives addition Proteins presents in treated albumen was similar to the fresh albumen Increase in susceptibility to trypsin with increasing pressure	[10]

(1)*ΔH*: Enthalpy of denaturation; EAI: Emulsifying activity index; ESI: Emulsion stability index; EW: Egg white; FC: Foaming capacity; FS: Foaming stability; *G'*: Elastic modulus; *G"*: Viscous modulus; HP: High pressure; LWE: Liquid whole egg; SH groups: Sulfuryl groups; WHC: Water holding capacity.

Table A3. Effect of ultrasound treatments on microorganisms' inactivation in liquid whole egg.

Microorganism	Egg product	Power (W)	Time (min)	Temperature (ºC)	Volume (mL)	Log $_{10}$ reductions	References
Salmonella Enteritidis H4267	Liquid whole egg	–	1 – 30	< 20 °C	25	0 – 2.3	[20]
Salmonella Enteritidis CB 919 Lux AB	Liquid whole egg	40	1 – 3	20	12.5	0.17 – 0.28	[21]
Salmonella Typhimurium ATCC 14028	Liquid whole egg	–	15 – 30	20	–	< 0.5	[22]
Listeria seeligeri NCTC 11289	Liquid whole egg	24.6, 34.6, 42.0	5	< 30 ºC	10	No inactivation	[23]
Escherichia coli K12 DH 5α	Liquid whole egg	24.6, 34.6, 42.0	5	< 30 ºC	10	1 – 2	[23]

Table A4. Effect of ultrasound treatments on physiochemical and functional properties of egg products and egg derivatives.

Egg product	Conditions	Results	References
Liquid whole egg	80 % amplitude, 1 – 30 min, not exceed 20 °C, 20 kHz	Similar L* and lighter colour Decrease a* and b*	[20]
Liquid egg white	0 – 480 W, 10 min, not exceed 25 ºC, 20 Hz, 200 mL	Increase in FC Reduction of viscosity, FS and surface tension Increase followed by a decrease in solubility, exposed SH groups and surface hydrophobicity Modification of aggregation state and surface morphology	[24]
Liquid egg white	80 W, 5 – 15 min, 20 ºC, 40 kHz	No significant change in pH Increase in FC Decrease in FS	[25]
Egg white thick fractions	17 W, 12 – 30 min, 55 Hz	Increase in turbidity Degradation of three-dimensional protein network Intact protein denaturation thermograms	[26]
Egg white proteins	600 – 1200 W, room temperature, 15 min, 40 kHz, ≈ 360 mL (Ultrasonic bath) 15 min, 25 ºC, 20 kHz, ≈ 360 mL (Probe-type)	Increase in total and exposed SH groups Decrease in particle size and particle size distribution	[27]
Egg white proteins solution (0.1 – 10 wt.%)	95 % amplitude, 0.25 – 2 min, 20 kHz, ≈ 50 mL	Reduction of pH, protein particle size, aggregate size and intrinsic viscosity Improved emulsifying properties No differences in primary structure Changes in emulsion droplet size	[28]

Egg white solutions (10 % w/w)	5 – 20 min, 25 ºC, 20 kHz, ≈ 360 mL (Ultrasound probe)	No significant effect in pH and exposed SH groups Improved foaming and emulsifying properties; antioxidant and antimicrobial activities Increase in solubility, total SH groups and digestibility by alkalase Modification of proteolytic pattern	[29]
Egg white solutions (10 % w/w)	40 % amplitude, 2 – 20 min, 25 ºC, 20 kHz, ≈ 180 mL	Increase in solubility, zeta potential, foaming and emulsifying properties Decrease in particle size diameter and polydispersity index value Increase followed by a decrease of hydrolysis degree, total and exposed SH groups Surfaces consistently smooth with some concavities Changes in proteolytic pattern and protein secondary structure	[30]
Egg white solutions (10 % w/w)	20 % amplitude, 20 min, 20 kHz, 5 mL	Increase in surface hydrophobicity, ES and particle size distribution Decrease in solubility, foaming properties, apparent viscosity and consistency index No significant change in total SH groups, conductivity and transition temperatures No exposed SH groups found Peaks temperature were not significantly modified Gels with predominates elastic behaviour No evident changes in the gel structure EW microstructure seemed strongly aggregated	[31,32]
Egg yolk solution (1:1)	0 – 300 W 10 min (ultrasound for 3 s, stop for 3 s) 80 mL	Increase in FC, EA, free SH groups and zeta-potential Decrease in FS and average particle size Increase followed by a decrease in ES and gel properties Decrease followed by an increase in surface hydrophobicity No obvious changes in EY protein bands Aggregation of yolk LDL Partial dissociation of yolk granules.	[33]

Ovalbumin solution (5 % (w/v) total solids)	60 – 90 % amplitude, 20 – 40 min, 20 kHz, ≈ 100 mL	Increase in FC, EA, surface hydrophobicity, exposed SH groups, particle size and polydispersity index Decrease in surface net charge No significant change in FS, ES, molecular weight and secondary structure Partly unfold and change in tertiary structure Higher gelation temperatures	[34]

[(1)]EA: Emulsifying activity; ES: Emulsion stability; EW: Egg white; EWP: Egg white proteins; FC: Foaming capacity; FS: Foaming stability; LDL: Low-density lipoprotein; SH groups: Sulfuryl groups.

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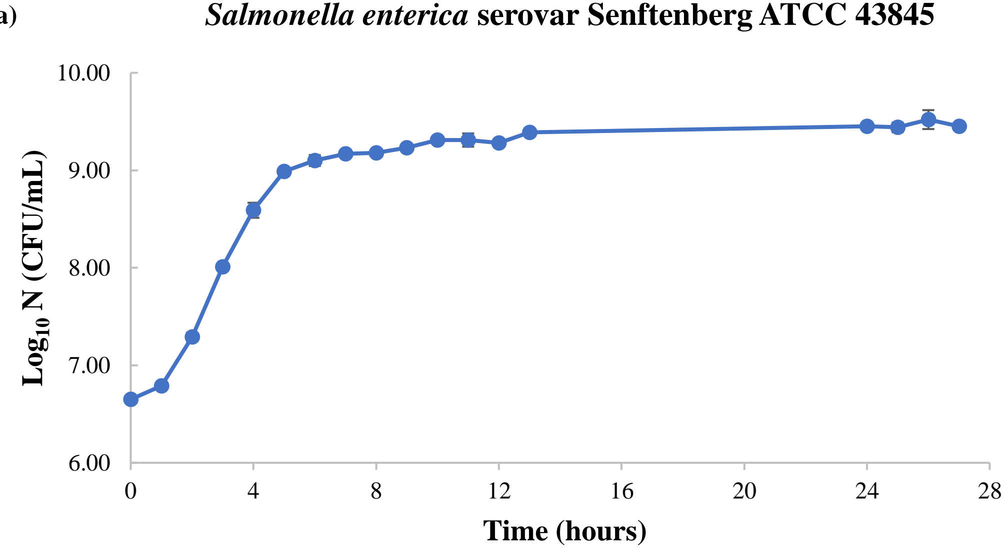
Annex B



