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# Quasi-Stable Slurries for the Determination of Trace Elements by Graphite Furnace Atomic Absorption Spectrometry

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# Abstract

High-pressure homogenization using a new flat valve homogenizer in combination with enzymatic digestion with a crude protease was investigated as a means of releasing Se compounds from zoological and botanical matrices prior to slurry introduction GF-AAS. Timed trials with four zoological certified reference materials (CRMs), three botanical reference materials (RMs), and a food crop indicated that Se release was quantitative after homogenization or became quantitative within 1 h of digestion at 60°C. For each of the zoological RMs, three passes through the homogenizer in the presence of protease provided a quantitative release of Se and incubation with the enzyme was not necessary. No separation of the Se between the liquid phase and the particulate phase was evident even after several days of subsequent storage at 4°C. The botanical matrices were more resistant to Se release and required up to 1 h of digestion with protease at 60°C. Alternatively, 10 passes through the homogenizing valve (in the presence of the enzyme) resulted in the quantitative release of analyte.

The same technique was employed on five animal feed samples. For each of the animal feeds, ten passes through the homogenizer combined with 0.5 h enzymatic digestion at 60°C was sufficient to provide a quantitative release of Se.

A new model of homogenizer equipped with ceramic homogenizing valve was evaluated in terms of analyte metal contamination levels within the final sample dispersion. For Cr, Cu, Fe, Mn and Pb, contamination levels fell within the range that can be either ignored or readily compensated for when determining levels of these analytes in biological matrices. The level of iron was greatly reduced when compared with levels introduced by the previous model.

Five animal feed samples and four wood pulp samples, were investigated for their content of Cu, Fe and Mn using high-pressure homogenization as the sample preparation technique prior to GF-AAS. Dispersions of dried animal feeds were sub-sampled reliably after 7 days of storage at 4°C.

Trials on pulp samples indicated that pulps could be sub-sampled reliably after 1 day of storage. However, further optimization studies will need to be performed to increase the homogeneity of the dispersions.

# RÉSUMÉ

L'homogénéisation à haute pression combinée à une digestion enzymatique par une protéase brute a été évaluée comme moyen de libération des composés du Se à partir de matrices zoologiques et botaniques, préalablement à l'analyse par SAA-FG. Pour ce faire, un nouvel homogénéisateur à valve plate a été utilisé. Des essais chronométrés avec quatre échantillons de référence certifiés (ERC) d'origine zoologique, trois échantillons de référence (ER) d'origine botanique et un échantillon de plante alimentaire ont montré que la libération du Se était quantitive après homogénéisation ou qu'elle devenait quantitative en 1h de digestion à 60°C. Pour chacun des ER zoologiques, trois passages à travers l'homogénéisateur en présence de protéase ont permis une libération quantitative du Se, sans que l'incubation avec l'enzyme soit nécessaire. Aucune séparation du Se entre la phase liquide et la phase particulaire n'a pu être mise en évidence, même après plusieurs jours consécutifs de conservation à 4°C. Les matrices botaniques étaient plus résistantes à la libération du Se et ont nécessité jusqu'à 1h de digestion en présence de protéase à 60°C. En alternative, 10 passages à travers la valve d'homogénéisation et en présence d'enzyme ont permis une libération quantitative de l'élément.

La même technique a été utilisée avec cinq échantillons d'aliments pour animaux. Pour chacun de ces aliments, dix passages à travers l'homogénéisateur, combinés à 0.5h de digestion enzymatique à 60°C étaient suffisants pour permettre une extraction quantitative de Se.

Un nouveau modèle d'homogénéisateur équipé d'une valve d'homogénéisation en céramique a été évalué en considérant le niveau de contamination métallique de l'élément dans la dispersion finale. Pour Cr, Cu, Fe, Mn et Pb, les niveaux de contamination se situaient dans une échelle pouvant être soit ignorée, soit facilement compensée au moment de la détermination des niveaux de ces éléments dans les matrices biologiques. Le niveau de fer était réduit de façon importante, comparativement au modèle précédent.

Cinq échantillons d'aliments pour animaux et quatre échantillons de pulpe de bois ont été étudiés pour leur teneur en Cu, Fe et Mn, en utilisant l'homogénéisation à haute pression pour la préparation des échantillons avant analyse par SAA-FG. Les dispersions des aliments secs pour animaux étaient rééchantillonnées efficacement après 7 jours de conservation à 4°C.

Des essais sur les échantillons de pulpe ont montré que les pulpes pouvaient être rééchantillonnées de manière efficace après un jour de conservation. Cependant, des études supplémentaires d'optimisation devront être mises en œuvre pour augmenter la stabilité des dispersions.

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# Introduction

## 1.1 Trace Elements

### 1.1.1 Definition

More than ninety different elements from hydrogen to uranium occur naturally. How many of them are essential to life? Some are easy to detect; whereas other elements occur in such small quantities in living tissues that for a long time their concentrations were not determined accurately; they were often mentioned as occurring at "trace" levels (conventionally considered to occur at concentrations of less than one ppm, part per million). Although exceedingly sensitive techniques have been developed that provide low to sub parts per billion (ppb) limits of detection, that are virtually specific for the analyte, and that permit precise determinations of virtually all elements, the term 'trace element' still remains popular in current usage. Some authors define a trace element as any substance that occurs at concentration of 1 mg or less in 1 kg of tissue (Cotzias and Foradori, 1969). Another definition was provided by Forssen (1972), trace elements are those elements that occur in tissues at concentration less than 0.01% of dry mass.

For a long time, the term "trace element" also suggested an uncertainty as to the biological significance of the element. Probably for the latter reason iron and iodine, although they can occur within the above–mentioned concentration ranges, are generally considered to be distinct from the group of trace elements.

The following elements are generally included among the trace elements: fluorine, aluminum, silicon, vanadium, chromium, manganese, cobalt, nickel, copper, zinc, arsenic, selenium, rubidium, molybdenum, cadmium, tin, mercury, and lead.

### 1.1.2 Classification

From a dietary point of view, trace elements are most conveniently classified into three groups: essential, possibly essential, and non-essential.

Criteria that an element must satisfy in order to be classified as essential for life are (Cotzias and Foradori, 1969):

1. it is present in healthy tissues of all living organisms.
2. its concentration from one individual to others of the same specie is fairly constant.
3. its withdrawal from the diet reproducibly induces the same structural and physiological abnormalities regardless of the species under study.
4. supplementation of the diet with the trace element either prevents or reverses these abnormalities.
5. the abnormalities induced by deficiency are always accompanied by specific biochemical changes.
6. these biochemical changes can be prevented or cured when the deficiency is prevented or remedied.

As recently as the mid 1950s, only five trace elements (in addition to iron and iodine) had been identified as being essential: manganese, cobalt, copper, zinc, and molybdenum. Research over the course of the following two and a half decades added several other elements, including fluorine, silicon, vanadium, chromium, nickel, selenium and tin. However, it should be noted that all trace elements are potentially toxic when the limits of safe exposure are exceeded. On the other hand, the well-known toxicity of an element is no bar to its being essential.

### 1.1.3 Function

How trace elements play their essential role in biological systems is only poorly understood. The search for an explanation of their physiological mode of action has emphasized their association with enzyme systems. Enzymes affected by metals can be categorized in two groups: metalloenzymes and metal-enzyme complexes, on the basis of the strength of the binding between the metal(s) and protein.

A metalloenzyme contains a metal as an integral part of its molecule structure (in a fixed ratio per molecule of protein), the specific and unique chemical nature of the metal-protein interactions apparently confers both stability and reactivity to the molecule.

Metal-enzyme complexes comprise a large group of enzymes, which are more loosely associated with metal ions, the criterion of association being the activation of catalysis. The metal is believed to act as a temporary link between the enzyme and the substrate during the chemical reaction. In addition to stabilizing the enzyme-substrate complex, the metal can also stabilize the reaction products – thus facilitating the reaction.

In addition to enzymatic catalysis, trace elements are involved in oxidation-reduction and transport processes, in membrane permeability, in the function of subcellular organelles such as mitochondria, in nerve conduction and in muscle contraction. They also appear to play a role in the synthesis and structural stabilization of both proteins and nucleic acids.

## 1.2 Trace Analysis

A trace metal analytical procedure should, ideally, possess the following attributes:

- possess a limit of detection (LOD) that is sufficient to answer the question being posed,
- be relatively fast,
- be relatively inexpensive,
- be applicable to a wide range of different samples,
- be relatively specific,
- be suitable for most analytical laboratories (which suggests no need for special equipment),
- be capable of providing information simultaneously on many elements,
- possess a relatively wide linear dynamic working range, and
- be accurate (be amenable to testing with Certified Reference Materials, CRMs)

Historically, gravimetric and titrimetric methods were first employed for trace element analysis. However, it was necessary to employ a number of time-consuming and error prone steps including separation and preconcentration to increase the concentration of the target trace constituent sufficiently to permit its unambiguous detection and/or quantitation using these techniques. It was not,

therefore, until sensitive analytical instruments became widely available that practical and accurate trace element analysis became possible.

In the 1930s optical emission spectrometric methods of analysis were developed. Initially flames were employed as excitation-atomization sources, followed by furnaces, arcs and sparks. Colorimetric-molecular-spectrophotometric methods were introduced at about the same time. These approaches include the related techniques of nephelometry, turbidimetry, and molecular fluorometry. Practical electrochemical methods were introduced in the late 1930s and early 1940s. The most commonly used eletrochemical approaches are polarography and anodic stripping voltametry.

It was not, however, until the introduction of atomic absorption spectrometry and modern neutron activation and related methods that routine trace element analysis became very popular. More recently the introduction of the plasma atomizer-excitation sources for use with optical emission and mass spectrometry have greatly expedited trace element analysis methods.

### 1.2.1 Atomic Absorption Spectrometry

Atomic absorption spectrometry (AAS) is generally considered to be a mature technique, and at present, is the most widely used technique for the determination of trace elements. The positive features of this technique remain the relative freedom from spectral interference and the modest purchase and operating costs. The approach is relatively simple and the instrumentation is available widely. It can be found in analytical trace metal laboratories and in a variety of biological, clinical, and environmental research, metallurgical and routine analytical establishments.

AAS involves the study and measurement of the absorption of radiant energy by free atoms. Free atoms are generated by an atomizer, which may be a flame or an electrothermal (furnace) atomizer. Radiation from a suitable light source such as a hollow cathode lamp (containing the element to be determined) is directed through the resulting population of free atoms that attenuate the incident radiation. The degree of attenuation (absorption) of the incident radiation is proportional to the

concentration of the analyte atoms within the light path. Absorption by free atoms at other (non-absorbing) wavelengths is essentially zero.

Atomic absorption detection limits depend on the type of atomizer used and on the sample matrix. Generally the levels of analyte element are in the range of micrograms per milliliter to sub nanograms per milliliter, the latter being the case when a furnace atomizer is employed. These limits of detection are usually adequate for determination of trace metals in a wide variety of samples.

**Flame Atomization**

A flame is formed using air, oxygen, or nitrous oxide as the oxidant gas and hydrogen, acetylene, coal gas, or butane as the fuel gas. Most often a mixture of air and acetylene provides the support gases to give a stable flame front that is maintained some 3-7 mm above the burner head. A liquid sample is aspirated into nebulizer where turbulent gas flows cause most of the sample to coalesce and to deposit on the sides of the chamber. The larger droplets are discarded via a drain. Only the smallest liquid droplets are carried up through the nebulizing chamber and into the flame where the solvent is evaporated. The solid residue of microcrystals is decomposed and free atoms are generated.

Flames are very inefficient atomizers. It has been estimated, for instance that approximately one atom in a million atoms of the sample element is actually transformed to the free atomic state. All other atoms are not reduced to free atoms quickly enough, and they pass through the light path unreduced and do not contribute to the atomic absorption signal.

**Hydride Generation Atomization**

The resonance wavelengths of a number of elements (principally metalloids and non metals) are in the vacuum UV region (less than 200 nm). Air appreciably absorbs radiant energy at these wavelengths so that the intensity of the radiation is greatly diminished. In consequence, the background signal is the most severe in this region making measurement of the atomic absorption by these elements difficult. For a number of these elements the problem can be overcome because fortunately they

form volatile hydrides. These hydrides can be generated by mixing the sample with a solution of strong acid plus $NaBH_4$.

$MX_n + nNaBH_4 + 2H^+ \rightarrow MH_n \uparrow + nNaX + nBH_3 + H_2 \uparrow$

Total reaction time is typically between 10 sec and 2 min.

The volatile hydride is swept into a silica tube that has been interposed within the optical light path. At the temperature of the tube the hydride is decomposed to free atoms of the analyte metal. The rapid release of molecular hydrogen into the quartz tube sweeps air from the light path. The absorption signal is then measured in the normal fashion.

The advantage of this technique is that the high background of the flame is eliminated because no flame is used in the process. The disadvantage is that hydride formation depends on the valence of the element being determined, and that only a few elements can be determined in this way. This technique has been widely applied to only certain species of the following elements: As, Pb, Sn, Bi, Hg, Ge, Sb and Se.

**Electrothermal (Graphite Furnace) Atomization**

Electrothermal (graphite furnace) atotomization is probably the most sensitive atomic spectrometry technique that is routinely available to the analyst. A small quantity (typically 20 μl) of sample is deposited into the interior of a hollow graphite tube (furnace) which is mounted horizontally within the optical beam of the instrument. In successive stages, the tube is heated (electrical resistance) to cause:

- solvent evaporation (drying stage);
- matrix decomposition (charring stage);
- analyte atomization (atomization stage); and
- furnace cool-down (and vapour clean out prior to introduction of the next sample)

The direct introduction of sample into the graphite tube makes differences in the physical properties (viscosity, solvent composition etc.) between sample and standard virtually insignificant. A second advantage of this design is that

volatilization of the analyte atoms occurs into a confined space so that the atom residence time within the optical beam is appreciably longer that with flame or plasma techniques. The limits of detection are at least an order of magnitude (often two or three orders) lower than with either flame AAS or plasma-atomic emission spectrometry (AES). In addition, this technique has the advantage that much of the matrix can be removed during the charring stage of the analysis, which makes the interference level less severe in the final determination stage.

### 1.2.2 Background Correction for AAS

The two main sources of background absorption in AAS arise from the scattering of the resonance radiation by solid particulate matter and the absorbance by molecular species formed during the sample matrix decomposition. The background interferences result in overestimates of the analyte concentration.

The scattering of light by particles of solids and liquids obeys Raleigh's stray light law in which a scattering coefficient is given by

$$\tau = P/P_0 = 24\pi^3 N v^2/\lambda^4$$

*i.e.* the magnitude of the effect is directly proportional to

-the number of particles (N) per unit volume

-the square of the particle volume (v)

-and is inversely proportional to the 4 th power of the light wavelength ($\lambda$)

(i.e. $\tau$ increases 256 folds from 800nm to 200 nm)

Historically, the initial attempt to provide reference measurements was achieved by incorporating a deuterium continuum source into the spectrometer's optical system. The absorbance measured with the hollow cathode lamp includes the contributions from the analyte, matrix and scattering components while with the deuterium lamp there is essentially no residual atomic absorption. Measurements of absorbance from both sources are subtracted to provide a background corrected atomic absorbance. While this technique is often adequate in many analytical applications, it will clearly fail should the matrix components present discrete line spectra.

Furthermore, no simple, single tunable source is capable of adequately covering the entire ultra violet-visible range required for atomic absorption and whenever multiple sources are employed careful optical alignment is imperative to ensure identical spatial distributions of source radiations within the furnace/flame.

Modern physics has demonstrated that the wave length of atomic spectral lines can be shifted by applying magnetic fields (Zeeman effect). By employing the Zeeman effect to modify the atomic absorption wavelength profile, the residual background absorbance at the analyte wavelength, can then be measured separately. Measurement of the background plus analyte signal can then be subtracted by the absorbance of the background.

Since only a single source is required, there remain none of the optical alignment problems encountered with multiple source schemes. Furthermore, the Zeeman effect is generally applicable to all spectroscopic transitions and so encompasses the entire wavelength range. However, for Zeeman atomic absorption, there is the complication in that calibration curves can become curvilinear at higher concentration. This means that two different concentrations can give the same absorbance, so the technique is analyte concentration limited.

### 1.2.3 Summary

As summarized in Table 1.1, only a limited number of trace elements in plasma or serum can be determined successfully by flame AAS. By contrast, the majority of analyte elements can be determined routinely with graphite furnace-AAS techniques, whereas others require hydride generation AAS procedures.

**Table 1.1** Atomic Absorption Techniques Used for the Determination of Trace Elements in Human Plasma or Serum

| Element | FAAS | GF-AAS | HGAAS | Dilution | Digestion | Preconcention |
|---|---|---|---|---|---|---|
| Li | X | X | | X | | |
| Al | | X | | X | | |
| V | | X | | X | | |
| Cr | | X | | X | [X] | [X] |
| Mn | | X | | X | | |
| Co | | X | | | X | X |
| Ni | | X | | X | [X] | [X] |
| Cu | X | X | | X | X | X |
| Zn | X | X | | X | | |
| As | | X | X | | X | X |
| Se | | X | X | | X | X |
| Rb | X | X | | | X | X |
| Mo | | X | | | X | X |
| Ag | | X | | | X | X |
| Cd | | X | | | X | X |
| Sb | | X | X | | X | X |
| Hg | | | | | X | X |

Note: [ ] = optional; FAAS = flame-AAS; GF-AAS = graphite furnace AAS; HGAAS = hydride generation AAS ( Versieck J. and Cornelis R., 1988)

### 1.3 Sample Preparation / Introduction for Trace Analysis

Sample preparation has always been viewed as a manual and cumbersome technology that is often regarded as "low tech". Today, analytical techniques are increasingly generic, there are a variety of ways to perform sample preparation, many of which are virtually sample-specific. Sample preparation remains one of the more time-consuming, cumbersome and error-prone aspects of the analytical procedures and as such, it has long been the "bottleneck" in the overall process. Usually, the time needed for the preparation of the samples exceeds by an order of

magnitude the instrument time needed for the actual measurement. The time and effort required during an analysis become critical factors in determining the feasibility and cost of an analytical method.

