Simultaneous quantitation of peptides and phosphopeptides by capLC-ICP-MS using the Agilent 8800 Triple Quadrupole ICP-MS

Application note

Proteomics

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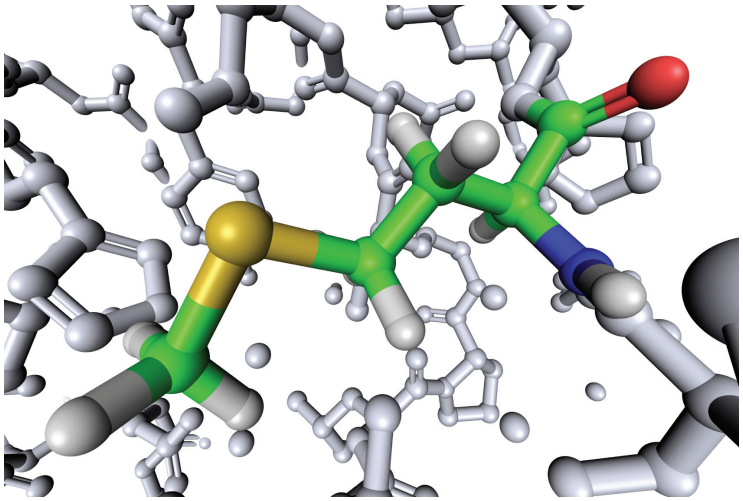

Abstract

In combination with capillary LC (capLC) separation, the Agilent 8800 Triple Quadrupole ICP-MS, operating in MS/MS mass shift mode, was used for the trace measurement of phosphorus (P) in phosphopeptides and sulfur (S) in S-containing peptides. The lowest absolute detection limits ever reported for the analysis of S- and P-containing species (11 fmol and 6.6 fmol, respectively) using LC-ICP-MS were achieved. Effective interference removal was confirmed by good agreement with the theoretical isotope ratio for S. Excellent peak profiles and signal to noise (S/N) ratios were observed for both P and S. The great potential of triple quadrupole capLC-ICP-MS for sensitive and simultaneous absolute quantitation of P- and S-containing peptides using non-specific standards is demonstrated.

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Introduction

LC-MS/MS is used for the quantification of target proteins in pharma/biopharma and clinical research. The approach generally relies on the use of synthetic isotopically labeled proteins and peptides, which are used as internal standards for the quantification of the corresponding target compound. In contrast, LC-ICP-MS enables non-species specific quantitation of proteins and peptides by measuring the heteroatoms S and P contained in the target compounds. In this way, different compounds containing the heteroatom may be used and thus can be quantified using a single non-proteinaceous heteroatom-containing compound as a generic standard. Unfortunately, detection limits (DLs) for P and S are compromised by their high ionization potential and by multiple polyatomic interferences with single quadrupole ICP-MS (ICP-QMS) – even when the collision/reaction cells (CRC) are employed. Sector field high resolution ICP-MS (HR-ICP-MS) has also been applied, but so far no ICP-MS instrument has achieved DLs (especially for sulfur) equivalent to compound DLs reported using ESI-MS/MS with isotopically labeled synthetic peptides. Table 1 lists published data for P and S DLs using LC-ICP-MS, showing the ICP-MS technology and separation technique used. In this work, the Agilent 8800 Triple Quadrupole ICP-MS (also referred to as ICP-QQQ) was applied to the determination of proteins and peptides by the absolute quantitation of P and S heteroatoms.

Compared to conventional or ICP-QMS, the Agilent 8800 features an additional quadrupole mass filter (Q1), situated in front of the Octopole Reaction System (ORS^3) cell and quadrupole mass filter (now called Q2). In this tandem MS configuration, Q1 operates as a mass filter, allowing only the target analyte mass to enter the cell, and rejecting all other masses. Because plasma and matrix ions are eliminated by Q1, this ensures that the reaction processes in the ORS^3 are precisely controlled and so accurate measurements can be performed even in complex, high matrix samples, while sensitivity is significantly increased. Chromatographic separation was performed by an Agilent cap-LC system coupled to the 8800 ICP-QQQ.

