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Absolute Quantification of Human Serum Transferrin by Species-Specific Isotope Dilution Laser Ablation ICP-MS

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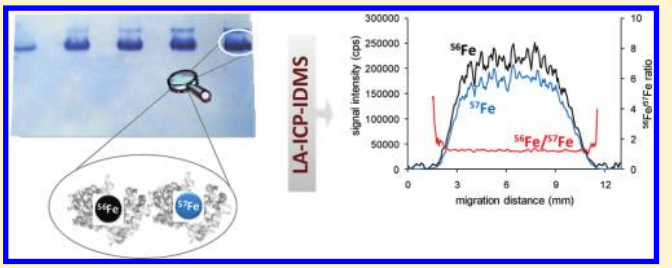
Supporting Information

ABSTRACT: We report for the first time the absolute quantification of a metalloprotein separated by nondenaturing gel electrophoresis (GE) using laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) in combination with species-specific isotope dilution mass spectrometry (IDMS). The proposed method is based on the use of an isotopically enriched ^{57}Fe-transferrin complex to quantify natural transferrin (Tf) in human serum samples. First, the saturation process of Tf with natural abundance or isotopically enriched ^{57}Fe was accomplished by using freshly synthesized Fe-citrate solutions. The stability of the metal-protein complex as well as its stoichiometry was investigated by spectrophotometry and ICP-MS, demonstrating a satisfactory stability over a period of at least one month and a molar ratio Fe:Tf of 1.94 ± 0.09, which is close to the expected value of 2. The species-specific IDMS method was compared with external calibration using the Fe-Tf (absolute Tf amount between 2 and 10 μg) and different sample preparation procedures (stained and nonstained gels) as well as two laser ablation strategies (single line ablation in the direction perpendicular or horizontal to the electrophoretic migration) were evaluated. The proposed species-specific GE-LA-ICP-IDMS method was tested for the analysis of a serum certified reference material (ERM-DA470k/IFCC). The results were in good agreement with the certified value with relative standard deviation values in the range of 0.9−2.7% depending on the data treatment procedure used. Furthermore, the analysis time has been drastically reduced in comparison with previous approaches to less than 15 min. The quantification by species-specific GE-LA-ICP-IDMS allowed us to obtain accurate and precise results not only by analyzing the protein spot in the middle position but also in the adjacent ablation line to the center.



INTRODUCTION

Metals are present in more than one-third of all proteins, playing important biological functions. Thus, in recent years many research efforts have been focused on the comprehensive study of metalloproteins, mainly dealing with the structure and functions of the metal sites as well as the biological implications of those metal-biomolecule interactions. Metalloproteomics has become a key part of the more general concept of metallomics.[1,2] The main analytical tools for the analysis of metalloproteins and metal-binding proteins include chromatographic or electrophoretic separation of the target biomolecule and their subsequent identification by molecular mass spectrometric techniques, using an electrospray (ESI) or a matrix-assisted laser desorption ionization (MALDI) source.[3,4] Several alternative strategies have been developed to investigate the chemical speciation of the metal bound to the protein. Such techniques are based on the online coupling of high performance liquid chromatography (HPLC) with an elemental detector, particularly with an inductively coupled plasma-mass spectrometer (ICP-MS).[1,5,6] Unfortunately, the chromatographic selectivity of HPLC methods is crucial here but not always enough to separate all the proteins in complex biological samples before final elemental detection.

However, gel electrophoresis (GE), with its unique ability to resolve thousands of proteins in a single run, is a powerful tool used routinely in biochemical, medical, and molecular biology laboratories today.[7,8] In fact, such routine tool has contributed significantly to clarify key bioscience issues, opening many new biological and clinical avenues. Classical detection methods in GE employ more or less specific chemical reactions, resulting in visible spots in the gel. Then, the analyte biomolecule can be extracted from the spot in the gel and characterized by molecular MS.[9] One of the main limitations of GE techniques is to achieve in this way the absolute quantification of the corresponding protein. Therefore, it would be desirable to have a solid sample introduction device able to transport directly the separated protein from the spots to the ICP-MS for detection. It has been demonstrated that natural presence of a heteroelement in a given protein enables the application of elemental MS in the field of metalloproteomics, making possible a robust and sensitive approach to protein quantification.[6,10] So far, however, protein quantification studies are scarce due to their complexity.[4−6]

Laser ablation (LA) ICP-MS is currently regarded as one of the most versatile techniques for trace element and isotopic analyses of solid materials.[11] Additionally, since its first introduction by

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Neilsen et al.,[12] the combination of GE with LA-ICP-MS has been successfully applied for the detection of phosphorylated proteins, selenoproteins, and other metalloproteins[13−15] as well as for the imaging of total metals in biological tissues.[16] Previous studies on the application of GE with LA-ICP-MS have been mainly focused on the optimization of the basic conditions for the effective separation of metal-binding proteins, focusing on their stability during GE and postseparation gel treatment.[15,17] Furthermore, while qualitative and semiquantitative analyses are almost routine in LA-ICP-MS, accurate quantitative analysis still remains an important challenge for a large variety of solid samples. In particular, current calibration methods developed for metalloprotein determinations using GE-LA-ICP-MS include the use of known metal spiked gels or introducing a standard metal solution into the laser ablation cell.[18,19] However, those approaches are external calibration methods and neither consider the behavior of proteins under the conditions used for electrophoresis nor compensate for possible distribution inhomogeneities in a protein spot.

