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Exploring the Potential of Electrospray-Orbitrap for Stable Isotope Analysis Using Nitrate as a Model

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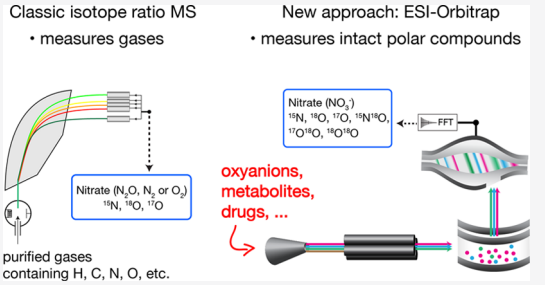
ABSTRACT: Widely used isotope ratio mass spectrometers have limited capabilities to measure metabolites, drugs, or small polyatomic ions without the loss of structural isotopic information. A new approach has recently been introduced that uses electrospray ionization Orbitrap to measure multidimensional isotope signatures of intact polar compounds. Using nitrate as a model compound, this study aims to establish performance metrics for comparisons with conventional IRMS at the natural abundance level. We present a framework on how to convert isotopolog intensities to δ values that are commonly used in the isotope geochemistry community. The quantification of seven nitrate isotopologs provides multiple pathways for obtaining the primary N and O δ values including non-mass-dependent O isotope variations, as well as opportunities to explore nonrandom isotopic distributions (i.e., clumping effects) within molecular nitrate. Using automation and the adaptation of measurement principles that are specific to isotope ratio analysis, nitrate $\delta^{15}N_{AIR}$, $\delta^{18}O_{VSMOW}$, and $\delta^{17}O_{VSMOW}$ were measured with a long-term precision of 0.4‰ or better for isotopic reference materials and purified nitrate from environmental samples. In addition, we demonstrate promising results for unpurified environmental samples in liquid form. With these new developments, this study connects the two largely disparate mass spectrometry fields of bioanalytical MS and isotope ratio MS, thus providing a route to measure new isotopic signatures in diverse organic and inorganic solutes.



INTRODUCTION

Stable isotope variations allow unique insights into natural processes because they can be used to relate actions that take place at the atomic level to effects on much larger scales. Typically, stable isotope ratios of polar solutes are measured after the analyte has been converted into a low molecular weight gas by combustion, pyrolysis, microbial fermentation, or other chemical treatments. The gas is then examined on a magnetic sector isotope ratio mass spectrometer (IRMS).[1] However, in these methods, much of the intramolecular isotopic information is erased during the formation of gases and by destructive ionization in the IRMS. Despite complementary techniques,[2,3] a significantly different and more sensitive approach is needed to access the native isotopic "fingerprints" that are recorded by intact molecules. Such patterns have long been thought to be potentially useful tools to elucidate processes and mechanisms that shape the functioning of cells and ecosystems.[4]

Liquid chromatography mass spectrometry (LC/MS) studies performed over the last decade point to a plausible path via soft ionization techniques such as electrospray ionization (ESI).[5−9] Recent work on unlabeled amino acids and oxyanions shows that in particular, ESI coupled to a quadrupole-Orbitrap analyzer (ESI-Orbitrap) is a promising technology for analyzing the isotopic information of intact polar compounds at a natural abundance level.[10−13] However, important gaps exist between these proof-of-concept studies and a method suitable for routine usage. For example, a framework for converting the isotopolog intensities to δ values that are commonly reported in the isotope geochemistry community has yet to be established. In addition, previous work has relied on manual injection of samples, limiting throughput, and precision (i.e., related to human errors or interruption of the spray). In this study, we improve ESI-Orbitrap-based isotopic spectrometry for analyzing natural-abundance isotopic composition of intact polar compounds and compare it to well-established IRMS techniques.

Specifically, we use nitrate as a reference model in this study. Nitrate (NO_3^-) is one of the simplest polar compounds, and it is a key component of the global cycling of nitrogen in natural and human-impacted environments.[14,15] The biogeochemical research community provides a firm foundation for the analysis and interpretation of nitrate isotopes. Optimized methods[16−19]

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and a suite of calibrated international reference materials[20−22] make nitrate our preferred model for developing isotope ratio measurements by ESI. 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) reveal how nitrogen moves among living and nonliving forms in the biosphere and geosphere. For example, the isotope ratios can reveal microbial pathways of nitrification and denitrification, as well as sources and fate of natural and anthropogenic nitrogen inputs to the environment.[23,24]

In this paper, we report measurement principles that are designed for automated and reproducible analysis of isotopes in intact molecules by ESI-Orbitrap, with application to nitrate. This is achieved by (1) reconfiguring and fine tuning of technologies that are routinely used as mass spectrometers in metabolomics with the aim of enabling their use for isotope ratio analysis, (2) combining measurements performed separately with and without the dominant isotopolog peak, (3) calibrating data with respect to nitrate isotopic reference materials, and (4) testing the method with different types of sample solutions of varying complexity including diluted brines and groundwater samples. The focused study of nitrate provides a significant increment of technical advancement for isotopic spectrometry by ESI-Orbitrap with approximately 10-fold improvement of sample throughput, sensitivity, and isotopic accuracy, as well as bringing the technology to environmental samples.[12] The replication and long-term accuracy of these analyses show that the ESI technology is approaching the stage of practical applications with capabilities not realized by previous methods, including comprehensive molecular isotopic characterization, small sample sizes, and simple pre-treatment in some cases.

EXPERIMENTAL SECTION

Reference Materials and Environmental Samples. Isotopic reference materials were provided by the United States Geological Survey (USGS; Reston, VA). USGS32, USGS34, and RSIL-N11 (N11) were provided as KNO_3 salts, and USGS35 as $NaNO_3$ salt.[20−22] Environmental samples archived from previous studies included (1) concentrated brines and soil leachate solutions from desert salt accumulations in representative arid regions on Earth[25,26] and (2) fresh groundwater samples from different regions in the US (see Table S2).[26−28] Desert soil leachates were prepared by mixing dry soil with deionized water and filtering.[25,26] Groundwater samples were collected from wells, filtered, and stored cold or with hydroxide as a preservative (pH 11−12). The groundwater samples were typical of aquifers affected by agriculture or urban land use and had relatively high NO_3^- concentrations (500−1300 μM) with varying proportions of Cl^-, SO_4^{2-}, and dissolved inorganic carbon, as described elsewhere.[26−28] For selected samples, NO_3^- was isolated from mixed salt solutions by trapping on large-volume AG1X8 ion-exchange resin columns followed by gradual elution with 0.5 M KCl to separate anions.[29] The KCl-KNO_3 eluents, as well as some of the reference materials, were passed through AG-MP50 resin columns in the Ag form to remove Cl and exchange K for Ag and then freeze-dried to produce $AgNO_3$ salts.[26]

For ESI analysis, solid samples of reference materials and purified environmental nitrate samples were prepared first as 100 mM stock solutions in a 1:1 mixture of LC/MS-grade water and methanol. Aliquots of the stock solutions were then diluted to 50 μM with LC/MS-grade methanol for isotopic analysis. For "dilute and shoot" analysis of environmental samples, the original aqueous leachates, brines, and groundwater samples were diluted to 1 μM nitrate with LC/MS-grade methanol and analyzed directly by ESI. For a list of nitrate isotopologs, relative abundances, representative mass spectra, and related definitions, see Table 1 and Figure 1.

