Dry Ashing or Wet Digestion ? A Comparative Study for Estimation of Zinc and Calcium in Freshwater Fish Samples by Atomic Absorption Spectrometry

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Summary: Different sample treatment procedures such as dry ashing and wet digestion are commonly used to decompose biological materials and to bring the elements of interest into the measurable form. A number of reagents such as *aqua regia*, HNO_3 / H_2O_2, HNO_3 / $HClO_4$ *etc.* are used to solubilize metals from biological matrix. In this study, performance of dry ashing and wet digestion methods has been compared for the estimation of zinc and calcium levels in the whole body freshwater fish samples. Flame atomic absorption spectrometry has been used as an analytical tool to quantify the metals. Results based on dry ashing and wet digestion methods for zinc differ significantly. Wet digestion method gives mostly higher zinc values compared to dry ashing method which indicates the possible losses of zinc during the ashing of fish samples at elevated temperature,i.e. 500°C. Higher recoveries of calcium in the whole body freshwater fish samples have been obtained following wet digestion procedure using strontium as releasing agent compared to calcium determination following dry ashing method (with and without strontium) and wet digestion method without strontium which suggests the use of a releasing agent (strontium) minimizes the chemical interferences of phosphate while determining calcium by flame atomic absorption spectrometry.

Introduction

Fish has occupied a respectable place in human diet. Flesh of fish is generally white and soft. In terms of food value, it is of equal rank or mostly superior having less amount of fat and more or equal proteins as compared to other fleshes consumed as food by human beings [1]. Recent research has indicated that fish has anticancerous effects, minimized risk of heart diseases and has resulted enhanced life expectancy. The living fishes are categorized into three distinct classes, Agntha, chondrichthyes and Osteichthyes. Approximately three fifth of the known living vertebrates are fish. There are some 30,000 species of fish of which approximately 2500 are freshwater [2].

All forms of aquatic animals require inorganic elements, the minerals, for their normal life processes. Unlike most terrestrial animals, fishes have the ability to accumulate metals not only from their diet but also from their external environment in both freshwater and seawater. The concentration of minerals in the body of aquatic organisms depends upon food source, environment, species, stage of development and physiological status of the animals [3]. Mineral elements which are usually found essential for human beings include iron, iodine, calcium, chloride, chromium, cobalt, copper, fluorine, manganese, molybdenum, potassium, selenium, sodium, sulphur and zinc whereas lead, cadmium, mercury and nickel are considered to be toxic [4]. Studies have shown that metals such as Zn, Mn, Pb, Co, Ni, Cu, Cr etc. in freshwater fishes are at very low levels [5].

One of the most important issues in evaluating the influence of trace constituents (ppm or lower) in problems of environmental concerns is the capability to make accurate, rapid and unbiased determinations of the elements present at this level. Sample preparation for trace analysis is one of the main issues affecting accuracy and precision. Contamination of samples by metal contaminants and/or loss of the analyte during sample preparation step are the major cause of errors. Traces of heavy metals in biological samples may be determined either by dry ashing or by wet chemical method. Most analytical measurements using highly sensitive methods (AAS, ICP-MS, ICP-AES *etc.*) are performed on solutions of the sample. So that decomposition of the sample is therefore an initial step of several analytical methods. Decomposition methods for trace metal determination can generally be classified into three different groups i.e., wet decomposition, combustion and fusion. Wet decomposition and ashing procedures are the subjects for further investigation in this project.

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Table-1:Comparison of results for zinc estimated in freshwater fish samples following dry ashing and wet digestion methods (F-test, t-test, α 0.05, n=3)

Sample No.	Zn Concentration* ($\mu g\ g^{-1}$ dry wt)		% Recovery by wet digestion in relation to dry ashing method	$F_{cal} = s_1^2/S_2^2$	t_{cal} value	Significantly different?
	Dry ashing	*Wet digestion*				
1	116.3 ± 10.2	122.1 ± 5.8	105	3.1	1.602	No
2	85.0 ± 8.8	115.4 ± 23.6	135	7.3	5.762	Yes
3	122.9 ± 17.8	140.4 ± 10.2	114	3.0	3.765	Yes
4	95.4 ± 15.6	92.5 ± 15.1	97	1.0	2.140	No
5.	114.3 ± 22.8	96.3 ± 4.3	84	28.1	0.925	No
6.	90.0 ± 2.2	120.6 ± 4.6	134	4.5	11.378	Yes
7.	106.3 ± 18.4	135.0 ± 21.4	127	1.4	5.423	Yes
8.	94.6 ± 9.5	136.2 ± 44.3	144	21.9	5.724	Yes
9.	96.7 ± 8.8	132.5 ± 12.2	137	1.9	8.995	Yes
10.	97.5 ± 13.8	98.1 ± 5.6	101	6.0	0.146	No
11.	96.7 ± 6.9	111.3 ± 3.3	115	4.4	4.4749	Yes
12.	118.3 ± 32.8	109.6 ± 9.0	93	13.3	1.402	No

