CRM REPORT

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J. Chem. Metrol. 18:1 (2024) 83-94

journal of chemical metrology

Homogeneity and short-term stability of a candidate matrix reference material from human hair for trace element measurements

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(Received March 24, 2024; Revised May 19, 2024; Accepted May 20, 2024)

Abstract: In the current project, the National Institute of Standards (NIS), Egypt, initiated a preliminary investigation to create a certified reference material for certain elements, including Fe, Mg, Mn, Al, Cu, and Zn, in human hair. This reference material would be a quality control sample for trace element determination. This paper studied homogeneity and short-term stability as critical parameters for producing certified reference materials. ICP-OES and AAS were used to measure human hair powder samples, and the data was statistically evaluated for normality and outliers, which resulted in the measurement results being normal after removing the outliers. Also, sample homogeneity was evaluated and assessed using analysis of variance (ANOVA), which revealed that the human hair samples produced were homogenous and stable during transportation. This CRM mainly intends to develop methods and check instrumental performance, analytical trueness, and accuracy related to trace element analysis in human hair and similar matrices.

Keywords: Biological samples; reference materials; homogeneity; stability; elemental analysis; ICP-OES.

1. Introduction

Identifying trace elements in human hair is becoming increasingly common for tracking environmental exposure, determining dietary status, analyzing intoxication, and making medical diagnoses. The difficulty of removing just exogenous origin components from hair, the weak correlation between the elements' contents in the hair and other tissues, and the subpar quality of some elements' analytical results have raised concerns about this usage.

Certain enzymes, active proteins, vitamins, and hormones in the human body depend on trace elements as essential constituents. Trace element overabundance can potentially harm the human body, but a deficiency in essential trace elements can lead to aberrant tissue architectures and physiological functioning [1]. Human trace element determination in clinics uses blood and hair as detection samples. However, blood detection is prone to contamination, leading to cross-contamination and cumbersome

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The article was published by ACG Publications
http://www.acgpubs.org/journal/journal-of-chemical-metrology January-June 2024 EISSN:1307-6183
DOI: http://doi.org/10.251135/jcm.111.2403.3156
Available online: May30, 2024

sampling. It is also affected by environmental and nutritional factors. The human body did not sustain any surface harm from the hair, and it was simple to sample and preserve. Simultaneously, the components in hair can more accurately mirror human elements throughout time than the elements in blood, making analysis and detection more accessible and stable. Hair is now considered an essential material for detecting trace elements in the human body due to its ease of analysis [2]. Many analytical techniques, such as atomic absorption spectrometry AAS, atomic absorption spectrophotometry, flame atomic absorption spectrometry, inductively coupled plasma mass spectrometry ICP-MS and inductively coupled plasma atomic emission spectrometry ICP-OES are used for trace element measurements and detection [3-6]. Nevertheless, processing the samples using these techniques was exceedingly laborious, time-consuming, and challenging to operate. They could not be widely promoted or used in routine testing or medical visits to rural areas due to their stringent requirements for the experimental setting. In addition, the equipment that is currently in use is costly, and its functionality in challenging and isolated locations is constrained. A detection approach that is easily accessible, effective, quick, accurate, portable, affordable, and capable of handling many elements is therefore desperately needed.

The worldwide environmental issue of toxic metal poisoning of air and water impacts hundreds of millions. Concerns about heavy metal contamination in food affect both human and animal health [7]. Elements and metals, like other environmental toxins, can be naturally present and persist in the environment. Human exposure to toxic elements cannot be avoided, and several studies have indicated gender variations in metal toxicity. Biological human scalp hair mineral analysis is increasingly suggested to assess human exposure to ambient mineral contaminants and as a diagnostic tool for associated health issues [8]. Scalp hair is a suitable material for biopsies due to its advantageous properties. It is simple to collect and store. An effective method for studying long-term changes in trace element levels involves analyzing hair samples using many precise and sensitive analytical techniques to determine multiple minor and trace elements. Using appropriate analytical quality control in this type of work is crucial. A hair reference material is essential for systems that lose electrons and form metal cations, which can bind to important macromolecules. Various acute and chronic harmful effects of heavy elements impact different human organs. Heavy metal toxicity can lead to several complications, such as gastrointestinal and kidney dysfunction, anomalies in the neurological system, skin lesions, damage to blood vessels, malfunctioning of the immune system, birth deformities, and the development of cancer. Simultaneous exposure to numerous metals can lead to cumulative effects [9].

