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METHOD VALIDATION FOR COPPER DETERMINATION IN HUMAN HAIR SAMPLES THROUGH GRAPHITE FURNACE ATOMIC ABSORPTION SPECTROMETRY

Nicoleta MATEI[a], Semaghiul BIRGHILA[a]*, Simona DOBRINAS[a], Alina SOCEANU[a], Ana-Maria ILIE[a], Viorica POPESCU[a]

ABSTRACT. A simple, cheap, and sensitive analytical method was validated for the determination of copper in human hair after microwave digestion. Method validation parameters such as linearity, precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ) were determined. A graphite furnace atomic absorption spectrophotometer has been used. The developed method was linear in the concentration range of 2 - 20 µg/L with a 0.9979 coefficient of determination. The recoveries obtained for the copper ranged from 90.46-94.96%, with a precision not exceeding 3.95% relative standard deviation (RDS%) and system suitability test with RSD% lower than 1.58%. LOD was found to be 0.05632 µg/g and LOQ 0.18745 µg/g. The analyzed samples were from healthy humans and the study shown similar concentration of copper in hairs collected from adult, teenager men and female. The proposed method was considered adequate for the determination of copper in hair samples.

Keywords: *GFAAS, hair, copper, validation*

INTRODUCTION

Copper is an indispensable element of life and a dynamic, anti-infectious, antiviral, anti-inflammatory mineral. The adult human body contains about 75 mg of copper. The body mobilizes it in cases of microbial

[a] *Ovidius University of Constanta, Faculty of Applied Sciences and Engineering, Department of Chemistry and Chemical Engineering, 124 Mamaia Blvd., RO-900527, Constanta, Romania, nmatei@univ-ovidius.ro, sbirghila@univ-ovidius.ro, sdorbinas@univ-ovidius.ro, asoceanu@univ-ovidius.ro, anamarianiculescu@yahoo.com, vpopescu@univ-ovidius.ro*
* *Corresponding author: sbirghila@univ-ovidius.ro*

aggression in infections. Copper helps our bodies to produce energy, to form important neurotransmitters and our connective tissue, supports the production of melanin in our skin and helps the transport of iron in the body. Consumption of foods that contain copper can prevent certain diseases or deficiencies (AIDS, leukemia, osteoporosis, stomach ulcer, allergies) [1].

Copper is widespread in foods, fruits and meat: in the liver, shells, nuts, vegetables, in most cereals, cocoa powder and in grapes [2]. The amount of copper in water varies depending on the natural mineral content of the water and the pH or the presence of copper pipes in the systems.

For children, adequate intakes between 400μg Cu/day (7—11 months aged) to 1100 μg Cu/day (< 18 aged girls), and 1300 μg Cu/day (< 18 aged boys) respectively, are proposed by EFSA (European Food Safety Authority) [3]. It has been found that the presence of zinc and vitamin C inhibits the retention of copper, and alcohol consumption can amplify the deficit. The deficiency of copper is mainly caused by genetic disorders and is manifested by anemia [1, 4-7], the discoloration of the hair and skin, bone demineralization, spontaneous fractures, increased blood cholesterol, brain degeneration, damage of the nervous system.

The copper toxicity determined by food intake is considered impossible, but it occurs because of water consumption with increased copper concentrations or those with professional exposure. The symptoms of acute copper poisoning are nausea, vomiting, diarrhea and abdominal pain, liver and kidney lesions, coma [1]. Wilson disease is a disorder of copper metabolism, based on a low serum ceruloplasmin level and a high urinary excretion of copper.

Hair is a bioindicator that offers important indices about nutritional imbalances in the body. It is used to detect chronic intoxication before symptoms appear, because concentrations of toxic metals are ten times higher in hair than in other biological materials [7]. The correct functioning of enzymes in the body is conditioned by vitamins and minerals. If minerals are missing, proteins cannot work correctly. Copper is an essential element of some enzymatic systems involved in hemoglobin production, carbohydrate metabolism and the formation of cross-linking between collagen, elastin and keratin fibers in the hair [8].

In medical analysis laboratories, the copper content of clinical samples is determined by ICP-MS [8-12], ICP-OES [13], ICP-AES [14,15] or AAS [8, 9, 16]. For the analysis of the copper of the hair, the AAS technique is recommended, preceded by an acidic digestion of the samples because thus the organic matter is effectively removed [7, 17]. GFAAS has wide application for trace elements, low operational and instrumental costs, is an accessible technique in most routine laboratories and it’s easy to operate [9, 18].