Selection of the optimal sample preparation/introduction procedure for an analysis requires many considerations. These considerations include the type of sample (e.g. solid, liquid, gas), the levels for the analyte(s) that are anticipated, the required accuracy and precision, the quantity of sample that is available to the experimenter and the number of determinations that can be performed per hour.

Most methods currently used in trace metal analysis require that the sample be presented to the instrument as a liquid as is the case for atomic absorption spectrometry. For the AAS analysis of most biological, environmental or food samples (in which trace elements are of great research interest), it is usually necessary to perform a digestion of the sample, in order to: (i) release the analyte element from the organic matrix into a form that is detectable by the AAS technique; (ii) make a true solution of the sample.

Despite successive improvements in the AAS technique in terms of instrumentation, measurement and quantitation methodologies, sample preparation has remained little changed during the last few decades. Since the early 1980's, a greater awareness of the sample introduction process and the development of a variety of sample introduction techniques has resulted in an even greater research activity in this area. Among the different strategies, microwave digestion of the sample and solid sampling techniques have attracted the greatest attention. Each is considered below.

### 1.3.1 Microwave-Assisted Digestion

Abu-Samra *et al.* (1975) first reported the use of microwaves as a heat source for wet chemical digestion methods. Since then, the microwave digestion technique has gradually gained widespread acceptance as a rapid and efficient method of sample preparation. Using this technique, has reduced digestion dramatically (by a factor of 2-5). In addition, this approach has reduced contamination, the consumption of reagent and sample, and has reduced the loss of volatile species and improved safety

(Kinston and Jassie, 1988). In the early 90's, the introduction of high temperature/pressure digestion devices provided a further impetus to the evolution of the technique (Dunemann and Meinerling, 1992). The advantages of the microwave digestion technique have led to its application as an effective sample preparation method for a wide range of sample matrices.

**Closed Microwave Digestion**

The closed digestion technique involves placing the sample in a sealed vial (or bomb), typically constructed of a fluorinated polymer, such as polytetrafluoroethylene or perfluoroalkoxy polymer. After adding the digestion reagents, the bomb is sealed tightly and placed in the microwave oven to be irradiated with microwave energy.

The major advantage of the closed microwave digestion is the high heating efficiency that can be achieved. Heating causes an increase in pressure, due to the evaporation of digestion acids and the gases evolved during the decomposition of the sample matrix. This benefits the digestion process by increasing the boiling point of the reagents, which aids the breakdown of the sample matrix. However, the excessive build-up of pressure, especially during the digestion of samples with a high content of organic matter, can lead to the rupture of sealed vessels. For this reason, most digestion bombs are fitted with pressure relief valves, designed to open if the pressure becomes excessive, and thus maintain safety. If venting does occur, sample losses are likely and a less efficient digestion can result from the loss of volatilized acid vapors. Considerable studies have been performed to find ways of controlling or reducing pressure built-up during the digestion process (Banuelos and Akohoue, 1994; Gluodenis and Tyson, 1992; Reid *et al.*, 1995).

**Open Digestion Techniques**

Open digestion systems operate at atmospheric pressure and so do not suffer from the problems associated with a rapid pressure build-up. However, they do require an effective fume removal system. The potential loss of volatile species is minimized by the re-condensation of vapors in a reflux column positioned above the

sample flask. The open vessel approach permits the addition of digestion reagent at any stage of the procedure, which can increase the effectiveness of the digestion. This is a distinct advantage over closed methods where the addition of reagents cannot be achieved without cooling and opening the vessels.

A disadvantage of the early open digestion system was that only one sample could be digested at a time. More recently, a two – or six-cavity open microwave digestion unit has become available commercially. Other features include the ability to program the power output/desired temperature to each sample independently (Prolabo, 1995).

As an example, microwave digestion techniques have been applied successfully to the digestion of biological samples prior to selenium determination. Many different microwave digestion procedures for the determination of selenium have described in the technical literature. Banuelos and Akohoue (1994) investigated a number of different reagent combinations with and without a pre-digestion stage. Using a simple nitric acid digestion, a Se recovery of only 23% was obtained for a National Institute of Standards and Technology (NIST) standard reference material (SRM, Wheat Flour). Recoveries were improved to 80% after a nitric acid and hydrogen peroxide digestion with a 4-h pre-digestion (but only 57% of the Se was recovered in the absence of a pre-digestion stage). Prolonged heating or the addition of hydrochloric acid did not increase the recoveries. Mizushima *et al.* (1996) reported that the successful determination of Se in NIST Bolvine Liver SRM was achieved by digestion with nitric acid, but that the results for (Internation Atomic Energy Agency. Fish Flesh certified reference material were low. Selenium determinations have also been carried out successfully using open nitric acid and hydrogen peroxide digestion procedures for NIST Bolvine Liver and Mixed Diet SRM. Whereas results for NIST Total Diet SRM were slightly low (Ducros *et al.*, 1994).

### 1.3.2 Slurry Technique

In recent years there has been considerable interest in procedures that avoid complete digestion, such as solid sampling analysis. A particular field of interest, within solid sampling analysis, has been the application of AAS to the analysis of

solid samples using a slurry introduction technique. In this technique, a stabilized suspension of solid particles is analyzed in an exactly analogous fashion to a conventional liquid solution. The technique is known as slurry atomization. This strategy combines the advantage of solid and liquid sampling, because the sample can be transported into the atomizer by conventional liquid handling equipment following a minimal pretreatment of the sample. This approach minimizes the potential contamination from the chemicals and minimized operator intervention in the process of suspending/dissolving the sample. Compared to solid sampling, the main advantage of slurry atomization is the ability to calibrate the instrumental response with aqueous standards.

**Atomization Methods**

Most of the work that has been reported on the slurry sampling of biological materials has been performed by AAS using either flame or electrothermal (furnace) atomization, the latter being the more popular approach.

Conventional flame nebulization systems are prone to blocking when slurries containing large particles are continuously nebulized into the instrument. Frequently, the effects have been attributed to sample transport effects. Solid particles and agglomerates generated from slurries containing large particles can be rejected preferentially as they traverse the spray chamber. The degraded analytical response for slurries can also be explained in part by incomplete atomization of the particles as they pass through the flame region. The composition of the support gases and resulting flame temperatures also have an appreciable effect on atomization efficiency (De Benzo *et al.*, 1991).

In contrast to flame and inductively coupled plasma (ICP) atomization of slurries, efficiencies obtained by furnace atomizations do not seem to vary with particle size. (De Benzo *et al.*, 1991). Solid samples are probably analyzed with fewer problems using GF-AAS (graphite furnace AAS) than by any other spectroscopic technique because the sample remains within the atomization chamber for a longer time. This makes it more likely that the solid particles of varying sizes and volatilities will be more fully decomposed. By integrating the analytical signal over the course of the

atomization stage of the furnace heating sequence, now routine in GF-AAS, troubles from large particles are minimized or avoided (Bradshaw *et al.*, 1989). Slurry sampling GF-AAS techniques are frequently applied to samples that previously had been difficult to analyze. Its relatively ease of use, the commercial availability of instruments and the ease of adapting laboratory systems to nearly all commercial autosamplers for GF-AAS systems are among the main reasons for its increasing application of this technique to numerous analytical tasks.

**Particle Size**

The mean particle size and the particle size distribution of the slurry is of particular importance for the efficiency and the repeatability of the nebulization. The introduction of slurries by nebulization into flames (Carrion *et al.*, 1987; De Andrade *et al.*, 1990; Lopez-Garcia *et al.*, 1991; Vinas *et al.*, 1993) and plasmas (Ebdon *et al.*, 1990; Goodall *et al.*, 1993) as well as into hydride systems (Madrid *et al.*, 1989; Calle *et al.*, 1991) require extremely small particle sizes, typically < 3μm, that typically are only achieved with vigorous milling procedures (Goodall *et al.*, 1993).

The slurry technique for GF-AAS can be performed with significantly larger particle sizes that approach the particle sizes obtained by conventional grinding techniques. The data reported for particle sizes comprise a broad range from ≤ 20-50μm and include particles that are as large as 100μm. Smaller mean particle sizes of 10-20 μm can be anticipated to improve the precision of the analytical results, but are only mandatory if the element(s) to be determined are not distributed homogeneously throughout the solid matrix (Miller-Ihli, 1992). If the analytes are quite homogeneously distributed in the solid material, significantly larger particle sizes up to about 500 μm can be tolerated.

Successful determinations have been reported for a variety of metals in zoological, botanical, and food samples that have been ground with conventional techniques, more vigorous grinding has been reported to improve the precision of metal determinations in soils, sediments and coal fly ash (Dolinsek *et al.*, 1991). Many of the materials to be analyzed, e.g. standard and certified reference materials already

have mean particle sizes in the optimal range (< 50-100 μm) for slurry sampling or even less. Thus it was often possible to use these materials directly for slurry preparation without prior grinding. However, whenever additional grinding is needed, it is very important to minimize contamination from the grinding devices.

Takuwa *et al.* (1998) reported the optimal precision of the analytical results was obtained after 60 min of grinding of plant samples, when 100% of the sample particles had diameters less than 50μm.

Bermejo-Barrera *et al.* (1998) reported that human hair powder with a mean particle size of 0.8μm was suitable for slurry GF-AAS determination of Al and Mn.

Meeravali and Kumar. (1998) used ceramic motor and piston for additional grinding of CRMs, and the particle size was found to be 15-30 μm for NIST SRM 1566a Oyster Tissue and 30-40 μm for GBW 08504 Cabbage and NIST SRM 1571 Orchard Leaves.

Januzzi *et al.* (1997) applied the slurry technique to the determination of Se in fish by GF-AAS. A sample was sieved through 250, 105 and 53 μm screens. The particle size distribution was 22.1% for 250 μm, 62.4% for 105 μm, and 9.8% for 53 μm. The repeatability of the signals from the slurries prepared from the coarse fractions was worse than those from the finest fraction. In addition, higher recoveries were obtained by employing the 53 μm particle size than with the 250 and 105 μm fractions.

**Dispersion Agent and Stability**

The stabilities of dispersions of solid samples within a liquid medium can be monitored readily by measuring with repeatability of the determination with time. Sometimes, when analyzing certain types of biological samples, it is advantageous to include a surfactant in the slurry liquid to assist in wetting and dispersing the fine powder. Without the surfactant, the powder can have a tendency to float and to agglomerate. Similar effects have not been observed for particles of more polar character including finely ground rocks and minerals for which wetting and flotation agents have not been found to be particularly useful (Willis, 1974).

The classical approach to maximizing slurry stability has been to increase the viscosity of the medium by adding dispersing agents such as Viscalex (Littlejohn *et al.*, 1985; Stephen *et al.*, 1985) or glycerol (Hoenig *et al.*, 1986). The use of viscosity modifiers have stabilized the slurry, ensuring that a homogeneous suspension can be maintained, without the requirement for external stirring during the withdrawal of a suitable aliquot for analysis.

Fuller and Thompson (1977) and Fuller *et al.* (1981) were the first to apply this technique for the determination of several elements. Suspensions containing approximately 2% (v/v) Viscalex gel was reported to be stable for several days. Littlejohn *et al.* (1985) and Stephen *et al.* (1985) applied the same technique for the determination of Pb in spinach using an autosampler. Yu *et al.* (1990) used a similar stablization technique for Pb determination in various NIST plant standard reference materials (SRMs) by manual injection. The material was ground to particle sizes less than 50 μm. For a Citrus Leaf SRM slurry (0.3% m/v) containing Viscalex HV30 (1.5% v/v) a stability of greater than 3 h was reported. However, it was reported (Miller-Ihli, 1988) that the use of Viscalex can cause problems with the repeatability of autosampler pipettings.

Another slurry stabilizing agent is glycerine. Hoenig and coworkers (1986, 1989) used mixtures of glycerine, methanol, nitric acid and matrix modifiers for the preparation of slurries from different geological and biological materials to determine various elements using an autosampler. Optimal results were recorded with 1+1 glycerine-demineralized water for environmental samples and glycerine-methanol (1:1 v/v) for lyophilized animal tissues. It was observed that these suspensions were stable for up to 1 h thus permitting introduction via an autosampler.

Bendicho and De Loos-Vollebregt (1990a, 1990b) attempted to stabilize slurries of glass materials with solutions containing 5-80% glycerol in the autosampler cup. The slurry was stirred then left in the autosampler cup without further mixing for automated injection. The authors reported that these slurries were very unstable at low concentrations of glycerol, whereas the high concentrations of glycerol caused repeatability problems with the autosampler. They concluded that for high density

materials such as glass powder, glycerol was unsuccessful as a stabilizing agent because the distribution of the particles was not homogeneous unless the slurry was continuously stirred.

In order to maintain an even distribution of particles in slurries, a method has been reported (Miller-Ihli, 1988) that utilizes ultrasonic agitation using a titanium probe inserted directly into the autosampler cup. This approach seemed to circumvent the need for stabilization of slurries using dispersing agents.

From the literature concerning modes of ultrasonication, the stability of the slurry appears to be of minor or even of no importance, if the agitation is performed directly in the sample tray and continued during sampling. However, in some cases, there is some elapsed time between the termination of ultrasonication and injection because of the different techniques applied. Therefore, some studies have been performed on the stability of slurries after ultrasonication. These studies provided addition information about the extent of analyte extraction from the solid into the liquid phase of the slurry ( Dobrowolski and Mierzwa, 1992, 1993; Mierzwa and Dobrowolski, 1994)

Lima *et al.* (1999) chose 0.2% v/v $HNO_3$ + 0.04% v/v Triton X-100 as media for suspending the fish slurries, since the medium provided low blank values and avoided sample flotation.

Vinas *et al.* (1999) also reported that acceptable analysis results were obtained using Triton X-100 (0.1% m/v) as surfactants for As determination of baby food slurries. In addition, foaming was prevented by adding one drop of a silicon antifoam agent.

For determinations of Al and Mn in human scalp hair, Bermejo-Barrera *et al.* (1998) studied the stability of slurries using different wetting agents including Triton X-100, Viscalex HV40 and glycerol. While improvement in the analysis precision were provided by either Triton X-100 or glycerol, the authors selected glycerol as most adequate agent due to problems related to the volumetric measurement of Triton X-100 solution.

**Modifier**

Modifiers are added to the sample solution/suspension to modify the physical characteristics of the sample and to increase the temperature at which it is volatized. The usefulness of matrix modifiers in slurry-GF-AAS is controversial and has been discussed in the literature. Some examples are given below.

Hinds and Jackson (1987) used matrix modifiers to overcome interferences due to organic carbon in soil slurries. They compared the addition of palladium nitrate and palladium chloride as chemical modifiers for the determination of Pb in solutions and soil slurries. They concluded that a mixture of Pd and Mg nitrates had the advantage of being applicable to a variety of analyte elements (Hinds and Jackson, 1990).

Lynch and Littlejohn (1989) found the Pd modifier to be superior to ammonium dihydrogen phosphate for Pb determination in food slurries. Haraldsen and Pougne (1989) used Mg nitrate as a modifier for the determination of Be in coal slurries to permit ashing temperatures of up to 1800°C. Ohta *et al.* (1990) determined Cd in various biological materials with a Mo tube electrothermal atomizer. Matrix modification was performed by adding 1 µl aliquots of a sulfur solution (10 mg/ml) in carbon disulfide.

Dolinsek *et al.* (1991) determined Cd and Pb in geological and plant materials. It was reported that for the analysis of plant materials, an ammonium dihydrogen phosphate modifier was optimal.

For the determination of As in different matrices, matrix modification was also employed. Lopez and Hernandez (1990) used Ni nitrate for Fe (III) oxide pigments. Pd-Mg nitrate was used by Bermejo-Barrera et al. (1994) for analysis of mussels. Bendicho and Sancho (1993) determined Se in wheat flour and reported the usefulness of a Pd/Mg matrix modifier and isothermal atomization. However, Miller-Ihli (1994) reported that frequently, the use of matrix modifiers for slurries appeared to be unnecessary. However, in some cases, greater amounts of matrix modifier than for aqueous solutions were required to achieve good results.

For the analysis of technical materials Krivan and coworkers (Friese and Krivan, 1995, Schneider and Krivan, 1995) found that only for a few elements (Cr, Cu, Fe,

and Mn in silicon nitride and Al, Cd, and Si in zirconium dioxide based materials) was the use of matrix modifiers necessary.

Lopez-Garcia *et al.* (1993) reported the use of rapid furnace programs and the addition of HF for the determination of Cr, Cu, and Pb in slurries of diatomaceous earth. Hoenig and Gillissen (1993) demonstrated that the use of rapid furnace programs for several metals in CRMs gave results comparable to conventional approaches using matrix modification, but that in all cases peak evaluation was mandatory.

