

# Development of candidate reference materials for the measurement of lead in bone

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**Abstract** The production of modest quantities of candidate bone lead (Pb) reference materials is described, and an optimized production procedure is presented. The reference materials were developed to enable an assessment of the interlaboratory agreement of laboratories measuring Pb in bone; method validation; and for calibration of solid sampling techniques such as laser ablation ICP-MS. Long bones obtained from Pb-dosed and undosed animals were selected to produce four different pools of a candidate powdered bone reference material. The Pb concentrations of these pools reflect both environmental and occupational exposure levels in humans. The animal bones were harvested post mortem, cleaned, defatted, and broken into pieces using the brittle fracture technique at liquid nitrogen temperature. The bone pieces were then ground in a knife mill to produce fragments of 2-mm size. These were further ground in an ultra-centrifugal mill, resulting in finely powdered bone material that was homogenized and then sampled-scooped into vials. Testing for contamination and homogeneity was performed via instrumental methods of analysis.

**Keywords** Reference materials · Instrumental analysis · Trace elements · Lead · Grinding

## Abbreviations

RM	reference material
NYS	New York State
DOH	Department of Health
PT	proficiency testing
UCM	ultra-centrifugal mill
SD	standard deviation
ETAAS	electrothermal atomization atomic absorption spectrometry
ICP-MS	inductively coupled plasma mass spectrometry

## Introduction

Lead (Pb) is a naturally occurring element that is also a ubiquitous environmental pollutant due to its widespread and varied use. In the past, the primary sources of Pb exposure in the USA included gasoline emissions, drinking water supplied via Pb plumbing, and the solder used for food cans. Today, Pb is rarely found in gasoline, solder, or interior household paints. However, human exposure to high levels of Pb can still occur in older (i.e., pre-1950s) US housing that contains deteriorating Pb-based paint [1, 2]. Other sources of exposure include traditional “folk” medicines, Pb-crystal glassware, and Pb-glazed ceramic containers that can release Pb into acidic beverages such as orange juice [1, 2]. In 2007, millions of imported children’s toys were recalled by manufacturers after determination that their painted coatings contain levels of Pb in excess of 600 µg/g.

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Exposure to Pb is most commonly assessed (e.g., for screening and for diagnostic purposes) via a blood Pb measurement [3]. Lead in the blood compartment predominantly reflects recent exposure to external sources, but it can also include a contribution from Pb that has been remobilized from bone [4]. The characteristic residence time of Pb in blood (i.e., the time for half of a given amount of Pb to be removed from blood) has been estimated to be 1 month [5], but more recent work suggests that it could be as short as 10–12 days, when recirculation among blood, tissue, and bone stores is considered [6]. The characteristic residence time of Pb in bone is measured in years. In adults, almost 90% of the total Pb body burden (75% in children) is in bone [7–9]. Bone Pb is, therefore, used as a biomarker of long-term Pb exposure, and is typically assessed using *in vivo* measurements of Pb in bone via x-ray fluorescence spectrometry (XRF) [10]. *In vitro* measurements of bone Pb can be made with instrumental methods based on electrothermal atomic absorption spectrometry (ETAAS), inductively coupled plasma optical emission spectrometry (ICP-OES), ICP mass spectrometry (ICP-MS), laser ablation (LA)-ICP-MS, and XRF [11, 12]. Applications of *in vitro* analysis include validation of *in vivo* measurements, environmental monitoring, analysis of biopsy samples, and archeological, agricultural, and forensic applications. It should be noted that Pb is not uniformly distributed in the bone compartment: concentrations differ among various bone types (i.e., cortical and trabecular), between the ends and shafts of long bones, along the shafts of long bones, and cross-sectionally within long bone shafts [8, 13–18].

In analytical chemistry, well-characterized, matrix-specific reference materials (RMs) are typically used to assess measurement techniques and then to assure their accuracy and precision. According to the International Organization for Standardization (ISO), the key properties of RMs are that they be sufficiently homogeneous and well-established to be useful for the calibration of an apparatus, for the estimation of the parameters of a measurement method (e.g., sensitivity), or for the assignment of a particular measurement property (e.g., a Pb concentration) to a sample of material for which the measurement property is not yet known [19]. Uses for RMs also include validation of a new method, testing of an existing method for a new matrix, and estimation of the uncertainty in an analytical method [20–23]. Ideally, RMs also have long-term stability, are accompanied by matrix composition data, and exist in sufficient supply to be useful [24].

There are several categories of RMs defined by ISO: primary standards, certified reference materials (CRMs), proficiency testing materials (PTMs), and quality control materials (QCMs) [19]. A CRM has one or more certified values that are known with uncertainties at stated levels of confidence, that are traceable to System International (SI)

units (thus making the CRM traceable). RMs without certificates may have target or informational values. RMs can be categorized as single-substance RMs (including primary standards) or matrix RMs [20, 22]. Single-substance RMs are pure chemicals, or solutions of pure chemicals, that have well-characterized values for chemical purity, concentration, melting point, viscosity, etc., and are used mainly to calibrate analytical instrumentation. Matrix-based RMs are usually ‘real-world’ samples containing the element(s) of interest in the form(s) found in the natural environment.

Matrix-based RMs have matrices and analyte concentration(s) that are close to those of the ‘unknown’ samples to be analyzed. Matrix-based RMs are introduced at the beginning of the run and undergo the same analytical process as do the unknown samples. Matrix-based RMs allow an assessment of the entire analytical process, rather than just the instrumental phase. RMs are sometimes produced because CRMs are not available for a particular matrix/analyte combination. Also, use of RMs obviates the high cost associated with the use of a large amount of a limited-supply CRM for the creation or validation of a new method, or for routine use as a QCM.

