nature protocols

https://doi.org/10.1038/s41596-024-00981-5

Protocol

Check for updates

A guide to precise measurements of isotope abundance by ESI-Orbitrap MS

Kristýna Kantnerová[1,4], Nils Kuhlbusch[2,3,4], Dieter Juchelka[2], Andreas Hilkert[2], Sebastian Kopf[1] & Cajetan Neubauer[1]

Abstract

Stable isotopes of carbon, hydrogen, nitrogen, oxygen and sulfur are widespread in nature. Nevertheless, their relative abundance is not the same everywhere. This is due to kinetic isotope effects in enzymes and other physical principles such as equilibrium thermodynamics. Variations in isotope ratios offer unique insights into environmental pollution, trophic relationships in ecology, metabolic disorders and Earth history including climate history. Although classical isotope ratio mass spectrometry (IRMS) techniques still struggle to access intramolecular information like site-specific isotope abundance, electrospray ionization–Orbitrap mass spectrometry can be used to achieve precise and accurate intramolecular quantification of isotopically substituted molecules ('isotopocules'). This protocol describes two procedures. In the first one, we provide a step-by-step beginner's guide for performing multi-elemental, intramolecular and site-specific stable isotope analysis in unlabeled polar solutes by direct infusion. Using a widely available calibration solution, isotopocules of trifluoroacetic acid and immonium ions from the model peptide MRFA are quantified. In the second approach, nitrate is used as a simple model for a flow injection routine that enables access to a diverse range of naturally occurring isotopic signatures in inorganic oxyanions. Each procedure takes 2–3 h to complete and requires expertise only in general mass spectrometry. The workflows use optimized Orbitrap IRMS data-extraction and -processing software and are transferable to various analytes amenable to soft ionization, including metabolites, peptides, drugs and environmental pollutants. Optimized mass spectrometry systems will enable intramolecular isotope research in many areas of biology.

Key points

- Carbon, hydrogen, nitrogen, oxygen and sulfur have naturally occurring stable isotopes. Variations in their relative distribution within molecules can provide information relevant to environmental biology, metabolism, food and drug origins and climate history.
- This protocol describes how to use electrospray ionization–Orbitrap mass spectrometry to achieve precise and accurate molecular quantification of isotopically substituted molecules ('isotopocules'). It serves as a citable reference for optimized Orbitrap IRMS data-extraction and -processing software.

Key references

Eiler, J. et al. *Int. J. Mass Spectrom.* **422**, 126–142 (2017): https://doi.org/10.1016/j.ijms.2017.10.002

Neubauer, C. et al. *Int. J. Mass Spectrom.* **434**, 276–286 (2018): https://doi.org/10.1016/j.ijms.2018.08.001

Hilkert, A. et al. *Anal. Chem.* **93**, 9139–9148 (2021): https://doi.org/10.1021/acs.analchem.1c00944

Jang, C. et al. *Cell* **173**, 822–837 (2018): https://doi.org/10.1016/j.cell.2018.03.055

Neubauer, C. et al. *J. Am. Soc. Mass Spectrom.* **34**, 525–537 (2023): https://doi.org/10.1021/jasms.2c00363

[1]University of Colorado Boulder & Institute for Arctic and Alpine Research (INSTAAR), Boulder, CO, USA. [2]Thermo Fisher Scientific GmbH, Bremen, Germany. [3]University of Münster, Münster, Germany. [4]These authors contributed equally: Kristýna Kantnerová, Nils Kuhlbusch. e-mail: 123caj@gmail.com

Introduction

Variations in intramolecular distribution of stable isotopes arise across all scales of biology—from the molecular level to the biosphere—based on kinetic and equilibrium isotope fractionation principles. Subtle differences in isotope ratios (e.g., $^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$ and $^{34}S/^{32}S$) may occur in all structural positions of natural and human-made compounds. Intramolecular isotopic fingerprints can differentiate between identical chemicals formed in different processes[1–4].

There are many applications in which isotopic composition is useful, for example, in detecting doping in sports[5] or food fraud (e.g., distinguishing between real and counterfeit manuka honey)[6]. Intramolecular isotope-related information can also be used to assess whether and how environmental pollutants are degraded in groundwater, detect metabolic disorders in medicine and decipher ecological networks (trophic interactions and food chains)[7]. In some cases, molecular isotopic records can be preserved for long time periods, for example, in long-lived cells and proteins that have a slow turnover in a living organism (neurons and histones, respectively[1]), as well as in archaeological, geological and extraterrestrial specimens.

Throughout the manuscript, we refer to isotopically substituted molecules by the generic term 'isotopocule'[8]. This term includes two other widely used subtypes defined by the International Union of Pure and Applied Chemistry[9,10]: isotopologues ('isotopic homologues') = molecules that differ only in their isotopic composition (e.g., water with 'light' hydrogen, $^{1}H_2O$, and with 'heavy' hydrogen, $^{2}H_2O$); and isotopomers ('isotopic isomers') = molecules that differ in position of the isotopic substitution (e.g., isotopomers of nitrous oxide $^{15}N^{14}N^{16}O$ and $^{14}N^{15}N^{16}O$).

Although these subtle differences in definitions have been known theoretically, it was only with high-resolution mass spectrometry (MS) fragmentation experiments that it became possible to get experimental isotopic information for molecular fragments, and in certain cases even specific structural positions.

Traditional techniques for natural-abundance stable-isotope analysis

There are three techniques that have been used for natural-abundance stable-isotope analysis: infrared laser spectroscopy, magnetic sector mass spectrometers and nuclear magnetic resonance (NMR) spectroscopy.

Isotope ratio laser spectroscopy in the infrared region is based on specific rotational-vibrational absorption spectra of isotopocules. It has been used to, for example, distinguish anthropogenic and biogenic sources of greenhouse gases in the atmosphere[11] but is limited to detection of low-molecular-weight gases and volatile compounds.

Magnetic sector field-based isotope ratio MS (IRMS) coupled with chromatography is sensitive enough to analyze compounds in complex and dilute samples. However, it fails to resolve intramolecular isotope patterns for a wide variety of molecules, except for a few highly specialized methods (e.g., site-specific analysis of carbon isotopes in amino acid carboxyl groups[12]).

NMR instruments can achieve position-specific isotope analysis (PSIA) of selected molecules and isotopes but at the cost of large quantities of purified samples (0.1–10 mmol[13]), which is prohibitive for most applications. However, it is a non-destructive technique and can serve as a valuable reference method for calibration of stable-isotope analysis by other techniques[3].

These traditional techniques are often unable to observe relevant intramolecular isotopic records in bioanalytes with the required sensitivity and site specificity. In addition, IRMS and NMR methods usually cannot analyze isotope ratios of multiple elements within the same experiment (a table comparing the techniques can be found in ref. 9). Hence, a large part of the information that could potentially be harvested from isotopic fingerprints for biosciences has not been measureable until now.

High-resolution MS

Soft-ionization techniques coupled with high-resolution accurate-mass MS hold promise to enable such long-envisioned progress[9]. Recent results using electrospray ionization (ESI)-Orbitrap systems demonstrate that using fragmentation of the analyte, intramolecular

isotope ratios can be measured with both high accuracy and high precision on instrumentation that has commonly been used in metabolomics, proteomics and stable isotope–labeling studies[14–19]. Multiple kinds of non-destructive sample ionization sources, as well as the flexibility to tune the instrument to almost any mass range, ensure accessibility for a broad variety of target analytes. High-resolution accurate-mass MS thus can differentiate many isotopic variants of a molecule.

'Bioanalytical' mass spectrometers such as ESI-Orbitrap mass spectrometers have been designed for the analysis of very small amounts of samples (e.g., improved ion sources and detectors to enhance sensitivity in the case of limited amounts of extracted peptides or enzymes). This drastically decreases the lower limit of quantification for obtaining quantitative compound-specific and site-specific isotopic data. The sample size can be a hindering factor for certain sample types (e.g., those of archaeological origin) when using the traditional techniques. When comparing ESI-Orbitrap MS to NMR, the difference in lower limit of quantification can be up to five orders of magnitude[15].

For a long time, it has been known that shifts in metabolic branching—caused by a disease-associated change in enzyme regulation, a metabolic disorder or a fundamentally different metabolism (animal, plant or microbe)—should profoundly change certain predictable site-specific isotope ratios in metabolites[1,20]. Using L-tyrosine as an example, its biosynthesis in plants through the shikimate pathway results in the enrichment of the oxygen atom in the *p*-position with ^{18}O compared to tyrosine synthesized in animals, which is produced by the hydroxylation of L-phenylalanine[21]. By examining the ^{18}O values, it becomes possible to distinguish tyrosine derived from plants or animals.

The exploration of ESI-Orbitrap MS for IRMS could render experimental access to intramolecular isotope ratios, which are a powerful source of biological information yet remain essentially unexplored. Classical IRMS and ESI-Orbitrap IRMS also enable the detection and analysis of naturally occurring, multiply isotopically substituted molecules ('clumped isotopes') in biological (but also abiotic) samples and the obtainment of the so-far unknown information that they can provide[22].

ESI-Orbitrap IRMS has been used by only a small number of researchers thus far. Most analytes that are important in biology and environmental sciences have not yet been studied. In the following text, we provide a guide for beginners who want to learn how to use ESI-Orbitrap MS for IRMS. We highlight recent developments and improvements to the technique and then provide step-by-step protocols to help extend its applications.

Development of ESI-Orbitrap IRMS

Protocols for ESI-Orbitrap IRMS are key to connecting research areas of isotopic MS between the life sciences and geosciences. Instruments used in bioanalytical MS and IRMS have developed rather independently for several decades, leading to largely independent technological, analytical and methodological innovations (Fig. 1).

There are many analytical applications in which stable-isotope-labeled compounds are added as internal standards for quantitation. Stable-isotope labeling has also been used to study cellular metabolism, protein turnover, human physiology, microbiomes and many other topics[23]. Labeling studies using ESI-Orbitrap MS have undergone many advances in recent years, including quantifying small label quantities[24–26]. However, the methods used in these labeling studies are not directly applicable to the study at natural abundance. They are often performed under unperturbed and ideal conditions in simplistic laboratory-model systems that are not representative of natural ecosystems or human metabolism. Even when isotope-labeling studies are performed in mesocosm experiments (outdoor environments with controlled conditions) or with patients, they are prone to isotopic artefacts, which makes the data not easy to interpret, and can be costly and time consuming. For example, the distribution and excretion of labeled compounds can be affected by a patient's health status, and the isotopic effects of the label can alter the metabolism of the studied organism. The technique that we describe here has the advantage that it does not require any experimental labeling. It traces isotopes at natural abundance by quantifying naturally occurring isotope fractionations.

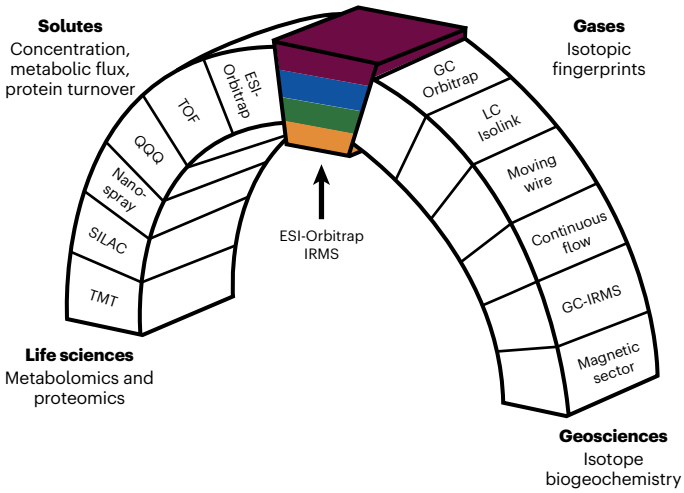


Fig. 1 | Like a keystone in a bridge, ESI-Orbitrap IRMS protocols connect MS techniques and instrumentation between the life sciences and geosciences. Techniques commonly used in quantitative proteomics: ESI; SILAC, stable isotope labeling by amino acids in cell culture; TMT, tandem mass tag. Mass analyzers: QQQ, triple quadrupole; TOF, time of flight. Separation techniques: GC, gas chromatography; LC, liquid chromatography.

Recent work has extended the traditional use of ESI-Orbitrap MS in the life sciences toward geosciences, because ESI-Orbitrap MS enables precise analysis of stable isotope ratios in intact metabolites, drugs and other polar solutes. The general approach has been demonstrated first by analyzing the amino acid methionine, specifically its site-specific ^{13}C isotope content[15].

The underlying IRMS technology, measurement principles and software have since been refined in studies of oxyanions, such as nitrate and sulfate[17,19]. These compounds are well suited for method development and benchmarking of ESI-Orbitrap IRMS because they have been widely studied in environmental isotope research. For example, the stable isotopic composition of nitrate has been important for distinguishing nitrogen sources in terrestrial and marine ecosystems[27].

ESI-Orbitrap IRMS methods have already been applied to various analyte types, including primary metabolites such as acetate[28], other amino acids[29] and other oxyanions[30,31]. An exciting link between ESI-Orbitrap IRMS and proteomics has recently also been reported in a study of amino acids of bone collagen from seals, other mammals and birds[18,32]. It has been found that bone collagen from seals has an unusually high $^{2}H/^{1}H$ ratio that cannot originate from the diet of these animals alone. In addition, approaches related to the protocols described in this article have been used with gas chromatography coupled to the Orbitrap MS to study nonpolar compounds[29,33–35].

Improved sample introduction for precision IRMS

The precision of isotopocule ratios analyzed by ESI-Orbitrap IRMS is constrained by the stability of sample introduction techniques, which are essential for ensuring a steady signal from analyte ions of sufficient duration. Most importantly, the stability of ionization during sample introduction is crucial. If the ionization is not consistent or if certain isotopocules are preferentially ionized over others, it can introduce bias in the isotopocule ratios. In some cases, sample introduction techniques may involve a separation step at the inlet. For example, liquid chromatography (LC) or gas chromatography may be coupled with IRMS for separation of compounds before entering the mass spectrometer[35]. This separation can enhance the precision and accuracy of isotopocule ratio measurements.

Precision and accuracy of obtained data were systematically further improved from initial direct infusion studies[15,16] by introducing IRMS strategies like sample/standard comparison and referencing schemes. This includes an automated flow injection method detailed in this protocol, but also a dedicated dual-syringe infusion system connected

to a programmable switching valve for applications demanding the highest precision (e.g., in clumped-isotope geochemistry). Further development has been ongoing to combine flow injection and syringe infusion for routine IRMS applications with high precision and increased sample throughput.

Improved data-acquisition settings

Another factor limiting the precision of isotopocule ratios is the statistical variation in sampling the ion population for analysis. This variation can be described mathematically by a Poisson distribution and is often referred to as shot noise or Poisson noise[19]. This means that the level of uncertainty in measuring an isotopocule ratio is dependent on the ion counts of the two signals in the ratio and additional non-Poisson noise sources[36]. However, higher ion counts require longer acquisition time and a higher sample consumption. To be able to analyze low-abundance isotopocules of target analytes with sufficiently good precision (i.e., ion counts as high as possible but with counting time as low as possible), instrument and ion extraction parameters were optimized.

Figure 2 shows the basic principles that were followed during the ion count optimization. After electrospray ionization, usually only a small fraction of the analyte enters the MS system as molecular ions through the ion-transfer tube. It is important that the sample solution is as pure as possible to ensure that most of the ions that enter the MS system are molecules of the target analyte. In the next step, ions in a selected mass range are transmitted through the quadrupole mass filter. The mass range is optimized to let through only relevant isotopocules and filter out any contaminant ions, taking into account physical limits of the quadrupole (i.e., ion transmission function). The transmitted ions are collected in an ion trap up to a certain count limit called the 'AGC target' (automatic gain control). The time that the ion collection takes is called 'injection time' (IT). Usually only a small fraction of the transmitted ion beam is stored in the ion trap.

Then, the ions are injected into the Orbitrap where they are analyzed. The Orbitrap mass analyzer consists of a spindle-shaped inner electrode surrounded by a pair of bell-shaped outer electrodes. Applying a high voltage between the inner and outer electrodes traps the ions

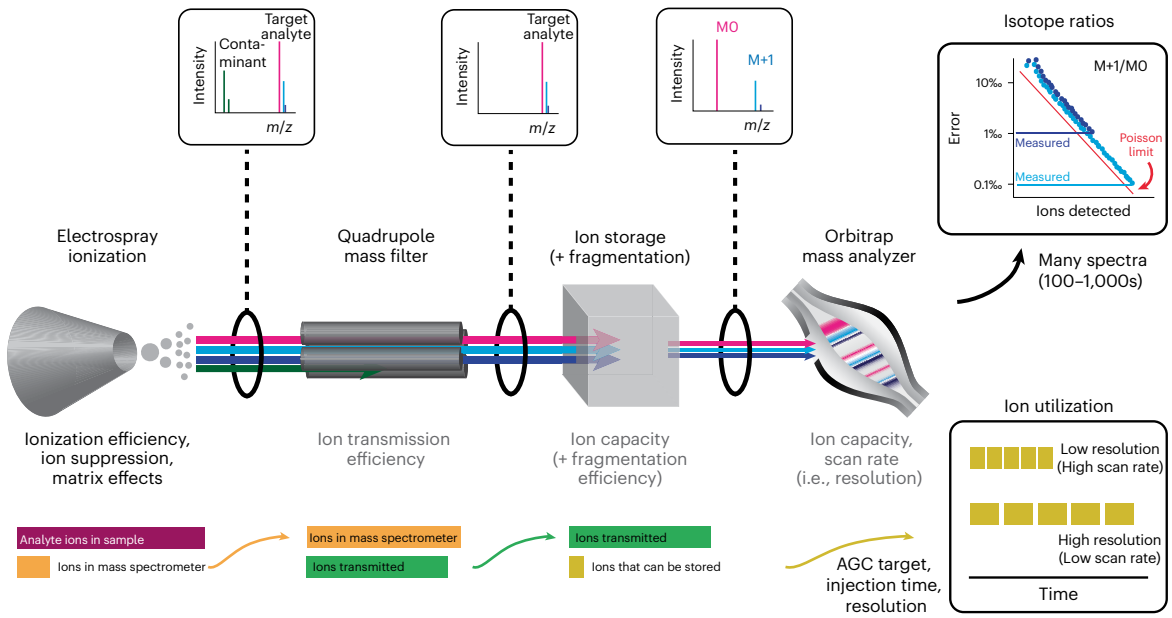


Fig. 2 | Basic measurement principles of ESI-Orbitrap IRMS. The purity of the target analytes in the sample and/or Orbitrap scans is important for precise measurement of isotopocule ratios. In addition, key instrument parameters, such as the quadrupole mass window, AGC target and Orbitrap resolution (which is inversely proportional to the Orbitrap scan rate), need to be optimized to achieve optimal performance. After the quadrupole mass filter, three isotopocules are detected: the main unsubstituted ions M0 (pink) and two singly substituted ions M+1 (light and dark blue). Any isotopocule ratio analysis (here, M+1/M0) is fundamentally limited in precision by ion counting (Poisson statistics).

in clouds oscillating between the two outer electrodes. The oscillation frequency is directly proportional to the mass-to-charge ratio (m/z) of the ions and is determined by recording the image current induced on the outer electrodes and processing the resulting transient signal with fast Fourier transform.

