CHAPTER 4

RESULTS AND DISCUSSION

4.1 Physical Analysis of Microplastic presence in Cockles and Mussels

The physical characterization of potential microplastics found on the tissue of cockles and mussels from Tanjong Karang, Kuala Selangor and Sebatu, Melaka were observed under light microscope (magnification 10x and 40x) and fluorescence microscope (magnification: 40x) after obtaining optimum digestion with nitric acid. The potential microplastics can be observed under light microscope due to its size range (<5 mm), but for better resolution, the microplastics can be observed using fluorescence microscope to get clearer image of microplastics presence on the tissue biota. The size of microplastics captured under light and fluorescent microscope within 10 µm to 50 µm (< 1 mm). The observation and characterization were done according to Hidalgo-Ruz and co-author (2012). For acid digestion, 69-70% of HNO_3 was widely used prior to visual sorting (Claissens et al., 2013). Visual sorting is commonly utilized to distinguish microplastics from biota (Jensen, 2017; Rummel et al., 2016), based on physical attributes such as shape, size colour and appearance (Devriese et al., 2015; Hall et al., 2015; Nadal et al., 2016; Romeo et al., 2015; Taylor et al., 2016; Terepocki et al., 2017; Vendel et al., 2017). However, the possibility of microplastics being captured within tissues making the microplastic difficult to be isolated. Therefore, prior to visual separation, oxidative, acid, alkaline or enzymatic-based digestion of tissue and gut contents is most often used (Claissens et al., 2013). However, in this experiment, the digestion using HNO_3 was sufficient since the temperature used for tissue digestion was not too high (85 ^{o}C) and the length of exposure towards the heat was not too long (20 minutes) compared to thermal degradation process which requires temperature exceed 200 ^{o}C to cause polymer degradation of plastics (Ibrahim, Abdelbagi, Ahmed and Ahmed, 2018). Furthermore, the main objective of using HNO_3 in this experiment was to achieve optimum tissue digestion of cockles and mussels which is supported by Nuelle et al. (2014) where it reported that applying strong organic acids such as HNO_3 associated with an incredibly good results for tissue digestion regarding biota tissue, mainly consisting of carbohydrates, proteins and fats. Addition to that, in this experiment, tissue digestion using HNO_3 yielding images of potential microplastics contaminant on cockles and mussels' tissues under light microscope.

4.1.1 Presence and physical characterization of microplastics on cockles and mussels from Tanjong Karang, Kuala Selangor.

Results shows the presence of microplastic in both samples harvested from Tanjong Karang, Selangor under light and fluorescence microscope. The characterization of potential microplastics images on the cockles and mussels' tissues were observed and portrayed as in Figure 4.1, Figure 4.2, Figure 4.3 and Figure 4.4. The characteristics of microplastics found under light microscope in cockles' tissues from Tanjong Karang, Kuala Selangor in Figure 1 shows (a) subangular with jagged fragment, (b) irregular shape with grooves, (c) elongated with broken edges shaped, (d) subangular fragment, (e) spheruloid-shaped pellet and (f) thin elongated filament. The colour variant observed were red, grey, black and crystalline clear (Figure 1). The crystalline clear colour of microplastics may originated from the plastic packaging (Wen, et al., 2018). Meanwhile, Figure 4.2 shows characteristics of microplastics found in the cockles under fluorescent microscope such as (a) degraded fragment (b) subangular jagged fragment (c) irregular shape with groove (d) degraded fragment with broken edges (e) subangular fragment and (f) spheruloid-shaped pellet. The colour variant observed under fluorescent microscope were orange, black, yellow, red and grey (Figure 4.2). In a study carried by Sarijan et al. (2019), microplastics found on the gastrointestinal tract of various species at Skudai River, Malaysia was characterized as fibrous, small foam, thin filament and a different colourful mixture of film and degraded fragment such as red, blue, pink, purple and white. However, the colour of microplastics can sometimes misidentified under light microscopic due to the uncertainty of exposure period and environmental conditions. Basically, microplastics that expose to the environmental conditions in uncertain periods such as physical abrasion, solar radiation and biological processes may lead to physical changes and colour variants in the microplastics (Fahrenfal et al., 2019). The most abundant characteristics found on the cockles' tissues from Tanjong Karang were degraded fragment with irregular shape as shown in Figure 4.1 and Figure 4.2.

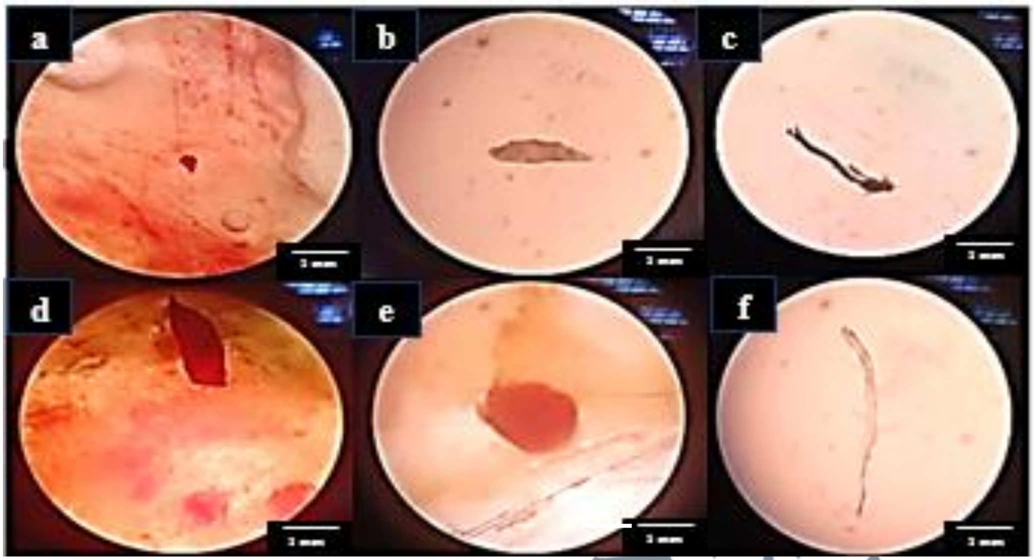

Figure 4.1: The characterization of potential microplastics on cockles' tissues from Tanjong Karang, Selangor under light microscope (magnification: 40x) which were a) subangular jagged fragment b) irregular shape with groove c) elongated fibre with broken edges d) subangular fragment e) spheruloid-shaped pellet f) thin elongated filament. The colour variant observed were red, grey, black and crystalline clear.

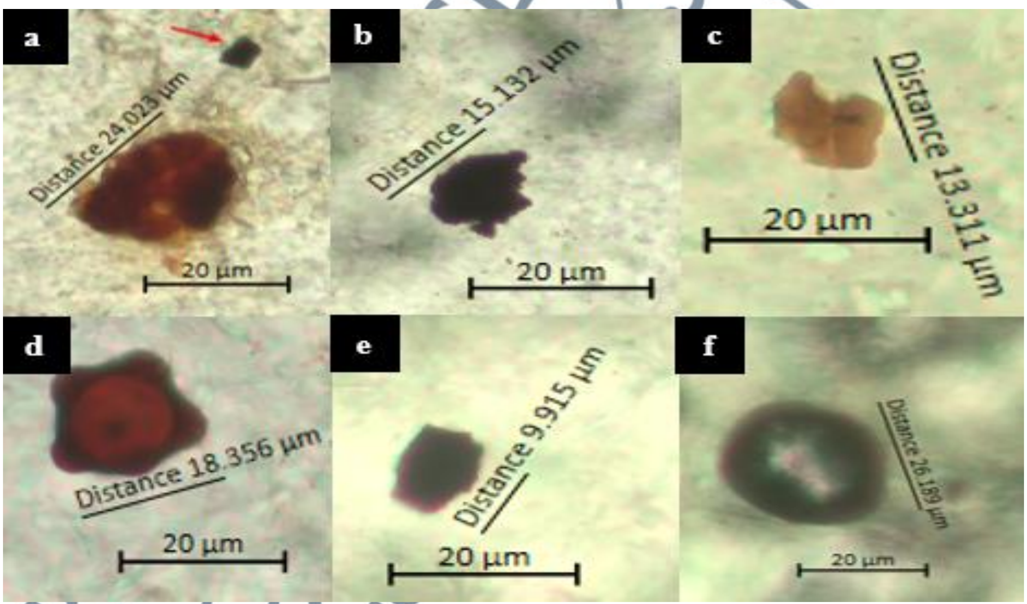

Figure 4.2: The characterization of potential microplastics on cockles' tissues from Tanjong Karang, Selangor under fluorescent microscope (magnification: 40x) which were a) degraded fragment b) subangular jagged fragment c) irregular shape with groove d) degraded fragment with broken edges e) subangular fragment f) spheruloid-shaped pellet. The colour variant observed orange, black, yellow, red and grey.

