

# Estimate of Uncertainty of Measurement from a Single-Laboratory Validation Study: Application to the Determination of Lead in Blood

MARINA PATRIARCA,\* MARCO CASTELLI, FEDERICA CORSETTI, and ANTONIO MENDITTO

**Background:** Lead is an environmental pollutant, and human exposure is assessed by monitoring lead concentrations in blood. Because the main source of environmental exposure has been the use of leaded gasoline, its phase-out has led to decreased lead concentrations in the general population. Therefore, validated analytical methods for the determination of lower lead concentrations in blood ( $<150 \mu\text{g/L}$ ) are needed. In addition, new ISO standards require that laboratories determine and specify the uncertainty of their results.

**Methods:** We validated a method to determine lead in blood at concentrations up to  $150 \mu\text{g/L}$  by electrothermal atomic absorption spectrometry with Zeeman background correction according to EURACHEM guidelines. Blood samples were diluted (1:1 by volume) with 2 mL/L Triton X-100.  $\text{NH}_4\text{H}_2\text{PO}_4$  (5 g/L) and  $\text{Mg}(\text{NO}_3)_2$  (0.5 g/L) were used as modifiers. Matrix-matched standards were used for calibration.

**Results:** We determined the limits of detection ( $3.1 \mu\text{g/L}$ ) and quantification ( $9.4 \mu\text{g/L}$ ). Repeatability and intermediate imprecision within the range 35–150  $\mu\text{g/L}$  were  $<5.5\%$  and  $<6.2\%$ , respectively. We assessed trueness by use of certified reference materials, by recovery tests, and by comparison with target values from other reference methods (candidate external quality assessment samples). The expanded uncertainty ranged from 20% to 16% (with a confidence level of 95%) depending on concentration.

**Conclusions:** This study provides a working example of the estimate of uncertainty from method performance data according to the EURACHEM/CITAC guidelines. The estimated uncertainty is compatible with quality specifications for the analysis for lead in blood adopted

in the US and the European Union.

© 2004 American Association for Clinical Chemistry

Lead pollution in the environment and workplace has been an ongoing matter of concern. It has been addressed in national and European legislation and directives (1–4) aimed at protecting workers, the general population, and especially young children from the potentially harmful effects of exposure to lead (5,6). The most reliable biomarker of recent lead exposure is the measurement of the concentration of lead in blood, and although invasive, it is still used for both occupational monitoring programs and population surveys. Several techniques can be applied to the determination of lead in blood, such as anodic stripping voltammetry, electrothermal atomic absorption spectrometry (ETAAS),<sup>1</sup> and inductively coupled plasma mass spectrometry (ICP-MS) (7,8).

In most industrialized countries improvements in working conditions and actions taken to minimize the environmental exposure to lead (such as the phase-out of leaded gasoline and improvements in food preparation and packaging) have led to a substantial reduction of lead concentrations in blood in both workers and the general population (9). In the US, a reduction of 78% in the mean lead concentration in blood (from 128 to 28  $\mu\text{g/L}$ ) was observed in phase 1 of the third National Health and Nutrition Examination Survey (1988–1991;  $n = 12\,119$ ) compared with the second such survey (1976–1980;  $n = 9832$ ) (10), and was later confirmed in additional studies (11). In 1998, blood lead concentrations ranging from 5 to 132  $\mu\text{g/L}$  were reported for a sample of 214 nonexposed British individuals (12). In Italy as well, a recent study (13) reported mean (SD) values for lead in blood of 45.1

Clinical Biochemistry Laboratory, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy

\*Author for correspondence. Fax 39-0649903686; e-mail marina.patriarca@iss.it.

Received December 4, 2003; accepted May 17, 2004.

Previously published online at DOI: 10.1373/clinchem.2003.029223

<sup>1</sup> ETAAS, electrothermal atomic absorption spectrometry; ICP-MS, inductively coupled plasma mass spectrometry; EQAS, External Quality Assessment Scheme; SI, International System; CRM, certified reference material; RM, reference material; LoD, limit of detection; LoQ, limit of quantification; RSD, relative SD; and SDp, pooled SD.

(27.4)  $\mu\text{g/L}$  for males and 30.6 (16.7)  $\mu\text{g/L}$  for females compared with median values of 86  $\mu\text{g/L}$  (2.5th–97.5th centiles, 30–230.5  $\mu\text{g/L}$ ) for males and 53.5  $\mu\text{g/L}$  (20–151.5  $\mu\text{g/L}$ ) for females between 1992 and 1996 (14).

However, attention is now being given to the potential health effects of long-term exposure to low lead concentrations, such as cognitive disorders and effects on renal function and blood pressure (15–19). The assessment of such risks is still based on the measurement of the blood lead concentration; therefore, the quality of analytical data at such low concentrations is of the utmost importance. Collaborative work carried out within the framework of the “European Network of Organizers of External Quality Assessment Schemes (EQAS) related to Occupational and Environmental Medicine” (EU Contract SMT 4-CT98-7522) has highlighted large differences in performance among laboratories for the determination of lead in blood at concentrations of 100, 400, and 700  $\mu\text{g/L}$  (20), and few data are available describing the degree of comparability of laboratory results at concentrations  $<100$   $\mu\text{g/L}$ . Currently, in many fields of analytical work, laboratories are formally required (sometimes by law) to demonstrate their competence by seeking accreditation to the appropriate national or international standards, such as CLIA '88 in the US (21) and ISO/IEC 17025 (22) or ISO 15189 (23) in the European Union and other parts of the world. Such standards require laboratories to use validated analytical methods and well-defined internal quality-control procedures and to participate in proficiency testing schemes. In addition, the ISO standards recommend that traceability to International System (SI) units and the uncertainty (24) of the measurements be established whenever possible. Additional guidance on the estimate of the uncertainty of measurement in analytical chemistry is detailed in the EURACHEM/CITAC Guide (25), in particular with respect to the use of performance data already available within a laboratory provided that appropriate quality-assurance measures are in place. Such data should generally include information on the overall precision of the method, the assessment of any significant bias, and estimates of the uncertainties associated with other relevant sources, such as residual matrix effects. A key issue in such an approach is the ability to document the size of any systematic bias affecting the measurement and the uncertainty associated with such an estimate. This can be difficult in occupational and environmental laboratory medicine because the concentrations of analytes to be determined in complex matrices are often close to detection limits and few certified reference materials (CRMs) are available. However, laboratories are still required to perform such analyses and to give an indication of their quality.