By increasing the AGC target, the IT is increased, and more ions are sampled from the transmitted ion beam. However, having too many ions in the Orbitrap can result in artefacts caused by interactions between the ions (space-charge effects)[37]. For example, ion coalescence is a process in which ions of two species with a similar m/z synchronize in their oscillation[38,39]. This leads to a mass shift and suppressed signals of both species, or even complete and irreversible merging of the two signals in the mass spectrum. Therefore, AGC target values have to be carefully selected to prevent these interferences.

Modern Orbitrap instruments allow data acquisition with Orbitrap resolution between 7,500 and 480,000 at m/z 200 (between 16- and 1,024-ms transient length, respectively). The Orbitrap resolution of the resulting mass spectrum is dependent on the length of the recorded transient. However, increasing the transient length also decreases the scan rate and therefore the ions counted in a given amount of time. To take full advantage of the parallel fill and capabilities of Orbitrap mass spectrometers, the IT needs to be balanced with the Orbitrap resolution. Both parameters can limit the achievable precision. As a rule of thumb, it is desirable to use an Orbitrap resolution at which the target isotopocules are baseline-resolved (or better). Sample concentration and AGC target can then be matched to enable efficient ion counting.

Improved data extraction and analysis

The new IRMS workflows created a need for specific data-extraction, -reduction and -analysis software. The IsoX software has been developed in collaboration with Thermo Fisher Scientific to extract relevant isotopic data from Orbitrap RAW files. It is programmed in C# (.NET) and reads RAW files generated by the instrument after Fourier transform of time-domain signals of ions oscillating in the Orbitrap (transients) by using the Thermo Scientific RawFileReader (github.com/thermofisherlsms/RawFileReader; Fig. 3). IsoX (version June 2022) reports the extracted data as tab-separated values within designated .isox files. Observed ion counts for isotopocule peaks are calculated by using the following approximation:

$$\text{ions.incremental} = \frac{\text{intensity}}{\text{peakNoise}} \times 3 \times \sqrt{\frac{240,000}{\text{resolution}}} \times \sqrt{\text{microscans}} \qquad (1)$$

where ‘intensity’ is the signal intensity for an isotopocule peak, ‘peakNoise’ is the noise associated with that isotopocule peak, ‘resolution’ stands for the resolution settings used (called ‘Orbitrap resolution’, defined at m/z 200) and ‘microscans’ represents the number of microscans in the data acquisition method. The ion counts are saved as ‘ions.incremental’ in the .isox files. The designations ‘intensity’, ‘peakNoise’, ‘resolution’ and ‘microscans’ refer to column labels in the .isox files for data extracted from the RAW files. The constant 3 is a rounded approximation of the number of charges corresponding to the noise at the resolution settings used, which has been experimentally determined[16]. If required, the ion counts can be recalculated by using any other constant or equation using the respective columns stored in the .isox files.

Subsequently, scripts using the open-source isoorbi R package read the .isox files (but the files could be read by any other data-evaluation software using custom scripts). For data reduction, isoorbi offers convenient, custom filters to remove outliers and select subsets

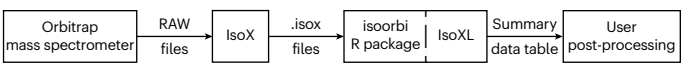


Fig. 3 | Workflow of isotope ratio data processing. The RAW data from the Orbitrap mass spectrometer can be processed by using the following software that has been custom-written for the ESI-Orbitrap IRMS analysis: IsoX software that generates .isox files, which can be processed by using R scripts or functions available in the isoorbi R package or the IsoXL graphical user interface. As a result, a simple summary data table is provided.

of the data, as well as functions to calculate isotopocule ratios and aggregate the results in a summary table. The software combination thereby provides a foundational tool for platform-independent and efficient stable-isotope analysis for data acquired by ESI-Orbitrap. The isoorbi functionalities have also been extended into a web-based graphical user interface (GUI; a link to the GUI can be found here: isoorbi.isoverse.org/articles/isoxl_demo.html) that provides click-button access to analyze data quality and reduce the .isox data into a summary table of isotopocule ratios that can be downloaded as a spreadsheet. Within this protocol, the last data-processing step (user post-processing) is performed by using offline .qmd scripts that are based on the isoorbi package and rendered in RStudio. A flowchart showing the functions provided within the isoorbi R package can be found in the supplementary information section (Supplementary Fig. 1).

Applications and limitations of ESI-Orbitrap IRMS

Notably, the protocols described here have improved sensitivity compared to other approaches for compound-specific and site-specific intramolecular stable isotope analysis. This opens research opportunities in which the sample size has been traditionally limiting, for instance, in sports doping control, clinical research, degradation studies of environmental pollutants and isotopes in biomolecules preserved in fossil specimens[9]. In addition, the possibility for simultaneous analysis of multiple isotopocule ratios can refine and complement existing methods in food authentication and other forensic applications. The analysis of intact molecular ions by using soft-ionization techniques simplifies the analysis of 'clumped isotopes', accessing isotopocule ratios with yet unknown but possibly transformative significance. These can potentially give new insights and refine our existing models of global biogeochemical cycles, as well as metabolic pathways in organisms.

Analyte and solvent

Any analyte soluble in adequate solvents and ionizable by ESI can be studied by the ESI-Orbitrap IRMS protocols described here. Technical specifications of the Thermo Scientific Orbitrap Exploris mass spectrometer limit the possible target compounds to those with $m/z > 40$. In addition, the upper limit of a target m/z is limited by the mass resolution of the instrument in use. The exact limit is determined by the mass resolution necessary to baseline-resolve peaks of all required isotopocules and the m/z of the target molecule. For example, the baseline resolution of the ^{2}H-caffeine and ^{13}C-caffeine isotopocules (3-mDa mass difference) at m/z of 195 requires Orbitrap resolution of 240,000 (defined at m/z 200). Although most solvent systems should be applicable, certain combinations of solvent and analytes could lead to undesirable isotopic exchange during the ionization. If a carbonyl compound is in a solvent system that promotes keto-enol tautomerization (e.g., specific pH conditions), the isotopic composition of the H positions adjacent to the carbonyl group may be compromised via exchange with hydrogen from the solvent[40]. This exchange can result in altered isotopic ratios and affect the accuracy of isotope ratio measurements. To minimize isotopic exchange during ionization in the presence of carbonyl-containing compounds, it is crucial to use carefully selected solvents and optimize ionization conditions.

Furthermore, PSIA using the ion-routing multipole of Orbitrap mass spectrometers for high-energy collisional dissociation (HCD) was proven to be feasible for different amino acids[15,33]. However, not every analyte makes a good PSIA target. The unique fragmentation pattern of a molecule determines and limits the molecular sites that can be accessed for PSIA. In addition, some analytes (e.g., sterols) have fragmentation spectra that can be difficult or impossible to assign unambiguously for comprehensive structural isotopic analysis.

Equipment considerations

The protocols describe parameters for a Thermo Scientific Orbitrap Exploris Isotope Solutions and a Thermo Scientific Vanquish Neo ultra-high-performance liquid chromatography (UHPLC) system on the target compounds trifluoroacetate (TFA), the peptide MRFA and nitrate. Orbitrap technology-based MS instruments other than the Orbitrap Exploris series may require more

adjustments in parameters related to the ion source (gas flows and spray voltage) and the scan settings (AGC target and RF lens) to fine-tune the output. Methods for other UHPLC systems should be set according to the particular instrument model.

Experimental design

The protocol showcases experimental setups that describe two fundamental procedures (Fig. 4). The procedures are not analyte specific; that is, the analytes shown in Procedure 1 can be analyzed by using Procedure 2 and vice versa. The two procedures are direct infusion analysis and flow injection using an UHPLC system with an autosampler.

Procedure 1

Direct infusion is a process in which the analyte is introduced into the ionization source via a syringe and a syringe pump, resulting in continuous flow of the analyte into the mass spectrometer. Direct infusion is a good choice during method development when ionization and detection conditions need to be optimized. Procedure 1 describes how to do this by using an easily accessible Orbitrap MS calibration solution FlexMix, analyzing its two compounds: (i) TFA in the negative ESI mode and (ii) the peptide MRFA (Met-Arg-Phe-Ala) and its amino acid fragments (MS^2 analysis) in the positive ESI mode.

For isotope analysis of TFA, the mass range 110.0–118.0 (m/z) is selected and scanned for the main unsubstituted ions (containing only the light isotopes), ^{13}C-isotopocules and ^{18}O-isotopocules of TFA. The objective is to obtain $^{13}C/^{12}C$ and $^{18}O/^{16}O$ isotope ratios of TFA and analyze the shot noise of the acquired data.

For isotope analysis of MRFA, the precursor at m/z 262.636 corresponding to doubly positively charged MRFA ions is selected because its signal is more intense than that of the singly charged MRFA ion (at m/z 524.264). The molecular ions are fragmented in the HCD cell, and the fragment ions are measured in the mass range 40–300 (m/z). Data are analyzed for unsubstituted (containing only the light isotopes, called 'M0') immonium fragment ions of alanine, arginine, methionine and phenylalanine at m/z 44, 70, 104 and 120, respectively,

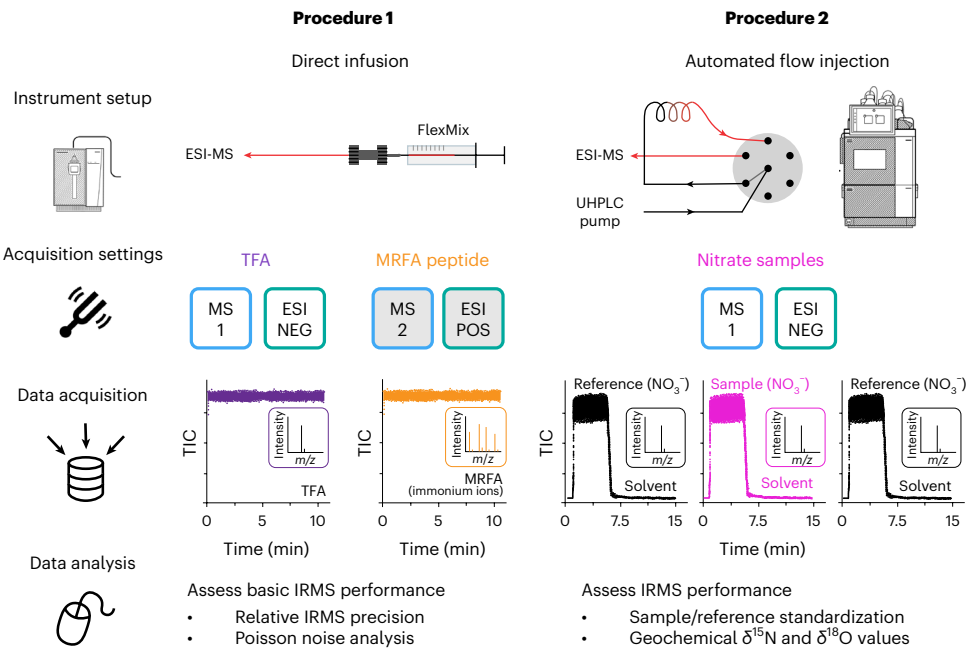


Fig. 4 | Overview of presented procedures. Procedure 1 describes a direct infusion of trifluoroacetic acid (TFA) and the peptide MRFA from a commercial calibration solution, and Procedure 2 describes an automated flow injection of nitrate dissolved in methanol. The compounds have been selected as suitable examples only to showcase the sample introduction techniques, but the techniques are not analyte specific.

and relevant ^{2}H-, ^{13}C-, ^{15}N-, ^{33}S- and ^{34}S-isotopocules of the immonium fragment ions. The objective is to obtain ^{2}H/^{1}H, ^{13}C/^{12}C, ^{15}N/^{14}N, ^{33}S/^{32}S or ^{34}S/^{32}S isotope ratios (H and S isotope ratios for methionine only) and the shot noise of the acquired data.

Negative ESI is an appropriate choice for any analyte that possesses functional groups or chemical properties favoring the generation of negatively charged ions (e.g., oxyanions, compounds with acidic and basic functional groups and halogenated compounds). When analyzing your selected compound, choose a mass range that encompasses the primary, unsubstituted isotopocule, as well as the singly (and, optionally, doubly) substituted isotopocules. Be sure to include an additional margin of ≥0.5 Da on both ends of the mass range.

Procedure 2

Flow injection is a process in which the analyte is introduced into the flow of solvent from a sample vial by using an automatic injector. Flow injection is a good choice if a method is fully optimized and a large number of samples is to be analyzed. Procedure 2 describes the stable isotope analysis of the model analyte nitrate (NO_3^-) by applying the flow injection using an UHPLC system with an autosampler. The mass range 61.2–67.0 (m/z) is selected and scanned for the main unsubstituted ions, ^{15}N-isotopocules, ^{17}O-isotopocules and ^{18}O-isotopocules of nitrate. The objective is to obtain the ^{15}N/^{14}N, ^{17}O/^{16}O and ^{18}O/^{16}O isotope ratios of a nitrate isotopic reference material (USGS32) and express its stable isotope composition in the geochemical δ scale, applying a second nitrate isotopic reference material (USGS35) analyzed within the same measurement sequence. When analyzing your selected compound, choose a mass range that encompasses the primary, unsubstituted isotopocule, as well as the singly (and, optionally, doubly) substituted isotopocules. Be sure to include an additional margin of ≥0.5 Da on both ends of the mass range.

Standards, sample purity and replicates

When conducting stable-isotope analysis, acquiring suitable stable-isotope reference materials is essential. When selecting these materials, prioritize those that hold certifications and traceability to international standards for ensured accuracy and reliability of isotopic values. In addition, choose reference materials with a matrix similar to your sample matrix to enhance result accuracy and representativeness. Lastly, confirm that the isotopic composition range of the selected reference materials covers the expected isotopic composition of your samples. If possible, using at least two reference materials is advisable for a two-point calibration.

The accuracy of isotopocule ratio measurements relies on the purity of the target analytes within the sample. The presence of contaminants that preferentially ionize may hinder the analysis of the intended compound. Using the quadrupole mass filter allows the removal of less-abundant contaminant ions with distinct m/z values. In cases in which there is uncertainty regarding solution purity or stability, creating fresh solutions to mitigate potential issues related to data quality is advisable.

The number of samples and analytical replicates depend on various factors, including the research objectives, experimental design and available sample volumes. Increasing the number of sample replicates enhances the statistical robustness of your results; therefore, having each experimental condition represented by at least two samples is recommended. For complex designs, more replicates may be necessary.

Regarding analytical replicates, determining the precision needed to answer your research questions is important. Although analyzing each sample three times is recommended, a greater number of replicates per sample may be necessary for improved precision. We suggest integrating routine replicates into quality control procedures to systematically monitor and ensure the reliability of your analytical process.

Analysis by Orbitrap MS

There are several MS platforms and LC systems that can be used to perform the stable-isotope analysis. In this protocol, we describe settings for Thermo Scientific Orbitrap Exploris 240

and 480 Isotope Solutions with a standard original equipment manufacturer (OEM) syringe pump and a Thermo Scientific Vanquish Neo UHPLC system. However, the protocols can be implemented also with a Thermo Scientific Q Exactive Orbitrap MS instrument with a compatible syringe pump or with a custom LC flow inlet setup.

Expertise needed to implement the protocol

The sample-handling portions of this protocol should be approachable for a researcher trained in analytical sample preparation. The Orbitrap MS system requires participation of an experienced mass spectrometrist, for example, a scientist used to performing routine ESI-Orbitrap mass calibration and system maintenance, to ensure that the instrumentation is performing at a level suitable for stable-isotope analysis. Acquisition and interpretation of the stable-isotope data as described here requires access to an Orbitrap mass spectrometer (ideally the Orbitrap Exploris series), analytical expertise and a basic working knowledge of data science software such as R scripts.

Using the protocol as a guide to study isotopes in new analytes and research contexts

This article provides a beginner's guide to precision-isotope measurements by ESI-Orbitrap IRMS. It illustrates the basic measurement and data-analysis workflows that should be applicable to a diverse range of polar solutes. Optimized ESI-Orbitrap IRMS protocols can be deceptively simple. However, each analyte can differ in aspects that may be critical for IRMS, and these need to be considered during experimental design and protocol validation.

Many experimental details can lead to distortions of isotopic distributions and thus affect the accuracy and precision of IRMS analyses. These include:

- Chemical reactivity of the analyte during sample preparation and MS analysis (e.g., solubility, adduct speciation and partial degradation during ionization)[41]
- Matrix effects, such as ion suppression and isotopic exchange during ionization[42]
- Near-isobaric interferences and other chemical noise (e.g., contaminants from solvents, laboratory equipment or sample preparation) entering the Orbitrap mass analyzer, potentially leading to space-charge effects[29]
- Kinetic isotope effects and changes in the precursor and product ion population in collision-induced fragmentation reactions[9,42]
- Isotopic fractionation associated with separation techniques (e.g., LC)[14]

Figure 5 outlines some of the questions and keywords that should be considered when starting new projects. It is helpful to gain experience in ESI-Orbitrap IRMS with model analytes, such as the showcased TFA, amino acids and nitrate, for which isotopic reference materials are readily available (e.g., from the United States Geological Survey (USGS), the National Institute of Standards and Technology and the International Atomic Energy Agency). Matrix effects and ion suppression present a risk for isotopic artefacts and in some cases can be compensated for by matrix matching between samples or the addition of volatile pH modifiers such as formic acid or ammonium acetate[43].

