Discovering Nature's Fingerprints:
Isotope Ratio Analysis on Bioanalytical Mass Spectrometers

A Perspective written by participants of the workshop "Nature's Fingerprints", which was held in November 2021 at the Hanse Institute for Advanced Study, Delmenhorst, Germany.

Cajetan Neubauer[1], Kristýna Kantnerová[1], Alexis Lamothe[2], Joel Savarino[2], Andreas Hilkert[3], Dieter Juchelka[3], Kai-Uwe Hinrichs[4], Marcus Elvert[4], Verena Heuer[4], Martin Elsner[5], Rani Bakkour[5], Maxime Julien[6], Merve Öztoprak[7], Stefan Schouten[7], Shohei Hattori[8], Thorsten Dittmar[9]

1 University of Colorado Boulder & Institute for Arctic and Alpine Research (INSTAAR), USA

2 University Grenoble Alpes, CNRS, IRD, INRAE, Grenoble-INP, IGE, Grenoble, France

3 Thermo Fisher Scientific GmbH, Bremen, Germany

4 MARUM Center for Marine Environmental Sciences, University of Bremen, Germany

5 Department of Chemistry, Technical University of Munich, Garching, Germany

6 GFZ German Research Center for Geosciences, Potsdam, Germany

7 NIOZ Royal Netherlands Institute for Sea Research, Texel, Netherlands

8 International Center for Isotope Effects Research, School of Earth Sciences and Engineering, Nanjing University, Nanjing, China

9 Institute for Chemistry and Biology of the Marine Environment (ICBM), University of Oldenburg, Oldenburg, Germany

Keywords: Isotopologue, isotopolog, isotopomer, isotopocule, Orbitrap, FT-ICR, electrospray, proteomics, metabolomics, lipidomics, clumped isotopes

Abstract

For a generation or more, the mass spectrometry that developed at the frontier of molecular biology was worlds apart from isotope ratio mass spectrometry, a label-free approach done on optimized gas-source magnetic sector instruments. Recent studies show that electrospray-ionization Orbitraps and other mass spectrometers widely used in the life sciences can be fine-tuned for high-precision isotope ratio analysis. Since isotope patterns form everywhere in nature based on well-understood principles, intramolecular isotope measurements allow unique insights into a fascinating range of research topics. This perspective introduces a wider readership to current topics in stable isotope research with the aim to discuss how soft-ionization mass spectrometry coupled with ultra-high mass resolution can enable long-envisioned progress. We highlight novel prospects of observing isotopes in intact polar compounds and speculate on future directions of this adventure into the overlapping realms of biology, chemistry, and geology.

Introduction

While isotopes of the same chemical element have nearly the same chemical reactivity, it is also true that they have noticeable physical differences. In many processes, isotope effects cause changes in isotope ratios – often called “isotope fingerprints” – that are characteristic and can be interpreted once quantified with enough precision. The study of stable isotope fingerprints is often overlooked by mass spectrometrists. It is an elegant scientific tool that yields insights into a fascinating range of fundamental research questions (Figure 1). In addition, the knowledge of isotopic patterns can be applied to address important practical and societal problems.

Earth scientists have long recognized that isotopes are a key to the secrets of our planet. For example, an anomaly in the relative abundance of sulfur isotopes in rocks provides strong evidence for an early anoxic atmosphere and allows dating of the evolution and rise of oxygenic photosynthesis (Figure 2).[1] Global warming is another prime example for a phenomenon that has been first identified by observing stable isotopes.[2] Long-term environmental isotope records in ice cores, tree rings, and sediments yield climate proxies that represent some of our best tools to infer changing interactions between the atmosphere, hydrosphere, lithosphere, and biosphere. For example, air trapped in glacier ice has been analyzed to determine past concentrations and isotopic composition of the greenhouse gases carbon dioxide, methane, and nitrous oxide.[3] Based on isotope records, past climate transitions can be linked to changes in the source of greenhouse gases and their effects on mean global temperature and sea level. The isotope data also show that emission levels of greenhouse gases since the industrial revolution have been unprecedented in the last

800,000 years, indicating that the present rise in the mean global temperature is caused by human activity.

Biogeochemists study the molecular mechanisms of life in natural habitats. Here, isotope fingerprints in organic molecules can reveal information about source materials, transformation mechanisms, and fluxes. This is because isotope fingerprints, being created and changed as a direct consequence of physical and (bio)chemical processes, are the result of reaction rate differences that lie at the heart of all natural transformations (Infobox I). Changes in isotope fingerprints can thus yield insights into chemical mechanisms. This has been useful to study the fate of organic pollutants, including petroleum products and chlorinated solvents.[4] In addition, isotope ratios can serve as fingerprints to trace fluxes from their sources through organisms, microbial communities, ecosystems, or between environmental compartments.[5] This aspect of isotope fingerprints has proven to be useful especially in ecology revealing trophic relationships in food webs, such as nutrient transfer between plants and the soil microbiome.[6] Via the study of fossils, the same concepts even enable insights into the diet and evolution of our earliest human ancestors that lived millions of years ago.[7]

Natural isotope patterns offer solutions for a range of practical problems. A notable topic is food fraud, which is a global issue in the food and beverage industry. Fraudsters substitute genuine ingredients with cheaper alternatives. Such activity may pose risk to human health or at least mislead consumers and undermine their trust. The botanical origin of ethanol is a classic exemplar of how understanding of intramolecular isotope patterns in metabolites has enabled the rational design of tests.[8] Ethanol in beverages inherits its isotopic content from hexoses,[9] whose carbon isotope composition in turn has been shaped by the CO_2 assimilation mechanism of the plant (Figure 3).[10] Similarly, forensic analysis of stable isotopes is a valuable tool to distinguish natural flavors from synthetic ones, to determine geographic sources of natural or semisynthetic drugs, to solve crime cases, to determine sources of explosive materials, and to reveal doping in sports.

These vignettes illustrate that exact observation of stable isotopes offers unique opportunities when it comes to studying life in a natural context. In the following sections, we will introduce well-established techniques to measure isotope ratios, with the aim of explaining their strengths and limitations. We will then explore how soft-ionization mass spectrometry can overcome certain fundamental technical limitations. Finally, we will discuss the potential of current developments to expand the utility of isotope analytics in the life sciences.

