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Dissertação do 2º ciclo de Estudos Conducente ao Grau de Mestre

IgE-binding capacity of crustacean allergens as affected by food processing and *in vitro* digestion

Bruno Carriço Sá

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Bruno Filipe Carriço de Sá

2023

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Resumo

Os crustáceos são importantes fontes alimentares, mas também são responsáveis por um número impressionante de reações alérgicas, estimando-se que afetem até 2,5% da população mundial. A alergia a crustáceos é uma das alergias alimentares mais graves, sendo responsável por desencadear sintomas clínicos que podem levar à anafilaxia. Os crustáceos englobam um número significativo de espécies, a maioria delas apresentando panalergénios (proteínas alergénicas), como tropomiosinas ou arginina cinases, que já foram identificadas em espécies como caranguejos, camarões ou lagostas. O objetivo deste trabalho foi investigar o efeito de alguns métodos comuns de processamento alimentar (fervura, cozedura, fritura) nas estruturas dos alergénios de 9 espécies diferentes de crustáceos e na sua capacidade de ligação às IgE, fornecendo assim dados importantes para uma melhor compreensão da alergenicidade das proteínas dos crustáceos. Além disso, simulou-se *in vitro* a digestão gastrointestinal, utilizando como modelo de estudo a lagosta americana, com o intuito de avaliar um possível efeito sinérgico dos métodos de processamento com a digestão, o qual pode afetar a reatividade do alergénio às IgE. As estruturas e os perfis proteicos foram estudados com SDS-PAGE, e a capacidade de ligação de IgE foi investigada através da realização de imunotransferência e ELISA, usando soros de pacientes alérgicos a crustáceos como plataformas de imunoreconhecimento.

No que diz respeito aos efeitos dos tratamentos de processamento alimentar na alergenicidade proteica a capacidade de ligação de IgE dos alergénios foi muito variável. Por exemplo, a tropomiosina, que se esperava ser muito resistente ao calor, apresentou resultados contraditórios dependendo da espécie de crustáceo em estudo. No entanto, os alergénios de espécies como camarão e lagostim parecem ter aumentado as suas ligações às IgE quando submetidas ao processamento térmico (exemplo, cozedura a 100 ºC), provavelmente devido à destruição da estrutura tridimensional da proteína e que pode revelar novos epítopos. Estes factos também foram confirmados por ELISA, com amostras processadas destas espécies tendo aumentado significativamente a ligação às IgE, enquanto as proteínas alergénicas em caranguejos e lagostas não tiveram aumento (mantêm) na ligação total de IgE, provavelmente devido a possuírem cascas duras. Os resultados da digestão revelaram que a fase gástrica permite um elevado grau de degradação dos alergénios, provavelmente devido à presença de alergénios sensíveis à pepsina (mesmo no caso das tropomiosinas). Quanto à fase intestinal, parece que alguns alergénios e/ou peptídeos reativos podem manter a sua alergenicidade na fase gástrica, e que muito provavelmente alguns peptídeos também podem sobreviver à digestão intestinal.

Também foi visível que as amostras processadas e digeridas apresentaram maior número de peptídeos digeridos de proteínas de alto peso molecular, provavelmente devido ao desdobramento proteico causado pelos métodos de processamento, promovendo uma ação mais eficiente das enzimas digestivas.

Em resumo, este trabalho foi capaz de preencher algumas lacunas existentes na literatura, dando uma visão mais global sobre como os diferentes processamentos alimentares e de como o processo de digestão afetam a estrutura do alergénio e a sua capacidade de ligação à IgE, como indicadores-chave para a alergenicidade.

Palavras-chave: crustáceos; alergia alimentar; ligação de IgE; processamento alimentar; digestão

Abstract

Crustaceans are important food sources, but they are also responsible for an impressive number of allergic reactions, being estimated to affect up to 2.5% of the world's population. Crustacean allergy is one of the most severe food allergies, being responsible for eliciting clinical symptoms that can lead to anaphylaxis. Crustaceans encompass a significant number of species, most of them presenting panallergens, such as tropomyosins or arginine kinases, which have already been identified in species like crabs, shrimps, or lobsters. Owing to its importance, the study of crustaceans is the focus of this work, which aimed to investigate the effect of some common processing methods (boiling, baking, frying) on the structures and IgE-binding capacity of allergens from 9 crustacean species, thus providing key data for a better understanding of protein allergenicity. Additionally, a simulated in vitro gastrointestinal digestion protocol was applied, with American lobster as a case study, to evaluate a possible synergistic effect of the processing methods with digestion that can affect allergen IgE-reactivity. Protein profiles and structures were studied with SDS-PAGE, and the IgE-binding capacity was investigated by performing both immunoblotting and ELISA using the sera of crustacean-allergic patients as immunorecognition platforms.

In what concerns to the effects of food processing treatments on protein allergenicity, it was verified that the IgE-binding capacity of allergens was very variable. For example, tropomyosin that was expected to be very heat-resistant, presented contradictory results depending on the crustacean species in study, as assessed by immunoblotting. However, species such as shrimp and scampi seemed to have enhanced IgE-binding when submitted to thermal processing (e.g., boiling), probably due to the protein unfolding that can reveal novel epitopes. These facts were also confirmed by ELISA, with processed samples of these species having significantly increased IgE-binding, whereas the allergenic proteins in crabs and lobsters had no increase (maintain) in total IgE-binding, probably due to their hard shells. Digestion results revealed that the gastric phase enable a high degree of allergen degradation, probably due to the presence of pepsin-sensitive allergens (even in the case of tropomyosins). As for the intestinal phase, it seems that some allergens and/or reactive peptides can retain allergenicity from the gastric phase, and that most likely some peptides may survive the intestinal digestion as well. It was also visible that processed and digested samples had a higher number of digested peptides from high molecular weight proteins, probably due to the protein unfolding caused by the processing methods, promoting a more efficient action of digestive enzymes.

In summary, this work was able to fill some existing gaps in the literature, giving a more global overview on how distinct food processing treatments and the digestion process affects allergen structure and its IgE-binding capacity, as key indicators for allergenicity.

Keywords: crustaceans; food allergy; IgE-binding; food processing; digestion

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List of abbreviations

Acryl/Bis - acrylamide/bis-acrylamide
AK – Arginine kinase
APS – ammonium persulfate
ATP - adenosine triphosphate
BCA - Bicinchoninic acid
BSA – bovine serum albumin
BAT - basophil activation test
CFSAN - Centre for Food Safety and Applied Nutrition
CRD - component-resolved diagnosis
DBPCFC – Double blind placebo-controlled food challenge
DNA – Deoxyribonucleic acid
IgE- Immunoglobulin E
ELISA - Enzyme-linked immunosorbent assay
EU – European Union
FAO - Food and Agriculture Organization of the United Nations
FDA – US Food and Drug Administration
HPP - high pressure processing
LC-MS – Liquid chromatography coupled to mass spectrometry
MLC – myosin light chain
MS – mass spectrometry
MW – Molecular weight
N/A – not applicable
PCR - Polymerase chain reaction
PEF - Pulsed electric field
PMSF - phenylmethylsulphonyl fluoride
PUV - pulsed ultraviolet light
RBL – rat basophilic leukaemia
RNA – ribonucleic acid
rpm – revolutions per minute
SCP – Sarcoplasmic calcium-binding protein
SD - standard deviation
SDS-PAGE - Sodium dodecyl-sulphate polyacrylamide gel electrophoresis
SGF – simulated gastric fluid
SIF – simulated intestinal fluid
SSF – simulated salivary fluid

SOFIA - State of World Fisheries and Aquaculture

SPT – Skin-prick test

TBS - Tris-buffered saline

TBST - Tris-buffered saline with 0.1% Tween 20 detergent

TEMED - N, N, N′, N′ -Tetramethyl ethylenediamine

Th1 - type 1 helper T cell

Th2 - type 2 helper T cell

TM - tropomyosin

TMB - Tetramethylbenzidine

UHPLC/Q-TOF-MS - ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry

USA – United States of America

UV/Vis – Ultraviolet/Visible

WHO/IUIS - World Health Organization and International Union of Immunological Societies

Theoretical Part

1 Introduction

Seafood occupies a key role in human nutrition. Seafood species are considered to be rich sources of highly assimilated proteins, polyunsaturated fatty acids (e.g., omega-3), vitamins and zinc, thus contributing to boost the immune system, as well as the heart and brain functions (Khan et al., 2019; Muthukumar et al., 2020). In global terms, the consumption of seafood is substantially dependent on several factors, namely cultural or financial conditions, as well as availability, being a relatively common food source across the world, especially in seaside countries or regions. Due to their health benefits and favourable organoleptic characteristics, the consumption of seafood has been rising. Consequently, there has been an increasing international trade of seafood products (Lopata et al., 2010), causing a higher availability and affordability of crustacean products. Recently, the European Commission, through the State of World Fisheries and Aquaculture (SOFIA 2022), reported that the sector of global fisheries and aquaculture production is predicted to play an even more crucial role in providing food and nutrition in the future. In 2020, the wild capture and the aquaculture production provided 178 million tonnes of aquatic animals, mostly due to the growth of aquaculture. From the same report, it is also possible to conclude that the annual consumption of seafood almost doubled in the last 60 years, being near the 20.2 kg per capita in 2020 (FAO, 2022).

The market globalization, the development of more efficient methods of food transportation and conservation, as well as the application of more strict and standardized rules in quality control are certainly contributing to the growth of seafood consumption worldwide. Countries with large populations such as China, Japan or the USA have observed that (Lopata et al., 2010) the population on the countryside has increased their consumption of seafood products. In Portugal, the consumption of seafood is very high, ranking Portugal with the second or third position in terms of largest consumption of seafood in Europe or World, respectively, with a live weight of 59 kg per capita (Almeida et al., 2015). That fact can be also attributed to its large coast, which promotes a great production/capture of seafood and high availability, as well as a cultural tendency for the consumption of this kind of food products, most likely due to our cultural heritage, and/or our Mediterranean diet based on traditional dishes.

“Seafood” is a very broad term, meaning that it is used to comprise all edible marine species, being typically associated with bony and cartilaginous fish in the phylum of Chordata, and with shellfish. The latter includes two phyla, namely the molluscs and the crustaceans, mostly due to the presence of shell or shell-like exoskeletons in most shellfish species (Khora, 2016; Ruethers et al., 2018). Crustaceans consist of a group of sea animals belonging to the Arthropoda phylum, comprising species like shrimps and crabs. Although

the crustacea taxon includes various orders, most of the species consumed by humans belong to the Decapoda order (**Table 1.1**). Within this order, species typically possess 5 pairs of legs on their thoracic body and 5 pairs of swimmerets located on their abdomen or tail (Lee et al., 2012). It is worth mentioning that, commonly, shrimp and prawn are interchangeable terms, used to refer to the same species in different countries.

Table 1.1 - List of some of the most common crustacean species (adapted from Fernandes et al. (2018)).

Order	Suborder	Infraorder	Superfamily	Family	Species	Scientific name
Decapoda	Dendrobranchiata		Penaeoidea	Penaeidae	Whiteleg shrimp	*Litopenaeus vannamei*
					Speckled prawn	*Metapenaeus monoceros*
					Indian white prawn	*Fenneropenaeus indicus*
					Giant tiger prawn	*Penaeus monodon*
					Jinga shrimp	*Metapenaeus affinis*
					Green tiger prawn	*Penaeus semisulcatus*
				Solenoceridae	Argentine red shrimp	*Pleoticus muelleri*
					Razor mud shrimp	*Solenocera melantho*
					Coastal mud shrimp	*Solenocera crassicornis*
					Knife shrimp	*Haliporoides triarthrus*
				Aristeidae	Scarlet shrimp	*Aristaeopsis edwardsiana*
	Pleocyemata	Caridea	Palaemonoidea	Palaemonidae	Common prawn	*Palaemon serratus*
		Astacidea	Nephropoidea	Nephropidae	Norway lobster	*Nephrops norvegicus*
					European lobster	*Homarus gammarus*
		Achelata		Palinuridae	Caribbean spiny lobster	*Panulirus argus*
		Brachyura	Portunoidea	Portunidae	Smooth swimming crab	*Portunus pelagicus*
					Velvet swimming crab	*Necora puber*
			Cancroidea	Cancridae	Edible crab	*Cancer pagurus*
Scalpelliformes	Scalpellomorpha			Pollicipedidae	Goose barnacle	*Pollicipes pollicipes*

Most of the edible shrimp/prawn species belong to the Penaeidae family (e.g., *Penaeus vannamei*), which are the ones better described in terms of allergenicity. The Caridea taxon species only differ from the Penaeidae in some anatomic and behavioural aspects, such as their brooding methods (Poore, 2004) and are scarcely used in nutrition because of their smaller size (Lee et al., 2012; Ruethers et al., 2018). However, there are some exceptions, like the *Macrobrachium rosenbergii* that grows larger, and has reported consumption and allergenicity (Jirapongsananuruk et al., 2008).

According to the World Fisheries and Aquaculture Report (FAO, 2020), the global aquaculture production of crustaceans, in 2018, was around 9.4 million tonnes, with over half of that amount being whiteleg shrimp (*Penaeus vannamei*). As for the crustacean consumption, it was reported that from 1990 until 2017, there was an average annual

growth, per capita, of 2.9%. However, crustacean species are well known for their allergenic potential, representing an important health risk for a significant portion of the world's population.

1.2 Crustacean food reactions

Due to the increasing consumption of crustaceans, there is a growing possibility of the occurrence of adverse reactions in humans, which can be caused by toxins, parasites, viruses, bacteria, hidden ingredients, additives (non-immunological reactions) or proteins (immunological reactions).

Within the non-immunological reactions, there are some cases that can be highlighted. For example, the biogenic amines are biologically active compounds produced during the microbial enzymatic decarboxylation of specific amino acids, producing amines. The ingestion of these compounds can cause clinical symptoms in sensitive individuals, such as nauseas or heart palpitations (Tabanelli et al., 2018). Biogenic amines have been reported in shellfish, including histamine (Biji et al., 2016; Lopata et al., 2010; Muthukumar et al., 2020) and cadaverine (Zhao et al., 2007), which are markers for potential degradation of the quality and safety of the crustaceans for human consumption. Crustaceans (e.g., xanthid crab) have also been reported as potential vectors of various toxins, such as saxitoxin (Deeds et al., 2008; Li & Persson, 2021) or palytoxin (Deeds & Schwartz, 2010). The first is responsible for a paralytic shellfish poisoning and saxitoxin puffer fish poisoning, which can occasionally lead to fatal conditions (Dorantes-Aranda et al., 2017). Conversely, the latter can target Na^+/K^+ membrane pumps by altering their selectivity, causing various clinical symptoms such as vomiting, bitter/metallic taste, difficulty in breathing, renal failure, and in some cases, death (Deeds & Schwartz, 2010).

In a similar way to any other food, crustaceans are susceptible to microorganism contamination, causing potentially life-threatening clinical symptoms to the consumer. For instance, crustaceans are liable to be contaminated by the well-known *Salmonella* spp. bacteria. *S. weltevreden,* capable of infecting humans (Koonse et al., 2005), was reported to account for 21% of the *Salmonella* presence in aquaculture shrimp water between 2001 and 2003 (Koonse et al., 2005). The most common *Salmonella* strains are known to cause diarrhoea and gastroenteritis (being non-fatal), while some strains can cause complications such as septicaemia, and lead to fatal outcomes (Gut et al., 2018). Crustaceans are also known for bioaccumulating heavy metals (cadmium – Cd, lead – Pb, mercury - Hg), which can cause severe health risks to humans, namely increasing the risk for cancer development (Haseeb-ur-Rehman et al., 2023; Hu et al., 2023).

Concerning the immunological reactions (allergies), they are mediated by the immune system. In the case of food allergies, they are normally mediated by the immunoglobulin E (IgE) and can affect any individual and at any stage of life. Food allergies are most likely developed during childhood, although they can also appear in adulthood or even during elderhood. In the case of allergies to crustaceans, they typically appear later in childhood, as most young children do not eat crustaceans.

1.3 Crustacean food allergy

1.3.1 Mechanism of pathophysiology

Contrarily to other food allergies that can be resolved with the maturation of the immune system, such as the case of egg and/or milk allergies (Sackesen et al., 2019), the allergies towards crustaceans may not fade away throughout adulthood, meaning that they tend to be life-persisting health conditions (Ayuso et al., 2010). The development of crustacean allergy is rather difficult to predict (similarly to other food allergies), and its mechanism of pathophysiology is very complex and not yet well understood.

In fact, there are some proteins that have the capacity to trigger the immune system of sensitised individuals zThis represents the first stage of food allergy (sensitisation), which can be followed or not by subsequent elicitation. On re-exposure to the same allergen(s) through the consumption of the offending food, the allergen transport to the mucosa will cause the binding of the allergens to the specific IgE on the surface of mast cells and basophils, triggering the release of mediators (cytokines, interleukins, histamine), and inducing a cascade of immunological events. When both stages occur, there is the development of a food allergy (Vickery et al., 2011). Besides sensitisation *via* oral ingestion, it is worth noting that it can also occur following skin contact or inhalation of vapour containing proteins, that can happen during cooking or other processing methods (Khan et al., 2019; Pedrosa et al., 2015; Ruethers et al., 2018). Following this exposure, patients may experience airway respiratory symptoms, dermatitis, itching, among others, although is normally less dangerous than the ingestion pathway (Lopata et al., 2010).

The analysis of IgE epitopes can help better understand allergies in specific patients, including the severity of allergic reactions, the (un)tolerance to processed foods, the likelihood of children retaining or not their allergy in adults, or the development of proteins for immunotherapy (Ayuso et al., 2010).

1.3.2 Clinical relevance

From a clinical perspective, allergy symptoms can usually appear within few minutes up to 2 hours of exposure, although delayed immunological responses (>24 h) might also occur. Allergic reactions to crustaceans range from moderate to potentially fatal, often evolving multiple organs/systems, thus resulting in severe and systemic immunological responses. Clinical symptoms can appear after the ingestion of crustaceans, although reactions can occur *via* other pathways, such as the respiratory tract or skin contact. In fact, inhalation of crustacean vapours, during food preparation/processing, has been commonly described as being able to induce patient sensitisation and/or clinical symptoms (Matricardi et al., 2016). In the gastrointestinal tract, the individual may experience nausea, vomiting, abdominal pain, and cramps. On the skin, itching, hives, redness, rashes, or swelling, known as angioedema, may occur. In the respiratory system, cases of occupational asthma or dermatitis have been reported, with varying degrees of severity (Zhao, Timira, et al., 2022). Life-threatening anaphylactic reactions are another clinical symptom of crustacean allergy, which often result in emergency hospitalization (Matricardi et al., 2016).

1.3.3 Allergy management

Crustacean ingestion by allergic patients can occur due to several reasons, such as inaccurate labelling of food products, presence of hidden crustacean residues (for example, lack of quality control in shared production lines, cross-contamination occurrence) during manufacturing, the patient not being aware of self-health condition, or underestimation of the disorder. Therefore, there are several ways to address this issue. The complete avoidance of crustacean ingestion is typically the recommended preventive action for the allergic patients, but this measure alone may not be enough since the absence of allergens does not always depend on the patient. Rigorous quality control in manufacturing and transport of seafood, strict laws on labelling, educating the masses and good healthcare systems are all efficient measures that can help decrease the number and severity of allergic reactions. Alternative approaches, such as oral and subcutaneous immunotherapies are being actively investigated. However, it is important to note that currently there are no immunotherapies for crustacean allergies that may have yielded satisfactory results (Khan et al., 2019).

Due to their importance as a food allergen source, the crustaceans were firstly recommended for mandatory labelling by the *Codex Alimentarius* to protect crustacean-allergic consumers (FAO/WHO, 1985). Although there are few laws regarding food allergen management, recommendations on prevention (e.g., how to avoid cross-contamination) are

established by organizations such as *Codex Alimentarius*, which sets up standards for food-related topics, including food allergy, transport or labelling (FAO/WHO, 2023). Based on these recommendations, different authorities can establish their own country or area-specific guidelines. Presently, crustaceans are recognised as part of the nine key food allergen sources by the Food and Agriculture Organization (FAO) of the United Nations, which are the 9-group of foods (crustaceans, fish, eggs, milk, wheat, peanuts, tree nuts, sesame, and soybean) (CFSAN-FDA, 2023) responsible for more than 90% of the allergic reactions reported. Therefore, crustaceans are of mandatory labelling in several countries/regions (Gendel, 2012). In the European Union, the Regulation (EU) No 1169/2011 (enforced since December 2014) issued the mandatory labelling of 14-groups of allergenic foods, including crustaceans, which must be highlighted from the rest of the list of ingredients (EU, Regulation No 1169/2011). Hence, the study and understanding of crustacean allergens, and the development of strategies to not only minimize their allergenic potential, but also to improve the quality of life of individuals who suffer from this condition is obviously of great importance.

1.3.4 Prevalence of crustacean allergy

The general consensus is that shellfish allergy must be around 0.5-2.5% (Khan et al., 2019) of the global population. Regarding the geographical location, shellfish allergy prevalence tends to be higher in Asian countries. In Europe, coastal countries are the ones with increased reports of shellfish allergy (Khan et al., 2019). This is supported by the greater consumption of seafood in both Asian and coastal countries. In what concerns shellfish, crustacean-induced allergy is reported to be more common when compared to molluscs (**Table 1.2**).

Table 1.2 - Prevalence of shellfish food allergy (crustaceans vs molluscs) considering data from all continents and age groups, and comparing different allergy prevalence determination methods (Moonesinghe et al., 2016)

Method	Crustaceans	Molluscs
Questionnaire-based	0.1%-5.5%	0.4%-1.5%
Sensitisation rates	0%-10.3%	0%
Oral food challenges	0%-0.9%	0.1%

It is worth mentioning that, although that seems to be true, especially with oral food challenges data (0-0.9% for crustaceans vs 0.1% for molluscs), there are few studies that report crustacean and mollusc prevalence separately, and a lot of reports only evaluate shrimp/crustacean allergy, which decreases the number of studies that can be considered

to support these conclusions, hindering their accuracy (Moonesinghe et al., 2016). Crustacean allergy is a complicated health condition. Thus, the actual determination of its prevalence has proven to be complex and still at a very preliminary stage. Each patient can be sensitised to different allergens and/or foods, often experiencing possible cross-reactivity with allergens and/or proteins of other species, meaning that the severity of the clinical symptoms may vary.

The lack of knowledge of patients on this issue is also a factor that contributes to the unreliable data. Individuals with some kind of food adverse reactions (including food allergies), often tend to exacerbate self-clinical symptoms, which restricts the correct classification of crustacean allergy. Additionally, the cultural differences or incomplete information on medical history are all factors that make it harder to correctly describe/classify patient allergy and to gather reliable information on the prevalence of crustacean allergy. Since most prevalence studies are based on surveys/questionaries made to general population, which often results on self-reported allergy, meaning that the data is most likely inflated. The use of data on specific IgE-binding and skin prick tests (SPT) is more accurate, but it only provides information on the prevalence of crustacean sensitisation. This means that individuals can have IgE-binding to crustacean allergens, but it may not be enough to induce the immunological processes (e.g., the release of immunological mediators such as histamine) necessary for the development of clinical symptoms. Only symptomatic individuals are considered allergic, and therefore the IgE-binding information, although a valuable indicator, is not enough to diagnose crustacean allergy. On the other hand, most prevalence studies on crustacean allergy are done to certain social groups, on a specific region or age group, introducing unwanted bias to the results. Open food challenges and double-blind placebo-controlled food challenges (DBPCFC) would be the most adequate methods, but they are logistically hard to perform since they require a hospital environment and patients that are willing to submit themselves to experiencing unwanted allergic symptoms (Zuidmeer et al., 2008). From the available data, most also report the prevalence of shellfish allergy, instead of crustacean allergy, constituting another constrain in determining the correct values.

Regarding age-specific prevalence, it is believed that shellfish allergy, unlike some other food allergies, is persistent, not being resolved into adulthood, even with some people reporting the development of shellfish allergy as adults (Sicherer & Sampson, 2018). Data supports this belief, with a report from Denmark stating higher prevalence of shrimp allergy in 22 year-olds (0.2%) when compared to 3 year-olds and younger (0%), using oral food challenge, and confirmed by double-blind placebo-controlled food challenges (Osterballe et al., 2005). This is confirmed in questionnaire-based studies, for example in America, with self-reported shellfish allergy prevalence in children going from 0.06% to 2.0%, whereas

adult results fluctuated between 1.7% and 9.0% (Moonesinghe et al., 2016). Using more reliable tests, like the immunoCAP, the sera from 4522 allergic patients from 13 countries belonging to the European Union, USA, and Australia, were evaluated towards different allergenic foods. In this study, it was possible to determine the prevalence of crustacean-allergic patients using shrimp as model food, which reached an overall proportion of 5.4% and with countries (e.g., Italy) presenting values as high as 10.2% (Burney et al., 2010).

It is important to remember that different studies may have different conclusions, depending on the parameters chosen. Besides the already mentioned social and geographical bias, the choice of a method of prevalence determination, number of individuals, confidence intervals, or the restrictions for the elimination of certain inconclusive or incomplete results can vary, and, consequently, have an impact on the results for the determination of actual prevalence of crustacean allergy.

1.3.5 Diagnosis

Allergy diagnosis comprises different steps, which usually begins with a well-documented clinical report on adverse responses to food, followed by specific exams, such as skin-prick tests and specific IgE quantification, and if there are any doubts, clinicians can also perform oral food challenges. More recently, methods like the component-resolved diagnosis (CRD), which uses specific allergens to better define the potential allergy, or the basophil activation tests (BAT) through the stimulation of basophils collected from allergic patients with specific food extracts/components have been advanced as more effective diagnostic tools (Matricardi et al., 2016). However, all these methods have different *pros* and *cons*, and since they evaluate distinct parameters, they can be combined to help in getting a differential diagnosis.

The diagnosis of a food allergy can be difficult to assess because contradictory results can be obtained depending on the method used, the species analysed and patient-specific characteristics. For example, it is well reported that shellfish allergy is more common in adults, as evidenced in **Figure 1.1**. Nevertheless, the mapping of the IgE epitopes has shown that sensitisation is significantly greater in children (for tropomyosin, frequency of allergen recognition in children was 94%, whilst in adults was 61%) (Ayuso et al., 2010).

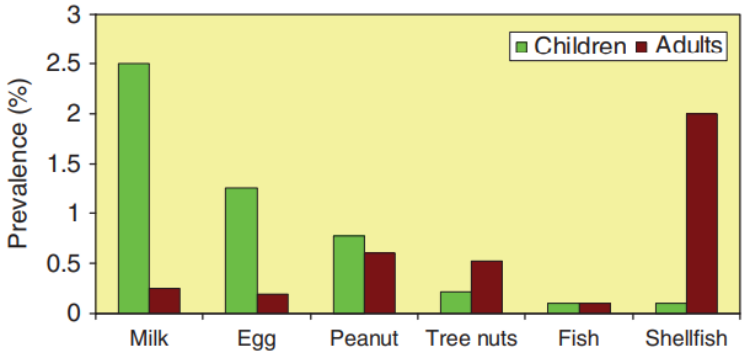

Figure 1.1 - Results of the prevalence of a self-reported allergy survey in children and adults (retrieved from (Lopata et al., 2010)).

1.3.6 Relevant crustacean allergens

The causative agents of crustacean allergies are the allergens (proteins or glycoproteins), which are natural compounds of foods. In general, food allergens are commonly described as small structures (10-70 kDa) possessing compact conformations, although more recently, structures of higher order have been increasingly described in new allergens (Costa, Bavaro, et al., 2022; Costa, Villa, et al., 2022). Food allergens are also defined as presenting high stability towards heat and high resistance to pepsin (gastric digestion). However, there are several potent allergens that are heat-labile and pepsin-sensitive, meaning that other unknown factors may also contribute to increase the allergenic potential of some proteins. Food allergens possess conformational (that depend on the structure of the allergenic protein) and/or sequential/linear (depends on the protein primary sequence) epitopes that are the binding regions responsible for the IgE recognition (Costa, Bavaro, et al., 2022; Costa, Villa, et al., 2022).

Considering that crustaceans include a huge number of different species, the number of allergenic proteins inducing potential allergic reactions is also high. In terms of diversity, crustacean allergens are included in different protein families, being most of them encompassed in tropomyosins, arginine kinases (AK), myosin light chain (MLC), sarcoplasmic calcium-binding proteins, among others.

1.3.6.1 Tropomyosins

Tropomyosins are a family of proteins with 284 amino acids, having a molecular weight around 33-39 kDa, and an isoelectric point of 4.2. Regarding their structure, tropomyosin is a coiled-coil homo-dimeric structure (Ruethers et al., 2018), with two parallel α-helices, allowing the binding of seven actin monomers. Additionally, the interaction of a repeated

sequence of seven amino acids with hydrophobic residues every first and fourth positions was reported (Ruethers et al., 2018). All of these properties may contribute to its known thermostability that allows tropomyosin to retain its immunoreactivity (Kamath et al., 2013). This protein is mainly, but not exclusively, found in the muscle cells of both vertebrates and invertebrates, where it interacts with both actin and myosin, contributing to the regulation of muscle contraction, cellular morphology and motility (Zhao, Timira, et al., 2022).

Despite its presence in vertebrates, this protein has only shown allergenic potential in invertebrate organisms, such as crustaceans and molluscs. Tropomyosin is the main crustacean allergen, having been confirmed in 16 crustacean species by WHO/IUIS (2023) (**Table 1.3**).

Table 1.3 - List of crustacean tropomyosins classified as food allergens by the WHO/IUIS (2023).

