Application Note: 30076

Simultaneous Phosphorus and Sulfur Speciation by HPLC Interfaced with High Resolution ICP-MS

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Key Words

- ELEMENT 2
- Chromatography
- High Resolution ICP-MS
- Phosphorus and Sulfur Speciation
- Phosphorylation
- Proteomics

Abstract

A highly sensitive method for the simultaneous speciation of phosphorus and sulfur is described. Phosphorus and sulfur containing molecules are separated by High Performance Liquid Chromatography (HPLC) and detected on-line by High Resolution Sector Field Inductively Coupled Plasma Mass Spectrometry (ICP-SFMS). With a resolution of 4000 (R = m/Δm), phosphorus and sulfur are completely resolved from all polyatomic interferences, resulting in high selectivity. This allows the use of gradient organic mobile phases of up to 100% acetonitrile, which would normally lead to worsened analytical performance, due to the formation of spectral interferences. By using high mass resolution simple and clear spectra are obtained without creating new interferences. Detection limits of 0.06 to 0.19 ng g^{-1} P and 1.3 to 1.9 ng g^{-1} S are obtained, corresponding to absolute amounts of 0.6 to 1.9 pg P and 13 to 19 pg S. Sensitivities of up to 1700 cps / ng g^{-1} P and up to 1100 cps / ng g^{-1} S are achieved. The investigated compounds are deoxyribonucleotides, peptides, l-methionine and *N*-acetyl-dl-methionine. Analytical precisions are between 2.3 and 3.8% RSD. Phosphorus and sulfur can be simultaneously detected within one chromatographic run with a scan duty cycle of 99.8%. This work shows the potential of ICP-SFMS for the determination of phosphorylation states in proteins.

Introduction

Phosphorus and sulfur containing molecules play an important role in biochemistry and proteomics. Phosphorus is present in the backbone of the DNA and RNA chain and in phospholipids. It is involved in energy storage processes and protein phosphorylation. The sulfur containing amino acids cysteine and methionine are found in proteins. Some other examples of sulfur containing biomolecules are: thiaminepyrophosphate, liponamide, acetyl coenzyme A, glutathione and *S*-adenosylmethionine.

The classical techniques for the investigation of phosphorus and sulfur in these biomolecules are ESI-MS (electrospray ionization mass spectrometry) and MALDI-MS (matrix assisted laser desorption/ionization mass spectrometry). Other techniques employed are based on the incorporation of radioisotopes (^{32}P, ^{33}P or ^{35}S) and subsequent monitoring of the b radiation emitted. More recent developments for the determination of phosphorus and sulfur compounds are the hyphenation of HPLC with ICP-MS[1-15] and gel electrophoresis followed by laser ablation with ICP-MS detection[16-19].

The fundamental limitation in the detection of phosphorus and sulfur by ICP-MS is the existence of polyatomic interferences formed in the ICP ion source; for example: $^{15}N^{16}O^+$, $^{14}N^{17}O^+$, $^{14}N^{16}O^1H^+$, $^{12}C^{18}O^1H^+$ and $^1H_3{}^{12}C^{16}O^+$ at mass ^{31}P (Figures 1 and 2), $^{16}O^{16}O^+$, $^{14}N^{18}O^+$ and $^{15}N^{16}O^1H^+$ on mass ^{32}S (Figure 3) and $^{16}O^{18}O^+$ and $^1H_2{}^{16}O_2{}^+$ on mass ^{34}S (Figure 4).

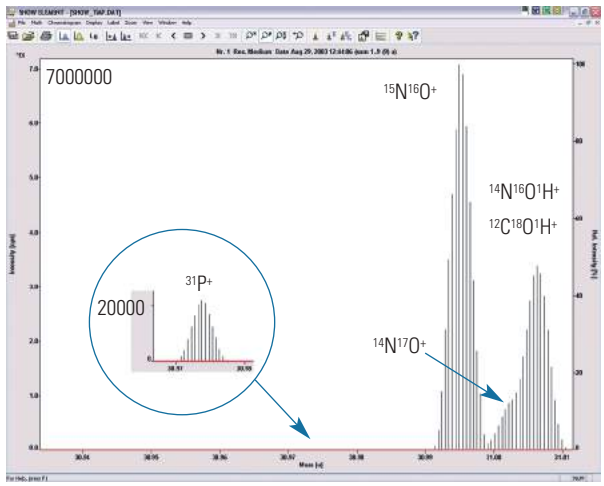

Figure 1: Phosphorus interferences in acetonitrile.

