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DETERMINATION OF POLYCYCLIC AROMATIC HYDROCARBON METABOLITES (1-HYDROXYPYRENE AND 2-NAPHTHOL) BY GAS CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY IN SOME ROCKY SHORE MOLLUSCS CONTAMINATED WITH CRUDE OIL

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ABSTRACT: After an oil spill accident occurred along the coast of Rayong Province, Thailand in July, 2013, we investigated the effect of crude oil and polycyclic aromatic hydrocarbons (PAHs) on intertidal rocky shore molluscs in Ao Prao (Prao Bay), Samed Island. Gas chromatography-tandem mass spectrometry (GC-MS/MS) was used to determine 1-hydroxypyrene (1-HOP) and 2-naphthol (2-NAP) concentrations in three intertidal rocky molluscs; whelk (*Morula musiva*), coil snail (*Planaxis sulcatus*) and rock oyster (*Saccostrea cucullata*). We used those metabolites as biomarkers to identify molluscs contaminated by petroleum hydrocarbons. Samples were collected 5 times during August 2013 to May 2015. Results showed that 1-HOP and 2-NAP were detected in the mean concentration range of 0.039–1.421 and 0.001–0.403 ng/g wet weight, respectively. 1-HOP concentrations in an intertidal rocky shore predator, whelk (*M. musiva*), were highest among all species probably due to its feeding behaviours. Metabolite concentrations in all molluscs decreased over time. In conclusion, 1-HOP is a suitable biomarker in intertidal rocky shore molluscs contaminated with petroleum hydrocarbon, and GC-MS/MS is an effective equipment to detect these metabolites.

Keyword: 1-HOP, 2-NAP, bioaccumulation, biomarkers, GC-MS/MS, trophic chain

INTRODUCTION

PAHs are a class of high molecular weight organic compounds and compose of multiple carbon aromatic rings (Ma *et al*., 2010). Coastal marine environments can be contaminated by these compounds during incomplete combustion of fossil fuels in the atmosphere or by oil spills in the seawater (Pereira *et al*., 1999). These substances are readily assimilated by marine organisms through the skin, gills or gastro-intestinal tract and, they are bioaccumulated in organism's fatty tissues causing severe illness, abnormalities or death (Connell, 1988; Fux *et al*., 2007). After entering the animal body, PAHs undergo a series of biotransformation processes. They are rapidly transformed into more hydrophilic metabolites that are excreted; thus, marine organisms exposed to these compounds show only trace amounts of PAHs in their tissues (Varanasi *et al*., 1991; Baumard *et al*., 1999).

Pyrene and naphthalene are major constituents of PAHs, and both are listed as hazardous substances by the USEPA (Pengchai *et al*., 2009). Pyrene is metabolized to 1-hydroxypyrene (1-HOP), and naphthalene is metabolized to 2-naphthol (2-NAP); these specific compounds alter the metabolism process of marine organisms (Shin *et al*., 2011). Measurements of 1-HOP and 2-NAP in excretory products of animals have gained considerable attention as a potential biomarker for PAHs exposure. This method has been widely used as a biomarker to study PAHs exposure in biota (Jongeneelen, 2001; Lim and Shin, 2013). Biomarker studies using l-hydroxypyrene have previously been reported with liquid matrices such as fish bile, blood and urine (Schaller *et al*., 1993; Lin *et al*., 1994). However, this approach cannot be utilized when dealing with small tissue samples from marine benthic invertebrates (Chaisuksant *et al*., 1999; McElroy *et al*., 2000). The presence of this petroleum hydrocarbon can be detected using the gas chromatography-mass spectrometry (GC-MS). As part of this techniques, the gas chromatography-tandem mass spectrometry analysis (GC-MS/MS) is a more precise method that has been widely used in several organisms (Blahova *et al*., 2010). GC-MS/MS is an advanced and highly selective mass spectrometry used to identify and quantify PAHs metabolite products, as a monitoring routine of pollution by petroleum (Uhl, 1997; Blahova *et al*., 2010).

In July 27, 2013, ca. 50,000 liters of crude oil leaked at the Single Point Mooring (SPM) while discharging from a vessel to the Refinery, 20 kilometres southeast of Map Ta Phut seaport, Rayong Province, Thailand. The strong wind and current drove spilled oil eastward, which shortly reached Ao Prao (Prao Bay), of semi-closed bay at the northwest coast of Samed Island. This crude oil covered more than 600 m of the intertidal zone of Ao Prao coast for more than 1-week resulting in serious damage to the intertidal ecosystem of the bay (Pongpiachan *et al*., 2018).

