Occurrence of aliphatic and polyaromatic hydrocarbons (PAHs) in Mytillus galloprovincialis from the traditional market in Marseille, France, by Gas Chromatography triplequadropole tandem Mass Spectrometry (GC-QQQ/MS)

M Y Azis¹,²,³, Yelmiza¹,², L Asia², A Piram², B. Buchari1, P Doumenq¹ and A D Syakti³

¹Analytical Chemistry Laboratory, Department of Chemistry, Institute of Technology Bandung, Jl. Ganesha No. 10, Bandung 40132, Indonesia
²Environmental Analytical Chemistry Laboratory (LCE), FRE-CNRS 3416 Equipe MPO, Europôle de l’Arbois-Bâtiment Villemin BP 80 Aix en Provence Cedex 4, France
³Center for Maritime Bioscience Studies-Institute for Research and Community Service, Jenderal Soedirman University, Jl. Dr. Suparno, Purwokerto 53123, Indonesia

*E-mail: yudis_chempd@yahoo.com

Abstract. Mediterranean mussel, Mytilusgalloprovincialis collected from the traditional market in Marseille, France, have been analysed using GC-QQQ/MS for their hydrocarbons (n-alkanes and polyaromatic hydrocarbons (PAHs)) extent with two different solvent extraction, such as heptane:dichloromethane (HEP:DCM; 1:1) and heptane:acetone (HEP:ACE; 1:1). The results showed hydrocarbons yielded from heptane:acetone extraction were 28335 μg.kg⁻¹ mussels dw (Σ n-alkanes C₁₅-3₄) and 202 μg.kg⁻¹ mussels dw (ΣPAHs) while the yield from heptane:DCM extract was lower ca. 27026 μg.kg⁻¹ mussels dw and 133 μg.kg⁻¹ mussels dw respectively from the Σ n-alkanes C₁₅-3₄ and ΣPAHs. High hydrocarbon levels can be affected by the presence of lipids or other metabolites in mussels that have the same polarity with hydrocarbon compounds which has interfered the measurement. Several ratio parameter of n-alkanes and PAHs source in the mussels were evaluated to assess the origins of their hydrocarbons in mussels from which we suggested origins of hydrocarbons were pyrolytic and biogenic rather than petrogenic.

1. Introduction
Petroleum hydrocarbons compounds i.e. aliphatic hydrocarbons and polyaromatic PAHs that known as toxic pollutants to the environment e.g. rivers, lagoons, and marine, and some of them have mutagenic and carcinogenic properties [1,2,6,7]. Those compounds may be derived from petroleum or oil spills refinery product (petrogenic sources), incomplete combustion of organic matter, and biomass fossil fuels (pyrolytic source) [1,3,6,7,10]. For that reason, petroleum hydrocarbons analysis within the organisms are important to monitoring activity of pollutant in environment as one integrative approach [14]. This study used Mediterranean mussel (M.galloprovincialis) to monitor marine ecosystems quality. Hydrocarbons can absorb into sediment via particulate or colloid and potentially ingested by marine benthic organisms in marine environment [2,9][2,4,5,8,9]. Bivalves such as Mussels had used
as sentinel organisms of marine pollution and have the capability to reflect the environment quality which related to their habitat, food chains, and pollutant hydrophobicity properties that have the tendency to be absorbed into their living tissues [2,9]. *Mytilus* families widely used since 1990’s and become one of the most successful model organisms for time-integrated responses to complex mixture of pollutants [14], also as bioindicator reference of hydrocarbons level [8,13]. This study focus on the hydrocarbons content in mussel from traditional market that have direct impact to human, because as we know that the traditional market sell unknown source of mussels which can be from pollutant site or propre site for human consumption.

The significance of this study related to the extraction, fractionation, and analysis method using GC-QQQ/MS [1,2,6,7,10]. Quadropole can be used in scanning or filtering mode. Triple quadropole system contains of three quadropoles. Quadropole 1 and 3 as mass filter while quadropole 2 as collision cell [15]. Several studies used heptane: dichloromethane (1:1 v/v) [1,6,7,9,10] and heptane:acetone (1:1, v/v) to extract the hydrocarbons [4,13]. Thematrice of mussels tissues are more complex than sediment and can influence the interference effect to separate the hydrocarbons content from their matrices. This study aimed to analyze the content of hydrocarbons in *M. galloprovincialis* mussel species that derived from traditional markets in Marseille, France, by using protocol analysis of hydrocarbon sediments and to compare the different extraction solution to extract hydrocarbons that can be optimized using GC-QQQ/MS.

