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Novel Approaches to the Determination of Trace Elements by Atomic Absorption Spectrometry

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ABSTRACT

Two perceived limitations of conventional atomic spectrometry were addressed in these studies. One limitation is that the time required for sample preparation can exceed the actual analysis time by two or more orders of magnitude. High pressure homogenization in combination with high speed blending was evaluated for the preparation of slurries which could be directly analyzed by graphite furnace atomic absorption spectrometry (GF-AAS). Cadmium, copper and lead concentrations were successfully determined in certified reference materials (CRMs) of biological origin and frozen cervine liver and kidney. By capping the flat valve head of the homogenizer with a ruby disc, metal contamination introduced by the processing was reduced appreciably (but not eliminated) and the procedure was extended to the determination of chromium, iron, manganese and nickel in botanical CRMs and air dried animal feeds. The one problematic analyte element proved to be selenium which was consistently underestimated with this procedure. However, the combination of high pressure homogenization and partial enzymatic digestion with a crude protease alone or admixed with lipase or cellulase, released Se-residues from zoological and botanical CRMs so that Se could be accurately determined by slurry introduction GF-AAS. This technique was also applied successfully to freeze-dried, fresh and boiled fish tissues. The principal advantages of the slurry preparation technique are its speed, simplicity and lack of operator intervention.

The second limitation involved the loss of chemical speciation information during analysis. To preserve this speciation information, individual chemical species were separated chromatographically then detected in the column eluate using a novel all silica T-tube interface coupled with AAS. The advantages of the current interface design over previous prototypes were the compatibility with both organic or aqueous mobile phases and the low limits of detection (LODs) for Ag, Cd, Cu or Zn. The feasibility of the approach was demonstrated by monitoring levels of metal analyte bound to individual metallothionein isoforms which had been partially resolved by size exclusion or ion exchange HPLC. Whereas the metallothionein-I (MT-I) isoform was enriched in Cu relative to the MT-II isoform, the reverse was true for Zn. Also, a Ag-saturation procedure rapidly and efficiently replaced the bound Cd, Cu and Zn in these polypeptides.

RÉSUMÉ

Deux principales restrictions perçues par l'utilisation conventionelle de spectroscopie atomique sont abordées dans ces études. Une restriction est que le temps requis pour la préparation des échantillons destinés au dosage par spectrométrie atomique peut excéder le temps requis pour le dosage actuel par deux ordres de magnitude ou plus. L'homogénéisation à haute pression jumelée à un mélange à haute vitesse a été évaluée pour la préparation de suspensions homogènes analysées directement par spectrométrie d'absorption atomique (SAA). Des concentrations de cadmium, cuivre et plomb ont été déterminées avec succès sur des échantillons de références certifiées (ERC) d'origine biologique ainsi que de foie et rein de cervidés. L'application d'un disque de rubis à la surface plate de l'extrémité de la valve de l'homogénéisateur a réduit de façon appréciable la contamination métallique sans toutefois l'éliminer complètement. Cette même procédure a été utilisée avec succès pour la détermination de concentrations de chrome, fer, manganèse, nickel et de cuivre sur des ECR d'origine botanique ainsi que sur des moulées séchées à l'air. Le sélénium dans ces matrices s'est montré l'élément le plus difficile à estimer avec cette procédure. Cependant, une homogénéisation à haute pression combinée à une digestion enzymatique partielle par une protéase seule ou en combinaison avec une lipase ou cellulase a permis de déloger le sélénium des ECR d'origine zoologique et botanique et de l'analyser de façon précise par l'introduction de suspensions SAA-FG. Cette technique a d'ailleurs été appliquée avec succès sur différents tissus de poisson lyophilisés, frais et bouillis. Les principaux avantages de la préparation de suspensions homogènes sont la vitesse, simplicité et la faible nécessité d'intervention de l'opérateur.

La seconde restriction implique la perte d'information chimique sur la spéciation durant l'analyse. Pour préserver cette information, les espèces chimiques furent séparées par chromatographie (CLHP) et par conséquent furent détectées dans l'éluat en utilisant une nouvelle interface de silice (en forme de T) jumelée au SAA. Les avantages de cette interface par rapport aux prototypes précédents sont d'une part la compatibilité avec les phases organiques ou aqueuses et d'autre part les basses limites de détection (LOD) du Ag, Cd, Cu, ou Zn. La possibilité de cette approche a été démontrée en contrôlant les niveaux des métaux liés aux isoformes individuelles de métallothionein partiellement résolues par échange d'ion CLHP. Alors que l'isoforme de la métallothionein-I (MT-I) a été enrichie en cuivre relativement à l'isoforme MT-II, le contraire s'applique pour le zinc. Finalement, une procédure de saturation à l'argent s'est avérée rapide et efficace pour remplacer le Cd, Cu, et Zn liés dans ces polypeptides.

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Chapter 1. Introduction

As a general approach to trace element determinations, atomic spectrometry has been widely used for some years. Yet improvements in procedures and techniques continue to evolve at a rapid rate. In the area of sensitivity, mass spectrometry is now able to determine elements at the sub part-per-billion level in solutions. Today, inductively coupled plasma (ICP) optical emission spectrometry is able to measure 20 - 60 elements simultaneously in less than one minute. However, such successes in the field of atomic spectrometry have brought with them new challenges. Two challenges in particular are attracting the research community's attention for they address major problems/weaknesses with atomic spectrometry. Whereas, the methodologies for the actual measurement and quantitation steps have evolved rapidly the same cannot be said for the sample preparation stage of the analysis which has remained, with few exceptions, virtually unchanged during the last few decades. The time required for sample preparation exceeds the actual measurement by two to three orders of magnitude. The problem is that atomic spectrometry techniques require that the analyte be presented to the instrument as isolated atoms (or as ions in the case of MS). It is also the case that the analyte typically must not interact appreciably with other components of the sample. The second problem is that conventional atomic spectrometry, while it is capable of determining the total content of analyte trace element within a sample, provides virtually no information on the form(s) of that element. The studies described in this thesis have attempted to address facets of both of these shortcomings.

1.1. Sample Introduction in Trace Element Analysis

Sample preparation and introduction procedures that avoid classical digestion methods prior to atomic spectrometry continue to attract considerable interest since they are considered to be one of the major limitations of analytical methods based on flame, furnace, or plasma atomization. With great sensitivity and multielement analytical capabilities, many of these spectrometers are also capable of fully automated sampling, data acquisition, calibration, data reduction, storage, and report form generation. But,

these analytical advantages are frequently offset by the tedious, error-prone and time consuming dry-ashing (fusion) or wet dissolution steps which are required for preparation of solid samples for most, ICP, MS, flame and furnace analyzers. In general, the time required for sample preparation can exceed the actual instrument time by two or more orders of magnitude. Moreover, the more labor intensive the sample pre-treatment, the more prone to errors the analysis becomes.

Samples are conventionally introduced to these spectrometers as small volumes of liquids that have been prepared by solubilizing a homogeneous sub-sample of the bulk material within a suitable liquid carrier. Two common approaches to sample preparation involve either wet- or dry-ashing (fusion) with the objective of removing the organic portion of the matrix, by volatilization and/or oxidation, from the sub-sample. To be in an acceptable liquid solution form typically requires that $\leq$ 2g sub-samples of extremely heterogeneous solid sample be refluxed with hot nitric acid for anywhere from a few hours up to one day. Perchloric acid has been used to accelerate the dissolution process for some solids, but this reagent has proven to be especially hazardous for high fat materials. Alternatively for plant materials, complete dissolution may require the use of hydrofluoric acid (McCurdy, *et al.*, 1990; Bendicho and de Loos-Vollebregt, 1991).

Microwaves are capable of accelerating the wet digestion process for individual samples of approximately 1 g or less. However, most commercially available microwave-based digestion systems have limited "parallel" throughput rates in terms of the numbers of samples which can be processed concurrently and are known to be intolerant to perchloric acid on a routine basis. On-line microwave-assisted digestion presents its own problems: how to control the build-up of pressure when slurry samples are digested with acid in an flow injection (FI) system.

In addition to the tedium, safety hazards, and expense (labor costs and the expense of high purity reagents) of wet-ashing and fusion methods, there are several possible sources of inaccuracy: contamination by reagents, analyte volatilization losses due to overheating (e.g., selenium and mercury), loss by unexpected precipitation or co-precipitation reactions (e.g., involving silicates or sulfates), or loss by retention on container surfaces. Inaccuracy can be minimized if skill, knowledge, and careful attention

to detail are employed when controlling the course of fusion and wet-ashing. This can be achieved by carefully optimized, element-specific wet-ashing and storage chemistries and specially selected storage containers which are required to stabilize the target analyte in solution. However, for simultaneous multi-element analysis, there is not any single truly error-free, multi-element wet-ashing procedure. Nor is there a unique solution storage container which is optimal for all elements of interest. Considering all of the possibilities for error, the time and expense involved, it is not surprising that sample preparation is generally considered to be the weakest link in the chain of steps for the analysis of heterogeneous solid materials by flame, furnace, or plasma atomization methods (Sneddon, 1990).

The use of graphite furnace atomic absorption spectrometry (GF-AAS) for the direct analysis of solids (Langmyhr, 1979; Carnrick, *et al.*, 1986) or slurries (Hoenig, *et al.*, 1986 and Olayinka, *et al.*, 1986) presents several advantages over conventional wet oxidation and dry ashing sample preparation procedures. The simplification of the general analytical procedure has been achieved by the use of direct analysis of solid samples without any chemical pretreatment of the sample. Several reviews have addressed the solid sampling technique from a general point of view (L'vov, 1976; Langmyhr and Wibetoe, 1985). Specific features of solid sampling have been noted throughout the literature. The advantages (Bendicho and de Loos-Vollebregt, 1991) of using solid sampling can be summarized as follows:

i. reduced sample pre-treatment and hence, an increase in the speed of the overall analytical procedure;
ii. decreased risk of contamination, an essential requirement when trace levels of metals are to be determined;
iii. fewer possibilities of analyte losses during the sample pre-treatment or retention by insoluble residues;
iv. the use of corrosive and hazardous chemicals is avoided

Several manufacturers of electrothermal atomizers have developed atomizers designed specifically for the direct analysis of solid samples. These designs include tube furnaces,

platforms, cups and probes. However, there are several limitations which have prevented this method from becoming more widely accepted. Researchers have repeatedly reported (Miller-Ihli, 1988) the following problems:

i. high background levels;
ii. difficulties with calibration;
iii. problems with micro-weighting and sample introduction into the atomization device;
iv. difficulties with less volatile analyte elements;
v. necessity for matrix-matched standards and/or matrix modifier;
vi. analyte atomization efficiencies which are particle size dependent;
vii. build-up of residual carbonaceous materials within the atomizer;
viii. sample-dependent peak shapes;
ix. sampling errors related to inhomogeneity;
x. degraded precision relative to conventional sample preparation/introduction.

The introduction of a suspension of the finely powdered sample (slurry) combines both the advantages of liquid and solid sampling (Stephen, *et al.*, 1985). This technique was first used by Brady and co-workers (1974) to determine Zn and Pb in leaves and marine sediments. This procedure overcomes some of the problems associated with direct solid sampling as it permits sample introduction using micropipettes and autosamplers, which are used routinely in conventional liquid sampling. Another remarkable feature of slurry introduction is that the same atomizers that are used for liquids can be used for the atomization of slurries. Wall atomization and platform atomization with tube-type atomizers have been the most frequently reported devices for slurry sampling. The most critical factor in the slurry technique is the maintenance of the slurry's homogeneity while the sample is introduced. Slurry atomization retains all of the best features of flame, furnace, and plasma analyses while minimizing the time, effort, cost and safety hazards involved with the preparation of heterogeneous solid samples. Moreover, in terms of homogenizing effect, slurry atomization offers an excellent chance of evolving into a

procedure that assures the delivery of an accurate representative sub-sample of heterogeneous solid material for direct flame, furnace, and plasma atomization analysis.

1.1.1. Methods for Slurry Preparation

A slurry is prepared by adding a liquid diluent to a solid material that has been ground to a small and preferably uniform particle size, possibly sieved and weighed. The quantity of solid material that is weighed depends on the anticipated concentration of the analyte and on the dilution in the final volume of the slurry. For somewhat heterogeneous samples, preparing a more concentrated slurry can improve the precision.

It is necessary to assure that the slurry is homogeneous and stable (at least over the course of the sampling) in order to have a representative aliquot transferred/injected into the flame or furnace. Stability of the slurry can be improved with thixotropic agents such as Viscalex (polyoxyethylene) or glycerol. These additives serve to increase the density of the liquid phase thus decreasing the settling times of the denser solid particles. Also a small particle size has proven to be the most critical factor in slurry stability. For flames and plasmas, a median slurry particle diameter of ≤ 8 μm is acceptable for efficient aerosol transport if spray chamber designs are optimized for slurries. For the more commonly employed unoptimized spray chambers, the median particle diameter is suggested to be $\leq$ 2 μm (Bendicha and de Loos-Vollebregt, 1991). The slurry particle size restrictions for graphite furnace atomic absorption applications are not as severe as for nebulizer systems. In GF-AAS applications, median particle diameters on the order of 20 -30 μm are acceptable.

Holcombe and Majidi (1989) studied the volumetric errors for slurries sampling. Errors unique to the volumetric sampling of slurries can be attributed to a number of sources that may not be present when removing sample aliquots from homogeneous solutions. One such error results from the settling of particles in the solution containing the suspension. Assuming that complete and uniform particle dispersion exists when the solution is being mechanically agitated, the time between stopping the agitation and actual removal of a sample aliquot can determine the repeatability of the mass sampled. In most

instances, particles are not normally buoyant in the suspending media and will eventually either float to the surface or sink to the bottom of the container and no sample will be found in the bulk of the solution. The errors associated with slurry sampling are random in character and originate from the finite number of particles that are withdrawn, variation in the mass of the particles collected in the sampling volume and any variation in relative analyte concentration from particle to particle.

Magnetic Agitation and Vortex Mixing

In earlier work, magnetic agitation and vortex mixing were used widely with the slurry technique. Typically, the slurry was prepared in a beaker and it was stirred (usually for 3-5 min.) in order to achieve a uniform suspension of the solid material in the liquid carrier. As soon as the stirring action was stopped, a sample aliquot was withdrawn and transferred to the atomizer (Slovak and Docekal, 1981). It was also possible to withdraw the sample aliquot while the suspension was continuously stirred (Jackson and Newman, 1983).

The effectiveness of magnetic stirring and vortex mixing depends on the sedimentation rate of the suspended material. Hinds *et al.* (1985) reported that differential sedimentation of samples contributed to an incomplete recovery of Pb. The error associated with sedimentation was a function of both particle size and the presence of particles having different compositions. As a result, large errors can be expected when the analyte is predominantly distributed in particles of high density, which undergo rapid sedimentation. After magnetic agitation or vortex mixing, slurries are often introduced into the atomizer with a micropipette because of the difficulties of incorporating these suspensions into an autosampler. Lynch and Littlejohn (1989) described a miniature magnetic stirring device that could be used in combination with an autosampler. Slurries were homogenized in the autosampler cups using small magnetic bars coated with polytetrafluoroethylene (PTFE). The system performed well for the determination of Pb in freeze-dried samples.

Ultrasonic Agitation

Ultrasonic agitation has also been used as an effective means of homogenizing suspensions prior to GF-AAS. Ultrasonic agitation has been used in combination with both manual and automated introduction of the slurry. An advantage of this approach relative to magnetic agitation and vortex mixing is that the analyte of interest can be partly extracted into the liquid phase, owing to the ultrasonic action, when the slurry is prepared in an acidic medium. Generally, ultrasonic agitation is more effective than other agitation techniques. This is important when the analyte is predominantly located in the larger sample particles that undergo rapid sedimentation. Miller-Ihli (1988) reported improved recoveries for the determination of Fe in wheat flour and bovine liver certified reference materials (CRMs) when the sample was sonicated. In the next year, Miller-Ihli (1989a) automated the system, combining ultrasonic agitation and sample introduction with the autosampler. A similar design has been reported by Carnrick *et al.* (1989) and is commercially available from Perkin-Elmer Corporation. Automated slurry introduction in combination with ultrasonic agitation has been used by Epstein *et al.* (1989) to study the sources of variability in measurements. They concluded that precision depended on both the extractive yield of the analyte and the homogeneity of the sample. Precision of 2-10% was obtained by Bradshaw and Slavin (1989) when determining Se, Pb and Tl in coal and coal ash. The time required for each determination was less than 1 min. using ultrasonic agitation of the slurry and the elimination of the pyrolysis step of the furnace program. (Jordan, *et al.*, 1989).

Gas Mixing

Bendicho and de Loos-Vollebregt (1990a.) described a procedure in which efficient homogenization of the slurry was achieved by passing an Argon stream through a narrow capillary tube introduced into the slurry medium. This procedure permitted slurry formation directly in the sample cups. The homogenization procedure required a "bubbling time" with the Ar stream. The precision obtained for the autoinjector for the determination of Cu, Cr, Mn, Fe and Ni in different samples of glass was about 6%. This procedure was easy to accomplish and did not require the use of stabilizing agents or

special devices for agitation. However, the effectiveness of mixing depended largely on both the particle size and the characteristics of the solid sample.

Pre-digestion of Slurries

Pre-treatment of the slurry can increase the efficiency of extraction of the target analyte into the liquid phase. In contrast to the extensive sample pre-treatment inherent in conventional fusion and wet digestion procedures, the pre-digestion of a slurry only requires a partial decomposition of sample, hence it is less time-consuming. Fagioli and co-workers (1986) were the first to use partial wet oxidation of several zoological and vegetable materials with concentrated sulfuric acid, followed by direct sampling of the resulting carbonaceous slurry. As a sample preparation technique, the procedure was much faster and more reliable than procedures based on dry ashing or extensive wet oxidation. Slurry pre-digestion can even be accomplished in the sample cups of an autosampler. For the analysis of sediments, Hoenig *et al.* (1989) reported difficulties in stabilizing slurries containing large particles. To solve these problems, pre-digestion was performed by adding a small amount of concentrated nitric acid to the sampling cups containing the slurry. Partial extraction of the analytes into the liquid supernatant fraction of the slurry was observed. The pre-digestion step significantly improved both the precision and accuracy of the results. A rapid pre-digestion procedure has been described by Bendicho and de Loos-Vollebregt (1990b) for the determination of several metals in glass. Pre-digestion of the slurry was effected by adding diluted hydrofluoric acid (3% v/v) followed by gas mixing during sampling (Bendicho and de Loos-Vollebregt, 1990a). The pre-digestion step was accomplished in the sample cups of an autosampler, 3 min. being sufficient to achieve an efficient extraction of the analytes from the powdered glass (15 μm particle diameter). This approach provided a 2-fold improvement in precision for the determination of Cu, Co, Cr, Mn, Fe and Ni, in comparison with analyses performed without pre-digestion. Cold extraction using dilute hydrofluoric acid has been useful for those materials in which the analyte is predominantly contained within the siliceous fraction (Bendicho and de Loos-Vollebregt, 1991).

1.1.2. Improvements in Slurry Stability

Slurry preparation in an aqueous carrier is rarely suitable because most powdered materials undergo rapid sedimentation. Sedimentation of the suspended material usually occurs after mixing the slurry. The sedimentation rate depends on the densities of the diluent and solid materials, the viscosity of the diluent medium and the diameter of the sample particles.

The slurry can be stabilized using a highly viscous liquid medium such as Viscalex HV30 (Stephen, *et al.*, 1985; Fuller and Thompson, 1977) or glycerol (Hoenig and Van Hoeyweghen, 1986; Miller-Ihli, 1988). In addition, non-ionic surfactants, and organic solvents of high viscosity have been used as slurry stabilizing agents. The stabilization capability of these reagents largely depends on sample characteristics and particle size. As pointed out by Majidi and Holcombe (1990), the time interval between complete mixing of the slurry and the removal of an aliquot for analysis can be increased when a highly viscous medium, with a density similar to that of the particles is sampled. Fuller and Thompson (1977) used a thixotropic thickening agent to stabilize slurries prepared from rock samples. The procedure consisted of adding sodium hexametaphosphate as the wetting agent and ammonia to neutralize the medium. The slurry was stable for several days.

Stabilizing agents can also be useful when an autosampler is used to introduce the sample into the atomizer, as the slurry can be left in the sampling cups without further homogenization (Hoenig and Van Hoeyweghen, 1986). Littlejohn *et al* (1985), observed that the stabilization of the slurry was dependent on both the particle size and the concentration of the stabilizing agent. The rate of solid particle deposition was retarded owing to the high viscosity of the medium, when the Viscalex concentration was greater than 2-3%. On the other hand, the maximum slurry concentration to be used depends on the concentration of the stabilizing agent. Stephen *et al.* (1985) found that the maximum slurry concentration was 10% when a stabilizing agent concentration of the order of 3% was used for slurries prepared from a spinach sample (50 μm particle size). Similar behavior was observed when glycerol was used as the stabilizing agent.

Problems have been reported with the use of viscous media for slurry preparations. The sample aliquot can be inefficiently pipetted into the atomizer when the concentration of the stabilizing agent is high. The sample can remain around the dosing hole, which can degrade the precision. This problem has been observed using micropipettes (Fuller and Thompson, 1977) and autosamplers (Stephen, *et al.*, 1985). The use of an autosampler also requires a cleaning procedure for the capillary between each successive determination (Stephen, *et al.*, 1985; Miller-Ihli, 1988). It is also necessary to have an additional pyrolysis step in order to remove the excess of stabilizing agent (Fuller and Thompson, 1977; Hoenig and Van Hoeyweghen, 1986). Stabilizing agents are usually inefficient when the slurry contains particles of high density (Bendicho and de Loos-Vollebregt, 1990b).

It has been reported that glycerol causes interference(s) for Cd and Pb determinations, even under isothermal atomization conditions (Bendicho and de Loos-Vollebregt, 1991). Owing to carbon build-up, formed by pyrolysing the glycerol, both Cd and Pb absorption signals were shifted to lower temperatures, which caused non-isothermal atomization. These problems can be minimized by cooling the slurry to 5° C, and using the smallest possible amount of glycerol.

Apart from stabilizing agents, the addition of wetting and antifoaming compounds can improve the dispersion of the slurry. The application of wetting and dispersing additives was discussed by Tsalev *et al.* (1990) in an extensive review of chemical modification in GF-AAS. In slurry analysis, Triton X-100 is useful for dispersing solid particles that might otherwise tend to float on the top of the liquid (Priesner, *et al.*, 1981; Miller-Ihli, 1988).

1.1.3. Slurry Nebulization

1.1.3.1. Influence of Particle Size

The particle size of the solid material used to make a slurry can influence the stability, deposition and atomization efficiency of the slurry. These in turn can influence both accuracy and precision. Fuller *et al.* (1981) reported that the precision achieved for slurry sample introduction was largely influenced by particle size, although this was not a critical factor in achieving effective atomization. Relative atomization efficiencies

approaching 100% were obtained for several metals in titanium ores and silicate rocks when the diameter of the solid particles was less than 25μm. Under those conditions, calibration could be carried out using aqueous standards. The decreased influence of particle size on atomization is an advantage of GF-AAS relative to other atomic spectrometry techniques (flame-AAS and ICP-OES) when used in combination with slurry sampling. Several researchers have reported incomplete recoveries of lead from soil. Owing to the slower vaporization of the larger particles, Pb was inefficiently atomized within the observation time. Pipetting efficiency was only 80% when the mean particle diameter was greater than 50μm. To obtain a higher precision among replicate determinations, Karwowska and Jackson (1987) used solid samples with particle sizes of less than 20μm for their atomization studies of Pb from a synthetic soil matrix. In contrast, Miller-Ihli (1988) reported that better precision was achieved for particles in the range 250-600μm, than for small particles. Stephen et al. (1987) observed an improved precision for the analysis of liver tissue if the finest fraction of the sample (particle diameter less than 21μm) was used.

It is generally agreed that smaller particles facilitate sample preparation and improve recoveries. Errors associated with large particles (diameter >100μm) result mainly from difficulties maintaining a homogenous distribution of particles in suspension and from the lower pipetting efficiencies associated with larger sized particles. To minimize these errors grinding of the original sample is usually required. The optimum range for particle size depends on the sample composition.

1.1.3.2. Influence of Slurry Concentration

Another important factor for the successful application of the slurry introduction technique is the slurry concentration. Samples of high analyte content can be analyzed more readily by the slurry technique than by direct solid sampling because the slurry can be easily diluted. The dilution of the slurry, however, can only be carried out within a limited range. A degradation in precision occurs when working with highly diluted slurries because only a small number of particles are actually transferred to the instrument per

increment of sample. If the analyte content of the original sample is very low, the concentration of solid in the slurry preparation must be increased accordingly, although pipetting efficiency can deteriorate if slurries are too concentrated. Another factor to be considered is the increase in the severity of the matrix effects that can arise when the slurry concentration is increased. Lynch and Littlejohn (1989) established that the variations among replicated determinations for an optimum slurry was less than 4% when working with particles of less than 50μm diameter, provided that the concentration of solids in the slurry was less than 10% (m/v). The accuracy deteriorated somewhat when the slurry concentration was above 5%, as a result of the excess of matrix. Slurry concentrations greater than 5% caused inefficient deposition of the slurry aliquot.

Holcombe and Majidi (1989) have characterized the errors associated with slurry sampling. According to these authors, errors can arise from uncertainties in the sample volume, the number of particles in the sample volume and from variations in the mass of the individual particles. Errors can be minimized by working with small particles, concentrated slurries and a narrow distributions of particle sizes.

1.1.3.3. Comparison of Nebulization Techniques

Flame and electrothermal atomic spectrometric methods of analysis have achieved considerable popularity over the last few decades. However, a disadvantage of conventional approaches is that the vast majority of the current methods require that the sample to be in a solution. Thus, for refractory materials, the potential speed of these techniques is compromized by complex, and often time-consuming dissolution procedures. Several researchers (Willis, 1975; Harrison and Juliano, 1971; Kashiki and Oshima, 1970) have demonstrated that an alternative approach is feasible. Samples that had been ground to a fine particle size and suspended with agitation, in an aqueous or organic medium, resulted in slurries which could be injected directly into conventional flame. Willis (1975) outlined the limitations of this slurry preparation that could be aspirated into a flame and noted that the samples had to be ground to a mean particle size of less than 10 μm. Moreover, the slurries required constant agitation in order to achieve a homogenous suspension.

Others (Fuller and Thompson, 1977; Fuller, et al., 1981) have developed methods for overcoming these limitations. These studies demonstrated that the use of electrothermal atomization, which can tolerate larger particles, in concert with a thixotropic agent, to stabilize the resulting suspensions, was preferable to methods using aqueous suspensions and flame atomization.

Willis (1975) compared the atomization efficiency, E, obtained from a slurry with that obtained from an aqueous solution containing the same concentration of the analyte element. The ratio E, where E = signal from slurry/signal from aqueous solution, was used to quantify the effects.

Flame atomization

Conventional flame nebulizers are prone to blocking when slurries are nebulised continuously. Pulsed nebulization, with 100 μL aliquots, has been used to circumvent this problem (Fuller, 1976). During initial experiments, significantly higher absorbance readings were obtained for slurries, with only a slight worsening of precision, when the flow-spoiler in the spray chamber was removed.

The effects of variations in particle size were determined for chromium and vanadium in samples of illuminate and rutile using a nitrous oxide-acetylene flame as the atomizer. High values for E (0.3-0.5) were obtained for slurries containing particles with mean diameters less than 10μm. When the mean particle size was increased above 25 μm values of E fell to below 0.1. This effect can be attributed, in part, to sample transport effects. In general, for the nebulizer/spray chamber designs used in commercial atomic-absorption spectrometers, only droplets with diameters less than 10 μm are efficiently transported to the flame. Therefore, the larger, solid particles and agglomerates from slurries will be preferentially rejected as they pass through the spray chamber. The poor analytical response with slurries can also be explained, in part, by the incomplete atomization of the particles when they reach the flame. Therefore, the flame composition and flame temperature would be expected to have an appreciable effect on atomization efficiencies. For aqueous solutions, manganese would normally be determined with an air-acetylene flame. However, it has been observed that the hotter nitrous oxide-acetylene

flame significantly increased the values of E, even for slurries with a small mean particle size (Novak, Jr. and Browner, 1980).

Inductively coupled plasma atomization

It might be expected that the hotter thermal environment of a plasma would result in enhanced signals relative to signals generated by flames. However, the inability of the available concentric and cross-flow nebulizers to cope with slurries resulted in a uniform lack of success in initial attempts to use an ICP source, even when using pulsed nebulization with discrete samples of 50-100 µL.

To overcome this problem, a cross-flow slot type nebulizer, similar to the design described by Wolcott and Sobel (1987), was tested with the result that slurries exceeding 10% m/V could be analyzed. This nebulizer enabled a study of particle-size effects to be carried out in a manner similar to experiments for the other atomization techniques. The atomization efficiency, for samples of small particle size, was significantly higher than that observed using the nitrous oxide-acetylene flame. However, the predominant limitation, as was the case with the flame, was still one of sample transport efficiency.

Electrothermal atomization

Electrothermal atomization was studied with 20 µL aliquots of the slurry. In this instance, the values of E were not characterized by the same decreases with increasing mean particle size that had been observed with flame and ICP atomization. Instead, for the range of particle sizes studied, there is a wide scatter in the values of E about unity. The error in volume involved in pipetting 20 µL sample aliquots of the slurry was negligible compared with the overall variations among replicate analyses (Fuller and Thompson, 1977). It was concluded that the relative standard deviation of the analytical measurement was affected principally by the particle size. The theoretical numbers of particles were calculated by assuming spherical particles with as average diameter (for the range covered) and a density of 5g/ml. The actual numbers of particles were determined by transferring ten consecutive 20µL aliquots to a microscopy slide, counting the numbers of particles in each instance and calculating the mean and relative standard deviations. The

results observed in the experiments for the number of particles agreed well with the calculated results, and demonstrated that as the particle size decreased, the number of particles in a sample aliquot increases dramatically. These results are also in general agreement with results reported by Langmyhr (1979) for the determination of mercury (II) by electrothermal atomization in an iron (II) alloy containing chromium and lead. Therefore, the principal prerequisite for successful slurry introduction-electrothermal atomization methods is that the sampling error must be minimized in order to achieve optimal analytical precision.

1.1.4. **Chemical Modification**

Slurry sampling in GF-AAS permits the use of chemical modifiers. The interaction between the chemical modifier and the solid sample particles is greater than for direct solid sampling. Most of the work on chemical modification for the slurry technique has been carried out in order to stabilize highly volatile elements such as Pb and Cd (Hoenig and Van Hoeyweghen, 1986; Ebdon and Lechotycki, 1987; Hinds and Jackson, 1988; Olayinka, *et al.*, 1986).

Karwowska and Jackson (1987) studied the release mechanism of Pb from alumna particles as a function of the location of analyte in the particles. A mixture of $(NH_4)_2HPO_4$ and $Mg(NO_3)_2$ was recommended as chemical modifiers, and successful calibration with aqueous standards was reported. They presumed that in soil samples, Pb is adsorbed on to the particles through covalent bonding with O and OH. Marecek and Synek (1990) studied the effect of the Ga occluded within or adsorbed to the surface of alumna samples. An increase in the signal peak height was attributed to the delay in the atomization caused by the alumna particles. Hinds and Jackson (1988) used a mixture of montmorillonite and humic acid to simulate the behavior of a soil matrix. A chemical modification study was also carried out for Pb. Among the modifiers studied, the best performance was obtained with a mixture of magnesium and palladium nitrates. This chemical modifier leads to similar appearance times for the atomic absorption signal of slurries and aqueous standards. Palladium was efficiently reduced by the organic matter present in the soil (Hinds, *et al.*, 1988). For the determination of Pb in soil, other procedures which

incorporated chemical modification such as oxygen ashing, pre-slurry ashing in a muffle furnace or the addition of nitric acid were less suitable. The characteristics of the resulting Pb peaks differed appreciably from those obtained with aqueous standards (Hinds and Jackson, 1988).

