Different Digestion Methods for Determination of Heavy Metals in Fish Muscles and Gills by FAAS

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Abstract - Through the use of the analytical method known as *Flame Atomic Absorption Spectroscopy (FAAS)*, we have analyzed three fish species, two of which were freshwater fish, while one of them has been sea fish. The analyzed fish species were: *Cyprinus carpio*, *Oncorhynchus mykiss* and *Dicentrarchus labrax*. In the fish muscles and gills using three digestion methods, we have quantitatively determined the presence of these types of heavy metals: Fe, Cu, Zn and Cr. The obtained results show a concentration over the permissible levels for Fe and Zn in the analyzed samples, while the concentrations of Cu and Cr are within the permissible levels. A significant difference of accumulated metals in the two analyzed fish tissues was also observed. Heavy metals are also determined in the habitat of freshwater fish, including some other water parameters, such as pH, temperature, dissolved oxygen and water conductivity.

Keywords: Flame Atomic Absorption Spectroscopy, Fish, Heavy Metals, Muscles, Gills.

1. INTRODUCTION

Consuming fish provides an important source of protein, polyunsaturated fatty acids (PUFA), liposoluble vitamins and essential minerals, which are associated with health benefits and normal growth [5].

Fish is important source of protein and it also provides essential omega 3-fatty acids (docosahexaenoic and eicosapentaenoic acids) that help to maintain cardiovascular health by playing a major role in the regulation of blood clotting and vessel constriction [9].

Fish is considered as one of the most significant indicators of metal pollution [1]in aquatic environment. Fish may absorb dissolved elements and heavy metals from surrounding water and food. Heavy metals could be found in water at the trace levels. Nonetheless, these constituents are very toxic and tend to accumulate in a long period of time [4].

Metals entering the aquatic ecosystem can be deposited in aquatic organisms through the effects of bioconcentration, bioaccumulation via the food chain process and become toxic when accumulation reaches a substantially high level. In fish, which is often at the higher level of the aquatic food chain, substantial amounts of metals may accumulate in their soft and hard tissues [7].

Therefore, World Health Organization (WHO) and European Community recommend controlling toxic metal ions in food sources in order to guarantee food safety and Flame Atomic Absorption Spectrometry (FAAS) is widely used and preferred for determination of toxic elements [1].

Heavy metal toxicity can result in damaged or reduced mental and central nervous function, lower energy levels, and damage to blood composition, lungs, kidneys, liver and other vital organ.

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The harmful effect of trace elements when consumed above the recommended limit can be toxic (acute, chronic or sub-chronic), and heavy metals can be neurotoxic, carcinogenic, mutagenic or teratogenic. The general symptoms of humans related to metal [e.g., Cd, Pb, As, Hg, Zn, Cu and aluminium (Al)] poisoning include vomiting, convulsions, paralysis, ataxia, hemoglobinuria, gastrointestinal disorder, diarrhea, stomatitis, tremor, depression and pneumonia [3].

In our study we have analyzed three species of fish, two of them were freshwater inhabitants, while the third it was seawater inhabitant.

The analyzed fish species were:

- *Cyprinus carpio*
- *Oncorhynchus mykiss*
- *Dicentrarchus labrax*

In each fish sample we have analyzed these types of heavy metals: Fe, Cu, Cr, Ni and Zn, and that in two fish tissues (muscles and gills).

The type of Flame Atomic Absorber that we have used for heavy metal determination was Perkin Elmer AA 300.

TABLE1

Permissible levels of some heavy metals in the fish according to some international organizations

	Fe	Cr	Pb	Cu	Zn	Cd	Ni	References
FAO (1983)			0.5	30	30	0.05		FAO (1983)
FAO/WHO Limit			0.5	30	40			FAO/WHO(1989)
WHO 1989	100		2	30	100	1		Mokhart (2009)
EC			0.2			0.05		EC (2005)
England			2	20	50	0.2		MAFF (2000)
FEPA		0.15- 1.0	0.2	20	50	0.2	0.5-1.0	FEPA (2003)

2. MATERIAL AND METHODS

2.1. Sampling

Sampling is done based on the sampling standard procedures, the recommendations given by different international organizations and institutions regarding the method of sampling and preparation of the fish specimens.