Allergen	Species (Scientific name)	Species (Common name)	Approximate MW (kDa)
Cha f 1	*Charybdis feriatus*	Crab	34
Cra c 1	*Crangon crangon*	North Sea shrimp	38
Exo m 1	*Exopalaemon modestus*	White legged freshwater shrimp	38
Hom a 1	*Homarus americanus*	American lobster	34
Lit v 1	*Litopenaeus vannamei*	White shrimp	36
Mac r 1	*Macrobrachium rosenbergii*	giant freshwater prawn	37
Mel l 1	*Melicertus latisulcatus*	King prawn	38
Met e 1	*Metapenaeus ensis*	Shrimp	34
Pan b 1	*Pandalus borealis*	Northern shrimp	37
Pan s 1	*Panulirus stimpsoni*	Spiny lobster	34
Pen a 1	*Penaeus aztecus*	Brown shrimp	36
Pen i 1	*Penaeus indicus*	Shrimp	34
Pen m 1	*Penaeus monodon*	Black tiger shrimp	38
Por p 1	*Portunus pelagicus*	Blue swimmer crab	39
Pro c 1	*Procambarus clarkii*	Red swamp crayfish	36
Scy p 1	*Scylla paramamosain*	Mud crab	38

Tropomyosins are classified as panallergens since they have highly conserved structures among different non-related species, being responsible for more than half of specific IgE-binding in crustacean allergic patients' sera (Ruethers et al., 2018; Zhao, Timira, et al., 2022). According to the IgE epitope mapping, there are reports that indicate that tropomyosin is the allergen most likely to contribute to the persistence of shrimp allergy through adulthood. Tropomyosin is estimated to be the most frequent primary sensitiser of crustacean allergy, since the IgE present in the sera of allergic patients recognise tropomyosin more consistently than other allergens. In fact, tropomyosin is reported to be 30% more IgE-recognised when compared to arginine kinase (AK), myosin light chain (MLC) and sarcoplasmic calcium-binding protein (SCP) (Ayuso et al., 2010).

1.3.6.2 Arginine kinase

Arginine kinase is an enzyme that plays a role in the energy metabolism of invertebrates by regulating the ATP levels in cells. Structurally, it consists of a monomeric, water soluble (Zhao, Timira, et al., 2022) and thermolabile (Khan et al., 2019) protein, with an isoelectric point of 6.0 and 359 amino acids corresponding to a molecular weight of 38-45 kDa. Regarding its secondary structure, the N-terminal zone is reported to have an α-helix domain, whilst the C-terminal is constituted by an α-β domain. AK is one of the most relevant crustacean allergens, following tropomyosin, with WHO/IUIS (2023) confirming 7 crustacean species containing AK as an allergen (**Table 1.4**). Arginine kinase represents 10-51% of positive IgE binding in shrimp-allergic individuals, which means it has no clear classification as major or minor allergen (Zhao, Timira, et al., 2022).

Table 1.4 - List of crustacean arginine kinases classified as food allergens by the WHO/IUIS (2023).

Allergen	Species (Scientific name)	Species (Common name)	Approximate MW (kDa)
Cal b 2	*Callinectes bellicosus*	Warrior swimming brown crab	40
Cra c 2	*Crangon crangon*	North Sea shrimp	45
Lit v 2	*Litopenaeus vannamei*	White shrimp	40
Mac r 2	*Macrobrachium rosenbergii*	giant freshwater prawn	40
Pen m 2	*Penaeus monodon*	Black tiger shrimp	40
Pro c 2	*Procambarus clarkii*	Red swamp crayfish	40
Scy p 2	*Scylla paramamosain*	Mud crab	40

1.3.6.3 Myosin light chain

Myosin light chain is, similarly to tropomyosin, a part of the muscle contraction structure, promoting the hydrolysis of ATP to move actin filaments (Khan et al., 2019). Myosin is a multimeric protein, with two heavy chains (of around 200 kDa) that bind to four light chains (of 18-20 kDa), and binds Ca^{2+} through its EF-hand domain (helix-loop-helix) (Ruethers et al., 2018; Zhao, Timira, et al., 2022). So far, 6 MLC proteins were identified and included in the WHO/IUIS (2023) list of allergens (**Table 1.5**).

Table 1.5 - List of crustacean myosin light chains classified as food allergens by the WHO/IUIS (2023).

Allergen	Species (Scientific name)	Species (Common name)	Approximate MW (kDa)
Cra c 5	*Crangon crangon*	North Sea shrimp	17.5
Pro c 5	*Procambarus clarkii*	Red swamp crayfish	18
Scy p 3	*Scylla paramamosain*	Mud crab	18
Hom a 3	*Homarus americanus*	American lobster	23
Lit v 3	*Litopenaeus vannamei*	White shrimp	20
Pen m 3	*Penaeus monodon*	Black tiger shrimp	20

MLC causes IgE sensitisation in less than one third of individuals, and it is considered as crustacean minor allergens (IgE-binding frequency < 50% (Caraballo et al., 2021)). Most of the known epitopes of this allergen are conformational, which is related to its relative heat and pH stability, since conformational epitopes do not survive thermal processing in heat labile proteins (Zhao, Timira, et al., 2022).

1.3.6.4 Sarcoplasmic calcium-binding protein

Also found in muscles of invertebrates, sarcoplasmic calcium-binding protein contains EF-hand domain, with helix-loop-helix motifs. This protein controls the calcium concentration in the cytosol, and is a part of calcium binding mechanisms, regulating muscular contraction and relaxation by moving Ca^{2+} from myofibrils (Khan et al., 2019; Ruethers et al., 2018). This family of proteins has molecular weight around 20-25 kDa, and isoelectric points of 4.6 (**Table 1.6**). Until now, five SCP have been identified and classified as food allergens in different species of crustaceans.

Table 1.6 - List of crustacean sarcoplasmic calcium-binding proteins classified as food allergens by the WHO/IUIS (2023).

Allergen	Species (Scientific name)	Species (Common name)	Approximate MW (kDa)
Cra c 4	*Crangon crangon*	North Sea shrimp	25
Lit v 4	*Litopenaeus vannamei*	White shrimp	20
Pen m 4	*Penaeus monodon*	Black tiger shrimp	20
Pon l 4	*Pontastacus leptodactylus*	Narrow-clawed crayfish	24
Scy p 4	*Scylla paramamosain*	Mud crab	20

1.3.6.5 Miscellaneous allergens

In addition to the main crustacean allergen families described so far, there are other proteins with confirmed potential to cause sensitisation in allergic individuals. These allergens are considered minor, as less than 50% of the IgE of sensitised individuals react to them. Therefore, the classification of minor allergen does not account with the potential severity of the allergic reactions, as patients reactive to them may also experience severe allergic responses. Within crustacean species, proteins like triosephosphate isomerase, troponin, fatty acid binding protein, hemocyanin, among others, are also classified as food allergens. So far, more than 15 allergens from different families and distinct species have been characterised and included in the list of allergens from WHO/IUIS (2023) (**Table 1.7**).

Triosephosphate isomerase consists of a dimeric protein with 26-29 kDa that participates in glucose energy metabolism as an enzyme. At least five of these proteins (Arc s 8, Cra c 8, Pen m 8, Pro c 8, Scy p 8) were identified as crustacean allergens.

Troponin is a protein complex that is involved in muscle contraction regulation and consists of three subunits (C, I and T), two of which have the potential for IgE-binding capacity in allergic patients (C and I). On one hand, and in a similar way to myosin light chain, troponin C performs its function through the binding of calcium ions. On the other hand, troponin I contributes to muscle regulation by binding to actin, blocking actin and myosin interaction and inhibiting muscle contraction. (Ruethers et al., 2018). So far, four of these proteins (3 troponin C and 1 troponin I) have been classified as food allergens in crustaceans.

Table 1.7 - List of some crustacean proteins classified as food allergens by the WHO/IUIS (2023).

Allergen family	Allergen	Species (Scientific name)	Species (Common name)	Approximate MW (kDa)
Triosephosphate isomerase	Arc s 8	*Archaeopotamobius sibiriensis*	Crayfish	28
	Cra c 8	*Crangon crangon*	North Sea shrimp	28
	Pen m 8	*Penaeus monodon*	Black tiger shrimp	27
	Pro c 8	*Procambarus clarkii*	Red swamp crayfish	28
	Scy p 8	*Scylla paramamosain*	Mud crab	28
Troponin C	Cra c 6	*Crangon crangon*	North Sea shrimp	21
	Hom a 6	*Homarus americanus*	American lobster	20
	Pen m 6	*Penaeus monodon*	Black tiger shrimp	16.8
Troponin I	Pon l 7	*Pontastacus leptodactylus*	Narrow-clawed crayfish	30
Fatty Acid Binding Protein	Pen m 13	*Penaeus monodon*	Black tiger shrimp	20
	Lit v 13	*Litopenaeus vannamei*	White shrimp	15
Hemocyanin	Pen m 7	*Penaeus monodon*	Black tiger shrimp	76
Glycogen phosphorylase-like protein	Pen m 14	*Penaeus monodon*	Black tiger shrimp	95
Filamin C	Scy p 9	*Scylla paramamosain*	Mud crab	90
Ovary development-related protein	Eri s 2	*Eriocheir sinensis*	Chinese mitten crab	28.2

Fatty acid binding protein, as the name suggests, can bind to lipids, which allows this protein to contribute to the transport and metabolism of cytosolic long chain fatty acids. It is a small protein, having a molecular weight of 15 kDa (Múnera et al., 2022) and only two were identified as allergens in crustaceans. Hemocyanin is a heat-stable, hexameric (each subunit having around 75 kDa) protein complex, that is a part of the oxygen transport and storage mechanism. Glycogen phosphorylase-like protein, filamin C, and ovary development-related protein are also confirmed allergens by the WHO/IUIS (2023). However, these allergens are rare and still scarcely investigated, and seldom referred to in literature, meaning that it is very difficult to find additional information on these allergens.

It is worth mentioning that, typically, minor allergens have less IgE recognition by patient sera (15-23% for triosephosphate isomerase or 12-29% for troponin), when compared to major allergens like tropomyosin or myosin light chain (72-98% for tropomyosin and 19-55% for myosin light chain) (Ruethers et al., 2018), a fact that contributes to “major” and “minor” allergen definitions. Furthermore, other proteins have been reported as possible

crustacean allergens, but they are still under investigation concerning their allergenic and physiochemical properties and have not been confirmed by WHO/IUIS (2023). In particular, that is the case for myosin heavy chain (Saenz de San Pedro et al., 2023), phosphopyruvate hydratase (Yang et al., 2021) and titin (Kamath et al., 2014), among others.

In summary, a lack of crustacean allergen information is noticeable, aligned with the incomplete or contradictory reports on allergen sources, the difficulty and inconsistency in allergen definition, which are factors that lead to the need for further investigation of crustacean proteins as potential allergens.

1.3.7 Cross-reactivity phenomenon

Cross-reactivity is defined as to the binding of the same IgE molecule to different allergens with common structural features, meaning that the same repertoire of IgE can recognise, bind, and induce an immune response to similar allergenic molecules (homologues) (Costa & Mafra, 2022; Popescu, 2015). When a patient has a confirmed allergy, it is common to experience some cross-reactivity with allergens from other non-related species, indicating the potential to elicit an allergic reaction to other food that the patient was not aware of. Cross-reactivity might be the consequence of structural or sequential similarities, originated by the immunorecognition of similar epitopes (Cheng et al., 2021).

Most crustacean allergens, such as tropomyosin, have been extensively studied in terms of cross-reactivity among invertebrates, specifically crustaceans, molluscs, insects, mites, fish, and parasites. Analysing the sequence of tropomyosin in molluscs and crustaceans, it was reported that 49% of the primary structure of tropomyosin is conserved between both groups of species. Within crustaceans, there is as much as 89% conservation of the primary sequence among the species (Kamath et al., 2013). Also, crustacean tropomyosins have high sequence homology with cockroaches and dust mites (over 75% in both cases), which can be explained by their phylogenetic closeness (Ruethers et al., 2018) and confirmed cross-reactivity (Klueber et al., 2020).

Arginine kinase is also a proven allergen in various invertebrates. Apart from crustaceans, its potential to elicit allergic reactions has been confirmed in molluscs (Khan et al., 2019), mites, cockroach, moths and silkworm (**Figure 1.2**). Structural similarities between crustacean and mollusc arginine kinase have been reported (Ayuso et al., 2010; Ruethers et al., 2018) sharing up to 54% of sequence homology (Zhao, Timira, et al., 2022). In contrast, sarcoplasmic calcium-binding protein has only 15-21% of sequence homology among crustaceans and molluscs, which probably indicates a lower probability of cross-reactivity (Khan et al., 2019).

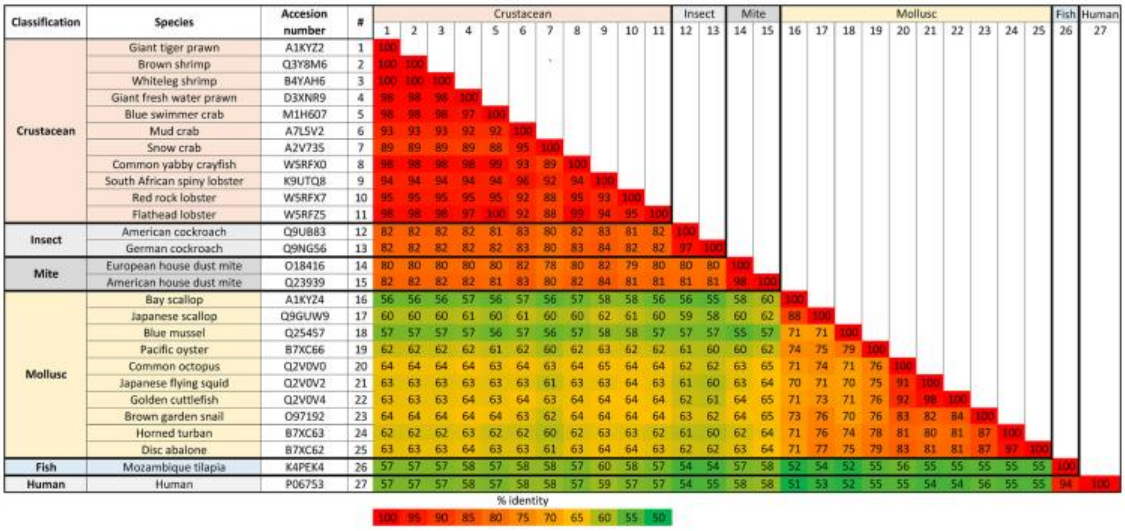

Figure 1.2 - Sequence homology (in %) of tropomyosin between crustacean, insect, mite and mollusc species, as well as fish and humans, reflecting the possibility of cross-reactivity (retrieved from Ruethers et al. (2018)).

1.4 Processing effect on crustacean allergens

Since ancient times, it has been well established that processing treatments have the ability to change the state and physicochemical properties of foods, thus contributing to increase their stability, shell-life, organoleptic properties (aroma and flavour) and principally their safety (elimination of microbiology hazards) (Vanga et al., 2017). However, with the advance of novel and more efficient processing technologies, the specific effects of processing treatments on protein allergenicity are still quite unknown, especially considering that within a food there are different allergens, each one often presenting distinct physicochemical properties (Costa, Bavaro, et al., 2022; Costa, Villa, et al., 2022).

One way of decreasing the allergenicity of foods is by using food processing technologies that change allergen structure and/or sequence. The destruction of conformation epitopes (by denaturing/altering conformational structure) or linear ones (breakdown of protein primary structures) can be efficient in diminishing the allergenicity of certain proteins, although not all allergens behave alike. In fact, some proteins are known to form aggregates, or the denaturing states can uncover linear epitopes, both factors that can contribute to increase protein allergenicity. Regarding the impact of food processing on protein allergenicity, there are still effects to be determined, especially on how proteins behave under a specific processing treatment or a combination of food treatments (hurdle technology) (Costa, Bavaro, et al., 2022; Costa, Villa, et al., 2022; Khan et al., 2019). Therefore, it would be beneficial to submit food to procedures that reduce, or even eliminate their allergenicity, thus improving patients' safety and their quality of life. Anyhow, the study

of allergies at a molecular and clinical levels, and the complexity, specificity and uniqueness of each patient allergy makes it hard to accomplish a world without food allergy.

1.4.1 Thermal processing

1.4.1.1 Boiling, baking, and frying

Crustaceans can be consumed in different manners: raw, partially, or fully cooked (Muthukumar et al., 2020). The choice of preparation often depends on factors such as geographical location, cultural practices, and/or to the specificities of the species of crustaceans. Traditional thermal processing methods include boiling, baking, grilling, or frying, being mainly used to eliminate pathogens (ensuring food safety) and to achieve/increase specific sensory properties (Cheng et al., 2021; Khan et al., 2019). However, it is important to note that the cooking process can affect the characteristics of the food, including the properties of its proteins. Various phenomena, such as denaturation/renaturation or aggregation can occur during processing, and their impact on allergenicity may greatly vary depending on the extent to which the epitopes and their ability to bind IgE are affected (Costa, Villa, et al., 2022).

In general, thermal-stable proteins, such as tropomyosin, are more prone to resist heat treatments and to retain or increase its immunoreactivity. In fact, some reports indicate an allergenicity enhancement after thermal processing of crustacean tropomyosin, with heat-processed samples having increased IgE-binding capacity, basophil activation and cutaneous responses (wheal size) in skin-prick tests (Costa, Villa, et al., 2022). This is most likely caused by the exposure of hidden linear epitopes or the generation of neo-epitopes, due to processes such as aggregation, formation of noncovalent bonds, or refolding (Cheng et al., 2021; Khan et al., 2019).

On the other hand, arginine kinase and other heat-labile proteins are more susceptible to the effects of thermal processing. This may result in significant changes in the tridimensional structure of proteins and in the destruction of conformational epitopes, which leads to a reduction in the IgE-binding capacity and, consequently, a decrease in their allergenicity (Costa, Villa, et al., 2022). In fact, it was found that heating arginine kinase above 80 °C leads to a significant decrease or complete elimination of its IgE-binding ability, mainly due to the complete unfolding of AK. In addition, AK could not be extracted from various cooked shrimps (*L. vannamei*), being mainly attributed to modifications, degradation, or aggregation of the protein within the food matrix during the heating process (Zhao, Timira, et al., 2022).

After subjecting shrimp to heat-processing and digestion with trypsin, the released peptides were analysed by mass spectroscopy. From this analysis, it has been found that

the quantity of peptides released from the major crustacean allergens varied (e.g., tropomyosin, arginine kinase, myosin light chain, and sarcoplasmic binding protein). Specifically, the number of peptides released from arginine kinase and sarcoplasmic calcium-binding protein significantly decreased in the baked and fried shrimp compared to the raw sample. Conversely, the number of tropomyosin peptides was lower, indicating that tropomyosin is more resistant to heat processing, thus preserving its structural integrity. Additionally, protein sequencing has shown that, due to its intrinsic allergenicity and higher number of known epitopes, tropomyosin exhibited the largest number of fragments and had the highest epitope matches among the four allergens studied (Xu et al., 2022). It is also interesting to mention that one of tropomyosin's fragments was common in all three samples and corresponded to a dominant T-cell epitope, even though most fragments were raw-specific. Overall, heat processing primarily reduced the reactivity of epitopes located in segments maintaining native secondary structures, while enhancing the IgE-binding capacity through non-native refolded regions.

A study conducted on the allergenicity of crab and white shrimp indicated that thermal processing, such as boiling and frying, generally led to a decrease in the quantity of allergens as detected by SDS-PAGE. However, it was observed that tropomyosin showed resistance to these treatments. The intensity of the bands associated with tropomyosin increased after the samples were subjected to these processing methods, and significant denaturation of this protein was only observed after autoclaving at much higher temperatures. This suggests that tropomyosin is resistant to the temperatures typically used in food processing. Regarding the IgE-binding capacity, as determined by ELISA, a general decrease in reactivity of IgE was reported for various allergens in shrimp. Nonetheless, some reactivity remained, likely due to the presence of smaller molecular weight peptides containing preserved reactive epitopes. In contrast, crab samples showed a different trend, with a 23% increase in reactivity after frying and minimal changes after boiling and autoclaving, indicating that the crab shell provides protection during processing (Arwani et al., 2022).

1.4.1.2 Glycation (Maillard Reaction)

Another relevant reaction is the glycation or Maillard reaction. It occurs when amino acid residues chemically interact with sugar at moderate/high temperatures. The effect of Maillard reaction has been previously reported for crustacean allergens. Tropomyosins, which are abundant in lysine residues, can readily react with reducing sugars at moderate/high temperatures, leading to the formation of Maillard products. It has also been reported that some tropomyosin epitopes contain lysine residues (Zheng et al., 2011), which

is likely to be one of the reasons for the reported decreased IgE-binding capacity after glycation (Yuan et al., 2017; Zhang et al., 2019). In fact, glycation (with glucosamine) has also been associated with the unfolding of tropomyosin, as well as cross-linking phenomena, changing tropomyosin's structure, and influencing its allergenicity (Yuan et al., 2017). Arabinose-induced glycation in crab has been shown (Han et al., 2018) to alter the structural properties of both tropomyosin and arginine kinase, increasing beta-turn structures and hydrophobic regions in both allergens, as well as beta-sheets in arginine kinase. In the referred report, it was also concluded that glycation induced the reduction of disulphide bonds in arginine kinase, key components of protein structure (Han et al., 2018).

In summary, it has been demonstrated that glycation is able to alleviate allergenicity in crustaceans, as shown by the reported decrease in immunoreactivity of both glycated arginine kinase and tropomyosin with various sugars. However, it has also been described that glycation may be able to both induce aggregation of allergens, and contribute to the formation of neoepitopes, which can increase immunoreactivity, for example, for tropomyosin (Costa, Villa, et al., 2022).

Further research is needed to investigate the observed tropomyosin variants in this study and to confirm the presence and impact of Maillard products following the heating of shellfish (Kamath et al., 2013). Furthermore, the effects of different sugars (Fu et al., 2019), as well as the immunological and clinical effects of glycation as a method to reduce allergenicity are also topics still lacking information.

1.4.2 Non-thermal processing

1.4.2.1 Acid treatment

The information on the effect of acid treatment on food allergenicity is still very limited. The application of acid treatment as a food processing method includes the use of acidic compounds, which are not sustainable. Besides, the acid treatment is a process that cannot be efficiently controlled, and it has the potential to inflict large amounts of damage to the sensorial, nutritional, and shelf-life properties of food systems. However, despite a low potential for the food industry, it is still worth analysing the effects of acid treatment for meal preparation. One way to introduce acidic treatment in a confirmed safe way is by using vinegar (contains acetic acid). Crustaceans soaked in vinegar have been related to induce low IgE-reactivity for allergic patients as assessed by SPT (Perez-Macalalag & Sumpaico, 2006), being potentially correlated with the modulation of conformational epitopes in tropomyosin when comparing different pH values and soaking periods (Lasekan & Nayak, 2016). Nonetheless, these reports cannot really guarantee the effectiveness of the acid treatment, with an incomplete explanation on the reason for their results on the first case,

and a heterogeneous distribution of tropomyosin on the samples due to different solubility, which influences conclusions.

1.4.2.2 Enzymatic hydrolysis

The enzymatic treatments have the potential to decrease the allergenicity of proteins by altering their conformation, destroying both conformational and sequential epitopes. The use of enzymes to process foods is very safe to consumers since it does not include the addition of any unknown or possibly hazardous compound. Besides, the enzymatic hydrolysis is compatible with other methods, although it depends on several factors like pH, temperature, substrate concentration, etc (Costa, Villa, et al., 2022), which is likely to improve the reduction/mitigation of food allergenicity (Khan et al., 2019). Processing methods inducing protein hydrolysis often include the use of digestive enzymes, such as pepsin or trypsin (Huang et al., 2010; Mejrhit et al., 2017), as well as other technological enzymes (alcalase, papain, etc).

To achieve the main goal of reducing/eliminating the allergenic potential of foods, there are two pathways: breaking down/cleaving proteins into smaller peptides or amino acids by destroying protein structure and sequence; or through internal cross-linking (between amino acids of the protein of interest) or cross-linking to other proteins/compounds, with both processes causing alterations to allergen structure (Cheng et al., 2021). There have been some productive attempts to reduce protein allergenicity by cross-linking process. It was shown that by cross-linking tropomyosin with laccase, laccase/coffee acid and transglutaminase of *Metapenaeus ensis* (shrimp), there was a decrease in the IgE-reactivity, leading to the promotion of some immunological changes that reduced allergenicity, by lowering cellular degranulation or balancing the Th1/Th2 ratio (Ahmed et al., 2020). In fact, the IgE-binding capacity of tropomyosin was decreased >30% after cross-linking with transglutaminase (Yuan et al., 2017). Other enzymes, such as tyrosinase and horseradish peroxidase have also shown potential in decreasing the allergenicity of crustaceans, inducing 34.5% and 63.5% reduction of tropomyosin IgE-binding capacity, respectively (Cheng et al., 2021).

1.4.2.3 High pressure processing

Pressure application to food is already a well-established method of processing that has been indicated to improve food safety and sensorial characteristics, as well as extending the shelf-life of food products (Costa, Villa, et al., 2022). However, since the methods based on pressure application (e.g., high pressure processing – HPP) are considered novel

technologies, it is still uncertain how they can impact the allergenicity of different proteins. In the case of some crustacean allergens like tropomyosins, which seem to be pressure-labile, the application of pressure processing seems to contribute to decrease their allergenic potential, especially when pressure methods are combined with the application of heat (Costa, Villa, et al., 2022; Long et al., 2015).

The application of HPP using 100-800 MPa seems to alter the conformational properties of proteins, inducing phenomena of unfolding, aggregation among others, by damaging/breaking weak non-covalent bonds and interactions, like hydrogen bonds. Regarding the potential mitigation of crustacean allergens, HPP has shown results in decreasing protein IgE-binding capacity, depending on the amount of time and pressure applied (for example, too much pressure can re-expose IgE-reactive epitopes) (Long et al., 2015). HPP seems to be able to reduce the IgE binding capacity (and potentially allergenicity) by facilitating further enzyme action during digestion (Khan et al., 2019). When combining heat and pressure, there are reports of a ~74% decrease in the IgE-reactivity of shrimp (55 ºC, 500 MPa, 10 min) (Long et al., 2015) showing the great potential of this pressure/temperature association for mitigating crustacean protein allergenicity.

1.4.2.4 Cold plasma

Another innovative technique that has been progressively used in food processing is the cold plasma. This technology involves the use of non-heated plasma, making it useful for food processing, since it does not use heat and thus avoiding the formation of possibly undesired organoleptic alterations on specific foods. Plasma formation includes the generation of ionized species (oxygen and nitrogen reactive species, for example) that are able to interact with the food matrix and to affect protein properties (Zhao, Timira, et al., 2022). Cold plasma has applications on food safety and technology, allowing microorganism elimination and inducing changes in protein conformation. Therefore, it has been analysed for potential food allergen mitigation by changing IgE-binding epitopes, which seems to have already generated promising results. In this sense, a 89% decrease in reactivity has been achieved for soybean allergens in protein isolates (Meinlschmidt et al., 2016).

Regarding crustaceans, prawn has been submitted to cold plasma and a ~18% reduction in the IgE-binding capacity of tropomyosin was observed, following modifications of the secondary and tertiary protein structures. However, the information on the effects of cold plasma technology on crustacean allergenicity is limited, and the mechanism that induces reactivity reduction is still not well understood (Ekezie et al., 2019). At this moment, cold plasma can potentially be useful for food allergen mitigation, most likely in combination with

other techniques, such as enzymatic hydrolysis, but further investigation is still necessary for a more effective application (Ekezie et al., 2019).

1.4.2.5 Ultrasound

Another environmentally friendly procedure that has been tested for its potential for allergenic mitigation is the use of ultrasound methodologies. High-intensity ultrasound viability for dehydration, for example, is already proven. This technology is based on the application of high-energy sound waves (with a frequency up to 100 kHz) that allow the formation and collapse of sonication bubbles. The collapse of these cavities releases energy and pressure into the surrounding area, causing internal agitation that impacts the non-covalent bonds, altering protein interactions, allergen epitopes and protein IgE-binding capacity (Khan et al., 2019; Zhao, Timira, et al., 2022).

For crustaceans, the content of tropomyosin of *Litopenaeus vannamei* was decreased following sonication procedures, per multiple reports (Dong et al., 2020; Li et al., 2011). This reduction was established as being up to 76%, with some degree of reduced allergenicity (Dong et al., 2020). Conversely, this work seems to contradict other reports indicating that ultrasound by itself has not shown the ability to mitigate the allergenic potential of tropomyosins (Chen et al., 2013; Costa, Villa, et al., 2022). However, when ultrasound is combined with heat treatments, ultrasound seems to allow proteins to be more accessible for the heat treatment. For shrimp, a reduction of more than 80% was observed for the IgE-binding capacity of tropomyosin with ultrasound and heat combined treatment, which facilitated the formation of smaller peptides from tropomyosin through protein denaturation (Li et al., 2006).

Regarding ultrasound viability as a food processing method to mitigate crustacean allergenicity, it shows good indications in allergen content, IgE-binding and allergenicity reduction. However, ultrasound methods can change food texture properties, more specifically, hardness. A 15% (heating and ultrasound) and 50% (only ultrasound) increase in hardness was observed for shrimp, which may not be desirable (Li et al., 2011). Moreover, as mentioned above, there are still some inconsistencies and gaps on the known effects of ultrasound on food, showing there is still work to be done in order to turn sonication into a cost-effective, reliable and safe food processing technique, especially to reduce allergenic proteins (Zhao, Timira, et al., 2022)

1.4.2.6 Gamma irradiation

Irradiation with gamma rays is already acknowledged as an useful and safe processing technology in the food industry, being its use allowed in 60 countries (Kume et al., 2009)

mainly with applications on food microbiological control. One of the properties of this method that makes it well accepted is its innocuous relationship with food properties, causing little to no modifications, which is advantageous for the food industry.