Hoenig and Van Hoeyweghen (1986) used a mixture of $(NH_4)_2HPO_4$, $Mg(NO_3)_2$ and HNO_3 as chemical modifiers for the determination of Pb and Cd in plant tissue, sediment and lyophilized animal material. Diammonium hydrogen phosphate has also been used for the determination of Cd in olive leaves and mussels (Ebdon and Lechotycki, 1987).

For As, $Ni(NO_3)_2$ and a mixture of $Ni(NO_3)_2$, $Mg(NO_3)_2$ and HNO_3 (Ebdon and Pearce, 1982) have been used. $Ni(NO_3)_2$ has also been used as a modifier for Se in selenized yeast (Thompson and Allen, 1981). Ebdon and Parry (1988) reported that $Ni(NO_3)_2$ performed better than either $Cu(NO_3)_2$ or $Pd(NO_3)_2$ as a chemical modifier for the determination of Se in coal.

Fine graphite powder can be used for chemical modification in the slurry technique. Carbon black has been used as a chemical modifier for the determination of Be and Mo in beryllium oxide and molybdenum disulfide (Bendicho and de Loos-Vollebregt, 1991), respectively. The addition of carbon black prevented the interference of silica and resulted in approximately a four-fold signal enhancement (390%). Likewise, this modifier enhanced Al and Fe absorption signals by a factor of 1.5 when determined in aluminum oxide and iron oxide, respectively. Graphite powder has been added to slurries consisting of simulated waste for the determination of Mo, Ru, Rh, and Pd (Schmiedel, *et al.*, 1989).

The possibility of eliminating both the chemical modifier and the pyrolysis step in slurry sampling has also been studied. This approach requires the use of isothermal conditions for atomization and Zeemen background correction in order to minimize matrix interferences. Bradshaw and Slavin (1989) reported the rapid determination of As, Pb, Tl and Se in coal and coal fly ash matrices by automated ultrasonic agitation and elimination of the pyrolysis step. The precision for replicates of slurries varied from 2 to 10%, depending upon the amount of solid sample delivered to the furnace.

Bendicho and de Loos-Vollebregt (1990a) have studied the influence of chemical modifiers such as magnesium nitrate, palladium nitrate and a mixture of both, in the atomization of Cu, Co, Cr, Mn, Ni and Fe in several glass materials. Scanning electron microscopy demonstrated that the pyrolysis step caused an increase in particle size of the slurry sub-sample on the platform and therefore, could be omitted. Chemical modifiers did not have any perceptable effect on recovery. Further research is required to establish the conditions under which rapid analysis can be carried out omitting both the pyrolysis step and chemical modifiers, yet without compromizing analytical performance.

1.1.5. Contamination

Although slurry methods do not usually employ large quantities of reagents, there is opportunity for wear contamination to occur during sample preparation (prior to suspension in a suitable carrier solvent). Metals can be introduced into the sample from the Polytron homogenizer, McCrone Micronising Mill, milling aids, or shaking beads used for particle size reduction. The Polytron homogenizer is normally composed of hardened stainless steel. For trace metal determinations, the Polytron homogenizer should be outfitted with a Teflon bushing instead of a brass one. Within the sensitivity limits of flame-AAS, Sneddon (1990) did not detect measurable wear contamination for Fe, Cr, and Ni, when the Teflon bushing was employed with the hardened stainless steel homogenizer head to process beef liver and oyster tissue certified reference materials. This absence of Fe, Cr, and Ni contamination was observed despite the homogenizer's stainless steel blades.

1.1.6. Calibration

Calibration in slurry analysis can be accomplished using the same approaches as for direct solids analysis. But, in contrast to direct solid sampling, the slurry technique often permits aqueous standards to be used for calibration. Quantification using aqueous standards has been carried out successfully in combination with different kind of atomization. Platform atomization and integrated absorbance quantification (Ebdon and Evans, 1987; Miller-Ihli, 1988), platform atomization and peak absorbance quantification

(Hoenig and Van Hoeyweghen, 1986), wall atomization and integrated absorbance quantification (Slovak and Docekal, 1981) and wall atomization and peak absorbance quantification (de Benzo, *et al.*, 1988) have all been reported. Integrated absorbance measurements have provided accurate results, even for wall atomization (Slovak and Docekal, 1981). The standard addition method or calibration using standard slurries were required for calibration when wall atomization was used (Carrion, *et al.*, 1988). Platform atomization does not always guarantee good accuracy when aqueous standards are used for calibration. The use of a chemical modifier was sometimes necessary in order to match the absorption signals produced by the analyte when in the aqueous standard with the signal for the same quantity of analyte in the slurry. (Hinds and Jackson, 1988).

The implementation of Zeeman background correction (Bradshaw and Slavin, 1989), Smith-Hieftje background correction (Ebdon and Parry, 1988) or wavelength modulation (Miller-Ihli, 1988) facilitates slurry analysis because they permit effective correction for the high background absorbances that can arise from the atomization of the matrix. The use of stabilized temperature platform furnace (STPF) conditions, as proposed by Slavin and co-workers (1983) has been recommended in order to eliminate spectral interferences. A variety of different sample matrices including soils and sediments have demonstrated that the STPF conditions can be successfully used for slurry atomization (Miller-Ihli, 1989b).

1.1.7. Applications

Flame and Inductively Coupled Plasma

The successful application of the slurry technique for practical analyses, using nebulizer-based systems, depends on satisfying two criteria. As the signal response is strongly dependent on particle size, some method of reproducibly grinding the sample to particle sizes of less than 10 μm (and preferably less than 5 μm) must be used. The second criteria is that the signal response varies with the sample matrix. Typically atomization of slurries produce signals which are only 30 - 50% of the signal produced by equivalent amount of analyte in an aqueous solution. Thus, standardization is very important. For accurate analysis, closely matched standards must be used. The standard-additions

procedure is frequently not applicable to slurry analysis as the elements in the sample and the added standards are present in different physical states which typically are characterized by different atomization efficiencies. However, methods of atomic emission spectroscopy based on excitation DC and IC plasma have been reported (Albers and Sacks, 1987). It would be expected that the hotter environment of the plasma would give rise to improved signal response for slurries compared with cooler air-acetylene and dinitrogen oxide-acetylene flames. But the same predominant effect of sample transport efficiency, as with the flames, has been found with these plasma excitation methods. (de Benzo, et al., 1991).

Electrothermal AAS

With electrothermal atomization, the problem of standardization is not as critical as with the nebulizer-based systems. As the signal response is within $\pm$ 20% of the aqueous response it would be feasible for practical analyses to employ aqueous standards provided that the samples have been ground to a particle size of less than 25μm.

Slurry sampling has been used widely for analytical determinations, and applied to many different matrices including biological materials, environmental samples, foodstuffs, glasses, plant tissues and sediments. The slurry procedure is rapid and reproducible. It has acceptable accuracy and is applicable to flame, electrothermal and ICP methods of atomization. For nebulizer-based systems, the variation in signal response with particle size and matrix means that, for practical analyses, closely matched standards must be used. However, with electrothermal atomization it is possible to use aqueous standards and obtain acceptable results, as there is a greater tolerance to particle size differences. This technique is, therefore, of use in situations: (i) that do not require the highest accuracy and precision; (ii) in which trends, rather than absolute results, are required; (iii) in which large numbers of samples are to be analyzed; and (iv) in which the samples can be ground to a fine powder.

Overall, the slurry introduction technique has several advantages over conventional sample preparation methods, including microwave digestion. The most important advantages are speed and simplicity, including reduced sample preparation time, decreased

analyte loss through volatilization prior to analysis, reduced analyte loss related to retention by an insoluble residue, reduced sample contamination, and elimination of hazards associated with the use of strong acids. Therefore, slurry methods have evolved rapidly as a promising approach to minimizing the sample preparation of heterogeneous solid materials for flame, furnace, and plasma atomization using either optical or mass spectrometric detection. The slurry technique, however, remains in an early stage of development. In order for widespread acceptance and routine use of slurry methods to occur, additional improvements are needed in terms of particle size reduction and improved particle vaporization for some materials, reduced individual slurry preparation time, development of rapid "parallel" methods of slurry preparation for ~30 samples at once, and especially the minimization of "wear-metal" contamination from homogenizer, mill, or bead methods of slurry preparation. The ultimate goal is the development of a rapid, accurate procedure which can handle large numbers of heterogeneous solid samples efficiently, which is free of contamination, and which can be calibrated with nothing more than aqueous standards.

1.2 Chromatographic Approaches to Trace Element Speciation

The second general problem associated with many methods for trace element determinations is the lack of information that they provide regarding the original physical and chemical forms of the analyte element within the sample. Typically, this "chemical speciation" information is often destroyed during the sample preparation stage of many methods. Metal speciation has become an important area of inquiry because of its importance to the understanding of the fate and effects of metals in the environment. It is a valid approach to determine the total concentration of metals in studies concerned with mass balance. However, studies that attempt to relate to analyte metal concentrations with biological effects require knowledge of the physical-chemical forms (i.e. species). At one time a predictor of potential environmental effects was considered to be that concentration of the metal(s) that was not removed by the 0.45 μm membrane filter. The problem is that pronounced biological effects, such as toxicity to aquatic organisms, are often caused by a few specific chemical species. The biological effect can be exacerbated by other metals and ligands in solution. The solution to this general problem requires a multi-variable approach. There is one popular approach to the determination of the different physical-chemical forms (chemical species) of an analyte element within a complex environmental or biological matrix. An automated chromatographic separation has been coupled with an on-line detection process which is both sensitive yet highly selective for the analyte. This approach requires that identities of the analytes not be compromized by either the isolation, separation or detection steps. Thus, for this approach to be practicable, analyte species must be 'non-labile' to the extent that they result in species-resolved responses from the detection system. Moreover, the analyte is diluted appreciably within the chromatographic mobile phase during the separation sequence. Indeed, the sensitivity of detection remains the principal limitation of many current methods.

1.2.1 Trace Element Speciation

The challenge of trace element speciation is that it is related to the concept of essential trace elements in human nutrition. Most trace elements are characterized by a surprisingly narrow range of optimal activity. For certain trace elements such as selenium

or fluorine, a 4-5 fold change in concentration (expressed as cumulative intake of the element/day for a 70 kg man) is sufficient to convert overt signs of deficiency to toxicity. A further change of less than an order of magnitude in either direction is sufficient to cause death. Although the quantities which characterize the limits of the separate regions are different for different elements, the relatively narrow window of optimal intake is characteristic of many trace elements. The term 'trace element' is used to denote those elements (other than carbon, hydrogen, oxygen, nitrogen, sulfur, and phosphorus) which typically are present in a natural biological/environmental matrix at sub-milligram per kilogram levels. Included are not only metallic elements but also nonmetals, which tend to exist as anionic species in an aqueous environment. Also, the term "trace element" avoids the complication of changes in the metallic/nonmetallic character with changing oxidation state. The component compounds containing the trace element analyte are typically present at trace or ultra-trace levels in a complicated biological/environmental matrix. They can be stable ions or molecules, labile or reactive species which are not at thermodynamic equilibrium with the rest of the sample, or even ions or molecules which are weakly or strongly associated with other components of the sample. However, the trace element component of a group of analytes can act as a marker or tag which can be exploited analytically using highly selective detection systems. These analytes may be small neutral volatile molecules, simple cationic or anionic species, more complex charged species or even biopolymers. They will be present at trace levels, in a biological matrix which may be anything from natural waters to biological tissues (Marshall, 1988).

The determination of the individual physicochemical forms represents a formidable challenge for the experimentalist. The information on the component chemical species can provide additional insight into the bioavailability of that element to a biological process or an intact organism. The ability to quantify traces of organic xenobiotics, of reactive intermediates and metabolites, or even enzymes and proteins have continued to evolve at a rapid pace. However, it is only more recently than inorganic and organometallic chemists have begun to make appreciable progress with the general problem of trace element speciation.

All methods for trace analysis typically involve a series of sequential steps in which the analytes are isolated from the sample matrix by extraction and concentration. Frequently, the extract is partially purified prior to an analytical separation step. Each analyte is then quantified with a sensitive detection system by a predetermined relationship between some physical property of the component and its chemical concentration in the original sample. Methods for analytes containing a trace element follow the same general steps. One successful approach to trace element speciation has been to couple an automated chromatographic separation of the analytes with a sensitive and highly selective means of detection.

1.2.2 Chromatographic Detectors for Trace Element Speciation

An ideal chromatographic detector for trace element speciation studies would possess the following desirable characteristics:

i. element specificity (with no chemical or sample matrix effects)
ii. high sensitivity (low to sub-ng limits of detection based on the sample injected into the chromatography)
iii. compatibility with a variety of mobile phases and associated flow rates
iv. direct on-line detection and determination of all elements of interest, such that there is the capability for 'n' simultaneous element specific chromatograms
v. must not appreciably degrade the chromatographic performance of the system
vi. ease of automation and operation

Atomic spectrometry (AS) and mass spectrometry (MS) are two of the most popular detection techniques that provide the requisite combination of selectivity and sensitivity to detect environmentally relevant levels of trace element containing analytes. Although both techniques were initially developed for discrete samples, there has been continued progress on the problems of coupling these detectors with flowing streams of chromatographic eluate. A series of related atomic spectrometric techniques which include atomic absorption, atomic emission, and atomic fluorescence spectrometry can be directly interfaced with flowing streams of chromatographic eluate.

1.2.2.1 Atomic Absorption Spectrometry

As a single element technique, atomic absorption spectrometry (Slavin, 1988) detects isolated analyte-free atoms in the gaseous phase. The limitation of detecting only one element at a time results from the use of hollow cathode lamps which were designed to provide spectral emissions for a single analyte element. The positive features of this technique include the relative freedom from spectral interferences and the low purchase and operating costs. Flame atomic absorption spectrometry employs a stationary flame front aligned with and positioned below the optical beam of the spectrometer to provide the requisite energy to atomize the trace element analyte. However, electrothermal atomization techniques appreciably improved the sensitivity (typically, by two to three orders of magnitude). Moreover, only a small volume of sample (typically 20 μL) is required. The sample is added to the interior of a hollow graphite tube, which is positioned within the optical beam of the spectrometer, and which is heated electrothermally in stages to volatilize the solvent, ash the matrix, and atomize the analyte. The major limitation of the GF-AAS technique are the many physical and chemical interferences have been reported extensively in the literature. Interferences can now be controlled with a combination of platform techniques (Falk, 1988), newer high-quality graphite materials, and accurate background correction techniques (Slavin and Carnick, 1988).

There has been continued progress in developing a multielement AAS instrument. One successful approach has used an echelle polychromator (spectrometer) and a zenon arc lamp to provide simultaneous detection of 16 elements and compatibility with either flame or furnace operation (Harnly and Garland, 1988). Commercial AAS instruments which focus the emissions from up to four hollow cathode lamps through a graphite tube and monitor the resulting signals with four separate photomultiplier tubes are also available (Retzik, 1988).

Relative to other atomic spectrometric techniques, AAS is characterized by rather narrower linear dynamic ranges which may complicate or even limit the use of the multielement AAS approach to speciation. This limitation can be minimized by resorting to an alternate calibration technique developed specifically for transient signals (Sperling, *et al.*, 1991).

1.2.2.2. Atomic Emission Spectrometry

Atomic emission spectrometry (Broekaert, 1987) is fundamentally different from AAS in that the analyte is detected/determined by the radiation emitted from free atoms of analyte trace elements. Various atom reservoirs have been developed to produce emission spectra from atoms. Atoms or ions of analyte acquire extra energy during collisions within a plasma which can be produced by chemical, electrical, or even by the interaction of an electromagnetic field with a noble gas such as argon. The production of a plasma in the absence of combustion processes requires some means of ionizing the gaseous medium. Electrically produced plasmas accelerate electrons to sufficient velocities so that their collisions with gas atoms result in ionizations and a cascade effect results. Alternatively, a plasma can be sustained by inductive coupling with a magnetic field generated by a radiofrequency source. In this case, the ionization process initiated by providing 'seed' electrons from a Tesla coil. Several formats of AES have become popular, including microwave induced plasma (MIP), direct current plasma (DCP) and inductively coupled plasma (ICP) (Boumans, 1987; Wolnik, 1988). These spectrometers are operated in the continuous mode. Sample atomization occurs within the spatially confined plasma which is characterized by high operating temperatures (6000 to 10,000°C) and produces a wealth of emission lines. A high-resolution monochromator is required to isolate the emission line of interest. The principal advantage of AES over AAS is not one of increased sensitivity, but rather that several lines corresponding to emissions from different elements can be monitored simultaneously or scanned sequentially in rapid succession. With a suitable data acquisition capability, this technique offers the possibility of multielement detection and access to several heteroatoms which are not directly available by AAS. Not only can several element-specific chromatograms be produced from the same analysis, but, in theory at least, the elemental composition of an eluting analyte can also be estimated. Relative to AAS, ICP-optical emission spectrometry (ICP-OES) provides comparable limits of detection for most elements. The ICP approach is advantageous for elements that form refractory oxides and for elements that are characterized by low ionization energies (Slavin, 1986).

1.2.2.3. Mass Spectrometry

A mass spectrometer is an analytical device that determines analytes by separating and quantifying ions according to their mass-to-charge ratio (m/z). The ions are generated inducing fragmentations of polyatomic species or by inducing either the loss or the gain of an electron. Once formed, the ions are separated according to their mass/charge ratio and finally detected. The result of ionization, ion separation, and detection is a mass spectrum that can provide molecular weight or even structural information. Mass spectrometry when coupled with an efficient atomization device provides unsurpassed limits of detection coupled with an applicability to virtually all trace elements. As a detection technique, MS also remains unsurpassed in terms of the quantity of information available to the analyst from a single chromatographic run. In the past, attempts were made to couple liquid chromatography with mass spectrometry. These attempts did produce limited success. More recently, the development of efficient ionization techniques has made LC-MS routine. The ability of electrospray ionization mass spectrometry to directly analyze compounds from aqueous or aqueous/organic solution has established the technique as a convenient mass detector for liquid chromatography.

As an important tool in biochemical research, researchers have developed this technique into what it is today, a highly sensitive tool that is capable of analyzing small and large molecules. Advances in chemical technology have been the engine powering the biotechnology industry. Analytical chemists have added fresh impetus to bioresearch with two new mass spectrometry ionization tools, electrospray and matrix-assisted laser desorption/ionization (MALDI). The commercial availability of these instruments has made routine the analysis of compounds including proteins, peptides, carbohydrates, oligonueleotides, natural products, and drug metabolites, offering picomole to femtomole sensitivities and enabling the direct analysis of biological fluids with a minimum amount of sample preparation. Mass spectrometry permits the analysis of these small and large biomolecules through 'mild' desorption and ionization methods. Their utility now extends beyond simple molecular weight characterization. Noncovalent interactions, protein and peptide sequencing, DNA sequencing, protein folding, in vitro drug analysis, and drug

development are among the areas to which mass spectrometry is being applied (Siuzdak, 1996).

Inductively coupled plasma mass spectrometry, as a detector for trace element speciation, has a unique separating power. In comparison with other methods of detection it offers some advantages. It has the capability of multielement detection, of performing analyses using isotope dilution, excellent sensitivity, and a wide linear dynamic range. However, several problems can arise when it is coupled with LC. Mobile phases with a high salt content can erode the sampler and skimmer, which often forces a change in the aperture configuration. This effect regulates the ion beam and thereby the sensitivity. Also mobile phases containing a large content of organic solvent cause plasma instability and may quench it. Possible solutions to this problem include cooling the spray chamber in order to prevent large amounts of organic solvent from entering the plasma (Tomlinson, *et al.*, 1995).

1.2.3. Speciation by Automated Liquid Chromatography

Automated LC encompasses a range of separatory techniques in which the components of partially purified sample extract are equilibrated between an active stationary phase and a liquid mobile phase during passage through the column. Because of differences in their relative affinities for the stationary vs. the mobile phase, components of the sample travel through the column at different rates and are resolved from each other by the time they reach the column exit. To be analytically useful, the separatory process must be highly reproducible and there must be some means of detecting the analyte(s) as they exit the column. Typically, automated LC instruments rely on dedicated detectors which respond to as broad a range of analytes as possible. For the most part, popular LC detector such as UV, refractive index, and fluorescence have been used only sparingly for speciation studies principally because of a lack of sensitivity. Yet, for ultra-trace studies, the resolving power of automated LC offers several attractive features. Relative to GC, LC is compatible with a much broader range of analytes because the constrains on volatility and thermal stability are less server and the sample capacity of the LC technique is greater (200 μL injections are common). The wide latitudes in choosing not only the

stationary phase, but the composition of the mobile phase and influencing the elution mode make LC a versatile technique for the separation of both labile and non volatile analytes. Additionally, the composition of the mobile phase can be varied during the chromatographic run to enhance the separation. Finally, for separations by ion exchange, it is sometimes advantageous to perform the chromatography at elevated temperature. However, relative to GC, it is appreciably more difficult to efficiently transfer liquid column eluate in a continuous and pulseless manner to either the reservoirs of atomic detectors or to the ion sources of mass detectors. Despite these difficulties, the resolving capabilities of LC have prompted many group to successfully develop interfaces to transfer analyte(s) from the liquid mobile phase to a variety of spectrometric detectors (Marshall and Momplaisir, 1995).

1.2.3.1. High-Performance Liquid Chromatography-Flame Atomic Absorption Spectrometry

Conventional flame atomic absorption spectrometry (FAAS), in which the column eluate is transferred directly to the nebulizer via a short section of capillary tubing, using either solvent compensation (Sherwood, *et al.*, 1985) or deliberately operating the nebulizer in a starved mode (Slavin and Schmidt, 1979) provides moderately enhanced instrumental responses. With conventional FAAS, aqueous sample is aspirated, typically at 4 to 6 ml/min, into a nebulizer chamber, where it is mixed with fuel and oxidant gases, then transported as a fine mist through a slotted burner head and ignited below the optical beam of the instrument. Even under optimal conditions, the analyte nebulization/atomization process, which must occur within a few milliseconds, is very inefficient (5 to 10%). Any improvements in the efficiency of transfer/atomization processes will appreciably improve the limits of detection of the technique. Other techniques which increase response some 3- to 5-fold include slotted-tube atom traps (STATs) (Hansen, *et al.*, 1992) or slotted tube atom retarders which increase the residence time of the analyte within the optical beam of the instrument. A possible difficulty with STATs constructed of silica is their relatively short lifetime within flames. Alkali metal atoms/ions are thought (Brown and Taylor, 1985) to migrate onto hot

exposed silica surfaces forming sodium silicates. On cooling, the silica contracts heterogeneously, causing fractures. Coating of outer tube surfaces with a refractory oxide (lanthanum, vanadium or aluminum, oxide) has been reported (Brown, *et al.*, 1987) to prolong the lifetime of quartz surfaces exposed to flame.

For conventional nebulization, a decrease in the aspiration rate into the FAAS nebulizer reduces the signal height but increases the atomization efficiency. Other nebulizers have been developed to generate a more homogeneous sample aerosol. A thermospray device was developed in which the safety diaphragm of an AAS nebulizer was replaced with a heating block which had been modified to accept a 0.15 mm i.d. stainless-steel tube (Mo, *et al.*, 1988). The resulting aerosol was transferred by the fuel and oxidant gases to a conventional burner head. Design improvements (Larsen and Blais, 1993) have reduced the limits of detection (LODs) for Cd, Pb and Zn appreciably. In an alternative approach, liquid was nebulized in a Meinhard concentric nebulizer and extensively desolvated in a heated desolvation chamber prior to passing into a conventional burner head (Gustavsson, 1989). Yet other approaches have involved hydraulic high pressure nebulization (HHPN) (Weber and Berndt, 1990) and a transport device consisting of platinum spirals mounted at the periphery of a rotating circular platform (Ebdon, *et al.*, 1987). In operation, fractions (30-60μL) were collected on each spiral, desolvated in a microburner, atomized within a conventional flame, and the analyte was trapped in a STAT mounted within the optical beam of the AAS instrument. Absolute LODs at the sub-nanogram level for Zn, Cu, Pb and Cd were reported for this device. For a flow injection system, reducing the flow rate from 4.5 to 1 mL/min resulted in a 9% decrease in peak height but a threefold increase in peak area for the same quantity of analyte (Wolf and Stewart, 1979). Several groups have transferred the liquid column eluate directly to the capillary inlet of the AAS nebulizer assembly (Suzuki, 1980; Richards, 1991). Yet optimal LC flow rates of 1 to 2 mL/min are appreciably less than the typical AAS aspiration rates. Two remedial approaches have been described: chromatographic effluent can be supplemented with solvent by means of a T-connector dipping into a solvent reservoir (Sherwood, *et al.*, 1985) or the nebulizer can be deliberately operated in a starved mode using a pertrifluoroethylene injection funnel

connected via capillary tubing to the nebulizer (Slavin and Schmidt, 1979). Column eluate was collected on a drop former. The droplets fell into the funnel and were aspirated into the flame. Air compensation added via a T-connector apparently increased calcium response in a flow injection analysis (FIA) system relative to either solvent compensation or the starved mode of operation (Garcia, *et al.*, 1987). The use of air compensation resulted in a 'pulsed' nebulization (Ebdon, *et al.*, 1985). Air was drawn into the chamber/burner which ran dry as these air segments passed through this assembly.

1.2.3.2. High-Performance Liquid Chromatography-Non-Flame Atomization

Progress has also been made with non-flame atomization devices, including modified graphite atomizers which can be operated for extended periods at temperatures sufficient to atomize volatile elements. Column eluate was transferred to the furnace by pneumatic (Nygren, *et al.*, 1988) or ultrasonic (Stupar and Frech, 1991) nebulization. Electrically heated quartz T-tubes coupled with thermospray nebulization have proven to be efficient atomization devices for alkylead compounds (Blais and Marshall, 1989), as well as for organoarsenicals and organoselenium compounds (Blais, *et al.*, 1990; Blais, *et al.*, 1991) in predominately methanolic mobile phases. The principal limitation of these interface designs remained the requirement for a methanol-rich mobile phase (>60% methanol) to support the combustion process. A quartz interface which is compatible with aqueous mobile phases has been reported (High, *et al.*, 1992) for the determination of Cd-bound metallothionein. A somewhat different design (Momplaisir, *et al.*, 1994), which is compatible with either an aqueous or methanolic mobile phases, provided sub-ng LODs for a variety of organoselenium (Lei and Marshall, 1995) and organoarsenic analytes (Momplaisir, *et al.*, 1994) in aqueous or methanolic high-performance liquid chromatography (HPLC) column eluate. A somewhat similar quartz T-tube design has also been used to detect metal analytes in supercritical carbon dioxide (Wang and Marshall, 1994).

1.2.4. Applications

Thirty-five years ago a concerted effort was undertaken to elucidate a possible biological role for cadmium akin to that which was then becoming known for zinc. This resulted in the discovery of a low molecular weight protein, metallothionein, which was initially isolated from horse kidney. Metallothioneins (MTs) are now known to be a group of heat stable, single-chain polypeptides of low molecular mass (6,000-8,000 Da) containing some 61 amino acids including 20 cysteine residues. This class of metal-binding polypeptide is ubiquitous throughout the animal kingdom and non-translationally synthesized analogs have been detected in more than 85 species of plants and microorganisms.

The biological functions of metallothioneins are closely related to their chemical structures which consist of two distinct adamantane-like metal binding clusters (Kägi and Kojima, 1987). The 20 cystieine residues can bind 7 divalent metal ions in two separate metal-thiolate clusters (M_3Cys_9 and M_4Cys_{11}) buried as 'mineral cores' in the interior of two globular domains formed by the carboxyl (α-domain) and the amino-terminal (β-domain) of the polypeptide chain. Although metallothioneins occur in multiple isoforms, the number of which vary with the source, mammalian MTs typically occur in only two isoforms. MT-I and MT-II which differ at neutral pH by a single negative charge. Even if the biological functions of MTs are not yet fully understood there are several lines of research that are being investigated actively:

i. regulation of zinc and copper metabolism.

The protective effects of MT against metal toxicity have been studied extensively both in animal experiments and in cell culture systems. Detoxification is by no means universally accepted as the primary function of MT (Webb, 1987; Templeton and Cherian, 1991). The high endogenous hepatic levels of zinc and copper bound MT in fetal and early neonatal life in several mammals suggest that MT may serve as an intracellular storage protein for these essential metals during perinatal development. The evidence for any specific roles for MT in the gastrointestinal absorption of zinc and copper and also in the hepatotoxicity of copper in certain genetic diseases is critically discussed by Bremner (1993). At very high oral doses of zinc, the intestinal content of both MT and another

cysteine-rich intestinal protein (CRIP) are increased. The latter protein is considered to regulate zinc absorption (Hempe and Cousins, 1992). Direct evidence for a causative role of MT in metal resistance has come from studies of copper sensitivity. A series of copper-resistant hepatoma cell lines have been reported to demonstrate a level of resistance proportional to the cellular concentration of Cu-MT (Freedman and Peisach, 1989).

ii. induction of MT synthesis by heavy metal ions, organic compounds and oxidative stress and protection against toxicity.

Both metals and glucocorticoids can directly induce MT synthesis. Divalent metals such as cadmium and zinc are the best know inducers of MT synthesis. Metal responsive elements and glucocorticoid responsive elements have been identified in MT genes. In addition to these inducers, several other compounds (which do not even bind with MT), oxidative stress and inflammatory agents have been shown to induce MT synthesis in various systems. Several mechanisms have been proposed for such induction of MT synthesis and they are thought to involve lipid peroxidation with free radical formation, release of cytokines during inflammation and changes in tissue distribution of zinc (Cherian and Chan, 1993).

iii. different pathways of degradation.

Earlier studies suggested that the turnover rate of MT depended on the species of metals bound to it. The turnover rate of Cu, Zn-MT is faster than Cd-MT ($t_{1/2}$ 15-20hr vs. 80hr). In addition the half-life for Cu-MT is less in Zn-deficient rats than those fed with a Zn-sufficient diet (Bremner *et al.*, 1978). Interestingly, the half life of MT isoform MT-1 is less than that of MT-2 (Anderson *et al.*, 1978; Suzuki and Maitani, 1983).

To quantitate metallothionein (MT) in complex biological materials several methods requiring largely different expenses in time, laboratory skills and instrumentation have been developed. The majority of these methods exploit the unique characteristics of MT namely its binding capacity for metals, high content of thiol groups and antigenic properties. However, in order to measure MT, one has to consider that each of these methods has its own inherent limitations.

As one of the methods for metallothionein determination, chromatographic methods consist of a separation step followed by quantification of either the protein or the metal content in the MT specific elution volume. By measuring metals, useful information on the metal content of the protein is obtained. Most favorably metal determinations can be performed in conjunation with the separation step (Suzuki, 1980 and 1991). Previously, chromatographic separation was performed by conventional gel filtration with Sephadex G-75. This approach has been replaced by the faster and more selective high-performance liquid chromatography (HPLC) of similar or even better specificity such as ion exchange (Suzuki, *et al.*, 1983; Klaassen and Lehman-McKeeman,, 1991) or hydrophobic interactions (Klauser, et al., 1983; Richards, 1989). Additionally, these techniques permit the quantification of MT isoforms. The selectivity of a particular chromatographic method depends, in large part, on the resolution, being best in reverse phase HPLC. In general, chromatographic methods seem to underestimate MT levels (Dieter, *et al.*, 1986) possibly due to unspecific interaction with the column matrix. The detection limits of chromatographic methods by analyzing SH groups via colorimetric methods or MT-bound metals via AAS are approximately 200 and 2.5ng MT, respectively (Summer and Klein, 1993).

