Effect of high pressure processing on fatty acid and amino acid content of New Zealand clams

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Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

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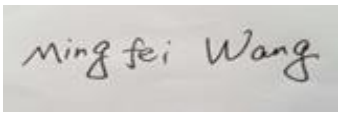

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List of abbreviations

HPP	high pressure processing
FA	fatty acid
UFA	unsaturated fatty acids
SFA	saturated fatty acids
PEF	pulsed electric field
FDA	drug Administration
ACP	aerobic plate count
TVBN	total volatile base nitrogen
UHP	ultra high pressure
P. zelandica	*Panopea zelandica*
TBA	2-Thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
CL	cardiolipin
MUFA	monounsaturated fatty acid
PUFA	polyunsaturated fatty acid
FAME	fatty acids methyl ester
DHA	docosahexaenoic
EPA	eicosapentaenoic acid
AA	amino acid
FAA	free amino acid
EAA	essential amino acid
NEAA	non-essential amino acid
HMW	heavy molecular weight
MHC	myosin heavy chain
LC-PUFAs	long-chain polyunsaturated fatty acids

GC	gaschromatography
FID	Flame Ionisation Detector
TDA	tridecanoic acid
SPE	solid phase extraction
IS	internal standard
ANOVA	Analysis of variance

Abstract

High pressure processing (HPP) treatment is considered to be a popular non-thermal processing technology. It benefits food producers as it may maintain or increase the nutritional value of food and extend shelf life. Previous researchers have documented that HPP treatment could significantly change the physical, chemical and microbial properties of food. However, only few studies have examined the effects of HPP on chemical properties of seafood. These studies have reported variations of lipid and fatty acid aspects in seafood in terms of their oxidation level and content composition. However, no literature has investigated the effect of HPP on amino acid content. Hence, the aim of the current study was to investigate the effect of HPP treatment on the content of amino acids and fatty acids in two species of New Zealand clams, storm shell and tuatua. HPP treatment was carried out at 100, 200, 300, 500, and 600MPa, 18°C, for 5 seconds and 10 minutes.

In the current research, the content of unsaturated fatty acids (UFA) and saturated fatty acids (SFA) in pressure treated storm shell clams significantly decreased at 300 and 600MPa, respectively, when held at 5s and 10min compared to controls. In comparison, TuaTua showed more significant changes in fatty acid content than storm shell when compared to controls. Specifically, in Tua Tua, a significant drop ($P<0.05$) in the total content of UFA occurred at 200MPa when held for 5s, and the value decreased significantly ($P<0.05$) in SFA at 500 and 600MPa when held for 5s. Moreover, treatment Tua Tua for 10min resulted in significantly increased ($P<0.05$) UFA and SFA at relatively lower pressures (100, 200, 300MPa) compared to controls. Comparison of pressurization times of 10 minutes and 5 second, revealed that the longer pressurization time (10 min) of Storm Shell clams caused a significant decrease ($P<0.05$) in both UFA and SFA content at relatively high pressures of 500 and 600MPa, with a significant increase ($P<0.05$) in SFA evident with 100MPa treatment. However, no significant changes in fatty acid contents were found in HPP treated Tua Tua clams when compared to pressurization time 10min to 5s. In general, almost all significant changes in fatty acid content of both Storm Shell and Tua Tua clams mainly occurred in the content of C18:0, C23:0, C24:0, C20:5n3, and C20:6n3 fatty acids.

Nineteen free amino acids, including 7 essential and 12 non-essential amino acids were identified in both storm shell and Tua Tua clams. HPP treatment of Storm Shell clams for 5s and 10min at all pressure conditions (100-600MPa) resulted in significantly decreased

($P<0.05$) total essential amino acid (EAA) content when compared with the control. Moreover, only some significant increases ($P<0.05$) in total content of non-essential amino acid (NEAA) of Storm Shell were found in samples treated at 100, 200, and 300MPa for 5s and 10min compared to control. However, a significant decrease ($P<0.05$) in the content of EAA in Tua Tua was observed at 500 and 600MPa for both of 5s and 10min treatments compared to control. Additionally, compared to control, no significant changes were found in the total content of NEAA with 5s treatment of Tua Tua, but some significant decrease ($P<0.05$) in NEAA occurred at 500 and 600MPa when pressure-treated for 10min. The effect of pressurization times of 5s and 10min under the same pressure conditions were also compared. In storm shell clams, there were significant increases ($P<0.05$) in total EAA and NEAA content at 100MPa treatment for 10min when compared to 5s treated samples and a significant decrease ($P<0.05$) at 300, 500 and 600MPa treatments for 10min compared to 5s treatment. As for Tua Tua, the significant increase ($P<0.05$) in the content of EAA at 200MPa, 10min and increased significantly of content of NEAA at 300MPa for10min can be observed when compared to 5s treated samples. Besides, the significant decrease in the content of EAA and NEAA ($P<0.05$) can be found at both 500 and 600MPa, 10min when compared to 5s treatment samples.

Table of Contents

Lists of Figures

Lists of Tables

Chapter 1 Introduction and objectives

Traditional thermal processing and sterilization technologies cannot satisfy consumer demands for environmentally friendly, safe, delicious and nutritious processed foods. These traditional methods mainly involve thermization that may affect the color, aroma, taste, texture, nutritional quality, and functional properties of foods. Non-thermal processing not only can kill microorganisms in food, but also can retain the nutrients, texture, color and freshness of food. Modern technologies without thermal processing include high pressure processing (HPP), pulsed light, supercritical CO_2, ultrasonic wave, pulsed electric field (PEF) and plasma treatment (Zhang et al, 2011). The earliest HPP research was conducted in America about 30 years ago (Huang, Wu, Lu, Shyu & Wang, 2016). In the year 2000, HPP was regarded as the most promising new technology by the United States Food and Drug Administration (FDA) (Duan, Wang & Ma, 2011). The widespread application of HPP has since boosted its market demand. The output value of global HPP market at the end of 2015 has approached $10 billion annually (Huang, Wu, Lu, Shyu & Wang, 2016).

As a pioneer of non-thermal technology, High-Pressure Processing (HPP) treatment has excited the amusement in the seafood industry in terms of controlling microorganisms and improving the processing of seafood like shellfish, oysters, and crustaceans. According to Raghubeer (2007), HPP is a non-thermal food preservative technology which has been widely used on clams, lobsters, shrimp, cod, mussels, crabs, and hake. HPP can reduce the need for manual shucking, by releasing the abductor muscle from the shell. Oysters that are fully or partly shucked using HPP are comparatively less damaged than mechanical shucking. Ready-to-eat seafood meals are also being processed through HPP nowadays. According to Angsupanich (2008), HPP can influence the hydrophobic and electrostatic interactions of all types of structures in proteins. Cruz-Romero et al., (2004) demonstrated that HPP treatment influenced the functional properties of food ingredients. It can strongly influence macromolecular phenomena, such as protein unfolding, and structural configuration. Specifically, pressure-treated protein may be changed as a result of disulfide bond binding. On the other hand, protein probably also can be reacted by exposed hydrophobic groups on the protein to form oligomers. Besides, because of the presence of 2-mercaptoethanol, complete dissolution of the aggregates can be achieved. Therefore, the addition of 2-mercaptoethanol, a reducing agent that destroys disulfide bonds, can dissolve high molecular weight protein complexes.

In terms of structure changes, Campus (2010) indicated that to some extent high pressure treatment inhibits calpain activity, and retards or avoids the cytoskeletal proteins degradation, such as desmin. Cruz-Romero et al., (2006) also pointed out that the interactions between proteins or free fatty acids were mainly achieved by electrostatics, van der Waals forces, hydrogen bonds, and hydrophobic forces. HPP could influence these forces and lead to lipid hydrolysis, accelerating the release of the free amino acids. The change in total content of fatty acids may result in structural and chemical changes when exposed to high pressure.

HPP also increases shelf life of seafood. For instance, Rode & Hovda (2016) proved that treated salmon, cod and mackerel with pressure treatment at 500 MPa for 120s can extend their shelf life. Hughes, Perkins, Yang, & Skonberg (2016) also pointed out that there were no big changes in terms of aerobic plate count (APC), total volatile base nitrogen (TVBN), K-value, biogenic amines after 35 days of storage, whereas, APC and TVBN increased, and biogenic amines detected. Huang, Wu, Lu, Shyu & Wang (2016) also reported that HPP was the most successful commercialized non-thermal processing technology that could eliminate food pathogens at room temperature and extend the shelf life of food circulated through the cold chain.

Previous studies have documented that the HPP treatment can significantly change the features of food in terms of some aspects (micro, chemical and physical). However, only few previous studies have examined the effects of HPP on chemical properties of seafood. These studies have reported variations of lipid and fatty acid aspects in seafood in terms of their oxidation level and content composition. However, no literature has investigated the effect of HPP on amino acids content. Hence, the main purpose of current project was to investigate the effect of HPP treatment on the content of amino acids and fatty acids in two species of New Zealand clams, storm shell and Tua Tua. HPP treatment was carried out at 100, 200, 300, 500, and 600MPa, 18°C, for 5 seconds and 10 minutes. As mentioned above, if the research question should be further elaborated, amino acid could be studied solely in terms of the effect of HPP on chemical properties.

Chapter 2 Literature review

2.1 High Pressure Processing (HPP)

High-pressure processing technology has been known as ultra high pressure treatment (UHP) or high hydrostatic pressure (HHP) (Campus, 2010). In food processing plants, HPP generally refers to UHP. Processing involves putting the packaged food into a high-pressure container, then the water acts as a medium to conduct the pressure to the products. Qin Zeng (2011) demonstreated that HPP can lead to the destruction or formation of non-covalent bonds in food, such as hydrogen bonds, ionic bonds, and hydrophobic bonds. These varations of structure in terms of bonds can lead to biopolymer material inactivation, denaturation or gelatinzation, contained enzymes, proteins and starches. This processing technique can potentially generate development of new food that are shelf stable.

Hiperbaric (2013) reported that HPP is an effective non-thermal food preservative technology. It is a technique related to a cold pasteurization technique. The products are introduced into a vessel, and a high level of isocratic pressure between 100 to 1000 MPa (industries up to 800Mpa) is applied by water (Figure 1). The absence of heat treatment can help retain the original freshness of food. Hence, HPP can maintain the nutritional properties and sensorial properties of food products. To some extent, HPP can almost eliminate all bacteria, moulds and yeasts, without causing nutritional damage and flavor changes like high temperature preservation. HPP can destroy the higher structure of bacterial protein by destroying the non covalent bonds, which can lead to protein coagulation and enzyme inactivation. Ultrahigh pressures can also cause disruption of cell membrane, resulting in various cell damage such as outflow of chemical components and so on. These factors lead to the death of microorganisms. The effect of HPP is impacted by many factors, such as the species of microorganism, cell morphology, temperature, time, pressure and so on. Generally, the higher pressure, the better the preservation effect. But the longer pressure time under the same pressure does not necessarily improve the effect of preservation. In recent decades, increasing food products have been treated by HPP techonolgy, including drinking, meat, fruit, vegetable, jam and salad (Houška & Silva, 2017).

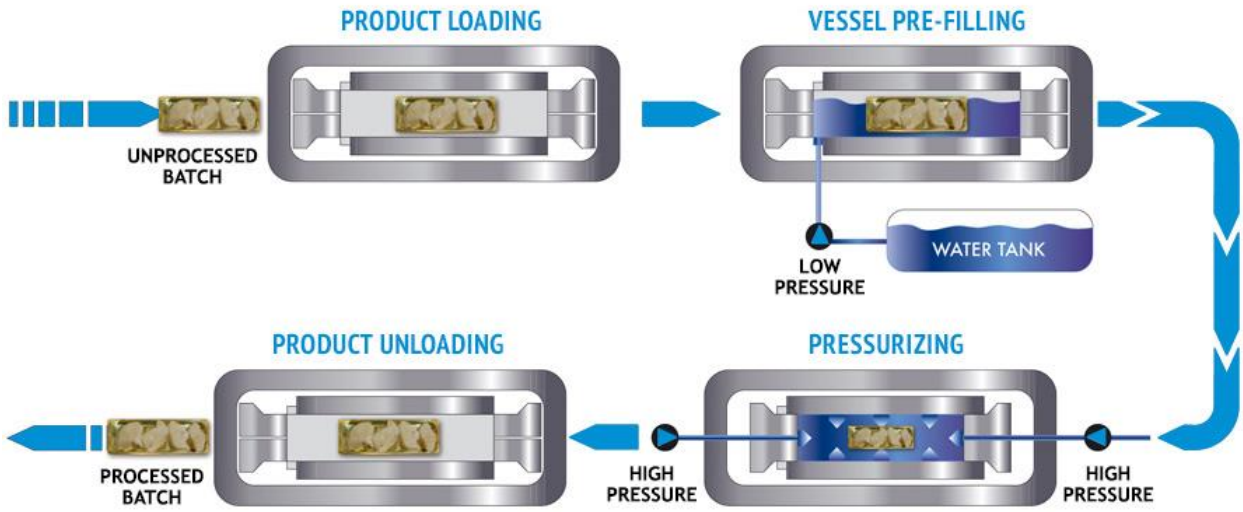

Figure 1. Diagram depicting HPP processing (Hiperbaric, 2013)

2.2 Benefits of using HPP treatment in seafood

HPP can damage the cell membranes and cell walls of microorganisms, change cell morphology, affect intracellular enzyme activity, transport intracellular nutrients and wastes, and kill spoilage bacteria in foods. As a physical method, it can kill pathogenic bacteria without or with the addition of chemical preservatives, and ensure the safety of food and prolonging the shelf life of foods. Additionally, as a non-thermal processing method, HHP does not have drastic changes in temperature during the sterilization process, does not destroy covalent bonds, has little impact on small molecule substances, and maintain the original color, smell, taste, function and nutrition of food (Koutchma, 2014).

2.2.1 Meat separation

López-Caballero, Pérez-Mateos, & Montero (2010) reported that HPP can be used to facilitate extraction of meat from oyster almost 100 percent. It helps operators to work on the small part of seafood that is really difficult to obtain.

- It reduces the use of labor in meat separation (Lopez-Caballero, M. E.-M. 2010)
- Almost 100 percent meant recovery from fish (Lopez-Caballero, M. E.-M. 2010)

- There is not found any physical damage in fish's meat from the shucking knives (Lopez-Caballero, M. E.-M. 2010)
- Yield weight increases the hydration of raw protein (Lopez-Caballero, M. E.-M. 2010)

Product quality improvement (Raghubeer, 2007)

2.2.2 Mollusk Shucking

The application of HPP helps avoid the handling of mollusks that may introduce contamination, and decreases the potential dangers when people use a knife when opening the shells (HSU, 2006). George (2008) claimed that HPP was able to reduce the need for manual shucking, by allowing the abductor muscle to be separated from the shell. This reduces the demand for extra labor to shuck the seafood including shellfish and oysters.

2.2.3 Effects on microorganisms

HPP can reduce the *Vibrio* spp-population effectively. According to Murchie, et al., (2006), use of mollusk shucking and digitization that has also included *Vibrio* inhibition, can work combined to achieve seafood products with good quality, such as the shelf life increased of the fresh oysters. They treated oyster at 400 MPa, 7℃, for 5min and found that HPP treatment reduced the number of all the target microorganisms (total live bacteria, H_2S-producing microorganisms, lactic acid bacteria, *Brochothrix thermosphacta*, and coliforms), the specific number around 5-log units. (M. Perez-Wona, 2012).

2.3 Clams

Bivalves are significant in the global shellfish market, especially in New Zealand. For example, geoduck clams (*Panopea zelandica*) which came from New Zealand, were placed in the New Zealand Quota Management System in 2006, and the total allowable catch can reach up to 40.5 tonnes. In recent years, geoduck clams have shown the potential for commercial development. Experts identified that *P. zelandica* was a major species to meet the New Zealand aquaculture industry's ambitious goal of reaching NZ$1 billion by 2025 (Le, et al. 2016). Additionally, Wen et al., (2018) pointed out that

molluscs are important to the New Zealand market, likes oyster, clam, and mussel. According to the FAO Fisheries and Aquaculture - Fishery Statistical Collections, the total world mollusc production was 23.8 million tonnes in 2015, with major contributions coming from oyster, clam, and mussel.

Clams are bivalves, which can burrow deeply into the sand or mud. Because of the different geographical positions, they have different shapes, sizes, and color (Gosling 2008). Morphologically, molluscs have a white soft tissue that is covered by a pair of hard shells (Defeo, 1996). As seen in Figure 2, clams comprise of seven constituent parts: shell, gills, foot, labial palps and alimentary canal gonads, heart and haemolymph vessels, and excretory organs (Gosling 2008). The edible parts of clams include the muscle, mantle and foot.

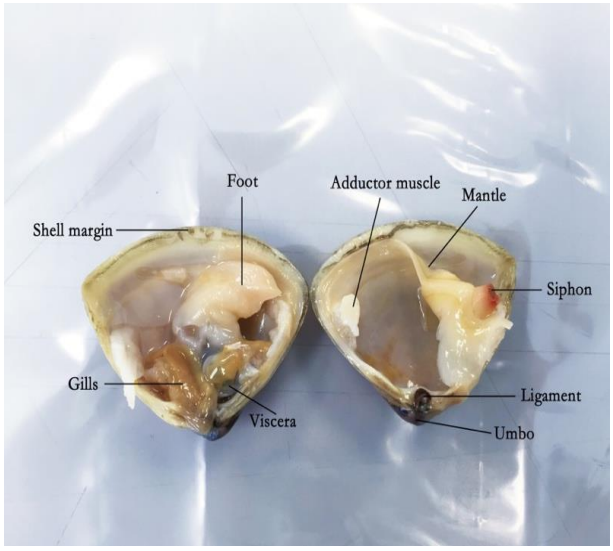

Figure 2. Figure 2. Parts of clam

2.4 Background of New Zealand Clams

On a few surf beaches in New Zealand, the large-scale bivalve fauna are dominated by surf clams (Cranfield, 2001). Cranfield (2001) also reported that there were seven main species that can be caught in New Zealand beaches: "Mesodesrnatidae (*Paphies donacina*), Mactridae (*Spisula aequihtera*, *Mactra murcshisoni*, and *M. discors*) and Veneridae (*Dosinia anus*, *D. subrosea*, and *Bassina yateo*)."

From 1986 to 2001, the New Zealand Fishing Industry granted 16 special permits to 16 locations around the New Zealand coast: Clifford Bay, Cloudy Bay, Rabbit Island, Nelson, Marlborough, Manawatu coast, an Foxton et al. (Cranfield, 2001). These locations were developed for clam har vesting. Cloudy Bay Clams Limited played a leading role in the

field of surf clams harvesting in New Zealand. The unique environmental conditions in Cloudy Bay (Figure 3) make it suitable for growth of high quality clams.

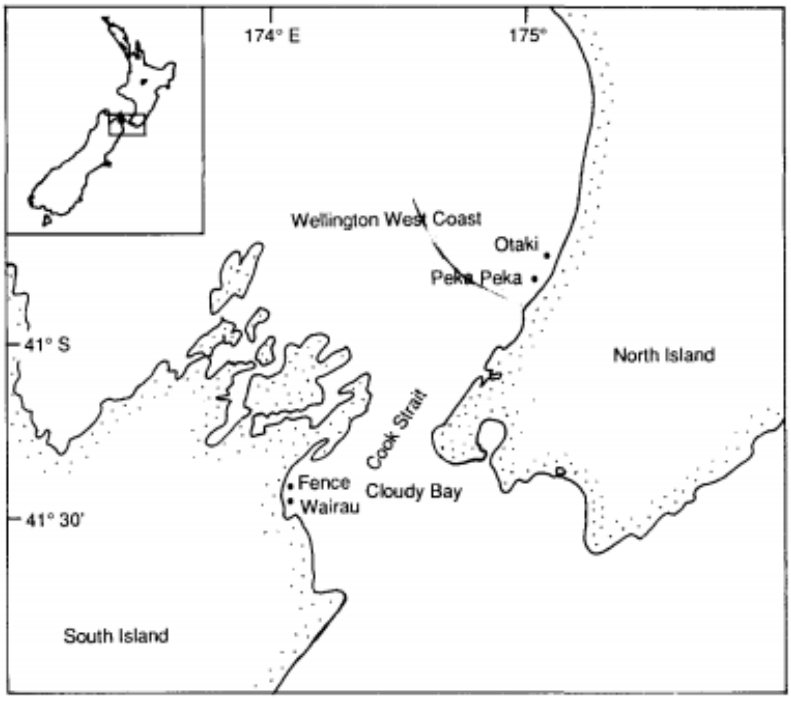

Figure 3. Map of Cloudy Bay, Southern Island, NZ

2.5 Composition of clam meat

2.5.1 Lipid

Lipids are the essential structural and functional components of food. The main components of lipids are triacylglycerol, fats, and oils. Lipids can contribute to flavor, odor, and texture of food (Frankel, 2014. & Shahidi & Zhong, 2010). Nutritionally, lipids are the main source of calories and the nutritional value and organoleptic properties of clams mainly come from lipids, and this has led to a high demand for clams in global markets (Orban et al, 2006).

