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Isotope ratio MS

Isotope ratio analysis of nitrate using Orbitrap Exploris Isotope Solutions

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Introduction

Nitrate (NO_3^-) is a key component of the global cycling of nitrogen in natural and human-impacted environments. Analysis and interpretation of nitrate isotopes can reveal microbial pathways of nitrification and denitrification, as well as sources and fate of natural and anthropogenic nitrogen inputs to the environment.[1-4] Isotope ratio analyses are investigating the relative abundances of the three heavier isotopes in nitrate (^{15}N, ^{18}O, and ^{17}O) in comparison to the more abundant, lower mass isotopes (^{14}N and ^{16}O).

In this technical note we demonstrate how Thermo Scientific™ Orbitrap Exploris™ Isotope Solutions allow for comprehensive isotope analysis of nitrogen and oxygen within intact molecular nitrate ions. This includes non-mass-dependent oxygen isotope relationships and doubly substituted isotopologue abundances (i.e., clumped isotope effects).

Isotope ratios by Orbitrap MS technology

Orbitrap Exploris Isotope Solutions enables 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 relative to a reference with known isotope ratios, which allows reporting of results relative to international standards.

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Equipment and methodology

The Orbitrap Exploris Isotope Solutions for nitrate analyses presented here includes the Thermo Scientific™ Orbitrap Exploris™ 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

The Dual Syringe Inlet system measurement setup includes two 500 µL HPLC syringes (gauge 22, flat tip) in the syringe pump, which are filled with reference and sample solution respectively (see Figure 1). Liquid solutions are delivered to the Orbitrap MS system through a diverter valve at a flow rate of 4 µL/min. Switching the diverter valve allows the alternating introduction of sample and reference into the Orbitrap Exploris MS. For data acquisition, reference/sample/reference bracketing is carried out for 10 min/block (e.g. 4 blocks of reference and 3 blocks of sample = 70 min run).
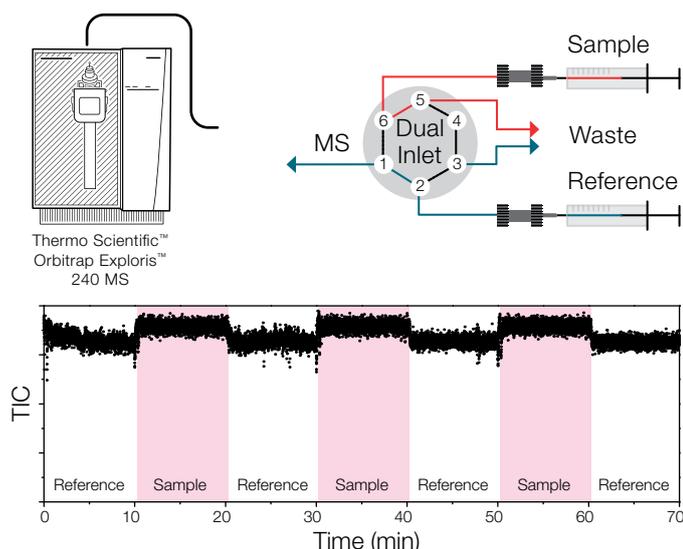

Figure 1. Dual Inlet measurement with marked times when the valve switches between standard and sample

