Anal Bioanal Chem (2011) 401:657–666
DOI 10.1007/s00216-011-5104-2

ORIGINAL PAPER

Absolute protein quantification by LC-ICP-MS using MeCAT peptide labeling

Diego Esteban-Fernández · Christian Scheler · Michael W. Linscheid

Received: 4 March 2011 / Revised: 6 May 2011 / Accepted: 12 May 2011 / Published online: 29 May 2011

Abstract Nowadays, the most common strategies used in quantitative proteomics are based on isotope-coded labeling followed by specific molecule mass spectrometry. The implementation of inductively coupled plasma mass spectrometry (ICP-MS) for quantitative purposes can solve important drawbacks such as lack of sensitivity, structure-dependent responses, or difficulties in absolute quantification. Recently, lanthanide-containing labels as metal-coded affinity tag (MeCAT) reagents have been introduced, increasing the interest and scope of elemental mass spectrometry techniques for quantitative proteomics. In this work one of the first methodologies for absolute quantification of peptides and proteins using MeCAT labeling is presented. Liquid chromatography (LC) interfaced to ICP-MS has been used to separate and quantify labeled peptides while LC coupled to electrospray ionization mass spectrometry served for identification tasks. Synthetic-labeled peptides were used as standards to calibrate the response of the detector with compounds as close as possible to the target species. External calibration was employed as a quantification technique. The first step to apply this approach was MeCAT-Eu labeling and quantification by isotope dilution ICP-MS of the selected peptides. The standards were mixed in different concentrations and subjected to reverse-phase chromatography before ICP-MS detection to consider the column effect over the peptides. Thus, the prepared multi-peptide mix allowed a calibration curve to be obtained in a single chromatographic run, correcting possible non-quantitative elutions of the peptides from the column. The quantification strategy was successfully applied to other labeled peptides and to standard proteins such as digested lysozyme and bovine serum albumin.

Keywords Quantitative proteomics · ICP-MS · MeCAT · Lanthanide labeling · Absolute quantification

D. Esteban-Fernández (✉) · M. W. Linscheid
Department of Chemistry, Humboldt-Universitaet zu Berlin,
Brook-Taylor-Str. 2,
12489 Berlin, Germany
e-mail: diesteban77@yahoo.es

C. Scheler
Proteome Factory AG,
Magnus-Str. 11,
12489 Berlin, Germany

Introduction

After the great progress achieved in qualitative proteomics, the quantitative dimension emerges as the new challenge. The measure of the protein quantity or the change in this quantity can provide important information related to the status or the changes in a biological system. The quantification of the proteome can reveal alterations on the normal biological state or even point out biological markers in important diseases. Though matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF) or electrospray ionization–mass spectrometry (ESI-MS) techniques are crucial in the identification of peptides and proteins, their application to quantitative analysis present some important drawbacks such as the differential response of proteins and peptides depending on size, hydrophobicity, matrix, or solvents. Stable isotope-coded labeling strategies [1] have been used to overcome these disadvantages and nowadays methodologies based on molecular mass spectrometry techniques are widely used for quantitative proteomics. Nevertheless, absolute quantification or sensitive detection of low-abundance proteins together with high abundant ones are still unfinished business.

In the last years, the outlook about protein quantification has changed noticeably with the incorporation of elemental mass spectrometry techniques to this field [2]. Screening of multiple heteroatoms naturally present or introduced as labels in biological samples as well as absolute quantifications are potential applications of ICP-MS in proteomic analysis [3]. Therefore, heteroatoms such as S [4], P [5, 6], Se [7], I [8], As [9], Pt [10], or lanthanides [11] among others, have been analyzed in proteomic studies and almost any biomolecule is ICP-detectable, either by nature or via labeling [12]. Also, interesting applications derived from the precise isotope abundance measurements have been developed [13, 14]. For both quantification and bio-speciation studies the separate determination of the species is paramount. Hence, separation techniques as HPLC or CE are frequently hyphenated with ICP-MS. The lack of structural information offered by ICP-MS cannot be compensated using chromatographic standards, with the additional use of molecular mass spectrometry techniques being mandatory. In fact, studies have been published taking advantage of the powerful analytical tool consisting in the combined use of mass spectrometry techniques with such different and complementary ionization sources [15, 16].

Stable isotopes covalently bound or incorporated in vivo have been widely used for quantification employing specific molecule mass spectrometry. The typical strategy consists of labeling two portions of the sample with heavy and light isotopes and measuring the mix by mass spectrometry after separation by reverse-phase liquid chromatography. Both labeled samples usually coelute due to its similar chemical properties only showing a mass shift related to the number of heavy isotopes in the label. Relative quantification can be carried out measuring the intensity ratio between heavy and light peaks of each peptide. The labeling can take place after or before an enzymatic digestion, the latter being better for post-translational modifications or protein isoforms studies. Stable isotope labeling by amino acids in cell culture (SILAC) [17] or the introduction of ^{18}O using $H_2{}^{18}O$ in the enzymatic digestion [18], are examples of metabolic and enzymatic labeling, respectively. However, the chemical labeling is the most extended strategy and methodologies as ICAT (isotope-coded affinity tag) [19], ICPL (isotope-coded protein label) [20], iTRAQ (isobaric tag for relative and absolute quantification) [21] or PROTEIN-AQUA (protein absolute quantification) [22] are capable to quantify in relative and even in absolute mode. Trying to obtain a bigger mass shift some other lanthanide-containing complexes which also increase the multiplex capacity have been introduced [23].

