BIOREFINERY PROCESS DEVELOPMENT FOR RECOVERY OF FUNCTIONAL AND BIOACTIVE COMPOUNDS FROM LOBSTER PROCESSING BY-PRODUCTS FOR FOOD AND NUTRACEUTICAL APPLICATIONS

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Abstract

Australia is the world's largest producer of lobster after the USA and Canada with annual production around 10,000 tons, valued at approximately $400 million. The Australian lobster processing industry annually produces about 3,000 tons of by-products including heads, shells, and livers not only costing about $500,000 for disposal treatments, but also considering environmentally unfriendly. Continued production of lobster processing by-products (LPBs) without any attempt for efficient utilisation will represent both financial and environmental challenges to the Australian lobster processors. However, analysing chemical composition of Australian LPBs showed these biomaterials are rich in proteins (43.5, 41.1, and 29 % in heads, livers, and shells respectively), chitin (25 and 19.8 % in shells and heads), lipids (24.3 % in livers), and minerals (36 and 31.6 % in shells and heads). These components could be economically recovered for applications in food and nutraceutical products by developing appropriate processes using emerging technologies. As there is the global demand for lobster products while the lobster extracts have a wide range of applications in several aspects, utilisation of Australian LPBs for production of functional and nutraceutical ingredients would not only generate economic benefits but also reduce the waste problems.

Although lobster livers contain a high proportion of protein and lipids, utilisation of this material faces a significant challenge due to its Arsenic (240 mg/kg) and Cadmium (8 mg/kg) content over the regulatory limits for fishery products (2 mg/kg). However, using supercritical carbon dioxide (SC-CO_2) extraction for recovery of lipids from lobster livers has shown significant advantages. Nearly 94 % of the lipids in lobster livers were recovered by the SC-CO_2 extraction at 35 MPa, 50 ^oC, in 4 hours. The extracted lipids contained approximately 31.3 % polyunsaturated fatty acids (PUFAs) in which long chain ω-3 fatty acids (DHA, EPA) accounted for 58 %. As compared with the lipids extracted by the Soxhlet method, PUFAs in the SC-CO_2 lipids was fourfold higher (31.3 vs 7.8 %) while this value for ω-3 fatty acids were six fold (18 vs 3.1 %). Moreover, the SC-CO_2 lipids were rich in carotenoids (astaxanthin 41.6 μg/mL, β-carotene 70.4 μg/mL). Particularly, inorganic Arsenic and Cadmium contaminants of the SC-CO_2 lipids were very low compared with the regulatory limit of Australian New Zealand Food Standard (ANZFS) (0.05 and under 0.01 mg/kg vs 2 and 0.5 mg/kg, respectively). Total amount of Arsenic in the SC-CO_2 lipids was fourteen fold lower than that in the Soxhlet lipids (2.2 vs 31 mg/kg) whereas this figure for

Cadmium was twenty-seven fold. With very low heavy metal contaminants, the extracted lipids could be used for food and nutraceutical applications.

Several available techniques could be applied for protein recovery from lobster heads but isoelectric solubilisation and precipitation (ISP) coupled with ultrasound performed as a highly effective method. For protein recovery by the ISP method, cold-base extraction (4 ^{o}C) is usually preferable but extraction protein recovery (EPR) obtained in this study was relatively low (63.2 %). Although hot-base extraction could improve with a significant increase in EPR from 63.2 to 85.1 %, the harsh conditions of the hot extraction (85 ^{o}C) could degrade protein quality. Ultrasonic extraction exhibited its high efficiency with a significantly high EPR compared with the cold-base extraction (63.2 vs 90.5 %) and the ultrasonic EPR was equivalent or even higher than that of hot extraction (85.1 vs 90.5 %). Ultrasonic extraction was considerably affected by water ratio indicated by the fact there was a significant difference in the EPR between water ratio of 6 and 8 mL/g. High EPR (99 %) could be even achieved in very short time (5 minutes) as water ratio of 8 mL/g was used. Over 82 % protein in the extracted solution was efficiently recovered by isoelectric precipitation at the pH 3.5. Adding chitosan with dose of 200 – 250 mg/L could improve precipitation protein recovery (PPR, 86.6 %). Protein recovered by the ultrasonic extraction contained more protein than by the hot-base extraction (83.2 vs 78.6 %) whereas essential amino acids (EAAs) of ultrasonic-extraction protein was significantly higher than hot-extraction protein (38 vs 30.4 %). Moreover, digestibility of the protein extracted by ultrasound was significantly higher than that of the hot-extraction protein (78.4 vs 58.4 %), which was comparable to that of egg protein (88.1 %).

In contrast to protein in lobster heads, proteins in lobster shells are tightly associated with chitin intensified by mineralisation for a strong shell structure. Since lobster shells contain high chitin with several excellent functional properties and versatile biological activities which have many useful applications in agriculture, food, cosmetics, pharmaceuticals, and biomedicine, production of chitin from lobster shells has become a great interest. Although several approaches could be applied for removal of protein and minerals from lobster shells, chemical deproteinisation and demineralisation is the most common method. However, this method has several drawbacks such as potential negative impacts to the environment, negative effects on physicochemical properties of final products, not allowing the recovery of food compatible protein and minerals. In this study, protein in lobster shells was efficiently removed by microwave-intensified enzymatic deproteinisation with alcalase to shells ratio 1 %, 55 ^{o}C, and pH 8.0 for 90 minutes. High efficiency

of the microwave over the conventional process was indicated by its high weight loss (30.3 vs 24.6 %) and deproteinisation degree (85.8 vs 58.03 %) but low residual protein in deproteinised shells (65.4 vs 96.4 mg/g). Moreover, protein recovered from the microwave process had good functionalities (solubility over 85 %, water binding 2.5 fold higher than that of egg protein, fat binding 2.3 mL/g, emulsification 51.3 % and foaming capacity 91.3 %), high nutritional quality (74 % protein, 34 % EAAs, low lysine/arginine ratio: 0.7), which could be used as a functional or nutrient ingredient in food products. In addition, the generation of enzymatically deproteinised shells (EDPS) with low protein residues could facilitate a sequential demineralisation step.

Although demineralisation of the EDPS with strong inorganic acids has high efficiency, this process is environmentally unfriendly and harmful for chitin quality caused by deacetylation and depolymerisation. Alternatively, demineralisation of the EDPS with lactic acid intensified by microwave has indicated several significant advantages. The process was optimised at a mild condition (lactic acid to shell (LA/S) ratio of 18 mL/g, 100 ^{o}C, and 23 minutes) achieving a high degree of demineralisation (99.2 %). Chitin obtained from the optimised process had low residues of protein (1.6 %) and mineral (0.99 %), which is suitable for advanced applications. Its mineral residue was tenfold lower than that of chitin demineralised by the stirring method (0.99 vs 10 %). As compared with chitin conventionally demineralised by lactic acid with LA/S ratio of 50 mL/g, 100 ^{o}C in 60 minutes, mineral residue of lobster chitin was 3.7 fold lower (0.99 vs 3.7 %) or it was comparable to chitin demineralised by kitchen microwave with LA/S ratio of 200 mL/g, 121 ^{o}C in 30 minutes (0.99 vs 0.22 %). The chitin obtained by this demineralisation process had a chemical structure very similar with the high quality chitin based on analysis from the FTIR-spectra. Particularly, this process also allowed the recovery of minerals from lobster shells with a good mineral profile (87 % calcium) potentially useful as another value-added product.

Extracts recovered from Australian LPBs were demonstrated with high quality attributes, which show good potential for food and nutraceutical applications. Lobster chitin with high DA (89.9 %) and high binding capacity of fat and cholesterol tested on various oils (7.5 – 16.1 g/g for fat and 258.7 mg/g for cholesterol) indicates its promising application for weight loss and cholesterol management products. Fat, cholesterol binding property over its high-quality counterparts demonstrates the great potential of lobster chitin for fat blocker production. Minerals recovered from lobster shells under the form of lactate salt with a good profile (87 % calcium) show this product could be directly marketable as a food ingredient since calcium lactate has several

applications in the food industry. Moreover, high solubility and bioavailability of lobster calcium over a commercial dietary calcium supplement (60.6 vs 8.3 % and 11.3 vs 1.9 %) demonstrated its potential in the production of calcium supplements. Lobster proteins with high protein content (74 - 83.2 %), rich in EAAs (34 - 39 %), and easy digestibility (78.4 - 96.9 %) could be directly marketable as lobster protein powders (LPPs). Possessing pleasant flavours, application of LPPs for flavouring purposes was demonstrated by food panellists. LPPs (1 %) formulated with lobster lipids generated pleasant flavours in the lobster flavour blends. Strong flavour of lobster lipids showed its promising application as flavorants. Using 2 % lobster lipids for production of infused lobster oils or 4 % for salt plated with lobster lipids were demonstrated by the evaluation panel. Particularly, richness in PUFAs (31.3 %), omega-3 (18 %), carotenoids (astxanthin 41.6 μg/mL and β-carotene 70.4 μg/mL) of lobster lipids demonstrated this product could be directly marketable as a dietary supplement.

Production of functional and nutraceutical lobster extracts from Australian LPBs was found to be not only promising at the laboratory scale but also feasible at the industrial scale. Economic feasibility was analysed for production of lobster extracts from different LPBs (livers, heads, and shells) in three production scales (A, B, and C equivalent to using 30, 52.2, and 74 % of Australian LPB availability, respectively) using the commercial simulation software, Superpro Designer. The result shows production of extracts from lobster shells by the integrated process required more total capital investment (TCI) than that from other LBPs (heads or livers) in all scales (17.414 vs 6.418 and 3.379 million USD, 22.872 vs 11.010 and 3.919 million USD, 27.559 vs 13.584 and 4.412 million USD for scales of 30, 52.2, and 74 %, respectively). However, production of lobster extracts by the integrated process was found to be the most financially interesting since this process could generate the highest net present value (NPV, 37.665 vs 15.193 and 5.777 million USD, 81.637 vs 42.729 and 9.535 million USD, 126.502 vs 72.504 and 13.975 million USD for scales of 30, 52.2, and 74 %, respectively). Production at the large scale (using 74 % of LPBs) was more feasible than at the small scales (using only 30 or 52.2 % of LPBs) found in all LPBs (livers, heads, and shells). This was determined by the increases in revenues and profitability due to upscaling over the rise in its TCI and AOC (eg. TCI of lipid production increased by 16 - 30.6 % while its NPV grew by 65 – 141.9 %). Although the cost of LPBs with 1,000 USD/ton was applied for all the processes, their profitability was still high. Even in the worse scenario with only 30 % of Australian LPB availability for utilisation, these industrial productions were still feasible indicated by their positive NPVs and not-over-three-year payback time (4.608 million USD and 2.63 years, 13.270 million

USD and 2.20 years, and 36.697 million USD and 2.22 years for production extracts from lobster livers, heads, and shells, respectively). All these results reveal that investing on these lobster projects are financially attractive due to the low risk with the low TCIs and AOCs and the short payback time.

Declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Signed.....

Date 28/02/2017

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List of publications

1. Nguyen, T. T.; Zhang, W.; Barber, A. R.; Su, P.; He, S., **2015.** Significant enrichment of polyunsaturated fatty acids (PUFAs) in the lipids extracted by supercritical CO_2 from the livers of Australian Rock lobsters *(Jasus edwardsii)*. *Journal of Agriculture and Food Chemistry,* A-H, doi: 10.1021/jf5059396

2. Nguyen, T. T., Zhang, W., Barber, A. R., Su, P., & He, S., **2016**. Microwave-intensified enzymatic Deproteinization of Australian Rock lobster shells *(Jasus edwardsii)* for the efficient recovery of protein hydrolysate as food functional nutrients. *Food and Bioprocess Technology*, 1-9, doi:10.1007/S11947-015-1657-y

3. Trung T. Nguyen, Andrew R. Barber, Wei Zhang, Paul Smith, Xuan Luo, **2017**. Application and optimization of the highly efficient and environmentally-friendly microwave-intensified lactic acid demineralization of deproteinized Rock lobster shells (*Jasus edwardsii*) for chitin production. *Food and Bioproducts Processing,* 102, 367-374.

4. Trung T. Nguyen, Kendall Corbin, Andrew R. Barber, Wei Zhang, **2017**. Lobster processing by-products as valuable bioresource of marine functional ingredients, nutraceuticals, and pharmaceuticals. *Bioresources and Bioprocessing*, (submitted manuscript with the publisher's accepted letter enclosed).

5. He, S., Nguyen, T., Zhang, W., & Peng, S., **2016**. Protein hydrolysates produced from rock lobster *(Jasus edwardsii)* head: emulsifying capacity and food safety. *Food Science and Nutrition*, 1-9, doi: 10.1002/fsn3.352.

6. Lorbeer, A. J.; Lahnstein, J.; Bulone, V.; Nguyen, T.; Zhang, W., **2015.** Multiple-response optimization of the acidic treatment of the brown alga *Ecklonia radiata* for the sequential extraction of fucoidan and alginate. *Bioresource Technology.*

The following above publications have evolved from this doctoral dissertation: 1-5

Abbreviations

LPBs - Lobster Processing By-products

SC-CO_2 - Supercritical Carbon Dioxide

PUFAs - Polyunsaterated Fatty Acids

ANZFS - Australian New Zealand Food Standard

ISP - Isoelectric Solubilisation and Precipitation

EPR - Extraction Protein Recovery

PPR - Precipitation Protein Recovery

EAAs - Essential Amino Acids

EDPS - enzymatically deproteinised shells

CGLS - Cooked Ground Lobster Shell

LSPH - Lobster Shell Protein Hydrolysate

LHB - Lobster Head By-product

LHP - Lobster Head Protein

LA/S - Lactic Acid to Shell ratio

SFE - Supercritical Fluid Extraction

SC-CO_2 - Supercritical Carbon Dioxide

FTIR - Fourier Transform Infrared Spectroscopy

NMR - Nuclear Magnetic Resonance

DA - degree of acetylation

LPPs - Lobster Protein Powders

DFC - Direct Fixed Capital

TPDC - Total Plant Direct Cost

TPIC - total plant indirect cost

TCI - Total Capital Investment

AOC - Annual Operation Costs

NPV - Net Present Value

ROI - Return On Investment

CHAPTER 1 INTRODUCTION AND LITERATURE REVIEW

1.1 Global lobster processing industry generates a large amount of by-products

In 2004, the global production of lobster yielded 165,367 tons (Holmyard & Franz, 2006) which was an estimated value at $3.32 billion. Over the last decade, these figures have been on the rise with 304,000 tons (captures and aquaculture) in 2012 (Sabatini, 2015). Lobster production can be found across the world; however, the majority of production is concentrated in only three countries: Canada (34%), America (29%), and Australia (11%) (Figure 1.1) (Annie & McCarron, 2006) with the four main commercial lobster species including the American lobster *(Homarus Americanus)*, Tropical or Spiny lobster *(Panulirus sp)*, Rock lobster *(Jasus sp)*, and European lobster *(Homarus gammarus)*.

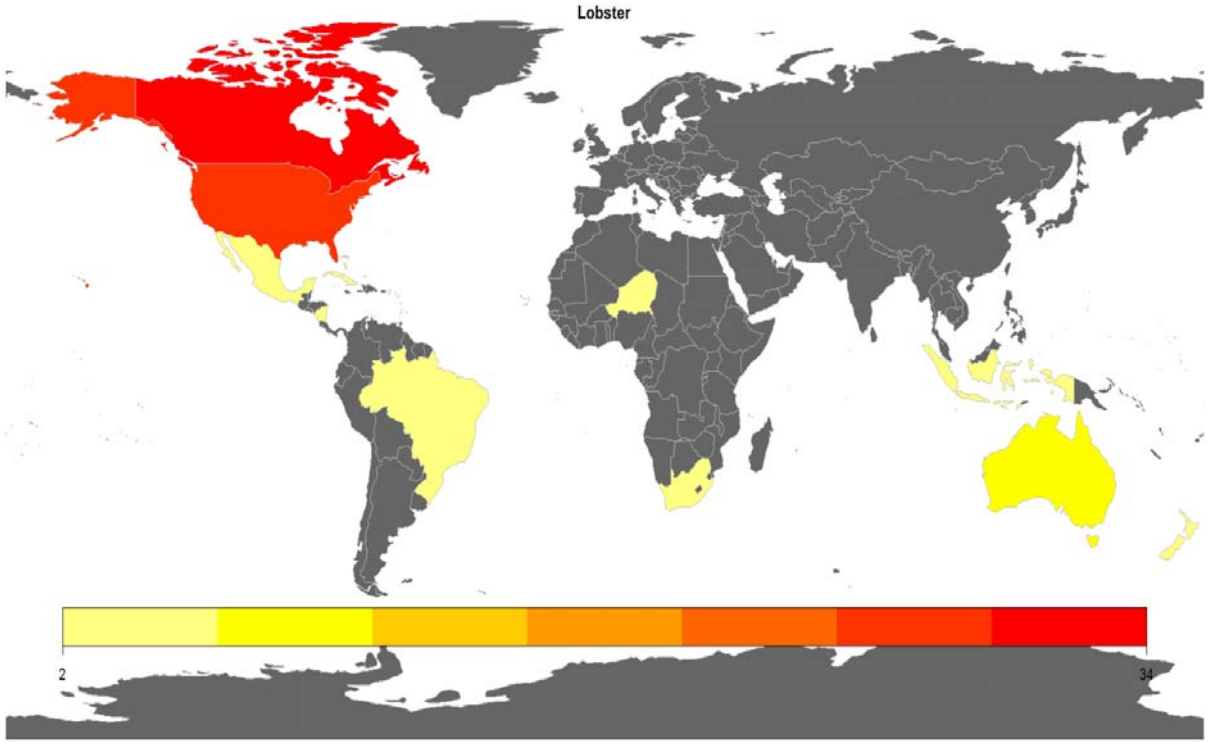

Figure 1.1 Major lobster producing countries in the world with their contribution to the global production

The most abundant species produced in the world is the American lobster which is mainly harvested in Canada and America (Figure 1.2) (Annie & McCarron, 2006). In 2012, both Canada and America produced 140,000 tons of lobsters (Thériault, Hanlon, & Creed, 2013) with the majority (74,790 tons, valued at $ 662.8 million) originating from Canada (Ilangumaran, 2014). The second

most readily available commercial species is Spiny lobster accounting for 38% of the global production while this contribution of the Rock lobster is 6%. This species is predominantly harvested from Australia, which includes four main commercial species: Western rock lobster (60%), Southern rock lobster (30%), Tropical rock lobster (8%), and Eastern rock lobster (2%) with the total yield about 9,650 tons annually (Gary, 2012). The term 'Rock lobster' has been used to describe lobster species such as *Jasus* and *Panulirus* which are caught by Australian lobster fishery (Holmyard & Franz, 2006).

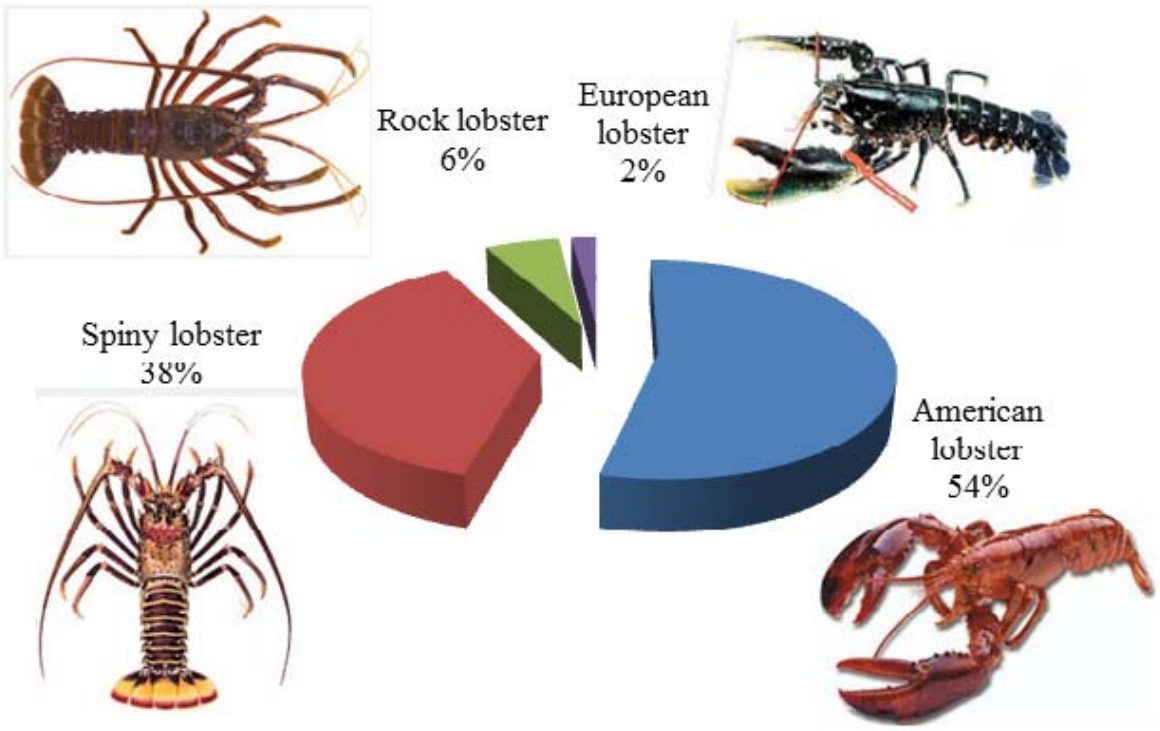

Figure 1.2 The four main commercial lobster species in the world

As lobster are consumed globally but are predominantly produced in a few countries, there is a rapidly growing export market of lobsters. Although live lobsters are preferred by consumers around the world, the export of live lobsters is limited due to its high cost, complexity, and high rates of mortality and loss during shipment. In contrast, processing lobsters has several advantages such as ease in transport and storage, extended shelf life, availability of the products, convenience in food preparation, and potential to add value to raw products. The ease in handling and increase in profits has resulted in over half of the landed lobster in the major lobster producing countries such

as Canada, America, and Australia being processed (Barker & Rossbach, 2013; Denise & Jason, 2012; Ilangumaran, 2014).

Lobsters are commercially processed into various products such as fresh lobster meat, picked lobster meat, canned lobster, lobster medallion, whole cooked lobsters, and frozen lobsters (Holmyard & Franz, 2006). In the commercial production of lobster, a substantial amount of inedible parts are removed including heads, shells, roe, and livers (Figure 1.3) which are traditionally discarded. Types and proportion of lobster processing by-products (LPBs) vary depending on the processing process (Table 1.1) but on average accounts for around 75 % of the starting material. As a result of this, the annual estimate of LPBs produced by Canada, America, and Australia was about 50,000 tons.

Figure 1.3 Different by-products generated from the commercial lobster processing industry

To maximise the production yield and profit, lobster processors have begun to utilise LPBs for processing products such as lobster tomalley, lobster roe, lobster concentrate, and lobster meat paste (Holmyard & Franz, 2006). However, the amount of LPBs being utilised is still very limited due to lack of the efficient and standardised techniques to transform these materials into a marketable form. Thus, the vast majority of LPBs is discarded at a cost to lobster processors at around $150 per

ton in Australia (Knuckey, 2004). This not only costs lobster processors a large sum of money (estimated about $7.5 million per year) for waste treatment and management, but also is considered to be environmentally unfriendly. As a result, the utilisation of these LPBs for recovery of marine functional and nutraceutical ingredients for incorporation into value-added products could bring significantly economic and environmental benefits.

Table 1.1 Types and rates of LPBs generated from the commercial processing industry

Lobster processing industry	By-products	By-products proportion	References
Canning production of Canadian lobsters	Lobster body	45 %	Ross (1927)
Production of Canadian lobster meat	Lobster head, hard carapace, viscera, mandibles, and gills	> 75 %	Y. Tu (1991)
Production of Brazilian lobster tails	Lobster head (cephalothorax)	75 %	Vieira, Martin, Saker-Sampaiao, Omar, and Goncalves (1995)
Fresh meat picked of Australian rock lobster	Lobster head, shell, and viscera	60 %	Lien (2004)
High hydrostatic pressure production of American lobster meat	Lobster shells, viscera, residual meat	75 – 80 %	Denise and Jason (2012)

1.2 LPBs as a bioresource of marine functional ingredients, nutraceuticals, and pharmaceuticals

In recent years, fishery resources have decreased significantly because of overfishing coupled with poor management. In response to this serious situation, many great efforts have been made on utilisation of fishery processing by-products in general and LPBs in particular. The most common traditional utilisation of LPBs is used for soil amendment (Cousins, 1997). This could be an informal method for disposal of LPBs with efforts for utilisation but bring no economic benefits for lobster producers. Recently, LPBs have been identified as an important bioresource for recovery of marine functional, nutraceutical, and pharmaceutical ingredients (Table 2) since these by-products

are rich in protein, chitin, lipids, minerals, and astaxanthin (Trung T. Nguyen, Zhang, Barber, Su, & He, 2015; Trung T. Nguyen, Zhang, Barber, Su, & He, 2016; Y. Tu, 1991).

Table 1.2 Overviewing utilisation of LPBs for recovery of functional, nutraceutical, and pharmaceutical ingredients

Lobster processing by-products	Functional ingredients, nutraceuticals, and pharmaceuticals	Suggested application fields	References
Lobster shells (carapace)	Chitin, chitosan	Water treatment	Gustavo, Galo, and Alexei (2005) Pathiraja (2014)
	Chitin, chitosan, chitin-oligosaccharides, chitosan-oligosaccharide	Agriculture	Borges et al. (2000) Falcón, Ramírez, Márquez, and Hernández (2002) J. C. Cabrera and Van Cutsem (2005) Falcón Rodríguez et al. (2010) Ilangumaran (2014)
	Chitosan, chitosan film	Food processing and preservative	Defang, Gang, Pengyi, and Zhiwei (2001) Garcıa et al. (2015)
	Water-soluble chitosan, chitosan particles (Micro and Nano)	Pharmacy	Safitri, Fajriah, Astriandari, and Kartika (2014) De la Paz et al. (2015)
	Chitosan film	Biomedicine	Malho et al. (2014) Qi (2015)
	Carotenoprotein	Aquafeed	Dauphin (1991); Simpson, Dauphin, and Smith (1993); Y. Tu (1991); Y. Tu et al. (1991)
	Astaxanthin	Food, nutraceutical, pharmaceutical; feed additive	Auerswald and Gäde (2008); Denise and Jason (2012); Gäde and Auerswald (2005)
	Proteins	Food and nutraceutical	Trung T. Nguyen et al. (2016); Oviedo, Garcia, Mendez, and Henriques (1982)
	Flavours and nutrient broth	Crackers, biscuits	Lien (2004)

Lobster heads (cephalothorax)	Body meat, breast meat, and leg meat	Lobster paste, canned products	Ross (1927)
	Lobster meat	Gourmet food products	Samuel P. Meyers and Machada (1978)
		Feed additive	Daniel (2008)
	Lobster protein hydrolysate	Flavour enhancer, protein supplement	Vieira et al. (1995)
Lobster roes	Raw roe	Lobster paste, canned products	Ross (1927)
Lobster livers (hepatopancreas)	Raw liver	Lobster paste, canned product,	Ross (1927)
	ω-3 rich lipids	Lobster oils, infused oils	Trung T. Nguyen et al. (2015); Tsvetnenko, Kailis, Evans, and Longmore (1996)
Lobster blood (haemolymph)	Phenol oxidase	Anti-microbial proteins	Fredrick and Ravichandran (2012)
	Crustin	Anti-microbial proteins	Battison, Summerfield, and Patrzykat (2008); Pisuttharachai et al. (2009)
	Bioactive fragment	Pharmaceutical and/or cosmetic treatment of viral and other neoplastic or pre-neoplastic mammalian tissue lesions	Bayer (2015)

1.2.1 Proteins

By-products from lobster processing are an excellent source of proteins. For example, the lobster liver (green) contains up to 41 % protein on dry basis (Trung T. Nguyen et al., 2015). Furthermore, the residual body meat, breast meat, and leg meat recovered from lobster heads (cephalothorax) are a major source of protein, which accounted for up to 20 % of the lobster weight (Vieira et al., 1995). In addition, lobster shells (carapace) constituted by a large amount of proteins (about 25 %) are another potential source for protein mining (Trung T. Nguyen et al., 2016).

The amino acid profile of lobster protein is comparable to that of red meat protein but it contains more nonprotein nitrogen (amino acids, small peptides, trimethylamine oxide (TMAO), trimethylamine, creatine, creatinine, and nucleotides) from 10 - 40 %. Thus, lobster protein is more palatable than meat proteins (Vazhiyil Venugopal, 2009). As compared with other marine species such as finfish, proteins derived from lobster, shrimp, crab, and krill generally contain larger amounts of arginine, glutamic acid, glycine, and alanine, this makes crustacean proteins more palatable than finfish proteins. Moreover, the nutritional value of crustacean protein is equal to or better than that of milk protein (casein) and red meat proteins because of its ideal essential amino acid pattern (Vazhiyil Venugopal, 2009). Lobster proteins are rich in all the essential amino acids (EAA) with its proportion approximately 41.2 % for protein in lobster head meat (Vieira et al., 1995) and 34 % for lobster shell protein (Trung T. Nguyen et al., 2016). The nutritional value of lobster protein is fortified significantly when it naturally combines with large amount of astaxanthin (295 μg/g) as a powerful antioxidant to generate a protein complex known as carotenoprotein found in lobster shells with high proportion (16 %) (Y. Tu et al., 1991).

Apart from its high delicacy, palatability, and nutritional values, lobster proteins have also been shown to have functional properties, which may be advantageous industrial applications. For example, protein hydrolysates produced by enzymatic hydrolysis of lobster head meat have been shown to have excellent wettability, high solubility, and emulsification (Vieira et al., 1995). Similarly, lobster shell protein (LSP) recovered by aqueous extraction showed good foaming and emulsification properties together with excellent solubility (over 93 %) which is independently from the pH value and the ionic strength of the solution used (Oviedo et al., 1982). More recently, LSP recovered during the microwave-intensified enzymatic deproteinisation for chitin production was found to have a positive effect on water binding of meat proteins (Trung T. Nguyen et al., 2016). Beef mince added with 2 % of LSP resulted in its water binding capacity increased significantly by 32 %, which was 2.5 times higher compared with the addition of egg white protein. In addition, the emulsifying property of lobster protein hydrolysate (LPH) (69.7 m^2/g) was significantly higher than that of cow gelatine (50.3 m^2/g) used as a common commercial emulsifier in the food industry (He, Nguyen, Zhang, & Peng, 2016).

1.2.2 Chitin and chitin derivatives

Chitin is a cationic linear polysaccharide composed of β-(1~4)-linked *N*-acetyl-D-glucosamine monomers (Figure 1.4a) and is the second most abundant biopolymer only after cellulose in the

biosphere (Kumar, 2000; Kurita, 2006). Chitin is present in lobster shells with contents of 16 - 23 % in the form of α-chitin (Lien, 2004; Rinaudo, 2006; Y. Tu, 1991). Chitosan represents a family of N-deacetylated chitin with various degrees of deacetylation (Figure 1.4b) while chito-oligosaccharides (COS) are derived from chitin and chitosan by either chemical or enzymatichydrolysis.

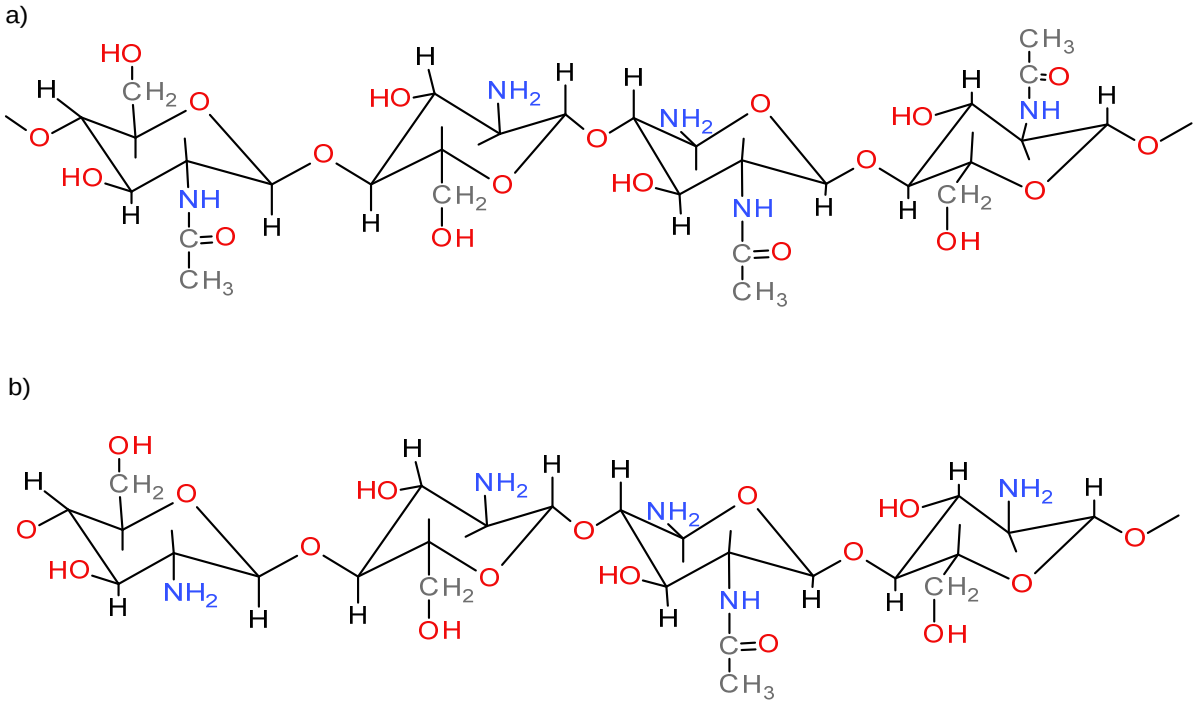

Figure 1.4 The chemical structure of the main polymer present in lobster shells, chitin (a) and the N-deacetylated form of chitin, chitosan (b)

LPB such as lobster shell waste is abundant and rich in chitin attributing to its importance as a potential source of commercial chitin. The utilisation of LPB for recovery of chitin has received great interest since chitin is a natural biopolymer with high biodegradability, biocompatibility and nontoxicity (Karagozlu & Kim, 2015). Derivatives generated from chitin such as chitosan and COS have significant potential economic values with more than 200 applications in water treatment, food, agriculture, healthcare products, environmental sector, pharmaceuticals, and biomedicine (Kaur & Dhillon, 2013; Muzzarelli, 1989; Sandford, 1989; Synowiecki & Al-Khateeb, 1997).

1.2.3 Lobster lipids

Australian LPB such as lobster cephalothorax has been used as a raw material for lipid recovery. High yield of lipids (19.4 %) has been extracted from this material and the extracted lipids were rich

in polyunsaturated fatty acids (PUFAs) with 22.6 % in which ω-3 fatty acids accounted for 50.8 % (Tsvetnenko et al., 1996). More recently, lobster livers generated from the Australian lobster processing industry were studied for lipid extraction (Trung T. Nguyen et al., 2015). Australian rock lobster livers were reported to contain about 24.3 % of lipids but this material was found to be contaminated with arsenic (240 mg/kg) and cadmium (8 mg/kg) posing hazardous potentials for direct consumption. Nearly 94% of lipids in lobster livers were recovered using supercritical carbon dioxide (SC-CO_2) extraction with very low heavy metal contamination. The heavy metal contamination level measured met Australian New Zealand food standard. Moreover, the extracted lipids were rich in PUFAs (31.3 %) with ω-3 fatty acids accounting for 58 %. The findings in this study suggest extracted lipids from lobster livers by SC-CO_2 may be used in food or nutraceutical applications.

1.2.4 Astaxanthin

Astaxanthin is the oxygenated derivatives of carotenoids occurring widely and naturally in marine organisms including crustaceans (lobster, shrimp, crab, and krill) and fish (salmon, sea bream). Astaxanthin as one of the first pigment isolated and characterised from lobster (Kuhn & Soerensen, 1938). By-products generated from lobsters, shrimps, crabs, crayfish and krill are a vital source of natural carotenoids, mainly astaxanthin (Sachindra, Bhaskar, Siddegowda, Sathisha, & Suresh, 2007). Apart from containing large proportions of mineral salt (15 – 35 %), proteins (25 – 50 %), chitin (25 - 35 %) (J. E. Lee & Peniston, 1982), and lipids (19.4 – 24.3 %) (Trung T. Nguyen et al., 2015), LPBs are also constituted of carotenoids. Astaxanthin content in lobster by-products compared with other crustaceans is shown on Table 1.3 and its content varies depending on species, season, and environmental grown conditions. Astaxanthin exists in a free form and/or in a complex form known as carotenoprotein. Since LPB contains high proportion of astaxanthin, this biomaterial has been utilised for recovery of astaxanthin (Auerswald & Gäde, 2008; Gäde & Auerswald, 2005). Two processes have been patented for astaxanthin extraction from lobster heads (Kozo, 1997; Sunda, Zhang, Zhang, Zhang, & Qishan, 2012). Most recently, shells of American lobster derived from the high hydrostatic pressure process have been studied for recovery of food grade astaxanthin by Denise and Jason (2012). Astaxanthin is a high value product and has been increasingly marketed as a functional food ingredient with prices ranging between 3,000 - $12,000 per kg (Lordan, Ross, & Stanton, 2011).

Table 1.3 Total astaxanthin content in processing by-products of lobster and other crustacean species

Source	Total astaxanthin (mg/100g)	References
Shrimp *(P. borealis)*	4.97	Torrissen, Tidemann, Hansen, and Raa (1981)
Crawfish *(P. clarkii)*	15.3	S.P. Meyers and Bligh (1981)
Backs snow crab *(Ch. Opilio)*	11.96	Shahidi and Synowiecki (1991)
Lobster *(Homarus Americanus)*	9.8	Y. Tu (1991)

1.3 Various applications of lobster functional ingredients, nutraceuticals, and pharmaceuticals

1.3.1 Lobster protein: dietary protein supplement, food functional ingredients or flavourings

The recovery of edible meat from lobster by-products is not novel. However, the use of these by-products as a source of sustainable proteins for food products has been less explored. In one study, the meat (body meat, breast meat, leg meat, roe, and liver) recovered from Canadian lobster by-products was to create a canned food product and lobster paste (Ross, 1927). Although the final products prepared from these protein sources were flavour-rich, nutritious, and palatable, the feasibility for commercialisation was impractical as the methods for residual meat recovery was inefficient. To address this key problem, a process was developed for the recovery of food-grade lobster meats from spiny lobster heads by-product by freezing the heads and later cutting them for meat picking. The recovered lobster meat was sold as a high-value, gourmet food products (Samuel P. Meyers & Machada, 1978).

The actual flavour of lobster, itself is considered a highly valued product which may be extracted and sold, creating an additional processing stream for lobster waste. The practice of converting of LPBs into natural lobster flavours has been standardised and is now an industrial practice. The cephalothorax of Brazilian lobster by-product *(Panulirus spp.)* has been utilised for lobster flavour production by enzymatic hydrolysis of lobster head meat (Vieira et al., 1995). Hydrolysed lobster protein could be used as flavour enhancers for various formulated food products. Australian lobster shells have also been used for the recovery of flavours by either frying lobster shells with edible oils for production of infused lobster oil or cooking lobster shells to extract nutrient broth for cracker

biscuit production (Lien, 2004). Lobster protein hydrolysate (LPH) has also proposed to be used as either a dietary protein source or functional ingredients in the food industry because it contains all EAAs in high concentration and possesses good functional properties such as high solubility, excellent wettability, and emulsification. In another study, the key aroma components derived from cooked tail meat of American lobster *(Homarus Americanus)* was investigated by G. H. Lee, Suriyaphan, and Cadwallader (2001). In this study, 3-methylbutanal, 2,3-butanedione, (Z)-heptenal, 3-(methylthio)propanal, 1-octadien-3-one, and (E,Z)-2,6-nonadienal were identified as key aroma components of cooked American lobster tail meat with high odour intensities. The proteins recovered from lobster shells were also characterised and trialled various applications. Water-soluble LSP extracts exhibited favourable functional properties such as high solubility, foaming capacity, and emulsification. All of these physical properties are advantages for use as a food functional ingredient or as a protein supplement in food (Oviedo et al., 1982). More recently, LSP recovered during chitin production by enzymatic deproteinisation of lobster shells with high ratio of EAAs was shown to have a potential use as a dietary protein supplement (Trung T. Nguyen et al., 2016). Due to having very low lysine/arginine ratio, LSP could be incorporated into meat products to reduce lipidemic effects of meat protein. Moreover, LSP has the potential to be used as a food functional ingredient due to its high water, oil-binding capacity besides its foaming and emulsification. LPH recovered from Australian LPB have also shown to have higher emulsifying properties compared to cow gelatine as a commercial emulsifier used in food industry (He et al., 2016).

1.3.2 Lobster chitin, chitosan, and their derivatives as natural biopolymers for multiple applications

In recent decades, a greater knowledge of chitin chemistry accompanied with the increased availability of crustacean shells as by-products of the crustacean processing industry have led to significant development and wide application of chitin and its derivatives produced by several pathways. In particular, derivatives from chitin such as chitosan and COS are highly valued compounds as they have more than 200 commercial applications (Kaur & Dhillon, 2013; Muzzarelli, 1989; Sandford, 1989; Synowiecki & Al-Khateeb, 1997). In addition, the biopolymers generated from marine crustaceans, lobster chitin, chitosan and their derivatives have been used in multitude of industries including water treatment, agriculture, food production, pharmaceuticals, and biomedicine (Table 1.4).

Table 1.4 Various applications of lobster chitin, chitosan, and their derivatives

Application sectors	Examples	References
Water treatment	Removal of mercuric ion	Peniche-Covas, Alvarez, and Argüelles-Monal (1992)
	Removal of reactive dyes from aqueous solution	Juang, Tseng, Wu, and Lee (1997); Juang, Tseng, Wu, and Lin (1996)
	Removal of heavy metals (Cu, Hg)	Gustavo et al. (2005)
	Dye absorbents for treatment of industrial effluent	Pathiraja (2014)
Agriculture	Seedling growth and anti-mycorrhizal in tomato crop	Iglesias, Gutierrez, and Fernandez (1994)
	Fungicides on plant fungal diseases	Pombo (1995)
	Amending media and seeds for inhibition of pathogen fungus growth	Borges et al. (2000)
	Induce systemic resistance agents in tobacco plants	Falcón et al. (2002)
	Elicitors of plants defence reactions	J. C. Cabrera and Van Cutsem (2005)
	Plant growth regulators	Hipulan (2005)
	Inducing defensive agents	J.-C. Cabrera, Boland, Cambier, Frettinger, and Van Cutsem (2010); Falcón Rodríguez et al. (2010)
	Plant protection	Ilangumaran (2014)
Food	Organic polymer flocculants	Defang et al. (2001)
	Chitosan/clay film for food and medical industries	Casariego et al. (2009)
	Biodegradable packages	Hudson, Glaisher, Bishop, and Katz (2015)

	Antioxidants	Garcıa et al. (2015)
Pharmaceuticals	Direct compression excipients for pharmaceutical application	Mir et al. (2008)
	Co-diluent in direct compression of tablets	Mir et al. (2010)
	Water-soluble lobster chitosan salt as materials for drug carriers	Cervera et al. (2011)
	Targeted drug delivery film	Bamgbose et al. (2012)
	Bio mouth spray for anti-halitosis	Safitri et al. (2014)
	The stability and safety of lobster chitosan salts	De la Paz et al. (2015); Lagarto et al. (2015)
	Natural additive for pharmaceuticals	Sayari et al. (2016)
Biomedicine	Immobilization of multi-enzyme extract	Osinga, Tramper, and Wijffels (1999)
	Novel hybrid biomaterials for medical application	Malho et al. (2014)
	Biomimetic functional materials	Qi (2015)

1.3.2.1 Water treatment

One of common applications of chitin and its derivatives is for the treatment of water. Chitin and chitosan with their high absorbance, chelating and affinity properties have been used as coagulation agents, chelating polymers, or bio-absorbents in water treatment for decades. In particular, chitin and chitosan prepared from lobster has shown high affinity for metal chelating. Peniche-Covas et al. (1992) reported lobster chitosan could effectively remove mercuric ions from solutions. Lobster chitosan was later used successfully for separation of heavy metals (Cu, Hg) (Gustavo et al., 2005). The heavy metal removal efficiency of lobster chitosan was comparable to that of commercial resin. Lobster chitin/chitosan has been used for the removal of reactive dyes (vinyl sulfone and chlorotriazine) from aqueous solution (Juang et al., 1997). Lobster chitin/chitosan produced from this study had significantly high adsorption of reactive dyes capacity (50 – 500 mg/L) compared with a conventional absorbent (activated carbon). The result of this study has been recently

amplified by a study of Pathiraja (2014) where lobster chitin was used as dye absorbents for effective treatment of industrial effluent.

