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► **To cite this version:**

Eduardo Paredes, Veronique Malard, Claude Vidaud, Emilie Avazeri, Richard Ortega, et al.. Isotopic variations of copper at the protein level in neuronal human cells exposed in vitro to uranium. *Analyst*, 2019, 144 (20), pp.5928-5933. 10.1039/C9AN01081E . hal-02447119

**HAL Id: hal-02447119**

**<https://hal.science/hal-02447119v1>**

Submitted on 21 Jan 2020

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# **Isotopic variations of copper at the protein level in neuronal human cells exposed in vitro to uranium**

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28 **Abstract**

29 The study of isotopic variations of endogenous and toxic metals in fluids and tissues is a recent  
30 research topic with an outstanding potential in biomedical and toxicological investigations.  
31 Most of the analyses have been performed so far in bulk samples, which can make the  
32 interpretation of results entangled, since different sources of stress or the alteration of different  
33 metabolic processes can lead to similar variations in the isotopic compositions of the elements  
34 in bulk samples. The downscaling of the isotopic analysis of elements at the sub-cellular level,  
35 is considered as a more promising alternative. Here we present for the first time the accurate  
36 determination of Cu isotopic ratios in four main protein fractions from lysates of neuron-like  
37 human cells exposed *in vitro* to 10 $\mu$ M of natural uranium for seven days. These protein  
38 fractions were isolated by Size Exclusion Chromatography and analysed by Multi-Collector  
39 Inductively Coupled Plasma Mass Spectrometry to determine the Cu isotopic variations in each  
40 protein fraction with regard to the original cell lysate. Values obtained, expressed as  $\delta^{65}\text{Cu}$ ,  
41 were  $-0.03 \pm 0.14 \text{ ‰}$  ( $U_c, k=2$ ),  $-0.55 \pm 0.20 \text{ ‰}$  ( $U_c, k=2$ ),  $-0.32 \pm 0.21 \text{ ‰}$  ( $U_c, k=2$ ) and  $+0.84 \pm$   
42  $0.21 \text{ ‰}$  ( $U_c, k=2$ ) for the four fractions, satisfying the mass balance. The results obtained in this  
43 preliminary study pave the way for dedicated analytical developments to identify new specific  
44 disease biomarkers, to get insight into the knowledge of stress-induced altered metabolic  
45 processes, as well as to decipher metabolic pathways of toxic elements.

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## 56 **Introduction**

57 The study of the isotopic signatures of endogenous elements, such as Cu, Zn, and Fe, in body  
58 fluids/ tissues and *in vitro* cultured human cell lines is a recent research topic which has shown  
59 an outstanding potential for biomedical investigations. (1,2,3) In particular, Cu and Zn isotopic  
60 signatures seem to be promising tools for the diagnosis of cancer (4,5,6,7) and  
61 neurodegenerative diseases, (8,9,10,11) as well as for the follow-up of patients. (12) *In vitro*  
62 cultured human cell lines are powerful tools to help identifying the altered metabolic processes  
63 leading to disease-induced isotopic variations, (13) as well as performing other metabolic or  
64 toxicological studies. (14,15,16,17) Isotopic fractionations may occur during the redistribution  
65 of an element among different chemical species, as predicted by *ab initio* calculations. (18) The  
66 stress-induced alteration of the metabolic processes involving any of these chemical species  
67 may lead to a modification of the element isotopic signatures. Since the altered metabolic  
68 processes may depend on the source of stress (disease, toxic element, etc), the chemical species  
69 undergoing isotopic signature variations may differ. Most of the studies performed so far have  
70 been based on the isotopic analysis of the element in the bulk sample, but two articles recently  
71 published (19,20) have demonstrated the potential of the determination of the isotopic  
72 signatures at the sub-cellular level.