The steps necessary to produce a matrix-based RM are similar to those used for a CRM, except that RMs are usually produced in smaller quantities than CRMs, and CRMs have more stringent stability requirements (they must be stable for longer periods of time) than do RMs. Homogeneity of an RM has to be established if the RM is to be distributed in a proficiency testing (PT) scheme, but the smaller amount of RM material (relative to a CRM) makes homogeneity easier to establish and maintain. In addition, the RM needs to be stable only for the duration of the PT scheme, and not for the years required of a CRM [22]. The particular steps required to produce a CRM vary somewhat among the institutions engaged in developing CRMs, but all of the institutions share several important steps [20–22, 25–28]. Statistical protocols for the certification of RMs in interlaboratory studies have been published [29].

Two CRMs are currently available for Pb in bone, both provided as standard reference materials (SRM) by the National Institute of Standards and Technology (NIST; Gaithersburg, MD): SRM 1400 Bone Ash and SRM 1486 Bone Meal. The Pb concentration is low in both SRMs relative to the range of values measured in humans; SRM 1486 is certified for Pb at  $1.335 \pm 0.014$   $\mu\text{g/g}$ , and SRM 1400 is certified at  $9.07 \pm 0.12$   $\mu\text{g/g}$ . According to its certificate, SRM 1486 Bone Meal was produced from material obtained from the Espoma Company, Millville, NJ. The manufacturer describes their bone meal product as: “... a by-product from meat (rendering factories). It consists of ground animal bones that have been cooked and sterilized, and then ground into a meal” (<http://www.espoma.com/>). It

has a brown color reflective of a high organic content, including collagen (which is an intrinsic part of the bone matrix), fat, and possibly residual tissue. SRM 1400 Bone Ash is also obtained from commercial sources (Monsanto Co., St. Louis, MO) but it was dry-ashed at high temperature, resulting in a white color and negligible (<1%) organic content.

While the matrix of SRM 1486 Bone Meal is a suitable matrix for dry bone, bone ash (as in SRM 1400) is less useful for methods that are applied to the analysis of dried bone samples. Although some investigators have employed dry ashing of bone samples as part of their analysis for Pb [30–32], dry ashing is time-consuming (>48 h) and has the potential for contamination and for losses of volatile elements [33].

Additional bone RMs certified for Pb content at various concentrations with identical matrices are desirable for validation, quality control, and calibration of instrumental methods for both *in vivo* and *in vitro* measurements of Pb in bone. The creation of such candidate RMs requires sources of bones that are enriched with endogenous Pb, so that the element is physiologically bound to the bone. Over several years, we have amassed a substantial quantity of bones from animals that were orally dosed with Pb as part of the New York State Department of Health's (NYS DOH) PT program for blood Pb (as described in detail below).

Once collected, such bones must be ground into a fine powder, so that the RM is sufficiently homogeneous for certification and distribution purposes. One approach to the grinding of hard bones that has previously been investigated [34] is the brittle-fracture technique (BFT) in which bones are frozen to  $-196\text{ }^{\circ}\text{C}$  with liquid nitrogen. This freezing increases the hardness and brittleness of the bone material [35]. Force can then be used more efficiently to shatter the material into smaller fragments suitable for further grinding.

Irrespective of the grinding method used, there are several considerations in the choice of a suitable grinding protocol. The first is to minimize metal contamination, given that as gross contamination can occur during grinding, from any deposits remaining from previously processed samples, and also from contact with the grinding surfaces of the device(s) used [28, 36]. Another consideration is to maximize the amount of material that can be processed at one time. Investigators in applied studies typically grind only small amounts (ca. 1 g) of material [16, 37–38]. For such a small mass of material, the BFT can be performed using a mixer mill, a device capable of accommodating <10 g of material. Larger-scale (200–500 kg) CRM projects, however, are generally undertaken by specialist facilities [26, 39].

The primary goal of this study was to investigate various grinding technologies used for creating a fine, homogenous

bone powder of sufficient quality to produce a candidate RM, and eventually a CRM, for Pb in bone.

## Experimental

### Sources of bones

For initial pilot studies and optimization of the bone-grinding protocol, cow bones obtained from a local butcher were used. For the production of the RMs, various long bones were obtained post mortem from farm animals (two cows and approximately 30 goats) from herds maintained by the NYS DOH. Some of these animals had been periodically dosed with Pb, in some cases over several years, to create blood Pb pools for distribution in the NYS DOH PT program for blood Pb (a program that has been in operation for more than 30 years). Twenty to 30 goats were used for the PT program between 1987 and 2000 and ranged in age from 3 to 13 years. Several cows have also been dosed with Pb and used by the program because they can deliver a larger volume of blood, necessary to produce multiple inhouse QC pools. The animals were orally dosed a few days before each scheduled PT event with Pb in the form of Pb acetate packed in a gelatin-coated capsule. The objective of the dosing regimen is to produce physiologically bound blood Pb at concentrations ranging from low levels (<1  $\mu\text{g}/\text{dL}$ ) to levels associated with acute Pb toxicity in humans (60–80  $\mu\text{g}/\text{dL}$ ). Whole blood is collected into blood bags and aliquoted into tubes for distribution to participant PT laboratories that perform the analysis for blood lead. In addition to their use in the blood Pb PT, these pools are also used to assess performance for erythrocyte protoporphyrin (EP), a biomarker of Pb toxicity. More detailed descriptions of the PT program have been given elsewhere [3, 40].

