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ARTICLE

Rapid and Simultaneous Determination of Essential Minerals and Trace Elements in Human Milk by Improved Flame Atomic Absorption Spectroscopy (FAAS) with Microwave Digestion

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A method for the simultaneous and economical determination of many trace elements in human milk is developed. Two multi-element hollow cathode lamps (HCLs) were used instead of single-element HCLs to improve the sample throughput of flame atomic absorption spectroscopy (FAAS). The microwave digestion of milk is optimized prior to detection, and the performance characteristics of the improved analysis method are identified. Clinical samples are detected by both FAAS and inductively coupled plasma−optical emission spectroscopy (ICP−OES) for methodology evaluation. Results reveal that the proposed FAAS with multi-element HCLs could determine six essential minerals and trace elements within 15 min. This method provides a linear analytical range of 0.01−10 mg L^{-1}. For Ca, Cu, Fe, Mg, Mn, and Zn, the limits of determination are 1.5, 3, 1.8, 2.2, 2.1, and 1.3 μg L^{-1}, respectively. The mean relative standard deviations (RSDs) of intra- and interassays are lower than 7%. Excellent operational characteristics of rapidity, simplicity, and economy make the proposed method a promising one for the quantification of trace elements in human milk in clinics of underdeveloped areas.

KEYWORDS: Atomic absorption spectroscopy (AAS); human milk; microwave digestion; multi-element; trace elements

INTRODUCTION

Human milk is an essential nutrient source for infants and young children because it provides all of the necessary macronutrients (such as proteins, lipids, and carbohydrates) and micronutrients (such as elements, vitamins, and enzymes) (*1*). It is recognized as an excellent source of Ca and can supply moderate amounts of Zn and Fe, small quantities of Mg, and trace amounts of Mn and Cu (*2*). Human milk is also a source of immune agents that can, among other functions, hold intestinal diseases in check, which is almost as important as nutrition itself. This is particularly true in the case of early childhood because milk is the only source of nutrients during the first few months of a baby's life. Accurate data on the concentrations of trace elements in human milk throughout lactation are important in formulating nutritional requirements for infants and in understanding the physiology of milk secretion. Thus, quantifying trace metals in milk is of particular concern when combining complex emulsion-like matrices and low concentration levels of metal ions.

Several analytical techniques are available for the determination of trace elements. Atomic spectrometry, for example, has been established as the mainstay of trace element analysis in biological samples and dairy products. Flame atomic absorption spectrometry (FAAS) (*3*), electrothermal atomic absorption spectrometry (ET−AAS) (*4*), inductively coupled plasma−optical emission spectroscopy (ICP−OES) (*5*, *6*), and inductively coupled plasma−mass spectroscopy (ICP−MS) (*7*, *8*) are the predominant techniques currently in use. ICP−MS provides the most satisfactory sensitivity and accuracy; unfortunately, expensive devices and rigid operating protocols greatly limit its prevalence in routine clinical laboratories (*9*). High detection costs have also hindered the application of ICP−OES and ET−AAS in rural clinics, although they have good accuracy and working ranges and even simultaneous multi-elemental capability (*6*, *10*, *11*).

It is imperative to develop a rapid, accurate, and economical method for trace element determination to meet the needs of rural clinical laboratories. FAAS is a simple and practical method and has been widely applied for biological samples and food detection (*12*, *13*). Unfortunately, the sequential light source mode of traditional FAAS complicates the detection procedure and increases detection time when multi-element detection is performed, which is mainly determined by the intrinsic characteristics of single-element hollow cathode lamps (HCLs). A recently developed multi-element HCL technique is promising for rapid multi-element detection, because it is unnecessary to shift light sources during the detection process, thus increasing detection stability and efficiency (*14*). However, the multi-element HCL technique requires rigid optical splitting systems that can eliminate the

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Table 1. Instrumental Conditions for FAAS Determination

multi-element HCL	wavelength (nm)	current (mA)	slit (nm)	height (nm)	air flow (L/min)	C_2H_2 flow (L/min)
Ca/Cu/Zn	239.9/324.8/213.9	3	0.3	6	6.8	1.5
Fe/Mg/Mn	248.3/202.6/279.5	6	0.3	6	6.8	1.5

interferences between many elements. Although improving the holographic grating of HLCs has accelerated the application of multi-element HCLs, their clinical applications have not been thoroughly specified.