Glossary

IRMS: Isotope Ratio Mass Spectrometry. Traditionally done on gas-source magnetic sector instruments; increasingly possible on other MS systems.

BSIA: Bulk Stable Isotope Analysis. A whole leaf, soil, fingernail samples, or similar is analyzed for an isotope ratio of a chemical element.

CSIA: Compound-Specific Isotope Analysis is a branch of isotope analysis aimed at determining the isotopic composition of a particular chemical compound.

PSIA: Position-Specific Isotope Analysis is a branch of isotope analysis aimed at determining the isotopic composition of a particular atom position in a molecule.

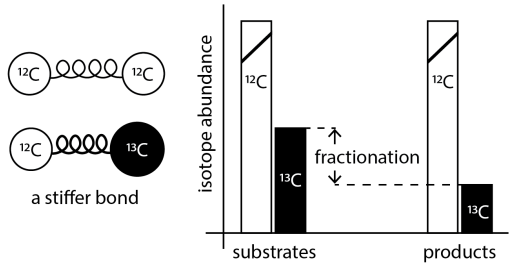
Isotopocules ("isotopically substituted molecules"): molecules of the same chemical compound that differ either in their isotopic sum formula or the structural configuration of isotopes; not defined by IUPAC yet.[11]

Isotopologues ("isotopic homologues"): molecules of the same chemical compound that differ in their isotopic sum formula. Isotopologues have distinct exact masses.[12]

Isotopomers ("isotopic isomers"): molecules of the same chemical compound that have the same isotopic sum formula but differ in the structural configuration of the isotopes. Isotopomers have the same exact mass.[12]

Clumped isotopes: a designation used in the geochemistry community for isotopocules with more than one heavier isotope (multiply substituted isotopocules).[13]

Infobox I: Isotope effects



Substitution of a light isotope by a heavier one in a chemical bond reduces the zero-point energy of the molecule (a measure of the vibrational energy that a molecule preserves at absolute zero, 0 K).[14] This brings along differences in physical and chemical properties of each isotopically distinct molecule (isotopocule). These isotope effects cause fractionation during reactions that leads to relative partitioning of the isotopes between products and substrates.

There are two main types of fractionation[15]:

1) ***equilibrium fractionation*** for systems at thermodynamic equilibrium

2) ***kinetic fractionation*** occurs during key rate-determining steps and is caused by a difference in activation energy for lighter and heavier isotopocules (usually lighter isotopocules react faster)

Infobox II: What are delta values?

To bring natural isotope variations to a scale that is easier to grasp and talk about, the δ notation has been introduced in the late 1940s (here expressed for carbon)[16]:

$$\delta^{13}\mathrm{C} = \frac{(\ ^{13}\mathrm{C}\ /\ \ ^{12}\mathrm{C})_{\mathrm{sample}}}{(\ ^{13}\mathrm{C}\ /\ \ ^{12}\mathrm{C})_{\mathrm{reference}}} - 1$$

By this classic convention:

1. Isotopes are measured as ***ratios***, rather than abundances (e.g., atomic percent), because not all isotopes may be experimentally observed.
2. Isotope ratios of samples are normalized relative to a ***reference standard*** to enable inter-laboratory comparisons.
3. The δ values are typically ***reported in permil*** (‰; i.e. raw values multiplied by 1,000), as natural isotopic variations are small.

For elements that have more than two stable isotopes, the relationship of several heavier-to-lighter isotope ratios should in theory be linear (mass-dependent). However, ***mass-independent fractionations*** (MIF) can be found under specific conditions. They are typically denoted as “cap delta” (see Δ^{33}S in Figure 2).[17]

Glass Ceilings Of Current Approaches

Before trying to measure isotope fingerprints on bioanalytical mass spectrometers, it is necessary to develop a sense for approaches that have proven to be effective in isotope analytics. This also helps to understand which technical limitations isotope experts currently face in their research.

Isotope analyzers take advantage of physical differences between isotopes and generally belong to one of three types:

- ***Isotope ratio mass spectrometry*** (IRMS) utilizes the differences in mass-to-charge ratios.[18]

- ***Laser absorption spectroscopy*** (LAS) resolves differences in molecular rotational-vibrational frequencies.[19]

- ***Isotopic nuclear magnetic resonance*** (isotopic NMR) relies on differences in magnetic spin.[20]

Each approach has distinct strengths and is implemented to ensure precision measurements of isotope fingerprints. For example, IRMS in general quantifies isotopes after conversion of analytes into gaseous molecules at very high accuracy and with good sensitivity. This is achieved by using an optimized introduction system for samples and references on a sector mass spectrometer. Commonly, IRMS combines ionization of volatile analytes (usually by electron impact) with a momentum separator (usually an electromagnet) and a set of ion detectors (usually Faraday cups).[18] An overview of currently available technologies for stable isotope analysis and their specifications is provided in Table 1.

Innovations in IRMS have been shaped by the needs of the stable isotope research community for robust precision measurements, leading to the development of strategies to ensure accurate data quality (Infobox III). Time-honored measurement principles and best practices are key for determining natural isotope fingerprints. They have been the basis for scientific progress on the isotopic information that can be measured and predicted ever more specific, revealing more closely the isotope fractionation processes as they occur in nature. A notable advance has been the transition from ***bulk*** stable isotope analysis of samples ("elemental analyzer" IRMS; BSIA) to ***compound-specific*** isotope analysis (CSIA) in the 1990s and further inroads to measure ***position-specific*** isotope analysis (PSIA; Figure 4). However, progress in isotope analytics has come to a state where adaptations that are rooted in traditional gas-source IRMS are facing barriers (e.g., insufficient mass resolution, upfront chemical preparation and transformation of the samples into a suitable form for the IRMS) that make it hard to address some of the most pressing research needs. A common theme across research disciplines is an aspiration of obtaining position-specific insights into polar analytes at low concentrations.

Infobox III: How to obtain reliable isotope ratios?

