Organic and Total Mercury Concentration in Fish Muscle and Thermodynamic Study of Organic Mercury Extraction in Fish Protein

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ABSTRACT MeHg and total mercury concentrations were determined in the muscle tissues of four fish species (*Cyprinus carpio*, *Rutilus frisii*, *Carassius auratus* and *Esox lucius*) from Anzali wetland (Guilan, Iran). Fish with the highest amount of MeHg was selected to determine the thermodynamic parameters of MeHg extraction. The extractions process was performed in the range of temperatures 331.15 to 367.15 K and at atmospheric pressure. Results show the extraction of MeHg from SH groups of sulfhydryl proteins was an endothermic process with a positive value for entropy and Gibbs free energy changes at the room temperature. Significant difference was found between MeHg content at T=367.15 K and other temperatures. Correlation coefficients results showed that the mercury concentration in muscle tissue was significantly related to the length and weight of fish (p≤0.01). Also, thermodynamic parameters of methylmercury extractions had significant correlation (p≤0.05) with length and weight of the six fish specimen.

Key words: *Methylmercury, Thermodynamic, Extraction, Fish protein*

1 INTRODUCTION

Mercury is one of the most toxic heavy metals commonly found in the global environment, including lithosphere, hydrosphere, atmosphere and biosphere. Hg exists in three oxidation states: metallic or elemental (Hg^0), mercurous (Hg_2^{2+}) and mercuric (Hg^{2+}). A series of complex chemical transformations allows the three-oxidation states of Hg to cycle in the environment (Barbosa *et al.*, 2001; Watras, *et al.*, 1992).

Elemental Hg is the most common form of Hg found in the atmosphere. Most of the Hg encountered in all environments is in the form of inorganic mercuric salts and organo-ercurics, with the sole exception of atmosphere (Hg^0, Hg_2^{2+} or Hg^{2+}). The mercuric salts (mercuric chloride, mercuric hydroxide and mercuric sulfide) are the prevalent forms existing in the environment and methylmercury chloride and methylmercury hydroxide are main forms of organic compounds, together with other organo-mercurics (i.e., dimethylmercury and phenyl mercury) existing in small fractions (USEPA, 1997).

Mercury can be present as a trace contaminant in all environmental compartments as a result of both natural and anthropogenic activities and also re-emitted (Horvat, 2001). Determination of mercury compounds in the

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environment is important because of toxicity and ability to bio-accumulate in many organisms.

The toxic effects and metabolic behavior of mercury is largely dependent on its chemical form. Monomethylmercury (MMHg) is by far the most toxic and the most commonly occurring organo-mercury compound, and is recognized as a major environmental pollution issue and health hazard for humans. Methylmercury (MeHg) has a high affinity to sulfhydryl groups (-SH) of proteins and this causes its rapid absorption in living organisms. It easily penetrates through biological membranes and is bio-magnified through the trophic chain.

Organic mercury enters into fish body by direct uptake from water and through food chain in comparison with inorganic mercury (IHg). As a result, the average proportion of MMHg over total Hg in fish tissues can be up to 95% (Horvat, 2001). Contamination of seafood is the major route of exposure for to MMHg. Therefore, humans and wildlife, such as bald eagles, kingfishers, otter and mink, which feed on fish, are particularly at risk because of the potential for MeHg to bio-accumulate in freshwater fish (USEPA, 1997 and Commonwealth, 1996).

Trace concentrations of mercury compounds in fish body are not just related to their concentrations in the environment, but fish can also affected by other factors, including its size, age, sex, growth rate, and reproductive condition (WHO, 1976). Distribution of MeHg takes place via the bloodstream to all tissues in the body. In the muscle, mercuric ions have also strong tendency to bond to SH groups on cysteine amino acids of the protein molecules and are stored there (Yawei *et al.,* 2005).

Several studies have been done on mercury effect in various organisms in Iran, including river otters (Solgi *et al.*, 2013), green tiger prawn (Hosseini *et al*., 2013), and silver carp (Jafarpour

et al., 2013). In our previous study, the extraction of IHg of sulfhydryl proteins was obtained as an endothermic process with positive value for entropy and Gibbs free energy in room temperature (Astani, 2011).

