Master's thesis

NTNU
Norwegian University of Science and Technology
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Innovative processing technologies in the valorization of rest raw materials

The potential of Microwave, Ultrasound and High-Pressure Processing as pre-treatment technologies for the valorisation of mechanically deboned chicken residual through enzymatic hydrolysis

Master's thesis in MTKJ
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Abstract

Enzymatic hydrolysis of food processing by-products is a gentle, non-toxic and highly specific strategy for utilization of unexploited protein sources into added-value peptide-rich formulations, towards mitigation of food waste footprint. However, mild treatment conditions, better suited for such high-value applications, are often associated to long processing times and limited extraction yields. Innovative technologies, such as high-pressure processing (HPP), microwave heating (MW) and sonication (US), can contribute to the valorisation of food residuals through enzymatic hydrolysis by enhancing the availability of cleavage sites through biomatrix restructuration and protein unfolding. Thus, the resulting hydrolysates exhibit improved/standard techno-functionality and health-promoting effects, besides higher process yields.

The objective of this work was to investigate the potential of innovative technologies (MW; US; HPP) to enhance the bioactive and functional properties of the hydrolysates obtained from enzymatically hydrolysed mechanically deboned chicken residual (MDCR).

In the present study, this was conducted by applying MW, US and HPP as pre-treatments of mechanically deboned chicken residual (MDCR) prior to enzymatic hydrolysis using an industrially relevant enzyme. After enzyme inactivation (95 ℃; 15 min), the hydrolysis reaction media was separated into a filtered aqueous, lipid, solid, particulate and a semi-solid multicomponent phase. The weight of all phases was recorded for the different experimental conditions. The protein concentration in the hydrolysates at 0.5, 5, 15, 30 and 60 min of hydrolysis and in the total reaction volume after hydrolysis (FHV) was determined. For the FHV of specific trials, the protein solubility of the hydrolysate at pH 2, 4, 6 and 11 was determined, as well as the DPPH radical scavenging (antioxidant) activity at protein concentrations of 0.5, 1, 2, 4 and 6 mg/mL. The investigated technologies present specific advantages with regards to the hydrolysate properties. In order to produce hydrolysates with increased protein concentration (after 60 min of hydrolysis), HPP should be applied at pressures of 400 MPa or higher (15 min; 25 ℃). The best option for enhanced DPPH radical scavenging activity (4 and 6 mg/mL protein concentration) would be US pre-treatment at 900 W (30 min, T < 30 ℃). Finally, hydrolysates with increased protein solubility over pH could be achieved with a microwave treatment of 1 min (40 ℃; 2.45 GHz) or HPP treatment, however longer treatment times would be needed (15 min). Apart from the mentioned recommendations the results suggest that several of the technologies hold the potential to induce the formation of peptides with enhanced functionality apart from protein solubility. A US pre-treatment at 600 W (30 min, T < 30 ℃) and a MW pre-treatment for 1 min (40 ℃; 2.45 GHz) seem to hold the potential of inducing the formation of peptides with higher lipid associating properties, as does HPP treatments at 200 MPa (15 min; 25 ℃) which also present antioxidant properties (4 mg/mL).

Preface

This report is written as part of the TBT4900 Biotechnology Mater Thesis (Spring 2019) with preliminary title *Role of innovative processing technologies in the valorization of rest raw materials* and final title *The potential of Microwave, Ultrasound and High-Pressure Processing as pre-treatment technologies for the valorisation of mechanically deboned chicken residual through enzymatic hydrolysis*. The research project is the result of a collaboration between NTNU and Nofima and aims to investigate the potential of innovative processing technologies for re-utilizing rest raw materials and residuals generated in the poultry industry. The introduction aims to both motivate on the relevance for such investigations but also provide an in-depth review of the technologies applied within this work, as well as their mechanism of action. The subsequent sections provide details on the experimental procedures, choice of process conditions during the various pre-treatments, results, discussion and conclusion on each separate technology, and a common section for future work.

Several expressions of gratitude are appropriate. Big thanks to my supervisor and co-supervisors Turid Rustad, Estefanía Noriega Fernàndez and Izumi Sone for contributing with helpful feedback, corrections and support during the project. Gratitude is also shown to Estefania and Izumi for the warm welcome and pleasant stay in Stavanger at Nofima. Finally, a big thanks to both Mette Risa Vaka and Rebeca Garcia Alvarez for helping me during the many hydrolysis. I hope both your dogs are in good health.

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List of acronyms

Table 1: The presented table provides a list of all acronyms used in the present work and their associated full form.

Acronym	Full form
MDC	Mechanically deboned chicken
MDCR	Mechanically deboned chicken residual
US	Ultrasound
MW	Microwave
HP	High pressure
HPP	High-pressure processing
t	Time
T	Temperature
rpm	Revolutions per minute
g	Gram
L	Litre
min	Minute
s	Second
h	Hour
DH	Degree of hydrolysis
ND	Not detected
EtOH	Ethanol
ATP	Adenosine triphosphate
Trp	Tryptophan
Tyr	Tyrosine
Met	Methionine
Cys	Cysteine
His	Histidine
Phe	Phenylalanine
Arg	Arginine
Asn	Asparagine
Gln	Glutamine
Asp	Aspartate
Pro	Proline
Ala	Alanine
Val	Valine
Lys	Lysine
Ile	Isoleucine
Thr	Threonine
Leu	Leucine
Glu	Glutamate
Gly	Glycine

1 Introduction

1.1 Increasing formation of food by-products and residuals and their potential utilization and valorisation

1.1.1 Current situation, driving forces and potential

Traditional diets in developing countries have been based on cereal and coarse grains, consumption patterns are however changing. Increasing income causes the intake of meat, eggs and fish to increase fast (Pingali 2007). In Asia alone the consumption of animal protein increased by 225% between 1961 and 2007 (Boland, Rae et al. 2013). While meat production in developing countries remains high, the per capita consumption of meat and meat-products is low as compared to developed nations. During the 80s, Europe produced most meat (40% of global) as compared to Asia (21%). Now 41% of the global meat production is constrained to the Asian continent (Ali and Pappa, 2011). In countries such as Norway the production and consumption of chicken meat, beef, eggs and fish are on the rise (InnovasjonNorge 2018, Landbruksdirektoratet 2018). The general trend in Europe indicates an increase in meat consumption as of overall, while meat consumption per capita has remained relatively constant since the early 90s. However, there seems to be clear shift towards higher poultry and pig meat consumption (Kanerva 2013).

40-60% of animal and fish weight does not comprise the main product, such as fillets, and thus is seen upon as waste (Aspevik, Oterhals et al. 2017). Globally, 1.3 billion tons of food and one third of the total global food production is wasted every year, costing the world economy close to €750 billion (Dahiya, Kumar et al. 2018). In Europe every year 88 million tons of food-waste is generated with an associated cost of €143 billion (FUSIONS 2016). Traditional applications of the waste are burning, incineration, composting and animal feed (Jain, Dhakal et al. 2017).

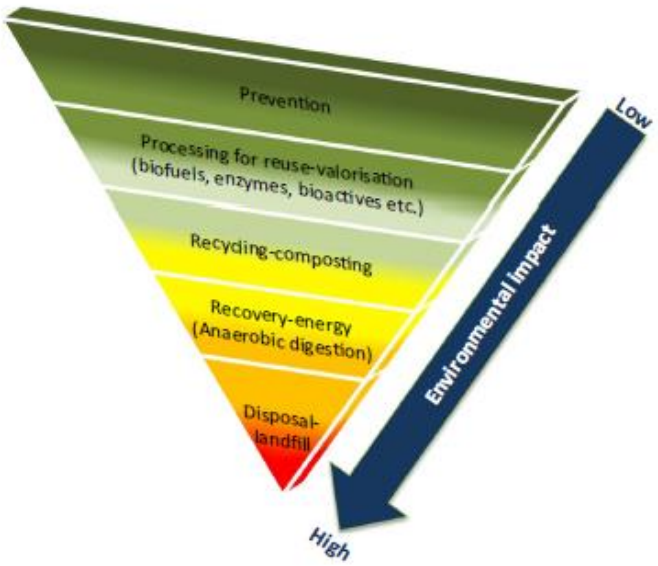

Figure 1: Environmental impact associated with different waste handling actions (Lin, Pfaltzgraff et al. 2013).

There are different environmental impacts resulting from different waste applications (Figure 1). Land fillings are often the least desirable action due to formation of methane which is a potent climate gas produced during anaerobic digestion of food components. Another disadvantage is the release of heavy metals which might accumulate in soil and groundwater (Girotto, Alibardi et al. 2015). Thermal treatments have been applied in combination with anaerobic digestion to handle the waste and at the same time produce biogas and an effluent applicable as fertilizer. However, while the biogas-production compensates for the energy used during the thermal treatment, the high moisture content in the waste makes this option a low-efficiency solution (Edwards and Daniel 1992, Girotto, Alibardi et al. 2015). Recycling food residuals is another option, either using them as fertilizer or animal feed. As food-waste generally is highly nutritious, animal feed might be a desirable option. However, there are strict regulations for premarket approval, limiting its commercial exploitations (Dahiya, Kumar et al. 2018). In addition, the production of animal feed from food by-products is regarded as a low value solution (Rustad 2003).

The increasing amount of food-related waste is receiving significant attention as proper uses are required in order to avoid devastating environmental and health effects. Plans and legislations launched by the European Union intend to change the current perception of these residuals as waste into resources applicable for value-added products. One of the regulations the EU has imposed on member states was to reduce the amount of land fillings by 35% within 2016. Another is to stepwise reduce food waste in the EU by 30% within 2025 and further by 50% within 2030. In addition, an

action plan to initiate a circular economy has been established, in order to boost jobs creation, investment, economic growth and to achieve a carbon neutral and resource efficient economy. Essential in the vision of a circular economy is sound and efficient waste management systems (Directorate-General 2010, Union 2017, Union 2019).

Valorization of food waste is a new concept in light of a circular economy, which offers high-value alternatives beyond that of land fillings and composting for the full exploitation.

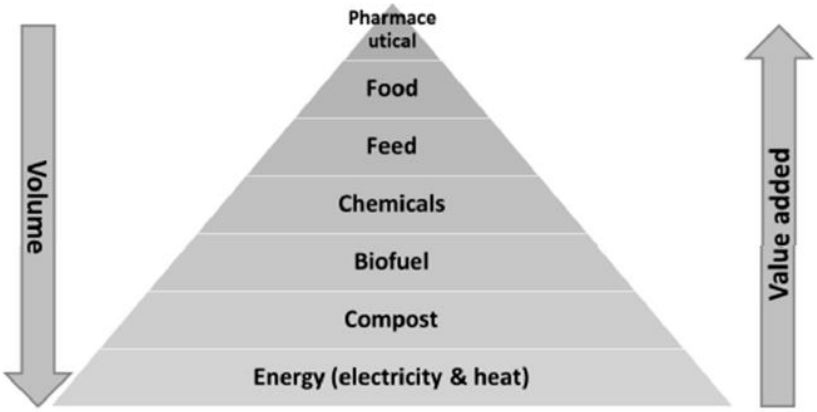

Figure 2: Pyramid of value for the different products one might generate from by-products. While the value of the products increase upwards in the pyramid, todays production-volumes decreases towards the top of the pyramid (Berbel and Posadillo 2018).

Non- or under-utilization of residuals and other food processing by-products causes a loss of potential revenues and might even be associated with increased cost due to high disposal fees. It has been estimated that 11 and 7% of the gross income from beef and pork respectively (United States Dept. of Agriculture Economic Research Service), comes from the residuals. However, these residuals comprise between 66 and 52 % of the total live weight (Jayathilakan, Sultana et al. 2012). Compared to the above-mentioned utilization of residuals, better profitability is found in applications intended for human consumption, biotechnological uses and pharmaceutical applications (Rustad 2003). However, in order to climb the steps illustrated in Figure 2 there are obstacles to overcome.

Development and commercialization of the high-value products are complex, expensive and risky and beyond the technological obstacles are legislations as well as consumer demands. The potentially marketable component present in the by-product must first be extracted from the matrix through combinations of biochemical, chemical or physical treatments and further modified into the higher value food products or other products. This without introducing microbial hazards and still

making sure that the final product follow food regulations and meet consumer preferences. Well-established and novel extraction technology used to recover biomolecules from by-products have recently received much research attention. However, in order to both make the final product affordable for the consumer and ensuring economic feasibility production-wise, the operating conditions and technology itself must be optimized with regards to the extraction of the desired component (Baiano 2014). Adding value to by-products earlier regarded as waste implies a certain degree of innovation. Several novel technologies have emerged in food industry recently. Ultrasound and microwave have for example been pointed out as the most convenient ones in an economic, processing and energy-efficient point of view (Pojić, Mišan et al. 2018). In Europe, the EU has developed an elaborated framework governing the use of residuals from fisheries, aquaculture, livestock and poultry industries. Type and quality of each material is important to define further use in food/feed applications according to the hygiene rules for food of animal origin. Processing must thereafter be implemented according to regulations relevant for subsequent utilization, for example obeying food hygiene regulations if processing into human consumption (Aspevik, Oterhals et al. 2017).

The expected increase in the world's population (to almost 10 billion within 2050) is one of the driving forces towards the formulation of sustainable solutions for valorization of food residuals. Enhancing the efficiency of food systems, generating less food-waste through redesigning food supply chains and introducing innovative and sustainable technology which might both reduce food losses and waste and allow for recycling resources are believed to be important (Nations 2017). A good prerequisite is that food waste is a reservoir of carbohydrates, proteins, lipids and functional foods, i.e. a well-suited raw material for further valorization. Possibilities for valorisation into production of biofuels, enzymes, bioactive compounds and other molecules has been reported and is illustrated in Figure 3 (Girotto, Alibardi et al. 2015).

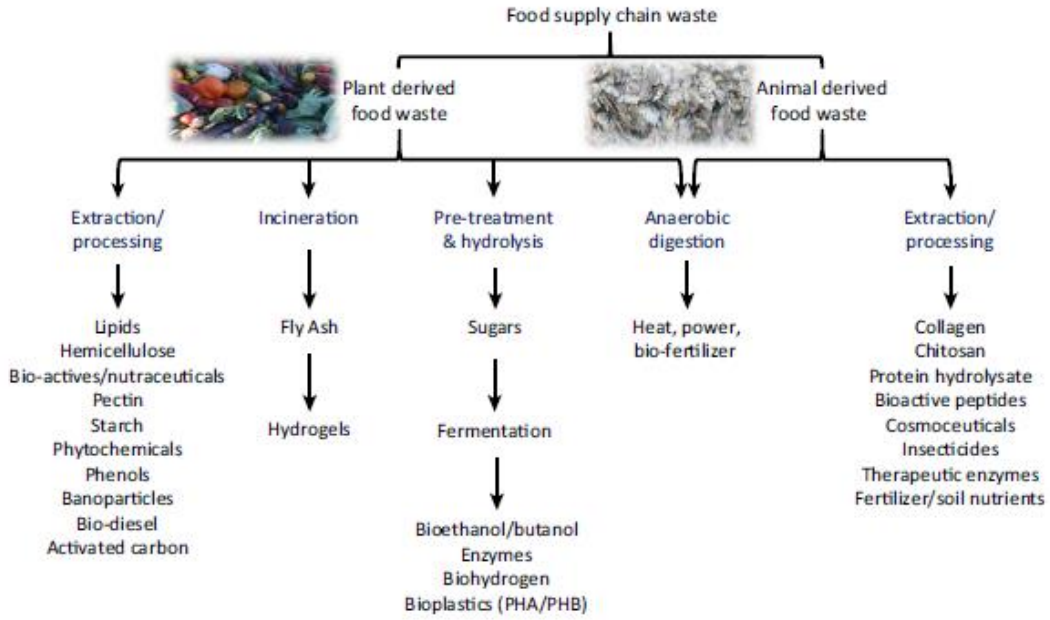

Figure 3: Possible applications for different types of side streams generated within food production (Girotto, Alibardi et al. 2015).

Animal by-products are in general high in protein but is often discarded due to aesthetic reasons (Lai, Khong et al. 2017). However most protein fractions might be extracted and used in hydrolysate production and further applied as food ingredients (McCarthy, O'Callaghan et al. 2013, Lai, Khong et al. 2017). While there are multiple bottlenecks, the potential is high. In fact, the global functional food market has been estimated to 168 billion US$ (2010) with USA holding the largest market segment followed by Europe and Japan. Functional food is a concept first promoted in 1984 by Japanese scientists who investigated relationships between nutrition, sensory satisfaction, fortification and modulation of physiological systems. Functional foods could enter every food category. Today this term is used to market food fortified with technologically developed ingredients (Siro, Kápolna et al. 2008, Grasso, Brunton et al. 2014).

The fact that consumers to a larger degree couple diet and health might urge them towards foods with additional benefits. However, there are different attitudes towards functional foods, new products and technology. In Europe there are great variations in consumer perception towards functional foods. Denmark often poses negative attitudes towards foods with health claims, while Finland and Sweden display more moderate responses. Another important factor regarding consumer acceptance is taste. In general, consumers do not offer taste for health benefits. Therefore superior tasting functional foods seem as a necessity for any new product (Grasso, Brunton et al. 2014). Foods fortified with proteins originating from marine by-products, is sometimes associated

with perception of bitterness when consumed by humans, making them unfavourable for consumer acceptance (Maehashi and Huang 2009). Another important bottleneck associated with consumer and market is the price of the product; in fact the large price gap between conventional and functional foods has been reported to make them inaccessible to consumers, even though consumers seem willing to pay more for functional foods (Grasso, Brunton et al. 2014). In addition, to reach the consumer, technology must be mature enough to allow for processing which ensures economic viability.

One of the high potential applications of fish waste/by-products is the production of protein hydrolysates through enzymatic treatment considering the large volume of proteinaceous waste in the form of heads, scales, bones and viscera from the fish processing (Jayathilakan, Sultana et al. 2012). Poultry residues are often dominated by head, skin, feet and viscera and might also serve as a source of protein hydrolysates (Lai, Khong et al. 2017). The by-products and residuals for example associated with fish processing are often used as fertilizer or feed, however these uses are associated with low profitability (Rustad 2003).

1.1.2 Revalorization of proteins present in the by-products and the advantages of enzymatic hydrolysis

Which method or process to be applied for by-product valorization is dependant both on the raw material to be treated and the targeted product. A wide variety of methods is available for protein extraction from by-products including solvent extraction, supercritical fluid extraction, subcritical water extraction, thermal treatments, enzymatic hydrolysis and chemical extraction. Today the most relevant methods are either chemical or enzymatical extraction. Chemical methods are usually based on extraction with isopropanol or acid/alkaline based (Ghaly, Ramakrishnan et al. 2013). The most commonly applied chemical method is based on the extraction with isopropanol. Undesirable issues associated with the final product include low solubility or dispersibility in foods and poor emulsification properties, which often is desirable in food products. Besides, safety issues related to solvent traces in the final product limit its applicability for human consumption. Both acid and alkaline methods have been applied to extract proteins from by-products (Kristinsson and Rasco 2000). The application of acid and alkali are however associated with destruction of certain amino acids and racemization, resulting in a significant process loss (Kristinsson and Rasco 2000, Ghaly, Ramakrishnan et al. 2013).

Enzymatic hydrolysis may be conducted by utilizing either endogenous or exogenous enzymes. The use of endogenous enzymes simply relies on enzymes already present in the tissue/material which is to be processed. This has the advantage that no commercial enzymes must be added. Disadvantages include longer processing times and difficulties with process and product control. The use of exogenous enzymes allows higher process and product control (Shahidi, Han et al. 1995). Essentially, addition of exogenous enzymes reduces system complexity, which originates from several factors. First, the composition of reaction components is usually very diverse as peptide fragments are both product and reactant. Second, there is a multiplicity of reaction types, as both parallel and sequential peptide bond cleavage occurs. Third, further complexity is achieved by the presence of reaction networks. Single components in one network might affect components in different networks both through inhibition and promotion of enzymatic reactions (Qi and He 2006). The lower number of components and networks influencing and dominating an exogenous enzyme process allows better product and process control as opposed to systems dominated by several endogenous enzymes. In addition to the mentioned complexities, heterogenicity in the substrate and raw material would contribute to difficulties improving the product, especially if the process is continuously operated. Heterogenicity in the raw material would not only influence through the substrate content but also through content and composition of endogenous enzymes.

Enzymatic hydrolysis of proteins might be applied to improve the physico-chemical, techno-functional, nutritional properties and bioactivity of the original proteins (Qi and He 2006).
For human consumption, there are roughly two groups of peptides, based on their properties, which may be produced by cleavage of protein peptide bonds yielding shorter peptides. The first group is characterized by short-chain peptides with a characteristic amino acid composition and a defined molecular size and are desirable for specific formulations. In humans, absorption of di- and tri-peptides are considered more efficient when compared to free amino acids due to the presence of specific transport systems. Some amino acids are also insoluble when in free form (Clemente 2000). This group is suitable for nutritional purposes, which for example may be relevant for elderly or immunocompromised subpopulations unable to efficiently digest proteins or for infants (Aluko 2018). The second group of peptides are also short-chain peptides which possess a sequence of amino acids which are responsible for a certain biological activity, not enabled when in the native protein. Therefore, enzymatic hydrolysis is required to liberate the active peptide sequence from the originating protein. These peptides may influence physiological functions and produce health

benefits. Among these benefits are reduction in blood pressure and oxidative stress relief. This group is known as the bioactive group. While dietary applications are favoured by low molecular weight peptides, improvement of techno-functional properties usually requires larger peptides, often peptides with more than 20 amino acid residues (Silvestre 1997). Protein hydrolysates can also improve techno-functional properties in foodstuff, to which degree is dependent on how, and if, interactions between protein, water and other components in the food matrix occur. Interactions between the mentioned components depend on both extrinsic (e.g. temperature, pH and ionic strength) and intrinsic (e.g. protein composition, protein chain length, protein rigidity, content of charged groups on protein and protein concentration) factors. Among the alterations in techno-functional properties that arise from protein hydrolysis, solubility, water-holding capacity, gelation and coagulation, emulsification, foaming and surface hydrophobicity have been reported (Panyam and Kilara 1996).

1.1.3 The composition and structure of major proteins present in mechanical deboned chicken residual

The protein-rich chicken residue from mechanical deboning (MDCR) has been recognized as a valuable enricher in food-applications (Sun, Zhao et al. 2010, Lafarga and Hayes 2014). The residue is formed in a process where a ground slurry of chicken meat and bones are placed into a mechanical deboner and pressure is applied to separate meat and soft tissue from the remaining fraction which is often rich in bone particles, muscle, connective tissue and fat (Kijowski and Niewiarowicz 1985). Such residues are reported to contain from 16 to 20% protein where the major fraction is collagen. 18% of the protein content is reportedly muscle associated proteins, namely sarcoplasmic and myofibrillar protein (McCurdy, Jelen et al. 1986). These proteins could be extracted and utilized for higher-value applications.

The myofibril is the major organelle in muscle cells. Sarcoplasmic proteins are also present inside the muscle cells often as soluble enzymes (Skaara and Regenstein 1990). The basic unit of a myofibril is the sarcomere. The sarcomere is a protein structure containing different components which perform different functions; however, the overall function is contraction. Actin is the major protein component in the thin filament and is anchored to an element called Z-disks and furthermore protrude towards the centre of the sarcomere. Myosin is the major component of the thick filament

and is connected to an element called the M-line which denoted the mid-point of the sarcomere (Kerth 2013). This is illustrated in Figure 4.

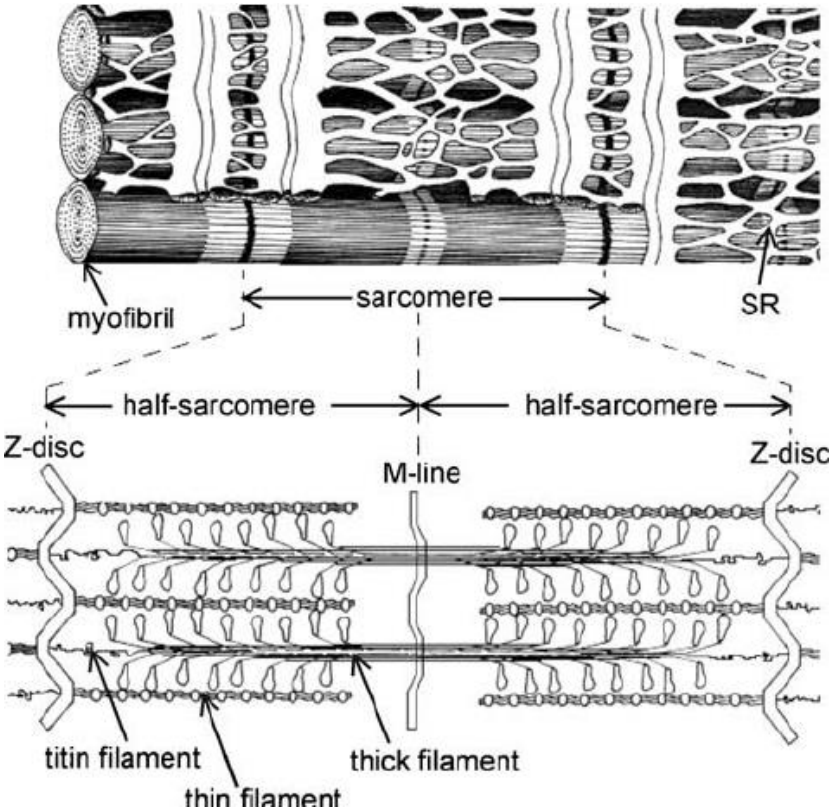

Figure 4: Illustration of the structure and position of the major myofibrillar components actin (thin filament), myosin (thick filament), the Z-disk and the M-line (Stehle, Solzin et al. 2009).

Myosin is the most abundant protein component in muscle tissue (For schematic structure see Figure 5). Myosin is an oligomer containing two heavy chains, two light chains and two heads. Areas on the myosin molecule naturally denoted to movement are reportedly susceptible to proteolytical digestion (Marked Swivel and Hinge in Figure 5) (Kerth 2013).

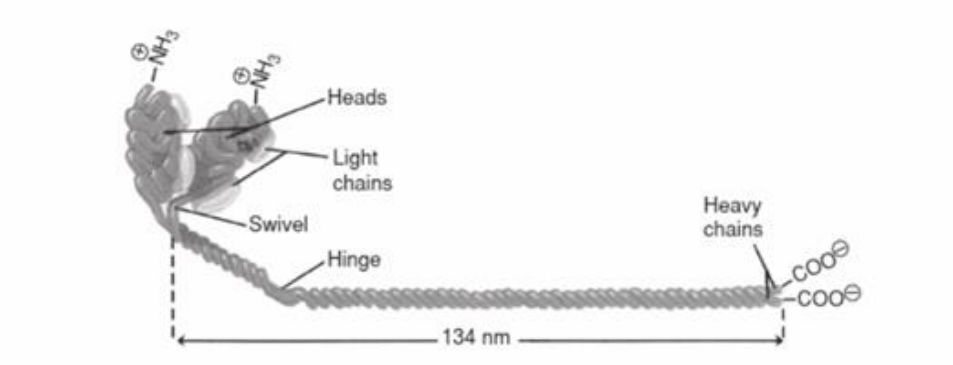

Figure 5: Illustration of the various sections of the myosin molecule (Kerth 2013).

In the thick filament, myosin molecules are associated such that the tails are oriented towards each other, generating a region with only heavy chains, namely the bare zone. Every thick filament, with myosin heads protruding in all surrounding directions, is surrounded by six thin filaments. Myosin comprises approximately 45% of the total myofibrillar protein content. There might be slight differences in amino acid composition and thereby molecular weights and physio-chemical properties between species, but the overall differences are small between different vertebrates. The amino acid content found in the myosin rod is consistent with that of an alpha-helical structure. The amino acid content of the myosin head is such that a globular structure would be favourable, in addition the myosin heads is rich in -SH groups (about 13 in each). There is also indication of a hydrophobic site in the globular head fragment of myosin (Skaara and Regenstein 1990).

While myosin is the main component of the thick filament, the thin filament is composed of both structural and regulatory proteins. However, the main thin filament component is the structural protein actin. In monomeric form actin takes a globular structure (G-actin). At physiologic ionic strength the monomeric actin is assembled into a filamentous structure (F-actin, as illustrated in Figure 6). Two F-actin structures generate the basis of the thin filament. Also present in the thin filament are two regulatory proteins, namely troponin and tropomyosin (Kerth 2013).

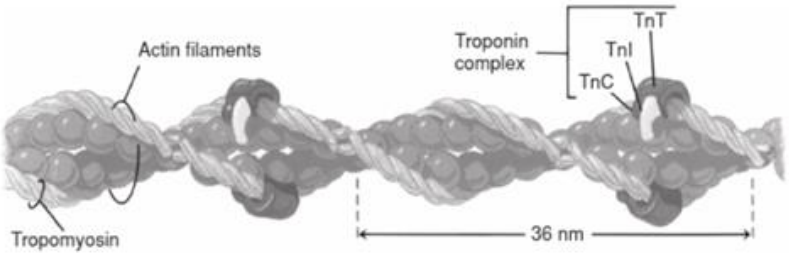

Figure 6: Illustration of F-actin filaments associated with tropomyosin and troponin (Skaara and Regenstein 1990).

The thin filament is anchored to the Z-disk (Figure 4), while the M-line is associated with the thick filament. The thin filaments surround the thick filaments and are arranged in a symmetrical pattern. In fact, the M-line connect adjoining thick filaments and the Z-disk connect adjacent sarcomeres (Kerth 2013). There are several proteins present in addition to those mentioned. A group of proteins not directly responsible for contraction in muscle-cells are the sarcoplasmic proteins, comprising about 30-35 % of the total muscle proteins. These proteins are located inside the muscle cell and are soluble in water or at low ionic strengths. Specific proteins within this category are oxidative enzymes, myoglobin and other heme pigments, enzymes responsible for glycolysis and lysosomal enzymes (Owens, Sams et al. 2000). Intermediate proteins are a group of proteins which in general run in perpendicular to the length of the muscle fibre sustaining the three-dimensional structure and shape of the sarcomere and the myofibril (Kerth 2013).

Connective tissue, skin and tendon is high in collagen which dominates the protein fraction of residuals obtained from mechanical deboned chicken (Engel and Bächinger 2005) (McCurdy, Jelen et al. 1986). The tissues associated with a mechanical function often possess collagen type 1. Other tissues such as bones and tooth dentin which require higher stiffness also contain, in addition to an organic matrix dominated by collagen, an inorganic component (carbonated hydroxyapatite) forming a high stiffness composite material. In tendons or cornea collagen is associated with other organic materials such as proteoglycans (Fratzl 2008).

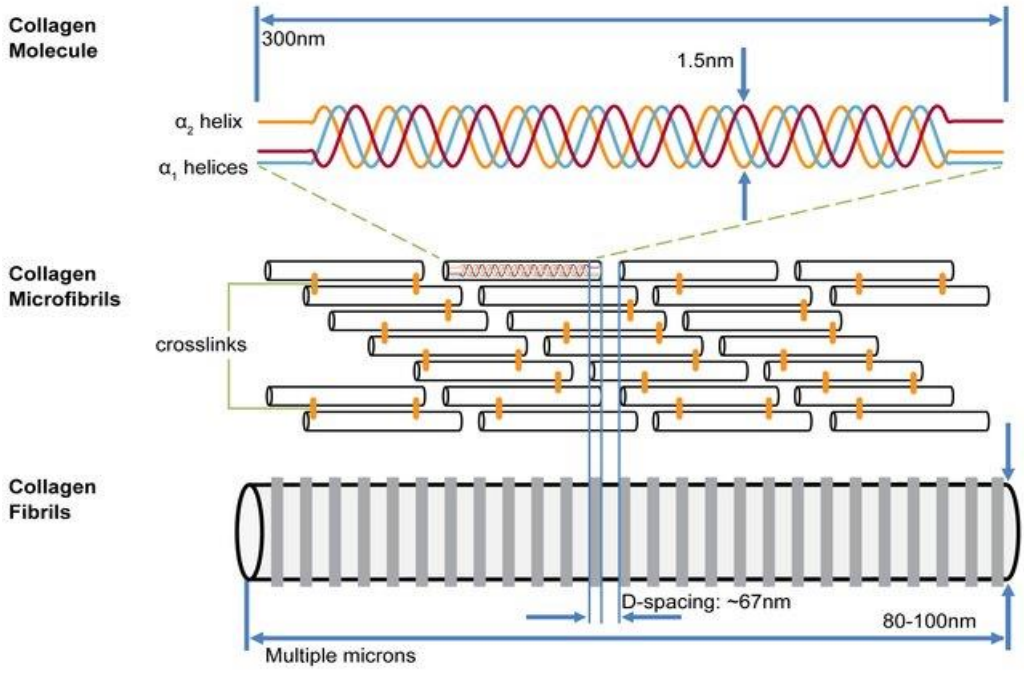

Figure 7: An overview of the collagen structure at a molecular, microfibrillar and fibrillar level (Canelón and Wallace 2016).

The collagen molecule is found at the lowest structural level and is a triple-helical protein chain. The individual chains within the molecule display a repeating amino acid sequence of Gly – X – Y, where X and Y are frequently proline and hydroxyproline, respectively. The three helices are staggered by one amino acid residue relative each other and super coiled along a common axis into the right-handed triple helix observed for the collagen molecule. The side-chains of the residues in the X and Y position play important roles, one of them due to the fact that they point out of the helix and might interact with other components during fibril formation (Engel and Bächinger 2005). These molecules are further staggered parallelly into collagen fibrils.

Most collagen found in animals is in insoluble form and of type 1. In fact, collagen type 1 seems to have distinct features such as a greater degree of internal crystallinity as well as a wide distribution of fibril diameter. How the collagen fibrils are packed seems complex, however the 67 nm staggering is often observed. The association between collagen molecules seems driven by hydrophobic and electrostatic interaction where a 234 – amino acid residue occurring with pseudo periodicity seems to play an important role when collagen molecules are associating. Further stabilization between collagen molecules is obtained through cross-linking (Fratzl 2008).

Together with glycoproteins, collagen compose the extracellular matrix which associates with the cell wall (sarcolemma) and their role is to keep the cells (e.g. muscle cells) together. This layer of collagen is named endomysium. Furthermore, these bundles of muscle fibres surrounded by the endomysium are coated with an additional layer of connective tissue namely the perimysium. The resulting structure is called a fascicle. Finally, multiple muscle fascicles are encased by the epimysium, forming and encapsulating the whole muscle. The epimysium is continuous around the muscle and joins the perimysium and the endomysium at the ends, ultimately thickening at junctions with tendons where muscle connects (Kerth 2013). The three mentioned layers of collagen allows the muscle to form a strong but still elastic structure and determines the solubility traits of the tissue. Unmodified collagen fibres tend to be relatively soluble upon heat treatment. Collagen insolubility mainly results from stable cross-linkages (Kerth 2013).

1.1.4 Motivating the introduction of a pre-treatment before hydrolysing MDCR enzymatically

Poultry residues have been associated with functional and bioactive properties which might facilitate addition of value upon for example enzymatic hydrolysis (Brandelli, Sala et al. 2015). More specifically, protein hydrolysates obtained from MDCR treated enzymatically have been associated to both elevated antioxidative properties and other health promoting effects (Sun, Zhao et al. 2010, Lafarga and Hayes 2014). However, even if a raw material has a high content of proteinaceous compounds such as collagen and myofibrils, extraction using exogenous proteolytical enzymes could be associated with low yields. Enzymatic hydrolysis using exogenous enzymes on the residues from mechanically deboned chicken is one example of a low yield process, and similar results are obtained from residuals originating from fisheries (Ghaly, Ramakrishnan et al. 2013, OLIVEIRA, FRANZEN et al. 2015).

Within other sectors of biorefining, namely those focusing on lignocellulosic biomass, pre-treatment of the raw material is applied before enzymatic hydrolysis due to the compact and stable structure of the raw material. The overall goals with a pre-treatment of lignocellulosic biomass is to increase the access of enzymatic cleavage sites (reduced cellulose crystallinity through H-bond disruption) and breakdown the cross-linked matrix (hemicellulose and lignin interactions are dissociated), which increases porosity and accessibility of the enzyme and subsequently the hydrolysis yield (Berbel and Posadillo 2018). The rationale behind such a pre-treatment might be transferable to food by-products as higher yields and shorter treatment times would be desirable, e.g. for enzymatic hydrolysis of MDCR. However, a thorough understanding of the residual structure and how a specific physical, chemical or biological pre-treatment affect the food matrix components is essential in order to properly design a well-functioning overall process.

Innovative technologies hold possibilities regarding processing both of foods and food residuals. Microwave (MW) treatment allows for a advanced volumetric treatment where the temperature might be increased from the inside of the material potentially offering lower temperature gradients and a higher efficiency, thus avoiding the deleterious effects associated with thermal degradation of food components due to excessive temperatures (Pereira and Vicente 2010). Innovative non-thermal technologies, e.g. high-pressure processing (HPP) or ultrasound (US), have the potential to inactivate microorganisms without elevating the temperature significantly, thus maintaining high quality food characteristics (Pereira and Vicente 2010). These innovative technologies (MW, US and HP) have been reported to increase the susceptibility of different proteinaceous substrates upon subsequent or during simultaneous enzymatic hydrolysis and could potentially be used to increase extraction

yields, alter the product peptide properties and reduce process times when treating mechanically deboned chicken residuals (Alemán, Giménez et al. 2011, Ma, Huang et al. 2011, Uluko, Zhang et al. 2013, Blayo, Vidcoq et al. 2016, Nguyen, Zhang et al. 2016, Bruno, Kudre et al. 2019).

1.2 Microwave technology (advanced volumetric heating)

1.2.1 General introduction on microwave technology

Microwaves (MWs) are a part of the electromagnetic spectrum. The wavelength of MWs range from 3000 m to 1 mm, the corresponding frequency range from 100 kHz to 300 GHz. Today, the general acceptance is that MWs causes dielectric heating in biological systems as dielectric material absorbs MW energy and the absorbed energy is transformed into heat because of internal resistance towards rotation. In addition to this heating effect, there are reports in literature that suggest the existence of a non-thermal effect, i.e. effects that cannot be explained by a temperature increase alone. There is however some controversy in this regard (Shamis, Croft et al. 2012).

In an electromagnetic wave two components are present, an electric field (Volt/m) and a magnetic field (amperes/m). Regarding a workload energy source, the electric field is especially important. An electric field originates due to the presence of a charged body and exerts a certain field stress at a given point in space. An example of an electrical field is that found between two charged plates in a capacitor. The electric field intensity is a vector between the two capacitor plates and is frequently drawn as “lines of forces”, as illustrated in Figure 8. These lines represent the path a charged particle would like to follow as a result of the force of the electrical field (Meredith 1998). How a material behaves when in an electric field is characterized by the material complex permittivity (Chen, Ong et al. 2004).

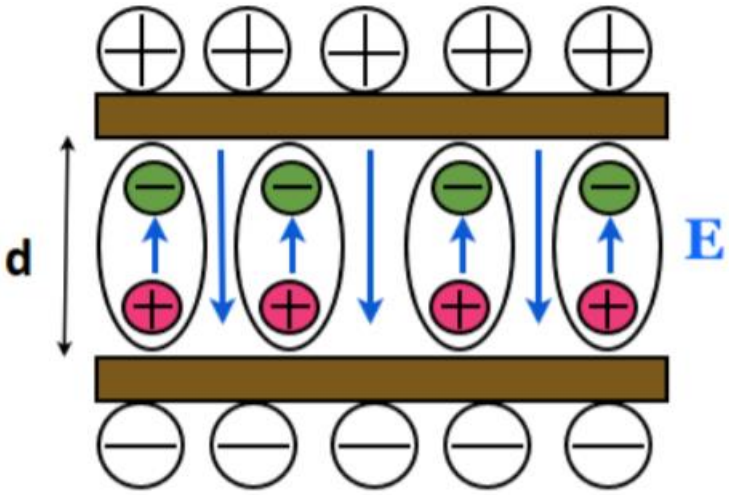

Figure 8: Illustration of how charged particles (green and red circles) are reoriented when affected by an external electric field (Kholimatussadiah and Prijo 2018).

Capacitors are often used to easily illustrate how electric fields behave. A capacitor consists of two metal plates which carry equal but opposite charges and is used to store electrical energy. Today they are important components in electric equipment such as radios, clocks, cell phones, etc. When the capacitor is charged with for example a battery, one of the plates will acquire a negative charge (-Q) while the other obtains a positive charge of the same size (+Q). If there was no loss between the two plates, they could essentially store this energy forever. But because there in fact is a loss between them, the difference in charge will after a certain time diminish. To which degree this occurs depends on the material present between the two plates. Thus the capability of the capacitor to maintain the difference in charges without loss is expressed in capacitance and is dependent of the material placed between the plates (Lillestøl, Hunderi et al. 2006).

In an alternating electric field, the material in the field (between the two plates of the capacitor) might be influenced. For example, if the material between the two parallel plates in a capacitor, as illustrated in Figure 8, is exposed to an electric field the presence of charged particles, which are not free to move within the material may be polarized and shift their orientation back and forth as the electric field alternates. This is in fact a current, namely a displacement current, and currents or moving electric charges may generate magnetic fields (Meredith 1998).

When microwaves are applied in order to heat a material, the effect is determined by the capability of the material to absorb microwave energy and convert it to heat. This is determined by the above mentioned complex permittivity or the dielectric properties (Chandrasekaran, Ramanathan et al. 2013). The dielectric constant might also be referred to as relative permittivity and in such case be relative vacuum. The permittivity of free space vacuum is the force between two charged particles parted by vacuum. However, if the two charges (e.g. in a capacitor) was to be separated by something different than vacuum, for example the residuals from mechanically deboned chicken, the force between the charged plates would increase by a factor called the relative permittivity.

By placing something different than vacuum between two charged plates the force exerted from one plate on the other increases, this indicates an increase in relative permittivity. This is due the response of the material when present in an electric field. There is in general four mechanisms of how a material might be influenced or polarized, and thus alter the force moving from one plate to the other in a capacitor. Electronic polarization occurs when electrons are displaced with respect to the nucleus, which happens to some degree for all substances. Atomic polarization occurs if atoms are moved within molecules or crystals. For molecules that carry a permanent dipole orientation polarization might occur. These molecules will try to re-orient themselves in the electromagnetic

field. The final mechanism is ionic polarization and is due to the movement of free ions (Ryynänen 1995). How these four mechanisms are affected by the alternating electric field frequency is presented in Figure 9. As displayed, these mechanisms are often associated with different frequencies as some are slow and require lower frequencies to dominate, while others are fast and prevail at very high frequencies. This said, there is furthermore two general ways a material may interact with electric and magnetic fields. Energy might be stored, or it might be dissipated. For electric field interaction this is expressed in the complex permittivity where the real part is ascribed to the storage effect while the imaginary part, to dissipation. The value of these numbers are closely related the four mechanisms mentioned regarding energy storage and dissipation and is included in Figure 9 (red line is the loss factor/dissipation factor, blue line is the storage factor) (Chen, Ong et al. 2004).

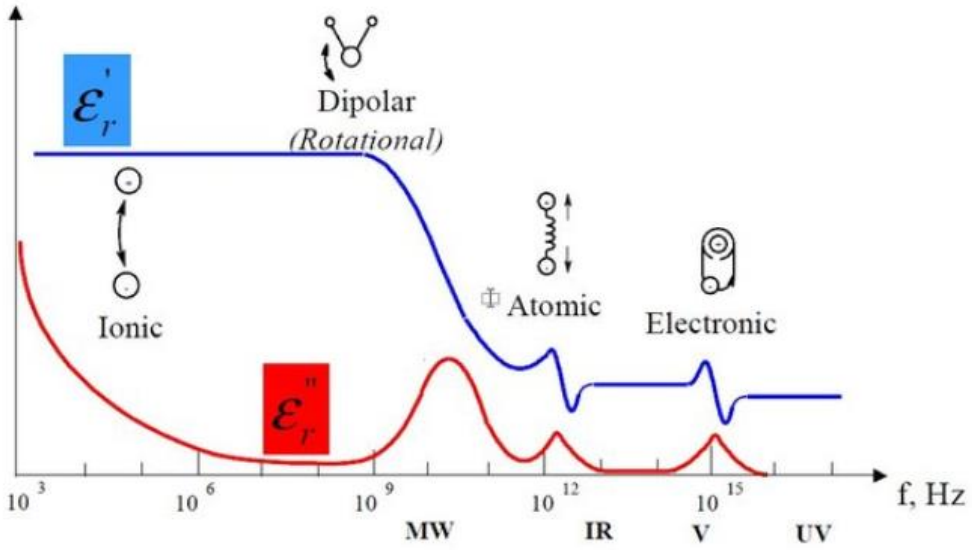

Figure 9: Overview of the four major mechanisms a material can be influenced through when exposed to an alternating electric field and how these mechanisms are affected by varying the frequency of the field (Pérez Cesaretti 2012).