Submitting crustaceans and other foods to gamma radiation is expected to cause conformational alterations on proteins that may experience phenomena like cross-linking, aggregation, or fragmentation (Cheng et al., 2021; Khan et al., 2019). As mentioned above, these phenomena are expected to destroy the IgE-binding epitopes, contributing to mitigate protein allergenicity. Mechanistically, gamma irradiation can influence allergens, either by photon energy or by the production of reactive oxygen species (Cheng et al., 2021). This influence is dose-dependent, with a positive correlation between the amount of radiation used and the damage on proteins, with reports of 50% reduction in the immunoreactivity of shrimp (Byun et al., 2002). Additionally, this method can, similarly to ultrasound, be used in combination with heat, with irradiation exposing epitopes and heat destroying them, and, in consequence, exacerbating the reduction of protein allergenic potential (Mou et al., 2014). It was referred previously that this technology is already in use, and even if it is not completely efficient for allergenicity mitigation, it can be complemented with other food processing technology. The main issue with this technology would be the public opinion, since there is some bias regarding the use of gamma irradiation to process food (Khan et al., 2019).

1.4.2.7 Other non-thermal processing methods

In summary, besides the methods mentioned so far, there are other non-thermal processing methodologies that need to be fully investigated. Pulsed electric field (PEF), pulsed ultraviolet light (PUV), microwaving, electron beam irradiation (Cheng et al., 2021), ohmic processing (Pereira et al., 2021) or fermentation (Zhao, Timira, et al., 2022) are all methodologies that are being analysed for their potential to modify the allergenic potential of proteins and how they are presented to the allergic patients in a reactive state or not.

1.5 Digestibility of crustacean allergens

The effect of gastrointestinal digestion on allergenic proteins is of most importance, since it is the digestive system that metabolizes food allergens, being the only processing mechanism that is common to all patients. However, despite being a common process to practically all humans, the gastrointestinal digestion can vary significantly among individuals (Gámez et al., 2015). Chemical, physical, and enzymatic processes all occur during gastrointestinal digestion, with varying effects on protein allergenicity.

1.5.1 Processing methods and their synergic relation with digestion

As already mentioned, thermal and non-thermal food processing methods can alter the structure and the physiochemical properties of allergens, and there are some methods that work well together to exacerbate the effects. For instance, as mentioned above, gamma irradiation can expose epitopes, increasing the effectiveness of allergenicity mitigation by heat processing. This phenomenon also happens in gastrointestinal digestion, with processing methods such as thermal treatment, high pressure processing and ultrasound having been reported to enhance the subsequent digestion, while reducing allergenicity, most likely due to the destruction of epitopes because of the induced structural alterations (e.g. cross-linking, aggregation, denaturation) (Faisal et al., 2019; Liu et al., 2018; Zhang et al., 2018). However, these treatments may not be enough to eliminate the clinical symptoms in patients, as there is some degree of allergen resistance, meaning that not all proteins are degraded alike, either by proteins preserving the integrity or by being fragmented in peptides containing reactive epitopes. In fact, small peptides of 3-5 kDa can maintain partial immunoreactivity and still cause mast cell degranulation, with the possibility of triggering allergic reactions and subsequent clinical symptoms (Costa, Villa, et al., 2022; Khan et al., 2019).

1.5.2 Proteolytic action of gastrointestinal digestion enzymes

From a molecular perspective, when allergens are ingested, they enter the stomach where the acidic environment and the action of pepsin cause the breakdown and alteration of protein structure of most allergens. Even though the digestive enzymes can breakdown the allergenic proteins, and in most cases, reducing their IgE-binding capacity by affecting their structure and IgE-reactive epitopes. Conversely, the digestion process can lead to the exposure or creation of new epitopes due to protein refolding and unequal breakdown with small peptides presenting 3-5 kDa have been reported to be recognised by specific IgE, even though most allergenic proteins are transformed into harmless peptides. The remaining allergens, along with some newly formed ones, are transported to the duodenum as part of the partially digested food. In the small intestine, the digestion proceeds with the fluids from the pancreas and the bile from the liver. These fluids contain enzymes, such as proteases, that will further breakdown the peptides into amino acids or smaller peptides. Still, one has always to consider that the efficiency of the gastrointestinal digestion may not be 100%, and part of the proteins may preserve their full integrity by the end of the process. This means that both digested (small size peptides) and intact proteins may still reach the intestinal lumen in an IgE-reactive state (Costa, Villa, et al., 2022).

The effect of gastrointestinal digestion on crustacean tropomyosins is somewhat variable, with tropomyosin from some species being pepsin-sensitive, whereas others are pepsin-resistant (Costa, Villa, et al., 2022; Liu et al., 2011). One possible explanation for these contradictory reports is the fact that different crustacean species may present higher tropomyosin sequence variability. These differences in homology might explain the similarities and variations in resistance between some species (Gámez et al., 2015), in addition to patient-specific digestion characteristics, including body temperature, that may alter the activity of digestive enzymes. Therefore, it is generally accepted that crustacean tropomyosins are resistant to gastric digestion (pepsin), but degraded under intestinal digestion conditions (trypsin and chymotrypsin), which helps these proteins/peptides to preserve some IgE-binding capacity (Huang et al., 2010; Klueber et al., 2020; Liu et al., 2011). Still, some allergenicity reduction, as confirmed by basophil activation test and skin-prick test, has been reported (Gámez et al., 2015).

Although most digestion studies are focused on tropomyosin, as it is the main crustacean allergen (followed by arginine kinase) and considered as panallergen among different species (crustaceans, molluscs, and vertebrates), arginine kinases are another family of proteins of great interest. Reflecting this fact, it is reported that arginine kinase is moderately resistant to trypsin and chymotrypsin actions, but susceptible to pepsin showing it strongly differs from tropomyosin in digestibility characteristics (Chen et al., 2013). Another study reported similar results, evidencing that the IgE-binding capacity of arginine kinase was significantly reduced after simulated gastric digestion but not after simulated intestinal digestion (Yu et al., 2013).

Regarding another group of allergenic proteins, the *in vitro* gastrointestinal digestion of the sarcoplasmic calcium-binding protein has also been assessed, revealing that this protein is almost completely degraded after 1 hour of being submitted to this treatment (Zhao, Zhu, et al., 2022). The effect of digestion on SCP significantly differs from tropomyosin and arginine kinase digestion, as the former has shown to have little resistance to the action of digestive enzymes. Normally, the IgE-binding capacity of SCP is eliminated at the end of the gastrointestinal digestion. Other remarks include a comparison between filamin C and triosephosphate isomerase allergens in red swamp crayfish, in which it was concluded that filamin C is less sensible to the digestion process, even though both allergens have common epitopes and similar thermal and pH resistance (Yang et al., 2017).

1.6 Methods used for protein analysis

As seen above, the study of allergenic proteins is of the most importance to better understand the mechanism of food allergy, as well as how to manipulate proteins to be less

allergenic. The wide variety of available methods to study proteins allows for different perspectives, which contributes to increase the accessible information. Therefore, the more it is known about this condition, the more prepared the patients, the food industry and the clinicians will be to deal with food allergy. However, due to the available equipment, intrinsic unique characteristics of each methodology and to the possible interpretation of the results, there are some inconsistencies in the available literature, requiring further studies to confirm some conclusions. Some of these methods that are further discussed below, include immunoblotting and ELISA, used to evaluate the IgE-binding capacity of allergens, as well as other *in vitro* and *ex vivo* techniques, such as the basophil activation test. Other important and informative techniques include the liquid chromatography coupled to mass spectrometry (LC-MS), which can be used for the identification of allergens by determining their amino acid composition or respective peptides. These are key techniques that must be considered when studying allergen physicochemical properties.

1.6.1 Methods to evaluate the IgE-binding capacity of allergens

1.6.1.1 Immunoblotting

The binding of IgE to specific proteins is a crucial event on food sensitisation and subsequent development of food allergy. In fact, the presence of IgE in the serum of an individual is commonly considered as an indicator of the allergic sensitisation, since IgE is produced in the first phase of food allergy development. Although this concept is commonly accepted among clinicians and researchers, sensitisation without elicitation does not mean clinically relevant allergy. Still, the investigation of the presence of IgE in the serum of a potentially allergic individual is part of the clinical diagnostic and explored in therapeutic tools. Thus, understanding this mechanism is useful and can improve the quality of life of the allergic patients.

By using the IgE of allergic patients as the primary antibody, and the allergens sources/allergenic foods as samples, it is possible to visualize the antibody-antigen interaction, with the antibody being the IgE and the antigen being the epitopes of the allergens. Analysing the IgE-binding by means of immunoblotting can be useful to investigate the effect of processing methods and digestion, to identify allergens, to study allergic patient characteristics, to better understand how sensitisation evolves into allergy, specifically how does IgE-binding can be related to allergenicity, and to provide information to the development of clinically relevant methods, such as immunotherapy and hypoallergenic foods. Immunoblotting is a protein analysis method that is based on the antigen-antibody interaction. This method can qualitatively identify the presence of proteins

on a complex sample, considering that the primary antibody has high specificity to target antigens (e.g., protein). To increase the specificity of this interaction, this step is preceded by the blocking of unspecific binding-sites with innocuous proteins and followed by the introduction of a secondary antibody to bind with the primary antibody-target antigen complex. Finally, the membrane with the adhered proteins is revealed, allowing for the user to observe the proteins to which the antibodies were able to bind (Costa et al., 2024).

There are several reports in which this type of study was performed with these objectives, specifically for crustacean allergens. For instance, the IgE-reactivity of tropomyosin (the main crustacean allergen) from crab and shrimp was studied by immunoblotting (Abramovitch et al., 2013). In this case, sera from at least 12 crustacean-allergic patients showed IgE-reactivity to extracts from both shellfish species, with significant differences when comparing heat-processed to raw samples. In fact, it could be noted that, in general, cooked samples showed more intense bands in the immunoblotting, when compared to their raw counterparts. Additionally, it is also noticeable that different patients have different profiles, indicating that each person reacts differently and can be allergic/sensitised to different crustacean species and/or allergens, and with different intensity. As mentioned above, immunoblotting can play a role in investigating diverse food allergy aspects. In addition to the referred study on the effect of food processing, there is also a report (Akimoto et al., 2021) that used immunoblotting as a part of a study on two potential new crustacean allergens. It is believed that the conducted research may be able to contribute to refine crustacean allergy diagnosis and treatment, as these two proteins are believed to be somewhat specific to more severe cases of shrimp allergy.

1.6.1.2 ELISA

ELISA is a well-known method, already established as one of the most useful tools regarding crustacean allergy. On one hand, it is traditionally used as a quantitative detection method for the protection of patients. By detecting and quantifying allergens in foods, labels can be improved, allowing for patients to be properly informed when acquiring processed food products. On the other hand, it can also be useful in detecting and quantifying allergen with IgE-binding capacity. Whereas the immunoblotting can qualitatively evaluate the IgE-binding to certain allergens, the ELISA can be used to quantify the antigen-antibody interaction. For instance, the IgE-binding capacity of different allergens upon food processing treatments can be measured by ELISA. However, since allergy is a complex disorder that is affected by many factors, in some cases there are somewhat contradictory results, making their confirmation with other methods of the highest importance. Even though work is being made in that direction, the specificity of each patient, crustacean species, and allergen can significantly alter the results. One report indicates, by ELISA, that

heat-processing can, for both prawn and crab, significantly increase the IgE-binding capacity of allergenic proteins using the sera from more than 20 patients (Abramovitch et al., 2013). Conversely, other report suggests that IgE-reactivity of allergen decreases with cooking, also using the sera of more than 20 patients (Jarupalee et al., 2018). It is likely that this incongruencies are caused by the fact that different crustacean species were studied, in addition to different patient sera. Even though the results are inconsistent, it shows the importance of further and broader studies, that can better understand the differences among crustacean species, methods, and patients in the study of IgE-binding as an indicator of allergenicity. Additionally, ELISA can also be used as complementary technique in studies regarding novel allergens (Liu et al., 2021), allowing the quantification of IgE-binding as an important step in confirming new proteins as having allergenic potential.

1.6.2 Methods to test the allergenicity of different proteins

The term protein allergenicity reflects a more refined concept, which regards the biological process that occurs in vivo during an allergic reaction. In general, it is considered that the use of in vitro biological assays (cellular models) can provide a functional analysis of the specific elicitation cell activation by allergen mediated specific IgE crosslinking, which can be measured by mediator release or upregulation of cellular surface molecules. Although such strategies can be more laborious and expensive than the previous approaches, the human basophil activation tests (BAT), the humanised rat basophilic leukaemia (RBL) mediator release assay and the mast cell models can be considered as in vitro surrogates of the allergic reaction that happens in vivo in allergic patients (Costa, Villa, et al., 2022).

1.6.2.1 BAT

Despite the presence of IgE being an important indicator of allergic sensitisation, it does not directly reflect allergenicity, because an individual can be sensitised, but without experience clinically relevant symptoms. This means that the individual is sensitised to specific allergens but not allergic to. Therefore, other methods are necessary to complement the information gained by immunoblotting and ELISA. Mediator release assays are the preferred type of methodology to achieve this goal. If significant amounts of mediators are being released, it is almost certain that the patient is experiencing symptoms, since the mediators are the molecules that elicit the clinical symptoms. Assays like BAT (basophil activation test) can provide data on protein allergenicity as affected by processing and/or digestion effects. For example, on crab, a report suggested that pressure and heat

treatment (which is believed to decrease IgE-binding) can decrease the surface expression of basophils. Additionally, this effect was enhanced by digestion, indicating that both the heat and pressure processing followed by the digestion decreased the allergenicity of crab (Liu et al., 2021).

BAT can also be used as a diagnostic tool to evaluate the potential of basophils extracted from sensitised/allergic individuals to react towards allergenic proteins from crustaceans, thus predicting the possibility of an allergic reaction being elicited.

1.6.2.2 RBL

Other mediator release assays are also commonly used to test the allergenicity of different foods, namely crustaceans. Normally, the humanised rat basophilic leukaemia (RBL) mediator release assay is based on the use of basophils extracted from rats which were modified to recognise the human IgE through the expression of humanized specific FcƐII receptors. This type of assays has been used to study the allergenic potential of tropomyosins from different sources, namely shrimp, mealworm and chicken, as calibrator proteins in functional biological assays for tropomyosin allergenicity assessment of novel animal foods (Klueber et al., 2020). As other example, this assay was also used to study the effect of glycation on the allergenicity of shrimp tropomyosin. (Lv et al., 2022) concluded that the glycation of tropomyosin with ribose could significantly inhibit the release of cytokines and mediators from RBL-2H3 cells, thus reducing the allergenicity of shrimp.

1.6.3 Methods to test allergen composition

1.6.3.1 LC-MS

Although a lot of data is provided by both IgE-binding and allergenicity analysis, if the goal is to deeply understand allergies, it is also necessary to better understand the physicochemical composition of allergens and their behaviour towards food processing treatments. Therefore, to confirm the presence of specific allergens to study them, and to evaluate the effects of external phenomena to these proteins, it is essential to study their composition, both in their native and their processed/digested state. For that purpose, LC-MS is the usual method, allowing the separation of allergens and the identification of their primary sequence composition with high accuracy. Even though this method is most frequently used for allergen detection in foods, LC-MS has the potential for analysing digested and/or processed allergens (Abramovitch et al., 2013; Liu et al., 2021). (Xu et al., 2022) evaluated the effect of heating on the structural properties of shrimp allergens, namely tropomyosin, arginine kinase, myosin light chain and sarcoplasmic calcium-binding

protein, in trypsin digests of raw, fried, and baked shrimp extracts by ultrahigh-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC/Q-TOF-MS). Heat processing modified the number of peptides released from the 4 allergens, with tropomyosin providing a detectable number of released peptides and epitope changes. The arginine kinase also revealed an epitope-matched sequence as a common differentiating peptide for heat processing.

2 Objective

In this work, the study of crustacean allergens was intended. The studied allergens include the well-known tropomyosin and arginine kinase, but also other allergens, such as triosephosphate isomerase or sarcoplasmic calcium-binding protein. The protein structure of allergens was studied by SDS-PAGE in non-denaturing conditions, and IgE-binding capacity was evaluated by ELISA and immunoblotting. One of the main goals was to study the effect of food processing on crustacean allergens, for which an extensive search for different crustacean species at local markets was intended to have a good representation of the most consumed crustaceans in Portugal. In this regard, popular heat-processing methods, such as boiling, oven-cooking, grilling, or frying, as well as the use of lemon juice (marination) were studied, since they are typically used to process most crustaceans at home or in restaurants. Other specificities of these processing methods were also analysed, such as the effect of the duration of the processing or the possible passage of allergens to boiling water and/or frying oil, following reports of patient sensitisation due to cooking vapour inhalation. Additionally, this work also aimed to evaluate if the identification and the immunorecognition of different allergens (e.g., tropomyosins) would be similar in distinct crustacean species as assessed by immunoblotting and ELISA with sera of patients allergic to crustaceans.

The application of a harmonised simulated gastrointestinal protocol (INFOGEST 2.0) using the American lobster as a case study was also important in investigating the effects of the digestion on this crustacean as affected by different processing treatments, aiming at inferring on a possible synergistic effect of digestion and processing methods, with the goal of understanding how to decrease protein allergenicity in crustaceans. Furthermore, since some common allergens (e.g., arginine kinase and tropomyosin) are important markers of possible sensitisation and actual crustacean allergy, an evaluation of the effect of digestion on these proteins was also performed.

In summary, with this work it was intended to fill some of the gaps that exists in the literature as most reports only focus on specific purified crustacean allergens.

Experimental Component

3 Methodology

During this work, good laboratory practices were followed. All food processing steps were performed in a specialized laboratory, using chemically decontaminated material and equipment to avoid unintended cross-contaminations. Whenever necessary, all disposable consumables were previously sterilised by autoclaving (121ºC, 15 min). All reagents were of analytical grade or specific for biological experiments.

3.1 Sampling and crustacean processing treatments

Sampling was done in a way that allowed to simulate how patients can be exposed to allergens, expressly in how the crustaceans were obtained and processed. Thereby, crustaceans were obtained at local markets or supermarkets. In addition, it was also taken into consideration that there are several edible crustacean species, and most patients can be allergic to more than one, due to cross-reactivity. Therefore, 9 crustacean species were selected based on their popular consumption among consumers, while assuring to gather species from the broadest range of taxonomic families. Afterwards, they were brought to the laboratory, where they were processed. The chosen crustacean species, as well of all the samples obtained and considered for analysis are described below (**Table 3.1**). All crustaceans were obtained frozen, apart from the spiny spider and brown crabs that were acquired alive and then killed at -80ºC. The *Vannamei* shrimp, black tiger shrimp, Argentine red shrimp and scampi had their shells, heads and tails removed after processing, since those portions are not commonly consumed. Likewise, for the species of American lobster, spiny spider crab and brown crab, only the edible meat portions inside the shells were stored and analysed (**Figure 3.1**). In the case of barnacles, only the soft parts were grinded, stored and further analysed. For the smooth swimming crab, the procedure was a bit different, since only the legs were acquired and from where the edible meat was extracted.

The processing treatments were selected based on the most commonly home/restaurant-used techniques, such as boiling, frying, oven-cooking, and grilling. Recipes on how to prepare each crustacean were consulted and a list of different processing treatments (time/temperature of processing) were defined for each species, as described in **Table 3.1**. Prior to any potential processing treatment, a sample of each raw (unprocessed) crustacean species was collected to serve as a reference. As one of the main objectives of the study was to evaluate what happens to allergens during the processing treatments, parts of the cooking liquids were also collected, namely the water used to boil crustaceans, was well as the oil used to fry them or the marination juices. These liquid samples were immediately collected using the same time points of the solid samples

(crustacean meat) and immediately frozen at -20 ºC. The crustacean meats were grinded/homogenised as much as possible, and immediately frozen at -20 ºC for later analysis.

Table 3.1 - List of all crustacean species considered for analysis, with their most common names, scientific names, and processing methods performed on each species.

Common name(s)	Scientific name	Food processing treatment
Vannamei shrimp 	*Litopenaeus vannamei*	Raw; Boiled (100 ºC) - 2 min; Boiling water - 2 min; Fried (180 ºC) - 3 min; Frying oil - 3 min; Grilled - 3 min
Black tiger shrimp/ Giant tiger prawn 	*Penaeus monodon*	Raw; Boiled (100 ºC) - 4 min; Boiling water - 4 min; Fried (180 ºC) - 4 min; Frying oil - 4 min; Grilled - 4 min
American lobster 	*Homarus americanus*	Raw; Boiled (100 ºC) - 15 min; Boiling water - 15 min; Fried (180 ºC) - 5 min; Frying oil - 5 min; Grilled - 20 min; Raw and marinated in lemon juice - 1.5h; Marination juice (lemon juice) of raw lobster; Marinated in lemon juice (1.5 h) + grilled - 5 min; Marinated in lemon juice (1.5 h) + oven cooked - 15 min
Argentine (red) shrimp 	*Pleoticus muelleri*	Raw; Boiled (100 ºC) - 3 min; Boiling water - 3 min; Fried (180 ºC) - 5 min; Frying oil - 5 min; Grilled - 3 min
Blue swimming crab 	*Portunus pelagicus*	Raw; Steam cooked 5 min; Boiled (100 ºC) - 5 min; Boiling water (5 min); Grilled 5 min; Oven cooked (180 ºC) - 15 min; Oven-cooking water (15 min).
(Goose) Barnacles 	*Pollicipes pollicipes*	Raw; Boiled (100 ºC) - 3 min; Boiling water - 3 min; Boiled (100 ºC) - 10 min; Boiling water - 10 min)
Spiny spider crab 	*Maja squinado*	Raw; Boiled (100 ºC) - 10 min; Boiling water - 10 min
Brown/Edible crab 	*Cancer pagurus*	Raw; Boiled (100 ºC) - 10 min; Boiling water - 10 min
Scampi/shrimp-lobster/Norway lobster 	*Nephrops norvegicus*	Raw; Boiled (100 ºC) - 5 min; Boiling water - 5 min; Fried (180 ºC) - 5 min; Frying oil - 5 min; Grilled - 5 min; Oven-cooked (180 ºC) - 15 min Raw and marinated in lemon juice - 1.5 h; Marination juice (lemon juice) of raw lobster; Marinated in lemon juice (1.5 h) + grilled - 5 min; Marinated in lemon juice (1.5 h) + oven-cooked - 15 min

As described below (**Table 3.1**), lemon juice was used to marinate, during 1.5 h at room temperature, the edible portions of two species of crustaceans, namely the American lobster and scampi. Since lemon juice is commonly used in crustacean preparation/cooking, it was aimed to verify if there were any changes induced by the citric acid on the protein structure and/or IgE-binding capacity of crustacean allergens.

Figure 3.1 - Example of in-lab processing for sampling the edible meat portion of American lobster, which were collected to sample tubes and further stored at -20 ºC. Head and paws were removed.

3.2 Sera from crustacean-allergic patients

To assess the IgE-binding capacity of the crustacean allergens, pool-sera from 14 crustacean allergic individuals were used, 4 from female donors and 10 from male donors. All patients presented specific IgE at least towards one crustacean species, as determined by immmunoCap, although most of them are reactive to more than one species. According to clinical data, some patients have clinically relevant symptomatology towards crustacean consumption. All of them have other pathologies, like allergic rhinitis or asthma. All details are presented in **Table 3.2.** The sera were acquired from PlasmaLab International (Everett, WA, USA).

3.3 Protein extraction

After preparing and processing all crustacean species, all solid samples were submitted to protein extraction. For this purpose, around 150 mg of each solid sample were weighted into 2.0 mL sterile reaction tubes, using an analytical balance. Subsequently, 1.5 mL of extraction buffer were added to each tube, following incubation. To improve the efficiency of protein extraction, two incubation methods were performed, one at 60 ºC for 2 hours or at 4 ºC overnight (around 16 hours). Additionally, different extraction buffers were retrieved for literature and further used for testing distinct crustacean species, namely (1) Tris-HCl

100 mM pH 8.0; (2) 0.02% NaN_3 in H_2O; (3) PBS 0.2 M pH 7.4; (4) ultrapure H_2O pH 9.0; (5) ultrapure H_2O pH 12.0; (6) NH_4HCO_3 100 mM and (7) Tris-HCl 100 mM + SDS 4% pH 7.6 (Lasekan & Nayak, 2016; Villa et al., 2020; Walczyk et al., 2017).

Following the addition of the extraction buffer, which should be in a 1:10 proportion, the procedure was as follows: incubation with shaking at 900-950 rpm, either at 60 ºC for 2 hours or 4 ºC overnight, using a thermomixer (Thermomixer Comfort, Eppendorf AG, Hamburg, Germany), with occasional extra vigorous vortexing of the samples.

Table 3.2 – Summarized patient information used for testing the IgE-binding capacity of crustacean species submitted to different food processing treatments.

Patient	Gender	Reported IgE reactivity	Specific IgE (kUA/L) (ImmunoCap FEIA)	Relevant Information	Other pathologies
#1	Male	Crab Lobster Total	6.985 7.5 215.9	-	Allergic rhinitis
#2	Female	Shrimp Total	26 1392	Clinically relevant symptomatology	Allergic rhinitis
#3	Female	Shrimp Lobster Total	19.3 13.7 1260	Clinically relevant symptomatology	Allergic rhinitis
#4	Male	Crab Shrimp Total	52.2 64.1 153	-	Allergic rhinitis
#5	Female	Crab Total	33.9 8623	Clinically relevant symptomatology	Asthma, eczema, food allergies, Multi-allergies
#6	Male	Crab Shrimp Total	3.22 5.39 3602	Clinically relevant symptomatology	Allergic rhinitis, Multi-allergy
#7	Female	Shrimp Total	19.7 1769	Clinically relevant symptomatology	Allergic rhinitis
#8	Male	Crab Lobster Shrimp Total	32.7 36.5 40.1 73	-	Allergic rhinitis
#9	Male	Crab Shrimp Total	46.7 60.1 7049	Clinically relevant symptomatology	Asthma, eczema, food allergies, Multi-allergies
#10	Male	Shrimp Total	5.28 4015	Clinically relevant symptomatology	Asthma, eczema, Multi-allergies
#11	Male	Shrimp Crab Lobster Total	39.4 32.3 35.7 76	-	Allergic rhinitis
#12	Male	Shrimp	4.25	-	Asthma, eczema, Multi-allergies
#13	Male	Shrimp Crab Lobster Total	1.79 3.82 2.04 133	Clinically relevant symptomatology	Allergic rhinitis
#14	Male	Shrimp Crab Total	29.9 27.3 64	-	Allergic rhinitis

Afterwards, a centrifugation was performed at 9000×*g* for 30 min (room temperature) (Heraeus FRESCO 17 Centrifuge ThermoScientific, Germany). The pellet was discarded,

and the supernatant was centrifuged again, using the same conditions, to remove any potential particles in suspension. Whenever necessary, the samples were also filtered (sterile syringe filter 0.45 µm polypropylene, VWR International, LLC, Radnor, Pennsylvania, USA). At the end, the supernatant was stored at -20 ºC until further use.

3.4 Protein quantification

Following extraction, the protein content of each solid and liquid sample was quantified by two methods, namely by direct quantification of the protein by UV/Vis spectrophotometry and by using a protein quantification kit containing bicinchoninic acid (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, Waltham, Massachusetts, USA).

3.4.1 UV/Vis spectrophotometry

Protein content of each sample was determined by UV/Vis spectrophotometry, measuring the absorbance of samples at different wavelengths: 280 nm (proteins), 260 nm (DNA), 230 nm (RNA) and 320 nm (potential interferents) and using a microplate of reduced volume (LVIS microplate, SPECTROstar Nano, BMG LABTECH, Ortenberg, Germany) that allows the direct reading of the samples. To perform the measurements, 3 µL of each protein extract was directly pipetted into the LVIS microplate and introduced into a plate reader (SPECTROstar Nano, BMG LABTECH, Ortenberg, Germany). The data was analysed and processed by the MARS Data Analysis Software (SPECTROstar Nano, BMG LABTECH, Ortenberg, Germany) using the pre-defined Protein280 protocol. The determination of total protein content by UV/Vis quantification was used either to dilute the samples in a normalized fashion for further analysis, or as a pre-quantification to facilitate the protein quantification using the BCA method.

3.4.2 Protein quantification by BCA

Most of the protein samples used for SDS-PAGE, immunoblotting and ELISA analysis were diluted after considering the results of the BCA protein quantification method. The assay was performed following the instruction manual on the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA, USA) for the microplate procedure, with a few changes. Briefly, this kit uses the BSA (Bovine Serum Albumin) at a 2.0 mg/mL in a 0.9% aqueous NaCl solution containing 0.05% sodium azide as standard protein solution.

Using the BSA, a calibration curve was prepared considering 7 calibrants: 2.0 mg/mL, 1.5 mg/mL, 1.0 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, and 0.025 mg/mL (**Table 3.3**).

Table 3.3 - Standard solutions preparation summary for the calibration of the BCA protein quantification assay, with the desired final concentration.