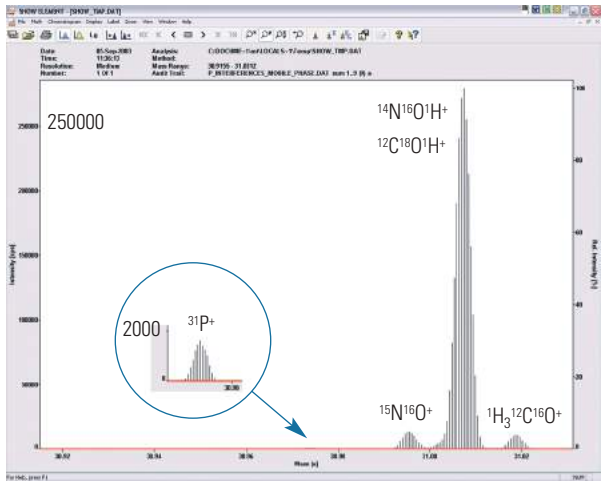

Figure 2: Phosphorus interferences in 4 mmol L^{-1} ammonium acetate and 2% methanol.

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	P SPECIATION OF DEOXYRIBONUCLEOTIDES	S SPECIATION OF SULFUR COMPOUNDS	P & S SPECIATION OF PHOSPHORYLATED PEPTIDES
Stationary phase	Discovery RP Amide C16	Discovery RP Amide C16	Discovery RP Amide C16
Mobile phase	4 mmol L^{-1} ammonium acetate, pH 6.4, 2% methanol (v/v)	0.1% trifluoroacetic acid, 10% acetonitrile, 89.9% water (v/v/v)	A = 0.1% trifluoroacetic acid in water (v/v) B = 0.1% trifluoroacetic acid in acetonitrile (v/v)
Isocratic / Gradient	Isocratic	Isocratic	Gradient: 10% B to 100% B (3-15 min)
Sample loop	10 µL	10 µL	10 µL
Flow rate	200 µL/min	200 µL/min	200 µL/min
Nebulizer	PFA µ-Flow-LC	PFA µ-Flow-LC	PFA µ-Flow-LC
Sample gas	1.09 L/min Ar	0.69 L/min Ar	0.69 L/min Ar
Additional Gas	---	0.04 L/min O_2	0.04 L/min O_2
Spray chamber	Quartz, double pass, room temperature	Quartz, double pass, peltier-cooled to -2 °C	Quartz, double pass, peltier-cooled to -5 °C
Injector	Quartz, 1.75 mm ID	Quartz, 1mm ID	Quartz, 1 mm ID
Cones	Ni sampler and Ni-X skimmer	Pt sampler and Pt-X skimmer	Pt sampler and Pt-X skimmer
RF power	1450 W	1450 W	1450 W
Isotopes monitored	^{31}P	^{32}S	^{31}P and ^{32}S
Resolution	Medium (4000)	Medium (4000)	Medium (4000)

Table 1: Instrument settings for the analysis of deoxyribonucleotides, sulfur compounds and phosphorylated peptides.

One approach used to reduce these interferences is the application of collision/reaction cell ICP-MS. Helium has been reported as collision gas for reducing interferences on ^{31}P[6]. But due to the stability of the O_2^+ interference, helium is not an effective collision gas for the determination of ^{32}S or ^{34}S. While xenon is useful for reducing interferences at sulfur, it has the disadvantage of reducing sensitivities for other elements. Therefore, either a single, non-ideal collision gas has to be used for all elements or ideal collision gases have to be changed repeatedly in a single chromatographic run, leading to long delays.

A simpler approach is the application of High Resolution Sector Field ICP-MS (ICP-SFMS). This technique avoids the use of any collision gas. The principle of ICP-SFMS is to physically resolve the analyte ions from interferences by their small difference in mass. A resolution of 4000 is sufficient to completely resolve phosphorus and sulfur from all interferences even in 100% organic solvents. Other advantages of ICP-SFMS are the outstanding sensitivity and a very high signal-to-noise ratio, which result in extremely low detection limits.