Accuracy and comparability are crucial for proper functioning, effective process control, and consistent measurement of results. To achieve all these, reliable metrological approaches are obtained by certified reference materials according to ISO/ICE 17034 and ISO Guide 35 requirements. CRMs are crucial in quality assurance, conducting precise research, validating test methods, and verifying results. The present work aims to study homogeneity and short-term stability as key parameters for candidate-certified reference material production using natural human hair for certain element measurements.

2. Experimental

2.1. Sample Collection and Cleaning

This project aimed to produce a certified reference material using natural human hair collected by various donors from different areas such as Giza, Beni Suef, and Fayoum in Northern Egypt. The material was processed under controlled temperature and humidity conditions and bottled in amber dark glass vials. The material consists of uncolored and untreated human hair collected from local hairdressers and transported to the NIS site in sealed plastic bags to avoid environmental contamination during transport [10].

A large amount of human hair was cleaned using the following procedure:1) The hair was placed in a plastic container, washed with Milli-Q ultra-pure water, and added a nonionic detergent (Triton X-100). 2) rinsing of the hair. A major part of the mixture, Milli Q/Triton X-100, was poured out. Then, several liters of clean water were poured and shaken well with the hair. The water was then removed while the hair remained in the container. This step was performed several times. 3) drying of the hair: the hair

was removed from the plastic container and spread over nylon sieves. Drying was performed at room temperature 25 °C for four days [10].

2.1.2. Hair Sample Processing

When the hair material was dry, it was manually cut using stainless steel scissors. Care was taken to get pieces that could be fed into the mill. The ball mill instrument was used to grind the samples, as shown in Figure 1(a). Small pieces of samples were placed in the oven at 60 °C overnight. Then, samples were ground by a ball mill to obtain the powder samples [2], as illustrated in Figure 1(b).

a) b)

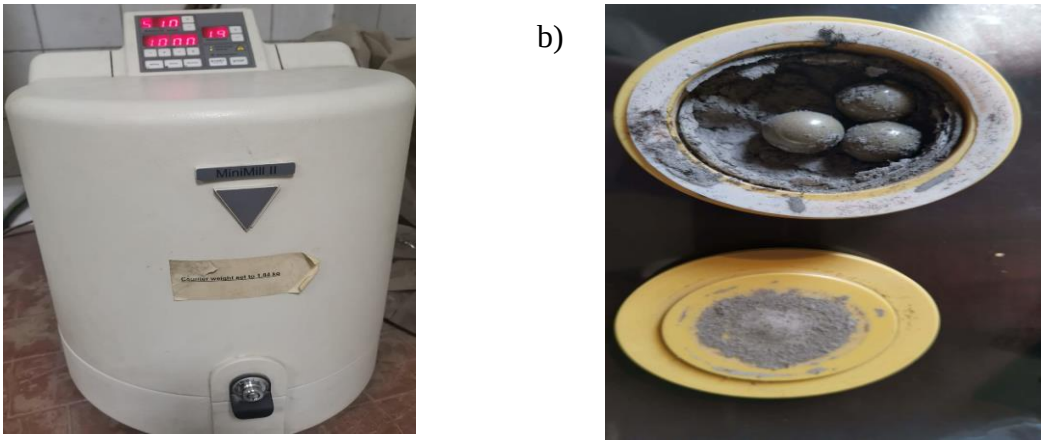

Figure 1. (**a**) Ball mill instrument, (**b**) Ground samples

2.1.3. Packing and Storage

Hair powder was packed into 30 mL borosilicate glass vials. All vials were cleaned with a 2% nitric acid solution in water and rinsed with Milli-Q water before being dried in a drying cabinet before filling. Each vial was consistently filled with approximately 3.0 grams. Once the vials were filled, they were closed. The vials were Labeled and stored in a desiccator at 25°C ± 2°C because human hair is highly hygroscopic.

2.2. Sample Preparation Chemicals and Reagents

All chemicals and reagents used in the study were of analytical grade. The Millipore (USA) Milli-Q system was used to obtain ultrapure water with a resistivity of 18.2 MΩ.cm. The Triton X-100 was manufactured by Alfa Aesar in Germany. The nitric acid produced by Merck in Germany has a purity of 69%. Hydroxy peroxide with a purity level of 30% was obtained from International Co, Egypt, and acetone for liquid chromatography was obtained from Merck, Germany.

2.3. Analytical Equipment

Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES), ICPS-5800 from Agilent, USA. An atomic absorption spectrometer, ZEEnit 700, from Analytik Jena, Germany, is equipped with a flame atomizer. Each element had its hollow cathode lamp used as a radiation source with a characteristic current. A deuterium lamp was used to automatically correct the background for flame atomizer measurements. Drying Oven with a maximum temperature of 250°C from Heraeus, Germany.

