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Journal:	*Journal of Analytical Atomic Spectrometry*
Manuscript ID:	JA-ART-02-2014-000049.R1
Article Type:	Paper
Date Submitted by the Author:	02-Apr-2014
Complete List of Authors:	Praamsma, Meredith; University at Albany, Dept of Environmental Health Sciences Parsons, Patrick; New York State Dept of Health, Lab of Inorganic and Nuclear Chem; University at Albany, Environmental Health Sciences

Characterization of calcified reference materials for assessing the reliability of manganese determinations in teeth and bone

Meredith L. Praamsma[ab] and Patrick J. Parsons[*ab]

[a] *Laboratory of Inorganic and Nuclear Chemistry, Wadsworth Center, New York State Department of Health, P.O. Box 509, Albany, NY 12201-0509, USA.*

[b] *Department of Environmental Health Sciences, School of Public Health, The University at Albany, P.O. Box 509, Albany, NY 12201-0509, USA*

E-mail: pparsons@wadsworth.org

Phone: 518-474-5475

Fax: 518-473-2895

Abstract

As interest increases in human biomonitoring for exposure to manganese (Mn) *via* analysis of bone and teeth, there is a growing need for calcified reference materials (RMs) certified for Mn content. Here, several existing calcified RMs and synthesized hydroxyapatite (HA) RMs were digested with acid and subsequently analyzed for Mn by graphite furnace atomic absorption spectrometry (GFAAS) and dynamic reaction cell inductively coupled plasma mass spectrometry (DRC-ICP-MS). Based on GFAAS and DRC-ICP-MS results, mean values and expanded uncertainties were assigned to four New York State (NYS) Bone RMs (05-01 through 05-04), and six synthesized HA standards. Consensus values and expanded uncertainties for Mn content were assigned to NIST SRM 1400 Bone Ash (16.5 ±2.3 μg g^{-1}) and NIST SRM 1486 Bone Meal (1.1 ±0.5 μg g^{-1}) by combining GFAAS and DRC-ICP-MS results with literature values. These powdered RMs were also prepared for solid sampling techniques by pressing them into pellets for subsequent analysis by laser ablation (LA-) ICP-MS. The "dilution" effect from adding a chemical binder to the pellets was quantified by both GFAAS and DRC-ICP-MS, and the decrease in Mn mass fraction was found to be proportional to the amount of binder added. The distribution of Mn across the RM pellets was determined by LA-ICP-MS to assess homogeneity and suitability for use as solid standards. The bone RMs were more heterogeneous than the synthesized HA RMs, but the latter may be a less optimal matrix-match due to the absence of organic material.

Introduction

Due to its potential to cause neurotoxic effects,[1] monitoring human exposure to manganese (Mn) has typically been performed by analyzing biological fluids such as blood and urine. However, there has been recent interest in using calcified matrices to monitor Mn exposure. For example, *in vivo* bone Mn measurements have been performed to assess welding fume exposure[2] and *ex vivo* tooth Mn measurements have been used to monitor time-related exposures, particularly *in utero* and during early childhood.[3-5] Tooth dentin and enamel grow in layers, so particular areas within the tooth represent specific points in time. These areas may be analyzed by techniques such as laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS), which is able to perform spatial elemental analysis. Prenatal and postnatal Mn exposures have been studied in deciduous teeth using LA-ICP-MS to measure

the Mn content.[3, 4, 6] However, assessing the accuracy of LA-ICP-MS methods for quantifying Mn in teeth and bones is a challenge due to the absence of matrix-matched reference materials (RM) that are certified for Mn content. The inorganic mineral matrix of teeth is composed of calcium hydroxyapatite (hereafter referred to just as HA) with the general chemical formula $Ca_{10-x}(H)_x(PO_4)_6(OH)_{2-x}$ with 1-4% of the tooth being CO_3^{2-}.[7] Additionally, trace elements such as Cl, F, Mg, and Na are present within the HA structure.[8] It is desirable for RMs used in tooth analyses to have a similar HA chemical structure. Bone is chemically and structurally similar to teeth in that it is also composed of HA,[8] so it could possibly be used as a substitute for a tooth RM, of which none exist.

The National Institute of Standards and Technology (NIST, Gaithersburg, MD) supplies two bone Standard Reference Materials® (SRM): SRM 1400 Bone Ash and SRM 1486 Bone Meal. These SRMs are readily available, but the certificate of analysis provides only non-certified values for Mn. This means that there is no stated level of confidence in the value provided on the certificate, which is a disadvantage when assessing method accuracy. Another alternative to using a tooth RM is producing synthesized HA supplemented with Mn. Indeed, there are several ways to prepare HA with common chemicals and laboratory equipment.[9]

The principal aim of the current study was to assign robust values for Mn to several existing bone RMs and six new HA RMs. The assigned values could then be used for quantifying the Mn content of tooth and bone samples by LA-ICP-MS. For the two existing NIST bone SRMs, 1400 and 1486, Mn data have been reported in previous studies; therefore, the data collected in this study were combined with those from the literature to assign a consensus value and associated uncertainty for Mn in these materials. The New York State (NYS) bone RMs 05-01 through 05-04 were produced originally for the determination of Pb in bone and consist of two bovine (cow) and two caprine (goat)-based materials.[10] Certified reference values and expanded uncertainties for Pb were assigned to each NYS RM, but not for other elements.[11] These materials were also circulated to 30 participants in a previous interlaboratory study of Pb in bone.[11] Informational values for other trace elements (including Mn) were obtained from the interlaboratory study of these NYS RMs. The interlaboratory data for Mn are reported here for comparison to an assigned value established as part of the current study. Six new synthesized HA standards supplemented with Mn were also produced for this study, and the Mn content was characterized after

production to allow for a multi-point calibration curve to be used in LA-ICP-MS techniques. The powdered bone and synthesized HA materials were analyzed for Mn using (a) graphite furnace atomic absorption spectrometry (GFAAS) and (b) dynamic reaction cell (DRC-) ICP-MS.