Recently, a new chemical label called metal-coded affinity tag (MeCAT) has been developed in our group for quantification purposes [24]. MeCAT analytical robustness and suitability for biological applications have been demonstrated, [25] and its fragmentation behavior has been described [26]. This lanthanide-containing reagent consist of the chelating agent 1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid (DOTA) including a cysteine reactive maleimide group. DOTA's capacity to harbor one lanthanide atom allows employing ICP-MS for the detection of MeCAT-labeled peptides and proteins, taking advantage of the excellent detection limits of this detector. Although the structural independent response of ICP-MS does not provide much information, it is the ideal signal for quantitative determinations. Thus, the same concentration of different proteins labeled with the same number of MeCAT tags should present identical responses. Therefore, the quantitative determinations become easier since the same calibration curve for all the species labeled with the same reagent can be employed. Furthermore, determination in very different concentration ranges and quantification by isotope dilution analysis (IDA) is possible due to the wide dynamic linear range and the multielemental capacity of ICP-MS, respectively.

Quantifications at the protein [27] and peptide [11] level have been performed using lanthanide-containing labels. Although work with intact labeled proteins is extremely interesting, manipulation of labeled peptide is much more straightforward and versatile. Liquid chromatography and molecule-specific mass spectrometry techniques are easily applicable to the separation and detection of peptides while intact proteins usually require separation by gel electrophoresis and final proteolytic digestion for mass spectrometry identification. In both cases, a complete labeling as well as a precise knowledge of the biomolecule/label stoichiometry is required to obtained reliable quantitative data.

Quantification of MeCAT-labeled peptides or proteins by direct infusion or by FIA (flow injection analysis) ICP-MS can be tackled using external calibration with elemental standards. However, usually, a separation technique such as reverse-phase LC must be applied prior to detection by ICP-MS. The unavoidable losses of biomolecules in the LC column cannot be determined with elemental standards since do not interact to the same extend with the stationary phase than large biomolecules. Also, employing species-unspecific IDA, the losses on the chromatographic column should be considered to obtain accurate and precise determinations. Another important problem arising from the need to build a calibration curve applying chromatography is the time consuming measurement of each calibration point in individual runs.

This work proposes a novel quantification strategy based on MeCAT-labeled standard peptides. For this purpose, known synthetic peptides were labeled and purified from the excess of MeCAT reagent. The subsequent quantification of the pure labeled peptides was performed by direct infusion ICP-MS using IDA. The characterized labeled standards were used to construct a calibration curve for the metal loaded in the MeCAT reagent using RP (reverse phase) chromatography. The expected losses of the standard

labeled peptides and sample labeled peptides are very similar. Furthermore, the different retention times of the employed standard peptides enabled the measurement of several peptides with different concentrations obtaining the calibration curve in only one chromatographic run [28].

Experimental section

Protein digestion and labeling

Standard lysozyme from hen egg white (Fluka, Germany) and bovine serum albumin (BSA; Sigma Aldrich, Germany) were prepared in 50 mM ammonium bicarbonate (Carl Roth, Karlsruhe, Germany) at 2.5 mg/mL. Trypsin (sequencing grade, Promega, Germany) was added in the ratio 1:100 trypsin/protein, and incubated for 4 h at 37 °C. A second aliquot of trypsin was added and the solution was incubated overnight at 37 °C. All the containing peptides and proteins solutions were prepared in Protein Lobind tubes (Eppendorf AG, Germany).

The resulting peptides were reduced before labeling with threefold excess of tris(2-carboxyethyl)phosphine (TCEP, Sigma Aldrich, Germany) per disulfide bridge for 30 min at 50 °C. The labeling was carried out in 100 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, Acros Organics, Geel, Belgium) at pH 7, 6 M urea (Riedel-de Haën, Seelze, Germany) and tenfold excess per cysteine of MeCAT-Eu (Proteome Factory AG, Berlin, Germany) for 2 h at 37 °C.

Six different synthetic standard peptides (Table 1, Schafer-N, Copenhagen, Denmark) also were labeled following the same protocol and using MeCAT-Eu. These cysteinyl-standard peptides are present in BSA, transferrine or lysozyme sequences and were selected because they have different retentions in reverse-phase chromatography and were successfully identified by ESI-MS.

In both samples, final labeled peptide concentrations around 20 μM were obtained.

Purification and quantification of the standard peptides

Labeled peptides were used as calibration standards to determine the concentration of labeled peptides in different samples. For this purpose, the excess of MeCAT-Eu reagent was separated from the labeled peptides followed by ICP-MS quantification. An HPLC system (Agilent 1200 series, Germany) with a RP-C18 column (Zorbax SB-C18, 12.5× 2.1 mm, 5 μm, Agilent), see Table 2, was used for the purification. The collected fractions were dried in a Speed Vacuum device and reconstituted in 50 μL 5% ACN (acetonitrile), 0.1% FA (formic acid). The purity of the obtained solution was checked by LC-ICP-MS (Element XR, Thermo Fisher Scientific, Bremen, Germany) configured with a desolvating membrane nebulizer MCN-6000 (CETAC, USA), working in low-resolution mode. The identity of the labeled peptides was confirmed by electrospray ionization ion trap Fourier transform ion cyclotron resonance (ESI-IT-FTICR, Thermo Fisher Scientific, Bremen, Germany).

IDA was employed as quantification technique to determine the concentration of each labeled peptide after purification by direct infusion ICP-MS. The Eu quantification allows calculating the concentration of the peptide once the stoichiometry is known. Eu_2O_3 enriched in the isotope 151 (Eurisotop, Saint-Aubin Cedex, France) was used to prepare an isotopically enriched solution of about 10 mg L^{-1} which was validated in our laboratory (^{151}Eu 97.55%, ^{153}Eu 2.45%). The preparation and validation procedure consisted of solubilization of the Eu_2O_3 in HNO_3 5%, filtration through 25 μm nylon filters and quantification of the resulting solution at least five times over 1 month. Isotope abundance was also determined and compared with the supplier's report.