73 We have previously developed a procedure for the accurate isotope ratio determination of U, Zn  
74 and Cu in bulk SH-SY5Y human neuroblastoma cell samples, after differentiation of the cells  
75 into neuron-like cells and exposure to low concentrations of natural U for seven days. (21,22) In  
76 the current study, isotopic analysis was downscaled at the protein level for the first time. To  
77 meet this aim, additional analytical efforts were required to isolate the protein fractions  
78 containing U, Zn and Cu, in combination with accurate isotope ratio measurements of the very  
79 small amounts of elements contained in these fractions. In these sense, we developed a method  
80 dedicated to the isotopic analysis of Cu, Zn and U in different protein fractions of cell lysates  
81 obtained by Size Exclusion Chromatography (SEC), after exposure of SH-SY5Y neuron-like  
82 cells to 10  $\mu$ M of natural U for seven days. Only Cu isotope ratio results are presented, since it

83 was not possible to accurately determine the isotope ratios of Zn and U in the protein fractions,  
84 as discussed in the following.

85

## 86 **Experimental**

### 87 **Reagents and solutions**

88 All the aqueous solutions were prepared using ultrapure water (resistivity > 18.2 MΩ cm at 25  
89 °C) from a Milli-Q® system (Millipore). Eagle's minimum essential medium (EMEM, ATCC,  
90 30-2003), F12 medium (Life Technologies, 21765-029), fetal bovine serum (FBS, ATCC, 30-  
91 2020) and penicillin/ streptomycin (Gibco-Thermo Fisher Scientific, 15070-063) solutions were  
92 used to prepare the culture medium for cell growing and exposure experiments. Retinoic Acid  
93 (RA, Sigma-Aldrich, R2625) and 12-O-tetradecanoylphorbol-13-acetate (TPA, Sigma Aldrich,  
94 P8139) were used for cell differentiation. TrypLE Express 1X/EDTA (Gibco-Thermo Fisher  
95 Scientific, 12605-010) was used for cell trypsinization. Phosphate buffer saline (PBS, pH 7.4)  
96 free of CaCl<sub>2</sub> and MgCl<sub>2</sub> (Gibco 10010-015) was used to wash the cells after trypsinization. A  
97 100mM ammonium acetate (Normapur grade, VWR Prolabo) solution in ultrapure water was  
98 used as mobile phase for the SEC experiments.

99 Plasma Pure Plus 34-37% HCl and Plasma Pure Plus 67-70% HNO<sub>3</sub> ultrapure reagents (SCP  
100 Science) were used for the sample preparation steps and for the preparation of solutions  
101 analyzed by Multi-Collector Inductively Coupled Plasma Mass Spectrometry (MC-ICPMS).  
102 IRMM-3702 [ $n(^{66}\text{Zn})/n(^{64}\text{Zn}) = 0.56397 \pm 0.00030$  ( $U_c$ ,  $k=2$ )] and ERM®-AE633  
103 [ $n(^{65}\text{Cu})/n(^{63}\text{Cu}) = 0.44563 \pm 0.00042$  ( $U_c$ ,  $k=2$ )] isotopic certified reference materials (i-CRM)  
104 traceable to SI units were purchased from the Institute for Reference Materials and  
105 Measurements (IRMM, Geel, Belgium). The ERM®-AE633 i-CRM was used to prepare a  
106 bracket solution analyzed just before and after the samples for Cu isotope ratio measurements.  
107 This bracket solution and the samples were spiked with the IRMM-3702 i-CRM, which was  
108 used as an internal standard for the correction of mass discrimination.