2.4. Preparation of Powder Hair Samples

2.4.1. Dry Mass Determination

The dry mass determination was checked throughout the whole process. It was carried out using approximately 0.20 g of the powder sample parallel to the sample weighing for the analysis. The samples were weighed and dried at 105 °C for two hours. The dried samples were weighed, and the mass difference was calculated. This process was repeated until the mass became constant [10].

2.4.2. Sample Preparation and Measurements

Approximately 0.3g of the sample was weighed in a glass flask. 4 mL of concentrated nitric acid was added cautiously with gentle mixing. 2 mL of 30% H_2O_2 was added gradually with slow heating to complete the dissolution of the sample. The samples were boiled at 120 ºC till complete dissolution. The vessels were left to cool down to room temperature, and then the solution was transferred to a 50 ml measuring flask and diluted with deionized water [11]. Blank samples were created with equivalent quantities of all reagents. Also, spike samples were prepared using equivalent procedures to confirm the accuracy of AAS and ICP-OES measurements. Table 1 illustrates the measurement set-up conditions of ICP-OES.

Table 1. Setup conditions of ICP-OES

Parameter	**Condition Set**
Read time (s)	3
RF Power (KW)	1.2
Stabilization Time (s)	10
Viewing Mode	Axial
Viewing Height (mm)	8
Nebulizer Flow (L/min)	0.7
Plasma Flow (L/min)	12
Aux Flow (L/min)	1

2.5. Homogeneity Study

To confirm the homogeneity of the powder samples, they were divided into groups with equal size, and every group was uniquely identified before measurements. Hair-labeled bottles are divided into 12 groups (A, B, C, D, E, F, G, H, I, J, K, and L). Each group contains five bottles of approximately 3 grams of powder sample. To achieve adequate sample representation, a stratified random selection technique was employed to choose 10% from each group. The selected samples were divided into three portions. Each portion was prepared and measured five times by an inductivity-coupled plasma optical emission spectrometer (ICP-OES). The homogeneity study's experimental setup is shown in Figure 2.

One-way analysis of variance (ANOVA) was performed to evaluate the homogeneity of the RM hair samples, determine within-sample variability and between-sample variability, and calculate the uncertainty brought on by sample heterogeneity [12-13].

2.6. Stability Study

Stability testing is required to determine the requirements for sample dispatch (short-term stability) and storage (long-term stability). Temperatures during transportation can rise to 60 °C, especially during the summer. If transport at ambient temperature is used, stability against these conditions must be proven.

2.6.1. Short-term Stability

An isochronous design was utilized to conduct stability investigations on powder samples. A random stratified sampling approach was adopted to select several units from the entire batch produced. These units were then stored for varying lengths of time (0, 1, 2, 3, and 4 weeks) at different temperatures (-20°C, 20°C, and 60°C). The study employed two units for each time/temperature combination, totaling 16 units. After the isochronous storage period, the samples were digested using a blend of nitric acid and hydrogen peroxide before being analyzed simultaneously under repeatability conditions via an atomic absorption spectrometer. [10, 14].

