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The Determination of Trace Transition Elements in Biological Tissues Using Flameless Atom Reservoir Atomic Absorption[†]

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The development of an analytical technique generally applicable to the determination of Ag, Cd, Co, Cu, Fe, Mn, Ni, Pb, V, and Zn in all biological tissues is described. All of these elements may readily be determined in tissue samples of less than 0.5 g using flameless atom reservoir atomic absorption with a Perkin-Elmer heated graphite atomizer. Some of the operational characteristics of this atomizer system are discussed. The utility of the method is illustrated by analyses of selected marine biota and of NBS standard orchard leaves.

INTRODUCTION

The analysis of biological tissues for their concentrations of trace transition elements has long been a problem of interest to environmental scientists.¹ Many techniques, such as absorption spectrophotometry, polarography, emission spectrography, gas liquid chromatography, flame atomic emission and absorption, and neutron activation have been employed for such analyses.¹⁻³ Most of the analytical procedures that have been developed utilizing these techniques have been concerned with the analysis of a single element or at most two or three elements. However, it is desirable to determine

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a number of elements simultaneously in the same sample, and this has been achieved by the application of atomic absorption,⁴⁻⁸ optical spectrography⁹⁻¹⁴ and neutron activation analysis.¹⁵⁻¹⁶

The flame atomic absorption determination method has been extensively utilized in trace transition element analysis of biological tissues.⁴⁻⁸ Atomic absorption is rapid, simple, relatively free from interferences, and inexpensive in equipment requirements as compared to most other techniques. However, its application has been limited because the attainable sensitivities both in terms of concentration and absolute mass are somewhat poorer than those that may be obtained with, for example, neutron activation analysis.

The recently developed flameless atom reservoir atomic absorption technique has improved the attainable sensitivity of atomic absorption spectrophotometry by at least an order of magnitude for most elements.^{17,18} The increase in sensitivity is such that for many elements this technique offers detection limits at least as good as, and often better than those attainable with any other currently available elemental analysis method. Thus, the flameless atom reservoir atomic absorption technique appears to be ideally suited to the development of a comprehensive analytical method for tissue samples. In this paper we describe the development of such a method for determination of a range of transition elements, and its application to tissue samples of marine origin.

EXPERIMENTAL

Reagents and apparatus

A Perkin-Elmer Model 403 atomic absorption spectrophotometer equipped with a Sargent-Welch Model SRG recorder, a deuterium arc background corrector and a Perkin-Elmer HGA-70 heated graphite atomizer were employed.

The heated graphite atomizer electronics were modified to enable selective volatilization analysis to be carried out.¹⁹ A modification was also employed which allowed the use of the static method of analysis to improve the sensitivity.²⁰ In our system, the solenoid valve is activated automatically by a relay at the commencement of the atomization cycle and a secondary timer-controlled relay allows the gas flow to be stopped for between 0 and 30 sec, independently of the atomization time. This replaces the manual operation of the solenoid employed earlier.²⁰

All temperature settings used in the graphite atomizer are reported in terms of the applied voltage measured directly across the atomizer terminals, and the approximate corresponding temperature determined from the instrument manual is indicated in parentheses (Table I).

All sample injections were made with Eppendorf microliter pipettes with disposable plastic tips.

Nitric acid and methylisobutyl ketone and glacial acetic acid were purified by distillation from a silica still, and ammonia solution was prepared by dissolution of the gas in double-silica-distilled, de-ionized water. Ammonium pyrrolidine dithiocarbamate was recrystallized from alcohol-water, and its solution was extracted with several portions of ketone before use. Containment vessels and extracting funnels throughout the analysis were of fused quartz, Teflon, or high-density polyethylene.