Meanwhile, for mussels's tissue observation under light and fluorescent microscope, the characterisation of microplastics found were described as thin elongated filament, degraded fragment, spheruloid shaped pellet, jagged fragment with irregular surface, and linear fracture (Figure 4.3 and Figure 4.4).

The colour variant observed from Figure 4.3 and Figure 4.4 were red, black, grey, yellow, brown and orange. The most microplastics' colour found on both cockles and mussels' tissues were grey and red. Three common shapes of microplastics found on the tissue of cockles and mussels from Tanjong Karang, Kuala Selangor were filament or fibres, fragment and pellet. These three prevalent types of microplastics found on the shellfish with variety of colours were associated with the particles originated from the degradation of intact plastic debris, which then fed by the species, dominating and accumulating in the tissues due to filter feeding properties. This intact debris may be associated with the abundance of offshore activities such as commercial fishery, waste or sewage disposal, navigation activities and shellfish culture site as key marine-based sources that contribute to the accumulation of plastic debris which end up in the tissue of filter feeders (Thushari and Senevirathna, 2020). Furthermore, environmental atmospheres, such as pH, temperature, salinity and physical abrasion caused by waves, tides and currents may lead to the diversity in the characteristics and distribution of microplastics in water environment (Zhang et. al., 2019). Others microplastics may also come from the manufacturing and packaging processes (Chaplin, Carpéné, and Mercader, 2018).

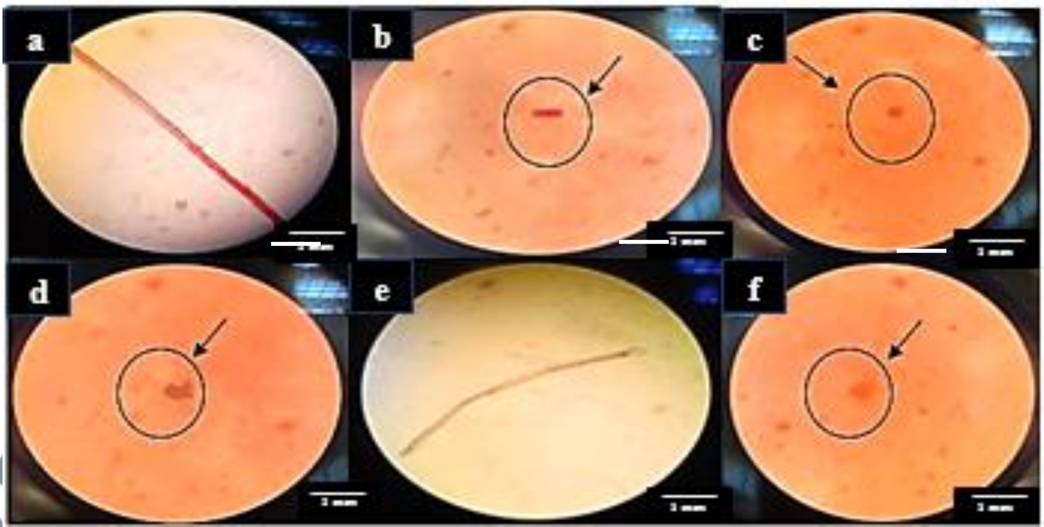

Figure 4.3: The characterization of potential microplastics on mussels' tissues from Tanjong Karang, Selangor under light microscope (magnification: 40x) which were a) thin elongated filament b) degraded fragment c) spheruloid-shaped pellet d) jagged fragment with irregular surface e) long linear fracture f) irregular spheruloid shaped pellet. The colour variant observed were red, orange and brown.

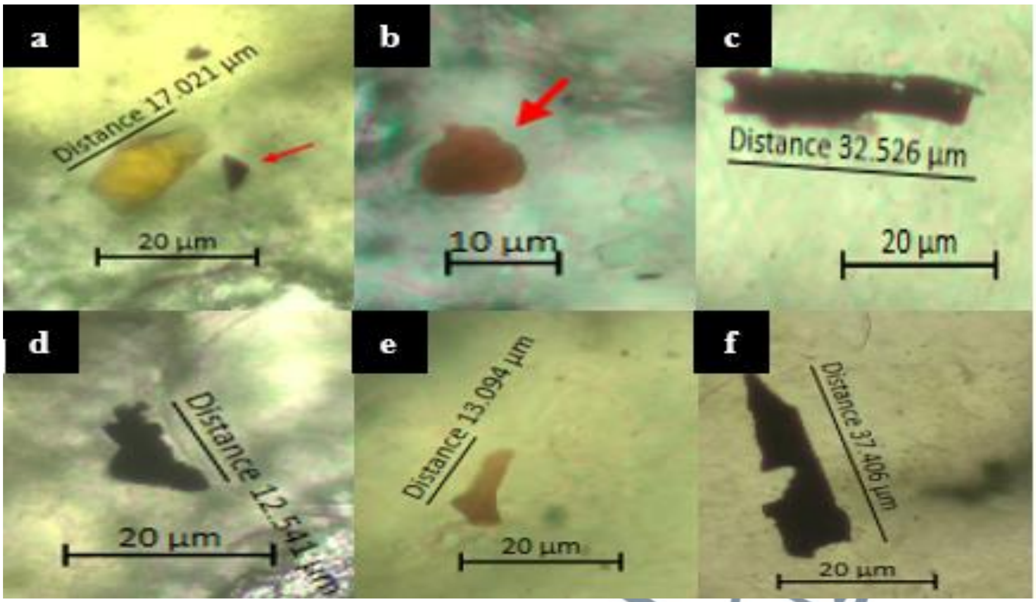

Figure 4.4: The characterization of potential microplastics on mussels' tissues from Tanjong Karang, Selangor under fluorescence microscope (magnification: 40x) which were a) & e) degraded fragment b) irregular spheruloid shaped pellet c) thick-elongated filament d) jagged fragment with irregular surface f) degraded fragment with linear fracture. The colour variant observed were yellow, red, brown and black.

As Selangor river flows in the direction of south east and trespassing about 110 km in distance before eventually passing the Straits of Malacca (Othman, Chowdhury, Wan Jaafar, Faresh, and Shirazi, 2018) making it the industrialization area focusing on mining activities that would contribute to plastic waste degradation into the sea. The most prevalent type of microplastics found in the filter feeders were polyethylene (88.4 %), followed by polypropylene (9.3 %) and polyethylene terephthalate (2.3 %) (Karbalaei et al., 2019). Therefore, it is speculated that the abundance of microplastics in the tissues of cockles and mussels were attributed to the presence of residence homes, jetty, ports and industries. The occurrence of microplastics in Tanjong Karang may be associated with the activities at the port, residential and industrial areas as well as fishing activities at the jetty. According to Lusher et al. (2015), fishing and shipping activities could be the factors of microplastics occurrence in less densely populated and non-urbanised areas.