The aim of this study is the validation (linearity and range, limit of detection, limit of quantification, precision, and accuracy) of a simple, cheap, and sensitive analytical method for the determination of copper content in hair samples through graphite furnace atomic absorption spectrophotometry (GFAAS). Determining the copper content in different human hairs can be used as an index of exposure to this potentially toxic element or as information on a person's health status.

RESULTS AND DISCUSSION

The graphite furnace atomic absorption spectrophotometry applied in this study was tested for system suitability by aspirating five replicates of copper samples. The best conditions are adequately selected. The system suitability was performed to provide that the testing system (instrument, reagents, and analyst) is appropriate for the copper analysis in hair samples. The primary parameters can include repeatability (%RSD) for five absorbance readings. The RSD% values are presented in Table 1.

Table 1. System suitability testing of copper determination in hair by GFAAS

Copper standard (µg/L)	Absorbances reading					RSD%
2	0.093	0.087	0.087	0.085	0.088	1.58
5	0.171	0.171	0.168	0.170	0.172	0.74
9	0.293	0.290	0.293	0.293	0.262	1.45
13	0.416	0.420	0.416	0.420	0.420	0.45
17	0.539	0.538	0.536	0.538	0.541	0.27
20	0.659	0.661	0.665	0.665	0.674	0.76

The proposed and validated analytical method was used to determine the copper in 12 hair samples. The hair samples, including 9 normal and 3 dye, were collected from both sexes aged from 12 to 75. The results are presented in tables 2-5.

The RSD% for copper absorbance readings should not be more than 2.0% [19]. Results presented in Table 1 demonstrated the suitability of the complete system for the copper analysis in hair samples.

Table 2. Intra-day repeatability of the proposed method

Sample	Theoretical copper concentration (µg/g)	Measured copper concentration (µg/g)* ±sd	Relative standard deviation (%)
Standard 1	13	12.8±0.352	2.75
Standard 2	20	20.5±0.170	0.83
Hair sample	-	11.4±0.362	3.18

* mean of six measurements

The homogeneity variance test (applied for the lowest and the highest concentration values of proposed concentration range, each of them measured by ten times) established a value of 3.24. F test was applied with the following acceptance criterion: F value is 5.35 higher than 3.24 value. This means that no significant differences were found between the variances of the concentration range limits.

The calibration curve for copper was constructed by plotting the absorbances versus concentrations. Linearity is observed in a concentration range from 2 µg/L to 20 µg/L of copper. The least squares method showed the linearity of the proposed method obtaining a linear regression equation y = 0.0324x and the determination coefficient (R^2) equal to 0.9979. The value of R^2 showed excellent linearity of the calibration curve for the method with LOD = 0.0563 µg/g and LOQ = 0.1874 µg/g.

Table 3. Inter-day reproducibility for copper standard solutions for proposed method

Sample	Day	Theoretical copper concentration (µg/L)	Measured copper concentration (µg/L)* ±sd	Relative standard deviation (%)
Standard 1	1st	13	12.90±0.101	0.79
	2nd	13	12.93±0.249	1.93
	3rd	13	12.85±0.204	1.59
Standard 2	1st	20	20.40±0.191	0.94
	2nd	20	20.30±0.416	2.05
	3rd	20	20.90±0.211	1.01

* mean of six measurements

Table 4. Inter-day reproductibility for different hair samples

Sample	Day	Measured copper concentration (µg/g)* ±sd	Relative standard deviation (%)
adult male hair sample	1st	11.4±0.362	3.18
	2nd	11.2±0.356	3.18
	3rd	11.3±0.205	1.82
adult female hair sample	1st	199.09±4.200	2.11
	2nd	191.80±4.089	2.14
	3rd	196.30±3.788	1.93
teenager male hair sample	1st	130.0±5.018	3.86
	2nd	133.2±5.261	3.95
	3rd	127.2±1.869	1.47
teenager female hair sample	1st	11.31±0.321	2.84
	2nd	11.41±0.416	3.65
	3rd	11.27±0.373	3.31

* mean of six measurements

Table 5. Levels of copper in hair samples

Hair sample	Treatment	Concentration (µg/g) ± confidence interval
adult male - 46 years old	normal	11.24±2.230
adult male - 50 years old	normal	45.42±2.206
adult male - 75 years old	normal	50.50±0.891
teenager male - 15 years old	normal	130.17±0.638
teenager male - 18 years old	normal	64.27±2.196
adult female - 44 years old	dyed	197.21±0.064
adult female - 58 years old	dyed	145.06±0.283
adult female - 61 years old	dyed	142.18±0.393
adult female - 21 years old	normal	74.17±1.038
adult female - 29 years old	normal	98.59±1.321
teenager female - 13 years old	normal	11.33±0.826
teenager female - 12 years old	normal	56.06±2.583