Our laboratory has been particularly concerned with the quality of analytical results for the determination of lead in blood because of our involvement with the European surveys of the general population (26, 27). This has led us, as the organizers of a national EQAS for lead in

blood, to investigate the performance of analytical methods (28, 29), to evaluate and certify reference materials (RMs) (30, 31), and to assess the performance of Italian laboratories in this field (32, 33). In this context, the aim of the work reported here was to estimate the uncertainty of our own measurements of low concentrations of lead in blood and to provide a practical example of how data from validation studies can be used for such purposes. In addition, we discuss the different approaches taken to demonstrate traceability to SI units.

## Materials and Methods

### INSTRUMENTATION

Lead in blood was determined by use of a Perkin-Elmer SIMAA 6000 atomic absorption spectrometer equipped with Zeeman background correction, a transverse heated graphite atomizer, and an EDL System 2 lamp for lead. This instrument, because it has an Echelle polychromator and a photodiode array detector, is capable of multielement analyses, using up to four different light sources, but was used in its single-element capacity for this work. In addition to UP-grade argon, the instrument was fed with air as the alternative gas to be used in the ashing step. Automatic pipettes (Gilson, France) with valid calibration certificates and class A volumetric glassware were used for all dilutions.

### REAGENTS, REFERENCE MATERIALS, AND SOLUTIONS

During the study, two certified lead stock standard solutions from Merck KgaA at mean (SD) concentrations of 1008 (2) mg/L and 999 (5) mg/L were used. According to the supplier, traceability of the stock solutions to SI units was assured by traceable gravimetric procedures or by measurement with high-precision ICP-optical emission spectroscopy calibrated against SRM 3128 “lead standard solution” (NIST).

Five working lead standards were prepared by adding to bovine blood, collected in EDTA, known amounts of lead. Intermediate standard solutions at 0, 0.625, 1.25, 2.50, and 3.75 mg/L were prepared in aqueous  $\text{HNO}_3$  (2 mL/L; 65% Suprapure; maximum lead concentration, 1 ppb; Merck) from the 1008 (2) mg/L stock standard solution. We diluted 2 mL of each intermediate standard solution to 50 mL with bovine blood to obtain matrix-matched lead standards at 0, 25, 50, 100, and 150  $\mu\text{g/L}$ . The blood solutions were divided into 1-mL aliquots in Eppendorf vials and stored at  $-20$  °C. The uncertainty of the concentrations of the working standards, calculated according to the EURACHEM/CITAC guide (25) using the spreadsheet method (34), was 0.6%.

A modifier was prepared by dissolving 2.5 g of  $\text{NH}_4\text{H}_2\text{PO}_4$  (99.999%) and 0.25 g of  $\text{Mg}(\text{NO}_3)_2 \cdot 6 \text{H}_2\text{O}$  (99.999+%), both from Aldrich, in 150 mL of ultrapure water. The solution was further purified by the addition of 2 mL of 20 g/kg ammonium pyrrolidine-dithiocarbamate (Merck) in water and extraction with chloroform (Carlo Erba Reagenti); the purified solution was then

brought to a volume of 500 mL. Triton<sup>®</sup> X-100 (Fluka; 2 mL/L) was used to dilute blood samples. Ultrapure water, at 18.2 MΩ/cm, obtained from a Millipore (Elix 3/MilliQ) combined system for water purification, was used throughout the study.

Two CRMs, BCR CRM 194 (Community Bureau of Reference), with a target value of 126 (4) μg/L, (Institute of Reference Materials and Methods, Geel, Belgium) and NIST SRM 955b, with a target value of 40.4 (1.5) μg/L, were used for validating the method.

Other RMs, Seronorm<sup>™</sup> Trace Elements Whole Blood, Level 1 (lot no. 404107) and Level 2 (lot no. MR9067), prepared from human blood, were obtained from Sero AS and reconstituted with 5 mL of ultrapure water according to the manufacturer's instructions. Internal control materials at concentrations of 85 μg/L (medium) and 129 μg/L (high) were prepared by mixing known amounts of Seronorm Trace Elements Whole Blood, Level 1 and Level 2.

Human blood samples obtained from 50 individuals undergoing screening programs for blood lead and stored frozen at -20 °C were also included in the validation study. The samples were anonymous and provided by clinicians for the assessment of lead exposure, for which informed consent was provided.

For recovery studies, the 999 mg/L lead stock standard solution was diluted with aqueous 20 mL/L HNO<sub>3</sub> to obtain solutions containing 0.62 and 1.24 mg/L. We diluted 1 mL of 20 mL/L HNO<sub>3</sub> or 1 mL of each solution containing added lead to 25 mL with bovine blood to obtain a sample with no added lead and two solutions, one containing 25 μg/L lead and the other 50 μg/L lead.

Blood samples provided as part of EQAS (35–37) were also analyzed to provide additional information on the trueness of the method at different concentrations and its long-term performance.

#### GRAPHITE FURNACE ATOMIC ABSORPTION ANALYSIS

A blank (ultrapure water), blood samples, and matrix-matched working standards were diluted 1:1 with 2 mL/L Triton X-100 by use of a Gilson automatic pipette and were analyzed in duplicate. The autosampler was programmed to pipette a volume of 15 μL of modifier, followed by 15 μL of the diluted blood sample or standard, and the two (total volume, 30 μL) were simultaneously injected into the furnace. The instrument settings are shown in Table 1, and the graphite furnace program is shown in Table 2. A calibration curve was obtained daily as follows: the absorbance given by the blood with no added lead was subtracted from the readings for the other calibration materials, and the regression line of the absorbances (*y*) vs concentrations of the working standard solutions (*x*) was calculated, with the condition imposed that the intercept = 0, i.e., the regression line was forced through the origin. This procedure was adopted to improve the precision of measurements of low concentrations of lead because the observed variations of the

**Table 1. Instrument settings.**

Wavelength	283.3 nm
Signal measurement	Peak area
Replicates	2
Slit	0.7 nm
Sample volume	15 μL
Modifier volume	15 μL
Read delay	0.0 s
Read time	3.0 s

intercept values (typically, ±0.001 absorbance units × s) would have caused variations >5% at lead concentrations <25 μg/L.

## Results

#### LIMITS OF DETECTION AND QUANTIFICATION

The limits of detection (LoD) (38) and quantification (LoQ) (38) were initially calculated from the results of 10 within-run measurements of a water blank diluted in the same manner as the blood samples (reagent blank). The LoD (mean + 3 SD) was 0.9 μg/L, and the LoQ (mean + 10 SD) was 2.0 μg/L. However, because the presence of a blood matrix is likely to diminish precision, additional information was obtained from the analysis, under the same conditions, of a bovine blood sample with very low lead content (12 μg/L). The SD of these measurements was 0.9 μg/L. Combining this SD value with the mean lead concentration observed in the reagent blank (0.4 μg/L), we calculated the method LoD and LoQ as 3.1 and 9.4 μg/L, respectively.

