Orbitrap Exploris Isotope Solutions: Using multiple microscans to enhance precision and accuracy for the ratios of minor isotopologues

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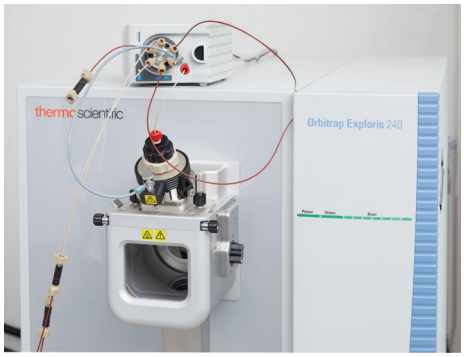

Introduction

For highly precise and accurate isotope ratio measurements using Thermo Scientific™ Orbitrap Exploris™ Isotope Solutions, various instrument settings and parameters must be considered. In this **prior publication,** the importance of balancing Orbitrap™ scan parameters and sample introduction techniques have been discussed. In this technical note we describe data processing of multiple microscans for improved isotope ratio analysis of low abundance isotopologues.

Isotope ratios by Orbitrap MS technology

Orbitrap Exploris Isotope Solutions enable measurement and calculation of isotope ratios directly from the relative abundances of a compound's isotopologues. Intact molecular ions are produced by electrospray ionization (ESI) and delivered to the Orbitrap analyzer. In addition, controlled fragmentation of the molecular ions can be used to deduce site-specific isotope compositions of organic compounds. Isotope ratios of unknown samples are analyzed in comparison to a reference with known isotope ratios, which allow reporting of results relative to international standards.

Equipment and methodology

The Orbitrap Exploris Isotope Solution presented here includes the Thermo Scientific™ Orbitrap Exploris™ 480 MS and data evaluation package for isotope ratio MS. Two sample introduction methods developed for sample/reference comparison are available:

1. Dual Syringe Inlet system based on a syringe pump and a diverter valve
2. An automated In-flow Injection approach utilizing the Thermo Scientific™ Vanquish™ Neo UHPLC System

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Each of the sample introduction systems deliver an analyte solution to the ESI source of the Orbitrap Exploris mass spectrometer. During ESI the analyte molecules are ionized and transferred to the gas phase for mass analysis. As ions exit the ESI source, they are filtered by Advanced Quadrupole Technology (AQT; Figure 1). The AQT allows a selected mass range to be analyzed. Ions are then passed into the ion-routing multipole (IRM) for storing and optional fragmentation. The number of ions included in every package is controlled by the Automatic Gain Control (AGC). The time needed to collect an ion package is called Injection Time (IT).

After the IT, the selected ion package or their fragments are injected by the C-Trap into the Orbitrap mass analyzer. The analyzer consists of a spindle-shaped central electrode around which ions orbit and an enclosing pair of bell-shaped outer electrodes. Please note, the collection of the next ion package begins while the prior package is still being analyzed to guarantee an efficient use of Orbitrap analysis time. The ions oscillate in-between the two outer electrodes with a frequency inversely proportional to the square root of their individual mass-to-charge ratios (*m/z*). The motion of ions induces an image current on the outer electrodes. The image current is recorded over a certain duration of time called analysis time (AT). Longer analysis times (longer transients) result in higher resolved mass spectra. A single Orbitrap cycle consisting of the IT and the AT of an ion package can be referred to as a microscan. (Figure 2) The result of every microscan (image current over time) is called a transient. It is processed using enhanced Fourier transform (eFT) to obtain the *m/z* and abundance of each ionic species including isotopologues.

