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Copper determination by ETAAS in fish tissue cytosols with minimal sample pretreatment

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Abstract

The metal concentrations in the fish tissue cytosols offer an important parameter in environmental monitoring studies as possible indicators of metal bioavailability in ambient water. The aim of this study was to examine the possibility of direct Cu determination in the cytosols of chub gills and intestines by electrothermal atomic absorption spectrometry (ETAAS) with minimal sample pretreatment and without the use of modifiers. Tube wall and platform atomizations were tested. When tube wall atomization was applied, a progressive decline of Cu absorbance was observed with increasing firing count. On the other hand, stability of Cu absorbance throughout the graphite tube lifetime (up to 250 firings) was obtained with platform atomization. Signal stability, together with excellent measurement repeatability (≤5%) and acceptable Cu recovery from spiked samples (77-89%), make ETAAS with platform atomization applicable to the determination of low Cu concentrations (<27 μg L^{-1}) in environmental samples with complex organic matrices, such as undigested fish tissue cytosols, even without the use of chemical modifiers.

INTRODUCTION

During the fall and spring seasons of 2005 and 2006, a metal pollution study was carried out in the Sava River (Croatia) in the course of the European Union Sixth Framework Programme project (SARIB project, INCO-CT-2004-509160). Among others, metal bioavailability in the river water was assessed using the bioindicator organisms (1,2). Fish are often used as bioindicators of water pollution with metals (3), and since the European chub (*Squalius cephalus* L.) is the fish species widespread in European freshwater, it was selected for this study. Two representative tissues, gills and intestine, were chosen for the metal determinations. As organs in direct contact with the ambient water and ingested food, they are expected to respond quickly to changes in metal exposure (4).

For environmental analyses, determination of total metal concentrations in digested tissues is the usual procedure. From an analytical point of view, the problem of matrix complexity is thereby eliminated, but valuable information on subcellular metal distribution and trophically available metal levels is lost. In our study, metals were therefore determined in tissue cytosol fractions, which contain heat-sensitive proteins (such as enzymes) and heat-stable proteins (such as metallothioneins) (5). The presence of increased metal concentrations in the fraction containing heat-sensitive proteins (potentially metal sensitive fraction) is expected to be associated with some biological impairment (6). Metals sequestered by metallothioneins, on the other hand, are considered detoxified, and thus not available to more sensitive cellular fractions (4). The cytosolic metal concentrations, as potential indicators of metal bioavailability in

ambient water, represent the important and very interesting parameter in environmental monitoring studies.

To avoid the analytical interferences of a complex organic matrix, tissue cytosols should be digested prior to Cu determination. However, microwave digestion requires the use of additional chemicals (mineral acid, hydrogen peroxide) and expensive equipment. It is, therefore, a costly and time-consuming procedure, as well as a potential source of sample contamination. It should also be pointed out that the concentrations of metals in the cytosols of fish tissues are rather low (1, 2), and any additional sample dilution could result in concentrations below the instrument detection limit. Considering these facts, we measured the concentrations of several metals (Fe, Zn, Mn, Cu, and Cd) directly in undigested gill and intestine cytosols to avoid possible sample contamination and over-dilution, and to make the procedure as simple and low-cost as possible. Iron and Zn were measured by flame atomic absorption spectrometry (AAS), while Mn, Cu, and Cd were measured by electrothermal atomic absorption spectrometry (ETAAS) due to their low concentrations in diluted cytosols (Cu and Mn <30 $\mu g\ L^{-1}$; Cd <2 $\mu g\ L^{-1}$) (1, 2). Metal determination by ETAAS in fish tissue cytosols without pretreatment has, to our knowledge, never been reported in the literature. However, the concentrations of Al, Cd, Co, Cr, Cu, Fe, Mn, Mo, Ni, Pb, and Se were measured in different types of undigested samples (milk, urine, serum, blood, wheat flour slurry, coconut water) but usually with the use of various chemicals as dilution or modifier solutions. The oxidant mixture (HNO_3+H_2O_2) was most frequently applied, but the use of ethanol, tertiary amines, Triton® X-100 and Pd was also reported (7-15).