109 In-house natural uranium powder ( $U_3O_8$ ) was dissolved in  $0.5 \text{ mol L}^{-1} \text{ HNO}_3$  to prepare a natural  
110 uranium stock solution at 150 mM. An intermediate U solution (pH = 8-8.5) was then prepared  
111 by 1:5 v/v dilution of this solution in a buffer solution containing  $0.1 \text{ mol L}^{-1} \text{ NaHCO}_3$   
112 (analytical reagent, Normapur),  $0.1 \text{ mol L}^{-1} \text{ Na}_2\text{CO}_3$  (99.95% extra pure, Acros Organics),  $0.15$   
113  $\text{mol L}^{-1} \text{ NaCl}$  (puriss. p.a., Sigma-Aldrich), and  $0.05 \text{ mol L}^{-1} \text{ TRIS}$  (ultrapure grade  $\geq 99.9\%$ ,  
114 Sigma-Aldrich) in ultrapure water. This dilution was performed by dropwise addition of the U  
115 stock solution into the buffer solution to avoid U precipitation. Finally, the exposure solutions  
116 ( $10 \text{ }\mu\text{M}$  of U) were prepared by diluting the intermediate U solution into an appropriate volume  
117 of the culture medium consisting in an equal mix of EMEM and F12 mediums supplemented  
118 with 10% FBS and 1% penicillin/ streptomycin.

#### 119 **Differentiation of cells, exposure to natural uranium and cell lysis.**

120 Human SH-SY5Y (ATCC, CRL-2266, Batch 59740436) cells were first grown and  
121 differentiated into neuron-like cells. Cells were grown in  $175 \text{ cm}^2$  flasks at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  for  
122 10 days. The culture medium was replaced with fresh medium every 3-4 days. Afterwards, the  
123 cells were passaged by trypsinization using TrypLE Express 1X/EDTA and then seeded at  
124  $25000 \text{ cells cm}^{-2}$ . The differentiation into neuron-like cells was performed according to the  
125 method developed by Presgraves *et al.* (23) For this, the culture medium was replaced with  
126 fresh medium containing  $10 \text{ }\mu\text{M}$  RA, and cells were incubated for 3.5 days. This solution was  
127 then replaced with fresh medium containing  $80 \text{ nM}$  TPA and left for 3.5 days again. The  
128 effectiveness of the cell differentiation was assessed by phase contrast microscopy and western  
129 blot showing neurite outgrowths and tyrosine hydroxylase expression in differentiated cells.  
130 (24)

131 The differentiated cells were exposed to freshly-prepared  $10 \text{ }\mu\text{M}$  U exposure solutions for 7  
132 days, with exposure solution renewal once after 3 days. After exposure, cells were trypsinized,  
133 collected, washed twice with PBS, and finally counted using a Vi-CELL XR 2.04 cell viability  
134 analyzer (Beckman Coulter).

135 Cell proteins were extracted using a mechanical method. For this, cells were suspended at  
136  $30 \times 10^6$  cells per mL in  $0.02 \text{ M}$  TRIS pH 7.5 containing anti-proteases (Roche cocktail-EDTA

137 free) and 0.002 M spermine base (Sigma). The cells were disrupted by one shot at 1000 bars  
138 using a cell disruption system (Constant Systems) and then incubated for 30 min at 4 °C. The  
139 extracts were then ultra-centrifuged (1 h at 100,000 g) and the supernatants collected. Aliquots  
140 of the supernatants were stored at -20 °C until use.

#### 141 **Separation and detection of metal enriched protein fractions**

142 A Smartline chromatographic system (KNAUER, Berlin, Germany) equipped with a dual Pump  
143 1000 was used for the chromatographic separation of metal enriched protein fractions by SEC.  
144 The column was a 4.6 x 150 mm BEH 200 (Waters, Milford, USA) with 1.7 µm particle size  
145 and 200 Å pore size. The mobile phase consisted in a 100 mM ammonium acetate solution in  
146 ultrapure water, and the flow rate was 0.3 mL min<sup>-1</sup>. Cell lysates (20 µL) were manually  
147 injected in the column with a Rheodyne injector valve (model 9725). The column was coupled  
148 to a UV detector (Smartline PDA detector 2800) connected in series to a quadrupole ICPMS (q-  
149 ICPMS, X7, Thermo) through a 1-meter length PEEK tubing (i.d: 125 µm). The monitoring of  
150 metal enriched protein fractions was performed by UV at λ = 280 nm, and by q-ICPMS by  
151 following the signal of the isotopes <sup>65</sup>Cu<sup>+</sup>, <sup>66</sup>Zn<sup>+</sup> and <sup>238</sup>U<sup>+</sup>. The integration time per  
152 chromatographic point in q-ICPMS was 10 ms, and approximately 5800 points per isotope were  
153 acquired for a run time of 10 min.

