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# Development of a non-denaturing 2D gel electrophoresis protocol for screening *in vivo* uranium-protein targets in *Procambarus clarkii* with laser ablation ICP MS followed by protein identification by HPLC–Orbitrap MS

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ABSTRACT

Limited knowledge about *in vivo* non-covalent uranium (U)-protein complexes is largely due to the lack of appropriate analytical methodology. Here, a method for screening and identifying the molecular targets of U was developed. The approach was based on non-denaturing 1D and 2D gel electrophoresis (ND-PAGE and ND-2D-PAGE (using ND-IEF as first dimension previously described)) in conjunction with laser ablation inductively coupled plasma mass spectrometry (LA-ICP MS) for the detection of U-containing proteins. The proteins were then identified by μbore HPLC–Orbitrap MS/MS. The method was applied to the analysis of cytosol of hepatopancreas (HP) of a model U-bioaccumulating organism (*Procambarus clarkii*). The imaging of uranium in 2D gels revealed the presence of 11 U-containing protein spots. Six protein candidates (*i.e.* ferritin, glyceraldehyde-3-phosphate dehydrogenase, triose-phosphate isomerase, cytosolic manganese superoxide dismutase (Mn-SOD), glutathione S transferase D1 and H3 histone family protein) were then identified by matching with the data base of crustacea Decapoda species (*e.g.* crayfish). Among them, ferritin was the most important one. This strategy is expected to provide an insight into U toxicology and metabolism.



## 1. Introduction

Uranium (U), a chemo- and radio-toxic element, can be found in aquatic ecosystems because of its natural occurrence and/or anthropogenic activities, particularly from the nuclear fuel cycle such as wastes from mining and milling of U ore [1]. In surface water and groundwater, U concentration ranges from the low 10 ng $L^{-1}$ to the high 1.5 mg $L^{-1}$ levels [2]. Although ubiquitous in the environment, U has no known metabolic function in animals and is regarded as a nonessential element. Its chemistry in aerobic media is characterized by a high solubility of the hexavalent form, uranyl ion ($UO_2^{2+}$), favoring binding to oxygen and nitrogen atoms in biomolecules (*e.g.* proteins, peptides, nucleic acids, and metabolites) [3].

Several data showed U toxicity after chronical exposure as it accumulated in animal organs such as bone, liver, and kidney [4,5]. The mechanisms of the U toxicity are poorly explored. Their understanding requires the identification of U molecular targets among which proteins have been shown to play an important role [6]. The acquisition of information on the identity of U-protein complexes remains challenging because of the electrostatic nature of the binding and their low abundance in biological materials because of the low exposure levels. The analytical methods developed to investigate U-containing protein complexes were based on liquid chromatography and/or electrophoresis with detection by elemental and molecular mass spectrometry [7–10].

The preservation of coordinate and multi-subunits structure of metalloproteins requires non-denaturing conditions. Detergents and reducing reagents used in denaturing gel electrophoresis protocols (*e.g.* SDS and DTT) can break the native conformation of a metalloprotein and cause the loss of metal ions. Detection of metalloprotein in organisms was achieved using non-denaturing gel electrophoresis and elemental imaging techniques (*e.g.* LA-ICP MS and X-ray spectrometry) [11–13]. Recently, U-protein complexes in a crayfish species (*Procambarus clarkii*), a U-bioaccumulating model organism [14–16], were quantitatively studied by 1D non-denaturing isoelectric focusing (ND-IEF) gel electrophoresis coupled

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to LA-ICP MS [17]. The analysis of the U-rich protein bands allowed the identification of 42 potential protein targets by a proteomic approach. The complexity of this information was reduced by restricting the taxonomy entry to crustaceans (crayfish, lobster and crabs) reducing the number of potential protein targets to 3. In order to improve the level of confidence for proteins identified as U targets, the resolution of the purification methodology has to be maximized for a better specificity.

The objective of this work was to develop a non-denaturing 2D gel electrophoresis protocol producing U-rich spots of sufficient purity to correlate unambiguously the presence of uranium and the identity of the protein present. As the first dimension, *i.e.* ND-IEF was already optimized in our previous study [17], we focused in this work on the 2nd dimension PAGE separation. Therefore, the 1D PAGE conditions *i.e.* denaturing or non-denaturing and acrylamide percentage in the gels were firstly compared to optimize the separation conditions using hepatopancreas (HP) samples. Then, the cytosols of HP were analyzed using 2D protocol, *i.e.* combining ND-IEF and ND-PAGE. 2D PAGE gels were then imaged by LA-ICP MS to locate the U-containing protein spots. The latter were submitted to a proteolytic digestion followed by μRPC-ESI MS/MS of the produced peptides to identify the proteins.

## 2. Experimental procedures

### 2.1. Instrumentation

Elemental analysis of gels was achieved by coupling a UP213 laser ablation system (Electro Scientific Industries, Freemont, CA) with an Agilent 7700 ICP MS (Agilent, Tokyo, Japan). 500 mL $min^{-1}$ of helium gas was used to transport ablated material into the plasma of ICP MS through a T-connector under dry plasma conditions. The ICP MS was equipped with a Micromist nebulizer mounted into a Scott spray chamber. The laser ablation was performed in a focused spot mode using a Nd:YAG laser source (wavelength: 213 nm) with 20 Hz repetition frequency and 100% energy delivered. The laser beam spot size and scan speed were set to 250 μm and 100 μm $s^{-1}$, respectively.

μRPC-ESI-MS/MS analysis was performed with an Agilent 1100 capillary HPLC system (Agilent, Tokyo, Japan) and an electrospray LTQ Orbitrap Velos mass spectrometer (ThermoFisher Scientific, Bremen, Germany).

ND-IEF gel electrophoresis was carried out in an IEF-SYS system (Biostep GmbH, Meinersdorfer, Germany). ND-PAGE and ND-2D-PAGE gel electrophoresis were carried out in a TV100 system (Biostep GmbH, Meinersdorfer, Germany). All the gels were imaged with an AGFA ARCUS II Scanner (Agfa, Belgium). For LA-ICP MS analysis, unstained gels were dried using a Hoefer GD 2000 slab gel dryer (Amersham Biosciences).