The main objective of our study was to solve the problems encountered as a consequence of the complex organic matrix in the direct Cu determination in undigested fish tissue cytosols. The comparative measurements on tube wall and platform atomization were performed by ETAAS without use of chemical modifiers. Due to decreased background, improved long-term stability and minimal chemical interferences, the use of platforms is recommended for the measurement of metals in samples with complex matrices (16). However, we first attempted to measure Cu by applying tube wall atomization, due to lower cost of simple graphite tubes compared to tubes with integrated platforms. The results obtained with tube wall and platform atomization were compared, and more favourable approach for Cu measurement in the undigested fish tissue cytosols was additionally analytically characterized. The detection and quantification limits, measuring ranges, repeatability, and Cu recovery from spiked samples are listed, providing new information about the direct metal determination in cytosolic types of samples by ETAAS (17).

EXPERIMENTAL

Isolation of Gill and Intestine Cytosols

The gills, ranging from 0.5 to 1.0 g, and the intestines, ranging from 1.0 to 2.0 g, were obtained from specimens of European chub (*Squalius cephalus* L.) caught in the Sava River (Croatia), cut up, and diluted in five volumes of cooled homogenizing buffer (1, 2). The applied gill homogenization buffer (GHB) was 100 mM Tris-HCl/Base buffer (Sigma Chemical Co., USA) with a pH equal to 8.1 at 4°C, and supplemented with the reducing agent, 1 mM dithiotreitol (Sigma Chemical Co., USA). The intestine homogenization buffer (IHB) additionally contained inhibitors of proteolitic activity,

0.5 mM phenylmethylsulphonyl fluoride (PMSF) and 0.006 mM leupeptine (LEU) (18). High purity water (Milli-Q™ Water System, Millipore, Madrid, Spain) was used for the preparation of the homogenization buffers. Homogenization was performed in ice-cooled tubes using a Glas-Col homogenizer (USA), set at 6,000 rpm. The homogenates were then centrifuged in the Sorval RC28S centrifuge (Kendro, USA) at 50,000×g for 2 h at 4°C. After the centrifugation, supernatant (S50), which is a water-soluble cytosolic tissue fraction, was separated from the pellet, then five times diluted with Milli-Q water (1:5), and deep frozen until subsequent metal determination. Diluted cytosols contained proteins [determined according to Lowry et al. (19)] in a concentration of approximately 2-4 mg mL^{-1} (1, 2).

Standard and Reference Solutions

Regarding the protein content, it was impossible to match the composition of the standard solutions and the cytosol samples. Thus, Cu calibration standards were prepared by the appropriate dilutions of a certified Cu standard solution (1,000 mg L^{-1}, ICP standard in 2-3% HNO_3, Merck, Darmstadt, Germany) with five times diluted GHB and IHB (as described above). The reference solution SW-HM-47, distributed by Vituki (Budapest, Hungary) as a part of the Qualco Danube intercomparison study, was used to examine the possible influence of the calibration standard composition on Cu determination. This reference solution is an acidified sample of river water with an assigned Cu concentration of 14.1 μg L^{-1}.

Instrumentation

A Varian SpectrAA 220 (Australia) deuterium-corrected atomic absorption spectrometer, equipped with a Varian GTA 100 graphite tube atomizer, an autosampler,

and multielement hollow cathode lamp (Cu/Zn), was employed in this study. Argon (99.99% purity) was used as a purge gas. The instrumental parameters are presented in Table I. The Cu absorbance signal was calculated by using the peak area mode to minimize potential differences in the atomization rate and signal shape between the calibration standards and the samples. It was shown experimentally that differences in matrix composition can alter peak width and height; therefore, absorbance integration used in the peak area mode produces smaller variations in the signals than peak height measurement (20). The cytosolic Cu concentrations were measured by tube wall atomization, using partitioned pyrolitically coated graphite tubes, and by platform atomization, using the universal tubes with integrated platforms. The heating programs for tube wall (21) and platform atomization are presented in Table II. There is time delay between the temperature of the tube and the temperature of the platform, and therefore the heating program used for graphite tubes with platforms involved higher drying, ashing, and atomization temperatures compared to the heating program used for tube wall atomization. A cool-down step is also included at the end of the platform heating program to ensure that the temperature of the platform is the same for each sample injection (22).

RESULTS AND DISCUSSION

Effect of Tube Ageing

At first, Cu determination was performed directly in undigested cytosols by a well-established ETAAS method with tube wall atomization, which is commonly used for Cu measurement in acidified solutions (21). The method was applied to more complex sample matrices without any adjustments and without use of modifiers. The obtained

results initially seemed to give reliable information on Cu concentrations in fish tissue cytosols. Excellent repeatability was achieved after Cu determination in duplicates, with relative standard deviations (RSD) mostly below 3%, while low background absorbance pointed to complete removal of the complex matrix during sample ashing at 800°C (Figure 1a). However, with an increasing number of tube firings, the decrease of Cu absorbance signal became obvious (Figure 1b). It was, therefore, impossible to compare the results obtained with the increasing number of firings with those obtained with the new graphite tube.