To date, there is no instrument available that is fully optimized for ESI-Orbitrap IRMS. For precise isotopocule quantification, it is therefore necessary to pay special attention to instrumental variables that can lead to distortions in isotopic distributions and ratios[42]. These include the transmission efficiency of the quadrupole, space charge effects such as ion coalescence in the Orbitrap mass analyzer, performance of the automatic gain control (AGC target) and signal processing (e.g., the enhanced Fourier transform). Developing ESI-Orbitrap systems that are fully optimized for IRMS will further improve the overall quality of the IRMS data and make it easier to acquire and process IRMS data and compare results between laboratories for validation.

How to evaluate the precision and accuracy of ESI-Orbitrap IRMS datasets

As new protocols focus more on standardization of obtained data, we can acquire more precise and accurate site-specific and intramolecular isotopic information. Workflows less targeted on specific compounds, such as those using coupled online liquid chromatographic separation, can be used to survey and screen for isotopic anomalies in biological and environmental

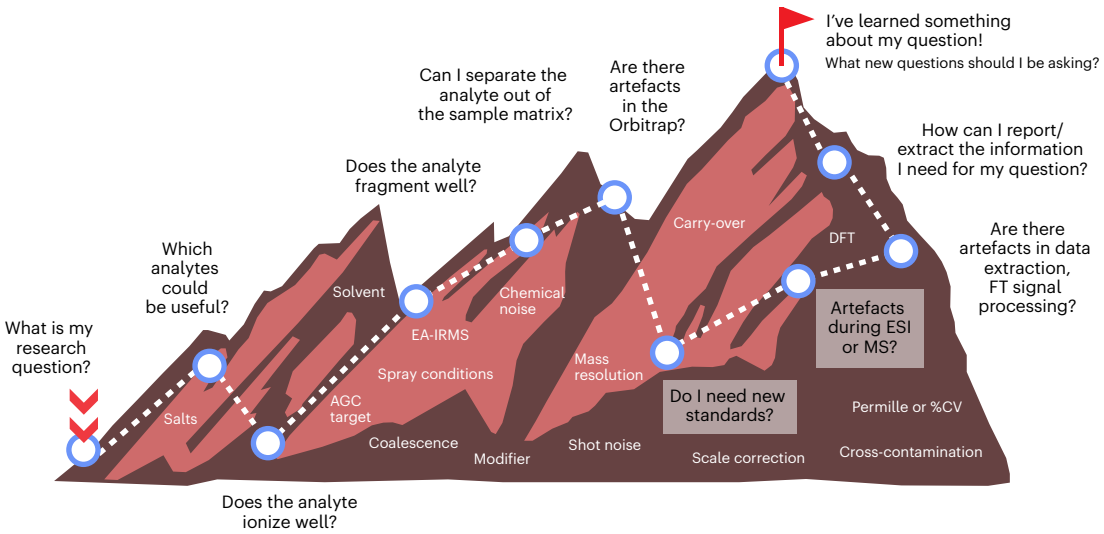


Fig. 5 | Adapting the presented protocols to new analytes and research contexts. The figure shows example questions that should be asked and key processes that should be taken into account while extending this protocol to new analytes and new research topics. The terms in white are defined and discussed in the main text in this section, except for the following: DFT, density-functional theory methods, which can be used to predict isotope fractionation or reaction mechanisms; EA-IRMS, elemental analyzer coupled with IRMS; %CV, the coefficient of variation, a statistical measure of variability in data). FT, Fourier transform.

samples. Such approaches might be easily compatible with existing metabolomics and proteomics workflows in some cases[18]. However, less-targeted workflows are more likely to produce artefacts[29,35]. Therefore, we need to develop robust metrics for emerging ESI-Orbitrap IRMS data collections. These metrics should take into account the approach used (e.g., compound-specific or site-specific intramolecular IRMS) and the uncertainty of the reported ratios. A first proposition of the precision and accuracy levels is shown in Fig. 6.

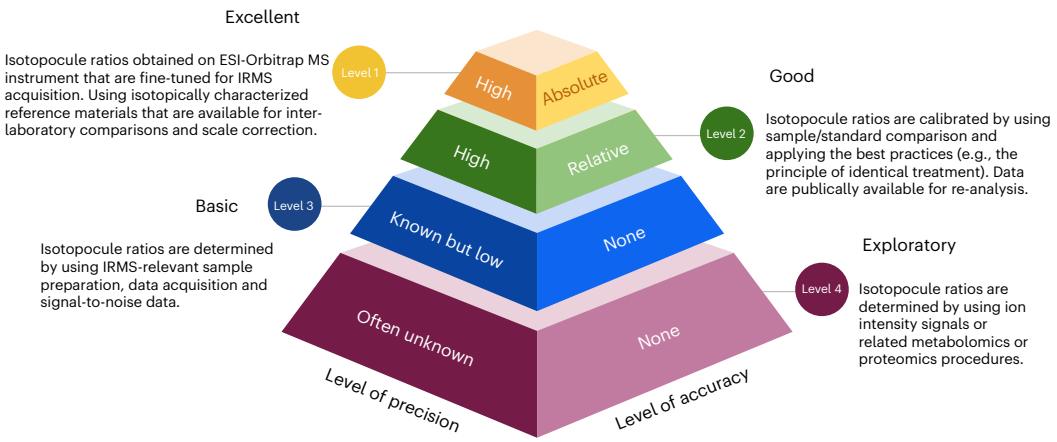


Fig. 6 | Proposed broad categories to assess the robustness of emerging ESI-Orbitrap IRMS workflows. The four levels, exploratory (magenta), basic (blue), good (green) and excellent (yellow), cover stages of precision and accuracy of obtained stable-isotope data with increasing data robustness. Level 4 can serve as a rapid assessment tool for gaining initial insights into a sample set and identifying samples with distinct isotope ratios. These distinctive samples can then be prioritized for more in-depth analysis at higher levels.

Materials

Reagents

Calibration solution, Procedure 1

▲ **CRITICAL** The solution can either be bought ready-made or be made up in the laboratory.

- Pierce FlexMix calibration solution (Thermo Scientific, cat. no. A39239)
  ▲ **CRITICAL** According to the manufacturer, keep the FlexMix solution always at room temperature, not in a refrigerator or freezer. Refer to Reagent setup for instructions to make the solution yourself. The ingredients are listed below.
- TFA (≥99.0% (GC); any suitable supplier)
- MRFA (Sigma-Aldrich, cat. no. M1170-1MG)
- ESI-compatible solvent (e.g., methanol; LC-MS grade; any suitable supplier) (e.g., LiChrosolv; EMD Millipore, cat. no. 106035)

Sample solution, Procedure 1

- Analyte of interest
  ▲ **CRITICAL** Any analyte can be used as long as it fulfills the requirements described above under Using the protocol as a guide to study isotopes in new analytes and research contexts. Avoid using high concentrations of new analytes; we recommend starting with a concentration <50 µM.
- ESI-compatible solvent (e.g., methanol; LC-MS grade, any suitable supplier) (e.g., LiChrosolv, EMD Millipore, cat. no. 106035)
  ▲ **CRITICAL** Any solvent can be used as long as it is compatible with the ESI source. Avoid using solvents that are not compatible with PEEK capillaries (PEEK may experience swelling when exposed, for example, to dimethyl sulfoxide or tetrahydrofuran).

Solvents for Procedure 2

All solvents should be of a suitable purity grade and can be bought from any supplier.

- Water (LC-MS grade, sulfate or nitrate ≤10 parts per billion) (e.g., LiChrosolv; EMD Millipore, cat. no. 115333)
- Methanol (LC-MS grade) (e.g., LiChrosolv; EMD Millipore, cat. no. 106035)
  ▲ **CAUTION** Methanol and its vapors are flammable. Methanol is highly toxic to humans in high concentrations and can cause blindness and damage to the liver, kidneys, central nervous system and heart or be fatal. Wear personal protective equipment, keep it away from any heat and use it in a well-ventilated area.
- Isopropanol (LC-MS grade, any suitable supplier)
  ▲ **CAUTION** Isopropanol is a flammable and mildly toxic liquid. Wear personal protective equipment, keep it away from any heat and use it in a well-ventilated area.

Standards for nitrate analysis

▲ **CRITICAL** Nitrate analysis is the example used. If you are performing a different analysis, you need to obtain appropriate stable-isotope reference materials. When choosing reference materials, opt for reference materials that are certified and traceable to international standards. Certification ensures the accuracy and reliability of isotopic values. In addition, choose reference materials with a matrix similar to your sample matrix to obtain more accurate and representative results. Lastly, ensure that the isotopic composition range of the reference materials encompasses the anticipated isotopic composition of your samples. It is recommended that at least two reference materials be used for a two-point calibration, whenever possible.

- Nitrate reference materials, USGS32 and USGS35 (USGS, usgs.gov/labs/reston-stable-isotope-laboratory/reference-materials-and-calibration-services)
- (Optional, if USGS32 and USGS35 are not available) Any high-purity KNO_3 or $NaNO_3$ salt or prepared solution such as Nitrate Standard for ion chromatography (IC) (Sigma-Aldrich, cat. no. 74246-100ML)

- Formic acid (HPLC grade, any suitable supplier)
  ▲ **CAUTION** Formic acid is a flammable liquid that can cause serious eye damage or skin burns. Wear personal protective equipment, keep it away from any heat and use it in a well-ventilated area.

Samples for nitrate analysis

▲ **CRITICAL** Any aqueous solution having a content of chloride ions lower than 100 µM can be used. At higher concentrations, chloride ions may suppress the ionization of analyte molecules and can contribute to corrosion of the electrospray needle and other components of the mass spectrometer. Possible sources of contamination are solvents, laboratory equipment and sample-preparation methods.

Equipment

General

- Orbitrap Exploris Isotope Solutions (Thermo Scientific, cat. no. IQLAAMGAATFARBMBNP; or Orbitrap mass spectrometers with similar performance: Orbitrap Exploris MS, Orbitrap Tribrid MS and Orbitrap Q Exactive series)
- Low-flow needle insert: NG, HESI LOFLO needle insert, 50 µm, ID35G (Thermo Scientific, cat. no. OPTON-30139)

Procedure 1: direct infusion

- Syringe pump (usually a part of the mass spectrometer setup, otherwise obtained through Thermo Fisher Scientific to ensure correct communication software with the mass spectrometer)
- Syringe, 500 µl (Thermo Scientific, cat. no. 1248730)
- Syringe adapter kit (Thermo Scientific, cat. no. 70005-62011), connecting the syringe with the ESI inlet (syringe adapter kit: 1/16-inch OD 0.005-inch ID red PEEK tubing, ferrules, fittings, union and 1/16-inch OD 0.030-inch ID Teflon tubing; 1/16-inch OD 0.0025-inch ID natural PEEK tubing, ferrules and fittings)

Procedure 2: flow injection analysis

- Thermo Scientific Vanquish Neo UHPLC system (thermofisher.com, cat. no. VN-S10-A-01; or similar)
- Split sampler NT (Thermo Scientific, cat. no. VN-A10-A)
- Binary Pump N (Thermo Scientific, cat. no. VN-P10-A-01)
- Split sampler sample loop, 25 µl (Thermo Scientific, cat. no. 6252.1940)
- (Optional) nanoViper fingertight fittings, 20 µm (Thermo Scientific, cat. no. 6041.5260)

Software

- Tune application 4.0 (or newer; Thermo Fisher Scientific)
- Xcalibur 4.5 data system (or newer; Thermo Fisher Scientific)
- SII for Xcalibur 1.5.1 (or newer; Thermo Fisher Scientific)
- FreeStyle 1.8 data-visualization application (or newer) or Qual Browser (Thermo Fisher Scientific)
- LibreOffice/MS Excel
- Internet browser (any)
- R 4.2 (or newer; a free software environment for statistical computing and graphics; https://www.r-project.org)
- RStudio data science solution (https://posit.co)
- IsoX application (Isotopocule data eXtraction from Orbitrap RAW files; version June 2022 or newer; see Software setup for more information)

Reagent setup

Sample preparation for Procedure 1, direct infusion

There is no need to prepare solutions to perform Procedure 1 if you use the Pierce FlexMix calibration solution. If you want to implement this with a custom-made solution of TFA or MRFA,

prepare your analyte in HPLC-grade methanol or other ESI-compatible solvent (e.g., acetonitrile or isopropanol), aiming for a concentration between 1 and 50 µM. Make sure that the analyte has dissolved completely before introducing the solution into the ESI source; you can centrifuge the solution or use a filter to remove any particles that may block the ESI needle. The custom-made solutions can be stored at 4 °C and re-used for ~1 month. Over longer periods, there can be contamination of the solution (e.g., from the container material), change in concentration because of solvent evaporation if the container is not tightly sealed or chemical reactions with impurities or contaminants. If there is any uncertainty about the purity or stability of the stored solutions, we recommend preparing fresh solutions to minimize potential issues with data quality.

Procedure 2: flow injection analysis

Prepare stock solutions of the nitrate salts: dissolve each salt in a water/methanol mixture (1:1, vol/vol) to obtain the concentration of 100 mM (the recommended total volume of each solution is 1 ml). Dilute the stock solutions prepared in the first step by a factor of 1/2,000 by using methanol to achieve a final concentration of 50 µM (e.g., dilute 5 µl of each stock solution into 10 ml of methanol). The solutions can be stored at 4 °C or frozen over several months.

If you use the Nitrate Standard for IC solution (1,000 mg/l NO_3^-, ~16 mM), dilute it by using methanol to achieve a final concentration of 50 µM (e.g., dilute 30 µl of the Nitrate Standard for IC solution into 10 ml of methanol).

Equipment setup

General

We use Thermo Scientific Orbitrap Exploris 240 and 480 Isotope Solutions systems. Make sure that the ESI source is equipped with a low-flow needle insert. Using a high-flow needle insert with flow rates <10 µl/min will result in spray instability. Before the isotopocule ratio measurements, we recommend performing a full mass and system calibration in both negative and positive ionization modes by using the FlexMix solution. To do so, follow the guidance in the corresponding mass spectrometer operating manual (e.g., Orbitrap Exploris Isotope Solutions Getting Started Guide, pages 3–16 and following).

Procedure 1: direct infusion

The sample is delivered from a syringe by using a syringe pump with a direct infusion line going into the ESI source (Fig. 4). Make sure that the syringe pump has contact with the plunger and that the syringe adapter does not leak.

▲ **CAUTION** The infusion line must go through the grounding point between the ESI probe and the syringe pump to provide protection from high voltage.

Procedure 2: flow injection analysis

We use the Thermo Scientific Orbitrap Exploris 240 Isotope Solutions system coupled to a Vanquish Neo UHPLC system (Fig. 4). The Vanquish system in this protocol does not serve for chromatographic separation of analytes; it uses only its autosampler and UHPLC pump functionalities. Before starting, confirm that all used solvent reservoirs are filled with appropriate solvents. For the Vanquish Neo, use the solvents listed in the table below ('weak' and 'strong' designations relate to the ability of a solvent to elute compounds from an HPLC column):

Port	Solvent
Pump port B: weak inner needle wash; weak wash port	Methanol
Pump port A: strong inner needle wash; strong wash port	Water
Rear seal wash	Isopropanol/water (3:1, vol/vol) + 0.1% formic acid (vol/vol)

1. Make sure that the correct solvent types are set and calibrated in the instrument control software.
2. Make sure that the UHPLC system is correctly configured (e.g., by running the procedure E01 – Initialize System Setup) with separation column type set to 'Linear Column', fluidics set to 'Nano/Cap' and the workflow set to 'Direct Injection'.
3. Equip the UHPLC autosampler with a sample loop that enables long-duration sample introductions for ≥7 min. For the Vanquish Neo, we use 25-µl injections with a flow rate of 4 µl/min to ensure the required analysis time.
4. Load the autosampler with the prepared solutions of nitrate salts, as well as a vial filled with the pure solvent for blank injections.
   ▲ **CRITICAL** The diameter of all capillaries connecting the UHPLC autosampler with the mass spectrometer should be adjusted to the applied flow rate. Thin capillaries are necessary to prevent unwanted mixing, long flushing time and too low a pressure for pump operation. For the Vanquish Neo, nanoViper fingertight capillaries with an inner diameter of 20 µm were used.

Software setup

An IsoXL demo graphical user interface (GUI) for data analysis can be accessed via an internet browser (isoorbi.isoverse.org/articles/isoxl_demo.html). Descriptions of other aspects of the software setup are described below under RStudio and IsoX setup.

RStudio

Install the isoorbi R package from CRAN (cran.r-project.org/web/packages/isoorbi/index.html, version 1.3.0 or newer). A quick start guide and description of the available functions for custom data analysis can be found at isoorbi.isoverse.org. A flowchart of the functions is shown in the supplementary information (Supplementary Fig. 1).

IsoX setup

A demo version of IsoX for the analysis of data collections described in the protocol article will become freely available through Thermo Fisher Scientific. Access to a demo version of IsoX can be obtained as follows:

1. Request access to the demo SharePoint environment at the e-mail address documentation.bremen@thermofisher.com.
2. Accept the received invitation within 6 d.
3. Go to thermofisher.sharepoint.com/sites/OrbitrapExplorisIsotopeSolutionsDemoVersion and log in with your created Microsoft password to access the software.

Tune software for direct infusion analysis (Procedure 1)

The direct infusion analysis is done manually in the Tune software. There is no need to set up an instrument method by using the Xcalibur method editor. However, once optimized for your analysis, the scan parameters can be saved in Tune under Favorites or within a method file by using the Xcalibur method editor for automated data collection.

Instrument method for flow injection analysis (Procedure 2)

▲ **CRITICAL** Within the LC instrument method editor, make sure that the keep loop in line command is activated. To do so, go to the tab 'Script Editor' on the left side of the method editor and change the value in line 3 ('Neo.SamplerModule.Sampler.KeepLoopInline') to 'Yes'.

For the flow injection analysis, create an instrument method in the Xcalibur method editor. The length of the method should be sufficient to fully flush the sample loop of the UHPLC autosampler to prevent any carry-over between sample and standard injections. Instrument methods for the Vanquish Neo typically include 5–8 min of flushing time resulting in an instrument method of 15 min total.

In the method, set the MS parameters according to the following table. For the ion source settings, select the 'Use ion source settings from Tune' option. This will take over the current, optimized ion source settings from the Tune software.

