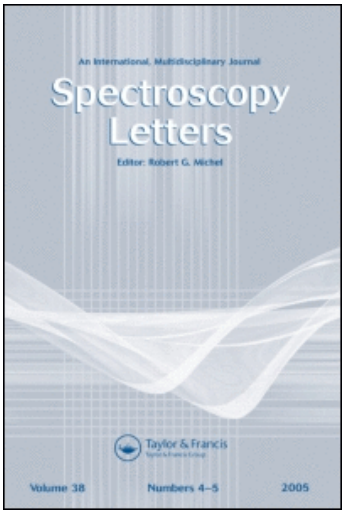

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Determination of Toxic Elements in Muscle Tissues of Five Fish Species Using Ultrasound-Assisted Pseudodigestion by Electrothermal Atomic Absorption Spectrophotometry: Optimization Study

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Abstract: A simple and rapid method is described for the determination of As, Cd, and Pb in muscle tissue of five freshwater fish species by ultrasound-assisted pseudodigestion (USD). A Plackett-Burman experimental design was used as a multivariate strategy for the evaluation of the effects of varying several variables at once. The variables such as sonication time (ST), sample mass of muscle tissues (SM), temperature of ultrasonic bath (T), nitric acid (A1), and mixture of acid and oxidant (A2) have been studied. From these studies, some variables showed significant effect on % recovery, and they were further optimized by a 2^3+star central composite design, which involved 16 experiments. Optimum values of the variables were selected for the development of USD to determine the contents of As, Cd, and Pb in muscle tissue of five fish species, used as bioindicators for Lake Manchar (Sindh, Pakistan) to know whether consumption of these fishes threatens human health. The determination of three toxic elements under study was carried out by electrothermal atomic absorption spectrometry (ETAAS). The accuracy of the optimized procedure was evaluated by analysis of certified reference materials DORM-2 (Dogfish Muscle Certified Reference Material for Trace Elements) and by comparison with conventional wet acid digestion methodology. No significant differences were observed for $p = 0.05$ when comparing the values obtained by the proposed USD method and conventional digestion method (CDM) (paired *t*-test). The average relative standard deviation of

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the USD method varied between 4.05%, 7.53%, and 4.55% for As, Cd, and Pb, respectively (n = 10).

Keywords: Arsenic, cadium, lead, Plackett-Burman experimental design, fish, ETAAS

INTRODUCTION

Analysis of aquatic organisms has been increasingly used as a direct measure of the abundance and availability of elements in the environment and has led to the adoption of the bioindicator (biomonitor) concept.[1] Analysis of metals and metalloids in biological samples by atomic absorption spectrometry (AAS), electrothermal atomic absorption spectrometry (ETAAS), atomic emission spectrometry (AES), or inductively coupled plasma mass spectrometry commonly requires the total dissolution of the sample to avoid the adverse effects on the analysis of the matrix in which the element is present.[2] Determination of trace quantities of toxic elements in aquatic animals requires the use of sensitive and selective techniques such as ETAAS, using sample preparation strategies addressed to shortening and simplifying the stages previous to analysis.[3,4]

The determination of elements in complex samples by AAS generally requires the destruction of the sample matrix to render a solution of the analyte ready for analysis. To obtain a solution suitable for AAS analysis for instance, biological, environmental, and geological samples, conventional wet acid digestion procedures entail tedious and time-consuming manipulations that involve the use of mixtures of corrosive and sometimes explosive acids and long heating periods.[5–8]

A solution that has demonstrated major potential for speeding up and simplifying sample treatment is by using ultrasound energy. In analytical chemistry, ultrasonic radiation has been used infrequently, although it could be a powerful tool for accelerating various steps in the analytical process.[9] Thus, ultrasound is of great help in the pretreatment of solid samples, as it facilitates and accelerates operations such as the extraction of organic and inorganic compounds. In solution, ultrasonic energy causes acoustic cavitations, which are bubble formations and their subsequent implosion. The collapse of bubbles created by the sonication of solutions generates extremely high local temperatures and pressure gradients leading to enhanced chemical reactivity. The combined effect of extremely high temperatures and pressures at the interface of the sonicated solution and the solid matrix, along with the oxidative power of strong acids, results in high extractive power. The temperature of these hot spots is about 5000°C—similar to the surface of the sun.[10]

This phenomenon makes ultrasonic extraction compatible with the traditional methods without the utilization of extreme experimental conditions

such as high temperature and high pressure.[11] These localized high-energy environments formed by the application of ultrasound to solutions can be employed for the purpose of extracting elemental analytes from solid matrices, so ultrasonic acid leaching, extraction, and digestion is an expeditious, inexpensive, and efficient alternative to conventional techniques and, in some cases, even to supercritical fluid and microwave-assisted techniques, as demonstrated by application to both organic and inorganic analytes in a wide variety of samples.[12–15] The efficiency of the wet acid digestion procedures can be enhanced with the use of ultrasound irradiation, which has also been applied for total element determination in environmental and industrial hygiene samples by single-element and multielement analysis.[16–18] Different acid mixtures have been tried and a simplex optimization procedure was used to determine optimum sonication periods and temperatures.[19]