### 2.2. Reagents

Water (18 MΩ cm) was obtained from a Milli-Q system (Millipore, Bedford, MA). Unless stated otherwise, all other analytical reagent grade chemicals were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). The U stock solution (1000 μg $mL^{-1}$ in 4% $HNO_3$) was purchased from SCP science (Courtaboeuf, France). Standard proteins including thyroglobulin (bovine thyroid, > 90%, 660 kDa), ferritin (equine spleen, 450 kDa), transferrin (human, > 98%, 80 kDa), bovine serum albumin (fraction V, > 96%, 66 kDa), ovalbumin (chicken egg white, > 98%, 45 kDa), Mn-superoxide dismutase (*Escherichia coli*, 40 kDa) and all the gel electrophoresis reagents including 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris, > 99%), 30% acrylamide/bis-acrylamide solution, *N,N,N′,N′*-tetramethylethylenediamine (TEMED), ammonium persulfate (APS), dithiothreitol (DTT), agarose and glycine ( > 99%) were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). The immobilized pH gradient strips (7 and 13 cm, pH 4–7) and immobiline DryStrip cover fluid were from GE Healthcare (Orsay, France).

### 2.3. Crayfish samples

All crayfish (*P. clarkii*) used in this study were adult intermoult males coming from the Vigueirat swamp of Camargue (France) and were acclimatized one month to laboratory experimental conditions. Animals were then exposed for 10 days to uranium concentrations: 0 (controls) and 600 μg $UL^{-1}$ of depleted uranium (as uranyl nitrate). Details of exposure were described by Al Kaddissi et al. [14]. After U exposure, animals were sampled from each tank, sacrificed and dissected, and the HP samples (0.2 g) were subjected to a subcellular fractionation protocol using 250 mM sucrose, 25 mM HEPES, 7.4 pH buffer (4 mL) as described by Frelon et al. to recover cytosolic fraction [16]. The protein concentrations of the cytosolic fraction were quantified using a BCA Protein Assay Kit (Sigma-Aldrich) based on an external standard calibration curve method. The absorbance (545 nm) of the protein solutions was measured by a STAT FAX 303 Plus Microstrip Reader (Block Scientific Inc., Bohemia, USA). Cytosols were stored at −80 °C until use.

### 2.4. Procedures

U-protein complexes cytosols were prepared from hepatopancreas (HP) of waterborne U-exposed crayfish (*P. clarkii*) based on our previous work [16,17]. HP is chosen because it is the place where digestion, absorption of nutrients, storage of reserves, detoxification and synthesis of digestive enzymes take place [18].

#### 2.4.1. Gel electrophoresis conditions

Polyacrylamide gels were hand-cast in gel cassettes (20 × 20 or 8 × 8 $cm^2$) using commercial 30% acrylamide/bis-acrylamide solution (37.5%/1%, w/v). For SDS-PAGE, gels contained 6% acrylamide/bis-acrylamide, 375 mM Tris (pH 8.8), 0.01% SDS, 0.01% APS and 0.001% TEMED solutions. 50 μL of samples were directly loaded into the well of gels, and a program (80 V for 0.5 h and 100 V for 3.5 h) was carried out in the SDS–Tris–glycine buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3). Gels were run in duplicate, one for protein bands visualizing by means of 0.1% coomassie brilliant blue (CBB) or silver staining method, and the other unstained-one for LA-ICP MS imaging analysis after drying (80 °C for 1 h) by a Hoefer GD 2000 slab gel dryer on 3 MM Chromatography Paper (Whatman, VWR, Pessac, France). After the drying process, the gels were covered by Saran film (Dow Chemical Company) to avoid potential contamination until LA-ICP MS analysis. Standard proteins (*i.e.* thyroglobulin, ferritin, transferrin, bovine serum albumin and ovalbumin) were used as molecular weight markers in gel electrophoresis experiments.

For non-denaturing gel electrophoresis (*i.e.* ND-IEF, ND-PAGE and ND-2D-PAGE), reagents including urea, DTT, CHAPS and IPG buffers were removed from standard protocols of IEF and SDS-PAGE [19]. Detailed experimental processes of ND-IEF gel electrophoresis were described elsewhere [17]. In brief, 200 or 100 μL of cytosols were directly loaded onto the IPG strips (pH 4–7, 13 or 7 cm) and left overnight for re-hydration. Then, a regular IEF program (60 V for 0.5 h, 250 V for 0.5 h, 500 V for 0.5 h, a linear gradient up to 3000 V for 3 h, and finally 3000 V until 20 kVh) was operated. After the focusing process, one IPG strip was left to air-drying for LA-ICP MS analysis, and two parallel strips were used for the separation in the second non-denaturing dimension

(*i.e.* ND-2D-PAGE). The ND-PAGE protocol was used based on the modification of the described SDS-PAGE gel electrophoresis protocol. All equipments were cleaned with Milli-Q $H_2O$ to remove residual SDS reagent. For ND-2D-PAGE, IPG strips after ND-IEF gel electrophoresis were equilibrated in 0.375 M Tris–HCl, 20% glycerin, pH 8.8 for 10 min. Then, the equilibrated strips were transferred to the top of polyacrylamide gels for the separation in the second dimension. Other experimental conditions, such as electrophoretic program were the same as described above.

*2.4.2. Optimization of PAGE protocol conditions for cytosolic samples*

To evaluate the resolution of PAGE and the stability of U-protein complexes, three protocols were investigated using HP cytosol: protocol (1) ND-PAGE with no addition of reagents to the cytosolic samples, protocol (2) SDS-PAGE with moderately denaturing sample preparation, and protocol (3) SDS-PAGE with a precipitation step additionally to protocol (2). Protocol (2) consisted in incubating the cytosol (100 μL) with an equal volume of denaturing buffer (8 M urea, 4% CHAPS, 65 mM DTT, 0.01% bromophenol blue) at 56 °C for 45 min. Protocol (3) allowed cytosolic (100 μL) proteins precipitation with 10% trichloroacetic acid (TCA) in ice-cold acetone, three times acetone washing, centrifugation (12,000 rpm at 4 °C, × 3) and dissolution of the protein pellet in the denaturing buffer (100 μL).

After the comparison of denaturing and non-denaturing PAGE protocols for cytosolic sample, a series of uniform polyacrylamide gels (4%, 6%, 8%, 12% and 16%) were compared for the best resolution. 50 μL of HP cytosols were directly loaded into the slots of gels and separated as described above. All the gels were then analyzed by LA-ICP MS.

*2.4.3. U recovery analysis of ND-PAGE*

To access the U recovery of ND-PAGE, 50 μL HP cytosol was directly diluted with 2% $HNO_3$ to 2 mL, and another 50 μL-aliquot was separated by ND-PAGE. After the electrophoretic process, the gel piece ($0.5 \times 7.5$ $cm^2$) was air-dried and soaked into 2% $HNO_3$ (2 mL) for 24 h. Finally, the amounts of U, Fe and Cu were quantitatively measured by ICP MS in the raw cytosol and polyacrylamide gel.