Parameter	Value
Scan type	Full scan
Orbitrap resolution	15,000
Scan range (*m/z*)	61.2–67.0
RF lens (%)	70 for Orbitrap Exploris 120 and 240 MS (100 for Orbitrap Exploris 480 MS)
AGC target	Custom
Normalized AGC target (%)	30 (corresponds to 3×10^5 in the absolute mode)
Maximum injection time	Custom
Time (ms)	1,000
Microscans	5
Source fragmentation	Off
Use EASY-IC	Off

▲ **CRITICAL** The settings suggested in the table are optimized for the Orbitrap Exploris MS series. If using a different instrument class and especially when measuring other compounds than described here, some parameters might have to be adapted (e.g., AGC target and Orbitrap resolution). Especially when using Tribrid instruments, be aware of the differences in AGC. Always check the total ion current (TIC) × IT (the ion count estimate in Orbitrap) to make sure that the Orbitrap is not being overfilled; otherwise, various space-charge effects may occur. The TIC × IT values should be close to the set AGC target. We recommend using a normalized AGC target of 1% on Tribrid instruments.

Setting up a sequence for Procedure 2

Use the sequence setup in Xcalibur to create a sequence with alternating injections of the two nitrate solutions from the autosampler tray (Extended Data Fig. 1).

1. Begin the sequence with a blank injection followed by five injections of the standard solution to let the system stabilize/equilibrate and then add alternating injections of the sample and standard solutions. End with a blank injection.
2. Select the path of the instrument method prepared in the prior paragraph and the path to which the result files will be saved.
3. Fill the file names, sample names and positions accordingly.

Procedure 1: direct infusion analysis

▲ **CRITICAL** Make sure that the used FlexMix solution passes the standard mass and system calibration, as described in Equipment setup (e.g., there is no major contamination from previous analyses).

TFA (FlexMix, negative mode)

Acquiring the data

● **TIMING 30–60 min**

1. Check that the Orbitrap mass spectrometer is set up as described in Equipment setup, Procedure 1.
2. Fill the syringe with the FlexMix solution and place it into the syringe pump.
3. In the Tune application, set 'Application Mode' to 'Small Molecules'.
4. Set 'Polarity' to 'Negative'.
5. Under Syringe settings, set 'Flow Rate' to 4 µl/min and 'Volume' to 500 µl.
6. On the 'ION SOURCE' tab, set the initial parameters according to the following table:

Parameter	Value
Pos Ion Spray Voltage (V)	3,400
Neg Ion Spray Voltage (V)	2,400

Parameter	Value
Sheath Gas (Arb, arbitrary units)	2
Aux Gas (Arb)	2
Sweep Gas (Arb)	0
Ion Transfer Tube Temp (°C)	320
Vaporizer temperature (°C)	0

7. On the 'DEFINE SCAN' tab, set the parameters according to the following table:

Parameter	Value
Scan type	Full scan
Orbitrap resolution	15,000
Scan range (*m/z*)	110.0–118.0
RF lens (%)	40 for Orbitrap Exploris 480 MS (70 for Orbitrap Exploris 240/120 MS)
AGC target	Custom
Normalized AGC target (%)	100 (corresponds to 1×10^6 in the absolute mode)
Maximum injection time	Custom
Time (ms)	1,000
Microscans	1
Source fragmentation	Off
Use EASY-IC	Off

▲ **CRITICAL STEP** The settings suggested in this table are optimized for the Orbitrap Exploris MS series. If using a different instrument class and especially when measuring other compounds than described here, some parameters might have to be adapted (e.g., AGC target and Orbitrap resolution). Especially when using Tribrid instruments, be aware of the differences in AGC. Always check the TIC × IT (the ion count estimate in Orbitrap) to make sure that the Orbitrap is not being overfilled; the TIC × IT values should be close to the set AGC target. We recommend using a normalized AGC target of 1% on Tribrid instruments.

8. Set the instrument to 'System ON' and start the syringe pump. Wait until the 'Ion Transfer Tube Temp' stabilizes.
9. Optimize the signal stability by fine-tuning the ion source parameters (spray voltage and gas flows) to gain a high sensitivity and low signal deviation (the relative standard deviation (RSD) parameter <10%). For the FlexMix solution with the described MS setting, the following values are expected: TIC of ~1×10^8, ion IT of ~3 ms and scan rate of ~4 scans/s. The spray current should be stable at maximum of 0.1 µA.
   ◆ **TROUBLESHOOTING**
10. Confirm that the peaks of monoisotopic TFA (M0) and the ^{13}C- and ^{18}O-substituted isotopocules are present and resolved in the mass spectrum window. The expected peaks for M0 and the ^{13}C- and ^{18}O-substituted isotopocules in the selected mass range are displayed in Fig. 7.
    ▲ **CRITICAL STEP** The ^{17}O- and ^{13}C-substituted isotopocules will not be resolved at the applied Orbitrap resolution (15,000). Empirical data on acetate show that due to the small intensity of the ^{17}O peak, accurate results for the ratio of ^{13}C/M0 can be obtained despite the ^{13}C peak not being baseline-resolved from the ^{17}O peak[28]. However, in the case of molecules with higher oxygen content, larger differences in ^{17}O abundance and very high-precision measurements, the effect of the interference of ^{13}C and ^{17}O peaks might not be negligible. In these cases, a higher Orbitrap resolution setting can be used to resolve the peaks (note that this will result in a much lower scan rate), or a correction on the content of the ^{17}O-substituted isotopocule for the ^{13}C-substituted isotopocule can be made on the basis of the content of the ^{18}O-substituted isotopocule, assuming mass-dependent fractionation. The doubly ^{13}C-substituted isotopocule interfering with the ^{18}O-substituted isotopocule can be disregarded at this point because of its low abundance.

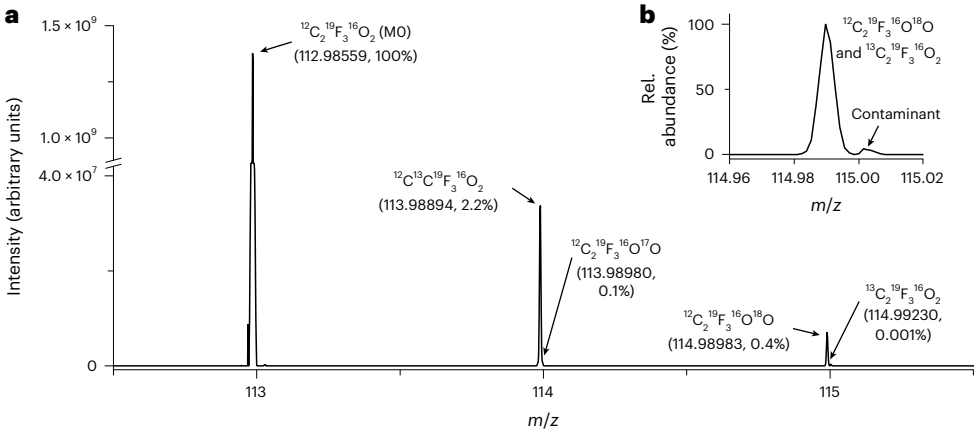


Fig. 7 | Mass spectrum of TFA in the scan range specified in Procedure 1. a, The whole mass range is shown. **b**, An insert showing the mass range around *m/z* 115 in detail. The ^{17}O- and ^{13}C-substituted isotopocules at *m/z* 114 are not resolved at the applied Orbitrap resolution (15,000), as well as the ^{18}O- and doubly ^{13}C-substituted isotopocules at *m/z* 115 (the small contaminant in this area is well resolved from the TFA isotopocules and does not hinder the analysis). In parentheses, expected theoretical *m/z* values are shown, together with the relative intensities of the respective isotopocules. Experimental values should not be different by more than 0.1 mDa from the accurate, theoretical values. rel, relative.

11. In the upper input field, fill in the file path where the data will be saved and the file name.
12. Open the menu under 'Instrument Method' and fill in information on the 'Sample Name', 'Comment'.
13. On the left side of the menu, select 'Continuous Acquisition' and enter under 'Minutes': 5.
14. Select the 'Go to standby when finished' check box.
15. If the signal stability is good, start the data acquisition by pressing the camera icon in the upper bar.

    ■ **PAUSE POINT** The samples can be discarded or used for further analysis. If the samples are required for further analysis at a later point, they should be stored at 4 °C for a maximum of 1 month. Data analysis can be done at any time.

Data processing

● **TIMING 60–90 min**

16. Open the generated RAW file in FreeStyle (or Qual Browser).
17. Check that the signal is stable (no strong drift, disturbances or jumps in the signal and the RSD parameter <10%) to ensure a high-precision measurement and that all required TFA isotopocules are present and resolved in the mass spectra.
18. The next step is the extraction of peak data from the RAW files for isotopocule ratio calculation. This is done by using the IsoX software. IsoX requires an isotopologs.tsv file as input (Extended Data Fig. 2; isotopolog = isotopic homolog in American spelling). Create the .tsv file by using a text editor or the IsoXL demo GUI.
    - *Text editor*. Paste the measured masses from the RAW files into any text-file-editing software (e.g., Notepad, Notepad++, LibreOffice and Excel).
    - *IsoXL demo GUI*. Open the link isoorbi.isoverse.org/articles/isoxl_demo.html and use the 'IsoX Input File' card on the left; this uses calculated masses based on relative atomic weights.

      ▲ **CRITICAL STEP** Make sure that after editing, the file is still saved in the 'tab-separated values' format as shown in Extended Data Fig. 2.
19. In FreeStyle (or Qual Browser) and the text editor, check if the masses of all TFA isotopocules (column 'Mass') detected in the measured data are within the tolerance windows specified

in the isotopologs.tsv file (column 'Tolerance [mmu]'). Change the mass and/or tolerance values if necessary and save the isotopologs.tsv file.

20. For the IsoX data extraction, place the isotopologs.tsv file into the same folder as the RAW files.
21. Open the IsoX software and load in the file path to the RAW files.
22. Make sure that the boxes 'Combine Output' and 'Simplify Output' are unchecked (for more information on these options, check the IsoX_howto.pdf file that is in the folder where IsoX is installed).
23. Run the IsoX data extraction by pressing the 'Start' button. IsoX will create .isox files for every RAW file in the selected folder.

    ▲ **CRITICAL STEP** When rerunning the IsoX data extraction on previously extracted RAW files, make sure to delete all .isox files created beforehand and the processed_files_individual.log file; otherwise, the RAW files recorded in the .log file will not be re-processed.

    ◆ **TROUBLESHOOTING**

    ■ **PAUSE POINT** Further data evaluation can be done at any time.
24. Carry out further evaluation of the created .isox files with any data evaluation tool. Multiple options are listed in Software setup. Here, we describe data evaluation based on the script 'Procedure_1.1_TFA.qmd' (it can be downloaded from the GitHub repository specified in Data availability).
25. Place the .isox file in a folder called 'data' that is located in the same directory as the .qmd file.
26. Open the .qmd file in RStudio.
27. Press the 'Render' button in RStudio. The script reads the .isox file from the 'data' folder and performs the following steps for data treatment (the script 'Procedure_1.1_TFA.qmd', lines 57–73):
    - *orbi_flag_satellite_peaks()*. The IsoX software extracts all peaks detected in the given tolerance window. This can result in multiple peaks being extracted for a single isotopocule if there is another peak present and the tolerance window is set too broad. This function flags all satellite peaks for filtering out so that only the most abundant one is used for ratio calculation (assuming that the other peak is less intense).
    - *orbi_flag_weak_isotopocules(min_percent = 10)*. Reusing a single isotopologs.tsv input file for IsoX extraction of data belonging to multiple experiments or compounds can cause very-low-abundance isotopocules to be detected in a minor part of scans. The data of these isotopocules are typically not of interest but are present in the result files. Therefore, these weak isotopocules are filtered out for the data evaluation if they are detected in <10% of scans.
    - *orbi_flag_outliers(agc_fold_cutoff = 2)*. The number of ions injected into the Orbitrap is controlled by AGC target. Significant under- or overfilling of the Orbitrap can affect the measured ratios because of decreasing signal-to-noise values or space-charge effects (the TIC × IT values should be close to the set AGC target). In this example (*agc_fold_cutoff = 2*), the filtering function removes outlying scans that have >2× or <0.5× the average number of ions in the Orbitrap analyzer. To prevent outlying scans from affecting overall data quality, Orbitrap filling is estimated by calculating TIC × IT, and scans deviating by more than the set criterion from average TIC × IT are flagged and filtered out from later data treatment.
28. Open the created output files: 'Procedure_1.1_TFA.html', summarizing the data treatment and showing a shot-noise plot, and 'shot_noise_tfa.xlsx', which includes the isotopocule ratios, shot-noise limit ('shot_noise.permil') and relative standard error ('ratio_rel_se.permil') data. The shot noise of the measurement is estimated by using equation (2), where N_{iso} is the ion count for the heavy isotopocule, and N_{BP} is the ion count for the isotopocule used as the base peak.

$$\text{Shot noise } (‰) = 1,000 \times \sqrt{\frac{N_{iso} + N_{BP}}{N_{iso} \times N_{BP}}} \qquad (2)$$

29. Open the additional plots created by the script; they are stored as .png and .pdf files in a 'plots' folder. These include a shot-noise plot similar to the ones shown in Anticipated results, Procedure 1, as well as plots for diagnostic purposes visualizing the different data-treatment steps described in Step 27 (e.g., 'Procedure_1.1_TFA_fig-tfa-satellite-peaks-1' files visualizing the satellite peak filtering).
30. The data are plotted against the effective number of ions counted in the analysis ('n_effective_ions') and/or analysis time ('time.min'). Carefully check the plots. If the quality of the obtained data is satisfactory, the relative standard error should closely follow the theoretical shot-noise limits—see Anticipated results, Procedure 1.

MRFA (FlexMix, positive mode, MS^2)

Acquiring the data

● **TIMING 30–60 min**

31. In the Tune application, set 'Application Mode' to 'Peptides'.
32. Set 'Polarity' to 'Positive'.
33. Under Syringe settings, set 'Flow Rate' to 4 µl/min and 'Volume' to 500 µl.
34. On the 'ION SOURCE' tab, set the same initial parameters as for TFA.
35. On the 'DEFINE SCAN' tab, set the parameters according to the following table:

Parameter	Value
Scan type	MS^2 scan
Precursor (*m/z*)	263.5
Precursor charge state	2
Isolation width (*m/z*)	4
Collision energy type	Normalized
HCD collision energy (%)	50 (corresponds to ~20 V (Orbitrap Exploris MS series) or 20 eV (Q Exactive Orbitrap MS series))
Orbitrap resolution	120,000
Scan range mode	Define *m/z* range
Scan range (*m/z*)	40–300
RF lens (%)	40 for Orbitrap Exploris 480 MS (70 for Orbitrap Exploris 240/120 MS)
AGC target	Custom
Normalized AGC target (%)	1,000 (corresponds to 10^6 in the absolute mode)
Maximum injection time	Custom
Time (ms)	1,000
Microscans	10
Source fragmentation	Off
Use EASY-IC	Off

▲ **CRITICAL STEP** The settings suggested in the table are optimized for the Orbitrap Exploris mass spectrometer series. If using a different instrument class and especially when measuring other compounds than described here, some parameters might have to be adapted (e.g., AGC target and Orbitrap resolution). Especially when using Tribrid instruments, be aware of the differences in AGC. Always check the TIC × IT (the ion count estimate in Orbitrap) to make sure that the Orbitrap is not being overfilled; the TIC × IT values should be close to the set AGC target. We recommend using a normalized AGC target of 5% on Tribrid instruments.

36. Set the instrument to 'System ON' and start the syringe pump.
37. Optimize the signal stability by fine-tuning the ion source parameters (spray voltage and gas flows) to gain high sensitivity and low signal deviation (the RSD parameter <10%). For the FlexMix solution with the described mass spectrometer setting, the following values are expected: TIC of ~1×10^7, ion IT of ~30 ms and scan rate of ~0.4 scans/s. The spray current should be stable at a maximum of 0.1 µA.

◆ **TROUBLESHOOTING**

38. Confirm that the immonium ion peaks of all four amino acids are present and resolved in the mass spectrum window. All expected peaks in the selected mass range are displayed in Fig. 8.
39. In the upper text field, fill in the file path where the data will be saved and the file name.
40. Open the menu under 'Instrument Method' and fill in information on the 'Sample Name', 'Comment'.
41. On the left side of the menu, select 'Continuous Acquisition' and enter under 'Minutes': 10.
42. Select the 'Go to standby when finished' check box.
43. If the signal stability is good, start the data acquisition by pressing the camera icon in the upper bar.
44. (Optional) To determine the repeatability of the results obtained with this procedure, carry out steps in Acquiring the data multiple times. Note that the file name of each replicate should end with '...Replicate_X', where X is the number of the replicate. This part is used for replicate assignment during data processing with the provided script 'Procedure_1.2_MRFA_replicates.qmd'.

    ■ **PAUSE POINT** The samples can be discarded or used for re-analysis if needed. If the samples are required for analysis at a later point, they should be stored at 4 °C for a maximum of 1 month. Data analysis can be done at any time.

Data processing

● **TIMING 60–90 min**

45. Open the generated RAW file in FreeStyle (or Qual Browser).
46. Check that the signal is stable (no strong drift, disturbances or jumps in the signal and the RSD parameter <10%) to ensure a high-precision measurement and that all required isotopocules of the immonium ions are present and resolved in the mass spectra.
47. The next step is the extraction of peak data from the RAW files for isotopocule ratio calculation. Follow Steps 18–23 in Procedure 1. The structure of the isotopologs.tsv file for MRFA is shown in Extended Data Fig. 3.

    ■ **PAUSE POINT** Further data evaluation can be done at any time.
48. Carry out further evaluation of the created .isox files with any data evaluation tool. Multiple options are listed in Software setup. Here, we describe data evaluation based on scripts. To analyze the shot noise of a single replicate (similar to the prior experiment on TFA), use the script 'Procedure_1.2_MRFA_shotnoise.qmd'. For the evaluation of multiple replicates, the script 'Procedure_1.2_MRFA_replicates.qmd' can be used (both can be downloaded from the GitHub repository specified in Data availability).