A second aim of the current study was to characterize the powdered bone and synthesized HA materials with specific regard to LA-ICP-MS. For LA-ICP-MS analysis, a powdered material is typically pressed into a pellet and a chemical binder may be added to make it more robust. Pressing the powder into a disc provides a flat surface to analyze, which is ideal for LA-ICP-MS. However, element concentrations could vary across the surface of the pellet depending on the homogeneity of the powdered material. Thus, the distribution of Mn and Ca, the element often used to normalize analyte counts in calcified matrices, was assessed by ablating the material in different areas of the pellet surface. Also, the effect on Mn concentration from adding binder to the powders was quantified, as binder incorporates added mass to the powdered materials. This investigation was carried out by digestion of the pressed pellet followed by solution-based analyses for Mn by GFAAS and DRC-ICP-MS.

Experimental

Reagents and reference materials

Doubly de-ionized (DI) water (>18 MΩ cm) produced from a NANOpure DIamond™ (Barnstead, Waltham, MA) unit was used for all analytical work along with concentrated HNO_3 distilled in-house with a DuoPUR sub-boiling acid still (Milestone, Shelton, CT). Additionally, 30 wt% Baker Analyzed® A.C.S. Reagent Grade H_2O_2 was used in digestions (J.T. Baker, Phillipsburg, NJ). A 1000 mg L^{-1} PE Pure Mn stock standard traceable to NIST SRM 3132 Manganese Standard Solution was used for calibration. The modifier used in GFAAS analysis was 0.015% (w/v) $Mg(NO_3)_2 \bullet 6H_2O$ (99.999% metals basis, GFS Chemicals® Inc., Columbus, OH), 0.1% (v/v) Triton® X-100 (Sigma Ultra Grade, Sigma-Aldrich®, St. Louis, MO), and 0.2% (v/v) HNO_3. The DRC-ICP-MS diluent contained 0.005% (v/v) Triton® X-100, 0.5% (v/v) HNO_3 and 5 µg L^{-1} Ga (GFS Chemicals® Inc.).

The RMs used in this study included: (a) NIST SRM 612 Trace Elements in Glass (b) NIST SRM 1400 Bone Ash, (c) NIST SRM 1486 Bone Meal, (d) NYS RM 05-01, 05-02, 05-03, 05-04, and (e) synthesized HA. NYS RM 05-01 and 05-02 are powdered bovine bones, while 05-03 and 05-04 are

powdered caprine bones. SRM 1400 and SRM 1486 are provided in glass bottles containing ~50 g of material, while the NYS RMs are provided in plastic vials containing ~5 g of material. HA was synthesized according to a protocol described by Ugarte *et al*.,[12] whereby ~25 mL of 1 mol L^{-1} $Ca(NO_3)_2 \bullet 4H_2O$ (99.999%, GFS Chemicals® Inc.) is added dropwise to ~25 mL of 0.48 mol L^{-1} $NH_4H_2PO_4$ (TraceSELECT ≥99.9999%, Sigma Aldrich), after adjustment of both solutions to pH 10 with concentrated NH_4OH (Veritas Redistilled, GFS Chemicals® Inc.). An aqueous Mn stock standard was used to supplement the $NH_4H_2PO_4$ solution in various amounts to obtain six HA standards of Mn mass fractions at 0, 1.0, 2.0, 5.3, 10.6, and 45.5 μg g^{-1}.

Instrumentation and calibration

A. Graphite furnace atomic absorption spectrometry

A PerkinElmer® (PE, Shelton, CT) AAnalyst™ 800 GFAAS equipped with a THGA graphite furnace and longitudinal Zeeman background correction was used to determine Mn at the 279.5-nm line. The autosampler for sample deposition was a Model AS-800. The instrument was operated with AA WinLab32™ software from PE. The method used was a modified version of a previous method developed for the determination of Mn in blood and validated for a PE Model 4100ZL THGA platform. The atomization temperature was optimized for calcified matrices (1900 °C instead of 1950 °C).[13] Mn working standards were diluted from a 1.0-mg L^{-1} intermediate solution to obtain 0, 5, 10, 20, 50, and 100 μg L^{-1}. Different calibration strategies were compared: (a) simple aqueous standards (b) standards that also contained a digested calcified material *e.g.*, bone, teeth or synthesized HA, to achieve matrix matching and (c) standard additions (limited to SRM 1400). For approach (b), each calibrator contained 100 μL of the working standard, 100 μL of a matrix digest, and 800 μL of modifier. For approach (c), the curve included a sample blank and three additional sample aliquots spiked with 10, 20, and 50 μg L^{-1} of Mn. A procedural (digestion) blank was subtracted from all standards and samples (1.0 μg L^{-1} on average).

B. Inductively coupled plasma mass spectrometry

A PE ELAN® DRC™ II ICP-MS was used with the ELAN software (version 3.3). The DRCII was equipped with a PE quartz cyclonic spray chamber (P/N WE025221) and a Meinhard® glass concentric nebulizer (Golden, CO). Data were collected in the DRC mode with NH_3 gas flowing at 0.45 mL min^{-1} and with an RP*q* of 0.7. The DRC-ICP-MS method was previously described and validated for the determination of Mn in urine.[13] Mn working standards were diluted from a 1.0-mg L^{-1} intermediate solution to produce 0, 2.5, 5, 10, 25, and 50 µg L^{-1} (plus a 100 µg L^{-1} standard when needed). Calibration strategies compared were (a) simple aqueous standards and (b) matrix-matched standards containing a digested calcified material. Each DRC-ICP-MS matrix-matched calibration standard contained 250 µL of working standard, 250 µL of a matrix digest, and 4500 µL of diluent. A procedural blank was subtracted from all standards and samples (0.2 µg L^{-1} on average).

