Extraction, Characterisation and Formulation of Natural Blue Pigment from Black-Footed Abalone (*Haliotis iris*)

Soniya Mohammadi

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Abstract

An ubiquitous and important factor in everyday life is the colour which invigorates substances. Most pigments used in food and cosmetics are made synthetically, and nearly all blue pigments available commercially are synthetic. This thesis aims to extract natural blue pigments from abalone (*Haliotis iris*) shells to provide as an alternative colour to synthetic pigments.

In this project, blue pigment was extracted from the shell of abalone by a solvent extraction technique and purified by solid phase extraction, which yielded 500 mg of pigment from 5 kg abalone shell. The cytotoxicity of the extracted blue pigment was tested for food and cosmetics applicability by using a Cell Counting Kit-8 (CCK8) *in vitro test*, and the minimum lethal dose was determined to be about 5000 µg/ml. Stability of the aquatic blue pigment was tested by exposing the pigment to different light regimes (Indoor light, UV light and sunlight), extreme temperatures (25, 50, 75 and 100°C), different pH values (2, 4, 6, 8, 10 and 12), oxidising agents (H_2O_2), reducing agents (Na_2SO_3), food additives (starch, citric acid, sodium tartrate, glucose anhydrous, sucrose and sodium chloride) and metal ions (Al^{3+}, Ca^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, K^{+}, Mg^{2+}, Zn^{2+}). It was found that, the aquatic blue pigment was unstable when exposed to the lights (Indoor light, UV light and sunlight) and high temperature (100°C), as well as in the presence of reducing agents and some metal ions (Cu^{2+} and Fe^{2+}). Microencapsulation was then used to enhance the stability of the pigment. Different formulations consisting of Gum Arabic, maltodextrin DE 11-12 and DE 17-19 mixtures as wall material were used to retain the colour intensity of the pigment. Particle morphology of encapsulated pigment was characterised by scanning electron microscopy. All particles obtained were spherical and smooth with different sizes. However, the smallest particle belonged to formulations containing Gum Arabic. In addition, formulations containing Gum Arabic had higher stability in comparison with other formulations, which did not have Gum Arabic. The extracted aquatic blue pigment can have potential applications in food and cosmetics. To demonstrate its use, hand creams and lipsticks containing the pigment were formulated.

In conclusion, aquatic blue pigment was successfully extracted from black-footed abalone (*Haliotis iris*) shells and formulated for use in cosmetics. The stability of the pigment, when exposed to various environmental conditions was significantly improved after microencapsulation. While the results from this project present a promising stepping-stone for developing a commercial natural aquamarine pigment, further studies are required to increase the extraction yield to make the process commercially viable.

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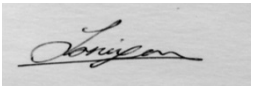
Attestation of Authorship

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

Signed: 

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Thesis aims and structure

Adding blue colour to food and cosmetics has been associated with the use of synthetic dyes, which are not popular and can cause allergies and other harmful effects in some users. Developing a natural blue colour that can be made at industrial scales can provide a better alternative to brilliant blue and indigotine, which are the only synthetic dyes approved by the US Food and Drug Administration (FDA). Having a strong paua aquaculture industry in New Zealand where paua shells are by-products that are otherwise discarded, has provided the opportunity to conduct this research. This aligns with New Zealand government's bio-prospecting framework (https://www.beehive.govt.nz/release/ bioprospecting-questions-and-answers). Therefore, the aim of this thesis is to extract a natural blue pigment from paua shells for applications in food and cosmetic products. The specific objectives of this thesis are to extract, separate and purify aquatic blue pigment from paua shells, investigate the chemistry, safety and solubility of the pigment, the stability of the pigment in various conditions, microencapsulate the pigment and prepare conceptual cosmetic products with the pigment to illustrate its suitability for industrial applications. This thesis consists of 5 chapters. **Chapter 1** 'Introduction and literature review' provides background historical information about pigment use, colour development, different types of colours, chemistry of blue colour, extraction procedures used for different pigments, methods of purification and microencapsulation of pigments, molluscans shell structure and general information about paua. **Chapter 2** 'Extraction, separation, purification and chemistry of aquatic blue pigment from paua, how the blue pigment was extracted from paua shells and purified using a solvent extraction method. Different analytic techniques were used to verify the purity of the pigment. **Chapter 3** 'Evaluation of stability and safety of aquatic blue pigment from paua consisting of 2 parts. The first section discusses the pigment stability in response to different environmental and chemical conditions, such as the presence of oxidants, reducers, food additives, metal ions, exposure to elevated temperatures, exposure to dark and different sources of light (indoor light, UV light and sun light) and in solution with various pH values(2, 4, 6, 8, 10 and 12). In the second section, an *in vitro* toxicity test is discussed to provide an insight into the safety of the pigment.

Chapter 4 is titled 'Microencapsulation and formulation of aquatic blue pigment from paua.' In my literature review and preliminary experiments, I found that the pigment might be unstable when exposed to the indoor light, UV light and sunlight as well as temperatures(100 °C). Therefore, to improve the stability, the pigment was protected and sheltered from the harsh environment. This objective achieved through microencapsulation technology that is explained in this chapter. Furthermore, in this chapter, conceptual cosmetic products are developed using the encapsulated pigment to illustrate its application in pharma- and nutraceutical industries. Discussion of the results and a conclusion is presented in **Chapter 5.** The final chapter of this thesis discusses overall outcomes of this project and how the aquatic blue pigment can be introduced as a natural alternative to synthetic blue pigments used in pharma- and nutraceutical industries. Finally, a conclusion is drawn and the limitations of this thesis along with its future potential are discussed.

Chapter 1. Introduction and literature review

1.1. Introduction

Looking at the surrounding environment, one can easily realise how vital colour (pigment) is in our lives. Colour is so significant in most of our foods and other products that increase demand and approval of the food in the industry (Babolhavaeji, 2015). Therefore, foods, pharmaceuticals and other products that are colourless or have unattractive natural colours require more colours. Some companies use colorants in their products to attract specific age groups such as children to increase their product sales. Natural and synthetic colours have been used to enhance products' appearance to be acceptable by consumers (Gultekin & Doguc, 2013). The reason for using synthetic dyes is that they are easily produce, inexpensive, and highly stable (Liamas et al., 2009).

1.1.1. History of pigment extraction and synthesis

In the past, natural organic pigments were obtained from plant sources (e.g., berries, fruit skin, seeds, roots and trees) and animal sources (e.g., crushed molluscs and insects). Purple colour, for example, was extracted from the glands of shellfish. Natural red colourants were extracted from animals (e.g., lac insect and cochineal) or vegetables (e.g., madder). Leuteolin, flavonoids and apigenin were yellow pigments derived from flowering plants. Haematite and ochre were coloured minerals responsible for the brown, red and yellow colours used in paintings. Around 1000 BC, the Egyptians developed synthetic inorganic pigments (e.g. Alexandria blue/Egyptian blue from a mixture of silicate of copper and calcium) (Christie, 2015). In the early history of humans, most natural dyes that were used for textile fabrics were unstable when exposed to light and with washing. However, particular dyes had better quality through using iron, tin, aluminium, copper or chromium as a fixing agent (mordents). Mordents were insoluble in water, which meant that they could be washed without leaching pigments. Natural pigments were unstable as well as being difficult and expensive to produce. Hence, in the 1800s, synthetic dyes were discovered and produced in commercial quantities. Synthetic dyes were

easy to produce and apply, providing good quality colour, cost effective and more versatile than natural dyes. The first synthetic dye produced was picric acid, which was derived from treating indigo with nitric acid in 1771. Picric acid was mostly used as an explosive, but in its wet form also was used as a skin dye. In 1856, synthetic purple dye (Aniline purple/ Mauveine) was discovered by Sir William Henry Perkin. Initially, he was planning to develop an antimalarial drug by synthesising quinine from the bark of cinchona (south American tree). However, the product was expensive to produce. During that time, he developed a black precipitate from reactions of aniline with potassium dichromate as an oxidising agent. He found that the purple dye could be extracted by dissolving the black precipitate in organic solvents (ethanol and water) (Gordon & Gregory, 2012). In fact, Mauveine was obtained from an impurity in aniline that is called toluidine, which contained organic compounds. It was a desirable colour in the fashion houses of Paris and London (Christie, 2015).

Azo dyes are synthetic colours, which have vivid colours (red, orange and yellow) due to the delocalisation of electrons within their structure. The first azo dye, 4-aminoazobenzene (brown), was produced from aromatic amine and nitrous acid that had a poor quality. However, in 1861, better quality azo dyes were obtained from *m*-phenylene diamine. In 1876, chrysoidine (the first orange azo dye in the market) was extracted from a reaction between *m*-phenylenediamine and diazotized aniline. Also, the reaction between diazotized sulfanilic acid, respectively, with 1-naphthol, 2-naphthol, *N*,*N*-dimethylaniline and diphenylamine gave a range of orange colours (I, II, III and IV). Intense and light colours could be obtained from lakes by precipitation of Lake Colourant to an inorganic mineral substrate. This method is applicable for water soluble synthetic dyes. After precipitation into inorganic minerals, for instance, barium sulphate and alumina, anionic dyes became insoluble in water. These cationic dyes were treated with antimony potassium tartrate or tannin to produce soluble pigments. After this process red and yellow azo pigments (β-naphthol red and Hansa yellow) were discovered and marketed (Christie, 2015).

During the 20th century, phthalocyanines (green and blue pigment) were discovered, which was a major discovery. In 1928, chemists in Scotland isolated blue impurities from the reaction of ammonia and phthalic anhydride yields. The phthalocyanine structure was one of the first significant molecules that was elucidated by X-ray. In the same year, a unique blue colour was extracted from copper phthalocyanine with high yields and low cost. During the 20th century, the use of reactive dyeing became a common practice in textile dyeing. Reaction between functional groups of reactive dyes and polymer molecules after dye application led to coloured fibre formation, which was faster than traditional methods. In 1954, a type of dye was discovered that had 1,3,5-triazinyl group (fibre reactive dyes) and was commonly used for cellulosic fibres and others such as nylon, silk and wool (Christie, 2015). In the late 20th century, more pigments and dyes were produced. However, their introduction to the market declined rapidly due to toxicity issues (Christie, 2015).

1.1.2. How colour is perceived by our eye

Photons (beam of light) contain a frequency that is perceived as colours by our eyes. The amount of energy of a photon relies on the specific light (colour) frequency. When lights hit an object, some get absorbed by the objects and the residual light that is not absorbed, is reflected into our eyes as a colour (Bloomfield, 2016). For instance, when blue light is absorbed in carotenoids, red colour is reflected. When red colour is absorbed, the blue colour is perceived that makes the object look blue. Thus, a blue colour results from a substance that absorbs red light at 600 (Figure 1-1) (Nielsen, 2010).

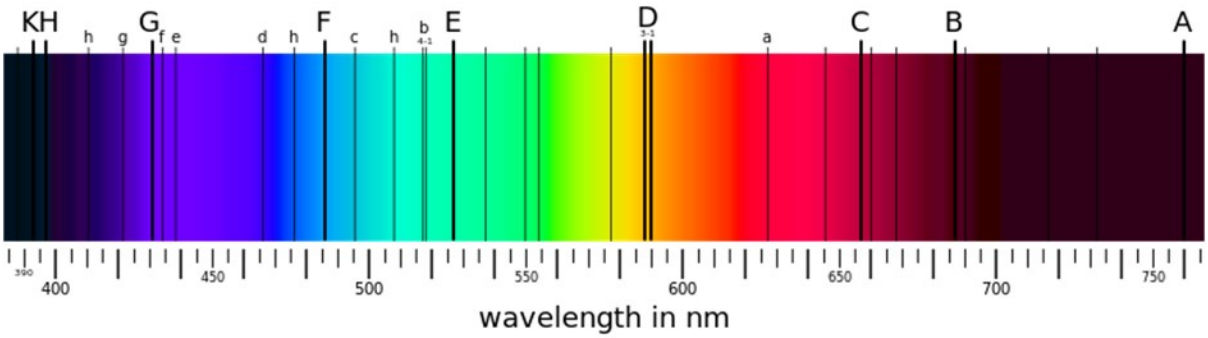

Figure 1-1 Colour perception visible absorbance spectrum (adopted from Number of different wavelengths in the visible spectrum reaching Earth, 2017).

The energy of an absorbed photon is equal to the energy difference between molecular orbitals (e.g. σ, n, or π). The photon is absorbed by an electron, which is moved to a higher energy level. Molecules can have high and low energies. Molecules with lower energy often have π → π* and n → π* energy transitions and can absorb visible light and therefore appear as pigments. π-Bond conjugation can lead to a decrease in the level of energy (highest occupied molecular orbital (HOMO)→ lowest unoccupied molecular orbital (LUMO) for π → π* to result as blue light (Kumar, 2006). Blue pigments normally contain ionic charges, heteroatoms (-Cl, =O, -OH and −NH2), aromatic rings and π-bond conjugations. Auxochromic (heteroatoms) can produce bathochromic shifts by decreasing the transition energy. As an example, blue colour can be formed by interaction between carotenoids and amino acids or the interaction between carotenoids and iodine or transformation of carotenoids to cation (Liaaen-Jensen & Kildahl-Andersen, 2008).

1.1.3. The safety of synthetic colours

Synthetics pigments are widely used these days and their consumption has increased by 500% in the past 50 years. They are cheaper, more stable and are more accessible than natural colours (Panchal, Sapal, Padhiar & Deshmukh, 2015). Nevertheless, synthetic food colorants have been known to cause immune system disorders, problems in children, such as attention-deficit/hyperactivity disorder (ADHD), attention deficit disorder (ADD) and aggression as well as allergies (Arnold, Lofthouse, & Hurt, 2012; Gultekin & Doguc, 2013). The ingestion of food delivers the greatest foreign antigenic load that can challenge the immune system. Another use of artificial colours is in pharmaceutical and cosmetics that can be ingested or absorbed through skin. Molecules of artificial colours are tiny and can bind to body proteins and activate the immune system. Digestion of synthetic colours can lead to inflammatory cascade activation, which can causes intestinal stimulation to antigens, causes neurobehavioral disorders, autoimmunity and cross-reactivity.

Azo dyes are synthetic organic colorants with azo –N=N– groups that have been used widely in toys, plastics, leathers, cosmetics and foods due to low cost and resistance to fading (Ahlstrom, Eskilsson & Bjorklund, 2005). In anaerobic conditions, they change into aromatic amines that have been found to be carcinogenic for human and animals (Ahlstrom, Eskilsson, & Bjorklund, 2005). In 1895, Ludwig Rehn, a German surgeon found that aromatic amines cause urinary bladder cancer in men. Other studies showed that the risk of developing cancer can be increased by aromatic amines (Yu, Skipper, Tannenbaum, Chan, & Ross, 2002). Digestion (food and sucking of toys by children) and skin absorption (use of cosmetics and wearing of azo dyed clothes) are the main exposure to azo compounds. Azo dyes have been associated with at least 20 carcinogenic aromatic amines.

Some food colourants can be hazardous and cause toxicity and carcinogenic effects on human body. Food colourants have been classified into three different varieties: natural, from natural eatable sources, for instance anthocyanins, nature-identicals, that are obtained from synthesis of chemicals (e.g. riboflavin and β-carotene) and artificial pigments that are not derived from nature but created by chemical synthesis (e.g. indigos, chinophthalon derivatives, xanthene, triarylmethane and the azo compounds). On the other hand, a common food colorant, tartrazine, has been reported to cause sleep disturbance in children, as well as restlessness and irritability (Rowe & Rowe, 1994). Kokum red and safflower yellow have been reported to have clastogenic impacts on the bone marrow cells of femora (Agarwal, Mukherjee, & Chakrabati, 1994)

Brilliant Blue and Patent Blue V (Sulphan blue/food blue 5), which are triphenylmethanes, are artificial colours, which have been used in food, cosmetics and pharmaceuticals such as dental staining, lymphangiography and lymph node biopsy). Brilliant blue has been permitted by USA Food and Drug Administration (FDA) and has been used in many developed countries. However, Patent blue is not permitted for food in many countries such as USA, Canada, New Zealand, Australia and Japan. Triphenylmethane dyes have inhibitory effects on mitochondria respiration in human cell. They can cause different health problems, such as, metabolic acidosis, refractory shock and death. Triphenylmethane dyes are used commonly in several products such as facial cleansers, pre and after

shaved products, hair dyes, oral rinses, tooth pastes, shampoos and more (Lucova, Hojerova, Pazourekova, & Klimova, 2013).

Prolonged use of some synthetic colours can result in helath issues, for instance abnormalities in offspring, blindness, mental retardation, damages in liver, spleen, kidney and the brain, tumour, cancer, allergic reaction, asthma, indigestion and anaemia (Ashida, Hashimoto, Tsuji, Kanazawa, & Dannoi, 2000);(Moutinho, Bertges, & Assis, 2007). In a previous study, brilliant blue was tested on rats to determine its toxic effects on different body organs (Mohamed & Abd, 2016). It resulted in decreased number of red blood cells, reduced concentration of haemoglobin and weight loss. It also caused a decrease in the activity of superoxide dismutase and glutathione-*S*-transferase in liver and blood. However, it caused an increase in albumin, protein, creatinine, urea, bilirubin, activity of alkaline phosphatase, aspartate and serumalanine aminotransferase (Mohamed and Abd 2016). Thus, natural compounds are better perceived by the public.

1.2. Natural colours

Natural pigments are present in our environment such as sea, plants and animals. Some pigments, for instance pigments from vegetables and fruits, are naturally presents in our foods and possess antioxidant properties that are beneficial for our health. Colour is an important factor in food and cosmetics. Some food and cosmetics require vivid colours to be more attractive to consumers. Colour can be used in food to judge its quality (freshness) and also enhance the food delight. For instance, when consumers encounter food that has unattractive colour, they suspect that the food is spoiled or has poor quality. Some colours are associated with some foods (e.g. fruits and vegetables), such as strawberry with red colour, banana yellow, carrot orange and celery green. Our psychological and physiological expectation are created by food colours during the time of education, traditions, experiences and also the environment (Dufoss, 2006).

In most plants, anthocyanins are the source of purple, red and blue colours are used in food. Their colours may differ in many conditions, such as the presence of trace metals, solvent, pH, structure,

intra- and intermolecular interactions. Anthocyanidins are polyphenolic compounds with 15 carbons that are one of the most important classes of flavonoid- based plant pigments (Jin & Mumper, 2010).

Quinoids and quinines contain aromatic rings with carbonyl groups. Anthraquinonoid have different colours at different pH. In acidic or neutral conditions they appear orange or red and in alkaline pH they appear blue. They can be derived from different microorganisms such as *Streptomyces peucetius* (Di Marco, A., Gaetani, M., Orezzi, P., Scarpinato, B. M., Silvestrini, R., Soldati & Valentini, 1964). Natural anthraquinonoids are not used commonly in food and beverages because they are not coloured at low pH (Jin & Mumper, 2010).