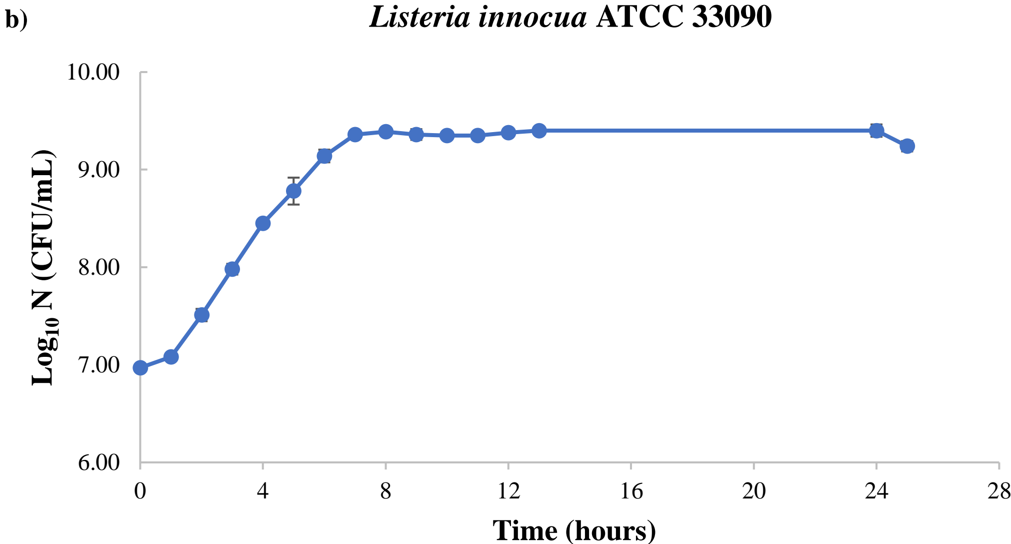


Figure B1. Growth curves (Log_{10} N vs time) of *Salmonella enterica* serovar Senftenberg (ATCC 43845) (a) and *Listeria innocua* (ATCC 33090) (b).

Annex C

Table C1. Standard curve equation and linear correlation parameters of Bradford microassay.

Parameter	Standard curve equation	R^2	Used for results from:
Bovine serum albumin (BSA) [x = g BSA/L y = absorbance (595 nm)]	y = 0.772x + 0.049	0.997	Chapter IV
	y = 0.903x + 0.022	0.996	Chapter V
	y = 0.810x + 0.009	0.991	Chapter VI
	y = 0.831x + 0.006	0.996	Chapter VII

Table C2. Standard curve equation and linear correlation parameters for lipid secondary oxidation determination.

Parameter	Standard curve equation	R^2	Used for results from:
TBARS [(1)] [x = μM MDA y = absorbance (538 nm)]	y = 0.051x + 0.003	0.999	Chapter V
	y = 0.051x + 0.004	0.999	Chapter VI
	y = 0.053x + 0.003	1.000	Chapter VII

[(1)]TBARS: Thiobarbituric acid-reactive substances; MDA: Malondialdehyde.

Annex D

Figure D1. Images of egg white samples packaged after each treatment. From left to right: raw egg white, egg white treated by commercial thermal pasteurization (55.6 ºC/6.2 min), shorter thermal pasteurization (55 ºC/3 min), combined treatments at 50 MPa/5 min – 55 ºC/3 min and 90 MPa/5 min – 55 ºC/3 min.

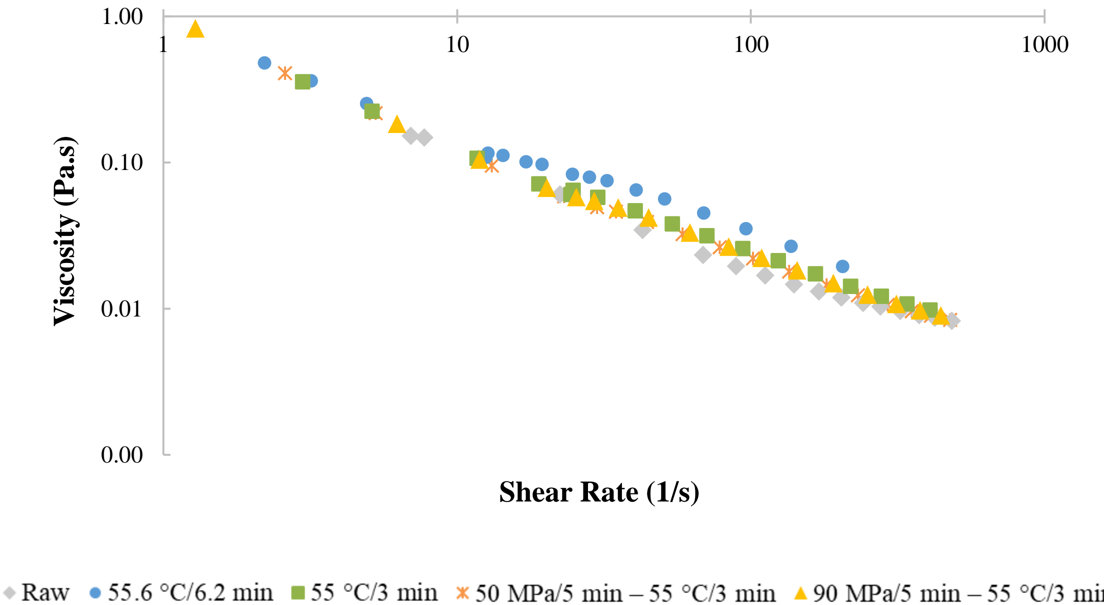


Figure D2. Flow chart of raw egg white and egg white treated by thermal pasteurization and combined processes (moderate pressure followed by thermal pasteurization).

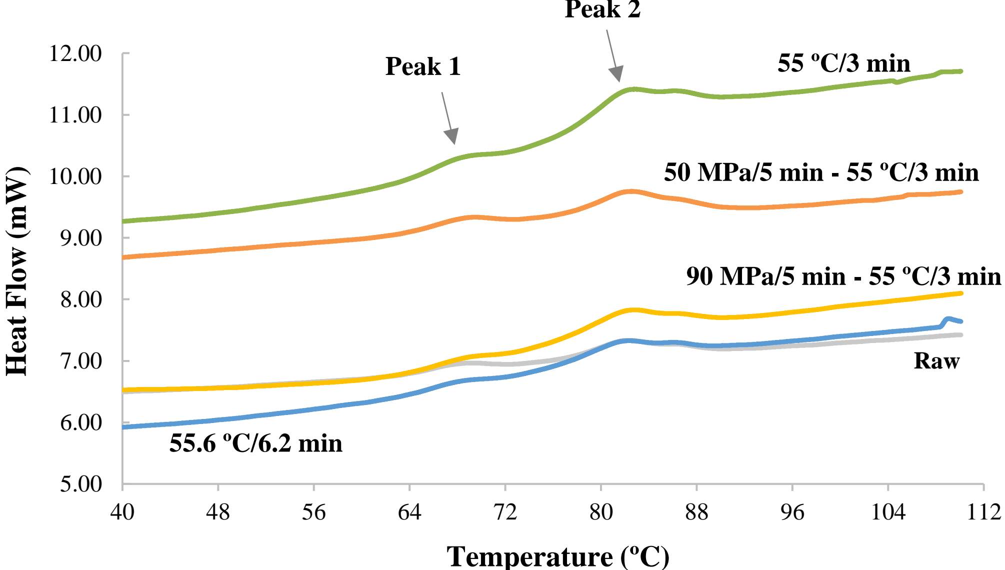


Figure D3. Differential scanning calorimetry (DSC) thermogram of raw egg white and egg white treated by thermal pasteurization and combined processes (moderate pressure followed by thermal pasteurization). Samples were heated from 5 to 110 °C at a rate of 10 C/min. Thermogram is a representative run.