High usage of plastic material these days such as face mask, gloves, personal protective equipment as well as food plastic packaging also increase the likely hood of microplastic contaminations in shellfish. Disposable face masks that are made from polystyrene and polypropylene (Aragaw, 2020) can leach out into the sea which taken up by filter feeders such as cockles and mussels unintentionally. These products may potentially enter the aquatic and earthbound environments in various way including flooding, leaching, littering and blowing therefore introducing to the environmental chaos caused by microplastics contaminants (Chaplin et al., 2018). Meanwhile, plastic food packaging also received high attention due to deliveries. A study reported that the most prevalent characteristics of microplastics found in Malaysian marine water were fragment or fibre type and high density polymer (>1.02 g cm^{-3}) such as polyester, polystyrene, polyamide, polyvinyl chloride, polyethylene and polypropylene (Karbalaei et al., 2019). Food plastic packaging that widely consumed during pandemics also originated from plastic polymers such as polyethylene terephthalate, polyethylene, polypropylene, polystyrene and even polyvinyl chloride (Windsor et al., 2019). The accumulation of these polymers of plastics in human food chain can cause hazards toxicity and affect human health. Therefore, the identity of microplastics polymer on cockles and mussels' landings from Tanjong Karang, Kuala Selangor were further analysed and confirmed by using FTIR spectroscopy.

4.1.2 Physical characterization of microplastics on cockles and mussels from Sebatu, Melaka.

The potential microplastics were observed on the cockle and mussels from Sebatu, Melaka under light and fluorescent microscope as shown in Figure 4.5, Figure 4.6, Figure 4.7 and Figure 4.8 respectively. The characterization of microplastics found on cockles' tissues (Figure 4.5 and Figure 4.6) were described as degraded fragment with irregular surface, jagged fragment with broken edges, thin elongated filament and spheruloid shaped pellet. The colour variant portrayed under light and fluorescence microscope were grey, yellow, red, orange, brown, green and greyish black. Almost all the microplastics found on the cockles' tissues were degraded fragments with irregular surface and shared the grey colour mostly as shown in Figure 4.5.

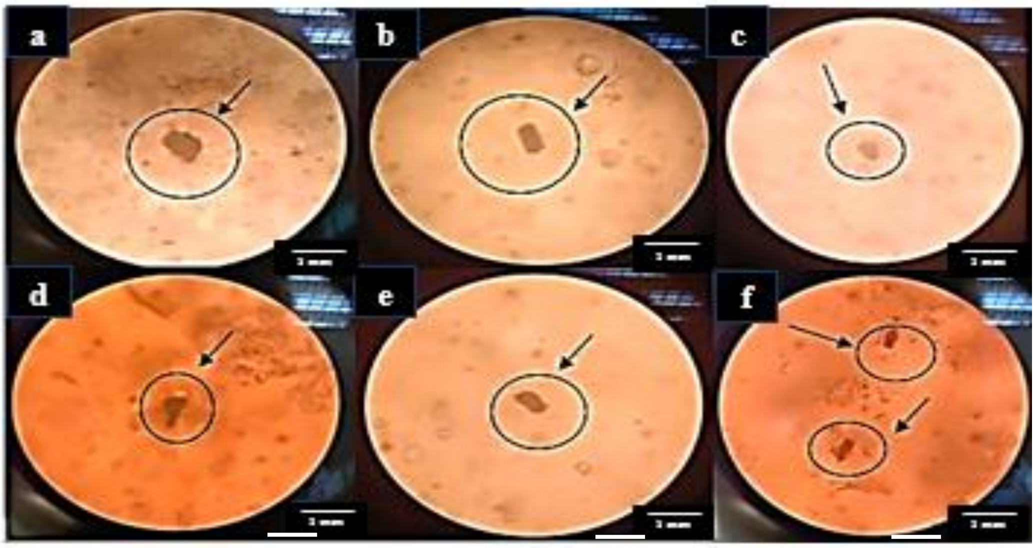

Figure 4.5: The characterization of potential microplastics on cockles' tissues from Sebatu, Melaka under light microscope (magnification: 40x) which were a) degraded fragment with irregular surface b), e) and f) degraded fragment c) spheruloid-shaped pellet d) jagged fragment with broken edges. The colour variant observed were black and grey only.

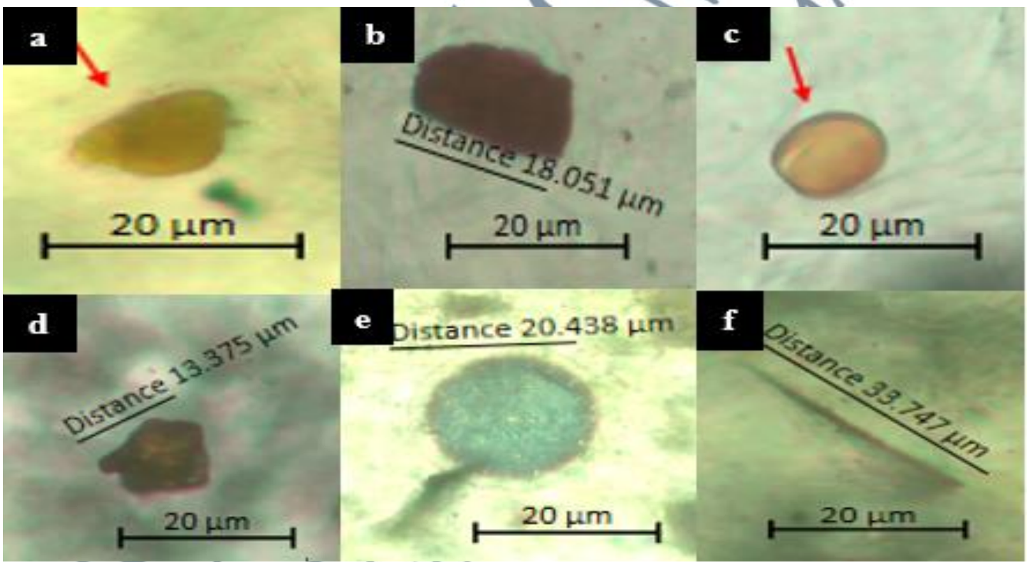

Figure 4.6: The characterization of potential microplastics on cockles' tissues from Sebatu, Melaka under fluorescent microscope (magnification: 40x) which were a) degraded fragment with irregular surface b) degraded fragment c) and e) spheruloid-shaped pellet d) jagged fragment with broken edges f) thin elongated filament. The colour variant observed were yellow, red, orange, brown, green and greyish black.

As for mussels' tissues from Sebatu, Melaka, the result in Figure 4.7 obtained under light microscope showing physical characteristics such as (a) degraded fragment with conchoidal fractures, (b) thin elongated filament with broken edges, (c) and (d) spheruloid shaped pellet with regular surface, (e) subangular jagged fragment and (f) degraded fragment. The colour variant observed in Figure 4.7 were red, black and grey. As for observation under fluorescence microscope as shown in Figure 4.8, the characteristics of microplastics found on mussels' tissues were (a) clear cylindrical fragment, (b), (c) and (f) degraded fragment, (d) subangular jagged fragment and (e) thin elongated filament with colour variant of crystalline clear, black, orange, red and green. However, the spheruloid shape pellet was dominantly found in mussels' tissues with mostly grey and red in colour. This indicates that dark colour possessed by microplastics such as black, grey, and red were ingested by filter feeders due to having the appearance of food compared to light colours (Zhao et al., 2015). Melaka, which strategically located at Straits of Malacca, is the sea pathway connecting China Sea and Indian Sea. The Strait is not just only rich in aquatic resources, but also known as the busiest shipping lanes in the world (Evers et al., 2006). Therefore, this strategic site is high likely to cause microplastics contamination due to the world trade shipping route and rapid industrial areas.