Isotope dilution mass spectrometry (IDMS) is internationally regarded as an absolute measurement method directly traceable to the International System of Units.[20] In contrast to other calibration strategies, the analytical result is not affected by signal drifts, matrix effects or analyte losses. The quantification of metalloproteins by HPLC-ICP-MS can be improved by the addition of an isotopically enriched protein at the beginning of the sample preparation procedure (species-specific spiking). In this way, current analytical procedures can be validated as the chromatographic separation, analyte losses,or transformations of the species will not affect the final results.[20−22] The use of IDMS in LA-ICP-MS can correct not only for such possible losses and transformations of the species but also for some common fractionation and matrix effects that cannot be controlled using other calibration procedures. Thus, the combination of LA-ICP-MS and IDMS has been successfully applied for the direct determination of trace elements in solid samples, such as soils and sediments, road dusts, hair samples, petroleum products, silicon wafers, etc.[23−25] So far, only one publication[26] dealing with the use of IDMS combined to GE-LA-ICP-MS for the analysis of metalloproteins (superoxide dismutase) has been reported. However, in such study, the absolute quantification capabilities of IDMS were not demonstrated, since the analysis of reference materials to validate the quantification approach was lacking.

Therefore, the aim of this work is the development and validation of a species-specific LA-ICP-IDMS methodology to achieve a sensitive, fast, and accurate absolute quantification of transferrin (Tf) metalloprotein in serum samples. Transferrin has been selected as model metalloprotein due to its important role in the iron metabolism of the human body. Physiologically, the majority of cells in the organism acquire iron from a well-characterized plasma glycoprotein; transferrin. The most common analytical approaches for absolute quantification of Tf in serum samples are based on separations by chromatography[5] or electrophoresis[27] techniques followed by an iron-specific detector such as ICP-MS. The proposed method is based on the use of an isotopically enriched ^{57}Fe-Tf complex to quantify natural Tf in human serum samples, after nondenaturing one-dimensional GE separation (1D-PAGE). A critical evaluation of novel external calibration strategies and the species-specific IDMS quantification method, in terms of precision, accuracy, and analysis time is presented. The proposed species-specific GE-LA-ICP-IDMS method was validated analyzing a certified reference serum material.

Table 1. Operating Conditions of the ICP-MS and Laser Ablation System

ICP-MS	thermo element 2
RF Power	1325 W
cooling gas	15.5 L min^{-1}
auxiliary gas	0.8 L min^{-1}
nebulizer gas (Ar)	0.9 L min^{-1} (ICP-MS)
	0.6 L min^{-1} (LA-ICP-MS; wet plasma)
cones	Ni (skimmer and sampler)
isotopes	^{13}C, ^{32}S, ^{34}S, ^{54}Fe, ^{56}Fe, ^{57}Fe, ^{58}Fe, ^{69}Ga, ^{71}Ga
sample time	0.01 s
mass window	100%
samples per peak	10
LA system	**CETAC LSX-213**
laser energy	100% (~5.6 mJ)
repetition rate	20 Hz
spot size	200 μm
scan speed	40 μm s^{-1}
ablation mode	single line scan
carrier gas (He)	0.8 L min^{-1}

EXPERIMENTAL SECTION

Standards, Reagents and Samples. All chemicals and reagents used in this work were of analytical grade and their detailed description is included as Supporting Information. The spike solutions of ^{57}Fe (94.42 ± 0.01% isotopic abundance) and ^{34}S (99.96 ± 0.01% isotopic abundance) were characterized in terms of isotopic composition and concentration by ICP-MS and reverse ICP-IDMS, respectively, and Fe and S concentrations were found to be 12.73 ± 0.26 μg g^{-1} for Fe and 66.81 ± 1.63 μg g^{-1} for S. The certified reference material (CRM) ERM-DA470k/IFCC was employed for the validation of the proposed quantification methodology.

Instrumentation. *Gel Electrophoresis.* The separation of protein standards and human serum samples was performed using a vertical mini-gel electrophoresis system (Mini-PROTEAN Tetra Cell, Bio-Rad Laboratories, Hercules, CA, U.S.). Gels were handcast in mini-gel cassettes as described in the Supporting Information. The running conditions were 300 V for 20 min or until bromophenol blue dye had migrated to the bottom of gel. For external calibration analyses a volume of 10 μL Fe-Tf standard (absolute amount of protein between 2 μg and 10 μg) was loaded into the gel. The serum proteins separation by 1D-PAGE was performed under nondenaturing conditions, avoiding the possible loss of metals associated to proteins.