Linear tetrapyrrole alkaloids are compounds that are responsible for the blue colour in bruises (Frankenberg & Lagarias, 2003). They contain all colours of the visible spectrum. These compounds have also been extracted from blue marine ascidians, marine bacterium and *Propionibacterium shermanii tetanomorphum*. Tetrapyrrole has been used for confectionary pigmentation. However, due to low stability, their use is restricted for other food and beverages. Some indole alkaloids are also blue which have been extracted from berries and woods. These pigments are unstable in extreme pHs and exposure to light. It has been mostly used for textiles (Newsome, Culver, & Breemen, 2014). Pyridine Alkaloids have been extracted from microbial sources, such as deep sea marine microorganism (*Shevanella violacea and Rheinheimera)*; (Kobayashi, Nogi, & Horikoshi, 2007). Nicotine blue, huito juice and Gardenia blue are examples of pyridine alkaloids.

Azulenes are the only blue organic chromophore that do not have heteroatoms. These compounds have been isolated from plant essential oils, fungi and marine Gorgonian species. Azulene compounds are lipophilic with low stability and low molar absorptivity (Wang, Yan, Fu, Parekh, & Yu, 2003). Metalloprotein and organometallics have been found in natural sources. Arthropods and crustaceans contains blue blood instead of red, because they have hemocyanin (oxygen transporting proteins) rather than haemoglobin (Magnus, Ton-that, & Carpenter, 1994). Hemocyanin has a copper active site

for binding to oxygen. Bacteria and plants also contain other blue proteins. However, metalloprotein and organometallics are not ideal blue colours to be used as food colourants. This is due to their low stability in low temperature and low pH.

1.2.1. Pigment extraction

Pigments can be extracted in many different ways. Conventional organic solvent extraction techniques, such as pressurised liquid extraction, percolation, counter-current extraction, maceration (soaking) and Soxhlet extraction are universal techniques for pigment extraction.

These techniques can provide rapid extraction of chemicals. However, they require high amounts of solvents that may cause molecule transformation or denaturation. Addition of hot water to the solvent increases the extraction. However, it may destroy thermolabile molecules. To increase the extraction rate, enzymes such as pectinase, cellulases or xylanases can be used. To avoid oxidation and thermal denaturation of pigments, samples can be freeze dried, frozen or kept in water vapour saturated atmospheres. The pH of the solution must be controlled for the extraction in aqueous solutions to prevent chemical transformation in alkaline or acidic conditions. Sometimes, stability of the pigment can be improved by changing the aqueous phase to solvents, such as *sec*-butanol. Table 1-1 shows a summary of different pigment extraction techniques.

1.2.2. Pigment purification

Pigments are required to be purified and isolated from other impurities (produced during extraction) for use in food and cosmetic industries. There are different techniques for purification of pigments such as adsorption chromatography (column chromatography), ion exchange chromatography partition chromatography, molecular exclusion and solid phase extraction (SPE).

Column chromatography is a method that contains a glass tube with alumina (Al_2O_3) or silica (SiO_2) inside. It is normally used to purify a sample from product mixture. Column chromatography contains stationary (static part) and mobile (moving part) phases. Mobile phase with high polarity are called

reversed phase column chromatography. Compounds with more polarity travel slower than other compounds with lower polarity that results in a slower elution (Levinson, 2001).

Table 1-1 A summary of the extraction of different pigments.

Case study	Pigment	Colour	Extraction procedure	Reference
1	Chlorophyll a and b and β,β-carotene	Green-yellow	Sample was soaked in acetone at 2 different temperatures (20°C and boiling). With the aid of vacuum microwave assisted extraction (VMAE) and Microwave assisted extraction (MAE), the pigment extracted. The extracts were filtered and dried by rotatory evaporator.	(Pasquet et al., 2011)
2	Marennine	Blue-green	Marine diatom *Haslea ostrearia* was frozen with NH_4HCO_3 buffer in liquid nitrogen and crushed. The separated coloured solution was centrifuged, filtered and freeze-dried. Then the extract was purified by ion exchange chromatography.	(Pouvreau et al., 2006)
3	(*3S,3'S*)-*trans*-astaxanthin	Red	Sample was crushed in acetone. The mixture was centrifuged and the extract was saponified with NaOH. The pigment was purified by column chromatography.	(W. Sun et al., 2015)
4	Chlorophyll, carotenoids and flavonoids	Green and yellow	Seaweed was soaked in the mixture of methanol and ethanol for 4hours at 60°C and filtered. Alternatively, the seaweed was soaked in the same solvents for 48 hours without heat, filtered and the extract was dried by rotatory evaporator.	(Renita. A & Davuluri, 2015)
5	Carotenoid	Orange-red	Pigments from shrimps were extracted by soaking in acetone. The extract was homogenised where petroleum ether and water were mixed with the extract. Then, the petroleum ether layer was separated from the solution. The mixture was filtered through anhydrous sodium sulphate and evaporated by rotatory evaporator. The pigment was dried in a vacuum oven.	(Sanchez-camargo et al., 2011)
6	Aquamarine blue pigment	Blue	Samples (shells of abalone) were steeped in acetic acid. After 24 hours the solution was filtered through filter paper	(Cai *et al*., 2011)

			and the crude extract was obtained by ethyl acetate.	
7	Anthocyanin	Red	Strawberries were homogenised and steeped in ethanol. The mixture was filtered and dried by rotatory evaporator.	(F. Sun *et al.*, 2013)

In this technique, the compound is loaded from top through the column that is eluted with gravity or by applying pressure which can be time consuming (Meyers, 2000). Another technique for separation is ion exchange chromatography where the stationary phase contains resin coating with covalently ions (cation/anion) that bonded to it. The ions of opposite charge (in mobile phase) electrostatically attracted to the resin. During passing the mobile phase through the stationary phase electrostatically, some ions are discharged and others bound (Levinson, 2001). Partition chromatography is a purification technique that contains non-volatile liquid as a stationary phase at the surface of the solid in the column. The sample is passed through the column by mobile phase (liquid/gas). Then with highly soluble component, the solute is eluted from the column (Levinson, 2001). Molecular exclusion is another method of separation in which the sample travels through a porous gel in form of liquid or gas. This technique is suitable for large particles as the size of the gel pores is designed for large particles to pass. On the other hand, small particles infuse easily to the gel and pass slowly through the column which can limit the separation of the particles (Levinson, 2001).

The faster alternative is SPE, which separates specific analytes particularly from mobile phase such as liquid, fluid or gas to the solid phase. The extraction of purified pigments can then occur by fluid, liquid or thermal desorption from the solid phase. SPE system is made of disposable cartridges (open syringe barrel/short column) with silica as a sorbent (Poole, 2003). SPE is a promising purification method that uses less solvent and time. It is also a simple and cheaper process than liquid chromatography (Li et al., 2016).

1.2.3. The stability of natural pigments

A key aspect of colour quality is its stability for application in cosmetics and food industries. This is because, the processing and manufacturing of the final product can cause deterioration of the

pigment (Tierno & Galarreta, 2016). Different factors such as oxygen, light, temperature, pH, metal ions, sugar, sulphite salts, ascorbic acids and enzyme activity can affect the pigment stability and lead to fading or colour alterations. For example oxidation and metal ion loss are reported to occur in chlorophylls, hydration and oxidation in anthocyanin and oxidation in carotenoids (Ghidouche, Rey, Michel, & Galaffu, 2013). In a study, absorbance of the aqua marine blue pigment decreased more than 50% after one day of exposure to the sun light. Also, its absorbance decreased by increase in concentration of oxidant and reducer (H_2O_2 and Na_2SO_3) which had changed the pigment chemical structure (Cai *et al.*, 2011). In another example, astaxanthin, which is an unsaturated pigment, can degrade by oxidation and temperature during manufacturing and storage which also produces undesirable aromas and flavours (Higuera-Ciapara, Felix-Valenzuela, Goycoolea, & Argüelles-Monal, 2004). One approach to increase pigment shelf life and its stability is through encapsulation techniques that shield the pigments from exposure to pH, light, temperature and other factors.

1.2.4. Microencapsulation of pigments for increased stability.

The process of microencapsulation entraps biologically active compounds inside a liquid vesicle or solid particles to protect and stabilise the compound and allow control over their release. This technique is inexpensive and a rapidly developing technology (Gouin, 2004). The produced microcapsules can be of different shapes and sizes, which range from sub micrometres to millimetres. The shape and size depends on the type of techniques and materials used. The aims of microencapsulation in the food industry are: (1) shielding the core material from degradation by decreasing its reactivity to the surrounding environment such as light, air, heat and moisture, (2) decreasing the transfer rate/evaporation of core material to the outside environment (3) programming the release of encapsulates at certain time or slowly (4) covering the flavour of core material, (5) achieving uniform distribution of small quantities of encapsulate in the host material and (6) separation of elements in a mixture to prevent interaction (Desai & Park, 2005) . There are many techniques to form capsules, such as phase separation, ionic gelation, encapsulation by rapid

expansion of supercritical fluid (RESS), freeze drying, emulsification and spray drying techniques (Table 1-2). The most widely used technique for microencapsulation in food industries is spray-drying which provides stable and dry products. Spray drying is flexible, economical and produces good quality particles. Wall materials such as maltodextrin, gum acacia and alginate can be used in spray drying. In microencapsulation, materials are mixed and homogenised in different ratios. The mixture is then put into the spray dryer and is atomised. A hot stream contacts the atomised mixture that leads to evaporation of water. Then the microcapsules are collected in a chamber (Desai & Park, 2005).

Table 1-2 A summary of techniques used for selected microencapsulated pigment.

Technique	Procedure	Advantage/limitation	Case study	Core material	Wall material	Reference
Coacervation (phase separation)	a. Formation of oil (lipophilic active)/water emulsion. b. Homogenisation of the emulsion c. Production of immiscible phases with centrifugation spray drying of samples	✓ Small size of spheres × Unstable coacervates × Complex method. × Time consuming × Expensive	1	Carotenoid	Poly (viny1 alcohol) and poly (viny1 alcohol)/poly(D,L-lactic acid) membranes	(Z. M. Sun, Poncelet, Conway, & Neufeld, 1995)
			2	β-Carotene	Whey protein isolate (WPI) and gum acacia	(Jain, Thakur, Ghoshal, Katare, & Shivhare, 2015)
			3	Curcumin	Gelatin and gum Arabic	(Zuanon, Malacrida, & Telis, 2013)
Ionic gelation/ ion-induced gelation	Formation of capsule by ionic interaction between core and wall materials that are extruded within an ionic solution	✓ Extreme condition of pH and temperature are excluded ✓ Organic solvents are excluded × capsules contain big pores that can be destroyed easily	1	Betalain	Sodium alginate (Emulgel CP 3792), Bovine serum albumin (BSA)	(Kunjachan, Jose, & Lammers, 2014), (Otalora, Carriazo, Iturriaga, Osorio, & Nazareno, 2016)

Encapsulation by rapid expansion of supercritical solution (RESS)	a. Dispersion of wall and core material in supercritical fluid (CO_2) b. Spraying of the super critical solution c. Precipitation of the shell materials onto the actives ✓ Organic solvent-free particles × Poor solubility of high molecular weight compounds in CO_2 × Requires highly soluble core and wall materials in supercritical fluid		1	Anthocyanin	Bixin-rich extract and polyethylene glycol (PEG) and a co-solvent (ethanol)	(Debenedettl, Tom, Kwauk, & Yeo, 1993) (Santos & Meireles, 2013)
Emulsification	a. Dissolve actives and emulsifiers in oil/water b. Mix oil and water phases under shear	✓ This technique is suitable for both polar and nonpolar compound × Capsule formation is uncontrollable	1	C.I. Pigment Blue Allyloxy nonyl-phenoxypropanol- polyoxyethyleneetherammonium sulfonate (ANPS) (Fu, Xu, Du, Tian, & Zhang, 2011)		
Spray drying	1. Dispersion of core material in wall material 2. Atomisation 3. Dehydration	✓ Fast technique ✓ Cost effective ✓ Variety of wall materials ✓ Good stability ✓ Good encapsulation efficiency ✓ One step process × Operating under elevated temperature	1	Anthocyanin	a. Combination of gum Arabic and maltodextrin b. Combination of maltodextrin and gelatin and maltodextrin	(Akhavan Mahdavi, Jafari, Assadpour, & Ghorbani, 2016) (Osorio et al., 2010)
			2	Anthocyanin	Maltodextrins [Stardri 10 (10DE), Glucodry 210 (20–	(Ersus & Yurdagel, 2007)

					23DE) and MDX 29 (28–31 DE)].	
			3	Violet pigment	Gum Arabic	(Venil, Aruldass, et al., 2015)
			4	Flexirubin-type	Gum Arabic (GA) and κ-carrageenan (KC)	(Venil, Khasim, Aruldass, & Ahmad, 2015)
			5	Anthocyanin	Maltodextrin of gum acacia and tricalcium phosphate	(Nayak & Rastogi, 2010)
Freeze drying	a. Dispersion of core and wall materials in water. b. Homogenization of the sample. c. Freeze the sample. d. Drying under low pressure.	✓ Good for thermosensitive compounds × Time consuming	1	Anthocyanin	Gum Arabic, maltodextrin,	(Mahdavee Khazaei, Jafari, Ghorbani, & Hemmati Kakhki, 2014)
			2	Fucoxanthin	Maltodextrin and Tween–80	(Indrawati, Sukowijoyo, Dumilah, Wijayanti, & Limantara, 2015)
			3	Potassium Norbixinate and Curcumin	Maltodextrin DE20	(Baesso, Neto, Marcolino, & Matioli, 2013)
			4	Red pigment	Maltodextrin D.E. 10, Maltodextrin D.E. 20, and gum Arabic	(Selim, Khalil, Abdel-Bary, & Abdel-Azeim, 2008)

1.3. Pigments extracted from Mollusc's shell

In recent years, marine animals especially molluscs that have bright interesting colours, have attracted attention in the scientific community. Shells of molluscs contain a variety of pigments (Comfort, 1950). Marine gastropod molluscs (e.g. abalone) have a single shell with spiral structure and muscular foot (Li, T and Zeng, K, 2013). Molluscs use their mineral skeletons for protection from predation, navigation, light reflection, temporary ion storage and detoxification. The shell is made up of 95% calcium carbonate precipitated as crystalline polymorphs such as aragonite and calcite with the remaining 5% being organic materials. Most gastropods have their shell made of aragonite rather than calcite. However, the polymorphic shell of abalone, particularly *Haliotis iris,* is composed of both calcite and aragonite. Abalone shell deposition initiates at the raised area of the shell. The tremata (pore) series on the shell develops almost parallel to the shell outline. The outer layer of the shell is thin (100-200 nm) non-calcified proteinaceous organic periosracum that protects the shell from endobionts, epibionts and corrosion by seawater. The layer underneath (second layer) is composed of crystals (each enclosed by an organic glycoprotein matrix of calcite) which is called prismatic calcite (100 nm diameter). The most inner layer is made up of uniform flat nacreous aragonites with thickness of 300-400 nm. Each of these aragonites are covered by organic compounds (calcified) to give strength to the shell. The edge of the shell is thicker and is made up of aragonite. Along the shell length, conchiolin organic layer is present which is heavier in weight.

Molluscan shells contain acid-soluble pigments that are reported to be similar to porphyrins (organic compound with large heterocyclic ring structures). *Haliotis cracherodii* shell has an acid soluble blue pigment. Comfort (1949) reported that the pigment might be pyrroles (heterocyclic organic compound). However, its absorbance and visible spectrum was similar to indigotin and indigos. There are two groups of naturally occurring pyrroles: porphyrins with four membered rings and bile pigments with rings arranged in a chain. Degradation of haem produces linear tetrapyrroles in animals. However, tetrapyrroles are absent in molluscs. In most molluscs haem is limited to cytochrome

prosthetic groups, helicorubin, haemoglobin, radular myhaemoglobins, and enzymes. Some marine shells contain linear pyrroles, which were first discovered by Krukenberg (1883, 1886) and by Schulz (1904). Shells of several mollusc species contains high amounts of free porphyrin. Some other species such as pelecypod and gastropods contain uroporphyrin. Porphyrins occurs only in Archaeogastropoda but not in other genera such as Haliotis or Patella. Haliotis contain linear pyrroles. The reason for shell pigmentation in molluscs is unclear. Uroporphyrin is present in high concentration which is a known protoporphyrin by-products of haem. Acid solution of shells with porphyrin groups will extract different pigments such as red, violet and blue which can be separated by chromatography. The blue section with porphyrin-like fluorescence perform as a mesobiliviolin (bile pigment).

1.4. The significance of paua in New Zealand

Abalone in New Zealand is called Paua by Maori who have been harvesting paua for food for over 800 years (Somerville, Krkosek, & Hepburn, 2014). Paua is a very significant aspect to the art of Maori. Paua shells are used in carving and jewellery. It also has high economical export value in New Zealand due to its nutritionals value and other valuable by-products (Hadi, Gutierrez, Alfaro, & Roberts, 2014). Paua is related to the genus *Haliotis*. *Haliotis* is a Latin name that means sea ear that is given to the paua because of its ear shape of its shell. There are 100 -130 species of abalone in the world (Philip, 2016). New Zealand has three abalone species that naturally occur: white-footed paua (*Haliotis virginea*), yellow-footed paua (*Haliotis australis*) and black-footed paua (*Haliotis iris*) (Allsopp, Lafarga-de la Cruz, Aguilar, & Watts, 2011). They are found throughout the country's coastline, including Stewart Island and the Chatham Islands. The black-footed paua is larger than the other two species and only species that is currently farmed in New Zealand. Its shell length can grow to about 180 mm. The New Zealand paua shell is the only abalone with brilliant multi colours in the world that makes them distinct from other abalones. At the moment, the New Zealand industry is based on about 12 farms. The largest farm is OceaNZ Blue Paua Ltd that is placed at NIWA's Bream Bay Park facility in

Northland (Philip, 2016). Developing high value products from paua and the paua industry's by-products can further expand paua's export value.

1.5. Conclusion

In conclusion, food and cosmetics are in high demand. Most of the food and cosmetics require colours to attract consumers. In the past, most of the colour were obtained synthetically. However, recently people prefer natural colours due to health issues that are caused by artificial colours. Natural colours have been extracted in the last years by organic solvent extraction, such as soaking and percolation. Then, they were purified by chromatography. Extracted natural pigment can easily degrade by light, temperature, pH, oxidant and reducers. Thus, natural pigments require to increase their shelf life by making a protective environment around them such as encapsulation.