ICP-SFMS has been widely used for the speciation of metals, e.g. aluminum[20], calcium[21-23], chromium[23-26], manganese[23], vanadium[25,27], iron[19,23,25,27-30], nickel[25,26,31], copper[19,23,25,26,29,32-35], zinc[19,23,25,26,29,33,34-36], arsenic[37-41], selenium[23,38,42-45], rhodium[46], palladium[46], cadmium[25,26,33-35,47], tin[23,48], antimony[26], tellurium[38], neodymium[49], gadolinium[15], platinum[22,46,50], mercury[51], lead[23,26], thorium[25] and uranium[25]. It has also been used for the speciation of silicon[52] and non-metals like iodine[5,26,53], phosphorus[1-5,12,13,16-19,52] and sulfur[2,8-11,14-16,18,19,22,27,33-35].

This application note shows the potential of HPLC interfaced to ICP-SFMS for the investigation of phosphorus and sulfur containing biomolecules and evaluates this technique through the analysis of deoxyribonucleotides, sulfur-containing molecules and peptides.

Experimental

The Thermo Scientific ELEMENT 2 High Resolution Sector Field ICP-MS was used with the Organic Matrix Kit (part number: 1067531; including an oxygen mass flow controller, platinum-tipped sampler and skimmer cones and a 1.0 mm quartz injector). A new design of Peltier cooled spray chamber with software based temperature control was used in order to reduce the solvent load in the plasma by decreasing the spray chamber temperature.

For acetonitrile containing solvents, oxygen was added in the spray chamber in order to maintain a stable plasma and to avoid carbon deposition on the cones. The highly increased oxides (e.g. NO, NOH, COH, O_2, H_2O_2) resulting are completely resolved from phosphorus and sulfur with a mass resolution of 4000.

The HPLC system consisted of the Thermo Scientific Surveyor™ LC Pump, containing a 4-channel in-line degasser and a six-port injection valve (Model 9125 from Rheodyne, Rohnert Park, CA) with a 100 µL PEEK™ sample loop. Separation was achieved using a Discovery™ RP Amide C16 column (150 x 2.1 mm, 5 µm, from Supelco®, Bellefonte, PA).

The HPLC and ICP-MS were connected with a PEEK capillary (0.25 mm ID), a PFA µ-flow nebulizer and a trigger cable (all contained in the HPLC Connection Kit, part number: 1159340). Data acquisition by the Element Software can be triggered by either contact closure or a 5 volts TTL signal from the HPLC system. In this work contact closure of the injection valve was used. Chromatograms can be automatically exported on-line in ASCII, ANDI (AIA netCDF), GRAMS™, Xcalibur™ and Spectacle compatible file formats. In this work, data evaluation was performed in GRAMS/AI v.7 software using peak heights. The instrument settings for different separation tasks are summarized in Table 1. Sample gas and torch position were optimized by tuning on the ^{74}Ge signal of the mobile phase containing 50 ng g^{-1} germanium.

Results and Discussion

With a mass resolution of 4000, all polyatomic interferences on phosphorus and sulfur are eliminated. This is demonstrated for phosphorus in Figures 1 and 2, which show that the ^{31}P peak at m/z 30.974 is completely resolved from interferences originating from different mobile phases. In acetonitrile, the most abundant interferences are $^{15}N^{16}O^+$, $^{14}N^{17}O^+$, $^{14}N^{16}O^1H^+$ and $^{12}C^{18}O^1H^+$ (Figure 1).

Even in this solvent, consisting largely of just carbon and nitrogen, phosphorus can be resolved from the carbon and nitrogen based interferences. In a 2% methanol solution containing 4 mmol L^{-1} ammonium acetate, an additional interference from $^1H_3{}^{12}C^{16}O^+$ can be detected (Figure 2).

Figure 3 shows that ^{32}S is resolved from all interferences with a resolution of 4000. The most abundant interferences on ^{32}S in acetonitrile are $^{16}O^{16}O^+$, $^{14}N^{18}O^+$ and $^{15}N^{16}O^1H^+$. Also ^{34}S is resolved from all interferences, mainly $^{16}O^{18}O^+$ and $^1H_2{}^{16}O_2{}^+$ (Figure 4). This enables the analysis of isotopically enriched sulfur compounds, which is necessary for sulfur tracer studies.

