

**DETERMINATION OF COPPER IN BIOLOGICAL MATERIALS
BY ATOMIC ABSORPTION SPECTROMETRY****A. Kotulanová, J. Komárek***Department of Analytical Chemistry, Masaryk University, Kotlářská 2, 61137 Brno, Czech Republic**Received 11-09-2001***Abstract**

Dry ashing in muffle furnace and ashing in low pressure oxygen plasma were compared with acid digestion in pressure vessels, solubilization with tetramethylammonium hydroxide and slurry sampling for the determination of copper in animal liver tissue samples by atomic absorption spectrometry. The ashing in the muffle furnace and in the oxygen plasma were proposed for decomposition of small pieces of liver. These samples were inserted in quartz microbowls and imitated the human liver tissue by biopsy.

Introduction

Copper is the third most abundant trace element in the body, following zinc and iron. There are a lot of disorders, which are associated with the toxic effects of copper accumulated in any tissues such as the liver, brain, cornea and kidney or copper deficiency. These disorders include Wilson's disease, Menkes disorder and Indian Childhood Cirrhosis.^{1,2}

Conventional sample preparation of biological materials for atomic absorption spectrometric analysis involves solubilization of the analyte and complete or partial decomposition of the matrix typically achieved by dry ashing or wet digestion techniques using oxidative acids.³ The simplest dry ashing technique for preparation of tissue specimens is performed in a muffle furnace at < 600 °C, usually with some type of ashing aid.^{4,5} For small samples acceleration of the reaction can be accomplished by heating at < 150 °C in an atmosphere of RF-generated oxygen at low pressure.⁴ Low temperature and low pressure ashing minimizes losses of volatile elements. The oxygen plasma contains ions, excited atoms and radicals that have a high oxidising power and are effective in decomposing the organic matter at low temperature. Wet digestion methods include decomposition by acids carried out in open vessel on a hot plate and in closed vessels at elevated pressure with thermal or microwave heating. The majority of procedures involve the application of nitric acid.⁴ An alternative approach to solubilize the biological material is the use of tetramethylammonium hydroxide (TMAH).^{3,6,7}

Slurry sampling electrothermal AAS has been extensively used for the analysis of biological tissues to simplify sample treatment and to avoid some problems associated with dissolution procedures.^{8,9} In order to ensure that a representative aliquot of slurry is injected into the furnace using an autosampler, the slurry must be either stabilized or homogenized. Stabilization has been attempted by the addition of dispersing agents such as glycerol,¹⁰ Viscalex HV-30,¹¹ nitric acid and Triton X-100¹²⁻¹⁷ while homogenization has been achieved by using agitation techniques such as ultrasonic agitation,^{8,12-17} magnetic stirring,¹⁸⁻²¹ gas bubbling,^{22,23} and vortex mixing.^{24,25}

In this paper, various types of decompositions as well slurry sampling technique for the determination of copper by electrothermal atomic absorption spectrometry (ETAAS) and flame AAS in animal liver samples were compared in order to assess the possibility of application for analysis of human biopsy.

Experimental

Chemicals

Stock standard solution containing $1.003 \text{ g l}^{-1} \text{ Cu}^{2+}$ was prepared by dissolution of 1 g of the high purity metal in 10 ml HNO_3 and by dilution to 1 l with water. The solution was standardized with EDTA titration on murexid.

A commercial solution of 10% tetramethylammonium hydroxide (Lachema) and Triton X-100 (Sigma) were used for sample preparation. All other chemicals used were of analytical grade purity.

Instruments

The measurements were carried out on a Perkin-Elmer 3030 AA spectrometer connected with a HGA 400 graphite furnace and an AS-1 autosampler. A Perkin-Elmer Intensitron Cu hollow-cathode lamp was operated at 20 mA (324,7 nm). Deuterium background correction was used. 20 μl of sample was dried 20 s at 140 °C with a 20 s ramp, decomposed 20 s at 800 °C with a 5 s ramp and atomized 3 s at 2300 °C with temperature-controlled maximal power heating and stop flow of argon. The electrographite tubes were cleaned for 2 s at 2650 °C with a 1 s ramp. For higher concentrations of copper, the flame acetylene-air (flow rates: 22.5 l min^{-1} air, 2 l min^{-1} acetylene) was used.