For In-Flow Injection measurements, the Vanquish Neo UHPLC System is coupled to the Orbitrap Exploris MS. The UHPLC pump module delivers a constant flow of LC/MS-grade methanol at a flow rate of 4 µL/min. The autosampler is equipped with a 25 µL sample loop for In-Flow Injections, resulting in 6-8 min wide plateau peaks. Each injection results in a total analysis time of 15 min per sample to ensure that no sample is left in the loop. The presented workflow setup comprises two alternative injection patterns to set up a sequence of In-Flow Injections. Either single injections of reference and sample can be alternated (see Figure 2) or injection blocks of multiple reference/sample injections can be alternated, both of which allow for isotope ratio determination by bracketing.
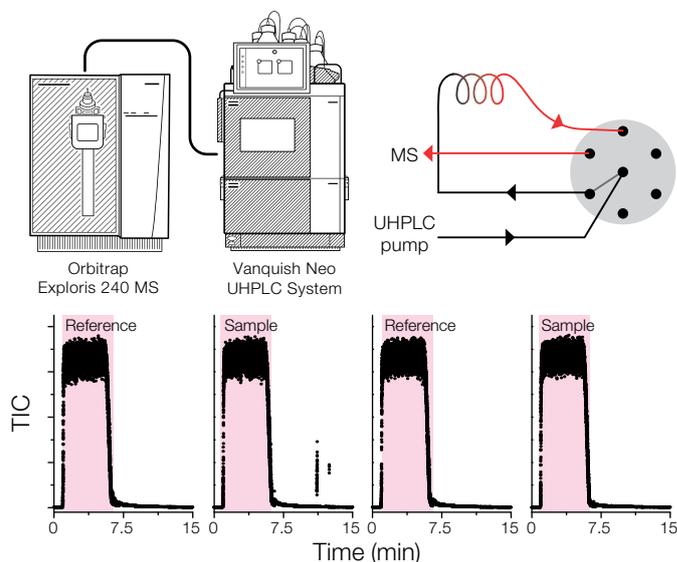

Figure 2. In-Flow Injection measurement showing alternating injections of sample and reference, and system flushing with methanol

Orbitrap Exploris MS settings

Source parameters and settings used for the isotope ratio analysis are listed in Table 1.

Table 1. Ion source settings for Orbitrap Exploris 240 MS

Ion source settings for Orbitrap Exploris 240 MS	
Sheath gas flow rate	2
Auxiliary gas flow rate	1
Sweep gas flow rate	0
Spray voltage	~ 2.7 kV (negative ionization mode)
Spray current (observed)	≤ 0.1 µA
Capillary temperature	280 °C
RF Lens	100 %

Two different methodologies were applied for isotope ratio analysis utilizing different scan ranges of the Orbitrap Exploris MS quadrupole mass filter:

1. 'M0' experiment - includes the predominant monoisotopic base peak consisting of the light isotopes ($^{14}N^{16}O_3$)

2. 'noM0' experiment - excludes the monoisotopic base peak amplifying the minor peaks, including doubly substituted isotopologues

The scan parameters used for the experiments are listed in Table 2.

Table 2. Scan parameters used for isotope ratio analysis with the Orbitrap Exploris 240 MS

Scan type	Full scan
Scan ranges (isolation range)	m/z 61.2–67 ('M0') and m/z 62.4–68 ('noM0')
Resolution	R = 15,000 ('M0') or 30,000 ('noM0')
Polarity	negative
Microscans	5
Lock masses	off
Automatic gain control (AGC) target	300,000
AGC prescan mode	-1
Maximum injection time	1000 ms

Data acquisition and evaluation

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

The resulting RAW files are processed by Thermo Scientific™ IsoX™ Software to extract all relevant parameters for the calculation of isotope ratios. To determine the number of ions (N) entering the analyzer, the signal-to-noise ratio (S/N_P) of a given spectral peak in a single scan is processed by formula (1).[5,6,7]

(1)

$$N = \left(\frac{S}{N_P}\right)\left(\frac{C_N}{z}\right)\sqrt{\frac{R_N}{R}}$$

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. For processing of multiple RAW files, a combined IsoX Software output file can be created.

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

Calculation of δ-values was performed using Microsoft™ Excel™. Presented samples and standards data were measured against a solution of working standard. Formula (2) shows the calculation of δ-values against a standard for isotope ratios of $^{15}N/^{14}N$.

(2)

$$\delta^{15}N_{Sample/Standard} = \left(\frac{R(^{15}N^{16}O_3/^{14}N^{16}O_3)_{Sample}}{R(^{15}N^{16}O_3/^{14}N^{16}O_3)_{Standard}} - 1\right) \cdot 1000 \, [‰]$$

Nitrate isotopic compositions were calculated and reported relative to the international reference materials, i.e. atmospheric N_2 nitrogen (AIR) for $^{15}N/^{14}N$, and Vienna Standard Mean Ocean Water oxygen (VSMOW) for $^{18}O/^{16}O$ and $^{17}O/^{16}O$.