In general, the relaxation frequency of the different polarization mechanisms is as follows. Electronic polarization involves the movement of the smallest mass and is therefore the fastest to re-obtain its regular, random movement as the influence of the electric field ceases. This essentially means that the mechanism is obtained even for very high frequencies, however for lower frequencies the overall contribution is smaller as a small amount of time is used to influence this polarization. Atomic polarization involves the movement of a larger mass and the response in an alternating electric field is therefore slower. Thus, at certain high frequencies this mechanism will drop of as there is not

enough time for a proper response. Further at lower frequencies orientation polarization dominates, which in general has lower relaxation frequencies than atomic polarization and is therefore not present at high frequencies. The last and slowest mechanism is ionic polarization and due to inertia does this mechanism only contribute to a materials response to an electric field at low frequencies. All these mechanisms represent the materials response to an electric field, thus as the frequency increases the different mechanisms will decrease and reduce the relative permittivity. However, the complex part of permittivity might increase at higher frequencies, this for example at frequencies where orientational polarization is exploited maximally (First red peak in Figure 9) (Içier and Baysal 2004).

In addition to different polarization mechanisms and frequency, factors such as temperature and moisture may affect the dielectric properties of a material. Increasing the temperature will, if the frequency applied is lower than the relaxation frequency, cause the loss factor (imaginary part) to increase (Içier and Baysal 2004). This is illustrated in Figure 10. However, increasing the temperature and thermal disorder in a system reduces the electric ordering of polar molecules, and thus their susceptibility to an electric field (Lillestøl, Hunderi et al. 2006).
Moisture exists in a material in different ways, and this will influence the associated relaxation frequency. While free water has a relaxation frequency of about 18 GHz, bound water possess a relaxation frequency of 100 MHz (Delgado, Kulisiewicz et al. 2012).

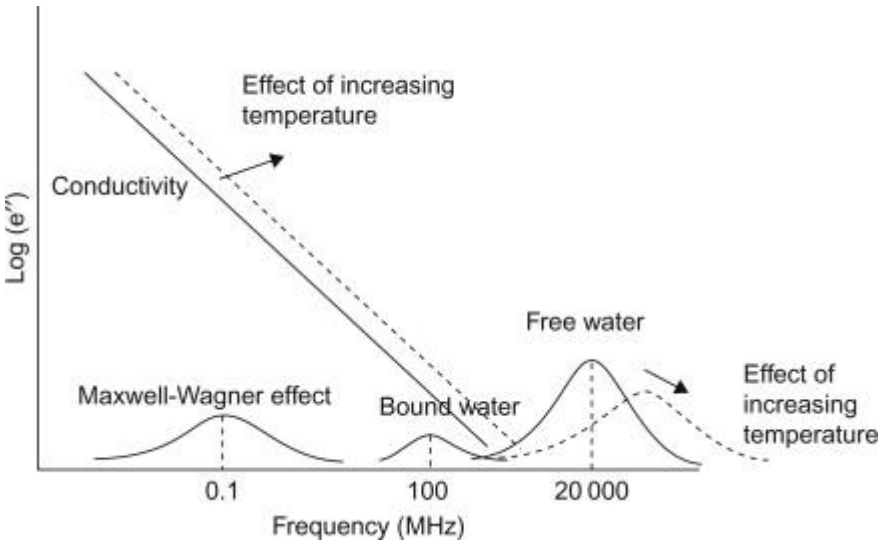

Figure 10: Illustration of how the complex permittivity loss factor is affected by elevating the system temperature (Delgado, Kulisiewicz et al. 2012).

Water in intercellular areas possess dielectric properties like those of free water. Properties of bound water on the other hand relate more to those of ice (Delgado, Kulisiewicz et al. 2012).

1.2.2 General on microwave technology and its effect on specific biopolymers

The most applied frequency in food applications is 2.45 GHz and is used in order to generate heat in high moisture foods (Osepchuk 2002). At this frequency the photon energy is approximately 0.0016 eV which is not sufficient to alter chemical bonds (Ma, Tai et al. 2010). As a comparison, the bond energy of hydrogen bonds is 0.040 – 0.044 eV (de la Hoz, Diaz-Ortiz et al. 2005). However, heat might at some point cause structural changes in biopolymers such as proteins. A protein in its native state is folded into a compact and ordered configuration. The overall tertiary and quaternary structures are a result of the different secondary structure, which further depends on the primary structure. This final structure displays the energetic minimum and thus the most stable confirmation, which is achieved through different bonds and interactions such as hydrogen bonds, van der Waals interaction, ionic interactions and hydrophobic interactions. Thermal modification is initiated by elevated thermal motion which at some point overcome the mentioned intra and intermolecular forces which has been stabilizing the protein's native confirmation. Eventually, as thermal motion increases, all protein groups are reorganized, hydrophobic groups are moved from the interior and exposed to the polar environment. This unfolding increases with temperature and generally secondary and tertiary structures are lost at 80 ℃. At this point a fully unfolded random coil is adopted. In addition, the unfolding is no longer reversible, and such irreversibility is achieved due to the disruption of disulphide bonds and chemical modification of sidechains. Denaturation is often associated with aggregation, and a particularly strong driver toward aggregation is the exposure of hydrophobic groups, since hydrophobic interactions offer a state of increased system entropy (Davis and Williams 1998).

Even though microwaves do not contain enough energy to directly alter chemical bonds, it is assumed that non-thermal effects might occur as polar groups could absorb energy which increase the kinetic energy and fluctuations of such segments, further affecting for example protein structures (Han, Cai et al. 2018). In fact, studies show that microwave treatment could both promote and reduce formation of disulphide bonds and different secondary protein structures (Han, Cai et al. 2018). Bohr and Bohr (2000) illustrate non-thermal effects induced by microwaves on proteins, and attribute this to topological excitations of the proteins. In fact, this is currently a topic of debate in enzymatic catalysis and has caught the interest of the communities at the interface between physics and biology. Nonlinear excitations including topological excitations and discrete breathers are

proposed to have active roles in protein functional dynamics (Piazza and Sanejouand 2008).

1.2.3 On the application of microwave as either pre or simultaneous treatment for enzymatic hydrolysis of proteinaceous substrates

When considering the application of microwaves as a pre-treatment for subsequent hydrolysis several potential beneficial effects should be taken into account: enhanced enzymatic access through increased number of cleavage sites (measured through DH), higher concentration of released peptides in the final solution (i.e. increased protein concentration) and enhanced properties of the peptides with regards to their functionality or bioactivity. As described above, thermal effects are bound to influence the protein substrate if certain temperatures are reached, besides non-thermal effects. A selection of reported results on application of microwaves as pre-treatment before enzymatic hydrolysis is presented in Table 2.

The reported changes in protein structure might not only alter the substrate's susceptibility to proteolytic degradation during hydrolysis, but also they might alter the enzymatic activity if microwaves are applied during hydrolysis, since the structure of enzymes is closely linked to activity (Han, Cai et al. 2018). Coupling microwaves with enzymatic reactions requires high control on parameters such as temperature because local hotspot formation might easily denature the enzyme. Despite this, significant synergisms could be achieved between enzymes and microwaves (Pauzi, Pz et al. 2017). One possibility is that absorption of electromagnetic energy by polarizable domains in the protein could induce certain modifications with regards to structure, which could promote interaction between enzyme and substrate (Rejasse, Lamare et al. 2007). A selection of reported results on the application of microwaves during enzymatic hydrolysis is presented in Table 3.

Table 2: Various results obtained when applying MW as a pre-treatment prior to enzymatic hydrolysis of different proteinaceous substrates.

Substrate	Enzymes(s)	Conditions	Results/Conclusions	Author(s)
Milk protein concentrate	Alcalase Trypsin Neutrase Alkaline protease Flavourzyme	**Pre-treatment:** f: 2.45 GHz P: 800 W T: T < 50 ℃ t: 0, 1, 3, 5 min **Hydrolysis:** T: Optimal pH: Optimal t: 180 min **Control:** No pre-treatment	• **DH%:** Significant increased for Neutrase, alkaline protease, Flavourzyme when MW was applied as pre-treatment. • **Protein solubility:** Increase toward pre-treatment time. Similar for all enzymes.	(Uluko, Zhang et al. 2013)
Milk protein concentrate	Pepsin + Trypsin	**Pre-treatment:** f: 2.45 GHz P: 800 W T: T < 90 ℃ t: 10 min **Hydrolysis:** T: 37 ℃ t:2 h + 2 h **Control:** No pre-treatment	• **DH%:** Significantly higher than that of control. • **DPPH:** Higher activity for pre-treated than control samples. .	(Uluko, Zhang et al. 2015)
Fish frame	Alcalase	**Pre-treatment:** f: 2.45 GHz P: 800 W T: 55, 90 ℃ t: 2-10 min **Hydrolysis:** T: 55 ℃ t: 15 min **Control:** No pre-treatment.	• **DH%:** Pre-treatment at 55 ℃ does not influence DH with increasing treatment time. Pre-treatment at 90 ℃ increases DH with increasing treatment time. • **Protein solubility:** Pre-treatment at 55℃ do not influence solubility with increasing treatment time. Pre-treatment at 90℃ reduces solubility with increasing treatment time.	(Ketnawa and Liceaga 2017)
Sweet potato protein powder	Pepsin Pancreatin	**Pre-treatment:** f: n/g P: 700 W T: n/g t: 2 min **Hydrolysis:** T: 37 ℃ t: 1, 2 h **Control:** No pre-treatment.	• **DH%:** Microwave treatment significantly increased DH compared to the control. • **Peptide weight distribution:** Lower quantity of large peptides (> 10 kDa) compared to the control. Higher quantity of small peptides (3-10 kDa) compared to control.	(Zhang, Huang et al. 2019)

Abbreviations and units

f: Frequency [GHz], P: Power [W], T: Temperature [℃], t: time [min] [h], n/g: Not specified

Table 3: Various results obtained when applying MW simultaneously as enzymatic hydrolysis of different proteinaceous substrates.

Substrate	Enzymes(s)	Conditions	Results	Author(s)
β-lactoglobulin	Pronase α-chymotrypsin	**MW-assisted:** P: 15, 30 W f: 2.45 GHz T: 40 ℃ t: 20, 30 min **Control:** Conventional heating	• **DH%: Pronase**: No effect compared to the control. • **DH%: α-chymotrypsi**n: Significantly lower than the control.	(Izquierdo, Alli et al. 2005)
Bovine serum albumin Cytochrome c β-casein	Trypsin	**MW-assisted:** P: 850 W f: 2.45 GHz T: 37, 50 ℃ t: 1, 3, 5,10, 30 min and 2, 16 h **Control:** Conventional heating	• **Rate of proteolysi**s: MW-assisted tryptic digestion of BSA, cytochrome c, β-casein proceeds at virtually identical rates as observed when applying conventional heating.	(Damm, Nusshold et al. 2012)
Lobster shell	Alcalase	**MW-assisted:** P: 40 W f: n/g T: 55 ℃ t: 30, 60, 90 min **Control:** Conventional heating	• **Rate of protein solubilisation:** Amount of protein solubilized from the solid lobster shell significantly increased when MWs were applied as compared to conventional heating.	(Mazinani and Yan 2016) (Nguyen, Zhang et al. 2016)
Fish frame	Alcalase	**MW-assisted:** P: 20% power with 50% duty cycle at 1200 W f: n/g T: 50-55 ℃ t: 3, 5, 15min **Control:** Conventional heating	• **DH%:** MW-assisted hydrolysis significantly increased DH% as compared to the control. • **Protein solubility:** Certain conditions did not display changes while other indicated reduction/enhancement when microwave was applied during hydrolysis. • **Surface hydrophobicity:** Significantly lower or equal when MW was applied as compared to the control. • **Foaming and emulsifying properties:** In general, significantly higher for hydrolysates obtained from microwave-assisted hydrolysis.	(Nguyen, Jones et al. 2017)

Abbreviations and units

f: Frequency [GHz], P: Power [W], T: Temperature [℃], t: time [min] [h], n/g: Not specified

Whether microwaves promote enzymatic activity by applying it as a pre-treatment on the substrate or simultaneously appears to be dependent on the enzyme and substrate. However, these could be due to temperature variances at the microscopic level affecting both enzyme and substrate. Furthermore, MW pre-treatment on residuals obtained from mechanically deboned chicken residuals might influence other components present in the raw material than proteins. The organized biomatrix could influence the effect of MW as a pre-treatment before enzymatic hydrolysis. Raghavan and Kristinsson (2014) reviewed the effect of microwave on lipids and it seems MWs could induce lipid oxidation. Moreover, some results indicate that there might be compounds such as antioxidants present in the biological system which could reduce this effect. MW has been reported as beneficial in processing of fish, meat and foods due to the high response of free water. However, reports on the effect of microwaves on meat, fish, minced meat or other high protein food products prior to enzymatic hydrolysis are scarce, as most treatments are conducted with isolated protein components in dilute suspensions.

1.2.4 The choice of MW process conditions for the treatment of MDCR prior to enzymatic hydrolysis

By-products such as mechanically deboned chicken residuals contain large amounts of proteins. This protein fraction is further dominated by collagen, although they also contain smaller amounts of muscle tissue mainly comprised of myofibrils and sarcoplasmic proteins. Applying microwaves to pre-treat this type of material could pose processing similarities to cooking (aggregate formation through cross-linking, hydrophobic interactions, etc.) due to the thermal effect, which has been associated with reduced proteolytic digestion (Gatellier and Santé-Lhoutellier 2009). Uluko, Zhang et al. (2013) reported that pre-treatments on milk protein concentrate with short MW treatments (1, 3 and 5 min, T < 50 ℃) increased both protein solubility and DH% when treatment time increased. Meanwhile, thermal treatment of milk proteins at 65 ℃ has been show to only cause minor denaturation for treatment times ranging between 10 and 30 min (Qian, Sun et al. 2017). This indicates that slight unfolding of proteins could be beneficial when proteins are targeted for subsequent proteolytic degradation. Important to note is that concentrations would probably influence the results as high protein concentrations would easily facilitate protein interactions and aggregate formation.

In muscle tissue phenomena such as granulation and gel formations occur at certain temperatures,

which are closely associated to structural alterations of the different protein components. Kijowski and Mast (1988) investigated at which temperatures protein denaturation occurred in chicken breast and thigh muscles. Five transition temperatures were observed at 57 °C, 63 °C, 67 °C, 73 °C and 78 °C and found to correspond to the denaturation of myosin, sarcoplasmic proteins, collagen and F-actin. Denaturation of myosin is often associated with several temperature peaks due to complex denaturation, this is also the case for collagen, which often displays broader temperature ranges for transition due to differences in tissues such as endomysium, perimysium and epimysium (Bernal and Stanley 1986). Murphy and Marks (2000) investigated the effect of heat on minced chicken breast properties such as protein solubility and molecular size distribution. While overall protein solubility decreased as temperature increased between 40 and 80°C the presence of low-molecular weight species increased when the 40 °C treatment was conducted. The presence of low-molecular species disappeared at 50 °C. Investigations conducted by Chang, Wang et al. (2011) on beef semitendinosus muscle with conventional heat treatment and microwaves found that granulation seemed to occur first at 50 °C and increased upon higher temperatures. Granulation is thought to be a result of both collagen shrinkage and myofibrillar denaturation. No granulation was observed at 40 °C. Granulation and gel formation is regarded as non-favourable for a subsequent hydrolysis. The work done by Zhang and Barbut (2005) gives an indication of the temperature at which gel formation starts in chicken breast meat. The elastic modulus of the different chicken meats did not change much until a temperature of 48 °C was achieved. However, between 48 and 55 °C a rapid increase occurred. This increase is explained by the denaturation of proteins resulting in the onset of structure formation, which results in the highest storage modulus obtained by heating seen at a temperature of 75 °C. Upon subsequent cooling the storage modulus continues to increase due to the formation of hydrogen bonds.

Thus, the pre-treatment of proteinaceous materials rich in collagen and muscle tissue should preferable avoid effects such as granulation and gel formation, which is reported at temperatures close to 50 °C. In fact, the presence of low-molecular weight species at 40 °C, possibly originating from myosin dissociation, could display beneficial characteristics such as increased cleavage sites, if enzymatic accessibility is sufficient. As for treatment time is 1, 5 and 10 min chosen for further investigation in the present work and are based on the parameters applied by Bruno, Kudre et al. (2019).

1.3 Ultrasound technology

1.3.1 General introduction on ultrasound technology

Ultrasound (US) refers to mechanical waves at frequencies above those which humans can hear. Depending on the frequency, US may be divided into three categories; Power US (16-100 kHz), high frequency US (0.1-1 MHz) and diagnostic US (1-10 MHz) (Zhang, Wang et al. 2018).
The types of mechanical waves which might occur in gasses, liquids and solids are either longitudinal or shear waves. In longitudinal waves the particles move back and forth parallel to the wave. In shear waves, the particles move at right angles to the direction of the wave propagation, i.e. up and down if the wave propagates in the horizontal direction. While longitudinal waves frequently occur in soft tissue, shear waves often occur in hard tissues such as bone (O'Brien Jr 2007).

Figure 11: Illustration of the particle movement association with longitudinal waves (top) and shear waves (bottom) (O'Brien Jr 2007).

Neither longitudinal nor shear waves produce any net flow of the medium. The particles are simply displaced for a certain amount of time, before returning to their original position only to be displaced again. The speed of the particles is higher in the areas where there is no displacement and close to zero in the regions with the most pronounced displacement (negative or positive maxima). The movement of particles in soft tissues form areas where particles concentrate and areas less concentrated, also known as areas of compression (high pressure) and rarefaction (low pressure), respectively. For simple linear waves, the acoustic pressure (rarefaction and compression areas) and the particle velocity are in phase (Leighton 2007). In real waves protruding through for example soft tissue different factors will cause small or large deviations to the mentioned in-phase relationship.

Ultrasound applied in water and soft tissues causes different effects but the most reported one is acoustically generated cavitation. When ultrasonical waves propagate through the tissue, a

mechanical strain can be generated. If this strain is significant around gas or vapor bubbles present in the medium, then cavitation can occur (Leighton 2007). The overall effect ultrasound poses on gas or vapor bubbles may be sorted into two categories; Inertial cavitation and non-inertial cavitation. The non-inertial cavitation is associated to a process where low energy effects occur (e.g. microstreaming due to water streaming close to an oscillating gas bubble). Inertial cavitation is associated to high-energy effects such as the formation of free radicals and shock waves. In order to generate inertial cavitation, the vapor or gas bubble must undergo a violent collapse. Whether this can occur or not depends on multiple factors, but it can be illustrated through the two main forces acting on the bubble, namely bubble stiffness and inertial forces.

As a volume of gas or vapor is compressed there will be forces, for convenience called bubble stiffness, acting and resisting the deformation. This resistance will in fact tend the bubble to expand. The potential energy associated with the highly compressed state will, as the bubble expands, be converted to kinetic energy which acts on the surrounding water and pushes it away. Because there is a certain delay (inertia) in the response from the surrounding liquid, the bubble can expand over its equilibrium radius. Eventually, the slightly compressed surroundings will start once again to compress the vapor or gas bubble (Crum 1984). When an acoustic field is applied on vapor or gas bubbles, in addition to the previously mentioned forces (inertial force from surrounding liquid and stiffness from the gas incompressibility), an acoustic pressure function is present. If the dynamics of the bubble collapse is dominated by the force from the acoustic pressure field, inertial cavitation occur. One might visualize this by realizing that the bubble needs to reach a certain size during expansion before collapsing fast enough to get ahead of the stiffness working against the bubble compression. While the surrounding water does not offer a quick enough compression, the assistance from an acoustic sound field might (Leighton 2007). The frequency of the acoustic field will also affect which bubble diameters are able to undergo inertial cavitation. There is a critical size range of bubble radii, which increases with increasing acoustic pressure amplitude and decreasing frequency, which will undergo inertial cavitation. Bubbles outside this critical range will tend to not undergo inertial cavitation (Leighton 2007). This is illustrated in Figure 12.

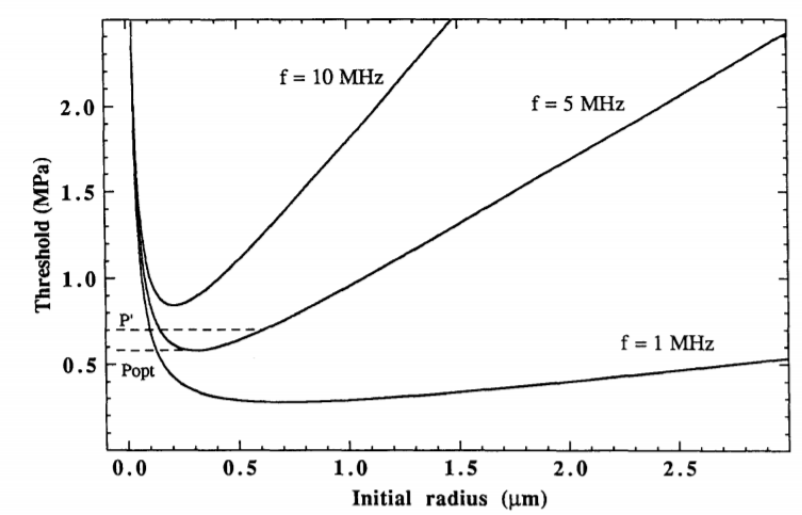

Figure 12: The relationship between acoustic pressure, initial bubble radius and how US frequency affects the threshold for inertial cavitation. Above the frequency curves inertial cavitation is favoured in water with a certain content of bubbles (Apfel and Holland 1991).

The overall cavitation activity (both inertial and non-inertial effects) depends on the size and number of bubbles present, power applied and frequency. Increasing both power and frequency seem to increase inertial cavitation. However, this effect seems to be observed only up to a certain point at which inertial cavitation stops (Hatanaka, Yasui et al. 2001, Lee, Yasui et al. 2018). Another important factor is that the initial amount and changes in gas content causes completely different cavitational effects (Sutkar and Gogate 2009). Furthermore, increasing power does not cause a linear increase in acoustic pressure (indicator on inertial cavitation), which may be attributed to two factors. First, a greater fraction of applied energy is transferred into higher frequency components as power increases. Acoustic signals in the MHz range are for example produced from a low kHz fundamental in strongly cavitational environments, with less inertial cavitation induced at higher frequencies (in general). Second, the acoustic pressure may be shielded by bubble clouds (very high inertial cavitation effect within small volumes) and therefore only extend a very short distance from the transducing surface (Hodnett, Choi et al. 2007). The region of shielding seem to extend with increasing power setting (Hodnett and Zeqiri 2008).

1.3.2 General on US technology and its effect on specific biopolymers

Different effects are reported when ultrasound is applied to biological materials. Yu, Zeng et al. (2016) reported the reduction in gelatine particle size followed by enhanced enzymatic hydrolysis, which was attributed to the physical effects of ultrasound (not radicals). However, Yang, Li et al. (2017) found protein solubility and subsequent hydrolysis to be enhanced when defatted wheat

germ protein was treated with ultrasound. These effects in addition to protein loosening and unfolding was attributed to sonochemical effects and thus, to the presence of H_2O_2 and free radicals. Disruption of disulphide and hydrogen bonds are also reported and might cause matrix expansion, thus facilitating subsequent enzymatic hydrolysis (Zhang, Ma et al. 2015). Another effect associated to ultrasound is the direct scission of polymer chains, however chain rupture has been reported and seem to favour polymers with high molecular weight in suspension (Pu, Zou et al. 2017). While proteins and carbohydrates have frequently been studied, the studies on the effects of ultrasound on lipids are scarce. However, many of the factors known to affect lipid degradation are also present during ultrasound processing such as high temperatures, light and free radicals (Pingret, Fabiano-Tixier et al. 2013).

1.3.3 On the application of ultrasound as either pre or simultaneous treatment for enzymatic hydrolysis of proteinaceous substrates

US can as already indicated be beneficial when applied as a pre-treatment followed by enzymatic hydrolysis. Effects apart from those already mentioned are reduction of aggregates, pore formation and removal of indigestible outer-layer tissues (Wang, Yan et al. 2018). Increased number of cleavage sites and enzyme accessibility have been reported. A short summary of reports on the application of US as a pre-treatment to enzymatic hydrolysis is presented in Table 4.

Most enzymes are proteins, and as seen US might alter protein conformation. Enzymatic activity often depends heavily on the configuration of the active site; thus, the activity of an enzyme might be affected by US. However, there seems to be no general link between structural changes and the specific US treatment. Further, while it is believed that effects associated with inertial cavitation alter protein conformation, expose enzymatic active sites and unfold the polypeptide chain, which might enhance interaction between enzyme and substrate, there is not any specific US effect linked to the different protein conformation changes. However, US is able to alter the enzymatic activity as different effects affect different enzymes with high specificity. Ma, Wang et al. (2015) investigated the use of US on the activity of polygalacturonase and discovered that a certain treatment enhanced the activity. This might be a result of shear forces, heat or free radicals formed from the collapsing bubble and further affecting the enzyme structure and activity. The effect seen on the enzyme was irreversible (Ma, Wang et al. 2015). However, Jadhav and Gogate (2014) reported, when investigating the activity of a lipase treated with ultrasound, a two-fold activity increase followed by a steady decrease after 60 min, thus displaying reversibility. A selection of results reported in literature on the simultaneous effect of US on different proteases and substrates is presented in Table 5.

Table 4: Various results obtained when US is applied as a pre-treatment before enzymatic hydrolysis of various proteinaceous substrates.

Substrate	Enzymes(s)	Conditions	Results	Author(s)
Tilapia muscle	Flavourzyme	**Pre-treatment:** f: 20 kHz P: 10, 70 W t: 15, 30, 45 min T: 55 ℃ **Hydrolysis:** t: 60 min T: 55 ℃ **Control:** Untreated sample	• **DH%:** Pre-treatment with US reduced DH% as compared to the control. • **DPPH**: Lowest for the control. Higher when US was applied as pre-treatment.	(Kangsanant, Murkovic et al. 2014)
Fish head	Protamex	**Pre-treatment:** f: 20 kHz P: 750 W T: n/g t: 5, 10, 15 min A: 40% **Hydrolysis:** T: 55 ℃ t: 2 h **Control:** Untreated sample	• **DH%:** In general, US enhanced DH% as compared to the control. • **Protein extraction/protein solubility**: Increased with US treatment time and was higher than that of the control for all treatment times.	(Bruno, Kudre et al. 2019)
Rice protein	Alcalase	**Pre-treatment:** f: 20, 28, 35, 40, 50,60 kHz P: 300 W per generator T: 30 ℃ t: n/g **Hydrolysis:** T: 50 ℃ t: 1 h **Control:** Untreated sample	• **Protein solubility**: Ultrasound significantly improved peptide concentration in the hydrolysate as compared to that of the control. • **Multiple simultaneous frequencies**: Multiple frequencies applied simultaneously seemed to offer a slight additional enhancement in peptide solubilisation.	(Yang, Li et al. 2017)
Potato protein	Alcalase	**Pre-treatment:** f: 20/40 kHz P: 250 W/L T: 25 ℃ t: 10 min **Hydrolysis:** T: 30, 40, 50, 60 ℃ t: 2 h **Control:** Untreated sample	• **Reaction rate constant during hydrolysis**: Significantly higher for all temperatures when US was applied as a pre-treatment as compared to that of the control.	(Cheng, Liu et al. 2017)

Abbreviations and units

f: Frequency [kHz], P: Power [W] or volumetric power [W/L], T: Temperature [℃], t: time [min] [h], n/g: Not specified.

Table 5: Various results obtained when US is applied during enzymatic hydrolysis of different proteinaceous substrates.

Substrate	Enzymes(s)	Conditions	Results	Author(s)
Tilapia muscle protein	Flavourzyme	**US-assisted:** P: 10, 70 W f: n/g T:55 ℃ t: 60 min **Control:** Conventional heating during hydrolysis	• **DH%:** US-assisted hydrolysis reduced DH%. Enzyme exposure to US alone only caused a 6% reduction in enzyme activity. • **Possible gelation:** The substrate treated with US appeared more viscous than the control. The temperature during the treatment could have induced substrate network-formation.	(Kangsanant, Murkovic et al. 2014)
Rice protein	α-amylase	**US-assisted:** P: n/g f: 20, 28, 35, 40, 50 kHz T: 50 ℃ t: 3 h **Control:** Conventional heating during hydrolysis	• **Protein concentration:** US-assisted hydrolysis significantly enhanced the protein extraction rate for all frequencies compared to that of the control. • **Frequencies:** For protein extraction it seems beneficial to apply low frequencies (20 or 35 kHz instead of 40 or 50 kHz for instance). Application of dual frequency modes seems to offer enhanced extraction rates compares to single modes. • **Surface hydrophobicity**: Ultrasound enhances surface protein hydrophobicity.	(Yang, Li et al. 2018)

Abbreviations and units

f: Frequency [kHz], P: Power [W], T: Temperature [℃], t: time [min] [h], n/g: Not specified.

1.3.4 The choice of US process conditions for the treatment of MDCR prior to enzymatic hydrolysis

Applying an ultrasound treatment on residuals from mechanically deboned chicken residuals will not only differ from the literature presented in Table 4 and Table 5 due to other biomolecules being present, but also due to the organized biomatrix, which could influence the effect of ultrasound as a pre-treatment before enzymatic hydrolysis. Similarly to other proteinaceous substrates treated with US general trends are scarce. Kang, Gao et al. (2017) investigated alterations in water holding capacity, tenderness, water distribution and myofibril denaturation during brining of solid beef assisted by US. While the brine itself caused some denaturation, both water holding capacity and myofibril denaturation increased for a US treatment of 30 min in combination with power above a certain level. Even though myofibrillar fragmentation increased, myosin oligomer formation generated through S-S bond formation also occurred. While S-S bond formation and associated aggregation would probably restrict a subsequent hydrolysis, increased water holding capacity and myofibrillar fragmentation would possibly benefit a subsequent hydrolysis. The combination of smaller particles of substrate (increased fragmentation of myofibrils) and more water present would lead to better accessibility for the proteolytic enzymes, which depends on the substrate access in the liquid phase. Increased water holding capacity could also indicate protein denaturation, which promotes water-protein interaction and thus increased protein solubility and extraction yield.

The results of Kang, Gao et al. (2017) refer to inertial cavitational effects, as also commonly reported for suspensions or solids treated with US. Non inertial cavitational effects are far milder than those associated to inertial cavitation and are believed to promote effects such as mass-transfer through for example microstreaming. According to Leighton (2007), the energy associated to the hotspot generated by inertial cavitation is not important for the effect seen by US, as the associated energy is very low. The remaining main effects are radical formations and the shockwave or rebound pulse, as well as the effect associated to non-inertial cavitation such as microstreaming. Another mild effect associated with cavitation is micro jets. Bubbles associated with solid surfaces often collapse asymmetrically where a liquid jet protrude through the collapsing bubble and onto the solid surface where a corroding effect occur. This is often called micro jets and is a mild effect.

The hot-spot formed during inertial cavitation might form the basis for thermal decomposition of water and can cause formation of hydroxyl radicals, hydrogen anions and hydrogen peroxides (Rehman, Jawaid et al. 2016). Previous investigations on myofibrils have displayed result indicating both enhanced and reduced proteolytic digestibility when exposed to oxidation and hydroxyl radicals

(Grune, Jung et al. 2004, Morzel, Gatellier et al. 2006). Suzuki, Kaneko et al. (1991) investigated the effect of hydrogen peroxide on myofibrils. The results suggested that oxygen free radicals may work by oxidation of SH groups, however it was emphasised that other oxygen radical effects such as protein-protein crosslinking and protein chain scission still could occur. When treating porcine muscle myofibrillar protein Park, Xiong et al. (2007) found protein unfolding and reduction of myosin heavy chain to occur. The effect was more pronounced with increasing hydrogen peroxide concentration.

Collagen exposed to radical generating systems can induce damage to the material, resulting in fragmentation and higher susceptibility for enzymatic degradation (Hawkins and Davies 1997). Liu, Jiang et al. (2014) investigated the effect of treating tilapia skin collagen type 1 with hydrogen peroxide. The results showed that strength and resistance of collagen decreased exponentially with heating time (53 ℃). First the ordered structure of collagen and its crystallinity was reduced, destroying bundle structures. Further fibre bundles were loosened and microfibres exposed and dissociated (Liu, Jiang et al. 2014). Collagen has also been found to be susceptible to fragmentation by superoxide anion. Seemingly, hydroxyl radicals in the presence of oxygen, cleave collagen into smaller peptides. Hydroxyl radicals in absence of oxygen however trigger polymerization of collagen through cross-link formation (Monboisse and Borel 1992).

Water radicals seem to offer varying results. However, the effect from radicals originating from water could potentially explain the myofibrillar fragmentation and enhanced water holding capacity observed by Kang, Gao et al. (2017) which was mentioned in the beginning of this section.

Another inertial-cavitational effect that could be responsible for the effect described by Kang, Gao et al. (2017) is the shock-wave associated to the collapsing bubble, which is generated subsequently the implosion. Biological tissue is acoustically inhomogeneous, which causes wave refraction and scattering, resulting in highly variable movement in the material. Shock-wave technology has been applied in meat tenderization treatment (Bolumar and Toepfl 2016). Shock-waves travel fast through fluids that resemble water, and as most meats contain about 75% water, pressure waves travel through meat, and at places where there are differences in acoustic impedance energy momentum is transferred so that muscle structure may be teared. A shock wave is much like sound waves which travel through a medium with oscillating positive acoustic pressure followed by a negative pressure region. However, a shock wave is primarily a single front wave with very high-pressure, followed by a negative pressure. The negative pressure is usually not as significant as the high-pressure component. The pressure difference still being large offers a significant effect. This has been reported to significantly increase tenderness of beef, fragment myofibrils, increase intermyofibrillar spaces, increase protein solubility as well as increase brine absorption of turkey breast (Bolumar and

Toepfl 2016). Meat treated with shockwaves seems to fragment myofibrils through physical tearing close and parallel to the Z-line (Figure 4). The endomysial space also seems to be increased by shock-wave treatment, likely due to disruption of collagen fibril network. Thus, shock waves seem to tenderize through both disrupting myofibrils and collagen (Bolumar and Toepfl 2016). Also the effect of shock waves seems to potentially be responsible for the effects seen by Kang, Gao et al. (2017), as myofibril fragmentation, tenderness, increase in protein solubility and in brine absorption have been reported and overlap with the effects mentioned. However, the effect of shock-waves and water radicals are based on sufficient inertial cavitation, and in solid food this might not be the case.

Duerkop, Berger et al. (2018) demonstrates the importance of vapor bubbles close to proteins in liquid phase by increasing the protein concentration, and at some point, the vapor/liquid interphase seemed to get saturated with proteins. As the bubbles collapsed inertially, protein aggregation reached a limit upon a certain protein concentration. Similar effects on cell lysis induced by inertial cavitation are reported by Miller, Miller et al. (1996). Neither formation of radicals nor mechanical shear seem to influence significantly aggregation. Proteins present at the surface of an oscillating bubble are forced, as this bubble collapses, inward towards the centre where high temperatures and pressure are formed. It would seem obvious that these proteins would denature and possibly entangle and forming aggregates. This would also explain why no more aggregation occurs when there is no more bubble surface for the proteins to occupy. Results suggest that close approximation to the collapsing bubble is important for the protein alteration. Inertial cavitation in materials such as soft tissue has been investigated both with regard to frequency and acoustic pressure threshold (Vlaisavljevich, Lin et al. 2015). Interestingly, the threshold seems to be independent of material stiffness but decreases with decreasing frequencies, which is in line with the trend shown in Figure 12. However, even though the threshold is lowered by decreasing frequencies, the acoustic threshold in tissues such as tendon, liver and tongue is close to that of degassed water (15% O_2). In order to generate inertial cavitation in the mentioned soft tissues approximately three times the acoustic pressure needed in gas saturated water is required (Vlaisavljevich, Lin et al. 2015. This might relate to the general low gas nucleation in biological tissues, thus high acoustic pressures are required to initiate inertial cavitation. However, tissues such as lungs are often high in gas nuclei and inertial cavitation are observed at lower acoustic pressures. Another factor which could explain the elevated acoustic pressures required in tissue is the restricted movement offered to the bubbles (Miller, Miller et al. 1996).

The effects associated with inertial cavitation could explain the results of Kang, Gao et al. (2017). However, a low amount of pre-existing bubbles, which require very high acoustic pressures, in

addition to the importance of close association to the collapsing bubble might be proof that inertial cavitation is not accountable for the observed effect. An interesting result mentioned by Miller (1985) is that individual pressure-pulses and their associated induced strain seem to accumulate during multiple pulse exposures, causing a gradual fatigue. Frenkel, Kimmel et al. (2000) reported on intercellular space widening as an effect that neither seems thermally induced, nor due to inertial cavitation. It is suggested that the formation of transverse shear waves is most likely responsible for the space increase between cells. The mechanism might be illustrated by cells forced into each other before being pulled apart by the different wave characteristics. Hancock, Smith et al. (2009) treated mice calf muscle with ultrasound and investigated, among other things, the structure of fibre bundles and the reversibility of the changes. Samples treated displayed enlarged gaps between the muscle fibres compared to the control. The gaps were reversible after 72 h. Disrupted connective tissue was observed between the gaps in addition to disrupted capillaries. This research might further suggest non cavitational mechanisms being responsible for the results obtained by Kang, Gao et al. (2017).

While the exact mechanism behind the results reported by Kang, Gao et al. (2017) remains uncertain, the results seem interesting with regards to a subsequent hydrolysis. Several works where raw materials with characteristics like those of MDC residuals were treated with ultrasound will be evaluated. Most research articles focus on brining of meat products and thus, the results are influenced by a high ionic strength. The effect of salt is mentioned by Kang, Zou et al. (2016), it seems to function by reducing the repulsion between myofibrillar molecules, allowing molecular relaxation and increasing the free sulfhydryl content and dissolution. Not all proteins are stabilized by salt-ions as the ions compete with water molecules in charged-group bonding and thereby might decrease solubility as ionic strength is increased. The salt might, at least partly, explain the increased protein solubility reported by Kang, Gao et al. (2017). Kang, Zou et al. (2016) report that when treating beef *longissimus dorsi* with ultrasound, the total sulfhydryl content decreased, which was further accompanied by a reduction in myosin heavy chain content and formation of high molecular weight aggregates. This seemed to occur for most power settings with increasing treatment time, however the reduction was not significant for a treatment time of 30 min. The loss of total sulfhydryl content could possibly be favoured by increased ionic strength. Surface hydrophobicity increased for all treatment times and power applied. This might be beneficial for subsequent hydrolysis as it indicates protein denaturation but might be exaggerated by the elevated ionic strength. Work done by Wang, Kang et al. (2018) where *m. semitendinosus* were treated with US (20 kHz, 5 ℃, 20 and 40 min, 25 W/cm^2) accelerated degradation upon subsequent storage. This was attributed to better access for endogenous enzymes. The results upon storage indicated increased degradation of the proteins desmin and troponin-T. Desmin is an important protein responsible for extending between Z-disks in

adjacent sarcomeres. Troponin is present in the thin filaments together with actin. Regarding collagen in association with beef, there are reports on the formation of US induced staggering and structural disordering which is further associated with a loose arrangement (40 kHz, 1500 W, 20 ℃, 10-60 min). The particle size reached a minimum after 20 min of treatment and increased slightly upon further processing (Chang, Xu et al. 2012).

Based on presented literature which report on desirable US effects on raw materials similar to MDCR, it would seem preferable to choose a treatment time between 20 and 40 min. Therefore, a treatment time of 30 min is chosen for the US trials in the present work. Furthermore, to avoid the thermal effects (gel formation and granulation) mentioned during the discussion of MW pre-treatment conditions (Section 1.2.4), temperatures higher than 30 ℃ should be avoided to ensure minimal intervention. Independently on which mechanism dominates and dictates the effect of a US treatment, a variation in power settings seems to be favourable. This ensures that an effect would be observable at lower power settings if, for example, higher power settings avoid the associated US effect through, for example, bubble shielding. Also, the power setting is highly variable between various US systems and therefore a high degree of uncertainty is associated with adapting power settings applied in trials employing different equipment.

1.4 High-pressure processing technology

1.4.1 General introduction on high-pressure processing technology

High-pressure processing (HPP) relies on a liquid medium to transmit pressure to the sample. The pressure acts uniformly throughout the mass and is not dependent on size, shape or composition (Khan, Ahmad et al. 2017). This is illustrated in Figure 13. A multitude of changes occur in systems at elevated pressures, e.g. changes in boiling point, melting point, density, viscosity, solubility, conductivity and the dielectric constant. In addition, effects such as changes in secondary and/or tertiary and/or quaternary structures in large complex compounds are reported (Zhang, Wang et al. 2018).

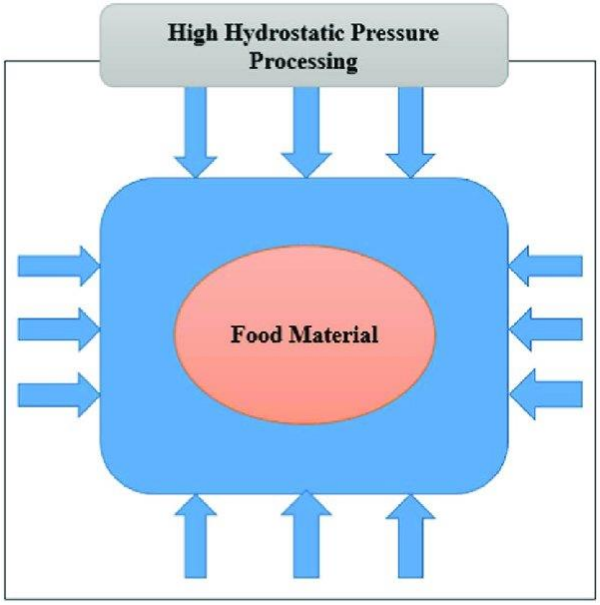

Figure 13: The application of HPP on a system and how the functioning forces affect a material is illustrated (Khan, Ahmad et al. 2017).

1.4.2 General on high-pressure processing and its effect on specific biopolymers

Two principles are often used to explain the effect elevated pressure has on e.g. alteration of protein structures. However, in complex systems such as biomatrices where equilibrium seldomly is reached is the first principle, Le Châteliers principle, less applicable than the second, the transition state theory (Martinez-Monteagudo and Saldaña 2014). The second principle states that if the molar volume of the intermediate species is different than the molar volume of the reactive species, the reaction velocity can increase or decrease by increased pressure. Thus, if the intermediate or product

species is less voluminous compared to the initial species, the reaction rate will increase upon elevated pressure. The opposite would be the case if the reactant was less voluminous. Elevated pressure will in other words benefit reactions with overall negative reaction volume and negative activation volume (Martinez-Monteagudo and Saldaña 2014).

Most literature consider the activation volume as an indicator on whether a reaction is promoted or inhibited by pressure and is mainly composed of two types of interactions (Martinez-Monteagudo and Saldaña 2014). The first type of interactions causing a basis for volumetric changes is molecular reorganization. Examples are alterations in chain-chain interactions that exist within the molecule. For example, a van der Waals interaction could be exchanged by a hydrogen bond and cause molecular reorganization and volumetric changes. The second term represents changes in solvent-molecule interactions. For example, cavities contribute to the overall volume, and might originate from e.g. imperfect packing of hydrophobic amino-acid residues localized in the interior of protein molecules. These surfaces, which are associated with non-polarity causes a reduction in hydration volume (Gekko and Hasegawa 1986).

Changes in bond types create one of the fundaments for volumetric alterations.
As pressure is increased the distance between certain atoms are changed. Weak interactions where the bond energy is dependent on distance is especially affected by changes in pressure (Martinez-Monteagudo and Saldaña 2014). Among these are coulomb, van der Waal, hydrogen bonds, solvation and hydrophobic interactions. Often the formation of hydrogen bonds is associated with a negative volume change. Formation of charged groups from ionic interactions is also associated to reduction in volume. The reason for this volume change is believed to be due to elevated electrostriction of the solvent molecules around the newly formed charged species. Association between non-polar groups seems to vary. While association between aliphatic groups seems to generate a positive volume change, association between aromatic groups generates a negative change in volume (Heremans 1982). It is generally accepted that high pressure only affects non-covalent bond types (Okamoto, Kawamura et al. 1990).