Quantification by external calibration

The elution gradient to resolve the standard labeled peptides was optimized for the RP-C18 column (Polaris 3 C_{18}-Ether, 150×1 mm, 5 μm, Varian). The chromatographic system was coupled to an ICP-MS equipped with a MCN-6000 nebulizer (CETAC, USA) to remove organic solvents from the eluent. With the elution conditions given in Table 3, peptides p2, p3, p4, and p6 could be resolved.

Thus, a mix of these four peptides in different concentrations was used to obtain an external calibration in a single chromatographic run. Calibration curves from 2 to 30 μg L^{-1}

Table 1 Synthetic standard peptides

Name	Amino acid sequence
p2	QNCDQFEK
p3	WWCNDGR
p4	DDPHACYSTVFDK
p5	SHCIAEVEK
p6	SCHTGLGR
p7	QEPERNECFLSHK

Table 2 Standard labeled peptides purification

Mobile phase A:	1% ACN. 0.1% FA	
Mobile phase B:	90% ACN. 0.1% FA	
Flow:	100 μL/min	
Injection volume:	200 μL	
Time (min)	**% B**	**Operation**
90	5	Cleaning
30	50	Fraction collection

Table 3 Experimental conditions for reverse-phase LC separation of labeled peptides

Mobile phase A:	1% ACN. 0.1% FA
Mobile phase B:	90% ACN. 0.1% FA
Flow:	40 μL/min
Injection volume:	2–5 μL
Post-column addition:	5 μg L^{-1} Pr at 3–5 μL/min
Elution program 1	
Time (min)	**% B**
0	5
2	5
3	15
5	20
40	23
43	50
Elution program 2	
Time (min)	**% B**
0	15
10	18
70	25
72	40

were tried successfully. Moreover, Pr was added post-column with a syringe pump as internal standard (Table 3, elution program 1). All the Eu chromatographic peaks were referred to the intensity of Pr to produce the calibration curve.

Post-column recovery determination

The recoveries of the standard labeled peptides from the Polaris 3 RP-C18 column were determined by species-unspecific isotope dilution analysis. The post-column addition of 5 μg L^{-1} elemental enriched Eu (^{151}Eu 97.55%, ^{153}Eu 2.45%) at 3 μL min^{-1} was carried out using a syringe pump. Mass bias and mass flow were calculated before every sample to ensure the accuracy and precision of the determinations. Mass bias factor was estimated using the exponential model while mass flow was calculated injecting 200 μL of 1 μg L^{-1} natural Eu with an external loop after the chromatographic column.

Results and discussion

Quantification of the purified standard labeled peptides by direct infusion IDA

The quantification of the purified labeled standard peptides was carried out by IDA. The spike/sample concentration ratio was around 1:1. The main problem found in the measurement of these labeled peptides was related to the introduction of the sample into the ICP-MS. The high hydrophobicity of some of the selected peptides led to strong interactions with tubes and glass components of the ICP-MS introduction system. Only using small amounts of organic solvents as ACN in the preparation of the diluted samples the peptides could be swept away properly. Thus, bad reproducibility was obtained for samples diluted with H_2O, 0.1% FA, with the addition of at least 5% ACN being necessary to ensure the correct measure of the labeled peptides. In the specific case of peptide p6, the low retention time in the C18 column (Fig. 1) indicates its low hydrophobicity and explains the good results obtained not only diluting the peptide in 5% ACN, 0.1% FA but also using aqueous solutions without organic solvents.

Considering the important effect of the solvent composition in the data reproducibility of the diluted samples measured by direct infusion ICP-MS, the solvent in which the purified peptides were reconstituted previously to the final dilution was also studied. The reconstitution of the dried labeled peptides after the purification step in pure H_2O or in different amounts of ACN did not affect the results in terms of precision and accuracy as long as the final dilution contains at least 5% ACN (data not shown). This behavior demonstrates that the homogeneity of the initial peptide solutions was not affected by the solvent composition, with the proper rinse of the sampling tubes being the main cause of the observed instability.

The peptides were determined three times over 3 weeks and each sample was prepared in triplicate. Desolvating membrane MCN-6000 and Meinhard-type nebulizers were compared resulting in similar quantitative results. Both were compatible with samples prepared in 5% ACN. In Table 4, the quantitative determinations of the labeled standard peptides using the MCN-6000 nebulizer are shown.

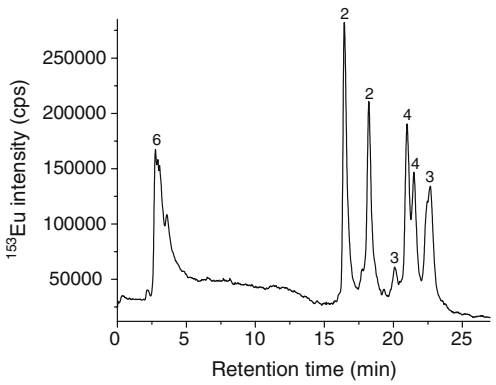

Fig. 1 LC(RP)-ICP-MS chromatogram of the calibration mix consist of the standard labeled peptides p2, p3, p4, and p6. Experimental conditions detailed in Table 3 (elution program 1)

Table 4 Quantification results by isotope dilution ICP-MS of the purified solutions containing standard labeled peptides

	Labeled Peptide					
	p2	p3	p4	p5	p6	p7
Batch 1 (n=3)	53.28	65.10	81.75	151.82	54.85	130.4
Batch 2 (n=3)	52.75	68.14	81.72	150.42	54.52	124.39
Batch 3 (n=3)	54.76	68.04	83.24	152.04	54.79	125.16
Average (μg L^{-1})	53.60	67.09	82.24	151.43	54.72	126.65
Std. dev. (μg L^{-1})	1.04	1.73	0.87	0.88	0.18	3.27
RSD (%)	1.94	2.57	1.06	0.58	0.32	2.58

External calibration curve for chromatographic applications

The quantification of peptides eluted from a chromatographic column entails the determination of the material loss inside the column. This can be achieved using specific internal standards of known concentration which allow the calculation of losses of each species. However, the preparation of individual internal standards is not feasible for complex samples containing a large number of peptides.

