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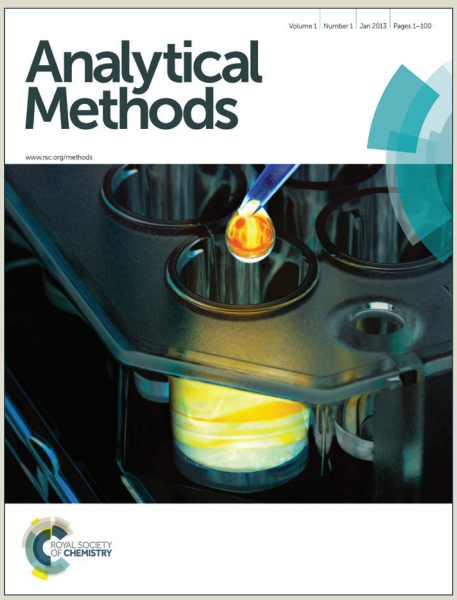

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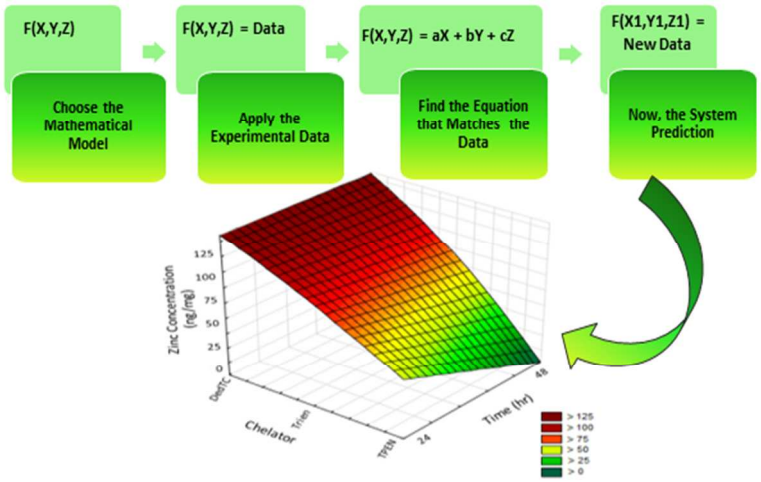
Graphical abstract



General Chelating Action for Copper, Zinc and Iron in Mammalian Cells

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ABSTRACT: The high-accuracy determination of trace metals in biological systems is a crucial step for the elucidation of their role in these systems. We investigated the influence of the most commonly used intracellular metal chelators, N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), triethylenetetramine (Trien) and N,N-diethyldithiocarbamate (DeDTC), on the concentration of Cu, Fe and Zn in mammalian cells. We analyzed the influence of chelator type, concentration and time of exposure on cultured cells. The obtained data were used to formulate a general equation for evaluating how each metal concentration is influenced by experimental conditions. For this purpose, an analytical method was improved to determine Cu, Fe and Zn concentrations in cells using graphite furnace atomic absorption spectroscopy with low sample handling and direct injection of the solid (SS GF AAS). We used non-adherent human lymphoma U937 cells as a model, and these cells received each chelator at different concentrations and exposure times. We used a factorial design to determine models to describe Cu, Fe and Zn concentrations in cells. Analyses using cubic equation models showed that chelator type is the most relevant factor for the three metals. Our results suggest that chelation therapy in cultured cells changes intracellular Cu, Fe and Zn concentrations in a more complex manner than currently described in the literature. For Cu, Zn and Fe variations inside the cell, chelator type is important, and for zinc, time of exposure and concentration of the chelator are also important.

Abbreviations: ET AAS, electrothermal atomic absorption spectrometry or GF AAS, graphite furnace AAS; SS GF AAS, solid sample graphite furnace AAS; F AAS, flame atomic absorption spectrometry; ICP AES, inductively coupled plasma atomic emission spectroscopy; ICP MS, inductively coupled mass spectrometry; ICP OES, inductively coupled plasma optical emission spectrometry; LA ICP MS, laser ablation inductively coupled mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; TPEN, N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine; Trien, N,N'-Bis(2-aminoethyl)ethane-1,2-diamine or Triethylenetetramine or TETA; DeDTC, N,N-diethyldithiocarbamate; SRM, certified reference material or standard reference material.

