Bovine liver sample preparation and micro-homogeneity study for Cu and Zn determination by solid sampling electrothermal atomic absorption spectrometry

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1. Introduction

Solid sampling electrothermal atomic absorption spectrometry (SS-ETAAS) has received attention due to several favorable characteristics (e.g., high sample introduction efficiency, low sample consumption, in situ analyte–matrix separation during the pyrolysis stage, rapid and low-cost analytical procedure, high selectivity and sensitivity and long residence time, and improvement of the atomization efficiency) [1,2]. The possibility of using micrograms of materials is one of the favorable characteristics of this technique, and for this reason, it has been applied to investigate microsample homogeneity of certified reference materials (CRM) [3–7]. The SS-ETAAS is also recommended when little quantity of sample is available [8–10], and
due to its high sensitivity, ultra-trace analyte concentrations have been determined in high-purity and technological products [11–14].

Besides its good characteristics, the SS-ETAAS technique also presents some drawbacks that need to be overcome to facilitate its application in routine analysis [2]. The spectrometer calibration is one important point that deserves attention. In the majority of methods, CRM are used for calibration. However, it has been shown in a number of more recent publications that calibration is often possible with aqueous reference solutions [15–19]. In both situations, CRM are necessary for analytical quality control.

Homogeneity is considered to be the most important prerequisite for CRM, mainly for small sub-sample analysis. Homogeneity of sample can be estimated by calculating the homogeneity factor according to: \( H_{e} = R_{HOM} \cdot m^{1/2} \), where \( H_{e} \) = homogeneity factor, \( R_{HOM} \) = relative standard deviation, and \( m \) = sample mass. Homogeneity factors below 10, determined in the small sample masses, are considered sufficient for the homogeneity of the materials [20]. Most of the organic materials show \( H_{e} < 10 \) [7], which makes them recommendable for micro-analytical techniques. However, the smallest recommended sample sizes for commercially available CRM or reference materials (RM) are within 100–500 mg [6], whereas the sample mass required for SS-ETAAS determinations is frequently smaller than 1 mg. Considering this drawback, efforts can be directed to investigate the microsample homogeneity of materials to assure their representativeness, even for low sample masses, like those adopted in SS-ETAAS.

Good homogeneity is a prerequisite for direct determination of elements in solid samples [20]. The information about homogeneity of a material is not an absolute one and should be discussed, taking into account factors such as the nature of the material, the sample size, and the concentration of the elements. It is well known that sample homogeneity affects strongly the precision and accuracy of analytical results, mainly when small masses are used [21].

Several authors have demonstrated the improvement in precision when the particle size is reduced [22,23]. Notwithstanding, if the sample is subdivided into smaller and smaller sub-samples, it becomes more and more heterogeneous because the statistical probability of finding the same mean concentration is lower. When the homogeneous material becomes a heterogeneous one due to the small sub-sample, there is a minimal representative sample mass that is an important parameter, which must be considered and well-established to guarantee accurate results [23].

In the present work, a systematic study for bovine liver sample preparation for copper and zinc determination using solid sampling electrothermal atomic absorption spectrometry (SS-ETAAS) is proposed. The main parameters investigated were sample pretreatment involving grinding process, particle size, sample size, and their relationship with the microsample homogeneity, to get precise and accurate results.

2. Experimental

2.1. Instrumentation

All determinations were performed with a Model ZEEnit® 60 atomic absorption spectrometer (Analytik Jena AG, Jena, Germany) equipped with a transverse-heated graphite tube atomizer and an inverse and transverse two-field and two-field mode Zeeman effect background corrector. Pyrolytically coated transverse-heated graphite tubes and pyrolytically coated boat-type solid sampling platforms (Analytik Jena) were used throughout.

A mechanical solid sampling accessory (SSA-6 Z; Analytik Jena AG, Germany) with pre-adjusted micro-tweezers was used to transfer the boat-type platform from the microbalance (Auto Balance AD-4; Perkin-Elmer, Norwalk, CT, USA) to the graphite tube. A stainless steel microspatula was used to transfer the samples to the pyrolytic boat-type platform. Using less sensitive analytical lines for both elements and the three-field mode Zeeman effect background correction for Cu, it was possible to work with up to 150 \( \mu \)g of samples.