The second option is an external calibration of the whole procedure. Though in most cases the use of elemental standards is suitable, peptide quantification needs standards that interact similarly with the stationary phase, with standard labeled peptides being well suited for this task. Thus, the calibrated signal takes into account possible losses in the LC column, while the change in the instrumental response due to changing experimental conditions such as solvent composition is corrected by post-column addition of an elemental internal standard (Pr).

The consecutive injection of all concentrations used to build the calibration curve means spending a lot of time only in the calibration process. The challenge of building the calibration curve in a single chromatographic run has been tackled by taking advantage of the different retention times of the standard labeled peptides. For this purpose, a calibration mix consisting of standard labeled peptides in different concentrations has been prepared. The complete chromatographic resolution of the peptides contained in the calibration mix was the first requirement to achieve this goal.

Usually, all the MeCAT-labeled peptides elute from a RP column before 30% ACN is reached in the mobile phase. This chromatographic behavior explains the shallow gradients employed to resolve the standard labeled peptides. Furthermore, they allow working with small amounts of organic solvent maintaining almost constant the high sensitivity of the ICP-MS during the whole analysis. The experimental conditions employed to resolve peptides p6, p2, p4, and p3 by reverse-phase LC are detailed in Table 3 (Elution program 1). Double peaks were detected for some labeled peptides (Fig. 1), due to the resolution of the diastereomers from the MeCAT labeling reaction where the bond between maleimide and cysteinyl thiol creates a new chiral center. This explanation can be applied to peptide p4 in accordance with the LC-ESI-IT-FTICR data. In the case of p2, the first peak corresponds to the expected labeled peptide, while the second one to its pyroglutamyl form. Finally, the minor first peak of the peptide p3 was identified as a group of labeled impurities of the synthetic product, while the correct p3 sequence was found in the big peak at higher retention time. This diastereomeric separation has been previously observed for MeCAT-labeled peptides separated with RP chromatography [26]. However, the separation of isomeric MeCAT-labeled proteins never has been achieved in preceding works using 1D and 2D-SDS-PAGE techniques [24]. Alternatively, the formation of diastereomers can be avoided using a new MeCAT derivative that it is being developed currently in our group, which consist of the same complex with an iodoacetamide reactive group instead of the maleimide moiety. Although this new promising reagent yields no isomers in the cysteine labeling, it is necessary check its stability, robustness, and labeling efficiency before its application in quantification tasks.

The final post-column recoveries of the standard labeled peptides were determined by species-unspecific IDA [29]. This quantification technique does not consider the losses of the sample inside the column and for this reason it is suitable for the quantification of the variations in the expected concentration due to these column effects. Using the experimental settings described in the experimental section, recoveries between 90.9% and 96.9% were achieved measuring peptide mix of about 10 μg L^{-1} just after its preparation (Fig. 2). As in the case of total content determinations by direct infusion, 5% ACN was added to every sample injected in the LC. However, after 2 h, the same solutions presented very different recovery values, indicating instability over time of the standard peptide samples. This behavior was confirmed several times with different preparations of the same peptide mixture noticing an important decrease of the recovery over time. On the other hand, this effect was not observed in the quantification of the purified standard peptides by direct

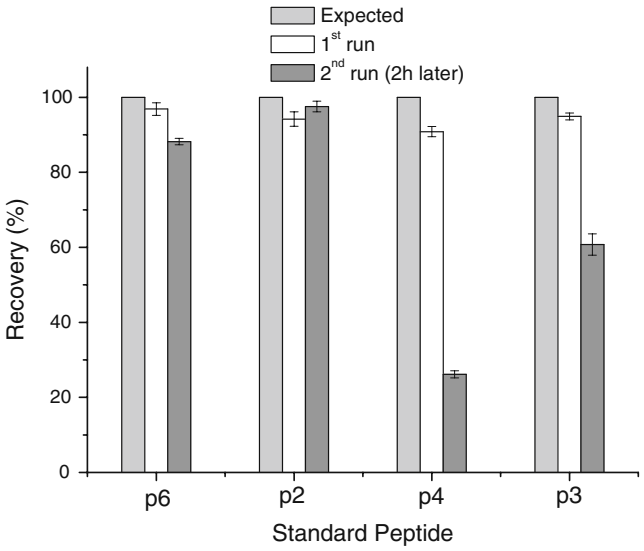

Fig. 2 Standard labeled peptide recoveries from a reverse-phase column

infusion ICP-MS, with the only difference being the use of low-binding material. Therefore, the problem seems to be related with the material of the vials used in the auto-sampler of the LC system which are not made of low-binding material. Therefore, losses of the peptides in the vials could be significant, working in the low microgram per liter concentration range. Although the problem was overcome measuring the calibration mix and the samples just after the preparation, new vials are being tested to avoid this problem. As can be seen in Fig. 2, the more hydrophobic peptides are more affected reaching recoveries as low as 26.2% for peptide p4.

The calibration curves were build injecting a mix of peptides p6, p2, p4, and p3 containing 30, 15, 8, and 2 μg L^{-1} respectively (Fig. 1). Trying to cope with the recovery problems described previously, the standard solutions were measured just after its preparation. The resulting calibration curves presented *R*-square values ranging from 0.993 to 0.999 and standard deviations lower than 0.06 μg L^{-1} were calculated for all the calibration points. The best observed limits of detection were around 1.6 fmol. The use of contaminated LC systems due to the previous use of ammonia buffers can increase the Eu background and adversely affect the limits of detection. The successful development of this calibration curve points to similar losses in the column for the four peptides although have very different hydrophobicities. Furthermore, it is a proof of the structural independency of the ICP-MS signal.

