

## Isotope ratio MS

## Uncover new dimensions of isotope ratio analysis with Thermo Scientific Orbitrap MS technology

### Introduction

Isotope ratio mass spectrometry (IRMS) is a powerful tool for determining subtle isotopic variations within a sample, providing unique insights to understand a wide range of questions from biochemical cycles, ecology, paleoclimate reconstructions through to food authenticity and doping control. In classical IRMS the isotope ratio of the sample is determined after the analyte has been converted into a low molecular weight gas (e.g. CO<sub>2</sub>, SO<sub>2</sub>, N<sub>2</sub>) by combustion, pyrolysis, microbial fermentation or chemical treatments. This concentrates all isotopic information of sample compounds in very few signals allowing high precision analysis of isotope ratios. Consequently, the intramolecular isotopic information is lost. A new, comprehensive approach to IRMS using electrospray ionization (ESI) Thermo Scientific™ Orbitrap™ MS technology gives access to a wide range of isotopic information from a variety of polar compounds in liquid samples. In contrast to classical IRMS methods, Thermo Scientific™ Orbitrap Exploris™ Isotope Solutions enable the calculation of isotope ratios directly from the relative abundances of the compounds isotopologues, measured as intact molecular ions.



Orbitrap Exploris Isotope Solutions

Intact molecular ions can be subjected to controlled collision induced fragmentation by the Orbitrap Exploris MS to gather additional site specific isotopic information. Studies of natural systems and biochemical processes require analyzing natural abundance and slightly enriched isotope ratios with high accuracy and precision. A new methodology has been developed, which leverages the high-resolution accurate mass (HRAM) and high sensitivity of the Orbitrap Exploris MS. The workflow comprises a dedicated sample introduction concept, distinctive method setup and a data evaluation framework to convert isotopologue intensities to isotope ratios and corresponding  $\delta$  values that are commonly used in the isotope community.

Isotope ratio analysis by ESI Orbitrap technology allows the investigation of:

1. Isotope ratios at natural abundance: direct measurements of individual analytes in liquid samples and simultaneous acquisition of all major and some minor isotopologues, enabling abundance determination of  $^{13}\text{C}$ ,  $^{15}\text{N}$ ,  $^{18}\text{O}$ ,  $^{17}\text{O}$ ,  $^2\text{H}$ ,  $^{34}\text{S}$ ,  $^{33}\text{S}$ ,  $^{36}\text{S}$  and more,
2. Position specific isotope ratios: controlled fragmentation of organic molecular species that enables unique position specific isotope analysis, and
3. Measurements of isotopologues with multiple rare isotope substitutions ('clumped isotopes').

### Analysis of oxyanions – nitrate as a model

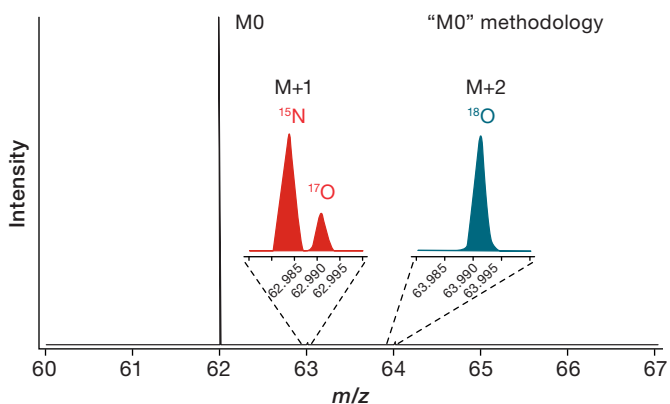
The benefits of using ESI Orbitrap technology for isotope ratio analysis can be demonstrated by looking at nitrate and its 20 distinct stable isotopic variants. Table 1 shows a list of nitrate isotopologues, with M0 representing the monoisotopic nitrate containing the light isotopes  $^{14}\text{N}$  and  $^{16}\text{O}$ , M+1 representing a single substitution of the heavy isotopes  $^{15}\text{N}$  or  $^{17}\text{O}$ , etc.

When using Orbitrap Exploris Isotope Solutions, the monoisotopic nitrate containing the light isotopes as well as three isotopologues that contain ions with a single substitution of the heavy isotopes are analyzed in a single run (Figure 1).