Sampling of *Cyprinus carpio* is done in the longest river in Kosovo known as Drini i Bardhë (Location:

Vërmicë - Southern Kosovo), *Oncorhynchus mykiss* we have taken from the fish pond (Location: Poslisht - Southern Kosovo) and *Dicentrarchus labrax* sampling is done in the Adriatic Sea (Republic of Albania) and imported in Kosovo for commercial purposes.

The fish was weighed and the body length was measured. The foil-wrapped sample is placed in a food grade plastic bag. After that, the sample is placed on the ice and quickly transferred to the laboratory.

Water sampling has been done in the same points as the fish were taken, excluding the seawater.

Sampling of water from the habitat of *Oncorhynchus myccis* is done at the entrance of the fish pond

2.2. Sample preparation

Before started the sample treatment, the fish were initially dried at 65 º C and then the process of dissection and tissue homogenization was started. We have done this to ensure a homogenous sample

The working tools we used were washed with soap and water and rinsed with hexane between samples to avoid cross-contamination. Then the samples are stored at -4 ºC temperature until the beginning of the analysis.

Water samples are placed in unused polyethylene bags, and then the water is treated with HNO3 65%. In the respective sampling points, we have analyzed some water parameters such as pH, temperature, dissolved oxygen (DO) and water conductivity, while analyzes of heavy metals from fish tissues and water have been done at the UBT Research Laboratories.

2.3. Calibration standard curve

After the fish sample preparation, we have prepared series of standards for all the analyzed heavy metals with known concentrations (0.05 ppm, 0.1 ppm, 0.5 ppm, 1 ppm, 10 ppm and 100 ppm). All chemicals used were of the highest purity and all solutions were prepared using double distilled water. Stock metal solutions, 1000 mg/L, were used for the preparation of standard and working solutions. All standard and sample measurements were repeated three times and the average of three measurements was calculated automatically. Finally the calibration curves has been constructed, and the correlation coefficient values for each element were: Fe (r^2) = 0.99830, Cu (r^2) = 0.99069, Cr (r^2) = 0.97712 and Zn (r^2) = 0.96778. The results of fish samples were expressed as mg/kg (ppm).

For water, we have prepared series of standards for all the analyzed heavy metals with known concentrations (0.05 ppm, 0.1 ppm, 0.5 ppm, 1 ppm, 10 ppm and 100 ppm). All standard and sample measurements were repeated three times and the average of three measurements was calculated automatically. Finally the calibration curves has been constructed, and the correlation coefficient values for each element were: Fe (r^2) = 0.99939, Cu (r^2) = 0.99416, Cr (r^2) = 0.97607 and Zn (r^2) = 0.94660. The results of water samples were expressed as mg/L (ppm).

2.4. Digestion Methods

The procedure and methods of digestion have been made according to the description given by Ranasinghe P et al (2016).

The digestion methods used in this study were acid digestion and dry ashing. The methods of the sample digestion with acids have been: the digestion with HNO3, the digestion with aqua regia solution (HCl : HNO3), while during dry ashing the sample was heated in a muffle furnace and then the residual ash was treated with HNO3.

2.4.1. HNO3 digestion

In the beginning of the HNO3 digestion, the fish sample (1.0 g) was placed in a 25 ml beaker. About 10 ml of concentrated NHO3 (69% w/w) was poured into the beaker. The beaker is covered with watch glass and placed on the magnetic stirrer/hot plate. At the beginning we kept the temperature at 40 ºC for one hour and then the temperature was maintained at 140ºC for another 3h. The residue is treated with the acid and the mixture is cooled in the room temperature. After that, the double distilled water was added into the beaker and the sample is filtered with by filter paper (Whatman No.1 grade). At the end, the sample ready for examination is stored at 4ºC until the heavy metal determination by FAAS.