In this work, the extraction of total mercury (THg) and MeHg in the muscle of four fish species was performed and the thermodynamic parameters of MeHg extractions from SH groups of cysteine amino acid were determined for two species. Also, the effects of age, weight, and length of fishes on the mercury contents were studied.

2   MATERIALS AND METHODS
2.1 Fish Sampling
Fishes were collected from the Anzali wetland (Iran). They were photographed and had their total length (cm) and weight (g) recorded before dissecting them to collect their muscles. Fish age was estimated from scale samples. Muscle samples, taken with a knife from the posterior left part of the fish body, were dried in an oven at 343.15 K for 24 h and then homogenized.

Feeding habits and approximate trophic position in the aquatic food web of the species are given in Table 1. Data on fish age, weight, length, mean values of THg and MeHg are summarized in Table 2.

2.2 Apparatus and procedures
MeHg and THg contents in fish muscle were measured by the method proposed by Ubillús *et al.* (2000), in which 5 ml of 10 M KOH was added to test tubes containing 0.5 g of the dried sample and the mixture was introduced into a circulating water bath for 25 min. The temperature variation of water was controlled within0.02 K. (Lauda Bath Ecoline RE 104, Germany).The samples were digested at a range of temperatures (331.15 to 367.15 K), then filtered by filter paper and transferred into a l00 ml volumetric flask. The tubes were washed with 1% (w/v) NaCl solution, then 15 ml of 65%

HNO$_3$ and 1 ml of oxidizing solution (1 g of potassium dichromate, 20 ml of nitric acid and deionized water to l00 ml) were added and then the volume was adjusted to 100 ml with 1% (w/v) NaCl solution. The solutions were used for determination of MeHg and THg by 0.3% (w/v) NaBH$_4$ and 1% (w/v) NaBH$_4$ solutions, respectively, as reduction solutions. An atomic absorption spectrophotometer (Analytic Jena HS 60) by a system Nov AA 400 cold vapour and a hollow cathode mercury lamp (lamp current: 3.0 mA, slit width: 1.2 nm) was used for determination of mercury concentrations.

Table 1 Fish species, their feeding habits and approximate trophic position in the aquatic food web

Common name	Scientific name	Family	Feeding strategy
Common carp	*Cyprinus carpio*	Cyprinidae	Herbivore/omnivore. Juveniles feed on zooplankton, phytoplankton and hard-shells. Adults feed largely on other macrobenthos
Kutum roach	*Rutilus frisii*	Cyprinidae	omnivore feeds on aquatic insect larvae, crayfish and pelecypoda
Goldfish	*Carassius auratus*	Cyprinidae	Herbivore/omnivore. Feeds on aquatic plants and insects, shellfishes
Northern pike	*Esox lucius*	Esocidae	Piscivore. Adults feed mainly on fish, also feed heavily on frogs and crayfish. Cannibalistic as juveniles.

Table 2 Age, weight, length and Hg content of samples under investigation

Species	Age (years) Range Mean	Weight (g) Range Mean	Length (mm) Range Mean	THg ($\mu g\ g^{-1}$) Range Mean ± SD	MeHg ($\mu g\ g^{-1}$) Range Mean ± SD
Common carp	2-4 2.6	132-660 354.4	250-378 317.6	0.016-0.248 0.179±0.02	0.010-0.180 0.146±0.01
Kutum roach	4-5 4.4	750-900 804	420- 470 443	0.052-0.324 0.200±0.06	0.038-0.272 0.170±0.02
Goldfish	2-5 3.6	62-506 245.3	155-325 243.7	0.003-0.165 0.115±0.05	0.003-0.107 0.084±0.03
Northern pike	1-5 2.8	178-1250 666.8	305-580 443.93	0.028-0.575 0.366±0.09	0.016-0.395 0.285±0.05

2.3 Data analysis

Data was analyzed using linear correlation and regression. Differences between averages of MeHg and THg contents in each temperature for each fish species were analyzed with one-way ANOVA followed by the least significant difference (LSD) post hoc for multiple comparisons. The relationships between fish size, mercury contents and extraction thermodynamic parameters were explored using linear regression analyses. Data were analyzed using the SPSS software, version 19.

2.4 Theoretical framework

Abiotical formation of MeHg involves the transfer of a methyl radical by methylcobalamine (Craig, 1986). The SH functional group of cysteine amino acid is active site for bonding MeHg and mercuric mercury with sulfhydryl proteins. One of the essential reactions of the SH group is with MeHg and mercuric ions (Shahbazi, 2006; Huheey, 1983). MeHg-S bonding energy in fish muscle tissue is enthalpy type.

