Determination of the total petroleum hydrocarbons in aquatic products by fluorescence spectrophotometry

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Abstract. The paper studied the determination of total petroleum hydrocarbon (TPHs) in aquatic products by fluorescence spectrophotometry method. To be suitable for the detection of TPHs in various aquatic products, the pretreatment conditions of the approach were optimized, such as the saponification temperature, saponification time, the adding order of anhydrous ethanol, the addition amount of extraction agent. The established method showed a good linearity in the TPHs range 0.0 ~ 40.0 μg/mL (R2 = 0.9965) with the limit of detection (LOD) 1.36 μg/mL and the precision of 6.1 % (relative standard deviation, RSD). The addition standard recoveries were 72.8 % ~ 88.4 %. Finally, the method was successfully applied in detection of TPHs in the various aquatic products.

1. Introduction
Petroleum hydrocarbons (PHs) are mixtures of various hydrocarbons (including n-alkanes, branched alkanes, cycloalkanes, aromatics) and other derivative, such as sulfide, nitride, naphthenic acid. With the development of the economy, people's demand for energy is expanding, and oil has become one of the most important energy sources [1]. In the process of exploitation, processing and utilization, oil increasingly enter the soil and the ocean, causing the pollution and the destruction of the environment [2,3]. As one of the widely existing organic pollutants in the environment, the excessive PHs entering into the soil and the ocean will accumulate in the body of marine organisms and finally enter into the human body along with the food chain, endangering human health [4,5]. Therefore, it is great significance to study and detect the PHs in aquatic products to ensure the quality and safety of aquatic products and reduce the harm to human being caused by the PHs.

Sun [6] found that most wild fish and crustacean in the Yellow Sea area near Jiangsu province were moderately polluted by PHs. Xia [7] studied that the PHs in clams were easily affected by the oil in sediments and water bodies. Guan [8] researched the PHs in the cultivation environment of the river crabs including the water quality and the sediment, found that the cultivation water quality was seriously polluted and the pollution index of the river crabs increased from 1.31 in 2013 to 2.42 in 2016, which indicated that some river crabs were heavily polluted by PHs. A variety of detection methods of PHs...
have been extensively developed with infrared spectrophotometry (IR) [9-11], ultraviolet and visible spectrophotometry (UV) [12,13], fluorescence spectrometry (FL) [14-16], gas chromatography (GC) [17-19], gas chromatography-tandem mass spectrometry (GC-MSA) [20-22]. IR and UV have low sensitivity, while GC and GC-MS are not suitable for the detection of the total petroleum hydrocarbons (TPHs). To avoid these disadvantages, fluorescence spectrometry is adopted in this paper to detect TPHs with the advantages of facile to operation, good stability and high sensitivity.

Herein we optimized that the pretreatment conditions of the approach, such as the saponification temperature, saponification time, the adding order of anhydrous ethanol, the addition amount of 

2. Materials and methods

2.1. Sample collection

The aquatic products mainly involved fresh samples, for instance Lophiomus sp., Argyrosomus argenteus, Dasylakis akaja, Psenopsis anomala, Trichurus lepturus, Pleuronectidae sp., Coelorhynchus multispinulosus Katayama, Caranx equula, Muraenesox sp., Lepidotrigla microptera, Heterodontus, Anguilla marmorata, Dentex tumifrons, Konosirus punctatus, Raja porosa, Decapterus maruadsi, Ilisha elongate, Scomberomorus sp., Thamnacon sp., Calappa sp., Charybdis japonicas, Jjaponicus, Lateolabrax japonicas, Cynoglossidae sp., Lestidium anago, Zeus japonicas, Jjaponicus, Lateolabrax japonicas, Cynoglossidae sp., Sardinella sp., Pampus argenteus, Scorpaena sp., Lestidium prolixum, Mackerel, Loligo chinensis, Sepiella maindroni, Octopodidae sp., Penaeus sp., Oratosquilla sp., Calappa sp., Charybdis japonica, Portunus trituberculatus, Ovalipes punctatus, Ruditapes philippinarum, Mercenaria, Ostrea sp., Mytilus sp., Sinonovacula constricta, containing fish, shrimp, shellfish and so on. In all, 52 kinds of aquatic products and 312 samples were collected. After the samples were transported back to the laboratory in the car refrigerators, thawed, homogenized, and kept at -20 °C for further using.