#### LINEAR RANGE AND SENSITIVITY

We assessed the linearity of calibration curves up to 150 μg/L and the variation in sensitivity over the lifetime (8 different runs and 440 firings) of a graphite tube. The results are shown in Fig. 1. The correlation coefficient (*r*) was ≥0.999. The mean (SD) sensitivity as *m*<sub>0</sub> (the mass of the analyte, in pg, required to produce an integrated absorbance of 0.0044 absorbance units × s) was 41.8 (0.9) pg for blood samples and 39.3 (0.8) pg (*n* = 5) for aqueous standards in 2 mL/L HNO<sub>3</sub> analyzed under the same conditions. This accounts for a difference in sensitivity of ~6%. Therefore, calibration with matrix-matched stan-

**Table 2. Graphite furnace program.**

Step	T, °C	Ramp time, s	Hold time, s	Gas	Internal flow, mL/min	Read
1	40	1	20	Argon	250	
2	120	30	30	Argon	250	
3	250	30	30	Argon	250	
4	550	30	60	Air	50	
5	550	1	20	Argon	250	
6	1600	0	6	Argon	0	Yes
7	2400	1	6	Argon	250	
8	20	1	6	Argon	250	
9	2400	1	10	Argon	250	

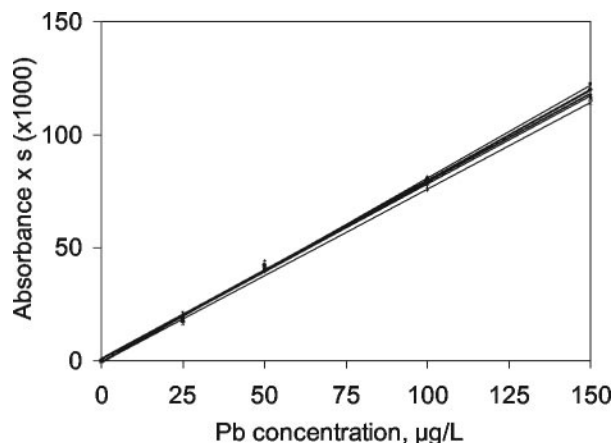


Fig. 1. Calibration curves obtained over the lifetime of a graphite furnace (8 different runs; 440 firings).

dards was still deemed necessary for accurate measurements.

#### PRECISION STUDIES

We assessed repeatability (within-run precision) by analyzing 17 control samples (concentrations range, 12–132  $\mu\text{g/L}$ ) 10 times in the same run. The trend of the percentage relative standard deviation (RSD) with increasing concentration is shown in Fig. 2. These results are comparable to those obtained in previous studies in our

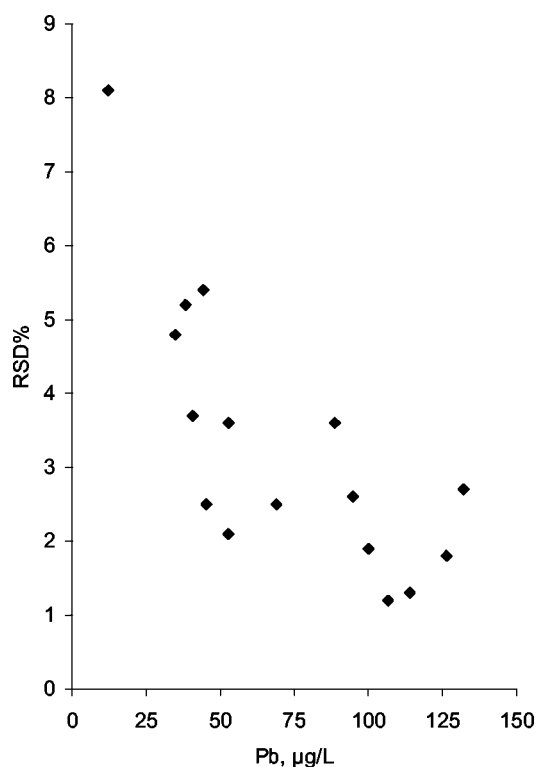


Fig. 2. Trend of RSD with increasing concentration in repeatability studies.

laboratory (28, 29) and to those reported by others (39–41).

The Seronorm Level 1 and the internally prepared medium and high control samples were used to study intermediate precision over a period of  $\sim 16$  months. The analyses were performed by one operator on 29 occasions. The means (SD) concentrations (RSD in parentheses) were, respectively, 34.8 (2.6)  $\mu\text{g/L}$  (7.5%) for Seronorm Level 1, 85.5 (5.1)  $\mu\text{g/L}$  (6.0%) for the medium control, and 128.4 (6.8)  $\mu\text{g/L}$  (5.3%) for the high control. The results are plotted as a control chart in Fig. 3. These values are comparable to those reported in the recent literature for similar concentrations (39–41).

An additional study of intermediate precision was carried out with 50 human blood samples, all of which were analyzed on two different occasions over a period of 2 weeks. The mean (SD) concentration was 44.1 (32.9)  $\mu\text{g/L}$  (range, 12.0–139.1  $\mu\text{g/L}$ ). Intermediate precision was calculated as the pooled SD (SD<sub>p</sub>), according to the formula:

$$SD_p = \sqrt{\frac{\sum_{i=1}^n (x_{i1} - x_{i2})^2}{2n}}$$

and was 2.1  $\mu\text{g/L}$  (4.7%). To compare these data with those obtained on control samples as reported above, we calculated the SD<sub>p</sub> separately for three concentration intervals (12–35  $\mu\text{g/L}$ ,  $n = 29$ ; >35–90  $\mu\text{g/L}$ ,  $n = 14$ ; and >90–139  $\mu\text{g/L}$ ,  $n = 7$ ); the SD<sub>p</sub> values were 1.9  $\mu\text{g/L}$  (8.4%), 2.5  $\mu\text{g/L}$  (4.5%), and 2.0  $\mu\text{g/L}$  (1.8%), respectively. For a subset of these samples ( $n = 20$ ), the results were available for lead determinations carried out 2.5 years before by a different operator using the same procedure and instrumentation. The mean (SD) lead concentration in this subset was 71.2 (34.6)  $\mu\text{g/L}$  (range, 17.4–140.5  $\mu\text{g/L}$ ). The SD<sub>p</sub> was 2.2  $\mu\text{g/L}$  (3.0%). The distribution of the differences between measured values for the same sample as a function of mean lead concentration is shown in Fig.