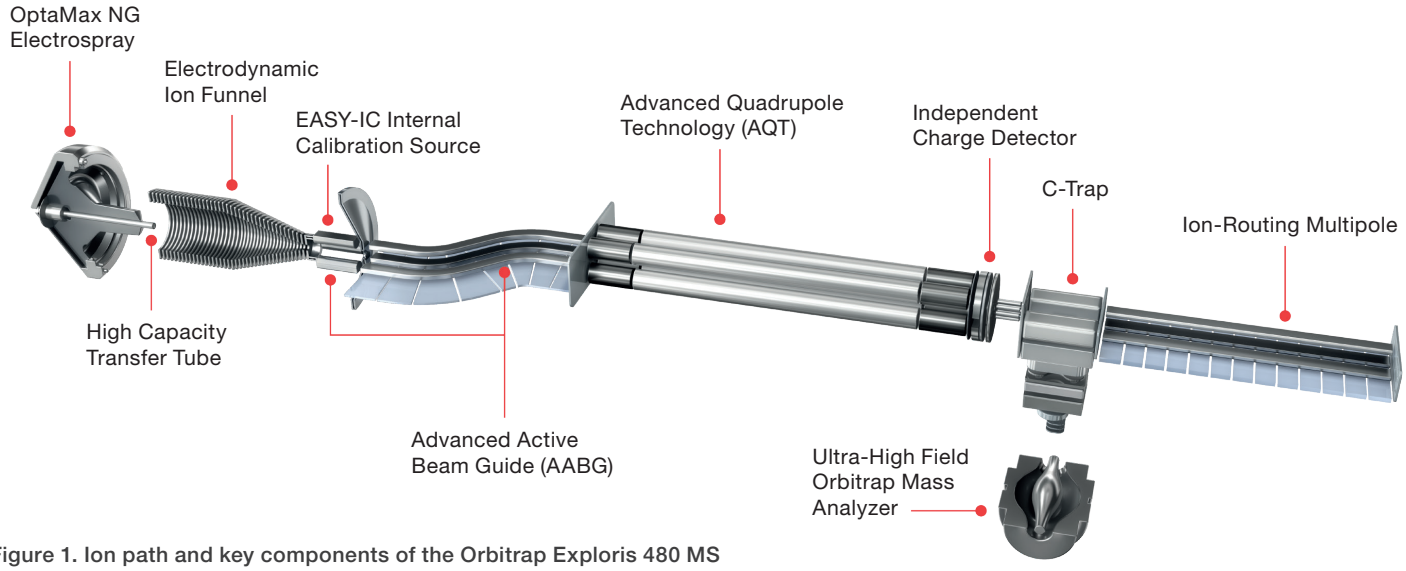

Figure 1. Ion path and key components of the Orbitrap Exploris 480 MS

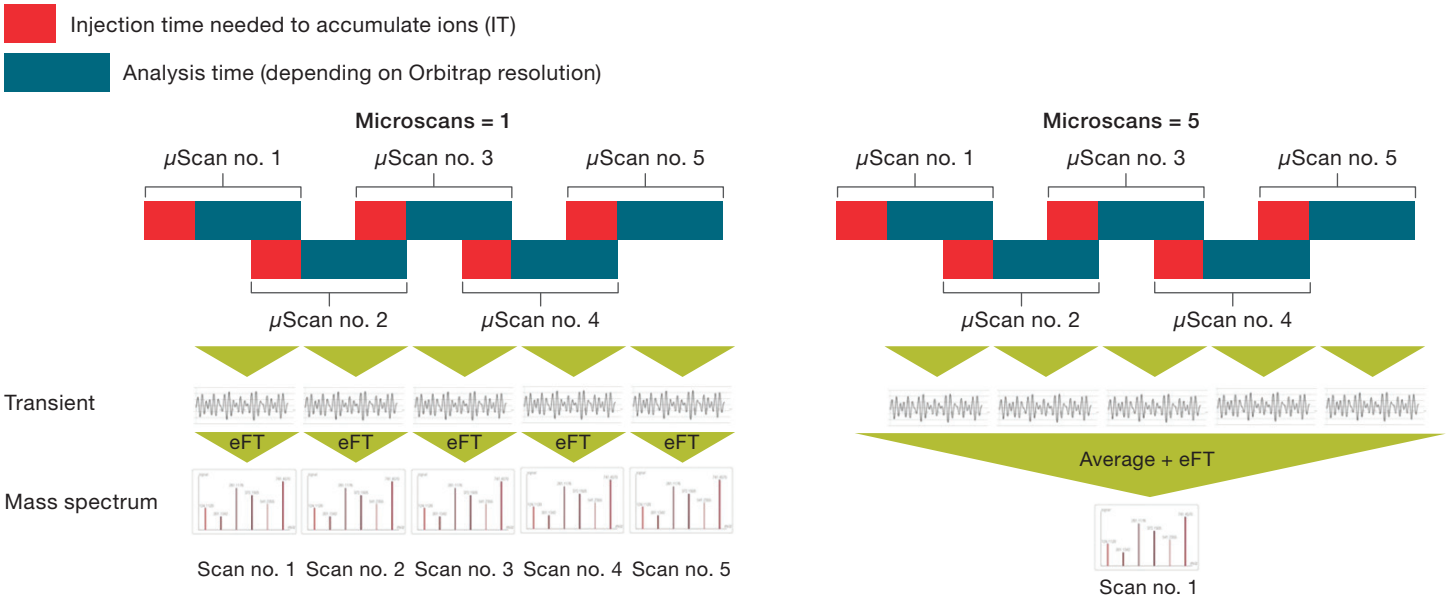

Figure 2. Orbitrap cycle and data processing using individual microscans (μScan) and combined microscans

Using standard settings (Microscans = 1), every transient is processed by eFT resulting in a full mass spectrum for the desired *m/z* range (Figure 2, left). The full process used to ultimately create a single mass spectrum is referred to as “scan”. In the Orbitrap result file (RAW files) the mass spectrum of every acquired scan can be displayed.

In addition to this standard procedure Orbitrap Exploris Isotope Solutions offer the opportunity to combine multiple microscans into a single scan. Here the transients of multiple microscans are summed up prior to the eFT data processing. In this way multiple microscans result in just a single scan (Figure 2, right, Microscans = 5). The resulting mass spectrum of this single scan contains the information of all the summed microscans. Benefits of this procedure to isotope ratio measurements will be discussed in the results paragraph.

Data acquisition and evaluation

Thermo Scientific™ Xcalibur™ Software is used for instrument setup and data acquisition. Every measurement results in a RAW file that is processed by Thermo Scientific™ IsoX™ Software to extract all relevant parameters for the calculation of isotope ratios. The resulting IsoX Software output files, including all the data and parameters needed for the further evaluation steps, are simple tab-delimited files and can be opened as spreadsheets. For processing of multiple RAW files, a combined IsoX Software output file can be created.

Further processing of the IsoX Software output files can be performed using commonly used data science statistical computing programs. R scripts are used for the evaluation of the presented data. Isotope ratios calculated by the R scripts are saved in different data formats to enable flexible data evaluation.

