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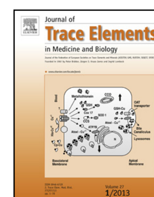

Analytical methodology

ICPMS analysis of proteins separated by Native-PAGE: Evaluation of metaloprotein profiles in human synovial fluid with acute and chronic arthritis

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ABSTRACT

The role of trace elements bound to proteins in the etiology and pathogenesis of rheumatoid arthritis (RA) remains unclear. In this sense, the identification and detection of metalloproteins has a strong and growing interest. Metalloprotein studies are currently carried out by polyacrylamide gel electrophoresis (PAGE) associated to inductively coupled plasma mass spectrometry (ICPMS), and despite that complete information can be obtained for metals such as Fe, Cu and Zn, difficulties due to poor sensitivity for other trace elements such as Sn, As, etc, are currently faced. In the present work, a simple and fast method for the determination of trace metals bound to synovial fluid (SF) proteins was optimized. Proteins from SF (long and short-term RA) were separated in ten fractions by native PAGE, then dissolved in nitric acid and peroxide hydrogen, and analyzed by ICPMS. Fifteen metals were determined in each separated protein fraction (band). Adequate calibration of proteins molecular weight allowed stablishing which protein type were bound to different metals.

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1. Introduction

Trace element determination in biological samples is a routine protocol in specialized laboratories for clinical diagnosis and toxicology of chemical elements [1–4]. Some trace elements are cofactors of enzymes in the organism and despite they are found at trace levels in the body, abnormal levels of them could cause serious diseases [5–7]. Approximately 30% of proteins present in a biological system contain metal or metalloid ions in their structures, and about 40% of these elements are crucial to maintain biological functions of proteins. These facts support the increasing interest in investigations concerning metal and metalloid ions bound to proteins in biochemical, environmental, pharmacological, and medical sciences [8].

Human synovial fluid (HSF) is considered a dialysate of plasma to which mucin has been added during its passage through the synovial membrane [9]. A number of studies have investigated the role of trace elements in the etiology and pathogenesis of rheumatoid arthritis (RA) [10–14]. As can be seen in the literature, the alterations in the concentrations of trace elements in the HSF of patients with RA are somehow inconsistent [6]. In a previous work, we evaluate the metallic content (as elemental profile) in HSF after a single-step sample preparation procedure with formic acid [10]. However, it is still unknown the relationship between the elemental profile and the protein content in articular diseases.

RA is a systemic inflammatory process characterized by synovitis of peripheral joints and subsequent joint destruction. Assessment of disease activity is based on the count of tender and swollen joints, the measurement of erythrocyte sedimentation rate (ESR) or serum levels of acute phase reactants such as C-reactive protein (CRP), and the pain score of patients [15]. Investigations on trace elements and disease activity score in patients with RA, showed that trace elements can be linked to the severity of the disease [16].

One analytical approach used to evaluate metalloproteins consists in isolating proteins from the sample of interest, and then analyzing by atomic spectrometry for the detection of metals or metalloids bound to them [8]. In this sense, high-performance

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Table 1
Instrument settings and data acquisition parameters for ICP-MS.

Instrument	Elan DRC-e (Perkin-Elmer SCIEX. Thornhill. Canada)
Sample uptake rate (μL min^{-1})	400
Sample introduction	Nebulizer model PFA-ST.
RF power (W)	1100
Gas flow rates (mL min^{-1})	Nebulizer. 0.87
Interface	Ni cones (sampler and skimmer)
Standard mode	^{27}Al,^{47}Ti, ^{51}V, ^{53}Cr, ^{54}Fe, ^{55}Mn, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{66}Zn, ^{75}As ^{82}Se, ^{88}Sr,^{111}Cd, ^{118}Sn, ^{138}Ba.
Scanning mode	Peak hopping
Dwell time (ms)	50 in standard mode
Number of replicate	3
Mode Detector	Dual

liquid chromatography (HPLC), capillary electrophoresis (CE) and polyacrylamide gel electrophoresis (PAGE)-based techniques (native or denaturing), have been proposed combined with elemental analysis methods [8,17–19]. Inductively coupled plasma-mass spectrometry (ICPMS) has some well known advantages, that make it suitable for biomonitoring studies [3,20–23]. In association with laser ablation (LA-ICPMS), several applications have been described for the analysis of metallic species bound to proteins that were separated with PAGE [24]. More recently, on-line coupling between PAGE and ICPMS was described for quantitative evaluation of intracellular metal binding properties of metallochaperones HpHypA and HpHspA in *Escherichia coli* cells [25].

The principle and theory of PAGE, the know-how for reproducibly producing a smooth linear gradient, and application in native PAGE (nPAGE) were used to these aims. In particular, pore gradient gel electrophoresis (PGGE) is one of the most reliable methods for estimating the relative molecular weight (Mr) of native multimeric proteins by electrophoresis [26]. Nevertheless, issues regarding calibration and sensitivity have been documented in those approaches [24].

The aim of this work was to develop a screening methodology for metal-containing proteins in HSF using nPAGE and ICPMS. The effects of the different steps in the analysis (sample preparation, separation, post-separation treatments) were evaluated by ICPMS. In the crucial steps, we focused on both issues-achieving for better extraction, and characterization of proteins, while maintaining the integrity of metal-protein binding. This method was applied for the analysis of metal distribution through the protein pattern obtained by nPAGE of human synovial fluid from RA patients.