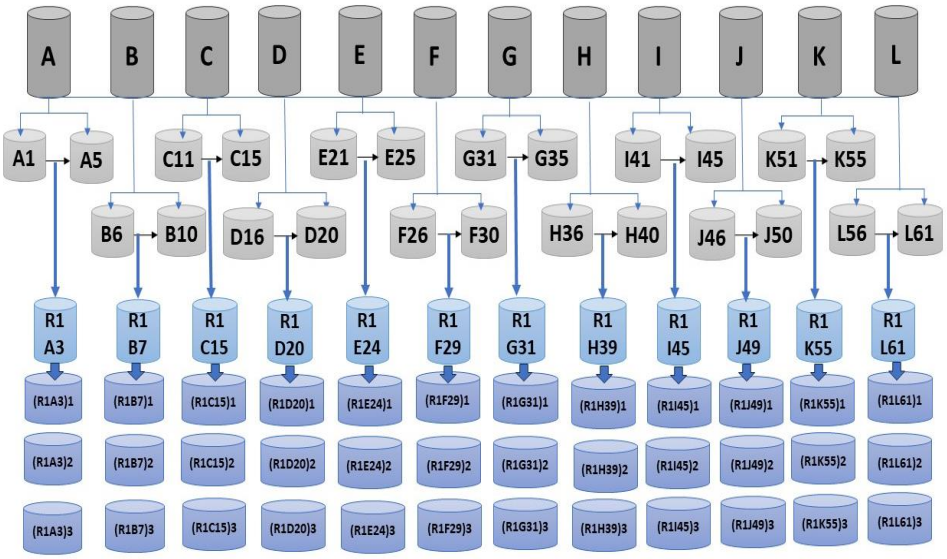

Figure 2. Homogeneity study's experimental setup

3. Results and Discussion

3.1. Calibration and Traceability

External calibration of an inductivity-coupled plasma atomic emission spectrometer (ICP-OES) was performed using NIS Standard solutions for magnesium, manganese, aluminum, copper, zinc, and iron. The intensity was measured after line overlap and matrix interferences were eliminated. Calibration solutions were made by diluting standard solution CRMs (1000 mg/L) in deionized water with 1% (v/v) HNO3 for calibrating ICP-OES. The concentrations were 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, and 5.0 mg/L for iron (Fe), manganese (Mn), zinc (Zn), copper (Cu), magnesium (Mg), and aluminum (Al). Table 2 shows different wavelengths of the elements measured by ICP-OES. The calibration curve of each element was established by plotting the concentration of the standard solution against the net intensity measured, as shown in Figure 3, which reveals that the calibration curves were linear as the R^2 of the regression line is > 0.99.

Table 2. Wavelengths of the elements measured by ICP-OES

Element	Wavelength (nm)
Al	396.152
Cu	327.395
Fe	238.204
Mg	279.553
Mn	257.610
Zn	213.857