Table 1. Isotopologs of Nitrate[a]

mass range	m/z	abundance [ppm]	isotopolog
M0	**61.9884**	**989,242**	**$^{14}N^{16}O_3$**
M + 1	**62.9854**	**3637**	**$^{15}N^{16}O_3$**
	62.9926	**1127**	**$^{14}N^{17}O^{16}O_2$**
M + 2	63.9896	4.1	$^{15}N^{17}O^{16}O_2$
	63.9926	**5951**	**$^{14}N^{18}O^{16}O_2$**
	63.9968	0.4	$^{14}N^{17}O_2{}^{16}O$
M + 3	**64.9897**	**21.9**	**$^{15}N^{18}O^{16}O_2$**
	64.9938	<0.1	$^{15}N^{17}O_2{}^{16}O$
	64.9968	**4.5**	**$^{14}N^{17}O^{18}O^{16}O$**
	65.0010	<0.1	$^{14}N^{17}O_3$
M + 4	65.9939	<0.1	$^{15}N^{17}O^{18}O^{16}O$
	65.9969	**11.9**	**$^{14}N^{18}O_2{}^{16}O$**
	65.9981	<0.1	$^{15}N^{17}O_3$
	66.0011	<0.1	$^{14}N^{17}O_2{}^{18}O$
M + 5	66.9939	<0.1	$^{15}N^{18}O_2{}^{16}O$
	66.9981	<0.1	$^{15}N^{17}O_2{}^{18}O$
	67.0011	<0.1	$^{14}N^{17}O^{18}O_2$
M + 6	67.9981	<0.1	$^{15}N^{17}O^{18}O_2$
	68.0011	<0.1	$^{14}N^{18}O_3$
M + 7	68.9981	<0.1	$^{15}N^{18}O_3$

[a]Isotopolog abundances are based on stochastically distributed isotope abundances corresponding to the primary standards AIR N_2 and VSMOW: ^{14}N = 99.6337%, ^{15}N = 0.3663%, ^{16}O = 99.76206%, ^{17}O = 0.03790%, and ^{18}O = 0.20004%.[44] The isotopologs in bold were measured in this study.

MS Instrumentation. For all the analyses, we used a Q Exactive HF Orbitrap (Thermo Fisher Scientific) with an Ion Max API source, which was connected to a HESI-II probe (Thermo Fisher Scientific) with a low flow needle for flow rates of 1−10 μL/min. The following ionization settings were used: sheath gas flow rate: 2, auxiliary gas flow rate: 0, sweep gas flow rate: 0, spray voltage: ~2.7 kV (negative ionization mode), spray current (observed): <0.2 μA, capillary temperature: 275 °C, S-lens RF level: 70, and auxiliary gas heater temp (actual): 40 °C.

To maximize the range of measurements that could be done, two different types of scans were performed, one of which included the predominant monoisotopic base peak consisting of only the light isotopes ("with M0") and one of which excluded the base peak so other minor peaks could be resolved more efficiently ("without M0"). The following mass spectrometer settings were used. Scan type: selected ion monitoring (SIM); scan ranges (isolation range): m/z 61.2−66 ("with M0") and m/z 62.2−67 ("without M0"); resolution: R = 15,000 or 30,000; polarity: negative; microscans: 5; lock masses: off; automatic gain control (AGC) target: 300,000; AGC prescan mode: −1; and maximum injection time: 1000 ms. Profile mode data were collected using the Q Exactive HF Tune software. The Xcalibur software (Thermo Fisher) was

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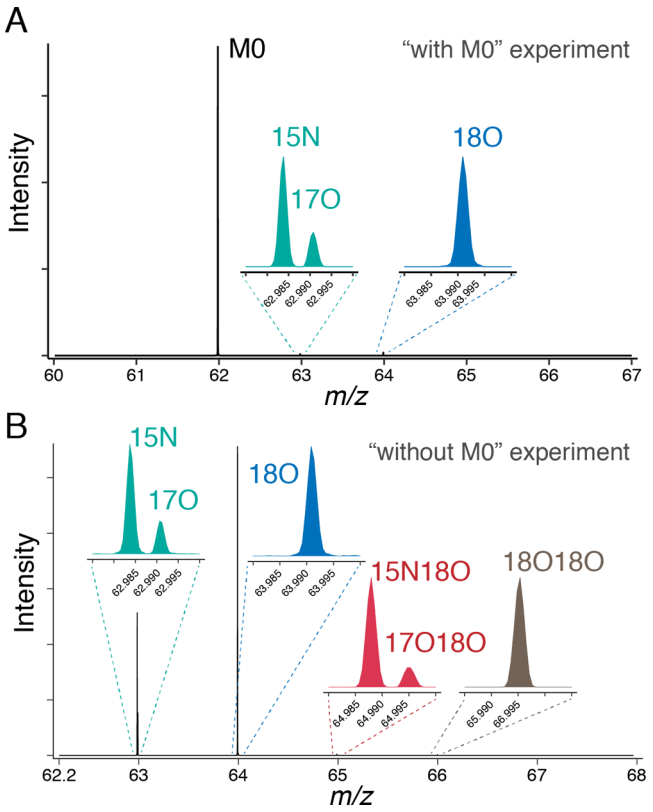

Figure 1. Mass spectra of NO_3^- in (A) "with M0" and (B) "without M0" experiments. Rare isotope substitutions are indicated (e.g., $^{18}O^{18}O$ is equivalent to $^{14}N^{18}O_2{}^{16}O^-$).

used for data acquisition by flow injection and automation of the dual inlet.

For most tests, we chose a nitrate concentration of 50 μM because this concentration did not limit the scan rate of the Orbitrap mass analyzer in the "without M0" experiments. The observed injection times were typically 0.4 ms in the "with M0" experiment and 25 ms in the "without M0" experiment when using 50 μM nitrate solutions (AGC target 300,000). The "dilute and shoot" of 1 μM solutions resulted in 4−7 ms injection time in the "with M0" experiment. The maximum Orbitrap scan rates were approximately 24 Hz at R = 15,000 and 14 Hz at R = 30,000 (at m/z 200).

Flow Injection. An UltiMate 3000 RSLCnano HPLC system (Thermo Fisher Scientific) was coupled to the Q Exactive HF Orbitrap for "flow injection" of nitrate solutions. The HPLC loading pump was used to carry LC/MS-grade methanol at a flow rate of 4 μL/min. The 6-port valve of the autosampler was equipped with a 20 μL loop for flow injection, resulting in a 6 min wide plateau peak. The total analysis time per sample was set to 15 min to prevent cross contamination. Sequences were set up in alternating blocks of four injections each for reference and for samples to improve precision and to allow drift correction. A sequence was started always with a block for conditioning. At the end of each block, an injection of 1:1 (v/v) HPLC-grade methanol:water was used to maintain a stable spray.

