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Short communication

Preliminary evaluation of a microwave-assisted metal-labeling strategy for quantification of peptides via RPLC–ICP-MS and the method of standard additions

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ABSTRACT

NIST has performed preliminary research on applying a calibration methodology based on the method of standard additions to the quantification of peptides via reverse-phase liquid chromatography coupled to inductively coupled plasma mass spectrometry (RPLC–ICP-MS). A microwave-assisted lanthanide labeling procedure was developed and applied to derivatize peptides using the macrocyclic bifunctional chemical chelator DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid), which significantly improved the lanthanide labeling yield and reduced reaction times compared to benchtop labeling procedures. Biomolecular MS technologies of matrix-assisted laser desorption ionization (MALDI)-MS and electrospray ionization (ESI)-MS/MS were used in concert with ICP-MS to confirm the results of microwave labeling, sample cleanup and standard additions experiments for several test peptides. The calibration scheme is outlined in detail and contextualized against complementary high accuracy calibration strategies currently employed for ICP-MS detection of biomolecules. Standard additions experiments using native, non-isotopic peptide calibrants confirm the simplicity of the scheme and the potential of applying a blending (recombined sample and spike) procedure, facilitating calibration via co-elution of lanthanide labeled peptides. Ways to improve and fully leverage the analytical methodology are highlighted.

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1. Introduction

Peptidomic and proteomic studies, respectively, rely on either the identification and detection of endogenous (bioactive) peptides, or peptides derived from enzymatically digested proteins in biological samples [1]. Concentrations of endogenous peptides are typically low in biological extracts, making the biomarkers challenging to detect and quantify in the presence of high abundance proteins, or even in the presence of fractional amounts of degraded high abundance proteins (proteolytic peptides). Quantification of peptides using classical radioimmunoassay or enzyme-linked immunosorbent assay (ELISA) techniques are restricted by the resolution required to quantify specific (modified) forms of a peptide, such as acylation, glycosylation and phosphorylation products [2]. Tandem mass spectrometry approaches provide adequate resolution to identify such post-translational modifications, but dynamic range and signal suppression can be an issue for peptide quantification, which is mostly accomplished using differential approaches where heavy and light isotopic internal standards are employed to derive estimates for relative amounts of peptides [3]. For absolute quantification of peptides via tandem mass spectrometry (MS), the AQUA^{TM} approach can be applied, using a peptide internal standard calibrant of the same form as the peptide of interest but modified isotopically (in ^{13}C or ^{15}N) [4]. Recently, Winter et al. [5] described the development of minimally permutated peptide (MIPA) calibrants for absolute quantification of peptides on similar platforms. In each technique a near ideal internal standard is spiked into the system such that multiple reaction monitoring (MRM) of the specific MS/MS transitions yields estimates for the absolute amount of peptide.

Liquid chromatography inductively coupled plasma mass spectrometry (LC)–ICP-MS presents an alternative platform for absolute quantification of proteins and peptides, employing ICP-MS detectable elements as proxies to measure biomolecules [6,7]. Proxy elements can be present in the analyte's native molecular structure or attached using derivatization procedures. Provided the that the ICP-MS can "see" the targets of interest, the robust ICP ionization source and the entire detection platform is less affected by signal suppression and compound-specific ionization differences over a wider dynamic range, relative to the soft ionization MS platforms (e.g. electrospray ionization MS/MS) [7,8]. This advantage is paid for with the destruction of molecular information and

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loss of additional mass-spectral specificity provided by MS/MS, so peptides measured via LC–ICP-MS using the same element signal require baseline resolution of analytes during the chromatographic separation step.

In this preliminary study, a calibration scheme based on the method of standard additions was developed and tested for absolute quantification of peptides via reverse-phase (RP) LC–ICP-MS, wherein a suite of test peptides and calibrants were derivatized and labeled with lanthanide metals prior to quantification. A microwave-assisted peptide bioconjugation and metal labeling approach was evaluated and applied to effect an improvement in peptide bioconjugation and chelation reaction times, and increase chemical labeling efficiencies. No real-world samples were measured in this initial study, but the methodology is being evaluated as to its potential application for absolute quantification of endogenous urinary peptides that serve as early onset indicators of acute kidney injury and organ transplant rejection [9–11].

1.1. High accuracy ICP-MS quantification strategies applicable to the absolute quantification of peptides

It is beyond the scope of this article to provide a comprehensive discussion on absolute quantification and detection of biomolecules (proteins, including metalloproteins, and peptides) by ICP-MS or biomolecular mass spectrometries, and communicate the associated analytical workflows. Interested readers are referred to several key articles and method-specific papers on these subjects that discuss current biomolecule labeling strategies [12–21], assay approaches [22,23], and quantification approaches [7,24–35]. Isotope dilution analysis (IDA) and the method of standard additions (MSA), discussed in more detail below, are the best strategies to employ for high accuracy quantification of peptides by LC–ICP-MS or ESI-MS/MS, as they more successfully handle drift, signal suppression and matrix effects compared to calibration methods based on external calibration or simple internal standardization. Considering specifically absolute quantification of biomolecules with ICP-MS, the current literature [24–35] shows that the isotope dilution analysis (IDA) strategies including species-specific, label-specific and post-column IDA have been applied successfully, and more frequently, to the quantification of proteins rather than peptides. The method of standard additions is scarcely mentioned [26] in conjunction with ICP-MS quantification of biomolecules of either type.

1.2. The method of standard additions (MSA)

The method of standard additions (MSA) has been applied to the absolute quantification of peptides and proteins using LC–MS/MS and MALDI-MS to help mitigate the matrix effects imposed during the measurement these analyte types in serological [36,37] and cellular [38] samples. This method has been neglected for absolute quantification of biomolecules in ICP-MS, likely due to a lack of suitable standards for peptides, and issues with metal loss (when measuring non-covalently bound trace elements in proteins). The quantification approach is favorable in terms of mass spectral linear dynamic range; a typical 3–10× addition of calibrant ensures that response differences between unspiked and spiked samples only span a single order of magnitude. Disadvantages of MSA quantification approaches include worse precision relative to isotope dilution quantification approaches and doubling of the analytical samples, making it less appealing for chromatography. A throughput advantage is gained relative to traditional split and spike standard addition approaches when unique lanthanide labels are employed. This facilitates co-elution of sample and standard, which reduces the number of analytical runs and minimizes column and ICP source drift relative to a (serial in time) LC–ICP-MS experiment. The combined sample and spike MSA calibration approach tested in this study is outlined in subsequent sections.

