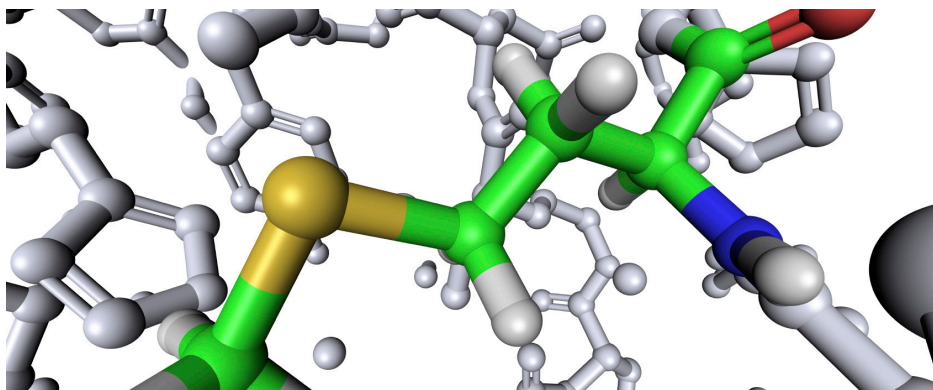


Simultaneous Quantification of Peptides and Phosphopeptides by capLC-ICP-MS

Trace measurement of phosphorus and sulfur using the Agilent 8800/8900 ICP-QQQ



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Introduction

LC-MS/MS is used for the quantification of 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 compound-specific standards for the quantification of the corresponding target compound. In contrast, the high temperature plasma ionization source used in ICP-MS ensures that elemental response is practically independent of the original form of the compound in which the element occurs. This enables non-species specific (or compound independent) quantitation of compounds by measuring the signal for an element contained in the target compound. In this way, different proteins and peptides containing the heteroatoms S and P can be quantified using a known S- or P-containing compound as a generic standard. Unfortunately, with conventional quadrupole ICP-MS, the detection limits (DLs) for P and S are compromised by their high ionization potential and by multiple polyatomic interferences. Sector field high resolution

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8900 ICP-QQQ



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.

Note that low level measurement of P and S is of interest in other applications, including pharmaceutical development and trace quantification of pesticide residues in food and environmental samples.

Compared to conventional or ICP-QMS, the 8800/8900 ICP-QQQ features an additional quadrupole mass filter (Q1), situated in front of the Octopole Reaction System (ORS) 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 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 ($\geq 99\%$ 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 $\geq 95\%$.

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.

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

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

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 $\mu\text{L}/\text{min}$, 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.

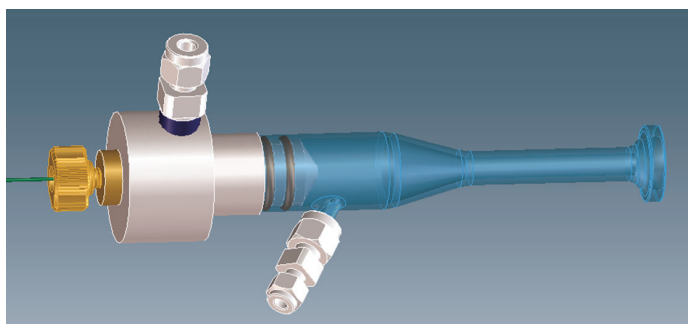


Figure 1. Agilent capillary LC interface kit (G3680A).