Chapter 2. Extraction and Purification of Aquatic Blue Pigment from Paua Shells

2.1. Introduction

Marine gastropods, such as abalone have a single shell with spiral structure and muscular foot (Li, T and Zeng, K, 2013). Shell of abalone, particularly *Haliotis iris* (paua), is composed of both calcite and aragonite. Paua shell deposition initiates at the raised area of the shell, called the umbo. The thickness of the shell increases with age (Eljdlo, Dgglwlrqdo, Derxw, & Duwlfoh, 2016). The outer layer of the shell is thin (100-200 nm) non-calcified proteinaceous organic periosracum that protects the shell from endobionts, epibionts and corrosion by seawater and other substances. The underneath layer (second layer) is composed of crystals (each enclosed by organic glycoprotein matrix of calcite), which is called prismatic calcite. The most inner layer is made up of uniform flat nacreous aragonites. The inner layer of the shell has different shades of colour. Paua shells are used for many applications, including jewelleries and traditional Maori carvings and their natural colours has the potential to be used in different industries, such as food and cosmetics. The majority of the blue pigments that are used are synthetic and paua shell is used for the first time for extraction of natural blue pigment. Paua shells contain high amounts of calcite, which is soluble in acid (Lund & Foglers, 1975). Therefore, due to acid solubility of paua shell, organic solvent extraction was used to extract pigment from the shell of paua. Acetic acid is an organic solvent and weak acid that was used for extraction of pigment(Cai et al., 2011).Thus, In this project to extract blue pigment from the paua shell same solvent has been used. After extraction of the blue pigment, it needs to be purified. One of the most rapid and simple methods in purification, is solid phase extraction (SPE) (Denev et al., 2010).

2.2. Chapter aims

The aim of this chapter is to extract natural blue pigment from paua shell and to purify it for further physicochemical analyses and formulation.

2.3. Methodology

2.3.1. Materials

Paua shells were supplied by OceaNZ Blue, Moana, Ruakaka, New Zealand. Glacial acetic acid was from Fisher scientific, UK, Whatman filter paper (qualitative 1, 70mm) from GE Healthcare life science, New Zealand, Sodium hydroxide (NaOH) pellets from environmental control product (ECP) Ltd, Auckland, New Zealand, Formic acid from May and Baker Ltd, UK and methanol from Fisher scientific, UK. For solid phase extraction: LC-18 packing 60ml, 10 g cartridges were purchased from Sigma-Aldrich, New Zealand. TLC plate were purchased from Macherey-Nagel, Germany. All other reagents and chemicals were of analytical grade.

2.3.2. Preparation/Extraction of paua pigment

A method used for extraction of aquatic blue pigment from other abalone species was used with some modifications (Cai *et al.*, 2011). About 5 kg of paua shells (*Haliotis iris)* were crushed into small pieces in a mortar and pestle. The crushed paua shells were then placed in a milling machine (Retsch, PM 100) for 5 minutes at 400 rpm or until a fine paua shell powder was obtained. The shell powder was soaked/steeped in 20L glacial acetic acid (5% v/v) for 24 hours (the solution was stored in a plastic container in dark). After 24 hours, the solution was filtered (11 µm pore size) to separate solids and the liquid containing the pigment. Sodium hydroxide (4M) was then added to the liquid containing pigment, to adjust its pH to 10 and thereof left for 30 minutes to precipitate. After 30 minutes, the precipitated solution was filtered (11 µm pore size). The solid precipitate was trapped on the filter paper and was placed in 1L glacial acetic acid (10% v/v) to dissolve the pigment. The solution (pigment in 10% acetic acid) was then centrifuged (Eppendorf, centrifuge 5810R) at 4000 rpm at 10°C for 4 minutes to separate the dissolved filter papers from the pigment solution. The centrifuged sample was transferred into a brown bottle, covered with aluminium foil and stored at 4°C for the next step.

2.3.3. Purification of the paua blue pigment

Solid phase extraction (SPE) method with some modifications was used to purify the pigment (Denev et al., 2010). Elution of the pigment is normally drawn with gravity; however, it is time consuming. Therefore, an in-house modification was used where a 60 ml catheter tip syringe was connected to the back of the SPE column (Figure 2-1)**.** This allowed air to be pumped through the column to speed up the process with the flow rate of 15 ml/min. To purify the sample, 100% methanol was first loaded into the SPE column from the tip of the catheter tip syringe and eluted with the aid of air. This was followed by the addition of MilliQ (ultrapure water) to clean the column and fix the silica particles within the column. Then, the sample solution was loaded and the pigment was attached to the silica whereas the unwanted solutions were eluted out. The column was washed with 50% v/v methanol to elute the rest of impurities and leave the compounds of interest in the silica. Finally, the purified blue pigment was eluted with 98% v/v methanol and 2% v/v formic acid. The purified pigment solution in methanol was placed in a 100 ml round flask, and the solvent was removed by the rotary evaporator to give the purified pigment as a blue powder (500 mg).

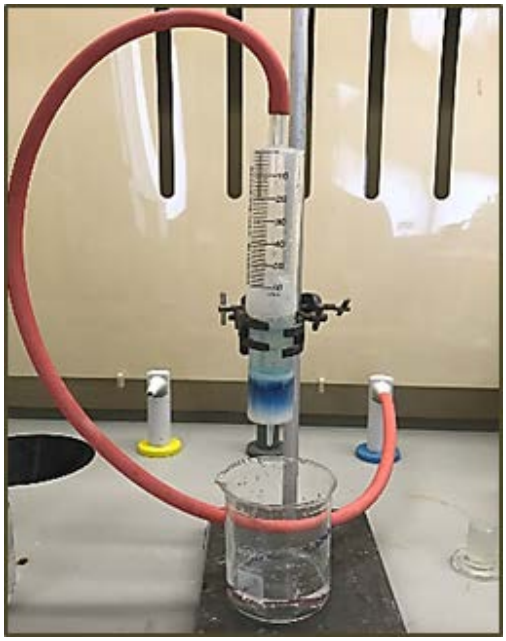

Figure 2-1. Solid phase extraction column (SPE).

2.3.4. Characterisation of the blue pigment

2.3.4.1. Purity analysis of the blue pigment

The purity of the pigment was analysed by high performance liquid chromatography (HPLC) and thin layer chromatography (TLC). The pigment was first analysed by HPLC with gradient elution (solvent A: 15% milliQ, solvent B: 80% acetonitrile and solvent C: 5% (94.6% NH_3COOH, 0.4% HCOOH and 5%MeCN)). The injection volume was 10µL, flow rate was 1 ml /min and detection was at 612 nm. The purified aquatic blue pigment was applied to silica gel GF 254 TLC-plates using H_2O/methanol (3.5:1.5, v/v) as mobile phase and the spot was determined by UV light at 254 nm. The retention factor of the pigment was calculated by Retention factor formula (Rf = distance travelled by solute/distance travelled by solvent).

2.3.4.2. Spectral analysis of the blue pigment

Aquatic blue pigment was analysed by 2 different spectroscopic techniques; Fourier transform infrared spectroscopy (FTIR) from thermos scientific (Nicolet iS10) and UV-vis spectroscopy (Ultrospec 7000). FTIR spectra of the pigment was obtained by placing a small amount of pigment on the diamond stage of spectra and the result was recorded at a resolution of 4 cm^{-1}. Aquatic blue Pigment was dissolved in H_2O and wave scanned between 300-700 nm by spectrophotometer in 1.5 mL polystyrene cuvettes alongside corresponding blank.

2.3.4.3. Morphology of the blue pigment

A small amount of dried pigment was placed on the stage of the scanned electron microscopy (SEM) (Thermos scientific, HITACHI, SU-70) and the pigment was coated with platinum by ion sputter (HITACHI, E-1045). Then, the pigment was scanned by SEM to obtain shape of the pigment.

2.3.4.4. Elemental analysis of the blue pigment

For element analysis of the pigment, scanned electron microscopy (SEM) and inductively coupled plasma mass spectrometry (ICP-MS) (7700 ICP-MS) were used. A small amount of dried pigment was

placed on the stage of the scanned electron microscopy (SEM) and the pigment was coated with platinum by ion sputter (HITACHI, E-1045). Then, the elements of the pigment were identified by SEM. In the second analysis, the aquatic blue pigment was dissolved in 5% nitric acid (HNO_3). The solution turned blue, however a fine white particulate substance was observed settling out of the solution. The pigment solution was analysed in semi-quantitative mode by scanning the entire mass spectrum (6-260 m/z) for the desired elements on an Agilent 7700 ICP-MS in Helium mode to reduce polyatomic interferences. A semi-quant standard was used to calibrate the instrument (Agilent Technologies). In semi-quant analysis mode, the results are generally accurate to +/- 15%. All results are in µg/g.

2.4. Result and Discussion

2.4.1. Preparation/Extraction of paua pigment

Aquatic blue pigment was extracted by reaction between acetic acid ($C_2H_3O_2$) and calcium carbonate $CaCO_3$ of the paua shell (Equation 1) (Cai et al., 2011). After 24 hours, the calcium carbonate reacts with acetic acid to form calcium acetate and unreacted material was separated from the solution by filtration pigment. After addition of the NaOH to the solution of ($Ca(C_2H_3O_2)_2$), Ca^{2+} was replaced by Na^+ and to form sodium acetate in a solution containing pigment. On the other hand, Ca^{2+} reacted with OH^- to form insoluble $Ca(OH)_2$. After a second filtration, the crude blue pigment was stuck on the filter paper and $C_2H_3O_2$ was removed at the same time.

$$2(C_2H_3O_2) + CaCO_3 \rightleftharpoons H_2CO_3 + Ca(C_2H_3O_2)_2$$

$$2NaOH + Ca(C_2H_3O_2)_2 \rightleftharpoons 2Na(C_2H_3O_2) + Ca(OH)_2$$

Equation 1. Reaction of the paua shell powder with acetic acid and NaOH

2.4.2. Characterisation of the blue pigment

2.4.2.1. Purification of the paua blue pigment

By passing the pigment through SPE column (reversed phase), ions and other impurities (Hydrophilic molecules) were eluted first due to the column that is hydrophobic, which absorbed hydrophobic molecules and leave hydrophilic molecules. After absorption of the hydrophobic molecules to the column, methanol attached to the pigment and eluted from the column.

2.4.2.2. Purity analysis of the blue pigment

According to Figure 2-2 in HPLC, the pigment gave a sharp peak at retention time of 6 minutes as well as a tiny peak next to it. The sharp peak represents the aquatic blue pigment. However, the short peak might be another compound in the pigment, which might be due to impurity of the pigment. The aquatic blue pigment gave a spot on the TLC plate with retention factor of 0.6. The spot was travelled close to the solvent front, which might be due to low polarity of the pigment. However, there was scattered compound around the spot, which indicates impurity of the pigment.

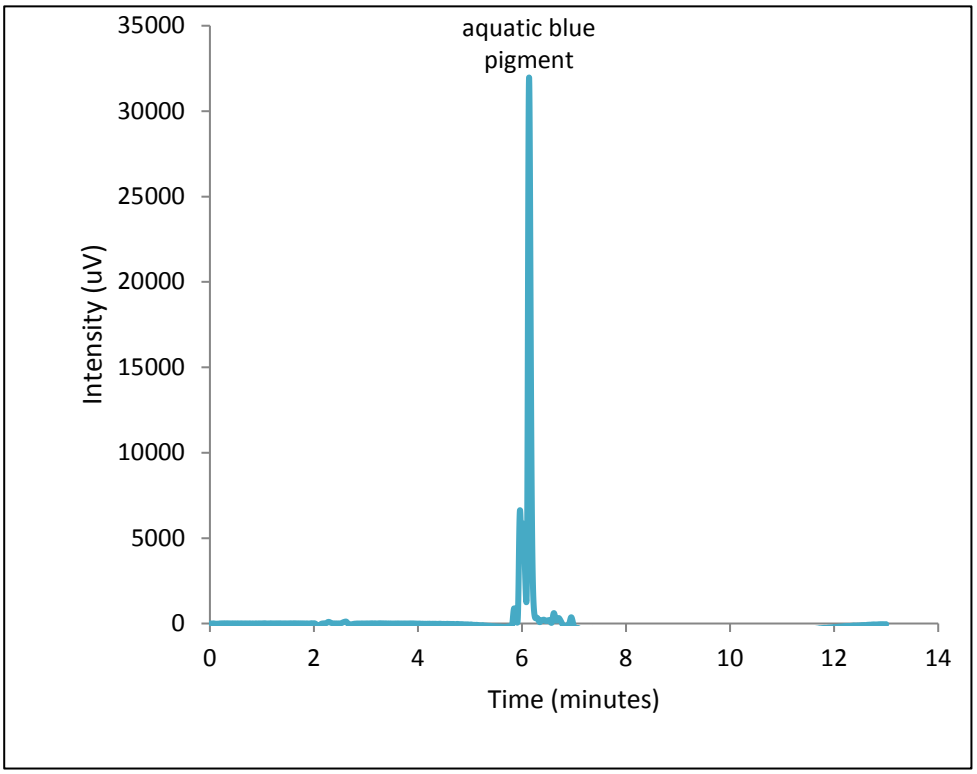

Figure 2-2. HPLC chromatogram of aquatic blue pigment of paua shell.

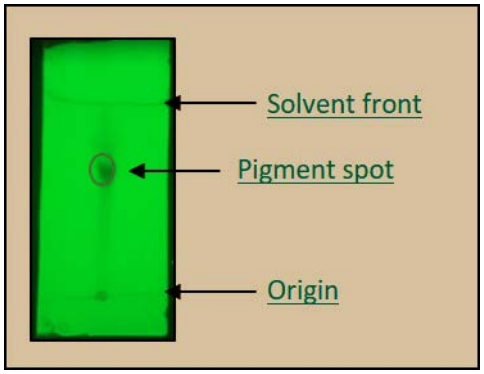

Figure 2-3. The spot of aquatic blue pigment on the TLC plate.

2.4.2.3. Morphology of the blue pigment

Pigment's photo was captured by SEM at 15 kV with magnification of 18.9 mm x1K. In Figure 2-4, pigment has same structure in most areas. However, there are a few round particles, which might be due to the impurity of the pigment.

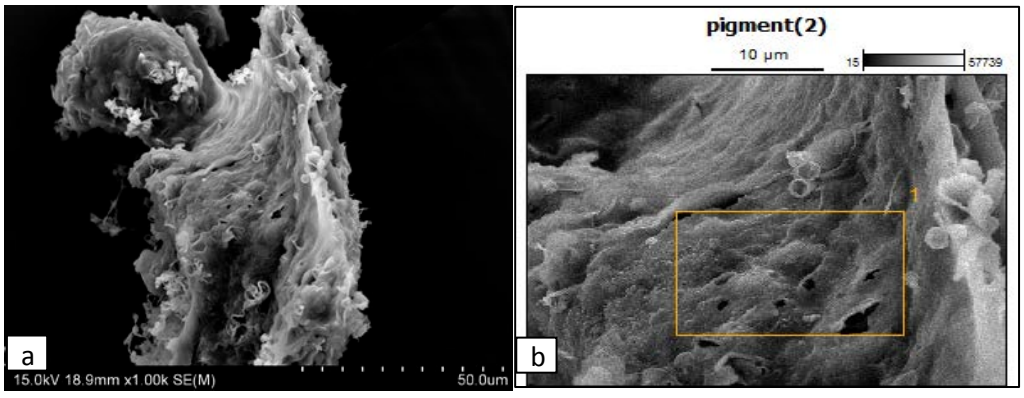

Figure 2-4. (a) Scanned Electron microscopy (SEM) of the aquatic blue pigment. (b) Section of the element analysis.

2.4.2.4. Spectral analysis of the blue pigment

As is stated in Figure 2-5, the ultraviolet–visible absorption spectrum (200–700 nm) of the aquatic blue pigment showed a strong absorbance between 600 to 620 nm. The peak maximum was at 612

nm. Therefore, the absorbance of the following tests was measured at 612 nm. FTIR spectra can provide information about functional groups of unknown samples (Chen, Gu, LeBoeuf, Pan, & Dai, 2002). According to the Figure 2-6, the IR spectra of the aquatic blue pigment shows a broad peak at 3440 cm^{-1} represents hydrogen bonding due to the presence of water. The sharp peak at 2920 and 2850 cm^{-1} both are related to the alkane groups (C-H) which normally show 2 bands on IR spectra. The peak at 1590 cm^{-1} is due to vibration of C=C bonds. The next peak at 1460 cm^{-1} is from alkanes group with vibration of CH_2 and CH_3. The obtained peaks of the pigment were in similar regions that the previous study got their peaks (Cai et al., 2011). However, they had an extra peak for C=O group.

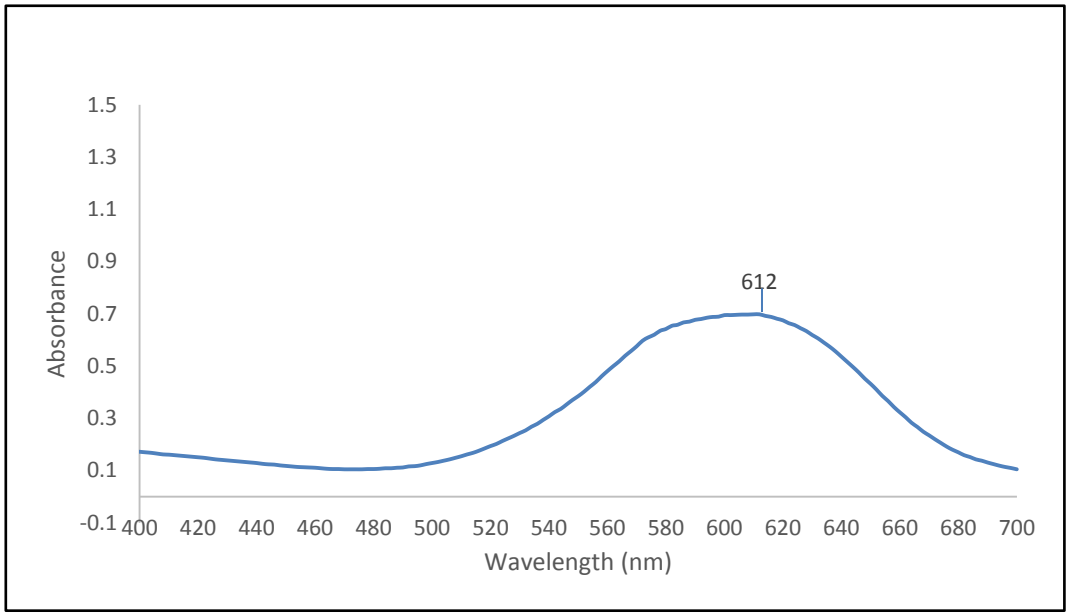

Figure 2-5. Ultraviolet–visible absorption spectrum of aquatic blue pigment from paua shell (*Haliotis iris*).