All measurements were based on integrated absorbance values, controlled by Windows NT® software. Argon 99.998% (vol/vol) (Air Liquide Brasil, São Paulo, Brazil) was used as purge gas.

A freeze drier (Savant Model Novalyph NL 150, USA), operating at liquid nitrogen temperature, and a domestic microwave oven (Consul 300W, Brazil), in combination with a stove, were used for sample drying.

A ball mill grinder 8000 M Spex CertiPrep (SPEx, Metuchen, NJ, USA), equipped with vials and tungsten carbide balls, and a cryogenic mill with a self-contained liquid nitrogen bath (SPEx Model 6800 Freezer Mill), operated at liquid nitrogen temperature, were used in order to comminute the samples. After grinding, the particle size distribution was measured using a Mastersizer S polydisperser particle analyzer (Malvern Instruments, Malvern, UK) with an MS-17 automated sample dispersion unit.

The bovine liver samples and the CRM (NIST 1577b) were digested in a closed vessel microwave oven (Microwave 3000; Anton Paar, Graz, Austria). Sample masses around 200 mg were mixed with a diluted oxidant mixture (1 mL of HNO\(_3\)+2 mL of H\(_2\)O\(_2\)+3 mL of H\(_2\)O) and the following heating program was performed (temperature (°C), ramp (min), hold (min)): (140, 5, 1), (180, 8, 2), and (220, 4, 10). Copper and Zn were also determined using a flame atomic absorption spectrometer (Model AAS 5-FL, Analytik Jena AG, Germany) equipped with an AS 52 autosampler (Analytik Jena AG).
2.2. Reagents and solutions

High-purity deionized water (resistivity = 18 MΩ cm) obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA) was used throughout. Analytical reagent-grade HNO₃ (Synth, SP, Brazil) was distilled in quartz sub-boiling stills (Marconi, Piracicaba, SP, Brazil). Nitric acid and H₂O₂ (Titrisol, Merck, Darmstadt, Germany) were used for sample digestion. All solutions and samples were stored in decontaminated polypropylene bottles (Nalge Company, Rochester, NY, USA).

Analytical calibration solutions for Zn and Cu were prepared by suitable dilution of stock standard solutions containing 1000 mg L⁻¹ ZnCl₂ and CuCl₂ (Titrisol, Merck, Darmstadt, Germany). Solutions containing 1000 mg L⁻¹ Ir (Na₂IrCl₆; Merck), W (Na₂WO₄·2H₂O; Merck), and Rh (RhCl₃; Aldrich) were used to deposit the permanent chemical modifier onto the boat-type graphite platform, according to previous work in the literature [24,25].

The CRM bovine liver NIST SRM 1577b (National Institute for Standards and Technology, Gaithersburg, MB, USA) was used for validation.

2.3. Samples

Approximately 5 kg of bovine liver, collected at a slaughterhouse in São Paulo (Brazil), were initially washed with Milli-Q® water, cut, and separated in four parts. All of them were frozen at −10 °C. Two parts were defrosted and submitted to different drying processes: (i) freeze drying for 48 h; and (ii) drying in a household microwave oven (30 W), for 45 min, followed by drying in a stove (60 °C) until constant mass, for approximately 48 h.

2.4. Sample grinding

Samples were ground in a ball mill using a flask and two balls made up of tungsten carbide. Masses around 5 g of bovine liver were ground for different times (5, 15, 30, and 60 min).

For cryogenic grinding, the sample (~2.0 g) was placed in a polycarbonate vial closed with two stainless steel end plugs, which was immersed in a liquid nitrogen bath inside the mill. After the pre-cooling step, the high-frequency motion of a stainless steel impact, located inside this vial, pulverizes the sample. The grinding tube was cleaned after each use to eliminate cross-contamination. The program used was 5 min of pre-cooling followed by 2 min of grinding and 1 min of cooling. This cycle was repeated three times for each sample mass.

2.5. Procedure

All glassware and polypropylene bottles were cleaned with detergent solution, soaked in 10% (vol/vol) HNO₃ for 24 h, rinsed with water, dried in a class 100 laminar flow hood, and stored in a closed polypropylene container.

Pyrolysis and atomization curves were obtained for both elements using aqueous solution containing 1.5 mg L⁻¹ Cu²⁺ and 3 mg L⁻¹ Zn²⁺ in 0.1% vol/vol HNO₃ and solid bovine liver, with and without permanent chemical modifiers (250 μg of W+200 μg of Rh) [24] and (200 μg of Ir) [25]. For the solid samples, masses around 70 μg were weighed onto the platform to optimize the heating program for both elements.