Lipid oxidation can influence the quality and acceptability of meats and other muscle foods when handling, processing, storage and cooking. This can result in color changes, drip loss, abnormal taste, texture variation and the production of potentially toxic compounds (Chaijan, 2008). Frankel (2014) further reported that lipid oxidation not only induces undesirable rancid flavors in foods but can also affect nutritional quality and safety negatively.

The occurrence of lipid oxidation results from inorganic and biological catalysts in food, such as metal ions and enzymes (Linares, Berruga, Bórnez, and Vergara 2007). The main strategies of limiting the lipid degradation degree include avoiding heat, light, enzyme inactivation, and metal ions elimination.

According to Maqsood and Benjakul, (2010), fatty fish species of nutritional importance include clams, oysters, and mussels. Their high level of the essential n-3 polyunsaturated fatty acids, such as eicosapentaenoic acid (C20: n5) and docosahexaenoic acid (C22: n6) are always preferred by many consumers. However, this makes them oxidize easily, and may result in lipid oxidation that is associated with rancidity and nutritive value damaged.

Similarly, Yarnpakdee et al., (2012) reported that undesirable flavor of seafood products generally reduces consumer acceptability making them unsuitable for sale. These negative effects are mainly associated with lipid oxidation. The major substrate for lipid oxidation is membrane phospholipid. Oxidation can be catalysed by heme protein, iron as well as lipoxygenase.

The extent of lipid oxidation can be assessed using sensory methods, peroxide value, conjugated dienes, and 2-Thiobarbituric acid (TBA) values (Frankel, 2014). The thiobarbituric acid reactive substances (TBARS) method is considered the most fast and effective method for the measurement of lipid oxidation (Frankel, 2014., Maqsood, Abushelaibi, Manheem, Al Rashedi, & Kadim, 2015., Shahidi & Zhong, 2010). As a major product of lipid oxidation, Malondialdehyde (MDA) is always regarded as a vital indicator about the detection of the extent of lipid oxidation. Specifically, the value comes from the reaction between thiobarbituric acid and MDA is always used to represent the extent of lipid oxidation (Frankel, 2014).

2.5.2 Fatty acids

Straight chain aliphatic carboxylic acids with 4 or more carbon atoms are referred as fatty acids (Wood et al., 2008). Fatty acid composition of many seafood contains at least 80% omega-3-fatty acids or their derivatives, wherein 5,8,11,14,17-eicosapentaenoic acid (EPA) and 4,7,10,13,16,19-docosahexaenoic acid make up at least 75% by weight of the total fatty acids (Breivik, Dahl & Krokan, 1997). Specifically, polyunsaturated fatty acid (PUFA) represented 42.9% of total FA of PL in the *R. philippinarum* clam. The main

PUFA were C20:4n-6, C20:5n-3 and C22:6n-3. C22:6n-3 that accounted for about 10% of total FA, 17.5% for C20:4n-6 and 8.6%, and 22.6% for C20:5n-3, and C18:0. CL (cardiolipin), was mainly (over 75%) composed of four FA: C22:6n-3, C20:5n-3, C16:0 and C18:0, with saturated FA (SFA) accounting for less than 30% of total FA. Moreover, CAEP was mainly composed of C16:0, representing 69.7% of total FA in *R. philippinarum* clams. Two others remaining SFAs were C17:0 and C18:0, making up about 3.5% and 4.5% of total FA. Additionally, LysoPC was mainly composed of SFA in *R. philippinarum* clams accounting for around 72.1%. Monounsaturated fatty acid (MUFA) occupied for 23.3% of total FA. The main monounsaturated fatty acid (MUFA) were C16:1n-7 and C18:1n-9 accounting for 10.4% and 12.6% total FA.

C22:6n3 (DHA) and C20:5n3 (EPA) are all engaged in maintaining structural and function of cell membrane. Different from mammals, DHA and EPA have more vital functions in terms of marine animals. (Sargent et al., 1999).

From a nutritional and health perspective, the three types of ratios that could be the important indicators, including PUFA: SFA, MUFA: SFA, and n-6: n-3. According to the report by Scollan et al. (2006), these three types of rations have been extensively considered as an index of healthy eating fats. Meanwhile, Haffner, (2006) announced that a relatively high ratio of PUFA to SFA (≥ 0.4) can weaken the adverse impact in terms of saturated fat, such as risks of cardiovascular disease and metabolic syndrome. Additionally, relatively high level of MUFA can prevent increase in blood cholesterol levels (Garaffo et al., 2011). The best PUFA ratios (PUFA/SFA) are probably close to 0.2, which is similar to dietary ratios of Cn−6: Cn−3. Chemical similarities of the three-long chain PUFA can result in competitive interactions, especially for EPA and FA. “Specifically, EPA competitively inhibits the formation of eicosanoids from FA. Therefore, eicosanoid actions in the body are determined by the ratio of FA/EPA” (Sargent et al., 1999). Relative high ration of FA/EPA can enhance the cardiovascular functions.

2.5.2.1 Saturated Fatty Acids

Saturated fatty acids mainly comprise of: (a) straight chain; (b) even number of carbons; (c) chain length containing 12-22 carbons. For example, myristic acid with 14 carbons is designated as C14:0 (meaning 14 carbons without double bonds), palmitic acid with 16

carbons is C16:0, and stearic acid with 18 carbons C18:0 (Frankel, 2014). The British HMSO (1994) recommended that the value of fat intake should be reduced to 30% or less of total intake of the energy in the best situation, and energy intake of saturated fatty acids limited to 10%.

The GC fatty acids methyl ester (FAME) method has been used to identify fatty acids in oysters, clams and squids (Abdulkadir and Tsuchiya, 2008). They reported that 29 fatty acids in clam samples, with the major saturated fatty acids being C16:0, C18:0, and C18:0 anteiso, that accounted for over 80% of total SFA. Besides that, C14:0, C15:0, C17:0, C21:0, C26:0, and C28:0 accounted for about 15% of total SFA. Similarly, Hanus et al, (2009) pointed out that the most abundant SFAs in clams were C16:0 and C18:0 accounting for 31% and 32% total FA, respectively.

2.5.2.2 Unsaturated Fatty Acids

Unsaturated acid can be classified as MUFA and PUFA. MUFA mainly contains palmitoleic (C16:1) and oleic (C18:1) (Banskalieva, Sahlu, & Goetsch, 2000). Oleic acid (C18:1), for example, has one double bond in cis configuration in the 9-carbon position is designated as C18:1 n-9. The "n" refers to the number of carbons from the terminal end of the 18-carbon chain. Obviously, the biggest difference between SFA and UFA is number of double bonds. Comparatively, PUFA has two or more double bonds, and is separated by a single methylene group or 1,4-diene structure (Frankel, 2014). Linoleic (C18:2), linolenic (C18:3) and arachidonic (C20:4) acids are representative acids (Banskalieva, Sahlu, & Goetsch, 2000). For instance, Linoleic has two cis double bonds in the 9, 12 carbon positions, and designated as C18:2 n-6 (9,12). Similarly, linolenic acid has three double bonds in the 9,12, and 15 carbon positions, and designated as C18:3 n-3 (9,12,15). As for arachidonic acid, it has four double bonds in the 5, 8, 11, 14 carbon positions, and designated C20:4 n-6 (5,8,11,14). Additionally, docosahexaenoic (DHA) and eicosapentaenoic (EPA) belong to PUFA group. DHA is always extracted and added into food and health products, because of its nutritional benefits to visual and brain functions (Frankel, 2014).

Hanus et al, (2009) reported that EPA (C20:5n3) and DHA (C20:6n3) acids were dominant fatty acids in clams, accounting for about 27% and 18% of total unsaturated

fatty acids respectively. Moreover, Abdulkadir and Tsuchiya, (2008) similarly reported that the predominant fatty acids were C20:4n6, C20:5n3 and C22:6n3. These three fatty acids accounted for around 70% of total UFA. The remaining fatty acids were C18:3 n3, C18:3n6, C20:3n3, C22:4n3, C22:5n3, and C22:6n3.

2.5.3 Amino acids

Non-essential and essential amino acids (AA)

Traditionally, AA have been classified as nutritionally essential (indispensable) or non-essential (dispensable). Nutritionally, essential amino acid (EAA) are defined as those AA which cannot be synthesized by animal cells and must be taken in the diet. On the other hand, nutritionally non-essential AA (NEAA) are those AA, which can be synthesized, and do not need to be provided in the diet (Wu, 2013). Wu, 2013 reported that the dietary NEAA requirements for people include arginine, glutamine, glutamate, and proline. These NEAA can maintain metabolism, promote protein synthesis, and generate essential non-protein metabolites.

Chen et al. (2012) reported that glutamic, alanine, and glycine are the main EAA in mollusks, that are considered as major taste-active amino acids. Specifically, three bivalve mollusks (clam, oyster, and paphia) were analyzed for their amino acid profile. The results showed that the amino acid composition of these three mollusks were very similar. Although glutamic, alanine, and glycine, the main EAA are similar, the contents of glycine, alanine, and tryptophan were different. Moreover, they pointed out that the tryptophan was the limiting amino acid in clams.

Many free amino acids (FAA) can affect the flavor of seafood. Yoshikawa et al. (2008) reported that the main taste-active FAAs are glycine, alanine, proline, and glutamine acid. They mainly provide four different flavors, including umami, sweetness, bitterness, and sulphur-containing flavours. Kube et al., (2007) reported that the dominant FAAs in the bivalve taxa were alanine, glycine, and taurine, which constituted about 50–90% of the total FAAs. Similarly, Sokolowski et al., (2003) pointed out that in clam meat, alanine, glutamic acid, arginine, and glycine were the four major FAAs, and usually greatly impact seafood taste. Chen and Zhang, (2007) demonstrated that taste active values (TAV) was a useful index to evaluate the individual taste active compounds in the food matrix.

Specifically, although the content of some FAAs occupied a small percentage in clams or oyster, the impact on taste were fierce due to their low threshold values. In clams, glutamic acid, alanine, glycine, aspartic acid, and arginine had high TAV values. The highest taste values which contributed by FAAs were glutamic acid and aspartic acid, followed by arginine, glycine, and alanine (Fuentes et al., 2009). Additionally, Lioe et al., (2005) found that amino acids with TVA more than 1.0 were considered to contribute to food taste. Their study showed that glutamic acid, alanine, glycine provided different flavors with umami and sweet tastes. Similarly, arginine contributes to seafood taste in clams and other seafoods. Besides, Lioe et al., (2005) further found that tyrosine and phenylalanine probably did not have any direct impact on taste significantly but could significantly enhance the taste of umami.

As for some other functions, Sokolowski et al., (2003) pointed out that glycine and taurine were considered the most important amino acids in regulating intracellular osmolarity in clams. Similarly, Chen and Zhang (2007) reported that glycine and alanine were important in regulation of intracellular osmolarity in clams. Sokolowski et al., (2003) found that the concentrations of glycine and alanine will be change with osmotic pressure variation of the external environment. Table 1 shows the composition and taste attributes of each amino acid in Oyster, Paphia and Clam.

Table 1. Table 1. The composition and taste attributes of each amino acid in Oyster, Paphia and Clam. (Chen et al. 2012).

Amino acid	Clam (mg/g)	Oyster (mg/g)	Paphia (mg/g)	Taste attribute [z]
Asp	0.21 (0.01) [a]	0.43 (0.01) [b]	0.28 (0.00) [c]	Umami (+)
Thr	0.21 (0.01) [a]	0.21 (0.01) [a]	0.42 (0.01) [b]	Sweet (+)
Ser	0.06 (0.00) [a]	0.35 (0.01) [b]	0.09 (0.00) [c]	Sweet (+)
Glu	0.96 (0.03) [a]	0.92 (0.03) [a]	1.60 (0.02) [b]	Umami (+)
Pro	0.08 (0.00) [a]	0.48 (0.02) [b]	0.17 (0.00) [c]	Bitter/sweet (+)
Gly	1.04 (0.02) [a]	1.39 (0.04) [b]	4.16 (0.04) [c]	Sweet (+)
Ala	2.50 (0.04) [a]	1.12 (0.04) [b]	1.76 (0.02) [c]	Sweet (+)
Cys	0.00	0.00	0.08 (0.00)	Bitter/sweet/sulfurous (−)
Val	0.12 (0.00) [a]	0.10 (0.00) [b]	0.15 (0.00) [c]	Bitter/sweet (−)
Met	0.08 (0.00) [a]	0.04 (0.00) [b]	0.10 (0.00) [c]	Bitter/sweet/sulfurous (−)
Ile	0.08 (0.01) [a]	0.04 (0.00) [b]	0.10 (0.00) [c]	Bitter (−)
Leu	0.15 (0.01) [a]	0.08 (0.00) [b]	0.16 (0.00) [a]	Bitter (−)
Tyr	0.19 (0.02) [a]	0.27 (0.01) [b]	0.17 (0.00) [a]	Bitter (−)
Phe	0.13 (0.02) [a]	0.06 (0.00) [b]	0.13 (0.00) [a]	Bitter (−)
Lys	0.18 (0.01) [a]	0.13 (0.01) [b]	0.28 (0.00) [c]	Bitter/Sweet (−)
His	0.07 (0.00) [a]	0.08 (0.00) [b]	0.09 (0.00) [c]	Bitter (−)
Arg	0.74 (0.03) [a]	0.23 (0.01) [b]	1.13 (0.01) [c]	Bitter/sweet (+)

2.6 Effects of HPP on the chemical properties of seafood

2.6.1 Effects of HPP on protein

The modification of myofibrillar proteins causes variation in sea fish texture. HPP can impact the structure of protein. Specifically, the HPP can modify sea fish texture through changing their protease activity, water bond, gelatin of myosin and sarcoplasmic proteins (Montero, 2009). Similarly, Sun (2014) also demonstrated that one of the main effects of HPP treatment is to influence electrostatic and hydrophobic interactions. Through changing the electrostatic and hydrophobic, the structure of protein can be variated, and their function will be changed probably.

According to Rehbein (1997), HPP treatment can result in enzymatic, protein and structural modifications in seafood. HPP treatment of proteins can cause conformational changes that can significantly influence functionality (Chapleau, 2011). Ramirez-Suarez and Morrissey (2006) claimed that HPP treatments at pressures of 275 and 310 MPa, for 2, 4, and 6 min had a significant impact on protein. Specifically, HPP could generate the heavy molecular weight (HMW) polypeptides formation and myosin heavy chain (MHC) band reduction. The change in disulfide bond is the most important factor, which can lead to these variations. Gilleland et al. (1997) pointed out that HPP treatment of surimi can induce disulfide bonding that can change texture. Specifically, in cells, some proteins need disulfide bonds to maintain their structure and functions. Through using some physical and chemical methods (heating, pressing, adding oxidizing agent, adding reducing agent), disulfide bonds can be changed, by either breaking or recombining. Once the changes in the disulfide bonds occurred, the structure of protein will be changed, and resulted in either protein enhancement, minimization or variation. Cruz-Romero et al., (2004) demonstrated that pressure generated the remarkable protein denaturation. When the pressure over than 300MPa, the speed of protein denaturation was accelerated. Specifically, fresh oyster treated at 100 to 800 MPa with 10 min (20℃) could lead to denaturation of protein to some extent. Relative to untreated oysters, protein contents decrease when the pressure increased up to 800 MPa.

Campus (2010) indicated that to some extent high pressure treatment inhibited calpain activity and prevented the degradation of cytoskeletal proteins such as desmin. In addition,

H. Simonin et al. (2012) suggested that muscle proteins are vulnerable of oxidation that result in the EAA content loss and protein digestibility weaken.

Researche carried out to determine amino acid content in mollusks are summarized in Table 2. Chen and Zhang (2007) reported that alanine, glutamic acid, arginine, and glycine were the major free amino acids in clam meat. Similarly, Sokolowski, Wolowicz et al. (2003) also pointed out that in Baltic clams (*Macoma balthica* L), free amino acids were mainly composed of Ala, Gln, Arg, Gly and Orn, which accounted for approximately 80% of the total free amino acids. Chen and Zhang (2007) and Sokolowski, Wolowicz et al. (2003) further reported that the glycine and alanine were important in regulating intracellular osmolarity in clams. Additionally, a few hydrophobic amino acids can be found in some mollusks such as cysteine, isoleucine, phenylalanine, and tyrosine (Chen and Zhang (2007). Different flavors can be conferred by amino acids. Yimdee and Wang (2016) demonstrated that in the fish sauces, the umami taste mainly comes from glutamic acid and aspartic acid; sweetness from threonine, serine, glycine, and alanine; and bitterness from valine, isoleucine, leucine, phenylalanine, lysine, histidine, and arginine. Some non-essential amino acids can contribute to bitter flavour. In addition, Peinado, Koutsidis et al. (2016) reported that in fish powder the of bitter and sweet flavors mainly originated from methionine and lysine, although glycine also contributed to sweet as well. Sour taste comes primarily from aspartic acid.

Table 2. Free amino acids content in mollusks

Species	Free amino acids	Results	Reference
Clam (Meretrix meretrix)	Ala, Glu, Arg and Gly	• Hydrophobic amino acids present in small amounts include cysteine, isoleucine, phenylalanine, and tyrosine. • Alanine, glutamic acid, arginine, and glycine were the main free amino acids in clam meat. • Glycine is important in regulating intracellular osmolarity in clams.	Chen and Zhang (2007)
Baltic clam (Macoma balthica L.)	Ala, Gln, Arg, Gly, and Orn	• Free amino acids are mainly composed of Ala, Gln, Arg, Gly and Orn, which accounted for around 80% of the total free amino acids. • The concentration of Gly probably can be regulated by gonadal development and reproduction. • The function of Ala is associated with intracellular osmoregulation.	Sokolowski, Wolowicz et al. (2003)
Fish sauces from various Asian countries.	Free amino acids	• The umami taste mainly comes from glutamic acid and aspartic acid; sweetness from threonine, serine, glycine, and alanine; and bitterness from valine, isoleucine, leucine, phenylalanine, lysine, histidine, and arginine. Some non-essential amino acids contributed to bitter flavour.	Yimdee and Wang (2016)

Mountain trout *(Salmo trutta macrostigma Dumeril)*	Met, Gly, Aspartic acid and Lys	• methionine (bitter/sweet), glycine (sweet), aspartic acid (sour), and lysine (sweet/bitter)	Gunlu and Gunlu (2014)
Fish powder (Croda International plc, UK)	17 AAs (like Ala, Gly, Val, Leu, Ile, Thr, Ser, Pro, Asp, Met, Glu, Phe, Lys, et al,)	• 17 AAs were identified and quantified in different fish powder hydrolysates (FPHs). Lysine, leucine, glutamic acid and alanine were the most abundant in FPHs.	Peinado, Koutsidis et al. (2016)
Squid (Sepioteuthis lessoniana, Loligo bleekeri, Loligo edulis, and Todarodes pacificus)	Thr, Ser, Gly, Ala, Pro, Arg and Glu	• Thr, and Ser were identified as unique taste-active components, which are only present in squid species, although found at very low levels.	Kani, Yoshikawa et al. (2008)

2.6.2 Effect of HPP on lipid oxidation

Oshima and others (2011) reported that HPP can result in deactivation and degradation of enzymes in fish muscle and lead to avoiding of the lipids oxidation. Table 3 summarizes studies on the influence of HPP in terms of the lipid oxidation of seafood.

Yagiz et al. (2009) reported that treatment of Atlantic salmon at 300MPa, 25°C for 15min significantly ($P<0.05$) decreased the degree of lipid oxidation when stored frozen at day 4 and day 6 compared to the control. Similarly, Aubourg et al. (2010) treated salmon at 135, 170, and 200 MPa for 30 s, and stored the frozen samples for 6 days. They showed that at day 6, the lipid oxidation value significantly decreased compared to control group. As for cold-smoked salmon, HPP treatment with 300 MPa at 10°C for 30 min significantly reduced the TBARS value to about 7.08 ml/mol when compared with control (Lakshmanan et al.,2005). Ramirez-Suarez & Morrissey (2006) further showed that minced albacore muscle with HPP treated under 275 and 310 MPa (2, 4 and 6min) also resulted in significantly decreased TBA values compared to the control (except for 275MPa, 2 min). HPP treatment at 310 MPa, the significantly decreased TBA value with minimal increment even after 11 days.

