Published in final edited form as:

Appl Radiat Isot. 2013 August ; 78: 57–61. doi:10.1016/j.apradiso.2013.04.012.

Calcium Isolation from Large-Volume Human Urine Samples for ^{41}Ca Analysis by Accelerator Mass Spectrometry

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Abstract

Calcium oxalate precipitation is the first step in preparation of biological samples for ^{41}Ca analysis by accelerator mass spectrometry. A simplified protocol for large-volume human urine samples was characterized, with statistically significant increases in ion current and decreases in interference. This large-volume assay minimizes cost and effort and maximizes time after ^{41}Ca administration during which human samples, collected over a lifetime, provide $^{41}\text{Ca}:\text{Ca}$ ratios that are significantly above background.

Keywords

^{41}Ca analysis; accelerator mass spectrometry; biological samples; calcium oxalate; human urine

INTRODUCTION

An extremely rare isotope of calcium, ^{41}Ca is an effective tool to track calcium release from bone (Denk et al., 2006, 2007; Elmore et al., 1990; Freeman et al., 1997; Johnson et al., 1994). ^{41}Ca has a very low natural abundance ($\sim 10^{-15}$) and a very long half-life (1.03×10^5 years), which enables it to be utilized as a quasi stable isotope analyzed by accelerator mass spectrometry (AMS) (Brown et al., 2005). Once injected into the human body, virtually all of the ^{41}Ca that remains in the body after 3 months resides in bone (Cheong et al., 2007; Weaver et al., 2009). $^{41}\text{Ca}:\text{Ca}$ ratios in urine can be used in translational research to help

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^eWork performed while student undergraduate research participant at Argonne National Laboratory

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determine, for example, whether cadmium in mainstream cigarette smoke stimulates calcium release from bone in humans (Bhattacharyya, 2009; Ebert McNeill et al., 2012). Administration of 10 to 100 nCi ^{41}Ca via injection or oral administration is sufficient to evaluate skeletal ^{41}Ca release for the lifetime of the subject – a feature unique to this long-lived isotope (Denk et al., 2006, 2007; Fitzgerald et al., 2005).

A number of low-volume methods to isolate calcium for AMS analysis are reported that use 30- to 50-ml samples of urine, serum, or saliva, with carrier calcium added to improve sample handling (Fitzgerald et al., 2005; Freeman et al., 1995; Freeman et al., 1997; Lin et al., 2004). However, addition of carrier calcium significantly decreases the $^{41}\text{Ca}:\text{Ca}$ ratio and therefore shortens the time after ^{41}Ca administration that a study produces samples with ratios significantly above background. Although one large-volume method is reported (Cheong et al., 2007), it requires expensive filtration devices that need to be cleaned for re-use. The purpose of this technical note is to characterize a simplified large-volume calcium isolation protocol for AMS, compare it to the existing protocol, and demonstrate its transfer to an outside laboratory, providing the scientific basis for its implementation in clinical studies using ^{41}Ca .

MATERIALS AND METHODS

To generate the most valid comparisons, each protocol was carried out in the laboratory that was most proficient in that particular protocol, i.e., Standard Protocol, C Weaver/Purdue University; Modified Protocol, MH Bhattacharyya/Argonne National Laboratory or SK Hui/University of Minnesota.

Standard Large-Volume Protocol

Cheong et al. (2007) report the one published protocol that isolates calcium for AMS analysis using large volumes of human urine. According to this protocol, urine samples are collected in acid-washed containers, mixed thoroughly, and ammonium hydroxide (concentrated; 14.5 M) is added (~10 ml per liter of urine). Samples are mixed well and pH is checked to ensure it is at least pH10. Saturated ammonium oxalate (~5% w/v) is added (50 ml per liter of urine) and mixed well. Samples are allowed to stand overnight to allow calcium to precipitate as calcium oxalate. The supernatant is decanted, leaving the precipitate and some urine. The precipitate is collected on the filter of a vacuum filter apparatus [Nalgene polysulfone (PSF) filter holder with 1 liter receiver and AcetatePlus filter (5.0 micron, 47mm, GE Water & Process Technologies)] and rinsed at least 3 times with 1% ammonium oxalate. The filter is transferred to a weighing boat, covered partially with another one to prevent contamination, and dried in an oven at ~37° C. The dried precipitate is transferred on the filter to a container for dissolution, in preparation for ^{41}Ca analysis by AMS at the Purdue University PRIME Lab. Each filter apparatus is cleaned free of ^{41}Ca using 10% Contrad 70 detergent solution and rinsed with deionized water and ultrapure water prior to re-use with another sample.