To calculate the δ-values against international primary reference materials, known values of the working standard ($\delta^{15}N_{(Standard/Air)}$) and the measured sample values ($\delta^{15}N_{(Sample/Standard)}$) were evaluated using equation (3).

(3)

$$\delta^{15}N_{Sample/Air} = \delta^{15}N_{Sample/Standard} + \delta^{15}N_{Standard/Air} + \frac{\delta^{15}N_{Sample/Standard} \cdot \delta^{15}N_{Standard/Air}}{1000}$$

Reference material

The following reference material was analyzed to provide method and referencing framework for nitrate isotope analysis: USGS35 ($NaNO_3$) and USGS32, USGS34, RSIL-N11 (N11) (all KNO_3). [8-10] Solid samples of reference materials were prepared as 100 mM stock solutions in a 1:1 mixture of water and methanol. Aliquots of the stock solutions were then diluted with methanol as working solution for isotopic analysis. The concentration of the working solution used for the shown data was 50 µM if not stated differently. All solvents used during the studies were of LC/MS-grade purity.

Results

The nitrate molecular ion consists of three oxygen and one nitrogen atom. Different combinations of the two elements' stable isotopes result in 20 isotopologues of nitrate. Table 3 lists the most abundant isotopologues in the range of m/z 61 to 66. M0, the most abundant isotopologue (98.9 %), refers to the monoisotopic molecule consisting of only the light isotopes ^{14}N and ^{16}O. All other isotopologues show single or multiple substitutions by the heavy isotopes ^{15}N, ^{17}O and ^{18}O. Based on their cardinal masses, the isotopologues can be grouped as M+X (Table 3). Using Orbitrap MS technology the four most abundant isotopologues can be detected in a single run (Figure 3). Typically, a scan range of m/z 61.2-67 is selected in the so called 'M0' methodology. The peaks are labeled according to the heavy isotope substitutions present in the corresponding nitrate ion.

Table 3. Abundance and *m/z* of nitrate isotopologues in the range of 61-66 *m/z*.

Cardinal mass	Isotopologue	Substitutions	m/z theor.	Abundance [ppm]
M0	$^{14}N^{16}O_3$	**Monoisotopic (M0)**	**61.9884**	**989.242**
M+1	$^{15}N^{16}O_3$	^{15}N	**62.9854**	**3637**
		^{17}O	**62.9926**	**1127**
M+2	$^{14}N^{17}O^{16}O_2$	$^{15}N^{17}O$	63.9896	4.1
	$^{14}N^{18}O^{16}O_2$	^{18}O	**63.9926**	**5951**
	$^{14}N^{17}O_2{}^{16}O$	$^{17}O^{17}O$	63.9968	0.4
M+3	$^{15}N^{18}O^{16}O_2$	$^{15}N^{18}O$	**64.9897**	**21.9**
	$^{15}N^{17}O_2{}^{16}O$	$^{15}N^{17}O^{17}O$	64.9938	< 0.1
	$^{14}N^{17}O^{18}O^{16}O$	$^{17}O^{18}O$	**64.9968**	**4.5**
	$^{14}N^{17}O_3$	$^{17}O^{17}O^{17}O$	65.001	< 0.1
M+4	$^{15}N^{17}O^{18}O^{16}O$	$^{15}N^{17}O^{18}O$	65.9939	< 0.1
	$^{14}N^{18}O_2{}^{16}O$	$^{18}O^{18}O$	**65.9969**	**11.9**
	$^{15}N^{17}O_3$	$^{15}N^{17}O^{17}O^{17}O$	65.9981	< 0.1
	$^{14}N^{17}O_2{}^{18}O$	$^{17}O^{17}O^{18}O$	66.0011	< 0.1