The objective of this study was to determine concentration of PAHs metabolites: 1-HOP and 2-NAP in three different trophic levels of intertidal rocky shore mollusc species: predator, *Morula musiva* (whelk), herbivore *Planaxis sulcatus* (coil snail) and filter feeder, *Saccostrea cucullata* (rock oyster) from Ao Prao (Prao Bay) after the oil spill incident using GC-MS/MS technique.

MATERIALS AND METHODS

Sampling Sites

Sampling was conducted in August 2013, December 2013, March 2014 and July 2014 with an additional sampling in May 2015 at two intertidal rocky shore located at Ao Prao (Prao Bay) (12°34′11″N 101°26′48″E) and Khao Laem Ya (12°35′26″N 101°25′26″E), located northeast of the oil spill incident (12°28′23″N 101°07′38″E). Khao Laem Ya, a reference site which is a natural park, is located about 3 kilometres from Ao Prao rocky shore (Fig. 1). Oil spill covered the entire rocky shore of Ao Prao longer than one week and no longer than a month (favoured by the southwest monsoon and wave actions). The heights of the waves at the time of the spill were around 1 to 2 meters and the wind speed was around 15 to 35 km per hour (Casarotto *et al*., 2014). Khao Laem Ya was used as a reference site because it is located close to Ao Prao and species composition of macrobenthos living in its intertidal rocky shore are similar to those at Ao Prao (Ganmanee, unpublished data). Furthermore, this reference site was not impacted by the spill.

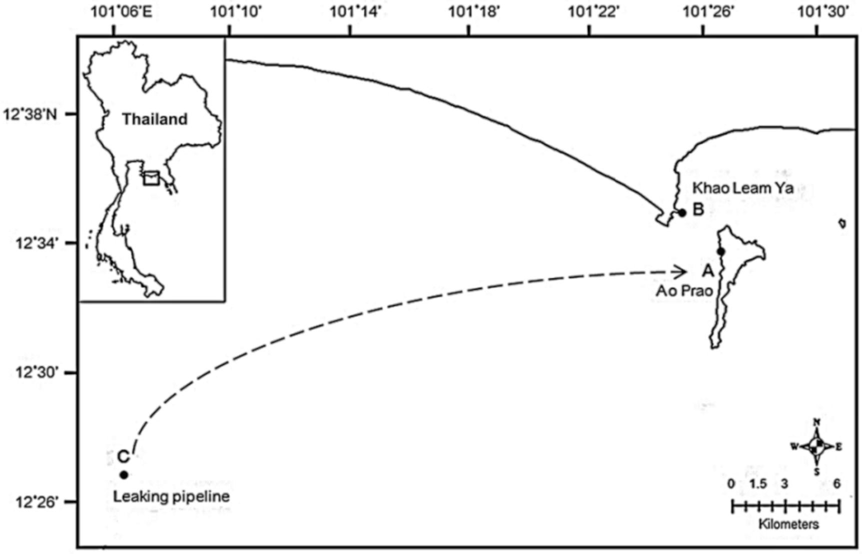

Figure 1. Map of the sampling sites. (A) Ao Prao (Prao Bay), Samed Island (impacted site), (B) Khao Laem Ya (reference site) and (C) Incident site. Dash line illustrates the pathway of oil spill.

Sample collection

From each sampling site, 20 individuals of each species (n=20) were collected. The target animals were three intertidal rocky shore molluscs; predatory whelk (*Morula musiva*), herbivore coil snail (*Planaxis sulcatus*) and rocky oyster (*Saccostrea cucullata*). Samples were placed in plastic bags (after ~1 h) and stored at –20°C for further analysis. Different animals were used in each trial.

Sample preparation

The extraction and clean-up of metabolites in soft tissues were performed as described by Lim and Shin (2013). In the laboratory, frozen mollusc tissues were thawed to reach room temperature (~27°C). Two grams (wet weight) of the mollusc tissues were homogenized for 5 min. We added 5.0 ml of 2 M KOH and sonicated for 10 min. The solution was hydrolyzed in a hot oven at 90°C for 60 min. Then, we adjusted the solution to pH 2-3 with 5 ml of 2 M HCl. For extraction, we added 10 ml of ethyl acetate and 2 g of sodium chloride. The solution was then vortexed for 2 min and centrifuged at 3,660 ×g RCF for 5 min. To collect the inorganic layer, we cleaned up the sample with 50 mg of primary secondary amine (PSA) sorbent and dried it by nitrogen gas for 15 min (filtered through a column of activated carbon). Finally, 50 µl of N, O-bis (trimethyl silyl) trifluoroacetamide (BSTFA) and 50 µl of ethyl acetate were added and heated in the oven at 60°C for 30 min. A 2 µl of the solution was injected into the GC system.