Following electrophoresis, the gel cassettes were dismantled and the mini-gels were stained in freshly prepared and filtered Coomassie staining solution (10% acetic acid, 20% methanol) for 30 min. Next, the gels were destained in 10% acetic acid with 50% methanol until the gel background was clear. The gels were then placed in glycerol for 1 min and dried for at least 3 h in an oven at 70 °C on filter paper (Whatman paper) and covered with a transparent film (Saran Film) to protect them against contamination. Nonstained gels were carefully transferred into glycerol

(as the stained gels), but neither Coomassie solution nor acetic acid and methanol were added. For LA-ICP-MS measurements, the filter papers with the gels were transferred into the ablation cell and, in all cases, LA-ICP-MS analyses were performed on the same day of gel preparation to minimize any possible diffusion of the proteins within the gel or into the sample substrate.

Spectrophotometer (UV−vis). An UV−Vis spectrophotometer (Thermo Spectronic GENESYS 20, Thermo Scientific, Bremen, Germany) and an Agilent HPLC system model 1100 (Agilent Technologies, Tokyo, Japan) with a diode array detector were used for the studies of Tf saturation with Fe.

LA-ICP-MS. Element-specific detection was carried out using a double-focusing sector field ICP-MS (Element 2, Thermo Fisher Scientific, Bremen, Germany) at medium mass resolution (R = 4000), in order to avoid spectral interferences on Fe and S determination. For laser ablation analyses, a CETAC LSX-213 laser system (Cetac Technologies, Omaha, Nebraska, U.S.) was employed. The optimized conditions used for ICP-MS and LA-ICP-MS measurements are listed in Table 1. Taking into account the relatively long analysis time of the spots in the gels (around 5 min) as well as the homogeneity of the samples within the spots, each isotope is counted during one time slice of 100 ms per 2 s to improve the ions statistic and, therefore, to obtain a better precision in the isotope ratio measurements. The LA-ICP-MS coupling was carried out in wet plasma conditions using a homemade Y-piece of glass. The laser-generated aerosol was transported through a high-purity tube (Tygon tubing) into the ICP torch by the He carrier gas of the ablation cell. Before the introduction into the plasma, the laser-generated aerosol was mixed with a liquid aerosol (nebulized by means of a concentric nebulizer). This dual-flow introduction system enables a complete and easy optimization of the ICP-MS (gas flow rates, torch position, lens voltages, etc.) for high sensitivity by nebulizing a 1 ng g^{-1} multielement tuning solution. Moreover, LA-ICP-MS coupling was optimized daily using a SRM NIST 612 glass standard for high sensitivity, background intensity, and the $^{238}U/^{232}Th$ signal ratio that should be close to 1 to ensure a low fractionation effect due to the ICP ionization efficiency. $^{248}ThO/^{232}Th$ signal ratio was also measured for controlling oxide formation, being always below 0.5% at the selected optimized conditions. In addition, during laser ablation analyses, the plasma was kept under wet conditions by the continuous nebulization of a 10 ng g^{-1} Ga standard solution, which was used to correct for mass bias during the measurement of Fe isotope ratios.

Procedures. *Iron Saturation: Apo-Tf Standard Incubated with Fe-Citrate.* For the saturation of the Tf standard, a solution of ~31 μM Apo-Tf (typical Tf concentration in human serum samples) was prepared in a physiological medium, which consists of 50 mM Tris and 150 mM NaCl (pH 7.4). This solution was diluted 1:1 with a model solution containing 20 mM ammonium bicarbonate, 150 mM NaCl and 300 μM sodium citrate (pH 7.4). The Apo-Tf was then saturated with an appropriate amount of a freshly synthesized Fe-citrate solution (Fe-citrate was prepared at pH 7.4 using 0.03 g of an Fe standard solution and trisodium citrate 1 M to final concentrations of 100 μM Fe, being the optimum Fe-to-citrate molar ratio 1:1000[22,28]). The mixture was finally thoroughly stirred and incubated at room temperature during 1 h. Then, possible low weight impurities and the Fe-citrate excess after Tf incubation were removed by ultrafiltration through an Amicon centrifugal device (molecular cutoff of 10 kDa) at 14000 g. The protein fraction (retained in the upper side of the filter) was reconstituted with the physiological medium previously used in the incubation and then centrifuged again. Finally, the Fe-Tf complex was reconstituted in the model solution by inversion of the Amicon filter and a brief centrifugation at 1000 ×g. It should be stated that the same procedure used for the incubation of natural Fe-citrate with Apo-Tf standards was used for the incubation of real life serum samples.

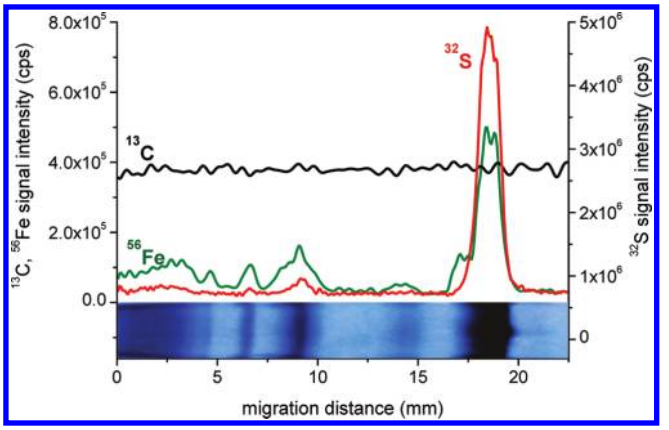

Figure 1. Electropherogram obtained by LA-ICP-MS for the analysis of a certified human serum sample (ERM-DA470k) after GE separation (nondenaturing 1D-PAGE, Coomassie stained gels).