*2.4.4. LA-ICP MS analysis*

ICP MS parameters (torch position, carrier gas flow rate and ion lenses voltage) were tuned for each set of experiments using a 1 ng $mL^{-1}$ solution of Y, Li, Tl and Ce. He was used as reaction gas in the collision cell mode with a flow rate of 10 mL $min^{-1}$. The isotopes of $^{238}U$, $^{55}Mn$, $^{56}Fe$, $^{58}Ni$, $^{59}Co$, $^{63}Cu$ and $^{64}Zn$ were monitored. Each IPG strip or polyacrylamide gel was scanned three times in parallel by LA-ICP MS. For elemental imaging of entire 2D gels, the 2D gel was divided and cut into ten uniform pieces ($4 \times 1.5$ $cm^2$), and each gel piece was scanned line by line with a distance of 750 μm. Then the collected data were processed with Matlab 7 software (MathWorks, Meudon, France) for $^{238}U$, $^{55}Mn$, $^{56}Fe$, $^{58}Ni$, $^{59}Co$, $^{63}Cu$ and $^{64}Zn$ imaging, and the individual images were regrouped to obtain the full size image using Adobe Photoshop CS5 software (Adobe Systems).

*2.4.5. Identification of in vivo U-protein targets by μRPC-ESI-MS/MS*

After elemental analysis, the selected protein spots/bands containing U were excised from stained gels. The gel pieces were washed with 500 μL $H_2O$ for 10 min, and then three times with 50 mM $NH_4HCO_3$, 50% acetonitrile (ACN) for CBB destaining, or using silver destaining protocol [20]. Following, the proteins in gels were reduced and alkylated by addition of 2 μL, 100 mM DTT at 56 °C for 45 min and 3 μL, 100 mM iodoacetamide at room temperature for 20 min in the dark, respectively. After washing with 100% ACN and air-drying, 1 μL trypsin solution (50 ng $\mu L^{-1}$, proteomics grade, Sigma-Aldrich) was added for overnight digestion at 37 °C. The peptides were extracted with 50% ACN, 2% formic acid (FA) solution and the solutions of peptide mixtures were stored at −20 °C for μRPC-ESI-MS/MS analysis. 8 μL of peptide mixtures were injected and separated into a Vydac C18 column (150 mm × 1 mm ID, 5 μm, Alltech/Grace, Templemars, France). The flow rate was 50 μL $min^{-1}$ of 0.05% FA in $H_2O$ (solvent A) and ACN (solvent B). The gradient program lasted for 3 min at 2% B and then increased to 80% in 45 min. Between each run, the column was equilibrated at least 10 min at 2% B. The mass spectrometer was operated in the positive ion mode and the $m/z$ 350–2000 range was scanned with a resolution set at 100,000 ($m/\Delta m$, fwhm (full width at half-maximum) at $m/z$ 400). The spray voltage and capillary temperature were respectively set at 3.3 kV and 300 °C. Ten most intense ions of each full scan were selected to be fragmented using collision induced dissociation (CID) at normalized collision energy of 35% and analyzed by an Orbitrap mass analyzer. The raw data were analyzed using Xcalibur 2.1 software (ThermoFisher Scientific). For protein identification, raw data files were converted to Mascot generic format (mgf) files and searched in National Center for Biotechnology Information (NCBInr) database via Mascot Search 2.4 (Matrix Science) with Other Metazoa as taxonomy. Mascot search parameters included trypsin as the proteolytic enzyme with one missed cleavage. The peptide and MS/MS tolerances were set at 10 ppm and 0.1 Da, respectively. The modifications of carbamidomethyl and methionine oxidation were selected. Peptide charges were set to +1, +2 and +3. A minimal Mascot score of 25 and two different peptides were set for protein identity validation.

## 3. Results and discussion

*3.1. Assessment of in vivo U-protein complexes stability by SDS-PAGE, ND-PAGE and LA-ICP MS*

$UO_2^{2+}$ ion is a linear and hard Lewis metal cation able to coordinate up to six hard donor ligands in the plane. Its highly oxophilic property favors strong interaction with functional groups such as carboxyl and phosphoryl groups of amino acid side chain [3]. Therefore, proteins are regarded as one of the most important biomolecules to coordinate, transport and detoxify $UO_2^{2+}$ [3]. Generally, proteins should have various chemical affinities for $UO_2^{2+}$ depending on their binding domains as demonstrated by *in vitro* studies [21–23]. U-protein complexes are expected to dissociate in the presence of detergents and reducing agents used in GE, although one report claims their stability to some extent [7]. Therefore, the stability of U-protein complexes was first studied with the HP cytosol sample (4.2 mg $mL^{-1}$ of protein) using three different protocols under denaturing and non-denaturing sample preparations and electrophoretic conditions.

Fig. 1 shows the results of HP cytosol analyzed by ND-PAGE (Fig. 1a) and SDS-PAGE (Fig. 1b and c). In the ND-PAGE electropherogram (Fig. 1a), five U peaks can be detected, as peak 1 (0 cm), peak 2 (1.2 cm), peak 3 (3.5 cm), peak 4 (5.6 cm) and peak 5 (6.5 cm). The asymmetric shape of peak 1 suggests that these U-biomolecules are stacked at the top of the gel because of their extremely large size (> 660 kDa). On the other side of gel, most of the U (> 80%) was found at 6.5 cm (< 45 kDa) migration distance near the dye front. In Fig. 1b, the decrease of U signal at 0 cm of *ca.* 2-fold and the disappearance of peak 3 suggest that these U-protein complexes are disrupted by the denaturing buffer. The increased front tailing of peak 5 hampered the detection of peak 4. Compared with ND-PAGE, the U intensity of peak 2 (~450 kDa) was nearly unchanged revealing a relatively stable or abundant U-