2. Material and Methods

2.1. Sampling
Approximately 3 kg wet mussels, *M. galloprovincialis* have taken from Noailles traditional market, Marseille, France. Fresh seafood market is located about 300 meters from Mediterranean port. After purchased, the wet mussel stored in freezer -20°C.

2.2. Chemicals
n-heptane (HEP), acetone (ACE), and dichloromethane (DCM) for organic solvent (Suprasolve, GC analysis grade (Merck, Pessac, France)). Individual deuterated standards for PAHs ([2H10]Anthracene, m/z 188 [2H12]Chrysene, m/z 240 (CIL Cluzeau, Andover, MA, USA) and individual labeled standards of n-alkane ([2H40] n-nonadecane, m/z 66 (PESTANAL Sigma-Aldrich, St Quentin, Fallavier, France) and 1-eicosane (n-C20) (Dr.Ehrenstrofer Laboratories, Augsburg, Germany). 16 PAHs US EPA (PAH mix 25) standar mix solutions(Dr. Ehrenstonfer Laboratories, Augsburg, Germany). Silice gel-60 and Alumine gel-60 (Merck, Darmstadt, Germany, size 200-300 mesh for each of them.

2.3. Sampel Preparation
The tissues taken and placed in buchner. Water content in wet tissues removed by gravitation for 30 minutes [3], then wetmass weighed with microbalance (Perkin Elmer AD2Z, Marseille France, weighed approximately ± 250 gr). The tissues were freeze-dried under pressure 0.080 bar and freeze at -50°C for 48 h. Then it cutted and crashed by mortar to small size and the dry mass weighed and stored under temperature ambient before the extraction [8,13].

2.4. Extraction and Purification
Hydrocarbons extracted from mussel tissues by using protecole extraction of hydrocarbons for sediments [1,6,7,11,12]. 10 gram freeze-dried mussels tissues accurately weighed and placed into pre-cleaned cellulose extraction thimble by soxtherm apparatus for 3.5 h with 150mL HEP:DCM (1:1 v:v) solvent extraction. Beside that, same extraction method has been done, with another solvent extraction, 150mL HEPT:ACE (1:1, v:v). 0.5 mL each internal surrogate deuterated standard solutions of hydrocarbons (n-alkane (nonadecane-d40 (15 mg/L)) and PAH (anthracene-d10 (20mg/L); chrysene-d12 (20mg/L)) added into sample before the extraction. Then, the extract concentrated to 2 mL with
rotary evaporator (Heindolph, Laborata 4000 efficient, Krackeler Scientific, Pessac, France) and evaporated under a gentle nitrogen stream to obtain the total extractable organic matter (EOM). The extract (EOM) weighed on a microbalance (Perkin-Elmer AD2Z) and part of EOM dissolved in a minimum of HEP. The fractionation of aliphatic and aromatic hydrocarbons using 1.0 x 3.0 cm borosilicate glass column with 8 g of silica gel and 8 g of alumina (bottom and top, respectively), both adsorbents activated with 5% H2O. Saturated hydrocarbon fraction or n-alkanes fractions eluted with 30 mL HEP and 20 mL HEP : DCM (90:10, v:v). For PAHs fraction or fraction 2 eluted with 40mL HEP:DCM (80:20, v:v). Both of fraction evaporated on rotary evaporator at 0.5mL. Then, dry fraction (F1 and F2) weighed and obtained the total hydrocarbon content. Fraction 1 dilluted with 300μL HEP and for fraction 2 dilluted in 300μL DCM.