Dual Syringe Pump ("Dual Inlet"). To enable the continuous delivery of a reference and a sample in alternating mode, a digitally controlled syringe pump (Fusion 100, Chemyx) loaded with two 500 μL HPLC autosampler syringes (gauge22, flat tip; Thermo Fisher Scientific) was coupled to a 6-port valve (Rheodyne). The reference and sample were pumped simultaneously at a flow rate of 4 μL/min. After conditioning the system for at least 10 min, reference/sample/reference comparisons were carried out for 30 or 70 min by switching the valve every 10 min.

Data Processing. A custom-built FTStatistic software module of the Thermo Fisher Scientific data processing software suite was used for the unsupervised extraction of ion intensities from RAW files. The software is available upon request from A. Hilkert. R (version 3.6.3), RStudio, and the R-packages dplyr, tidyr, and ggplot2 were used for data analysis. Final evaluation and calculation of δ values were performed in Microsoft Excel. An example analysis is provided in the SI.

Calculation of δ-Values. Delta (δ) is defined as $\delta_{sa/STD} = R_{sa}/R_{STD} - 1$ or $[R_{sa} - R_{STD}]/R_{STD}$, where sa represents a sample, STD represents the primary standard, and R is the isotope abundance ratio ($^{15}N/^{14}N$, $^{18}O/^{16}O$, and $^{17}O/^{16}O$). Nitrate isotopic compositions were calculated and reported relative to the international standards, atmospheric N_2 nitrogen (AIR), and Vienna Standard Mean Ocean Water oxygen (VSMOW).

Isotopolog abundance ratios of samples were measured against those of a reference solution of a working nitrate laboratory standard RSIL-N11 (N11; see Table S2 in the SI). Provisional $\delta^{15}N$ values of a sample ("sa") relative to the N11 lab standard were obtained using isotopolog signals measured by ESI in experiments "with M0", for example:

$$\delta^{15}N_{sa/N11} = \frac{(^{15}N^{16}O_3/^{14}N^{16}O_3)_{sample}}{(^{15}N^{16}O_3/^{14}N^{16}O_3)_{N11}} - 1 \quad (1)$$

These values were then expressed relative to the $\delta^{15}N_{AIR}$ scale using the independently known value of $\delta^{15}N_{N11/AIR}$ (1-point calibration):

$$\delta^{15}N_{sa/AIR(1)} = \delta^{15}N_{sa/N11} + \delta^{15}N_{N11/AIR} + \delta^{15}N_{sa/N11}\cdot\delta^{15}N_{N11/AIR} \quad (2)$$

The accuracy of the results was tested and improved in some experiments using additional reference samples (e.g., USGS32 and USGS34) with much higher or lower δ values than those of N11 to adjust for δ-scaling effects (2-point calibration):

$$\delta^{15}N_{sa/AIR(2)} = \delta^{15}N_{RM1/AIR} + [\delta^{15}N_{sa/labstd} - \delta^{15}N_{RM1/labstd}]_{meas} \cdot[\delta^{15}N_{RM2/AIR} - \delta^{15}N_{RM1/AIR}] /[\delta^{15}N_{RM2/labstd} - \delta^{15}N_{RM1/labstd}]_{meas} \quad (3)$$

where RM1 and RM2 are nitrate isotopic reference materials with contrasting values (e.g., N11, USGS32, and USGS34) and labstd is an arbitrary reference material analyzed repeatedly in a given sample set to monitor machine performance and drift. For experiments in the current study, we used N11 as a substitute for both labstd and RM1 in eq 3. Analogous formulas to eqs 1−3 were used to obtain $\delta^{18}O_{sa/VSMOW}$ and $\delta^{17}O_{sa/VSMOW}$ from measurements of the $^{14}N^{18}O^{16}O_2$ and $^{14}N^{17}O^{16}O_2$ peaks relative to the $^{14}N^{16}O_3$ peak ("M0").

To benchmark δ values for isotopologs measured in the "without M0" experiment, we leveraged information known independently from gas-source IRMS measurements. For instance, $\delta^{18}O_{sa/N11}$ was calculated from $^{15}N^{16}O_3$ and

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$^{14}N^{18}O^{16}O_2$ isotopologs measured "without M0" using $\delta^{15}N_{sa/N11}$ known from IRMS (note, however, that $\delta^{15}N_{sa/N11}$ can also be determined by ESI; eq 1):

$$\delta(^{18}O/^{15}N)_{sa/N11} = \frac{(^{14}N^{18}O^{16}O_2/^{15}N^{16}O_3)_{sample}}{(^{14}N^{18}O^{16}O_2/^{15}N^{16}O_3)_{N11}} - 1 \quad (4)$$

$$\delta^{18}O_{sa/N11} = \delta(^{18}O/^{15}N)_{sa/N11} + \delta^{15}N_{sa/N11} + \delta(^{18}O/^{15}N)_{sa/N11}\cdot\delta^{15}N_{sa/N11} \quad (5)$$

The "without M0" experiment is conceptually novel and requires appropriate calibration procedures. 1- or 2-point calibrations were applied to the δ scale that was directly measured, for example, for $\delta(^{18}O/^{15}N)$ using USGS32 and N11. These two materials have a similar $\delta^{18}O$ but are suitable for calibration here because they display a big difference in $^{14}N^{18}O^{16}O_2/^{15}N^{16}O_3$ due to their difference in $\delta^{15}N$. A more detailed description of δ value calculations, including a formal derivation of equations and a sensitivity analysis of deviations associated with approximating δ values from isotopolog intensities, is available in the SI.

RESULTS AND DISCUSSION

Observable Isotopic Species. There are 20 stable isotopic species of nitrate (NO_3^-) with distinct masses (Table 1). The monoisotopic peak (M0; representing nitrate containing only the light isotopes ^{14}N and ^{16}O) as well as three peaks that contain ions with a single substitution of ^{15}N, ^{17}O, or ^{18}O is readily detected by ESI-Orbitrap (Figure 1). M0 contains almost all of the nitrate ions (98.9%). Other rare isotopologs are within the noise and not detected in these spectra because the Orbitrap has a limited capacity for ions per scan.

To study rare isotopologs, the monoisotopic peak can be eliminated by adjusting the quadrupole mass filter,[11] a setting which we call the "without M0" experiment. This enrichment allows the detection of the doubly substituted species $^{15}N^{18}O^{16}O_2$, $^{14}N^{17}O^{18}O^{16}O$, and $^{14}N^{18}O_2{}^{16}O$. Analysis of such "clumped" isotopes has gained prominence in geochemistry, for example, in carbonate clumped isotope thermometry.[30] Nitrate clumped isotopes, however, have never been measured before. This highlights that quantifying intact polar solutes can open new avenues for research.