Preparation of the Isotopically Enriched Fe-Saturated Tf. Concerning the synthesis of ^{57}Fe-Tf, 250 μL of an Apo-Tf standard solution in a physiological medium was diluted 1:1 with the model solution used for incubations. The Apo-Tf was then saturated with the ^{57}Fe-citrate solution previously synthesized (following the same incubation procedure used for the natural abundance Fe-citrate). The same strategy explained above was also applied for the purification of the ^{57}Fe saturated Tf. The isotopic composition of the ^{57}Fe-Tf spike was calculated by Fe isotope ratio measurements at medium resolution using the ICP-MS. The elemental concentrations of the isotopically enriched Fe-saturated Tf were determined by reverse ICP-IDMS using the corresponding standards of natural isotopic composition. The ^{57}Fe abundance was found to be 94.77 ± 0.12% and the obtained total concentration of Fe was 3.51 ± 0.05 μg g^{-1}. To demonstrate that the synthesized ^{57}Fe-Tf complex can be used over a large time scale for the analysis of different samples, its stability was studied over a period of one month. No significant changes on the isotopic composition were observed (more detailed information is presented in the Results and Discussion Section). Additionally, as previously described by del Castillo Busto et al.,[21] no replacement of ^{57}Fe by natural abundance Fe in the ^{57}Fe-Tf complex was found.

RESULTS AND DISCUSSION

Uptake of Fe by Tf under Physiological Conditions. Iron saturation of Apo-Tf has to be first established to avoid Fe isotopes exchange when applying IDMS to speciation analyses. Thus, the molar ratio Fe:Tf was first investigated using spectrophotometry and ICP-MS, both for the natural Fe-Tf and the isotopically enriched ^{57}Fe-Tf to demonstrate the Fe saturation. Furthermore, the complex stability and stoichiometry were evaluated spectrophotometrically. The detailed description of the methodology of Fe uptake used for the sample preparation is included as Supporting Information as well as the relationship

Table 2. Determination of Tf Concentrations in a Serum Reference Material by External Calibration GE-LA-ICP-MS[a]

sample preparation	laser ablation strategy	CRM [Tf][b] (g L^{-1})	RSD (%)	LOD Tf (g L^{-1})	analysis time[c]
stained gel (n = 3)	horizontally	2.45 ± 0.76	31	0.7	1.5 h
stained gel (n = 30)	horizontally	2.21 ± 0.10	5	0.7	18 h
stained gel (n = 3)	perpendicularly	2.07 ± 0.30	14	0.7	1.5 h
nonstained gel (n = 3)	perpendicularly	2.25 ± 0.18	8	0.2	4.5 h

[a] Comparison of different ablation strategies and sample preparation procedures. The precision of the measurements was calculated based on 1s-standard deviation from three independent replicates. [b] CRM ERM-DA470k: certified value 2.36 ± 0.08 g L^{-1} Tf [c] Including 5-points calibration and one sample (triplicate measurements)