Presented samples and standards data were measured against a reference solution. Formula (1) shows the calculation of δ-values against a standard for isotope ratios of $^{33}S/^{32}S$ as an example. The reference value of $R(^{33}S/^{32}S)$ of the prior and the following reference block were averaged by implementing bracketing referenciation.

$$(1)\quad \delta^{33}S_{(Sample/Reference)} = \left(\frac{R(^{1}H^{33}S^{16}O_4/^{1}H^{32}S^{16}O_4)_{Sample}}{R(^{1}H^{33}S^{16}O_4/^{1}H^{32}S^{16}O_4)_{Reference}} - 1 \right) \cdot 1000\ [‰]$$

Sulfate isotope ratio analysis as a model

In this technical note the use of multiple microscans for isotope ratio measurements will be discussed using sulfate as a model compound. Using multiple microscans during isotope ratio analysis can be useful for any high-quality analysis of low abundance species in Orbitrap mass spectra. In addition to improving the signal to noise ratio and peak resolution, this approach will reduce size of the output files and increase precision and accuracy of low abundance peaks.

Sulfate is measured as monohydrogen sulfate (HSO_4^-) in negative ESI mode. Typical ion source settings used to achieve a stable and intense signal for sulfate on an Orbitrap Exploris 480 MS are listed in Table 1. To achieve similar results for sulfate isotope ratio measurements on Thermo Scientific™ Orbitrap Exploris™ 120 MS and Orbitrap Exploris™ 240 MS adjustment of the gas flows and RF-Lens settings is required.

Table 1. Ion source settings for Orbitrap Exploris 480 MS

Sheath gas flow rate	0
Auxiliary gas flow rate	7
Sweep gas flow rate	0
Spray voltage	~ 2.4 kV (negative ionization mode)
Spray current (observed)	<0.2 µA
Capillary temperature	280 °C
RF lens	70 %

The HSO_4^- molecular ion consists of four oxygen, one sulfur and one hydrogen atom. Different combinations of the elements` stable isotopes result in multiple isotopologues distributed roughly proportional to their natural abundance. Table 2 lists the most abundant isotopologues that are detectable with the ‘M0’ methodology during sulfate measurements.