1.3. Microwave-assisted lanthanide labeling of peptides

The peptide labeling strategy applied utilized an amide targeting bifunctional chelator (BFC) based on DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) NHS ester to place lanthanide labels [12–14,16–21] on peptides prior to detection via RPLC–ICP-MS. The reader is referred to a review that covers the use of several different DOTA BFCs for the labeling of peptides specifically [39]. There are several advantages to labeling peptides with rare earth elements. Using the DOTA NHS ester BFC any amide-containing peptide can be derivatized to produce a response in ICP-MS detection space. After derivatization, numerous lanthanide elements (or corresponding purified isotopes) can be chelated to the derivatized analyte, lending favor to multiplexing samples or independent tracking of biomarkers of interest in recombined analytical samples. Lanthanide complexes of DOTA are among the most kinetically stable and inert [40] and can survive harsh method steps. This stability is important when ICP-MS quantification of peptides using the metal surrogate is the end goal. Disadvantages should not be ignored. Incomplete tagging or non-specific binding is an issue with any small chemical tag. Bifunctional chelators can decompose easily and offer slow reaction kinetics, which is one of the driving factors for attempting to speed up the metal labeling reactions using microwave chemistry in this study. Microwave heating increases molecular and ionic diffusion in the sample through dipole rotation and ionic conduction heating mechanisms, and is particularly suited to the derivatization of peptides and other molecules with strong dipole moments; speed (faster reaction kinetics), less side reactions, higher and purer product yields, and better reproducibility are well known [41]. These benefits are routinely leveraged in microwave synthesis of peptides [42] and have been applied to the peptide bioconjugation and metal chelation reactions using bifunctional chelators such as DOTA macrocyclics [43,44].

2. Experimental

2.1. Microwave-assisted peptide labeling

Water soluble DOTA NHS Ester was purchased from Macrocyclics, Inc. (Dallas, TX). Lanthanides of Tb, Tm and Ho were derived from single element spectrochemical solutions of the NIST SRM 3100 series of Standard Reference Materials (NIST, Gaithersburg, MD). Test peptides angiotensin I (DRVYIHPFHL, MW 1296 g/mol), angiotensin II (DRVYIHPF, MW 1046 g/mol), bradykinin (RPPGFSPFR, MW 1060 g/mol) and a MARCKS peptide clip (FKKSFKL, MW 897 g/mol) were purchased as high purity (>99% pure) lyophilized powders from Anaspec, Inc. (Freemont, CA). A MARS X microwave system (CEM Inc., Matthews, NC) was utilized for the microwave-assisted peptide bioconjugation and metal chelation experiments. A custom program was developed to monitor the temperature (via fiber optic probe) in a beaker of high purity deionized water that housed up to nine capped 2.0 mL microcentrifuge reaction tubes that were fully immersed in the water and symmetrically spaced in a round, PTFE turret. The bioconjugation part of the labeling process (derivatizing peptides with DOTA) required buffering the samples to a nominal pH of 8, using either a 0.1 mol/L triethylamine/acetic acid buffer (angiotensin (I and II), and bradykinin experiments), or a 0.1 mol/L bicarbonate/acetic acid buffer (MARCKS experiments). A 0.1 mol/L K_2HPO_4 buffer was used for bradykinin MSA tests. Peptides were reacted with the DOTA NHS ester for 5–10 min using a temperature ramp and hold program to a maximum of

60 °C, at 300 W of applied power. Subsequently, the heated water was exchanged for room temperature water, the samples were temporarily removed from the system, and Ho, Tb or Tm spikes (prepared in aqueous 0.1% mass fraction formic acid solutions) were introduced to perform the chelation reactions at a nominal pH of 6. The samples were then subjected to a second round of microwave irradiation (5 min) to chelate the metal to the DOTA-derivatized peptides. A typical experiment used a volume of 0.1–0.4 mL of peptide sample and spike solutions (0.1–5 mg/mL), 0.65–0.75 mL of DOTA solution (1–10 mg/mL) and 0.65–0.75 mL of metal solution (150 mg/mL), such that the total solution volume was kept under 2 mL.

2.2. Sample cleanup procedures

Solid phase extraction (SPE) using Strata X (Phenomenex, Inc., Torrance, CA) and Oasis HLB (Waters, Inc. Milford, MA) polymeric reverse phase media were employed to clean up the peptide samples after metal labeling. The procedure consisted of activating 500 mg of SPE sorbent with isopropanol (6 mL), rinsing with water, loading the microwave sample, performing two successive 3 mL washings with high purity, deionized water to remove the free lanthanides, and finally eluting the peptides with 2 mL of 100% isopropanol into LoBind™ microcentrifuge tubes (Eppendorf, Hauppauge, NY).

2.3. Standard additions experiments for peptides

Aqueous, buffered working peptide standards were prepared from the lyophilized solid peptide and a dilution was performed to create a lower concentration sample that was split (using equal volumes) into an unspiked and a spiked sample fraction. The spike sample received an additional aliquot of working peptide standard to increase the concentration in the spike sample roughly 4-fold relative to the unspiked sample, which received a dummy spike consisting of an equal volume of buffer absent peptide. The unspiked and spiked peptide samples were carried through the two-step microwave bioconjugation and chelation procedures outlined above, using different lanthanides. The resultant metal-labeled, unspiked and spiked samples were combined either prior to SPE cleanup (MARCKS peptide experiments) or after SPE cleanup (bradykinin experiments).

2.4. Mass spectrometric detection

Microwave-assisted peptide labeling efficiencies were evaluated by MALDI-MS, using an Applied Biosystems (Foster City, CA) Voyager-DE STR workstation operating in positive, linear mode. Approximately 1 μL of the SPE-processed peptide sample (angiotensin (I and II) and bradykinin standards) was spotted onto pre-dried matrix spots residing on the sample stage. The matrix was derived from 3,5-dimethoxy-4-hydroxycinnamic acid (AHCA) dissolved in 50:50 isopropanol:water, containing 0.1% mass fraction trifluoroacetic acid. One hundred fifty shots were used to generate each MALDI mass spectrum and at least three cumulative spectra were collected across each sample spot to evaluate matrix-related spectral variation within a sample.

Electrospray ionization (ESI) mass spectrometry was used to verify microwave bioconjugation and chelation of native peptides using the MARCKS peptide standard, and to perform standard additions measurements, where peptides labeled with different metals were blended for the purposes of effecting a MSA calibration. The SPE samples (isopropanol pull-down fractions) were diluted with water, acidified (0.1% formic acid) and directly infused into an Applied Biosystems (Foster City, CA) 4000 Q-TRAP LC/MS/MS system at 10 μL/min. No chromatographic separations were performed. Q1, enhanced resolution, and enhanced product ion (MS/MS) scans were collected.

