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Determination of total hydrocarbon and its relation to amino acid found in two bivalve edible species from Alexandria and El Ismailia coast, Egypt.

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ABSTRACT

Organic contamination can be viewed as the secondary dispersions of organic compounds from various sources into the global circulation across different spheres of the environment; this study was conducted to assess the role of total hydrocarbon on two species of edible bivalve; *Pinctada radiata* and *Ruditapes decussatus*. Due to their microphage capacity, bivalves have great biologic importance as they clean the environment of harmful microorganisms and decaying organic compounds which result in water purification, water filtering. Waste materials derived from animals metabolism coalesce in larger aggregates which sink and deposit onto the bottom, thus enabling the bivalvular shells to contribute to developing and securing adequate conditions for the productive cycle of marine environment. The aim of this work was to investigate the present status of the contamination by total organic hydrocarbons in two species of bivalves collected from (Alexandria and Ismailia), Egyptian Mediterranean Sea Coast in winter season, 2015 (November). The spatial distribution for total hydrocarbon suggested that most of the contaminants may originate from urban runoff, municipal wastes and petroleum industries. Only two of the essential amino acids (L-Lysine, and L-Leucine) were reported in clam; *Ruditapes decussatus* collected from (Ismailia and Alexandria) and three of the essential amino acids were reported in oyster; *Pinctada radiata* that were collected from Alexandria; (L-Arginine, L-Lysine, and L-Leucine). In the present results it is concluded that the total amount of amino acid in the two species of bivalve (oyster and clam), followed the arrangement of a.a. in oyster which is collected from Alexandria> a.a. in oyster which is collected from Ismailia> a.a. in clam that were collected from Alexandria>a.a. in clam that were collected from Ismailia; as 4.45805 × 15.4649 = 68.9432> 3.3436 × 15.1117 = 50.5274 > 3.0089 ×15.6026 = 46.9466 > 2.9164 ×15.1932 = 44.30944 µMol/gm; respectively.The total hydrocarbon in tissue of both bivalve species is less than that reported by ATSDR,1995, with range of (0.40-1.3 µg/g) <3.0 µg/g.

Indexing terms/Keywords

Key wards: amino acids, bivalve, seawater, total hydrocarbon.

Academic Discipline And Sub-Disciplines

Ecology- pollution-bivalve –hydrocarbons –ecosystem-biodiversity.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are groups of hydrophobic organic compounds with two or more fused aromatic rings, are introduced into the environment via natural and anthropogenic processes (La flame and ITES, 1988, NRC, 1985). Some PAHs such as benzo(a)pyrene and benz(a)anthracene, have mutagenic and carcinogenic properties (IARC, 1983),)PAHs accumulate in aquatic organisms,particularly in invertebrate species that have a low metabolizing capability (Forster *et al*., 1988) and have been detected in marine mammal tissue (Hellon *et al*., 1990; Hellon *et al*., 1991). PAHs are suspected of inducing cancer in marine and fresh water fish (Varanasi *et al*., 1987; BLACK & Baumann, 1991; Myers *et al*., 1991). Several possible sources for PAHs in the environment exist (Fouchecourt *et al*.,1999 and Mc Elroy *et al*., 1989). Polycyclic aromatic hydrocarbons can result from natural processes, but anthropogenic activity is generally considered to be the major source of PAH input into the environment. Concerning natural sources pyrolytic PAHs can be generated by forest or grass fires. Marine seeps can release hydrocarbon compounds into seas and oceans and natural compounds can derive from biogenic precursors.

The presence of organic compounds and their harmful derivatives makes water unfit to consumption. Maintaining a supply of pure water for ever increasing population is already a daunting challenge all over the world while the organic contaminants are aggravating the challenges further [Chen *et al.*, 2007; Wilcox *et al.*, 2009]. The organic substances of biological origin also result from either excreta or wastes from of living organisms, from municipal waste decomposition, etc. Among the dissolved organic substances in water, polycyclic aromatic hydrocarbons (PAHs), halogenated aromatic and aliphatic hydrocarbons are the major classes [Al-Mudhaf *et al.*, 2009]. The input of organic contaminants from natural sources does not increase the way they are increasing from anthropogenic sources. Human activities are increasing the consumption of the already known organic compounds all over the world for meeting diverse needs while organic pharmaceuticals, pesticides, paints and coloring materials, cosmetics, with novel properties are synthesized every day for many purposes. The quantity and diversity of anthropogenic organic contaminants that enter the fresh water system and polluting it are accumulating [Loos *et al.*, 2009].

