

# Comparison of Zinc Reduction with Platinum Reduction for Analysis of Deuterium-Enriched Water Samples for the Doubly Labeled Water Technique

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## Abstract

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**Objective:** Isotope ratio mass spectrometry of hydrogen and oxygen is frequently used to determine total energy expenditure (TEE) using doubly labeled water. Conventionally, hydrogen isotope ratio is determined in hydrogen gas generated from water samples using zinc reduction. We compare this with a new automated platinum method to determine the ratios of hydrogen isotopes in deuterium-enriched water samples.

**Research Methods and Procedures:** The platinum method of sample preparation was compared with the zinc method in three ways: analytical variation in deuterium enrichment (within sample;  $n = 51$ ), analytical variation in TEE estimates (within sample set;  $n = 10$ ), and level of agreement of TEE estimates between both methods ( $n = 14$ ).

**Results:** For the zinc method, the standard deviation for multiple sets of triplicate  $^2\text{H}_2\text{O}$  sample analysis was  $\pm 4.36\%$  and  $\pm 2.07\%$  for platinum. The correlation between TEE estimates when sample sets were analyzed in duplicate was  $r = 0.89$  for zinc and  $r = 0.83$  for platinum. The intercept and slope of the regression line were significantly different from the line of identity for duplicate TEE estimates by zinc but were not different from the line of

identity for platinum. After correction for the intra-assay variation of each method, the correlation between zinc and platinum for TEE was 0.77, and the intercept, but not the slope, of the regression was significantly different from the line of identity. The mean difference between the zinc method and the platinum method was 56 kcal/day, and the 95% confidence interval was  $-438$  to 550 kcal/day.

**Discussion:** These data suggest that the platinum method is at least as reliable as the zinc method as a sample preparation technique for isotope ratio mass spectrometry of deuterium-enriched water samples. The platinum method is also less costly and less labor-intensive than the zinc method.

**Key words:** zinc, platinum, reduction, deuterium, energy expenditure

## Introduction

Determination of energy expenditure is fundamental to investigations of human energy metabolism, particularly when examining the adaptation of energy metabolism to conditions such as growth and development, aging, overfeeding, underfeeding, and physical activity. The generally accepted technique for measuring energy expenditure in free-living human subjects is the doubly labeled water method. Used in a variety of circumstances, the popularity of this method in human studies has grown rapidly in the last 15 years through its ease of use and noninvasive nature.

The principle underlying the doubly labeled water technique is based on the kinetics of two stable isotopes of water, namely  $^2\text{H}_2\text{O}$  and  $\text{H}_2^{18}\text{O}$ . It is assumed that water labeled in the oxygen position is eliminated from the body as both  $\text{CO}_2$  and  $\text{H}_2\text{O}$ , whereas that labeled in the hydrogen position is eliminated as a function of water turnover only (1). The difference between the two elimination rates is, therefore, a measure of  $\text{CO}_2$  flux. Oxygen consumption can

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be calculated from the rates of CO<sub>2</sub> production, together with food quotient data (measured or theoretical), thus allowing rates of energy expenditure to be derived based on the equation of de Weir (2).

One of the technical limitations of the doubly labeled water method is the laborious, expensive, and time-consuming nature of <sup>2</sup>H analysis of water samples. Conventionally, deuterium-enriched water samples are prepared for isotope ratio mass spectrometric analysis off-line by the zinc reduction method (3). At high temperatures the zinc reacts quantitatively with water, producing H<sub>2</sub> with an isotopic composition equal to that of the water. More recently a less complicated on-line method has been established that equilibrates water with hydrogen gas in the presence of a platinum catalyst (4). Comparison of these two methods has previously been examined using five deuterium-enriched water standards (5). The same author also reported rates of energy expenditure by the two methods in a single volunteer (5). However, there have not been any other substantial studies that have compared the new platinum equilibration method with the conventional zinc reduction technique. Additional information and comparisons of the two methods are needed for the platinum equilibration method to be accepted as an alternative to the zinc method for preparation of deuterium-enriched water samples for isotope ratio mass spectrometry. Therefore, the objective of this study was to compare total energy expenditure (TEE) by doubly labeled water arising from either the zinc or platinum equilibration procedures and to determine whether these methods of <sup>2</sup>H<sub>2</sub>O sample preparation yield comparable results for estimates of TEE in human subjects.