154 To estimate the molecular weight (MW) range of the protein fractions, the calibration of the  
155 column was performed by injection of 10 mg L<sup>-1</sup> standards of Aprotinin (6.5 kDa), Ovalbumin  
156 (43 kDa), Conalbumin (75 kDa), Monoclonal antibody (150 kDa), Ferritin (440 kDa) and  
157 Thyroglobulin (669 kDa) diluted in the mobile phase. The signal was monitored by UV at λ =  
158 280 nm and the retention time (average of 3 replicates) was determined.

159 For the collection of the main protein fractions, 100 injections of cell lysates (20 µL) were  
160 performed, and the 100 eluate volumes corresponding to the same Cu protein fraction (F1, F2,  
161 F3 and F4) were pooled before Cu isotopic analysis. For comparison, 500 µL of cell lysates  
162 were subjected to isotopic analysis without protein separation step. For matrix matching  
163 between the original lysate and the Cu protein fractions, the original lysate was diluted in the  
164 appropriate volume of the mobile phase before sample preparation for isotopic analysis.

165 Furthermore, procedural blanks were prepared by subjecting the same volume of the mobile  
166 phase to the analytical procedure.

### 167 **Sample preparation for isotopic measurements**

168 Sample preparation and analysis were performed at the *Laboratoire de développement*  
169 *Analytique, Nucléaire, Isotopique et Élémentaire* (LANIE, CEA). The labware used for sample  
170 preparation previous to isotopic measurements was systematically pre-washed with acid  
171 solutions in accordance with clean lab practices. A 120 x 52 x 70 cm glovebox (Ateliers de  
172 Technochimie, Ivry sur Seine, France) was purposely designed to perform all sample  
173 preparation steps in order to protect samples from atmospheric contamination. This glovebox  
174 was made of polyvinyl chloride (PVC) and was equipped with High Efficiency Particulate Air  
175 (HEPA) filters to avoid contamination by dust particles.

176 The sample preparation for the procedural blanks (mobile phase) and the samples (protein  
177 fractions and cell lysate diluted in the mobile phase) was similar to that published elsewhere.  
178 (21) First, the samples/ procedural blanks were evaporated at 80 °C on a heating block. The  
179 residues were then acid digested in 15 mL closed Savillex vessels at 85 °C for 2 h after the  
180 addition of 1 mL of 67-70% HNO<sub>3</sub>. The acid was then evaporated at 85 °C until dryness and the  
181 residue was re-dissolved in 1 mL of 3 M HNO<sub>3</sub> for the purification of U, Cu and Zn according  
182 to a protocol described elsewhere. (21)

### 183 **Isotope ratio measurements**

184 All isotope ratio measurements were performed with a Neptune Plus MC-ICPMS (Thermo  
185 Fisher Scientific, Darmstadt, Germany) equipped with 9 Faraday detectors fitted to 10<sup>11</sup> Ω  
186 resistors. The sample introduction system consisted of an OpalMist nebulizer at around 10 μL  
187 min<sup>-1</sup> (Glass Expansion, Melbourne, Australia) coupled to an Apex HF desolvation system  
188 (Elemental Scientific, Omaha, USA). ‘Jet’ sampler and X-type skimmer cones adapted to dry  
189 plasma conditions (25) were employed. The cup configuration used can be found elsewhere.  
190 (21)

191 Preamp gain calibration was performed daily. The Zn hydride formation rates were measured by  
192 monitoring the signal at  $m/z$  69 (<sup>68</sup>Zn<sup>1</sup>H<sup>+</sup>) at the beginning of each measurement session. ZnH<sup>+</sup>



193 formation rates were 0.005-0.008% and the interference of  $^{64}\text{Zn}^1\text{H}^+$  on  $^{65}\text{Cu}^+$  was systematically  
 194 corrected. A background correction was performed by running a 2%  $\text{HNO}_3$  solution before the  
 195 samples, procedural blanks and bracketing solutions. Three procedural blanks were run at the  
 196 beginning of the session and the average signal measured for the procedural blanks was  
 197 subtracted to the signal of the samples.