1.3.2.2 Agriculture

Several modes of action have been proposed for how chitin and its derivatives can be used to stimulate the plant grow and improvement of crop yields. Apart from the direct effect on plant nutrition and plant growth stimulation, chitin and chitin-derived products exhibit toxicity to plant pests and pathogens, induce plant defences, and stimulate the growth and activity of beneficial microbes (Sharp, 2013). Chitin, chitosan, and their derivatives prepared from lobster shells is also no exception, as they have been trialled as fungicides, biocides, and bio-stimulants.

For instant, lobster chitosan performed high anti-fungal activity as fungicides when it was tested on two phytogenic fungi (Pombo, 1995). Other biological effects of lobster chitosan on bio-control plants were reported by Borges et al. (2000). The author used chitosan prepared from lobster chitin to amend agar and coat tomato seeds together with menadione sodium bisulphite for inhibition of pathogen fungus growth and disease development on tomato plant seeds. The results indicated lobster chitosan significantly inhibited the fungus and diminished disease occurrence in the roots from seeds, which had been treated. In another study lobster chitin was shown to have positive effects on seedling growth and mycorrhizal infection of tomato crop (Iglesias et al., 1994). Furthermore, chitin extracted from Cuban lobster shells has been shown to have biological functions. Researchers at the University of Havana prepared chitosan from lobster chitin to coat tomato seeds as well as encapsulate somatic embryos in order to design artificial seeds for accelerating yields. Under laboratory conditions, the coated seeds exhibited considerably higher rates of germination and growth compared with the non-coated seeds. From this study it was concluded that the lobster-shell-derived chitosan served as a biological stimulant yielding better seed germination, increased plant height, enhanced stem thickness and dry biomass yield (Hareyan, 2007).

Chitin, chitosan, and COS recovered from lobster shells have also been studied for their biocide and biostimulant activities for agricultural applications. A study carried out by Falcón et al. (2002) investigated the use of lobster chitosan and chitosan oligomers for crop protection. Both lobster chitosan and its enzymatic hydrolysate exhibited high pathogen-resistant activity against *Phytophthora parasitica* on tobacco plants at low concentrations ranging from 5 – 500 mg/L. With this success, the study was extended by preparing various chitosan and chitosan-oligomers from

Cuban lobster chitin. Both lobster chitosan and its derivatives were tested for antimicrobial activity (versus fungus and oomycetes) and their ability to induce defensive and protective responses in tobacco and rice plants. Some of the lobster chitosan derivatives were found to be active protectants against infection for both cultivars at field scale (Falcón Rodríguez et al., 2010).

In another study, Peters, Sturz, and MacLeod (2006) used raw lobster shells and compost as a soil amendment to supply nutrients for plants and as a biological control method for soil-borne fungi pathogenic to potatoes. The results showed that lobster shells served as a nutrient source for plant growth, enhanced beneficial soil microbial communities, suppressed soil-borne diseases, and was an organic production process. Following this success, Ilangumaran (2014) studied isolation of soil microbes for bioconversion of lobster shells into chitin and chitin-oligomers for plant disease management. The extracted bioproducts showed significant induction of disease resistance in *Arabidopsis* confirming the findings in previous studies.

1.3.2.3 Food

Chitin recovered from spiny lobster shells has been studied for production of low molecular weight chitosan for food application using gamma irradiation. Lobster chitosan produced from this process had high antioxidant activity with the potential for use as a food preservative (Garcıa et al., 2015). Chitin and chitosan have also been used as bio-flocculants or functional biomaterials (membranes, films, and packages) in the food industry (processing, packaging or storage). Chitosan prepared from lobster was used for recovery of solids in food processing plants (Defang et al., 2001). Lobster chitosan films were used as the film matrix in combination with clay micro/nanoparticles for preparation of chitosan/clay films (Casariego et al., 2009). With its significant improvement in physical property (water solubility, water vapour, oxygen and carbon dioxide permeability, optical, mechanical and thermal properties), the lobster chitosan/clay film was proposed for use in food and medical industries. Apart from retarding moisture migration and the loss of volatile compounds, reducing the respiration rate, and delaying changes in textural properties, another advantage of chitosan films is that it is biodegradable. Thus, chitosan films are environmentally friendly alternatives to synthetic, non-biodegradable films which may be further modified to create biodegradable food packages (Hudson et al., 2015).

1.3.2.4 Pharmaceuticals and biomedicine

Recently, the applications of chitin, chitosan and their derivatives in pharmaceuticals and biomedicines have received more attention because they are not only biocompatible, biodegradable, and non-toxic (Jeon & Kim, 2000) but also exhibit biological and physiological characteristic with known medical benefits. For example these polymers and their derivatives are antioxidant, antimicrobial, anticancer, immune-stimulant, hypocholesterolemic, hypoglycaemic, angiotensin-I-converting enzyme (ACE) inhibition, and anticoagulant (Wijesekara & Kim, 2010). Furthermore due to these known beneficial properties chitin and chitin derivatives may be used as food additives, anti-cholesterol agents, and as dietary supplements.

Apart from that, chitin and chitin derivatives also possess several other physical properties that are favourable for their applications in pharmaceutical and biomedical sectors. Chitin and chitosan prepared from lobster were studied on their deformation and compaction properties for use as pharmaceutical direct compression excipients (Mir et al., 2008). In comparison with other established direct compression excipients (microcrystalline cellulose), lobster chitin/chitosan performed better at both tendency to plastic deformation and compression behaviour. This result indicates that lobster chitin and chitosan have a potential use as co-excipients for direct compression applications.

The most commonly used chitosan derivatives used in drug delivery are the water-soluble lobster chitosan acid salts (Cervera et al., 2011). Lobster chitosan salts prepared by spray-drying have a higher tendency toward sphericity, which are good excipients for pharmaceutical applications. Moreover, lobster chitosan acid salts maintain their physical, chemical and microbiological characteristics for a period of twelve months when stored correctly at room temperature in a dry place (De la Paz et al., 2015). Apart from being stable for extended periods (one year), the toxicity level of lobster chitosan acid salts is negligible. This was indicated by a study investigating the single and repeated dose toxicity of chitosan and its salts (lactate and acetate) on rats. At oral doses of 2000 mg/kg, no fatalities or changes in the general behaviour of the rats in both the acute and repeated dose toxicity studies was observed (Lagarto et al., 2015). This came to a conclusion that chitosan obtained from lobster shells may be safe for use in the pharmaceutical industry.

The welling potential of lobster chitosan film in various solvents was determined for use as targeted drug delivery or drug film (Bamgbose et al., 2012). Since it can generate a membrane with structure porous and stability in several organic solvents, lobster chitosan film could be incorporated in

devices for development of targeted drug delivery. Recently, novel hybrid biomaterials for medical application was also prepared from lobster chitosan (Malho et al., 2014). A bifunctional protein was successfully attached to lobster chitin to generate biosynthetic materials with advanced functional properties. Bio-inspired chitin/protein nanocomposites were developed using lobster chitin nanofibers and recombinant chitin-binding resilin (Qi, 2015). Furthermore, chitosan nanoparticles with its anti-oxidant, anti-microbes in combination with high absorption have been used as pharmaceutical ingredients. Recently, Safitri et al. (2014) reported chitosan nanoparticles produced from lobster had positive results in prevention and treatment of halitosis. With the significant results, lobster chitosan nanoparticles were used as an active ingredient for production of bio mouth spray anti-halitosis. Ongoing advances such as these could considerably expand the use of lobster chitin nanofibers-based composites and functional materials.

1.3.3 Lobster lipids as a natural source of ω-3 fatty acids for nutraceuticals and pharmaceuticals

Although lipids have often been condemned, the use of lipid and its products has drawn a dramatic interest in recent years due to findings related to their health effects. A part from enhancing flavor, texture, and mouthfeel to foods, lipids also provide essential fatty acids (eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), arachidonic acid (AA), and γ-linolenic acid), fat-soluble vitamins (A, D, E, K) and other minor components (phospholipids, tocopherols, tocotrienols, carotenoids, sterols, and phenolic compounds) (Rizliya & Mendis, 2014; Shahidi, 2006). Particularly, the role of carotenoids, EPA and/or DHA in heart health, mental health, brain and retina development has been well documented (Alabdulkarim, Bakeet, & Arzoo, 2012; Guerin, Huntley, & Olaizola, 2003; Swanson, Block, & Mousa, 2012; Eiji Yamashita, 2013) and such lipid constituents have been regconised as nutraceuticals and pharmaceuticals for improving human health. By this reason, fish oils have been used for enriching DHA and EPA of many food products such as powder milk formulate, salad oil, fruit beverage, vegetable juice (Kolanowski & Berger, 1999), dairy products (Kolanowski & Weißbrodt, 2007), soft goat cheese (Hughes, Brian Perkins, Calder, & Skonberg, 2012) or cookies (Jeyakumari, Janarthanan, Chouksey, & Venkateshwarlu, 2016). Lobster oils have a potential use as a source of natural ω-3 fatty acids for many fortified products since they have been demonstrated to contain PUFAs and ω-3 fatty acids as high as that of fish oil (menhaden) or krill oils (Albalat et al., 2016; Tsvetnenko et al., 1996). Moreover, higher bioavailability of fatty acids derived from crustacean oils compared with those of fish oils (Köhler, Sarkkinen, Tapola, Niskanen, & Bruheim, 2015) together with the anti-oxidant superior of

crustacean oils provided by carotenoids make them to be applied as a novel and beneficial food ingredient (Tetens, 2009) or as oil supplement (Köhler et al., 2015). With a significant richness in astaxanthin, PUFAs, and ω-3 fatty acids, lobster oil was suggested for use as a dietary supplement (T. Trung Nguyen, 2017) since oils produced from fish livers are often considered as an important source of vitamins A and D with several therapeutic properties (Gunstone, 2006; Rizliya & Mendis, 2014). In addition, lobster liver oil contains very strong specific flavors combined with the unique and inherent ability of oils for absorbing and preserving flavors would be a promising application for flavor industry. For this reason, lobster lipids were investigated for production of infused lobster oil, salt plated with lobster flavors, and lobster seasoning and obtained promising results (T. Trung Nguyen, 2017).

1.3.4 Astaxanthin as a powerful antioxidant

Oxidative molecules or free radicals such as hydroxyls, peroxides, and reactive oxygen species generated during normal aerobic metabolism are necessary for sustaining life processes. However, under certain conditions or periods of exposure such as physiological stress, air pollution, smoking, chemical inhalation or exposure to UV light, the increased production of these free radicals can be detrimental. This threat arises due to the highly reactive nature of free radicals with essential cellular components such as proteins, lipids, carbohydrates, and DNA (Di Mascio, Murphy, & Sies, 1991). As a result of oxidative damage through a chain reaction know as oxidative stress, proteins and lipids are oxidised while DNA is severely damaged. It has been suggested that diseases such as macular degeneration, retinopathy, carcinogenesis, arteriosclerosis, and Alzheimer may be induced by such damages (Maher, 2000).

The human body controls and reduces oxidation by self-producing enzymatic antioxidants including catalase, peroxidase, super oxide dismutase and other antioxidant activity molecules. However, the level of these compounds in many cases is not sufficient to protect the body against oxidative stress and an additional supplement of water soluble antioxidants (vitamin C) and lipophilic antioxidants (vitamin E, carotenoids: beta-carotene and astaxanthin) is required. The use of astaxanthin as an antioxidant has been receiving significant attention because it possesses superior antioxidant activity. Antioxidant activity of astaxanthin was found to be 10 times higher than that of zeaxanthin, lutein, canthaxanthin, and β-caroten and 100 times compared with vitamin E (Miki, 1991). With its superior antioxidant activity, astaxanthin has been used as a natural antioxidant in edible oil (Rao, Sarada, & Ravishankar, 2007), nutraceuticals (Guerin et al., 2003) and cosmetics (Tominaga,

Hongo, Karato, & Yamashita, 2012). Particularly, astaxanthin has shown great potential for promoting human health and the prevention/treatment of various diseases (Table 1.5). Its efficiency has been proven in over 65 clinical studies and featured in over 300 peer-reviewed publications (Eiji Yamashita, 2013). It should be noted that while other carotenoids can act as a prooxidant under specific condition such as high oxygen and partial pressure, there is currently no information available regarding astaxanthin.

Table 1.5 Effects of astaxanthin on promotional human health

Human health	Health benefits	References
Neurovascular protection	Decreases oxidation of red blood cells, decreases of incidences of ischemic stroke, and improves memory and learning	Yook et al. (2015); Zhang, Zhang, Wu, et al. (2014); Zhang, Zhang, Zhou, et al. (2014)
Eye fatigue relieve	Reduces eye fatigue relieve in subjects suffering from visual display syndrome	Kajita, Tsukahara, and Kato (2009); Kidd (2011); Nagaki, Mihara, Tsukahara, and Ono (2006); Serrano and Narducci (2014); Seya, Takahashi, and Imanaka (2009)
Immune system booster	Have an immunomodulating effect, strong immune system stimulator, anti-tumour, very effective for autoimmune conditions such as rheumatoid arthritis	B. Chew, Park, Chyun, Mahoney, and Line (2010); B. P. Chew and Park (2004); Jyonouchi, Sun, Tomita, and Gross (1995); Nir and Spiller (2002); Park, Chyun, Kim, Line, and Chew (2010)
Cardiovascular health	Improve blood lipid profiles, decrease blood pressure, protection from hypertension and stroke, reduces the consequences of a heart attack and vascular inflammation, reduces the area of infarction and the damage, reduces the area of infarction and the damage	Fassett and Coombes (2011, 2012); Gross and Lockwood (2005); Guerin et al. (2003); Hussein et al. (2006); Hussein et al. (2005); Iwamoto et al. (2000); Miyawaki, Takahashi, Tsukahara, and Takehara (2008)
Liver health and metabolic syndrome	improves blood lipids and increases adiponectin, prevents fatty liver disease, reduces the risk for atherosclerotic plaque,	Kindlund and BioReal (2011); Kishimoto, Yoshida, and Kondo (2016); Shen et al. (2014); Yilmaz et al. (2015)

	inhibits progression of fatty liver disease, restores insulin-glucose balance, increases fat burning and decreases inflammatory markers	
Diabetes and Kidneys	Reduces glucose toxicity and kidney inflammation; improve pancreatic function, insulin resistance and insulin sensitivity	Naito et al. (2004); Ni et al. (2015); Savini, Catani, Evangelista, Gasperi, and Avigliano (2013); Uchiyama et al. (2002)
Fertility	Improve sperm parameters and fertility	Comhaire, Garem, Mahmoud, Eertmans, and Schoonjans (2005); Donà et al. (2013); Mina, Mohammad, and Hamid (2014)
Muscle resilience	Enhances power output, endurance and recovery after exercise; prevents muscle damage and muscle atrophy	Earnest, Lupo, White, and Church (2011); Malmstena and Lignellb (2008); E Yamashita (2011)
Capillary circulation	Improves blood flow and capillary integrity; reduces blood cell oxidation and risk of thrombosis	Kanazashi et al. (2013)
Skin aging defence	Prevents UV induced wrinkle formation, skin sagging, and age-spots; improve skin elasticity and skin dryness	Seki, Sueki, Kono, Kaoru, and Eiji (2001); Tominaga et al. (2012); Eiji Yamashita (2005)

1.4. Industrially applicable techniques for efficient recovery of functional and bioactive compounds from LPBs

1.4.1 Ultrasound-intensified extraction of functional and nutritional proteins

Commercial lobster processing for fresh lobster meat, picked lobster meat or canned lobster generates large amounts of lobster by-products containing residual meat, which is frequently not recovered by hand, or using mechanical equipment. This underutilisation of the lobster results in a waste highly valuable protein, which must be disposed of accordingly, frequently at a cost to the producer. Several possibly techniques for the recovery of proteins from crustacean, fish or meat processing by-products have been considered. One of the most conventional methods used to transform fishery by-products into a marketable and consumer friendly products is the use of enzymes (Tong-Xun & Mou-Ming, 2010). Enzymatic hydrolysis with endogenous or added

proteolytic enzymes may be used. Although, the use of added enzymes allows good control of the hydrolysis and even improves or modifies the physicochemical, functional, and sensory properties of the native protein (V. Venugopal & Shahidi, 1995), the extent of reaction could be difficult to control. However, the slow rate of hydrolysis, generation of short-chain peptides, loss of functionality of the native proteins, and the absence of homogeneous hydrolysates are other major limitations of this process (Kristinsson & Rasco, 2000). Moreover, enzymes used often require an inactivation step and thus cannot be reutilised which considerably rises processing costs (Kristinsson & Rasco, 2000). In addition, low yield, taste defects, and economic feasibility are still major issues for using enzymatic hydrolysis on industrial scale. Other approaches, which have been explored, are the use of chemicals. The use of acidic or alkaline solutions for degrading proteins into peptides of varying sizes is a non-selective and rapid method used. However, the severe conditions (HCl 6 N, 118 ^{o}C, 18 hours or pH 12.5, 95 ^{o}C, 20 min) used for chemical hydrolysis can have negative consequences such as racemization, bitter taste, reduced nutritional quality and poor functionality, resulting in products of lower value, i.e. fertiliser (Chobert et al., 1996).

A more promising approach is the technique of isoelectric solubilisation and precipitation (ISP). The shifting in pH of the solutions used during this processing induces solubility of residual proteins while concurrent separation of lipids and removal of inedible parts such as shells, membranes, bones, scales, skin, not intended for human consumption (Gehring, Gigliotti, Moritz, J.C., & Jaczynski, 2011). Apart from its high efficiency of protein recovery, this process also generates proteins of high quality, which still maintain their functional properties and nutritional value (Chen, Tou, & Jaczynski, 2007; Gigliotti, Jaczynski, & Tou, 2008; Nolsoe & Undeland, 2009; Taskaya, Chen, Beamer, Tou, & Jaczynski, 2009; Taskaya, Chen, & Jaczynski, 2009c) (Reza Tahergorabi, Beamer, Matak, & Jaczynski, 2012). Since the ISP process is simple and quick, it has been used for the recovery of fish proteins at both laboratory and pilot scales using batch mode (Choi & Park, 2002; Kim, Park, & Choi, 2003; Kristinsson & Hultin, 2003; Mireles Dewitt, Gomez, & James, 2002; Undeland, Kelleher, & Hultin, 2002). Furthermore, the presence of dioxin and polychlorinated biphenyls (PCBs), one of the most predominant bio-toxic compounds in fish protein has been found to reduced significantly in the ISP-recovered proteins (Marmon, Liljelind, & Undeland, 2009). When applied to meat processing by-products such as beef (Chen, Nguyen, Semmens, Meamer, & Jaczynski, 2007; Mireles Dewitt et al., 2002) or chicken (R. Tahergorabi, Beamer, Matak, & Jaczynski, 2011; R. Tahergorabi, Sivanandan, & Jaczynski, 2012), high yields of

protein recovery were still achieved. With its significant advantages over conventional methods, the ISP process could have a great potential for application on protein recovery from LPBs.

Recently, ultrasound based extractions have been shown to be an effective technique for improving the rate of various extraction processes (Lebovka, Vorobiev, & Chemat, 2011; Majid, Nayik, & Nanda, 2015; Vilkhu, Mawson, Simons, & Bates, 2008). Ultrasound processing disrupts cells and creates micro-cavities in the tissue, which enhances the surface area and thus the penetration of the solvent into the material, mass transfer, and improves protein release. The use of ultrasound in protein extraction from fish, meat, and beef by-products resulted in higher extraction yields with reduced processing time and solvent consumption compared with the use of ISP alone (Chemat & Khan, 2011; Saleem, Hasnain, & Ahmad, 2015; Vardanega, Santos, & Meireles, 2014; Vilkhu et al., 2008). Therefore, recovery of protein by ultrasound-assisted extraction has been scaled up to an industrial level due to their high economic feasibility (Álvarez & Tiwari, 2015; Z.-c. Tu et al., 2015).

1.4.2 Supercritical fluid extraction for recovery of rich-ω-3 lipids and astaxanthin

Solvent extraction is the most common method for lipid or astaxanthin extraction (Sindhu & Sherief, 2011; Tsvetnenko et al., 1996). In this method, organic solvents including acetone, ethyl acetate, hexane, isopropanol, methanol, methyl ethyl ketone, ethanol, dichloromethane, dimethyl sulfoxide, or chloroform may be used for the extraction. Although some of these solvents can be used to extract lipids for food applications, others such as dichloromethane, dimethyl sulfoxide, and chloroform cannot be used due to their toxicity (FDA, 2010). Regardless of the solvent used there is increasing public awareness of the hazards related to the use of any organic solvents in extraction and the possibility of solvent contamination in the final extracts. Apart from that, high demands for natural astaxanthin and bioactive lipid components such as ω-3 fatty acids, physterols, tocopherols, and tocotrineols have stimulated the search for green and sustainable extraction methods (Delgado Vargas & Paredes-Lopez, 2003). One such approach that is being considered is the use of supercritical fluid extraction techniques.

In recent years, supercritical fluid extraction (SFE) has become an important technology for extracting high quality lipids from fishery processing by-products (Letisse, Rozieres, Hiol, Sergent, & Comeau, 2006; N. Rubio-Rodríguez et al., 2008). Furthermore, it is an effective separation technique in the production of nutraceutical supplements and functional foods (Parajó, Dominguez, Moure, & Diaz-Reinoso, 2008; Reverchon & De Marco, 2006). The operational conditions of SFE

is also favourable from an environmental and industrial processing viewpoint. SFE can extract bioactive nutraceuticals at moderate temperatures in a non-oxygen environment with very low lipid oxidation, selectively extracts low polar lipid compounds and does not co-extract polar impurities such as some organic derivatives containing heavy metals (Nuria Rubio-Rodríguez et al., 2012).

Supercritical carbon dioxide (SC-CO_2) extraction is presently being evaluated as a promising technology compared to conventional methods (López-Cervantes, Sánchez-Machado, & Rosas-Rodríguez, 2006) due to its ability to extract the heat-sensitive, easily oxidised compounds (PUFAs, ω-3 fatty acids, astaxanthin) without the use of toxic solvents. Moreover, CO_2 is a generally recognised as safe (GRAS), relatively cheap and easy to evaporate from the matrix and extracts (Mercadante, 2008; Reverchon & De Marco, 2006; Sahena et al., 2009). The high extraction yields achieved using this technique is achieved due to the high diffusivity and solubility but low viscosity of SC-CO_2. In contrast to the products extracted from the conventional methods, the SC-CO_2 extracts are rich in nutraceuticals with high purity. Several authors have used SC-CO_2 to extract lipids and carotenoids from vegetables (Filho et al., 2008; Hardardottir & Kinsella, 1988; Mendes et al., 1995; Silva, Gamarra, Oliveira, & Cabral, 2008) and animal matrices (Froning et al., 1990; Hardardottir & Kinsella, 1988; Letisse et al., 2006; Tanaka & Ohkubo, 2003). The SC-CO_2 extraction technique has also been studied for extraction of lipids and astaxanthin from crustacean processing waste such as shrimp by-products (Charest, Balaban, Marshall, & Cornell, 2001; Felix-Valenzuela, Higuera-Ciapara, Goycoolea, & Arguelles-Monal, 2001; Kamaguchi et al., 1986; Lopez, Arce, Garrido, Rios, & Valcarcel, 2004). Recently, ω-3-rich lipids have been recovered with high yield (94 %) from rock lobster livers by SC-CO_2 extraction (Trung T. Nguyen et al., 2015).

1.4.3 Microwave-intensified production of chitin and chitin derivatives

Chitin occurs in lobster shells by closely associating with proteins, minerals, and pigments, which need to be completely removed and separated from chitin during the extraction process. Although the conventional methods removes nearly all these compounds from the shells, the use of strong chemicals and high temperatures during the process can cause deacetylation and depolymerisation reactions leading to inconsistent physical properties of the extracted chitin (Jung et al., 2007; Kjartansson, Zivanovic, Kristbergsson, & Weiss, 2006; Percot, Viton, & Domard, 2003). In addition, the proteins and minerals removed cannot be used because of undesirable reactions between amino acids and the alkaline medium as well as racemization (Synowiecki & Al-Khateeb, 2003). Moreover, the conventional method requires large volumes of water for washing steps,

generating a huge amount of waste water (Wang & Chio, 1998). To circumvent these issues, various biological processes have been developed for the production of chitin from crustacean shells by fermentation or using commercially available enzymes (Giyose, Mazomba, & Mabinya, 2010; Jung et al., 2007; Manni, Ghorbel-Bellaaj, Jellouli, Younes, & Nasri, 2010; Oh, Kim, Jung, & Park, 2007; Sini, Santhosh, & Mathew, 2007; Sorokulova, Krumnow, Globa, & Vodyanoy, 2009; Xu, Gallert, & Winter, 2008). However, these proposed new methods require a longer production time (8 – 72 hours) while their removal degrees (deproteinisation, demineralisation) are relatively low.

Recently, microwave has emerged as a promising as non-conventional energy source for performing organic synthesis. Heat generated from microwave irradiation accelerates chemical reactions and enhances the rate of enzyme-catalysed reactions so spectacularly that it cannot be explained by the effect of rapid heating alone (De La Hoz, Diaz-Ortiz, & Moreno, 2005). Apart from thermal effects, microwave irradiation is accompanied with several non-thermal effects such as overheating, hot spots, selective heating, highly polarizing field, and mobility and diffusion. Microwave-assisted extraction has been proven to be an efficient technique due to its high ability to intensify processes, low usage of extraction chemicals, and shorter extraction time (Teo, Chong, & Ho, 2013).

Microwave technology has been used as an environmentally-friendly and cost-effective method for chitin production by assisting demineralisation of deproteinised shrimp shells with lactic acid (Valdez-Pena et al., 2010). This process produced high yields of chitin with low residual minerals (0.2 %), thus demineralization of lobster shells by the microwave process was also optimized for chitin production obtaining promising results such as high degree of demineralization, low residues, and recovery of lobster minerals (Trung T. Nguyen, Barber, Luo, & Zhang, 2017). The rate and yield of extraction can be further exploited by combining multiple processing approaches such as the microwave technology has been employed for intensifying the rate of enzymatic hydrolysis of lobster shells (Trung T. Nguyen et al., 2016). Microwave irradiation has also been demonstrated as highly efficient in chemical deacetylation of chitin into chitosan. The degree of deacetylation in chitosan production within 5.5 min of microwave irradiation was as high as it was deacetylated at 121 ^{o}C, 15 psi for 4 hours (Sahu, Goswami, & Bora, 2009). To generate chitosan with high solubility or desired functional properties, microwave irradiation has been extensively utilised for the chemical modification of chitosan (Ge & Luo, 2005; Huacai, Wan, & Dengke, 2006; Liu, Li, Li,

& Fang, 2004). Particularly, the microwave has been demonstrated as a green and sustainable technology for which may be used for the degradation of chitin (Ajavakom, Supsvetson, Somboot, & Sukwattanasinitt, 2012; Roy, Mondal, & Gupta, 2003) and chitosan (Garcıa et al., 2015; Li et al., 2012; Petit, Reynaud, & Desbrieres, 2015; Wasikiewicz & Yeates, 2013) generating low molecular weight chitosan or chito-oligomers with a wide range of applications.

1.5 Hypothesis

The hypothesis of this study is that valuable components of LPBs can be economically recovered for food and nutraceutical applications using ultrasound, microwave, and/or SC-CO_2 extraction.

1.6 Aims and objectives

The main aim of this study is to recover functional and bioactive compounds from Australian LPBs for food and nutraceutical applications. To achieve this aim, three specific objectives were addressed (1) to develop a biorefinery process for the recovery of functional and bioactive ingredients from Australian LPBs; (2) to characterise the lobster extracts and demonstrate their potential to be integrated in food and nutraceutical applications; (3) to determine the economic feasibility for industrial extraction and utilisation of lobster functional and bioactive compounds, based on techniques developed and optimised in this study.

1.7 Significances

The results obtained from this study provide significant insight into how to efficiently and economically recover functional and bioactive compounds from LPBs by the use of microwave, ultrasound, and SC-CO_2 extraction. This work highlights the advantages to using these cutting-edge, green technological approaches and investigates the composition, chemical properties and potential applications of the compounds extracted. The findings and techniques used herein may be industrially applicable for recovery of functional compounds not only from lobster by-products but also from other crustacean species. Overall, this work has the potential to increase revenue and create jobs for the lobster industry by converting unwanted and underutilised by-products into a profitable source for food and nutraceutical applications.

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CHAPTER 2 SIGNIFICANT ENRICHMENT OF PUFAs IN THE LIPIDS EXTRACTED BY SUPERCRITICAL CO_2 FROM THE LIVERS OF AUSTRALIAN ROCK LOBSTERS *(JASUS EDWARDSII)*

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2.1 Introduction

Polyunsaturated fatty acids (PUFAs) have been extensively reported in the literature as being beneficial to human health (FAO, 2010; Sahena et al., 2009; Sampath & Ntambi, 2007). In particular, omega-3 (ω-3) or highly unsaturated fatty acids (HUFAs) are believed to play an important role in the prevention of certain health problems such as diabetes, allergies, and some types of cancers, as well as having anti-thrombotic, anti-arrhythmic and anti-inflammatory effects (Morales, Munío, Gálvez, Guadix, & Guadix, 2013). Fishery processing by-products have been recognised as an important potential source of ω-3 fatty acids, which could be used in the production of ω-3 rich lipids. According to a previous investigation by Tsvetnenko, Kailis, Evans, and Longmore (1996), one major source of PUFAs in the Australian Rock lobster *(Panulirus cygnus)* is the hepatopancreas (liver), but this inedible part is usually removed during processing and discarded. Australia is the largest producer and exporter of Rock lobster in the world (Tsvetnenko et al., 1996) annually producing more than 3,000 tons of lobster processing wastes including heads, shells, and livers, in which the lobster liver accounts for 2 – 5 %. However, these materials are often disposed of as biowaste with a cost to the processor of up to AUD 150 per ton (Knuckey, 2004). Because of the cost and environmental burdens, the Australian lobster processing industry is examined how to utilise these processing co-products, especially the Rock lobster liver, as a valuable potential resource for the production of ω-3 rich lipids.

In recent years, processing co-products from different types of fish including tuna (Chantachum, Benjakul, & Sriwirat, 2000), herring (Aidos, Van-der-Padt, Boom, & Luten, 2002), salmon (Wu & Bechtel, 2008), walleye Pollock (Wu & Bechtel, 2009), and shrimp (Sachindra, Bhaskar, &

Mahendrakar, 2006) have been extensively studied for lipid extraction, however, very little work has been done on Rock lobster liver. It is also important to improve the current extraction procedure of marine co-products to produce lipid with a high concentration of PUFAs and low contaminants of heavy metals.

Currently, there are several available technologies for the extraction of lipids and pigments from crustacean processing co-products. The most common technology is solvent extraction (Meyers & Bligh, 1981; Sachindra et al., 2006; Sahena et al., 2009). The solvent selection, a decisive factor on the quality of the extracted lipids, is crucial. Lipids are generally soluble in non-polar solvents that have been traditionally used for lipid extraction (Mercadante, 2008). Several organic solvents including acetone, ethyl acetate, hexane, isopropanol, methanol, methyl ethyl ketone, and ethanol are used in the food industry while others such as dichloromethane, dimethyl sulfoxide, and chloroform cannot be used due to their toxicity (FDA, 2010). There is, however, increasing public awareness of the health, environmental and safety hazards related to the use of organic solvents for lipid extraction, and the possible solvent contamination of the final lipid products. These issues have stimulated the interest to develop alternative extraction methods (Delgado-Vargas & Paredes-López, 2002). As such, supercritical fluid extraction (SFE) has become an important technology for extracting high quality lipids (Andrich, Nesti, Venturi, Zinnai, & Fiorentini, 2005; Cheng, Shieh, Wang, Lai, & Chang, 2012; Follegatti-Romero, Piantino, Grimaldi, & Cabral, 2009; Walker, Cochran, & Hulbert, 1999) from fishery processing co-products (Amiguet et al., 2012; Letisse, Rozieres, Hiol, Sergent, & Comeau, 2006; Rubio-Rodríguez, Diego, Beltrán, & Jaime, 2008) and is also performed as an effective separation technique in the production of nutraceutical supplements and functional foods (Parajó, Dominguez, Moure, & Diaz-Reinoso, 2008; Reverchon & De Marco, 2006). SFE can be employed at moderate temperature and provides an oxygen free media in reducing lipid oxidation during the extraction process. In addition, it allows for the selective extraction of low polar lipid compounds, avoiding the co-extraction polar impurities such as some organic derivatives with heavy metals (Rubio-Rodríguez et al., 2012). Supercritical carbon dioxide (SC-CO_2) extraction is the common SFE technology since this technique is able to extract the heat-sensitive, easily oxidised compounds such as PUFAs and omega-3 (Amiguet et al., 2012; Andrich et al., 2005; Cheng et al., 2012; Follegatti-Romero et al., 2009; López-Cervantes, Sánchez-Machado, & Rosas-Rodríguez, 2006; Rubio-Rodríguez et al., 2008) without any contaminated residues in the products. Moreover, CO_2 is a generally recognised as safe (GRAS) solvent type, which is relatively cheap and easy to separate from the solid matrix and extracts (Mercadante, 2008;

Reverchon & De Marco, 2006; Sahena et al., 2009). The excellent extraction performance of the SC-CO_2 extraction is a result of the low viscosity of SC-CO_2, high diffusivity and solubility. In contrast, conventional extraction processes such as Soxhlet extraction produce dilute extracts and contain materials that are easily oxidised. Furthermore, the subsequent steps of solvent separations can lead to the degradation of the target products. Several authors have used SC-CO_2 successfully to extract lipids and carotenoids from vegetable (Filho et al., 2008; Hardardottir & Kinsella, 1988; Mendes et al., 1995; Silva, Gamarra, Oliveira, & Cabral, 2008) and animal matrices (Froning et al., 1990; Hardardottir & Kinsella, 1988; Letisse et al., 2006; Tanaka & Ohkubo, 2003). Recent studies have used the SC-CO_2 extraction of lipids and astaxanthin from crustacean processing waste such as shrimp by-products (Charest, Balaban, Marshall, & Cornell, 2001; Felix-Valenzuela, Higuera-Ciapara, Goycoolea, & Arguelles-Monal, 2001; Kamaguchi et al., 1986; Lopez, Arce, Garrido, Rios, & Valcarcel, 2004). To our knowledge, no work has been done on the SC-CO_2 extraction of lipids from Rock lobster liver. Therefore, the PUFAs proportion and heavy metal contamination, which are major concerns for potential consumers of Rock lobster lipids extracted by SC-CO_2 and Soxhlet, have not yet been investigated. The objectives of this study were to optimise the SC-CO_2 extraction of lipids from Australian Rock lobster liver, with the aims of obtaining the lipids with enriched PUFAs, comparing them with the lipids extracted from conventional processing methods, and evaluating the PUFAs profiles and heavy metal content of the extracted lipids for potential health benefits and to confirm that they meet food safety standards.

2.2 Materials and methods

2.2.1 Materials

Fresh livers of Australian Rock lobsters were provided by Ferguson Lobster Company in South Australia. Food grade carbon dioxide (CO_2) gas used for SFE was supplied by CoreGas, South Australia.

2.2.2 Methods

2.2.2.1 Proximate analysis of lobster liver

According to the AOAC (2006) methods, moisture was determined by oven drying at 105 ^oC (AOAC 950.46) until a constant weight was obtained, while ash content was quantified by incineration in a muffle furnace at 600 ^oC (AOAC 920.153). Lipid content was determined by the

Soxhlet extraction method (AOAC 991.36), and protein content was determined by the micro Kjeldahl method (AOAC 928.08).

2.2.2.2 Preparation of rock lobster liver for extraction

One kilogram of fresh lobster liver was held frozen (Thermo Scientific, TSE series -86C Ultra Low Temperature freezer) at - 80 ^{o}C for 8 hours. The frozen lobster liver was transferred to a plastic box with a large surface before it was freeze-dried at - 85 ^{o}C, 15 mTorr for 48 hours by a Benchtop freeze dryer (Virtis model BT6KEL#301804). The freeze-dried lobster liver was collected and stored in the freezer at - 80 ^{o}C for one week before it was used for the lipid extraction.

2.2.2.3 Supercritical CO_2 extraction of lipids from Rock lobster livers

Extractions were carried out by the laboratory supercritical CO_2 extraction system (Applied Separation model Spe-ed SFE-2#7071) equipped with 100-mL and 1000-mL extraction vessels. The 100-mL extraction vessel was packed with around 10 g of freeze-dried lobster liver for each batch of extraction and the void volumes at two ends of the extraction vessel were completed with washed sand and glass wool layers. A 20-minutes static time was used for making a good contact between the sample and the supercritical solvent before the needle valve was opened to release the extracted lipid. For extraction, CO_2 at supercritical state was continuously drained through the extraction vessel and the extracted oil was collected in a pre-weighed dark glass bottle. The CO_2 mass flow rate was maintained at around 0.434 kg/h and the time for extraction was 240 minutes. The extraction temperature of the oven, valve, and vessel was set at 50, 60, and 50 ^{o}C, respectively while the extraction pressure was adjusted to 25, 30, and 35 MPa as experimental design. The lipid-collecting bottle was weighed every hour to calculate lipid recovery. Extractions at every condition were carried out in three replicates.

2.2.2.4 Soxhlet extraction of lipids from rock lobster liver

Soxhlet extraction of lipids from Rock lobster liver was carried out at the National Measurement Institute (NMI) of Australia. Ten grams of freeze-dried Rock lobster livers were homogenised thoroughly before it was placed in a Soxhlet thimble. The thimble containing the sample was then placed in a hot extraction beaker while 90 mL of diethyl ether was added and locked into the hot extraction unit. Extraction was carried out using a pre-programed period of approximately 4 hours with a temperature of 40 ^{o}C. The amount of crude lipids obtained by this method was 2.43 gram /10.0 gram of the freeze-dried lobster liver.

2.2.2.5 Fatty acid analysis

The fatty acid composition of the lipids extracted by SFE and Soxhlet was analysed at NMI. Extracted fats were converted into fatty acids methyl esters (FAME), separated, then measured on a Hewlett-Packard 6890 gas chromatograph equipped with a 50 cm capillary column (0.32 mm internal diameter SGE, Victoria, Australia) coated with 70% (w/w) cyanopropyl polysilphenylene-siloxane (BPX-70) (0.25 μm film thickness), which was fitted with a flame ionisation detector. Helium was the carrier gas (flow rate 60 ml/min) and the split-ratio was 20:1. The injector temperature was set at 250 ^{o}C and the detector temperature at 300 ^{o}C. The initial oven temperature was 140 ^{o}C and programmed to rise to 220 ^{o}C at 5 ^{o}C per minute and held for up to 3 minutes. FAMEs were identified based on the retention time of standards obtained from Nucheck Prep Inc. (Elysian, MN, USA) using Chemstation software. An external standard of 463 from NuCheck Prep Inc was analysed and used for calibration.

2.2.2.6 Heavy metals and inorganic arsenic analysis

The content of heavy metals and inorganic arsenic was determined using standard methodsVL247 Ver. 9.1 (NMI, 2013b) and NT 2.56 (NMI, 2013a) described by National Measurement Institute of Australia.

2.3 Results and discussion

2.3.1 Composition of Australian Rock lobster livers

2.3.1.1 Chemical composition

The chemical composition of Australian Rock lobster liver was analysed. As shown in Figure 2.1, the two major components of the Australian Rock lobster livers obtained in this study are protein (41.1 % by dry weight) and lipids (24.3 % by dry weight). The lipid content obtained in this study is higher than the 19.4 % lipid content in the previous study reported by Tsvetnenko et al. (1996). This could be caused by either different species of lobster being used in the experiments or by other environmental factors. Compared with the lipid content of menhaden, which is an Atlantic fish caught primarily for production of fish oil and meal (9.13%), the lipid content in Rock lobster liver was nearly three times higher. Moreover, Tsvetnenko et al. (1996) also reported that the lipids recovered from Rock lobster cephalothorax were rich in PUFAs of which HUFAs accounted for 50.8 %. The results indicated that Australian Rock lobster liver, a relatively readily available lobster

processing by-product, could be a valuable but inexpensive resource for the extraction of ω-3-rich lipids.

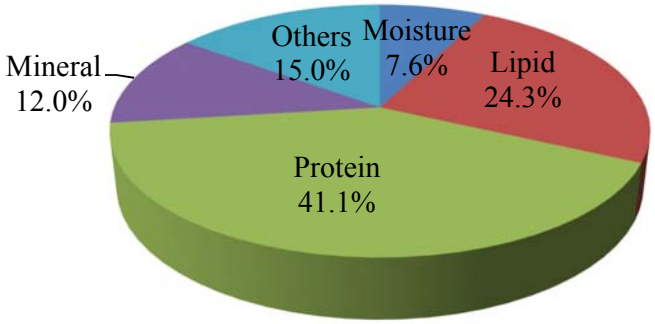

Figure 2.1 Chemical composition of freeze-dried Australian Rock lobster liver *(Jasus ewardsii)*

2.3.1.2 Heavy metal composition

The concentration of heavy metals found in Australian Rock lobster livers is shown in Table 2.1. Copper was detected in very high amounts of approximately 1000 mg/kg. This value for copper is several times higher than 151 mg/kg in veal liver which in turn is regarded as a very high copper content food (WHO, 1996; WHO, FAO, & IAEA, 1996). Consuming just 10 – 15 g of rock lobster liver could meet the daily requirement for copper suggested by the WHO/FAO. Copper is an essential mineral required for bone and connective tissue production, and for coding specific enzymes that possess the function of eliminating free radicals. A deficiency in copper can lead to osteoporosis, joint pain, and lowered immunity since copper is essential for the absorption of iron (Ness, 2014). Apart from having a rich copper content, Australian Rock lobster liver also has a high content of zinc (170 mg/kg) and selenium (16 mg/kg) which are beneficial for human health (WHO, 1996). While zinc is good for skin care, healing of wounds, prostate disorders, colds, weight loss, appetite loss, pregnancy, diarrhoea, respiratory infections, and malaria (Deshpande, Joshi, & Giri, 2013), selenium is one of the most effective mineral antioxidants since it actually prevents the formation of new free radicals by participating in various cellular reactions that lower the peroxide concentration in the cellular body (Chan, Gerson, & Subramaniam, 1998). Therefore, Australian Rock lobster liver could be an excellent dietary source of zinc and selenium for human demands.