The effect of selenocysteine (Sec) in this context should be noticed. The binding constant of selenium to mercury (Se-Hg) is bigger than sulfur to mercury (S-Hg), but there are two reasons showing that only S-Hg reaction is dominant: (1) the ratio of cysteine (Cys) to selenocysteine (Sec) is relatively high because the Sec is a very rare amino acid in protein structure, (2) because of the very higher binding constant and rareness of Sec, it is reasonable that we assume all selenocysteines were saturated by Hg during association/dissociation reactions.

2.5 Thermodynamic framework

The system of dried fish muscle samples and KOH 10 M solution in the sealed assay tube could be assumed as a closed system in thermal and mechanical equilibrium. Most of the mercury compounds in fish muscle tissue are covalently bound to sulfhydryl proteins.

KOH 10 M solution can break covalent bonds of the system, following the heat energy during the chemical reactions. Covalent bonds also are broken between the sulfur atoms of sulfhydryl proteins and mercury compounds.

3 RESULTS AND DISCUSSION
3.1 Factors affecting mercury accumulation in fish

The concentrations of different kinds of mercury compounds varied with fish species (Table 2), which is related to variation in extracted thermodynamic parameters of mercury compounds. Calculation of thermodynamic parameters was performed based on the maximum values of MeHg concentrations in each fish species.

Mercury content is affected in fish muscle tissues by some factors. It is clear that diet and living environment expressively influence content of mercury in muscle tissues of fishes (Sarica, 2005). Results show that the highest contents of THg (0.366 µg g^{-1} in dry matter) and MeHg (0.285 µg

g^{-1} in dry matter) belonged to the muscle of northern pike. The living environment (wetland's bed) of northern pike probably influences contamination of its tissues. In the other hand, the lowest contents of mercury were found in the muscle of goldfish, whose diet consists mainly of phytoplankton. Generally, muscle tissue of predatory fishes contained significantly higher contents of mercury than that of non-predatory fishes.

Predatory fishes such as northern pike almost lie in the end of aquatic dietary pyramid. Northern pike eats small fish such as goldfish and white bream. Fishes at lower trophic levels contained the lowest contents of mercury. Dietary pyramid represents bioaccumulation of THg and MeHg with increasing trophic levels.

Beside diet, it is believed that size and age of fish are the possible effective factors on mercury level in fish tissues. The relative ages for the fish were determined based on the fish length and weight (Ikingura, 2003). However, there was no significant relationship between the THg and MeHg contents in fish muscle tissues and either fish age, weight or length among individuals of any fish species (Table 2). But there was a strong relationship between THg and MeHg contents with fish mean length (r = 0.702) and weight (r = 0.622) across all species (Table 2). Also the same correlations were seen for organic mercury (r = 0.614 for the total length and r = 0.684 for the weight, at p < 0.01). Because of the narrow range of fish age (1-5 years), age did not influence the mercury content in the tested tissues. Although only few samples were analyzed, the results suggest that in some cases it is possible to predict fish mercury levels from the relationship with fish weight or length.

3.2 Concentration equilibrium constant of the extraction process

In this work, we also studied the thermodynamic of the MeHgextraction from muscle tissue of three northern pike and three

kutum roach. MeHg concentrations of the examined fish muscle tissues were measured at T=331.15 to 367.15 K temperature range. These concentrations were used for calculation of concentrationequilibrium constant (K_c) the breaking Hg-S chemical bond of protein-MeHg molecules of the muscle at the corresponding temperatures. Equilibrium constant, K_c, calculated from the eq. (1) as follow:

$$K_C = \frac{C_2}{C_1} \tag{1}$$

where C_1 is the equilibrium concentration of non-extracted MeHg and C_2 is the equilibrium concentration of extracted MeHg in solution. C_2 concentrations ($\mu g\ g^{-1}$) were calculated at the corresponding temperatures based on experimental data of CVAAS apparatus as follow:

$$\text{MeHg} (\mu g\ g^{-1}) = \frac{C \times V \times DF}{B} \tag{2}$$

where C in $\mu g\ L^{-1}$ unit, is the MeHg concentration measured of fish muscle by calibration curve; V is the extract volume (L); B is the sample weight (g) and DF is the dilution factor.