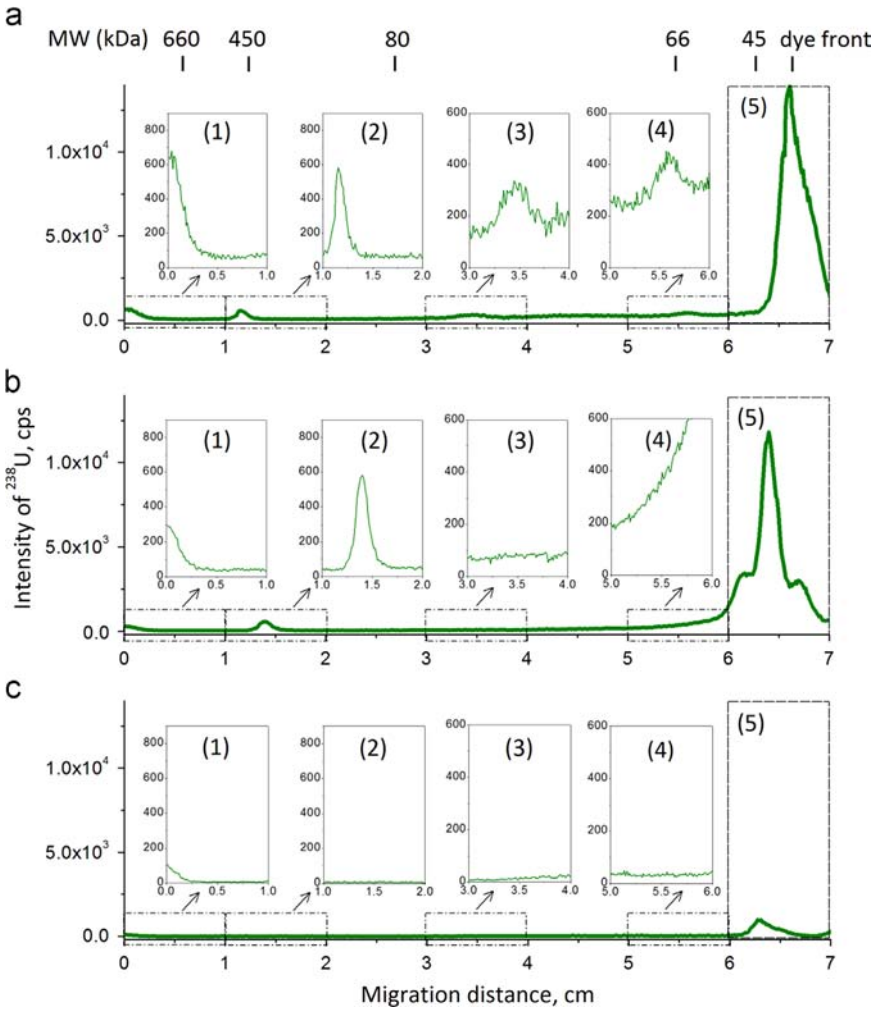


**Fig. 1.** $^{238}$U LA-ICP MS electropherograms of HP cytosol of U-exposed crayfish analyzed by (a) ND-PAGE with protocol 1, and denaturing SDS-PAGE with (b) protocol 2 and (c) protocol 3. 6% polyacrylamide gels are used. 1, 2, 3 and 4 are the magnified parts in the square dashed line. Molecular weight markers (660, 450, 80, 66 and 45 kDa as native protein molecules) are used as an approximate reference for ND-PAGE, as the migration of proteins in ND-PAGE not only depends on their molecular size but also on their charge because of the non-denaturing conditions.

**Table 1.**
Relative percentage of metals left in gels compared with ND-PAGE (protocol 1, $n=3$)

| Method | Relative percentage (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | U | Mn | Fe | Ni | Co | Cu | Zn |
| SDS-PAGE/protocol 2 | $80 \pm 6$ | $67 \pm 1$ | $40 \pm 14$ | $69 \pm 13$ | $66 \pm 1$ | $52 \pm 10$ | $64 \pm 13$ |
| SDS-PAGE/protocol 3 | $0.5 \pm 0.1$ | $0.4 \pm 0.1$ | $1.3 \pm 0.4$ | $0.8 \pm 0.1$ | $0.6 \pm 0.1$ | $0.5 \pm 0.1$ | $0.5 \pm 0.1$ |

protein species under denaturing electrophoretic conditions of protocol 2. Furthermore, its shift from 1.2 cm to 1.4 cm migration distance suggested that these biomolecules brought more negative charges and moved further toward the anode. The profile of peak 5 (82% of total U for protocol 1, 85% for protocol 2) emphasized either the change of cytosolic U-protein complexes or an improved resolution under denaturing electrophoretic condition. $80.2 \pm 5.5\%$ of U was found in SDS-PAGE electropherograms using protocol 2 (Table 1) compared to results obtained by means of protocol 1 confirming that detergent and reducing reagents were less favorable for maintaining the integrity of U-protein complexes *in vivo*. The same conclusion can be drawn under extreme denaturing condition as only 0.5% of U is left in the gel (Fig. 1c) in the same manner for the others metals (Table 1). Therefore, non-denaturing gel electrophoresis (protocol 1) was selected to maximize the stability of U-protein complexes during separation protocol.

### 3.2. ND-PAGE-LA-ICP MS analysis for in vivo screening of U-protein complexes in U-exposed crayfish

In order to obtain the maximum separation efficiency, different uniform polyacrylamide gels (*i.e.* Fig. 2a: 4%, Fig. 2b: 6%, Fig. 2c: 8%, Fig. 2d: 12% and Fig. 2e: 16%) were prepared and compared for the separation of U-protein complexes in HP cytosol of U-exposed crayfish. Fig. 2 shows that between 3 and 5 U peaks can be detected under different electrophoretic conditions. Their migration distances are shortened with the increasing acrylamide percentage