Modified Large-Volume Protocol

A modified protocol for isolating calcium from large-volume urine samples was developed at Argonne National Laboratory. Steps in the modified protocol and the rationale for each are presented in Table 1. Four steps in the precipitation differ from the above standard protocol: using heated ammonium oxalate, adding sodium acetate, adding ammonium oxalate *before* adjusting the pH with ammonium hydroxide, and washing the precipitate thoroughly, by centrifugation, with dilute (0.5%) ammonium oxalate. Finally, precipitation was carried out in the original urine collection bottle, transferred to a 50-ml centrifuge tube, and all subsequent steps were carried out in that tube, including washing, drying, storing, and shipping the precipitate for analysis of $^{41}\text{Ca}:\text{Ca}$ ratio by AMS at the Purdue PRIME Lab.

Split-Sample Comparison

MH Bhattacharyya provided training in the modified protocol to personnel in the laboratory of SK Hui at the University of Minnesota. After training was complete, nine individual ^{41}Ca -containing human urine samples from a University of Minnesota study were each split. One 400-ml portion of each was shipped to Purdue University for calcium precipitation by their standard protocol in the laboratory of C Weaver; the remaining portion of each was retained at the University of Minnesota for calcium precipitation by the modified protocol in the laboratory of SK Hui. All precipitates were analyzed for $^{41}\text{Ca}:\text{Ca}$ ratio by AMS at the Purdue PRIME Lab (method below). The University of Minnesota study was approved by the University of Minnesota Institutional Review Board (IRB). Signed consent forms were obtained from all study subjects.

Between-Study Comparison

This comparison evaluated the extent to which improvements achieved via the modified protocol persisted when samples were obtained from separate studies. Thirty-six ^{41}Ca -containing human urine samples (500–700 ml per sample), obtained from a study of postmenopausal women conducted by MH Bhattacharyya at Argonne National Laboratory, were processed by the modified large-volume protocol in MH Bhattacharyya's laboratory. Twenty-six ^{41}Ca -containing human urine samples (400 ml urine per sample), obtained from a study of postmenopausal women conducted by C Weaver at Purdue University, were processed by the standard large-volume protocol in C Weaver's laboratory. All precipitates were analyzed for $^{41}\text{Ca}:\text{Ca}$ ratio by AMS at the Purdue PRIME Lab. The samples for this comparison, identified retrospectively, comprised the 62 samples from the latter two studies that were analyzed during the same AMS run on the same two sample wheels. Each study was approved by the appropriate Institutional Review Board (IRB). Signed consent forms were obtained from all study subjects.

Analysis of $^{41}\text{Ca}:\text{Ca}$ Ratios by AMS

Each calcium precipitate was dissolved in 50 mL of 0.25M HNO_3 solution and chromatographically purified by cation-exchange using Bio-Rad AG 50W-X8 resin. Calcium fluoride (CaF_2) was precipitated with hydrofluoric acid (HF), washed, dried in a vacuum oven, loaded into aluminum sample holders, and inserted into the ion source of the

accelerator mass spectrometer (AMS) to obtain the $^{41}\text{Ca}:\text{Ca}$ ratio in the Purdue PRIME Laboratory (Jackson et al. 2001). The mean value of each $^{41}\text{Ca}:\text{Ca}$ isotope ratio was determined by normalizing the measured value against the measured values of two standards, one measured before and one measured after each unknown. The two standards used had a $^{41}\text{Ca}:\text{Ca}$ ratio of either 3.7×10^{-10} or 2.0×10^{-9} . Each sample was measured multiple times, and the uncertainty was calculated from counting statistics and systematic errors (Elmore et al., 1984).