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Organic contaminants are mostly human-induced chemicals entering into natural fresh water through pesticide use, industrial chemicals, and as by-products of degradation of other chemicals and persist long enough in the environment to cause harmful effects. They tend to accumulate in reservoirs such as water, soil, sediments etc. From these reservoirs, they are remobilized through various processes, switch form or speciation and become available to the biological food chain. In this way, these contaminants tend to bio accumulate and bio magnify exhibiting toxicity and other related outcomes – mutagenicity, carcinogenicity and teratogenicity - resulting into chronic and acute disorders [Barnes *et al*., 2008]. Organic contaminants are composed basically of hydrocarbons both from anthropogenic and natural sources. Many contaminating hydrocarbons exist in the environment but carcinogens, mutagens and teratogens among them are the most closely monitored [Barnes et al., 2008]. Effluents from municipal sewage treatment plants, industrial sources, storm sewer systems, mining and construction sites. are examples of point sources. In contrast, organic contaminants from non-point-sources are diffused over broad geographical scale in a relatively uniform environmental concentration explicitly delineated into spatial or temporal patterns. Consequently, the management of non-point organic contaminants is difficult. Among non-point-sources are agricultural runoff, urban runoff and atmospheric wet and dry depositions [Baranowska *et al*., 2005].

Organic contaminants of biological origin are natural organic contaminants of which alkaloids and terpenoids are prominent. Synthetic organic contaminants are generated through reaction among different chemical species, which subsequently get discharged into the fresh water - examples include DDT, Polychlorinated biphenyls (PCBs) Chlordane, and Dieldrin [Zhang *et al*., 2003]. Some organic contaminants are easily broken down upon entry into the environment, but others are very persistent, popularly termed as, Persistent Organic Pollutants (POPs) which are of particular concern because of the long term risks they pose. POPs, due to their persistent nature, become widely distributed geographically to pose adverse effects to human health and the environment [Pal *et al*., 2010]. The most common pesticide among the fresh water organic contaminants are DDT, Diedrin, Aldrin, Chlordane, Endrin, Toxaphene, and Heptachlor [Cuyno *et al*., 2001; Fernandez-Cornejo *et al*., 1998]. Poly Aromatic Hydrocarbons (PAHs) are produced through burning of common fossil fuels, such as coal, petroleum and natural gas as well as from biomass fuels such as fuel wood, animal excreta. Naphthalene, an ingredient in dyeing industry, aluminium smelting industry and lubricant oils as well as wood procession industry, enters into water, mainly through discharges and spills during the storage, transportation and disposal of fuel oil and coal tar and incomplete combustion of organic compounds [Rosenfeld and Plumb, 1991].

Burning of gasoline, garbage or biomass causes release of benzopyrene into the environment. It also releases from burning of tar during road construction, from wood preservative creosote and from glues used in electrical components (Chao and Ting-lin, 2011).The environmental release of benzene occurs from industries such as rubber processing, dyeing and washing, pharmaceuticals, and agrochemicals. Underground gasoline storage tanks also leak benzene into ground water [Adeyemi *et al*., 2009]. Agrochemicals are predominantly organic in nature and diverse in classes, nature and applications. They constitute the major anthropogenic source of organic contaminants into the fresh water system [Vedal, 2010]. Some of the persistent organic contaminants, including DDT, move from the point of application through the atmosphere and translocate from relatively warm regions to get condensed at colder, higher latitudes through a process known as global distillation effect. This explains the deposition of organic compounds at high concentrations in the Arctic region which is free from usage of such compounds [Kidd *et al*., 1998; Stern *et al*., 2005]. Benzene is water soluble, to some extent, and can seep into groundwater as well. Among the POPs, polychlorinated biphenyles (PCBs) were wonder materials with a wide range of applications. In fresh surface water or ground water, most of the PCBs adhere to suspended sediment particles and remain as such for years [Welfinger-Smith and Carpenter, 2011]. Petroleum hydrocarbons, and particularly the more toxic aromatics and heterocyclics, accumulated by marine animals interact with cells and tissues to produce a variety of lesions (Roubaland Collier, 1975). Many hydrocarbons are irritants and cause localized inflammatory responses. In oysters Crassosrrea gigas from the Amoco Cadiz oil spill site, the most common histopathology was an inflammatory response in mantle and gill tissues (Neff and Haensly, 1982). Mussels *Mytilus edulis*, transplanted to a bay that was heavily contaminated with oil from the Amoco Cadiz spill, developed accumulations of lipid droplets (Wolfe *et al*., 1981).

Stainken, (1976) reported generalized leucocytosis in the mantle of soft-shell clams *Mya arenaria* exposed in the laboratory to oil. He also observed glycogen depletion and cellular vacuolization in several tissues of exposed clams. A wide variety of some histopathological lesions have been reported in invertebrates exposed to petroleum in the laboratory or field (Malins, 1982). There are several reports in the literature of increased incidence of apparently cancerous tumors in populations of bivalve mollusks from oil-contaminated environments (Bayne *et al*., 1985).

Material and Methods

The sampling cruise took place in November 2015, from two areas in Alexandria total of eighty samples from both species (*Pinctada radiata* and *Ruditapes decussatus*); (fourty/species) from the beginning of the El Mex in the west to the Abu Quir in the east. Fourt/species were collected from two sampling sites that were chosen in Ismailia goverenrate (two locations in lake Temsah) to provide an adequate as possible geographical coverage of the study area. Two different eidible bivalve species were collected from these areas. *Pinctada radiata* and *Ruditapes decussatus* from the presented sites. The specimens were collected and wrapped in three sheets of clean, heavy duty aluminum foil, where the dull side of the foil was in contact with the sample and the samples were then kept in a deep-freezer at –20 C.