A thermo (Franklin, MA) X7 collision cell ICP-MS was used to monitor lanthanide signals for microwave labeled peptides. A Dionex (Sunnyvale, CA) ICS-3000 dual pump LC system was used to interface liquid chromatography to ICP-MS. Separations were performed on a Phenomenex, Inc. (Torrance, CA) Luna C18(2) HST column (2.5 mm × 3 mm × 100 mm) using a flow rate of 0.325 mL/min and the flow rate was reduced to approximately 0.050 mL/min prior to ICP-MS, using a passive splitter. A linear peptide separation gradient 98.5%/1.5% volume fraction water/acetonitrile to 1.5%/98.5% volume fraction water/acetonitrile over 24 min was used to separate peptides, and no TFA modifier was used, in order to avoid low pH conditions and potential loss of metal on column from the chelation center of the labeled peptides. A combination of Pt cones, a cooled peek spray chamber (−5 °C) and oxygen introduction were used to handle the increased carbon loading of the ICP source imposed by the LC gradient. No collision cell gases were employed.

3. Results and discussion

3.1. Results for microwave labeling experiments

The main advantages of performing microwave labeling experiments (reduced reaction times and increased labeling yields) have been outlined; several results are now highlighted for angiotensin I, angiotensin II and bradykinin. The MALDI mass spectra in Fig. 1 shows the angiotensin II/Tm labeling results for non-irradiated (Fig. 1a) and microwave-irradiated (Fig. 1b) peptide control samples (with no DOTA or lanthanides introduced), a 10 min microwave reaction performed at 300 W and ramp to 60 °C (Fig. 1c), and a room temperature bioconjugation and chelation experiment (Fig. 1d) performed over the course of 4 h (2 h for derivatization and 2 h for metal chelation). Peptide control data show the native peptide peak (m/z = 1047 $[M+H]^+$ peak); no microwave-induced degradation products were observed. Fig. 1c and d shows the DOTA-derivatized and lanthanide-labeled product peptide peak at m/z = 1599 $[M+DOTA+Tm-2H]^+$, reflecting the bioconjugation and metal chelation steps, and loss of the NHS leaving group from DOTA-NHS. Count rates (based on MALDI-MS peak heights for reactant (native) and product peptides were used to determine crude estimates of the relative amount of peptide labeled. This concentration independent proxy for peptide labeling efficiency or yield assumes identical SPE recoveries and MS ionization efficiencies for both the unlabeled peptide and its metal-labeled complement). Referring to Fig. 1c, the microwave process produced a metal-labeled (product) peptide ion at m/z = 1599 that was roughly 55% of the base peak for the unlabeled (reactant) peptide ion (m/z = 1047), compared to approximately 15% of the base peak (Fig. 1d) for the room temperature reaction. The data communicate the relative amount of peptide labeled (calculated as the quotient of product counts to total reactant + product counts); 37% and 13%, respectively, for microwave versus room temperature labeling.

A full parametric optimization of the microwave parameters impacting peptide derivatization and metal chelation was not performed in this preliminary study, but two time trials (5 or 10 min temperature ramp to 60 °C) were performed to test the effect of microwave irradiation time on the peptide bioconjugation step, while holding the microwave irradiation time constant at 5 min for the secondary metal chelation step. The irradiation times and temperatures were chosen to fall within typical limits used in microwave synthesis of peptides. Fig. 2 displays example MALDI-MS spectra for angiotensin I. Control data (10 min microwave irradiation time, Fig. 2a) show the native peptide peak

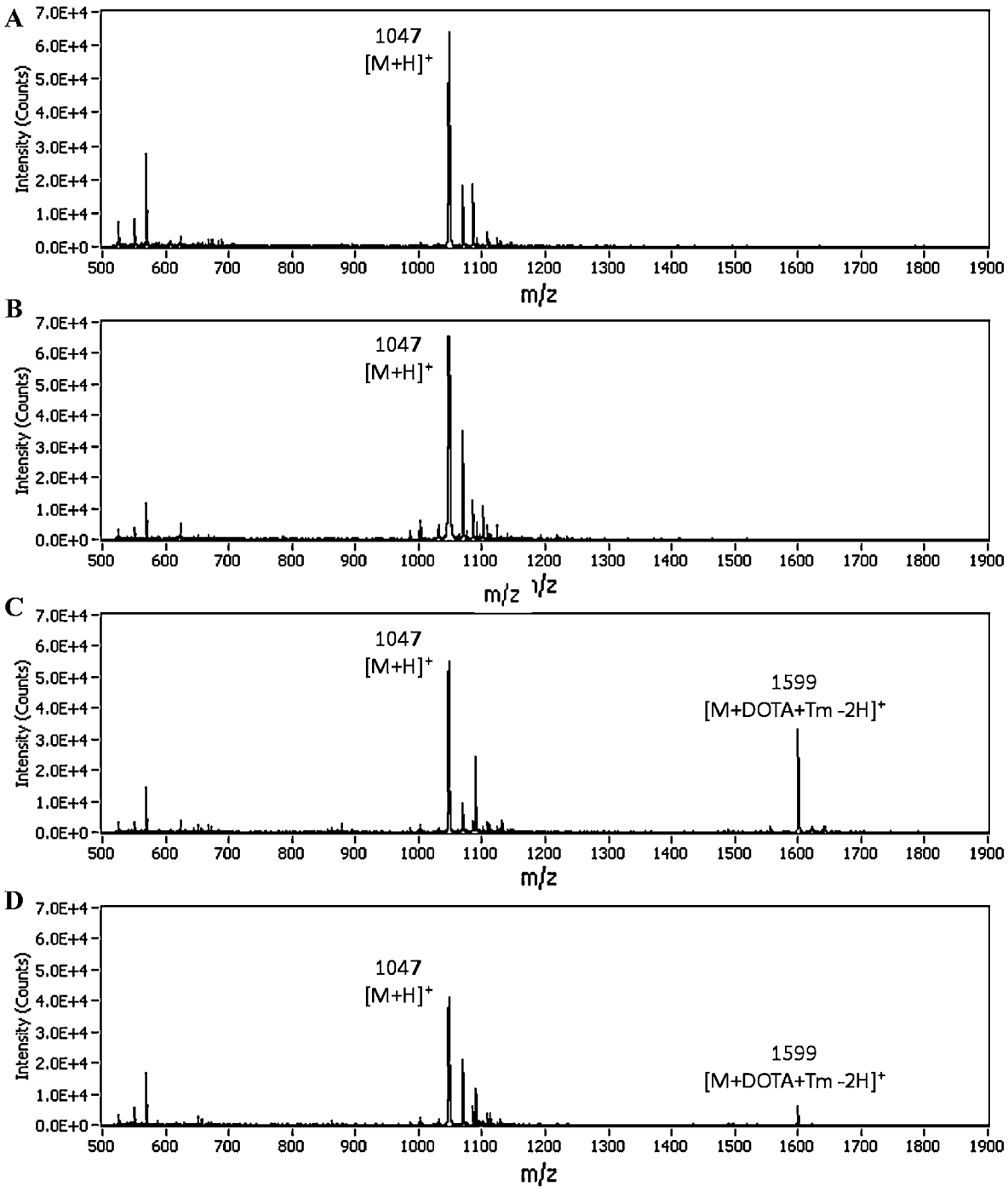

Fig. 1. Example MALDI-MS spectra for Tm-labeled angiotensin II. (a) Non-irradiated and (b) 10 min microwave-irradiated peptide control samples showing the $[M+H]^+$ peak at $m/z = 1047$. (c) 10 min microwave bioconjugation/5 min microwave chelation showing the $[M+DOTA+Tm-2H]^+$ peak at $m/z = 1599$. (d) 4 h room temperature Tm labeling experiment.