However, Amanatidou et al. (2000) found that TBARS values would not be affected after treatment of fresh Atlantic salmon at pressures up to 200 MPa. Moreover, Cruz-Romero, Kerry, & Kelly (2008) demonstrated that TBARS value significantly increased after 31 days chilled storage after treatment at 260 MPa, 400 MPa, or 600 MPa, with treatment at 600 MPa having the highest TBARS value. Yagiz et al. (2009) also indicated that excessive pressure may not be beneficial for controlling lipid oxidation. However, when Atlantic Salmon was processed at gentle pressure levels with 150 MPa and 300 MPa for 15 min, samples had significantly lower oxidation value compared to control sample. Campus (2010) reported that cod muscle pressurized at 200–600MPa for 15 and 30 min caused significant increase on lipid oxidation. Similarly, when a mixture which contains sardine oil and defatted sardine meat was processed with high-pressure with 100MPa for 30min and 60min, the TBA value of samples increased significantly with processing time after freeze storage when compared to the control. However, if only treated sardine oil with 500MPa, the extent of lipid oxidation reached significantly decreased. Sequeira-Munoz et al., (2006) drew a different conclusion. In their study, TBARS values for HPP treated samples rose significantly ($P < 0.05$) with pressure and pressurization time (15

and 20min) compared with the control and all other pressure conditions (100, 140, 180, and 200MPa), except for samples treated with 100 MPa for 15 min. Similarly, Ohshima et al (1992) pointed out that when cod muscle was processed by high pressure with 202, 404 and 608 MPa for 15 and 30 min, the peroxide values of the extracted oils rose ($P<0.05$) with increasing pressure and pressurization time. Yagiz et al. (2007) further found that Rainbow trout treated at 300, 450, and 600 MPa for 15 min, the extent of lipid oxidation increased as pressure increased beyond 300MPa. The TBA value increased significantly at 450, and 600MPa with values ranging from 6μmolMDA / kg to 70μmol MDA / kg after 6 days of storage.

Most of the studies above demonstrate that HPP can promote lipid oxidation by working as a catalyst for lipid oxidation (Bajovic, Bolumar, & Heinz, 2012; H. Ma et al., 2007; Orlien et al., 2000; Vázquez et al., 2013; Wang et al., 2013). P. Cheah & D. Ledward (1996) confirmed that the minimum critical pressure to initiate catalysis of lipid oxidation was around 300-400MPa. Some research showed that the release of haem molecules through membrane disruption can trigger lipid oxidation (P. Cheah & D. Ledward, 1996; Orlien et al., 2000). The variation in protein structure of metmyoglobin with high-pressure can lead to the pressure increased between the different haem molecules and eventually cause membrane disruption of haem molecules. The main drawback of lipid oxidation by HPP treatment is the loss of volatile flavours of meat. Lipid is one of precursor, which can generate the volatile flavour compounds. Therefore, increased lipid oxidation can bring negative impacts on sensory quality (Mottram, 1998; Toldrá, Flores, & Sanz, 1997).

Table 3. Impact of HPP on the lipid oxidation of seafood.

Types	HPP Parameters	Results	References
Cold-smoked salmon	Processed under 200 and 300 MPa with 15, 30 min at 10°C.	Relatively high pressure of 300 MPa can increase lipid oxidation to a greater extent. Treatment for 30min at 200 MPa resulted in higher TBARS value than 15 min	Lakshmanan et al. (2005)a
Atlantic salmon	Processed at 150 MPa and 300 MPa (15 min) at 25 °C	After 4 and 6 days of frozen storage, 300 MPa samples had significantly lower oxidation compared to control samples.	Yagiz et al. (2009)
Mahi mahi	Pressurized with 150, 300, 450, and 600 MPa for 15 min	300 MPa resulted the highest (significantly) lipid oxidation.	Yagiz et al. (2007)
Rainbow trout	Treated with 150, 300, 450, and 600 MPa for 15 min	The extent of lipid oxidation significantly increased as pressure increased beyond 300MPa.	Yagiz et al. (2007)
Salmon	Treated with 135, 170, and 200MPa for 30 s	170 and 200MPa treatment resulted in significantly higher TBARS values compared to 135MPa treatment.	Aubourg et al. (2010)a
Cod muscle	0, 200MPa, 400MPa, 600MPa and 800MPa	Treatment of samples over 400 MPa significantly decreased the oxidative stability of lipids of cod muscles.	Angsupanichand & Ledward (1998)
Cod muscle	202, 404, and 608MPa for 15 and 30 min	The peroxide value of Cod muscle extracted oils rose significantly with grew pressure and pressurization time.	Ohshima et al (1992)
Cod muscle	200-600MPa for 15-30 min	Increased significantly in lipid oxidation	Campus (2010)
Sardine oil and defatted sardine meat	100MPa to 500MPa, 30-60 min	TBARS value of sardine oil and defatted sardine meat at 100MPa in cold storage increased significantly with processing time compared to control. There was a minimal oxidation extent (significantly), when the sample treated under 500MPa compared to other treatments (100,200,300,and 400MPa)	Campus (2010)
Minced albacore muscle	275, 310MPa for 2, 4, and 6 min	All HPP treatments (except for 275 MPa, 2 min) resulted in significantly lower TBA value	Ramirez-Suarez & Morrissey (2006)

		than control at day 0. Meanwhile, treatment at 310MPa resulted in significantly lower TBARS value than 275MPa, except at day 4 and day 11.	
Vacuum packaged cold-smoked cod	Processed under 400MPa, 500MPa and 600 MPa with 5 and 10 min	No significant differences	Montiel, De Alba, Bravo, Gaya, and Medina (2012)
Raw carp fillets (Cyprinus carpio)	The pressure of 100, 140, 180, 200MPa were applied on samples with 15, 20 min	The values of TBA increased significantly with pressure and pressurization time	Sequeira-Munoz et al., (2006)

2.6.3 Effect of HPP on fatty acid composition

The composition and content of fatty acids in seafood directly affect the sensory evaluation and nutritional value of seafood products (Wood et al., 2008). Seafood is a good contributor of n3 long-chain polyunsaturated fatty acids (n-3 LC-PUFAs) that can help in preventing some diseases. According to Véronique et al. (2008), eel, salmon, swordfish and halibut had a high content of n-3 LC-PUFAs, with average values of 1434, 3625, 3358, 2654 mg n-3 LC-PUFAs/100 g fresh weight respectively. The intake of ω-3 fatty acid can effectively decrease the risk of coronary heart disease. Meanwhile, the concentration of ω-3 fatty acids was very important as well, especially the ratio of ω-6/ω-3. According to Simopoulos (2008), high ratio of ω-6/ω-3 promoted the pathogenesis of many sickness, containing angiocardiopathy, cancer, and inflammatory and autoimmune diseases. The ratio of n-6/n-3 to prevent chronic diseases has been recommended to be less than 4 (Wood et al., 2004).

Table 4 summarizes studies on the impact of HPP on the fatty acid content of seafood. Pazos et al. (2015) reported that Atlantic Mackerel treated at 125, 150, 175, and 200MPa for 0 min can significant decrease total content of fatty acids compared to the control. Additionally, samples treated at 150 MPa, leaded to significantly lower ($P<0.05$) fatty acid content compared to other treatment conditions (125, 175, and 200MPa). Similarly, Kaur et al. (2013) also found that Black Tiger shrimp under HPP treatment with 100, 270, and 435 MPa for 5 min can result in a significant drop in fatty acids content after 25 days

of frozen storage. Moreover, Canto et al. (2015) treated Caiman meat with HPP at 200, 300, and 400MPa at 20°C for 10min, and reported that the content of all n-3 fatty acids decreased significantly at all pressure conditions ($P<0.05$) such as C18:3n3, C20:5n3, C22:5n3, and C22:6n3.

However, Yangzi et al. (2009) reported that when Atlantic salmon was processed by 150 MPa, 15 min at 25 °C, the content of C16:0 significantly increased at 100MPa compared to the control. Similarly, Sequeira-Munoz (2006) also reported that raw carp fillets treated by HPP with four different conditions (100, 140, 180 and 200 MPa) at 4 °C for 15 and 20 min can lead to significantly increased amount of FA with increased pressurization.

Some studies have reported that HPP did not significantly change fatty acid content. For example, Cruz-Romero, Kerry & Kelly (2007) reported that the fatty acids content of oyster was almost same as control, when treated with 260MPa/3 min, 500MPa/5 min, or 800MPa/ 5 min. Similarly, Gomez-Estaca et al., (2007) treated cold-smoked sardine at 300MPa at 20°C for 15min and did not found any significant impact in terms of fatty acid content compared to the control sample. Turbot muscle processed by 100, 140, 180, and 200MPa at 4°C for 30 min also could not find any significant differences in FA content (Chevalier et al., 2008).

Table 4. Effect of HPP on fatty acid content of seafood.

Types	HPP Parameters	Results	References
Oyster	260MPa/3 min, 500MPa/5 min or 800MPa/ 5 min 0 min at 10°C.	no significant differences	Cruz-Romero, Kerry & Kelly (2007)
Atlantic salmon	150 MPa and 300 MPa for 15 min at 25 °C	The main source of total saturated fatty acids was 14:0, 16:0 and 18:0. C16:0 had a Significant increase at 150Mpa	Yagiz et al. (2009)
Cold-smoked sardine	300MPa at 20°C for 15min	300MPa had no significant decreased on fatty acid content compared to control group	Gomez-Estaca et al., (2007)
Turbot muscle	100, 140, 180, and 200MPa at 4°C for 30 min	No significant effect on fatty acid content with all treatment conditions	Chevalier et al. (2008)
Black tiger shrimp	100 270, and 435 MPa, room	After storage at -21°C for 25 days, the content of free fatty	Kaur et al. (2013).

	temperature for 5min	acid significantly decreased at 270 and 475Mpa.	
Mackerel and hemin	600MPa, 10min	No significant effect on fatty acid content	Figueiredo et al. (2015)
Caiman meat	200, 300, and 400MPa at 20°C for 10min	Lower ($P < 0.05$) content of C22:5n3, C22:6n3, C20:5n3, and C18:3n3 than control in Caiman meat. Total Omega-3 content drpped ($P < 0.05$) at 200, 300, and 400MPa compared to control.	Canto et al. (2015)
Raw crap fillets	100, 140, 180 and 200 MPa with 15 and 20 min at 4 °C	100MPa is a great pressure for maintaining the fatty acid, The amount of FA released significantly increased with increased pressurization and longer pressurization time.	Sequeira-Munoz (2006)
Atlanic Mackerel	125, 150, 175, and 200MPa for 0 min	Fatty acid content in Atlanic mackerel significantly decreased compared with control group at all pressure conditions (125, 150, 175 and 200 MPa). Samples treated at 150MPa was significantly lower than 125, 175 and 200 MPa.	Pazos et al. (2015)

Interactions between proteins and free fatty acids mainly due to the hydrophobic forces, van der, hydrogen bonding electrostatic, and Waals. Sequeira-Munoz (2006) reported that HPP could result in a change in these forces that can lead to lipid hydrolysis that accelerates the release of fatty acids. Therefore, the content of fatty acids may increase with increase in pressure. However, Leyton, Drury, & Crawford (1987) demonstrated that compared with SFA, UFA was easier to be oxidized. With pressure increased, the level of oxidation increased as well and resulted in a decrease in fatty acid content. Similarly, Gray (1978) and Shahidi and Zhong (2010) also showed that the PUFA in meat may use the free radical chain mechanism to react with molecular oxygen and form fatty acyl hydroperoxides and other primary products of oxidation. This can decrease content of the PUFA. Pereda, Ferragut, Quevedo, Guamis, and Trujillo (2008) further reported that the decrease in fatty acid content might be due to fatty acid oxidation and acidification. Additionally, Bolumar et al., 2011; Frankel, 2005; McArdle et al., 2010 believed that to some extent, the decrease in total content of fatty acids may result in structural and chemical changes when exposed to high pressure. Specifically, in muscle foods, HPP can

disrupt cell membranes, facilitate and induce the interactions between unsaturated membrane lipids and cellular enzymes. This can probably result in a significant decrease in fatty acid content. Moreover, McArdle et al. (2011) reported that Omega-6 polyunsaturated fatty acids are the resistant of physical-chemical changes which induced by HPP. The change in Omega-6 PUFA commonly can be observed when pressures are above 300MPa.

2.7 Effects of HPP on the physical properties of seafood

2.7.1 Drip loss

According to Kingsley (2007), controlling and maintaining lower drip loss in shucked seafood is very important in terms of quality. From the legal perspective, drip loss should not exceed by 10 % for bivalve mollusks. Drip loss has been reported to significantly increase proportionally with the use of HPP (Ando, 2006). Similarly, Campus (2010) also pointed out that HPP could inhibit the calpain activity and prevent the degradation of cytoskeletal proteins such as desmin, increasing the drip loss (reducing the water-holding capacity). Proteolysis and protein oxidation were the major contributors to drip loss.

2.7.2 Color

According to the study of Gerge (2008), HPP did not affect color of oysters. A small informal panel has noted that an HPP treatment on oysters would not result in significant changes of color compared to the control (Hutrado, 2009). Chéret, (2005) investigated the effect of HPP on sea bass fillet treated at 0, 100, 200, 300, 400, and 500MPa for 5 min, then stored frozen for 0, 7 and 14 days. They found that lightness significantly increased, but resulted only a little change of hue when the pressure increased up to 500MPa

Gómez-Estaca et al (2007) found that treatment of cold-smoked dolphinfish at 300MPa, significantly increased in the value of yellowness (b*) and lightness (L*), while significantly decreased with redness (a*) compared to the control. Similarly, Ramirez-Suarez & Morrissey (2006) reported that minced tuna muscle treated at 275MPa and 310MPa for 2 min, 4 min, and 6 min, significantly increased L*, a* and b* values

compared to the control. Similarly, Atlantic salmon fillets treated with 150, 300, 450, and 600MPa resulted in significantly decreased redness and significantly increased lightness (Yagiz and others, 2005).

2.7.3 Texture

Table 5 summarizes studies on the impact of HPP on the texture of seafood. Kaur, Rao, & Nema (2016) reported that black tiger shrimp treated between 300-600MPa, from 3 - 15 min resulted in significantly increased with hardness, chewiness and springiness compared to control. Yagiz et al. (2009) also showed that Atlantic salmon muscle treated at 150-300MPa can lead to significantly increased springiness and chewiness. Treatment at 300MPa significantly increased hardness. Similarly, Ramirez-Suarez & Morrissey (2006) reported that the minced albacore muscle treated at 275MPa resulted in some positive changes, including significantly increased tenderness, chewiness, juiciness, and springiness. However, at 310 MPa, hardness significantly increased. Treatment of cold smoked salmon at 400 and 900MPa for 10, 20, and 30s, can result in a significant increase in hardness compared to control. Meanwhile, treatment of cold smoked salmon at 900MPa for 10s, 20s, and 30s significantly increased hardness compared to 400MPa treatment (Gudbjornsdottir, Jonsson & Hafsteinsson, 2010).

Table 5. The changes in texture of seafood with HPP treatment.

Product	Conditions	Main effects	Reference
Black tiger shrimp	300-600MPa, 3 min-15 min	Significantly increased hardness, chewiness, and springiness.	Kaur, Rao, &Nema (2016)
Atlantic salmon muscle	150-300MPa	Springiness and chewiness significantly increased	Yagiz et al. (2009)
Minced albacore muscle	275 and 310MPa	Significantly increased tenderness, chewiness, juiciness, and springiness at 275MPa. Hardness significantly increased at 310MPa	Ramirez-Suarez & Morrissey (2006)

Cold smoked salmon	400, 900MPa, 10, 20, 30s	Hardness significantly increased	Gudbjornsdottir, Jonsson & Hafsteinsson, 2010

Chapter 3 Materials and Methods

3.1 Pretreatment of clams

Two types of live clams, Storm Shell (*Mactra murchisoni*) and TuaTua (*Paphies donacina*), were purchased from the Cloudy Bay Clam Limited company located at the northeast of New Zealand's South Island. The samples were sent by air freight. Samples were collected from the airport and transferred to the AUT lab with gel ice packs in plastic foam boxes to keep chilled temperature. Due to the different sizes of clams (Strom shell: 5-6 pieces per kg & Tua Tua: 14-16 pieces per kg (http://cloudybayclams.com/)), five Storm Shell (Figure 4) and ten TuaTua (Figure 5) clams were packed in high-density polyethylene bag (Iconpack, Australia, supplied by Duuningham's Auckland) and vacuum sealed using a vacuum packaging machine (DZ-400/T). Each bag was labelled to indicate different replicates and pressure processing treatments. Vacuum packed samples were stored in polystyrene boxes containing ice blocks and subjected to HPP treatment on the following day.

Figure 4. Storm Shell (Mactra murchisoni) and Tua Tua (Paphies donacina)

3.2 HPP processing

The two species original clams (with shells) were subjected to HPP treatment and used the Multivac HPP 160 (Figure 5) (Multivac company, Germany) which is located at Food Bowl, Auckland, New Zealand. Food bowl is an open access facility operated by NZ Food Innovation Auckland, part of the NZ Food Innovation Network, and created to support the growth of food and beverage businesses. During HPP treatment, water is an essential media which can transfer the pressure to samples. The initial temperature of

water was around 7-8°C, and with pressure increase, the temperature of water slightly increased, but not more than 20°C. The pressures employed were at five different levels: 100, 200, 300, 500, and 600 MPa, respectively, and holding times used were at 5 seconds and 10 min (under majority of the pressure levels, 5s treatment has been able to generate some chemical changes. As for 10min, quite longer pressurization time probably can bring some potential variations than usual). There was a total of three replicates for each pressure treatment group (five Storm Shell clams per replicate and tenTuaTua clams per replicate). Moreover, control samples comprised of five independent replicates for each pressure treatment condition. After depressurization, all samples were transported back to AUT the same day and stored at - 20 °C (Fisher & Paykel) prior to further analysis.

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Figure 5. HPP instrument used.

3.3 Preparation of clams after HPP processing

After HPP treatment, clam shells were removed, and samples placed into high-density polyethylene bag and vacuum packed. Samples were then stored frozen at -20 °C. Frozen clams were freeze dried with a freeze dryer (Christ Alpha 2-4 LD), made by John Morris Scientific Ltd.) for 36 hours (Figure 6). As freeze drying of samples are carried out at quite low temperatures, chemical properties of samples are almost unchanged.

Subsequently, the freeze-dried samples were ground into powder, packed in high density polyethylene bags, and stored at - 20 °C prior to further analysis.

Figure 6. It is the Freeze dryer

3.4 Fatty acid analysis

The method of measurement of total fatty acids was carried out with Juárez et al. (2008). Used acid hydrolysis of lipids of sample to release free fatty acids. Then esterified into fatty acid methyl ester (FAME) and extracted into toluene for GC analysis. Ground freeze dried sample (20 mg) was placed into a clean 4ml brown glass vial and added the internal standard (2g/L tridecanoic acid (10µL)) with a glass syringe. Toluene (490 µL) and freshly prepared 5% methanolic HCl (750 µL) (dilute 5mL concentrated HCL with methanol into 100mL) were added and the mixture vortexed thoroughly. The headspace of the tube was filled with nitrogen then incubated at 70°C in a heating block for 2 hours. After 2 hours, took out the tube and cooled down to room temperature. Then, 6% K_2CO_3 (1 mL) and toluene (500µL) were added and vortexed thoroughly. Subsequently, the mixture was centrifuged (1100 x g for 5 min) and placed liquid into a 1.8 mL brown glass autosampler vial after removed the organic phase (containing a low volume glass insert and cap). 6% aqueous K_2CO_3 was prepared by dissolving 6g anhydrous K_2CO_3 in 100

mL of ultrapure water. 5% methanolic HCl was prepared by adding 5mL concentrated HCl to 95 mL methanol. 2g/L tridecanoic acid (TDA) in toluene was prepared by diluting a 10g/L stock solution (200 µL of stock solution measured using a glass syringe was diluted in 800 µL toluene). The 10 g/L TDA stock solution was prepared by measuring 12 mg TDA powder into a brown glass vial and volume of toluene added in ml to be added was obtained by dividing TDA mass by 10 (1.2 ml). This 2g/L TDA internal standard solution was stored at -20°C.