Quantification of labeled peptides

Two labeled standard peptides, p5 and p7, were selected as first samples to perform a quantification based on the multi-peptide external calibration. The mentioned standard labeled peptides were subjected to a reverse-phase separation using the same elution gradient employed to build the calibration curve (Table 3 elution program 1). The separated peptides are shown in Fig. 3a and b. The two peaks observed in the p5 chromatogram (Fig. 3a) correspond to the mentioned diastereomers generated in the MeCAT labeling. In the case of p7, up to four peaks can be observed due to the expected isomers and the partially conversion of the N-terminal glutamine residue to the pyroglutamic acid. Using RP-ESI-IT-FTICR (data not shown), the two first peaks were identified as the diastereomeric forms of the original labeled peptide while the second pair of peaks match with the expected masses for the pyroglutamylated labeled peptide.

The quantification results for both standard labeled peptides are presented in Table 5. Good values in terms of accuracy and precision were obtained for both samples confirming the applicability of the developed multi-peptide external calibration to the quantification of peptides.

Quantification of peptides from tryptic digestions

Finally, the applicability of the developed quantification methodology has been tested with samples of higher

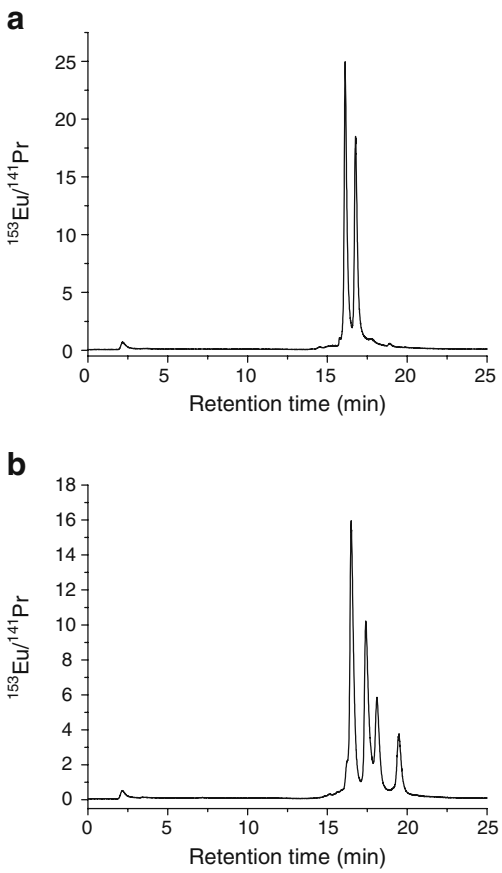

Fig. 3 LC(RP)-ICP-MS chromatogram of the labeled standard peptides **a** p5 and **b** p7

Table 5 Quantification results of standard labeled peptides, lysozyme and BSA by LC(RP)-ICP-MS using multi-peptide external calibration

Sample	Labeled Peptide	Theoretical concentration ($\mu g\ L^{-1}$)	Measured concentration ($\mu g\ L^{-1}$)	Standard Deviation ($\mu g\ L^{-1}$)	Recovery (%)
Standard peptides	p5 (n=3)	16.30	17.34	0.28	106.37±1.61
Standard peptides	p7 (n=3)	16.15	16.98	0.10	105.09±0,60
Lysozyme	A (n=3)	14.49	14.02	0.32	96.74±2.28
BSA	B (n=3)	17.36	16.67	0.46	95.99±2.62
BSA	C (n=3)	17.36	16.86	0.45	97.13±2.58
BSA	D (n=3)	17.36	16.42	0.39	94.56±2.27

complexity as lysozyme and bovine serum albumin, containing 8 and 35 cysteine residues, respectively. Though in most cases higher resolution in the separation is necessary, one-dimensional liquid chromatography can be enough to tackle the separation of the tryptic digested of single proteins playing with the elution parameters. Especially in the case of BSA, the high number of labeled peptides forces to extend the elution gradient to achieve enough chromatographic resolution. Thus, some labeled peptides were resolved and used for the determination of the protein concentration.

First, the feasibility of the multi-peptide external calibration was tested for digested lysozyme. This protein contains eight cysteines and more complex chromatograms than in the case of single-labeled peptides are expected. Reverse-phase ICP-MS chromatogram of lysozyme-labeled peptides is presented in Fig. 4a. From all the detected labeled peptides, only those completely resolved can be