2.5. Analysis by capillary gas chromatography coupled to mass spectrometry (GC/MS)
Both of fractions (F1 and F2) analyzed by GC-QQQ/MS (Autosystem XL GC and TurboMass triple quadrupole mass spectrometer, Perkin Elmer, USA). Chromatographic conditions follows: splitless injection (30 s), Elite 5MS capillary column (30 m x 0.25 mm i.d. x 0.25 μm). The GC oven temperature programmed from 40 °C (isotherm 2 min) then raised to 120 °C (45 °C.min⁻¹) and then raised to 310 °C (5 °C.min⁻¹) and finally held isothermally for 20 min. Helium (1mL.min⁻¹) used as carrier gas in constant flow mode. For injector temperature 50 °C (isotherm 0.1 min) to 250 °C (20 °C/min) for reduce solvant peaks. The mass spectrometer operated in the electron ionization (EI) positive ion mode (70 eV) and simultaneously scanned in both Full Scan and Selected Ion Monitoring (SIFI mode). MS scanned with comparing with NB library reference to confirm and matching their retention times and fragmentation profiles against corresponding standards. Detection of n-alkanes (m/z 71), 8 PAH target ions (Acenaphthene, m/z 152; Acenaphthylene, m/z 154; Phenanthrene, m/z 178; Anthracene, m/z 178; Fluoranthene, m/z 202; Pyrene, m/z 202; Benzo(a)anthracene, m/z 228; Chrysene, m/z 228; Indeno(123cdi) pyrene, m/z 276; Benzo(ghi)perylene, m/z 276). internal labeled standard of PAHs (Anthracene-d₁₀, m/z 188 and Chrysene-d₁₂, m/z 240) and n-alkanes (nanodecane-d₄₀, m/z 66) to evaluate hydrocarbons analysis.

2.6. Quality Control
Calibration Curves have been performance with external standards methods at different concentrations levels using diluted individual labeled hydrocarbons standards (i.e. 0.05; 0.2; 0.5; 1.5; 2; 3;3.5 mg/L). In duplicate samples have been calculated that the mean recovery are 97% (HEP:ACE ; 1:1, v/v) and 93% (HEP:DCM, 1:1,v/v). For PAHs are anthracene-d₁₀ and chrysene-d₁₂ respectively (82%; 89%(HEP:DCM ; 1:1, v/v) and 90%; 101% HEP:ACE ; 1:1,v/v).The coefficient of correlation was 0.99 forall labeled standard compounds.

3. Result and discussion
Extractable Organic Matter (EOM) concentrations, Total Hydrocarbons Content (THC), quantities of F1 and F2, comparing between THC and MOE, F1 and F2 from two different solvant extraction are given gravimetrically in Table 1. The solvant extraction(HEP:ACE ; 1:1 ; v/v) solvant has a lot EOM, THC and fractions content then HEP:DCM (1:1, v/v).

Total hydrocarbon content from both extract are < 1 mg.g⁻¹ generally. The proportions of THC in EOM are 18 % for HEP:DCM (1:1),v/v and 53% for (HEP:ACET (1:1), v/v). Fraction saturated (F1) has more quantity then F2. Comparing of F1/F2 with solvant extraction HEP :ACE (1:1), v/v) more abundance than HEPT :DCM (1:1), v/v).

Generally, n-alkanes are nonpolar compounds and semipolar for PAHs, that can extracted and separated using fractionation method with comparing different polarity of eluent. GC/MS analysis with Single Ion Monitoring (SIM) mode has found 20 peaks n-alkanes (n-C₁₅₋₃₄) and 8 peaks identical of PAHs (Figure 1 and Figure 2).
According from chromatograms, both of chromatograms have similar profile. Besides \(n\)-alkanes derivatives, isomer found from \(n\)-alkanes such Pristane, Phytane which indicate the presence of crude oils that might be from natural (biogenic or diagenetic) and isomer from \(n\)-alkenes, squalene which derived from microbial degradation [1]. Figure 1 showed that there are two high peaks (\(n\)-C\(_{29}\) and \(n\)-C\(_{31}\)) in the last chromatograms and \(n\)-C\(_{17}\), \(n\)-C\(_{18}\), \(n\)-C\(_{19}\) and \(n\)-C\(_{20}\) have high peaks in the beginning.
Figure 2. Capillary column gas chromatograms of the polycyclic aromatic hydrocarbon fraction with HEP:DCM (1:1, v/v) (above) and HEP:ACE (1:1, v/v) (under) as solvant extraction. Phe: Phenanthrene; An: Anthracene; Fl: Fluoranthene; Pyr: Pyrene; BzA: Benzo(a)Anthracene; Chry: Chrysene; IndP: Indeno (123 cdi) pyrene; BghiP: Benzo(ghi)Perylene.