In complex food matrices there are multiple components present, which through different interactions will affect the overall compressibility of such systems. For example, the compressibility of chicken breast appears to be similar to ham at higher pressures, while at lower pressures chicken breast is more compressible (Min, Sastry et al. 2010). One explanation could be the higher content of

air in chicken breast, which will dissolve and reduce the volume as the pressure is increased. Another factor influencing systems such as chicken breast and ham is the high moisture content. Compressibility of liquids is relatively high at low pressures as there is a considerable amount of free volume between the molecules. When the pressure is increased this free volume rapidly diminishes and together with repulsive forces acting between molecules, further compressing is limited. A frequent food component is fat. Compared to water, chicken fat for example exhibits a high compressibility for pressures up to 100 MPa, a similar compressibility up to 300 MPa, but a reduced compressibility upon further pressurization up to 700 MPa (Min, Sastry et al. 2010). The high compressibility observed up to 100 MPa is attributed to the relatively loose packing of fat molecules. At pressures above 300 MPa, the free space in fats is probably occupied and chemical and physical resistance decreases further compressibility (Min, Sastry et al. 2010). High compressibility up to 100 MPa has also been observed for chicken breast, however a further pressure increase is associated to compressibility lower than that of water (Min, Sastry et al. 2010). The physical compression of compounds such as proteins is known to alter their structure (Pérez-Andrés, Charoux et al. 2018). The compressibility of proteins is closely related to their inherent flexibility (Gekko and Hasegawa 1986). Globular proteins in general display a positive compressibility due to a strong compression of voids within the structure (Heremans 1982). Fibrous proteins are on the other hand far less compressible (Gekko and Hasegawa 1986). As a general rule, protein denaturation occur at pressures of 100-1200 MPa with an associated midpoint transition between 400-800 MPa (Pérez-Andrés, Charoux et al. 2018). While thermal protein denaturation is known to occur through strong thermal fluctuations eventually transferring nonpolar groups from the hydrophobic protein core to the exterior, increasing pressure causes the opposite effect (Knorr, Heinz et al. 2006). Pressure denaturation of proteins occur as polar water molecules are forced into the protein interior. It is further believed that the mechanism relies on a loss of interaction between the non-polar groups which causes the molecule to unfold (Knorr, Heinz et al. 2006). To which degree the protein unfold depends on its flexibility and thus ability to compensate for the loss of non-covalent bonds.

1.4.3 On the application of high-pressure processing as either pre or simultaneous treatment for enzymatic hydrolysis of proteinaceous substrates

High-pressure processing could be applied prior to enzymatic hydrolysis and protein unfolding may expose more sites for enzymatic cleavage and increase access in a complex biomatrix. Marciniak, Suwal et al. (2018) reports on the use of HPP as both a pre-treatment and a simultaneous treatment when producing bio-active peptides with different enzymes on different proteinaceous substrates. Even though benefits seem evident, the nature and properties of substrate and enzyme should be considered when the choice of HPP parameters is made. According to literature, when HPP is applied as a pre-treatment hydrolysis should be conducted shortly thereafter, as the structural alterations are reversible (Marciniak, Suwal et al. 2018). Another interesting result is that higher pressures not necessarily results in quicker unfolding (Marciniak, Suwal et al. 2018). Pressurization may also result in protein-protein interactions and can further result in aggregates. For complex substrates, lipid-lipid and lipid-protein aggregation could occur, which may cause difficulties in downstream processing. A selection of results on the pre-treatment of proteinaceous substrates with HPP prior to enzymatic hydrolysis is presented in Table 6.

As happens with protein substrates, high pressure could also affect the catalytic effect of enzymes. This will depend on the magnitude of the volume changes, in addition to the reversibility of the changes. If high pressure is applied simultaneously to the catalysis, the overall effect will be influenced by changes in substrate, enzyme and the interaction between them. Both reversible enhancements and reductions of enzymatic activity are reported when using HP, possibly through either changing substrate specificity, or by changing rate-limiting molecular structures. The rate-determining step of an enzymatic reaction may be influenced by pressure by changing the substrate conformation, such as polymer unfolding or gelatinization. There is also evidence suggesting that pressure can change enzyme specificity, which may alter the product composition (Knorr, Heinz et al. 2006). Moreover, conditions such as temperature, time, properties of the solvent, pH, ionic strength and the concentration of substrate will play important roles in the enzyme activity (Marciniak, Suwal et al. 2018). The ideal conditions for the simultaneous use of HPP will have to be a compromise between enzyme activity and substrate denaturing as both probably would be affected. A selection of results on simultaneous HPP and enzymatic hydrolysis is presented in Table 7.

Table 6: Various results obtained when HPP is applied as a pre-treatment prior to enzymatic hydrolysis of different proteinaceous substrates.

Substrate	Enzymes(s)	Conditions	Results	Author(s)
Insoluble bovine collagen type 1	Alcalase Collagenase Thermolysin Proteinase K Pepsin Trypsin	**Pre-treatment:** P: 600 MPa T: n/g t: 15 min **Hydrolysis:** T: Optimal t: 4 h **Control:** Untreated sample	• **DH%:** Applying HPP as a pre-treatment increased the DH% for Alcalase and collagenase. The effect of the pre-treatment was enzyme dependant.	(Zhang, Olsen et al. 2013)
Whey protein	Trypsin	**Pre-treatment:** P: 300 MPa T: 25 ℃ t: 15 min **Hydrolysis:** T: 37 ℃ t: 135 min **Control:** Untreated sample	• **DH%:** Applying a HPP pre-treatment significantly enhanced the DH%. • **Protein solubility**: The pre-treatment reduced the protein solubility to approximately 75%.	(Blayo, Vidcoq et al. 2016)
Egg white (EW) protein	Trypsin	**Pre-treatment:** P: 350, 450, 550 MPa T: 15-25 ℃ t: 5-15 min **Hydrolysis:** T: 37 ℃ t: 120 min **Control:** Untreated sample	• **DH%:** The DH% increased with an increase in both pressure level and pressure holding time when compared to the control.	(Singh and Ramaswamy 2014)

Abbreviations and units

P: Pressure [MPa], T: Temperature [℃], t: time [min] [h], n/g: Not specified.

Table 7: Various results obtained when HPP is applied simultaneously to enzymatic hydrolysis of various proteinaceous substrates.

Substrate	Enzymes(s)	Conditions	Results	Author(s)
Fish gelatin	Alcalase Collagenase Trypsin Pepsin	**HP-assisted:** P: 100, 200, 300 MPa T: Optimal t: 15-30 min **Control:** Atmospheric pressure	• **DH%:** Increased with all enzymes for the HP-assisted hydrolysis as compared to the control hydrolysis.	(Alemán, Giménez et al. 2011)
Anchovies powder	Flavourzyme Alcalase Marugoto E Protamex	**HP-assisted:** P: 300 MPa T: 50 ℃ t: 1 h **Control:** Atmospheric pressure	• **DH%:** Significantly higher for all enzyme combinations when hydrolysis was conducted at 300 MPa as compared to the control. • **Protein solubility:** Increased significantly when hydrolysis was conducted during the 300 MPa pressurization, this holds for all enzyme combinations. as does the result mentioned on DH.	(Kim, Son et al. 2016)
Soy protein isolate	Flavourzyme	**HP-assisted:** P: 100-600 MPa T: 50 ℃ t: 15 min **Control:** Atmospheric pressure	• **DH%:** HPP-assisted hydrolysis at 100 MPa significantly increased the DH%. However, the DH% declined at higher pressures and was equal to the control at 600 MPa. • **Protein solubility:** HPP applied during hydrolysis resulted in a significant increase in protein solubility at pH 4 and 7 as compared to that of raw soy protein extract. However, this was even more enhanced when HPP was applied as pre-treatment.	(Meinlschmidt, Brode et al. 2017)

Abbreviations and units

P: Pressure [MPa], T: Temperature [℃], t: time [min] [h], n/g: Not specified.

1.4.4 The choice of HPP process conditions for the treatment of MDCR prior to enzymatic hydrolysis

In order to apply high pressure as a means for increasing the number of enzymatic cleavage sites, changing the cleavage sites available and increasing accessibility in a proteinaceous material such as MDCR, some sort of structural alteration is required. As shown in Table 6 and Table 7, changes in protein structure which enhance/reduce substrate digestibility or enzyme activity seem to be specific regarding applied pressure, time and temperature. Therefore, in order to choose appropriate system conditions, treatments conducted on similar material should be investigated. Buckow, Sikes et al. (2013) stated that HP might cause various alterations on proteins present in muscles, which is dependent on pressure magnitude, temperature and treatment duration. Exactly which mechanisms that causes changes in meat texture and water holding capacity is not entirely understood. Other apparent changes caused by high pressure on food structures are for instance colour modifications due to changes in myoglobin structure and content. This might generate a cooked-like appearance of fresh meat treated with HP, however high moisture foods often appear unaltered.

Myosin is as previously mentioned the major myofibrillar protein with both globular heads rich in -SH groups and a helical rod. F-actin is stabilized through hydrophobic and electrostatic interactions, another component of the thin filament, tropomyosin, interacts with F-actin through a positive charge present in an F-actin groove (Von der Ecken, Müller et al. 2015). Other proteins, in addition to those mentioned, are also present in the myofibrillar structure. Collagen is present in muscular tissue and constitutes the major protein fraction in MDCR. However, collagen is found to be unaffected by high pressure (Fernandez-Martin 2007). Since myosin and F-actin present globular structures, it seems feasible that a high-pressure response could occur during certain treatments. Also, water soluble sarcoplasmic proteins often display a globular structure which potentially could contain void spaces which might be influenced by high pressure. More uncertainty is however associated with pressure induced alterations of the helical rod section of myosin.
In a review by Aubourg (2018), the extractable content of sarcoplasmic proteins was found to both decrease at elevated pressures (400 MPa) but also to be non-affected by HPP. Another review on pressure effects on myofibrillar proteins revealed that dissociation of myosin into monomers seemed to be favoured at elevated pressures (Cheftel and Culioli 1997). This due to the positive volume change associated to the formation of the dimer, therefore the volume change is negative upon dissociation and favoured upon elevated pressures. Even though there is a connection between myosin and actin which only can loosen upon addition of ATP, the length of the filaments is often reduced upon elevated pressures. This might indicate that high pressure acts in the same way as

muscle contraction, which could cause fragmentation similar to that occurring upon rigor.
F-actin seems to denaturate at relatively low pressures, often at 100 MPa. The myosin light chains (helical) seem to be unaffected by pressure, but at pressures around 300 MPa myosin denaturation seems to occur, which presumably refers to denaturation of globular domains. The solubilization of these myofibrillar structures is reported to increase upon high pressure treatment, which depends on process parameters, although pressures ranging 150-300 MPa seem to favour enhanced solubility and subsequent extraction. The proteins extracted are reportedly actin, tropomyosin, troponin C and M-proteins. Myosin heavy chains are not reported to solubilize before pressures of 300 MPa are reached. Other results suggest that myosin fragments do not solubilize even at pressures close to 500 MPa even though partial denaturation is observed (Aubourg 2018).

Ma and Ledward (2004) treated beef *longissimus dorsi* (200-800 MPa; 20-70 ℃; 20 min) and found collagen to be relatively unaffected by pressure, being still intact at 800 MPa. Furthermore, results regarding myosin and actin coincide well with those mentioned in the section above. At 20 ℃ myosin seems to denature at pressures close to 400 MPa, while actin denatures at 200 MPa. A new structure seems to be formed upon the denaturation of myosin and actin. This is also reported by Sikes, Tornberg et al. (2010). Further pressure increase does not dissolve this new complex formed, indicating that it is not stabilized by weak bonds. The formation of this complex could explain why, as mentioned earlier, no elevated protein extraction was found even though myosin had denatured. It therefore seems beneficial to avoid this complex formed at higher pressures. However, the thermal transition temperature of the pressure induced complex is close to 50 ℃ which could be beneficial when conducting enzymatic hydrolysis at a similar temperature.

As temperature and pressure display contradicting effects on the raw material, room temperature is considered a sensible choice when conducting HPP as a treatment prior to enzymatic hydrolysis of MDCR (Fernandez-Martin 2007). The treatment time of myofibrillar substrates vary mainly between 5 and 30 min (Aubourg 2018). Therefore, a treatment time of 15 min is chosen for further investigation in the present work. To investigate the denaturation of the major protein constituent within the MDCR, namely that of actin (~100-200 MPa) and myosin (~400 MPa), and how this affects subsequent enzymatic hydrolysis, the pressures 100, 200 and 400 MPa are chosen for further investigation. However, 600 MPa is also further investigated in the present work as it might shed light on the proteolytic susceptibility of the pressure-induced protein complex reported to form at high pressures.

2 Objectives

The objective of this work is to investigate the potential of innovative technologies (high-pressure processing, HPP; microwave, MW; ultrasound, US) to enhance the bioactive and functional properties of the hydrolysates obtained from enzymatically hydrolysed rest raw materials (mechanically deboned chicken residual, MDCR). Specific activities are included in the following.

- Effect of innovative technologies (HPP, MW and US) implemented as pre-treatments on the enzymatic hydrolysis of rest raw materials (MDCR) by using an industrially relevant enzyme.
    - HPP: 100, 200, 400 and 600 MPa; 15 min; mild temperature
    - MW: 1000 W; 2.45 GHz; 40 ℃
    - US: 300, 600 and 900 W; 15 min; T < 30 ℃; 67/170 kHz ± 2 kHz
- To characterize the hydrolysates: composition (e.g. protein content), physico-chemical and properties (protein solubility), bioactivity (e.g. antioxidant activity).

3 Materials and methods

3.1 Materials

Chemical reagents applied for analysis were NaOH (MERCK, >99%), Na_2CO_3 (Riedel-de Haën, analytical grade), Folin and Ciocalteus phenol reagent (MERCK, 1.09001.0100), $CuSO_4x(H_2O)_5$ (Sigma Aldrich, ≥98%), Bovine Serum Albumin (Sigma, EC: 232-936-2, [9048-46-8]), 2,2-Diphenyl-1-picrylhydrazyl (95%, CAS: 1898-66-4, ThermoFisher Alfa Aesar), hydrochloric Acid 25% (MERCK, 1.00316.1000), ethanol (96%, Kemetyl Norge AS, EG.nr. 200-578-6) and potassium sodium tartrate (1.77 M, VWR International, The Tintometer Ltd., LOVI466101).

An industrially relevant enzyme and residuals from mechanically deboned chicken carcasses kindly supplied by Norilia were used for the experimental trials. The residuals were collected on 30.01.2019 at 09.00 AM and transported by car in an insulated bag with cooling elements, thus ensuring a temperature between 0-4 ℃. When the raw material arrived at Nofima it was kept at 3 ℃ before further processing. The raw material was minced with a MADO Garant type MTK662 (MADO GmbH, Germany, Dornhan) for 10 s at low speed followed by 10 s at high speed. Thereafter, 500 g of minced residue was vacuum packed (99%) in double 29.5 x 20.0 cm sous-vide bags using WEBOMATIC FH-S Supermax C V3 vacuum machine (Webomatic, Germany, Bochum). Packed samples were stored at -40 ℃. Prior to experimental trials, the minced samples were thawed at 4 ℃ overnight.

3.2 Pre-treatment processing

3.2.1 Innovative pre-treatments (MW; US; HPP)

Pre-treatment of the thawed minced samples was conducted by microwave (MW), ultrasound (US) or high-pressure processing (HPP), as summarised in Figure 14. The control samples were not subject to any pre-treatment.

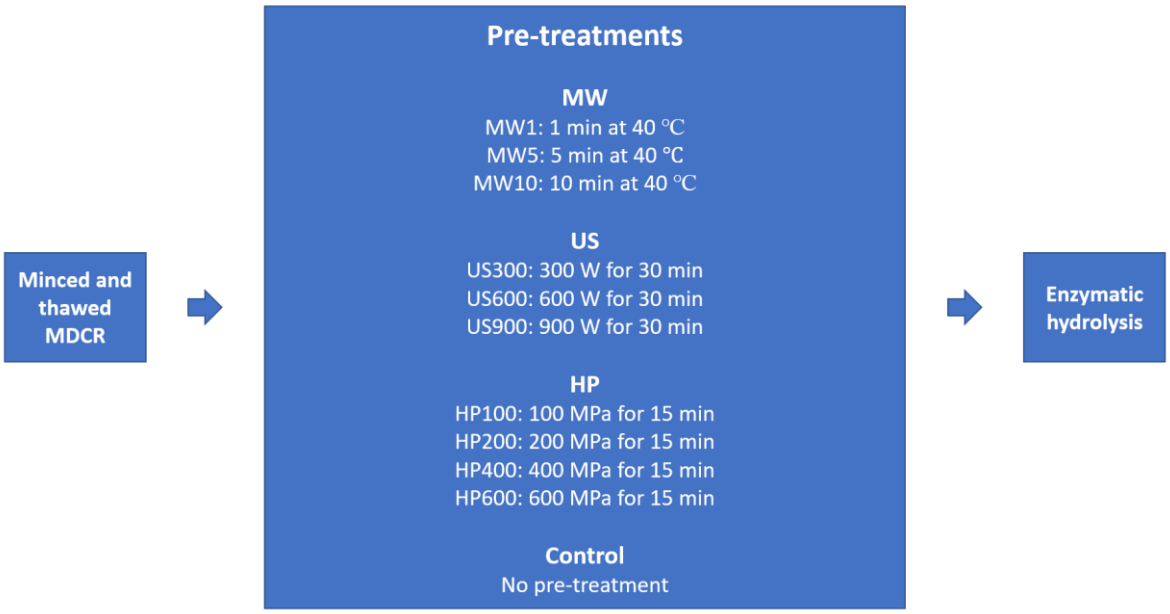

Figure 14: Flowchart illustrating the various pre-treatment conditions applied in the experimental trials in the present work and the operations conducted prior to and after the pre-treatments.

3.2.2 Microwave pre-treatment

MW treatment was performed in a microwave-heated batch autoclave (Gigatherm AG, Flawil, Switzerland) operating at 2.45 GHz. The apparatus was configured such that a power of 1000 W was generated from a single water-cooled magnetron. The raw-material sample was adapted to a flat, cylindrical shape with a diameter of approximately 16.5 cm, as illustrated in Figure 15. Three thermal sensors were inserted to monitor the temperature evolution in the sample. The mentioned sensors were placed at 1, 8 and 12 cm respectively from the sample edge, along a pre-defined and constant trajectory and depth (approx. 0.5 cm below surface) (the position of the sensors are illustrated in Figure 15 as blue circles).

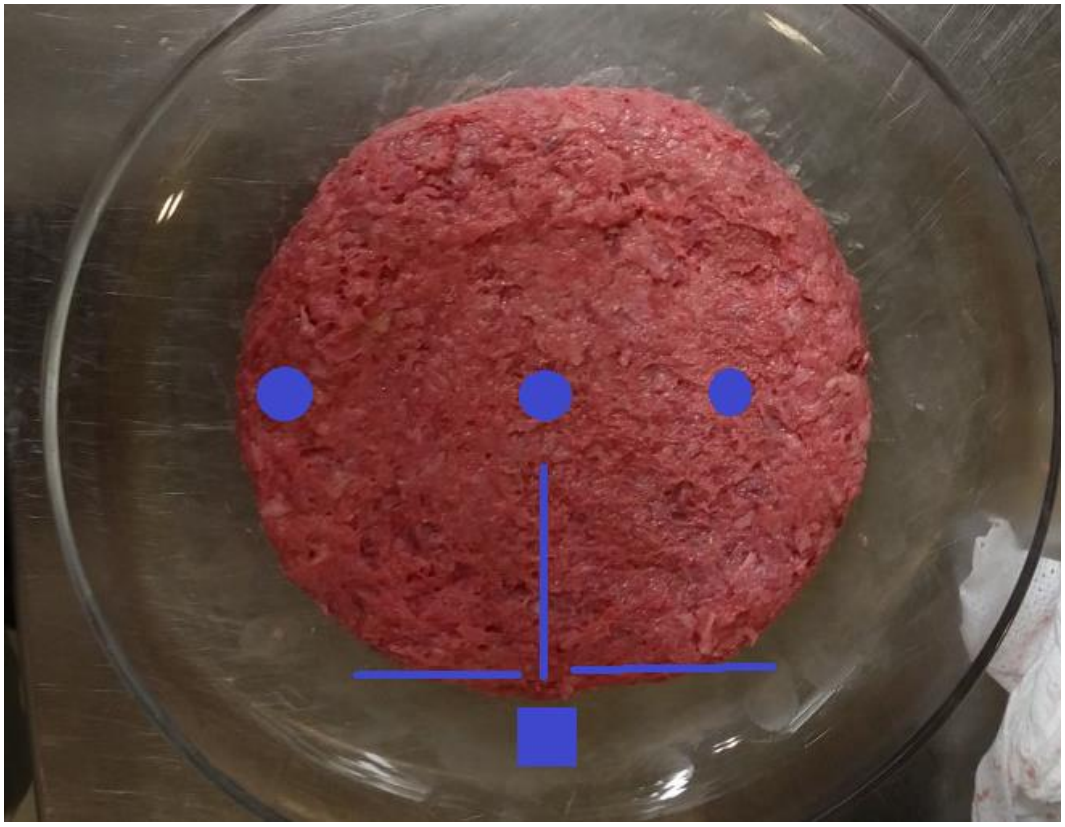

Figure 15: The figure illustrates the shape of the MDCR sample and the positions for the three thermal sensors (blue circles). The blue square and the straight lines indicate a fixed point and angle used to ensure a constant sample position in the apparatus.

The software (TwinCAT 2.11.000) enabled power-adjustment such that an average temperature of 40 ℃ was achieved after 154-190 s. Treatment times of 1, 5 and 10 min were applied from the point at which an average temperature of 40 ℃ was reached. Plots displaying temperature evolution towards exposure time can be found in Appendix A.

3.2.3 Ultrasound pre-treatment

US treatment was conducted using a BT 130H bench top system (UPCORP, Illinois, USA), with a maximum power of 1000 W through transducers bonded to the tank bottom. The transducers are further configured to two banks, each of an effect 500 W but operating at 67 kHz and 170 kHz. Thus, half the effect is originating from each bank and frequency. The system furthermore allows for different frequency sweep rates for a 4 kHz sweep output. In this work a maximum sweep rate of 1000 Hz was used as it was recommended by the manufacturer for viscous solutions. This essentially

implies that the output frequency in the apparatus changes with a rate equal to 1000 Hz around 67 kHz and 170 kHz with ±2 kHz. The external/second sous-vide bag was removed before the treatment and the sample packed in a single vacuum bag was placed at a pre-defined location in the bath (centre) and approximately 2.5 cm above the tank bottom, as illustrated in Figure 16.

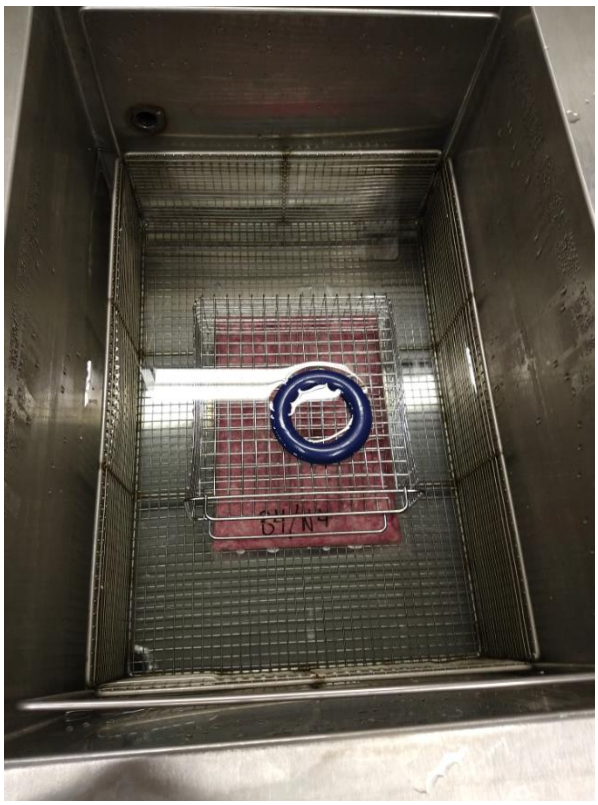

Figure 16: The figure presents the configuration of the sample (packed in one vacuum bag) in the US water bath prior to treatment.

In the present work, US powers of 300, 600 and 900 W were investigated. The initial temperature of the water bath was chosen such that a temperature of 29-30 ℃ (Anritsu HD-1250K Thermometer, Atsugi, Japan) was reached at the end of each pre-treatment. A fixed exposure time of 30 min was selected for the US pre-treatments.

3.2.4 High-pressure processing pre-treatment

High-pressure processing (HPP) pre-treatments at 100, 200, 400 and 600 MPa were conducted using a high hydrostatic pressure machine QFP 2L-700 (Avure Technologies Inc., Columbus, USA). Samples (in two vacuum-packing bags) were placed in a treatment-chamber filled with distilled water.

Software (RSView runtime 1500 Ver. 06.02.20.00) further allowed programming for rapid pressurization and depressurization. Plots displaying both pressure and temperature evolution inside the treatment chamber are available in the Appendix B. 15 min holding time at the previously mentioned pressures was selected.

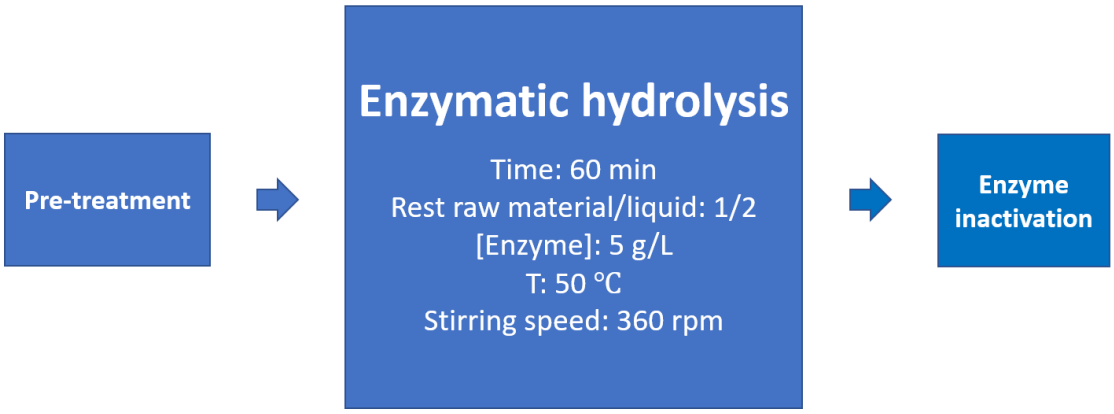

Figure 17: The flowchart illustrates operations conducted prior to and after the enzymatic hydrolysis. The conditions applied during the enzymatic hydrolysis is also presented.

3.3 Enzymatic hydrolysis

Enzymatic hydrolysis of control and pre-treated samples were conducted in a 2 L Radleys ReactorReady (Radleys, Saffron Walden, UK) mounted with a Heidolph Hei-Torque400 (Heidolph, Germany, Schwabach) with stirring speed set at 360 rpm. The temperature in the jacketed reactor was maintained at 50 ℃ by means of an external circulating water bath. 990 mL of distilled water was initially added to the reactor and allowed to warm up to 50 ℃. The operations conducted prior to and after the enzymatic hydrolysis is illustrated in Figure 17. When the temperature in the water reached 50-51 ℃ (Anritsu HD-1250K Thermometer, Atsugi, Japan), the pre-treated or control sample was added. Further, when the temperature of the reactor media (990 mL distilled water and the sample) reached 50 ℃, 10 mL of an enzyme solution (0.5 g enzyme per mL) was added and this point

was considered the start of the 60 min hydrolysis. The enzymatic hydrolysis set-up is illustrated in Figure 18.

Figure 18: The figure illustrates the enzymatic hydrolysis set-up used in the present work during experimental trials.

Samples of 15 mL were taken at 0.5, 5, 15, 30 and 60 min. After 60 min the remaining reactor volume was drained into a crystallization bowl and inactivated. For both, samples and final hydrolysis volume, the enzyme was inactivated by microwave treatment (Menumaster Commercial Microwave Model DEC18E2 ACP, 1800 W, Cedar Rapids, USA) allowing rapid boiling of the medium (within 30 seconds for the 15 mL samples and 4 min for the final hydrolysis volume) and subsequent heating at 95 ℃ in a water bath for 15 min. After enzyme inactivation, the final hydrolysis volume was allowed to cool down for 30 min under cold running water. An overview of the operations conducted prior and after the enzyme inactivation is presented in Figure 19.

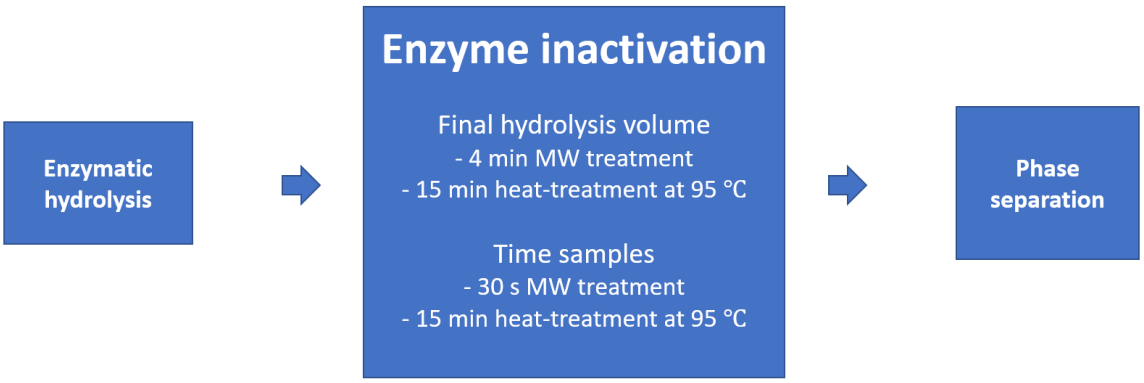

Figure 19: The figure illustrates the operations conducted prior to and after the enzyme inactivation. The conditions applied for inactivation of the time samples and final hydrolysis volume are also presented.

3.4 Separation of phases

The samples taken at different time intervals during the enzymatic hydrolysis (time-samples) and the final hydrolysis volume were centrifuged after cooling down. The final hydrolysis volume was centrifuged at 4,400 rpm (Thermo Scientific Heraeus multifuge x3 FR, Osterode, Germany; bucket Thermo Scientific FIBERLite F14-6x250LE (USA)) and 25 ℃ for 20 min. The time-samples were centrifuged at similar conditions however a different bucket (Thermo Scientific FIBERLite F15-8x50cy, USA) was applied. After centrifugation, approximately 2 mL of the aqueous phase of time-samples was filtered with an acetylated cellulose syringe filter (w/0.45µm, VWR international, Art. Nr. 514-0063) and stored at -40 ℃ for further analysis.

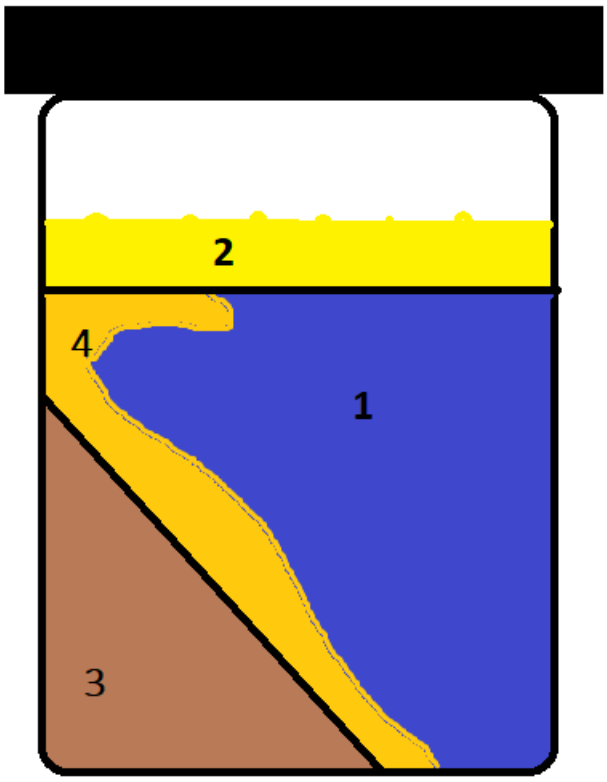

Figure 20: The various phases present after the centrifugation of the final hydrolysis volume is illustrated. 1: The aqueous phase which also contains suspended particles. 2: The lipid phase. 3: The solid phase. 4: The multicomponent phase. The aqueous phase (1) is later filtered yielding a solid particulate and a filtered aqueous phase/hydrolysate.

The various phases present in the final hydrolysis volume is presented in Figure 20. For the final hydrolysis volume, the phases were separated into an unfiltered aqueous, solid, lipid and multicomponent phase after centrifugation by gently pouring the medium through a sieve into a phase-separation funnel. The multicomponent phase was entrapped in the sieve while the lipid-water mixture continued into the funnel and was allowed 15 min to form immiscible phases and then separated into an aqueous and lipid phase. The solid phase was held back in the centrifugation bottle. The aqueous phase was vacuum-filtered (Pall filter systems, Depth Filters, Material nr. 1426045) using a Büchner filtration system to remove the remaining solid particulate. The solid, filtered aqueous and lipid phases were stored at -40 ℃. The solid, unfiltered aqueous, filtered aqueous, lipid and multicomponent phases were weighed. An overview of the various operations conducted after enzyme inactivation for both the final hydrolysis volume and the various time-samples is presented in Figure 21.

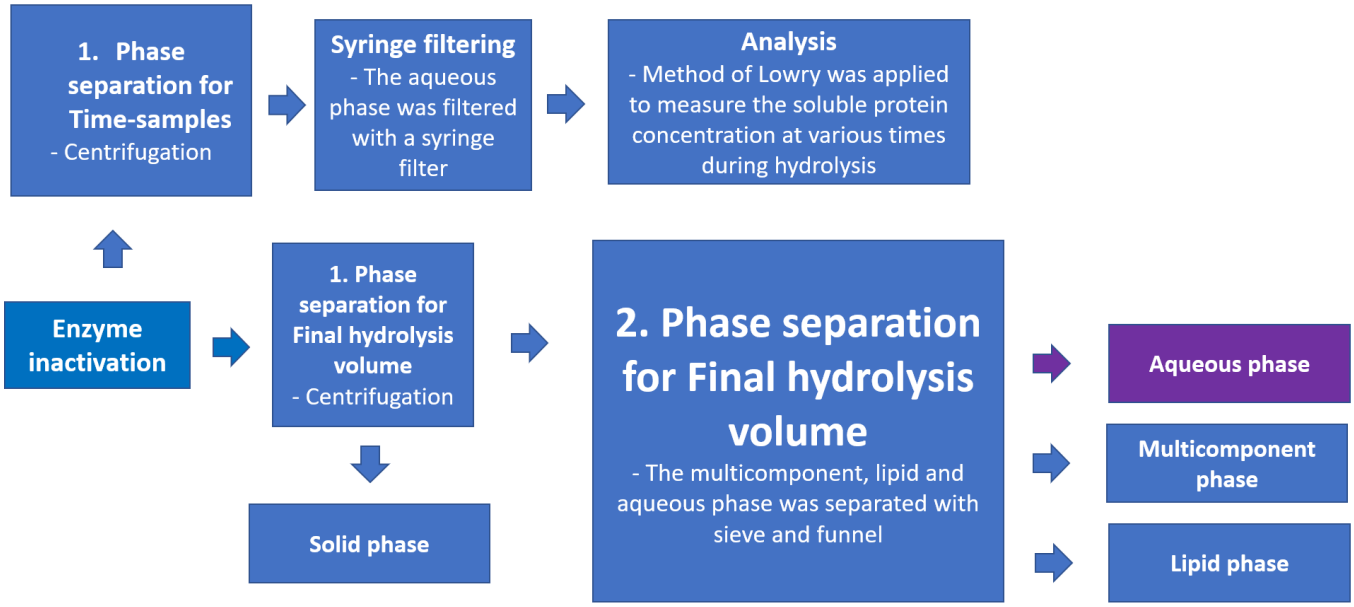

Figure 21: The flowchart presents an overview of the various operations conducted after the enzyme inactivation of either the final hydrolysis volume or the time-samples.

3.5 Analytical methods

The protein concentration of the time-samples was analysed through the method of Lowry, this is illustrated in Figure 21. An overview of other analysis performed with the filtered aqueous phase from the final hydrolysis volume is presented in Figure 22.

3.5.1 Soluble protein content

The protein concentration in the time-samples (syringe-filtered aqueous phase) and in the final hydrolysis volume (vacuum and syringe filtered aqueous phase) was determined by the method of Lowry, Rosebrough et al. (1951) with bovine serum albumin as standard. For the spectrophotometric measurements, a Shimadzu UV-mini 1240 spectrophotometer (Shimadzu, Japan) was used. Absorbance was measured at 750 nm.

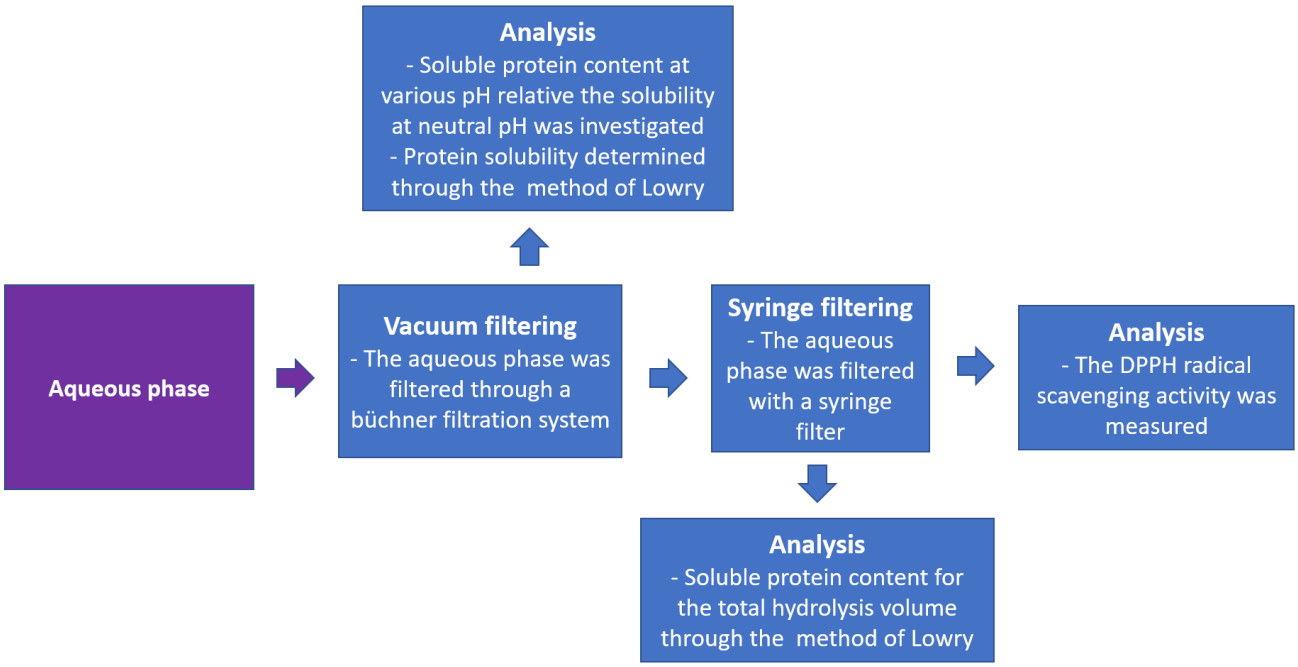

Figure 22: The figure illustrates the operations conducted between obtaining the unfiltered aqueous phase from the inactivated final hydrolysis volume, to performing various analysis.

3.5.2 The DPPH radical scavenging activity

The DPPH radical scavenging activity was determined according to Shimada, Fujikawa et al. (1992) as described by Thiansilakul, Benjakul et al. (2007) with slight modifications. A solution of 0.15 mM DPPH in 96% EtOH was prepared the day before analysis and stirred at 4 ℃ overnight in the dark. 1.5 mL of the diluted hydrolysate sample was added to 1.5 mL of 0.15 mM DPPH and mixed vigorously with a vortex for 10 s. The samples were left at room temperature in the dark for 60 min. The absorbance was then read at 517 nm. The blank was prepared in the same way as the hydrolysate samples, but 1.5 mL of distilled water was used instead of proteinaceous sample. DPPH radical scavenging was calculated according to Equation 1.

$$\text{Radical scavenging activity}(\%) = \frac{B-A}{B} * 100\%, \quad \textit{(Equation 1)}$$

Where A is the measured absorbance of the hydrolysate sample and B is the measured absorbance of the blank. The apparatus was calibrated with diluted hydrolysate (1.5 mL hydrolysate sample diluted with 1.5 mL distilled water), as the proteins present will contribute to the absorbance measured in the sample but not in the blank (Cumby, Zhong et al. 2008).

3.5.3 Protein solubility against pH

4.38-5 mL of the final hydrolysis volume was mixed with distilled water and HCl (1, 0.1 and 0.01 M) or NaOH (1 and 0.1 M) to obtain the pH 2, 4, 6 or 11. The total volume and the protein concentration after pH adjustment was 25 mL and 8.2-8.6 g/L, respectively. After either the base, acid or water were added to the samples they were mixed vigorously with a vortex for 5 s. When the desired pH was achieved, the samples were left at room temperature for 30-60 min and then centrifugated at 10,000 rpm for 15 minutes at 20 ℃ (Thermo Scientific Heraeus multifuge x3 FR, Osterode, Germany; Bucket: Thermo Scientific FIBERLite F15-8x50cy (USA)). After centrifugation, 120 µL were pipetted immediately from the clear supernatant. The soluble protein content was then determined through the method of Lowry. In order to compare the different pre-treatments with the control, the soluble protein concentration at various pH (2, 4, 6 and 11) was normalised against the soluble protein concentration at neutral pH (6.9-7) also diluted to 25 mL with distilled water instead of acid/base and the following procedure was kept equal. The soluble protein content at a respective pH relative to the soluble protein content at neutral pH was calculated as described in Equation 2.

$$\frac{\text{Undiluted protein concentration at certain pH } \left[\frac{\text{g}}{\text{L}}\right]}{\text{Undiluted protein concentration at neutral pH } \left[\frac{\text{g}}{\text{L}}\right]}, \quad \text{(Equation 2)}$$

3.5.4 Statistical analysis

For statistical analysis, Excel and the function T.TEST were used. The function returns the probability associated with a Student t-test. The function has syntax T.TEST(array1, array2, tails, type). Array1 and array2 are the first and second data set, respectively. The term “Tails” indicates whether 1 or 2 distribution tails are applied (</> or ≠) and the term “type” indicates which type of test is to be conducted. In this work, a two tailed distribution (≠) and a type 3 test was performed. A type 3 t-test assumes two unequal sample variances. Limit for significance was set as $\alpha \leq 0.05$.

4 Results and discussions

4.1 The control samples and samples not added exogenous enzymes

4.1.1 The results and discussion on the effect of exogenous enzyme addition on the protein solubilisation and the phase contents

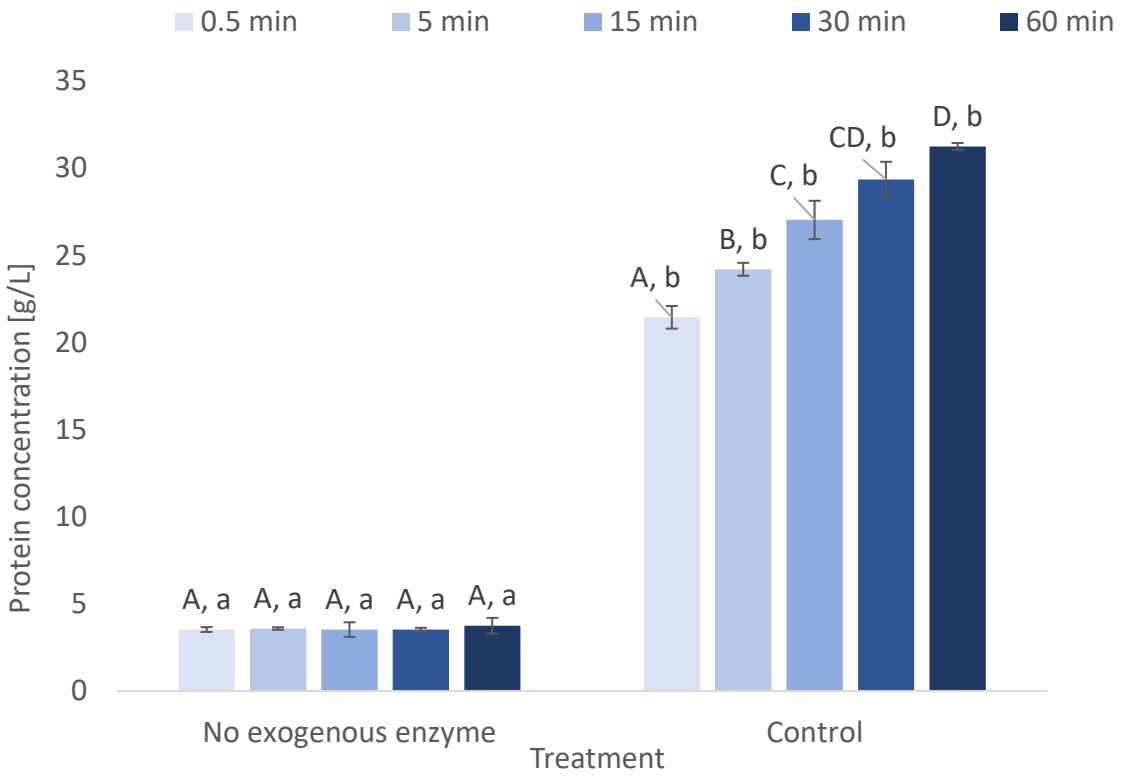

Figure 23: The figure presents the evolution of protein concentration against hydrolysis time for the control (no pre-treatment but presence of exogenous enzymes) and the trials where no exogenous enzymes were present. Significant differences at similar conditions among different treatments is indicated by different lower-case letters. Significant differences at varying conditions within a certain treatment is indicated by different upper-case letters.