Sample preparation and dissolution

All tissue samples were freeze-dried to constant weight before analysis. Until the recent development of a solid sample atomization technique,²¹ each of the commercially available atom reservoirs has required that the sample be in solution for introduction into the reservoir. The development of the method described here was, therefore, based upon the use of dissolved samples for the analysis. Although it would seem that the necessity for utilizing dissolved samples may be obviated by the direct use of solid samples, our preliminary experience with solid biological tissue samples leads us to believe that this may not be true, except in limited instances. The results of our study of solid sample analysis will be reported in greater detail elsewhere. However, it appears that difficulties such as the long drying and ashing time required, matrix interferences, the lack of dynamic range, and nonreproducible volatilization of the non-uniformly dispersed samples may reduce the effectiveness of this technique. It would therefore appear that for general analysis of varied samples with a requirement for high accuracy and precision, the dissolution of the sample will still be necessary.

Many methods for the ashing and dissolution of biological tissues have been employed.^{22,23} During analysis for trace transition metals, the major problem has often been that of either contamination of the sample with impurities in the reagents used or the loss of metal from the sample during ashing. To minimize both of these possibilities, we have chosen to wet-ash the organic matrix with nitric acid alone in pure fused silica conical flasks.⁴ The flasks are heated on a hotplate and each flask is covered with a silica bulb stopper to maintain the acid vapor within the flask as long as possible. Contamination of the samples by this procedure is extremely small as the nitric acid may be obtained in a highly purified state by distillation from a silica still. Oxidation is somewhat slow when using nitric acid alone. However, we have always been able to achieve complete dissolution of the samples if the digestion is prolonged for a period of several days, except when small quantities of silica residues were left from some siliceous organisms. The possible loss of metal during the ashing was investigated using standard additions or

TABLE I
Recommended settings for Perkin-Elmer HGA-70 heated graphite atomizer^a

	Sequence					
	Drying ^b		Ashing		Volatilizing	
	Time (sec)	Voltage ^c	Time (sec)	Voltage ^c	Time (sec)	Voltage ^c
Copper	30	0.4 (~ 100°C)	15	3.0 (~ 900°C)	20	7.0 (~ 2100°C)
Iron	30	0.4 (~ 100°C)	15	3.6 (~ 1150°C)	25	8.0 (~ 2200°C)
Zinc	30	0.4 (~ 100°C)	15	2.0 (~ 300°C)	15	7.5 (~ 2150°C)
Cadmium	30	0.4 (~ 100°C)	15	1.5 (~ 250°C)	15	6.5 (~ 1950°C)
Vanadium	30	0.4 (~ 100°C)	45	5.0 (~ 1300°C)	35	8.5 (~ 2300°C)
Nickel	30	0.4 (~ 100°C)	15	3.0 (~ 900°C)	25	8.0 (~ 2200°C)
Lead	30	0.4 (~ 100°C)	20	1.5 (~ 250°C)	20	7.0 (~ 2100°C)
Silver	30	0.4 (~ 100°C)	15	2.0 (~ 300°C)	20	7.5 (~ 2150°C)
Manganese	30	0.4 (~ 100°C)	20	2.0 (~ 300°C)	20	7.0 (~ 2100°C)
Cobalt	30	0.4 (~ 100°C)	15	3.6 (~ 1150°C)	20	8.0 (~ 2200°C)

^a For determination of aqueous 0.1N HNO₃ solution of metals.

^b Drying times are for 20-ml injections. For smaller volumes, drying time may be decreased. For larger volumes, drying time should be increased (e.g., 100-ml injection, 60 sec drying).

^c Voltage as read between + and - terminals of the atomizer.

radiochemical tracers. No loss of Zn, Pb, Co, Ag, Sn, Fe, Mn, Cr, Cd, Ni, or Cu was observed.

Analysis of the aqueous digest solution by direct injection into the heated graphite atomizer.

It would be ideal if aliquots of the aqueous solution of the ashed biological tissue could be used directly for injection and analysis with the heated graphite atomizer. However, our experience has been that this is not possible for most samples when determining trace transition elements. The complicated matrix composition of the solution does not normally permit accurate determinations to be carried out by this means. The concentrations of most transition elements in biological tissues are almost always very small compared to the concentrations of the major metallic elements, particularly sodium, potassium, calcium, and magnesium. Thus, an aliquot of the ashed solution of most samples which contains sufficient transition metal for analysis by atom reservoir atomic absorption also contains relatively large quantities of other inorganic salts. These salts are atomized at the same time as the transition elements and due to their mass they give rise to considerable non-specific molecular and scattering absorption which interferes with the determination. A small amount of such interference can be compensated for by the use of a deuterium arc background corrector, but in practice the magnitude of the interfering absorption was found to be too great in many analyses and the correction was no longer effective.

