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Lana Abou Zeid, Albert Pell, Marta Garcia Cortes, H el ene Isnard, Pascale Delangle, et al.. Determining the selectivity of a tetra-phosphorylated biomimetic peptide towards uranium in the presence of competing cations through the simultaneous coupling of HILIC to ESI-MS and ICP-MS. *Analytical and Bioanalytical Chemistry*, 2023, 415, pp.6107-6115. 10.1007/s00216-023-04884-4 . cea-04190954

HAL Id: cea-04190954

<https://cea.hal.science/cea-04190954v1>

Submitted on 30 Aug 2023

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1 **Determining the selectivity of a tetra-phosphorylated biomimetic peptide**
2 **towards uranium in the presence of competing cations through the**
3 **simultaneous coupling of HILIC to ESI-MS and ICP-MS**

4
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17 KEYWORDS: Uranium, phosphorylated peptide, selectivity, competing ions, HILIC, mass
18 spectrometry/ICP-MS

22 Abstract

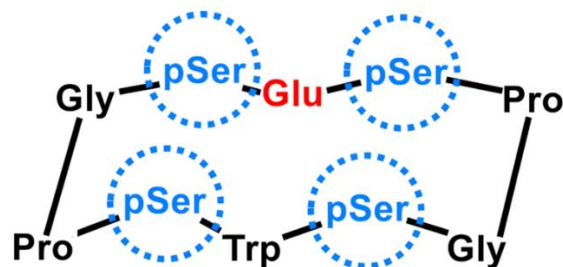
23 The cyclic tetra-phosphorylated biomimetic peptide (pS1368) has been proposed as a promising starting
24 structure to design decorporating agent of uranyl (UO_2^{2+}) due to its affinity similar to that of osteopontin
25 (OPN), a target UO_2^{2+} protein *in vivo*. The determination of this peptide selectivity towards UO_2^{2+} in the
26 presence of competing endogenous elements is also crucial to validate this hypothesis. In this context,
27 the selectivity of pS1368 towards UO_2^{2+} in the presence of Ca^{2+} , Cu^{2+} and Zn^{2+} was determined by
28 applying the simultaneous coupling of hydrophilic interaction chromatography (HILIC) to electrospray
29 ionization (ESI-MS) and inductively coupled plasma (ICP-MS) mass spectrometry. Sr^{2+} was used as
30 Ca^{2+} simulant, providing less challenging ICP-MS measurements. The separation of the complexes by
31 HILIC were firstly set up. The selectivity of pS1368 towards UO_2^{2+} was determined in the presence of
32 Sr^{2+} , by adding several proportions of this latter to $\text{UO}_2(\text{pS1368})$. UO_2^{2+} was not displaced from
33 $\text{UO}_2(\text{pS1368})$ even in the presence of a ten-fold excess of Sr^{2+} . The same approach has been undertaken
34 to demonstrate the selectivity of pS1368 towards UO_2^{2+} in the presence of Cu^{2+} , Zn^{2+} and Sr^{2+} as
35 competing endogenous cations. Hence, we showed that pS1368 is selective towards UO_2^{2+} in the
36 presence of Sr^{2+} , but also in the presence of Cu^{2+} and Zn^{2+} . This study highlights the performance of
37 HILIC-ESIMS/ICPMS simultaneous coupling to assess the potential of molecules as decorporating
38 agents of UO_2^{2+} .

39

40

41 1. Introduction

42 Elements such as Ca, Cu and Zn are essential to humans, as they are mandatory to many biological
43 functions and present in some enzymes and proteins key binding sites [1]. Others such as uranium (U)
44 which is ubiquitous in the earth's crust have no known biological role, making U toxicity of great
45 concern [2]. Indeed, natural U (U_{nat}) exhibits mainly chemical toxicity governed by the interactions of
46 its uranyl (UO_2^{2+}) form with target biomolecules *in vivo* [3]. However, the mechanisms in which UO_2^{2+}
47 is involved at the cellular and molecular levels are still poorly understood. Research is still in progress
48 to characterize the coordination sites of proteins responsible for their affinity towards UO_2^{2+} , which is
49 of prime importance to further develop selective decorporating agents in the event of contamination [4].
50 To be able to design an efficient decorporating agent, it is essential to determine its selectivity towards
51 the target element in the presence of endogenous ions that are potential competitors [5]. Recently, a
52 tetra-phosphorylated cyclic biomimetic peptide pS1368 (Fig. 1), exhibiting an affinity for UO_2^{2+} similar
53 to that of the hyper-phosphorylated protein osteopontin (OPN), has been proposed as representative of
54 the coordination sites of UO_2^{2+} in this protein [6].



55
56 **Fig. 1** Structure of the cyclic tetra-phosphorylated peptide pS1368 considered in this work. Adapted from Ref.6.
57 The selectivity of pS1368 towards UO_2^{2+} in the presence of Ca^{2+} , the cation mainly bound to OPN [7],
58 was deduced by comparing the affinity constants of $UO_2(pS1368)$ and $Ca(pS1368)$. These latter were
59 determined using fluorescence spectroscopy and it was found that the affinity of pS1368 for Ca^{2+} is
60 significantly lower than for UO_2^{2+} , with $K^{pH 7.4}(Ca(pS1368)) = 10^2$, more than nine orders of magnitude
61 lower than $K^{pH 7.4}(UO_2(pS1368)) = 10^{11.3}$ [6]. These data suggest that pS1368 could be highly selective
62 towards UO_2^{2+} in the presence of physiological amounts of Ca^{2+} . To be able to propose pS1368 as a
63 promising starting structure for UO_2^{2+} decorporation *in vivo*, the determination of its selectivity when

64 Ca^{2+} and UO_2^{2+} are in a competing complexation reaction is crucial as well as in the presence of other
65 endogenous competing ions, such as Cu^{2+} and Zn^{2+} .

66 In this work, the methodology that we previously developed [8], based on the simultaneous coupling of
67 HILIC to ESI-MS and ICP-MS, was applied to determine in a single step the selectivity of pS1368
68 towards UO_2^{2+} in the presence of competing ions at physiological pH. Knowing that OPN binds
69 primarily Ca^{2+} to ensure its biological function [7], this latter cation is the most relevant competing ion
70 for UO_2^{2+} binding to OPN *in vivo*. Therefore, the evaluation of the selectivity of the model peptide
71 pS1368 towards UO_2^{2+} in the presence of Ca^{2+} appears as an essential first step. We chose Sr^{2+} as Ca^{2+}
72 simulant since it has a similar coordination number and ionic radius [9] and exhibits very close
73 physicochemical properties [10], while ensuring less challenging ICP-MS measurements.

74 The conditions to separate UO_2^{2+} and Sr^{2+} complexes by HILIC [11] were set up. The selectivity of
75 pS1368 towards UO_2^{2+} was then determined in the presence of Sr^{2+} and in a single step, by on-line
76 identifying the separated complexes using ESI-MS and simultaneously quantifying them by ICP-MS
77 [12]. We evaluated then the influence of Sr^{2+} on UO_2^{2+} complexation by pS1368. Through the same
78 approach, we determined the selectivity of pS1368 towards UO_2^{2+} in the presence of Sr^{2+} , Cu^{2+} and Zn^{2+}
79 as competing ions, since they are known to be highly competitive for hard metal cation binding in the
80 blood [13]. To our knowledge, such an analytical approach to assess in a single step the selectivity of a
81 chelating molecule that may be considered as a potential decorporating agent is described for the first
82 time.