Table 2.1 Heavy metal contents in livers of Australian Rock lobster (on dry weight basis), regulatory limits, and WHO/FAO recommendation

Trace elements	Australian Rock lobster livers (mg/kg)	Regulatory limits (mg/kg)	WHO/FAO recommendation (mg/kg)
Antimony	0.028	-	-
Arsenic (inorganic form)	0.67	2.0	-
Arsenic (organic & inorganic form)	240	-	-
Cadmium	8.7	2.0	-
Copper	1000	-	10 - 12 mg/day
Lead	0.024	0.5	-
Mercury	0.28	0.5	-
Selenium	16.0	-	46 - 61 µg/day
Tin	< 0.01	250	-
Zinc	170	-	35 - 45 mg/day

However, four other elements including lead (Pb), cadmium (Cd), arsenic (As), and mercury (Hg) that are generally considered to be hazardous to human health at low to medium concentrations (Davidowski, Grosser, & Thompson, 2009) have also been found in Australian Rock lobster livers. The amount of Pb and Hg were just half of the regulatory limit (0.5 mg/kg) of seafood products, but the total As and Cd were quite high with the content of 240 and 8.7 mg/kg, respectively (Table 2.1). The high contamination of these toxic heavy metals could be caused by a large amount of As and its derivatives presenting in sea water due to natural processes (Smedley & Kinniberg, 2002) and pollution as many species of fish and shellfish can bioaccumulate As and toxic heavy metals (Authority, 2010; NSW-Food-Authority, 2010). In spite of contamination with high levels of As, the vast majority of As and its derivatives in Australian Rock lobster livers are in organic forms which are harmless (Authority, 2010) and its limit is not specified in the food code. By contrast, soluble inorganic arsenic compounds, which are highly toxic (Hedegaard, Hansen, Larsen, & Sloth, 2005), are present in very small amounts of about 0.67 mg/kg compared the regulatory limit of 2 mg/kg. However, the content of Cd in Rock lobster livers is four times higher than the maximum

value recommended for seafood according to our study. This high Cd content should be brought into account when Rock lobster liver is directly consumed or utilised as a raw material for food. As result of this, Rock lobster liver is usually removed during lobster processing to avoid cross-contamination of toxic heavy metals. An advanced technology is required to ensure the extracted lipids have significantly reduced contaminants.

2.3.2 Optimisation of SC-CO_2 extraction of lipids from Rock lobster livers

SC-CO_2 extraction has been applied to several materials to investigate extraction yield. A number of processing parameters including pressure, temperature, CO_2 rate, and extraction time have been reported to have significant influence on lipid recovery, with extraction pressure and temperature being the most important factors. Several optimum conditions have been suggested for SC-CO_2 extraction of lipids from different materials but these conditions could be changed depending upon the nature of the materials being treated. In the literature, the suggested pressure usually ranges from 20 – 40 MPa and the temperature varies from 40 – 60 ^oC. In the present work, extractions of lipids from Australian Rock lobster livers were carried out with pressures from 25 MPa to 35 MPa for one to four hours at 50 ^oC and the CO_2 flow rate of 0.434 kg/h in order to find the efficient condition for lipid extraction from this material. Although the SC-CO_2 extraction can be carried out at the mild temperature for better prevention of lipid oxidation, the results from initial studies carried out at the same temperature as the Soxhlet extraction (40 ^oC) indicated that lipid recovery was very low (only 32.17 % after two hours of extraction). Therefore, a slightly higher extraction temperature of 50 ^oC was chosen to minimise negative effects on the PUFAs profile but to achieve a high enough lipid recovery (over 90 %).

As shown in Figure 2.2, the extraction curves obtained in this study fit well with the empirical model suggested by Kandiah and Spiro (1990), with an assumption that two diffusion stages, which are based on the amount of lipids accessible to the supercritical CO_2, could control the process. At the early stage, the content of most accessible lipids is high and thus, the extraction rate is high. As can be seen in Figure 2.2, the initial extraction rate, or the lipid recovery, increases significantly with applied pressure. When the extraction pressure rose from 25 to 35 MPa, the lipid recovery increased significantly by about 30 % (47.30 % to 75.86 %) during the first two hours of extraction. Up to 90 % of the lipids in Rock lobster livers relative to the Soxhlet extraction could be recovered in this stage once the pressure of 35 MPa was applied, but for the extraction pressure of 30 and 25 MPa these values were just 75 and 67 %, respectively. This result indicates that the internal mass

transfer is negligible and the process is controlled by the lipid solubility in SC-CO_2. Since both solvent density and capacity increase with pressure, a rise in pressure leads to an increase in the lipid solubility of the solvent. However, at the second stage, the remaining lipids which are less accessible to the solvent, were extracted much more slowly because of the considerably higher internal mass transfer resistance (Rubio-Rodríguez et al., 2008). This can be clearly observed in Figure 2.2 when only around 4, 13, and 19 % of the lipids were recovered during the last two hours of extraction with the pressure of 35, 30, and 25 MPa, respectively. The results indicate that using high pressure for SC-CO_2 extraction of lipids from Rock lobster livers can shorten the extraction time since the extraction rate increases with pressure.

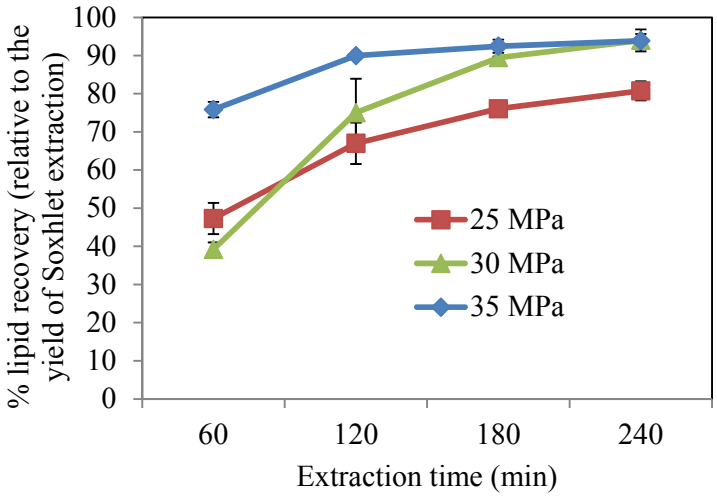

Figure 2.2 Lipid recovery extracted by SC-CO_2 at different pressures over extraction time

Although the amount of recovered lipids can be increased with an extended extraction time at all observed pressures, the maximum value of lipid recovery was found at four hours of extraction. The lipid recovery for four-hour extraction at 35 and 30 MPa was approximately 94.0 %, whereas this value for 25 MPa was reduced to 80.76 %. The result, that lipid recovery increased with higher extraction pressure, is in agreement with the previous investigation by Sahena et al. (2010). Therefore, SC-CO_2 extraction of lipids from Rock lobster livers using high pressure not only shortened the extraction time but also improved the extraction yield.

It is also observed in Figure 2.2 that the appropriate time for extraction is significantly different among the extraction pressures. Though four hours of extraction seems to be an appropriate time for the pressure of 30 MPa, this time is clearly not suitable for 35 MPa. This is because the lipid recovery for the pressure of 35 MPa at two hours and four hours was not significantly different

(nearly 90.0 % and 94.0 %). The prolonged extraction time could lead to considerably increase in processing costs and in the case of extraction at 35 MPa, the two hours of extraction is clearly more economic than others. In the overall consideration of extraction yield, extraction time, and PUFAs content, the SC-CO_2 extraction at 35 MPa and 50 ^oC for two hours extraction was chosen as the efficient condition for lipid extraction from freeze-dried rock lobster liver.

2.3.3 Significant improvement in nutritional profile of the SC-CO_2 extracted lipids

2.3.3.1 PUFAs-rich lipids extracted by SC-CO_2 extraction

Lipid extraction with SC-CO_2 has been suggested as a promising alternative for producing lipids with a high amount of PUFAs. CO_2 at supercritical condition possesses superior mass transfer characteristics and this technique involves the use of a non-oxidant atmosphere and mild temperature, which could prevent the oxidation of PUFAs. In current research, the lipids extracted from Australian lobster livers by SC-CO_2 extraction and Soxhlet were analysed and their fatty acid profiles were compared. It can be observed in Figure 2.3 that there are significant differences in the fatty acid composition of the two extracted lipid mixtures. While the majority of total fatty acids in the Soxhlet-extracted lipids are saturated fatty acids (SFAs) and mono-unsaturated fatty acids (MUFAs), its PUFAs are very low, at only around 7.8 %. In contrast, the SC-CO_2-extracted lipids have a very high proportion of PUFAs (31.3 %), which is greater than the level of MUFAs (29.4 %) and equivalent to SFAs. Notably, the level of PUFAs in the SC-CO_2-extracted lipids is four times higher than that in the Soxhlet-extracted lipids, and considerably higher compared to 22.6 % of PUFAs in the previous work of Tsvetnenko et al. (1996) who extracted lipids from Rock lobster *(Panulirus cygnus)* cephalothorax using a mixture of methanol/chloroform solvent and then evaporated under vacuum. This result is also supported by the study of Cheung, Leung, and Ang (1998) who compared the fatty acid composition of lipids extracted from brown seaweed by SC-CO_2 and Soxhlet. Their work also showed that the lipid oxidation of PUFAs had been minimised under low oxidising conditions used in SC-CO_2 extraction, therefore the obtained lipids contained higher PUFAs.

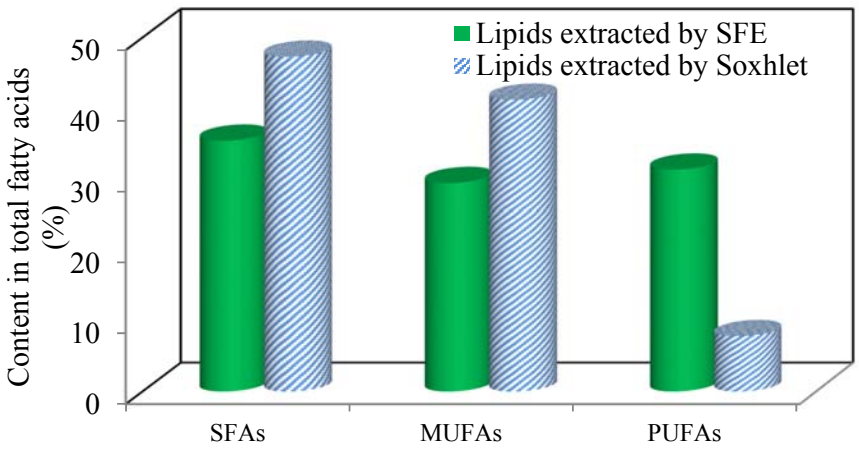

Figure 2.3 Differences in fatty acid composition of the lipids extracted by SC-CO_2 and Soxhlet method

Apart from significantly enriching PUFAs, the SC-CO_2-extracted lipids also have a significantly higher proportion of ω-3 fatty acids, which account for 18 % of total fatty acids, six times higher compared with the 3.1 % of ω-3 fatty acids found in the lipids recovered by the Soxhlet method (Figure 2.4). This result is similar to the study of Cheung et al. (1998), who discovered that ω-3 fatty acids levels in the SC-CO_2-extracted lipids from brown seaweed were significantly higher than those in the Soxhlet-extracted lipids, and concluded that ω-3 fatty acids were extracted more effectively with SC-CO_2 extraction than with the Soxhlet. More specially, ω-3 HUFAs such as DHA, EPA, and DPA with their biochemical activities which may be useful in the prevention and treatment of several disorders and diseases including coronary heart disease, rheumatoid arthritis, asthma, cancers, diabetes and others (Pamela, 2001), were the most dominant in PUFAs of the lipids extracted by SC-CO_2 extraction. These ω-3 HUFA concentrations were seven times higher than those in the Soxhlet-extracted lipids were and they comprised more than 95 % of the total ω-3 fatty acids in the SC-CO_2-extracted lipids. The maintenance of a high amount of PUFAs in the composition of the extracted lipids with the vast majority of these being ω-3 HUFAs provides a significant advantage for the SC-CO_2 extraction technique for extracting ω-3-rich lipids from the livers of Australian Rock lobster.

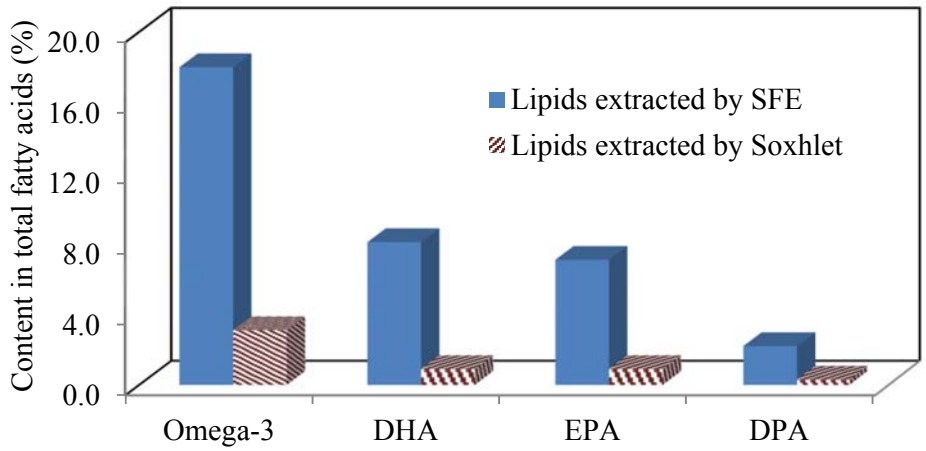

Figure 2.4 Contents of omega-3 in the lipids extracted by SC-CO_2 and Soxhlet method

The investigation on the evolution of PUFAs and ω-3 fatty acids of the SC-CO_2-extracted lipids over the extraction time (Figure 2.5) indicated that these fatty acid contents are relatively constant in the first three hours and slightly increase in the fourth hour. The extraction time does not have a significant impact on the fatty acid profile.

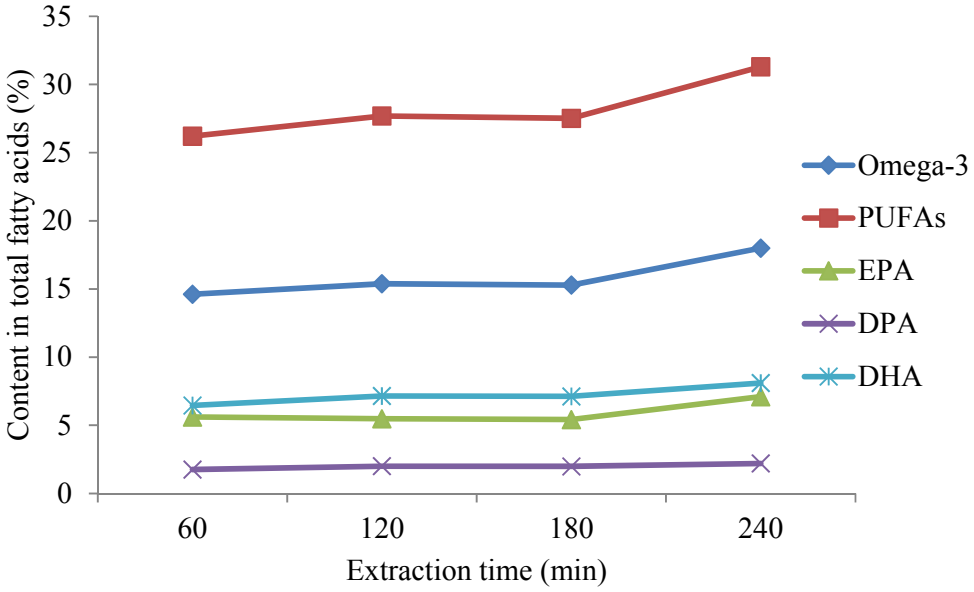

Figure 2.5 The evolution of PUFAs, omega-3 fatty acids, DHA, EPA, and DPA the over extraction time

2.3.3.2 Significantly lower toxic heavy metals in the SC-CO_2 extracted lipids

More than 50 different arsenic species have been found in marine environments and arsenic derivatives, such as arsenobetaine, being the main species and water soluble form, have been detected in fish (Ackley, Bhymer, Sutton, & Caruso, 1999). Therefore, seafood is considered as a major dietary source for arsenic exposure (Hedegaard et al., 2005). In our current work, the heavy metal content of the extracted lipids have been specially taken into account since some toxic heavy metals were detected over the regulatory limit in the raw materials used for extracting the lipids. This becomes more necessary because a considerable amount (4.3 – 10.5 ppm) of arsenolipids, which are non-polar lipids bound with As, have been found in ten different fish lipids (Schemeisser, Goessler, Kienzl, & Francesconi, 2005). Table 2.2 shows the concentration of heavy metals detected in the lipids extracted by the SC-CO_2 extraction and by the Soxhlet. The lipids extracted by both methods contain very small amounts of heavy metals compared with the raw materials and the regulatory limits while their beneficial elements such as Zn and Se are still quite high. The content of these heavy metals in the extracted lipids changed significantly from 0.5 times to 870 times depending on the method used for the lipid extraction and the types of trace elements. The low contamination of heavy metals in the extracted lipids is a result of these methods utilising non-polar solvents for extraction, which usually solubilise mainly the non-polar compounds and leave behind the polar compounds.

Table 2.2 Heavy metal contents in the lipids extracted by SC-CO_2 (50 ^oC, 35 MPa, 4 hours, CO_2 flow rate 0.434 kg/h), Soxhlet (40 ^oC, 4 hours), and the regulatory limits

Trace elements	Lipids extracted by SC-CO_2 (mg/kg)	Lipids extracted by Soxhlet (mg/kg)	Regulatory limits (mg/kg)
Antimony	< 0.01	< 0.01	-
Arsenic (inorganic form)	0.05	0.22	2.0
Arsenic (organic & inorganic form)	2.2	31	-
Cadmium	< 0.01	0.27	2.0
Copper	13	150	-
Lead	0.012	0.022	0.5
Mercury	< 0.01	0.012	0.5

Selenium	0.25	2.6	-
Tin	0.19	< 0.01	250
Zinc	12	18	-

However, this work has been specifically focused on an investigation of toxic heavy metals since contamination with these compounds can render the material unusable due to potential risks of excessive ingestion that may lead to a decline in mental, cognitive and physical health. As shown in Figure 2.6 and Figure 2.7, there are significant differences in the amount of toxic heavy metals in the lipids extracted by SC-CO_2 and Soxhlet techniques. The content of all toxic heavy metals in the SC-CO_2-extracted lipids were considerably lower than those in the Soxhlet-extracted lipids. While the concentration of Pb in the SC-CO_2-extracted lipids was just half of that in the Soxhlet-extracted lipids (0.022 mg/kg), the content of inorganic arsenic in the former was four times lower compared with the amount of 0.22 mg/kg in the latter. Notably, the content of Cd in the SC-CO_2-extracted lipids (under 0.01 mg/kg) was 27 times lower than that in the Soxhlet-extracted lipids, whereas the concentration of total arsenic was nearly 15 times lower (2.2 mg/kg compared with 31 mg/kg). This trend is strongly supported by the recent investigations by Rubio-Rodríguez et al. (2012). Their results indicate that SC-CO_2 extraction is an effective technology for extracting fish lipids with greatly reduced contamination with toxic heavy metals.

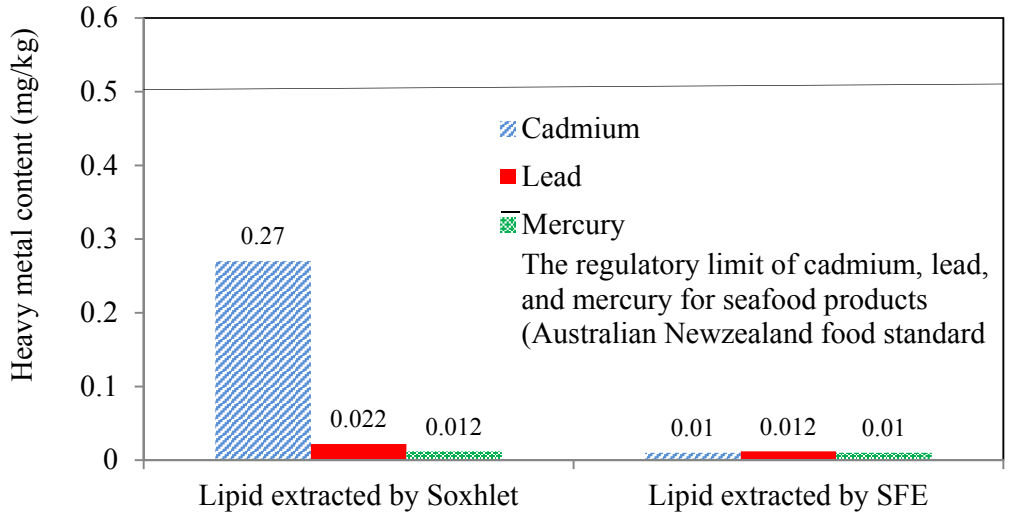

Figure 2.6 Contents of cadmium, lead, and mercury in the lipids extracted by SC-CO_2 and Soxhlet methods

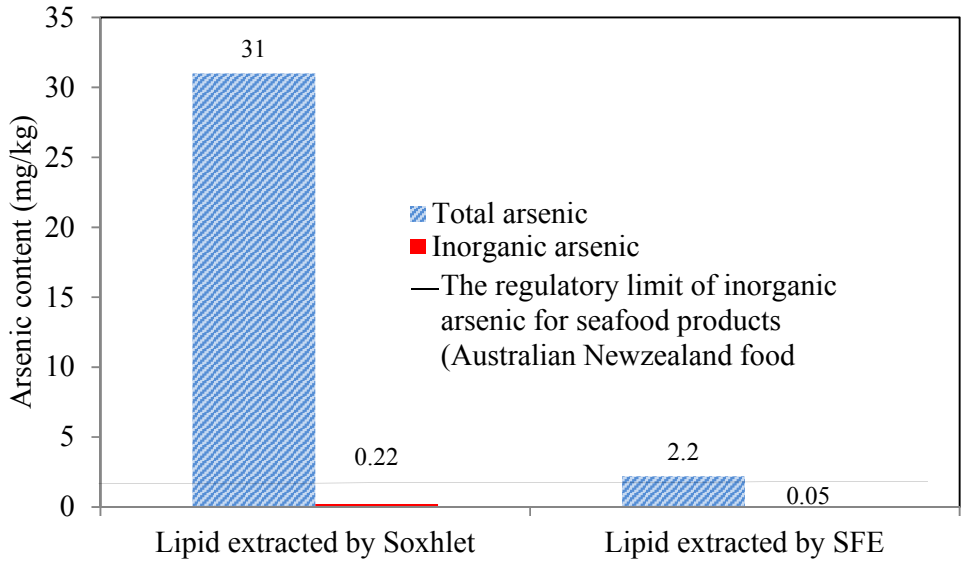

Figure 2.7 Contents of total and inorganic arsenic in the lipids extracted by SC-CO_2 and Soxhlet methods

2.3.3.3 Lipid profiles

Table 2.3 illustrates the fatty acid composition of the lipids extracted by SC-CO_2 (50 ^oC, 35 MPa, 0.434 kg/h) and by Soxhlet from Australian Rock lobster livers. It needs to be highlighted that there are significant differences in the fatty acid composition of the lipids extracted by these two methods. While the highest proportion in the Soxhlet-extracted lipids was SFAs (47.3 %) followed by MUFAs (41.2 %) and PUFAs (7.8 %), these contents in the SC-CO_2-extracted lipids were 35.4 % of SFAs, 29.4 % of MUFAs and 31.1 % of PUFAs, respectively. The former was rich in SFAs and MUFAs but very poor in PUFAs while the latter has very high amounts of PUFAs dominated by ω-3 (18 %) and ω-6 (13.3 %). The differences in fatty acid profiles of these two lipids could be explained by differences in conditions used for extracting the lipids. While the Soxhlet method employed organic solvents such as diethyl ether for extraction in this study, the SC-CO_2 extraction used CO_2 at supercritical condition that has high diffusion and very low surface tension compared to liquid solvents (Karale Chandrakant, Dere Pravin, Hondre Bharat, Sachin, & Kote Amol, 2011). These novel properties could be a favourable condition for PUFAs extraction. This point was confirmed by Cheung et al. (1998) that the yield of omega-3 fatty acids extracted by SC-CO_2 extraction at higher pressure (31.0 and 37.9 MPa) was significantly higher than that in the Soxhlet. However, both the lipids have diversified fatty acids in which oleic is the most dominant in their composition (20.5 % for SC-CO_2-extracted lipids and 28.4 % for Soxhlet-extracted lipids). Palmitic

is abundant in SFAs (16.7 % for SC-CO_2-extracted lipids and 22.5 % for Soxhlet-extracted lipids) whereas DHA, EPA, and Arachidonic are three main fatty acids in PUFAs accounting for up to 75 %.

Table 2.3 The fatty acid composition of the lipids extracted from liver of Australian Rock lobster using SC-CO_2 (50°C, 35 MPa, 4 hours, CO_2 flow rate 0.434 kg/h) and Soxhlet (40°C, 4 hours)

Fatty acids		**Lipids extracted by SC-CO_2 (%)**	**Lipids extracted by Soxhlet (%)**
Saturated fatty acids (SFAs)		*35.4*	*47.3*
C4:0	Butyric	< 0.1	< 0.1
C6:0	Caproic	< 0.1	0.3
C8:0	Caprylic	< 0.1	< 0.1
C10:0	Capric	< 0.1	< 0.1
C12:0	Lauric	< 0.1	0.1
C14:0	Myristic	4.3	5.7
C15:0	Pentadecanoic	1.9	2.2
C16:0	Palmitic	16.7	22.5
C17:0	Margaric	2.0	2.6
C18:0	Stearic	8.1	10.6
C20:0	Arachidic	1.4	1.9
C22:0	Behenric	0.7	0.9
C24:0	Lignoceric	0.1	0.3
Mono-unsaturated fatty acids (MUFAs)		*29.4*	*41.2*
C14:1	Myristoleic	< 0.1	< 0.1
C16:1	Palmitoleic	5.5	7.6
C17:1	Heptadecenoic	0.3	0.3
C18:1	Oleic	20.5	28.4
C20:1	Eicosenic	2.3	3.4

C22:1	Docosenoic	0.5	0.8
C24:1	Nervonic	0.4	0.7
Poly-unsaturated fatty acids (PUFAs)		*31.3*	*7.8*
C18:2w6	Linoleic	1.8	1.8
C18:3w6	gamma-Linolenic	< 0.1	< 0.1
C18:3w3	alpha-Linolenic	0.5	0.2
C20:2w6	Eicosadienoic	1.0	0.9
C20:3w6	Eicosatrienoic	0.2	< 0.1
C20:3w3	Eicosatrienoic	0.2	0.8
C20:4w6	Arachidonic	8.0	1.2
C20:5w3	Eicosapentaenoic (EPA)	7.1	0.9
C22:2w6	Docosadienoic	0.3	0.4
Omega 3	fatty acids (ω-3)	18.0	3.1
Omega 6	fatty acids (ω-6)	13.3	4.7
C22:4w6	Docosatetraenoic	2.0	0.4
C22:5w3	Docosapentaenoic (DPA)	2.2	0.3
C22:6w3	Docosahexaenoic (DHA)	8.1	0.9
Total mono trans fatty acids		0.6	0.5
Total poly trans fatty acids		3.2	3.2
P:M:S ratio		0.9:0.8:1	0.2:0.9:1

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CHAPTER 3 HIGHLY EFFICIENT RECOVERY OF NUTRITIONAL PROTEIN FROM LOBSTER HEAD BY-PRODUCTS BY ULTRASONIC EXTRACTION

3.1. Introduction

The Australian lobster processing industry yields over 3,000 tons of lobster processing by-products (LPBs) including heads, shells, and livers annually (He, Nguyen, Zhang, & Peng, 2016). Currently this material (LPBs) is discarded as biowaste with a disposal cost of $150 AUD per ton. As globally lobster consumption continues to increase so does the amount of waste generated. Currently the disposal costs for lobster waste in Australia are estimated to be $500,000 AUD. With the increase in the number of lobsters processed in conjunction with increasing fees for disposal there is real risk of environmental problems arising due to improper disposal of waste. One solution to this problem may to find alternative, higher value uses for this waste material. For example, LPBs such as lobster head by-product (LHB) are composed of lobster meat (up to 20 %) which is rich in high quality proteins (Vieira, Martin, Saker-Sampaiao, Omar, & Goncalves, 1995). The proteins derived from LHB have been shown to have nutritional value (containing all of essential amino acids with high proportion) and excellent functionality (solubility, wettability, water/oil binding, emulsifying capacity) (He et al., 2016; Nguyen, Zhang, Barber, Su, & He, 2016; Vieira et al., 1995). Due to this attributes it has been suggested the proteins may be used for dietary protein supplements, food functional nutrients, or flavour enhancers. As a result, utilisation of LHB for protein recovery is a practical solution for reduction in operational costs while increasing potential profits.

Previous studies have investigated the conversion of LHB to lobster protein hydrolysate (LPH) using either enzymatic hydrolysis (Vieira et al., 1995; Yang & Lee, 2000) or chemical hydrolysis (He et al., 2016). The hydrolysis processes used in these studies are both simple and industrially applicable, however, the rate of hydrolysis was found to be unpredictable, yielding short-chain peptides with loss of functionality (Sanmartín, Arboleya, Villamiel, & Moreno, 2009). Enzymatic hydrolysis resulted in low protein recovery and the proteins that were recovered had taste defects, which would be unfavourable in food products. Moreover, the harsh conditions of chemical hydrolysis (HCl 6 N, 118 ^{o}C, 18 hours for acidic hydrolysis or pH 14, 121 ^{o}C, 15 psi, 20 minutes for alkaline hydrolysis) generates protein hydrolysates with a bitter taste, racemization, reduced nutritional quality, and poor functionality. The poor characteristics of these protein products in

conjunction with the synthesis of undesired by-products makes them undesirable. As a result, the final products of the chemical hydrolysis are mainly used as fertilisers.

With the limitations imposed by traditional processing methods, newly innovated techniques are required before the extraction of proteins from lobster waste is economical. Two methods that may be considered include isoelectric solubilisation and precipitation (ISP) and ultrasound. The ISP method has been used for recovery of proteins from fish at pilot and industrial scales (Chiranjib, Arijit, Alfredo, Reza, & Sudip, 2015; Reza Tahergorabi & Jaczynski, 2014) and beef and chicken processing by-products (Mireles Dewitt, Gomez, & James, 2002; R. Tahergorabi, Sivanandan, & Jaczynski, 2012). Therefore, this technology may also work for LHBs. In the ISP process, proteins are solubilised and then precipitated for recovery by pH inducing, while simultaneously lipids and other inedible parts are separated allowing protein recovery with lower lipids and other impurities (Gehring, Gigliotti, Moritz, J.C., & Jaczynski, 2011). Unlike other methods, the proteins may be easily recovered without compromising the yield or quality of protein recovered (Y. C. Chen & Jaczynski, 2007b; Taskaya, Chen, Beamer, & Jaczynski, 2009). Proteins recovered from the ISP process maintain their functional properties and nutritional value (Y. C. Chen, Tou, & Jaczynski, 2007; Gigliotti, Jaczynski, & Tou, 2008; Nolsoe & Undeland, 2009; Taskaya, Chen, Beamer, Tou, & Jaczynski, 2009; Taskaya, Chen, & Jaczynski, 2009c). Moreover, the ISP process also allows recovering proteins with significantly lower contaminations (L. Lansdowne, Beamer, & Jaczynski, 2009; LR Lansdowne, Beamer, Jaczynski, & Matak, 2009; Marmon, Liljelind, & Undeland, 2009), which could be used for functional foods or nutraceutical products (Reza Tahergorabi, Beamer, Matak, & Jaczynski, 2012; Reza Tahergorabi, Matak, & Jaczynski, 2015). With all these significant advantages, the ISP may be useful for protein recovery from LHBs.

Ultrasound based extractions have been shown to be an effective technique for improving the rate of various processes (Lebovka, Vorobiev, & Chemat, 2011; Majid, Nayik, & Nanda, 2015; Vilkhu, Mawson, Simons, & Bates, 2008). Ultrasound processing disrupts cells and creates micro-cavities in the tissue, which enhances the surface area and thus the penetration of the solvent into the material, mass transfer, and improves protein release. The use of ultrasound in protein extraction from fish, meat, and beef by-products resulted in higher extraction yields with reduced processing time and solvent consumption (Chemat & Khan, 2011; Saleem, Hasnain, & Ahmad, 2015; Vardanega, Santos, & Meireles, 2014; Vilkhu et al., 2008), which could be scaled up to an industrial level due to their high efficiency (Álvarez & Tiwari, 2015; Z.-c. Tu et al., 2015). It has

been suggested that this process may be further exploited by the addition of chitosan for improvement protein recovery. Chitosan has been used as an effective bio-flocculent for soluble protein recovery from surimi wash water (S. Wibowo, Velazquez, Savant, & Torres, 2005; Singgih Wibowo, Velazquez, Savant, & Torres, 2007) and carotenoprotein recovery from the waste water generated from shrimp chitin production (Trang, 2010). Protein-chitosan complexes (PCCs) generated from these processes showed higher protein efficiency ratio and net protein ratio than those of casein (Singgih Wibowo, Savant, et al., 2007) while the PCCs have been studied for functional foods (De Souza et al., 2014), improving emulsion stability (Zinoviadou, Scholten, Moschakis, & Biliaderis, 2012) or bioavailability (El-Beltagy & El-Sayed, 2012). The overall aim of this work is to improve protein recovery from LHBs using ultrasound for intensifying protein extraction coupled with chitosan as bio-flocculants for enhancing protein precipitation. The process efficiency was evaluated by comparing to the yields achieved using ISP. Furthermore, the molecular weight, digestibility, amino acid profile, and composition of the extracted proteins was investigated.

3.2. Materials and methods

3.2.1 Materials

Raw LHBs (3.1a) were blended at Ferguson Australia Pty Ltd (16 Circuit Dr, Hendon SA, Australia) to obtain the crushed form (3.1b) by before they were delivered to Flinders University (Registry road, South Australia, Australia) on frozen condition.

a) b)

Figure 3.1 Australian lobster head by-product before (a) and after (b) blending

Upon arrival, the samples were homogenised using an Express Blender (Blendtec, Model ES3 XPress, Ser. No. XPR-25558). The homogenised material was stored at -70 ^{o}C until analysed.

3.2.2 Chemicals

Crab shell chitosan with deacetylation degree over 85 %, concentrated hydrochloride, sodium hydroxide (pellet), and glacial acetic acid were purchased from Sigma Aldrich (Australia).

3.2.3 Composition analysis of Australian LHB

The moisture content was determined by oven drying (105 ^{o}C) blended LHB until obtaining a constant weight was achieved (AOAC 950.46). The ash content was determined by incinerating the samples in a muffle furnace at 600 ^{o}C for 15 hours, as previously described (AOAC 920.153). The lipid content was measured using the Soxhlet extraction method and protein quantified by the method of Micro Lowry using Bio-Rad protein assay. The method described by Simpson and Haard (1985) was followed to measure the amount of chitin in each sample. Heavy metal and inorganic arsenic were determined by the National Measurement Institute of Australia (NMI, 2013a, 2013b).

3.2.4 Molecular weight of extracted proteins

The molecular weight of extracted lobster proteins were determined using a Bio-Rad precast gel. Protein extract (10µl at a concentration of 20 mg/ml) was mixed with 30 µl of 4x loading-buffer containing 4 mg DTT per 100µl buffer. Samples were heated at 95 °C for 2 min using a heating block and then 20 µl loaded into the gel. Dual colour protein was used as a standard (5 µl). The applied voltage was 200 V, and the run time was 30 min. The gel was imaged using a Bio-Rad Gel-Doc EZ Imager.

3.2.5 Protein recovery from LHB

Blended LHB (10 g) was mixed with cold MilliQ water (4 ^{o}C) at specified ratios (4, 6, and 8 mL/g) in the 200-mL flasks. The mixtures were adjusted from pH 8.5 to either pH 10, 11, 12, or 13 using NaOH (10 N). For samples extracted using ultrasonic intensification the mixtures were sonicated in the cold room (4 °C) for 5, 15, or 30 min using a sonifier (Branson Ultrasonics, Model 250/450, Ser. No. BH60167) with a constant duty cycle, output control at level 8 (high frequency (20 kHz), maximum power 200 watt (level 9)). Protein extraction (30 min) using conventional methods were conducted at either 4 °C for cold extraction or 85 °C for hot extraction using a hot plate stirrer.

After separating the insoluble residues with cheesecloth, both the volume and protein concentration of filtrates were determined for calculating extraction protein recovery (EPR):

$$\text{EPR (\%)} = \frac{\text{Protein in the extracted solution (g)}}{\text{Protein in sample (g)}} \text{ x } 100$$

The extracted solution was then adjusted pH to desired values (pH 3.0 – 5.5) using HCl 10N, 2N and chitosan solution (1 % chitosan solubilised in acetic acid 1M), in which the final chitosan concentrations were 0 – 400 mg/L. The suspension was stirred for 5 minutes using a multiple stirrer and left standing for 30 minutes before centrifugation at 5,000g, 4 ^{o}C, and 5 minutes for obtaining precipitate. The precipitates were freeze-dried and referred as lobster head proteins (LHP), which were weighed for calculating the precipitate protein recovery (PPR).

$$\text{PPR (\%)} = \frac{\text{Precipitated protein (g)}}{\text{Protein in the extracted solution (g)}} \text{ x } 100$$

3.2.6 Lobster protein solubility, digestibility, and amino acid composition

3.2.6.2 Digestibility

The digestibility of lobster proteins was determined using the method of Mohamed, Zhu, Issoufou, Fatmata, and Zhou (2009) with slight modifications and egg white protein (EWP) was used for the positive control. Protein extracts (10 mg) with known protein concentrations (P_S) were digested with 1 mg trypsin in 5 mL of 0.1 M Tris-HCl buffer pH 7.6. The samples were incubated at 37 °C for 2 hours. The hydrolysis reaction was stopped by adding 5 mL of trichloroacetic acid (TCA) 50 % (v/v) and centrifuged at 9,000*g* for 10 min using Beckman Coulter Centrifuge (Allegra X-12R, SN: ALX08813). The amount of digested protein in the supernatant (P_D) was determined by using the Micro Lowry method. Protein digestibility (PD) was calculated as follows:

$$\text{PD (\%)} = \frac{P_D}{P_S} \text{ x } 100$$

3.2.6.3 Amino acids composition

The amino acid profile of the lobster protein extracts was determined at the Australian Proteome Analysis Facility (APAF), Macquarie University (Sydney).

3.2.7 Statistical analysis

Excluding the amino acid profiling analysis, all other experiments were carried out using three technical replicates and the data was analysed using a Statistical Analysis System (SAS) 9.1 (Inc., 2004). Duncan's multiple range test was performed to determine significant differences ($p < 0.05$).

3.3. Results and discussion

3.3.1 Australian LHB as a rich-protein source for recovery

3.3.1.1 Chemical composition of LHB

Australian LHB is composed predominantly of protein (43.5 % w/w), minerals (31.6 %, w/w), and chitin (19.8 %, w/w) (Table 3.1). Compared to other crustacean processing by-products, Australian LHB protein content is higher than crab (42.9 %) (Beaulieu, Thibodeau, Bryl, & Carbonneau, 2009) and some species of lobsters (Table 3.1) but lower than shrimp heads (54.5 %) (Trung & Phuong, 2012). The protein derived from the residual meats in Australian LHB accounted for 40 % of the total protein, which would be an excellent protein source for recovery.

Table 3.1 Approximate of Australian LHB compared with American LHB

Components	**Australian Rock LHB** (%)	**American LHB*** (%)
Protein	43.5	39
Chitin	19.8	20
Lipids	0.3	2.2
Minerals	31.6	34

*Reported by Y. Tu (1991)

3.3.1.2 Heavy metal composition of LHB

As some parts of lobster waste (i.e. lobster liver) are heavily contaminated with Arsenic (As) and Cadmium (Cd), the heavy metal composition of the lobster heads was determined. As illustrated in Table 3.2, the heavy metal content of the lobster heads was significantly lower than the livers. Particularly, the levels of toxic heavy metals were very low, under 0.01 mg/kg for Mercury (Hg) and approximately 0.057 mg/kg for Lead (Pb). The level of inorganic As and Cd was 0.35 and 0.31 mg/kg, respectively, which is under the legal limit for fish products (under 0.5 mg/Kg for Pb, Hg and 2.0 mg/kg for As, Cd) as regulated by Australian New Zealand Food Standard (ANZFS).

Therefore, Australian LHBs would be a safety source for protein recovery for food and nutraceutical products.

Table 3.2 Comparison of the heavy metal content in LHB, lobster liver, and the ANZFS

Trace elements	LHB (mg/kg)	Lobster liver (mg/kg)	ANZFS (mg/kg)
Antimony	< 0.01	0.028	-
Arsenic (inorganic)	0.35	0.67	2
Arsenic (total inorganic & organic)	93	240	-
Cadmium	0.31	8.7	2
Copper	46	1000	-
Lead	0.057	0.024	0.5
Mercury	< 0.01	0.28	0.5
Selenium	1.8	16.0	-
Tin	< 0.01	< 0.01	250
Zinc	78	170	-

3.3.2 Protein recovery using ultrasonic extraction compared to conventional extraction methods

3.3.2.1 Ultrasound-intensified extraction of LHP

The proteins in LHBs were extracted at alkaline conditions (pH 10 – 13) using sonication and stirring (30 min) and the EPR values calculated (Figure 3.2).

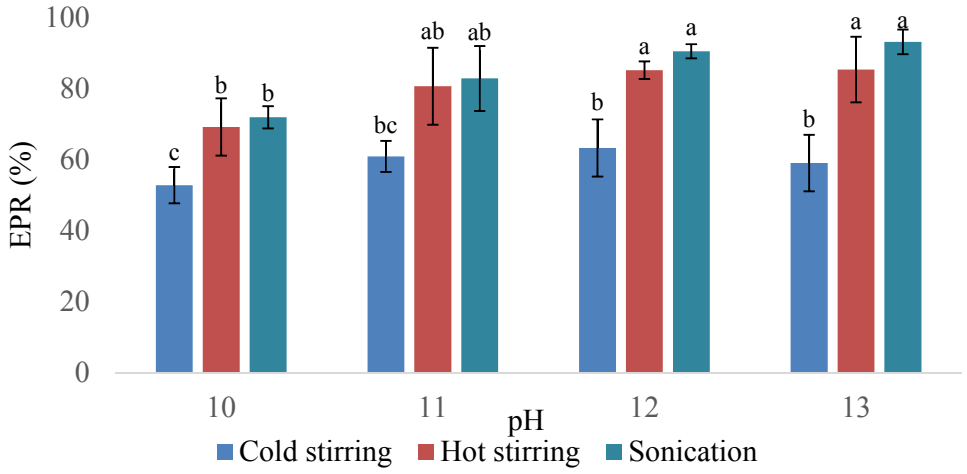

Figure 3.2 Extraction protein recovery (EPR) of the sonic extraction at different pH values compared with other conventional extraction methods. The results shown are the average of three replicates.