### Research Methods and Procedures

The study used data collected from children and adults who were previously involved in studies of energy metabolism in our laboratory. Rates of energy expenditure using the doubly labeled water technique were monitored routinely in these subjects as an integral part of longitudinal studies of energy metabolism. The protocol for the doubly labeled water technique was in accordance with recommendations by the International Atomic Energy Agency report (6). Briefly, subjects reported to the General Clinical Research Center after an overnight fast, and a baseline urine sample was obtained. Subjects then consumed an oral dose of doubly labeled water, prescribed according to body mass (0.15 g of H<sub>2</sub><sup>18</sup>O and 0.075 g of <sup>2</sup>H<sub>2</sub>O per kg of body mass). For each subject's dose, a weighed 1:400 dilution of the dose was prepared and stored at -70 °C for later analysis by isotope ratio mass spectrometry. Similarly, samples of the water used for dilution were also stored at -70 °C for later analysis. Urine samples were collected from subjects at the beginning (Day 1) and end (Day 14) of the doubly labeled water study period. Two samples were obtained on each

occasion, at least 3 hours apart, and all samples were stored in sealed containers at -70 °C.

Samples were prepared and analyzed in triplicate by isotope ratio mass spectrometry. On one occasion, only the CO<sub>2</sub> equilibration technique was used to prepare samples for <sup>18</sup>O-enrichment analysis (7). These <sup>18</sup>O analyses were used in all subsequent calculations. Samples for <sup>2</sup>H analysis were prepared for isotope ratio mass spectrometry by the two methods under comparison: using the conventional zinc equilibration method and using the more recent platinum system.

For the zinc equilibration method, hydrogen gas was prepared from the samples using an off-line zinc reduction method previously described by Wong and Klein (8). 3 μL of sample (water, diluted dose, or urine) were dispensed into quartz reduction vessels over a stream of nitrogen gas. The base of the vessels contained 100 mg of zinc (Biogeochemical Laboratories, University of Indiana, Bloomington, IN). Samples were frozen with liquid nitrogen, and the vessels were evacuated and then sealed. Reduction of the samples was achieved by heating the vessels at 500 °C for 30 minutes. The resulting hydrogen gas was introduced into a dual inlet, stable isotope ratio mass spectrometer (OPTIMA; Micromass Inc., Beverly, MA) and analyzed for deuterium enrichment.

For the platinum method, equilibration of the samples with hydrogen gas was achieved in the presence of a platinum catalyst using an on-line system. We used a commercially available sample preparation device (Multiprep; Micromass Inc.), specifically for use with dual inlet, stable isotope ratio mass spectrometers. The computerized system, comprising a temperature controlled sample rack, an auto sampler, and a water trap, was configured to equilibrate hydrogen with water samples for <sup>2</sup>H analysis. 200 μL of sample (water, diluted dose, or urine) were dispensed into reaction vials (Alltech, Deerfield, IL) containing glass capillary tubes filled to a depth of 3 mm with platinum black (Aldrich, Milwaukee, WI). The vials were sealed with septum-lined caps (Alltech) and were incubated at 40°C for 6 hours. Hydrogen gas was automatically injected into the vials and, following the equilibration reaction, the preprogrammed automatic sampler transferred the equilibrated gas from the headspace of the vial to the water trap for removal of any water vapor. The drying process took an average of 4 minutes, after which the gas was transported from the water trap to the inlet system of the mass spectrometer (OPTIMA) for deuterium enrichment analysis.

The equation applied to calculate CO<sub>2</sub> production utilized individual dilution spaces and turnover rates for both <sup>18</sup>O and <sup>2</sup>H (using either zinc or platinum data), as previously described (9). To convert CO<sub>2</sub> production to energy expenditure, the equation of de Weir (2) was used, assuming a constant food quotient of 0.88.