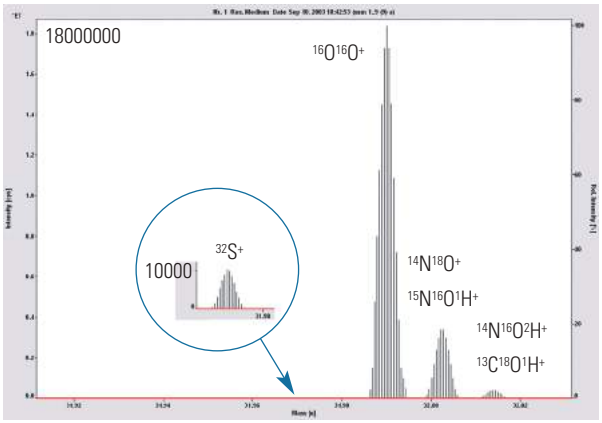

Figure 3: ^{32}S interferences in acetonitrile.

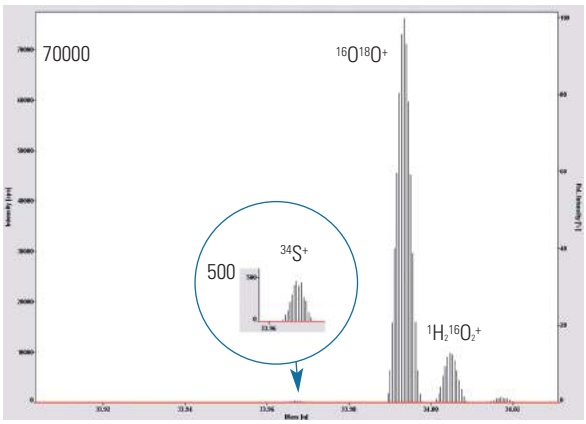

Figure 4: ^{34}S interferences in acetonitrile.

By using Medium Resolution (m/Δm = 4000), ^{31}P, ^{32}S and ^{34}S are completely resolved from all carbon and nitrogen based polyatomic interferences, resulting in extraordinary specificity. Simple and clear spectra are obtained without creating new interferences and without changing any ICP-MS conditions.

Phosphorus Speciation in a Mixture of Deoxyribonucleotides

In order to show the potential of HPLC interfaced to Sector Field ICP-MS for phosphorus speciation, a mixture of four deoxyribonucleotides (dAMP, dTMP, dGMP and dCMP) has been analyzed with this technique, using the parameters listed in Table 1. A chromatogram obtained after the injection of a mixture containing 50 ng g^{-1} P of each deoxyribonucleotide is shown in Figure 5.

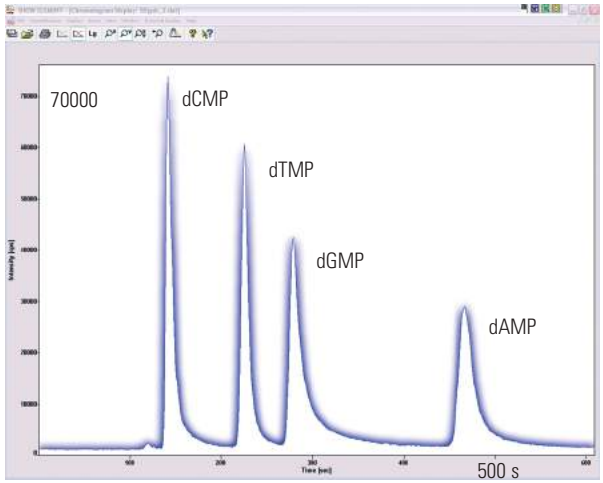

Figure 5: Online P chromatogram of a mixture containing 50 ng g^{-1} P of each deoxyribonucleotide.

The figures of merit, obtained by evaluation of peak heights, are shown in Table 2.

	RETENTION TIME [min]	CALIBRATED RANGE [ng g^{-1} P]	RSD [%] height*	RSD [%] time**	SENSITIVITY [cps/ng g^{-1} P]	LOD [ng g^{-1} P]	LOD [pg P]
dCMP	2.4	1 - 200	2.6	0.6	1681	0.11	1.1
dTMP	3.7	1 - 200	3.8	0.4	1276	0.07	0.7
dGMP	4.6	1 - 200	3.4	0.8	1013	0.19	1.9
dAMP	7.7	1 - 200	2.9	0.7	581	0.06	0.6

* n = 5 (10 ng g^{-1} P) ** n = 19

Table 2: Figures of merit for the determination of deoxyribonucleotides.