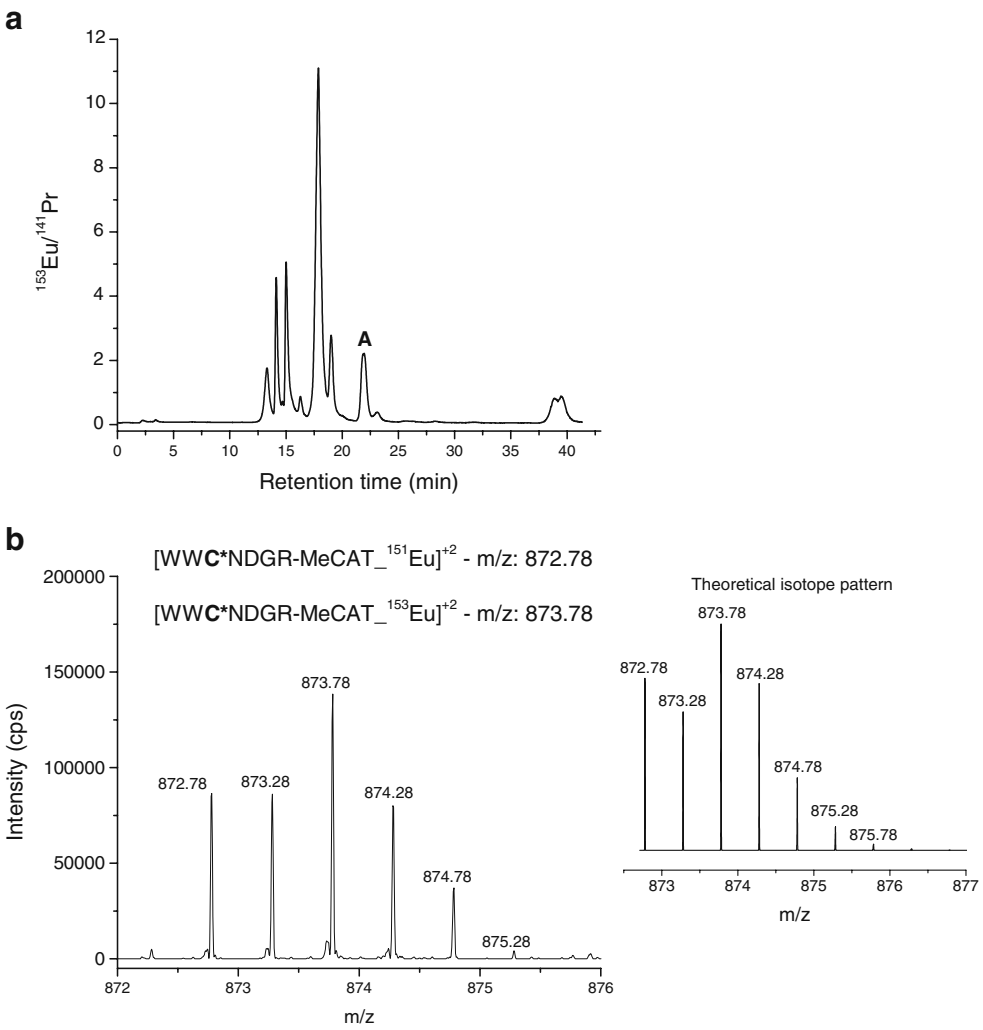

Fig. 4 **a** LC(RP)-ICP-MS chromatogram of a lysozyme tryptic digest labeled with MeCAT-Eu. **b** Experimental and theoretical LC(RP)-IT-FTICR spectra of peptide A

used for quantification in LC-ICP-MS. The peptide WWCNDGR (labeled as A in Fig. 4a) satisfies this requirement and matches with the peptide p3 employed in the calibration mix. In this case, no impurities were detected as in the case of the synthetic standard peptide. In addition, complete labeling of the studied protein was achieved using tenfold excess of the label multiplied by the number of cysteines, as R. Ahrends et al. [24] demonstrated in previous works. LC(RP)-ESI-IT-FTICR analysis allowed to check the correct digestion and labeling of the sample. The two steps used in the digestion procedure helped to achieve a complete digestion and no miss cleavages of the target peptide A were found. On the other hand, MeCAT/peptide stoichiometry also was confirmed by molecule-specific mass spectrometry (Fig. 4b). This parameter is essential for the subsequent calculation of peptide concentration in the sample. The exact masses detected with high-resolution MS confirm the ratio 1:1 peptide/MeCAT-Eu and showed the expected isotope pattern resulting from the interaction of the peptide A and both Eu isotopes. Table 5 shows the quantification results of the peptide A. Considering the observed 1:1 stoichiometry and the complete digestion of the studied peptide, the concentration measured for the peptide A match up with the concentration of the whole protein. Again, good accuracy and precision were obtained demonstrating that not only standard labeled peptides but also digested proteins can be quantified applying the new developed methodology based on MeCAT-labeled multi-peptide standards.

The developed quantification methodology was also applied for the quantification of BSA. The number of cysteines in the BSA is more than four times higher than in lysozyme, with a longer gradient to resolve the labeled peptides (Table 3, elution program 2) being necessary. As in the case of lysozyme parallel LC-ICP-MS and LC(RP)-ESI-IT-FTICR analysis were performed. No unlabeled BSA peptides were identified and no miss cleavages were found in 80% of the identified labeled peptides. These results confirm the suitability of the digestion and labeling procedures for different proteins and match with previous results [24]. Three labeled peptides without miss cleavages were resolved in the LC-ICP-MS chromatogram (peptides B, C, and D in Fig. 5). Peptides C and D present a slight resolution of their diastereomers while peptide B appears as a single Gaussian