Introduction

The study of trace elements and their functions in biological systems requires methodologies that precisely quantify each of the metals in these systems.[1] Various analytical methods have been developed to quantify trace metals, including Cu, Fe and Zn in biological matrices, such as cells and tissues.[2-8] In several studies of Cu, Fe and Zn quantification, researchers have found that the excess or absence of these metals is associated with neurodegenerative diseases[9-12] and metabolic control.[13] Elemental analyses of Cu, Fe and Zn in mammalian cells in culture or from tissue plays an important role in evaluating how these metals affect diseases and cellular metabolism. Therefore, choosing the analytical method that results in high accuracy is very important for the reliability of biological research.[14] The use of chelants is equally important for evaluating the effect of some metals on metabolism[15,16] or cancer[17] and for studying disease treatments.[18-25]

Various analytical methods are widely used in determining Cu, Fe and Zn content in biological samples, including total reflection X-ray fluorescence spectrometry (TXRF),[26,27] mass spectrometry with inductively coupled plasma (ICP MS),[4,28,29] graphite furnace atomic absorption spectrometry (GF AAS)[30-32] and flame atomic absorption spectrometry (F AAS).[33,34]

Elemental determination in solid samples is commonly performed after previously treating the matrix, and this critical step requires time, is expensive and occasionally results in errors due to excessive sample manipulation. GF AAS allows for direct introduction of solids (SS GF AAS), and in some cases, a simple milling step can result in good precision and accuracy.[35,36] Among other advantages, GF AAS enables the determination of trace and ultra-trace elements. The heating program allows the sample to be thermally pre-treated, causing matrix and analyte separation, especially during the pyrolysis step; a suitable heating program, coupled with the use of chemical modifiers, permits equipment calibration with standard aqueous solutions, which partly addresses the lack of a sufficient quantity of calibrant materials with similar chemical compositions and the small amount of the analyte of interest.[32,37] Furthermore, the requirement of only small sample quantities is an important advantage in the basic determination in biological samples. The Cu, Fe and Zn

concentrations in biological samples such as rice, cereals, vitamins,[38,39] proteins,[40] nuts and seeds,[41] has been studied by several authors. These authors determined the composition of these samples using solid GF AAS. In most cases, it was possible to use an aqueous calibration, and in some cases, chemical modifiers were not required. Thus, these previous studies served as a starting point for the method developed in our study for cell samples, which contain a rich organic matrix.

The concentration of iron, copper and zinc in mammalian U937 cells by SS GF AAS was assessed after administering the chelator TRIEN, DeDTC or TPEN in the culture medium at different concentrations and exposure durations. Statistical analyses were performed to investigate correlations between exposure durations, concentrations and the type of chelator employed. This model establishes a better understanding of the effect of chelators widely used for biological research.

EXPERIMENTAL

Instrumentation

The determination of Cu, Fe and Zn concentrations was performed on an atomic absorption spectrometer with a graphite furnace, model ZEEnit 600 (Analytik Jena AG, Jena, Germany), equipped with a background correction based on the Zeeman effect. A solid sampling acessory was purchased from the same manufacturer. A tube of transversely heated pyrolytic graphite and a pyrolytic graphite-type boat (Analytik Jena) platform were used. The standard aqueous solutions and certified reference material (CRM or SRM) of the National Institute of Standards and Technology (NIST) 1643e were transferred to the platform using a micropipette. Cell samples and CRM NIST 1547 were transferred using a titanium spatula.

The hollow cathode lamps for Cu, Fe and Zn and the spectrometer were operated according to the conditions listed in Table 1. The purging and shielding gas was high-purity argon (99.9992%, Air Products). Heat program optimization was performed using pyrolysis curves and atomization in a standard aqueous solution and the certified reference material NIST 1547. Integrated absorbance signals (for the standard aqueous solution) and the ratio of the integrated absorbance and heavy mass (for the solid matrix cell) were used to construct the analytical calibration curves. The experimental conditions and heating program used to determine Cu, Fe and Zn concentrations with SS GF AAS are shown in Table 1.

Heat program optimization with the standard aqueous solutions was performed using a 10 µL solution 50 µg L^{-1} Cu, 20 µg L^{-1} Fe, and 10 µg L^{-1} Zn. CRM NIST 1547 (3.7 ± 0.4 mg Kg^{-1} Cu, 218 ± 14 mg Kg^{-1} Fe and 17.9 ± 0.4 mg Kg^{-1} Zn) was performed using approximately 100 mg, 30 mg and 35 mg of the material for Cu, Fe and Zn, respectively.