83 **2. Experimental part**

84 **2.1. Chemicals**

85 Acetonitrile (ACN, CH_3CN , LC-MS grade) and ammonia NH_3 (20-22%) were purchased from VWR
86 prolabo (Briare le canal, France). Ammonium acetate ($\text{NH}_4\text{CH}_3\text{CO}_2$), toluene ($\text{C}_6\text{H}_5\text{CH}_3$, purity > 99.7
87 %) and ethylenediaminetetraacetic acid (EDTA) tetrasodium salt dihydrate ($\text{Na}_4\text{C}_{10}\text{H}_{12}\text{O}_8\text{N}_2 \cdot 2\text{H}_2\text{O}$,
88 99.5% purity) were supplied by Sigma Aldrich (Saint Quentin Fallavier, France). The cyclic tetra-
89 phosphorylated peptide pS1368 (MW 1291.9 g mol^{-1}) was supplied by Cambridge peptides (Cambridge,

90 UK) and synthesized following the procedure developed by the CIBEST team [6]. Ultrapure water (18.2
91 MΩ cm at 25°C) was obtained from Milli-Q purification system (Merck millipore, Guyancourt, France).
92 Certified uranium (U), strontium (Sr), copper (Cu), zinc (Zn), yttrium (Y) and bismuth (Bi) standard
93 solutions (1000 μg mL⁻¹ in HNO₃ 2% w/w), were provided by the SPEX Certiprep Group (Longjumeau,
94 France). L-Tryptophan (L- Trp, 99% purity) was purchased from Acros organics. Nitric acid solutions
95 (HNO₃ 2%) were prepared by diluting sub-boiled HNO₃ in ultrapure water. Sub-boiled HNO₃ was
96 prepared by distilling HNO₃ 65% (Merck, France) with an evapoclean unit from Analab (France).

97 **2.2. Preparation of stock solutions, contact solutions, samples and standard solutions**

98 **2.2.1. Stock solutions of uranium, metals and pS1368**

99 The stock solution of natural uranium (U_{nat}) was obtained by diluting in ultrapure water an in-house U_{nat}
100 solution previously prepared by dissolving uranium oxide powder (U₃O₈) in 0.5 mol L⁻¹ HNO₃[14], to
101 achieve a uranium concentration of 10,000 μg mL⁻¹ (5 x 10⁻² mol L⁻¹). The stock solutions of Sr, Cu and
102 Zn were prepared by evaporating to dryness 10 mL of the corresponding SPEX solutions on a hot plate
103 at 95°C, and by recovering the residue with 1 mL of 2% HNO₃ to achieve a metal concentration of 5 x
104 10⁻² mol L⁻¹. The concentration of each stock solution was measured by ICP-MS by external calibration
105 using SPEX standard solutions as calibrants.

106 The pS1368 stock solution was prepared by dissolving 4 mg of the peptide powder in 1 mL of an aqueous
107 solution of NH₄CH₃CO₂ (20 mmol L⁻¹, pH ~ 7) to reach a concentration of 3 x 10⁻³ mol L⁻¹. The pS1368
108 concentration was determined by external calibration using HILIC coupled to UV/VIS in series with
109 ESI-MS, by quantifying the Trp contained in the sequence of the peptide (UV absorption at λ = 280 nm)
110 [8].

111 **2.2.2. Contact solutions**

112 ***1UO₂²⁺:2pS1368:xSr²⁺*** solutions were obtained by mixing a very small volume of stock solution of U_{nat}
113 (~1.6 μL) with 125 μL of pS1368 stock solution, to reach a UO₂²⁺ concentration of 5x10⁻⁴ mol L⁻¹.

114 Different contact solutions of following proportions ***1UO₂²⁺:2pS1368:xSr²⁺*** (x = 0 to 10) were obtained
115 by addition of 0 - 16 μL of Sr stock solution. The pH was adjusted to 7.4 with NH₃ using a micro-

116 electrode. All the contact solutions were systematically prepared the day before the analysis and stored
117 at 4°C.

118 $1\text{UO}_2^{2+}:2\text{pS1368}:1\text{Sr}^{2+}:1\text{Cu}^{2+}:1\text{Zn}^{2+}$

119 In a first step, the stability of Cu^{2+} , Zn^{2+} and Sr^{2+} was verified in a solution containing the three elements
120 prepared in 20 mmol L^{-1} of $\text{NH}_4\text{CH}_3\text{CO}_2$. By measuring their concentrations for three consecutive days
121 off-line by ICP-MS, precipitation of Cu^{2+} was observed through the formation of a blue-green
122 precipitate, while this was not the case for Zn^{2+} and Sr^{2+} (See Electronic Supplementary Material Table
123 S1). To overcome this issue, a solution containing Cu^{2+} , Zn^{2+} and Sr^{2+} was prepared in 60 mmol L^{-1} of
124 $\text{NH}_4\text{CH}_3\text{CO}_2$ and the concentration of the metals was monitored for three days by ICP-MS (See
125 Electronic Supplementary Material Table S2). In these conditions, it could be observed that Cu^{2+} , Zn^{2+}
126 and Sr^{2+} were stable under their soluble form. Hence, the contact solution of
127 $1\text{UO}_2^{2+}:2\text{pS1368}:1\text{Sr}^{2+}:1\text{Cu}^{2+}:1\text{Zn}^{2+}$ was obtained by adding 3 μL of stock solutions of U_{nat} , Sr, Cu and
128 Zn in 250 μL of pS1368 stock solution prepared in $\text{NH}_4\text{CH}_3\text{CO}_2$ 60 mmol L^{-1} , to reach a concentration
129 of 5×10^{-4} mol L^{-1} for all elements. The pH was adjusted to 7.4 with NH_3 using a micro-electrode. The
130 contact solution was prepared the day before the analysis and stored at 4°C.

131 2.2.3. *Working samples*

132 The working samples were freshly prepared just before analysis by diluting the contact solutions in the
133 mobile phase made of 72/28 ACN/ H_2O v/v and 20 mmol L^{-1} $\text{NH}_4\text{CH}_3\text{CO}_2$, to reach a UO_2^{2+}
134 concentration of 10^{-4} mol L^{-1} . The concentration of pS1368 in the working samples was calculated by
135 weight, by considering the stock solution concentration determined by UV/VIS. The total concentration
136 of UO_2^{2+} and metal(s) in the working samples was measured by ICP-MS as described in the section 2.3.

137 2.2.4. *Standard solutions*

138 The standard solutions were prepared in the working mobile phase by mixing in the adequate proportions
139 the U_{nat} stock solution and the Sr SPEX solutions, both at 10 000 $\mu\text{g mL}^{-1}$, to yield five concentration
140 levels ranging from 6 to 42 $\mu\text{g mL}^{-1}$ for U_{nat} and 6 to 92 $\mu\text{g mL}^{-1}$ for Sr. The tetra-phosphorylated peptide
141 pS1368 was added to the standard solutions in an equimolar ratio with respect to UO_2^{2+} in order to

142 prevent the hydrolysis of this latter, that is likely to occur in a non-complexing medium [15] but also to
143 obtain specific standard solutions of UO_2^{2+} and Sr^{2+} peptidic complexes [8].