Experimental

Reagents and materials

Ultra pure water (Millipore, Tokyo, Japan), HPLC and analytical reagent grade acetonitrile and formic acid (Wako Pure Chemical, Ltd., Osaka, Japan). ICP-MS standards (SPEX CertiPrep, Inc., Metuchen, NJ, USA). Bis(4-nitrophenyl) phosphate (BNPP, 99% purity) and methionine (≥ 99% 82 purity) (Sigma Aldrich, Steinheim, Germany). Amino acid sequences of the phosphopeptide standards were LRRApSLG and KRSpYEEHIP, and the S- containing peptide standards were ACTPERMAE and VPMLK. All peptides were purchased from AnaSpec (Fremont, CA, USA) with purity ≥ 95%.

Table 1. Critical comparison between detection limits obtained for the analysis of S- and P-containing species using different ICP-MS instruments

	Phosphorus			Sulfur		
Instrument	**Separation technique**	**DL (compound)**	**Reference**	**Separation technique**	**DL (compound)**	**Reference**
ICP-QMS Collision cell (He)	capLC LC capLC CE	110 fmol 0.97 pmol 63 fmol 19 fmol	1 2 3 2			
ICP-QMS Collision cell (Xe)				nanoLC CE	1.5 pmol 2.6 pmol	6 7
ICP-QMS DRC (O_2)	LC	2.2 pmol	4	microLC microLC LC	2.7 pmol 3.1 pmol 2.3 pmol	8 9 4
HR-ICP-MS	capLC capLC	100 fmol 50 fmol	5 10	microLC capLC capLC	9 pmol 11 pmol 10 pmol	11 12 13
ICP-MS Triple Quad	capLC	6.6 fmol	This paper	capLC	11 fmol	This paper

Preparation of P- and S-containing standards

Calibration standards containing P and S at 0, 25, 50, 100 and 200 ng/mL (as the element) using BNPP and methionine were prepared in a matrix of 1% mobile phase B (which was the starting point of the LC gradient).

Preparation of the phosphopeptide solutions

To confirm the retention time and purity of each peptide, three separate solutions (100 ng/mL , as the element) were prepared. Mixture 1 contained LRRApSLG and ACTPERMAE and mixture 2 contained KRSpYEEHIP and VPMLK. Both mixtures contained approximately 100 ng/mL of BNPP and methionine. Mixture 3 was prepared after 1:1 mixing of mixtures 1 and 2 by weight.

Instrumentation

capLC system

An Agilent 1200 Series with Agilent Zorbax SB C18, 5 µm, 150 x 0.3 mm reversed phase capillary column was used. Mobile phases A and B consisted of water and acetonitrile respectively. Both phases contained 0.1% formic acid and 10 ng/mL Ge (used as ICP-MS ISTD). Injection volume was 1-2 µL. LC flow rate was 5 µL/min, and the LC gradient was 0-3 min: 1 % B, isocratic. 3-35 min: 1-60% B linear.

capLC-ICP-MS interface

The capLC column was connected to the ICP-MS using the Agilent capLC interface (Agilent G3680A capillary LC interface kit, Figure 1), which consists of a total consumption nebulizer inside a small quartz spray chamber.