The results of K_c of the examined fish muscle at the corresponding temperature are tabulated in Table 3. The results show theequilibrium concentrations of MeHg (C_2) increase with temperature. The plot of the ln K_c versus $1/T$ shows that the extraction process of

MeHg of sulfhydryl proteins is an endothermic process. Pearson correlation coefficient (r) was calculated using SPSS 19 and statistical significance $p<0.01$. The plots of ln K_c versus $1/T$ were shown for two fish samples in Figure 1. All of other samples were found to have linear correlation over the entire range of temperatures (336.15 to 361.15) K.

3.3 Variance analysis calculation of mercury concentration at different temperature

ANOVA-1 was used to compare MeHg and THg concentrations at different temperatures for each fish species. Results showed significant differences among extracted MeHg means at different temperatures (P<0.05). In all fishes, MeHg concentration at T=367.15K was significantly different from that in other temperatures. In the other words, there wasn't any significant difference in free MeHg concentration when temperature changed from 331.15K to 361.15K, but the temperature 361.15K induced significant difference. Therefore, MeHg concentration has significant difference at T= 367.15K with the other temperatures. At this temperature, there is sufficient heat for breaking all covalent MeHg-S bonds; so, at maximum temperature MeHg concentration is highly different from the other temperatures. Same results were observed in ANOVA-1 analysis of THg means in these fish muscle tissues.

Table 3 Computed extraction equilibrium constants at the corresponding temperature in Kelvin

T	N. pike 3 ln K_c	N. pike 4 ln K_c	N. pike 5 ln K_c	K. roach 1 ln K_c	K. roach 2 ln K_c	K. roach3 ln K_c
361.15	0.958	0.821	1.436	1.621	1.090	1.398
357.15	0.430	0.018	0.191	0.104	0.873	0.512
351.15	-0.856	-0.548	-0.841	-0.419	0.120	0.189
346.15	-1.105	-1.039	-1.326	-0.937	-1.133	-1.234
341.15	-1.514	-2.102	-1.966	-1.728	-1.846	-1.891
336.15	-2.492	-2.804	-2.330	-3.979	-3.512	-2.823

The maximum values of MeHg and THg in the six fish species are tabulated in Table 4. The results show that the northern pike and kutum roach can concentrate MeHg in its muscle tissues much more than IHg level reported in our previous work (Astani et al., 2011).
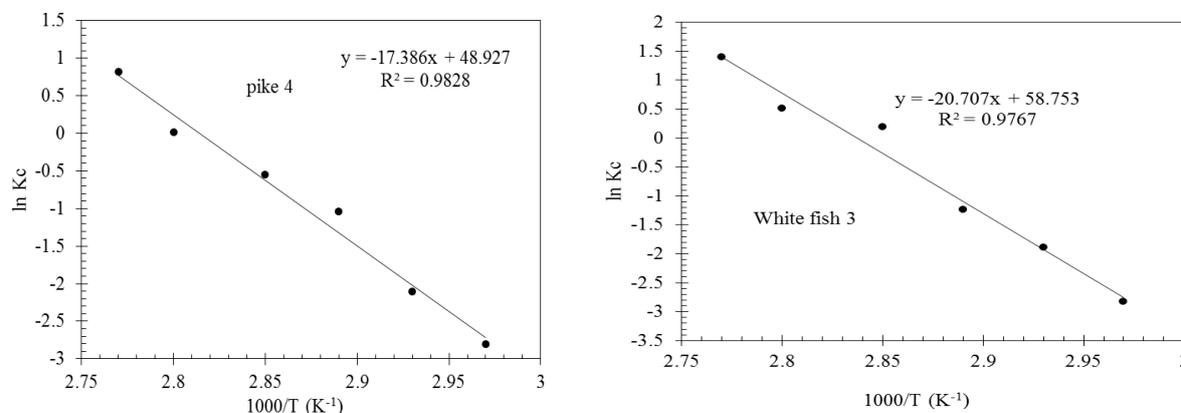

Figure 1 The plots of ln K_c versus 1/T for a selected northern pike and kutum roach