144 **2.3. Selectivity of pS1368 towards UO_2^{2+} in the presence of competing metals**

145 Firstly, the quantification method previously developed using the simultaneous coupling of HILIC-
146 ESIMS/ICPMS [8] was applied to evaluate the selectivity of pS1368 towards UO_2^{2+} in the presence of
147 Sr^{2+} .

148 The total content of UO_2^{2+} and Sr^{2+} , denoted as $[\text{UO}_2^{2+}]_{\text{total}}$ and $[\text{Sr}^{2+}]_{\text{total}}$, were measured in each sample
149 using ICP-MS based on external calibration, by introducing the samples in Flow Injection Analysis
150 (FIA) mode. The samples containing several UO_2^{2+} :2pS1368: $x\text{Sr}^{2+}$ proportions were injected into the
151 column in duplicate. The Sr^{2+} and UO_2^{2+} complexes were then separated, on-line identified by ESI-MS
152 and the area of the chromatographic peaks obtained by ICP-MS was totally integrated for further
153 quantification of the complexes by the means of external calibration.

154 The quantitative distribution of UO_2^{2+} and Sr^{2+} among the separated complexes, expressed in percent
155 (%), was calculated as the ratio of the concentration of UO_2^{2+} or Sr^{2+} complexes to the total concentration
156 of these latter in the sample, according to the Equation 1.

$$157 \quad \% \text{ M(Ligand)} = \frac{[\text{M(Ligand)}]}{[\text{M}]_{\text{total}}} \text{ (Equation 1)}$$

158 With M being UO_2^{2+} or Sr^{2+} .

159 After each run, around 20% of unbound UO_2^{2+} remained adsorbed on the surface of the stationary phase.
160 Therefore, successive injections of pS1368 were performed to elute this residual fraction.

161 Secondly, the method was applied to determine the selectivity of pS1368 towards UO_2^{2+} in the presence
162 of competing Cu^{2+} , Zn^{2+} and Sr^{2+} ions. To do this, a model sample containing a mixture of the metals in
163 an equimolar ratio with respect to UO_2^{2+} was considered and denoted
164 1UO_2^{2+} :2pS1368:1 Sr^{2+} :1 Cu^{2+} :1 Zn^{2+} .

165 The sample was introduced in FIA into the ICP-MS to determine the total content of each element
166 through the integration of the total area of the corresponding peaks obtained by recording the signal of
167 the $^{64}\text{Zn}^+$, $^{63}\text{Cu}^+$, $^{88}\text{Sr}^+$, $^{238}\text{U}^+$ isotopes. The complexes were further separated, on-line identified by ESI-
168 MS and simultaneously quantified by ICP-MS. Duplicate injections of the sample were performed. The

169 quantitative distribution of UO_2^{2+} and the metals among the separated complexes, expressed in percent
170 (%), was calculated as the ratio of the peak area of UO_2^{2+} and metal complexes to the peak area of their
171 total elemental content in the sample, according to the Equation 2.

$$172 \quad \% \text{ M(Ligand)} = \frac{A_{\text{M(Ligand)}}}{A_{\text{M total}}} \text{ (Equation 2)}$$

173 With M being UO_2^{2+} , Sr^{2+} , Cu^{2+} and A being the peak area.

174 After each run, successive injections of pS1368 were performed to elute the residual fraction of UO_2^{2+}
175 potentially adsorbed on the surface of the stationary phase.

176 In all cases, a control sample containing only UO_2^{2+} at $10^{-4} \text{ mol L}^{-1}$ and two equivalents of pS1368 was
177 considered.

178 **2.4. Instrumentation**

179 *2.4.1. Hydrophilic interaction liquid chromatography*

180 An ultimate 3000 UHPLC⁺ Dionex/ThermoFisher scientific (Courtaboeuf, France) module, made of a
181 degasser, a dual RS pump, an RS autosampler, a column compartment and an RS diode array detector,
182 was used. The metal and UO_2^{2+} complexes were separated using a YMC Triart Diol column (100 x 2
183 mm; 1.9 μm). Mobile phases of different compositions were obtained by online mixing of solvent A
184 (60/40 ACN/ H_2O v/v containing 20 mmol L^{-1} $\text{NH}_4\text{CH}_3\text{CO}_2$) and solvent B (80/20 ACN/ H_2O v/v
185 containing 20 mmol L^{-1} $\text{NH}_4\text{CH}_3\text{CO}_2$) in the adequate proportions. Separations were run in isocratic
186 elution mode at a flow rate of 300 $\mu\text{L min}^{-1}$ and the sample injection volume was 3 μL .

187 The retention factor k of the analytes was calculated following the equation (3):

$$188 \quad k = \frac{(t_R - t_0)}{t_0} \text{ (Equation 3)}$$

189 Where t_R is the retention time (min) of the analyte, determined by HILIC-ESI-MS, t_0 is the void time of
190 the toluene as unretained marker ($10^{-4} \text{ mol L}^{-1}$, $V_{inj} = 1 \mu\text{L}$), determined by HILIC-UV/VIS at $\lambda = 254$
191 nm.

192 The selectivity and resolution factors of the separations, α and R_s , respectively, were calculated based on
193 the equations 4 and 5:

$$194 \quad \alpha = \frac{k_2}{k_1} \text{ (Equation 4)}$$

195
$$R_s = 1.18 \times \frac{t_{R2} - t_{R1}}{W_{0.5h1} + W_{0.5h2}} \text{ (Equation 5)}$$