Most proposed methodologies adopt a digestion process to eliminate organic matrices, which usually involves several steps and contaminations. This, however, could be a serious obstacle for obtaining accurate data of trace quantities of elements (*15*, *16*). Microwave-based acid digestion procedures are well-established and are fast becoming popular because of their rapid speed, lower acid consumption, and high digestion efficiency (*15*, *17*). Although the procedure has the advantage of fast and effective digestion operation, it requires the participation of concentrated acids. Thus, the digestion protocol should be optimized prior to detection (*18*).

In this experiment, the microwave digestion protocol is optimized and a FAAS is mounted with two multi-element HCLs to determine the six essential minerals and trace elements in human milk. The analytical performance of this method is evaluated, and clinical samples are detected by both FAAS and ICP−OES for further methodology evaluation.

MATERIALS AND METHODS

Instruments. A Multiwave 3000 microwave sample preparation system (Anton Paar, Graz, Austria) equipped with up to eight high-pressure quartz vessels was used in this study. A BH5100h atomic absorption spectrophotometer (Bohui, Beijing, China) was equipped with two multi-element HCLs (Ca/Cu/Zn and Fe/Mg/Mn) (Shuguangming Electronic, Beijing, China). An Optima 3000 ICP−OES was obtained from PerkinElmer (Waltham, MA), and the HF-6100D air compressor used was from Haofu Mechanics (Beijing, China).

Reagents. All chemical reagents were of analytical grade. Working analytical solutions for Ca, Cu, Fe, Mg, Mn, and Zn were prepared immediately before use by serial dilution of stock reference solutions containing 1000 mg L^{-1} of each element (National Research Center for Reference Materials, Beijing, China) in a gradient as necessary. The accuracy of the study was determined using two reference materials, National Institute of Standards and Technology (NIST) standard reference material (SRM) non-fat milk powder SRM-1549 and whole milk powder RM-8435. NaCl (0.6 M), HNO_3 (65%, v/v), H_2O_2 (30%, v/v), $HClO_4$ (70%, v/v), H_2SO_4 (98%, v/v), C_2H_2 (98%, v/v), and Ar (99.9%, v/v) were obtained from the chemical industry (Beijing, China). Milli-Q water (18.2 MΩ cm) was used to prepare all solutions throughout the work. The calibration blank was prepared by diluting 20 mL (1 + 1) HNO_3 and 10 mL (1 + 1) HCl in a 500 mL polymethylpentene (PMP) volumetric flask to volume with 18 MΩ Milli-Q water. The rinse blank was prepared by acidifying reagent water in an acid-cleaned flask bottle to concentrations of 2% (v/v) HNO_3 + 2% (v/v) HCl. All glass apparatuses were soaked in a 10% (m/v) nitric acid solution for 48 h, thoroughly rinsed with Milli-Q water, and then dried in a dust-free environment before use.

Collection and Storage of Samples. Human milk samples were collected from 758 healthy lactating women (aged 22.5−34.7 years old, with an average age of 28.4) admitted to Southwest Hospital between May 2006 and December 2009. All participants gave signed informed consent, and this study was approved by the Ethics Board of the Third Military Medical University. An 8 mL aliquot was retained from a whole right breast collection conducted according to a strict protocol (*19*). The mother first cleaned the nipple and the surrounding area with Milli-Q water while wearing disposable talc-free trace-element-free gloves. Each sample was collected by a manual pump (Hollister, Libertyville, IL), an electric pump (Medela, Inc., McHenry, IL), or by hand expression into the container supplied with the pumping device. The milk collection kit was repeatedly acid-washed in 5% distilled HNO_3 before sampling. Immediately after collection, the milk was transferred into special metal-free (royal blue top) BD Vacutainer tubes (Franklin Lakes, NJ), placed in a styrofoam cooler on ice for delivery to the laboratory, and stored at −20 °C after the vials were wrapped tightly in plastic. All of the samples were numbered, divided into two equal parts, and then stored at −20 °C until analysis by FAAS and ICP−OES.