It is crucial for isotope researchers to be familiar with four time-honored measurement principles [21], thus being able to compare data from different days, different instruments, and/or different laboratories:

(1) ***Sample/reference comparisons:*** Many factors can negatively affect long-term stability of raw isotope ratios. To account for this, sample measurements are bracketed by analyses of reference standards with known isotope compositions (two-point calibration). Often, an additional working standard is analyzed in regular intervals to monitor and correct for drift in the data quality.

(2) ***Principle of identical treatment:*** Samples and reference materials should go through the same preparation steps and be analyzed under the same instrumental conditions (not always possible for complex matrices).

(3) ***Zero enrichment:*** Using the same material in sample/reference comparisons allows crucial insights even in the absence of suitable reference materials. All measured isotopocule signals are expected to yield a 0 ‰ difference (± experimental uncertainty).

(4) ***International reference materials:*** The highest level of reproducibility between different laboratories (and over long periods of time) is achieved by calibrating isotope ratios relative to a carefully selected and prepared community standard.

Table 1. **Currently available technologies for stable isotope analysis and their specifications.**

technology*	stable isotope analysis			sample			elements/ simultaneous multi-element analysis	reference
	bulk	position-specific	clumped isotopes	state	amount	purity / coupling		
Isotope Ratio Mass Spectrometry (IRMS)								
IRMS	yes	limited	some	solid/ liquid/gas	10 nmol - 10 µmol	HPLC / GC	many / no	[22]
HR-IRMS	yes	limited	yes	gas	tens of µmol	pure	many / no	[23]
ESI-Orbitrap	yes	yes	yes	liquid/gas	0.1-100 nmol	HPLC / GC	many / yes	[24]
Laser Absorption Spectroscopy (LAS)								
CRDS, TDLAS, QCLAS	yes	yes	yes	gas, volatile	< 10 µmol	as is / purified‡	many / yes	[25]
isotopic Nuclear Magnetic Resonance (NMR)								
NMR	no	yes	no	liquid/gas	0.1-10 mmol	pure	H, C, N, O / no	[20]

‡purified into a defined matrix in case of spectral interferences; possible coupling with automatic preconcentration units

*** HR-IRMS** – Ultra high-resolution isotope ratio mass spectrometry
ESI-Orbitrap MS – electrospray-ionization Orbitrap mass spectrometry
CRDS – cavity ring-down spectroscopy
TDLAS – tunable diode laser absorption spectroscopy
QCLAS – quantum cascade laser absorption spectroscopy

Biological metabolites. The use of isotope analytics in biological research is hindered by a legacy that stems from the beginnings of IRMS: if one wants to analyze isotopes in metabolites, the polar analyte needs to be broken down into a gaseous molecule, which is in turn analyzed for its isotopic content.[18] Sophisticated strategies have been implemented to accomplish this task for different biomolecules via combustion, pyrolysis, microbial, chemical, and enzymatic conversion. Often, CSIA can be achieved via the on-line coupling of IRMS with chromatographic separation by GC (interfaced via a pyrolysis or combustion tube) – a workhorse technology that has been continuously optimized.[26] For biological analytes that are not amenable to derivatization and GC separation, a LC coupling of gas-source IRMS has been developed that uses water or other carbon-free solvents during chromatography and a post-column oxidation to convert analytes into CO_2 (Figure 5). This system has been successfully applied in CSIA of volatile fatty acids and amino acids [27,28] but can even be modified to enable carbon PSIA of carboxyl groups in amino acids by using the ninhydrin reaction for on-line conversion of the analyte into CO_2.[29] Many more methods based on gas-source IRMS have been invented to improve the isotopic analysis in life science applications. Examples include high-temperature GC-IRMS, moving wire micro combustion, and microEA-IRMS (EA: elemental analysis).[30]

While these gas isotope measurements by IRMS and LAS are suitable for important research questions, they share a common fundamental limitation. The native structure of non-gaseous analytes is destroyed during the conversion step and structural (position-specific) information that may be most diagnostic of biological processes is lost. Isotopic NMR does measure position-specific isotope information in native polar compounds but has a much decreased sensitivity relative to MS. Also, the technique requires many milligrams of pure analyte, which is not suitable for most biological questions.[31]

Environmental pollutants. Sources and fate of anthropogenic contaminants in natural environments are vital input information to assess pollution risks. Often, a first-order question comes: is a pollutant of emerging concern degraded at all or does it simply get diluted over time? And if it is degraded, which biotic or abiotic mechanisms are involved? Such questions often pose a daunting task to be revealed by merely monitoring concentration dynamics of the contaminant in complex environmental compartments.[32] The use of isotopic variations, such as $^{13}C/^{12}C$, $^{2}H/^{1}H$, and $^{15}N/^{14}N$, within an organic contaminant is a powerful tool to reveal (bio)degradation over the course of its in-situ breakdown[33] or to assign its source origin.[34] CSIA approaches have been successful for (i) highly volatile contaminants such as chlorinated solvents that can be easily purged and concentrated on a solid phase; and (ii) heavily contaminated areas next to production sites (e.g., explosives) associated with high concentrations in the parts-per-million range. However, larger and less volatile contaminants

occurring in the parts-per-trillion range remain a major challenge for environmental chemists. First they are more difficult to extract from water. Second, they may be not sufficiently volatile to be amenable for GC-IRMS, yet not polar enough to be separated by LC-IRMS without organic solvents. Third, isotope effects during natural degradation are only manifested in the reactive bond(s) so that isotope changes in the compound average become smaller for analytes of larger molecular size.[35] Hence, for many contaminants that derive from pesticides, pharmaceuticals and consumer products isotope changes may become too small to be informative - calling for position-specific analysis - while major efforts are necessary to develop GC-IRMS analysis [33] - calling for new liquid injection-based approaches.