Plots with different letters are statistically significant, p< 0.05. The error bars are the standard deviations.

Regardless of the pH used ultrasonic extraction achieved higher EPR values compared to conventional extraction methods (cold or heat based). Compared to the yields achieved under cold stirring extraction, the EPRs of sonicated extraction was significantly higher (19.1 – 34 %); however, the increase in yield was less significant compared to that of heat-based extraction. The increase in protein extracted is likely due to the physical and mechanical changes that occur when samples are treated with ultrasound (Awad, Moharram, Shaltout, Asker, & Youssef, 2012). As shown in Figure 3.2, the highest yield of protein solubilisation was achieved at pH 13, which is consistent with the findings in another crustacean study investigating protein extraction (Y-C Chen, Tou, & Jaczynski, 2009).

3.3.2.2 Effect of water ratio on the ultrasonic extraction

The ultrasonic extraction of proteins was further optimised by trialling different processing parameters such as the water to LPB ratio and the time of extraction (Figure 3.3). The water to LPB ratio was found to have a significant effect on the yield of EPR that could be achieved. An increase in the water to LPB ratio from 4 to 8 mL/g brought the EPR value from 72 to 99 % within 5 min. However, lengthening the extraction time from 5 to 15 min did not improve the EPRs of the process. Therefore, it can be concluded that the water to LHB ratio has a greater effect on the EPR than does the time of the extraction.

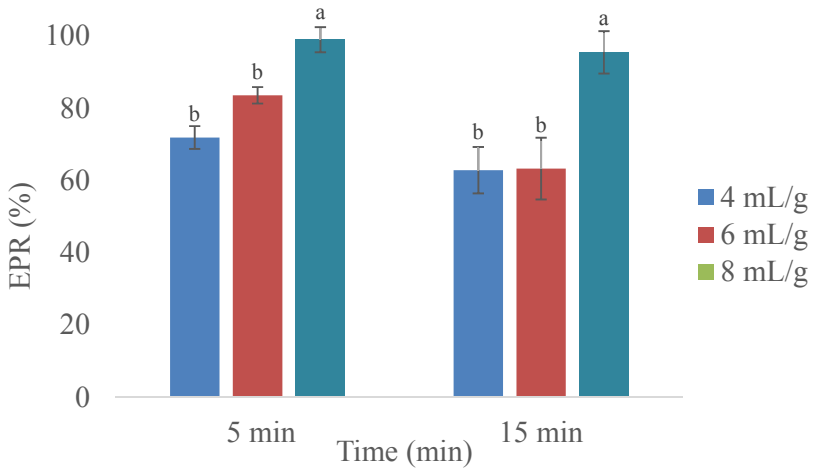

Figure 3.3 Extraction protein recovery (EPR) of lobster head at different water to LHB ratios (mL/g) and time of extraction (min). The results shown are the average of three replicates. Plots with different letters are statistically significant, p< 0.05. The error bars represent the standard deviations.

3.3.2.3 Isoelectric precipitation of LHP enhanced by chitosan

The proteins solubilised using sonication were recovered by adjusting the pH of the solution so the proteins had zero electrostatic charge for precipitation and separation, this is called the isoelectric point. To determine the optimal pH for LHP recovery, the precipitation process was tested at a range of pH values (3.5 – 5.5) and the protein precipitation recovery (PPR) values calculated (Figure 3.4). Although protein was recovered at every pH tested the highest PPR value was achieved at pH 3.5 (82.3 %) indicating that more acidic solutions are better for the precipitation of LHP.

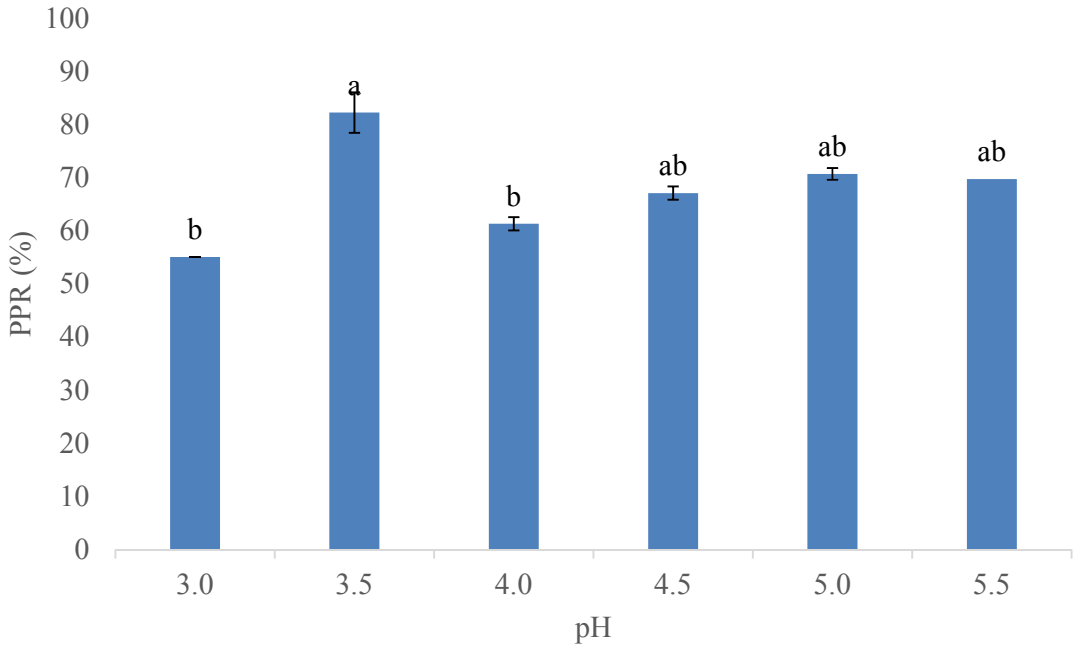

Figure 3.4 Effect of various precipitation pH values on the PPR. The results shown are an average of three replicates. The plots with different letters are statistically significant, $p < 0.05$. The error bars represent the standard deviations.

For improving the PPR, the use of various chitosan concentrations during precipitation were also investigated. As shown in Figure 3.5, adding chitosan had significant effects on the precipitation of LHP. Although the use of chitosan with low (50 – 100 mg/L) and high concentration (350 – 400 mg/L) could not improve the PPR, the use of chitosan with a dose of 200 – 250 mg/L could bring the PPR to higher value (86.6 %). This result is consistent with the previous studies reporting that using an appropriate chitosan dose during the precipitation could improve protein recovery from whey protein (Kennedy, Paterson, Taylor, & Silva, 1994), fish protein (Singgih Wibowo,

Velazquez, et al., 2007), or shrimp protein (Trang, 2010). Chitosan with its glucosamine back bone containing functional groups offers it a poly-cationic property working as a polyelectrolyte which may be ionisable and have the ability to destabilise or enhance the flocculation of the constituents in the aqueous medium (Gebelein & Carraher, 1994). Chitosan has worked as a flocculent, which is adsorbed on the surfaces of adjacent colloids to bind them together or chitosan bears an opposite charge to the suspended material adsorbed, thereby reduce the potential energy of repulsion between adjacent colloids.

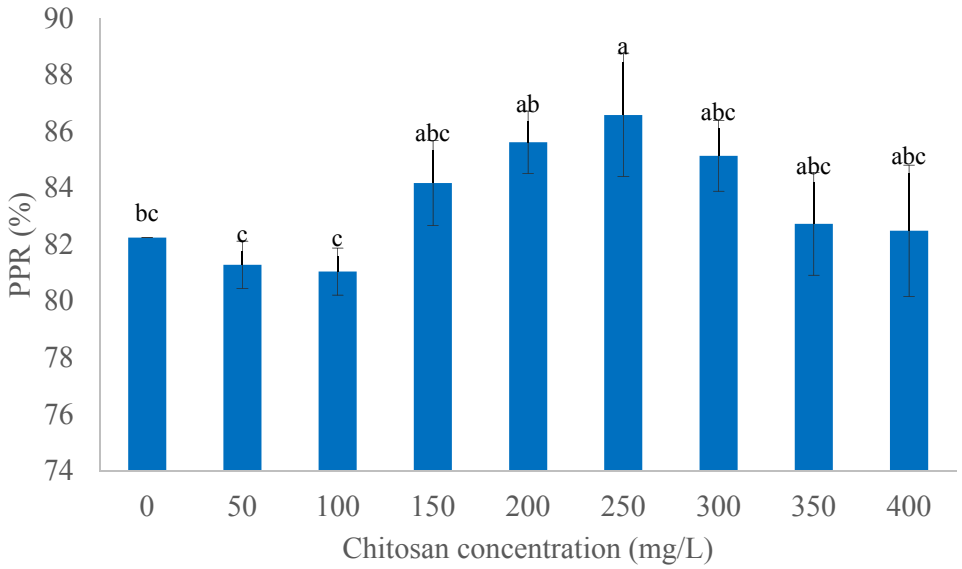

Figure 3.5 Effect of chitosan concentration and sediment time on protein precipitation. The results shown are the average of three replicates. The plots with different letters are statistically significant, $p < 0.05$. The error bars are the standard deviations.

3.3.3 Quality of protein extracted by the ultrasound

3.3.3.1 Protein-rich ultrasonic extract

Approximate in chemical composition of LHP shows the ultrasonic extract contained high proportion of protein up to 83.2 % (Table 3.3), which is higher than heat based extracted protein (78.6 %). Moreover, lipid contents of the ultrasonic-extracted LHP was low (3.4 %), which would be an advantage in quality since lipids can be oxidised during processing or storage and this would affect functionalities and nutritional quality of protein (A. R. Shaviklo, 2015; G. R. Shaviklo, Thorkelsson, Arason, & Sveinsdottir, 2012). High protein content demonstrated these LHPs could be marketed as lobster protein powder (A. R. Shaviklo, 2015).

Table 3.3 Chemical composition of LHPs extracted by ultrasound and hot stirring

Components	LHP extracted by ultrasound* (%)	LHP extracted by hot stirring (%)
Moisture	6.6 ± 0.03	7.6 ± 0.04
Protein	83.2 ± 2.6	78.6 ± 4.3
Lipids	3.4 ± 0.6	4.7 ± 0.7
Minerals	6.8 ± 0.1	9.1 ± 0.1

*Results are an average of three replicates with their standard deviations

The molecular weight (MW) of the proteins recovered using these two extraction methods was determined using electrophoresis (Figure 3.6). The ultrasonic-extracted protein (lane 3) had MW from 50 – 150 KDa, which was higher than the hot-extracted protein, 25 – 37 KDa (lane 2). The higher MW of the ultrasonic-extracted protein suggests that mild conditions of ultrasonic extraction (4 ^{o}C, 5 min) did not degrade the proteins during processing thus the quality and structure of the proteins is retained. However, as heat-based extractions are more severe the protein may be degraded and hydrolysed, generating low MW proteins and peptides, which could limit the uses and applications of the extract. For example, low molecular weight proteins and hydrophobic peptides produce a bitter taste (Lee & Shahidi, 2007).

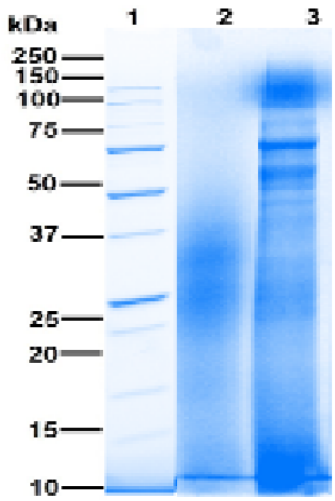

Figure 3.6 Molecular weight of hot-extracted protein (lane 2) and the ultrasonic-extracted protein (lane 3) is determined using electrophoresis

3.3.3.2 Chemical scores and amino acid availability of the ultrasonic-extracted LHP

The nutritive value of a protein depends upon its capacity to provide nitrogen and amino acids in adequate amounts to meet the requirements of an organism. Thus, evaluating protein quality by comparing its amino acid profile with human amino acid requirements is the most logical approach (FAO/WHO, 1990). In this study, amino acid availability, chemical scores, and limiting amino acids of the LHPs were determined for quality evaluation by comparison of their essential amino acid patterns with the reference protein for adult humans recommended by WHO/FAO (1990). As compared with protein recovered by the hot extraction, total EAAs in the ultrasonic-extracted protein was significantly higher (38.9 vs 30.4 %), Table 3.4. All EAAs of the ultrasonic-extracted protein as well as their chemical scores were significantly higher than those of the hot-extracted protein with isoleucine, leucine, and methionine nearly doubled. These levels of EAAs were double the values suggested for adults by FAO/WHO with the exception of lysine and methionine. Methionine was the limiting amino acid of LHPs indicated by its low chemical score but the lack of methionine was more pronounced in the hot-extracted protein than in the ultrasonic-extracted protein (0.41 vs 0.85). The harsh processing conditions of hot extraction could play a role in the availability of methionine and other amino acids. The availability of amino acid refers to the chemical integrity of the amino acid and its influence to processing by heat, high pH, oxidation, etc. (Venugopal, 2009). Apart from its high efficiency for protein extraction, the ultrasonic process also extracts protein with high nutritional quality indicated by its significantly high EAAs over those of the hot extract, which exceed the amount recommended for adult humans.

Table 3.4 Amino acid composition of LHPs recovered from Australian LHB by ultrasonic or hot extractions

Amino acids[1]	**Sonic extract LHP** (mg/g)	**Chemical scores for sonic extract LHP**	**Hot extract LHP** (mg/g)	**Chemical scores for hot extract LHP**	**FAO/WHO[2]**
Alanine	21.5		27.8		
Arginine	25.2		25.9		
Aspartic acid	49.5		38.4		
Glutamic acid	65.0		62.4		

Glycine	19.8		33.1		
Histidine	15.4		9.9		-
Isoleucine*	26.9	2.07	17.9	1.38	13
Leucine*	39.1	2.06	24	1.26	19
Lysine*	25.3	1.58	16.1	1.01	16
Methionine*	14.4	0.85	7	0.41	17
Phenylalanine	28.4		21.2		
Proline	19.2		30.2		
Serine	19.5		24.1		
Threonine*	23.7	2.63	21.7	2.41	9
Tyrosine	23.8		15.8		
Valine*	28.0	2.15	25.3	1.95	13
Total essential amino acids (TEAA)	172.8		121.9		
TEAA suggested by FAO/WHO					87
Proportion of essential amino acids	38.9		30.4		
Lysine/Arginine ratio	1.0		0.6		

[1]Amino acids with * are EAAs

[2]FAO/WHO (1990)

Moreover, lobster protein recovered by ultrasonic extraction contained large proportion of glutamic and aspartic acids, which are responsible for umami taste (Guichard & Salles, 2016; Imm & Lee, 1999). Since umami enhances the palatability of a wide variety of foods (Beauchamp, 2009; Yamaguchi, 1998), this suggested lobster proteins could be used as a naturally containing glutamate

source for production of tastier and healthier products. In addition, low Lysine/Arginine ratio (1.0) is another advantage of LHPs

3.3.3.3 Digestibility of the sonic-extraction LHP

Protein digestibility is used to evaluate protein quality. The digestibility of the LHPs recovered using different extraction methods are shown in Figure 3.7. LHPs recovered by the sonic extraction had a high rate of digestibility (77.7 %) and was nearly as high as that of EWP (88.1 %) which is considered to be one of the most digestible proteins. However, protein recovered by the sonic extraction was more digestible than protein recovered by the hot extraction method (77.7 vs 58.4 %). Significantly high digestibility of protein recovered by ultrasound over hot extraction could be explained by the fact that processing treatment can limit digestibility. Exposure of food proteins to certain processing conditions, particularly harsh conditions, could induce some major chemical changes such as racemization of L-amino acids to D-isomers and concurrent formation of cross-linked amino acids such as lysine-alanine (Venugopal, 2009). Racemization rate varies depending on the amino acid, presence of other proteins, pH, processing conditions, and temperature. Since D-isomers are less absorbed than L-amino acids, racemization impairs digestibility and thus the nutritional quality.

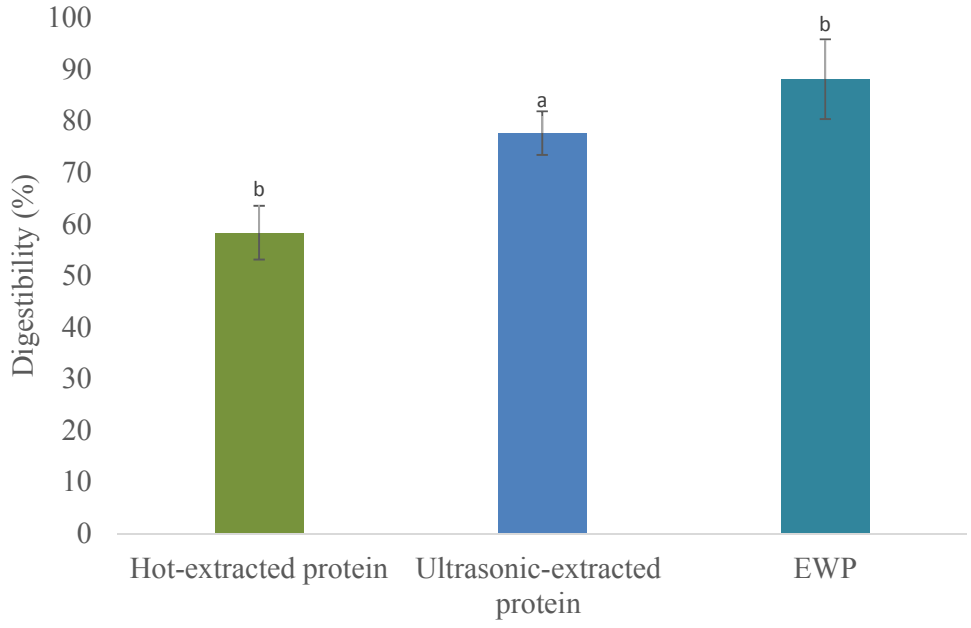

Figure 3.7 Digestibility of LHPs recovered from different methods compared with egg protein. The results shown are the average of three replicates. The plots with different letters are statistically significant, $p < 0.05$. The error bars are the standard deviations.

3.3.3.4 Safety of LHP recovered by sonic and hot extractions

To ensure that the extracted proteins are safe for food applications, the LHPs were analysed for their heavy metal composition. As shown in Table 3.5, regardless of the methods used for extraction, all LHPs had very low heavy metal levels. The sonic-extraction LHP was 74 % lower in Cadmium than that in the hot-extraction LHP. Furthermore the values for Lead and Mercury were 47.6 and 10.5 % lower, respectively. These results are consistent with the previous studies of Bendicho and Lavilla (2000); Hristozov et al. (2004), and Deng, Feng, and Qiu (2009) which reported that ultrasound could facilitate heavy metal removal. Particularly, toxic heavy metals such as inorganic Arsenic, Cadmium were determined to be low, at 0.18 and 1.2 mg/kg, respectively. These values were under the limit for fish products regulated by ANZFS. Therefore, LHPs recovered by both the sonic and hot extractions are safe for food applications.

Table 3.5 Heavy metal composition of LHPs recovered by the sonic and hot extractions

Trace elements	**Ultrasonic extract LHP (mg/kg)**	**Hot extract LHP (mg/kg)**	**ANZ Food Standard (mg/kg)**
Antimony	< 0.01	0.03	-
Arsenic (inorganic)	0.18	0.18	2
Arsenic (total)	70 ± 1	69	-
Cadmium	0.89 ± 0.02	1.2	2
Copper	38 ± 1	38	-
Lead	0.10 ± 0.006	0.21	0.5
Mercury	< 0.01	0.16	0.5
Selenium	0.95 ± 0.24	0.31	-
Tin	< 0.01	0.37	250
Zinc	49 ± 1	72	-

* Results are averages of triplicate with their standard deviations

3.4 Conclusion

Australian LHB is rich in proteins, which could be extracted as a high-value product. Although the hot extraction could recover significantly more protein than the cold extraction, protein recovered from this method was lower nutritional quality than that of the ultrasonic extraction. In contrast, the sonic extraction could recover protein with very high extraction efficiency at mild condition in short time while the extracted protein had significantly high nutritional quality indicated by its availability and digestibility. The sonic-extracted protein contained all EAAs with high chemical scores and was easily digestible and food safety, which suggested that LHP could be used as nutrients for food products.

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CHAPTER 4 MICROWAVE INTENSIFIED ENZYMATIC DEPROTEINISATION OF AUSTRALIAN ROCK LOBSTER SHELLS (*JASUS EDWARDSII*) FOR EFFICIENT RECOVERY OF PROTEIN HYDROLYSATE AS FOOD FUNCTIONAL NUTRIENTS

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4.1 Introduction

Protein and chitin are two potentially valuable components of Australian lobster processing by-products. Protein and chitin have been reported to make up about 29 % and 23 % on dry weight basis of typical lobster processing by-products, respectively (Tu et al., 1991). The potential economic value and relatively high volume of these by-products offer significant value-added opportunity for the Australian lobster processing industry if chitin and protein in the lobster processing by-products can be economically recovered. However, the recovery and utilisation of these two components from Australian lobster processing by-products for food application have not yet been realised. Lobster by-products are currently used for the production of aqua-feed and bio-fertiliser with low commercial value, or directly discarded with a disposal costs to the industry around $150 per ton. Therefore, the utilisation of Australian lobster processing by-products for the recovery of protein and chitin for food applications will reduce operational costs and improve the sustainability of the Australian lobster industry.

Chitin and its derivatives such as chitosan and chito-oligosaccharides (COS) have high economic value because of their desirable physical properties and versatile biological activities with many applications including biomedicine, pharmaceuticals, food, and agriculture (Hayes, 2012). Although chitin can be found in various species, crustacean shells are the sole major resource used for the production of industrial chitin (Xu, Gallert, & Winter, 2008). Shells generated from the lobster processing industry have the potential to offer an inexpensive commercial chitin source because of high chitin content and relatively large quantities generated. The potential value of lobster by-products has already been demonstrated by the use of chitin and its derivatives extracted from lobster shells for biocide and bio-stimulant applications (Sharp, 2013).

Lobster shells have also been identified as an important animal protein source that contains 29 % protein with carotenoprotein making up as much as 16 % of dry lobster shell (Tu, 1991). Carotenoprotein is a complex of protein and carotenoid pigments, mainly astaxanthin (Cremades et al., 2001) that possesses high antioxidant activity and may assist in several health benefits (Higuera-Ciapara, Felix-Valenzuela, & Goycoolea, 2006). Moreover, peptidic fractions generated from enzymatic hydrolysis of carotenoprotein have been reported to possess good functionalities and high antioxidant activity (Sila et al., 2014). In spite of these developments, it has still lacked a simple but efficient process, which could fully recover protein and chitin for value-added products.

To fill this gap, a biorefinery process has been proposed to recover the protein as the first product by enzymatic deproteinisation before extracting chitin as the second product. Previously, the protein generated from chemical deproteinisation of lobster shells was not usually recovered due to its incompatibility for food application. The use of proteolytic enzymes for deproteinisation of lobster shells could allow the recovery of functionally and nutritionally valuable protein hydrolysates suitable for food application (Gagné & Simpson, 1993). Protein hydrolysates derived from crustacean shells have been reported to contain bioactive peptides used as pharmacological tools or as growth stimulating agents in animal feed (Cudennec, Ravallec-Ple, Courois, & Fouchereau-Peron, 2008). Furthermore, a growing body of scientific evidence suggests that crustacean protein hydrolysates and peptides possess several biological functions promoting human health and prevent some chronic diseases (Lordan, Ross, & Stanton, 2011). Although a variety of enzymatic processes have been developed for deproteinisation of crustacean shells, the industrial use is still limited because of the inefficient removal of proteins and long processing times (Juan et al., 2014). Improving enzymatic deproteinisation of crustacean shells such as lobster shells by enhancing protein removal and reducing reaction time could make this process more industrially feasible.

Recently, microwave-assisted extraction (MAE) has been reported as an efficient technique for extraction of bioactive compounds with high yield, low usage of solvents, and reduced extraction time (Xiao, Han, & Shi, 2008). Microwave has also been used for the pre-treatment of biological materials to improve the enzymatic reaction rate (Roy, Mondal, & Gupta, 2003). The pre-treatment of materials with microwave has been reported to generate final products with high bioactivities and good functionalities (Lin et al., 2010). Microwave has also been used as an effective method to intensify enzymatic reactions such as enzymatic hydrolysis of starch and protein (Horikoshi, Nakamura, Kawaguchi, & Serpone, 2015; Lukasiewicz, Marciniak, & Osowiec, 2009). However,

there have been no reports on microwave-intensified enzymatic deproteinisation (MIED) of lobster shells during chitin extraction and recovery of lobster shell protein hydrolysate (LSPH) for food applications. The aim of this work is, therefore, to develop an efficient microwave-intensified enzymatic process for deproteinisation of lobster shells to recover protein while providing the suitable residue materials for subsequent chitin recovery by comparing with the conventional enzymatic process. The LSPH generated from this new process were further evaluated for their physicochemical functions relevant to food applications.

4.2 Materials and methods

4.2.1 Materials

Shells of Australian rock lobster *(Jasus edwardsii)* were supplied by Ferguson Australia Pty Ltd as cooked and ground lobster shells (CGLS) with 49.9 % of particle size passing through a 250-µm laboratory sieve.

Alcalase 2.4 L FG, food grade proteolytic enzyme, was supplied by Novozymes Australia Pty Ltd. All the other chemicals used are of analytical grade.

4.2.2 Chemical composition of Australian Rock lobster shells

Moisture was determined by oven drying of Australian lobster shells at 105 ^{o}C (AOAC 950.46) until a constant weight was obtained, while ash was quantified by incineration in a muffle furnace at 600 ^{o}C (AOAC 920.153). Lipid content was measured using supercritical CO_2 extraction reported by Dionisi, Hug, Aeschlimann, and Houllemar (1999). Protein content was determined using the method of Synowiecki and Al-Khateeb (2000). Chitin was determined using the method described by Simpson and Haard (1985).

4.2.3 Enzymatic deproteinisation of lobster shells by microwave-intensified process

The CGLS (5 g) were mixed with MilliQ water (4 mL/g) in a 250-mL round bottom flask. The pH was adjusted to 8.0 using lactic acid (40 %) and preheated at 55 ^{o}C (input energy 70 W, stirring 95 %) for 5 minutes by a microwave extraction system (Milestone Microwave Laboratory Systems, Model START SYNTH, serial number 131154) to satisfy the optimal pH and temperature for the enzyme alcalase. This microwave system was installed with two built-in sensors including an ATC-FO temperature sensor and infrared sensor to control the temperature with accuracy 99 ± 0.5 %. The alcalase was added with the enzyme/shell ratio 1 % (v/w). The CGLS was then enzymatically

deproteinised by setting up firstly 55 ^{o}C (input energy 40 W, stirring 95 %) for a desired time (30, 60, and 90 minutes) and then at 95 ^{o}C (input energy 200 W, stirring 95 %) for 5 minutes to inactive alcalase. After vacuum filtration with sintered metal filter, the residual solid was washed with MilliQ water (10 mL) and then oven-dried to a constant weight (referred to as enzymatically deproteinised shells, EDPS). The dried EDPS was weighed to calculate the weight loss and determine residual protein content while the protein concentration of the filtrate (referred as LSPH) was determined to calculate the degree of deproteinisation using the following formula.

$$\text{DD (\%)} = \frac{\text{Protein in LSPH}}{\text{Total protein in lobster shells}} \text{ x } 100$$

4.2.4 Enzymatic deproteinisation of lobster shells by conventional incubation

The CGLS (5 g) were mixed with MilliQ water (4 mL/g) in a 250-mL conical flask followed by adjusting pH to 8.0 using lactic acid (40 %). The mixture was preheated in a waterbath at 55 ^{o}C for 15 minutes. The enzymatic deproteinisation of CGLS was then carried out at 55 ^{o}C for the desired time (30, 60, and 90 minutes) by placing the flask in the waterbath (Ratek, Model ET22, serial number 908023262). The reaction mixture was manually shaken for 10 seconds every 30 minutes during this process. After deproteinisation, the flask was boiled for 5 minutes to inactivate alcalase. Subsequent steps were carried out as the same manner as the microwave-intensified enzymatic deproteinisation process described above to obtain the LSPH.

4.2.5 Physicochemical properties of LSPH

4.2.5.1 Protein solubility

The solubility of LSPH was determined by dispersing 50 mg of sample in 5 ml MilliQ water with the solution pH adjusted to 2, 4, 6, 8, and 10 using HCl 1 M or NaOH 1 M. The solution was magnetically stirred for 30 minutes at room temperature (20 ± 3 ^{o}C) followed by centrifugation at 990 x g for 10 minutes (Beckman Coulter, Allegra X-12R, SN: ALX08813). The protein contents in the supernatants were measured by the method of Lowry, Rosebrough, Farr, and Randall (1951) while the total protein of LSPH was determined by Kjeldahl method (AOAC, 2000).

$$\text{Solubility (\%)} = \frac{\text{Protein content in supernatant}}{\text{Total protein content in sample}} \text{ x } 100$$

4.2.5.2 Water absorption capacity

The ability of LSPH to impact the water absorption capacity of meat products was determined by the method described by Geirsdottir et al. (2011) with some modifications. LSPH (0.1 g) was added to 4.9 g beef mince to produce the beef mince with LSPH while the blank sample was prepared solely using beef mince (5 g). Both the beef mince with and without LSPH were placed in 50-mL centrifuge tubes and mixed with 1 g MilliQ water (W_A) by vortex for 1 minute. After the mixture was left standing on ice for 30 minutes, free water was separated by centrifugation at 3000 x g for 10 minutes (Beckman Coulter, Allegra X-12R, SN: ALX08813). The weight of the tubes before (WT_B) and after separation of free water (WT_A) were recorded to calculate the water absorption capacity of the beef mince.

$$\text{Water absorbance capacity (\%)} = \frac{WT_B - WT_A}{W_A} \times 100$$

4.2.5.3 Oil absorption capacity

Oil absorption capacity of LSPH was measured using the procedures described by Beuchat (1977) with slight modifications. One gram of sample (Ws) was mixed with 10 mL canola oil (O_A) in a 50-mL centrifuge tube by vortex for 30 seconds. The sample was then allowed to stand at room temperature (20 ± 3 ^oC) for 30 minutes before it was centrifuged at 5000 x g for another 30 minutes (Beckman Coulter, Allegra X-12R, SN: ALX08813). Free oil (O_F) was separated by decantation at a 45 degree angle and the volume of free oil was measured in a 10 ml graduated cylinder.

$$\text{Oil absorbance capacity (mL/g)} = \frac{O_A - O_F}{W_S} \times 100$$

4.2.5.4 Emulsifying capacity and stability

Emulsifying capacity and stability were measured using the method described by Lawal (2005) with a slight modification. Five millilitre of LSPH solutions (dissolved in MilliQ water) at 10 mg/mL and 5 mL of canola oil were homogenised at 13500 rpm for 60 seconds using a homogeniser (Ika, Ultra-Turrax T25). The emulsions were centrifuged at 1100 x g for 5 minutes (Beckman Coulter, Allegra X-12R, SN: ALX08813). The height of the emulsified layer and that of the total contents in the tube was recorded. The emulsifying capacity was calculated as follows.

$$\text{EA (\%)} = \frac{\text{Height of emulsified layer in the tube}}{\text{Height of the total contents in the tube}} \times 100$$

Emulsifying stability (ES) was measured by heating the emulsions in a water bath at 80 ^{o}C for 30 min before centrifuging at 1100 x g for 5 minutes.

$$ES\ (\%) = \frac{\text{Height of emulsified layer after heating}}{\text{Height of the total contents in the tube}} \times 100$$

4.2.5.5 Foaming capacity

The foaming capacity was determined using the method of Coffman and Garcia (1977) with slight modifications . Ten millilitre of LSPH solution (dissolved in MilliQ water) at 10 mg/mL were foamed by a homogeniser (Ika, Ultra-Turrax T25) at 8000 rpm for 60 seconds. Volumes of the solutions before (V_1) and after homogenisation (V_2) were recorded. The percentage volume increase referred to as index of foam capacity was calculated based on the following equation.

$$\text{Index of foam capacity } (\%) = \frac{V_2 - V_1}{V_1} \times 100$$

The foaming samples were left stand at room condition for 60 minutes and volume of samples after standing (V'_2) were determined. The foaming stability was determined by the following equation.

$$\text{Index of foam stability } (\%) = \frac{V'_2 - V_1}{V_1} \times 100$$

4.2.5.6 Amino acids analysis

Amino acid composition of LSPH was analysed at Australian Proteome Analysis Facility (APAF), Macquarie University, Sydney.

4.2.5.7 Statistical analysis

Except for amino acid profile, all other experiments were carried out in three replicates. The obtained data was subjected to an analysis of variance using a Statistical Analysis System (SAS) 9.1 (Inc., 2004). Duncan's multiple range test was performed to determine the significant ($p < 0.05$) difference among means. Levene test was also used to verify whether the data satisfy the ANOVA condition and Kruskall-Wallis test has been applied for the data not satisfying ANOVA condition.

4.3 Results and discussion

4.3.1 Australian Rock lobster shells rich in protein and chitin

Australian Rock lobster *(Jasus edwardsii)* contains a large proportion of minerals (36 %) while its lipid content is very low (0.6 %). Two significant components of Australian Rock lobster shells with high potential economic value are protein and chitin accounting with respective contents of 29 % and 25 %. These contents are close to 29 % protein and 23 % chitin, reported in American cooked lobster waste *(Hamarus Americanus)* (Tu et al., 1991).

4.3.2 Microwave intensified enzymatic deproteinisation (MIED) of lobster shells

4.3.2.1 Weight loss

The weight losses of shells during enzymatic deproteinisation by the microwave and conventional incubation are shown in Figure 4.1. In both cases, lobster shells were clearly susceptible to weight loss due to enzymatic reaction. Proteins constituted in the lobster shells could be hydrolysed during the process and quickly released into the solution leading to the weight loss (Valdez-Pena et al., 2010). As shown in Figure 4.1, the weight loss increased with the treatment time from 30 minutes to 90 minutes for both processes but the weight loss of the 60-minute treatment was not significant compared with those of the 30-minute and 90-minute treatment. The highest value of weight loss was found at 90 minutes of deproteinisation with values of 30.3 % for the microwave-intensified process and 24.6 % for the conventional incubation. The weight losses of the lobster shells in the microwave-intensified process were considerably ($p < 0.05$) higher than those in the conventional incubation at all the investigated times (26.1 %, 28.2 % and 30.3 % for the microwave-intensified process vs 22.5 %, 23.7 % and 24.6 % for the conventional incubation, respectively). The significant differences in the weight loss by the two deproteinisation methods could closely relate to the enhanced protein removal ability as a result of proteolytic hydrolysis of shells intensified by microwave (Horikoshi et al., 2015).

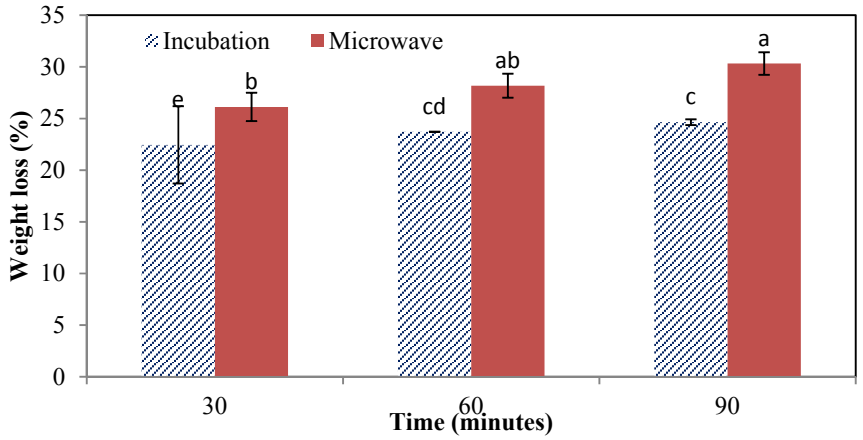

Figure 4.1 Weight loss of Australian rock lobster shells during enzymatic deproteinization by microwave-intensification and incubation (results are means of three replicates; plots with different letter are statistically significant different with P < 0.05; the error bars are the standard deviations)

4.3.2.2 Deproteinisation degree

The percentage of protein removed from the lobster shells by proteolysis during enzymatic deproteinisation was defined as the deproteinisation degree. As shown in Figure 4.2, there are significant differences in the deproteinisation degree between the samples treated for 30 minutes and 90 minutes but insignificant for the 60-minute treatment with others. The highest deproteinisation degrees in both processes were obtained at treatment of 90 minutes (85.8 % for the microwave-intensified process and 58 % for the conventional incubation). The deproteinisation degrees of the microwave-intensified process were significantly ($p < 0.05$) higher than those of the conventional incubation at all the treatment times. In fact, these deproteinisation degrees increased by 18 – 27.8 % in the microwave-intensified process compared with those in the conventional incubation. A considerable increase (27.8 %) was found at 90 minutes of deproteinisation. The higher deproteinisation degree indicates that more proteins were released under the microwave heating. This result is in accordance to the previous studies of Horikoshi et al. (2015) and De La Hoz, Diaz-Ortiz, and Moreno (2005) where enzymatic reaction rates were reported to increase several times under microwave field relative to conventional heating.

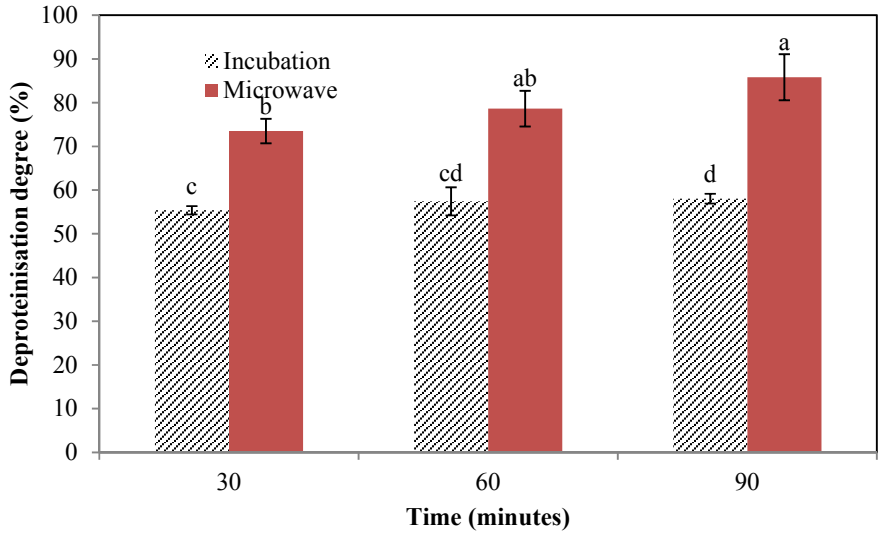

Figure 4.2 Deproteinisation degree of lobster shells during enzymatic deproteinisation by microwave-intensification and incubation (results are means of three replicates; plots with different letter are statistically significant different with P < 0.05; the error bars are the standard deviations)

The higher values of both the weight loss and the deproteinisation degree indicate that enzymatic deproteinisation of the lobster shells by the microwave-intensified process is far more effective compared with the conventional incubation. However, to further confirm this result as well as to demonstrate the ease for further chitin recovery from the EDPS, the residual proteins in these materials were also investigated.

4.3.2.3 Residual proteins in the EDPS

Figure 4.3 shows the residual proteins in the EDPS generated from the microwave-intensification and conventional incubation. The residual proteins in the EDPS reduced significantly with the increase in deproteinisation time and these values together with the removed proteins could make a protein mass balance in the range of 92 – 107 %. At all the investigated times, the microwave-intensified process had the residual protein values significantly ($p < 0.05$) lower than those of the conventional incubation by 17 % - 32.2 %. The lowest residual proteins (65.4 mg/g) occurred at 90 minutes of deproteinisation by the microwave-intensified process. With the low residual proteins, the EDPS could be directly demineralised to obtain chitin or further mild deproteinisation followed by demineralisation to recover purified chitin.

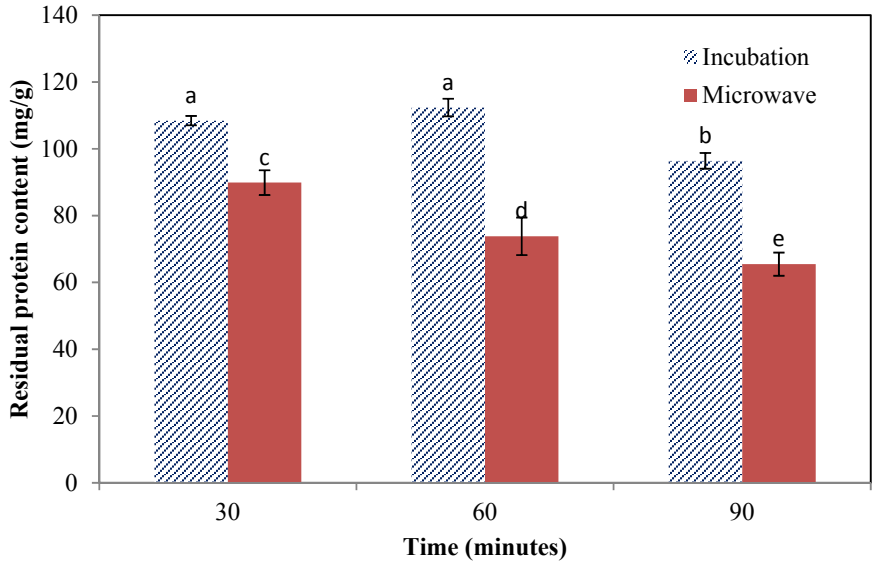

Figure 4.3 Residual protein content (mg/g) in the EDPS obtained during enzymatic deproteinisation by microwave-intensification and conventional incubation (results are means of three replicates; plots with different letter are statistically significant different with $P < 0.05$; the error bars are the standard deviations)

4.3.3 Functional property of LSPH

4.3.3.1 Solubility of LSPH

Solubility values for the LSPH and reference protein (egg white protein, EWP) prepared in house by spray-drying of chicken egg purchased from local supermarket after removing the yolk) in a wide range of pH 2 - 10 were investigated. As shown in Table 4.1, LSPH has very high solubility (91.7 %). This result is similar to that of Vieira, Martin, Saker-Sampaiao, Omar, and Goncalves (1995) where protein hydrolysate derived from lobster by-products was reported to have good solubility. In contrast to EWP, LSPH solubility was not significantly affected by pH change. Its solubility was over 85.5 % in a wide range of pH (2 – 10) while this value for EWP was only 77.7 % in the same pH range. The solubility of LSPH was higher than that of EWP with an exception at pH 2 and 10 (beyond effective food applications).