Table 2. Abundance (out of 10^6) and *m/z* of sulfate`s isotopologues in the range of 96–105 *m/z*

Cardinal mass	Accurate mass	Isotopologue	Heavy isotope substitution(s)	Abundance
M0	96.9601	$^{1}H^{32}S^{16}O_4$	-	940,592
M+1	97.9595	$^{1}H^{33}S^{16}O_4$	^{33}S	7,427
M+1	97.9643	$^{1}H^{32}S^{17}O^{16}O_3$	^{17}O	1,433
M+2	98.9559	$^{1}H^{34}S^{16}O_4$	^{34}S	42,084
M+2	98.9644	$^{1}H^{32}S^{18}O^{16}O_3$	^{18}O	7,632
M+4	100.9601	$^{1}H^{34}S^{18}O^{16}O_3$	$^{18}O^{34}S$	333

M0, the most abundant isotopologue, refers to the monoisotopic molecule consisting of only the light (major) isotopes ^{32}S,^{16}O and ^{1}H. Typically, a scan range of 93–105 is selected for these measurements. The peaks are labeled according to the heavy isotope substitutions present in the corresponding sulfate ion and contain both single and double substitutions by the heavy isotopes ^{33}S, ^{34}S, ^{17}O and ^{18}O (Figure 3). Based on their cardinal masses, the isotopologues can be grouped as M+X (Table 2). Raw data acquired by 'M0' methodology can be used to calculate the isotope ratios of all singly substituted species over the unsubstituted (M0) from a single run. In these 'M0' experiments the M0 peak is the so called "base peak" for isotope ratio calculations.

The scan parameters used for sulfate 'M0' experiments are listed in Table 3.

Table 3. Scan parameters used for sulfate isotope ratio analysis with the Orbitrap Exploris 480 MS

Scan type	Full scan
Scan ranges (isolation range)	*m/z* 93–105
Resolution	45,000
Polarity	Negative
Lock masses	Off
Automatic gain control (AGC) target	300,000
Maximum injection time	1,000 ms

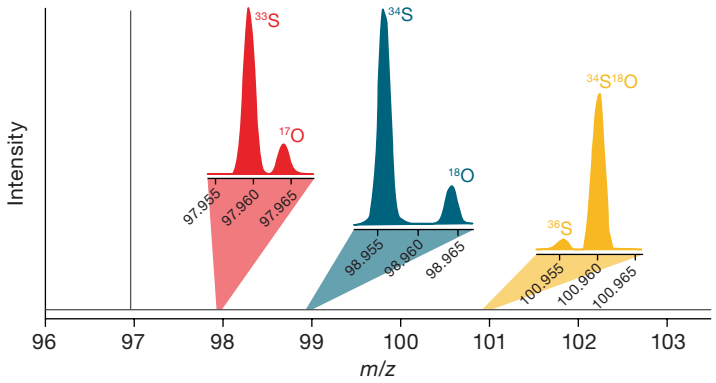

Figure 3. Mass spectrum of sulfate with 'M0' methodology (scan range 93–105 *m/z*). The peaks are labeled according to the heavy isotope substitutions in the corresponding sulfate ion

How different microscan settings improve isotope ratio analysis of sulfate

The effect of different microscan settings on peaks of differing abundance will be discussed in the following section. For simplicity we will focus on sulfate M0-scans, where: M0 is the major unsubstituted base peak; M+1 is the singly ^{33}S-substituted isotopologue medium abundance peak and M+4 is the $^{34}S^{18}O$-doubly substituted low abundance peak. Only these three isotopologue peaks will be used for further discussion.

Adding the transients of multiple ion packages leads to a linear increase of the peak signals (*S*) with the number of transients. The spectral noise (*N*) only increases by the square root of the number of transients, resulting in an increase in *S/N* by the square root of the number of microscans.[X] Figure 4 shows the increase in *S/N* for the three sulfate isotopologue peaks.