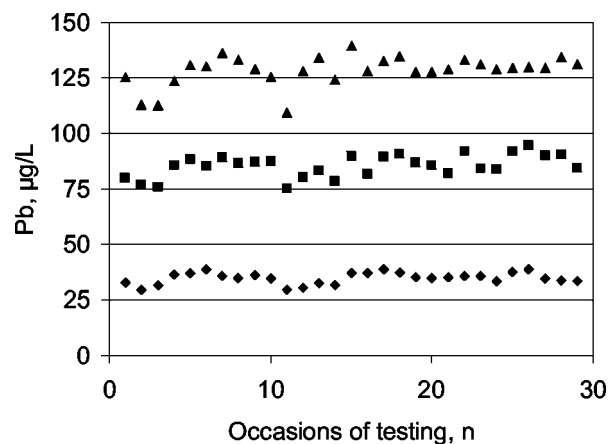


Fig. 3. Intermediate precision study on control samples.

▲, high control; ■, medium control; ◆, Seronorm Level 1.

4 as Bland–Altman plots (42) for the whole group and the subset. These data did not indicate a difference in precision between control and patient samples, and the subset provided additional information on the robustness of the method.

#### TRUENESS

Trueness was assessed from the analysis of CRMs, from recovery studies, and by comparison with target values of other RMs (candidate EQA samples). All samples were analyzed at least 10 times each within the same run (repeatability conditions).

#### ANALYSIS OF CRMs

Two CRMs were available for the determination of lead in blood at concentrations within the range applicable to the present method: BCR CRM 194 (IRMM) for the European Union and SRM 955b Level 1 (NIST) for the US. Both were analyzed in this study to estimate trueness. In addition to

different physical states (lyophilized vs frozen liquid), the target values of these CRMs have been assigned by different approaches. For BCR 194, the target value was obtained in 1985 as the consensus mean of the values provided by 23 expert laboratories using different techniques [flame atomic absorption spectroscopy, ETAAS, anodic stripping voltammetry, and isotope-dilution-MS] (31). According to the certificate accompanying SRM 955b, its target values were determined in 1998 by isotope-dilution-ICP-MS, a technique not available in 1985, and confirmed by ETAAS. According to ISO Guide 35 (43), both approaches are valid; however, under appropriate conditions of use, isotope-dilution-ICP-MS has the potential of a “primary method of measurement” (44). The results (mean, SD, and RSD) of the analysis of the two CRMs are reported in Table 3. For each CRM, the ratio between each measured value and the certified value was calculated. The mean ratio ( $R_m$ ) and its RSD are also reported in Table 3. The agreement with the target value was excellent for BCR CRM 194 ( $R_{m1} = 1.001$ ), whereas for NIST SRM 955b Level 1, we observed a  $R_{m2}$  of 1.139, indicating an apparent positive bias of the method at this concentration.

#### RECOVERY STUDIES

Recovery studies were performed on blood samples to which lead had been added. The traceability of the added amounts (target values) was documented by use of data from the manufacturer’s certificate for the stock standard solution and from the calibration certificates of volumetric glassware and pipettes. The results of analysis of the sample without added lead and the two blood samples with lead added (25 and 50  $\mu\text{g/L}$ ), as the mean (SD), are given in Table 4. For each lead-enriched sample, the difference between each measured value and the mean concentration of the sample without added lead was calculated.  $R_m$  was obtained as the mean of the ratios between each difference and the target value. The  $R_m$  value and RSD are given in Table 4 for each sample without added lead. In both recovery studies,  $R_m$  was  $>1$ .

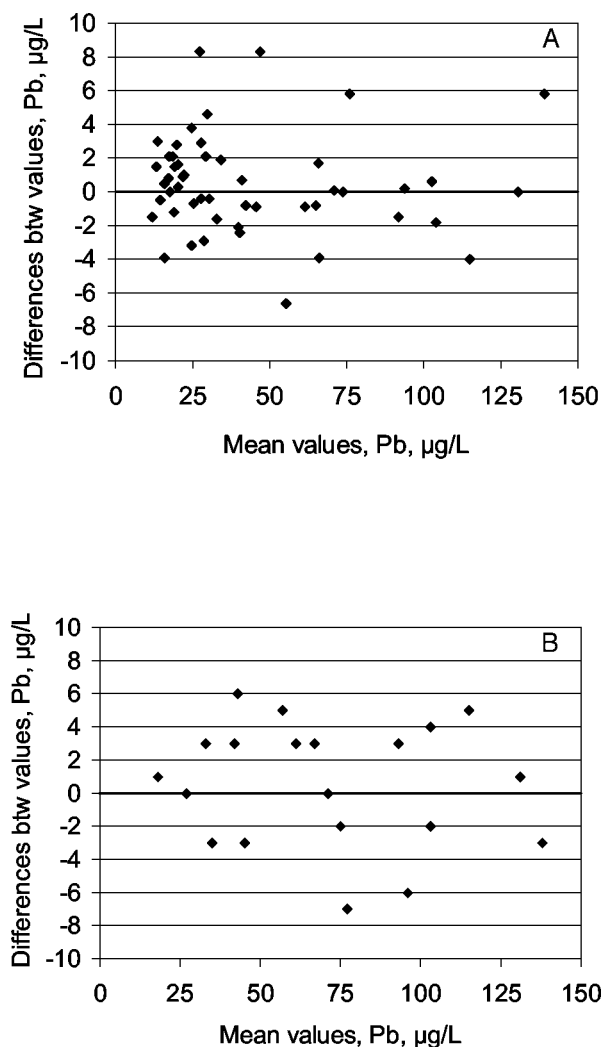


Fig. 4. Intermediate precision study on human blood samples. (A), duplicate analyses, 10–15 days apart; (B), duplicate analyses, 2.5 years apart.

Table 3. Analysis of CRMs.

	BCR <sup>a</sup> CRM 194	NIST SRM 955b Level 1
Certified value $\pm$ U(95%), <sup>b</sup> $\mu\text{g/L}$	126 $\pm$ 4	40.4 $\pm$ 1.5
$u(\text{CRM})^c$	2.04	0.76
Mean, $\mu\text{g/L}$	126.1	45.8
SD, $\mu\text{g/L}$	2.3	2.3
RSD, %	1.8	5.1
n	10	18
Ratio to certified value ( $R_m$ )	1.001	1.139
RSD of $R_m$ , %	1.8	5.6

<sup>a</sup> BCR, Community Bureau of Reference.