The treatment, Pb dosing, and euthanasia of these animals, as well as the procedures for harvesting the long bones, and the final disposal of the carcass, are conducted in accordance with a defined protocol that is periodically reviewed and reapproved by the Wadsworth Center's Institutional Animal Care and Use Committee (IACUC). The attending veterinarian is responsible for the health and care of all animals. As each animal approaches the end of its natural life expectancy, or if an animal becomes sick or injured, the attending veterinarian administers euthanasia via lethal injection. During the subsequent necropsy, long bones from both the thoracic (i.e., humerus, radius-ulna, metacarpal) and pelvic (i.e., tibia, femur, metatarsal) limbs are harvested. The calcaneus and patella are also collected, along with samples of skull, vertebrae, and rib. The kidney, brain, and liver are also collected.

## Bone preparation

For removal of the marrow from the diaphyses, bone shafts are separated from epiphyses with a reciprocating autopsy saw (Stryker, Kalamazoo, MI)<sup>1</sup>. Stainless steel scalpels are used to remove most of the overlying fur, skin, and muscle from each bone. Tendons are removed by scraping of the bone with custom-fabricated tantalum scrapers. Remaining blood, tissue, and marrow deposits are removed after an overnight soaking in 30% (v/v) hydrogen peroxide (J. T. Baker, Phillipsburg, NJ). Bovine bones, because of their larger mass, undergo an additional step of physical reduction in size after the initial hydrogen peroxide cleanup, either with the autopsy saw or with the combination of the BFT and a hammer, in order to ease marrow removal and defatting. Bones were defatted by soaking in diethyl ether (J. T. Baker) for 1–2 h. The bones were air-dried to remove all traces of ether, and then stored in a freezer at  $-80\text{ }^{\circ}\text{C}$  (Forma Scientific, Marietta, OH) prior to grinding. Ground bone samples were freeze-dried in a Freezemobile 6 (The Virtis Company, Inc., Gardiner, NY). Bovine bones obtained from the butcher were cleaned in the same manner as those obtained from the Pb-dosed animals.

## Grinding apparatus

Several commercially available mills (Retsch, Inc., Haan, Germany) were evaluated at the facilities of Glen Mills, Inc. (Clifton, NJ), the US distributor. A Model SM 2000 heavy-duty knife mill was assessed for initial coarse grinding of bone fragments. Material is introduced into the SM 2000, via a 20-cm hopper that feeds to a vertical cutting area containing several staggered tungsten carbide cutting tips that trap the material against fixed stainless steel cutting bars. Small fragments are expelled through selectable screen grates of 1–10 mm that are placed at the base of the mill. The use of successively smaller grates reduces the particle size accordingly. The SM 2000 is supplied with a wooden ram to drive feedstock sample into the mill; but we replaced it with a custom-built Teflon ram of the same dimensions, so as to reduce the potential for sample contamination from the wood. Coarse-ground bone material is collected into a stainless steel bucket that is lined with a large Ziploc<sup>®</sup> plastic bag.

Other mills were evaluated for ability to produce a fine bone powder from the coarse ground material. We evaluated the Retsch Model PM 400 planetary ball mill and the Retsch Model S100 centrifugal ball mill. In the

S100 centrifugal mill, the bone material and a number of ceramic balls are placed in a single sealed container (so as to minimize sample loss) that is subsequently oscillated along an axis. During this procedure, material is propelled along the sides of the container, where it is crushed by impact with several balls. Repetition of the passes with successively smaller balls produce a finer grind. The PM 400 planetary mill operates in a similar fashion, except that it can accommodate four individually rotating jars that are secured to a platform. The platform rotates in a direction opposite to that of the jars' motion. For both the S100 and the PM 400, ceramic jars and balls can be used, helping to minimize metal contamination.

We also evaluated the Retsch Model RM 100 mortar grinder, which is an automated mortar and pestle, and the Retsch laboratory jet mill (jet mills were used to create a radioactive bone ash CRM [26]). In a jet mill, the material is simultaneously circulated in a vertical plane and propelled in a circular path; in this movement trajectory, it collides with more material that is entering the mill, and is shattered into smaller particles. Jet mills minimize contamination, by using the material to grind itself.

The ZM 200 ultra-centrifugal mill (UCM) was also evaluated. The UCM contains a titanium metal rotor and a titanium screen. Material less than 2 mm in diameter is introduced through the center of the rotor, and centripetal force drives it into the screen, where it shatters. The UCM can accommodate samples cooled to liquid N<sub>2</sub> temperature.

Mixer mills (SPEX 800 and Retsch MM 200) (both from SPEX, Metuchen, NJ) were also evaluated for production of powders from individual samples. The SPEX 800 was selected for further study. In a mixer mill, bone samples are placed in a container typically constructed from agate, stainless steel, or from ceramic materials, with one or two balls made from the same material. The container is then secured in a device that shakes it rapidly. Cryogenic grinding can be accomplished by freezing the filled container prior to operation of the mill. Unfortunately, mixer mill containers hold only ca. 10 g of material, necessitating many repetitions piecemeal for larger batch sizes, and increasing the potential for contamination and inhomogeneity.

## Grinding method development

We quantified the amount of bone that is lost during each step of the final, optimized milling procedure by weighing several batches of powder before and after each pass. Sometimes, during processing, the mills would jam and would have to be stopped. The partially processed bone material was collected, and the mill was cleaned before being restarted.