ICP-MS

An Agilent 8800 Triple Quadrupole ICP-MS (ICP-QQQ) was used. Oxygen (Ar:O₂ 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 $\mu\text{L}/\text{min}$. Integration time was 150 ms (one point per peak) for ³¹P, ³²S, and ³⁴S. The 8800 ICP-QQQ 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 $\mu\text{L}/\text{min}$, 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 8800/8900 ICP-QQQ 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 ³²S⁺ 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 O₂. 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 ³¹P⁺ to pass through, rejecting interferences at all other masses (including m/z 47). ³¹P⁺ is converted to PO⁺ in the cell by reaction with O₂. 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: (¹⁵N¹⁶O⁺, ¹⁴N¹⁶OH⁺, ¹⁴N¹⁷O⁺, ¹²C¹⁸OH⁺ on ³¹P⁺ and ¹⁶O₂⁺, ¹⁵N¹⁷O⁺, ¹⁵N¹⁶OH⁺, ¹⁴N¹⁸O⁺ on ³²S⁺) and Q2 (⁴⁷Ti⁺, ³²S¹⁴NH⁺, ³²S¹⁵N⁺, ¹⁵N¹⁶O₂⁺, ¹²C³⁵Cl⁺ on ³¹P¹⁶O⁺ at m/z 47, and ⁴⁸Ti⁺, ³⁶Ar¹²C⁺, P¹⁶OH⁺, ³¹P¹⁷O⁺, ⁴⁸Ca⁺ on SO⁺ at m/z 48), are shown in each diagram.

Note also that without the presence of Q1, reaction product ions derived from P and S would interfere with each other, which could affect 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 8800/8900 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 all isotopes of S enter the cell and react to produce SO reaction product ions which overlap each other (e.g. ³²S¹⁸O⁺ overlaps the ³⁴S¹⁶O⁺ product ion at m/z 50).

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 8800. ICP-QQQ DLs achieved were 0.6 ng/mL for ³¹P and 1.2 ng/mL for ³²S with BECs of 9 ng/mL for ³¹P and 5 ng/mL for ³²S. 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 ³²S, a 34/32 ratio matching the theoretical value for ³⁴/₃₂S 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 ³⁴/₃₂S 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.

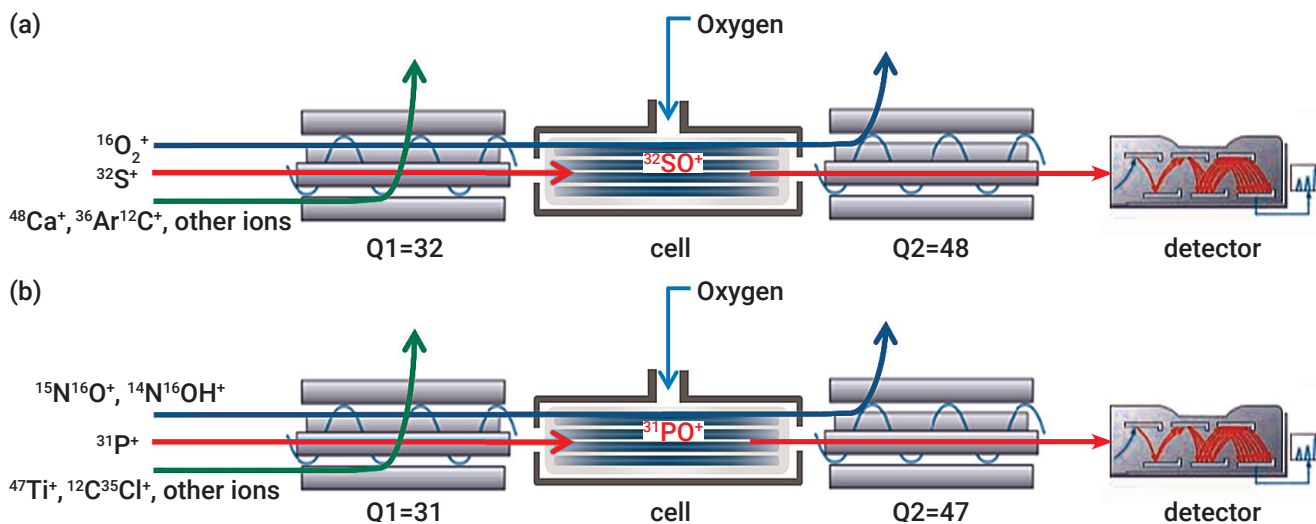


Figure 2. 8800/8900 ICP-QQQ MS/MS operation in mass shift mode to remove interferences on S (a) and P (b).