As shown in Figure 23, enzymatic hydrolysis with no pre-treatment, i.e. the control for pre-treated samples, has in this work been found as an efficient method for peptide extraction from MDCR. A significant increase in solubilized peptides was obtained after 0.5, 5, 15 and 60 min of enzymatic hydrolysis. On the other side, without enzyme addition there was not any significant effect between 0.5 min and 60 min, indicating a low inherent protein solubility and low endogenous enzyme activity in MDCR at the treatment conditions. Halim, Yusof et al. (2016) reported a trend similar to what is observed in this present work when hydrolysing fish proteins, namely a sharp increase in protein

concentration during the first minutes of hydrolysis. Fonkwe and Singh (1996) investigated enzymatically hydrolysis of mechanically deboned turkey residue with papain at 60 ℃. In general, the solubilised protein content seemed significantly lower than that of the control in the present work. This could be due to the temperature applied as the proteinaceous constituents within the raw material are reported to denaturate between 50 and 60 ℃ (Murphy and Marks 2000). However, the application of different enzymes and variations within the raw material might also contribute to the observed differences.

In trials added exogenous enzymes there is an effective protein solubilization, as seen in Figure 23, this significantly affects the contents of the various phases obtained after the enzyme inactivation. The final hydrolysis volume is separated into a solid, multicomponent and a liquid phase. The liquid phase is further separated into an unfiltered aqueous and a lipid phase. The aqueous phase is then filtered into a filtered aqueous phase and a particulate phase. The content of the lipid associated phases (the lipid and multicomponent phase), the filtered aqueous phase and solid phases (particulate and solid) for the control and trials without addition of exogenous enzymes are presented in Figure 25, Figure 26 and Figure 26, respectively.

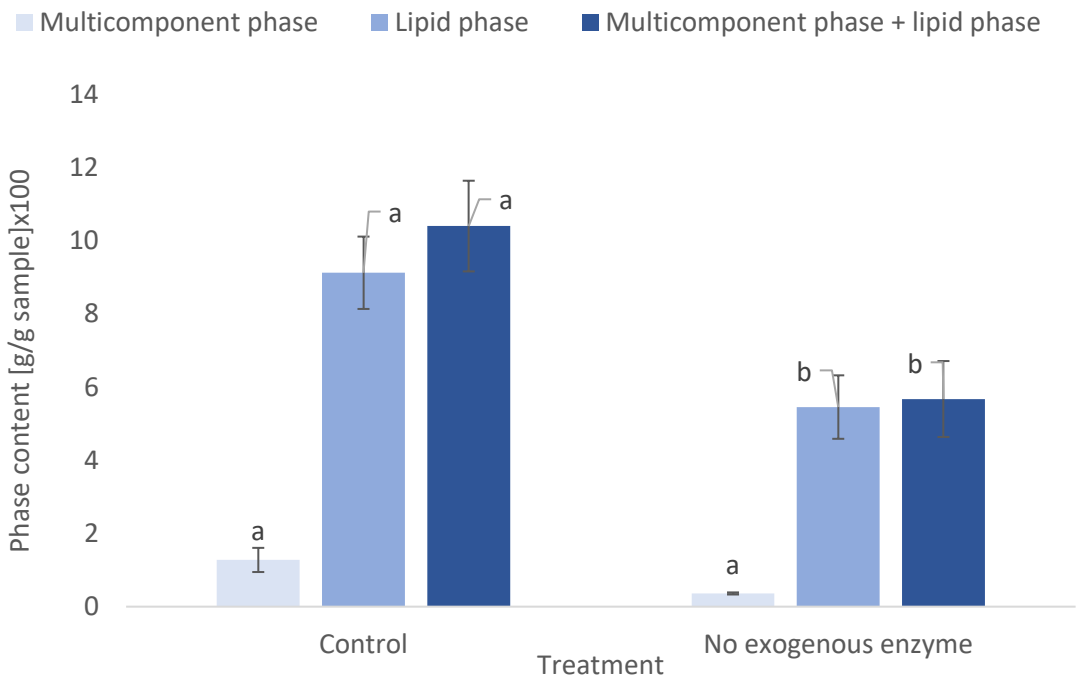

Figure 24: The figure presents the content of the multicomponent and lipid phase obtained from trials where exogenous enzymes were applied (control) or not (no exogenous enzyme). Significant differences for similar phases among different treatments is indicated by different lower-case letters.

As shown in Figure 24, the lipid and lipid + multicomponent content increased for the control as compared to trials where no exogenous enzymes were present. The formation of soluble peptides could enhance the association between proteins and lipids, thereby increasing the lipid and lipid+multicomponent contents. Even though there is no significant increase in the multicomponent content between control samples and trials absent of exogenous enzymes, there seem to be an increase in the multicomponent phase. This could together with the increase of the lipid phase indicate that peptides able to associate with the lipids are formed during the enzymatic hydrolysis.

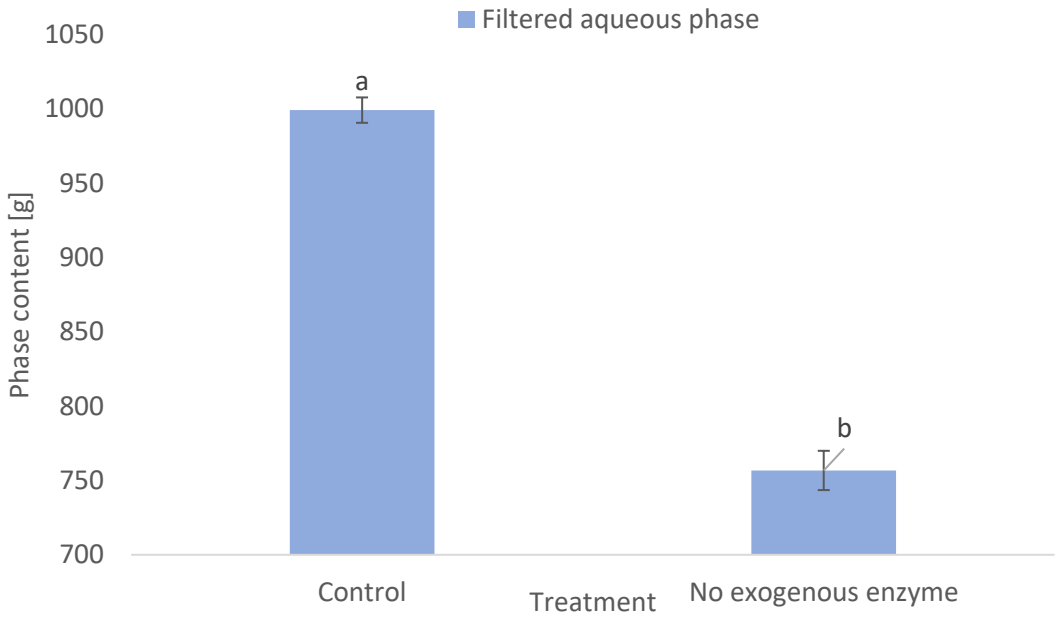

Figure 25: The figure presents the content of the filtered aqueous phase/hydrolysate obtained from the trials where exogenous enzymes were applied (control) or not (no exogenous enzyme). Significant differences for similar phases among different treatments is indicated by different lower-case letters.

The addition of exogenous enzymes significantly increased the filtered aqueous phase content, as presented in Figure 25. This could be due to the thermally induced formation of proteinaceous network structures from undissolved proteins in trials lacking exogenous enzymes. Such structures can form upon the heat-treatment mimicking enzyme inactivation and could further entrap water and thereby reduce the content of the filtered aqueous phase for trials without exogenous enzymes. In fact, formation of proteinaceous networks are reported to start in chicken meat for temperatures between 48 and 55 ℃ (Zhang and Barbut 2005). The rapid protein solubilisation seen upon addition of exogenous enzymes (Figure 23) therefore seem to dissolve the proteins present before any significant network structures are formed.

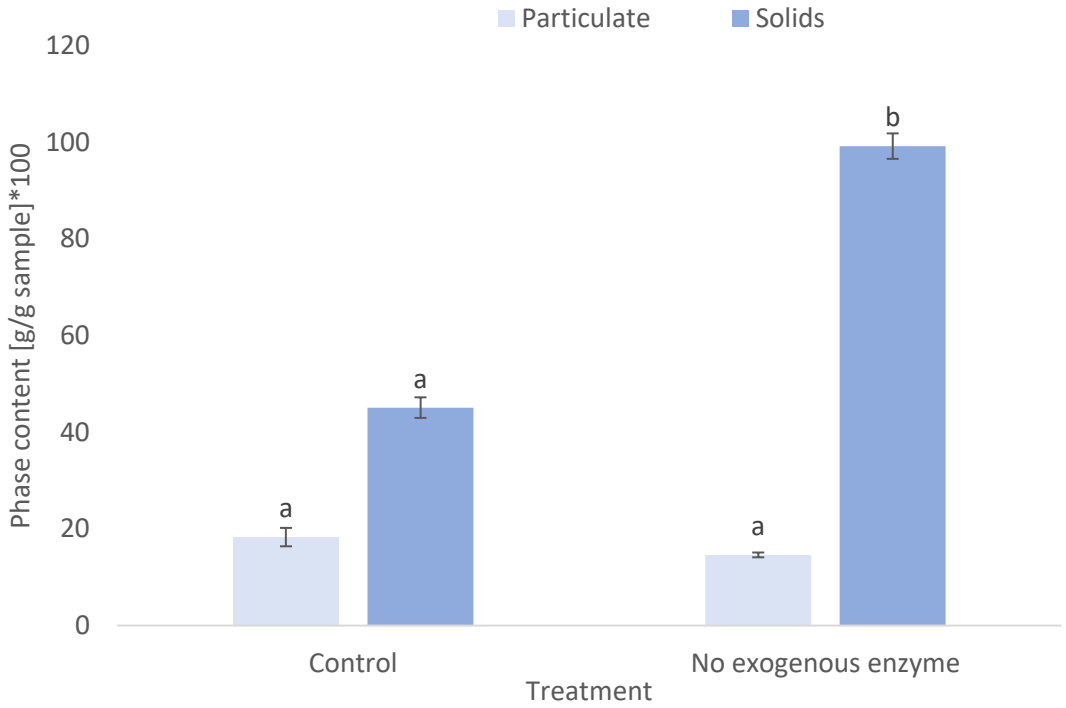

Figure 26: The figure presents the content of solids and particulate (filtered from the aqueous phase) from trials where exogenous enzymes were applied (control) or not (no exogenous enzyme). Significant differences for similar phases among different treatments is indicated by different lower-case letters.

While the particulate content seems to be unaffected, the solid phase content is significantly reduced upon addition of exogenous enzymes, as presented in Figure 26. Upon addition of exogenous enzymes, the reduction of solid phase content, as seen in Figure 26, and the increase in lipid content, as seen in Figure 24, could possibly be due to liberation of lipids as solid protein structures are degraded by active exogenous enzymes. Also, for the trials with no exogenous enzymes present, the heat-treatment operation designated for enzyme inactivation could generate proteinaceous network structures which entrap lipids, and thereby reducing the lipid phase content. However, some of the increased lipid content could be due to the presence of peptides with certain properties allowing protein-lipid interaction. Tveit (2014) reports that hydrolysates produced from MDC by a commercial enzyme contained soluble peptides with low content of hydrophobic amino acids like leucine, phenylalanine, isoleucine and lysine, which probably cause minor peptide-lipid association. Also, increasing the DH% has been found to elevate the lipid phase after hydrolysis on behalf of a phase containing a mixture of all components. This indicates a reduction of peptides associating with lipids due to higher content of charges per chain length which occur upon elevated DH%. A high content of charges would interact unfavourably with the lipid phase making these

fractions would be non-miscible. Formation of peptides favouring lipid-protein structures is however enzyme dependant (Šližyte, Daukšas et al. 2005). Therefore, the results obtained in the present work could be influenced by peptides and lipids interacting after the hydrolysis. However, even if peptides are lost through the multicomponent or lipid phase, variations of these phase contents could indicate variations in the product peptide properties. To be noted is that the multicomponent phase in the present work is not an emulsion in the usual sense, but rather a structure not miscible in the lipid or aqueous phase with some rigidity allowing maintenance of structure during and after centrifugation. Thus, in order to avoid misinterpretations is the phase in the present work referred to as a multicomponent phase. But for later discussion regarding peptide properties should it be noted that certain characteristics are associated with peptide-lipid interactions. Peptides consisting of at least 20 amino acids, a hydrophobic domain, a hydrophilic domain and region(s) where similar peptides can interact favourably with each other and form a film which can withstand mechanical and thermal motion are believed to be prerequisites for interaction between peptides and lipids in an aqueous environment (Kristinsson and Rasco 2000).

4.1.2 The results and discussion on the DPPH radical scavenging activity for the control hydrolysates

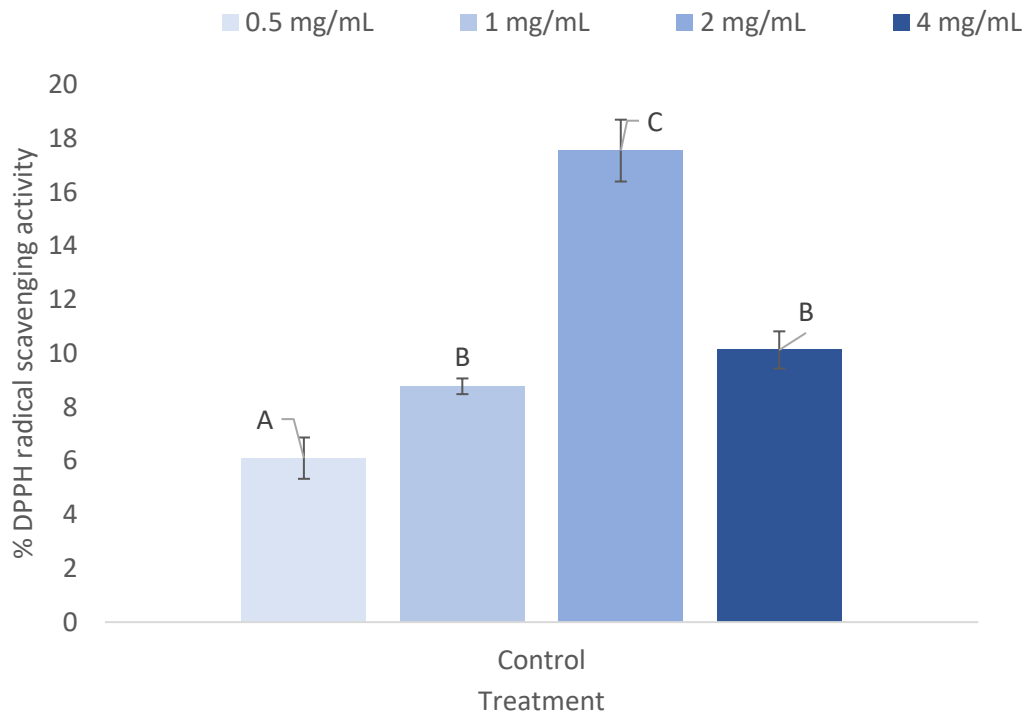

Figure 27: The figure presents the DPPH radical scavenging activity for the control hydrolysates at protein concentrations 0.5, 1, 2 and 4 mg/mL. Significant differences at varying conditions within a certain treatment is indicated by different upper-case letters.

The DPPH radical scavenging for the control hydrolysates with protein concentrations 0.5, 1, 2 and 4 mg/mL is presented in Figure 27. There was a linear increase in the DPPH radical scavenging activity when peptide concentration was increased between 0.5-2 mg/mL (R^2>0.988). However, a significant reduction in the DPPH radical scavenging activity was observed when the peptide concentration further was increased to 4 mg/mL. The potential peptides present in meat contain sequences of amino acids with health effects such as antioxidant activity (Udenigwe and Howard 2013). In general, high antioxidant properties are correlated to peptides of high hydrophobic group content (Saiga, Tanabe et al. 2003). Dávalos, Miguel et al. (2004) reported that Trp, Tyr and Met showed the highest antioxidant activities. Next was Cys, His and Phe. The remaining amino acids (Arg, Asn, Gln, Asp, Pro, Ala, Val, Lys, Ile, Tre, Leu, Glu, Gly) did not display any antioxidant properties. Shorter peptides are reported to allow higher rates of radical interaction and therefore are correlated to better antioxidant activity, as compared to longer chain peptides (Onuh, Girgih et al. 2014). Saiga, Tanabe et

al. (2003) identified several peptides displaying antioxidant properties from myofibril hydrolysates. In fact, Udenigwe and Howard (2013) observed that myosin and actin potentially were the richest sources of peptides displaying antioxidant activity from chicken proteins. However, myofibrillar proteins are reported to be relatively robust towards hydrolysis compared to sarcoplasmic proteins, which is associated to the spatial structure of actomyosin (Khantaphant, Benjakul et al. 2011). However, the DPPH radical scavenging activity of myofibrils might still be enhanced through enzymatic hydrolysis, which also applies for sarcoplasmic proteins although the effect is less significant than for myofibrils (Borawska, Darewicz et al. 2016).

There are several methods applicable in order to investigate the radical scavenging activity of peptides. Scavenging of the DPPH radical evaluates to what degree the antioxidant peptide displays activity as a hydrogen donor. However, there are constraints associated to the DPPH radical scavenging activity as a measurement of antioxidant properties. Serpen, Gökmen et al. (2012) suggest that the DPPH radical, being relatively hydrophobic compared to other radicals such as the ATBS radical, could interact less with polar antioxidant compounds than for example the ATBS radical. Ethanol is in the present work used as a solvent during DPPH radical scavenging activity measurements and could potentially participate in the precipitation of proteins present in the hydrolysate. This could be explained by the solvent interacting with certain amino acids present within the peptides. Ethanol interactions with glycine, asparagine and histidine are unfavourable thus decreases their solubility, while ethanol interaction with hydrophobic amino acids such as leucine and phenylalanine are favourable (Yoshikawa, Hirano et al. 2012). Glycine is usually considered one of the three amino acids within the collagen backbone. Tveit (2014) reported on a hydrolysate produced from MDCR rich in the amino acids associated with collagen. If the protein hydrolysate contains peptides and proteins comprised of amino acids which unfavourably interact with ethanol, this could cause precipitation during the assessment of DPPH radical scavenging activity. Precipitated proteins could further reduce apparent scavenging activity of the sample, not only as antioxidant peptides precipitate from the liquid phase, but also as precipitate absorb light at the wavelength often used during such investigations (Cumby, Zhong et al. 2008). This negative effect could get more pronounced as protein concentration increases (Dissanayake, Ramchandran et al. 2013). The above mentioned effects could explain the drop in DPPH radical scavenging activity observed when increasing peptide concentration from 2 to 4 mg/mL, as presented in Figure 27. Centenaro, Salas-Mellado et al. (2014) investigated the DPPH radical scavenging of fish muscle hydrolysates obtained with Flavourzyme and α-chymotrypsin. The radical scavenging of the hydrolysate produced with α-chymotrypsin increased between 0.5-2.5 mg/mL but dropped between 2.5-5.0 mg/mL. Meanwhile, hydrolysates produced with Flavourzyme displayed an increasing DPPH radical scavenging activity

when protein concentration was increased between 2.5 and 5.0 mg/mL. An explanation could be that Flavourzyme cleave bonds which produce peptides of a more hydrophobic nature as compared to α-chymotrypsin.

4.1.3 The results and discussion on the relative protein solubility at pH 2, 4, 6 and 11 for the control hydrolysates

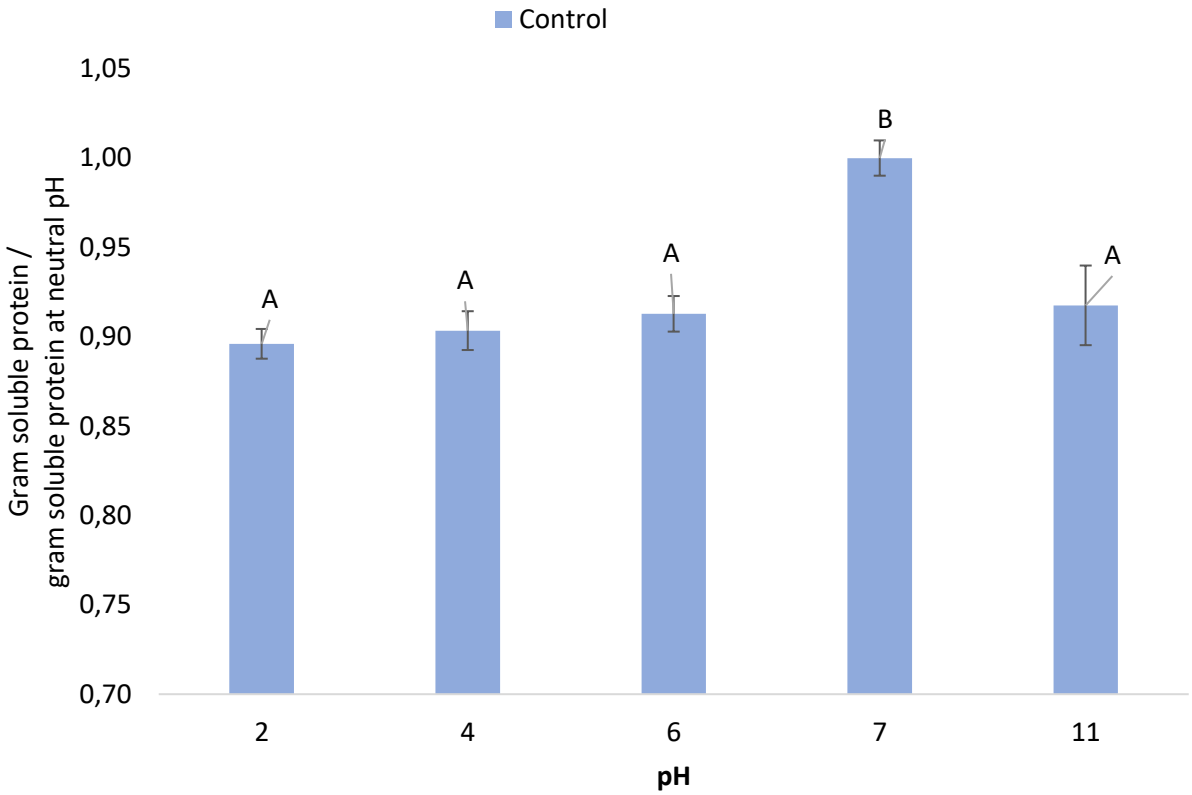

Figure 28: The figure presents the relative protein solubility at pH 2, 4, 6, 7 and 11 for the control sample hydrolysates/filtered aqueous phases. Significant differences at varying conditions within a certain treatment is indicated by different upper-case letters.

In this present work the relative protein solubility of the control samples varied significantly between neutral pH and pH 2, 4, 6 and 11, as displayed in Figure 28. However, no significant differences between the relative protein solubility at pH 2, 4, 6 or 11 was observed. Protein solubility could vary for several reasons but probably due to changes in the content of charged or hydrophobic groups per peptide chain length. As the net charge is shifted from zero at the pI, protein solubility increases. Thus, certain proteins or peptides in the aqueous phase of the hydrolysate exhibits reduced solubility

at pH 2, 4, 6 and 11, this due to fewer groups interacting with water at these pH values. Food industry often prefers protein hydrolysates with high protein solubility over a wide pH as highly soluble proteins provide a homogenous dispersibility of the molecules in colloidal systems and enhance interfacial properties (Thiansilakul, Benjakul et al. 2007). As proteins are cleaved during the enzymatic hydrolysis some of the hydrophobicity in the chain is converted as two-end carbonyl and amino groups are formed. Hydrophobic regions do not favour solute-solvent (in water) interactions and thereby do not contribute to dissolving proteins. Hydrophobic regions can instead interact and form aggregates. Higher content of polar and charged components allows more frequent solute-solvent interaction thus increasing the soluble fraction (Halim, Yusof et al. 2016). The pH will determine the charged state of weakly acidic and basic side-chain groups in the peptides and therefore peptide solubility (Kristinsson and Rasco 2000).

The results presented in this work are in line with the results of Gbogouri, Linder et al. (2004) when investigating the solubility of salmon by-product (head) hydrolysates produced with Alcalase, where protein solubility increased from pH 3, with a peak at pH 6-7 before declining until a pH of 11. When producing hydrolysates from salmon muscle with Alcalase, Kristinsson and Rasco (2000) also observed reduced solubility of the hydrolysate (5% DH) at low (1-3) and high (11) pH as compared to neutral pH. Souissi, Bougatef et al. (2007) also conducted investigations with Alcalase on fish proteins and observed a similar trend on evolution of protein solubility towards pH as that reported by Kristinsson and Rasco (2000) for acidic and alkaline conditions (6.62 DH%). Thus, the protein solubility of hydrolysates produced from raw materials exhibiting similarities to MDCR display reduced solubility at pH different than neutral. What seem to be a general trend is that the highest protein solubility improvement through enzymatic hydrolysis for such raw materials is seen between pH 4 and 6. This indicates the presence of parental proteins with low charge content at these conditions and it overlaps with the pI of myosin and actin (Collins and Elzinga 1975, Lin and Park 1998). In the present work, the specific peptides responsible for the precipitation observed at e.g. pH 4 and 6 could therefore originate from either myosin or actin. However, the liberated peptides present in the hydrolysate do not necessarily display the same isoelectric properties as the parental proteins. Several peptides present in the product could either be dominated by basic amino acids, and thus precipitating at alkaline conditions, or dominated by acidic amino acids and precipitating at acidic conditions. However, a significant loss in protein solubility is in this work already seen at pH 6, which could indicate presence of relatively large peptide species. Both myosin and sarcoplasmic proteins generate peptide fragments with relatively high molecular weight upon enzymatic hydrolysis, which could indicate formation of peptides resistant for further proteolytical degradation. This is reported by Kristinsson and Rasco (2000) and Souissi, Bougatef et al. (2007) who also observed

reduced solubility at alkaline and acidic conditions compared to neutral pH, however the reduction in protein solubility seemed more gradual to what observed in the present work. The rapid solubility loss against pH seen in this work highly resemble the results obtained by Diniz and Martin (1997) at relatively low DH%. This also suggests that peptides with higher molecular weights are present, which in generally are more sensitive to changes in pH as compared to shorter peptides. As pH is further reduced other peptides with isoelectric points lower than pH 6 could dominate the relative protein solubility. The reduced peptide solubility at pH 11 observed in this work could be due to a high gelatin content. While gelatin present at neutral pH often is soluble, this content could be partially precipitated at alkaline conditions. Gelatin formed from collagen through mild processing such as heat treatment could display a pI of 9.4 (Poppe 1992). Besides, due to the complexity of the raw material might other basic peptides be responsible for the reduced solubility observed at pH 11.

4.2 The results and discussion on the effect of pre-treating samples with microwave (MW)

4.2.1 Protein solubilisation during the enzymatic hydrolysis after MW pre-treatments

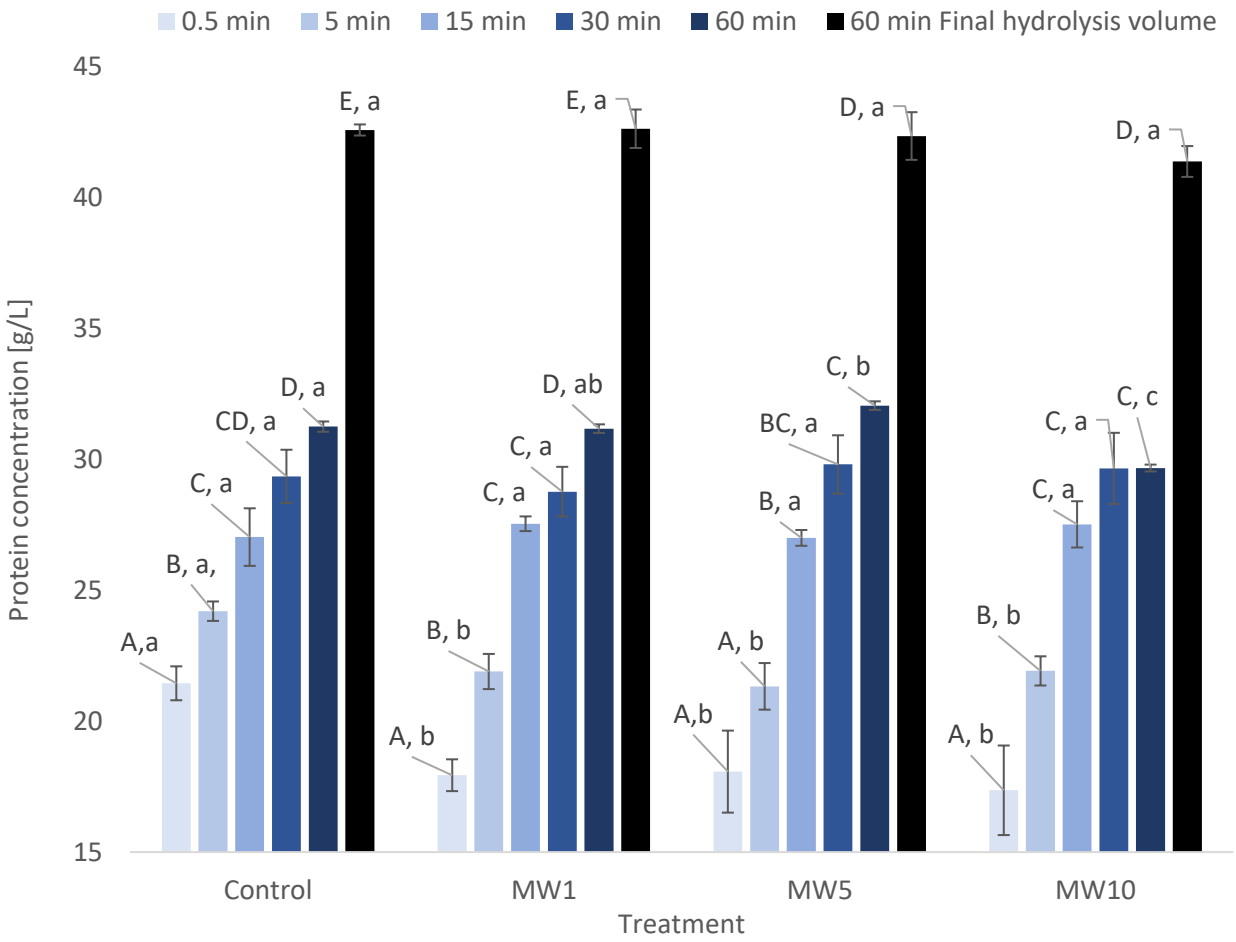

Figure 29: The figure presents the protein concentration at 0.5, 5, 15, 30 and 60 min of hydrolysis for the control, MW1, MW5 and MW10 trials. In addition, the protein concentration observed after enzyme inactivation of the final hydrolysis volume is presented for the mentioned trials. Significant differences at similar conditions among different treatments are indicated by different lower-case letters. Significant differences at varying conditions within a certain treatment is indicated by different upper-case letters.

According to the Figure 29, applying microwaves prior to the enzymatic hydrolysis (1, 5 and 10 min; MW1, MW5 and MW10; 1000 W; 40 ℃; 2.45 GHz) significantly reduces the protein solubility after 0.5 min of hydrolysis, as compared to the control without pre-treatment, which indicate some structural alteration of the proteinaceous substrates. For the 0.5 min sample, there is no significant difference among the microwave pre-treatment times 1, 5 or 10 min (MW1, MW5 or MW10) which could be due to the small difference in absorbed microwave energy (See Appendix A). The protein

concentration in pre-treated samples was still significantly lower than that of control samples after 5 min of hydrolysis. However, after 15 min of hydrolysis, there was no significant difference in the protein concentration between pre-treated samples and the control samples. In fact, while the protein solubilisation seemed to increase steadily for the control samples, there was a sudden increase in solubilised proteins between 5 and 15 min for all microwave treated samples, which compensates the low initial protein solubilisation, as compared to the control samples. The solubilisation patterns following 15 min of hydrolysis vary among the MW treated samples but only significantly after 60 min of hydrolysis (samples). MW5 show a significant increase as compared to MW10 and control samples, while MW10 exhibits a significant decrease as compared to the rest of conditions. The soluble protein concentration measured in the final reactor volume after hydrolysis (60 min final hydrolysis volume samples) does not significantly differ among the treatments or control, but it seems to gradually decrease with increasing MW treatment time. There is a significant difference in the protein concentrations measured between the total reaction volume after hydrolysis (60 min final hydrolysis volume) and the time-samples withdrawn after 60 min of hydrolysis. This could be due to varying release of collagen or other proteinaceous compounds from the large content of solids present during enzyme inactivation of the remaining hydrolysis content (final hydrolysis volume). This could hide the slight but significant effect observed in the 60 min samples. Another factor influencing the 60 min final hydrolysis volume might be the potential loss of peptides due to the formation of the multicomponent phase after enzyme inactivation and centrifugation.

4.2.2 Phase contents obtained after enzyme inactivation of the final hydrolysis volumes from samples pre-treated with MW

The amount of solid and particulate phases, filtered aqueous phase, lipid and multicomponent phases originating from the control, MW1, MW5 and MW10 samples are presented in Figure 30, Figure 31 and Figure 32, respectively. In general, no significant differences of solid or particulate content were observed among the various treatments, including the control samples.

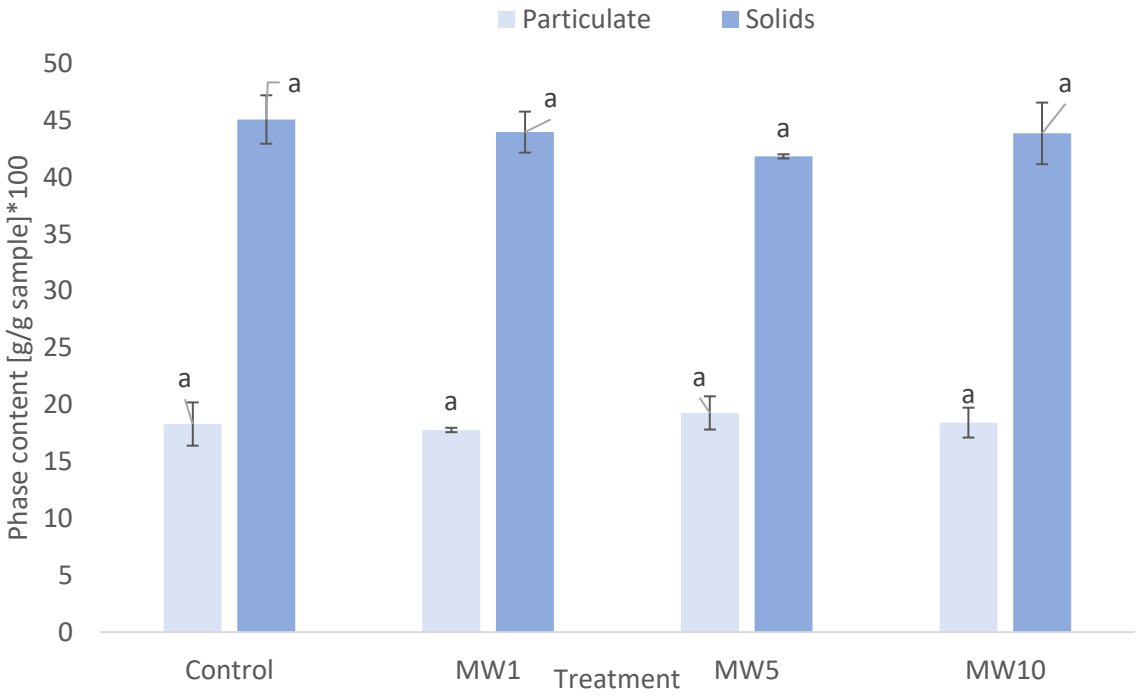

Figure 30: The figure presents the particulate and solid phase contents from the control, MW1, MW5 and MW10 trials. Significant differences among similar phases between different treatments are indicated by different lower-case letters.

There were also no significant differences in the filtered aqueous content across the various MW pre-treatments or as compared to the control samples.

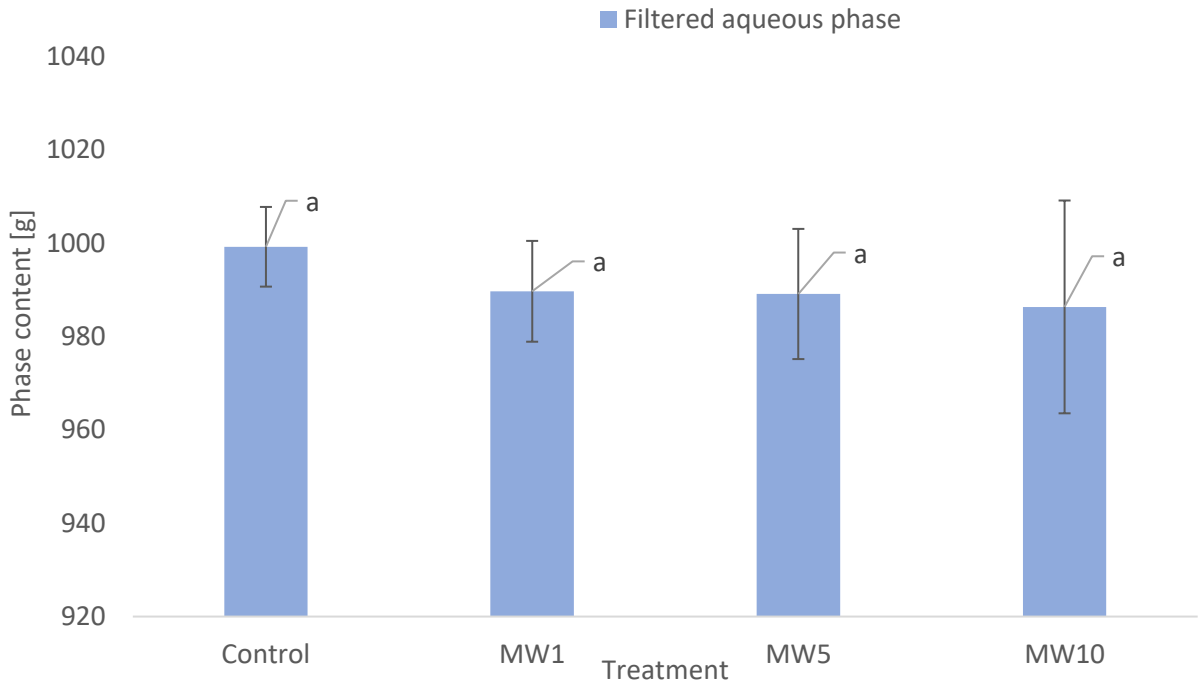

Figure 31: The figure presents the filtered aqueous phase content for the control, MW1, MW5 and MW10 samples. Significant differences among similar phases between different treatments are indicated by different lower-case letters.

Finally, there were no significant differences in the total lipid content or lipid phase + multicomponent phase content among the treatments/control, but significant differences were observed in the multicomponent content depending on the MW pre-treatment applied. MW1 samples showed a significantly higher multicomponent content as compared to the MW5 and MW10 samples. However, these results do not vary significantly from the control samples.

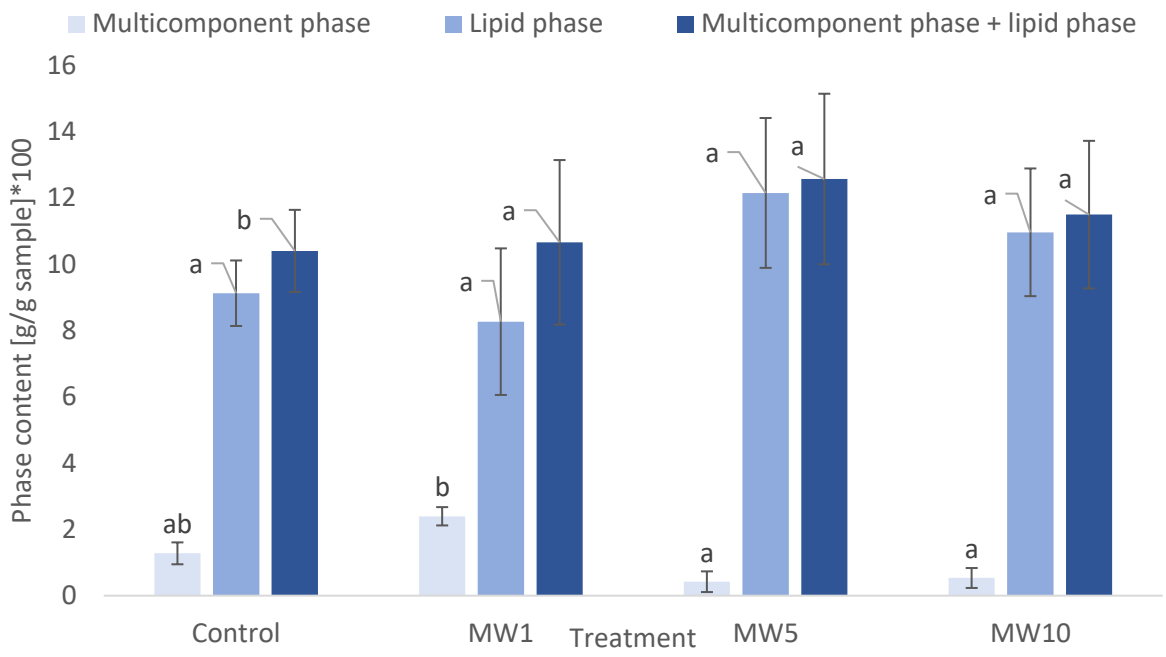

Figure 32: The figure presents the contents of the lipid and multicomponent phases in addition to the sum of these phases for the control, MW1, MW5 and MW10 samples. Significant differences among similar phases between different treatments are indicated by different lower-case letters.

4.2.3 The DPPH radical scavenging activity observed in the hydrolysates of samples pre-treated with MW

The DPPH radical scavenging activity in the hydrolysates (filtered aqueous phase) of the control, MW1 and MW10 at various peptide concentrations is presented in Figure 33. A linear dependence between the DPPH radical scavenging activity and the peptide concentration of 0.5-2.0 mg/mL (R^2>0.988) was observed for the control, MW1 and MW10. The protein concentrations of 0.5, 1 and 2 mg/mL presented no significant variations among treatments/control. However, 4 mg/mL presented significant differences among the applied treatments/control. MW1 samples displayed a significantly lower DPPH radical scavenging activity as compared to both MW10 and the control samples. MW10 samples presented a significantly higher radical scavenging activity with respect to MW1 samples, but this value was still lower than that of the control samples.