10 gm bivalve tissue (wet weight) was treated with 30 gm of sodium anhydrous sulfate and the mixture was blended at high speed for 5 minutes. Then the mixture was extracted with a Soxhlet extractor with 200 ml of methanol for 8 hours (UNEP/IOC/IAEA, 1981). Then, 0.7 M KOH (20 ml) and distilled water (30 ml(were added to the flask and the reflux was continued for 2 hours to saponify the lipids. The content of the extraction flask was extracted in a separatory funnel with 80

ml/3hexane. Then the extracts were combined, dried with anhydrous sodium sulfate and filtered through glass wool. The hexane fraction was concentrated with a rotary evaporator down to about 15 ml at 30 C followed by concentration with nitrogen gas stream down to a volume of 1 ml. A chromatography column was prepared using a 50 ml burette; 10 gm of silica gel was transferred into the column, followed by 10 g of alumina and finally 1 g of sodium sulfate. The extract (1 ml) was sequentially eluted from the column with 20 ml of hexane for the saturated aliphatic fraction (F1). Then 30 ml of hexane and dichloromethane (90:10) for the unsaturated and aromatic hydrocarbons fraction (F2). F1 and F2 were concentrated using stream of nitrogen for instrumental analysis. To control the analytical reliability and assure recovery efficiency and accuracy of the results, 7 analyses were conducted on PAH compound reference materials, HS-5 (sediment) provided by NRC-IMB of Canada and SRM-2974 (Freeze-dried clam tissue provided by NIST of USA.The laboratory results showed recovery efficiency ranged from 89-110% with coefficient of variation (CV) of 10-14% and standard deviation (SD) of ± 7-15. All solvents were pesticide grade purchased from Merck. Blanks of 1000 fold concentration were analyzed by Gas Chromatography with a flame ionization detector (FID). The Gas Chromatographer was a Hewlett Packard HP 5890- series II equipped with split/splitless injector and a fused silica capillary HP-1 (30 m,0.3 mm, 0.17 mm) 100% dimethylpolysiloxane.The temperature was programmed from 50-190 C with rate of 5C min–1 and was, then,maintained at 290C for 25 min. Nitrogen was used as a carrier gas at a flow of 1.3 ml min–1.

Materials for detection of amino acids in bivalve tissue: Acetonitrile (LC grade), methanol (LC grade), and phenol (p.a. grade), were purchased from Sigma-Aldrich (St. Louis, MO). Borate buffer, OPA and FMOC reagents and standard solutions of mixture of 15 amino acids (10, 25, 100, 250 and 1000 nmol cm-3) were obtained from Agilent Technologies (Waldbronn, Germany). Hydrochloric acid, used for the preparation of 6 mol dm-3 and 0.1 mol dm–3 HCl, was obtained from Lach-Ner (Neratovice, Czech Republic). Sodium phosphate monobasic was purchased from Acros Organics (New Jersey, USA). Nitrogen gas was purchased from Messer Technogas (Belgrade, Serbia). LC grade water was produced by a Heming ID-3 system (Belgrade, Serbia). The reference material of a complete fodder mixture for piglets was purchased from the National Reference Laboratory of the Central Institute for Supervising and Testing in Agriculture (Brno, Czech Republic). Apparatus Vacuum hydrolysis tubes (19 mm×100 mm) were obtained from Pierce (Rockfors, IL).Cellulose membrane syringe filter (0.22 μm pore size), screw cap vials and screw caps were purchased from Agilent Technologies (Waldbronn, Germany). Blue-labelled filter discs (quant.) grade: 391 were obtained from Munktell (Bärenstein, Germany). The hydrolysis was performed using a Reacti-ThermTM heating/stirring module (Thermo Scientific, Rockford, IL), while the evaporation procedure also included a Reacti-Vap TM Evaporator (Thermo Scientific, Rockford, IL).The analysis was performed on an Agilent 1260 Infinity liquid chromatography system equipped with a μ-degasser (G1379B), 1260 binary pump (G1312B), 1260 standard autosampler (G1329B), 1260 thermostated column compartment (G1316A), 1260 diode array and multiple wavelength detector (G1315C), and a Zorbax Eclipse-AAA column (150 mm× 4.6 mm, i.d., particle size 5 μm) (Agilent Technologies, Santa Clara, CA).