Table 4.1 Functional properties of LSPH recovered from enzymatic deproteinisation of lobster shells by microwave-intensified process (alcalase to shells ratio 1% (v/w), irradiated at 55 ^{o}C (input energy 40 W), stirring 95 % for 90 minutes) in a range of pH from 2 to 10

Functional properties (%)	Protein*	pH range				
		pH 2	pH 4	pH 6	pH 8	pH 10
Solubility	LSPH	$88.6^{a} \pm 6.4$	$86.4^{a} \pm 0.4$	$85.5^{a} \pm 3.5$	$89.5^{a} \pm 3.1$	$91.7^{a} \pm 4.1$

EC	EWP	$92.3^{a} \pm 4.4$	$77.7^{b} \pm 9$	$81.3^{b} \pm 8.8$	$81.3^{b} \pm 7$	$95.5^{a} \pm 5.9$
	LSPH	$49^{a} \pm 3.4$	$50.2^{a} \pm 1.8$	$48^{a} \pm 1.1$	$51.3^{a} \pm 3.9$	$46.5^{a} \pm 3.5$
ES	EWP	$48.7^{b} \pm 1.8$	$51.1^{ab} \pm 0.9$	21.4 ± 3.2^{c}	$22.5^{c} \pm 2.2$	$56.3^{a} \pm 5.1$
	LSPH	0^{d}	$28.5^{b} \pm 3.9$	0^{d}	18 ± 2.3^{c}	$45.7^{a} \pm 3.3$
FC	EWP	$42.2^{b} \pm 4.4$	$46.1^{b} \pm 2.5$	$20^{c} \pm 3.6$	$20.33^{c} \pm 3$	$55.8^{a} \pm 4.7$
	LSPH	$14.7^{d} \pm 5.0$	$91.3^{a} \pm 8.1$	$54^{b} \pm 5.3$	$33.3^{c} \pm 5.8$	$53.3^{b} \pm 5.8$
FS	EWP	$45.6^{c} \pm 2.6$	$57.3^{b} \pm 4.6$	$24.2^{d} \pm 6.2$	$20^{d} \pm 0$	$80^{a} \pm 0$
	LSPH	$13.3^{d} \pm 5.8$	$85.3^{a} \pm 9.2$	$46.7^{b} \pm 5.8$	$33.3^{c} \pm 5.8$	$50^{b} \pm 0$
	EWP	$1.3^{d} \pm 1.2$	$33.3^{b} \pm 5.8$	$20^{c} \pm 0$	$14^{c} \pm 2.0$	$73.3^{a} \pm 5.8$

The high solubility of LSPH over a wide range of pH could be explained by smaller peptides (molecular weight less than 10 KDa) produced by enzymatic hydrolysis of lobster shell protein, increasing polar and ionisable groups of protein hydrolysates for better interaction with water (Mutilangi, Panyam, & Kilara, 1996). The excellent solubility of the LSPH indicates good potential for food applications since solubility does not only influence emulsification and foaming capacities but also has significant effects on sensory properties (Wilding, Lillford, & Regenstein, 1984).

4.3.3.2 Water absorption capacity of beef mince with LSPH addition

Water absorption capacity, the ability of LSPH to imbibe water and retain it against gravitational force within a beef mince matrix, is presented in Figure 4.4. Addition of 2 % LSPH to the beef mice, its water absorption capacity rose significantly by up to 90 %, increased by 32 %. It was 2.5 times higher than that of adding with EWP. This result is consistent with the results of the study of Kristinsson (1998) when salmon fish protein hydrolysate and egg albumin were used in salmon mince patties. The significant improvement in water absorption of the beef mice with addition of LSPH could be explained by the presence of polar groups such as -COOH and/or -NH_2; these functional groups were likely to have a substantial effect on water absorption capacity (H. G. Kristinsson & B. A. Rasco, 2000).

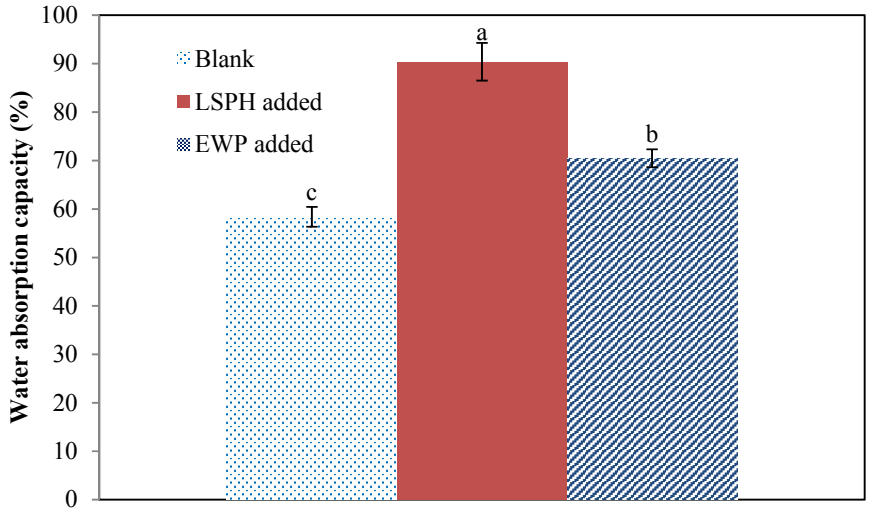

Figure 4.4 The effect of LSPH and EWP at 2 % on water absorption capacity of beef mince compared with the blank (results are means of three replicates; the error bars are the standard deviations)

Water absorption capacity of proteins added to muscle tissue is of great importance to the food industry because retaining water in a food system often improves its texture, reduces its overall energy content per unit mass and reduces the overall cost of the product. The functional properties of proteins in a food system depend on the water-protein interaction, and the final outcome greatly depends on how well the protein binds and holds water in a food system (H. G. Kristinsson & B. A. Rasco, 2000). With its excellent water absorption within a protein matrix, the LSPH has potential for use as a water-binding agent in meat products to improve cooking yield, drip losses and/or as cryo-protective agents.

4.3.3.3 Oil absorption capacity of LSPH

The quantity of oil directly bound to protein is expressed as oil absorption property. As illustrated in Figure 4.5, LSPH had oil absorption capacity lower than that of EWP (2.31 mL/g compared with 3.66 mL/g) but it was comparable to that of egg albumin (2.36 mL/g) tested with soybean oil by Hordur G Kristinsson and Barbara A Rasco (2000). The oil absorption capacity of LSPH could be influenced by bulk density of the protein and peptide sizes (Kinsella & Melachouris, 1976). The capacity of LSPH to absorb oil is an important functional property which is useful in some applications required for the meat and confectionary industries (Hordur G Kristinsson & Barbara A Rasco, 2000).

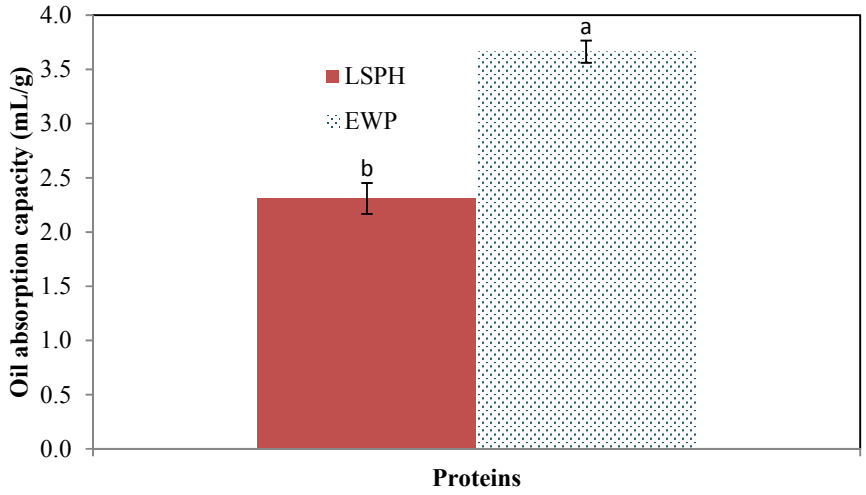

Figure 4.5 Oil absorption capacity (mL/g) of LSPH and EWP (results are means of three replicates; the error bars are the standard deviations)

4.3.3.4 Emulsification capacity and stability of LSPH

In contrast to EWP, the emulsification capacity of LSPH was not affected by pH change, slightly fluctuating around 50 % in a range of pH 2 – 10 (Table 4.1). The excellent solubility of LSPH over a wide range of pH would be expected to give the positive results for its emulsification capacity (Klompong, Benjakul, Kantachote, & Shahidi, 2007). With high solubility, LSPH can quickly diffuse and adsorb at the interface to have positive effects on the emulsification capacity. EWP had very low emulsification capacity at pH 6.0 and 8.0, which coincided with its low solubility at these pH values. Although LSPH had greater initial emulsification capacity than EWP, its emulsification stability was lower than that of EWP; the lower stability is likely to relate to the lower molecular weight of LSPH (less than 10 KDa) as smaller peptides are less effective in stabilising the emulsions (Klompong et al., 2007).

4.3.3.5 Foaming capacity and stability of LSPH

Similar to EWP, foaming property of LSPH was significantly influenced by pH change. LSPH had a higher foaming capacity than that of EWP with an exception at pH extremes (pH 2 and 10) (Table 4.1). The highest foaming capacity of LSPH was found at pH 4 (91.3 %) while the LSPH solubility was low at this pH value. Thus, it seems that the effect of composition and net charge of peptides on the foaming properties outweigh that of its solubility. Foam generated from LSPH was more stable than that from EWP (foaming reduction of LSPH around 6 % compared with 24 % of EWP at pH

4). Foaming is responsible for the desired texture of many food products, thus LSPH with its ability to form and stabilise foam could find applications in fish based soufflés and pâté for example.

4.3.4 Amino acids composition of LSPH

The nutritional value of food depends on the type and amount of amino acids available for body functions (El-Beltagy & El-Sayed, 2012). Although biological parameter such as the protein efficiency ratio or net protein utilisation are common for assessing protein quality, the protein quality assessment can also be carried out by an analysis of its essential amino acid content or chemical score (Ovissipour, Benjakul, Safari, & Motamedzadegan, 2010). Chemical score provides a nutritive value estimate of a protein by comparing the levels of essential amino acids between the test protein and reference protein. In this study, determination of chemical score and comparison of essential amino acid content of the LSPH was based on the reference protein recommended by WHO/FAO (1990) for adult humans (FAO/WHO, 1990). LSPH produced by microwave-intensified process contains 74 % of protein including 34 % of essential amino acids over total amino acid content (Table 4.2). The amount of essential amino acids in LSPH was twice the values of those suggested by FAO/WHO with the exception of methionine. Therefore, the essential amino acid composition of LSPH meets or exceeds the essential amino acid recommendation for adult humans. Moreover, LSPH contain very high amount of arginine (45.4 mg/g) that is important for its participation in protein synthesis and other physiological functions such as detoxification and energy conversion (Cao, Zhang, Hong, & Ji, 2008) and plays an important role in cardiovascular disease treatment (Niittynen, Nurminen, Korpela, & Vapaatalo, 1999). Lysine/arginine ratio has also used as an indicator for evaluation of protein nutritional value (Oomah & Mazza, 2000). LSPH has a very low lysine/arginine ratio (0.69) compared with meat protein (13.78) (Sidransky, 1990). The low lysine/arginine ratio of LSPH suggests that it could be used in meat products to reduce lipidemic and atherogenic effects induced by meat protein on human health. With all these nutritional attributes, LSPH could be potentially used as a supplement to poorly balanced dietary proteins or meat proteins.

Table 4.2 Amino acid composition of LSPH recovered from enzymatic deproteinisation of Australian Rock lobster shells by microwave-intensified process (alcalase to shells ratio 1 % (v/w), irradiated at 55 ^{o}C (input energy 40 W), stirring 95 % for 90 minutes)

Amino acids	Amount (mg/kg)	Chemical score	Suggested by FAO/WHO 1990

Alanine	33.2		
Arginine	45.4		
Aspartic acid	55.4		
Glutamic acid	81.0		
Glycine	31.9		
Histidine	18.1		-
Isoleucine*	25.3	1.95	13
Leucine*	38.3	2.02	19
Lysine*	31.1	1.94	16
Methionine*	13.0	0.76	17
Phenylalanine	28.3		
Proline	34.2		
Serine	28.1		
Threonine*	29.2	3.24	9
Tyrosine	25.3		
Valine*	33.4	2.57	13
Total amino acids	551.2		
TEAA suggested by FAO/WHO			87
Total essential amino acids (TEAA)	188.4		

4.4 Conclusion

Australian rock lobster shells are rich in protein and chitin that could be economically recovered in a novel biorefinery process. Enzymatic deproteinisation of lobster shells intensified by microwave was shown highly efficient than that with conventional incubation, achieving higher deproteinisation degree and low residual protein content. The lobster shell protein hydrolysate produced by microwave-intensified process has excellent functionalities in term of its solubility, water absorption, oil absorption, emulsification, foaming, and nutritional values for food applications. Compared with undeproteinised shells and the conventionally deproteinised shells, the

microwave-deproteinised shells have significantly lower residual proteins, which could be easily used for further chitin recovery. The microwave-intensified enzymatic deproteinisation process is effective and could be considered for protein recovery from lobster shells at large scale.

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CHAPTER 5 APPLICATION AND OPTIMISATION OF THE HIGHLY EFFICIENT AND ENVIRONMENTALLY FRIENDLY MICROWAVE-INTENSIFIED LACTIC ACID DEMINERALISATION OF AUSTRALIAN ROCK LOBSTER *(JASUS EDWARDSII)* SHELLS FOR CHITIN PRODUCTION

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5.1. Introduction

Chitin, a linear homopolysaccharide composed of N-acetyl-D-glucosamine residues linked by β-1,4 bonds, is the second most abundant natural biopolymer after cellulose. Chitin and its derivatives such as chitosan, chito-oligosaccharides are biomolecules of significant potential economic value with a wide range of applications including environment, agriculture, food, pharmaceuticals, biomedicine, and health care products (Kaur & Dhillon, 2013). Lobster shells have been identified as a source of chitin (approximately 25 % by dry weight), which is another potential material for the commercial production of chitin (Nguyen, Zhang, Barber, Su, & He, 2016). In lobster shells, chitin is tightly associated with proteins, minerals, and pigments which should be completely removed during chitin recovery, particularly for biological applications. To remove the associated minerals from crustacean shells, strong inorganic acids such as HCl, HNO_3, H_2SO_4, and H_3PO_4 have been used but hydrochloric acid is the preferred reagent (Woo-Jin, Gyung-Hyun, Kuk, Kim, & Park, 2005). Demineralisation with these corrosive acids is costly and hazardous to both workers and the environment (Ameh, Abutu, Isa, & Rabiu, 2014). This conventional demineralisation process consumes a large amount of water for extensive sequential washing, therefore generating a large volume of harmful effluent water that is costly for neutralisation and treatment. Moreover, harshly acidic treatments may lead to partial deacetylation and depolymerisation of chitin resulting in inconsistent physiological properties of the final product (Jung et al., 2007).

An alternative method to this harsh treatment is the use of organic acids for mineral removal during chitin production (Chemat et al., 2014; Greene, Robertson, Young, & Clyburne, 2016; Ramírez et al., 2015). Although organic acids are not as strong as inorganic acids, their mineral removal efficiency from crustacean shells could be comparable to that of hydrochloric acid (Mahmoud, Ghaly, & Arab, 2007). Using organic acids for demineralisation could bring several advantages

such as less potential environmental impacts, improved preservation of the physicochemical property of the purified chitin (Das & Ganesh, 2010), and the possibility for recovery of organic salts for use as food preservatives (Pattison & Von Holy, 2001; Sallam, 2007) or anti-icing agents (Bang & Johnston, 1998; Jin, Kishita, Moriya, Enomoto, & Sato, 2002). Although several organic acids could be used for demineralisation of crustacean shells, the preferred one is lactic acid since it can be produced economically and sustainably from low cost biomass such as cheese whey (Ghaffar et al., 2014). However, using lactic acid alone for demineralisation of crustacean shells required a prolonged time for demineralisation (6 hours) while the mineral residue in the obtained chitin were relatively high (4.6 %) (Mahmoud et al., 2007).

Demineralisation of crustacean shells in combination with other techniques has been demonstrated to obtain high efficiency (Hong, Jiangfeng, & Jie, 2014). Recently, microwave intensification for extraction processes has been extensively applied because microwave has several significant advantages over conventional heating such as high efficiency (Sahu, Goswami, & Bora, 2009), low consumption of solvents (Khaled et al., 2015), and reduced extraction time (Ajavakom, Supsvetson, Somboot, & Sukawattanasinitt, 2012; Chen, Xie, & Gong, 2007; Won, Choi, & Cha, 2009; Xiao, Han, & Shi, 2008). Microwave technology has also been used as a mild treatment process for chitin recovery from shrimp wastes (Contreras-Esquivel, Pena-Valdez, Balvantin-Garcia, & Flores-Davila, 2009; Flores, Barrera-Rodríguez, Shirai, & Durán-de-Bazúa, 2007). Most recently, shrimp heads demineralised by microwave with lactic acid at a ratio of 200 mL/g, 121 ^{o}C for 30 minutes showed very low residual minerals (0.2 %) in the extracted chitin (Valdez-Pena et al., 2010). However, the use of large volume of lactic acid and high temperature in the process due to a lack of optimisation could be major limitations for its industrial applications while the use of this process for more difficult demineralisation crustaceans as Rock lobster shells, so far, has not yet been investigated. The aim of this study was to apply the microwave-intensified lactic acid demineralisation for chitin production from the enzymatically deproteinised lobster shells (EDPS) generated from the innovative process published in our earlier publication (Nguyen et al., 2016). The process was then optimised using response surface methodology with Box-Behnken design to investigate the impacts of three key process parameters (lactic acid to shell (LA/S) ratio, temperature, and time) on the demineralisation degree. A valid model as well as the optimum condition for the process was determined while chitin and minerals obtained from the process were characterised to evaluate their purity and quality as the process efficiency confirmation.

5.2. Materials and methods

5.2.1 Materials

Shells of Australian rock lobster *(Jasus edwardsii)* were supplied by Ferguson Australia Pty Ltd as cooked ground lobster shells (CGLS). Particle size of CGLS has 50 % passing through a 250-µm laboratory sieve. Food grade DL-lactic acid 88.1 % (syrup) was purchased from Purac Bioquimico South Australia. Commercial crab chitin was purchased from Sigma-Aldrich in Australia.

5.2.2 Chemical composition of EDPS and extracted chitin

Moisture was determined by oven drying 0.5 gram of samples at 105 °C (AOAC 950.46) until obtaining a constant weight while their ash were quantified by incineration in a muffle furnace at 600 °C (AOAC 920.153). Protein content of samples was measured using the method of Synowiecki and Al-Khateeb (2000) with some modifications while chitin was determined using the method described by Simpson and Haard (1985). Lipids were calculated using Soxhlet extraction method.

5.2.3 Microwave demineralisation of Rock lobster shells

Rock lobster shells were firstly deproteinised by the method developed by Nguyen et al. (2016) to generate enzymatic deproteinised shells (EDPS). Dry EDPS (5 g) were mixed with lactic acid 7.5 % at the desired LA/S ratios (10, 15, and 20 mL/g) in a 500-mL round bottom flask. The mixture was demineralised by microwave at designated temperatures (50, 75, and 100 ^{o}C) using the Milestone Microwave Laboratory Systems (Model START SYNTH, serial number 131154, 1200 W, equipped with the LCD screen for setting process parameters and built-in with ATC-FO temperature and infrared sensors for temperature control) for a certain period of time (10, 20, and 30 minutes). Residual solids were separated and washed with running tap water until the pH of the rinse water reached approximately 7. The residue was further deproteinised by microwave with 25 mL NaOH 0.5 M at 100 ^{o}C for 30 minutes before depigmentation using 50 mL ethanol followed by stirring with 50 mL NaOCl 0.315 % for bleaching. The solid was washed and oven-dried at 65 ^{o}C for 6 hours to obtain chitin. Both chitin yield in each experiment run and its mineral residue were determined to calculate demineralisation degree. The sample for control was demineralised at LA/S ratio of 18 mL/g, 67 ^{o}C for 23 minutes using a hot plate stirrer while other steps were conducted in the same manner as in the microwave process.

5.2.4 Calculating demineralisation degree and mineral composition analysis

Chitin around 0.5 gram (W_S) placed in the pre-weighed crucibles (W_C) was burnt in a muffle furnace that reached 600 ^oC within 2 hours and maintained at this temperature for 10 hours. After cooling in a desiccator, the crucibles with minerals ($W_{C.M}$) were weighed for the calculation of mineral content (MC) as follows:

$$MC\ (\%) = \frac{W_{C.M} - W_C}{W_S} \times 100$$

And demineralisation degree (DD) was determined using the method described by Younes et al. (2012):

$$DD\ (\%) = \frac{(W_{DPS}.M_{DPS} - W_{CT}.M_{CT})}{W_{DPS}.M_{DPS}} \times 100$$

M_{DPS} and M_{CT} are mineral contents of deproteinised shells and chitin

W_{DPS} and W_{CT} represent the mass of deproteinised shells and chitin

Composition of lobster minerals obtained from the microwave-intensified demineralisation lactic acid process was analysed using inductively coupled plasma mass spectrometry **(**ICP-MS) as described by Shi, Francis, Machado, and Wu (1995).

5.2.5 FTIR spectroscopy

FTIR spectrum of lobster chitin produced by the microwave demineralisation was obtained using the method of Prabu and Natarajan (2012) with slight modification. All samples were scanned with Fourier transform infrared spectroscopy (FTIR) spectrophotometer (PerkinElmer, Frontier, Australia) using with an attenuated total refraction (ATR) method at speed 0.2 cm/s, resolution of 4 cm^{-1} and 12 accumulations with the absorbance in a range from 650 to 4000 cm^{-1}.

5.2.6 Experimental design

To investigate the effects of LA/S ratio (X_1), temperature (X_2), and microwave time (X_3) on the efficiency of demineralisation, a three-factor Box-Behnken design for response surface methodology (RSM) was used (Bezerra, Santelli, Oliveira, Villar, & Escaleira, 2008). The nature of Box-Behnken design is suitable for the exploration of quadratic response surfaces and generates a second-degree polynomial model, which in turn is used to optimise a process with only a small

number of experiments. The design was developed using the R program with the support of RSM package (Lenth, 2009) to generate experiments with the range and levels of variables as shown in Table 5.2. The complete design consisted of 15 combinations from these three factors including three replicates at the centre points. A second order polynomial was fitted to the experimental data using R program with the RSM package to estimate the response of the dependent variable and predict the optimal condition. The second order polynomial was described as follow:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2$$

Where Y is the predicted response; X_1, X_2, X_3 are independent variables; b_0 model constant; b_1, b_2, b_3 are linear coefficients; b_{12}, b_{23}, b_{13} are cross product coefficients; and b_{11}, b_{22}, b_{33} are the quadratic coefficients.

5.2.7 Data analysis

To analyse the resulting data including estimating the response surface, testing its lack of fit and generating response plots of the fitted surface, R software version 3.0.2 (2013-09-25) was used with the support of the RSM package version 2.03 (2013-2-21).

5.3. Results and discussion

Mineral was a major component of Rock lobster shells accounting for 36 % followed by protein and chitin (Table 5.1) (Nguyen et al., 2016). Since the EDPS still contained a small amount of protein residues (6.5 %) due to the incomplete enzymatic deproteinisation, a further deproteinisation step was integrated in the process. Mineral content in the EDPS after deproteinisation was significantly higher than that in the original shells (62 % vs 36 %) indicating that the enzymatic deproteinisation could not remove minerals from Rock lobster shells.

Table 5.1 Chemical composition of Rock lobster shells (RLS) and enzymatically deproteinised shells (EDPS)

Components	RLS (%)	EDPS (%)
Moisture	9.4 ± 1.6	3.9 ± 0.2
Protein	29 ± 1.6	6.5 ± 0.3

Mineral	36 ± 0.06	62 ± 0.2
Lipids	0.6 ± 0.04	-
Chitin	25 ± 1.1	27.6 ± 0.4

The EDPS was demineralised by microwave under various LA/S ratios, temperatures, and times with the full experimental design and corresponding data are shown in Table 5.2. The obtained data was analysed to set up a mathematical relationship between the demineralisation degree and process parameters.

Table 5.2 Three-factor Box Behnken design with observed and predicted responses of demineralisation degree (%)

Run order	Factors			Response	
	Actual values			Demineralisation degree (%)	
	LA/S Ratio (mL/g)	Temperature (^oC)	Time (min)	Observed	Predicted
1	10	50	20	86.3	83.9
2	20	50	20	98.1	99.0
3	10	100	20	84.4	83.9
4	20	100	20	99.8	99.0
5	10	75	10	79.1	81.1
6	20	75	10	96.0	96.1
7	10	75	30	82.0	83.0
8	20	75	30	98.2	98.0
9	15	50	10	95.0	95.5
10	15	100	10	98.1	95.5
11	15	50	30	96.9	97.4
12	15	100	30	98.7	97.4
13	15	75	20	98.6	98.3

14	15	75	20	97.6	98.3
15	15	75	20	95.8	98.3

5.3.1 Model equation

The effects of LA/S ratio, temperature, and time together with their interactions on the demineralisation degree were described in equation: $Y = 23.0166667 + 9.125X_1 – 0.445X_2 + 0.9466667X_3 + 0.0074X_1X_2 – 0.0035X_1X_3 – 0.0013X_2X_3 – 0.2701667X_1^2 + 0.0025533X_2^2 – 0.0175417X_3^2$. This regression equation was obtained using the result generated by analysis of experimental data with RSM function (Lenth, 2009) (Table 5.3). The validation of a model is usually evaluated through its coefficient of determination (R^2) and a model is considered as good fit once its R^2 value reaches at least 0.80 (Joglekar & May, 1987). High coefficient of determination ($R^2 = 0.981$ and adjusted $R^2 = 0.947$) of the obtained regression equation indicates that it was adequately representative for the real relationship between dependent and independent variables under experimental conditions.

Table 5.3 Regression results between the degree of demineralisation and variables

	Df	**Parameter estimate b_i, b_{ii}, or b_{ij}**	**Probability**
Intercept		23.0166667	0.1862523
X_1	1	9.1250000	0.0005272 ***
X_2	1	-0.4450000	0.1125126
X_3	1	0.9466667	0.1045051
X_1 X_2	1	0.0074000	0.2990045
X_1 X_3	1	-0.0035000	0.8351879
X_2 X_3	1	-0.0013000	0.7008325
X_1^2	1	-0.2701667	0.0004578 ***
X_2^2	1	0.0025533	0.1129051
X_3^2	1	-0.0175417	0.0885461

$R^2 = 0.981$ Adjusted $R^2 = 0.9469$ P-value = 0.0008814 *** $P < 0.001$

The regression equation reveals that the linear terms such as LA/S ratio (X_1) and microwave time (X_3) had positive influences on the demineralization degree (Y) while the temperature (X_2) showed negative effect. In contrast, two quadratic terms of LA/S ratio (X_1^2) and time (X_3^2) had negative influences whereas this of temperature (X_2^2) positively influence on the demineralisation degree. On the other hand, the interaction term of LA/S ratio-temperature (X_1X_2) was positive but those of LA/S ratio-time (X_1X_3) and temperature-time (X_2X_3) were negative. The highest value of the estimated regression coefficient for LA/S ratio compared with temperature and time indicates that this parameter was the most influential factor with a significant effect (*P = 0.0005*) on the process (Table 5.3). The significance of fit was also evaluated by carrying out analysis of variance (ANOVA) with results shown in Table 5.4. The lack of fit of the model compared with its pure error was insignificant *(P = 0.434)* revealing that the obtained model fitted to the experimental data. Since temperature was insignificant, this term and its interactions were deprived from the regression equation to obtain the refined model: $Y = 4.345192 + 9.773654X_1 + 0.840385X_3 – 0.275538X_1^2 – 0.018635X_3^2$ with $R^2 = 0.9580$ and adjusted $R^2 = 0.9412$. The refined model was used for calculating the predicted values as displayed in Table 5.2 and determining the optimum condition for the process by solving the refined model combined with analysing the response surface plots.

Table 5.4 Analysis of variance (ANOVA) for quadratic model of demineralisation degree

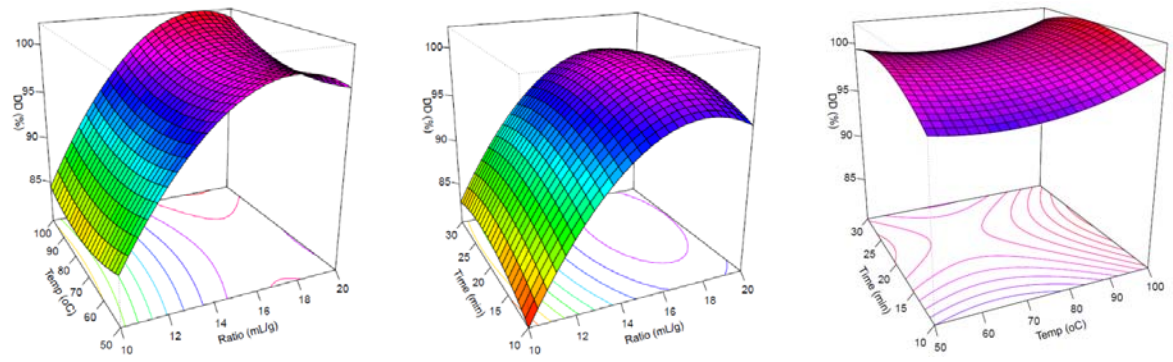
Source of variation	Degree of freedom (Df)	Sum of squares (SS)	Mean squares	F value	Significance Pr (>F)
FO (X_1,X_2,X)	3	462.87	154.290	60.4980	0.0002375
TWI (X_1, X_2, X_3)	3	3.97	1.323	0.5168	0.6876620
PQ (X_1, X_2, X_3)	3	192.23	64.076	25.1246	0.0019222
Residuals	5	12.75	2.550		
Lack of fit	3	8.72	2.908	1.4445	0.4340244
Pure error	2	4.03	2.013		

5.3.2 Analysis of response surface plots for the optimum conditions

The response surface plots were generated by keeping one variable constant at the central point while changing the other two variables within the experimental domain. The effects of X_1, X_2, and

X_3 on the response were evaluated by analysis of these plots. As shown in Figure 5.1a, the response was as a function of X_1 and X_2 but X_1 was far more influential than X_2. An increase in LA/S ratio could bring a considerable rise in the demineralisation degree while the effect of temperature was not clear at the low LA/S ratio. The estimated demineralisation degree was only around 85 % at 10-mL/g ratio even temperature rising to 100 ^oC. This could be because lactic acid at low ratio might not be sufficient to react with the large amount of mineral molecules in Australian rock lobster shells at 36 % reported by Nguyen et al. (2016). However, the effect of temperature became clearer at high LA/S ratio indicating that temperature might enhance the process once sufficient lactic acid was present as mentioned in the previous report of Synowiecki and Al-Khateeb (2003). Therefore, it explains for the observed trends that an increase in the LA/S ratio accompanied with a growth in temperature resulted in a considerable increase in the demineralisation degree. The highest demineralisation degree was found at temperature around 100 ^oC accompanied with the LA/S ratio around 18 mL/g.

In contrast to X_1, the effect of X_3 on the response was insignificant in the experimental range but the high value of X_1 combined with the appropriate value of X_3 could bring the response to the highest value (Figure 5.1b). The maximum demineralisation degree was found around the 18-mL/g LA/S ratio for the 23-minutes microwave. Since the effects of temperature and time were insignificant, their interaction effect on the response was not considerable within the experimental range. Although the demineralisation degree could not significantly improve with increases in time and temperature, the high ratio of LA/S in combination with appropriate temperature and time could bring the demineralisation degree to a higher value (Figures 1c). All analyses of the response surface plots revealed that the process obtained the optimised point at condition of 18 mL/g LA/S ratio, 100 ^oC, and 23 minutes. The accuracy of the model was further tested by conducting the microwave-intensified lactic acid demineralisation using the optimum conditions. Since demineralisation degree obtained from this replicated process was 99.2 % that is close to the predicted value (100 %), the obtained mathematical model was capable of predicting the studied response.



a) b) c)

Figure 5.1 Response surface plots showing the demineralization degree affected by LA/S ratio and time (a), LA/S ratio and temperature (b), time and temperature (c)

5.3.3 Demineralisation efficiency of the microwave-intensified process compared with the conventional method

The microwave-intensified process after optimisation was used for demineralisation of the deproteinised lobster shells at the optimum condition to generate chitin with mineral residue as shown in Table 5.5. Although the mild condition (LA/S ratio of 18 mL/g, 100 ^{o}C, and 23 minutes) was used for the process, mineral residue of the obtained chitin was only around 1 %. This mineral residue amount was significantly lower than that of 3.7 % in shrimp chitin demineralised by conventional heating at 50 mL/g of LA/S ratio, 100 ^{o}C, for 60 minutes reported by Mahmoud et al. (2007). Moreover, the result obtained in this study was comparable to that of 0.22 % in shrimp chitin demineralised by microwave at 200 mL/g of LA/S ratio, 121 ^{o}C, for 30 minutes as investigated by Valdez-Pena et al. (2010). As compared with the lobster chitin demineralised by conventional heating at LA/S ratio of 18 mL/g, 100 ^{o}C for 23 minutes as the control, mineral residue in chitin obtained from the microwave-intensified process was ten folds lower than that of chitin generated from the conventional process (0.99 vs 10 %). In addition, this process could also remove 55.1 % of residual protein in EDPS, which could serve as a step for further deproteinization to obtain purified chitin. The high demineralisation efficiency of the microwave process over the conventional process could be explained by a combination of its efficient thermal and non-thermal effects. As opposed to conventional heating methods relying on convection current and thermal conductivity which is slow and introduced from the surface, microwave heating occurs at a molecular level and on the whole material thus offers a much faster reaction rate (Collins & Leadbeater, 2007; De La Hoz, Diaz-Ortiz, & Moreno, 2005). Due to microwave energy efficiently interacting with the polar solvent as lactic acid, there would be effects on the mobility and diffusion

leading to effective contact for effective demineralisation. A part from its environmentally friendliness due to using organic acid and allowing to utilise demineralised solution for calcium recovery, high efficiency of the microwave process indicated by its significant reduction in chemical consumption would be another important factor which makes this process is more environmentally friendly.

Table 5.5 Chemical composition of the microwave-intensified-demineralisation chitin compared with the conventional-demineralisation chitin prepared at the optimal condition

Components	Chitin prepared by conventional demineralisation (%)	Chitin prepared by microwave demineralisation* (%)
Moisture	6.7 ± 0.5	6.2 ± 0.1
Protein	2.2 ± 1.2	1.6 ± 0.3
Mineral	10 ± 0.5	0.99 ± 0.05

5.3.4 FTIR spectroscopy of chitin

FTIR is one of the most important and widely used techniques for structural analysis of chitin and chitin derivatives. The FTIR spectrum was obtained by passing infrared electromagnetic radiation through chitin prepared at optimum conditions. As shown in Figure 5.2, the FTIR spectra of chitin display a series of narrow absorption bands, which correspond to the frequency of the vibration of a molecule part, and thus it allows qualitative identification of certain bond types in the sample for physicochemical characterisation of chitin and chitin derivatives. The FTIR spectrum of lobster chitin has several major peaks that are very similar to the spectrum of commercial chitin produced from crab shells. This indicates that the physicochemical property of the extracted chitin could be close to that of commercial chitin (Kumirska et al., 2010) although further work would be required to demonstrate its effectiveness in practical applications. Purity of the lobster chitin was also determined by analysis of its chemical composition. As shown in Table 5.5, the lobster chitin prepared at optimal condition contains very low residual minerals (0.99 %) and residual protein (1.6 %). With this high purity, the lobster shell chitin could be used for advanced applications.

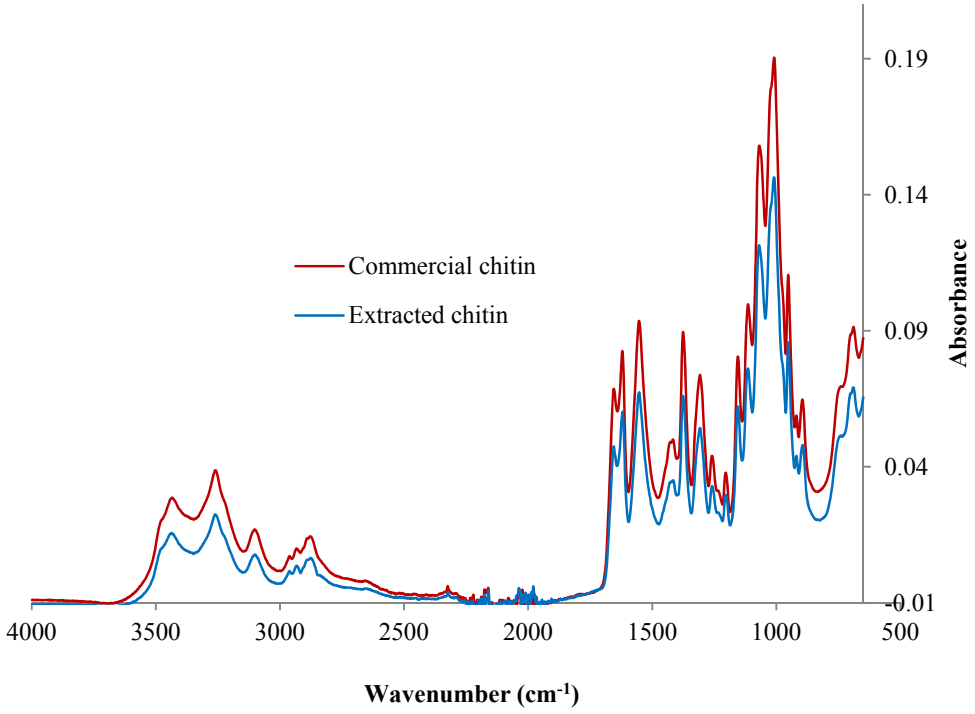


Figure 5.2 FTIR spectrum of the lobster shell chitin obtained by the microwave-intensified demineralization compared with commercial chitin produced from crab shells

5.3.5 Lobster mineral profile

Apart from generating the high quality chitin, the microwave-intensified lactic acid demineralisation of lobster shells also allows to utilise the demineralised solution (pH 3.76) for recovery of lobster minerals (lactate salts) with a good profile (Figure 5.3). Since the lactate salts obtained from this process had a calcium-rich profile (up to 87 % calcium in its mineral composition), they could be considered as another potential value-added product. This is indicated by the fact that calcium-rich organic salts could be used for calcium deficiency treatment (Sunyecz, 2008) or used as mineral supplements (Edwards, Dixon, Friel, & Hall, 2010). Moreover, since alkaline salts of carboxylic acids such as lactate calcium has antimicrobial activity and de-icing ability, they can be used as food preservatives (Pattison & Von Holy, 2001; Shelef, 1994) and bake powder in bakery industry (Darby et al., 1956) or as anti-icing agent (Sapienza, Johnson, & Ricks, 2005).

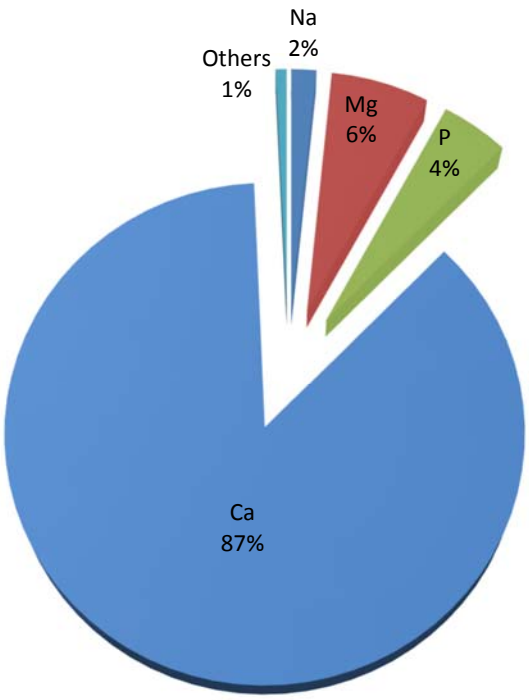


Figure 5.3 Profile of minerals recovered from the microwave-intensified lactic acid demineralisation of Australian rock lobster shells (results are mean of triplicates)

5.4 Conclusion

Demineralisation of EDPS using the microwave-intensified lactic acid achieved nearly complete demineralisation (99.2 %) at mild condition (LA/S ratio of 18 mL/g, 100 ^{o}C, and 23 minutes). The LA/S ratio is the dominant factor significantly affecting the demineralisation efficiency of the process. Chitin obtained from the optimised process had low residues of minerals (0.99 %) and protein (1.6 %) while its chemical structure was similar with that of commercial chitin. Moreover, minerals recovered from the microwave process could be used for another value-added product due to its calcium-rich profile calcium (87 % calcium). The significant results obtained from the optimised process indicates that high quality chitin could be produced in a more environmentally-friendly and sustainable process.

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CHAPTER 6 APPLICATIONS OF FUNCTIONAL AND NUTRACEUTICAL EXTRACTS RECOVERED FROM LOBSTER PROCESSING BY-PRODUCTS

6.1 Introduction

Several functional and nutraceutical ingredients of Australian LPBs have been efficiently recovered by the industrially applicable technologies developed in this study (chapters 2-5), which demonstrated good functionalities and bioactivities, high nutritional quality, and food safety. In this chapter, the applications of these lobster extracts will be explored as food functional ingredients or bioactive nutraceuticals for several examples of value-added products.

6.1.1 Lobster chitin

Chitin is a natural biopolymer possessing biological properties with known health benefits. Apart from naturally occurring in crustacean shells, insects and fungi, this biopolymer also exists in grain, yeast, bananas, and mushrooms consumed daily as stable foods for a normal life-long human diet (Dare, 1998). Chitin provides nutritional fibre functioning as one of the keys to a healthy and cancer free offered by several biological activities of natural biopolymers (Dare, 1998; Reddy & Yang, 2015). Chitin and its derivative, chitosan, provide potential capacity for "fat fighting". The ingestion of chitin and chitosan could prevent the absorption of fat, cholesterol, and lipids into the body by binding with these lipids in the stomach and small intestine for ultimate elimination of these materials (Prajapati, 2009). Chitin can even bind to cholesterol that is produced by the liver (Carolyn, 1999). Chitin binds fat with fivefold its weight (Gades & Stern, 2002; Venugopal, 2016) and more effectively than any other fibres (Darrell, 2008). Consumption of chitin and chitin derivatives as dietary fibres have been demonstrated with several health benefits such as weight loss (Darrell, 2008; Essays, 2013; Mhurchu et al., 2004), cholesterol management and/or lowering blood pressure (Khoushab & Yamabhai, 2010; Maezaki et al., 1993). Chitin and chitosan help in the treatment of irritable bowel syndrome (IBS), constipation and other digestive disorders, inhibition of tumours and dental plaque (Rudloe & Rudloe, 2009). Chitin could be used as nutritional oral composition since consumption of chitin micro-particles obtained by micro-fluidisation with an average diameter of between 1 and 100 μm could improve regulatory immune response (Mackenzie, 2011). Due to their significant potential health benefits, chitin and its derivatives have been suggested for use as nutritional fibre for aged people (Kerch, 2015). In this study, chitin

obtained from lobster shell by the microwave-intensified process demonstrated with high purity and good physicochemical properties, which has a potential use as a nutraceutical ingredient for fat and cholesterol binding.