2.4.2. Aqua regia digestion (HCl-HNO3)

The aqua regia solution was initially prepared by mixing of the concentrated HCl and HNO3, in the ratio 3:1 and 1 ml HClO4. Then 1.0 g of fish sample

was digested in 10 ml of aqua regia solution for 3hr at 60 ºC.

2.5. Dry ashing

For dry ashing, 1.0 g of fish sample initially is dried at 105 ºC, and then the vessels were placed in the furnace (Box Type Resistance Furnace SX-2.5-12), gradually heated to 550ºC starting from the room temperature (50ºC every 30 min) and ashed for 2 h. After cooling the residues are treated with 8 ml of concentrated HNO3 and then dissolved and filtered.

3. RESULTS AND DISCUSSION

3.1. Results

TABLE 2

Mean concentration of heavy metals after three replications in water habitat of *Cyprinus carpio* and *Oncorhynchus myccis*

Sampling point	Temp.ºC	Dissolved O2 (mg/L)	O2 saturation (%)	pH	Conductivity (µs/cm)	Fe (mg/l)	Cu (mg/l)	Zn (mg/l)	Cr (mg/l)
The River Drini i Bardhë	13.8	9.0	93.5	8.58	433	0.137	0.490	0.723	ND
Fish Pond (Poslisht)	9.6	8.7	85.4	7.79	286	0.096	0.050	0.528	ND

TABLE 3

Fish characteristics and sampling points

Fish name	Length/mm	Weight/g	Sampling points
Oncorhynchus mykiss	400	457.90	Fish Pond (Poslisht - Southern Kosovo)
Cyprinus carpio	270	258.80	The River Drini i Bardhë (Southern Kosovo)
Dicentrarchus labrax	300	337.13	Adriatic Sea (Durrës - Albania)

TABLE 4

Mean concentration of heavy metals after three replications in two analyzed tissues of *Oncorhynchus mykiss*

Fish name	Fish tissue	Digestion method	Fe (mg/kg)	Cu (mg/kg)	Zn (mg/kg)	Cr (mg/kg)

Oncorhynchus mykiss	Muscle	HNO3	6.32	3.56	134.92	ND
		Aqua regia (HNO3+HCl)	12.6	3.44	89.16	ND
		Dry ashing	11.24	3.20	76.44	ND
	Gills	HNO3	71.6	3.52	317.84	ND
		Aqua regia (HNO3+HCl)	84.28	3.44	326.04	ND
		Dry ashing	30.16	3.20	161.72	ND