Procedure:Samples of bivalve (oysters and clams) were analyzed for their amino acid content. Preparation of protein hydrolysates. The fodder samples were ground to pass through a 0.5 mm sieve. The samples were then hydrolyzed by two different procedures. First, 0.1–1.0 g was weighed (equivalent to 10 mg nitrogen content) into a screw capped test tube and 2 cm3 of 6 mol dm-3 HCl was added. The tubes were capped and the samples were hydrolyzed for 24 h at 110 °C. After the hydrolysis, the mixtures were evaporated to dryness under vacuum. The hydrolysates were reconstituted in 2 cm3 of 0.1 mol dm-3 HCl (Thermo Scientific Pierce GC and HPLC Technical Handbook, 2008). In the second procedure, samples of the same mass were weighed into vacuum hydrolysis tubes and 7 cm3 of 6 mol dm-3 HCl with 0.1 % of phenol were added and mixed gently. The hydrolysis was realised in a Reacti-ThermTM heating/stirring module for 6 h at 150 °C. After the hydrolysis, the samples were cooled to room temperature and evaporated to dryness using a Reacti-ThermTM heating/stirring module and Reacti-VapTM Evaporator, at 70 °C under a stream of nitrogen. The residues were quantitatively transferred into 50 cm3 volumetric flasks using 0.1 mol dm-3 HCl. The solutions were filtered through quantitative filter paper into glass tubes and the filtrates were purified using 0.22 μm pore size, cellulose membrane syringe filters (Thermo Scientific Pierce GC and HPLC Technical Handbook, 2008).

HPLC Determination: The chromatographic conditions employed were in accordance with the Agilent method,25 except for mobile phase A, which consisted of 5.678 g of Na2HPO4 per 1 dm3 water, adjusted to the pH 7.8 with a 6 mol dm-3 HCl solution (buffer strength 40 mmol dm-3). The mobile phase B was acetonitrile–methanol–water (45:45:10, vol %). Briefly, the hydrolyzed samples or the solutions the standard amino acid mixture were automatically derivatised with OPA and FMOC by programming the autosampler (1). draw 2.5 μl from vial 1 (borate buffer), (2). draw 0.5 μl from sample (position X),(3). mix 3 μl in air, max. speed, 2×, (4). wait 0.5 min, 5. draw 0 μl from vial 2 (water, uncapped vial), 6. draw 0.5 μl from vial 3 (OPA), 7. mix 3.5 μl in air, max speed, 6×, 8. draw 0 μl from vial 2 (water, uncapped vial), 9. draw 0.5 μl from vial 4 (FMOC), 10. mix 4 μl in air, max speed, 6×, 11. draw 32 μl from vial 5 (water), 12. mix 18 μl in air, max speed, 2× and 13. inject). After derivatisation, 0.5 μl of each sample was injected into a Zorbax Eclipse-AAA column at 40 °C, with detection at λ1 = 338 nm and λ2 = 262 nm. The separation was performed at a flow rate of 2 cm3 min-1 employing a solvent gradient (vol. %,) as follows: 0 min, 0 % B, 1.9 min, 0 % B, 18.1 min, 57 % B, 18.6 min, 100 % B, 22.3 min, 100 % B, 23.2 min, 0 % B and 26 min, 0 % B. Twenty amino acid standards including alanine (Ala),arginine (Arg), aspartic acid (Asp), cystein (Cys), glutamic acid (Glu), histidine (His), isoleucine (Ile), leucine (Leu),lysine (Lys), Ornithine (Orn), Tryptophan (Trp), Cystine ((Cys)2), glycine (Gly), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine, (Thr), tyrosine (Tyr), valine (Val) and c-aminobutyric acid (GABA), were obtained from Sigma Corporation. Other reagents such as acetic acid were all analytically pure. Pure water was prepared by Milli-Q super pure water system. DBCEC was synthesized in the laboratory (You *et al.*, 2009).

Results and discussion:

In the present study, samples (20) of clam collected from Ismailia, in November 2015, showed the presence of two of the essential amino acids; L-Lysine and L-Leucine, in the amount of 1.69496×15.1932=25.751866 and 1.22146×15.1932=18.557886 µMol/gm with a retention time of ; 19.843 and 24.46; respectively. The total amount of amino acids of the present samples was reported as 2.9164×15.1932= 44.309448µMol/gm. Whereas the amino acids that did not show any presenceas; Aspartic acid, glutamic acid, asparagine, DL-serine, Glutamine, Glycine, DL-Threonine,L-Alanine,L-Arginine, Tyrosine, DL-2-Amino-N-Butyric , Tryptophan, phenyl alanine and IsoLeucine. In the samples of clams (20) collected from Alexandria, in November, 2015, showed the presence of two EAA; L-Lysine, L-Lucine as; 9.0063×15.6=140.52169, 2.108×15.6=32.8902 µMol/gm; respectively, with a retention time of; 24.475, 19.85' respectively, with total amino acids as; 3.0089×15.6 µMol/gm. In the samples of oysters (20) collected from Alexandria,in November,2015, showed the presence of three essential amino acids(EAA); L-Arginine,L-Lysine, and L-Luecine as; 8.1864×15.4645=126.598 µMol/gm, 2.10686×15.4649=32.58237 µMol/gm and 1.53256×15.4649-23.70088 µMol/gm, with the retention time of 17.111,19.856 and 24.474; respectively, with the total amount of 4.458 ×15.4649=68.94252 µMol/gm. In the samples of oysters (20) collected from Ismaelia in November, 2015, showed the presence of two essential amino acids (EAA); L-Lysine and L-Leucine in the amount of 1.9325×15.1117 and 1.41093×15.1117µMol/gm with a retention time of 19.824 and 24.438; respectively, with a total amount of amino acids of 3.3434×15.1117µMol/gm. It leads us to conclude that the total amount of amino acid in the two species of bivalve –oyster and clam- followed the arrangement of oyster which is collected from Alexandria> oyster which is collected from Ismailia> clam that was collected from Alexandria> clam that was collected from Ismailia; as 4.45805×15.4649 =68.9432> 3.3436×15.1117=50.5274 > 3.0089×15.6026=46.9466 > 2.9164×15.1932=44.30944 µMol/gm; respectively.