Relations to Conventional Isotope Scales. The measurements described here differ in important aspects from two approaches that are common in various scientific disciplines. Intact polar molecules such as metabolites are often studied using artificially enriched isotope tracers. The abundances of isotopically labeled compounds are quantified by mass isotopomer analysis or related metabolic flux analyses.[31,32] In contrast, natural isotopic variations occur without experimental interference and are orders of magnitude smaller. Natural isotopic differences are thus usually measured after converting a polar analyte into a gas to achieve the necessary precision. Relative stable isotope abundances are then quantified as ratios in comparison to those of an internationally accepted reference, expressed in parts per thousand (per mil; ‰). For example, the nitrogen-15 content of nitrate in a sample normally is determined by converting the nitrate to another compound (e.g., N_2 and N_2O), and the IRMS results are reported as a $\delta^{15}N$ value, using nitrogen in air as the primary reference ($\delta^{15}N$ of air $N_2 \equiv 0$):

$$\delta^{15}N = \frac{(^{15}N/^{14}N)_{sample} - (^{15}N/^{14}N)_{AIR}}{(^{15}N/^{14}N)_{AIR}} = \frac{(^{15}N/^{14}N)_{sample}}{(^{15}N/^{14}N)_{AIR}} - 1 \quad (6)$$

When we are now measuring intact nitrate, each N and O isotope can be found in multiple isotopologs. In the simplest case, ^{15}N occurs in 10 species that have distinct masses (Table 1). This combinatorial complexity is a fundamental property of compounds containing elements with more than one atomic mass, and it includes isotopic fingerprints that are largely unexplored.[33] Any deviations from the stochastic isotopolog pattern are exceedingly small for the most abundant isotopologs of unlabeled nitrate. It can thus be approximated that

$$\frac{^{15}N}{^{14}N} = \frac{^{15}N^{16}O_3}{^{14}N^{16}O_3} \quad (7)$$

This relation can be used to define nitrate $\delta^{15}N$ based on measuring nitrate isotopologs from a sample and a reference. Because atmospheric N_2 cannot be measured directly by ESI, an additional calculation step is needed to express $\delta^{15}N$ values in the established international reference frame versus air N_2 (see Experimental Section and the SI).

The "without M0" experiment enriches rare isotopologs about 100-fold in the Orbitrap, with corresponding increases in counting statistics (shot noise limit). For example, $^{14}N^{18}O^{16}O_2/^{15}N^{16}O_3$ can be constrained with a precision of ±0.1‰ (1 RSE) within 15 min. Such tightly determined ratios offer much potential for isotope analysis. For instance, unusual isotopolog ratios can be leveraged to obtain familiar isotope ratios, using a relation of this kind

$$\frac{^{18}O}{^{16}O} = \frac{^{14}N^{18}O^{16}O_2}{^{15}N^{16}O_3}\cdot\frac{^{15}N}{^{14}N} \quad (8)$$

The link to conventional bulk isotopic ratios here is established by adding a single independent parameter for the bulk isotopic composition. This can be a $\delta^{18}O$ or $\delta^{15}N$ value determined in the "with M0" experiment, thus using ESI as a stand-alone method. For benchmarking "without M0" experiments, we can alternatively utilize δ values independently known from gas-source IRMS analysis (see also Experimental Section and the SI).

Necessary Requirements for IRMS Fidelity. Isotope ratios need to be measured with high accuracy and precision to learn about natural systems. Appropriate measurement principles have been refined for IRMS over decades. In the following, we bring these principles to the ESI-Orbitrap and report several features that are critical for its use as an isotope ratio mass spectrometer.

The measurement was automated, which improves reproducibility and sample throughput and decreases the sample amount needed per measurement. Most important for isotope ratios is that automation should keep electrospray conditions as stable as possible during data collection. We configured an HPLC system to sequentially deliver samples by "flow injection" via a continuous flow to the electrospray needle (Figure 2). After stabilization for 1−2 h, this setup allows

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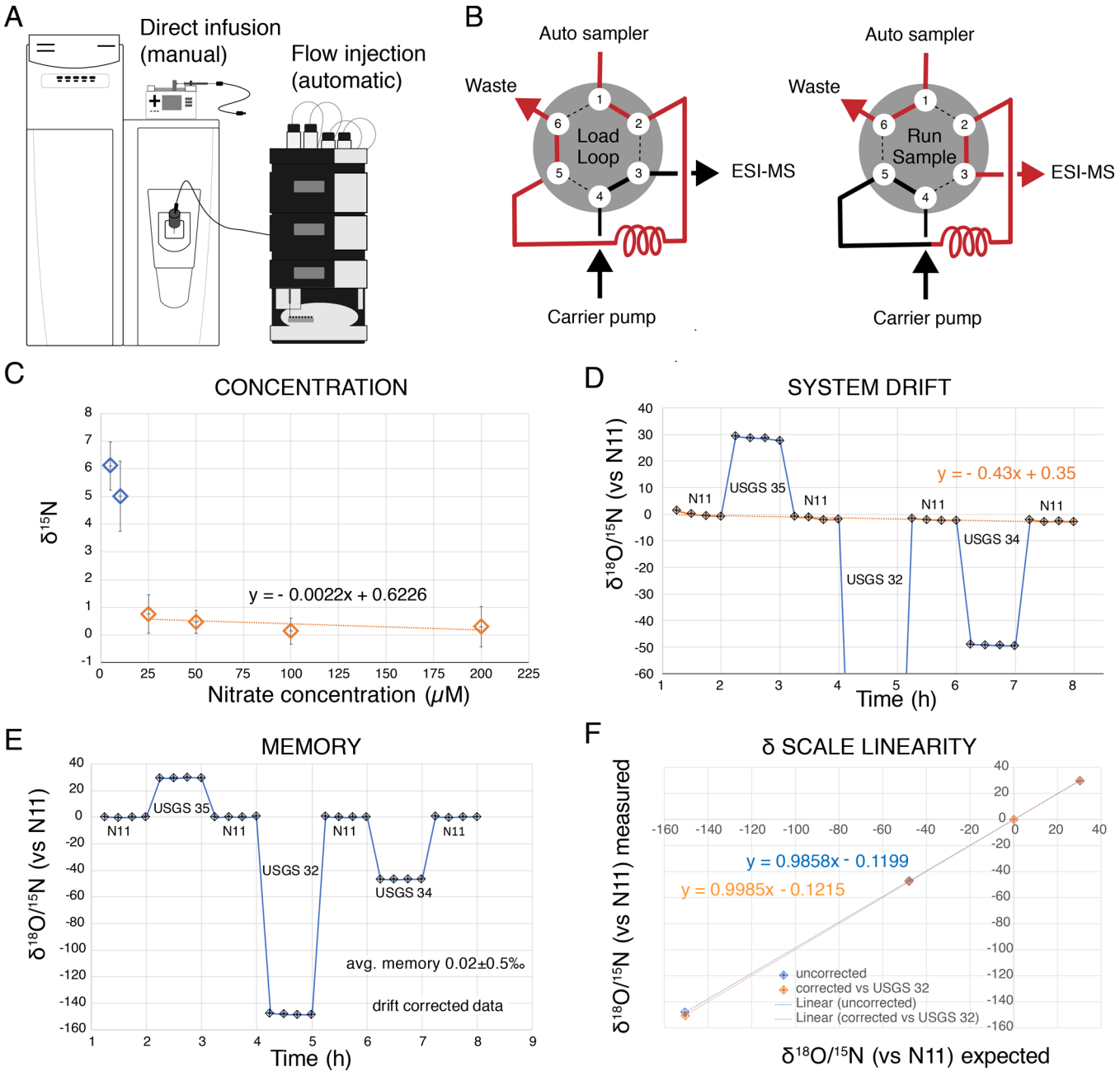