Precision was determined by analyzing results from the intra-day repeatability and inter-day reproductibility for the known concentrations of copper standard solutions (n= 6). Simultaneous replicates at different times, on different hair samples, were performed during the study period, in the same way as for standard solution to obtain more representative values [20]. The relative standard deviations are based on Horwitz equation which suggest that $RSD<2^{(1-0.5\lg c)}$, where c is the concentration of the analyte. The obtained %RSD values for the standard solution of copper and for hair samples, were lower than those obtained using the Horwitz equation (table 2 - 4). Also, according to the acceptance criteria established by the Commission of the European Communities all the calculated RSD values were lower than 15%, demonstrating an adequate precision [21].

By combining the data obtained, it can be found that with the increase in the number of samples analyzed, the confidence in the precision of the measurements made increases; with the increase of analytical variability, no deterioration of RSD% is observed.

The accuracy values obtained for the different additions of standard solutions containing 7.20 µg/g, 18.08 µg/g and 36 µg/g of copper are 92.91%, 94.96% and 90.46%, respectively. These results (table 6) highlight the fact that the recovery obtained is between 90.46-94.96%, falling within the acceptance criterion for fortification tests which requires recovery values within the range of 80 to 110 % [20]. It is evident that the method is accurate within the desired range.

Table 6. The results for accuracy at the copper determination from hair using the GF AAS technique.

Sample	A multielement stock solution concentration added (calculated) µg/g	Concentration recovered µg/g	Recovery (%)
adult male hair sample	-	11.03	
	+7.20	17.72	92.91
	+18.08	28.2	94.96
	+36.00	43.6	90.46

Method Applicability

Copper concentrations detected in studied hair samples (Table 5) are comparable and some are higher as those reported in other studies [6, 9-16, 18, 22-24]. The data obtained indicate that higher amounts of copper are found in adult female hair samples; the amount are higher probably due to the dyed hair. These differences in the composition of colored or untreated hair are because coloring process leads to the opening of the hair cuticle and the partial exchange of element between the hair and the dye [25, 26].

Copper concentrations in studied hair samples were higher in the female subjects than male subjects. This probably can be assigned to the different hormonal balance between sexes. The copper level increased from teenager female to adult female (normal treatment of hair), but it decreased from teenager man to adult male. This can be explained by the high intake required during adolescence period for teenager man and in case of teenager female was observed a different behavior due to the beginning of menstruation [4].

Grzegorz Izydorczyk and co. publish a study about hair mineral analysis in the population of Poland. They found that the copper levels are higher on female than male as in our study (Table 5) and smoking, occupation, dietary habits, local crops consumption are the factors that influences the cooper level in hair [27].

The copper content in hair samples is influenced by a series of factors such as: metabolism, lifestyle, health status, geographical and economical living conditions as well the type of hair, and hair treatment [2, 28 - 30].

CONCLUSION

The data obtained from the validation procedure shows that the proposed GFAAS method is accurate and corresponds to the criteria of linearity, repeatability, intermediate precision, LOD and LOQ, implemented by Council Directive 96/23/EC. Validation was performed by studying analytical curve linearity (R^2=0.9979) and range (2 - 20 µg/L), estimated limit of detection (LOD) - 0.05632 µg/g and limit of quantification (LOQ) - 0,18745 µg/g. The method accuracy ranged from 90.46-94.96% and the precision measured as intra-day repeatability and inter-day reproductibility did not exceed 3.95% RDS%. The system suitability test was performed with RSD% lower than 1.58%. This study confirms that the proposed method is suited to copper analysis from human hair samples. It was concluded that the copper concentration in the dyed hair samples is higher than the normal hair samples. Also, the data obtained indicated that higher amounts of copper may be required for growing of teenagers.

EXPERIMENTAL SECTION

Sample Preparation

The human hair samples were collected from healthy adults and teenagers in the age range of 12 -75. Each sample was cut in 5 mm length with sterilized stainless-steel scissors and packaged in polyethylene bags. In the laboratory, one gram of each sample was washed with detergent, then rinsed with ultrapure water, acetone and dry for 6 h at 80^0C. The ground dry hair sample (50 mg) was accurately weighed into a PTFE vessel and 2.5 ml of HNO_3 65 % was added (suprapur HNO_3 65 %, Merck KGaA, Darmstadt, Germany). The vessel was closed, shacked, and placed inside the microwave digestion system for 15 min at 170^0C, table 7. At the end of digestion, the samples were removed from the digestion oven, cooled at room temperature, and diluted to 50 ml final

volume with deionised water. Finally, the solutions were filtered using a 0.45 µm pore size filter. Mineralisation was carried out in a Microwave Digestion System (Berghof Speedwave®, ENTRY, Germany), in high-pressure PTFE vessel, using a standard acid digestion protocol for hair [31]. The blanks were submitted to the same digestion procedure mentioned above.