Instrumental analysis

Experiments were conducted to establish a prcedure for preparing standard calibration curves for determination of 1-hydroxypyrene (1-HOP) and 2-naphthol (2-NAP) standards. The calibration curve used in the gas chromatography-tandem mass spectrometry (GC-MS/MS) was obtained using 2 g wet weight of the *S. cucullata* tissue from Ang Sila oyster commercial farm where low concentration of PAH has been reported with 0.05, 0.50 and 2.50 ng of 1-HOP and 2-NAP standards (Boonyatumanond *et al*., 2006). The oyster tissue from Ang Sila commercial farm was chosen for making calibration curve because the area should have less contamination by PAH as pointed out by Boonyamanond *et al*. (2006). Intensive input of PAH was from urban environment and coastal oil refinery. The limit of detection (LOD) for 1-HOP and 2-NAP analysed was 0.05 ng based on an assayed control samples of 2 g (A signal-to-noise ratio (S/N) of three is generally accepted for estimating LOD). Procedural blanks were performed for each set of samples. After extraction and clean up, we injected the final solution into the GC-MS/MS. To determine the presence of the metabolites 1-HOP and 2-NAP in our mollusc samples, the GC-MS/MS conditions based on Shin and Lim (2011) and Lim and Shin (2013). Contrary to them, we used a gas chromatograph Bruker GC-MS TQ with a splitless injector (Bruker Daltonics Inc. Chemical and Applied Markets, West Warren Avenue Fremont, CA, USA) to obtain all mass spectra of our samples. The GC instrument run with helium as the carrier gas, and the analytical column was a HP-5MS column (cross - linked 5 % phenylmethylsilicon, 30 m × 0.25 mm I.D.×0.25 µm F.T). Moreover, the column temperature for analyses programmed started at 100°C, held for 1 min, then to 320°C at 20°C/min and held for 5 min. The ion source was operated in the electron ionization mode (EI; 70 eV) at 250°C. Collision Induced Dissociations (CID) of PAHs metabolites were studied using a two-section mass spectrometer. The collision gas employed was argon. Finally, to corroborate the 1-HOP and 2-NAP metabolites detected by the GC-MS/MS in all our samples, GC-MS/MS based on the ions was selected in this study (Table 1).

Table 1. Parameters of 1-HOP and 2-NAP on MRM mode.

Compound	Precursor ion	Product ion	Collision Energy
1-HOP	290	275	15
	290	274	25
	290	259	25
2-NAP	216	201	10
	216	203	10
	201	181	10

RESULTS

The concentration ranges and mean values of the PAHs metabolites (1-hydroxypyrene (1-HOP) and 2-naphthol (2-NAP)) in all mollusc species from Ao Prao (Prao Bay) are shown in Fig. 2. Initially, the mean concentrations of 1-HOP and 2-NAP metabolites ranged from 0.039 to 1.421 ng/g wet weight and 0.001 to 0.403 ng/g wet weight, respectively. The mean concentrations of 1-HOP and 2-NAP were detected in all mollusc species at Ao Prao but, the mean concentrations decreased over time and were almost null 1-year after the oil spill contamination. On the other hand, at Khao Laem Ya (reference site), 1-HOP and 2-NAP concentrations were never detected in any of the mollusc species.

The highest PAHs concentration for 1-HOP was found in *M. musiva* at 1.421±0.227 ng/g wet weight in August 2013, while *P. sulcatus* had the lowest concentration of 0.039±0.009 ng/g wet weight during the same month. The lowest concentration of 1-HOP was found in *S. cucullata* (0.094±0.009 ng/g wet weight) in December 2013 and it was the lowest among the three species (Fig. 2).

2-NAP was detected in the highest mean concentration (0.396±0.053 ng/g wet weight) in *S. cucullata* gathered in August 2013. The lowest concentration (0.001±0.000 ng/g wet weight) was detected in *M. musiva* and not detected in *P. sulcatus* in August 2013. In December 2013, 2-NAP concentrations were detected in all mollusc species.