* Mean ± standard deviation; F_{tab} = 19.0; t_{tab} = 2.132.

The most commonly used reagent for wet digestion are mineral and mostly oxidizing acids. Wet ashing has the advantage of being effective on both inorganic and organic materials. It often destroys or removes the sample matrix, thus helping to reduce or eliminate some type of interferences. Strong acids commonly applied for wet decomposition of different materials are HCl, H_2SO_4, HNO_3, $HClO_4$, some times with the HF. With H_2SO_4, sulphates are formed like $CaSO_4$, $BaSO_4$ and $SrSO_4$ which are difficult to break. Mixtures of acids used for wet decomposition include HNO_3/HCl, HNO_3/H_2SO_4, HNO_3/$HClO_4$/H_2SO_4, HNO_3/HCl/HF, HF/boric acid and HNO_3/HF/boric acid. Strong acid digestions using nitric acid or mixture of acids (*aqua regia*) are commonly used to bring the greater part of the metals in a sample into solution [6]. Garraud *et al.* [7] used nitric acid and H_2O_2 to digest biological reference materials for the determination of trace metals by ICP-MS.

In recent years, pollution of freshwater resources by various types of wastes containing toxic heavy metals and accumulation of these metals in freshwater fishes have raised concern about their possible health hazard. Such a situation has necessitated the reason to find out accurate and reliable analytical procedures for the determination of metallic elements in freshwater fishes. Cited chemical literature on metal levels in freshwater fishes indicates that various authors have either selected dry ashing method or wet chemical method as sample preparation procedure for the determina-tion of metals [5, 8-10]. This study was aimed to carry out experiments to compare the performance of dry ashing and wet digestion procedures for the determination of zinc and calcium in whole body freshwater fish samples by flame atomic absorption spectrometry.

Results and Discussion

Determination of Zinc

Zinc concentrations in twelve whole body freshwater fish samples were estimated by flame atomic absorption spectrometry following dry ashing and wet digestion procedures. Table 1 shows the comparison of the results for zinc estimated by dry ashing and wet digestion procedures. F-test was applied to compare the variability in results of obtained by these methods whereas t-test was used to compare the mean Zn values obtained by the dry ashing and wet digestion methods. Variations in estimated zinc levels in different samples may be attributed to natural composition of the fishes analyzed. This comparison indicates that the results obtained by two methods differ significantly. Higher zinc values were obtained by wet digestion method compared to dry ashing procedure which may be due to the Zn losses during the ashing process of the dried fish samples at higher temperature i.e. 500^0C. It may be concluded that wet digestion procedure involving the use of an acid mixture such as *aqua regia* may be used for the accurate determination of zinc in freshwater fish samples by FAAS.

Determination of Calcium

Atomic absorption spectrometry suffers from a number of interferences. Chemical interference, or the chemical combination of the element of interest with other elements in the sample or the flame, is probably the most important interference in flame

Table 2: Calcium concentration in whole body freshwater fish samples determined by FAAS following dry ashing and wet digestion methods (with and without addition of strontium as releasing agent; mean ± standard deviation, n = 3).