196 Being analyte 2 more retained than analyte 1. $W_{0.5}$ corresponds to the full width half-maximum of each
197 peak.

198 2.4.2. *Mass spectrometers*

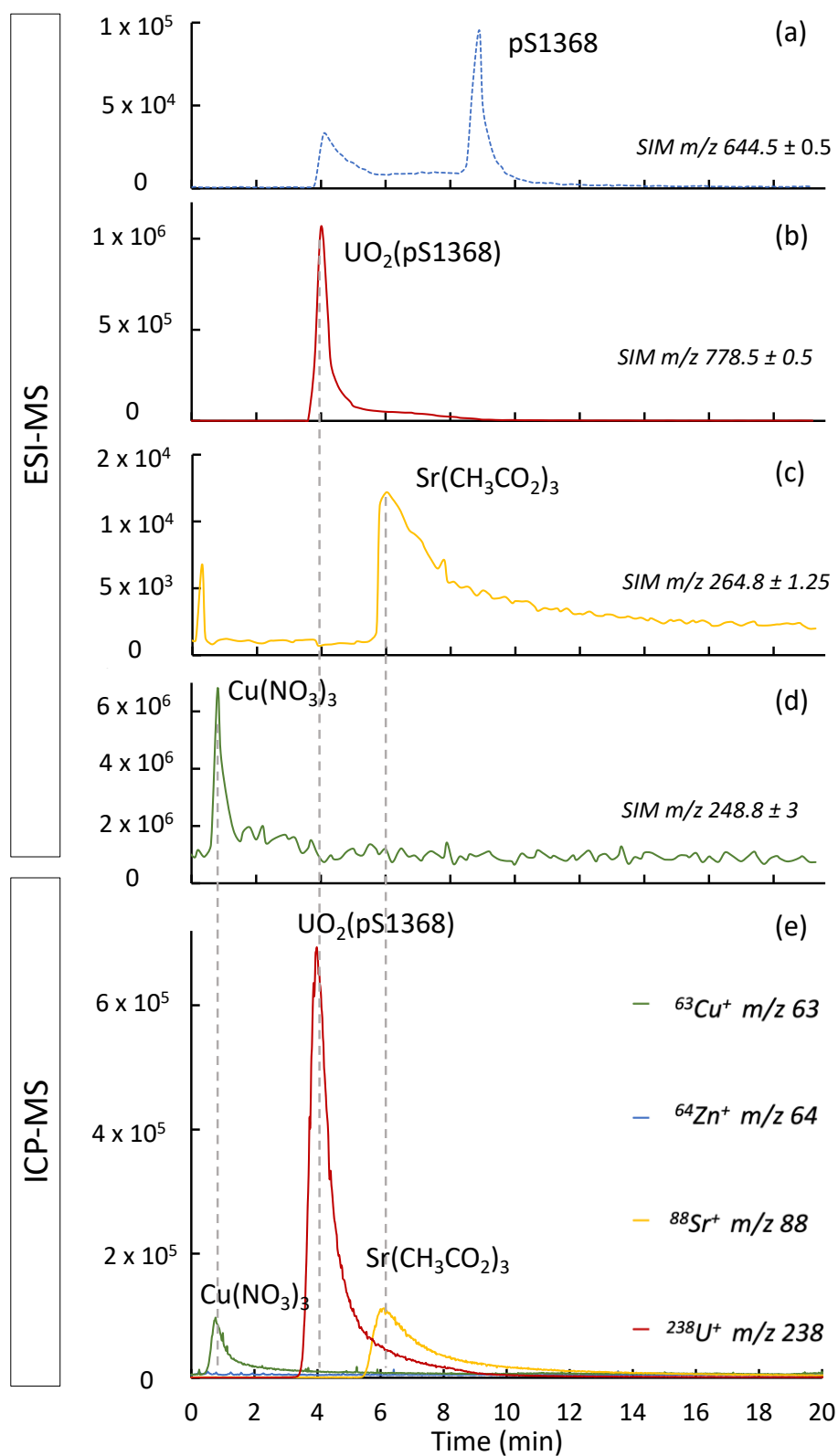
199 The ESI mass spectrometer was a triple quadrupole TSQ Quantum Ultra™ (Thermo Fisher scientific,
200 San Diego CA, USA) equipped with an H-ESI II ionization probe. All mass spectra were recorded in
201 negative ionization mode by applying the following parameters: spray voltage -3.5 kV, probe
202 temperature 120°C and capillary temperature of 360°C. Direct ESI-MS injection of samples was firstly
203 undertaken in order to identify the most abundant molecular ions. Double charged species of
204 $\text{UO}_2(\text{pS1368})$ and free pS1368 were observed, being $[\text{UO}_2(\text{pS1368})\text{-4H}]^{2-}$ and $[\text{pS1368}\text{-2H}]^{2-}$, in
205 agreement with previous studies [6,8] while mono-charged metal complexes of Sr^{2+} and Cu^{2+} were
206 detected, being $[\text{Sr}(\text{CH}_3\text{CO}_2)_3]^-$ and $[\text{Cu}(\text{NO}_3)_3]^-$, respectively. For the sake of clarity, the complexes
207 were denoted $\text{UO}_2(\text{pS1368})$ and $\text{M}(\text{ligand})$ with $\text{M} = \text{Sr}^{2+}$, Cu^{2+} along the manuscript, by omitting the
208 charge. Throughout the HILIC-ESI-MS coupling, mass spectra were acquired using full scan (m/z 400-
209 1500) and single ion monitoring (SIM) by selecting the m/z ratio associated to the most abundant isotope
210 of each species (spectral width: $m/z \pm 0.5$, unless stated otherwise), being the free peptide pS1368 and
211 $\text{UO}_2(\text{pS1368})$ or metal(ligand) complexes previously determined.

212 The ICP mass spectrometer was a single quadrupole XSeriesII (Thermo Fisher Scientific). The sample
213 introduction system consisted of a perfluoroalkoxy PFA-ST nebulizer operating at $146.7 \mu\text{L min}^{-1}$, and
214 a quartz cyclonic spray chamber thermostated at 3 °C. In order to prevent any carbon deposition due to
215 the use of organic solvents, additional 8 mL min^{-1} oxygen flow rate was introduced in the plasma,
216 through an “additional gas port” located in the spray chamber [16,17]. Furthermore, platinum skimmer,
217 sampler cone and a 1 mm inner diameter injector were used. The tune parameters were daily optimized.
218 Chromatograms were recorded by following the signal of $^{238}\text{U}^+$, $^{88}\text{Sr}^+$, $^{63}\text{Cu}^+$, $^{64}\text{Zn}^+$, $^{209}\text{Bi}^+$ and $^{89}\text{Y}^+$ with
219 an integration time of 90 ms for each isotope, knowing that ^{209}Bi and ^{89}Y were used as internal standards.
220 The simultaneous coupling of HILIC to ESI-MS and ICP-MS was performed according to the setting
221 up described in our previous work [18].

222 **3. Results and discussion**

223 **3.1. Definition of HILIC conditions to separate UO_2^{2+} and metal complexes**

224 By applying the chromatographic conditions set up in our previous study [8], a YMC triart diol column
225 and a mobile phase composed of 72/28 ACN/ H_2O v/v and 20 mmol L^{-1} $\text{NH}_4\text{CH}_3\text{CO}_2$ allowed the
226 successful separation of complexes of UO_2^{2+} , Sr^{2+} and Cu^{2+} following the injection of the model sample
227 $1\text{UO}_2^{2+}:2\text{pS1368}:1\text{Sr}^{2+}:1\text{Cu}^{2+}:1\text{Zn}^{2+}$. Although the Zn^{2+} complex was not detected by ICP-MS neither
228 by ESI-MS, successive injections of EDTA allowed to recover the whole Zn^{2+} that was adsorbed on the
229 surface of the stationary phase [19]. The chromatograms obtained simultaneously by ESI-MS and ICP-
230 MS are shown in Fig. 2.



231

232 **Fig. 2** Separation of UO_2^{2+} and metal complexes by HILIC simultaneously coupled to ESI-MS and ICP-MS.