Figure 2. Automation enables high fidelity IRMS. (A) Coupling of routine LC and ESI-Orbitrap suitable for automation. (B) Schematic of a 6-port valve used for loading and infusing samples. (C) Concentration dependence of $\delta^{15}N$ of a sample (USGS35) in the "with M0" experiment. The concentration of the reference (also USGS35) was 50 μM. (D) An isotope ratio from the "without M0" experiment ($^{14}N^{18}O^{16}O_2/^{1516}O_3$) that showed a pronounced experimental drift. (E) Characterization of memory effects. (F) Linearity of the δ scale.

about 100 sample injections for isotope analysis per day. One injection requires less than 50 μL of a 50 μM sample (<155 ng of NO_3^-), with room for further improvements. These sample throughput and sensitivity are comparable to well-established methods for nitrate isotope analysis, like the denitrifier method.[19]

This automation enables systematic tests to improve the fidelity of ESI-Orbitrap as an IRMS. For instance, long series of replicate analyses showed that in some datasets, isotope ratios were impacted by a small but significant system drift over time, which can be characterized and corrected using multiple blocks of reference injections (Figure 2D; Experimental Section and SI). A memory test with differences in δ values of up to 149‰ between sample blocks showed no significant memory effects with an average of 0.02 ± 0.5‰ (Figure 2E and SI). Experiments also indicate that δ values are constant over a range of nitrate concentrations. For example, $\delta^{15}N$ is constant for samples between 25 and 200 μM in comparisons with a reference at 50 μM (Figure 2C). For lower sample concentrations, the reference should be diluted as well. This is in line with the "principle of identical treatment", which conveys that isotope measurements become more accurate with lesser differences between the sample and reference injection.

Validation with Reference Materials. To assess the precision and accuracy of this new setup, three USGS nitrate

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Table 2. Flow Injection of USGS Reference Materials, δ Values in ‰ ($\pm$1 SD, n = 3)[a]

sample	$\delta^{15}N_{AIR}$ ESI[a]	$\delta^{15}N_{AIR}$ IRMS[d]	$\delta^{18}O_{VSMOW}$ ESI[a]	$\delta^{18}O_{VSMOW}$ IRMS[d]	$\delta^{17}O_{VSMOW}$ ESI[a]	$\delta^{17}O_{VSMOW}$ IRMS[d]	$\Delta^{17}O$ ESI[a]	$\Delta^{17}O$ IRMS[d]
"with M0": denominator is $^{14}N^{16}O_3$								
USGS35	2.6 ± 0.5	2.7	56.8 ± 0.1[c]	56.8	51.1 ± 0.5	51.1	21.6 ± 0.4	21.6
USGS32	(178.0 ± 0.1)[b]	180	25.2 ± 0.3[c]	25.3	13.0 ± 0.3	13.0	−0.1 ± 0.3	−0.2
USGS34	−2.2 ± 0.2	−1.8	(−28.1 ± 0.3)[b,c]	−27.8	(−16.8 ± 0.4)[b]	−14.8		
"without M0": denominator is $^{15}N^{16}O_3$								
USGS35			56.8 ± 0.4	56.8	51.5 ± 0.5	51.1	21.9 ± 0.4	21.6
USGS32			(28.0 ± 0.3)[b]	25.3	(14.4 ± 0.4)[b]	13.0		
USGS34			−27.7 ± 0.3	−27.8	−15.0 ± 0.1	−14.8	−0.6 ± 0.2	−0.3

[a]Values are averages of analyses done on three different days, with four replicates each. See also the SI. [b]USGS32 or USGS34 in combination with N11 were used for 2-point calibration (values in parentheses are the pre-scaled 1-point calibration data). Average δ scale correction factors were 1.012 ($\delta^{15}N$) and 0.933 ($\delta^{17}O$) for "with M0" and 1.015 ($\delta^{18}O$) and 1.010 ($\delta^{17}O$) for "without M0". [c]$\delta^{18}O$ values in the "with M0" experiment were shifted by −0.36‰ relative to N11 (see text for discussion). [d]See Table S2 in the SI (KNO_3 and $NaNO_3$ data).

reference materials were measured against our lab standard N11 (Table 2). Sequences with four sample replicates on three different days revealed an average precision in the "with M0" experiments of 0.23‰ for $\delta^{15}N_{AIR}$, 0.4‰ for $\delta^{18}O_{VSMOW}$, and 0.3‰ for $\delta^{17}O_{VSMOW}$. The "without M0" experiment yielded $\delta^{17}O_{VSMOW}$ and $\delta^{18}O_{VSMOW}$ values with <0.4‰ precision (using $^{15}N^{16}O_3$ as a denominator); these values include error propagation of a $\delta^{15}N_{sa/N11}$ precision of ±0.23‰ to fully reflect uncertainties associated with using ESI as a stand-alone technique, as $\delta^{15}N$ from IRMS was used here to calculate $\delta^{17}O_{VSMOW}$ and $\delta^{18}O_{VSMOW}$ to benchmark the procedure. Overall, this precision is promising and within the requirements of many research applications.

This performance well below 1‰ isotopic precision for nitrate $\delta^{15}N_{AIR}$, $\delta^{17}O_{VSMOW}$, and $\delta^{18}O_{VSMOW}$ provides a first starting point for developing dedicated referencing schemes and procedures that are optimized for accurate nitrate isotopolog analysis. For example, we can account for a subtle but reproducible contraction of the $\delta^{15}N$ scale, relative to the values established by conventional IRMS. The scale contraction on $\delta^{15}N$ was approximately 1.2‰ per 100‰. This was observed on several instruments across multiple laboratories.[12] Correcting this scale contraction by 2-point calibration improved the accuracy of the reported $\delta^{15}N_{AIR}$ (see Experimental Section and the SI). Scale corrections were also applied for the other δ values. Measured $\delta^{18}O$ values determined in this study were most consistent with independent data for reference materials (Table 2) and other samples (see below) if the lab standard N11 was assigned a $\delta^{18}O$ value of +26.7‰, which is slightly higher than the expected value of +26.3‰ ($\delta^{17}O$ was not reassigned; see also Table S2). Measured $\delta^{18}O$ values determined "with M0" required an additional offset of 0.36‰ to match independent data more consistently. Causes of these minor offsets could include uncertainties in the reference data[22,34] and artifacts of the ESI-Orbitrap system (see also section about Current Limitations of Technology Development). With isotopic referencing schemes and calibrations, most measured values were consistent with the expected values within ±1 SD (Table 2).[34] These benchmarking efforts highlight that with further technical refinements ESI-Orbitrap can become a new generation of highly accurate IRMS technology.