Tube ageing was previously reported as one of the main factors that can affect metal determination by ETAAS (23). This problem had to be solved to obtain more reliable Cu measurements, and thus the stability of the Cu absorbance signal was compared for tube wall and platform atomization. Cu concentrations were recorded periodically in the selected samples of gill and intestine cytosols during the tube utilization, approximately up to 200 tube firings. The study was performed separately for gill and intestine cytosols to establish if their different matrices damage the tube at different rates. For intestine cytosols the same sample (IN-1, Table III) was used for both tube wall and platform atomization measurements, so the starting absorbance was the same in both cases (Figure 2). For gill cytosols, due to the small sample volume, two samples of different concentrations were used, G-1 for tube wall atomization and G-2 for platform atomization (Table III). When tube wall atomization was used, a progressive decline of Cu absorbance signal was observed (Figure 2). After ~200 firings, the signal decreased approximately 30%, and the rates of tube impairment caused by gill and intestine cytosol matrices were comparable. When tube wall was replaced with platform atomization, the Cu absorbance signal recorded after ~200 firings was comparable to

the signal obtained with the new tube. It varied only slightly (signal RSD 3-4%) throughout tube usage (Figure 2). A possible explanation for signal decline is that the determination of trace metals in biological samples with complex organic matrices gives rise to the formation of carbonaceous residues inside the tube, which could seriously alter the effectiveness of the drying and ashing temperatures (24).

According to Welz et al. (25), the analytical useful lifetime of graphite tubes is defined by the number of firings which can be made until the analytical signal drops to about 80% of its initial value, and/or the RSD begins to deteriorate significantly. The severe effect of tube ageing observed when Cu is measured in chub tissue cytosols using tube wall atomization significantly reduced the tube lifetime. Based on our analyses, it is limited to approximately 130-150 firings (Figure 2). However, even within this limited number of firings, the signal was not stable, but continually decreasing (Figure 2). Therefore, unlike tube wall atomization, the use of platform atomization can be recommended for Cu measurements in undigested fish tissue cytosols even without the use of modifiers, since it provides longer stability of the absorbance signal. Based on our practical experience, graphite tubes with integrated platforms can be used up to 250 firings without signal deterioration.

Method Characterization

Since the main purpose of each analytical measurement is to obtain reliable information about the analyte content in the analyzed sample (26), it is important to characterize the method performance whenever applied to new types of samples. For the characterization of the Cu determination in undigested chub tissue cytosols by ETAAS with platform atomization, the limit of detection, limit of quantification, linearity of

measuring range, repeatability, and recovery from spiked samples were assessed mainly following the International Recommendation R100 (27). Five times diluted cytosols of six gill and five intestine samples were used, with the Cu concentrations ranging from 7.25-16.66 μg L^{-1} and 18.51-26.45 μg L^{-1}, respectively (Table III). Due to the differences in matrix composition of gill and intestine cytosols, method characterization was performed separately for each type of cytosol.

Calibration

The preliminary results indicated that Cu concentrations in the five times diluted cytosols of chub tissues mainly fall into the concentration range of 10-20 μg L^{-1}. Since it is optimal that the calibration range encompasses values up to 150% of the expected measurements (28), the linearity was tested up to the Cu concentration of 27 μg L^{-1}, which was also roughly indicated as the upper calibration limit for Cu determination by ETAAS (21). According to some recommendations (29), it is sufficient to calculate the regression coefficient as a measure of linearity; and when it reaches at least 0.999, it can be assumed that the analytical signal is proportional to the measured analyte concentration in the defined calibration range. The regression coefficients for Cu calibration straight lines were equal to 0.997 and 0.998 for gills and intestine calibrations, respectively (Figure 3 a-b), and thus nearly complied with the requirements for linearity. However, better fit was achieved when the quadratic calibration curve was applied, with higher regression coefficients (r=0.9999 in both cases) and lower intercepts (a=0.003-0.004). The slopes of the quadratic calibration curves (b=0.013-0.014) were comparable for both calibrations (Figure 3 a-b). The differences in the composition of calibration standards obviously did not affect the size of Cu absorbance signal.