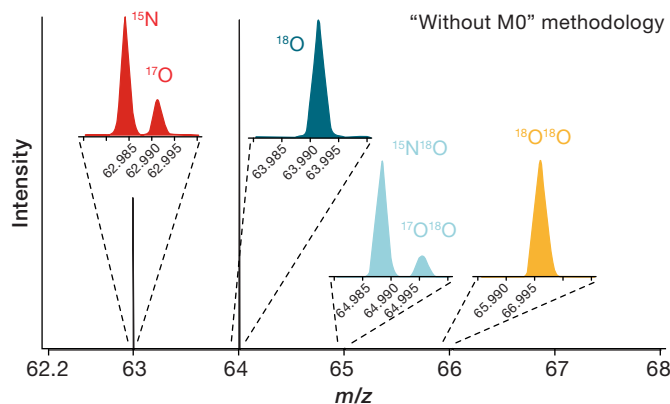
Due to the finite capacity of the Orbitrap, a methodology excluding the base peak is required to optimize measurements of minor isotopologues. To prevent the M0-ion from entering the Orbitrap when using the so called "without M0" methodology the scan range of the Orbitrap Exploris MS is shifted to higher mass-to-charge ratios ( $m/z$ ). This way the Orbitrap's capacity can be used more efficiently for ions of the minor isotopologues resulting in higher precision for the ratios of multiple substituted nitrate-species i.e.  $^{15}\text{N}^{18}\text{O}^{16}\text{O}_2$ ,  $^{14}\text{N}^{17}\text{O}^{18}\text{O}^{16}\text{O}$ , and  $^{14}\text{N}^{18}\text{O}_2^{16}\text{O}$  (Figure 2).

**Table 1. List of nitrate isotopologues from M0 to M+4 with corresponding mass to charge ratios and abundances**

| Mass range | $m/z$   | Abundance [ppm] | Isotopologue   |
|------------|---------|-----------------|--|
| M0         | 61.9884 | 989,242         | $^{14}\text{N}^{16}\text{O}_3$                         |
| M+1        | 62.9854 | 3637            | $^{15}\text{N}^{16}\text{O}_3$                         |
|            | 62.9926 | 1127            | $^{14}\text{N}^{17}\text{O}^{16}\text{O}_2$            |
| M+2        | 63.9896 | 4.1             | $^{15}\text{N}^{17}\text{O}^{16}\text{O}_2$            |
|            | 63.9926 | 5951            | $^{14}\text{N}^{18}\text{O}^{16}\text{O}_2$            |
|            | 63.9968 | 0.4             | $^{14}\text{N}^{17}\text{O}_2^{16}\text{O}$            |
| M+3        | 64.9897 | 21.9            | $^{15}\text{N}^{18}\text{O}^{16}\text{O}_2$            |
|            | 64.9938 | <0.1            | $^{15}\text{N}^{17}\text{O}_2^{16}\text{O}$            |
|            | 64.9968 | 4.5             | $^{14}\text{N}^{17}\text{O}^{18}\text{O}^{16}\text{O}$ |
|            | 65.0010 | <0.1            | $^{14}\text{N}^{17}\text{O}_3$                         |
| M+4        | 65.9939 | <0.1            | $^{15}\text{N}^{17}\text{O}^{18}\text{O}^{16}\text{O}$ |
|            | 65.9969 | 11.9            | $^{14}\text{N}^{18}\text{O}_2^{16}\text{O}$            |
|            | 65.9981 | <0.1            | $^{15}\text{N}^{17}\text{O}_3$                         |
|            | 66.0011 | <0.1            | $^{14}\text{N}^{17}\text{O}_2^{18}\text{O}$            |



**Figure 1. Mass spectra of  $\text{NO}_3^-$ , with the predominant monoisotopic base peak (M0),  $^{14}\text{N}^{16}\text{O}^{16}\text{O}^{16}\text{O}$**



**Figure 2. Mass spectra of  $\text{NO}_3^-$ , without the predominant monoisotopic base peak (M0)**

In the case of nitrate, the signal of the  $^{15}\text{N}$ -isotopologue is used as the base peak in the “without M0” methodology. The gathered ratios of minor isotopologues to  $^{15}\text{N}$  can be correlated to M0 using the known ratio of  $^{15}\text{N}/\text{M0}$  from the M0 experiment. By combining the data from multiple experiments, we increase the range of accurate and precise isotope ratio analysis via Orbitrap Exploris Isotope Solutions.

