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Compensation of gradient related effects when using capillary liquid chromatography and inductively coupled plasma mass spectrometry for the absolute quantification of phosphorylated peptides

Daniel Pröfrock*, Andreas Prange

Marine Bioanalytical Chemistry, Institute for Coastal Research, GKSS Research Centre Geesthacht, Max-Planck Str. 1, 21502 Geesthacht, Germany

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ABSTRACT

The application of reversed phase liquid chromatography (RP-LC) hyphenated to inductively coupled plasma mass spectrometry (ICP-MS) for the accurate quantification of bio-molecules via covalently bound hetero atoms such as phosphorus is restricted, due to the known effects of increasing amounts of organic solvents on the ionization behavior of certain elements. An approach for the compensation of variations in the elemental response, due to changes in the solvent composition during the RP gradient separation of phosphorylated peptides is described, which includes the application of a second, matched reversed gradient, that is mixed post-column with the RP column outflow before entering the LC–ICP-MS interface. The experimental design allows the application of gradient separations, while the element-specific detection is carried out under isocratic conditions with a constant organic solvent intake into the plasma. A constant elemental response is a general pre-requisite for the application of ICP-MS for the absolute quantification of peptides via their hetero atom content, especially when no corresponding high purity standards are available or natural mono-isotopic hetero element tags are utilized. As complementary technique LC–electrospray ionization linear ion trap mass spectrometry (ESI-QTRAP-MS) has been used for peptide identification and to elucidate their phosphorus stoichiometry. Highly reproducible separations have been obtained with retention time and peak area RSDs of 0.05% and 7.6% ($n = 6$), respectively. Detection limits for phosphorus of 6 $\mu g\,L^{-1}$ (6 pg absolute), have been realized, which corresponds to approximately 200 fmol of an average molecular weight, singly phosphorylated peptide. In addition an automatic routine for flow injection analysis (FIA) at the end of each chromatographic separation has been developed, to calibrate each chromatographic separation, which allows absolute quantification of the separated species, whenever their tag stoichiometry is known. Phosphorylated peptides as well as tryptic protein digests have been used as model compounds for method development and to demonstrate the applicability of the proposed setup for phosphopeptide quantification on the basis of simple inorganic phosphorus standards.

1. Introduction

Besides the qualitative analysis of a proteome, its quantitative determination is of growing interest, since only the quantity of proteins or changes in their abundance reflect the status of and changes inside a defined biological system [1]. As the basic structure of a protein is genetically encoded the real time dynamic and regulation of its structure as well as its functions are generally depending on specific post-translational modifications, which cause for example their phosphorylation [2–5]. Up to 30% of all proteins in an eukaryotic cell are supposed to be phosphorylated at any one time, indicating the biological importance of this process [5]. Recently Kirkpatrick et al. stated, that the ongoing large scale identification of proteins and post-translational modifications has enhanced the understanding of cellular processes, however there is still an inherent lack of quantitative information, associated with these datasets [6].

A number of stable isotope based labels with different chemical properties such as ICAT [7], ITRAQ [8–12], ICPL [13,14], IDBEST [15] or techniques such as SILAC, which allows to incorporate labelled aminoacids into proteins during cell culture experiments [16,17] have been recently introduced for the comparative investigation of different sample states, allowing the *relative quantification* of differentially expressed proteomes. As an alternative natural occurring, ICP-MS detectable elements such as sulfur or phosphorus represent powerful "tags", which can be utilized for selected applications within the field of protein biochemistry or proteomics, such as qualitative and quantitative protein phosphorylation analysis. As

* Corresponding author. Tel.: +49 0 4152 87 2846; fax: +49 0 4152 87 1875.
E-mail addresses: daniel.proefrock@gkss.de, HProefrock@gmx.de (D. Pröfrock).

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an element-specific detector ICP-MS features some unique properties with respect to calibration and quantification of bio-molecules since it provides a much larger linear dynamic detection range (up to 9 orders of magnitude) compared to organic MS techniques. In comparison to the ESI or MALDI ionization process, the main advantage of the ICP as an ion source is its compound independency, since its sensitivity is proportional to the number of detectable atoms inside the molecule investigated. Therefore, a compound independent calibration and quantification can often be performed, without the need for specific standards [18]. As a consequence the utilization of ICP-MS for the determination of bio-molecules such as phosphorylated proteins and peptides via their covalently bound (hetero)elements or via chemical element labels, represents a current emerging strategy within the field of absolute protein or peptide quantification [19–21].

Wind et al. pioneered the field of ICP-MS assisted protein phosphorylation analysis by using capillary LC hyphenated to either elemental and molecule specific MS as real complementary techniques, allowing the fast screening of complex samples for such functionalities [22,23] or the quantification of protein phosphorylation degrees [24]. The development of new interface systems helped to overcome some of the instrumental limitations of the first approach described by Wind et al. allowing the direct nebulisation inside the ICP torch without the detrimental effects of, e.g. direct injection high efficiency nebuliser (DIHEN) nebulisers. This helps in particular to overcome the well known problems related with the introduction of reversed phase gradients, containing high amounts of organic solvents into the ICP, namely carbon buildup, signal suppression or plasma instabilities, without the need to use techniques such as membrane desolvation [25].