Sample Digestion and Detection. A total of 3 mL of thawed human milk samples was added to the digester, followed by 3 mL of HNO_3 and 1 mL of H_2O_2. The mixture was shaken gently and then incubated for 6 min at room temperature. The digester was sealed tightly and placed in the container. The microwave energy program used for the combustion procedure was as follows: (1) 1400 W for 10 min, (2) 0 W for 2 min, (3) 1400 W for 8 min (optional step), and (4) 0 W for 20 min for cooling, if step 3 was applied. During these steps, the cooling fan was set at level 2 (level 3 was applied only for cooling). The volume of the digests was then adjusted to 25 mL with deionized water after sample digestion.

Under the working conditions of the instruments described in **Table 1**, the standard, sample, and calibration blank solutions were determined by multi-element HCL-mounted FAAS. All results were calculated automatically by software using absorbance as ordinates and concentration as the abscissa after working curves were plotted.

Method Validation and Clinical Sample Detection. First, calibration curves were constructed, and linearity was determined by serially diluting stock trace element solutions with 0.5% HNO_3. The analytical characteristics, including precision, accuracy, analytical sensitivity, and detection limit, were then evaluated in depth. The clinical samples were analyzed by the FAAS method and ICP−OES by following the guidelines of the manufacturer. Calibration curves were visualized with Origin (version 8.0) software. Bland−Altman analyses were performed with MedCalc, version 11.1.

RESULTS AND DISCUSSION

Optimization of Sample Digestion Protocols. Digestion protocols are key factors that guarantee the efficiency of microwave digestion. Our previous experiments and those from other researchers have proven that a concentrated acid mixture of $HNO_3 + H_2O_2$ has relatively higher efficiency compared to combinations of HNO_3, $HNO_3 + HClO_4$, and $HNO_3 + H_2SO_4$. In this experiment, the optimal ratio of 4 mL of mixture (3 mL of HNO_3/1 mL of H_2O_2) produced the best results when 3 mL of human milk was digested.

Calibration Curves and Linearities. The calibration curves were established using the standard solutions of analyte elements prepared in 5% (m/v) HNO_3 by diluting 1000 mg L^{-1} stock solutions. Curves were created by plotting the detected absorbance versus element concentrations. A correlation coefficient of > 0.998 was considered acceptable for the aim of the study. For the six trace elements, the correlation coefficients were above the minimum required value for the method. The respective upper linearity ranges are given in **Table 2**. Within the measuring range, the deviations from theoretical values did not exceed 5%, demonstrating a good correlation between the element concentration and absorbance. To match the linearity ranges of the calibration curves, human milk sample concentrations higher than the upper limits of the linear calibration range were diluted (5-fold or more) to appropriate concentrations before detection.

Precision and Accuracy. The accuracy of the method was assessed by the two SRMs. The FAAS results for Ca, Cu, Fe, Mg, Mn, and Zn in SRM 1549 non-fat milk powder and the SRM 8435 whole milk reference are summarized in **Table 3**. The concentrations obtained for SRM 1549 and SRM 8435 agree well with reference values. No statistical differences existed

Table 2. Parameters of Calibration Equations

	Ca	Cu	Fe	Mg	Mn	Zn
slope (SE)[a]	3.158 (0.312)	0.087 (0.001)	0.325 (0.004)	8.497 (0.821)	31.91 (1.22)	41.72 (2.61)
y intercepts (SE)	0.108 (0.306)	0.005 (0.0007)	−0.599 (0.021)	0.590 (0.031)	−0.007 (0.002)	0.333 (0.084)
r^2[b]	0.9995	0.9998	0.9983	0.9988	0.9997	0.9982
upper linearity	10 mg L^{-1}	400 μg L^{-1}	400 μg L^{-1}	8 mg L^{-1}	200 μg L^{-1}	10 mg L^{-1}

[a] SE = standard error. [b] All of the *r* values were higher than 0.998, indicating good consistency.