Most procedures proposed by analytical chemists are optimized by development of univariate methodology (one variable at each time); this simple optimization methodology is supposed to have an easier interpretation. However, univariate methodology is not so reliable because of involvement of excessive experimentation and amount of reagent, and is time consuming, too, and even doing so we cannot observe interaction among variables. Means of optimization such as classic single-at-a-time approach is also unsuitable when a large number of possible variables have to be studied. Procedures involving optimization by multivariate techniques have been increasingly used as they are faster, more economical and effective, and allow more than one variable to be optimized simultaneously.[20–22] Interest in the use of multivariate optimization method has increased substantially in recent years.[23]

There is a need for relatively quick, simple, environmentally friendly, and cost-effective methods for determining the toxic elements in reasonable numbers of samples; therefore, we adopted ultrasound energy to enhance the action of acid-oxidants to decompose organic matrixes and for accurate determination of total As, Cd, and Pb in fish muscles. For this purpose, a Plackett-Burman experimental design was used as a multivariate strategy for the evaluation of the effects of varying several variables at once. Parameters influencing USD, such as intensification time (in ultrasonic bath), different acid mixtures, and temperature of ultrasonic bath, were fully investigated. From these studies, some variables showed significant effect on % recovery, and they were further optimized by a 2^3+star central composite design, which involved 16 experiments optimum values of the variables were selected for the development of the acid pseudodigestion method to extract toxic elements from fish muscles. ETAAS was used to determine As, Cd, and Pb. The proposed method was validated against NRCC DORM-2. An acid digestion induced by electric hot plate was used to obtain the total element concentration and also for comparative purposes.[24]

MATERIALS AND METHODS

Apparatus

A Perkin-Elmer model 700 (Norwalk, CT, USA) atomic absorption spectrometer, equipped with a graphite furnace (HGA-400), pyrocoated graphite tube with integrated platform, an autosampler (AS-800), and deuterium lamp as background correction system was used for As, Cd, and Pb measurements. Hollow cathode lamps (Perkin-Elmer) operating at recommended current were used for all cases; all conditions of instrumental parameters are summarized in Table 1. A Retsch vibrating ball mill (Haan, Germany), equipped with zircon cups (15 mL in size) and zircon balls (7 mm diameter) was used to pulverize and reduce the particle size of the dried muscles of fish samples. The ultrasonic extractions were carried out with an ultrasonic bath capacity 4L (Sonicor, model No. SC-121TH, Sonicor Instrument Corp., Copiague, NY, USA), programmable for temperature ranging from 0°C to 90°C with intensification frequency of 35 kHz.

Reagents

All the chemicals used were of analytical grade. Ultrapure water, resistance 0.05 $\mu s\ cm^{-1}$, was obtained from an Elga purification device (Elga Lab Water, Bucks, UK). Standard solutions of As, Cd, and Pb were prepared by dilution of certified standard solutions (1000 ppm, Fluka Kamica, Buchs, Switzerland) of corresponding elements in 1 M $HNO_3 \cdot Mg(NO_3)_2$ and $Pd(NO_3)_2$

Table 1. Measurement conditions for ETAAS

	As[a]	Cd[a]	Pb[b]
Lamp current (mA)	10	7.5	7.5
Wavelength (nm)	193.7	228.8	283.3
Slit-width (nm)	0.7 L	0.7 L	0.7 L
Cuvette	Tube^	Tube	Tube
Carrier gas (mL/min)	200	200	200
Sample volume (μL)	10	10	10
Temperature program			
Dry	140$ 15/5*	140 15/5	140 15/5
Ash	1300 10/20	850 10/20	700 10/20
Atomization	2300 0/5	1650 0/5	1800 0/5
Cleaning	2600 1/3	2600 1/3	2600 1/3

^Pyro/platform tube; $Temperature °C; *Ramp/hold (s).
[a]$Mg(NO_3)_2$, $Pd(NO_3)_2$ (0.01–0.015 mg) as chemical modifier for As, Cd.
[b]0.2 mg $NH_4H_2PO_4$ as chemical modifier for Pb.

were used for As and Cd, and $NH_4H_2PO_4$ was used for Pb as chemical modifiers (Merck Ltd., Poole, Dorset, UK). Concentrated nitric acid 65% and hydrogen peroxide 30% were spectroscopic grade (Merck, Darmstadt, Germany). The certified reference material for trace metals NRCC DORM-2 (Dogfish Muscle) from the National Research Council of Canada (Ottawa, Ontario, Canada) was used for validation of the proposed method.

Sample Collection and Pretreatment

Three hundred individual samples of five freshwater fish species (*Chitala chitala*, *Mastacembelus armatus*, *Glossogobius giuris*, *Ompok pabda*, and *Oreochromis mossambicus*) were collected during June 2005 to may 2006 from five stations along Lake Manchar (Sehwen Sindh, Pakistan) (26°3′N: 67°6′E) on a monthly basis. All fish species were collected at size 20–45 cm with weight range 750–1500 g and assigned individual identification numbers. At the end of each sampling effort, all the samples were wrapped in plastic bags, placed in polyethylene bags, held in an ice box, and refrigerated at −4°C until further analysis. The samples once in the laboratory were homogenized using a mixer, and dried for 48 h in an oven at 65°C to constant weight. Then, they were ground in the vibrational agate ball mill for 5 min using a power of 60%. The powdered samples were sieved through a nylon sieve to obtain particle size <65 μm. The powder obtained upon sample preparation was stored in closed polyethylene tubes and maintained in a refrigerator at 4°C until analysis. The 300 fish targets were established to provide five composite samples of each fish species. These numbers are enough to establish, with high confidence, the mean contaminant concentration in the fish tissues.