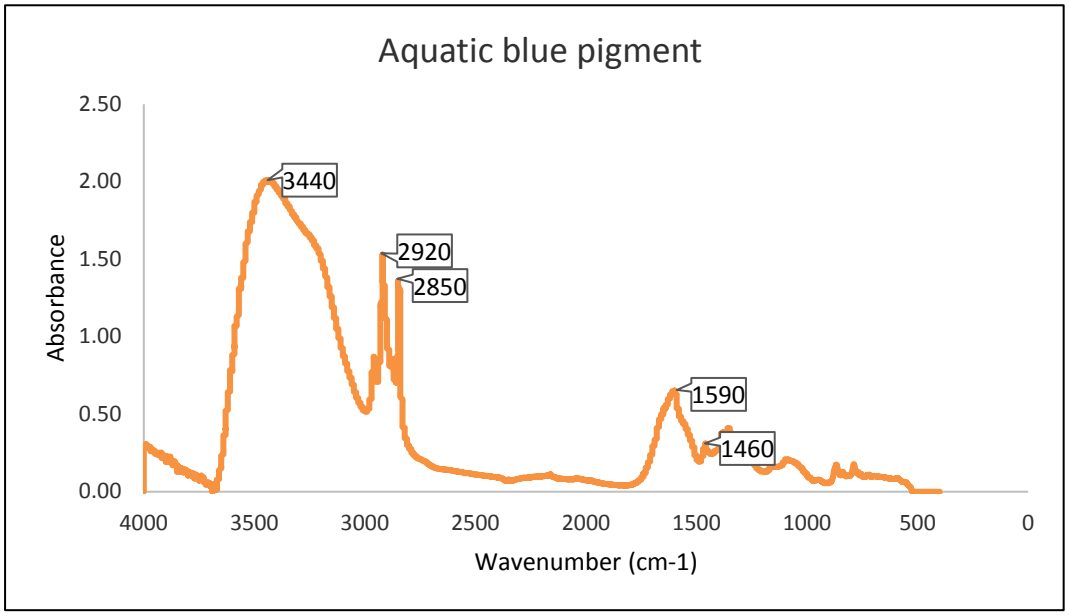

Figure 2-6.The IR spectra of aquatic blue pigment from paua shell.

2.4.2.5. Elemental analysis of the blue pigment

Elemental analysis was carried out using SEM Element Analyser and ICP-MS. According to the Figure 2-7 and Table 2-1, Carbon has the highest percentage of atoms in the pigment. The second most abundant elements were O and Ca. Zr also had high percentage in the pigment. However, the presence of Zr was thought to be due to contamination of the SEM. Further analysis by ICP-MS confirmed the absence of Zr. There was also 0.87% and 1.24% of S and Si respectively in the pigment.

According to the Figure 2-8, 66 different elements were detected by ICP-MS. 0.257g of Ca was present in 1g of the aquatic blue pigment, which was the highest amount of the elements in the pigment. This was due to the shell of the paua is made of 95% calcium carbonate. The second most elements were Na (0.001g) and Cl (0.001g) in 1g of pigment. The rest of elements were less than 0.0007g.

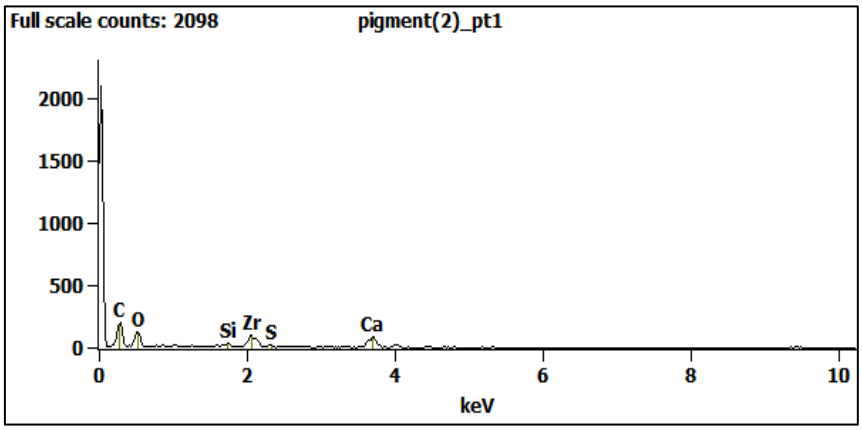

Figure 2-7. Elements analysis present in pigment by SEM.

Table 2-1. Table of presence of different elements in the aquatic blue pigment that is analysed by SEM.

	Elements					
	C	O	Si	S	Ca	Zr
Net counts	1313	770	125	173	1088	1552
Weights %	27.85	22.37	1.13	1.83	19.87	26.96
Atoms %	50.35	30.36	0.87	1.24	10.76	6.42

Table 2-2. Presence of different elements in the pigment that detected by ICP-MS.

	Elements	Conc. (µg/g)		Elements	Conc. (µg/g)		Elements	Conc. (µg/g)
1	**Li**	35.617	23	Ge	0.004	45	**Nd**	0.006
2	**Be**	<0.095	24	As	0.344	46	**Sm**	0.002
3	**B**	10.100	25	Br	19.651	47	**Eu**	0.003
4	**Na**	1259.224	26	Rb	0.124	48	**Gd**	0.003
5	**Mg**	756.135	27	Sr	239.716	49	**Dy**	0.001
6	**Al**	2.066	28	Zr	0.022	50	**Ho**	0.000
7	**Si**	613.838	29	Nb	0.000	51	**Er**	0.000
8	**P**	205.868	30	Mo	0.000	52	**Tm**	0.000
9	**S**	797.198	31	Ru	0.000	53	**Yb**	<0.001
10	**Cl**	1263.741	32	Rh	0.040	54	**Lu**	0.000
11	**K**	153.129	33	Pd	0.895	55	**Hf**	0.001
12	**Ca**	257490.573	34	Ag	0.016	56	**Ta**	0.001
13	**Ti**	0.399	35	Cd	0.000	57	**W**	0.000
14	**V**	0.026	36	In	0.000	58	**Re**	0.000
15	**Cr**	4.724	37	Sn	0.034	59	**Os**	<0.002
16	**Mn**	41.670	38	Te	<0.059	60	**Pt**	0.001
17	**Fe**	6.457	39	I	43.206	61	**Au**	0.018
18	**Co**	0.433	40	Cs	0.037	62	**Hg**	0.008
19	**Ni**	3.003	41	Ba	2.254	63	**Pb**	8.271
20	**Cu**	9.427	42	La	0.010	64	**Bi**	0.022
21	**Zn**	47.046	43	Ce	0.048	65	**Th**	0.000
22	**Ga**	0.437	44	Pr	0.002	66	**U**	0.207

2.5. Conclusion

In conclusion, the aquatic blue pigment was extracted from paua shell (*haliotis iris*) by 5% acetic acid. SPE column was used to purify the extracted pigment. However, after analysis of the pigment by HPLC and TLC plate, the aquatic blue pigment was not ultra-pure. This was due to the HPLC that showed 1 sharp peak as well as 1 small peak next to it which generally 1 peak is for 1 compound. The other reason was the analysis of the pigment by TLC plate, which showed a scattered spot instead of a single spot. Thus, the solid phase extraction is not a good method for separation of different pigments and compounds. The pigment absorbance was detected at 612 nm at UV-Vis spectra, which

is normally blue. Information on the functional groups in the pigment was obtained by FTIR. There was one alcohol group (OH) and three other groups that maybe related to alkane groups (C-H). SEM element analyser determined different elements such as C, O, Ca, S, Si and Zr. However, existence of Zr was due to contamination of SEM. 66 elements were identified by MS-ICP, which were present in the pigment. The pigment was high in Ca (0.257 g/g) that was due to the shell of the paua is 95% Calcium carbonate.

Chapter 3. Stability and safety of aquatic blue pigment

3.1. Introduction

A key aspect of quality in food products is to preserve stable and intense colours during processing and manufacturing (Tierno & Galarreta, 2016). Pigments, especially natural pigments, are unstable and degrade easily in many ways, such as exposure to lights, oxidatants, high temperatures, additives, metallic ions, sugars and acids (Ersus & Yurdagel, 2007; Rodriguez-Amaya, 2016). When organic pigments are exposed to light or air, chemical degradation occurs in photoactive pigments, which is called photo oxidation (Sai, Leung, Zádor, & Henkelman, 2014). Similarly, the aquatic blue pigment extracted from paua can undergo degradation. Therefore, it is necessary to investigate the effect of light, temperature and additives on the stability of the pigment.

Currently, consumer attention has been attracted to the natural food and cosmetics due to health issues that are caused by artificial food additives, such as pigment and cosmetics with artificial colours. Most natural colours have low toxicity that can be good substitutes for artificial colours (Ersus & Yurdagel, 2007). In a previous study, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the toxicity of the tattoo pigment on human cells (Falconi et al., 2009). MTT assay is a test to assess viability of cells after exposing the cells to the compounds (Falconi et al., 2009). In MTT assay yellow tetrazole changes to dark blue in living cells (Mosmann, 1983). Due to the blue colour of the MTT and the aquatic blue pigment, MTT is not a good choice to be used for the cytotoxicity of the pigment as both have similar absorbance. Thus, Cell Counting Kit-8 (CCK8) could be used instead. CCK8 is a nonradioactive material that is used for determination of cell viability in cytotoxicity assay and cell proliferation. The sensitivity of detection of Cell counting kit-8 is also more than other tetrazolium salt such as MTS, XTT and MTT (“Cell Counting Kit - 8,” 2017).

3.2. Chapter aims

The first aim of this chapter is to determine the stability of the aquatic blue pigment when exposed to different chemicals and conditions. The second aim is to prove the non-toxicity of the aquatic blue pigment.

3.3. Methodology

3.3.1. Materials

Ethanol (C_2H_6O), hydrogen chloride (HCl), aluminium chloride ($AlCl_3$), hydrated magnesium chloride $MgCl_2{\cdot}6H2O$, cupper (II) chloride ($CuCl_2$), potassium chloride(KCl), D-glucose anhydrous ($C_6H_{12}O_6$) and starch were purchased from Thermo fisher scientific, potassium dihydrogen phosphate (KH_2PO_4) from BDH chemicals, sodium hydroxide pellets (NaOH) from Environmental Control Products (ECP) laboratory and research chemicals, sodium hydrogen carbonate ($NaHCO_3$) from Panreac quimica SA, hydrogen peroxide (H_2O_2) from Fisher scientific, sodium sulphite (Na_2SO_3) from BDH chemicals, calcium chloride dihydrated ($CaCl_2H_4O_2$) from VWR international BVBA, Iron (II) sulphate ($FeSO_4$) AR from ECP laboratory and research chemicals, Iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) extra pure from Scharlau, zinc chloride ($ZnCl_2$) from Prolabo, Citric acid ($C_6H_8O_7$) from Riedel-de Haen, sodium tartrate ($C_4H_4O_6Na_2$) from BDH chemical ltd, Sucrose ($C_{12}H_{22}O_{11}$) from Sigma life science and Sodium chloride (NaCl) from VWR international BVBA,

Cell culture medium (RPMI 1640, no phenol red), L-glutamine (200 mM; 100 mL), Penicillin-Streptomycin (10,000 U/mL; 100 mL), Trypan Blue Solution, 0.4% and TrypLE™ Express Enzyme (1X), no phenol red all supplied from Life Technologies (Thermo Fisher Scientific), Sterile filtered foetal bovine serum (FBS) from Medica Pacifica (Auckland, NZ), Embryonic Kidney; Human (HEK 293) from ATCC (In vitro Technology, NZ) and CCK8 was supplied from Sigma-Aldrich.

3.3.2. Pigment stability studies

3.3.2.1. Photo stability of the aquatic blue pigment

To determine the effect of light on the aquatic blue pigment, 20 mg of dried pigment was dissolved in 25 ml ethanol: H_2O (1:1) (v/v). The pigment solution was then exposed to different sources of light namely ultraviolet light (UV) with the wavelength of 260 nm, indoor light, sunlight and in darkness. Samples were monitored over 10 days and the absorbance was recorded at 612 nm.

3.3.2.2. Thermostability of the aquatic blue pigment

The effect of temperature on the pigment was examined by adding an aqueous solution of aquatic blue pigment in capped test tubes placed in a water bath (25, 50, 75 and 100°C) for up to 3 hours and the temperature was mainained by a thermometer. The absorbance of the pigment was recorded after 1, 2 and 3 hours.

3.3.2.3. pH stability of the aquatic blue pigment

The stability of the pigment was studied at different pHs (2, 4, 6, 8, 10 and 12) at room temperature (25°C) . At specific time points (1, 2 and 3 hours), samples were centrifuged at 3000 rpm for 4 minutes and the absorbance was recorded at 612 nm.

3.3.2.4. Redox stability of the aquatic blue pigment

The effect of oxidisers (H_2O_2) and reducers (Na_2SO_3) on the stability of the pigment were tested by mixing the pigment solution with H_2O_2 and Na_2SO_3. These mixtures were incubated at room temperature (25°C) for 1 hour and the absorbance of them was recorded at 612 nm.

3.3.2.5. Stability of the aquatic blue pigment in various food additives

Effect of food additives (starch, citric acid, sodium tartrate, glucose anhydrous, sucrose and sodium chloride) on the aquatic blue pigment stability was conducted by mixing the food additives with the pigment solution (0.65g/ml ethanol (50%)) to make the final food additive concentration of 5 mM. Then the mixtures were centrifuged at 10°C at 4000 rpm for 4 minutes. The mixtures were stored at

room temperature (25°C) and the absorbance of the aquatic blue pigment was obtained at 612 nm after 6, 12 and 24 hours.

3.3.2.6. Stability of aquatic blue pigment in metal ions

Effect of metal ions (Al^{3+}, Ca^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, K^{+}, Mg^{2+}, Zn^{2+}) on the aquatic blue pigment stability was studied by mixing them with the pigment solution to make the final concentration of 5 mM. The mixtures were then centrifuged at 10°C at 4000 rpm for 4 minutes and stored at room temperature (25°C). Then, the absorbance of the aquatic blue pigment was obtained at 612 nm after 6, 12 and 24 hours.

3.3.3. In vitro toxicity of the pigment

3.3.3.1. Preparation of Cells for cytotoxicity assay

Culture medium was removed from HEK 293. 10 mL of sterile pre-warmed (37°C) phosphate buffered saline (PBS) was used to wash the cells. Then the flask was shaken gently and let the cells to immerse in the PBS for 30 seconds. Finally, the PBS was removed from the flask. TrypLE Express solution (2 ml) was added to the flask to treat the cells. After 30 seconds, 5 ml medium was used to stop trypsinization. The solution was transferred to a centrifuge tube and centrifuged at 1200 RPM for 5 minutes to separate supernatant from the cells. After 5 minutes, the supernatant of the solution was discarded and 4 ml new completed culture medium was added to the tube containing cells, to resuspend the cells.

3.3.3.2. Cell Counting

A total of 10 µL of the cell suspension was mixed with 10 µL of Trypan Blue and was placed on one side of the haemocytometer. The number of cells was determined under the microscope by counting at least four squares. Then average number of the cells in four squares were generated and the density of cell in the re-suspended cell suspension was calculated.

3.3.3.3. Cell seeding

After determining the number of cells (108 x 10^4 cells/ml), cell suspension was diluted with medium to reach 5 x 10^4 cells/mL. To 96 well plates (24, 48 and 72 hours), 100 µl of cell suspension (5 x10^4 cells/mL) were seeded to half of the wells (5000 cells/well) where the other half was filled with cell culture medium and PBS was added to the wells around the plates to sustain the humidity. Then, all plates were incubated at 37°C with 5% carbon dioxide for 24 hours. There was one extra 96 well plate for blank.

3.3.3.4. Preparation and seeding of the different concentration of the pigment

A stock solution of pigment was prepared by dissolving 0.123 g of pigment in 2 ml complete medium, and different concentrations were prepared by serial dilutions. Each well contained 100 µl cell culture that required 10 µl of pigment (total 110 µl). The 0.123 g/2 ml pigment was diluted with complete culture medium to reach 5000, 4000, 3000, 2000, 1000, 500, 100, 50, 20, 10 µg/ml. After 24 hours incubation, 10 µl of different concentrations of pigment were added to the cells and incubated for 24, 48 and 72 hours at 37°C. After 24, 48 and 72 hours incubation, 10 µl CCK8 was added to each well and incubated for 4 hours at 37°C. After 4 hours the absorbance for cell viability was measured at 450 nm.

3.4. Result and Discussion

3.4.1. Pigment stability studies

3.4.1.1. Photo stability of the aquatic blue pigment

When the aquatic blue pigment was exposed to the sun light, after 1 day, the absorbance of the pigment decreased about 80% and over 10 days the pigment bleached completely (Figure 3-1). In both UV and indoor lights, samples were stable on the 1st day. However, after 5 days the absorbance of the pigment in UV light was decreased to 50% and the pigment in indoor light decreased 40% of the original. On day 10, pigment intensity in UV light decreased to 15% and pigment in indoor light reduced to 30-35%. The pigment samples stored in dark were stable, but the intensity was reduced to 70%

after 10 days. Thus, the result of the light stability indicates that the pigment is not stable in sunlight, UV light, indoor light and slightly stable in dark.

Most natural pigments lose their colour when exposed to light. The extent of light degradation usually depends on the wavelength of the light (Mahdavee Khazaei et al., 2014). One of the most important mechanisms of degradation of photoactive chemicals is photo-oxidation that occurs when organic compounds are exposed to light or air. Changes in chemicals leads to breaking of the π conjugation and decrease in absorbance in the process of photo bleaching/photolysis (Sai, Leung, Zádor, & Henkelman, 2014). Photolysis is breaking of chemical bonds by photons. Presence of oxygen in many organic chemicals causes increased instability. When photons are absorbed, triplet oxygen of molecules changes to single oxygen, which is defined as oxidation. Degradation of organic molecules also occurs by the formation of hydroxyl radicals. At the surface of the Earth, photon absorption is at 290-700 nm range. The absorbed photon contains energy that is transferred to electrons of molecules and alters their configuration by exciting electron from ground state to the higher energy level (excited state). Excited molecules are unstable in the presence of water and oxygen, which can then hydrolyse or oxidise (Lagzi, Meszaros, Gelybo, & Leelossy, 2014).

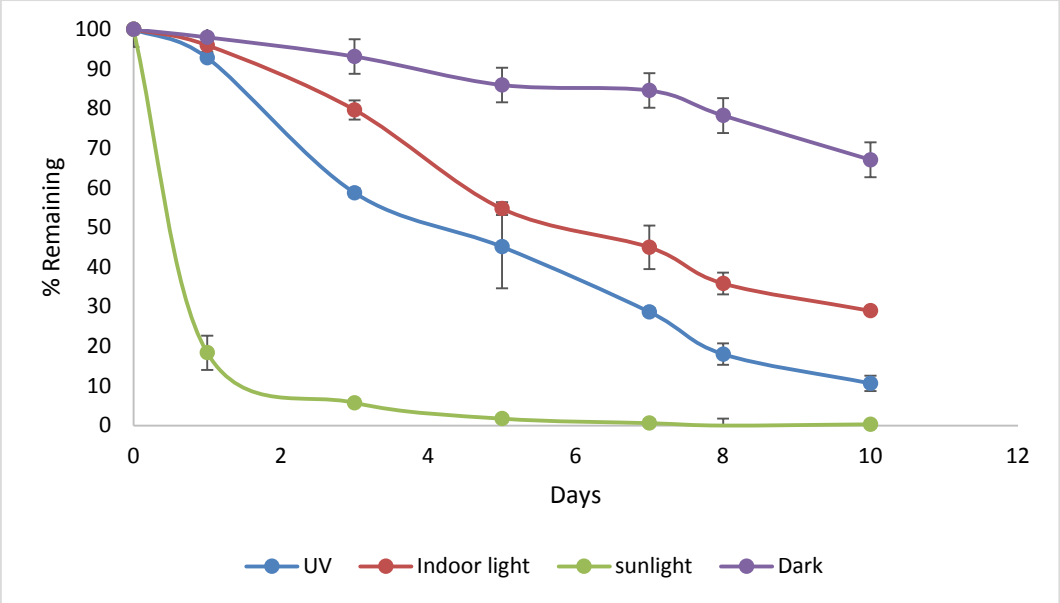

Figure 3-1 Effect of light from different sources on the stability of the aquatic blue pigment (n=3 ± SD).