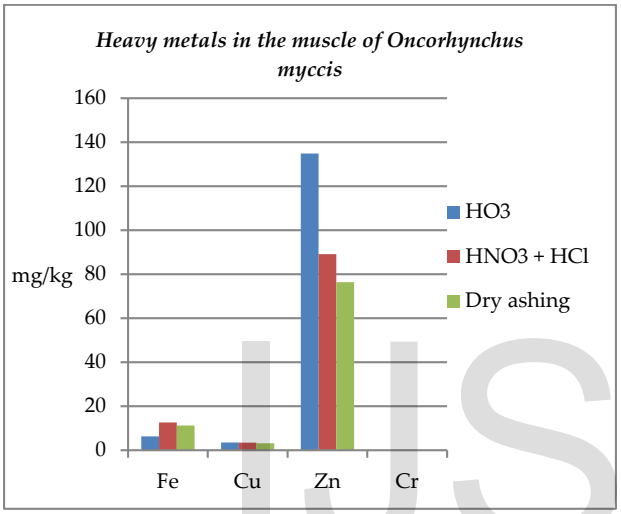

Fig. 1 Concentration of heavy metals in the muscle of *Oncorhynchus myccis*

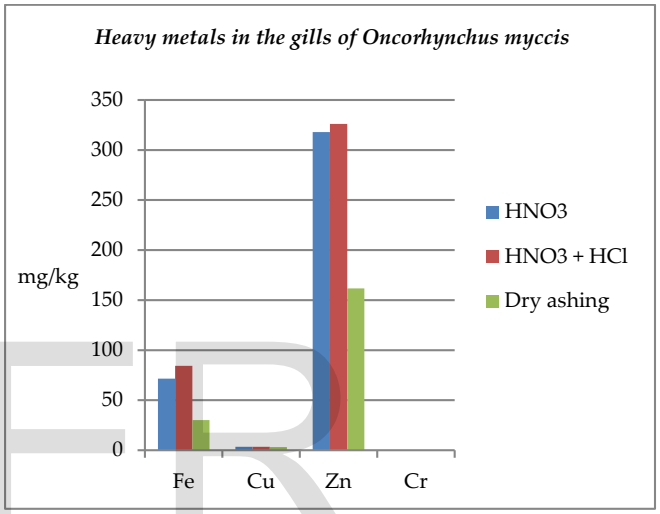

Fig. 2 Concentration of heavy metals in the gills of *Oncorhynchus myccis*

TABLE 5

Mean concentration of heavy metals after three replications in two analyzed tissues of *Cyprinus carpio*

Fish name	Fish tissue	Digestion method	Fe (mg/kg)	Cu (mg/kg)	Zn (mg/kg)	Cr (mg/kg)
Cyprinus carpio	Muscle	HNO3	6.6	3.24	88.72	ND
		Aqua regia (HNO3+HCl)	13.28	3.28	94.04	ND
		Dry ashing	6.44	3.08	92.72	ND
	Gills	HNO3	72.48	3.12	393.24	ND
		Aqua regia (HNO3+HCl)	53.92	3.28	365.56	ND
		Dry ashing	13.12	3.12	360.76	ND

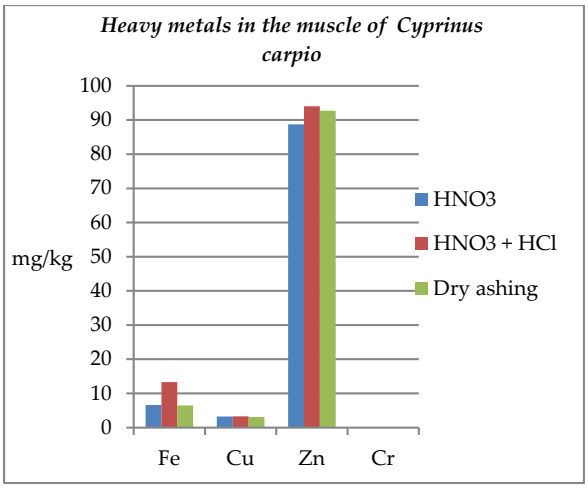

Fig. 3 Concentration of heavy metals in the muscle of *Cyprinus carpio*

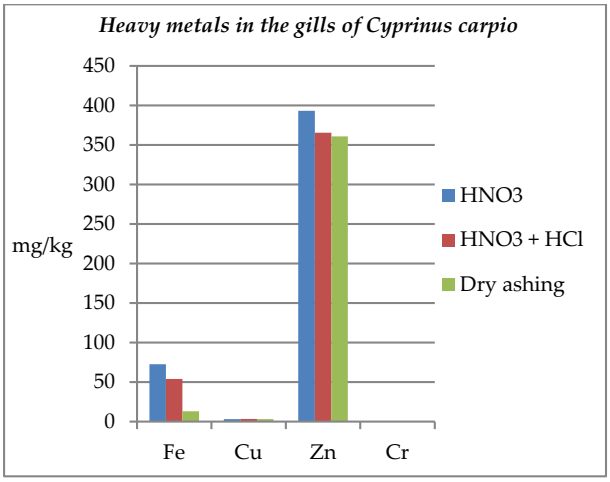

Fig. 4 Concentration of heavy metals in the gills of *Cyprinus carpio*

TABLE 6

Mean concentration of heavy metals after three replications in two analyzed tissues of *Dicentrarchus labrax*