Purification of crude MT isolates and each isoform (MT-1 and MT-2) prior to RP-HPLC can be achieved using a two-step column chromatographic procedure: (i.) Gel-permeation chromatography is performed on a Sephadex G-75 column (5.0 cm i.d. x 60 cm), eluted with 10 mM Tris-HCl, pH 8.6. (ii.) The fractions of interest are pooled and applied to a DEAE-Sephadex A-25 or DEAE-Sephacel column (2.6 cm i.d. x 20 cm) and eluted with a 500 mL linear gradient of 10 - 300 mM Tris-HCl, pH 8.6 (Richards, 1991). Classical gel filtration chromatography is not suitable for detection and isolation of labile zinc-binding ligands. However, when the gel was equilibrated with a constant concentration of zinc, 100% of zinc in the applied samples was recovered (Evans and Johnson, 1979). Two forms of Zn-metallothioneins (MT-1 and MT-2), which were induced by cadmium injection, were purified from rabbit liver. After dissolving the precipitate with tris buffer, supernatants were subjected to Sephadex G50 and DEAE Sephardex Fast Flow column chromatography to effect purification of the

metallothioneins. The content of Cd as well as Cu in the samples was negligible. Both Zn-MT and Cd-MT had a similar amino acid composition (Li, *et al.*, 1993). The fractions containing the MT isoforms were combined then subjected to RP-HPLC. The type of column that has been used routinely to separate the MT isoforms is a μBondapak C_{18} packing contained in a polyethylene cartridge (8 mm i.d. x 100 mm, 10-μm particle size) housed in a Z-module or RCM 8 x 10 radial compression device (Klauser, *et al.*, 1983). Steel columns containing different types of silica-based revered-phase packings including C_{18}, C_8 and C_3, with particle sizes ranging from 3 to 10 μm have also been popular (Klauser, *et al.*, 1983; Suzuki, *et al.*, 1983). There are several advantages to using radically compressed cartridge columns to effect the separation of MT isoforms. The flow through a polyethylene cartridge column can be maintained at a relatively high rate (3.0 mL/min) without sacrificing column efficiency. Therefore, the time required to separate the MT isoforms is reduced compared to steel columns, which are typically run at lower flow rates (1.0-2.0 mL/min). Moreover, the higher flow rate is better suited to detection methodology involving AAS. The mobile phase used to elute MT isoforms from the reversed-phase column consisted of an equilibration buffer (buffer A) such as 10 mM NaH_2PO_4, pH 7.0 and an elution buffer (buffer B) that contained an organic modifier such as 60% acetonitrile dissolved in buffer A. Metallothioneins were eluted with a gradient formed by mixing an increasing amount of buffer B with buffer A over a specified period of time (Klauser, *et al.*, 1983). In addition to acetonitrile, other organic modifiers such as methanol and *n*-propanol have been included in the mobile phase used to elute MT isoforms from reversed-phase columns (Richards, 1989). The choice of buffer should be considered carefully. Both acidic and neutral buffer systems have been employed to separate MT isoforms by RP-HPLC. The acidic buffer system most commonly used consists of 0.1% (w/v) trifluoroacetic acid (TFA) with acetonitrile as the organic modifier (Klauser, *et al.*, 1983). Alternatively, neutral buffer systems can be applied to the separation of MT isoforms (Van Beek and Baars, 1988). Because silica-based reversed-phase column are unstable above pH 8.0, the pH of the buffers used should not exceed 7.5. Thus, the choice of a RP-HPLC column, an acidic versus a neutral buffer system, the type of organic modifier for the mobile phase, and the type of gradient are all to be

considered when formulating a strategy to isolate MT isoforms from a particular sample. Since no single configuration will be applicable to all sample types, modifications in these parameters will have to be made routinely.

As detection, absorbance in the UV range (200-220nm) can be used as both a qualitative as well as a quantitative means of detection of MT isoforms. By monitoring absorbance at 214 nm it is possible to demonstrate differences in the isoform pattern for MTs purified from the livers of different eukaryotic species (Richards and Steele, 1987). As for UV absorbance, detection of MT-associated metals by AAS can be both qualitative as well as quantitative. An AAS can be used to survey the metal composition of individual MT isoforms separated by RP-HPLC. The effluent from the column was fed directly into the nebulizer of an atomic absorbance spectrophotometer set to detect the atomic absorbances for zinc, cadmium or copper. However, a separate injection of 100 μg of MT in 100 μl of 10 mM NaH_2PO_4, pH 7.0, was made for the determination of each metal (Richards, 1989).

1.3. Objectives of This Research

The primary objective of slurry sampling is to reduce the time and effort involved in solid sample preparation. The sample preparation might have applications for flame, furnace, and plasma atomization techniques. However, in the development of slurry methods, it is also important to avoid low atomization efficiencies and the attendant requirement to use correction factors and excessive matrix effects. It is further desirable to avoid the need for sieving a sample. Otherwise, a heterogeneous material may fractionate and yield an appreciably altered composition along with distorted analytical results. The generic term 'homogenizer' has been applied to any piece of equipment that disperses and/or emulsifies (including a turbine blade mixer, an ultrasonic probe, a high-shear mixer, a colloid mill, a blender or even a mortar and pestle). For purposes of this thesis, a more precise definition of a homogenizer is a device consisting of a positive displacement pump and a homogenizing valve that forms a restricted orifice through which product flows. The successful use of a homogenizer to prepare emulsions and/or dispersions of meats suitable for transmissions FTIR spectrometry has been reported (Dion, *et al.*, 1994) recently. A high-pressure flat valve homogenizer to prepare a milk-like emulsion of meat was introduced. In this type of homogenizer, the dispersed phase is reduced by forced passage through a narrow gap of various geometries. The model EF-B3 is operated at a pressure of approximately 138MPa when supplied with an inlet air pressure of 689.4 KPa which implies very high energy input and very short residence time in the valve. Droplet disruption in the valve can be attributed to turbulence, shearing stress, and cavitation depending on the geometry of the valve and pressure drop across the orifice. To our knowledge, high-pressure homogenization has not been studied as a rapid preparatory technique for sample preparation prior to slurry introduction atomic spectrometry. The objectives of this study were as follows:

i. to evaluate high-pressure homogenization as a rapid sample treatment procedure to provide a quasi-stable emulsion/dispersion of soft biological tissues or powdered certified reference materials which could be analyzed for cadmium, copper or lead by GF-AAS;

ii. to evaluate the slurry preparation/introduction technique for the determination of other analyte elements (including Cr, Ni, Mn, Fe and Se) in biological tissues;
iii. to extend the applicability of the technique to selected botanical samples;
iv. to reduce the levels of heavy metal contamination introduced into the product slurry by processing at high pressure.

The second part of this thesis is concerned with the application of a novel silica T-tube to the detection of selected heavy metals in aqueous flowing streams. Recently, an interface device (a silica T-tube design) was developed in our lab to couple HPLC with atomic absorption spectrometry. This device was developed for the detection of arsenic or selenium compounds in aqueous column elute and employs a flame in tube combustion to liberate the analyte element from its organic cage. In operation, the analyte in a liquid carrier is nebulized by thermospray effect. The aerosol is combusted in a heated pyrolysis chamber. The combustion products are swept into a hydrogen rich atmosphere where they are thermally converted to their corresponding hydrides. These volatile hydrides are then swept up into the optical tube of the interface which is interposed within the optical beam of the detector. It is postulated that variants of this same design could be used as an on-line device to destroy organic materials in solubilized tissue and fluid samples. Traditionally, silica-T tube interfaces have only be applied to the determination of As, Se, Sn, Hg, Pb, Sb, Bi and Te; trace elements which are considered to form stable hydrides. The objectives of the studies with the interface are as follow:

i. to assess the performance characteristics of this new device for the detection of Zn, Cd, Pb, Hg and Ag in flowing liquid streams;
ii. to explore the feasibility of monitoring HPLC eluate for protein-bound heavy metal analyte(s).

Chapter 2
Slurry Preparation by High Pressure Homogenization for the Determination of Heavy Metals in Cervine Liver and Kidney and in CRMs by GF-AAS

2.1 High- Pressure Homogenization

A cursory survey of the literature will reveal that "homogenizer" as a term has become a generic descriptive applied to any piece of equipment that disperses and/or emulsifies (including a turbine blade mixer, an ultrasonic probe, a high shear mixer, a colloid mill, a blender or even a mortar and pestle). For the purpose of this thesis a more precise definition of a homogenizer would be a device consisting of a positive displacement pump and a homogenizing valve that forms a restricted orifice through which product flows. The successful use of a homogenizer to prepare emulsion and/or dispersion of meats suitable for transmissions FTIR spectrometry has been recently reported (Dion, *et al.*, 1994). In this type of homogenizer, the dispersed phase is reduced by forced passage through a narrow gap of various geometries. The model EF-B3 is operated at a pressure of approximately 138MPa when supplied with an inlet air pressure of 689.4 KPa which implies very high energy input and very short residence time in the valve. Droplet disruption in the valve can be attributed to turbulence, shearing stress, and cavitation depending on the geometry of the valve and pressure drop across the orifice. To our knowledge, this type of homogenization has not been studied as a rapid technique for sample preparation prior to slurry introduction atomic spectrometry. For flame AAS or other aspiration/nebulization based spectroscopic techniques, a major difficulty with the introduction of slurries containing larger particles remains the tendency to clog the burner head. To counter this problem electrothermal atomization has been used. In addition to offering excellent sensitivities, it is less prone to the adverse effects of larger particles. This is so because of the operator selectable length of time of the atomization stage of the determination. Additionally, background interferences can be minimized with the judicious use of an ashing cycle coupled with appropriate matrix modifiers. Of great interest would be the development of this high-pressure homogenization as a rapid sample treatment procedure to provide a quasi-stable emulsion/dispersion of soft biological tissue or

powdered certified reference materials (CRMs) which could be analysed for heavy metals such as cadmium, copper or lead by GF-AAS.

2.2 Experimental Procedures

2.2.1 Samples

- Fresh cervine (deer or moose) liver or kidney tissue, frozen at -20 °C, were provided by Prof. D.J. Ecobichon, Department of Pharmacology and Therapeutics, McGill University.
- Certified reference materials (CRMs) were purchased from the National Research Council of Canada (NRCC), or from the US National Institute of Science and Technology (NIST).

2.2.2 Sample Preparation

Frozen (-20 °C) cervine (deer or moose) liver or kidney tissue was thawed and a 1 cm-wide transverse section of the organ was excised with a stainless steel scalpel, accurately weighed (≈ 2g) and transferred to 50 mL beaker together with 20 mL of 1 + 9 (v/v) ethanol-water containing 0.25% (m/m) tetramethylammonium hydroxide (TMAH). For certified reference materials, an accurately weighed sample (≈0.1 g) was added directly to 20 mL of the same solvent combination [1 + 9 (v/v) ethanol-water containing 0.25% (m/m) TMAH] in the beaker. The mixture was macerated/blended (20,000 RPM, 60 s, Tekmar SDT Tissuemizer, Cincinnati, OH) and the resulting suspension was then processed through the 20 ml capacity flat valve homogenizer (EmulsiFlex Model EF-B3, Avestin, Inc., Ottawa, ON) capable of developing 130 MPa. Each slurried sample was re-processed through the homogenizer three more times. Dilutions, when required, were performed with solvent mixture which had not been homogenized.

Prior to ICP-MS determinations for Cd, Pb and Cu, frozen tissues were thawed, cut into thin strips and ground to a paste in a meat grinder. Accurately weighed aliquots of paste (6 g liver or 3 g kidney) were digested at room temperature with 70% HNO_3 (25

mL) until gas evolution had ceased then heated at 80 °C until a clear yellow solution was obtained. The resulting digests were diluted prior to analysis. Digestions and analyses were performed by the Research and Productivity Council of New Brunswick, Fredericton, N.B.

2.2.3 GF-AAS Operation

A Varian Model 300 GF-AAS instrument (equipped with an autosampler, conventional hollow cathods lamps and Zeeman background correction) was used to determine cadmium, copper or lead using a hot injection technique. Analytical operating parameters for each analyte element are presented in Table 1.

2.2.4 Calibration

Quantitation was performed using both the method of external standards (ES) and standard additions (SA). ES consisted of appropriately diluted processed reagent blank, and up to four levels of standard that were prepared automatically by the sample introduction device. Background corrected peak area responses, resulting from three replicate injections of each diluted standard were used to define the best fit regression equation. For SA calibrations, 10 µL aliquots of each emulsion/dispersion were amended with 2, 5 or 10 µL of aqueous standard chosen to result in a range of peak areas including signals which were at least twice the signal for the unamended sample. The data were modelled by least squares linear regression. Quantitation was performed by dividing the Y-intercept of the regression equation by the slope of that equation and the overall standard error of estimate (SEest) was calculated from:

$$SEest = (SE^2_{Y\text{-}int} + SE^2_{slope})^{1/2}$$

The Student T-test was used to identify significant differences between the slopes or between the Y-intercepts of regression for different sample matrices. The F-test was used to detect significant differences between regression models.

Table 1. Graphite furnace operating parameters for the determination of Cd, Pb or Cu in test slurries.

Analyte	Modifier	Furnace Program
Cd	5 μL of 0.1 M $NH_4H_2PO_4$	Ar purge gas (3 L/min) on, 70 °C, ramp to 120 °C over 50 s, to 300 °C over 25 s, 600 °C for 25 s, Ar off, 1800 °C, read 2 s, Ar on, 2,600 °C 2 s, 60 °C 13 s.
Pb	5 μL of 3.0 M $Pd(NO_3)_2$	Ar purge gas (3 L/min) on, 70 °C, ramp to 120 °C over 50 s, to 300 °C over 75 s, 950 °C for 30 s, Ar off, 2,400 °C, read 2 s, Ar on, 60 °C 13 s.
Cu	5 μL of 1 % (m/m) NH_4NO_3	Ar purge gas (3 L/min) on, 70 °C, ramp to 120 °C over 50 s, to 300 °C over 25 s, 900 °C for 25 s, Ar off, 2,300 °C, read 2 s, Ar on, 2600 °C for 2 s, 60 °C 13 s.

a. Analyses for Cd were performed at 228.8 nm (0.5 nm slit width) with a partition tube.

b. Analyses for Pb were performed at 283.3 nm (0.5 nm slit width) with a platform tube.

c. Analyses for Cu were performed at 324.8 nm (0.1 nm slit width) with a partition tube

2.3 Results and Discussion

2.3.1 Protein Solubilization

In the past, conventional approaches to protein solubilization have often relied on a combination of high ionic strength and a strong base (alcoholic KOH or aqueous NaOH). For certain volatile heavy metals (As, Cd, Cu, Hg, Mn, Pb, Se and Zn) it was thought that the slurry preparation technique would prove useful for both flow injection - quartz T-tube atomization (Momplaisir, *et al.*, 1994) as well as for electrothermal atomisation - AAS. While still providing reliable analytical data, the appreciably lower limits of detection for these analytes (relative to conventional flame AAS and plasma optical emission spectrometries) result in less severe constraints on the maximum dilution which can be incurred during sample preparation. However, a limitation of the quartz interface devices is that the presence of alkali metal and alkaline earth cations in the mobile phase must be minimized/avoided in order to prolong their useful lifetime. The success of alkylammonium hydroxides as tissue solubilizers and the miscibility of these compounds with organic media suggested that these bases might be substituted for alkali metal hydroxides as effective solubilizers for a variety of biological matrices.

2.3.2 Homogenizer

Typically, conventional automated homogenizers and microfluidisers employ a pressure driven inlet and outlet ball check valve and seat design to pump the liquid feed stock through the homogenizing valve which makes them incompatible with particulate matter. A simplified homogenizing plug valve featuring a flat-faced valve stem to control the aperture between the valve stem and seat coupled to a pneumatic pressure multiplier circumvents the need for check valve assemblies. With this design (Figure 1) the sample chamber is filled with the suspension, the sample inlet entry port is sealed and the sample is processed by actuating the three way pneumatic flow controller. After increasing the aperture, the homogenate can then be aspirated back into the sample chamber by reversing the flow controller. Subsequent passes were typically performed with a slightly larger gap setting.

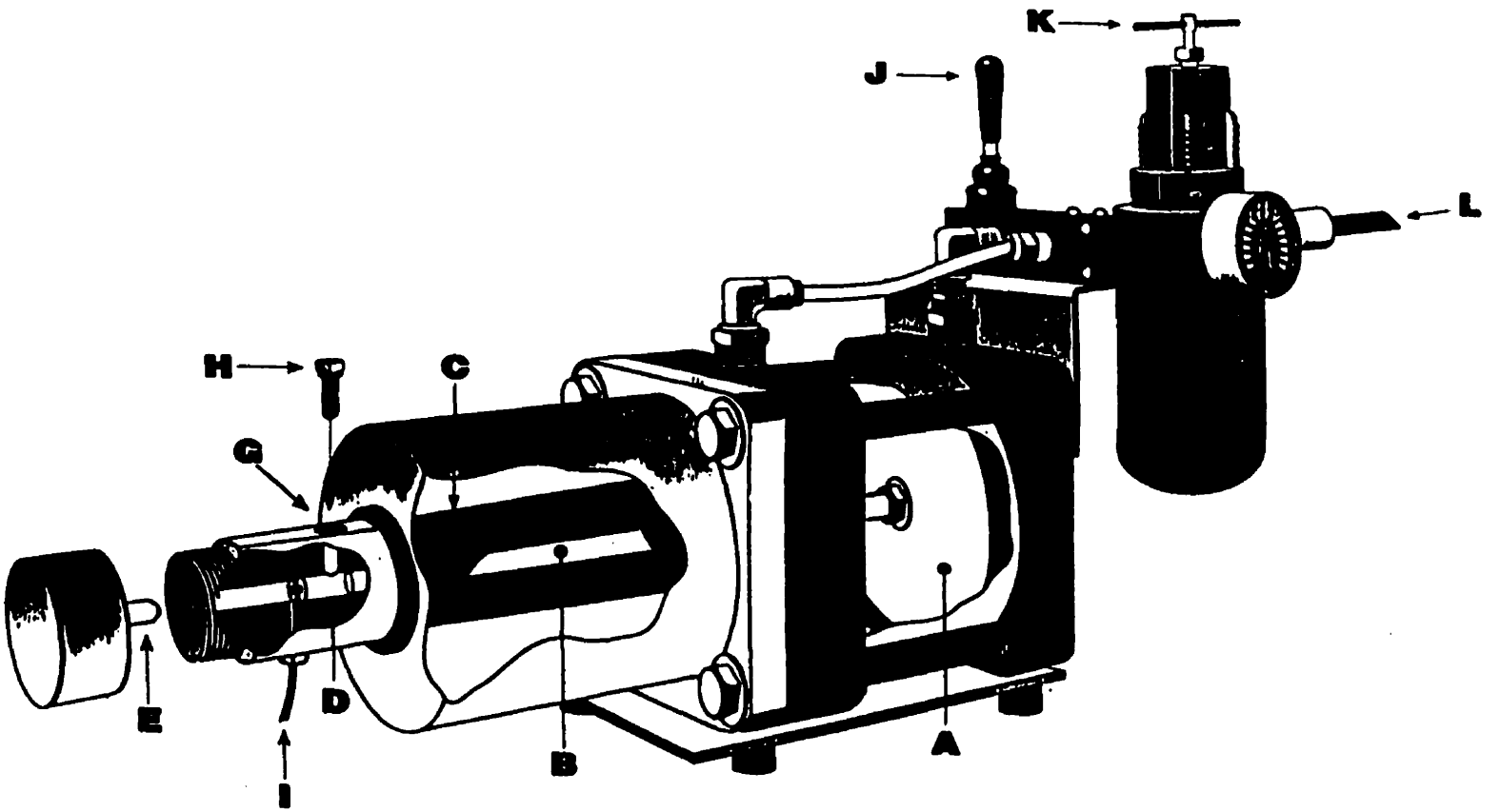

Figure 1. A low dead volume flat-valve homogenizer. A, pneumatic pressure multiplier; B, stainless steel piston; C, high-density polyethylene-Viton[tm] seal; D, low dead volume sample chamber; E, flat faced valve stem; F, fine-threaded screw cap; G, inlet; H, inlet cover screw; I, outlet; J, three-way pneumatic valve controller (reprinted from Dion, *et al.*, 1994).

2.3.3 Contamination

The envisaged strategy was to macerate the sample matrix with aqueous tetramethylammonium hydroxide - ethanol in a polytron type device and then to subject the blended mixture to homogenization. In initial studies, determinations were restricted to GF-AAS. It was considered that the capacity to vary both the duration and the temperature of the ashing cycle offered greater possibilities to selectively volatilize components of the sample matrix which might interfere in the subsequent analyte atomization stage of the determination. A serious limitation of the procedure became evident when solvent blanks were processed. Prior to determination, these blanks had been subjected to either (i.) the high speed blending procedure followed by homogenization or (ii.) to the blending procedure alone or (iii.) homogenization alone or (iv.) were untreated. As summarized in Table 2, appreciable quantities of Pb and lesser quantities of Cu were mobilized/solubilized during the mixing - homogenizing processes. These concentrations were estimated by additions of analyte standard into the processed solvent mixture. For convenience, the observed analyte levels have been expressed as if they had been present in either 2 g or 0.1 g of sample. Whereas the background levels of analyte Cd, Pb and Cu in the aqueous TMAH-ethanol solvent mixture were acceptably low, blending at high speed for 60 s increased only the levels of Pb (four fold). Processing the solvent mixture through the homogenizer appreciably increased analyte levels in all cases. Moreover, the net effect of the two procedures was approximately additive. Despite the high analyte levels introduced into the samples by the homogenizer, it was judged worthwhile to assess the stability of the resulting homogenates and their suitability for GF-AAS. It was envisaged that if the feasibility trials were successful, modifications could subsequently be made to the homogenizer design to reduce the contamination appreciably.

2.3.4 Certified Reference Materials

Six powdery certified reference materials were chosen as substrates for the preliminary evaluation of the suitability of the slurry preparation procedures. The variations in the certified concentrations of Cd, Pb and Cu among the six reference

Table 2. Analyte concentration in solvent mixture (μg/g ± 1 relative standard deviation from the mean), calculated as if the 20 mL solvent had contained either 0.100 g or 2.000 g sample following various mixing treatments.

	Cadmium		Copper		Lead	
Treatment	**2 g sample**	**0.1 g sample**	**2 g sample**	**0.1 g sample**	**2 g sample**	**0.1 g sample**
none	n.d.[a]	n.d.	0.0005	0.010	0.0049	0.0620
high speed blending (60s)	n.d.	n.d.	0.0005	0.0100	0.0198	0.395
homogenization (4 sequential passes)	0.001	0.020	0.015	0.300	0.069	1.38
high speed blending (60s) + homogenization	0.002 ± 23%	0.042 ± 23%	0.0154 ± 16%	0.324 ± 7.0%	0.076 ± 9.2%	1.58 ± 4.9%

[a]. n.d. = none detected above the apparent analyte concentration in distilled deionized water used to zero the instrument.

materials were 2023-fold (0.013-26.3 μg/g), 77-fold (0.37-10.4 μg/g) and 196-fold (2.34-439 μg/g) respectively. Despite the high analyte backgrounds introduced by the slurry preparation, there was good agreement (Tables 3-5) between the determined concentrations of cadmium, lead or copper in these materials, as estimated by the method of standard additions, and the corresponding certified reference values. No statistically significant differences between the measured analyte levels and the certified values were evident. The largest discordance (|accepted value - experimental value| / accepted value) between the determined concentration and the certified value was < 27% for Cd, < 23% for Pb and < 18% Cu. The mean uncertainty (as measured by the mean relative standard error of estimate) for the different reference materials was ± 7.5% for Pb, ± 8.7% for Cu and ± 8.7% for Cd. Longer term stability of the sample preparations were assessed by resampling with fresh sample after one month (Table 3-5, DORM-1, DORM-2, oyster tissue, lobster hepatopancreas, bovine liver and bovine muscle). Only for lead determinations in the DORM-2 CRM (certified concentration, 0.065 μg/g), were the data not sufficiently repeatable to be reported.

2.3.5 Frozen Cervine Tissues

The slurry preparation procedure was also applied to frozen fresh cervine tissue. Three liver and three kidney samples from white-tailed deer were analysed. Also, two samples of liver and kidney tissue from moose were analysed. These tissues had been analyzed previously by conventional acid digestion followed by quantitation by ICP-MS. The measured concentrations of Cu, Pb and Cd in these tissues are summarized in Tables 6-8. Duplicate entries for the same matrix represent separate analyses performed at approximately 1 month intervals. Again, there was good general agreement between the two sample preparation/determination techniques. Only single estimates of analyte concentrations by ICP-MS were available. However, duplicate preparations / determinations of the Cd, Pb and Cu levels in two reference materials (DORM-1 and DOLT-1 CRM) had demonstrated that the techniques were being performed reliably. The degree of discordance between the two techniques for Cu and Cd analyte was < 22% in all cases and < 15% in all but one case. For Pb, the discordance was greater, <31%, in part

Table 3. Cd concentrations (μg/g) in certified reference materials (CRMs) as determined by slurry introduction GF-AAS with calibration by standard additions or external standards.

Certified Reference Material[a]	Standard Additions (± 1RSEE[b])	External Standards (± 1RSD[c])	Certified Value (μg/g)
DORM-1	0.109 ± 13%	0.113 ± 6.0%	0.086 ± 14%
	0.107 ± 6.2%[d]	0.094 ± 14%[d]	
DORM-2	0.053 ± 21%	0.046 ± 22%	0.043 ± 19%
	0.040 ± 4.5%[d]	0.040 ± 6.9%[d]	
Bovine Liver	0.48 ± 6.0%	0.53 ± 5.6%	0.44 ± 14%
Bovine Muscle	0.010 ± 15%	0.046 ± 22%	0.013 ± 85%
	0.016 ± 6.0%[d]	0.006 ± 17%[d]	
Oyster Tissue	3.98 ± 3.6%	3.94 ± 2.1%	4.15 ± 9.0%
Lobster Hepatopancreas	26.7 ± 3.0%	27.2 ± 3.7%	26.3 ± 8.0%

[a] defatted dogfish muscle reference materials (DORM-1, DORM-2) and liver hepatopancreas (TORT-1) were purchased from the Nation Research Council of Canada. Bovine muscle powder (SRM 8414), bovine liver powder (SRM 1577a) and oyster tissue (SRM 1566a) were purchased from the National Institute of Standards and Technology (NIST), U.S. Dept. of Commerce.

[b] relative standard error of estimate

[c] relative standard deviation based on 3 replicate determinations of unamended slurry.

[d] slurry was prepared then sampled one month of storage in the dark at room temperature in sealed vials.

Table 4. Pb concentrations (μg/g) in certified reference materials (CRMs) as determined by slurry introduction GF-AAS with calibration by standard additions or external standards.

Certified Reference Material[a]	Standard Additions (± 1RSEE[b])	External Standards (± 1RSD[c])	Certified Value (μg/g)
DORM-1	0.31 ± 8.0%		0.4 ± 30%
	0.43 ± 9.0%[d]	0.12 ± 9.2%[d]	
Bovine Liver	0.15 ± 7.0%		0.135 ± 11%
Bovine Muscle	0.32 ± 7.4%	0.53 ± 10%	0.38 ± 63%
Oyster Tissue	0.35 ± 6.9%	0.37 ± 12%	0.37 ± 3.8%
Lobster Hepatopancreas	11.79 ± 8.0%	12.03 ± 2.8%	10.4 ± 19%
	9.79 ± 4.6%[d]	10.1 ± 2.6%[d]	

[a] defatted dogfish muscle reference materials (DORM-1) and liver hepatopancreas (TORT-1) were purchased from the Nation Research Council of Canada. Bovine muscle powder (SRM 8414), bovine liver powder (SRM 1577a) and oyster tissue (SRM 1566a) were purchased from the National Institute of Standards and Technology (NIST), U.S. Dept. of Commerce.

[b] relative standard error of estimate

[c] relative standard deviation based on 3 replicate determinations of unamended slurry.

[d] slurry was prepared then sampled one month of storage in the dark at room temperature in sealed vials.

Table 5. Cu concentrations (μg/g) in certified reference materials (CRMs) as determined by slurry introduction GF-AAS with calibration by standard additions or external standards.

Certified Reference Material[a]	Standard Additions (± 1RSEE[b])	External Standards (± 1RSD[c])	Certified Value (μg/g)
DORM-1	4.48 ± 14%	6.29 ± 10%	5.22 ± 6.3%
	4.44 ± 5.0%[d]	4.25 ± 0.8%[d]	
DORM-2	2.21 ± 12%	2.78 ± 3.4%	2.34 ± 6.8%
	2.01 ± 6.6%[d]	2.43 ± 4.2%[d]	
Bovine Liver	124.4 ± 7.5%	116.1 ± 3.1%	158 ± 4.4%
Bovine Muscle	2.46 ± 7.6%	2.80 ± 0.7%	2.24 ± 20%
	2.35 ± 8.4%[d]	2.94 ± 4.7%[d]	
Oyster Tissue	54.9 ± 15%	77.8 ± 5.7%	66.3 ± 0.4%
Lobster Hepatopancreas	425.2 ± 2.8%	411.8 ± 2.8%	439 ± 5.0%

[a] defatted dogfish muscle reference materials (DORM-1, DORM-2) and liver hepatopancreas (TORT-1) were purchased from the Nation Research Council of Canada. Bovine muscle powder (SRM 8414), bovine liver powder (SRM 1577a) and oyster tissue (SRM 1566a) were purchased from the National Institute of Standards and Technology (NIST), U.S. Dept. of Commerce.

[b] relative standard error of estimate

[c] relative standard deviation based on 3 replicate determinations of unamended slurry.

[d] slurry was prepared then sampled one month of storage in the dark at room temperature in sealed vials.

Table 6. Cadmium concentrations (μg/g) in cervine liver or kidney as determined by slurry introduction GF-AAS with calibration by standard additions or by external standards.

Sample	Std. Addns. [a] (± 1RSEE)	Ext. Stds. [b] (± 1RSD)	ICP -MS [c]
B2-Liver	0.304 ± 2.3%	0.257 ± 4.8%	0.285
B2-Kidney	2.20 ± 4.1%	2.15 ± 2.5%	1.78
B4-Liver	0.59 ± 2.1%	0.57 ± 2.5%	0.57
	0.58 ± 3.4%[d]	0.57 ± 5.2%[d]	
B4-Kidney	3.04 ± 4.8%	3.12 ± 0.9%	3.48
	3.29 ± 3.4%[d]	3.23 ± 2.0%[d]	
B6-Liver	1.36 ± 3.7%	1.44 ± 2.4%	1.27
B6-Kidney	8.69 ± 3.9%	8.41 ± 1.4%	8.47
B10-Liver	10.9 ± 5.1%	11.3 ± 1.3%	10.7
	10.5 ± 4.9%[d]	10.3 ± 1.4%[d]	
B10-Kidney	17.1 ± 4.6%	17.5 ± 1.1%	16.3
	16.5 ± 3.7%[d]	16.3 ± 1.2%[d]	
B11-Liver	3.35 ± 2.9%	3.45 ± 2.1%	3.03
B11-Kidney	11.8 ± 8.0%	12.2 ± 1.2%	11.4

[a] Calibration by standard additions (± 1 relative standard error of estimate)

[b] Calibration by external standards (± 1 relative standard deviation)

[c] Determinations were performed by the Research and Productivity Council of New Brunswick, Fredericton, N.B.