2.2. Instrument.

Fluorescence spectrophotometer (960CRT) was purchased from Shanghai Precision Instrument Co., Ltd. High speed centrifuge (CF16RX II) was purchased from HITACHI company, and the termovap sample concentrators (N - EVAPTM11) was purchased from Organomation Associates. Digital display water bath constant temperature oscillator (SG-8016C) was from Shanghai Shuoguo Electronic Technology Co., Ltd. Turbine mixers were purchased from IKA.

2.3. Reagents.

The calibration oil of 20-3# with the concentration of 1.00 mg/mL were charged from the State Oceanic Administration of Environmental Protection. Anhydrous ethanol, sodium hydroxide and sodium chloride were all purchased from Sinopharma Chemical Reagent Co. Ltd. Cyclohexane (HPLC) were from J.T.baker, and dichloromethane (HPLC) from Macron Fine Chemicals. Ultrapure water was 18.2 MΩ·cm. Other reagents were analytical degrade without special instructions.

2.4. The Sample pretreatment.

Homogenized samples were accurately weighed of 2.5000~3.0000 (±0.0001 g) in the saponification flasks with the volume of 100 mL, then 20 mL of sodium hydroxide solution with the concentration of 6 mol/L were added into the flasks. The reaction systems were subjected to shock and saponify for 10 h at 37 °C in the dark, which were shocked every 0.5 h for 1 min in the first 4 hours. Then 20 mL anhydrous ethanol was added into the systems, shake well and react for 4 hours at 20 °C in the dark. Blank
experiments were also performed with the same operations. Saponification liquids were transferred into the 500 mL separatory funnels, 10 mL dichloromethane, 30 mL saturated sodium chloride and 80 mL ultrapure water saturated sodium chloride were added successively. After being shaken vigorously for 3 min deflating carefully, the stock solutions were standing for 0.5 h. The reaction systems were divided into two layers and the lower layers were collected. Another 10 mL methylene chloride solutions were added into the separatory funnel, and the reaction systems were extracted again. The two extracting solutions were combined and concentrated under the condition of nitrogen blowing. 5.0 mL of cyclohexane was accurately added to dissolve the residue, then the solutions were mixed for further detection.

2.5. The calibration curve.  
0.0 µL, 10.0 µL, 20.0 µL, 40.0 µL, 100.0 µL, and 200.0 µL of the calibration oil (1.00 mg/mL) were measured in saponified flasks and operated in accordance with the samples. Taking the relative fluorescence intensities of the calibration oil removing the ones of the blank samples as the vertical and the concentration of calibration oil as the horizontal to draw the calibration curve.

2.6. Determination.  
The relative fluorescence intensities of the calibration solutions and the samples were measured at the emission wavelength of 360 nm under the excitation wavelength of 310 nm. The slit width was set as 10 nm, and the voltage was 400 V with the integral time of 1.0 s, and the delay time of 0.0 s. According to the calibration curve, the total petroleum hydrocarbons in the actual samples (mg/kg) were calculated.

2.7. Calculation formula.  
The total petroleum hydrocarbons in the samples were calculated as follows:  
\[ X = \frac{C \times V}{m} \]  
(1)

In the formula (1), X, C, V and m denoted the content of total petroleum hydrocarbons in the sample (mg/kg), the concentration of total petroleum hydrocarbons (µg/mL) calculated from the calibration curve, the constant volume (mL) and the weight of the aquatic product (g), respectively.