A serial dilution of the FAME standard（Supelco 47885-U，Sigma Aldrich, Sydney, Australia） in 1.5mL vials was carried out for construction of the calibration curve. The first vial contained undiluted FAME standard, at a concentration of 10g/L. Five other different concentrations of standards were prepared: 5g/L, 2.5g/L, 1.25g/L, 0.625g/L, and 0.3125g/L. The standard curve for each fatty acid was constructed.

For FAME analysis, the Shimadzu GC2010 GLC equipped (Figure 7) was constituted with the Flame Ionisation Detector (FID), split injector and AOC-20i auto-injector. The column (0.25mm x 30m x 0.25µm) which produced by Restek Ltd in USA was used. Nitrogen was considered as a carrier gas with 43 Pa, and the flow rate maintained at 7 ml/min. The initial temperature of the oven of GC was 140 °C, increased to 245 °C at a rate of 5 °C/min, and held for 15 minutes at this temperature.

When the peak area of each fatty acid was obtained, the area of individual fatty acid was divided by the TDA internal standard peak area. Then this value was divided by the concentration of prepared TDA solution (2g/L). This value is the correction factor. Subsequently, each fatty acid peak area from clam sample will be divided by the correction factor. This is the real peak area for each fatty acid. Then using the different standard curves of individual fatty acids, the concentration of fatty acid was calculated. Additionally, as different fatty acids were present in different ratios in the FAME standard, the concentration value was multiplied by the corresponding ratio. The unit of fatty acid was expressed as mg/g.

Figure 7. Shimadzu GC2010 gas chromatograph used in this study equipped with a Flame Ionisation Detector.

3.5 Amino acid analysis

Freeze dried clam samples (0.05g) was weighted into a centrifuge tube with 1 mL of methanol added, and vortexed thoroughly. The mixture was centrifuged at 2000 xg for 2 mins. Subsequently, the EZfaast kit (Phenomenex®, USA) was used to extract amino acids (manual shown in Appendix). The specific steps involved firstly, mixed 50uL sample with 100uL internal standard (IS) (0.2mM Norvaline+10% N-propanol) into a small glass vial, and then used 1.5mL syringe (with absorbable particle) to take in mixture and empty the vail slowly about 1-2min (the amino acid had been absorbed). Then 200uL N-propanol was added into the previous empty glass vial and used 1.5mL syringe (which was used before with absorbable particle) to absorb the N-propanol completely about 1-2min. Thirdly, 200uL eluting medium (sodium hydroxide and N-propanol) was added into that empty glass vial, and used 0.6mLsyringe to push the sorbent into the glass vail; Fourth, 50uL chloroform was added into the glass vial, and vibrated the mixture 5s-8s; Fifth, 100uL iso-octane was added into glass vial, and shocked 5s; Sixth, 100uL 1N hydrochloric acid was added in to the glass vial.

Amino acid (AA) derivatives were extracted and quantified by Shimadzu GC2010 gas chromatography (Figure 8) which is constituted with a Flame Ionization Detector (GC-

FID), a split injector, an AOC-20i auto-injector, and equipped with a ZBAAA GC column (10m x 0.25mm x 0.25um) obtained from Phenomenex in USA. The equipment parameters for free amino acid analysis are as follows: Initial oven temperature was set at 80℃, then the temperature was increased to 320℃ over 9.5min (25℃/min), and held at this temperature for 3 min. Additionally, nitrogen/air was used as carrier gas with a column flow at 1.46mL/min.

As for the standard curve, the ratio of AA peak area to IS peak area was used for construction of the final standard curve (x-axis: concentration; Y-axis: ratio).

Figure 8. GC-FID used for amino acid for amino acid analysis.

3.6 Statistical analysis

The final data was processed by Excel 2016 and statistical analysis was conducted by using XLSAT Software 2010 (Addin soft, USA). Analysis of variance (ANOVA) was performed at a significance level at 0.05 to analyze the effect of HPP treatment (control, 100, 200, 300, 500, and 600 MPa) on fatty acids, and amino acids. When the result of ANOVA was significant (p-value < 0.05), the Fisher's least significant difference test was used to determine the significant differences.

Chapter 4 Result and discussion

4.1 Fatty acid analysis

4.1.1 Effect of different HPP treatments on Storm Shell fatty acids content

Table 6. Fatty acid composition (mg/g) of New Zealand Storm Shell clams after HPP treatments at 100MPa, 200MPa, 300MPa, 500MPa and 600MPa).

Fatty Acid	Pressure (MPa)	Control	Time 5s	Time 600s	F value pressure	F value time	F value pressure*time
UFA	0	23.52±5.18	23.52±5.18^{bc}	23.52±5.18^{b}	4.635**	12.741***	1.820
	100	23.52±5.18^{A}	20.98±1.35^{abA}	20.92±0.53^{bA}			
	200	23.52±5.18^{B}	24.75±0.50^{cB}	20.10±3.48^{abAB}			
	300	23.52±5.18^{B}	19.13±2.02^{aA}	17.00±0.76^{aA}			
	500	23.52±5.18^{B}	21.55±2.20^{abB}	20.10±4.27^{abAB}			
	600	23.52±5.18^{B}	21.55±2.64^{abB}	17.29±2.10^{abA}			
SFA	0	28.30±2.12	28.30±2.12^{b}	28.30±2.12^{b}	19.043****	0.003	13.216****
	100	28.30±2.12^{A}	28.30±2.13^{bA}	34.68±0.66^{cB}			
	200	28.30±2.12^{A}	28.28±0.87^{bA}	26.59±2.53^{abA}			
	300	28.30±2.12^{AB}	28.40±0.80^{bAB}	24.84±0.43^{aA}			
	500	28.30±2.12^{B}	27.76±2.33^{bB}	25.59±2.93^{abA}			
	600	28.30±2.12^{B}	24.65±2.35^{aA}	25.84±1.64^{abA}			
UFA:SFA	0	0.83±0.12	0.83±0.12^{bc}	0.83±0.12^{c}	13.246****	23.258****	4.666**
	100	0.83±0.12^{B}	0.74±0.08^{abAB}	0.60±0.02^{aA}			
	200	0.83±0.12^{AB}	0.88±0.03^{cB}	0.73±0.07^{bcA}			
	300	0.83±0.12^{B}	0.67±0.05^{aA}	0.68±0.03^{bA}			
	500	0.83±0.12^{A}	0.78±0.05^{bA}	0.78±0.08^{cA}			
	600	0.83±0.12^{AB}	0.88±0.11^{cB}	0.75±0.05^{bcA}			

n6	0	0.85±0.00	0.85±0.00^{a}	0.85±0.00^{b}	6.383****	6.029***	4.198**
	100	0.85±0.00^{B}	0.81±0.01^{aA}	0.92±0.02^{bB}			
	200	0.85±0.00^{A}	1.14±0.24^{bB}	0.85±0.15^{bA}			
	300	0.85±0.00^{B}	0.79±0.05^{aB}	0.70±0.09^{aA}			
	500	0.85±0.00^{A}	0.91±0.15^{aA}	0.85±0.09^{bA}			
	600	0.85±0.00^{A}	0.91±0.20^{aA}	0.88±0.08^{bA}			
n3	0	15.92±3.77	15.92±3.77^{c}	15.92±3.77^{b}	7.579****	12.417***	2.337
	100	15.92±3.77^{B}	13.02±1.01^{abA}	13.38±0.30^{bA}			
	200	15.92±3.77^{AB}	16.79±0.70^{cB}	12.89±2.29^{bA}			
	300	15.92±3.77^{B}	12.28±1.92^{aAB}	10.33±0.79^{aA}			
	500	15.92±3.77^{B}	15.21±1.92^{bcB}	13.93±2.86^{bA}			
	600	15.92±3.77^{B}	14.58±2.17^{abcAB}	13.44±1.06^{bA}			
n6:n3	0	0.06±0.01	0.06±0.01^{aA}	0.06±0.01^{aA}	0.863	0.807	0.251
	100	0.06±0.01^{A}	0.06±0.00^{aA}	0.07±0.00^{aA}			
	200	0.06±0.01^{A}	0.07±0.00^{aA}	0.07±0.02^{aA}			
	300	0.06±0.01^{A}	0.07±0.01^{aA}	0.07±0.01^{aA}			
	500	0.06±0.01^{A}	0.06±0.01^{aA}	0.06±0.01^{aA}			
	600	0.06±0.01^{A}	0.06±0.01^{aA}	0.07±0.01^{aA}			

Note: Values are means ± standard deviation. The letters with a, b, c, and d in the same column represent significant differences at $p < 0.05$ separated by Fisher ANOVA test about “Pressure”. The letters with A and B in the same column means the significant differences at $p < 0.05$ separated by Fisher ANOVA test about “Holding time”. $P < 0.0001$, $P < 0.001$, $P < 0.01$ and $P < 0.05$ were presented as “****”, “***”, “**” and “*” respectively for level of significance and nothing means no statistically significant.

Table 7. Fatty acid composition (mg/g) of New Zealand Storm Shell clam after HPP treatments at 100MPa, 200MPa, 300MPa, 500MPa and 600MPa).

Fatty Acid	pressure	Control	Time 5s	Time 600s	F value pressure	F value time	F value pressure*time
Capric (C6:0)	0	2.9±0.65	2.9±0.65^{cd}	2.9±0.65^{bc}	12.051****	2.173	2.917*
	100	2.9±0.65^{A}	3.11±0.20^{dA}	3.20±0.30^{cA}			
	200	2.9±0.65^{B}	2.29±0.19^{aA}	2.33±0.34^{aAB}			
	300	2.9±0.65^{B}	2.85±0.05^{bcdB}	2.52±0.5^{abA}			
	500	2.9±0.65^{A}	2.55±0.20^{abA}	2.93±0.53^{bcA}			
	600	2.9±0.65^{A}	2.61±0.33^{abcA}	3.01±0.36^{cA}			
Myristic (C14:0)	0	2.6±1.0	2.6±1.0^{bc}	2.6±1.0^{c}	6.882****	1.488	0.553
	100	2.6±1.0^{A}	2.56±0.17^{bcA}	2.57±0.14^{bcA}			
	200	2.6±1.0^{A}	2.65±0.07^{cA}	2.48±0.77^{bcA}			
	300	2.6±1.0^{A}	2.48±0.14^{cdA}	2.55±0.19^{bcA}			
	500	2.6±1.0^{A}	2.06±0.55^{bA}	1.88±0.61^{abA}			
	600	2.6±1.0^{A}	2.22±0.27^{aA}	1.86±0.35^{aA}			
Palmitic (C16:0)	0	8.05±1.75	8.05±1.75^{ab}	8.05±1.75^{b}	44.396****	0.359	3.845**
	100	8.05±1.75^{A}	9.58±1.85^{dB}	10.97±0.16^{cC}			
	200	8.05±1.75 ^{A}	8.55±0.55^{bA}	7.75±1.21^{bA}			
	300	8.05±1.75^{A}	8.69±0.2^{bA}	8.47±0.50^{bA}			
	500	8.05±1.75^{C}	7.38±1.25^{abB}	6.45±1.18^{aA}			
	600	8.05±1.75^{A}	6.26±1.65^{aA}	6.20±0.82^{aA}			
Stearic (C18:0)	0	9.75±0.05	9.75±0.5^{abc}	9.75±0.05^{b}	8.030****	1.460	23.442****
	100	9.75±0.05^{A}	8.64±1.51^{aA}	12.6±0.9^{cB}			
	200	9.75±0.05 ^{A}	9.85±0.6^{bcA}	9.18±0.65^{bA}			
	300	9.75±0.05^{B}	9.74±0.61^{abcB}	7.92±0.35^{aA}			
	500	9.75±0.05^{A}	10.53±1.91^{cB}	9.48±0.75^{bA}			
	600	9.75±0.05^{AB}	9.03±0.63^{abA}	9.87±0.63^{bB}			
	0	1.33±0.25	1.33±0.25^{a}	1.33±0.25^{b}			
	100	1.33±0.25^{AB}	1.25±0.22^{aA}	1.56±0.07^{cB}			

Tricosanoic (C23:0)	200	1.33±0.25^{A}	1.25±0.17^{aA}	1.19±0.23^{bA}	5.448***	0.622	7.233****
	300	1.33±0.25^{B}	1.31±0.17^{aB}	0.94±0.1^{aA}			
	500	1.33±0.25^{A}	1.34±0.15^{aA}	1.25±0.05^{bA}			
	600	1.33±0.25^{A}	1.25±0.18^{aA}	1.30±0.09^{bA}			
Lignoceric (C24:0)	0	3.7±0.2	3.7±0.2^{ab}	3.7±0.2^{b}	12.395****	0.511	8.656****
	100	3.7±0.2^{AB}	3.15±0.55^{aA}	3.74±0.05^{bB}			
	200	3.7±0.2^{A}	3.70±0.15^{abA}	3.66±0.2^{bA}			
	300	3.7±0.2^{B}	3.35±0.35^{abB}	2.45±0.2^{aA}			
	500	3.7±0.2^{A}	3.91±0.55^{abA}	3.62±0.18^{bA}			
	600	3.7±0.2^{A}	3.29±0.49^{aA}	3.61±0.20^{bA}			
Palmitoleic (C16:1)	0	3.35±1.65	3.35±1.65^{b}	3.35±1.65^{b}	3.833**	5.684*	1.165
	100	3.35±1.65^{AB}	3.66±0.28^{bB}	2.99±0.25^{abA}			
	200	3.35±1.65 ^{A}	3.67±0.11^{bA}	2.85±1.3^{abA}			
	300	3.35±1.65^{A}	3.09±0.2^{bA}	3.35±0.25^{bA}			
	500	3.35±1.65^{A}	2.59±0.9^{aA}	2.32±1.15^{aA}			
	600	3.35±1.65^{B}	2.97±0.65^{bB}	2.23±0.65^{aA}			
Oleic/Elaidic (C18:1n9)	0	0.53±0.05	0.53±0.05^{ab}	0.53±0.05^{b}	18.285****	3.757	5.159**
	100	0.53±0.05^{A}	0.57±0.03^{bA}	0.66±0.08^{cB}			
	200	0.53±0.05 ^{A}	0.58±0.03^{bA}	0.55±0.10^{bA}			
	300	0.53±0.05^{AB}	0.57±0.05^{bB}	0.48±0.00^{abA}			
	500	0.53±0.05^{A}	0.49±0.05^{aA}	0.45±0.10^{abA}			
	600	0.53±0.05^{B}	0.45±0.10^{aAB}	0.42±0.03^{aA}			
cis-11-Eicosenoic (C20:1n9)	0	0.95±0.05	0.95±0.05^{abc}	0.95±0.05^{a}	18.817****	5.271*	3.744*
	100	0.95±0.05^{A}	1.12±0.09^{cB}	1.29±0.08^{bC}			
	200	0.95±0.05^{AB}	0.78±0.14^{aA}	0.99±0.04^{aB}			
	300	0.95±0.05^{A}	0.87±0.10^{abA}	0.90±0.05^{aA}			
	500	0.95±0.05^{A}	0.98±0.08^{bcA}	0.90±0.15^{aA}			

	600	0.95±0.05^{A}	0.90±0.16^{abA}	0.90±0.01^{aA}			
	0	0.85±0.00	0.85±0.00^{a}	0.85±0.00^{b}			
	100	0.85±0.00^{AB}	0.81±0.01^{aA}	0.92±0.02^{bB}			
cis-8,11,14-	200	0.85±0.00^{A}	1.14±0.24^{bB}	0.85±0.15^{bA}	6.383****	6.029***	4.198**
Eicosatrienoic	300	0.85±0.00^{B}	0.79±0.05^{aB}	0.70±0.09^{aA}			
(C20:3n6)	500	0.85±0.00^{A}	0.91±0.15^{aA}	0.85±0.09^{bA}			
	600	0.85±0.00^{A}	0.91±0.20^{aA}	0.88±0.08^{bA}			
	0	11.35±3.55	11.35±3.55^{b}	11.35±3.55^{c}			
	100	11.35±3.55^{A}	9.95±0.95^{abA}	10.29±0.35^{bcA}			
cis-5,8,11,14,17-	200	11.35±3.55^{AB}	11.68±0.55^{bB}	8.45±1.85^{abA}	5.711***	9.703**	2.368
Eicosapentaenoic	300	11.35±3.55^{B}	8.25±1.25^{aA}	6.9±0.6^{aA}			
(C20:5n3)	500	11.35±3.55^{A}	9.93±1.45^{abA}	9.5±2.7^{bcA}			
	600	11.35±3.55^{B}	10.25±2.1^{abB}	8.8±0.85^{abA}			
	0	1.78±0.25	1.78±0.25^{b}	1.78±0.25^{b}			
	100	1.78±0.25^{A}	1.81±0.50^{bA}	1.67±0.47^{bA}			
Erucic (C22:1n9)	200	1.78±0.25^{A}	1.78±0.18^{bA}	1.50±0.60^{aA}	1.760	1.473	1.242
	300	1.78±0.25^{A}	1.45±0.35^{aA}	1.25±0.31^{aA}			
	500	1.78±0.25^{A}	1.37±0.36^{aA}	1.67±0.18^{abA}			
	600	1.78±0.25^{B}	1.73±0.14^{abB}	1.43±0.50^{aA}			
	0	4.55±0.25	4.55±0.25^{bc}	4.55±0.25^{b}			
	100	4.55±0.25^{B}	3.09±0.24^{aA}	3.09±0.35^{aA}			
cis-4,7,10,13,16,19-	200	4.55±0.25^{AB}	5.11±0.17^{cB}	4.45±0.55^{bA}	35.424****	11.270***	4.016**
Docosahexaenoic	300	4.55±0.25^{B}	4.15±0.70^{bB}	3.45±0.25^{aA}			
(C22:6n3)	500	4.55±0.25^{AB}	5.28±0.63^{cB}	4.4±0.45^{bA}			
	600	4.55±0.25^{A}	4.33±0.39^{bA}	4.61±0.27^{bA}			

Note: Values are means ± standard deviation. The letters with a, b, c and d in the same column represent significant differences at $p < 0.05$ separated by Fisher ANOVA test for "Pressure". The letters with A and B in the same row indicate significant differences at $p < 0.05$ separated by Fisher ANOVA test for "Holding time". $P < 0.0001$, $P < 0.001$, $P < 0.01$ and $P < 0.05$ were presented as "****", "***", "**" and "*" respectively for level of significance.

As seen in Table 7, there were 13 fatty acids (6 SFAs and 7 UFAs) identified in New Zealand storm shell clams treated by HPP at 100, 200, 300, 500, and 600 MPa for 5s and 600s. The major saturated fatty acids were C16:0 and C18:0, and the main UFAs were C20:5n3 and C22:6n3. This finding was similar to Yagiz et al. (2009); Kaur et al. (2013); and Canto et al. (2015) in terms of Atlantic salmon, Black Tiger shrimp, and Caiman meat respectively. The changes in fatty acid content with different HPP treatment conditions varying in pressure and holding time will be discussed below.

4.1.1.1 Effect of UFA

Effect of pressure at 5s holding time

The 5s pressure treated sample almost did not have any significant changes in UFA compared to control except at 300MPa treatment (Table 6). The total UFA content under 300MPa significantly decreased when compared with the control ($P<0.05$). Kaur et al. (2013) reported that HPP treated Black Tiger shrimp at 270MPa, 5 min had a significant decrease in UFA after storage for 25 days. Specifically, in the current study (Table 7), the content of C20:5n3 and C22:1n9 significantly decreased at 300MPa compared to the control. Shahidi and Zhong (2010) explained that high pressure could accelerate oxidation with molecular oxygen forming fatty acyl hydroperoxides and other primary products of oxidation that can result in the decrease in UFA.

Effect of pressure at 600s holding time

Holding the pressure for 600s only resulted in a significant decrease in UFA content at 300MPa (Table 6). Specifically (Table 7), the content of C20:3n6, C20:5n3, C22;1n9, and C22:6n3 significantly decreased ($P<0.05$) at 300MPa compared to the control. Similarly, Canto et al. (2015 reported that processed Caiman meat samples with 300 MPa (10min) at 20°C for also leaded to a significant decrease in C20:5n3. C22:5n3, and C22:6n3 compared to control. Leyton, Drury, & Crawford (1987) explained that longer pressurization time could lead to increased level of oxidation, and a decrease in fatty acids.

Effect of holding time

As seen in Table 6, there was only a significant change in fatty acids content at 600MPa due to increase of pressure holding time from 5s to 600s. Specifically (Table 7), the

content of C16:1, C20:5n3, and C22:1n9 significantly decreased ($P<0.05$) when held for 600s. No studies have reported this. Cruz-Romero, Kerry & Kelly (2007) reported that HPP treatment of oyster at 800 MPa, at 20°C, for 5 and 0min, resulted in no significant changes of content between two different holding times.