3.4.1.2. Thermostability of the aquatic blue pigment

Another important factor affecting the stability of most natural pigments is temperature (Jenshi, Saravanakumar, Aravindhan, & Suganya, 2011). Stability of the aquatic blue pigment was analysed under various temperatures (25, 50, 75 and 100°C) over 3 hours. The effect of different temperatures on the extracted aquatic blue pigment from the paua shells were recorded at 612 nm. The results expressed that the aquatic blue pigment was almost 90% stable at 25°C and 50°C in 1, 2 hours, which decreased after 3 hours to 85% (Figure 3-2). However, at 75°C and 100°C, the pigment was degraded. Having said that, colour changes were not visible with naked eye and spectrophotometric analysis showed that the pigment decreased to 85%, 80% and 78% at 75°C and 74, 66 and 60% at 100°C, respectively, after, 1, 2 and 3 hours. This indicates that the aquatic blue pigment was not stable at high temperatures at various time points. As shown in Figure 3-3, temperature could affect on the stability of the dry aquatic blue pigment, which decreased its stability to 53% after 3hours. This study showed that by increasing temperature and time, some changes may occur in the aquatic blue pigment, which could result in decrease in the absorbance of the pigment.

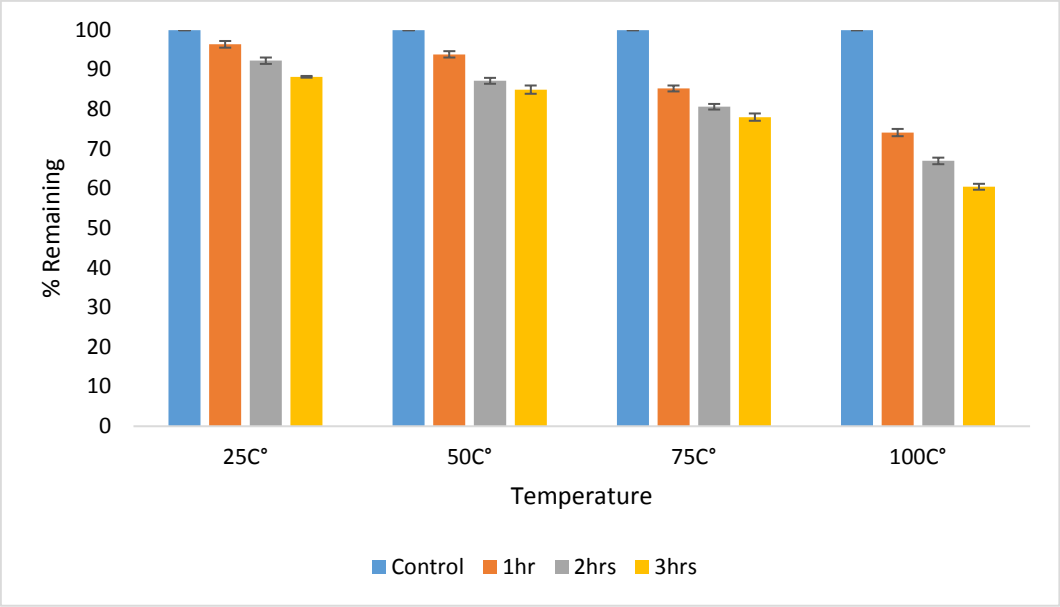

Figure 3-2. Effect of temperature on the stability of the aquatic blue pigment (liquid) (n=3 ± SD).

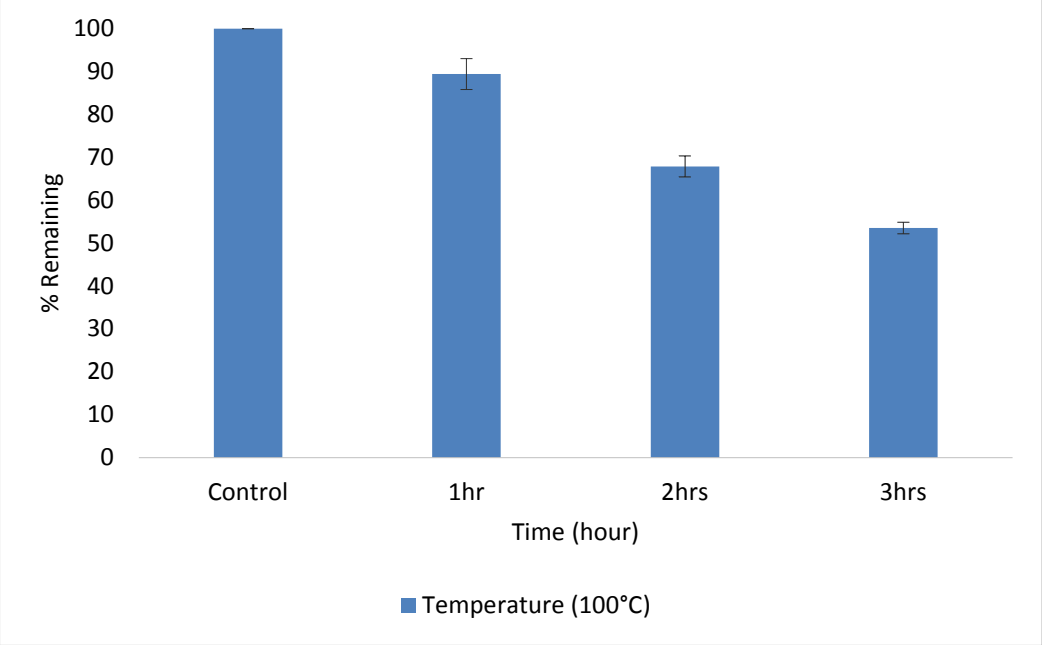

Figure 3-3. Effect of temperature (100°C) on dry aquatic blue pigment (n=3 ± SD).

3.4.1.3. Effect of pH on the stability of the aquatic blue pigment

One of the most important factors affecting the stability of pigments is pH. pH plays a significant role in destabilisation of pigments. The stability of the aquatic blue pigment was analysed in various buffers (pH 2, 4, 6, 8, 10 and 12) (Figure 3-3). The pH stability test was performed at room temperature (25°C) for 1, 2 and 3 hours and the absorbance was recorded at 612nm. After addition of different buffers to the aquatic blue pigment, the colour of the samples with the pH 10 and 12 had changed due to precipitation. However, samples with pH 2, 4, 6 and 8 had stable colour. absorbance of samples at pH 2, 4, 6 and 8, after 1, 2 and 3 hours storage, remained the same and the aquatic blue pigment was almost stable up to 90% (Figure 3-2). However, for samples in pH 10 and 12 after 1 hour storage, the absorbance decreased to about 40% after which no further decrease in absorbance was observed.

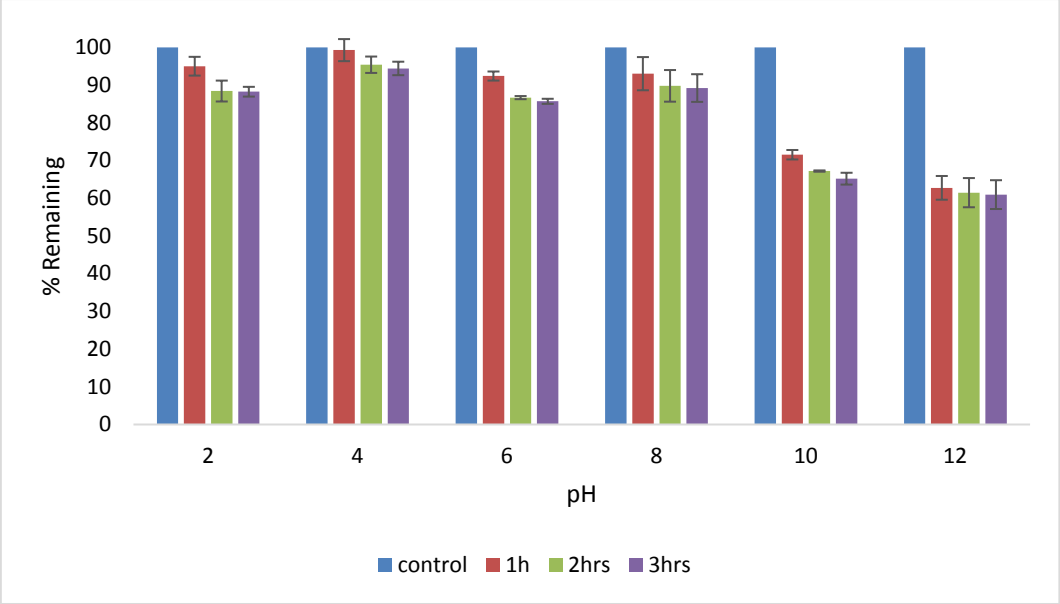

Figure 3-4. Effect of different pH (2-12) on the stability of the aquatic blue pigment (n=3 ± SD).

3.4.1.4. Redox stability of the aquatic blue pigment

According to the Figure 3-4, increasing concentration of the H_2O_2 resulted in a decrease in absorbance of the pigment from 100% to 10%. This is because H_2O_2 can oxidise the pigment and change the structure of the pigment that leads to degradation and bleaching of the pigment. Sodium sulphite (Na_2SO_3) is dechlorinating and reducing agent where one part of oxygen requires to be neutralized by 8 part of sodium sulphite (Streans, 2006). In this experiment, increasing the concentration of Na_2SO_3 had little effect on the pigment stability and the absorbance of the pigment remained about 90% (Figure 3-5). This indicates that the aquatic blue pigment was not reduced by the reducing agent.

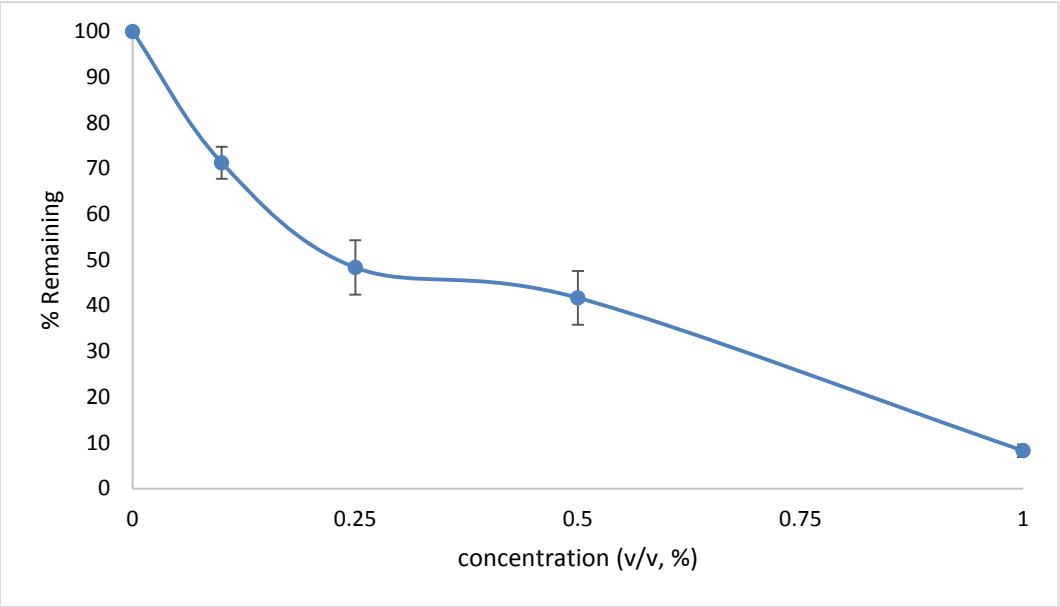

Figure 3-5. Effect of H_2O_2 on stability of the aquatic blue pigment (n=3 ± SD).

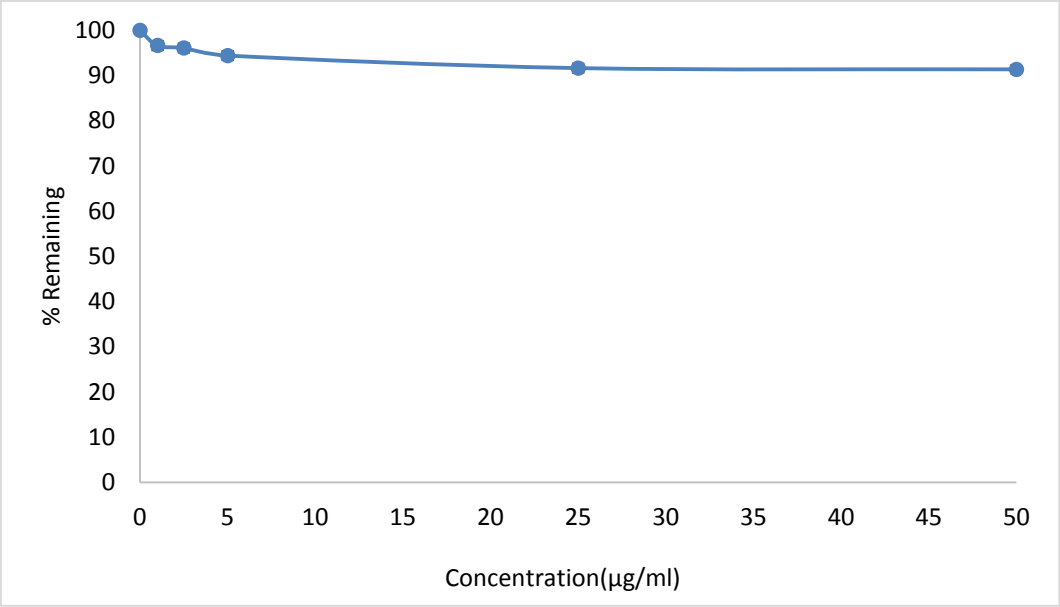

Figure 3-6. Effect of Na_2SO_3 on stability of the aquatic blue pigment(n=3 ± SD).

Generally, the chemical structure of natural pigments can be changed by oxidisers or reducers ,which leads to bleaching of the pigment (Pan et al., 2009). Hydrogen peroxide is a strong oxidant that contains 3 free radicals; Hydroxyl Radical ($OH^{\bullet -}$) a strong non-specific oxidant, which reacts with organic and inorganic compounds, perhydroxyl radical ($HO_2^{\bullet}$) a moderate oxidant that is significant in chain propagation of hydrogen peroxide reactions and superoxide anion ($O_2^{\bullet -}$), which is responsible for organic contaminants degradation (Petri, Watts, Teel, Huling, & Brown, 2011). Reaction between hydroxyl radical and organic compounds occurs in three ways; direct electron transfer, addition to

multiple bonds and abstraction of hydrogen which results in organic radical product that reacts with water and other compounds and degrade.

3.4.1.5. Stability of the aquatic blue pigment in various food additives

Stability of the paua blue pigment was analysed in 6 different food additives in different time points (6, 12 and 24 hours). After addition of the food additives, in few samples, such as starch precipitation occurred. On the other hand, and starch sample was time consuming to dissolve in the pigment. Due to these issues, the samples were centrifuged at 4000 rpm, 10°C for 4 minutes and the absorbance reading was recorded. After 6 and 12 hours, the paua aquatic blue pigment was more than 90% stable in the food additives. However, after 24 hours the pigment had 3-4% reduction. This was occurred because of precipitation and for the second time the samples were centrifuged at 4000 rpm, 10°C for 4 minutes.

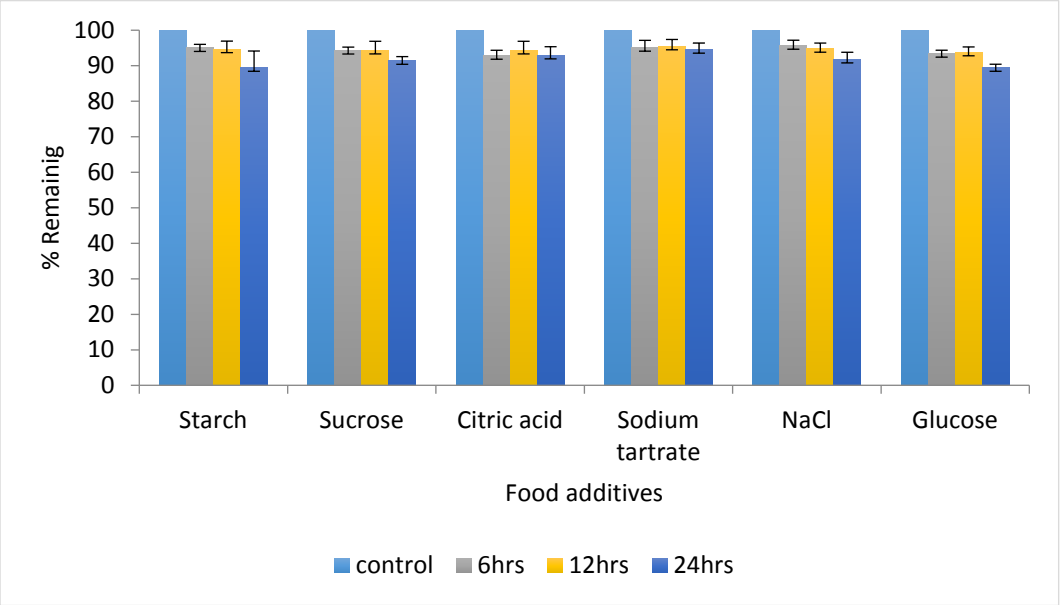

Figure 3-7. Effect of different food additives on stability of the aquatic blue pigment (n=3 ± SD).

3.4.1.6. Stability of aquatic blue pigment in metal ions

The paua blue pigment was analysed in 8 different metals in different time points (6, 12 and 24 hours). After addition of the metals to the pigment, precipitation occurred. Therefore, the samples were centrifuged at 4000 rpm, 10°C for 4 minutes and the absorbance reading was recorded after 6 and 12

hours, pigment in Al^{3+}, Zn^{2+} , Ca^{2+} , Mg^{2+} , K^{+} and Fe^{3+} was more than 80%stable. However, samples of Cu^{2+} and Fe^{2+} had a massive reduction after 6 and 12 hours. This was due to high precipitation which occurred, and because of this issue, both Cu^{2+} and Fe^{2+} samples were centrifuge every time before reading absorbance which lead to some of the pigment settle at the bottom of the centrifuge tubes. After 24 hours, all metal samples centrifuged once more, due to precipitation. After 24 hours, the blue colour was retained in most of metals more than 65%.