3. Results and discussion  
3.1. Optimum the reaction conditions  
3.1.1. Study of the saponification reaction. Compared with shellfish, fish aquatic products were difficult to saponify completely at room temperature. Figure 1 described the effects of saponification versus different saponification temperatures and times. The results showed that fish samples were complete saponification at 25 °C for 14 h or even longer, as shown in Figure 1(a) ~ 1(i). When the saponification temperature increased to 30 °C, the saponification time could reduce to 12 h (Figure 1(a1) ~ 1(h1)). When the saponification temperature continued to increase to 37 °C, the saponification rates of the samples were significantly increased. After 8 h ~10 h (Figure 1(a2) ~ 1(g2)), the saponification solutions were transparent, which suggested the samples could be completely saponified. However, as the temperature further rising, TPHs with low boiling points would be lost during the saponification due to their volatilization. Shrimp and crab could also be saponified completely under the same conditions. In summary, the aquatic products were saponified at 37 °C for 10 h in this work.
Figure 1. The effects of saponification reaction under the different times and different temperatures. 25 °C (a) 0 h, (b) 1 h, (c) 2 h, (d) 4 h, (e) 6 h, (f) 8 h, (g) 10 h, (h) 12 h, (i) 14 h; 30 °C (a₁) 0 h, (b₁) 1 h, (c₁) 2 h, (d₁) 4 h, (e₁) 6 h, (f₁) 8 h, (g₁) 10 h, (h₁) 12 h, and 37 °C (a₂) 0 h, (b₂) 1 h, (c₂) 2 h, (d₂) 4 h, (e₂) 6 h, (f₂) 8 h, (g₂) 10 h, respectively.

3.1.2. Influences of the anhydrous ethanol addition order. In order to shorten the reaction time, Bo [23] simultaneously added anhydrous ethanol and sodium hydroxide into the saponification flasks, and the saponification time reduced to 5 h ~ 6 h. The influences of the anhydrous ethanol addition order on the detection were researched and the results were shown in figure 2. As clearly can be seen from Figure 2, the relative fluorescence intensities of anhydrous ethanol latter addition (the black points in the Figure 2) were higher than that of anhydrous ethanol first addition (the red points in the Figure 2) at the same concentration of TPHs. Furthermore, as the concentration of TPHs increased, the distinctions enlarged, which may be resulted from some PHs removing along with the anhydrous ethanol volatilization from the reaction system during saponification. Therefore, it was adopted that the reaction systems should be cooled the temperature to 20 °C after saponification at 37 °C for 10 h, then the anhydrous ethanol was added into the systems.

Figure 2. Influences of the anhydrous ethanol addition order (Red points: first adding and blank points: latter adding, respectively).

3.1.3. Impacts of water addition volume. Wang [24] found that adding water into the reaction systems during the extraction was conducive to reducing the background interference and improving the accuracy of the results. Zhang [25] resulted that the volume of water was too small to high of the blank despite the obvious stratification, and too large to avoid the emulsification while the blank value decreased. Figure 3 presented the impacts of the ultrapure water adding volume during the extraction operation. As obviously shown in Figure 3(a), the lower layer had a large number of suspended solids.
with the addition volume of ultrapure water of 40 mL. As the volume addition of ultrapure water increased to 60 mL and 70 mL, the suspended solids in the lower layer were decreased (Figure 3(b) and Figure 3(c)). As the volume addition of ultrapure water increased to 80 mL, the lower layer exhibited transparent and clarification (Figure 3(d)). Whereas the volume addition of ultrapure water further increased to 90 mL and 100 mL, the lower layer began to emulsify (Figure 3(e) and Figure 3(f)), which was consistent with the reported. Finally, the optimal volume addition of the ultrapure water was 80 mL during the extraction in this paper.

3.2. Linear range, detection limit, recovery and precision of the established method

It was performed that saponification, extraction, concentration, reconstitution and determination under the optimum reaction conditions. Each sample was tested two times in parallel to ensure the reliability of the results. The detail addition standard experiments were conducted as follows. 5.0 mg/kg, 25.0 mg/kg and 50.0 mg/kg of the calibration oil were added to the negative aquatic products as the blank samples, respectively. 6 samples were measured in parallel with each concentration. Meanwhile, blank experiments were conducted at the same time.

The results were list in Table 1, which displayed the method kept well linear with the TPHs in the concentration range of 0.0 ~ 40.0 μg/mL (R²=0.9965). The average recoveries were 72.8 %, 84.6 % and 88.4% at the low, medium and high concentrations of TPHs, respectively. The precision of the method was 6.1 %, which was calculated by the eleven times results of the blank samples in parallel. The limit of detection (LOD) is 1.36 μg /mL (S/N=3). The results suggested that the method established in our work presented the good accuracy and the favourable precision, which indicated it could be applied to detect the TPHs in real samples.