($m/z = 1297$ $[M+H]^+$ peak). Fig. 2b (10 min microwave derivatization time) and Fig. 2c (5 min microwave derivatization time) show the DOTA-derivatized and lanthanide-labeled product peptide peak at $m/z = 1850$ $[M+DOTA+Tm-2H]^+$. The complementary bar graph in Fig. 3 summarizes the results for repeat bioconjugation time trials using angiotensin I $m/z = 1849$: $m/z = 1297$ product and reactant counts to estimate the relative amount of labeled peptide. Despite the somewhat large within-spot ratio uncertainties (potentially an artifact of analyte heterogeneity in the MALDI matrix), the relative peptide labeling yield is higher and the trial repeatability is improved when applying the shorter 5 min irradiation time for the DOTA bioconjugation step. This counterintuitive result is likely related to the slope of the temperature ramp to 60 °C, which is steeper for 5 min versus 10 min irradiation during the peptide bioconjugation step. However the data are preliminary; full parametric optimization of the microwave labeling scheme is needed, including both the bioconjugation and chelation step irradiation times, and the reaction temperature. Collectively, the data presented in Figs. 1–3 indicate that the bioconjugation and chelation of peptides is improved using microwave irradiation from both a speed and efficiency standpoint, but concrete conclusions cannot be drawn until more data is collected. Absolute labeling yields

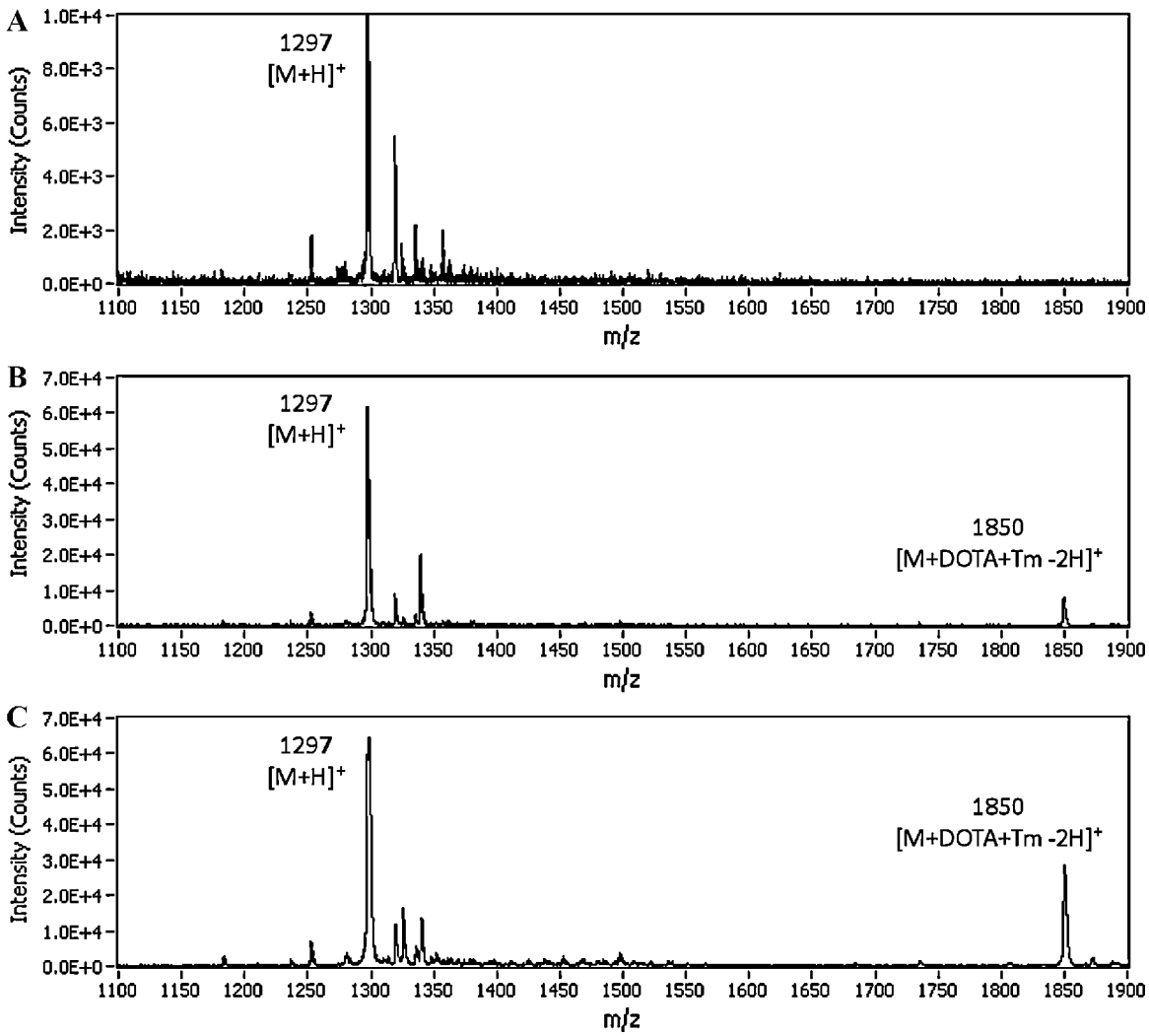

Fig. 2. Example MALDI-MS spectra of Tm-labeled angiotensin I. (a) 10 min microwave-irradiated peptide control sample showing the $[M+H]^+$ peak at $m/z = 1297$. (b) 10 min microwave bioconjugation/5 min microwave chelation showing the $[M+DOTA+Tm-2H]^+$ peak at $m/z = 1850$. (c) 5 min microwave bioconjugation/5 min microwave chelation.

cannot be determined until comprehensive SPE recovery studies are completed using an AQUA standard to measure the loss of peptide reactant and a lanthanide-labeled AQUA standard to measure creation of peptide product.