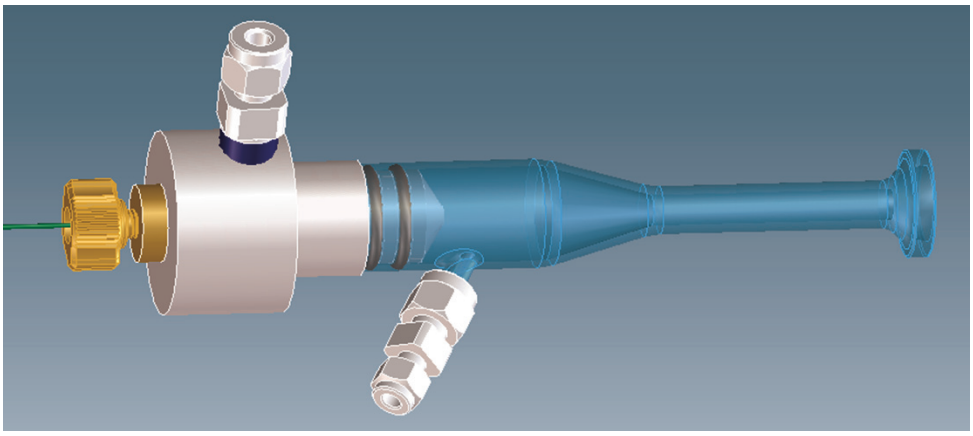

Figure 1. Agilent capillary LC interface kit (G3680A)

ICP-MS

An Agilent 8800 Triple Quadrupole ICP-MS was used. Oxygen ($Ar:O_2$ mixture, 8:2) was added to the nebulizer gas at 80 mL/min. Cell gas: oxygen at 0.35 mL/min. Sample flow rate 5 µL/min. Integration time was 150 ms (one point per peak) for ^{31}P, ^{32}S and ^{34}S. The Agilent 8800 was optimized for lowest P and S DLs using a standard containing 100 ng/mL P and S in 30% acetonitrile with a flow rate of 5 µL/min, introduced with a syringe pump. Operation of the LC and integration of the chromatographic peaks was performed using the Agilent ICP-MS MassHunter software.

Removal of interferences on S and P using the Agilent 8800 in MS/MS mode

The ICP-QQQ was operated in MS/MS mass shift mode to remove interferences on P and S. In Figure 2, the upper diagram (a) shows Q1 set to *m/z* 32 which allows $^{32}S^+$ to pass through to the cell, rejecting interferences at all other masses (including those at *m/z* 48). S^+ is converted to SO^+ in the cell by reaction with O_2. Q2 is set to *m/z* 48, which allows SO^+ to pass to the detector, rejecting all the original interferences on S at *m/z* 32. Interferences at *m/z* 48 were previously rejected by Q1. This is the key to the interference removal power of the QQQ configuration — it allows controlled, accurate, and interference-free measurement of reaction product ions.

The lower diagram (b) in Figure 2 shows the settings for P determination: Q1 is set to *m/z* 31, which allows $^{31}P^+$ to pass through, rejecting interferences at all other masses (including *m/z* 47). $^{31}P^+$ is converted to PO^+ in the cell by reaction with O_2. Q2 is set to *m/z* 47 to allow the reaction product PO^+ to pass to the detector. Interferences at *m/z* 47 were previously rejected by Q1. The interferences removed by Q1: ($^{15}N^{16}O^+$, $^{14}N^{16}OH^+$, $^{14}N^{17}O^+$, $^{12}C^{18}OH^+$ on $^{31}P^+$ and $^{16}O_2{}^+$, $^{15}N^{17}O^+$, $^{15}N^{16}OH^+$, $^{14}N^{18}O^+$ on $^{32}S^+$) and Q2 ($^{47}Ti^+$, $^{32}S^{14}NH^+$, $^{32}S^{15}N^+$, $^{15}N^{16}O_2{}^+$, $^{12}C^{35}Cl^+$ on $^{31}P^{16}O^+$ at *m/z* 47, and $^{48}Ti^+$, $^{36}Ar^{12}C^+$, $P^{16}OH^+$, $^{31}P^{17}O^+$, $^{48}Ca^+$ on SO^+ at *m/z* 48), are shown in each diagram.

Note also that without the presence of Q1, polyatomic species derived from P and S would interfere with each other, likely affecting P/S ratios, measurement of P- and S-containing species (leading to errors in the calculation of the degree of phosphorylation), and S isotope ratios (e.g. leading to errors in isotope dilution calculations). Using the 8800 in MS/MS mass shift mode, reaction product ions retain the correct isotopic pattern of the parent ions – this is not the case in reaction cell ICP-QMS. In ICP-QMS there is no Q1, so both isotopes of S enter the cell and react to produce SO reaction product ions which overlap each other.