To overcome the difficulty of weighing the same mass of solid sample, the ratio between absorbance signal and the sample mass was plotted against the temperature to establish the pyrolysis and atomization curves. The same strategy was used to find the best pyrolysis time.

Calibrations curves were obtained by using two different ways: the first one was based on the use of reference solutions containing 0.5–3 mg L⁻¹ Cu²⁺ and 1–6 mg L⁻¹ Zn²⁺ in 0.1% vol/vol HNO₃, and the other one was based on increasing masses of the bovine liver CRM weighing masses of 60–150 μg directly onto the boat-type platform.

To investigate the homogeneity of the material, determinations of Cu and Zn were performed using increasing bovine liver sample masses (9–150 μg) dried in the microwave/stove and ground in the ball mill for 5 min and in the cryogenic mill for 6 min, or freeze–dried and ground in the ball mill for 5 min. For comparison, the same study was accomplished with the CRM.

3. Results and discussion

3.1. Heating program optimization

The pyrolysis and atomization temperatures are the most critical parameters that need to be optimized for direct analysis by SS-ETAAS. In general, high amounts of matrix are introduced into the graphite furnace, claiming a very effective pyrolysis step.

The pyrolysis and atomization temperatures for Cu and Zn in aqueous solution and in solid bovine liver, in the absence and presence of 250 μg of W+200 μg of Rh or 200 μg of Ir as chemical modifiers, are shown in Table 1. In the presence of W+Rh or Ir, and bovine liver matrix, pyrolysis temperatures for Cu were 1300 °C. These pyrolysis temperatures are 300 °C higher than without modifier, showing the effectiveness of the chemical modifiers in the presence of solid matrix. For W+Rh, an atomization temperature higher than 2200 °C was necessary. However, a temperature above 2200 °C is not recommended due to the volatilization of the Rh film deposited onto the graphite platform, diminishing the lifetime of the permanent chemical modifier [24]. For Ir, calibration with aqueous solution was impossible, probably...
Table 1
Pyrolysis and atomization temperatures for Cu and Zn in aqueous solution and bovine liver in the presence and absence of permanent chemical modifiers

<table>
<thead>
<tr>
<th>Chemical modifier</th>
<th>Cu</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>W+Rh</td>
</tr>
<tr>
<td>Aqueous solution</td>
<td>$T_p$ ($^\circ$C)</td>
<td>1100</td>
</tr>
<tr>
<td></td>
<td>$T_a$ ($^\circ$C)</td>
<td>2300</td>
</tr>
<tr>
<td>Bovine liver</td>
<td>$T_p$ ($^\circ$C)</td>
<td>1000</td>
</tr>
<tr>
<td></td>
<td>$T_a$ ($^\circ$C)</td>
<td>2300</td>
</tr>
</tbody>
</table>

$T_p$=pyrolysis temperature; $T_a$=atomization temperature.

due to the difference in pyrolysis temperatures for aqueous solutions (1100 °C) and solid matrix (1300 °C). On the other hand, in the absence of a chemical modifier, the pyrolysis temperatures for Cu were practically the same in aqueous solution and solid matrix. Considering the good performance presented by Cu in the absence of a chemical modifier, this condition was chosen for the subsequent studies.

For Zn, a significant increase in the pyrolysis temperature was observed in the presence of W+Rh or Ir (Table 1). Considering the volatility of Zn, this thermal stabilization is important to guarantee effective organic matrix decomposition. Although both chemical modifiers have presented a good thermal stabilization for Zn, better performance regarding aqueous calibration was found for W+Rh. Pyrolysis and atomization temperatures of 700 °C and 1700 °C, respectively, were chosen.

The instrument parameters and optimized temperature program for Cu and Zn determinations in bovine liver materials are presented in Table 2.

3.2. Effect of sample grinding

The particle size distribution of bovine liver was shown to be directly related to the grinding time. Comparing the particle size for bovine liver dried in microwave/stove and ground in a ball mill during 5 and 30 min, it was possible to observe a reduction of 33% in the particle size. The average particle size for sample ground during 5 min was around 130 μm and for 30 min was around 90 μm. When the freeze-dried sample was ground in a ball mill for 5 min, the average particle was around 90 μm. For samples ground in a cryogenic mill during 6 min, it was around 64 μm, independent of the drying procedure.