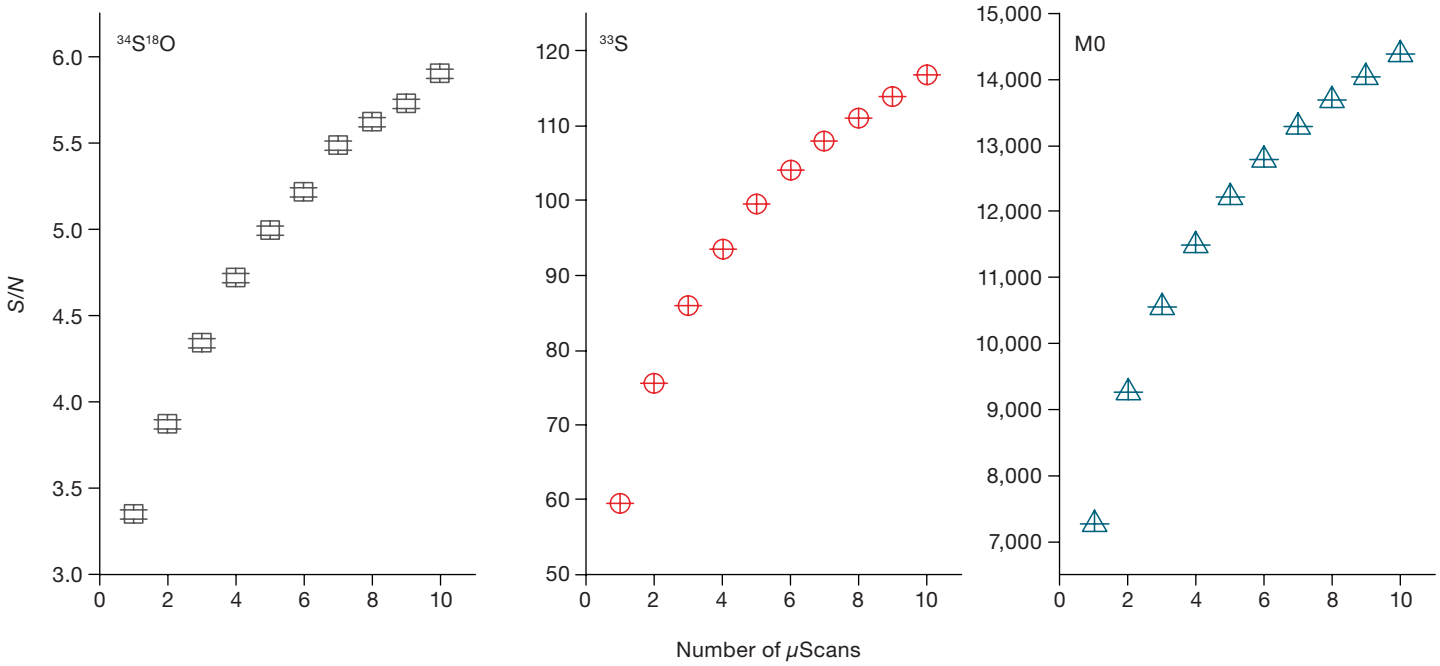

Figure 4. Increase of *S/N* for three different sulfate isotopologue with increasing number of microscans used

What is the actual impact of higher *S/N*?

During the data processing of an Orbitrap measurement, peaks with low *S/N* (<10) are treated differently to those with medium and high *S/N*. The spectral peak resolution can be taken into account to determine how a peak was treated during data processing (magnitude mode vs. absorption mode).[Y] Figure 5 shows density plots of the peak resolutions for the three discussed sulfate isotopologues acquired using different numbers of microscans with a nominal Orbitrap resolution of 45,000. Since this resolution is defined for a nominal mass of 200 *m/z* the peak resolutions plotted in Figure 5 often exceed this value. For the low abundance peak of $^{34}S^{18}O$-substituted sulfate, a bimodal distribution can be seen in the density plot at low numbers of microscans (1–4). At higher microscan numbers the peak resolution increases as a result of increased use of absorption mode. A slight increase in peak resolution can also be observed for the medium abundance peak of ^{33}S-substituted sulfate when going from low to high numbers of microscans. For high intensity peaks like M0, the peak resolution is unaffected by an increase in the number of microscans.