Initially, DLs were measured in a high organic matrix (30% acetonitrile, delivered continuously via the syringe pump) to assess the interference removal efficiency of the Agilent 8800. DLs achieved were 0.6 ng/mL for ^{31}P and 1.2 ng/mL for ^{32}S with BECs of 9 ng/mL for ^{31}P and 5 ng/mL for ^{32}S. These were significantly lower in aqueous solution, which suggests the presence of P and S in the acetonitrile. To confirm interference removal on S, the 34/32 ratio was measured. Since interferences are much higher on ^{32}S, a 34/32 ratio matching the theoretical value for $^{34/32}S$ indicates effective removal of interferences. The 34/32 ratio in the blank (0.0484 ±0.0017) agreed well with the standard (0.0487 ±0.0012). The IUPAC value for $^{34/32}S$ is 0.0447 ±0.0025. The difference between observed and theoretical is due to mass bias (the 8800 ratios were not mass bias corrected). The -4.0% to -4.3% mass discrimination factor matches that reported in the literature [14-16], demonstrating that all interferences on S are effectively removed by the 8800, and that S is present in the acetonitrile.

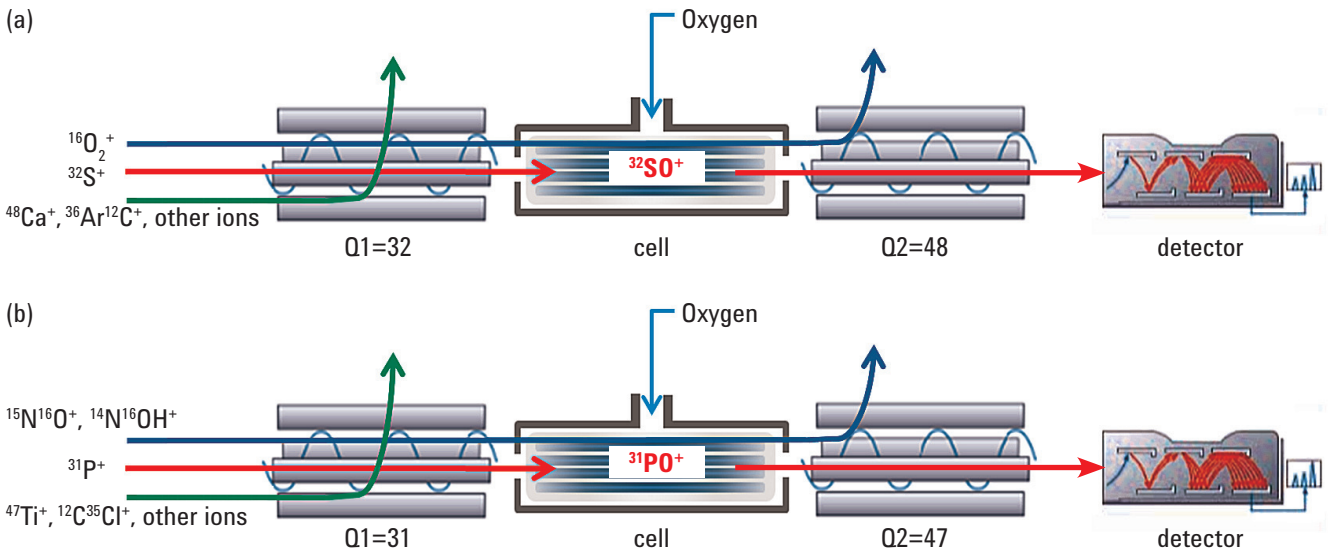

Figure 2. 8800 ICP-QQQ MS/MS operation in mass shift mode to remove interferences on S (a) and P (b)

Results and discussion

Switching to capLC-ICP-MS operation, calibration standards containing 0, 25, 50, 100 and 200 ng/mL of both P and S (BNPP and methionine, respectively) were injected and measured: excellent linearity and RDSs of <4% were obtained (Figure 3).