Samples for the Between-Study Comparison were analyzed before those for the Split-Sample Study, and a new detector/software combination was in place at the Purdue AMS when samples for the Split-Sample Comparison were run. This new detector/software combination tolerated higher levels of interference and did not generate a separate value for tails, as reflected in results reported in Table 2, Split-Sample Comparison. All AMS analyses *within* a given comparison (i.e., Split-Sample Comparison, Between-Study Comparison) were conducted over a single two-day period, with each set of samples present on the same one or two sample wheels. This approach ensured that all samples within a given comparison were run under similar instrument conditions. In addition, for the Split-Sample comparison, a sample prepared by one method was analyzed and then immediately followed by a sample prepared by the other method, with the order randomized, to decrease any effects of fluctuations in the run conditions.

Additional Methods

To determine total recovery of urine calcium in the precipitate, samples of urine were diluted with 0.1 N HCl/0.9% LaCl_3 , and urine calcium concentrations were determined by flame atomic absorption spectrometry (Varian SpectrAA 220, air/acetylene flame, deuterium background correction) (Varian Analytical Instruments, Sugar Land, TX) (Trudeau and Freier, 1967). Ca standards were prepared in 0.1 N HCl/0.9% LaCl_3 . Elemental analyses for Mg and K were conducted by ICP-MS (Thermo Fisher iCAP 6300 ICP mass spectrometer).

RESULTS

The recovery of urine calcium in the oxalate precipitate using the modified protocol was nearly complete: $96\% \pm 3\%$ (mean $\pm$ SD, $n=3$ separate urine samples, each from a different individual). Regarding purity, calcium oxalate monohydrate accounted for $66 \pm 14\%$ of the total weight of the precipitate (mean $\pm$ SD, $n=3$ separate urine samples). Magnesium precipitated along with calcium for both protocols: Mg:Ca ratios (weight percent) [mean $\pm$ SE (n)] for the modified vs. standard protocols were $9\% \pm 6\%$ (9) vs. $37\% \pm 5\%$ (9) for the urines from the Split-Sample Comparison and $34\% \pm 8\%$ (33) vs. $76\% \pm 31\%$ (8) for urines from the earlier Between-Study Comparison.

Table 2 presents AMS results that were obtained when calcium oxalate was precipitated by the modified protocol vs. the standard protocol, for two separate comparisons. Beyond its simplicity, one of the biggest advantages of the modified protocol was the significant increase in average ion current. For the split-sample comparison, the average ion current was 20% higher for the modified than the standard protocol samples ($p<0.05$, two-tailed, independent t-test for groups with unequal variances), and twice as many samples prepared

by the modified protocol had high ion currents (>500 nA) (Table 2). For the between-study comparison, this advantage resulted in about half as many modified protocol samples with low current (<150 nA) and five times as many with high current (>500 nA). Most important, no samples prepared by the modified protocol fell in the ‘unreliable’ category. In contrast, two of the 26 samples prepared by the standard protocol had currents <75 nA, typical of the ~5–10% of standard protocol samples whose results are not used because ion current values are too low.

The overall interference rate in the detector – counts from everything that is not ^{41}Ca – was significantly lower for the modified protocol samples in the Split-Samples Study, even though their total ion current values were higher (Table 2). Similarly for the Between-Study Comparison, the *relative* amount of interference (cps per nA ion current) was also lower for the modified protocol samples, because their ion currents were higher: 0.24 cps/nA vs. 0.34 cps/nA for modified vs. standard protocol samples, respectively. In addition, for the Between-Study Comparison, which gave output on tails rate, samples prepared by the modified method had a count rate in the tails region that was significantly lower than did those prepared by the standard method (116 cpm vs. 183cpm) ($p < 0.05$, two-tailed, independent t-test for groups with unequal variances) (Table 2). This decrease in tail was largely due to the significantly lower K:Ca ratio of the calcium precipitate for these samples (weight percent): $0.44\% \pm 0.1\%$ (33) vs. $3.6\% \pm 1.0\%$ (8) for the modified vs. standard protocol samples, respectively (mean $\pm$ SE for number of samples in parentheses) ($p < 0.05$). In fact, the spectra from anode 3 in the gas ionization detector (Knies and Elmore, 1994) showed that the ^{41}K peak was *much smaller* than the ^{41}Ca peak for a modified protocol sample, while the ^{41}K peak for a comparable standard protocol sample was *somewhat larger* than the ^{41}Ca peak (data not shown). When the two protocols were applied to the *same* samples as in the split-sample comparison, a substantive decrease in K:Ca ratio was still achieved with the modified protocol (mean weight percent): $0.08\% \pm 0.03\%$ (8) vs. $0.54\% \pm 0.20\%$ (8) for the modified vs. standard protocol samples, respectively (mean $\pm$ SE for number of samples in parentheses).