Carlosena *et al.*(1997) studied use of some chemical modifier for determinations of Cr, Co and Ni in vegetables. It was reported that a mixture of $HNO_3$-$H_2O_2$ acts as an oxidant modifier that permits one to dispense with conventional chemical modifiers (e.g., Mg and Pd); moreover, its presence affords a high pyrolysis temperature for Cr and Co.

In Lima's (Lima *et al.* 1999) study, a tungsten carbide-rhodium coating on the integrated platform of a transversely heated GF-AAS was used as a permanent chemical modifier for the determination of Cd in fish slurries. The permanent modifier increases the tube lifetime by 50-90% compared with untreated integrated platforms. Also, there is less loss of sensitivity during the atomizer lifetime when compared with the use of a conventional modifier so that there is, a decreased need for re-calibration during routine analysis.

**Standardization and Accuracy**

Different methods have been employed for the direct analysis of slurries: comparisons with commercial standard reference materials, calibration with the method of standard additions and measurements against aqueous standard solutions. For nebulizer-based procedures, the variation in signal response with particle size and matrix require that closely matched standards be used for accurate analysis. By contrast, it is possible to use aqueous standards for electrothermal atomization and to obtain acceptable results (De Benzo *et al.*, 1991).

The accuracy of the slurry method for determinations in biological samples has been verified by different approaches. These include: recovery tests and the method of

standard additions, the comparison of results generated with independent analytical methods and the use of standards or certified reference materials (CRMs) - the latter two methods have been applied most frequently (De Benzo *et al.*, 1991).

Specifically for biological samples, a great variety of CRMs are available. In consequence, the accuracy of analytical methods has been verified mainly against these standards. Many studies reported that the accuracy of the slurry method compares favorably with the accuracy of other methods for these types of materials (De Benzo *et al.*, 1991).

### 1.3.3 Summary

**Slurry Sampling *vs.* Solid Sampling**

Direct solid sampling is desirable if only a small amount of sample is available or if there is an interest in the distribution of the analyte within the solid matrix. Most solid-sampling insertion devices are manually operated and require skill to obtain acceptable precision. Additionally, there is a requirement for repeated micro-weighings for a single analysis. The use of either matrix-matched solid standards or the method of standard additions might also be required.

Slurry sample introduction is quite straightforward when using a conventional liquid autosampler device that also permits the ready addition of matrix modifiers. It has the further advantages of facile dilution, convenient sample transfers and facilitated calibration with aqueous standards that collectively provide both accurate and precise results.

**Slurry Sampling *vs.* Sample Digestion**

Compared to conventional sample digestion, the preparation of slurries has minimal requirements for additional chemicals and equipment as well as a relatively small laboratory sample size. The preparation can be relatively simple and environmental friendly in that much less chemicals are typically employed. It also has advantage for the determination of trace elements in non-biological materials such as ores, and silicates, that can be difficult to decompose by classical chemical digestion, as well

as determinations of ultra-trace analytes if the digestion stage is prone to contamination.

## 1.4 High-Pressure Homogenization

### 1.4.1 High-Pressure Homogenizer

The term "homogenizer" has become a generic descriptor 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).

A high-pressure homogenizer is an apparatus that applies a pre-selected pressure to fluid materials that are to be processed into emulsified products. Usually, the instrument is equipped with a stainless steel cylinder block and a plunger to force the fluid through a tiny orifice. The fluid then impacts on an adjustable flat-faced valve. The purpose of homogenization is to physically break up the solid particles or liquid globules in the carrier fluid under reproducible conditions of pressure and the gap distance between the orifice and the valve. The passage of fluid through the minute orifice under high pressure and controlled flow subjects the fluid to conditions of high turbulence and shear that provide an efficient mechanism for particle or droplet size reduction.

### 1.4.2 High-Pressure Homogenization of Milk

The high-pressure homogenization technique was first employed in the dairy industry. Milk is an oil-in-water emulsion, with fat globules dispersed within a continuous skim milk phase. When raw milk is permitted to stand, the fat globules coalesce, rise and form a layer of cream. Homogenization is a mechanical treatment of the fat globule suspension in raw milk that results in a decrease in the mean diameter and a corresponding increase in the numbers and mean surface areas of the globules. The net result, from a practical view, is a appreciably reduced tendency of resulting globules to cream. Homogenization does not affect the nutritional quality of the product and is performed entirely for aesthetic purposes (University of Guelph, 1999).

Auguste Gaulin's patent in 1899 described a 3 piston pump in which product was forced through one or more hair-like tubes under pressure. There have been more than 100 patents issued since, all directed to producing smaller mean particle sizes with the expenditure of as little energy as possible. To understand the mechanism, consider a conventional homogenizing valve processing an emulsion such as milk at a flow rate of 20,000 L/hr at 14 MPa (2100 psi). As it first enters the valve, the velocity of the liquid is about 4 to 6 m/s. It then moves into the gap between the valve and the valve seat where the velocity is increased to 120 m/s in about 0.2 ms. The liquid then moves across the face of the valve seat and exits in about 50 sec. The homogenization phenomena is completed before the fluid leaves the area between the valve and the seat, and therefore emulsification is initiated and completed in less than 50 sec. The entire process occurs between 2 pieces of steel in a steel valve assembly. The product may then pass through a second stage valve similar in design to the first stage. While most of the fat globule size reduction takes place in the first stage, there is a tendency for clumping or clustering of the reduced fat globules. The second stage valve efficiently separates these clusters into individual fat globules (University of Guelph, 1999).

It seems likely that a combination of two theories, turbulence and cavitation, can explain the reduction in size of the fat globules during the homogenization process.

*Turbulence:* Energy dissipation within the liquid passing through the homogenizing valve, generates intense eddies of turbulence of the same size as the average globule diameter. Globules are thus torn apart by these eddy currents reducing their average size (University of Guelph, 1999).

*Cavitation:* There is a considerable pressure drop with the charge of velocity of fluid. The liquid cavitates because its vapor pressure is attained. Dissolved air is expelled from the solution to form bubbles which expand rapidly then contract. The high velocity gives liquid a high kinetic energy which is disrupted in a very short period of time. Dissipation of this energy leads to a high energy density (energy per volume and time). Cavitation generates further eddies that would produce disruption of particles.

### 1.4.3 The Application of High-Pressure Homogenization to Cell Disruption

A lot of biological molecules are inside the cell, including many metalloenzymes and metalloproteins, and for biological assay or biochemical/chemical analyses, they must be released from the cell before determination. This is achieved by cell disruption (lysis). Cell disruption is a sensitive process because of the cell wall's resistance to the high osmotic pressure inside them.

High-Pressure homogenization has been applied as a mechanical cell disruption method. Cell disruption is accomplished by three different mechanisms: impingement on the valve, high liquid shear in the orifice, and sudden pressure drop upon discharge, causing finally an explosion of the cell. The technique is suitable for large-scale applications. Following the rationale first employed by Dunnill (1972), the application of this high pressure device to cell disruption has found to be very useful for industrial applications.

The Manton-Gaulin APV homogenizer is one of the most widely used high-pressure disruption devices in industry. Using it to disrupt baker's yeast, Hetherington *et al.* (1971) reported disruption to be proportional to the number of passes and the operating pressure.

Sauer *et al.* (1989) studied the disruption capabilities of the Microfluidizer (a high-pressure homogenizing device) using both recombinant and nonrecombinant strains of the bacterium *E. coli*, and they reported the device achieved excellent disruption (e.g. 95% to 98% disruption at an homogenizer operating pressure of 95 Mpa with recombinant *E. coli* NM989 with only two to three passes through the homogenizer).

### 1.4.4 Slurry Preparation by Means of High-Pressure Homogenization

Recently, high-pressure homogenization has been evaluated for the preparation of quasi-stable dispersions/slurries suitable for FTIR (Dion *et al.*, 1994) or GF-AAS (Tan *et al.*, 1996a, 1996b). The advantage of this approach to sample preparation were the ease and speed of the slurry preparation and the fact that analyte metals were extracted quantitatively into the liquid phase during the preparation so that no analyte segmentation was detected within the slurry even after standing for several

days storage at 4 °C. Certified reference materials (CRMs), frozen cervine 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 amount of contaminating analyte metals introduced into the sample by the homogenization operation. Contamination was reduced appreciably, but not eliminated entirely, by capping the flat face of the stainless-steel homogenizing valve with a ruby, alumina or sapphire disc (Tan *et al.*, 1996b).

## 1.5 Selenium, an Ultra-Trace Essential Element

Selenium was first discovered in 1818 by a Swedish chemist, Berzelius. Early interest in selenium by nutritionists concerned its high concentration in certain range plants and the consequent toxicosis induced in animals that grazed on these plants. For a long time, Se was considered to be toxic for human and animals, causing severe poisoning in some regions of the world (Franke, 1934). In recent years many exciting research results have indicated that Se, depending on its concentration, can influence mammalian metabolism in a variety of ways; the essentiality of selenium to homeotherms has become the focus of more recent attention (Schwarz and Foltz, 1957; Rotruck *et al.* 1973; Flohe *et al.* 1973; Keshan disease research group, 1979a, 1979b;) This element is now known to be an ultra-trace essential element which is required by laboratory animals, food animals and humans, but the range between dietary requirements and toxic levels is surprisingly narrow (National Research Council, 1980).

### 1.5.1 Analysis of Se

Sensitive assays are required to determine the quantities of total selenium that is present albeit at low concentrations in many biological and food crops,. A variety of analytical methods have been applied for the determination of trace amounts of selenium in various materials. These include fluorimetry, atomic absorption spectrometry, neutron activation analysis, optical emission as well as a variety of

mass spectrometric methods of detection that are frequently combined with chromatographic separation techniques.

**Fluorometry**

The fluorometric method using diaminonaphthalene (DAN) to form a piazselenol type derivative has been one of the most popular methods for Se analysis (Cousins, 1960). This method involves conversion of Se, present in various oxidation states within the sample, to Se (IV), and reaction with DAN to form benzopiazselenol, a fluorescent product that can be quantified fluorometrically. The chief advantages of the DAN procedure are its good sensitivity and its relatively low cost. Nevertheless, the method has certain disadvantages. It entails the tedious nitric-perchloric acid digestion, which is labor intensive and can be hazardous. In addition to a quantitative release of Se from the sample matrix the analyte must be in the tetravalent state prior to reaction with DAN.

A second potential problem involves interferences by fluorescent degradation products from the reagent DAN itself (Dye *et al.*, 1963).

**Atomic Absorption Spectrometry**

Comparative studies have indicated that AAS is an attractive choice for the determination of selenium. The best AAS sensitivity [lowest limit of detection (LOD)] for Se are currently achieved using GF-AAS (0.05 to 3 mg/liter). Conventional flame atomic absorption spectrometry has not been generally suitable for the determination of Se in biological samples due to the relatively high LOD (ca. 0.1 ppm). It can only be used for samples containing high concentration of this analyte. The very short residence time of the atomized element in the analytical flame and the relatively low signal to noise ratio for flame AAS are the factors that limit the sensitivity of the technique.

Hydride generation - AAS provides the advantage of good sensitivity (ca. 0.01 ppm) but requires that great care be taken to ensure complete sample digestion and conversion of selenium to selenite (Lloyd *et al.*, 1982; Bye *et al.*, 1983). However,

it doesn't work for certain Se-compound. Additionally, it suffers from possible interference due to other elements that can also form hydrides (e.g. As, Cu, Sb).

Improved sensitivity has been obtained using graphite furnace –AAS (Alfthan and Kumpalainen, 1982; Kumpalainen *et al.*, 1983; Lopez-Garcia, 1995, 1996; Januzzi, 1997). This method avoids the problems associated with wet acid digestion by employing high temperature oxidation within a graphite furnace. The use of high temperature ashing reduces interference that result from nonspecific absorption of organic compounds and non-Se salts, but can increase analyte losses due to the volatilization of Se under such conditions. This problem can be minimized with the use of modifiers (salts that stabilize). In practice, GF-AAS has LODs for Se of approximately 0.003 ppm and with the use of Zeeman-effect background correction sensitivities approaching 0.001 ppm have been reported (Carnick *et al.*, 1983; Januzzi *et al.*, 1997).

### 1.5.2 Sample Preparation for Se Determination

Numerous accurate techniques for the determination of Se in biological materials have been developed. However, almost all these methods require a prior sample digestion to decompose organic matter. A complete digestion of the samples is necessary prior to the determination of Se because, on one hand Se is frequently incorporated within proteins in the form of selenomethionine and/or selenocysteine and must be released (Welz, *et al.*, 1984). On the other hand, some of the techniques (GC-MS, HGAAS, HG-ICP-MS) require chelation or hydride formation, which are convenient only for total digestion, though GC can achieve partial separation prior to MS detection.

The digestion methods for Se commonly involve use of strong oxidants such as $HNO_3$ and $HClO_4$ to decompose the Se-containing compounds. Moreover, to avoid losses of the volatile Se, which typically are in reduced forms, oxidizing conditions must be maintained throughout the digestion procedure (Oster and Prellwitz, 1982; Koh and Benson, 1983).

Microwaves can also accelerate the wet ashing process for individual samples of approximately 1 g or less. However, most commercially available microwave-based

digestion systems have a limited "parallel" throughput rate in terms of numbers of samples that can be processed simultaneously and are not configured to handle sub-samples as large as 10-30g.

Conventional acid digestion methods are time consuming and require constant operator attention to minimize analyte losses in the process or the addition other contaminants. It involves using strong oxidizing acid and once the analysis has been completed, there remains the problem of waste disposal that contains appreciable quantities of hazardous chemicals.

## 1.6 Heavy Metal in Paper Pulp

### 1.6.1 Role of Metals in Pulp Processing

Totally Chlorine Free (TCF) bleaching was first introduced commercially in the pulp and paper industry around 1990, and the rate of its development in recent years has been very rapid. The necessity for a reduction in the use of chlorine gas for the bleaching of pulp is no longer a question of debate. Increasingly, market pressure is driving pulp mills towards reduced emissions of all effluents with the eventual goal a completely closed mill. Closure of a mill will be difficult to achieve if the recycling streams contain chloride ions because of their high corrosivity. A TCF sequence will permit mills to operate with the least pollution of the environment whilst the development of the necessary recycling processes are carried out. Additionally, a TCF bleaching process will not cause the formation of any organo-chlorine matter in the pulp or in waste streams (Troughton and Sarot, 1992).

Hydrogen peroxide is an attractive candidate for use in TCF sequences. It is extensively used in the pulp and paper industry as a bleaching reagent, particularly for brightening of mechanical pulps. It is decomposed to give water and oxygen only, so it is ideally suited to applications where the effect on the environment of effluents has to be minimal (Troughton and Sarot, 1992).

Metal ions present in wood and pulp have a great effect on the efficiency of bleaching wood pulps with oxygen and hydrogen peroxide. Because of their intrinsic ability to easily change oxidation state, transition metals naturally present in wood pulp can catalytically disproportionate hydrogen peroxide into water and

oxygen, through radical intermediates. Among the transition metals, Mn, Fe and Cu are those most commonly found in wood and are therefore of most significance for bleaching with hydrogen peroxide (Bryant *et al.*, 1992).

In both acidic and alkaline media, hydrogen peroxide will react with transition metals. For example, hydrogen peroxide will oxidize iron (II) ions to iron (III), generating hydroxyl radicals. Further reaction with hydrogen peroxide will generate other radicals including the perhydroxyl radical. Oxygen, water and iron (II) are the end product (See reaction equations 1-7). The generated oxygen is not effective as a delignifying agent and is lost to the system. The iron (II) can react further, so the cycle destroys the hydrogen peroxide. The hydroxyl radical intermediate is a highly reactive species. It is electrophilic and will readily delignify pulps. Unfortunately, it also readily attacks cellulose rapidly destroying the physical properties of the pulp.

Peroxide Decomposition Reactions (Troughton, 1992):

$$Fe(II) + H_2O_2 \longrightarrow Fe(III) + \cdot OH + OH^- \quad (1)$$

$$H_2O_2 + \cdot OH \longrightarrow \cdot OOH + H_2O \quad (2)$$

$$Fe(III) + \cdot OOH \longrightarrow Fe(II) + H^+ + O_2 \quad (3)$$

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$$2\,H_2O_2 \longrightarrow 2\,H_2O + O_2 \quad (4)$$

Additional reactions:

$$Fe(II) + \cdot OH \longrightarrow Fe(III) + OH^- \quad (5)$$

$$Fe(II) + \cdot OOH \longrightarrow Fe(III) + OOH^- \quad (6)$$

$$Fe(III) + OOH^- \longrightarrow Fe(II) + \cdot OOH \quad (7)$$

Iron is not the only transition metal that catalyses the generation of hydroxyl radicals and the decomposition of hydrogen peroxide. Manganese and copper are known to be particularly active at alkaline pH whereas titanium is more active at acid pHs.

Therefore, effective use of hydrogen peroxide for bleaching or delignification of wood pulp requires the removal or deactivation of transition metals. With a

controlled metal ion profile it is possible to achieve a good bleaching result with hydrogen peroxide, or other bleaching agent such as ozone and oxygen.