Bovine bone from both dosed and undosed animals was used to determine whether any gross Pb contamination was

<sup>1</sup> The use of trade names is for informational purposes only, and does not imply an endorsement by the New York State Department of Health.

introduced during the grinding procedure. For each bone, separately, a ‘grab’ sample was obtained after each grinding step and analyzed for Pb using ETAAS (see below). Contamination from elements other than Pb was also a concern, since we anticipated that these candidate RMs could later be analyzed for additional elements of interest to other researchers and laboratories. With the BFT, size reduction is achieved rapidly, thereby minimizing exposure time to metallic components in the mills, so that non-Pb contamination is less of a concern. The metal compositions of various components of the knife mill and UCM are shown in Table 1, indicating those non-Pb contaminants that are of potential concern. We purchased the UCM configured with titanium components substituted for stainless steel, so as to limit metal contamination during the UCM step to a single element (Ti) that was not considered central to our project. Avoidance of stainless steel whenever possible was thought to be desirable, because contamination by that material would likely introduce cobalt, manganese, and other trace elements that could be of potential interest to future workers.

#### Analytical measurements

Lead content in bone was determined by an ETAAS method previously developed by Zong et al. [41]. We also used an analytical method based on externally calibrated ICP-MS, and via isotope dilution ICP-MS, as described in detail elsewhere [42]. Additional trace elements were measured by externally calibrated ICP-MS. Samples of bone were digested in high-purity concentrated nitric acid (GFS Chemicals, Powell, OH) in clean 50-mL polypropylene conical vials (Starstedt, Newton, NC), using either microwave-assisted heating for 1 h with a maximum temperature of 70 °C or overnight at room temperature as described previously [42]. Using either method, the final solutions were free of particles and clear in color. During each analytical run, similar digests of SRMs 1486 Bone

Meal and SRM 1400 Bone Ash were analyzed in order to monitor method accuracy and to ensure that each run remained statistically controlled. The results of a particular run were only accepted if the SRM result and uncertainty in that result overlapped the certified value and the stated uncertainty of the SRM.

For ETAAS bone Pb measurements, we used a Perkin-Elmer (Shelton, CT) Model Z5100 atomic absorption spectrometer equipped with an HGA 600 graphite furnace and a transverse Zeeman background-correction system. Pyrolytically coated graphite tubes (Perkin-Elmer, part number BO505057) with a preinstalled forked platform were used with the HGA 600. A Model AS-60 autosampler was used to deposit a 10- $\mu$ L aliquot on the L’vov platform. The ETAAS instrument operated under version 3.0 of Perkin-Elmer’s WinLab software. Calibration standards were prepared from serial dilutions of 1,000 mg/L “pure lead atomic absorption standard” (Perkin-Elmer, Norwalk, CT). The ETAAS modifier solution contained 2% (m/v) Ca (NO<sub>3</sub>)<sub>2</sub> (Sigma-Aldrich, St. Louis, MO) and 20% (m/v) NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> (J.T. Baker). The final modifier contained 20 g/L calcium and 0.2% (m/v) NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> in 0.2% (v/v) HNO<sub>3</sub>.

For ICP-MS measurements, we used a Perkin-Elmer SCIEX Model DRC Plus equipped with a Meinhard nebulizer (WE024371) and cyclonic spray chamber (WE025221) supplied by Meinhard Glass Products (Golden, CO). Contamination studies were conducted using an external calibration method for Zn, W, Mn, and Co. Calibration and internal standard solutions were purchased from High Purity Standards (Charleston, SC) or from SPEX (Metuchen, NJ). Homogeneity testing was conducted with a double isotope dilution method [42]. Samples were spiked with a quantity of SRM 991 Lead-206 Spike Assay and Isotopic purchased from the National Institute of Standards and Technology (NIST) (Gaithersburg, MD) sufficient to create a <sup>206</sup>Pb/<sup>208</sup>Pb of 2 for NYS RM 05–01 and 1 for NYS RM 05–02 through 04. Ten replicate measurements of 1,000 sweeps were employed to maximize precision of the isotope ratio measurements. Mass bias was corrected through analysis of SRM 981 Natural Lead (Isotopic) and SRM 982 Equal Atom Lead (Isotopic) Standard (Gaithersburg, MD).

**Table 1** Principal metal constituents of components of the mills used in this study

Retsch SM 2000 knife mill		Retsch ZM 200 ultra-centrifugal mill	
Cutting bars	Fe, Cr, Mn	Rotor	Ti
Cutting tips	W, Co, Ti, Ta	Bushings in rotors	Fe, Cr, Mn, Mo
Rotor disk	Fe, Mn, Cr, Ni	Titanium sieves	Ti, Fe
Door insert	Fe, Mn, Cr, Ni		
Hopper	Fe, Mn, Cr, Ni		
Housing	Al (<1% Cu, Zn, Fe, Ti, Mn, Mg)		

Data were derived from the manufacturer’s product literature

## Results

### Preliminary grinding studies

Several steps proved necessary to produce a suitably fine and homogenous powdered bone material from intact long bones. The first step, reduction of the bones to smaller (<5 cm), more manageable pieces that could be introduced into a cutting mill, was achieved by freezing of the bone material with liquid nitrogen and then fracturing and

shattering them with a steel hammer (also known as the BFT approach). Intact frozen bone was placed into a robust plastic bag to prevent direct contact between the bone and the steel hammerhead, and thus avoid a potential source of contamination.