C. Laser ablation inductively coupled plasma mass spectrometry

A New Wave™ Research UP-213 Laser Ablation System (Fremont, CA) with MEO Laser Ablation System Software (version 1.14.3.0) was used to characterize the homogeneity of Mn and Ca in pressed pellets of the bone and synthesized HA RMs. This system emits a laser beam at a wavelength of 213 nm from a Nd:YAG solid-state source. Helium gas was swept through the sample chamber and Ar, as a make-up gas, was introduced with a y-connector after the sample chamber. The LA system was coupled to the PE ELAN DRCII ICP-MS and Mn monitored at *m/z* 55 and Ca at *m/z* 43. As with solution analyses, DRC mode was used with a NH_3 gas flow rate of 0.45 mL min^{-1} and an RP*q* of 0.7. Counts from line scans 1000 µm in length were obtained at a 20-µm s^{-1} scan rate, 55-µm spot size, 20 Hz repetition rate, and at 100% laser power. Given the small amount of Mn in these samples, a high repetition rate and 100% laser power were necessary to maximize the Mn signal.

Sample digestion and preparation

Powdered samples (100-200 mg) of the six bone RMs and six synthesized HA standards were dried for two hours at 105 °C in an oven and then weighed (0.0001 g balance readability). Approximately 100 mg was weighed for synthesized HA and 200 mg for the powdered bone materials. To each sample, 2 mL of concentrated HNO_3 were added and left to digest at room temperature for 24 hours. Then, 1 mL of 30 wt% H_2O_2 was added and left at room temperature for another 24 hours. The final step in the

digestion protocol was microwave-assisted heating at atmospheric pressure with a Microwave Accelerated Reaction System 5® (MARS 5®, CEM Corporation, Matthews, NC). Modified 15-mL polypropylene conical tubes (Becton Dickinson, Franklin Lakes, NJ) with holes drilled into the caps for venting were used as the sample vessels. The MARS 5® was ramped to 83 °C over 15 minutes, and this temperature was held for 60 minutes. A similar open-vessel digestion procedure was previously validated for trace element analysis of biological tissues and has been used with good success.[14] The addition of 2 mL HNO_3 was enough to digest the synthesized HA, but all three steps were required to digest the powdered bone materials. After microwave digestion, the digestates were diluted with DI water in glass Class A volumetric flasks (10 mL for five synthesized HA standards 0-4, four NYS RMs, and SRM 1486; 50 mL for SRM 1400 and synthesized HA standard 5). In preparation for GFAAS analysis, 100 µL of sample was diluted with 900 µL of modifier. In preparation for DRC-ICP-MS analysis, 250 µL of sample was diluted with 250 µL of DI water and 4500 µL of diluent.

Pellet preparation

Powders for analysis by LA-ICP-MS were pressed into pellets to provide an even, compacted surface. Approximately 750 mg of powdered RM sample was used for each pellet. Chemplex® Liquid-Binder™ Grinding/Binding Additive (Palm City, FL) was added to each powdered sample in a volume of 0.75 mL for the HA standards and 0.5 mL for the NYS RMs, SRM 1400, and SRM 1486. The liquid binder was combined with the powder using an agate mortar and pestle. The mixture of binder and powder was placed between two stainless steel 13-mm pellet dies and pressed for 1 minute under 6 tons of pressure with a Carver® 4350 Manual Pellet Press (Wabash, IN).

Chemplex® Liquid-Binder™ Grinding/Binding Additive is a polymer with general chemical formula $(C_6H_9ON)_n$ which is dissolved in CH_2Cl_2 solvent. With addition of a binder, pelletized material is able to endure handling and use without breakage. However, 1 mL of binder adds ~100 mg of mass to the pelletized material. Theoretically, adding 0.75 mL of liquid binder to 0.75 g of powdered sample would be expected to decrease the Mn mass fraction by ~10% for the HA standards, while 0.5 mL of binder would decrease the Mn mass fraction of the NYS RMs, SRM 1400, and SRM 1486 by ~7%. The decrease in Mn mass fraction was verified experimentally by analyzing raw powder without binder as described above under “Sample digestion and preparation” and pressed pellets with binder by GFAAS and DRC-ICP-MS

following acid digestion. Pressed pellets prepared with binder were dried in an oven for two hours at 105 °C and then divided into four pieces that were weighed and digested as described above. This procedure was followed for synthesized HA standards 1, 2, 4, and 5, and SRM 1486. The pressed pellet digestates were diluted in modifier/diluent as outlined above and then analyzed for Mn using both GFAAS and DRC-ICP-MS.

Calculations and statistics

The expanded uncertainty (U) was calculated by multiplying the standard uncertainty (u) by a coverage factor (k) according to the following equation:

$$U = ku = k\sqrt{SD^2/n}$$

where k is calculated as the two-tailed Student's t-statistic for a 95% confidence interval for $n-1$ degrees of freedom and n is the number of measurements. Statistical calculations were performed with Prism® (version 6.0a, GraphPad Software, Inc., La Jolla, CA). LA-ICP-MS data were reduced and integrated using a non-commercial freeware package (Iolite, version 2.12, School of Earth Sciences, University of Melbourne, Melbourne, Australia) run through Igor Pro (version 6.21, WaveMetrics, Inc., Lake Oswego, OR). Mean values from the line scans were calculated without the rejection of any outliers. Method detection limits (MDL) defined as 3SD, and limits of quantitation (LOQ) defined as 10SD, were calculated as described below.

Results and Discussion

Method performance

A. Graphite furnace atomic absorption spectrometry

Both matrix-matched and aqueous calibration strategies were evaluated for the determination of Mn in powdered bone materials by GFAAS. The calibration line R^2 values were all >0.997. Comparing matrix-matched and aqueous calibration lines, the average ratio of the two slopes was 1.02 ±0.11 (n = 12), which was not a statistically significant difference (p = 0.6630, unpaired t-test), and which suggests the absence of any major matrix effect. The MDL and LOQ for Mn were calculated based on ten between-day Mn results from a HA matrix and were 0.3 μg g^{-1} and 0.8 μg g^{-1}, respectively. Method repeatability

(%RSD, n = 10 days) was 7% at 1.3 μg g^{-1} Mn in a bone matrix. The average characteristic mass (m_0) was 3.5 ±0.1 pg for matrix-matched calibration and 3.6 ±0.2 pg for aqueous calibration (n = 12).