### 1.6.2 Analysis of Heavy Metals in Pulp Fibers

To beneficially control the metal profile, analyses must be performed to provide the information of metal content in a wood pulp. The metals present in a wood pulp vary according to the wood species and the soil in which it was grown.

When using the TAPPI (Technical Association of the Pulp & Paper Industry) standard methods (TAPPI, 1988), the metal contents are determined by AAS with calibration standards. Sample preparation involves either dry ashing or more frequently, wet digestions with strong acids. Typically, pulp fibers are wet-ashed to convert/volatilize the carbon to carbon dioxide. The inorganic residual ash that is present in (or has been re-dissolved in) strong acid, is then analyzed by AAS.

The wet digestion with refluxing strong acids (hydrochloric or nitric acid/$H_2O_2$) must be performed with appreciable operator care because of corrosive nature of these oxidants. Extreme care must also be exercised with dry-ashing techniques to avoid analyte volatilization losses. Again for pulp matrices, sample preparation remains the most time consuming aspect of the overall analysis procedure.

## 1.7 Research Objective

The objectives of this study were as follows:

1. To evaluate high-pressure homogenization combined with partial enzymatic digestion as a rapid sample treatment procedure to provide a quasi-stable dispersion of certified reference materials or animal feed that can be analyzed for Se by GF-AAS.
2. To evaluate the performance of a new model of homogenizing instrument in terms of metal (Cr, Cu, Fe, Mn, Pb) contamination introduced by the instrument during the process of high-pressure homogenization.
3. To evaluate the slurry preparation/introduction technique for the determination of other elements (Cu, Fe, Mn) in animal feed.
4. To extend the applicability of the technique to wood pulp samples.

# 2

# Enzymatic Digestion – High - Pressure Homogenization Prior to Slurry Introduction GF-AAS for Selenium Determination in Plant and Animal Tissues

## 2.1 Introduction

A previous study (Tan and Marshall, 1997) had established that a high-pressurehomogenization of solid samples in the presence of aqueous tetramethylammonium hydroxide (TMAH) – ethanol did not release all of the selenium analyte into the aqueous phase. Determinations performed on slurries of eight different CRMs consistently underestimated the concentration of Se in these materials. However, if the homogenization was performed in the presence of protease and the resulting homogenate was digested at 37°C for 4-8 h, recoveries in the resulting digests became quantitative. Whereas zoological CRMs required up to 4h of enzymatic hydrolysis, botanical CRMs proved to be more resistant and required up to 8h of hydrolysis. The addition of the protease to the sample suspension prior to homogenization (i.) accelerated the hydrolysis somewhat relative to additions of protease post homogenization and (ii.) resulted in less variation (decreased relative standard deviation) among replicate analyses. Since determinations of the supernatant fraction after several days of storage at 4°C provided estimates that agreed with the certified concentration in these reference materials, it was suggested that the analyte had been transferred quantitatively to the liquid phase. In the current report, reference is made to the release of the analyte into a form that can be detected by the GF-AAS technique without implying whether the analyte has been transferred quantitatively to the liquid phase or whether a fraction remains bound to solids.

The objectives of the current studies were: to evaluate the performance of a new homogenizing instrument and to develop a more rapid sample preparation for the determination of Se in biological materials by GF-AAS. There have been few reports of the determination of Se in slurried samples (Ebdon and Parry, 1988; Bradshaw and Slavin, 1989; Wagley *et al.*, 1989; Bendicho and Sancho, 1993).

Recent reports (Cabrera *et al.*, 1995; Lopez-Garcia *et al.*, 1996) indicate that the approach is promising for this analyte as well. Prolonged enzymatic digestion with a crude protease has been used (Gilon *et al.*, 1995) to liberate component selenoamino acids from proteins. This approach seemed promising as a post-homogenization sample preparation.

## 2.2 Experimental Procedures

### Reagents

Tris[hydroxymethylamino]methane (TRIS) and tetramethylammonium hydroxide (TMAH) were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA) and aqueous Se solution (sodium selenite, 1000 μg/ml, traceable to a NIST primary standard) was purchased from SCP Chemical Co. (St-Laurent, Qué.).

Samples: CRMs were purchased from the National Research Council of Canada (DOLT-2, DORM-2, TORT) or the US National Institute of Science and Technology (Durham Wheat Flour (DWF), SRM #1538; Whole Egg Powder, SRM #8415 (Egg) ). The rape seed sample and two flour reference materials (QCIV and QCV) that had been subjected to round-robin Se determinations by six laboratories was kindly supplied by B. Gowalko, Canadian Grain Commission, Winnipeg, Man.

### Sample Preparation

Reference materials (RMs) were stored in a dessicator until use. Rape seed was ground, to pass a 0.5 mm screen, in a Tecator Cyclotec sample mill, Tecator AB, Höganäs, Sweden. Sample (approximately 0.2 g of CRM or plant tissue) weighed to the nearest 0.1 mg was added directly to 10 ml of 5% (v/v) ethanol-0.03M TRIS containing 20 mg crude protease (Type XIV, ex Streptomyces griseus, Sigma Catalog # P-5147, Sigma Chemical Co., St. Louis, MO, USA). The resulting suspension was then processed three times through the flat valve homogenizer (EmulsiFlex Model C5, Avestin, Inc., Ottawa, ON) capable of developing 137.9 MPa (20,000 psi) when provided with compressed air (689.5 KPa, 100 psi). This unit was capable of re-circulating the processed fluid back through the

homogenizing valve. The homogenates, in 50 ml Erlenmeyer flasks were then digested for up to 3 h at 60°C with gentle agitation every 15 min. Alternatively, solid sample was suspended in 90% (v/v) water ethanol containing 0.25% (w/v) TMAH. The suspension was sonicated at low power for up to 15 min then processed as above.

**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 sodium selenite in the presence/absence of co-injected bovine serum albumin. 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 1,400°C. Furnace operating parameters are presented in Table 2.1.

**Calibration**

GF-AAS quantification was performed by both the method of external standards (ES) and by standard additions (SA). ES consisting of appropriately diluted processed solvent blank plus protease, and up to four levels of standard were prepared automatically by the sample introduction device. Background corrected peak area response, resulting from three replicate injections of each diluted standard was used to define the best fit regression equation. For standard addition (SA) calibrations, 10 ml aliquots of processed fluid was amended with 2, 5, or 10 ml of aqueous standard chosen to result in a range of peak areas including signals which were one-half and at least twice the signal for the unamended sample. The data were modelled by least squares linear regression. Quantification 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:

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

Particle Size Determinations: Particle size/shape characterizations were performed with a Galai CIS-100 instrument. The distributions of the numbers of particles as a function of length/area/volume, in TRIS buffer-ethanol suspensions of three CRMs before and after processing through the homogenizer were recorded. To detect concentration dependent associations/agglomerations of the particles, the sample suspension was diluted ten-fold with TRIS buffer-ethanol and the determination was repeated and after a second ten-fold dilution the distribution of particle sizes was determined a third time.

### 2.3 Results and Discussion

The homogenizing instrument (EmulsiFlex Model C5) possessed several desirable features that were not available in the prototype model that had been used for previous studies. These included (i.) the ability to recirculate the product dispersion back through the homogenizing valve, (ii.) a higher operating pressure [137.9 MPa (20,000 psi) *vs.* 68.9 MPa (10,000 psi)] and (iii.) a ceramic homogenizing valve (to minimize metal contaminations within the final product). An initial characterization of the influence of high-pressure homogenization on the sizes of particles was performed using laser granulometry. The Galai CIS-100 instrument was capable of determining the length, cross sectional area and volume of irregularly-shaped particles that fell within the range of 0.5-1200 nm in length. The distribution of the volumes of the particles within the sample was of special interest since it was considered that volume (rather than mean length or mean cross sectional area) would relate more closely to mass and therefore to the concentrations of individual analytes. It was possible that much of the mass of the sample might be included in a relatively few particles of larger volume. Arbitrarily, three CRMs (DORM-2, DWF and Egg) were chosen for analysis by granulometry. Homogenization at 103.4 MPa produced a dispersion that was characterized with respect to the mean length, mean cross sectional area and mean volume of the particles. Distributions of the numbers of particles as a function of length, area and volume were also recorded. The means of these parameters for each of the three CRMs before and after processing

are recorded in Table 2.2. Homogenization appreciably reduced the mean length, area and the volume of the particles in all three matrices. However, particles within the flour sample proved to be more resistant to size reduction. More importantly, the relative standard deviations associated with the means were not changed appreciably by processing. This indicated that the approximately normal distributions of particle- length, area and volume were not broadened appreciably by processing. This lack of any appreciable change in the form of the distribution can be observed in Figures 2.1-2.3 that present distributions of the numbers of particles as a function of particle volume for the dogfish muscle (DORM-2), Durham wheat flour and the whole egg powder samples respectively. Plots of the cumulative percent by volume vs. particle length that are included in each of these figures also indicate an approximately normal distribution (symmetrical s-shaped plots) for the egg and DORM-2 matrices. Ten passes of the flour sample through the homogenizer did result in some broadening of the particle volume distribution yet the distribution remained approximately Gaussian and the mean volume was reduced to approximately one eighth by the processing. Each suspension, before and after processing, was diluted either 10-fold or 100-fold with solvent then re-analysed. Since the resulting particle size/cross sectional area / volume distributions remained essentially unchanged by the dilution there was no evidence for concentration-dependent particle agglomeration or flocculation within the suspension.

Because crude enzyme isolates tend to be more resistant to heat-induced denaturation, this enzyme isolate offered the possibility that the rate of hydrolysis might be accelerated by performing the incubation at elevated temperature. In preliminary trials with 0.02g of enzyme suspended in TRIS buffer, sufficient hydrolytic activity remained in the suspension after 2 h incubation at 75°C, to cause the quantitative release (at 37°C) of the selenium from DORM-2 and Egg CRM. Somewhat arbitrarily, in all subsequent trials, 0.2 g samples were digested at 60°C with 20 mg enzyme for 0, 0.5, 1, 2 or 3 h. For the four zoological matrices (egg, DORM, DOLT and TORT), the release of Se was achieved by three passes through the homogenizer (Table 2.3). Prior to homogenization, each suspension of sample plus enzyme in TRIS buffer had been sonicated for 15 min at low setting, to

completely wet the sample matrix. Homogenization at 103.4 MPa (post sonication for 15 min) served to release the Se into an AAS-detectable form. Relative to the previous model, the higher operating pressure of the current homogenizer caused efficient liberation of Se from each of the four zoological matrices. It is unclear whether the analyte was released into the liquid phase quantitatively or whether processing reduced the particle size sufficiently to permit efficient pyrolysis/atomization of analyte directly from the solid phase. Within experimental error, all of the Se analyte in the TORT matrix was detected after one day of storage of the homogenate at 4°C. Similarly, a quantitative recovery of Se in the DOLT-2 homogenate that had been digested enzymatically for 0.5 h was observed even after 6 days of storage of the homogenate at 4°C. In both trials, storage caused no apparent segregation of the analyte between the liquid and solid phases suggesting that the analyte had been transferred quantitatively to the liquid phase.

Companion sample preparations were also generated with two zoological matrices (DORM-2 and DOLT-2) in the absence of the protease enzyme. An objective for previous studies had been to define a single solvent system for homogenization that could be applied to all samples for the determination of all metals; work had focussed on 90% (v/v) water ethanol containing 0.25% (m/v) of TMAH. The results for Se determinations in slurries that had been prepared in the absence of the protease (Table 2.4) indicated that the recovery of Se was not influenced perceptibly by the number of passes through the homogenizing valve, by the duration of the sonication procedure (0, 5 or 15 minutes) prior to homogenization or by the solvent used to prepare the slurry. In all cases, the recovery of Se from these reference materials remained incomplete. Quantitative recoveries were achieved only if the enzyme was present during the homogenization (Table 2.4). The reason for the beneficial effect of the added enzyme on Se recovery is not known. Storage of the resulting slurries at 4 °C for 12h caused appreciable segregation within the DORM-2 (but not within the DOLT-2 sample). Determinations of Se in the supernatant fractions from DOLT-2 slurries post 12h storage at 4°C indicated that virtually all the Se that was detected initially was present in the supernatant fraction. By contrast, for the DORM-2 matrix an appreciable fraction of the total Se that was

detectable immediately post preparation remained with the solids fraction after 12 h storage at 4°C.

In contrast to the zoological reference materials, the botanical RMs proved to be somewhat more resistant to the homogenization induced release of Se. Three passes through the homogenizing valve in the presence of protease released Se quantitatively from the rape seed sample and but was inefficient for all three flour samples (Table 2.5). Homogenization coupled with enzymatic digestion at 60°C during 1h resulted in a quantitative liberation of the analyte from all three flour samples. Quantitative recoveries from each of the flour samples was also achieved if the sample was processed through the homogenizer several times. This was accomplished conveniently by operating the homogenizer in the recycle mode during 2 min (<10 passes). For the DWF matrix that had been homogenized continuously during 2 min, ten days of storage at 4°C did not result in a perceptible decrease in the Se content in the supernatant fraction. Similarly, once complete enzymatic release from the QCIV and QCV samples had been achieved (1h), subsequent storage at 4°C for 2 or 3 days did not result in any apparent segregation of the Se between the supernatant fraction and the solids.

The principle shortcoming of the homogenization technique is considered to be the decreased precision associated with the introduction of slurries. The precision was improved slightly with increased digestion time but apparently not with increased processing.

## 2.4 Conclusions

High-pressure homogenization provides a rapid sample preparation technique for soft biological materials prior to GF-AAS. Reliable determinations of the selenium content in the resulting slurry can be achieved provided that the particle volume has been reduced sufficiently and that the matrix is sufficiently homogeneous. This work has also demonstrated that enzymatic digestion with a crude protease can be accelerated by incubation at elevated temperature (60°C) to liberate Se from biological matrices. However, Se-release can be achieved more rapidly by sufficiently reducing the size of the particles within the sample matrix. Multiple

passes through the homogenizing valve were sufficient to generate a quasi-stable slurry that could be sampled reliably for Se even after several (1-10) days of storage at 4°C post preparation.

**Table 2.1.** Furnace operating parameters[a] for determinations of selenium.

| Element | selenium |
|---|---|
| wavelength / nm | 196.0 |
| lamp current / A | 10 |
| slit width / nm | 1.0 |
| injection T / °C | 60 |
| pre-injection | yes |
| Temperature of last dry step (10s) / °C | 250 |
| charring sequence | 10 s ramp to 1,400°C, 40 s hold. |
| cool down | None |
| Atomization | 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) $Pd(NO_3)_2$ + 2.5% (m/m) citric acid] for 10 µl sample |

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

**Table 2.2.** Means of the length of particles, their cross sectional areas and volumes (per unit volume of solvent) for three CRMs prior to and post homogenization at 103.4 MPa.

| Matrix (prior or post 1, 5 or 10 passes through the homogenizer) | Length ($\mu m \pm 1SD^a$) | Area ($\mu m^2 \pm 1SD$) | Volume ($\mu m^3 \pm 1SD$) |
|---|---|---|---|
| **DORM-2** | | | |
| Prior | 8.9 ±21.0 | 139.8 ± 98.5 | 206.4 ± 120.9 |
| Post x 1 | 4.1 ± 4.0 | 12.4 ± 8.2 | 17.8 ± 11.1 |
| **DWF** | | | |
| Prior | 17.0 ± 33.2 | 140.1 ± 89.4 | 202.9 ± 107.2 |
| Post x 1 | 8.4 ± 15.1 | 91.6 ± 64.8 | 137.4 ± 87.6 |
| Post x 5 | 3.4 ± 5.7 | 41.9 ± 32.4 | 67.0 ± 29.6 |
| Post x 10 | 4.2 ± 3.5 | 13.6 ± 14.1 | 28.2 ± 22.1 |
| **Egg** | | | |
| Prior | 38.2 ± 44.4 | 89.8 ± 58.2 | 162.1 ± 78.1 |
| Post x 1 | 8.2 ±21.0 | 62.0 ± 41.7 | 103.3 ± 30.6 |

[a]SD = standard deviation

**Table 2.3.** Percent recoveries (± 1 standard deviation) of selenium from certified zoological reference materials determined after 15 min of sonication at low speed followed by 0, 0.5, 1.0, 2.0 or 3.0 h of enzymatic digestion[a] (or following digestion plus several days of storage at 4°C

| Matrix | Hours of Digestion at 60°C | | | | | Certified Concentration (μg/g ± 1SD) |
|---|---|---|---|---|---|---|
| | 0 | 0.5 | 1.0 | 2.0 | 3.0 | |
| Solvent blank (TRIS) + protease | 23 ±7.6 (ng/10 ml) | | | | | |
| Whole Egg Powder | 108 ± 9% | 104 ± 5% | 106 ± 4% | 108 ± 8% | | 1.39 ± 0.17 |
| | 88 ± 3% | 98 ± 6% | | 88 ± 3% | | |
| DOLT-2 | | 100 ± 2% | 100 ± 5% | | 95 ± 2% (2d) | 6.06 ± 0.49 |
| | | 103 ± 4% (6d) | 104 ± 2% (6d) | 103 ± 6% (6d) | | |
| DORM-2 | 96 ± 6% | 100 ± 7% | 96 ± 8% | | | 1.40 ± 0.09 |
| TORT | 110 ± 4% | 103 ± 5% | 100 ± 6% (3d) | | | 6.9 ± 6.8 |
| | 110 ± 9% (1d) | 106 ± 5% (1d) | 100 ± 7% (1d) | | | |

[a]Each sample was processed through the homogenizer three times.