The highest mean concentration (0.403±0.073 ng/g wet weight) was detected in *S. cucullata*, followed by *P. sulcatus* (0.305±0.076 ng/g wet weight) and *M. musiva* (0.227±0.079 ng/g wet weight). Finally, 2-NAP concentrations were not found in any of the molluscs after December 2013 (Fig. 2).

The chromatogram shows the peak symmetry of different target compounds. Their peaks appear in the chromatograms at different retention times. The retention times of standard solutions 1-HOP-TMS and 2-NAP-TMS were 11.04 and 6.50 min, respectively (Fig. 3).

The ions selected in this study from Gas chromatography-mass spectrometry with selected ion monitoring (SIM), Mass spectra analyses of 1-HOP-TMS and 2-NAP-TMS were obtained (Fig. 4). N, O-bis (trimethyl silyl) trifluoroacetamide (BSTFA), was used as derivatization reagents to compare repeatability, sensitivity and stability (Fig. 4). Confirmations of tract 1-HOP-TMS and 2-NAP-TMS were performed by two MS characteristic ions from multiple reaction monitoring modes (MRM). This increases the selectivity for detection and characterization of metabolites in mollusc samples. 1-HOP-TMS showed product molecular ion at m/z (mass-to-charge ratio) 259, 274, 275 while 2-NAP-TMS showed at m/z 181, 201, 203 (Fig. 5). Ion characteristics of the target compound preferably distinguish this compound from any others with similar retention times. The extracted ion chromatograms for this ion were used for the quantification.

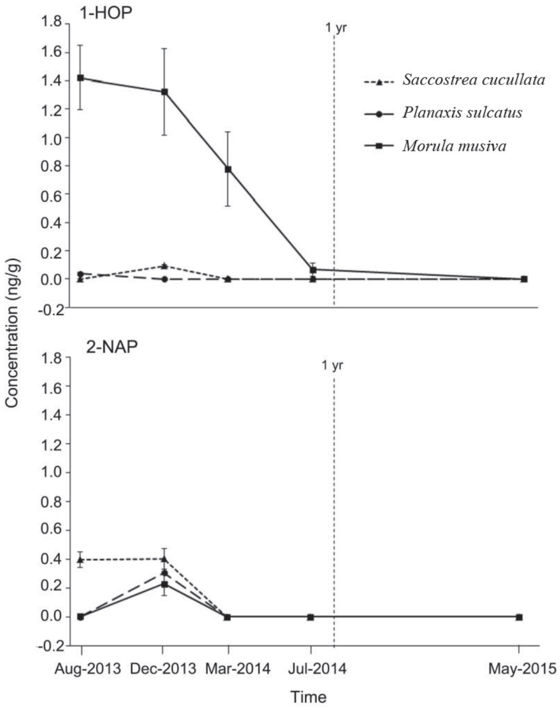

Figure 2. Mean concentrations of the metabolites 1-HOP and 2-NAP in mollusc samples (n=20) from Ao Prao (Prao Bay) (impacted site) by GC-MS/MS. (ng/g wet weight ± SE).

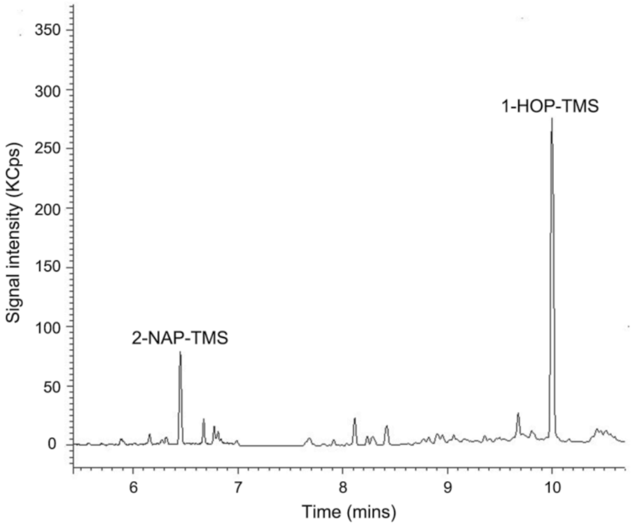

Figure 3. Chromatogram of the standard solutions 1-HOP-TMS and 2-NAP-TMS in rock oyster (*Saccostrea cucullata*) from GC-MS/MS.