B. Dynamic reaction cell inductively coupled plasma mass spectrometry

The calibration line R^2 values were >0.999 and the average ratio of matrix-matched and aqueous calibration line slopes was 1.04 ±0.06 (n = 4). The difference between these two calibration strategies was not statistically significant (p = 0.6571, Mann-Whitney test), again suggesting no major matrix effect. The MDL for Mn determined in digested powdered samples was 0.1 μg g^{-1} by DRC-ICP-MS and the LOQ was 0.5 μg g^{-1}, based on ten within-day results for a base HA material. The MDL and LOQ for Mn by LA-DRC-ICP-MS were calculated based on the gas blank and were 0.1 μg g^{-1} and 0.3 μg g^{-1}, respectively.

Assigned values for Mn in calcified materials

A. NIST SRM 1400 Bone Ash

NIST SRM 1400 Bone Ash powder was digested in quadruplicate. Upon digestion in concentrated HNO_3 fine gray particulates were observed in the digestate indicative of incomplete digestion for silicate. Although the NIST certificate recommends that SRM 1400 be dissolved with HF, HNO_3, and $HClO_4$,[15] here only concentrated HNO_3 was used to avoid exposure to more hazardous HF and $HClO_4$. The four digestates were analyzed for Mn on three days by GFAAS and two days by DRC-ICP-MS using a matrix-matched calibration curve. Analysis by standard additions was also performed with GFAAS on a single day for each of the four digested aliquots. These data were combined to yield a single value of 15.5 ±1.4 μg g^{-1} for NYS. These results are presented in Table 1 (a) along with values for Mn from the literature for comparison (b).

The literature values reflect a range of analytical methods that includes: GFAAS, ICP- optical emission spectrometry (OES), instrumental neutron activation analysis (INAA) and NAA using k_0-factors. All of these studies report values that range from 16.1 to 17.3 μg g^{-1}, and agree to within 7%. The data for Mn in SRM 1400 reported in our study and based on GFAAS was found to be in good agreement with the range of values reported by others. By contrast, the result obtained here by DRC-ICP-MS was found to exhibit a negative bias of 14% relative to the consensus value (Table 1 (c)), suggesting perhaps there are uncorrected matrix effects from bone ash despite using the matrix-matched calibration standards. Two other studies also reported variation in SRM 1400 Mn concentrations depending on analytical conditions.

Using GFAAS, Acar *et al*.[16] found a Mn concentration of 15.1 ±0.8 μg g^{-1} with a La modifier compared to 16.7 ±0.4 μg g^{-1} with a La/Pd/citrate modifier. With ICP-OES, Borkowska-Burnecka *et al*.[17] found a Mn concentration of 10.4 ±0.4 μg g^{-1} without cloud point extraction sample preparation compared to 16.9 ±1.4 μg g^{-1} with cloud point extraction. The cloud point extraction served to separate Ca from the analytes of interest. It is conceivable that the matrix effects reported here for Mn are also related to the calcium-rich matrix, and use of matrix-matching fails to overcome them completely when using DRC-ICP-MS.

A consensus reference value for Mn content in NIST SRM 1400 Bone Ash was calculated by combining the un-weighted mean of the NYS data plus those results reported in the four literature studies, where a statement of uncertainty was reported (Table 1 (c)). The NIST informational value was not included in this calculation as there is no statement of uncertainty provided on the certificate of analysis. Expanded uncertainty was calculated by taking the square root of the sum of the u^2 of the NYS GFAAS mean and each literature study. The consensus value is 16.5 ±2.3 μg g^{-1}, giving a range of 14.2-18.8 μg g^{-1} as the 95% confidence interval. This range is large with 14% relative *U*, showing just how difficult the determination of Mn in calcified matrices is, but the use of at least three different instrumental techniques lends credibility to this range established for the Mn content in SRM 1400.

B. NIST 1486 Bone Meal

NIST SRM 1486 Bone Meal was digested in quadruplicate. As with SRM 1400, the dissolution approach recommended by NIST for SRM 1486 is a combination of HF, HNO_3, and $HClO_4$.[18] Using the less hazardous digestion procedure described previously, fat deposits from SRM 1486 remained on the polypropylene tube walls after completion, but these are not considered significant for trace element analysis. Each aliquot was analyzed for Mn on two days by GFAAS and on two days by DRC-ICP-MS. The data are shown in Table 2 (a). The GFAAS and DRC-ICP-MS results are 11% different. The mean values for Mn in SRM 1486 obtained by GFAAS and DRC-ICP-MS were combined to yield an Mn value of 1.2 ±0.1 μg g^{-1}, which is consistent with other values reported in the literature (Table 2 (b)).

As with SRM 1400, a variety of analytical methods were used to produce the Mn data for SRM 1486 reported in the literature including: flame (F-) AAS, ICP-OES, INAA, and LA-ICP-MS. With SRM 1486 having a lower concentration of Mn, greater “relative” variability of 32% is reported in the literature, with Mn values from 0.96 to 1.32 μg g^{-1}. A consensus value of 1.1 ±0.5 μg g^{-1} was calculated by

combining the NYS mean value with values from studies that reported sufficient data to estimate *u* (Table 2 (c)). The expanded uncertainty is defined as the square root of the sum of the u^2 of the NYS mean and five literature studies.[12, 19-22] The 95% confidence interval is 0.6-1.6 μg g^{-1} and all of the mean values in Table 2 overlap with this range. The all-method mean *U* is 45% for SRM 1486, which is triple that of SRM 1400. Inflation of *U* is to be expected at this lower Mn concentration, but should be improved for better assessments of method accuracy at such low levels.