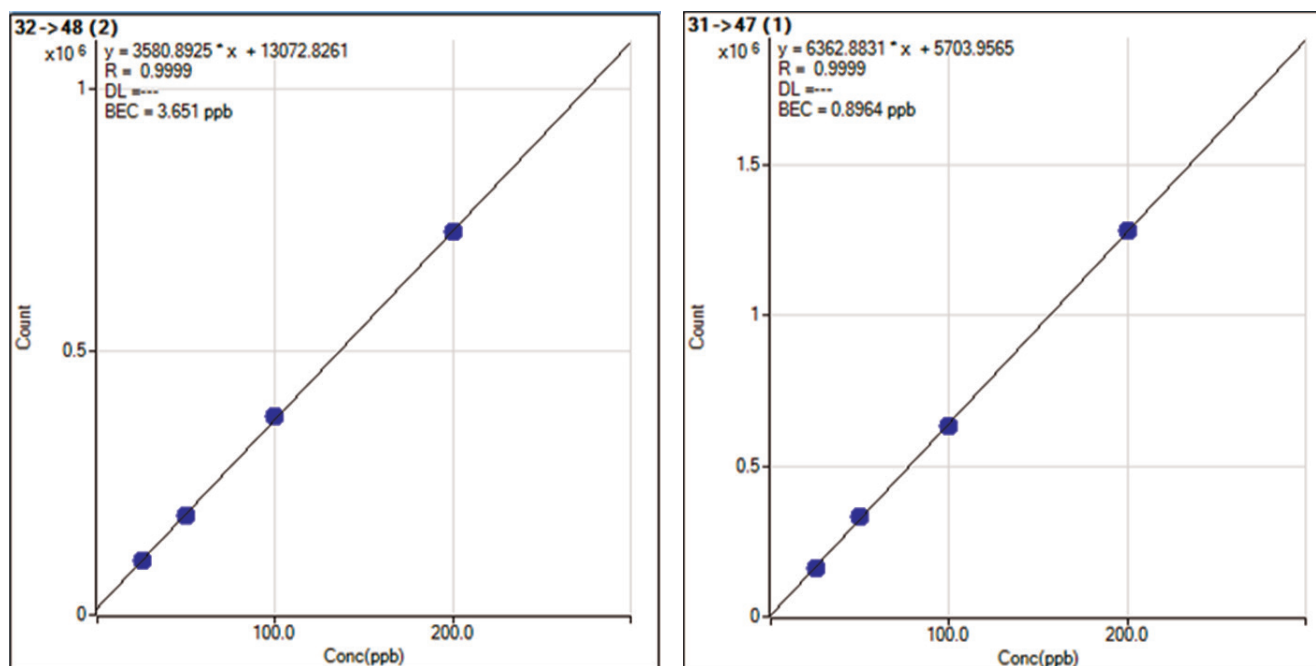


Figure 3. Calibration plots for ^{32}S and ^{31}P (methionine and BNPP, respectively) at 25, 50, 100 and 200 ng/mL (as element). Injection volume = 2 μL , flow rate = 5 $\mu\text{L}/\text{min}$, gradient given in text.

Results and discussion

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

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 8800 ICP-QQQ 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).