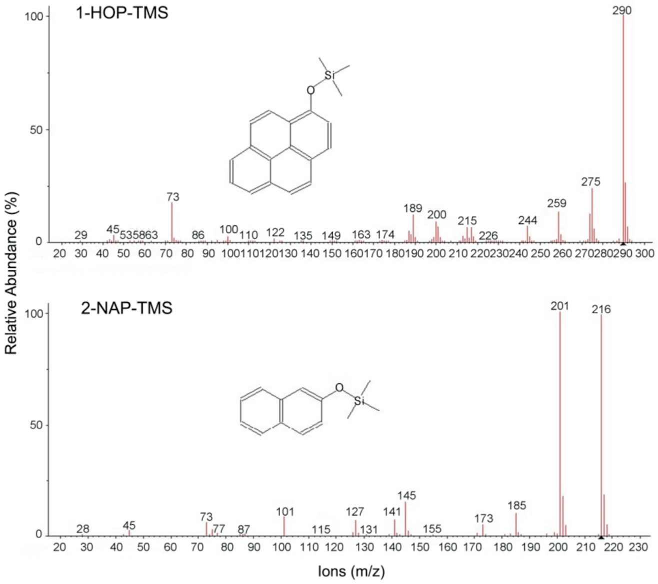

Figure 4. Mass spectra of 1-HOP-TMS and 2-NAP-TMS in rock oyster (*Saccostrea cucullata*) from the GC-MS/MS.

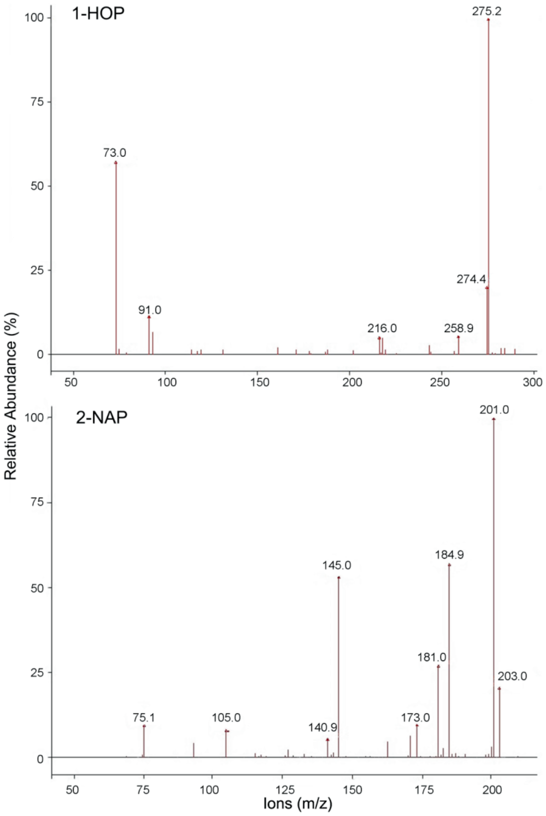

Figure 5. Mass spectra of product of 1-HOP-TMS and 2-NAP-TMS in rock oyster (*Saccostrea cucullata*) from the GC-MS/MS.

DISCUSSION

PAHs are the compounds that can be detected in organisms contaminated by crude oil (Meador *et al*., 1995). In this study, 1-hydroxypyrene (1-HOP) was selected to be one of the biomarkers because it represents the highest concentrations of pyrene compared with the others PAHs compounds previously recorded (Jongeneelen, 2001; Tairova *et al*., 2009). Additionally, to 1-HOP, we also used 2-NAP as a biomarker. This is the first time that 2-NAP is used in molluscs contaminated by the oil spill. 2-naphthol (2-NAP) metabolite is the most representative component of naphthalene and is a commonly known biomarker used in various organisms (Li *et al*., 2008).

Results from this study showed decreasing concentrations of 1-HOP and 2-NAP in mollusc tissues over time and the concentrations of 2-NAP (a metabolite of naphthalene) was lower than 1-HOP (a metabolite of pyrene) for the same time (Fig. 2). Maillacheruvu and Pathan (2009) reported that naphthalene was more easily biodegradable than pyrene. In addition, we found that mean concentrations of 1-HOP and 2-NAP differ among all target species. Several studies have shown higher bioaccumulation levels of PAHs metabolites in marine top predators (Jackson *et al*., 1989; Peterson, 2001; Nácher-Mestre *et al*., 2010). This bioaccumulation level depends on the metabolic rate (Jørgensen *et al*., 2005) and feeding behaviours (Baumard *et al*., 1999) of the animal.