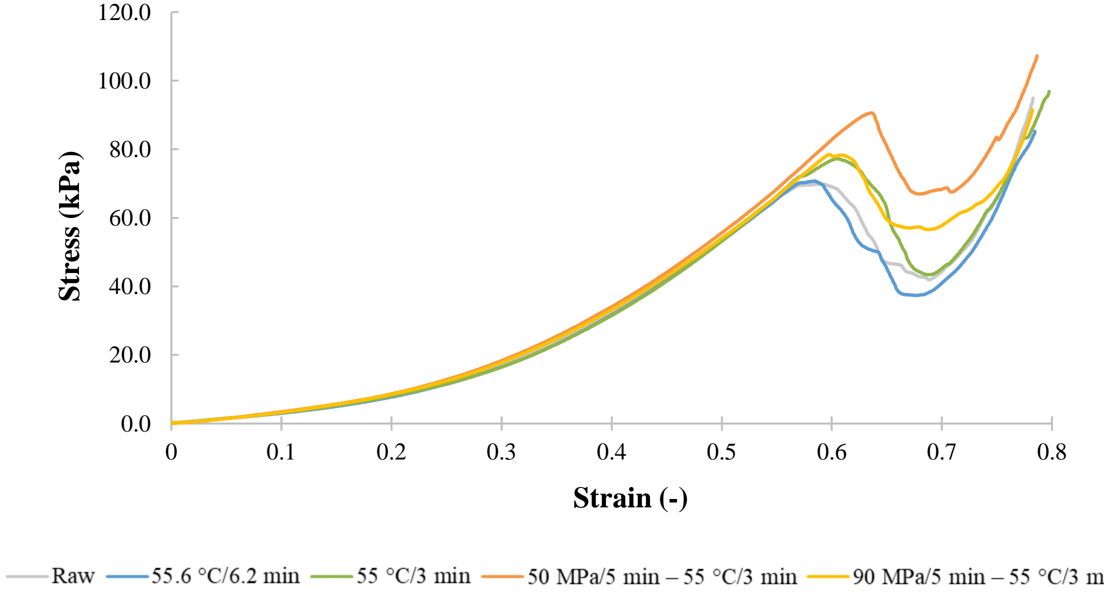


Figure D4. Examples of typical stress-strain curves obtained for egg white heat-induced gels prepared with raw and treated egg white.

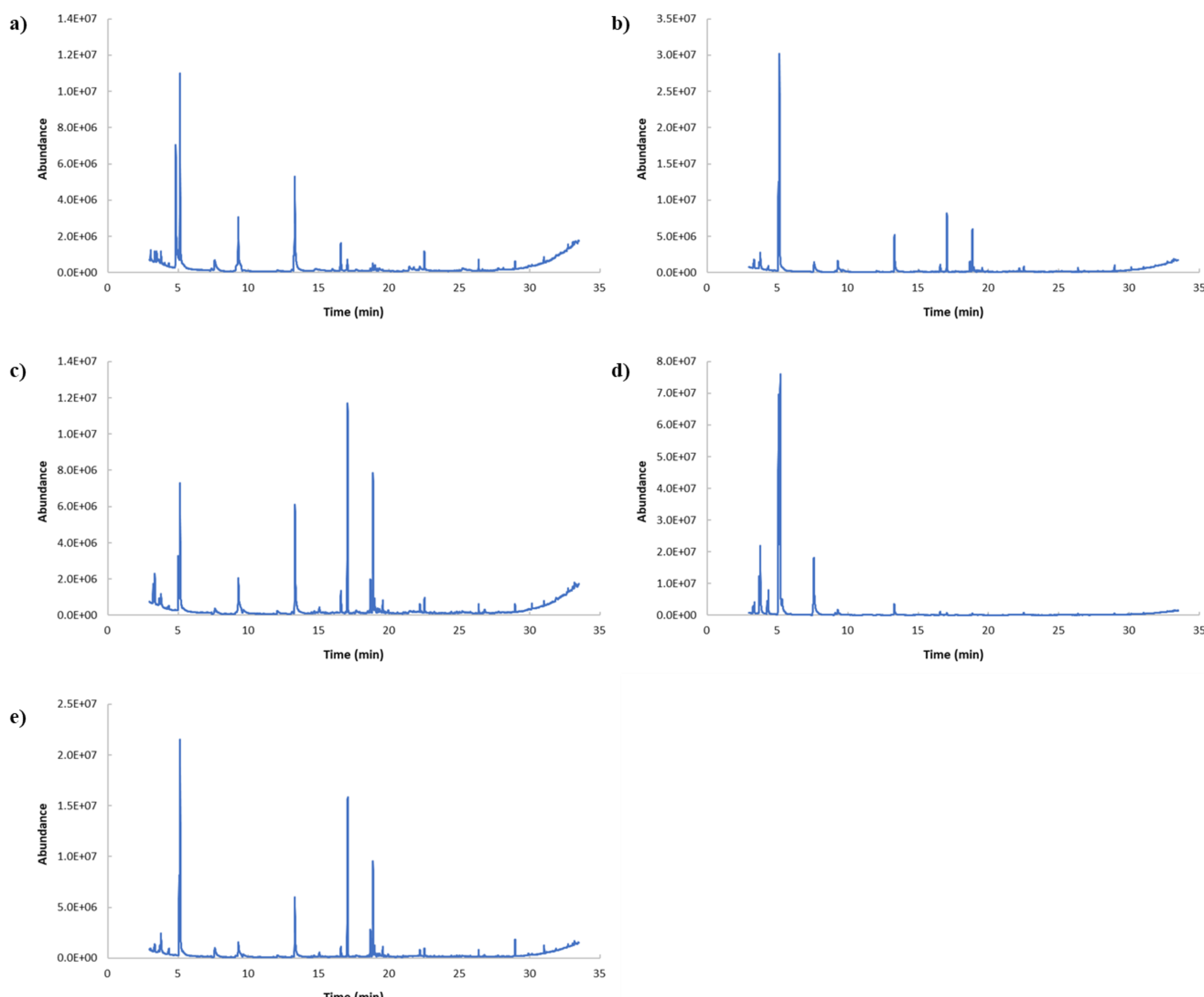


Figure D5. Examples of chromatograms of volatile compounds of raw egg white (a) and egg white treated by thermal pasteurization (55.6 ºC/6.2 min (b) and 55 ºC/3 min (c)), and combined processes (50 MPa/5 min – 55 ºC/3 min (d) and 90 MPa/5 min – 55 ºC/3 min (e)). Chromatogram is a representative run.