Standards	Diluent (H_2O) (µL)	BSA (µL)	Source of BSA	Calibrants BSA (µg/mL)
A	0	300	Stock	2000
B	100	375	Stock	1500
C	400	325	Stock	1000
D	350	325	C dilution	500
E	350	325	D dilution	250
F	350	325	E dilution	125
G	400	100	F dilution	25
H	400	0	N/A	0 (Blank)

Following the protocol, 20 µL of each standard concentration and of the unknown samples were added to each microplate since the protocol suggested a volume between 10 µL (for a working range of 125-2000 µg/mL) and 25 µL (for a working range of 20-2000 µg/mL). After adding the standards and samples to a 96-well plate, a working solution was prepared by mixing 50 parts of BCA reagent A with 1 part of reagent B (20 mL/plate of reagent A + 400 µL/plate of reagent B). Reagent A contained sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in a 0.1M sodium hydroxide solution, while the reagent B contained 4% cupric sulphate. Two-hundred microliters of this working solution were added to each plate-well, followed by gentle microplate mix and coverage of the wells with some parafilm. Then, the microplates were let to incubate at 37 ºC in dark conditions for 30 min. After incubation, the microplates were let at room temperature for 20 min before reading in the plate reader (SPECTROstar Nano, BMG LABTECH, Ortenberg, Germany). All standards and unknown samples were analysed in *n*=3 replicates per microplate (**Figure 3.2**).

The results were processed using the MARS Data Analysis Software (SPECTROstar Nano, BMG LABTECH, Ortenberg, Germany). The absorbance values from the calibration curve were analysed by a linear function $\boldsymbol{y=mx+b}$ (**Equation 1**) and the unknown samples read against the respective calibration curve.

$$y = mx + b \quad \textbf{(Equation 1)}$$

where m is the slope and b is the intersection with the y-axis.

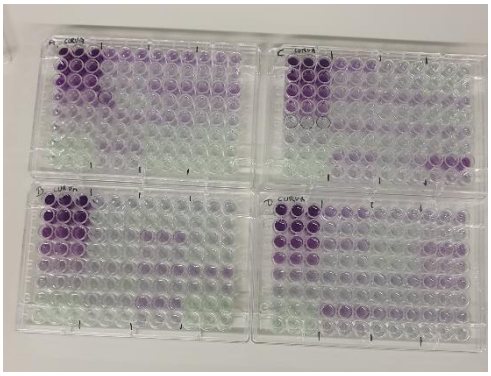

Figure 3.2 - Example of four BCA plates right after reading the absorbance at 562 nm. The first three columns correspond to the calibration curve, and the remaining wells are unknown samples read in triplicate.

3.5 SDS-PAGE

3.5.1 Gel preparation

To prepare the polyacrylamide gels for the SDS-PAGE (1 mm thick), the necessary reagents were mixed in the following amounts (**Table 3.4**).

Table 3.4 - Protocol used to prepare discontinuous SDS-PAGE gels, with 12% or 15% acrylamide for the separating gel and 5% polyacrylamide for the stacking gel.

Discontinuous SDS-PAGE gels	**Separating gel (2 gels)**		**Stacking gel (2 to 5 gels)**
	12%	15%	5%
H_2O (mL)	4.200	2.880	5.833
Separating gel buffer 4x (mL)	3.000	3.000	-
Stacking gel buffer 4x (mL)	-	-	2.500
Acryl/Bis 37:5:1 solution (mL)	4.800	6.000	1.667
SDS 10% (µL)	120	120	100
TEMED (µL)	12	12	20
APS 10% (µL)	60	60	50
Bromophenol blue 10% (µL)	-	-	20
Total volume (mL)	**12**	**12**	**10**

The amounts defined in **Table 3.4** are intended to prepare 2 gels, although the reported amounts can be multiplied to prepare a higher number of gels. The separating gel would contain either 12% or 15% acrylamide, depending on the intended purpose, whereas the stacking gel was always used with 5% of this compound. The glass plates were carefully cleaned with ethanol and checked for any marks, being further arranged within cassettes

(**Figure 3.3**). Around 5 mL of the separating gel solution was then introduced in the cassette sandwich to make each gel.

The 10% APS solution needs to be prepared freshly. The addition of APS and TEMED is necessary for the polymerization process, which means that these 2 reagents need to be added to the gel formulation just before distributing the gel. The polymerization takes around 1.5 h in anaerobic conditions, that is accomplished by adding 1 mL of 100% isopropanol to the top of the gel, which in turns helps to eliminate bubbles and protect the gel from oxygen. When the separating portion is polymerized, the isopropanol is removed (washed) and around 1.5 mL of the stacking gel mixture is added to the top of the separating gel, with the APS and TEMED reagents inducing the polymerization process. Combs (typically 15-well) are placed to allow the formation of wells. At the end of the 1.5-hour polymerization, which is visible due to the formation of a line between the two portions, the gels are wrapped in wet paper, with the combs still inserted, introduced in a plastic bag, and stored overnight at 4 ºC.

Figure 3.3 - Representation of a typical setup when gels were being prepared. The flasks contain the separating (closer to the camera) and the stacking gel solutions. In the figure, the separating gel is polymerizing inside the assembly.

3.5.2 Sample preparation

After the extracts were diluted to the desired final concentration (e.g. 5 mg/mL), 10 µL of the diluted sample is mixed with 10 µL of loading buffer (Laemmli Sample Buffer 2x, Bio-Rad Laboratories, Inc., Hercules, CA, USA), with (denaturing conditions) or without (non-denaturing conditions) 50 mM of beta-mercaptoethanol, that worked as a reducing agent, breaking the disulfide bridges of the proteins (thus destroying the conformational structure of proteins).

When performing SDS-PAGE in denaturing conditions, the mixture of the samples with Laemmli Sample Buffer 2x and 50 mM of beta-mercaptoethanol was heated at 95 ºC for 5 min. On the other hand, if SDS-PAGE is performed in non-denaturing conditions, the mixing of the loading buffer plus the sample is immediately distributed in the wells without any denaturing step (heat + denaturing agent).

3.5.3 Gel electrophoresis

The SDS-PAGE was performed in a Mini-PROTEAN® Tetra System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The pre-prepared gels were introduced in the running modules, which were closed and inserted in the electrophoresis tank. Subsequently, an electrophoresis buffer is used to help maintain pH and temperature conditions, as well as providing ions that allow the circulation of the electrical current. For the SDS-PAGE runs, 1x Tris/Glycine/SDS buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) is used to fill the electrophoresis tank until the adequate mark, depending on the number of gels that are being run. After making sure the gels were properly covered in 1x TGS buffer, the combs are removed, and the wells are loaded.

Firstly, 3 µL of the Precision Plus Protein Dual Color Standard 10-250 kDa (Bio-Rad Laboratories, Inc., Hercules, CA, USA) is loaded into the first well of each gel, followed by 10 µL of the Laemmli buffer and sample mixture (for non-denaturing conditions) or the Laemmli buffer + beta-mercaptoethanol and sample mixture (for denaturing conditions). For the denaturing conditions, the sample plus the loading buffer is heat treated at 95 ºC for 5 min and then loaded in the gel. After finishing the sample loading, the electrophoresis tank is connected to the power supply (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and set at 130 V, until the samples reached the separating gel, which usually takes between 10-15 minutes. The voltage is raised to 160 V and the gels continue to run for 45 to 55 minutes, ensuring that the gels are always adequately covered with the TGS buffer. The end of the run is easily identified by the position/separation of the bands of the protein marker and by the line of bromophenol that determines the front run of the gel (**Figure 3.4**).

At the end of the run, the gels were removed from the tank, and placed in a recipient containing Coomassie Staining Solution (1.25 g of Coomassie G-250, 500 mL of methanol, 100 mL of glacial acetic acid, 400 mL of ultrapure water) for 10 minutes, with shaking. After the staining process, the staining solution was removed, and replaced with distaining solution (50 mL of methanol, 70 mL of glacial acetic acid, 880 mL of ultrapure water), which was renewed 2 to 3 times. Then, the gel was distained for several hours. If distaining overnight, the gels were placed at 4ºC and revealed the next day.

3.5.4 Gel revelation and image acquisition

The gel is placed on a white tray and introduced inside the Gel Doc™ EZ Imager (Bio-Rad Laboratories, Inc., Hercules, CA, USA) to obtain the image of the gel. The system is defined with Coomassie protocol, and an image is acquired and processed by the Image Lab 5.2.1 software (Gel Doc™ EZ Imager, Bio-Rad Laboratories, Inc., Hercules, CA, USA).

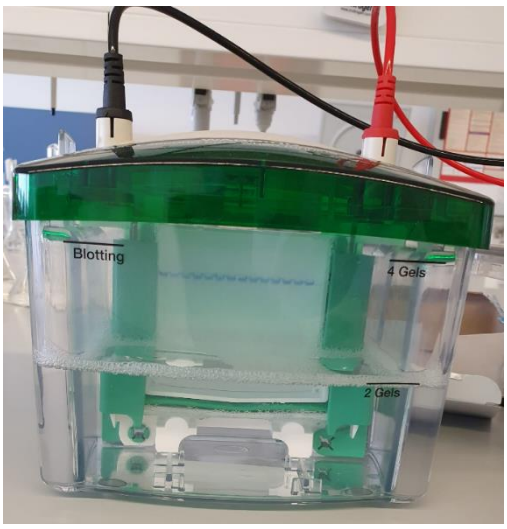

Figure 3.4 - Example of an SDS-PAGE run of 2 gels in the Mini-PROTEAN® Tetra System (Bio-Rad Laboratories, Inc., Hercules, CA, USA), where the front of the run is identified by the blue colour of the bromophenol solution.

3.6 Immunoblotting

For immunoblotting, the first step consisted of performing the same steps as described in section 3.5 (SDS-PAGE). Some minor alterations can be used when running the gels for immunoblotting, such as using less amount of protein or by changing the amount of protein marker. In this work, the gels for the immunoblotting were run in similar conditions except for the quantity of marker, which was 4 µL. At the end of the run, a nitrocellulose membrane 0.2 µm transfer pack (Bio-Rad Laboratories, Inc., Hercules, CA, USA) is placed on a tray of the blot system (Trans-Blot® Turbo™ Transfer System, Bio-Rad Laboratories, Inc., Hercules, CA, USA). Depending on the number of blots, 2 mini gels can be blotted in a Midi format nitrocellulose membrane, or 1 gel in a Mini format nitrocellulose membrane following the instructions of the membrane pack. Afterwards, the tray is closed and inserted in the Trans-Blot® Turbo™ Transfer System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The transfer process from the gel to the membrane was then performed using the automatic turbo protocol, that consisted of applying 2.5 A, up to 25 V (that usually remained at around 20 V) for 7 min for Mini format or 10 min for Midi format membranes (**Figure 3.5**).

Following the transfer, the gel is discarded (checking first that the transfer was efficient), and the membranes are carefully moved into recipients (when using Midi format membranes with 2 gels, it is necessary to separate the membranes by cutting in half). The membranes are then washed with TBST 1x (0.1% Tween 20) 3 times, for 10 min each, with constant shaking. The TBST 1x (0.1% Tween 20) solution is prepared by diluting 10x TBS pH 7.4 solution with ultrapure water. The 10% TBS was in-house prepared, by mixing 12.1 g of Tris-base, 29.1 g of NaCl in a final volume of 1 L using ultrapure H_2O, after adjusting the pH to 7.4 with 6 M HCl. After the washing steps, the membranes were blocked with a blocking agent, which needs to be carefully selected based on the target proteins that are intended to be immunorecognised. As the target proteins belong to crustaceans, the selected blocking agent was milk powder. Therefore, the membranes were incubated with 30 mL of TBST 1x (0.1% Tween 20) + 2% of milk powder, for 1 hour, at room temperature with constant agitation. When the blocking phase was finished, the membranes were washed as described previously. After the 3-washing steps, the membranes were incubated with the primary antibody, which in this case was the pool of sera from 14 patients reported to be allergic to crustaceans (section 3.2). The sera were diluted 1:30 in 4.5 mL of incubation buffer (TBST 1x (0.1% Tween 20 + 2% milk powder)) and added to each membrane to incubate overnight at 4 ºC with soft shaking. The next day, the membranes were washed again (3-times with TBST 1x (0.1% Tween 20)) and further incubated with secondary antibody (anti-human IgE) diluted 1:20000 in 30 mL of incubation buffer (TBST 1x (0.1% Tween 20 + 2% milk powder)) for 2 hours at room temperature and constant shaking. After incubation, the membranes were washed again in the same conditions and revealed with 1.5 mL of Clarity™ Western ECL (Bio-Rad Laboratories, Hercules, CA, USA) by mixing 750 µL Clarity Western Luminol/Enhancer Reagent and 750 µL Clarity Western Peroxide Reagent on a ChemiDoc system (Bio-Rad Laboratories, Hercules, CA, USA) for some minutes.

Figure 3.5 - Gels and membranes on the transfer apparatus, after the end of the transfer process.

3.7 Stripping

Occasionally, the incubation of the membranes with primary or secondary antibodies is not fully efficient, thus producing low intensity signals. In those cases, it is possible to confirm if that happened due to low IgE-binding to those proteins, or if there was some problem with either antibody, or with the dilution used for them. Therefore, in those specific cases, the membranes can be stripped with a specific solution. The stripping solution allows the removal of the antibodies and blocking agent that were already bounded to the membrane but leaving the membrane with the adhered proteins. Therefore, after analysis the membranes can be washed 3-times with TBST 1x (0.1% Tween 20) for 10 min each with constant agitation. At the end of the 30 minutes of washing, the membranes can be stripped with the stripping solution (1 g of SDS, 10 mL of Tween 20, 990 mL ultrapure water, adjusted to pH 2.2). Stripping is like the washing steps, as the membranes are incubated 3-times for 10 minutes each at room temperature with striping solution, renewing the solution each time. Then, the membranes are washed again 3-times TBST 1x (0.1% Tween 20) in the same conditions. At the end of the stripping step, the immunoblotting protocol can be restarted with the blocking step.

3.8 ELISA (Enzyme-linked immunosorbent assay)

Enzyme-linked immunosorbent assay (ELISA) was also performed aiming at evaluating how the IgE-binding capacity (increase/decrease) of crustacean allergens is affected by food processing. The proposed ELISA was developed in an indirect format. In summary, in indirect ELISA, the proteins are adsorbed to the bottom of the plate wells, which is followed by blocking and incubation with primary and secondary antibodies. Secondary antibody is coupled with an enzyme, and after the addition of the substrate, there is the formation of blue colour due to the reaction between the enzyme and the substrate. Finally, the reaction is stopped by acid (sulfuric acid), modifying the blue colour to yellow, which is quantified by spectrometry, indicating the amount of protein in each sample (Villa et al., 2022).

Indirect ELISA was performed in high protein-binding capacity polystyrene 96-well plates (Nunc MaxiSorp™ flat-bottom, Thermo Scientific, Rochester, NY, USA) to ensure maximal protein coating capacity, thus enabling hydrophobic-ionic binding interactions (Pereira et al., 2021). Each sample was previously diluted in coating buffer (pH 9.6) (10 mM Na_2CO_3, 30 mM $NaHCO_3$, 15 mM NaN_3) and pipetted in triplicate, summarizing n=6 replicates in 2 independent plates. Therefore, the ELISA was carried out in n=4 microplates with one blank sample in each one (3 wells) as negative control. A volume of 100 µL of sample in coating solution at a concentration of 1 mg/mL was pipetted to each well and left for a 2-hour

incubation at room temperature to allow for the immobilization of the proteins onto the plate wells. After this incubation, the microplates were washed 3-times, with 200 µL of the washing solution (PBS 0.01 M, pH 7.5). This solution was composed of 50 mL of PBS 0.2 M, 1 mL of Tween 20 and 949 mL of ultrapure water. The removal of the washing solution must be done thoroughly by throwing the liquid out and hitting the plate firmly into some lint-free paper, thus guaranteeing the removal of all the liquid inside the wells. The subsequent washing steps were all performed as described. Afterwards, the blocking step was initiated with the addition of 200 µL of coating buffer (+ 2% milk powder) to each well (**Figure 3.6**), at room temperature, for a period of 2 hours. To guarantee enough quantity for all wells, 2 g of milk powder were solubilized in 100 mL of coating buffer. The blocking step contributes to the decrease of unspecific binding, with milk protein occupying unspecific binding sites, which could yield inconsistent and inaccurate results (Villa et al., 2022). At the end of the 2-hour incubation period, the washing step was done 3-times in the previously described conditions to remove unbound milk protein and the remains of the blocking solution. Then, the samples were incubated with primary antibody, which in this case was 875 µL of pool sera from crustacean-allergic patients added to 34.15 mL of assay buffer with 2% milk powder (1:40 dilution). One hundred microliters of the antibody solution were put in each well, and left incubating overnight at 4 ºC to allow the IgE from the sera to bind to the allergenic proteins in the plate. It is worth mentioning that the assay buffer contained 100 mL of PBS 0.2 M, 400 µL of Tween 20 and 300 mL of ultrapure water.

The next day, the plates were washed 3-times as previously described with the goal of removing any excess unbound IgE, thus decreasing the chances of having an artificial inflation of the results. The microplates were further incubated with the second antibody (anti-human IgE) in a 1:20000 dilution (i.e., 1.8 µL of anti-human IgE + 36 mL of assay buffer, 2% milk powder) for 1 hour at room temperature. This detection antibody captures the primary antibody + allergen complex. The washing step was again repeated 3-times, removing excess antibody and enzyme, and 50 µL of the substrate solution (3, 3′,5 ,5′-TMB Liquid Substrate, Sigma-Aldrich) were added to each well, inducing the development of a blue colouration. This blue colouration is consequence of the reaction between the enzyme coupled to the secondary antibody and the added substrate, generating the blue colour product. After adding the substrate, the colour development took around 15-20 min, being further interrupted with 50 µL of 1 M sulphuric acid (H_2SO_4). The plates were then introduced in a plate reader (SPECTROstar Nano, BMG LABTECH, Ortenberg, Germany) and their absorbance was measured at 450 nm with the ELISA protocol, allowing for quantification of the antigen-antibody binding in the wells.

Figure 3.6 - Blocking solution being pipetted into the plates during the ELISA assay, with a multichannel.

3.9 In vitro gastrointestinal digestion protocol

The *in vitro* digestion was performed following the INFOGEST 2.0 protocol with some changes (Brodkorb et al., 2019). For the gastrointestinal digestion, 3 samples were selected, namely raw and 2 processed species were analysed.

3.9.1 Preparation steps

The day before the digestion, around 500 mg of the sample were weighed to 29 independent flat bottom glass vials (previously decontaminated). Although it would be ideal to weigh the samples on the digestion day, the methodology is very time-consuming due to the number of samples, and it is more important to finish the digestion within the same day. Additionally, on digestion day (before it started), the simulated fluids were heated at 37 ºC and the enzymes were prepared as described in **Table 3.5**.

3.9.2 Protocol

Regarding the digestion protocol, the samples were submitted to the oral, gastric, and intestinal phases. The samples were submitted to different timings of each phase. Consequently, not all samples were submitted to the gastric and/or intestinal digestion. The

way each one of the 29 samples were submitted to the simulated digestion is represented in the following chronogram (**Table 3.6**).

Table 3.5 - Protocol for the preparation of both digestive enzymes and bile solutions.

	Mass (mg)	Final volume (mL)	Solvent
Pepsin	30.74	6	H_2O
Pancreatin	3000	9	SIF
Bile*	768	8	SIF

*Bile solution needed some agitation at 37ºC to solubilize. SIF – simulated intestinal fluid.

Table 3.6 - Chronogram showing the time of each digestive phase for every sample that was considered for analysis. The samples are organised sequentially, from T0 (time 0 min) to T21 (time 242 min). The R1 samples are replicate samples (for example, T21R1 means that this sample is the replicate of sample T21), but the R2 correspond to samples without the addition of digestive enzymes. C1 and C2 are control samples, C1 as a negative control with no food, and C2 as the pH control sample.

Sample	Salivary phase (min)	Gastric phase (min)	Intestinal phase (min)
T0	0	-	-
T1	2	-	-
T1R	2	-	-
T2	2	0	-
T3	2	5	-
T4	2	10	-
T5	2	15	-
T6	2	20	-
T7	2	30	-
T8	2	45	-
T9	2	60	-
T10	2	90	-
T11	2	120	-
T11R1	2	120	-
T11R2	2	120	-
T12	2	120	0
T13	2	120	5
T14	2	120	10
T15	2	120	15
T16	2	120	20
T17	2	120	30
T18	2	120	45
T19	2	120	60
T20	2	120	90
T21	2	120	120
T21R1	2	120	120
T21R2	2	120	120
C1	2	120	120
C2	2	120	120

In the beginning, the sample C2 was used to determine the adequate pH – 3.0 for the gastric phase, and 7.0 for the intestinal phase. The digestion process itself started with the addition of 400 µL of simulated salivary fluid (SSF), 97 µL of H_2O and 3 µL of $CaCl_2(H_2O)_2$, followed by mechanical pressure with a glass rod for 30 seconds to replicate chewing and an incubation at 37 ºC for 2 minutes.

At the end of the 2 minutes, the gastric phase was initiated, starting with 800 µL of simulated gastric fluid (SGF). To the mixture of the partially digested material and simulated gastric fluid, a pre-determined amount of 6 M HCl was added to reach a pH of 3.0, using the reference volumes of C2 sample. Of the pepsin solution, the quantity used was 160 µL, followed by 1 µL of $CaCl_2(H_2O)_2$. Ultrapure water was added considering the difference of HCl to 40 µL and finished the gastric phase with an incubation of 2 hours at 37 ºC with smooth agitation. At the end of the incubation, 100 µL of 1 M NaOH were used to reach a pH value above 7.0, which stops pepsin activity, and consequently halts the gastric digestion process.

The samples that entered intestinal phase were initially treated with 850 µL of simulated intestinal fluid (SIF), and the previously determined amount of 1 M NaOH that would allow a final pH of 7.0. Moreover, 250 µL of the bile solution, 4 µL of $CaCl_2(H_2O)_2$ and 500 µL of pancreatin solution were further included in the mix, completed with H_2O for a final volume of 4 mL. Lastly, the samples were submitted to a 37 ºC incubation for 2 hours with smooth agitation. In due time, the intestinal phase was terminated with the addition of 50 µL of either 0.1 M PMSF (protease inhibitor) or 0.1 M Pefabloc.

After all the samples were digested, they were centrifuged (Centrifuge 5810 R, Eppendorf AG, Hamburg, Germany) for 15 min, at 9000*xg* and 4 ºC. This centrifugation was performed mainly to remove the remaining solid debris of the digested samples and obtain a clearer supernatant with protein content for further analysis.

4 Results and Discussion

4.1 Determination of total protein by UV/Vis spectrophotometry

Protein quantification by UV/Vis spectrophotometry consists of the determination of the protein content by estimating the absorbance at a wavelength of 280 nm. Samples containing proteins are introduced in a microplate (LVIS microplate, SPECTROstar Nano, BMG LABTECH, Ortenberg, Germany), and the concentration was determined at 280 nm (maximum absorption of proteins), mainly due to the contribution of tryptophan and tyrosine residues, without the addition of any external reagents. This characteristic differs from most

protein quantification methodologies, making UV/Vis spectrophotometry as a fast, cheap, and simple method for protein screening, although less sensitive. In fact, there are some potential interferents in most protein extracts (e.g., the presence of colour, solid impurities), which means that this method is less reliable (less accurate) to quantify proteins.

The results of protein extracts from different samples by UV/Vis spectrophotometry is presented in Tables 4.1 to 4.4. The raw barnacles extracted with different buffers allowed to obtain good concentrations of total protein, presenting yields ranging from 10.83 to 17.03 mg/mL (**Table 4.1**). In general, the extraction of protein performed at 60 ºC promoted slightly higher yields, except for the raw barnacles extracted with PBS 0.2 M that presented a higher yield when the protocol was performed at 4 ºC overnight. Still, the differences in terms of total protein amount were small, meaning that they are comparable among them.

Table 4.1 - Protein concentration estimated by UV/Vis spectrophotometry of raw barnacles extracted with different buffers, using 2 protocols: 60 ºC/2 h or 4 ºC overnight (mean ± SD (mg/mL) of 2 independent extractions/per buffer and per protocol).

Buffer for protein extraction	**Raw barnacles extracted at 60 ºC for 2 h**	**Raw barnacles extracted at 4 ºC overnight**
	Concentration (mg/mL)	**Concentration (mg/mL)**
Tris-HCl 100 mM pH 8.0	13.16 ± 0.03	13.81 ± 0.13
0.02% NaN_3	15.07 ± 0.04	12.64 ± 0.08
PBS 0.2 M pH 7.5	10.83 ± 0.03	13.99 ± 0.05
ultrapure H_2O pH 9.0	12.51 ± 0.16	12.73 ± 0.10
ultrapure H_2O pH 12.0	13.07 ± 0.08	12.29 ± 0.01
NH_4HCO_3 100 mM	14.57 ± 0.04	13.25 ± 0.03
Tris + SDS pH 7.6	17.03 ± 0.03	14.78 ± 0.18

In the case of boiled barnacles (10 min) extracted with different buffers, the total amount of protein was in general lower than for the extracts obtained from raw barnacles (**Table 4.2**). Like for the raw barnacles, the extraction of protein performed at 60 ºC promoted slightly higher yields, except for the boiled barnacles extracted with Tris-HCl 100 mM that had a higher yield at 4 ºC overnight. From these results, any of the selected buffers seemed to provide extracts with good concentration for both raw and boiled barnacles.

Table 4.2 - Protein concentration estimated by UV/Vis spectrophotometry of boiled barnacles (10 min) extracted with different buffers, using 2 protocols: 60 ºC/2 h or 4 ºC overnight (mean ± SD (mg/mL) of 2 independent extractions/per buffer and per protocol).

Buffer for protein extraction	**Boiled barnacles (10 min) extracted at 60 ºC for 2 h**	**Boiled barnacles (10 min) extracted at 4 ºC overnight**
	Concentration (mg/mL)	**Concentration (mg/mL)**
Tris-HCl 100 mM pH 8.0	5.25 ± 2.32	6.80 ± 0.22
0.02% NaN_3	6.86 ± 0.07	6.45 ± 0.38
PBS 0.2 M pH 7.5	7.17 ± 1.30	6.97 ± 0.17
ultrapure H_2O pH 9.0	6.65 ± 0.07	6.10 ± 1.61
ultrapure H_2O pH 12.0	9.25 ± 1.48	7.61 ± 0.92
NH_4HCO_3 100 mM	7.95 ± 0.01	7.28 ± 0.04
Tris + SDS pH 7.6	12.45 ± 0.39	9.89 ± 0.11

The total amount of protein of different crustacean species submitted to distinct food processing methods extracted with Tris-HCl 100 mM pH 8.0 buffer is presented in **Table 4.3**. Even though, this buffer does not yield the extraction with the highest quantity of protein, other factors came into consideration when choosing the buffer. For example, Tris + SDS severely affected the integrity of the sample. Additionally, the preliminary studies with other species (e.g., vannamei shrimp, not shown), and the analysis of the proteins extracted by different buffers by SDS-PAGE and immunoblotting (further presented in section 4.3) did not correlate well with the results presented herein. Therefore, Tris-HCl 100 mM pH 8.0 was chosen for this study as it presented the most satisfactory results and it was already considered as a highly reliable buffer.

Samples were all extracted with Tris-HCl 100 mM pH 8.0 buffer following the protocol of incubation at 60 ºC per 2 h. The total protein content for all samples was within the same order of magnitude, ranging from 4.5 to 23.0 mg/mL and with the spiny spider crab boiled for 10 min presenting the highest protein value. Within each species, the variation in protein content was less than 5 mg/mL, independently on the type of food processing. Regarding the cooking liquids, the water from boiling most species presented good content of proteins (1.5 to 6.1 mg/mL), whose variation is greatly influenced by the species being processed and by the duration of boiling process. The frying oils and the marination juices presented high values for protein content, which was not expected. In fact, most proteins are water soluble, not lipophilic, meaning that those values might be due to interferents and not to the proteins.

Table 4.3 - Protein concentration estimated following quantification by UV/Vis spectrophotometry for all crustacean samples, including boiling water and frying oil (mean ± SD (mg/mL) of 2 independent extractions).