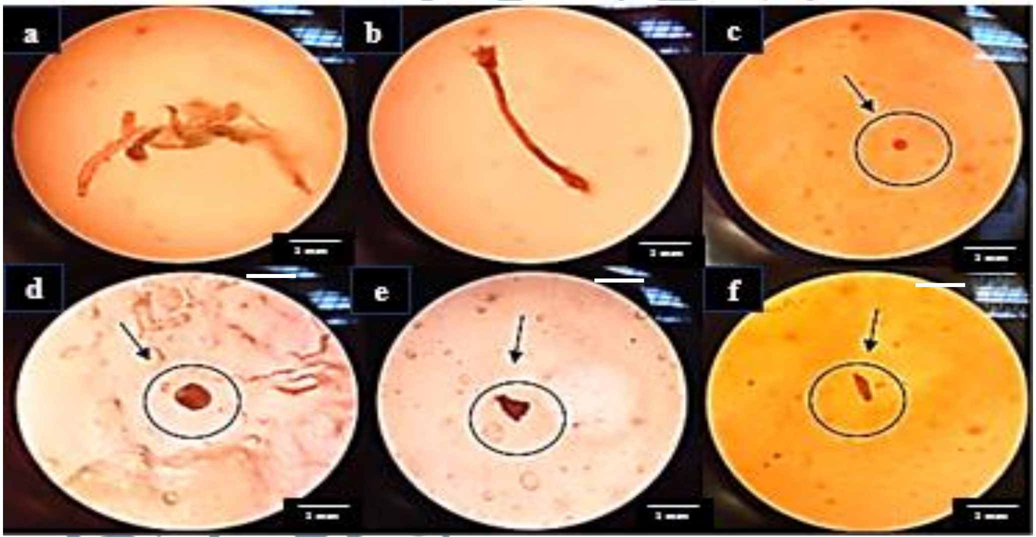

Figure 4.7: The characterization of potential microplastics on mussels' tissues from Sebatu, Melaka under light microscope (magnification: 40x) which were a) degraded fragment with conchoidal fractures b) thin elongated filament with broken edges c) and d) spheruloid-shaped pellet e) subangular jagged fragment f) degraded fragment. The colour variant observed were red, black and grey.

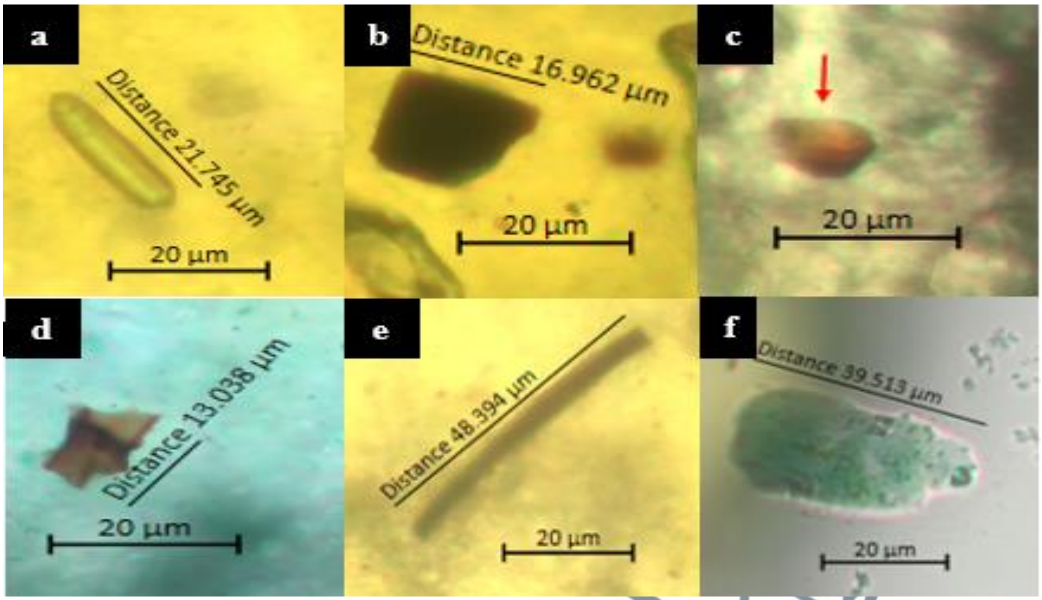

Figure 4.8: The characterization of potential microplastics on mussels' tissues from Sebatu, Melaka under fluorescent microscope (magnification: 40x) which were a) clear cylindrical fragment b), c) and f) degraded fragment d) subangular jagged fragment e) thin elongated filament. The colour variant observed were crystalline clear, black, orange, red and green.

Zhao et al. (2015) also stated that the predominance of coloured microplastics was observed in subsurface in estuaries of urban area. Different colours may give an idea about the origin of the plastic materials (Khalik et al., 2018). The occurrence of black or dark grey colour of microplastics may be associated with degradation of synthetic fibres, tyres and fishing gears due to strategic sites of Melaka which at the centre of Straits of Malacca (Zaki et al., 2021). The presence of microplastics indicates straight sources of multiple commercial activities and the primary route for ship, boats and yachts (Omar et al., 2018). As Melaka is a strategic site for high economic growth, industrialization and urbanisation, therefore, they produced higher yield of waste. Its inappropriate waste management system can contribute to microplastics contamination of marine environments. Filter feeders like cockles and mussels would unintentionally ingest the microplastics that sunk into the bottom of the sea or unintentionally ingest prey that already contaminated with microplastics. Small sized plastic particles attract these filter feeders that mistakenly ingest them as food. This indicates the potential biomagnification, bioaccumulation and trophic transfer of microplastics among organisms in the food web, which may eventually transfer to humans through shellfish consumption (Zaki et al., 2021). Colour separation may not lead us to confirmation of the debris found. Therefore, to confirm the microplastics presence on the cockles and mussels' tissues from Sebatu, Melaka, FTIR analysis were done and compared with the spectrum of the standard of polystyrene, polyethylene and polypropylene.

4.1.3 Physical characterization of microplastics on seawater samples

The area of seawater samples collected were within the coordinate of the cockles and mussels sampling site (Table 3.1). The seawater samples were collected at the area of sampling site to clarify the purity of the water in the habitat of filter feeders live. Since shellfish are filter feeders which feed by trapping suspended debris and food fragments from water surrounding, normally by transferring the water over a specific filtering structure (Larsen, 2020), therefore shellfish plays a significant role in clarifying water (Borthagaray and Carranza, 2007). Based on Figure 4.9, there were no traces of microplastics found on the seawater samples. The zone of collecting seawater samples may associated with the shellfish habitat living therefore contributing to the clarification of water with no presence of microplastics as the filter feeders have played their role in mechanism of filtering out tiny particles involuntarily. Filter feeders like cockles, mussels and oysters can filter out small fragments or particles and even toxins out of the water and enhance water clarity (Kennedy, 2019). The images obtained under light microscope were the accumulation of salt crystals due to the density separation method using sodium chloride (NaCl). Other than that, microplastics buoyancy may also associated with this un-identifiable results as the plastics particles depend on the sinking processes when they reach the ocean due to biofouling (Alfaro-Núñez et al., 2021). Biofouling and other interactions of aquatic biota such as fragmentation, degradation or leaching of additives may enhance the sinking process of plastic particles (van Sebille et al., 2019). Hence, the sunk microplastics particles at the ocean's floor may be ingested and filtered by shellfish bivalves to enhance the water clarity, therefore the presence of microplastics in the seawater samples at the cultivation site of shellfish samples cannot be detected. However, the salt particles obtained from the physical analysis were further analysed using FTIR spectroscopy to out rule the presence of microplastics particles in the seawater samples.

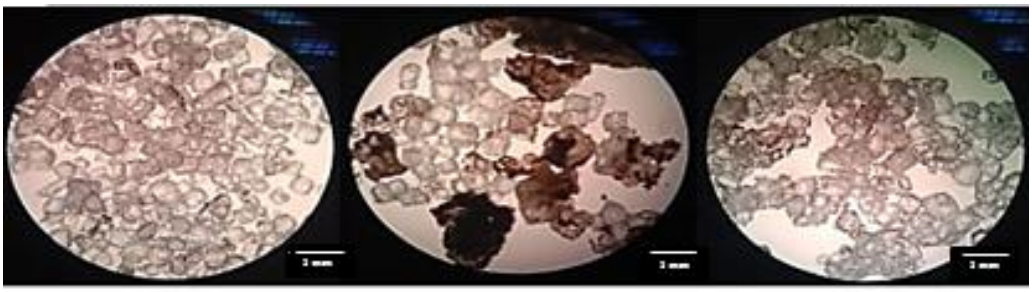

Figure 4.9: Images of seawater samples under light microscope. No traces of microplastics were found on the seawater samples. Images show salt crystals prior to density separation using sodium chloride (NaCl).