233 Chromatograms acquired by ESI-MS using SIM mode, centered on the m/z ratios of the characteristic ion of

234 pS1368 and considering most abundant isotope of the different elements involved in the complexes (a-d), and (e)

235 superposition of the elution profiles recorded by ICP-MS, following the signal of the most abundant isotopes

236 $^{238}\text{U}^+$, $^{88}\text{Sr}^+$, $^{63}\text{Cu}^+$ and $^{64}\text{Zn}^+$, with an integration time of 90 ms. Sample: $1\text{UO}_2^{2+}:2\text{pS1368}:1\text{Sr}^{2+}:1\text{Cu}^{2+}:1\text{Zn}^{2+}$.
 237 Column YMC Triart Diol 100 x 2 mm; 1.9 μm . Mobile phase: 72/28 ACN/ H_2O v/v with 20 mmol L^{-1}
 238 $\text{NH}_4\text{CH}_3\text{CO}_2$, flow rate: 300 $\mu\text{L min}^{-1}$, $V_{\text{inj}} = 3\mu\text{L}$.
 239 From the elution profiles registered by ICP-MS (Fig 2e), the retention factors of the peaks of $^{63}\text{Cu}^+$,
 240 $^{238}\text{U}^+$ and $^{88}\text{Sr}^+$ were 0.5, 4.3 and 6.6, respectively. By comparison of these retention factors with those
 241 obtained from the ESI-MS chromatograms (Fig 2b-d), the peaks could be attributed to $\text{Cu}(\text{NO}_3)_3$,
 242 $\text{UO}_2(\text{pS1368})$ and $\text{Sr}(\text{CH}_3\text{CO}_2)_3$, respectively. Hence, Sr^{2+} and Cu^{2+} were under the form of acetate or
 243 nitrate complexes under these conditions, UO_2^{2+} was found as a peptidic complex, while the chemical
 244 form of Zn^{2+} could not be identified due to its adsorption on the stationary phase.

245 3.2. Selectivity of pS1368 towards UO_2^{2+} in the presence of Sr^{2+}

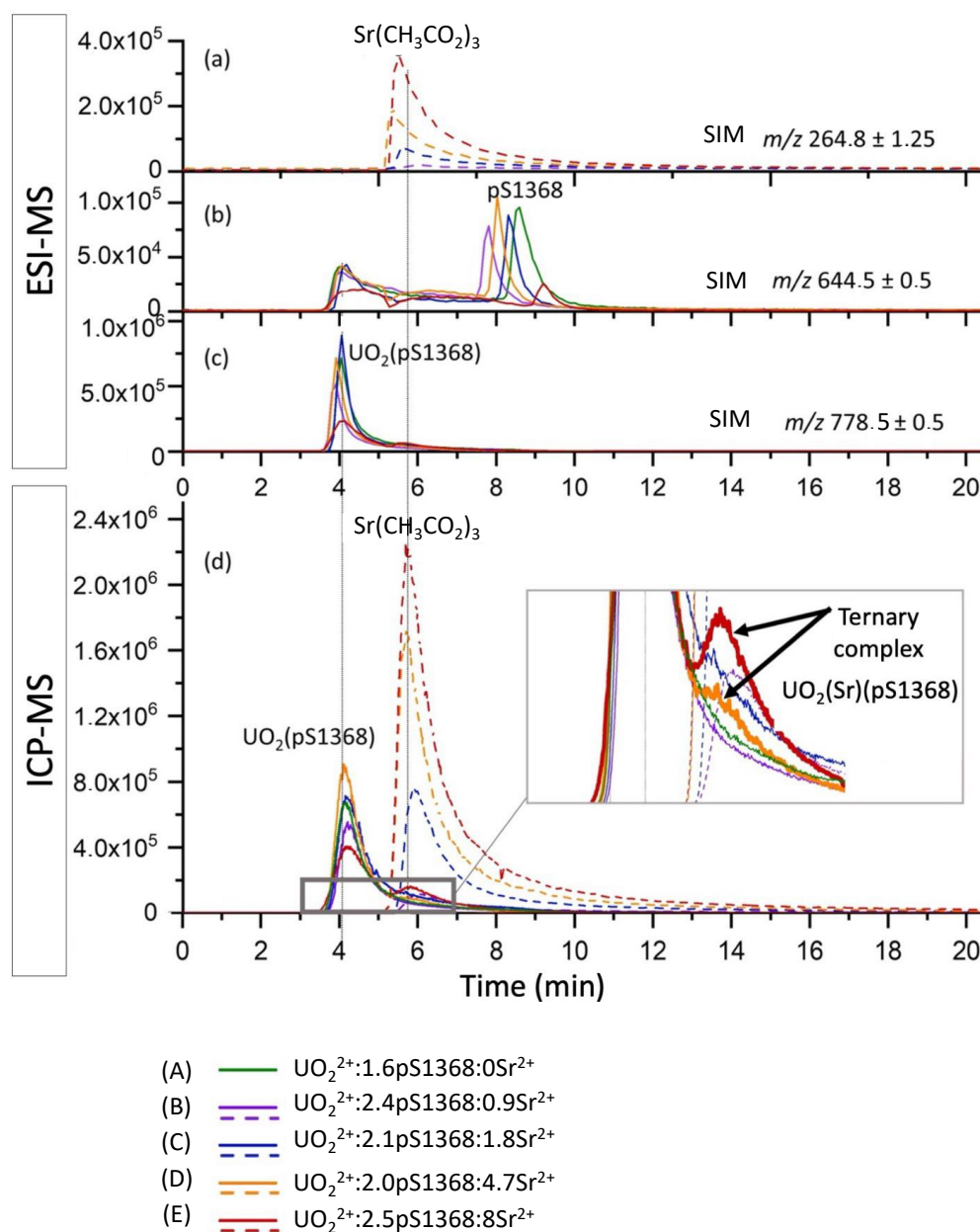
246 Since pS1368 is representative of UO_2^{2+} coordination sites in OPN that binds primarily Ca^{2+} to ensure
 247 its biological function [7], the evaluation of the selectivity of pS1368 towards UO_2^{2+} in the presence of
 248 Ca^{2+} is a crucial first step. To meet this aim, several equivalents of Sr^{2+} , used as Ca^{2+} simulant, were
 249 added to the $\text{UO}_2(\text{pS1368})$ complex at pH 7.4, according to $1\text{UO}_2^{2+}:2\text{pS1368}:x\text{Sr}^{2+}$ ($0 \leq x \leq 10$).
 250 The total UO_2^{2+} and Sr^{2+} concentrations in each sample measured by ICP-MS, as well as the targeted
 251 and measured $1\text{UO}_2^{2+}:2\text{pS1368}:x\text{Sr}^{2+}$ proportions are listed in table 1.

252 **Table 1:** Total UO_2^{2+} and Sr^{2+} concentrations measured by ICP-MS using external calibration in samples
 253 injected in duplicate and in FIA mode, relative deviation (%) of the values obtained for the two replicates,
 254 measured concentration of pS1368, targeted and measured $1\text{UO}_2^{2+}:2\text{pS1368}:x\text{Sr}^{2+}$ proportions.