Although the particle diameter had been higher than obtained by other authors [26], the results obtained in this work showed that for particle sizes below 90 μm, the sample homogeneity is guaranteed. Previous researches showed that particle size is less important in the analysis of biological and botanical samples because the analytes are often more weakly retained in these materials, and more easily leached from the matrix than in other kind of samples [27].

3.3. Sample mass size and sample preparation effect

Lower sample masses used in an analytical procedure have a direct effect on the sample homogeneity, which can affect the sample representativeness, precision, and accuracy of the results.

The influence of sample mass on the precision and accuracy of Cu and Zn determinations by SS-ETAAS is shown in Figs. 1 and 2, which represent the recoveries and standard deviation of the bovine liver samples prepared in this work and the CRM. For this purpose, 10 sample mass intervals with bovine liver masses between 9 μg and 150 μg were chosen, and each interval represents the average concentration and standard deviation of five sub-samples ($n=5$): mass interval 1 (9–20 μg), mass interval 2 (21–30 μg), mass interval 3 (31–40 μg), mass interval 4 (41–50 μg), mass interval 5 (51–60 μg), mass interval 6 (61–70 μg), mass interval 7 (71–80 μg), mass interval 8 (81–90 μg), mass interval 9 (91–100 μg), and mass interval 10 (101–150 μg). Copper and Zn reference concentration values indicated in Figs. 1 and 2 (dark line) with the respective standard deviations (dotted line) were established by using digested solutions of the bovine liver samples prepared by different drying and grinding procedures and the CRM and determination by FAAS.

From Fig. 1a–d, it is obvious that precision and accuracy are degrading when small sample masses are used. The best homogeneity factors (<10), with good precision and accuracy, were obtained for masses higher than 50 μg. In a general way, this behavior was observed

Table 2
Instrumental conditions and heating program for the transversally heated graphite atomizer

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature ($^\circ$C)</th>
<th>Ramp ($^\circ$C s$^{-1}$)</th>
<th>Hold (s)</th>
<th>Argon flow (L min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drying</td>
<td>130</td>
<td>10</td>
<td>10</td>
<td>1.0</td>
</tr>
<tr>
<td>Pyrolysis</td>
<td>1000$^a$, 700$^b$</td>
<td>100</td>
<td>20$^a$, 30$^b$</td>
<td>1.0</td>
</tr>
<tr>
<td>Auto zero</td>
<td>1000$^a$, 700$^b$</td>
<td>0</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Atomization</td>
<td>2300$^a$, 1700$^b$</td>
<td>2300$^a$, 1700$^b$</td>
<td>6$^a$, 5$^b$</td>
<td>0</td>
</tr>
<tr>
<td>Cleaning</td>
<td>2400$^a$, 2200$^b$</td>
<td>1200</td>
<td>3</td>
<td>1.0</td>
</tr>
</tbody>
</table>

$^a$ Cu.

$^b$ Zn.
for all samples independent of the sample pretreatment. The precision and accuracy observed for Cu were best for samples dried in microwave/stove using 5 min of grinding in a ball mill (Fig. 1b). However, very similar results were also obtained when the samples were ground in a cryogenic mill (Fig. 1d).

The sample preparation procedure affected significantly the Zn homogeneity. In Fig. 2a and c, results are shown for Zn determination in the CRM and freeze–dried bovine liver ground in a ball mill, respectively; both exhibit the same behavior. But for samples dried in the microwave/stove and ground in a ball mill (Fig. 2b), the use of low sample mass not only affected the precision, but also the accuracy, while for the other two samples (Fig. 2a and c), only the precision was affected. However, when the samples dried in the microwave/stove were ground in a cryogenic mill, their behavior became similar to freeze–dried samples. These results can be attributed to the freeze-drying process that occurs at low temperature. At this low temperature (−196 °C), the tissue structure probably became more fragile, facilitating the homogenization process [28]. Problems caused by temperature rise and sieve clogging during grinding of spices with high fat content have been reported. Cryogenic grinding has been used to solve problems of agglomeration during grinding at room temperature. This sample preparation method was used to prepare plant reference materials by slurry ET AAS [29].