Nitrate that forms via photochemical reactions in the atmosphere carries a characteristic anomaly, "mass-independent" fractionation of $^{16}O/^{17}O/^{18}O$.[35] The anomaly commonly is expressed as $\Delta^{17}O$, the excess ^{17}O over what is normally expected based on ^{18}O content ($\Delta^{17}O \cong \delta^{17}O - 0.52 \times \delta^{18}O$). The precision of $\Delta^{17}O$ is limited by the precision of the $\delta^{17}O$ and $\delta^{18}O$, which can be constrained by ESI most efficiently in the "without M0" experiment, in combination with independently known $\delta^{15}N$ values. The obtained $\Delta^{17}O$ values agree to within ±0.4‰ with the values from gas-source IRMS (Table 2). It is important to note, however, that previous calibrations and definitions of $\Delta^{17}O$ in nitrate may not be entirely consistent, which may affect accuracy comparisons. While existing methods for $\Delta^{17}O$ measurements by gas-source isotope ratio mass spectrometry can be more precise, ESI-Orbitrap makes this measurement readily accessible and enables exploration of $\Delta^{17}O$ variations that range from near 0‰ in biogenic nitrate to more than 30‰ in atmospheric nitrate. Additionally, ^{17}O anomalies could also be detected from doubly substituted, oxygen-containing isotopologs such as $^{14}N^{17}O^{18}O^{16}O$ and $^{14}N^{18}O_2{}^{16}O$, which are only accessible by ESI-Orbitrap. Together, these tests with nitrate reference materials suggest that ESI-Orbitrap enables comprehensive and high-accuracy isotopic analysis of nitrate and likely other oxyanions such as sulfate.[12]

Current Limitations of Technology Development. The proposed ESI technology aims to build a technology bridge that will make it possible to use a sophisticated bioanalytical technique (ESI-Orbitrap MS) for powerful geochemical concepts (isotopic fingerprints in molecules). Progress on this effort is necessarily incremental, as all components of sample preparation, analysis, and data interpretation need to be adapted or reinvented. Some limitations we now encounter are likely linked to data acquisition and signal processing. For example, the stability of an electrospray can vary between days and sometimes even samples and needs to be better controlled. Also, more technical development is needed to better control the quantification of isotopolog signals. The automatic processing procedure of the Orbitrap signal has been developed for untargeted mass spectrometry, which has very different performance requirements than isotope ratio analysis.[36] This may explain the variabilities we notice in certain signals that seem prone to artifacts at the sub-per mil isotopic accuracy level, most notably, $^{14}N^{18}O^{16}O_2$ in the "with M0" experiment. $\delta^{18}O$ values in the "with M0" experiment were consistently shifted on average by −0.36‰ relative to the "without M0" experiment (Table 2 and SI). This unexplained impact on the quantification of the $^{14}N^{18}O^{16}O_2$ signal in the "with M0"

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Table 3. Flow Injection Analyses of $AgNO_3$ from Desert Salt Leachates, δ Values in ‰ (±1 SD, n = 3)[d]

sample	$\delta^{15}N_{AIR}$ ESI[a]	$\delta^{15}N_{AIR}$ IRMS[c]	$\delta^{18}O_{VSMOW}$ ESI[b]	$\delta^{18}O_{VSMOW}$ IRMS[c]	$\delta^{17}O_{VSMOW}$ ESI[b]	$\delta^{17}O_{VSMOW}$ IRMS[c]	$\Delta^{17}O$ ESI[b]	$\Delta^{17}O$ IRMS[c]
Atacama (N14362)	−0.5 ± 0.3	−0.2	54.3 ± 0.2	54.3	49.5 ± 0.2	49.4	21.3 ± 0.1	21.2
Antarctica (N17499)	−16.6 ± 0.1	−16.4	81.0 ± 0.2	80.8	73.6 ± 0.6	73.6	31.5 ± 0.5	31.6
Death Valley (N13315)	3.2 ± 0.3	2.9	nd	nd	nd	nd	nd	nd

[a]"With M0". [b]"Without M0", denominator is $^{15}N^{16}O_3$. [c]See Table S2 in the SI ($AgNO_3$ data). [d]N11 was used as a lab standard. Applied δ scale correction factors for 2-point calibration were 1.012 ($\delta^{15}N$), 0.998 ($\delta^{18}O$), and 0.980 ($\delta^{17}O$) (see Table S1).

Table 4. Dual Inlet "Dilute and Shoot" Analyses of Environmental Samples, δ Values in ‰ (NO_3^- = 1 μM; n = 1; "with M0")

sample	$\delta^{15}N_{AIR}$ ESI	$\delta^{15}N_{AIR}$ IRMS[f]	$\delta^{18}O_{VSMOW}$ ESI[d]	$\delta^{18}O_{VSMOW}$ IRMS[f]	$\delta^{17}O_{VSMOW}$ ESI[d]	$\delta^{17}O_{VSMOW}$ IRMS[f]	NO_3^- mmol/L[e]	Cl^- mmol/L[e]
Atacama (N14362)[a]	−0.8	0.0	54.9	55.1	54.8	49.8	44	82
Death Valley (N13315)[a]	3.4	3.5	25.5	24.2	20.6	20.7	57	nd
Death Valley (N14685)[a]	−0.6	−0.8	33.9	34.3	30.3	30.8	24	329
Namibia (N16854)[a]	8.2	9.1	28.8	28.6	19.6	21.0	36	3689
Antarctica (N17499)[a,c]	−16.1	−16.2	83.6	82.5	78.8	74.5	86	nd
UAE Sabkha (N13825)[a]	11.6	10.6	32.6	32.2	23.9	25.5	173	6974
New Mexico groundwater (N13993)[b]	8.4	6.8	−0.5	0.7	1.0	0.4	0.5	17.3
California groundwater (N17008)[b]	8.9	7.7	3.4	3.6	4.6	3.3	1.0	0.4
New York groundwater (N13819)[b]	5.7	7.3	2.8	3.5	0.0	1.8	0.5	0.3
Maryland groundwater (N10177)[b]	1.8	2.7	2.2	1.5	−0.4	0.8	1.3	0.4

[a]Concentrated leachate and brine samples. [b]Fresh groundwater samples. [c]This dilution had NO_3^- = 50 μM. [d]Blank corrected using sample data (see text for discussion). [e]NO_3^- and Cl^- concentrations are given for original samples before dilution. [f]See Table S2 in the SI.

experiment motivates an ongoing detailed investigation on the Orbitrap signal processing.