For those elements that are relatively involatile, selective volatilization analysis is possible, whereby the major interfering salts are removed at a low temperature and then the element of interest is atomized at a higher temperature.^{19,24} However, there is almost always some co-volatilization of the analysis element during the volatilization of the major salts. The quantity of the analysis element co-volatilized was found to be critically dependent upon the total salt content of the solution²⁴ and also upon the major element, both cation and anion, composition of the dissolved salt matrix. As both the total salt content and the matrix composition may be extremely variable in the ashed solutions of biological tissues, the selective volatilization technique is not applicable, except in instances where a routine analysis is to be performed for a series of tissue samples of identical compositional types. In addition, the large concentrations of calcium often present in biological tissues make the major salt matrix more refractory than was the case in a previous investigation of sea water analysis by this technique.²⁴ Thus, it was necessary to raise the ashing temperature to effectively remove the scattering interference and consequently co-volatilization of the analysis element was increased and the sensitivity reduced.

In the light of the above difficulties, it was found necessary to extract the transition elements from the major elements before determination. This extraction also provides an increase in the overall sensitivity of the analysis. The ash from as little as 1 g of biological tissue, depending upon the actual tissue type, often requires at least 20 ml of nitric acid for complete dissolution. After extraction, this volume can be reduced, and a larger proportion of the sample may be introduced into the atomizer for each analysis.

Solvent extraction of transition metals

The solvent extraction of transition elements as their pyrrolidine dithiocarbamates has been extensively utilized in the separation of these elements from the alkali and alkaline earth metals prior to analysis by flame atomic absorption.⁴ This extraction technique was adopted for the current study, as it had already been shown to be quantitative for Zn, Pb, Co, Ni, Cd, Fe, Mn, Sn, Cu, and Ag extracted into methylisobutyl ketone or *n*-amylmethyl ketone from a digest solution of various marine animal tissues.⁴ In the present work, we have also found the ashing and extraction technique to be quantitative for vanadium.

The ketone solution of the metal pyrrolidine dithiocarbamates may be injected into the heated graphite atomizer for analysis of the various metals.

TABLE II
Approximate detection limits using the HGA-70^a

Approximate detection limits (ng/g)		
	Analysis of ketone extract (5-mcl injections)	Analysis of extract after reversion to aqueous solution (100-mcl injections)
Ag	1.2	0.02
Cd	1.8	0.03
Co	30	0.5
Cu	30	0.5
Fe	25	0.4
Mn	25	0.4
Ni	120	2.0
Pb	20	0.3
V	300	5.0
Zn	0.6	0.01

^a 1 g sample extracted into 15 ml ketone, reverted to 5 ml of aqueous solution.

However, the detection limits are not significantly better than those that could be obtained simply by aspirating the ketone directly into the flame atomizer, as we have done in previous investigations⁴ (Table II). This lack of sensitivity is due to the necessity for restricting the volume of ketone injected to 5 mcl or less. Due to the low surface tension of organic solvents as compared to water, ketone, when injected into the atomizer, spreads out on the floor of the tube. If more than 5 mcl are injected, some of the ketone is lost from the ends of the atomizer and the analysis is not reproducible. Multiple additions of 5 mcl of ketone at a time, with drying between injections, can be used to increase the total sample weight introduced into the atomizer. However, the reproducibility of analysis was much reduced when using multiple injections. Therefore, it was decided that the transition metals should be reverted back to aqueous solution before analysis. Currently under assessment are modified graphite tubes which eliminate loss of organic solvents during drying. When these tubes become more freely available, reversion back to aqueous solution should prove unnecessary for most analyses. Modified tubes were not, however, available for this investigation.