2. Materials and methods

2.1. Instrumentation

An inductively coupled plasma mass spectrometer, Perkin Elmer SCIEX, ELAN DRC-e (Thornhill, Canada) was used. The argon gas with purity of 99.996%, was supplied by Air Liquide S.A. (Córdoba, Argentina). An HF-resistant and high performance perfluoracetate nebulizer model PFA-ST, was used. Before changing to the micro-concentric nebulizer, a performance check for sensitivity, oxide and doubly charged ion formation, using a conventional PTFE cross flow nebulizer and a Scott-type spray chamber was carried out. The ICPMS operative conditions are listed in Table 1.

A water-cooled gel electrophoresis system model Wide Dual Mini-Vertical DCX-800C, from CBS-Scientific, Company Inc. (USA) was used for protein separations.

2.2. Reagents

Ultrapure water with a resistivity of 18.2 MΩ cm produced by an Easy pure RF system from Barnstead (Dubuque, IA, USA) was used for all preparations. A PTFE sub-boiling acid distiller (Distillacid, Berghof Products + Instruments GmbH, Germany) was used to obtain double-distilled nitric and hydrochloric acids.

Hydrogen peroxide 30% Merck (Germany), tris(hydroxymethyl)aminomethane (Tris) (99.85%, Across Organics, New Jersey, USA), ammonium persulfate, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), native sample buffer: 62.5×10^{-3} mol L^{-1} Tris-HCl, pH 6.8; 40% glycerol; 0.01% bromophenol blue, bis-acrylamide 2%, and acrylamide (Bio-Rad, USA), were used. Multi-element calibration standard solution N° 1 and N° 5, Hg standard solution, and setup/mascal solutions from Perkin Elmer Pure Plus, Atomic Spectroscopy Standard (Norwalk, USA), were used for instrument and method calibrations.

Total proteins and albumin quantifications were carried out with a Beckman spectrophotometer DU 520. The Biuret reagent ($CuSO_4$ 1×10^{-3} mol L^{-1}, sodium-potassium tartrate 32×10^{-3} mol L^{-1}, and sodium hydroxide 250×10^{-3} mol L^{-1}) was employed for total protein determination and was obtained from GT Lab (Rosario, Argentina). Bovine serum albumin (BSA) powder, lyophilized, defatted and deionized, purity grade >99% was provided by Fedesa-UNSL S.A. (San Luis, Argentina). For molecular weight determination, a standard curve was draw with the relative mobility of a High Molecular Weight Calibration Kit for native electrophoresis, GE Healthcare UK Limited, (Buckinghamshire, UK).

For the external calibration against aqueous standards, the solutions were prepared in 1%v/v nitric acid. The concentrations of the elements were 5.0, 10.0, 20.0, 40.0 and 80.0 μg L^{-1}. As internal standard, 20 μg L^{-1} Rh were added to all solutions including the samples.

2.3. Samples

The analyzed samples were 21HSF taken from patients with acute RA symptoms (n = 6) and patients with RA with treatments lasting between 2 and 10 years (n = 15). All samples used in this investigation were aspirated aseptically with a stainless steel needle from the joint of patients attending the orthopedic and physical medicine clinics of the hospital. The samples were then placed onto 15-mL polypropylene metal-free centrifuge tubes. The particulate material and cellular content of all fluids were removed by centrifugation at 3000*g* for 15 min. The supernatants were collected and maintained at −80 °C to prevent denaturing. The collected samples were available after the relevant consents according to Moyano et al. [10].

2.4. Total protein and albumin quantification

Total protein and albumin quantifications in HSF were determined using Biuret spectrophotometric method [27]. A solution of 1.0 g L^{-1} of BSA in ultrapure water was prepared as stock standard. Standards for a calibration curve ranged between 1–100 μg L^{-1} were prepared and were reacted with biuret regent and incubated for 15 min at 37 °C. Then standards were read at 540 and 625 nm. HSF samples were analyzed under described the same experimental conditions.

2.5. Native-PAGE

The native PAGE gradient gels were made in glass plates (10 × 15 cm) filled with two solutions of acrylamide/bis-acrylamide (20 and 5.25% T respectively), that were delivered with peristaltic pumps to generate a gradient from 18.70 to 6.25% of acrylamide