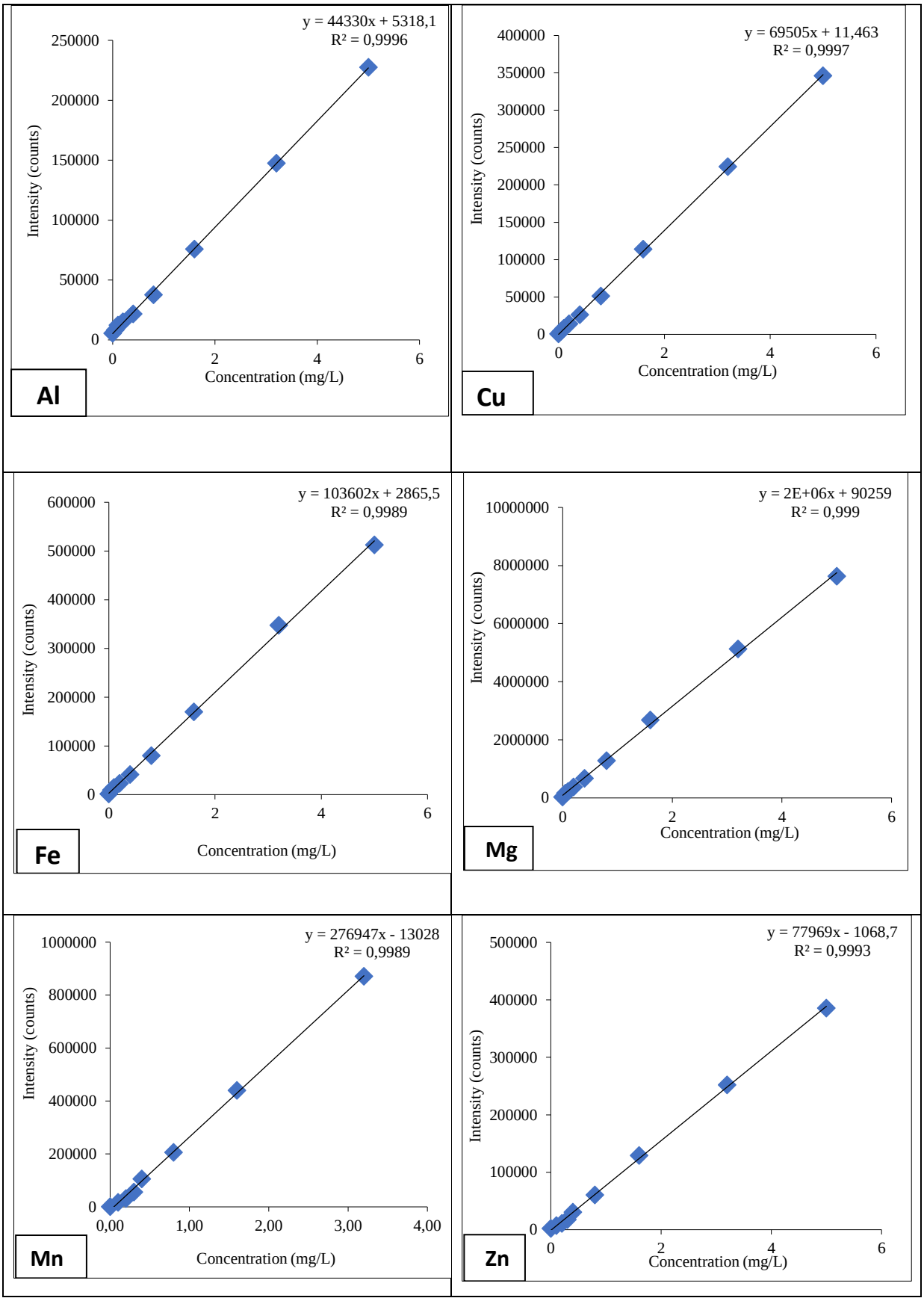

Figure 3. Calibration curves of the elements measured by ICP OES

3.2. Homogeneity Assessment Study

A fundamental requirement for any reference material is the consistency and uniformity of the various units. It is important to determine if the difference between units is substantial compared to the uncertainty of the certified value. It is irrelevant if this difference between units is significant compared to the analytical variation. ISO 17034 mandates that Reference Material producers measure the variation between units. This element is addressed in inter-unit homogeneity investigations. The certified value's uncertainty remains unaffected by within-unit homogeneity as long as the minimum sample intake is met. However, it sets the minimum aliquot size that corresponds to the unit. Quantifying the homogeneity within a unit is essential to establish the minimal sample size required [16-18].

The consistency among units was assessed to confirm that the CRM-certified values apply to all units produced within the specified uncertainty. The samples were divided into 12 groups, each containing five units. Twelve units were selected using a random stratified sampling scheme covering the whole batch produced. From each unit, three independent aliquots were taken and digested. A one-way analysis of variance (ANOVA) was used to assess the measurement results.

Table 4 shows the homogeneity results of human hair samples, which reveal that the values of $F_{calculated}$ are smaller than those of $F_{critical}$ for α equals 5%. As a result, the batch of human hair is sufficiently homogeneous to serve as control samples.

The method repeatability standard deviation S_{wb} and between unit standard deviation S_{bb} of the material homogeneity were calculated according to the following equations [19]:

$$S_{wb} = \sqrt{MS_{within}} \quad (1)$$

$$S_{bb} = \sqrt{\frac{MS_{between} - MS_{within}}{n}} \quad (2)$$

where $MS_{between}$ is the mean square for the between-sample variability, MS_{within} is the mean square for the within-sample variability, and n is the number of measurements taken for each selected sample [10-13].

Table 4. Results of analysis of variance for the homogeneity study of human hair batch.

Analyte	$F_{calculated}$	***P value***	$F_{critical}$	S_{bb}
Fe	1.27432	0.16424	1.5085	0.0184
Mn	1.00000	0.47838	1.5050	1.12719E-10
Cu	1.29516	0.15476	1.5240	0.0020
Zn	1.18124	0.27076	1.5878	0.0025
Mg	1.10262	0.34044	1.5260	0.0035
Al	1.51525	0.07288	1.6001	0.0061

3.3. Statistical Assessment of Measurement Results

The measurement results were subjected to a normality test to confirm that the data is normally distributed. Tests show that, except manganese (Mn), all measured element results were normal after the outliers were eliminated. The p-value for the manganese is less than 0.05, which indicates that the data was not normal. The results of the measured elements that give normal behavior are shown in Figure 4. The Grubbs test was used to check the presence of outliers. G is calculated using the following equation:

$$G = \frac{\left| x_{suspect} - \bar{x} \right|}{s} \quad (3)$$

where $x_{suspect}$ is the outlier value, S is the standard deviation of the data set, including the outlier value, and $\bar{x}$ is the mean value of the measurement results.

The value of G obtained from the measurements is compared with the theoretical values of G listed in statistical tables for n measurements. A confidence interval of 95% is used. Figure 5 provides a graphical representation of the outlier's test. The test reveals that the p-value is ≥ 0.05. This means that the calculated G is less than the theoretical G, and the data set contains no outliers [20].