6.1.2 Lobster minerals

Calcium is an essential macro-mineral for structural integrity of teeth, the strength of bones in addition to playing a vital role in regulating critical functions of nerve impulses, muscle contractions and the activities of enzymes (Etcheverry, Grusak, & Fleige, 2012; Tremblay & Gilbert, 2011). Calcium deficiency has been linked to some chronic diseases such as osteoporosis, fluorosis, hypertension, and reduced ability of blood clotting and cancer (Amalraj & Pius, 2014). The average intake of calcium required is about 900 mg/day for adults and 1200 mg/day for both adolescents and the elderly suggested by FAO/WHO 2001. Intake of calcium via diet is important for human health in general, particularly for those people who are in the periods of maximum growth, such as in childhood, adolescence or during lactation (Bosscher, Dyck, Robberecht, Caillie-Bertrand, & Deelstra, 1998; Ünal, El, & Kiliç, 2005). Although milk and dairy products are known as the most common and trusted source for calcium intake, many Asian people do not take milk due to lactose indigestion and intolerance, which make them intolerant to milk and its products (Jung, Shahidi, & Kim, 2009).

Seafood and its products serve as another alternative for dietary calcium supplement. Regular intake of seafood over 250 g per week has been found to associate with greater bone mineral density (Zalloua et al., 2007). The intake of small fish with bones has increased calcium bioavailability in rats; small fish have been considered as an important source of dietary calcium, especially for those people with low intakes of milk and dairy products (Larsen, Thilsted, Kongsbak, & Hansen, 2000). Therefore, seafood processing by-products such as fish bone and crustacean shells could be excellent sources for recovery of dietary calcium, which could be recovered as a food ingredient (Jung et al., 2009; S. K. Kim & Mendis, 2006). Inclusion of fish bone and crab processing by-products in feed as calcium supplement for Atlantic cod was found to have positive effects on weight gaining (Toppe, Aksnes, Hope, & Albrektsen, 2006). Recently, fish bones have been utilised as a highly bioavailable calcium source for growing pigs, in which piglets fed with the diets formulated with fish bone had significantly higher calcium absorption than that of the control (Malde, Graff, et al., 2010). With such success, calcium generated from salmon and cod bone was tested for its bio-absorption in young healthy men showed positive results (Malde, Bügel, et al.,

2010). Therefore, apart from demonstrating calcium derived from bone meal and oyster shells for use in several food processing processes (Jung et al., 2009), minerals generated from seafood processing by-products have been suggested for using as a source of nutraceutical ingredients (Menon & Lele, 2015). In this study, minerals obtained from lobster shell during the chitin production have a calcium-rich mineral profile that could be further invested as a source of dietary calcium for food applications or supplement.

6.1.3 Lobster lipids

Fish oil is a virtually unique source of natural long-chain (LC) omega-3 fatty acids, comprising eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which delivers higher level and more balanced proportion of omega-3 fatty acids compared to other sources such as flax and algal oil (Pike & Jackson, 2010; Rizliya & Mendis, 2014). Fish oil has been used for enriching omega-3 in several food products such as powder milk formulate, salad oil, fruit beverage, vegetable juice (Kolanowski & Berger, 1999), dairy products (Kolanowski & Weißbrodt, 2007), soft goat cheese (Hughes, Brian Perkins, Calder, & Skonberg, 2012) or flavour enhancer ingredients for flavour formulation (Peinado, Koutsidis, & Ames, 2016). More importantly, fish oil produced from fish liver is an important source of vitamins A and D with several therapeutic properties, thus fish liver oil has been used as a dietary supplement (Gunstone, 2006; Rizliya & Mendis, 2014). In this study, lobster lipids have been produced from lobster livers by SC-CO_2 extraction with high level of PUFAs and omega-3 (DHA and EPA) (Nguyen, Zhang, Barber, Su, & He, 2015). Moreover, the extracted lipids had very strong lobster flavour that could be used as lobster flavouring agents for production of infused lobster oil, salt plated with lobster flavours or incorporation with lobster protein and other ingredient to produce lobster seasoning.

6.1.4 Lobster proteins and protein hydrolysate

Concentrated proteins and hydrolysates are two common products recovered from seafood processing by-products with potential applications in many value-added food products. Since these products usually contain over 65 % protein, they have been used in the food industry for developing reconstructed and ready-to-eat products (Shaviklo, 2015). Due to their high nutritional quality indicated by high delicacy, palatability, and nourishing characteristics (Venugopal, 2009), these fish protein products have been used as ingredients for nutrients supplementation (Nobile et al., 2016; Vikøren, Nygård, Lied, Rostrup, & Gudbrandsen, 2013) or malnourished treatment (Nesse, Nagalakshmi, Marimuthu, & Singh, 2011). Fish protein has also been used in several food products

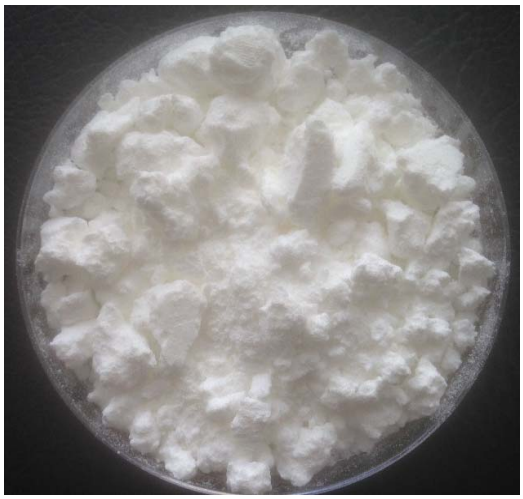
as a functional ingredient for enhancing water retention (Ibarra et al., 2013), functioning as cryo-protectives (Cheung, Liceaga, & Li-Chan, 2009; Dey, Dora, Raychaudhuri, & Ganguly, 2013; Ruttanapornvareesakul et al., 2005), lowering fat binding capacity of food products (He, Franco, & Zhang, 2015), even as a constituted ingredient of functional food (Tahergorabi, Matak, & Jaczynski, 2015). Moreover, since fish protein hydrolysates (FPHs) contain several bioactive ingredients, the use of these FPHs for nutraceuticals and pharmaceuticals products has been demonstrated (Anil, Athapol, & Punchira, 2013; Guérard et al., 2010; Khora, 2013; Rasika, 2013). In particular, some fish proteins possess a strong flavour, an attractive attribute in the food industry, thus various seafood flavour formations have been produced from fish protein such as shrimp (Teerasuntonwat & Raksakulthai, 1995), fish (Imm & Lee, 1999; Peinado et al., 2016), lobster (Lee, 2007). Most recently, apart from generating a pleasant flavour, lobster protein has demonstrated with high nutritional quality (Nguyen, Zhang, Barber, Su, & He, 2016) and excellent functionality (He, Nguyen, Zhang, & Peng, 2016). Thus, it is a great potential for investigation of lobster protein as a functional, nutritional, and flavourful ingredient in formulated food products.

6.2 Materials and methods

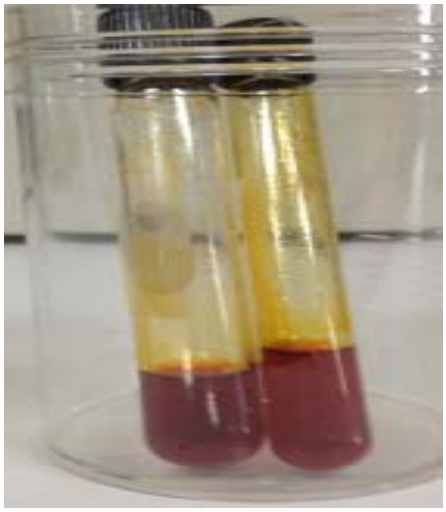
6.2.1 Materials

All lobster products (Figure 6.1) used in this section were recovered from Australian LPBs using the processes developed in this study including lipids (extracted by the developed in chapter 2), proteins (head and shell proteins extracted by the developed in chapters 3 - 4), and chitin and minerals (extracted by the developed in chapter 5). High quality chitin (crab shell) and chitosan (from crab shell, minimum 85 % deacetylation) were purchased from Australian Sigma Aldrich. Calcium complex supplement (calcium carbonate, Healthy care) and Fat Blocker (chitosan formulated with 9 % Ascorbic acid, Thompson's) were bought from Chemist Warehouse (Adelaide, Australia). Salmon and lobster spread, butter, oils (canola, olive, peanut, sunflower, and vegetable) were purchased from Coles Supermarkets Australia Pty Ltd (Adelaide, Australia). Infused lobster oil was supplied by Ferguson Australia Pty Ltd.

a)



b)



c)

d)

Figure 6.1 Products recovered from Australian LPBs: a) lobster chitin, b) lobster minerals, c) lobster lipids, and d) lobster protein

Other materials used for comparison are commercial products, which are readily available in the domestic market. High quality chitin and chitosan were purchased from Sigma Aldrich. High quality calcium supplement and fat blocker products were bought from Chemist Warehouse. Salmon and lobster spread, butter, oils (canola, olive, peanut, sunflower, and vegetable) were bought from Coles. Infused lobster oil was supplied by Ferguson Australia Pty Ltd.

6.2.2 Characterisation and functional tests for lobster extracts

6.2.2.1 Chitin

Degree of acetylation

The degree of acetylation (DA) of lobster chitin was determined using solid-state ^{13}C nuclear magnetic resonance (^{13}C NMR) (Duarte, Ferreira, Marvao, & Rocha, 2001). ^{13}CCP-MAS NMR spectra were obtained by recording at 100.62 MHz on a Bruker 400 MHz spectrometer with a spinning rate of 5 kHz for solid samples. DA of lobster chitin was calculated using the relative resonance intensities of ring carbon and methyl carbon as follows:

$$DA = \frac{I_{CH3}}{\frac{I_{C1} + I_{C2} + I_{C3} + I_{C4} + I_{C5} + I_{C6}}{6}}$$

Fat binding

The fat binding capacity of lobster chitin were determined following the method of Cho, No, and Meyers (1998) with some slight modifications. Briefly, samples (50 mg) were dispersed with 1 mL oil in 2-mL centrifuge tubes by vortexing for 60 seconds. The tubes incubated at ambient temperature for 30 minutes with intermittent shaking for 5 seconds every 10 minutes before centrifugation at 3,500 rpm for 25 minutes. The supernatant and bound fat were determined for calculation of fat binding capacity (FBC) as follows:

$$FBC\ (g/g) = \frac{\text{Fat bound (g)}}{\text{Sample weight (g)}}$$

Cholesterol binding

Cholesterol binding of lobster chitin was determined using the method of Tong, Guan, Wang, Xu, and He (2011) with some slight modifications. Cholesterol was solubilised in hexane with known concentration and then added to chitin samples (50 mg) in the pre-weighed Eppendorf tubes. The tubes were sealed and shaken at room temperature for 8 hours to allow the establishment of the equilibrium binding. The suspensions were then centrifuged (14,000 rpm, 10 minutes) and the supernatant collected. The uptake of cholesterol was determined by measuring the concentration of cholesterol present in the supernatant using the spectrophotometric method described by Y. Park (1999).

6.2.2.2 Lobster lactate salts

Chemical composition

The moisture content was determined by oven drying samples at 105 ^{o}C until a constant weight was achieved. The total mineral content was quantified by incinerating 500 mg of sample in a muffle furnace 600 ^{o}C for 12 hours. The lipid content was determined using Soxhlet extraction and protein was quantified using the method of Micro Lowry.

Mineral profile

The mineral profile was determined using inductively coupled plasma mass spectrometry **(**ICP-MS) as described by Shi, Francis, Machado, and Wu (1995). Briefly, samples (50 mg) were mixed with 3 mL of Nitric acid, incubated overnight, and then heated for 1 hour (100 °C). After cooling, hydrochloric acid (2 mL) was added and heated for 1 hour (100 °C). Peroxide (1 mL) was added to cool samples before heating at 100 °C for a further hour and then made up to 50 mL with MilliQ water for analysis by ICP-MS.

Solubility

The solubility of lobster minerals was determined following the method described by Chaiwanon, Puwastien, Nitithamyong, and Sirichakwal (2000) with slight modifications. Samples (W_S) were added to MilliQ water (4 mL per gram) and then vortexed for 60 seconds. The solutions was centrifuged at 14,000 rpm for 10 minutes to separate the soluble and insoluble fractions. The insoluble residues (R_I) were collected and dried at 105 ^{o}C until a constant weight was achieved. The solubility of the lobster minerals (SLM, %) was calculated as follows:

$$\text{SLM (\%)} = \frac{W_S - R_I}{W_S} \times 100$$

Bioavailability

Bioavailability of lobster calcium was determined by the method of Shen, Luten, Robberecht, Bindels, and Deelstra (1994). This is a two-part method which includes a gastric stage and an intestinal stage as described below.

- Gastric stage: Samples (2.5 g) containing known amount of calcium (Ca_T) were homogenised with 22.5 mL of MilliQ water. The homogenate was adjusted to pH 2.0 with HCl (6 N) before adding

pepsin for pepsin-HCl digestion (0.5 g pepsin per 100 g samples). The mixture was incubated at 37 ^{o}C for 2 hours in an orbital shaking incubator.

- Intestinal stage: Segments of dialysis tube (molecular mass cut-off value 12000 – 14000 Da) containing 25 mL of MilliQ water and an amount of $NaHCO_3$ equivalent to the titratable acidity of the combined pepsin digest pancreatin–bile salts mixture to pH 7.5 was placed in a 100-mL beaker filled with 20 g of the pepsin digest. The beaker was incubated in waterbath (37 ^{o}C) for 30 minutes. The pancreatic-bile salt mixture prepared by dissolving pancreatin (3g) and bile salt (7g) in 1 L $NaHCO_3$ 0.1M (5 mL) was added incubated for 2 hours. After incubation, the dialysis tubes were removed and rinsed with MilliQ water. The calcium concentration in the dialysates (Ca_B) and in the soluble fraction (Ca_{SF}) were determined using the ICP-MS method. The bioavailability and solubility of calcium in samples was calculated as follows:

$$\text{Bioavailability (\%)} = \frac{Ca_B}{Ca_T} \times 100 \qquad \text{Calcium solubility (\%)} = \frac{(Ca_B + Ca_{SF})}{Ca_T} \times 100$$

6.2.2.3 Total carotenoids and astaxanthin

The astaxanthin content in lobster lipid extracts were determined using the method described by Arar (1997) with some modifications. Samples and standards were diluted in acetone (90 % v/v) prior analysis. A Shimadzu HPLC system equipped with UV-Vis detector and Luna C18 reversed phase column (250mm x 4.6mm, 5um pore size) was used. The mobile phase was a mixture of 60 % solvent A (80 % methanol, 20 % 0.5 M ammonium acetate), 30 % of solvent B (acetonitrile 90 %), and 10 % ethyl acetate (HPLC grade). The flow rate was set at 1mL/min. Commercial astaxanthin and beta-carotene was used as standards.

6.2.2.4 Protein quality evaluation

Lobster protein quality was evaluated based on amino acids composition (Consultation, 2011), EAA index (Oser, 1959), chemical scores (Henderickx, 1963), and digestibility (Akeson & Stahmann, 1964). Protein efficiency ratio (PER) was estimated using to the following regression equation proposed by Alsmeyer, Cunningham, and Happich (1974):

$$PER = -0.468 + 0.454\ (\text{Leucine}) - 0.105\ (\text{Tyrosine}).$$

6.2.3 Commercial assessment of lobster proteins and lipids

The ease of use, product bulking, and sensory properties of lobster protein and lipids were assessed to demonstrate end-use applications by a panel (six panellists) including food experts from The Centre of Marine Bioproducts Development (Flinders University, South Australia), South Australian Research and Development Institute (SARDI), and Ferguson Australia Pty Ltd (lobster company). Three lobster-formulated products including infused lobster oils, salt plated with lobster lipids, and lobster flavour seasoning prepared at SARD facility were used for the evaluation that was carried out at Ferguson Australia Pty Ltd.. Sensory evaluation of lobster oil was directly determined based on its flavour and colour while salt plated with lobster lipids and lobster seasoning were evaluated by mixing lobster products (6.5 g) with noodles (74 g) in boiling water (300 mL). The flavour and taste of lobster flavoured noodle soup was assessed by each member in the panel.

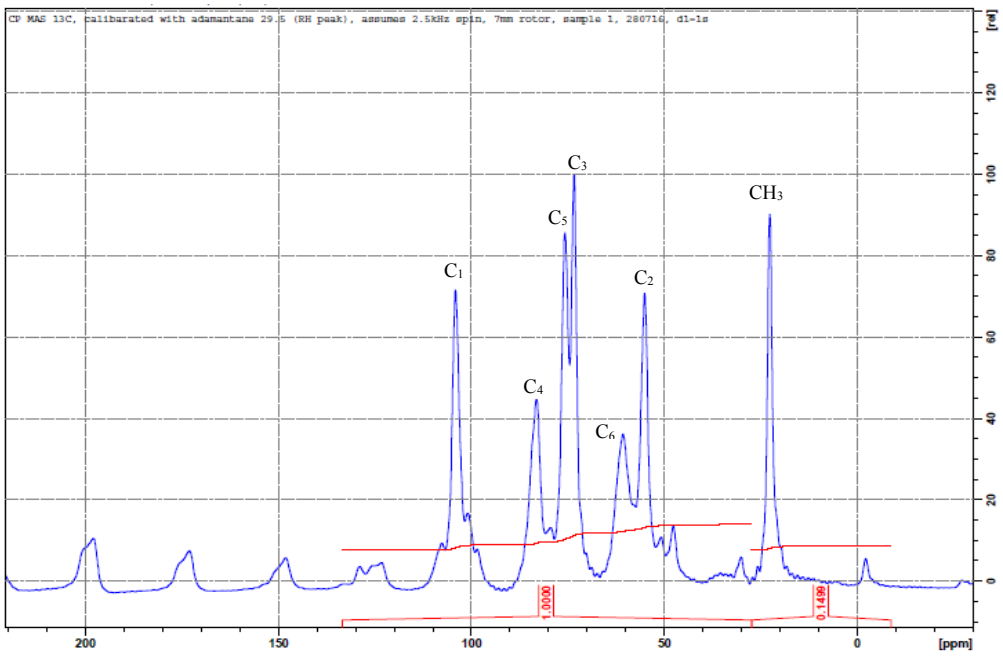
6.3 Results and discussion

6.3.1 Lobster chitin produced by the microwave-intensified processes

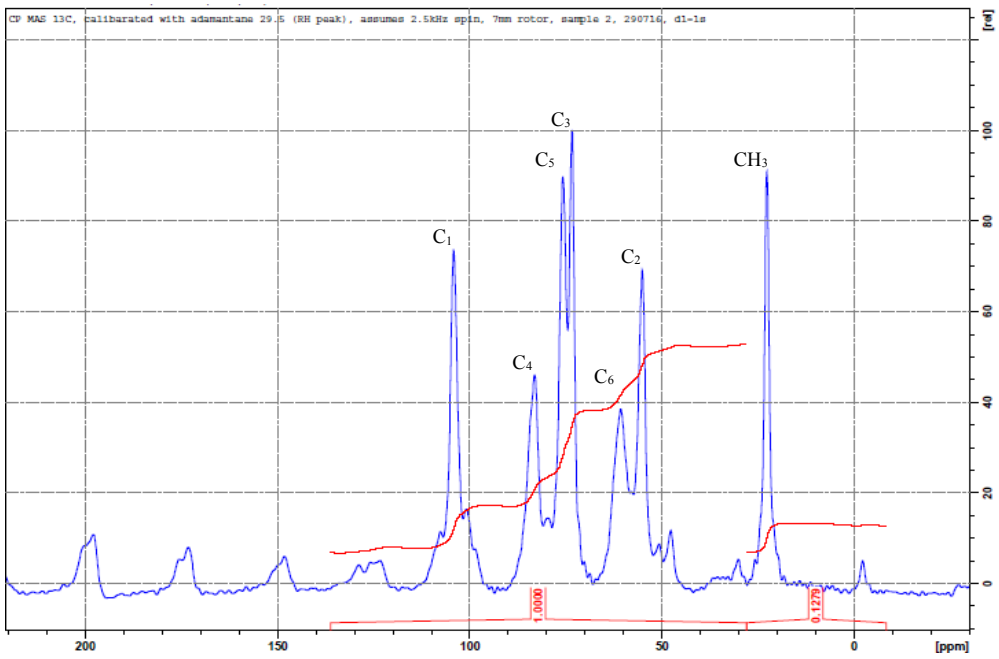
6.3.1.1 High acetylation degree of lobster chitin indicating its good physicochemical properties

Apart from characterisation in composition and chemical structure for evaluation its quality, lobster chitin was also analysed testing in the physicochemical properties useful for demonstrating its potential applications by determination of its DA based on NMR technology. As shown in Figure 6.2, the NMR-spectrum of lobster chitin (Figure 6.2a) recovered using microwave extraction was found to be similar to that of commercial chitin (Figure 6.2b). For example, in both spectra have several major peaks representing chemical bonds of different carbon groups existing in both chitins but the relative resonance intensities between ring carbon and methyl carbon of lobster chitin was higher than that of high quality chitin. As defined, this ratio is the DA of chitin that was determined to be higher in the lobster chitin (89.9 %) compared to commercially available chitin (76.8 %). The higher DA of lobster chitin over the commercial one tested could be a result of mild conditions used for production (Younes & Rinaudo, 2015). DA is one of the most important chemical characteristics of chitin and chitin derivatives, which could influence on their performance in many applications (Kumirska et al., 2011; Sorlier, Denuzière, Viton, & Domard, 2001) and there is a positive relationship between DA and physicochemical properties. DA of chitin and chitosan has significant effects on several properties such as solubility, conformation and dimensions (Schatz, Viton, Delair, Pichot, & Domard, 2003), biodegradability (Kurita, Kaji, Mori, & Nishiyama, 2000), and

biocompatibility (Schipper, Olsson, Hoogstraate, Vårum, & Artursson, 1997) of the polymer. Changes in chitin DA also affect the activity and therefore the application of the polysaccharide. Applications in which chitin with modified DA may be used include muco-adhesion (Mao et al., 2004), drug delivery (Kofuji et al., 2005), gene delivery (Lavertu, Methot, Tran-Khanh, & Buschmann, 2006), tissue engineering (Tığlı, Karakeçili, & Gümüşderelioğlu, 2007), wound healing (Ueno, Mori, & Fujinaga, 2001), antimicrobial activity (Andres, Giraud, Gerente, & Le Cloirec, 2007; Hongpattarakere & Riyaphan, 2008). High DA indicates physicochemical properties of lobster chitin were well preserved during production, which could offer some specific functional and biological properties for being exploited in nutraceutical applications.



a)



b)

Figure 6.2 ^{13}C-NMR spectra of lobster chitin recovered by the microwave-intensified process (a) compared with a commercially available chitin standard (b)

6.3.1.2 Lobster chitin has a high affinity for fat and cholesterol binding

The lobster chitin produced by microwave intensification was determined to have high quality (low protein and mineral residues) and good physicochemical properties (high DA). Based on these results, potential nutraceutical applications of the chitin were investigated, such as its use as an active ingredient for fat and cholesterol binding. Results from this study showed that lobster chitin has high fat binding properties; however, its binding capacity varied significantly depending on the type of oil-based product used. The binding capacity was highest for butter (16.1 g/g), relatively high for vegetable oil (13.1 g/g), and lower for other oils such as canola, olive, peanut, and sunflower (around 8 g/g) (Figure 6.3), which is conclusive with a previously published study (Hélène, 2015). The variation in the binding of different oils could be due to differences in saturation patterns. For example, butter has significantly higher levels of saturated fatty acids (SFAs) (61 %) compared with other oils (1.5 - 2.9 %) while chitin and its derivatives was also found to bind to lauric, myristic, and palmitic fatty acids better than other fatty acids (Santas, Espadaler, Mancebo, & Rafecas, 2012).

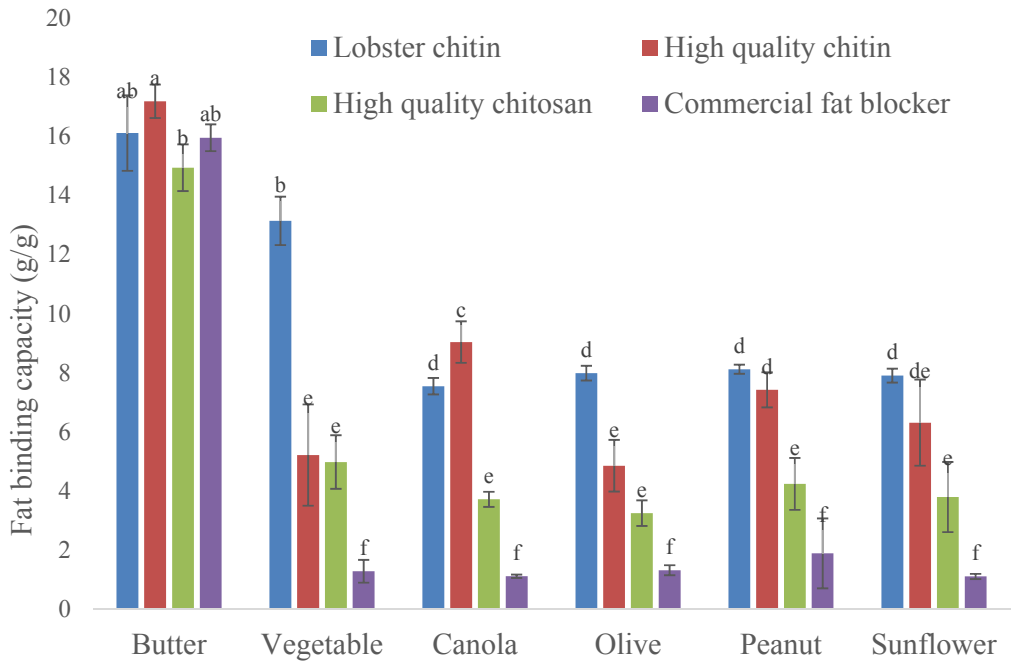


Figure 6.3 The fat binding capacity (g/g) of lobster chitin to six different types of oil-based products was compared to other commercial products. The results shown are the average of three replicates. Plots with different letters are statistically significant, p< 0.05. The error bars are the standard deviations.

Furthermore, compared to other excellent fat binding ingredients such as high quality chitin, chitosan and Fat Blocker the fat binding capacity of lobster chitin was higher (vegetable, olive, peanut, and sunflower). Excluding butter, binding of chitin group to other oils was significantly higher than that of chitosan group. High fat binding of chitin samples over chitosan samples could be explained by high DA of chitin compared with chitosan (Zhang et al., 2010). Particularly, lobster chitin bound to oils 1.1 - 2.5 fold better than commercial chitin with an exception for canola oil while this significance was 4.2 – 10 fold for fat blocker and 2 - 2.6 for commercial chitosan. High fat binding of lobster chitin over commercial chitin, chitosan, and fat blocker indicates its potential applications for weight loss products.

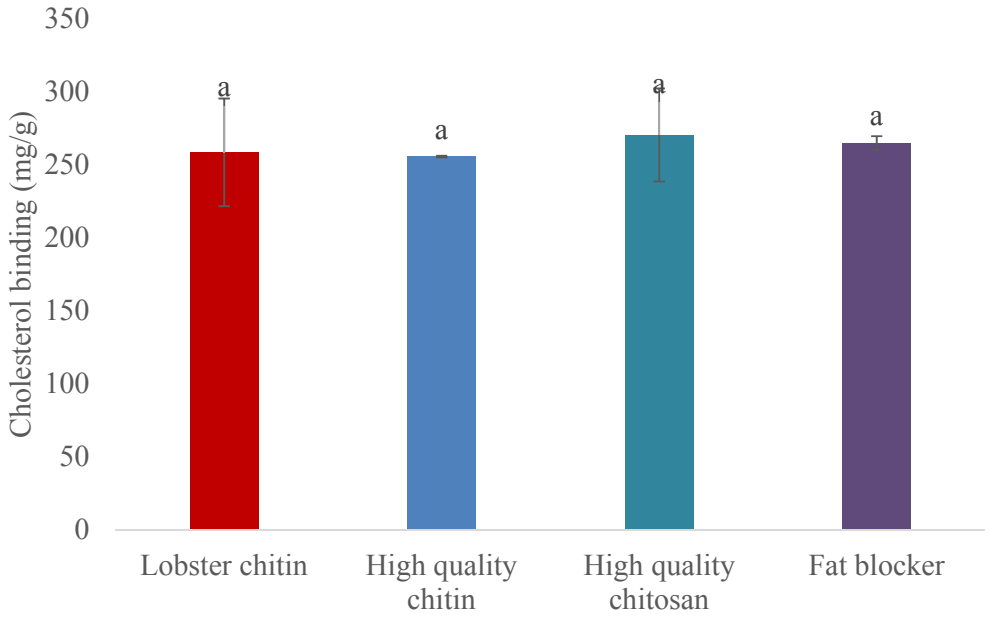


Figure 6.4 The level of cholesterol binding (mg/g) of lobster chitin compared with other commercial products. The results shown are the average of three replicates. Plots with the same letters are statistically insignificant, p< 0.05. The error bars are the standard deviations.

Chitin and chitin derivatives have been shown to reduce cholesterol extensively highlighted in the literature with several promising results (Azuma, Ifuku, Osaki, Okamoto, & Minami, 2014; Azuma et al., 2015). As shown in Figure 6.4, lobster chitin bound well to cholesterol (258.7 mg/g), which is similar to previously published work finding that chitin could reduce cholesterol between 6 and 42 % (Ylitalo, Lehtinen, Wuolijoki, Ylitalo, & Lehtimäki, 2002). Lobster chitin bound to cholesterol as well as other commercial ingredients (high quality chitin, chitosan, and fat blocker) known as excellent cholesterol-binding ingredients. This significant result in combination with high fat-binding capacity over commercial products demonstrates potency of the lobster chitin for weight loss and cholesterol management products.

6.3.2 Lobster lactate salts recovered from lobster shells during demineralisation by the microwave-intensified process

6.3.2.1 Lobster lactate salts have a calcium-rich profile demonstrating its potential application as lactate calcium

The demineralised solution generated from the microwave intensified lactic acid demineralisation (MILAD) was spray-dried for recovery of lobster lactate salts. This organic salt contains very low levels of protein and lipids (1.4 and 0.4 %, respectively) but are rich in minerals accounting for 72.4

% (Table 6.1). Therefore, this product is referred to as lobster minerals with a potential use as a source of dietary minerals since several studies on minerals obtained from fish and crustacean processing by-products have demonstrated their effects on weight gaining (Toppe et al., 2006) and growing (Malde, Graff, et al., 2010). Especially, minerals obtained from crustacean shells have been demonstrated as an excellent source of calcium for various applications including bioactive calcium substances, dietary calcium supplement, and calcium fortifier (Jung et al., 2009; S. K. Kim & Mendis, 2006; Malde, Bügel, et al., 2010; Malde, Graff, et al., 2010).

Table 6.1 Chemical composition of lobster lactate salts recovered from lobster shells by the MILAD process

Components	**Content*** **(%)**
Moisture	17.7 ± 0.23
Minerals	72.4 ± 0.3
Protein	1.3 ± 0.04
Lipids	0.4 ± 0.06
Others	9.9 ± 0.3

* The results shown are the average of three replicates with standard deviations.

Further characterisation in mineral profile shows that calcium is the major element of lobster minerals (Figure 6.5). Calcium accounts for 87 % of the total minerals with other elements present in lower abundance: Mg (6 %), P (4 %), and Na (2 %). Calcium has been extensive demonstrations for using as an essential macronutrient for daily intake up to 1200 mg (Jung et al., 2009). With its high mineral content and richness in calcium, lobster minerals could be marketable as calcium lactate, which has many applications in the food industry (Irshad et al., 2016; Y. Kim et al., 2009; Luna-Guzmán & Barrett, 2000; Martin-Diana et al., 2004; Udomkun, Mahayothee, Nagle, & Müller, 2014). Moreover, calcium extracted from fish minerals have been demonstrated to have high bioavailability (Malde, Graff, et al., 2010) and good bio-absorption (Malde, Bügel, et al., 2010), which may be potentially useful as a source for a dietary calcium supplement.

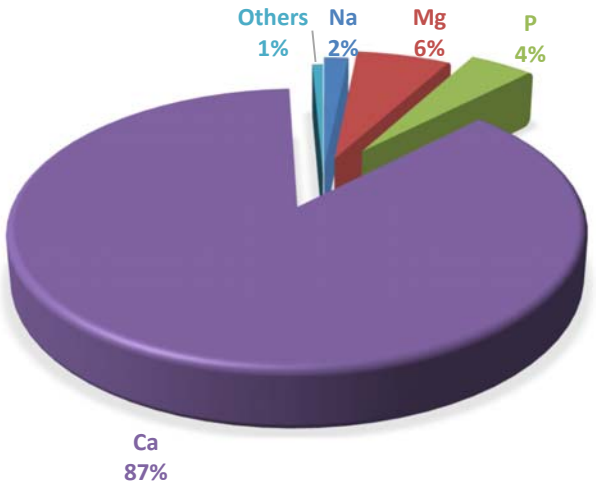


Figure 6.5 Mineral profile of lobster lactate salts recovered from lobster shells by the MILAD was analysed using ICP-MS

6.3.2.2 Calcium of lobster lactate salts are highly soluble and bioavailable

One of the most important parameters in which the quality of mineral extracts is scored is their solubility. As shown in Figure 6.6, lobster lactate salts are highly soluble (98.4 %). Compared with a commercial calcium complex supplement, lobster lactate salts are fivefold more soluble (98.4 vs 18.6 %). Moreover, the solubility of lobster calcium was over sevenfold higher than that of calcium of commercial supplement complex (60.0 vs 8.3 %). As there is a positive relationship between calcium solubility and its bioavailability (Etcheverry et al., 2012), lobster minerals may be a good source of calcium for dietary supplements

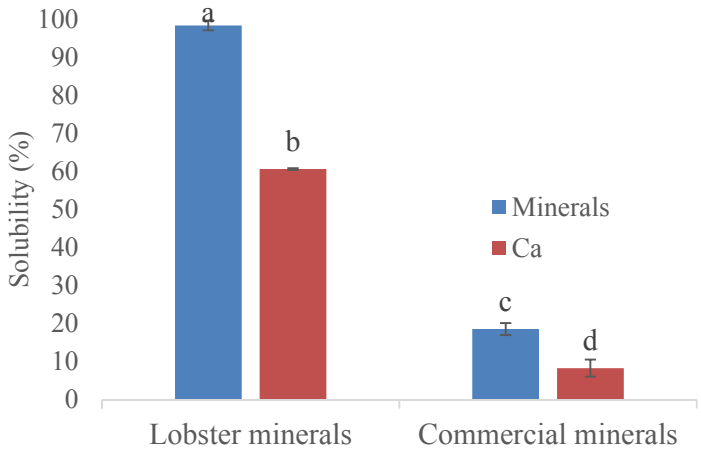


Figure 6.6 Solubility of lobster lactate salts with its calcium compared with a commercial calcium complex supplement. The results are shown as mean of tree replicates with standard deviation. Plots with the same letters are not statistically significant with confident 0.05)

For further investigation of its potential application as dietary calcium supplement, the bioavailability of lobster calcium was also investigated. As defined, bioavailability is the degree to which the amount of an ingested nutrient is absorbed and available to the body. Apart from having good solubility (60.6 %), lobster calcium had relatively high bioavailability compared with that of dietary calcium complex supplement (Figure 6.7). The bioavailability of lobster calcium was 5.9 fold higher than that of commercial calcium complex supplement (11.3 vs 1.9 %). This result is consistent with previous studies comparing adsorption of fish bone calcium with calcium derived from other sources (calcium carbonate, calcium citrate) using either growing pigs or dialysis carried out by Malde, Graff, et al. (2010) and Luu and Nguyen (2009) with conclusion that calcium obtained from fish bone was highly bioavailable or even higher than calcium from other sources. Calcium derived from fish demonstrated with high efficiency on bone mineralisation without adverse effects, thus fish calcium has been suggested for human consumption as dietary supplement (Flammini et al., 2016). Therefore, a number of commercial products containing fish calcium has been marketed as dietary calcium supplements such as Kalsio (TechNyFlex, 2010) and calcium fish bone powder (NutriZing, 2016). Being rich in calcium with relatively high solubility and bioavailability, lobster minerals could be marketable as a calcium supplement.

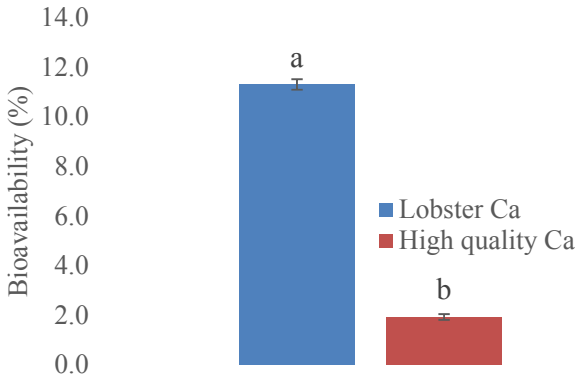


Figure 6.7 Bioavailability of lobster calcium compared with a dietary calcium of a commercial supplement

6.3.3 Lobster lipids and proteins with high nutritional quality possibly marketable as food nutrients or dietary supplement

6.3.3.1 Enriching in PUFAs, omega-3, astaxanthin, and carotenoids, lobster lipids possibly marketed as dietary supplement

Addition to characterisation of fatty acids for nutritional evaluation, total carotenoids and astaxanthin of lobster lipids were also identified and quantified with its chromatogram against beta-

carotene and astaxanthin standards displayed in Figures 7.3. There were several peaks generated in the lobster lipid chromatogram (Figure 7.3 a) indicating that lobster lipids contained several types of carotenoids with various concentration. Based on retention time of beta-carotene standard in the chromatogram (Figure 7.3 b), lobster lipids contained a considerable amount of β-carotene with the concentration of 70.4 (µg/mL). Particularly, over 59 % of carotenoids are astaxanthin (41.6 µg/mL) with its retention time as shown in the Figure 7.3 c. Astaxanthin is a powerful antioxidant (J. S. Park, Chyun, Kim, Line, & Chew, 2010; Sila et al., 2013) and has several benefits on human health (Higuera-Ciapara, Felix-Valenzuela, & Goycoolea, 2006; Hussein et al., 2005), containing astaxanthin with high concentration in its composition could make lobster lipids become much more valuable (Kajita, Tsukahara, & Kato, 2009; Yamashita, 2013). Moreover, enriching in astaxanthin and carotenoids could help lobster lipids stabilise for a prolonged self-life because of their super antioxidative activity (Rao, Sarada, & Ravishankar, 2007).

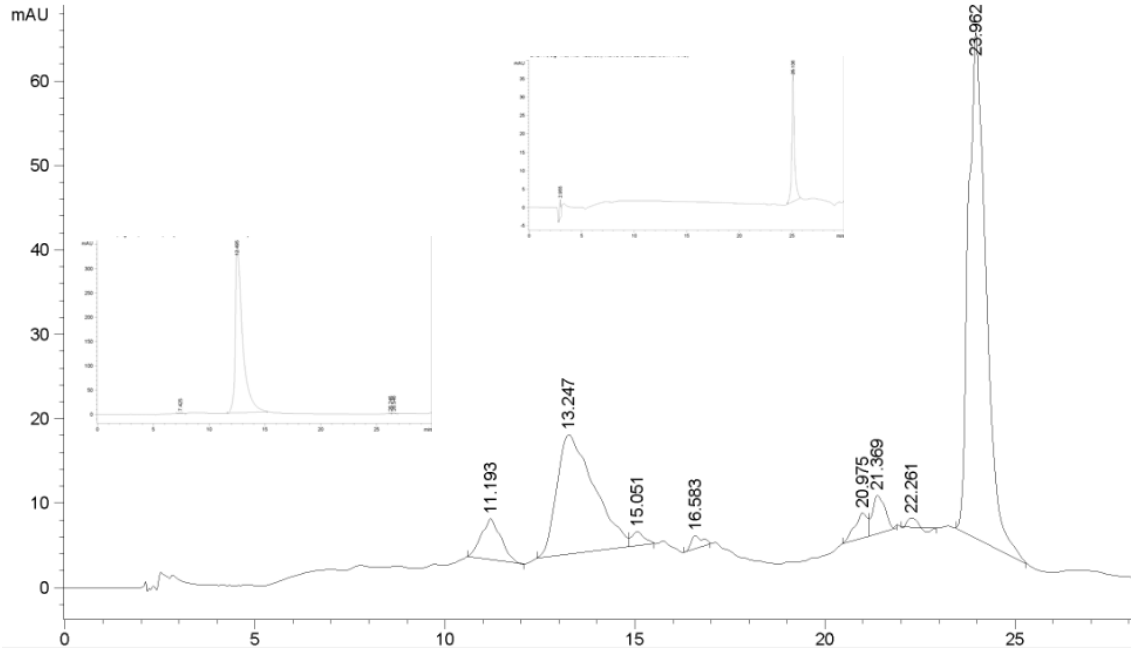


Figure 6.8 Chromatograms of lobster lipids with astaxanthin and beta-carotene standards used for identifying and determining carotenoids present in lobster lipid extracts

6.3.3.2 Lobster proteins have high nutritional quality and digestibility

Apart from being demonstrated with high nutritional quality indicated by containing all EAAs with significant amounts greater than the suggested intake values for adults suggested by FAO/WHO (1990), quality of lobster proteins was also confirmed by their enriching in protein (83.2 % for LHP and 74 % for LSPH) over the recommended value for commercial fish protein powder (65 %)

(Shaviklo, 2015). The need for a dietary protein is for both EAAs and dietary nitrogen, thus protein quality is generally decided by its amino acid concentration and ratio. The greater ratio of EAAs occurs in a protein, the greater biological value or quality it is. As shown on Table 6.2, both LHP and LSPH had EAAs ratio of 38.9 and 34.2 % which are in the suggested range for dietary protein (33.3 – 53.6 %) (Ige, Ogunsua, & Oke, 1984). Since methionine is a slightly deficient in lobster protein indicated by their low chemical scores (85 % for LHP and 76 % for LSPH), methionine is the only limiting amino acid of lobster proteins. However, this limitation could be overcome by formulating lobster proteins with other protein sources rich in methionine.

Table 6.2 Quality of lobster proteins determined by amino acid composition, protein efficiency ratio, and digestibility

Quality attributes	LPH (%)	LSPH (%)	Suggested values (%)
Protein	83.2	74	> 65[a]
Total EAAs	38.9	34.2	33.3 – 53.6[b]
PER	14.8	14.3	> 2[c]
Digestibility	78.4	96.9	88.1[d]
Chemical scores	85	76	-
Limiting aa	methionine	methionine	-

[a] Protein content suggested for commercial fish protein powder

[b] EAA suggested for dietary protein

[c] The protein efficiency ratio (PER) value of protein denoted with good to high quality

[d] Digestibility of egg protein

The PER has also been used for evaluating the quality of protein in food and is the standard in Canada (Agency, 2016). It is suggested that protein with a PER value over two have good to high quality. Lobster proteins with the estimated PER values of 14.7 for LHP and 14.3 for LSPH could be denoted with high quality. Meanwhile, digestibility of proteins indicates the susceptibility of its peptide bonds to hydrolysis, which varies with proteins. Both lobster proteins had high digestibility possibly due to the absence of strong collagenous fibres and tendons, which are common in protein of land animals. Digestibility of LSPH was significant higher than that of egg protein (96.9 vs 88.1 %) while this of LHP was comparable (78.4 vs 88.1 %). This could be explained by their

differences in molecular weights (MW). LSPH recover by hydrolysis is fragmented proteins (MW 10 KDa) while LHP could contain larger proteins (MW 50 – 150 KDa). In contrast to intact proteins, protein hydrolysates are highly soluble and do not form viscous solutions resulting more effectively absorbed in the gastrointestinal stage (Venugopal, 2009). By these significant advantages, protein hydrolysates have been demonstrated as useful nutrients supplements (Nobile et al., 2016; Vikøren et al., 2013) or potential treatment for malnourished people (Nesse et al., 2011). Therefore, the obtained lobster proteins could be possibly marketable as lobster protein powders or food ingredients for formulated products.