[d] Homogenates were re-sampled and reanalyzed after one month of storage in the dark at room temperature.

Table 7. Lead concentrations (μg/g) in cervine liver or kidney as determined by slurry introduction GF-AAS with calibration by standard additions or by external standards.

Sample	Std. Addns. [a] (± 1RSEE)	Ext. Stds. [b] (± 1RSD)	ICP -MS [c]
B2-Liver	0.036 ± 8.0%	0.005 ± 3.7%	0.03
B2-Kidney	0.146 ± 5.0%	0.123 ± 3.9%	0.12
B4-Liver	0.032 ± 9.0%	0.005 ± 4.9%	0.04
	0.033 ± 4.5%[d]	0.027 ± 10.1%[d]	
B4-Kidney	0.048 ± 8.0%	0.052 ± 3.4%	0.07
	0.059 ± 7.9%[d]	0.048 ± 16.9%[d]	
B6-Liver	0.029 ± 7.0%	0.005 ± 2.4%	0.03
B6-Kidney	0.065 ± 8.0%	0.035 ± 3.9%	0.07
B10-Liver	0.345 ± 12%	0.338 ± 2.9%	0.32
	0.312 ± 4.4%[d]	0.306 ± 4.0%[d]	
B10-Kidney	0.059 ± 7.0%	0.073 ± 1.2%	0.07
	0.053 ± 7.7%[d]	0.043 ± 12.9%[d]	
B11-Liver	0.020 ± 5.0%	n.d.	0.03
B11-Kidney	0.043 ± 9.0%	0.005 ± 2.4%	0.04

[a] Calibration by standard additions (± 1 relative standard error of estimate)

[b] Calibration by external standards (± 1 relative standard deviation)

[c] Determinations were performed by the Research and Productivity Council of New Brunswick, Fredericton, N.B.

[d] Homogenates were re-sampled and reanalyzed after one month of storage in the dark at room temperature.

Table 8. Copper concentrations (μg/g) in cervine liver or kidney as determined by slurry introduction GF-AAS with calibration by standard additions or by external standards.

Sample	Std. Addns. [a] (± 1RSEE)	Ext. Stds. [b] (± 1RSD)	ICP -MS [c]
B2-Liver	49.4 ± 4.8%	45.0 ± 0.7%	47.1
B2-Kidney	3.04 ± 6.8%	2.14 ±10.%	3.11
B4-Liver	44.0 ± 3.1%	49.2 ± 1.0 %	40.5
	40.6 ± 1.7%[d]	41.5 ± 2.3%[d]	
B4-Kidney	3.10 ± 8.1%	2.69 ± 8.2%	3.17
	3.04 ± 4.9%[d]	3.25 ± 3.3%[d]	
B6-Liver	83.3 ± 7.0%	83.0 ± 0.1%	93.8
B6-Kidney	4.93 ± 2.8%	4.77 ± 1.9%	4.66
B10-Liver	3.42 ± 3.3%	2.83 ± 5.3%	3.30
	3.54 ± 7.4%[d]	3.08 ± 12.%[d]	
B10-Kidney	4.56 ± 6.7%	4.02 ± 6.0%	4.39
	4.48 ± 6.7%[d]	4.02 ± 6.0%[d]	
B11-Liver	37.0 ± 2.3%	32.9 ± 1.4%	43.3
B11-Kidney	3.14 ± 2.1%	4.02 ± 6.0%	3.28

[a] Calibration by standard additions (± 1 relative standard error of estimate)

[b] Calibration by external standards (± 1 relative standard deviation)

[c] Determinations were performed by the Research and Productivity Council of New Brunswick, Fredericton, N.B.

[d] Homogenates were re-sampled and reanalyzed after one month of storage in the dark at room temperature.

because of number of significant digits used to report the data.

2.3.6 Linear Regression Models

In Table 9 the means of the slopes of the best fit regression lines for standard additions of each analyte to liver, to kidney, to CRM or to homogenized solvent mixture are compared. There were no significant differences in mean slopes of the regression lines among the different sample matrices for the same analyte provided that the analyses were performed on the same day. Moreover there were no significant differences among the slopes for the six different reference materials which, in turn was not significantly different from the slope of an external standards calibration curve for the same analyte. Collectively, these observations suggest that, for these matrices at least, a single standard additions calibration could have been used to quantify analyte concentrations in any of the samples. An external standards calibration technique might also have been equally effective. However, for CRMs containing lower analyte concentrations ([Cd] < 0.04 and [Pb] < 0.4,) this was not the case. Arbitrarily for CRMs, 0.1 g samples had been slurried. For [Cu] in CRMs, the level of discordance between the measured and reference values was up to 31% and appreciably larger than the corresponding discordance between the measured [Cu] by standard additions and the reference value (< 22%).

For fresh tissues, 2 g sample were slurried. The degree of discordance for the [Cd] as determined by ICP-MS and external standards GF-AAS (Table 4), was < 21% in all cases (mean relative difference, 7.1 ± 6.1%) and < 15% in 13 of 14 cases. Similarly for [Cu] (Table 4), the relative difference between the two techniques was < 31% in all cases (mean relative difference 12 ± 9.4%) and ≤ 17% in 13 of 14 cases. By contrast, for Pb, the discordance was excessive. This is not surprising given that the [Pb] in 8 of the 10 samples, as determined by ICP-MS, was less than the background level introduced by the homogenization procedure. Collectively, the results determined by external standards calibration were reliable provided that analyte levels in the sample were appreciably greater than the contamination introduced by the slurry preparation procedure.

Table 9. Means of slopes (± 1RSD[a]) of the best fit linear regression models for standard additions of Cd, Pb or Cu to liver, kidney, certified reference materials or processed blank solvent mixture.

Analyte	Liver	Kidney	CRMs	Solvent Blank	liver + kidney + CRM
Cd	1.002 ± 0.55%	1.013 ± 3.06%	1.010 ± 1.02%	1.005 ± 2.49%	1.009 ± 1.38%
Pb	0.1855 ± 0.91%	0.1819 ± 0.78%	0.1858 ± 1.55%	0.1837 ± 1.33%	0.1847 ± 1.46%
Cu	0.2572 ± 1.54%	0.2576 ± 0.77%	0.2593 ± 0.54%	0.2574 ± 1.00%	0.2585 ± 0.82%

[a] ± one relative standard deviation of the slope from the mean

2.3.7 Slurry Stability Trials

The stability of the slurries with time were assessed by performing analyses (by the method of standard additions) for Cd, Pb and Cu in five reference materials and four cervine tissues. Aliquots were removed from the undisturbed preparations two days and after six days post preparation and analyzed. The results as determined by standard additions calibration are recorded in Tables 10 and 11. For convenience, the values representing 0 days post preparation from Tables 3-5 have been reproduced in column 3 of these tables. For certified reference materials, there was no convincing evidence for any significant change in the apparent analyte concentrations over the six day trial. For each sample matrix, if the results for each analyte from the three different times were treated as replicates, the mean relative standard deviation (RSD) was in 12 of 14 cases lower than the mean relative standard error of estimate (RSEE) associated with the individual determinations of that group. In the other two cases, the RSD (± 22 and ± 20%) was appreciably smaller than the uncertainty associated with the reference value (30 and 63% RSD). In only one case (Cu in DORM-2) did the apparent analyte levels decrease with time to the extent that there was a 22% difference between the determined concentration at day 6 and the reference value. However this decrease was not statistically significant at the 95% level of confidence.

For the cervine tissues, again there was no appreciable change in the apparent analyte concentrations with time. The degree of discordance between the ICP-MS result and the determined value at day 6 was less than 12% for all cases but one (Pb in the B4-liver sample). The apparent changes in [Pb] in the B4-liver sample with time were erratic (mean [Pb] = 0.047 ± 28% vs. 0.04 by ICP-MS) reflecting, in part, the high background levels introduced by the slurry procedure relative to the Pb analyte level in this sample. By contrast, the mean RSD for all 12 analyte-sample matrix combinations was ± 9.6%. Collectively, these results indicate that there was no segregation of the analyte within the sample over the six day trial. It seems certain that at least a high proportion of the analyte was extracted into the liquid phase during sample processing. For these matrices at least, short-term slurry storage had no discernible effect on the analyte apparent concentrations.

Table 10. Variations in apparent analyte concentrations (μg/g ± 1RSEE) with time in unmixed slurries.

Sample	Analyte	0 days	2 days	6 days	certified value
DORM-1	Cd	0.109 ± 13%	0.109 ± 5.7%	0.107 ± 8.3%	0.086 ± 14%
	Pb	0.31 ± 8.0%	0.46 ± 6.7%	0.33 ± 10%	0.4 ± 30%
	Cu	4.48 ± 14%	4.59 ± 4.9%	4.73 ± 4.8%	5.22 ± 6.3%
DORM-2	Cd	0.053 ± 21%	0 053 ± 4.6%	0.054 ± 8.3%	0.043 ±19%
	Cu	2.21 ± 12%	2.00 ± 10.1%	1.82 ± 7.1%	2.34 ± 6.8%
Bovine Muscle	Cd	0.010 ± 50%	0.015 ± 42%	0.007 ± 37%	0.013 ± 85%
	Pb	0.32 ± 7.4%	0.36 ± 7.4%	0.47 ± 8.8%	0.38 ± 63%
	Cu	2.46 ± 7.6%	2.51 ± 8.8%	2.42 ± 8.1%	2.84 ± 20%
Oyster Tissue	Cd	3.98 ± 3.6%	3.85 ± 3.0%	3.87 ± 4.1%	4.15 ± 9.0%
	Pb	0.35 ± 6.9%	0.38 ± 7.8%	0.31 ± 9.7%	0.37 ± 3.8%
	Cu	54.9 ± 15%	57.8 ± 14%	58 2 ± 7.8%	66.3 ± 6.5%
Lobster HepPn[a]	Cd	26.7 ± 3.0%	25.8 ± 4.7%	28.3 ± 3.8%	26.3 ± 8.0%
	Pb	9.79 ± 4.6%	10.2 ± 8 8%	10.5 ± 4.7%	10.5 ± 19.0%
	Cu	425.2 ± 2.8%	415.2 ± 3.1%	406 ± 3.6%	439. ± 5.0%

[a] HepPn = hepatopancreas

Table 11. Variations in apparent analyte concentrations (μg/g ± 1RSEE) with time in unmixed slurries.

Sample	Analyte	0 days	2 days	6 days	ICP-MS
B4-liver	Cd	0.59 ± 2.1%	0.49 ± 4.4%	0.52 ± 5.4%	0.57
	Pb	0.033 ± 4.5%	0.050 ± 7.7%	0.059 ± 8.8%	0.04
	Cu	40.6 ± 1.7%	41.6 ± 2.3%	42.7 ± 1.8%	40.5
B4-kidney	Cd	3.04 ± 4.8%	3.39 ± 2.9%	3.41 ± 2.4%	3.48
	Pb	0.048 ± 8.0%	0.064 ± 5.1%	0.066 ± 6.4%	0.07
	Cu	3.10 ± 8.1%	3.64 ± 9.2%	2.78 ± 10.6%	3.17
B10-liver	Cd	10.9 ± 5.1%	10.6 ± 3.4%	10.4 ± 3.8%	10.7
	Pb	0.312 ± 4.4 %	0.302 ± 3.1%	0.307 ± 3.8%	0.32
	Cu	3.42 ± 3.3%	3.64 ± 9.2%	3.71 ± 7.4%	3.30
B10-kidney	Cd	17.1 ± 4.6%	21.4 ± 4.6%	15.8 ± 4.8%	16.3
	Pb	0.053 ± 7.7%	0.057 ± 7.7%	0.068 ± 6.6%	0.07
	Cu	4.56 ± 6.7%	4.60 ± 7.3%	4.65 ± 6.6%	4.39

2.4 Conclusions

In summary, these results indicate that the high pressure homogenization is capable of generating emulsions/dispersions of CRMs of animal origin or of fresh soft tissues which can be reliably sub-sampled and analysed by GF-AAS for Cd, Pb or Cu. This is true providing that the levels of the analyte in these matrices are appreciably greater than the levels of contamination generated by the homogenization procedure. Attractive features of this approach include the speed (60 s blending followed by 4 passes through the homogenizer - less than 3 minutes total), simplicity of sample processing and the apparent stability of the preparation. The repeatability of the determinations preformed (over six days) on the same homogenate indicate that at least a high proportion of the analytes had been extracted into the liquid phase during sample processing. Moreover, the similar evidence would get from the results of longer term stability of sample preparation which was assessed by resampling with fresh sample after one month storage The cost of the homogenizing device is appreciably less than the cost of a commercial microwave digester. The homogenizer is easily cleaned between samples by processing fresh solvent. However, for the technique to become more widely applicable, background levels of analyte introduced by the homogenizer would have to be reduced substantially. It was anticipated that modifications to the flat faced valve stem of the homogenizer would reduce levels of contamination appreciably.

Chapter 3.
Modifications to the Homogenizer for the Determination of Heavy Metals in Zoological and Botanical CRMs and Animal Feeds by GF-AAS

3.1 Modifications to the Homogenizer

The preparation/introduction of slurried samples continues to attract considerable attention because of the ease with which quasi-stable preparations can be generated and their compatibility with conventional liquid handling techniques. Within the general field of solid sampling analysis (Langmyhr, 1979; Miller-Ihni, 1992 and 1993), it is the use of slurried samples which has become the most popular approach to trace element determination. The studies described in Chapter 2 had demonstrated that copper, cadmium and lead concentrations could be reliably determined in soft organ tissues or in zoological reference materials by direct slurry introduction into the graphite furnace-atomic absorption spectrometer (GF-AAS). Moreover, there was no evidence, over 6 days, of any analyte metal segmentation within solutions which resulted from a combination of high speed blending and homogenization. In addition, the instrumental response to analyte metal could be calibrated with aqueous external standards. However, the applicability of the technique was limited by the appreciable levels of contamination introduced into the sample by the homogenization step. It was postulated that the principle sources responsible for the contamination were the stainless steel surfaces which were exposed to the homogenized fluid, particularly the flat face of the demountable valve head. As such, modifications to the homogenizer were necessary in order to reduce the contamination during the homogenization process. By capping the valve head with an inert surface capable of withstanding the impact of the jet of fluid exiting the homogenizing orifice might reduce the levels of contamination appreciably. It had been reported (Miller-Ihli, 1992) previously that zirconium oxide beads had been used to reduce the particle size and to mix particulate solids. Moreover silicon nitride or boron carbide provide good abrasion resistance, even for the relatively lower pressure requirements of pistons and check valves for high performance liquid chromatography (HPLC). Sapphire, ruby and zirconium oxide are preferred over other ceramic materials for their superior resistance.

The objectives of the studies described in this chapter were: (i.) to evaluate the efficiencies of various materials as caps to reduce the levels of contamination introduced by the high pressure processing technique; (ii.) to evaluate the slurry preparation/introduction technique for the determination of other analyte elements (including Cr, Ni, Mn, Fe and Se) in biological tissues; (iii.) to extend the applicability of the technique to selected botanical samples.

3.2 Experimental Procedures

3.2.1 Samples:

- CRMs were purchased from the National Research Council of Canada (NRCC), or the US National Institute of Science and Technology (NIST).
- Samples of animal diet mixtures destined for a zoo were chosen to contain a variety of plant and animal materials including timothy grass, bamboo leaves, whole smelts, cricket chow and a mixture (contents unspecified) formulated for panda bears. Animal feed supplements were composed of mixed forage corps. Animal feed samples were generously supplied by Dr. E.R. Chavez, Animal Science, Macdonald Campus of McGill University.

3.2.2 Sample Preparation:

As described in section 2.2.2 (page 39).

3.2.3 Homogenizer

The flat-faced head of the valve stem and screw cap assembly of the homogenizer (Emulsifex Model EF-B3, Avestin, Inc., Ottawa, ON, Canada, Figure 2) was modified by gluing a polished 4 mm diameter x 2 mm thick disk of: (i.) ruby manufactured from a ruby sphere (from an HPLC check valve) or (ii.) tungsten carbide manufactured from a 6-12 mm diameter sphere (Spex Inc., Metuchen, NJ, USA) or (iii.) zirconia (Optimize Technologies Inc., Portland, OR, USA) or (iv.) sapphire manufactured from an HPLC

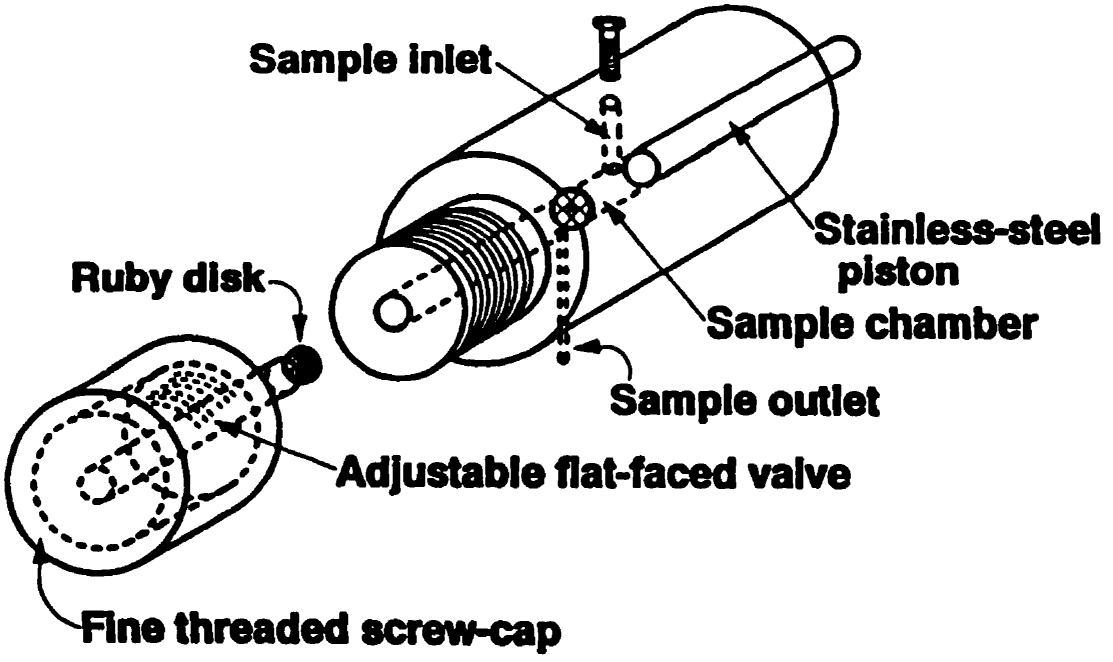

Figure 2. Enlarged view of the homogenizing valve and sample compartment of the Model EF-B3 homogenizer.

piston or (v.) poly-methacrylate (Spex Inc., Metuchen, NJ, USA) to the polished surface of the flat-faced head. Sample macerate was transferred to the sample chamber via the inlet port which was then sealed with a fine-threaded screw cap. The stainless steel piston (connected to a pneumatic multiplier) then forced the fluid through an aperture and the homogenate was collected from the sample outlet. Each sample was re-processed three more times with the valve stem retracted slightly to provide a slightly larger gap.

3.2.4 Flame-AAS

Prior to determinations for Cu, Fe and Mn, feed samples were dried to constant weight and ground to pass a 1 mm screen. Accurately weighed aliquots of ground plant material (approximately 2 g) were digested at room temperature in a perchlorate fume hood with 25 ml of (4 +1 v/v) 70 % HNO_3 $HClO_4$ until gas evolution had ceased, then heated at 80°C until a clear yellow solution was obtained. The resulting digests were diluted prior to analysis. Digistions and analyses by flame AAS were performed by Ms. Denise Gaulin, Animal Science, Macdonald Campus of McGill University.

3.2.5 GF-AAS

Analyses for chromium, copper, iron, manganese, nickel or selenium were performed using a hot injection technique on a Varian Model 300 GF-AAS system equipped with an autosampler, conventional hollow cathode lamps and Zeeman effect background correction. Analytical operating parameters for each analyte element are presented in Table 12.

3.2.6 Calibration

As described in section 2.2.4. (page 40).

Table 12. Graphite furnace operating parameters for the determination[a] of Cr, Cu, Fe, Mn and Ni.

	Chromium	Copper	Iron	Manganese	Nickel
wavelength (nm)	357.9 // 429.0	324.7 // 244.2	386.0	403.1 // 279.5	232.0
lamp current (amps)	7	4	5	5	4
slit width (nm)	0.2	0.1 // 0.5	0.2	0.2 // 0.2	0.2
injection T (^{o}C)	60	60	60	60	60
pre-injection	no	no	yes	yes	no
last dry step, 5s (^{o}C)	no	no	250	250	no
charring sequence	20 s ramp to 1450^{o}C, 40 s hold	5 s ramp to 900 ^{o}C, 20 s hold.	10 s ramp to 1100 ^{o}C, 21 s hold	10 s ramp to 1200 ^{o}C, 20 s hold	5 s ramp to 800 ^{o}C, 20 s hold
cool down	7 s ramp to 40^{o}C, 10 s hold	none	none	none	none
atomisation	1.2 s ramp to 2,400^{o}C 8 s hold	1.0 s ramp to 2,300 ^{o}C, 2.0 s hold	1.2 s ramp to 2400^{o}C 4.0 s hold	0.7 s ramp to 2200^{o}C, 2 s hold	0.9 s ramp to 2400 ^{o}C, 4 s hold
measurement time	8 s	3 s	6 s	3 s	4 s
matrix modifier	3 µl of 20 g/L $Mg(NO_3)_2$ for 10 µL sample	5 µL of 1% (m/m) NH_4NO_3 for 10 µL sample	5 µL of (500 mg/L Pd + 2.5% citric acid) for 10 µL sample	5 µL of (500 mg/L Pd + 2.5% citric acid) for 10 µL sample	none

[a] Each step of the furnace programs (with the exception of the read step) was performed in the presence of Ar purge gas (3 l/min).

3.3 Results and Discussion

3.3.1 Addition of a Ruby disk to the Homogenizer

A disk of ruby which was manufactured by grinding and polishing a 4 mm diameter ruby ball, was glued temporarily to the flat face of the demountable valve head. Solvent mixture, 20 mL, was blended then homogenized four successive times (in the presence or absence of the ruby disk) prior to GF-AAS analysis for Cr, Cu, Fe, Mn, Ni and Se. Analyte concentrations (Table 13) were expressed as if the solvent had contained 0.100 g of sample. The reduction in heavy metal content of the homogenized fluid was, in all cases, reduced appreciably (some 2 to 30-fold). Nonetheless, the level of contamination remained appreciable even in the presence of the ruby cap.

3.3.2 Zoological Materials

The slurry preparation technique, with the ruby capped homogenizer, was then applied, in preliminary studies, to five biological certified reference materials (CRMs) and to four frozen liver and kidney tissues from moose and ring tail deer. As was reported previously, the resulting homogenates appeared to be uniform and contained no visible particulate material. Analyses for chromium, iron, manganese and nickel in the five CRM homogenates (with calibration by standard additions) provided estimates (Table 14 and 15) which did not differ significantly from their certified concentrations. No analyte Ni was detected in the bovine muscle CRM (certified concentration 0.05 μg/g), however, this concentration was less than half the [Ni] introduced into the homogenate during preparation. The repeatability of the analyses, as measured by the relative standard error of estimate (RSE_{est}), did not indicate any problems with the repeatability of transfer of any of the homogenates to the graphite tube, however as the concentration of analyte approached the level of contamination, the percent discord. (|certified value - experimental value / certified value|), which was always less than 20%, tended to increase.

Frozen liver and kidney tissue were analysed only for Cr and Mn. GF-AAS estimates of [Cr] in homogenates were consistently higher (mean +14.4%) than estimates determined by conventional acid digestion ICP-MS. However, the [Cr] in these tissues was approximately one-half the contamination concentration from the homogenization

Table 13. Apparent analyte concentrations (μg/g sample) in 20 mL solvent mixture (± EDTA) following various mixing treatments. Concentrations in the homogenized fluid are expressed as if the solvent mixture had contained 0.100 g of sample.

homogenizing treatment	**Aluminum**	**Cadmium**	**Chromium**	**Copper**	**Iron**	**Lead**	**Manganese**	**Nickel**	**Selenium**
4 sequential homogenizations s steel head	42.12	0.020	0.89	15.0	56.94	1.38	2.31	3.57	1.34
4 sequential homogenizations ruby disk (RD)	21.52	0.03	0.20	0.70	13.99	0.28	0.39	0.11	0.68
4 sequential homogenizations RD + EDTA	23.10	0.03	0.35	1.16	14.59	0.23	0.43	0.10	0.67
EDTA amended solvent blank	0.42	n.d.[a]	0.07	0.31	0.26	0.08	0.05	n.d.[a]	n.d.[a]

[a] none detected above background in unhomogenized solvent blank.

Table 14. Chromium and iron concentrations (μg/g ± 1 standard error of estimate[a], expressed as a percentage) in zoological certified reference materials (CRMs) and cervine liver and kidney with calibration by standard additions. Reported concentrations have been corrected for analyte contrations (Table 13) introduced by the ruby capped homogenizer.

	Chromium		Iron	
Matrix[b]	**Experimental**	**Certified[c]**	**Experimental**	**Certified[c]**
DORM-1	3.50 ± 0.37	3.6 ± 11.1	59.0 ± 4.9	63.6 ± 8.3
DORM-2	33.53 ± 3.75	34.7 ± 15.9	130.7 ± 3.5	142. ± 7.0
Bovine muscle	0.081 ± 22.	0.071 ± 54	63.1 ± 5.1	71.2 ± 12.9
Oyster tissue	1.24 ± 2.7	1.43 ± 32.2	540.2 ± 5.8	539.1 ± 2.8
Hepatopancreas	3.0 ± 0.5	2.4 ± 25.0	172.5 ± 6.6	186.0 ± 5.9
B4-liver[d]	0.126 ± 1.8	0.10		
B4-kidney[d]	0.114 ± 0.6	0.11		
B10-liver[d]	0.095 ± 2.7	0.08		
B10-kidney[d]	0.11 ± 18.1	0.10		

[a] Standard error of estimate: $SE_{est} = (SE^2_{Y\text{-int}} + SE^2_{slope})^{1/2}$

[b] Defatted dogfish muscle reference materials (DORM-1, DORM-2) and liver hepatopancreas (TORT-1) were purchased from the Nation Research Council of Canada. Bovine muscle powder (SRM 8414) and oyster tissue (SRM 1566a) were purchased from the National Institute of Standards and Technology (NIST), U.S. Dept. of Commerce.

[c] μg/g (± 1 standard deviation, expressed as a percentage)

[d] Cervine tissue, the reference values were determinated by ICP-MS following digestion of the sample with 70% HNO_3.

Table 15. Manganese and nickel concentrations (μg/g ± 1 standard error of estimate[a], expressed as a percentage) in zoological certified reference materials (CRMs) and cervine liver and kidney with calibration by standard additions. Reported concentrations have been corrected for analyte contrations (Table 13) introduced by the ruby capped homogenizer.

	Manganese		Nickel	
Matrix[b]	**Experimental**	**Certified[c]**	**Experimental**	**Certified[c]**
DORM-1	1.14 ± 5.97	1.32 ± 19.7	1.33 ± 8.80	1.20 ± 25.0
DORM-2	3.26 ± 4.34	3.66 ± 9.29	17.2 ± 9.8	19.4 ± 16.0
Bovine muscle	0.32 ± 4.58	0.37 ± 24.3	n.d.	0.05 ± 80.0
Oyster tissue	11.78 ± 5.1	12.3 ± 12.2	2.19 ± 10.2	2.25 ± 19.6
Hepatopancreas	24.0 ± 0.53	23.4 ± 4.27	2.29 ± 4.89	2.3 ± 3.01
B4-liver[d]	4.11 ± 0.84	4.40		
B4-kidney[d]	1.57 ± 3.37	1.62		
B10-liver[d]	2.30 ± 2.78	2.73		
B10-kidney[d]	3.39 ± 0.29	3.41		

[a] Standard error of estimate: $SE_{est} = (SE^2_{Y\text{-int}} + SE^2_{slope})^{1/2}$

[b] Defatted dogfish muscle reference materials (DORM-1, DORM-2) and liver hepatopancreas (TORT-1) were purchased from the Nation Research Council of Canada. Bovine muscle powder (SRM 8414) and oyster tissue (SRM 1566a) were purchased from the National Institute of Standards and Technology (NIST), U.S. Dept. of Commerce.

[c] μg/g (± 1 standard deviation, expressed as a percentage)

[d] Cervine tissue, the reference values were determination by ICP-MS following digestion of the sample with 70% HNO_3.

process. The percent discord for [Mn] in these homogenates was appreciably less (mean -6.5 %) reflecting the fact that [Mn] in these tissues was appreciably greater than the 0.39 μg/mL introduced by the homogenizer.

3.3.3 Botanical Materials

The slurry preparation technique with the ruby capping homogenizer was then applied to four botanical CRMs and to five dried animal feeds. The processed fluids were then analyzed for copper, iron and manganese since these metals are monitored routinely in animal feeds. In contrast to the uniform homogenates of zoological materials, the botanical materials resulted in suspensions containing some residues of fibres and particulate matter. Two other solubilizing procedures, prior to homogenization, did not change the characteristics of the homogenate appreciably and were not investigated in detail. In the first pre-homogenization treatment, dried plant tissue, suspended in tris buffer (pH 8.0), was extracted, at 60-65°C, with hexadecyltrimethylammonium bromide and EDTA during 30 min. Alternatively, dried material was incubated at 37°C, with 1% cellulase and 3% macerase/pectinase at pH 5.0 for 2h.

During the subsequent metal determinations by GF-AAS, no attempt was made to resuspend solids, rather, only the supernatant fraction was sampled. The degree of discord [(accepted-experimental)/accepted] between the experimental results and the certified concentrations of the CRMs or the flame-AAS results for the feed samples again was the acceptably small. For [Cu] (Table 16), the mean discord between the experimental results and accepted values was +4.4 ± 11.6% and for [Mn] (Table 17) the mean discord was -1.7% ± 11.5%. By contrast, the mean discord for [Fe] (Table 18) was somewhat greater, -11.6% ± 8.7%, suggesting that all the analyte Fe might not have been extracted into the liquid phase. To test this hypothesis, a fresh aliquot (approximately 0.1g) of each sample was homogenized in the presence of 50 mg disodium EDTA. The resulting determinations of [Fe] in homogenates prepared in the presence of Na_2EDTA are reported in Table 18. For [Fe] in homogenates prepared with Na_2EDTA amended solvent mixture, the mean discord was reduced to -6.0% ± 9.7%. Despite the apparent slight improvement

Table 16. Cu concentrations (μg/g ± 1 standard error of estimate, expressed as a percentage) in botanical certified reference materials and in animal feeds as determined in the supernatant fraction of the slurry suspension sampled immediately or 10 days post preparation. Reported concentrations have been corrected for analyte concentrations (Table 13) introduced by the ruby capped homogenizer process.

Matrix[a]	Experimental[b]	Experimental[c]	Reference[d]
pine needles	3.47 ± 9.1	3.21 ± 8.1	3.0 ± 10.0
corn stalk	6.79 ± 4.5	7.25 ± 8.2	8.0 ± 12.5
apple leaves	5.99 ± 6.3	5.14 ± 6.2	5.64 ± 4.3
corn bran	2.78 ± 8.2	2.19 ± 7.3	2.47 ± 16.2
303-5	3.3 ± 8.4	3.2 ± 3.3	3.5
299-5	19.5 ± 2.6	18.0 ± 8.2	17.5
158-5	21.0 ± 2.4	20.0 ± 5.6	18.5
314-5	15.7 ± 3.5	15.9 ± 3.4	14.1
307-5	79.4 ± 14.4	84.2 ± 7.2	88.4

[a] Standard reference materials composed of pine needles (SRM 1575), corn stalk (SRM 8412), apple leaves (SRM 1515) and corn bran (SRM 8433) were purchased from the National Institute of Standards and Technology (NIST), U.S. Dept. of Commerce.