Performing CSIA of many organic micropollutants is hindered by the sample amounts required by current instrumentations (see Table 1). These are not possible to meet without extensive sample preparation. The necessary effort can be illustrated for the corrosion inhibitor 1*H*-benzotriazole. This omnipresent contaminant occurs in river water typically at concentration around 100 ng/L and requires enrichment by factors exceeding 10^5 times for accurate $\delta^{13}C$ and $\delta^{15}N$ measurements on GC/combustion/IRMS.[36] While extraction techniques are available for large samples using sorbents, the selectivity for commercially available sorbents is often insufficient. In other words, obtaining clean extracts that meet the stringent criteria of CSIA is not possible due to co-extraction of interfering natural organic matter. Therefore, extensive efforts have been invested in meticulous analytical procedures that validate extraction procedures from large samples,[37] synthesis and evaluation of selective sorbents,[36] preparative purification procedures,[38] and multidimensional chromatography and custom-made instrumentation.[39] All these efforts to overcome the shortcomings of the limited sensitivity of IRMS make CSIA approaches for newly emerging environmental contaminants increasingly tedious, cost-ineffective, and time consuming.

Biogeochemical cycles. Our understanding of Earth as an evolving living system owes much to isotope analytics. Isotope ratios have been widely used as a tool to search for evidence of early life in ancient rocks. Certain biological lipids can be preserved as hydrocarbons over geologic time and their isotope composition provide ever more specific information about the past biosphere, including mass extinction events and the emergence of new types of metabolism.[40] The sustained habitability of the planet over the eons has been shaped by the global cycling of chemical elements and concomitant formation of redox gradients. Isotopic studies of inorganic oxyanions, such as sulfate (SO_4^{2-}), nitrate (NO_3^-), and phosphate (PO_4^{3-}), are particularly informative because they reveal diagnostic signatures for distinct types of microbial metabolism that underpin the functioning of all ecosystems.

Although the reconstruction of NO_3^- and SO_4^{2-} isotope records from polar ice cores has been achieved by IRMS, required sample sizes are several tens of nanomoles for NO_3^- and several micromoles for SO_4^{2-}, which limit obtaining high-resolution records.[41] For small multi-elemental ions such as oxyanions, chemical transformations are especially problematic. They can lead to complex procedures, which add time, cost, and increase the required sample size. Alternatively, oxyanions can be introduced to the ion source of an inductively coupled plasma (ICP), where the analyte is dissociated prior to isotopic analysis of the element of interest (usually done by multi-collector inductively coupled plasma mass spectrometry; MC-ICP-MS).[42] Either way, isotopocule information is lost during bond breaking. This is particularly detrimental for the study of information contained in molecules with more than one isotopic substitution (referred to as "clumped isotopes").[43]

Forensic applications. Food and doping analysis laboratories are continually looking into improvements to increase their detection capabilities.[44] The competing aims of fraudsters and testing laboratories resemble an evolutionary arms race, and thereby foster diagnostic innovations and counter-adaptations. Isotope analysis offers a unique and valuable perspective. Sources of a compound that are otherwise identical tend to have a specific isotopic composition that reflects their production. In other words, metabolites administered from exogenous sources are often isotopically distinct from endogenous material. A prime example is the misuse of anabolic substances such as testosterone that can be administered in such a way as to obscure a positive doping test result. Based on initial longitudinal screening tests, suspicious samples are routinely analyzed by IRMS on androgen metabolites.[45]

Despite enormous efforts to reveal and control for biological variables such as genotype and diet, doping science is challenging because analytical tests need a very high single-test specificity (e.g., >99%) to have an acceptable false-positive rate. A key step to advance anti-doping testing could be to gain access to more specific tests. In the realm of isotope analytics, a desirable option is to achieve PSIA and standardize this information to metabolic precursors and degradation products. Increased sample throughput for IRMS is also beneficial, so that the false-positive rate of a diagnostic test can be known more confidently from the analysis of larger cohorts.[46] Advances of isotopocule analysis first pioneered in anti-doping analytics could be transferable to biological samples in clinical research.

This brief section on established isotope fingerprinting techniques and applications highlights technical challenges of IRMS that commonly occur across a diverse range of research questions:

- Missing access to position-specific isotope information because of degradation of the original analyte
- Specialized instrumentation needed for different analytes and/or chemical elements because limited mass range can be observed and resolved simultaneously
- Time required and sample throughput when analytes cannot be introduced directly, but need to be modified (e.g., derivatized, converted)

Novel Approaches Using Bioanalytical Mass Spectrometers

While the sample ionization dictates the scope and utility of any MS experiment up front by restricting which classes of substances are available for analysis, it is actually the combination of the ionization technique with a mass analyzer and detector that ultimately determines the quality and reliability of the measurement. Since the rise of soft-ionization techniques, the development of mass analyzers and detectors has run largely independent of improvements of the magnetic sectors used for IRMS. However, there have been large analytical gains in mass analyzers that are widely used in bioanalytical research. This includes improvements in figures of merit that are important for isotope ratio measurements, most notably abundance sensitivity, linear dynamic range, speed and mass resolving power [47,48]. With dedicated efforts, it is now becoming possible to reconcile these two areas of mass spectrometry.

Depending on the physics of mass analysis, analyzers can be divided into quadrupole, magnetic sector, ion trap, time-of-flight (TOF), and Fourier transform mass spectrometers (FTMS). Among FTMS instruments, the most notable are ion cyclotron resonance (FT-ICR) and the Orbitrap mass analyzers. These components are often further combined to allow workflows that select analytes and create molecular fragments (MS/MS or MS^2), for example, for metabolite identification using quadrupole-TOF MS/MS experiments. The design of bioanalytical ESI-MS systems has been shaped by the need for increased resolution and speed, and better ratiometric measurements. The most notable drivers towards improved isotope quantifications are: 1) chemical labeling strategies in proteomics that utilize stable isotopes to allow sample multiplexing [49]; 2) improved assignment of chemical sum formulas based on isotopic fine structure [50]; 3) more accurate concentration determinations by isotope dilution [51]; and 4) the precise quantification of low levels of isotopic tracers.[52] It has been speculated that these advances could eventually close the gap to gas-source IRMS that is classically done on magnetic sectors.[50,53]

Technological advances can be synergistic and it is thus timely to ask to what degree bioanalytical mass spectrometers can be repurposed to study natural isotope fingerprints.