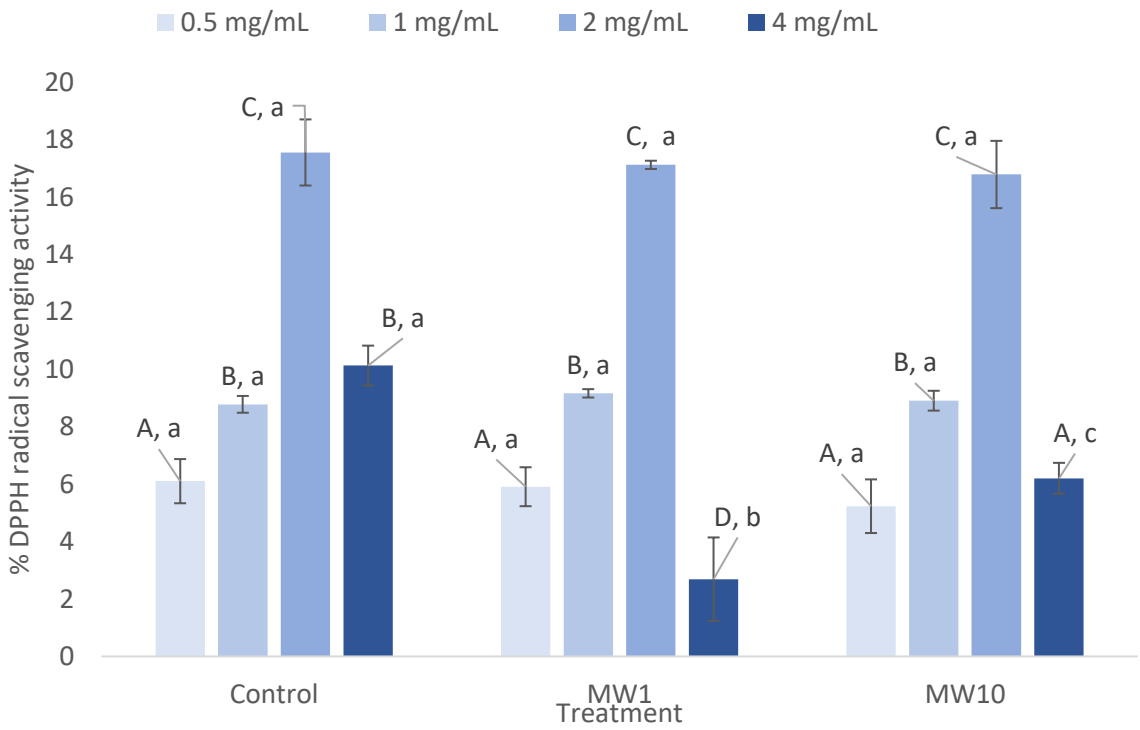

Figure 33: The figure presents the DPPH radical scavenging activity for the control, MW1 and MW10 hydrolysates/filtered aqueous phases with protein concentration 0.5, 1, 2 and 4 mg/mL. Significant differences at similar conditions among different treatments are indicated by different lower-case letters. Significant differences at varying conditions within a certain treatment is indicated by different upper-case letters.

4.2.4 The relative protein solubility observed for hydrolysates of samples pre-treated with MW

The protein solubility of the hydrolysates (filtered aqueous phases) from the control, MW1 and MW10 treatments at pH 2, 4, 6 and 11, as compared to the protein solubility at neutral pH, is presented in Figure 34. The relative protein solubility for the control samples was not found to be different at pH 2, 4, 6 or 11. MW1 samples displays significantly higher relative protein solubility at all pH values as compared to that of the control, but these values were not significantly different to those of the MW10 pre-treatment at any pH value. Similarly, MW10 samples displayed significantly higher relative protein solubility as compared to that of the control at all pH except pH 4, where protein solubility was not statistically different with regards to that of the control and MW1. The relative protein solubility of MW1 samples was similar at pH 6 and 11 and significantly higher than the values at pH 2 and 4, which were similar among themselves. The relative protein solubility of MW10 samples followed the same trend as MW1 samples. The result indicates that a MW pre-treatment followed by hydrolysis generates peptides with similar protein solubility against pH, irrespective of the MW pre-treatment time applied.

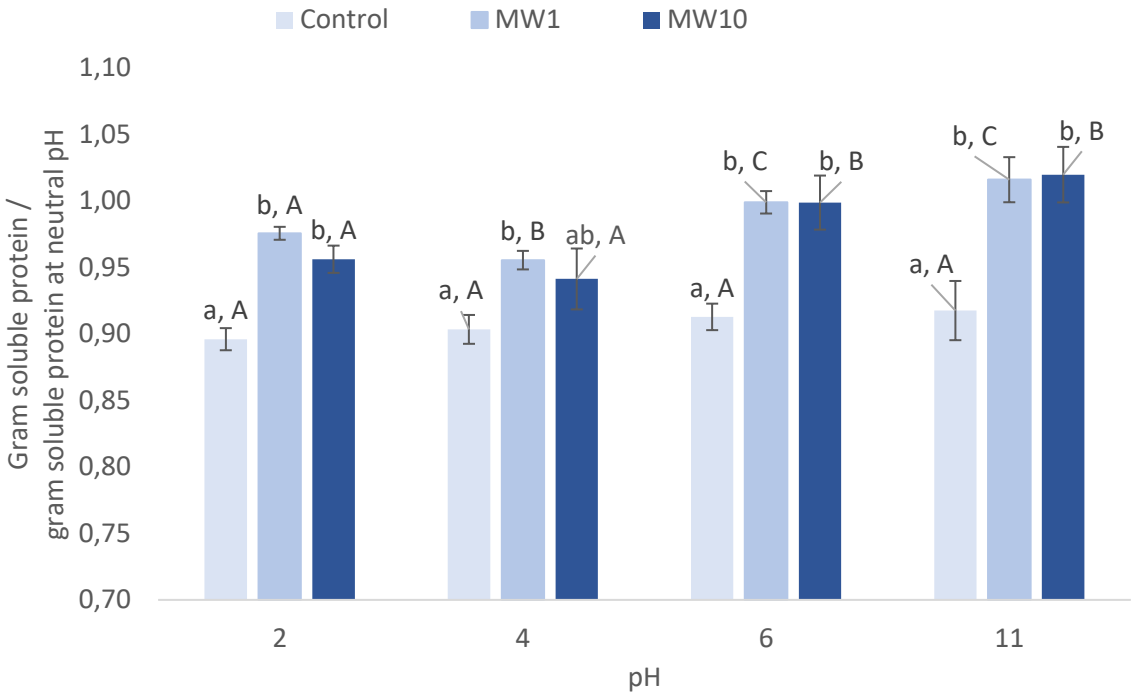

Figure 34: The figure presents the relative protein solubility at pH 2, 4, 6 and 11 for the hydrolysates/filtered aqueous phase from the control, MW1 and MW10 trials. Significant differences at similar conditions among different treatments are indicated by different lower-case letters. Significant differences at varying conditions within a certain treatment is indicated by different upper-case letters.

4.2.5 Discussion of MW results

The solubilized protein content in the control was significantly higher than for MW10 samples at 60 min of hydrolysis. Furthermore, there seemed to be a stop in protein solubilisation for MW10 samples between 30 and 60 min of hydrolysis. An explanation for this is protein aggregation, which suggests that the effect from the pre-treatment and the thermal conditions in the reactor (50 °C; optimal temperature for the enzyme) could have been too severe on the proteinaceous components in the raw material. Protein aggregates are reported to be highly resistant to further enzymatic breakdown, and could also decrease the peptide content in the aqueous phase and thus, the extraction yield (Šližyte, Daukšas et al. 2005). Mohr (1980) mention that proteins might denature and precipitate during hydrolysis, but this would depend on both the type of protein and conditions applied. The cause of the effect observed in the present work could be a combination of both the pre-treatment, the hydrolytic activity of the enzymes and the thermal effect from the reactor. As a general trend muscle protein solubility is negatively affected by microwave treatment through aggregation of originally soluble proteins (YOWELL and FLURKEY 1986, Kong, Tang et al. 2008, Wu and Mao 2008). The MW energy absorbed during pre-treatment is presented in Appendix A and Table 8. The MW energy absorbed was significantly higher when the raw material was treated for 10 min as compared to the 1 min pre-treatment, but this was not noticeable for the protein concentrations until a hydrolysis time of 60 min was reached. However, this suggests that the initial alterations of protein structures caused by the various MW pre-treatments were different, leading to different bonds being hydrolysed by the enzyme, with peptides present after 30-60 min of hydrolysis capable of forming aggregates. Such structural alteration could be attributed the thermal effect associated with long MW pre-treatment time or temperature (Murphy and Marks 2000). The alterations could also be non-thermal and associated to the differences in absorbed MW energy (George, Bilek et al. 2008). What should especially be noted is the similarity of protein solubilisation throughout the course of hydrolysis for MW1, MW5 and MW10 samples, which only seemed to differ for the 60 min samples. It seems as the effect posed from the pre-treatment causes a similar effect of denaturing independent of treatment time, but the additional affect from MW5 or MW10 seem to eventually contribute in a positive or negative manner. There would seem as there is a fine balance between desirable and non-desirable outcomes when MW is applied. And as shall be seen in the following there seem to be differences in the product peptide properties.

The protein content in the filtered aqueous phase after enzyme inactivation of the total hydrolysis volume was influenced not only by the enzymatic hydrolysis, but also by the formation and separation of the various phases. The presence of functional peptides stabilized in the

multicomponent phase or antioxidant peptides in the lipid phase may have caused their reduced contents in the aqueous product as compared to that seen in the 60 min samples. This will be covered further later in this discussion.

Why the 60 min time samples display significant differences in protein concentration, while the inactivated total hydrolysis volume does not, could be several. One explanation would be differences in solid content when inactivating the time-samples as compared to the total hydrolysis reaction volume. As stated earlier, the content of collagen is relatively high in MDCR (McCurdy, Jelen et al. 1986). The hydrolysate of MDCR is reported to contain significant amounts of amino acids associated with collagen (Tveit 2014). The thermal solubilisation of collagen could occur during the hydrolysis, during the enzymatic inactivation or partially during both processes (Yannas 1972). Upon thermal (enzyme) inactivation of the final hydrolysis volume, the solid to liquid ratio could be higher than that in the time-samples. Thus, the potential peptide release when heat treating these samples with different composition was not similar. This could explain the significant increase observed between protein concentration in 60 min time-samples and 60 min final hydrolysis volume samples. The significant differences of protein concentration in 60 min samples could either have disappeared due to variances in the amount of proteins solubilized upon enzyme inactivation/heat-treatment, or due to the association of certain species through the multicomponent or lipid phase during the separation of phases. Increasing the MW treatment time seemed to negatively affect the protein concentration after inactivation of the final hydrolysis reaction volume. However, the extent was still not great enough to display an effect on the solid phase contents, as the solid and the particulate contents not changed significantly. A higher aggregate content would probably have been visible through increased particulate or solid phase contents. Interestingly, the MW5 samples presented a significantly higher protein concentration after 60 min of hydrolysis, with a reduced solid fraction and increased particulate content. This could indicate favourable conditions for solubilisation of major proteinaceous components within the raw material, as the particulate are suspended particles which indicate higher content of lower density compounds. Murphy and Marks (2000) observed increasing contents of low molecular weight species when heat treating chicken breast patties. Thus, the enhanced protein concentration observed for MW5 samples in the present work could be due to the release of such species. When Murphy and Marks (2000) further increased the temperature to 50 ℃ these species were lost and could indicate the scenario occurring upon to severe heat treatment as that for the late stage of hydrolysis in the present work.

The multicomponent phase content of MW1 samples were significantly higher than that for MW5 and MW10 samples, which indicates the presence of peptides with different properties. Moreover, it is noteworthy that the multicomponent phase of MW1 dropped when increasing the pre-treatment

time to 5 min (MW5), but the lipid phase + multicomponent phase content seemed to increase. This could indicate that the lipid fraction still was associated to certain peptides, even though not generating lipid-protein structures present within in the multicomponent phase. Further increasing the pre-treatment time to 10 min maintained the low multicomponent phase content observed for MW5, while the lipid fraction seemed to decrease slightly. This could indicate that protein-lipid interaction decreased when increasing treatment time from 5 to 10 min and could be connected to the reduction in protein concentration observed after 60 min of hydrolysis. This as hydrophobic peptide domains are lost through hydrophobically driven protein-protein association and thus reducing the release of species able to associate with the lipid phase. Kristinsson and Rasco (2000) connected interactions between peptides and lipids to the combination of hydrophilic and hydrophobic properties observed for protein hydrolysates, which allow proteins to adsorb to the surface of oil droplets and interact with water at the same time. This might prevent oil droplets from coalescing and could generate stable structures present in e.g. the multiphase content. Pacheco-Aguilar, Mazorra-Manzano et al. (2008) further suggest that small peptides could be efficient absorbers at the liquid-oil-interphase, however when compared to larger peptides they are not as efficient in reducing the interfacial tension which is necessary in order to form emulsions, but could potentially contribute to elevated contents of lipid phase. However, partial hydrolysis of myofibrils are found to generate peptides interacting with both lipid and aqueous layers which could explain the observations on increasing multicomponent phase for the MW1 samples (Smith and Brekke 1985). It could however be possible that further denaturing, which might be the case for MW5 and MW10 induce the formation of peptides merely absorbing at the liquid-lipid interphase as mentioned by Pacheco-Aguilar, Mazorra-Manzano et al. (2008). While the presence of such peptides could contribute to varying contents of multicomponent and lipid phases, the pre-treatment could also affect the actual release of lipids from the biomatrix during the enzymatic hydrolysis. After the enzymatic hydrolysis, undigested proteins aggregated during enzymatic inactivation could also entrap lipids, thereby affecting the contents of lipid and multicomponent phase.

The presence of peptides with varying properties was not only indicated by differing phase contents, but also the DPPH radical scavenging activity. As shown in Figure 33, applying MW as a pre-treatment can alter the antioxidant properties of the hydrolysates. The apparent drop of antioxidant properties could be caused by charged and polar groups interacting unfavourably with ethanol (used in the present work during the measurement of the DPPH radical scavenging activity). Ethanol is a relatively non-polar solvent as compared to water (Yoshikawa, Hirano et al. 2012). Unfavourable interactions between peptides and ethanol could cause precipitation and thereby increase light absorbance at certain wavelengths, but also reduce the DPPH radical scavenging activity as the relevant peptides

did not offer scavenging activity due to precipitation (Cumby, Zhong et al. 2008). This effect could further be accelerated by increasing the hydrolysate protein concentration (Dissanayake, Ramchandran et al. 2013). As the aromatic and non-polar side chains of Trp, Tyr, Met and Phe are associated to high antioxidant properties, the enhanced multicomponent phase content could explain the reduction of DPPH radical scavenging activity observed for MW1 samples (Dávalos, Miguel et al. 2004). As the side chains which are known to display high antioxidant properties and interact favourably with the DPPH solvent, also could contribute to favourable interactions with the lipid phase or as a tension reducer by interacting favourably with both the lipid and aqueous phase simultaneously. Such peptides present in the product of MW1 could therefore generate protein-lipid structures and contribute to the content of the multicomponent phase. As the MW treatment time was increased from 1 to 10 min, the multicomponent phase content was reduced significantly while the DPPH radical scavenging activity increased significantly as compared to MW1 samples. This could indicate that groups capable of scavenging the DPPH radical are released and a higher content was available in the hydrolysate. However, the scavenging activity was still not higher than that of the control. This could be explained by the apparently high content of the lipid phase as groups interacting favourably with lipids also interacts relatively favourable with ethanol. Therefore, peptides able to scavenge the DPPH radical could be stabilized within the lipid phase. The apparent aggregation observed in the late stage of the MW10 hydrolysis could also contribute to the observed DPPH radical scavenging activity. These protein-protein interactions could be due to hydrophobic interactions that reduced the content of DPPH radical scavenging groups per gram of hydrolysate and therefore, reduce the scavenging abilities observed for MW10-hydrolysates.

Applying MW as a pre-treatment significantly enhanced the peptide solubility at pH 2, 4, 6 and 11, as compared to the control, except for the MW10 hydrolysates exposed to pH 4. Enhanced solubility is often associated with increased DH% or reduced molecular weights which increase the charges present at both acidic and alkaline conditions, as additional carboxylic and amide groups are introduced upon hydrolysis of the peptide bond (Halim, Yusof et al. 2016). Thus, the increasing content of low molecular weight species present upon exposure of raw materials containing constituents like that of MDCR to 40 ℃, as mentioned by Murphy and Marks (2000), could generate a hydrolysate with peptides originating from small proteins, which further have the potential to increase the presence of peptides with high charge content per chain length in the hydrolysate. The improved solubility at various pH values explained by an increased number of charged/polar groups would also suit the reduced DPPH radical scavenging activity, since a high content of charged groups would interact unfavourably with ethanol but could elevate solubility at various pH values. Regarding the slight and not significant reduction in relative protein solubility at pH 2 and 4 seen for MW10

samples compared to those of MW1; If certain peptide species in the product of MW1 were associated to lipids and present in the multicomponent phase and therefore not in the MW1 aqueous phase, the relative solubility at low pH would be enhanced if these peptides exhibited a high acidic group content. Moreover, if such peptides were present in MW10 samples, they would cause the solubility to decrease for acidic conditions and thus, reduce the relative solubility.
As there was both a (slight) solubility reduction at low pH and an increase in DPPH radical scavenging activity for MW10 as compared to MW1 samples, this could indicate that groups able to interact favourably with both the polar aqueous and non-polar lipid phases were increasingly present in MW10 as compared to MW1 samples. The lipid phase + multicomponent phase also seemed to increase while the multicomponent phase content remained stable between MW5 and MW10 samples. This could indicate a hydrolysate increasingly dominated by hydrophobic peptides.

Interestingly, a pH of 4.5-5.5 often holds the highest potential for enhanced protein solubility in raw materials such as that applied in the present work (Smith and Brekke 1985). This is often related to the isoelectric properties of myofibrillar proteins (Collins and Elzinga 1975, Lin and Park 1998). Myofibrillar proteins are usually related to peptides with high potential both regarding functional and antioxidant properties when compared to sarcoplasmic protein, which could indicate that variations in solubility at acidic conditions (pH 2 and 4), changes in DPPH radical scavenging activity and protein-lipid properties are caused by modification within the structure of these proteins (Smith and Brekke 1985, Borawska, Darewicz et al. 2016). However, while these components are reported to potentially offer peptides with desirable properties, there is often limitations with regards hydrolysis of the actomyosin complex (Khantaphant, Benjakul et al. 2011). This motivates the application of a pre-treatment such as MW to generate structural alterations of the native protein confirmation which offer new cleavage sites for the enzyme. The mechanism this occur through is thermal, and during heat treatment, initial denaturation of proteins may occur with little or no apparent loss in solubility; this step is then usually followed by aggregation and solubility loss (Li-Chan, Nakai et al. 1984). The aggregated proteins are highly resistant to enzymatic breakdown, which reduces the yield of solubilised components (Šližyte, Daukšas et al. 2005). Cai, Feng et al. (2018) investigated how microwave treatment affected both sarcoplasmic and myofibrillar proteins and found that both microwave power and treatment time had direct effects on the protein solubility. However, aggregates formed from sarcoplasmic proteins required lower power or time to form as compared to myofibrillar proteins. In the present work, a gradual decrease (not significant) in the protein concentration was observed for the final hydrolysis volume samples. The significant reduction in protein concentration observed for MW10 samples after 60 min of hydrolysis could be due to the reported aggregation of sarcoplasmic proteins upon the final stages of hydrolysis. The increase in

DPPH radical scavenging activity seen for MW10 compared to MW1 samples and the declining 60 min protein concentration could suggest the presence of hydrophobic protein surfaces at some point during the enzymatic hydrolysis, which further could cause aggregation through hydrophobic interactions. Feng, Xue et al. (2017) investigated changes in surface hydrophobicity of actomyosin heated by microwave to an average temperature of 35 °C. Surface hydrophobicity increased gradually with treatment time until 40 min was reached at which the surface hydrophobicity dropped, suggesting aggregation. This confirms the possibility of the actomyosin complex to denature and form aggregates through hydrophobic interactions. It should be stated that this investigation was conducted in an actomyosin solution, and the complex would probably be more stable within the natural biomatrix. This is illustrated through the "polymer in a box" metaphor, as the denaturation of a protein inside the matrix is confined by the presence of all the surrounding structures as well. However, as mentioned by Tornberg (2005), certain components within the matrix might denature and connect to other structures, and in such a way favour aggregate formation. Tornberg (2005) proposed that this first occur through denaturation of sarcoplasmic proteins, which is in agreement with the work of Cai, Feng et al. (2018). This could explain what seem as a sudden drop in protein solubility between MW5 and MW10 and the hydrolysis-times of 30-60 min. An early denaturing of sarcoplasmic proteins, linking other structures into larger complexes would most certainly upon further actomyosin denaturing set the stage for precipitation. In order to avoid this the pre-treatment would have to favour early degradation of sarcoplasmic proteins, but also favour the subsequent degradation of the myofibrillar structures, which are reportedly relatively non-soluble (Smith and Brekke 1985). One option to reduce what could be a negative effect from the denaturing sarcoplasmic proteins is pure water washing prior to the enzymatic hydrolysis which would remove the water soluble sarcoplasmic species and conditions could be optimised regarding the myofibrillar proteins (Lin and Park 1996). While the sarcoplasmic proteins might denature and link structures together, so might other components within the raw material. Work done by Konno, Yamamoto et al. (2000) display interesting effects of heat (3-5 min; 40 °C) on carp myofibrils and subsequent chymotryptic digestion (0-60 min). The digestion patterns presented by heated myofibrils were clearly different than that of the control, as in the present work. Observations of new molecular weight species indicated that new cleavage sites were exposed on the myosin rod. What also should be noted regarding the mentioned work is that the solubility of the salt-soluble fraction (myofibrils) was reduced by 50 % when the material was heated at 40 °C for 3 min. However, this was relative the original solubility in a high ionic strength solution. Myofibrils in pure water are insoluble at low ionic strength (Smith and Brekke 1985) and therefore might the effect of heat on myofibrillar digestion be none-observable. However, the digestion of the control sample (no heat treatment) performed by Konno, Yamamoto et al. (2000) was conducted in ionic strength not

reported to affect myofibrillar solubility (Smith and Brekke 1985) and presented the formation of reduced molecular weight species. Even though some of the work of Konno, Yamamoto et al. (2000) present the solubilisation of myofibrils through elevation of ionic strength, it display important points. Both the 3 and 5 min heat treatment displayed the presence of major myofibrillar species containing significant hydrophobic domains (myosin S-1 and actin) when investigated after the digestion, thus was this treatment time not sufficient to aggregates these structures which would make them disappear into higher molecular weight species. However, the myosin S-1 fragment seemed to occur at later stages of hydrolysis when treatment time was increased. This could indicate that unfavourable conditions are increasingly occurring and could potentially explain the reduction in solubility for MW10 at 60 min of hydrolysis as potentially this fragment causes some aggregation of species which originally were soluble.

Work done by Ketnawa and Liceaga (2017) bear some resemblance to the present work. A fish frame slurry was pre-treated with microwave (55 or 90 ℃; 2, 4, 6 or 8 min + 10 min for heating) before hydrolysed by Alcalase (55 ℃; 15 min). However, autolytic activity was inactivated (90 ℃; 15 min) prior to the pre-treatment. No significant effects were seen on degree of hydrolysis, protein solubility or protein recovery when pre-treatment time was changed. This could be due to the initial inactivation step used to ensure low autolytic activity. Work done by Bruno, Kudre et al. (2019) present many similarities to the present. A MW pre-treatment time of 5, 10 and 15 min (without heating period which in the present work was ~ 3 min) was applied on a fish head slurry before hydrolysed enzymatically (55 ℃; 2 h). The two shortest pre-treatment times resulted in a significantly higher DH%, indicating exposure of more cleavage sites, while the highest treatment time not resulted in an increase compared to the control. This suggest that certain denaturation was induced by the pre-treatment increased the number of enzymatic cleavage sites, while a prolonged treatment reversed this. This matches with the findings for MW1, MW5 and MW10 in the present work, where MW10 through aggregation very likely would correlate to the reduction in DH% observed by Bruno, Kudre et al. (2019). The work of Bruno, Kudre et al. (2019) also investigated extraction oil from the raw material. The longest pre-treatment time reduced the oil extraction yield. This was attributed reduced release of lipids due to a lower protein hydrolysis caused by heat aggregation. Released oil could also be entrapped in newly formed aggregates, which might form if partially degraded proteins form aggregates during e.g. enzyme inactivation. This could explain the results observed in the present work, where MW10 seem to present a reduced lipid content as compared to that of MW5. MW5 could therefore present a pre-treatment offering a high number of peptide cleavage sites which liberates a high content of lipids. Why the milder treatment of MW1 generates a higher content of multicomponent phase could be due to the earlier mentioned formation of protein-lipid

structures. This is reported to reduce the pure lipid phase (Šližyte, Daukšas et al. 2005). But it could also be due to a low release of initially entrapped lipids within the matrix of the raw material.

4.3 The results and discussion on the effect of pre-treating samples with ultrasound (US)

4.3.1 Protein solubilisation during the enzymatic hydrolysis after US pre-treatments

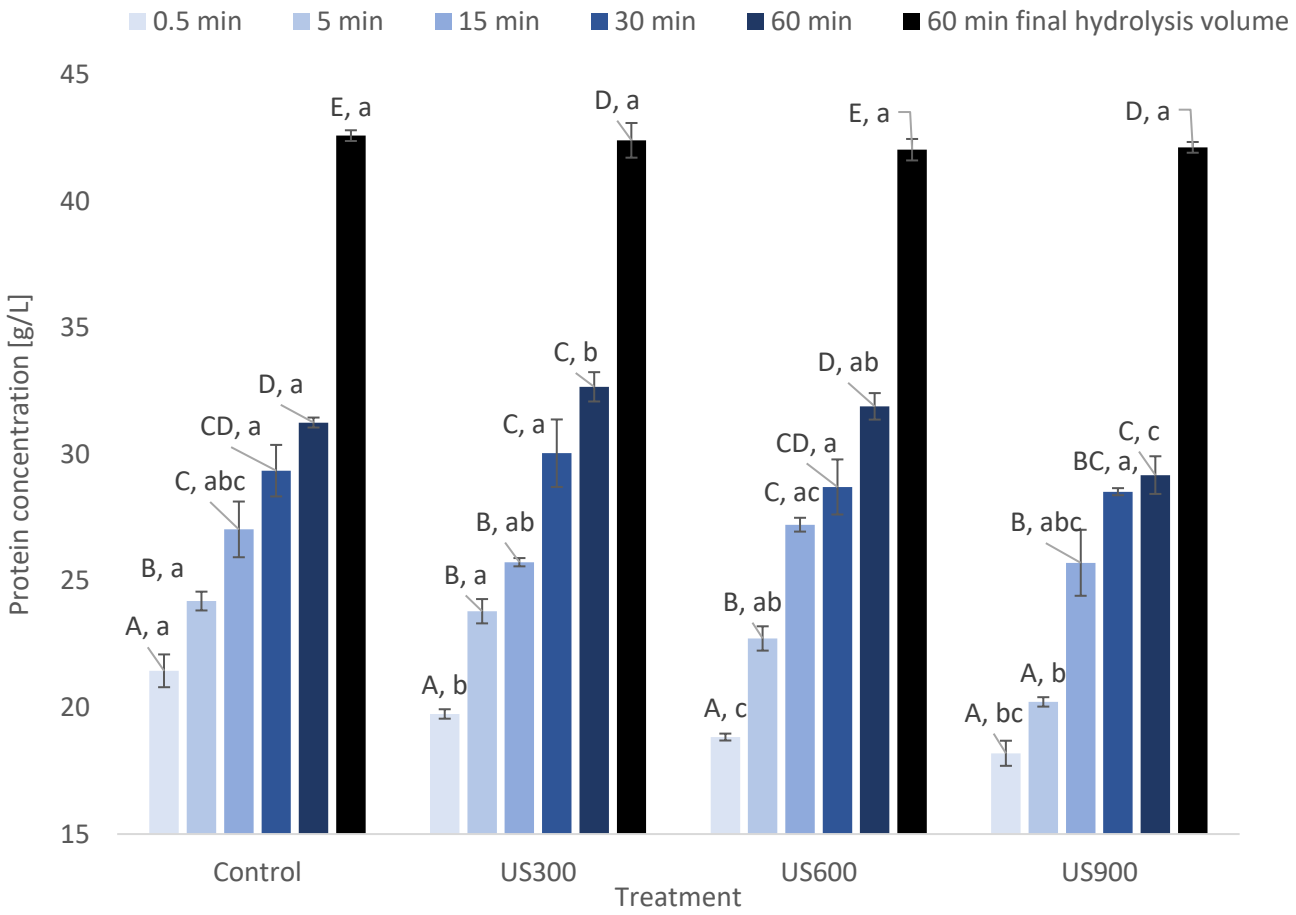

Figure 35: The figure presents the protein concentration at 0.5, 5, 15, 30 and 60 min of hydrolysis for the control, US300, US600 and US900 samples. In addition, the protein concentration observed after enzyme inactivation of the final hydrolysis volume is presented for the mentioned trials. Significant differences at similar conditions among different treatments are indicated by different lower-case letters. Significant differences at varying conditions within a certain treatment is indicated by different upper-case letters.

As presented in Figure 35, applying US (300, 600 and 900 W; US300, US600 and US900; 68/170 kHz; 30 min) as a pre-treatment significantly reduced the protein concentration measured after 0.5 min of hydrolysis, indicating a structural alteration of the proteinaceous components in the raw material. The protein concentration after 0.5 min of hydrolysis for US900 (900 W) was significantly lower than that for the control but statistically similar the two other US pre-treatments. The protein concentration after 5 min of hydrolysis followed a similar trend. However, as US pre-treatment

power declined, the protein concentration for US300 and US600 samples seemed to gradually approach the protein concentration observed for the control. US300 did in fact not exhibit any significant reduction in protein concentration compared to the control at 5 min of hydrolysis. The protein concentration of US600 samples at 5 min of hydrolysis was statistically similar to that for the rest of conditions. The protein concentration after 5 min of hydrolysis for US900 was significantly lower than that for the control but not significantly different than the protein content solubilised after 0.5 min of hydrolysis within the same treatment. This indicates that US900 causes an effect on the raw material at the early stages of hydrolysis where there are few cleavage sites able to release a significant mass of peptides available for the enzyme. At 15 min of hydrolysis, the only difference in the peptide concentration among treatments was observed for US300 and US600 treatments, with the highest content found for the latter. After 30 min of hydrolysis, there is no significant difference in dissolved peptide content among the different pre-treatments and control. After 60 min of hydrolysis, US300 displayed a significantly higher protein concentration as compared to the control; US900 presented a significantly lower protein concentration than all other treatments; and US600 was not significantly different to the US300 samples or the control. Soluble protein content present in the total hydrolysis reaction volume was similar among all the treatments and significantly higher than the protein concentration for 60 min of hydrolysis samples within each treatment.

The different time samples taken during the hydrolysis of the control were significantly different until the 30 min sample, which was statistically similar to the protein concentration in the 15 and 60 min sample; this trend was however affected by the US pre-treatments. In fact, increasing the ultrasound power from 300 to 600 W significantly increased the protein concentration obtained after 15 min, while the value at 900 W was statistically similar to both US300 and US600 treatments. Besides, for the US300 samples, the protein concentrations at 60 and 30 min of hydrolysis was statistically similar, as it was also for 5 and 15 min hydrolysis samples, else did the protein concentration increase significantly with each subsequent time step. For the US600 pre-treatment, the 30 min protein concentration was statistically similar to the levels observed for 15 min of hydrolysis, while other samples were significant different (higher for each subsequent time step). For US900, the protein concentration after 0.5 and 5 min of hydrolysis was statistically similar; in addition, the protein concentration at 30 min of hydrolysis was statistically similar to the levels at 15 and 60 min of hydrolysis. The protein concentrations among other time points were significantly different (higher for each subsequent time step).

4.3.2 Phase contents obtained after enzyme inactivation of the final hydrolysis volume from samples pre-treated with US

The particulate and solid phase content, the filtered aqueous phase content, the multicomponent and lipid phase content of the various US trials and the control is presented in Figure 36, Figure 37 and Figure 38, respectively. The mentioned contents originate from the final hydrolysis volumes. There are no significant differences in solid phase or particulate phase contents among the control and the different treatments.

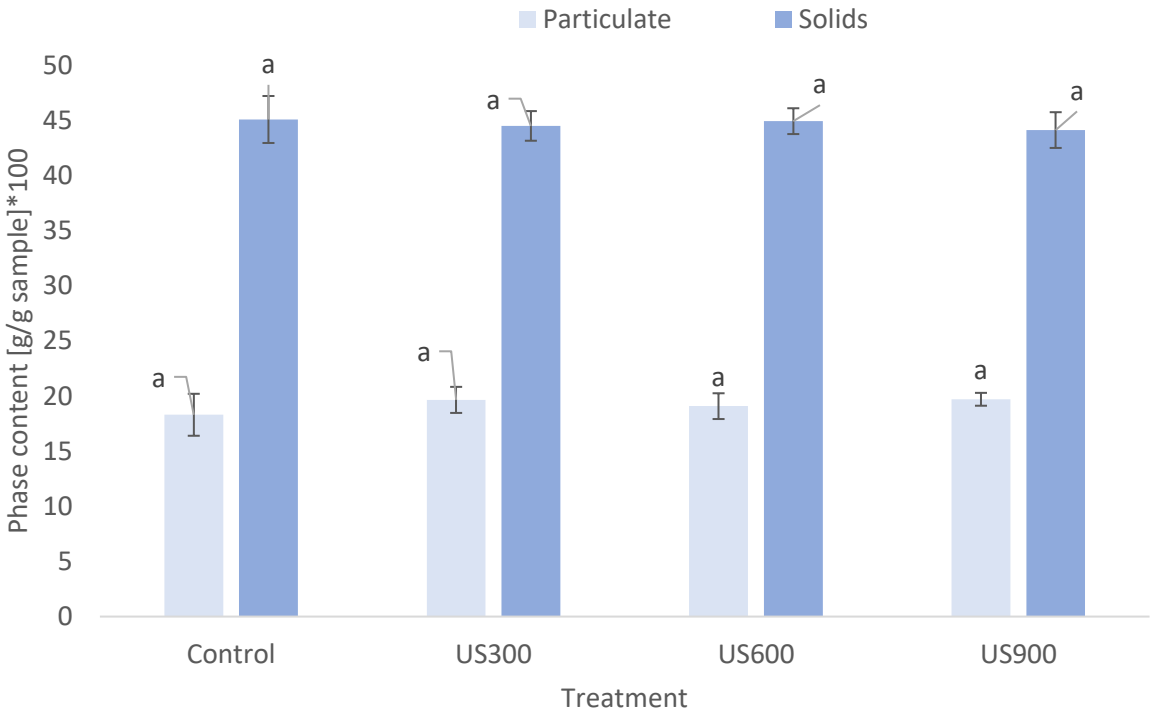

Figure 36: The figure presents the particulate and solid phase contents from the control, US300, US600 and US900 trials. Significant differences among similar phases between different treatments are indicated by different lower-case letters.

The filtered aqueous content for the various US trials and the control is presented in Figure 37. There are no significant differences among the different treatments.

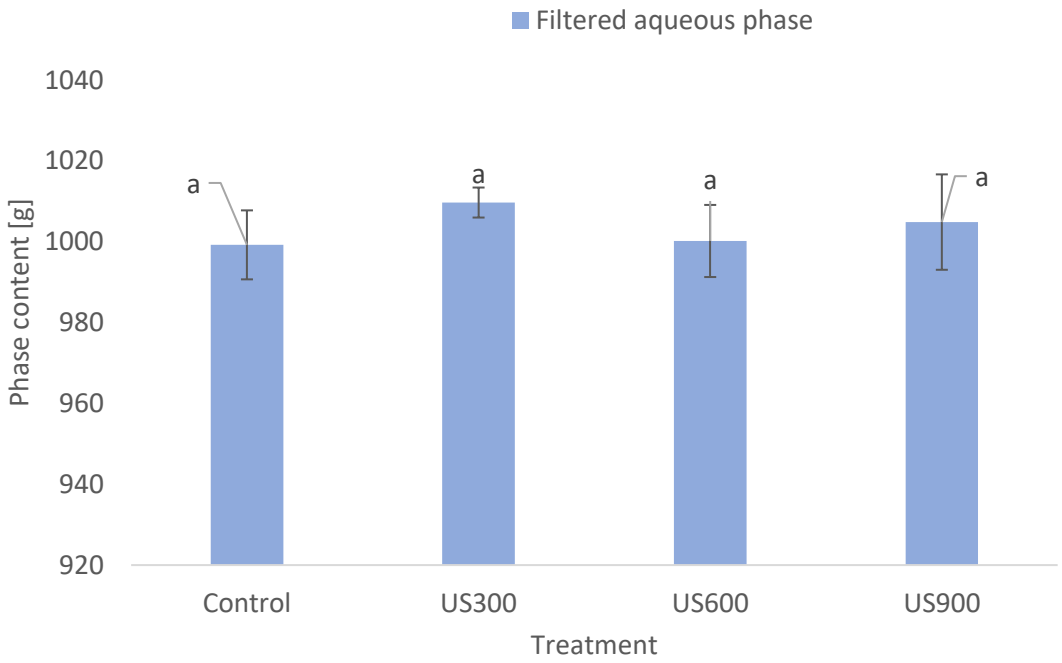

Figure 37: The figure presents the filtered aqueous phase/hydrolysate content for the control, US300, US600 and US900 samples. Significant differences among similar phases between different treatments are indicated by different lower-case letters.

Even though no significant differences for the multicomponent phase contents were found across the various US-treatments and control (Figure 38), US treatments displayed an enhancing effect in general. The multicomponent phase content seemed to increase with US300 and US600 treatments, as compared to the control, while decrease between the US600 and US900 treatments. However, the multicomponent content for US900 samples still presented seemingly similar values to that observed for US300 treatments. In addition to affect the multicomponent phase content, the lipid fraction obtained with the US900 treatment was significantly higher than that obtained with the US300 treatment, while the amount of multicomponent phase seemed to be similar. The US600 and control samples displayed lipid phase content statistically similar to the US900 and US300 samples.

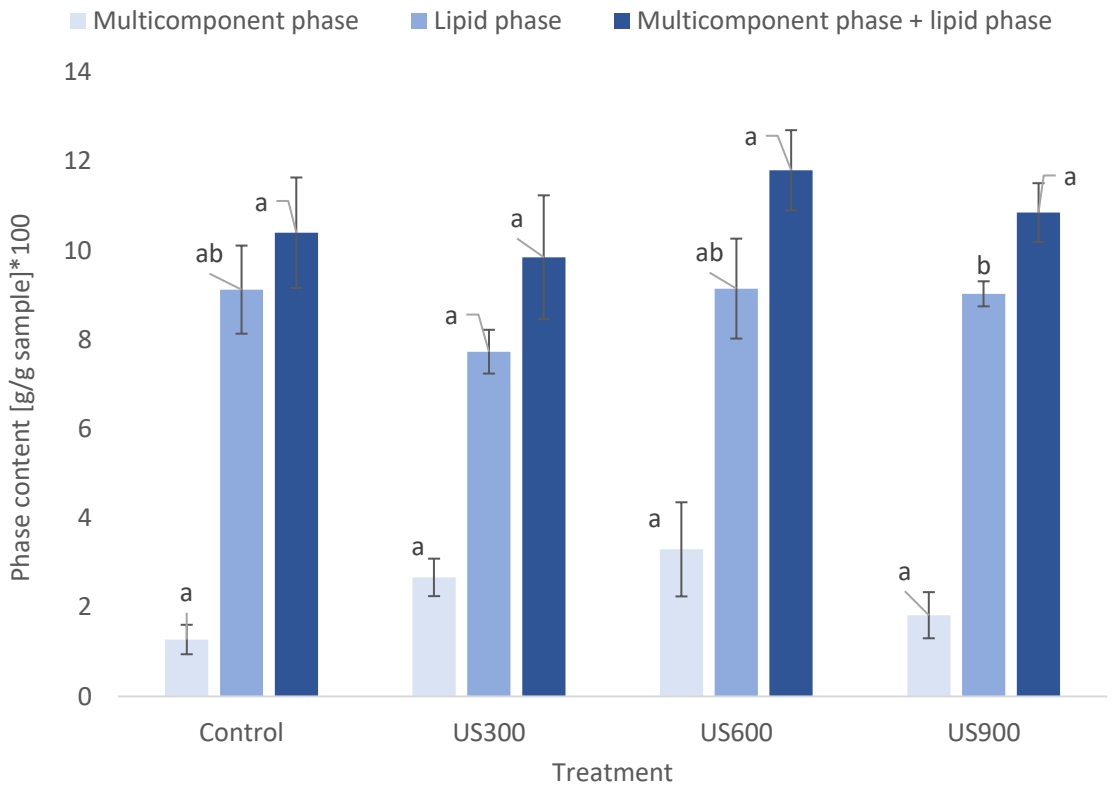

Figure 38: The figure presents the contents of the lipid and multicomponent phases in addition to the sum of these phases for the control, US300, US600 and US900 samples. Significant differences among similar phases between different treatments are indicated by different lower-case letters.

4.3.3 The DPPH radical scavenging activity observed in hydrolysates from samples pre-treated with US

The DPPH radical scavenging activity of hydrolysates from the different US pre-treatments and control is presented in Figure 39. For a protein concentration of 0.5, 1.0 and 2.0 mg/mL, there was a linear and significant increase in DPPH radical scavenging activity for increasing protein concentration (R^2>0.982). This was observed for all investigated treatments. There was no difference in DPPH radical scavenging activity for concentrations of 0.5, 1.0 or 2.0 mg/mL across the different treatments. However, when investigating concentrations above 2.0 mg/mL, a decrease in DPPH radical scavenging activity was observed. Increasing the peptide concentration to 4.0 mg/mL and 6.0 mg/mL caused significant reductions in DPPH radical scavenging for all the treatments as compared to 2.0 mg/mL for the same treatment. For the US300 treatment and a peptide concentration of 4.0 mg/mL, the DPPH radical scavenging was significantly lower than the corresponding ones for the control and US900, the latter showing the highest value. At a peptide concentration of 6.0 mg/mL, only the hydrolysates from the US900 treatments resulted in a detectable DPPH radical scavenging activity.

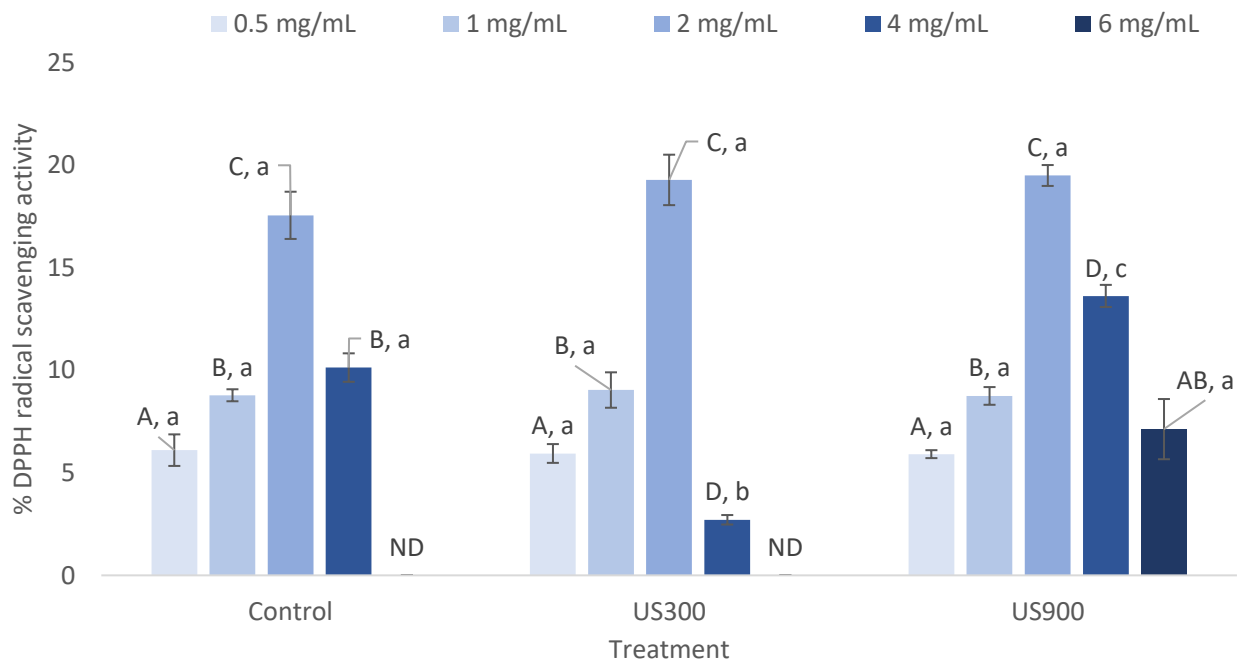

Figure 39: The figure presents the DPPH radical scavenging activity for the control, US300, US600 and US900 hydrolysates/filtered aqueous phases with a protein concentration of 0.5, 1, 2, 4 and 6 mg/mL. ND indicates that no DPPH radical scavenging activity was detected. Significant differences at similar conditions among different treatments are indicated by different lower-case letters. Significant differences at varying conditions within a certain treatment is indicated by different upper-case letters.

4.3.4 The relative protein solubility of hydrolysates from samples pre-treated with US

The relative protein solubility of the hydrolysates from the control, US300 and US900 treatments at pH 2, 4, 6 and 11 is illustrated in Figure 40. The relative protein solubility for the control was not found to vary significantly among pH 2, 4, 6 or 11. The relative peptide solubility of US300 hydrolysates was significantly higher at pH 11 as compared to pH 2. The relative solubility of US300 hydrolysates at pH 4 and 6 were statistically similar to each other but also to the relative peptide solubility for US300 at pH 2 and 11. The US900 hydrolysates displayed significantly higher relative protein solubility at pH 6 and 11 as compared to pH 2 and 4. However, there was no statistical difference between the relative solubility at pH 2 and 4.