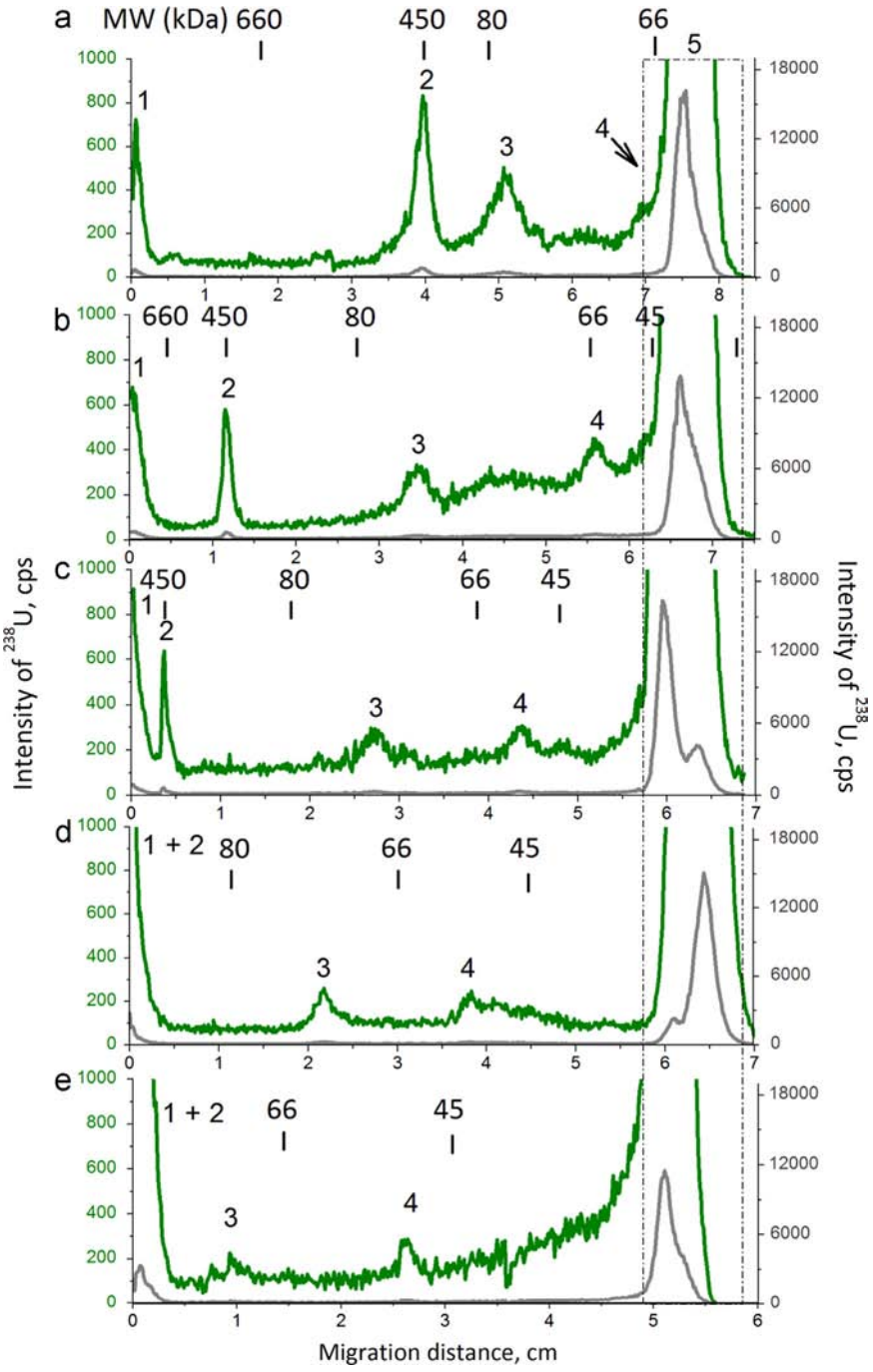


**Fig. 2.** $^{238}$U ND-PAGE-LA-ICP MS electropherograms of HP cytosol from U-exposed crayfish. (a) 4%, (b) 6%, (c) 8%, (d) 12% and (e) 16% polyacrylamide gels are used. 1, 2, 3, 4 and 5 are $^{238}$U peaks detected in the gels. Green (0–1000 cps) and gray (0–19000 cps) traces represent $^{238}$U intensity for the same analysis at different scales. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

from 4% to 16% which is the limit of uniform polyacrylamide gels. This confirms that the migration of U-protein complexes is correlated with the pore size of gels. These five peaks can only be observed in Fig. 2b and c simultaneously indicating that 6% and 8% polyacrylamide gels are optimal for further analysis. U percentages of peaks 1, 2, 3, 4 and 5 are calculated to be $1.4 \pm 0.3\%$, $2.2 \pm 0.6\%$, $1.7 \pm 0.7\%$, $1.5 \pm 0.1\%$ and $82.4 \pm 5.4\%$ of the total U area in all electropherograms, respectively. In addition, their molecular weights are estimated to be $>450$ kDa, 450 kDa, 80–66 kDa, 66–45 kDa and $<45$ kDa utilizing the native standard proteins in Fig. 3. These qualitative and quantitative data suggest that *in vivo* U-protein complexes are dominantly of low-molecular weight ($<45$ kDa) in HP cytosol of U-exposed crayfish which tends to be consistent with the previous SEC results (*ca.* 60%) although the recoveries of SEC and ND-PAGE are different [16,17].

In 8% polyacrylamide gel (Fig. 3), the protein bands (Fig. 3a) corresponding to U peaks (Fig. 3b and c) (the same electropherogram but with different intensity scales) can be distinguished after CBB-staining confirming the presence of U-protein complexes. Compared with the control sample (gray line in Fig. 3b and c) (crayfish without U exposure), significant U signals (green line in Fig. 3b and c) generated by U-protein complexes could be