Sample	**Protein content**
***Vannamei* shrimp**	**Mean** ± SD (mg/mL)
Raw	9.91 ± 0.72
Boiled (100 ºC) - 2 min	4.44 ± 0.76
Boiling water - 2 min	6.09 ± 0.05
Fried (180 ºC) - 3 min	5.51 ± 0.86
Frying oil - 3 min	54.23 ± 1.95
Grilled - 3 min	6.36 ± 0.80
Black tiger shrimp	
Raw	8.85 ± 0.91
Boiled (100 ºC) - 4 min	6.46 ± 0.80
Boiling water - 4 min	1.63 ± 0.00
Fried (180 ºC) - 4 min	10.59 ± 1.12
Frying oil - 4 min	27.01 ± 0.23
Grilled - 4 min	11.13 ± 1.03
American lobster	
Raw	10.17 ± 0.95
Boiled (100 ºC) - 15 min	6.97 ± 0.95
Boiling water - 15 min	11.63 ± 0.00
Fried (180 ºC) - 5 min	7.22 ± 0.73
Frying oil - 5 min	44.74 ± 1.48
Grilled - 20 min	6.08 ± 0.67
Raw and marinated in lemon juice - 1.5 h	12.11 ± 0.86
Marination juice (lemon juice) of raw lobster	35.26 ± 0.33
Marinated in lemon juice (1.5h) + grilled - 5 min	12.24 ± 1.04
Marinated in lemon juice (1.5h) + oven cooked - 15 min	10.28 ± 1.10
Argentine (red) shrimp	
Raw	16.64 ± 1.05
Boiled (100 ºC) - 3 min	12.70 ± 1.4 2
Boiling water - 3 min	4.72 ± 0.01
Fried (180 ºC) - 5 mi	14.65 ± 1.62
Frying oil - 5 min	42.12 ± 4.12
Grilled - 3 min	15.46 ± 1.49
(Goose) Barnacles	
Raw	13.16 ± 0.03
Boiled (100 ºC) - 3 min	7.82 ± 1.30
Boiling water - 3 min	1.52 ± 0.02
Boiled (100 ºC) - 10 min	6.17 ± 1.08
Boiling water - 10 min	4.71 ± 0.02
Blue swimming crab	
Raw;	13.15 ± 0.21
Steam cooked - 5 min	11.22 ± 0.59
Boiled 5 min	10.98 ± 0.81
Boiling water - 5 min	4.87 ± 0.09
Grilled 5 min	13.43 ± 1.88
Oven cooked - 15 min	13.68 ± 1.53
Oven-cooking water - 15 min	46.99 ± 0.37
Spiny spider crab	
Raw	10.37 ± 1.51
Boiled (100ºC) - 10 min	22.93 ± 2.73
Boiling water - 10 min	4.50 ± 0.05
Brown crab	

Raw	14.50 ± 1.70
Boiled (100ºC) - 10 min	19.41 ± 2.13
Boiling water - 10 min	5.87 ± 0.17
Scampi	
Raw	10.47 ± 1.08
Boiled (100ºC) - 5 min	8.98 ± 1.11
Boiling water - 5 min	5.92 ± 0.02
Fried (180ºC) - 5 min	13.73 ± 2.59
Frying oil - 5 min	21.64 ± 0.01
Grilled - 5 min	9.56 ± 1.08
Oven-cooked - 15 min	9.75 ± 1.22
Raw and marinated in lemon juice - 1.5 h	11.60 ± 1.27
Marination juice (lemon juice) of raw lobster	34.42 ± 0.72
Marinated in lemon juice (1.5 h) + grilled - 5 min	10.84 ± 1.3\\\
Marinated in lemon juice (1.5 h) + oven-cooked - 15 min	16.74 ± 1.75

The light scattering due to the presence of suspended impurities (physical interferents), as the samples were neither filtered nor centrifuged, might contribute to the reported values. Likewise, the chemical interferents probably also played a role, since all samples had some colour, which meant they could absorb in the visible spectrum. In the case of the marination juices, which are rich in citric acid from the lemons, the protein values were also high. This might be related with potential interferents rather than to the actual presence of proteins. The content of protein was also determined by UV/Vis spectrophotometry for the samples that were in vitro digested. **Table 4.4** presents the values of protein for the in vitro gastrointestinal digestion of American lobster (raw, raw and marinated in lemon juice 1.5 h and marinated 1.5 h + grilled 5 min) at different time points, following salivary (T0-T1R), gastric (T2-T11R2) and intestinal (T12-T21R2) phases.

All tubes had a starting quantity of sample of 500 mg, except for tube C1 that functioned as the negative control (no samples) of the digestion as only fluids and enzymes were added. The values are all in the same order of magnitude, presenting low degree of variation in terms of protein content. The values for the T11R2 and T21R2 correspond to the sample in salivary/gastric and salivary/gastric/intestinal, but with no digestive enzymes added, meaning that the samples were probably less degraded, thus disabling to retrieve higher amounts of protein. In the salivary phase, all samples presented high yield of protein ranging from 50-65 mg/mL. interestingly, when analysing the samples from gastric phase the total amount seems to be half of the salivary phase, fact that might be justified by the activity of pepsin, which cleaves proteins. The addition of pancreatin in the intestinal phase is probably responsible for increasing again the total amount of each digested sample, as this mix of enzymes contributes to increase the yield of proteins.

In general, and since BCA is a more adequate methodology for protein quantification, it was the preferred methodology for evaluating the protein content of each sample before further analysis. However, the preliminary determination of the protein content by UV/Vis

was of great utility, as they were used as a guideline to dilute samples to values of protein concentration which were closer to the optimal range of quantification of the BCA method, as well as serving as a confirmation of the BCA results.

Table 4.4 - Protein concentration estimated following quantification by UV/Vis spectrophotometry for the American lobster digestion samples.

		American lobster - protein concentration (mg/mL)		
Sample	**Time points (min)**	**Raw**	**Raw and marinated in lemon juice (1.5h)**	**Marinated in lemon juice (1.5h) + grilled (5 min)**
Salivary				
T0	0	49.87 ± 0.67	64.88 ± 2.26	61.27 ± 0.33
T1	2	49.46 ± 0.52	61.65 ± 2.22	53.69 ± 0.75
T1R	2	50.01 ± 0.99	62.46 ± 2.75	61.60 ± 0.91
Gastric				
T2	0	23.49 ± 0.01	28.97 ± 0.20	27.04 ± 0.08
T3	5	25.14 ± 0.21	33.05 ± 0.21	32.70 ± 0.25
T4	10	25.96 ± 0.13	32.51 ± 0.10	32.55 ± 0.10
T5	15	26.82 ± 0.09	34.00 ± 0.01	31.15 ± 0.15
T6	20	26.69 ± 0.11	35.41 ± 0.35	30.93 ± 0.05
T7	30	27.10 ± 0.15	35.45 ± 0.93	35.37 ± 0.33
T8	45	28.98 ± 0.86	36.73 ± 0.66	36.09± 0.49
T9	60	29.21 ± 0.04	38.71 ± 0.24	36.84 ± 1.03
T10	90	29.48 ± 0.08	37.41 ± 0.40	36.55 ± 0.59
T11	120	29.46 ± 0.09	37.01 ± 0.03	38.18 ± 0.03
T11R1	120	28.85 ± 0.15	36.81 ± 0.01	36.89 ± 0.32
T11R2	120	23.87 ± 0.10	28.83 ± 0.09	22.05 ± 0.05
Intestinal				
T12	0	61.95 ± 2.88	53.19 ± 1.16	61.18 ± 4.96
T13	5	57.70 ± 0.22	60.72 ± 0.73	68.44 ± 0.32
T14	10	60.96 ± 1.09	62.47 ± 3.11	65.15 ± 2.23
T15	15	57.72 ± 1.54	62.87 ± 1.14	65.02 ± 1.89
T16	20	60.37 ± 0.04	63.23 ± 0.01	65.09 ± 2.16
T17	30	61.84 ± 1.35	64.16 ± 5.22	63.21 ± 3.89
T18	45	62.74 ± 1.64	60.30 ± 1.10	64.74 ± 0.19
T19	60	61.98 ± 2.29	63.32 ± 5.67	62.60 ± 2.88
T20	90	56.47 ± 0.96	65.98 ± 3.68	65.68 ± 0.38
T21	120	63.39 ± 4.44	63.79 ± 1.81	67.07 ± 0.05
T21R1	120	65.49 ± 3.06	62.17 ± 7.61	66.10 ±0.03
T21R2	120	18.26 ± 0.17	18.79 ± 0.26	18.54 ± 0.74
C1	242	58.81 ± 9.48	58.68 ± 3.06	65.03 ± 0.03
C2	242	59.23 ± 3.60	64.04 ± 2.55	65.25 ± 0.06

C1, without food; C2, pH control of entire digestion (food+enzymes); T11/T11R1 and T21/T21R1; duplicates; T11R2/T21R2, tubes without digestive enzymes.

Therefore, UV/Vis spectrophotometry allowed for an enhanced application of the BCA method, reducing the probability of having to repeat assays, whilst saving time and reagents and reducing the cost per sample analysis. Additionally, BCA proved ineffective in quantifying the frying oil and lemon juice samples, due to the presence of interferences, making UV/Vis the only accessible method for quantification of these samples.

4.2 Determination of total protein by BCA method

The Pierce™ BCA Protein Assay Kit is based on the use of bicinchoninic acid to promote the biuret reaction, which is a common reaction between proteins and copper ions for protein quantification. In alkaline conditions, proteins can reduce the cupric ion (Cu^{2+}) to the cuprous ion (Cu^{+}), corresponding to the biuret reaction. The formation of Cu^{+} reflects the amount of protein in a sample, as the extension at which the biuret reaction occurs depends on the content of protein in solution. This means that a high quantity of proteins increases the extension of the biuret reaction, since the working reagents of BCA kit contains cupric ions. In this process, there is the chelation of one cuprous ion with two BCA molecules, forming a purple, water-soluble complex with intense absorbance at a wavelength of 562 nm. Since this method can be used for different proteins, the quantification is performed considering the concomitant quantification of known concentrations of a BSA (Bovine Serum Albumin) used as standard, which enables the construction of a calibration curve and allows the determination of the protein content of each sample. Hence, the effects of interferences on the final concentration values are attenuated, contrarily to the direct measurement by UV/Vis spectrophotometry. This method depends on the presence of some amino acids such as tyrosine, tryptophan, and cysteine; reducing agents and some strong acids and bases, which are some common interferences on the results (Pierce BCA Protein Assay Kit Instructions, Thermo Fisher Scientific, Waltham, Massachusetts, USA) (Thermo Fisher Scientific, 2020).

Table 4.5 presents the BCA results of the concentrations of the samples that were used to evaluate buffer efficiency in extracting protein from crustaceans, namely barnacles. In what concerns the raw barnacles, the protein concentrations ranged from 3.6 to 16.9, which are lower than what was observed with UV/Vis spectrophotometry, specifically when considering the lowest value obtained for UV was 10.8 mg/mL. When comparing the extraction at 60 ºC with the one at 4ºC, it is visble that the results are only slightly different similarly to what was suggested by the UV. Therefore, apart from the extracts obtained with Tris-HCl 100 mM pH 8.0 that presented a greater discrepancy (5.11 to 14.48 mg/mL), the extraction method does not seem to induce a lot of differences in the total amount of protein.

As for the boiled barnacles (10 min), although they present significant differences from their raw counterparts in some samples (e.g., Tris-HCl 100 mM pH 8.0 and Tris-HCl 100 mM + SDS 4% pH 7.6 at 4 ºC overnight), this fact does not seem to be related with the thermal processing, since the higher value of protein concentration varies between the raw and boiled barnacles, which contradicts what was suggested for UV/Vis spectrophotometry. Comparing the different extraction methods, the samples extracted at 60 ºC seem to have

higher yields, apart from PBS 0.2M pH 7.5 buffer sample, which was also verified to happen in raw barnacles for UV/Vis analysis.

Table 4.5 - Protein concentration estimated following quantification by BCA method for raw and boiled barnacles (10 min) extracted with different buffers, using 2 protocols: 60 ºC/2 h or 4 ºC overnight (mean ± SD (mg/mL) of 2 independent extractions/per buffer and per protocol).

Buffer for protein extraction	Raw barnacles	Boiled barnacles (10 min)
	Concentration (mg/mL)	Concentration (mg/mL)
60 ºC for 2 h		
Tris-HCl 100 mM pH 8.0	5.11 ± 0.41	8.35 ± 0.14
0.02% NaN_3	10.62 ± 0.07	9.73 ± 0.22
PBS 0.2 M pH 7.5	13.37 ± 0.16	3.65 ± 0.36
ultrapure H_2O pH 9.0	5.91 ± 0.07	7.71 ± 0.04
ultrapure H_2O pH 12.0	7.89 ± 0.18	11.04 ± 0.14
NH_4HCO_3 100 mM	12.37 ± 0.81	12.05 ± 0.09
Tris + SDS pH 7.6	16.89 ± 0.72	15.83 ± 0.71
4 ºC overnight		
Tris-HCl 100 mM pH 8.0	14.48 ± 0.07	5.29 ± 0.08
0.02% NaN_3	7.51 ± 0.12	6.80 ± 0.18
PBS 0.2 M pH 7.5	15.83 ± 1.41	12.44 ± 0.88
ultrapure H_2O pH 9.0	5.06 ± 0.09	5.71 ± 0.25
ultrapure H_2O pH 12.0	6.16 ± 0.00	7.01 ± 0.05
NH_4HCO_3 100 mM	10.11 ± 0.35	7.87 ± 0.20
Tris + SDS pH 7.6	14.57 ± 0.35	8.14 ± 0.03

The total amount of protein of different crustacean species submitted to distinct food processing methods extracted with Tris-HCl 100 mM pH 8.0 buffer and incubated at 60ºC for 2 hours was evaluated by BCA and is presented in **Table 4.6**. The protein concentration for all samples varied between 1.45 and 6.81 mg/mL, which are all values on the same order of magnitude, making them more comparable. The sample with the highest value was the water of oven-cooked blue swimming crab, and the solid samples with the highest protein content being the vannamei and the black tiger shrimps.

It is worth noting that, in similarity to what was seen for some liquid samples on UV/Vis spectrophotometry, there could be some interferences that affect the results, although BCA is, in theory, much less influenced by external interferents than the UV/Vis protein quantification. In this case, the frying oils and the marination juices were not analysed, since it proved difficult to submit these samples to the BCA method in a satisfactory manner. When considering the samples submitted to marination with lemon juice, the American lobster showed higher yields in marinated samples than in non-marinated ones, whereas scampi did not present many differences between the marinated and non-marinated samples, which can indicate that BCA was not directly affected by this treatment. In general, many raw samples had superior yields than their processed counterparts, which was

expected, since these procedures probably alter the protein conformation by inducing processes of denaturing or even breakdown of protein, influencing the BCA quantification.

Table 4.6 - Protein concentration estimated following quantification by the BCA method for all crustacean samples, including boiling water and frying oil. The samples were quantified in different assays. In the case the sample was quantified in all three assays, the most distant result was not considered (highlighted in red). If the sample was only quantified in one of the assays, the standard deviation was not determined (N/A). Most frying oils were also not quantified, because the properties of these samples cause difficulties in performing BCA.

Sample	**Protein content**
***Vannamei* shrimp**	**Mean** ± SD (mg/mL)
Raw	5.63 ± 0.34
Boiled (100 ºC) - 2 min	2.82 ± 0.13
Boiling water - 2 min	3.62 ± 0.01
Fried (180 ºC) - 3 min	3.52 ± 0.23
Frying oil - 3 min	N/A
Grilled - 3 min	5.05 ± 0.19
Black tiger shrimp	
Raw	5.19 ± 0.02
Boiled (100 ºC) - 4 min	3.67 ± 0.12
Boiling water - 4 min	1.57 ± 0.06
Fried (180 ºC) - 4 min	3.79 ± 0.02
Frying oil - 4 min	N/A
Grilled - 4 min	5.29 ± 0.26
American lobster	
Raw	1.71 ± 0.03
Boiled (100 ºC) - 15 min	1.93 ± 0.03
Boiling water - 15 min	2.16 ± 0.04
Fried (180 ºC) - 5 min	1.70 ± 0.01
Frying oil - 5 min	N/A
Grilled - 20 min	1.45 ± 0.24
Raw and marinated in lemon juice - 1.5 h	3.40 ± 0.03
Marination juice (lemon juice) of raw lobster	N/A
Marinated in lemon juice (1.5h) + grilled - 5 min	3.35 ± 0.21
Marinated in lemon juice (1.5h) + oven cooked - 15 min	3.25 ± 0.09
Argentine (red) shrimp	
Raw	4.63 ± 0.12
Boiled (100 ºC) - 3 min	3.55 ± 0.07
Boiling water - 3 min	3.59 ± 0.09
Fried (180 ºC) - 5 mi	3.70 ± 0.11
Frying oil - 5 min	N/A
Grilled - 3 min	2.36 ± 0.12
(Goose) Barnacles	
Raw	4.6 ± 0.16
Boiled (100 ºC) - 3 min	2.45 ± 0.11
Boiling water - 3 min	1.66 ± 0.13
Boiled (100 ºC) - 10 min	1.71 ± 0.09
Boiling water - 10 min	1.75 ± 0.07
Blue swimming crab	
Raw;	3.53 ± 0.14
Steam cooked - 5 min	3.03 ± 0.01
Boiled 5 min	2.71 ± 0.18

Boiling water - 5 min	1.57 ± 0.03
Grilled 5 min	3.05 ± 0.14
Oven cooked - 15 min	2.94 ± 0.08
Oven-cooking water - 15 min	6.81 ± 0.21
Spiny spider crab	
Raw	3.36 ± 0.13
Boiled (100ºC) - 10 min	4.19 ± 0.02
Boiling water - 10 min	1.83 ± 0.05
Brown crab	
Raw	3.08 ± 0.01
Boiled (100ºC) - 10 min	2.55 ± 0.07
Boiling water - 10 min	1.77 ± 0.04
Scampi	
Raw	4.82 ± 0.03
Boiled (100ºC) - 5 min	2.49 ± 0.06
Boiling water - 5 min	1.68 ± 0.02
Fried (180ºC) - 5 min	2.50 ± 0.18
Frying oil - 5 min	N/A
Grilled - 5 min	2.83 ± 0.03
Oven-cooked - 15 min	2.87 ± 0.23
Raw and marinated in lemon juice - 1.5 h	4.24 ± 0.51
Marination juice (lemon juice) of raw lobster	N/A
Marinated in lemon juice (1.5 h) + grilled - 5 min	3.33 ± 0.08
Marinated in lemon juice (1.5 h) + oven-cooked - 15 min	2.66 ± 0.11

The total protein amount of digested American lobster (raw, raw and marinated in lemon juice 1.5 h and marinated 1.5 h + grilled 5 min) at different time points, following salivary (T0-T1R), gastric (T2-T11R2) and intestinal (T12-T21R2) phases was also determined by the BCA protein quantification method (**Table 4.7**).

In contrast to UV/Vis spectrophotometry, BCA results suggest that there is some variation between samples in their total protein amount, and there is not much specific information that can be retrieved from these results. It is noticeable that the C1 sample has lower values than most intestinal phase samples, due to having no food. Additionally, it is also visible that intestinal phase samples have higher yields of protein content, probably due to the addition of pancreatin, which adds a lot of protein content to the sample in the form of enzymes. That would also explain the significant amounts of protein on sample C1, for example for marinated in lemon juice (1.5h) + grilled (5 min) American lobster (29.87 mg/mL). Contrarily to the UV/Vis results, the raw lobster presented lower values for total protein amount, especially in the salivary phase. This might be explained by the fact that during this 2 min of salivary digestion, there are no enzymes degrading the sample, which does not allow to the protein to be released/degraded. The addition of pepsin in the gastric and pancreatin in the intestinal phases promotes the degradation of tissues and further release of protein/peptides, which are responsible for increasing the yield of total protein in digested samples.

Table 4.7 - Protein concentration estimated following quantification by BCA method for the American lobster digestion samples.

		American lobster - protein concentration (mg/mL)		
Sample	**Time points (min)**	**Raw**	**Raw and marinated in lemon juice (1.5h)**	**Marinated in lemon juice (1.5h) + grilled (5 min)**
Salivary				
T0	0	0.25 ± 0.02	19.61 ± 0.54	12.17 ± 0.24
T1	2	0.65 ± 0.01	20.54 ± 1.23	15.34 ± 0.28
T1R	2	3.00 ± 0.60	22.20 ± 0.36	17.40 ± 0.87
Gastric				
T2	0	5.80 ± 2.29	2.49 ± 0.13	8.61 ± 0.16
T3	5	8.56 ± 0.37	9.56 ± 0.60	14.63 ± 2.48
T4	10	11.56 ± 0.22	9.83 ± 0.44	15.26 ± 0.49
T5	15	13.80 ± 0.38	10.73 ± 0.29	18.31 ± 0.94
T6	20	13.80 ± 0.00	13.69 ± 0.71	18.31 ± 0.87
T7	30	4.23 ± 0.11	15.15 ± 0.41	12.24 ± 1.07
T8	45	5.80 ± 0.26	16.50 ± 0.88	15.08 ± 0.80
T9	60	9.64 ± 0.54	18.30 ± 1.34	14.38 ± 1.00
T10	90	10.58 ± 0.47	20.17 ± 1.37	16.17 ± 0.28
T11	120	13.75 ± 0.83	22.66 ± 1.52	22.69 ± 1.20
T11R1	120	14.81 ± 0.58	26.60 ± 3.29	16.69 ± 0.25
T11R2	120	8.58 ± 0.11	13.98 ± 1.97	8.72 ± 0.14
Intestinal				
T12	0	16.56 ± 0.42	29.83 ± 3.03	53.84 ± 0.76
T13	5	15.64 ± 0.17	29.70 ± 0.62	39.83 ± 0.37
T14	10	20.27 ± 1.05	34.07 ± 0.52	45.31 ± 2.64
T15	15	23.05 ± 2.12	34.88 ± 1.43	40.35 ± 1.21
T16	20	26.70 ± 2.30	27.41 ± 2.54	36.78 ± 1.62
T17	30	37.93 ± 1.93	23.67 ± 0.76	40.20 ± 1.10
T18	45	36.31 ± 1.82	24.41 ± 0.45	58.28 ± 1.42
T19	60	40.57 ± 0.74	14.10 ± 0.34	61.70 ± 2.03
T20	90	36.02 ± 2.01	14.26 ± 0.22	63.97 ± 2.17
T21	120	17.65 ± 0.56	18.09 ± 0.27	55.71 ± 0.81
T21R1	120	22.02 ± 3.34	15.70 ± 0.06	44.26 ± 0.63
T21R2	120	23.44 ± 2.18	14.54 ± 0.14	2.75 ± 0.04
C1	242	11.85 ± 0.45	6.81 ± 0.43	29.87 ± 2.40
C2	242	13.89 ± 0.11	11.24 ± 0.30	44.29 ± 0.52

4.3 Evaluation of protein extraction using different buffers

To better analyse the effect of food processing and to maximise the amount of protein extraction, some crustacean species were submitted to different extraction buffers as well as to distinct extraction conditions, in terms of temperature and time of incubation. Therefore, different common buffers and incubation temperatures/time intervals were applied to extract protein from raw and boiled samples of barnacles, as a case study. The protocols included testing incubation temperature/period of 60 ºC for 2 h and 4 ºC overnight with selected buffers. The extraction buffers tested were Tris-HCl 100 mM pH 8.0, ultrapure H_2O pH 9.0, ultrapure H_2O pH 12.0, 0.02% NaN_3, NH_4HCO_3 100 mM, Tris-HCl 100 mM +

SDS 4% pH 7.6, PBS 0.2 M pH 7.5. The results for the total amount of protein extracted using the referred conditions are presented in sections 4.1 and 4.2, being further used to normalise the total quantity of protein used to run the gels/immunoblots.

In general, barnacles submitted to 4 ºC/overnight extraction have a higher quantity and intensity of bands when compared to their 60 ºC/2 h extracted counterparts, independently on their processing state. Comparing the buffers, the SDS-PAGE analysis of barnacles seems to indicate that most extraction buffers have similar behaviour, although the ultrapure H_2O at pH 12.0 slightly outperforms the remaining buffers. For the raw barnacles, this buffer allowed to extract proteins with higher efficiency, presenting bands with higher intensity, independently on the temperature and extraction time. Contrarily to literature, and despite PBS 0.2 M pH 7.5 being the buffer mostly used to extract proteins, barnacles extracted with this buffer presented the worse performance, especially in the case of raw barnacles (**Figure 4.1A**, lanes 7 and 14). The protein extracts using the Tris+SDS pH 7.6 buffer exhibited slightly different profiles than the remaining 6 buffers, often presenting bands at different molecular weights. This distinct profile is even more marked in boiled barnacles, compared to raw counterpart. For the boiled barnacles (10 min), all buffers allowed extracting proteins with good intensity, being more efficient when samples were extracted at 4 ºC overnight (**Figure 4.1B**). Also in this case, the buffers exhibiting better performance were the ultrapure H_2O pH 12.0 and the Tris+SDS pH 7.6.

For barnacles, the protein profiles observed in SDS-PAGE were somewhat distinct depending on the buffer used, even though there are some key similarities. For instance, despite putting the same amount of total protein per lane (25 µg), the intensity of the bands was visibly lower in raw barnacles, with the most intense band appearing at around 37 kDa, which are suspect to be tropomyosin. Less intense bands at around 15, 25 and 100 kDa are also observed. Depending on the buffer used, some bands at 45-50 kDa, 60-75 kDa and above 150 kDa are also visible (**Figure 4.1A**). In comparison, boiled barnacles present several of these bands but with a greater intensity, especially the bands at 37 kDa and 45 kDa, independently of the temperature or time of the extraction (**Figure 4.1B**). The bands appearing above 100 kDa in the raw barnacles are less intense in the boiled barnacles, probably resulting from protein denaturation. Conversely, there are more bands appearing at 20-25 kDa in boiled samples (but not in raw barnacles), which might either be a consequence of the boiling process to the proteins, causing fragmentation, or an enhanced performance of the extraction method in thermally processed samples.

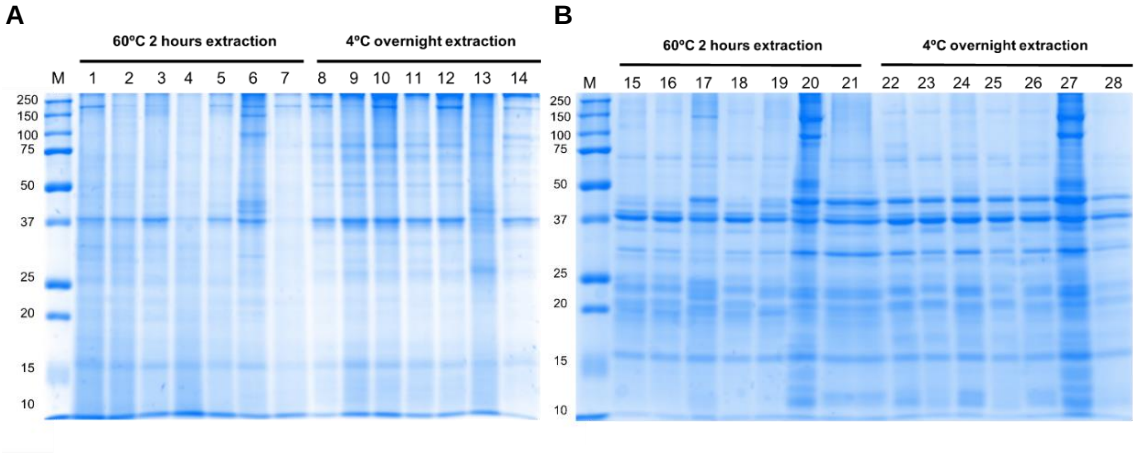

Figure 4.1 - SDS-PAGE gels in non-denaturing conditions of raw barnacles (A) and boiled barnacles (B) submitted to protein extraction with different buffers (total protein 25 µg) and protocols of 60 ºC/2 h and 4 ºC/overnight. Legend: lane M – Precision Plus Protein Dual Color Standard 10-250 kDa (Bio-Rad Laboratories Inc., Hercules, CA, USA); lanes 1, 8, 15, 22 – protein extracted with Tris-HCl 100 mM pH 8.0; lanes 2, 9, 16, 23 - protein extracted with ultrapure H_2O pH 9.0; lanes 3, 10, 17, 24 - protein extracted with ultrapure H_2O pH 12.0; lanes 4, 11, 18, 25 - protein extracted with 0.02% NaN_3; lanes 5, 12, 19, 26 - protein extracted with NH_4HCO_3 100 mM; lanes 6, 13, 20, 27 - protein extracted with Tris + SDS pH 7.6; lanes 7, 14, 21, 28 - protein extracted with PBS 0.2 M pH 7.5.

Despite most bands presenting molecular weights at 15-25 kDa (troponin C/myosin light chain/sarcoplasmic calcium binding protein), 37 kDa (tropomyosins), 42-45 kDa (arginine kinases), 75-100 kDa (hemocyanin/glycogen phosphorylase-like protein) being associated with known allergens, it is important to evaluate their IgE-binding capacity. This means that a buffer should be selected based on its capacity to extract (i) the highest quantity of total proteins and (ii) most of the allergenic proteins. Therefore, the same extracts were submitted to immunoblotting analysis (**Figure 4.2**).

In non-reducing conditions, the immunoblotting results evidenced that the extracts with higher IgE-binding capacity were the ones extracted at 60 ºC (2 h incubation), specifically with Tris-HCl 100 mM pH 8.0, ultrapure H_2O pH 9.0 and ultrapure H_2O pH 12.0 buffers (lanes 1, 2 and 3, **Figure 4.2**), although the 0.02% NaN_3 buffer also reveal interesting results in boiled barnacles (lane 4, **Figure 4.2B**). The band at 37 kDa manifested the higher intensity, which might indicate that there are probably 2 different proteins involved, tropomyosin (37 kDa) and arginine kinase (42-45 kDa), both considered as panallergens of crustaceans. There are some other bands showing IgE-binding capacity at some degree, namely the band at 75 kDa that is likely to be a hemocyanin or the IgE-reactive bands at 15-25 kDa, which are likely to be troponin C, myosin light chain and/or sarcoplasmic calcium binding protein (**Figure 4.2**).