**Table 2.4.** Mean percent recoveries (± 1 standard deviation) of Se from DORM-2 or DOLT-2 CRM post 5-, 8- or 10-sequential passes through the homogenizing valve in the absence of protease enzyme.

| Matrix | Homogenization | | | Solvent | Sonication prior to homogenization |
|---|---|---|---|---|---|
| | 5-passes | 8-passes | 10-passes | | [Time (min)] |
| DORM-2[a] | 77 ± 6% | 81 ± 9% | 71 ± 4% | TMAH[b] | 0 |
| | | 76 ± 2% | | TMAH | 5 |
| | | | 64 ± 8% | TRIS[c] | 15 |
| | | | 66 ± 3% | | |
| DOLT-2[a] | 86 ± 4% | 72 ± 10% | 80 ± 7% | TMAH | 5 |
| | | 82 ± 4% | 85 ± 1% | | |
| | | | 83 ± 4% | TRIS | 15 |

[a]Certified concentration is quoted in Table 2.3.

[b]TMAH = 90% (v/v) water-ethanol containing 0.25% (m/v) of tetramethylammonium hydroxide

[c]TRIS = 5% (v/v) ethanol-0.03M tris[hydroxymethyl]aminomethane

**Table 2.5.** Percent recoveries of selenium (± 1 SD based on 3 replicate samples) from botanical reference materials determined after 15 min. sonication followed by 0, 0.5, 1.0, 2.0 or 4.0 h of enzymatic digestion at 60°C (or following enzymatic digestion plus several days of storage at 4°C).

| Matrix | Hours of Digestion at 60 °C | | | | | Certified concentration (μg/g ± 1SD) |
|---|---|---|---|---|---|---|
| | 0 | 0.5 | 1.0 | 2.0 | 4.0 | |
| Solvent blank + protease | 23 ±7.6 (ng/10 ml) | | | | | |
| Durham wheat flour (DWF ) | 82 ± 2% [a,c] | 87 ± 5% [a] | 92 ± 9% [a] | 97 ± 3% [a] | | 1.23 ± 0.09 |
| | 86 ± 8% [a] | 91 ± 6% [a] | 86 ± 8% [a] | 90 ± 4% [a] | | |
| | 117 ±9% [b] (10d) | 107 ±13% [b] (10d) | 107 ± 8 [b] (10d) | 91 ± 6% [b] (10d) | | |
| | 92 ± 11% [b] | 92 ± 11% [b] | 97 ± 9% [b] | | | |
| Milled Wheat Flour (QCIV) | 52 ± 11 [a,c] | 77 ± 16% [a,c] | 87 ± 13% [a] | 94 ± 7% [a] | 91 ± 8% (2d) | 0.87 ± 0.02 |
| | | 95 ± 8% [b] | 92 ± 9% [b] | 92 ± 5% [b] | | |
| | 91 ± 15% [b] | 91 ± 7% [b] | 100 ± 9% [b] | 108 ± 10% [b] | | |
| | 101 ± 2% [b] | 109 ± 6% [b] | 109 ± 6% [b] | 105 ± 5% [b] | | |
| Milled Wheat Flour (QCV) | 55 ± 14% [a,c] | 60 ± 12% [a,c] | 90 ± 5% [a] | 115 ± 9% [a] | 115 ±7% [a] ( 3d) | 1.11 ± 0.07 |
| | 77 ± 20% [a] | 71 ± 20% [a,c] | 105 ± 13% [a] | 105 ± 8% [a] | | |
| | 99 ± 12% [b] | 86 ± 17% [b] | 92 ± 4% [b] | 102 ± 4% [b] | | |
| | 73 ± 3% [a,c] | 86 ± 17% [a] | 87 ± 10% [a] | | | |
| Rape Seed | 99 ± 12% [b] | 104 ± 10% [b] | 92 ± 4% [b] | 102 ± 4% [b] | | 0.23 ± 0.25 |

[a]Samples were subjected to three successive passes through the homogenizing valve or [b] to continuous homogenization during 2 min.

[c]Significantly different from the certified concentration at the 95% level of confidence.

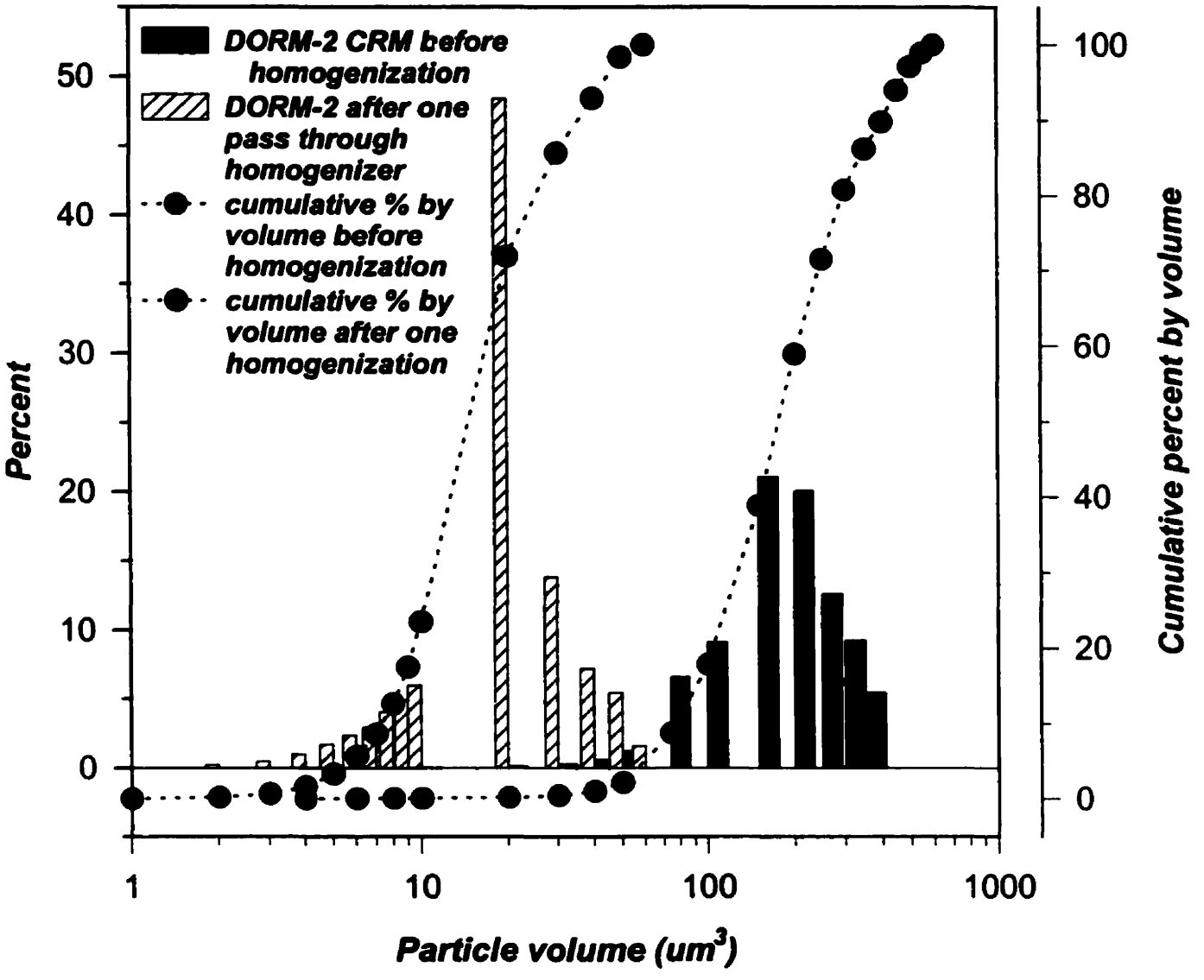


**Figure 2.1** Distribution of the number of particles as a function of particle volume for dog fish muscle (DORM-2)

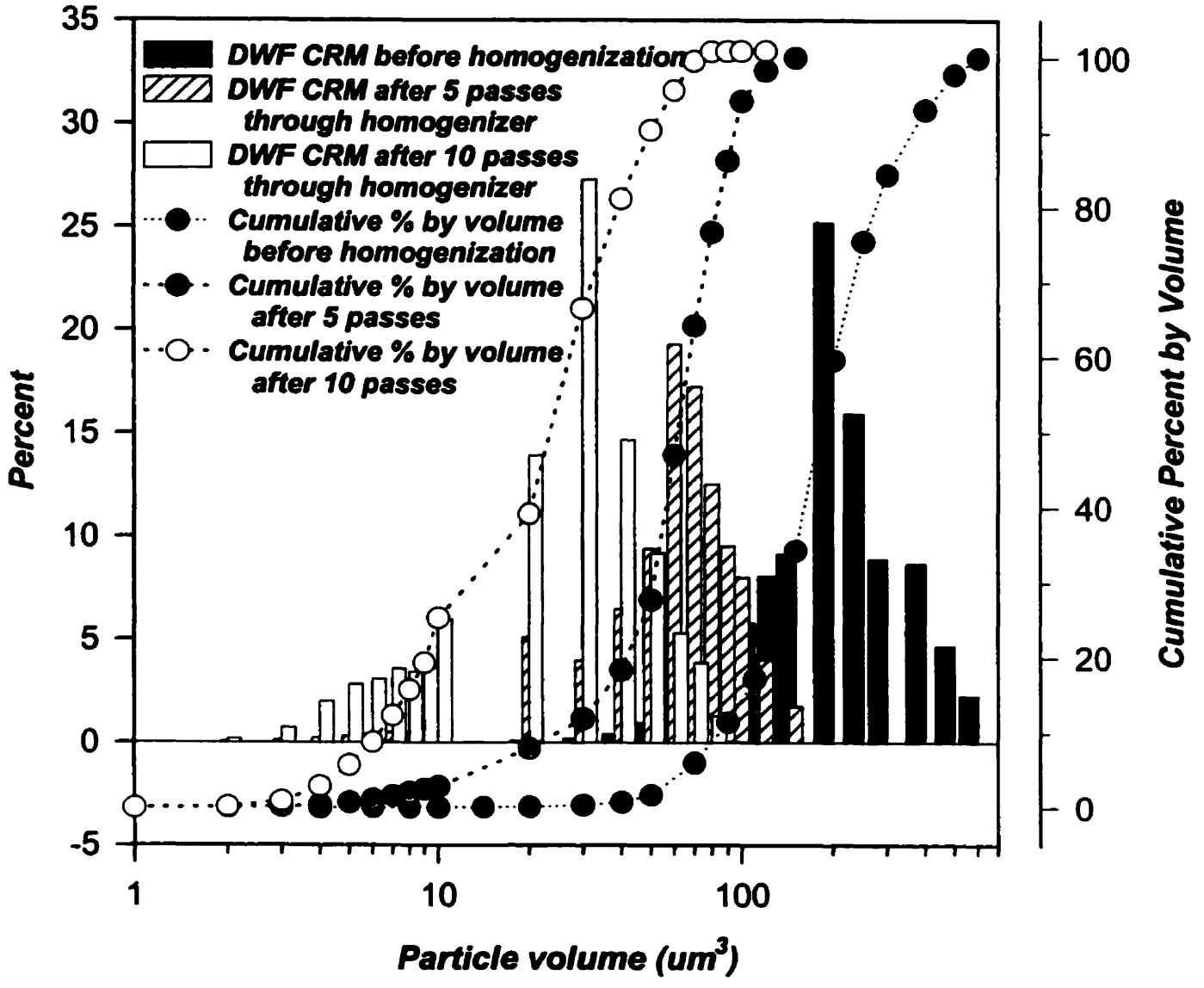


**Figure 2.2** Distribution of the numbers of particles as a function of particle volume for Durham wheat flour (DWF).

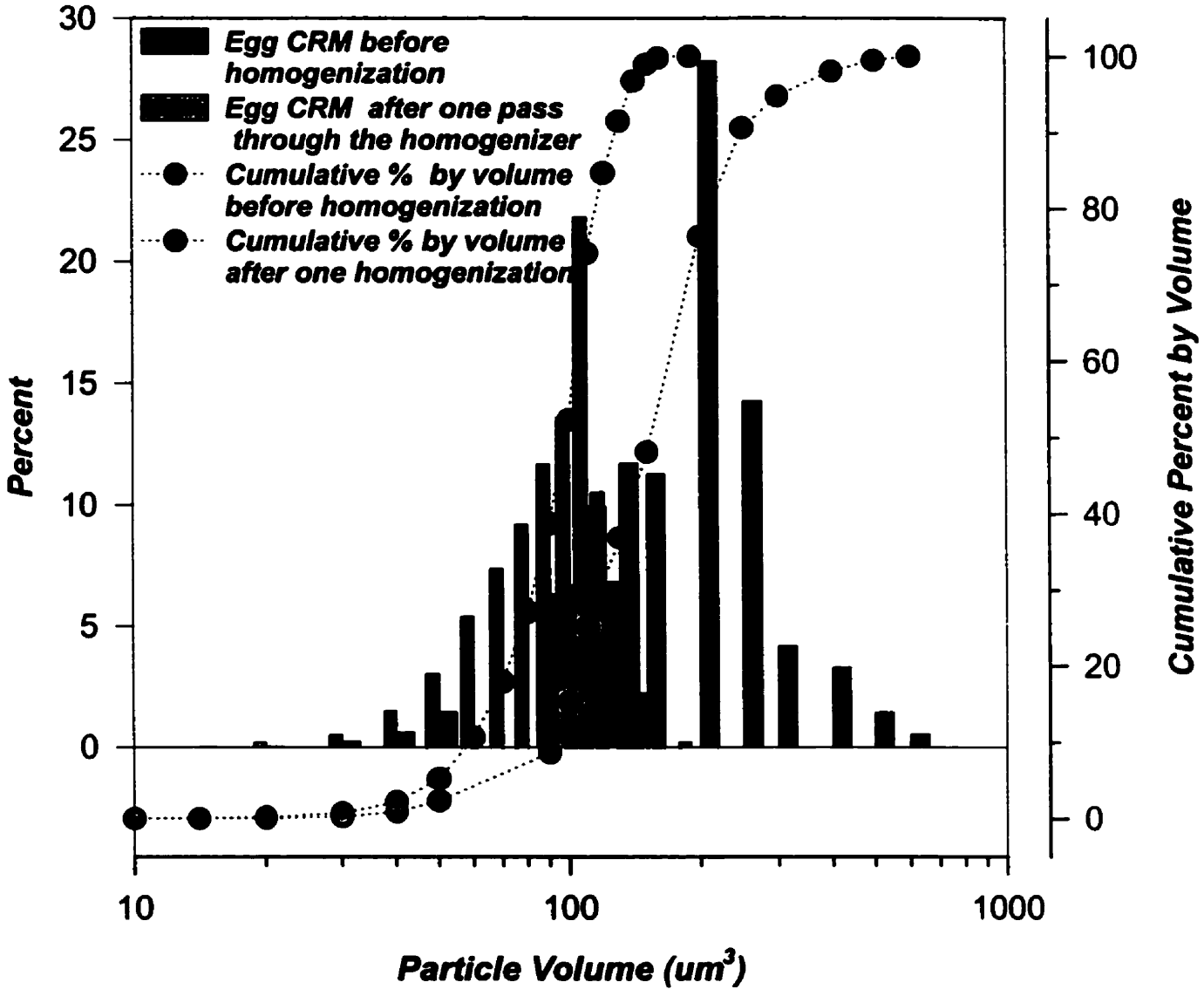


**Figure 2.3** Distributions of the numbers of particles as a function of particle volume for the whole egg powder (Egg).

# 3

# High-Pressure Homogenization Slurry Preparation Prior to Heavy Metal Determinations in Animal Feeds and Wood Pulps by GF-AAS

## 3(I). Metal Contamination from the New Homogenizer

### 3(I).1 New Model of Homogenizer Incorporating a Ceramic Homogenizing Valve

Previous studies (Tan, 1996a, 1996b) had demonstrated that the principal limitation of the high-pressure homogenization technique was the quantity of contaminating analyte metals introduced into the sample by the homogenization process. The new model of homogenizing instrument (EmulsiFlex Model C5) possessed several desirable features that were not available in the prototype model that had been used for previous studies. Included among the modifications, was a ceramic homogenizing valve that was anticipated to minimize metal contaminations in the final product.

Prior to employing the slurry preparation technique, investigation were performed to determine the level of metal contaminations introduced by the EmulsiFlex Model C5 homogenizer.

### 3(I).2 Experimental Procedure

**Reagents**

Aqueous metal standard solutions (1,000 μg/ml, traceable to NIST primary standard) were purchased from SCP Chemical Co. (St-Laurent, QC). Tetramethylammonium hydroxide (TMAH) was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Dilution of metal standard solutions were performed with ethanol-water (1 + 9 v/v) containing 0.25% (m/m) of TMAH.

**Sample preparation**

The solvent mixture, ethanol-water (1 + 9 v/v) containing 0.25% TMAH, was processed through the high-pressure homogenizer *EmulsiFlex Model C5* four or more times at the working pressure of 103.4 MPa. This was accomplished by operating the homogenizer in the recycle mode.