As shown in Table 3 (top), all nine Split-Urine samples gave the same ^{41}Ca : Ca ratio independent of the method applied for preparation of the calcium precipitate. In addition, the ratio measurements were highly precise independent of protocol, with mean RUC values of 3.5% to 4.0%. In contrast, the ^{41}Ca : Ca ratio measurements for the Between-Study Comparison samples were not as precise, in particular for samples prepared by the standard protocol. The latter samples had mean RUC values of 8.2% to 9.0% -- two- to three-fold higher than RUC values for the modified protocol samples (Table 3, bottom). The standard protocol samples retained the higher uncertainty values even when the ^{41}Ca :Ca ratios were matched as closely as the two datasets would allow (Subset 1 vs. Subset 1A, Table 3, bottom).

DISCUSSION

The modified protocol reported here was devised based on the early literature on gravimetric analysis of calcium in biological samples (McCrudden, 1910; Stohl and Pedley, 1921; Washburn and Shear, 1932). It offers an optimal method to obtain a calcium oxalate

precipitate from a large volume of human urine that served well for ^{41}Ca analysis by accelerator mass spectrometry at Purdue University. A cumbersome and expensive filtration step was eliminated, while significantly increasing the quality of the AMS measurement.

Compared to previously published low-volume protocols (Fitzgerald et al., 2005; Freeman et al., 1995; Freeman et al., 1997; Lin et al. 2004), the modified protocol generates a large amount of calcium precipitate without addition of exogenous calcium. While addition of stable calcium to low-volume samples produces a consistent sample size and uniform current output for the AMS measurement, this addition sacrifices sensitivity by decreasing the $^{41}\text{Ca}:\text{Ca}$ ratio. The modified protocol minimizes the amount of ^{41}Ca that needs to be administered to subjects and maximizes the amount of time after dosing during which samples have $^{41}\text{Ca}:\text{Ca}$ ratios that are well above background. The latter feature is significant for studies that require long-term follow up of subjects, for example, for research into cancer survivor's bone health.

The sets of samples compared in this study were each measured during the same time period during the same AMS run under similar foil and ion conditions. This approach minimized differences that might otherwise be attributable to differences in instrument parameters. Despite the fact that the samples prepared with the modified method had higher ion currents, values for both total interference rate (cps) for the split-urine samples and tails rate (cpm) for the between-study samples were significantly lower ($p < 0.05$). The decrease in tails rate would translate to a significant decrease in background. The mean K:Ca ratio for the standard protocol samples from the between-study comparison (3.6% w/w) was at least seven-fold higher than for all other samples in Table 3 (0.5% w/w). These higher K:Ca ratios would translate to greater interference from ^{41}K , as documented by the AMS spectra, most likely contributing to the higher uncertainty values obtained for these standard protocol samples (Table 3, bottom).

Simplified, faster chemical processing, higher ion currents, and lower interference rates are key factors for translation of this technology to clinical applications. The modified protocol described in this report was recently implemented in a clinical trial whose aim is to gain insight into the acute (short-term) and prolonged (long-term) bone complications associated with radiation and chemotherapy in cancer survivors (SK Hui, data not shown). By extension, this detailed and focused technical report on sample methodology will help others to test the application of ^{41}Ca in a clinical laboratory setting as a diagnostic tool to monitor patients' bone health, with the ultimate goal to limit morbidity from fracture and improve quality of life among cancer survivors and the elderly.

Acknowledgments

We would like to acknowledge Mr. Tom Kubley, Purdue University, for organizing operation of the AMS during these measurements; Ms. Anna Kempa-Steczko, Purdue University, for preparing the oxalate precipitates; and Dr. Manju Sharma, University of Minnesota, for helping with urine chemistries. Research for MHB, JJM, and SPC was funded by GHSU start-up funds (Departments of Orthopedic Surgery and Medicine) and Phillip Morris USA, Inc. and Phillip Morris International. Research at Purdue University (GSJ, CMW, JE) was funded by Grant P50-AT00477 from the National Institutes of Health. Research at University of Minnesota (SKH) was funded by the National Institute of Child Health and Human Development (1K12-HD055887-01), National Institute of Arthritis and Musculoskeletal Disease (RO3 AR055333-02), and Cancer Center Support Grant P30 CA77398.