### Concept for analysis of isotope ratios

Isotope ratios in natural abundance need to be measured with high precision. In order to achieve high precision comparable with classical IRMS by using Orbitrap MS technology, isotopologue signals are measured for 6 to 8 minutes. However, complex sample preparation and conversion of molecular compounds into simple gases with subsequent removal of by-products (such as water, oxygen etc.) is not required. Isotope ratios are calculated directly from the relative abundances of the isotopologues of compound ions. These ions are produced by ionizing intact or fragmented molecules and can be used to deduce molecular-average or site-specific isotopic composition. In addition, Orbitrap Exploris Isotope Solutions has been specifically developed for isotope ratio analysis of liquid samples with the dual syringe inlet – “dual inlet” system for sample-reference comparison. This enables isotope ratios of unknown samples to be analyzed relative to a reference, ensuring accuracy of the isotope ratio results, reported relative to international standards. For singly substituted isotopologues of oxyanions such as nitrates, Orbitrap Exploris Isotope Solutions achieve subpermil precision.

### Technology overview

Orbitrap Exploris Isotope Solutions offer a dedicated workflow for delivering isotope ratio data based on Orbitrap MS technology that includes:

- Thermo Scientific™ Orbitrap Exploris™ 120/240/480 MS
- Data evaluation package for Isotope Ratio MS
- Dual Syringe Inlet

For automation and higher throughput, the Thermo Scientific™ Vanquish™ Neo UHPLC System can be coupled to Orbitrap Exploris Isotope Solutions.

The first step in sample analysis is the soft ionization technique known as ESI, which allows for direct analysis without unintentional fragmentation. Determining isotope ratio data from the ratios of intact isotopologues of molecular ions can provide a better understanding of the processes of molecule formation and provides new geochemical proxies for understanding the world around us. With an ESI source, polar molecules can be introduced as liquid solutions without preparatory chemical derivatization or conversion, making preparation and analysis of samples faster and simpler.

The principle of ESI includes dispersion of a fine spray of charged droplets, solvent evaporation and in the final step, ion ejection from the highly charged droplets into the gaseous phase (Figure 3).