Reagents and solutions

Standard aqueous solutions of Cu, Fe and Zn were prepared from serial dilutions of $Cu(NO_3)_2$ (Fluka), $Fe(NO_3)_3$ (ULTRA Scientific) and $Zn(NO_3)_2$ (Sigma Aldrich) stock solution at 1000 mg L^{-1} in a 1% (v/v) nitric acid medium. High-purity deionized water was obtained with the Milli-Q® ultra purification system (Millipore, Beldford, MA), and the solutions were stored in sterile vials.

Table 1. Instrumental Parameters and Heating Program Used to Determine Cu, Fe and Zn Concentrations in Mammalian U937 Cells with SS GF AAS

	Cu	Fe	Zn
Wavelength (nm)	216.5/324.8	302.1/248.3	213.9/307.6
Lamp current (mA)	4.0	6.0	4.0
Bandpass (nm)	0.8	0.8	0.8
Drying step			
Temperature (°C)	130	130	125
Ramp (°C s^{-1})	10	10	10
Hold (s)	10	45	25
Argon flow (L min^{-1})	1.0	1.0	1.0
Pyrolysis step			
Temperature (°C)	1200	1200	700
Ramp (°C s^{-1})	100	100	100
Hold (s)	20	20	40
Argon flow (L min^{-1})	1.0	1.0	1.0
Atomization step			
Temperature (°C)	2300	2300	2200
Ramp (°C s^{-1})	1300	1000	1200
Hold (s)	6	6	6
Argon flow (L min^{-1})	0	0	0/0.25
Cleaning step			
Temperature (°C)	2600	2500	2500
Ramp (°C s^{-1})	1100	800	1100
Hold (s)	6	8	6
Argon flow (L min^{-1})	1.0	1.0	1.0

Sample preparation

The cell cultures were manipulated using sterile, disposable, non-pyrogenic plasticware and maintained at 37°C in a 5% CO_2 atmosphere at a relative humidity of 80%. Nonadherent U937 human promonocytic cells from ATCC (American Type Culture Collection, USA) were grown in 75 cm^2 bottles in RPMI 1640 culture medium (Sigma) with 10% fetal bovine serum (FBS; Gibco), 100 U/mL penicillin and 10.0 µg/mL streptomycin (Sigma). The cells were routinely reinnoculated onto plates at a density of 4 x 10^5 cells/mL. The cells received treatment with the chelators N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), triethylenetetramine (Trien) and N,N-

diethyldithiocarbamate (DeDTC) at concentrations of 5-50 µM, and the cells were incubated for 24 or 48 hours at 37°C with 5% CO_2 and 80% humidity. The cells were cultured in triplicate, and control cells were maintained without the addition of chelators for 24 and 48 hours.

After the respective treatment periods, the cells were collected in sterile Falcon® tubes and centrifuged at 218 *g* for 3 minutes. The supernatant was discarded, and the cell pellet was washed 3 times with 3 mL of PBS-EDTA buffer (2.7 mM KCl, 137 mM NaCl, 10 mM Na_2HPO_4, 10 mM NaH_2PO_4, and 0.53 mM EDTA, adjusted to pH 7.4), followed by two washes with 3 mL of PBS (phosphate buffered saline). After washing, the Falcon® tubes containing the cell pellet were kept in an oven at 60°C for 10 days or until completely desiccated. Then, the cells were crushed with a mini plastic pestle in a laminar flow hood until a fine and homogeneous powder was obtained.

RESULTS AND DISCUSSION

To understand the chelator action in cultured cells, a precise analytical method for determining Cu, Fe and Zn content is necessary. We used atomic absorption spectroscopy with a graphite furnace and the direct injection of solids (SS GF AAS) to develop such a method. Our method enables fast, precise and direct measurements of a solid sample without pre-treatment or excessive cell manipulation.

Sample pyrolysis and atomization temperatures

The thermal behavior of Cu, Fe and Zn in standard aqueous solution and in solid material (SRM 1547, peach leaf) was assessed using pyrolysis temperature curves (Tp) and the atomization temperature (Ta) of the elements in the absence of chemical modifiers.