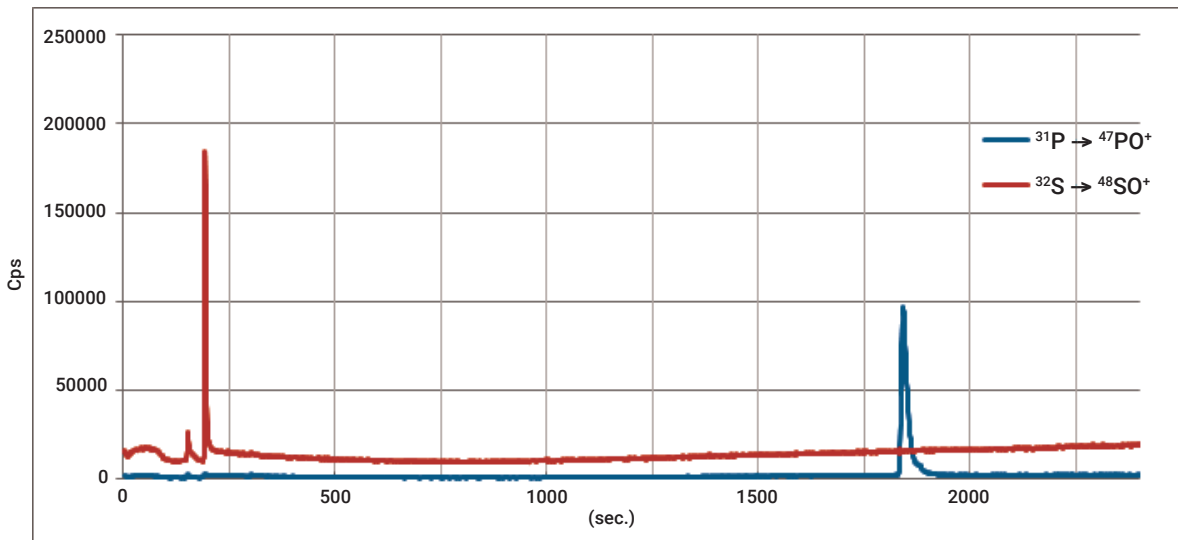


Figure 4. Chromatogram obtained for the 50 ng/mL standard.

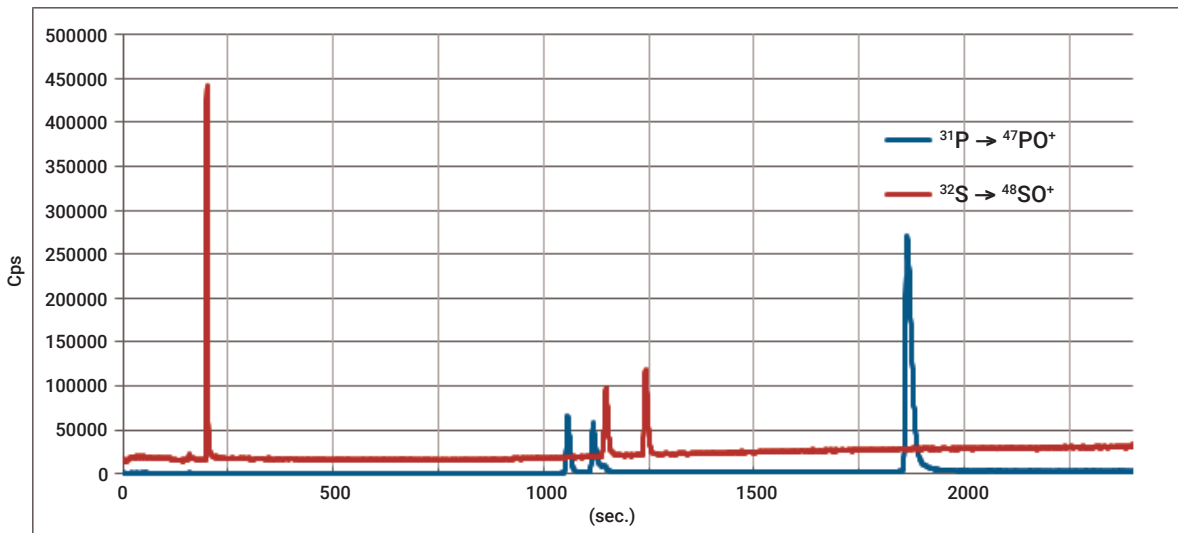


Figure 5. Chromatogram of mixture 3 containing LRRApSLG, ACTPERMAE, KRSpYEEHIP and VPMLK plus BNPP and methionine.

Conclusion

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.

A significantly lower detection limit for sulfur should be achievable using the Agilent 8900 ICP-QQQ #100 (Advanced Applications Configuration), as this newer instrument has a re-engineered argon gas flow path that utilizes low sulfur (and silicon) materials. This has the effect of minimizing the elemental background for these two elements.

Operating in MS/MS mass shift mode, the 8800/8900 ICP-QQQ eliminates interferences on both P and S by reacting the analytes with O₂ and measuring the oxide ions produced. Effective interference removal on S was confirmed by good agreement with the theoretical isotope ratio for S. Because correct isotope patterns are retained with ICP-QQQ, isotope dilution calibration can be used to correct for S sensitivity changes due to gradient dilution.

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 can also be applied in fields such as pharmaceutical research (drugs and metabolites).

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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