We found that use of the SM 2000 knife mill was the only feasible method for coarse grinding of the bone fragments that were produced using the BFT. Bone fragments were cooled with liquid nitrogen immediately before they were introduced into the knife mill. Two successive passes with grate sizes of 10 mm and then 2 mm were required to produce coarsely ground bone materials suitable for the next step. During the first pass, the frozen bone material was found to shatter rapidly through the 10-mm grate. However, for the second pass, the bone material circled around the center of the milling area before passing through the 2-mm grate.

Several fine-grinding methods were found to be suboptimal in performance, during our exploratory pilot studies. In initial tests with the S100 centrifugal mill and with the PM 400 planetary mill, little change in the size of the particles was achieved after operation for 1 h. We observed that some of the bone material formed a thin film instead of a powder. Application of the BFT in combination with use of the mills that had stainless steel jars and balls resulted in some size reduction, but the output was not fine enough for our purposes. In addition, the color of the material changed from white to gray, possibly due to contamination from chromium and/or iron. Even when used with the BFT, the RM 100 mortar grinder was found to reduce the bone particle size only slightly; it was, therefore, not considered further. Initial results with the laboratory jet mill showed that while this device did reduce the size of the bone material during the first few passes, it did not readily produce powdered material suitable for input to an RM. Although a jet mill can be configured for cryogenic grinding, a special setup is necessary to reduce condensation from the cooled material. Such a configuration was not available to us.

The ZM 200 ultra-centrifugal mill (UCM) was found to be the most successful in producing a fine and homogeneous bone powder suitable for our purposes. One drawback of the UCM is that, even with liquid N<sub>2</sub> cooling, substantial heating of the bone powder can occur, resulting in a burnt product. To prevent this, we removed the powdered bone from the UCM after about 100 g had been produced. Repeated raising of the cover to remove the product inevitably resulted in loss of material: bone dust visibly contaminated the laboratory workbench. For operator safety and to avoid contamination of the entire laboratory, the UCM was operated in a fume hood. The operator also wore an approved filter-type face mask to avoid inhalation of fine bone particles.

### Final optimized grinding protocol

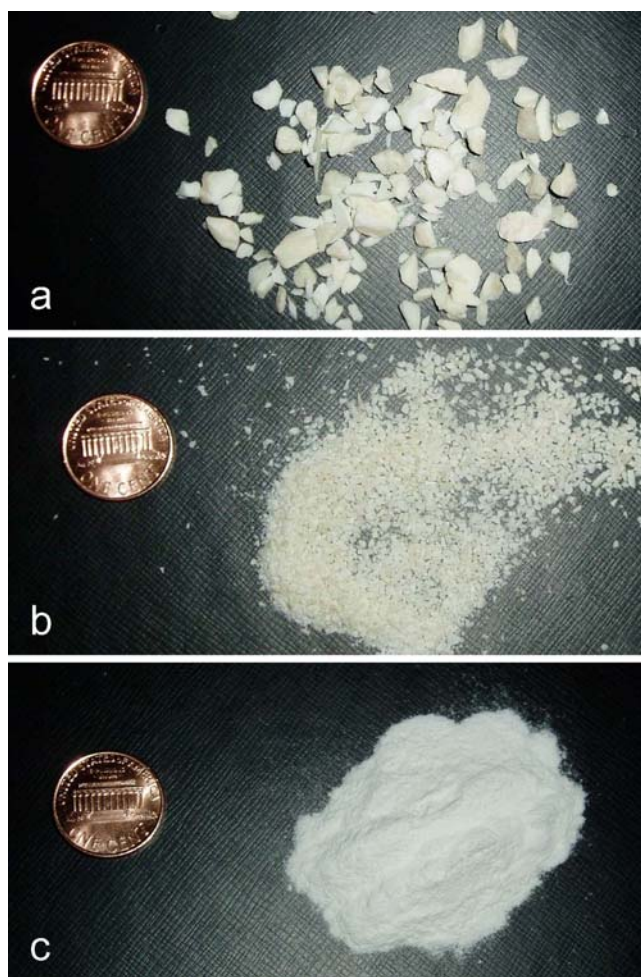
The principal goal of this study was to develop an optimized protocol for the grinding of bone into a powered material suitable for an RM. The protocol that we finally adopted is described below. In order to minimize potential cross contamination of the mills from any bone material remaining from a previously processed pool, the materials being ground should be processed in order of increasing Pb concentration.

For coarse grinding, a stock of cleaned and defatted bone fragments previously prepared using a standard hammer with the BFT, and stored at  $-80\text{ }^{\circ}\text{C}$ , is divided into batches of ca. 1 kg each. Prior to coarse grinding, approximately 400 g from the 1-kg batch is placed on a polypropylene tray and immersed in liquid N<sub>2</sub> until the liquid N<sub>2</sub> stops boiling. Excess liquid N<sub>2</sub> is then poured off, and the bone fragments are inserted into a SM 2000 knife mill equipped with a 10-mm screen. Although the SM 2000 is supplied with a wooden ram for sample loading, we used a custom-built Teflon ram, to reduce the potential for contamination from the wood. After completion of the coarse grinding, the 10-mm screen is replaced with the 2-mm screen. Then, approximately 200 g of the coarse ground (10-mm) bone material is refrozen to liquid N<sub>2</sub> temperature before being reintroduced into the knife mill. After the 2-mm coarse grind is complete, the bone material should be stored at  $-80\text{ }^{\circ}\text{C}$ . Between one 1-kg batch and the next, all of the bone material from previous batch should be removed, and the milling components should be cleaned with doubly deionized water (rated at 18 M $\Omega$  cm; Millipore, Bedford, MA) and then with methanol (J. T. Baker).