Ashing of samples were performed in muffle furnace (Veb Elektrobád Frankenhausen MLW) and in the low-pressure oxygen plasma in Plasma machine IP 1101 (International Plasma Corporation, California). The autoclaves ZA-1 of JZD Pokrok Zahnašovice with Teflon vessel were used for wet digestion.

Procedures

Pretreatment of liver samples

The samples of bovine, pork and chicken liver tissues were lyophilised and finely grained by means of a vibrating ball mill Vibrom 2S (Vibrom, Czech Republic) with liquid nitrogen. The homogeneous tissues were stored in pre-cleaned polyethylene vials. Small pieces (10 mg) of animal liver samples were thinly sliced with stainless surgical scalpel and were inserted in quartz microbowls (Fig.1). These samples imitated the human liver tissue by biopsy. The samples were dried to a constant weight.

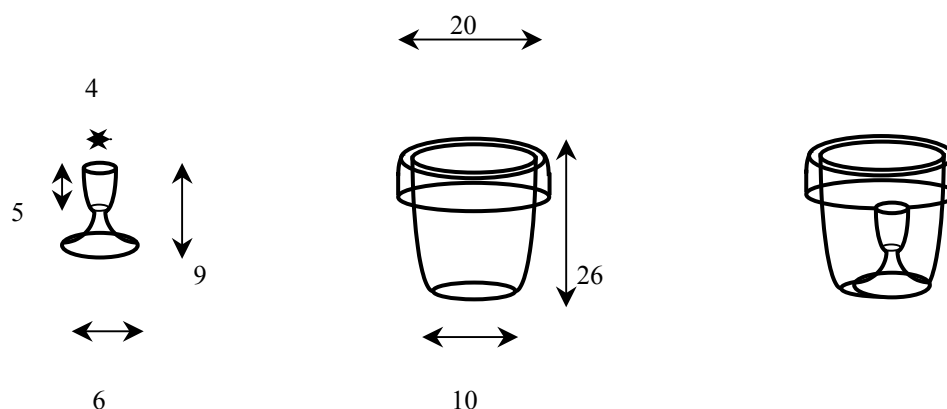


Fig. 1. Quartz microbowl in quartz crucible (size in mm)

Digestion in the autoclave

100 mg of finely grained liver sample was weighed into a PTFE digestion vessel and 2 ml of concentrated nitric acid were added. The bomb was closed and the decomposition was carried out at 140 °C for 4.5 h. After cooling, the decomposed sample was diluted to the 25 ml by bidistilled water.

Ashing in the muffle furnace

100 mg of fine-grain liver sample was put on the quartz flat plate and was ashed at 550 °C for 17 h. After cooling, the ash was dissolved in 2 ml of 3 mol l⁻¹ nitric acid and diluted at a suitable volume by bidistilled water. Quantification was performed using a calibration curve. Standards and blanks contained the same concentration of nitric acid as the samples.

For 10 mg of sample the microbowl with dried tissue was inserted in the quartz crucible and samples were ashed at 550 °C for 17 h.⁵ Due to possible contamination, the crucible was covered by quartz lid (Fig.1). The ash was dissolved in 2 ml of 3 mol l⁻¹ nitric acid. By ashing bovine liver, it was necessary that the lid was risen to allow air to get into the sample.

Ashing in the low-pressure oxygen plasma

The microbowls with 10 mg of dried tissues in the quartz crucibles were placed on the quartz flat plate (6x12cm). The samples were ashed for 5 h in Plasma machine IP 1101. The ash was dissolved with 2 ml of 3 mol l⁻¹ nitric acid. Calibration was performed by using standard solutions in 3 mol l⁻¹ nitric acid.

Tissue solubilization with tetramethylammonium hydroxide (TMAH)

1 ml of 10% TMAH was added to the 100 mg of liver sample. The reaction between the TMAH solution and the liver tissue occurred at room temperature for 48 h. The solution was shaken from time to time. After the reaction, the samples were diluted to 10 ml with bidistilled water. Calibration was obtained by using standards in 1% TMAH.

Slurry technique by ETAAS

10 mg of fine-grain samples were accurately weighed into the polyethylene vials and 4 ml of 0.75 mol l⁻¹ nitric acid containing 0.05% Triton X-100 was added. The slurries were homogenised by stirring with a PTFE coated magnetic bar for 30 min. The homogenous slurry was immediately added into graphite furnace by AS-1 autosampler. Using of standard additions method performed quantification.