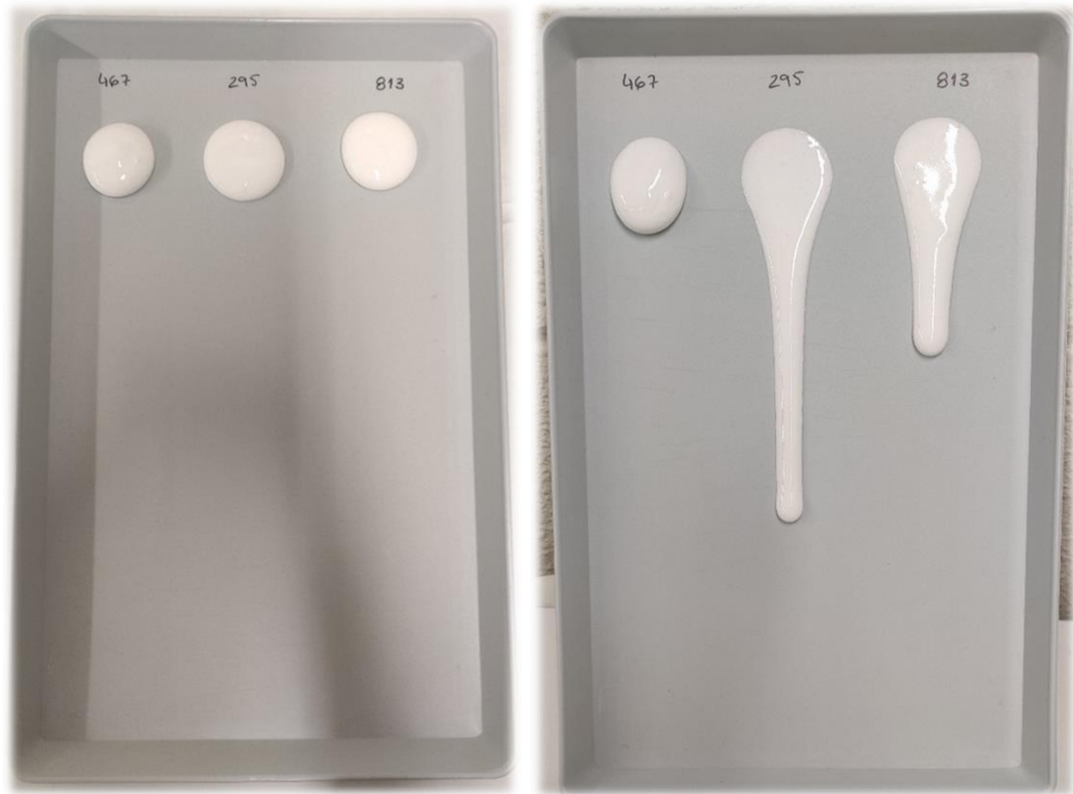


Figure D6. Images of meringue dough prepared with treated and non-treated egg white and its consistency (on an inclined plane, 45 º). From left to right: 467 – meringue batter produced with non-treated EW; 295 – meringue batter produced with EW treated at 55.6 ºC/6.2 min; 813 –meringue batter produced with EW treated at 90 MPa/5 min followed by 55 ºC/3 min.

Meringues produced with non-treated EW

Meringues produced with EW treated at 55.6 ºC/6.2 min

Meringues produced with EW treated at 90 MPa/5 min followed by 55 ºC/3 min

Figure D7. The visual picture of cooked meringues prepared from non-treated and treated egg white.

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Sensory evaluation of meringues

Sex: M / F

Age: ____

Evaluate **meringues samples**: **295**, **467**, **813**, **individually**, according to your appreciation of the product, using the following **hedonic scale from 1 to 9**:

1. Dislike extremely
2. Dislike very much
3. Dislike moderately
4. Dislike slightly
5. Neither like nor dislike
6. Like slightly
7. Like moderately
8. Like very much
9. Like extremely

	Sample 295	Sample 467	Sample 813
Visual appearance	____	____	____
Aroma (1)	____	____	____
Surface colour	____	____	____
Interior colour (after breaking)	____	____	____
Texture (2)	____	____	____
Crunchiness (3)	____	____	____
Flavour	____	____	____
Global acceptability	____	____	____

(1) Aroma is the detection of volatile compounds as they enter the nasal passage and era understood by the olfactory system.
(2) Texture when breaking by hand.
(3) Crunchiness when crunching/chewing.

After sensory analysis, rate the samples according to your preference, from the one you liked least to the one you liked the most, filling out the diagram below with the code for each sample:

1

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(the one you liked least) (___) < (___) < (___) (the one you liked the most)

Notes (optional):

____
____
____
____
____

2

Figure D8. Example of the sensory analysis sheet of meringues.

Figure D9. Images of the meringues sensory analysis tables.

Annex E

Figure E1. Images of egg yolk samples packaged after each treatment. From left to right: raw egg yolk, egg yolk treated by commercial thermal pasteurization (60 ºC/6.2 min), shorter thermal pasteurization (60 ºC/3 min), combined treatments at 50 MPa/5 min – 60 ºC/3 min and 90 MPa/5 min – 60 ºC/3 min.

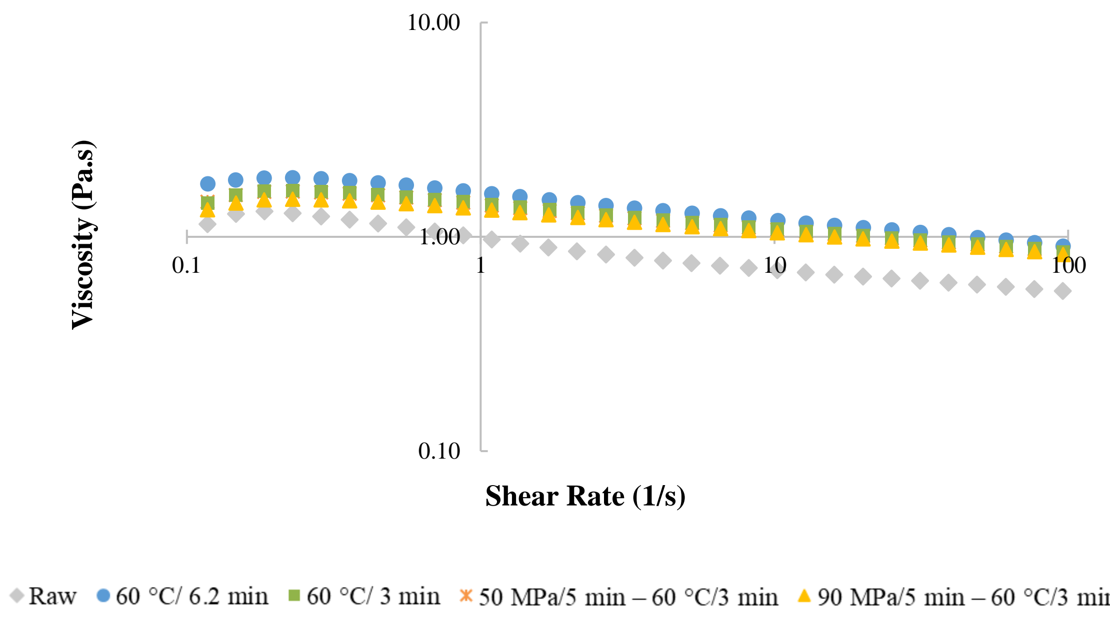


Figure E2. Flow chart of raw egg yolk and egg yolk treated by thermal pasteurization and combined processes (moderate pressure followed by thermal pasteurization).