Setting the heating program is essential for accurately analyzing samples by SS GF AAS. The samples may be placed into the graphite furnace with minimal or no pre-treatment by applying suitable atomizing and pyrolyzing temperatures.[42-44] After evaluating the thermal behavior of copper and iron in the standard aqueous solution and CRM NIST 1547 solution, we determined that the best pyrolysis temperature for both metals was 1200°C. At this temperature, we observed a transient signal without the formation of a double peak, and a low relative standard deviation. For atomization, 2300°C was determined to be the ideal temperature for both metals. Before optimizing the heating program for zinc, its thermal behavior was investigated in two steps: first, the pyrolysis temperature was varied from 500 to 900°C to select a temperature range in which a marked loss of analytical signal was observed. A 600-850°C range was selected based on the analytical signal intensity. Then, we evaluated the analytic signal profile within the previously established pyrolysis temperature range with a pyrolysis step of 10 to 40 seconds. After assessing the profile and analytical signal intensity, 700°C and 40 seconds were selected as the optimal conditions for the pyrolysis step. Significant differences in the analytical signals were not observed when the heating increase of the pyrolysis step varied from 50 to 200°C/s.

Table 2 shows the calibration curves with the standard aqueous solution and the solid U937 cell matrix for determining Cu, Fe and Zn content with the optimized heating program. Table 2 also presents the parameters of the analytical calibration curves and the correlation coefficients.

Table 2. Parameters for the Analytical Calibration Curves Obtained with Standard Aqueous Solutions and a Solid Cell Matrix

Metals	Linear Equations	R^2
Cu[a]	A = 0.0423 C + 0.0195	0.9992
Cu[b]	A = 0.0431 C + 0.0324	0.9935
Fe[a]	A = 0.151 C + 0.0492	0.9988
Fe[b]	A = 0.154 C + 0.144	0.9927
Zn[a]	A = 0.00152 C + 0.00133	0.9993
Zn[b]	A = 0.00159 C + 0.00245	0.9971

[a] standard analytical solution, [b]U937 cells

By comparing the slopes obtained using a linear equation for the analytical curves with a standard aqueous solution and solid cell matrix (Table 2), the effect caused by the matrix could be evaluated. The ratios obtained from the slopes of the curves were approximately one (1) for the three metals (copper: 0.98, iron: 0.98, and zinc: 0.95), indicating a low matrix effect and confirming the possibility of calibration using standard aqueous solutions.

We also evaluated the limit of detection (LOD), limit of quantification (LOQ), characteristic mass (m_0) and precision. The LOD and LOQ were estimated according to the IUPAC recommendations at a confidence level of 99.8%.[45] Precision was expressed in relation to the coefficient of variation estimated by 12 independent measurements with solutions of copper (25 µg L^{-1} and 0.5 mg L^{-1}), iron (35 µg L^{-1} and 0.25 mg L^{-1}), and zinc (4.0 µg L^{-1} and 0.25 mg L^{-1}). Whereas the analyzed cells were submitted to different treatments to increase or decrease intracellular concentrations of Cu, Fe and Zn, the analytical parameters were obtained using the two absorption wavelengths for each metal, which are 324.8 and 216.5 nm for Cu, 248.3 and 302.1 nm for Fe, and 213.9 and 307.6 nm for Zn. Table 3 shows the analytical sensitivity parameters for the metals at both wavelengths.

Table 3. Analytical Parameters Developed for Determining Cu, Fe and Zn Content in Cells by SS GF AAS

Metal and Wavelength (nm)	Linear range (ng)	LOD (μg g^{-1})	LOQ (μg g^{-1})	m_0 (pg)	VC (%)
Cu 324.8	0.05 – 0.75	0.048	0.16	22	6.4
Cu 216.5	0.33 – 10	0.52	1.9	80	1.7
Fe 248.3	0.05 – 0.50	0.024	0.078	37	5.1
Fe 302.1	0.5 – 7.0	0.087	0.29	31	2.2
Zn 213.9	0.04 – 0.16	0.0069	0.017	0.079	4.2
Zn 307.6	2.5 – 100	2.1	6.7	2714	2.0

VC – variation coefficient

Table 4. Determining Cu, Fe and Zn Content in CRM Using SS GF AAS (n = 3)

	CRM NIST	Certified value	Observed value	
Cu	1643e	22.76 ± 0.31	21.33 ± 0.29	μg L^{-1}
Cu	1547	3.7 ± 0.4	3.4 ± 0.1	mg Kg^{-1}
Fe	1643e	98.1 ± 1.4	101 ± 4	μg L-1
Fe	1547	218 ± 14	224 ± 11	mg Kg^{-1}
Zn	1643e	78.5 ± 2.2	65 ± 24	μg L-1
Zn	1547	17.9 ± 0.4	17.3 ± 0.4	mg Kg^{-1}

CRM: certified reference materials

The accuracy of a method can be determined by certified reference materials, interlaboratory tests, and comparing and testing the methods of addition and recovery. In the proposed method, the accuracy was evaluated by determining Cu, Fe and Zn content in CRM 1643e and 1547, as shown in Table 4.