Table 4 The mean values of MeHg and THg in the examined fishes

Sample	C_{max} (µg g^{-1}) of MeHg	C_{max} (µg g^{-1}) of THg	L (cm)	W (g)
Northern pike 3	0.268	0.426	530	1200
Northern pike 4	0.395	0.575	580	1250
Northern pike 5	0.271	0.392	470	794
Kutum roach 1	0.255	0.296	420	750
Kutum roach 2	0.246	0.260	440	750
Kutum roach 3	0.272	0.322	455	830

3.4 Thermodynamics of the extraction process

The thermodynamic parameters of the MeHg extraction in the muscle tissues of three northern pike and three kutum roach were obtained. The parameters such as enthalpy change (ΔH) and entropy change (ΔS) were derived from the van't Hoff equation as follow:

$$\ln K_C = \frac{-\Delta H}{RT} + \frac{\Delta S}{R} \qquad (3)$$

ΔS and ΔH were calculated from the intercept and slope of linear plot of ln K_c versus $1/T$, respectively. Gibbs free energy change (ΔG) was calculated from the equation $\Delta G = \Delta H - T\Delta S$, where T is the absolute temperature and R is the universal constant. The computation of ΔG, ΔH and ΔS of the breaking of cysteine MeHg-S covalent bonds on the sulfhydryl proteins of fish muscle are possible by calculating of K_c at $T = 336.15$ to 361.15 K.

The results of the thermodynamic parameters of MeHg extraction are described in Tables 5 and 6. The positive values of ΔH suggest an endothermic process for the extraction of MeHg of sulfhydryl proteins that are related to the required energy for S-Hg breaking bond in cysteine amino acid. Also, the positive values of entropy change (ΔS) show an increasing randomness at the breaking for Hg-S covalent bonds of cysteine amino acids on the sulfhydryl proteins of fish muscle. The change in enthalpy and entropy of reaction were calculated in the 336.15 to 361.15 temperature range. They are not in standard condition. So, calculated Gibbs free energy change of reaction depends on

temperature. Table 6 shows the computed mean values of Gibbs free energy change of reaction at different temperatures. At low temperature, ΔG values are positive in both fish species that indicate a non-spontaneous process for the extraction. But for high temperatures, the Gibbs free energy mean value of reaction is negative that show the extraction process is spontaneous. Computed results for mean ΔG indicated that entropy production in S-H breaking bond of cysteine amino acid and temperatures were two more important factors that affected the Gibbs free energy of extraction. Comparison of the effect of these factors show the temperature is the most important. In summary, the less value of Gibbs free energies indicate the existence of close competition among enthalpy and entropy of extraction process and temperature can inverse the sign of ΔG.

Factors such as diet, size and age of fish in addition to influencing the mercury content in the muscle, they can also directly or indirectly affect the thermodynamic parameters of extracting mercury species. The results showed that the mercury concentration in the fish muscle was generally positively correlated with the weight or length of the fish. However, a relatively strong negative correlation between

the extracted thermodynamic parameters of MeHg and the total length and weight of the fish was evident (Table 7). Based on the results, the extraction of thermodynamic parameters of MeHg decreases with increasing total length and weight of the fish.

According to our previous study (Astani, 2011), thermodynamic parameters of IHg had a significant negative correlation with fish total length of all tested fishes (these data are presented in Table 7 for comparison). They also showed a small negative correlation with the fish weight. However, the correlation between ΔH, ΔS, and ΔG contents of MeHg was small (coefficient less than 0.7).

The highest extracted ΔH, ΔS, and ΔG contents of MeHg was found in kutum roach with total length of 420 mm and weight 750 g, whereas the northern pike with total length of 530 mm and weight 1200 g had the lowest extracted ΔH, ΔS, and ΔG contents of MeHg. With increase in fish length and weight, mercury contents of muscle also increased. All fish samples were digested at temperature ranging 331.15 to 367.15 K for 25 minutes. The fish with low concentration and smaller size required more thermal energy for breaking covalent Hg-S bonds than that with higher concentration and larger size.