For isotope researchers, this is an attractive new strategy. It empowers the field to study isotopes in intact polar molecules that could not be directly analyzed before, and collaboration with the wider mass spectrometry community could propel research on isotope fingerprints into the essentially unexplored world of "isotopocules". This field has first been entered by atmospheric chemists.[11]

Opening access to natural isotope patterns by ESI-MS

Advances in ESI-MS have inspired several protocols for isotopic label quantifications. For instance, isotope pattern matching (IPM) is used in proteomics to measure incorporation of stable C and N isotopes [54,55], or to determine the elemental composition of a peptide.[56] Occasionally, studies explore the use of TOF and Orbitrap LC-MS systems for quantification of low levels of deuterium incorporation during lipid biosynthesis.[57,58] Systematic work towards lowering the quantification limit into the range of natural-level isotopic variation is worthwhile for another reason. Label-free ESI-MS could become a step change for IRMS because it bypasses laborious and partially destructive sample preparation steps that are required otherwise.[57]

In recent years, this niche area of mass spectrometry received a boost of confidence when targeted exploratory tests showed that, with appropriate instrument settings and data analysis, the combination of ESI and Orbitrap mass analyzers is in principle capable of providing access to isotopic measurements at the level of natural variations.[59,60] These benchmarking tests can be extended to evaluate the utility of other soft-ionization MS systems (APCI, DESI, MALDI) coupled to Orbitraps or other mass analyzers such as quadrupole TOF and FT-ICR. Advancements of this research area face a practical dilemma, however, since essentially all components of bioanalytical MS systems differ from well-established and proven IRMS workflows. Progress to date has additionally been hindered because IRMS expertise is fragmented across diverse research areas that usually have little overlap with relevant areas of mass spectrometry such as proteomics.

Untargeted molecular surveys of isotope contents FTMS

Due to its unparalleled high mass resolving power, FT-ICR-MS has become the tool of choice for the study of the most chemically complex natural samples. "Inseparable mixtures", such as dissolved organic matter (DOM) extracted from ocean water, requires very high mass accuracy of <0.1 ppm for molecular formula attribution. This is particularly challenging because the natural samples consist of at least hundreds of thousands if not millions of different substances.[61] Recent work indicates that random *m/z* errors can be eliminated and mass accuracies of 0.01 ppm be achieved by averaging mass spectra from independent environmental samples.[62] Interestingly, averaging also improved intensity ratios of

isotopologues to a degree where measured deviations were within the range of natural isotope fractionation effects.[62]

Measuring isotopes in complex samples comes with new challenges. For example, non-traditional standardization becomes necessary. In analogy to $\delta^{13}C$, a ^{13}C abundance relative to a calculated isotopologue abundance ($\Delta^{13}C$) has been proposed.[62] This "top-down" isotopocule analysis may be extended to Orbitrap FTMS. It has conceptual similarities for efforts in labeling studies that aim at improving spectral accuracy (i.e., accuracy in measuring the abundances of isotopic peaks).[63] The directness of the top-down approach for CSIA makes it potentially very useful to survey isotopic fingerprints across diverse compound classes. With continued improvements the approach could find use in many sample types and topics (e.g., atmospheric aerosols, dietary fingerprints).

Targeted isotopocule analysis

The analysis of purified compounds by direct infusion is a well-controlled starting point to develop measurements of isotope fingerprints by FTMS. Often, isotopic reference materials for benchmarking experiments can either be obtained or generated relatively easily (e.g., using an elemental analyzer to measure $\delta^{13}C$). By now, optimized infusion systems for isotopic analysis have been devised based on workflows established in classic IRMS.[24] These include a "*dual inlet*" system consisting of two syringes and a software-controlled switching valve to enable very stable infusion of reference materials and a sample (Figure 6). For higher throughput, the autosampler of an ultra-performance liquid chromatography (UPLC) system can be repurposed to enable automated "*flow injection*" that enables continuous electrospray in between sample injections. In combination with software used to extract ion counts, these setups can be employed to systematically study the performance of isotopic measurements for different analytes, sample types, instrumental settings, and how to cope with adverse conditions such as matrix effects.

The precision of isotope ratio measurements is fundamentally limited by the number of ions that can be observed in the two isotopocules that are used to calculate a ratio (shot-noise limit). The way in which to convert signal-to-noise into ion counts has been studied for Orbitrap mass analyzers.[59,64] However, more work will be needed to firmly establish shot-noise and thermal-noise estimates at the fundamental level. With appropriate adjustments to the sample introduction and data acquisition, mass spectra without fragmentation (MS^1) close to limits from counting statistics can be collected for many intact polar analytes. NO_3^- and SO_4^{2-} isotopic reference materials that can directly be measured by IRMS and ESI-Orbitrap provide the strongest indication to date that highly accurate natural level isotopic analysis on ESI-Orbitrap instruments is possible.[59,60,65] It would be valuable to compare the

isotopic accuracy of ESI in combination with other modern mass analyzers. Unfortunately, we are not aware that a rigorous analysis has yet been done on current TOF and FT-ICR systems.

Since in ESI-Orbitrap, both ionization and ion detection are different from the classical gas-source IRMS, the new platform offers access to polar analytes at high sensitivity. Initial publications on model compounds such as oxyanions, amino acids, and organic acids demonstrate improved sensitivity in the nanomolar range.[24,66] They also highlight other promising methodological advances, such as CSIA with simultaneous measurements of isotopes of several elements.

Intramolecular isotopocule analysis

Major innovations in bioanalytical mass spectrometry over the last decade have enabled the implementation of new MS data acquisition workflows (e.g., SWATH, BoxCar). Since ESI delivers intact polar solutes for subsequent isotope analysis by MS, similar opportunities exist to develop procedures that take advantage of instrumental strengths, while minimizing physical or instrumental trade-offs. This could be particularly useful for intramolecular isotopic analysis, which has been challenging to do by MS for the vast majority of analytes.