These limitations are unlikely to remain at the level experienced today as it may still be possible to make systematic progress by adapting underlying procedures or instrument components. The major next questions for future research applications are rather whether similar accuracy can be obtained from environmental samples and whether new biogeochemical information will become measurable. The following is a first rigorous attempt at these questions, using vetted samples for benchmarking.

Validation with Environmental Samples. Nitrate occurs in many diverse environments on Earth. The use of ESI-Orbitrap as IRMS hence hinges on its ability to measure isotopes reliably in the face of influences of the sample other than the analyte that is being measured (i.e., matrix effects). There are well-established protocols to isolate nitrate as silver salts from diverse sample types, including groundwater and brines.[21,26,37,38] To gain insights into how the ESI method can be used for environmental samples in the absence of interfering anions, we measured several samples with known and highly variable $\delta^{15}N$, $\delta^{18}O$, and $\delta^{17}O$ values that were prepared as $AgNO_3$.

A selection of three $AgNO_3$ samples was analyzed by flow injection (n = 4) against N11 in the $AgNO_3$ form. This sequence was repeated on three different days to evaluate the long-term reproducibility. The reproducibility in the "with M0" experiments was <0.3‰ for $\delta^{15}N_{AIR}$. In the "without M0" experiments, $\delta^{17}O_{VSMOW}$ and $\delta^{18}O_{VSMOW}$ were also determined with reproducibilities of <0.5 and <0.3‰, respectively, with $^{15}N^{16}O_3$ as a denominator (Table 3). Most results fall within ±1 SD of the expected values after being calibrated using $AgNO_3$ reference values and δ scaling factors from Table S1. These tests illustrate that the ESI-Orbitrap can be used for isotope analysis of analytes from complex geological or biological samples in combination with suitable extraction methods.

ESI-Orbitrap could be useful for high-throughput studies. To characterize its performance in a screening mode, seven $AgNO_3$ samples representing natural nitrate with a wide range of isotopic compositions, including variable $\Delta^{17}O$, were analyzed by a single injection. The sample Antarctica (N17449) was injected several times during the sequence and used to determine δ scaling factors for 2-point calibration. Each block of four samples was bracketed by two blocks of reference injections (N11 in the $AgNO_3$ form). The average precision of the reference injections was ±0.9‰ (1 SD) for $\delta^{15}N_{AIR}$ in the "with M0" experiment and also for $\delta^{18}O_{VSMOW}$ in the "without M0" experiment; the precision for $\delta^{17}O_{VSMOW}$ in the "without M0" experiment was ±0.4‰ (1 SD). Most data fall within ±2 SD to the expected values (Table S1). The performance for oxygen isotopes in these tests was more robust in the "without M0" experiment. Reported $\delta^{18}O$ and $\delta^{17}O$ values were thus calculated using the $^{14}N^{18}O^{16}O_2/^{15}N^{16}O_3$ and $^{14}N^{17}O^{16}O_2/^{15}N^{16}O_3$ ratios together with independently known $\delta^{15}N$ values (Table S2). These tests of environmental samples provide a proof-of-concept demonstration that ESI-Orbitrap can yield reliable results for environmental samples, adding crucial evidence that it can become a broadly useful IRMS technology.

"Dilute and Shoot". Results summarized above were obtained from various types of environmental samples that had been prepared in the form of purified $AgNO_3$. This purification is time-consuming and can itself introduce uncertainties (e.g., blanks and fractionations) that are comparable to analytical uncertainties in the method validation discussed above. Much

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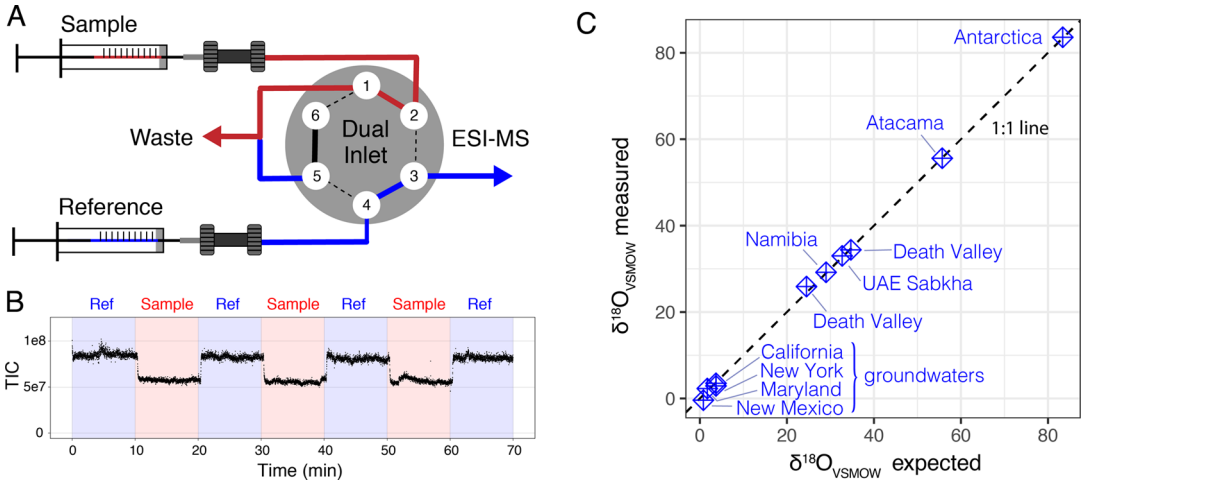

Figure 3. Dual-inlet system and ESI-Orbitrap results for "dilute and shoot" isotopic analyses of environmental samples. (A) Schematic diagram of the dual-inlet system. (B) Representative total ion chromatogram (TIC). (C) $\delta^{18}O_{VSMOW}$ of nitrate from environmental waters diluted to 1 μM in methanol and measured by dual inlet (see Table 4 for data and explanation).

more practical would be a method that does not require prepurification for routine isotopic analyses of NO_3^-. This is not a trivial task, as there can be adverse effects of the sample matrix on isotope ratio measurements.[39] In addition, salts can be deposited during ESI so that the spray needle tip and the capillary transfer tube need to be exchanged, the mass spectrometer be vented, and the ion optics be cleaned.[40]

Electrospray ionization is able to detect minute quantities of analytes, down into the low-attomolar range (10^{-18} mol).[41] It is therefore possible to dilute nitrate samples substantially for isotopic analysis. In some environmental sample types, dilution may allow to overcome the negative impacts of interfering ions because dilution decreases the competition of the analyte and matrix for ionization.[42] To this end, we conducted preliminary tests with some of the brines, soil leachate solutions, and representative groundwater samples with different relative concentrations of NO_3^- and other ions (Table 4).