198 The so-called modified sample-standard bracketing (m-SSB) approach, (21,26) which combines  
 199 the classical sample-standard bracketing and the inter-element correction approaches, was used  
 200 to determine the  $\delta^{65}\text{Cu}$  values. For this, the samples were bracketed by ERM<sup>®</sup>-AE633 solutions  
 201 and the Cu concentrations in the samples and the bracketing solutions were matched to less than  
 202 50 % difference. Both the samples and the bracketing solutions were spiked with IRMM-3702  
 203 at the same Zn (internal standard) concentration. For the determination of  $\delta^{65}\text{Cu}$  in a sample,  
 204  $(\delta^{65}\text{Cu})_{\text{analyte}}$  and  $(\delta^{66}\text{Zn})_{\text{spike}}$  were calculated using a classical sample-standard bracketing  
 205 as shown in equations 1 and 2:

$$206 \quad (\delta^{65}\text{Cu})_{\text{analyte}} = \left( \frac{(r_{65/63})_{\text{sample}}}{(r_{65/63})_{\text{bracketing}}} - 1 \right) \times 1000 \quad (1)$$

$$207 \quad (\delta^{66}\text{Zn})_{\text{spike}} = \left( \frac{(r_{66/64})_{\text{sample}}}{(r_{66/64})_{\text{bracketing}}} - 1 \right) \times 1000 \quad (2)$$

208 Where  $(r_{65/63})_{\text{sample}}$  and  $(r_{66/64})_{\text{sample}}$  are the measured  $^{65}\text{Cu}^+ / ^{63}\text{Cu}^+$  and  $^{66}\text{Zn}^+ / ^{64}\text{Zn}^+$  ratios  
 209 in the sample, whereas  $(r_{65/63})_{\text{bracketing}}$  and  $(r_{66/64})_{\text{bracketing}}$  are the average of the  
 210 measured  $^{65}\text{Cu}^+ / ^{63}\text{Cu}^+$  and  $^{66}\text{Zn}^+ / ^{64}\text{Zn}^+$  in the two bracketing solutions run just before and after  
 211 the sample. The final  $\delta^{65}\text{Cu}$  in the sample (protein fractions or original lysate) with regard to the  
 212 ERM<sup>®</sup>-AE633 solution was obtained by subtracting  $(\delta^{66}\text{Zn})_{\text{spike}}$  to  $(\delta^{65}\text{Cu})_{\text{analyte}}$ . With  
 213 these results, the  $n(^{65}\text{Cu})/n(^{63}\text{Cu})$  isotope ratio in the samples was calculated. Finally, results  
 214 were expressed as  $(\delta^{65}\text{Cu})_{\text{Fi}}$ , corresponding to the relative difference in parts per mil between  
 215 the  $n(^{65}\text{Cu})/n(^{63}\text{Cu})$  isotope ratio in the protein fraction i and the  $n(^{65}\text{Cu})/n(^{63}\text{Cu})$  isotope ratio in  
 216 the original lysate:

$$217 \quad (\delta^{65}\text{Cu})_{\text{Fi}} = \left( \frac{(r_{65/63})_{\text{fraction } i}}{(r_{65/63})_{\text{original lysate}}} - 1 \right) \times 1000 \quad (3)$$

218 Where  $(r_{65/63})_{fraction\ i}$  is the  $n(^{65}\text{Cu})/n(^{63}\text{Cu})$  isotope ratio determined in the protein fraction  $i$   
219 and  $(r_{65/63})_{original\ lysate}$  is the  $n(^{65}\text{Cu})/n(^{63}\text{Cu})$  isotope ratio determined in the original lysate.  
220 The methods used for U and Zn isotope ratio measurements can be found elsewhere (21) and are  
221 not described in this article, since only Cu isotope ratios could be accurately determined in the  
222 protein fractions.