In general, at pH 2 there was not significant differences in relative protein solubility among the various treatments. At pH 4, US300 displayed higher relative protein solubility as compared to US900 and the control, which were statistically similar. At pH 6 and 11 the relative protein solubility was significantly higher for US300 and US900 as compared to the control, while there were not significant differences among the investigated US treatments at these pH values.

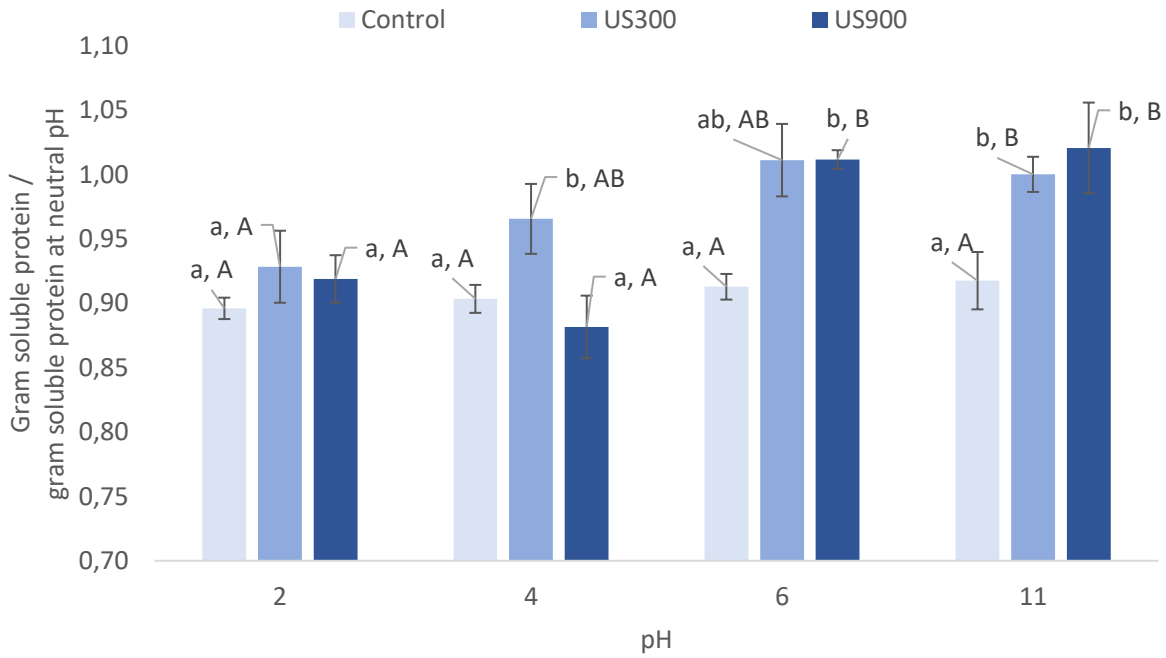

Figure 40: The figure presents the relative protein solubility at pH 2, 4, 6 and 11 for the hydrolysates/filtered aqueous phases from the control, US300, US600 and US900 samples. Significant differences at similar conditions among different treatments are indicated by different lower-case letters. Significant differences at varying conditions within a certain treatment is indicated by different upper-case letters.

4.3.5 Discussion of the US results

Applying US as a means of pre-treatment caused several changes in the course of protein solubilisation during the enzymatic hydrolysis. The protein concentration obtained after 0.5 and 5 min of hydrolysis gradually decreased when increasing the power of the US treatment. As mentioned in Section 1.3.4 could this be due to any of the many effects posed by ultrasound on the MDCR system. But as the inertial cavitational effect could seem to be a minor contributor in such systems the "push-pull" mechanism caused by the alternating positive/negative acoustic pressures from the US-wave could explain the slow protein solubilisation in the early stages of hydrolysis. Ricce, Rojas et al. (2016) investigated whether vacuum packing carrot slices (or not) during US treatment had any effect on subsequent drying and rehydration. Samples treated directly in liquid medium seemed to benefit from the surrounding water as it flushed disrupted structures from the biomatrix and thereby increased porosity, enhanced drying and accelerated rehydration in the trials that followed. Samples vacuum-packed did not seem to benefit from this effect and were even slower to rehydrate than the control samples. This observation could advocate for the pushing-pulling effect of US where structures of low stiffness (certain proteins or carbohydrates) in the biomatrix are influenced, gradually disrupted and flushed out by the pulsing water (Pieczywek, Kozioł et al. 2017). When observing the early development for the US900 samples, namely the protein concentrations after 0.5 and 5 min of hydrolysis, protein solubilisation seems to have halted when compared to the other treatments and the control. Thus, specific proteins within the MDCR could be influenced in similar ways as the pectin in the carrots of Ricce, Rojas et al. (2016), and if not removed continuously could a "clogging" effect occur and beneficial effects such as increased porosity would be absent (Wu, Du et al. 2011). This could explain the slow initial protein solubilisation as entangled/partially aggregated structures would be slower to dissolve, probably due to low enzyme accessibility. Meanwhile, in the present work, the US900 treatment also seemed to pose a negative effect on the protein concentration observed after 60 min of hydrolysis. US waves could potentially cause a combination of effects within the MDCR. First by posing a "push-pull" effect on complex components such as the myofibrillar structures and cause gradual fatigue and denaturation as suggested in section 1.3.4. The idea on gradual fatigue of certain structures by US was proposed by Miller (1985). Meanwhile, as bubbles and water seem essential for inertial cavitation, water soluble proteins such as sarcoplasmic or extracellular proteins could potentially be exposed to such mechanisms and thus aggregate at faster rates compared to the myofibrillar proteins, which only is exposed to the milder "push-pull" mechanism. Chang, Wang et al. (2015) treated vacuumed packed beef with US (40 kHz; 1500 W) and discovered shrunken sarcomeres, intracellular cavities and protein aggregates within the

extracellular space and the observations seemed to increase with treatment time (10-60 min). This could indicate that certain components required to maintain the elongated configuration of the sarcomere were partially disrupted while the proteins dissolved in aqueous environments were more prone to aggregation. Therefore, the work of Chang, Wang et al. (2015) does not reject the possibility of both inertial cavitation and push-pull mechanisms occurring simultaneously in such systems. Meanwhile, in the present work the US300 samples displayed two fast phases of protein solubilisation and did not experience the slow phases observed for the US900 samples. Also, the final protein concentration for the US300 samples were higher than compared to the control samples. One explanation is that the gradual increase of US power increases both the disruption of the sarcomeres and protein aggregation. A specific combination of these effects could introduce the differences in protein solubilisation observed for the US300 and US900 samples. The effect of sarcomere shrinkage could be due to denaturation of important structural proteins within or between the sarcomeres. Wang, Kang et al. (2018) treated *m. semitendinosus* with US (20 kHz; 5 ℃; 20-40 min; 25 W/cm^2) and observed accelerated degradation upon subsequent storage. This was attributed to better access for endogenous enzymes and could explain the significantly increased protein concentration observed for the US300 samples after 60 min of hydrolysis in the present work. The results of Wang, Kang et al. (2018) indicated increased degradation of the proteins desmin and troponin-T. Desmin is an important protein responsible for extending between the Z-disks in adjacent sarcomeres. Troponin is present in the thin filaments together with actin. Such findings could indicate that the "push-pull" mechanism is expressed between the sarcomeres through denaturation of proteins responsible to maintain their close association, in this case desmin. Kang, Gao et al. (2017) found myofibrillar fragmentation to increase with both US power and treatment time and desmin degradation was at a treatment time of 30 min heavily dependent on the power applied. Thus, desmin could be one of the proteins responsible for the altered protein solubilisation patterns observed for the various US treatments applied in the present work. This could potentially cause myofibrillar fragmentation which enhance enzymatic accessibility and increase the dissolved protein concentration as seen for the US300 samples after 60 min of hydrolysis. Further increasing power could potentially cause aggregation of protein species present in the sarcoplasm or extracellular space thereby reducing the benefits caused by the push-pull mechanism.

The combination of the US and thermal effect in the reactor could also have resulted in the slow protein solubilisation observed during the late stages of the US900 hydrolysis (30-60 min). The effect of temperature on relevant proteins is discussed in the MW discussion Section 4.2.5. Interestingly, what might have been the case for the US samples is aggregation of sarcoplasmic/extracellular

proteins through inertial cavitation in compartments with high aqueous and bubble content (where inertial cavitation might be favoured). However, the temperature applied in the present work could have increased such effects. The proteins present at such locations are reported to link other proteinaceous structures together (Tornberg 2005) and could contribute to the slow protein solubilisation phases observed for the US900 samples.

The significant increase in protein concentration observed for each treatment after enzyme inactivation/heat-treatment is believed to be due to similar causes as those discussed in Section 4.2.5 and will not be mentioned further in this Section.

Irrespective of the mechanism, the US pre-treatments induced structural alterations in the proteins within the raw material which upon subsequent enzymatic hydrolysis generated peptides capable of affecting the multicomponent phase content. This could be due to the formation of peptides able to interact favourably with both the polar and lipid phases. Proteolysis of myofibrillar proteins has been found to enhance the emulsifying properties as protein structures able to associate with both the aqueous and lipid phase are formed (Smith and Brekke 1985). While formation of emulsions often requires certain peptide size, it is suggested that smaller peptides could efficiently absorb to liquid-oil-interphases (Pacheco-Aguilar, Mazorra-Manzano et al. 2008). This could explain the observed trend in the present work where a gradual increase of US power changed the product peptide properties. First for the US600 samples, the peptides seemed able to stabilise lipids in the increased content of the multicomponent phase. But as power and denaturation progressed to that of the US900 samples, the product peptides no longer associated to structures in the multicomponent phase, and instead caused a significant increase in the lipid phase as observed for the US900 samples. Elevation in pure lipid content (higher content of pure lipids as compared to mixed phases) is often associated with increased DH% as the peptide size is reduced and the charge content per chain length is higher, but also because a higher content of lipids are released from the matrix (Kristinsson and Rasco 2000, Šližyte, Daukšas et al. 2005). The presence of peptides with protein-lipid associating properties seemed to be declining for the US900 samples compared to those of the US600 samples. However, the lipid content was significantly higher than that seen for US300 samples while the multicomponent phase content seemed relatively alike. This could suggest that more lipids were released from the solid phase upon enzymatic hydrolysis while the declining protein concentration could through aggregation have compensated for this, such that no significant difference was seen in solid or particulate phase content. Another possibility is that peptides containing significant hydrophobic domains could have associated to the lipid phase and resulted in the declining protein concentration seen after 60 min of hydrolysis for the US900 samples.

Applying US as a pre-treatment was found to both reduce and increase the DPPH radical scavenging activity relative the control. A suggestion for the reduction in the DPPH radical scavenging activity within each treatment/control as protein concentration was increased is given in Section 4.2.5 but will be repeated briefly for convenience. A high content of charged residues within a peptide is believed to interact unfavourably to the solvent applied (ethanol) during radical scavenging activity investigations, these groups are in general believed to present low DPPH radical scavenging activity and might exaggerate this effect by precipitating and absorbing light at the wavelength applied during the trials (Dávalos, Miguel et al. 2004, Cumby, Zhong et al. 2008, Yoshikawa, Hirano et al. 2012, Dissanayake, Ramchandran et al. 2013).

The significant reduction observed for the US300 samples (4 mg/mL) compared to that of the control could thus be due to the loss of radical scavenging groups to lipid-protein structures in the multicomponent phase. The significant increase observed for the US900 samples (4 and 6 mg/mL) compared to the control could be due to a higher content of hydrophobic peptides in the product aqueous phase. Some of which might be present in the lipid phase which was significantly increased as that seen for the US300 samples. These observations do not contradict the development of the protein concentration throughout the hydrolysis, and the reduction seen for the US900 samples at 60 min of hydrolysis could be due to peptide loss through the lipid phase or aggregation but seem to occur as hydrophobicity increase. But as mentioned by Bruno, Kudre et al. (2019), variations in lipid extraction and thus the lipid phase content is affected by the overall degradation of matrix structures. First, a high degree of hydrolysis and protein solubilisation would release a high content of lipids originally unextractable. Second, a high degree of hydrolysis and protein solubilisation would also reduce the formation of aggregates that might form upon prolonged high temperature hydrolysis or during enzyme inactivation which can entrap lipids. Third, a high degree of hydrolysis and protein solubilisation would indicate high charge content per chain length and therefore unfavourable interactions with the released lipid phase. The US300 samples seemed to increase the multicomponent phase as the lipid phase content decreased and there was seemingly no change in solid or particulate content which rule out that additional protein-aggregates containing lipids were present in these phases. However, there was a slight increase in the aqueous product as compared to the control, US600 and US900 samples. This could indicate the presence of lipid-peptide structures enriching this phase and thereby contributing to the reduction observed for the lipid phase content and similar structures might be responsible for what seem to be a slight increase in the multicomponent phase. One possibility is the disruption of structures which liberate phospholipids. Hydrolytically liberated phospholipids have been reported to associate with the protein containing phases and might therefore be present in the hydrolysate (Slizyte, Nguyen et al. 2004). However, when increasing the US power, the aqueous phase content seemed to drop from the levels observed

for the US300 samples to those similar to the control samples. This could indicate the presence of peptides favouring formation of protein-lipid structures allowing their presence in the multicomponent phase instead of the aqueous phase as US power was increased (to 600 W). This seemed to occur solely on the behalf of the aqueous phase content, suggesting altered peptide properties or an altered effect on lipid structures. However, for the US900 samples the multicomponent phase seemed similar to that of the US300 samples, while the lipid phase content was significantly higher than that of the US300 samples, this also seemingly on the account of the aqueous phase product. Which together with the significantly increased DPPH radical scavenging activity seen for the US900 samples (4 and 6 mg/mL) and significantly reduced protein concentration after 60 min of hydrolysis suggests that peptides containing groups interacting relatively favourable with lipids (and ethanol) might contribute to the enhanced lipid phase.

It might seem as a the content of hydrophobic peptides gradually increased as the power applied during US pre-treatment was increased. Zou, Xu et al. (2018) investigated how applying a US treatment (20 kHz; 20 min; T < 8 ℃) affected the properties of actomyosin. The content of near-surface or surface charges was found to increase when the treatment power was increased. However, this was only up to a certain threshold were the application of higher power levels reduced the surface charge content observed on the actomyosin molecules. The same trend was observed for surface hydrophobicity. It should be noted that both surface charge content and surface hydrophobicity was maintained at levels higher than that of the control (no US treatment) at the power level above those found to result in the peak of surface hydrophobicity and surface charge content. The same was seen for emulsion activity, while emulsion stability declined at every subsequent US power. Even though the investigations of Zou, Xu et al. (2018) were done on actomyosin in an aqueous suspension, the findings display similarities to the present work. To be noted is that no enzymatic hydrolysis was conducted subsequent the US treatment, however the groups present at the protein surfaces would be those (probably) available for an enzyme and thereby relevant. The effect of ultrasound on the actomyosin molecule seemed to cause denaturation which up to certain point elevates both charged and non-charged species, which would coincide with the gradually enhanced multiphase content seen for US300 and US600 samples in the present work. The surface hydrophobicity, charge content and emulsion activity, peaked at the same power setting and the subsequent power causes a drop in the mentioned properties (still presenting values higher than for the control), which present similarities to the trend seen between US600 and US900 in the present work. It must be noted that emulsion related properties were not investigated in the present work, but such structures could have contributed to enhanced multiphase contents.

Exactly how the results of Zou, Xu et al. (2018) would affect an subsequent hydrolysis would obviously depend on many factors but because varying (increasing then decreasing) levels of hydrophilic + hydrophobic groups were present at the surface of the protein complexes it could certainly explain the results obtained in the present work. In the present work, the thermal effect in the reactor could have caused a combined contribution which explain the product peptide properties of the US900 samples seemingly dominated by hydrophobic residues (interpreted through increase of DPPH radical scavenging and elevated lipid content). This as both the thermal and US effect seemingly promotes exposure of hydrophobic surfaces on the proteins present in the MDCR. Meanwhile, the US effect also seemed to promote exposure of hydrophilic groups which could contradict the negative thermal effect seen for samples treated with MW.

Enzyme accessibility could also be promoted by US. In fact, Zou, Zhang et al. (2018) treated actomyosin with US (20 kHz; 20 min) and found the treatment to immediately increase myofibrillar fragmentation. The content of free actin increased significantly 0 h after the treatment indicating dissociation of the actomyosin complex. The mentioned work was conducted with an actomyosin suspension, and thus the US mechanisms at play might differ from those displayed in the present work. Meanwhile, the fact that actin and myosin often are considered the richest sources of peptides displaying antioxidant and functional properties within raw materials such as that treated in the present work and the observed results of the lipid-associated phases and the DPPH radical scavenging activity might suggest that they are affected by the US pre-treatments (Smith and Brekke 1985, Udenigwe and Howard 2013, Borawska, Darewicz et al. 2016).

The relative protein solubility observed for the US300 and US900 samples at pH 4 suggest the presence of peptides with varying properties due to US power. In general, a higher DH% is associated with elevated protein solubility at various pH. This is due to the increased number of charges per chain length as peptide bonds are hydrolysed (Halim, Yusof et al. 2016), which seem to be the case for US300 and US900 hydrolysates at pH 6 and 11. However, the peculiar results of relative protein solubility against pH observed between the control, US300 and US900 suggest that some structures in the product not are affected by the pre-treatment, some depend on power while some seem independent on power. The similarities and differences in the protein solubility at various pH seen for US300, US900 and control does not necessarily originate from the same peptides. However, the results could indicate that US affect specific proteins within the raw material. Inertial cavitation, producing significant chemical and mechanical effects is reported to affect the main components within the present raw material, as discussed in Section 1.3.4. However, the seemingly specific effect seen in the present work could advocate for the push-pull effect mentioned in the work of Ricce,

Rojas et al. (2016). In the mentioned work the elastic component (in that specific case pectin) become particularly vulnerable to the continued back-forth oscillation posed by US and eventually dissolved when in direct contact with water. The push-pull mechanism could through a gradual fatigue of protein structure cause denaturing in the raw material, while other structures are spared. Wang, Yang et al. (2017) observed this type of specificity when treating actomyosin with ultrasound. Over a certain treatment period myosin seemed to exhibit slight denaturation while the structure of actin completely disappeared. This, together with the effect on desmin and troponin as mentioned by Kang, Gao et al. (2017) and Wang, Kang et al. (2018), the changes in sarcomere structure mentioned by Chang, Wang et al. (2015), suggest that such specificity could be due to an push-pull mechanism. This while proteins present in aqueous dominated environments could be susceptible for structural alteration through inertial cavitation.

Kangsanant, Murkovic et al. (2014) and Misir and Koral (2019) are among few who have investigated the potential of US as a pre-treatment or a simultaneous treatment for enzymatic hydrolysis of substrates dominated by muscle proteins. Kangsanant, Murkovic et al. (2014) produced fish (fillet) protein hydrolysate through enzymatic hydrolysis (55 ℃; 1 h) after an ultrasound pre-treatment (10 or 70 W; 15, 30 or 45 min). The pre-treated fish homogenate resulted in a lower DH% when compared to the control. Kangsanant, Murkovic et al. (2014) mention that the pre-treated homogenate appeared more viscous than the non-treated homogenate. This indicates protein-protein interaction prior to the enzymatic hydrolysis, possibly through hydrophobic interactions. Exposure of hydrophobic groups is indicated in the present work through enhanced DPPH radical scavenging activity. In fact, both powers applied by Kangsanant, Murkovic et al. (2014) increased the DPPH radical scavenging activity. However, the DH% was reduced which indicate the presence of fewer cleavage sites probably due to aggregation which also the viscous pre-treated homogenate suggests. Meanwhile there were similarities between the present work and the work of Kangsanant, Murkovic et al. (2014), especially when observing the results for the US900 samples, even though aggregation only seem slight after 60 min of hydrolysis.

Misir and Koral (2019) investigated the use of US during (assisted) (60 ℃; 1 h; US power or frequency not specified) enzymatic hydrolysis of rainbow trout by-products with Alcalase. However, as Alcalase has been displayed to exhibit increased catalytic activity in the presence of US, it is difficult to state the effect of US on the raw material (Ma, Huang et al. 2011). Meanwhile, Misir and Koral (2019) presented yields seemingly increased by US-assisted hydrolysis as compared to the control. However, no significant change (except for valine) was observed in the amino acid composition between conventional hydrolysis and US-assisted hydrolysis suggesting minor effect on the structure of the raw material, and the observed effect could be due either to enhanced Alcalase activity or increased

substrate accessibility within the matrix, this without causing any significant denaturation. The work by Misir and Koral (2019) therefore seem to present results which carry similarities to those found during the US300 treatment in the present work, where there seem to be a slight increase in dissolved proteins after 60 min of the enzymatic hydrolysis. In fact Misir and Koral (2019) observed increased DH between 30 and 60 min of hydrolysis when applying US during the proteolysis. The US-assisted hydrolysis further generated a hydrolysate which significantly increased both oil-binding and foaming capacity, which even though not measured in the present work, could draw lines toward the observed contents of multicomponent and lipid phases in the present work.

4.4 The results and discussion on the effect of pre-treating samples with high-pressure processing (HPP)

4.4.1 Protein solubilisation during the enzymatic hydrolysis after HPP pre-treatments

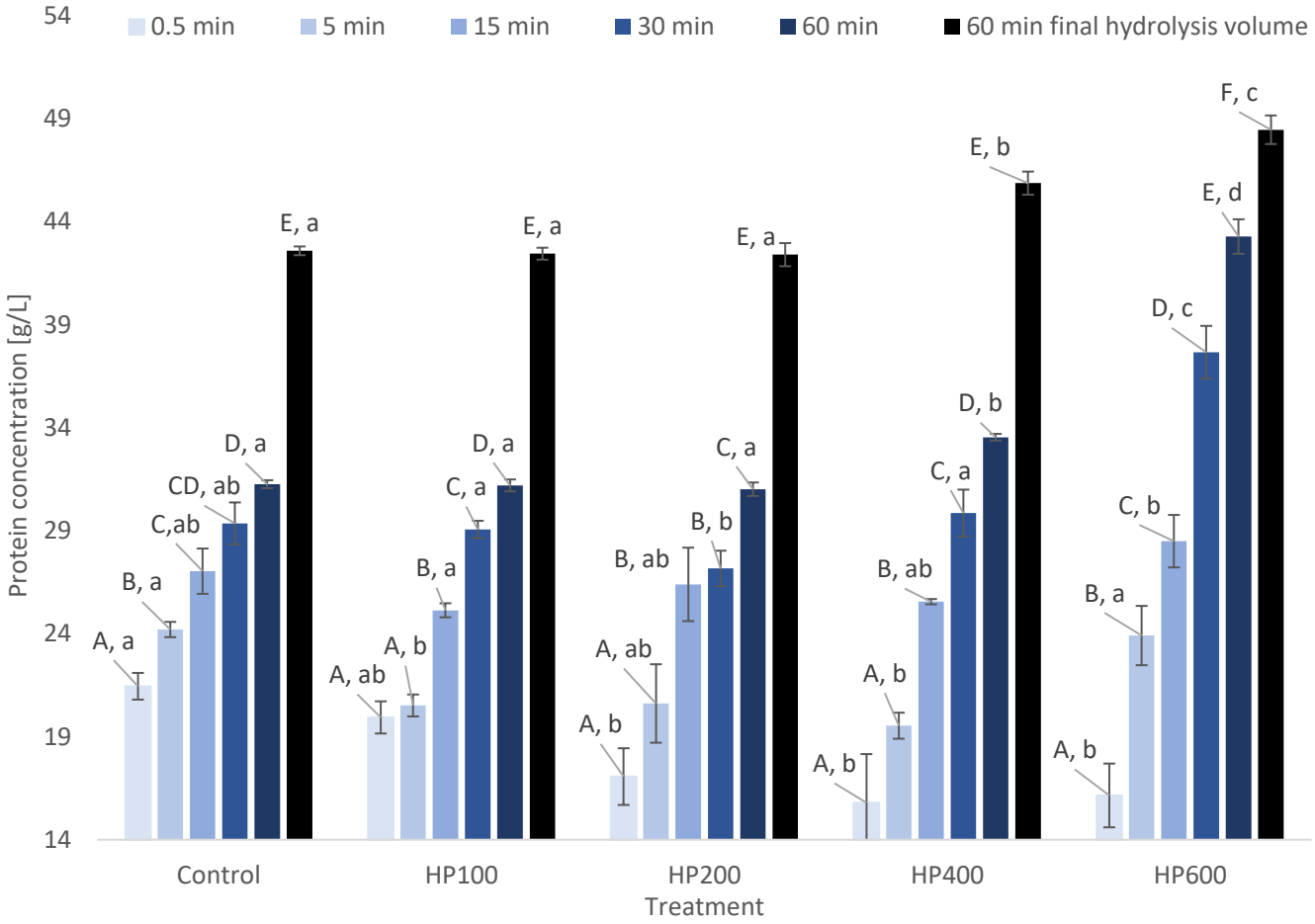

Figure 41: The figure presents the protein concentration at 0.5, 5, 15, 30 and 60 min of hydrolysis for the control, HP100, HP200, HP400 and HP600 samples. In addition, the protein concentration observed after enzyme inactivation of the final hydrolysis volume is presented for the mentioned samples. Significant differences at similar conditions among different treatments are indicated by different lower-case letters. Significant differences at varying conditions within a certain treatment is indicated by different upper-case letters.

The protein concentration in the control and HPP (100, 200, 400 and 600 MPa; HP100, HP200, HP400 and HP600; 15 min) pre-treated samples at various points during enzymatic hydrolysis is presented in Figure 41. Applying a pressure of 100 MPa (HP100) during HPP pre-treatment of the MDCR samples caused the soluble peptide content measured at 0.5 min of hydrolysis to drop but not significantly as compared to the control. Further increasing the pre-treatment pressure to 200, 400 and 600 MPa

(HP200, HP400 and HP600) caused the initial soluble peptide content to significantly decrease as compared to the control, which indicates structural alterations of the proteins within the material. The general trend observed after 5 min of hydrolysis seems to be a reduction in the content of dissolved peptides for all HP treatments apart from HP600, which not was significantly different from the control despite the low peptide solubility observed after 0.5 min of hydrolysis. The protein concentration in HP200 was not significantly different to that in other treatments after 5 min of hydrolysis, while HP100 and HP400 displayed significantly lower protein concentrations when compared to the levels in the control. After 15 min of hydrolysis the only significant difference among treatments was observed for the higher protein concentration in HP600 samples as compared to that within HP100 samples, while all other treatments were statistically similar to these contents. When the hydrolysis time was further increased to 30 and 60 min, increased pressure during pre-treatment seem to benefit the protein extraction. The HP400 samples displayed significantly higher content of solubilised proteins as compared to the control and lower pressures but presented significantly lower protein content than that of the HP600 samples. This was also observed after 30 and 60 min of hydrolysis when these treatments were compared, but also for the 60 min final hydrolysis volume samples among the various treatments. Within HP600 samples there was a significant increase in solubilised peptides at each point of hydrolysis but also between the 60 min and 60 min final hydrolysis volume samples indicating the release of soluble proteins during enzyme inactivation. HP400 displayed a similar trend to that observed for HP600 except for the 0.5 and 5 min hydrolysis points having similar protein concentrations, indicating a slow initial rate of hydrolysis. Reducing the pre-treatment pressure to 200 MPa reduced the final protein concentrations seen for both the 60 min and 60 min final hydrolysis volume samples to levels similar that of HP100 and the control, but also reduced the significant difference between the 15 and 30 min of hydrolysis peptide content. Reducing the pre-treatment pressure further to 100 MPa created a protein concentration pattern similar to that of the control, except for the 0.5 and 5 min samples displaying statistically similar protein concentrations.

4.4.2 Phase contents obtained after enzyme inactivation of the final hydrolysis volume from samples pre-treated with HPP

The particulate and solid phase content, the filtered aqueous phase content, the multicomponent and lipid phase content for the various HPP samples and the control is presented in Figure 42, Figure 43 and Figure 44, respectively. There were no significant changes in particulate or solid phase content as the pressure applied during the pre-treatment was altered.

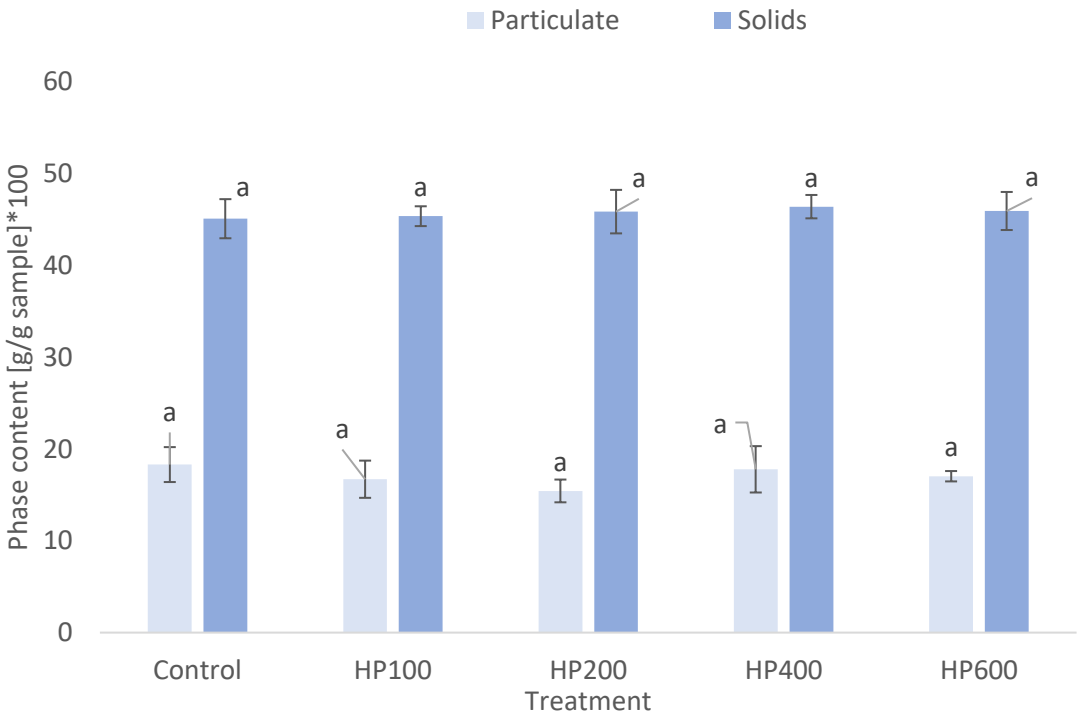

Figure 42: The figure presents the particulate and solid phase contents from the control, HP100, HP200, HP400 and HP600 samples. Significant differences of similar phases among different treatments are indicated by different lower-case letters.

The filtered aqueous product / hydrolysate was significantly reduced when hydrolysis was conducted after applying pre-treatment pressures above 200 MPa when compared to the control and HP200. HP100 displayed no significant difference in aqueous phase content as compared to the other treatments.

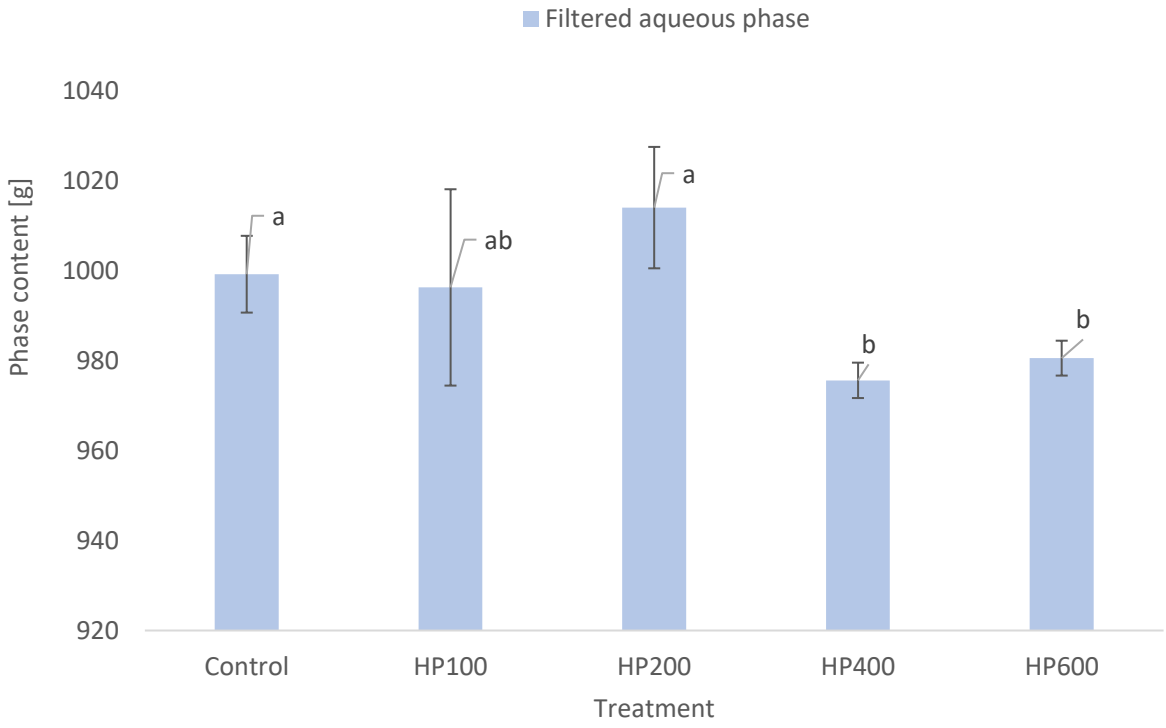

Figure 43: The figure presents the filtered aqueous phase/hydrolysate content for the control, HP100, HP200, HP400 and HP600 trials. Significant differences of similar phases among different treatments are indicated by different lower-case letters.

The multicomponent phase content was significantly lower for HP600 when compared to HP400 and the control, but not different to the measured content of HP200 or HP100. This could indicate that lower pressures during pre-treatment, namely HP100 or HP200, could promote multicomponent phase formation when compared to the control, HP400 and especially HP600. Meanwhile the multicomponent phase contents measured for HP100 and HP200 were not significantly different that of HP600, HP400 and the control. The lipid fractions for HP400 and HP600 were similar but significantly greater than that of HP200, HP100 and the control. The lipid content of HP200 was significantly lower compared to that of the content of the control while the content from HP100 was statistically similar to both. The lipid phase + multicomponent phase content varied between certain treatments. HP400 presented a lipid phase + multicomponent phase content significantly higher than that of HP200 and the control but was statistically similar the content in HP100 and HP600. Besides, there was no statistical difference in the lipid phase + multicomponent phase content among the various treatment.

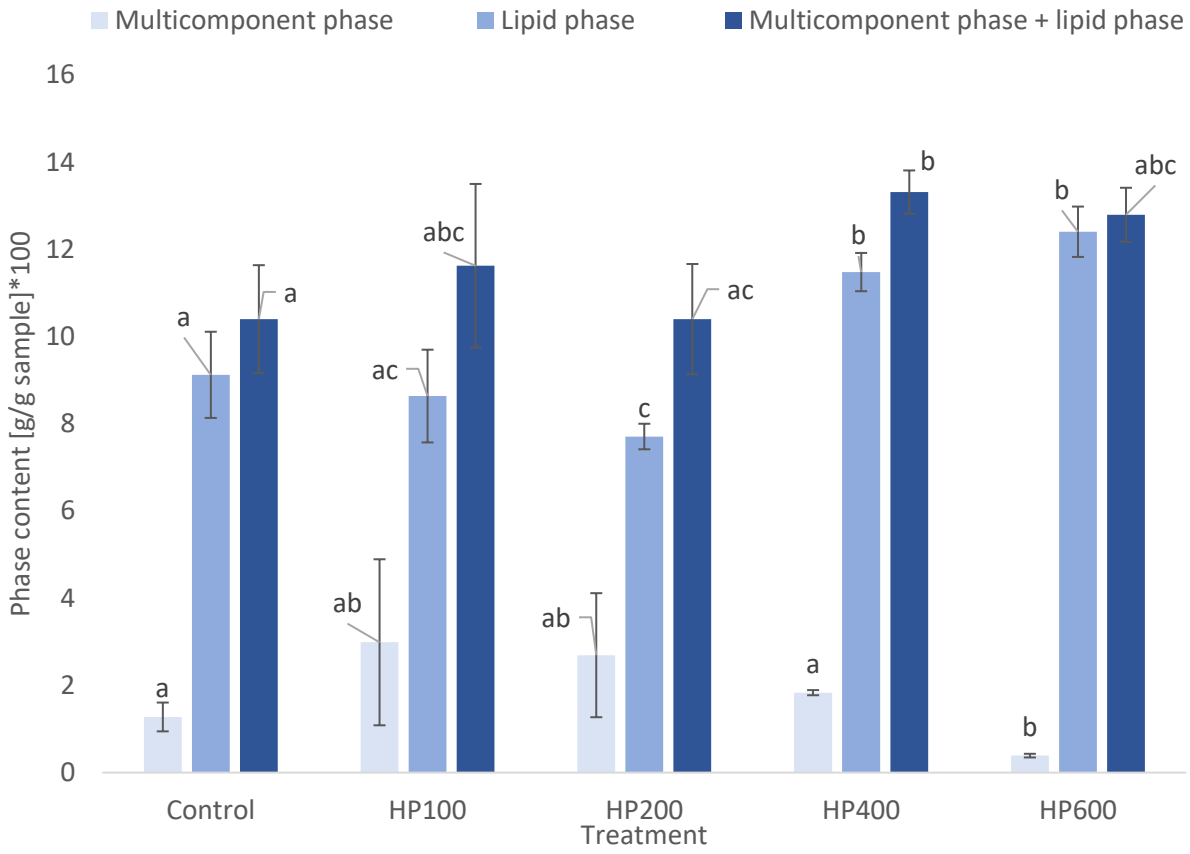

Figure 44: The figure presents the contents of the lipid and multicomponent phases in addition to the sum of these phases for the control, HP100, HP200, HP400 and HP600 samples. Significant differences of similar phases among different treatments are indicated by different lower-case letters.

4.4.3 The DPPH radical scavenging activity observed in hydrolysates from samples pre-treated with HPP

The DPPH radical scavenging activity of the hydrolysates from the control, HP100, HP200 and HP600 with protein concentrations 0.5, 1, 2 and 4 mg/mL is presented in Figure 45. The DPPH radical scavenging activity for the control and HP100 was statistically similar at all concentrations. Changes were observed at certain peptide concentrations when pre-treatment pressure was increased further. Hydrolysates from HP200 and HP600 with concentrations 0.5 and 1 mg/mL displayed statistically similar scavenging activity as that measured in the hydrolysate of the control and HP100. However, the scavenging activity measured for the HP200 hydrolysate with a peptide concentration of 2 mg/mL was significantly lower than that seen for HP100 and control hydrolysates at the same concentration. When increasing the hydrolysate concentration to 4 mg/mL a significantly higher scavenging activity was observed for HP200 hydrolysates when compared to the control and HP100 at the same concentration. HP600 generated a hydrolysate where protein concentrations of 0.5 and 2 mg/mL yielded statistically similar DPPH radical scavenging. This scavenging activity was the lowest measured at a protein concentration of 2 mg/mL for any hydrolysate.

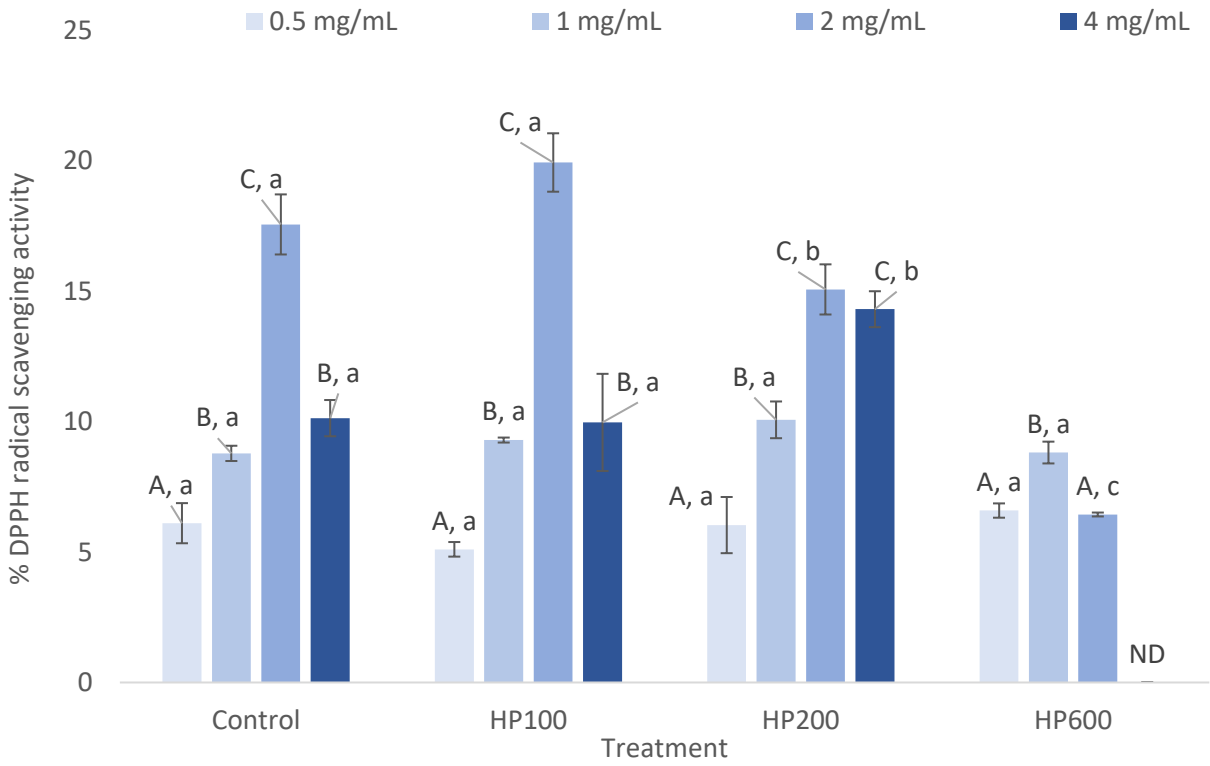

Figure 45: The figure presents the DPPH radical scavenging activity for the control, HP100, HP200 and HP600 hydrolysates/filtered aqueous phases with protein concentration 0.5, 1, 2 and 4 mg/mL. Significant differences at similar conditions among different treatments are indicated by different lower-case letters. Significant differences at varying conditions within a certain treatment is indicated by different upper-case letters.

4.4.4 The relative protein solubility of hydrolysates from samples pre-treated with HPP

The relative protein solubility of the hydrolysates from the control, HP200 and HP600 at pH 2, 4, 6 and 11 is presented in Figure 46. The relative protein solubility of peptides originating from a hydrolysis where the MDCR was pre-treated with either HP200 or HP600 was significantly higher than that of the control over all pH investigated. In addition, the relative protein solubility of HP200 samples was significantly greater than that of HP600 samples at pH 6. Else at pH 2, 4 or 11 there was no significant difference between the HP200 or HP600 samples.

For HP200 the relative protein solubility was significantly higher at pH 11 and 6 as compared to the solubility measured at pH 2 and 4. At these two pH pairs there was no statistical difference in protein solubility. The relative solubility for HP600 hydrolysates was significantly higher at pH 11 as compared to the relative solubility at other pHs. Furthermore, hydrolysates from HP600 exhibited a solubility which was significantly higher at pH 6 relative to pH 4, but both were statistically similar to the relative solubility measured at pH 2.