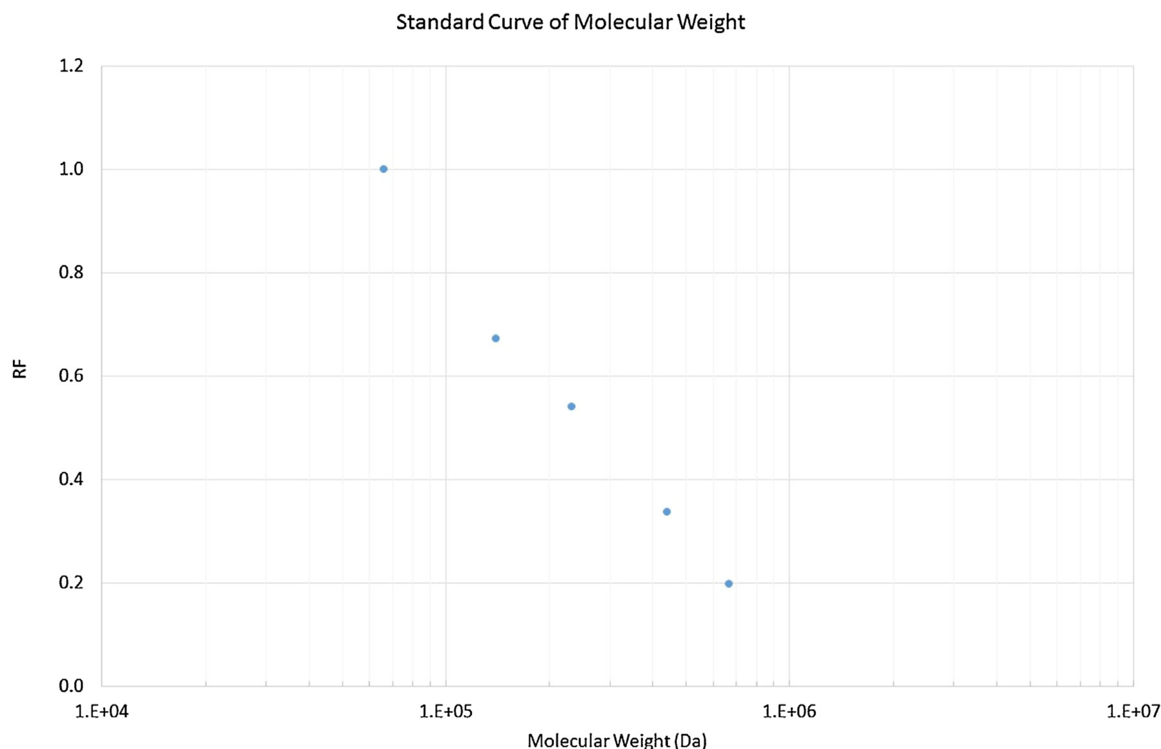

Fig. 1. Correlation for Molecular Weight vs. Relative Mobility.

Table 2
Molecular weight of protein standards used for estimation of molecular weight of proteins of HSF.

Standard	Molecular weight (Daltons)	Log MW	Rf
Bovine Serum Albumin	66,000	4.82	1.00
Bovine Heart Lactate dehydrogenase	140,000	5.15	0.67
Bovine Liver Catalase	232,000	5.36	0.54
Equine Spleen Ferritin	440,000	5.64	0.34
Porcine Thyroid Thyroglobulin	669,000	5.82	0.20

bis-acrylamide. Tris-HCl (1.5 mol L^{-1}, pH 8.8) and Tris-glycine (0.025 mol L^{-1}, pH 8.3) were used as gel and running electrode buffers respectively. Parameters optimized during the separation procedure were applied voltage (45–65 V per gel), dilution of samples (1:2, 1:4, 1:8, 1:16 and 1:32 in sample buffer Bio-Rad), and sample volume loaded per lane (5–20 μL). Optimal conditions were: constant voltage 60 V per gel, sample dilution 1:16, for 10 μL sample volume.

2.5.1. Gel staining

After gel electrophoresis separation, the proteins can be visualized and located in the gel by using a staining procedure [28–31]. Gels were stained with Coomassie Brillant Blue R-250 (in 40% methanol and 10% acetic acid) solution during 1 h. After that, the gel distained was performed with a mixture of methanol:acetic acid:water (40:10:50) to remove background and scanned at 600 dpi resolution in TIFF image format. Gel images were processed with Adobe Photoshop CS3 (version 10.0) to obtain the best contrast for densitometry analysis through software. Best photo scans were taken and their relative optical densitometry (ROD) and percentage amount of protein fraction in HSF (%PF) was done using Image J (version 1.45) software program. The molecular weights of the protein fractions in HSF (Fig. 1) was determined through comparison of its electrophoretic relative mobility (Rf) with that proteins contained in the molecular weight marker (Table 2). The results were expressed as mean ± standard deviation for all data sets. Differences between short-term and long-term groups were evaluated using Student's t-test. A probability of less than 0.05 was assumed to be significant, as can be seen in Table 3.

In this work, the staining solution was prepared without phosphoric acid since it was demonstrated that metal releasing from proteins is prevented [30].

2.5.2. Dissolution of protein fractions and ICP-MS analysis

As an alternative to the decomposition process, a novel method for the dissolution of gel bands (protein fractions) was optimized. The protein fractions (10 bands at the same Rf, and in replicate runs for each protein) and a portion of gel without fractions (used as blank) were manually excised with a glass cover-slip and then placed into 15 mL plastic tubes.

The observation of the obtained solutions enabled qualitative conclusions about the preferred conditions which were: 800 μL of concentrated nitric acid and 800 μL of hydrogen peroxide were added. The tubes were placed in a water bath heated to 60 °C until complete dissolution (2 h), obtaining transparent sample solutions. After that, solutions were brought to a final volume of 8 mL with ultrapure water and stored at 4 °C until analysis.

A variety of trace elements are found in bone including Cu, Zn, Mn and Sr. The participation of trace elements, especially Cu, Mn and Zn, in the normal development and maintenance of the skeleton is, at least in part, related to their catalytic functions in organic bone matrix synthesis or in the functioning of cells of bone or cartilage [32–34]. Sn is contained in osseous tissue, as it has high affinity for bone, and is a strong chelating agent with various proteins. Although there are important alterations in plasma and synovial fluid concentrations of Se and Cu in RA, these alterations were not observed in OA.

The simultaneous determination of Al, As, Ba, Cd, Co, Cr, Cu, Mn, Ni, Pb, Sn, Sr, Ti, V and Zn in HSF was considered both to establish a mark of them in RA, and to monitor their concentration associated to protein content.

Table 3
Electrophoretic study of HSF.