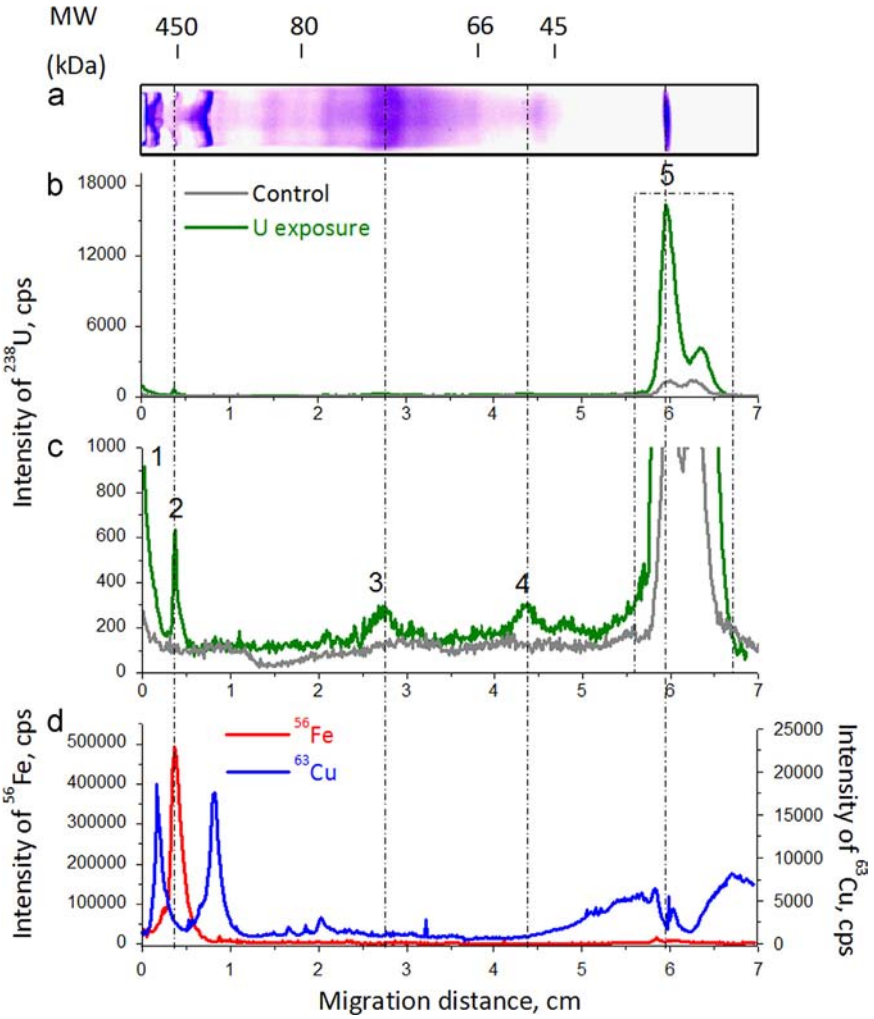


**Fig. 3.** (a) CBB-stained ND-PAGE gel. (b, c) The same $^{238}$U LA-ICP MS electropherograms in different $^{238}$U intensity scales. (d) $^{56}$Fe and $^{63}$Cu LA-ICP MS electropherograms. The gray and green lines respectively represent the HP cytosol from control and U-exposed crayfish. 8% polyacrylamide gels are used for electrophoretic separation. 1, 2, 3, 4 and 5 are $^{238}$U peaks detected in the gels. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

observed. Fe and Cu signals (Fig. 3d) were also monitored as they co-migrated with U by ND-IEF gel electrophoresis in our previous work [17]. The results show one Fe peak at the same migration distance (0.4 cm) as U peak 2 in Fig. 3c and ferritin standard, and a minor Cu peak overlapped with peak 5 (migration distance, 5.9 cm). Furthermore, these findings were confirmed by the 6% and 4% polyacrylamide gels (Fig. S1).

As a preliminary attempt, the proteins in bands corresponding to U peaks in Fig. 3a are identified by μRPC-ESI-MS/MS. After restricting taxonomy information to crustacean animals (*e.g.* crayfish, shrimps, lobsters and crabs), 1, 3, 2, 4 and 1 proteins were identified for peaks 1, 2, 3, 4 and 5, respectively (Table S1). The animal species of some identified proteins do not belong to *P. clarkii* species probably because of its incomplete genetic information in database. An Fe-containing protein, ferritin, was identified at the position of peak 2.

Under the optimized ND-PAGE condition, the U recovery was measured and determined to be 43–49% for both HP samples, and the Fe and Cu recovery was respectively *ca.* 39% and 20%. However, the lack of resolution of ND-PAGE may hamper the detection and identification of minor U species. Thus to assure the formal identification of the protein targets of uranium, the purity of U-protein complexes had to be improved. For this purpose, the non-denaturing IEF protocol developed earlier [17] was combined with the ND-PAGE optimized (8% polyacrylamide) in this work.

### 3.3. ND-2D-PAGE-LA-ICP MS for U-protein complexes in vivo screening

Fig. 4a shows the typical U image where 11 focused U spots can be detected. Among these spots, 8 are near the anode side (pI 4.0–5.2) and the other 3 are near the cathode side (pI 6.0–7.0). This distribution also matched with the CBB-stained 2D gel (Fig. 4b) and was well confirmed by the ND-IEF (Fig. 4c) and ND-PAGE (Fig. 4d) electropherograms obtained in parallel. These results indicated that most of U is bound *in vivo* to acidic proteins (pI < 6). Even if the resolution was lower, 6% polyacrylamide gel allowed confirming the presence of U spots 2, 3, 5, 6 and 7 (Fig. S2). Combined with the U recovery (*ca.* 64%) of ND-IEF [17], around one third of U in raw cytosols was estimated to be left in the 2D gels. The loss might happen during the transfer from the 1st to the 2nd dimension (*ca.* 35% of total U) induced by the neutral and positive-charged U-biomolecules and the extreme large protein molecules which still stayed in IPG strip. It can also be provoked by the adsorption of U species on the IPG gel matrix and/or insufficient equilibration time as already suggested by others [24]. Based on all experimental data, the 2D gel protocol was repeatable according to ferritin position (pI and MW) allowing us to further identify proteins under U-rich spots.

Six others metals such as Mn, Fe, Co, Ni, Cu and Zn were also imaged in order to investigate the potential interaction of