4.2 Chemical analysis using FTIR spectroscopy

A study carried out by Erni-Cassola et al. (2019), stated that the most plentiful manufactured plastic polymers found in seawater were polypropylene, polyethylene and some forms of polystyrene which are less dense than water, where they can sink at the bottom of the sea when bio-fouled due to the increased density. Multiple types of plastic polymer that are denser than seawater ($\rho > 1.02$ g cm^{-1}) which can cause sinking, which makes them most prevalently found in the deep ocean's floor (Woodall et al., 2014). Filter feeders like cockles and mussels who live at the bottom of the sea may unintentionally ingest them thus circulating microplastics in the food chain. The potential of microplastics presence in the tissues of shellfish bivalves are obtained by physical analysis using microscope. However, to prevent misidentification and overestimation of microplastics presence during observation physically, chemical analysis using infrared spectroscopy is needed to substantiate the chemical and polymer identification (He et al., 2018). Overall, the results from both samples cockles and mussels show overlapping spectrum with the standards but were slightly different on certain wavelength as the microplastics found in samples had been through chemical changes since it is high processing product and have been added with other substances throughout processing. It is crucial to note that polymer in plastics have been undergo various processing and rarely used in their pure form, as most plastics produced prior to commercial use utilize colourants, additives, plasticizers and other substances to enhance and improve polymer properties (Vinay Kumar et al., 2021). Therefore, these substances tend to alter the spectrum of the polymer in the samples making slight differences with the spectra of the microplastics' standards obtained. Secondly, the prolonged presence of microplastics exposure to the environment which intact with various factors such as physical decomposition (heating or cooling, wetting or drying or abrasive forces), biofouling by the action of microorganisms and aging of the polymer leading to the changes of polymer properties thereby altering their spectra in FTIR analysis (Kerr et al., 2013).

4.2.1 FTIR analysis on cockles and mussels' tissues from Tanjong Karang, Selangor

Polyethylene are presence into two groups, which are high-density polyethylene and low-density polyethylene. Both types are heat resistant made from petroleum and considered as safe for food packaging. However, they can potentially become jeopardize the human health if harmful substances are presence such as bisphenol A (BPA) or phthalate (Proshad et al., 2017). Moreover, previous study also reported a long time exposure of high density polyethylene towards UV ray of sunlight may bring harmful towards human health (Okunola et al., 2019). Polypropylene, on the other hand, a semi-transparent plastic and stronger than polyethylene, is considered safe for food and drinks packaging (Proshad et al., 2017) and it also the safest among all plastics due to the absence of bisphenol A (BPA). Polypropylene is unlikely to leach into food unless being exposed extensively with the UV ray of sunlight. As for polystyrene, it contains benzene that is carcinogenic towards human and its long-time effect even for a little exposure of styrene can be neurotoxic and lead to carcinogenic, cytogenetic and hematological effects (Dowty et al., 1976). However, the Plastics Foodservice Packaging group identified that the styrene exposure from the use of food intact packaging made from polystyrene is extremely low with the calculated daily intake estimation at 6.6 micrograms per person daily in which 10 000 times below the safety limit set by US Food and Drug Administration (US FDA CFR, 2019). Since these three types of plastics polymers (polyethylene, polypropylene and polystyrene) are commonly used by the industries, therefore they are selected as standards in this experiment for comparison with the spectrum obtained by the samples of cockles and mussels from two different sites in West Coast Peninsular Malaysia, Tanjong Karang, Selangor and Sebatu, Melaka. Figure 4.10 shows the overlapping spectrum of microplastics polymer standards which are polyethylene, polypropylene and polystyrene as reference to the presence of microplastics on cockles and mussels' landings.

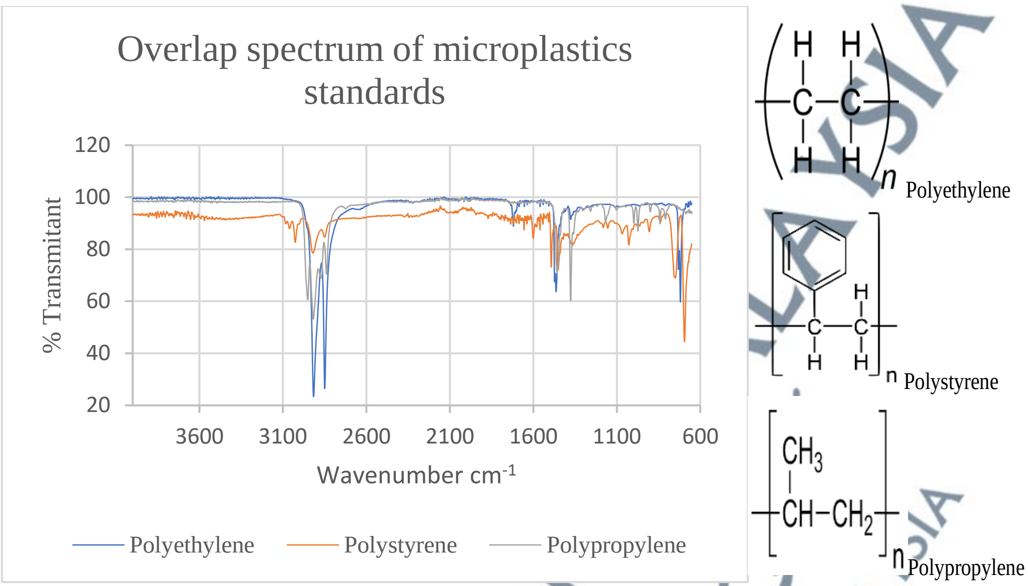

Figure 4.10: The overlapping spectrum of microplastics standards; a) Polyethylene (blue), b) Polystyrene (orange), and c) Polypropylene (grey) respectively. Images on the right shows chemical structure of microplastics standards consists of polyethylene, polystyrene and polypropylene.

From Figure 4.10, the functional groups presence in polystyrene were detected at wavenumber in the range between 2800 and 3000 cm^{-1}, also falls in the range of 1300 and 1500 cm^{-1}. The other two standards of polyethylene and polypropylene also shared the spectrum close with polystyrene when the peak obtained falls in the region within the range of polystyrene standard. Basically, the mid-IR spectrum is the most widely used in sample analysis and it is divided into four regions (Coates, 2000) which are; 1) The single bond region (2500-4000 cm^{-1}), 2) The triple bond region (2000-2500 cm^{-1}), 3) The double region (1500-2000 cm^{-1}) and 4) The fingerprint region (600-1500 cm^{-1}). The spectrum shown below 1500 cm^{-1} is fingerprint region (Nandiyanto et al., 2019). Table 4.1 is highlighting the characteristics of FTIR bands on its functional group on various wavenumber (cm^{-1}). Although polyethylene and polypropylene are both polyolefins, they can be readily distinguished and identified by FTIR (Thompson et al., 2009). When compared the spectrums of all microplastics standards, polystyrene has the highest transmittance for the functional group at wavenumber between 2800 cm^{-1} and 3000 cm^{-1}. This may be due to the density of the polystyrene which are the highest compared to polyethylene and polypropylene which are 1.04 g/cm^3, 0.86 g/cm^3 and 0.85 g/cm^3 respectively.

Among all, polystyrene has the highest density which are denser than seawater (1.02 g/cm^3) thus making it sunk deeper at the sea while the other two standards have density lower than seawater thus floating at the surface of the water (Cronin and Ouellet, 2016).