[b] Supernatant fraction from the slurry suspension was analyzed immediately after preparation.

[c] Supernatant fraction from the slurry suspension was sampled 10 days post preparation.

[d] For CRMs, μg/g ± 1 SD (expressed as a percentage); feed samples were analyzed by flame-AAS following digestion in HNO_3.

Table 17. Mn concentrations (μg/g ± 1 standard error of estimate, expressed as a percentage) in botanical certified reference materials and in animal feeds as determined in the supernatant fraction of the slurry suspension immediately after or 10 days post preparation. Reported concentrations have been corrected for analyte concentrations (Table 13) introduced by the ruby capped homogenizer process.

Matrix[a]	Experimental[b]	Experimental[c]	Reference[d]
pine needles	648.0 ± 1.3	621.4 ± 8.7	675. ± 2.2
corn stalk	15.6 ± 2.8	15.9 ± 13.3	15.0 ± 2.5
apple leaves	51.8 ± 2.2	50.9 ± 4.0	54.0 ± 5.6
corn bran	2.12 ± 5.0	2.11 ± 6.2	2.6 ± 11.4
303-5	14.05 ± 5.6	14.2 ± 6.2	13.9
299-5	51.3 ± 5.5	49.8 ± 12.0	66.3
158-5	111.5 ± 3.8	117.0 ± 9.2	120.0
314-5	40.6 ± 4.7	34.25 ± 7.1	37.6
307-5	204.5 ± 4.6	201.2 ± 7.6	220.1

[a] Standard reference materials composed of pine needles (SRM 1575), corn stalk (SRM 8412), apple leaves (SRM 1515) and corn bran (SRM 8433) were purchased from the National Institute of Standards and Technology (NIST), U.S. Dept. of Commerce.

[b] Supernatant fraction from the slurry suspension was analyzed immediately after preparation.

[c] Supernatant fraction from the slurry suspension was sampled 10 days post preparation.

[d] For CRMs, μg/g ± 1 SD (expressed as a percentage); feed samples were analyzed by flame-AAS following digestion in HNO_3.

Table 18. Fe concentrations (μg/g ± 1 standard error of estimate, expressed as a percentage) in botanical certified reference materials and in animal feeds as determined in the supernatant fraction of the slurry suspension immediately after or 10 days post preparation. Reported concentrations have been corrected for analyte concentrations (Table 13) introduced by the ruby capped homogenizer process.

Matrix[a]	Experimental[b]	Experimental[c]	Experimental[d]	Reference[e]
pine needles	170.2 ± 10.5	176.0 ± 13.0	184.3 ± 5.2	200 ± 5.0
corn stalk	113.8 ± 4.3	111.4 ± 2.4	124.1 ± 3.6	139 ± 10.8
apple leaves	78.94 ± 6.4	75.9 ± 4.3	85.9 ± 8.0	83 ± 6.4
corn bran	11.29 ± 9.7	12.1 ± 8.3	13.4 ± 9.0	14.8 ± 12.2
303-5	68.62 ± 5.9	65.2 ± 6.1	69.4 ± 8.4	78.1
299-5	364.7 ± 8.8	379.1 ± 12.0	371.5 ± 10.1	425
158-5	14.66 ± 9.3	11.5 ± 4.2	14.2 ± 11.0	12.2
314-5	56.05 ± 6.5	53.9 ± 7.2	57.0 ± 5.3	64.7
307-5	921.3 ± 3.6	942.4 ± 14.5	925.1 ± 8.1	1027

[a] Standard reference materials composed of pine needles (SRM 1575), corn stalk (SRM 8412), apple leaves (SRM 1515) and corn bran (SRM 8433) were purchased from the National Institute of Standards and Technology (NIST), U.S. Dept. of Commerce.

[b] Supernatant fraction from the slurry suspension was analyzed immediately after preparation.

[c] Supernatant fraction from the slurry suspension was sampled 10 days post preparation.

[d] Samples were slurried in 20 ml of 1 + 9 (v/v) ethanol-water containing 0.25 % (m/m) tetramethylammonium hydroxide (TMAH) and 50 mg Na_2EDTA.

[e] For CRMs, μg/g ± 1 SD (expressed as a percentage); feed samples were analyzed by flame-AAS following digestion in HNO_3.

(decreased discord) in estimates of [Fe] in homogenates prepared with Na_2EDTA, the larger absolute standard deviations (±10%) associated with these means precludes a conclusion that this additive had any significant influence on iron extraction into the homogenizing solvent. To determine whether Na_2EDTA had any influence on levels of contaminants mobilized during the slurry preparation sequence, solvent blank amended with Na_2EDTA was homogenized then analyzed for metal analytes. The results are recorded in Table 13. The concentrations of Al, Cr and Cu were increased appreciably whereas the concentrations of Cd, Fe, Mn, Pb and Ni were not changed greatly when compared with apparent analyte metal levels in solvent blank plus solvent blank homogenized in the presence of the ruby disk.

3.3.4 Stability

The apparent stability of crude homogenates with respect to metal analytes, was also monitored with time. The supernatant fractions of homogenates were reanalyzed for Cu, Fe and Mn after 10 days of contact, at room temperature in sealed containers, with the particulate fraction. The results are recorded in Table 16-18. The degree of discord for [Cu], [Fe] and [Mn] determinations in the homogenates after 10d was -2.4 ± 9.1% (vs. +4.4% ± 11.6 at day 0), -11.1% ± 8.6 (vs. -11.6% ± 8.7 at day 0) and -7.7% ± 9.6 (vs. -1.7% ± 11.5 at day 0) respectively. Although individual estimates changed slightly with time, there was no appreciable evidence for any consistent change in analyte concentrations in the supernatant fractions. These results indicate that homogenates can be stored, at room temperature, for up to 10 days without any Cu, Fe or Mn segregation.

3.3.5 Selenium Determination

Determinations of selenium were also attempted on the homogenates. However, for either zoological or botanical CRMs, the estimated [Se] was appreciably less than the certified values even for calibrations by standard additions. Also there was no evidence of any analyte loss prior to the atomization stage as judged by the signal graphics software which provided a continuous display of the Se-signal over the course of the furnace

program. For wheat-based feed samples (≈10 μg Se/g), previous studies (Lei and Marshall, 1995) had suggested that virtually all of the Se was protein-bound and that neither selenate, selenite, selenomethionine nor selenocystine was present in detectable quantities. Presumably, protein-bound Se in the botanical CRMs is not liberated efficiently by the ashing cycle of the electrothermal program.

3.3.6 Calibration

Although the analyte determinations reported in Tables 14 and 15 were calibrated by standard additions, there were no significant differences among the slopes of the calibration plots for the regressions of peak area on the quantity of analyte standard added back into each of the matrices (Table 19). Moreover, the slopes of calibration plots for standard additions to solvent blank which had not been homogenized or had been homogenized in the presence or absence of the ruby cap were not significantly different from each other. Thus, there was no evidence for any matrix effect in any of the feed samples, the cervine tissues or the reference materials. Calibrations performed with standards added to solvent blank were used to determine the levels of contamination due to processing. These values proved to be repeatable indicating the external aqueous standards could have been used equally well for calibrations.

3.3.7 Other Options for Capping Modifications to the Homogenizer

For the purposes of comparison, it was of interest to evaluate other possible materials as caps for the flat face of the demountable valve head. Separate disks composed of zirconia, tungsten carbide, or poly-methacrylate, which had been manufactured by grinding and polishing a 6-12 mm diameter grinding balls, were glued temporarily to the flat face of the demountable valve head. Similarly, the sapphire disk was generated from a used HPLC piston. Solvent mixture, 20 mL, was homogenized four successive times (in the presence or absence of the test disk) prior to GF-AAS analysis for Al, As, Cd, Cr, Cu, Fe, Mn, Ni, Pb and Se. Analyte concentrations (Table 20) were expressed as if the solvent had contained 0.100 g of sample. The heavy metal content of the homogenized fluid was,

Table 19. Means of slopes [a] (± 1 RSD) of the best-fit linear regression models for standard additions of Cu, Fe or Mn to solvent blank or to slurries of zoological or botanical CRMs or of animal feeds. Only determinations peformed in the same working day were included in these calculations.

Analyte	Zoological SRMs (n=5)	Botanical CRMs (n=4)	Animal Feeds (n=5)	Solvent Blank [b] (n=3)	Mean (blanks + feeds + CRMs)
Cu (244.2 nm)	2.60 ± 1.3%	3.22 ± 3.8%	3.05 ± 1.3%	3.06 ± 0.3%	3.11 [c] ± 3.4% (n=12)
Fe (386.0 nm)	6.74 ± 2.5%	6.77 ± 2.7%	6.82 ± 1.1%	6.87 ± 1.0%	6.75 ± 1.9% (n=17)
Mn (403.1 nm)	8.38 ± 2.2 %			8.57 ± 4.6%	8.46 ± 3.2 % (n=8)
Mn (279.5 nm)		1.28 ± 1.2%	1.30 ± 1.0%	1.30 ± 2.3%	1.30 ± 1.4 % (n=12)

[a] Slopes were determined from linear regression of the mean peak area (absorbance sec) for 3 replicate determinations of each of ≥4 additions of aqueous standard to the homogenate or to the solvent.

[b] Aqueous analyte standard added to solvent blank or to solvent blank homogenized in the presence or absence of the ruby disk.

[c] Mean of slopes for botanical CRMs + feeds + solvent blanks. [Cu] in zoological CRMS had been determined some 45 d previously and were not included in the calculation.

Table 20. Apparent analyte concentrations (μg/g sample) in 20 ml solvent mixture following various mixing treatments. Concentrations in the homogenized fluid are expressed as if the solvent mixture had contained 0.100 g of sample.

	Aluminum	Arsenic	Cadmium	Chromium	Copper	Iron	Lead	Manganese	Nickel	Selenium
unhomogenized solvent blank	0.32	n.d[a].	n.d.	n.d.	n.d.	0.40	n.d.	n.d.	n.d.	n.d.
4 sequential homogenizations with:										
stainless steel head	42.12		0.02	0 89	15.0	56.94	1.38	2.31	3.57	1 34
polymethacrylate cap	7.92	n.d.	0.02	1.80	n.d.	5.20	n.d.	0.10	n.d.	n.d.
ruby cap	21.52	n.d.	0.03	0.20	0.70	13.99	0.28	0.39	0.11	0.68
sapphire cap	3.64	n.d.	n.d.	1.80	0.80	4.90	n d.	0 10	n.d	0.60
tungsten carbide cap	15.32	n.d.	0.04	4.40	1.40	38.6	2.00	0.40	0.10	n.d.
zirconia cap	0.42	n.d.	0.02	4.00	1.20	15.7	n.d.	0.20	0.10	0 20

[a] n.d. = none detected above the apparent analyte concentration in distilled deionized water to zero the instrument

in all cases, reduced appreciably. Nonetheless, contamination remained appreciable for several elements, even in the presence of the poly-methacrylate or the sapphire cap.

3.4 Conclusions

The results indicate that high pressure homogenization is capable of generating emulsions/dispersions of soft organ tissues, dried animal feeds and zoological/botanical CRMs which can be reliably sub-sampled during 10-d of storage (botanical CRMs, feeds). The elements which can be determined in these slurries include Cu, Cr, Mn, Ni, and Fe. By contrast, estimates of [Se] in biological CRMs were consistently lower than their certified values, suggesting that release of this analyte from the protein matrix during the charring/ashing sequence was inefficient since there was no evidence of any analyte loss prior to the atomization stage as judged by the signal graphics software which provided a continuous display of the Se-signal over the course of the furnace program. The addition of EDTA to the solvent prior to processing did not perceptibly improve the recoveries of iron from either the botanical CRMs or from the animal feeds but did increase the levels of contamination. The presence of the ruby cap on the valve head of the homogenizer, appreciably attenuated but did not eliminate contamination introduced by the processing. Other capping materials, such as tunsten carbide, zirconia, saphire and poly-methacrylate, were also studied. Although ruby was the good choice for the determination of heavy metals in zoological/botanical CRMs and animal feeds, subsequent studies indicated that the sapphire disk and the poly-methacrylate disk minimized the levels of contamination. Sample preparation proved to be rapid (approximately 3 min) and the homogenizer was readily cleaned between samples by processing fresh solvent. In summary, the technique presents a rapid means of sample preparation but, at present, can only be applied to samples which contain analyte levels in excess of the levels of contaminants introduced by processing.

Chapter 4
Determination of Selenium in Plant and Animal Tissue by Slurry Introduction GF-AAS following Enzymatic Digestion and High Pressure Homogenization

4.1 Enzymatic Digestion

In chapter 2 and 3 high pressure homogenization was evaluated for the preparation of quasi-stable dispersions suitable for direct introduction into a graphite furnace-atomic absorption spectrometer. The advantages of this approach to sample preparation were: the ease and speed of the slurry preparation, which required less than 1 min, and the fact that analyte metals were apparently extracted quantitatively into the liquid phase during the preparation so that no analyte segmentation was detected within the slurry even after several days of storage. Certified reference materials (CRMs), frozen liver and kidney and dried animal feeds of botanical origin were analyzed successfully for Cd, Cr, Cu, Mn, Ni and Pb but not for Se. The principal limitation of the high pressure homogenization technique was the quantity of contaminating analyte metals introduced into the sample by the homogenizing operation. Contamination was reduced appreciably, but not eliminated entirely, by capping the flat face of the stainless steel homogenizing valve with a disk of inert material. Suitable materials included ruby, sapphire, zirconia, tungsten carbide and polymethacrylate.

The objectives of the studies described in this chapter were to develop efficient alternative slurry preparation techniques for the determination of Se in biological materials. Homogenization, in combination with partial enzymatic digestion with a crude protease alone or admixed with lipase or cellulase, was investigated as a means of releasing Se-residues from zoological and botanical matrices prior to slurry introduction GF-AAS. Although there have been few reports of the determination of Se in slurried samples (Ebdon and Parry, 1988; Bendicho and Sancho, 1993), a recent report (López-García, *et al.*, 1996) indicates that the approach is promising for this analyte as well. Prolonged enzymatic digestion with a crude protease fraction has been used (Gilon, *et al.*, 1995) to liberate component selenoamino acids from proteins. This approach seemed promising as a pre-homogenization sample preparation.

4.2 Experimental Procedure

4.2.1 Reagents

Tris(hydroxymethylamino)methane (tris) was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA) and aqueous Se solution (1,000 μg/mL, traceable to NIST primary standard) was purchased from SCP Chemical Co. (St-Laurent, Québec).

4.2.2 Samples

- CRMs were purchased from the National Research Council of Canada (NRCC), or the US National Institute of Science and Technology (NIST).
- Samples of animal diet mixtures destined for a zoo were chosen to contain a variety of plant and animal materials including timothy grass, bamboo leaves, whole smelts, cricket chow and a panda bear mixture (contents unspecified). Animal feed supplements were composed of mixed forage crops. Animal feed samples were generously supplied by Dr. E.R. Chavez, Animal Science, Macdonald Campus of McGill University.

4.2.3 Sample Preparation

Samples of certified reference materials (CRMs), dried feeds or supplements (ground, to pass a 0.5 mm screen, in a Tecator Cyclotec sample mill, Tecator AB, Höganäs, Sweden), were accurately weighed (approximately 0.2 g) and added directly to 10 ml of ethanol-0.03M tris(hydroxymethyl)aminomethane (1 + 19 v/v, pH 7.5) containing either 20 mg crude protease (Type XIV, Sigma Chemical Co., St. Louis, MO, USA), 20 mg protease plus 20 mg lipase (Type VII , Sigma Chemical Co.), or 20 mg protease plus 20 mg cellulase (Cellulysin®, Calbiochem-Novabiochem Corp., La Jolla, CA, USA). The resulting suspensions were then processed through the 20 mL capacity flat valve homogenizer (EmulsiFlex Model EF-B3, Avestin, Inc., Ottawa, ON). Each slurried sample was re-processed through the homogenizer three more times. The homogenates, in 50 mL Erlenmeyer flasks were then digested, at 37 °C, with gentle agitation for 4-8h.

4.2.4 Homogenizer

The valve stem of screw cap assembly of the homogenizer was modified by gluing a polished 4 mm diameter x 2 mm thick poly-methacrylate disk manufactured by Spex Inc., Metuchen, NJ, USA. Sample + enzyme suspension was transferred to the sample chamber via the inlet port which was then sealed with a fine-threaded screw cap. The stainless steel piston (connected to a pneumatic multiplier) then forced the fluid through an aperture and the homogenate was collected from the sample outlet. Each sample was re-processed three more times with the valve stem retracted slightly to provide a slightly larger gap setting.

4.2.5 Se-determinations (hydride generation or fluorescence)

Feed samples were dried to constant weight and ground to pass a 1 mm screen. Accurately weighed aliquots of ground feed or freeze-dried fish tissue (approximately 2 g) were digested at room temperature in a perchlorate fume hood with 25 mL of (4 +1 v/v) 70 % HNO_3 $HClO_4$ until gas evolution had ceased, then heated at 80 °C until a clear yellow solution was obtained. The resulting strong acid digests were analysed by hydride generation-AAS or fluorescence of the piazselenol derivative after conversion of the analyte residues to Se(IV). Digestions and analyses by fluorescence were performed by Ms. Denise Gaulin, Animal Science, Macdonald Campus of McGill University.

4.2.6 GF-AAS

Selenium determinations were performed using a hot injection technique on a Varian Model 300 GF-AAS system equipped with an autosampler, pyrolytically coated platform graphite tubes, a conventional Se hollow cathode lamp and Zeeman effect background correction. Ashing - atomization curves were generated for Se standard in the presence/absence of co-injected biological sample. At temperatures <2,300 °C, the Se atomization signal was somewhat broadened by the presence of biological materials but was sharpened (and did not tail) for atomizations at 2,400 °C. In the presence of the palladium - citric acid modifier, no loss in the Se signal was observed at an ashing temperature $\leq$1,400 °C. Analytical operating parameters are presented in Table 21.

Table 21. Furnace operating parameters[a] for determinations of selenium.

	Selenium
wavelength (nm)	196.0
lamp current (amps)	10
slit width (nm)	1.0
injection T (°C)	60
pre-injection	yes
last dry step, 10s (°C)	250
charring sequence	10 s ramp to 1,400 °C, 40 s hold.
cool down	none
atomisation	0.6 s ramp to 2,400 °C, 5.0 s hold
measurement time	5.6 s
matrix modifier	5 µL [0.5% (m/m) Pd, 2.5% (m/m) citric acid] for 10 µL sample

[a] Each step of the furnace programs (with the exception of the read step) was performed in the presence of Ar purge gas (3 L/min).

4.2.7 Calibration

Calibrations were performed by the method of standard additions (SA) or by the method of external standards (ES) as described in section 2.2.4 (page 40).

4.3 Results and Discussion

4.3.1 Enzymatic Digestion

Previous attempts to determine Se in biological materials by slurry introduction GF-AAS of high pressure homogenates (Chapter 3) were not successful using a variety of furnace programs. Yet there was no evidence of any analyte loss prior to the atomization stage as judged by the signal graphics software which provided a continuous display of the Se-signal over the course of the furnace program. Since a high proportion of the analyte element in biological materials is considered to be protein-bound, it was decided to evaluate partial enzymatic hydrolysis as a means of liberating bound analyte residues. Somewhat arbitrarily, it had been decided initially to attempt to develop a single combination of mixed enzymes which hopefully would be applicable to all sample matrices. Previous studies (Forsyth and Marshall, 1983) had indicated that a combination of crude proteases and lipases efficiently hydrolysed avian egg yolk. Initial studies were limited to this combination of enzymes. The mixture of crude enzymes was suspended in 10 mL tris buffer (pH 7.5) then sequentially passed four times through the poly-methacrylate capped homogenizer to furnish a digestion control homogenate. Relative to a distilled deionized water blank, this "zoological" control sample contained 0.11 mg/kg ± 12.6% Se and 0.10 mg/kg ± 11.3% Se after 4 and 8h of digestion respectively (Table 22) when it was assumed that the digests had contained 0.200g of sample. Similarly, a control homogenate composed of protease alone contained 0.044 mg/kg ± 12.6% after 4h. A crude cellulase was substituted for the lipase in the enzyme mixture and the digestions were performed in analogous fashion to furnish alternative enzymatic digestion control samples. The "botanical" control samples contained 0.048 mg/kg ± 14.2% and 0.051 mg/kg ± 12.0% after 4 and 8h of digestion respectively (Table 22) and the solvent blank + protease alone contained 0.044 ± 12.6% again assuming that the digests had contained 0.200g of sample. Thus, virtually all of the Se in control digests originated with the lipase

Table 22. Selenium concentrations (± 1 RSD based on 3 replicate samples) in certified reference materials determined immediately after 4 or 8h of enzymatic digestion or following digestion plus 10d storage.

Matrix	4h Digestion	8h Digestion	4h Digestion + 10d Storage	8h Digestion + 10d Storage	Certified Concentration
Solvent blank + protease + lipase	0.11 ± 12.6%	0.10 ± 11.3%	0.12 ± 10.2%	0.10 ± 13.6%	
Solvent blank + protease		0.044 ± 12.6%			
Solvent blank + protease + cellulase	0.048 ± 14.2%	0.051 ± 12.0%	0.040 ± 23.2%	0.045 ± 12.2%	
Oyster Tissue [a]	2.18 ± 12.7%	2.27 ± 11.6%	2.16 ± 11.4%	2.15 ± 12.6%	2.21 ± 10.9%
DORM-2 [a]	1.38 ± 9.2%	1.35 ± 11.6%	1.34 ± 6.6%	1.33 ± 11.0%	1.40 ± 6.4%
Bovine Muscle [a]	0.067 ± 22.6%	0.066 ± 20.5%	0.067 ± 20.2%	0.070 ± 21.4%	0.076 ± 13.2%
Apple Leaves [b]	0.043 ± 12.7%	0.045[c] ± 19.9%	0.047 ± 19.5%	0.041 ± 12.7%	0.050 ± 18.0%
Corn Bran [b]	0.034[c] ± 12.5%	0.036 ± 14.9%	0.033 ± 15.3%	0.036 ± 16.5%	0.045 ± 17.8%
Corn Stalk [b]	n.d.[d]	n.d.[d]	0.025[c] ± 47.0%	0.011[c] ± 52.2%	0.016 ± 50.0%

[a] Reported concentrations have been corrected for the [Se] in the protease + lipase control homogenate.
[b] Reported concentrations have been corrected for the [Se] in the protease + cellulase control homogenate.
[c] No [Se] above the LOD (0.010 µg/g) was was detected in one of the three aliquots.
[d] No [Se] above the LOD (0.010 µg/g) was was detected in any of the three aliquots.

and/or the protease. Based on $3S_r$/slope, the corresponding method limit of detection (LOD) for digestions with mixed protease lipase, with protease cellulase and with protease alone were 0.020, 0.010 and 0.010 μg/g.

4.3.2 Enzymatic Digestion Time

In preliminary experiments, three biological CRMs and one animal feed, suspended in 10 mL tris-buffer, were digested with a combination of protease and lipase for up to 16h at 37 °C. Each suspension was homogenized immediately prior to or directly after digestion then analysed by GF-AAS. The results are presented in Figures 3 and 4. The TORT-1 results and the animal feed results of Figures 3 and 4 respectively have been displaced by 0.4h for clarity of presentation. For all four substrates, homogenization prior to digestion (closed symbols) generally resulted in higher recoveries relative to homogenization post digestion (open symbols) although the differences were only rarely statistically significant. Moreover, the differences tended to decrease with longer digestion time. Presumably, homogenization initially exposed more of the protein component to the enzyme. On the other hand, homogenization post digestion immediately prior to GF-AAS did not significantly improve the precision of the determination (as judged by the relative standard deviation associated with three replicate measurements performed on each of three digests). In general, there was a gradual but small improvement in precision with increased length of digestion (somewhat more evident in the plant samples of Figure 4). For both the dogfish muscle (DORM-1) and the liver hepatopancreas (TORT-1) marine tissue samples of Figure 3, 4h of digestion at 37 °C were sufficient to liberate the Se quantitatively. The results after 16h would seem to indicate that hydrolysis of plant samples was virtually complete at 8h of hydrolysis (Figure 4). After sampling for GF-AAS, the plant digests were stored at 4 °C for 5d then reanalysed. No effort was made to resuspend solid materials, instead a portion of each supernatant fraction was transferred directly to the sampling cup. The recovery of Se from each of the supernatant fractions was quantitative (Figure 5) indicating that the crude protease was active at the storage temperature and that there was no apparent segmentation of the Se residues between the

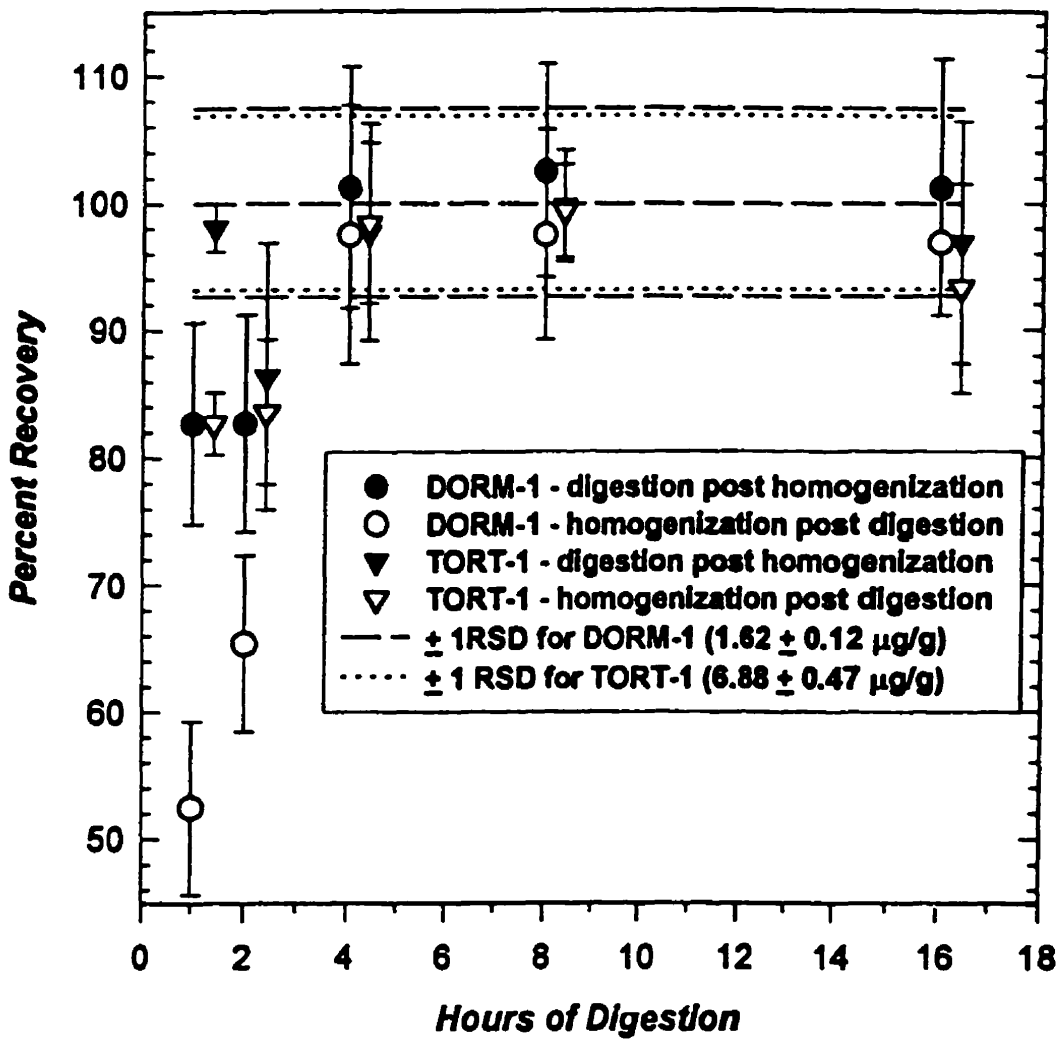

Figure 3. Variation in percent recovery of Se (± RSD based on three replicate samples) from DORM-1 or TORT-1 certified reference materials *vs.* hours of enzymatic digestion with protease plus lipase prior to (open symbols) or post (closed symbols) high pressure homogenization. For clarity of presentation, the TORT-1 results have been displaced by 0.4h.

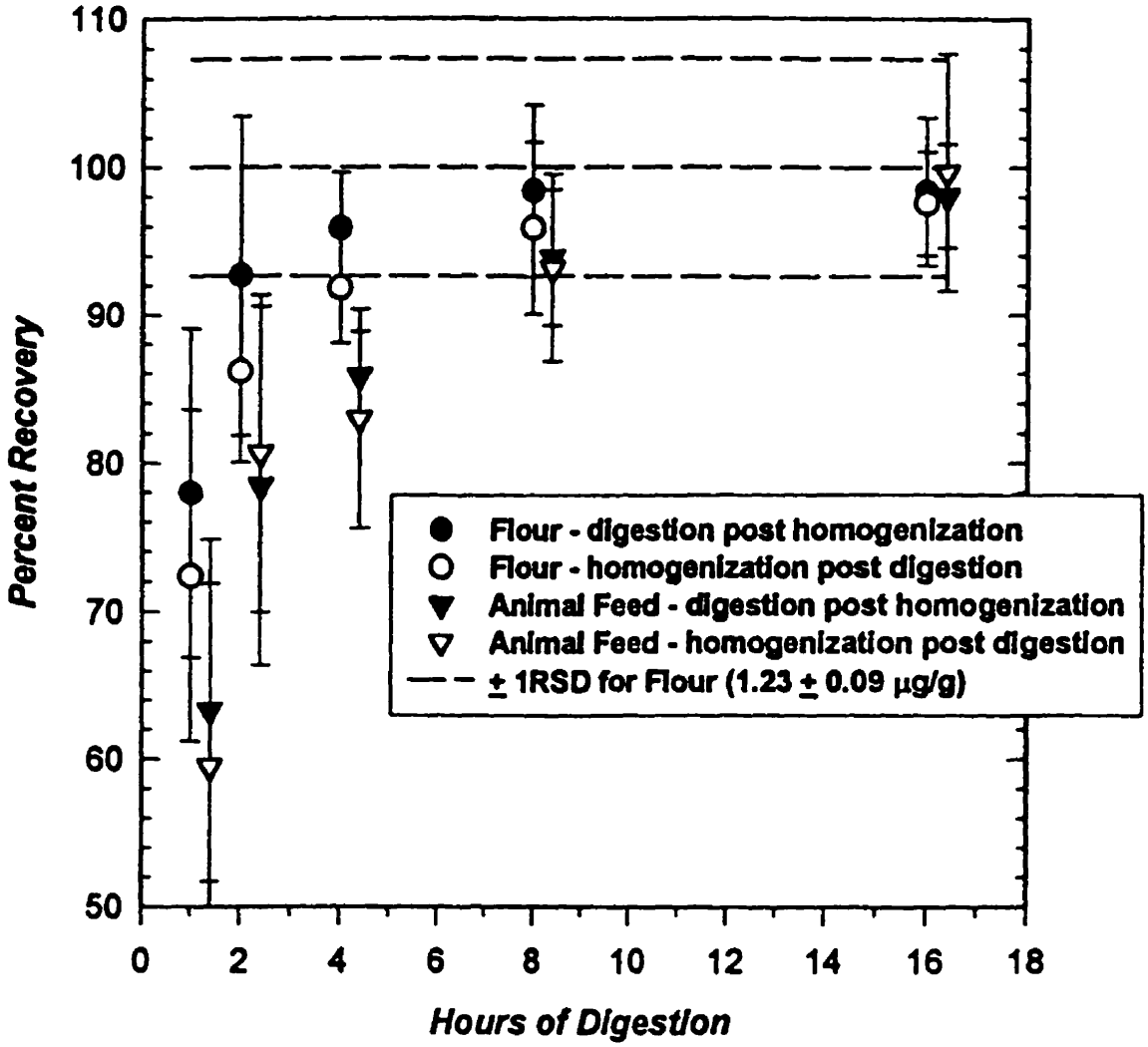

Figure 4. Variation in percent recovery of Se (± RSD based on three replicate samples) from Durham wheat flour (NIST SRM 8436) or a ground animal feed sample *vs.* hours of enzymatic digestion with protease plus cellulase prior to (open symbols) or post (closed symbols) high pressure homogenization. For clarity of presentation, the animal feed results have been displaced by 0.4h.