Peptides possess unique physiochemical characteristics that may affect overall peptide labeling yield. One example is hydrophilicity, which is related to polarity, or peptide dipole moment strength that could affect peptide reaction kinetics through stronger or weaker dielectric heating under conditions of microwave irradiation. Angiotensin I and bradykinin labeling studies were conducted, and relative yields were compared using the MALDI-MS approach for samples processed under identical sample preparation and microwave conditions (applying the 10 min bioconjugation and 5 min Tm chelation procedure). The labeling yields and method reproducibilities were approximately $13 \pm 4\%$ and $34 \pm 6\%$ ($k = 2$, 95% confidence intervals), respectively, for angiotensin I and bradykinin, based on three independent trials of the overall microwave labeling, chelation, sample cleanup and MS detection processes. A significant difference in peptide labeling efficiency was observed, favoring bradykinin, despite the large uncertainties observed for the independent trials. This is likely related to differences in peptide polarity and peptide charge, which impact the overall dipole moment vectors of the respective molecules, and in turn, peptide kinetic motion in the oscillating electric field. Bradykinin possesses a net charge of 2 at the derivatization pH (pH ≈ 8), and an average hydrophilicity of 0.1, based on the Hopp and Woods scale [45], compared to angiotensin I, which possesses a net charge of 0.2, and an average hydrophilicity of −0.5. For reference, the hydrophilicity scale ranges from −3.5 to 3.5. An RPLC–ICP-MS elution order study (data not shown) was used to confirm that more polar, Tm-labeled bradykinin ($T_r = 11$ min) eluted before Tm-labeled angiotensin I ($T_r = 12.5$ min).

3.2. Description of standard additions calibration scheme

One goal of the study was to design an MSA approach that leverages some of the benefits of both the traditional ID-MS and the post-column ID-MS quantification methodologies, by developing ways to deal with the problem of the lack of species-specific ICP-MS amenable calibrants for traditional ID-MS, while eliminating the recovery factor correction requirements for post-column ID-ICP-MS. The conceptual calibration scheme developed for RPLC–ICP-MS quantification of peptides is outlined in Fig. 4. First, the sample is split into two equal volume or mass portions and a known amount of peptide calibrant is added to the spiked sample (typically 3–10×), while the unspiked sample is volume or mass corrected by adding a dummy aliquot of the same buffer (less peptide) used for dissolution of the calibrant. The main point of emphasis is that the calibrant is a native, purified peptide, not an isotopic variant (in C, H, N or O or an ICP-MS detectable element). Next, the peptides

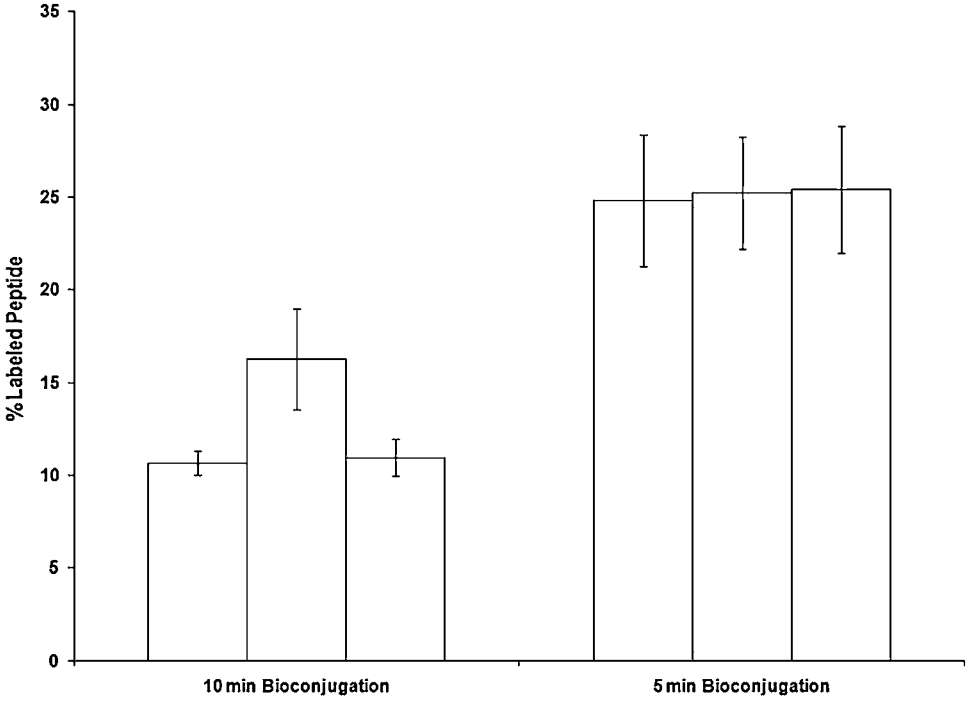

Fig. 3. Results for microwave time trials for triplicate 10 min and 5 min DOTA/angiotensin I bioconjugation reactions, using a fixed Tm chelation time of 5 min. Uncertainty bars represent within-MALDI spot standard uncertainty for triplicate 150 shot acquisitions.

are derivatized through a bioconjugation reaction with DOTA NHS ester and the derivatized peptides are chelated using either two different lanthanide elements, or alternatively, two purified isotopes of the same lanthanide element. This process facilitates labeling the derivatized peptides in the unspiked and spiked sample pools with unique metal identifiers. Once unique element or isotope identifiers are assigned to sample and spike, the samples can be recombined to achieve a significant time throughput advantage relative to performing serial separation and detection of the unspiked and spiked samples. Blending the samples also minimizes chromatographic and ICP-MS instrumental drift, which increases accuracy, because each co-eluting unspiked sample/spiked sample peak set efficiently produces the two-point calibration data used for quantification. These blended sample approaches are routinely applied in differential proteomics measurements [3], and are effectively leveraged for the MSA. The scheme outlined accommodates multiplex peptide analyses, and variable spike concentration analyses that are theoretically only limited by the number of unique peptide calibrants introduced into the system. Practically, quantification of a small mixture of peptides should be possible. An RPLC–ICP-MS chromatogram would produce lanthanide signals for metal-labeled peptides with a ratio of approximately unity for peptides lacking a complementary peptide calibrant, and would produce lanthanide ratios greater than unity for each specific calibrant peptide spiked into the system (refer to the conceptual chromatogram in Fig. 4). This calibration-effected signal offset offers advantages for rapid identification of analyte peaks in complex chromatograms.