Total of $n$-alkanes ($n$-C$_{15-34}$) and PAHs respectively were 27026 and 133μg.kg$^{-1}$tissues.dw for those extracted with HEP:DCM (1:1, v/v) and for HEP:ACE (1:1, v/v), $\Sigma n$-C$_{15-34}$and $\Sigma$PAHs respectively have value 28335 and 202μg.kg$^{-1}$tissues.dw (see Table 2). The high level of $n$-alkanes content have the possibilities that originated from disrupting of lipid and metabolites, or these mussels have high contamination in mussels tissues from Mediterranean sea.
Table 2. Concentrations of \( n \)-alkanes, pristane and phytane and PAHs in \( \textit{M. galloprovincialis} \) from traditional market, Noailles, Marseille, France (\( \mu \text{g.kg}^{-1} \) tissues dw)

| Compounds      | (HEP:ACE) | (HEP:DCM) |
|----------------|-----------|-----------|
| \( n \)-C\(_{15} \) | 442       | 654       |
| \( n \)-C\(_{16} \) | 663       | 712       |
| \( n \)-C\(_{17} \) | 1775      | 1737      |
| Pristane       | 940       | 1082      |
| \( n \)-C\(_{18} \) | 1794      | 1682      |
| Phytane        | 2023      | 1541      |
| \( n \)-C\(_{19} \) | 2288      | 2244      |
| \( n \)-C\(_{20} \) | 2039      | 1885      |
| \( n \)-C\(_{21} \) | 1541      | 1412      |
| \( n \)-C\(_{22} \) | 1725      | 1568      |
| \( n \)-C\(_{23} \) | 1424      | 1289      |
| \( n \)-C\(_{24} \) | 1075      | 953       |
| \( n \)-C\(_{25} \) | 971       | 634       |
| \( n \)-C\(_{26} \) | 574       | 375       |
| \( n \)-C\(_{27} \) | 1103      | 1126      |
| \( n \)-C\(_{28} \) | 535       | 525       |
| \( n \)-C\(_{29} \) | 2009      | 2400      |
| \( n \)-C\(_{30} \) | 589       | 521       |
| \( n \)-C\(_{31} \) | 3332      | 3352      |
| \( n \)-C\(_{32} \) | 449       | 398       |
| \( n \)-C\(_{33} \) | 699       | 714       |
| \( n \)-C\(_{34} \) | 346       | 222       |
| \( \Sigma n \)-C\(_{15-34} \) | 28335     | 27026     |
| Phenanthrene   | 24        | 13        |
| Anthracene     | 9         | 8         |
| Fluoranthen    | 93        | 52        |
| Pyrene         | 62        | 45        |
| Benz(a)anthracene | 3     | 4         |
| Chrysene       | 8         | 9         |
| Indeno(1233cdi)pyr | 1       | 1         |
| Benz(ghi)perylen | 2       | 1         |
| \( \Sigma \text{PAH} \) | 202       | 133       |