The Factorial Design

The original Plackett-Burman approach is based on balanced incomplete blocks[25] and suggests designs for 8, 12, 16, and so fourth, variables or factors. For the evaluation of five factors at two levels, a Plackett-Burman design with only 16 experiments was described instead of the $2^5 = 32$ required for a full factorial design.[26] This work was carried out using Minitab. (release 13 of Minitab) Version 5.1.[27,28]

For performing the current factorial design test, five parameters were chosen as variables and tested at two levels, (+) representing the maximum level and (−) the minimum level (Table 2). The system responses were evaluated for As, Cd, and Pd contents of a certificate reference material DORM-2. Sixteen experiments were carried out to complete the design matrix, the resulting values (1–16) being the % recovery of all three analytes under study (shown in Table 3), showing the average value of 10 replicates.

Table 2. Variable and levels used for the Plackett-Burman and central composite designs in the factorial design

Variable	Symbol	Low level (−)	High level (+)
HNO_3/mL[a]	A1	1	3
Ultrasound or sonication time/min	ST	1	5
Sample mass/mg	SM	100	500
Ultrasonic water-bath temperature/°C	T	40	80
HNO_3:H_2O_2/mL[a]	A2	1	3

[a]The concentration of HNO_3 and H_2O_2 was 65% and 30%, respectively, for all experiments.

Central 2^3+Star Orthogonal Composite Design

Having screened out the variables that did not have a significant effect on the response, the remaining three or two factors were optimized to provide the maximum element recovery. A central 2^3+star orthogonal composite design with 6 degrees of freedom and involving 16 experiments was performed to optimizing the variables A2, T, and ST for the As, Cd, and Pb determination in muscle tissues (Table 4).

Table 3. Plackett-Burman design (n = 16) for the significant variable determination

						% Recovery (mean ± SD)		
Run	A1	ST	SM	T	A2	As	Cd	Pb
1	+	−	−	−	+	23.1 ± 2.0	27.1 ± 2.7	21.0 ± 1.9
2	+	+	−	−	−	48.2 ± 4.2	57.7 ± 4.5	36.2 ± 2.3
3	+	+	+	−	−	40.3 ± 3.2	54.7 ± 3.8	33.8 ± 2.6
4	+	+	+	+	−	75.1 ± 5.9	71.6 ± 5.2	71.0 ± 4.3
5	−	+	+	+	+	96.7 ± 4.6	97.8 ± 4.2	96.1 ± 6.5
6	+	−	+	+	+	73.0 ± 6.5	85.2 ± 5.6	72.6 ± 5.3
7	−	+	−	+	+	98.8 ± 7.7	98.9 ± 6.1	97.8 ± 7.2
8	+	−	+	−	+	21.8 ± 2.1	24.6 ± 2.7	19.1 ± 1.3
9	+	+	−	+	−	85.8 ± 8.5	77.3 ± 7.6	74.0 ± 5.2
10	−	+	+	−	+	49.3 ± 3.9	76.4 ± 6.0	40.2 ± 4.1
11	−	−	+	+	−	51.2 ± 4.9	56.5 ± 4.3	49.2 ± 4.0
12	+	−	−	+	+	76.2 ± 5.1	83.2 ± 5.7	73.6 ± 4.3
13	−	+	−	−	+	51.4 ± 2.5	68.5 ± 3.8	38.9 ± 2.8
14	−	−	+	−	−	19.1 ± 1.9	23.2 ± 1.9	14.2 ± 1.1
15	−	−	−	+	−	50.5 ± 4.7	53.9 ± 5.3	50.5 ± 4.3
16	−	−	−	−	−	20.3 ± 1.8	25.0 ± 1.7	16.0 ± 1.1

Table 4. Central 2^3 orthogonal composite design (n = 16) for sonication time/ultrasonic water-bath temperature/HNO_3:H_2O_2