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Highlights

- A method was optimized to precipitate calcium oxalate from large-volume urine samples
- The modified protocol resulted in AMS measurements with significantly higher ion current and decreased interference
- Result is lower effort and cost and higher sensitivity for bone remodeling studies

Table 1**Modified Large-Volume Protocol for Calcium Isolation from Human Urine for ^{41}Ca Analysis by AMS^a**

Step	Detailed Description (per 100 ml urine)	Explanation
1. Acidify urine in collection bottle	Add 7.5 ml 12 N HCl to urine collection bottle, acidifying urine to ~0.8M acid.	Acidified urine can be stored at room temperature, nonbiohazardous, calcium maintained in solution
2. Add hot ammonium oxalate	Add 25 ml saturated ammonium oxalate (~5%) heated to 90°C	Precipitation from hot solution produces purer precipitate than from room temperature solution
3. Add sodium acetate	Add 25 ml 10% sodium acetate	Improves precipitate properties
4. Mix well	Mix by rotation and inversion of urine bottle	Initiates precipitation process ^b
5. Add ammonium hydroxide	Add ~10 ml conc. ammonium hydroxide (14.5 M). Verify pH 11 using pH paper; add more NH_4OH if needed.	
6. Mix well	Mix by rotation and inversion of urine bottle	
7. Place in cold room overnight	Tilt urine bottle at 45° angle and place in cold room. Allow to settle overnight.	Precipitate settles in corner for easy decanting of supernatant ^b
8. Decant supernatant into waste container, leaving precipitate (ppt) plus ~20 ml supernatant	Self-explanatory	Ppt transfer need not be complete – final AMS measurement, ratio of ^{41}Ca to Ca, is unaffected by recovery
9. Transfer ppt from urine collection bottle to 50-ml centrifuge tube	Suspend ppt in the ~20 ml residual supernatant and pour into tared 50-ml PE centrifuge tube	
10. Complete transfer of ppt to 50-ml tube and centrifuge	Rinse urine bottle with 5% ammonium oxalate twice (2×10 ml) to complete transfer of ppt to centrifuge tube. Centrifuge (2000×g, 5 min) and decant supernatant.	
11. Wash ppt 2x with dilute ammonium oxalate and centrifuge	Suspend ppt in 20 ml 0.5% ammonium oxalate (1:10 dilution of 5% solution), centrifuge as above, pour off supernatant. Repeat.	Removes sodium and potassium, including ^{41}K , from calcium oxalate precipitate
12. Dry ppt with acetone	Add 20–30 ml acetone and thoroughly suspend ppt, centrifuge, pour off supernatant, allow ppt to dry. Re-weigh tube and subtract tare weight to obtain weight of dried ppt.	Final 50-ml centrifuge tube, with precipitate, can be capped and shipped for AMS analysis

^aTypically applied to ~700-ml urine sample stored in 1-L wide-mouth polyethylene bottle. Amounts in Steps 1–5 are ‘per 100 ml urine’; amounts in remaining steps refer to entire precipitate, independent of starting urine volume.

^bIf precipitate was formed at pH 3–5, Ca was pure (no Mg), but precipitates did not consistently settle by gravity.

Table 2AMS results for urine samples processed by modified vs. standard protocol^a

Parameter	Modified Protocol	Standard Protocol
Split-Sample Comparison^b		
Samples Analyzed: n	18	18
Ion Current: nA	543 ± 21 [*]	452 ± 34
Low Current <150 nA: n (%)	0	0
High Current >500 nA: n (%)	11 (61)	5 (28)
Unreliable ^d <75 nA: n (%)	0	0
Interference ^e : cps	452 ± 45 [*]	694 ± 65
Between-Study Comparison^c		
Samples Analyzed: n	36	26
Ion Current: nA	431 ± 24 [*]	307 ± 34
Low Current (<150 nA): n (%)	2 (6)	5 (19)
High Current (>500 nA): n (%)	15 (42)	3 (12)
Unreliable (<75 nA): n (%)	0 (0)	2 (8)
Interference ^e : cps	105 ± 5	104 ± 3
Tails ^f : cpm	116 ± 10 [*]	183 ± 30

^{*} Mean for modified protocol significantly different from mean for standard protocol (twotailed, independent T-test for groups with unequal variances; $p < 0.05$).