Table 5 Thermodynamic parameters of MeHg extraction

Sample	ΔH(kJ mol^{-1})	ΔS(J K^{-1} mol^{-1})
Northern pike 3	132.45 ±1.48	373.6±4.3
Northern pike 4	141.40±1.00	398.0 ±2.9
Northern pike 5	145.21±2.20	409.8±6.3
Mean value	139.69±1.56	393.8±4.5
Kutum roach 1	192.23±3.31	544.0±9.5
Kutum roach 2	184.88±2.15	524.2±6.2
Kutum roach 3	168.38±1.45	477.7±4.2
Mean value	181.83±2.30	515.3±6.6

Table 6 Gibbs free energy change in kJ mol^{-1} of MeHg extraction reaction at difference temperature

T	336.15	341.15	346.15	351.15	357.15	361.15
Kutum roach	8.61±1.48	6.04±4.3	3.46	0.88	-2.21	-4.27
Northern pike	7.31	5.35	3.38	1.41	-0.96	-2.53

Table 7 Correlation coefficient of thermodynamic parameters (kJ mol^{-1}) of organic and inorganic mercury extraction with length and weight

Parameter	Organic mercury			Inorganic mercury		
	ΔH^0	$T\Delta S^0$	ΔG^0	ΔH^0	$T\Delta S^0$	ΔG^0
Length	-0.884	-0.887	-0.859	-0.947	-0.931	-0.901
Weight	-0.793	-0.796	-0.779	-0.699	-0.722	-0.407

The system containing muscle tissue of fish and KOH 10 M solution in the sealed assay tube was closed and the chemical reactions in this system occurred at constant atmospheric pressure. In each experiment, the system was exposed to heat energy for 25 minutes. In each temperature, some part of this heat energy is taken to break Hg-S bonds. Therefore, part of total heat energy absorbed in this process is consumed for breaking of Hg-S bonds which is equal to enthalpy change of organic or inorganic mercury extraction. So, the total heat energy absorbed by the system is the same for all samples. However, in breaking Hg-S bonds in fish tissues, there is a relationship between the mercury concentration and the total absorbed heat energy. The total heat energy absorption is greater in bigger fish (with high mercury concentrations) than the small fish (with low mercury concentrations). Therefore, the bigger fish has greater computed *ΔH*, *ΔS*, and *ΔG* values.

3   CONCLUSION

Comparing the MeHg and THg concentrations in the muscle of four freshwater fish species, the northern pike showed a higher concentration. The muscle of the northern pike and kutum roach, which had the highest amount of MeHg, were digested over 331.15-367.15K range of temperatures. The obtained data was used to calculate the equilibrium constant as a function of temperature. Pearson correlation coefficient (r) was used to prove linear relation between the inverse temperature and ln K_c. Thermodynamic parameters of Hg dissociation from thiol (SH) group of cysteine amino acid

was investigated. This study highlights the importance of contaminating concentration of Hg compounds and its transfer to human diet.

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غلظت جیوه آلی و معدنی در بافت ماهیچه‌ای ماهی و مطالعه ترمودینامیک استخراج جیوه آلی از پروتئین ماهی

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چکیده غلظت متیل جیوه و جیوه کل در بافت ماهیچه‌ای چهار گونه مـاهی در اکوسیسـتم مـرداب انزلـی تعیـین شـد. ماهی‌هایی با غلظت زیاد متیل جیوه برای به‌دست آوردن پارامترهای ترمودینامیکی استخراج متیـل جیـوه انتخـاب شـد. فرایند استخراج در محدوده دمایی ۳۳۱/۱۵ تا ۳۶۷/۱۵ کلوین در فشار محیط انجام شد. نتـایج نشـان داد کـه اسـتخراج متیل جیوه از گروه SH پروتئین‌ها در بافت ماهیچه‌ای ماهیان در دمای یک اتاق یک فرایند گرماگیر بوده که آنتروپـی آن در حال افزایش و انرژی آزاد گیبس مثبت دارد. اختلاف معنی‌داری بین مقدار متیل جیوه در دمای ۳۶۷/۱۵ کلوین با سـایر دماها مشاهده شد. تجزیه و تحلیل ضرایب همبستگی پیرسون نشان داد که غلظت جیوه در بافت ماهیچه‌ای نمونه‌هـا بـه صورت معنی‌داری با طول و وزن ماهی‌ها با سطح اطمینان ۹۹ در صد ارتباط دارد. هم‌چنـین پارامترهـای ترمودینـامیکی استخراج با سطح اطمینان ۹۵ درصد با طول و وزن ماهی ارتباط دارد.

کلمات کلیدی: متیل جیوه، ترمودینامیک، استخراج، پروتئین ماهی