Method Detection Limits and Measuring Ranges

For the determination of the detection and quantification limits (LOD and LOQ, respectively), Cu concentrations were measured consecutively 10 times in the blank solutions, and their compositions are provided in Table I. The LOD was calculated on the basis of three standard deviations of the blank (27), and the LOQ on the basis of 10 standard deviations of the blank (30).

For gill calibration (standards prepared with GHB; see section above), the Cu LOD amounted to 0.27 μg L^{-1} (Figure 3a), while for intestine calibration (standards prepared with IHB; see section above) the LOD amounted to 0.82 μg L^{-1} (Figure 3b). The presence of two additional components in the intestine calibration standards, PMSF and leupeptine, evidently increased the noise (standard deviation of the blank). Therefore, the measuring range, which is defined as the range of Cu concentrations from the LOQ value to the upper limit of the calibration range (26), is broader for the gill calibrations with the lower limit set at 1 μg L^{-1}. The lower limit of the measuring range for the intestine calibrations was set at 3 μg L^{-1}.

Repeatability

The repeatability was determined for the gill and intestine calibration standards (see above) with four concentrations selected within the calibration range. It was also established for six gill and five intestine cytosol samples. The indicator of the measurement repeatability was RSD (%) of 10 consecutively measured Cu concentrations in each calibration standard solution and in each cytosol sample (27).

The obtained RSDs (%) of Cu measurements in standard solutions were <5%, whether the calibration standards were prepared with GHB or IHB (Table IV). The repeatability observed for the Cu determination in undigested cytosols was also excellent, and the RSDs were mainly ≤5% for both gill and intestine cytosol samples (Table IV).

Influence of Calibration Standard and Sample Composition on Cu Recovery

The influence of calibration standard composition on Cu recovery was tested using the reference acidified river water sample (SW-HM-47), with the assigned Cu concentration of 14.1 μg L^{-1} and the range of acceptance from 11.3-16.9 μg L^{-1}. Copper concentration in SW-HM-47 (obtained when the calibration curve was prepared with the acidified standards) was 14.7 μg L^{-1}. Similar Cu concentrations were obtained from two calibration curves, prepared with gill and intestine homogenization buffers (described above), and amounted to 14.8 and 15.2 μg L^{-1}, respectively. Based on comparable results, whether the calibration curve was created with acidified solutions (104%) or homogenization buffers (105-108%), it can be concluded that the complex composition of the calibration standards used in this study did not affect the accuracy of the Cu measurement.

Since the certified reference material with matrix corresponding to gill or intestine cytosol was not commercially available, the reliability of Cu determination in the cytosols using platform atomization was tested by establishing Cu recovery from the spiked samples. The volumes of 300 μL of five-time diluted gill or intestine cytosols were mixed with the appropriate volumes of Cu calibration standards. Although the cytosols were additionally diluted with calibration standards, the Cu recovery still should indicate if the complex cytosol matrix affects the measurement reliability.

Copper recovery from spiked gill cytosols (Table V) was 77±9%, while it was higher for spiked intestine cytosols (89±2%). Unlike the components of the homogenizing buffers, the organic matrix clearly caused the decrease of Cu recovery. Still, the recoveries of added Cu from both the gill and the intestine cytosols can be regarded as acceptable results for environmental monitoring studies, in which the comparability of the results is more important than the absolute accuracy.

CONCLUSION

The severe deterioration of Cu absorbance signal as a consequence of tube ageing was observed during the direct Cu measurements in undigested fish gill and intestine cytosols by ETAAS with tube wall atomization. Due to the longer signal stability (up to 250 tube firings), excellent repeatability, and acceptable recovery from spiked samples, ETAAS with platform atomization can be recommended for the determination of low Cu concentrations in fish tissue cytosols, requiring neither previous sample digestion nor use of additional modifiers.

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FIGURE CAPTIONS

Fig. 1. Illustration of Cu peak profile for gill cytosol sample (G1) obtained by tube wall atomization: **(a)** in the new tube; **(b)** after approximately 150 firings.

Fig. 2. Dependence of individual Cu absorbance signals periodically measured in gill and intestine cytosols on the number of tube firings for tube wall and platform atomizations, as indicated in the legend.