<sup>b</sup> Expanded uncertainty, as provided by the manufacturer.

<sup>c</sup> Standard uncertainty of the certified value, calculated dividing the expanded uncertainty (U) provided by the manufacturer by the corresponding value of  $k$  (1.96) for a 95% confidence interval.

**Table 4. Recovery studies.**

	Sample without added lead	Sample with added lead	Recovery, %
Added lead = 25 µg/L			
Target value of added lead ± U(95%), <sup>a</sup> µg/L			25 ± 0.16
Mean measured value, µg/L	12.1	40.6	28.5
SD, µg/L	1.0	1.5	
RSD, %	8.1	3.7	
n	10	10	10
Mean ratio to target value ( $R_m$ )			1.147
RSD of $R_m$ , %			5.3
Added lead = 50 µg/L			
Target value of added lead ± U(95%), µg/L			50 ± 0.24
Mean measured value, µg/L	12.1	69.0	56.9
SD, µg/L	1.0	1.7	
RSD, %	8.1	2.5	
n	10	10	10
Mean ratio to target value ( $R_m$ )			1.150
RSD of $R_m$ , %			1.7

<sup>a</sup> Expanded uncertainty, as provided by the manufacturer.

#### ANALYSIS OF OTHER RMs

In addition to the specific experiments designed to document the trueness of the method by comparison with CRMs with stated uncertainty, we examined the information available from the analyses of other RMs. Nine control samples, prepared for two different EQAS, were analyzed as part of homogeneity and/or certification studies before their distribution to the participants. The target values were obtained either from the data provided by reference laboratories (Scheme A) or from the results provided by all participants (Scheme B). The mean measured value, the SD, RSD, the target value, and the ratio between measured and target value ( $R_s$ ) for each sample are reported in Table 5. A substantial difference from the target value was observed only for the sample at the lowest lead concentration (16 µg/L). However, in this case, the robustness of the target value assigned from consensus data is questionable because such a concentration would be close to or below the detection limits of many of the analytical methods used for routine purposes.

**Table 5. Results of analysis of other RMs.**

RM	Target value, µg/L	Measured value, µg/L	SD, µg/L	RSD, %	n	$R_s$
A-1	35.2	34.7	1.7	4.8	10	0.986
A-2	40.1	45.2	1.2	2.6	10	1.127
A-3	94.0	94.8	2.4	2.6	10	1.009
A-4	97.6	100.2	1.9	1.9	10	1.027
B-1	16	12	0.7	5.8	10	0.750
B-2	51	52.6	1.9	3.6	10	1.031
B-3	54	52.5	1.1	2.1	10	0.972
B-4	107	106.6	1.2	1.2	10	0.996
B-5	119	114	1.3	1.1	10	0.958

#### ESTIMATE OF THE UNCERTAINTY OF LEAD DETERMINATIONS

According to the Eurachem/CITAC Guide (25), the uncertainty of measurement can be estimated from the performance data available from validation and monitoring programs, provided that appropriate quality-control measures are in place and care has been taken to include the relevant sources of uncertainty in the validation study. In such case, the following information can be used to estimate the uncertainty of measurement: (a) the best available estimate of overall precision [ $u(P)$ ]; (b) the best available estimate of overall bias ( $R_m$ ) and its uncertainty  $u(R_m)$ , calculated from the analysis of CRMs and the recovery of amounts of the pure analyte added to samples; and (c) the best estimate of any other components of uncertainty associated with effects incompletely accounted for in the development and validation study, e.g., differences or inhomogeneities in the matrix composition of the real samples submitted for analysis.

For this method, the concentration ( $c$ ) of lead in blood can be represented by a simple mathematical model:

$$c = \frac{A - A_0}{b}$$

where  $A$  is the absorbance of the sample,  $A_0$  is the absorbance of the blank, and  $b$  the slope of a linear calibration function. The main factors affecting the measurement (sample preparation; instrument calibration; day-to-day variation; batches of reagents, standards, and control materials; matrix effects) were expected to be satisfactorily taken into account by the long-term precision studies and detailed studies of the trueness of the method. No other contribution (item c in the previous paragraph) was judged necessary at this stage. The approach followed to combine the information from the

performance studies for the estimate of uncertainty of measurement is outlined below. All terms were expressed as RSD (i.e., relative standard uncertainties).

#### UNCERTAINTY ASSOCIATED WITH THE BEST

##### AVAILABLE ESTIMATE OF OVERALL PRECISION

$u(P)$  was evaluated from the RSD of the results obtained in the intermediate precision studies. The RSD obtained for the three control samples analyzed for the intermediate precision study were compared by use of the  $F$ -test (95% confidence level). Only the RSDs for the control samples Seronorm level 1 and high were found to be significantly different, suggesting that  $u(P)$  varied with concentration. This information was integrated with the data available from the studies on human samples described earlier for three corresponding concentration intervals. The following values for  $u(P)$  were obtained for each concentration range:  $u(P)_1 = 0.082$  (12–35  $\mu\text{g/L}$ );  $u(P)_2 = 0.062$  (>35–90  $\mu\text{g/L}$ ); and  $u(P)_3 = 0.052$  (>90–150  $\mu\text{g/L}$ ), by combining the RSD for each control sample with the relative SDp given above for the human samples (square root of the sum of the squares).

#### UNCERTAINTY OF THE BIAS ESTIMATE CALCULATED FROM THE ANALYSIS OF CRMS AND RECOVERY TESTS

Two CRMs were analyzed to estimate trueness.  $u(R_m)$  was calculated for each CRM with the following formula, taking into account the relative standard uncertainty of the certified value (first term) and the relative uncertainty of the measured value as the RSD of the mean (RSDM, second term):

$$u(R_{m1}) = R_m \times \sqrt{\left[\frac{u(\text{CRM})}{c(\text{CRM})}\right]^2 + \left[\frac{\text{RSDM}}{\sqrt{n}}\right]^2}$$

For BCR CRM 194,  $u(R_{m1})$  was calculated as shown below, using the data from Table 3.

$$u(R_{m1})_{\text{BCR 194}} = 1.001 \times \sqrt{\left(\frac{2.04}{126}\right)^2 + \left(\frac{0.018}{\sqrt{10}}\right)^2} = 0.017$$

For NIST SRM 955b Level 1,  $u(R_{m2})$  was 0.026.