**Sample Analysis**

After homogenization, the processed solvent mixture was analyzed for trace metals. Analyses for chromium, copper, iron, manganese, and lead were performed using a hot injection technique on a Varian Model 300 GF-AAS system equipped with an autosampler, a pyrolytically coated partition or platform graphite tube, a conventional hollow cathode lamp and a Zeeman effect background correction system. Analytical operating parameters are presented in Table 3.1 and 3.2.

**Calibration**

GF-AAS quantification was performed by the method of external standards. External standards consisting of reagent blank, and up to four levels of standard were prepared automatically by the GF-AAS autosampler. Background corrected peak area responses resulting from three replicate injections of each diluted standard were used to define the best fit regression equation.

### 3(I).3 Results and Discussion

A previous study (Tan, 1996a) had demonstrated that levels of trace metal contaminations that were introduced into the dispersion by processing was reduced appreciably but not eliminated entirely by capping the flat face of the stainless-steel homogenizing valve with either a ruby disc or with various ceramic materials. In the case of homogenizations performed with the ruby capped homogenizing piston, Cr, Cu, Fe, Mn, and Pb concentrations in the processed solvent mixture was reduced 4-20 fold when compared to concentrations that were determined in solvent that had been processed with a bare stainless steel piston. But still, for certain elements, the level of contamination remained appreciable even in the presence of the ruby cap. Moreover, there was also high probability of Al contamination introduced into homogenized fluid by the ruby cap.

As summarized in Table 3.3, the new model, equipped with the ceramic homogenizing valve significantly decreased the metal content in the processed blank solvent mixture. The concentration of five metals, Cr, Cu, Fe, Mn and Pb, in the processed fluid fell in the range between 0.3 to 16.7 ppb, with the lowest for Pb, highest for Fe.

Each element was determined by GF-AAS several times on different days. Sample was prepared fresh on each day that analyses were performed. The results were summarized in Table 3.4. For Pb, the reproducibility was not very good, presumably the result of the very low level of the analyte in the sample solution (0.3 to 0.8 ppb). For the same reason, determinations performed on the same day resulted in elevated relative standard deviations (RSDs, *i.e.* 0.8 ppb ± 68.7%). However, there was appreciably less Pb in the solvent mixture processed by the ceramic homogenizing valve than in solvent mixture that had been processed with the ruby capped homogenizing valve, and much less in the solvent that had been processed with the stainless steel valve.

For Mn, not only were the determinations performed on different days, but also the solvent mixture was processed through the homogenizer for varying times. Initial investigations were performed on fluid that had been processed for four sequential times. Additional studies were performed to compare the Mn level in fluid homogenized for one time or for 30 sec in a recycle mode (approximately 6 times). As described in Chapter 2, when applying the high-pressure homogenization to biological samples for slurry preparation prior to GF-AAS, samples might have to be homogenized in the recycle mode to achieve a quantitative release of the analyte metal from the matrix. By comparing the metal burdens observed in a solvent blank that had been processed with a single pass *vs.* processing with several sequential passes, changes in the levels of metal contamination would be evident. Moreover, the levels of contaminations were anticipated to reflect the number of passes through the homogenizer. This strategy was performed for Mn determinations. Data obtained for analyses performed on different days agreed with each other, 0.6 ppb on Day 1, 0.7 ppb on Day 2, and analyte concentrations for samples of solvent that had been processed four sequential times agreed well with solvent that had been processed for 30 sec in the recycle mode (0.6 ppb). However, the solvent that had been homogenized only once contained levels of Mn at 0.2 ppb ± 14%. This suggested that the analyte metal might have accumulated with longer homogenizer processing times. On the other hand, a relatively stable level of Mn contamination might be reached within a specific duration of processing.

Similar investigation was performed with Cu. Whereas solvent that had been subjected to only a single pass through the homogenizer provided copper concentrations that contained approximately 1 ppb, whereas processing in the recycle mode for 20 sec (≈ 4 times), 30 sec

(≈ 6 times), 1 min (≈ 12 times) were characterized by Cu level 2.2, 2.96, and 4.1 ppb respectively. As was the case for Mn, the data for 4 sequential passes, 20 sec (≈ 4 times) and 30 sec in the recycle mode (≈ 6 times) were in close agreement, whereas Cu contamination in solvent that had processed continuously for 1 min increased somewhat. Previous studies had demonstrated that for most zoological samples, processing by homogenization for three sequential times was sufficient to release analyte metal quantitatively into a form that was detectable by GF-AAS whereas botanical samples required extended processing times. The duration of the contact time between the sample and the homogenizer was anticipated to increase the metal contamination in the processed sample. However, typical concentrations of heavy metal in most of the botanical samples can be anticipated to be sufficiently high that the levels of contamination introduced by homogenization process can be ignored. The concentrations of both Mn and Cu in the homogenized solvent mixture were appreciably lower (Mn, 0.7 ppb; Cu, 2.6 ppb) than the corresponding concentrations observed for homogenizations with four sequential passes through the ruby capped homogenizer (Mn, 2.0 ppb; Cu, 3.5 ppb).

For Cr, behavior that was similar to both Mn and Cu (reported above), was obtained for sample that had been processed once , for 30 sec, and or 1 min., (2.1 ppb, 5.6 ppb, and 7.5 ppb). However, levels of chromium contamination generated by processing with the ruby capped homogenizing valve were not appreciably greater than levels generated by the ceramic homogenizing valve. The source of this contamination remained unclear.

Of the different metals, iron provided the greatest levels of contamination. Despite the fact that the new model homogenizer was equipped with ceramic homogenizing valve, most of the other parts of this instrument were composed of stainless steel. It was to be anticipated that Fe contamination levels in the homogenized fluid would be higher than for other heavy elements. Determinations of Fe indicated that levels varied from 12.5 ppb, to 16.7 and 18.7 ppb, for analyses that were performed on different days. Each solvent mixture was homogenized four times successively. When using the stainless steel homogenizing valve, contamination induced by processing was as high as 285 ppb, whereas processing with the ruby capped device resulted in Fe concentrations of 70.0 ppb. Apparently, the ceramic valve did reduce the Fe contamination level appreciably. Despite the introduction of Fe, accurate analysis can still be performed on real samples by correcting the total Fe concentration in the

slurried sample for the relatively low Fe contamination, or more simply use the homogenized solvent mixture as the blank for analyses of real samples.

### 3(I).4 Conclusions

When compared with levels of metal contamination introduced by the previous model, the EmulsiFlex Model C5 homogenizer equipped with ceramic homogenizing valve has the advantage of reducing levels of the metal contamination within the final dispersion. The contamination levels of five elements (Cr, Cu, Fe, Mn and Pb) fell within the range that can be either ignored or readily compensated for when determining trace metal in biological matrices. This approach is especially valuable for the screening of large numbers of samples, where simple, fast, and relatively accurate analyses are required. The generation of dispersions by high-pressure homogenization is a rapid sample preparation technique that to date, has consistently resulted in the quantitative transfer of analyte to the liquid phase. The practical result of this analyte transfer is that the supernatant fraction of the product dispersion can be sampled even several days after preparation without any apparent loss (segmentation of analyte between the liquid and solid phases).

The most appreciable improvement made by the new model was the greatly reduced level of Fe contamination in the homogenized fluid, which had been of major concern with previous model.

Metal contamination levels was increased by passing the fluid through the homogenizer several times. So, when applying the technique to real samples, care should be taken not to over-process the suspension. When the analyte element has been released quantitatively from the matrix, the homogenization should be stopped. Studies to date have demonstrated that three sequential passes of suspensions from zoological samples were sufficient for quantitative release, whereas botanical matrices can require up to ten passes (less than two minutes in the recycle mode).

**Table 3.1.** Graphite furnace operating parameters for the determination[a] of chromium, copper and iron.

| | Chromium | Copper | Iron |
|---|---|---|---|
| λ (nm) | 357.9 // 429.0 | 324.7 // 244.2 | 386.0 |
| lamp current | 7 amps | 4 amps | 5 amps |
| slit width (nm) | 0.2 | 0.1 // 0.5 | 0.2 |
| injection T °C | 60°C | 60°C | 60°C |
| pre-injection | no | no | yes |
| charring sequence | 20 s ramp to 1450 °C, 40 s hold | 5 s ramp to 900°C, 20 s hold. | 10 s ramp to 1100°C, 21 s hold |
| cool down | 7 s ramp to 40°C, 10 s hold | none | none |
| atomization | 1.2 s ramp to 2,400 °C, 8 s hold | 1 s ramp to 2,300°C, 2 s hold | 1.2 s ramp to 2400°C, 4.0 s hold |
| measurement | 8 s | 3 s | 6 s |
| matrix modifier | 3µl of 20 g/l $Mg(NO_3)_2$ | 5 µl of 1 % (m/m) $NH_4NO_3$ | 5 µl of (500 mg/l Pd+ 2.5 % citric acid) |

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

**Table 3.2.** Graphite furnace operating parameters for the determination[a] of manganese and lead.

| | Manganese | Lead |
|---|---|---|
| λ (nm) | 403.1 // 279.5 | 283.3 |
| lamp current | 5 amps | 4 amps |
| slit width (nm) | 0.2 // 0.2 | 0.5 |
| injection T°C | 60°C | 70°C |
| pre-injection | yes | yes |
| charring sequence | 10 s ramp to 1200 °C, 20 s hold | 0.5 s ramp to 900°C, 30 s hold |
| cool down | none | none |
| atomization | 0.7 s ramp to 2200 °C, 2 s hold | 0.5 s ramp to 2400 °C, 4 s hold |
| measurement | 3 s | 2.8 s |
| matrix modifier | 5 µl of (500 mg/l Pd + 2.5 % citric acid) | 5 µl of (500 mg/l Pd + 2.5% citric acid) |

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

**Table 3.3** Analyte concentration (ppb) in solvent mixture that had been processed through the homogenizing instrument[a] fitted with various homogenizing valves.

| Analyte Element | Homogenizing Valve | | |
|---|---|---|---|
| | Ceramic Valve | Ruby Disk | Stainless Steel Head |
| Chromium | 3.3 | 1 | 4.5 |
| Copper | 2.6 | 3.5 | 75 |
| Iron | 16.7 | 70.0 | 284.7 |
| Manganese | 0.7 | 2.0 | 11.6 |
| Lead | 0.3 | 1.4 | 6.9 |

[a]Solvent mixture was processed through the instrument four times.

**Table 3.4** Analyte concentrations (ppb) (± 1 relative standard deviation expressed as a percentage, based on triplicate determinations) in solvent mixture that had been processed through the ceramic homogenizing valve (*EmulsiFlex Model C5*) for varying times.

| Analyte Element | Homogenization Time / Mode | | | | | |
|---|---|---|---|---|---|---|
| | 1 Time | 4 Times [a] | 4 Times [a] | 20 sec [b] | 30 sec [b] | 1 min [b] |
| Lead | | 0.33 ±9% | 0.8 ±68.7% | | | |
| Manganese | 0.2 ±14% | 0.6 ±5.8% | 0.7 ±10.1% | | 0.6 ±2.1% | |
| Copper | 1.0± 16.7% | 2.6±5.2% | | 2.2 ±7.8% | 2.96 ±4% | 4.1 ±5.6% |
| Chromium | 2.1 ± 2.6% | 3.2±9.5% | 3.3 ±1.6% | 4.2 ±14.6% | 5.6 ±6.8% | 7.5 ±2.2% |
| Iron | | 16.7±5.2% | 12.5 ±8.9% | | 18.7 ±8.1% | |

[a]Determinations were performed on different days with freshly prepared solvent mixture.

[b]Solvent mixture passed through the homogenizer continuously in recycle mode.

## 3(II). Application of the Slurry Preparation Technique to the Determination of Trace Elements in Animal Feeds

### 3(II).1 Introduction

To characterize a novel analytical procedure, it is important to assess the accuracy of the results. Generally, three approaches to accuracy assessment have been employed:

1. Analysis of certified reference materials (CRMs).
2. Recovery studies.
3. Use of a completely independent technique to assess the concentration of the target analyte.

The use of CRMs (reference materials for which there is common agreement on the concentrations of target analytes that have been determined in advance using several different techniques) is much preferred. In previous studies (Tan, 1996a, 1996b), the concentrations of selected trace metals in a variety of CRMs of botanical and zoological origin had been determined by means of rapid slurry preparation prior to GF-AAS determination. The same techniques were also applied to the analysis of selected biological samples. However, due to the metal contamination induced by the homogenizing instrument, the analytical results had to be corrected for analyte concentrations that had been introduced by the homogenizing process. By employing the new model homogenizer, it was anticipated that trace analysis of real sample following slurry preparation would be more accurate.

Enzymatic digestion - high - pressure homogenization for slurry preparation prior to GF-AAS determinations of selenium, had only been performed on reference materials, that contained relatively high levels of selenium. However, in most real biological samples, the content of selenium is much lower. It was important to evaluate the applicability of the new technique to real samples.

Five kinds of animal feeds with various matrices were investigated for their contents of Cu, Mn, and Se. Since no reference data was available on analyte element concentrations in these samples, it was decided to employ microwave digestion, as a separate technique for sample preparation prior to GF-AAS determination. Evaluation of the new slurry technique could

then be performed by comparing the analyte concentrations as determined by either sample preparation technique.

Meeravali and Kumar (2000) compared slurry sampling *vs.* open focused microwave digestion for determination of Cu, Mn and Ni in algae using transverse heated-GF-AAS. They reported that no significant differences were evident between the results obtained by either of these methods, when the student's t-test was applied at the 95% confidence level. For all the three analyte elements, the limits of detection obtained by rapid slurry atomization were lower than that provided by microwave digestion.

Mierzwa *et al.* (1998) analyzed tea leaves using slurry sampling GF-AAS and microwave assisted acid digestion ICP-AES for determinations of Ba, Cu, Fe, Pb and Zn. The accuracy of both methods was verified by using CRMs; the recoveries of analytes varied from 91% to 99% for slurry sampling, and from 92.5% to 102% for microwave digestion ICP-AES. The results for Ba and Pb determination were calculated using the standard addition method, and the results of Cu, Fe and Zn from external standard method were based on aqueous standards. These two studies both concluded that slurry sampling GF-AAS is relatively fast technique that can provide analytical results with satisfying accuracy. Meeravali and Kumar reported slurry sampling had the advantage of lower limit of detection and lower process blank. However, neither study reported on the stability of the slurry (i.e. analysis post sample storage).

### 3(II).2 Experimental Procedure

#### Reagents

Aqueous metal standard solution (1,000 μg/ml, traceable to NIST primary standard) was purchased from SCP Chemical Co. (St-Laurent, QC).

Tetramethylammonium hydroxide (TMAH) and tris(hydroxymethyl) amino methane) (TRIS), were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA).

Crude protease (Type XIV) was purchased from Sigma Chemical Co. (St. Louis, MO. USA).

Nitric Acid (63%, m/m) was purchased from ACP Chemical Co., Montreal, QC.

**Samples**

Animal feed samples that had been destined for a local zoo, were kindly provided by Professor E. Chavez, Department of Animal Science, McGill University.

**Sample Preparation**

**(1).Primary Treatment**

Animal feed samples were dried at 125°C for 72 h, then ground to pass 0.5 mm screen in a Cyclotec sample mill (Tecator, Hoganas, Sweden).

**(2).SlurryPreparation**

For determination of Cu and Mn, approximately 0.2 g of accurately weighed, dried and ground sample was added directly to 50 ml of ethanol-water (1 + 9 v/v) containing 0.25% TMAH (m/m) in a 50 ml Erlenmeyer flask. The resulting suspension was sonicated at low power setting for 10 minutes in order to completely wet the sample, then it was processed through the EmulsiFlex Model C5 homogenizer for 6 min (approximately 10 times) in the recycle mode. The working pressure of the homogenizer was maintained at 103.4 MPa.

For determination of Se, approximately 0.2 g of accurately weighed, dried and ground sample was added directly to 10 ml of 5% (v/v) ethanol-0.03M TRIS containing 20 mg crude protease. The resulting suspension was then processed through the homogenizer (103.4 MPa working pressure) for 1 min in the recycle mode (approximately 8 times). The homogenate, in 50 ml Erlenmeyer flasks was then enzymatically digested at 60°C for 0.5 h.

**(3).Microwave Digestion**

Microwave digestions were performed using a focussed open digestion systems with a Prolabo Type 3.6 Microdigester. The digester features a focussed microwave source and an open digestion flask. The microwave frequency was 2.45GHz. The power input was controlled (between 10% and 100% in 1% increments) independently for each of the three flasks. The instrument was also equipped with a suction-scrubbing unit that was capable of aspirating the acidic volatiles away from the neck of each flask.

Dried ground feed sample (approximately 0.4 g) was added directly to the microwave digestion flask, followed by 15 ml of 63% (m/m) $HNO_3$ (ultra-pure grade) using the pump

that was attached to the microwave system. The sample was digested for 10 min at 20% power, and then paused to permit the sample to cool down (approximately 20 min). Subsequently, 3 ml of $H_2O_2$ was added to the sample via the pump and the sample was digested for a further 40 min at 40% power. The resulting clear solution was diluted to 25 ml with de-ionized distilled water.