The zinc method was compared to the platinum method at the following three levels:

- 1) Analytical variation for deuterium enrichment was assessed from multiple sample reanalysis using the within-method variation for the same sample analyzed in triplicate using either the zinc or platinum method. For each method, the average standard deviation and coefficients of variation from triplicate analysis across a range of deuterium enrichments was used.
- 2) Analytical variation for assessment of TEE was assessed in 10 subjects (2 adults; 8 children) using the within-method variation for estimates of TEE when the same set of samples from the same subject were analyzed on two separate occasions using either the zinc or platinum method. For each method, the standard deviation of the difference between measurement 1 and measurement 2, known as the “repeatability coefficient” (10), for pairs of energy expenditure data in the 10 healthy individuals was calculated.
- 3) The level of agreement for TEE using either the zinc or platinum methods for determining TEE was examined in 14 healthy individuals (2 adults; 12 children) using the statistical methods recommended by Bland and Altman (10). In addition, the relationship between estimates of TEE by each method was examined by correlation and regression analysis. The correlation between estimates of TEE by each method was adjusted for the intra-assay variation of each method by dividing the correlation coefficient by the square root of the product of the correlation coefficients of duplicate estimates of TEE by each method, i.e.,  $r(\text{Zn} - \text{Pt}) / \sqrt{[r(\text{Zn} - \text{Zn}) \times r(\text{Pt} - \text{Pt})]}$ .

## Results

### *Analytical Variation for Deuterium (within Sample)*

We analyzed data from 51 sets of samples generated from doubly labeled water studies, including water standards, diluted dose standards, baseline urine samples, and urine samples collected on Days 1 and 14 after dosing. Each sample was analyzed in triplicate using the zinc or platinum methods. For the zinc method, the average standard deviation for multiple sets of triplicate  $^2\text{H}_2\text{O}$  sample analysis was  $\pm 4.36\%$ , which was equivalent to approximately 0.8% of the mean enrichment of the water, dose, and urine samples. For the platinum equilibration method, the average standard deviation was  $\pm 2.07\%$ , which was equivalent to approximately 0.35% of the mean enrichment.

### *Analytical Variation for TEE Using the Zinc and Platinum Methods (within Sample Sets)*

The repeatability of TEE using the zinc and platinum methods for  $^2\text{H}_2\text{O}$  analysis is presented in Table 1. For both methods, the mean difference in energy expenditure between measurement 1 and measurement 2 was not significantly different from zero, thus satisfying the criterion for calculating repeatability coefficients. The repeatability coefficient (i.e., the standard deviation of the difference between measurement 1 and measurement 2) for the zinc method was  $\pm 169$  kcal/day and for the platinum method was  $\pm 225$  kcal/day. There was no significant difference in TEE values when using the zinc compared to the platinum method by paired  $t$  test (1854 vs. 1769 kcal/day;  $p = 0.4$ ). Repeatability of the dilution space ratios for each method is given in Table 2. The dilution space ratio coefficients of

**Table 1.** TEE in 10 subjects when the same samples were analyzed in triplicate on two occasions by the zinc and on two occasions by the platinum equilibration methods

Subject	TEE (kcal/day)					
	Zinc			Platinum		
	Analysis 1	Analysis 2	$\Delta$	Analysis 1	Analysis 2	$\Delta$
1	1739	1512	227	1234	1051	183
2	1587	1839	-252	1732	2266	-534
3	1710	1658	52	1273	1292	-19
4	2004	2134	-130	2327	2289	38
5	2283	2642	-359	1777	2081	-304
6	1854	1789	65	1827	1713	114
7	1984	1921	63	2085	2014	71
8	1349	1398	-40	1452	1431	21
9	1929	1961	-32	1927	1823	104
10	1892	1897	-5	1956	1830	126
<b>Mean (SD)</b>	1833 (255)	1875 (346)	-41.1 (169)	1759 (351)	1779 (414)	-20 (225)

**Table 2.** Dilution space ratio data in nine subjects when the same samples were analyzed in triplicate on two occasions by the zinc and on two occasions by the platinum equilibration methods