				% Recovery (mean ± SD)		
Run	ST	T	A2	As	Cd	Pb
1	−	−	−	24.70 ± 1.2	27.30 ± 2.7	17.90 ± 2.0
2	+	−	−	45.50 ± 3.0	58.60 ± 3.6	36.54 ± 3.5
3	−	+	−	55.80 ± 2.8	56.80 ± 4.2	57.20 ± 2.6
4	+	+	−	78.20 ± 5.1	86.10 ± 4.9	75.60 ± 4.8
5	−	−	+	26.20 ± 1.2	30.20 ± 2.7	27.20 ± 3.6
6	+	−	+	54.50 ± 4.6	79.10 ± 4.6	49.50 ± 2.4
7	−	+	+	87.60 ± 4.8	77.80 ± 4.8	75.20 ± 3.6
8	+	+	+	98.85 ± 3.8	98.83 ± 4.4	97.84 ± 2.6
9	$-a^1$	${}^bf_0$	${}^cf_0$	45.00 ± 3.4	59.50 ± 4.6	29.40 ± 2.1
10	$+a^2$	${}^bf_0$	${}^cf_0$	73.70 ± 5.2	71.20 ± 3.2	54.30 ± 3.0
11	${}^af_0$	$-b^1$	${}^cf_0$	29.70 ± 2.0	36.02 ± 2.6	26.80 ± 4.7
12	${}^af_0$	$+b^2$	${}^cf_0$	92.40 ± 5.6	94.50 ± 3.2	90.04 ± 2.7
13	${}^af_0$	${}^bf_0$	$-c^1$	24.70 ± 1.3	28.50 ± 2.1	17.01 ± 2.9
14	${}^af_0$	${}^bf_0$	$+c^2$	79.60 ± 5.8	81.30 ± 2.8	78.60 ± 2.3
15	${}^af_0$	${}^bf_0$	${}^cf_0$	74.50 ± 5.2	76.50 ± 4.3	69.80 ± 4.6
16	${}^af_0$	${}^bf_0$	${}^cf_0$	72.80 ± 6.3	78.30 ± 1.7	66.70 ± 2.3

${}^af_0 = 3$ min, ${}^bf_0 = 60°C$, ${}^cf_0 = 2$ mL.
$a^1 = -0.36359$ min, $a^2 = 6.36359$ min.
$b^1 = 26.3641°C$, $b^2 = 93.6359°C$.
$c^1 = 0.3182$ mL, $c^2 = 3.68179$ mL.

The factors that were shown to be insignificant by the Plackett-Burman design were fixed at convenient values. The CRM DORM-2 and fish muscle mass was 200 mg for all runs.

Ultrasound-Assisted Pseudodigestion Method

For ultrasound-assisted pseudodigestion optimization, HNO_3 (A1), mixture of acid-oxidant HNO_3:H_2O_2 (A2) (1:1 v/v), intensification or sonication times (1–5 min), and the temperature of ultrasonic bath ranging between 40°C and 80°C were tested. To evaluate the efficiency of the process, the results obtained with the USD were compared with those obtained from conventional digestion method (CDM).

In order to optimize the different analytical variables, 10 replicates of DORM-2 and duplicate samples of dried powdered fish of each batch at minimum level (−) and maximum level (+) for SM were taken in Pyrex flask, (25 mL capacity), concentrated mixture of acids A1 and A2 were added at two levels, minimum (−) and maximum (+), separately, then the flasks were placed inside the ultrasonic water bath and were subjected to

ultrasonic energy at 35 kHz for different time intervals (1–5 min). The temperature range of ultrasonic water bath was 40°C to 80°C. After sonication for different time intervals, the supernatant liquid was evaporated to approximately 0.5 mL final volume on an electric heating plate. Final solution was made up to 10 mL with 1 M HNO_3 and subjected to sonication again for 2 min and made upto 25 mL with 1 M HNO_3 and filter. Finally, solutions were collected in a polyethylene flask for the determinations of As, Cd, and Pb by ETAAS (Table 5). Blanks were also treated in the same way. After studying the effect of the different variables involving in USD method, the remaining collected fish samples were prepared according to optimized conditions prior to determining the analytes under study.

Conventional Digestion Method

An acid digestion method induced by electric hot plate was used in order to discover the total content of the elements and also for comparative purposes. Replicate three samples of DORM-2 and duplicate 200 mg subsamples of dried powder of fish (n = 300) were weighed in 50 mL Pyrex flasks. Five milliliters of a freshly prepared mixture of concentrated HNO_3:H_2O_2 (1:1, v/v) was added to each flask, and solutions were heated on an electric hot plate at 80°C for 2–3 h until the clear transparent digests were obtained. Final solutions were made up to 25 mL with 2 M HNO_3 and collected in polyethylene flask for the determinations of As, Cd, and Pb by ETAAS. Blank digestions were also carried out. The concentrations were obtained directly from calibration graphs after correction of the absorbance for the signal from an appropriate reagent blank.

ETAAS Determinations

As, Cd, and Pb determinations were carried out by ETAAS under optimum conditions (Table 1). In order to obtain the optimum pyrolysis and atomization temperatures for As, Cd, and Pb, chemical modifier such as $NH_4H_2PO_4$ was used as a chemical modifier for Pb determination, and a solution containing

Table 5. Validation of the proposed method against DORM-2 (mg kg^{-1} dried basis) n = 10

Elements	Certified ($x \pm s$)	CDM ($x \pm s$)	%RSD	USD ($x \pm s$)	%RSD	t_{crit} = 2.262
As	18 ± 1.1	18.11 ± 0.85	4.7	17.793 ± 0.72	4.05	0.146
Cd	0.043 ± 0.008	0.0443 ± 0.006	13.82	0.0425 ± 0.0032	7.53	0.704
Pb	0.065 ± 0.07	0.0654 ± 0.005	7.65	0.0636 ± 0.0029	4.55	0.212

$Pd(NO_3)_2$ and $Mg(NO_3)_2$ was used as modifier for Cd and As determinations, according to the recommended conditions of the instrument manufacturer. For certified and collected samples, the appropriated modifiers were mixed and injected simultaneously into pyrocoated graphite tubes using autosampler AS-800.