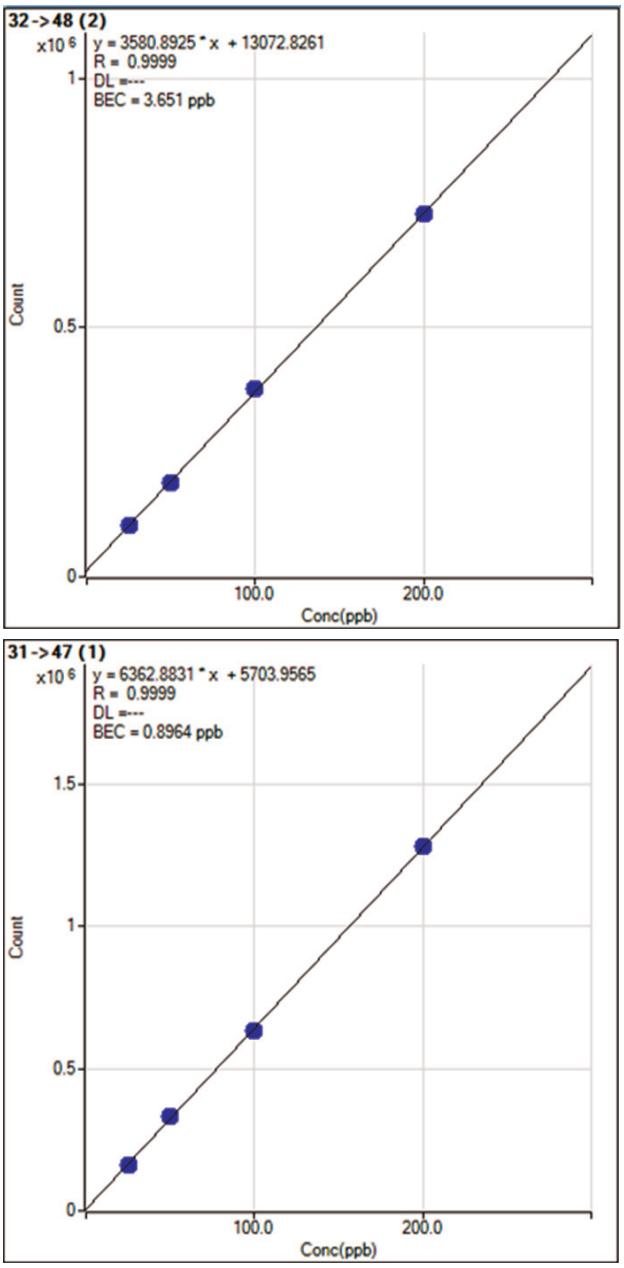

Figure 3. Calibration plots for ^{32}S and ^{31}P (methionine and BNPP, respectively) at 25, 50,100 and 200 ng/mL (as element).
Injection volume = 2 µL, flow rate = 5 µL/min, gradient given in text.

The chromatogram obtained for the 50 ng/mL standard is shown in Figure 4. Signal to noise (S/N) ratio observed was excellent for both elements. In LC-ICP-MS operation, the background due to P and S in the acetonitrile mobile phase can be effectively subtracted, allowing the detection power of the Agilent 8800 to be accurately measured. DLs obtained were 0.10 ng/mL P and 0.18 ng/mL S (6.6 fmol P, 11 fmol S absolute). We believe these to be the lowest absolute DLs ever reported for S and P by LC-ICP-MS, as summarized in Table 1.

Measurement of phosphopeptides and S-containing peptides

The LC-ICP-MS system was then used to measure phosphopeptides and S-containing peptides. The retention time for each peptide was calculated by two different solutions — mixtures 1 and 2 as described in the experimental section. Mixture 3, which contained approximately 45 ng/mL of each of the peptides and 105 ng/mL (as P and S) of the standard was measured under the same instrumental conditions. The chromatogram obtained from mixture 3 is shown in Figure 5. Both peptides and the standard for both P and S are clearly shown. Only the pTyr-containing peptide exhibited a small shoulder, likely corresponding to a phosphopeptide impurity, accounting for 9% of the signal. S/N ratios observed were excellent (no smoothing performed). The average DL for the S-containing peptides (0.26 ng/mL S), was slightly higher than that obtained for the methionine standard (0.18 ng/mL S), possibly due to slightly wider base peak widths (24 s) than those observed for the methionine standard (9 s). In the case of P, the average detection limit obtained (0.33 ng/mL P) was significantly higher than that obtained for the BNPP standard (0.10 ng/mL P), because phosphorus suffers a sensitivity enhancement in the presence of carbon (gradient elution).