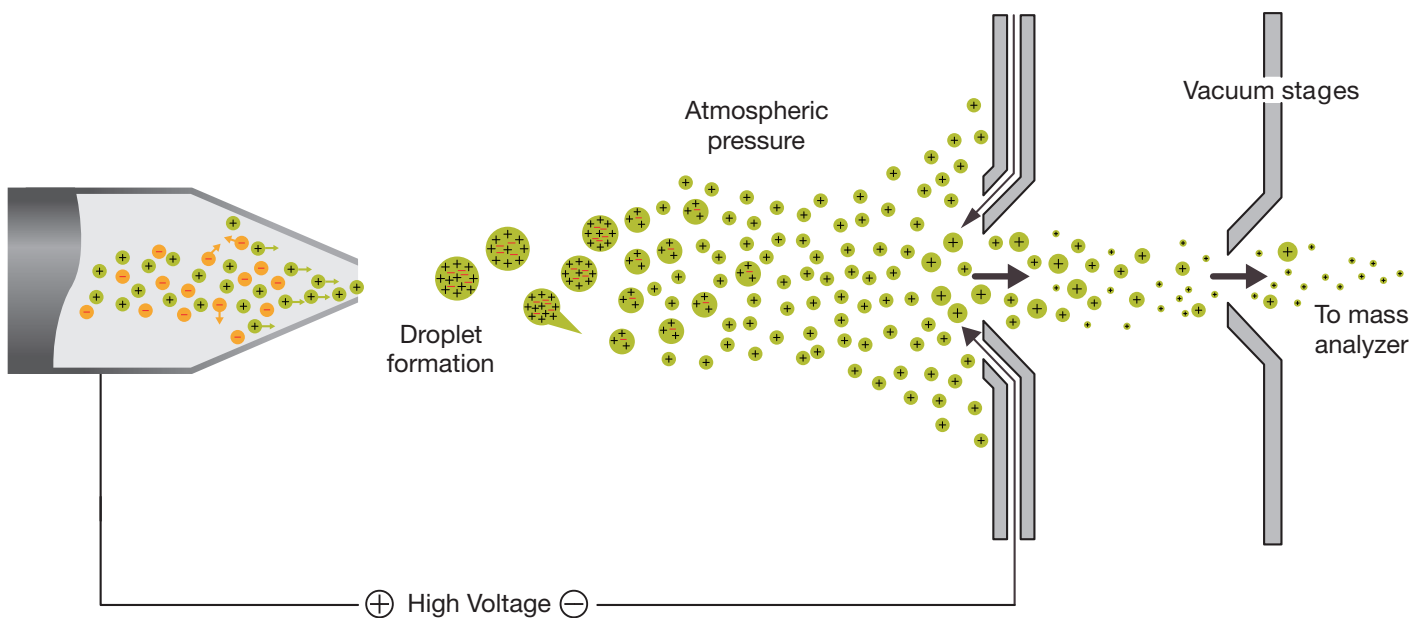


Figure 3. In ESI, the sample is aerosolized to a fine mist of charged droplets

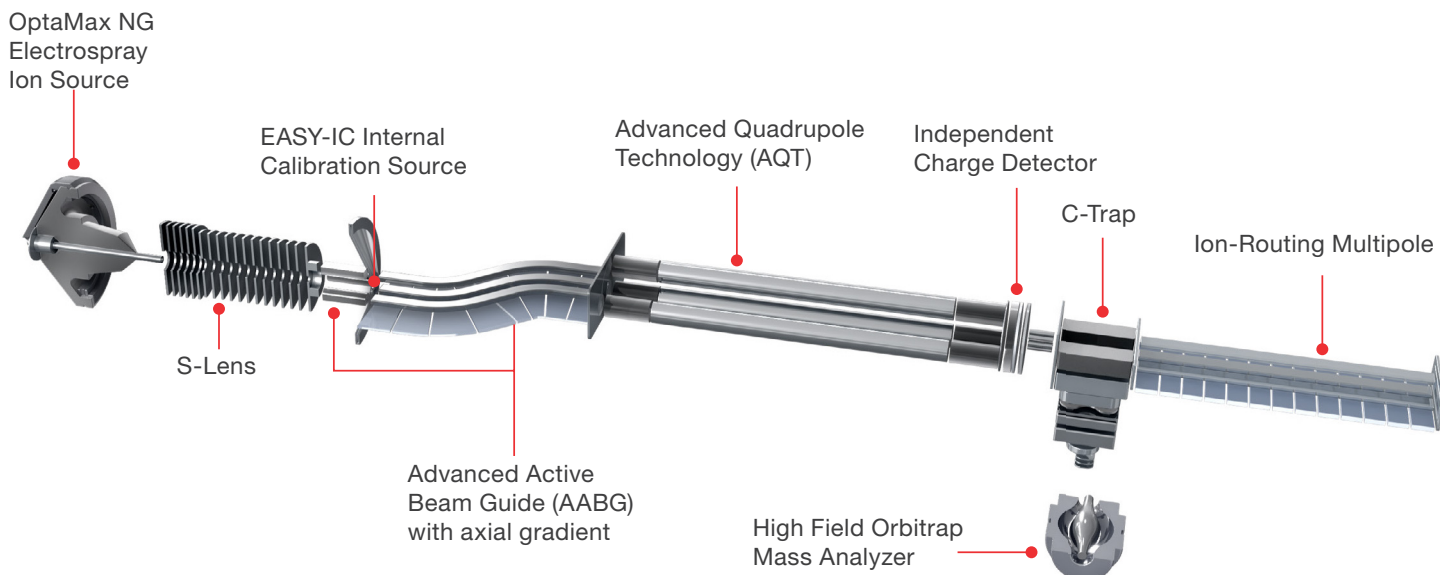


Figure 4. Ion path and key components of Orbitrap Exploris 120 and Orbitrap Exploris 240 mass spectrometers

As ions exit the ESI source, they are filtered through the Advanced Quadrupole Technology (AQT; Figure 4). The AQT allows a selected mass range to be analyzed. Ions are then passed into the ion-routing multipole by the C-Trap for storing and optional fragmentation by energetic collisions. After which, the selected ions or their collisionally-induced fragments are injected 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 between which the ions oscillate, at a frequency that is inversely proportional to the square root of the specific ion's mass-to-charge ratio,  $m/z$  of each ion. This oscillation of ions induces an image current on the outer electrodes, which is then processed using fast Fourier transform analysis to obtain the  $m/z$  and abundance information for each analyte species.