Subject	Dilution space ratio					
	Zinc			Platinum		
	Analysis 1	Analysis 2	$\Delta$	Analysis 1	Analysis 2	$\Delta$
2	1.09	1.07	0.02	1.01	0.99	0.02
3	1.07	1.07	0.00	1.04	1.03	0.01
4	1.08	1.05	0.03	1.04	1.01	0.03
5	1.01	1.10	-0.09	1.07	1.04	0.03
6	1.05	1.07	-0.02	1.07	1.05	0.02
7	1.08	1.12	-0.04	1.02	1.07	-0.05
8	1.04	1.07	-0.03	1.04	1.05	-0.01
9	1.05	1.10	-0.05	1.06	1.05	0.01
10	1.03	1.06	-0.03	1.05	1.07	-0.02
<b>Mean (SD)</b>	1.06 (0.03)	1.08 (0.02)	0.02 (0.04)	1.04 (0.02)	1.04 (0.03)	0.004 (0.03)

variation for the zinc and platinum methods were 2.3% (SD 1.7) and 1.5% (SD 0.9), respectively. (Subject 1 was excluded from the analysis, because ratios were less than 1.0 and were therefore out of the physiological range). The dilution space ratio was significantly higher using zinc compared to platinum (1.07 vs. 1.04;  $p = 0.04$ ).

#### **Agreement between Zinc and Platinum Equilibration Methods**

The relationship between TEE derived using the zinc method and TEE derived from the platinum method is shown in Figure 1A. After correcting for the intra-assay variation of each method, the correlation between the zinc method and the platinum method for rates of energy expenditure was  $r = 0.77$  (Figure 1A). The regression between TEE by the two methods of analysis was significantly different from the line of identity for the intercept ( $p = 0.04$ ) but not for the slope ( $p = 0.05$ ) (Figure 1A). A limits of agreement approach to the corrected data (10) gave a mean difference between the zinc method and the platinum method of 56 kcal/day, with a 95% confidence interval of -438 to 550 kcal/day (Figure 1B).

### **Discussion**

Analysis of stable isotopes of water was established over half a century ago and has subsequently been simplified and automated. Before analysis can begin, however, water samples require equilibration with the gas phase so that the isotopic information of the water is transferred into the gas phase. The oxygen component of water exchanges isotopic information with  $\text{CO}_2$  and reaches equilibrium relatively quickly. For hydrogen, however, equilibrium is less easily

achieved—the fractionation factor is low and precise temperature control is required (11). Consequently, conventional methods of sample preparation for hydrogen analysis have tended to be laborious and time-consuming. The data presented in this paper compared the conventional zinc reduction method with the more recently developed platinum method of sample preparation for isotope ratio mass spectrometry analysis of  $^2\text{H}$ -enriched water samples generated by the doubly labeled water technique. Previous work comparing the zinc method with the platinum method was limited by a small data set, i.e., five comparisons of deuterated water standards and TEE estimates from just one volunteer (5). The present data are therefore the most comprehensive available to suggest that the hydrogen isotopic composition of water samples can be measured equally well, if not better, using the platinum equilibration method compared with the conventional zinc method.

We compared the performance of the platinum method with the zinc method in three ways: the within-sample analytical variation for deuterium enrichment, the within-sets of samples analytical variation for TEE, and the level of agreement between the zinc and platinum methods. The analytical variation for deuterium enrichment by the platinum method was almost half that by the zinc method ( $\pm 2.07\%$  vs.  $\pm 4.36\%$ ), and linear regression analysis showed that duplicate analysis of TEE estimates and dilution space ratios by the platinum method were more acceptable than were those by the zinc method. High association, however, does not necessarily imply good agreement. To overcome this problem, we also assessed how close the points on plots of duplicate TEE values lay to the respective lines of identity for zinc data and the platinum data (if the points follow the line of identity, then the level of agreement