Table 1. The calibration equations, linear range, precision, recovery and LOD of The method.

| Analyses | Linear range / μg/mL | The calibration equations | R² | Spiked /mg·kg⁻¹ | Recovery/ % | Precision / % | LOD /μg·mL⁻¹ |
|----------|----------------------|---------------------------|----|----------------|-------------|--------------|---------------|
| TPHS     | 0.0 ~ 40.0           | y = 0.9080 + 0.0448 x    | 0.9965 | 5.0, 25.0, 50.0 | 72.8, 84.6, 88.4 | 6.10 | 1.36 |

3.3. Detection of TPHs in real samples

Table 2 list the results of TPHs detected by the established method in various aquatic products. According to the data in the table, it was observed that all of the 32 shellfish samples were found to contain the TPHs pollutants, which may be caused by the anxiety of shellfish, and the results were consistent with the literature reports. 81.0 % of the cephalopod samples detected to suffer from the TPHs pollutants. The detection rates of crustaceans samples and fish samples were 68.3 % and 61.4 %, respectively. The detection rates of TPHs in different kinds of aquatic products collected in our work were more than 60 % (i.e. 68.9 %), demonstrating that different kinds of aquatic products were seriously polluted by TPHs, which should be required more attentions.
Table 2 The TPHs detected by the established method in different aquatic products.

| Species      | The sample quantity | Detection Numbers | Detection rates / % |
|--------------|---------------------|-------------------|---------------------|
| Shellfish    | 32                  | 32                | 100                 |
| Cephalopods  | 42                  | 34                | 81.0                |
| Crustaceans  | 41                  | 28                | 68.3                |
| Fish         | 197                 | 121               | 61.4                |
| Sum          | 312                 | 215               | 68.9                |

4. Conclusion
In summary, we developed the determination method of total petroleum hydrocarbons in various aquatic products based on fluorescence spectrophotometry. The pretreatment conditions of the approach were optimized, including the saponification temperature, the saponification time, the adding order of anhydrous ethanol, the addition amount of water. The results showed that the aquatic samples were saponified in water bath at 37 °C for 10 h, especially fish. Then the stock solutions cooled to 20 °C and added anhydrous ethanol to further saponify for 4 h. During the extraction process, 80 mL of ultrapure water was added to effectively avoid the suspensoid and the emulsification. The obtained method revealed a good linearity in the TPHs range 0.0~40.0 μg/mL (R²= 0.9965) with the limit of detection (LOD) 1.36 μg /mL, the precision of 6.1 % and the addition standard recoveries of 72.8 %~88.4 %. Finally, the method was applied in detection of the TPHs in the aquatic products involving the fish, the shellfish, the crustaceans and the cephalopods. The results demonstrated that different kinds of aquatic products were seriously polluted by TPHs, which should be paid more attentions.

Acknowledgments
This work was supported financially by the central public-interest scientific institution basal research fund (East China Sea Fisheries Research Institute) (No. L32201924480).

References
[1] Cai Y.T., Xu Y.B., Wu L.F. (2008) Study on the distribution and variation of TPH content in Marine culture organisms. Fujian aquatic products, 26: 40-43.
[2] Chagas-Spinelli A. C. O., Kato M. T., De Lima E. S., & Gavazza S. (2012) Bioremediation of a tropical clay soil contaminated with diesel oil. Journal of Environmental Management, 113, 510-516.
[3] Zhan Y. (2008) The harm of soil oil pollution in China and its countermeasures. Environmental pollution and prevention, 30: 91-93.
[4] Shang L.S., Sun Q., Xu H.Z., Xu X.R. (1997) The marine oil pollution and determination. Marine environmental science, 16: 16-20.
[5] Albaiges J., Farran A., Soler M., Gallifa, A., & Martin, P. (1987). Accumulation and distribution of biogenic and pollutant hydrocarbons, pbe's and ddt in tissues of western mediterranean fishes. Marine Environmental Research, 22:1-18.
[6] Sun J., Gu X.Y., Zhang A.Q., Wang X.R. (2010) Organism qualities and pollution assessment at the Yellow Sea of Jiangsu Province. Ocean science, 34:28-33.
[7] Xia P.Y., Yuan Q., Jiang M., Shen X.Q. (2011) Petroleum hydrocarbon pollution in the Meretrix meretrix habitat in Rudong tidal flat of Nantong. Journal of ecology, 30:2283-2289.
[8] Guan L., Lin C.C., Guo X.S. (2019) Investigation and evaluation of petroleum hydrocarbon pollution in the area of Chinese Mitten Crab farming. Aquaculture, 40:37-42.
[9] Ma H.W. (2010) Study on the method of determination of total petroleum hydrocarbon in soil by infrared spectrophotometry. Environmental science and technology, 23: 62-63.
[10] Ma X.L., Su L.N., Peng L., Wang L.L. Chen P. (2018) Determination of total petroleum hydrocarbons in low oil contaminated soil by infrared spectrophotometry with accelerated solvent extraction. Physical and chemical examination (Chemical Series), 54:388-392.
[11] Wang X., Ye J. Y., Huang L. P., Jiang K.M. (2019) Isolation and identification of hydrocarbon...
degradation bacteria from contaminated oil sludge. Environmental impact assessment, 41:100-104.