RESULTS

Ultrasound energy has been adopted to enhance the action of acid-oxidants to decompose organic matrixes to exclude inferences in biological samples due to high organic content. The interference problems most frequently occur in slurry and direct introduction of biological samples in graphite furnace due to high organic matter, as for form the reliable approaches for routine analysis. All blanks, standards, and sample solutions were made in 0.1 M nitric acid to minimize the matrix interferences. The proposed ultrasound-assisted pseudodigestion procedure has several variables. However, the factors that may potentially affect the extraction process are concentrated nitric acid (A1), sonication time (ST), sample mass of muscle tissues (SM), temperature of ultrasonic bath (T), and mixture of 65% nitric acid–30% hydrogen peroxide (A2). A two-level Plackett-Burman design, with 16 runs was carried out to determine the main factors of the USD. Table 2 shows the maximum and minimum values of each factor, and the results of the variance analysis (ANOVA) carried out on the data given in Table 3 are shown in Table 6. This value expresses the probability that a factor is due to random errors. The results of the design were also visualized by using standardized ($P = 95\%$) effects of Pareto chart separately for As, Cd, and Pb (Fig. 1).

DISCUSSION

Effects and Interactions of Variables

The complete analytical results obtained for each experiment are shown in Table 3 for As, Cd, and Pb, respectively. Certified sample of tissue was used for optimization purposes. Significance of the effects was verified by ANOVA and p value significance levels. This value indicates the probability of a factor due to random errors. A factor is considered significant if p is less than 5% (Table 6; Fig. 1).

Influence of Acid

From the results of the Plackett-Burman design (Table 3), and standardized effect in Pareto chart (Fig. 1), p values, as well as center composite design

Table 6. Analysis of data given table 3

	DF	SS	MS	F-ratio	p value
			As		
A1	1	2.4025	2.4025	0.0717743	0.7942157
ST	1	2766.76	2766.76	82.656469	3.778E-06
SM	1	48.3025	48.3025	1.4430287	0.2573283
T	1	6963.9025	6963.9025	208.04536	5.091E-08
A2	1	622.5025	622.5025	18.597153	0.0015312
A1*SM	1	21.16	21.16	19.383206	0.0217284
ST*SM	1	19.8025	19.8025	18.139695	0.0237363
A1*T	1	24.01	24.01	21.993893	0.0183289
ST*T	1	0.0225	0.0225	0.0206107	0.8949456
SM*T	1	0.49	0.49	0.448855	0.5508366
SM*A2	1	6.76	6.76	6.1923664	0.0886056
T*A2	1	259.21	259.21	237.44427	0.0005937
Error	3	3.275	1.0916667		
Total	15	10738.6			
			Cd		
A1	1	22.09	22.09	0.2263123	0.6444935
ST	1	3141.6025	3141.6025	32.185747	0.0002057
SM	1	0.16	0.16	0.0016392	0.9685018
T	1	4462.24	4462.24	45.715691	4.972E-05
A2	1	1256.7025	1256.7025	12.874929	0.0049441
A1*SM	1	17.64	17.64	1.6808004	0.2855353
ST*SM	1	0.3025	0.3025	0.0288232	0.8759907
A1*T	1	96.04	96.04	9.1510243	0.0565315
ST*T	1	513.0225	513.0225	48.882563	0.0060068
SM*T	1	0.49	0.49	0.0466889	0.8427866
SM*A2	1	12.6025	12.6025	1.2008099	0.3532489
T*A2	1	304.5025	304.5025	29.014054	0.0125353
Error	3	31.485	10.495		
Total	15	9858.88			
			Pb		
A1	1	0.16	0.16	0.0041689	0.949791
ST	1	1844.7025	1844.7025	48.065413	4.03E-05
SM	1	8.7025	8.7025	0.2267516	0.6441759
T	1	8344.8225	8344.8225	217.43199	4.121E-08
A2	1	817.96	817.96	21.312697	0.0009556
A1*SM	1	1.44	1.44	1.8762215	0.2642819
ST*SM	1	0.0025	0.0025	0.0032573	0.9580757
A1*T	1	0.64	0.64	0.8338762	0.4284867
ST*T	1	12.6025	12.6025	16.420195	0.0270727
SM*T	1	0.3025	0.3025	0.3941368	0.5746706

(*continued*)

Table 6. Continued

	DF	SS	MS	F-ratio	p value
			Pb		
SM*A2	1	1.69	1.69	2.2019544	0.2344846
T*A2	1	364.81	364.81	475.32248	0.0002112
Error	3	2.3025	0.7675		
Total	15	11400.138			

DF, degrees of freedom; SS, sum of squares; MS, mean squares; p value, probability level.