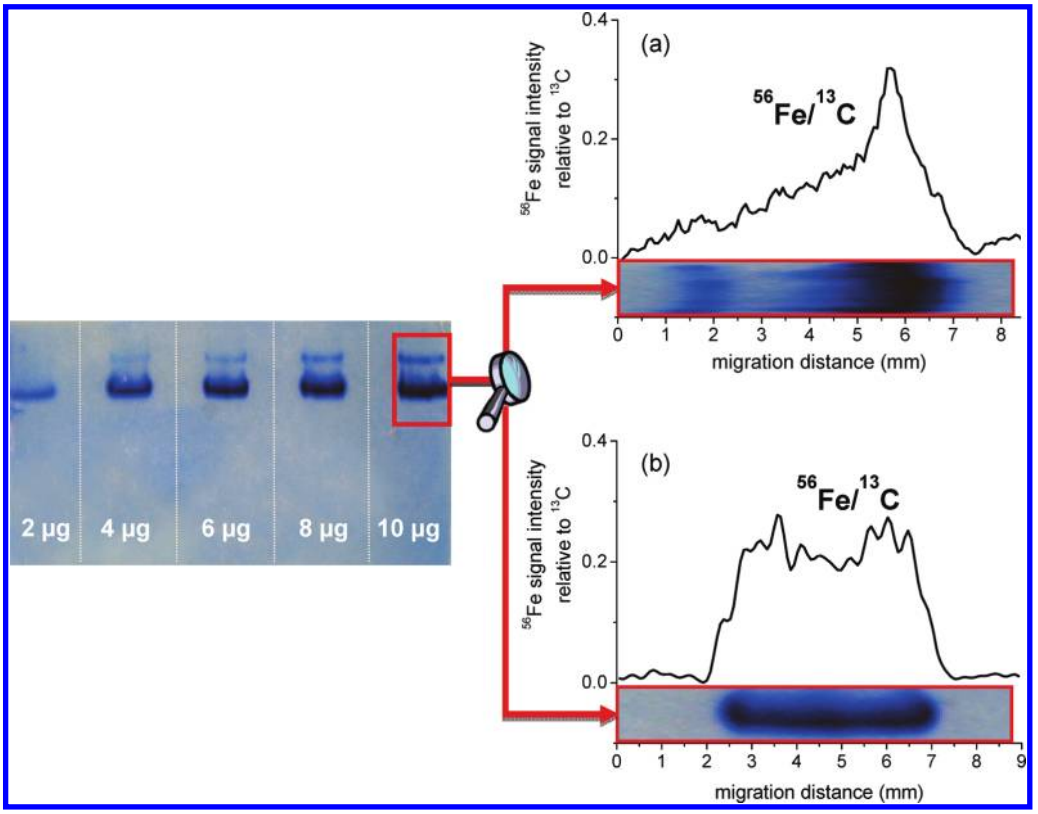

Figure 2. Nondenaturing gel after Coomassie blue staining of saturated Tf and ^{56}Fe/^{13}C profiles obtained by LA-ICP-MS. (a) Ablation of the protein spot horizontally to migration direction; (b) ablation of the protein spot perpendicular to migration direction.

observed between the absorbance measured at 465 nm and the Fe concentration used for incubation (Figure S1 of the Supporting Information, SI). The molar ratios of natural Fe:Tf and the ^{57}Fe:Tf spike were found to be 1.94 ± 0.09 and 1.88 ± 0.10, respectively, (close to the theoretical molar ratio of 2) and the Fe-Tf complex was stable during at least 4 weeks.

Quantification of Tf by External Calibration GE-LA-ICP-MS. The proposed species-specific IDMS method of quantification was compared with a conventional external calibration determination using our Fe-Tf standard solution. First, different sample preparation procedures and ablation strategies were evaluated. The external calibration was performed by using different concentrations of Fe-Tf to consider the behavior of the proteins under the selected electrophoretic conditions. The signal of ^{13}C (homogeneously distributed in the acrylamide gel) was employed as an internal standard to correct for any instrumental signal drift occurring during the ablation process. In all cases, triplicates of each Tf concentration (absolute amount between 2 and 10 µg) were submitted to gel electrophoretic separation using nondenaturing 1D-PAGE. Generally, after separation of proteins and Coomassie staining, the gel lanes were scanned by the laser beam in the electrophoretic migration direction, obtaining the corresponding electropherogram for the Fe, S, and C signal intensities as a function of the migration distance. For quantification purposes, signals were integrated with the *Origin* program and a reference serum sample (certified for total Tf) was analyzed. In this way, the accuracy and precision of LA-ICP-MS analyses was evaluated. Figure 1 shows the electropherogram obtained for the analysis of the CRM (3 µg of Tf). As can be seen, Fe was detected coordinated to Tf (~9 mm) as well as to albumin (~18 mm) to a high extent and to the immunoglobulins (0−5 mm) to a lower extent.

Four different external calibration approaches were investigated for the analysis of the certified serum sample, varying the ablation strategy (laser beam horizontal or perpendicular to the electrophoresis migration direction) and the sample preparation

(stained and nonstained gels). Table 2 shows a summary of the results obtained in these experiments aiming at the quantification of Tf in the CRM. Additionally, this table shows for comparison the observed required analysis time (LA-ICP-MS measurement time, excluding sample preparation time) and the limits of detection (LODs) for each calibration strategy. LODs were calculated by using the 3s criterion ($3s_b/S$), where s_b is the standard deviation of 5 independent measurements of the blank value (ablation of a pure polyacrylamide gel without protein spots) in cps and S is the sensitivity for the corresponding analyte isotope obtained by measuring the serum reference material. As can be seen in Table 2, the Tf concentrations determined by external calibration GE-LA-ICP-MS agreed quite well with the certified value (within the given uncertainties) in all cases. However, the required analysis time as well as the accuracy and precision were strongly dependent upon the conditions selected for the sample preparation and ablation strategy.

The Tf concentration determined by external calibration in stained gels using a single line ablation strategy (only one line per protein spot) in the same direction of migration (denoted as horizontally) showed results in agreement with the certified value, but a high standard deviation is apparent (relative standard deviation, RSD, ~30%). Figure 2a shows the electropherogram obtained for the analysis of a 10 μg Tf spot by LA-ICP-MS. As expected, increasing the number of analysis (10 single line ablations per protein spot) the precision obtained was significantly better (5% RSD). However, the sample analysis time (including a 5-point calibration and triplicate measurements of standards and sample) increase from 1.5 to 18 h for the multiple line ablation strategy. Thus, a different ablation strategy was investigated to overcome the inhomogeneous distribution of the protein inside the spot and its possible diffusion (due to the electrophoretic separation). As can be observed in Figure 2a, although no perfectly defined borders were visible for protein spots, the maximum intensity for ^{56}Fe signal was always identified at the center of the spot. Therefore, a single line ablation in the direction perpendicular of electrophoretic migration (only one line in the middle of each protein spot) was selected. Figure 2b collects the electropherogram obtained for the analysis of a 10 μg Tf spot using the perpendicular ablation strategy. As can be seen, ^{56}Fe profile was better defined than that obtained with the horizontal ablation and a 2-fold reduction in RSD value was found for the perpendicular strategy compared to the horizontal one (14% and 31% RSD, respectively). Such improvement in the precision could be attributed to a better counting statistics (the laser beam stays longer on the protein spot) and to a more homogeneous Fe distribution throughout the spot in this direction. It should be noted that the precision obtained for Tf quantification in this case was significantly worse than that for multiple ablation (14% vs 5%), but the analysis time was drastically reduced.