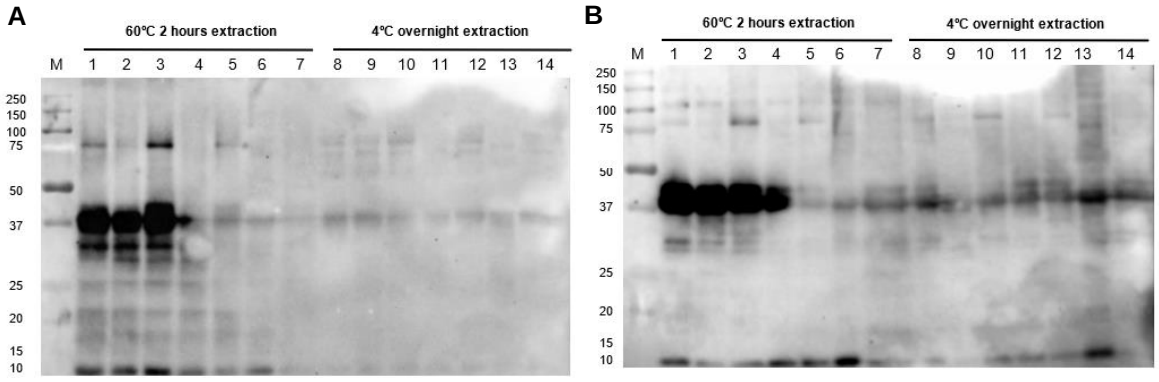

Figure 4.2 - Immunoblotting membranes incubated with pool sera from crustacean-allergic patients as primary antibody, run in non-denaturing conditions of raw barnacles (A) and boiled barnacles (B) submitted to protein extraction with different buffers (total protein 25 µg) and protocols of 60 ºC/2 h and 4 ºC/overnight. Legend: lane M – Precision Plus Protein Dual Color Standard 10-250 kDa (Bio-Rad Laboratories Inc., Hercules, CA, USA); lanes 1, 8, 15, 22 – protein extracted with Tris-HCl 100 mM pH 8.0; lanes 2, 9, 16, 23 - protein extracted with ultrapure H_2O pH 9.0; lanes 3, 10, 17, 24 - protein extracted with ultrapure H_2O pH 12.0; lanes 4, 11, 18, 25 - protein extracted with 0.02% NaN_3; lanes 5, 12, 19, 26 - protein extracted with NH_4HCO_3 100 mM; lanes 6, 13, 20, 27 - protein extracted with Tris + SDS pH 7.6; lanes 7, 14, 21, 28 - protein extracted with PBS 0.2 M pH 7.5.

Considering all the information from the SDS-PAGE and the immunoblotting, it was decided to choose the Tris-HCl 100 mM pH 8.0 with a 2-hour incubation at 60ºC as the method to extract the remaining crustacean species, as this buffer is considered as one of the most versatile. Although the SDS-PAGE analysis showed less intensity of bands at 60 ºC/2 hours extraction using the Tris-HCl 100 mM pH 8.0 buffer, the immunoblotting allowed to verify that the IgE-binding capacity of the proteins was significantly higher when extracting the proteins with the referred buffer and under 60 ºC/2 h incubation, which is far more relevant to study the allergenic potential of proteins. Additionally, it obviates the drawbacks of using the 4 ºC overnight extraction protocol since it is a very time-consuming process, requiring a more prolonged usage of equipment and “overnight” was not a specific amount of time, that varied between 16 and 20 hours. It was also considered to extract all crustaceans with the 7 buffers and incubation methods to implement the better method for each crustacean, but it was found to be time-consuming and cost-demanding due to the unnecessary use of material, reagents, and equipment. Therefore, the selection of barnacles was based on the unusual typology of this crustacean, being considered as the case study and assuming that the remaining crustaceans would follow a similar behaviour.

In summary, the choice of Tris-HCl 100 mM pH 8.0 as the extraction buffer was due to its demonstrated consistency for extracting protein from the remaining crustaceans. In consequence, the Tris-HCl 100 mM pH 8.0 was more reliable in yielding better results,

considering the large variety of species with different protein profiles and physicochemical properties, as well as providing satisfactory results regarding the IgE-reactivity of crustacean allergens as assessed by immunoblotting analysis.

4.4 Effect of different food processing treatments on protein IgE-binding capacity

After extracting all crustacean samples with the Tris-HCl 100 mM pH 8.0 buffer, SDS-PAGE and immunoblotting analysis was conducted to characterize the protein profile on different crustacean species, and to evaluate the effect of different processing methods (mostly common heat treatments) on the protein properties and IgE-binding capacity of crustacean allergens. Additionally, it was also sought to evaluate the passage of proteins to the cooking water during the boiling process, and the possibility of the water containing any proteins with allergenic potential, which would align with the reported patient sensitisation following exposure to aerosolized allergens (Lopata & Jeebhay, 2013). Furthermore, it was also intended to verify the reported possibility of allergens being transferred to the frying oil (Sj & Sankar, 2021).

4.4.1 Vannamei (Litopenaeus vannamei) and black tiger (Penaeus monodon) shrimps

Vannamei shrimp is one of the most vastly consumed crustacean species, encompassing more than half of all the crustacean production globally, as of 2018 (FAO, 2020). Black tiger shrimp is also a well-known, although less available crustacean used as a food source. Therefore, these two species were included in this study (**Figure 4.3**). For *vannamei* shrimp, it was observed that the raw sample had an intense band at around 18 kDa (**Figure 4.3A**, lanes 1 and 13), that was suspected to be either a myosin light chain 2 (Lit v 3) or a sarcoplasmic calcium-binding protein (Lit v 4), since it was IgE-reactive when tested with the pool-sera of the crustacean-allergic patients (**Figure 4.3B**, lane 13). In fact, this might indicate that the band is probably the combination of both allergens. The 18 kDa band is also present and IgE-reactive in all the processed samples of *vannamei* shrimp, namely in boiled (lanes 2 and 14), fried (lanes 4 and 16) and grilled (lanes 6 and 18) ones (**Figure 4.3B**). Boiling (100 ºC) appeared to reduce the IgE-binding capacity of the allergen, whereas frying (180 ºC) seemed to enhance it, which can be related to its higher temperature. This dissimilarity does not seem to be related to the hydrophobicity of the proteins, given that it was neither present in the boiling water nor in the frying oil. Other observation made was that a band (around 37 kDa) appeared in the processed samples, suspected to be tropomyosin (Lit v 1), the most common crustacean panallergen, but it was

not apparent in the raw *vannamei* shrimp. This phenomenon was not expected but it was probably caused by the extraction method chosen.

It is also worth mentioning that this 37 kDa protein was also visible on the SDS-PAGE in the boiling water (lane 3) and frying oil (lane 5) samples (**Figure 4.3A**), meaning that part of the tropomyosin can migrate to the water or oil. Although with weaker signal compared to their presence in the processed meat of *vannamei* shrimp, this protein has some degree of IgE-binding capacity both in the boiling water and frying oil. In general, the SDS-PAGE analysis allowed to identify different protein profiles, of both allergenic and non-allergenic proteins, confirming that there are significant differences in the effect of heat processing methods on proteins.

Regarding the black tiger shrimp, the 18 kDa band was also present in raw and processed samples, with the frying lane band (lanes 10 and 22) being more distinct due to the lower intensity of this band, which contrasts with that observed for *vannamei* shrimp (**Figure 4.3**). Nevertheless, the 18 kDa band is very intense both in the boiled and grilled meat of black tiger shrimp, being probably a combination of more than one allergen, namely a myosin light chain 2 (Pen m 3), sarcoplasmic calcium-binding protein (Pen m 4) and/or cytoplasmic fatty acid binding protein (Pen m 13). Additionally, it was also observed that the tropomyosin band (Pen m 1) is present in most lanes of processed black tiger shrimp, but not on the raw sample, which is well correlated to the *vannamei* shrimp observations. However, for black tiger shrimp, this allergenic protein was transferred to the boiling water (lanes 9 and 21), showing higher IgE-reactivity in the immunoblotting (**Figure 4.3**). There is also a protein present in both SDS-PAGE and immunoblotting at around 27 kDa in both boiled (lanes 8 and 20) and fried (lanes 10 and 22) samples, that is likely to be triosephosphate isomerase (Pen m 8), another known black tiger shrimp allergen. For this crustacean, frying oil is characterized as having no proteins (lanes 11 and 23), allergenic or not, although that could happen due to the difficulty in pipetting the oil samples into the SDS-PAGE wells, as they do not mix properly with the 2x Laemmli buffer (**Figure 4.3**).

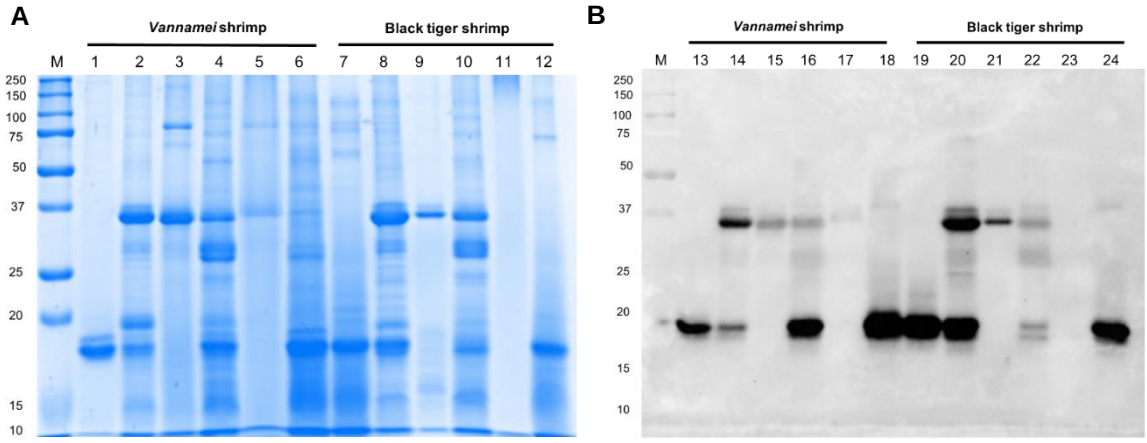

Figure 4.3 - SDS-PAGE gel (A) and immunoblotting membrane (B) run in non-denaturing conditions of *vannamei* shrimp and black tiger shrimp after different heat processing methods (total protein 25 µg) submitted to protein extraction with Tris-HCl 100 mM pH 8.0 buffer at 60 ºC/2 h. The membrane was incubated with pool-sera from crustacean-allergic patients as primary antibody. Legend: lane M – Precision Plus Protein Dual Color Standard 10-250 kDa (Bio-Rad Laboratories Inc., Hercules, CA, USA); lanes 1 and 13 - raw *vannamei* shrimp; lanes 2 and 14 - boiled *vannamei* shrimp 2 min; lanes 3 and 15 - boiling water of *vannamei* shrimp 2 min; lanes 4 and 16 - fried *vannamei* shrimp 3 min; lanes 5 and 17 - frying oil *vannamei* shrimp 3 min; lanes 6 and 18 - grilled *vannamei* shrimp 3 min; lanes 7 and 19 - raw black tiger shrimp; lanes 8 and 20 - boiled black tiger shrimp 4 min; lanes 9 and 21 - boiling water black tiger shrimp 4 min; lanes 10 and 22 - fried black tiger shrimp 4 min; lanes 11 and 23 - frying oil black tiger shrimp 4 min; lanes 12 and 24 - grilled black tiger shrimp 4 min.

4.4.2 American lobster (Homarus americanus)

Like the previous crustaceans, the American lobster was also studied, being submitted to heat treatment and marination with lemon juice (**Figure 4.4**). Starting by comparing the raw American lobster with the heat-processed samples, there were no significant differences observed. Both raw and processed samples presented a protein profile with few intense bands, with only the bands around 36 kDa and 75 kDa being more noticeable, but with some faint bands at 17, 25 and 100 kDa. The band at 36 kDa seems to encompass 2 proteins, probably corresponding to a tropomyosin (Hom a 1) with stronger signal and to an arginine kinase (although there is no official identification of this allergen) (WHO/IUIS, 2023), for both raw and heat processed samples. The band at 75 kDa was not present in the SDS-PAGE for boiled lobster (lane 2) but it was present in the boiling water (lane 3), indicating that it was almost completely transported to the boiling water (**Figure 4.4A**). The fried lobster (lane 14) showed some IgE-reactivity towards this protein, indicating a possible allergen, most likely hemocyanin (**Figure 4.4B**). In this case, it was possible to see the tropomyosin in the boiling water (lanes 3 and 13), but not in frying oil (lanes 5 and 15).

Apparently, although the differences in band intensity are not that big in SDS-PAGE, the IgE-binding capacity of different allergens was higher for processed samples (lanes 12 and 14) than for the raw sample (lane 11) (**Figure 4.4B**), indicating an enhanced IgE-binding capacity for the tropomyosin upon suffering heat processing, thus confirming what is reported in the literature (Abramovitch et al., 2013; Kamath et al., 2013).

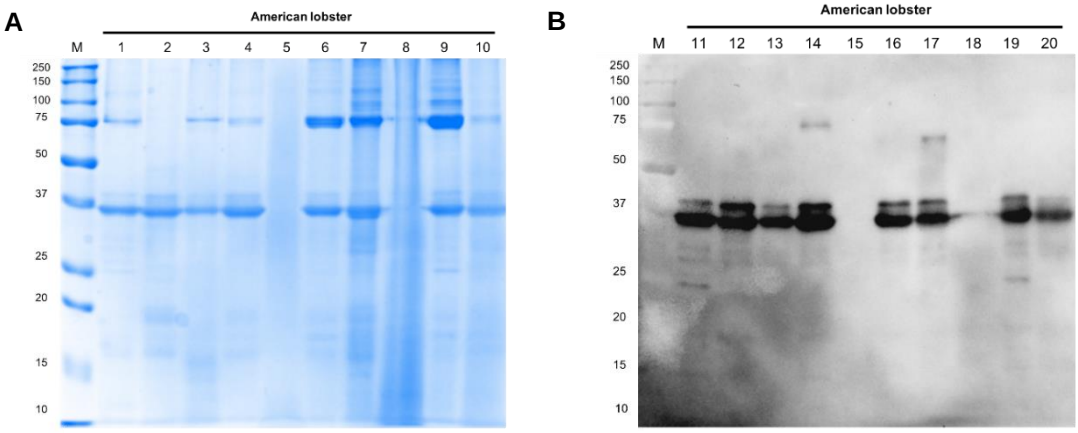

Figure 4.4 - SDS-PAGE gel (A) and immunoblotting membrane (B) run in non-denaturing conditions of *American lobster* after different heat processing methods (total protein 25 µg) submitted to protein extraction with Tris-HCl 100 mM pH 8.0 buffer at 60 ºC/2 h. The membrane was incubated with poo-sera from crustacean-allergic patients as primary antibody. Legend: lane M – Precision Plus Protein Dual Color Standard 10-250 kDa (Bio-Rad Laboratories Inc., Hercules, CA, USA); lanes 1 and 11 - raw American lobster; lanes 2 and 12 - boiled American lobster 15 min; lanes 3 and 13 - boiling water American lobster 15 min; lanes 4 and 14 - fried American lobster 5 min; lanes 5 and 15 - frying oil American lobster 5 min; lanes 6 and 16 - grilled American lobster 20 min; lanes 7 and 17 - American lobster raw and marinated in lemon juice (1.5 h); lanes 8 and 18 - lemon juice from raw and marinated American lobster (1.5 h); lanes 9 and 19 - American lobster marinated in lemon juice (1.5 h) and grilled 5 min; lanes 10 and 20 - American lobster marinated in lemon juice (1.5 h) and oven-cooked 15 min.

In what concerns the use of lemon juice, the tropomyosin band (36 kDa) did not suffer significant changes when comparing both band intensities for SDS-PAGE and immunoblotting, either for raw samples (lanes 1 and 11 vs lanes 7 and 17), or posteriorly heat-processed by grilling (lanes 6 and 16 vs lanes 9 and 19) (**Figure 4.4**). The only differences seem to be related with the 75 kDa protein that, in SDS-PAGE, has enhanced intensity, but it does not show significant IgE-binding capacity in most samples appearing only in the fried sample (lane 14). Despite that, as mentioned before, it cannot be rule out that this protein might be an allergen, because it would be possible that this weak 75 kDa band might be a hemocyanin. However, the absence of IgE-binding on the other samples

could be caused by the fact that most of the IgE in the pool sera might not be reactive towards this allergen. In the lemon juice it was not possible to verify the presence of any proteins that was recovered in marinating the raw American lobster. Additionally, by consulting the Allergen data base of WHO/IUIS, it was found that allergens for American lobster also include troponin C and myosin light chain, possibly corresponding to the bands visible at around 20-25 kDa, which are clearer in raw lobster (lane 11) (**Figure 4.4**).

4.4.3 Argentine shrimp (Pleoticus muelleri) and Blue swimming crab (Portunus pelagicus)

Other common type of shrimp that is widely consumed is the Argentine shrimp. For this crustacean, it was not possible to make any conclusions regarding the effect of heat processing, since only a few low-intensity bands were visible, at around 20 kDa and 37 kDa in the solid samples, including raw (lane 1) and fried (lane 4) shrimp after SDS-PAGE analysis (**Figure 4.5A**).

In the immunoblotting, there are no reactive IgE-bands, which is consistent to the lack of bands also verified in the SDS-PAGE (**Figure 4.5B**). The absence of bands for this crustacean is most likely due to low protein concentration, and not the SDS-PAGE runs, since the remaining crab samples have visible bands. Other attempts of extracting higher quantities of protein from the samples of Argentine shrimp fail to provide better results both by SDS-PAGE or immunoblotting. Still, it is important to highlight that the BCA quantification of Argentine shrimp extracts presented appropriate amounts of protein, being contradictory to the results of the gels. This indicated that the referred samples have enough protein for running the samples with a 5 mg/mL concentration, which should be more than enough to obtain visible bands.

The blue swimming crab legs were also acquired as an easily available crustacean source. It is well-known that tropomyosin is the most relevant crustacean allergen, being responsible for more than half of IgE-binding in allergic patients (Ruethers et al., 2018; Zhao, Timira, et al., 2022). With that fact in mind, and following the tendency of the previously analysed crustaceans, it was possible to observe that tropomyosin is also present in this crustacean as the main allergen (Por p 1) (**Figure 4.5**).

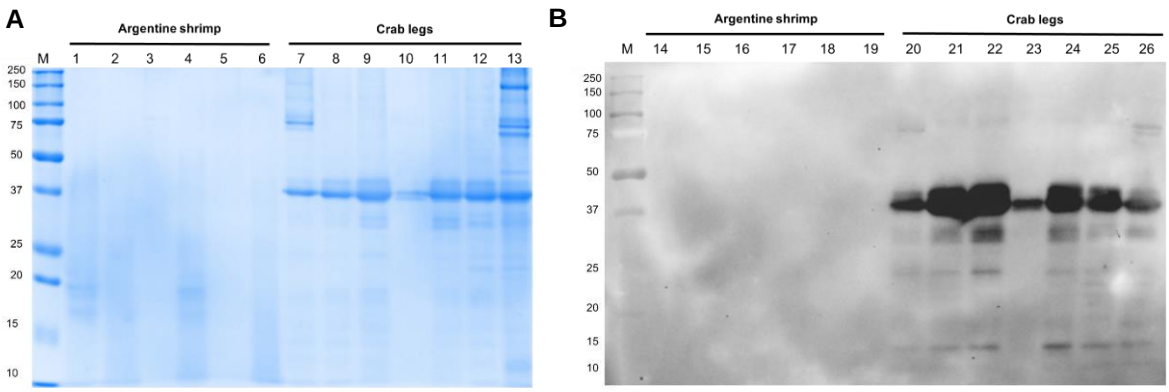

Figure 4.5 - SDS-PAGE gel (A) and immunoblotting membrane (B) run in non-denaturing conditions of Argentine shrimp and blue swimming crab legs after different heat processing methods (total protein 25 µg) submitted to protein extraction with Tris-HCl 100 mM pH 8.0 buffer at 60 ºC/2 h. The membrane was incubated with pool-sera from crustacean-allergic patients as primary antibody. Legend: lane M – Precision Plus Protein Dual Color Standard 10-250 kDa (Bio-Rad Laboratories Inc., Hercules, CA, USA); lanes 1, 14 - raw Argentine shrimp; lanes 2, 15 - boiled Argentine shrimp 3 min; lanes 3, 16 - boiling water Argentine shrimp 3 min; lanes 4, 17 - fried Argentine shrimp 5 min; lanes 5,18 - frying oil Argentine shrimp 5 min; lanes 6, 19 - grilled Argentine shrimp 3 min; lanes 7, 20 - raw crab legs; lanes 8, 21 - steam cooked crab legs 5 min; lanes 9, 22 - boiled crab legs 5 min; lanes 10, 23 - boiling water crab legs 5 min; lanes 11,24 - grilled crab legs 5 min; lanes 12, 25 – oven-cooked crab legs 15 min; lanes 13 and 26 – oven-cooking water crab legs 15 min.

SDS-PAGE analysis showed an intense tropomyosin band, with other band just above the 37 kDa mark in all solid samples. This protein is probably an arginine kinase, which is another common crustacean panallergen (~42 kDa), as observed in lanes 7, 8, 9, 11 and 12 (**Figure 4.5A**), with no significant differences between band intensity, apart from a slightly weaker band for raw crab (lane 7). Other significant bands appear at 75 kDa, specifically in raw blue swimming crab (lane 7) and oven cooking water (lane 13), indicating that it might be a hydrophilic protein that is fragmented during heat processing, which would also justify the appearance of lower molecular weight bands (<25 kDa) on the lanes containing heat-processed samples (e.g., lanes 11 and 12) (**Figure 4.5A**). Considering the immunoblotting analysis, the heat-processing enhances the IgE-binding of 37 kDa-band (lanes 21 and 22) when compared to its raw counterpart (lane 20, **Figure 4.5B**). Nevertheless, when comparing these results to the ones obtained by SDS-PAGE, it is possible to conclude that the apparent strong band can, in fact, be comprised by two bands (probably tropomyosin and arginine kinase), that appear together due to bad resolution or excess of protein, which could be solved by the increasing the percentage of acrylamide in the SDS-PAGE gels or by diluting samples to a lower final concentration, respectively. Even so, tropomyosin heat-resistance is probably the main cause of this observation, since

arginine kinase is not as resistant to heat processing, and therefore it would make sense for arginine kinase to be somewhat fragmented, being a possible justification for the reactivity visible on various lower molecular weight bands (around 30 kDa, 25 kDa and 12 kDa). Therefore, these bands could correspond to smaller fragments of arginine kinase, as well as to fragments from other allergens, such as the 75 kDa IgE-reactive protein, suspected to be hemocyanin. However, these lower molecular weight bands might also be other allergens, such as myosin light chain (~20 kDa), sarcoplasmic calcium binding protein (20-25 kDa), triosephosphate isomerase (~28 kDa), troponin C (16-20 kDa), fatty acid binding protein (15-20 kDa) or ovary development-related protein (28 kDa), all of them confirmed as crustacean allergens by WHO/IUIS. Nonetheless, the one identified allergen in blue swimming crab is the tropomyosin (Por p 1), meaning that it is difficult to confirm their identity.

The analysis of the boiling water sample also allowed to identify a relatively intense band on immunoblotting at 37 kDa (lane 23, **Figure 4.5B**), confirming the possibility of tropomyosin being transferred to the cooking water, contributing to the allergic reactions to crustacean vapours during cooking. Other interesting inference that could be retrieved from analysing the treatments used to process this crustacean regards the difference between dry and moist heat cooking methods. In moist-heat processes, such as steam cooking (lane 21) and boiling (lane 22) that use liquid and/or steam to heat the food have slightly more intense bands, consequence of higher IgE-binding capacity of allergens than the dry-heat cooked samples (lanes 24 and 25) that have been grilled or oven-cooked (**Figure 4.5B**). In moist heat methods, the heat transference happens by conduction, meaning that the energy occurs by direct contact. In dry-heat, it involves the circulation of hot air by convection phenomenon. These distinct ways of transferring heat to the food may have an impact on how much energy reaches the proteins, inducing variable consequences to the protein structure, thus potentially affecting the epitopes and their IgE-binding capacity. This possibility has been presented relatively to milk allergy (Zenker et al., 2019), but there were no definitive conclusions on the matter.

4.4.4 Goose barnacles (Pollicipes pollicipes), spiny spider crab (Maja squinado) and brown crab (Cancer pagurus)

The effect of different processing treatments was also tested on goose barnacles, spiny spider crab and brown crab, which are less commonly consumed, but they are widespread known crustaceans (**Figure 4.6**). The processing of barnacles was investigated with two different times of boiling (3 and 10 minutes). With the SDS-PAGE analysis, no significant differences between the two times of boiling could be observed (lanes 2 and 4), but the

boiling water of barnacles for 10 minutes (lane 5) had more protein than its 3-minute counterpart (lane 3), with bands showing at 37-40 kDa, 20-25 kDa, and 70 kDa, which are the same bands exhibited in the processed samples (**Figure 4.6**). This fact is probably due to the longer time of boiling being a more intense process and giving more time to the protein to migrate to the water. Boiled barnacles also presented some proteins that seemingly were not transported to the water, such as the band at 16 kDa, some high molecular weight bands (between 100-150 kDa), and a more intense band at around 30 kDa. This could be a consequence of the higher hydrophobicity of these proteins, or perhaps the location in the cells and/or tissues, making them less prone to migrate to the boiling water. When comparing the raw (lane 1) to the boiled samples (lanes 2 and 4), it is also clear that the bands in the raw barnacles are fainter, though the same proteins seem to appear in both sample types. When doing the evaluation of the IgE-binding capacity, it is evidenced that the raw barnacles (lane 12) have the most intense bands, namely proteins at the 20-25 kDa range, at 37-40 kDa, and 75 kDa. The suspected allergens are the same as of previous crustaceans, such as myosin light chain (20-25 kDa), tropomyosin and arginine kinase (37-40 kDa) or hemocyanin (75 kDa) are possible allergens (**Figure 4.6A**). As for the processed samples (lanes 13 and 15), the bands seem to be less IgE-reactive, but appearing at the same molecular weights (**Figure 4.6B**). If the 37 kDa protein is tropomyosin, and not arginine kinase, it conflicts with most of the conclusions advanced for other crustaceans, and to what is reported in the literature regarding the fact that tropomyosin resists to heat processing, and its IgE-binding in most cases is enhanced. A characterization of this allergen would be necessary, namely by mass spectrometry, to confirm its protein sequence and to draw further conclusions. It should also be mentioned that, in accordance with SDS-PAGE, the absence of proteins in boiling water after boiling 3 minutes matches up with no IgE-reactivity in immunoblotting (lane 14), whereas the boiling water of 10 min (lane 16) seems to have received some reactive allergenic proteins (**Figure 4.6B**).

In what concerns to spiny spider crab, SDS-PAGE analysis shows the presence of high molecular weight proteins, with more than 75 kDa, in both raw (lane 6) and boiled (lane 7) samples (**Figure 4.6A**). Processed spiny spider crab also has a faint band at around 40 kDa, which can be consequence of some degree of breaking down the high molecular weight proteins visible on the raw sample. The boiling water has no intense bands, with a light band at around 75 kDa. When comparing these results to immunoblotting, there is almost no reactivity on both solid samples (raw – lane 17 and boiled – lane 18) (**Figure 4.6B**), which can indicate that the bands visible on SDS-PAGE may be non-allergenic, or simply not reactive to any of the patients whose serum was in the pool. However, there is a strong IgE-reactivity towards the protein at 37 kDa in the boiling water. This result does not correlate with the one observed in SDS-PAGE, since this treatment shows no bands. This

could mean that the samples were too diluted for SDS-PAGE, but there was a strong IgE-binding to the low amount of protein present in that range, which can be traces of tropomyosin, arginine kinase, or fragments from the higher molecular weight proteins.

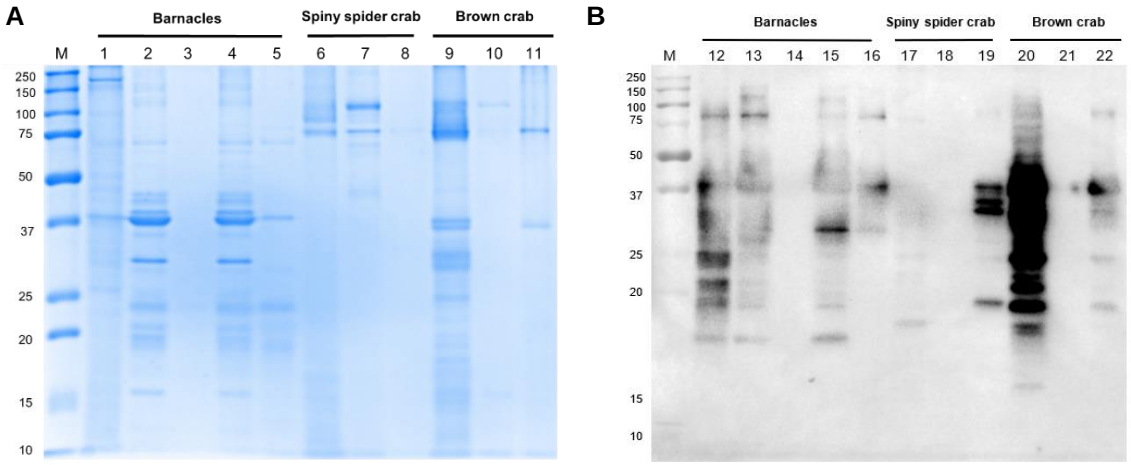

Figure 4.6 - SDS-PAGE gel (A) and immunoblotting membrane (B) run in non-denaturing conditions of barnacles, spiny spider crab and brown crab after different heat processing methods (total protein 25 µg) submitted to protein extraction with Tris-HCl 100 mM pH 8.0 buffer at 60 ºC/2 h. The membrane was incubated with pool sera from crustacean-allergic patients as primary antibody. Legend: lane M – Precision Plus Protein Dual Color Standard 10-250 kDa (Bio-Rad Laboratories Inc., Hercules, CA, USA); lanes 1, 12 - raw barnacles; lanes 2, 13 - boiled barnacles 3 min; lanes 3, 14 - boiling water barnacles 3 min; lanes 4, 15 - boiled barnacles 10 min; lanes 5, 16 - boiling water barnacles 10 min; lanes 6, 17 - raw spiny spider crab; lanes 7, 18 - boiled spiny spider crab 10 min; lanes 8, 19 - boiling water spider crab 10 min; lanes 9, 20 - raw brown crab; lanes 10, 21 - boiled brown crab 10 min; lanes 11, 22 - boiling water brown crab 10 min.