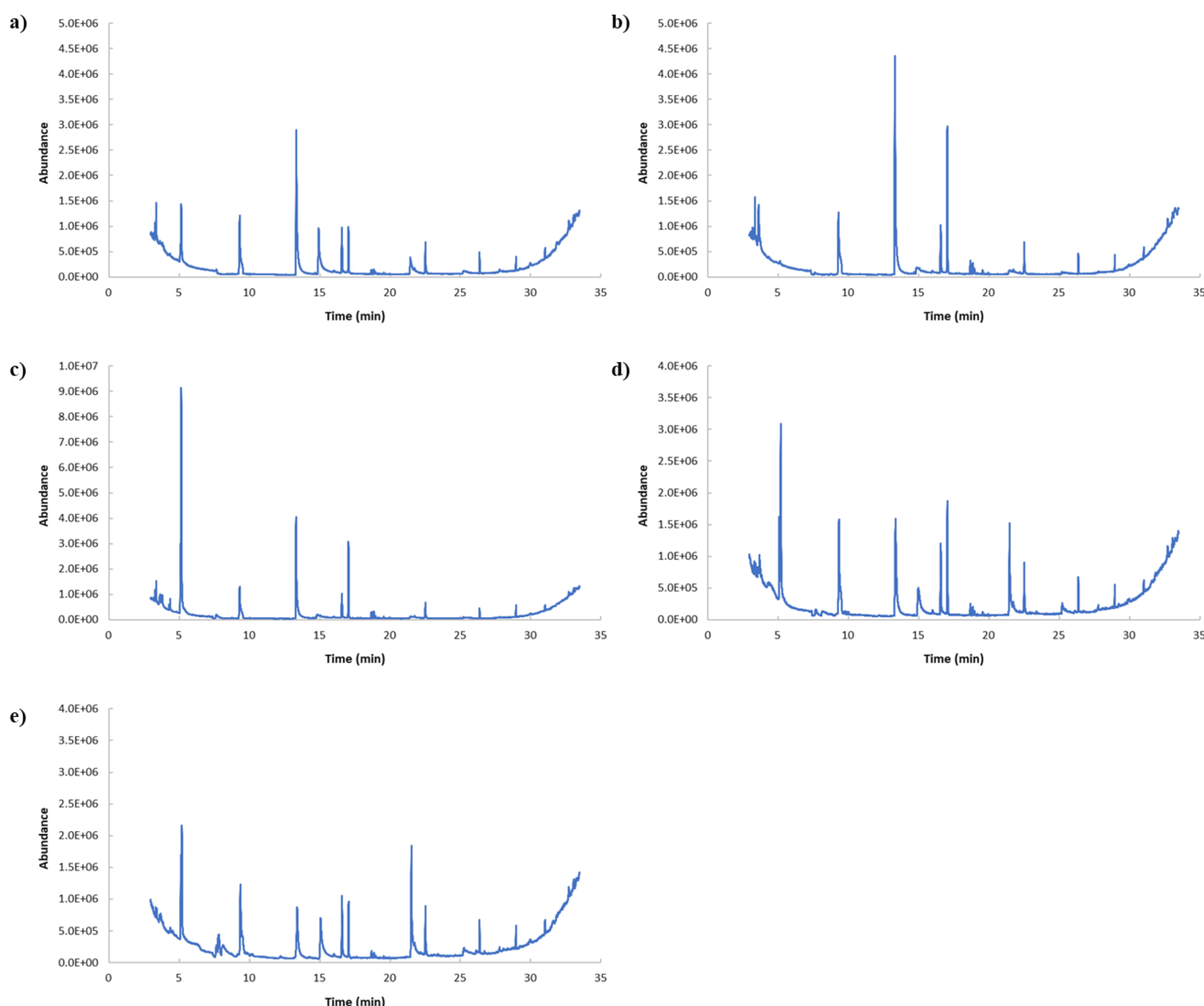
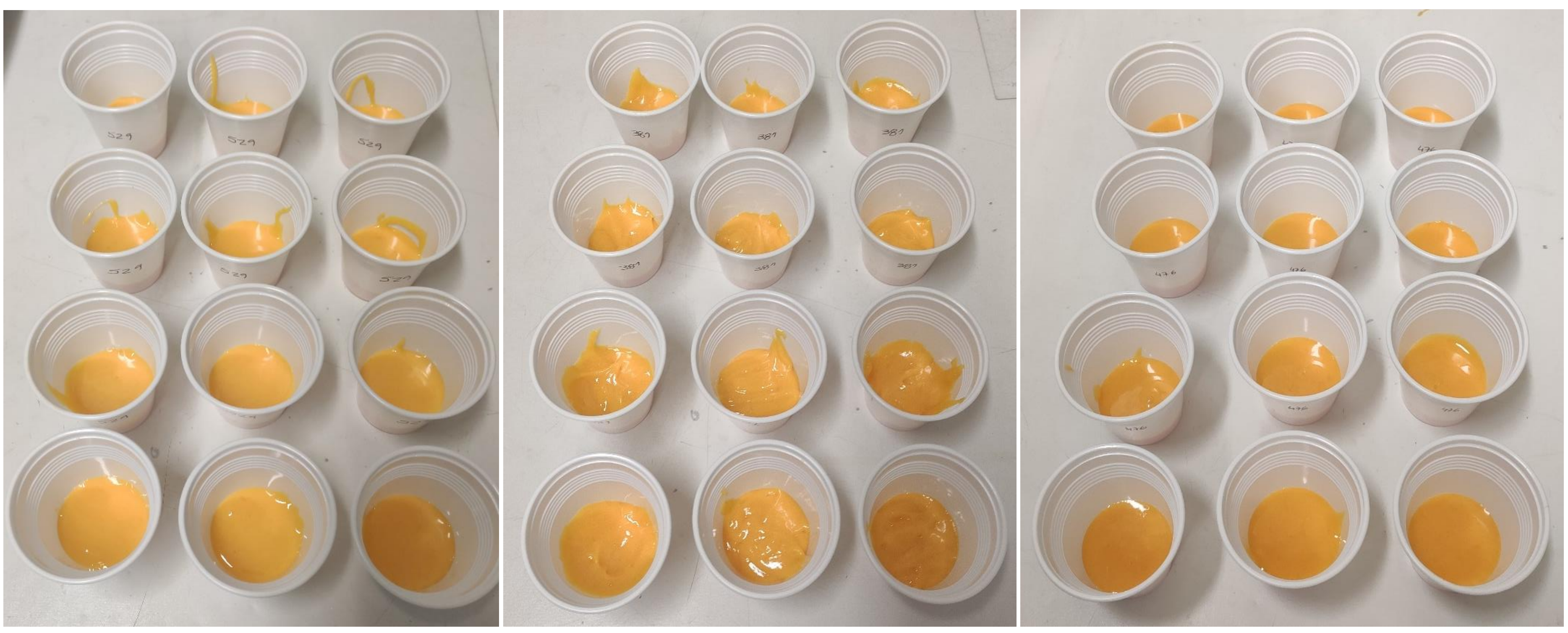


Figure E3. Examples of chromatograms of volatile compounds of raw egg yolk (a) and egg yolk treated by thermal pasteurization (60 ºC/6.2 min (b) and 60 ºC/3 min (c)), and combined processes (50 MPa/5 min – 60 ºC/3 min (d) and 90 MPa/5 min – 60 ºC/3 min (e)). Chromatogram is a representative run.