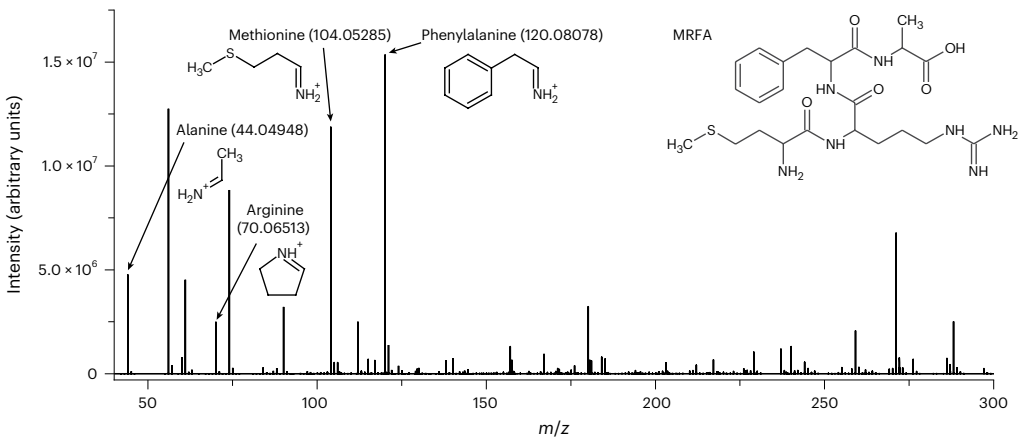


Fig. 8 | Mass spectrum of immonium ions from amino acids in MRFA in the scan range specified in Procedure 1. In parentheses, expected theoretical *m/z* values are shown. Experimental values should not be different by more than 0.1 mDa from the accurate, theoretical values.

49. For both scripts, place the .isox files in a folder called 'data' that is located in the same directory as the .qmd file. For the script 'Procedure_1.2_MRFA_shotnoise.qmd', place only one file into the 'data' folder for which shot-noise analysis should be done.
50. Open the .qmd file in RStudio.
51. Press the 'Render' button in RStudio. The script performs the steps for data treatment described in Step 27 of this protocol. The script 'Procedure_1.2_MRFA_shotnoise.qmd' reads the .isox files in the 'data' folder and creates two output files: 'Procedure_1.2_MRFA_shotnoise.html', summarizing the data treatment and showing a shot-noise plot, and 'shot_noise_MRFA.xlsx', which includes the isotopocule ratios, shot-noise limit ('shot_noise.permil') and relative standard error ('ratio_rel_se.permil') data. In addition, plots for diagnostic purposes visualizing the different data-treatment steps are generated, as described in Step 27 of this procedure.
52. The data are plotted against the effective number of ions counted in the analysis ('n_effective_ions') and/or analysis time ('time.min'). Carefully check the plots. If the quality of the obtained data is satisfactory, the relative standard error should closely follow the theoretical shot-noise limits—see Anticipated results, Procedure 1.
53. (Optional) Open and render the script 'Procedure_1.2_MRFA_replicates.qmd' in case .isox files from multiple replicates need to be read in. It creates two output files: 'Procedure_1.2_MRFA_replicates.html', summarizing the data treatment, and 'MRFA_data_replicates.xlsx', which includes the isotopocule ratios ('ratio'), shot-noise limit ('shot_noise_permil') and relative standard error of the mean (s.e.m.) ('ratio_relative_sem_permil') for the $^{13}C/M0$ ratio in every replicate.
    - Shot noise is calculated in the script as described in Step 28 above.
    - For ratio calculation, the 'sum' method is used as default. The ion counts for the different isotopocules in every scan are summed up for every compound in every replicate. Ratios are calculated in the script on the basis of these summed ion counts.
    - As a base peak for the calculation of the isotopocule ratios, the monoisotopic (M0) isotopocule is used with the default settings. It has the highest signal intensity, consisting only of the lighter isotopes (^{12}C, ^{1}H, ^{14}N and ^{16}O in the case of the alanine immonium ion).
    - The relative standard error of the ratios calculated for each replicate is estimated by using the ratios calculated for every scan.

Procedure 2: flow injection analysis

Acquiring the data

● **TIMING 15 min per injection**

1. Check that the UHPLC-Orbitrap MS system is set up as described in Equipment setup: Procedure 2.
2. Set the initial parameters in the instrument control software according to Software setup.
3. Set the ion source parameters according to the following table as starting values and wait until the 'Ion Transfer Tube Temp' stabilizes.

Parameter	Value
Neg Ion Spray Voltage (V)	2,700
Sheath Gas (Arb)	2
Aux Gas (Arb)	2
Sweep Gas (Arb)	0
Ion Transfer Tube Temp (°C)	280
Vaporizer temp (°C)	0

4. Perform one or more manual injections of the analyte to optimize the ion source parameters to gain high sensitivity and low signal deviation (the RSD parameter). For the 50 µM solution of nitrate in methanol with the described MS setting, TIC > 6×10^8 is to be

expected, and the ion source tuning should aim for an RSD <10%. The spray current should be stable at a maximum of 0.1 µA.

◆ **TROUBLESHOOTING**

5. Confirm that all peaks of nitrate isotopocules are present and resolved in the mass spectrum window. All expected peaks in the selected mass range are displayed in Fig. 9.
6. Once the signal stability is optimized, run the prepared sequence in Xcalibur.

■ **PAUSE POINT** The samples can be discarded or used for re-analysis if needed. If the samples are required for analysis at a later point, they should be stored at 4 °C or frozen over several months. Data analysis can be done at any time.

Data processing

● **TIMING 60–90 min**

7. Open the sequence in FreeStyle (or Qual Browser).
8. Check each of the sample injections for a stable signal during the injection plateau (Extended Data Fig. 4) and check that the duration of the signal is as expected with regard to injection volume and flow rate. The injection of nitrate solution should result in a duration of ≥5 min with a stable nitrate signal to ensure a high-precision measurement.
9. The next step is the extraction of peak data from the RAW files for isotopocule ratio calculation. Follow Steps 18–23 in Procedure 1. The structure of the isotopologs.tsv file for nitrate is shown in Extended Data Fig. 5.

■ **PAUSE POINT** Further data evaluation can be done at any time.

10. Carry out further evaluation of the created .isox files with any data evaluation tool. Multiple options are listed in Software setup. Here, we describe data evaluation based on the script 'Procedure_2_Nitrate.qmd' (it can be downloaded from the GitHub repository).
11. To run the script, place all .isox files in a folder called 'data' that is located in the same directory as the .qmd file. Add a sequence.csv file into the same 'data' folder. The sequence.csv file can be created by using the Xcalibur application if it is not available:
    - Open the used sequence file in the Xcalibur 'Sequence Setup'.
    - Go to *File* → *Export Sequence* and enter the 'data' folder.
    - Click OK to create the sequence.csv file.
12. Check isotope ratios and delta values of the reference materials in the code chunk '#Optional User Inputs' (lines 57–78). The code chunk defines the known isotope ratios of the international references, as well as the delta values known for the two used reference materials USGS35 and USGS32. If different reference materials were used, the values need to be modified in this part accordingly. Note that the file names of the RAW files have to

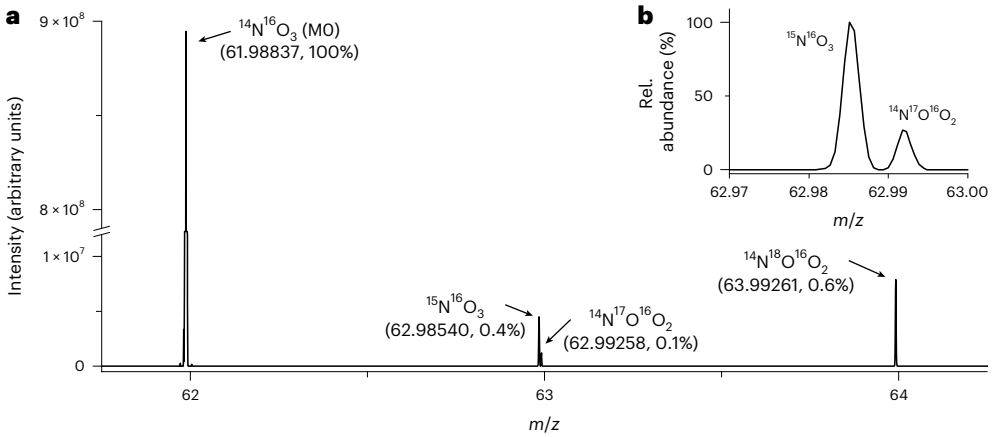


Fig. 9 | Mass spectrum of nitrate in the scan range specified in Procedure 2. a, The whole mass range is shown. **b**, An insert shows the mass range around *m/z* 63 in detail. The ^{15}N- and ^{17}O-substituted isotopocules at *m/z* 63 are fully resolved at the applied Orbitrap resolution (15,000). In parentheses, expected theoretical *m/z* values are shown, together with relative intensities of the respective isotopocules. Experimental values should not be different by more than 0.1 mDa from the accurate, theoretical values. rel, relative.

match the file names in the sequence.csv. In addition, the sample names in the sequence.csv must match the inputs for 'Reference' and 'Sample' in lines 61 and 62 of the script.
▲ **CRITICAL STEP** It is important to place all of the files in the same folder called 'data'; otherwise, the script will not process them.

Further data deconvolution

13. Open the .qmd file in RStudio.
14. Press the 'Render' button in RStudio. The script reads all .isox files in the 'data' folder and performs basic data-treatment steps as described in Step 27 of Procedure 1. In addition, the injection of nitrate in every RAW file is detected on the basis of time points that define the beginning and the end of each injection by using the *orbi_define_block()* function. Ratios are calculated for every autosampler injection as described in Step 53 of Procedure 1.
15. *Correction of the ratio*. The use of a sample/standard comparison in Procedure 2 enables a one-point calibration[17]. The calibration can be performed in two different ways that result in the same final result. Further information on the alternative ways can be found in the Supplementary Description and Supplementary Spreadsheet.
    (A) **Correction method 1**
        (i) Using a spreadsheet, calculate the correction factor for every injection of the standard by dividing the expected ratio of the standard with the ratio measured for that injection.
        (ii) Multiply the ratio measured for every sample injection with the average correction factor of the preceding and following standard injections.
        (iii) Use this corrected ratio to calculate a δ value versus a known ratio of any other reference (see the Supplementary Spreadsheet).
    (B) **Correction method 2**
        (i) Calculate δ values as described by Hilkert et al.[17], first versus a standard, and then convert this value into the δ scale of a stable-isotope primary reference material. The equations used for this are explained as follows. For calculating δ values, the relative difference in the natural-abundance isotope ratios of two materials is typically referred to as geochemical δ values. Delta (δ) is defined as

$$\delta_{\mathrm{sam/STD}} = R_{\mathrm{sam}}/R_{\mathrm{STD}} - 1 \text{ or } [R_{\mathrm{sam}} - R_{\mathrm{STD}}]/R_{\mathrm{STD}} \qquad (3)$$

        where 'sam' represents a sample, 'STD' represents a working standard and R is the isotope abundance ratio ($^{15}N/^{14}N$, $^{18}O/^{16}O$ and $^{17}O/^{16}O$). δ values are conventionally multiplied by 1,000 and reported as permille (‰)[44].
        (ii) For interlaboratory and international comparisons, these isotope ratios are typically compared on an international δ scale with different isotopic reference materials depending on the analyzed isotope. Because of limited access and volume of primary and secondary reference materials, other materials are used as in-house working standards with δ values determined against the international primary reference material ($\delta_{\mathrm{STD,int}}$). Use equation (4) to calculate the difference in isotope ratios of any unknown sample to the international primary reference material ($\delta_{\mathrm{sam,int}}$) on the basis of the measured difference of the sample and working standard ($\delta_{\mathrm{sam,STD}}$) and the known difference of working standard and primary reference material ($\delta_{\mathrm{STD,int}}$):

$$\delta_{\mathrm{sam,int}}\,(‰) = \delta_{\mathrm{sam,STD}} + \delta_{\mathrm{STD,int}} + \frac{\delta_{\mathrm{sam,STD}} \times \delta_{\mathrm{STD,int}}}{1,000} \qquad (4)$$

16. Use the script 'Procedure_2_Nitrate.qmd' to generate multiple output files. 'Procedure_2_Nitrate.html' summarizes the data treatment. The file 'nitrate_data_all.xlsx' includes the ratios, shot-noise limit ('shot_noise.permil') and relative standard error ('ratio_rel_se.permil') calculated for every isotopocule in every sample injection and the bracketing reference injections. The files 'table_nitrate_ratios.xlsx' and 'table_nitrate_deltas.xslx' summarize the results by using method 1 or method 2 for ratio correction.

Troubleshooting

Troubleshooting advice can be found below and in Table 1.

Table 1 | Troubleshooting table

Step	Problem	Possible reason	Solution
9 and 37 (Procedure 1), 4 (Procedure 2)	Large deviations in signal stability (the RSD parameter >>10%, low TIC, large IT, low scan rate and spray current >0.1 µA)	Possibly blockages or partial blockages of the ESI needle or the sample transfer line	Check the ESI needle or the sample transfer line for high back pressure and clean or exchange them if needed
23 (Procedure 1)	Data for some isotopocules are missing in the IsoX output (.isox) files	Mass entries in the isotopologs.tsv file probably do not correspond to masses detected during the analysis in the RAW files	Check the mass data in both files and correct as needed. The data acquisition can also be done by using a lock mass to prevent any shifts in the *m/z* domain

Data acquisition

The signal intensity is too low or is unstable

Large deviations in signal stability (strong drift, disturbances or jumps in the signal) can be seen when the RSD parameter is >>10% and/or the spray current is >0.1 µA. This can be caused by different factors. As the first step, a broader range of values for the three ion source parameters (ion spray voltage and flows of sheath and auxiliary gases) should be tested to check for an optimum, especially when analyzing a new compound. To simplify this process, the automated optimizations under the 'Optimization' tab in the Tune software can be used. If no parameter settings with sufficient signal intensity and stability can be found, the position of the ESI probe inside the source housing should be adjusted. As a final step, the ESI needle, the sample transfer line and the ion-transfer tube can be checked for high back pressure (blockages or partial blockages) and be flushed, cleaned or ultimately exchanged.

In the case of contamination, clean the sample transfer line and the ESI needle by flushing both with pure solvents (e.g., 1:1 (vol/vol) methanol/water) and clean the ion-transfer tube according to the instrument manual.

The data acquisition can be performed with a lock mass, in which a mass of usually the most abundant molecule is used to correct for any small change in the *m/z* domain. This can ensure more reliable extraction of isotopocule data from RAW files by using the entries in the isotopologs.tsv file.

Data processing

Scripts fail

The most common causes of script failure (resulting in error messages) are linked to the IsoX data extraction. Check all the IsoX output files, as well as the isotopologs.tsv file to make sure that each file contains data for the isotopocules of interest. For example, an error message describing a missing signal of the peak labeled as 'M0' will prohibit any ratio calculation.

Data quality

Low data quality suggested by the script filters or poor precision/accuracy

In the case of any flaws regarding data quality, the HTML file and general-feedback messages created by the script can serve as a starting point for troubleshooting (examples can be found in Anticipated results). Most flaws in data quality directly affect the analysis of low-abundance isotopocules. The user can confirm in FreeStyle (or Qual Browser) if the signal was not correctly extracted because of unmatching mass values in the .tsv file, or the isotopocule was not detected in the measurement. Poor precision and/or accuracy can be checked by analyzing a known sample and comparing the obtained data with known or expected values. If the data are recorded with poor precision or accuracy, try increasing the AGC target and/or the number of microscans to increase the signal-to-noise ratio of the low-abundance peaks.

Timing

General
Installing IsoX, R and RStudio software: usually 30–45 min

Procedure 1: direct infusion–TFA and MRFA
Setting up the instrument method: 15–30 min
Steps 1–15 and 31–44, acquiring the data: 30–60 min
Steps 16–30 and 45–53, data processing: 60–90 min (users with basic experience in R)

Procedure 2: flow injection–nitrate
Preparing solutions for measurement: 60 min
Setting up the instrument method: 30–60 min
Steps 1–6, acquiring the data: 15 min per injection
Steps 7–16, data processing: 60–90 min (users with basic experience in R)

Anticipated results

Direct infusion analysis of TFA (FlexMix, negative mode)
Because of counting statistics, the precision of any isotopocule ratio improves with increasing ion count of each species. The theoretical limit of the precision for any measurement can be estimated as the shot noise and is calculated by using equation (2). For ESI-Orbitrap IRMS, the most effective ways to increase ion counts are an increase in the AGC target (with concomitant higher IT) and the scan rate (decrease in the Orbitrap resolution), although Fourier transform interferences and space-charge effects have to be considered as well. From the acquired dataset, two meaningful isotopocule ratios can be calculated. During evaluation of Orbitrap IRMS data, the ratio of any two detected isotopocules can be calculated and analyzed. For most applications, the most abundant isotope species is used as a denominator for the ratio calculation. In the cases described in this protocol, the most abundant species is the unsubstituted analyte ion (containing only the light isotopes), also called 'M0'. In the case of the TFA data, if there is no significant isotope-clumping effect, the isotopocule ratio of ^{13}C-TFA/M0 can be calculated to determine the ^{13}C/^{12}C isotope ratio of TFA, and the ^{18}O-TFA/M0 accordingly for the ^{18}O/^{16}O isotope ratio. Results for both isotopocule ratios acquired by using Orbitrap Exploris 480 Isotope Solutions are displayed in Fig. 10.

The plots show how the relative standard error (data points) closely follows the theoretical shot-noise limit (the lines). Both decrease with increasing ion count during the analysis time (5 min). The isotopocule ratio ^{13}C/M0 reaches a target precision of 1‰ more quickly than ^{18}O/M0 because it is more abundant. To get to the relative standard error of 1‰, it takes ~0.3 min for ^{13}C (ratio versus M0) but 1.1 min for ^{18}O (Fig. 10b). By following the described procedure and using the recommended settings, the relative standard error of both isotopocule ratios should be significantly <1‰ after 5 min.

Results requiring troubleshooting intervention
If studies are being performed in different laboratories, it is important to bear in mind that results from these different labs can be directly compared only if they are obtained by using instruments with a comparable scan rate and Orbitrap filling as the Orbitrap Exploris MS series. For example, using instruments equipped with low-field (D30) Orbitrap analyzers (e.g., Q Exactive and Q Exactive Plus) will result in higher relative standard error after the same duration of measurement because of a lower scan rate and therefore a lower number of ions counted at the same Orbitrap resolution setting compared to a high-field instrument[45].