For the brown crab, some interesting results were verified. The SDS-PAGE analysis of the raw brown crab (lane 9) shows a wide-range of proteins, from more than 100 kDa, to less than 15 kDa, with somewhat strong bands at around 30-37 kDa. However, the boiled sample (lane 10) shows a weak protein profile, with just a few, low intensity bands of high molecular weight. In fact, even the boiling water (lane 11) presents at least two strong bands, at 75 and 37 kDa (**Figure 4.6A**). These conclusions can indicate that the boiling process is efficient in breaking down proteins in this crustacean, with most proteins getting fragmented or transferred to the boiling water. Studying the immunoblotting membrane exacerbates and confirms what was verified in the gel (**Figure 4.6B**). Raw brown crab (lane 20) has visible IgE-binding capacity from 100 to less than 20 kDa, meaning that there are a lot of proteins on this sample that have allergenic potential, as evidenced by their strong IgE-reactivity. Surprisingly, albeit confirming SDS-PAGE results, the boiled crab (lane 21)

shows no significant IgE-binding, probably due to the lack of proteins. Similarly, the boiling water shows some proteins with IgE-binding capacity to various molecular weights (lane 22), in accordance with the proteins present in the SDS-PAGE (lane 11). These results contrast to what has been seen in previous crustacean species. In this case, the raw sample shows the highest IgE-binding, and boiling seems to be almost completely efficient in destroying protein structure and epitopes, with a passage of the remaining allergens to the boiling water, leaving the solid sample with next to no allergenic potential for this pool of patients. Therefore, it appears that boiling is an effective way of protecting allergic patients in their consumption of brown crab. This comes to show that perhaps, although most crustacean species have an enhanced IgE-binding after heat processing, due to the resistance of tropomyosin, there are some exceptions in both the literature (Jarupalee et al., 2018) and in the present work. This indicates that the IgE-binding to allergens is a process which is highly dependent on the crustacean species, processing methods and patient allergic profile.

4.4.5 Scampi (Nephrops norvegicus)

Lastly, the effect of food processing with heat treatment and marination with lemon juice on the IgE-reactivity of scampi proteins was studied. In a similar way to what was been seen in both *vannamei* and black tiger shrimps (**Figure 4.3**), the raw sample (lane 1) did not present the protein band at the vicissitudes of the 37 kDa marker, but which was visible for most processed samples (**Figure 4.7**). Although presenting bands at other molecular weights, the possibility of the chosen extraction method not being adequately optimized for raw samples cannot be ruled out. This may result from a lower access to the protein content, and to more resistance from the matrix. The SDS-PAGE shows high molecular weight (75 kDa and around 125 kDa) and low molecular weight (16 kDa) bands in the raw scampi. Processed samples are characterized by having the typical tropomyosin band, with just less than 37 kDa, and another band with a slightly higher molecular weight, believed to be an arginine kinase, which is visible in boiled, fried and oven cooked samples (lanes 2, 4 and 7, respectively) (**Figure 4.7A**).

These solid processed samples have similar protein profiles in SDS-PAGE, with other common protein, at around 20 kDa, that may correspond to other allergens, as referenced before, such as myosin light chain or sarcoplasmic fatty acid binding protein. This similarity is reflected on immunoblotting, where these cooked samples (lanes 13, 15 and 18) have analogous IgE-binding capacity (**Figure 4.6B**). The amalgamation of the tropomyosin and arginine kinase bands in these lanes generates a high intensity, apparently single, band at around 37 kDa, indicating a high probability of sensitisation to scampi in the patients. Other

bands are also visible, including the band between 75 and 100 kDa, likely to be either hemocyanin (~75 kDa) or filamin C (~95 kDa).

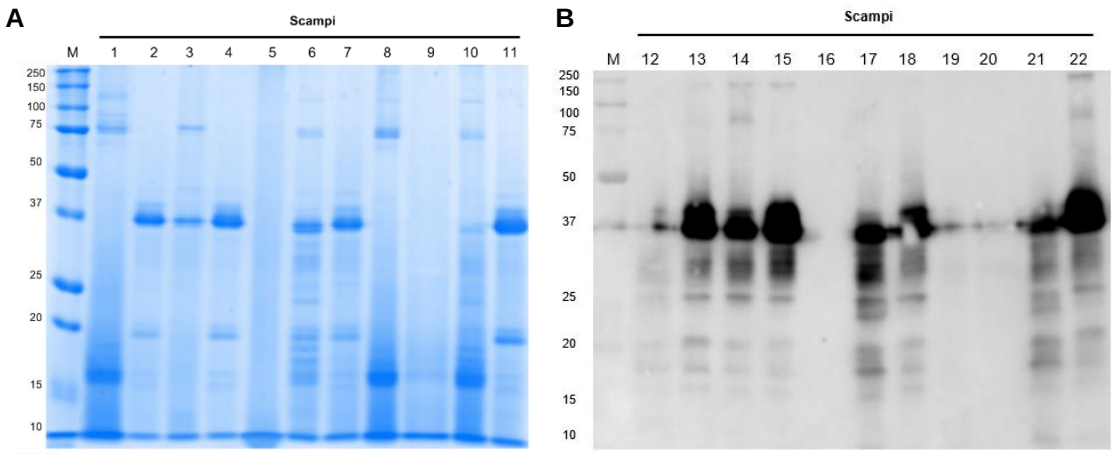

Figure 4.7 - SDS-PAGE gel (A) and immunoblotting membrane (B) run in non-denaturing conditions of scampi after different heat processing methods (total protein 25 µg) submitted to protein extraction with Tris-HCl 100 mM pH 8.0 buffer at 60 ºC/2 h. The membrane was incubated with pool sera from crustacean-allergic patients as primary antibody. Legend: lane M – Precision Plus Protein Dual Color Standard 10-250 kDa (Bio-Rad Laboratories Inc., Hercules, CA, USA); lanes 1 and 12 – raw scampi; lanes 2 and 13 – boiled scampi 5 min; lanes 3 and 14 – boiling water scampi 5 min; lanes 4 and 15 – fried scampi 5 min; lanes 5 and 16 – frying oil scampi 5 min; lanes 6 and 17 – grilled scampi 5 min; lanes 7 and 18 – Oven-cooked scampi 15 min ; lanes 8 and 19 – raw scampi marinated in lemon juice (1.5 h); lanes 9 and 20 – lemon juice (1.5 h) from raw scampi; lanes 10 and 21 – scampi marinated in lemon juice (1.5 h) and grilled 5 min; lanes 11 and 22 – scampi marinated in lemon juice (1.5 h) and oven-cooked 15 min .

There is some visible IgE-binding in lower molecular weights, in the 17-37 kDa range, which may be the result of the presence of several allergens, such as the ones referred above. It is also worth to analyse the boiling water (lanes 3 and 14) and frying oil (lanes 5 and 16) for their protein profile (SDS-PAGE) and IgE-binding capacity (immunoblotting). In fact, the boiling water has a band in the tropomyosin/arginine kinase range, with immunoblotting showing the two distinct bands, since the tropomyosin (lower molecular weight) presence seems to be less strong. The 75 kDa band seen in the raw sample appears to be present in the boiling water, probably due to a high hydrophilicity. Therefore, these proteins (and more of the lower molecular weight allergens) seem to have been transported to the boiling water during processing, causing high IgE-binding capacity, bringing up the possibility of patient sensitisation *via* inhaling the vapour of the crustacean boiling water. As for the frying oil, no protein or IgE-binding was visible, which can be related to the reasons mentioned above for other frying oils. Lastly, grilled scampi (lanes 6 and 17) has a clearly different profile than the other processed samples, with more low molecular

weight bands, and the suspected hydrophilic high molecular protein visible in both raw and boiling water samples (**Figure 4.7B**). This phenomenon could be a consequence of the high temperature dry-heat processing applied to this sample, causing the unequal fragmentation of higher molecular weight proteins, including the heat-labile protein, arginine kinase, which does not seem to be as present in this sample, indicating that this method was more intense in applying heat to the crustacean.

Scampi, just like American lobster, was chosen to test the effect of lemon juice on raw and processed samples, after marinating for 1.5 hours. Raw scampi marinated in lemon juice (lane 8) showed a similar protein profile to the non-marinated sample, as well as no significant IgE-binding capacity (lane 19), indicating that this method was not very effective in changing protein structure on raw samples. Lemon juice (lanes 9 and 20) also did not seem to contain high amounts of protein, with only a low intensity band at 15 kDa, and very limited IgE-reactivity. Marinated and grilled scampi has a very different protein profile in SDS-PAGE when compared to its non-marinated counterpart. The proteins with approximately 37 kDa in the grilled sample (lane 6) may have been fragmented by the action of the lemon juice, since the marinated and grilled sample (lane 10) has a much less visible 37 kDa band, and a more intense at 15 kDa, probably due to the accumulation of fragments of this molecular weight. By examining the immunoblotting, the IgE-binding capacity of the 37 kDa bands decreased slightly after marination (lane 17 vs lane 21) (**Figure 4.7B**), but the protein at around 30 kDa seems to have lost most of its reactivity with this change, with high probability of being the allergen triosephosphate isomerase. Finally, oven-cooking the marinated scampi (lanes 11 and 22) seems to impact the IgE-binding capacity of the allergen. A high quantity of tropomyosin and arginine kinase can be identified, with high IgE-binding and SDS-PAGE band intensity at 37 kDa, with some IgE-reactivity at the 15-37 kDa range, probably due to low amounts of the other allergens. When comparing the non-marinated scampi with marinated ones, (**Figure 4.7**, lanes 7 and 18 vs lanes 11 and 22) it was observed, for oven cooked samples, that there is a higher IgE-binding capacity in the marinated sample, indicating that in this case the lemon juice might have enhanced the reactivity of the proteins to the sera. Although this contradicts what was verified in the grilled sample, it was also seen that the non-marinated grilled and oven cooked samples had very distinct protein profiles, and therefore it could happen that the lemon juice interacts differently with distinct allergens, and therefore, since the allergen amount present in each sample is different, distinct results may emerge. Therefore, the effectiveness of this method in diminishing allergenicity is limited, and highly affected by patient allergy characteristics.

All in all, the work herein presented about the effects of food processing on the mitigation of crustacean allergenicity confirms the previously reported enhanced IgE-binding capacity of tropomyosin when submitted to heat, contributing to the study of different protein profiles

and IgE-binding abilities of different crustaceans. There are a lot of gaps in the study of crustacean allergenicity, specifically the effect of different processing methods, generating contradictory results, providing incomplete information and sub-investigating some topics. Therefore, this work helped fill some of those gaps. The sampling allowed for more authentic processing, using common methods that better replicate what happens in the real-life of allergic patients – for instance, some authors study purified allergens, whereas others only study the effect of processing on extreme conditions, inaccessible to the public. Additionally, the immunoblotting analysis is performed in non-denaturing conditions, reducing the additional processing the samples endure. Furthermore, there are not many reports that use several processing methods and crustacean species, inducing strong limitations on the comparison they can perform between different species and the effects of distinct treatments. Also, the frying oil and boiling water are not a well-explored area regarding allergenicity, although there are reports indicating their potential as allergen sources (Lopata & Jeebhay, 2013; Sj & Sankar, 2021).

4.5 Impact of simulated *in vitro* gastrointestinal digestion of crustaceans

The simulated i*n vitro* gastrointestinal digestion was performed on American lobster in three different processed states, namely raw, raw and marinated in lemon juice (1.5 h) and marinated in lemon juice (1.5 h) followed by oven-cooking for 15 min. The study of digestion was intended for other crustaceans, meaning that the American lobster was selected as the first one only because of availability, since it was collected in larger quantity during sampling. By selecting these samples, the objective was to evaluate the effects of gastrointestinal digestion combined with different processing treatments on this crustacean species. In this case, it was intended to investigate if the use of lemon juice for 1.5 h and/or the heat treatment (oven cooking for 15 min) had any synergistic effect during the gastrointestinal digestion, which had been previously reported (Yu et al., 2011).

4.5.1 Gastrointestinal digestion of raw American lobster

For the raw American lobster (**Figure 4.8**), a positive control was used in both gels and membranes (lane L), corresponding to the raw sample extracted with Tris-HCl 100 mM pH 8.0. Lane Pep served as the control for pepsin aiming at the comparison of this band with the remaining lanes of gastric digestion. For the intestinal phase, lanes T21R2 and C1 also worked as controls, with T21R2 being used to evaluate a non-enzymatic digestion, and C1

to serve as control of the enzymes, since it does not include the food, only digesting enzymes (pepsin from gastric and pancreatin plus the bile from intestinal phases).

In what concerns the salivary and gastric phases of raw lobster digestion, the SDS-PAGE analysis revealed the presence of both low and high molecular weight proteins, already studied earlier in this work (**Figure 4.8A**). In lane T0 (0 min salivary phase), there are a lot of proteins present, namely the tropomyosin Hom a 1 (~37 kDa), the myosin light chain 2 (Hom a 2) and troponin C (Hom a 3) (20-25 kDa), as well as other proteins at 1-20 kDa, 42-45 kDa (probably arginine kinase) and above 75 kDa (hemocyanin). The salivary phase (lanes T0 to T1) does not seem to have changed protein structure in any significant way, with identical band profile in both lanes. It is worth noting that the salivary phase lanes have a slightly different set of results when compared to the control lane. However, that difference is only noticeable on the band intensity, with the control lane protein bands being the same, only less intense, with this possibly being a consequence of the extraction method used for the sample in lane L, since the digested samples were not extracted.

At the beginning of the gastric phase (lane T2, corresponding to 0 min gastric digestion), the protein degradation becomes clearly visible, with a strong decrease of the intensity of relatively high molecular weight bands, with special incidence on the proteins between 20 and 50 kDa. New bands are formed, such as the ones at the 15 kDa and 30 kDa, which are most likely protein subunits or peptides resulting from protein fragmentation. There is a strong band at around 40 kDa, but that is probably pepsin, since it has the same molecular weight of this digestion enzyme (Lane pep). On the following lanes (from T3 to T11, corresponding to 0, 5, 10, 15, 20, 30, 45, 60, 90, 120 min of gastric digestion) there are also some differences when comparing to the T2 well, because of the action of pepsin. In the T2 well, the action of pepsin on the proteins is negligible since the activity of the enzyme was instantly interrupted at t=0 min of the gastric phase. As expected, the T2 lane presents proteins of high molecular weight, visible for example at 30 kDa, but the following lanes are clearly affected by the pepsin action, which promoted protein fragmentation. There is also some thickening of the bands at 10 kDa, since all peptides of <10 kDa appear here, showing the action of pepsin at breaking down proteins in smaller peptides. Therefore, with SDS-PAGE analysis it was possible to conclude that pepsin has a considerable role in breaking down crustacean proteins in digestion. The band corresponding to tropomyosin (~37 kDa) seems to be decreasing with increasing time of gastric digestion, being greatly reduced at 120 min of pepsin action. Additionally, it can be inferred that some proteins (such as the 75 kDa one) are more resistant to pepsin action, since in general, most proteins seem to resist to the final stages of the gastric phase. In summary, it seems that there are no considerable alterations on the protein profile during the gastric phase after the first 10 minutes (lane T4), with the changes on band intensity being probably due to slight variations on the

quantification of the protein content in each sample, generating slightly dissimilar concentrations and, consequently, band strength (**Figure 4.8A**).

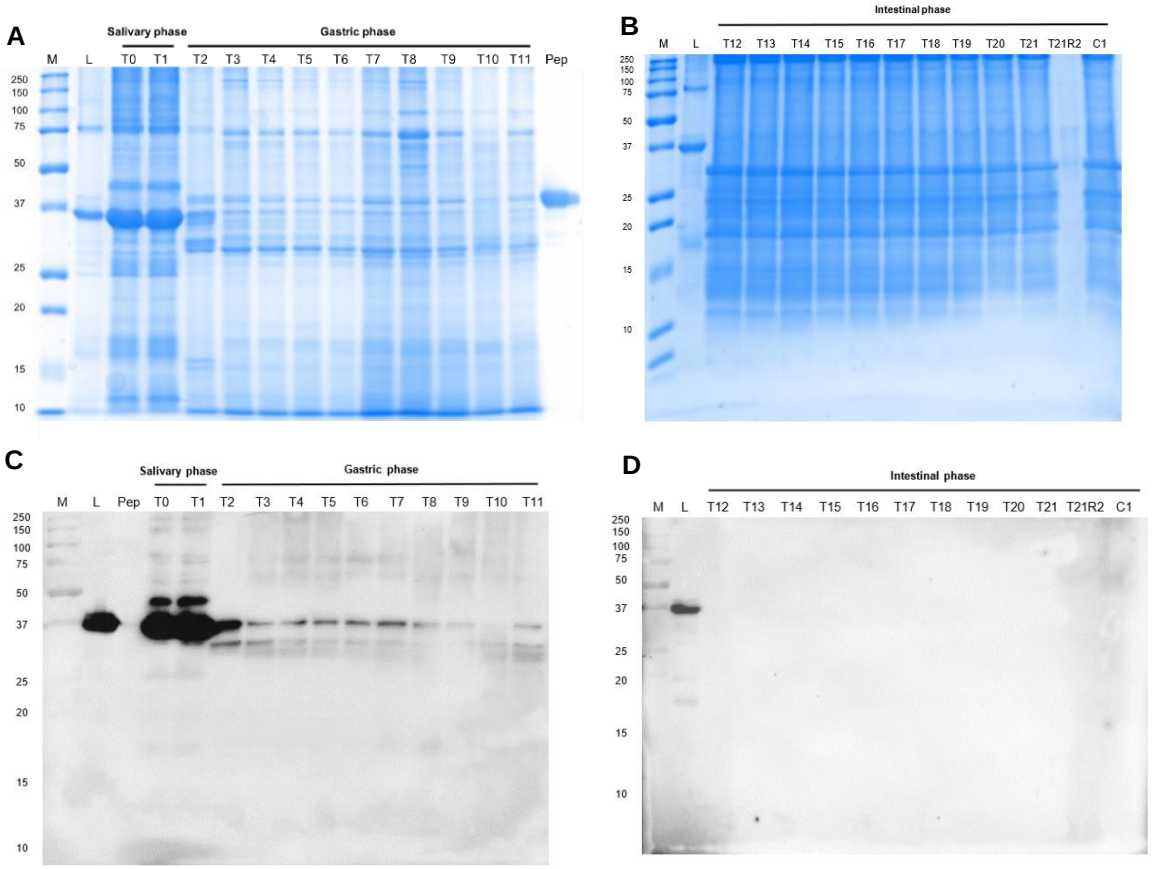

Figure 4.8 - Non-denaturing SDS-PAGE gels (A, B) and immunoblotting membranes (C, D) of the gastrointestinal digestion of raw American lobster. For salivary and gastric phases (A, C), discontinuous 5-12% acrylamide gels were used containing 25 µg of total protein, while for the intestinal phase (B, D), discontinuous 5-15% acrylamide gels were used containing undiluted protein, except for lanes L and T21R2, which contain 25 µg of total protein. The membranes were incubated with pool sera from crustacean-allergic patients as primary antibody. Legend: lane M – Precision Plus Protein Dual Colour Standard 10-250 kDa (Bio-Rad Laboratories Inc., Hercules, CA, USA); lane L – raw American lobster; lane Pep – Pepsin; lanes T0 and T1 – samples that were only submitted to the salivary phase with increasing times (0 and 2 min, respectively); lanes T2 to T11 – samples submitted to salivary (2 min) + gastric phase with increasing times (0, 5, 10, 15, 20, 30, 45, 60, 90, 120, respectively); lanes T12 to T21 – samples submitted to salivary (2 min) + gastric phase (120 min) + intestinal phase with increasing times (0, 5, 10, 15, 20, 30, 45, 60, 90, 120, respectively); lanes T21R2 – control sample with no enzyme; lanes C1 – control with no food sample..

The immunoblotting analysis (**Figure 4.8C**) shows some similarities and differences compared to the SDS-PAGE (**Figure 4.8A**). The incubation of the membranes with the pool-

sera of crustacean-allergic patients allowed to verify that there is a high IgE-binding capacity of at least two proteins at around 37 kDa and 42-45 kDa, probably tropomyosin (Hom a 1) and arginine kinase during the salivary phase, which does not seem to alter the protein structures (lanes T0 and T1). However, at the beginning of the gastric phase (lane T2), it is possible to observe a clear reduction in the intensity of one of the strongest bands (37 kDa), with the other one disappearing (42-45 kDa), probably due to a fast destruction of its structure. In the following lanes, with the increasing time-points of the gastric phase, the intensity of the 37 kDa-band is decreased, until it is greatly reduced at the end of gastric digestion (lane T11, 120 min). Additionally, there are some bands that are IgE-reactive at 30 kDa, which start to appear in lane T3 and remain present until lane T11 (**Figure 4.8C**). The results from SDS-PAGE and the respective immunoblot seem to be in good agreement, considering that it seemed that the highest effect of pepsin is observed from T2 to T3, although the bands continue to be less IgE-reactive with the increasing time-points of gastric digestion (**Figure 4.8A,C**, lanes T3-T11).

The SDS-PAGE analysis of the intestinal phase (**Figure 4.8B**) does not allow to retrieve many conclusions, since the pancreatin enzymes occupy most of the gel, as it is visible by comparing lane C1 (control containing the digestive enzymes and no food) with the remaining lanes (T12-T21). There are no significant differences among lanes, meaning that it is difficult to predict if there are still bands of protein from the American lobster during the intestinal phase. The control with no pancreatin (lane T21R2) was expected to present some bands, which was not the case. Therefore, between the raw lobster and the raw lobster digested with no enzymes, there is a clear decrease in the number of proteins that cannot be explained. When evaluating the results in the immunoblotting membrane (**Figure 4.8D**), no IgE-reactive bands were observed during the intestinal phase (T12-T21). Since there were some IgE-reactive bands at end of gastric phase (120 min), it was expected that at least lane T12 (0 min of intestinal digestion) would present some faint IgE-reactive bands. However, this did not occur, meaning that the intestinal phase was not effectively blocked by the pefabloc nor by the PMSF, which are the compounds recommended by the INFOGEST protocol to stop the enzymatic activity of pancreatin (Brodkorb et al., 2019). The lane T21R2 (complete digestion with no enzymes) was expected to present some IgE-reactive bands, which was not the case. In fact, this was in good agreement with the SDS-PAGE analysis, although no conclusion s can be advanced for this fact at this point. The gastrointestinal protocol should be repeated with this sample to confirm these unexpected results.

4.5.2 Gastrointestinal digestion of raw and marinated American lobster

The simulated gastrointestinal digestion of raw American lobster marinated in lemon juice (1.5 h) was performed and further analysed by SDS-PAGE and immunoblotting (**Figure 4.9**). In a similar way to what was seen in the gastrointestinal digestion of the raw lobster, the digested samples have more protein content than the positive control, specifically the T0 and T1 (0- and 2-min salivary phase, respectively) and whose reasons were already discussed in section 4.5.1. The simulated gastrointestinal digestion seems to produce identical effects on proteins to what was observed for the raw lobster (**Figure 4.8**), although the pattern of bands is slightly different in raw and marinated American lobster compared to its raw counterpart. No significant effect on proteins was observed during the salivary phase. The early gastric phase without pepsin effect (lane T2) has some effect on the proteins, diminishing the band intensity, with some accumulation of lower molecular weight peptides, as it can be seen in the 75-100 kDa range, with stronger band at the 10 kDa molecular weight. The band at 37 kDa is consistent with the reported in section 4.5.1, evidencing a decrease intensity of the band with increasing gastric time (**Figure 4.9A**).

In the immunoblotting (**Figure 4.9C**), the IgE-reactivity of tropomyosin (37 kDa) is decreased, as well as a slightly lighter protein, at around 30 kDa, possibly triosephosphate isomerase or fragments from the 75 kDa protein (possibly hemocyanin) that loses IgE-binding capacity after the addition of pepsin (lane T3), meaning that this protein is pepsin-sensitive. It is interesting that the protein at 100 kDa, observed in SDS-PAGE as being present in large quantities, shows no reactivity to the pool-sera of crustacean-allergic patients, meaning that this protein is most likely not an allergen or that the sera does not have IgE repertoire for the referred molecule. Additionally, the 20 kDa band seems to lose its IgE-binding capacity over time, specifically at the beginning of the gastric phase, indicating it is also susceptible to the action of pepsin. Once again, the analysis of the intestinal phase did not allow for many conclusions (**Figure 4.9B**). In an identical way to the raw lobster digestion, pancreatin seems to occupy the entire gel, with only the three previously mentioned bands being visible in the positive control. In SDS-PAGE, the lane T21R2 reveals a weakening of the 37 kDa band, indicating that the tropomyosin might have been susceptible to the non-enzymatic treatment of the digestion. The immunoblotting (**Figure 4.9D**), once again, only had strong bands in the positive control. Although presenting some signal, again the T21R2 (digestion with no enzymes) should present more IgE-reactive bands, since no enzymatic activity is expected in this lane. At this point, it cannot be ruled out the possibility of internal enzymatic activity of this sample, that might have some effect after 4 h of gastric and intestinal digestion of the lobster.

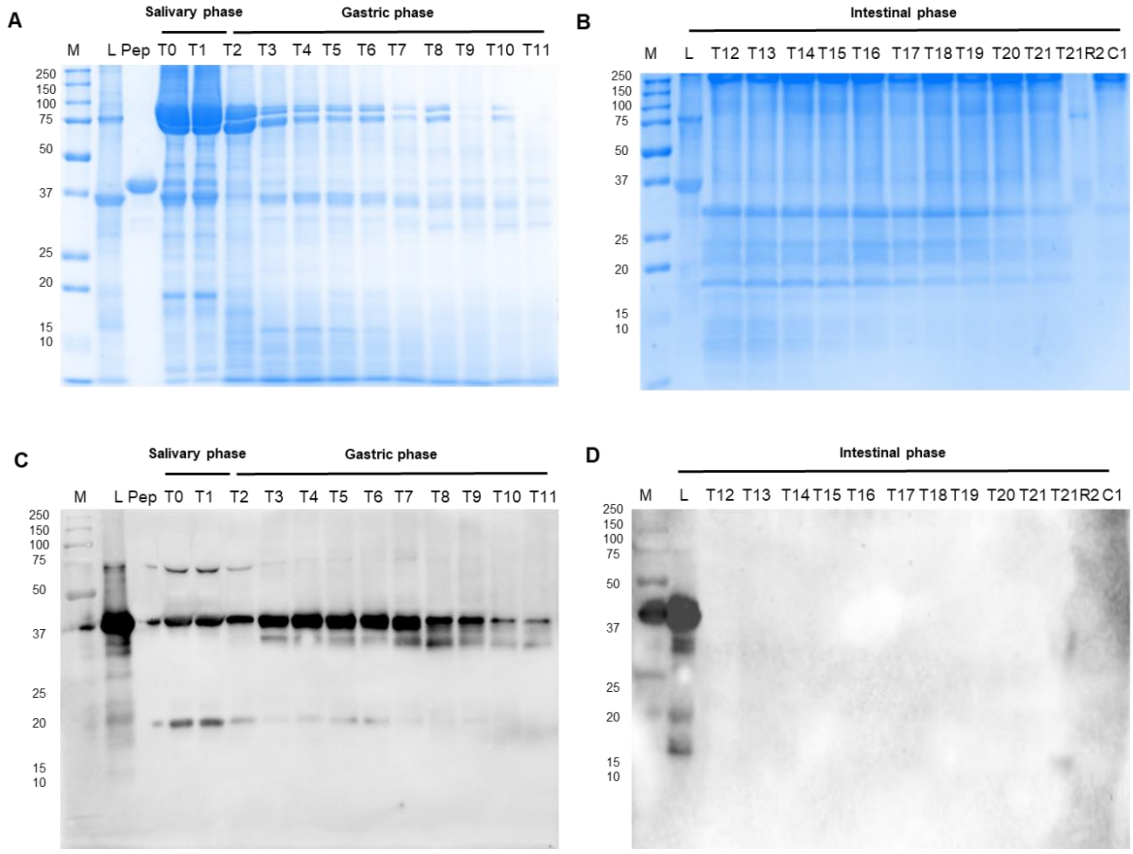

Figure 4.9 - Non-denaturing SDS-PAGE gels (A, B) and immunoblotting membranes (C, D) of the gastrointestinal digestion of raw and marinated in lemon juice (1.5 h) American lobster. For salivary and gastric phases (A, C), discontinuous 5-12% acrylamide gels were used containing 25 µg of total protein, while for the intestinal phase (B, D), discontinuous 5-15% acrylamide gels were used containing undiluted protein, except for lanes L and T21R2, which contain 25 µg of total protein. The membranes were incubated with pool sera from crustacean-allergic patients as primary antibody. Legend: lane M – Precision Plus Protein Dual Color Standard 10-250 kDa (Bio-Rad Laboratories Inc., Hercules, CA, USA); lane L – raw American lobster; lane Pep – Pepsin; lanes T0 and T1 – samples that were only submitted to the salivary phase with increasing times (0 and 2 min, respectively); lanes T2 to T11 – samples submitted to salivary (2 min) + gastric phase with increasing times (0, 5, 10, 15, 20, 30, 45, 60, 90, 120, respectively); lanes T12 to T21 – samples submitted to salivary (2 min) + gastric phase (120 min) + intestinal phase with increasing times (0, 5, 10, 15, 20, 30, 45, 60, 90, 120, respectively); lanes T21R2 – control sample with no enzyme; lanes C1 – control with no food sample

Although there are some very faint bands in T12 (0 min intestinal phase), they are almost negligible. Still, it is important to highlight that some IgE-reactive peptides might survive the gastrointestinal digestion and be present at molecular weight below 10 kDa, which might not be visible on these gels or membranes. This means that further analysis by mass spectrometry must be performed to confirm conclusions. In summary, even though

marinating the raw lobster slightly changed the protein profile, and apparently improved pepsin action, it is not possible to affirm for certain that there is a synergistic effect between the use of lemon juice and the digestion, requiring further analysis.