Groundwaters, leachates, and brines were diluted in pure methanol to 1 μM (62 pg/μL nitrate). We analyzed the diluted samples by direct infusion via a setup analogous to a "dual inlet" system used in gas-source IRMS (Figure 3A). This "dilute and shoot" method is a safe and simple way to study new unpurified environmental sample types that could damage or contaminate the flow injection system. Nitrate concentrations <1 μM were not desirable in our setup because nitrate blanks from reagents and waters typically were on the order of 0.2 μM under our lab conditions. Tests with USGS reference materials revealed a blank contribution of ∼18% for a 1 μM sample. Based on this estimate, we calculated empirical $\delta^{18}O$ and $\delta^{17}O$ values of 36 and 13‰, respectively, for the blank in the environmental sample set by applying a best fit between apparent and expected δ values (no constraint for $\Delta^{17}O$ was applied). This information was used to correct for background contributions at low nitrate concentrations. Because blank contributions are an important aspect of many research questions, alternative correction procedures should be evaluated in future studies.

Environmental samples with concentrations >500 μM nitrate and with up to a 100-fold excess of chloride over nitrate were analyzed using alternating 10 min infusions (40 pmol nitrate each) of the sample and reference (Figure 3). The average precision based on the agreement with expected values for $\delta^{15}N_{AIR}$ was 1.1‰ and for $\delta^{18}O_{VSMOW}$ was 0.7‰ (Table 4). A potential contaminant for the $^{14}N^{17}O^{16}O_2$ signal at a resolution of R = 15,000 is the ^{18}O isotopolog of bicarbonate. With a higher resolution (R = 30,000), the $\delta^{17}O_{VSMOW}$ was determined with an average precision of 2.2‰. The $^{14}N^{17}O^{16}O_2$ isotopolog requires further investigation of interfering masses and signal processing.

In sum, these tests with environmental nitrate illustrate that ESI-Orbitrap could become a very sensitive and useful tool to obtain initial overviews when many samples need to be analyzed. "Dilute and shoot" could be especially promising for studies of certain sample types, like nitrate in terrestrial ecosystems. Additionally, the dual inlet is a cost-effective way of turning a large number of existing ESI-Orbitrap instruments into isotope ratio mass spectrometers, providing a mechanism to kick-start and democratize new technology and method developments that can advance isotope analytics.

New Dimensions: Clumped Isotopes of Nitrate. The study of isotopes in intact molecules by ESI permits numerous opportunities to discover new isotopic signatures in polar organic and inorganic solutes. For example, nonstatistical isotopic distributions among nitrate molecules (i.e., "clumped isotopes") may encode how individual pathways of the biogeochemical N cycling interact and shape the nitrogen cycle.[43] To the first order, the three clumped isotope signals in the "without M0" experiment offer multiple paths to approximate ^{15}N, ^{18}O, and ^{17}O contents. Thus, the "without M0" experiment yields apparent δ values that can be skewed by isotope clumping and other processes. For example,

$$\frac{^{15}N}{^{14}N} \approx \frac{^{15}N^{18}O^{16}O_2}{^{14}N^{18}O^{16}O_2} \quad (9)$$

Measuring the nitrate reference materials in the "without M0" experiment confirms that such ratios indeed yield δ values that are close to the true $\delta^{15}N$ and $\delta^{18}O$ values (Table 5). However, it is possible that some of the apparent δ values are skewed by processes that cause non-stochastically distributed isotopolog populations. For USGS32, for example, it appears that $\delta(^{15}N^{18}O/^{18}O)$ is 5.6‰ lower than the actual $\delta^{15}N$, and $\delta(^{15}N^{18}O/^{15}N)$ is 1.6‰ lower than the actual $\delta^{18}O$. In contrast, the apparent $\delta(^{17}O^{18}O/^{18}O)$ value of the Antarctica

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Table 5. Exploration of δ Values (in ‰) Derived from Clumped Isotopes[a]

sample	$\delta^{15}N_{AIR}$ *via* δ(15N18O/18O)	$\delta^{15}N_{AIR}$ expected	$\delta^{18}O_{VSMOW}$ *via* δ(15N18O/15 N)	$\delta^{18}O_{VSMOW}$ expected
USGS35	3.2 ± 0.5	2.7	56.6 ± 0.5	56.8
USGS32	174.4 ± 0.3	180	23.7 ± 0.6	25.3
USGS34	−2.9 ± 1.0	−1.8	−27.9 ± 0.9	−27.8

[a]"Without M0", not scale corrected, assuming stochastic distribution in N11.

leachate analyzed as $AgNO_3$ salt was +8.7‰ higher than the actual $\delta^{17}O$ value. Whether these are indeed anomalies containing useful information about the formation processes of nitrate requires further studies. Nonetheless, our preliminary analyses of isotope clumping in nitrates suggest that new isotopic dimensions of nitrate can be measured with a precision that may be suitable to evaluate whether significant nitrate clumping effects manifest in nature.

CONCLUSIONS

This study illustrates how the ESI-Orbitrap can be repurposed as an isotopic ratio mass spectrometer. ESI-Orbitrap is capable of differentiating nitrates of different origins, such as from atmospheric, anthropogenic, and biogenic processes. Results indicate that the ESI-Orbitrap method can provide usable data for conventional isotope ratios in nitrate ($\delta^{15}N$, $\delta^{18}O$, and $\Delta^{17}O$), potentially on much smaller samples and with less sample preparation than previous methods. Measurements of seven nitrate isotopologs provide multiple pathways for calculating the conventional δ values including non-mass-dependent oxygen isotopic variation, as well as opportunities to explore nonrandom isotopic distributions (i.e., clumping) in nitrate. In summary, this report provides initial theoretical frameworks, measurement principles, and validation procedures for high-fidelity isotope measurements by ESI-Orbitrap on nitrate, with potential applications to diverse intact polar solutes.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.1c00944.

(1) Description of data sets and data processing, (2) supplemental tables, (3) derivation of equations for calculating δ values from ratios of isotopologs and sensitivity analysis associated with approximating δ values from isotopolog intensities (PDF)
(4) Spreadsheet with details on δ scale contraction/expansion, memory effects, linearity, and drift (XLSX)
(5) Spreadsheet with details on reproducibility, precision, accuracy of isotope measurements in nitrate reference materials for "with M0" experiments (XLSX)

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Author Contributions

A.H. and C.N. initiated the project and carried out the ESI-Orbitrap experiments. J.K.B. and S.J.M. supplied samples and closely participated in data analysis. K.A. developed key analysis software, and K.L.F. assisted in configuring the research prototype mass spectrometer in Bremen. X.T.W. suggested the project and provided advice on experiments, and S.H.K. established theory for δ value calculations. All authors participated in the interpretation of experimental data and writing of the manuscript.

Notes

The authors declare the following competing financial interest(s): A.H., K.L.F. and K.A. are employees of Thermo Fisher Scientific, which manufactures Orbitrap mass spectrometers as well as gas-source isotope ratio mass spectrometers.

Published data will also be made available through the BCO-DMO data repository (https://www.bco-dmo.org; project 851883).

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