Among all target species, we detected that whelk (*Morula musiva*) had the highest levels of the metabolite 1-HOP. This animal feeds on other smaller molluscs like, *Cellana toreuma*, *Lunella coronata* (Lam, 2002) but, as a major part of its diet are *Planaxis sulcatus* and *Saccostrea cucullata* (Taylor, 1990; Lam, 2002), On the other hand, the feeding behaviours of the grazer *P. sulcatus* and the filter-feeder *S. cucullata* could explain the remarkably low concentration levels of 1-HOP compared with *M. musiva*. Results from this research illustrates toxic levels associated with organisms of the food chain. There are several studies indicating crude oil spill accident impact to marine organisms such as phytoplankton, zooplankton, and fish in food chain (Gin *et al*., 2001). The amount of PAHs accumulations in the prey species may pass on to predatory marine organisms. Marine mammals, fish or other higher trophic level aquatic organisms are also exposed to PAHs through the ingestion of contaminated prey species (Takeuchi *et al*., 2009).

Knowing the relative level of PAHs exposure is important in understanding the link between PAHs exposure, tissues accumulation and abnormalities or death, particularly since PAHs exert their toxic effects after metabolism (Grundy *et al*., 1996; Moore *et al*., 1987). In addition, this study provides baseline data of PAHs, which can be used to assess environmental impact of marine oil spills in the future.

Kingston (2002) concluded that ecosystem and the marine community in high latitude needed up to three years to restore and recover after an oil spill of 49 million litres (known as “Exxon Valdez”) impacted the coast of Alaska and other nearby regions. However, our results showed that PAHs metabolites concentrations in all mollusc in this study decreased periodically and were almost null within one year after the accident. In Thailand where average temperature is much higher than temperate zone and as a consequence of this, the metabolic rate and oil degradation goes much faster. Ribicic *et al*. (2018) reported that the rate of biodegradation depends on temperature; increasing temperature of the water increases both the metabolic rate and amount of oil degradation. This illustrates that marine ecosystems and habitats affected by the high temperature of water in amounts of crude oil have no long-term effects. Nevertheless, in most oil disasters, strong wave action, seawater currents and winds direction contributes to a quick recovery process of the shoreline communities contaminated by this pollutant (Kingston, 2002). The weather for Rayong reflects the previously mentioned strong waves and winds. The heights of the waves at the time of the spill were around 1 to 2 meters and the wind speed was around 15 to 35 km/h contributed to a quick recovery in of the affected sites after the oil spill in Thailand (Casarotto *et al*., 2014). In ecosystems polluted by oil spill incidents, evaluation of PAHs metabolites found in contaminated animals provide accurate results and is an important tool for ecological risk assessment (Peters, 1995; Van der Oost *et al*., 2003). The impact of PAH on intertidal the marine rocky shore community is rarely documented.

Gas chromatography-mass spectrometry analyses (GC-MS) have been the most widely used methods in many studies involving marine organisms contaminated with PAH compounds (Pointet and Milliet, 2000; Poster *et al*., 2006; Prempramote *et al*., 2015). However, when comparing methods to detect specific PAHs metabolites found in organisms, GC-MS/MS is the most effective and analytically detailed. The main difference between these two methods is that GC-MS select ion monitoring mode is used to detect specific compounds (Ferrer *et al*., 2005; Prempramote *et al*., 2015) while, GC-MS/MS multiple selected monitoring mode is a highly selective mass spectrometric confirmation analysis method. Our present and previous research results demonstrate the function of different GC-MS and GC-MS-MS methods. GC-MS analysis is an acceptable alternative to full-scan mass spectrometry when full-scan mass spectra are incapable of detecting target compounds below the limits of quantification (Prempramote *et al*., 2015). The interfering compound is present in previous, maybe false negative results which can occur when interfering compounds are present at high relative concentrations. However, in present study, we found that GC-MS/MS isolate a target compound ion from matrix and then fragmented to generate spectra. Because of this, GC-MS/MS spectrum consists only of product ions from the selected precursor.

In our study, the GC-MS/MS play a predominant role in bioassays to detect metabolites in marine biota contaminated with petroleum. This is due to its inherent high selectivity and sensitivity which gives it the potential to identify metabolites. The retention time can help to differentiate between the PAHs metabolites 1-HOP and 2-NAP. The results of this study also showed that GC-MS/MS is an efficient and precise tool to detect occurrence of PAHs metabolites in intertidal rocky shore molluscs contaminated by crude oil. Finally, our work is the first study in Thailand using GC-MS/MS to determine concentrations of the PAHs metabolites. Therefore, we are able to evaluate oil spill pollution in intertidal rocky shore molluscs.

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