$u(R_m)$  was calculated from the recovery studies by use of the spreadsheet method (34) and taking into account the relative uncertainty of the amount of lead added to samples and the RSDM of the measured values for the samples with and without added lead.  $u(R_{m3})$  and  $u(R_{m4})$  were 0.027 and 0.025, respectively, for recovery of 25 and 50  $\mu\text{g/L}$  lead added to blood.

Because four estimates of trueness ( $R_m$ ) were available, each with its estimated uncertainty [ $u(R_m)$ ], these were combined according the following formula:

$$u_c(R_m) = \sqrt{\frac{u(R_{m1})^2 + u(R_{m2})^2 + \dots + u(R_{mn})^2}{n}}$$

The combined uncertainty was 0.024.

The significance of the mean bias estimated from the analysis of CRMs and lead-enriched samples could be

judged against the combined uncertainty associated with such estimate [ $u_c(R_m)$ ], according to:

$$\frac{|1 - \bar{R}_m|}{u_c(R_m)} \leq k$$

where  $k$  is the coverage factor applied to provide a confidence interval of  $\sim 95\%$ , selected from the  $t$ -distribution tables for the appropriate number of degrees of freedom and  $\bar{R}_m$  is the mean of the four estimates of the trueness (1.101). It was found that the mean positive bias observed was statistically significant when compared with the combined uncertainty associated with its estimate ( $k = 1.96$ ). Therefore, a correction should be applied to the individual results or, because the difference between measured and target values was small, the uncorrected bias was taken into account by increasing the uncertainty associated with its estimate according to (45):

$$u_c(R_m)' = \sqrt{\left(\frac{1 - \bar{R}_m}{k}\right)^2 + u_c(R_m)^2} = 0.060$$

#### UNCERTAINTY OF THE BIAS ESTIMATE, EVALUATED FROM THE ANALYSIS OF OTHER RMs

For comparison, the results of the analyses performed on other RMs and reported in Table 5 were also examined to derive information on the uncertainty of the bias estimate.

For the samples with concentrations between 35.2 and 119  $\mu\text{g/L}$ ,  $R_s$  ranged from 0.958 to 1.127, with a mean (SD) of 1.013 (0.052). This SD value can be taken as an approximate estimate of the uncertainty associated with the assessment of the method bias and compared with that obtained by more rigorous procedures, such as analysis of CRMs and recovery studies.

Similar exercises can be performed on archived EQA samples when other CRMs are not available to obtain at least a first estimate of the trueness of the method and its uncertainty, provided that sufficient confidence can be placed on the target values.

#### COMBINED UNCERTAINTY AND EXPANDED UNCERTAINTY

The overall relative uncertainty was obtained, according to concentration, by combining the corresponding  $u(P)$  contribution with  $u_c(R_m)$  for the three concentration ranges stated previously, giving the values of 0.102, 0.087, and 0.080. The expanded relative uncertainties, for a 95% confidence level, were calculated using  $k = 1.96$  and were 0.20, 0.17, and 0.16 for the concentrations intervals of 9.4–35, >35–90, and >90–150  $\mu\text{g/L}$ .

#### MONITORING OF METHOD PERFORMANCE: PARTICIPATION IN EQAS

Over the period covered by this study, blood samples were received as part of our laboratory's participation in an EQAS and were analyzed under the conditions applied in our laboratory for any patient specimen (two times each, under repeatability conditions). The individual re-

sults obtained for the eight samples with lead concentrations within the scope of this method are presented in Fig. 5 as the plot of the relative differences from the target values ( $y$ ) vs the target values ( $x$ ) and compared with the stated expanded uncertainty of the method. Twelve of the 16 results fell within the target value  $\pm$  uncertainty interval. Only the results for the two samples with concentrations  $<23 \mu\text{g/L}$  did not, but as mentioned previously, some doubt could exist for the robustness of target values assigned by consensus from data close to the detection limits of routine methods.

### Discussion

We present the results for a study aimed at validating a method for the determination of lead in blood and estimating the uncertainty of these measurements. The method is a further refinement of existing ETAAS methods in our laboratory (28, 29), adapted to the THGA furnace and focused on the determinations of low ( $<150 \mu\text{g/L}$ ) lead concentrations similar to those observed at present in samples from the general population. A furnace program was developed that was capable of using a considerably less diluted blood sample (1:1) than is typically analyzed. This was based on longer drying and charring steps and the use of an air-ashing step to prevent carbon build-up. Because the method presented here was intended for the accurate measurement of low blood lead concentrations, it should not be applied to the screening of occupationally exposed individuals. Blood samples with expected lead concentrations  $>150 \mu\text{g/L}$  would be better analyzed by other methods that use larger sample dilutions and shorter furnace programs.

The primary aim of the study was to show in practice how data already available to laboratories as a result of their quality-assurance procedures and in-house validation studies could be used to estimate the uncertainty of measurement. To obtain a reliable estimate of the method precision, which is often the main component of uncertainty, we studied intermediate precision over an extended period of time, using both control and patient

samples. The contribution to uncertainty attributable to imprecision was estimated as between 8.2% and 5.2%, depending on the lead concentration.

We assessed the bias of the method by different approaches: analysis of CRMs, recovery studies based on the addition to blood samples of lead concentrations with known uncertainties, and analysis of other RMs (e.g., archived EQA samples). This was done with the aim of demonstrating how such different approaches can be applied and to compare their outcomes. The example of the assay of lead in blood by ETAAS was well suited for this purpose because the properties and the state of the art of the technique are well documented, because both CRMs and pure standards are available, and because several well-established EQAS are active. The results of this study highlighted differences between the information provided by the analysis of the two available CRMs, whose target values were certified by different approaches and fell at opposite ends of the stated scope of the method. Recovery studies were then used to confirm the presence of a small positive bias, and the results were cumulated for the evaluation of the uncertainty component associated with bias ( $\pm 6.0\%$ ).

The strength of the traceability chain varies inversely with the uncertainty associated with the reference values used for comparison. However, to provide a valid and unbiased check of the measurement system, the RMs used in the process of validating a method should not only have traceable reference values but should resemble as closely as possible the samples to be analyzed. Several factors, such as chemical species of the analyte, concentration range, matrix effects, potential interferences, and physical status, may have a role and should be considered. As a complementary approach to the analysis of CRMs (or as an alternative, if none is available), the analysis of other RMs allowed assessment of the trueness of the method over the entire range of concentrations within its scope, providing a comparable estimate of the uncertainty component associated with bias ( $\pm 5.2\%$ ). It should be recognized, however, that this approach may not apply to every assay.