4.1.1.2 Effect of SFA

Effect of pressure at 5s holding time

The 5s pressure treated samples were mostly not significant for SFAs compared to the control (Table 6). Similarly, Canto et al. (2015) found that no significant difference ($P > 0.05$) in total SFA contents can be observed at 200, 300, and 400 MPa treatments. In the current study, when holding the pressure at 5s, there was a significant decrease in SFA at 600 MPa compared to the control. In Table 7, C14:0 significantly decreased at 600 MPa for 5s when compared to 0-500MPa. Gray (1978) and Shahidi and Zhong (2010) explained that under high pressure, SFA can easily use the free radical chain mechanism to react with molecular oxygen and form fatty acyl hydroperoxides and other primary products of oxidation.

Effect of pressure at 600s holding time

When the samples were pressure treated for 600s, the significant decrease ($P<0.05$) in total content of SFAs occurred at 300 MPa compared to the control (Table 6). As seen in Table 7, C18:0, C23:0, and C24:0 fatty acids significantly decreased at 300 MPa. Similarly, Yagiz et al. (2009) reported a significant decrease in C18:0 content when Atlantic salmon was treated at 300 MPa for, 15 min at 25°C. After storage for 2 days at 4°C, the content of C18:0 significantly decreased compared to the control and 150 MPa treated samples. Leyton, Drury, & Crawford (1987) explained that longer pressurization time could lead to increased level of oxidation, and a decrease in fatty acids.

In our current study, some significant increases ($P<0.05$) can be found as well, specifically in the content of C16:0, C18:0 and C23:0 fatty acids (Table 3) with 100MPa treatment for 600s. Similarly, Yagiz et al. (2009) reported that HPP treatment of Atlantic salmon at 150 MPa for 15min at 25°C resulted in significantly increased content of C16:0 and C18:0 compared to 300 MPa and control treatments, after storage for 6 days at 4°C. Sequeira-Munoz (2006) also found that treatment of raw carp fillets at 100 MPa at 4 °C, for 15 or 20min caused a significant increase of content of SFA compared to the control.

Effect of holding time

As seen in Table 7, there were more significant changes in fatty acids content due to pressure holding time. The total content of SFAs significantly changed at 100, 300, and 500 MPa. Specifically, treating samples at 100 MPa resulted in a significant increase at 600s compared to 5s and control samples. Similarly, Sequeira-Munoz (2006) found that treating Raw carp fillets with 100 MPa (15 and 20 min) at 4 °C can lead to higher content of SFAs with longer pressurization time. For example, the content of C16:0, C18:0 significantly increased ($P<0.05$) at 100 MPa for 600s compared to 5s and control samples. The content of C23:0 and C24:0 significantly increased at 500 MPa for 600s only compared to the 5s treatment group. Sequeira-Munoz (2006) further explained that longer pressurization time could lead to the lipid hydrolysis, accelerating the release of free amino acids.

In the current study, a significant decrease was evident at 300 and 500MPa, 600s compared to 5s and control samples. As shown in Table 7, it can be seen that the content of C6:0, C18:0, C23:0, and C24:0 had a fatty acid significantly decreased ($P<0.05$) at 300 MPa treatment for 600s compared to 5s and control samples. Besides, C14:0, C16:0 at 500MPa-600s had lower values ($P<0.05$) than 5s and control samples. Noticeably, C18:0 significantly decreased at 500 MPa for 600s compared to 5s. This result was quite different that reported by Cruz-Romero, Kerry & Kelly (2007) who reported that pressure and holding time would not bring any significant variations in terms of the SFAs content.

4.1.2 Effect of different HPP treatments on Tua Tua fatty acids content

Table 8. Fatty acid composition (mg/g) of New Zealand Tua Tua clams after HPP treatments at 100MPa, 200MPa, 300MPa, 500MPa and 600MPa.

Fatty Acid	Pressure	Control	Time 5s	Time 600s	F value pressure	F value time	F value pressure*time
UFA	0	18.51±0.85	18.51±0.85^{ab}	18.51±0.85^{a}	25.068****	13.070***	1.593
	100	18.51±0.85^{A}	20.27±0.93^{bAB}	22.26±0.44^{cB}			
	200	18.51±0.85^{A}	21.78±1.06^{cB}	23.20±0.58^{cB}			
	300	18.51±0.85^{A}	19.22±2.23^{abA}	20.63±1.26^{bA}			
	500	18.51±0.85^{A}	18.91±0.96^{abA}	19.32±0.46^{aA}			
	600	18.51±0.85^{A}	18.50±0.55^{aA}	18.50±1.47^{aA}			
SFA	0	28.74±1.56	28.74±1.56^{cd}	28.74±1.56^{b}	39.728****	1.957	3.690**
	100	28.74±1.56^{A}	30.59±0.93^{dAB}	32.46±0.98^{cB}			
	200	28.74±1.56^{A}	30.04±1.27^{dAB}	32.57±3.84^{cB}			
	300	28.74±1.56^{A}	27.46±3.39^{bcA}	29.85±1.34^{bA}			
	500	28.74±1.56^{B}	24.25±1.30^{aA}	23.76±1.32^{aA}			
	600	28.74±1.56^{B}	25.53±1.51^{abA}	23.05±1.71^{aA}			
UFA:SFA	0	0.65±0.06	0.65±0.06^{a}	0.65±0.06^{a}	17.533****	5.542*	2.291
	100	0.65±0.06^{A}	0.66±0.02^{aA}	0.69±0.02^{abA}			
	200	0.65±0.06^{A}	0.73±0.05^{bcA}	0.72±0.07^{bA}			
	300	0.65±0.06^{A}	0.70±0.03^{abA}	0.69±0.02^{abA}			
	500	0.65±0.06^{A}	0.77±0.04^{cAB}	0.82±0.05^{cB}			
	600	0.65±0.06^{A}	0.73±0.05^{bcAB}	0.80±0.02^{cB}			
n6	0	1.33±0.46	1.33±0.46^{c}	1.33±0.46^{c}	5.368***	0.521	2.104
	100	1.33±0.46^{B}	1.02±0.17^{bAB}	0.940±0.02^{abA}			
	200	1.33±0.46^{B}	0.97±0.01^{abA}	1.270±0.08^{bAB}			
	300	1.33±0.46^{B}	0.82±0.11^{aA}	0.942±0.09^{aA}			
	500	1.33±0.46^{B}	0.82±0.08^{aA}	0.958±0.07^{aA}			
	600	1.33±0.46^{B}	0.91±0.07^{abA}	0.817±0.15^{aA}			

	0	13.68±0.18	13.68±0.18^{a}	13.68±0.18^{a}			
	100	13.68±0.18^{A}	14.77±0.75^{aAB}	16.78±0.40^{cB}			
	200	13.68±0.18^{A}	17.12±1.02^{bB}	17.95±0.27^{dB}	22.219****	19.564****	2.576*
n3	300	13.68±0.18^{A}	15.05±0.25^{aAB}	16.58±0.78^{cB}			
	500	13.68±0.18^{A}	14..62±0.74^{aAB}	15.05±0.38^{bB}			
	600	13.68±0.18^{A}	14.53±0.13^{aA}	14.61±1.31^{abA}			
	0	0.10±0.03	0.10±0.03^{c}	0.10±0.03^{b}			
	100	0.10±0.03^{B}	0.07±0.01^{bA}	0.06±0.00^{aA}			
	200	0.10±0.03^{B}	0.06±0.01^{aA}	0.06±0.0^{aA}	1.998	1.513	3.060*
n6:n3	300	0.10±0.03^{C}	0.05±0.00^{aA}	0.05±0.00^{aA}			
	500	0.10±0.03^{B}	0.06±0.00^{aA}	0.06±0.00^{aA}			
	600	0.10±0.03^{B}	0.06±0.01^{abA}	0.06±0.00^{aA}			

Note: Values are means ± standard deviation. The letters a, b, c and d in the same column represent significant differences at $p < 0.05$ separated by Fisher ANOVA test about "Pressure". The letters A and B in the same column indicate significant differences at $p < 0.05$ separated by Fisher ANOVA test about "Holding time". $P < 0.0001$ presented as ∗∗∗∗for level of significance; $P < 0.0001$, $P < 0.001$, $P < 0.01$ and $P < 0.05$ were presented as "****", "***", "**" and "*" respectively for level of significance and nothing means no statistically significant.

Table 9. Fatty acid composition (mg/g) of New Zealand Tua Tua clams after HPP treatments at 100MPa, 200MPa, 300MPa, 500MPa and 600MPa.

Fatty Acid	Pressure	Control	Time 5s	Time 600s	F value pressure	F value time	F value pressure*time
Capric (C6:0)	0	4.45±0.57	4.45±0.57^{bc}	4.45±0.57^{d}	50.992****	1.825	3.155*
	100	4.45±0.57^{A}	4.51±0.45^{cA}	3.95±0.59^{cdA}			
	200	4.45±0.57^{B}	3.78±0.59^{bAB}	3.52±0.39^{bcA}			
	300	4.45±0.57^{B}	3.79±0.61^{bAB}	3.31±0.36^{bA}			
	500	4.45±0.57^{C}	1.93±0.15^{aA}	2.44±0.04^{aB}			
	600	4.45±0.57^{B}	2.38±0.35^{aA}	2.43±0.24^{aA}			
Myristic (C14:0)	0	1.30±0.07	1.30±0.07^{ab}	1.30±0.07^{bc}	18.454****	0.106	5.465***
	100	1.30±0.07^{A}	1.41±0.05^{bcA}	1.58±0.10^{dB}			
	200	1.30±0.07^{A}	1.51±0.13^{cAB}	1.55±0.16^{dB}			
	300	1.30±0.07^{A}	1.23±0.27^{abA}	1.43±0.03s^{cdA}			
	500	1.30±0.07^{A}	1.19±0.15^{aA}	1.12±0.18^{abA}			
	600	1.30±0.07^{B}	1.30±0.03^{abB}	1.03±0.16^{aA}			
Palmitic (C16:0)	0	8.30±0.07	8.30±0.07^{b}	8.30±0.07^{b}	51.829****	0.266	9.819****
	100	8.30±0.07^{A}	8.07±0.16^{bB}	10.99±0.30^{cC}			
	200	8.30±0.07^{A}	8.13±0.12^{bA}	10.44±0.95^{cB}			
	300	8.30±0.07^{A}	7.13±1.39^{aA}	9.27±0.39^{bA}			
	500	8.30±0.07^{A}	7.08±0.87^{aA}	6.90±0.79^{aA}			
	600	8.30±0.07^{B}	7.43±0.59^{aB}	6.26±0.70^{aA}			
Stearic (C18:0)	0	8.38±0.74	8.38±0.74^{b}	8.38±0.74^{cd}	13.138****	0.004	1.445
	100	8.38±0.74^{A}	8.07±0.64^{bA}	8.01±0.22^{cA}			
	200	8.38±0.74^{A}	8.13±0.68^{bA}	8.49±0.40^{dA}			
	300	8.38±0.74^{B}	7.13±0.78^{aA}	7.54±0.27^{bAB}			
	500	8.38±0.74^{B}	7.08±0.71^{aA}	6.89±0.16^{aA}			
	600	8.38±0.74^{B}	7.43±0.58^{abAB}	6.95±0.55^{aA}			
	0	0.83±0.25	0.83±0.25^{a}	0.83±0.25^{a}			
	100	0.83±0.25^{A}	0.88±0.02^{aA}	0.93±0.02^{aA}			

Tricosanoic (C23:0)	200	0.83±0.25^{A}	0.85±0.08^{aA}	0.98±0.02^{aA}	1.059	1.216	1.033
	300	0.83±0.25^{A}	0.82±0.11^{aA}	0.81±0.14^{aA}			
	500	0.83±0.25^{A}	0.85±0.13^{aA}	0.82±0.14^{aA}			
	600	0.83±0.25^{A}	0.83±0.04^{aA}	0.91±0.13^{aA}			
Lignoceric (C24:0)	0	5.49±0.21	5.49±0.21^{a}	5.49±0.21^{a}	24.819****	20.442****	12.986****
	100	5.49±0.21^{A}	5.56±0.72^{abA}	7.01±0.11^{cB}			
	200	5.49±0.21^{A}	6.56±0.27^{bB}	6.33±0.29^{bB}			
	300	5.49±0.21^{A}	6.19±0.52^{bcA}	7.48±0.45^{dB}			
	500	5.49±0.21^{A}	5.50±0.29^{aA}	5.59±0.32^{aA}			
	600	5.49±0.21^{A}	5.72±0.25^{abA}	5.48±0.28^{aA}			
Palmitoleic (C16:1)	0	1.58±0.11	1.58±0.11^{ab}	1.58±0.11^{ab}	10.091****	1.557	2.336
	100	1.58±0.11^{A}	1.62±0.07^{abA}	1.82±0.10^{bB}			
	200	1.58±0.11^{A}	1.82±0.08^{bB}	1.79±0.25^{bAB}			
	300	1.58±0.11^{A}	1.45±0.35^{aA}	1.44±0.29^{aA}			
	500	1.58±0.11^{A}	1.46±0.19^{aA}	1.26±0.32^{aA}			
	600	1.58±0.11^{B}	1.53±0.24^{aB}	1.21±0.19^{aA}			
Oleic/Elaidic (C18:1n9)	0	0.55±0.07	0.55±0.07^{bcd}	0.55±0.07^{ab}	22.994****	3.086*	10.664****
	100	0.55±0.07^{A}	0.60±0.07^{cdA}	0.72±0.04^{cB}			
	200	0.55±0.07^{A}	0.62±0.02^{dA}	0.67±0.10^{cA}			
	300	0.55±0.01^{AB}	0.46±0.08^{aA}	0.61±0.05^{bB}			
	500	0.55±0.07^{A}	0.55±0.06^{bcdA}	0.46±0.07^{aA}			
	600	0.55±0.07^{B}	0.53±0.04^{bB}	0.44±0.05^{aA}			
cis-11-Eicosenoic (C20:1n9)	0	1.40±0.28	1.40±0.28^{ab}	1.40±0.28^{ab}	12.579****	0.992	1.187
	100	1.40±0.28^{A}	1.67±0.07^{cB}	1.67±0.12^{cB}			
	200	1.40±0.28^{A}	1.55±0.11^{bcAB}	1.65±0.03^{bcB}			
	300	1.40±0.28^{A}	1.44±0.20^{abA}	1.33±0.18^{aA}			

	500	1.40±0.28^{A}	1.33±0.13^{aA}	1.26±0.14^{aA}			
	600	1.40±0.28^{A}	1.45±0.08^{abA}	1.34±0.18^{aA}			
	0	1.33±0.46	1.33±0.46^{c}	1.33±0.46^{c}			
	100	1.33±0.46^{B}	1.02±0.17^{bAB}	0.940±0.02^{abA}			
cis-8,11,14-	200	1.33±0.46^{B}	0.97±0.01^{abA}	1.270±0.08^{bAB}	5.368***	0.521	2.104
Eicosatrienoic	300	1.33±0.46^{B}	0.82±0.11^{aA}	0.942±0.09^{aA}			
(C20:3n6)	500	1.33±0.46^{B}	0.82±0.08^{aA}	0.958±0.07^{aA}			
	600	1.33±0.46^{B}	0.91±0.07^{abA}	0.817±0.15^{aA}			
	0	4.60±0.14	4.60±0.14^{a}	4.60±0.14^{a}			
	100	4.60±0.14^{A}	4.81±0.40^{aA}	5.72±0.18^{cB}			
cis-5,8,11,14,17-	200	4.60±0.14^{A}	5.23±0.06^{abB}	5.66±0.33^{cC}	4.997**	18.623****	4.162**
Eicosapentaenoic	300	4.60±0.14^{A}	4.88±0.60^{abA}	5.40±0.37^{bcA}			
(C20:5n3)	500	4.60±0.14^{A}	4.92±0.19^{abB}	4.87±0.12^{aAB}			
	600	4.60±0.14^{A}	5.00±0.11^{abA}	5.03±0.50^{abA}			
	0	1.55±0.14	1.55±0.14^{b}	1.55±0.14^{ab}			
	100	1.55±0.14^{A}	2.21±0.07^{cB}	2.15±0.31^{cB}			
Erucic (C22:1n9)	200	1.55±0.14^{A}	1.52±0.29^{bA}	1.84±0.29^{bcA}	20.752****	0.584	1.720
	300	1.55±0.14^{A}	1.45±0.32^{abA}	1.23±0.43^{aA}			
	500	1.55±0.14^{A}	1.60±0.16^{bA}	1.64±0.25^{bA}			
	600	1.55±0.14^{A}	1.08±0.44^{aA}	1.29±0.32^{abA}			
	0	9.08±0.32	9.08±0.32^{a}	9.08±0.32^{a}			
	100	9.08±0.32^{A}	9.96±0.44^{aB}	11.06±0.25^{cC}			
cis-4,7,10,13,16,19-	200	9.08±0.32^{A}	11.90±1.02^{bB}	12.28±0.17^{dB}	28.217****	13.817***	1.468
Docosahexaenoic	300	9.08±0.32^{A}	10.17±1.02^{bAB}	11.18±0.59^{cB}			
(C22:6n3)	500	9.08±0.32^{A}	9.70±0.57^{aAB}	10.18±0.45^{bB}			
	600	9.08±0.32^{A}	9.53±0.13^{aA}	9.58±0.88^{abA}			

Note: Values are means ± standard deviation. The letters a, b, c and d in the same column represent significant differences at $p < 0.05$ separated by Fisher ANOVA test about “Pressure”. The letters A, B and C in the same column indicate significant differences at $p < 0.05$ separated by Fisher ANOVA test about “Holding time”.

There were 13 fatty acids (6 SFA and 7 UFA) found in Tua Tua clams. This included C6:0, C14:0, C16:0, C18:0, C23:0, C24:0, C16:1, C18:1n9, C20:1n9, C20:3n6, C20:5n3, C22:1n9 and C22:6n3. The C16:0 and C18:0 were the major SFA, and C20:5n3 and C22:6n3 were the main UFA. This finding was similar with Yagiz et al. (2009); Kaur et al. (2013); and Canto et al. (2015). In terms of Atlantic salmon, Black tiger shrimp, and Caiman meat respectively. In this section, changes in fatty acids content of Tua Tua clams treated at five different pressure conditions and two different holding times will be described.

4.1.2.1 Effect of UFA

Effect of pressure at 5s

The 5s treated samples showed almost no significant changes in UFAs for all pressures except at 200 MPa (Table 8) compared to the control. Sequeira-Munoz (2006) showed that treated raw crap fillets at 200MPa, 4 °C for 15 and 20 min could generate significant decrease of content when compared to the control. In Table 9, it can be seen that only the content of C22:6n3 significantly increased ($P<0.05$) at 200 MPa.

Effect of pressure at 600s

At 600s treatment, there were more significant increase in fatty acids at 100, 200, 300MPa (Table 8). Similar results were only shown by Sequeira-Munoz (2006). The raw crap fillets were treated with 100, 140, 180, and 200 MPa for 15 and 20 min at 4°C had a significant increase in total content compared to the control. In the current study, as for individual UFA, the content of C18:1n9, C22:1n9, C20:5n3, and C22:6n3 had higher ($P<0.05$) values at 100MPa when compared to the control. C18:1n9, C20:5n3, and C22:6n3 showed a significant increase at 200MPa compared to the control. As for significant increase at 300MPa, C20:5n3 and C22:6n3 also showed changes.

Effect of holding time

In terms of effects of holding time, there were no significant changes between 5s and 600s treatments at the same pressure. Similarly, Cruz-Romero, Kerry & Kelly (2007). reported that HPP treatment of oyster at 260 MPa/3 min, 500 MPa/5 min and 800 MPa/ 5 min, at 10°C resulted in no significant differences in FA content. As shown in Table 9, although there was

no significant difference in total content, there were few significant changes at some pressure conditions. Taking the most obvious change as an example, the content of C16:1, C18:1n9, C20:5n3, and C22:6:3 increased significantly ($P<0.05$) with 600s treatment when compared to 5s treatment. The content of C18:1n9 and C20:5n3 also increased significantly ($P<0.05$) with 600s treatment under 200 MPa and 300 MPa respectively. However, C16:1 and C18:1n9 both presented a significant drop ($P<0.05$) at 600MPa.