Next, the fine grinding stage is accomplished with the ZM 200 UCM, which is configured with a 250- $\mu\text{m}$  titanium screen. Batches of coarsely ground bone material, ca. 100 g each, are cooled to liquid N<sub>2</sub> temperature, and passed through the UCM. After each batch has been processed, the UCM should be stopped, and its collection pan removed, and all of the processed bone powder therein transferred into a plastic bag. Any residual pieces of bone trapped in other parts of the UCM should be extricated and discarded. When the UCM has reached room temperature, all of its components should be cleaned with deionized water and then methanol, to remove residual bone powder before the next batch is processed. Photographs of the bone product at the various stages of the process described above are shown in Fig. 1.

### Production of RMs

The desired bulk mass of each RM pool was approximately 3 kg. The first pool of bones selected to create a base material (NYS RM 05–01) consisted of the major long bones (humerus, femur, etc.) collected from a single cow



**Fig. 1** Bone fragments at successive stages of the grinding process: **a** after first pass through the knife mill with the 10-mm screen, **b** after second pass through the knife mill with the 2-mm screen, **c** after passage through the ultra-centrifugal mill. Scale given by US penny = 19-mm diameter

that had not been previously dosed with Pb. The second pool (NYS RM 05–02) consisted of long bones from a single cow dosed with a total of 12.7 g Pb during its lifetime (cumulative dose). The third pool (NYS RM 05–03) consisting of the long bones from nine goats that had each received a cumulative lifetime dose of between 10 and 20 g Pb. The fourth pool (NYS RM 05–04) consisted of the long bones from 14 goats that had each received a cumulative lifetime dose of between 40 and 55 g Pb.

#### Bone losses during grinding

The results of the experiment to estimate the amount of bone lost during each step of the milling procedure are summarized in Table 2. Greater losses ( $p=0.003$ ) occurred with the UCM than with the knife mill, because of the repeated openings of the former to remove the (ca. 100 g) sub-batches, and the need for subsequent cleaning of the UCM as previously mentioned. Despite these small-scale

losses, total mass recovery for each batch of bone material was of the order of 95% or better.

#### Contamination assessment

Analysis of grab-samples of bovine bones originating from the undosed animal, obtained after each step, showed that no gross Pb contamination was being introduced by the grinding protocol (Table 3). The data obtained from the dosed bovine bone are also shown in Table 3. A one-way analysis of variance in the data from the dosed bovine bone with a Tukey–Kramer multiple comparison test was of borderline statistical significance ( $p=0.062$ ), suggesting possible differences between the Pb concentration measured at the end of one step and that at the end of the next. Table 3 suggests that the variability in the Pb concentrations decreases after each grinding step, indicative of better homogeneity as particle size decreases.

**Table 2** Sample loss during the production of bone reference materials

Material <sup>a</sup>	Mass loss resulting from processing step (%)			Mass recovered (%)
	Knife mill with 10-mm screen	Knife mill with 2-mm screen	Ultra-centrifugal mill with 1/4-mm screen	
Test grind (butcher bone)	1.4	3.3	2.2	93.1
NYS RM 05–03 sample A	0.5	1.6	2.3	95.6
NYS RM 05–03 sample B	0.1	1.3	2.6	96.0
NYS RM 05–03 sample C	0.4	1.6	2.6	95.4
NYS RM 05–04 sample A	0.2	1.2	2.6	96.0
NYS RM 05–04 sample B	0.0	1.0	2.5	96.5
NYS RM 05–04 sample C	0.1	1.0	3.7	95.2
NYS RM 05–04 sample D	0.3	1.1	3.1	95.5
Mean	0.4	1.5	2.7	95.4

<sup>a</sup> The initial mass of the test grind was 81 g. The initial mass of the NYS RM samples ranged from 669 to 1,215 g

**Table 3** Lead concentration of bovine bone after each step of the grinding protocol

	No. digests <sup>a</sup>	Bone Pb concentration <sup>b</sup> (µg/g dry weight)
Bone material from undosed cow (RM 05–01)		
1st pass through knife mill (10-mm screen)	3	1.2, 1.2, 1.1
2nd pass through knife mill (2-mm screen)	3	1.0, 1.1, 1.1
Pass through UCM (250-µm screen)	3	1.1, 1.1, 1.2
Bone material from dosed cow (RM 05–02)		
1st pass through knife mill (10-mm screen)	6	16.9 (1.6)
2nd pass through knife mill (2-mm screen)	6	15.8 (0.6)
Pass through UCM (250-µm screen)	7	15.5 (0.4)

<sup>a</sup>Number of independent digestions carried out<sup>b</sup>Triplicate digestion data (upper panel) or mean (SD) (lower panel)

The RMs were produced in order of increasing Pb concentration so as to minimize cross-contamination risks, with one exception. Three additional bones derived from the dosed animal that supplied material for NYS RM 05–02 were discovered in our repository. These bones were ground after the highest-Pb pool (NYS RM 05–04) had been produced. Thus, determination of the Pb concentration of the additional bovine material allowed us to check for cross-contamination, before we mixed this material with the existing pool of NYS RM 05–02. A Student's *t*-test showed no significant difference ( $p=0.127$ ) in Pb concentration between the two batches of NYS RM 05–02.