Limits of detection were calculated by dividing three times the standard deviation from 8 measurements of a low concentration standard by the slope of the calibration curve. The limits of detection obtained range between 0.6 to 1.9 pg P (0.06 to 0.19 ng g^{-1}) and are limited by effects from the chromatographic system, e.g. P contamination of the mobile phase and column washout effects after the injection of high concentration samples etc. With HPLC interfaced to a collision cell ICP-MS, detection limits of 30 to 60 pg P (3 - 6 ng g^{-1}) have been reported for the speciation of the same deoxyribonu-cleotides by Proefrock et al.[6]. For the speciation of phospholipids by HPLC interfaced to collision cell ICP-MS, detection limits of 210 to 1200 pg P have been reported by Kovacevic et al.[7]. The low limits of detection achievable with High Resolution Sector Field ICP-MS are due to the high sensitivity (600 to 1700 cps/ng g^{-1} P), high ion transmission and the specificity, caused by the unambiguous mass resolution of the analyte from polyatomic interferences. The different sensitivities obtained for different species are due to the different peak heights in the chromatogram.

A wide linear dynamic range (1 to 200 ng g^{-1} P) and good stability and precision (2 to 4% RSD) are also achieved. As an example, the calibration curve for dAMP is shown in Figure 6.

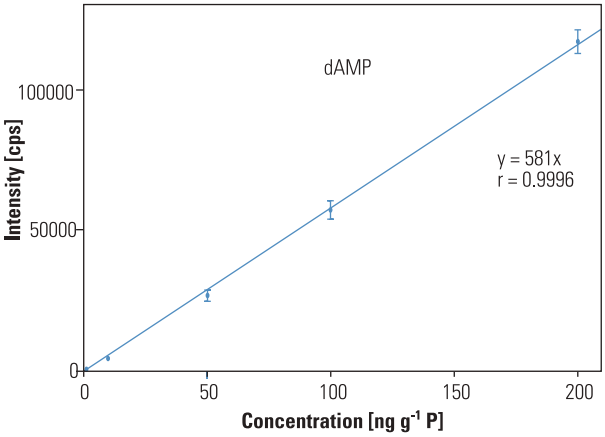

Figure 6: Calibration curve for dAMP.

Sulfur Speciation

l-Methionine and N-acetyl-dl-methionine were chosen as sulfur containing model compounds in order to evaluate the performance of HPLC interfaced to Sector Field ICP-MS for sulfur speciation. The chromatogram obtained after injection of a mixture containing 250 ng g^{-1} S of each species is shown in Figure 7. Figures of merit are listed in Table 3. Limits of detection range between 1.3 to 1.9 ng g^{-1} S, equivalent to 13 to 19 pg S and are limited by the sulfur content of the mobile phase. The sensitivity is approximately 1000 cps / ng g^{-1} S.

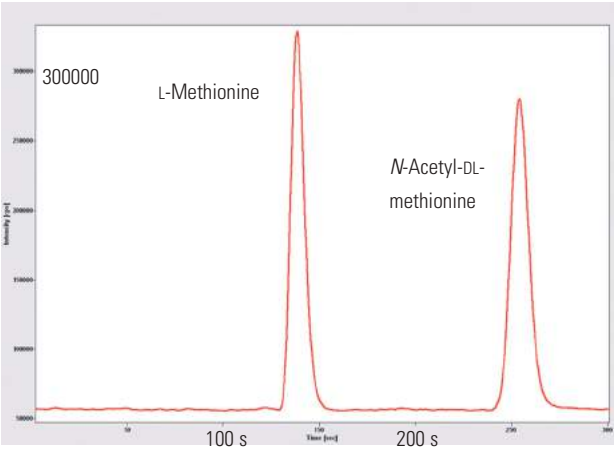

Figure 7: Online S chromatogram of L-Methionine and *N*-Acetyl-DL-methionine (each 250 ng g^{-1} S).