A Student's t-test indicated similarity between the certified and observed values for all metals at a confidence level of 95%, except for Cu in NIST CRM 1643e, which showed 99.9% agreement between values.

The sample preparation and manipulation with concentrated nitric acid and hydrogen peroxide required for analytical methods is a critical step in precise trace metal determination[14]. Sample contamination during this step can be derived from the air, impurities in the reactants or contaminated materials, loss by adsorption or volatilization elements, and incomplete decomposition of the samples. Here we used solid samplings allowing minimal manipulation of the sample without losing the characteristic sensitivity of GF AAS. Many authors successfully apply the solid sampling method in different solid samples (see Table S1 in Supporting Information for comparison) [38-41,46]. We have improved the methodology for the endogenous metal quantification (such as Cu, Fe and Zn) in order to use it in mammalian cell samples.

Factorial Design

We used a factorial design to determine models to describe Cu, Fe and Zn concentrations in cells after treatment with DeDTC, Trien and TPEN chelants. We sought to determine a mathematical model to use with our SS GF AAS method and to determine metal content in similar cell lines. We believe that the exposure time (*T*), the chelant type (*Q*), and the chelant concentration (*C*) are three important factors. For the exposure time, we used two durations, 24 hours and 48 hours. For the chelant type, three types of chelants were used, DeDTC, Trien and TPEN. We used two chelant concentrations, 5 μM and 50 μM. Table S2 (in Supporting Information) displays the results obtained using these factors (in triplicate).

The factorial design enabled a cubic regression model, shown as eq. (1):

$$\text{Model} = \beta_0 + \beta_1 \times \text{Q} + \beta_2 \times \text{C} + \beta_3 \times \text{T} + \beta_4 \times \text{Q}^2 + \beta_5 \times \text{QC} + \beta_6 \times \text{QT} + \beta_7 \times \text{CT} + \beta_8 \times \text{QCT} + \beta_9 \times \text{Q}^2\text{C} + \beta_{10} \times \text{Q}^2\text{T}. \quad (1)$$

where the parameters β_i are the regression coefficients. The β_0 is the typical metal content when no chelator was used. Note that in this model, some terms are missing, such as C^2, T^2 and Q^3, because three levels in T and C and four levels in Q are needed for inclusion. By applying the least squares method to the copper concentration values shown in Table S2 (Supporting Information) with model (1), we obtain eq. (2):

$$\text{Copper} = \underset{\pm 0.31}{2.03} - \underset{\pm 0.21}{17.58} \times \text{Q} - \underset{\pm 0.31}{1.48} \times \text{C} + \underset{\pm 0.31}{0.93} \times \text{T} + \underset{\pm 0.37}{16.28} \times \text{Q}^2 - \underset{\pm 0.21}{1.08} \times \text{QC} + \underset{\pm 0.21}{6.58} \times \text{QT} + \underset{\pm 0.17}{0.13} \times \text{CT} - \underset{\pm 0.21}{0.58} \times \text{QCT} + \underset{\pm 0.37}{1.83} \times \text{Q}^2\text{C} - \underset{\pm 0.37}{7.40} \times \text{Q}^2\text{T}. \quad (2)$$

The variance analysis of this model for copper is shown in Table S3. The adjusted error value (MS_{ADJ}) is higher than the value of $F_{1,23}$ when divided with the pure error (MS_{PURE}) by a factor of 2.79, indicating that the model adjusts only reasonably well when using the cubic model. In fitting the copper values to a quadratic model (the quadratic models are not included in this manuscript), MS_{ADJ}/MS_{PURE} is higher than $F_{4,24}$ by approximately 50-fold; thus, much better agreement can be obtained with the cubic model. The regression mean square (MS_{REG}) divided by the residual mean square (MS_{RES}) for the cubic model is MS_{REG}/MS_{RES}=1115. This value is approximately 495-fold higher than that of $F_{10,24}$ and indicates that the regression is significant. In equation (2), *CT* is the only term that can be discarded because the error is greater than the value of its regression coefficient. The larger terms for the copper concentration in the U937 cells are *Q*, Q^2, *QT* and Q^2T, suggesting that the relevant factors for copper analysis are primarily chelant type and, to less degree, exposure time.