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. Generally, the most abundant peak of a mass spectrum is the base peak. In 'M0' experiments the base peak is the M0 peak which is used for isotope ratio calculations. Different to conventionally used sector field MS, the Orbitrap Exploris MS does not analyze a continuous stream of ions but ion packages that are collected after the quadrupole mass filter and injected into the Orbitrap one by one. The size of these packages can be controlled by adjusting the target of the automatic gain control (AGC). The AGC target is an arbitrary number that correlates to the number of ions send to the Orbitrap-analyzer.
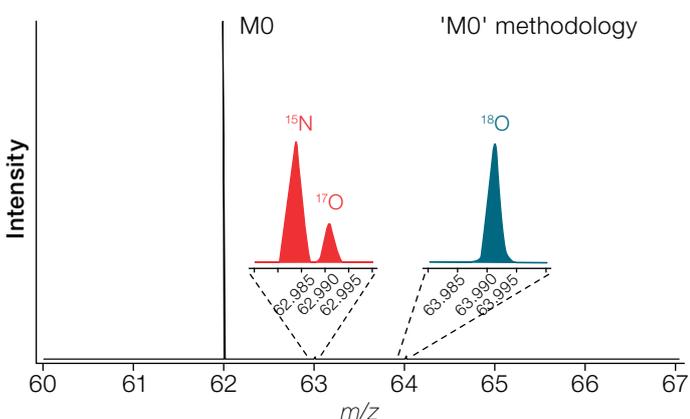

Figure 3. Mass spectrum of nitrate 'M0' methodology (scan range *m/z* 61.2-66). The peaks are labeled according to the heavy isotope substitutions in the corresponding nitrate ion.

In case of 'M0' experiments ~99 % of the ions within a single Orbitrap scan are M0 ions (see Table 3). All other isotopologues make the last 1 %, not letting enough ions of the clumped minor isotopologues enter the Orbitrap to be detected.

Shifting the scan range of the quadrupole mass filter to higher masses (e.g. *m/z* 62.2-68) excludes the M0 ion from the ion package entering the Orbitrap-analyzer. This so-called 'noM0' methodology, enables the detection and the calculation of isotope ratios for the three minor clumped isotopologues $^{15}N^{18}O^{16}O_2$, $^{14}N^{17}O^{18}O^{16}O_2$ and $^{14}N^{18}O_2{}^{16}O$. Figure 4 shows the

mass spectrum of a 'noM0' measurement. The peaks are labeled according to the heavy isotope substitutions in the corresponding nitrate ion.
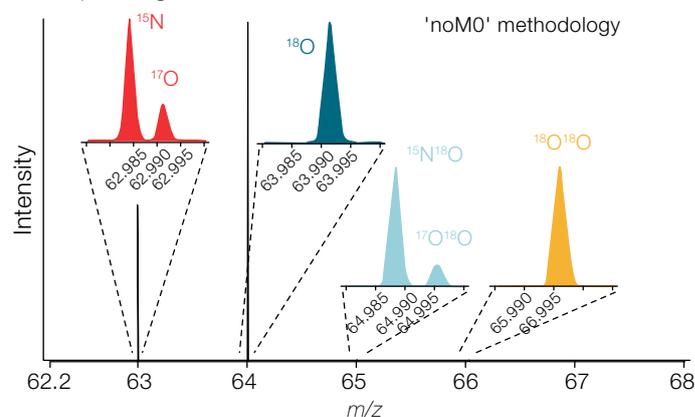

Figure 4. Mass spectrum of nitrate with 'noM0' methodology (scan range *m/z* 62.2-67). The peaks are labeled according to the heavy isotope substitutions of their corresponding nitrate isotopologue ion.

The M0 ion being excluded from entering the Orbitrap here allows the analysis of all other isotopologues at higher intensities. Being the most abundant species in these 'noM0' experiments, the peaks of the isotopologues with single substitutions of either ^{15}N or ^{18}O are typically used as base peak for isotope ratio calculation. The 'M0' methodology provides the link to the major isotopes (^{14}N and ^{16}O) by calculating the isotope ratios $^{15}N/^{14}N$ and $^{18}O/^{16}O$. Ratios of the clumped isotopes ($^{15}N^{18}O^{16}O_2$ or $^{14}N^{18}O_2{}^{16}O$, $^{14}N^{18}O^{17}O^{16}O$) over the major isotopes can be calculated using the base peak as the mediator.

The isotope ratios of nitrate isotopologues can be determined with high precision and accuracy by combining the data acquired in both experiments. Additional automation of the isotope ratio analysis is achieved utilizing the Vanquish Neo UHPLC System.