A first workflow has been demonstrated for methionine. It uses a narrow initial mass selection window to isolate intact molecular ions that are heavier due to the presence of one or more isotopic substitutions, followed by fragmentation (MS/MS).[60] The basic theory for predicting isotopic distributions resulting from the dissociation of individual isotopic peaks has been developed and can, in principle, be extended to MS^n.[67] These MS experiments are useful for structural studies, albeit relatively little utilized. Combined with precision measurements of isotopic abundances, they provide several interesting features. First of all, for many small metabolites, the isotopic distribution is dominated by monoisotopic ions. Isolating ions that have one or more of the rare isotopes effectively results in an isotopic enrichment that can speed up ratio analysis. Secondly, a small mass window selecting one nominal mass allows a well-defined subset of ions to pass, which adds specificity for the subsequent MS^2 analysis. At the same time, the quadrupole would create only small isotopic fractionation because the passing ions are near-isobaric (*m/z* typically differs by <10 mDa).

These intramolecular isotope studies currently require relatively pure analyte preparations (e.g., >90% of the ions entering the Orbitrap with a given quadrupole setting). This provides an opportunity to systematically work towards better understanding and refining all components of mass spectrometry systems for precision isotopocule analysis, from ionization sources to detectors and signal processing. It is important to point out that intramolecular isotope patterns should be most representative of isotope fractionation effects as they occur in biological systems. Technical advances to make them measurable in robust

and specific ways can have many applications. For amino acids in humans, for example, applications can range from insights into the origins of the early hominins[68], to biomedical research dissecting human/diet/microbiome interactions[69], and pinpointing aberrations in cancer metabolism [70].

What May Become Possible

Natural isotope fingerprints require predictions of where to carefully look for informative patterns. Isotope analysis thus mostly involves hypothesis-driven and highly targeted observations. Using isotopes as "thinking tools" can be uniquely powerful to interrogate biological systems across all scales, from elucidating enzymatic reactions in a test tube to quantifying the global elemental cycles in the ocean. Like a common currency, isotopes move freely between living and nonliving forms and thus are the universal tool to study life in the context of its natural environment.

Based on recent promising benchmarking studies, we expect that isotope fingerprints have great potential to complement current capabilities in metabolomics and proteomics, as well as to advance existing areas of IRMS that have reached limitations of the gas-source magnetic sectors. Natural stable isotope analysis is a high precision game that requires several orders of magnitude higher measurement precision than labeling experiments. In addition to artifacts caused by data acquisition and analysis (Infobox III), confounding factors throughout the experiment need to be controlled for or corrected).

The future utility of IRMS on bioanalytical mass spectrometers, such as soft-ionization Orbitrap and TOF instruments, therefore is going to depend on new methods being critically examined to understand their limitations and risk for artifacts. While the sources of artifacts in traditional isotope ratio analysis are well understood, the same cannot be said for this emerging area of isotope ratio analysis. This includes diverse factors that can alter the observed isotope distribution during sample preparation, ionization, mass analysis and detection as well as signal processing.[71] Important factors that need to be studied and controlled for are:

- Linear dynamic range of an instrument for specific isotope ratio analyses [47,71]
- Isotopic exchange or fractionation during sample preparation (e.g., in reactions with solvents, chemical derivatization) [72]
- Near-isobaric impurities that may overlap with the desired *m/z* values of the analyte [73]
- Isotopic fractionation associated with (liquid) chromatography [74]
- Kinetic isotope effects and changes in the precursor and product ions population in fragmentation reactions that can influence the isotope signatures [75]

If remaining technical issues can be adequately addressed, we may be seeing applications of isotopic techniques that have long been envisioned by isotope researchers.

IRMS in metabolomics. Metabolic flux analysis is successfully used to study the incorporation of ^{13}C from position-specific labeled substrates into biosynthetic products during microbial activity in order to elucidate metabolic pathways and associated fluxes.[76] In general, position-specific stable isotope probing (SIP) overcomes the limitation of uniformly labeled substrates, which impede the discrimination of the individual isotopic nature of each C in a given molecule. Using position specific-labeled substrates, the metabolic fate of amino acids and carbohydrates in marine and terrestrial environments has been reconstructed.[77–79] With regard to the different oxidation states of C in organic molecules, a preferential incorporation of particular C positions into biomass and discrimination of others has been observed. The position-specific incorporation is a direct consequence of the flux patterns of the central C metabolic network, which appears remarkably similar across all domains of life.[80] Metabolic flux analysis and modeling to determine intracellular flux patterns have been widely used in metabolic engineering, biotechnology, microbiology, and the environment.[79–81] It is conceivable that some of the insights gained from metabolic flux analysis can increasingly be complemented with observational studies, by taking advantage of "natural labeling" that creates isotopic fingerprints.

In metabolism studies, it is often desirable to quantify several analytes in a single analysis, and some trade-off with regards to isotopic accuracy may be acceptable. Initial attempts to couple LC with ESI-Orbitrap for IRMS are promising.[82] This approach is particularly attractive for biological samples from systems that cannot be controlled in a laboratory and in which labeling is prone to artifacts, which are often associated with biological isotope effects. For example, in human nutrition and clinical research, the investigation of unlabeled studies is particularly attractive. Suitable sample collections may already exist and natural-level isotope measurements could bypass the need for time-intensive and costly labeling studies in new cohorts.

IRMS in proteomics. There has been exciting progress in measuring isotopic label in amino acid fragments to measure protein turnover from labeled peptides.[83,84] The quantification of isotopes in immonium ions (protonated amino acid residues minus the carbonyl group), has recently been extended far beyond labeling studies into the natural isotopic abundance range.[85] This achievement is remarkable for two main reasons. Firstly, immonium isotopic analysis is compatible with established proteomics workflows. Secondly, isotope fingerprints in peptides are fundamentally important because they provide one of the few options that exist to make direct observations that connect metabolic activity with genetic information.

Assuming that important technical aspects can be worked out, the immonium isotopocules can open entirely new areas of applications for isotope fingerprinting. It opens a route for a large number of specialized proteomics laboratories in academia and industry to partake in cutting edge research of isotope fingerprints. By adding isotopic patterns as a new dimension to proteomics research we may be able to get a fresh perspective on established research topics by measuring isotopic fingerprints in amino acids from peptides. It is conceivable that one day proteomics laboratories could start exploring natural-level isotopic information by simply loading a different method file when starting a sample sequence.

Opportunities in established areas of IRMS. The described advances in isotope analytics have the potential to change how currently established areas of IRMS approach their research questions. This has been the case before with the development of laser spectroscopy systems for isotope analysis, finding numerous applications for greenhouse gas emissions and water isotopes.