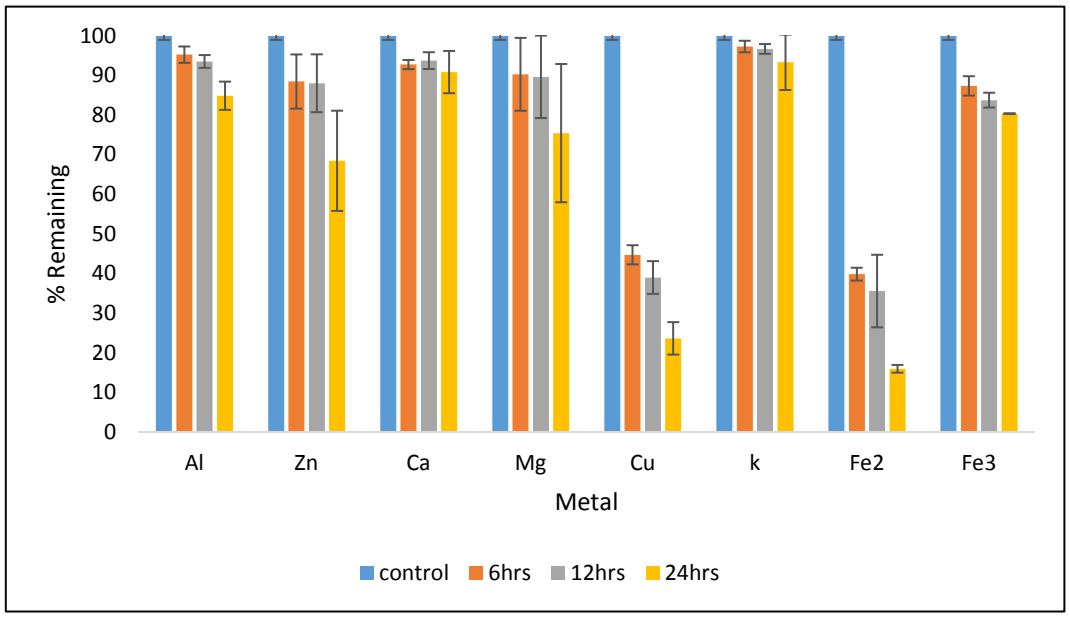

Figure 3-8. Effect of different metals on stability of the pigment (n=3 ± SD).

3.4.2. In vitro toxicity of the pigment

3.4.2.1. Effect of different concentration of aquatic blue pigment on human cell line (HEK 293)

To assess the safety of the pigment in vitro, HEK 293 cell line were exposed to different concentration of the aquatic blue pigment and the absorbance obtained at 450 nm. According to Figure 3-9, after 24 hours exposure, cells were almost 95% viable in up to 2000 µg/ml of the pigment. However, their viability decreased to about 75% in 3000 µg/ml of the pigment. Viability of the HEK 293 in 5000 µg/ml of the pigment also decreased to 55%. This indicates that, by increasing the concentration of the aquatic blue pigment, cell viability decreases. As seen in the Figure 3-10, after 48 hours exposure to the aquatic blue pigment cells were 90% resistant to the pigment in concentrations up to 1000 µg/ml.

However, cell viability was reduced to 80% at 2000 and 3000 µg/ml of the pigment. In 4000 and 5000 µg/ml pigment concentrations, cells viability decreased to 60% and 50%, respectively. Death of 50 % of the cells with 5000 µg/ml pigment indicates minimum lethal dose of the pigment in 24 hours. After 72 hours of exposure, HEK 293 cells were resistance to pigment concentrations up to 1000 µg/ml (Figure 3-11). However, cell viability decreased to 75% in 2000 µg/ml pigment concentration. Exposure of the cells to the 3000 and 4000 µg/ml of pigment over 72 hours decrease cell viability to 50% and in 5000 µg/ml to 35%. According to Figure 3-9, Figure 3-10 and Figure 3-11, HEK 293 cells retained up to 90% of their viability when exposure to 1000 µg/ml concentration of the pigment after 24, 48 and 72 hours. However, increasing the time and concentration of the pigment to over 24 hours and more than 2000 µg/ml, the cell viability decreases significantly.

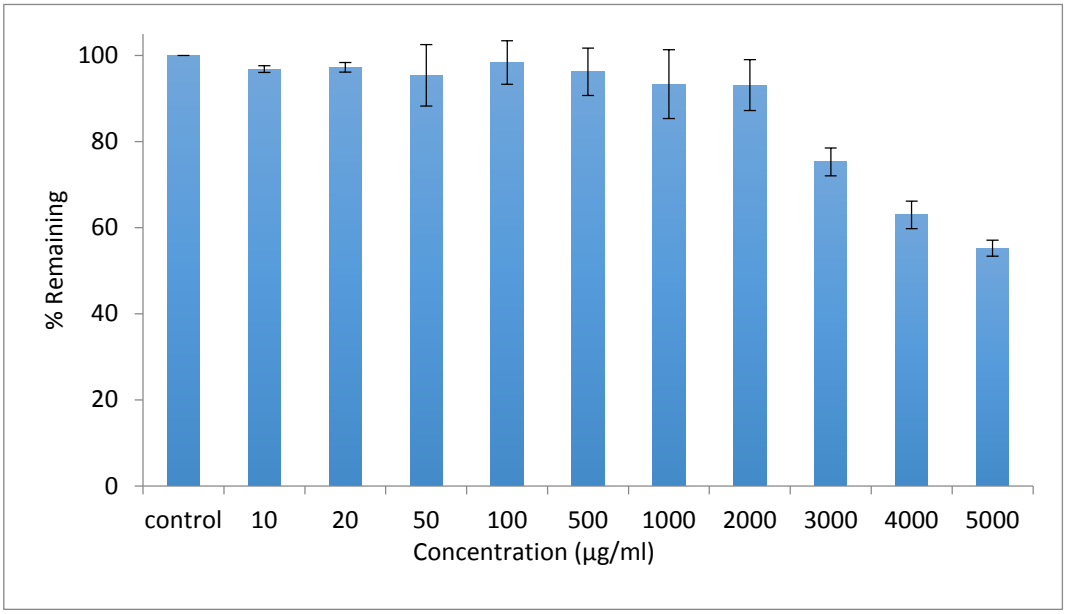

Figure 3-9. Effect of pigment on cell line (HEK293) after 24 hours (n=3 ± SD).

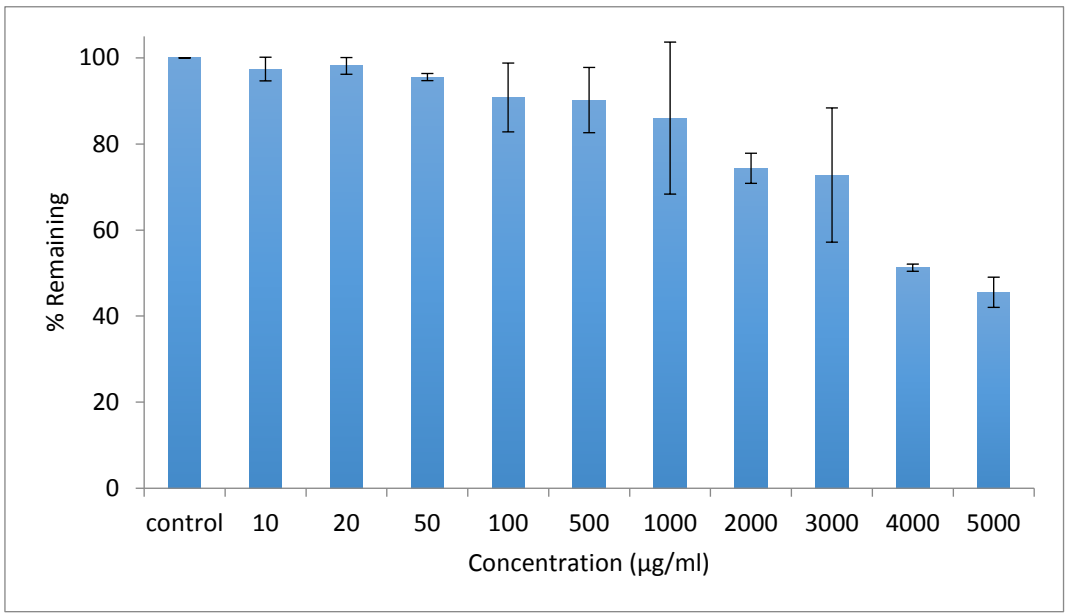

Figure 3-10. Effect of pigment on cell line (HEK293) after 48 hours (n=3 ± SD).

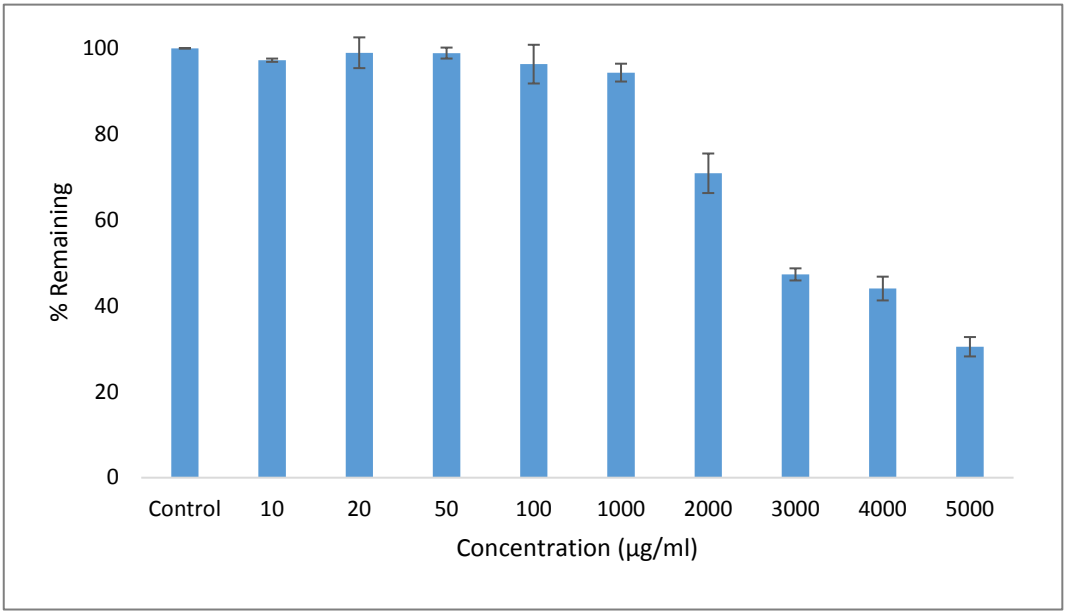

Figure 3-11. Effect of pigment on cell line (HEK293) after 72 hours (n=3 ± SD).

3.5. Conclusion

For the first time, different tests have been conducted on the aquatic blue pigment extracted from paua (Haliotis iris) shell to determine its stability when exposed to factors such as light, temperature, pH, oxidant and reducer, food additives, metals. The aquatic blue pigment lost its stability when exposed to the different types of light, such as sun light, UV light and indoor light. It was stable when stored in dark. However, the stability of the pigment in the dark decreased 30% over 10 days. Thus,

the aquatic blue pigment was not stable in light and was bleached. When the aquatic blue pigment was tested in different temperatures (25, 50, 75 and 100 °C), the pigment was stable at 25, 50 and 75°C after 3 hours. However, the absorbance of the pigment decreased 20% after 3 hours in 100°C. Therefore, the aquatic blue pigment is stable under high temperature. The aquatic blue pigment in pH stability test was stable in pH 2, 4, 6, 8 over 3 hours. However, the pigment stability reduced to almost 65% after 3 hours in pH10 and 12. These results indicate that the pigment is not stable in strong bases. In the redox stability, the absorbance of the pigment decreased with H_2O_2 and therefore the pigment oxidised. However, the Na_2SO_3 could not reduce the aquatic blue pigment and the pigment was stable. The blue pigment was highly stable in food additives over, 6, 12 and 24 hours. In the metal stability test, pigment was more than 80% stable when exposed to Al^{3+}, Zn^{2+}, Ca^{2+}, Mg^{2+}, K^{+} and Fe^{3+} over 12 hours. After 24 hours, the pigment absorbance of sample $Zn^{2+}$and Mg^{2} decreased to 60-70%. Pigment absorbance of Cu^{2+} and Fe^{2+} after 6 hours decreased to 40% and after 24 hours decreased to 20-25%. This result indicated that, the aquatic blue pigment is stable in most metals, except Cu^{2+} and Fe^{2+}. Aquatic blue pigment toxicity was tested on HEK 293. The results showed that the pigment was not toxic up to 1000 µg/ml after 72 hours. However, after 72 hours, exposure of the cell line to maximum concentration (5000 µg/ml) of pigment, the viability of the cell line decreased to 30%.

Chapter 4. Microencapsulation and Formulation of Aquatic Blue Pigment from Paua

4.1. Introduction

Colour is one of the most important aesthetic factors in foods and cosmetics. Pigments with high colour intensities can enhance product look and acceptability. Most pigments are available as crude powders or solutions. Water soluble pigments are preferred by the food industry due to easier handling and uniform distribution in aqueous preparations. In the cosmetic industry however, oil-soluble pigments are preferred as they can be dissolved in a variety of cosmetic oils, waxes and bases. Nonetheless, water soluble pigments have their place in the cosmetic preparations too, especially for those formulations that have colours in the aqueous phase.

Formulating crude pigments has several challenges. Typically, a formulation should retain or hold its colour during the shelf-life of the product in the specified storage condition. This usually means that the dye should be a stable molecule. However, most pigments are prone to photo-bleaching and chemical/oxidative degradation over time. Elevated temperatures can also affect the stability of pigments during the formulation processes that involve heat. One of the best ways to conserve the pigment from degradation is microencapsulation. Microencapsulation is defined as incorporation of core materials such as pigments, enzymes, drugs, cells and food components in form of solid, liquid or gas within a wall material that prevent the core materials from contacting the surrounding environment (Selim et al., 2008).

There are many wall materials for encapsulation of pigments, such as Gum Arabic, starch (maltodextrin), gellan gum, bees wax, stearic acids and gelatine (Zuanon et al., 2013). One of the most common wall materials used in food and cosmetics is Gum Arabic, which has high stability in the form of emulsion. Other useful wall materials are hydrocarbons such as maltodextrins which have high solubility and low viscosity and also form soft and spherical microcapsule that lead to high adhesion

between core and wall materials (Akhavan Mahdavi et al., 2016). There are several techniques of microencapsulation, such as spray drying, freeze drying, phase separation and ionic gelation. For aquatic blue pigment, a low temperature process is most suitable. In this thesis, Freeze drying was used as a microencapsulation procedure for aquatic blue pigment to prevent pigment deterioration. Freeze drying or lyophilisation is a simple technique with minimum pressure and temperature for encapsulation of water soluble components and thermosensitive compound, such as aquatic blue pigment. During freeze drying, wall materials and core materials are homogenised and lyophilised (Mahdavee Khazaei et al., 2014).

4.2. Chapter aims

The aim of this chapter is to increase the stability of the aquatic blue pigment by microencapsulation. This chapter also aims to produce cosmetic products using the extracted pigment as an example for its application.

4.3. Methodology

4.3.1. Materials

Gum Arabic from Sigma-Aldrich, USA, Maltodextrin (DE11-12), Maltodextrin (DE17-19) both from the Three Mac, New Zealand. Lipstick base (castor oil, NZ beeswax, almond oil and vitamin E), Glycerin, xanthan gum, caprylic glyceride, macadamia oil, almond oil, jojoba oil, stearic acid, Olivem 1000, geogard Ultra, Vitamin E and Glycerin based extract were purchased from Pure Nature, New Zealand. All other reagents were lab grade.

4.3.2. Microencapsulation procedure

An accurately weighed amount of aquatic blue pigment (500 mg) was dissolved in 20 ml H_2O. From this, pigment and 8 different mixtures of wall materials were prepared with the ration of (1:20) (w/w) (Table 4-1). Each wall material was dissolved in 40 ml H_2O. Then to each wall materials 2 ml pigment solution was added and mixed well. Each mixture was transferred to 50 ml centrifuge tube and frozen

by placing them in liquid nitrogen. After that, microencapsulated pigments were dried in a freeze dryer with vacuum (0.0010 mbr) at -80°C.

Table 4-1. Microencapsulation mixtures composition.

Formulation	Maltodextrin (DE11-12)	Maltodextrin (DE17-19)	Gum Arabic	pigment
1	1000 mg	-	-	50 mg
2	-	1000 mg	-	50 mg
3	-	-	1000 mg	50 mg
4	500 mg	500 mg	-	50 mg
5	330 mg	330 mg	330 mg	50 mg
6	167 mg	167 mg	667 mg	50 mg
7	167 mg	667 mg	167 mg	50 mg
8	667 mg	167 mg	167 mg	50 mg

4.3.3. Characterisation of microencapsulated pigment

4.3.3.1. Morphology of the microencapsulated pigment

A small quantity of dried microencapsulated pigment samples was placed on the stage of the scanned electron microscopy (SEM) (Thermos scientific, HITACHI, SU-70) and samples were coated with platinum by an ion sputter (HITACHI, E-1045). Then, the microencapsulated pigments were scanned for their morphology. Particle size was measured using Image J software on SEM pictures on 50 or more particles. The average size of particles for all formulations were then calculated.

4.3.3.2. FTIR analysis of the microencapsulated pigment

Microencapsulated pigments were analysed by Fourier transform infrared spectroscopy (FTIR) from thermos scientific (Nicolet iS10). FTIR spectra of the samples were obtained by placing a small amount

of sample on the diamond stage of spectra and the result was recorded in the wavenumber range of 500-4000 cm^{-1}.

4.3.4. Microencapsulated pigment stability studies

The stability of microencapsulated pigment samples (in liquid and dry forms) after exposure to an elevated temperature and varying sources of light was investigated. For both forms, 15 mg of each microencapsulated samples was placed in a 100°C water bath for 3 hours. The absorbance of the samples was obtained after 1, 2 and 3 hours at 612 nm. The effect of light on the stability of the microencapsulated pigment was analysed by placing liquid (12 mg of microencapsulated pigment in 240 µl H_2O) and solid form of the microencapsulated pigment under sun light, UV light, indoor light and in dark. After 1, 5 and 10 days the solid samples were dissolved in 240 µl H_2O and the absorbance of the solid and liquid samples were determined at 612 nm.

4.3.5. Incorporation of the extracted aquatic blue pigment in cosmetic formulations

To prepare a blue lipstick, 15.0924 g of lipstick base was melted at 75°C in a water bath. During the melting process, 0.083 g aquatic blue pigment that was previously dissolved in 15 ml ethanol (50%) and filtered through a micro pore filters (0.20mm) was added to the melted lipstick base and mixed well until ethanol was evaporated. The mixture was then transferred to the lipstick containers and left to set (Figure 4-1).