[12] Zhou L.H., Wu Y. (2004) Determination of petroleum in refinery wastewater by ultraviolet spectrophotometry. Petrochemical technology and applications, 22:456-458.

[13] Liang Y.Z. (2013) Screening algae strains of Chlorella sp. and Dunaliella salina with resistance to oil pollution and their lipid enrichment by using petroleum hydrocarbon. (Doctoral dissertation). Liaoning Normal University.

[14] Jiang C.J., Qiao Q.L., Cai Y.Q., Xu J. (2006) Kinetic features and threshold value of petroleum hydrocarbons for Ruditapes philippinarum. Marine fisheries, 28:314-320.

[15] Jiang F.H., Zhao M.L., Wang X.Y., Zheng L., Chen J.H., Wang X.R. (2012) Determination of petroleum hydrocarbon in mussels by fluorescence spectrophotometry with ultrasonic extraction. Marine environmental science, 31:906-909.

[16] Ren N., Zhu T., Fang Y., Wang Z.L., Feng R.N. Ji Y.Q. (2017) Monitoring and evaluation of heavy metals and petroleum hydrocarbons in organisms in coastal waters of Bohai bay in Tianjin. Ocean information, 32:37-43.

[17] Drozdova S., Ritter W., Lendl B., Rosenberg E. (2013) The Challenge in the determination of petroleum hydrocarbons in water by gas chromatography (hydrocarbon index). Fuel, 113:527-536.

[18] Wang S.W., Han W.J., Li H.L., Zhang F.J. (2008) Determination of total petroleum hydrocarbons in environmental waters by GC. Physical and chemical testing: chemical volume, 54:297-302.

[19] Zhou R.J., Ren X.R. (2019) Determination of extractable total petroleum hydrocarbons (C10~C40) in soil by GC combined with ultrasonic oscillation. Environment and development, 31:147-151.

[20] Yang H.J. (2013) Determination of petroleum hydrocarbon in soil and phytoremediation by rhizosphere bacteria. (Doctoral dissertation) Nanjing Normal University.

[21] Hua Z.T. (2013) The Influence of soil and petroleum hydrocarbon properties on the efficiency of petroleum contaminated soil remediation by solvent extraction. (Doctoral dissertation) Tianjin University.

[22] Li J.J., Bai G.M., Yu Y.X. (2017) Comparison between GC-MS method and infrared spectrophotometry for determination of petroleum hydrocarbon in water. Science and technology innovation, 31:156-157.

[23] Wang Q., Shao M.H., Tao P. (2006) Determination of total petroleum in halobios by fluorescence spectrophotometry. Journal of Dalian Maritime university, 32: 51-53.

[24] Zhang H.Y., Zhou D.Q. (2008) Study on fluorescence spectrophotometry test of petroleum hydrocarbon in aquatic products. Marine fisheries research, 29:106-111.

[25] Bo E.L., Guo N., Luo J. (2015) Rapid Determination of Petroleum Hydrocarbon Contaminants in Aquatic Products by Fluorescence Spectrophotometry. Liaoning Agricultural Sciences, 56: 27-30.