		Protein Fraction										
		1	2	3	4	5	6	7	8	9	10	Alb/Glob ratio
Short-term Rheumatoid Arthritis	ROD	1.00	0.27	0.06	0.05	0.32	0.28	0.18	0.19	0.31	1.03	**0.38**
	%PF	27.51 ± 1.10 [a]	7.15 ± 1.09	1.53 ± 0.16	1.17 ± 0.26	8.54 ± 0.54	7.54 ± 0.17	4.81 ± 0.23	5.21 ± 0.21	8.41 ± 0.08	28.13 ± 0.75	
Long-term Rheumatoid Arthritis	ROD	1.00	0.20	0.03	0.11	0.13	0.08	0.07	0.09	0.04	–	**1.34**
	%PF	57.33 ± 0.92	11.6 ± 0.63	1.46 ± 0.11	6.36 ± 1.01	7.31 ± 0.69	4.65 ± 0.26	3.85 ± 0.20	5.26 ± 0.71	2.18 ± 0.30	–	

ROD: Relative Optical Density.
%PF: Percentage amount of Protein Fraction in HSF based on a total protein concentration of 65,83 mg/mL and 80,83 mg/mL for long and short-term RA, respectively.

Table 4a
Estimated molecular weight of protein fractions of HSF of patients with short-term rheumatoid arthritis.

Fractions	Molecular weight (Daltons)	Rf
1	66,000	1.00
2	98,392	0.83
3	124,283	0.76
4	176,640	0.64
5	296,257	0.46
6	392,473	0.36
7	496,878	0.28
8	598,439	0.22
9	661,241	0.19
10 (end)	707,799	0.16
10 (beginning)	1,074,360	0.02

Table 4b
Estimated molecular weight of protein fractions of HSF of patients with long-term rheumatoid arthritis.

Fractions	Molecular weight (Daltons)	Rf
1	66,000	1.00
2	168,424	0.65
3	221,610	0.56
4	303,054	0.45
5	421,061	0.34
6	547,778	0.25
7	661,241	0.19
8	724,035	0.16
9 (end)	829,583	0.11
9 (beginning)	1,089,080	0.02

Table 5
Metal content of proteins from HSF of **a.** long-term rheumatoid arthritis **b.** acute rheumatoid arthritis.

a

	Protein Fraction			
Analyte	1	2	4	5
Al ($mg\,g^{-1}$)			0.38	0.81
Mn ($mg\,g^{-1}$)		0.84		
Co ($\mu g\,g^{-1}$)		2.35		35.17
Ni ($mg\,g^{-1}$)		0.67		
Cu ($mg\,g^{-1}$)			4.04	0.83
Zn ($mg\,g^{-1}$)		4.74		
Sr ($mg\,g^{-1}$)		0.93		
Cd ($\mu g\,g^{-1}$)	3.29	0.34	0.12	
Ba ($mg\,g^{-1}$)			0.1	
As ($\mu g\,g^{-1}$)		1.08		
Sn ($mg\,g^{-1}$)	0.72		0.88	

b

	Protein Faction					
Analyte	1	2	5	6	9	10
Al ($mg\,g^{-1}$)						2.44
Ni ($mg\,g^{-1}$)			0.14			
Cu ($mg\,g^{-1}$)	3.36	2.79	0.77	0.89		
Se ($\mu g\,g^{-1}$)					0.43	
V ($\mu g\,g^{-1}$)						81.12
Cr ($\mu g\,g^{-1}$)						55.22
Sn ($mg\,g^{-1}$)	1.55					0.24
Ti ($mg\,g^{-1}$)						0.22

3. Results

3.1. Electrophoretic separation of HSF proteins and characterization

The results of the electrophoretic study of HSF (Fig. 2) showed that protein fractions have a wide molecular weight range, from 66KD to 1089KD (Table 4a Table 4b). A better correlation was found between the distribution of the protein fractions and the duration of the effusion. The protein patterns and profiles of fluids of short-term and long-term were different each other. As can be seen in Table 4, are listed de values of molecular weight of each fraction both in HSF short-term rheumatoid arthritis and HSF long-term rheumatoid arthritis. This procedure showed that short-term HSF presents a pattern of fractions with 10 bands while long-term HSF exhibited 9 bands (Fig. 3).

In addition, the bands numbered as 2, 4, 5 and 6 of short-term sample were not in the pattern of the long-term sample. Similarly, the multiple bands included in fraction 10 of short-term sample were not present in the long-term sample pattern, where only the fractions designated as numbers 8 and 9 were present. The proteins profile showed significant differences in the γ-globulin zone (band 10).

A significant increase ($p < 0.0001$) of band 1 (albumin) %PF was observed from short-term to long-term samples. Similarly, albumin/globulin ratios exhibited a significant increase ($p < 0.0001$) from the short-term to long-term samples (Table 3) as expected for the acute infectious process. The causes of these changes in RA are the result of dietary deficiency or immune imbalance, this could be a result of the inflammatory condition, which is regulated by pro-inflammatory and immunoregulatory cytokines.

The results showed a greater relative abundance of proteins profile related to activation, inflammation and the immune response. Since neutrophils are the predominant cell type in HSF with RA, most proteins are neutrophil-related (i.e. azurocidin, defensin 2, neutrophil gelatinase associated lipocalin or the leukocyte elastase inhibitor) [35–37].