3.3. ESI-MS/MS and RPLC–ICP-MS results for MARCKS peptide characterization and standard additions experiments

Standard addition experiments were performed using the scheme outlined in Fig. 4 for a MARCKS peptide and peptide characterization and quantification results were obtained using ESI-MS/MS and RPLC–ICP-MS monitoring. Fig. 5 shows the resultant ESI-MS/MS mass spectra for the metal-lableled peptides. The enhanced resolution scan (Fig. 5a) confirms the presence of both Ho- and Tm-labeled peptides, as indicated by the 4 g/mol difference in m/z for the peak sets and the higher response for the Tm peptide (spiked) sample. Collision induced dissociation was applied to deconstruct the derivatized peptides and verify that the 4 g/mol difference carried through to the lanthanides. A collision energy study was performed (data not shown) using potentials of 60 V, 75 V, 90 V and 130 V to determine the optimal collision potential required to fragment the Tm-labeled peptide (m/z = 1468 [M+DOTA+Tm−2H+H_2O]$^+$) using enhanced product ion scanning mode. Maximum product ion yields for the DOTA-Tm tag (m/z = 571.3) and the native peptide (M+H, m/z = 897.7) products were obtained using a collision potential of 75 V. Employing a 90 V potential resulted in near 100% elimination of the labeled peptide, with a moderate reduction in product ion yields, relative to the 75 V case. Enhanced product ion scans collected for the m/z = 1464 and m/z = 1468 parent ions (Fig. 5b and c), using a collision potential equal to 90 V, produced the MARCKS peptide [M+H]$^+$ (m/z = 897.7) molecular ion, corresponding to the native, unlabeled peptide (MW ≈ 897 g/mol), and produced characteristic fragment ions of metal-incorporated DOTA at m/z = 567.3 [DOTA+Ho−2H+H_2O]$^+$ and m/z = 571.3 [DOTA+Tm−2H+H_2O]$^+$, respectively, for the Ho- and Tm-labeled peptide variants. Note again the 4 g/mol difference in the DOTA fragments, corresponding to the two lanthanides employed. Further MS/MS on the DOTA mass fragment ions using a collision potential of 130 V, produced the corresponding singly charged metal ions (Fig. 5d and e). The results obtained using the ESI-MS platform showed that the standard additions experiment worked as a proof-of-concept test, but the measured spike/unspiked sample signal ratio (3.57, refer to Fig. 5a) was biased lower than the expected ratio of 4, which translates to a 10% bias in the expected ratio, and correspondingly, a 17% bias in the absolute peptide data (Table 1). Complementary MSA data for MARCKS peptide were obtained with RPLC–ICP-MS. The chromatogram in Fig. 6 displays the Ho and Tm traces along with their corresponding oxides (inverted chromatogram). Addition of oxygen to the ICP source (to accommodate the RPLC mobile phase) resulted in the formation of significant levels of lanthanide oxides.

Table 1
ESI-MS and RPLC–ICP-MS results for MARCKs peptide standard additions experiments.

MARCKs MSA experiment	Measured ratio	Expected ratio	Ratio bias (%)	Measured peptide amount (μg)	Expected peptide amount (μg)	Amount bias (%)
ESI-MS	3.57	4.00	−10.7	119.5	102.4	16.7
ICP-MS (no oxide correction)	6.32	4.00	58.0	57.7	102.4	−43.6
ICP-MS (with oxide correction)	4.05	4.00	1.2	100.8	102.4	−1.6

Holmium oxide comprised approximately 51% of the total measured Ho signal, and similarly, TmO^+ comprised approximately 24% of the total measured Tm signal. The ICP-MS data (Table 1) showed poor agreement with expected values when oxide correction was neglected and good agreement with expected values when the lanthanide signals were corrected for oxide losses. Applying this correction was necessary because the measurement was standardless in ICP-MS detection space, and required a full accounting of the lanthanides in the eluting peptide peaks to produce accurate results. This oxide correction concept has been applied to the quantification of rare earth elements in glass samples using laser ablation ICP-MS [46].

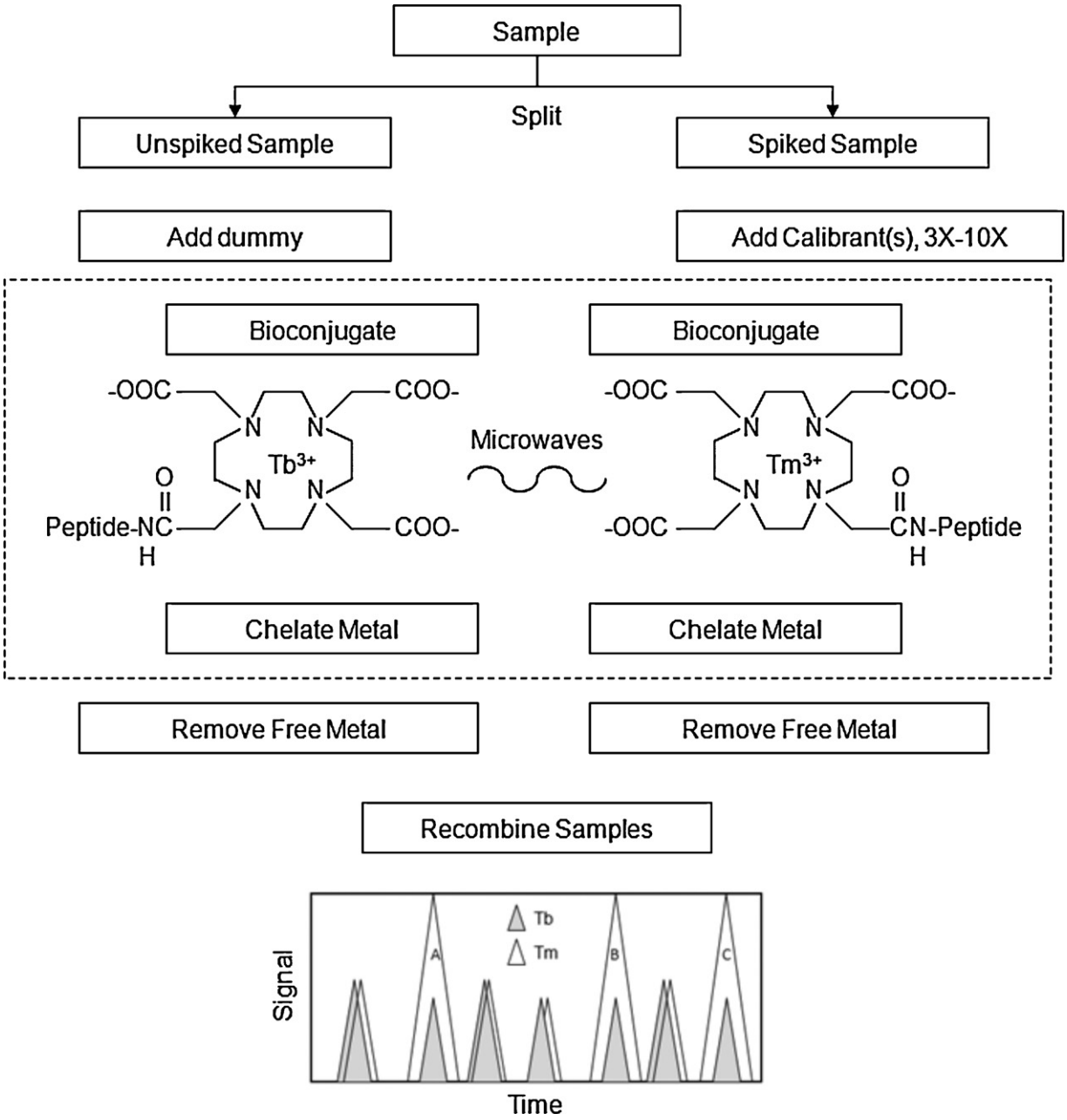

Fig. 4. Outline of calibration scheme and workflow for quantification of lanthanide-labeled peptides using the method of standard additions and RPLC–ICP-MS. The peaks labeled A, B and C in the conceptual chromatogram indicate peptide calibrants added to the spike portion of the sample and labeled with a lanthanide element.