(which will be developed later), it is clearly observed that high amount (+) of acid oxidant mixture (HNO_3:H_2O_2) provides a significantly higher recovery for As, Cd, and Pb. The concentrated HNO_3 alone at (+) level gives 85.8%, 77.3%, and 74.0% recovery of As, Cd, and Pb, respectively, at the optimum values of other variables ST, T (+) level, and SM (−) level. It was reported that oxidation of biological samples with high organic matter is usually incomplete with only HNO_3, so in this experiment we used H_2O_2 in combination with HNO_3, which yields clear solutions and improved recovery.

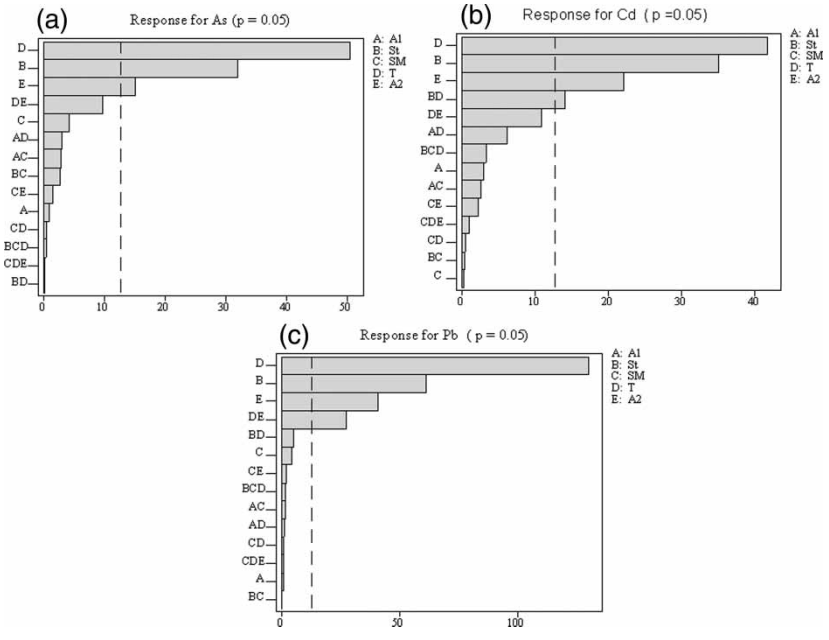

Figure 1. Pareto chart of the standardized effects: (a) arsenic, (b) cadmium, and (c) lead.

In addition, two-order interaction between A2 and temperature was significant for As and Pb, and A2 with ST is significant for the % recovery of Cd. It can be seen in runs 7 and 9 that with maximum acid volume of A1 at optimum values of other variables (i.e., ST and T at (+) levels and SM at (−) level) yields 13.0%, 21.6%, and 23.8% lower amount of As, Cd, and Pb, respectively, compared with those obtained from A2 at the same conditions.

Influence of Temperature

The increase in the ultrasonic water bath temperature showed a highly significant effect on the recovery of As, Cd, and Pb. Therefore, the temperature of the ultrasonic water bath was significant for the USD to extract elements under analysis from muscle tissues. While varying the temperature of ultrasonic bath in the range 40°C to 80°C, the maximum recovery of all these elements was observed at 80°C. It can be seen in experiment 13 that at (−) level of T with optimum values of other variables, A2 and ST (+) and SM (−), the % recovery of As, Cd, and Pb were only 51.4%, 68.5%, and 38.9%, respectively. It was clear that the temperature effects were significant for the recovery of all, especially for Pb.

Influence of Intensification Time

Intensification time is in the range 1–5 min, however it can be seen in Table 3 that the maximum recoveries of all three elements were achieved after 5 min at temperature 80°C in ultrasonic bath. The recovery of As, Cd, and Pb using ultrasonic-assisted pseudodigestion required a maximum 5 min to reach the same recoveries of all these elements obtained by CDM. Longer sonication time >25 min has no significant effect on the recovery of elements under study. This fact offers an important practical advantage as it reduces the duration for acid digestion, while in previous studies developed by El-Azouzi et al.[12] the sonication time was about 180 min.

Influence of Sample Mass

Sample mass [SM] was in the range of (+) and (−) level, that is, 100 and 500 mg of the same particle size <65 μm. This factor did not produce any significant effects. As seen in run 5 of the Plackett-Burman design, with (+) level of SM, the recovery of As, Cd, and Pd was only 2.1%, 1.1%, and 1.7% lower compared with the values of all three elements obtained in experiment 7, with low level (−) of (SM).

Estimated Effects and Interaction of Variables

The main effects and variance obtained are shown in Table 6. For all three elements, the higher significant estimated effect was found for variable T, in

the order Pb > As > Cd. The minimum main effects were observed for variable A1 and SM. The most relevant interaction between two variables was seen for T and A2. On the contrary, the least interaction was observed in A1 and SM. Therefore, it can be concluded that the temperature of ultrasonic bath, nitric acid and hydrogen peroxide mixture volume, and time for ultrasonic exposure exhibited significant influence on extraction efficiency of all three elements. However, the influence of the remaining two variables (A1 and SM) is less important in order to achieve quantitative recovery of As, Cd, and Pb.