It has been recently reported by several authors[17,26] that Coomassie blue staining of the gels prior to LA-ICP-MS analysis can be a metals contamination source, which increase the Fe background in the gel and masks the real Fe content of the protein. Moreover, the reproducibility of the staining and destaining stages is quite low due to the concentration of the exogenous Fe, strongly depends on the duration and homogeneity of each stage. Aiming at avoiding such contamination source we also investigated the use of an external calibration in nonstained gels (and the proposed ablation strategy perpendicular to the migration direction). Since the proteins tend to diffuse in the gel after the electrophoretic separation, some actions must be taken to fix them in the gel. In our case, denaturation of the protein both chemically (by lowering the pH to 3) and thermally (by heating to 70 °C) were investigated. More accurate and precise results with the latter thermal strategy were observed. Figure 3 shows the Fe and S calibration curves obtained by LA-ICP-MS using the perpendicular ablation strategy. It should be stressed that as the spot (without staining) is currently not visible in the gels, a larger area had to be analyzed to ensure the protein spot ablation in its maximum. Thus, now the ablation of 3 lines per protein spot was carried out in all cases. As can be seen in Table 2, such calibration strategy allowed us to obtain not only a Tf concentration in agreement with the certified value, but with a good precision (similar RSD value to that obtain with n = 30) and in a reasonable analysis time. Additionally, Tf limit of detection was found to be 0.2 g L^{-1}, a value well below LODs recently reported by other authors.[26]

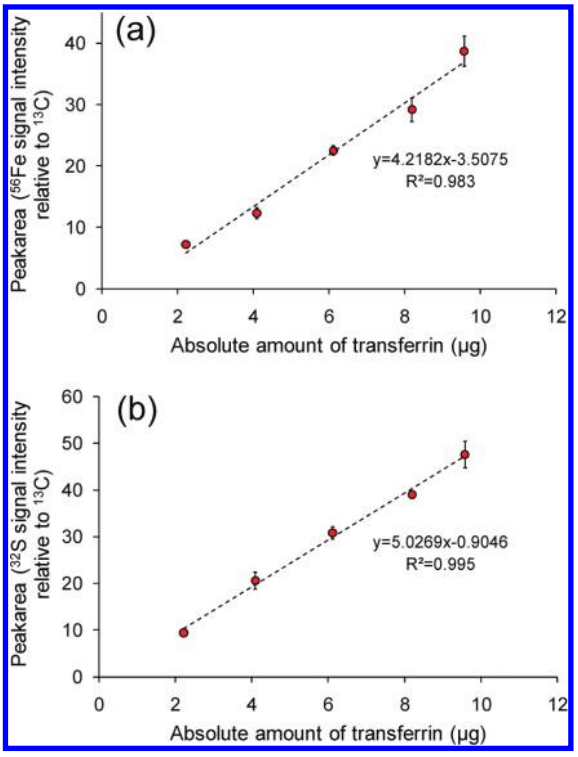

Figure 3. Calibration curves obtained for ^{56}Fe and ^{32}S (^{13}C as internal standard) by GE-LA-ICP- MS using a perpendicular ablation strategy (nondenaturing 1D-PAGE, nonstained gels). (a) ^{56}Fe; (b) ^{32}S.

Absolute Quantification of Tf by Species-Specific Isotope Dilution GE-LA-ICP-MS. Alternatively to external calibration, a species-specific quantification methodology based on the use of the previously synthesized isotopically enriched ^{57}Fe-Tf was tested. The ^{57}Fe-Tf spike was mixed up with the serum samples before the GE separation. Thus, matrix-matched quantification without any external standard with adequate corrections for all signal variations during the subsequent analysis (either derived from instrumental drift, varying mass ablation rates, etc.) was secured. Moreover, any possible analyte losses after the spiking (e.g., during sample handling and electrophoretic separation) will not affect the final concentration result. A successful isotope dilution analysis requires that the isotopically enriched ^{57}Fe-Tf added behaves chemically as the natural abundance Fe-Tf. Therefore, Apo-Tf saturated with natural Fe and isotopically

enriched ^{57}Fe-Tf were submitted to nondenaturing 1D-PAGE separations to compare their migration patterns. Figure 4a shows the protein spots observed corresponding to the natural and the isotopically enriched Fe-Tf standards, after Coomassie blue staining. As can be seen, the migration behavior was the same (expressed by a retention factor of $R_f = 0.25$), demonstrating that neither the charge nor the structure of the isotopically enriched protein was affected by the ^{57}Fe labeling procedure. In addition, it has to be stressed that no diffusion of the protein or protein cross contamination were observed.