In general, relative low pressures at 600s resulted in higher content of SFAs, while relative high-pressure at 600s caused lower content. It could be that high pressures applied can damage and change the physical and chemical properties, and longer pressurization times could further intensify these changes as reported by Canto et al. (2015).

4.1.2.2 Effect of SFA

Effect of pressure at 5s

The total content of SFAs significantly decreased ($P<0.05$) at 500 and 600MPa. When held at 5s Specifically, the content of C6:0, C16:0 and C18:0 significantly decreased at 500MPa, and C6:0 and C16:0 significantly decreased at 600MPa. Previous studies reported that 500, 600, 800 MPa with 3min, 5min, and 10 min presented no significant variation in SFAs content in oyster, Mackerel and hemin, but none of them explored the underlying reasons for the changes (Figueiredo et al. 2015; Cruz-Romero, Kerry & Kelly, 2007). Bolumar et al. (2011), Frankel (2005) and McArdle et al. (2010) believed that to some extent, the decrease in the content of SFAs may have resulted from structural and chemical changes of protein under high pressure.

Effect of pressure at 600s

When the samples were treated for 600s, the total SFA content significantly increased at 100 and 200 MPa and decreased at 500 and 600 MPa. Specifically, C14:0, C16:0. and C24:0 significantly increased at 100 and 200 MPa. Sequeira-Munoz (2006) found that treatment of raw carp fillets under 100 and 200 MPa for 15 and 20 min at 4°C leaded to the significant increase of total content of SFAs compared to control. However, with the higher pressure upon 500MPa and 600 MPa, there were significant decreases in total content of SFAs.

Specifically, in the current study, the content of C6:0, C14:0, C16:0, C18:0, and C24:0 (Table 9) decreased significantly at 500 and 600MPa when held for 600s compared to 5s.

Effect of holding time

As for the effect of holding time (F value with 1.957) it can be found (as shown in Table 8). that there were no significant changes between 5s and 600s treatment in total SFAs contente. Similarly, Cruz-Romero, Kerry & Kelly (2007) reported that when treated oyster with 800 MPa for 5 min and 0 min, no significant changes can be found in SFA content. However, there were still some significant differences in individual fatty acids. It can be seen from Table 9 that there were few significant increases ($P<0.05$) at relative low pressure with 600s treatment compared to 5s. Specifically, when samples were treated for 600s, the value of C14:0, C16:0, and C24:0 content significantly increased ($P<0.05$) at 100 MPa. Furthermore, C16:0 and C24:0 content also increased significantly ($P<0.05$) at 200 and 300 MPa respectively when treated with 600s. Additionally, only C14:0 and C16:0 significantly decreased ($P<0.05$) at 600 MPa treatment held for 600s.

4.2 Amino acids analysis

4.2.1 Effect of different HPP treatments on Storm Shell amino acids content

Table 10. Free amino acid composition (nmol/mL) of Storm Shell clams after pressure treatment at 100MPa, 200MPa, 300MPa, 500MPa and 600MPa.

Amino Acid	Pressure	Control	Time 5s	Time 600s	F value pressure	F value time	F value pressure*time
EAA	0	1030.88±57.46	$1030.88±57.46^{d}$	$1030.88±57.46^{d}$	25.891****	18.719****	11.656****
	100	$1030.88±57.46^{C}$	$547.98±25.39^{abA}$	$740.67±63.27^{cB}$			
	200	$1030.88±57.46^{B}$	$673.46±176.39^{bcA}$	$567.89±41.46^{bA}$			
	300	$1030.88±57.46^{C}$	$746.70±129.55^{cB}$	$605.46±36.60^{bA}$			
	500	$1030.88±57.46^{C}$	$500.32±125.82^{aB}$	$319.31±25.09^{aA}$			
	600	$1030.88±57.46^{C}$	$551.80±26.65^{abB}$	$306.38±45.41^{aA}$			
NEAA	0	5870.92±527.90	$5870.92±527.90^{a}$	$5870.92±527.90^{a}$	14.233****	5.687*	15.178****
	100	$5870.92±527.90^{A}$	$6706.27±152.43^{abB}$	$8438.63±933.10^{cC}$			
	200	$5870.92±527.90^{A}$	$7018.40±532.23^{bcB}$	$7154.50±767.76^{bB}$			
	300	$5870.92±527.90^{AB}$	$7706.60±576.72^{cB}$	$5392.63±73.28^{aA}$			
	500	$5870.92±527.90^{A}$	$6797.15±875.86^{abcB}$	$5731.09±644.67^{aA}$			
	600	$5870.92±527.90^{A}$	$5969.21±317.24^{aA}$	$5432.21±161.12^{aA}$			

Note: Values are means ± standard deviation. The letters a, b, c in the same column represent significant differences at $p < 0.05$ separated by the Fisher test for "Pressure". The letters A, B and C in the same column represent significant differences at $p < 0.05$ separated by Fisher test for "Holding time". $P < 0.0001$, $P < 0.001$, $P < 0.01$ and $P < 0.05$ were presented as "****", "***", "**" and "*" respectively for level of significance, and no "*" means not statistically significant.

Table 11. Essential Free amino acids composition (nmol/mL) of different Storm Shell clams after pressure treatment at 100MPa, 200MPa, 300MPa, 500MPa and 600MPa

Amino Acid	Pressure	Control	Time 5s	Time 600s	F value pressure	F value time	F value pressure*time
	0	188.478±22.692	188.478±22.692^{b}	188.478±22.692^{c}			
	100	188.478±22.692^{C}	104.184±2.817^{aA}	138.732±7.913^{bB}			
VAL	200	188.478±22.692^{A}	154.547±57.257^{bA}	134.882±7.671^{bA}	27.780****	14.341****	6.790****
	300	188.478±22.692^{B}	171.832±31.579^{bAB}	143.465±5.536^{bA}			
	500	188.478±22.692^{C}	105.873±25.702^{aB}	62.322±9.105^{aA}			
	600	188.478±22.692^{C}	113.941±13.043^{aB}	56.699±10.977^{aA}			
	0	177.666±38.671	177.666±38.671^{c}	177.666±38.671^{d}			
	100	177.666±38.671^{C}	92.635±11.827^{aA}	125.492±8.368^{cB}			
LEU	200	177.666±38.671^{B}	126.468±50.892^{bAB}	91.540±25.311^{bA}	17.816****	12.780***	6.502****
	300	177.666±38.671^{B}	130.508±24.597^{bA}	120.231±5.702^{cA}			
	500	177.666±38.671^{C}	88.154±21.318^{aB}	41.469±15.173^{aA}			
	600	177.666±38.671^{C}	88.814±2.874^{aB}	42.703±14.967^{aA}			
	0	138.652±4.159	138.652±4.159^{d}	138.652±4.159^{c}			
	100	138.652±4.159^{C}	65.565±1.849^{aA}	88.151±5.605^{bB}			
ILE	200	138.652±4.159^{B}	96.521±37.551^{bcAB}	86.692±2.233^{bA}	12.260****	9.594**	4.400**
	300	138.652±4.159^{B}	112.498±23.409^{cdAB}	81.628±32.923^{bA}			
	500	138.652±4.159^{C}	70.008±18.774^{aB}	44.184±8.265^{aA}			
	600	138.652±4.159^{C}	71.666±6.926^{abB}	40.495±6.673^{aA}			
	0	199.903±8.882	199.903±8.882^{bc}	199.903±8.882^{cd}			
	100	199.903±8.882^{AB}	154.437±16.675^{abA}	226.483±45.305^{dB}			
THR	200	199.903±8.882^{B}	135.232±10.347^{aA}	137.215±16.705^{bA}	12.767****	17.594****	19.105****
	300	199.903±8.882^{B}	217.116±35.964^{cAB}	162.622±18.054^{bcA}			
	500	199.903±8.882^{B}	159.312±46.982^{abB}	101.624±18.901^{aA}			
	600	199.903±8.882^{B}	218.571±24.448^{cB}	107.168±16.303^{aA}			
	0	85.070±11.996	85.070±11.996^{c}	85.070±11.996^{d}			
	100	85.070±11.996^{C}	42.755±7.147^{bA}	57.276±4.241^{cB}			
PHE	200	85.070±11.996^{B}	51.254±10.351^{bA}	40.791±6.661^{bA}	37.461****	3.325	7.538**
	300	85.070±11.996^{C}	47.368±9.396^{bB}	37.552±2.708^{bA}			
	500	85.070±11.996^{B}	26.908±7.426^{aA}	24.374±3.749^{aA}			
	600	85.070±11.996^{C}	29.375±3.855^{aB}	22.347±1.683^{aA}			

	0	105.804±8.777	105.804±8.777^{d}	105.804±8.777^{c}			
	100	105.804±8.777^{B}	23.112±11.298^{bcA}	32.225±3.817^{bA}			
MET	200	105.804±8.777^{C}	30.249±14.680^{cB}	15.426±6.702^{aA}	15.230****	0.343	4.999**
	300	105.804±8.777^{B}	15.191±5.660^{abA}	13.918±2.059^{aA}			
	500	105.804±8.777^{B}	10.148±1.265^{aA}	11.886±1.970^{aA}			
	600	105.804±8.777^{B}	10.512±1.136^{aA}	10.667±1.204^{aA}			
	0	135.304±11.846	135.304±11.846^{e}	135.304±11.846^{e}			
	100	135.304±11.846^{B}	65.290±8.314^{cdA}	72.306±10.294^{dA}			
LYS	200	135.304±11.846^{B}	79.192±12.740^{dA}	61.347±15.077^{cA}	43.812****	1.334	2.916*
	300	135.304±11.846^{B}	52.178±7.922^{bcA}	46.041±4.803^{bA}			
	500	135.304±11.846^{B}	39.920±8.498^{bA}	33.446±4.805^{aA}			
	600	135.304±11.846^{C}	18.912±2.402^{aA}	26.303±1.630^{aB}			

Note: Values are means ± standard deviation. The letters a, b, and c in the same column represent significant differences at $p < 0.05$ separated by the Fisher test for "Pressure". The letters A and B in the same column represent significant differences at $p < 0.05$ separated by Fisher test for "Holding time". $P < 0.0001$, $P < 0.001$, $P < 0.01$ and $P < 0.05$ were presented as "****", "***", "**" and "*" respectively for level of significance, and no "*" means not statistically significant.

Table 12 .Non-Essential Free amino acid composition (nmol/mL) of Storm Shell clams after pressure treatment at 100MPa, 200MPa, 300MPa, 500MPa and 600MPa.

Amino Acid	Pressure	Control	Time 5s	Time 600s	F value pressure	F value time	F value pressure*time
	0	1375.961±110.639	1375.961±110.639^{ab}	1375.961±110.64^{ab}			
	100	1375.961±110. 639^{A}	1729.129±129.972^{cB}	1847.523±220.343^{cB}			
SAR	200	1375.961±110. 639^{A}	1601.772±297.587^{bA}	1626.160±201.917^{bA}	16.082****	10.203**	5.102**
	300	1375.961±110. 639^{A}	1684.014±127.302^{bB}	1238.170±30.0837^{aA}			
	500	1375.961±110. 639^{AB}	1586.723±316.018^{bB}	1213.047±156.857^{aA}			
	600	1375.961±110. 639^{B}	1262.012±116.419^{aAB}	1160.610±104.054^{aA}			
	0	2910.250±285.067	2910.247±285.067^{a}	2910.247±285.067^{a}			
	100	2910.250±285.067^{A}	3418.639±231.476^{aA}	4277.684±566.568^{cB}			
GLY	200	2910.250±285.067^{A}	3372.629±956.729^{aA}	3462.138±283.746^{bA}	6.842****	0.632*	7.071*****
	300	2910.250±285.067^{A}	3567.557±338.311^{aB}	2664.130±89.224^{aA}			
	500	2910.250±285.067^{A}	3501.290±328.646^{aB}	3051.709±367.89^{aAB}			
	600	2910.250±285.067^{A}	3042.172±276.304^{aA}	3007.570±199.000^{aA}			
	0	23.223±6.490	23.223±6.490^{a}	23.223±6.490^{ab}			
	100	23.223±6.490^{A}	29.353±5.621^{abA}	59.285±22.525^{cB}			
b-AIB	200	23.223±6.490^{A}	29.961±3.315^{abA}	36.880±14.334$^{b\ A}$	13.541****	0.226	10.129****
	300	23.223±6.490^{A}	33.098±5.957^{bA}	24.740±9.315^{abA}			
	500	23.223±6.490^{B}	26.461±2.983^{aB}	14.478±5.313^{aA}			
	600	23.223±6.490^{B}	24.533±3.779^{aB}	13.951±3.968^{aA}			
	0	160.629±42.081	160.629±42.081^{a}	160.629±42.081^{b}			
	100	160.629±42.081^{A}	159.452±36.278^{aA}	274.476±17.131^{dB}			
SER	200	160.629±42.081^{AB}	137.432±41.132^{aA}	207.598±13.393^{cB}			
	300	160.629±42.081^{B}	292.955±36.372^{cC}	101.695±2.867^{aA}	16.510****	13.750***	48.163**
	500	160.629±42.081^{AB}	162.267±57.314^{aB}	84.466±23.454^{aA}			
	600	160.629±42.081^{A}	227.554±13.685^{bB}	164.560±20.294^{bA}			
	0	135.857±11.319	135.857±11.319^{c}	135.857±11.319^{e}			
	100	135.857±11.319^{C}	86.774±7.067^{abA}	109.820±7.614^{dB}			
PRO	200	135.857±11.319^{A}	110.670±41.400^{bcA}	105.152±9.250^{dA}	32.821****	15.989****	6.818****
	300	135.857±11.319^{B}	126.982±29.888^{cB}	89.492±7.035^{cA}			
	500	135.857±11.319^{C}	80.803±22.208^{aB}	36.871±3.352^{bA}			

	600	135.857±11.319^{C}	58.398±4.802^{aB}	27.766±4.099^{aA}			
ASN	0	53.749±7.402	53.749±7.402^{ab}	53.749±7.402^{b}	33.869****	7.388**	10.627****
	100	53.749±7.402^{A}	68.755±14.526^{bA}	100.642±5.438^{dB}			
	200	53.749±7.402^{A}	94.655±27.251^{cB}	79.587±15.025^{cAB}			
	300	53.749±7.402^{A}	94.813±15.906^{cB}	52.685±8.476^{bA}			
	500	53.749±7.402^{A}	52.280±18.428^{abA}	41.982±5.904^{bA}			
	600	53.749±7.402^{B}	40.670±9.925^{aB}	26.704±4.256^{aA}			
TPR	0	113.667±0.992	113.667±0.992^{ab}	113.667±0.992^{cd}	15.179****	8.693**	3.725*
	100	113.667±0.992^{C}	71.381±12.573^{aB}	62.361±4.924^{bA}			
	200	113.667±0.992^{A}	116.599±49.531^{bA}	117.446±20.591^{dA}			
	300	113.667±0.992^{A}	83.861±19.000^{abA}	93.812±17.229^{cA}			
	500	113.667±0.992^{AB}	114.800±33.035^{bB}	76.062±14.435^{bA}			
	600	113.667±0.992^{B}	83.848±15.518^{abA}	73.870±7.951^{bA}			
ASP	0	285.037±1.851	285.037±1.851^{a}	285.037±1.851^{a}	13.330****	2.333	30.546****
	100	285.037±1.851^{A}	355.350±84.726^{aA}	839.977±59.693^{cB}			
	200	285.037±1.851^{A}	546.060±104.079^{bAB}	679.181±209.762^{bB}			
	300	285.037±1.851^{A}	787.605±80.853^{cB}	368.763±20.836^{aA}			
	500	285.037±1.851^{A}	396.884±106.026^{aAB}	418.857±19.066^{aB}			
	600	285.037±1.851^{A}	406.267±80.007^{aA}	385.854±121.875^{aA}			
HYP	0	105.804±8.777	113.851±7.318^{b}	113.851±7.318^{c}	0.980	1.468	1.983
	100	105.804±8.777^{B}	80.836±12.353^{abA}	90.685±16.118^{bAB}			
	200	105.804±8.777^{A}	97.667±40.757^{abA}	69.643±4.637^{aA}			
	300	105.804±8.777^{A}	80.219±19.926^{abA}	78.097±24.973^{abA}			
	500	105.804±8.777^{B}	69.767±18.137^{aA}	76.328±4.041^{abA}			
	600	105.804±8.777^{B}	82.601±21.482^{abA}	65.391±7.640^{aA}			
GLU	0	376.548±100.735	376.548±100.735^{ab}	376.548±100.735^{bc}	45.890****	7.859**	11.896****
	100	376.548±100.735^{A}	537.004±51.180^{cB}	624.465±43.182^{eC}			
	200	376.548±100.735^{A}	422.112±113.276^{bA}	484.333±71.695^{dA}			
	300	376.548±100.735^{A}	657.139±69.199^{dB}	421.196±16.881^{cA}			
	500	376.548±100.735^{A}	375.415±86.530^{abA}	305.021±19.035^{bA}			
	600	376.548±100.735^{B}	321.038±72.197^{aA}	241.973±25.435^{aA}			
GLN	0	262.514±11.846	262.514±11.846^{abc}	262.514±11.846^{a}	11.416****	6.727*	2.047
	100	262.514±11.846^{B}	131.296±35.456^{aA}	141.064±22.861^{aA}			
	200	262.514±11.846^{AB}	440.899±151.973^{cB}	251.618±101.087^{aA}			
	300	262.514±11.846^{A}	253.331±51.352^{abA}	221.974±87.432^{aA}			

	500	262.514±11.846^{A}	404.879±157.159^{cA}	398.444±152.522^{bA}			
	600	262.514±11.846^{AB}	389.803±142.054^{bcB}	251.346±39.082 ^{aA}			
	0	59.680±13.426	59.680±13.426^{d}	59.680±13.426^{c}			
	100	59.680±13.426^{B}	38.305±5.756^{bcA}	72.306±6.107^{cB}			
TYR	200	59.680±13.426^{B}	47.947±12.740^{cdAB}	61.347±9.268^{cA}	35.400****	17.087****	8.359****
	300	59.680±13.426^{B}	45.018±7.164^{cA}	46.041±2.277^{bA}			
	500	59.680±13.426^{C}	25.576±6.507^{aB}	33.446±2.542^{aA}			
	600	59.680±13.426^{C}	30.314±5.205^{abB}	26.303±3.829^{aA}			

Values are means ± standard deviation. The letters a, b, c and d in the same column represent significant differences at $p < 0.05$ separated by the Fisher test for "Pressure". The letters A and B in the same column represent significant differences at $p < 0.05$ separated by Fisher test for "Holding time". $P < 0.0001$, $P < 0.001$, $P < 0.01$ and $P < 0.05$ were presented as "****", "***", "**" and "*" respectively for level of significance, and no "*" means not statistically significant.

In this study, the effects of HPP treatment on amino acids under different pressure conditions (100, 200, 300, 500, 600 MPa) and holding times (5s and 600s). As seen in Tables 8 and 9, a total of nineteen amino acids (valine, leucine, threonine, isoleucine, methionine, phenylalanine, lysine, sarcosine, glycine, b-Aminoisobutyric acid, serine, proline, asparagine, aspartic acid, thioproline, glutamic acid, hydroxyproline, tyrosine, and glutamine) were identified and quantified by EZ:faastTM, Phenomenex®, USA. Seven EAAs were identified (valine, leucine, isoleucine, methionine, phenylalanine, threonine, and lysine) (Table 11). According to the research by Peinado, Koutsidis et al. (2016), these amino acids are found in fish powder hydrolysates (FPHs). Lysine, leucine were the most abundant amino acids in this study. Phenylalanine content was the lowest value. This is supported by Chen and Zhang (2007). They indicated that hydrophobic amino acids such as cysteine, isoleucine, phenylalanine, and tyrosine were present in small amounts.

4.2.1.1 Essential amino acids

Effect of pressure after holding for 5s

As seen in Table 10, all 5s pressure treated samples had significant changes in EAAs content compared to the control. Specifically, all pressure treatments significantly decreased ($P<0.05$) EAAs content compared to control. Moreover, the relative higher-pressure treatments as 500 and 600 MPa had lower values, such. Similarly, H. Simonin et al. (2012) reported that the muscle proteins were easy to be impacted by oxidative reactions and leaded to EAA loss and protein digestibility decreased. Prooxidant and antioxidant factors can reduce EAA values. As for individual EAAs (Table 11), leucine, phenylalanine, methionine, and lysine had significant decrease ($P<0.05$) at all pressure conditions (100-600MPa) compared with the control. Valine and isoleucine content decreased significantly at 100, 500 and 600MPa. Noticeably, only threonine significantly decreased at 200 MPa. Cruz-Romero et la., (2004) reported that HPP treatment can change the functional properties of food ingredients and also can strongly affect macromolecular phenomena, such as protein unfolding, and structural configurations. Specifically, changes of protein after HPP originated from disulphide bonding and reaction of the exposed hydrophobic groups and eventually formed oligomers. Because of the presence of 2-mercaptoethanol, complete solubilisation of aggregates was achieved.