Table 3. Detected and Certified Concentrations of Elements Determined in Milk by FAAS[a]

	whole milk powder SRM 8435		non-fat milk powder SRM 1549	
element	detected value (mg kg^{-1})	certified value (mg kg^{-1})	detected value (mg kg^{-1})	certified value (mg kg^{-1})
Ca	9.096 ± 0.24	9.220 ± 0.49	1.22 ± 0.02	1.30 ± 0.05
Cu	0.44 ± 0.02	0.46 ± 0.08	0.67 ± 0.03	0.70 ± 0.10
Fe	1.82 ± 0.01	1.80 ± 1.1	1.72 ± 0.04	1.78 ± 0.10
Mg	0.828 ± 0.022	0.814 ± 0.076	0.118 ± 0.005	0.120 ± 0.003
Mn	0.18 ± 0.02	0.17 ± 0.05	0.24 ± 0.02	0.26 ± 0.06
Zn	29.2 ± 0.8	28.0 ± 3.1	45.2 ± 0.6	46.1 ± 2.2

[a] Numbers are mean concentration and confidence intervals (n = 5; p = 0.05).

between determined and certified values at the 95% confidence level. For each sample, duplication tests were repeated 20 times a day for intra-assays and repeated for 20 consecutive days in the same manner (three duplicates per day) for interassays. The relative standard deviations (RSDs) of intra- and interassays were 6.15 and 6.62% for Ca, 4.35 and 5.22% for Cu, 4.98 and 6.30% for Fe, 4.81 and 6.49% for Mg, 4.98 and 6.30% for Mn, and 4.98 and 6.30% for Zn, respectively. These coefficients of variation (CVs) were acceptable for clinical sample detection (CV < 7%), although the average interassay CV (6.06%) is slightly higher than that of the intraassay (5.07%).

Recovery tests were performed by adding diluted stock trace element solutions for accuracy assessment according to standard recovery test protocols. In this experiment, diluted single-element stock trace element solutions (with 0.5% HNO_3) of low, medium, and high concentrations were added and analyzed for five replicates. Recovery (%) is calculated as the ratio of the recovered concentration and added concentration, and average recovery (%) is the mean of high, medium, and low concentrations of standard materials. The results in **Table 4** indicate recoveries of different added concentrations ranging between 90.0 and 109.3% and average recoveries of each element ranging from 96.2 to 103.4%, which are superior to the ICP−MS results reported by Montesinos et al. (*20*). Moreover, the RSDs of recovery, including low, medium, and high concentrations of each trace element, were sufficiently low (< 2.5%) (see **Table 4**).

Limits of Detection. The limits of detection ($3\sigma/S$) and quantification ($10\sigma/S$) are 1.5 and 5 μg L^{-1} for Ca, 3 and 9.8 μg L^{-1} for Cu, 2.2 and 7.3 μg L^{-1} for Fe, 1.8 and 6 μg L^{-1} for Mg, 2.1 and 7 μg L^{-1} for Mn, and 1.3 and 4.3 μg L^{-1} for Zn, respectively. The detection and quantification limits were calculated according to International Union of Pure and Applied Chemistry (IUPAC) recommendations, where σ is the standard deviation (SD) of the calibration blank (n = 10) and S is the slope of the analytical curve. Although the detection limits are poorer than those of ET−AAS and ICP−MS in milk sample determination (*21*), the limits of detection and quantification for Fe, Cu, and Zn are higher than those from earlier developed FAAS techniques and even reached the detection limit levels of ICP−OES (*22*, *23*). The other two elements had similar sensitivities to traditional FAAS methods in the milk matrix (*5*).

Interference Evaluation. In this experiment, interferences by major elements (Na, K, and P) were assessed under optimal determination conditions. Results demonstrate that P (0.5 mg L^{-1}), Na (0.8 mg L^{-1}), and K (2.0 mg L^{-1}) had no significant effects on the results of Ca, Cu, Fe, Mg, Mn, and Zn.

Spectral interference is a major disadvantage that hinders the application of ICP−OES. This is not a serious problem in the traditional AAS method because the single-element HCL in the AAS system always provides an excellent line source. Although introducing multi-element HCLs may achieve cost savings and avoid the complication of carrying many lamps for operation or as spares, some criteria should be followed during the multi-element merging process, including spectral overlap, compatibility between elements, and acceptability of emission. The cathode materials and their relative ratios require careful adjustment for an optimum design lamp.

The results show that the spectral interference between elements from proposed multi-element HCLs was as low as that from traditional single-element HCL spectrometry, which is similar to earlier FAAS methods (*24*, *25*). In this experiment, two multi-element HCLs were adopted, one with Ca, Cu, and Zn and another with Fe, Mg, and Mn. Results indicate that a combination of two multi-element HCL produces less interference than a six-element HCL pattern. Thus, the proposed FAAS could greatly reduce the RSD during repeated tests and detection time by simplifying the detection process, thus enhancing the practicability of the method.