Table 7. Temperature program

Step	Temperature (^{0}C)	Ramp (min)	Time (min)
1	130	3	8
2	155	2	5
3	170	2	15
4	75	1	10

Instrumentation

The copper content was determined by graphite furnace atomic absorption spectrometry (GFAAS, model: ContrAA 800D, AnalytiK Jena Instruments, Germany) using standard calibration technique. Blank samples were prepared by adding same the reagents into a PTFE vessel without the sample and subsequently diluted in the same manner as described above. To reduce the risk of contamination, the whole glass was carefully cleaned and rinsed with ultrapure water.

To provide results directly proportional to the analyte concentration in the sample within a set range, calibration curve was prepared through 1000 ppm standard solution dilution (ICP multi-element standard solution IV, Merck, Germany). Deionized water (Direct Q UV, Millipore, approximately 18.0MΩ) was used in the preparation of all solutions. The solutions were kept in polyethylene vials. The graphite furnace parameters of analytical method are: 324.754 nm (wavelength – primary line), with 900^{0}C for pyrolysis and 2000^{0}C for atomization (without modifier), table 8. The atomic absorption spectrometer has a Xenon short arc lamp as a single light source.

Table 8. Graphite furnace parameters (platform)

Step	Name	Temperature (^{0}C)
1	Drying	80
2	Drying	90
3	Drying	110
4	Pyrolysis	350
5	Pyrolysis	900
6	Gas adaption	900
7	Atomize	2000
8	Clean	2450

Method Validation

In this study validation parameters such as linearity and range, limit of detection (LOD), limit of quantification (LOQ), precision, and accuracy were evaluated.

Linearity

The linearity of this method was evaluated by constructing a calibration curve in the range 2 µg/L - 20 µg/L.

A set of copper standard solutions were freshly prepared by appropriate dilution of the stock intermediate standard solution (20 µg/L) to get calibration curve. The appropriate volumes were measured from the intermediate stock solution automatically inserted with the autosampler in the GF-AAS. For each sample five readings were noted and the % relative standard deviation for absorbance for each copper standard solution were calculated. System suitability parameters were evaluated.

The homogeneity variance test was used to test the linearity range. To the obtained variances, the F test was applied to evaluate the significant differences of concentration range limits and to evaluate the regression and lack of fit significances [32].

Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ were calculated using the calibration data and regression statistic, using the formulas: LOD = a + 3 s(r); LOQ = a + 10 s(r); where s(r) is the residual standard deviation or residual standard error and a is the y-intercept [33]. Residual standard error is a statistical measure of the deviation of the data from the fitted regression line. It is possible to calculate a confidence interval for the predicted values using the calibration function, an interval that appears in the literature under the name “standard error of prediction”. The prediction interval provides an estimate of the uncertainly associated with the predicted values of x. The 13µg/L standard solution was used to evaluate the detection limit of the method and the absorbance of three measurements was used in the calculations.

Precision

Intra-day repeatability. The repeatability of the analytical method was determined by relative standard deviation (%RSD) for six determination of the copper standard solution (medium level concentration - 13µg/L and high-level concentration - 20µg/L) and an adult male hair sample, performed on the same day with an interval of an hour under the same experimental and laboratory conditions.

Inter-day reproducibility. Inter-day reproducibility is the estimation of variations in analysis when a method is used in the same laboratory, on the different day and different analyst or when a method is used within laboratories. The inter-day reproducibility is assessed by analyzing simultaneous replicates at different times, on different hair samples (adult male, adult female, teenager male and teenager female hair samples), were performed during the study period, in the same way as for standard solutions.

Accuracy

In this study, accuracy was demonstrated by performing additions of different concentrations of copper standard solution (7.20 µg/g, 18.08 µg/g and 36.00 µg/g) to a known pre-analyzed sample.

The % recovery of the added copper standard solution calculated as, %Recovery = [(Ct −Cs) / Ca] × 100, where Ct is the total analyte concentration measured after standard addition; Cs, analyte concentration in the sample; Ca, analyte concentration added to sample.

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