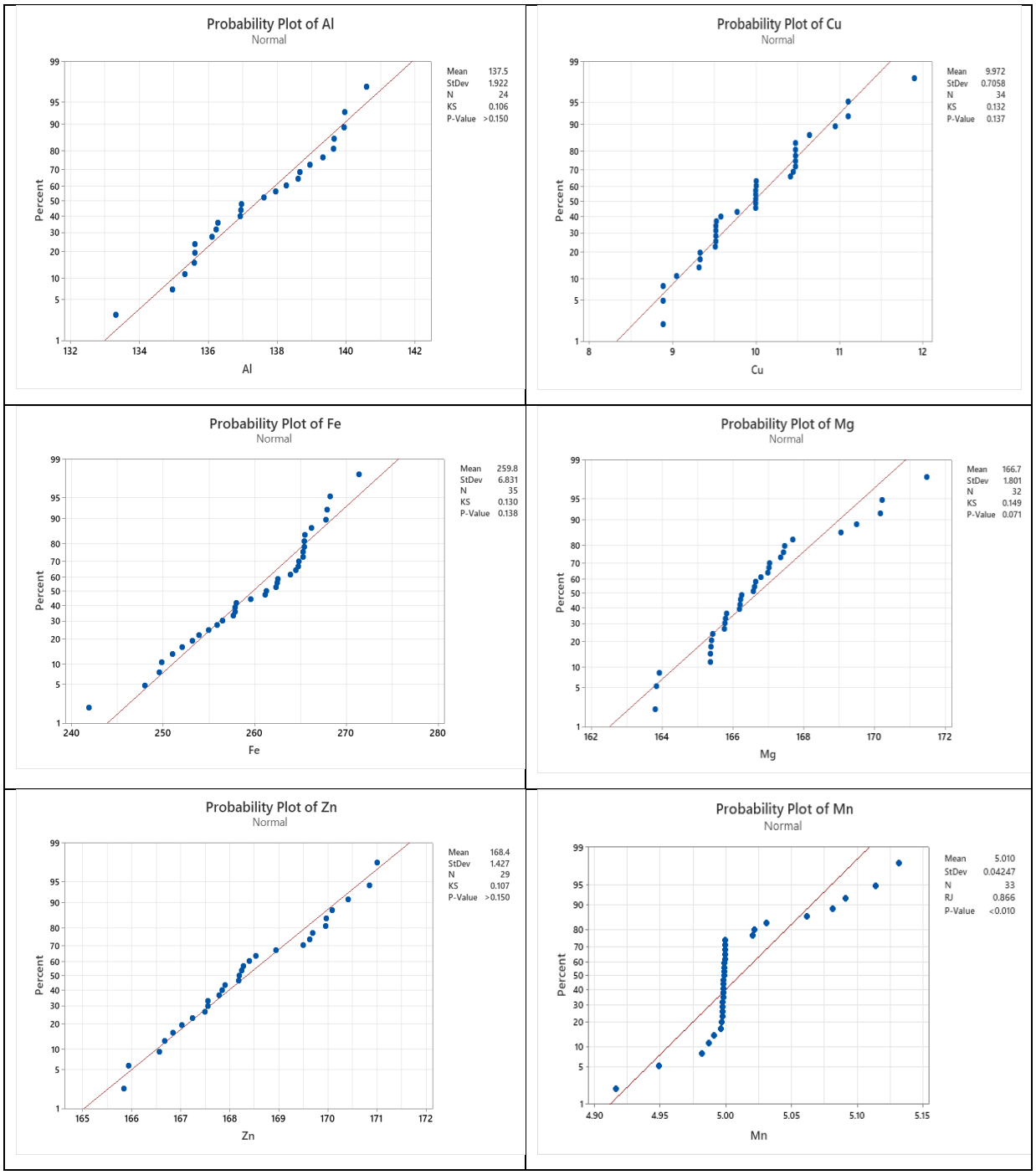

Figure 4. Probability plots of the measured elements

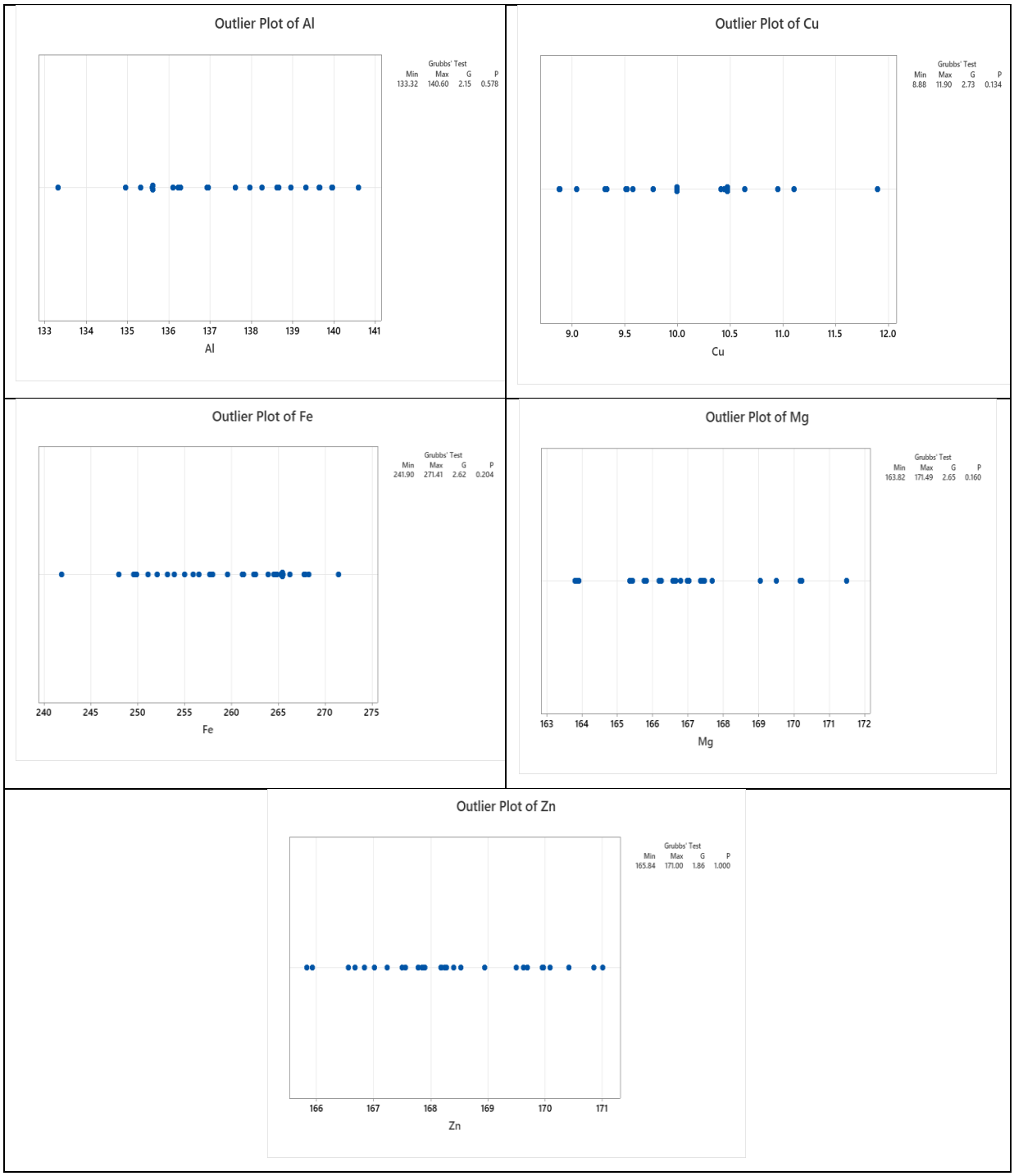

Figure 5. Outlier plots of the elements measure

3.4. Short-term Stability

The short-term stability of human hair powder samples was evaluated by measuring the elements at different temperatures/ time combinations. Grubb's test was used to check the presence of outliers in the measured data. Figure 6 illustrates a graphical representation of the short-term stability measured data, revealing no systemic trends were observed. Also, the significance of trends was checked using a t-test. The slope of the regression lines of the mass fractions of the measured elements was calculated at 20 ºC and 60 ºC [21-22].