Quality control by dual inlet measurements of nitrate with 'M0' methodology

There are three nitrate reference materials available from the United States Geological Survey (USGS): USGS32, USGS34 and USGS35. A typical workflow for a quality check of an Orbitrap MS system for isotope ratio analysis is the dual inlet analysis of USGS32 versus USGS35. According to Figure 1, alternating 10 min blocks of reference solution (USGS35, 50 µM) and sample solution (USGS32, 50 µM) are infused into the Orbitrap MS by the syringe pump. The measurement of four reference and three sample blocks within a single analysis results in 70 min runtime. USGS35 is used as reference with a reported $\delta^{15}N_{USGS35/Air}$ of 2.7 ‰. Using formula (3) the measured values of USGS32 as shown in Table 4 are calculated versus Air. The δ-value of each sample block is calculated against the average isotope ratio of the bracketing reference blocks. Three consecutive dual inlet analyses show $\delta^{15}N_{USGS32/Air}$ values in the range of 179.0-179.6, resulting in an accuracy of ≤ 1 ‰ to the reported value of 180.0 ‰.

Table 4. Three dual inlet analyses of USGS32 versus USGS35 using 'M0' methodology.

Analysis	Description	Ratio ($^{15}N/^{14}N$)	$\delta^{15}N_{USGS32/Air}$ [‰]	$\delta^{15}N_{USGS32/Air}$ [‰] Reported value = 180.0 ‰	Std.dev.
1	reference (USGS35)	0.00429			
	sample (USGS32)	0.00506	180.2		
	reference (USGS35)	0.00430			
	sample (USGS32)	0.00505	178.7	179.0	1.0
	reference (USGS35)	0.00430			
	sample (USGS32)	0.00506	178.3		
	reference (USGS35)	0.00431			
2	reference (USGS35)	0.00430			
	sample (USGS32)	0.00505	178.6		
	reference (USGS35)	0.00430			
	sample (USGS32)	0.00506	180.7	179.5	1.1
	reference (USGS35)	0.00430			
	sample (USGS32)	0.00506	179.2		
	reference (USGS35)	0.00430			
3	reference (USGS35)	0.00430			
	sample (USGS32)	0.00506	179.5		
	reference (USGS35)	0.00430			
	sample (USGS32)	0.00506	179.3	179.6	0.4
	reference (USGS35)	0.00430			
	sample (USGS32)	0.00506	180.1		
	reference (USGS35)	0.00430			

Referencing scheme: Analysis of international standards for nitrate

Combining the isotope ratio data of 'M0' and 'noM0' experiments and using N11 as a working standard all three USGS nitrate reference materials can be characterized using the Orbitrap MS technology. The analyses can be automated using UHPLC systems for In-Flow Injections of nitrate solutions. A typical injection sequence consists of alternating blocks with multiple reference/sample injections. One injection results in a run time of 15 min. Injection of nitrate solution provides 6-8 minutes of constant signal intensity for isotope analysis and is followed up with 7-9 min of loop-flushing to prevent cross contaminations (see Figure 2). Performing four injections per block and analyzing all three USGS standards (sample) versus N11 (reference) within a single sequence results in 28 injections and an analysis time of ~8 h for the 'M0' experiments. To get additional isotope ratio information of the minor isotopologues ($^{15}N^{18}O^{16}O_2$, $^{14}N^{17}O^{18}O^{16}O_2$ and $^{14}N^{18}O_2{}^{16}O$) such a sequence can be repeated with 'noM0' methodology.