Table 4.1: Characteristic of FTIR bands' functional groups on different wavenumber (cm^{-1})

Wavenumber (cm^{-1})	Functional groups
3400	O-H stretching vibrations of hydrogen-bonded hydroxyl groups in polymeric association
2930	Asymmetric aliphatic C-H stretch vibrations-methylene (CH_2)
2850	Symmetric aliphatic C-H stretch vibrations-methylene (CH_2)
1610	Aromatic ring (C=C in plane) stretching symmetric
1510	C=O stretching vibrations
1458	Asymmetric aliphatic C-H deformation of methylene and methoxyl
1430-1420	Aromatic C=C stretching vibrations
1370	Symmetric aliphatic C-H bending of methyl groups (CH_3)
1266	C-O stretch vibration (in lignin-gualacyl ring and C-O stretch)
1224	C-O stretch vibration (in lignin-gualacyl ring and C-O stretch)
1031	C-O-H deformation in cellulose
822	Aromatic out-of-plane-rings with 2 neighboring C-H groups
-534	Si-O-Al^{VI} vibrations (Al in octahedral coordination) of clay minerals
-468	Si-O-Si bending vibrations of clay minerals

Source: Oikonomoipoulos et al. (2010)

The presence of microplastics on cockles and mussels' tissues were confirmed as shown in the Figure 4.11 and 4.12 respectively. The mean spectrum peaks obtained by cockles from Tanjong Karang similar with the spectrum of the microplastics standards indicates that the presence of microplastics from physical analysis using light microscope are confirmed. From the graph, the peaks appear at the wavenumber between 2800 cm^{-1} and 3000 cm^{-1}, which also similar with polystyrene standard peaks (2918.5 cm^{-1}) indicating functional group of CH_2 (symmetrical stretching) and CH_2 (asymmetrical stretching) (Razak et al., 2018). The visible peak at 1700 cm^{-1} represents the functional group of C=O vibrations (Zarshenas et al., 2015). The subsequent peak at 1300 cm^{-1} shows NO_2 stretch with strong intensity. The absorption bands at 800 cm^{-1} and 900 cm^{-1} were associated with vinylidene C-H out of plane bend and trans C-H out of plane bend respectively (Jung et al., 2018). The spectrum that appeared in this fingerprint region originate in interacting vibrational modes resulting in a complex absorption pattern.

Nevertheless, this region is quite complex and is hard to interpret, but each organic compound exerts its own unique absorption pattern in this region. Therefore, a number of infrared spectrum will be used to verify a compound by comparing it with a sample of a known compound (Nandiyanto et al., 2019). Figure 4.12 shows the mean of mussels' tissues which also shared similarly close to the mean spectrum of cockles' tissues. When compared to the spectrum of the standards, the overlapping spectrum at the wavenumber 2800 cm^{-1} and 3000 cm^{-1} were assigned to the =C-H stretch similar with the spectrum of cockles' tissues mentioned above. This confirmed the presence of polyethylene, polypropylene and polystyrene in mussels' tissues as well as cockles' tissues that may be associated with the locations of Tanjong Karang, Kuala Selangor, in which it is heavily influenced by wastes from fishing and maritime activities as well as industrial and residential areas. Overall, polyethylene, polypropylene and polystyrene are the common plastics used abundantly in packaging industry (food and plastics bottles), textiles industry (Claessens et al., 2011; Di and Wang, 2018) and cosmetic products (Cole et al., 2011).

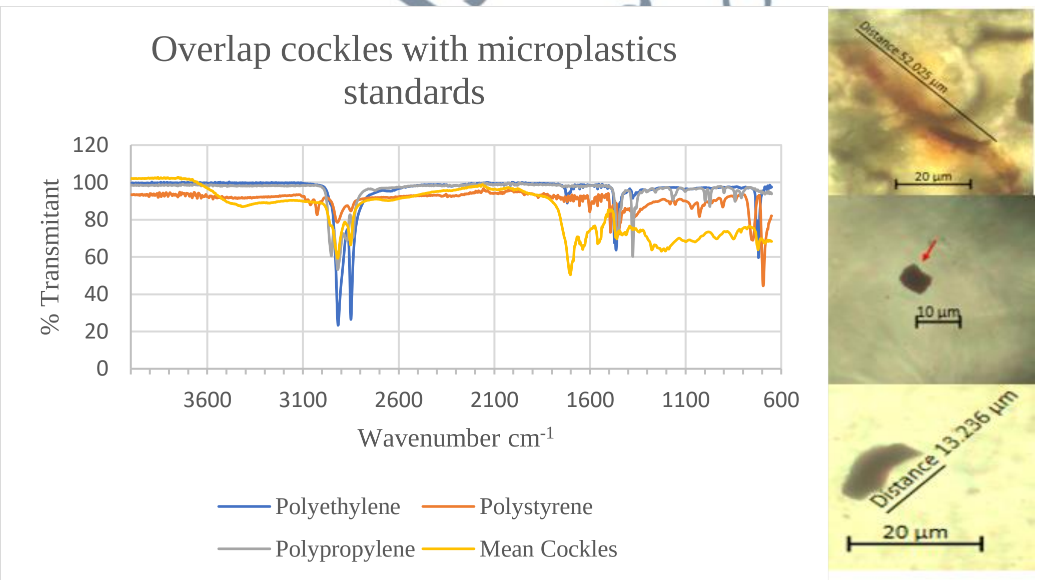

Figure 4.11: The mean spectrum of cockles (yellow) from Tanjong Karang overlapping with microplastics standards; a) Polyethylene (blue), b) Polystyrene (orange), and c) Polypropylene (grey) respectively. Images on the right show presence of microplastics on cockles' tissues under fluorescent microscope (magnification: 40x) with characteristics of thin elongated filament, degraded filament and subangular jagged fragment.

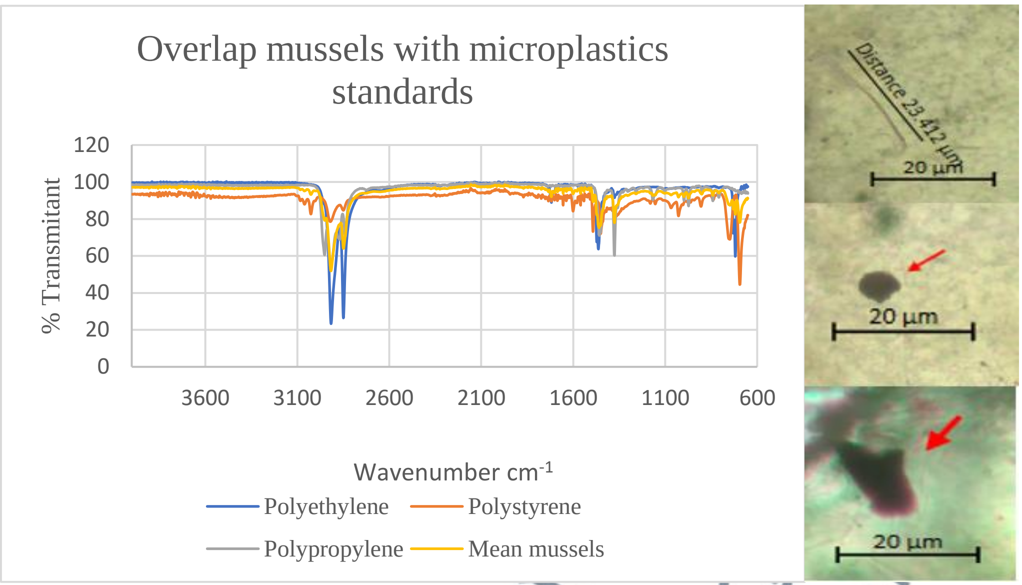

Figure 4.12: The mean spectrum of mussels (yellow) from Tanjong Karang overlapping with microplastics standards; a) Polyethylene (blue), b) Polystyrene (orange), and c) Polypropylene (grey) respectively. Images on the right show microplastics presence on mussels' tissues under fluorescent microscope (magnification: 40x) with characteristics of thin elongated filament, spheruloid shaped pellet and degraded fragment.

4.2.2 FTIR analysis on cockles and mussels' tissues from Sebatu, Melaka

Sebatu, Melaka has been associated with predominantly contamination by various pollutants including microplastics due to its strategic sites as busiest shipping routes, shipping activities and transportation (Looi et al., 2015). Therefore, it is expected to detect the occurrence of microplastics contaminants on cockles and mussels' tissues. The results (Figure 4.13) show similarity of mean spectrum peaks of cockles with the spectrum of the standards (polyethylene, polypropylene and polystyrene). As Melaka also known as eco-tourism region (Alam et al., 2014), other than its strategic sites as the medium routes in transportation and trading activities, it also well known for its historical stories and buildings, therefore, these features attract more tourists and increase population in Melaka area thus introducing more plastics in the environment either through food plastic packaging, textiles or domestic wastes.