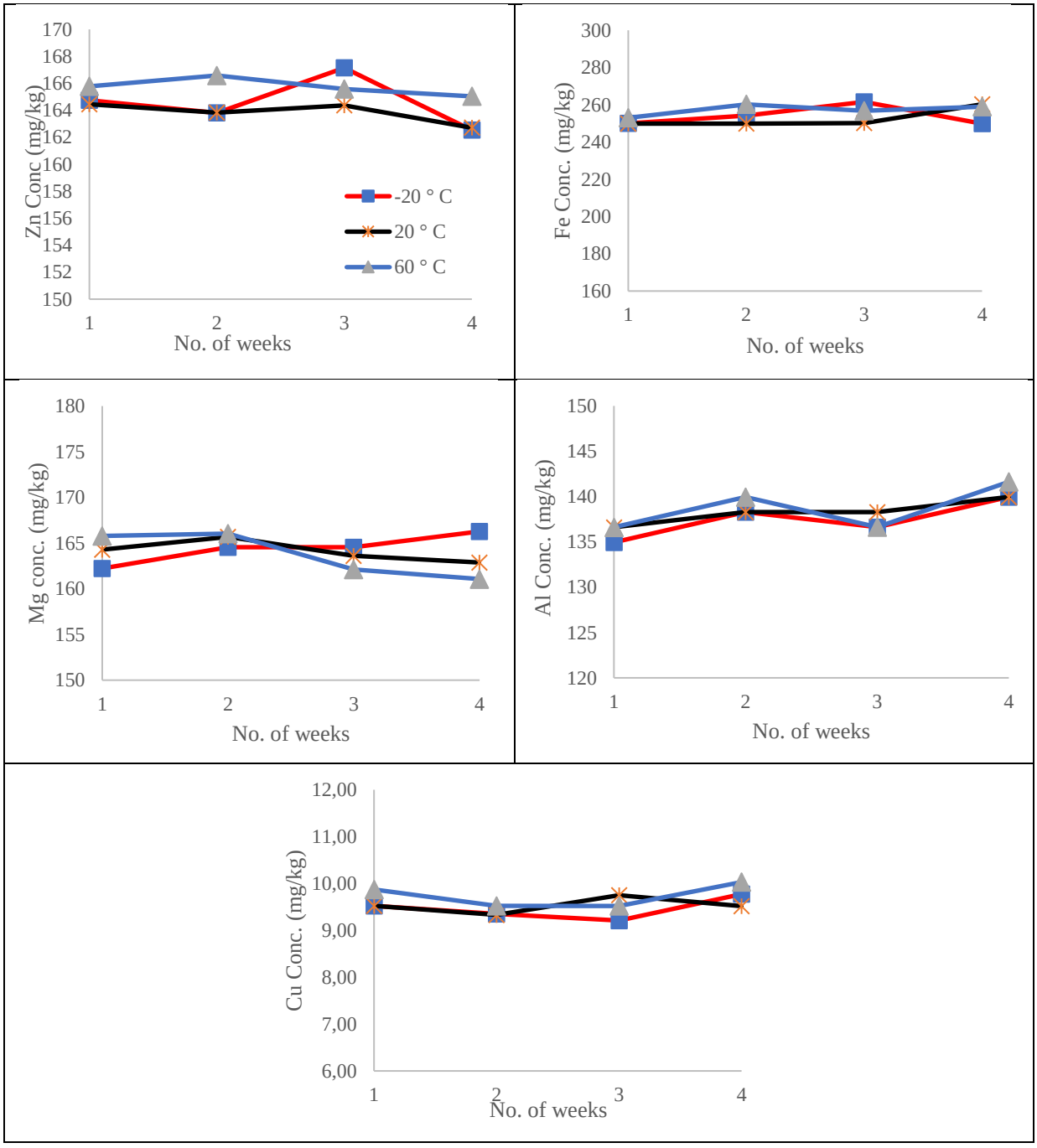

Figure 6. Short-term stability assessment of the measured elements**.**

Table 5 shows each element's slope values and standard error (S) at both temperatures. The results revealed that the slope was not significantly different from zero at both temperatures. The standard uncertainty of the short-term stability of the analytes according to the following equation:

$$u_{sts} = \frac{S_{x.y}}{\sqrt{\sum(x_i - \bar{x})^2}}$$

Where $S_{x.y}$ is the standard error calculated from the regression line analysis, x_i measurement result at time point i and $\bar{x}$ is the mean of all measurement results for all time points. $S_{x.y}$ is calculated as follows:

$$S_{x.y} = \sqrt{\frac{\sum(y - \hat{y})^2}{n - 2}}$$

Where y is the measured concentrations, $\hat{y}$ is the concentrations from the regression line, and n is the total number of measurements. The standard uncertainty of each element was calculated and presented in Table 5.

Table 5: The Slope, the standard error, and the uncertainty of the short-term stability

Analyte	20 ºC		60 ºC		u_{sts}
	Slope	S	Slope	S	
Al	-0.0024	0.0034	-0.0013	0.0032	0.0028
Zn	-0.0008	0.0045	0.0001	0.0066	0.0133
Cu	-0.0017	0.0200	-0.0015	0.0044	0.0221
Mg	-0.0112	0.0015	-0.0183	0.0014	0.0010
Fe	0.0099	0.0182	0.0032	0.0125	0.0051

4. Conclusions

The production of Certified Reference Materials (CRMs) requires the assessment of two critical parameters, homogeneity and stability, as per the standard ISO guide 35. In this study, we focused on evaluating the homogeneity and short-term stability of a batch of human hair powder using ICP-OES for measurements. The results were statistically analyzed, and it was found that the samples were uniform and stable during transportation at different temperatures. Further studies will be conducted to assess the long-term stability and characterization of the CRM in different laboratories. The measurement results will be evaluated, and the final assigned values and their associated uncertainties will be calculated. This CRM will be available to labs involved in elemental measurements as a control sample to verify the accuracy and correctness of their measurement results.

Acknowledgments

This study is part of the master's thesis of student Reham Elnaggar, and the study will continue to finish the certification steps for candidate human hair-certified reference material.

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