Table (1): Shows the amount of amino acids (µMol/gm) in the tissue of bivalve (*Pinctada radiata* and *Ruditapes decussatus*) collected from Alexandria and Ismailia, Egypt in November, 2015.

Locations	Amount of amino acid (µMol/gm)		
	L-Arginine	L-Lysine	L- Leucine
Oyster Alexandria	126.596	32.581	23.697
Clam Ismailia		25.751	18.558
Clam Alexandria		32.891	140.525
Oyster Ismailia		29.203	21.292

Table (1) represents the presence of essential amino acids in the two bivalves represented species with the highest amount of L- Leucine (140.525 µMol/gm) in the tissue of clam (*Ruditapes decussatus*) collected from Alexandria in November, 2015.Whereas, the L-Arginine (126.596 µMol/gm) was only represented in oyster collected from Alexandria in November,2015.L-Lysine ranged from 25.751 to 32.891 µMol/gm and it was reported in all samples in both bivalve species in the present study.

Table (2): Shows the total amount of amino acids in the two species of bivalve collected in November 2015 from Alexandria and Ismailia:

Species/Locations	Total amount (µMol/gm)
Oyster Alexandria	68.943
Clam Ismailia	44.309
Clam Alexandria	46.948
Oyster Ismailia	50.526

Table (2) represents the total amount of amino acids with ranging from 44.309 in clams collected from Ismailia to 68.943µMol/g in oyster collected from Alexandria. Oyster showed high amount of amino acids than that of the clam.

Table (3): Comparison of the total hydrocarbon in tissue of bivalve between the four studied groups according to the results (µg/g):

	Oyster Alex (n= 4)	Clam Alex (n= 4)	Oyster Ismailia (n= 4)	Clam Ismailia (n= 4)	p
Results					
Mean ± SD.	1.37 ± 1.36	1.31 ± 1.46	0.48 ± 0.60	0.40 ± 0.45	0.445
SEM	0.68	0.73	0.30	0.23	

Normally quantitative data was expressed in (mean ± SD) and was compared using F- ANOVA test

Table (3) reports the mean of total hydrocarbon in tissue of bivalve ranged between 1.4±0.45(µg/g) in clam collected from Ismailia to 1.37±1.36 (µg/g) in oyster collected from Alexandria in November, 2015, at P<0.45.

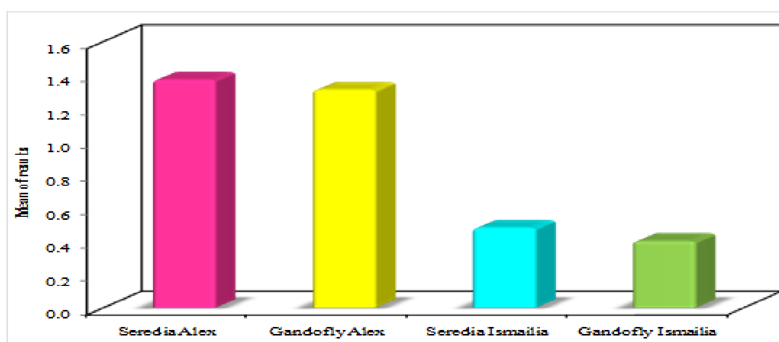

Figure (1):Comparison between the four studied groups according to results

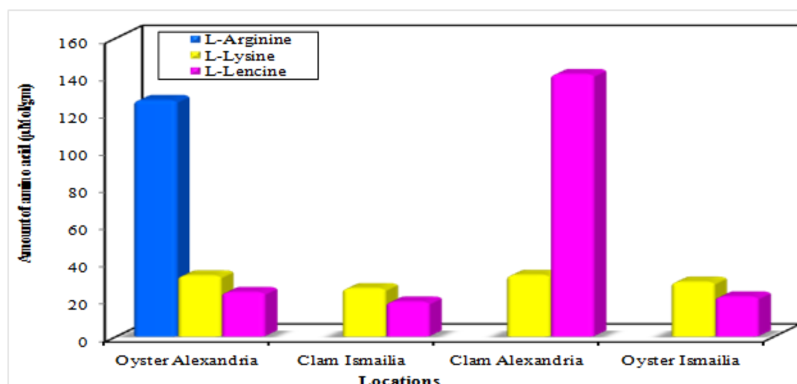

Figure (2): Shows the amount of essential amino acids found in in *Pinctada radiata* and *Ruditapes decussatus* collected in November, 2015 from Alexandria and Ismailia.