The similar spectrum peaks of the microplastic isolated from the samples were closely similar with the spectrum of cockles from Tanjong Karang, Kuala

Selangor due to the same species and the organic compound presence in the tissues. In addition, when comparing with the spectrum of the microplastics polymers' standards, the obvious peaks of cockles' tissues also similar at wavenumber between 2800 cm^{-1} and 3000 cm^{-1}, 1700 cm^{-1} and some spectrum on the fingerprint region. This proved the presence of microplastics such as polyethylene, polypropylene and polystyrene on cockles' tissues from Sebatu, Melaka. The spectrum obtained by the cockles' tissues were closely similar with mussels' tissues as shown in the Figure 4.14.

When compared the results of cockles and mussels from both Sebatu, Melaka and Tanjong Karang, Kuala Selangor, both shared similarity in terms of the presence of functional group and overlapping spectrum with the standards. Based on the results obtained, filter feeders like cockles and mussels can easily access to the accumulation of the microplastics in the seawater and directly ingest them when mistaken as food. These filter feeders cannot be selective and prevent from ingesting other particulates that may be resent unintentionally (Cole et al., 2013). Even though some organisms have developed adaptations and modifications to avoid the consumption of unwanted materials, that can prevent ingestion of particles larger than a specific size using modified mesh size of gill rakers and other anatomical components (Rahman, 2019), microplastics can still being consumed because these adaptations have occurred over thousand year ago compared to this current issue of microplastics that just raised concern globally for less than a century (Lusher et al., 2017).

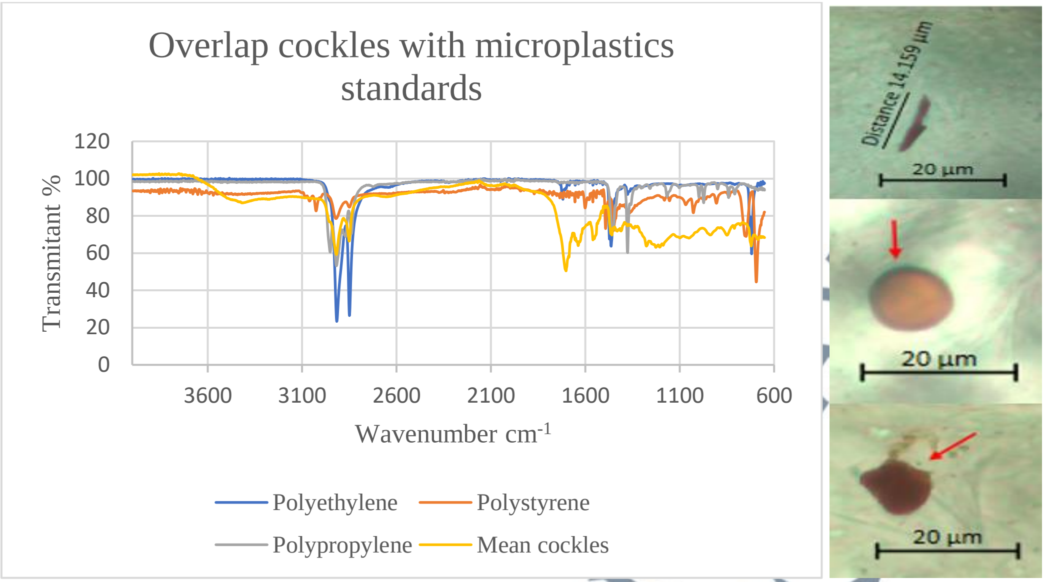

Figure 4.13: The mean spectrum of cockles (yellow) from Sebatu, Melaka overlapping with microplastics standards; a) Polyethylene (blue), b) Polystyrene (orange), and c) Polypropylene (grey) respectively. Images on the right show microplastics presence on cockles' tissues under fluorescence microscope (magnification: 40x) with characteristics of elongated filament, spheruloid shaped pellet and degraded fragment.

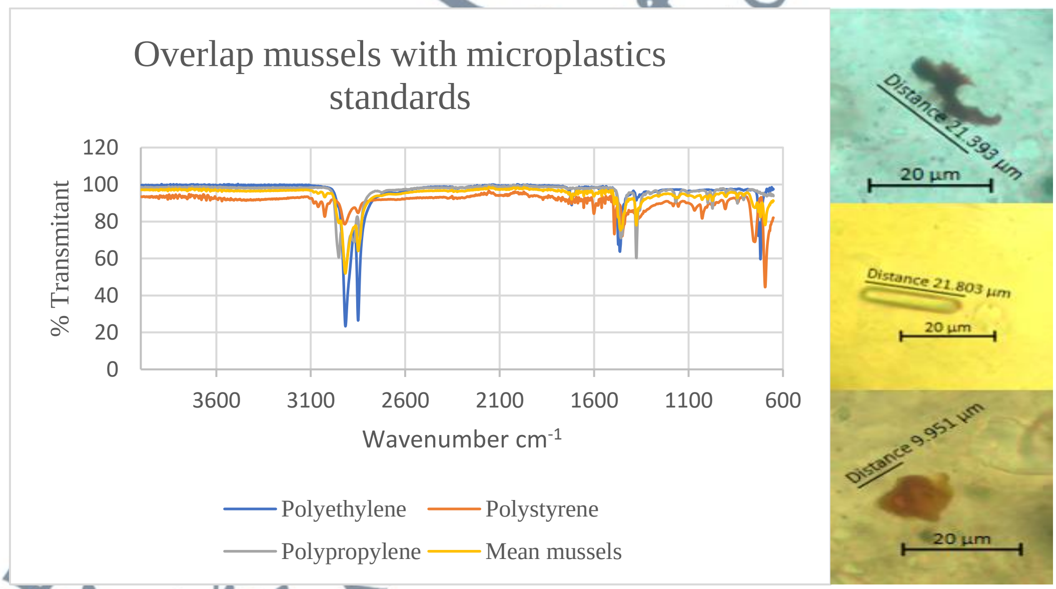

Figure 4.14: The mean spectrum of mussels (yellow) from Sebatu, Melaka overlapping with microplastics standards; a) Polyethylene (blue), b) Polystyrene (orange), and c) Polypropylene (grey) respectively. Images on the right shows microplastics presence on mussels' tissues under fluorescence microscope (magnification: 40x) with characteristics of subangular jagged fragment, thin elongated filament and degraded fragment.

4.2.3 FTIR analysis on seawater samples

As expected, the results obtained in FTIR analysis able to confirm the presumption of previous physical analysis where there was no presence of microplastics on the seawater samples from both sampling sites, Tanjong Karang, Kuala Selangor and Sebatu, Melaka. From Figure 4.15, the spectrum between 3000 cm^{-1} and 2800 cm^{-1} showed no overlapping spectrum of seawater samples with the standards of microplastics. This region is a very significant functional group for plastic polymers where this indicates the presence of functional group of aliphatic and aldehyde compound (Nandiyanto et al., 2019). Therefore, absence of these functional groups means absence of the polymeric substances crucial for the microplastics configuration. The absence of microplastics in seawater samples may be due to the presence of filter feeder's community at the sampling site which also play a big role in clarification of water. From the graph (Figure 4.15), the obvious spectrum of mean seawater was seen at the wavenumber 3400 cm^{-1} which associates with the O-H stretching vibrations (Oikonomoipoulos et al., 2010), as the presence of hydroxyl group (O-H group) was originated from H_2O compound in the seawater. The sample pre-treatment that was subjected to wet peroxide oxidation using hydrogen peroxide (H_2O_2) also contributes to the O-H stretching vibrations of hydrogen bonded hydroxyl groups in polymeric association. In microplastics standards, there were no O-H configuration in the functional group of polyethylene, polypropylene and polystyrene, therefore, this presence of O-H group denied the presence of microplastics in the seawater samples.

Other studies reported the presence and emergence of microplastics in Malaysian marine waters such as in Kuala Nerus and Kuantan port (Khalik et al., 2018), two selected beaches from Kuching namely Santubong and Trombol (Noik et al., 2015) and selected beaches in Kuala Terengganu such as Teluk Kemang, Batu Burok, Tanjung Aru, Seberang Takir and Teluk Likas (Fauziah et al., 2015). The occurrence of microplastics in these areas were associated with anthropogenic activities, industrialization areas and recreational sites. Other than that, the different method of accessing microplastics on the water samples and different sampling locations were also contribute to the contradict results of microplastics occurrence in these areas. However, for this study, filter feeders like cockles and mussels can filter out small fragments or particles and even toxins out of the water and enhance water clarity (Kennedy, 2019). As for seawater at Tanjong Karang, Selangor and Sebatu, Melaka, the areas on collecting the water samples were the cultivation sites for cockles and mussels sampling where these filter feeders contribute for filtering the water column of fragments or debris (Noren et al., 1999) by actively pumping water across the filtering organs, as most of the cultured marine-suspension feeders can trap small fragments and particles up to >4 µm with 100 % efficiency under optimal conditions (Riisgard, 2001).