Redissolution in aqueous solution

The most rapid means of returning trace metals in chelate form from organic solution to aqueous solution is by back extraction, usually with an acid solution. However, the nature of the equilibrium between aqueous and organic solvents of the metal pyrrolidine dithiocarbamates is such that even when strong acids were used for back extraction (e.g., 6N HNO₃), a proportion of some metals, e.g., Pb, Cd, V, remained in the organic phase. Therefore, the metals were returned to the aqueous phase by evaporating off the ketone solvent, destroying the organic components of the resultant matrix with nitric acid and dissolving the metals in dilute nitric acid solution. This procedure has been found to be quantitative and the resultant inorganic salts may be dissolved in as little as 5 ml volume or less.

Atomic absorption determination

The range of trace transition elements to be found in biological tissues is extremely large. Therefore, it was found advantageous to utilize the very large dynamic range offered by the parallel use of flame atomization and the heated graphite atomizer (Table III). Flame atomization atomic absorption can be carried out about twice as fast as flameless atom reservoir atomic absorption, so that the samples were initially monitored by this technique to determine whether or not concentrations of the various elements were below the optimum concentration range. For those elements whose concentrations were within

the optimum range for flame atomization analysis or were above this range (normally only Fe and Zn), analysis was carried out using standard flame atomic absorption conditions²⁵ and either dilutions of, or the undiluted, aqueous solutions. For those elements whose concentrations were below this range, normally Ni, Co, Cd, Pb, Ag, V and for most of our samples Cu, the undiluted aqueous solution or dilutions of these were utilized for analysis by injection into the heated graphite atomizer.

TABLE III

Optimum concentration ranges for flame and heated graphite atomizer analysis

	Concentration range (ng/ml) in analysis solution		ng/g Sample ^a	
	Flame	HGA-70	Flame	HGA-70
Ag	1,000- 10,000	0.004- 500	5,000- 50,000	0.02- 2,500
Cd	500- 5,000	0.006- 500	2,500- 25,000	0.03- 2,500
Co	2,000- 20,000	0.1 -1,000	10,000-100,000	0.5 - 5,000
Cu	2,000- 20,000	0.1 -3,500	10,000-100,000	0.5 -17,000
Fe	2,000- 20,000	0.1 -1,000	10,000-100,000	0.4 - 5,000
Mn	1,000- 10,000	0.1 - 400	5,000- 50,000	0.4 - 2,000
Ni	2,000- 25,000	0.5 -4,000	10,000-125,000	2.0 -20,000
Pb	4,000- 40,000	0.1 -2,000	20,000-200,000	0.3 -10,000
V	5,000-100,000	1.0 -5,000	25,000-500,000	5.0 -25,000
Zn	200- 3,000	0.002- 200	1,000- 15,000	0.01- 1,000

^a 1 g sample dissolved in final volume of 5 ml.

Injection volumes of from 5 mcl to 100 mcl were employed as necessary. The optimum conditions for analysis utilizing the heated graphite atomizer have been determined (Table I). For each metal, an ashing step was employed during which the furnace was heated to a temperature just insufficient to atomize any of the analysis element. For most samples, this step was unnecessary. However, the step was always utilized as a precaution to remove any volatile material and thus minimize the scattering signal during the analysis atomization. When using these conditions combined with the use of the deuterium arc background corrector, no interferences have been observed for a considerable range of samples. Chemical interferences were also absent as determined by the analysis of standard solutions of each analysis element in the presence of large excesses (5,000 fold) of each of the other elements determined.

Adopted procedure

The freeze-dried tissue samples were ashed as previously described.⁴ Samples of ca. 1 g dry weight in 100-ml fused silica conical flasks were used.