Targeted $1\text{UO}_2^{2+}:2$ pS1368: $x\text{Sr}^{2+}$	$[\text{UO}_2^{2+}]_{\text{total}}$			[pS1368] mol L^{-1}	$[\text{Sr}^{2+}]_{\text{total}}$			Measured $1\text{UO}_2^{2+}:2\text{pS1368}:x\text{Sr}^{2+}$
	$\mu\text{g mL}^{-1}$	(mol L^{-1})	Relative deviation (%)		$\mu\text{g mL}^{-1}$	(mol L^{-1})	Relative deviation (%)	
1:2:0	27.3 (1.1×10^{-4})	27.2 (1.1×10^{-4})	0.4	1.8×10^{-4}	0.0	0.0	0.0	1:1.6:0 (A)
1:2:1	16.4 (6.9×10^{-5})	16.7 (7.0×10^{-5})	1.3	1.6×10^{-4}	5.2 (5.9×10^{-5})	5.3 (6.0×10^{-5})	1.8	1:2.4:0.9 (B)
1:2:2	24.6 (1.0×10^{-4})	24.0 (1.0×10^{-4})	2.2	2.2×10^{-4}	16.3 (1.9×10^{-4})	16.4 (1.9×10^{-4})	0.2	1:2.1:1.8 (C)
1:2:5	23.2 (9.7×10^{-5})	22.1 (9.3×10^{-5})	4.6	2.0×10^{-4}	40.6 (4.6×10^{-4})	40.2 (4.6×10^{-4})	1.1	1:2.0:4.7 (D)
1:2:10	18.6 (7.8×10^{-5})	18.8 (7.9×10^{-5})	1.2	1.9×10^{-4}	55.0 (6.3×10^{-4})	54.9 (6.2×10^{-4})	0.1	1:2.5:8 (E)

255 As can be observed in table 1, the relative deviation between the measured concentrations of UO_2^{2+} and
 256 Sr^{2+} lies between 0.4 and 4.6% for UO_2^{2+} and between 0.1 and 1.8% for Sr^{2+} , showing good repeatability
 257 of the measurements.

258 Following this step, complexes were separated by HILIC, identified online by ESI-MS and
 259 simultaneously quantified by ICP-MS following the method described in the experimental part. The
 260 separation of the UO_2^{2+} and Sr^{2+} complexes contained in each sample was performed in duplicate, by
 261 applying the chromatographic conditions described in part 3.1. The chromatograms simultaneously
 262 acquired by ESI-MS and ICP-MS are shown in Fig. 3 for the five samples.



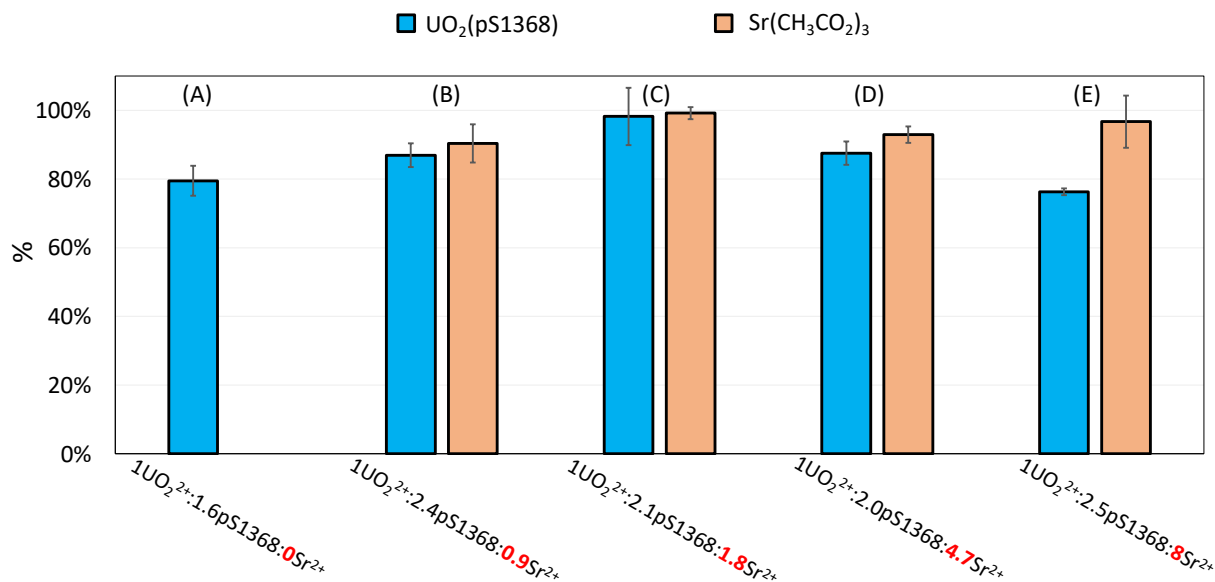
263
 264 **Fig. 3** Separation of UO_2^{2+} and Sr^{2+} complexes by HILIC simultaneously coupled to ESI-MS and ICP-MS.
 265 Chromatograms acquired by ESI-MS using SIM mode (a-c), centered on the m/z ratios of the characteristic ion
 266 of (a) $\text{Sr}(\text{CH}_3\text{COO})_3$, (b) free pS1368, (c) $\text{UO}_2(\text{pS1368})$ considering most abundant isotope of the different
 267 elements involved in the complexes and (d) superposition of the elution profiles obtained by ICP-MS, recording

268 the signal of $^{238}\text{U}^+$ (solid line) and $^{88}\text{Sr}^+$ (dotted line) with an integration time of 90 ms. Column YMC Triart Diol
269 100 x 2 mm; 1.9 μm . Mobile phase: 72/28 ACN/ H_2O v/v with 20 mmol L^{-1} $\text{NH}_4\text{CH}_3\text{CO}_2$, flow rate 300 $\mu\text{L min}^{-1}$,
270
$$V_{\text{inj}} = 3\mu\text{L}.$$

271 The peaks of $^{238}\text{U}^+$ and $^{88}\text{Sr}^+$ obtained by ICP-MS were attributed to $\text{UO}_2(\text{pS1368})$ and $\text{Sr}(\text{CH}_3\text{CO}_2)_3$,
272 based on the chromatograms recorded by ESI-MS. No peak corresponding to $\text{Sr}(\text{pS1368})$ was observed,
273 showing that Sr^{2+} remains mainly bound to acetate whatever the proportions. In all cases, the
274 chromatographic peak of the free fraction of pS1368 was observed by ESI-MS (Figure 3b). When the
275 number of Sr^{2+} equivalents was equal or higher than 4.7 compared to UO_2^{2+} (samples D and E), a tailing
276 appeared on the $\text{UO}_2(\text{pS1368})$ peak registered by ICP-MS, matching with the peak of $\text{Sr}(\text{CH}_3\text{CO}_2)_3$
277 (Figure 3d). This allowed to suggest the formation of a ternary complex, that can be explained by the
278 ability of pS1368 to coordinate UO_2^{2+} and Sr^{2+} ions through bridging phosphates [6]. In order to check
279 this hypothesis, additional experiments were carried out with the sample E containing the largest amount
280 of Sr^{2+} . This allowed us to identify the ternary complex, owing to the ESI mass spectrum extraction from
281 the tailing peak for acquisitions performed in SIM mode, by applying a mass filter centered on the m/z
282 ratio of the ternary complex $\text{UO}_2(\text{Sr})(\text{pS1368})$. The chromatograms recorded simultaneously by ESI-
283 MS and ICP-MS are shown in supporting information (See Electronic Supplementary Material Figure
284 S1). Hence, the addition of up to eight equivalents of Sr^{2+} compared to UO_2^{2+} are not sufficient to
285 displace the latter from $\text{UO}_2(\text{pS1368})$ and leads instead to the formation of a ternary complex
286 $\text{UO}_2(\text{Sr})(\text{pS1368})$. This observation is consistent with literature that reports the formation of ternary
287 complexes of UO_2^{2+} , as $\text{UO}_2(\text{Ca})(\text{CO}_3)$ [20].