Fish name	Fish tissue	Digestion method	Fe (mg/kg)	Cu (mg/kg)	Zn (mg/kg)	Cr (mg/kg)
Dicentrarchus labrax	Muscle	HNO3	92.04	3.52	82.48	ND
		Aqua regia (HNO3+HCl)	114.88	3.4	89.72	ND
		Dry ashing	113.4	3.32	73.88	ND
	Gills	HNO3	93.6	3.28	107.44	ND
		Aqua regia (HNO3+HCl)	117.48	3.36	141.64	ND
		Dry ashing	102.4	3.04	95.84	ND

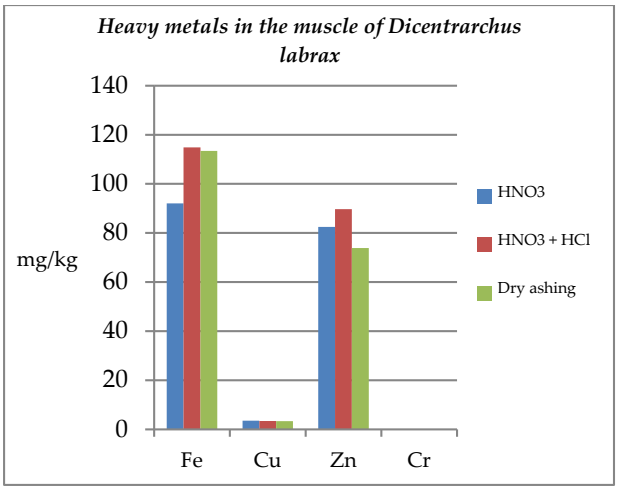

Fig. 5 Concentration of heavy metals in the muscle of *Dicentrarchus labrax*

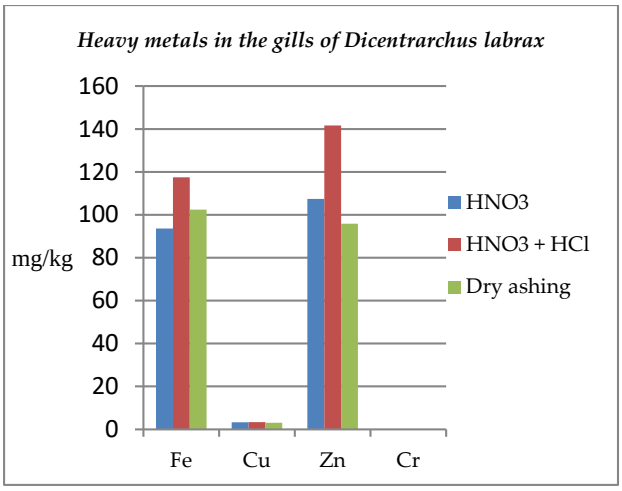

Fig. 6 Concentration of heavy metals in the gills of *Dicentrarchus labrax*

3.1 Discussion

3.1.2 Lead (Pb)

In none of the analyzed samples of the fish *Oncorhynchus mykiss* (tab.4), the concentration of Fe does not exceed permissible levels (100 mg/kg). Although in the gills we can see a relatively high Fe concentration compared to the fish muscles and that in the sample digested with aqua regia solution (HCl+HNO3) in combination with perchloric acid (HClO4).

In the fish *Cyprinus carpio* (tab. 5) the concentration of Fe does not exceed the permissible levels. Even in this case, a significant difference can be observed regarding the concentration of Fe in the gills compared to the fish muscles. The higher concentration of Fe we gained after the sample digestion with HNO3.

In almost all analyzed samples in the sea fish *Dicentrarchus labrax* (tab.6) the concentration of Fe has exceed the maximum permissible levels. We can see a higher concentration of Fe compared with two freshwater fish species, and even here as the most efficient method is shown the sample digestion with aqua regia solution (HCl+HNO3) in combination with perchloric acid (HClO4).

The source of iron in surface water is anthropogenic and is related to mining activities.

There are basically two different forms of iron over-accumulation, one due to excess extracellular iron and one due to excess intracellular iron. The most dramatic conditions of iron overaccumulation are called iron overload, also called hemochromatosis.

If it is not shielded properly, it can also catalyze the reactions involving the formation of radicals which can damage biomolecules, cells, tissues and the whole organism [6].