3.2. Elements associated to proteins

The analysis of dissolved bands (#1, 2, 5, 6, 9 and 10; bands of short-term RA) showed that the essential trace elements Cu and Se were associated to proteins in each band. Low levels of Se and elevated levels of Cu were observed in short-term RA patients. Acute-phase reactants, one of them ceruloplasmin, are released during inflammation and the ceruloplasmin release is associated with the increased plasma Cu level.

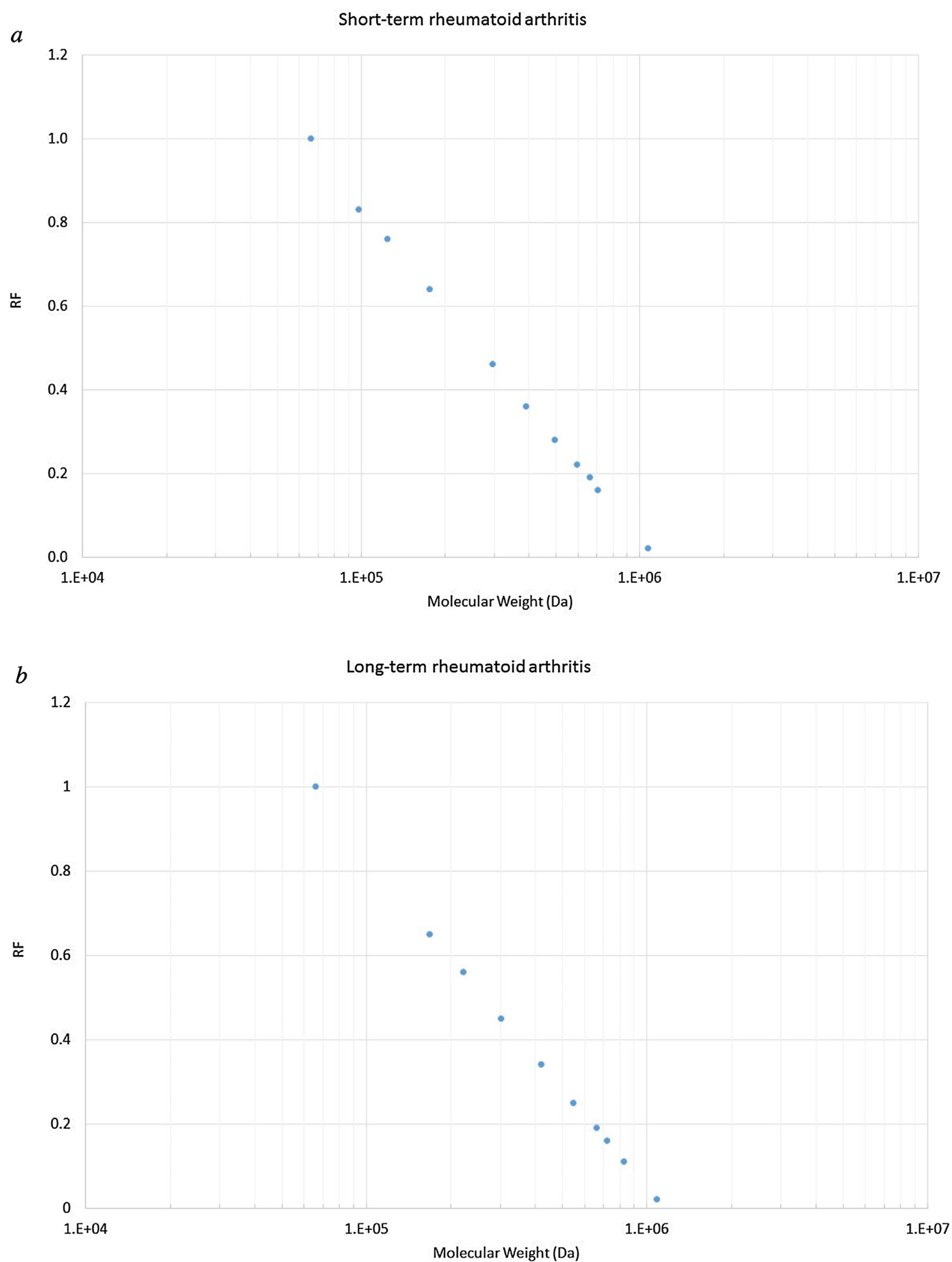

Fig. 2. Correlation for Molecular Weight vs. Relative Mobility for Short-term Rheumatoid Arthritis and Long-term Rheumatoid Arthritis.

The found level of Sn in all RA patients was significantly elevated (Fig. 3). The bands designated with numbers 1 and 4 (long-term sample) or 1 and 10 (short-term sample) showed high Sn levels. This element is contained in osseous tissue, has a high affinity toward bone with its strong chelating properties due to various proteins on it. A Sn (IV) chelate can quickly localize the bone surface and bind to hydroxyapatite, with a high uptake at the site of abnormal bone remodeling. Abnormal bone remodeling at joints because of RA will involve the elevated level of Sn^{4+} released from the bone matrix, which may trigger the overproduction of reactive oxygen species. In addition, the results showed no significant alterations of levels of Zn (band 2 of long-term sample) in RA patients.