ESI-Orbitrap and other bioanalytical MS instruments for precision isotope measurements can transform the way in which fossils are analyzed. Generally, CSIA provides information on food webs in modern ecology research, but it may become particularly useful in archaeology. CSIA has been applied to Neanderthal samples (<100,000 years old), but not to older samples. Chemical evidence exists that amino acids are preserved in fossils that are much older than that, providing a window into dietary studies way back to the earliest human ancestors (up to 4 million years old) and perhaps all of mammal evolution. Similar analytical protocols can find use in clinical samples, for example in nutrition research to develop biomarkers for diet intake and lifestyle.

Direct analysis of isotopocules of oxyanions (e.g., NO_3^-, SO_4^{2-}, PO_4^{3-}) can provide a welcome opportunity to revisit longstanding debates in our understanding of biogeochemical cycles of the elements in modern and ancient ecosystems. Simultaneous measurements of several oxyanions and their multiple isotopes may present exciting opportunities to obtain a critical understanding of the natural coupling of nitrogen, sulfur and carbon cycles, which are mostly studied separately but they are tightly interdependent in nature.

In environmental pollutant research, additional information from more elements and specific molecular positions would tremendously increase our capability of tracing contaminant sources, and of characterizing their environmental transformation. Identifying reactive sites in pollutant molecules from position-specific isotope fractionation would be a key step to pinpointing transformation pathways of natural attenuation. Position-specific isotope fractionation would also facilitate a sensitive detection of degradation-associated isotope

fractionation circumventing dilution in the molecular average. Non-reactive positions, in turn, may remain unchanged and preserve their original signature to enable source fingerprinting.

In enzymology, classic use of enzymatic isotope effects can be revisited with label-free isotopocule monitoring in reactions to test hypotheses and develop a more rigorous quantum-chemical understanding of enzyme mechanisms, for example with mutated or resurrected ancestral protein sequences. This knowledge could be translated to human studies of drug metabolism in pharmacology (ADME) without the need for labeled substances. In diagnostics, more specific tests may become attainable, ideally based on rational test design that is less limited by technical capabilities of IRMS to measure certain atomic positions. This may provide a path to design tests to differentiate organic from conventionally farmed foods – an area lucrative for fraudsters in the absence of established authenticity testing.

How could the envisioned technical synergies become a game changer for various scientific disciplines?

Today's soft-ionization mass spectrometers are a versatile tool to identify, quantify and spatially resolve molecules. The time could be ripe to add natural isotope fingerprints to this list. Isotopes bring an extra layer of anatomic (structural) and genetic (historical) information to our understanding of chemical, biological and geological processes. Intramolecular isotope research done to date provides only a first glimpse of a largely unexplored natural abundance isotopocule landscape. The adaptation of bioanalytical mass spectrometry to quantify isotopocules precisely is opening up this landscape across all the sciences.

What does variance in natural isotopocule abundances tell us about molecular pathways in human health and disease? What information about the lives and evolution of the earliest human ancestors could still be preserved in fossils? Are there hidden records in geological sediments that tell us new insights into the ancient past of how life and Earth co-evolved over billions of years? Is there a quantitative way to determine whether a chemical has been made by a living organism? These are some of the questions that could soon be addressed systematically with emerging isotope ratio analysis. The most interesting questions, however, are likely those raised by newcomers; researchers that are willing to venture into isotope analytics with an open mind, taking new approaches to discover questions no one has yet been able to ask.

Key takeaways:

- The workhorse precision isotope ratio technology (gas-source IRMS) has diverse limitations.

- For polar organic and inorganic molecules, sample introduction and requisite conversion to gases prior to analysis limit access to original molecular information.
- ESI-MS of intact analytes at the natural abundance level opens a new isotopic landscape across all sciences, including multi-dimensional and intramolecular isotopic fingerprints.
- In biomedical sciences, it provides an additional methodology for important mechanistic studies in humans which are not accessible with currently used methods.
- Navigating this maturing technology and its workflows to extract the maximum for effective use needs cross-disciplinary dialogue and user community engagement.

Acknowledgements

The authors thank Doris Meyerdierks for hosting our workshop at the Hanse-Wissenschaftskolleg. The authors' research on stable isotope fingerprints is supported by the following funding programs. C.N.: U.S. National Science Foundation (#2041539); K.K.: Swiss National Science Foundation (project number P500PN_206702); S.H. acknowledges start-up funding from Nanjing University, J.S.: LabEx OSUG@2020 ("Investissements d'avenir" – ANR10 LABX56), the ANR project ANR-15-IDEX-02 (IDEX-UGA), the IceMemory Foundation, the European Union (ERC DOC-PAST grant 101054558). Views and opinions expressed are however those of the authors only and do not necessarily reflect those of the European Union or the Research Council Executive Agency. Neither the European Union nor the granting authority can be held responsible for them.

References

(1) Ono, S. Photochemistry of Sulfur Dioxide and the Origin of Mass-Independent Isotope Fractionation in Earth's Atmosphere. *Annu. Rev. Earth Planet. Sci.* **2017**, *45* (1), 301–329.
(2) Broecker, W. S. Climatic Change: Are We on the Brink of a Pronounced Global Warming? *Science* **1975**, *189* (4201), 460–463.
(3) Hoefs, J.; Harmon, R. The Earth's Atmosphere--A Stable Isotope Perspective and Review. *Appl. Geochem.* **2022**, 105355.
(4) Vogt, C.; Dorer, C.; Musat, F.; Richnow, H.-H. Multi-Element Isotope Fractionation Concepts to Characterize the Biodegradation of Hydrocarbons - from Enzymes to the Environment. *Curr. Opin. Biotechnol.* **2016**, *41*, 90–98.
(5) Fry, B. *Stable Isotope Ecology*; Springer, New York, NY, 2006.
(6) Giesemann, P.; Rasmussen, H. N.; Liebel, H. T.; Gebauer, G. Discreet Heterotrophs: Green Plants That Receive Fungal Carbon through Paris-Type Arbuscular Mycorrhiza. *New Phytol.* **2020**, *226* (4), 960–966.
(7) Wynn, J. G.; Alemseged, Z.; Bobe, R.; Grine, F. E.; Negash, E. W.; Sponheimer, M. Isotopic Evidence for the Timing of the Dietary Shift toward C_4 Foods in Eastern African

Paranthropus. *Proc. Natl. Acad. Sci. U. S. A.* **2020**, *117* (36), 21978–21984.