Optimization by Central Composite Design

Acid mixture (A2) is efficient for the recovery of elements under study and has a strong interaction with sonication time (ST) and temperature of ultrasonic bath (T) as demonstrated by results of the Plackett-Burman factorial design (Table 3). These three factors were optimized to provide the maximum recovery of As, Cd, and Pb. A central 2^3+star orthogonal composite design with 6 degree of freedom involving 16 experiments was performed to optimize these variables. The factors that were shown to be insignificant by the Plackett-Burman design were fixed at convenient values; therefore, the mass of muscle tissues was 200 mg for all runs. The experimental field definition for this design is given in Table 2, and Table 4 shows the central composite design matrixes together with the response obtained for As, Cd, and Pb. A study of the estimated response surfaces for each pair of variables, (A2)/(ST), (A2)/(T) for As, Cd, and Pb showed the optimum values for each variable and for each element. Figure 2 indicates the contour diagram of the estimated response surfaces.

The comments for each element are as follows.

Arsenic and Lead

From the center composite design (Table 4), it was observed that the % recovery of As and Pb increased as a function of temperature, while at low level (−) the % recovery of As and Pb are 54.50% and 49.50% (run 6), respectively, although the other two variables A2 and ST were at (+) levels.

In this case, the USD efficiency is directly proportional to the temperature. The other effective variable is acid oxidant mixture volume A2 at (+) level, shown in Table 4, and counter surface estimated graph. It was noted that at (−) level of A2, the % recovery of As and Pb was obtained as 78.2% and 75.60%, respectively (run 4), although the other two variables ST and T were at optimum level. Figure 2 (b, c, h, i) clearly indicates the significance of A2 variable and its interaction with ST and T. At (+) level of A2, the percentage recovery of As and Pb was attained as 98.85% and 97.85% (run

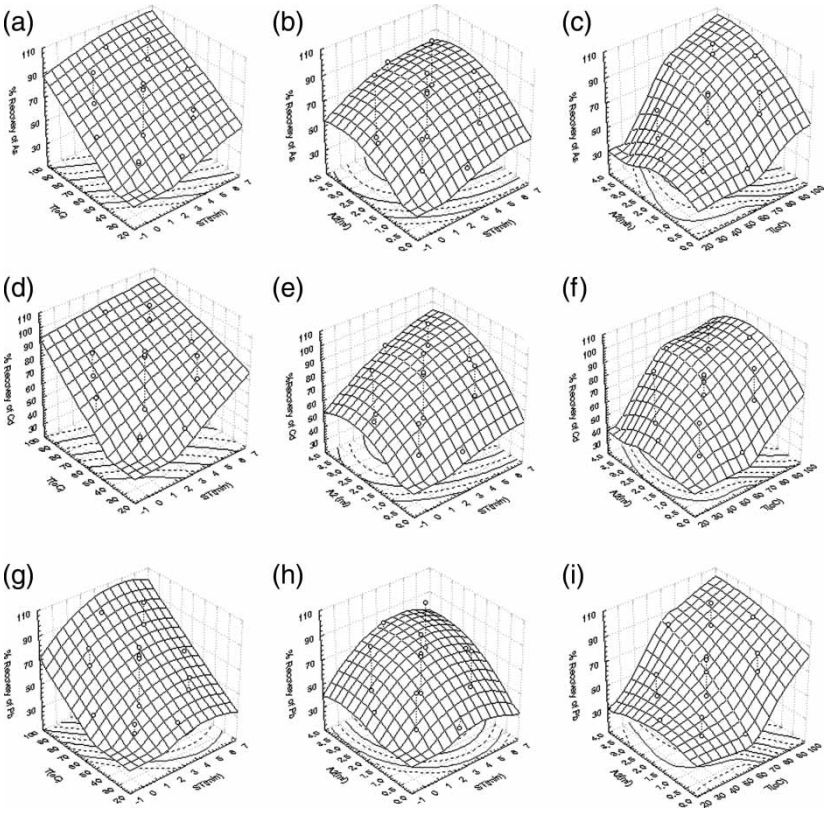

Figure 2. Three-dimensional surface and contour plot of % recovery: (a–c) for As, (d–f) for Cd, and (g–i) for Pb.

8), respectively. The third variable (ST) was also significant. It is shown in Table 4 (run 8) that at upper level (+), the percentage recovery of As and Pb has enhanced 11.15% and 22.75%, respectively, compared with the run 7. It is also indicated in the contour diagram of the estimated response surface (Fig. 2a, b, g, h) the interaction of ST with other variables.

Cadmium

It can be seen from Fig. 2 (d, f) that the USD efficiency increased when the temperature was higher; at low level (−) the percentage recovery of Cd was 79.1% (Table 4) (run 6). The % recovery of Cd was 86.1% at (−) level of A2, which is higher compared with As and Pb at the same condition (run 4) (Fig. 2e, f). The percentage recovery of Cd was the highest when both factors T and A2 were at the maximum levels. Figure 2 (d, e) indicates the

effect of the sonication time on the recovery of Cd from muscle tissues. It was observed that at low level (−) of ST, the recovery is 77.80%, which was 21.01% lower compared with the value obtained at high level of ST (run 7 and run 8).