288 Several attempts have been made to deconvolute the peak of $^{238}\text{U}^+$ in order to determine the proportion
289 of UO_2^{2+} involved in the ternary complex but did not lead to satisfactory results. Therefore, the
290 quantitative distribution of UO_2^{2+} and Sr^{2+} among the complexes formed in the different samples was
291 determined by applying Equation 1. For D and E samples, the UO_2^{2+} and Sr^{2+} proportions were
292 calculated based on the integration of the chromatographic peak areas including the one involved in the
293 ternary complex, meaning therefore that the quantitative distribution of UO_2^{2+} and Sr^{2+} in the pS1368
294 complexes was slightly overestimated.

295 In order to determine the effect of Sr^{2+} addition on the complexation of UO_2^{2+} by pS1368, these values
 296 were compared to the proportion of UO_2^{2+} measured in $\text{UO}_2(\text{pS1368})$ in the control sample containing
 297 $1\text{UO}_2^{2+}:2\text{pS1368}$, being 79.5 % with respect to total UO_2^{2+} (sample A, Fig. 4).



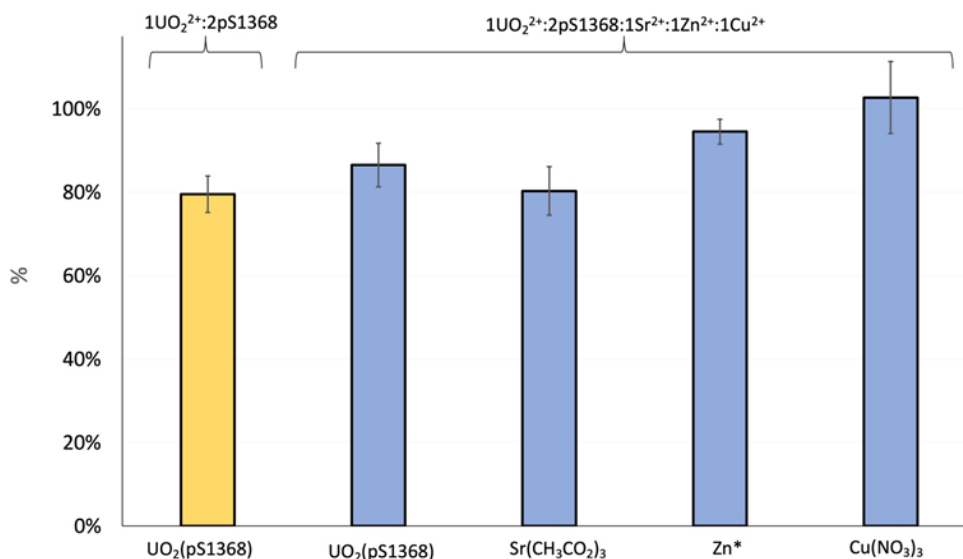
298
 299 **Fig. 4** Proportions of UO_2^{2+} and Sr^{2+} involved in $\text{UO}_2(\text{pS1368})$ and $\text{Sr}(\text{CH}_3\text{CO}_2)_3$ complexes for samples (A-E)
 300 corresponding to $1\text{UO}_2^{2+}:2\text{pS1368}:x\text{Sr}^{2+}$, with $x = 0-8$. The bars represent the mean value of two measurements
 301 of the same sample and the error bars the associated standard deviation.

302 Regardless the number of Sr^{2+} equivalents, at least 76% of UO_2^{2+} remains bound to pS1368. In addition,
 303 more than 90% of Sr^{2+} is in the form of an acetate complex in all the samples. From these results, the
 304 selectivity of pS1368 towards UO_2^{2+} was demonstrated even in the presence of an excess of Sr^{2+} , in
 305 accordance with the trend observed for the stability constants published previously [6], being $K^{\text{pH } 7.4}$
 306 ($\text{UO}_2(\text{pS1368})$) = $10^{11.3}$, much higher than $K^{\text{pH } 7.4}$ ($\text{Ca}(\text{pS1368})$) = 10^2 . Hence, the combination of four
 307 phosphate binding groups and the cyclic peptide skeleton of pS1368 that orients the latter towards the
 308 equatorial plane of UO_2^{2+} , induces efficient selectivity for UO_2^{2+} even in the presence of an excess of
 309 Sr^{2+} . Moreover, the low affinity of this peptide for Sr^{2+} as Ca^{2+} analogue, seems to indicate that Ca^{2+}
 310 would not bind the phosphorylated pSer residues of OPN, but instead the Aspartate (Asp) and Glutamate
 311 (Glu) residues [7]. This is consistent with the larger affinity constants observed for Ca^{2+} with similar
 312 model peptides presenting four glutamate/aspartate instead of four phosphoserine residues [21].

313 **3.3. Selectivity of pS1368 towards UO_2^{2+} in the presence of competing cations Cu^{2+} ,**
314 **Zn^{2+} and Sr^{2+}**

315 The selectivity of pS1368 for UO_2^{2+} in the presence of competing cations Cu^{2+} , Zn^{2+} in addition to Sr^{2+}
316 was determined by applying the same approach, using a model sample containing
317 $1\text{UO}_2^{2+}:2\text{pS1368}:1\text{Sr}^{2+}:1\text{Cu}^{2+}:1\text{Zn}^{2+}$. In the blood, typical concentrations of Ca^{2+} , Cu^{2+} and Zn^{2+} are 2.5
318 mmol L^{-1} , $50 \mu\text{mol L}^{-1}$ and $1 \mu\text{mol L}^{-1}$, respectively [13]. However, we chose an equimolar ratio of these
319 cations to induce similar competing effects compared to UO_2^{2+} , regarding their complexation ability of
320 pS1368.

321 The formed complexes were separated using the diol-grafted column and the mobile phase composed
322 of 72/28 ACN/ H_2O v/v with $20 \text{mmol L}^{-1} \text{NH}_4\text{CH}_3\text{CO}_2$. The quantitative distribution of UO_2^{2+} , Cu^{2+} and
323 Sr^{2+} within the different complexes, determined by ICP-MS as the ratio of the peak area of UO_2^{2+} and
324 metal complexes to the peak of the total elemental content of these latter in the sample, is shown in Fig.
325 5 for injections in duplicate. Since the Zn^{2+} only eluted owing to successive EDTA injections, the Zn^{2+}
326 quantitative distribution was determined by relating the sum of the areas of the peaks resulting from its
327 column recovery to the peak area of the total Zn^{2+} contained in the sample.



328 **Fig. 5** Proportions of UO_2^{2+} , Sr^{2+} and Cu^{2+} involved in the complexes separated by HILIC. *the complexed
329 proportion was calculated from the recovery of Zn^{2+} adsorbed on the column. The bars represent the mean value
330 of two measurements of the same sample and the error bars represent their standard deviation.
331

332 As it can be seen in Fig. 5, more than 80% of UO_2^{2+} is complexed by pS1368 while Sr^{2+} and Cu^{2+} exist
333 mostly (80-100%) as acetate and nitrate complexes, whereas the chemical form of Zn^{2+} could not be
334 identified due to the adsorption of this element on the column. We can also observe that the proportion
335 of UO_2^{2+} involved in $\text{UO}_2(\text{pS1368})$ in the model sample is close to the one in the control sample. We
336 can therefore conclude that UO_2^{2+} is not displaced from the $\text{UO}_2(\text{pS1368})$ complex by Sr^{2+} , nor by Cu^{2+}
337 or Zn^{2+} , being in equimolar proportions compared to UO_2^{2+} . This shows that pS1368 selectively binds
338 UO_2^{2+} in the presence of these competitors, confirming the interest of this peptide as a selective binding
339 site for the design of decorporating agent of UO_2^{2+} *in vivo*.