Fig. 3. Copper calibration curves obtained by ETAAS with platform atomization; LOD and LOQ values are denoted on the graph, as well as the regression equations: **(a)** Cu calibration standards prepared with GHB (Tris/DTT; see text); **(b)** Cu calibration standards prepared with IHB (Tris/DTT/PMSF/LEU; see text).

TABLE I
Common Instrumental Parameters for Cu Measurement in Undigested Gill and Intestine Cytosols by ETAAS With Tube Wall and Platform Atomization

Blank Gills	20 mM Tris HCl/Base, 0.2 mM DTT	**Background Correction**	Deuterium Lamp
Blank Intestine	20 mM Tris HCl/Base, 0.2 mM DTT, 0.1 mM PMSF, 1.2 μM leupeptine	**Copper Standards**	9.0 μg L^{-1} 18.0 μg L^{-1} 27.0 μg L^{-1}
Sampling Mode	Automix	**Calibration Algorithm**	Quadratic
Measurement Mode	Peak Area	**Sample Volume**	20 μL
Wavelength	324.8 nm	**Total Volume**	35 μL
Slit Width	1.0 nm	**[a]Copper Bulk Concentration**	18.0 μg L^{-1}
Lamp Current	7.0 mA	**Gas Type**	Argon

[a]Copper bulk concentration refers to the concentration of Cu standard solution which is used for preparation of the other two calibration standards in automix sampling mode.

TABLE II
Heating Programs for Cu Measurement by ETAAS With Tube Wall and Platform Atomization

[a]Heating Program for Tube Wall Atomization				[b]Heating Program for Platform Atomization			
Stage	**Temperature °C**	**Time s**	**Gas Flow L min^{-1}**	**Stage**	**Temperature °C**	**Time s**	**Gas Flow L min^{-1}**
Hot Inject	60			**Hot Inject**	100		
Drying	85	5.0	3.0	**Drying**	100	5.0	3.0
	95	40.0	3.0		210	25.0	3.0
	120	10.0	3.0		300	10.0	3.0
Ashing	800	5.0	3.0	**Ashing**	1000	5.0	3.0
	800	2.5	3.0		1000	30.0	3.0
	800	0.5	0.0		1000	2.0	0.0
Atomization	2300	1.1	0.0	**Atomization**	2600	0.8	0.0
	2300	2.0	0.0		2600	2.0	0.0
Clean	2800	2.0	3.0	**Clean**	2800	2.0	3.0
	2800	5.0	3.0		2800	3.0	3.0
				Cooling	40	21.9	3.0
					40	35.0	3.0

[a]Heating program for tube wall atomization was taken from Varian GTA manual (21).
[b]Heating program for platform atomization was recommended by Varian experts (personal communication).

TABLE III
Concentrations of Cu in Diluted Gill and Intestine Cytosols Measured by ETAAS With Platform Atomization ([a]n=10)

Gill Cytosol	Cu (μg L^{-1})	Intestine Cytosol	Cu (μg L^{-1})
G-1	14.03±0.56	IN-1	18.51±0.69
G-2	7.25±0.08	IN-2	21.55±1.18
G-3	8.55±0.11	IN-3	20.65±0.52
G-4	9.60±0.15	IN-4	19.24±0.41
G-5	16.66±0.45	IN-5	26.45±0.20
G-6	14.59±0.40		

[a]n refers to number of replicates

TABLE IV
Repeatability of Cu Measurements by ETAAS With Platform Atomization in Gill and Intestine Cytosols, as well as in two Types of Calibration Standards ([a]n=10)

		Gills (% RSD)	Intestine (% RSD)
[b]**Standards**	4 μg L^{-1}	**0.8**	**1.7**
	9 μg L^{-1}	**4.6**	**3.3**
	18 μg L^{-1}	**4.7**	**1.1**
	24 μg L^{-1}	**0.5**	**0.9**
[c]**Samples**	G-1/IN-1	**4.0**	**3.7**
	G-2/IN-2	**1.1**	**5.5**
	G-3/IN-3	**1.3**	**2.5**
	G-4/IN-4	**1.6**	**2.1**
	G-5/IN-5	**2.7**	**0.7**
	G-6	**2.7**	

[a]n refers to number of replicates.
[b]Standards for gill calibration prepared with Tris/DTT buffer; standards for intestine calibration prepared with Tris/DTT/PMSF/LEU buffer.
[c]Gill cytosol samples (G), intestine cytosol samples (IN).