**GF-AAS**

Concentrations of Se were determined in slurries or digests using procedures that were identical to those described previously (Chapter 2, section 2.2)

Similarly, concentrations of Cu or Mn were determined in slurries or digests using procedures that were identical to those described previously (Chapter 3, Section 3(I).2)

**Calibration**

Calibrations were performed as described in Chapter 3, section 3(I).2. However, for sample prepared by microwave digestion, calibrations was performed using de-ionized distilled water as blank and standard solutions were diluted with de-ionized distilled water. For determinations of Cu or Mn in homogenized samples, calibrations were performed using ethanol-water (1 + 9 v/v) containing 0.25% m/m TMAH as blank, standard solutions were diluted with the same solvent mixture. For determination of Se, ethanol-0.03M TRIS (1 + 19 v/v, pH 7.5) containing 0.2% (m/v) crude protease was used as blank as well as the solvent mixture for diluting aqueous standards.

### 3(II).3 Results and Discussion

The five animal feed samples that were investigated for their contents of Cu, Mn and Se, included full fat soybean, control diet, full fat soybean diet, flaxseed and flaxseed diet. Because there were no reference data on the metal analyte concentrations in these samples, microwave digestion was employed as an independent sample preparation method to provide a means of comparison. Since microwave digestion employed a strong acid in combination with an oxidizing agent, it can be considered to be a more conventional procedure. Since the main purpose of the study was to develop a simple and rapid sample preparation method

prior to trace element determinations in biological materials, microwave digestion, (that has been generally accepted as a rapid and widely applicable technique) could serve as a reference method.

The results of Cu, Mn, Se determinations in five animal feed samples are summarized in Table 3.5. For all of the five samples, trace element concentrations in sample processed by high-pressure homogenization (HOMO) agreed closely with the concentrations in solutions that had been prepared by microwave digestion (MW). A Student T-Test for the relative differences ((HOMO-MW)/MW) between the slurry technique and microwave digestion was used to compare the results from both techniques. The comparisons were performed for each element (Cu, Mn, Se) in different matrices (Table 3.6), and for each matrix (Full Fat Soybean, Control Diet, Soybean Diet, Flax Seed, Flax Seed Diet) regardless the analyte identity (Table 3.7). No significant differences at the 95% level of confidence ($p = 0.05$) were detected between results generated by either preparation method. An alternative parameter involves the fact that the confidence interval between upper 5% and the lowest 5% must include 0.0. (Table 3.6, and Table 3.7). The contentrations of Cu and Mn in all five animal feeds ranged from 15.3 to 121 ppm, whereas Se concentrations were appreciably lower than 1 ppm. Statistical comparisons demonstrated that there was no significant difference between the two sample preparation procedures (HOMO *vs.* MO) prior to GF-AAS determination for trace elements at either trace (ppm) or ultra-trace (sub-ppm) levels.

The relative differences [(HOMO-MW)/MW] between results for the homogenization procedure and for the microwave procedure were acceptably small in all cases. For copper concentrations, relative differences fell in the range between -3% (full fat soybean diet) to 13% (full fat soybean). For manganese concentrations, relative differences ranged between 0.4% (full fat soybean diet) to 13% (flaxseed diet). The concentrations of Cu and Mn in homogenized samples were not always higher than those in samples prepared by microwave digestion, suggesting that no appreciable levels of contamination had been introduced into sample slurries by processing.

For trace element determinations in biological materials it is frequently considered that calibrations by the method of standard additions can provide more accurate results. It might be expected that the results using different preparation methods (HOMO or MW) would be more concordant if the calibrations were performed by the method of standard additions.

The main problems with this approach are that this form of calibration is both labor intensive and time consuming. Calibrations must be repeated for each analyte and for each sample matrix.

Studies with animal feed samples were undertaken to evaluate the feasibility of high pressure homogenization as a rapid sample preparation method for biological materials when using the method of external standard as the calibration procedure. For results generated by both sample preparation procedures (Table 3.5), it can be observed that high-pressure homogenization technique provides estimates of concentrations that are comparable to concentrations determined after microwave digestion GF-AAS. However, microwave digestion still involves the use of strong acid, that must be performed in a properly ventilated fume hood in order to exhaust acid fumes that are liberated during the digestion. The digestion of multiple samples will require extended processing times because a maximum of three samples can be digested simultaneously. Once completed, the individual digestion flask must be cleaned carefully before the digestion of the next batch is started. Collectively the treatments result in an appreciably longer processing time than might have been anticipated by the length of the digestion time. By contrast, a maximum of 10 min was required for high-pressure homogenization of each sample plus clean up prior to the next sample. Rapid cleaning can be achieved by simply passing solvent through the instrument for a few minutes. The high pressure helps to flush out any contaminating residues from the interior surfaces.

The concentrations of Cu, Mn and Se in five animal feeds are summarized in Table 3.8, 3.9 and 3.10 respectively.

For determination of Cu, post high-pressure processing, dispersions were stored at 4°C for 3 days or 7 days prior to copper determinations by GF-AAS. The results demonstrated a lack of any significant difference in copper concentrations in samples that had been stored for 3 or 7 days. Collectively these results suggested no tendency for the analyte to segregate between the liquid supernatant fraction and the solids fraction over the course of the storage. For the samples that had been processed with high pressure then stored at 4°C for up to 7 days, the relative standard deviation (RSD) associated with replicate determinations were all acceptably low, $<\pm 8\%$. This low level of variation demonstrated good reproduciblity associated with the total analysis procedure. Prior to atomic spectroscopy, the sample

dispersion (that had been stored in a sealed glass container) was shaken gently then an aliquot was transferred to an instrumental autosampler cup by pipette. During the actual determination, no further stirring or mixing was performed. The mean results obtained from three replicate sub-samples from each sample matrix were characterized by low RSD (less than 5%), indicating a high level of repeatability for both the processing method and for the determinations.

For determination of Mn, one sample was investigated after one or two days of storage at 4 °C post preparation or immediately after microwave digestion for each of the different animal feeds. Again, there was good agreement between the Mn concentrations in slurries that had been stored for 1 day and 2 days. For manganese concentrations observed for different storage batches, the RSD was less than 6%, except for flaxseed diet, which was 13%. Good repeatability was also observed for the replicate readings of each sample during actual determinations.

The good repeatabiliities of the Cu and Mn determinations in slurried samples of animal feed indicate that the high-pressure homogenization is a very promising technique for sample preparation prior to GF-AAS analysis, especially for soft media including biological, environmental, and food samples that can require extended digestion times to generate a homogeneous liquid phase.

Typically, SRMs and CRMs have been defatted during preparation to improve their shelf life. It was necessary to evaluate the homogenization technique for samples containing fat, so as to extend the applicability of the procedure to real samples.

During the studies it was observed that the crude protease enzyme by itself did not result in any appreciable AAS reading, but its presence in the dispersion was able somehow to enhance the instrumental response to Se. For Se determinations, calibrations were performed using a TRIS buffer-ethanol solvent mixture amended with protease as the blank. Protease concentration in this blank was approximately the same as that in slurry samples.

Due to the low Se content in the samples (appreciably less than 1 μg/g), the RSDs associated with estimates of concentrations of this analyte in samples that had been prepared by either procedure were elevated relative to those for Cu or Mn. No segmentation between the supernatant liquid phase and the solid phase of the dispersion

could be detected after one day of storage. There was good agreement between the concentration of Se in microwave digested samples and Se in slurries prepared from the same sample matrix after 1 day of storage at 4 °C. Analogous behavior was observed (Chapter 2) for SRMs and CRMs. Thus, the inclusion of an appreciable fat component in some of the feed samples did not seem to interfere with the determinations. Dispersions of all the five feed samples gave reproducible results for Se, that matched with estimates obtained from microwave digests.

### 3(II).4 Conclusions

The results indicate that high-pressure homogenization can be used to rapidly generate dispersions of dried animal feeds that can be reliably sub-sampled during 7 days of storage and analyzed by GF-AAS for Cu, Mn and Se. This processing did not introduce appreciable levels of analyte contamination into the product slurry. Sample preparation proved to be rapid (a maximum 6 minutes was required to prepare 50 ml of sample) and the homogenizer was readily cleaned between samples by processing fresh solvent.

The repeatability of determinations performed on the same homogenate post different periods of storage indicated that at least a high proportion of each analytes is extracted into the liquid phase during processing. Results of determinations performed on different sub-samples of the same feed using slurry preparation prior to GF-AAS proved to be highly repeatable. The results of Se determinations indicated that the homogenization followed by enzymatic hydrolysis was a practical method for releasing Se from the sample matrix into a form that can be detected by GF-AAS.

**Table 3.5** Mean copper, manganese and selenium concentrations[a] in dried ground animal feeds that had been processed by high-pressure homogenization or by microwave digestion.

| Sample Preparation | Copper | | Manganese | | Selenium | |
|---|---|---|---|---|---|---|
| | HOMO[b] | MW[c] | HOMO | MW | HOMO | MW |
| Full Fat Soybean | 24.2 ±3% | 21.3 ±1% | 44.7 ±2% | 50.1 ±2% | 0.18 ±12% | 0.15 ±10% |
| Control Diet | 22.7 ±0% | 25.9 ±1% | 83.1 ±3% | 73.1 ±2% | 0.34 ±4% | 0.37 ±8% |
| Full Fat Soybean Diet | 23.6 ±2% | 24.4 ±2% | 120.8 ±6% | 121.3 ±1% | 0.32 ±11% | 0.38 ±8% |
| Flax Seed Diet | 30.4 ±3% | 31.0 ±1% | 111.9 ±13% | 99.2 ±1% | 0.42 ±9% | 0.55 ±5% |
| Flax Seed | 16.4 ±0% | 15.3 ±2% | 22.1 ±5% | 22.2 ±0% | 0.17 ±17% | 0.20 ± 8% |

[a]μg/g ± 1 relative standard deviation based on duplicate analyses performed on different days.

[b]High-Pressure Homogenization.

[c]Microwave Digestion

**Table 3.6** Student T-Test of the Relative Difference (RD = [(HOMO-MW)/MW] ) between slurry technique and microwave digestion (indexed by element for animal feeds).

| Element | Mean of RD | SD[a] | T | *p*-Value | 95% CI[b] |
|---|---|---|---|---|---|
| Cu | -0.06 | 0.19 | -1.50 | 0.1508 | (-0.15, 0.02) |
| Mn | 0.03 | 0.11 | 0.83 | 0.4297 | (-0.05, 0.11) |
| Se | -0.04 | 0.15 | -0.92 | 0.3817 | (-0.15, 0.06) |

[a]SD: Standard deviation

[b]CI: Confidence interval

**Table 3.7** Student T-Test of the Relative Difference (RD = [(HOMO-MW)/MW] ) between slurry technique and microwave digestion (indexed by animal feed matrix).

| Matrix | Mean of RD | SD[a] | T | *p*-Value | 95% CI[b] |
|---|---|---|---|---|---|
| Full Fat Soybean | 0.08 | 0.14 | 1.71 | 0.1301 | (-0.03, 0.20) |
| Control Diet | -0.06 | 0.13 | -1.28 | 0.2404 | (-0.16, 0.05) |
| Soybean Diet | -0.05 | 0.09 | -1.68 | 0.1377 | (-0.12, 0.02) |
| Flax Seed | -0.18 | 0.23 | -2.29 | 0.0556 | (-0.37, 0.01) |
| Flax Seed Diet | 0.04 | 0.09 | 1.14 | 0.2935 | (-0.04, 0.11) |

[a]SD: Standard deviation

[b]CI: Confidence interval

**Table 3.8** Copper concentrations[a] in dried ground animal feeds that had been processed by either high-pressure homogenization or by microwave digestion.

| Preparation Method | Full Fat Soybean | Control Diet | Full Fat Soybean Diet | Flax seed Diet | Flax seed |
|---|---|---|---|---|---|
| 3 d storage @ 4°C post homogenization[b] | 25.7 ± 0% | 23.6 ± 1% | 23.6 ± 1% | 21.5 ± 0% | 17.4 ± 1% |
| | 23.5 ± 3% | 21.6 ± 2% | 24.4 ± 1% | 20.1 ± 2% | 15.4 ± 5% |
| 7 d storage @ 4°C post homogenization[b] | 24.7 ± 1% | 21.8 ± 2% | 23.5 ± 1% | 19.7 ± 2% | 15. 7 ± 1% |
| | 22.8 ± 1% | 21.8 ± 2% | 23.1 ± 2% | 20.4 ± 1% | 17.2 ± 2% |
| Means of 3 d & 7 d of storage @ 4°C | 24.2 ± 3% | 22.7 ± 0% | 23.6 ± 2% | 20.4 ± 3% | 16.4 ±0% |
| Microwave Digestion | 21.3 ± 1% | 25.9 ± 1% | 24.4 ± 2% | 31.0 ± 1% | 15.3 ± 2% |
| | | | 23.6 ± 1% | 33.1 ± 5% | |

[a]μg/g ± 1 relative standard deviation based on three replicate determinations (for each analysis) or duplicate analyses performed on different days (for mean concentrations).

[b]High-Pressure Homogenization.

**Table 3.9** Manganese Concentrations[a] in dried ground animal feeds that had been processed by high-pressure homogenization or by microwave digestion.

| Preparation Method | Full Fat Soybean | Control Diet | Full Fat Soybean Diet | Flax seed Diet | Flax seed |
|---|---|---|---|---|---|
| 1 d storage @ 4°C Post Homogenization [b] | 45.2 ± 3% | 85.0 ± 2% | 125.9 ± 1% | 101.3 ± 2% | 22.9 ± 3% |
| 2 d storage @ 4°C Post Homogenization [b] | 44.2 ±4% | 81.2 ± 0% | 115.6 ± 1% | 122.5 ± 1% | 21.3 ± 1% |
| Mean of 1 d & 2 d of storage | 44.7 ±2% | 83.1 ± 3% | 120.8 ± 6% | 111.9 ± 13% | 22.1 ± 5% |
| Microwave Digestion | 50.1 ±2% | 73.1 ± 2% | 121.3 ± 1% | 99.2 ± 1% | 22.2 ± 0% |

[a] μg/g ± 1 relative standard deviation, based on three determinations (for each analysis) or duplicate analyses performed on different days.

[b] High-Pressure Homogenization.

**Table 3.10** GF-AAS determinations of selenium concentrations[a] in dried ground animal feeds post processing by high-pressure homogenization or by microwave digestion.

| Preparation Method | Full Fat Soybean | Control Diet | Full Fat Soybean Diet | Flax seed Diet | Flax seed |
|---|---|---|---|---|---|
| Homogenization[b] + 1d storage @ 4°C | 0.19 ± 12% | 0.33 ± 9% | 0.34 ± 2% | 0.44 ± 3% | 0.15 ± 18% |
| | 0.16 ± 12% | 0.35 ± 8% | 0.29 ± 0% | 0.39 ± 9% | 0.19 ± 10% |
| Mean of 1 d @ 4°C | 0.18 ± 12% | 0.34± 4% | 0.32 ± 11% | 0.42 ± 8.5% | 0.17 ± 17% |
| Microwave Digestion | 0.15 ± 10% | 0.37 ± 8% | 0.38 ± 8% | 0.48 ± 5% | 0.17 ± 8% |

[a]μg/g ± 1 relative standard deviation based on three determinations (for each analysis) or duplicate analyses performed on different days.

[b]High-Pressure Homogenization.

## 3(III). Application of the Slurry Preparation Technique to the Determination of Trace Elements in Wood Pulps

### 3(III).1 Trace metal analysis in wood pulp samples

As described in Chapter 1 (Section 1.6), during the paper making process, transition metal can exert undesirable effects during bleaching with hydrogen peroxide, oxygen or ozone. Improvements in the bleaching efficiency of these chemicals can be obtained by adjusting the free metal ion content in the pulp. To provide the information of metal content in pulp, metal analyses must be performed both prior to and post treatment. Sample preparation involves either dry ashing or an acid digestion, that are time consuming, error prone and require appreciable operator intervention.

Karadjova and Karadjov (1998) determined Cd and Pb in plant (poplar, clover, plantain) leaves using GF-AAS. Slurry preparation procedures using TMAH were compared with wet digestion (1 mol/l $HNO_3$ + 1.5 mol/l $H_2O_2$). It has been found that a single standard addition could be used for quantitative determination in the case of wet digestion GF-AAS and a standard addition to each analysed matrix was recommended in the case of slurry GF-AAS. For all plant leaves that were investigated, a good agreement was achieved between the concentration determined by wet digestion GF-AAS and slurry GF-AAS. The wet digestion method provided relative standard deviations (RSD) for Cd raging from 3% to 17% and for Pb from 2% to 16%. For the slurry method, the RSD values are in the range of 4-31% for Cd and 4-30% for Pb, depending, for both cases, on the magnitude of the measured concentrations.

Takuwa *et al.* (1997) used ultrasonic slurry sampling GF-AAS to determine Co, Ni and Cu in flowers, leaves stem and roots of four plant species (*Helichrysum candolleanum, Peristrophe decorticans, Blepharis diverspinia and Tephrosia burchelli*). The slurry sample method was also validated by comparing the results from slurry sampling with those from the analysis of decomposed samples. The two methods gave comparable precision and accuracy. The RSDs were in the range 1-14% for slurry sampling and 1-20% for decomposed materials. Accuracy was confirmed by analyses of certified plant reference materials, no significant difference from the certified values was found when the slurry sampling technique was used.