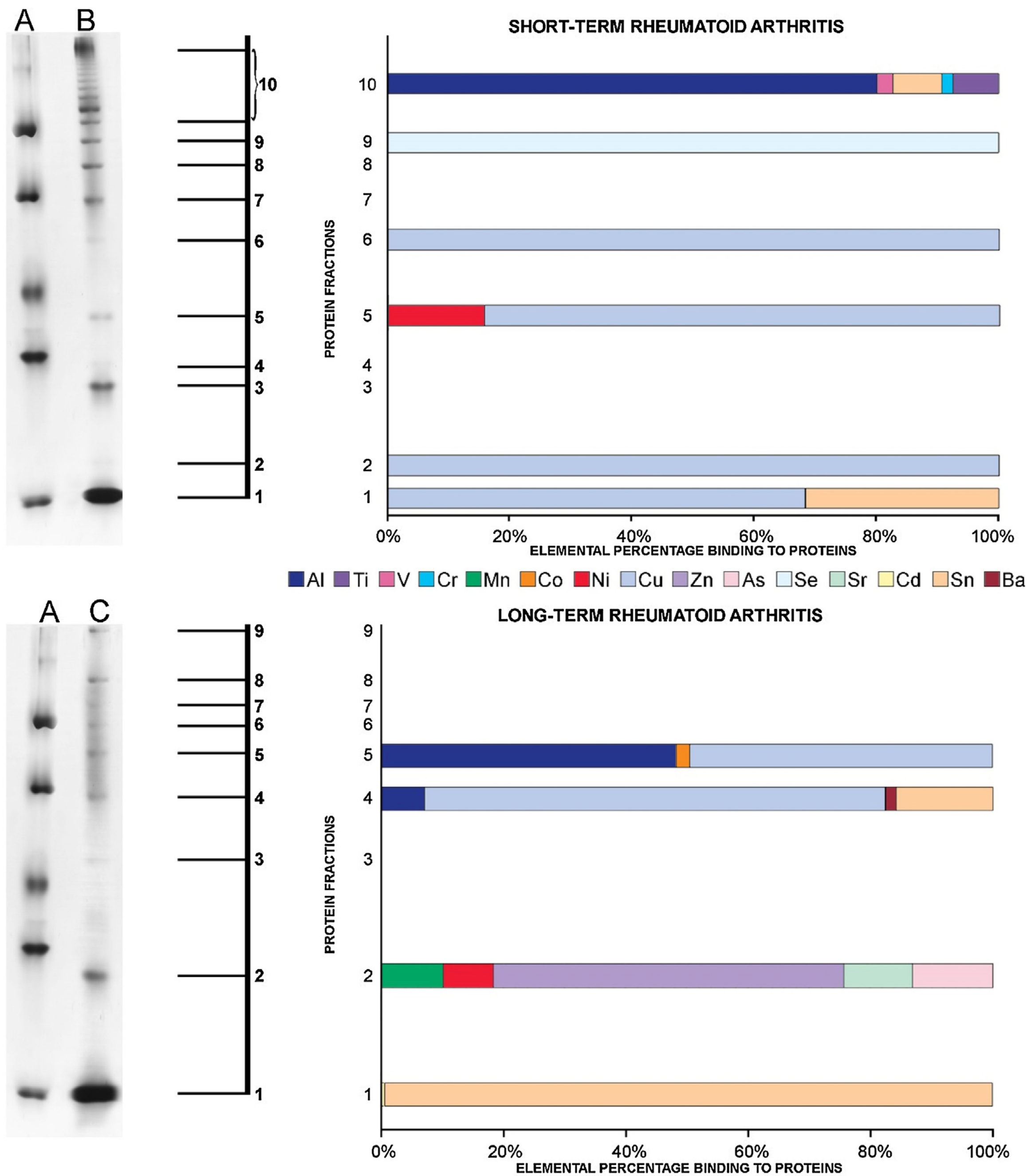

Fig. 3. Elemental profiles determined by ICP-MS in proteins (bands) of HSF with arthritis, separated by Native-PAGE.

3.3. Analytical performance and validation

Under the optimum conditions described above, the performance data of the system for elemental profile determination were established. Table 5 compares the protein patterns of fluids of short duration and long duration, with its corresponding element profile determined by ICP-MS.

The repeatability of the method was evaluated with 2 mL of blank sample solution (polyacrylamide gel) repeating this procedure 10 times. The relative standard deviation (R.S.D.) was calculated (from 1.3% for Se to 9.7% for Zn). The calibration graph using this method for elementary profile was linear with a correlation coefficient of 9.999 in all cases.

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated as the amount of trace element required to yield a net peak equal to three times the standard deviation of the background signal (3σ and 10σ criteria) (Table 6). Validation of ICPMS analysis took place through spike-recovery tests on albumin bands (ten pooled spots) obtained from a standard of BSA (section 2). The samples were treated as the recommended procedure and small amounts of standard multielemental solutions were added to three aliquots in ways to obtain low, medium and high level spikes, plus an aliquot without addition of standard. In all cases, the recoveries were quantitative from 87% for ^{27}Al to 115% for ^{47}Ti.

4. Discusion and conclusions

In this work a metalloprotein study was undertaken by polyacrylamide gel electrophoresis associated to ICPMS, with adequate

Table 6
Analytical figures of merit of the analytical method.

Analyte	Mass	R.S.D	LOD (mg g^{-1})	LOQ (mg g^{-1})
Al	7	4.2	0.03	0.10
Ti	47	1.5	0.05	0.16
V	51	4.8	0.0008	0.0025
Cr	53	3.5	0.001	0.039
Mn	55	9.0	0.02	0.07
Co	59	8.9	0.01	0.05
Ni	60	8.5	0.02	0.06
Cu	63	6.0	0.001	0.005
Zn	66	9.7	0.015	0.052
As	75	9.4	0.001	0.004
Se	82	1.3	0.24	0.78
Sr	88	6.0	0.006	0.020
Cd	111	8.7	0.0005	0.0017
Sn	118	7.5	7.45	25.73
Ba	138	9.6	0.03	0.11

Values expressed as mass of metal per mass of total protein in HSF.

sensitivity and accuracy. The study focused on the determination of trace metals bound to proteins found in synovial fluids of persons with either long or short-term RA.