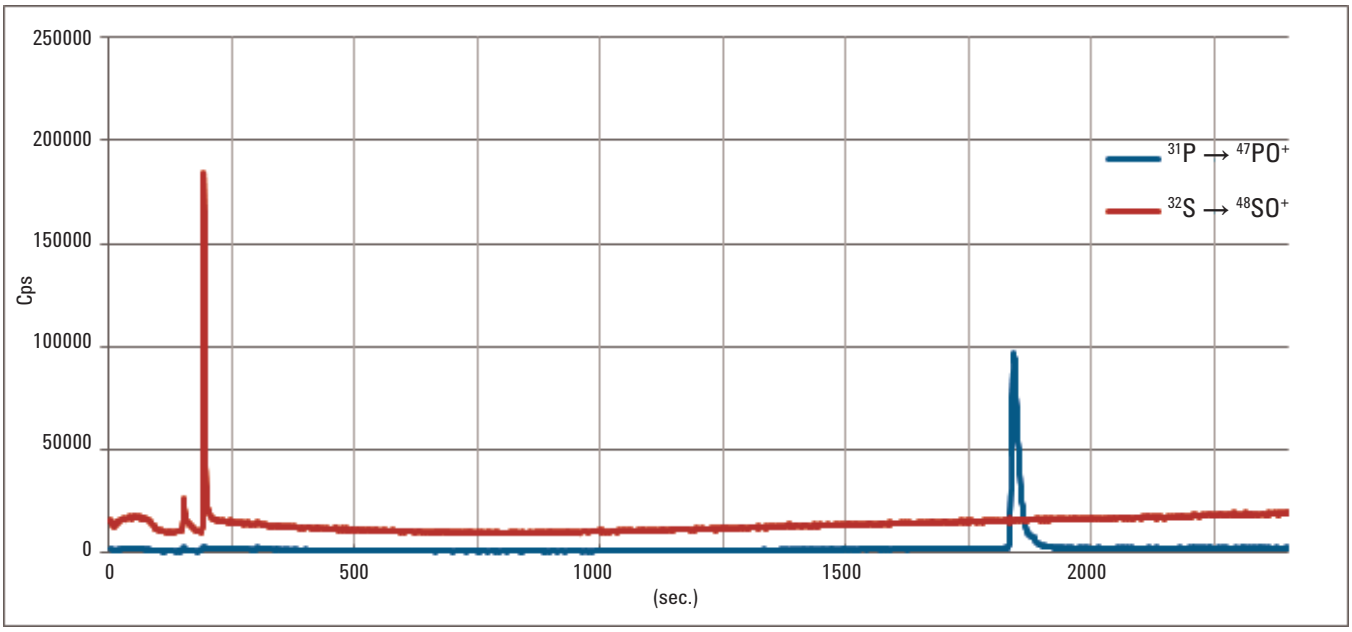

Figure 4. Chromatogram obtained for the 50 ng/mL standard

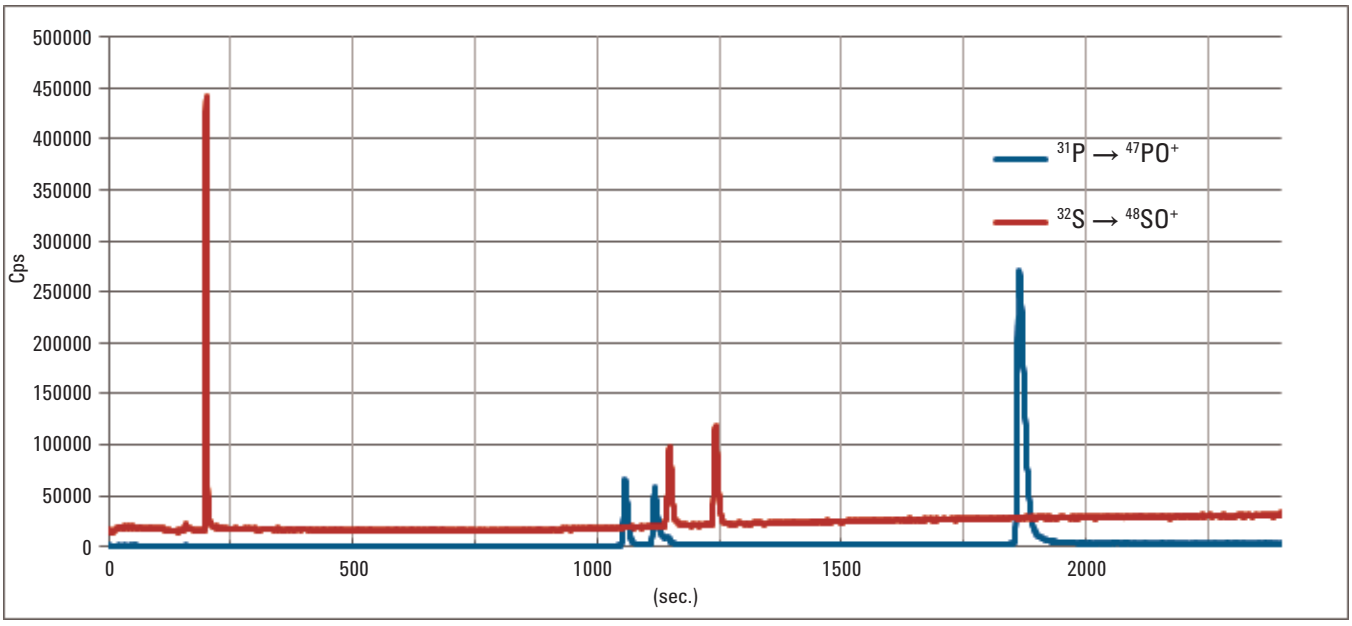

Figure 5. Chromatogram of mixture 3 containing LRRApSLG, ACTPERMAE, KRSpYEEHIP and VPMLK plus BNPP and methionine

Conclusions

Highly sensitive and interference free quantitation of ICP-MS-detectable elements naturally present in proteins will expand the use of LC-ICP-MS in proteomics. In combination with cap-LC separation, the Agilent 8800 Triple Quadrupole ICP-MS generated the lowest absolute detection limits ever reported for the analysis of S- and P-containing species (11 fmol and 6.6 fmol, respectively) using LC-ICP-MS.

Operating in MS/MS mass shift mode, the Agilent 8800 eliminated interferences on both P and S by reacting the analytes with O_2 and measuring the oxide ions produced. Effective interference removal on S was confirmed by good agreement with the theoretical isotope ratio for S, and because correct isotope patterns are retained with QQQ MS, isotope dilution correction of S for sensitivity changes due to gradient dilution can be performed.

Excellent peak profiles and S/N ratios were observed for both S and P species and peptides. This is the first time that generic standards have been applied to the simultaneous absolute quantification of peptides and phosphopeptides.

It is envisaged that this new powerful approach for P and especially for S detection will lead to the use of LC-ICP-MS in fields such as pharmaceutical research (drugs and metabolites), environment analysis (pesticides) and nanotechnology (characterization of engineered nanoparticles).

More Information

For a full account of this application see publication: Triple Quad ICPMS (ICPQQQ) as a New Tool for Absolute Quantitative Proteomics and Phosphoproteomics, Silvia Diez Fernández, Naoki Sugiyama, Jorge Ruiz Encinar, Alfredo Sanz-Medel, Anal. Chem., 2012, 84 (14), pp 5851–5857, Publication Date (Web): June 18, 2012

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Published November 28, 2012
Publication number: 5991-1461EN

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