### 223 **Uncertainty estimation**

224 The expanded uncertainty ( $U_c$ ,  $k=2$ ) of  $\delta$  values was estimated by quadratic propagation of two  
225 sources of uncertainty (see ref. 21 for details): the within-day measurement reproducibility  
226 ( $SD_M$ ) and the reproducibility associated to procedural blank correction ( $SD_{PB}$ ), as shown in the  
227 following equation:

$$228 \quad U_c = 2\sqrt{SD_M^2 + SD_{PB}^2} \quad (4)$$

### 230 **Results and discussion**

231 Under the experimental conditions used, the protein fractions from lysate injections eluted in  
232 less than 8 min, as shown in the UV profile of Figure 1. The column was previously calibrated  
233 with UV monitoring of standard proteins, which made it possible to assign the theoretical  
234 masses of the Cu, Zn and U protein fractions from their retention times (peak maxima). The  
235 different metal-containing protein fractions observed in the q-ICPMS chromatograms (Figure 1)  
236 corresponded to theoretical masses of approximately > 600, 110, 32 and 6 kDa for Cu, > 600, 33  
237 and 6 kDa for Zn, and > 600, 70 and 3-6 kDa for U, respectively. We estimated the relative  
238 proportions of metal in each one of the peaks by comparing the peak areas corresponding to the  
239 main Cu, Zn and U peaks from 14 SEC-q-ICPMS chromatograms. These relative Cu  
240 proportions for the peaks corresponding to > 600, 110, 32 and 6 kDa were  $38 \pm 5\%$  (SD,  $n=14$ ),  
241  $20 \pm 4\%$  (SD,  $n=14$ ),  $22 \pm 3\%$  (SD,  $n=14$ ) and  $21 \pm 4\%$  (SD,  $n=14$ ), respectively. The relative  
242 Zn proportions for the peaks corresponding to > 600, 33 and 6 kDa were  $39 \pm 5\%$  (SD,  $n=14$ ),  
243  $42 \pm 5\%$  (SD,  $n=14$ ) and  $18 \pm 6\%$  (SD,  $n=14$ ), respectively. A larger variability was found for  
244 the U peaks, being the relative U proportions for the peaks corresponding to > 600, 70 and 3-6

245 kDa of  $50 \pm 13$  % (SD, n=14),  $36 \pm 16$  % (SD, n=14) and  $13 \pm 4$  % (SD, n=14), respectively.

246 The proteomic analysis of these protein fractions was carried out with the aim of identifying the

247 U-target proteins, and the results are reported elsewhere. (27)

248 Four protein fractions F1, F2, F3 and F4 (dotted rectangles, Figure 1) corresponding to the main

249 Cu protein fractions were collected. For Zn and U isotope ratio measurements, F2 and F3 were

250 mixed after the element purification, since they corresponded to the same Zn and U peaks.

251 However, the accurate determination of the U isotope ratios was not possible because the

252 recovered U amounts were too small. In the case of Zn, the amounts in the protein fractions

253 were high enough for precise isotope ratio measurements. However, the Zn isotope ratios could

254 not be accurately determined because of the high procedural blanks obtained (from 200 to 350

255 ng), corresponding to over 50% of the total Zn amounts in the samples. These high procedural

256 blanks cannot be attributed to the sample preparation procedure for isotope ratio measurements,

257 which were found to be  $35 \pm 19$  ng, (21) and probably came from the mobile phase reagents

258 and/or the stationary phase. Indeed, high base lines were observed for Zn in the q-ICPMS

259 chromatograms (Figure 1).