TABLE V
Copper Recovery From Chub Gill and Intestine Cytosols Spiked With the Standard Solutions (Volumes of 300 μL of diluted gill or intestine cytosols were mixed with the appropriate volumes of Cu calibration standards; the results are referring to the ETAAS measurements with platform atomization.)

Sample	Added Cu	[c]Recovery of Added Cu	
	μg L^{-1}	μg L^{-1}	%
[a]**G-1**	10.91	8.07	**74.0**
G-2	5.14	4.38	**85.2**
G-3	8.18	6.80	**83.1**
G-4	9.60	7.44	**77.5**
G-5	16.62	13.20	**76.5**
G-6	9.60	5.81	**60.5**
		Average Recovery	**76.6±8.9**
[b]**IN-1**	12.00	10.59	**88.2**
IN-2	15.00	13.87	**92.4**
IN-3	15.00	13.29	**88.6**
IN-4	12.00	10.38	**86.5**
IN-5	15.00	13.49	**89.9**
		Average Recovery	**89.1±2.2**

[a] G - gill cytosol.
[b] IN - intestine cytosol.
[c] Average of three consecutive measurements.

Figure 1.

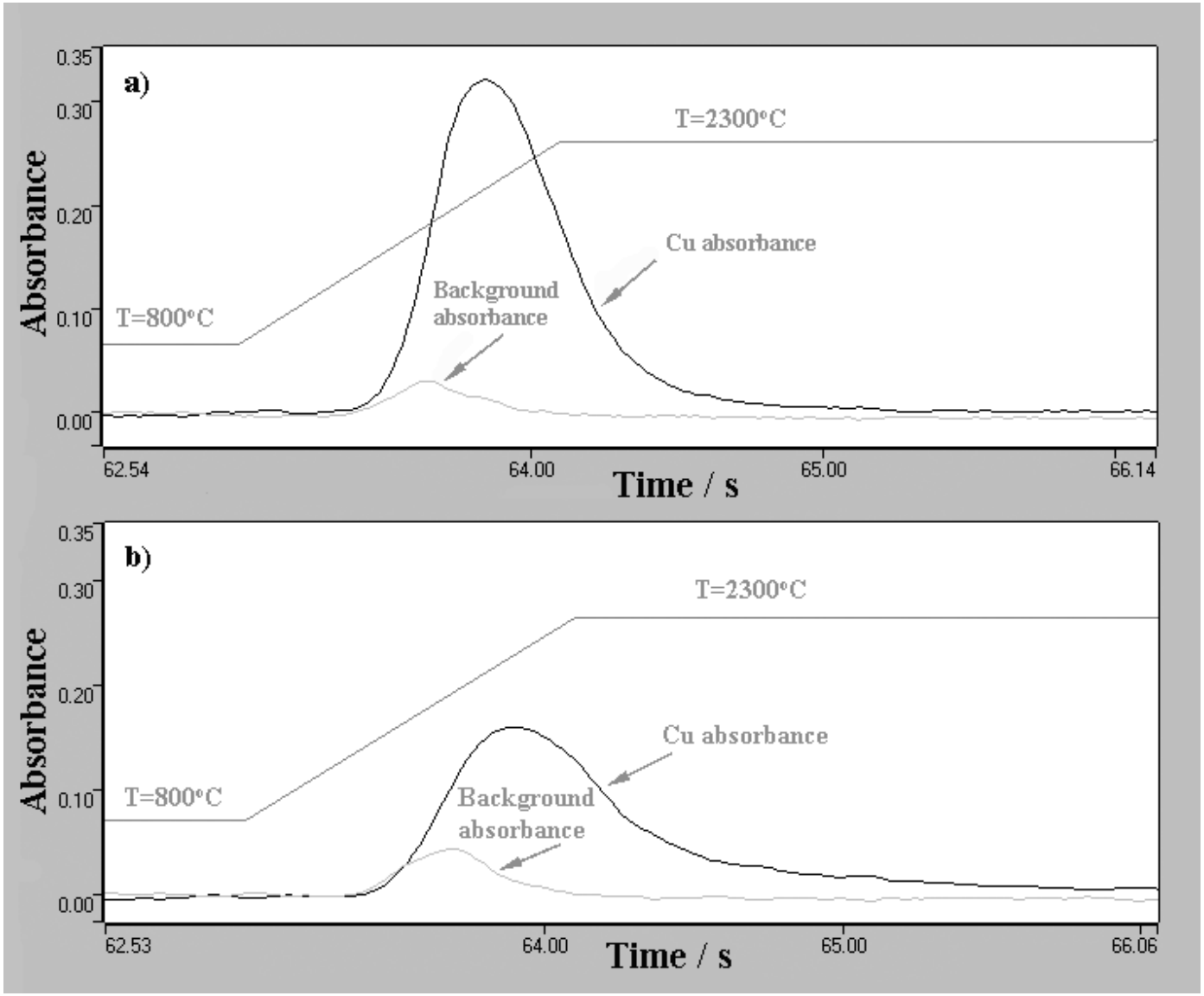

a)
Absorbance
0.35
0.30
0.20
0.10
0.00
T=2300°C
Cu absorbance
Background absorbance
T=800°C
62.54
64.00
65.00
66.14
Time / s
b)
Absorbance
0.35
0.30
0.20
0.10
0.00
T=2300°C
Cu absorbance
T=800°C
Background absorbance
62.53
64.00
65.00
66.06
Time / s

Figure 2.

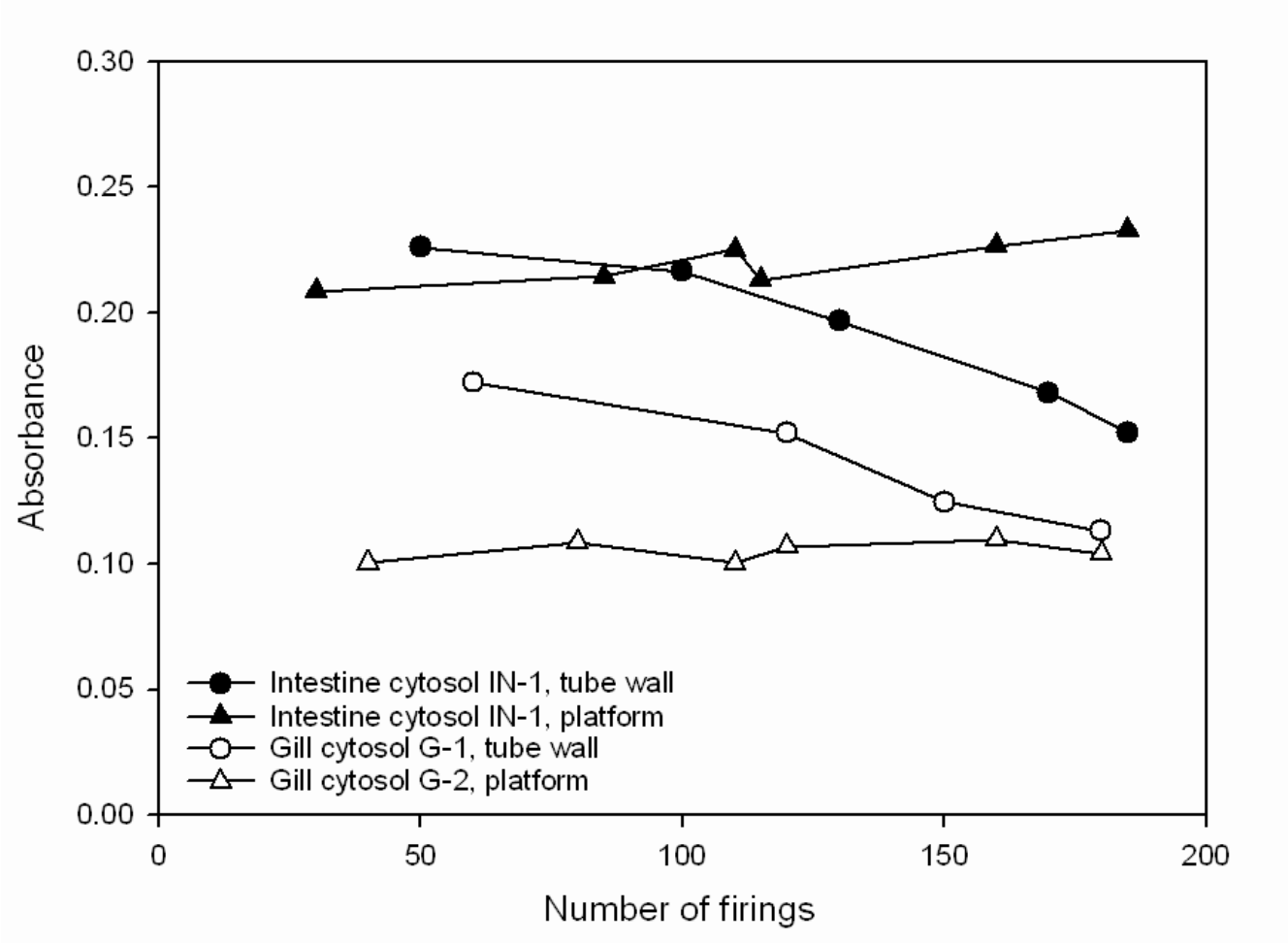
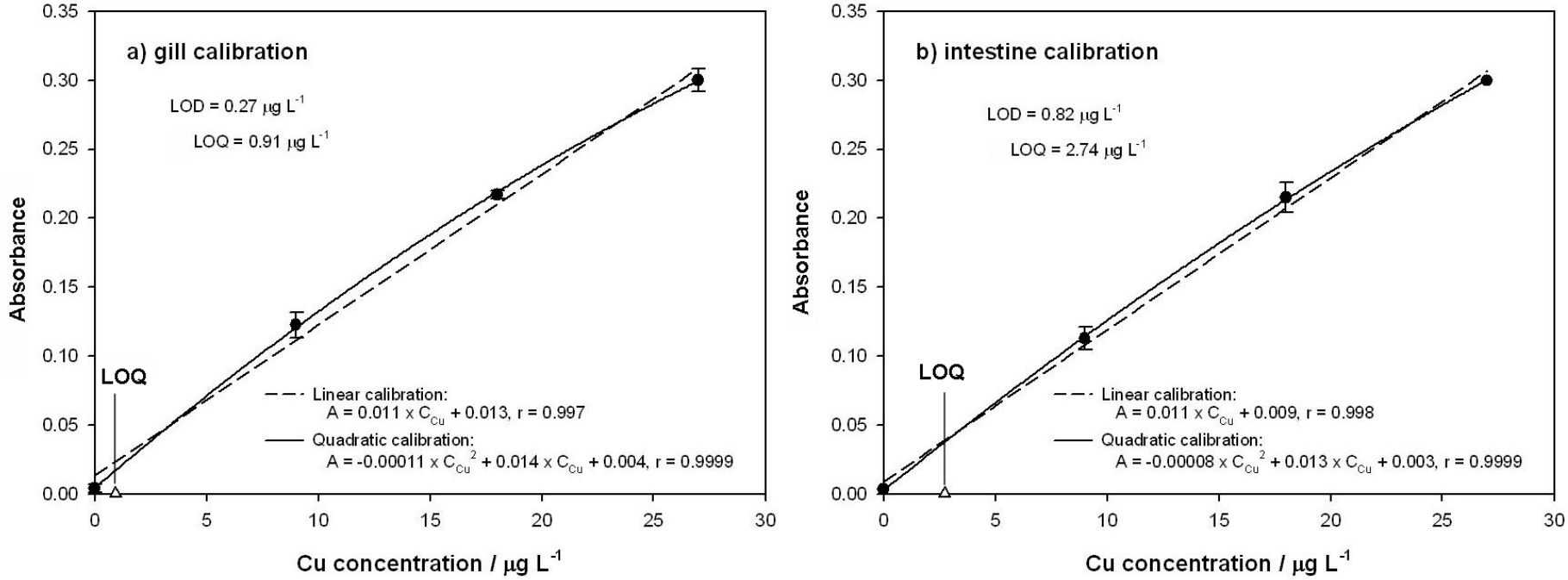

0.30
0.25
0.20
0.15
0.10
0.05
0.00
Absorbance
0
50
100
150
200
Number of firings
Intestine cytosol IN-1, tube wall
Intestine cytosol IN-1, platform
Gill cytosol G-1, tube wall
Gill cytosol G-2, platform

Figure 3.


a) gill calibration
LOD = 0.27 μg L-1
LOQ = 0.91 μg L-1
LOQ
Linear calibration:
A = 0.011 x CCu + 0.013, r = 0.997
Quadratic calibration:
A = -0.00011 x CCu2 + 0.014 x CCu + 0.004, r = 0.9999
Absorbance
Cu concentration / μg L-1
b) intestine calibration
LOD = 0.82 μg L-1
LOQ = 2.74 μg L-1
LOQ
Linear calibration:
A = 0.011 x CCu + 0.009, r = 0.998
Quadratic calibration:
A = -0.00008 x CCu2 + 0.013 x CCu + 0.003, r = 0.9999
Absorbance
Cu concentration / μg L-1