Effect of pressure after holding for 600s

Holding pressures for 600s resulted in significantly reduced EAAs content at all pressure conditions compared to the control. Specifically, threonine content decreased significantly at 200, 500, and 600 MPa. The remaining EAAs significantly decreased at all pressure conditions. The decrease is probably related to the structural changes in protein (Cruz-Romero et la., 2004).

Effect of holding time

As seen in Table 10, treatment at 100 MPa for 600s caused a significant increase in EAAs compared to 5s treatment. In addition, treatment at 300, 500, and 600 MPa for 600s significantly reduced EAAs compared to 5s treatment. Specifically (Table 11), the value of valine, leucine, isoleucine, phenylalanine, and threonine significantly increased ($P<0.05$) at 100 MPa compared to 5s treatment. Few previous studies have reported the effect of HPP on amino acids content in seafood. Only Cruz-Romero et la., (2008) reported that total protein content Pacific oysters (*Crassostrea gigas*) treated at 260 MPa for 3 min significantly increased at 260MPa for 3 min compared to the control, and this increase was attributed to proteolysis. Additionally, samples treated at 300, 500, and 600 MPa for 600s significantly decreased ($P<0.05$) valine, leucine, isoleucine, phenylalanine, and threonine content compared to "5s". As mentioned, oxidative reactions and structural changes can result in EAAs loss (H. Simonin et al. 2012. & Cruz-Romero et la., 2004)

4.2.1.2 Non-essential amino acids

In this section, changes in twelve NEAAs (sarcosine, glycine, b-Aminoisobutyric acid, serine, proline, asparagine, aspartic acid, thioproline, glutamic acid, hydroxyproline, tyrosine, and glutamine) will be discussed. Sarcosine, glycine, aspartic acid, glutamic acid, and glutamine are the four major NEAAs. Chen and Zhang (2007) also reported that glutamic acid and glycine are two major NEAAs in clam meat. In our study, the content of glutamic acid and glycine were approximately 500nmol/ml and 3200nmol/ml respectively. Glutamic acid contributes to umami taste and glycine contributes to mainly sweet taste. Moreover, Sokolowski, Wolowicz et al. (2003) found that in Baltic clam (*Macoma balthical*), free amino acids were mainly composed of Ala, Gln, Arg, Gly and Orn. These FAAs that

accounted for 80% of total free amino acids. In this study, glycine alone accounted for 50% of total amino acids.

Effect of pressure after holding for 5s

As seen in Table 10, the total content of NEAA significantly increased ($P<0.05$) at 200 and 300MPa treatments compared to the control. This is quite different from EAA results. Looking at changes in the individual NEAAs (Table 12), the content of b-Aminoisobutyric acid, glycine, serine, and glutamic acid significantly increased ($P<0.05$) at 300MPa, and only glutamine significantly increased at 200 MPa. Additionally, asparagine and aspartic acid significantly increased at 200 and 300 MPa. Campus (2010) indicated that to some extent high pressure treatment can inhibit calpain activity and prevent the degradation of cytoskeletal proteins such as desmin. This may explain why the content of NEAA is maintained at relative high-level, and even increased.

Effect of pressure after holding for 600s

Treatments for 600s and 5s, significantly increased total NEAA content compared to control (Table 10). Specifically (Table 12), the content of sarcosine, glycine, and b-aminoisobutyric acid significantly increased at 100 MPa. Serine, asparagine, aspartic acid, and glutamic acid content significantly increased ($P<0.05$) at 100 and 200 MPa. Campus (2010) reported that high pressure treatment can probably inhibit calpain activity and prevent the degradation of cytoskeletal proteins such as desmin.

Effect of holding time

The effect of pressure holding time on the total content of NEAA mainly occurred at 100, 300, and 500MPa. Treatment at 100 MPa for 600s, NEAA content compared to 5s treatment. Besides that, treatment at 300 and 500 MPa for 600s significantly decreased ($P<0.05$) the total NEAA content compared to 5s. Specifically (Table 12), the value of glycine, b-Aminoisobutyric acid, serine, proline, asparagine, aspartic acid, glutamic acid, and tyrosine significantly increased ($P<0.05$) at 100 MPa for 600s. A significant decrease in the content of glycine, serine, asparagine, aspartic acid, glutamic acid, b-aminoisobutyric acid, and thioproline occurred.at 300 or 500 MPa when at 600s compared to 5s. Noticeably, the content of sarcosine and proline both decreased significantly at 300 and 500MPa at 600s compared

to 5s. The longer treatment time at relative-high pressure significantly decreased NEAA content due to changes in protein structure. This is because HPP treatment can strongly affect macromolecular phenomena, such as protein unfolding, and structural configurations. Specifically, changes of protein after HPP originated from disulphide bonding and reaction of the exposed hydrophobic groups and eventually formed oligomers according to Cruz-Romero et al., (2004).

4.2.2 Effect of different HPP treatments on TuaTua amino acids content

Table 13. Free amino acid composition (nmol/mL) of Tua Tua clams after pressure treatment at 100MPa, 200MPa, 300MPa, 500MPa and 600MPa

Amino Acid	Pressure	Control	Time 5s	Time 600s	F value pressure	F value time	F value pressure*time
EAA	0	1264.66±121.12	$1264.66±121.12^{c}$	$1264.66±121.12^{c}$	30.620****	7.296**	6.390****
	100	$1264.66±121.12^{B}$	$1267.63±104.40^{cB}$	$1011.24±116.06^{bA}$			
	200	$1264.66±121.12^{B}$	$1076.04±344.99^{cbA}$	$1233.77±229.31^{cB}$			
	300	$1264.66±121.12^{AB}$	$1158.83±92.91^{cA}$	$1317.34±226.13^{cB}$			
	500	$1264.66±121.12^{C}$	$904.09±245.26^{abB}$	$567.75±25.65^{aA}$			
	600	$1264.66±121.12^{C}$	$775.39±65.91^{aB}$	$430.34±15.13^{aA}$			
NEAA	0	4986.05±530.97	$4986.05±530.97^{ab}$	$4986.05±530.97^{b}$	14.838****	3.741*	1.895
	100	$4986.05±530.97^{B}$	$5377.48±414.30^{bA}$	$5247.57±110.74^{bA}$			
	200	$4986.05±530.97^{A}$	$5084.96±1202.31^{bA}$	$5695.70±307.45^{cB}$			
	300	$4986.05±530.97^{A}$	$5278.91±702.00^{bA}$	$5079.80±573.91^{bA}$			
	500	$4986.05±530.97^{B}$	$4745.12±708.85^{aB}$	$4172.01±66.33^{aA}$			
	600	$4986.05±530.97^{C}$	$4132.44±212.94^{aB}$	$3802.52±181.84^{aA}$			

Note: Values are means ± standard deviation. The letters a, b and c in the same column represent significant differences at $p < 0.05$ separated by the Fisher test for "Pressure". The letters A and B in the same column represent significant differences at $p < 0.05$ separated by Fisher test for "Holding time". $P < 0.0001$, $P < 0.001$, $P < 0.01$ and $P < 0.05$ were presented as "****", "***", "**" and "*" respectively for level of significance, and no "*" means not statistically significant.

Table 14. Essential free amino acids composition (nmol/mL) of Tua Tua clams after pressure treatment at 100MPa, 200MPa, 300MPa, 500MPa and 600MPa.

Amino Acid	Pressure	Control	Time 5s	Time 600s	F value pressure	F value time	F value pressure * time
VAL	0	250.899±19.219	$250.899±19.219^{b}$	$250.899±19.219^{bc}$	26.584****	3.714	7.573****
	100	$250.899±19.219^{B}$	$254.515±25.792^{bB}$	$199.352±24.785^{bA}$			
	200	$250.899±19.219^{A}$	$230.568±78.556^{bA}$	$288.369±53.427^{cA}$			
	300	$250.899±19.219^{A}$	$244.918±22.309^{bA}$	$286.155±46.844^{cA}$			
	500	$250.899±19.219^{B}$	$203.198±58.385^{abB}$	$127.270±4.765^{aA}$			
	600	$250.899±19.219^{C}$	$162.524±13.316^{aB}$	$93.922±4.118^{aA}$			
LEU	0	254.899±28.669	$254.899±28.669^{c}$	$254.899±28.669^{bc}$	24.121****	6.621*	2.897*
	100	$254.899±28.669^{AB}$	$255.466±30.687^{cB}$	$207.387±25.338^{bA}$			
	200	$254.899±28.669^{A}$	$224.559±76.087^{bcA}$	$230.353±54.995^{bcA}$			
	300	$254.899±28.669^{A}$	$240.167±15.250^{cA}$	$264.068±45.220^{cA}$			
	500	$254.899±28.669^{C}$	$180.922±38.546^{abB}$	$128.774±7.796^{aA}$			
	600	$254.899±28.669^{C}$	$157.044±11.236^{aB}$	$102.268±1.265^{aA}$			
ILE	0	166.172±12.150	$166.172±12.150^{bc}$	$166.172±12.150^{c}$	37.842****	9.468**	8.266****
	100	$166.172±12.150^{B}$	$173.819±15.790^{cB}$	$128.889±15.641^{bA}$			
	200	$166.172±12.150^{A}$	$157.602±47.466^{bcA}$	$183.554±31.596^{cA}$			
	300	$166.172±12.150^{A}$	$168.532±15.130^{cA}$	$192.818±27.643^{cA}$			
	500	$166.172±12.150^{B}$	$131.017±36.563^{abB}$	$77.207±3.694^{aA}$			
	600	$166.172±12.150^{C}$	$103.842±8.844^{aB}$	$54.362±3.067^{aA}$			
THR	0	262.430±10.774	$262.430±10.774^{ab}$	$262.430±10.774^{bc}$	19.829****	0.994	10.438****
	100	$262.430±10.774^{AB}$	$270.909±20.931^{bB}$	$236.163±20.495^{bA}$			
	200	$262.430±10.774^{AB}$	$206.946±66.806^{aA}$	$303.212±50.871^{cB}$			
	300	$262.430±10.774^{A}$	$240.515±41.649^{abA}$	$295.735±60.393^{cA}$			
	500	$262.430±10.774^{B}$	$197.400±81.210^{aB}$	$109.245±13.179^{aA}$			
	600	$262.430±10.774^{C}$	$197.849±21.046^{aB}$	$111.435±6.320^{aA}$			
MET	0	80.993±19.403	$80.993±19.403^{d}$	$80.993±19.403^{c}$	26.650****	0.864	1.885
	100	$80.993±19.403^{A}$	$65.121±2.015^{cdA}$	$59.623±18.508^{bcA}$			
	200	$80.993±19.403^{A}$	$58.691±26.086^{cA}$	$43.459±19.910^{bA}$			
	300	$80.993±19.403^{A}$	$55.235±3.345^{cA}$	$69.770±19.815^{cA}$			
	500	$80.993±19.403^{B}$	$33.166±9.891^{bA}$	$24.354±1.340^{aA}$			

	600	80.993±19.403^{B}	16.701±2.868^{aA}	14.824±3.139^{aA}			
	0	118.265±12.842	118.265±12.842^{bc}	118.265±12.842^{c}			
	100	118.265±12.842^{B}	119.846±9.115^{cB}	87.723±7.962^{bA}			
PHE	200	118.265±12.842^{A}	99.140±28.810^{bA}	83.044±16.228^{bA}	32.694****	41.686****	2.121
	300	118.265±12.842^{B}	101.158±15.506^{bcAB}	93.649±13.621^{bA}			
	500	118.265±12.842^{C}	73.582±19.197^{aB}	36.893±10.106^{aA}			
	600	118.265±12.842^{C}	68.821±6.757^{aB}	43.824±4.025^{Aa}			
	0	131.000±13.600	131.000±13.600^{d}	131.000±13.600^{e}			
	100	131.000±13.600^{B}	127.955±4.203^{dB}	92.107±5.823^{cA}			
LYS	200	131.000±13.600^{A}	98.533±31.353^{bcA}	101.784±14.294^{cdA}	55.082****	32.563****	11.053****
	300	131.000±13.600^{A}	108.306±7.661^{cA}	115.145±20.861^{deA}			
	500	131.000±13.600^{C}	84.808±11.889^{abB}	64.009±10.470^{bA}			
	600	131.000±13.600^{C}	68.607±8.067^{aB}	97.101±2.067^{cA}			

Values are means ± standard deviation. The letters a, b, c and d in the same column represent significant differences at $p < 0.05$ separated by the Fisher test for "Pressure". The letters A and B in the same column represent significant differences at $p < 0.05$ separated by Fisher test for "Holding time". $P < 0.0001$, $P < 0.001$, $P < 0.01$ and $P < 0.05$ were presented as "****", "***", "**" and "*" respectively for level of significance, and no "*" means not statistically significant.

Table 15. Non-Essential free amino acid composition (nmol/mL) of Tua Tua clams after pressure treatment at 100MPa, 200MPa, 300MPa, 500MPa and 600MPa.

Amino Acid	Pressure	Control	Time 5s	Time 600s	F value pressure	F value time	F value pressure*time
	0	1098.289±133.804	1098.289±133.804^{ab}	1098.289±133.804^{c}			
	100	1098.289±133.804^{A}	1109.239±22.678^{bA}	1287.373±29.24^{dB}			
SAR	200	1098.289±133.804^{A}	1148.454±349.375^{bA}	1378.596±85.475^{eA}	21.516****	0.470	7.675****
	300	1098.289±133.804^{AB}	1161.473±148.225^{bB}	964.089±75.820^{bA}			
	500	1098.289±133.804^{B}	1038.092±161.983^{abB}	770.511±28.274^{aA}			
	600	1098.289±133.804^{B}	862.024±62.882^{aA}	796.037±54.243^{aA}			
	0	1787.746±186.335	1787.746±186.335^{c}	1787.746±186.335^{b}			
	100	1787.746±186.335^{A}	1667.228±128.592^{cA}	1683.888±41.031^{bA}			
GLY	200	1787.746±186.335^{A}	1457.626±224.710^{aA}	1533.736±188.431^{abA}	3.380*	2.259	3.287*
	300	1787.746±186.335^{A}	1569.221±101.514^{abA}	1593.058±209.242^{abA}			
	500	1787.746±186.335^{B}	1770.386±195.433^{cB}	1427.052±142.749^{aA}			
	600	1787.746±186.335^{B}	1504.651±123.431^{abA}	1420.836±148.916^{aA}			
	0	136.577±18.722	136.577±18.722^{d}	136.577±18.722^{c}			
	100	136.577±18.722^{A}	130.414±24.486^{dA}	120.498±11.614^{bcA}			
b-AIB	200	136.577±18.722^{A}	104.237±24.684^{bcA}	108.894±11.408^{bA}	14.220****	3.059	2.676*
	300	136.577±18.722^{B}	116.497±15.291^{cdB}	86.760±7.956^{aA}			
	500	136.577±18.722^{A}	83.516±4.952^{aA}	84.146±6.380^{aA}			
	600	136.577±18.722^{B}	89.753±5.903^{abA}	90.691±17.163^{aA}			
	0	265.018±15.373	265.018±15.373^{bc}	265.018±15.373^{bc}			
	100	265.018±15.373^{AB}	302.360±29.790^{cB}	257.830±13.903^{bA}			
SER	200	265.018±15.373^{AB}	230.337±95.367^{abcA}	341.363±66.563^{cdB}	16.396****	5.618*	3.593*
	300	265.018±15.373^{A}	276.807±68.455^{cA}	367.131±72.299^{dA}			
	500	265.018±15.373^{A}	151.807±89.750^{aA}	151.688±33.525^{aA}			
	600	265.018±15.373^{B}	195.488±29.493^{aA}	218.571±24.448^{bAB}			
	0	160.798±20.015	160.798±20.015^{ab}	160.798±20.015^{a}			
	100	160.798±20.015^{A}	276.183±135.489^{bcA}	157.567±36.803^{aA}			
PRO	200	160.798±20.015^{A}	259.761±127.999^{abcA}	367.046±126.867^{bA}	6.838**	2.247*	3.664*
	300	160.798±20.015^{A}	306.107±106.158^{cB}	168.588±23.681^{aA}			
	500	160.798±20.015^{A}	132.266±47.229^{aA}	143.310±81.832^{aA}			
	600	160.798±20.015^{A}	183.928±32.554^{abcA}	146.915±95.675^{aA}			
	0	110.531±30.371	110.531±30.371^{b}	110.531±30.371^{bc}			

	100	110.531±30.371^{A}	107.696±7.073^{bA}	98.334±8.988^{bA}			
ASN	200	110.531±30.371^{A}	109.169±34.401^{bA}	129.050±23.225^{cA}	22.554****	2.384	3.096*
	300	110.531±30.371^{A}	105.241±37.000^{bA}	108.099±6.709^{bA}			
	500	110.531±30.371^{B}	82.892±22.264^{abB}	50.151±6.521^{aA}			
	600	110.531±30.371^{C}	65.725±11.60^{aB}	45.003±2.882^{aA}			
	0	52.219±5.022	52.219±5.022^{ab}	52.219±5.022^{b}			
	100	52.219±5.022^{A}	61.696±19.939^{bA}	63.052±2.337^{dA}			
TPR	200	52.219±5.022^{A}	50.563±10.087^{abA}	56.343±7.101^{bcA}	30.012****	4.141*	0.993
	300	52.219±5.022^{AB}	49.845±8.216^{abA}	59.892±1.007^{cdB}			
	500	52.219±5.022^{A}	76.888±9.254^{cB}	85.134±5.277^{eB}			
	600	52.219±5.022^{B}	44.936±5.452^{aAB}	42.632±4.287^{aA}			
	0	160.515±34.180	160.515±34.180^{a}	160.515±34.180^{a}			
	100	160.515±34.180^{A}	193.117±25.058^{abA}	245.884±17.507^{cB}			
ASP	200	160.515±34.180^{A}	212.386±51.104^{bA}	215.510±13.492^{bcA}	14.584****	1.650	3.144*
	300	160.515±34.180^{A}	175.238±24.214^{abA}	196.717±38.245^{bA}			
	500	160.515±34.180^{A}	168.573±27.880^{aA}	163.108±18.439^{aA}			
	600	160.515±34.180^{A}	157.342±33.525^{aA}	132.895±10.062^{aA}			
	0	113.851±7.318	113.851±7.318^{ab}	113.851±7.318^{ab}			
	100	113.851±7.318^{A}	168.898±34.044^{bB}	97.828±9.1935^{aA}			
HYP	200	113.851±7.318^{A}	109.613±41.901^{aA}	151.340±44.529^{bcA}	3.002*	1.471	4.969**
	300	113.851±7.318^{A}	177.199±71.594^{bA}	119.765±9.584^{abA}			
	500	113.851±7.318^{A}	103.741±19.171^{aA}	107.061±10.786^{aA}			
	600	113.851±7.318^{A}	143.888±37.780^{abA}	166.826±55.796^{cA}			
	0	645.571±100.272	645.571±100.272^{bc}	645.571±100.272^{b}			
	100	645.571±100.272^{A}	594.958±9.455^{bA}	812.077±59.400^{cB}			
GLU	200	645.571±100.272^{A}	780.396±141.131^{cA}	792.351±48.620^{cA}	46.987****	4.083*	10.774****
	300	645.571±100.272^{AB}	772.628±62.522^{cB}	643.561±75.651^{bA}			
	500	645.571±100.272^{AB}	614.520±114.674^{bA}	810.032±120.545^{cB}			
	600	645.571±100.272^{C}	407.688±64.842^{aB}	328.749±29.599^{aA}			
	0	445.099±47.125	445.099±47.125^{a}	445.099±47.125^{bc}			
	100	445.099±47.125^{B}	652.460±75.119^{bC}	336.846±32.319^{aA}			
GLN	200	445.099±47.125^{A}	536.687±131.954^{abA}	541.581±54.011^{cA}	10.027****	5.799*	13.522****
	300	445.099±47.125^{A}	480.139±163.506^{aA}	680.235±83.833^{dB}			
	500	445.099±47.125^{AB}	463.891±63.952^{aB}	343.646±62.461^{aA}			
	600	445.099±47.125^{A}	420.609±91.922^{aA}	376.593±45.662^{abA}			

	0	69.831±0.786	69.831±0.786^{ab}	69.831±0.786^{b}			
	100	69.831±0.786^{A}	113.238±9.335^{cC}	86.396±8.378b^{cB}			
TYR	200	69.831±0.786^{A}	85.727±30.451^{bB}	79.894±16.028b^{cB}	38.899****	16.029****	2.507
	300	69.831±0.786^{A}	88.510±2.684^{bB}	91.906±15.155^{cB}			
	500	69.831±0.786^{B}	58.553±16.494^{aA}	36.208±5.883^{aA}			
	600	69.831±0.787^{B}	56.409±6.812^{aA}	36.774±2.696^{aA}			

Values are means ± standard deviation. The letters with a, b, c, and d in the same column represent significant differences at $p < 0.05$ separated by the Fisher test for "Pressure". The letters with A and B in the same column indicate significant differences at $p < 0.05$ separated by Fisher test for "Holding time". $P < 0.0001$, $P < 0.001$, $P < 0.01$ and $P < 0.05$ were presented as "****", "***", "**" and "*" respectively for level of significance, and no "*" means not statistically significant.