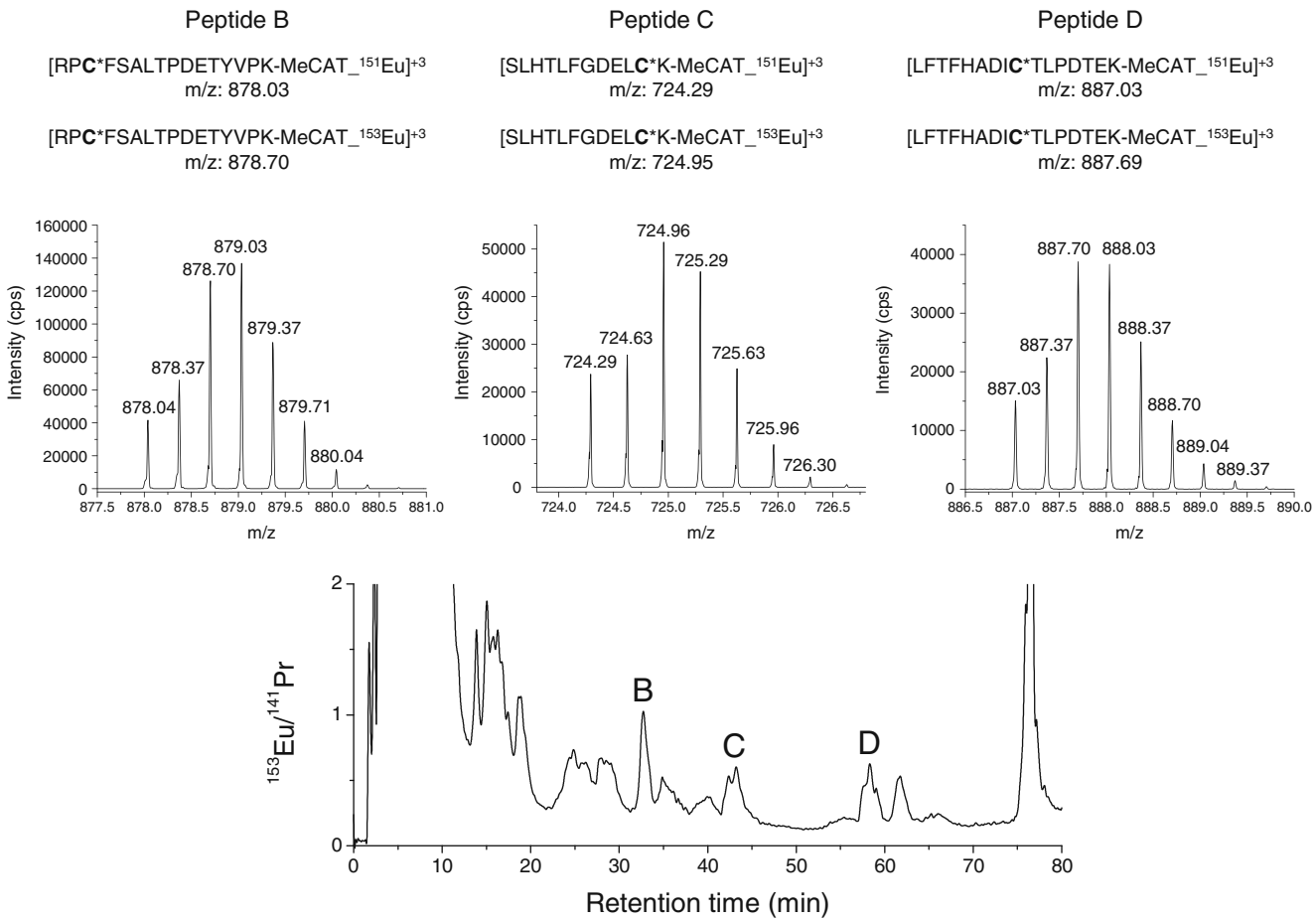

Fig. 5 LC(RP)-ICP-MS chromatogram of a BSA tryptic digest labeled with MeCAT-Eu. Experimental LC(RP)-IT-FTICR spectra of peptides B, C, and D

peak. At the beginning of the chromatogram, a huge peak corresponding to the required excess of MeCAT reagent, which is much higher than in the case of lysozyme due to the number of cysteines, can be observed. Combining the ICP-MS and the molecular mass spectrometry information, other incompletely resolved labeled peptides can be located. Peptides B, C, and D consist of one cysteine and the expected 1:1 stoichiometry was confirmed by ESI-MS (Fig. 5). Therefore, the concentration of each peptide should match up with the concentration of the intact protein. In Table 5 is presented the quantification results for BSA. For all the quantified peptides, recoveries higher than 94% and relative standard deviations lower than 3% were measured. Similar values were calculated for lysozyme (peptide A), with the precision obtained in the calculations for the standard labeled peptides p5 and p7 being higher.

In this work, samples of low and moderate complexity have been quantified using one liquid chromatography dimension. Unfortunately, samples of higher complexity may require additional separation steps and/or purification procedures to simplify the sample. The proposed multi-peptide calibration methodology has been successfully applied to relative complex samples as digested BSA and can be suitable for more complicated procedures involving several chromatographic separations. For this reason, new projects focused on the application of the multi-peptide methodology to bi-dimensional procedures are being developed. Although in the present work not very important sample losses were found, multidimensional separations increase noticeably the sample manipulation as well as losses. Thus, standard labeled peptides can be an interesting tool not only for the signal calibration but also for the evaluation of the sample losses. In addition, the inclusion of the mentioned new generation of MeCAT reagents can reduce the complexity of the chromatograms and improve the separations avoiding the diastereomeric double peaks.

Conclusions

Absolute quantification of peptides and proteins has been conducted using MeCAT labeling and LC-ICP-MS. Although MeCAT label has been studied extensively and its robustness and suitability for cysteine labeling was shown in previous works, the results presented now can be considered the first direct application of MeCAT to protein quantification. Moreover, it shows the potential of artificial elemental tags to perform absolute quantification based on ICP-MS.

The successful application of the presented strategy was possible due to the structure-independent response of the ICP-MS. The approach is designed to allow absolute quantification of species without the need of specific internal standards and can be applied to all the labeled peptides present in the sample. Furthermore, multiplex determinations of peptides and proteins can be tackled using the developed methodology since MeCAT reagents can be loaded with different lanthanides.

The quantification strategy proposed in this work corrects unknown losses of the labeled peptides on the column by subjecting the standards also to the chromatographic separation. The external calibration curve was obtained in one chromatographic run using a mix consisting of four standard labeled peptides in different concentrations.

The complementary use of ESI-MS techniques was paramount for the identification of the labeled peptides and proteins. Once again, the combined information obtained from ICP-MS and molecular mass spectrometry techniques is presented as a useful tool for proteomics studies.

Acknowledgements This work was financially supported by the Deutsche Forschungsgemeinschaft. D. E. acknowledges the European Commission for the post-doctoral Marie Curie Intra-European fellowship for career development under the seventh Framework Programme. The authors also want to thank Thermo Fisher Scientific for providing the Element XR mass spectrometer.