Results and discussion

Analysis of liver tissues in amount about 100 mg

Digestion in the autoclave, ashing in the muffle furnace and tissue solubilization with TMAH were used for the analysis of 100 mg of liver tissues. TMAH is an efficient reagent for solubilization of tissue samples.³ This method gives a clear homogeneous solution. The influence of TMAH on signal of copper was examined by ETAAS (Fig.2). With the increasing concentration of TMAH the absorbance signal decreased probably since copper forms precipitates with the basic reagent. The optimal concentration of TMAH was 1%.

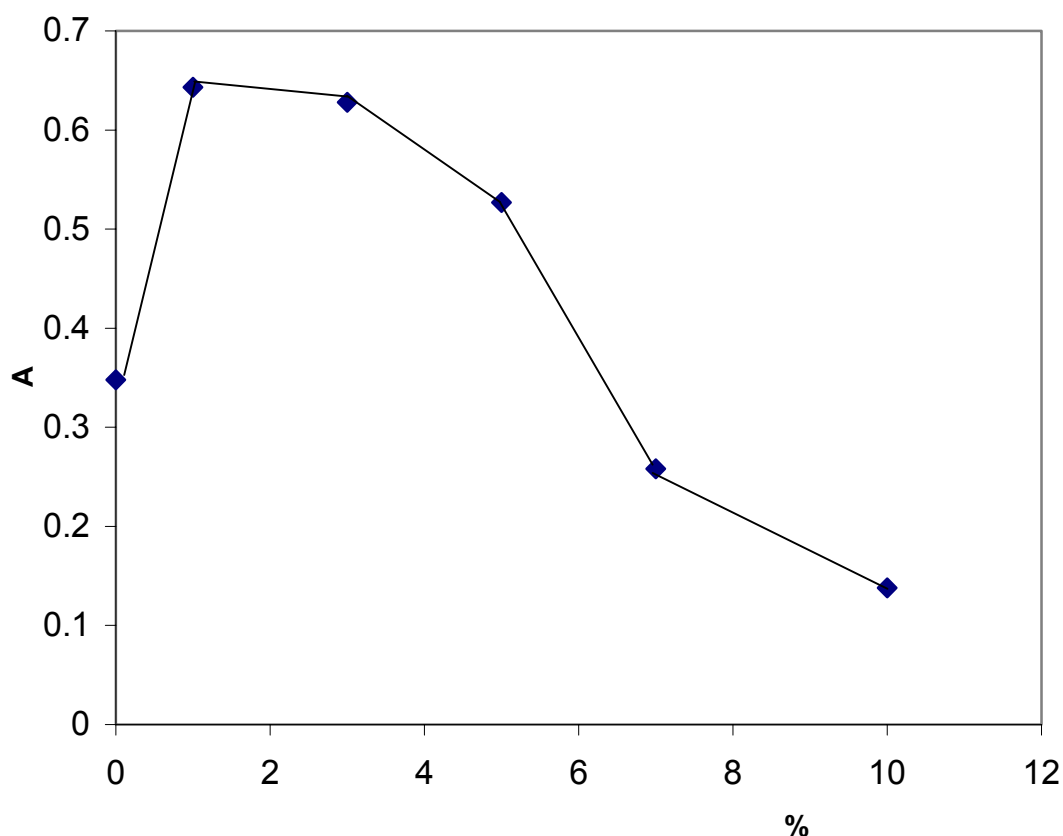


Fig. 2. Dependence of copper absorbance on concentration of TMAH by ETAAS

Analytical results of copper content for liver tissues in amount 100 mg are summarized in Table 1. Results obtained showed that measured concentrations are in good agreement. However, treatment of small sample amount in relative large vessels of autoclaves is not too suitable.

Table 1. Analytical results for liver tissues in amount 100 mg

Tissues	Method of decomposition	Measured value ^a $\mu\text{g g}^{-1} \text{Cu}$
Chicken liver	muffle oven	8.8±0.3
	autoclave	9.1±0.3
	TMAH	8.0±0.2
Pork liver	muffle oven	19.0±0.3
	autoclave	18.7±0.7
	TMAH	19.2±0.4
Bovine liver	muffle oven	120.0±2.0
	Autoclave	122.0±2.0
	TMAH	119.6±1.8

^a The results are expressed as mean and the standard deviation (n=6)