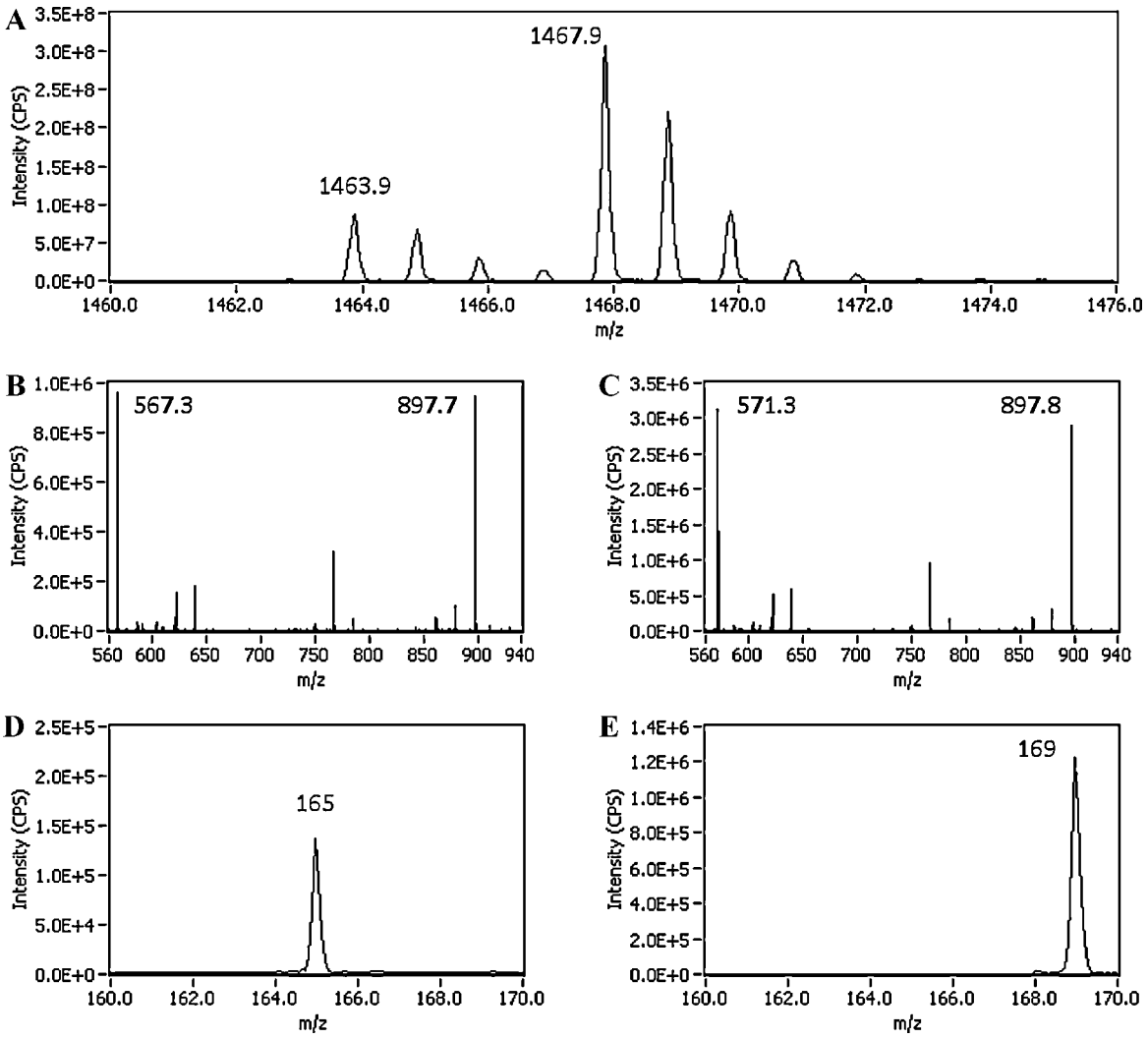

Fig. 5. ESI-MS/MS spectra for the MARCKS peptide standard additions experiment. (a) Enhanced resolution scan showing Ho-labeled and Tm-labeled MARCKS peptide peak sets differing by 4 g/mol, with m/z values at 1463.9 and 1467.9 representing $[M+DOTA+Ho-2H+H_2O]^+$ and $[M+DOTA+Tm-2H+H_2O]^+$, respectively. Enhanced product ion scans of the $m/z = 1463.9$ and $m/z = 1467.9$ ions collected using a collision potential of 90 V show respectively, in (b) the Ho-DOTA fragment $[DOTA+Ho-2H+H_2O]^+$ at $m/z = 567.3$ and MARCKS peptide $[M+H]^+$ peak at $m/z = 897.7$, and in (c) the Tm-DOTA fragment $[DOTA+Tm-2H+H_2O]^+$ at $m/z = 571.3$ and MARCKS peptide $[M+H]^+$ peak at $m/z = 897.8$. Enhanced product ion scans of the Ho-DOTA and Tm-DOTA fragment ions in (b) and (c) were collected using a collision potential of 130 V to verify that the 4 g/mol difference in the peptide peaks related to the corresponding lanthanides in (d) Ho^+ at $m/z = 165$ and (e) Tm^+ at $m/z = 169$.

3.4. RPLC–ICP-MS results for bradykinin standard additions experiments

Experiments were performed using bradykinin (MW 1060 g/mol) to test for recovery-related biases during the sample cleanup (free lanthanide removal) step. Terbium (Tb) and Tm were employed as the lanthanide pair system. The experimental design roughly followed the procedures outlined in the previous paragraph for MARCKS peptides, except the metal-labeled unspiked and spiked samples were not combined until after the SPE cleanup step. This facilitated removal of free lanthanides that could potentially undergo scrambling reactions [17] or metal substitutions at the DOTA chelation site. The experiment was conducted in a forward and reverse fashion, with either Tb or Tm in the spike sample, and the opposite lanthanide in the unspiked sample, to test the consistency of the entire method and detection platform to produce the expected 4-fold sensitivity difference between the unspiked and spiked samples. Fig. 7 displays two chromatograms corresponding to the forward and reverse standard addition experiments. The resultant oxide-corrected spike ratios and concentration data for the bradykinin experiments listed in Table 2 show reasonable agreement with expected values, but indicate that the calibration response was not conserved when the positions for the lanthanide pairs were switched between the unspiked and spiked samples. This is most likely related to incomplete metal-labeled peptide recovery prior to blending of unspiked and spiked samples. Large oxide corrections were required for both experiments. When Tb was employed as the spike, TbO^+ and TmO^+ respectively, comprised roughly 30% and 8% of the total signal for the corresponding lanthanide. When Tm was employed as the spike, TbO^+ and TmO^+ respectively, comprised approximately 73% and 27% of the total signal for the corresponding lanthanide.