Analytical Figures of Merit

Calibration was performed with a series of As, Cd, and Pb standards. Sensitivity (m) was the slope value obtained by least-squares regression analysis of calibration curves based on peak area measurements. The equations ($n = 5$) for the calibration curves are as follows:

$$Y = (0.0017 \pm 2 \times 10^{-5})\ (\text{As}) + (0.0179 \pm 0.00015)$$

$$Y = (0.0079 \pm 4 \times 10^{-4})\ (\text{Cd}) + (0.0012 \pm 0.0001)$$

$$Y = (0.0266 \pm 2 \times 10^{-3})\ (\text{Pb}) + (0.023 \pm 0.00057),$$

where Y is integrated absorbance, and As, Cd, and Pb are the arsenic, cadmium, and lead masses deposited in the furnace. The linear range of the calibration curve for As was 1.0–50 ppb, for Cd was 0.5–15 ppb, and for Pb was 1–30 ppb. The limit of detection (LOD) was defined as $3\,sm^{-1}$, s being the standard deviation corresponding with 10 blank injections and m the slope of the calibration graph equal to 2.58, 0.379, and 0.642 pg/10 μL for As, Cd, and Pb, respectively. The quantification limit (LOQ), defined as $10\,s/m$, was 0.862, 0.126, and 0.214 μg/L, respectively, for As, Cd, and Pb.

Application for Real Samples

The proposed methodology was applied to the analysis of muscle tissues of five freshwater fishes collected from Lake Manchar in Pakistan. This lake is heavily polluted with agricultural and industrial waste. A 200 mg mass of dried ground fish muscles sieved at <65 μm were subjected to extraction of the As, Cd, and Pb at optimized conditions of different variables. The mean element concentration expressed as $x \pm s$, where x is the mean and s is the standard deviation for n ∼ 20 measurements, is given in Table 7. Extraction efficiency, expressed as percentage, was calculated as the ratio between element content obtained with ultrasound treatment and those obtained with CDM, based on the equation

$$\%\text{Recovery} = \frac{[\text{Metals obtained by USD}]}{[\text{Metals obtained by CDM}]} \times 100.$$

No significant differences were observed for $p = 0.05$ when comparing the values obtained by the proposed USD method and CDM (paired t-test).

Table 7. Analytical results obtained for As, Cd, and Pb in fish muscles (mg kg^{-1} dried basis)

Metals	Species	CDM ($x \pm s$)	USD ($x \pm s$)	% Recovery	$t_{crit} = 2.098$
As	*Chitala chitala*	3.315 ± 0.289	3.273 ± 0.253	98.7	0.68
	Mastacembelus armatus	12.73 ± 0.0	12.50 ± 0.097	98.19	0.45
	Glossogobius giuris	3.675 ± 0.088	3.633 ± 0.038	98.85	0.047
	Ompok pabda	3.486 ± 0.115	3.434 ± 0.056	98.50	0.068
	Oreochromis mossambicus	2.620 ± 0.241	258 ± 0.201	98.51	0.63
Cd	*Chitala chitala*	0.118 ± 0.028	0.166 ± 0.008	98.31	0.772
	Mastacembelus armatus	1.061 ± 0.097	1.048 ± 0.082	98.77	0.639
	Glossogobius giuris	0.086 ± 0.008	0.085 ± 0.006	98.84	0.062
	Ompok pabda	1.794 ± 0.24	1.773 ± 0.22	98.8	0.122
	Oreochromis mossambicus	1.823 ± 0.39	1.801 ± 0.34	98.7	0.52
Pb	*Chitala chitala*	0.268 ± 0.060	0.258 ± 0.018	96.27	0.509
	Mastacembelus armatus	2.955 ± .673	2.86 ± .201	96.79	0.558
	Glossogobius giuris	0.873 ± 0.067	0.852 ± .057	97.59	0.122
	Ompok pabda	2.145 ± 0.121	2.098 ± .289	97.81	0.515
	Oreochromis mossambicus	3.218 ± 0.536	3.146 ± 0.217	97.76	0.593

CONCLUSIONS

A dynamic ultrasound-assisted pseudodigestion method has been proposed for the determination of As, Cd, and Pb from muscle tissue of fish samples. Rapid optimization of sample pretreatment is affected by many factors prior to determination of As, Cd, and Pb by ETAAS. The application of factorial design for factor screening shows that acid-oxidant mixture, temperature of ultrasonic bath, and the exposure to ultrasonic energy are the most significant variables for maximum recovery of As, Cd, and Pb from muscle tissue. By using such a design, we have been able to establish the optimum conditions for the determination of trace and toxic elements in biological samples. Furthermore, as atomic spectroscopy moves more toward multivariate analysis, the need for multivariate experimental design and optimization techniques becomes important in establishing a valid methodology. Here, 200 mg dried fish muscle in 3 mL HNO_3:H_2O_2 (1:1) at 80°C of ultrasonic water bath for

5 min sonication time at the (+) level are the optimized conditions to obtain quantitative results for As, Cd, and Pb. The results obtained for analytes under study were compared with those obtained by means of classic sample pretreatment based on conventional wet acid digestion induced by electrical hot plate. Thus, the proposed procedure is more economic due to minimum reagent and minimum time and also due to the reduction of nitrous vapors produced. The current work was focused on muscle tissue, but from a preliminary screening, it can be anticipated the USD procedure is also useful for other biological samples.

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