Sample No.	Ca Concentration (mg g^{-1} dry wt)			
	Dry Ashing		Wet Ashing	
	Without Sr	With Sr	Without Sr	With Sr
1	5.84 ± 2.09	32.89 ± 4.65	94.55 ± 8.74	125.07 ± 2.64
2	17.98 ± 1.13	67.93 ± 5.76	91.46 ± 3.33	122.32 ± 3.43
3	24.26 ± 3.75	71.20 ± 7.16	95.51 ± 4.74	150.88 ± 11.51
4	21.71 ± 2.72	56.89 ± 10.97	88.52 ± 6.31	123.43 ± 11.80
5	13.88 ± 1.81	43.97 ± 8.00	20.71± 8.34	126.59 ± 28.31
6	13.99 ± 6.46	36.78 ± 8.90	98.77 ±12.35	166.42 ± 16.69
7	15.09 ± 8.43	52.03 ± 5.71	81.63 ± 5.20	129.21 ± 2.60
8	26.95 ± 3.33	88.27 ± 17.90	101.39 ± 23.66	121.67 ± 6.83
9	31.35 ± 2.02	44.10 ± 3.46	88.70 ± 13.62	154.68 ± 17.98
10	24.17 ± 9.47	54.66 ± 12.03	82.07 ± 15.78	129.72 ± 6.80
11	24.00 ± 4.06	50.85 ± 2.06	92.31 ± 10.26	121.62 ± 11.76
12	21.29 ± 3.53	53.25 ± 3.21	78.86 ± 7.46	89.69 ± 14.33

methods. It directly affects the efficiency of production of neutral atoms in the flame and hence affects both absorption and emission in a similar manner [11]. One of the most common types of chemical interference is the formation of refractory compounds with the test element, usually by an anion in the aspirated solution [12]. The result is a decreased signal. For example, phosphate will react with calcium ions to produce calcium phosphate in air-acetylene flame. Less frequently, the presence of another action may result in a decreased signal. Chemical interferences, fortunately, can generally be minimized by adding an appropriate releasing agent. These agents either compete for the interfering substance or displace it from the test element. For example, phosphate interference with calcium absorption can be eliminated by adding a sufficiently high concentration (about 1% depending on the actual phosphate levels) of strontium or lanthanum chloride to the solution. The strontium or lanthanum will preferentially combine with the phosphate and prevent its reaction with the calcium or high concentration of EDTA can be added to form a chelate with the calcium and prevent its reaction with phosphate. Because addition of an external reagent can sometimes change the total composition, it should also be added to working metal standards. The use of high temperature flames will frequently eliminate chemical interferences. Phosphate interference on calcium, for example, does not occur in the nitrous oxide- acetylene flame [13].

In the present study, calcium concentrations in the whole body freshwater fish samples were determined by FAAS using air –acetylene flame following dry ashing and wet digestion method with and without the addition of a releasing agent i.e. strontium. Results are given in Table 2.

In general, higher values of calcium (in both cases of dry ashing and wet digestion method) obtained when strontium was used as releasing agent. It indicates that the use of strontium as releasing agent was effective to overcome the chemical interference of phosphate in whole body freshwater fish samples. 3000 ppm Sr concentration in each working calcium standard and the sample solution was used as suggested by Udoh [14]. In general, percent recoveries of calcium by these different procedures were found to be in the following order: wet digestion method (with Sr) > wet digestion method (without Sr) > Dry ashing method (with Sr) > Dry ashing method (without Sr). Higher recoveries of Ca by wet digestion method compared to dry ashing procedure may be due to greater solubilization of calcium matrix ($Ca_3(PO_4)_2$ in fish bones) in very strong oxidizing acid mixtures i.e. *aqua regia*. Table 3 presents the percent recovery of calcium in fish samples following wet digestion (with Sr as releasing agent) in relation to wet digestion method without Sr addition.

Keeping in view the results obtained by this study, the following conclusions may be made: i) A wet digestion method involving the use of an acid mixture such as *aqua regia* should be used to solubilize Zn and Ca from the whole body freshwater fish samples, ii) A releasing agent such as strontium

Table 3: Recovery of calcium by wet digestion with strontium addition *vs* wet digestion without strontium addition .

	Sample No. 1	2	3	4	5	6	7	8	9	10	11	12
% recovery of Ca by wet digestion (with strontium addition) in relation to wet digestion without strontium addition	132	133	158	139	124	118	158	120	174	158	133	114

Table 4: Data on freshwater fish samples analyzed for zinc and calcium.