4.5.3 Gastrointestinal digestion of American lobster marinated and oven-cooked

Lastly, the American lobster marinated in lemon juice (1.5 h) and oven-cooked 15 min was submitted to simulated gastrointestinal digestion (**Figure 4.10**). In this case, the analysis by SDS-PAGE was like the one of the raw and marinated sample (**Figure 4.9**), with visibly strong intensity bands at the 37, 40, 75 and 100 kDa molecular weights. In this case, the positive control (lane L) was less different of the salivary phase digestion samples (T0 and T1, 0-min and 2-min, respectively), and with the profiles of these lanes being like the ones in **Figure 4.9A** (lanes T0 and T1). In contrast with the other digestion processes, the lane T2 did not present many high intensity protein bands. In fact, the following samples (lanes T3, T4) had more intense bands, fact that might be related with the normalisation of the samples to present the same total protein amount (25 µg). In lanes T3 to T11, it was observed that high molecular weight bands (>100 kDa) seem to fade when the time-points of the gastric phase increases, meaning that this protein is susceptible to pepsin, fact that was also mentioned for the raw and marinated American lobster. Bands such as the one at 40 kDa that appear at 15 minutes of gastric digestion (lane T5) are probably fragments from the digested high molecular weight proteins (**Figure 4.10A**). The strong 37 kDa-band in the positive control seems to be practically gone at the end of the gastric treatment. Since this protein is probably tropomyosin, it contradicts what it was observed in the SDS-PAGE analysis of the previous digestions. On the other hand, the 75-100 kDa range proteins, in a similar way to the raw and marinated sample, are present in large quantities, but seem to be quickly broken down by the enzymatic activity. However, when analysing the immunoblotting results, the band of tropomyosin remain IgE-reactive until the end of the gastric phase (**Figure 4.10C**), being very strong in the salivary phase. There is no IgE-binding capacity of the highly concentrated 100 kDa protein although some IgE-reactivity to the 75 kDa-protein is observed. In range of 20 to 35 kDa, there are several reactive bands, which might be myosin light chain 2 (Hom a 2) and/or troponin C (Hom a 3), though some of the bands might also result from the degradation of higher molecular weight proteins. Additionally, more proteins seem to keep their allergenic potential until the end of gastric digestion compared to the gastric phase of the raw and the raw/marinated lobster. The 20 kDa band, for instance, was not present in raw (**Figure 4.10**) or it was significantly weaker

in raw and marinated lobster (**Figure 4.10**) membranes of the simulated gastric phase, which might need heat to activate its IgE-binding epitopes.

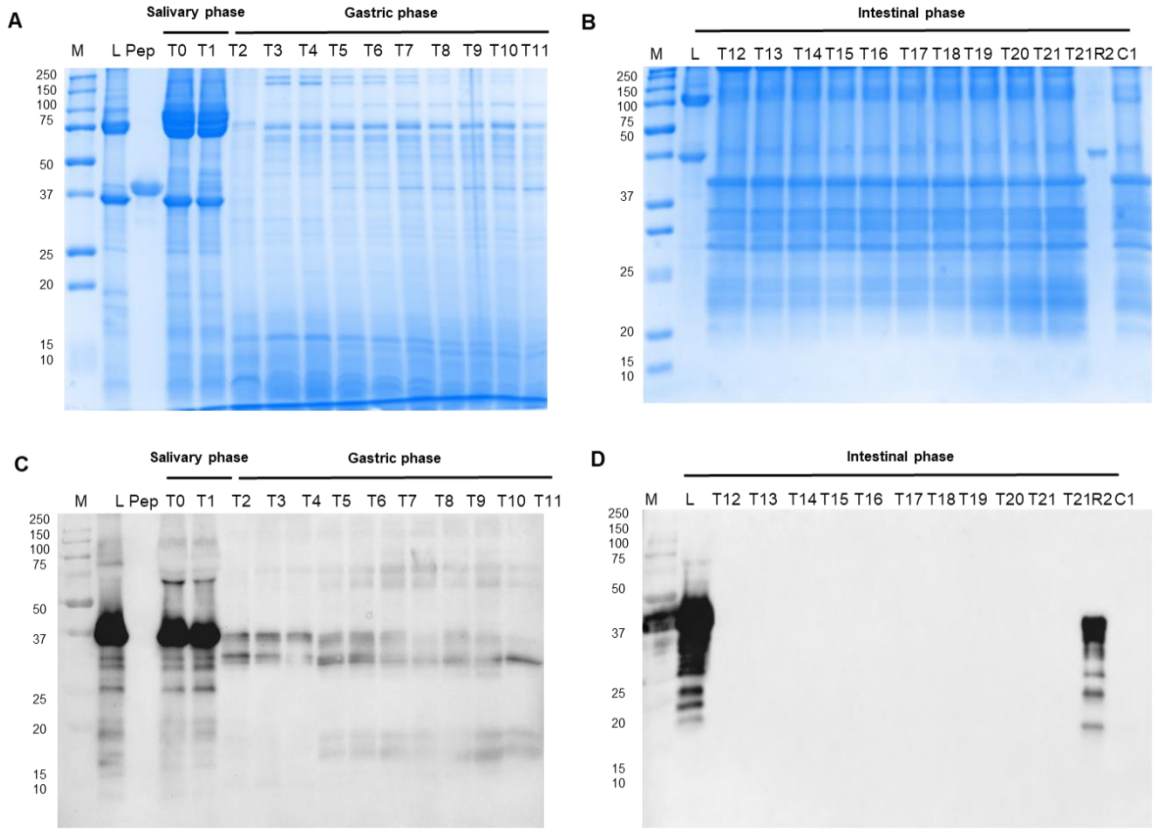

Figure 4.10 - Non-denaturing SDS-PAGE gels (A, B) and immunoblotting membranes (C, D) of the gastrointestinal digestion of American lobster marinated in lemon juice (1.5 h) and oven cooked 15 min. For salivary and gastric phases (A, C), discontinuous 5-12% acrylamide gels were used containing 25 µg of total protein, while for the intestinal phase (B, D), discontinuous 5-15% acrylamide gels were used containing undiluted protein, except for lanes L and T21R2, which contain 25 µg of total protein. The membranes were incubated with pool sera from crustacean-allergic patients as primary antibody. Legend: lane M – Precision Plus Protein Dual Color Standard 10-250 kDa (Bio-Rad Laboratories Inc., Hercules, CA, USA); lane L – raw American lobster; lane Pep – Pepsin; lanes T0 and T1 – samples that were only submitted to the salivary phase with increasing times (0 and 2 min, respectively); lanes T2 to T11 – samples submitted to salivary (2 min) + gastric phase with increasing times (0, 5, 10, 15, 20, 30, 45, 60, 90, 120, respectively); lanes T12 to T21 – samples submitted to salivary (2 min) + gastric phase (120 min) + intestinal phase with increasing times (0, 5, 10, 15, 20, 30, 45, 60, 90, 120, respectively); lanes T21R2 – control sample with no enzyme; lanes C1 – control with no food sample.

These facts, along with the previously discussed conclusion that heat processing can enhance the IgE-binding of allergens, may indicate that digestion is not enough to counter

that effect. Without further studies, perhaps repeating the digestion simulations with other heat-processed samples, there is no way of affirming with some degree of certainty that digestion has an antagonistic or synergistic effect on potentiating the IgE-binding ability of allergens in heat-processed crustaceans.

Contrarily to the two previous digestions, the intestinal phase of marinated and oven-cooked lobster evidenced a significant difference (**Figure 4.10D**). Although the SDS-PAGE results did not introduce new information (**Figure 4.10B**), the lane T21R2 had strong IgE-reactivity, being the most intense around 37 kDa, and with various lower molecular weight bands until 20 kDa (**Figure 4.10D**). Regarding this control with no enzymes, it finally produced the expected result, as most allergens were still reactive at the end of the digestion. Nevertheless, it was still expected to have some degree of IgE-reactivity on the remaining samples (lanes T12 to T21), which was not the case. It was found abnormal that the pancreatin effect was instantly so strong that it destroyed the proteins in such a manner that no IgE-reactivity was seen. It was expected that pancreatin activity would proceed in a time-dependent sequential process, similarly to what was verified for the pepsin in the gastric phase. After studying the three distinct lobster samples and having similar results in this regard, it was suspected that pancreatin was probably doing some unforeseen effects. Later, it was found out that other research teams using the INFOGEST protocol for studying allergens were having problems alike when performing the intestinal phase, and ended up concluding that the pancreatin, which a mixture of different enzymes (trypsin, chymotrypsin, lipases) was most likely to be the problem. It seems that the activity of these intestinal enzymes is not interrupted by neither PMSF 0.1 M nor Pefabloc 0.1 M, explaining the complete absence of IgE-reactivity even at T12 (0 min of intestinal phase) when analysis is later performed. Therefore, pancreatin does not seem to be adequate for this study, and an optimization of the protocol is necessary for intestinal phase analysis.

4.6 IgE-binding capacity of allergens assessed by ELISA as affected by food processing

The indirect ELISA was developed to target different crustacean allergens using as primary antibody the pool-sera of crustacean allergic patients that containing reactive IgE as previously assessed by immunoblotting. In general, the ELISA is set up using a set of calibrators, but in this case, it was not possible to construct a calibration curve with an exact concentration of all crustacean allergens, considering that there are several distinct proteins with IgE-binding capacity. Therefore, the ELISA only intended to evaluate the trend of the IgE-binding capacity of crustacean allergens, by comparing the absorbance values of the species submitted to different food processing treatments in relation to their raw

counterparts. **Figure 4.11**, **Figure 4.12** and **Figure 4.13** present the percentage of increase/reduction in the IgE-binding capacity of the different allergens submitted to distinct processing treatments as a relative proportion towards each raw counterpart.

The allergenic proteins identified in the vannamei shrimp by the pool-sera of crustacean allergic patients were the tropomyosins (Lit v 1), arginine kinases (Lit v 2), myosin light chains (Lit v 3) and sarcoplasmic calcium-binding proteins (Lit v 4), as assessed by immunoblotting (**Figure 4.3**). In the case of ELISA, the results showed that the IgE-binding capacity of vannamei shrimp allergens greatly increased when submitted to boiling for 2 min (267%), frying for 3 min (244%), or grilling for 3 min (71%). The ELISA also revealed that the water from boiling the vannamei shrimps presented an increase of more than 216% in the IgE-binding capacity of its allergens (**Figure 4.11**).

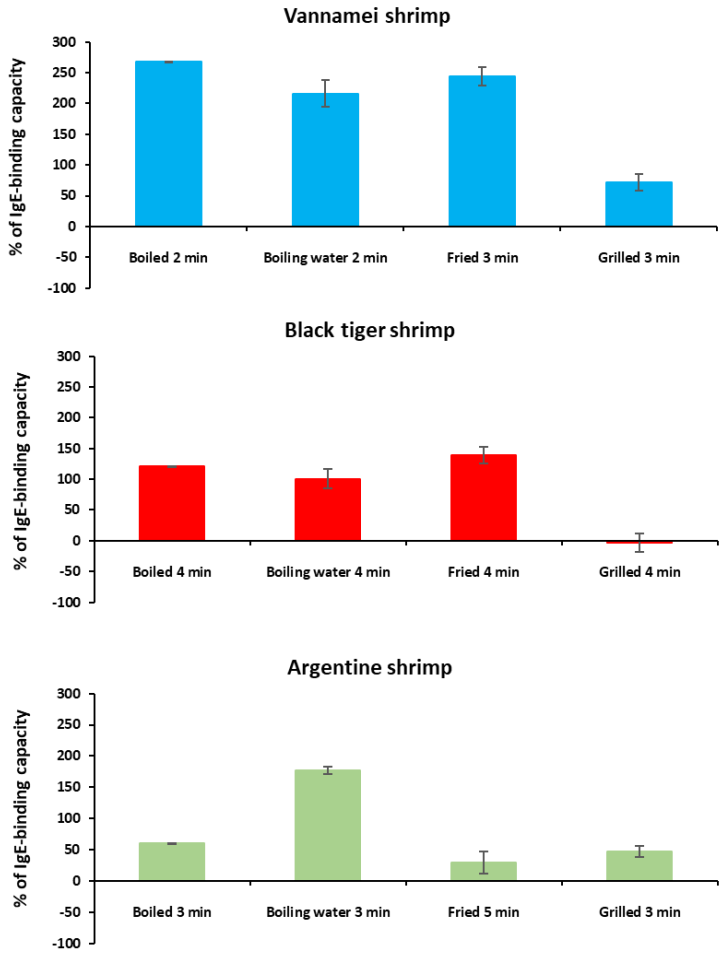

Figure 4.11 – Percentage of IgE-binding capacity of crustacean allergens as affected by food processing treatments for 3 shrimp species: vannamei shrimp, Black tiger shrimp and Argentine shrimp.

In fact, the main allergenic protein identified by immunoblotting in the water of boiling vannamei shrimp was tropomyosin (line 15, **Figure 4.3**), which means that this increase in IgE-binding capacity is greatly attributed to the high solubility of tropomyosin in water. In the case of black tiger shrimp, the identified allergens were Pen m 1 (tropomyosin, 37 kDa), Pen m 2 (arginine kinase, 40 kDa), Pen m 3 (myosin light chain 2, 20 kDa), Pen m 4 (sarcoplasmic calcium binding protein, 20 kDa), Pen m 6 (troponin C, 17 kDa) and Pen m 13 (cytoplasmic fatty acid binding protein, 20 kDa) as high intensity bands (**Figure 4.3**). These proteins might be responsible for the IgE-binding capacity observed by ELISA, whose results revealed 120-140% increasing immunorecognition for black tiger shrimp boiled or fried when compared to its raw counterpart (**Figure 4.11**). As for the vannamei shrimp, the water from boiling black tiger shrimp also presented increased (~100%) IgE-binding capacity, which can also be attributed to the passage of tropomyosin to the water (**Figure 4.3**). Contrarily to the previous treatment, the black tiger shrimp grilled for 4 min did not present any relevant alteration in terms of IgE-binding capacity, thus maintaining its IgE-reactivity (**Figure 4.11**).

In the case of Argentine shrimp, there are no officially identified allergens, fact that was confirmed by immunoblotting (**Figure 4.5**). However, by ELISA it was possible to evaluate the IgE-binding capacity of this crustacean and to confirm that an increased IgE-reactivity was observed when boiled 3 min (60%), fried (29%) and grilled (48%). Interestingly, the water from boiling Argentine shrimp presented the highest rising in IgE-binding capacity (~158%), fact that cannot be attributed to any allergen in specific (**Figure 4.11**).

Scampi also presented increased IgE-reactivity upon different food processing treatments, evidencing the highest IgE-binding capacity after grilling 15 min (151%) and oven-cooking 15 min (147%) (**Figure 4.12**). This might be related to the fact that both processing treatments use dry heat, which have been associated with a higher exposure of the epitopes to IgE thus increasing the allergenic potential of proteins in other matrices (e.g., peanut roasting). As assessed by immunoblotting (**Figure 4.7**), the main contributors for these increased IgE-reactivity correspond to bands around 37-40 kDa (tropomyosins and arginine kinases) and 20-25 kDa (most likely myosin light chains and sarcoplasmic calcium binding proteins). The results of ELISA also evidenced increased IgE-binding capacity for scampi boiled 5 min (107%) and fried 5 min (118%). Similarly, in other cases, the boiling water also evidenced high quantities of IgE-reactive allergens. To test a different processing, scampi was pre-treated with lemon juice and let for marinating 1.5 h. In this case, the raw and marinated scampi evidenced maintained or slightly decreased (10% reduction) IgE-binding capacity compared to its raw counterpart. Upon processing (grilling or oven-cooking), the IgE-reactivity of marinated scampi increased by 73% and 142%, respectively (**Figure 4.12**).

In the case of the American lobster, previous immunoblotting analysis evidenced reduced effect of food processing on the IgE-binding capacity of the allergens (**Figure 4.4**). the identified allergens were tropomyosin (Hom a 1, 34 kDa), myosin light chain (Hom m 3, 23 kDa) and troponin C (20 kDa). By ELISA, boiled lobster and the respective boiling water presented small IgE increases (<15%), which are not meaningful. The raw and marinated lobster also presented an increased IgE-reactivity (24%) compared to raw counterpart. However, taking the raw and marinated lobster as reference for the remaining treatments (grilling and oven-cooking), the IgE-binding capacity of allergens was maintained or slightly reduced (**Figure 4.12**). For the barnacles, the IgE-binding capacity of allergens (mainly tropomyosins, hemocyanin, myosin light chain and/or sarcoplasmic calcium binding proteins) was not significantly altered (<10%) independently on the food processing treatment. The ELISA results (**Figure 4.12**) are in good agreement with the data retrieved from immunoblotting analysis (**Figure 4.6**).

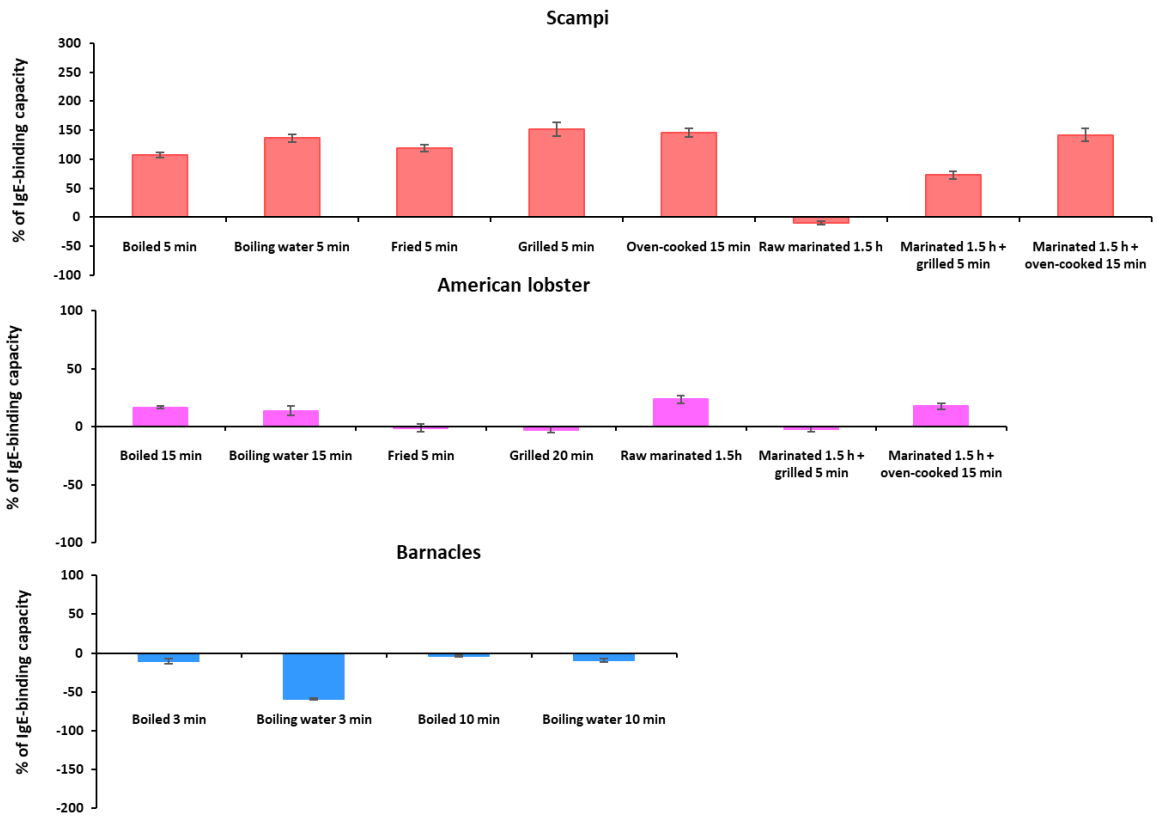

Figure 4.12 - Percentage of IgE-binding capacity of crustacean allergens as affected by food processing treatments for 3 shrimp species: scampi/shrimp lobster, American lobster, and goose barnacles.

The results from ELISA for the spiny spide crab and for the brown crab were somewhat contradictory, as the first had no significant alteration and the second presented a reduction of 61% in the IgE-binding capacity of their allergens upon boiling for 10 min. Comparatively, the boiling water of spiny spider crab had high amounts of allergens (77% increase), whereas the amount of allergens in brown crab boiling water was low (**Figure 4.13**). The allergen officially identified in the tropomyosin Por p 1 (39 kDa), although several bands around 15 kDa, 20-25 kDa, 37-40 kDa and 75 kDa appeared in the qualitative analysis of this crustacean (**Figure 4.5**). The blue swimming crab was also submitted to multiple food processing treatments, which had limited effect on the IgE-binding capacity (<12%) of most allergens (**Figure 4.13**), meaning that the allergenic potential of the referred proteins is not altered by food processing.

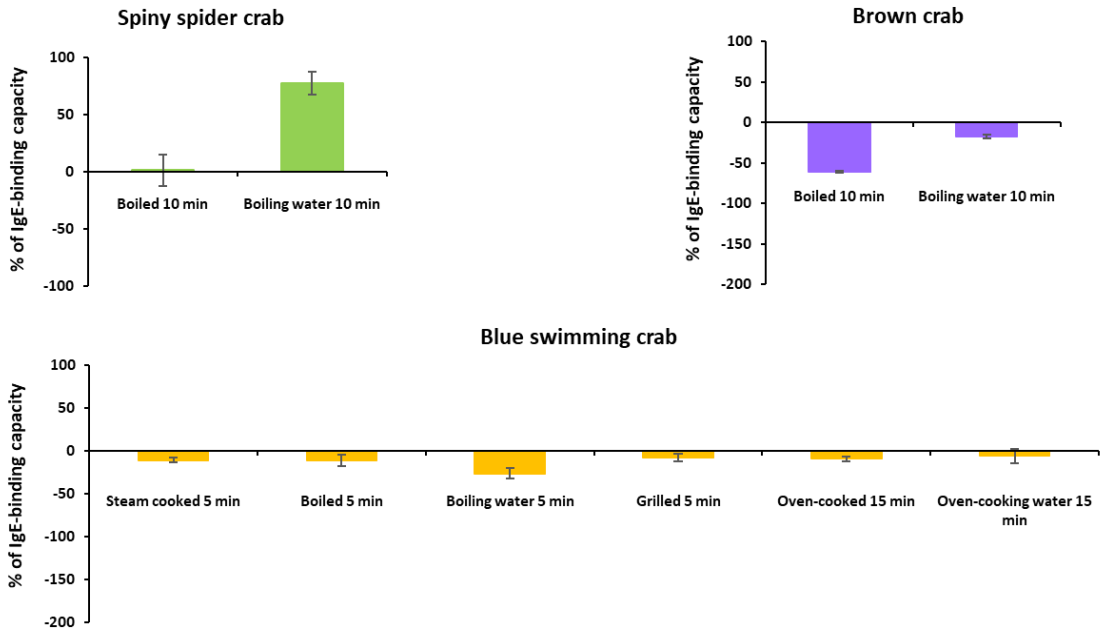

Figure 4.13 - Percentage of IgE-binding capacity of crustacean allergens as affected by food processing treatments for 3 shrimp species: spiny spider crab, brow crab and blue swimming crab.

In summary, the species presenting increased IgE-binding capacity of their allergens are mainly the shrimps (vannamei, Argentine and black tiger) and the scampi (also called shrimp lobster), which is genetically close related to shrimps. In these species, the shell is relatively easy to remove and the meat easy reaches the high temperatures used in the tested food processing treatments. In those cases, the raw species are less allergenic than the processed ones. Therefore, the allergens are probably suffering unfolding that reveals novel epitopes that are reactive to the IgE present in the pool sera of the crustacean allergic patients. On the contrary, crabs and lobster are by nature allergenic in their raw form,

meaning that they do not lose IgE-reactivity when submitted to processing. Besides, they are involved by their hard shells, meaning that the meat is somewhat protected from processing. This might justify the reason beyond the reported results, meaning that not all crustacean species behave alike and that there is ground for research.

5 Conclusion

In summary, the main goals of this work were fully achieved. In what concerns the effect of food processing on the allergenicity of crustaceans, it was seen that, in general, allergens behave differently towards distinct processing methods. SDS-PAGE in non-denaturing conditions evidenced alterations in protein structure during processing, for both suspected allergens and other proteins, and provided information on the behaviour of these samples when submitted to variable conditions. The use of sera of crustacean-allergic patients enable to identify the first step for food allergy development, which is the allergic sensitisation. Moreover, it allowed to test how the IgE present in that sera would react towards proteins submitted to different food processing treatments, including those using wet- and dry-heat. In some cases, the duration of the processing method (e.g., boiling) was also compared in the evaluation the effect of heat on allergens. As most studies normally use antibodies towards specific proteins (e.g., tropomyosin), in this work it was intended to have a more holistic overview on the behaviour of multiple allergens instead as single target proteins. In addition, the use of 9 crustacean species also contributed to a broader view of crustacean IgE-binding capacity. Furthermore, and since every patient have specific sensitisation patterns, a pool-sera of crustacean-allergic patients was used for immunoblotting and ELISA, which showed that thermal processing has the capacity of increasing or diminishing the IgE-binding capacity of some allergens upon different processing treatments. The use of locally available samples and widespread processing methods, allowed to this work to be closer to the reality of crustacean consumers, and therefore allergic patients.

The reactivity of tropomyosin, the main allergen for crustaceans, seems to be enhanced with heat, as boiling, frying, oven-cooking, and grilling contributed to increase tropomyosin IgE-binding capacity in most crustacean species. Because this work had a great variety of crustacean species, it allowed a broader range of analysis and a better representation of what consumers, specifically in Portugal, are subjected to. Therefore, it was suggested that brown crab, for example, had a different behaviour to heat treatment, with a significant reduction in the IgE-binding capacity of most allergens after boiling, which can indicate that each crustacean species should be treated as individual, and specific studies to each one is necessary if crustacean-allergic patients are to be adequately protected. As for the use of lemon juice, it seemed to have some potential in altering allergen structure and reducing its IgE-binding capacity, the results were very variable and seem to be species-specific, and therefore its applicability is limited, requiring further studies. Furthermore, the boiling water and frying oils were also investigated, and though frying oil was difficult to study due to the high presence of interferents, variable consistency and fast degradation, some interesting

results emerged from the analysis of the boiling waters. It is likely that allergens are transferred to water during processing (e.g., boiling), contributing to increase the risks of accidental exposure to cooking vapours that might trigger an allergic reaction by inhalation or skin contact. It is also worth mentioning that a pool-sera of 14 crustacean-allergic patients was used for both ELISA and immunoblotting analysis, meaning that the effect of patient-specific properties was minimised. In this work, it was possible to combine the identification of which allergens are more affected by processing treatments as assessed by immunoblotting with the determination of the ration of increase/reduction of IgE-binding capacity of allergens by ELISA.

In general, the results from immunoblotting and ELISA were well correlated, thus facilitating the attribution of a relative quantification on the total increase/decrease of allergen IgE-binding capacity. The shrimps (vannamei, Argentine and black tiger) and the scampi (shrimp lobster) were the species presenting increased allergen IgE-binding capacity upon most processing treatments. Conversely, crabs and lobster revealed to be more allergenic in their raw form, compared to shrimp species, meaning that they maintain their values for IgE-reactivity when submitted to processing. It became clear that there is a certain common behaviour among species that are more genetically close related, which might be associated with the typology of crustacean shells. Thin and easily to remove shells seemed to contribute to an increased IgE-reactivity of shrimp allergens, while hard shells protect the meat from processing, thus contributing to maintain their IgE-binding capacity. These results highlighted that crustacean species do not behave alike in terms of allergenic potential and it important to consider the effects of processing on individual species.

For the simulated *in vitro* gastrointestinal digestion (INFOGEST 2.0 protocol), the American lobster was used as a case study as raw, raw and marinated with lemon juice and marinated and oven-cooked (15 min), to try to see the possibly of promoting a synergic effect of the use of lemon juice and heat treatment with digestion. The salivary phase did not seem to have significant effects on any of the samples, neither on protein structure nor on IgE-binding capacity, probably due to the lack of enzymatic breakdown of proteins in this phase. As for the gastric phase, there was some protein degradation, with the decrease in the intensity of higher molecular weight bands and a slight increase in the intensity of lower molecular weight ones, suggesting that allergens are being breakdown to smaller peptides during this digestion phase. However, some proteins seem to be pepsin-resistant, and suffered insignificant decrease in their band intensity. At last, intestinal phase was generally difficult to study, with significant lack of bands for analysis, most likely due to the suspected problems with pancreatin. When comparing the food processing effect associated with digestion, it was visible that there were some differences between the raw and the processed samples. For example, high molecular weight proteins (>100 kDa) seemed to

have higher degradation during the gastric phase on the processed samples when compared to the raw ones, which can be due to processing being able to promote protein denaturation, thus allowing pepsin to be more efficient. However, that fact is not enough to confirm with the necessary degree of certainty that there is a synergistic effect of processing methods and digestion, demanding further analysis.

In summary, this work was able to fill some gaps that existed in the literature, giving a more holistic view of the different effects of common food processing methods and the digestion process on allergen structure and IgE-binding capacity, which are key indicators of allergenicity. By working with non-purified samples and limiting the processing that was performed on the samples, for example by using non-denaturing conditions on SDS-PAGE, this work enabled to see a more accurate representation of how patients can be in direct in contact with crustacean allergens. Additionally, diversified food processing treatments, crustacean species and patient sera on this work allows for a better comparison of all these factors, which have direct consequences on the allergenicity of crustaceans.

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