Since pulp is generated from the mechanically or chemically induced separation of the fibers from wood chips, it can be considered to be a form of plant matrix. The pulp matrix contains a high proportion of long fibers. This matrix is different from the reference materials that had been investigated previously; they were readily ground to a powder form. It was decided to evaluate the high-pressure homogenization procedure for pulp samples as a means of sample preparation prior to GF-AAS determination of metals. If the technique could be made to work on this kind of matrix, it would demonstrate that the homogenization technique is more widely applicable and would provide another attractive optional procedure for sample preparation of this matrix prior to metal determinations.

### 3(III).2 Experimental Procedure

#### Reagent

As described in section 3(II).2.

#### Sample

Wood pulp samples were provided by Dr.K. Thurbide, Pulp & Paper Research Institute, McGill University, and Dr. C. Heitner, Paprican (Pointe Claire, QC)

#### Sample Preparation

#### (1).Primary Treatment

Wood pulp samples were dried at 125°C for 72 h, then ground to pass 0.25 mm screen in a sample mill (General Electric, USA).

#### (2). Slurry Preparation

Approximately 0.1 g of accurately weighed dried and ground sample was added directly to 50 ml of ethanol-water (1 + 9 v/v) containing 0.25% TMAH (m/m) in a 50 ml Erlenmeyer flask. The resulting suspension was processed through the EmulsiFlex Model C5 homogenizer for 6 min (approximately 10 times) in the recycle mode. The working pressure of the homogenizer was maintained at 103.4 MPa.

**(3). Microwave Digestion**

Microwave digestions were performed using a focussed open digestion system [Prolabo Type 3.6 Microdigester, as described in Chapter 3, Section 3(II).2].

Dried ground pulp sample that had been weighed accurately (approximately 0.1 g), was added directly to the microwave digestion flask, followed by 15 ml of 63% (m/m) $HNO_3$ (ultra-pure grade) using the pump that was attached to the microwave system. The sample was digested for 10 min at 20% power, and then paused to permit the sample to cool down (approximately 20 min). Subsequently, 3 ml of $H_2O_2$ was added to the sample via the pump and the sample was digested for a further 35 min at 40% power. The resulting clear solution was diluted to 50 ml with de-ionized distilled water.

**GF-AAS**

Concentrations of Cu, Fe and Mn were determined in slurries or digests using procedures that were identical to those described previously [Chapter 3, Section 3(I).2].

**Calibration**

Calibrations were performed by both the methods of external standards and standard additions (as described in Chapter 2, Section 2.2). However, for samples prepared by microwave digestion, calibrations were performed using distilled water as blank and standard solutions were diluted with $H_2O$. For determinations of Cu, Fe and Mn in homogenized samples, calibrations were performed using ethanol-water (1 + 9 v/v) containing 0.25% (m/m) TMAH as blank, standard solutions were diluted with the same solvent mixture.

**3(III).3 Results and Discussion**

Four wood pulp samples that were investigated for their contents of Cu, Fe and Mn included a raw pulp (RP) of unknown origin, as well as pulp samples of jack pine (JP), balsam fir (BF) and an ash free filter pulp (AF) (purchased from Fisher Scientific). Because there were no data on the concentrations of metal analytes in these samples, microwave digestion was employed as an independent sample preparation method to provide means of comparison.

The results of Cu, Fe, Mn determinations in four wood pulp samples are summarized in Table 3.11. For each of the four samples, the apparent trace element concentrations in

sample that had been processed by high-pressure homogenization (HOMO) agreed closely with the concentrations in solutions that had been prepared by microwave digestion (MW). The relative difference [(HOMO-MW)/MW] between results for the homogenization procedure and for the microwave digestion procedure were acceptably small in all cases. If the sign of the relative difference was taken into account, the mean value of the relative differences of three metals in four pulp samples (RP, RP*,JP,BF) was 0.68%, which suggested no appreciable difference between the HOMO and MW methods, while HOMO technique provided the advantages of speed and simplicity. A Student T-Test for the relative differences ((HOMO-MW)/MW) between the slurry technique and microwave digestion was used to compare the results from both techniques. The comparisons were preformed for each element (Cu, Mn, Fe) in different matrices (Table 3.12), and for each matrix (RP, RP post 1 day of storage at 4°C, RP*, JP, BF) regardless the analyte identity (Table 3.13). No significant differences at the 95% level of confidence ($p$ = 0.05) were detected between results generated from either preparation method. An alternative parameter of significance involves the fact that the confidence interval between upper 5% and the lowest 5% must include 0.0 (Table 3.12 and Table 3.13).

The mean concentrations of Cu, Fe and Mn in homogenized samples were not significantly different from those in samples prepared by microwave digestion, indicating that no appreciable levels of contamination had been introduced into the sample slurries by processing.

Compared to the results of Karadjova and Karakjov (1998) and of Takuwa (1997), the current results were characterized by a decreased RSD (relative standard deviation). The RSDs based on analysis performed on different days were as follows: Cu, 1-7%; Mn, 0-6%; Fe, 0-8%. These results demonstrated that the high-pressure homogenization slurry technique provided good reproducibility for wood pulp analysis.

The mean value of relative standard deviations associate with all three analyte elements determinations was 3.1% for sample prepared by high-pressure homogenization (HOMO), and for those of MW, the mean value was 2.8%. This indicated that for the three elements, the repeatability of MW samples was slightly higher than that of HOMO samples. Whereas MW samples were aqueous solutions, HOMO's were aqueous dispersions.

Preliminary investigations were performed on raw pulp (RP). Calibrations were performed using both external standards method and standard additions method. By comparing the slopes of the calibration curves obtained for microwave digested matrices and homogenized matrices, it was found that the difference between the slopes had an appreciable influence on the final results. It was decided to apply standard addition calibration method to both microwave digested matrices and homogenized matrices, in order to minimize matrix effects. RP samples were also analyzed for their Cu and Mn content after 1 day of storage at 4 °C, post homogenization. The results were characterized by a lack of any significant differences in Cu or Mn concentration between samples that had been stored for 1 day or that had been analyzed promptly after preparation. (Cu 30.18 μg/g *vs.* 30.21 μg/g; Mn 64.5 μg/g *vs.* 63.9 μg/g)

However, prior to GF-AAS determination, the sample (either freshly prepared or stored) was gently hand shaken prior to sub-sampling into the autosampler cup. Mixing by Pasteur pipette within autosampler cup prior to transferring sample into graphite furnace was also important to achieve good repeatability (i.e. results with low relative standard deviation among replicates). By applying these mixing procedure (re-suspending solid fraction of the dispersion), to all the five homogenized pulp samples (three RPs, JP and BF), the analytical results in Table 3.11 demonstrated good repeatability, the highest RSD was 8% (Fe in JP), and the lowest 0% (Fe in BF).

For analysis of RP, two batches (RP and RP*, * after 1 day of storage at 4°C) of sub-sample were taken and milled on different days. The results of the homogenized RP from these two batches did not match with each other, whereas the results of homogenized and microwave digested RP within the same batch were characterized by good agreement. This implied that the whole pulp sample was not homogeneous, the metal contents varied within different regions of the gross sample. To reach the real average metal content in the whole sample, a larger size sub-sample should have been taken and milled.

Since there was no applicable sample blank for the pulp sample, Ash Free filter paper pulp (AF) was employed as a reference blank to investigate the extent of metal contamination induced by the sample process procedures. AF was treated by both the homogenizing procedure and the microwave digestion (except for Mn analysis, the MW result was somehow missing). As summarized in Table 3.11, the concentrations of Cu and Fe in

HOMO AF did not show significant differences in metal concentrations from those prepared by MW. The concentration of Mn in HOMO AF was very low compared to those in the other five pulp samples (less than 2%). These suggested no appreciable metal contamination was introduced into the sample during high pressure homogenization. However, when comparing the Fe with Cu and Mn in AF (in either HOMO AF or MW AF), Fe was appreciably higher than the other two metal concentrations. It was suspected that Fe contamination was induced by the milling process. Since there was no reference data for Fe concentration in AF, no conclusion can be made regarding Fe contamination.

While performing the high pressure homogenization of the samples, an aliquot of TMAH solvent mixture was also passed through the homogenizer under the same working conditions. The concentration of Cu, Mn and Fe in TMAH in Table 3.11 are expressed in units of ppb. Since Cu and Mn in TMAH were both very low, they were not considered when reporting the Cu and Mn content in pulp samples. But Fe concentrations of all five HOMO pulp samples (three RPs, JP and BF) were corrected the Fe in TMAH, although when compared with the Fe in these pulp sample, Fe in TMAH contributed, at most, less than 12% (JP) of the metal concentration in the final homogenate.

### 3 (III).4. Conclusions

The results indicated that the high-pressure homogenization procedure can be used as a rapid sample preparation technique for wood pulps prior to GF-AAS determinations for Cu, Fe and Mn. The technique can generate dispersions of wood pulps that can be reliably sub-sampled after one day of storage. The high-pressure homogenization process did not introduce appreciable levels of analyte contamination into the product slurry. Sample preparation proved to be rapid (a maximum 6 minutes was required to prepare 50 ml of sample) and the homogenizer was readily cleaned between samples by processing fresh solvent.

The method will require further optimization in terms of the stability of the sample dispersion. It would be advantageous to cause analyte metals to be transferred quantitatively to the liquid phase during the processing (as were the cases for CRMs and animal feeds), so that no further mixing/agitation of the sample would be required prior to the automated sampling stage by the instrument. Considering that a relatively larger uncertainty might be

acceptable to customers, the pre-sampling mixing step might be skipped entirely when only a rough estimation of the metal content is required.

**Table 3.11** Mean copper, manganese and iron concentrations[a] in dried ground wood pulps that had been processed by high-pressure homogenization or by microwave digestion then subjected to metal determinations by GF-AAS.

| Sample | Copper | | manganese | | iron | |
|---|---|---|---|---|---|---|
| | HOMO[b] | MW[c] | HOMO | MW | HOMO | MW |
| Raw Pulp (RP) | 30.2 ±1% | 33.2 ±1% | 63.9 ±2% | 65.2 ±4% | 252.4 ±8% | 258.5 ±0% |
| Raw Pulp[d] | 30.2±1%[d] | 33.4±7%[d] | 64.5±4%[d] | 63.5±2%[d] | | |
| Raw Pulp[e] (RP*) | 13.5 ±7% | 13.6 ±7% | 104.6 ±1% | 110.9 ±4% | 193.1 ±6% | 174.0 ±0% |
| Jack Pine (JP) | 9.7 ±1% | 10.0 ±1% | 70.4 ±1% | 65.5 ±7% | 185.8 ±8% | 188.4 ±2% |
| Balsam Fir (BF) | 6.0 ±2% | 6.4 ± 6% | 106.1 ±2% | 105.3 ±0% | 388.5 ±0% | 377.3 ±1% |
| Ash Free (AF) | 8.2 ±14% | 9.4 ±18% | 1.0 ±23% | | 64.2 ±1% | 58.3 ±5% |
| TMAH[f] | 6.9 ±18% | | 4.6 ±22% | | 43.1 ±14% | |

[a]μg/g ± 1 relative standard deviation based on duplicate analyses performed on different days.

[b]High-Pressure Homogenization.

[c]Microwave Digestion.

[d]Post 1 day of storage at 4°C.

[e]Separately milled sub-sample from the same matrix.

[f]Metal concentration expressed as ppb in TMAH solvent mixture that had been processed by homogenization for ten cycles.

**Table 3.12** Student T-Test of the Relative Difference (RD = [(HOMO-MW)/MW] ) between slurry technique and microwave digestion (indexed by element for wood pulp samples).

| Element | Mean of RD | SD[a] | T | *p*-Value | 95% CI[b] |
|---|---|---|---|---|---|
| Cu | -0.02 | 0.07 | -1.18 | 0.2649 | (-0.07, 0.02) |
| Mn | 0.00 | 0.05 | 0.32 | 0.7532 | (-0.02, 0.03) |
| Fe | 0.02 | 0.08 | -0.81 | 0.4429 | (-0.04, 0.08) |

[a]SD: Standard deviation

[b]CI: Confidence interval

**Table 3.13** Student T-Test of the Relative Difference (RD = [(HOMO-MW)/MW] ) between slurry technique and microwave digestion (indexed by wood pulp matrix).

| Matrix | Mean of RD | SD[a] | T | *p*-Value | 95% CI[b] |
|---|---|---|---|---|---|
| Raw Pulp (RP) | -0.04 | 0.05 | -2.18 | 0.0716 | (-0.09, 0.00) |
| Raw Pulp[c] (RP) | 0.03 | 0.05 | 1.58 | 0.1746 | (-0.02, 0.09) |
| Raw Pulp[d] (RP*) | 0.01 | 0.09 | 0.35 | 0.7380 | (-0.07, 0.09) |
| Jack Pine (JP) | 0.01 | 0.07 | 0.25 | 0.8085 | (-0.06, 0.07) |
| Balsam Fir (BF) | -0.01 | 0.04 | -0.42 | 0.6937 | (-0.05, 0.04) |

[a]SD: Standard deviation

[b]CI: Confidence interval

[c]Post 1 day of storage at 4°C

[d]Separately milled sub-sample from the same matrix

# 4

## Summary of Conclusions

The objectives of the study were to optimize novel procedures and techniques for rapid sample preparation prior to trace element determinations by GF-AAS and to apply them to samples of biological origin.

In chapter 2, high pressure homogenization (HPH) was demonstrated to provide a rapid sample preparation technique for biological materials prior to selenium determination by GF-AAS. Reliable determinations of the selenium content in the resulting slurry were achieved provided that the particle volumes have been reduced sufficiently and that the matrix is sufficiently homogeneous. This work has also demonstrated that enzymatic digestion with a crude protease can be accelerated by incubation at elevated temperature (60°C) to liberate Se from these matrices. However, Se-release can be achieved more rapidly by adequately reducing the size of the particles within the sample matrix. Multiple passes through the homogenizing valve were sufficient to generate a quasi-stable slurry that could be sampled reliably for Se even after several (1-10) days of storage at 4°C post preparation.

In chapter 3, section 3(I), investigations were performed to determine the levels of metal contamination induced by the EmulsiFlex Model C5 homogenizer. When compared with levels of metal contamination introduced by the previous model, the EmulsiFlex Model C5 homogenizer equipped with a ceramic homogenizing valve decreased levels of the metal contamination appreciably. The contamination levels of Cr, Cu, Fe, Mn and Pb fell within the range that can be either ignored or readily compensated for when determining the concentrations of these elements in biological matrices. This approach is especially valuable for the screening of large numbers of samples, where simple, fast, and relatively precise analyses are required. The most appreciable improvement made by the new model was the greatly reduced level of iron contamination in the homogenized fluid, which had been of major concern with previous model.

Levels of metal contamination were increased by passing the fluid through the homogenizer several times. Studies to date have demonstrated that three sequential passes of suspensions prepared from zoological materials was sufficient for a virtually

quantitative release of analyte, whereas botanical matrices can require up to ten sequential passes (less than two minutes in the re-cycle mode for 10 ml suspension).

In chapter 3, section 3(II), the applicability of the new technique to the analysis of biological sample was evaluated. The results indicate that: (*i.*) HPH can be used to rapidly generate dispersions of dried animal feeds and (ii.) these dispersions can be reliably sub-sampled after 7 days of storage and analyzed by GF-AAS for Cu, Mn and Se. This processing did not introduce appreciable levels of analyte contamination into the product slurry. Sample preparation proved to be rapid (a maximum 6 min was required to prepare 50 ml of sample) and the homogenizer was readily cleaned between samples by processing fresh solvent.

The repeatability of determinations performed on the same homogenate post different periods of storage indicated that at least a high proportion of each analyte was extracted into the liquid phase during the processing. Results of determinations performed on different sub-samples of the same feed using slurry preparation prior to GF-AAS proved to be highly repeatable.

The results of Se determinations indicated that the homogenization followed by enzymatic hydrolysis was a practical method for releasing Se from the sample matrix into a form that can be detected by GF-AAS. This sample preparation is effective not only for CRMs that are fat free and contain relatively high content of Se, but also for real samples that, very often, contain an appreciable component of fat and have an appreciably lower content of selenium.

In chapter 3, section 3(III), wood pulp samples were analyzed for their metal contents by applying the HPH technique prior to GF-AAS. The pulp matrix contained a high proportion of long fibers and is very different from the reference materials that had been investigated previously. The RMs were readily ground to a powder form. The results indicated that the high-pressure homogenization procedure can be used as a rapid sample preparation technique for wood pulps prior to GF-AAS determinations for Cu, Fe and Mn. The technique can generate dispersions of ground wood pulp that can be reliably sub-sampled even after 1 day of storage.

The method will require further optimization in terms of the stability of the sample dispersion. However, considering that a relatively larger uncertainty might be entirely

acceptable to industry, the pre-sampling mixing step might be skipped entirely when only a rough estimation of the metal content is required.

In summary, HPH provided a rapid sample preparation technique for biological materials prior to trace element determination by GF-AAS. When combined with partial enzymatic digestion, the procedure released the Se quantitatively from biological materials into forms that can be detected by GF-AAS. The HPH procedure can be employed with various biological matrices, including CRMs of both botanical and zoological origin, animal feeds, and wood pulp. For all those samples, the procedure did not induce appreciable analyte metal contamination during the process. The attractive features of this approach are its speed, simplicity 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.

For further study, the HPH technique could be evaluated for other biological materials. More work needs to be done on wood pulp samples, to achieve a more prolonged stability of the product dispersion, and to demonstrate that the procedure is applicable to pulps from different plant species.

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