Analytical Biochemistry 543 (2018) 37–42

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Evaluation of digestion methods for analysis of trace metals in mammalian tissues and NIST 1577c

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ARTICLE INFO

Keywords:
ICP-OES
Pancreatic cancer
Trace metals
Digestion method
NIST 1577c
ICP-MS

ABSTRACT

Digestion techniques for ICP analysis have been poorly studied for biological samples. This report describes an optimized method for analysis of trace metals that can be used across a variety of sample types. Digestion methods were tested and optimized with the analysis of trace metals in cancerous as compared to normal tissue as the end goal. Anthropological, forensic, oncological and environmental research groups can employ this method reasonably cheaply and safely whilst still being able to compare between laboratories. We examined combined HNO_3 and H_2O_2 digestion at 170 °C for human, porcine and bovine samples whether they are frozen, fresh or lyophilized powder. Little discrepancy is found between microwave digestion and PFA Teflon pressure vessels. The elements of interest (Cu, Zn, Fe and Ni) yielded consistently higher and more accurate values on standard reference material than samples heated to 75 °C or samples that utilized HNO_3 alone. Use of H_2SO_4 does not improve homogeneity of the sample and lowers precision during ICP analysis. High temperature digestions (> 165 °C) using a combination of HNO_3 and H_2O_2 as outlined are proposed as a standard technique for all mammalian tissues, specifically, human tissues and yield greater than 300% higher values than samples digested at 75 °C regardless of the acid or acid combinations used. The proposed standardized technique is designed to accurately quantify potential discrepancies in metal loads between cancerous and healthy tissues and applies to numerous tissue studies requiring quick, effective and safe digestions.

Introduction

Trace metals play a vital role in cellular processes as cofactors for enzymes, and in the creation of vascular tissue, cell proliferation and apoptosis [1]. The study of trace metals in normal cellular processes is critical to further our understanding of their role in abnormal situations [2]. Although many trace-metals were examined in this study, four (Cu, Zn, Fe and Ni) were chosen due to their significance in metabolism and, more specifically, disease [3]. Some examples of significant enzymes that utilize trace metals include: matrixmetalloproteinase 9 which, upon laceration of the epithelial tissue, utilizes copper to aid in clearance of oxidative damage by macrophages [4]; cytochrome C oxidase of the electron transport chain contains two Cu centers; superoxide dismutase, an important antioxidant, employs Zn or Cu [5]. Deciphering the role of metals in tumorgenesis may assist in better detection, diagnosis and treatment of cancer [6].

A review of the literature over the past 40 years reveals a lack of consistency in sample preparation and digestion techniques of tissue for ICP-MS/ICP-OES (Inductively Coupled Plasma Mass Spectrometry/ Optical Emission Spectrometry) analysis. Careful review of prior studies reveals significant differences in digestion protocols across a wide variation of trace metal concentrations reported for similar samples [7–13]. Many of the methods tested did not result in complete digestion of the sample and/or produced wide variation in results. A uniform digestion method is necessary for across laboratory comparison of patient tissue samples in order for accurate and precise information to be gleaned from the trace metal composition in tissues.

Moreover, the aforementioned digestion protocols lead to vastly different measurements of trace metals regardless of the specific instrument. Samples were run on both ICP-MS and ICP-OES concurrently to eliminate disparity based on instrumentation. For the purposes of clarity, only OES data is shown with the exception of human samples.

Background

The main focus of this study is to determine if the variations reported for trace element concentrations in human and animal tissues are due to differences in the tissues themselves or due to sample preparation. A common practice is to employ cross laboratory analysis of digested standard reference material (SRM) similar in matrix to your

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https://doi.org/10.1016/j.ab.2017.11.016
Received 25 April 2017; Received in revised form 17 November 2017; Accepted 20 November 2017
Available online 24 November 2017
0003-2697/

samples, but the use of ICP and limited number of certified standard reference materials for a wide range of elements to assess the metal load of mammalian tissues has thus far been limited [14–16]. Published literature has employed numerous heating techniques involving HNO_3, H_2O_2, aqua-regia and HF [10,17,18]. Some procedures suggest that complete digestion can be achieved with HNO_3 alone [7,19,20].

This work seeks to rectify missing information about differences between digestion methods and will show that complete digestions are only achieved using methods similar to digestion II described by Ashoka and colleagues [7], but at increased temperatures. Moreover this study bears to question how much variation in published data on metal loads is due to the sample or the sample preparation technique. As tissue metal research progresses, it is vital to accurately quantify abnormalities in metal loads of pathological tissues [21]. Determination of variability between patients and tissue types is critical and must not be confounded by variations in sample preparation, such as in digestion procedures [9]. Drug discovery, animal biology, forensic science and anthropology all utilize ICP technology for qualitative and quantitative analysis of sample composition making it a befitting technology for standardized metallome analysis.

Method

In this work we specifically focus on Cu, Zn, Ni and Fe and also examine a number of other lesser-studied metals in various tissue types. Early work by Mulay et al. [15] did not show significant differences in the abundance of the metals tested for cancerous and non-cancerous tissues of various types. More recent work concerning mammalian tissue varies widely in digestion protocol, postmortem time and instrument [14,22–24].