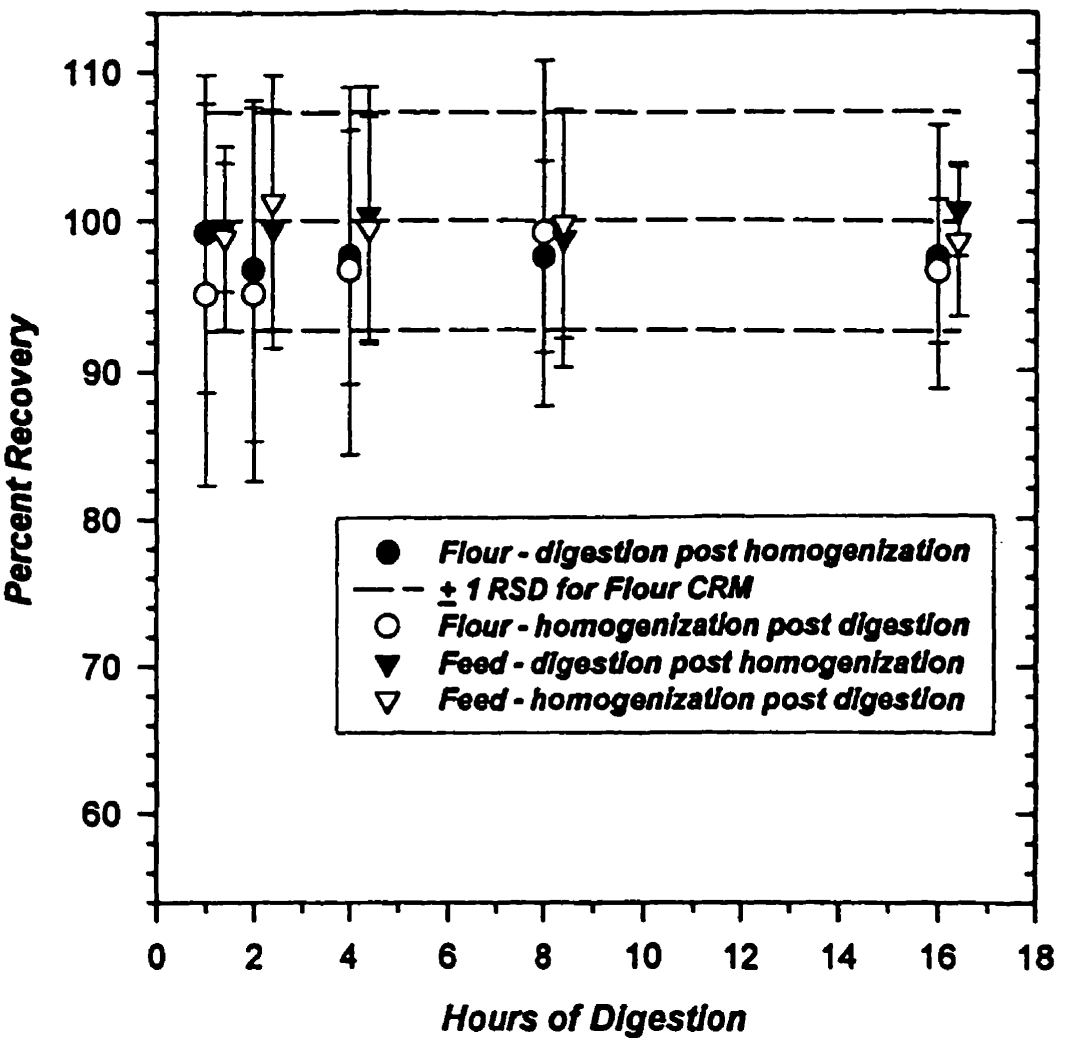

Figure 5. Influence of storage subsequent to slurry preparation on percent recovery of Se (± RSD based on three replicate samples) in Durham wheat flour (NIST SRM 8436) or a ground animal feed sample. For clarity of presentation, the animal feed results have been displaced by 0.4h.

liquid and solid phases of the crude digest. Somewhat surprisingly, the short-term repeatability of the procedure was not improved by the storage as evidenced by the S_r associated with replites. Repeatability continued to be somewhat improved for longer digestions at 37 °C.

4.3.3 Certified Reference Materials

Three replicate aliquots of each of six other certified reference materials were homogenized then digested for 4 or 8h prior to GF-AAS. The results, corrected for the [Se] in the appropriate zoological or botanical control digest are presented in Table 22. The digestion of the three biological CRM homogenates (liver hepatopancreas , dogfish muscle and bovine muscle) provided estimates which were not significantly different from their certified Se concentrations. However, the lower concentrations in the botanical CRMs (apple leaves, corn bran and corn stalk) resulted in estimates which, occasionally, were not different from the control concentrations. In the latter cases, the certified Se concentrations were less than the [Se] in control digests. Storage of the digests for 10d at 4 °C did not change the measured concentrations of analyte significantly (Table 22).

4.3.4 Dried Plant Materials

Three aliquots (approximately 0.2g) of each of eight dried ground feed supplements consisting of mixed forage crops were suspended in 10 mL buffer, homogenized then digested with the protease-cellulase combination for either 4 or 8h prior to Se determination by GF-AAS. Again, there was good agreement between the results (Table 23) for slurry introduction GF-AAS following 4 or 8h of enzymatic digestion and a single fluorescence determination following strong acid digestion and piazselenol formation. Storage of the digests for 10d at 4 °C did not change the measured concentrations of analyte significantly (Table 23). In only one feed supplement (sample 307-5, in which [Se] was appreciably less than in the botanical control digest) were the results of the two methods discordant.

Table 23. Selenium concentrations[a] (± 1 RSD for triplicate determinations of 3 replicate samples) in dried ground plant materials determined by piezselenol formation, immediately after 4 or 8h of enzymatic digestion or following enzymatic digestion plus 10d storage.

Sample	4h Digestion	8h Digestion	4h Digestion + 10d storage	8h Digestion + 10d storage	Strong Acid Digestion + Fluoresence
Solvent blank + protease + cellulase	0.048 ± 14.2%	0.051 ± 12.0%	0.040 ± 23.3%	0.045 ± 12.2%	
9-3	1.29 ± 10.6%	1.24 ± 9.9%	1.28 ± 8.1%	1.25 ± 9.0%	1.31
6-3	1.31 ± 12.3%	1.19 ± 9.0%	1.25 ± 9.1%	1.20 ± 8.9%	1.23
7-3	1.04 ± 12.3%	1.15 ± 9.0%	1.11 ± 6.6%	1.18 ± 6.5%	1.17
546-3	0.79 ± 8.2%	0.79 ± 11.1%	0.80 ± 9.6%	0.79 ± 10.5%	0.81
158-5	0.53 ± 9.2%	0.57 ± 8.9%	0.49 ± 11.0%	0.55 ± 7.2%	0.59
299-5	0.44 ± 13.7%	0.42 ± 8.2%	0.45 ± 18.2%	0.40 ± 10.7%	0.43
314-5	0.14 ± 16.0%	0.14 ± 13.2%	0.14 ± 20.8%	0.13 ± 10.7%	0.13
307-5	0.021 ± 23.5%	0.022 ± 24.1%	0.022 ± 24.2%	0.021 ± 20.9%	0.03

[a] Reported concentrations have been corrected for the [Se] in the protease + cellulase control homogenate

4.3.5 Matrix Effect

No matrix effects for Se determinations in any of the samples were detected. The slopes of the best fit regression lines for standard additions to homogenized protease-lipase or protease-cellulase enzyme suspensions in tris buffer, in the presence or absence of DORM-1, TORT-1, wheat flour, corn bran, or apple leaf CRM or to five of the feed supplements varied by less than 11% (relative standard deviation) provided that calibrations/determinations were performed on the same day. This observation suggested that a single calibration by standard addition(s) tc the enzyme mixtures would suffice to determine Se in any of the samples.

4.3.6 Fresh Fish Tissue

A single calibration curve generated by adding aqueous Se-standard to the botanical control homogenate was then used to determine the Se-content of freeze-dried freshwater fish filets which had been frozen fresh or boiled to simulate cooking following common native practice. Aliquots of the freeze-dried materials were digested enzymatically for 4 or 8h then analyzed by GF-AAS or digested with strong acids then analysed by hydride generation AAS (Table 24). Boiling the filet prior to freeze drying did not inhibit the enzymatic release of Se-residues from the matrix but apparently lowered the [Se] in the cooked product. There were no significant differences between the results after 4 and 8h of digestion or between GF-AAS and hydride generation results. However, the precision associated with replicate enzymatic digestion - GF-AAS Se-determinations (mean relative deviation nearly 15 ± 2%) was appreciably worse than the precision associated with hydride generation determinations (mean, 7 ± 4%) but typical of the replicate determinations of other experiments (mean relative deviation for the 36 determinations of Table 22, 14.4 ± 0.3, and 12.6 ± 0.5 for 29 of the determinations of Table 23). Thus, short-term repeatability (i.) was not adversely affected by the use of the single calibration curve but (ii.) can be anticipated to be somewhat degraded relative to other conventional procedures for Se-determination.

Table 24. Selenium concentrations (μg/g ±1 SEE[a]) in freeze dried fresh or boiled fish filet following 4 or 8h digestion with protease and lipase and GF-AAS.

Sample	4h Enzymatic Digestion	8h Enzymatic Digestion	Hydride Generation[b]
Lake trout (boiled)	3.56 ±20.9%	3.48 ±14.1%	3.60 ± 8.0%
Lake trout (fresh)	1.86 ± 11.9%	2.12 ± 13.2%	1.69 ±10.1%
Northern Pike (fresh)	2.48 ±16.6%	2.51 ±12.5%	2.75 ± 2.9%

[a] SEE, standard error of estimate based on three replicate determinations of three separate digests

[b] ± 1 RSD based on duplicate determinations. These determinations were performed by Dr. H. M. Chan, Centre for Nutrition and the Environment of Indigenous Peoples (CINE), McGill University.

4.4 Conclusions

The principal advantages of the enzymatic digestion procedure are its simplicity and speed relative to conventional acid digestion and the fact that it can be performed unattended. The conditions of digestion do not appear to be critical and there was no tendency of the liberated Se-residues to segregate within the resulting suspensions.

In Chapter 2, this technique of high pressure homogenization was demonstrated to be capable of generating emulsions/dispersions of CRMs of animal origin or fresh soft tissue which could be reliably sub-sampled and analysed by GF-AAS for Cd, Pb or Cu. In Chapter 3, the technique was extended to generate emulsions/dispersions of dried animal feeds and zoological/botanical CRMs which could be reliably sub-sampled during 10-d of storage and determine for Cu, Cr, Mn, Ni or Fe using a modification of ruby disk capping to the homogenizer. This modification appreciably decreased but did not eliminate contamination introduced by the homogenizing processing. In Chapter 4, this technique was combined with enzymatic digestion successfully to determine selenium in plant and animal tissue.

Even though this technique was applied to the determination of up to nine elements in both fresh and dried tissue of animal or plant origin, limitations remain. The method limit of detection remains compromised by the heavy metal contamination introduced by the homogenizing valve (even in the presence of an inert capping material). Certain of these capping materials, notably the poly-methacrylate, were characterized by limited lifetimes under the impact of processed fluid. Nonetheless there remains a wide scope for further improvements to this technique.

Chapter 5

A Silica T-tube Interface for the Determination, by AAS, of Cadmium, Copper Lead, Zinc, Mercury and Siliver in Flowing Liquid Streams

5.1 Silica T-tube Interface

As an approaches to the determination of the different physico-chemical forms (chemical species) of an analyte element within a sample matrix, a silica T-tube interface can be used to couple a flowing stream of liquid from an automated chromatographic separation with detection by AAS. As a result, the atomic spectrometer becomes a dedicated detector for the liquid chromatography. In an earlier design (Blais, *et al.*, 1990), liquid eluate was nebulized by thermospray effect into an expanded section of the lower T-tube which served as a pyrolysis chamber. The pyrolysis was effected in a hydrogen-rich atmosphere maintained by flow of O_2 and H_2 (which enter this chamber via separate gas inlets). The resulting pyrolysis products were entrained by the expanding gases into the upper optical tube which was interposed in the optical beam of the spectrometer. This design works very well with organic-rich mobile phases. There is, however, a new design for the on-line techinque which has several modifications and a simplification. In this newest design, provision is made to heat the outer skin of the pyrolysis chamber and the optical tube separately. To date, studies (Momplaisir, *et al.*, 1994) of the performance of the new device have been restricted to the detection of As and Se compounds in HPLC column eluate. The objectives of this study were two fold: to assess the performance characteristics of this new device for the detection of those elements which are not considered to form volatile hydrides of sufficient stability to be analytical useful such as Zn, Cd, Pb, and Ag in the flowing liquid streams and if successful, to apply the interface to the separation and detection of polypeptide/protein bound analyte metals.

5.2 Materials and Methods

5.2.1 Apparatus

The instrument consisted of a Varian Star 9000 HPLC pump, a six-port manual rotatory injection valve, a silica T-tube interface and a Varian AA5 atomic absorption spectrometer equipped with a deuterium background corrector. Transient instrumental responses [flow injection (FI) mode] were recorded on a Spectra Physics Model 4370 recording integrator. The spectrometer operating parameters used in these studies are recorded in Table 25. A normal hollow cathode lamp was used in each case and in all cases the operator-selectable slit-width was set at its widest setting, normally 1 nm.

5.2.2 Installation of the Interface

The all-quartz interface [Fig. 6(a)] was a modified T-tube design consisting of an upper optical tube (A, 12 x 0.9 cm i.d.) mounted within the optical beam of the AA spectrometer and a lower sample introduction tube (total length, 13 cm). The liquid eluate, contained within a 25 cm x 0.05 mm i.d. capillary silica tranfer line (F), was nebulized by thermospray effect into a pyrolysis chamber (B, 4.5 x 0.9 cm i.d.) which formed an expanded section of the sample introduction tube. The capillary line was supported by a quartz guide tube (G, 0.32 cm o.d.) which extended concentrically inside the thermospray tube (C, 6 x 0.4 cm i.d.) past the coil of high resistance heating wire (eight turns of 22-G Kanthal A-1 wire, 4 Ω/m). The thermospray tube heating coil served to superheat the liquid mobile phase contained within the capillary transfer line. The position of the guide tube within the thermospray tube and of the tip of the capillary transfer line within the guide tube were fixed with a 0.64-0.32 cm and a 0.32-0.16 cm stainless-steel Swagelok reducing union, respectively, and made gas tight with nuts [J, Fig. 6(b)] and Vespel ferrules [K, Fig. 6(b); Chromatographic Specialites, Brockville, Ontario, Canada]. The 0.64-0.32 cm reducing union [H, Fig. 6(b)] had been drilled out so as to form a collar around the guide tube and was fitted with a separate O_2 gas entry-port fabricated by silver soldering a 3 cm x 0.16 mm i.d. section of stainless-steel HPLC tubing into a 3 mm wide hole drilled into the side of the modified union.

Table 25. Atomic Absorption Spectrometer Operating Parameters

Analyte	Wavelength (nm)	Lamp Operating Current (Amperes)
Cd	228.8	7
Cu	324.8	4
Pb	217.0	9
Hg	253.7	5
Zn	213.9	6
Ag	328.1	4

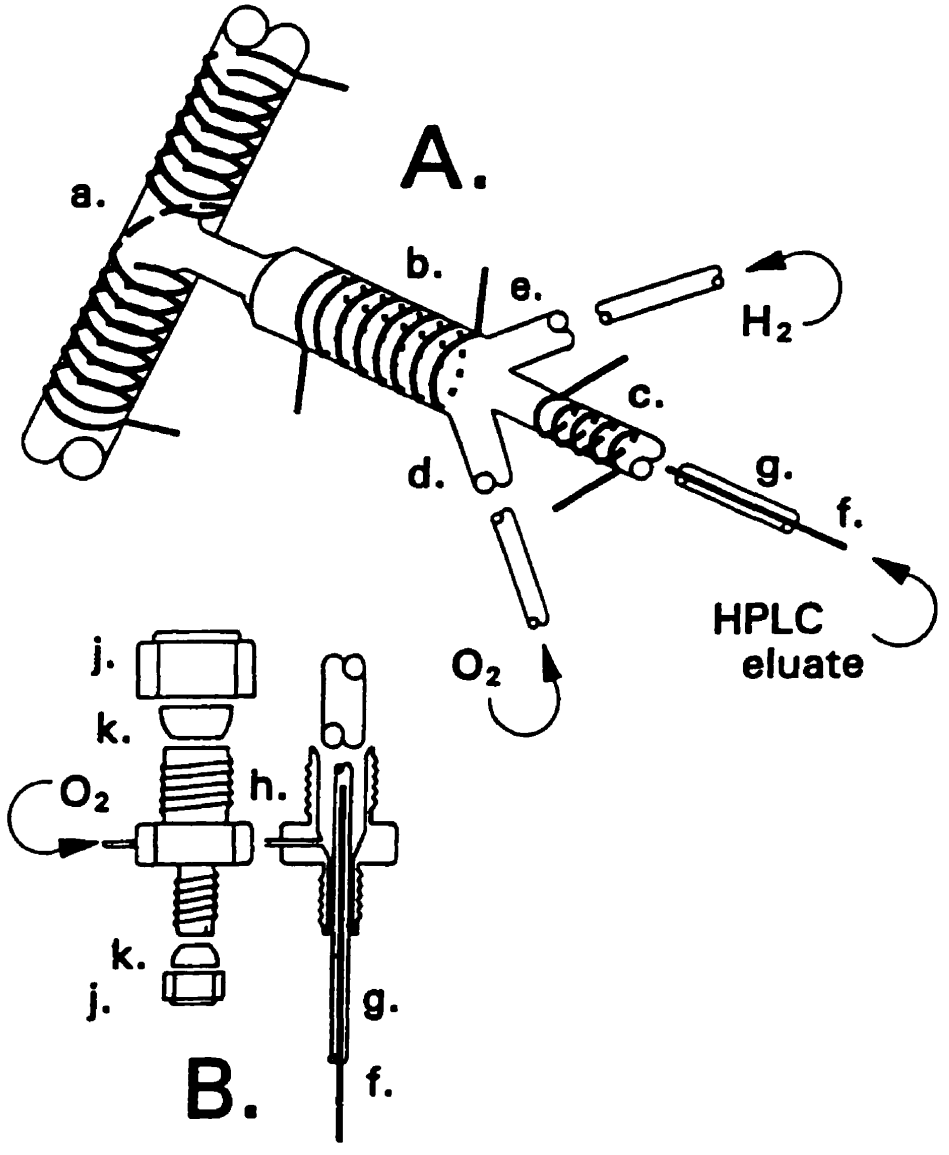

Figure 6. (A) All quartz T-tube interface comprising an upper optical tube, (a), and a lower sample introduction tube which consists of: (b), a pyrolysis chamber which is heated radiatively with a 8-turn coil of high resistance heating wire and is fitted with gas inlets for O_2 (d), H_2 (e) and (c), a thermospray tube jacketed with a separate 6-turn coil of heating wire. (B) Positioning of the ends of the transfer line and guide tubes is facilitated with a stainless-steel reducing union (h) whcih had been drilled out to accept a 1/8" O.D. quartz tube and fitted with a separate side arm, stainless-steel tube to serve as a supplementary gas inlet (j and k, nuts and Vespel ferrules, respectively)

5.2.3 Pyrolysis Process within the Interface

The pyrolysis process was supported by flows of O_2 and H_2 which were added *via* separate gas entry ports [(d) and (e), 6 cm x 0.64 and 0.4 i.d., respectively] which met the base of the pyrolysis chamber at an angle of approximately 45°. These gases were added to the interface *via* flow controllers, 0-500 mL and 0-2.5 L capacity, respectively (Matheson, Whitby, Ontario, Canada), connected to quartz insert tubes (8 x 0.32 cm o.d.) which were extended inside the gas entry ports to the base of the pyrolysis chamber. The inserts were constricted to a 0.2 cm orifice at the exit end, positioned within the gas entry port with Swagelok 0.64-0.32 cm reducing unions and made gas-tight with Vespel ferrules.

5.2.4 Heat Applied to the Surface of the Interface

Separate heating coils, fashioned from 22-gauge Kanthal high-resistance heating wire, were wrapped around the pyrolysis chamber and around the optical tube. The coils which were separately energized by an alternating current rectified by a variable transformer (Variac) were wrapped in refractory wool (Fibrefax, The Carborundrum Company, Niagara Falls, NY, USA) and encased in a shaped firebrick which was held in place with a hose clamp.

5.2.5 Operation of the Interface

To initiate the operation of the device, the pyrolysis chamber and thermospray tube were pre-heated to operating temperatures, then the H_2 flow rate was increased to 700 ml/min and the gas was ignited by holding an open flame to each exit of the optical tube. The flow of O_2 was initiated and slowly increased to the optimum flow rate of 50 mL/min had been reached. The flow rate of H_2 was decreased to cause the visible flames to recede within the interface (at approximately 150 mL/min), then again increased to the optimum interface operating conditions. The capillary line was positioned within the thermospray tube and finally the flow of mobile phase was rapidly ramped from 0 to the desired flow rate. To extinguish the diffused flame within the pyrolysis chamber, the flow of mobile phase was stopped, the capillary transfer line was withdrawn from the guide tube and the

O_2 flow to the pysolysis chamber was stopped. Finally the H_2 flow was interrupted and the interface was permitted to cool to room temperature.

5.2.6 Caution

The order of the procedural steps for igniting and extinguishing the pyrolysis flame should be followed rigidly. The interface should be positioned below an efficient hood capable of venting the exit gases directly to the outside.

5.2.7 Reagents

Aqueous metal standards were prepared by serial dilution with distilled, de-ionized water of concentrates prepared from analytical-reagent grate soluble salts [$Cd(NO_3)_2$, $ZnCl_2$, $Pb(NO_3)_2$, $Cu(NO_3)_2$, $Hg(NO_3)_2$ and $AgNO_3$].

5.3 Results and Discussion

5.3.1 Modification of the Interface

In the earlier design (Blais, *et al.*, 1990), the pyrolysis chamber was contained in a separate side-arm attached to the lower T-tube and atomization of the pyrolysis products was achieved by entraining the product gases through a separate micro-diffusion flame maintained just upstream from the optical tube. Within the current device, the atomization and pyrolysis processes are combined within the lower T-tube. The principle advantage of this design is its compatibility with volatile buffers contained either aqueous or organic mobile phases.

5.3.2 Optimization of the Operating Parameters

The experimental approach to the optimization of the interface operating parameters for the determination of Cd, Cu, Hg, Pb and Zn was to use a surface-response methodology. Since the eventual aim of these studies is to develop an HPLC detector, the peak area of the resulting transient AAS response to the injection of an aqueous solution of a soluble salt containing the analyte was chosen as the dependent variable to be optimized. Interface operating parameters, which were anticipated to influence the peak

area of the AAS response, included the mobile phase flow rate and the skin temperatures of the thermospray region, the pyrolysis region and the optical tube, as well as the length and inner diameter of the silica transfer-line. In preliminary experiments, it was determined that heating of the pyrolysis chamber, the optical tube and/or the thermospray tube above a critical temperature had only a minor and typically detrimental effect (increased noise) on the magnitude of the AAS response to injected Cd^{2+}, Cu^{2+} or Zn^{2+}. Only for Pb was the instrumental response improved by heating the optical tube to approximately 900°C. For the determination of Cd, Cu, Hg and Zn, the heating-coil of the optical tube was energized only slightly in order to minimize condensation, and the heating-coil around the pyrolysis chamber was maintained constant at 20 V. The two operating varibles which influenced peak area appreciably were the flow rates of H_2 and O_2 to the pyrolysis chamber. Although response surface methodologies are more time-consuming to perform than simplex optimization procedures, it was anticipated that the resulting mathematical models would provide supplementary information on the operation of the interface. In all cases, there was no significant lack of fit of the mathematical model to the analytical data at the 95% level of confidence.

5.3.3 H_2-rich Pyrolysis Reaction

With water as the mobile phase, a maximum response to each of the four analytes was achived with a H_2-rich mixture of support gases (Figure 7). For these response surface plots, the flow rate of O_2 increase from the centre of the circle to the circumference, the H_2 flow rate increases around the circumference in a counter-clockwise direction and analyte peak area increases with height above the circle. These plots clearly indicate that for each element (Cd, Cu, Pb and Zn), the detector response is appreciably more sensitive to variations in the flow rate of O_2 than to the flow rate of H_2 to the pyrolysis chamber, but that, overall, the response is only moderately sensitive to changes in these parameters. This operating characteristic of the interface is very desirable from the point of view of repeatability. The flow rates of support gases, which are predicted to result in analyte response maxima, are more readily visualized with contour maps generated from the same mathematical models (Figure 8). As indicated by these maps of

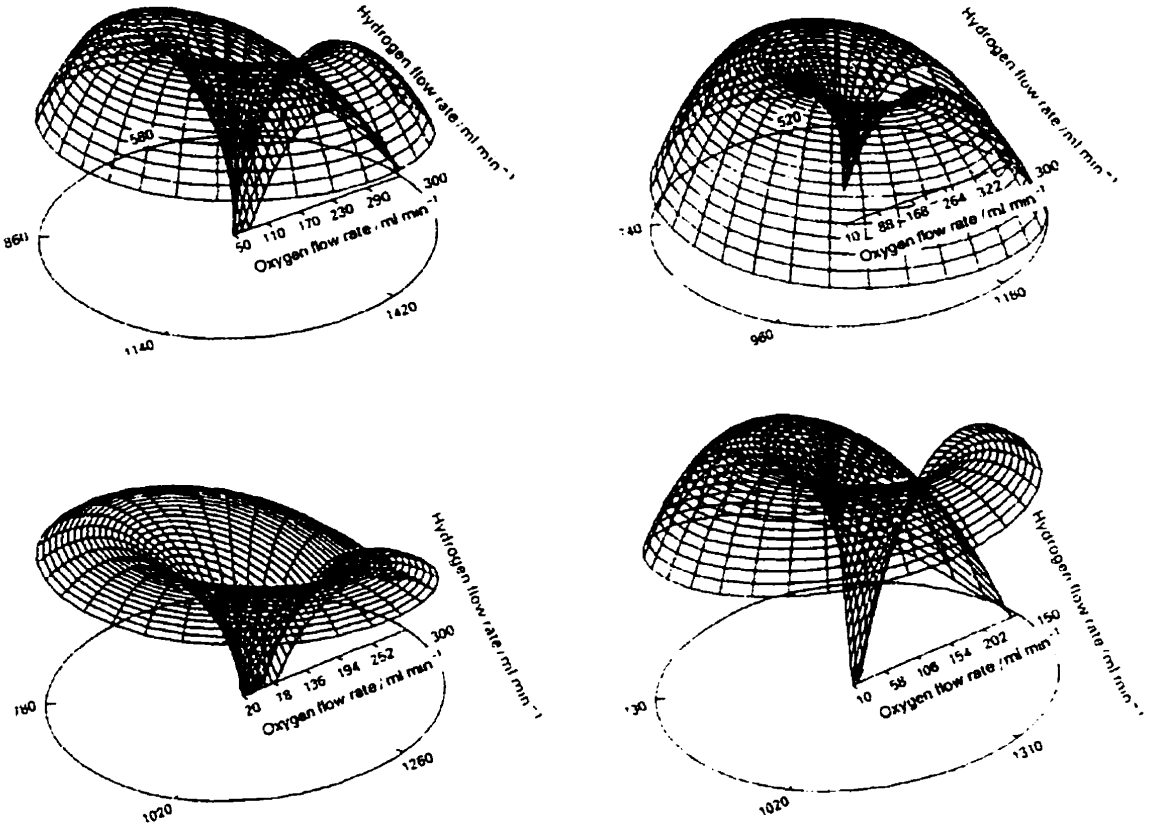

Figure 7. Response surface plots of the predicted AAS signal (peak area) for (a) Zn; (b) Cd; (c) Cu and (d) Pb as a function of the flow rate of O_2 (radius) and H_2 (circumference) support gases to the pyrolysis chamber of the interface

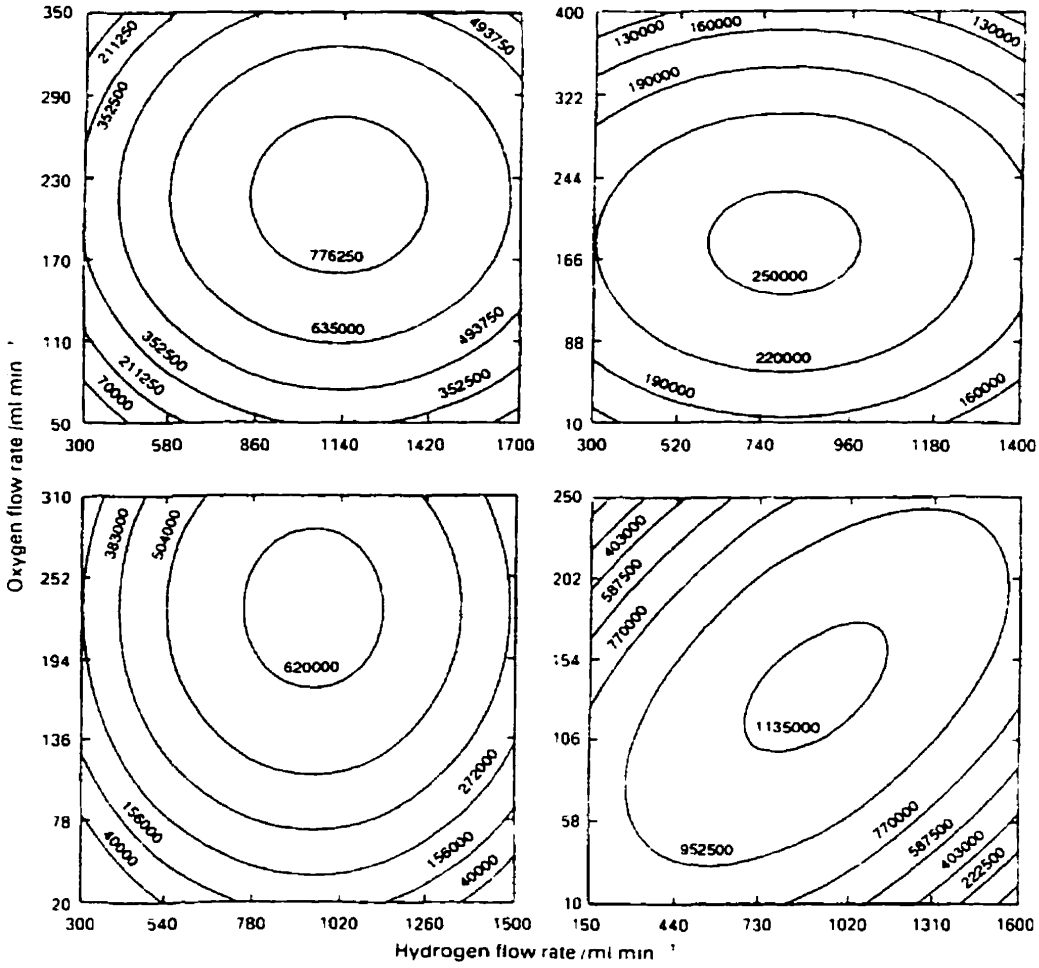

Figure 8. Contour maps of the predicted AAS signal (peak area) for (a) Zn; (b) Cd; (c) Cu and (d) Pb as a function of the flow rate of O_2 and H_2 support gases to the pyrolysis chamber of the interfce.

the detector response as a function of O_2 and H_2 flow rates to the pyrolysis chamber, a maximum for peak area was observed in each case. Whereas the scales of the horizontal axes of these plots are approximately equivalent, the ordinate scales have been chosen to span 5-6 contours of peak area. The optimal flow rates from the predictive models are recorded in Table 26. Interestingly, for the determination of Cd, Cu, Pb, Zn and Ag, the optimal predicted total flow rate of gases fell within a relatively narrow range (961-1346 mL/min), which was appreciably less than the optimal flow rates for either As (2050 and 170 mL/min of H_2 and O_2, respectively) or Se (2000 and 300 ml/min) in a water-based carrier, which had been observed previously (Momplaisir, *et al.*, 1994) with this interface. Moreover, for different elements, the predicated optimal ratio of H_2 to O_2 flow rates varied only slightly (between 4.1 and 5.7, Table 26) and in each case was appreciably greater than the stoichiometric requirement for complete combustion of the support gases. These results indicate a second role for the H_2 support gas, possibly as a source of hydrogen radicals which might serve to accelerate the decomposition of pyrolysis products to form free atoms of analyte. Alternatively, these radicals might capture oxyradicals, decreasing the formation of analyte oxide(s) and/or hydroxide(s).