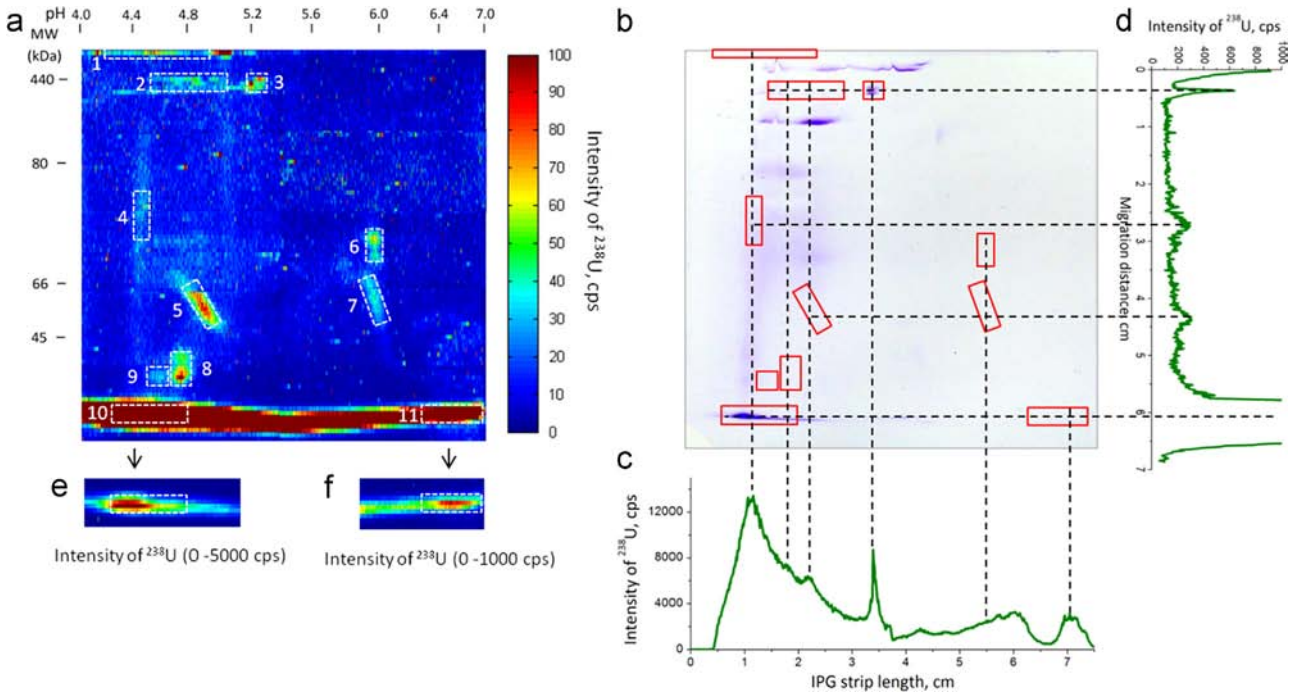


**Fig. 4.** (a) ND-2D-PAGE LA-ICP MS $^{238}$U image, (b) CBB-stained 2D gel, (c) ND-IEF and (d) ND-PAGE LA-ICP MS $^{238}$U electropherograms of HP cytosol from U-exposed crayfish. (e, f) The corresponding parts of spots 10 and 11 in (a) within the $^{238}$U intensity scale of 0–5000 and 0–1000 cps, respectively. The pH 4–7 IPG strips and 8% polyacrylamide gels are used for separation.

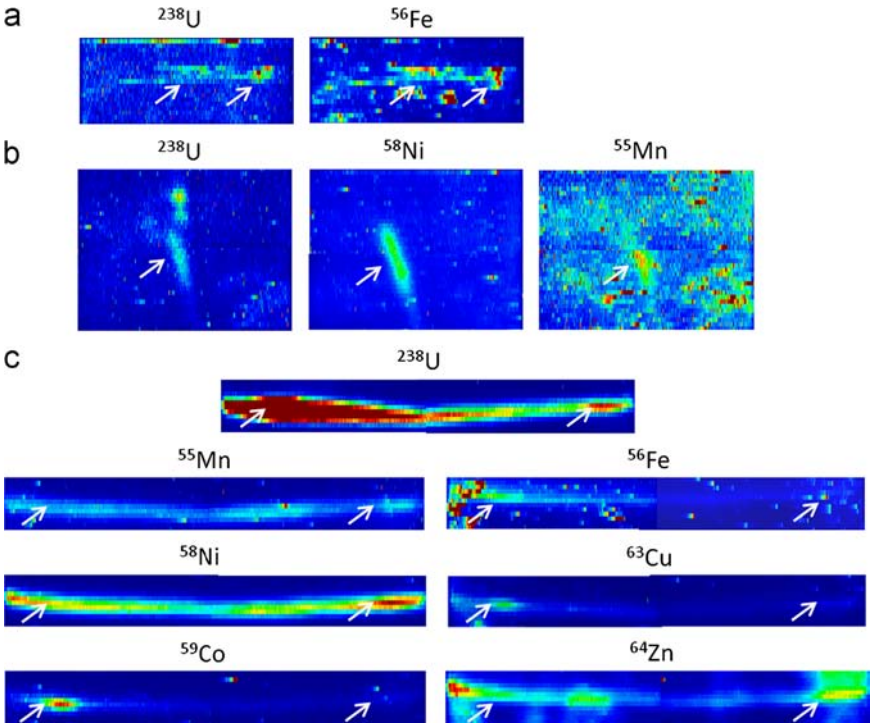


**Fig. 5.** Elemental LA-ICP MS image of spots (a) 2 and 3, (b) 7 and (c) 10 and 11 in 8% polyacrylamide gels of ND-2D-PAGE (Fig. 4) for HP cytosol from U-exposed crayfish. The arrows in (a), (b) and (c) stress the position of protein targets binding metals such as Mn, Fe and Ni.

metalloproteins with U. These metals can be regarded as potential *in vivo* competitors of U when their biocoordination chemistry is similar [25]. Fig. 5 shows obvious relationships of these metals with U in the 2D gel suggesting their coexistence *in vivo*. For instance, Fe was found at the same position as U with very similar patterns in spots 2 and 3 (Fig. 5a). This is not surprising as the *in vitro* interaction between U and Fe-containing proteins (*e.g.* transferrin and ferritin) is demonstrated elsewhere [22,23], and transferrin is believed to be an important protein involved in the U metabolic process [23]. Unexpectedly, Ni, Mn and U were found jointly in spot 7 (Fig. 5b) sharing similar spot shapes. To the best of our knowledge, no experimental studies have been reported to examine the possibility of U interacting with Ni- or Mn-containing proteins. Interaction of U with Fe, Ni and Mn was also supported by the ICP MS detection of these heteroelements co-eluting in size exclusion chromatography (Fig. S3). At the dye

**Table 2**
Protein identification by ND-2D-PAGE and μRPC-ESI-MS/MS in HP cytosol of U-exposed crayfish.

| Spot no. | Accession no. [a] | Score [b] | Sequence coverage (%) | Identified peptides | Protein (*Species*) |
|---|---|---|---|---|---|
| 2 | gi\|328900280 | 97 | 72 | 11 | Ferritin (*Procambarus clarkii*) |
| 3 | gi\|328900280 | 202 | 58 | 11 | Ferritin (*Procambarus clarkii*) |
| 4 | gi\|31338868 | 50 | 12 | 3 | Glyceraldehyde-3-phosphate dehydrogenase (*Procambarus clarkii*) |
| 5 | gi\|328900101 | 30 | 29 | 6 | Triose phosphate isomerase (*Procambarus clarkii*) |
| 8 | gi\|194346532 | 33 | 19 | 5 | Cytosolic manganese superoxide dismutase (*Procambarus clarkii*) |
| 9 | gi\|194346532 | 31 | 15 | 3 | Cytosolic manganese superoxide dismutase (*Procambarus clarkii*) |
| | gi\|31338868 | 144 | 39 | 12 | Glyceraldehyde-3-phosphate dehydrogenase (*Procambarus clarkii*) |
| | gi\|194346532 | 99 | 23 | 7 | Cytosolic manganese superoxide dismutase (*Procambarus clarkii*) |
| 10 | gi\|328900141 | 55 | 33 | 6 | Glutathione S transferase D1 (*Procambarus clarkii*) |
| | gi\|328900101 | 27 | 35 | 7 | Triose phosphate isomerase (*Procambarus clarkii*) |
| | gi\|323387836 | 43 | 25 | 2 | H3 histone family protein (*Procambarus clarkii*) |
| 11 | gi\|328900141 | 32 | 12 | 2 | Glutathione S transferase D1 (*Procambarus clarkii*) |

[a] NCBInr database accession number.
[b] Mascot score.

front positions (*i.e.* spots 10 and 11, Fig. 5c), all these metals could be detected rendering impossible to elucidate the identity of these metallobiomolecules because of insufficient resolution of the 2D gel.