4.2.2.1 Essential amino acids

Effect of pressure after holding for 5s

Changes in seven EAAs (valine, leucine, isoleucine, methionine, phenylalanine, threonine, and lysine) will be discussed. As seen in Table 13, significant decrease ($P<0.05$) appeared at relative-high pressure (500 and 600 MPa) for 5s compared to the control. H. Simonin et al. (2012) demonstrated that muscle proteins are easy to be impacted through oxidative reactions and caused EAA loss and protein digestibility. Prooxidant and antioxidant factors can decrease EAA values. Besides that, Cruz-Romero et la., (2004) attributed the decrease due to changes in protein structure. They pointed out that HPP treatment can change the functional properties of food constituents. HPP strongly affect macromolecular phenomena, such as protein unfolding, and structural configurations. Specifically, changes of protein after HPP originated from disulphide bonding and reaction of the exposed hydrophobic groups and eventually formed oligomers. Because of the presence of 2-mercaptoethanol, complete solubilisation of aggregates was achieved. Specifically (Table 14), the content of valine, leucine, methionine, phenylalanine, and lysine significantly decreased ($P<0.05$) at 500 and 600 MPa treatments for 5s compared to control. Isoleucine significant decreased ($P<0.05$) at 600 MPa when held at 5s. Only, threonine showed no significant changes.

Effect of pressure after holding for 600s

Treatments with longer holding time (600s), significantly decreased ($P<0.05$) the total value of EAA content at 100, 500, and 600 MPa compared to control (Table 13). Looking at the individual EAAs (Table 14), valine, leucine, and threonine significantly decreased ($P<0.05$) at 500 and 600 MPa. Methionine, phenylalanine, and lysine content significantly decreased at 100, 500, and 600 MPa. H. Simonin et al. (2012) explained that the muscle proteins are vulnerable to oxidative reactions that can result in loss of EAA and decrease protein digestibility, and prooxidant and antioxidant factors can reduce EAA values.

Effect of holding time

As seen in Table 13, the holding time significantly impacted the content of EAAs. 100,

500, and 600 MPa for 600s, the significantly decreased ($P<0.05$) total EAA content compared to 5s treatment. Treatment at 200 and 300 MPa for 600s significantly increased content compared to 5s. In terms of individual EAAs (Table 14), almost all EAA except methionine, significantly decreased ($P<0.05$) at 100, 500, and 600 MPa treatments for 600s compared to 5s treatment. A significant increase was only observed in threonine at 200 MPa, 600s compared to 5s treatment.

4.2.2.2 Non-essential amino acids

Effect of pressure after holding for 5s

Pressure treatments for 5s resulted in few significant changes (Table 13). The total content of NEAA only showed a significant decrease ($P<0.05$) at the highest pressure of 600 MPa compared to the control. The value of glycine, b-aminoisobutyric acid, serine, asparagine, and glutamic acid significantly decreased at 600 MPa ($P<0.05$).

Effect of pressure after holding for 600s

Through looking Table 13, 600s treatment resulted in a significant increase and decrease at 200 and 500 MPa respectively compared to control. Specifically (Table 15), sarcosine, proline, aspartic acid, tyrosine, and glutamic acid significantly increased at 200 MPa. Campus (2010) indicated that to some extent high pressure treatment inhibits calpain activity and prevents the degradation of cytoskeletal proteins such as desmin. Hence the content of NEAA is maintained at a relative high-level, or even increased. As for the significant decrease, the content of sarcosine, glycine, b-Aminoisobutyric acid, serine, asparagine, tyrosine, and glutamine significantly decreased at 500 MPa compared to control.

Effect of holding time

In general, the main significant changes in NEAA occurred for treatments at 200, 500, and 600 MPa for 600s compared to 5s (Table 13). Treatment at 200 MPa for 600s significantly increased NEAA content compared to 5s treatment. Serine, proline, and hydroxyproline (Table 15) content significantly increased ($p<0.05$) at 200 MPa. Treatments at 500 and 600

MPa for 600s significantly decreased NEAA content compared to 5s treatment. Specifically, sarcosine, glycine, asparagine, and glutamine significantly decreased ($P<0.05$) at 500 MPa. Moreover, serine, asparagine, thioproline, and glutamic acid significantly decreased ($P<0.05$) at 600 MPa for 600s compared to 5s. The increase was explained by Campus (2010) who reported that to some extent, high pressure treatment can inhibit calpain activity and prevent the degradation of cytoskeletal proteins such as desmin. This may explain why the content of NEAA is maintained at relative high-level, or even increased.

Chapter 5 Conclusion

The major applications of HPP in mollusks are shucking, crustacean meat extraction and increasing shelf life of the product. Most previous studies have investigated the effects of HPP on microbiological and quality aspects of food. However, the effect of HPP on the chemical property of seafood have yet to be understood. In the current study, two species of New Zealand clams (storm shell and TuaTua) were subjected to HPP and evaluated in terms of changes in AA and FA composition.

Amino acids can significant impact the nutritional value and flavor of food. The results in this study showed that sarcosine, glycine, aspartic acid and glutamic acid are the four most abundant amino acids in the sample. HPP treatment leaded to significantly decreased ($P<0.05$) content of EAA in storm shell clams when treated between 100 to 600MPa for both 5s and 10min compared to control. Moreover, few significant increases in NEAA were evident in 100, 200, and 300MPa treated storm shell samples for 5s and 10min compared to control. Additionally, the longer pressurization time of 10min at relative-lower pressure conditions may contribute to significant increases in the content of EAA and NEAA. Increased pressurization for a longer time can result in the loss of amino acids content. These changes can lead to nutrition loss value and change in flavor. Significant changes (increase or decrease) were mainly due to sarcosine, glycine, aspartic acid, glutamic acid, glutamine and valine content.

Although clams are not a significant source of fat, they do contain polyunsaturated fatty acids that have health benefits. In storm shell, only a significant decrease ($P<0.05$) in SFA and UFA content appeared at 300 and 600MPa treatments when held for 5s or 10min compared to control. As for Tua Tua, application of pressure treatments at 500 and 600MPa for 5s and 10min caused the significant decrease of the content of SFA compared to control. Moreover, there was a significant decrease in both SFA and UFA content when treated at 300MPa for 10min compared to the control. In addition, pressurization for a longer time (10min) caused a significant decrease ($P<0.05$) in both UFA and SFA content at relatively high-pressure conditions of 500 and 600MPa, and a significant growth ($P<0.05$) in SFA at 100MPa compared to 5s treatments.

Taken together, the result suggested that HPP treatment of clams can result in changes in fatty acids and amino acids composition. These changes are influenced by the type of clam processed, as well as pressurization conditions and holding time. Hopefully, results of current study could be useful to the New Zealand seafood industries because it will help processors choose appropriate HPP processing conditions that will maintain the nutritional value of clams.

Appendix:

EZ:faast™, Phenomenex®, USA user manual.

3.0 Sample Preparation Procedure

3.1 Setup

The EZ:faast kit packaging has been designed as an efficient workstation. It holds a reagent tray, a vial rack, a pipette rack and a section for sorbent tips and vials. To speed up sample preparation it is recommend that the workstation be arranged as shown in figure 1a. By following directions and markings on the reagent box by breaking it along perforations it can be transformed into a reagent tray. When the kit is not in use for several days, the reagent tray (figure 1b) may be conveniently removed and placed in the refrigerator.

3.2 Preparing the Eluting Medium

The volume of prepared Eluting Medium depends upon the number of samples to be analyzed during the day (200µL/sample). The eluting medium should be prepared fresh each day:

1. Use capped vials of appropriate size (not included) for preparation of the Eluting Medium
2. Combine 3 parts Reagent 3A (Eluting Medium Component I) with 2 parts Reagent 3B (Eluting Medium Component II) in an appropriate sized vial (see Table 2, page 5, for reagent volumes based on number of samples). Mix briefly.
3. Store prepared eluting medium during the day at room temperature. Discard any unused mixture at the end of the day.

Workstation Arrangement - (Figure 1)

To speed up sample preparation it is recommended that the workstation be arranged as shown below.

Figure 1a

Figure 1b

Table 2 - For your convenience check the table below to determine the volume of Eluting Medium components needed depending on your number of samples:

Number of Samples	Reagent 3A Eluting Medium Component I	Reagent 3B Eluting Medium Component II
2	300µL	200µL
4	600µL	400µL
7	900µL	600µL
12	1.5mL	1.0mL
14	1.8mL	1.2mL
19	2.4mL	1.6mL
24	3.0mL	2.0mL
29	3.6mL	2.4mL
34	4.2mL	2.8mL
39	4.8mL	3.2mL
44	5.4mL	3.6mL
49	6.0mL	4.0mL

3.3 Sample Preparation by SPE and Derivatization

Prepare Eluting Medium first; refer to section 3.2 for preparation protocol. The freshly prepared Eluting Medium vial may be placed in one of the empty slots in the reagent tray.

1. For each sample, line up one glass sample preparation vial in the vial rack (Figure 2). Be aware of some variability in vial opening and sorbent tip dimensions, which may prevent the tip from reaching to the bottom of the sample preparation vial.

Note: *Droplets of liquid in SPE tip or spilled sorbent particles will not affect the precision of the assay in any way.*

Glass Vial Line Up - (Figure 2)

For each sample, line up one glass sample preparation vial in the vial rack.

Figure 2

2. Pipette 100µL sample (serum, plasma, urine or other), and 100µL Reagent 1 (Internal Standard Solution) into each sample preparation vial.

Caution: The pH of biological samples is usually around 7. After the addition of Reagent 1 (Internal Standard) the mixture has the correct pH for successful loading onto the SPE tip as described in the next step. With other samples make sure that the sample + Reagent 1 mixture has a pH between pH 1.5 and pH 6.0!

Note: *Samples with amino acid concentrations higher than 10mmol/L (10µmol/mL; e.g. dark colored urine) should be analyzed by pipetting only 50µL (or 25µL) sample in the sample preparation vial instead of 100µL. Concentrations recorded as a result of the GC analysis will be half (one quarter) of the actual concentrations for these samples. Conversely, when low concentrations of amino acids have to be quantified, the volume of sample to be prepared should be 200µL or more.* ***The total amount of amino acids present in the sample to be loaded onto the SPE tip should not exceed 1.2 µmols.***

3. Attach a sorbent tip to a 1.5mL syringe and loosen the syringe piston; immerse the tip and let the solution in the sample preparation vial pass through the sorbent tip by SLOWLY pulling back the syringe piston, in SMALL steps.
4. Pipette 200µL Reagent 2 (Washing Solution) into the same sample preparation vial. Pass the solution SLOWLY through the sorbent tip and into the syringe barrel. Drain the liquid from the sorbent bed by pulling air through the sorbent tip. Detach the sorbent tip, and leave it in the sample preparation vial, then discard the liquid accumulated in the syringe.

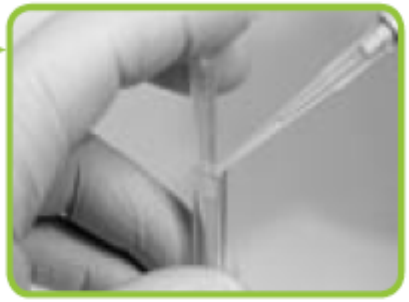
Note: *save the syringe, as it can be reused with many other samples. For convenience place it into the pipette rack.*

5. Pipette 200µL Eluting Medium (prepared fresh each day, section 3.2) into the same sample preparation vial.

Keep the Sorbent Tip in the Vial - (Figure 3)

Keep the sorbent tip in the sample preparation vial through steps 3-8, even while dispensing reagents.

Figure 3



6. Pull back the piston of a 0.6 mL syringe halfway up the barrel and attach the sorbent tip used in steps 3-8.
7. Wet the sorbent with Eluting Medium; watch as the liquid rises through the sorbent particles and stop when the liquid reaches the filter plug in the sorbent tip.
8. Eject the liquid and sorbent particles out of the tip and into the sample preparation vial. Repeat step 7 and 8 until the sorbent particles in the tip are expelled into the sample preparation vial. Only the filter disk should remain in the empty tip, see figure 4. Keep the syringe as it can be reused with many other samples.
9. Using the adjustable Drummond Dialamatic Microdispenser (included) transfer 50µL Reagent 4 into the sample preparation vial.

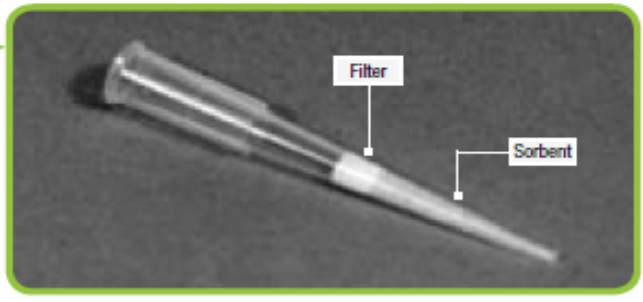
3.4 Optimizing Sample Preparation Time

For experienced users, sample preparation proceeds in 7-8 minutes per sample. This process can be further improved by preparing up to ten samples at a time. For example, at step 2 dispense Reagent 1 (and at later steps all other reagents) in ten vials successively, using the same pipette tip. At step 9, after dispensing Reagent 4, vortex 2-3 vials simultaneously. During each one minute wait at steps 10-12, prepare autosampler vials for sample transfer.

Sorbent Tip - (Figure 4)

Wet the sorbent with Eluting Medium and stop before it gets to the filter then eject the liquid and sorbent particles out of the tip.

Figure 4



4.0 Gas Chromatographic Analysis

4.1 Column For EZ:faast Physiological Amino Acid Analysis by GC-MS

The Zebron ZB-AAA GC column comes without a cage. Connect the ends of the column in the usual manner; rest the column coil on the oven bracket. Keep the pieces of thermal thread spaced evenly around the column coil. The maximum column temperature is 320/340°C.

Caution: Always use safety glasses while installing the GC column.

4.2 Instrument Settings:

GC	
Injection*	Split 1:15@250°C, 1.5-2µL (with hot needle, see section 4.5)
Carrier Gas	Helium 1.1mL/min constant flow
Oven program	30°C/min from 110° to 320°C
MS	
MS Source	240°C
MS Quad	180°C
Auxiliary	310°C
Scan Range:	45-450 m/z
Sampling Rate	2² (3.5 scans/s)

*When using a Shimadzu GC instrument, please increase the injector temperature to 300°C

4.3 Liners

Use the best deactivated liners supplied by the instrument manufacturer. Good results were obtained with FocusLiners™ (included; Phenomenex P/N AG0-4680; fits Agilent and Varian 1177 injectors). In general, the liner should carry a plug of silanized quartz or pesticide grade glass wool.

4.4 Injection

- Split injection at a ratio of 1:10 to 1:20 is recommended
- Injection volumes of 1.5-2µL are optimal

Quasi-splitless injection mode will produce a 5 to 10 fold increase in sensitivity with some instruments. In this mode, the split valve should be closed for an initial 5 to 7 seconds. Before selecting this injection mode it should be checked experimentally that no significant discrimination of late eluting amino acid derivatives takes place in comparison with common split injection. Alternatively, instruments equipped with EPC/AFC can be operated with double initial head pressure for 6-10 seconds.

4.5 Sampling

Both autosampler and manual sampling can be performed. If manual sampling is preferred, hot needle injection is recommended to prevent discrimination of components with high boiling temperatures. With this technique the sample plug is completely drawn into the syringe barrel, leaving the needle empty. The needle is inserted and kept in the hot injector for about two seconds before injection.

4.6 Calibration Standards

For quantitation purposes, mixtures of amino acid standards should be prepared following the Sample Preparation by SPE and Derivatization procedure described in this manual in Section 3.3. Standard mixtures should be stored in the freezer as some amino acids are not stable in solution. Three vials of different standard mixtures are included in the kit:

SD1: 23 amino acids, 200 nmoles/mL each, as follows:

AAA	ASP	GLY	LEU	PHE	THR
ABA	ßAIB	HIS	LYS	PRO	TYR
alLE	C-C	HYP	MET	SAR	VAL
ALA	GLU	ILE	ORN	SER	

SD2: Complementary amino acids not stable in acidic solution, 200 nmoles/mL each, as follows:

ASN	GLN	TRP

SD3: Complementary urine amino acids, 200 nmoles/ mL each, as follows:

APA	CTH	GPR	HLY	PHP	TPR

A typical chromatogram of a mixture of all three amino acid standard solutions included in this kit. Column and instrumental settings as specified in section 4.1-4.2.

Figure 5

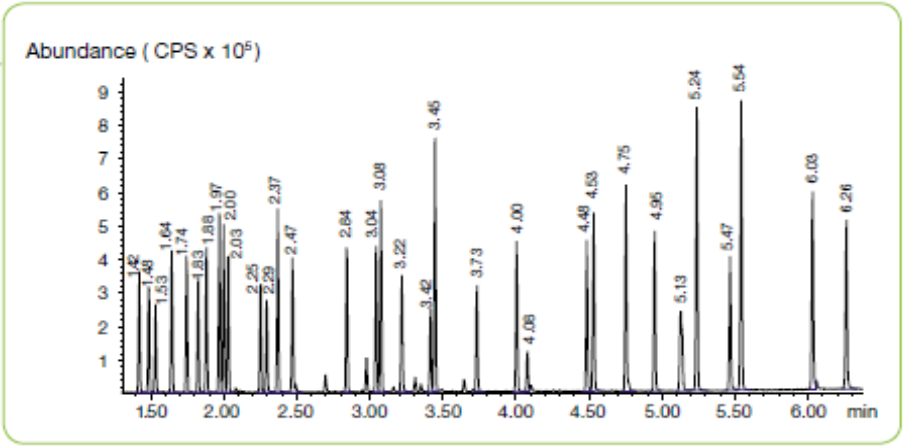


*Figure 5. Elution order: tR 1.42min ALA; 1.48 SAR; 1.53 GLY; 1.64 ABA; 1.74 VAL; 1.83 BAIB; **1.88 IS = NVAL**; 1.97 LEU; 2.00 alLE; 2.03 ILE; 2.25 THR; 2.29 SER; 2.37 PRO; 2.47 ASN; 2.84 TPR; 3.04 ASP; 3.08 MET; 3.22 HYP; 3.42 GLU; 3.45 PHE; 3.73 AAA; 4.00 APA; 4.08 GLN; 4.48 ORN; 4.53 GPR; 4.75 LYS; 4.95 HIS; 5.13 HLY; 5.24 TYR; 5.47 PHP; 5.54 TRP; 6.03 CTH and 6.26 C-C.*

4.7 Calibration Procedure

Use the following standard amino acid mixtures and make duplicate injections of each to generate the desired calibration:

Calibration Solution

I. 25µL of SD1 solution, plus 25µL of SD2 (and/or SD3 depending on your application), plus 100µL Reagent 1 (calibration level I:50 nmoles/mL)

II. 50µL SD1+ 50µL SD2 (and/or SD3) + 100µL Reagent 1 (calibration level II:100 nmoles/mL)

III. 100µL SD1+ 100µL SD2 (and/or SD3) + 100µL Reagent 1 (calibration level III:200 nmoles/mL).

The concentration of the internal standard (IS= Norvaline) in the sample prepared for gas-chromatographic analysis is 200 nmoles/mL (serum/urine/etc.).

Note: *Disregard the first 3-5 injections when performing method calibration or after replacing column or liner. These injections act as primers and mask active sites inside the liner and chromatographic column. Use subsequent duplicate runs for calibration.*

Remember: the SD1, SD2, and SD3 vials should be placed in the freezer after use! Allow standards to reach room temperature before use.

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