As a qualitative check for cross-contamination from other metals, the same digests from the undosed bovine

**Table 4** Analysis of bovine bone from an undosed animal, and used to assess zinc, tungsten, manganese, and cobalt contamination from mill components

	Zn	W	Mn	Co
1st pass through knife mill (10-mm screen)	76, 82, 74	0.02, 0.01, 0.01	0.7, 0.6, 0.5	42, 44, 46
2nd pass through knife mill (2-mm screen)	71, 75, 75	0.07, 0.09, 0.07	2.0, 2.5, 1.9	46, 47, 50
Pass through ultra centrifugal Mill (250-µm screen)	77, 74, 74	0.07, 0.06, 0.06	2.1, 2.2, 1.9	54, 51, 52

Digestions were performed in triplicate followed the analysis for Zn, W, Mn, and Co by ICP-MS and performed in a single run. The analytical data are reported as µg of measurand per g dry weight bone

bones that were previously analyzed for Pb by ETAAS, were analyzed in triplicate by ICP-MS for Zn, W, Mn, and Co. The results are summarized in Table 4. Due to the small number of analyses performed here, and information on only a few elements in bone, our results are somewhat limited. Qualitatively speaking, however, Zn concentrations remained constant. This was expected, since zinc is not a major component of either mill. Tungsten and Mn concentrations appeared to increase, from the first pass to the second in the knife mill. Tungsten is a major component of the SM 2000 knife mill cutting tips, and Mn is used throughout the knife mill (Table 1). There also appeared to be a small increase in Co concentration from the first to the second pass in the knife mill, and another after the UCM grinding, but more samples would need to be analyzed before we could attach statistical confidence to any of these observations. For these reasons, our RMs may not be suitable for the determination of W, Mn, or Co.

#### Lead concentration of reference materials

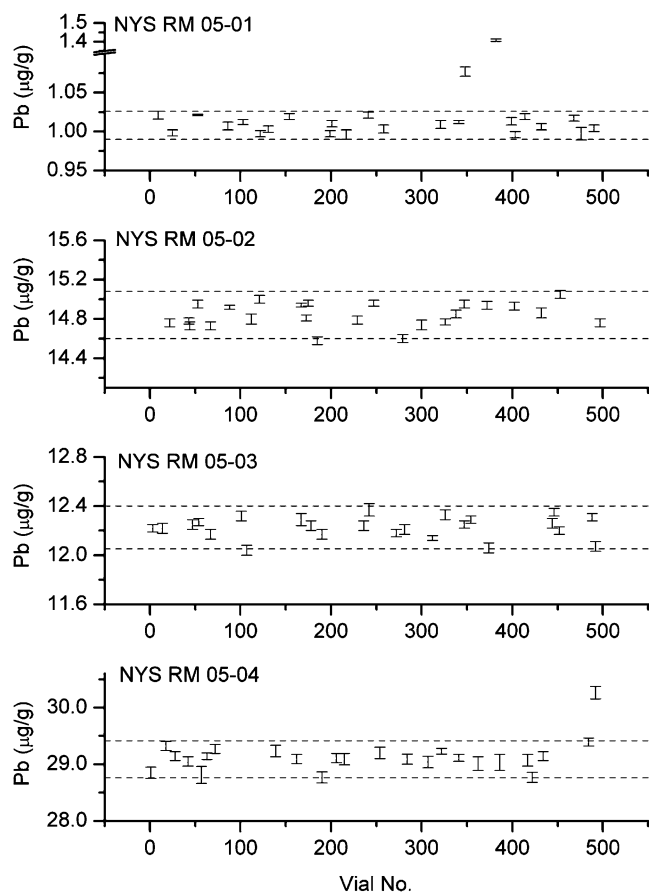
A Glen Mills T2F Turbula shaker-mixer was used to homogenize each of the four pools of bone RMs. Each bulk RM was subsequently sample-scooped, under clean conditions, into 500 vials, for a final weight of ca. 5 g each. The moisture content of NYS RMs 05–01 through 04 was about 4%. Details about each RM produced and the results of preliminary analysis by ETAAS are given in Table 5. Unexpectedly, NYS RM 05–02 had higher Pb content than did NYS RM 05–03. Figure 2 gives a graphical representation of homogeneity of each of the four candidate RMs, as determined by isotope dilution ICP-MS. The homogeneity testing protocol was developed from the recommendations of Ellison et al. [43]. For each of the four RMs, 24 samples (i.e.,  $3(\sqrt[3]{n})$ ) out of the total were selected at random from groups of vials stratified according to the fill order. Given

**Table 5** Description of four bone Pb reference materials produced in this study

Reference material	Source	No. animals used	RM mass produced (g)	ETAAS mean (SD) <sup>a</sup> Pb concentration (µg/g)
NYS RM 05–01	Cow	1	3,293	1.2 (0.1)
NYS RM 05–02	Cow	1	2,866	16.3 (0.6)
NYS RM 05–03	Goats	9	3,071	13.3 (0.4)
NYS RM 05–04	Goats	14	3,169	31.3 (0.7)

Mean bone lead values (µg/g dry weight) derived from 22 ETAAS measurements per RM

<sup>a</sup>SD includes factors due to digestion and within-run considerations



**Fig. 2** Lead concentrations in individual vials of NYS RMs 05–01 through 04, as determined by isotope dilution ID-ICP-MS, shown as the mean  $\pm$  1 standard deviation of the within-vial measurements. Dashed lines denote  $\pm$  2 standard deviations of the between-vial mean (excluding outliers: two in the top panel; one in the bottom panel)

that each vial contains only ca. 5 g of material, it was reasonable to assume that within-vial inhomogeneity is insignificant compared with between-vial inhomogeneity. As a result, a single 200-mg sample was analyzed from each vial, rather than duplicate samples per vial. The four RMs showed good homogeneity apart from two outliers for NYS RM 05–01 and NYS RM 05–04. The outliers could have resulted from analytical error or from bench contamination during the analytical procedure, as well as from inhomogeneity of the sample. It is recommended in such cases with a small percentage of outliers, that the outliers be excluded from the homogeneity term [44]. Analysis of a second aliquot taken from these vials gave results within 2 SD of the mean values listed in the table. The homogeneity of the RMs was thus sufficient for them to be judged fit-for-purpose.