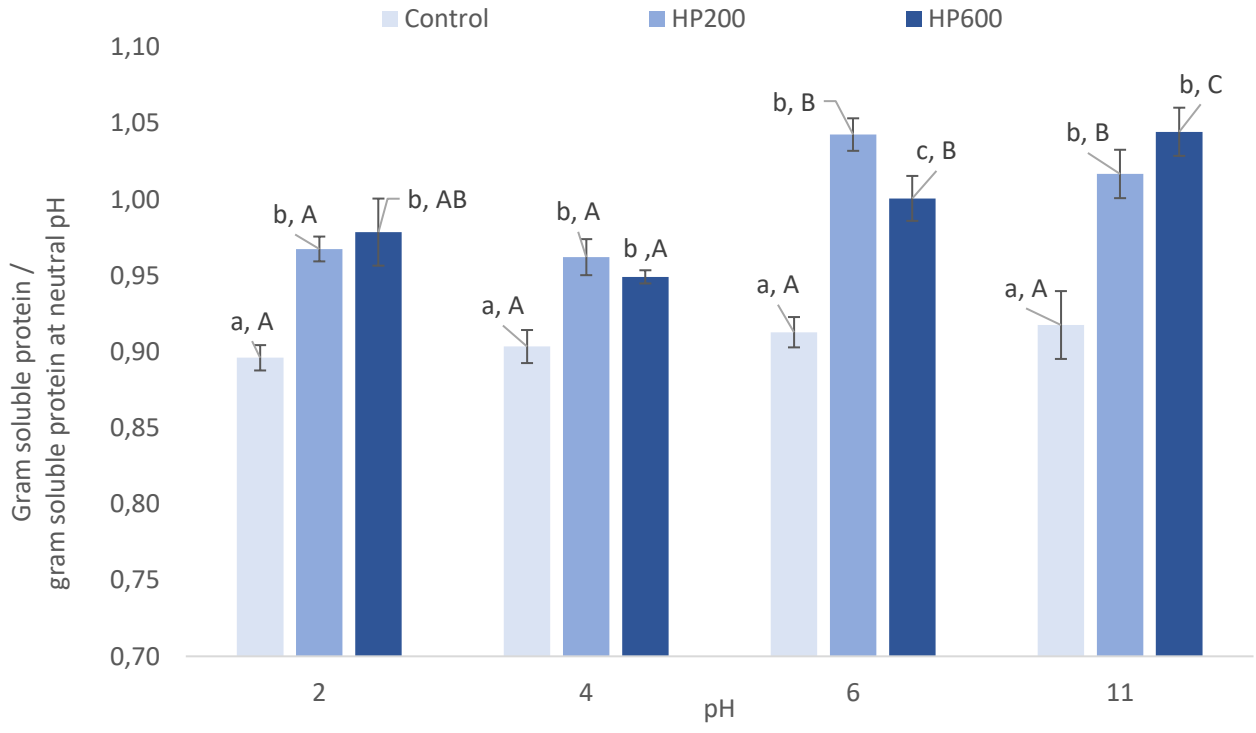

Figure 46: The figure presents the relative protein solubility at pH 2, 4, 6 and 11 for the hydrolysates/filtered aqueous phases from the control, HP200 and HP600 samples. Significant differences at similar conditions among different treatments are indicated by different lower-case letters. Significant differences at varying conditions within a certain treatment is indicated by different upper-case letters.

4.4.5 Discussion of the HPP results

The increased protein concentrations observed at the end of the HP400 and HP600 hydrolyses indicates that the protein structures were modified such that certain peptide cleavage sites were available in a way that when acted upon by the enzymes, a higher mass of peptides were released, either through a higher number of peptides or heavier peptides. Another possibility is that the biomatrix structure was altered, such that bonds not available in the control or upon treatment by US or MW for that matter, were made accessible for the enzymes after the HP treatment. This could occur through disruption of muscular structures upon HPP, enhancing enzyme access within the matrix. Worth mentioning is that two opposing effects occurred during the pre-treatment and hydrolysis. HPP is known to contradict the thermal effect seen on compounds such as proteins through volume reduction, while thermal fluctuations increase volume (Fernandez-Martin 2007). Therefore, as the material was treated during HPP certain structural alteration would occur through reduced compound and protein volumes. The structures would upon exposure to the temperatures in the reactor initially offer a certain number of enzyme cleavage sites which were acted upon. However, the compressed proteins would after a certain time start to express the thermal effect from the reactor environment thus exposing new cleavage sites for the enzyme. While some proteins in the HP treated raw material could have been insoluble after the pre-treatment, the thermal effect could “revive” their interaction with water and thereby enable proteolysis and peptide release into the aqueous phase. In such a way, the number of enzymatic cleavage sites every protein exposed to the polar environment and the enzyme, could increase because of the duality of effects brought by HPP and the reactor temperature.

While the duality of effects could have caused the overall protein extraction to increase, there are other possibilities for the observed increase in the final protein concentration as seen for HP400 and HP600. The peptides dissolved and enriching the aqueous phase for HP400 and HP600 must originate from a reduction in one of the other phases. However, as seen in Figure 42 there were no significant reductions in the solid or particulate phase contents. However, there were both reduced and elevated phase contents among the multicomponent, lipid and filtered aqueous phases indicating that the altered extraction yield could originate from these, maybe as new interactions between components were brought by HPP. The HP100 and HP200 samples exhibited gradually reduction in initial/0.5 min protein concentrations during hydrolysis and thus seem to offer alterations in protein confirmation which could benefit the formation of peptides able to form structures associated with the multicomponent phase. At the same time the lipid fraction gradually decreased indicating a possible entrapment of lipids in the multicomponent phase. Smith and Brekke (1985) found partially

proteolysis of myofibrils to generate peptides able to interact with both lipid and aqueous phases. Thus, potentially could peptides have been formed which associate with lipids and thereby increased the multicomponent phase, as observed for both HP100 and HP200 samples. However, this might also be due to less lipids actually released from the matrix which would explain lower content of lipids as these were entrapped in protein structures not affected by the enzymes, as mentioned by Bruno, Kudre et al. (2019).

The results obtained for HP400 and HP600 suggest something not seen before in the present work. Especially should it be noted that while the multicomponent phase content of HP400 samples was statistically similar to that of the control, the lipid phase content was significantly higher than that of the control. This could suggest a lipid phase enriched with hydrophobic peptides. While formation of emulsions often requires certain peptide size, it is suggested that smaller peptides might form upon higher DH% and could efficiently absorb to liquid-oil-interphases if containing certain amino acids (Pacheco-Aguilar, Mazorra-Manzano et al. 2008). Such peptides might have accompanied the lipid phase of HP400. But because there in addition was an increased protein concentration after 60 min of hydrolysis, it suggests that both lipids and proteins suddenly were present at levels higher than for HP100, HP200 and the control samples. The increase in lipid phase and final protein concentration seen for pressures above 200 MPa were accompanied by a significant reduction of aqueous product phase. This suggests that the structures responsible for the elevated lipid and dissolved protein contents originated from this phase and because it seemingly elevated lipid and protein levels, it is believed to be protein-lipid structures. These structures could be disrupted by the two highest pressures applied during pre-treatments and could possibly dissociate further during the enzyme inactivation. However, as this release of lipid and proteins was not observed through an elevated lipid fraction or an elevated 60 min final hydrolysis volume peptide concentration for the control, HP100 or HP200, it seems certain that pressure-induced destabilization was required to separate the components within these complexes. However, because the difference between the 60 min and 60 min final hydrolysis volume protein concentrations seemed to decrease upon elevated pressures applied during the pre-treatment, this could indicate that gradually less proteins were solubilised during the enzymatic inactivation and because collagen is reportedly unaffected by high pressure treatments (Gekko and Koga 1983), suggestions are that the proteins released during enzymatic inactivation were species affected by pressure, thus not of a collagenous type as indicated earlier in the present work. Chapleau, Mangavel et al. (2004) investigated how high-pressure treatments (10 min; 100,200, 300, 400, 500 and 600 MPa; 20 °C) affected the structure of myofibrils. Interestingly, two groups of proteins were affected by increasing pressure levels. Applying 100 MPa increased the size of both the two protein structures compared to the control (one originally large and one originally small structure), further increasing the pressure (200 MPa) decreased the size of the

originally largest protein structure until it disappeared for pressures above 200 MPa. The originally smallest structure displayed a steady increase in size as pressure was increased from 0.1-600 MPa. The disappearance of the largest structure mentioned by Chapleau, Mangavel et al. (2004) might be due to disruption of similar structures as those seemingly affected in the present work which thus would seem to be myofibrillar structures possibly containing entrapped lipids. Meanwhile, the smallest structure which gradually displayed an increasing size in the work of Chapleau, Mangavel et al. (2004) could be the structure mentioned by Hsu, Hwang et al. (2007) and Ma and Ledward (2004). Namely a heat labile structure (transition temperature close to 50 ℃) seemingly formed by aggregated myosin and actin through S-S and hydrogen bonds and not hydrophobic interactions.

Applying HP as a pre-treatment was found to both reduce and increase the DPPH radical scavenging activity relative to the control. A suggestion for the reduction in the DPPH radical scavenging activity within each treatment/control as protein concentration increased is given in Section 4.2.5 but will be repeated briefly for convenience. A high content of charged residues within a peptide is believed to interact unfavourably to the solvent applied (ethanol) during radical scavenging activity investigations, these groups are in general believed to present low DPPH radical scavenging activity and might exaggerate this effect by precipitating and absorbing light at the wavelength applied during the trials (Dávalos, Miguel et al. 2004, Dissanayake, Ramchandran et al. 2013) (Cumby, Zhong et al. 2008, Yoshikawa, Hirano et al. 2012). First, HP100 seemed to enhance the association between lipid and peptides. However, this might be due to the formation of protein structures entrapping lipids as an increased content of the large myofibrillar derivates was reported by Chapleau, Mangavel et al. (2004) when treating myofibrils with 100 MPa. The size of the mentioned structure seemed to gradually decrease when exposed to pressures above 100 MPa and disappeared when exposed to pressures higher than 200 MPa. However, such structures might explain the increased multicomponent phase content, at least for the HP100 samples. The course of protein solubilisation throughout the hydrolysis for HP100 exhibited variations from the control at 0.5 and 5 min of hydrolysis, however there were no differences at later stages of the hydrolysis. This indicates slight changes in parental protein structures. These structural alterations were not accompanied by any changes in the DPPH radical scavenging activity as compared to the control. The absence of effect on DPPH radical scavenging activity could be as the peptides present in the aqueous product of HP100 were similar to those present in the control. When the pre-treatment pressure was elevated further to 200 MPa, there was observed significant changes in the course of protein solubilisation during the enzymatic hydrolysis, suggesting further parental protein denaturation. While there were no changes in the solid contents, there seemingly was for the other phases. First to note is that the lipid phase seemingly decreased, suggesting that pure lipids could associate with the multicomponent phase.

However, there was no change in the multicomponent phase content as compared to what was observed for the HP100 samples. This could partially be explained if less proteins were needed to stabilize the certain amount of lipids within the multicomponent phase, but also due to what seemed to be an increase in the aqueous product content. As already mentioned, when applying pressures above 200 MPa during the pre-treatment, protein-lipid structures seemed to be destabilised and fractionated. However, for HP200 the opposite seemed to occur. An explanation for the observed increases in aqueous phase contents could be disruption of cellular membranes. Mammalian cell membranes are comprised of a double layer mainly composed of phospholipids and cholesterol (Spector and Yorek 1985). Upon hydrolysis of similar raw materials formation of emulsions has for example been associated to the presence of triglycerides while phospholipids mainly are located in the protein containing fractions (Slizyte, Nguyen et al. 2004). Thus, if the pre-treatment were to disrupt such structures, could the liberation of phospholipids contribute to the increase of lipid species present in the hydrolysate and thereby explain the increased content of aqueous phase for HP200 samples. Meanwhile, at higher pressures interactions might occur which favour the presence of phospholipids within either the multicomponent or the lipid phase. This as the aqueous phase decreases. But as mentioned, this decrease could be due to the disruption of such interactions already present irrespective of the pre-treatment. It would seem as such structures were present within the aqueous phase of the control, which was demonstrated by the HP600 samples (increase in protein concentration and lipid content on behalf of aqueous phase content), and it seems likely that certain pre-treatment conditions (HP200) could promote further formation of these.

The peptides able to form such associations could be responsible for the peculiar DPPH radical scavenging activity seen for HP200, where radical scavenging both increased and decreased significantly at certain protein concentrations as compared to the control. Such behaviour could be explained by peptides relatively unstable in ethanol while simultaneously displaying relatively high DPPH radical scavenging activity. More precisely, the peptides could display both hydrophilic and hydrophobic properties. This could also explain what seemed to be properties enabling firm association with lipid components as observed in the reduced lipid content for HP200, stable multicomponent phase and seemingly an elevated aqueous product. Xue, Xu et al. (2018) treated myofibrils with high pressure (9 min; 200 MPa; 25 ℃) and found the treatment to increase protein solubility, surface hydrophobicity and reactive -SH content. A parental protein presenting such properties would possibly enable formation of peptides which in fact could interact with both the aqueous and lipid phase and could therefore explain the results observed in the present work for HP200 samples. However, when the pre-treatment pressure was increased to 600 MPa in the present work, these properties seemed absent. For the HP600 samples the solubilisation during the hydrolysis occurred efficiently (significantly increased) between each time step, which could indicate

a high presence of low molecular weight species which easily were solubilised by the enzymes. This would further allow the formation of peptides with a high content of charges per peptide chain length. The DPPH radical scavenging activity for HP600 supports the presence of peptides of a highly hydrophilic nature, as unfavourable interaction with the solvent seemed to occur and the DPPH radical scavenging was low.

Both the HP200 and HP600 samples presented significantly increased relative protein solubilities over the investigated pH interval when compared to the control samples. Regarding the significant difference of the solubility observed at pH 6 between HP200 and HP600 could this originate from peptides released from the protein-lipid structure seemingly dissociated by pressures higher than 200 MPa. This could introduce species to the HP600 hydrolysate which exhibit lower peptide solubility at pH 6. The fact that both HP200 and HP600 hydrolysates contained peptides with relatively high solubility at pH 2, 4, 6 and 11 as compared to the control samples is probably due to structural alterations by the pre-treatment exposing more cleavage sites and thus increasing DH%. Elevated DH% has been associated with elevated peptide solubility (Halim, Yusof et al. 2016). For the HP600 samples, the hydrolysates seemed to exhibit relatively similar properties to that obtained for the HP200 samples, which might suggest that the increased pressures merely enhance accessibility within the matrix to similar cleavage sites. However, when observing the DPPH radical scavenging activity or the ability to form multicomponent phases, it would seem to not be the case.
These variations could be due to the presence of different peptides in the respective hydrolysates. Possibly from the protein structures apparently disrupted by pressures above 200 MPa, or from further denaturing of the same parental proteins, or a combination of both these factors.
Jung, de Lamballerie-Anton et al. (2000) presented results were high-pressure treated myofibrils displayed significantly enhanced solubility when exposed to pressures of 300 MPa and higher, which coincides with the size reduction and eventually disappearance of large myofibrillar structures observed by Chapleau, Mangavel et al. (2004). This enhanced solubility seemed to originate especially from low molecular weight species namely myosin light chains. This together with the gradual increase of low molecular weight species generated from sarcoplasmic proteins upon increasing treatment pressure, as mentioned by Villamonte, Pottier et al. (2016), could contribute to the effective protein solubilisation observed in the present work, for HP400 and HP600 samples. This would introduce species which originally displayed low molecular weight and upon enzymatic hydrolysis would yield short chained peptides, thus high solubility across pH and low DPPH radical scavenging activity due to high charge content and unfavourable ethanol interaction. In addition, the significant disruption of myofibrillar structures observed by Jung, de Lamballerie-Anton et al. (2000)

could increase the extraction of such species from the earlier enclosed organelles. Such disruption could also explain the increase in lipid content as these structures could hold entrapped oil. Meanwhile, the disappearance of the large myofibrillar protein structures mentioned by Chapleau, Mangavel et al. (2004) was accompanied by the gradual size increase of another. Investigations done by Hsu, Hwang et al. (2007) discovered that high pressure treatments above a certain threshold altered the native actomyosin structure and formed aggregates seemingly stabilised by S-S bonds. These complexes appeared to present structures which, even though aggregated, were scattered/spread and could possibly allow increased enzyme accessibility. Jung, de Lamballerie-Anton et al. (2000) treated meat in vacuum-sealed bags and exposed the samples to high pressure (260 s; 0.1-600 MPa; 10 ℃). Pressures above 130 MPa significantly increased the surface area of the myofibrils, which could correspond to the state of the scattered aggregates mentioned by Hsu, Hwang et al. (2007). Angsupanich, Edde et al. (1999) reported that aggregated structures of myofibrils were stabilised by hydrogen and S-S bonds, and seemingly heat labile compared to the native thermal stability of the respective constituents. This was in accordance with the work of Ma and Ledward (2004), who treated beef with high pressure (0.1-800 MPa; 20-70 ℃; 20 min) and found the actin and myosin structure modified at 200 MPa (20 ℃) and the actin structure gone after treatment at 400 MPa (20 ℃). In the work of Ma and Ledward (2004) a new structure/aggregate was reported to occur after treatment at 200 MPa (20 ℃) with thermal transition temperature of 50.1 ℃. The same was seen in pure myofibrils where actin and myosin were exposed to 400 MPa which seemed to fully alter their native structure into this new complex/aggregate, which further was stable up to 800 MPa. The gradual introduction of such an aggregate which seemingly present a structure with increased surface area could potentially benefit a subsequent hydrolysis. In addition, it would seem as aggregation occur through S-S and hydrogen bonds and not hydrophobic interactions might benefit a subsequent hydrolysis. This gradual alteration of the major MDCR constituents could potentially explain the differences and similarities observed among the HP200 and HP600 samples in the present work. However, it was probably accompanied by the release of smaller molecular weight proteins as described above. This would lead to both increased solubility over pH, with variations due to different extents of these phenomena, but also alterations in the DPPH radical scavenging activity due to the same reason.

Kim, Son et al. (2016) investigated how proteolytical degradation of fish powder proteins were affected by simultaneously applying high pressure (0.1 and 300 MPa; 50 ℃; 1 h). The hydrolysates produced at 300 MPa presented a content were most peptide species had a molecular mass lower than 5 kDa. It should be mentioned that HP might have a direct effect on the enzyme activity, thus reducing the comparability to the present work (Hendrickx, Ludikhuyze et al. 1998). Meanwhile, the

work done by Kim, Son et al. (2016) seemed to offer observations from the pressure-region which in the present work seemed to initiate the increased protein concentration seen after 60 min of hydrolysis. This was also observed by Kim, Son et al. (2016) through increased TSS (total soluble solids) and TWSN (total water soluble nitrogen) when conducting the hydrolysis at 300 MPa. This was observed for all the different combinations of enzymes applied. In addition was a considerable increase in DH% observed for the various enzyme combinations (the increase ranging between a 12-21 %) which indicates considerable increase in peptide bond exposure, which together with the high content of low molecular weight components would increase the charge content for the peptides in the hydrolysate and in such way could explain the increasing hydrophilic properties which seemed to dominate the hydrolysates obtained from the highest pressure treatments in the present work.

7 Conclusions

The results of this study indicate a gradual increase in protein denaturation with increasing MW pre-treatment time, which caused protein aggregation at the later stages of hydrolysis for samples subject to 10 min pre-treatments (MW10). For samples undergone 1 min MW pre-treatments (MW1), a significant increase in the multicomponent phase content was observed as compared to the samples pre-treated for 5 and 10 min (MW5 and MW10). Both MW1 and MW10 hydrolysates presented significant increases in protein solubility over pH as compared to the control samples. No significant increase in the DPPH radical scavenging activity was observed when samples were pre-treated with MW1 or MW10 and pre-treatment times longer than 10 min should be avoided. However, MW1 presents a rapid pre-treatment strategy for increased protein solubility over pH and might display desirable functional properties as interaction with lipids seemingly occur when low pre-treatment times are applied.

The results of this study indicate that the properties of the protein hydrolysates were gradually altered as US power was increased during the pre-treatment. The various powers (300 W, 600 W, 900 W; US300, US600, US900) applied increased the multicomponent content. Else, the amount of the different phases varied with US power. Applying the highest US power significantly reduced the protein concentration after 60 min of hydrolysis as compared to that of the control. On the other hand, the DPPH radical scavenging activity increased with increasing power. While the US300 samples displayed a reduced DPPH radical scavenging activity compared to that of the control, the US900 samples presented a significantly increased DPPH radical scavenging activity when compared to the control samples. The effect US posed on the raw material seemed to affect specific constituents, as protein solubility increased significantly independently of US power at pH 6 and 11, increased significantly dependant of US power at pH 4 (for US300), and was unaffected by US power at pH 2. Based on the findings in the present work, high power (900 W) US holds great potential as a pre-treatment technology prior to enzymatic hydrolysis for production of highly active DPPH radical scavenging hydrolysates without reducing the overall extraction yield. Moreover, low to medium power (300-600 W) US pre-treatments seem to favour the formation of peptides able to associate to lipids.

The effect of exposing the raw material to high pressure during the pre-treatments caused different effects on the measured properties depending on the pressure applied. The effects observed when exposing the samples to 100 MPa (HP100) during the pre-treatment were not significant as compared to the control. However, when applying higher pressures, significant changes in the hydrolysate properties were observed. A pre-treatment at 200 MPa (HP200) increased the aqueous

and multicomponent phase contents while it reduced the lipid phase content. Applying 200 MPa further increased the protein solubility over pH and the DPPH radical scavenging activity as compared to the control samples. At 600 MPa (HP600) the properties of the hydrolysates seemed to change, resulting in a significantly increased protein concentration in the final hydrolysis volume, increased protein solubility over pH, but reduced DPPH radical scavenging activity. Samples pre-treated at 400 MPa (HP400) also presented increased protein concentration in the final hydrolysis volume compared to the control, but not compared to the HP600 samples. The elevated protein content in the hydrolysates of samples pre-treated with pressures equal to 400 MPa and higher also displayed elevated lipid phase contents accompanied by reductions in the aqueous phase, suggesting disruption of certain protein-lipid structures causing the increase observed for the mentioned properties. This was observed simultaneously as the multicomponent phase decreased when pressure was increased from 400 to 600 MPa, possibly as lipid-protein structures was further disrupted. HPP is therefore found to present several potential advantages for pre-treatment of MDCR. Applying low pressures during the pre-treatment (200 MPa) result in peptides with increased solubility over pH, increased DPPH radical scavenging activity and with the potential ability to associate to lipids. Applying higher pressures (600 MPa) maintained high protein solubility and increased protein concentration in the final hydrolysis volume but presented low lipid association and DPPH radical scavenging properties.

8 Future work

In the present work the potential for valorising MDCR has been confirmed. Among the various technologies there are indications of enhanced protein extraction, antioxidant properties, lipid interactions and solubility over pH. However, undesirable interactions between certain components were observed after enzyme inactivation. Ideally, pure fractions of the aqueous product containing the product peptides, lipid phase and solids are obtained after the hydrolysis. This was however not the case as interactions between peptides and lipids formed structures affecting the multicomponent phase content. Thus, reducing the extraction of peptides with potentially desired properties, but also reducing the purity of the lipid phase itself. Thus, investigations should be conducted on the separation of the protein and lipid components within the raw material. This such that a treatment could be developed achieving full exploitation of both lipids and peptide fractions from the MDCR. Future investigations should also investigate further upon the properties of the lipid components within the MDCR as their exploitation would contribute toward economic viability. If a desirable fractionation of lipids and the proteinaceous components are achieved either prior to the hydrolysis, avoiding significant protein denaturation, or after the hydrolysis, the targeting of process conditions is still required in order to optimize the desired peptide properties. The results obtained in the present work offer good starting points for further investigation and optimization of the various properties obtained within the hydrolysate.

As previously specified, the investigation done in the present work on lipid-protein associations could be initial indicators for e.g. emulsifying properties or oil-binding properties. However, the methods applied are on the simple side, therefore a more thorough investigation on process conditions and their effect on such properties should be conducted. This also holds for antioxidant properties, and at least two different methods are often recommended in order to say anything certain about the inherent antioxidant properties of a material. As mentioned earlier in this work, scavenging of the ATBS radical is often associated with antioxidant peptides also displaying a hydrophilic character, and together with the DPPH radical investigation could these methods give an appropriate indication on the antioxidant properties of the hydrolysate. The investigations regarding the relative protein solubility in this work was conducted using the liquid aqueous phase containing some particulate. However, this states nothing about the overall protein solubility of the material. Therefore, further investigations on the protein solubility is recommended done using a certain content of hydrolysate powder (freeze-dried aqueous phase for example) which is dissolved in a certain amount of water before manipulating the pH. However, it should be emphasised that the results obtained in the present work is believed to display good indications on the general solubility properties of the

hydrolysates produced.

The pre-treatment conditions applied in the present work were compiled based on their reported effect on the major protein components within the MDCR raw material. Thus, other materials comprised of similar components would be receptive in a similar way upon similar conditions. An important advantage is that the applied conditions presented what seemed to be a gradual modification of the product properties, thus where the conditions neither to mild nor to severe. Regarding other major protein components within this raw material, especially collagen, will the enzymatic extraction of such a constituent require specific enzymes, namely collagenases. Other possibilities would be to achieve extraction through the pre-treatments and subsequent exposure in the thermal environment of the reactor. However, none of the applied pre-treatment technologies and their effects (except heat associated to microwave exposure) are reported to present extracting effects on collagen. Meanwhile, this is slightly debated as there are reports on collagen being both insensitive to pressure (Suzuki, Watanabe et al. 1993) and responsive (Potekhin, Senin et al. 2009, Nan, Zou et al. 2018). Thus, HPP might affect the thermal stability of the collagen molecules and presents another possibility for future investigations.

As mentioned in the introductions of the various technologies, the treatments might also be applied during the enzymatic hydrolysis. As the effect associated with the microwave treatment is thermal (at least), there would during simultaneous application be a compromise between the impact on enzyme and substrate. On the one hand the enzyme exhibits optimum activity at a certain temperature, this while the complex mixture of proteinaceous substrates responds differently at such a temperature. Certain combinations of enzyme and raw material might present desirable compromises, while other do not. In any case, such relationships would be highly specific and not dwelled upon further. Another possibility could be to apply an enzyme mixture with a broad temperature spectrum for optimum activity, and steadily increase the temperature in the hydrolysis reactor. In such a way would the gradual denaturation of each component (responding differently to a certain temperature) within the raw material be acted upon continuously and possibly benefit the extraction yield, while other specific properties are neglected. Simultaneous application of US and HPP are also options. In such cases must also compromises be expected, as both these technologies would pose different effects on the enzyme and the various proteinaceous substrates. However, certain combinations of enzyme, substrate and treatment condition might generate synergetic phenomena where enzyme activity is enhanced while denaturation of certain protein substrates occur in such a way that peptides with desirable properties are formed at high rates. However, as this would be highly dependent on enzyme and substrate, it will not be dwelled further upon in this

work but mentioned as a possibility.

As a final remark within this section will the issue on myofibrillar solubility be noted. These structures are reportedly insoluble at low ionic strength. As these structures might be sources to antioxidant and functional peptides, it would seem beneficial to increase their solubility throughout the treatment. Thus, in order to test whether the pre-treatment (and subsequent hydrolysis) increase the solubility of these constituents and their derivatives, one could perform the pre-treatment and then check the protein solubility in pure water. Next, one could filter out the insoluble (solids) content and check the solubility of this fraction in a solution with ionic strength reported to solubilise myofibrillar proteins. These fractions could further be investigated through SDS-PAGE as pre-treatment severity is gradually changed. This in order to investigate which parental proteins are affected and discover which structures are solubilised (in water) by the certain pre-treatments. Furthermore, the hydrolysates originating from samples pre-treated might also be investigated with SDS-PAGE in order to evaluate which of the structures are degradable for the applied enzyme.

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Appendix

A. Data for temperature development and heat absorption during the MW pre-treatments

The figures in the following subsections presents the temperature development recorded in the parallels of the various MW pre-treatments. Sensor 1 indicates the temperature development closest to the sample edge, sensor 2 the temperature development in the sample centre and sensor 5 indicates the temperature at a position in between sensor 1 and 2. This is further described in the materials and methods section. Average temperature of the three sensors is also presented in each Figure.

MW10 replicates

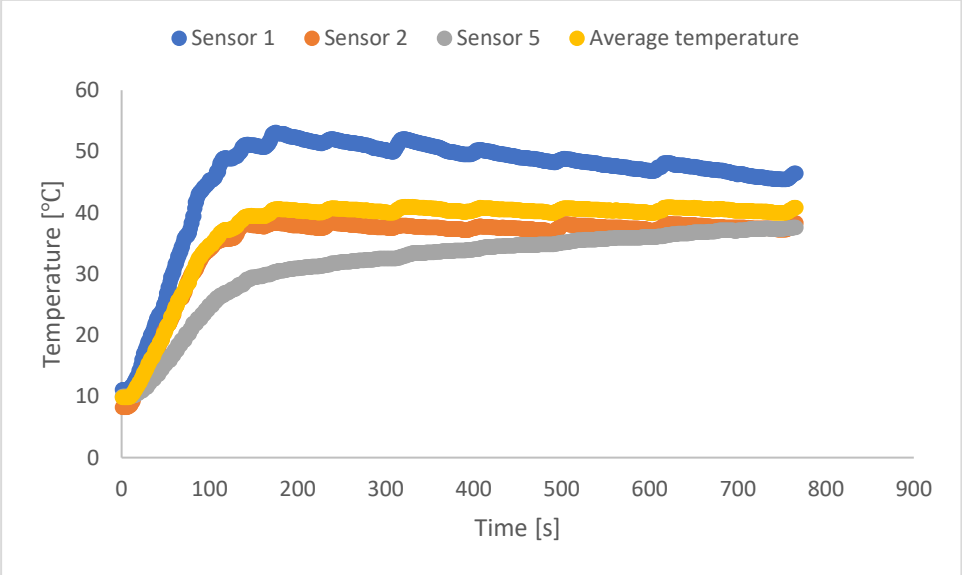


Figure 47: The figure illustrates the temperature development for pre-treatment MW10 replicate 1. Energy absorbed during the pre-treatment: 87.6 kJ. An average temperature of 40 ℃ was reached after 170 s.

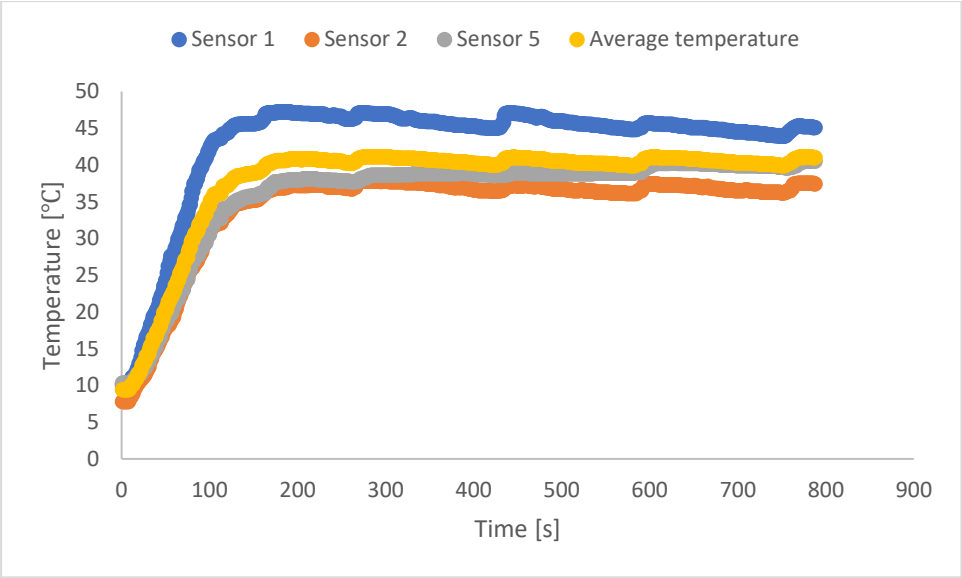


Figure 48: The figure illustrates the temperature development for pre-treatment MW10 replicate 2. Energy absorbed during the pre-treatment: 84.8 kJ. An average temperature of 40 ℃ was reached after 164 s.

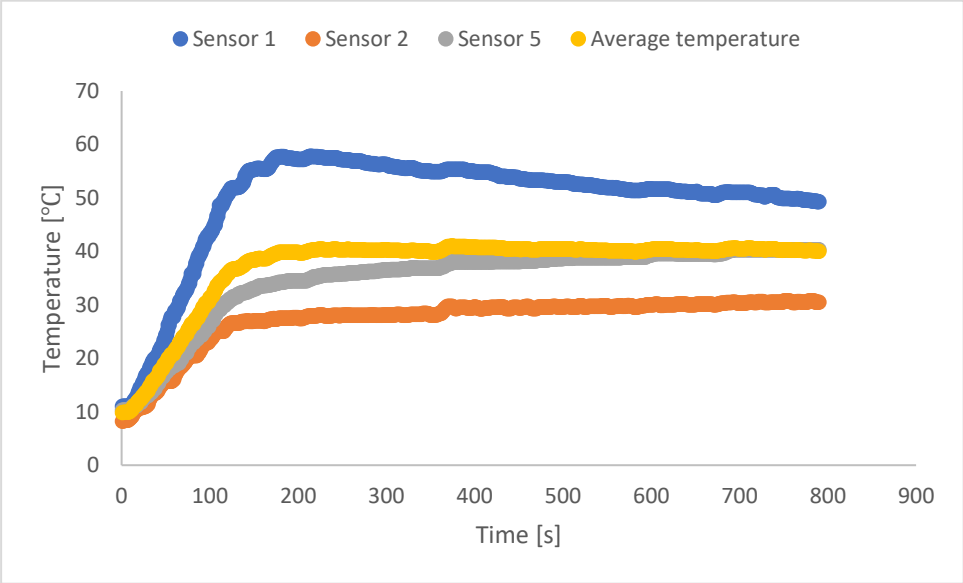


Figure 49: The figure illustrates the temperature development for pre-treatment MW10 replicate 3. Energy absorbed during the pre-treatment: 95.9 kJ. An average temperature of 40 ℃ was reached after 190 s.

MW5 replicates

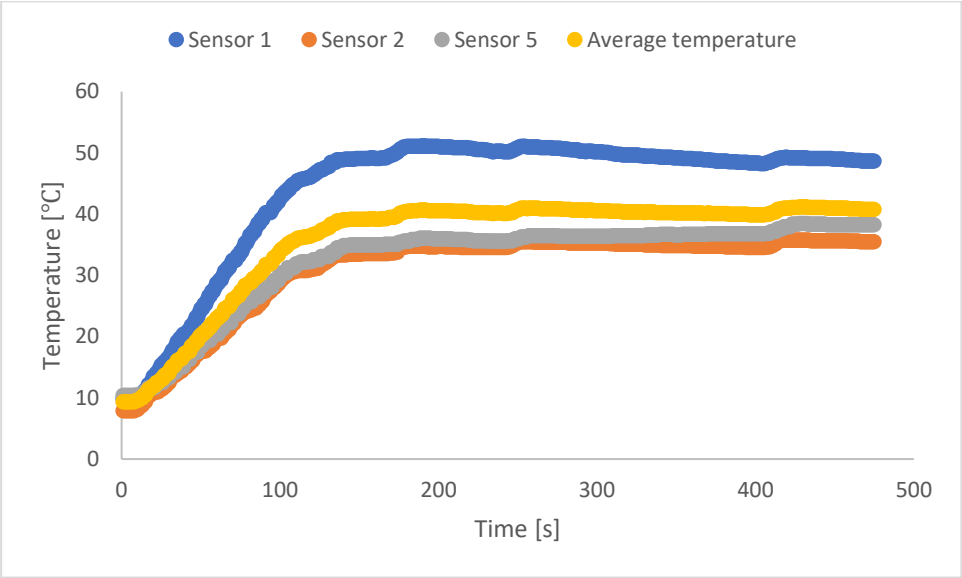


Figure 50: The figure illustrates the temperature development for pre-treatment MW5 replicate 1. Energy absorbed during the pre-treatment: 84.2 kJ. An average temperature of 40 ℃ was reached after 176 s.

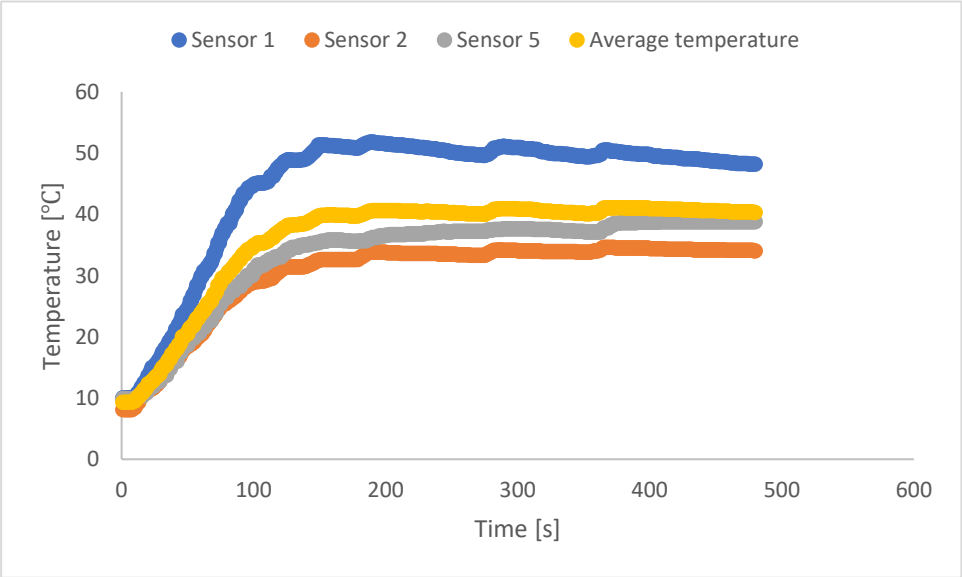


Figure 51: The figure illustrates the temperature development for pre-treatment MW5 replicate 2. Energy absorbed during the pre-treatment: 76.1 kJ. An average temperature of 40 ℃ was reached after 183 s.

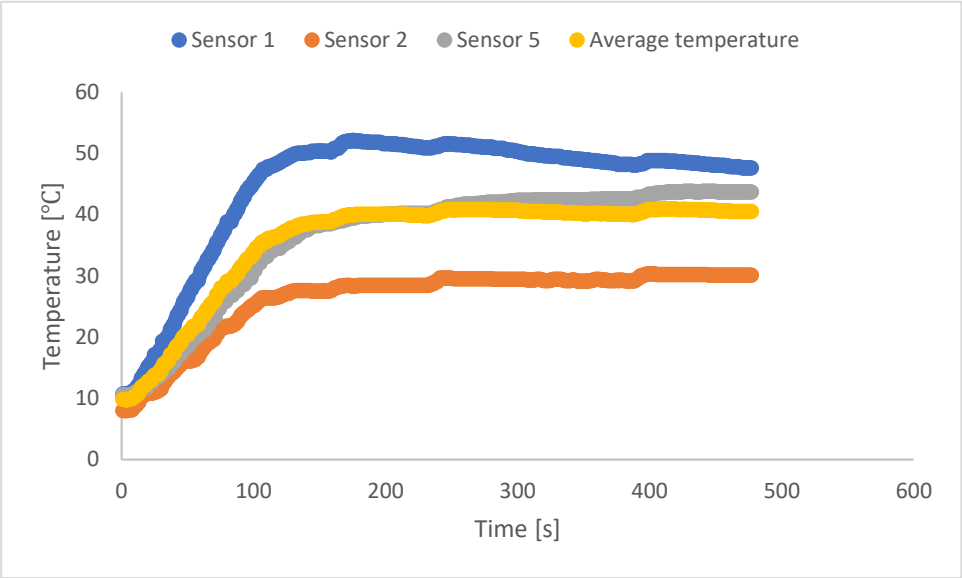


Figure 52: The figure illustrates the temperature development for pre-treatment MW5 replicate 3. Energy absorbed during the pre-treatment: 82 kJ. An average temperature of 40 ℃ was reached after 177 s.

MW1 replicates

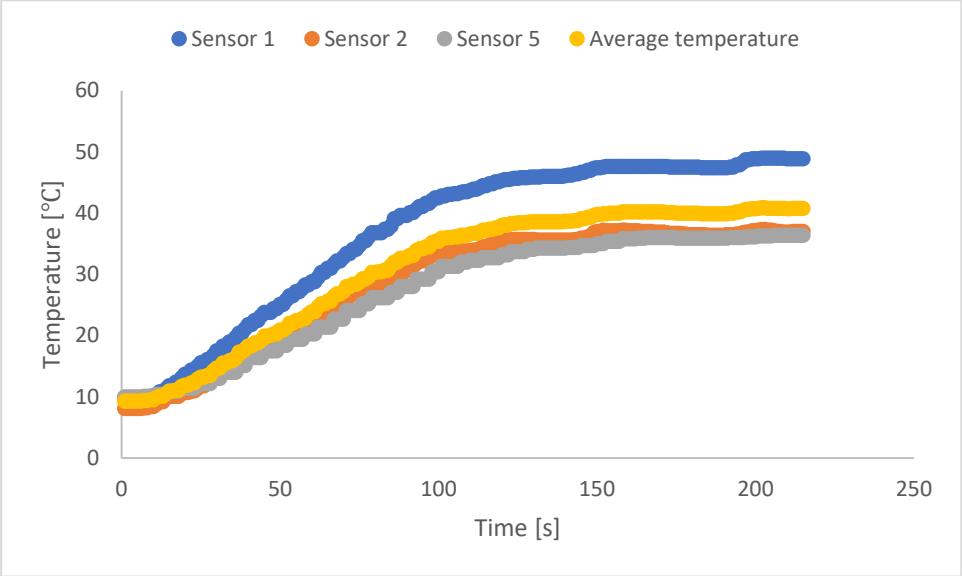


Figure 53: The figure illustrates the temperature development for pre-treatment MW1 replicate 1. Energy absorbed during the pre-treatment: 74.5 kJ. An average temperature of 40 ℃ was reached after 154 s.

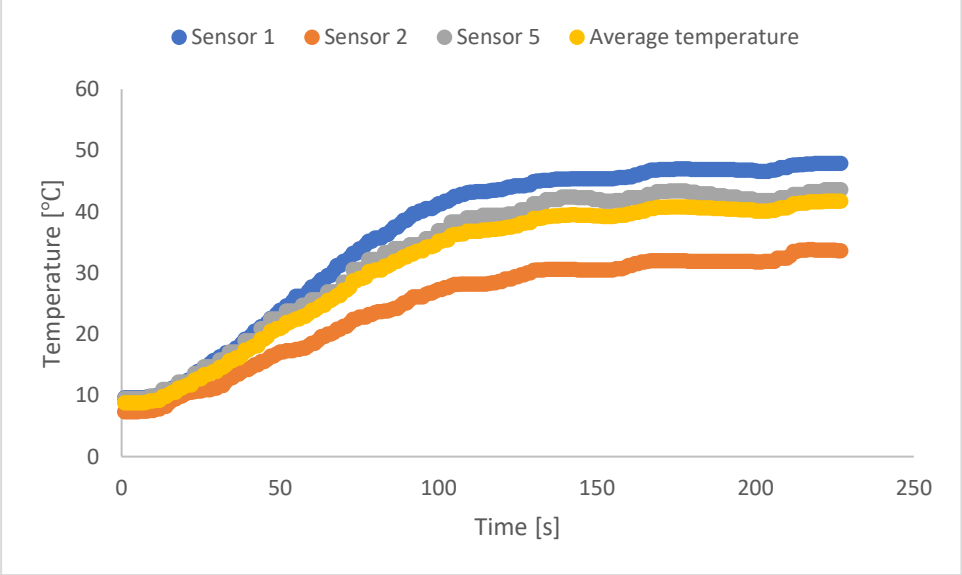


Figure 54: The figure illustrates the temperature development for pre-treatment MW1 replicate 2. Energy absorbed during the pre-treatment: 74.6 kJ. An average temperature of 40 ℃ was reached after 164 s.

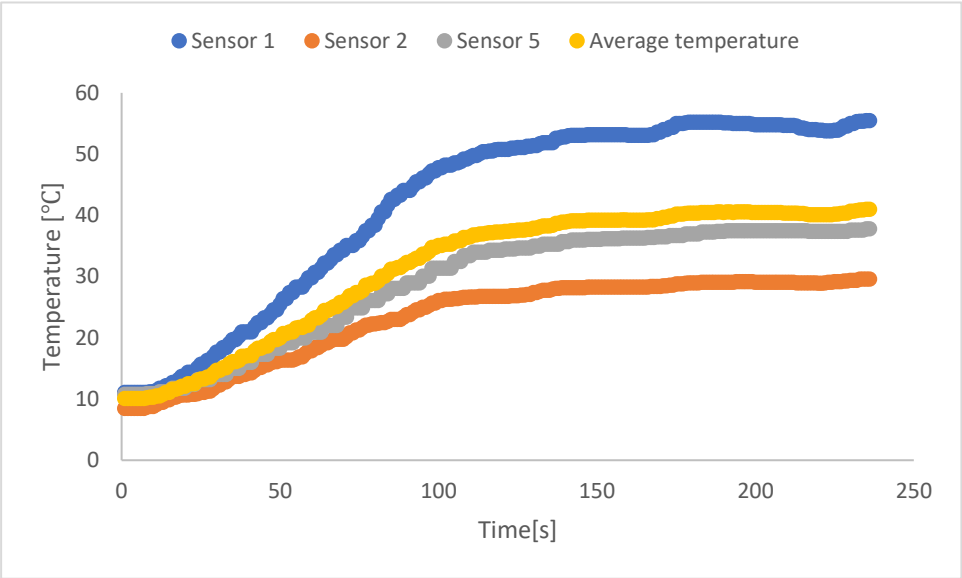


Figure 55: The figure illustrates the temperature development for pre-treatment MW1 replicate 3. Energy absorbed during the pre-treatment: 75.5 kJ. An average temperature of 40 ℃ was reached after 175 s.