Deviations from the optimal settings described in this protocol will result in a loss of precision. Extended Data Fig. 6 shows a comparison of the results acquired with an optimal

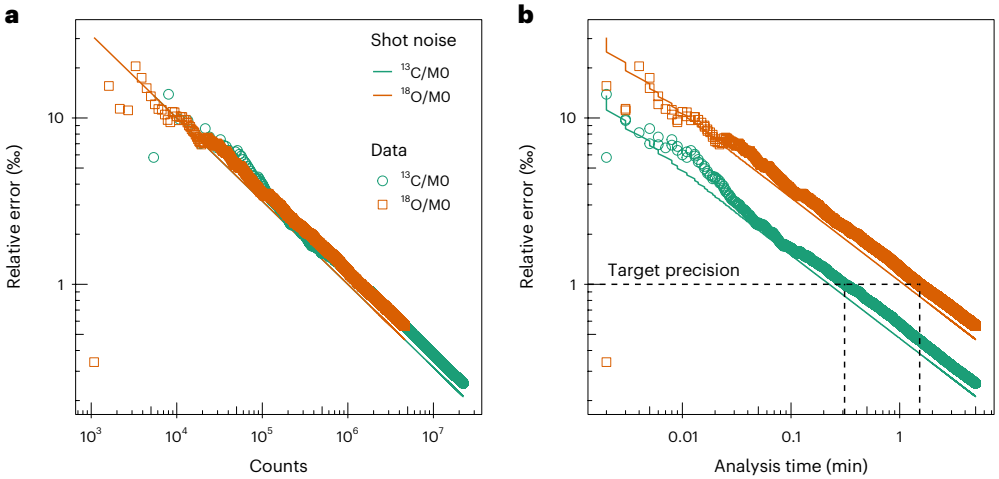


Fig. 10 | Shot-noise plots for the isotopocule ratios ^{13}C/M0 (green) and ^{18}O/M0 (orange) of TFA. a,**b**, The relative standard error values were calculated as described in the main text versus the count of the substituted isotopocule (**a**) and the analysis time (**b**). The plots show how the relative standard error (data points) closely follows the theoretical shot-noise limit (the lines), and both decrease with time (increasing ion count). Panel **b** highlights the time needed to reach the relative standard error of 1‰ (the target precision) for both ratios.

AGC setting (AGC target: 10^6; IT: ~0.75 ms) with a dataset acquired at significantly lower AGC settings (a minimum IT of 0.03 ms was used).

When using AGC target, always be aware of the maximum and minimum ITs. The maximum IT can be set in the MS settings tree, while the minimum IT is fixed at 0.03 ms. For the second dataset displayed in Extended Data Fig. 6 (data in green), a very low AGC target (10^4) was used at high TIC (~1.6×10^9). Here, the calculated IT would be ~ 0.006 ms. Because this value is lower than the minimum IT, an IT of 0.03 ms was used for all scans of that measurement instead. The same problem might occur if the calculated IT exceeds the maximum IT.

Extended Data Figure 7 displays a similar comparison for two Orbitrap resolution settings: 15,000 and 120,000. At higher resolution for both ratios, the relative standard error calculated after 5 min of analysis is significantly higher than at the optimal settings (the lower resolution). At 120,000, a longer analysis time would be necessary to achieve a matching precision.

MRFA (FlexMix, positive mode, MS^2)

In Extended Data Fig. 8, relative s.e.m. and shot-noise limits for immonium ions created from the MRFA peptide are shown. Data were acquired by using Orbitrap Exploris 480 Isotope Solutions. As in the case of TFA, the relative s.e.m. decreases with increasing ion count during the analysis time (10 min). Differences in the isotopocule ratio precision between the different amino acid immonium ion fragments are caused by their different relative abundances after MRFA fragmentation. Alanine and arginine show lower relative abundances than methionine and phenylalanine (Fig. 8), resulting in higher relative s.e.m. and shot noise.

If the analysis of the MRFA ion described in Procedure 1 is carried out multiple times, the repeatability of the measurement can be estimated. Figure 11 shows results of an example dataset with five replicate measurements. Because the error within one replicate is reduced as more ions are counted, the error on a ratio calculated by averaging (or summing) the replicate results (or ion counts) is reduced as well. The ion count ('ions.incremental') measured for the ^{13}C-substituted alanine immonium ions is less than half of the corresponding ion counts for arginine and methionine immonium ions. This results in an increased relative standard deviation of the five replicates for alanine of 0.39% compared to 0.13% and 0.11% for arginine and methionine, respectively. The isotopocule ratio of the phenylalanine immonium ion gives a repeatability of 0.13% despite the increased ion count (3–4× more) compared to methionine and arginine immonium ions. Here, the external error limits further improvement of the repeatability.

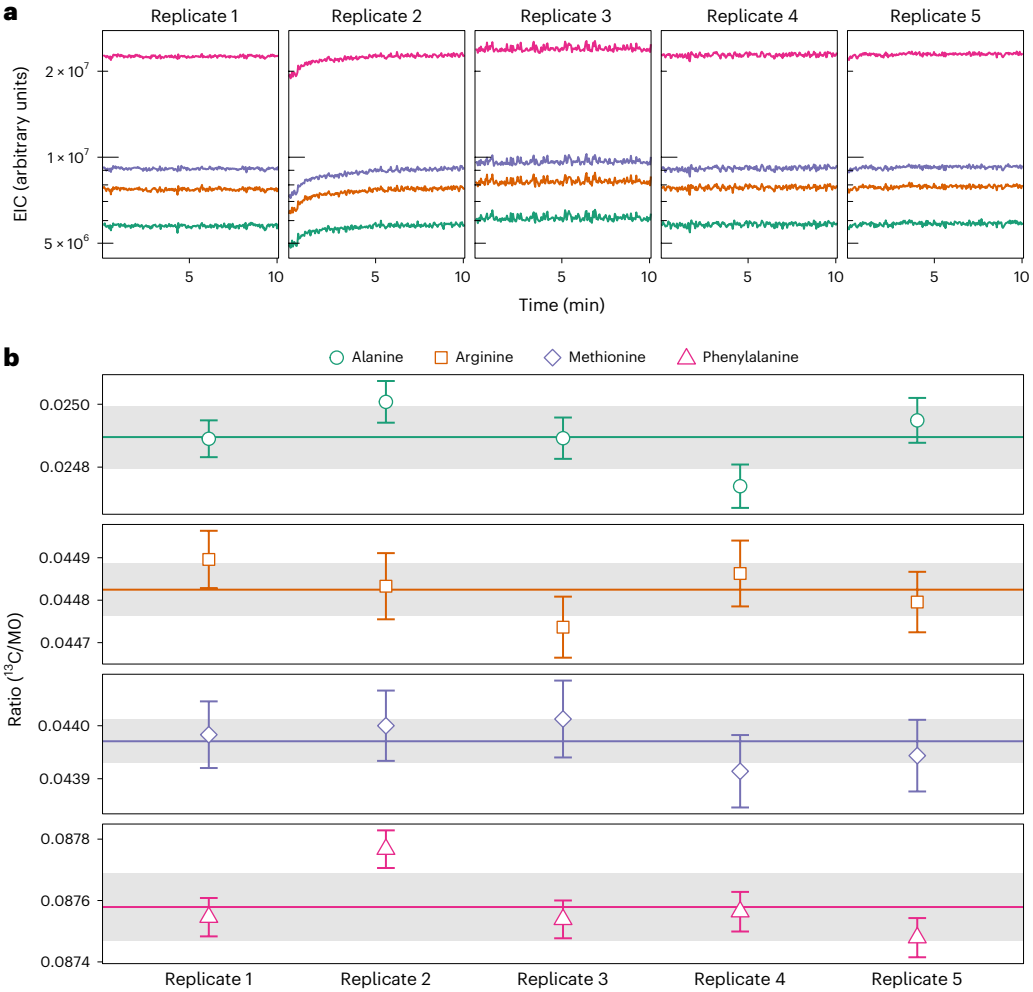


Fig. 11 | Replicate analysis of isotopocule ratios from immonium ions created from the MRFA peptide. Alanine (green), arginine (orange), methionine (purple) and phenylalanine (magenta). **a**,**b**, Extracted ion currents (EICs) for all amino acid immonium ions are plotted in **a**, and ratios of ^{13}C/M0 calculated for every replicate are shown in **b**. Error bars indicate the s.e.m. calculated for each time range (10 min, $n = 230$). The straight line shows the average of all five replicates, with gray boxes showing the standard deviation (s.d.). Key processing parameters are reported in files in the provided repositories (see Data availability).

Flow injection analysis of nitrate

The method 'Procedure 2: flow injection–nitrate' describes automated high-precision and high-accuracy isotopocule ratio analysis on the example of two nitrate reference materials (USGS32 and USGS35). The script used for the data evaluation extracts an exhausting amount of isotope data from the acquired dataset and offers a set of figures and tables as output files where the data can be seen.

All data-processing steps are performed by the script 'Procedure_2_Nitrate.qmd' automatically, providing feedback on the result of every single step to estimate the quality of the acquired dataset. For this purpose, the HTML file contains message boxes that provide information on the progress and outcome of the process. For example, for filtering minor signals, a message 'orbi_flag_satellite_peaks() is flagging minor signals (satellite peaks)…flagged *xx*/*xx* peaks in *x* isotopocules (*x*) as satellite peaks (*xx*%) in *xx* seconds' will be displayed, ensuring that only the most abundant signal extracted from a certain mass tolerance window is used for ratio calculation.

Table 2 | Isotopocule ratios of the nitrate reference material USGS32 obtained by Procedure 2 and experimental values before and after correction using the reference material USGS35

Sample	Isotopocule ratio	Ratio expected	Experimental ratio		Experimental ratio corrected	
			Average (n = 6)	%CV (rel. s.d.)	Average (n = 6)	%CV (rel. s.d.)
USGS32	^{15}N/M0	0.004338	0.005178	0.11	0.004345	0.10
USGS32	^{17}O/M0	0.001155	0.001381	0.24	0.001154	0.23
USGS32	^{18}O/M0	0.006171	0.008854	0.17	0.006180	0.14

rel., relative.

Table 3 | Experimental δ values of the reference material USGS32 obtained by Procedure 2 (six injections of USGS32 bracketed by seven injections of USGS35)

Isotopocule ratio	Reference	Reference ratio	Experimental ratio of USGS32, corrected	δ expected (‰)	δ measured (‰)	
					Average	s.d. (n = 6)
^{15}N/M0	Air-N_2	0.003676	0.004345	180.0	182.1	1.2
^{17}O/M0	VSMOW	0.001140	0.001154	13.2	12.1	2.4
^{18}O/M0	VSMOW	0.006016	0.006180	25.7	27.2	1.4

Table 4 | Experimental δ values of the reference material USGS32 obtained by Procedure 2 by using non-optimal ion-source conditions (six injections of USGS32 bracketed by seven injections of USGS35)

Isotopocule ratio	Reference	Reference ratio	Experimental ratio of USGS32, corrected	δ expected (‰)	δ measured (‰)	
					Average	s.d. (n = 6)
^{15}N/M0	Air-N_2	0.003676	0.004345	180.0	181.9	2.3
^{17}O/M0	VSMOW	0.001140	0.001156	13.2	14.3	3.1
^{18}O/M0	VSMOW	0.006016	0.006201	25.7	30.8	2.0

In conventional IRMS, a scheme using working standards and the sample/standard comparison is used to improve accuracy and precision (repeatability) of the measurement, by implementing various drift and linearity corrections. The same concepts were also found to be beneficial for ESI-Orbitrap IRMS. By alternately injecting a known standard material (here, the reference material USGS35) and an unknown sample (here, the reference material USGS32) in a short time frame, we can correct not only long-term stable systematic effects on the isotopocule ratios, like asymmetric quadrupole filtering or ion decay in the Orbitrap, but also many forms of drift (e.g., changing ESI conditions).

Table 2 shows data acquired by using Orbitrap Exploris 480 Isotope Solutions with Procedure 2. Because the analyzed sample is a well-characterized reference material, its expected and measured isotopocule ratios can be used to determine the accuracy of the measurement. Values in the column 'Ratio expected' were calculated on the basis of the isotope ratios of the primary reference materials Air-N_2 and Vienna Standard Mean Ocean Water (VSMOW) and USGS32 (reported by USGS; pubs.usgs.gov/publication/70188273).

All three measured isotopocule ratios differ significantly from the expected ratios, with a difference of up to 43% in the case of ^{18}O/M0. To correct this ratio via the sample/standard comparison, a correction factor was calculated for every injection of USGS35. In the next step, the average correction factor of the preceding and following USGS35 injections was used to correct the ratio calculated for every USGS32 injection. This referencing significantly improves accuracy, with only 0.18% difference between the corrected and the expected ratio of ^{18}O/M0, while also reducing the coefficient of variation (%CV) for all three isotopocule ratios.

Although the effect of referencing on the repeatability of an isotopocule ratio measurement (here expressed as %CV) appears to be minor for short sequences of measurement (as

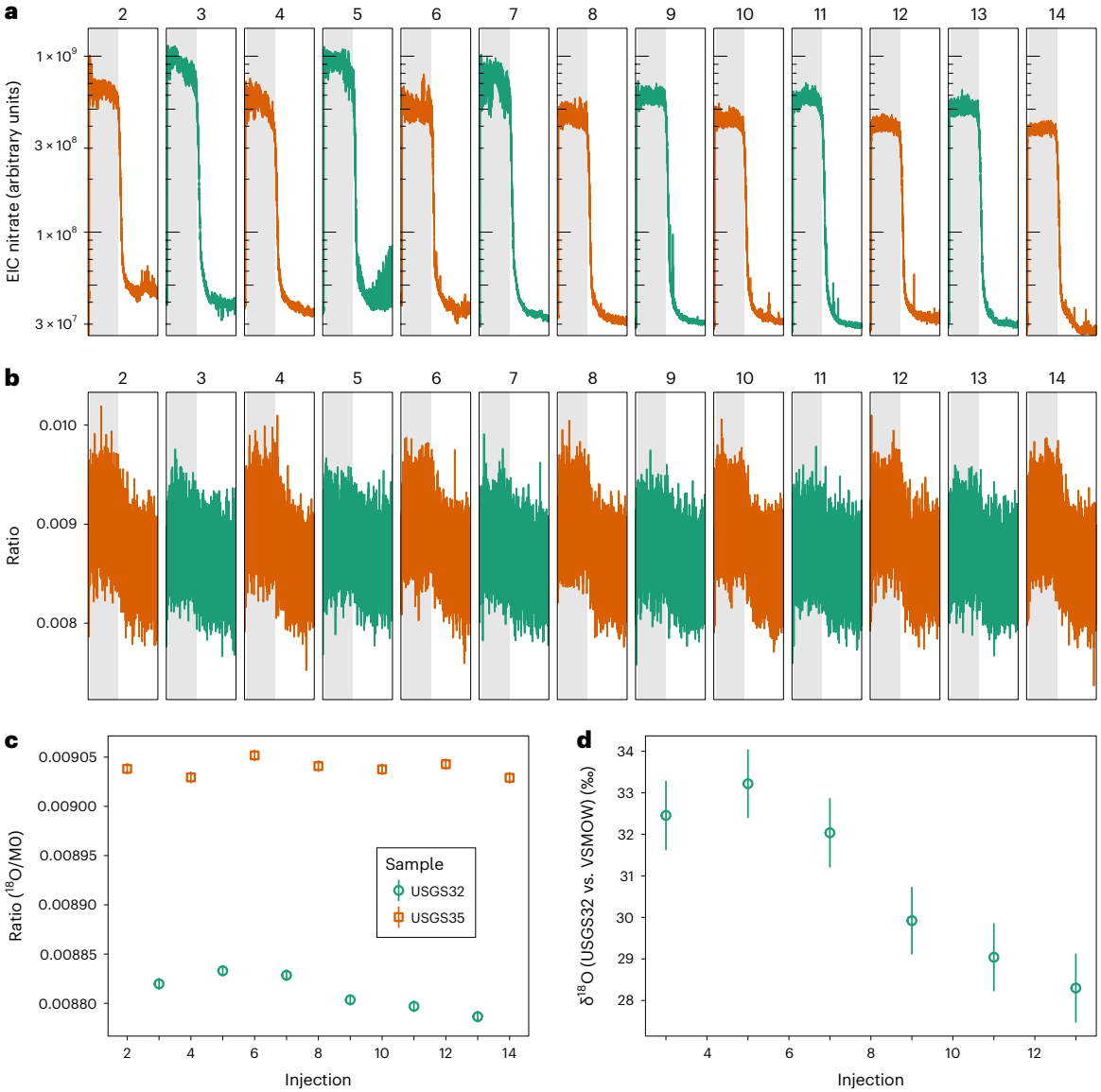


Fig. 12 | Results of nitrate isotopocule ratio analysis with non-optimal ion source settings. a, EICs for nitrate for injections 2–14 are plotted. **b**, Ratios of $^{18}O/M0$ during the injections. **c**, ratios of $^{18}O/M0$ calculated for every injection (alternating injections of USGS35 and USGS32). **d**, $\delta^{18}O$ of the USGS32 material, showing the drift of the δ values due to unstable ion-source conditions. The gray bars show time intervals of data used for calculation of the ratios and δ values.

demonstrated in Procedure 2), it becomes of greater importance for longer measurement sequences as well as monthly, yearly and interlaboratory reproducibility. For demonstration purposes, the sequence of alternating injections of USGS35 and USGS32 was extended to a total of 365 injections. For the resulting sequence of ~72 h, the average %CV of all three isotopocule ratios was reduced from 0.35% for the uncorrected ratios to 0.16% for the corrected ratio.

In many application fields of natural-abundance stable-isotope-ratio analysis, the ratios of unknown samples are compared to the ratios of primary reference materials like Air-N_2 ($^{15}N/^{14}N$) and VSMOW ($^{18}O/^{16}O$ and $^{17}O/^{16}O$). The relative difference of a sample to the primary reference material is typically referred to as its δ value (in permille)[44]. In Procedure 2, the δ values of USGS32 were calculated by using equation (3) and are listed in Table 3.

One of the most sensitive and crucial steps in the workflow of ESI-Orbitrap IRMS is the process of ionization. Careful tuning of the ion source settings, as described in Steps 9 and 37

in Procedure 1 and Step 4 in Procedure 2, is mandatory to achieve a stability that is sufficient for high-precision isotopocule ratio determination. To demonstrate this impact, a third dataset was acquired by using Procedure 2 with the ion source settings changed to non-optimal conditions right before the start of the sequence. The spray voltage was increased to 3 kV, and the sheath and auxiliary gas flows were set to 4 and 2, respectively. Although the TIC and TIC variation metrics indicated good spray conditions under the new settings, the spray current increased to >0.1 µA, and it was noisy and unstable. Table 4 shows the results from this third dataset.

The ratio of ^{15}N/M0 was only barely affected by the changes in the ion source settings, but both ^{17}O and ^{18}O isotopocules of nitrate were affected in accuracy and precision of the measurement. The TIC and the δ values (especially ^{18}O/M0) showed a drift throughout the sequence (Fig. 12). These results stress the significance of stable ion source conditions, with a low (<0.1 µA) and stable (the RSD parameter <10%) spray current being the main indicator for long-term stability.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The raw data supporting the findings of this study have been deposited to the MassIVE repositories with accession codes MSV000093222 (Procedure 1) and MSV000093223 (Procedure 2). Source data, data processing scripts and output plots and tables are available via a GitHub repository at github.com/isoverse/2023_kantnerova_et_al. Source data are provided with this paper.