## Discussion

The principal result of this study is an optimized method for producing RM-quality ground bone pools. The method as

presented here is the result of extensive research and development that assessed almost all of the practical and cost-effective bone grinding methods available today. We hope that the optimized protocol will be useful to other researchers who need to prepare a ground bone RM; it may also be useful to researchers who require bone RMs containing trace levels of elements other than Pb.

Neither the coarse nor the fine grinding methods adopted produced any substantial material loss or Pb contamination. Initial assessment of contamination from elements other than Pb produced data that probably warrant further investigation, but which do not appear to detract from the utility of the bone Pb RMs produced. More data are required before statistical confidence can be assigned to the changes suggested by the W, Mn, and Co data. If the changes do prove to be significant, they can, for the most part (i.e., with the possible exception of Co), be attributed to the longer contact time with the knife mill components during the second pass. During the first pass, most of the frozen bone material appears to quickly shatter on the cutting bars and tips, and it then passes through the 10-mm screen. During the second pass, however, the now-finer bone material takes longer to break, and it circulates around the rotor for several seconds before exiting the 2-mm screen. An additional possibility is that the ram used to force bone pieces into the mill produces contact of some of the material with the stainless steel surface of the hopper. If so, the use of the ram could be minimized. The assumption that the Zn, W, Mn, and Co content was uniformly distributed in the bone was not tested. Also, although we considered processing a CRM known to be homogeneous (SRM 1486, Bone Meal) through the mills, in order to assess contamination, the fineness of SRM 1486 would probably have resulted in a large proportion

**Table 6** Summary of results of interlaboratory study and ID-ICP-MS analysis of the four bone Pb reference materials produced in this study [42]

Reference material	Interlaboratory study consensus value <sup>a</sup> of Pb concentration ( $U_x$ ) ( $\mu\text{g/g}$ )	ID-ICP-MS certified value <sup>b</sup> Pb concentration of ( $U_c$ ) ( $\mu\text{g/g}$ )
NYS RM 05–01	1.08 (0.04)	1.09 (0.03)
NYS RM 05–02	15.3 (0.5)	16.1 (0.3)
NYS RM 05–03	12.4 (0.5)	13.2 (0.3)
NYS RM 05–04	29.9 (1.1)	31.5 (0.7)

<sup>a</sup> Consensus value based on robust mean of the interlaboratory study;  $U_x$  is the expanded uncertainty with coverage factor 2 calculated from the robust standard deviation. Methods for calculation are described in ISO 13528 [45]

<sup>b</sup> Certified values based on ID-ICP-MS;  $U_c$  is the expanded uncertainty with coverage factor 2 [42]

of the material clogging and becoming trapped in the mill, and not recovered. A future approach could be to grind a sample of bone in the mixer mill, and then analyze the processed powder. Comparison of the elemental composition from the bulk powder and from the powder reprocessed by the RM grinding protocol could allow us to identify additional metal contamination sources from the knife mill or the UCM.

Precision and accuracy of the ETAAS method for Pb were both acceptable although the precision was slightly poorer than previously reported [41]. The RMs produced by the method described here were found to have, as per our intent, bone Pb levels that encompassed a useful range of concentrations, reflective of environmental and occupational exposures in humans.

These new bone RMs, with Pb concentrations higher than in preexisting RMs will, we hope, be useful to other researchers in many scientific fields. For example, we have used pressed pellets of these NYS Bone Pb RMs with LA-ICP-MS analysis of bone to construct a four-point calibration line for quantitative Pb measurements [12]. Moreover, we used pressed pellets of the current NIST bone SRMs as quality control/validation materials in LA-ICP-MS [12].

The RMs described here were recently circulated in an interlaboratory study of the measurement of Pb in bone by *in vitro* methods, and reference values for each of the four materials were established by isotope dilution ICP-MS [42]. A brief summary of the results is given in Table 6. There was good agreement between the values reported by the participant laboratories and among different analytical instrumentation and methods, resulting in a relatively small uncertainty in the consensus mean calculated from the interlaboratory data. However, there was a small discrepancy between the consensus mean, determined from the interlaboratory study, and the certified value that was determined by ID-ICP-MS. This discrepancy was largely attributed to the efficacy of the various drying methods that were employed by the interlaboratory participants. The results indicate that these newly certified reference materials are valuable to workers studying lead in bone in the concentration range found in human subjects, and as such are fit-for-purpose. Future work includes distribution of the CRM material, in a pelletized form, in an interlaboratory study of the measurement of Pb in bone by *in vivo* XRF methods.

## Conclusion

An optimized protocol was developed for the production of modest quantities (<3.3 kg) of a candidate RM for use in bone Pb measurements. Long bones obtained from Pb-dosed animals and from an undosed animal were selected to produce four pools of powdered bone reference material having a range of Pb concentrations. Bones were harvested

post mortem, cleaned, defatted, and fragmented using the BFT at liquid nitrogen temperature. The bone fragments were then ground in a knife mill to produce coarse particles of <2-mm size, which were then further homogenized in an ultra-centrifugal mill, resulting in powdered bone that was sampled-scooped into vials. The four RMs have since been certified (CRMs) and are available to interested researchers upon request.

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