Distinctive Characteristics. Besides high accuracy and sensitivity, the proposed FAAS is economical, greatly reducing the cost of many single-element HCLs by mounting only two multi-element HCLs. In clinical detection, the calibration solution of various elements could be integrated into just one solution, providing lower reagent costs.

The proposed FAAS method is also easy to operate. Milk digestion could be automatically completed by microwave before simultaneous multi-element determination, which avoids the shift of the HCLs required by single-element-mounted FAAS during the determination of many elements (*26*, *27*). Moreover, the operating protocol of this practical method requires no complicated training courses, which fits the need of clinical laboratories.

Rapid detection is the third advantage of this proposed FAAS method, because it greatly shortens the multi-element analysis time; six elements could be analyzed within 10 s. The total analysis time, including microwave digestion (12 min), baseline adjustment (1 min), and real-time detection (< 1 min), is within 15 min, which is the same as that of ICP−OES, and is much shorter than conventional single-element FAAS (*28*).

Clinical Sample Detection and Comparison. We used Bland−Altman difference plot analyses to evaluate the correlation between the proposed method (FAAS) and a reference method (ICP−OES) on clinical sample detection (**Figure 1**) (*29*). The Bland−Altman difference plot analysis for Cu showed a mean (SD) difference (FAAS minus ICP−OES) of 0.4 (0.7) mg L^{-1}, and the ranges for the limits of agreement (from $d - 1.96S$ to $d + 1.96S$; from −15.8 to 15.1 μg L^{-1}) were sufficiently narrow. Similarly, Ca, Fe, Mg, Mn, and Zn had narrow ranges of the

Table 4. Recoveries of Trace Elements (n = 20)

element	original sample concentration (SE) (μg/mL)	added concentration (SE) (μg/mL)	recovered concentration (SE) (μg/mL)	recovery (SE) (%)	average recovery (SE) (%)
		0.030 (0.001)	0.029 (0.003)	96.67 (1.374)	
Ca	0.322 (0.017)	0.150 (0.004)	0.147 (0.006)	98.00 (2.112)	99.56 (1.697)
		0.300 (0.005)	0.312 (0.018)	104.00 (2.148)	
		0.050 (0.002)	0.053 (0.011)	106.00 (1.321)	
Cu	0.353 (0.021)	0.150 (0.004)	0.142 (0.05)	94.67 (1.651)	99.22 (1.353)
		0.500 (0.003)	0.485 (0.021)	97.00 (2.213)	
		0.050 (0.003)	0.053 (0.004)	106.00 (1.387)	
Fe	0.568 (0.021)	0.250 (0.006)	0.244 (0.011)	97.60 (2.263)	100.59 (2.055)
		0.600 (0.001)	0.589 (0.025)	98.17 (2.443)	
		0.010 (0.002)	0.009 (0.001)	90.00 (1.684)	
Mg	0.270 (0.013)	0.100 (0.001)	0.108 (0.012)	108.00 (2.215)	96.17 (1.775)
		0.200 (0.002)	0.181 (0.008)	90.50 (1.678)	
		0.050 (0.002)	0.049 (0.004)	98.00 (1.345)	
Mn	0.294 (0.015)	0.150 (0.004)	0.164 (0.006)	109.33 (1.894)	103.38 (1.891)
		0.250 (0.006)	0.257 (0.012)	102.80 (2.562)	
		0.100 (0.001)	0.106 (0.005)	106.00 (1.535)	
Zn	3.551 (0.012)	0.800 (0.003)	0.785 (0.019)	98.13 (1.671)	101.70 (1.681)
		2.500 (0.005)	2.524 (0.025)	100.96 (2.576)	