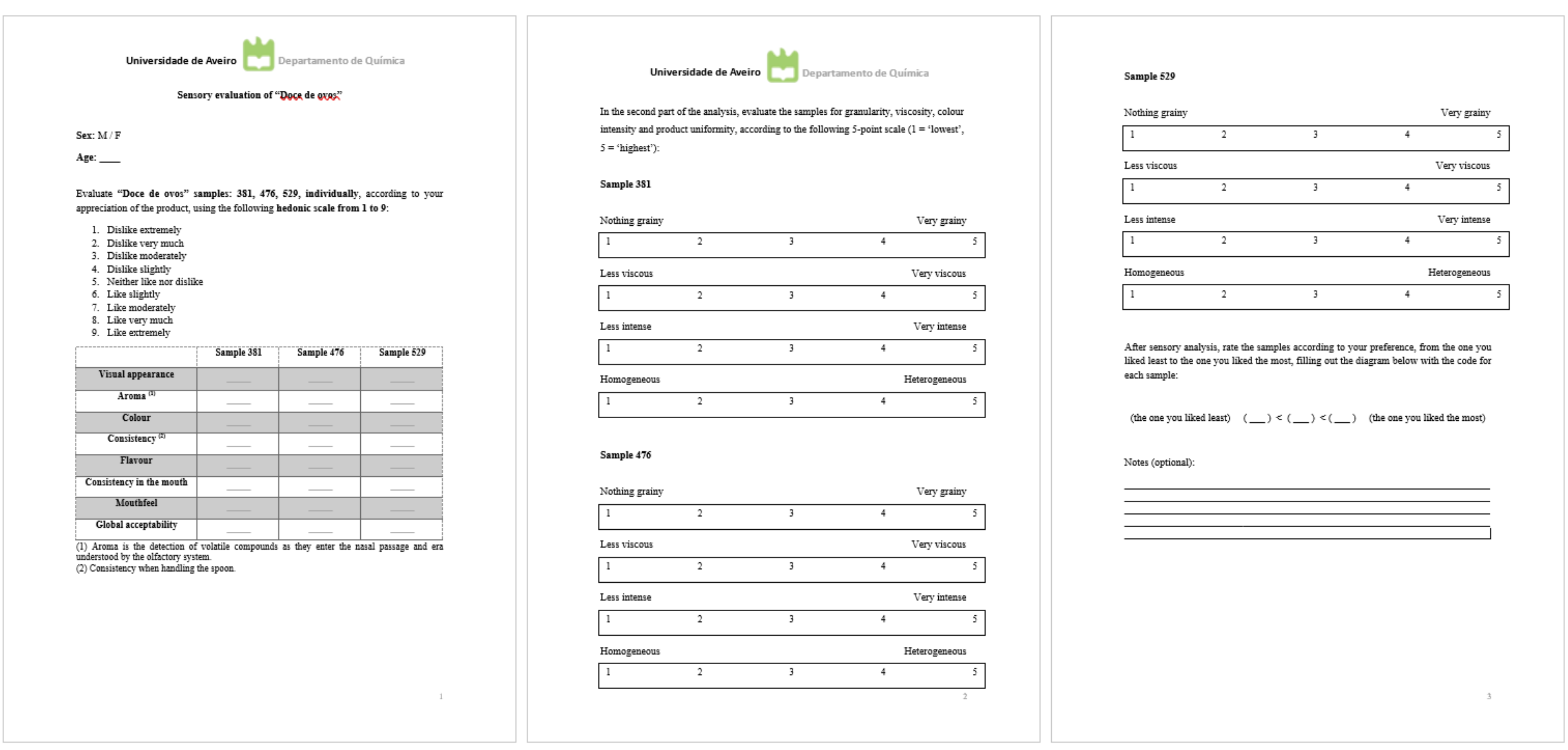


“Doce de Ovos” produced with non-treated LWE

“Doce de Ovos” produced with LWE treated at 60 ºC/3.5 min

“Doce de Ovos” produced with LWE treated at 200 MPa/5 min followed by 60 ºC/1.75 min

Figure E4. Images of “Doce de Ovos” prepared from non-treated and treated egg yolk.



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Sensory evaluation of "Doce de ovos"

Sex: M / F

Age: ____

Evaluate **"Doce de ovos" samples: 381, 476, 529, individually**, according to your appreciation of the product, using the following **hedonic scale from 1 to 9**:

1. Dislike extremely
2. Dislike very much
3. Dislike moderately
4. Dislike slightly
5. Neither like nor dislike
6. Like slightly
7. Like moderately
8. Like very much
9. Like extremely

	Sample 381	Sample 476	Sample 529
Visual appearance	____	____	____
Aroma (1)	____	____	____
Colour	____	____	____
Consistency (2)	____	____	____
Flavour	____	____	____
Consistency in the mouth	____	____	____
Mouthfeel	____	____	____
Global acceptability	____	____	____

(1) Aroma is the detection of volatile compounds as they enter the nasal passage and era understood by the olfactory system.
(2) Consistency when handling the spoon.

1

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In the second part of the analysis, evaluate the samples for granularity, viscosity, colour intensity and product uniformity, according to the following 5-point scale (1 = 'lowest', 5 = 'highest'):

Sample 381

Nothing grainy — Very grainy

1	2	3	4	5

Less viscous — Very viscous

1	2	3	4	5

Less intense — Very intense

1	2	3	4	5

Homogeneous — Heterogeneous

1	2	3	4	5

Sample 476

Nothing grainy — Very grainy

1	2	3	4	5

Less viscous — Very viscous

1	2	3	4	5

Less intense — Very intense

1	2	3	4	5

Homogeneous — Heterogeneous

1	2	3	4	5

2

Sample 529

Nothing grainy — Very grainy

1	2	3	4	5

Less viscous — Very viscous

1	2	3	4	5

Less intense — Very intense

1	2	3	4	5

Homogeneous — Heterogeneous

1	2	3	4	5

After sensory analysis, rate the samples according to your preference, from the one you liked least to the one you liked the most, filling out the diagram below with the code for each sample:

(the one you liked least) (___) < (___) < (___) (the one you liked the most)

Notes (optional):

3

Figure E5. Example of the sensory analysis sheet of "Doce de Ovos".

Figure E6. Images of the “Doce de Ovos” sensory analysis tables.

Annex F

Figure F1. Images of whole egg samples packaged after each treatment. From left to right: raw whole egg, whole egg treated by commercial thermal pasteurization (60 ºC/3.5 min), shorter thermal pasteurization (60 ºC/1.75 min), combined treatments at 50 MPa/5 min – 60 ºC/1.75 min and 200 MPa/5 min – 60 ºC/1.75 min.

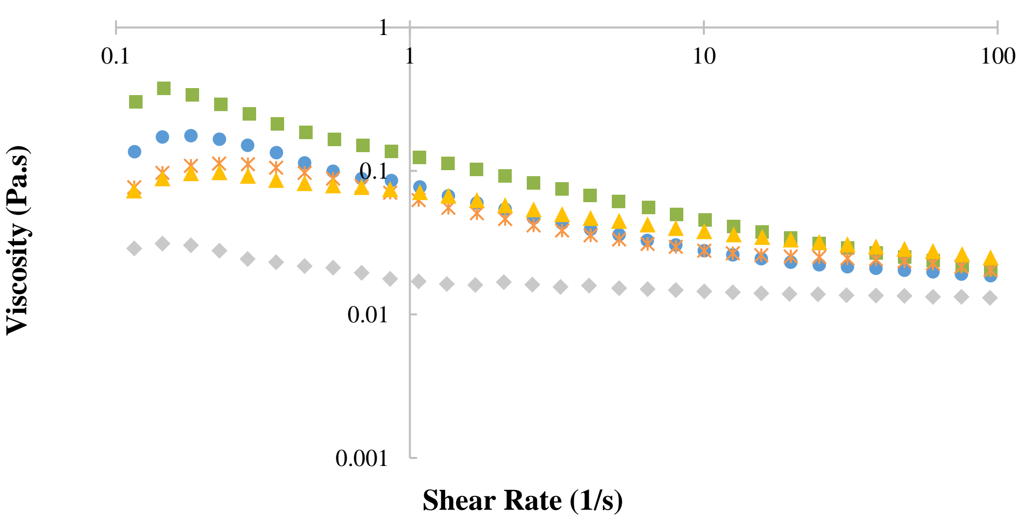

Figure F2. Flow chart of raw whole egg and whole egg treated by thermal pasteurization and combined processes (moderate pressure followed by thermal pasteurization).