The components of uncertainty associated with precision and trueness were of similar size except for the lowest concentration range, where the precision component was 1.4-fold larger than that associated with trueness. This shows that evaluations based solely on precision data are likely to underestimate the actual uncertainty associated with ETAAS measurements of lead in blood.

Because the uncertainty of measurement was estimated globally from the method performance data, rather than from the uncertainty contribution associated with each influence quantity described in the mathematical model of the measurement, further insight on the main factors contributing to the uncertainty of measurement was not possible. However, in a previous report, Kristiansen et al. (46) discussed the estimate of the uncertainty of measure-

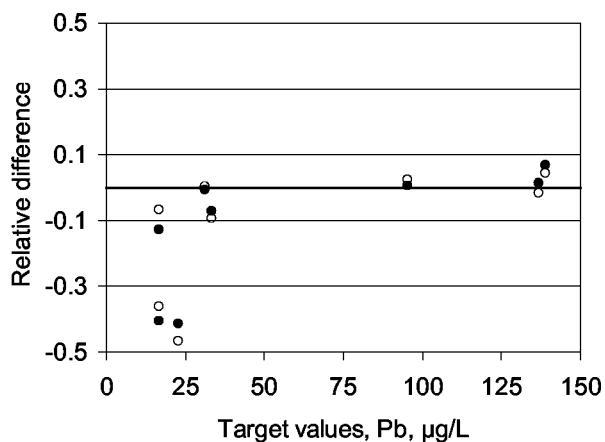


Fig. 5. Comparison of measured and target values for EQA samples.



ment in ETAAS from its individual components and used the measurement of lead in blood within the range 0.072–2.00  $\mu\text{mol/L}$  (15–415  $\mu\text{g/L}$ ) as an example. The uncertainties of the atomic and blank signals accounted for 75–90% of the total variance at low concentrations, whereas at the other end, the uncertainty of the calibration slope gave the largest contribution.

In conclusion, according to our study, an expanded uncertainty (95%) of 17% should be associated with measurements between 35 and 150  $\mu\text{g/L}$ , which increased to 20% between 9.4 and 35  $\mu\text{g/L}$ . This uncertainty value is compatible with the quality specifications for lead in blood adopted by CLIA '88 (21), which are  $\pm 40 \mu\text{g/L}$  or  $\pm 10\%$ , whichever is greater, and the Network of European Organisers of EQAS in occupational and environmental medicine (20). It also supports the view that carefully validated methods, using currently available technology, can provide even better results (47) because it is recognized that such limits are still too wide for laboratories investigating lead concentrations in blood of potentially exposed children (4). On the other hand, accurate assessment by ETAAS methods of the current blood lead concentrations for the general population may be difficult because concentrations are now close to the LoD of this technique. In this case, it would be desirable to apply more sensitive procedures or to confirm the accuracy of data by means of alternative analytical techniques (48).

We acknowledge the financial support of the Italian Ministry of Health, Project "Human Exposure to Xenobiotics with Potential Endocrine Activity: Evaluation of Reproductive and Developmental Risks" (Grant 99C/1999); the Italian Ministry of Health, Project "Validation of Strategies, Methods and Markers for Environmental and Biological Monitoring of Occupational Exposure to Xenobiotics" (Grant 176/2001); the Istituto Superiore di Sanità, Project "Metrology and Quality Improvement in Preventive, Environmental and Occupational Laboratory Medicine" (Grant RI 1036/2036); and the Istituto Superiore di Sanità, Project "The Clinical Laboratory in Preventive, Environmental and Occupational Medicine: Biomarkers, and Predictive Factors" (Grant RI1033/2033).

### References

1. European Economic Community. Council Directive 82/605/EEC of 28 July 1982 on the protection of workers from the risks related to exposure to metallic lead and its ionic compounds at work (first individual Directive within the meaning of Article 8 of Directive 80/1107/EEC). Off J No L 247;23/8/1982:12–21.
2. European Economic Community. Council Directive 98/24/EC of 7 April 1998 on the protection of the health and safety of workers from the risks related to chemical agents at work [fourteenth individual Directive within the meaning of Article 16(1) of Directive 89/391/EEC]. Off J No L 131;05/05/1998:11–23.
3. European Economic Community. Council Directive 77/312/EEC of 29 March 1977 on biological screening of the population for lead. Off J No L 105;28/04/1977:10–7.
4. Centers for Disease Control and Prevention. Screening young children for lead poisoning: guidance for state and local public health officials. Appendix C.1. Atlanta, GA: Centers for Disease Control and Prevention, 1997.
5. International Programme on Chemical Safety (IPCS). WHO Task Group on Environmental Health criteria for lead. Lead, Environmental Health Criteria 3. Geneva: WHO, 1977.
6. International Programme on Chemical Safety (IPCS). WHO Task Group on Environmental Health criteria for lead. Inorganic lead, Environmental Health Criteria 165. Geneva: WHO, 1995.
7. National Committee for Clinical Laboratory Standards. Analytical procedures for the determination of lead in blood and urine; approved guideline. NCCLS document C40-A. Wayne, PA: NCCLS, 2001:21.
8. Subramanian KS. Determination of lead in blood by graphite furnace atomic absorption spectrometry—a critique. Sci Total Environ 1989;89:237–50.
9. Menditto A, Chiodo F, Patriarca M, Morisi G. Ann Ist Super Sanita 1998;34:1–164.
10. Pirkle JL, Brody DJ, Gunter EW, Kramer RA, Paschal DC, Flegal KM, et al. The decline in blood lead levels in the United States. The National Health and Nutrition Examination Surveys (NHANES). JAMA 1994;272:284–91.
11. Pirkle JL, Kaufmann RB, Brody DJ, Hickman T, Gunter EW, Paschal DC. Exposure of the U.S. population to lead, 1991–1994. Environ Health Perspect 1998;106:745–50.
12. White MA, Sabbioni E. Trace element reference values in tissues from inhabitants of the European Union X. A study of 13 elements in blood and urine of a United Kingdom population. Sci Total Environ 1998;216:253–70.
13. Apostoli P, Baj A, Bavazzano P, Ganzi A, Neri G, Ronchi A, et al. Blood lead reference values: the results of an Italian polycentric study. Sci Total Environ 2002;287:1–11.
14. Menditto A, Chiodo F, Patriarca M, Morisi G. Esposizione al piombo: valutazione del rischio per la popolazione generale italiana negli anni '90. Ann Ist Super Sanita 1998;34:27–40.
15. Lanphear BP, Dietrich K, Auinger P, Cox C. Cognitive deficits associated with blood lead concentrations  $<10 \mu\text{g/dL}$  in US children and adolescents. Public Health Reports 2000;115:521–9.
16. Payton M, Riggs KM, Avron S, Weiss ST, Howard H. Relations of bone and blood lead to cognitive function: the VA normative aging study. Neurotoxicol Teratol 1998;20:19–27.
17. Siddiqui MK, Srivastava S, Mehrotra PK. Environmental exposure to lead as a risk for prostate cancer. Biomed Environ Sci 2002; 15:298–305.
18. Lin JL, Lin-Tan DT, Hsu KH, Yu CC. Environmental lead exposure and progression of chronic renal disease in patients without diabetes. N Engl J Med 2003;348:277–86.
19. Glenn BS, Stewart WF, Links JM, Todd AC, Schwartz BS. The longitudinal association of lead with blood pressure. Epidemiology 2003;14:30–6.
20. Taylor A, Angerer J, Claeys F, Kristiansen J, Mazarrasa O, Menditto A, et al. Comparison of procedures for evaluating laboratory performance in external quality assessment schemes for lead in blood and aluminum in serum demonstrates the need for common quality specifications. Clin Chem 2002;48:2000–7.
21. US Department of Health and Human Services. Medicare, Medicaid and the CLIA programs; regulations implementing the Clinical Laboratory Improvement Amendments of 1988 (CLIA). Final rule. Fed Regist 1992;57:149–62.
22. International Standards Organization. ISO/IEC 17025. General