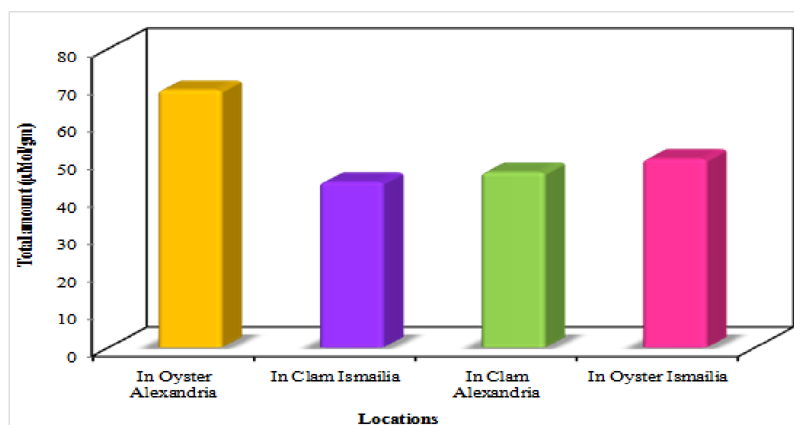

Figure (3): Shows the total amount of amino acids in the two species of bivalve in the two locations collected in November, 2015.

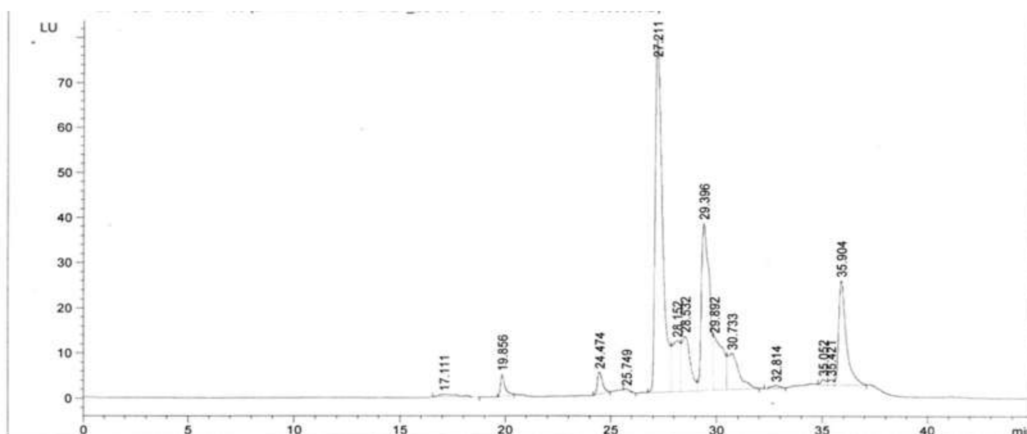

Figure (4): Using HPLC Showing the amino acid present in the oyster collected from Alexandria, in November, 2015.

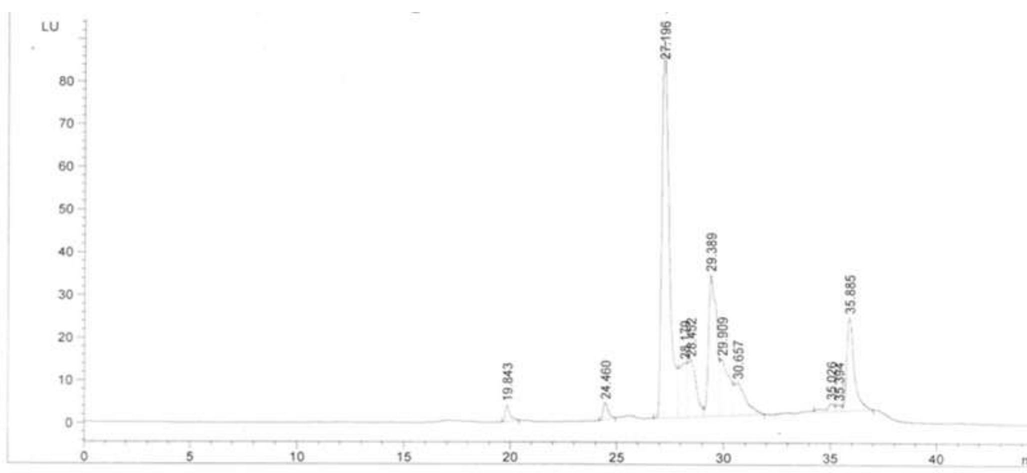

Figure (5): Using HPLC showing the amino acid present in the clam collected from Ismailia, in November,2015.