### 3.4. Identification of protein in U spots in vivo by μRPC-ESI-MS/MS

Proteins in U spots (in the square dashed lines in Fig. 4a) were tryptically digested in gel and analyzed by μRPC-ESI-MS/MS for protein identification. Table 2 lists only the proteins belonging to *P. clarkii*, and the left one (*i.e.* pseudohemocyanin-1) from other animals (*i.e.* crayfish, lobsters and shrimps) can be found in Table S2. Totally, 6 reliable protein candidates (*i.e.* ferritin, glyceraldehyde-3-phosphate dehydrogenase, triosephosphate isomerase, cytosolic manganese superoxide dismutase, glutathione S transferase D1 and H3 histone family protein) were picked up. Four of them were also identified in ND-PAGE gels (Fig. 3 and Table S1). Ferritin was identified in spots 2 and 3 with high sequence coverage (*i.e.* 72% and 58%). Considering overall results, ferritin can be considered as a target of U in HP cytosol of U-exposed crayfish even if the U-ferritin complex cannot be detected by molecular mass spectrometry because of the proteolysis denaturing protocol. Ferritin is a globular Fe-containing protein (450 kDa, pI 4–6) with a protein shell composed of 24 subunits, and the central cavity (8.0 nm internal diameter) of its molecule (12.5 nm external diameter) can carry up to 4500 Fe atoms [26–28]. Its shell is notable for its stability to heat (5–10 min, 70 °C) and to urea [28], which can explain its resistance (peak 2 in Fig. 1a and b) to gentle denaturing electrophoretic condition (incubation with denaturing buffer at 56 °C). As a Fe-storage and -cycling protein, ferritin maintains Fe in an available soluble form for use by cells (*e.g.* in oxygen transfer and electron transfer) which can be found in most of vertebrates and invertebrates from human to bacteria [26]. On the other side, ferritin is important for detoxification when excess Fe enters the cells. Except Fe, various metals (*e.g.* Ca, Zn, Cu and Cd) can also be complexed by ferritin [26,28]. The presence of *in vivo* U-ferritin complex was reported in the microorganism *Pyrococcus furiosus* [29]. On the basis of *in vitro* studies, it was proposed that $UO_2^{2+}$ can enter the central cavity region through the hydrophilic channels to find specific binding sites [27]. However, $UO_2^{2+}$ binding sites in ferritin molecules remain unexplored. Spots 2 and 3 in Fig. 4a most possibly belong to different ferritin high molecular weight oligomers [30] or posttranslational modification forms which cannot be detected in ND-PAGE. Their apparent molecular weights (450 kDa) and pI (4.4–5.3) are in accordance with the reported values (440 kDa and 5.0) for crayfish *Pacifastacus leniusculus* ferritin [31]. Our data confirm the *in vivo* existence of U-ferritin complex in HP cytosol of U-exposed crayfish, demonstrating the involvement of ferritin in the detoxification of excess of uranium.

Mn-SOD which is identified in spots 8, 9 and 10 (Fig. 4a) is a member of essential antioxidant enzymes for scavenging excess of reactive oxygen species in organisms. Unlike other animals, crustaceans (*i.e.* shrimps, lobsters and crabs) lack cytosolic Cu/Zn-SOD, but have a cytosolic Mn-SOD instead [32,33]. The theoretical molecular weight and pI of identified cytosolic Mn-SOD are respectively 31 kDa and 5.4 which are similar to measured ones (*i.e.* < 45 kDa and 4.4–4.8). Its analog in crab *Callinectes sapidus* was reported to be a dimeric protein and exists in a monomer–dimer equilibrium [32]. The distribution of cytosolic Mn-SOD in three spots can either be explained by a similar equilibrium in crayfish cytosol or by its posttranslational modification. So far, even if there is no report on the binding affinity of Mn-SOD and U, our work shows potential interactions between U and Mn-containing proteins (Fig. 5b and c) in spots 7 and 10 as hypothesized elsewhere [15] while only Mn-SOD is identified in spot 10. The absence of Mn in spots 8 and 9 may probably be explained by the too low intensity (*e.g.* 300 cps for spot 7) and the high background (*ca.* 150 cps). Among the remaining 4 proteins (Table 2), only glyceraldehyde-3-phosphate dehydrogenase was reported to have a strong and specific chemical affinity to $UO_2^{2+}$ and could be captured by $UO_2$-IMAC column from human kidney extract [9]. Ours results indicate that the *in vivo* U-binding glyceraldehyde-3-phosphate dehydrogenase complex can also be formed in crayfish HP cytosol after exposure to U excess. In addition, the identification of the phosphorylated protein (*e.g.* triose phosphate isomerase) as U target is consistent with the strong chemical affinity of U and phosphate group [34]. Finally the reliability of our methodology is supported by the findings of others groups for two of these proteins binding with U [9,29]. For proteins which have not been already reported by others to bind U, further *in vitro* studies would be necessary to validate their binding affinity with U, especially for proteins with a low sequence coverage or such as H3 histone family protein.

## 4. Conclusions

Non-denaturing 2D gel electrophoresis allowed the separation of *in vivo* U-protein complexes in HP cytosol of U-exposed crayfish at low dose. On the basis of the elemental analysis by LA-ICP MS and protein identification by μRPC-ESI-MS/MS, several U-protein targets (especially ferritin) could be sensitively detected and identified for the first time. Compared to the previously developed ND-IEF, the ND-2D electrophoresis protocol provided obviously a gain in terms of resolution and specificity but to the detriment of U

recovery (*ca.* 35% in ND-2D *versus* 65% in ND-IEF). On one hand, non-denaturing conditions of 2D electrophoresis limit the separation and analysis of some metalloproteins (neutral/positively charged proteins or of very high molecular weight) which cannot enter the 2D gels or focus well as a spot. On the other hand, the improved resolution led to an increase of the level of confidence for protein identification with higher sequence coverages (12–72%) determined only in crayfish species (*versus* 1–11% considering crayfish, lobster and crab species). Overall, this methodology is promising and it can be applied to other organs to understand U trafficking and detoxification mechanisms in U-exposed crayfish.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.04.065.

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