The separation of differentially expressed proteins contributes to the understanding of molecular factors of the disease better and paves the way for new markers. It was demonstrated that a common element expression profile can be identified in human patients with RA in practise. Correlation between metal occurrence and protein type (s) (in terms of its molecular weight) could be laid out. Differences were observed in the obtained profiles, demonstrating that a field study could be carried out with this method with ease. The elemental profile may be performed to test whether its concentration may serve not only as markers of rheumatoid arthritis and its remedies, but also as predictors of adverse outcomes.

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References

[1] A.A. Ambushe, R.I. McCrindle, C.M.E. McCrindle, Speciation of chromium in cow's milk by solid-phase extraction/dynamic reaction cell inductively coupled plasma mass spectrometry (DRC-ICP-MS), J. Anal. At. Spectrom. 24 (2009) 502–507.
[2] J.B.B. Da Silva, D.L.G. Borges, M.A.M.S. Da Veiga, A.J. Curtius, B. Welz, Determination of cadmium in biological samples solubilized with tetramethylammonium hydroxide by electrothermal atomic absorption spectrometry, using ruthenium as permanent modifier, Talanta 60 (2003) 977–982.
[3] L. Tormen, R.A. Gil, V.L.A. Frescura, L.D. Martinez, A.J. Curtius, Determination of trace elements in biological samples treated with formic acid by inductively coupled plasma mass spectrometry using a microconcentric nebulizer, Spectrochim. Acta B 65 (2010) 959–966.
[4] L. Tormen, R.A. Gil, V.L.A. Frescura, L.D. Martinez, A.J. Curtius, The use of electrothermal vaporizer coupled to the inductively coupled plasma mass spectrometry for the determination of arsenic, selenium and transition metals in biological samples treated with formic acid, Anal. Chim. Acta 717 (2011) 21–27.
[5] M.L. Mena, E. Moreno-Gordaliza, M.M. Gómez-Gómez, TCEP-based rSDS-pAGE and nLC-eSI-LTQ-mS/MS for oxaliplatin metalloproteomic analysis, Talanta 116 (2013) 581–592.
[6] M. Yazar, S. Sarban, A. Kocyigit, U.E. Isikan, Synovial fluid and plasma selenium, copper, zinc, and iron concentrations in patients with rheumatoid arthritis and osteoarthritis, Biol. Trace Elemen. Res. 106 (2005) 123–132.
[7] M. Su, T. Zhang, T. Zhao, F. Li, Y. Ni, X. Wang, et al., Human gouty arthritis is associated with a distinct serum trace elemental profile, Metallomics 4 (2012) 244–252.
[8] A. Sussulini, J.S. Becker, Combination of PAGE and LA-ICP-MS as an analytical workflow in metallomics: state of the art, new quantification strategies, advantages and limitations, Metallomics 3 (2011) 1271–1279.
[9] E. Pascual, V. Jovaní, Synovial fluid analysis, Best Pract. Res.: Clin. Rheumatol. 19 (2005) 371–386.
[10] F. Moyano, E. Verni, H. Tamashiro, S. Digenaro, L.D. Martinez, R.A. Gil, Single-step procedure for trace element determination in synovial fluid by dynamic reaction cell-inductively coupled plasma mass spectrometry, Microchem. J. 112 (2014) 17–24.
[11] G.M.M. Hashmi, M.H. Shah, Comparative evaluation of trace elements in the scalp hair of arthritis patients and healthy donors, Toxicol. Environ. Chem. 93 (2011) 2123–2134.
[12] G.M.M. Hashmi, M.H. Shah, Comparative assessment of essential and toxic metals in the blood of rheumatoid arthritis patients and healthy subjects, Biol. Trace Elem. Res. 146 (2012) 13–22.
[13] J. Aaseth, E. Munthe, Ø. Førre, E. Steinnes, Trace elements in serum and urine of patients with rheumatoid arthritis, Scand. J. Rheumatol. 7 (1978) 237–240.
[14] U. Tarp, H. Graudal, K. Overvad, E.B. Thorling, J.C. Hansen, Selenium in rheumatoid arthritis: a historical prospective approach, J. Trace Elem. Electrolytes Health Dis. 3 (1989) 93–95.
[15] K.W. Kim, B.M. Kim, H.W. Moon, S.H. Lee, H.R. Kim, Role of C-reactive protein in osteoclastogenesis in rheumatoid arthritis, Arthritis Res. Ther. 17 (2015).
[16] S. Taysi, I. Gulcin, R.A. Sari, S. Kuskay, N. Bakan, Trace elements and disease activity score in patients with rheumatoid arthritis, Pain Clin. 15 (2003) 435–439.
[17] H. Yamagiwa, G. Sarkar, M.C. Charlesworth, D.J. McCormick, M.E. Bolander, Two-dimensional gel electrophoresis of synovial fluid: method for detecting candidate protein markers for osteoarthritis, J. Orthop. Sci. 8 (2003) 482–490.
[18] M.A. Smith, S.K. Bains, J.C. Betts, E.H.S. Choy, E.D. Zanders, Use of two-dimensional gel electrophoresis to measure changes in synovial fluid proteins from patients with rheumatoid arthritis treated with antibody to CD4, Clin. Diagn. Lab. Immunol. 8 (2001) 105–111.