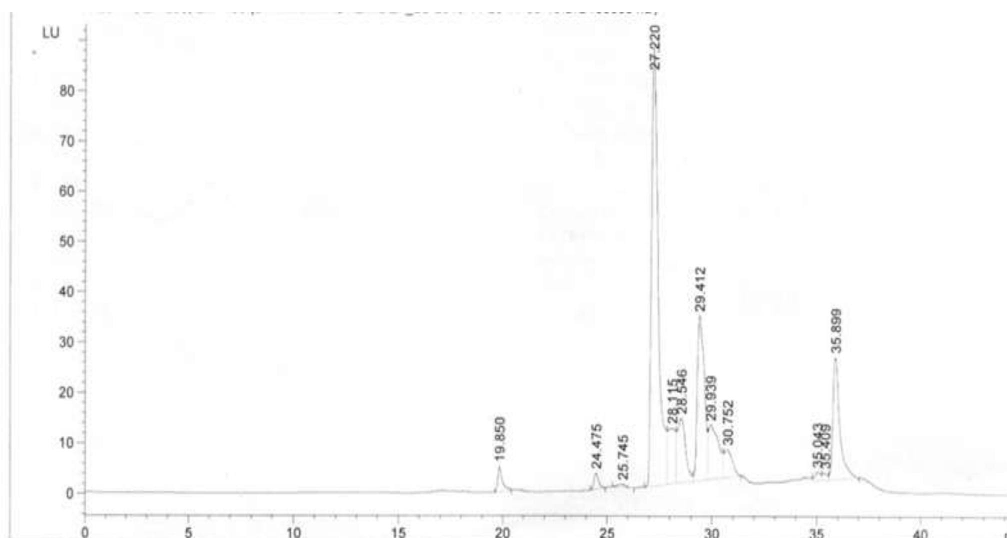

Figure (6): Using HPLC showing the amino acid present in the clam collected from Alexandria in November, 2015.

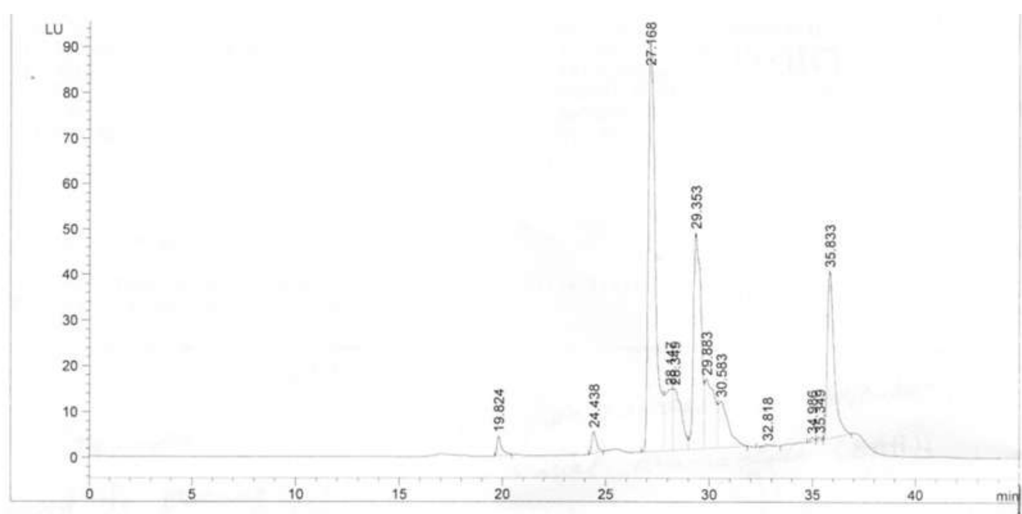

Figure (7): Using HPLC showing the amino acid present in the oyster collected from Ismailia in November, 2015.

It is well established that aquatic bivalves are the final accumulation site of water-borne constituents derived from natural sources in situ, surroundings, and artificial (domestic, urban-industrial and agricultural wastes) sources. Molluscs have been used for monitoring contaminants in the environment (Farrington *et al.*, 1983). The polycyclic aromatic hydrocarbons (PAHs) are of pyrolytic origin (Garrigues *et al.*, 1995; Benlahcen *et al.*, 1997). The most likely source for these pyrolytic PAHs is the grass fires and exhaust gases from cars (ElSikily *et al.*, 2002). In the present study, the results of the analysis represent average concentrations from total hydro carbon determinations. Among the essential amino acids, L-Lysine is found in the bivalve tissue (*Pinctada radiata* and *Ruditapes decussatus*) in the present study. The result revealed in this study showed that, the bivalve meat is a potential source for food value due to high quality protein, as well as balanced essential amino acids. Similar observations were made by Ajaya Bhaskar, who reported that, the total amino acids in *Perna viridis* is 95.76%, 98.4% in *C. madrassensis* and 65.17% in *M. casta* (Ajaya, 2002).