C. NYS Bone Reference Materials

Two aliquots from a single vial of each NYS bovine and caprine RM were digested and analyzed for Mn content by GFAAS and DRC-ICP-MS. One aliquot was analyzed by GFAAS over four days and the other over two days, while additional aliquots were analyzed by DRC-ICP-MS over one day and three days. Results from these analyses for Mn in the NYS RMs are shown in Table 3 (a). Hetter *et al.*[10] previously reported Mn mass fractions of 1.9, 2.1, and 2.2 μg g^{-1} for NYS RM 05-01 analyzed by ICP-MS operated in standard mode. Those prior data are consistent with values obtained in the current study, but there are no other results in the literature with which to compare. However, as a part of the Pb interlaboratory study conducted by NYS with the NYS RM materials, three laboratories reported Mn concentrations in addition to Pb. The results from these laboratories using ICP-MS, ICP-OES, and high resolution (HR-) ICP-MS are shown in Table 3 (b) for comparison. A mean value was assigned to each RM based on combining data from GFAAS and DRC-ICP-MS (Table 3 (a)), but data from the other three laboratories were not included since their reported results only contained limited information on analytical conditions. As can be seen in Table 3 (a) and (b), NYS RM 05-01 and 05-02 contain slightly higher Mn mass fractions than NYS RM 05-03 and 05-04, possibly reflecting the differences between the bovine and caprine sources. Previously, a between-vial homogeneity assessment was completed for Pb,[10] but homogeneity for Mn was not included. In the current study, only one other aliquot from a second vial of NYS RM 05-01 was digested and the Mn mass fraction was 1.8 μg g^{-1}. Although additional analyses from multiple vials would increase the robustness of the assigned value for Mn in the NYS RMs, the data presented here give an initial estimate as to their Mn content.

D. Synthesized hydroxyapatite standards

Three aliquots of each synthesized HA standard were digested and analyzed for Mn. The GFAAS data shown in Table 4 represent two days of analysis for the three digestates of each HA standard level, as do the data from DRC-ICP-MS. The mean Mn mass fraction is the un-weighted mean of the 12 results from the two techniques. The exceptions are standards 0 and 3, in which the digestates were analyzed on two days by GFAAS but only one day by DRC-ICP-MS (n = 9). The expanded uncertainty is <15% relative for each standard except for standard 0 where the mass fraction is close to the MDL. The Mn values for the HA standards are weighted toward the low μg g^{-1} since mass fractions below 10 μg g^{-1} are typical in human bones and teeth. However, some authors have reported higher values for Mn,[20] so the highest standard was synthesized at 45.2 μg g^{-1}. In comparing GFAAS to DRC-ICP-MS results, it is seen that at higher Mn concentrations, DRC-ICP-MS values are lower compared to GFAAS, which is consistent with SRM 1400 results.

The synthesis protocol used in this study typically yielded 1.8 g of HA material for each standard. This amount is enough to produce a pressed pellet and characterize the Mn content. On average, 101 ±10% (Table 4) of the expected Mn content was recovered in each standard, indicating that Mn is efficiently incorporated into the HA matrix. Standards 0 through 3 were white in color, but standards 4 and 5 appeared light blue. Paluskiewicz *et al*.[23] showed that Mn could be incorporated at levels up to 10,000 μg g^{-1} with the mineral phase remaining HA. Since the highest standard produced here was 45.2 μg g^{-1}, all standards were assumed to be HA despite color changes that are likely due to the formation of the +5 oxidation state of Mn.[23] The base (no Mn added) synthesized powder was analyzed by X-ray diffraction to confirm that its identity was HA.

Characterization of Mn in pellets pressed with binder

The Mn mass fractions found in the HA raw powder (without binder) and the pressed HA pellet (with binder) using (a) GFAAS and (b) DRC-ICP-MS are given in Table 5. Each value is the mean Mn mass fraction (±U) from four digestates analyzed on a single day. The binder “dilution” factor was calculated as the difference between Mn content of the pellet pressed with binder and the Mn content of the powder without binder, expressed as a percentage. The mean binder dilution factor was −12% (range: −4 to −16%) based on GFAAS and is close to the predicted value of −10%. By contrast, data obtained by

DRC-ICP-MS has a mean of −15% (range: −10 to −19%) suggesting a larger negative bias. Since the same digestates were analyzed by both GFAAS and DRC-ICP-MS, the larger bias of the latter must be assigned to instrumental sources.

A similar study was completed for SRM 1486 in which raw powder without binder and the pressed pellet with binder were prepared and analyzed by GFAAS (3 days) and DRC-ICP-MS (2 days). The mean Mn mass fraction of the raw powder (±*U*) was 1.28 (0.07) μg g^{-1} by GFAAS and 1.18 (0.12) μg g^{-1} by DRC-ICP-MS. The mean Mn mass fraction of the pressed pellet with binder (±*U*) was 1.19 (0.03) μg g^{-1} by GFAAS and 1.14 (0.05) μg g^{-1} by DRC-ICP-MS. The difference between the Mn in raw powder compared to pellet pressed with binder was 7% less by GFAAS and 3% less by DRC-ICP-MS in the latter. For the GFAAS data, the difference between the Mn content in the raw powder versus the pellet pressed with binder was statistically significant (p = 0.0267, Mann-Whitney test), but for the DRC-ICP-MS data, the difference did not reach significance (p = 0.5235, Mann-Whitney test). Based on the GFAAS data, adding binder to the pellet has a small but statistically significant dilution effect compared to unpelletized SRM 1486 Bone Meal and this is consistent with a predicted factor of 7%.

Analysis of calcified reference materials by LA-ICP-MS

The uniformity of Mn distribution was assessed within each bulk HA material by digesting multiple aliquots and analyzing them for Mn by DRC-ICP-MS under conditions of repeatability, to obtain the within-run precision. Also, multiple LA-ICP-MS line scans (n = 5) were taken on the surface of each RM pellet to assess Mn distribution after pressing. The location of the 5 line scans (A through E) for each of the five HA standards is shown in Figure 1. NIST SRM 612 Trace Elements in Glass is also shown in Figure 1 as a reference, since it is relatively homogenous. The NIST certificate of analysis for SRM 612 states that the target level of precision was better than 5%.[24] The plotted intensities of ^{55}Mn and ^{43}Ca for each line scan (A through E) are displayed in Figure 1 with a semi-log scale. The laser is firing for 50 seconds at a time when the “steady-state” signal is visible (A through E) and the laser is off for ~60 seconds at a time when the signal is returning to baseline.