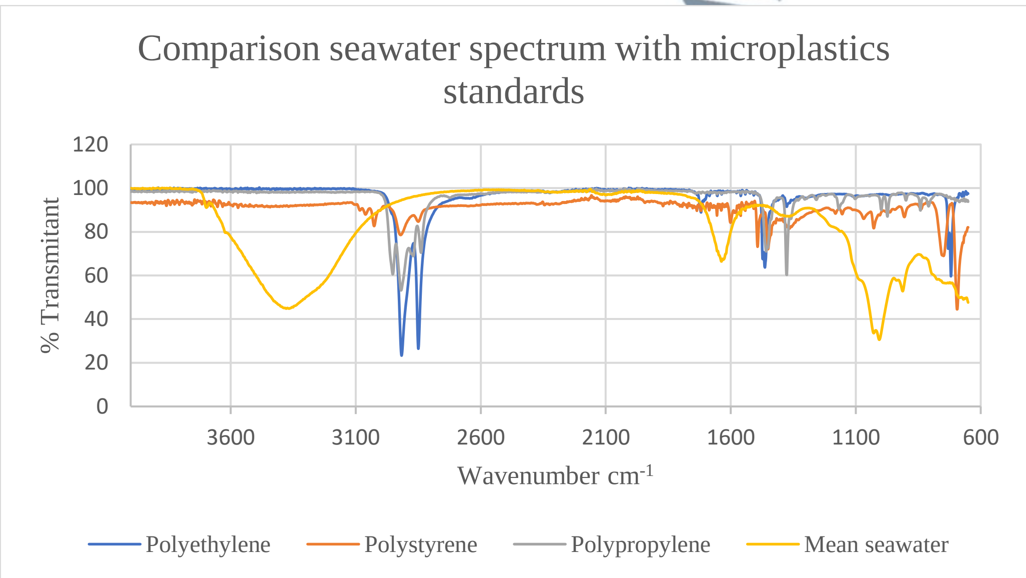

Figure 4.15: Comparison of seawater spectrum (yellow) with microplastics standard; a) Polyethylene (blue), b) Polystyrene (orange), and c) Polypropylene (grey) respectively.

4.3 Principal component analysis (PCA) on shellfish samples

The shellfish samples from FTIR analysis were analysed using Principal Component analysis (PCA) to compare the occurrence of microplastics presence between two different samples, cockles and mussels at two different sampling sites at West Coast Peninsular Malaysia, Tanjong Karang, Selangor and Sebatu, Melaka. PCA results from Figure 4.16 indicates that two variables (sample type and sampling sites) described the overall trend or spatial distribution of cockles and mussels of two sampling sites in two dimensional coordinates. Figure 4.16 shows principal component 2 (PC2) plotted against principal component 1 (PC1) in a PCA from the data set obtained from FTIR analysis on two samples, cockles and mussels from two sampling sites, Tanjong Karang and Sebatu, Melaka with PC1 and PC2 explaining >95% of the total variance. Most the samples displayed strong discrimination within each sample from two different sites with total variation of 96% thus clearly could discriminate two different samples, cockles and mussels from two strategic sites, Tanjong Karang and Melaka. This data supports the variation in the terms of microplastics distribution on cockles and mussels from Tanjong Karang and Melaka as the location were associated with offshore and anthropogenic activities that contribute to the high distribution of total variance (Looi et al., 2015). PC1, explaining total variance of 72%, had high loadings of cockles and mussels from Melaka which are positioned at the left side of the plot (negative direction). This component represents the correlation of the cockles and mussels came from Melaka which mainly associated with the global shipping route at Straits of Malacca (Zaki et al., 2021). PC2, explaining 24% of total variance, was dominated by cockles from Tanjong Karang and some sample of mussels from Tanjong Karang since these samples were positioned in positive direction (right hand-side of the score plot). The PC2 represent another cluster of cockles and mussels from Tanjong Karang which are in correlation when these samples located at Tanjong Karang with less trading activities compared to Melaka but focus on fishing and shipping activities (Lusher et al., 2015). Based on the PCA distributions, chances of detecting microplastic is higher in mussels harvested in Tanjong Karang and cockles harvested in Melaka in microplastic contamination environment compared to cockles harvested in Tanjong Karang and mussels harvested in Melaka, thus the result is inconclusive. More study on microplastics with more sampling number or larger scale at these two sampling sites are required to analyse the pattern of the microplastics' distribution. In terms of spatial distribution, the position of cockles and mussels were distributed inside the outlier, with some samples show similar fingerprinting, coming from two sampling sites, Tanjong Karang and Sebatu, Melaka with high microplastics abundance causing similarities in the same component. A few samples of cockles and mussels from Tanjong Karang and Melaka respectively were discriminated far apart from each other may be due to replication purposes. Seasonal changes, environmental conditions and also human error may lead to variations of microplastics occurrence (Zhang et al., 2019).

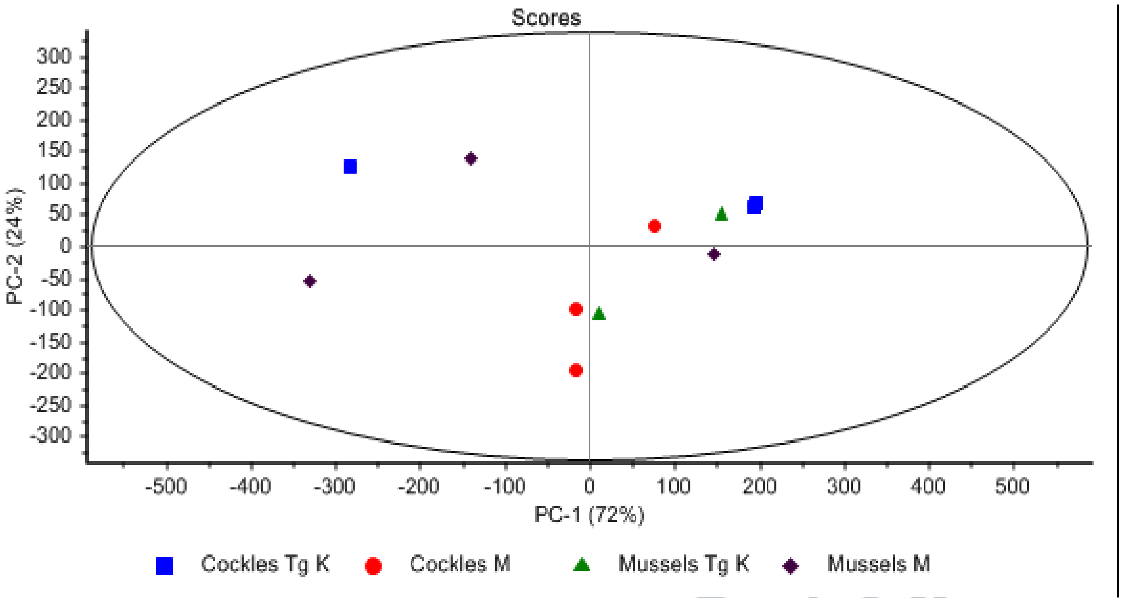

Figure 4.16: Score plot between the selected principal components obtained from applying FTIR data set on PCA analysis. Samples labelled with different colour and symbols indicating different shellfish samples from two sampling sites.

Overall results shows that there was significant different ($\rho< 0.05$) using one-way ANOVA between cockles and mussels in Tanjong Karang, Selangor as well as between two types of shellfish from Sebatu, Melaka. The difference in location contributes to different microplastics distribution between two sampling sites as PCA results shows all the data discriminates from each other and positioned in both positive and negative direction in the matter of first and second principal component.