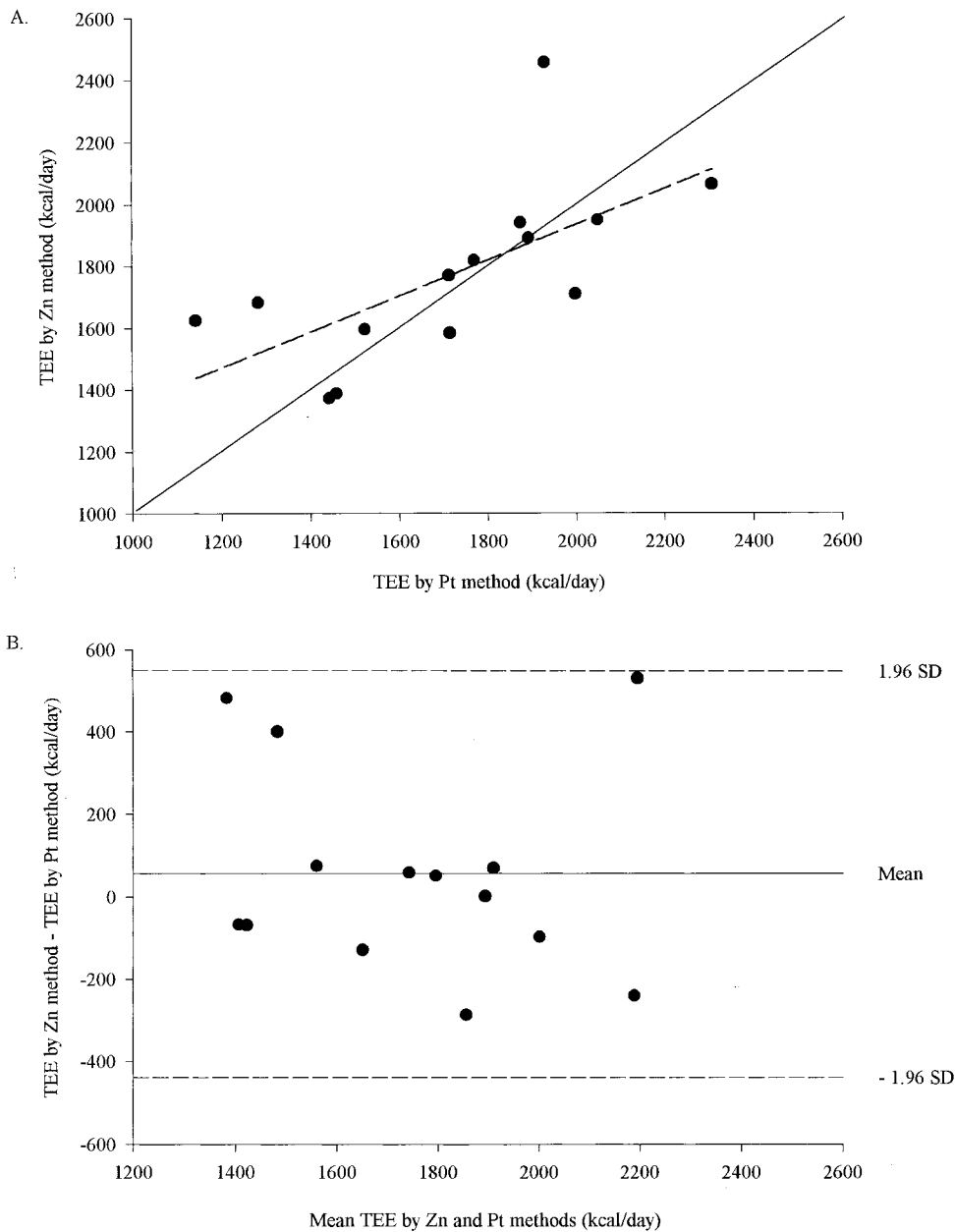


Figure 1. (A) Relationship between TEE by the zinc (Zn) method and TEE by the platinum (Pt) method. Regression line is broken (intercept = 775 (SE 335) kcal/day; slope = 0.58 (SE 0.19)); line of identity is continuous. (B) Difference vs. mean for TEE values using the zinc method and the platinum method. The broken line represents  $\pm 1.96$  SDs, and the continuous line represents the mean difference between methods.

is high). This rigorous approach, of using a correlation coupled with observing the nearness of the points to the line of identity, indicated that the reproducibility of TEE estimates by the platinum method was better than that of the zinc method. The use of the zinc method for estimating TEE has previously been criticized, because it underestimates the deuterium enrichment of urine samples (12). Indeed, the present analysis shows that dilution space ratios are signif-

icantly higher for the zinc method than for the platinum method (1.07 vs. 1.04), suggesting that the former may introduce some systematic error. However, this effect is small and did not affect the TEE values, which were not significantly different when using zinc vs. platinum. We also noted, however, that the zinc method was more imprecise than the platinum method, such that it is likely to be a source of random error.