The mean intensity and SD were calculated for the five line scans for each ablated material, which builds on data presented previously.[12] Ugarte *et al.*[12] evaluated the reproducibility of the Mn LA signal in their synthesized HA standards by ablating five 500-μm lines around the pressed pellet. Their

reported RSDs for $^{55}Mn/^{43}Ca$ were 9, 10, and 2% for standards containing 3, 11, and 29 μg g^{-1} of Mn, respectively. Here we report the %RSD data for the HA standards, and also for each of the bone RMs analyzed by LA-ICP-MS. Table 6 provides %RSD data based on LA-ICP-MS analyses for (a) ^{55}Mn counts for solid samples (b) $^{55}Mn/^{43}Ca$ count ratios for solid samples, and (c) DRC-ICP-MS analyses of digested bulk powdered bone samples for comparison. For the latter, RSDs range from 5 to 9%, except for where close to the MDL, *i.e.*, standard 0. The %RSD data based on using ^{55}Mn counts are generally greater than solution-based data. Taking the $^{55}Mn/^{43}Ca$ ratio improves the %RSD for the LA-ICP-MS analysis of HA standards. The LA-ICP-MS HA standard line scan RSDs range from 2 to 5%, in agreement with data reported by Ugarte *et al*.[12], except for standard 0, which is close to the MDL.

The precision of the LA data for bone RMs did not always improve upon taking the $^{55}Mn/^{43}Ca$ ratio. For SRM 1486 the RSD increases from 3 to 20% based on the $^{55}Mn/^{43}Ca$ ratio, which is due to line scan A (Figure 1). In line scan A, the Ca signal was only 225,000 cps whereas in line scans B through E it exceeded 320,000-360,000 cps, yet the mean Mn signal was consistently 2,400 in all five line scans. Excluding the data from line scan A reduces the RSD to 2% based on the $^{55}Mn/^{43}Ca$ ratio. It is important to note that the particle size given for SRM 1486 is up to 355 μm[18] and it contains fat and organic material that might make ablation inconsistent.

The laser RSD found for Mn in the NYS RMs suggests a lack of homogeneity. It is quite a typical occurrence for the Mn signal from the NYS RMs to spike from a few hundred counts to several thousands of counts. For NYS RM 05-01 and 05-02, there was one line scan in particular that caused the data to become imprecise, but even with removal of this one scan, the $^{55}Mn/^{43}Ca$ RSDs are 23 and 10%, respectively. The greater RSD found for NYS RM 05-01 and 05-02 may indicate that Mn content in bovine bones is less homogeneous than in caprine bones (NYS RM 05-03 and 05-04). A typical RSD for the NYS RMs without any large “contamination” spikes would be 20%. Suspected Mn contamination was reported from the grinding process during the RM preparation procedures,[10] which makes them less desirable for LA work for Mn. Out of all the bone RMs analyzed by LA-ICP-MS, SRM 1400 Bone Ash was found to produce the most precise and consistent data for Mn. The size distribution data for SRM 1400 particles are not reported,[15] but it is dry-ashed bone with very little organic material, which might improve the consistency of its ablation.

Conclusions

There is an urgent need for calcified matrix-based RMs, certified for Mn as tooth and bone samples are becoming increasingly important for Mn biomonitoring. In the absence of certified RMs, mass fractions and expanded uncertainties for Mn were calculated based on consensus values that included data from this study (with digestion and subsequent analysis by GFAAS and DRC-ICP-MS) and data reported in the literature for SRM 1400 (16.5 ±2.3 μg g^{-1}) and SRM 1486 (1.1 ±0.5 μg g^{-1}). Additionally, four NYS RMs (05-01: 1.9 ±0.2 μg g^{-1}, 05-02: 2.0 ±0.2 μg g^{-1}, 05-03: 1.5 ±0.2 μg g^{-1}, 05-04: 1.4 ±0.2 μg g^{-1}) and six synthesized HA standards were characterized for Mn content based on data from the current study. Although the assigned values were based on analysis of bulk powder material, these RMs have the potential to be used as standards for solid sampling techniques when pressed into pellets. Characterizing the Mn distribution across the pressed pellets using LA-ICP-MS showed that Mn in synthesized HA standards was more homogeneously distributed than Mn in bone RMs. This would make the HA standards supplemented with Mn more desirable for solid sampling techniques such as LA-ICP-MS. However, the synthesized HA may be a less accurate matrix-match to tooth and bone since it does not contain the organic component; therefore, the bone RMs may be better for validating solid sampling methods for calcified biological matrices. Further studies are needed to determine if bone and synthesized HA materials are fit for purpose as Mn standards in solid sampling analysis of teeth and bone.

Acknowledgement

The authors would like to thank Dr. Alexander Filatov, the Director of the X-ray Diffraction Center at the University at Albany, for conducting the X-ray diffraction analysis

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Table 1 Mn determination in NIST SRM 1400 Bone Ash – data from the literature and the current study

(a) Current study

Method	Sample preparation	Calibration mode	Mn (μg g^{-1})	*U* (μg g^{-1})	*U* (%)	*n*
DRC-ICP-MS	Digested	Matrix-matched	14.2	1.0	7	8
GFAAS	Digested	Matrix-matched	15.5	0.6	4	12
GFAAS	Digested	Standard additions	16.7	1.3	8	4
NYS mean value			15.5	1.4	9	24

(b) Literature

Method	Sample preparation	Calibration mode	Mn (μg g^{-1})	SD (μg g^{-1})	RSD (%)	*n*
GFAAS[16]	Digested	Aqueous	16.7	0.4[a]	2[a]	7
ICP-OES[17]	Digested	Aqueous	16.9	1.4	8	3
INAA[25]	Polyethylene bag	Cadmium ratio technique	16.1	0.8	5	6
INAA[22]	Polyethylene bag	Aqueous on filter paper	17.3	0.7	4	5
none specified[15]	None specified	None specified	17	--	--	--

(c) Consensus value

	Mn (μg g^{-1})	*U* (μg g^{-1})	*U* (%)	*n*
All method mean value	**16.5**	**2.3**	**14**	**45**