260 On the contrary, accurate results could be obtained for the Cu isotope ratios. The Cu amounts

261 were high enough for the measurement of isotope ratios with external precisions better than

262 0.15‰, and the uncertainty associated to the procedural blank correction ranged from 0.08 to

263 0.17‰ (2SD, n = 6, see ref. 21 for details on the calculation method). Figure 2 shows the

264  $(\delta^{65}\text{Cu})_{Fi}$  in the 4 protein fractions, reflecting the isotopic signature of Cu in each of the

265 fractions relative to the original lysate. Fraction 1 showed a similar isotopic signature as the

266 original lysate with  $(\delta^{65}\text{Cu})_{F1} = -0.03 \pm 0.14$  ‰ ( $U_c$ , k=2), whereas fractions 2 and 3 were

267 depleted in  $^{65}\text{Cu}$  with  $(\delta^{65}\text{Cu})_{F2} = -0.55 \pm 0.20$  ‰ ( $U_c$ , k=2) and  $(\delta^{65}\text{Cu})_{F3} = -0.32 \pm 0.21$  ‰

268 ( $U_c$ , k=2), respectively. Finally, fraction 4 was enriched in  $^{65}\text{Cu}$  with  $(\delta^{65}\text{Cu})_{F4} = +0.84 \pm 0.21$

269 ‰ ( $U_c$ , k=2). The mass balance was verified from these results using equation 5:

270

271 
$$(\delta^{65}\text{Cu})_{\text{original lysate}} = \frac{f_1(\delta^{65}\text{Cu})_{F1} + f_2(\delta^{65}\text{Cu})_{F2} + f_3(\delta^{65}\text{Cu})_{F3} + f_4(\delta^{65}\text{Cu})_{F4}}{100} \quad (5)$$

272

273 Where  $(\delta^{65}\text{Cu})_{F1}$ ,  $(\delta^{65}\text{Cu})_{F2}$ ,  $(\delta^{65}\text{Cu})_{F3}$  and  $(\delta^{65}\text{Cu})_{F4}$  are the  $\delta^{65}\text{Cu}$  in the protein fractions  
274 1 to 4 with regard to the original lysate, and  $f_1$ ,  $f_2$ ,  $f_3$  and  $f_4$  are the percentages of Cu in the  
275 protein fractions 1 to 4 (see above). The  $(\delta^{65}\text{Cu})_{\text{original lysate}}$  calculated when applying  
276 Equation 5 was  $-0.02 \pm 0.12 \text{ ‰}$ , which is not significantly different from the expected value of 0  
277 ‰.

278 The results obtained in this work clearly demonstrate a different isotopic distribution of Cu  
279 among the four protein fractions. Recently, two papers have demonstrated that different Cu  
280 pools of biological samples may show different isotopic signatures. In one of these articles, the  
281 Cu isotope ratios were determined in the bulk serum of healthy individuals and alcoholic  
282 cirrhosis patients, as well as in the exchangeable + ultrafiltrable (EXCH + UF) Cu fraction of  
283 the serum, representing the labile Cu pool, and the non-exchangeable + non-ultrafiltrable  
284 (NEXCH + NUF) Cu fraction containing the Cu bound to ceruloplasmin. (19) The results  
285 showed a heavier Cu isotope ratio in the EXCH + UF fraction compared to the bulk serum and  
286 the NEXCH + NUF fraction of healthy individuals, whereas this difference was not found in the  
287 patients, potentially reflecting a labile Cu deregulation linked to the disease. (19) In another  
288 article, the Cu isotope ratios were determined in sub-cellular fractions of the SH-SY5Y human  
289 neuroblastoma cell line, corresponding to the mitochondria and the rest of the cell lysate,  
290 demonstrating different isotopic signatures. (20) The present work can be considered as a step  
291 forward in the downscaling of Cu isotope ratio determinations at sub-cellular level, since we  
292 performed accurate Cu isotope ratio determinations at the protein level. These three studies open  
293 new perspectives on the identification of new disease-specific biomarkers. In previous  
294 publications, the study of the isotopic signature of the element as prognostic biomarker was  
295 performed on bulk samples. The main drawback of such approach is that similar isotopic  
296 variations in bulk samples can be induced by different types of diseases. For instance, similar  
297 depletions in the heavier  $^{65}\text{Cu}$  isotope have been found in the serum of cancer, (7) cirrhosis (28)  
298 or Wilson disease (8) patients. Furthermore, in some cases, the isotopic variations observed