For the analysis, a known amount of the ^{57}Fe-Tf spike was added to the certified human serum previously incubated with natural Fe-citrate for saturation. Samples were vortexed and left at room temperature during 5 min to guarantee the complete mixing and equilibration of Fe-Tf from the sample and the added isotopically enriched ^{57}Fe-Tf. Next, the CRM proteins were separated using nondenaturing 1D-PAGE and the nonstained gels were analyzed by LA-ICP-IDMS using the optimized ablation strategy perpendicular to the migration direction. As an example, Figure 4b shows the profile obtained for the analysis of the CRM (analyte/^{57}Fe-Tf spike ratio corresponding to a 1:1 proportion). Three different data treatment approaches to calculate the Fe isotope ratios were employed for comparison. First, ^{56}Fe and ^{57}Fe integrated areas within the protein spot along the LA-ICP-MS profiles (denoted as Integration in Table 3) were calculated. Second the ^{56}Fe/^{57}Fe point-by-point calculation of

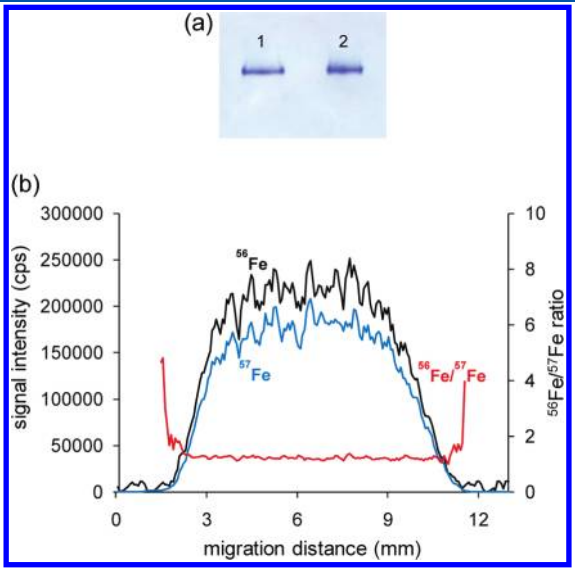

Figure 4. (a) Nondenaturing mini-gel after staining with Coomassie blue (2 μg Tf). Line 1- isotopically enriched ^{57}Fe-Tf; Line 2- natural abundance Tf. (b) Profiles obtained by LA-ICP-MS for ^{56}Fe, ^{57}Fe and their isotopic ratio using a mixture of the CRM and the isotopically enriched ^{57}Fe-Tf (13 μg natural Fe-Tf:14 μg ^{57}Fe-Tf).

average isotope ratios (point-by-point data in Table 3) was used to calculate the quantity of the natural Tf in the sample. Finally, a new strategy of isotopic ratios calculation recently described by Fietzke et al.[29] was also evaluated (corresponding to linear regression slope in Table 3). Here, we applied the described method for calculation of Fe isotope ratios using the INDEX LINEST function of MS EXCEL with the ^{56}Fe and ^{57}Fe signal intensities. This function calculates the statistics for a line by using the "least squares" method to determine a straight line that best fits the obtained data, and then returns an array that describes the line. In such a way, background correction and the subjective influence which may occur by setting the integration limits were avoided, simplifying enormously the evaluation of data. Although the linear regression slope treatment approach has been previously investigated for isotopic ratios measurements with a simultaneous detection by multicollector ICP-MS,[29,30] it should be highlighted that the present work shows, for the first time, the successful application of such signal processing using a slow signal acquisition detection.

Two mass bias correction approaches were investigated in this work by the measurement of natural abundance Fe isotope ratios in polyacrylamide gels of the human serum Tf standard (external correction) and by the continuous nebulization of a 10 ng g^{-1} natural abundance Ga standard solution (internal correction).[24] In both cases, the mass bias factor was calculated using the linear law model. The mathematical model used for the mass bias correction as well as the numerical differences observed between internal and external approaches are included as Supporting Information. As comparative studies showed similar results for internal and external mass bias correction strategies, the Ga standard solution was used for all the measurements. This means that the mass bias factor was performed in the same LA-ICP-MS profile by using a natural Ga standard with well-known isotopic composition. The final calculation of the GE-LA-ICP-IDMS results was carried out by using the conventional isotope dilution equation.[31]

The results obtained for the determination of Fe in the certified reference serum sample by species-specific GE-LA-ICP-IDMS are summarized in Table 3. As can be seen, the Tf concentration determined by the proposed species-specific IDMS method was in good agreement with the certified value, being the precision obtained significantly better than that obtained by external calibration; RSDs in the range of 0.9−2.7% depending on the data treatment procedure. The uncertainty for each individual measurement of Fe concentration was calculated according to the method proposed by Kragten.[31] A relative uncertainty (%) of 13.4% was obtained when external calibration was applied, whereas for the proposed species-specific IDMS methodology a relative uncertainty of 1.7% was found. Moreover, in order to study the influence of the different factors in the final uncertainty of the concentration values, we have calculated full uncertainty budgets for Fe in a representative analysis of the