^a Values with uncertainties are mean ± SE for number of samples analyzed, n. For values given as number of samples, n, percentages are shown in parentheses

^b Urines for Split-Sample Comparison were nine individual samples each split and processed by the two protocols, as described in text. Number of samples analyzed is 18 because each sample for a given protocol was again split, and each split was analyzed individually by AMS.

^c Urines for Between-Study Comparison encompass 62 samples: 36 ANL-Study samples and 26 Purdue-Study, as described in text.

^d "Unreliable" indicates sample produced < 75 nA of ion current, which translates to < 10 nA of analyzed beam on the high energy side. These currents are too low to give reliable results.

^e Interference is the total count rate of everything in the detector. Note that the new detector/software combination in place in the AMS when split-sample urines were run tolerated higher interference rates.

^f Tail value is the counts per minute of interferences that made it past the Purdue PRIME Lab hardware gates and were gated out by the data analysis software. Note that the new detector/software combination in place in the AMS when split-sample urines were run did not provide a separate tail value.

Table 3⁴¹Ca:Ca ratios for calcium precipitates prepared by modified vs. standard protocol^a

Protocol	Sample	⁴¹ Ca:Ca ×10 ¹⁰	%RUC
Split-Sample Comparison^b			
Modified	1	0.641 ± 0.022 (2)	3.5
Standard	1	0.656 ± 0.025 (2)	3.8
Modified	2	0.528 ± 0.019 (2)	3.7
Standard	2	0.560 ± 0.036 (2)	6.8
Modified	3	0.475 ± 0.022 (2)	4.6
Standard	3	0.478 ± 0.033 (2)	6.8
Modified	4	0.523 ± 0.014 (2)	2.7
Standard	4	0.538 ± 0.015 (2)	2.7
Modified	5	0.346 ± 0.013 (2)	3.8
Standard	5	0.332 ± 0.012 (2)	3.6
Modified	6	0.416 ± 0.025 (2)	6.0
Standard	6	0.423 ± 0.019 (2)	4.5
Modified	7	32.30 ± 0.75 (2)	2.3
Standard	7	28.76 ± 0.74 (2)	2.6
Modified	8	54.9 ± 1.4(2)	2.6
Standard	8	52.3 ± 1.3 (2)	2.6
Modified	9	3.18 ± 0.077 (2)	2.4
Standard	9	3.26 ± 0.092 (2)	2.8
Between-Study Comparison^c			
Modified	All samples (ANL study)	4.05 ± 0.150 (36)	3.7
Standard	All samples (Purdue study)	1.06 ± 0.096 (26)	9.0
Modified	Subset 1 (ANL study) ^d	2.01 ± 0.060 (3)	3.0
Standard	Subset 1 (Purdue study) ^d	1.46 ± 0.132 (12)	9.0
Modified	Subset 1 (ANL study) ^d	2.01 ± 0.060 (3)	3.0
Standard	Subset 1A (Purdue study) ^e	1.87 ± 0.153 (3)	8.2

^aValues for ⁴¹Ca:Ca ratios are mean ± UC (n), with UC = uncertainty of AMS measurement, as described in text; n = number of samples. %RUC = relative uncertainty expressed as percentage of mean.

^bSplit urine samples are the nine urine samples identified in Table 2, footnote b.

^cBetween-Study samples are the 62 urine samples identified in Table 2, footnote c. Because mean ⁴¹Ca:Ca ratio was higher for Argonne National Laboratory (ANL) study, Subsets 1 and 1A, which have closer mean values (as described in footnotes d and e), were also evaluated

^dSubsets 1 include all Between-Study urine samples with ⁴¹Ca:Ca ratios (×10¹⁰) between 1.0 and 2.1; they provide largest number of samples with better equality of ⁴¹Ca:Ca means for the two protocols.

^eSubset 1A includes the three samples from the Purdue set with the highest ratios; it provides the best possible equality of ⁴¹Ca:Ca means, although with a small number of samples in each group (3).