3.1.3 Copper (Cu)

In the tissues of the fish *Oncorhynchus mykiss* (tab.4), in none of the analyzed samples the concentration of Cu does not exceed the permissible levels (20-30 mg/kg). The concentrations are approximately low in both tissues and that in the three applied digestion methods.

The same applies to the other two fish species *Cyprinus carpio* (tab. 5) and *Dicentrarchus labrax* (tab.6), from the results of which we can see that the concentration of Cu are mainly low.

Copper is not poisonous in its metallic state but some of its salts are poisonous. Copper is a powerful inhibitor of enzymes. It is needed by the body for a number of functions, predominantly as a cofactor for a number of enzymes such as ceruloplasmin, cytochrome oxidase, dopamine β-hydroxylase, superoxide dismutase and tyrosinase.

Copper toxicity is increasingly becoming common these days. It is a condition in which a increase in the copper retention in the kidney occurs. Copper first start depositing in the liver and disrupts the liver's ability to detoxify elevated copper level in the body thus adversely affect nervous system, reproductive system, adrenal function, connective tissue, learning ability of new born baby, etc. Metallic taste in mouth, salivation, burning pain stomach, nausea, vomiting, vomiting matter will be blue in colour, cramps of legs or spasm, colicky abdominal pain, diarrhea, urine is inky in appearance, etc. [2].

3.1.4 Zinc (Zn)

In some of the analyzed samples (tab.4) of the fish *Oncorhynchus mykiss,* the concentration of Zn exceed the permissible levels (30 -100 mg/kg). In particular the high concentration of Zn has been encountered in the gills, including here a single sample of the muscle digestion with HNO3 which has exceeded the maximum permissible levels. The most efficient used method in the gills it was the digestion of the sample with aqua regia solution (HCl+HNO3) in combination with perchloric acid (HClO4). There is also a significant difference of this method comparing with dry ashing method.

There is also a significant difference between analyzed fish muscles and gills in sense of the Zn concentration at the fish *Cyprinus carpio* (tab. 5). The Zn levels in the gills are several times higher than permissible levels while in the muscle they are within permissible levels (30 – 100 mg/kg). In the gills, the higher concentration of Zn is achieved by using the sample digestion method with HNO3, although the difference in fact is very small comparing with aqua regia solution (HCl+HNO3) and dry ashing method.

In the fish *Dicentrarchus labrax* (tab.6) there is approximately same concentration of Zn in both analyzed tissues. The values that exceed the permissible levels were found in the gills and that with the method of the sample digestion with aqua regia solution (HCl+HNO3) in combination with perchloric acid (HClO4). In the muscle of the fish *Dicentrarchus labrax* it can be seen that the Zn values are within the permissible levels.

Taking up large doses of supplemental zinc over extended periods of time is frequently associated with copper deficiency. Whereas several other metals are well-known carcinogens, zinc is not generally considered to be a causative agent for cancer development. The induction of apoptosis by high levels of intracellular zinc has been shown in different tissues and cell types. Many studies indicate that zinc acts as a neuromodulator [8].

3.1.5 Chromium (Cr)

From the obtained results regarding to the concentration of Cr in all analyzed fish samples and using the three digestion methods, the concentration of Cr does not exceeded the permissible levels (0.15-0.1 mg/kg).

Human exposure to sufficiently high Cr concentration would result in potential harm through its toxic, genotoxic and carcinogenic effects. Chromium is one of eight metals in the top 50 toxic substances in the world in the data issued by the Agency for Toxic Substances and Disease Registry (ATSDR), and WHO has classified Cr as carcinogenic to human beings [10].

4. CONCLUSION

Pollution with some heavy metals (Fe and Zn) has been observed in different fish species, and this probably as a result of irresponsible act of anthropogenic factors. We have also seen that gills are the tissues where the bioaccumulation occurs with that much compared to fish muscles. In the end, it has been observed as the most efficient digestive method it was the combination of aqua regia (HNO3 + HCl) with perchloric acid (H3PO4), followed by HNO3 and dry ashing.

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