### Sample introduction methods

Orbitrap Exploris Isotope Solutions incorporate the concept of sample/reference comparison and supports two sample introduction methods:

1. Dual Syringe Inlet system utilizing the diverter valve option of the Orbitrap Exploris MS
2. In-flow Injection automated approach based on the Vanquish Neo UHPLC System

In Dual Inlet measurement setup (Figure 5), two syringes are filled with reference- and sample-solution respectively and flushed through the diverter valve by a single syringe pump. Switching the valve allows the alternating introduction of sample and reference into the Orbitrap Exploris MS. After conditioning the system, reference/sample/reference bracketing is carried out with 10 min blocks each. Figure 4 shows an example of a 70 min Dual Inlet measurement with 4 blocks of reference and 3 blocks of sample.



For In-flow Injection measurement (Figure 6), the Vanquish Neo UHPLC System is coupled to an Orbitrap Exploris MS. The Vanquish Neo binary pump can deliver a constant flow of LC/MS-grade methanol even at nano- and micro-flowrates. The autosampler is equipped with a loop for In-flow Injection, resulting in a 6-8 min wide plateau peak. After each injection the Vanquish Neo UHPLC System is flushed with pure methanol from the binary pump, resulting in total analysis time of 15 min per sample. The workflow setup comprises alternating injections of reference and sample. Alternatively, multiple blocks of sample and multiple blocks of reference can be analyzed for enhanced data statistics.

Dual Inlet setup allows efficient, high precision analysis while the automation of an In-flow Injection setup additionally enables the analysis of high quantities of samples with minimum user effort.

### Data collection and evaluation

Orbitrap Exploris Isotope Solutions deliver an isotope data collection and evaluation workflow. Isotope ratio measurements are executed using the following software tools:

1. Thermo Scientific™ Xcalibur™ Software for instrument setup and data acquisition

2. Thermo Scientific™ FreeStyle™ Application for data visualization, spectral confirmation, and accurate mass determination
3. Thermo Scientific™ IsoX™ Software for isotopologue data extraction from the Orbitrap Exploris MS RAW files

Before any data can be collected, ESI parameters are optimized within the Tune New Generation (TNG) Software to obtain optimal sensitivity and stability for isotope ratio analysis (Figure 7). Setting up measurements, instrument methods and sample sequences for the acquisition of RAW files is done via the Xcalibur Software.

Using the FreeStyle Application (Figure 8), sample composition is visualized and accurate  $m/z$  values are displayed for target analytes. For isotope ratio analysis, the RAW files are processed by the IsoX Software, utilizing the simple tab-delimited input file (isotopologs.tsv), which defines the compounds of interest and their isotopologues.

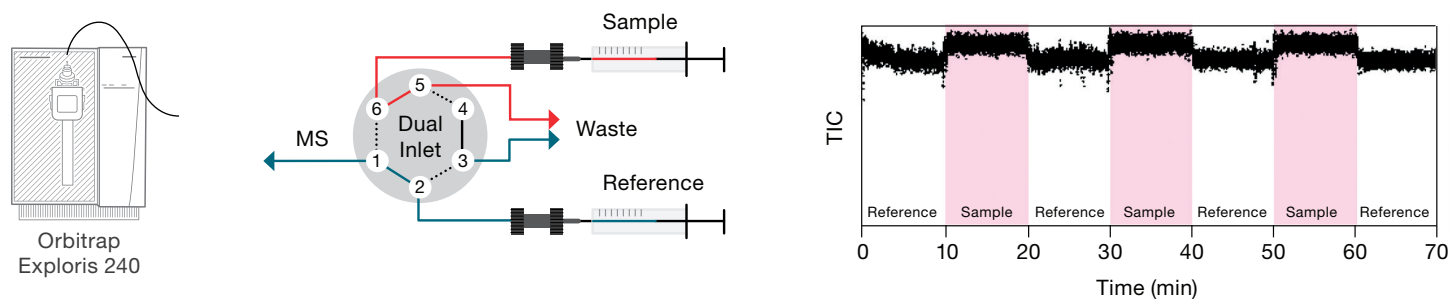


Figure 5. Dual Inlet measurement with marked times when the valve switches between standard and sample