Table 3. Determination of Tf Concentrations in a Serum Reference Material and Human Serum Samples by Species-Specific GE-LA-ICP-IDMS Using Three Different Data Treatment Approaches[a]

data treatment	CRM isotope ratio (^{56}Fe/^{57}Fe)	CRM [Tf][b] (g L^{-1})	human serum isotope ratio (^{56}Fe/^{57}Fe)	human serum [Tf][b] (g L^{-1})	analysis time[c]
integration	0.95 ± 0.03	2.40 ± 0.08	0.98 ± 0.02	2.48 ± 0.04	15 min
point-by-point	0.90 ± 0.01	2.28 ± 0.03	0.89 ± 0.01	2.24 ± 0.03	15 min
linear regression slope	0.92 ± 0.01	2.34 ± 0.02	0.93 ± 0.01	2.34 ± 0.04	15 min

[a] Standard deviation values are calculated from the mean of three measurements in three independent gels. [b] CRM ERM-DA470k: certified value 2.36 ± 0.08 g L^{-1} Tf [c] Triplicate measurements

serum CRM. The corresponding uncertainty budgets demonstrated that the most important contributor to the uncertainty was the measurement of the $^{56}Fe/^{57}Fe$ isotope ratio for the isotope dilution procedure and the measurement of ^{56}Fe and ^{13}C signals and the y-intercept of the calibration curve for the external calibration. The uncertainty budget obtained for a representative analysis of the CRM by species-specific LA-ICP-IDMS can be observed in Table S1 of the SI.

Additionally, the analysis time (LA-ICP-MS measurement time, excluding sample preparation time) was drastically reduced to less than 15 min. Moreover, the quantification by species-specific GE-LA-ICP-IDMS provided accurate and precise results not only by analyzing the protein spot in the center position but also in adjacent ablation lines to the center with deviations from the certified value below 5%. It should be stressed here that by using the linear regression slope data treatment a significant improvement in the precision (0.9% RSD) and in the accuracy of the Tf concentration values were obtained (as compared to the results obtained by the other tested integration and point-by-point approaches). In addition, although a different instrumentation as well as ablation strategies were employed in previous IDMS approaches for the analysis of soils, sediments and crude and fuel oil samples, the precision obtained for the direct analysis of gels by species-specific LA-ICP-IDMS was significantly better that precisions reported in previous work using IDMS and LA-ICP-MS, where RSD values in the order of 15% were found.[23,24]

Finally, once the validation of the optimum quantification method was guaranteed by the analysis of the reference serum sample, the analysis of real life human serum samples from healthy volunteers was performed. A triplicate of a pool of serum samples was separated in nondenaturing 1D-PAGE following the Procedures section and the nonstained gels were analyzed by species-specific GE-LA-ICP-IDMS. Table 3 collects the results obtained using the three data treatment approaches. As can be observed, the Tf concentration obtained showed typical values reported for healthy human serum samples (similar to those obtained for the CRM). Although the comparison of the absolute amount of Tf from healthy volunteers and patients suffering from a disease affecting its concentration (e.g., hypotransferrinemia) would be an interesting application of the IDMS method for the evaluation as potential biomarker, such samples are not currently available in our laboratory.

CONCLUSIONS

While common GE-LA-ICP-MS is not a particularly accurate method, species-specific GE-LA-ICP-IDMS has been demonstrated here for the first time to provide an accurate, precise, and time-effective strategy for eventual absolute direct determinations of metalloproteins in serum samples (even at concentration of protein in the low gram per liter range). Compared with alternative quantification methodologies, no calibration curves or standard reference materials are necessary using the ID proposed method. Moreover, the sample analysis time is reduced to less than 15 min and precision and accuracy obtained for the analysis of a CRM were significantly better than those obtained by external calibration. The appropriate selection of the laser ablation strategy (single line ablation in the direction perpendicular to the electrophoretic migration) and of sample preparation (nondenaturing 1D-PAGE and nonstained gels) was found to be crucial to ensure a good precision and accuracy. Therefore, this enriched isotopes based analytical approach offers a tremendous potential to achieve reliable and direct metalloproteins quantification after conventional 1D and 2D gel electrophoretic separations. The use of such species-specific IDMS methods applied to LA-ICP-MS could open the door to meet the quality assurance requirements urgently needed in quantitative heteroatom-tagged protein analyses.[6] This work opens new avenues first, to uncover further potentialities of species-specific GE-LA-ICP-IDMS for other metalloproteins and, second, to apply such quantification potential of metal-biomolecules in biochemical, molecular biology, and medical sciences (e.g., detection of multiple proteins on one spot, immuno-microarray experiments, microarray-based multiplexed detection studies, etc.).

ASSOCIATED CONTENT

Supporting Information. The Supporting Information details the Experimental section of the manuscript concerning the description of the standards, reagents and samples employed in this work as well as a detailed explanation of the gel electrophoretic instrumentation and experimental conditions used. Furthermore, it includes a critical description of the Results and Discussion section of the Fe uptake by Tf under physiological conditions. A detailed explanation of the two models employed for the mass bias correction as well as the full uncertainty budget calculated for Fe in a representative analysis by species-specific LA-ICP-IDMS of the serum CRM are also included as Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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