We then used the least square method to fit the cubic model (1) to the iron concentration values shown in Table S2. We obtained eq. (3):

$$\text{Iron} = \underset{\pm 0.65}{23.56} + \underset{\pm 0.46}{12.24} \times \text{Q} + \underset{\pm 0.65}{2.37} \times \text{C} - \underset{\pm 0.65}{3.88} \times \text{T} + \underset{\pm 0.79}{8.21} \times \text{Q}^2 + \underset{\pm 0.46}{12.30} \times \text{QC} - \underset{\pm 0.46}{1.18} \times \text{QT} - \underset{\pm 0.37}{2.07} \times \text{CT} - \underset{\pm 0.46}{2.94} \times \text{QCT} + \underset{\pm 0.79}{10.40} \times \text{Q}^2\text{C} + \underset{\pm 0.79}{3.26} \times \text{Q}^2\text{T}. \quad (3)$$

The variance analysis for the iron cubic model is presented in Table S4. MS_{REG}/MS_{RES}=245 when compared with $F_{10,25}$=2.24, indicating that the regression is significant. Furthermore, a comparison of MS_{ADJ}/MS_{PURE}=0.0957 with $F_{1,24}$=4.26 demonstrated that the model has an excellent agreement with the observed data. Note that in equation (3), the most relevant terms are *Q*, Q^2, *QC* and Q^2C, suggesting that the chelant type and its concentration are important factors and that the exposure time factor has a smaller contribution. The quadratic model provides MS_{ADJ}/MS_{PURE}=55.4 when fitted to the iron data, which is greater than $F_{4,24}$=2.78, indicating that the quadratic model is not appropriate for the iron concentration data.

Finally, we fitted the cubic model (1) to the zinc concentration data shown in Table S2. After applying the least square method, we observe the following eq. (4):

$$\text{Zinc} = \underset{\pm 1.4}{84.9} - \underset{\pm 1.0}{40.1} \times \text{Q} - \underset{\pm 1.4}{1.4} \times \text{C} + \underset{\pm 1.4}{2.0} \times \text{T} + \underset{\pm 1.7}{6.2} \times \text{Q}^2 - \underset{\pm 1.0}{17.1} \times \text{QC} + \underset{\pm 1.0}{7.0} \times \text{QT} - \underset{\pm 0.82}{2.37} \times \text{CT} - \underset{\pm 1.0}{2.6} \times \text{QCT} - \underset{\pm 1.7}{17.1} \times \text{Q}^2\text{C} - \underset{\pm 1.7}{11.2} \times \text{Q}^2\text{T}. \quad (4)$$

The variance analysis for the zinc model is shown in Table S5. A comparison between MS_{REG}/MS_{RES}=238 and $F_{10,25}$=2.24 indicated that the regression is significant. However, a comparison between MS_{ADJ}/MS_{PURE}=19.46 and $F_{1,24}$=4.26 indicated only reasonable fitting. For the quadratic model, MS_{ADJ}/MS_{PURE}=41.6 and $F_{4,24}$=2.78. Therefore, similar to the previous cases, the cubic model is a better option to fit the zinc concentration data. The larger terms in equation (4) are *Q*, *QC*, Q^2C and Q^2T, suggesting that all of the factors are important for zinc.

For all metals, we found that the cubic model (1) fit the data much better than the quadratic model. For iron, the fitting was excellent, and for copper and zinc, the fittings were reasonably good.

CONCLUSIONS

We developed a method to quantify Cu, Fe and Zn in mammalian cells in culture using U937 cells. We used direct solid injection with GF AAS (SS GF AAS) to avoid sample manipulation and acidic pre-treatment. The applied method was validated by application with two different certified reference materials. We investigated the limit of detection (LOD), limit of quantification (LOQ), characteristic mass (m_0) and precision. We used a factorial designed mathematical model to explain the application of commercial and widely used chelators in mammalian cells in culture. The chelators, DeDTC, Trien and TPEN, withdraw iron, copper or zinc, respectively, from intracellular medium. We investigated relevant factors to explain the action of chelators on metal content in cells using a cubic mathematical model obtained from our SS GF AAS quantification data. For zinc, all factors were important: concentration, chelator type and time of exposure to the chelator. For iron, the type and concentration of the chelator were important. For copper, only the type of chelator used in culture was important, regardless of the time of contact and the chelator concentration. Considering the employed concentration range (up to 50 µM), time of exposure (up to 48 h) and types of chelators (those most frequently used in the biological literature), we believe this work will help others

researchers conduct experiments using chelators in cell culture, taking into account their actions on trace metals, including Cu, Fe and Zn.

Supporting Information. Tables S1, S2, S3, S4, S5 and mathematic explanations for the formulas.

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Graphical Abstract