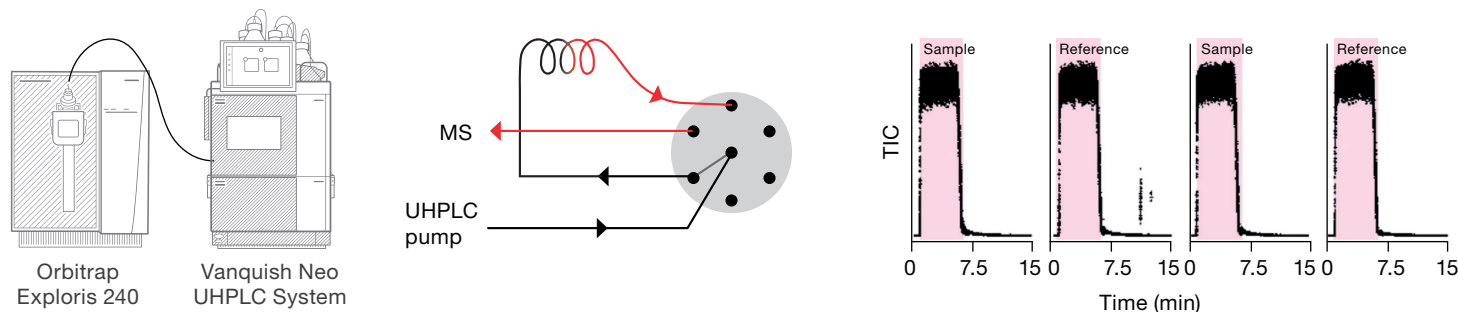


Figure 6. In-flow Injection measurement showing alternating injections of sample and reference, and the system flushing with methanol