3.5. Assessment of calibration biases

More experiments will be needed to fully evaluate the biases and uncertainties of the method at each stage of the analytical workflow. Peptide derivatization and metal labeling procedures, sample cleanup and analyte separation procedures, and MS ionization source and platform effects can all contribute to bias measurement results. The data suggest that utilizing purified isotopes of the same lanthanide element would help eliminate potential biases

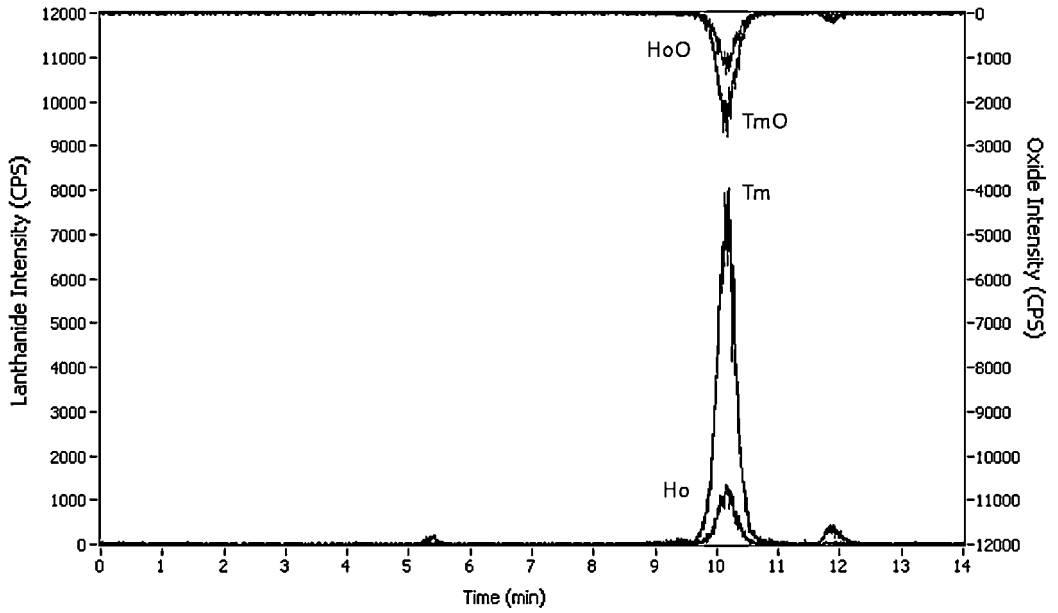

Fig. 6. RPLC–ICP-MS chromatograms for lanthanide and lanthanide oxides (inverted chromatogram) obtained for the MARCKs peptide standard additions experiment.

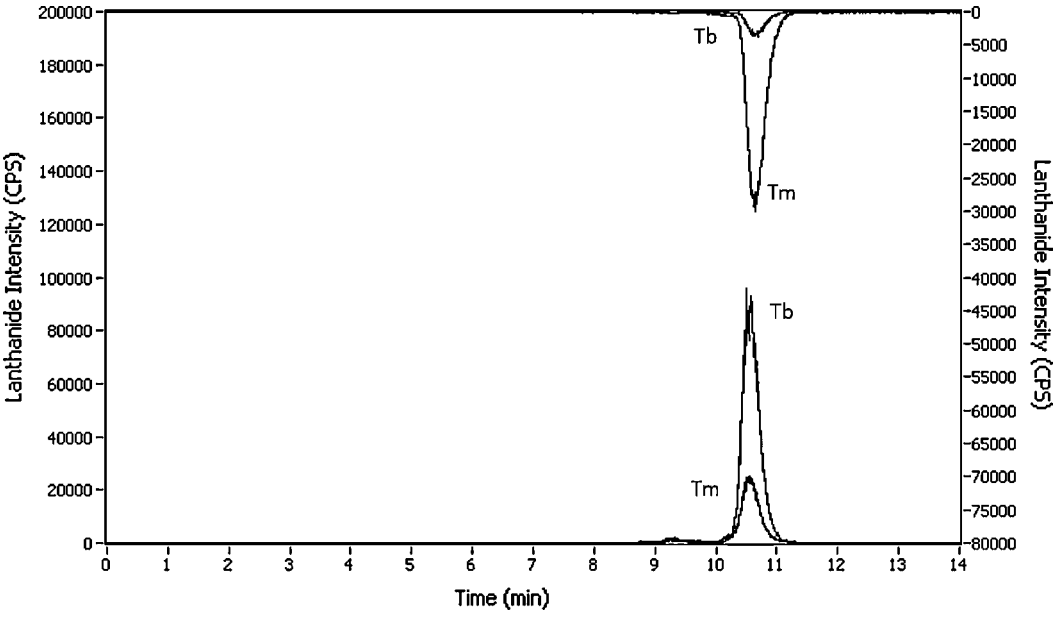

Fig. 7. RPLC–ICP-MS chromatograms for forward and reverse (inverted chromatogram) bradykinin standard additions experiments.

(different metal incorporation efficiencies) occurring at the metal labeling step, minimize recovery related biases during chromatographic cleanup and separation steps and minimize ICP-MS-related biases caused by differences in ionization efficiency, mass response and metal-oxide formation rates. The data for the MARCKS peptide experiments, wherein unspiked and spiked pools of metal-labeled peptides were blended after lanthanide labeling, but prior to SPE cleanup, suggests that scrambling of free lanthanides and chromatographic column effects are relatively minor concerns for calibration bias. The bradykinin MSA tests showed that blending unspiked and spiked samples after independent SPE processing may induce analyte recovery-related calibration biases.

Table 2
RPLC–ICP-MS results for forward and reverse bradykinin standard additions experiments.

Bradykinin MSA experiment	Measured ratio	Expected ratio	Ratio bias (%)	Measured peptide amount (μg)	Expected peptide amount (μg)	Amount bias (%)
Tb in spike	4.10	4.13	−0.7	329.4	326.5	0.9
Tm in spike	3.78	4.13	−8.3	366.5	326.5	12.2

4. Conclusions

The use of microwave energy to derivatize peptides and prepare them for ICP-MS detection warrants further study. Proof-of-principle research on the quantification of peptides via RPLC–ICP-MS showed that the standard additions calibration scheme using DOTA bioconjugation and lanthanide labeling has potential to serve as a complementary calibration strategy to LC-MS/MS quantification of peptides. Future work will involve optimizing all steps of the analytical workflow from derivatization to detection and performing method validation studies for quantification of peptides in NISTs clinical biofluid SRMs.

Disclaimer

Certain commercial equipment or instruments are identified in the article to specify adequately the experimental procedures; such identification does not convey recommendations or endorsements by the National Institute of Standards and Technology, nor does it imply that the equipment or instruments are the best available for the purpose.

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