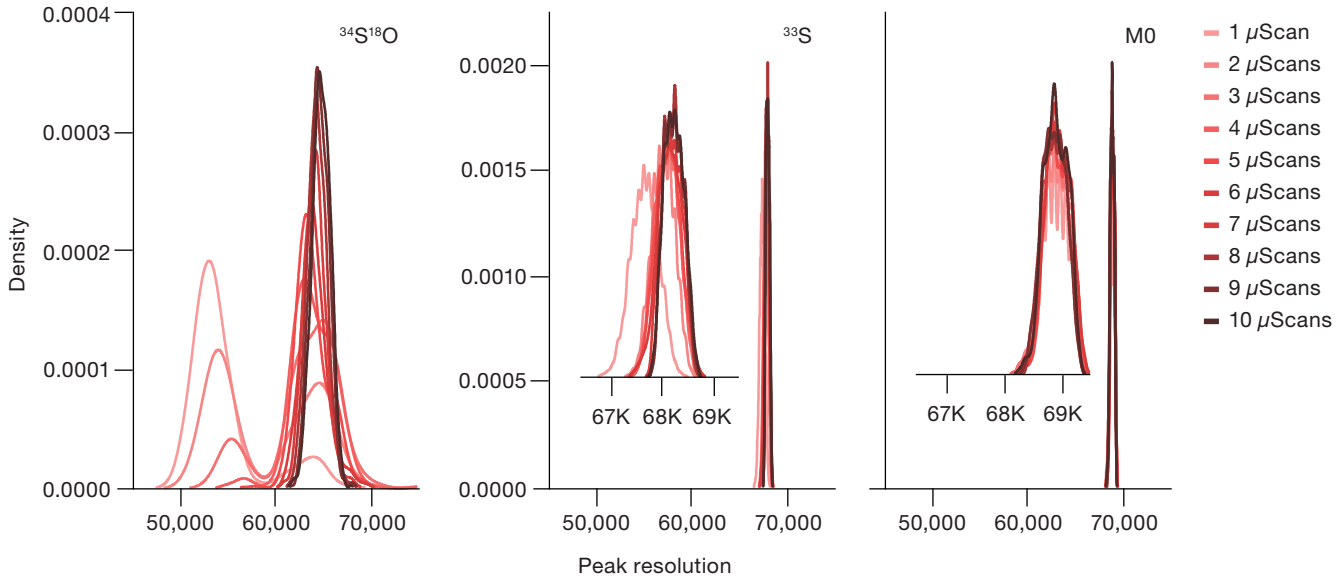

Figure 5. How different microscans settings effect the peak resolution of MS-peaks of sulfate-isotopologues with different abundances in M0-analyses. Density plot of the peak resolution resulting from individual scans after 10 min of continuous data acquisition

Due to the changes in intensity, which accompany decreasing resolution in magnitude mode, accurate determination of isotope ratios for low abundance species becomes increasingly difficult. To demonstrate this, a 50 μM solution of sodium sulfate in methanol was continuously infused into the MS using a single syringe. Ten minutes of data were acquired for each number of microscans in between 1 and 10. No sample-reference comparison was performed.

Figure 6 shows a δ-value of ^{33}S/M0 and $^{34}S^{18}$O/M0 calculated versus the measurement using 10 μScans plotted versus the number of microscans used. The presented results show how the use of up to 10 microscans for isotope ratio analysis with Orbitrap mass spectrometers can have a significant impact on the accuracy of the isotope ratio of low abundant isotopologues.

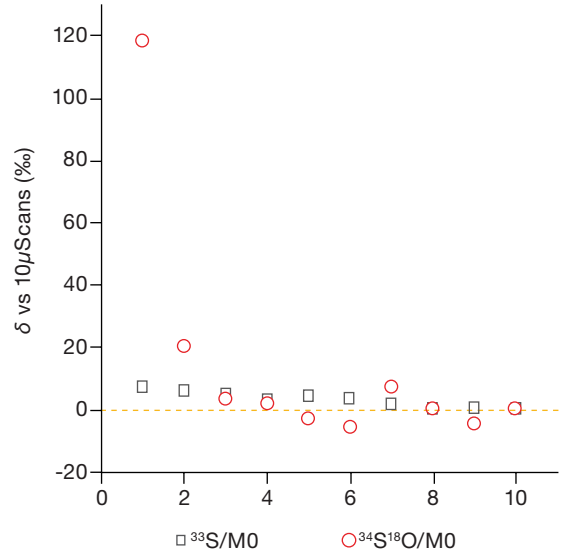

Figure 6. δ^{33}S/M0 and $^{34}S^{18}$O/M0 determined at different numbers of microscans versus 10 microscans. Orange dotted line indicating a difference of 0‰