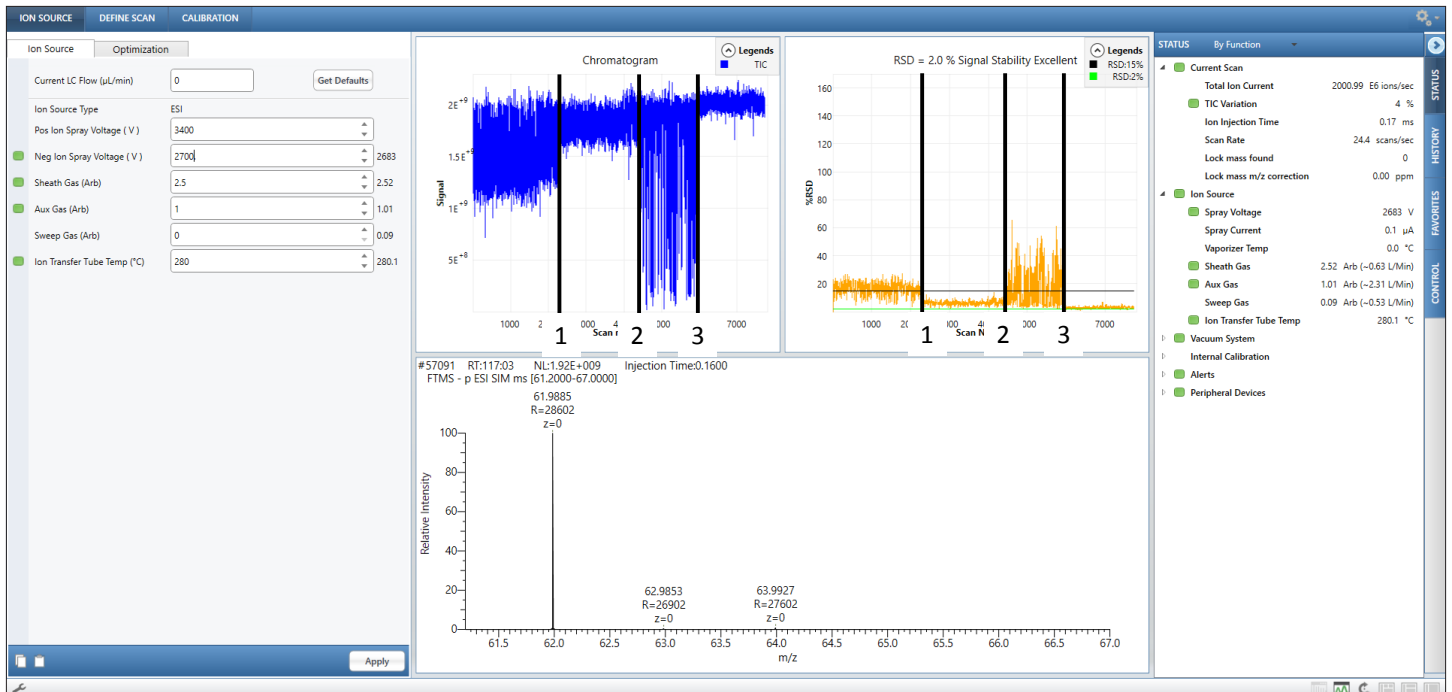


Figure 7. Tuning ESI parameters for optimizing the sensitivity and stability of the signal in TNG Software. The starting conditions were spray voltage (SV): 2400 V; Aux Gas (AG): 2; Sheath Gas (SG): 2.5. (1): SV increased to 2700 V. (2): AG increased to 3. (3): AG decreased to 1.