The relation between TEE by the zinc method of sample preparation and that by the platinum method was based largely on graphical techniques and calculations described by Bland and Altman (10). However, regression analysis was also considered; the intercept (775 kcal/day) was significantly different from zero. The offset between the zinc and platinum methods was similarly reflected in the limits of agreement approach recommended by Bland and Altman (10). This statistical method for assessing the level of conformity between the zinc and platinum methods gave a confidence interval for the differences between TEE estimates by the two methods (−438 to 550 kcal/day). The disadvantage of this approach is that the agreement between the methods appears poorer than it actually is, because the 95% confidence interval is twice as wide as the standard deviation, the most commonly used indicator of variation. Nevertheless, in our opinion the agreement between the zinc method and the platinum method was acceptable, given the intra-assay variation for each method. That is the between-method analytical “noise” was comparable to the within-assay “noise” for either method.

If used in isolation, there are certainly limitations to each of the statistical approaches used in our analysis of the data, and the correct single approach is not straightforward or obvious. However, the strength of our “aggregate” approach is that it allowed several statistical methods of comparison to be used in combination and, thus, probably provides the most complete way to compare the performance of the two methods. Taken together, therefore, statistical analyses of the data indicate that the performance of the zinc method is surpassed by the platinum method and that there is good agreement between the methods. Furthermore, our results show that water samples analyzed by the platinum method of reduction are closer to the interlaboratory mean precision for the doubly labeled water technique than samples analyzed by the zinc method (13).

Not only is the performance of the platinum method preferred to the zinc method, it is also favored because increased sample throughput can be achieved at considerably lower costs for supplies and technician time. In our laboratory, each subject generated a set of seven samples (baseline, two samples on Day 1, two samples on Day 14, and two standards), which were analyzed in triplicate. We estimate that the sample preparation time for the zinc reduction method is ~2 hours for each set of samples (i.e., 21 reaction tubes for seven samples analyzed in triplicate), consisting of 30 minutes to prepare the tubes and fill them with zinc, 60 minutes to load the samples and to freeze and evacuate the tubes, and 30 minutes for the reduction reaction. This is equivalent to 6 minutes of sample preparation time per reaction tube analyzed. In comparison, we estimate that sample preparation time for the platinum method is 15 minutes for an equivalent set of samples or 0.7 minute per tube (i.e., 8.5-fold improvement in efficiency). Regarding

equipment needs, the zinc preparation method required the use of specialized reaction tubes (\$100 each; they are reusable, but they do break and need repair or replacement) as well as an initial outlay for a sample preparation manifold, vacuum pump, and heating block. In addition, the zinc reduction uses zinc metal (\$0.30 per tube) and nitrogen gas (negligible cost per tube). The platinum method requires microtubes (\$0.05 per tube and reusable unless broken), platinum black (\$0.40 per tube and reusable), septa (\$0.26 per tube), and seals (\$0.08 per tube). The major feature that allows a great increase in sample output is the ability of the Multiprep system to analyze samples from an automatic sampler. This increased the number of tubes analyzed per day from 30 to more than 60. There is also the timesaving benefit of using equipment that did not require changing the batch of tubes every nine tubes and then restarting. The Multiprep system also allows some overlap of individual samples during analysis, which is not possible with the zinc reduction method.

Because our major finding was related to precision rather than the accuracy of the zinc method, these findings have very little implication on the quantitative accuracy of data previously reported from our laboratory using the zinc method. Even when recognizing the small difference in TEE estimates between the two methods, we do not currently have any evidence to support which number would be more correct. However, the current findings do imply that some of our previously reported data using the zinc method may contain greater inter- and intraindividual variation (random not systematic). However, the overall effect is small, because the greatest source of variation is physiological and not methodological. For example, we previously reported that the within-subject variation in TEE in adults was  $\pm 12\%$  (14). Using a value of 5% for methodological error in the doubly labeled water technique previously implied that the observed within-subject variation in TEE was mainly physiological at  $\pm 10.9\%$  (square root of  $12^2 - 5^2$ ). If we now assume that the methodological error in the doubly labeled water technique is  $\pm 2\%$ , then we project that if we repeated this study we would observe a variation in TEE of  $\pm 11.1\%$  (square root of  $10.9^2 + 2^2$ ), but the projected estimate of the physiological variation would be unaltered.

Therefore, the platinum method is a cheaper, faster, and more reproducible and efficient process, allowing a 2- to 3-fold increase in throughput, requiring less technical expertise for sample preparation, and, overall, a more analytically reliable and economical alternative to the zinc reduction method.

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