To check if the impact on the accuracy of isotope ratio measurements for low abundance isotopologues can be compensated by sample reference comparison, two sulfate materials were analyzed using the dual syringe inlet sample introduction. Both Na_2SO_4 materials were dissolved in methanol/H_2O (1:1, *v*:*v*) at a concentration of 100 mM and diluted with methanol to a concentration of 50 μM for analysis. For four different microscan settings (1, 2, 4 and 10) two dual inlet runs were performed for each. Every dual inlet run included 5 sample and 6 reference blocks with a block duration of 13 min. δ-values were calculated via bracketing. Figure 7 shows the results of all ten datapoints for the ^{33}S-isotopologue and the $^{34}S^{18}$O isotopologue plotted versus the number of microscans used and error bars indicating their standard deviation. Assuming a stoichastic distribution using the δ^{34}S values analyzed by the Orbitrap measurement and the ^{18}O value from EA-IRMS measurements, the expected δ-value was calculated for the $^{34}S^{18}$O-isotopologue, indicated by a black horizontal line in Figure 7 (right).

These results show that increasing the number of microscans significantly improves the accuracy of the determined ratio of $^{34}S^{18}$O/M0. No measurable improvement was made to the δ^{33}S/M0 isotopologue ratio.

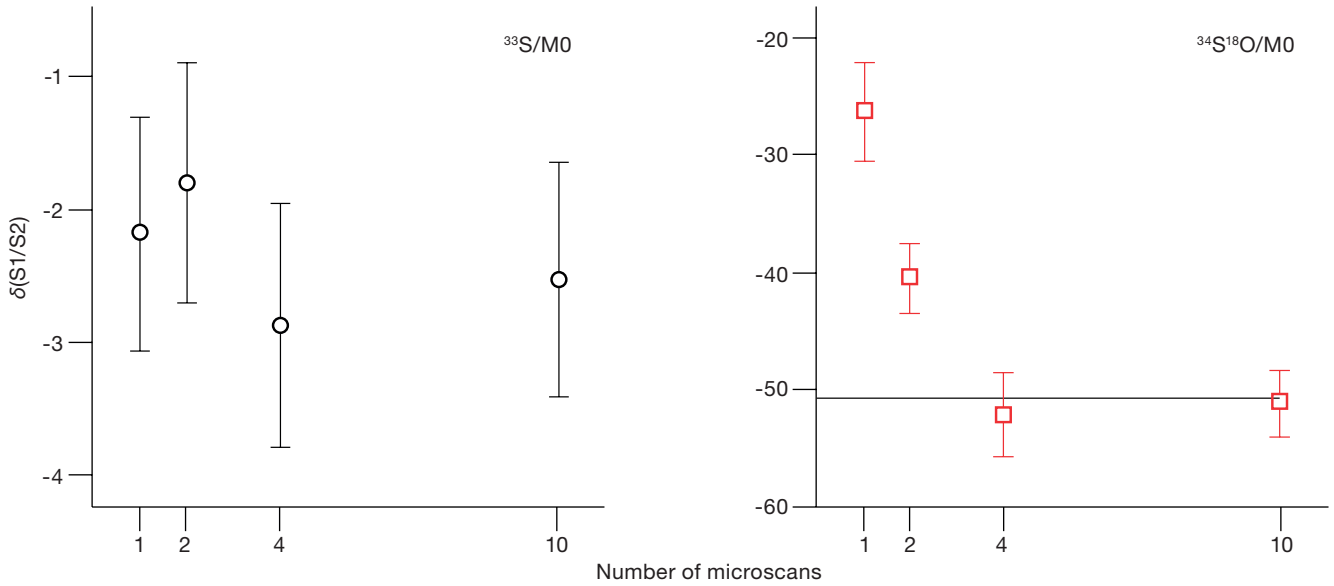

Figure 7. δ(S1/S2) for ^{33}S/M0 (left) and $^{34}S^{18}$O/M0 (right)

Conclusions

Orbitrap Exploris Isotope Solutions offer the opportunity for isotope ratio analysis of many different isotopologues including ones with very low abundances. Summing up the transients recorded for multiple ion packages analyzed by the Orbitrap prior to the data processing offers multiple advantages especially for small signals. The presented data of sulfate isotope ratio analysis shows how this methodology can improve signal-to-noise ratio, peak resolution and isotope ratio precision.

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