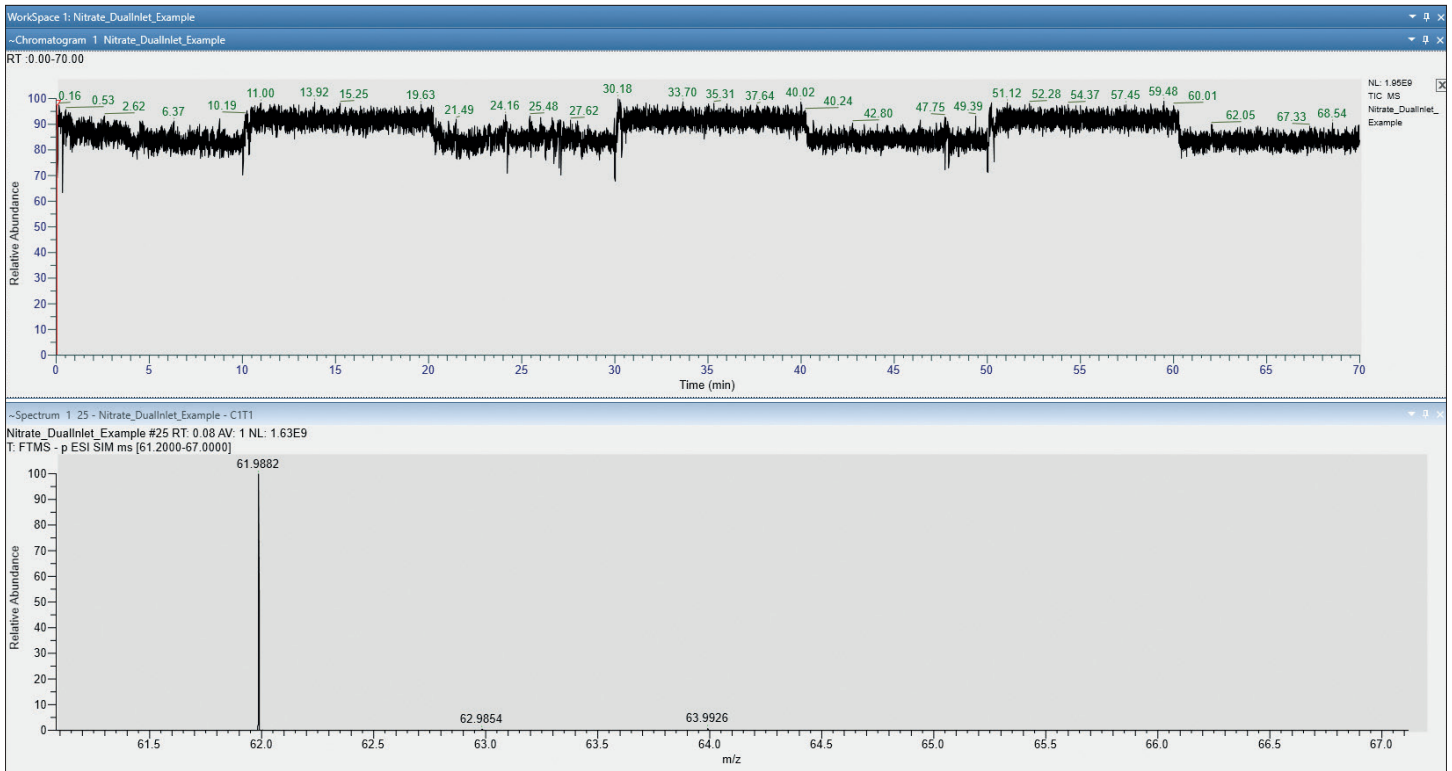


Figure 8. FreeStyle Application

The IsoX Software then extracts all relevant intensity and  $m/z$  information from the RAW files based on targets defined in the isotopologs.tsv file. The resulting IsoX Software output files, including all the data needed for the calculation of isotope ratios, are simple tab-delimited files and can be opened as spreadsheets. Isotope ratio calculation and further data processing can be performed using commonly used data science statistical computing programs (Figure 9). For processing of multiple RAW files (e.g. from In-Flow Injection measurements), a combined IsoX Software output file can be created.

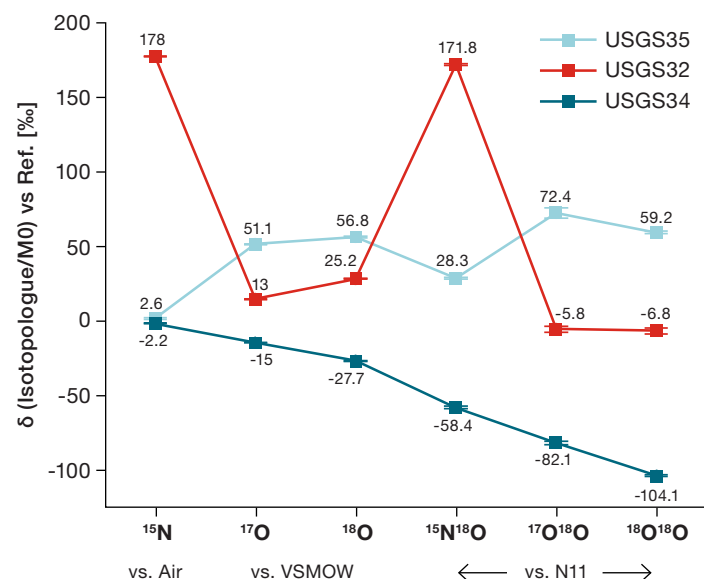


Figure 9. Plot with relative delta values of the isotopic reference materials USGS32, USGS34 and USGS35

## Applications

Orbitrap Exploris Isotope Solutions open new dimensions of isotope ratio analysis in a variety of applications, including:

1. Understanding **biogeochemical cycles** using multiple isotopologues of CNSOH bearing species (e.g. nitrate, sulfate, phosphate, acetate and other simple organics)
2. Understanding **redox states in the geological past** (e.g. different oxygen reservoirs in the geologic record, presence of microbial activity, atmospheric conditions, atmosphere-hydrosphere interactions) using sulphate isotopes in ice cores and ancient mineral deposits

3. Understanding **geological proxies and their formation** (e.g. formation of alkenones and fatty acids) using isotope ratios of multiple elements
4. **Deconvoluting complex metabolic pathways** by tracing metabolite transformations, e.g. disease prevention, personalized medicine and understanding microbial consortia interactions
5. Investigating **authenticity control of food and beverages** using isotope ratios of multiple elements and position specific isotope analysis, e.g. geographic origin of vanillin
6. **Identifying the use of performance enhancing drugs**

## Summary

Classical IRMS works by breaking down complex molecules in simple gases. In doing so, important intramolecular isotopic information is lost. This intramolecular isotopic information is important as it can provide a better understanding of the processes of molecule formation and provide new geochemical proxies for understanding the world around us.

Current technology for deriving intramolecular isotopic information is limited either by large sample sizes, long analysis times or limited applicability across a range of sample types.

Orbitrap Exploris Isotopes Solutions for isotope ratio analysis offer several key possibilities for deriving intramolecular isotopic information:

- Reduce the analysis times by analyzing the ratios of the multiple number of isotopologues in a single run
- Increase the productivity by removing matrix interferences and focusing the analysis on a selected scan range
- Acquire accurate isotopic information from minor isotopologues by amplifying their signals using the 'No MO' methodology
- Reduce sample sizes due to increased sensitivity compared to classical IRMS
- Preserve intramolecular isotopic information throughout the analysis utilizing soft ionization techniques
- Acquire position specific isotope information from functional groups through fragmentation of molecules

Learn more at [thermofisher.com/orbitrap-for-isotopes](https://thermofisher.com/orbitrap-for-isotopes)

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