To prepare a moisturising cream, 0.4 g xanthan gum, 5 g glycerin and 0.080 g pigment were dissolved in 71.1 g H_2O. This mixture (aqueous phase) was heated at 75°C and stirred until gel formation. In the oil phase, 2 g Macadamia oil, 2 g almond oil and 2 g jojoba oils were mixed together before 1.5 g stearic acid, 4 g olivem (emulsifier) and 5 g caprylic were added to the oils and the mixture was mixed at 75°C until homogenous. At the same temperature, the oil phase was added to the aqueous phase and mixed. The mixture was removed from the heat and the heat sensitive materials vitamin E (1 g)

and Glycerin based extract (1.5 g) were added at temperature below 40°C. Finally, the cream was left in the fridge for 24 hours to be set.

4.4. Result and Discussion

4.4.1. Characterisation of the microencapsulated pigment

As seen in Figure 4-1A, formulation 1 had spherical and smooth particles with an average size of 1.32 µm +/- 0.7. Formulation 2 (Figure 4-1B) had particles with similar morphology to formulation 1. However, formulation 2 particles were slightly smaller with an average size of 1.1 µm ± 0.7. Formulation 3 (Figure 4-1C) had tiny, smooth and spherical particles with uniform particle size and an average size of 0.703 µm ± 0.3. Formulation 4 and 5 (Figure 4-1E and D) both had similar spherical and smooth particles with an average size 1.41 µm ± 0.8 and 1.187 µm ± 0.5, respectively. Formulation 5 had slightly smaller particles than formulation 4, which may be due to the presence of Gum Arabic in formulation 5. According Figure 4-1F and G, formulation 6 and 7 had spherical, oval and bigger particles than other formulations with an average size of 1.72 µm ± 0.9 and 1.65 µm ± 1.1 respectively. A number of the particles observed under SEM were less than other formulations. Formulation 8 (Figure 4-1H) had particles in various sizes with spherical or oval shapes and an average size of 1.30 µm ± 0.8. Thus, Gum Arabic in formulation 3 was an effective encapsulation agent for the aquatic blue pigment, which formed a good barrier between the pigment and the surrounding environment. Gum Arabic had smaller, more spherical particles than other formulations.

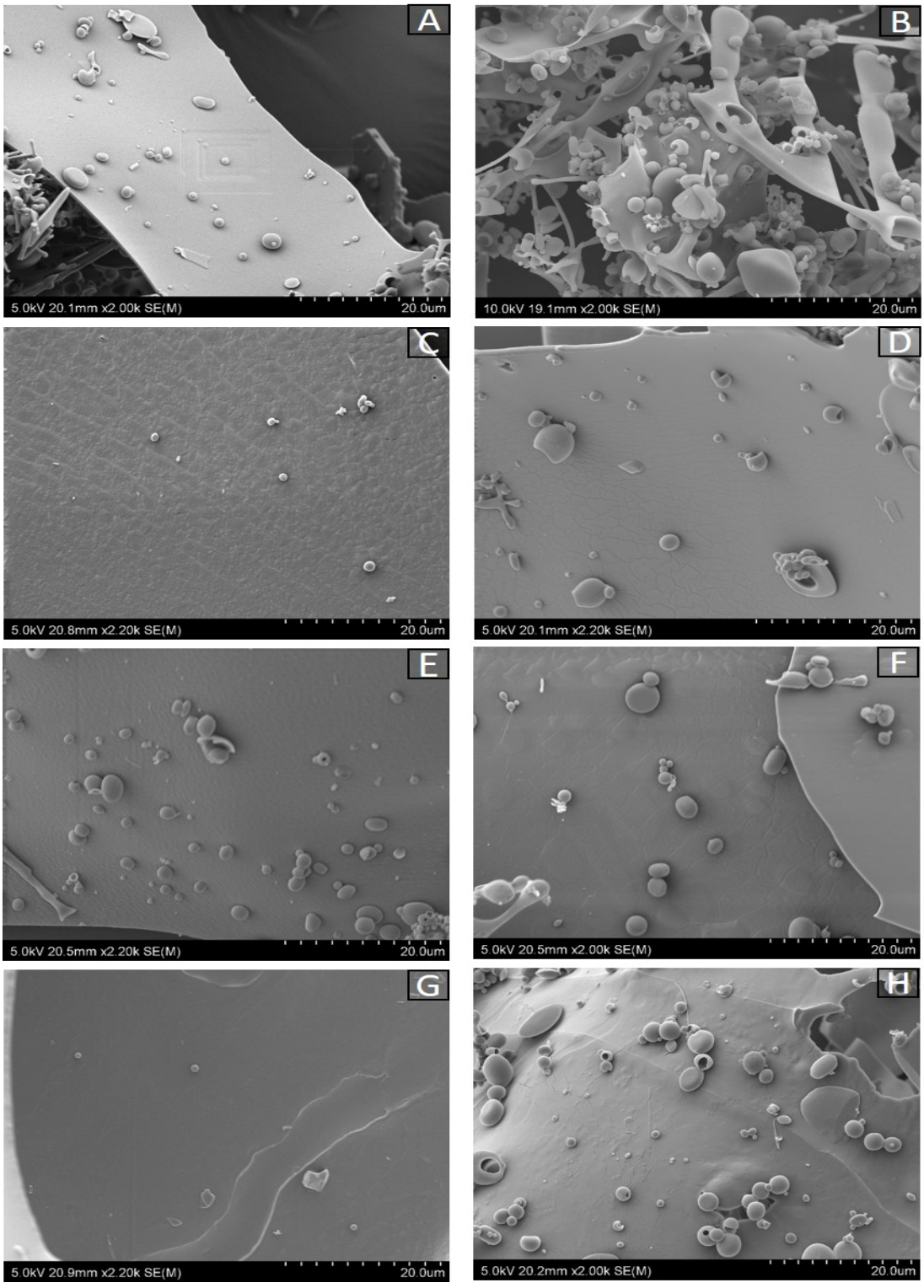

Figure 4-1. SEM picture of (A) formulation 1, (B) formulation 2, (C) formulation 3, (D) formulation 4, (E) formulation 5, (F) formulation 6, (G) formulation 7 and (H) formulation 8.

4.4.1.1. Spectral analysis of the microencapsulated pigment

FTIR was used to study the homogeneity of pigment within the particles and also to detect any chemical interaction between the materials. According to Figure 4-2, the IR spectra of all formulations and wall materials were at the similar regions and the peaks of the aquatic blue pigment were not visible in any of the formulations. This might be due to the ratio of the pigment to wall material, which was 1: 20 w/w also that, IR normally detects signals from surface of the matter. Thus, this indicates that the pigment had been encapsulated within the microparticles and IR could only hit the surface and detect the compounds at the surface of the formulations not in their core.

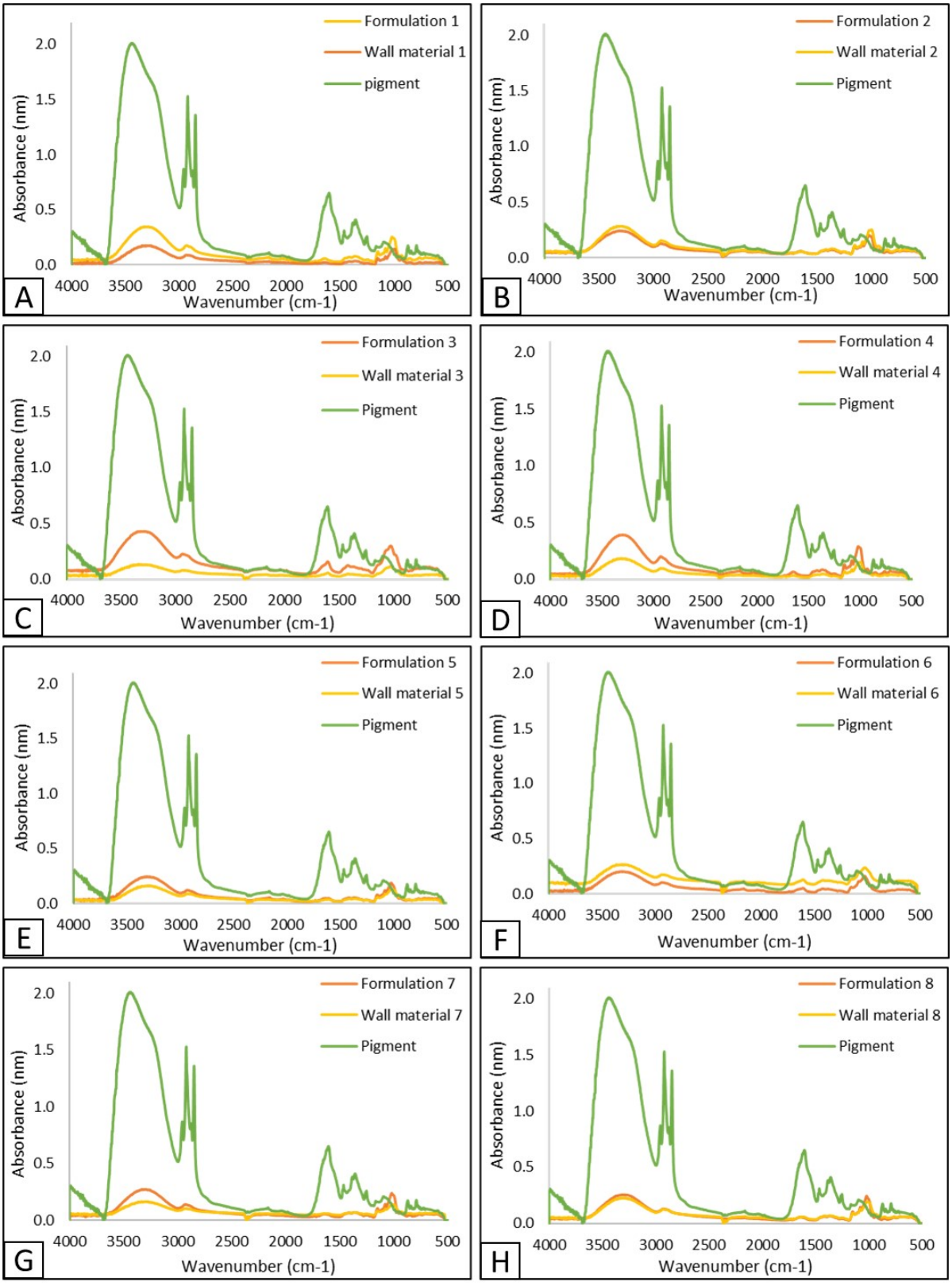

Figure 4-2. FTIR spectra of (A) pigment, formulation and wall material 1 , (B) formulation and wall material 2, (C) formulation and wall material 3, (D) formulation and wall material 4 , (E) formulation and wall material 5, (F) formulation and wall material 6, (G) formulation and wall material 7 and (H) formulation and wall material 8.

4.4.2. Photo stability of microencapsulated pigment

Stability of microencapsulated pigment in liquid form were analysed. According to Figure 4-3, the blue colour of the pigment in formulation 1 was retained and the stability of the pigment increased in comparison to non-microencapsulated pigment in indoor light from 28 ± 0.8% to 32± 2.5%, in UV from 10± 1.2% to 36 ± 1.3% and in sun light from 0 to 11 ± 3.1% at Day 10. Therefore, Maltodextrin (DE 11-12) was found to improve the stability of the aquatic blue pigment. As shown in Figure 4-3B, the blue colour of the pigment was retained and the stability of the pigment was increased by formulation 2, in indoor light from 28 ± 0.81% to 47 ± 5.26%, in UV from 10 ± 1.95% to 48 ± 6.51% and in sun light from 0 to 13 ± 3.61% at Day 10. Formulation 3 in Figure 4-3C could retain the blue colour of the pigment and its stability improved in indoor light from 28 ± 0.81% to 65 ± 5.56%, in UV from 10 ± 1.95% to 34 ± 2.88% and in sun light from 0 to 43 ± 1.39% at Day 10. The blue colour of the pigment in formulation 4 was also increased in indoor 28 ± 0.81% to 36± 0.57%, in UV from 10 ± 1.95% to 29 ± 0.26% and in sun light from 0 to 11 ± 0.50% at Day 10, which could help in retaining of the blue colour of the pigment. As shown in Figure 4-3E, the blue colour of the pigment in formulation 5 was retained and the stability of the pigment was increased in indoor light from 28 ± 0.81% to 73 ± 6.45%, in UV from 10 ± 1.95% to 41 ± 1.12% and in sun light from 0 to 48 ± 1.88% at Day 10. Formulation 6 in Figure 4-3F retained the blue colour of the pigment and could increase its stability in indoor 28 ± 0.81% to 63 ± 5.59%, in UV from 10 ± 1.95% to 36 ± 1.44% and in sun light from 0 to 23 ± 8.23% at Day 10. The blue colour of the pigment was retained by formulation 7 and its stability improved in indoor 28 ± 0.81% to 56 ± 2.11%, in UV from 10 ± 1.95% to 48 ± 3.45% and in sun light from 0 to 10 ± 1.37% at Day 10. In comparison of the formulation 8 to non-microencapsulated pigment in Figure 4-3H, formulation 8 also could preserve the blue colour of the pigment and could increase the stability of the pigment in indoor 28 ± 0.81% to 55 ± 4.09%, in UV from 10 ± 1.95% to 52 ± 6.72% and in sun light from 0 to 16 ± 7.53% at Day 10. Formulation 5 and 6 of storage in dark, in comparison to non-encapsulated pigment could increase the stability of the pigment from 67 ± 3.33% to 69± 4.67% and 72 ± 11.23% respectively.

However, formulation 1, 2, 3, 4, 7 and 8 had reduction in their stability, which might be due to the light that could penetrate to the formulations.

Stability of formulations in dry form were evaluated in Figure 4-4. When formulation 1 was stored in dark; it retained 78 ± 6.98% of its blue colour over 10 days. When it exposed to indoor light, UV light and sun light 76 5.87%, 64 ± 1.31% and 52 ± 4.88% of the colour remained after 10 days respectively. It is evident that this formulation increased the stability of the pigment. As shown in Figure 4-4B, when formulation 2 was stored in dark, 82 ± 5.69% of the blue colour was retained. When exposed to the indoor light, UV light and sunlight, 74 ± 8.17%, 76± 2.56%, 56 ± 4.88% of the blue colour was retained after 10 days respectively. This indicates that formulation 2 that contained could also increase the stability of the pigment. Formulation 3 (Figure 4-4C) could also improve the stability of the aquatic blue pigment. When formulation 3 was stored in dark, the blue colour was retained 85 ± 5.64%. it was also conserved the blue colour in exposed to indoor light 79 ± 0.9%, in UV light 81 ± 6.7% and sun light 53 ± 1.3% after 10 days. Also, in formulation 4 (Figure 4-4D), the stability of the pigment was increased and colour was retained 74 ± 2.4% when formulation 4 was stored in dark , 70 ± 5.9% in indoor light , 69 ± 3.8% in UV light and 56 ± 1.9% in sun light after 10 days. In formulation 5 (Figure 4-4E) stability of the pigment was improved which retained the blue colour 80 ± 4.3% in dark, 74 ± 1.9% in indoor light, 78 ± 2.1% in UV light and 57 ± 2.2% in sun light after 10 days. Formulation 6 (Figure 4-4F) provided high stability for the pigment in compare with non-microencapsulated pigment. Over 10 days storage of the formulation 6 in dark and exposure to the indoor light, UV light and sun light where its stability increased and the colour was retained from 87 ± 3.7%, 77 ± 2.6%, 76 ± 7.7% and 53 ± 5.9% respectively. Formulation 7 (Figure 4-4G) protected the blue colour of the pigment from fading 66 ± 0.5%, 68 ± 7.8%, 63 ± 3.1%, and 49 ± 3% when stored in dark, exposed in indoor light, UV light and sun light respectively. As stated in formulation 8 (Figure 4-4H), 72 ± 1.8%, 71± 4.1%, 67 ± 2.2% and 56 ± 3.3% the blue colour was retained when stored in dark, exposed to indoor light, UV light and sun light after 10 days respectively. All of the formulations gave high stability to the aquatic blue pigment, especially those which contained more gum Arabic. However, the most effective one was formulation

3, which contained only Gum Arabic. The high stability that the Gum Arabic has provided for the pigment was due to Gum Arabic acts as a protective colloid to the pigment (Krishnan, Bhosale, & Singhal, 2005). It has surface-active properties, high solubility and low viscosity. Its surface active and its solubility has enabled it to act as a good microencapsulation agent. Gum Arabic forms a dried film around the core materials and prevent it from contacting with surrounding environment (McNamee, O'Riordan, & O'Sullivan, 2001).

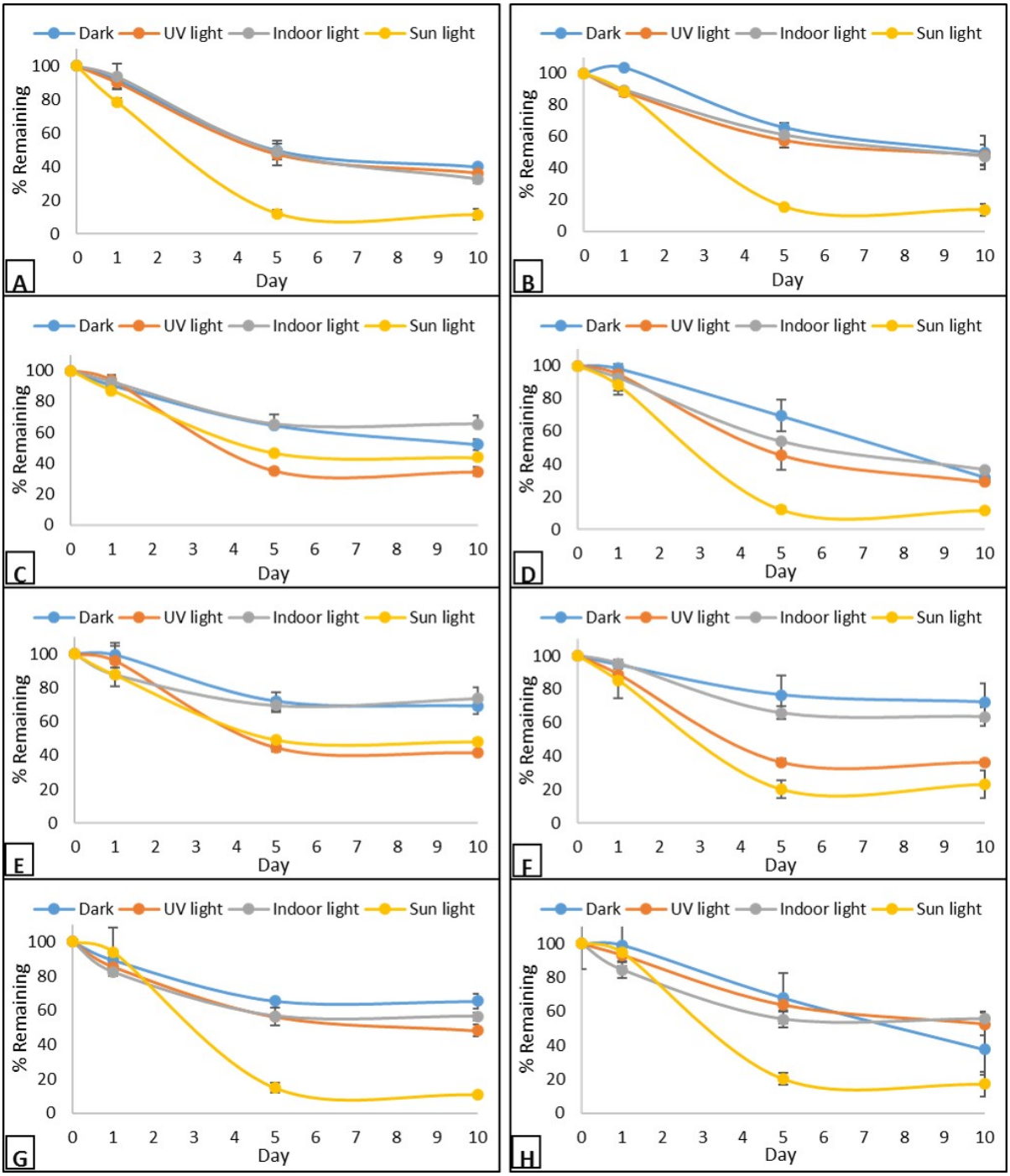

Figure 4-3. Effect of light from different sources on liquid formulation 1 (A), formulation 2 (B), formulation 3 (C), formulation 4 (D), formulation 5 (E), formulation 6 (F), formulation 7 (G), formulation 8 (H) (n=2 ± SD).