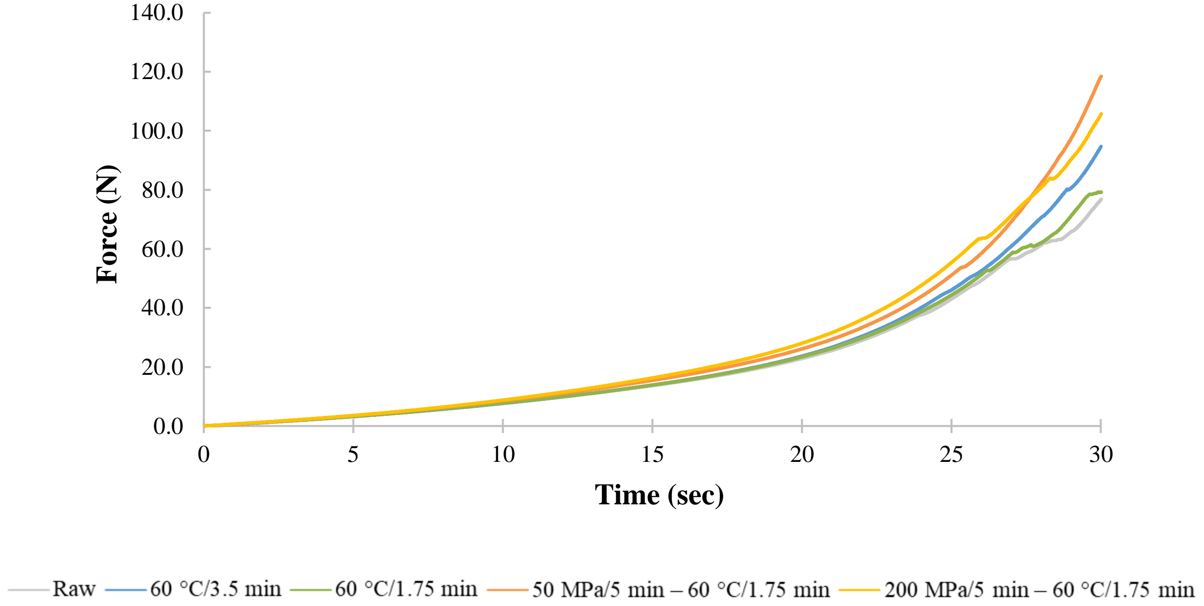

Figure F3. Examples of typical force-time curves obtained for whole egg heat-induced gels prepared with raw and treated whole egg.

Egg tart produced with non-treated LWE

Egg tart produced with LWE treated at 60 ºC/3.5 min

Egg tart produced with LWE treated at 200 MPa/5 min followed by 60 ºC/1.75 min

Figure F4. Images of egg tart prepared from non-treated and treated liquid whole egg.

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Sensory evaluation of egg tart in the oven

Sex: M / F

Age: ____

Evaluate **egg tart samples: 183, 674, 952, individually**, according to your appreciation of the product, using the following **hedonic scale from 1 to 9**:

1. Dislike extremely
2. Dislike very much
3. Dislike moderately
4. Dislike slightly
5. Neither like nor dislike
6. Like slightly
7. Like moderately
8. Like very much
9. Like extremely

	Sample 183	Sample 674	Sample 952
Visual appearance	___	___	___
Aroma [1]	___	___	___
Surface colour	___	___	___
Interior colour (after cutting)	___	___	___
Texture when cutting	___	___	___
Texture when cracking	___	___	___
Overall sensation when chewing	___	___	___
Flavour	___	___	___
Global acceptability	___	___	___

(1) Aroma is the detection of volatile compounds as they enter the nasal passage and era understood by the olfactory system.

After sensory analysis, rate the samples according to your preference, from the one you liked least to the one you liked the most, filling out the diagram below with the code for each sample:

1

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(the one you liked least) (___) < (___) < (___) (the one you liked the most)

Notes (optional):

2

Figure F5. Example of the sensory analysis sheet of egg tart.

Figure F6. Images of the egg tart sensory analysis tables.

Annex G

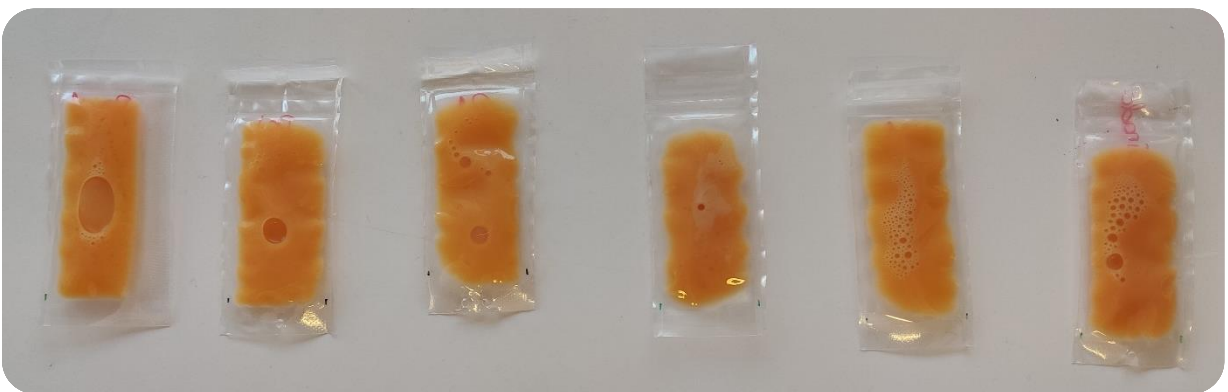

Figure G1. Images of whole egg samples packaged after each treatment. From left to right: raw whole egg, whole egg treated by commercial thermal pasteurization (60 ºC/3.5 min), shorter thermal pasteurization (60 ºC/1.75 min), moderate pressure (160 MPa/5 min), ultrasound (50 % amplitude/1 min), and combined treatment (160 MPa/5 min – 50 % amplitude/1 min – 60 ºC/1.75 min).

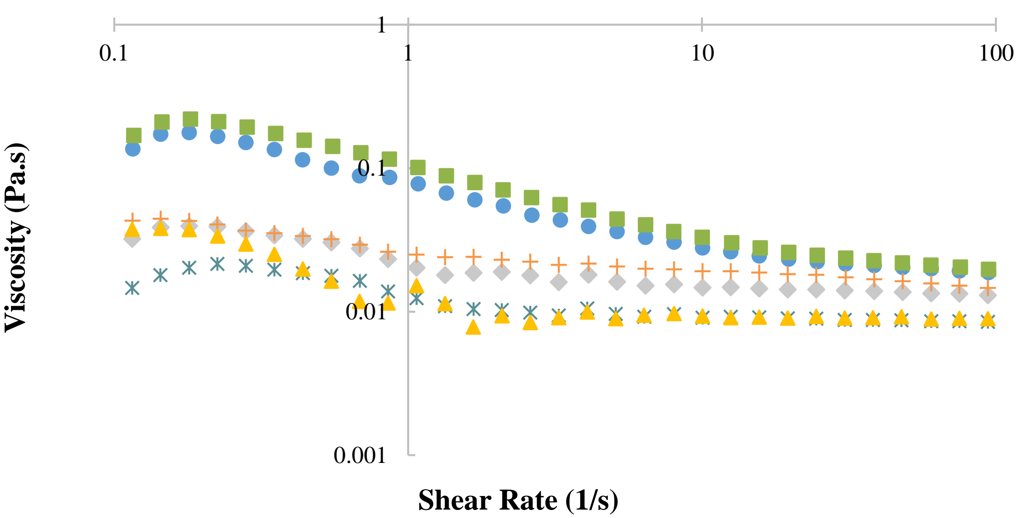

Figure G2. Flow chart of raw whole egg and whole egg treated by thermal pasteurization (60 ºC/3.5 min (commercial) and 60 ºC/1.75 min (shorter)), moderate pressure (160 MPa/5 min), ultrasound (50 % amplitude/1 min), and combined process (moderate pressure followed by ultrasound and followed by a shorter thermal pasteurization).