[a] reported as the expanded uncertainty, with %*U*

Table 2 Mn determination in NIST SRM 1486 Bone Meal – data from the literature and the current study

(a) Current study

Method	Sample preparation	Calibration mode	Mn (μg g^{-1})	*U* (μg g^{-1})	*U* (%)	*n*
GFAAS	Digested	Matrix-matched	1.3	0.1	7	8
DRC-ICP-MS	Digested	Matrix-matched	1.2	0.1	10	8
NYS mean value			1.2	0.1	11	16

(b) Literature values

Method	Sample preparation	Calibration mode	Mn (μg g^{-1})	SD (μg g^{-1})	RSD (%)	*n*
LA-ICP-MS[12]	Pressed into pellets	HA pellets	1.2	0.2[a]	16[a]	5
ICP-OES[19]	Digested	Aqueous	0.96	0.06	6	3
INAA[20]	Polyethylene bag	Aqueous on filter paper	1.08	0.12	11	4
ICP-OES[21]	Digested	Aqueous	1.02	0.24	24	9
INAA[26]	Polyethylene bag	Aqueous on filter paper	1.01	0.10	10	--
FAAS[27]	Digested	None specified	0.98	--	--	6
FAAS[28]	Digested	Aqueous	1.32	0.36	27	--
INAA[22]	Polyethylene bag	Aqueous on filter paper	1.01	0.11	11	6
None specified[18]	None specified	None specified	1	--	--	--

(c) Consensus value

	Mn (μg g^{-1})	*U* (μg g^{-1})	*U* (%)	*n*
All method mean value	**1.1**	**0.5**	**45**	**49**

[a] reported as the expanded uncertainty, with %*U*

Table 3 Assigned value and associated *U* for Mn in NYS bovine and caprine bone RMs

(a) NYS data

Method	Mn (μg g^{-1})	*U* (μg g^{-1})	*U* (%)	*n*
NYS RM 05-01				
GFAAS	1.8	0.2	13	6
DRC-ICP-MS	1.9	0.2	8	4
Mean	1.9	0.2	13	10
NYS RM 05-02				
GFAAS	2.0	0.2	10	6
DRC-ICP-MS	2.0	0.2	9	4
Mean	2.0	0.2	11	10
NYS RM 05-03				
GFAAS	1.5	0.1	9	6
DRC-ICP-MS	1.4	0.3	18	4
Mean	1.5	0.2	15	10
NYS RM 05-04				
GFAAS	1.4	0.2	12	6
DRC-ICP-MS	1.4	0.2	12	4
Mean	1.4	0.2	13	10

(b) Interlaboratory data[a]

Method	Mn (μg g^{-1})	SD (μg g^{-1})	RSD (%)
NYS RM 05-01			
ICP-MS	2.4	--	--
ICP-OES	1.8	0.1	6
HR-ICP-MS	2.2	0.1	4
NYS RM 05-02			
ICP-MS	2.0	--	--
ICP-OES	2.3	0.2	7
HR-ICP-MS	2.0	0.3	16
NYS RM 05-03			
ICP-MS	1.1	--	--
ICP-OES	1.4	0.1	5
HR-ICP-MS	1.3	0.1	9
NYS RM 05-04			
ICP-MS	2.0	--	--
ICP-OES	1.4	0.1	9
HR-ICP-MS	1.4	0.2	18

[a] Lab 1 ICP-MS; Lab 2 ICP-OES; Lab 3 HR-ICP-MS

Table 4 Mn content in synthesized HA standards

Standard	Theoretical spike Mn (μg g^{-1})	GFAAS Mn (μg g^{-1})	DRC-ICP-MS Mn (μg g^{-1})	Mean Mn (μg g^{-1})	*U* (μg g^{-1})	*U* (%)	Recovery (%)
0	0	0.1	0.1	0.1	0.2	138	--
1	1.0	1.1	1.2	1.2	0.1	11	117
2	2.0	2.0	1.9	2.0	0.2	8	98
3	5.3	5.5	5.3	5.4	0.8	14	101
4	10.6	9.8	9.5	9.6	1.0	11	90
5	45.5	46.6	43.8	45.2	2.8	6	99

Table 5 Mass fractions of Mn (±*U*) in synthesized HA standards with and without binder, plus the calculated percent binder dilution from (a) GFAAS and (b) DRC-ICP-MS analyses

(a) GFAAS

Standard	Mn (μg g^{-1}) without binder	Mn (μg g^{-1}) with binder	Binder dilution factor (%)
1	1.1 (0.3)	0.9 (0.1)	−16
2	1.9 (0.4)	1.8 (0.2)	−4
4	9.6 (2.4)	8.6 (1.7)	−10
5	45.8 (6.1)	38.6 (3.9)	−16

(b) DRC-ICP-MS

Standard	Mn (μg g^{-1}) without binder	Mn (μg g^{-1}) with binder	Binder dilution factor (%)
1	1.2 (0.3)	0.9 (0.1)	−19
2	1.9 (0.3)	1.7 (0.1)	−10
4	9.3 (2.0)	8.1 (1.7)	−13
5	43.5 (5.0)	35.3 (3.7)	−19

Table 6 Uniformity of Mn distribution in HA materials as characterized by RSD from three methods of calculation

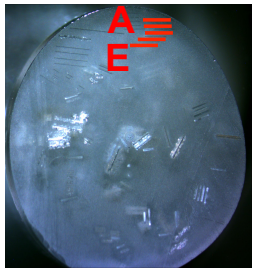
Sample	Laser Mn counts RSD (%)	Laser $^{55}Mn/^{43}Ca$ RSD (%)	Solution-based RSD (%)
Standard 0	37	30	61
Standard 1	14	4	9
Standard 2	29	5	6
Standard 3	13	2	9
Standard 4	9	4	9
Standard 5	21	3	5
NYS RM 05-01	102	109	--
NYS RM 05-02	119	120	--
NYS RM 05-03	22	30	--
NYS RM 05-04	17	12	--
NIST SRM 1400	12	7	5
NIST SRM 1486	3	20	9
NIST SRM 612	6	2	--