The essential amino acids (EAA) are; Phenylalanine, Lysine, Histadine, Methionine, Arginine, Leucine, Threionine, Isolucine, Valine, Tryptophan. Whereas the nonessential amino acids are (NEAA); Glycine, Serine, Glutamic acid, Cysteine, Glutamate, Alanine, Proline, Aspartate, Tyrocin, Aspargine. In the present study it was reported three of the EAA in all the samples collected from the two locations. The present study showed that the concentrations of total hydrocarbon are relatively low in comparison with other coastal seas. PAHs in the Mediterranean Sea are generally of pyrolytic origins; this indicates the frequent grass fire as a common source of these compounds together with the car exhaust fumes. Other sources such as petrogenic, non-petroleum industries, oil refineries, oil distribution and heavy ship traffic may be involved.

The determination of the amino acid composition of the proteins in food is of great importance (Wathelet, 1999). Namely, the amino acid level is an indicator of the nutritional value of food and fodder proteins (Heems *et al.*, 1998). As a laboratory technique, the analysis of amino acid plays an important role in biochemical, pharmaceutical and biomedical fields (Liu, 2000). Hitherto, several different methods have been developed for the determination of amino acids (Moore and Stein,1963; Gökmen *et al.*, 2012). Mostly, the methods were based on the technology developed by Moore and Stein (Moore and Stein ,1963), which includes post-column derivatisation and detection in the visible region on an amino acid analyser. These analyses are reliable, but costly and time-consuming. The HPLC technique, combined with pre-column derivatisation of amino acids, has become a very important method for the analysis of amino acids (Sarwar and Botting, 1993). It should be emphasized that pre-column derivatisation has gained wide acceptance and a number of different derivatisation reagents have been used (Bruton,1986; Li *et al.*, 2011).

All methods that involve analysis of numerous AA show critical separation of several compounds or matrix interference: for instance, Gln-His, Arg-Ala, HIS, PEA, TRM (Herbert *et al.*, 2006); Thr, Ala-Arg, Val-Met, Trp-His, Phe-MTA, Lys-ETA,CAD, PUT (Kutlán & Molnár-Perl, 2003), GABA-Pro, Trp, AGM,Orn-Lys-His (Krause *et al.*, 1995); Asp, Asn-Ser-Hyp, ammonium ion-AGM, Trp-Leu, SPD, TRM (Gómez-Alonso *et al.*, 2007); Hyp, Gly-His-Thr, Arg-Ala, HIM, Trp, Leu-Phe, TYM, SPD, CAD (Cejudo-Bastante et al., 2010); Glu-Asp, Val-Met, Phe-Iso-Leu, Lys-TRM,HIS-Tyr (Jia *et al.*, 2011). Pollution may cause alterations in the concentrations of the major free amino acids (Livingstone, 1985; Scholz, 1987; Hummel *et al.*, 1994; 1996) and also in enzymes involved in its metabolism (Narvia and Rantamäki, 1997). In this context, FAA may reflect metabolic status and provide information on the physiological condition of the organisms (Livingstone, 1985), functioning as a convenient index of stress. Indeed, not only total FAA, but also relative proportions of certain amino acids like taurine : glycine, the sum of serine and threonine or alanine have been used as general stress indicators (Zurburg *et al.*, 1989; Pranal *et al.*, 1995; Sokolowski *et al.*, 2003), also when considering metallic and organic polluted environments (Livingstone, 1985; Hummel *et al.*, 1994; 1996). Occasionally, other behavioural responses such burrowing capacity of *Macoma balthica* have been reported to be more sensitive indicator of stress than condition index and free amino acids (Duquesne *et al.*, 2004).

As general pattern, a decrease in total FAA and the sum of serine and threonine and an increase in t:g ratio were observed in molluscs as indication of environmental deterioration (Livingstone, 1985; Scholz, 1987; Hummel *et al*., 1996). The sum of serine and threonine was reported to be highly questionable when the effects of pollution were investigated in the mussel *Mytilus edulis* (Hummel *et al*., 1994). The most common pattern, therefore, consists in a decrease of free amino acids in tissues of bivalves exposed to contaminants that might be related to the fact that uptake of amino acids is reduced as a consequence of valve closure under such circumstances (Viarengo *et al*., 1980) that in turn might cause a reduction of protein metabolism. Only incidentally the opposite (decline in taurine and t:g ratio) occurred in molluscs facing crude oil-contaminated sediments (Augenfeld *et al*., 1980). It is important to highlight here that exposure to hydrocarbons may promote protein catabolism by destabilization of lysosomal membranes (Viarengo *et al*., 1992), with a concomitant increase of protein amino acids. PAHs composition, however, was described to represent a combination of fossil and pyrolytic sources (Labarta *et al*., 2005) and in specific locations, the uptake of oil hydrocarbons occurred over a background load of pyrogenic PAHs that partly accounting for the variability. They have pointed out the convenience of studying the oil spill impact using biochemical indicators (survival potential, lipid metabolism) and extending the pollution analyses to other hydrocarbons that might have an effect on metabolic routes. The importance of alanine in the osmoregulation processes of bivalves and their anaerobic metabolism have been widely described (de Vooys, 1991). Together with alanine, glycine is also an important organic compound playing a role in osmoregulatory processes of bivalves (Bishop *et al*., 1994).

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