Significant improvements in analytical instrumentation allow us to investigate many other metal concentrations at only a few μg/L and lower. Numerous digestion techniques of various foods, sediments, compost as well as animal and human tissues have recently been investigated [7,13,19,20] and generally indicate that a combination of HNO_3 and H_2O_2 heated for 24 h at 75 °C is sufficient to liberate most trace metals (method IV of Ashoka et. Al [7]). We tested and modified these above methods for sample preparation and wet tissue digestion but without the assistance of a microwave oven, thereby simplifying it and making it more accessible to a wide variety of laboratories. Further, we utilized five different digestion techniques and evaluated the completeness of each, both visually and chemically, with use of a NIST (National Institute of Science and Technology) standard.

Analytical conditions on the ICP-OES and ICP-MS are summarized in Table 1. Instruments were tuned according to standard methods whereby the ICP-OES was centered and maximized on Mn at 265 nm and the ICP-MS was optimized for the full mass range using a 1 ppb solution of Mg, In and U. The ICP-MS was also optimized to limit oxide interference using CeO/Ce and double charges using Ba^{++}/Ba to less than 3%. Series standards were developed from single element stock solutions obtained from either High-Purity Standards (Charleston, SC, USA) or from Alfa Aesar (Tewksbury, MA, USA) and diluted accordingly to make a linear series of calibration standards. Pig tissues were run along with certified reference material NIST 1577c, a synthetic matrix matched standard, and analytical blanks which were run every 10 samples to ensure both precision and accuracy as well as to account for any drift and assess carryover between samples. Additional runs included multiple NIST 1577c standards and some runs were entirely comprised of these standards digested in the five methods described.

Table 1
Five comparative methods used to digest 50 mg of wet tissue for ICP-OES analysis.

Method 1	Combined HNO_3 + H_2O_2 digestion for 12 h at 160 °C
Method 2	Multiple acid attack utilizing a combination of HNO_3 and H_2SO_4 160 °C
Method 3	HNO_3 digestion overnight at 85 °C
Method 4	HNO_3 digestion at 175 °C for 4 h followed by addition of H_2O_2 with an additional 2 h heating at 75 °C
Method 5	same as 1 but with 24 instead of 12 h incubation

Digestion of tissues

Organs were freshly harvested from a wild hog/domestic pig hybrid and immediately cooled with dry ice to minimize biochemical degradation in the cell composition from living tissue. The tissue was then stored at −80 °C until sample preparation.

To maximize surface area and homogenize the sample, we used a crushing technique using liquid nitrogen to completely freeze the sample and then crush it into a fine powder using an agate mortar and pestle. Unfortunately, adipose quickly thawed during the crushing process causing the fats to smear rendering the sample unusable. High temperature ashing techniques were explored and quickly abandoned because so much material was lost due to volatilization in an open vessel that only 1–2 mg maximum was usable from a sample of 100 mg or larger. After consultation with the tissue bank at Moffitt Cancer Center (Tampa, FL, USA) we quickly realized that biopsies are typically available in frozen weights of 50 mg or less and therefore sought a suitable alternative. Here we present the most pragmatic methods for wet tissue analysis.

We digested a series of pig tissues in 50 mg samples from pancreas, liver, kidney and lung along with NIST SRM 1577c. For all analyses, following addition of HNO_3, samples were digested for the designated incubation time and were allowed to cool before the addition of the second reagent (either H_2O_2 or H_2SO_4, see Table 1). HNO_3 at high temperature and pressure will effuse from the vessel if improperly cooled, leading to personal safety hazards and loss of material due to volatilization. Finally, after the second incubation period, samples were diluted to 10 mL using highly purified Milli-Q water.

Additionally, since closed instead of open vessels are used as seen in Ref. [22], the amount of material that can be volatilized and lost is reduced maximizing the ability to produce comparative data sets. We opted to cut the sample after minimal thawing from −80 °C using a ceramic knife and an HDPE non-porous cutting board to minimize metal contamination, similar to the procedures of Rahil-Khazen et al. [14]. The wet weight was recorded and the sample was subsequently placed in a PFA digestion vessel (Savillex corporation, 23 mL PFA pressure vessels for blanks and standards and 5 mL for samples), whereupon reagents were added immediately. The vessels were sealed and placed on a hot plate. Trace metal grade HNO_3 was doubly distilled and then used. ACS certified H_2O_2 was used.

Results

Sample homogeneity was initially checked visually. Samples digested with low heat failed to dissolve all sample material due to residue adhering to the side of the vessel even after decanting. Secondary heating with H_2O_2 after high temperature HNO_3 treatment efficiently oxidized lipids resulting in clear solutions free of precipitates. Furthermore, digestions work best when HNO_3 is added and heated prior to the addition of H_2O_2 thereby limiting loss of sample from effervescence and/or over-pressure which may result in catastrophic failure of the pressure vessel.

NIST 1577c was used as a standard reference material in order to test the aforementioned methods. As outlined previously, the metals analyzed were chosen due to their metabolic importance.

Additional tests incorporating HF, depicted below, adequately dissolved standards but failed to breakdown cancerous breast tissue. These images are notable for the prominent lipid micellation in the form of black precipitates even after addition of HF and heating. HF was therefore avoided due to the volatile and dangerous nature of this acid and other means were sought to digest fatty tissues. It was found that