- requirements for the competence of calibrating and testing laboratories. Geneva: ISO, 1999.
23. International Standards Organization. ISO 15189. Medical laboratories—particular requirements for quality and competence. Geneva: ISO, 2003.
  24. International Standards Organization. ISO guide to the expression of uncertainty in measurement. Geneva: ISO, 1993.
  25. EURACHEM/CITAC. Quantifying uncertainty in analytical measurement, 2nd ed (2000). <http://www.eurachem.ul.pt> (accessed ???).
  26. Morisi G, Patriarca M, Bortoli A, Mattiello G, Gelosa L, Fortuna E, et al. Italian results in a quality control program for the determination of lead in blood. EEC directive 312/29–3-1977. *Ann Ist Super Sanita* 1983;19:323–34.
  27. Morisi G, Patriarca M, Carrieri MP, Fondi G, Taggi F. Lead exposure: assessment of the risk for the general Italian population. *Ann Ist Super Sanita* 1989;25:423–36.
  28. Morisi G, Patriarca M, Macchia T. Caratterizzazione del metodo per la determinazione del piombo nel sangue in assorbimento atomico col fornello di grafite e la piattaforma di L'vov. *Ann Ist Super Sanita* 1982;18:587–90.
  29. Patriarca M, Morisi G. Metodo raccomandato per la determinazione del piombo nel sangue. *G Ital Chim Clin* 1987;12:51–6.
  30. Morisi G, Patriarca M, Taggi F. Comparable laboratory performances in the analysis of lead in control samples and in fresh human blood. *Ann Ist Super Sanita* 1989;25:417–22.
  31. Yeoman WB, Colinet E, Griepink B. The certification of lead and cadmium in three lyophilized blood materials. EUR 10380 EN. Luxembourg: Commission of the European Communities, 1985.
  32. Morisi G, Patriarca M, Taggi F. The interlaboratorial quality assurance program for blood lead determination. An evaluation of methods and results. *Ann Ist Super Sanita* 1989;25:405–16.
  33. Menditto A, Palleschi S, Minoprio A, Rossi B, Chiodo F, Patriarca M, et al. Quality assurance in biological monitoring of environmental exposure to pollutants: from reference materials to external quality assessment schemes. *Microchem J* 2000;67:313–37.
  34. Kragten J. Calculating standard deviations and confidence intervals with a universally applicable spreadsheet technique. *Analyst* 1994;119:2161–6.
  35. Menditto A, Patriarca M, Chiodo F, Morisi G. The Italian external quality assessment scheme for trace element analysis in body fluids. *Ann Ist Super Sanita* 1996;32:261–70.
  36. Taylor A, Briggs RJ. The Guildford trace elements external quality assessment scheme. *Ann Ist Super Sanita* 1996;32:253–9.
  37. Ramm-RH, Lehnert G, Angerer J. The German external quality assessment scheme in occupational and environmental medicine. *Ann Ist Super Sanita* 1996;32:247–51.
  38. EURACHEM Working Group. The fitness for purpose of analytical methods. A laboratory guide to method validation and related topics (1998). <http://www.eurachem.ul.pt/index.htm> (accessed ???).
  39. Parsons PJ, Slavin W. A rapid Zeeman graphite furnace atomic absorption spectrometric method for the determination of lead in blood. *Spectrochim Acta* 1993;48B:925–39.
  40. Jacobson EB, Lockitch G, Quigley G. Improved sample preparation for accurate determination of low concentrations of lead in whole blood by graphite furnace analysis. *Clin Chem* 1991;37:515–9.
  41. Bannon DI, Murashchik C, Zapf CR, Farfel MR, Chisolm JJ Jr. Graphite furnace atomic absorption spectroscopic measurement of blood lead in matrix-matched standards. *Clin Chem* 1994;40:1730–4.
  42. Bland JM, Altman DG. Statistical methods for assessing agreement between to methods of clinical measurement. *Lancet* 1986; i:307–10.
  43. International Standards Organization. ISO Guide 35. Certification of reference material. General and statistical principles, 2nd ed. Geneva: ISO, 1989.
  44. Bureau International des Poids et Mesures. Report of the 5th meeting of CCQM, 1999. [www.bipm.fr](http://www.bipm.fr) (accessed ???).
  45. Barwick JV, Ellison SLR. The evaluation of measurement uncertainty from method validation studies. *Accred Qual Assur* 2000; 5:47–53.
  46. Kristiansen J, Molin Christensen J, Nielsen JL. Uncertainty of atomic absorption spectrometry: application to the determination of lead in blood. *Mikrochim Acta* 1996;123:241–9.
  47. Parsons PJ, Geraghty C, Verostek MF. An assessment of contemporary atomic spectroscopic techniques for the determination of lead in blood and urine matrices. *Spectrochim Acta* 2001;56B: 1593–604.
  48. Patriarca M, Lyon TDB, Delves HT, Howatson AG, Fell GS. Determination of low concentrations of potentially toxic elements in human liver from newborns and infants. *Analyst* 1999;124:1337–43.