340 4. Conclusion

341 In this work, a dedicated analytical approach based on the implementation of the simultaneous coupling
342 of HILIC to ESI-MS and ICP-MS was successfully applied to the one-step determination of the
343 selectivity of pS1368 peptide towards UO_2^{2+} in the presence of the competing ions Sr^{2+} , Cu^{2+} and Zn^{2+} .
344 Sr^{2+} was used to mimic the behavior of Ca^{2+} during complexation by pS1368, since it has similar
345 physico-chemical properties, while providing less challenging ICP-MS measurement. The complexes
346 of UO_2^{2+} , Sr^{2+} , Cu^{2+} and Zn^{2+} were separated by HILIC using a YMC triart diol column and a mobile
347 phase composed of 72/28 ACN/ H_2O v/v with 20 mmol L^{-1} $\text{NH}_4\text{CH}_3\text{CO}_2$, identified online by ESI-MS
348 and simultaneously quantified by ICP-MS. In the presence of increasing number of Sr^{2+} equivalents,
349 more than 76% of UO_2^{2+} remains complexed by pS1368. Furthermore, when the number of Sr^{2+}
350 equivalents compared to UO_2^{2+} is equal or higher than 4.7, a ternary complex $\text{UO}_2(\text{Sr})(\text{pS1368})$ is
351 detected. These results led to the conclusion that pS1368 was selective towards UO_2^{2+} in presence of
352 Sr^{2+} , as Ca^{2+} simulant, in agreement with previous results obtained by spectroscopic techniques. The
353 selectivity of this peptide for UO_2^{2+} was also determined in the presence of endogenous competing
354 cations such as Cu^{2+} and Zn^{2+} in addition to Sr^{2+} , all being in equimolar ratio compared to UO_2^{2+} . Under
355 these conditions, UO_2^{2+} is not displaced from $\text{UO}_2(\text{pS1368})$. Hence, we demonstrated here that the
356 biomimetic peptide pS1368 is selective for UO_2^{2+} in presence of the three competing endogenous cations
357 $\text{Ca}^{2+}/\text{Sr}^{2+}$, Cu^{2+} and Zn^{2+} . The high affinity of the cyclic tetra-phosphorylated peptide pS1368 for UO_2^{2+}

358 and its selectivity with respect to Sr^{2+} and other endogenous metals thus confirm that pS1368 is an
359 attractive starting scaffold for the design of a selective decorporating agent of UO_2^{2+} *in vivo*.

360 The method that we developed shows to offer several advantages, in particular the possibility to directly
361 determine the selectivity of pS1368 towards UO_2^{2+} in the presence of endogenous cations, though a
362 competing reaction in the same sample and in one single step. This leads to a reduction of the sample
363 consumption, which is of prime importance for the screening of a large range of chemical or peptide
364 candidates. Overall, these findings highlight the powerful combination of HILIC to ESI-MS and ICP-
365 MS for acquiring in a single step complementary elemental and molecular information which is of prime
366 importance to assess the potential of a candidate molecule as decorporating agent of toxic elements.

367

368 **Acknowledgements**

369 The authors would like to acknowledge the Cross-Disciplinary Program on Instrumentation and
370 Detection of CEA, the French Alternative Energies and Atomic Energy Commission and the Cross-
371 cutting basic research Program (RTA Program) of the CEA Energy Division, for their financial support.

372

373 **Declarations**

374 The authors declare that they have no conflicts of interests/competing interests.

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- 443

444 7. Figure captions

445

446 **Fig. 1** Structure of the cyclic tetra-phosphorylated peptide pS1368 considered in this work. Adapted
447 from Ref.6.

448

449 **Fig. 2** Separation of UO_2^{2+} and metal complexes by HILIC simultaneously coupled to ESI-MS and ICP-
450 MS. Chromatograms acquired by ESI-MS using SIM mode, centered on the m/z ratios of the
451 characteristic ion of pS1368 and considering most abundant isotope of the different elements involved
452 in the complexes (a-d), and (e) superposition of the elution profiles recorded by ICP-MS, following the
453 signal of the most abundant isotopes $^{238}\text{U}^+$, $^{88}\text{Sr}^+$, $^{63}\text{Cu}^+$ and $^{64}\text{Zn}^+$, with an integration time of 90 ms.
454 Sample: $1\text{UO}_2^{2+}:2\text{pS1368}:1\text{Sr}^{2+}:1\text{Cu}^{2+}:1\text{Zn}^{2+}$. Column YMC Triart Diol 100 x 2 mm; 1.9 μm . Mobile
455 phase: 72/28 ACN/ H_2O v/v with 20 mmol L^{-1} $\text{NH}_4\text{CH}_3\text{CO}_2$, flow rate: 300 $\mu\text{L min}^{-1}$, $V_{\text{inj}} = 3\mu\text{L}$.

456

457 **Fig. 3** Separation of UO_2^{2+} and Sr^{2+} complexes by HILIC simultaneously coupled to ESI-MS and ICP-
458 MS. Chromatograms acquired by ESI-MS using SIM mode (a-c), centered on the m/z ratios of the
459 characteristic ion of (a) $\text{Sr}(\text{CH}_3\text{COO})_3$, (b) free pS1368, (c) $\text{UO}_2(\text{pS1368})$ considering most abundant
460 isotope of the different elements involved in the complexes and (d) superposition of the elution profiles
461 obtained by ICP-MS, recording the signal of $^{238}\text{U}^+$ (solid line) and $^{88}\text{Sr}^+$ (dotted line) with an integration
462 time of 90 ms. Column YMC Triart Diol 100 x 2 mm; 1.9 μm . Mobile phase: 72/28 ACN/ H_2O v/v with
463 20 mmol L^{-1} $\text{NH}_4\text{CH}_3\text{CO}_2$, flow rate 300 $\mu\text{L min}^{-1}$, $V_{\text{inj}} = 3\mu\text{L}$.

464

465 **Fig. 4** Proportions of UO_2^{2+} and Sr^{2+} involved in $\text{UO}_2(\text{pS1368})$ and $\text{Sr}(\text{CH}_3\text{CO}_2)$ complexes for samples
466 (A-E) corresponding to $1\text{UO}_2^{2+}:2\text{pS1368}:x\text{Sr}^{2+}$, with $x = 0-8$. The bars represent the mean value of two
467 measurements of the same sample and the error bars the associated standard deviation.

468

469 **Fig. 5** Proportions of UO_2^{2+} , Sr^{2+} and Cu^{2+} involved in the complexes separated by HILIC. *the
470 complexed proportion was calculated from the recovery of Zn^{2+} adsorbed on the column. The bars
471 represent the mean value of two measurements of the same sample and the error bars represent their
472 standard deviation.

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