5.3.4 Methodology to Optimize the Operation of the Interface

Whereas the response surfaces for Zn^{2+} and Cu^{2+} were moderately sensitive to both O_2 and H_2, the AAS response for Cd^{2+} was distinctly less sensitive to the flow rates of these gases (approximately 3-fold as judged by the magnitude of the contour intervals of the contour intervals of Figure 8) and of the two gases, the response was less sensitive to the flow rate of H_2 than of O_2. Of the four analyte metals, the response surface for Pb^{2+} was the most sensitive to changes in the flow rates of support gases (approximately 2-fold greater than either Zn^{2+} or Cu^{2+}). Using an analysis of the approach of steepest ascent, the contour map for Pb^{2+} also indicated that the ratio of H_2 : O_2 flow rates is a variable which appreciably influences analyte response. By contrast, the contribution of the interaction term (the cross-product of the O_2 flow rate x H_2 flow rate) to the optimized model for the other three elements was minor. Collectively, these observations indicate that, at least for

Table 26. Optimized interface operating conditions

	Optimal	Flow Rates	(mL/min)	
Analyte	**O_2**	**H_2**	**Total**	**H_2 :O_2Ratio**
Cd	178	785	961	4.4
Cu	229	950	1179	4.1
Pb	135	770	905	5.7
Zn	219	1127	1326	5.2
Hg	0	0	0	-
Ag	340	895	1235	2.6

these relatively volatile elements, a H_2-rich atmosphere is essential for efficient atomization. Total gas flows through the interface also play a role in the efficiency of detection.

5.3.5 Determination of Mercury

The optimum interface operating parameters for the determination of Hg in liquid flowing streams contrasted sharply with the optimized operating parameters for the other five elements (Cd, Cu, Pb, Zn and Ag). Maximum sensitivity was achieved when the flows of support gases to the interface were turned off. The optimum response for Hg was also dependent on the carrier solvent. A 6-fold increase in response was obtained if methanol was substituted for water as the carrier stream (Table 26). As indicated by the response surface contour maps (Figure 9) for this analyte in water or in methanol, a similar (but not indentical) thermospray heat input (voltage input) was optimal for both carriers but a somewhat lower combustion chamber heat input was required for the methanol carrier than for the water carrier. Somewhat surprisingly, optimal energy inputs were observed in both cases. An excess of heat applied to the thermospray tube and/or to the combustion chamber had a detrimental effect on the response. Moreover, the response contours were very nearly circular when account is taken of the difference in scales in these plots. The small negative slope associated with the positioning of the major axis of the elliptical contours of these plots might reflect the shorter residence time of the atomic Hg within the optical beam of the detector. Increased heat input to the system would result in a corresponding expansion in the volume of the vapours. As with the other analytes, except Pb, heating the optical tube of the interface did not change the response perceptibly.

5.3.6 Determination of Silver

For the detection of Ag contained in aqueous media, the optimization of the interface operating parameters was studied by using a univariate methodology. Analyte solutions were introduced into 1.0 mL/min of distilled water and transferred directly to the interface in a flow injection mode. Stepwise, univariate analysis of the influence, on Ag-response, of flow the rates of (i.) O_2, and (ii.) H_2 to the pyrolysis chamber and (iii.) the

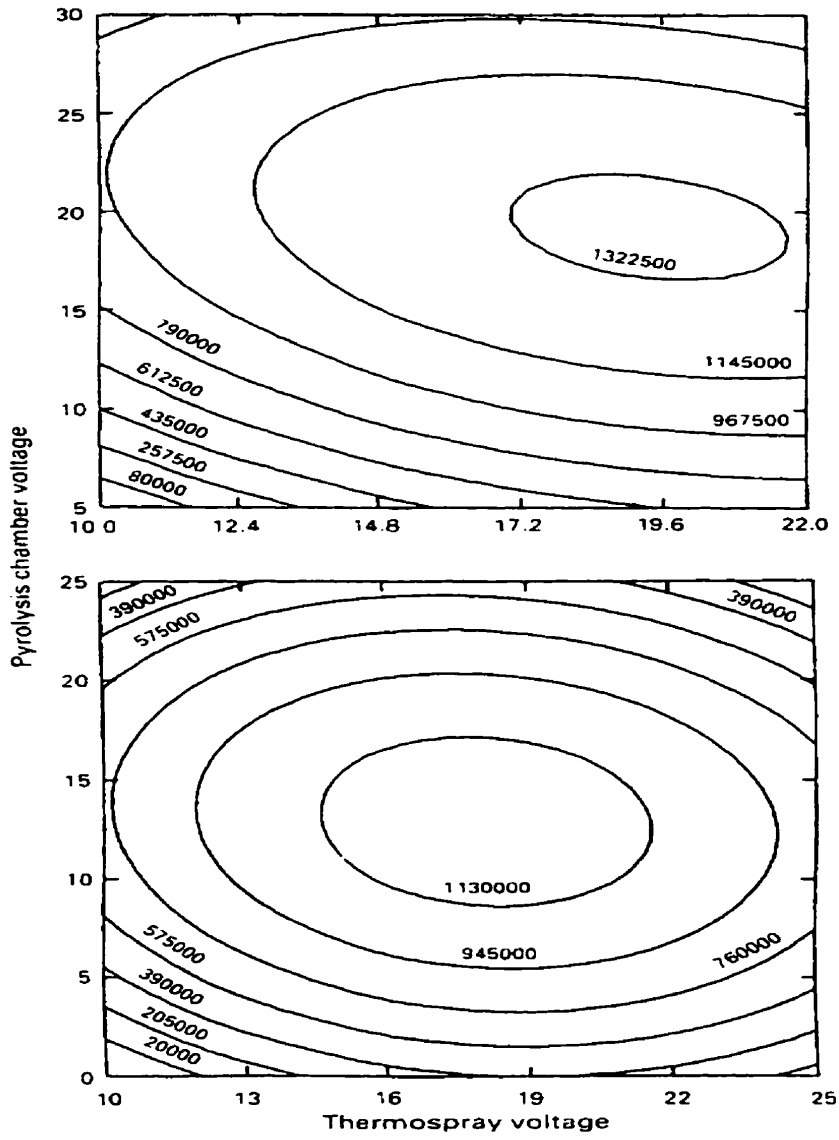

Figure 9. Contour maps of the predicted AAS response (peak area) to Hg contained in (a) water or (b) methanol as a function of the voltage applied to the pyrolysis chamber heating coil and the thermospray heating coil

skin temperatures of the thermospray tube, the pyrolysis chamber and the optical tube in the vicinity of the separately energised heating coils indicated that maximum peak area response to $AgNO_3$ was obtained with flow-rates of 340 and 895 ml/min for O_2 and H_2 respectively and 720, 1300 and 1120 °C for the temperatures of the thermospray tube, the pyrolysis chamber and the optical tube respectively. Typical responses are presented in Figure 10. The optimal H_2/O_2 ratio (2.6) for Ag detection, although slightly hydrogen-rich, was appreciably less than the optimal ratio for other volatile metals such as Cd, Cu, Pb and Zn (4.7-5.1).

5.3.7 Limits of Determination

Included in Table 27 are estimates of the absolute LODs determined from calibration curves (spanning 2 orders of magnitude and including at least one point near the LOD) using a first order error propagation model (Foley and Dorsey, 1984), assuming normally distributed baseline noise. This approach to estimating LODs presumes that the detector response in the FI mode can be modelled as a chromatographic peak. Also included in Table 27 are estimates of LODs for the elements using the more conventional model of three times the standard deviation of the baseline noise divided by the slope of the calibration plot. Low to sub-nanogram LODs were determined in every case.

5.4 Conclusions

The all-quartz interface coupling with AAS on-line detection technique offers several advantages over previous prototype designs. These advantages include (i.) compatibility with either organic or aqueous mobile phases containing volatile buffers, (ii.) detection of those heavy metals which are not considered to form hydrides with sufficient stability to be analytically useful, including Cd, Cu, Pb, Zn and Ag. and (iii.) LODs for Cd and Zn which approach those achieved by electrothermal AAS. A maximum instrumental response to each analyte element was achieved with a H_2-rich atmosphere in the pyrolysis chamber,suggesting an active role (other than as a fuel) for the H_2 in the atomization process.

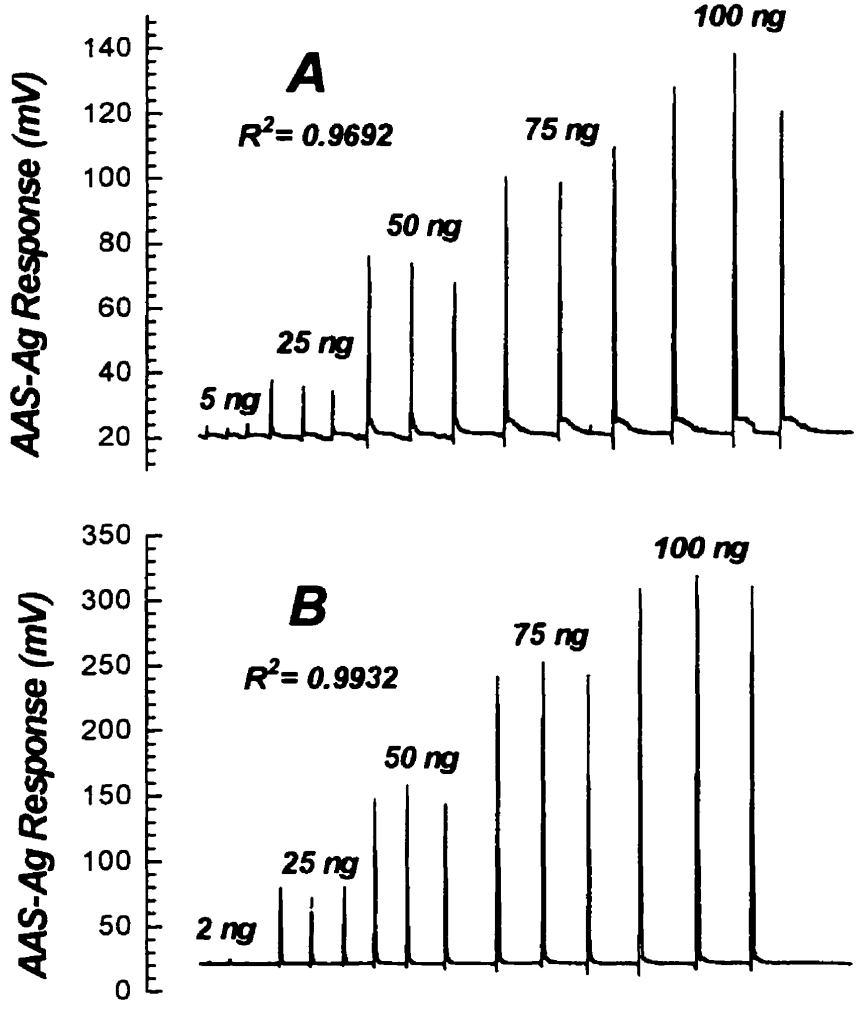

Figure 10. Silica T-tube AAS response to $AgNO_3$ standard flow injected into A, 10 mM tris buffer or B, distilled water.

Table 27. Calculated LODs for Cd, Cu, Pb, Zn, Hg and Ag standards under optimized operating conditions

Analyte	Mobile Phase	LOD (pmol)	
		A*	B**
Cd	H_2O	0.84	0 02
Cu	H_2O	1.4	0.16
Pb	H_2O	1.3	0.32
Zn	H_2O	0.60	0.03
Hg	H_2O	6.1	0.67
Hg	MeOH	1.0	0.38
Ag	H_2O		1.40
Ag	10 mM Tris buffer		9.30

* LOD = 3 $[s_b^2 + s_i^2 + (i/S)^2 Ss^2]^{1/2}/S$ where s_b is the standard deviation of the peak-to-peak baseline noise/5 (recorded over 10 min) and S, i, Ss ans s_i are the slop, intercept and their respective standard deviations of the calibration plot (obtained by linear regression).

** LOD = $3s_b / S$.

Chapter 6

Approaches to the Determination of Metallothionein(s) by HPLC-Silica T-Tube Interface-Atomic Absorption Spectrometry

6.1 HPLC-Silica T-Tube-AAS on Line Detection

Even though conventional atomic absorption and emission spectrometries have been used extensively for the study of metal-bound metallothioneins (Klaassen and Lehman-KcKeeman, 1991), this approach can suffer from a lack of sufficient sensitivity for the environmental monitoring of these analytes in certain tissues. The novel silica T-tube interface design (described in Chapter 5) have been used to couple HPLC with atomic absorption spectrometry and evaluated for the determination of As compounds (Momplaisir, *et al.*, 1994), and Se-compounds (Momplaisir, *et al.*, 1994; Lei and Marshall, 1995) in marine tissues and plants. In addition to As and Se, this interface provided sensitive responses (low to sub-ng limits of detection) to Cd, Cu, Hg, Pb, Zn and Ag. The desirable characteristics of this interface were considered to be its compatibility with volatile buffers contained in either aqueous or organic mobile phases, and the low cost and the robust character of the device which can be operated over several months without an appreciable change in response.

6.2 Metallothioneins

Metallothioneins (Hamer, 1986; Suzuki et al., 1993) are a group of heat stable, low molecular weight, cysteine rich, metal binding protein which are characterized by a unique amino acid sequence, spectroscopic features characteristic of tetrahedral metal-thiolate (-mercaptide) complexes and a strong avidity for Ag(I), Cu(I), Cd, Hg and Zn. Although first isolated from equine kidney cortex, this class of metal binding polypeptide is widely distributed throughout the animal kingdom and non-translationally biosynthesised analogs have been identified in more than 85 species of plants, yeasts and fungi. The most conserved features among the mammalian structural sub group of this class of protein are the twenty cysteines (cys) (of a total of 60-62 amino acid residues) which collectively bind seven divalent metal ions in two separate metal thiolate oligonuclear clusters (M_3Cys_9 and

M_4Cys_{11}) buried as "mineral cores" in the interior of the two globular domains formed by the carboxyl- (α-domain) and amino-terminal (β-domain) halves of the polypeptide chain (Kägi, 1992). Two other structural motifs have been identified for monovalent metals ($M_{12}Cys_{20}$ and $M_{18}Cys_{20}$). Mammalian (class 1) metallothioneins occur in two principal isoforms (conventionally labelled as MT-I and MT-II based on their order of elution from a DEAE-Sephadex column) although several sub-isoforms have also been separated by HPLC. (Kägi, 1992; Vasak, 1991).

Whereas the average apparent association (binding) constant for Zn(II), at pH 7.0, is in the range of 10^{11}-10^{12} M^{-1}, Cd(II), Cu(I) and Ag(I) are bound more tightly by several orders of magnitude and follow the general order found for inorganic thiolates Hg(II) > Ag(I) $\approx$ Cu(I) > Cd(II) > Zn(II) (Stillman, 1995; Otvos, *et al.*, 1992). Despite binding constants which, at pH 7, are nearly equal to EDTA in avidity and their position within the recesses of the enveloping protein, cluster-bound metal ions are somewhat mobile in exchange interactions with other metals in solution, with other ligands such as glutathione and with other MT molecules due principally to a cleft within each domain of the enveloping polypeptide. Elevated levels of this class of polypeptide are accumulated *in vivo* in response to exposure to a variety of metal salts, as well as oxidative stress, heat, solvents and steroids.

6.3 Objectives

The objectives of this study were threefold: (*i.*) To explore size exclusion and ion exchange HPLC as separatory techniques for the determination of individual isoforms of mammalian and molluscan metallothioneins (MTs); (ii.) to develop a sensitive method to quantify these polypeptides by detecting the Cd, Cu or Zn metal(s) bound to their surfaces and (iii.) to explore Ag-saturation as a means of determining the total quantities of these polypeptides.

6.4 Materials and Methods

6.4.1 HPLC-Silica Tube-AAS.

Mobile phase provided by a Varian model 9010 HPLC pump was delivered, at 1 ml/min, to the column via a manual 6-port rotary injection valve. Column eluate contained in a 0.05 mm x 25 cm section of silica capillary transfer line (SGE Industries, Austin, TX) was nebulized by thermospray effect, the silica T-tube interface as described in the previous Chapter. Spectrometer operating parameters are recorded same as in Table 25.

6.4.2 Size Exclusion HPLC

The 7.8 mm x 30 cm column of TSK Gel (G3,000PW_{XL}, Supelco Inc., Bellefonte, PA, USA) was eluted with 30 mM tris(hydroxymethyl)aminomethane (tris) buffer (pH 8.9) delivered at 1 ml/min.

6.4.3 Ion Exchange HPLC

The 7.8 mm x 7.5 cm column of Progel (Supelco Inc.) was eluted with a 2-step solvent program. The concentration of tris buffer (pH 7.5) was increased from 10 mM to 100 mM over 5 min, then further increased to 150 mM over 15 min. The column was subsequently regenerated with 10 mM tris buffer for 15 min prior to the next injection.

6.4.4 Standards and Samples

- Metallothionein, metallothionein-I and metallothionein-II (each nominally 5.7% (m/m) Cd and 0.7% Zn, isolated from rabbit liver and essentially salt-free), hemoglobin and tris(hydroxymethyl)aminomethane were purchased from Sigma Chemical Co. (St Louis, MO, USA).
- The freshwater mollusc (Anodonta grandis grandis) was collected from an experimental precambrian shield lake located in northwestern Ontario. The water in this lake (Chamberland, *et al.*, 1995) had been intentionally spiked, over a five years period, with Cd, to result in a mean concentration between 54-177 ng/L (below the 200 ng/L limit set by the Canadian Water Quality guideline for the protection of aquatic life in oligotrophic lakes). Mussel metallothionein fraction was prepared from a single specimen of whole mussel (*A. grandis*) soft tissue by homogenization in 5 volumes of 30 mmol/L tris(hydroxymethyl)methylamine buffer (pH 8.9). The soluble

fraction was partially purified by discarding thermo-coagulated protein material after 30 min incubation at 70 °C. The identity the metallothionein fraction in the supernatant was based on chromatographic behaviour which was identical to a metallothionein standard (3.5 μg/mL in 30 mmol/L Tris buffer) isolated from rabbit liver (Sigma Chemical Co., St Louis, MO, USA). These mussel samples were kindly provided by J. S. Blais, Agriculture Canada, Food Research and Development Centre, 3600 Casavant Blvd., St.-Hyancithe, Quebec.

6.4.5 Silver Saturation

MT standard 0.2-1 mg in 0.2 ml of tris buffer (10 mM) was incubated with 0.1 ml of 0.5% (m/v) $AgNO_3$ at 20 °C for 30 min with occasional vortex mixing. Hemoglobin, was provided by H. M. Chan, Center for Nutrition and the Enviroment of Indigenous Peoples (CINE), Macdonald Campus of McGill, 0.2 ml of 1 % (m/v) in 10 mM tris buffer was added to the crude incubation mixture, thoroughly mixed, then heated to 80-100 °C for 5 min. The crude reaction mixture was then centrifuged for 10 min at 10,000 rpm and the resulting crude supernatant fraction was directly analysed by HPLC.

6.4.6 Quantification

Quantification was performed by the method of external standards. Five working solutions were prepared by diluting concentrates of inorganic standards with mobile phase. The instrumental response to these standards and to crude supernatant spiked with standard were recorded in the flow injection mode.

6.5. Results and Discussion

6.5.1 Size Exclusion Chromatography

The utility of the interface as an on-line detector for HPLC was demonstrated by monitoring the eluate from a size exclution column. The system was evaluated using a rapid monitoring procedure to determine the levels of Zn, Cd or Cu bound to a crude metallothionein(s) fraction isolated from a freshwater mussel. In

preliminary experiments (in the absence of the column) it was demonstrated that: with minor adjustments of the interface operating conditions, (i.) responses for Cd or Zn standard, equivalent to those obtained with a water carrier, could be achieved with a 30 mmol/L tris buffer as the mobile phase carrier and (ii.) co-injection of Cd^{2+} or Zn^{2+} standard in 30 mmol/L tris buffer together with the crude metallothionein fraction isolated from the mollusc did not change the response of the detector to the added standard. Diverting a portion of the support gas to the supplementary O_2 inlet port on the reducing union did not result in a perceptible change in instrumental response to either inorganic standard or to the crude metallothionein isolate. Cadmium-specific chromatograms of metallothionein standard (3.5 μg of protein per mL) and the crude metallothionein fraction from the musssel are presented in Figure 11. Also included are Zn and Cu specific chromatograms of this isolate obtained under identical chromatographic conditions. Although it was not possilbe to detect the Cu bound to this fraction with certainty, quantification of the Cd and Zn (Table 28) was performed by comparison with peak area from standards determined in the FI mode. The metallothionein standard was found to contain 8.5% w/w of Cd and was apparently saturated with this metal (7.5 equivalents of Cd per mol of protein).

Another chromatographic trials was conducted with a molluscan MT fraction which had been saturated with Ag^{+} in the presence of 2-mercaptoethanol, treated with hemoglobin to complex the excess free and/or loosely bound metal ion and then heated to denature the hemoglobin which was subsequently removed by centrifugation. The supernatant fraction was then subjected to size exclusion HPLC-AAS (Figure 12). Although three fractions were clearly evident in the chromatogram of the Ag-labelled MTs

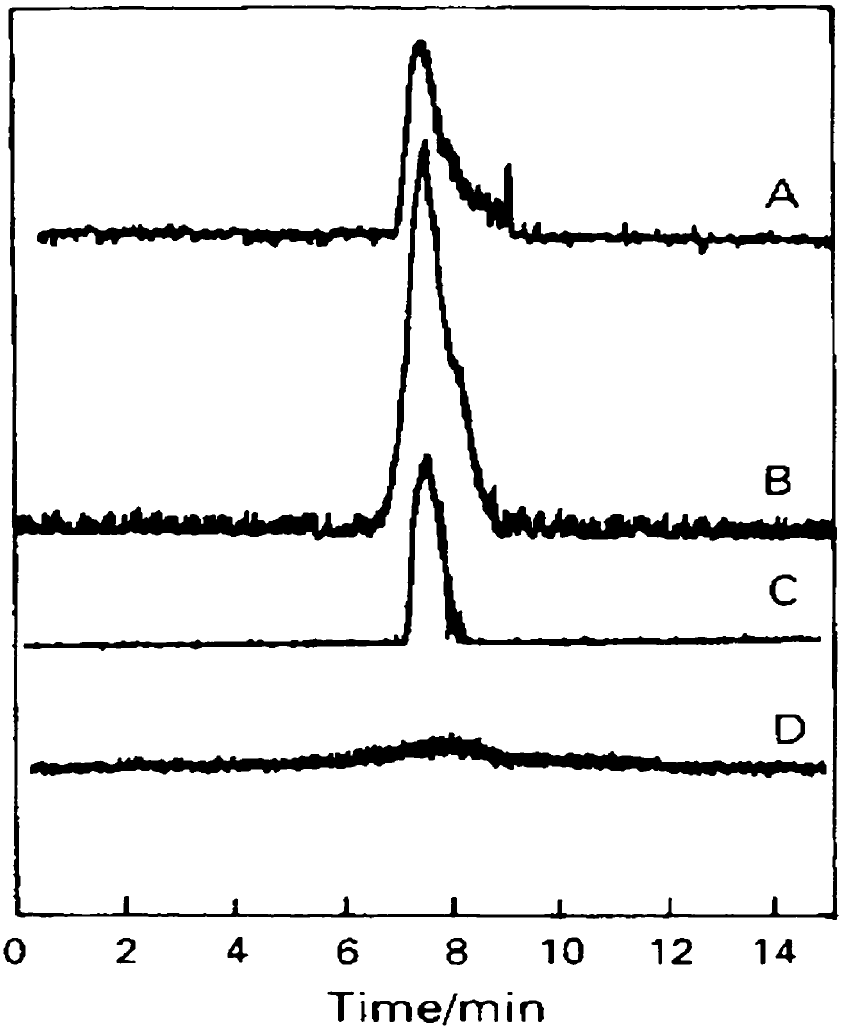

Figure 11. Size-exclusion chromatographys of A, Cd bound to commercial metallothionein standard from rabbit; B, Cd; C, Zn; and D, Cu bound to the crude metallothionein(s) fraction isolated from a single mussel

Table 28. Levels of Cd and Zn bound to metallothionein standard or to the crude metallothionein fraction (± 1 ralative standard deviation) from mussel

	Cd	Zn
Metallothionein standard/ng per ng of protein	0.0846* ± 4.3%	ND**
Crude extract/ng per 50 µL injection	59.4 ± 3.2%	20.9 ± 6.4%

*14.8 (± 4.3%) ng of Cd per 175 ng of protein = 0.132 nmol of Cd per 0.0175 nmol of protein.
** ND = not determined.

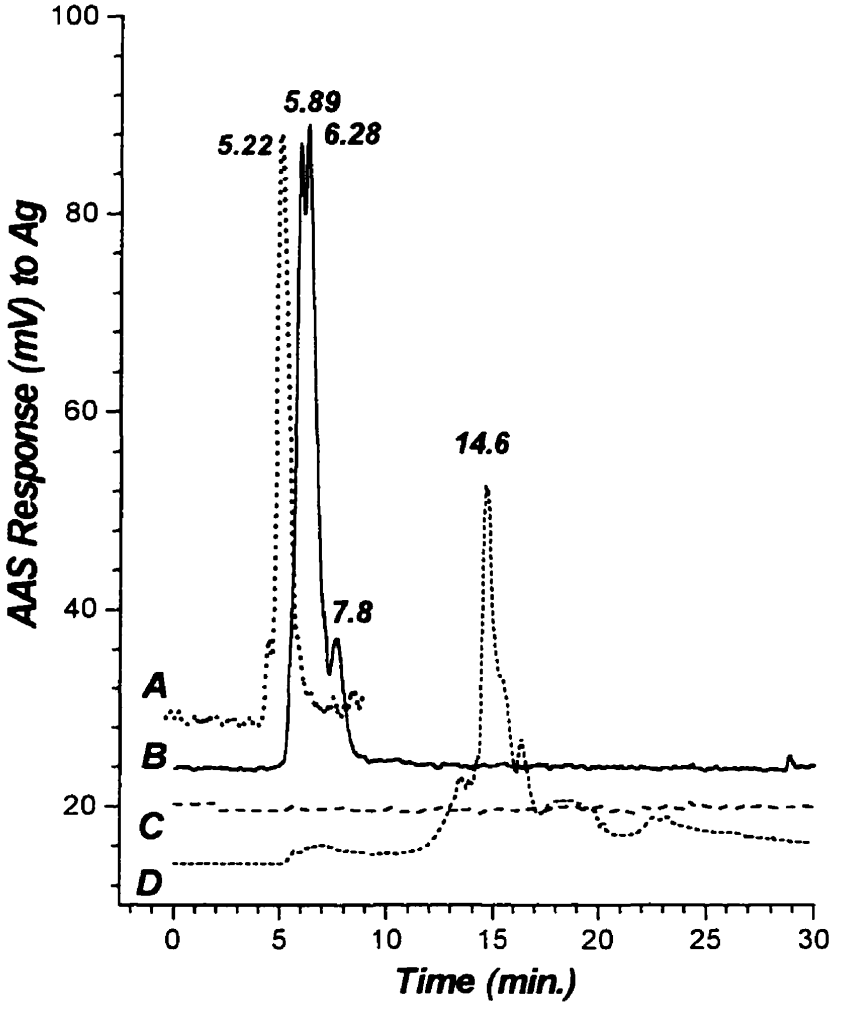

Figure 12. Ag-specific size exclusion chromatograms of: A, 1:1 Ag-haemoglobin complex; B, Ag-labelled molluscan MT fraction; C, 2-mercaptoethanol control and D, 1:1 Ag-mercaptoethanol complex.

[retention time, R_T 5.89, 6.28 and 7.80 min.], it was not possible to assign the fractions to individual isoforms. A separate chromatogram of Ag-hemoglobin complex (Fig. 12A, R_T 5.22 min) indicated that a maximum of 6 % of the Ag-response in the chromatogram of the MT fraction could have been due to this potential interferent. Similarly, the injection of 2-mercaptoethanol alone failed to release any column bound Ag-analyte (Fig 12C) and Ag-mercaptoethanol complex (Fig 12D) was appreciably more retained than any of the fractions in the Ag-labelled MT fraction. Separate Cd- or Cu-specific chromatograms of the supernatant fraction failed to detect the presence of bound or free residues of these metals in the Ag-labelled product mixture. Exchange was thus rapid and virtually complete.