Fig. 1 Intensities for ^{55}Mn and ^{43}Ca of five laser ablation line scans (A through E) in each of six different materials

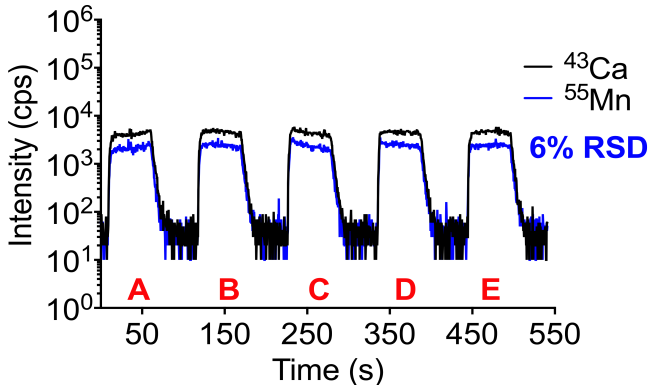
Characterization of calcified reference materials for assessing the reliability of manganese determinations in teeth and bone

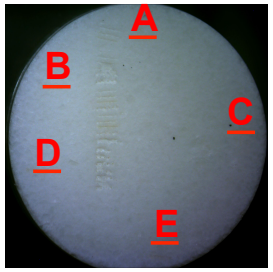
Meredith L. Praamsma and Patrick J. Parsons*

Robust values for manganese with expanded uncertainties were assigned to several calcified reference materials, which were evaluated for use in LA-ICP-MS analysis.

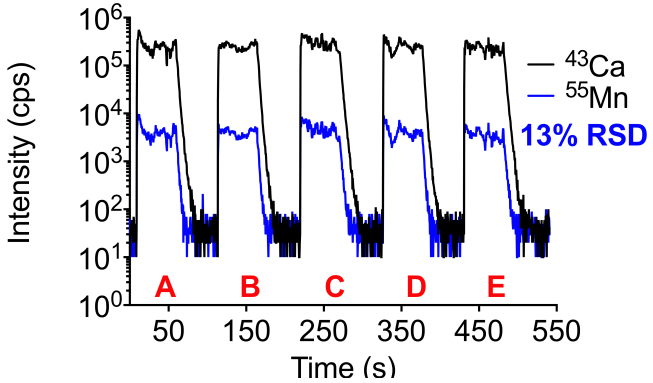


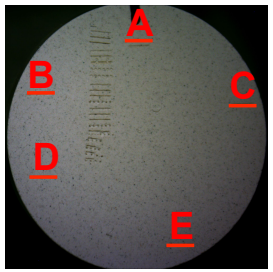
NIST SRM 612





HA Standard 3





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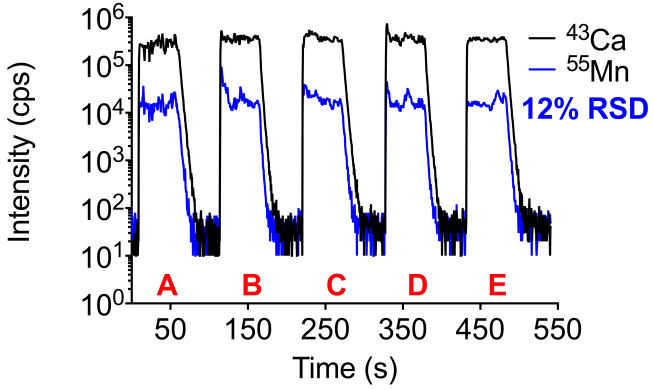

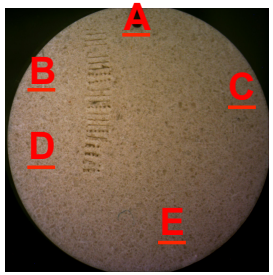

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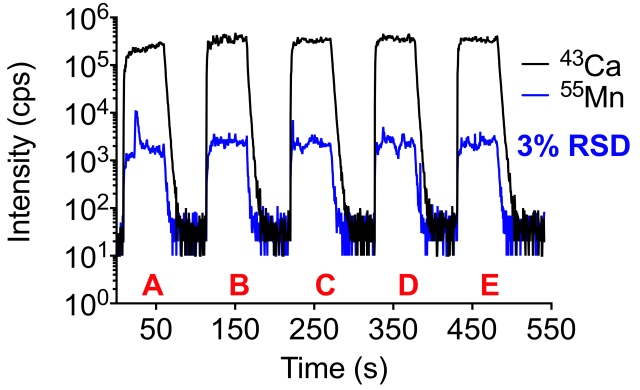
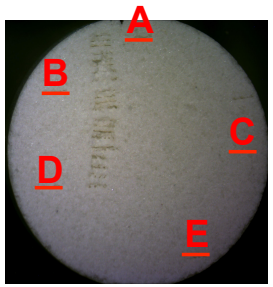
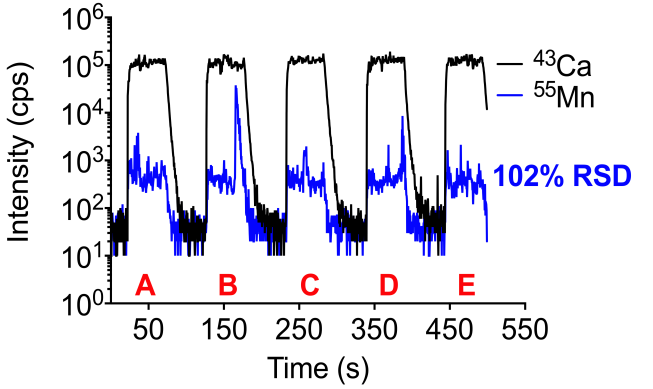
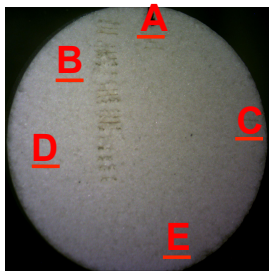




NYS RM 05-01





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