The mixed solvents HEP:ACE (1:1, v/v) known to be more polar than HEP:DCM (1:1, v/v). From this result, hydrocarbons content from \( \textit{M. galloprovincialis} \) were determined. The properties of mussels tissues are presumably more complex because of their macromolecules content such as lipid, protein, and other metabolite [4]. Such compounds are capable to interference while separating process and these metabolites macromolecules can affect the extraction yield since they have the same polarity with hydrocarbons compounds. For that reason, hydrocarbons can associated with this macromolecule and have the same ion fragmentation with ion target [4]. The method of fractionation need another method to separate these macromolecules before the hydrocarbons fraccionation process. One of method for macromolecules separation is permeable gel chromatography, which is the macromolecule can be removed from mussel tissues before the fractionation process. Our finding showed aliphatic and PAHs extraction yield within the same greater magnitude that compared to previous studies using sediment as environmental matrices. Partially concluded that the mussels from traditional market were potentially polluted by hydrocarbons [1,6,7 and 10]. Obviously, further research is needed to revalidate and to find out the effective method to separate another molecules prior for the environmental
In addition, high concentration of the hydrocarbons in *M. Galloprovincialis* has been observed, compare to another previous studies concerning PAHs in *Mytillus edulis* (27.6-442 μg.kg⁻¹ mussels dw) [12]; *M. galloprovincialis* from coastal of Saronikos, Gulf, Greece, (17PAHs: 219-1487 μg.kg⁻¹ mussels tissues dw) [13] and for n-alkanes in *M. galloprovincialis* from Galicia coast (n-C₈₃₅: 89.46-5098.01 μg.kg⁻¹ mussels tissues dw) [16].

7 ratios for n-alkanes used to assess the potential sources of hydrocarbons contamination: CPI (Carbon Preference Index), NAR (Natural alkane ratio), nC₁₈/phyt, nC₁₇/phyt, prist/phyt, TAR (Terrigenous Aquatic Ratio), and LMW/HMW (Low Molecular Weight/High Molecular Weight), while PAHsindices are derived from comparison identical molecular weight such as (anthracene/∑m/z 178; Fluorene/∑m/z 202; Bz(a) anthracene/∑m/z 228; indeno (123 cd)pyrene/∑m/z 176). Generally, there are two main sources of hydrocarbons, origin from anthropogenic activities and from biogenic process (natural process) or biological activities. Concern to biogenic source, it may be derived from plant or animal residual or both of them.

### Table 3. Characteristic of n-alkanes from different sources [1,6 and 7]

| Parameters | Origin from oil | Aquatic environment | Terrestrial environment |
|------------|-----------------|---------------------|------------------------|
| CPI (24-32) | ≈ 1 | >1 |
| n-C₂₉/n-C₁₇ | <1 | >1 |
| n-C₁₇/prist | >1 | <1 | >1 |
| n-C₁₈/Phyt | >1 | ≈ 1 | ≈ 1 |
| Prist/Phyt | <2 | >2 |
| NAR | ≈ 0 | >0.5-1 |
| TAR | <1 | >1 |
| LMW/HMW | >2 | ≈ 1 | <1 |

CPI: Carbon Preference index; TAR: Terrigenous/Aquatic Ratio; NAR: Natural n-alkane ratio; LMW/HMW (Low Molecular Weight/High Molecular Weight); Pr: Pristane; Phy: Phytane

Parameters index and their values of hydrocarbons are given at Table 3 for n-alkanes and Table 4 for PAHs.

### Table 4. Characteristics of PAH source from different source (μg.kg⁻¹ mussels tissues dw)

| Origin from oil | Origin from mix between oil and combustion product | Origin from combustion of fossil and plant |
|----------------|-----------------------------------------------|-----------------------------------------|
| An/∑₁₇₈ | < 0.1 | > 0.1 |
| Fl/∑₂₀₂ | < 0.4 | 0.4 – 0.5 | Mixed source from combustion of fossil |
|          |       |         | Cerosen combustion, charbone or biomass (herbal, wood…)
| BzA/∑₂₂₈ | < 0.2 | Mixed | > 0.35
| IndP/∑₂₇₆ | < 0.2 | 0.2 – 0.5 | Combusction result or flammable fossil liquid |
|          |       |         | combusction of charbone or biomass (wood, herbal) |
The profile of capillary column gas chromatograms of n-alkanes fraction in mussels extract between Σ m/z 176 and it also has the probability that those indice given indices < 0.5 (anthracene / Σ m/z 178, benzo (a) anthracene / Σ m/z 228; indeno (123cdi) pyrene / Σ m/z 202) parameters source of PAHs resulted from the two solutions of different extracts that came from oil which origin from n-alkanes isomer (Pristane/Phytane) < 2. The source n-alkanes that are produced by higher plants, marine animals and sedimentary bacteria.