6.5.2 Ion Exchange Chromatography

Ion exchange was also evaluated as a separatory technique for metallothionein(s). Commercial standards of metallothionein (containing both MT-I and MT-II) isolated from the liver of rabbits which had been exposed to Cd, as well as separate fractions enriched in either MT-I or in MT-II from the same source were separated using a 2-step gradient which increased the concentration of tris over time. Cd-specific chromatograms of these materials are presented in Figure 13. The chromatograms of this figure have been corrected for changes in the baseline with changing mobile phase (Figure 13 D). In addition to the MT-I and MT-II isoforms (R_T, 5.85 and 7.58 min respectively) which were only partially separated, two other fractions were also detected (R_T, 2.22 and 3.18 min). The MT-I enriched material contained the same 4 fractions, although this isolate was enriched in the MT-I isoform relative to the MT-II isoform (Table 29). By contrast the MT-II fraction contained only traces of Cd bound to the R_T, 2.22 min fraction, none of the R_T, 3.18 fraction and smaller amounts of Cd bound to the MT-I isoform (16%, Table 29). The identities of the R_T, 2.22 and 3.18 fractions, which collectively accounted for 21-24 % of the total Cd-response in the mixture and MT-I enriched fractions, are not known. These fractions might represent oligomers of MT or degradation products. Although short term Cd-, Cu- and Zn-chromatograms of solutions of these materials was highly

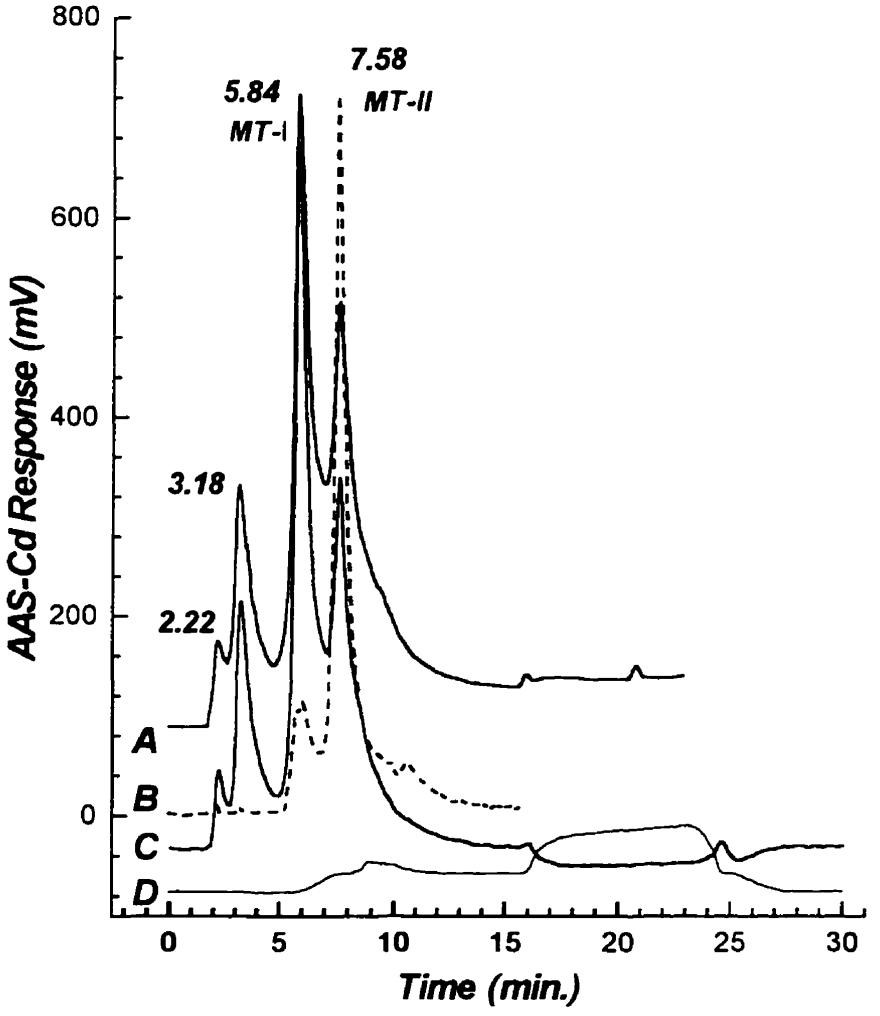

Figure 13. Cd-specific ion exchange chromatograms of: A, MT-I/MT-II mixture (0.5 μg), B, sample enriched in MT-II (0.5 μg), C, sample enriched in MT-I (0.5 μg) and D, solvent blank. Chromatograms A, B and C have been background (D) corrected.

Table 29. Proportions of components (% composition as estimated by background corrected relative peak areas from metal specific chromatograms) in MT mixture, and in fractions enriched in either MT-I or MT-II.

	AAS-Cd Response			AAS-Cu Response			AAS-Zn Response		
R_T (min)	Mixture	MT-I	MT-II	Mixture	MT-I	MT-II	Mixture	MT-I	MT-II
2.22	3	4		5			1		
3.18	18	20		32	48		8	6	3
5.85	38	45	16	48	35	56	18	14	5
7.58	40	32	84	15	15	44	38	44	32
9.43							34	36	60

repeatable, longer-term storage of standards in 10 mM tris buffer at 4 °C, resulted in a progressive loss of signal presumably reflecting the loss of analyte metal from the protein surface. For Cd monitoring, deuterium background correction was unnecessary.

6.5.3 Zn-specific Chromatograms

The Zn content of these materials was much lower than the Cd concentration and increases in the baseline signal over the course of the solvent program were appreciably worse (Figure 14A). The Zn-response (at 213.9 nm) of the detection system was reduced appreciably by the tris buffer and short-term noise proved to be more severe. Efforts to circumvent the dependence of the Zn-response on the mobile phase and to minimise the short-term shot noise were only moderately successful. The response of the detector at 307.7 nm proved to be too insensitive for our purposes however deuterium background correction reduced the change in the background signal (over the course of the solvent program) by about 50% while reducing the Zn signal by less than 5%. The Zn-response to 100 μg of the MT-I/MT-II mixture (Fig. 14D) indicated that this analyte was bound predominantly to two fractions, the MT-II isoform (R_T, 7.58) and a later eluting fraction (R_T, 9.43) which had not been observed in the Cd-specific chromatograms. The MT-I enriched fraction (Fig. 14B) contained 4 fractions [R_T, 3.18, 5.84 (MT-I), 7.58 (MT-II) and 9.43 min]. For the MT-II enriched material (Fig. 14C), more of the Zn was bound to the R_T, 9.47 fraction than to the MT-II isoform (R_T, 7.58). Only traces of the R_T, 3.18 fraction was detected and only a minor amount of the Zn (5%) was present in the MT-I impurity (Table 29).

6.5.4 Cu-specific Chromatograms

AAS-Cu chromatograms (Figure 15) for the ion exchange separation of these same solutions was appreciably different from the Cd- or Zn-responses. Only traces of Cu were detected in any of these materials. Chromatograms A, B and C represent responses to 100 μg of protein *vs* Cd-responses to 0.5 μg of protein in Figure 13. Approximately one-half of the Cu in the metallothionein mixture was associated with the MT-1 isoform and a

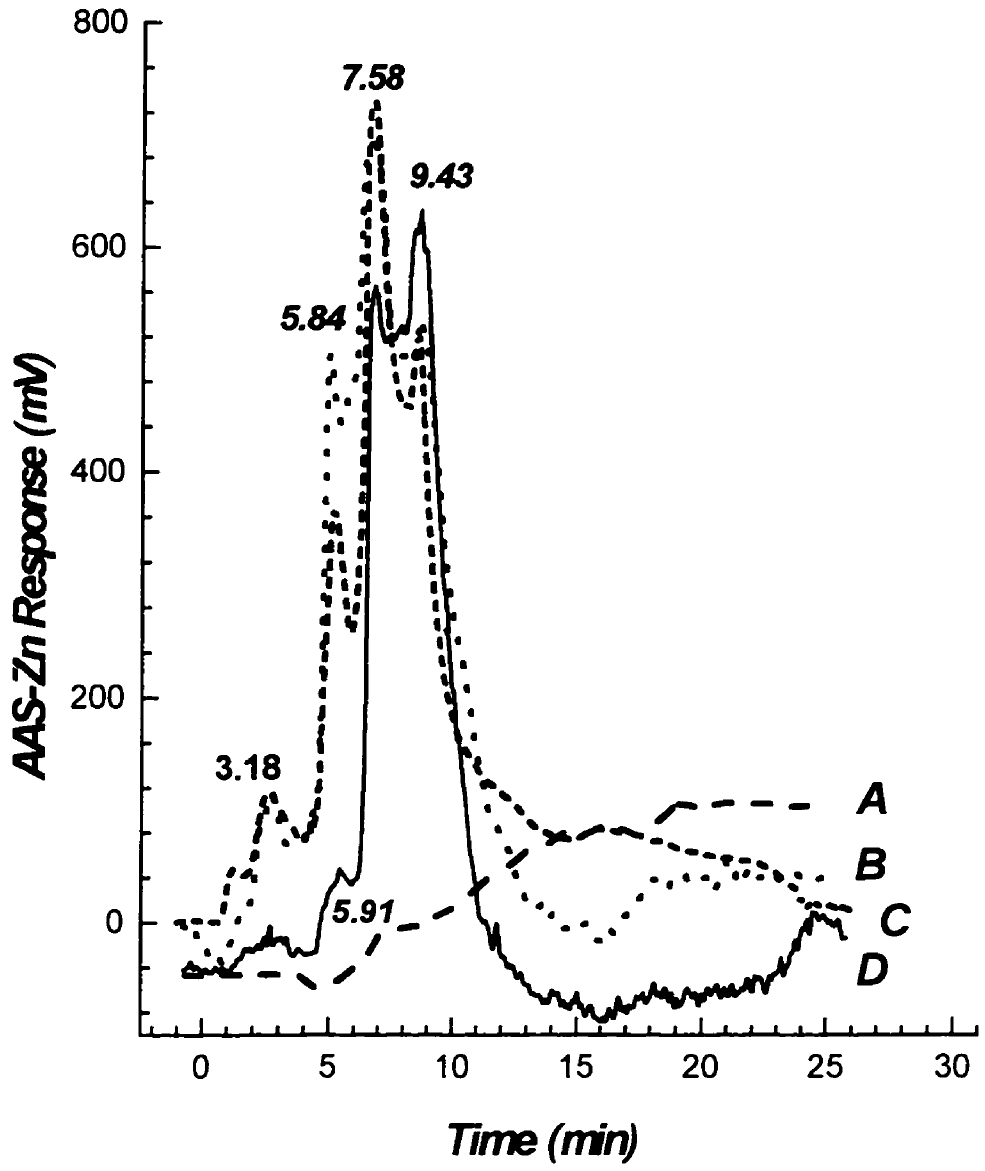

Figure 14. Zn-specific ion exchange chromatograms of A, solvent blank; B, MT-I enriched sample (125 μg); C, MT-II enriched sample (100 μg) and D, MT-I/MT-II mixture (125 μg). Chromatograms B, C and D have been background (A) corrected and for clarity, chromatogram D has been displaced vertically by 40 mV.

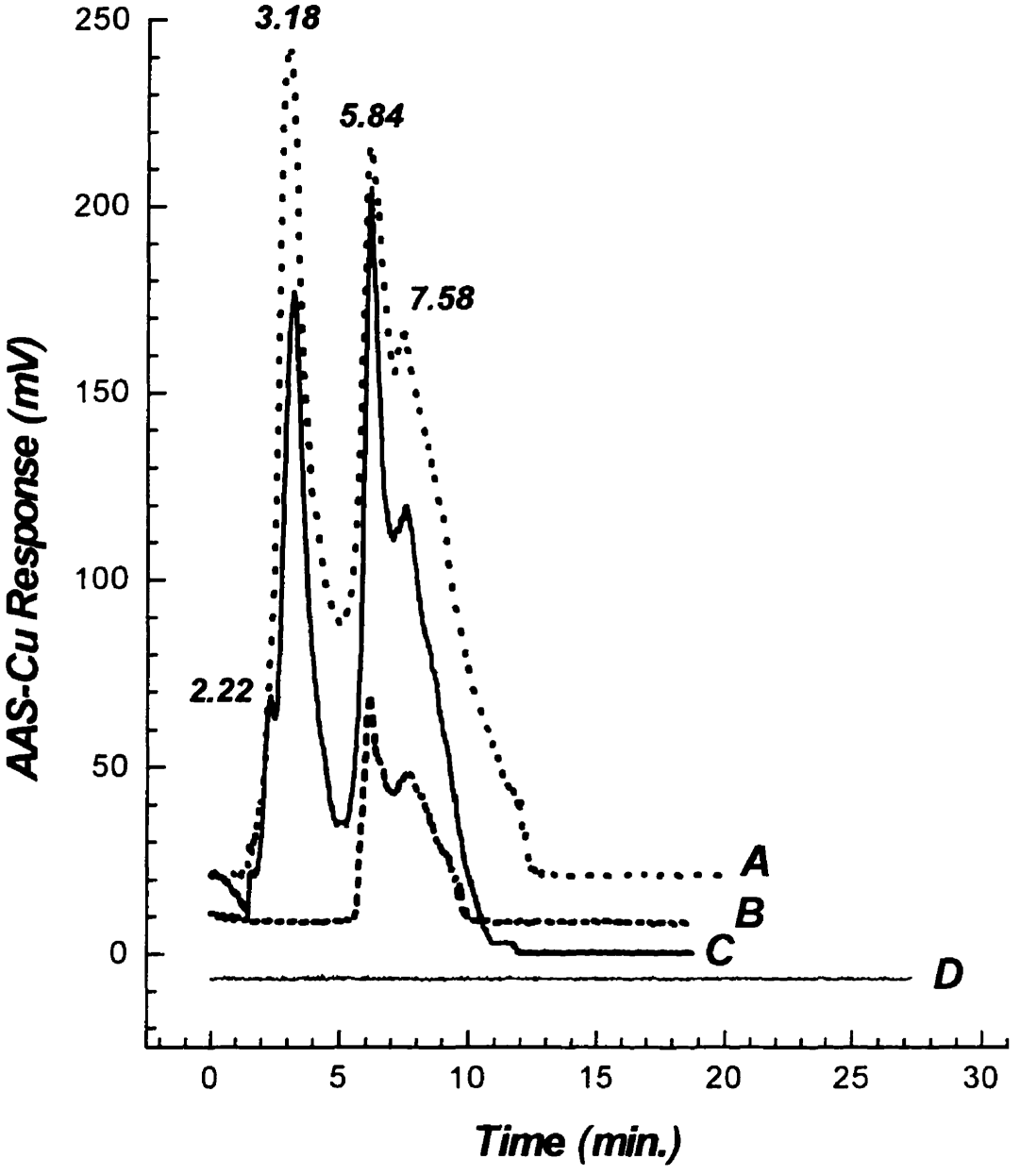

Figure15. Cu-specific ion exchange chromatograms of A, sample enriched in MT-I (100 μg); B, sample enriched in MT-II (100 μg); C, MT-I/MT-II mixture (100 μg) and D, solvent blank.

further one-third with the R_T, 3.18 min fraction (Fig. 15C). The MT-II isoform in this sample accounted for only 15% of the bound Cu. This is consistent with a previous report that MT-I contains more Cu than does the MT-II isoform (Chan and Cherian, 1992). Interestingly, for the MT-I enriched sample (Figure 15A), one-half of the bound Cu (48%) was associated with the 3.25 min fraction. The MT-II sample (Figure 15B) contained appreciably less Cu which was approximately equally distributed between the MT-I and MT-II isoforms. No free Cu ion was detected in any of these chromatograms.

6.5.5 Silver Saturation

To explore the proposed Ag-saturation technique, the mixed standard and the fractions enriched in each isoform were separately Ag-saturated using the procedure described above. The crude supernatant fraction from the hemoglobin separation was analyzed, without further purification, by ion exchange HPLC using the tris buffer-based solvent program (Figure 16). Perhaps not surprisingly, there was little separation of the components with these chromatographic conditions. The size exclusion separation of Ag-saturated molluscan MTs had suggested that the denaturation/centrifugation step efficiently removed hemoglobin from the crude reaction mixture. Cd_7-MT is known to bind up to 18 Ag ions and then to unwind with the loss of the polypeptide's tertiary structure (Stillman, 1995). Additionally, the addition of 4 units of positive charge (18 Ag *vs* 7 Cd/Zn) would be anticipated to change the chromatographic behaviour of the Ag-substituted product(s) and result in less anionic retention. The isoforms of silver-saturated denatured products can be anticipated to be difficult to separate in that these polypeptide chains vary by only a single aminoacid residue.

6.5.6 Operating Characteristics

In terms of operating characteristics, the relatively low operating temperatures of the interface resulted in an appreciable influence of the mobile phase on the response to analyte metals. Not only were the LODs increased by the tris buffer, changes in the mobile phase composition contributed appreciably to changes in the Zn-background signal over

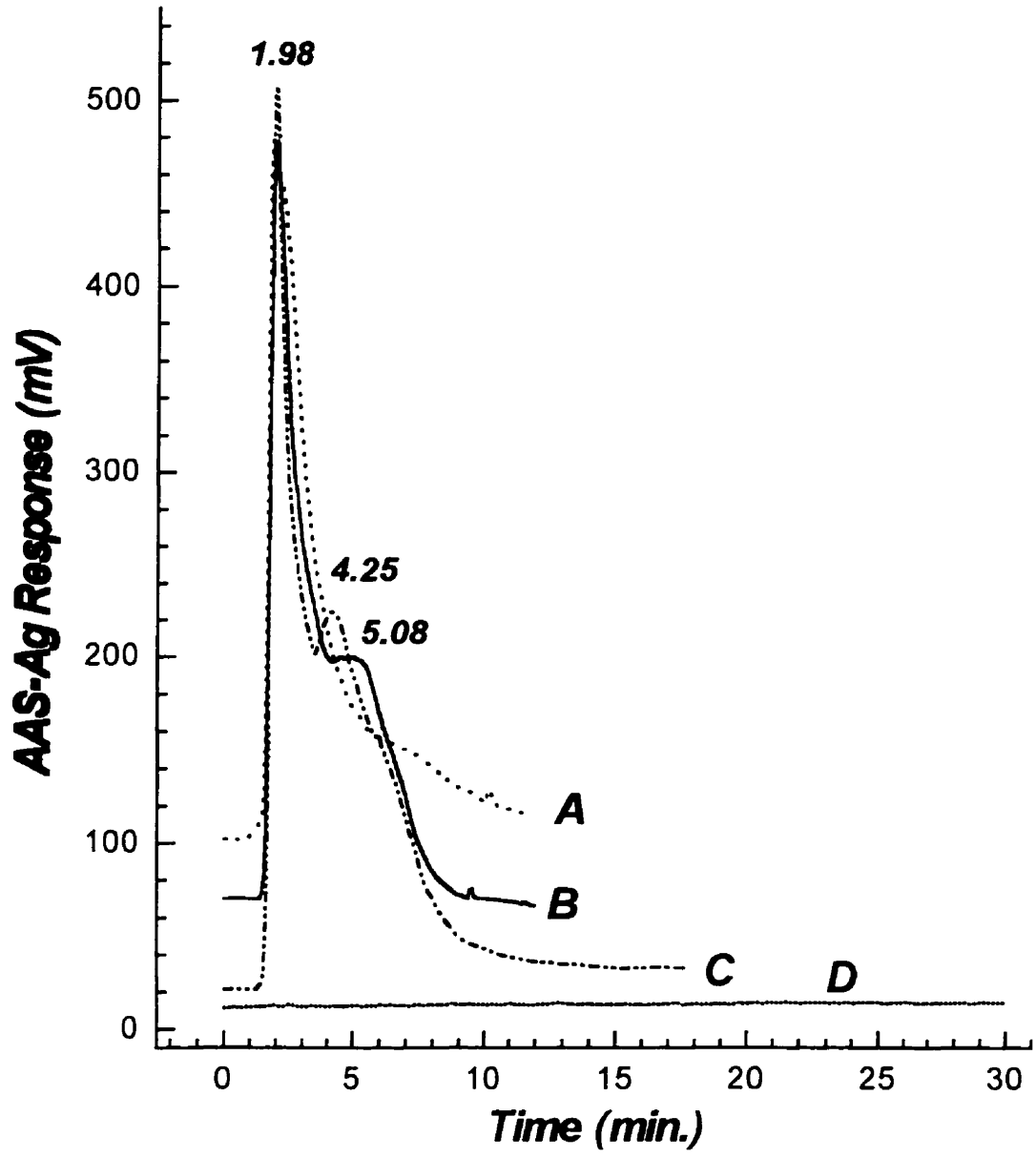

Figure 16. Ion exchange chromatograms of Ag-labelled: A, MT-I/MT-II mixture (100 μg), B, fraction enriched in MT-II (40 μg), C, fraction enriched in MT-I (40 μg) and D, solvent blank. Chromatograms A, B and C have been background (D) corrected.

the course of the solvent program (Fig. 14A) and modestly to the Cd-signal (Fig. 13D) but had no influence at longer wavelengths used for Cu and Ag (Figs. 15D and 16D). Only for Zn monitoring did this become a limiting factor in the current application.

6.6 Conclusions

Whereas the cadmium in the MT-I/MT-II mixture was approximately equally distributed between the two isoforms, the Cu in this mixture was predominantly associated with the MT-I isoform and the Zn was predominantly associated with the MT-II isoform. The MT-I enriched sample contained appreciable Zn and Cd but less Cu bound to the MT-II isoform (accounting for approximately 44, 33 and 12 % respectively of the total Zn-Cd- or Cu-response). By contrast, the MT-II enriched sample contained appreciably less metal-bound MT-I isoform (16% of the total Cd and 5% of the total Zn but no Cu). Three other fractions were also detected among the chromatograms. Whereas the two early eluting fractions (R_T 2.22, 3.18) contained all three metals, the R_T 9.43 min fraction contained only Zn. These fractions remain unidentified.

These preliminary studies were only partially successful in identifying rapid procedures to monitor/determine the metal content associated with separate isoforms (sub-isoforms) of mammalian metallothioneins, the quartz T-tube interface provided a sensitive yet inexpensive alternative to conventional atomic absorption or emission techniques. As reported previously, the operation of the device was compatible with conventional flows (1 ml/min) of HPLC column eluate containing volatile buffer. Moreover, the low LODs for a variety of metal analytes, including Cd, Cu and Ag, offers the possibility for monitoring metal binding biopolymers in a variety of environmental media by detecting the metal(s) bound to their surfaces.

Whereas the commercial substrates used for these studies were saturated with respect to metal loadings, isolates from environmental matrices might be undersaturated and susceptible to partial oxidation (during isolation) to form dissulfide bridges. To monitor analyte biopolymer levels in these isolates, the Ag-saturation technique

(performed in the presence of 2-mercaptoethanol to reactivate metal binding sites on the protein) seems to be a promising route. However, appreciably more study of the stoichiometry of the reaction and the chromatographic behaviour of the Ag-labelled products will be required.

Chapter 7

Summary of Conclusions

The objectives of these studies were to develop novel procedures and techniques to circumvent two perceived limitations of conventional atomic spectrometric analyses. Whereas the actual time for analysis has been shortened steadily by increasing automation, sample preparation techniques have changed little in recent decades. In chapter two, homogenization with a flat valve homogenizer, in combination with high-speed blending, was successfully applied to the preparation of slurries for the GF-AAS determination of cadmium, copper and lead concentrations in six certified reference materials and in frozen cervine liver and kidney. The stability of the slurry over time was not a problem since at least a very high proportion of the analytes was extracted into the liquid phase [ethanol-water (1 + 9 v/v) containing 0.25% m/m tetramethylammonium hydroxide] during sample processing.

The attractive features of this approach are its speed, simplicity of sample processing and the apparent stability of the resulting preparation. A further attractive feature is the lower cost of the homogenizing device relative to a commercial microwave digester. However, for the technique to become more widely applicable to other kind of biological samples and other analyte elements, background levels of metals introduced by the homogenizer will have to be reduced substantially.

In chapter 3, efforts were made to reduce the level of metal contamination introduced by the homogenization procedure. A modification to the flat-faced valve stem of the homogenizer was evaluated. By capping the flat valve head with a ruby disk, the levels of contaminating metals (Al, Cr, Cu, Fe, Pb, Mn, Ni and Se) introduced by processing were reduced appreciably (from two to thirty-fold). Moreover, other capping materials, such as tungsten carbide, zirconia, sapphire and poly-methacrylate were also studied. The modified homogenizer was then used to prepare slurries of soft tissues (liver and kidney), botanical and zoological CRMs and animal feeds which were analyzed for Cr,

Cu, Fe, Mn and Ni. Again, there was excellent agreement between the analyte concentrations as determined by slurry introduction GF-AAS and their certified values. However, the concentration of Se in CRMs was consistently underestimated when using the high-pressure slurry preparation/introduction technique. Also there was no evidence of any analyte loss prior to the atomization stage as judged by the signal graphics software which provided a continuous display of the Se-signal over the course of the furnace program. This indicated that the release of Se from protein matrix during the pyrolysis and ashing sequence was inefficient.

In chapter 4, homogenization, in combination with partial enzymatic digestion with a crude protease alone or admixed with lipase or cellulase, was investigated as a means of releasing Se-residues from zoological and botanical matrices prior to slurry introduction GF-AAS. For two zoological CRMs, one botanical CRM and one mixed animal feed sample, 4h of digestion at 37 ^{o}C were sufficient to liberate Se-residues quantitatively. Again, the resulting digests were stable to storage for up to 10 days. Enzymatic digestion was also applied successfully to freeze-dried fresh and boiled fish tissues. The principal advantages of the enzymatic digestion procedure are its simplicity and lack of operator intervention.

However, for the high pressure homogenization technique to become widely applicable, background levels of analyte introduced by the homogenizer will have to be reduced further. Even with the addition of an inert cap to the flat-faced valve stem of the homogenizer levels of metals introduced by processing can degrade the limit of detection for certain trace element analytes. As a suggestion for further study, other organic-based polymeric materials which are more resistant to the impact of a jet of processed sample fluid, would be one of the choices for further research into improvements to this technique.

The second perceived limitation to atomic spectrometry involves the loss chemical speciation information during analysis. To circumvent this problem, a combination of a chromatographic separation with *on-line* detection by atomic absorption spectrometry (AAS) was explored. In chapter 5, an all-silica T-tube interface for coupling flowing liquid streams with *on-line* monitoring by AAS was evaluated for the determination of Cd, Cu,

Pb, Zn, and Ag, elements which are not considered to form volatile hydrides of sufficient stability to be analytically useful. This interface provided several advantages over previous prototype designs including compatibility with either organic or aqueous mobile phases and limits of detection (LODs) for Cd and Zn which approached the LODs for the determination of these elements by GF-AAS.

In chapter 6, the utility of the high-performance liquid chromatography-AAS interface was demonstrated by monitoring the Cd, Zn, Cu and Ag bound to (*i.*) a crude metallothionein isolate (from a single freshwater mollusc) in gel permeation HPLC eluate or (*ii.*) individual metallothionein isoforms which had been partially resolved by ion exchange HPLC. With the low LODs for a variety of metal analytes, it is possible to monitor metal binding biopolymers in a variety of environmental media by detecting the metals to their surfaces.

For further study, the Ag-saturation technique seems to be a promising route to study metal binding proteins/polypeptides in environmental matrices. These analytes might be undersaturated with respect to metal loading and susceptible to partial oxidation to form disulfide bridges. However, for this approach to become more widely applicable, appreciably more study of the stoichiometry of the reaction and optimization of the chromatographic separation of these Ag-labeled analytes will be required.

Claims to Originality

1. A rapid sample preparation technique, high-pressure homogenization, was developed for soft tissues and used in combination with slurry introduction atomic spectrometry.

2. A novel modification to the homogenizing valve involved capping the stainless steel surface of the flat faced valve to reduce the levels of metal contamination introduced during processing.

3. High pressure homogenization was combined with partial enzymatic digestion using a crude protease alone or admixed with lipase or cellulase to release Se-residues from zoological and botanical matrices prior to slurry introduction GF-AAS.

4. The first reported study to assess the performance characteristics of a new interface device for the detection, in flowing liquid streams, of those trace elements (Cd, Zn, Pb and Ag) which are not considered to form volatile hydrides of sufficient stability to be analytical useful.

5. A sensitive method was developed to quantify individual isoforms of metal-binding polypeptides by detecting the Cd, Cu or Zn metal(s) bound to their surfaces and to explore Ag-saturation as a means of determining the total quantities of these polypeptides.

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IMAGE EVALUATION
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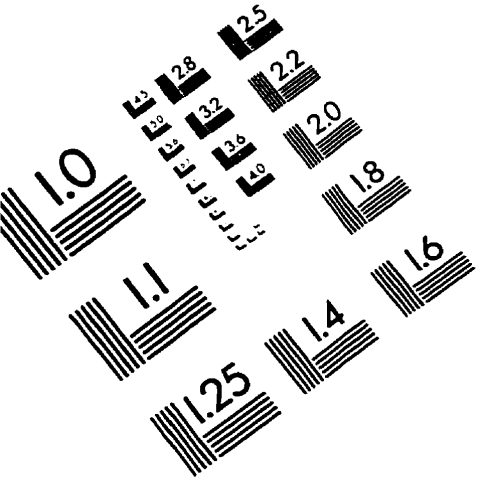

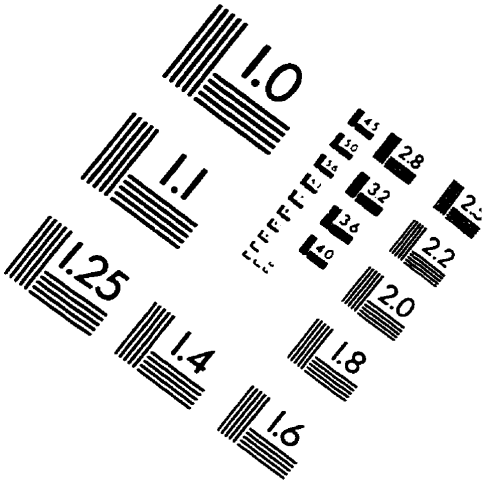

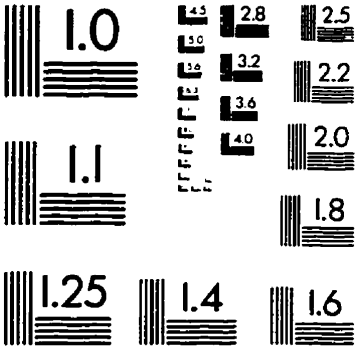

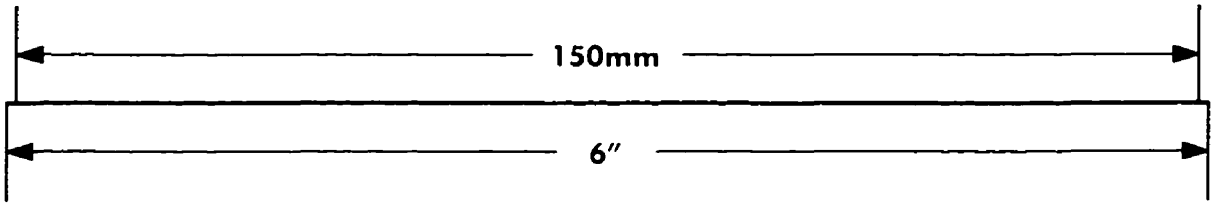

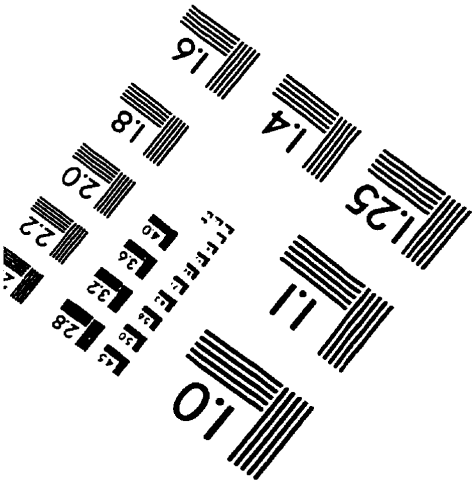

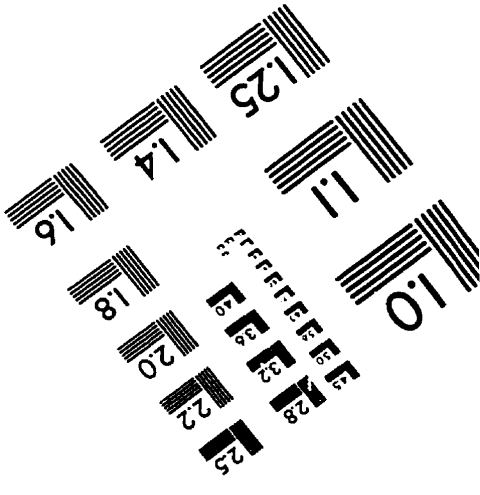

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