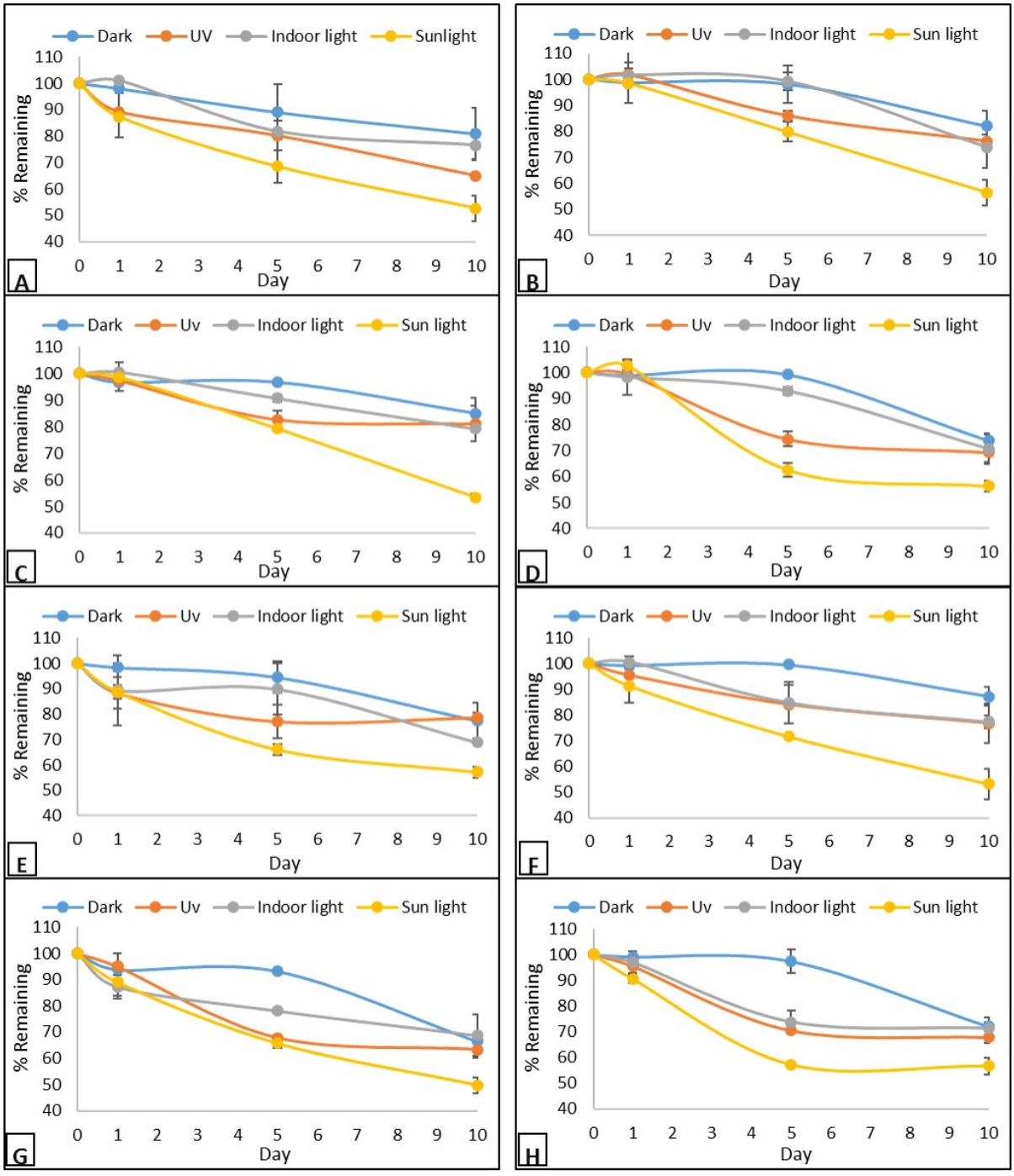

Figure 4-4. Effect of light from different sources on dry formulation 1 (A), formulation 2 (B), formulation 3 (C), formulation 4 (D), formulation 5 (E), formulation 6 (F), formulation 7 (G), formulation 8 (H) (n=2 ± SD).

4.4.3. Thermostability of microencapsulated pigment

The effect of temperature on the stability of the developed formulations in both dry and liquid form was investigated. In dry samples, the blue colour of formulation 3, 5, 6 and 8 was retained more than 90% when exposed to 100°C for 1 hour (Figure 4-5). Similarly, formulation 1, 2, 4 and 7 retained the blue colour 84 ± 0.9%, 87 ± 10.4%, 82 ± 1.2%, 81 ± 3.1% respectively. By increasing the time of exposure and after 3 hours, formulations 1, 2, 3, 5, 6, 7 and 8 retained 80± 5.2%, 68 ± 8%, 85 ± 13.1%, 87 ± 2.9%, 82 ± 3.9%, 69 ± 3.3%, 80 ± 0.6% of the blue colour respectively but, formulation 4 had decreased to 56% ± 1.43 .

In liquid form, the blue colour of all formulation was retained more than 78% after 1 hour at 100°C (Figure 4-6). After 3 hours exposure, the blue colour of the formulations 2, 3, 5, 6, 7 and 8 was retained 78 ± 4. %, 80 ± 4.8%, 75 ± 1.14%, 80± 7%, 74 ± 8.2% and 76 ± 6.1% respectively. However, formulation 1 and 5 had their stability decreased to 63 ± 10.1% and 64 ± 0.0% respectively. Therefore, by increasing time of exposure of the formulations to 100 °C, the stability of them decreases. However, these reductions were significantly smaller in comparison with unformulated original pigment stability, which was discussed in chapter 3 meaning that these formulations have indeed enhanced the stability of the pigment.

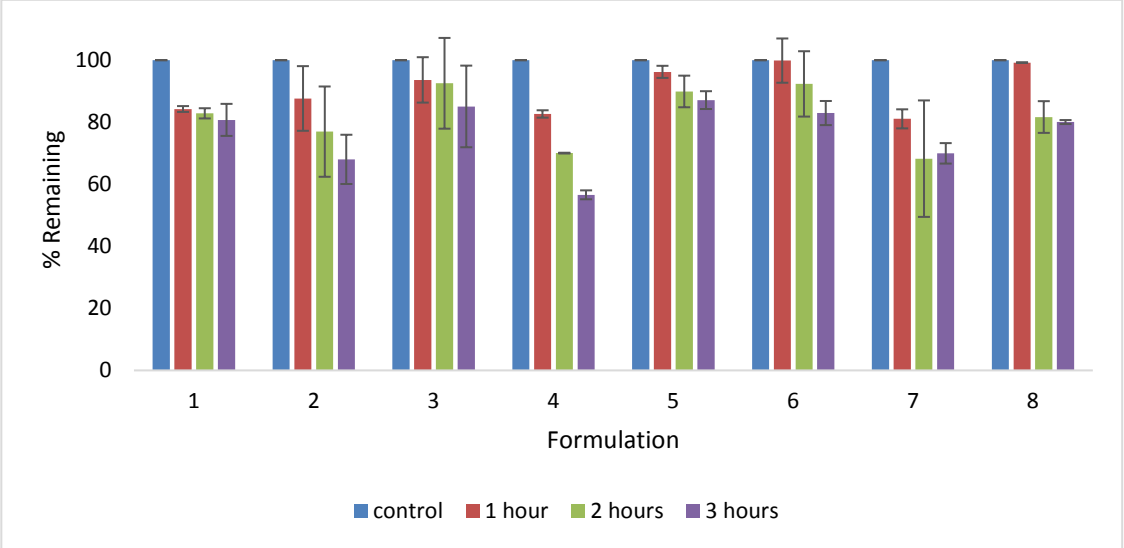

Figure 4-5. Effect of temperature (100 °C) on microencapsulated pigments (Dry) (n=3 ± SD).

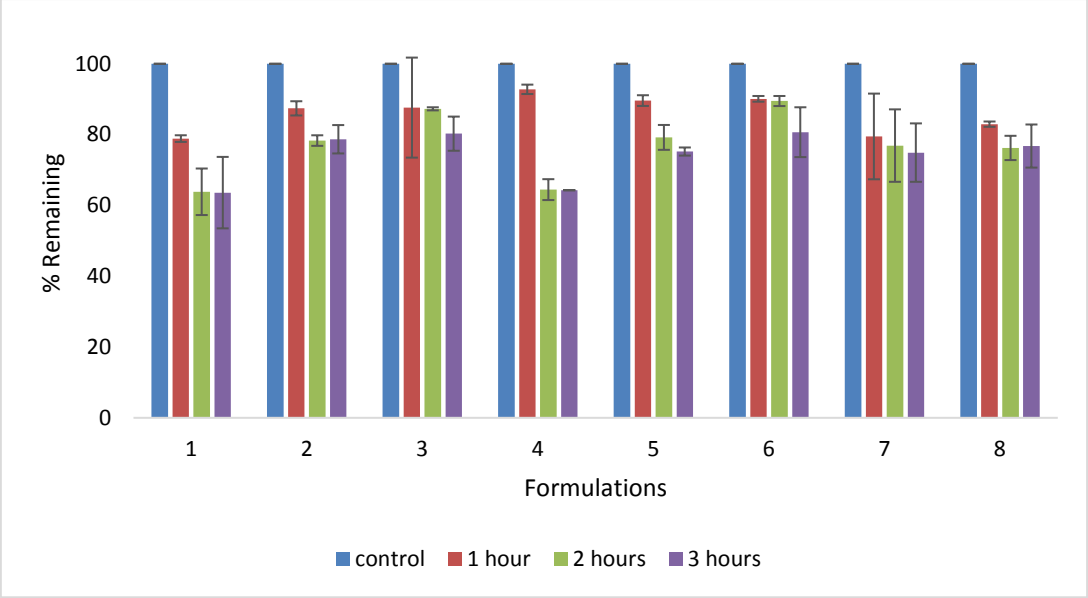

Figure 4-6.Effect of temperature (100°C) on microencapsulated pigments (liquid) (n=3 ± SD).

4.4.4. Lipstick and hand cream

As seen in Figure 4-19, after 24 hours storage of hand cream in the fridge after formulation, it was found that the presence of the aquatic blue pigment had satisfactory results. Both hand cream and the lipstick had a soft and creamy texture. The aquatic blue pigment provided a pleasant bright blue colour for both hand cream and lipstick. The blue colour remained stable over 3 months of storage at room temperature. Furthermore, the pigment had no effect on the stability of the formulations and both formulations retained their initial appearance with no changes in colour or odour throughout the storage period. In unpublished work being carried out in Drug Delivery Research Group in AUT, paua shell has been found to have antioxidant activity, which further adds to the value of this pigment to be used in niche skin care products.

Figure 4-7. Natural blue hand cream and lip balm containing aquatic blue pigment.

4.5. Conclusions

In conclusion, stability of the aquatic blue pigment was increased by microencapsulation of the pigment in different materials such as maltodextrin DE (11-12), maltodextrin DE (17-19) and Gum Arabic. Microencapsulation is a process that can be used to increase pigment stability in high temperature and exposure to light. Formulations with presence of Gum Arabic had higher stability than other formulations. SEM of the formulations showed the formation of spherical particles. Peaks of aquatic blue pigment were not detectable in all formulations. The aquatic blue pigment was used to formulate lipsticks and hand creams, which provided an appealing light blue colour for the cosmetics.

Chapter 5. General discussion and conclusions

5.1. General discussion

Colour is an important aesthetic factor in our life. Our emotional feelings such as happiness, sadness, energy, calmness and excitement can be enhanced by colours (Ou, Luo, & Wright, 2003). For example, coloured cosmetics have a positive effect on attractiveness of females and increase how pleased a woman can feel about her appearance (Etcoff, Stock, Haley, Vickery, & House, 2011).Colour also impacts on our perceptions such as taste of food. For example; in most fast food restaurants, red colour has been used to stimulate the appetite of costumers to eat more (Sing, 2007). There are 3 main colours: red, yellow and blue and other colours are developed from a mixture of main colours. Nearly all of the blue colour in the market are synthetic. The aim of this project was to develop a natural blue pigment from a natural source, paua (*Haliotis iris*) and preserving its colour by microencapsulation to be used in food and cosmetics.

In chapter 1, the literature was reviewed to provide background information about pigments, extraction procedures, safety and formulation. Firstly, the history of the pigments use and synthesis was discussed. The importance of natural and synthetic pigments was discussed. In the past, natural dyes were used mostly in textile industry. Due to instability of natural dyes synthetic pigment were developed, which were cheaper and easier to produce (Christie, 2015). Then, this chapter reviewed health issues that can be caused by synthetic pigments and how human attentions have been attracted to use more natural pigments in products. Different types of extraction and purification procedures as well as some of the stability issues associated with extracted natural pigment and various microencapsulation techniques for improvement of pigment stability were reviewed in this chapter. This chapter concluded that, a natural blue pigment could be extracted from paua shells to provide a natural alternative for synthetic blue colours. As a value added bi-product of paua

aquaculture industry in New Zealand, shell pigments can be extracted to be used in food and cosmetics.

In the second chapter, a solvent extraction technique was used to extract the aquatic blue pigment from paua shells. This extraction procedure yielded 500 mg pigment from approximately 5 kg of paua shells. The extracted pigment was then purified by solid phase extraction (SPE). High performance liquid chromatography detected a sharp peak to confirm the purity of the pigment. Maximum absorbance (λmax) of the aquatic blue pigment was determined at 612 nm in UV-Vis spectra. The aquatic blue pigment contained one alcohol group (OH) and 3 alkane groups (C-H) detected through FTIR analysis. There were different elements such as C, O, Ca, S and Si present in the pigment samples that were detected by SEM analyser. ICP-MS detected 66 different elements in the pigment. However, only calcium had significantly high concentration in the pigment that was 0.257 g/g. This high amount of calcium is due to the presence of 95% Calcium carbonate in paua shell.

The third chapter discussed the stability and safety of the aquatic blue pigment. The stability was analysed by exposing the pigment to environmental factors such as light, temperature, pH, presence of oxidant and reducer, food additives and metals. The aquatic blue pigment underwent photo-bleaching when exposed to the different sources lights such as sunlight, indoor light, UV light and even storing in the dark. It was also unstable in temperatures more than 50°C after 3 hours. The aquatic blue pigment was not stable in strong bases (pH 10 and 12). This was determined after pH stability in different pHs (2, 4, 6,8,10 and 12) but, the pigment was stable in pH values 2, 4, 6, 8 over 3 hours. In redox stability tests, the absorbance of the pigment was decreased after treatment with H_2O_2 and the pigment was oxidised. However, treatment with Na_2SO_3 did not affect the aquatic blue pigment and the pigment was stable. The presence of food additives such as starch, citric acid, sodium tartrate, glucose anhydrous, sucrose and sodium chloride did not affect the stability of aquatic blue pigment. In addition, pigment stability remained more than 70% after 24 hours exposure to different metals (Al^{3+}, Ca^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, K^{+}, Mg^{2+}, Zn^{2+}). However, addition of Cu^{2+} and Fe^{2+} resulted in 60% decrease

in colour of aquatic blue pigment after 6 hours. Embryonic kidney cells (HEK293) were treated with different concentrations of the aquatic blue pigment and the result indicated that the pigment did not affect cell viability in concentrations up to 1000 µg/ml after 24, 48 and 72 hours exposure. However, cell viability decreased to 30% after 72 hours when cells were exposed to maximum concentration (5000 µg/ml) of pigment.

Chapter 4 explored the efficacy of microencapsulation in increasing the stability of the pigment. A simple microencapsulation technique was used to protect the pigment from environmental conditions where different wall materials such as maltodextrin 11-12, 17-19 and Gum Arabic were used to create lyophilised microparticles. SEM of the formulations showed formation of spherical microparticles. Formulations that contained Gum Arabic had smaller particle size and higher stability than other formulations. Microencapsulation increased the stability of the pigment when exposed to different sources of light as well as in high temperature (100°C). FTIR analysis on microencapsulated pigment revealed the absence of pigment's peaks indicating successful encapsulation within the particles. Finally, the aquatic blue pigment was used to formulate a lipstick and hand cream as an example for its potential application. The pigment provided a pleasant light blue colour for both formulations and the blue colour of the pigment was stable over 3 months.

5.2. Limitation and future achievement

Time constrains was the major limitation in this work. The extraction and purification of the pigment was a time consuming method requiring weeks of lab labour to produce purified pigment in quantities sufficient for experimental use. The extraction yield was also limited and only about 500 mg aquatic blue pigment could be obtained from 5 kg of the paua shells which necessitated repeated extraction procedures that was extremely time consuming. This work was conducted as part of a one-year Master of Science degree and future studies should focus on reducing the time of extraction, increasing the yield and development of a better technique for purification of the pigment.

5.3. Conclusions

In conclusion, the aquatic blue pigment was successfully extracted from paua shells and formulated for use in cosmetics. The stability of the pigment when exposed to environmental conditions was significantly improved after microencapsulation.

In addition:

- aquatic blue pigment was extracted and purified using a solvent extraction and solid phase extraction technique,
- pigment stability in various environmental conditions was assessed,
- microencapsulation was used to enhance the stability of the pigment,
- in vitro cytotoxicity test of the pigment on human cell lines was conducted and the pigment was found to have a high 50% lethal dose (further toxicity studies maybe required) and
- the pigment was successfully incorporated into cosmetic formulations to demonstrate its applications.

While the results of this project are promising stepping-stones for developing a commercial natural aquamarine pigment, further studies are required to increase the extraction yield to make the process commercially viable.

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