Code availability

The isoorbi R package can be found at isoorbi.isoverse.org, github.com/isoverse/isoorbi and cloud.r-project.org/package=isoorbi. The IsoXL demo GUI can be found at isoorbi.isoverse.org/articles/isoxl_demo.html.

Received: 31 March 2023; Accepted: 8 February 2024;
Published online: 23 April 2024

References

1. Brenna, J. T. Natural intramolecular isotope measurements in physiology: elements of the case for an effort toward high-precision position-specific isotope analysis. *Rapid Commun. Mass Spectrom.* **15**, 1252–1262 (2001).
2. Eiler, J. M. The isotopic anatomies of molecules and minerals. *Annu. Rev. Earth Planet. Sci.* **41**, 411–441 (2013).
3. Gilbert, A. The organic isotopologue frontier. *Annu. Rev. Earth Planet. Sci.* **49**, 435–464 (2021).
4. Hinrichs, K.-U., Eglinton, G., Engel, M. H. & Summons, R. E. Exploiting the multivariate isotopic nature of organic compounds. *Geochem., Geophys., Geosyst.* **2**, 1052 (2001).
5. Piper, T., Geyer, H., Nieschlag, E., Bally, L. & Thevis, M. Carbon isotope ratios of endogenous steroids found in human serum—method development, validation, and reference population-derived thresholds. *Anal. Bioanal. Chem.* **413**, 5655–5667 (2021).
6. Guyader, S. et al. Combination of ^{13}C and ^{2}H SNIF-NMR isotopic fingerprints of vanillin to control its precursors. *Flavour Fragr. J.* **34**, 133–144 (2019).
7. O'Brien, D. M. Stable isotope ratios as biomarkers of diet for health research. *Annu. Rev. Nutr.* **35**, 565–594 (2015).
8. Kaiser, J. & Röckmann, T. Correction of mass spectrometric isotope ratio measurements for isobaric isotopologues of O_2, CO, CO_2, N_2O and SO_2. *Rapid Commun. Mass Spectrom.* **22**, 3997–4008 (2008).
9. Neubauer, C. et al. Discovering nature's fingerprints: isotope ratio analysis on bioanalytical mass spectrometers. *J. Am. Soc. Mass Spectrom.* **34**, 525–537 (2023).
10. Murray, K. K. et al. Definitions of terms relating to mass spectrometry (IUPAC Recommendations 2013). *Pure Appl. Chem.* **85**, 1515–1609 (2013).
11. Christen, A. Atmospheric measurement techniques to quantify greenhouse gas emissions from cities. *Urban Clim.* **10**, 241–260 (2014).
12. Fry, B., Carter, J. F., Yamada, K., Yoshida, N. & Juchelka, D. Position-specific ^{13}C/^{12}C analysis of amino acid carboxyl groups – automated flow-injection analysis based on reaction with ninhydrin. *Rapid Commun. Mass Spectrom.* **32**, 992–1000 (2018).
13. Akoka, S. & Remaud, G. S. NMR-based isotopic and isotopomic analysis. *Prog. Nucl. Magn. Reson. Spectrosc.* **120–121**, 1–24 (2020).
14. Neubauer, C. et al. Towards measuring growth rates of pathogens during infections by D_2O-labeling lipidomics. *Rapid Commun. Mass Spectrom.* **32**, 2129–2140 (2018).
15. Neubauer, C. et al. Scanning the isotopic structure of molecules by tandem mass spectrometry. *Int. J. Mass Spectrom.* **434**, 276–286 (2018).
16. Eiler, J. et al. Analysis of molecular isotopic structures at high precision and accuracy by Orbitrap mass spectrometry. *Int. J. Mass Spectrom.* **422**, 126–142 (2017).
17. Hilkert, A. et al. Exploring the potential of electrospray-Orbitrap for stable isotope analysis using nitrate as a model. *Anal. Chem.* **93**, 9139–9148 (2021).
18. Gharibi, H. et al. Proteomics-compatible Fourier Transform isotopic ratio mass spectrometry of polypeptides. *Anal. Chem.* **94**, 15048–15056 (2022).
19. Neubauer, C. et al. Stable isotope analysis of intact oxyanions using electrospray quadrupole-Orbitrap mass spectrometry. *Anal. Chem.* **92**, 3077–3085 (2020).
20. Abelson, P. H. & Hoering, T. C. Carbon isotope fractionation in formation of amino acids by photosynthetic organisms. *Proc. Natl Acad. Sci. USA* **47**, 623–632 (1961).
21. Fronza, G., Fuganti, C., Schmidt, H. L. & Werner, R. A. The δ^{18}O-value of the *p*-OH group of L-tyrosine permits the assignment of its origin to plant or animal sources. *Eur. Food Res. Technol.* **215**, 55–58 (2002).
22. Eiler, J. M. "Clumped-isotope" geochemistry—the study of naturally-occurring, multiply-substituted isotopologues. *Earth Planet. Sci. Lett.* **262**, 309–327 (2007).
23. Jang, C., Chen, L. & Rabinowitz, J. D. Metabolomics and isotope tracing. *Cell* **173**, 822–837 (2018).

24. Angel, T. E., Naylor, B. C., Price, J. C., Evans, C. & Szapacs, M. Improved sensitivity for protein turnover quantification by monitoring immonium ion isotopologue abundance. *Anal. Chem.* **91**, 9732–9740 (2019).
25. Su, X., Lu, W. & Rabinowitz, J. D. Metabolite spectral accuracy on Orbitraps. *Anal. Chem.* **89**, 5940–5948 (2017).
26. Llufrio, E. M., Cho, K. & Patti, G. J. Systems-level analysis of isotopic labeling in untargeted metabolomic data by X^{13}CMS. *Nat. Protoc.* **14**, 1970–1990 (2019).
27. Granger, J. & Wankel, S. D. Isotopic overprinting of nitrification on denitrification as a ubiquitous and unifying feature of environmental nitrogen cycling. *Proc. Natl Acad. Sci. USA* **113**, E6391–E6400 (2016).
28. Mueller, E. P., Sessions, A. L., Sauer, P. E., Weiss, G. M. & Eiler, J. M. Simultaneous, high-precision measurements of δ^2H and δ^{13}C in nanomole quantities of acetate using electrospray ionization-quadrupole-Orbitrap mass spectrometry. *Anal. Chem.* **94**, 1092–1100 (2022).
29. Hofmann, A. E. et al. Using Orbitrap mass spectrometry to assess the isotopic compositions of individual compounds in mixtures. *Int. J. Mass Spectrom.* **457**, 116410 (2020).
30. Csernica, T., Bhattacharjee, S. & Eiler, J. Accuracy and precision of ESI-Orbitrap-IRMS observations of hours to tens of hours via reservoir injection. *Int. J. Mass Spectrom.* **490**, 117084 (2023).
31. Peng, Y., Wang, Z. & Bao, H. Oxygen isotope analysis of phosphate by Electrospray Orbitrap mass spectrometry. *AGU Fall Meet. Abstr.* **2022**, V32B-0080 (2022).
32. Gharibi, H. et al. Abnormal (hydroxy)proline deuterium content redefines hydrogen chemical mass. *J. Am. Chem. Soc.* **144**, 2484–2487 (2022).
33. Wilkes, E. B. et al. Position-specific carbon isotope analysis of serine by gas chromatography/Orbitrap mass spectrometry, and an application to plant metabolism. *Rapid Commun. Mass Spectrom.* **36**, e9347 (2022).
34. Cesar, J., Eiler, J., Dallas, B., Chimiak, L. & Grice, K. Isotope heterogeneity in ethyltoluenes from Australian condensates, and their stable carbon site-specific isotope analysis. *Org. Geochem.* **135**, 32–37 (2019).
35. Zeichner, S. S. et al. Methods and limitations of stable isotope measurements via direct elution of chromatographic peaks using gas chromatography-Orbitrap mass spectrometry. *Int. J. Mass Spectrom.* **477**, 116848 (2022).
36. MacCoss, M. J., Toth, M. J. & Matthews, D. E. Evaluation and optimization of ion-current ratio measurements by selected-ion-monitoring mass spectrometry. *Anal. Chem.* **73**, 2976–2984 (2001).
37. Kharchenko, A., Vladimirov, G., Heeren, R. M. A. & Nikolaev, E. N. Performance of Orbitrap mass analyzer at various space charge and non-ideal field conditions: simulation approach. *J. Am. Soc. Mass Spectrom.* **23**, 977–987 (2012).
38. Gorshkov, M. V., Fornelli, L. & Tsybin, Y. O. Observation of ion coalescence in Orbitrap Fourier transform mass spectrometry. *Rapid Commun. Mass Spectrom.* **26**, 1711–1717 (2012).
39. Grinfeld, D. et al. Space-charge dynamics in Orbitrap mass spectrometers. *Int. J. Mod. Phys. A* **34**, 1942007 (2019).
40. Wang, Y., Sessions, A. L., Nielsen, R. J. & Goddard, W. A. Equilibrium ^{2}H/^{1}H fractionations in organic molecules: I. Experimental calibration of ab initio calculations. *Geochim. Cosmochim. Acta* **73**, 7060–7075 (2009).
41. Silverman, S. N. et al. Practical considerations for amino acid isotope analysis. *Org. Geochem.* **164**, 104345 (2022).
42. Claesen, J., Rockwood, A., Gorshkov, M. & Valkenborg, D. The isotope distribution: a rose with thorns. *Mass Spectrom. Rev.* https://doi.org/10.1002/mas.21820 (2023).
43. Taylor, P. J. Matrix effects: the Achilles heel of quantitative high-performance liquid chromatography-electrospray-tandem mass spectrometry. *Clin. Biochem.* **38**, 328–334 (2005).
44. Skrzypek, G. et al. Minimum requirements for publishing hydrogen, carbon, nitrogen, oxygen and sulfur stable-isotope delta results (IUPAC Technical Report). *Pure Appl. Chem.* **94**, 1249–1255 (2022).
45. Makarov, A., Denisov, E. & Lange, O. Performance evaluation of a high-field Orbitrap mass analyzer. *J. Am. Soc. Mass Spectrom.* **20**, 1391–1396 (2009).

Acknowledgements

K.K. was supported by the Swiss National Science Foundation (Project No. P500PN_206702). Parts of this protocol build on work enabled by a grant from the National Science Foundation (Award no. 2041539) to S.K. and C.N. We thank J. K. Böhlke for fruitful discussions about referencing of stable-isotope data and access to reference materials and K. Aizikov for guidance on programming IsoX. We also thank B. Davidheiser, A. Angert, N. Bernet, H. Hayen, R. Marks, S. Ono and L. Yu for their critical feedback on the draft manuscript and software.

Author contributions

All co-authors conceived, designed and developed the protocol. K.K., N.K. and C.N. performed the experiments, analyzed the data and wrote the manuscript. K.K., N.K., C.N. and S.K. prepared the data analysis scripts and software. D.J., A.H. and S.K. edited the manuscript. All co-authors read, commented on and accepted the final manuscript.

Competing interests

The authors declare the following competing financial interest(s). A.H. and D.J. are employees of Thermo Fisher Scientific GmbH, which manufactures Orbitrap mass spectrometers as well as gas-source isotope ratio mass spectrometers. N.K. is a PhD student at the University of Münster and is conducting his doctoral thesis employed as a PhD student/R&D scientist at Thermo Fisher Scientific GmbH on Orbitrap-based isotope ratio measurements. C.N. is a paid independent scientific consultant to Thermo Fisher Scientific Inc. for ESI-Orbitrap mass spectrometer–based isotope ratio measurements.

Additional information

Extended data is available for this paper at https://doi.org/10.1038/s41596-024-00981-5.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41596-024-00981-5.

Correspondence and requests for materials should be addressed to Cajetan Neubauer.

Peer review information *Nature Protocols* thanks Amy Hofmann, Yongbo Peng, Matthias Pilecky and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Related links

Key references using this protocol

Eiler, J. et al. *Int. J. Mass Spectrom.* **422**, 126–142 (2017): https://doi.org/10.1016/j.ijms.2017.10.002
Neubauer, C. et al. *Int. J. Mass Spectrom.* **434**, 276–286 (2018): https://doi.org/10.1016/j.ijms.2018.08.001
Hilkert, A. et al. *Anal. Chem.* **93**, 9139–9148 (2021): https://doi.org/10.1021/acs.analchem.1c00944
Jang, C. et al. *Cell* **173**, 822–837 (2018): https://doi.org/10.1016/j.cell.2018.03.055
Neubauer, C. et al. *J. Am. Soc. Mass Spectrom.* **34**, 525–537 (2023): https://doi.org/10.1021/jasms.2c00363

	Sample Name	Study	File Name	Path	Inst Meth	Position	Inj Vol
1	Blank_MeOH	Procedure_2_Nitrate	230706_01	C:\Xcalibur\data\2023\Nitrate_FlowInjection\FlowInjection_Sequence_USGS32vs35\data	C:\Xcalibur\methods\VanquishNeo\Nitrate\Nitrate_FlowInjection_ExampleMethod	B:B9	25.00
2	USGS35	Procedure_2_Nitrate	230706_02	C:\Xcalibur\data\2023\Nitrate_FlowInjection\FlowInjection_Sequence_USGS32vs35\data	C:\Xcalibur\methods\VanquishNeo\Nitrate\Nitrate_FlowInjection_ExampleMethod	B:B2	25.00
3	USGS32	Procedure_2_Nitrate	230706_03	C:\Xcalibur\data\2023\Nitrate_FlowInjection\FlowInjection_Sequence_USGS32vs35\data	C:\Xcalibur\methods\VanquishNeo\Nitrate\Nitrate_FlowInjection_ExampleMethod	B:C2	25.00
4	USGS35	Procedure_2_Nitrate	230706_04	C:\Xcalibur\data\2023\Nitrate_FlowInjection\FlowInjection_Sequence_USGS32vs35\data	C:\Xcalibur\methods\VanquishNeo\Nitrate\Nitrate_FlowInjection_ExampleMethod	B:B2	25.00
5	USGS32	Procedure_2_Nitrate	230706_05	C:\Xcalibur\data\2023\Nitrate_FlowInjection\FlowInjection_Sequence_USGS32vs35\data	C:\Xcalibur\methods\VanquishNeo\Nitrate\Nitrate_FlowInjection_ExampleMethod	B:C2	25.00
6	USGS35	Procedure_2_Nitrate	230706_06	C:\Xcalibur\data\2023\Nitrate_FlowInjection\FlowInjection_Sequence_USGS32vs35\data	C:\Xcalibur\methods\VanquishNeo\Nitrate\Nitrate_FlowInjection_ExampleMethod	B:B2	25.00
7	USGS32	Procedure_2_Nitrate	230706_07	C:\Xcalibur\data\2023\Nitrate_FlowInjection\FlowInjection_Sequence_USGS32vs35\data	C:\Xcalibur\methods\VanquishNeo\Nitrate\Nitrate_FlowInjection_ExampleMethod	B:C2	25.00
8	USGS35	Procedure_2_Nitrate	230706_08	C:\Xcalibur\data\2023\Nitrate_FlowInjection\FlowInjection_Sequence_USGS32vs35\data	C:\Xcalibur\methods\VanquishNeo\Nitrate\Nitrate_FlowInjection_ExampleMethod	B:B2	25.00
9	USGS32	Procedure_2_Nitrate	230706_09	C:\Xcalibur\data\2023\Nitrate_FlowInjection\FlowInjection_Sequence_USGS32vs35\data	C:\Xcalibur\methods\VanquishNeo\Nitrate\Nitrate_FlowInjection_ExampleMethod	B:C2	25.00
10	USGS35	Procedure_2_Nitrate	230706_10	C:\Xcalibur\data\2023\Nitrate_FlowInjection\FlowInjection_Sequence_USGS32vs35\data	C:\Xcalibur\methods\VanquishNeo\Nitrate\Nitrate_FlowInjection_ExampleMethod	B:B2	25.00
11	USGS32	Procedure_2_Nitrate	230706_11	C:\Xcalibur\data\2023\Nitrate_FlowInjection\FlowInjection_Sequence_USGS32vs35\data	C:\Xcalibur\methods\VanquishNeo\Nitrate\Nitrate_FlowInjection_ExampleMethod	B:C2	25.00
12	USGS35	Procedure_2_Nitrate	230706_12	C:\Xcalibur\data\2023\Nitrate_FlowInjection\FlowInjection_Sequence_USGS32vs35\data	C:\Xcalibur\methods\VanquishNeo\Nitrate\Nitrate_FlowInjection_ExampleMethod	B:B2	25.00
13	USGS32	Procedure_2_Nitrate	230706_13	C:\Xcalibur\data\2023\Nitrate_FlowInjection\FlowInjection_Sequence_USGS32vs35\data	C:\Xcalibur\methods\VanquishNeo\Nitrate\Nitrate_FlowInjection_ExampleMethod	B:C2	25.00
14	USGS35	Procedure_2_Nitrate	230706_14	C:\Xcalibur\data\2023\Nitrate_FlowInjection\FlowInjection_Sequence_USGS32vs35\data	C:\Xcalibur\methods\VanquishNeo\Nitrate\Nitrate_FlowInjection_ExampleMethod	B:B2	25.00
15	Blank_MeOH	Procedure_2_Nitrate	230706_15	C:\Xcalibur\data\2023\Nitrate_FlowInjection\FlowInjection_Sequence_USGS32vs35\data	C:\Xcalibur\methods\VanquishNeo\Nitrate\Nitrate_FlowInjection_ExampleMethod	B:B9	25.00

Extended Data Fig. 1 | Screenshot of a sequence file. This example shows the sequence to analyze nitrate by using the described flow injection method (USGS35: standard; USGS32: sample). Injections of USGS35 at the beginning of the sequence for equilibration are not shown.