6.3.4 High potency of lobster proteins and lipids in the lobster flavour formulated products

6.3.4.1 Sensory properties of lobster proteins and lipids

The sensory properties of extracted lobster proteins and lipids were evaluated prior to their incorporation into products. As shown in Table 6.3, lobster extracts have some specific sensory properties, which would be promising for applications in the formulated products. Very strong flavours of lobster lipids combined with an attractive colour demonstrated its potential use in several incorporated products since colour and flavour of food play an important role not only in the selection, but also in the determination of consumption, satiation, and ingestion (Dias et al., 2012). Particularly, cherry red-burgundy colour of lobster lipids could add high sensory value to products due to its high psychological impact of colour on sensory assessment (Spence, 2015). Furthermore, lobster proteins with strong or mild flavours combined with pleasant tastes could be used as flavour enhancer or seasoning.

Table 6.3 Sensory assessment of lobster proteins and lipids

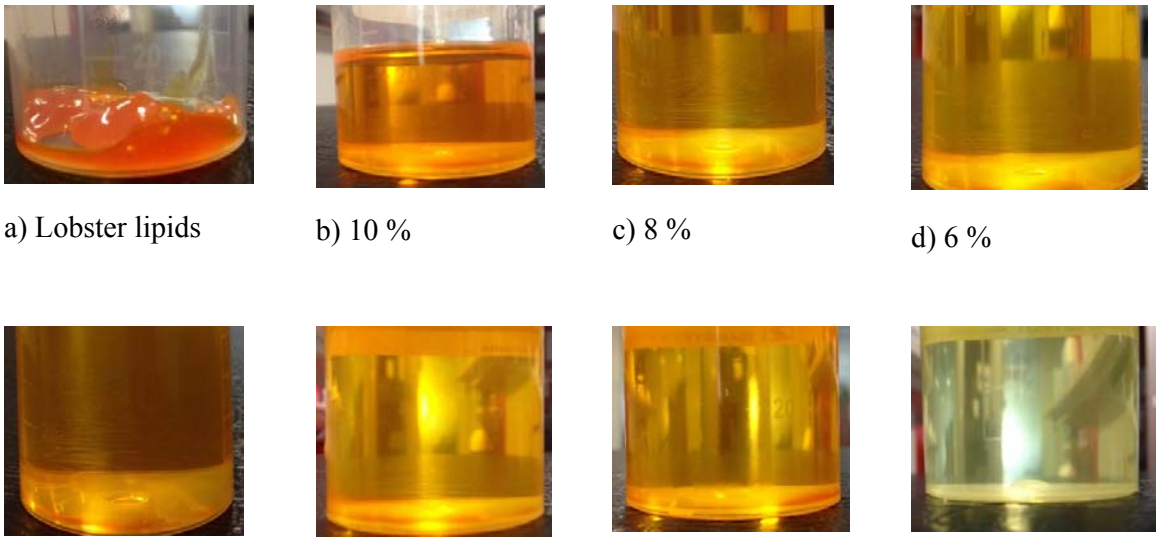
Properties	LSPH	LHP	Lobster lipids
Colour	Yellow/Cream	White/Straw	Cheery red-burgundy
Appearance	Flaky, crystalline	Free flowing powder	Syrupy liquid at room temperature and solid under refrigerated conditions
Aroma	Mild	Strong	Very strong odour reminiscent of lobster
Taste	Well balanced, strong flavour	Intense sharp (initially), then fishy	NA

6.3.4.2 Potency of lobster proteins and lipids in the formulated products

The potency of lobster proteins and lipids used as flavorants were evaluated. A number of formulated products were created and the final products evaluated by a food panel based on the flavour, taste, and colour.

Infused lobster oils

As lobster lipids have very strong lobster flavour and a tractive colour, their potential as soluble lobster flavorants in a liquid matrix was investigated. Olive oil was used as a carrier infused with lobster lipids at varying concentration 1 – 10 % (v/v) as shown in Figure 6.9. Based on the flavour and colour of the final product, the oil infused with 2 % of lobster lipids was assessed with the high potential for commercialisation as the infused lobster oil (Figure 6.9 f).



a) Lobster lipids b) 10 % c) 8 % d) 6 %

e) 4 % f) 2 % g) 1 % h) Olive oil

Figure 6.9 Olive oils infused with lobster lipids at different concentrations (1 – 10 %, v/v)

Salt plated with lobster flavours

Table 6.4 Composition of salt plated with lobster lipids

Ingredients	Concentration (%)
Salt	94
Lobster lipids	4

BHA	100 ppm
Silicon dioxide	2

Salt plated with lobster flavours was prepared with ingredients as shown in Table 6.4. Lobster lipids were plated on salt (sodium chloride) at a loading of 4 % (v/w). The salt acted as carrier to bind and dispense the hydrophobic liquid (lobster lipids) to create a dry flour compound. Butylated hydroxyl anisole (BHA) and silicon dioxide were also added to reduce oxidation and to prevent caking. The plated salt has attractive flavours representing for lobster in a dry flour form without caking (Figure 6.10a), which was obtained high acceptance from the panel.

a) b)

Figure 6.10 Salt plated with lobster lipids (a), lobster flavour seasoning formulated with lobster protein and the plated salt (b)

Lobster flavour seasoning

Lobster flavour seasoning was prepared by mixing with several other ingredients as described in Table 6.5. Evaluation was carried out on a series of lobster flavour seasoning formulated with various concentration of lobster protein (1 – 5 %, w/w) and plated salt (10 – 15 %, w/w) as key ingredients. The lobster flavour seasoning was organoleptically as an additive to Smith plain potato chips and in instant noodle soup.

Table 6.5 Lobster protein and lipids formulated with other ingredients for a lobster seasoning product

Ingredients	**Concentration (%, w/w)**
Salt	32
Maltodextrin	22

Sugar	15
Salt plated with lobster lipids	12
MSG	12
Garlic powder	2.5
Onion	2.5
Lobster proteins	1
Black pepper	0.5
Celery seed	0.3
Parsley flakes	0.2
Silicon dioxide	0.5 – 0.8

The combined use of lobster protein and lipids for formulation showed advantages since lobster protein provided base savoury notes and supported the aroma top notes presenting in lobster lipids. Particularly, the product formulated with 1 % of lobster protein and 12 % of plated salt offered the desired flavours with potency for commercialisation as lobster flavour seasoning (Figure 6.10b).

6.4 Conclusion

Lobster extracts demonstrated with good physicochemical properties, high nutritional quality, possessing certain attractive sensory properties or specific functionalities, which have good potential for food or nutraceutical applications. Lobster chitin with high fat and cholesterol binding which was comparable to commercial ingredients indicates its high potential applications for weight loss and cholesterol management products. Various applications of calcium lactate in the food industry indicate lobster minerals could be directly marketed as food ingredients or dietary calcium supplement due to its calcium-rich profile together with a high solubility and bioavailability. Lobster lipids and protein with their high nutritional quality (rich in PUFAs, omega-3, carotenoids, astaxanthin, EAAs, and high digestibility) could be directly marketable as dietary supplement or lobster protein powder. Moreover, these ingredients were successfully incorporated into the formulated products thank to their strong flavours, attractive colour, and pleasant tastes. Lobster oils, lobster flavour seasoning, and salt plated with lobster flavours produced from lobster lipids

(using 2 and 4 % for lobster oils and plated salts, respectively) and proteins (using 1 % for lobster flavour seasoning) were evaluated to have potentials for commercialisation.

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CHAPTER 7 ECONOMIC FEASIBILITY ANALYSIS FOR INDUSTRIAL PRODUCTION OF FUNCTIONAL INGREDIENTS AND NUTRACEUTICAL FROM AUSTRALIAN LOBSTER PROCESSING BY-PRODUCTS

7.1 Introduction

The Australian lobster production in 2015 was 10,307 tons (Peter, 2016) and over half the total volume of lobster was processed for the global demands (Gary, 2012), which produces lobster processing by-products (LPBs) including heads, shells, and livers estimated over 3,000 tons/year (He, Nguyen, Zhang, & Peng, 2016). Since LPBs are rich in protein, minerals, chitin, and lipids, which have been marketed as food functional ingredients, nutraceuticals or pharmaceuticals, studies on recovery of these valuable components, have obtained promising results. Australian lobster livers contained high lipids (24.3 %) which were efficiently recovered by supercritical carbon dioxide (SC-CO_2) with the recovery of 94 % (Nguyen, Zhang, Barber, Su, & He, 2015). The extracted lipids had very strong flavours which was found to have potential applications as lobster flavouring agents for production of lobster oils and salt plated with lobster flavours with a highly commercial value (Peinado, Koutsidis, & Ames, 2016). Moreover, the lipids recovered by the SC-CO_2 were rich in nutraceuticals (31.3 % PUFAs with omega-3 accounting for up to 58 %, astaxanthin 41.6 μg/mL, and β-carotene 70.4 μg/mL) that was found to potentially market as dietary supplement (Rizliya & Mendis, 2014).

Lobster heads, the major by-product of the Australian lobster industry, were studied for protein recovery by ultrasonic extraction. Protein of lobster head meats was found to be efficiently extracted by ultrasonic extraction in a very short time (99 % in 5 minutes) and precipitated for recovery of over 86 % protein. High quality of the extracted protein (83.2 % protein, 38.9 % EAAs, digestibility 78.4 %) demonstrated its high potential use as food nutrients or functional ingredients (He et al., 2016; Shaviklo, 2015; Vikøren, Nygård, Lied, Rostrup, & Gudbrandsen, 2013). Furthermore, a pleasant taste and flavour of LHP also indicated its commercial application for production of lobster seasoning and other formulated products.

Cooked ground lobster shells (CGLS) generated from the Australian lobster industry rich in chitin (25 %), protein (29 %), and minerals (36 %) were studied for protein recovery via deproteinisation during chitin production (Nguyen, Zhang, Barber, Su, & He, 2016). It was found that enzymatic

deproteinisation of CGLS could be intensified by microwave for economic recovery of 85 % lobster shells protein (LSP). Due to its high nutritional quality (high protein 74 % and EAAs 34 %, rich in arginine, low lysine/arginine ratio 0.7) and excellent functionality (high solubility, excellent water binding in the meat matrix), the extracted LSP has a potential use as food functional nutrients with high potential commercial values (Nguyen et al., 2016; Nobile et al., 2016; Rizliya & Mendis, 2014). Meantime, lobster minerals recovered from the sequential demineralisation also indicate their commercial potential as food ingredients or dietary calcium supplements due to the fact that lobster minerals had a calcium-rich profile (87 % calcium) and high solubility (over 60 %) as well as bioavailability. In addition, chitin generated from these microwave-intensified processes was found to have potential use as active ingredients in weight loss and cholesterol management products with high market values since it has excellent functional property (significantly high fat and cholesterol binding) and high quality (low protein and mineral residues, high DA).

Although the obtained results for production of functional and nutraceutical extracts from Australian LPBs (livers, heads, and shells) are promising, these studies were limited to the laboratory scale and has not been translated to the large-scale production. Moreover, applicability of SC-CO_2, ultrasound, microwave in the industrial production has still been very limited due to doubts about the high costs for initial investment and maintenance as well as complexity (Ciriminna et al., 2016; Galanakis, Barba, & Prasad, 2015; C. G. Pereira, Prado, & Meireles, 2013). Therefore, economic feasibility analysis for industrial production of lobster functional and nutraceutical extracts would be an important step to attract potential investors and/or justify business decisions to invest. To translate these laboratory results to the industrial production, a process simulation software could be used for economic evaluation of the process performance in silico before carrying out any expensive scale-up production (Petrides, Koulouris, & Lagonikos, 2002). The use of these process simulators offers the great opportunity to shorten the time required for industrial process development while these simulators allow comparing among several the process alternatives, thus many steps in the process design can be synthesised and analysed interactively in a short time. Moreover, they could computationally simulate the process to reduce unnecessary time for the scale-up process development or model and predict production costs for an industrial process. Superpro Designer, the proprietary software package, was specifically developed for the biotechnology industry, which successfully overcomes the weakness of the previously developed process simulation software for biotechnology processes. Many bioprocesses usually involve biomaterials and products whose exact physical properties, structure or chemical composition may

not be fully known, thereby affecting the accuracy of the simulation outcome. The large data base of specific feed stocks and unit operations in Superpro Designer successfully increases the accuracy of bio-process simulation, thus this software has been used to simulate and evaluate the economic feasibility of several different biological processes for industrial production of enzymes (Castro, Carvalho, Freire, & Castilho, 2010), microbreweries (Jones, Onwumelu, & Worstell, 2012), succinic acid (Lam, Leung, Lei, & Lin, 2014) or fish protein hydrolysate (He, Christopher, & Franco, 2015). Furthermore, it has also been applied for industrial feasibility analysis of emerging extraction technologies such as SC-CO_2 (C. Pereira, Rocha, FLP, & Mendes, 2013; Prado & Meireles, 2011; Rosa & Meireles, 2005), microwave (Ciriminna et al., 2016), and ultrasound (Santos, Veggi, & Meireles, 2010; Vieira, Cavalcanti, Meireles, & Hubinger, 2013). In this study, economic feasibility for industrial production of functional and bioactive ingredients from LPBs was analysed using SuperPro.

7.2 Process description

Promising results obtained from the laboratory scales for production of functional and nutraceutical ingredients from Australian LPBs suggested that these processes could be applied for the industrial scales. SuperPro Designer, the most common simulator widely used by several industries (pharmaceutical, food, biotech, specialty chemical, consumer product, mineral processing), with its significant advantages in translating from the laboratory scale to the industrial production was used for these simulations. The simulated processes were built by the powerful built-in tools. The batch process was used for all the models with the processing capacities relatively close to either the estimated quantities of LPBs annually generated by local lobster processors or the possible capacity of industrial extractors. The default setting with a maximum annual operating time of 7,920 hours per year was employed whereas the down time was used for regular maintenance and engineering work.

7.2.1 Process for SC-CO_2 extraction of lipids from lobster livers

Several significant advantages of SC-CO_2 extraction over conventional methods have been extensively reported at the laboratory scale but its applications for industrial extraction has still been very limited due to the high initial investment (Prado & Meireles, 2011). Recently, several studies on cost of manufacturing (COM) have revealed that the SC-CO_2 extraction process is very competitive in spite of high initial investment cost. The COM of the SC-CO_2 process is competitive with the COM of the conventional extractions once all involved costs are taken into account (Jesus

& Meireles, 2014; C. Pereira et al., 2013; C. G. Pereira et al., 2013). Particularly, the SC-CO_2 extraction could maximise its advantages when either the target compounds are thermolabile or the final products are required to have high purity (C. G. Pereira et al., 2013). In this study, lobster lipids recovered by the SC-CO_2 extraction significantly enriching in PUFAs, omega-3, carotenoids, and astaxanthin but low heavy metal contamination demonstrated its great potentials for industrial production. The laboratory-scale process developed by Nguyen et al. (2015) was used for simulation at a large scale with four main sections including material treatment, extraction, product recovery, and solvent recycle as illustrated in Figure 7.1.

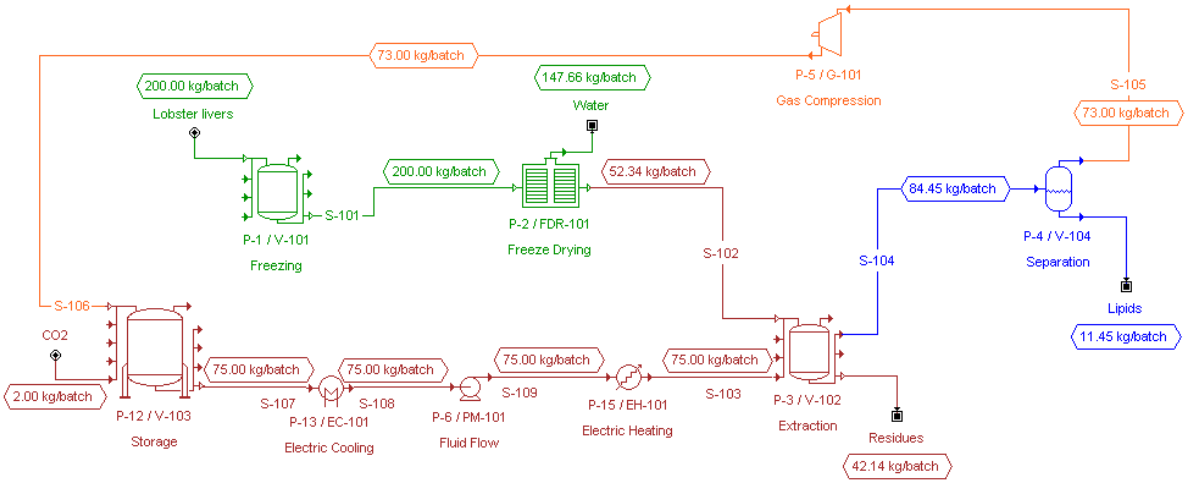

Figure 7.1 The simulated process for SC-CO_2 lipid extraction from lobster livers

Pre-treatment materials: fresh lobster should be freeze-dried for water removal to obtain the final dried material with 24.3 % of lipids. Therefore, fresh lobster livers (200 kg) were frozen at -18 ^oC for one hour in the equipment P-1/V-103 before freeze-drying at – 40 ^oC for six hours using the P-2/FDR-101.

Extraction: the freeze-dried lobster liver was transferred to the extractor (P-3/V-102) before loading CO_2 for extraction. The CO_2 fluid was cooled to around 5 °C by electric cooling (P-13/EC-101) for maintaining its incompressible liquid conditions (Sapkale, Patil, Surwase, & Bhatbhage, 2010). The CO_2 liquid was then pumped to a heating unit (P-15/EH-101) by the P-6/PM-101 unit, where it was heated to supercritical conditions (temperature > 31 ^oC and P > 7.4 MPa). Since the process was optimised at 50 ^oC and 350 MPa (Nguyen et al., 2015), CO_2 was heated and then pressurised to this optimum condition for extraction. Supercritical fluid (CO_2) then passes into the extraction vessel (P-

3/V-102), where it rapidly diffused into the material matrix and dissolved 94 % lipids within four hours of extraction.

Product recovery: The dissolved material was swept from the extraction cell into a separator (P-4/V-101) at lower pressure and the extracted lipids were settled out for recovery. A certain amount of CO_2 is lost at this stage due to absorption to the product and residue while the majority of released CO_2 moved to the recycle section.

Recycle: CO_2 was compressed by the P-5/G-101 unit during extraction process before it was transferred to the storage tank (P-12/V-107) for the subsequent batch. Amount of CO_2 lost with assumption of 2 % (Trees, 2009) was supplemented at this section. Annual material consumption, productivity, and batch information of the simulated process was summarised in Table 7.1.

Table 7.1 Annual feeding materials required and lobster lipids produced by the simulated SC-CO_2 lipid extraction from lobster livers

Feeding materials/Main products		Unit	Year (Unit/year)
Materials	Livers	Kg	249,800
	CO_2	Kg	2,498
Product	Lipids	Kg	14,301
Batch information	Recipe batch time (11.92 hours)	Batch	1,249
	Recipe cycle time (6.33 hours)		

7.2.2 Process for ultrasonic extraction protein from lobster heads

Ultrasonic extraction of protein from lobster head by-products was demonstrated to have several significant advantages at a laboratory scale such as high extraction recovery, short extraction time, and high quality protein, application of this method for a large-scale extraction process has still limited. In this study, nutritional LHP was efficiently recovered by ultrasonic extraction, which was simulated with three sections including pre-treatment, extraction, and recovery Figure 7.2.

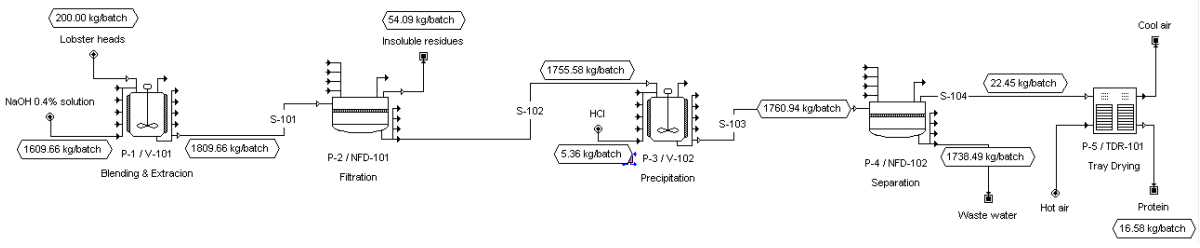

Figure 7.2 The simulated process for ultrasonic extraction protein from lobster heads

Pre-treatment: The feeding material used in this process was raw lobster heads including legs with an average weight of 150 – 300 g containing meats of body, breast and leg. These residual meats were good protein sources for recovery, thus raw lobster heads (200 kg) were blended in the P-1/V-101 tank for 15 minutes to obtain the homogenously blended lobster heads.

Extraction: Protein in the homogenised lobster heads was extracted by sonicating the homogenate with 8 volumes of NaOH 0.4 % for 5 minutes in the same tank (P-1/V-101). With the ultrasonic intensity applied for this extraction (duty cycle of 50 %, output control of 80 %), approximately 95 % protein of residual meats were extracted. The extracted protein solution was obtained by Nutsche filtration (P-2/NFD-101) for two hours to remove the insoluble residues.

Recovery: Protein was precipitated for recovery by adjusting pH of the extracted solution to isoelectric point, thus the extracted solution (pH ~ 13.5) was agitated with the concentrated HCl (10 N) at ratio of 0.05 - 0.1 % (v/v) in the P-3/V-102 tank for 30 minutes for protein precipitation (pH ~ 3.5), which precipitated 82.6 % protein in the extracted solution. The precipitated protein was separated by Nutsche filtration (P-4/NFD-102) for 30 minutes before tray-dryer was used in 3.5 hours, at 45 ^{o}C as suggested by Tu (1991) for retaining LHP. Annual material consumption, productivity together with batch information for the simulated process is shown on Table 7.2.

Table 7.2 Annual feeding materials required and LHP produced by the ultrasonic extraction from LHB

Feeding materials/Main products		**Unit**	**Year (Unit/year)**
Materials	Lobster heads	Kg	441,800
	NaOH 0.4 %	L	3,555,745
	HCl 10 N	L	11,848
Main product	LHP	Kg	36,448.5
Batch information	Recipe batch time (6.67 hours)	Batch	2,209
	Recipe cycle time (3.58 hours)		

7.2.3 Processes for the microwave-intensified extraction of protein, minerals, and chitin from CGLS

7.2.3.1 Microwave-intensified enzymatic deproteinisation of CGLS for LSPH production

The microwave-intensified enzymatic deproteinisation of CGLS was simulated using the laboratory results published by Nguyen et al. (2016). The simulated process was calculated for the scale of 150 kg per batch, which was feasible for the industrial-scale microwave extractors marketed by IDo

(2016). The process was simulated with three main sections including deproteinisation, separation, and drying as illustrated in Figure 7.3.

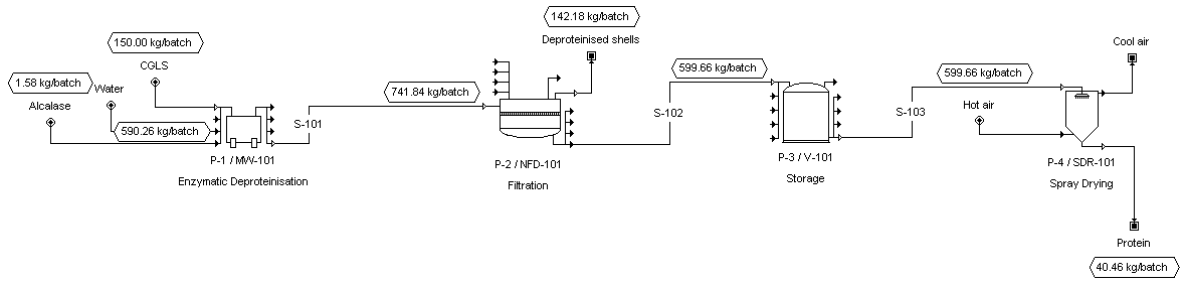

Figure 7.3 The simulated process for microwave-intensified enzymatic deproteinisation of CGLS for LSPH production

Deproteinisation: Starting material of this process was the dried CGLS composing of 29 % protein associated with other components such as minerals, chitin, and pigments (Nguyen et al., 2016). Deproteinisation of CGLS was carried out using alcalase at the E/S ratio 1 % (v/w) intensified by microwave (P-1/MW-101) at 55 ^{o}C, stirring 70 % for 90 minutes after CGLS was mixed with four volumes of warm water (55 ^{o}C) to obtain the homogenised slurry for deproteinisation. Approximately 85 % protein of CGLS were deproteinised at this step.

Separation: The extracted protein solution was obtained by Nutsche filtration (P-2/NFD-101) for one hour to separate the insoluble residues.

Drying: The tank (P-3/V-101) was used for collecting the extracted solution for spray drying by the unit (P-4/SDR-101) with the temperature of input/output air around 180/100 ^{o}C. Table 7.3 describes annual material consumption, productivity together with batch information for the simulated process.

Table 7.3 Annual feeding materials required and LSPH produced from the microwave-intensified enzymatic deproteinisation of CGLS

Feeding materials/Main products		**Unit**	**Year (Unit/batch)**
Materials	CGLS	Kg	475,050
	Alcalase	L	4,988
Main product	LSPH	Kg	128,144
Batch information	Recipe batch time (4.75 hours)	Batch	3,167
	Recipe cycle time (2.50 hours)		

7.2.3.2 Microwave-intensified demineralisation of DPS for mineral production

Deproteinised lobster shells (DPS) generated from the microwave-intensified enzymatic deproteinisation process containing high proportion of minerals was used as feeding material for this process as described by Nguyen, Barber, Luo, and Zhang (2017). The simulated processed was divided into three sections: demineralisation, separation, and drying as described in Figure 7.4.

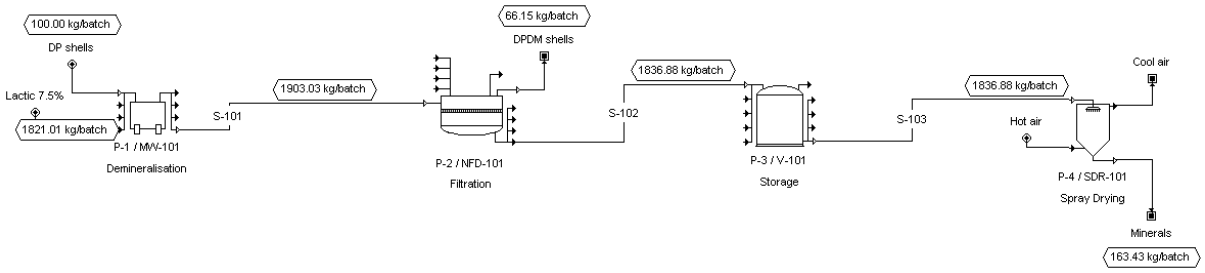

Figure 7.4 Simulated process for microwave-intensified demineralisation of DPS for mineral production

Demineralisation: minerals were removed by mixing DPS with lactic acid 7.5 % at a ratio of 18 mL/g as the optimum ratio for the microwave demineralisation. The process was then intensified by microwave in the P-1/MW-101 at 100 ^{o}C for 23 minutes. This step removed 99.2 % minerals of DPS.

Separation: After demineralisation, the insoluble residues was separated by the Nutsche filtration (P-2/NFD-101) for 30 minutes while the demineralised solution was collected by the tank P-3/V-101 during the spray-drying period.

Drying: Mineral powder was obtained after spray drying by the P-4/SDR-101 unit with temperature of input/output air about 180/100 ^{o}C for two hours. All details about annual material consumption, productivity, and batch information for the simulated process were summerised on Table 7.4.

Table 7.4 Annual feeding materials required and lobster lactate salts produced from the microwave-intensified demineralisation of DPS

Feeding materials/Main products		**Unit**	**Year (Unit/Year)**
Materials	DPS	Kg	316,700
	Lactic acid 7.5 %	L	5,767,124
Main product	Lobster lactate salts	Kg	517,597
Batch	Recipe batch time (4.50 hours)	Batch	3,167
	Recipe cycle time (2.50 hours)		

7.2.3.3 Microwave-intensified extraction of chitin from deproteinised demineralised shells (DPDMS)

Major component of the insoluble solid (DPDMS) generated from the demineralisation process was chitin but it still associated with residual protein and pigment (Nguyen et al., 2017). Therefore, further deproteinisation and depigment for chitin recovery are two main sections in the simulated process as described in Figure 7.5.

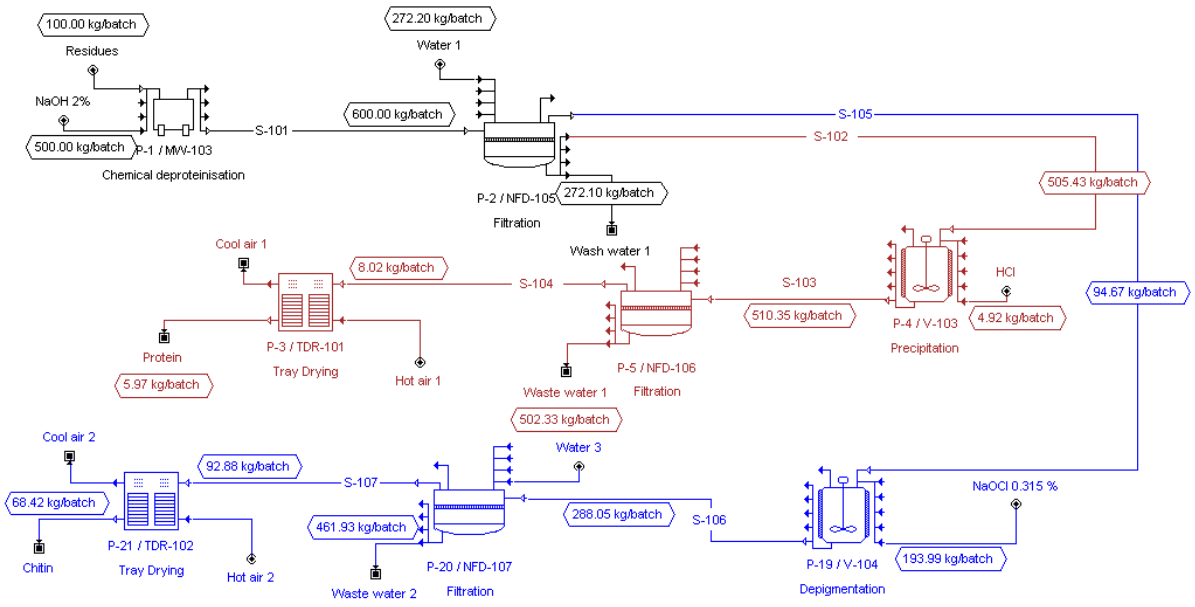

Figure 7.5 Simulated process for microwave-intensified production of chitin from DPDMS

Further deproteinisation: Mixing the insoluble residue with five volumes of NaOH 2 % for deproteinisation intensified by microwave (P-1/MW-103) at 100 ^{o}C for 30 minutes could remove 92.7 % residual protein. The residual solid and liquid phase were separated by Nutsche filtration (P-2/NFD-105) for one hour. Whereas the solid phase was moved to the next section for depigment, pH of the liquid phase was adjusted to the isoelectric point (pH ~ 3.5) in the tank (P-4/V-103) using concentrated HCl (10 N) for precipitation of 85 % protein. The precipitate was tray-dried by the unit P-3/TDR-101 at 45 ^{o}C for 3.5 hours.

Depigment and recovery: The residual solid was agitated with five volumes of NaOCl 0.315 % for 30 minutes in the tank P-19/V-104. The residual solid was separated and washed by the Nutsche unit (P-20/NFD-107) for 60 minutes before it was tray-dried in the P-21/TDR-102 unit at 70 ^{o}C for 4 hours. A summary for annual material consumption, productivity, and batch information is illustrated on Table 7.5.

Table 7.5 Annual feeding materials required and main products produced from the microwave-intensified chitin production from DPDMS

Feeding materials/Main products		Unit	Year (Unit/Year)
Materials	DP-DMS	Kg	197,800
	NaOH 2 %	L	989,000
	HCl 10 N	L	49,969
	NaOCl 0.315 %	L	383,708
Main product	LSPH	Kg	11,777
	Chitin	Kg	135,335
Batch	Recipe batch time (9.41 hours)	Batch	1,978
	Recipe cycle time (4.00 hours)		

7.2.3.4 Integrated process for microwave extraction of protein, minerals and chitin from lobster shells

The integrated process for biorefinery recovery of functional and nutraceutical components from CGLS illustrated in Figure 7.6 include three main sections: protein extraction, mineral extraction, and chitin recovery.

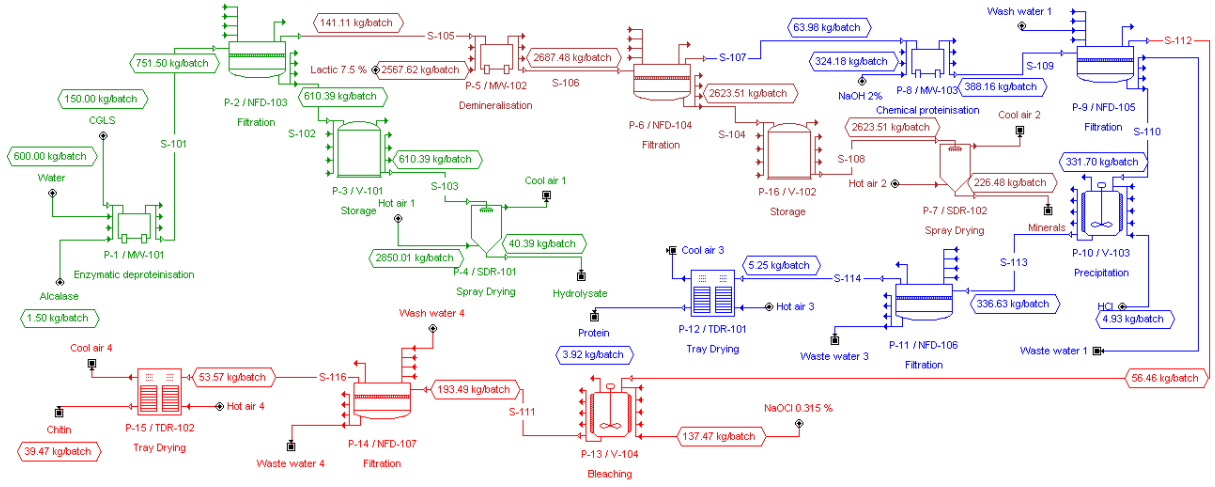

Figure 7.6 Integrated process for microwave-intensified production of LSPH, minerals, and chitin from

Protein extraction: Deproteinisation of CGLS using alcalase intensified by microwave could extract 85 % protein of CGLS. LSPH was obtained by spray-drying the deproteinised solution after filtration for separation of the insoluble residue.

Mineral extraction: Minerals were recovered from the deproteinised residue (DPS) using lactic acid intensified by microwave. This step could extract 99.2 % minerals od DPS for recovery by

separation of the demineralised solution for spray-drying while its insoluble residue was used for the next section.

Chitin recovery: Purifying the DPDMS by further deproteinisation and depigmentation could remove 92.7 % of residual protein while all its pigments were bleached. The residual solid was tray-dried for obtaining lobster chitin. Annual material consumption, productivity, and batch information for the integrated process was summerised on Table 7.6.

Table 7.6 Annual feeding materials required and main products produced from CGLS by microwave intensification

Feeding materials/Main products		Unit	Year (Unit/Year)
Materials	CGLS	Kg	222,450
	Alcalase	L	2,225
	Lactic acid 7.5 %	L	3,807,777
	NaOH 2 %	L	480,766
	HCl 10 N	L	7,311.19
	NaOCl 0.315 %	L	203,865
Main product	LSPH	Kg	65,699
	Lobster lactate salts	Kg	335,872
	Chitin	Kg	58,539
Batch	Recipe batch time (12.42 hours)	Batch	1,483
	Recipe cycle time (5.33 hours)		

7.3 Process simulation assumption

The economic feasibility analyses for industrial production of functional and nutraceutical ingredients from Australian LPBs were carried out using the Superpro Designer version 8.0 software developed by Intelligen Inc. (Petrides et al., 2002). The estimation of total capital investment, total production costs, and profitability of all the simulated processes were calculated using the built-in databank of the software. Since SC-CO_2, ultrasonic, and microwave are still emerging technologies, not all updated data for these processes are included in the software databank. Therefore, the economic feasibilities for these processes were analysed with assumptions that the industrial-scale units perform as well as the laboratory-scale units and both the yield and the extraction time of the large-scale processes are similar to the laboratory-scale ones when the processing parameters are kept constant. The industrial units were assumed to be operated at full capacity with the default time of 7920 hours per year, thus number batches per year for each simulated process were calculated based on its recipe batch time and recipe cycle time.

7.3.1 Supercritical

Calculations for the simulated SC-CO_2 extraction of rich-ω-3 lipids from lobster livers were based on the laboratory results published by Nguyen et al. (2015). The purchased cost for the large-scale SC-CO2 extractor was estimated using the information reported by Trees (2009) and C. Pereira et al. (2013) that the 50-L extractor priced at 500,000 USD. For being more cost-effective, CO_2 was recycled after extraction, thus it was filled with 1.5 volumes of the dried material for the first batch while the lost CO_2 (assumption with 2 %) was supplemented for each sequential batch. Since SC-CO_2 extraction is an environmentally friendly technology while CO_2 is nontoxic gas, thus the cost of waste treatment was considered null.

7.3.2 Ultrasound

The simulated process for ultrasonic extraction of nutritional protein from lobster heads was calculated by applying the laboratory results obtained in the chapter 3 of this study and the published results (He et al., 2016). The ultrasonic extractors at industrial scales were made by bonding the ultrasonic transducers to the external walls of the tanks as described by Keil and Swamy (1999) and Vinatoru (2001). The purchased costs of such ultrasonic extractors were estimated about 28,200 USD for a 1000-L unit (Vieira et al., 2013), which was used for calculating the purchased cost of an ultrasonic extractor in the simulated process.

7.3.3 Microwave

Although the use of microwave for intensifying extraction have obtained several significant advantages at laboratory scale, its industrial applicability has still very limited due to the high technical complexity of applying microwave in the large-scale equipment (Ciriminna et al., 2016). However, technical problems for producing large-scale microwave units were finally solved with some large-scale microwave units being installed for industrial extraction of natural products (Fidalgo et al., 2016; Petigny et al., 2014) showing that scale up of the microwave-intensified processes at the industrial scale is indeed possible and economically attractive (Li, Radoiu, Fabiano-Tixier, & Chemat, 2012).

The capital cost for industrial microwave extractor was calculated using the suggestions of Mujumdar and Zhonghua (2007) that the total purchased price of a microwave unit mainly associates with its power generator together with specially designed captivity estimated 1,500 – 5,500 USD/KW depending on its the system power, frequency, and complexity. Due to its

relatively high complexity applied for industrial extractors, the purchase cost of an industrial-microwave unit was selected at 3,700 USD/KW. The microwave powers for industrial microwave extractors used in these simulated processes were calculated according to proportion of material being processed, processing parameters but the maximum power was not over 150 Kw as advised by industrial microwave supplier (IDo, 2016). Energy consumed by an industrial microwave extractor was calculated using the method described by Hasna (2011). Maintenance and consumable costs of the industrial-microwave system is only with the microwave generator, magnetron. Since the magnetron usually last significantly longer than 5000 hours (Hasna, 2011), these costs were neglected for all the microwave processes.

7.4 Economic evaluation

Economic evaluation using simulation software as Superpro Designer has becoming a valuable tool to determine the feasibility of the developed processes, which was carried out to check the technical and economic attractiveness aside from providing the necessary support for implementing a feasible process. The economic analysis of a large-scale process usually involves in evaluating both the capital and operation cost related to the assembling and operation of the processing plant, respectively (Rostagno & Prado, 2013), however, economic evaluation contains a calculation about profitability are more preferable (Van Dael, Kuppens, Lizin, & Van Passel, 2015).

7.4.1 Capital investment

7.4.1.1 Fixed capital estimation

The processing facility was assumed to be built at an appropriate place where LPBs could be conveniently supplied by local lobster processors (e.g. Ferguson Australia Pty Ltd.). As such, the direct fixed capital cost (DFC, referring to the fixed assets of an investment such as plant and equipment) was estimated for the plan built in South Australia including total plant direct cost (TPDC, cost elements that are directly related to an investment); total plant indirect cost (TPIC, cost that are indirectly related to an investment such engineering, construction); and contractor's fee & contingency (CFC, miscellaneous costs). Superpro Designer uses a factor-based method to estimate all the capital investments associated with each section of a process as described in Table 7.7. These factors have been assigned default values that should be reasonable for most cases (Intelligen, 1991).

Table 7.7 Direct fixed capital estimation calculated by Superpro default setting

DFC = TPDC + TPIC + CFC			
TPDC	=	(1) + (2) + (3) + (4) + (5) + (6) + (7) + (8) + (9)	
	(1)	Equipment purchase cost (PC)	LPC + UPC
	(2)	Installation	ILE + IUE
	(3)	Process piping	0.35*PC
	(4)	Instrumentation	0.4*PC
	(5)	Insulation	0.03*PC
	(6)	Electrical	0.10*PC
	(7)	Buildings	0.45*PC
	(8)	Yard improvement	0.15*PC
	(9)	Auxiliary facilities	0.4*PC
TPIC	=	(10) + (11)	
	(10)	Engineering	0.25*TPDC
	(11)	Construction	0.35*TPDC
CFC	=	(12) + (13)	
	(12)	Contractor's fee	0.05*(TPDC+ TPIC)
	(13)	Contingency	0.1*(TPDC+ TPIC)

Equipment purchase cost (PC) includes listed equipment purchase cost (LPC) and unlisted equipment purchase cost (UPC) with UPC = 0.2*LPC. The PCs of almost units were calculated using the available updated databank whereas some equipment units which are not available in the databank such as extractors for SC-CO_2, microwave, and ultrasonic processes, estimations were carried out. The PCs for these extractors were calculated using information provided by industrial suppliers for a specific equipment unit required for the simulated process according to its capacity and scale. Vessels were priced with the assumption that these units were made of materials which are resistant to the dilute acids, bases, and the salty water for exposal to during operation. Installation costed by default includes installation of listed equip (ILE) and installation of unlisted equip (IUE) with IUE = 0.5*IUE. The estimated DFCs of different simulated processes are shown on Table 7.8. Among several processes used for production of lobster extracts, chitin production and the integrated production of chitin, protein, and minerals are two processes required the largest DFC while others needed significantly lower than half the DFC of these two processes. A long process with many steps combined with the relatively high complexity of industrial microwave extractors made the DFC of these processes become significant.