The results showed an apportionment of the n-alkanes level from biogenic sources, probable from aquatic organism or terrestrial environment with index (CPI, n-C17/Pr, n-C18/Phy, and TAR) > 1, for NAR is close to zero < 1, indicates for petroleum hydrocarbons, LMW/HMW< 1 usually represent n-alkanes that are produced by higher plants, marine animals and sedimentary bacteria.

The index indicates the source of PAHs in mussels from traditional market derived as natural or biogenic sources which origin from the burning of fossil or living creatures biosynthesis results with given indices < 0.5 (anthracene / Σ m/z 178, benzo (a) anthracene / Σ m/z 228; indeno (123cdi) pyrene / Σ m/z 176 and it also has the probability that those indices came from oil with indices > 0.5 (Fluorene / Σ m/z 202) parameters source of PAH resulting from the two solutions of different extracts that shown in Table 6.

### Table 5. Selected n-alkanes ratios in *M. galloprovincialis* (μg.kg⁻¹ mussels tissues dw)

| Mussels                  | CPI  | n-C17/Pr | n-C18/Ph | Pr/Ph | n-C29/n-C17 | NAR  | TAR  | LMW/HMW |
|--------------------------|------|----------|----------|-------|-------------|------|------|---------|
| *M. galloprovincialis*   | 3.0  | 1.6      | 1.1      | 0.7   | 1.4         | 0.4  | 1.5  | 0.7     |
| (HEP:DCM)               |      |          |          |       |             |      |      |         |
| *M. galloprovincialis*   | 2.6  | 1.9      | 0.9      | 0.5   | 1.1         | 0.3  | 1.4  | 0.8     |
| (HEP:ACE)               |      |          |          |       |             |      |      |         |

CPI: Carbon Preference Index = 2*(n-C25+n-C27+n-C29+n-C31)/(n-C24+2*(n-C26+n-C28+n-C30)+n-C32); NAR: Natural n-alkane ratio = ((Σn-C19,32)- 2Σ even n-C20,32)/(Σn-C19,32); TAR: Terrigenous/Aquatic Ratios = (n-C21+n-C29+n-C31)/(n-C15+n-C17+n-C19); Pris: Pristane; Phy: Phytane; LMW/HMW (Low Molecular Weight/High Molecular Weight) = (n-C15-21)/(n-C22-34) [1,6,7,10, 16]

### Table 6. Characteristic of PAH sources from *M. galloprovincialis* (μg.kg⁻¹ mussels tissues dw)

|                      | An/Σ178ᵃ | Fl/Σ202ᵇ | BzA/Σ228ᶜ | IndP/Σ276ᵈ |
|----------------------|-----------|-----------|------------|------------|
| *M. galloprovincialis* (HEP:ACE) | 0.27      | 0.53      | 0.32       | 0.29       |
| *M. galloprovincialis* (HEP:DCM) | 0.38      | 0.54      | 0.31       | 0.31       |

ᵃ An/Σ178 (Anthracene/ (Anthracene+Phenanthrene) ᵇ Fl/Σ202 (Fluoranthene/Fluoranthene+Pyrene) ᶜ BzA/Σ228 (Benz(a)anthracene/Benz(a)anthracene+Chrysene) ᵈ IndP/Σ276 (Indeno(123cdi)pyrene/Indeno(123cdi)pyrene+Benz(ghi)perylenes)

The profile of capillary column gas chromatograms of n-alkanes fraction in mussels extract between HEP:ACE (1:1, v/v) and HEP: DCM (1:1, v/v) as solvent extraction have same profile. n-C15-34 were found with different yield.

### 4. Conclusion

The extract solution of HEP:ACE (Σn-C15-34: 28 335 μg.kg⁻¹ tissues dw and Σ PAHs: 202 μg.kg⁻¹ tissues dw) better than HEP:DCM (Σn-C15-34: 27026 μg.kg⁻¹ tissues dw and Σ PAHs: 133 μg.kg⁻¹ tissues dw).
High hydrocarbons extract yield might be come from two possibilities (i). direct disruption of lipids and metabolites during analysis; (ii). the apportionment as source of n-alkanes and PAHs from the use of diagnostic molecular indices that indicated pyrolytic and biogenic, also petrogenic sources.

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