Using the accepted values for N11 the δ-values of $^{15}N/^{14}N$, $^{17}O/^{16}O$ and $^{18}O/^{16}O$ were calculated against the international references Air and VSMOW. Clumped isotope ratios are reported versus N11 assuming stochastic distribution for the working standard. The UHPLC system allows the atomization of 'M0' and 'noM0' experiments for a full characterization of nitrate isotope ratios as shown in Figure 5 with minimal user input.
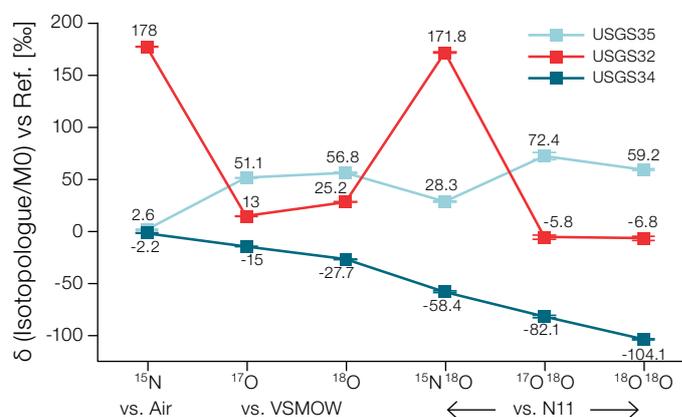

Figure 5. Exemplary data of isotope ratios of six of nitrate`s most abundant isotopologues measured for the three analyzed USGS-standards using the In-Flow Injection setup.

Method validation by environmental samples

Hilkert et al. [1] reports methodology tests and evaluation of nitrate analysis using Orbitrap technology. To validate the methodology, isotope ratio analysis of groundwaters, leachates and brines were performed as dilute and shoot measurements using the system. The analysis of environmental water samples for nitrate isotope ratios using the dual inlet dilute and shoot approach requires no prior purification of the samples. The dilution of the samples in methanol enabled analysis of water samples originally with > 500 µM of nitrate and high concentration of other anions such as sulfate and chloride. Even for samples with chloride concentrations of up to 100-folds higher than nitrate concentration no ion suppression in the ESI-source was observed. After the ionization, matrix interferences were removed by the quadrupole mass filter or by mass spectral resolution. As Figure 6 shows, the isotope ratios of $^{15}N/^{14}N$, $^{18}O/^{16}O$ and $^{17}O/^{16}O$ match the expected values from conventional IRMS approaches. The δ-values determined by Orbitrap MS technology show average accuracy of better than 1 ‰ for both $\delta^{15}N$ and $\delta^{18}O$; results obtained from single dual inlet runs.

The reader is referred to Hilkert et al. [1] for more details. Note: the analysis of isotope ratios in Hilkert et al. [1] was performed using the Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer with the UltiMate™ 3000 RSLCnano System. System performance is equivalent or better for Orbitrap Exploris Isotope Solutions with Orbitrap Exploris MS and Vanquish Neo UHPLC System.

Conclusions

By utilizing two different techniques for sample introduction and UHPLC in-Flow Injection, Orbitrap Exploris Isotope Solutions allow the isotope ratio analyses of nitrate in environmental samples with faster analysis time and reduced sample size. Soft ionization by ESI-technology enables the analysis of intact nitrate ions and determination of non-stochastic isotopic distributions for the clumping of heavy isotopes. This isotopic information, which would be lost during combustion in conventional nitrate-IRMS methodology, can open new dimensions in understanding the individual pathways of biogeochemical N-cycling.
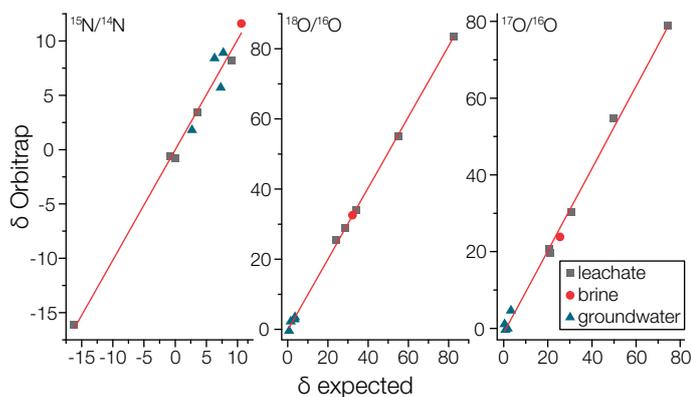

Figure 6. Plots of δ-values determined by Orbitrap MS versus expected values for ten environmental samples of different regional origins. Samples were analyzed using the Dual Syringe Inlet sample introduction system and 'M0' methodology.

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