Table 7.8 Direct fixed capital (DFC) of the large-scale production of different lobster extracts from Australian LPBs

Processes	**DFC distribution**			**DFC (USD)**
	TPDC (USD)	**TPIC (USD)**	**CFC (USD)**	

Lobster lipids	1,621,000	972,000	389,000	**2,982,000**
LHP	3,193,000	1,916,000	766,000	**5,875,000**
LSPH	4,085,000	2,451,000	981,000	**7,517,000**
Minerals	4,558,000	2,735,000	1,095,000	**8,388,000**
Chitin	7,373,000	4,424,000	1,770,000	**13,567,000**
Integrated	8,679,000	5,208,000	2,083,000	**15,970,000**

7.4.1.2 Total capital investment

The total amount of money needed to supply the necessary plant, manufacturing facility, and working capital for operation is defined as total capital investment (TCI). The TCI for an industrial production plant was described by Turton, Bailie, Whiting, and Shaeiwitz (2008) including DFC, working capital, and start-up cost as illustrated in Table 7.9. Total capital investment estimations charged to all the simulated processes were obtained using the default setting of SuperPro software, which were automatically adjusted according to the year of commencement (2017) with a 30-month construction period and a 4-month start-up period for a 15-year project lifetime. Generally, the large-scale production of functional and nutraceutical lobster extracts required small amount money for investment (under 10 million USD) (Table 7.9) but the required investment for chitin production and its integrated process were around 14,687,000 and 17,414,000 USD. The high TCI of chitin production and its integrated process over others because of their complexity with many steps which required high DFC, working capital, and start-up cost.

Table 7.9 Total capital investment (TCI) for the large-scale production of different lobster extracts from Australian LPBs

Processes	**Capital investment distribution**			**TCI (USD)**
	DFC (USD)	**Working capital (USD)**	**Start-up cost (USD)**	
Lobster lipids	2,982,000	248,000	149,000	**3,379,000**
LHP	5,875,000	249,000	294,000	**6,418,000**
LSPH	7,517,000	373,000	376,000	**8,266,000**
Minerals	8,388,000	370,000	419,000	**9,177,000**
Chitin	13,567,000	441,000	679,000	**14,687,000**
Integrated	15,970,000	646,000	798,000	**17,414,000**

7.4.2 Operating cost estimation

The facility-dependent costs such as equipment maintenance, depreciation, insurance, property taxes, and possible other overhead costs, which depend on the facility set-up, estimated using the software default settings. Utility costs were set at the current price applied for the intended location in South Australia with $ 0.216 per KWh for electricity and $ 2.42 per kilolitre for water. A basic labour rate of $ 30 per hour was used while this rate for the specific types of operators, the

associated supervision, supplies, administration, and overhead costs was $ 69 per hour. The default value of 15 % was also included in the labour costs for covering quality assurance and checking.

The cost for LPBs (livers, heads, and shells) was estimated based on the fact that Australian LPBs are currently discarded with disposal costs of $ 150 per ton (He et al., 2016) meaning that each ton of LPBs utilised would be subsidised $150 used for collecting, storing, and transporting LPBs from local lobster processing facilities to the plant. Therefore, no cost was applied to all feedstock of LPBs. Costs for other materials used in the simulated processes were estimated according to the quotes provided by domestic suppliers as described in Table 7.10. Since lactic acid 7.5 %, NaOH 0.4 %, and NaOCl 0.315 % were assumed to be diluted from the concentrated solutions or pellets carried out on site, their costs were estimated according to the sum of the mixed components.

Table 7.10 The price of materials used for the large-scale production of different lobster extracts from Australian LPBs

Materials		Unit	Price (USD)	Suppliers
Feeding materials	CO_2 liquid	Kg	1.6	Puregas
	Alcalase	L	168.94	Novozyme
	Lactic acid syrup (88.1 %)	L	7.504	Purac
	NaOH pellet	Kg	46	Chem-Supply
	HCl 36 %	L	12.8	Chem-Supply
	NaOCl 8 – 12.5 %	L	4.6	Chem-Supply
Consumable materials	Filtration fabric	m^2	2.6	

Waste disposal costs were neglected in these simulated processes since the effluent waters or gases generated from these processes were neither hazardous nor fairly expensively treated. Meanwhile, the residual biomass generated from these processes could be removed free-of-charge by other partners due to their applications in other commercial products such as aquaculture feeds, biofertiliser, and compost or soil conditioners. Labour charges and labour not directly associated with production were estimated by the simulator. Other company costs such as packaging, marketing, and research and development were not included in these calculations, thus the actual annual operating costs calculated for these processes are illustrated on Table 7.11.

Table 7.11 Annual operating costs (AOC) of the large-scale production of different lobster extracts from Australian LPBs

Processes	AOC distribution			AOC (USD)
	Raw materials (USD)	Labour depend (USD)	Facility depend & utilities (USD)	
Lobster lipids	2,000	2,715,000	572,000	**3,289,000**
LHP	655,000	2,079,000	1,105,000	**3,839,000**
LSPH	807,000	3,250,000	1,465,000	**5,522,000**
Minerals	2,850,000	1,015,000	1,791,000	**5,656,000**
Chitin	1,088,000	3,751,000	2,571,000	**7,410,000**
Integrated	3,320,000	3,615,000	3,179,000	**10,114,000**

Operation costs of chitin production and its integrated process were higher other processes. Patterns of cost distribution are different among the simulated processes. Since CO_2 is the only costed material but this material was recycled during process, the material cost for lipid production was very low (Figure 7.7). In contrast, a high ratio of lactic acid (18 mL/g) was used for mineral production resulting the material cost accounted for 50 % of its operation costs. Whereas labour is major cost in almost the processes, this cost was about 18 % in the mineral production. This could be explained by the simplicity and efficiency of the microwave process (reaction time 23 minutes).

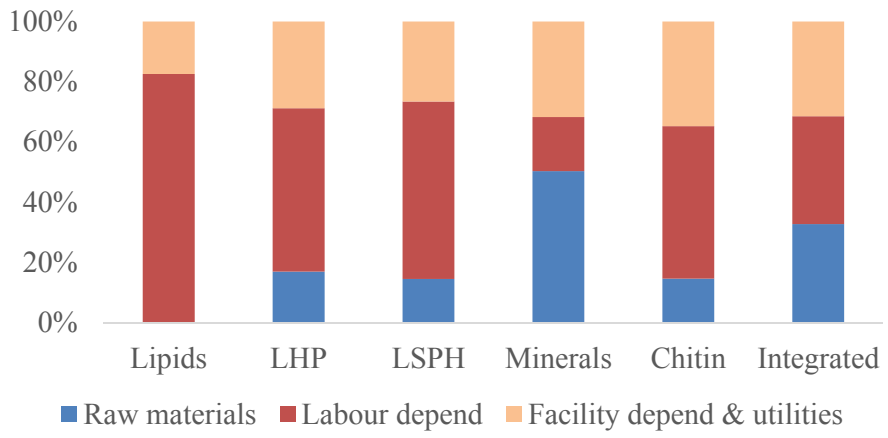

Figure 7.7 Distribution of cost patterns in the large-scale production of lobster extracts from different LPBs

7.4.3 Revenues and profitability analysis

The selling prices of the revenue streams obtained from the simulated processes including lobster lipids, protein, minerals, and chitin were calculated depending on the relative quality between lobster products and their respectively commercial counterparts as shown in Table 7.12. Calculation

for lobster lipid price was based on the fact that the extracted lipids (2 %) could be infused with olive oil to produce Lobster Oils marketed by Australia Pty Ltd with the rate of AUD 12 for a 100-mL bottle. Meanwhile, lobster protein were assumed to be sold for production of Salmon and Lobster spread products launched to the Australian market by Peck's at the price of AUD 1.88 for a jar (50 g) containing only 0.6 % of lobster extract. Thompson fat blocker sold at AUD 50 for a jar with 120 capsules (500 mg) was used for calculation of chitin price due to its high fat and cholesterol binding. Finally, the price of lobster minerals was determined based on the similarity between lobster lactate salts and calcium lactate commercialised by The Melbourne Food Ingredient Depot with the rate of AUD 26.5 for 200 g. Although it was hard to accurately determine the price of lobster functional and nutraceutical extracts, their enriching in nutraceuticals, high nutritional quality, similarity in chemical structure, and excellent physicochemical properties could be excellent indicators for calculation their prices. The price for lobster extracts was obtained after deducting all costs associated with production, using other ingredients, packaging, labelling, etc.

Table 7.12 Selling price of lobster extracts obtained from different simulated processes

Main products		**Referenced products**		
Lobster extracts	**Price (AUD/kg)**	**Commercial products**	**Units**	**Price (AUD/Unit)**
Lipids	500	Lobster oil*	L	120
Protein	308	Lobster spread**	Kg	37.6
Protein hydrolysate	150			
Minerals	26.5	Calcium lactate	Kg	132.5
Chitin	168	Fat blocker	Kg	833.3

*Only 2 % lobster protein used for production of lobster oil
**Only 0.6 % lobster protein used for production of lobster spread

Revenues of all lobster extracts obtained from the simulated processes and their respective AOCs were used for calculation of profitability (gross profits and net profits) (Table 7.13). The annual revenues that obtains before tax is defined as the gross profit while the one obtaining after tax is the net profit. Although lipid production had a low AOC, it generated less profitability (1,930,000 USD for gross profit and 1,441,000 USD for net profit). This is because its number of batches per year was low (1,249 batches) due to the long batch time (11.92 hours) and cycle time (6.33 hours). In contrast, production of LSPH and chitin obtained high profitability, which could be explained by the cost-effectiveness of the microwave-intensified processes combined with highly value-added of the extracted products. Furthermore, production of these lobster ingredients was more profitable

when it was integrated into one process where materials, processes and infrastructure could be shared among processes to be cost-effective.

Table 7.13 Profitability of different simulated processes for production of lobster extracts from Australian LPBs

Processes	Revenues (USD/Year)	AOC (USD/Year)	Gross profit* (USD/Year)	Net profit** (USD/Year)
Lobster lipids	5,219,000	3,289,000	1,930,000	1,441,000
LHP	8,234,000	3,839,000	4, 395,000	3,195,000
LSPH	14,032,000	5,522,000	8,510,000	5,820,000
Minerals	10,013,000	5,656,000	4,357,000	3,411,000
Chitin & LSPH	17,887,000	7,410,000	10,477,000	7,575,000
Integrated	20,871,000	10,114,000	10,757,000	7,971,000

*Gross profit = Revenues - AOC
**Net profit = Gross profit – Taxes (40 %) + Depreciation (10 %)

7.4.4 Economic feasibility of industrial production of functional and nutraceutical ingredients from Australian LPBs

Net present value (NPV), the gross margin, return on investment (ROI), and payback time were used as economic indicators for determining feasibility of all the simulated processes (Table 7.14). The NPV is the total value of future net cash flows during the project lifetime (discounted to reflect the value of money at the present year using the default interest rate of 7 %), which is usually used as an indicator of profitability (Van Dael et al., 2015). An industrial production process is considered interesting once it has a positive NPV is (Haim & Marshall, 1982). As seen from Table 7.14, the NPV of all the simulated processes are positive meaning that they are economically feasible. Among these processes, production of LSPH, chitin, and its integrated process are the most financially interesting since they had the highest NPV (over 31 million USD). Moreover, the high attractiveness of these processes were also indicated by their high gross margin (over 50 %), return on investment (ROI, 45 – 70 %), and short payback time (under 2 years). The percentage of annual revenues that become gross profit is defined as the gross margin while the payback time is the time for cumulative annual net profits to balance the TCI since processing operations commence; and the ROI is the annual percentage return on the TCI. These parameters were also used as economic indicators to demine economic feasibility of a production process (Lu, Hanwu, & Roger, 2015; Osorio-Tobón, Carvalho, Rostagno, & Meireles, 2016; Zabot, Bitencourte, Tres, & Meireles, 2017). Highly economic feasibility of these processes could be explained by their simplicity, high efficiency of the microwave intensification together with luxury of lobster products in high demand.

Table 7.14 Economic indicators of different simulated processes for production of lobster extracts from Australian LPBs

Processes	NPV (USD)	GM (%)	ROI (%)	Payback time (Years)
Lobster lipids	5,777,000	36.98	42.65	2.34
LHP	15,193,000	53.38	49.79	2.01
LSPH	31,417,000	60.65	70.41	1.42
Minerals	14,841,000	43.51	37.17	2.69
Chitin	37,320,000	57.57	51.58	1.94
Integrated	37,665,000	51.54	45.77	2.18

7.4.5 Feasibility of the projects in a scenario of variations in availability and cost of biomass

Although over 3,000 tons of LPBs are estimated to be annually produced in Australia, it would be impractical to collect all these by-products for utilisation since there are more than seven lobster processors scattered across the country. In this study, the simulation for lobster extract production was carried out at scales of 200 kg/batch for livers, 200 kg/batch for heads, and 150 kg/batch for shells, which totally utilised about 914.1 tons of Australian LPBs accounting for 30 % of the estimated amount (level A, Table 7.15). This could be practical since there are three large processors of lobster processing in Southern Australian including Southern Ocean Rock Lobster Pty Ltd, South Australian Lobster Company, and Ferguson Australia Pty Ltd that could provide enough amount of LPBs for these lobster-extract projects.

Table 7.15 Relative between the production scales of the simulated processes and availability of Australian LPBs

Scales[1]	Livers[1] (tons/year)	Heads[2] (tons/year)	Shells[3] (tons/year)	Total (tons/year)	Relative to estimated amount of biomass (%)
Level A	249.8	441.8	222.5	914.1	30.5
Level B	312.3	883.6	370.8	1,566.7	52.2
Level C	374.7	1,325.4	519.1	2,219.2	74.0

[1]Level A: 200 kg/batch; Level B: 250 kg/batch; Level C: 300 kg/batch
[2]Level A: 200 kg/batch; Level B: 400 kg/batch; Level C: 600 kg/batch
[3]Level A: 150 kg/batch; Level B: 250 kg/batch; Level C: 350 kg/batch

For more practical, production of lobster extracts with the larger scales (level B and C) utilising up to 52.2 and 74 % of the Australian LPBs was also analysed for further evaluation and comparison (Table 7.16). The growth of Australian lobster processing industry could lead to a significant increase in amount of LPBs to be utilised while both social and economic benefits of these lobster-extract projects could encourage for extending their production scales. In these cases, collecting

LPBs from other main lobster processors such as Waterman's Lobster Co. Potts Point, Australian Bay Lobster producers or Indian Ocean Rock Lobster located in other regions would be necessary. A number of costs such as storage, refrigeration, and transportation could become more significant thus the simulations were also carried out with an assumption that the cost for LPBs is 1,000 USD per ton.

Table 7.16 Economic feasibility of lobster extract production in different scenarios of by-product availability and costs

By-products	Material cost (USD/kg)	Scale* (kg/batch)		TCI* (USD)	AOC* (USD)	Revenue* (USD)	NPV* (USD)	GM (%)	ROI (%)	Payback time (Years)
Lobster livers	0	A	200	3.379	3.289	5.219	5.777	36.98	42.65	2.34
	0	B	250	3.919	3.389	6.381	9.535	46.88	54.28	1.84
	0	C	300	4.412	3.482	7.544	13.975	53.85	63.78	1.57
	1	A	200	3.402	3.539	5.219	4.608	32.19	37.96	2.63
	1	B	250	3.947	3.702	6.381	8.176	41.99	49.15	2.03
	1	C	300	4.446	3.856	7.544	12.345	48.88	52.24	1.72
Lobster heads	0	A	200	6.418	3.839	8.234	15.193	53.38	49.79	2.01
	0	B	400	11.010	5.310	16.463	42.729	67.74	69.57	1.44
	0	C	600	13.584	6.418	24.688	72.504	74.01	89.50	1.12
	1	A	200	6.458	4.281	8.234	13.270	48.01	45.37	2.20
	1	B	400	11.090	6.194	16.463	38.883	62.38	64.29	1.56
	1	C	600	13.704	7.743	24.688	66.735	68.64	82.91	1.21
Lobster shells	0	A	150	17.414	10.114	20.871	37.665	51.54	45.77	2.18
	0	B	250	22.872	13.380	34.784	81.637	61.53	64.86	1.54
	0	C	350	27.559	16.541	48.739	126.502	66.06	78.80	1.27
	1	A	150	17.435	10.336	20.871	36.697	50.48	44.96	2.22
	1	B	250	22.905	13.751	34.784	80.023	60.47	63.79	1.57
	1	C	350	27.606	17.060	48.739	124.243	65.00	77.53	1.29

*In million USD unit

Although production of lobster extracts at the larger scales (level B and C) required more TCI (16 - 30.6 %, 71.5 - 111.7 %, and 31.3 - 58.3 % for livers, heads, and shells, respectively) with the higher AOC in all most cases, increasing in their revenues due to up scaling was more significant than those of TCI and AOC (Table 7.16). As a result, their economic indicators, particularly the NPVs, rose remarkably (65 – 141.9 %, 181.2 – 377.2 %, and 116.7 - 235.9 % for livers, heads, and shells, respectively) while there were dramatical decreases in their payback times (2.34 to 1.84 and 1.57 years, 2.01 to 1.44 and 1.12 years, and 2.18 to 1.54 and 1.27 years for lobster livers, heads, and shells, respectively). All these significant results indicate that production at the B or C scale would be far more profitable than that of the A scale. Although the cost of LPBs with 1,000 USD/ton was applied for these processes, changes in their economic feasibility were insignificant indicated by the highly positive NPVs and short payback time. Even though the material cost applied for the limited condition with only 30 % of the LPB availability (the A scale), their NPVs were 4.608, 13.270, and 36.970 million USD for livers, heads, and shells, respectively while the payback times were not over three years (2.66, 2.20, and 2.18 years for lobster livers, heads, and shells, respectively).

7.5 Conclusion

Production of functional and nutraceutical extracts from Australian LPBs is not only promising at the laboratory scale but also feasible for up scaling to the industrial levels determined by the high NPVs and ROIs. This could be a result of the use of emerging technologies (SC-CO_2, ultrasound, and microwave) with the high efficiency for cost-effective production and high-quality products for highly commercial value, which significantly contributed to the high revenues but low AOCs of these processes. All the large-scale production of lobster extracts from Australian LPBs could generate profits but the use of lobster shells for production of extract by the integrated process was found to be the most financially interesting since it had the highest NPV at all scales (37.665, 81.637, and 124.243 million USD for scales of 30, 52.2, and 74 %, respectively). However, the TCI required for the integrated process was higher than that for other processes (lipids or LHP processes). Production of lobster extracts at the large scale was more profitable than the small scales indicated by a higher gross margin and a shorter payback time. These projects were still financially attractive as the cost of LPBs with 1,000 USD/ton was applied for the production. These projects were not only financially interesting but also low risk because they were still feasible with the worse scenario with only 30 % of LPB availability for utilisation. Therefore, investing on these projects not only obtains economic benefits but also contribute to solve processing waste problems.

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CHAPTER 8 KEY FINDINGS AND FUTURE DIRECTIONS

8.1 Key findings

The significant findings obtaining in this study provide necessary knowledge and technology for better utilisation of LPBs by economically and efficiently transforming these biomaterials into functional and bioactive compounds for highly added-value products. Applying these significant knowledge and technology for the industrial production of these functional and nutraceutical lobster extracts from Australian LPBs was found to be feasible. By such efficient utilisation of LPBs, the financial and environmental burdens of Australian lobster processors related to processing wastes are not only solved but several other economic and social benefits are also generated such as generating economic profits, better utilisation of LPBs for sustainable development, producing more food for the increasing global demand, diversifying lobster products, and creating more jobs. Moreover, these significant knowledge and technology could also be applied for other seafood processing by-products for better utilisation and reducing the processing waste problems. The key findings of this studied are summerised as follows:

8.1.1 Australian LPBs as valuable biomaterials for recovery of functional and bioactive ingredients

The goal of this study was to recover high-value functional and bioactive compounds from the underutilised resource, Australian LPBs. For this goal, the composition of three different Australian LPBs (lobster livers, lobster heads, and CGLS) was determined for identification their functional and nutraceutical components. Australian LPBs were found to be rich in proteins (43.5 % in heads, 41.1 % in livers, and 29 % in shells) which could be recovered. Another major component of the LPBs was determined to be minerals (36 % in shells and 31.6 % in heads), of which the dominate component was calcium (84 %). Apart from protein and minerals, chitin also contributed significantly to the composition of the LPBs studied (25 % in shells and 19.8 % in heads). Lipid is the second major component in lobster livers (24.3 %) but further analyses in heavy metal composition showed its Arsenic and Cadmium over the regulated limit for fish products (240, 8.7 mg/kg vs 2, 0.5 mg/kg) while these of other by-products were very low. Overall, it was concluded that Australian LPBs contain several functional and nutraceutical compounds which could be recovered cheaply, value adding to the Australian lobster industry. However, the utilisation of lobster livers should be carried out with attention on their Arsenic and Cadmium contaminants. All

results obtained in this section provide the key foundation for further developing efficient and economic processes for recovery of these valuable lobster components.

8.1.2 Highly efficient and environmentally friendly processed developed for recovery of functional and nutraceutical ingredients from Australian LPBs

8.1.2.1 Recovery of high quality lipids from lobster livers using the SC-CO_2 extraction (the first paper)

By analysis in chemical approximate and heavy metals it was determined that lobster livers are valuable materials for lipid recovery but the levels of Arsenic and Cadmium contaminants in this material pose a significant challenge for process development. Interestingly, the process developed in this study using the SC-CO_2 technique performed as an excellent process for lipid recovery from lobster livers in terms of both quality and extraction yield. Particularly, inorganic As and Cd contaminants in the SC-CO_2 extracted lipids were very low (0.05 and under 0.01 mg/kg), which meets Australian New Zealand Food Standard. As compared with the Soxhlet-extracted lipids, Cd content in the SC-CO_2 lipids was twenty-seven fold lower (0.01 vs 0.27 mg/kg) while this significance for total As was fourteen fold (2.2 vs 31 mg/kg). Moreover, high quality lobster lipids were also recovered with the high yield (94 %) at the optimised conditions of 35 MPa, 50 ^{o}C, and 4 hours. The extracted lipids contained approximately 31.3 % PUFAs in which ω-3 fatty acids (DHA and EPA) accounted for 58 %. PUFAs of the SC-CO_2 extracted lipids were fourfold (31.3 vs 7.8 %) higher than those of the Soxhlet-extracted lipids, whereas this significance for ω-3 fatty acids were six fold (18 vs 3.1 %). In addition, quality of the SC-CO_2 extracted lipids were significantly fortified by their enriching in β-carotene (70.4 μg/mL) and astaxanthin (41.6 μg/mL) due to widely commercial applications of these carotenoids. The knowledge gained from this section is necessary for the establishment of industrial production of lobster lipids in the future while it provides effective solutions for extraction of high quality products from the challenged biomaterials.

8.1.2.2 Recovery of nutritional proteins from lobster heads by ultrasonic extraction

To recover high quantity and quality proteins from lobster heads ultrasonic extraction was used, which performed as a highly efficient method for extraction of nutritional protein from lobster heads. The ultrasonic extraction was carried out as same manner as the cold-based extraction, which replaced stirring by sonicating. Although the experimental temperatures were the same, the yield of protein extracted by ultrasound was significantly higher that of the cold-based extraction (90.5 vs 63.2 %). While the protein yield of ultrasonic extraction was equivalent or even better than that of

the heat-based extraction (90.5 vs 85.1 %), the proteins recovered using ultrasonic extraction were of higher quality compared to the heat-extracted protein (high protein content 83.2 vs 78.6 %, rich EAAs 38 vs 30.4 %, and more digestibility 78.4 vs 58.4 %). It was also determined that the yield of protein extracted was improved by using the high water ratio (the EER achieved was 99 % in 5 minutes with a water ratio of 8 mL/g) while chitosan could be used for improvement protein precipitation with a dose of 250 mg/L (the PPR increased slightly from 82.3 to 86.6 %).

8.1.2.3 Recovery of functional and nutritional protein, chitin, and minerals from lobster shells using microwave intensification (the second and third papers)

The aim of this study was to develop an efficient and environmentally friendly process for the recovery of multiple high-value components in lobster shells. The lobster shell alone contributes to 35 % of the starting mass of lobsters, and is often discarded as waste in lobster processing. One of the limitations to reusing lobster shells is its recalcitrant nature. Lobster shells are composed of proteins, chitin, mineral, and pigments which are tightly associated together, creating a strong and hard structure for decomposition (deproteinisation, demineralisation, and depigmentation) during chitin production. While the chemical processes have several disadvantages (environmentally unfriendly, not allow to recover other value-added components, and degrading chitin quality), the use of bioprocesses alone (by fermentation or commercial enzymes) are the prolonged (8 – 72 hours) and incomplete processes (degrees of decomposition not high).

Almost complete deproteinisation of the lobster shell was achieved using microwave-intensification combined with commercial enzymes at moderate conditions (Alcalase enzyme to shells ratio 1 %, 55 ^{o}C, and pH 8.0). The use of the microwave for deproteinisation of lobster shell proved to be more efficient compared to using conventional methods in terms of the DD, (85.8 vs 58.03 %), the weight loss (30.3 vs 24.6 %), and the amount of residual protein in the EDPS (65.4 vs 96.4 mg/g). Moreover, these protein extracts were found to have excellent functionalities (solubility over 85 %, high water binding in the meat matrix) and nutritional quality (rich in protein 74 % and EAAs 34 %, low lysine/arginine ratio (0.7)). These high levels of EEAs observed meet the daily human requirements suggested by WHO/FAO, and therefore could be marketed as lobster protein powders (LPPs). It was determined that the removal of the majority of the proteins from the shell by using microwave irradiation facilitated the extraction of additional compounds. For example, by removing the proteins from the lobster shell minerals and chitin was more easily recovered from the residual low-protein shell fraction.

Demineralisation of deproteinised shells using lactic acid intensified by microwave irradiation with optimisation in this study obtained several significant advantages. High DD (99.2 %) achieved at a mild condition (LA/S ratio of 20 mL/g, 100 ^{o}C, and 23 minutes). Significance of this result over the conventional method was indicated by using far less chemical (20 vs 50 mL/g) and shorter time (23 vs 60 minutes) but generating chitin with considerably lower mineral residues (0.99 vs 3.7 %). Although the process was carried out on the harder material (lobster shells vs shrimp shells) at a milder condition (20 mL/g, 100 ^{o}C, and 23 minutes vs 200 mL/g, 121 ^{o}C, and 30 minutes), the mineral residues in the extracted chitin was comparable to that of chitin demineralised by kitchen microwave without optimisation (0.99 vs 0.22 %). Chitin demineralised by the microwave-intensified lactic acid had tenfold lower mineral residues than chitin demineralised at the same condition by the stirring method (0.99 vs 10 %). Moreover, chitin produced by the microwave process was well maintained its physicochemical property determined by high DA (89.9 vs 76.8 %) while it had the chemical structure similar to the high quality commercial chitin characterised by the FTIR technique.

Interestingly, the fat binding capacity of the-microwave-produced chitin was several fold higher than that of commercial fat blockers (4.3 - 10 fold depending on type of oils), chitosan (1.9 - 2.6 fold depending on type of oils), and high quality commercial chitin (1.1 - 2.5 fold for all tested oils with an exception for canola oil). Furthermore, cholesterol binding of the-microwave-produced chitin was as high as commercial ingredients (chitin, chitosan, and fat blocker) with the amount of 258.7 mg/g compared with 255.8, 270.6, and 264.9 mg/g respectively. These findings are of high commercial interest due to their potential applications for weight loss and cholesterol management products.

Apart from its high efficiency, cost saving, and generating high quality chitin, the microwave-intensified lactic acid process also allowed to recover lobster minerals with a calcium-rich profile (calcium accounting for up to 87 %) which is potentially used as a source of calcium. As compared with a commercial calcium supplement complex, lobster minerals were far more soluble (98.4 vs 18.6 %) while the solubility of lobster calcium was sevenfold higher than that of its counterpart (60.0 vs 8.3 %). Moreover, lobster calcium was found to have 5.9-fold higher solubility than that of commercial calcium complex (11.3 vs 1.9 %). These findings make lobster mineral become more interesting for its commercial applications as food ingredients or a dietary calcium supplement.

The significant results found in this section show the benefits of using microwave energy for intensifying the enzymatic or organic extraction of high-value compounds from samples, which are not easily amendable. This not only provides a novel approach for economic and environmentally friendly recovery of valuable components from crustacean shells but also generates the extracts with high quality and good functional properties.

8.1.4 Economic feasibility for the industrial production of functional and nutraceutical lobster extracts from Australian LPBs

The results achieved at the bench-scale for the extraction of functional and bioactive extracts from Australian LPBs was concluded to be promising. However, economic analyses were required before conclusions could be made on the viability and profitability of these processes at an industrial scale. Using the SuperPro Designer for simulating the large-scale production of lobster extracts (lipids, protein, minerals, and chitin) from all LPBs (livers, heads, and shells) was found to be economically feasible but production of lobster shell extracts by the integrated process was the most financially interesting at all scales (30, 52.2, and 74 % of Australian LPB availability). However, the total capital investment (TCI) required for lobster extract production by the integrated process from lobster shells was higher than from other LPBs (livers or heads) at all scales (30, 52.2, and 74 % of Australian LPB availability). Production of lobster extracts with the large scale (using 74 % of LPBs) was more feasible than the small scale (using only 30 or 52.2 % of LPBs) indicated by the significant increases in revenues and profitability compared with the rise in its TCI and operation costs. Although the cost of LPBs with 1,000 USD/ton was applied for these processes, their profitability were still high. Even though the cost of all LPBs used for the worse scenario with only 30 % LPB availability, changes in their economic feasibility were insignificant. These significant findings provide vital information about the technical and economic attractiveness aside from providing the necessary support for up scaling these processes in the future.

8.2 Future directions

8.2.1 Utilisation of protein-rich residues generated from the SC-CO_2 extraction for protein and peptides mining

Proteins derived from crustacean have been evaluated as a luxury protein because of their high delicacy, palatability, and rich in EAAs (Tou, Jaczynski, & Chen, 2007; Venugopal, 2009). Particularly, proteins recovered from LPBs (lobster heads, lobster shells) were demonstrated with high nutritional quality and excellent functional properties (wettability, high solubility, foaming,

and emulsification (He, Nguyen, Zhang, & Peng, 2016; Nguyen, Zhang, Barber, Su, & He, 2016; Oviedo, Garcia, Mendez, & Henriques, 1982; G. H. Vieira, Martin, Saker-Sampaiao, Omar, & Goncalves, 1995). In addition, protein hydrolysate or peptides derived from lobster have exhibited some specific bioactivities which could be used for nutraceutical or pharmaceutical products such as antibacterial (Christie et al., 2007), antimicrobial (Battison, Summerfield, & Patrzykat, 2008), insulin-like and insulin binding protein (Chandler et al., 2015). The residue generated from the SC-CO_2 extraction contains up to 54 % protein, which could be an excellent source for protein or peptides mining but it was found to be contaminated with Arsenic (328 mg/Kg) and Cadmium (14.7 mg/Kg). Removal of heavy metal from this biomaterial using chitin, chitosan in combination with ultrasound for protein recovery will be the future trend since chitin, chitosan were successfully used for heavy metal removal (Mohanasrinivasan et al., 2014; F.-C. Wu, Tseng, & Juang, 2010) while ultrasound was used for intensified the removal process (Dennis & Krishnan, 2004; Hristozov et al., 2004).

8.2.2 Developing more industrially applicable process for chitin production

Although the microwave-intensified process for chitin production from lobster shells was shown to be promising, the long processing time (over 12.4 hours for the scale-up process) significantly affect its industrial applicability due to its high operation costs. The process could be mainstreamed and simplified by combining the deproteinisation with demineralisation into one-step. A recent study of Baron et al. (2015) showed that demineralisation using weak acids coupled with deproteinisation using an ASP enzyme yielded high rates of demineralisation (99 %) and deproteinisation (95 %). Using other green and sustainable solvents such as CO_2 (Ramírez et al., 2015) or Gluconic acid (Juan et al., 2014) is another approach which should be investigated. This is because the mild conditions used in these processes could sustainably produce chitin with interesting functionalities and bioactivities.

8.2.3 Further investigating on pharmaceutical applications or clinical trials for these lobster extracts

8.2.3.1 LSPH potentially containing bioactive ingredients for nutraceutical or pharmaceutical applications

Protein hydrolysates derived from fish have been extensively reported possessing several bioactivities for numerous nutraceutical or pharmaceutical products (Chalamaiah, Dinesh kumar, Hemalatha, & Jyothirmayi, 2012; Elias, Kellerby, & Decker, 2008; Gómez-Estaca, Calvo, Sánchez-

Faure, Montero, & Gómez-Guillén, 2015; Mbatia, Ogonda, Muge, & Mulaa, 2014). Fractionating biological peptides from fish protein hydrolysates for potential use in major health issues was predicted with very high potentials (Vijaykrishnaraj & Prabhasankar, 2015) while several peptides derived from crustaceans have exhibited stimulating cholecystokinin release (Cudennec, Ravallec-Ple, Courois, & Fouchereau-Peron, 2008), ACE-inhibitory (Amado, González, Murado, & Vázquez, 2015), antioxidant (M. A. Vieira, Oliveira, & Kurozawa, 2016). Particularly, carotenoprotein, a complexes of protein and astaxanthin, usually occurs up to 16 % in lobster shells (Tu et al., 1991). Hydrolysed fractions of carotenoprotein demonstrated to possess several interesting bioactivities (Armenta & Guerrero-Legarreta, 2009; Senphan, Benjakul, & Kishimura, 2014; Sila, Nasri, & Bougatef, 2012; M. A. Vieira et al., 2016). LSPH contains fragmented proteins and peptides of shell protein with the MW around 10 KDa could be a source of bioactive peptides and carotenoids for nutraceutical or pharmaceutical applications. This would be the future study since applications of hydrolysed proteins for nutraceutical and pharmaceutical industries has become a great interest (Cheung, Ng, & Wong, 2015).

8.2.3.2 Lobster minerals enriching in calcium with high solubility and bioavailability potentially applied for prevention or treatment of osteoporosis

In many cultures, but especially Asian, seafood based products are the main source of calcium in the diet. Several studies on fish bone for dietary calcium supplement have found promising results in both vitro and vivo tests such as highly bioavailable (Luu & Nguyen, 2009; Malde, Graff, et al., 2010), well absorbed in human (Malde, Bügel, et al., 2010), high efficiency on human bone mineralisation without adverse effects (Flammini et al., 2016). However, the exploration on the use of fish bone calcium for prevention or treatment of osteoporosis has not been carried out. Lobster minerals have a calcium-rich profile with high solubility and bioavailability, thus further investigation on efficiency of lobster calcium on human bone mineralisation or the use of lobster calcium for prevention osteoporosis is required.

8.2.3.3 Lobster lipids enriching in omega-3, astaxanthin, and β-carotene for nutraceutical or pharmaceutical applications

PUFAs and long chain omega-3 fatty acids have been extensively studied with several bioactivities including anti-aging, anti-inflammation, anti-cholesterol, anticancer, cardiovascular prevention for biomedical or pharmaceutical applications (Gill & Valivety, 1997; La Guardia et al., 2005; Russo, 2009; Sahena et al., 2009). Moreover, carotenoids, particularly astaxanthin, possess several biological activities with well-known health benefits such as super antioxidant effects, anti-lipid

peroxidation, anti-inflammation, anti-diabetic activity, cardiovascular disease prevention, anticancer activity, and immuno-modulation (Ambati, Phang, Ravi, & Aswathanarayana, 2014; Higuera-Ciapara, Felix-Valenzuela, & Goycoolea, 2006; S. Wu et al., 2012; Yamashita, 2013). Recently, shrimp lipids were demonstrated as a source of cancer chemopreventive compounds due to their enriching in bioactive ingredients (Lopez-Saiz, 2013). Therefore, nutraceutical or pharmaceutical applications of lobster lipids should be further investigated since this lobster extract was found to enrich in PUFAs, omega-3, β-carotene, and astaxanthin with several known nutraceuticals and pharmaceutical applications.

8.2.3.4 Lobster chitin demonstrated with multiple biological activities for various applications

In previous studies, chitin and chitin derivatives from lobster were shown to have several biological properties such as antifungal, antibacteria, biostimulant, plant-growth promotion for seedling growth, plant growth regulator, crop protection and plant disease management (Hipulan, 2005; Iglesias, Gutierrez, & Fernandez, 1994; Ilangumaran, 2014; Pombo, 1995); antioxidant for food products (Garcıa et al., 2015); great healing for surgical materials (Hareyan, 2007); antihalitosis for bio-mouth spray (Safitri, Fajriah, Astriandari, & Kartika, 2014); antimicrobial and anti-proliferative activities against the colon cancer (Sayari et al., 2016). Lobster chitin obtained by the microwave-intensified process had high purity, good physicochemical and functional properties but its biological activities for regulating immune responses have not been investigated. Thus chitin supplementation reducing inflammation would be a further study of lobster chitin.

8.2.4 Production of chitin derivatives for the extended commercial applications

Chitin possesses several interesting functionalities and bioactivities for many applications but insolubility of chitin has significantly limited its applications. Production of chitin or chitin derivatives with the improved solubility have drawn much attention. Modifying chitin for obtaining chitin derivatives with the improved solubility would be the perspective trends because these processes could be easily conducted using physical, chemical or biological methods (Kurita, 1986). Generation of chitin with small particle sizes (micro-particles, nanofibers, nanowhisker, and nanofibril) could be one of the most potential approaches for chitin modification due to the simplicity of process and several significant advantages of chitin particles. Chitin particles could be easily and directly produced from chitin samples by the simple and efficient processes to obtain homogeneous chitin particles in large amounts (Ifuku et al., 2011; Ifuku et al., 2010; Ifuku, Yamada, Morimoto, & Saimoto, 2012). By significantly improving in dispersity, functionalities,

and bioactivies, chitin particles have applications in several industries: biomaterial production (Mushi, Utsel, & Berglund, 2014; Qi, 2015; J. Wu, 2014), food industry (M.-C. Li et al., 2016; Mackenzie, 2011), cosmetic (Pierfrancesco Morganti, 2010; Saumya Choudhary et al., 2015), pharmacy (P Morganti, Ciotto, & Gao, 2012; Pierfrancesco Morganti et al., 2014), medicine (Ghotloo et al., 2015; Ozdemir et al., 2006; Strong, 2013), and tissue engineering (Hassanzadeh et al., 2013).

Preparation of water-soluble chitin derivatives (chitosan, chito-oligosaccharides (COS)) by deacetylation or degradation of chitin intensified by microwave would be another area of research that should be investigated due to both the extended applications of water-soluble chitin derivatives and significant advantages of microwave energy. Chitosan and COS have numerous applications in water treatment, agriculture, food, nutraceutical, pharmaceutical, biomedicine, tissue engineering (Aam, Heggset, Norberg, Sorlie, & Varum, 2010; Je & Kim, 2012; Thomas, Venkatesan, Manivasagan, & Kim, 2015; Xia, Liu, Zhang, & Chen, 2011). Although, chitosan and COS have been traditionally obtained by conventional methods, the significant advantages of microwave over conventional heating (low chemical consumption, short extraction time, high efficiency in mass and heat transferring) reveals potentials of this technology for these chemical and biological processes (K. Li et al., 2012; Sahu, Goswami, & Bora, 2009; Wasikiewicz & Yeates, 2013).

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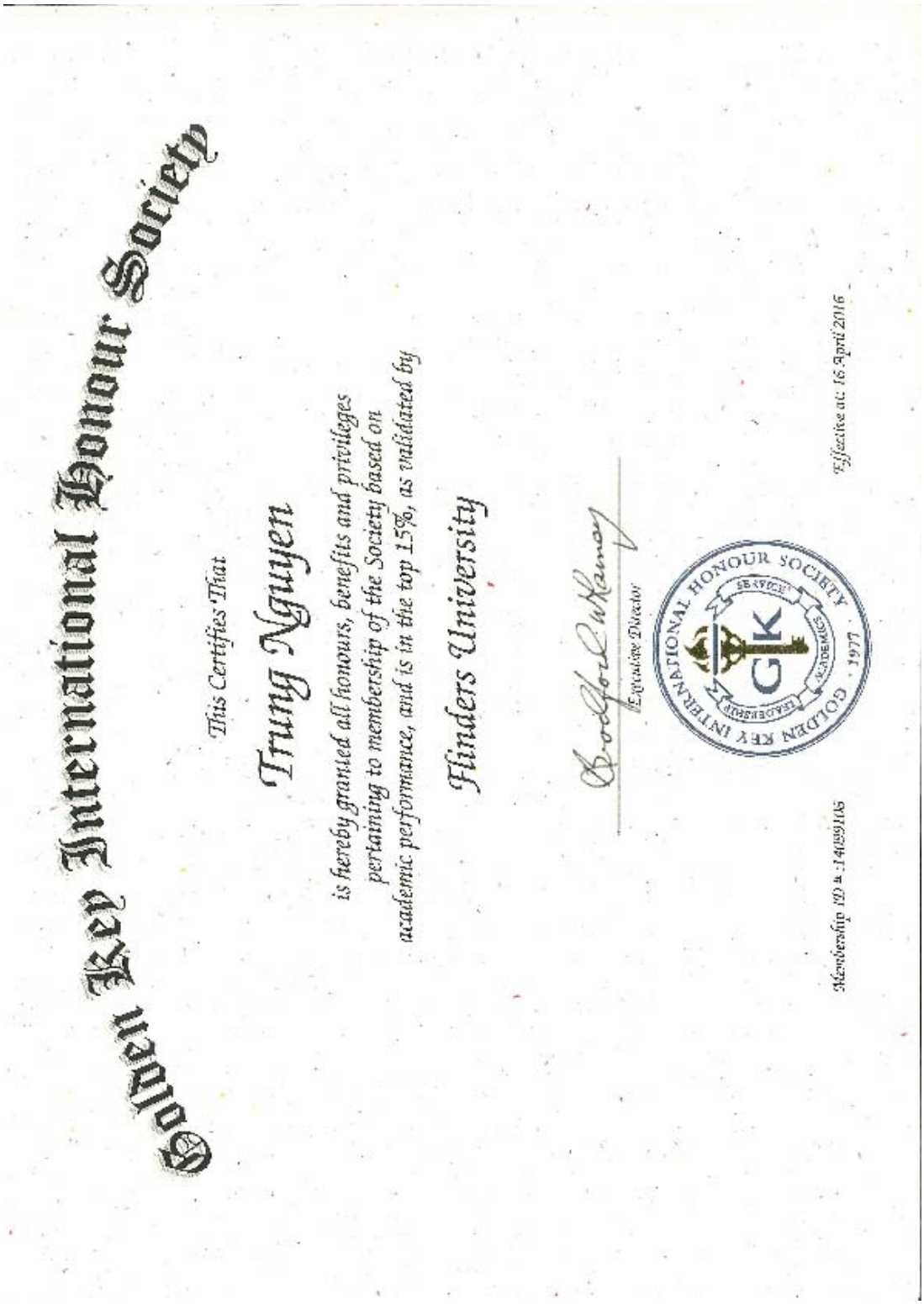
Appendices

1. Award from Golden Key International Honour Society



2. Midas award from head department of Flinders Medical Biotechnology