The solution of the ashed tissue sample was diluted to 100 ml, transferred to a 250-ml Teflon separatory funnel, 1 ml of glacial acetic acid was added and the pH was adjusted to ca. 3.0 by cautious addition of ammonia solution. A 10% aqueous solution of ammonium pyrrolidine dithiocarbamate (8 ml) was added. After mixing thoroughly, the pH of the solution was adjusted to 3.0–3.5 and the transition metals were extracted immediately into 15 ml of methylisobutyl ketone. After the two layers had separated, the organic phase was transferred quantitatively to a 100-ml fused silica conical flask. The aqueous solution was re-extracted with another 10 ml of methylisobutyl ketone and the organic phase was combined with that from the first extract. The silica flask was heated on a hotplate at ca. 100°C to evaporate the ketone. When the ketone had been completely evaporated, the resultant organic matrix was ashed with 5 ml of concentrated HNO₃. The solution was allowed to evaporate almost to dryness and 0.1 N nitric acid was added to make the volume up to about 3 ml. The solution was transferred quantitatively to a 5-ml graduated flask, made up to volume with 0.1 N nitric acid and transferred to a 10-ml polyethylene bottle for storage prior to atomic absorption analysis.

Analysis was carried out either by flame atomization of this solution or dilutions of it, or by flameless atom reservoir atomic absorption spectrophotometry utilizing injection volumes of 5–100 mcl and at least three replicate injections (see Tables I and III).

The atomic absorption spectrophotometer was calibrated with aqueous standards prepared from ultra-pure chemicals (Alfa Inorganic minimum 99% pure). Corresponding blank determinations and standards carried throughout the ashing, extraction and analysis procedure were utilized. The precision of the method depends upon the concentration of the element to be determined but in no instance was it worse than $\pm 10\%$ (standard deviation 3 replicates) except when samples had concentrations very close to the detection limits (signal/noise ratio of less than 10/1).

RESULTS AND DISCUSSION

The technique described above has been applied to the analysis of many samples of marine grasses, algae and sponges from a sub-tropical estuary. These samples represent a considerable spectrum of different tissue types particularly with regard to the degree of calcification. The results of these

analyses will be reported elsewhere. However, examples of the results obtained are reproduced in Table IV. In addition, samples of NBS standard orchard leaves (SRM-1571) have been analyzed for some elements (Table V). It may be noted that this standard reference material contains a fraction considerably more resistant to oxidation than we have found in marine biota samples

TABLE IV
Selected analyses of marine biota^a (mcg/g dry weight)

	Ag	Cd	Co	Cu	Fe	Mn	Ni	Pb	V	Zn
Marine grass										
<i>Thalassia</i>										
<i>testudinum</i>	0.15	0.6	0.94	3.72	64.4	81.4	6.6	1.65	6.15	23.2
Marine algae										
<i>Laurencia</i>										
<i>poitei</i>	—	0.5	0.13	3.70	175	50.3	1.5	0.86	4.94	15.4
<i>Penicillus</i>										
<i>capitatus</i>	—	0.4	0.05	0.86	81.1	33.4	0.6	1.18	7.03	8.57
Marine sponges										
<i>Chondrilla</i>										
<i>nucula</i>	0.03	2.0	0.87	15.1	215	9.67	8.5	0.87	13.5	50.0
<i>Haliclona</i>										
<i>molitba</i>	0.32	1.2	1.53	5.88	444	5.54	1.7	1.14	12.0	22.1

^a All results are based on the average of three or more sample injections.

TABLE V
Analysis of NBS standard orchard leaves SRM 1571

Element	Concentration determined ^a (mcg/g)	Certified concentration (mcg/g)
Fe	290	300 ± 20
Mn	87	91 ± 4
Pb	44	45 ± 3
Zn	24	25 ± 3
Cu	12	12 ± 1
Ni	1.4	1.3 ± 0.2
Cd	0.23 ± 0.02	0.11 ± 0.02

^a Average of at least four samples. Cd value is average of 10 samples.

from all but a very few species. It was, therefore, necessary in addition to the nitric acid to use a small quantity (1 ml) of perchloric acid to complete the oxidation, and hydrofluoric acid (2 ml) to dissolve the silica residue.

Analysis of these samples has shown the applicability of the current method to all biological samples and the method may be used to great effect when it is required to analyze a range of different samples. For analysis of samples of similar composition, more direct analytical procedures may be applicable, particularly when analysis for the less volatile elements only is desired. Development of such specific methods is currently being carried out particularly with reference to blood and urine samples.²⁶

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