References

1. Prange A, Pröfrock D (2008) Chemical labels and natural element tags for the quantitative analysis of biomolecules. J Anal At Spectrom 23:432–459
2. Sanz-Medel A, Montes-Bayón M, Fernández de la Campa MR, Ruiz Encinar J, Bettmer J (2008) Elemental mass spectrometry for quantitative proteomics. Anal Bioanal Chem 390:3–16
3. Bettmer J, Montes Bayón M, Ruiz Encinar J, Fernández Sánchez ML, Fernández de la Campa MR, Sanz Medel A (2009) The emerging role of ICP-MS in proteomic analysis. J Proteom 72:989–1005
4. Wind M, Wegener A, Eisenmenger A, Kellner R, Lehmann WD (2003) Sulfur as the key element for quantitative protein analysis by capillary liquid chromatography coupled to element mass spectrometry. Angew Chem Int Ed 42:3425–3427
5. Navaza AP, Encinar JR, Sanz-Medel A (2007) Absolute and accurate quantification of protein phosphorylation by using an elemental phosphorus standard and element mass spectrometry. Angew Chem Int Ed 46:569–571
6. Siethoff C, Feldmann I, Jakubowski N, Linscheid M (1999) Quantitative determination of DNA adducts using liquid chromatography/electrospray ionization mass spectrometry and liquid chromatography/high-resolution inductively coupled plasma mass spectrometry. J Mass Spectrom 34:421–426
7. Kryukov GV, Castellano S, Novoselov SV, Lobanov AV, Zehtab O, Guigo R, Gladyshev VN (2003) Characterization of mammalian selenoproteomes. Science 300:1439–1443
8. Shah M, Wuilloud RG, Kannamkumarath SS, Caruso JA (2005) Iodine speciation studies in commercially available seaweed by coupling different chromatographic techniques with UV and ICP-MS detection. J Anal At Spectrom 20:176–182
9. Wrobel K, Wrobel K, Caruso JA (2005) Pretreatment procedures for characterization of arsenic and selenium species in complex samples utilizing coupled techniques with mass spectrometric detection. Anal Bioanal Chem 381:317–331

10. Esteban-Fernández D, Gómez-Gómez MM, Canas B, Verdaguer JM, Ramírez-Camacho R, Palacios MA (2007) Speciation analysis of platinum antitumoral drugs in impacted tissues. Talanta 72:768–773
11. Rappel C, Schaumlöffel D (2009) Absolute peptide quantification by lutetium labeling and nanoHPLC-ICPMS with isotope dilution analysis. Anal Chem 81:385–393
12. Edler M, Jakubowski N, Linscheid M (2006) Quantitative determination of melphalan DNA adducts using HPLC-inductively coupled mass spectrometry. J Mass Spectrom 41:507–516
13. Bettmer J (2010) Application of isotope dilution ICP-MS techniques to quantitative proteomics. Anal Bioanal Chem 397: 3495–3502
14. Edler M, Jakubowski N, Linscheid M (2005) Styrene oxide DNA adducts: quantitative determination using 31P monitoring. Anal Bioanl Chem 381:205–211
15. Esteban-Fernández D, Montes-Bayón M, Blanco González E, Gómez-Gómez M, Palacios MA, Sanz-Medel A (2008) Atomic (HPLC-ICP-MS) and molecular mass spectrometry (ESI-Q-TOF) to study cis-platin interactions with serum proteins. J Anal At Spectrom 23:378–84
16. Wind M, Edler M, Jakubowski N, Linscheid M, Wesch H, Lehmann WD (2001) Analysis of protein phosphorylation by capillary liquid chromatography coupled to element mass spectrometry with 31P detection and to electrospray mass spectrometry. Anal Chem 73:29–35
17. Ong SE, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A, Mann M (2002) Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Mol Cell Proteom 1:376–386
18. Mirgorodskaya OA, Kozmin YP, Titov MI, Korner R, Sonksen CP, Roepstorff (2000) Quantitation of peptides and proteins by matrix-assisted laser desorption/ionization mass spectrometry using O-18 labeled internal standards. Rapid Commun Mass Spectrom 14:1226–1232
19. Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R (1999) Quantitative analysis of complexprotein mixtures using isotope-coded affinity tags. Nat Biotechnol 17:994–999
20. Schnidt A, Kellermann J, Lottspeich F (2005) A novel strategy for quantitative proteomics using isotope-coded protein labels. Proteomics 5:4–15
21. Wiese S, Reidegeld KA, Meyer HE, Warscheid B (2007) Protein labeling by iTRAQ: a new tool for quantitative mass spectrometry in proteome research. Proteomics 7:340–350
22. Gerber SA, Rush J, Stemman O, Kirschner MW, Gygi SP (2003) Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. Proc Natl Acad Sci USA 100:6940–6945
23. Whetstone PA, Butlin NG, Corneillie TM, Meares CF (2004) Element-coded affinity tags for peptides and proteins. Bioconjug Chem 15:3–6
24. Ahrends R, Pieper S, Kühn A, Weisshoff H, Hamester M, Lindemann T, Scheler C, Lehmann K, Taubner K, Linscheid MW (2007) A metal-coded affinity tag approach to quantitative proteomics. Mol Cell Proteomics 6:1907–1916
25. Ahrends R, Pieper S, Neumann B, Scheler C, Linscheid MW (2009) Metal-coded affinity tag labeling: a demonstration of analytical robustness and suitability for biological applications. Anal Chem 81:2176–2184
26. Pieper S, Beck S, Ahrends R, Scheler C, Linscheid MW (2009) Rapid Commun Mass Spectrom 23:2045–2052
27. Yan X, Xu M, Yang L, Wang Q (2010) Absolute quantification of intact proteins via 1,4,7,10-tetraazacyclodecane-1,4,7-trisacetic acid-10-maleimidoethylacetamide-europium labeling and HPLC coupled with species-unspecific isotope dilution ICPMS. Anal Chem 82:1261–1269
28. Edler M, Metze D, Jakubowski N, Linscheid M (2002) Quantification of silylated organic compounds using gas chromatography coupled to IICP-MS. J Anal At Spectrom 17:1209–1212
29. Rottmann L, Heumann KG (1994) Development of an on-line isotope dilution technique with HPLC/ICP-MS for the accurate determination of elemental species. Fresenius J Anal Chem 350:221–227