3.4. Analytical characteristics of the methods

The proposed method for Cu and Zn determination by using SS-ETAAS allowed analysis of solid bovine liver samples using calibration with aqueous solutions. For Cu determination, no chemical modifiers were used, while for Zn, 250 μg of W + 200 μg of Rh was used as permanent chemical modifier.

The calibration curves for Cu determination were obtained with reference solutions containing 5, 10, 20, and 30 ng of Cu in 0.1% (vol/vol) HNO₃ and by using increasing masses of CRM (bovine liver, NIST 1577b), as shown in Fig. 3a. In the case of Zn determination, the use of calibration curves obtained with aqueous solutions was possible only when 250 μg of W+200 μg of Rh was used as permanent chemical modifier.

The calibration curves for Zn determination were obtained with reference solutions containing 10, 20, 40, and 60 ng of Zn in 0.1% (vol/vol) HNO₃ and by using increasing masses of CRM (bovine liver, NIST 1577b), as shown in Fig. 3b. The slopes of the two calibration curves were essentially identical for both
Fig. 3. Analytical calibration curves: (a) Cu using aqueous solution, \( y = 0.003230x, R = 0.9976 \); and CRM, \( y = 0.003232x, R = 0.9984 \); and (b) Zn using aqueous solution, \( y = 0.00160x, R = 0.99757 \); and CRM, \( y = 0.00162x, R = 0.99989 \).

Fig. 2. Influence of sample mass on the precision and accuracy of Zn in: (a) bovine liver CRM (NIST 1577b); (b) bovine liver dried in microwave/stove and ground in a ball mill for 5 min; (c) freeze-dried bovine liver and ground in a ball mill for 5 min; and (d) bovine liver dried in microwave/stove and ground in a cryogenic mill for 6 min. The reference concentration (---) and standard deviation (----) values were obtained for each bovine liver (a, b, c, and d) determined by FAAS. For mass interval, see text.
elements, demonstrating that calibration against aqueous standards can be used without any problems.

The application of the permanent chemical modifier allowed to use the solid sampling platform for up to 670 heating cycles, while without modifier, the lifetime was approximately 350 cycles.

The characteristic mass, based on integrated absorbance, was 1.4 ng for Cu and 2.8 ng for Zn. The detection limits were calculated by considering the variability of 20 consecutive measurements of platform inserted without sample, according to \( (3S)/m \), where \( S \) is the standard deviation of the measurements and \( m \) is the calibration slope. The obtained values for Cu and Zn are 1.6 ng and 1.3 ng, respectively.

The reliability of the developed method was checked through the analysis of digested samples using determination by flame AAS. The results obtained from solid sampling analysis and digestion procedure are presented in Table 3. Good agreement (95% confidence level) was observed using the proposed method and conventional flame AAS. The relative standard deviations were lower than 6% (\( n = 5 \)).

Table 3

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Cu (mg kg^{-1})</th>
<th>Zn (mg kg^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SS-ETAAS</td>
<td>FAAS</td>
</tr>
<tr>
<td>I</td>
<td>221 ± 10</td>
<td>215 ± 6</td>
</tr>
<tr>
<td>II</td>
<td>223 ± 12</td>
<td>222 ± 4</td>
</tr>
<tr>
<td>III</td>
<td>232 ± 5</td>
<td>229 ± 6</td>
</tr>
<tr>
<td>IV</td>
<td>235 ± 10</td>
<td>235 ± 4</td>
</tr>
</tbody>
</table>

* Bovine liver mass >70 µg.

4. Conclusion

The sample pretreatment (drying and grinding) adopted for microanalysis by using SS-ETAAS can influence the homogeneity of the material. In general, bovine liver samples submitted to the cryogenic pretreatment showed good homogeneity, even for small sample masses. Considering Cu and Zn determination in bovine liver by SS-ETAAS, the best sample pretreatment is drying in a microwave oven (30 W) for 45 min followed by drying in a stove (60 °C) and grinding in a cryogenic mill for 6 min. The obtained results indicated the possibility of preparing a bovine liver reference material with good accuracy and precision for Cu and Zn determination by SS-ETAAS. Although commercially available CRM guarantee accuracy and precision only for sample masses higher than 100 mg, this study showed that masses lower than 150 µg can be used, assuring good accuracy and precision of the results.

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References