Energy absorbed during the MW pre-treatments

Table 8: The table present the energy absorbed during the various MW pre-treatment replicates, the average within each type MW pre-treatment, and the associated standard deviation. Significant differences are indicated by differing superscript letters.

Treatment	*Parallel 1*	*Parallel 2*	*Parallel 3*	*Avg*	*StD*
	[kJ]	[kJ]	[kJ]	[kJ]	[kJ]
MW1^{A}	74,5	74,6	75,4	74,83	0,49
MW5^{AB}	84,2	76,1	82,0	80,76	4,19
MW10^{B}	87,6	84,8	95,9	89,43	5,77

B. Data for pressure and temperature development during the HPP pre-treatments

The figures in the following subsections presents the temperature and pressure development recorded in the replicates of the various HPP pre-treatments. Both TOP and MID temperature are measured within the pressure chamber and no temperature set-point was applied. Else is the pressure set-point illustrated with a black graph and the actual pressure within the chamber with a red graph.

HP100 replicates

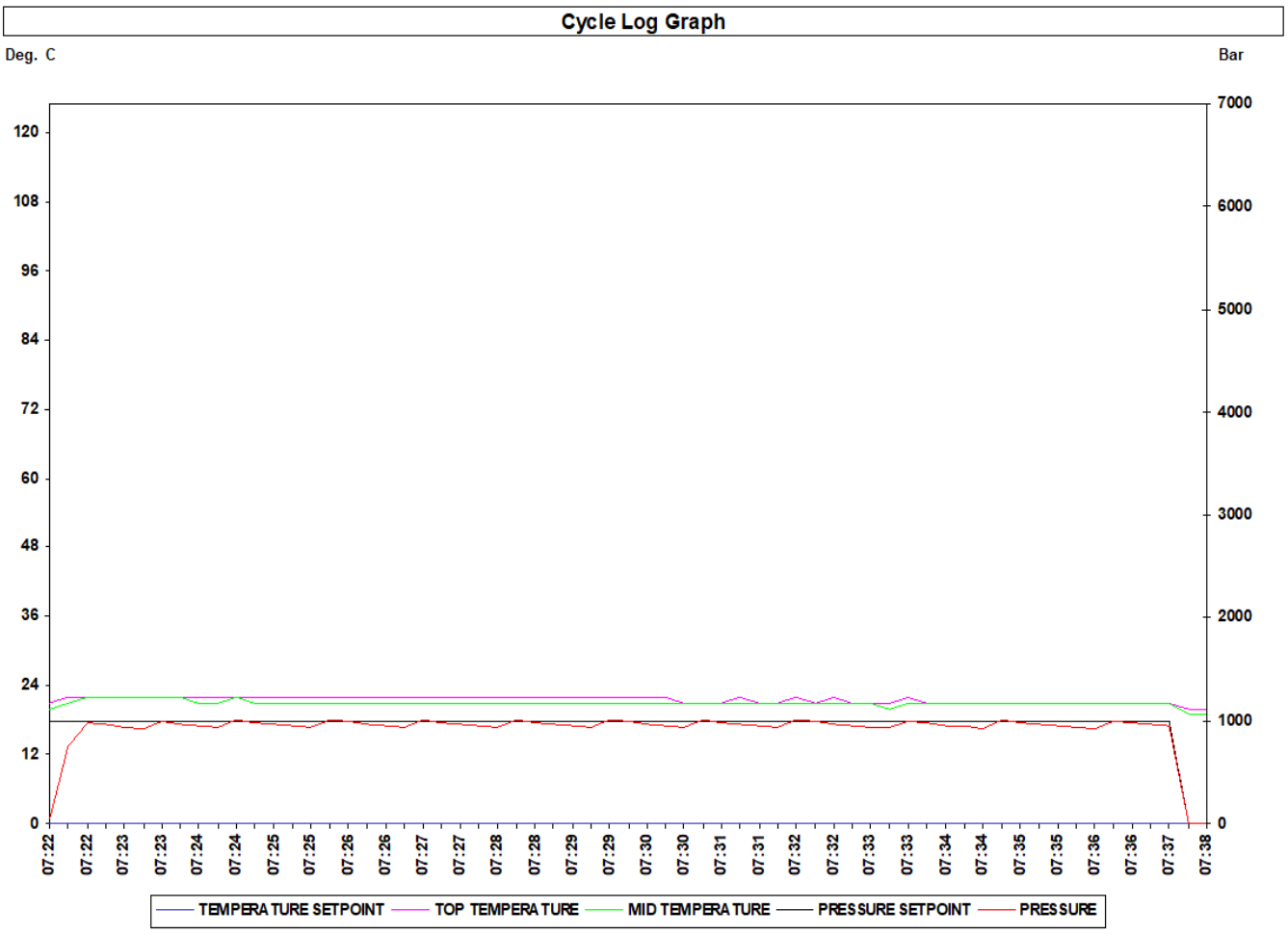


Figure 56: The figure illustrates the set-point and actual development of the pressure within the pressure-chamber of the HPP apparatus for HP100 replicate 1. In addition, two recordings of temperature within the pressure-chamber is illustrated through MID and TOP temperature.

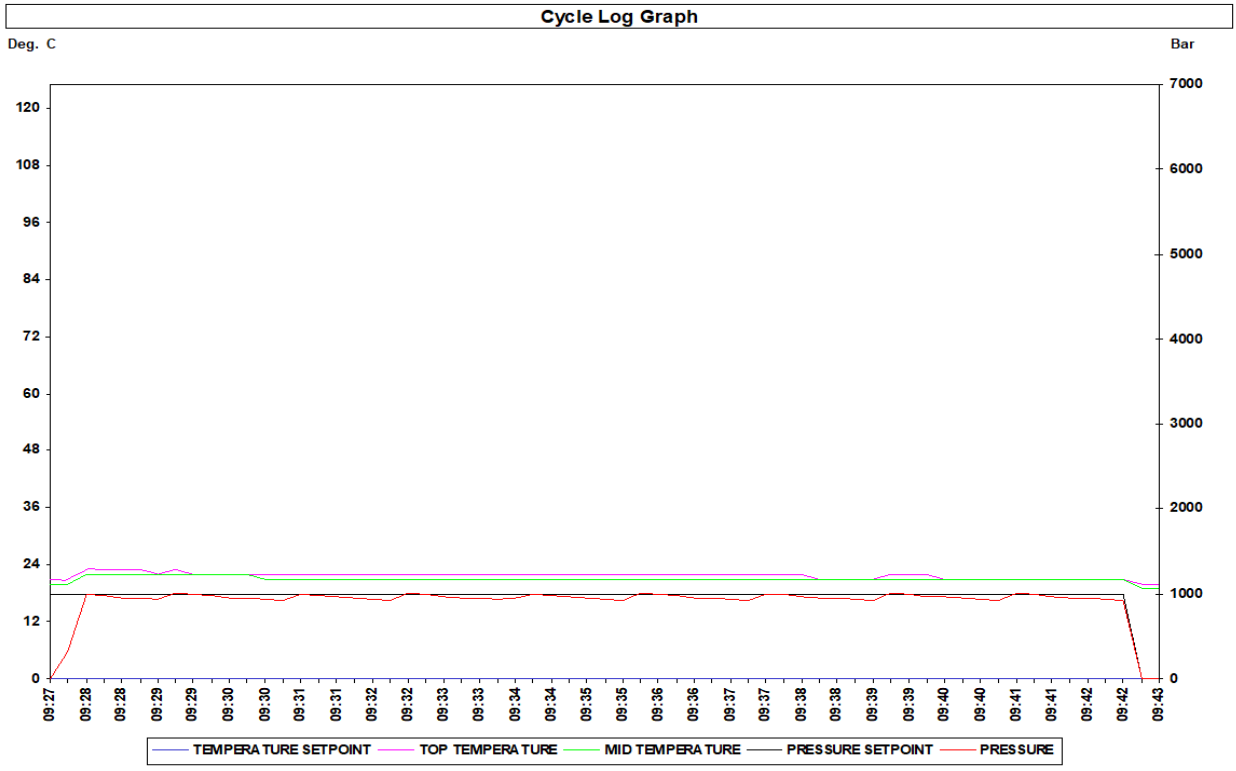


Figure 57: The figure illustrates the set-point and actual development of the pressure within the pressure-chamber of the HPP apparatus for HP100 replicate 2. In addition, two recordings of temperature within the pressure-chamber is illustrated through MID and TOP temperature.

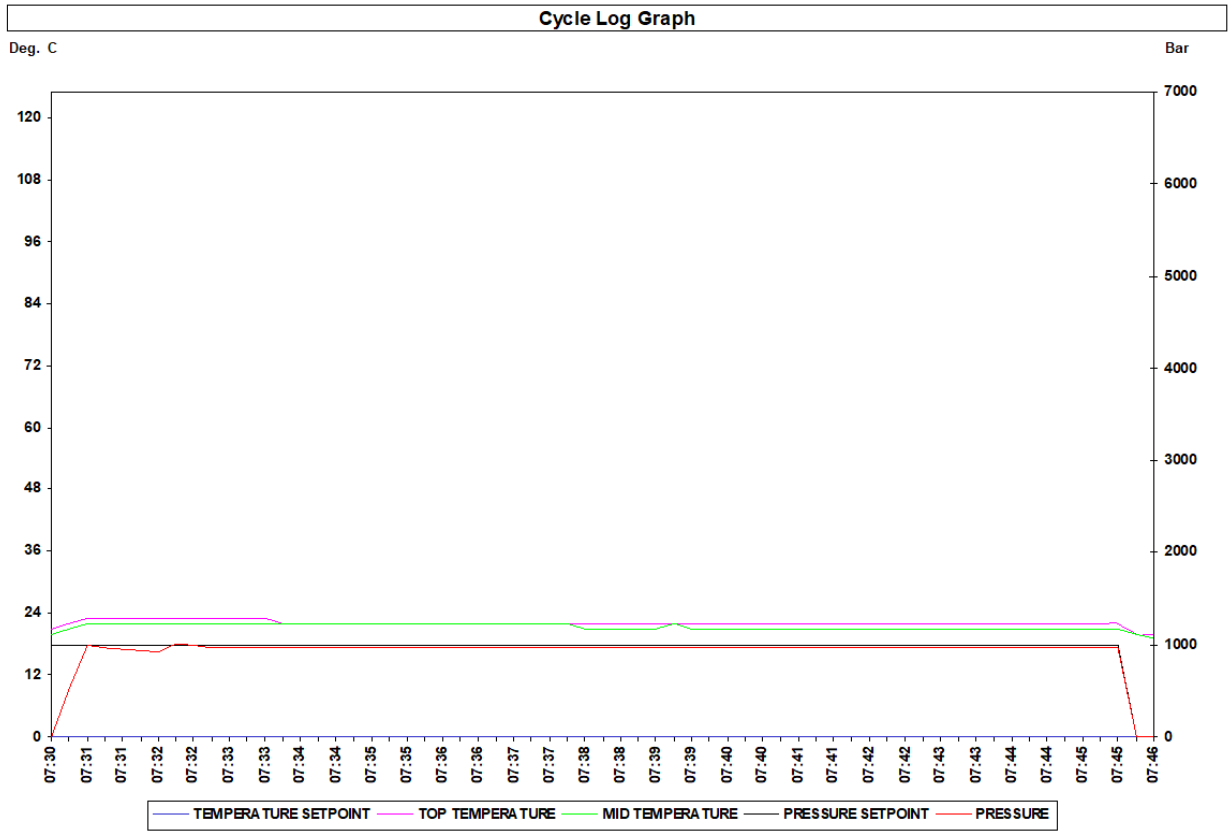


Figure 58: The figure illustrates the set-point and actual development of the pressure within the pressure-chamber of the HPP apparatus for HP100 replicate 3. In addition, two recordings of temperature within the pressure-chamber is illustrated through MID and TOP temperature.

HP200 replicates

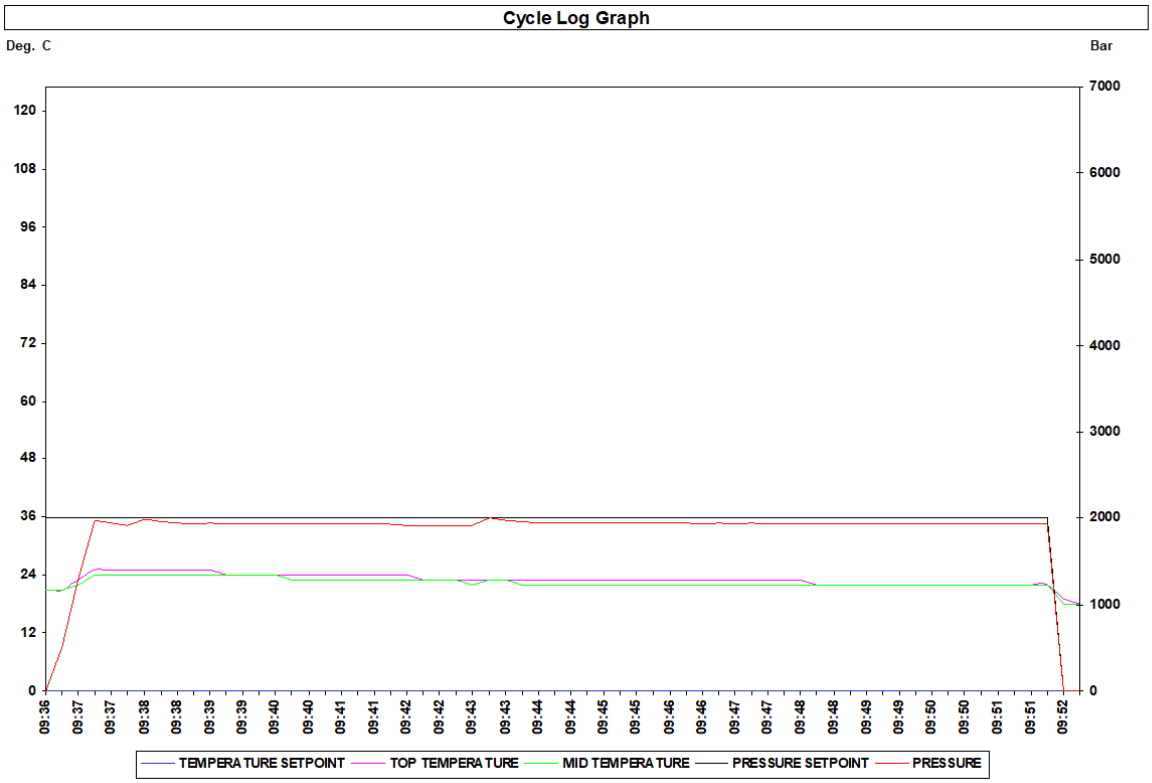


Figure 59: The figure illustrates the set-point and actual development of the pressure within the pressure-chamber of the HPP apparatus for HP200 replicate 1. In addition, two recordings of temperature within the pressure-chamber is illustrated through MID and TOP temperature.

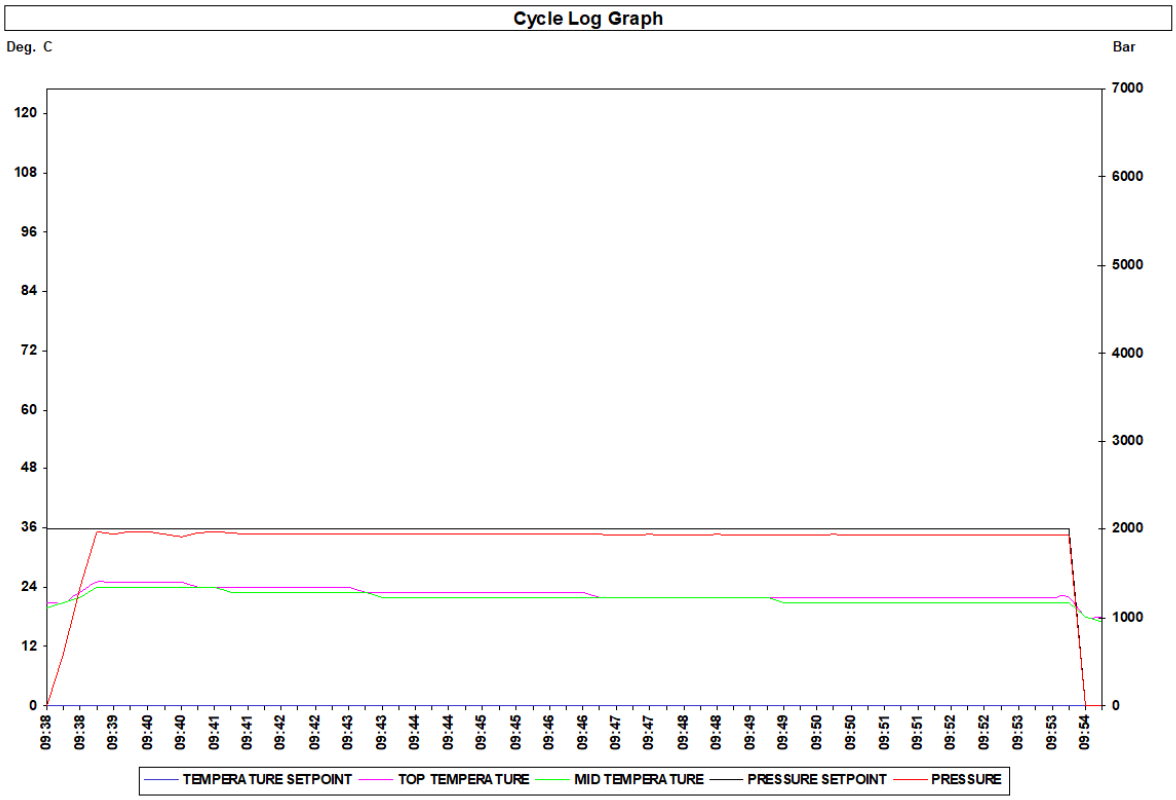


Figure 60: The figure illustrates the set-point and actual development of the pressure within the pressure-chamber of the HPP apparatus for HP200 replicate 2. In addition, two recordings of temperature within the pressure-chamber is illustrated through MID and TOP temperature.

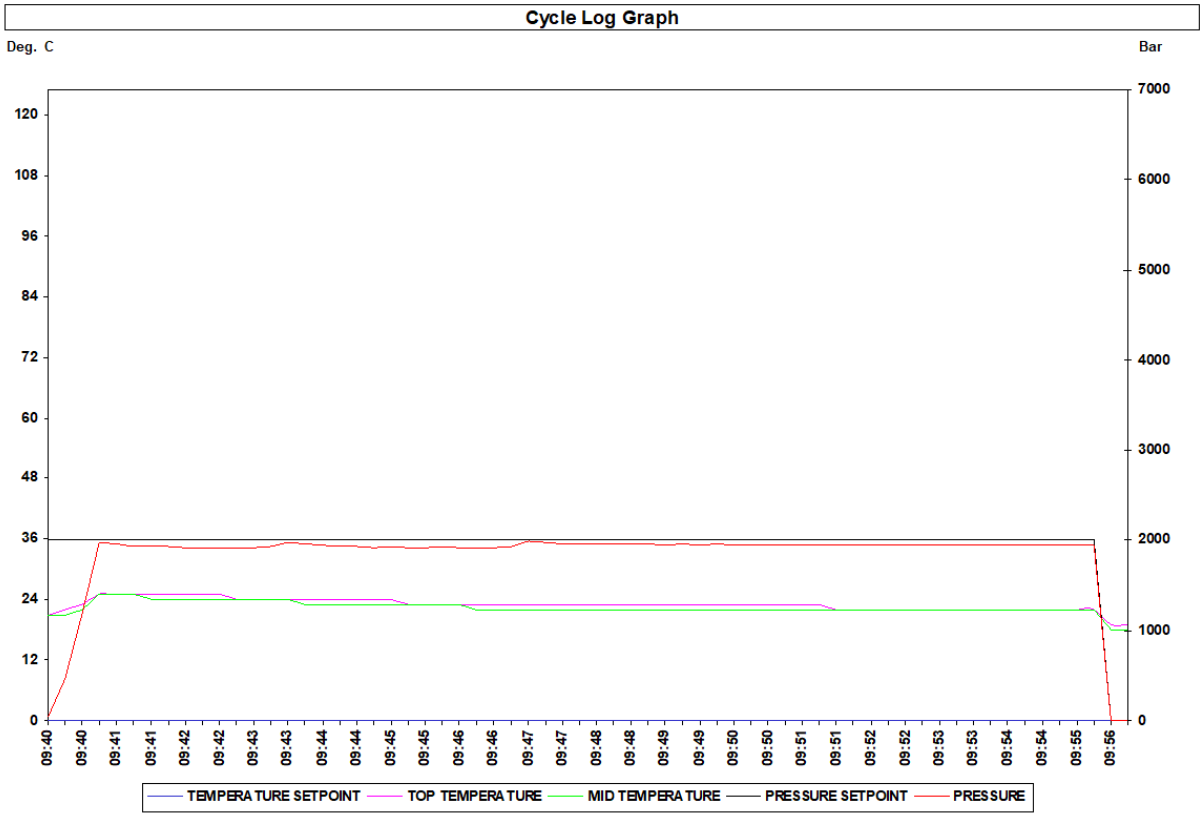


Figure 61: The figure illustrates the set-point and actual development of the pressure within the pressure-chamber of the HPP apparatus for HP200 replicate 3. In addition, two recordings of temperature within the pressure-chamber is illustrated through MID and TOP temperature.

HP400 replicates

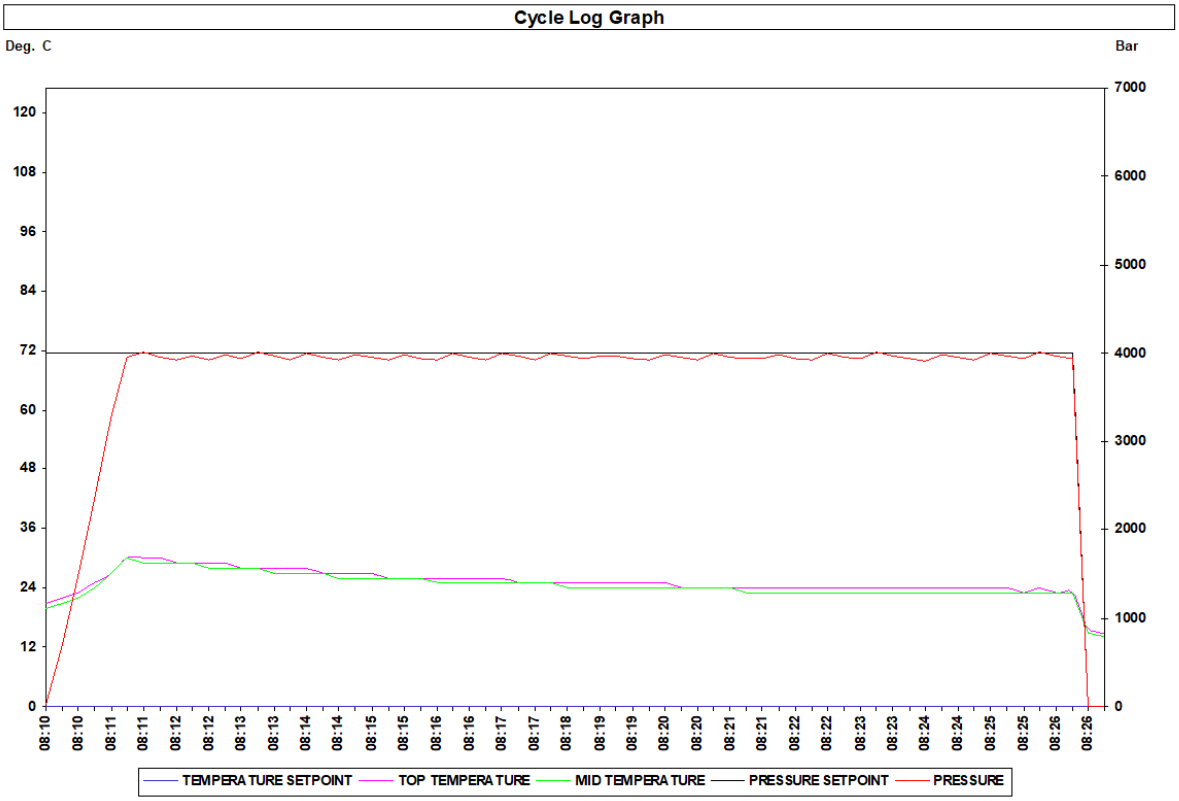


Figure 62: The figure illustrates the set-point and actual development of the pressure within the pressure-chamber of the HPP apparatus for HP400 replicate 1. In addition, two recordings of temperature within the pressure-chamber is illustrated through MID and TOP temperature.

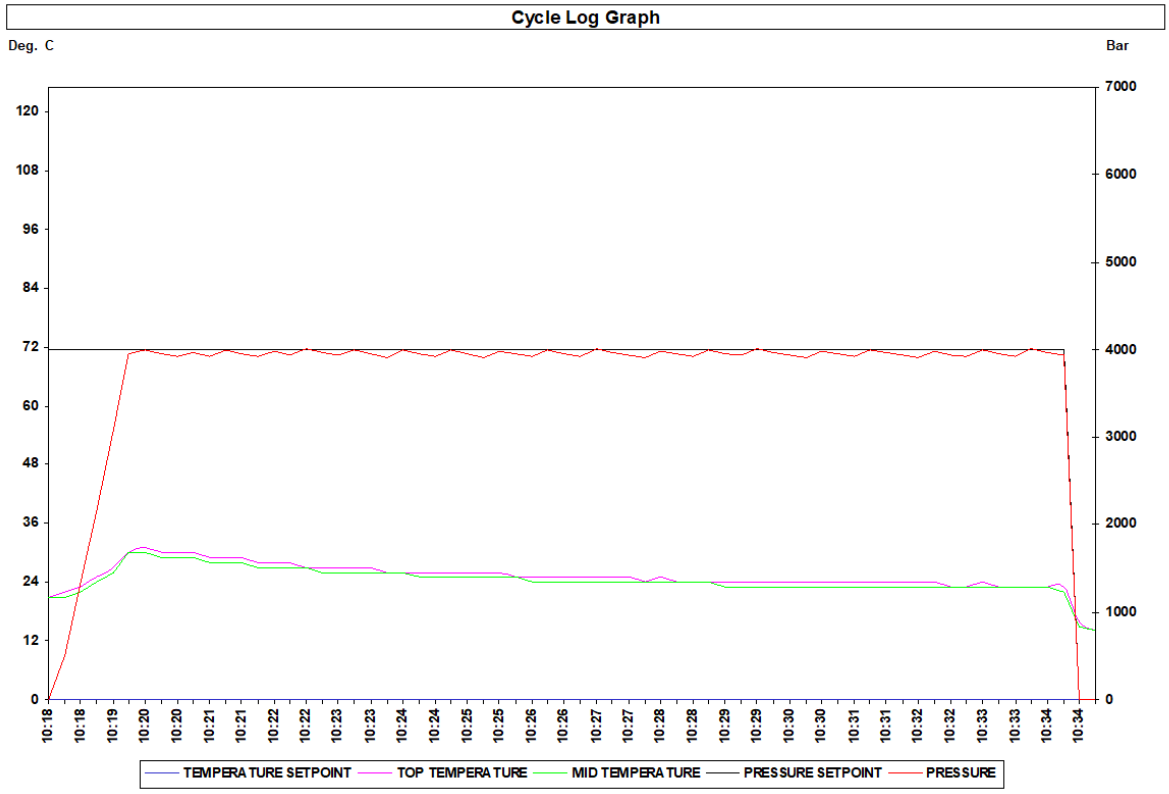


Figure 63: The figure illustrates the set-point and actual development of the pressure within the pressure-chamber of the HPP apparatus for HP400 replicate 2. In addition, two recordings of temperature within the pressure-chamber is illustrated through MID and TOP temperature.

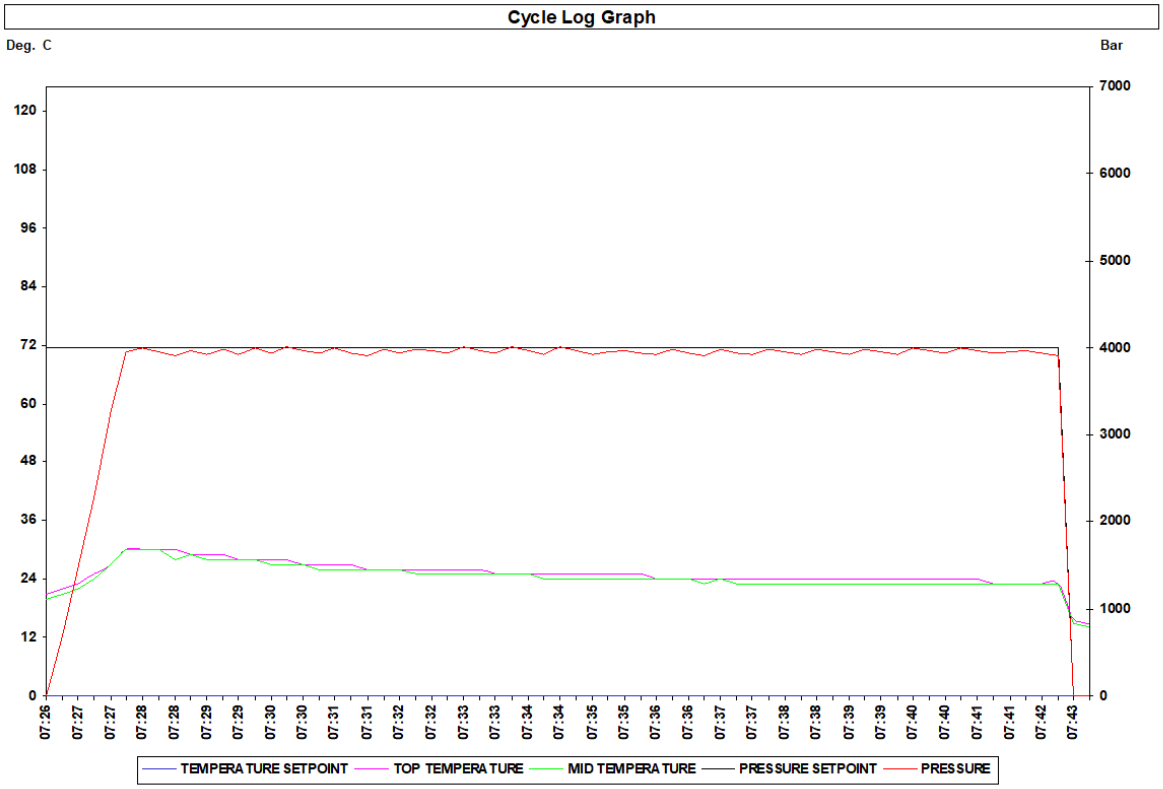


Figure 64: The figure illustrates the set-point and actual development of the pressure within the pressure-chamber of the HPP apparatus for HP400 replicate 3. In addition, two recordings of temperature within the pressure-chamber is illustrated through MID and TOP temperature.

HP600 replicates

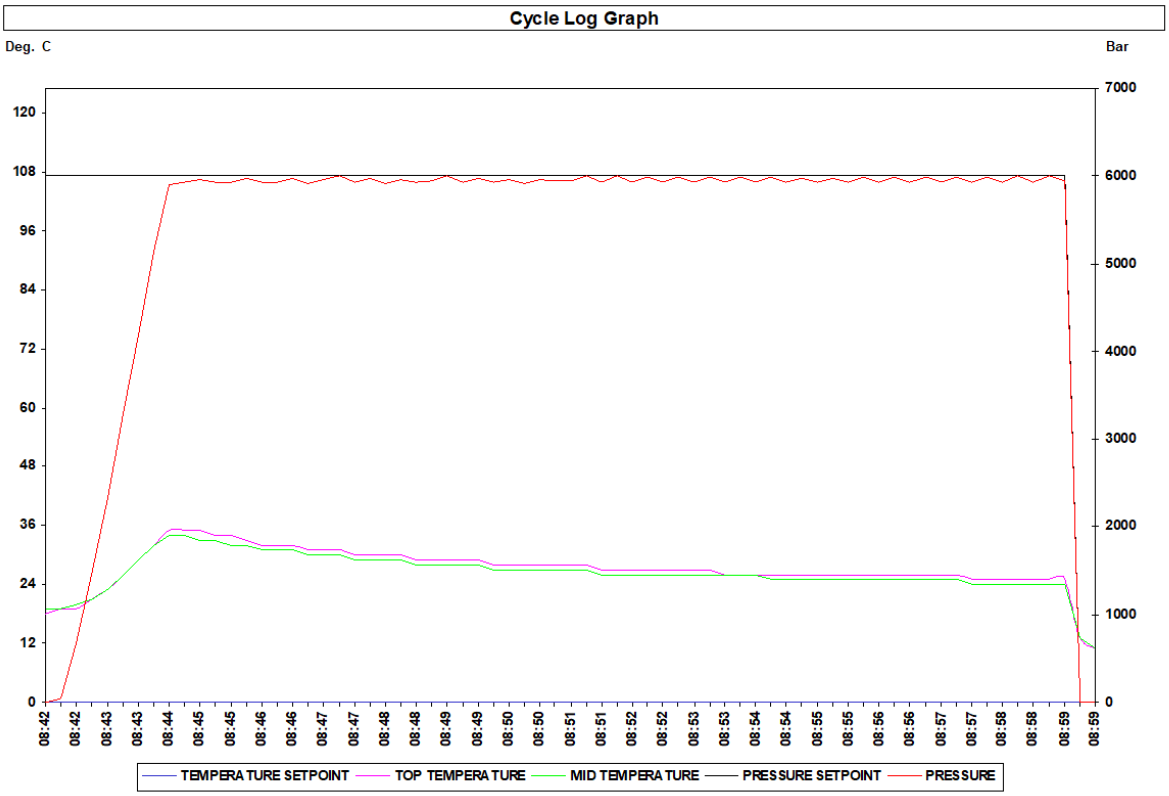


Figure 65: The figure illustrates the set-point and actual development of the pressure within the pressure-chamber of the HPP apparatus for HP600 replicate 1. In addition, two recordings of temperature within the pressure-chamber is illustrated through MID and TOP temperature.

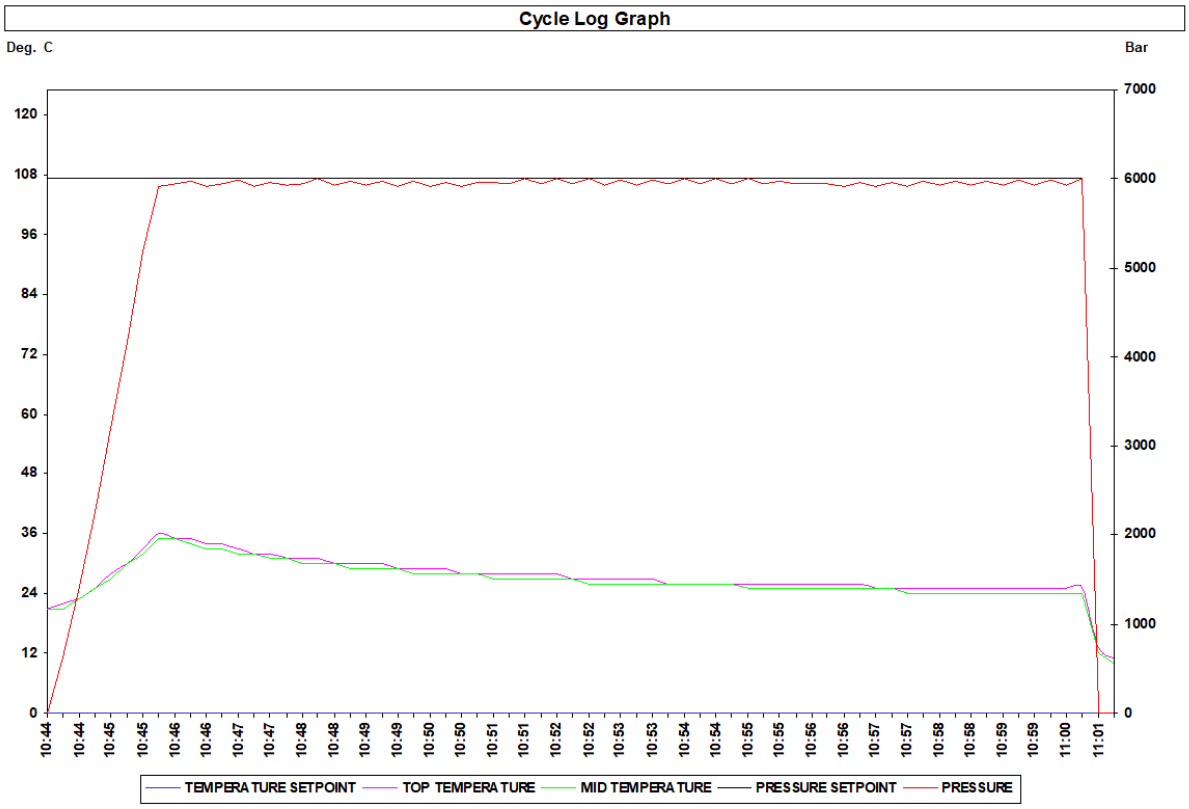


Figure 66 The figure illustrates the set-point and actual development of the pressure within the pressure-chamber of the HPP apparatus for HP600 replicate 2. In addition, two recordings of temperature within the pressure-chamber is illustrated through MID and TOP temperature.

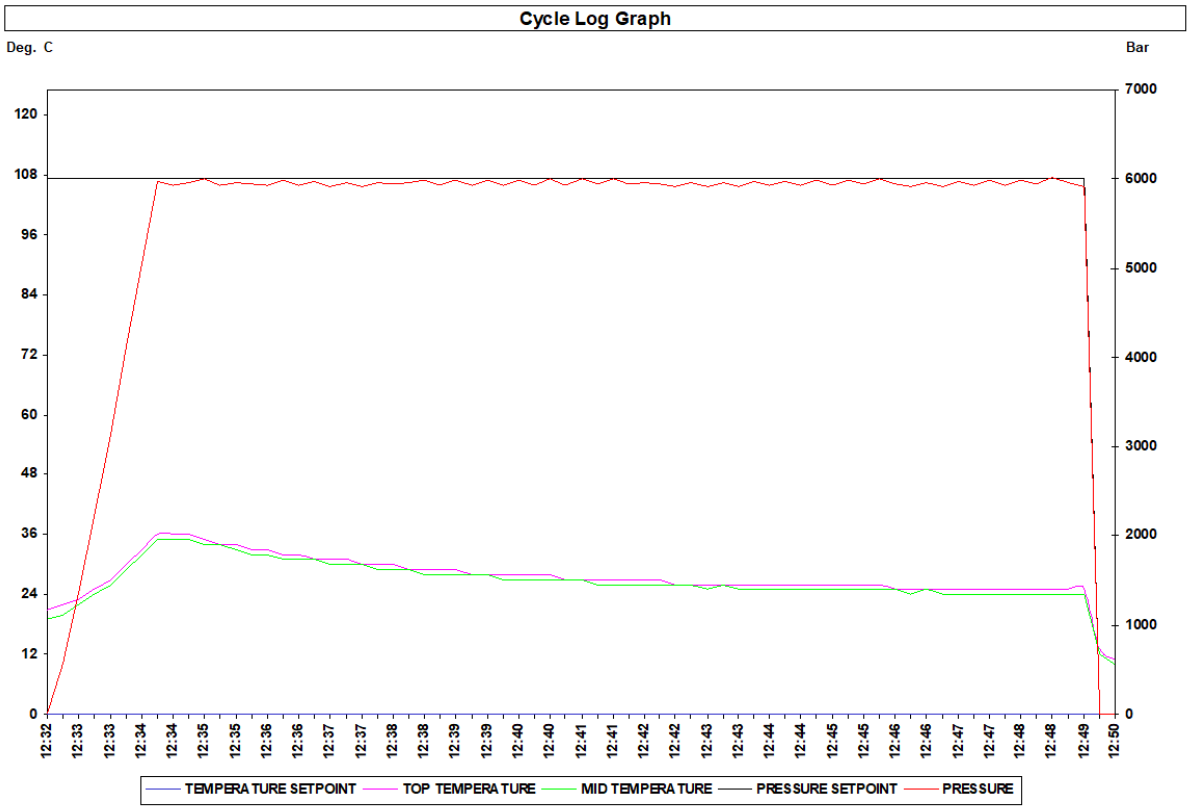
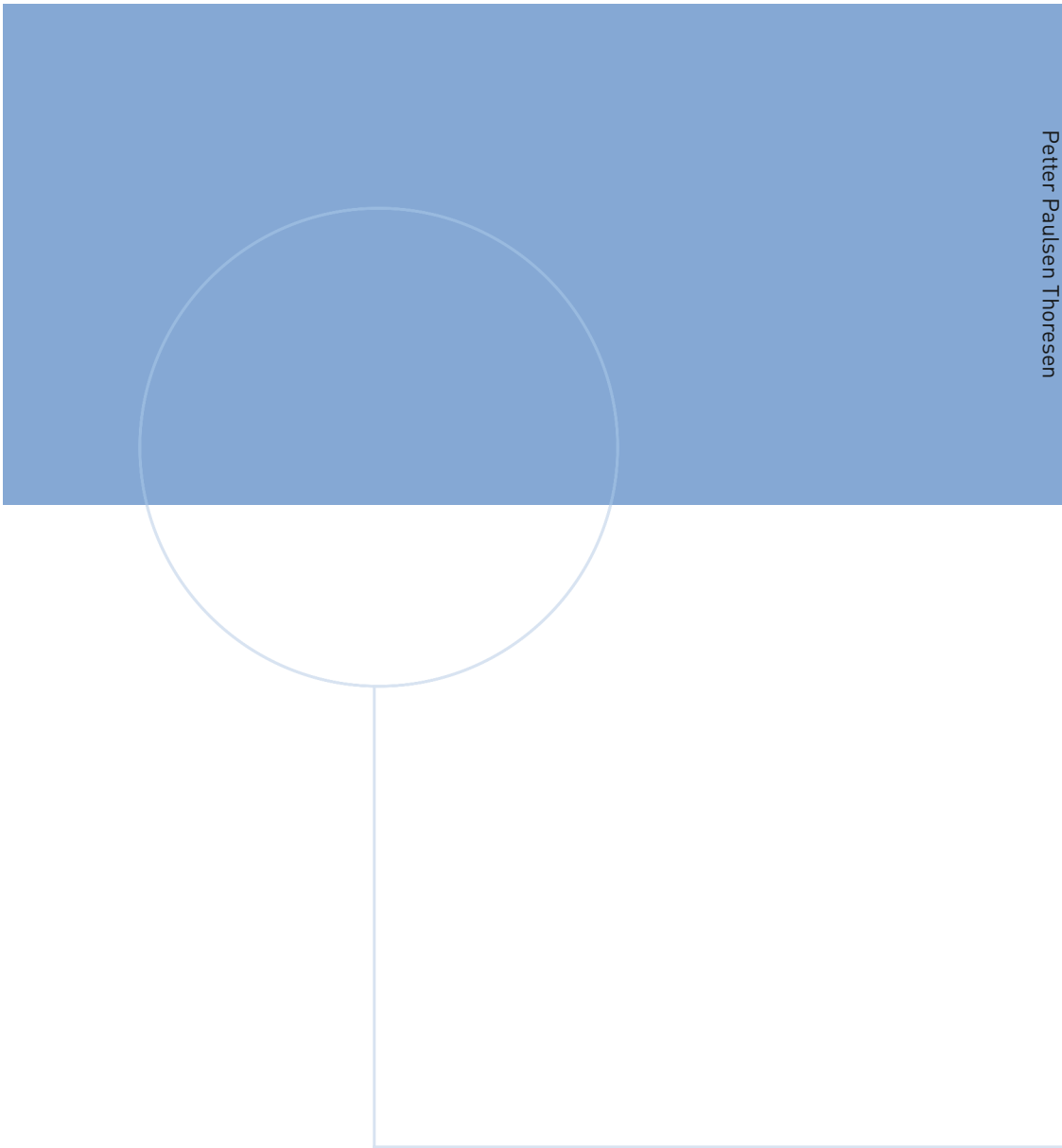


Figure 67: The figure illustrates the set-point and actual development of the pressure within the pressure-chamber of the HPP apparatus for HP600 replicate 3. In addition, two recordings of temperature within the pressure-chamber is illustrated through MID and TOP temperature.



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