Analysis of liver tissues in amount about 10 mg

Ashing in the muffle furnace, ashing in the low-pressure oxygen plasma and slurry sampling technique were used for the analysis of 10 mg of liver samples. The obvious advantage of slurry sampling by ETAAS is simplified sample treatment, which leads to increased speed of analysis, less risk of contamination and loss of the analyte. The introduction of slurry into the graphite tube can be performed in the same way as for liquid samples and the calibration with aqueous standards is more likely to be successful.¹⁰ Very small sample aliquots are used for slurry sampling. The procedure requires that the analyte is homogeneously distributed, otherwise the sampling error can be large. The homogeneity of the slurry must also be secured during the injection of sample into the atomizer. A dispersion agent, Triton X-100 has often been used for stabilizing, wetting and dispersion of slurry samples.^{10,14,18} According to some workers,¹⁴ medium of $0.75 \text{ mol l}^{-1} \text{HNO}_3$ was chosen. The effect of concentration of Triton X-100 on the Cu response was studied and 0.05% Triton X-100 was found to be optimal. A concentration of 0.05% Triton X-100 is needed to improve the wetting and dispersion of the slurries significantly. A high concentration of Triton X-100 (1%) made the slurry too viscous.

Analytical results of copper content for liver tissues in amount 10 mg are summarized in Table 2. Measured values for animal liver tissues are in good agreement. However, the precision of measurement is worse than for 100 mg amount of samples. This may be caused by the homogeneity of sampling amount. The obtained copper contents for different methods were in good agreement with approximately same precision.

Table 2. Analytical results for liver tissues in amount 10 mg

Tissues	Method of decomposition	Measured value ^a $\mu\text{g g}^{-1} \text{Cu}$
Chicken liver	Muffle oven	8.7 ± 0.5
	Oxygen plasma	8.7 ± 0.4
	Slurry technique	8.2 ± 0.4
Pork liver	muffle oven	18.4 ± 0.6
	slurry technique	18.4 ± 0.8
Bovine liver	muffle oven	118.3 ± 2.7
	slurry technique	119.3 ± 2.3

^a The results are expressed as mean and the standard deviation (n=6)

Suitable methods

All applied methods are suitable for the determination of copper in liver samples. The ashing in the muffle furnace and in the oxygen plasma are the most suitable methods for decomposition of small pieces of liver samples when 10 mg are sliced with scalpel as by biopsy. The accuracy of the proposed methods was proved with reference materials SRM 1577b (bovine liver) and CRM 361a (bovine kidney) analysed by both procedures (Table 3). Measured concentrations are in good agreement with certified values for the biological reference materials.

Table 3. Analytical results for reference materials.

Reference material	Certified value $\mu\text{g g}^{-1}$ Cu	Measured value ^a $\mu\text{g g}^{-1}$ Cu	
		Ashing in muffle furnace	Ashing in oxygen plasma
SRM 1577b ^b	160±8	166.0±4.0	165.0±4.2
CRM 361a ^c	16.9±0.2	16.5±0.3	16.3±0.4

^a Results are expressed as mean and the standard deviation (n=6)

^b National Institute of Standards and Technology, USA

^c Slovak Institute of Metrology, SR

Determination of copper in human liver

The level of hepatic copper for a healthy man is in the range 4-35 $\mu\text{g g}^{-1}$,^{5,26} for patients suffering Wilson's disease is this level much increased. The liver tissue from a patient suspected of Wilson's disease was analysed and found amount of copper was 440 $\mu\text{g g}^{-1}$ Cu in 3.8 mg of dry liver tissue obtained by biopsy.

Conclusions

Dry ashing in muffle furnace, ashing in low pressure oxygen plasma, acid digestion in pressure vessels, solubilization with tetramethylammonium hydroxide and slurry sampling were examined in details. Comparison of results for these methods by the determination of copper showed that all are suitable for analysis of animal liver tissues. The ashing in the muffle furnace and in the oxygen plasma with sampling in quartz microbowls were recommended for small pieces of liver samples in amount about 10 mg. These methods may be applied for the human liver tissues obtained by biopsy.

Acknowledgements

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Povzetek

Razgradnja vzorcev živalskih jeter za analizo bakra z atomsko absorpcijsko spektrometrijo je potekala v muflovki, kisikovi plazmi pod nizkim tlakom in s kislim razklopom v reaktorju pod nadtlakom. Primerjava rezultatov je pokazala, da sta primerni metodi za pripravo majhnih vzorcev jeter razgradnja v muflovki oziroma nizkotlačni kisikovi plazmi.