	RETENTION TIME [min]	CALIBRATED RANGE [ng g^{-1} S]	RSD [%]* height	RSD [%]* time	SENSITIVITY [cps/ng g^{-1} S]	LOD [ng g^{-1} S]	LOD [pg S]
L-M.	2.4	10 - 2000	2.3	0.6	1095	1.3	13
N-A.	4.3	10 - 2000	2.3	0.3	917	1.9	19

L-M. = L-Methionine; N-A. = *N*-Acetyl-DL-methionine; *n = 8 (250 ng g^{-1} S)

Table 3: Figures of merit for sulfur speciation.

Simultaneous Phosphorus and Sulfur Speciation of Phosphorylated Peptides

Unlike any other technique, both phosphorus and sulfur can be detected simultaneously in organic mobile phases by High Resolution Sector Field ICP-MS using identical instrument conditions. With a resolution of 4000, P and S analyte ions are resolved from all polyatomic interferences originating from the mobile phase. Therefore this technique, when interfaced to HPLC, is ideally suited for the simultaneous speciation of phosphorus and sulfur, e.g. for the investigation of phosphorylated peptides. In order to demonstrate this, a tryptic digest of casein was separated by HPLC with on-line phosphorus and sulfur detection by Sector Field ICP-MS. The chromatogram obtained is shown in Figure 8.

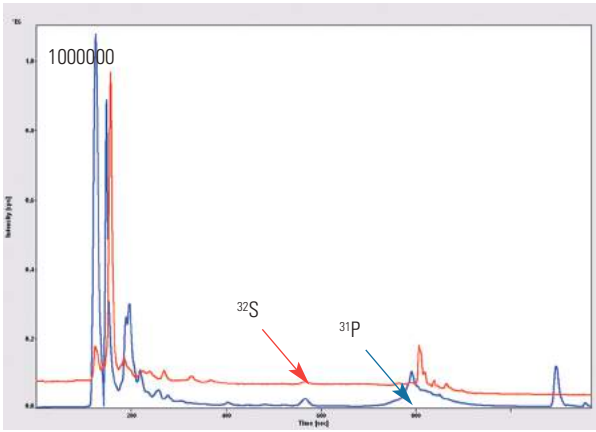

Figure 8: Online P and S chromatogram of a tryptic digest of casein, containing phosphorylated peptides.

The peaks of the ^{31}P trace correspond to phosphorylated peptides and the peaks of the ^{32}S trace correspond to peptides with sulfur containing amino acids. The chromatogram shows that, even with gradients from 10 to 100% acetonitrile (which are frequently used in proteo-mics), no drift of the baseline is observed as has been seen with other ICP techniques due to changing levels of interferences. The duty cycle (time spent actually acquiring data divided by the total analysis time) of the method used was 99.8%. Advantages of High Resolution ICP-MS for this application are:

- All polyatomic interferences are completely physically resolved from P and S with a single set of ICP-MS parameters
- Phosphorus and sulfur can be detected simultaneously without any collision gas
- Chromatographic gradients can be used and the variable organic content of the mobile phase does not lead to an increased baseline
- High sensitivity (up to 1700 cps / ng g^{-1} P and 1100 cps / ng g^{-1} S)
- The ^{31}P signal intensity is independent of the chemical form of phosphorus and proportional to the molar amount of phosphorus in the HPLC eluate

Conclusions

- With on-line HPLC/ICP-SFMS, the simultaneous speciation of phosphorus and sulfur has been demonstrated. The determination of phosphorus and sulfur (as a measure of the protein or peptide content) enables the determination of phosphorylation states.
- By using Sector Field ICP-MS, phosphorus and sulfur are completely resolved from all interferences, resulting in high specificity.
- Simple and clear spectra are obtained without creating new interferences.
- Very low limits of detection for phosphorus and sulfur speciation are possible with this technique. These low detection limits are due to the high sensitivity and the low background that is not influenced by polyatomic interferences.
- Gradients can be used, because the chromatographic phosphorus and sulfur baselines are not affected by interferences from different solvents.
- Organic solvents like acetonitrile are frequently used as a mobile phase in proteomics for the separation of peptides. Therefore the complete removal of interferences caused by organic solvents is important for biochemical applications.
- Since the analytes are completely resolved from all interferences by the small difference in mass, Sector Field ICP-MS is matrix independent, making it an ideal tool for speciation analysis.

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