Sample No.	*Hypophthalmichthys molitrix* 1	2	3	4	5	*Ctenopharyngodon idella* 6	7	8	9	10	11	12
Total length *(cm)*	13.2	13.4	12.8	13.5	12.6	13.0	12.2	18.5	15.4	11.5	15.3	17.0
Total Wet weight *(g)*	20.86	23.80	18.90	28.35	17.86	23.50	18.90	65.00	35.80	15.10	35.20	50.00
Dry weight *(g)*	4.04	5.81	3.93	5.81	3.64	4.80	3.80	13.21	7.60	3.00	7.50	10.38

should be added to the acid digested fish sample solutions in order to make accurate estimation of calcium by flame atomic absorption spectrometry when using air – acetylene flames.

Experimental

Five silver carp,(*Hypophthalmichthys molitrix*) samples of body length ranging from 12.6 cm to 13.5 cm and body weight from 17.86 g to 28.35g were obtained from Shah Mehmood Fish farm located at Shakh-i-Madina, Khanewal Road, Multan, Pakistan. Seven Grass Carp (*Ctenopharyngodon idella*) samples of body length ranging from 11.5 cm to 18.5 cm and body weight from 15.1g to 65.0 g from Faheem Fish farm located at Matital road near Multan, Pakistan using a cast net and were transported live in plastic containers to the Fisheries research laboratory, Institute of Pure and applied Biology Bahauddin Zakariya University, Multan, Pakistan.

Fishes were removed from plastic containers and killed, blotted dry with a paper towel and weighed on an electric digital balance to the nearest 0.01g weight on an electronic digital balance. Total body lengths were measured to nearest 0.01 cm using measuring tape. All measurements were made from tip of maxilla to the tip of longest caudle fin ray. These pre-mature and pre-weighed fishes were placed in pre-weighed aluminium foil tray for drying till constant weight in an electric oven at 100^{0}C. Then dry carcasses were crushed in an agate pestle and mortar. Table 4 presents the data on freshwater fish samples analyzed for zinc and calcium.

Analytical Reagent Grade chemicals supplied by Merck were used without further purification for the purpose of metal analysis. Deionised water was used to prepare standard and sample solutions. Stock standard solutions 1000 mg L^{-1} of each element were prepared by dissolving required amounts of the respective salts in water. These solutions were standardized against standard EDTA solution using Eriochrome Black T. Dilutions of the Standard solutions were made to prepare working acidic standards (0.1 M HNO_3 final molar concentration) in the measuring ranges.

Sample Preparation

Dry Ashing Method

Sample solutions were prepared as: "0.2 g of dried fish powder was ashed in a muffle furnace (RJM 1.8-10, China) at 500^{0}C for 5 hours. The ash contents were dissolved in 2 ml of a mixture of HNO_3:HCl (1:3) and heated slowly to dryness. Then 5ml of 1 M HNO_3 was added, filtered the solution (if necessary) and diluted upto 25 ml with deionised water . Sample solutions were stored in acid washed polyethylene bottles for further analysis". Solutions were also diluted with deionised water to bring the analyte in the measuring range.

Wet Digestion Method

Sample solutions were prepared as: "0.2 g of dried fish powder was digested in 5ml of *aqua regia* (HNO_3:HCl 1:3) and refluxed for half an hour. Sample mixture was cooled down to room temperature and was filtered if necessary. Sample solution was then diluted upto 25 ml with deionised

Table 5: Instrumental conditions for metal analysis by FAAS.

Parameters	Zn	Ca
Wavelength (nm)	213.8	422.7
Band pass (nm)	1.3	0.4
Lamp current (mA)	10.0	7.5
Fuel pressure (Kg/cm^2)	0.20	0.35
Burner height (nm)	7.5	10.0
Calibration range ($mg\ L^{-1}$)	0.3 -3.0	1.0 – 10.0
Flame composition [a]		
Oxidant pressure [b] (Kg/cm^2)		
Atomizer [c]		
Measurement Mode [d]		

a air: $C_2\ H_2$; b 1.60 ; c Standard Burner; d Absorbance.

water and stored in an acid washed polyethylene bottle for further analysis. Solution was also diluted to bring the analyte concentration in the measuring range.

Metal analysis by FAAS

Standard and sample solutions were aspirated into atomic absorption spectrophotometer (A-1800, Hitachi, Japan) and absorbance measurements were made for each element using specific instrumental conditions for FAAS (Table 5). Analysis of each sample was made in duplicate. Concentrations were computed in $\mu g\ g^{-1}$ using an IBM compatible personal computer. Calibration of the atomic absorption spectrometer was carried out periodically during analysis.

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