299 between the healthy and cancerous tissues do not lead to changes in the blood isotopic  
300 signatures, as observed for Zn isotope ratios in breast cancer patients. (5)

301

## 302 **Conclusions**

303 For the first time, the accurate determination of Cu isotope ratios was performed in intracellular  
304 protein fractions isolated by SEC, which represents a significant progress in the downscaling of  
305 isotope ratio determinations from the cellular level to the protein level. We evidenced  
306 differences in the Cu isotopic signatures in the four main protein fractions from SH-SY5Y  
307 neuron-like cell lysates. Since the Cu isotopic signatures in the proteins might be modified by  
308 stress sources altering the Cu metabolism, this work paves the way for the identification of new  
309 disease biomarkers as well as for the development of new strategies to get insight into the  
310 knowledge of stress-induced alteration of Cu metabolic processes, which can be critical in the  
311 development of diagnostic tools. However in this study, Zn and U isotope ratios could not be  
312 accurately determined in the protein fractions, and dedicated analytical developments are  
313 required to extend this approach to other elements than Cu, as well as different types of  
314 biological samples.

315 The upcoming major step is to perform isotopic analysis at the molecular level, through the  
316 study of isotopic fractionations of elements involved in different chemical species, in  
317 combination with *ab initio* theoretical calculations of these isotopic fractionations among these  
318 species. (18) This is key information for deeper investigation of stress-induced alteration of  
319 metabolic processes at the molecular level, as well as to aid deciphering the metabolic routes of  
320 toxic elements in a more specific manner.

321

## 322 **Conflicts of interest**

323 There are no conflicts to declare.

324

## 325 **Acknowledgements**

326 The authors would like to acknowledge the Transversal Toxicology Program run by the CEA  
327 DRF (France) and the financial support from this program. E. Paredes would also like to thank  
328 the CEA - Enhanced Eurotalents program, co-funded by the European Commission through the  
329 Marie Skłodowska-Curie COFUND program under the 7th Framework Program for research  
330 and technological development (FP7). The authors also acknowledge the CNRS  
331 Interdisciplinary Mission through the PEPS (Projet Exploratoire Premier Soutien) Faidora  
332 program (Faibles Doses, Risques, Alertes).

333

### 334 **Figure captions**

335 **Figure 1.** SEC chromatograms of cell lysates of the SH-SY5Y human neuroblastoma cell line  
336 differentiated into neuron-like cells and exposed to 10  $\mu\text{M}$  of natural U for seven days. The  
337 elution profiles correspond to the UV signal monitored at 280 nm and the q-ICPMS signals of  
338  $^{65}\text{Cu}^+$ ,  $^{66}\text{Zn}^+$  and  $^{238}\text{U}^+$  isotopes. For each q-ICPMS chromatogram, the signal relative to the  
339 maximum signal measured for the isotope throughout the chromatogram is plotted. The dotted  
340 rectangles show the 4 protein fractions collected for Cu isotopic analysis.

341

342 **Figure 2.**  $(\delta^{65}\text{Cu})_{Fi}$  values for the 4 Cu protein fractions with regard to the original lysate. The  
343 vertical bars correspond to the expanded uncertainty of the results ( $k=2$ ). The plain and dotted  
344 red lines indicate the  $\delta^{65}\text{Cu}$  value of the original lysate,  $(\delta^{65}\text{Cu})_{original\ lysate}$ , defined as 0 ‰,  
345 and its expanded uncertainty.

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