isotopologs.tsv

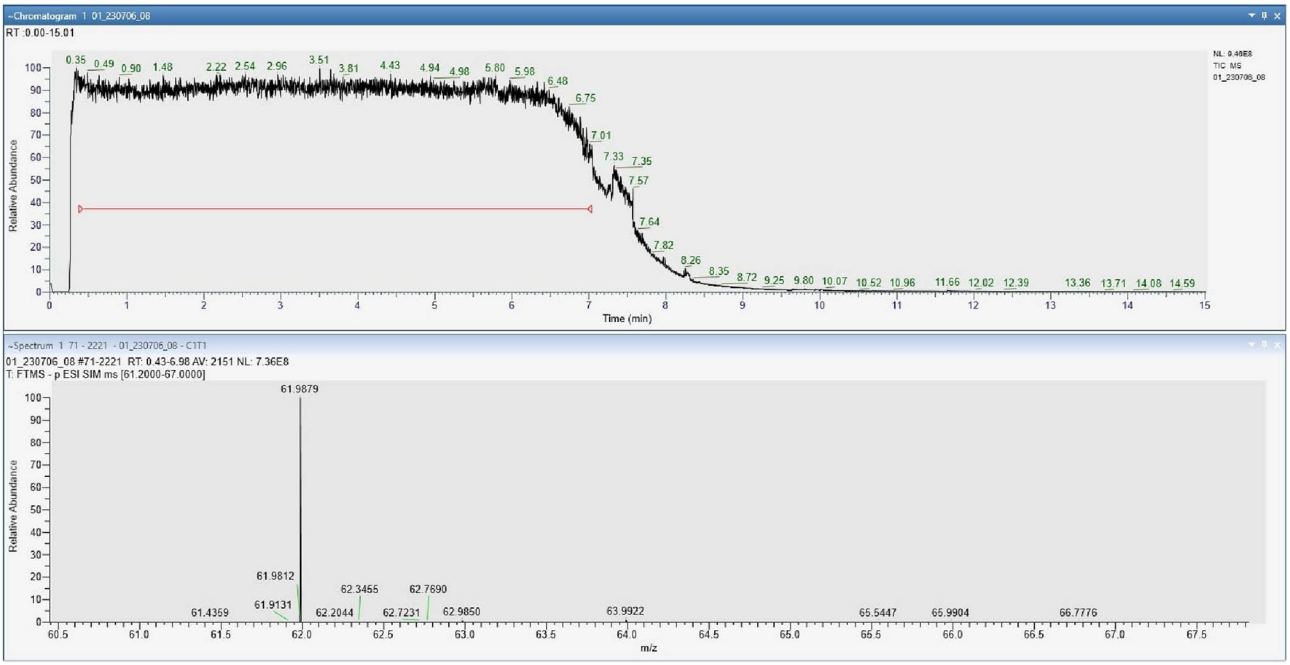
```
#Compound	Isotopolog	Mass	Tolerance	[mmu]	z
TFA	M0	112.98550	0.1	1
TFA	13C	113.98870	0.1	1
TFA	18O	114.98960	0.1	1
```

Extended Data Fig. 2 | Structure of the isotopologs.tsv file for TFA.

isotopologs.tsv

	#Compound	Isotopolog	Mass	Tolerance [mmu]	z
2	Ala_imm	M0	44.04940	0.1	1
3	Ala_imm	15N	45.04640	0.1	1
4	Ala_imm	13C	45.05270	0.1	1
5	Ala_imm	2H	45.05570	0.1	1
6	Arg_imm	M0	70.06500	0.1	1
7	Arg_imm	15N	71.06210	0.1	1
8	Arg_imm	13C	71.06840	0.1	1
9	Arg_imm	2H	71.07130	0.1	1
10	Met_imm	M0	104.05270	0.1	1
11	Met_imm	15N	105.04970	0.1	1
12	Met_imm	33S	105.05210	0.1	1
13	Met_imm	13C	105.05600	0.1	1
14	Met_imm	2H	105.05890	0.1	1
15	Met_imm	34S	106.04850	0.1	1
16	Phe_imm	M0	120.08050	0.1	1
17	Phe_imm	15N	121.07760	0.1	1
18	Phe_imm	13C	121.08390	0.1	1
19	Phe_imm	2H	121.08660	0.1	1

Extended Data Fig. 3 | Structure of the isotopologs.tsv file for immonium ions from MRFA.

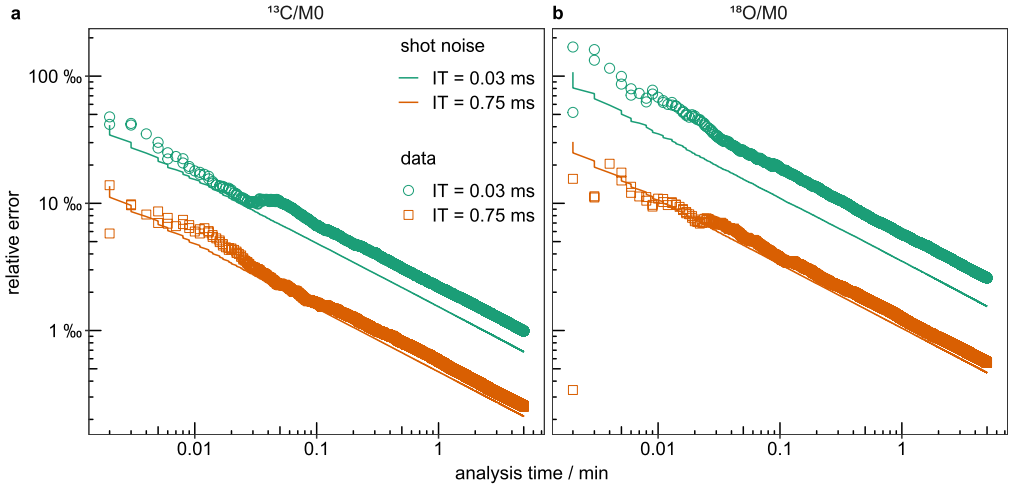


Extended Data Fig. 4 | Screenshot of a RAW file. The screenshot is from the FreeStyle software, showing the signal intensity (top) and the mass spectrum (bottom) for the flow injection analysis of nitrate during the stable signal plateau.

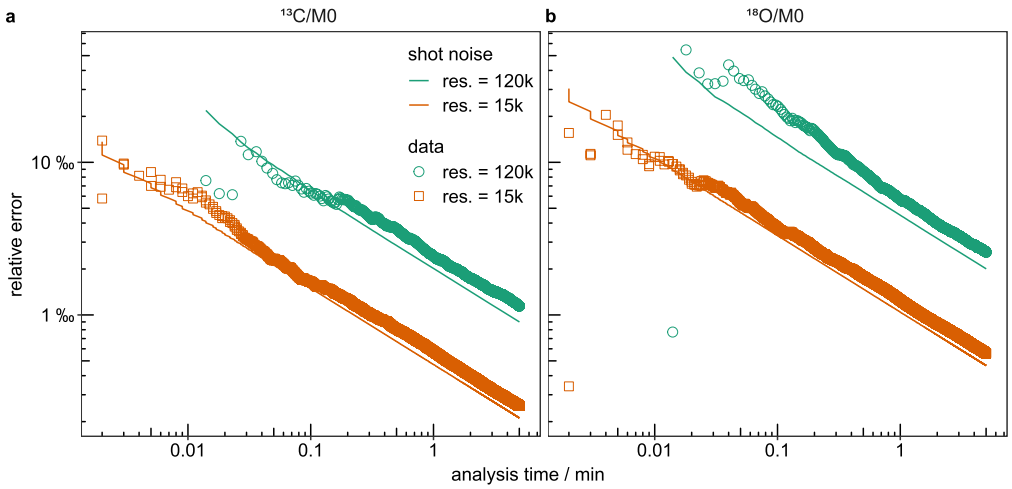
isotopologs.tsv

```
#Compound	Isotopolog	Mass	Tolerance [mmu]	z
NO3-	M0	61.9879	1	1
NO3-	15N	62.9849	1	1
NO3-	17O	62.9916	1	1
NO3-	18O	63.9922	1	1
```

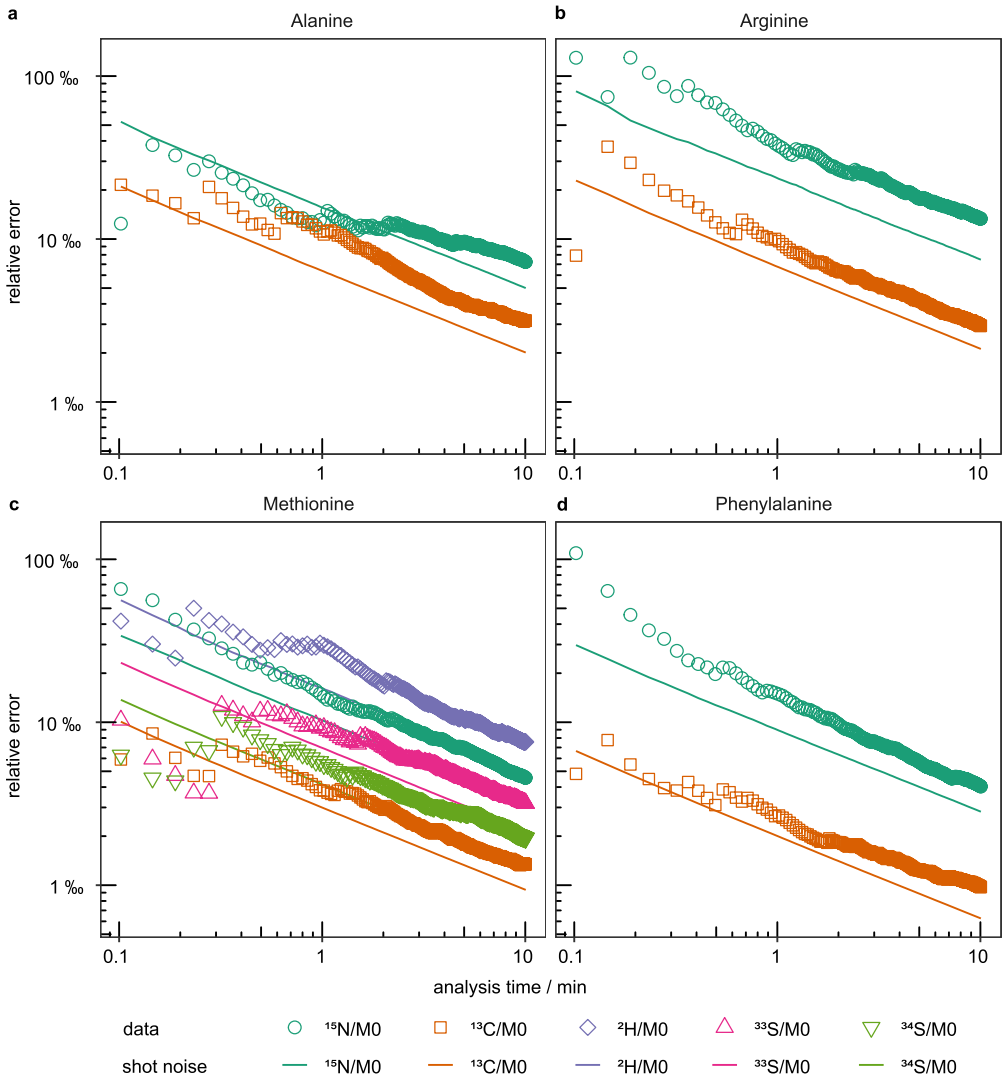
Extended Data Fig. 5 | Structure of the isotopologs.tsv file for nitrate.



Extended Data Fig. 6 | Shot-noise plots for TFA. a and **b**, Data for the isotopocule ratios ^{13}C/M0 (**a**) and ^{18}O/M0 (**b**). The data were obtained with two AGC target settings that resulted in different ITs: AGC target of 10^4 with IT = 0.03 ms (green, suboptimal) and AGC target of 10^6 with IT = 0.75 ms (orange, optimal). The optimal settings result in better precision (relative standard error, data points) within the same time, compared to the suboptimal settings. The lines depict the theoretical shot-noise limit.



Extended Data Fig. 7 | Shot-noise plots for TFA. a and **b**, Data for the isotopocule ratios ^{13}C/M0 (**a**) and ^{18}O/M0 (**b**). The data were obtained with two Orbitrap resolution settings: 120,000 (green, suboptimal) and 15,000 (orange, optimal). The optimal settings result in better precision (relative standard error, data points) within the same time, compared to the suboptimal settings. The lines depict the theoretical shot-noise limit.



Extended Data Fig. 8 | Shot-noise plots for isotopocule ratios of immonium ions created from the MRFA peptide. a–d, Alanine (**a**), arginine (**b**), methionine (**c**) and phenylalanine (**d**). The plots show decreasing relative standard error (the points) during the analysis time, which follows the theoretical shot-noise limit (lines). The offset between the individual isotopocules is based on their relative abundance ($^{13}C/^{12}C$ has the highest isotopic abundance ratio, whereas $^{2}H/^{1}H$ has the lowest).

nature portfolio

Corresponding author(s): Cajetan Neubauer

Last updated by author(s): 2024-01-22

Reporting Summary

Nature Portfolio wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Portfolio policies, see our Editorial Policies and the Editorial Policy Checklist.

Please do not complete any field with "not applicable" or n/a. Refer to the help text for what text to use if an item is not relevant to your study.
For final submission: please carefully check your responses for accuracy; you will not be able to make changes later.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed
○	● The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
○	● A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
○	○ The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
○	○ A description of all covariates tested
○	○ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
○	
●	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
○	○ For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
○	○ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
○	○ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
○	○ Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	● Tune application 4.0 (or newer; Thermo Fisher Scientific Inc.)
Data analysis	● FreeStyle 1.8 Data-Visualization Application (or newer) or Qual Browser (Thermo Fisher Scientific Inc.)

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

The raw data supporting the findings of this study have been deposited to the MassIVE repositories with accession codes MSV000093222 (Procedure 1) and MSV000092683 (Procedure 2). Source data, data processing scripts, and output plots and tables are available via a GitHub repository at github.com/isoverse/2023_kantnerova_et_al.

Human research participants

Policy information about studies involving human research participants and Sex and Gender in Research.

Reporting on sex and gender	
Population characteristics	
Recruitment	
Ethics oversight	

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

○ Life sciences ○ Behavioural & social sciences ◉ Ecological, evolutionary & environmental sciences

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	
Data exclusions	
Replication	
Randomization	
Blinding	

Behavioural & social sciences study design

All studies must disclose on these points even when the disclosure is negative.

Study description	
Research sample	
Sampling strategy	
Data collection	
Timing	
Data exclusions	
Non-participation	
Randomization	

Ecological, evolutionary & environmental sciences study design

All studies must disclose on these points even when the disclosure is negative.

Study description	This protocol describes how to use electrospray ionization (ESI)-Orbitrap mass spectrometry to achieve precise and accurate molecular
Research sample	FlexMix calibration solution (trifluoroacetate and MRFA peptide), it is widely available in labs with ESI-Orbitrap MS instruments
Sampling strategy	Not relevant.
Data collection	The data were recorded by Kristyna Kantnerova, Nils Kuhlbusch, and Gaietan Neubauer, using the Thermo Scientific Orbitrap Exploris
Timing and spatial scale	Not relevant.

Data exclusions	Not relevant.
Reproducibility	The analysis of the MRFA peptide has been replicated 5 times, all successful.
Randomization	Not relevant.
Blinding	Not relevant.

Did the study involve field work? ○ Yes ◉ No

Field work, collection and transport

Field conditions	
Location	
Access & import/export	
Disturbance	

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
○	◉ Antibodies
○	◉ Eukaryotic cell lines
○	◉ Palaeontology and archaeology
○	◉ Animals and other organisms
○	◉ Clinical data
○	◉ Dual use research of concern

Methods

n/a	Involved in the study
○	◉ ChIP-seq
○	◉ Flow cytometry
○	◉ MRI-based neuroimaging

Antibodies

Antibodies used	
Validation	

Eukaryotic cell lines

Policy information about cell lines and Sex and Gender in Research

Cell line source(s)	
Authentication	
Mycoplasma contamination	
Commonly misidentified lines (See ICLAC register)	

Palaeontology and Archaeology

Specimen provenance	
Specimen deposition	
Dating methods	

☐ Tick this box to confirm that the raw and calibrated dates are available in the paper or in Supplementary Information.

Ethics oversight	

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Animals and other research organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research, and Sex and Gender in Research

Laboratory animals	
Wild animals	
Reporting on sex	
Field-collected samples	
Ethics oversight	

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Clinical data

Policy information about clinical studies

All manuscripts should comply with the ICMJE guidelines for publication of clinical research and a completed CONSORT checklist must be included with all submissions.

Clinical trial registration	
Study protocol	
Data collection	
Outcomes	

Dual use research of concern

Policy information about dual use research of concern

Hazards

Could the accidental, deliberate or reckless misuse of agents or technologies generated in the work, or the application of information presented in the manuscript, pose a threat to:

No	Yes	
○	○	Public health
○	○	National security
○	○	Crops and/or livestock
○	○	Ecosystems
○	○	Any other significant area

Experiments of concern

Does the work involve any of these experiments of concern:

No	Yes	
○	○	Demonstrate how to render a vaccine ineffective
○	○	Confer resistance to therapeutically useful antibiotics or antiviral agents
○	○	Enhance the virulence of a pathogen or render a nonpathogen virulent
○	○	Increase transmissibility of a pathogen
○	○	Alter the host range of a pathogen
○	○	Enable evasion of diagnostic/detection modalities
○	○	Enable the weaponization of a biological agent or toxin
○	○	Any other potentially harmful combination of experiments and agents

ChIP-seq

Data deposition

☐ Confirm that both raw and final processed data have been deposited in a public database such as GEO.

☐ Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

Data access links

May remain private before publication

Files in database submission

Genome browser session
(e.g. UCSC)

Methodology

Replicates

Sequencing depth

Antibodies

Peak calling parameters

Data quality

Software

Flow Cytometry

Plots

Confirm that:

☐ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☐ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☐ All plots are contour plots with outliers or pseudocolor plots.

☐ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Instrument

Software

Cell population abundance

Gating strategy

☐ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.

Magnetic resonance imaging

Experimental design

Design type

Design specifications

Behavioral performance measures

Acquisition

Imaging type(s)

Field strength

Sequence & imaging parameters

Area of acquisition

Diffusion MRI ○ Used ○ Not used

Preprocessing

Preprocessing software

Normalization

Normalization template

Noise and artifact removal

Volume censoring

Statistical modeling & inference

Model type and settings

Effect(s) tested

Specify type of analysis:

Whole brain ROI-based Both

Statistic type for inference (See Eklund et al. 2016)

Correction

Models & analysis

n/a | Involved in the study

- Functional and/or effective connectivity
- Graph analysis
- Multivariate modeling or predictive analysis

Functional and/or effective connectivity

Graph analysis

Multivariate modeling and predictive analysis