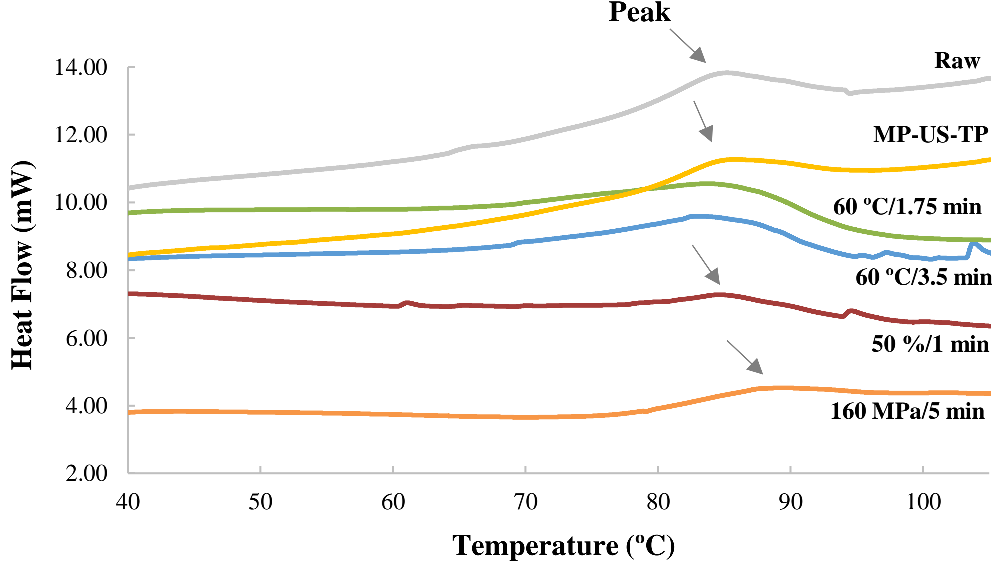

Figure G3. Differential scanning calorimetry (DSC) thermogram of raw whole egg and whole egg treated by thermal pasteurization (TP, 60 ºC/3.5 min and 60 ºC/1.75 min), moderate pressure (MP, 160 MPa/5 min), ultrasound (US, 50 % amplitude/1 min) and combined process (MP followed by US and followed by a shorter TP, MP-US-TP). Samples were heated from 30 to 120 ºC at a rate of 10 ºC/min. Thermogram is a representative run.

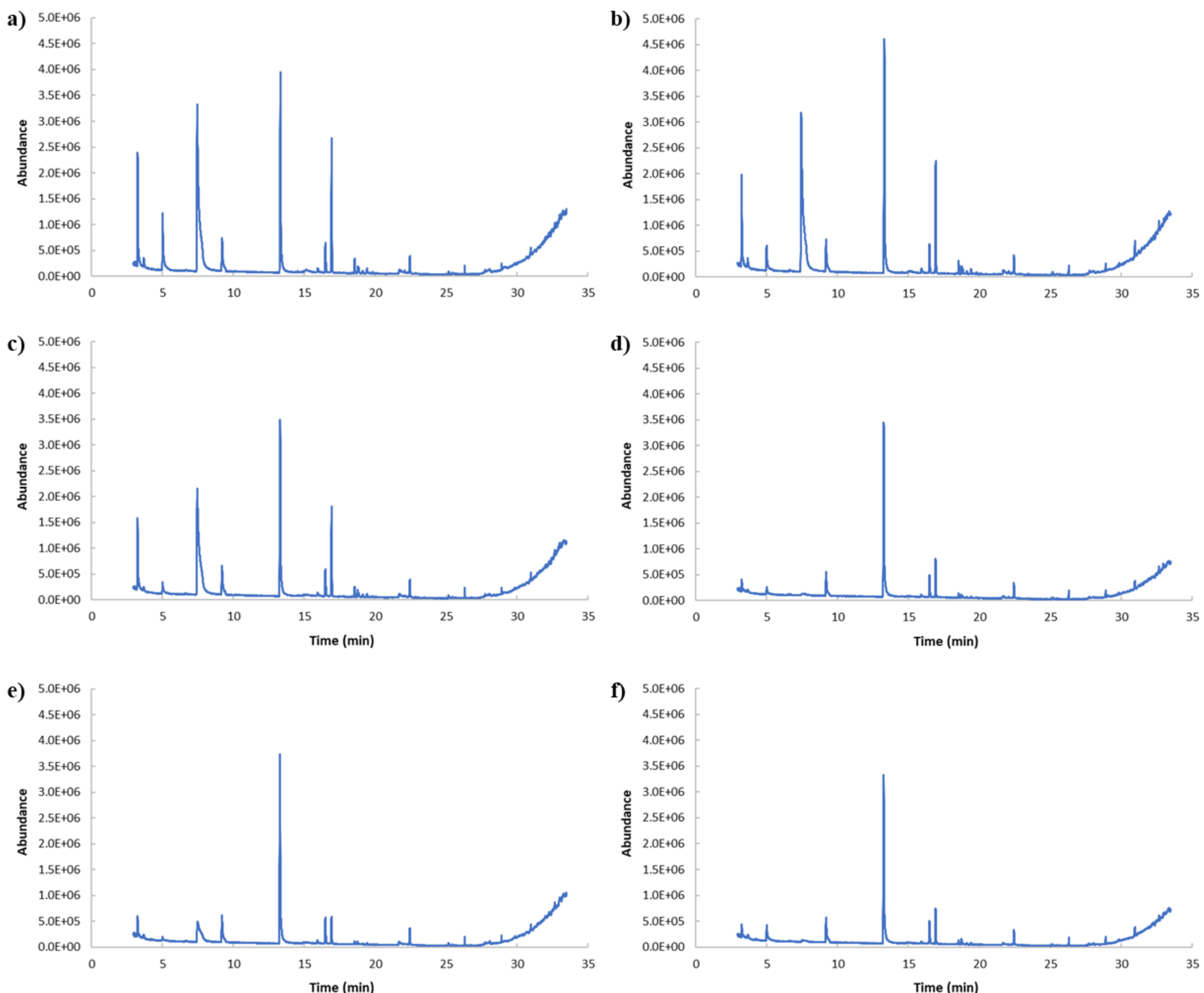

Figure G4. Examples of chromatogram of volatile compounds of raw whole egg (a) and whole egg treated by thermal pasteurization (60 ºC/3.5 min (b) and 60 ºC/1.75 min (c)), moderate pressure (MP, 160 MPa/5 min) (d), ultrasound (US, 50 % amplitude/1 min) (e), and combined process (MP followed by US and followed by a shorter TP, MP-US-TP (f)). Chromatogram is a representative run.