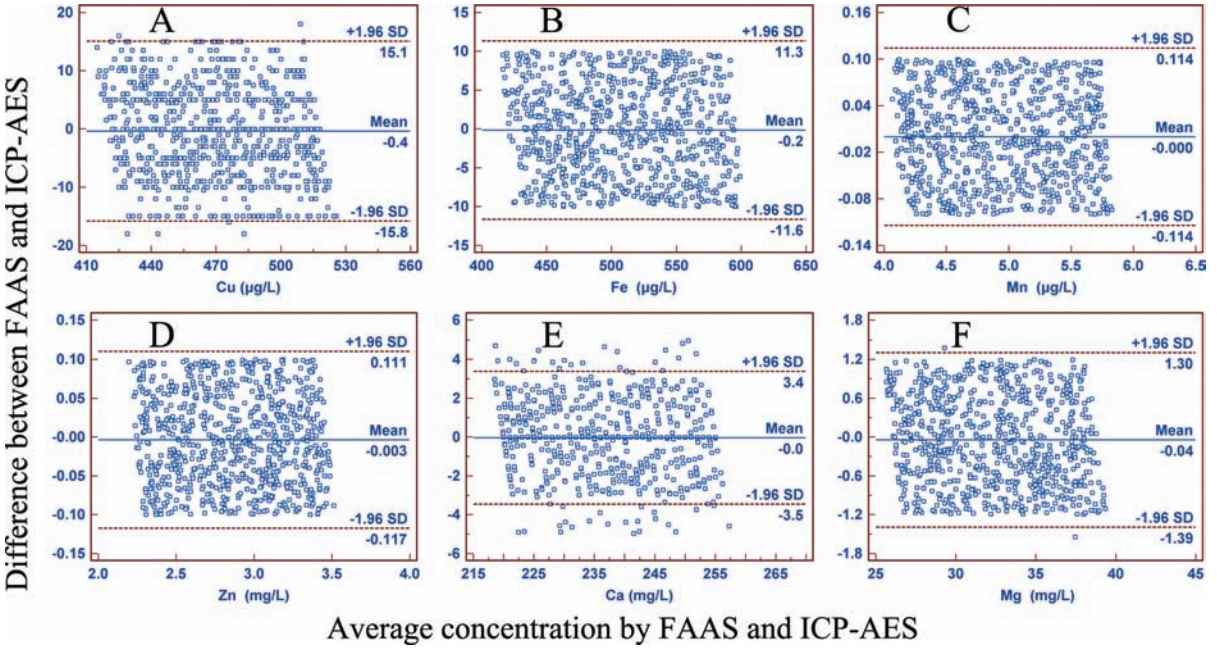

Figure 1. Bland−Altman difference plot for trace element results by FAAS and ICP−OES. (A) Bland−Altman difference plot comparing mineral and trace element concentrations obtained with FAAS against ICP−OES. (B−F) Bland−Altman difference plots comparing mineral and trace element concentrations of Fe, Mn, Zn, Ca, and Mg, respectively. The solid line represents the mean difference in quantitative measurement of essential minerals and trace elements between the methods, and the red dashed lines are mean ± 1.96 SD.

agreement limit, suggesting good agreement between these two methods.

Detection results of clinical human milk samples also verified the high reliability of this optimized FAAS assay. Of the 758 healthy lactating women's milk, the 95% confidence interval (CI) of the Cu concentration in sample varies from 420 to 518 μg L^{-1}, with an average of 468 ± 52 μg L^{-1}; Zn, from 2.24 to 3.36 mg L^{-1}, with an average of 2.81 ± 0.91 mg L^{-1}; Ca, from 220 to 255 mg L^{-1}, with an average of 238 ± 16 mg L^{-1}; Mg, from 26.1 to 38.8 mg L^{-1}, with an average of 31.2 ± 2.1 mg L^{-1}; Fe, from 420 to 596 μg L^{-1}, with an average of 481 ± 121 μg L^{-1}; and Mn, from 4.09 to 5.78 μg L^{-1}, with an average of 4.83 ± 1.21 μg L^{-1}. The essential minerals and trace element concentrations in breast milk are similar to those in previous reports (*30*, *31*).

In this experiment, an economical and simple method is developed to determine essential minerals and trace elements in human breast milk, and validation of the reliability, accuracy, and practicality of the method was performed by method evaluation and clinical sample detection on multi-element FAAS. In conclusion, the proposed method is simple, economical, accurate, and highly reliable and can be applied for the clinical detection of trace elements in biological samples from underdeveloped areas.

ABBREVIATIONS USED

AAS, atomic absorption spectrometry; CV, coefficient of variation; ET−AAS, electrothermal atomic absorption spectrometry; FAAS, flame atomic absorption spectroscopy; HCL, hollow cathode lamp; ICP−MS, inductively coupled plasma−mass spectroscopy; ICP−OES, inductively coupled plasma−optical emission spectroscopy; RSD, relative standard deviation.

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