As described in the literature the addition of a constant amount of carbon containing compounds (e.g. organic solvents) [26,27] or gases (e.g. nitrogen, helium) [28,29] to the ICP can lead to an improved ionization process for difficult to ionize elements such as S (enhancement under dry plasma conditions, decrease when using LC), As, Se or P, resulting in improved sensitivities. As a result even at low flow rates as used in nano- or capillary LC significant changes in the elemental response with changing gradient composition can be observed, which complicates accurate *quantitative* analysis of, e.g. phosphorus containing compounds, especially when no matched analyte standards are available.

To overcome this limitation first reports describe the utilization of mathematical correction functions derived by monitoring the influence on the phosphorus signal while running a blank organic gradient were both solvents have been spiked with the same amount of phosphorus [24,30]. However the acquisition of the data for the correction function is often time consuming and has to be repeated on a regular basis to account for instrumental changes. Different authors also describe the application of membrane desolvation to remove organic solvents from the sample aerosol, which results in a significant elimination of gradient related changes of the instrumental response [31,32]. However also the loss of late eluting phosphorylated peptides inside the membrane desolvator has been reported [22].

The application of *constant post-column sheath flows* to buffer solvent related changes in the elemental response during gradient elution has been already described in the recent literature. For the quantification of selenium containing peptides Giusti et al. adapt RP-nano-LC at a flow rate of 340 nL min^{-1} to the optimal flow rate of the used interface by utilizing a constant sheath flow of 3.8 μL min^{-1} 30% acetonitril. Gradients with 10–70% acetonitril have been applied, however even at nano-liter flow rates a small impact on the instrumental response especially at the end of the gradient has been observed [33].

Recently Navaza et al. described the application of a constant sheath flow of 5.5 μL min^{-1} containing 40% acetonitril to compensate gradient related changes in the ICP-MS response during RP-capillary LC separations at a flow rate of 3.5 μL min^{-1}. The approach has been used for the absolute quantification of phosphopeptides, using bis(4-nitro-phenyl)phosphate (BNPP) as internal phosphorus standard. This approach allows to partly compensate the impact of the acetonitril gradient. However at a higher organic solvent load an impact on the instrumental response has been observed, which might influence especially the accurate quantification of late eluting, hydrophobic phosphopeptides [34,35].

Here we describes a technical development to overcome the problems related with the application of a constant sheath flow for buffering gradient related effects during the capillary LC separation of phosphorylated peptides via utilizing a matched *reversed gradient post-column sheath flow*. This approach maintains the percentage of organic solvent, which finally enters the plasma at a constant level over the whole gradient. The instrumental setup allows the separation of the analytes using *gradient conditions*, while the element-specific detection is carried out under *isocratic conditions*. In addition an automated flow injection analysis (FIA) at the end of the gradient separation has been implemented, which allows the injection of a certified phosphorus calibration standard. Phosphopeptides as well as tryptic protein digests have been used to demonstrate the potential of this new generic approach for absolute quantification of phosphorylated peptide. To our knowledge this is the first report on the application of a reversed gradient sheath flow to compensate gradient related effects, when using capillary LC–ICP-MS for the absolute quantification of phosphorylated peptides.

2. Experimental

2.1. Chemicals and standards

Ultra pure water (18 MΩ cm) was prepared using a Millipore Elix 3/Milli-Q Element water purification system (Millipore, Milford, MA, USA). Methanol, acetonitril (Lichrosolv, hypergrade) and formic acid (100% suprapur) as well as single element standards of P, Y and Ce were obtained from Merck (Merck, Darmstadt, Germany). Trifluoroacetic acid (TFA), bis(4-nitro-phenyl)phosphate (BNPP) and Bovine α and β casein were obtained from Sigma (Sigma–Aldrich, Deisenhofen, Germany). A further phosphorus standard was obtained from Aldrich (Sigma–Aldrich, Deisenhofen, Germany). Ammonium bicarbonate was purchased from Fluka (Fluka, Buchs, Switzerland). Model phosphoproteins used during this study Protein Tyrosine Phosphatase Peptide Substrate (MW 1118.0, sequence ENDpYINASL), IR10 Insulin Receptor [1142–1153] (pY1151) (MW 1703.0, sequence TRDIYETDYpYRK), $pp60^{c\text{-}src}$ C-terminal Phosphoregulatory Peptide (MW 1543.7, sequence TSTEPQpYQPGENL) and Protein Phosphatase Peptide Substrate (MW 752.7, sequence RRApTVA) were purchased from Biomol (Biomol International, Hamburg, Germany). Trypsin was purchased from Promega (Promega Corporation, Madison WI, USA). Helium 5.6 (99.9996%) was used as collision gas inside the octopole reaction system. Argon 5.0 (99.999%) was used as plasma gas. A mixture of 10% oxygen (5.0) and 90% argon (4.8) was used as additional spray chamber gas. All gases were obtained from Air Liquide (Air Liquide, Lübeck, Germany). Solution and standards were prepared under clean room conditions (class 1000) in a clean bench (class 100) to avoid elemental contamination as well as particles which could block the micro-scale capillaries used.

2.2. Instrumentation

2.2.1. Capillary HPLC

An Agilent 1100 series capillary LC system consisting of two four channel on-line degassers, two capillary LC pumps, a cooled micro-wellplate autosampler, a column oven with a 6 port micro-