[19] S. Saito, M. Kawashima, H. Ohshima, K. Enomoto, M. Sato, H. Yoshimura, et al., Separation of metalloproteins using a novel metal ion contaminant sweeping technique and detection of protein-bound copper by a metal ion probe in polyacrylamide gel electrophoresis: distribution of copper in human serum, Analyst 138 (2013) 6097–6105.
[20] T. Zhao, T. Chen, Y. Qiu, X. Zou, X. Li, M. Su, et al., Trace element profiling using inductively coupled plasma mass spectrometry and its application in an osteoarthritis study, Anal. Chem. 81 (2009) 3683–3692.
[21] B.L. Batista, D. Grotto, J.L. Rodrigues, V.C. De Oliveira Souza, F. Barbosa Jr, Determination of trace elements in biological samples by inductively coupled plasma mass spectrometry with tetramethylammonium hydroxide solubilization at room temperature, Anal. Chim. Acta 646 (2009) 23–29.
[22] P.J. Parsons, F. Barbosa Jr., Atomic spectrometry and trends in clinical laboratory medicine, Spectrochim. Acta B 62 (2007) 992–1003.
[23] B.L. Batista, J.L. Rodrigues, J.A. Nunes, V.C. De Oliveira Souza, F. Barbosa Jr, Exploiting dynamic reaction cell inductively coupled plasma mass spectrometry (DRC-ICP-MS) for sequential determination of trace elements in blood using a dilute-and-shoot procedure, Anal. Chim. Acta 639 (2009) 13–18.
[24] D. Pozebon, G.L. Scheffler, V.L. Dressler, M.A.G. Nunes, Review of the applications of laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) to the analysis of biological samples, J. Anal. At. Spectrom. 29 (2014) 2204–2228.
[25] Y. Wang, L. Hu, X. Yang, Y.-Y. Chang, X. Hu, H. Li, et al., On-line coupling of continuous-flow gel electrophoresis with inductively coupled plasma-mass spectrometry to quantitatively evaluate intracellular metal binding properties of metallochaperones HpHypA and HpHspA in *E. coli* cells, Metallomics 7 (2015) 1399–1406.
[26] T. Yamaoka, Pore gradient gel electrophoresis: theory, practice, and applications, Anal. Chim. Acta 372 (1998) 91–98.
[27] C.V. Sapan, R.L. Lundblad, N.C. Price, Colorimetric protein assay techniques, Biotechnol. Appl. Biochem. 29 (1999) 99–108.
[28] M.S. Jiménez, L. Rodriguez, M.T. Gomez, J.R. Castillo, Metal-protein binding losses in proteomic studies by PAGE-lA-ICP-MS, Talanta 81 (2010) 241–247.
[29] M.S. Jiménez, L. Rodriguez, J.R. Bertolin, M.T. Gomez, J.R. Castillo, Evaluation of gel electrophoresis techniques and laser ablation-inductively coupled plasma-mass spectrometry for screening analysis of Zn and Cu-binding proteins in plankton, Anal. Bioanal. Chem. 405 (2013) 359–368.
[30] A. Raab, B. Pioselli, C. Munro, J. Thomas-Oates, J. Feldmann, Evaluation of gel electrophoresis conditions for the separation of metal-tagged proteins with subsequent laser ablation ICP-MS detection, Electrophoresis 30 (2009) 303–314.
[31] A. Hagège, T.N.S. Huynh, M. Hébrant, Separative techniques for metalloproteomics require balance between separation and perturbation, TrAC—Trends Anal. Chem. 64 (2015) 64–74.
[32] H.I. Afridi, T.G. Kazi, D. Brabazon, S. Naher, Association between essential trace and toxic elements in scalp hair samples of smokers rheumatoid arthritis subjects, Sci. Total Environ. 412–413 (2011) 93–100.
[33] Z. Zhang, J. Zhang, J. Xiao, Selenoproteins and selenium status in bone physiology and pathology, Biochimica et Biophysica Acta—Gen. Subj. 1840 (2014) 3246–3256.
[34] H.I. Afridi, T.G. Kazi, D. Brabazon, S. Naher, Interaction between zinc, cadmium, and lead in scalp hair samples of pakistani and irish smokers rheumatoid arthritis subjects in relation to controls, Biol. Trace Elem. Res. 148 (2012) 139–147.

[35] J. Mateos, L. Lourido, P. Fernández-Puente, V. Calamia, C. Fernández-López, N. Oreiro, et al., Differential protein profiling of synovial fluid from rheumatoid arthritis and osteoarthritis patients using LC-mALDI TOF/TOF, J. Proteom. 75 (2012) 2869–2878.
[36] L.M. Bittencourt, D.A.P.D. Lana, C. De, A.M. Pimenta, A.V. Dos Santos, A.P.F. Gonçalves, R. Augusti, et al., Determination of metal associated with proteins of wheat seed samples after sequential extraction procedure, J. Braz. Chem. Soc. 25 (2014) 264–270.
[37] S. Drynda, B. Ringel, M. Kekow, C. Kühne, A. Drynda, M.O. Glocker, et al., Proteome analysis reveals disease-associated marker proteins to differentiate RA patients from other inflammatory joint diseases with the potential to monitor anti-tNFü therapy, Pathol. Res. Practice 200 (2004) 165–171.