(8) Thomas, F.; Randet, C.; Gilbert, A.; Silvestre, V.; Jamin, E.; Akoka, S.; Remaud, G.; Segebarth, N.; Guillou, C. Improved Characterization of the Botanical Origin of Sugar by Carbon-13 SNIF-NMR Applied to Ethanol. *J. Agric. Food Chem.* **2010**, *58* (22), 11580–11585.

(9) Gilbert, A.; Silvestre, V.; Robins, R. J.; Tcherkez, G.; Remaud, G. S. A ^{13}C NMR Spectrometric Method for the Determination of Intramolecular $\delta^{13}C$ Values in Fructose from Plant Sucrose Samples. *New Phytol.* **2011**, *191* (2), 579–588.

(10) Gilbert, A.; Robins, R. J.; Remaud, G. S.; Tcherkez, G. G. B. Intramolecular ^{13}C Pattern in Hexoses from Autotrophic and Heterotrophic C_3 Plant Tissues. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109* (44), 18204–18209.

(11) Kaiser, J.; Röckmann, T. Correction of Mass Spectrometric Isotope Ratio Measurements for Isobaric Isotopologues of O_2, CO, CO_2, N_2 O and SO_2. *Rapid Communications in Mass Spectrometry*. 2008, pp 3997–4008.

(12) Murray, K. K.; Boyd, R. K.; Eberlin, M. N.; Langley, G. J.; Li, L.; Naito, Y. Definitions of Terms Relating to Mass Spectrometry (IUPAC Recommendations 2013). *Pure Appl. Chem.* **2013**, *85* (7), 1515–1609.

(13) Eiler, J. M. "Clumped-Isotope" geochemistry—The Study of Naturally-Occurring, Multiply-Substituted Isotopologues. *Earth Planet. Sci. Lett.* **2007**, *262* (3-4), 309–327.

(14) Urey, H. C. The Thermodynamic Properties of Isotopic Substances. *J. Chem. Soc.* **1947**, 562–581.

(15) Hoefs, J. Theoretical and Experimental Principles. In *Stable Isotope Geochemistry*; Hoefs, J., Ed.; Springer International Publishing: Cham, 2021; pp 1–48.

(16) Coplen, T. B. Guidelines and Recommended Terms for Expression of Stable-Isotope-Ratio and Gas-Ratio Measurement Results. *Rapid Commun. Mass Spectrom.* **2011**, *25* (17), 2538–2560.

(17) Savarino, J.; Romero, A.; Cole-Dai, J.; Bekki, S.; Thiemens, M. H. UV Induced Mass-Independent Sulfur Isotope Fractionation in Stratospheric Volcanic Sulfate. *Geophysical Research Letters* **2003**, *30* (21).

(18) Brand, W. A.; Douthitt, C. B.; Fourel, F.; Maia, R.; Rodrigues, C.; Maguas, C.; Prohaska, T. Gas Source Isotope Ratio Mass Spectrometry (IRMS). In *Sector Field Mass Spectrometry for Elemental and Isotopic Analysis*; Prohaska, T., Irrgeher, J., Zitek, A., Jakubowski, N., Eds.; 2015; pp 500–549.

(19) Michael Hollas, J. *Modern Spectroscopy*; John Wiley & Sons, 2004.

(20) Akoka, S.; Remaud, G. S. NMR-Based Isotopic and Isotopomic Analysis. *Prog. Nucl. Magn. Reson. Spectrosc.* **2020**, *120-121*, 1–24.

(21) Meier-Augenstein, W.; Schimmelmann, A. A Guide for Proper Utilisation of Stable Isotope Reference Materials. *Isotopes Environ. Health Stud.* **2019**, *55* (2), 113–128.

(22) Hayes, J. M.; Freeman, K. H.; Popp, B. N.; Hoham, C. H. Compound-Specific Isotopic Analyses: A Novel Tool for Reconstruction of Ancient Biogeochemical Processes. *Org. Geochem.* **1990**, *16* (4-6), 1115–1128.

(23) Eiler, J. M.; Clog, M.; Magyar, P.; Piasecki, A.; Sessions, A.; Stolper, D.; Deerberg, M.; Schlueter, H.-J.; Schwieters, J. A High-Resolution Gas-Source Isotope Ratio Mass Spectrometer. *Int. J. Mass Spectrom.* **2013**, *335*, 45–56.

(24) Hilkert, A.; Böhlke, J. K.; Mroczkowski, S. J.; Fort, K. L.; Aizikov, K.; Wang, X. T.; Kopf, S. H.; Neubauer, C. Exploring the Potential of Electrospray-Orbitrap for Stable Isotope Analysis Using Nitrate as a Model. *Anal. Chem.* **2021**, *93* (26), 9139–9148.

(25) Ostrom, N. E.; Ostrom, P. H. Mining the Isotopic Complexity of Nitrous Oxide: A Review of Challenges and Opportunities. *Biogeochemistry* **2017**, *132* (3), 359–372.

(26) Baczynski, A. A.; Polissar, P. J.; Juchelka, D.; Schwieters, J.; Hilkert, A.; Summons, R. E.; Freeman, K. H. Picomolar-Scale Compound-Specific Isotope Analyses. *Rapid Commun. Mass Spectrom.* **2018**, *32* (9), 730–738.

(27) Heuer, V. B.; Pohlman, J. W.; Torres, M. E.; Elvert, M.; Hinrichs, K.-U. The Stable Carbon Isotope Biogeochemistry of Acetate and Other Dissolved Carbon Species in Deep Subseafloor Sediments at the Northern Cascadia Margin. *Geochim. Cosmochim. Acta* **2009**, *73* (11), 3323–3336.

(28) McCullagh, J. S. O.; Juchelka, D.; Hedges, R. E. M. Analysis of Amino Acid ^{13}C Abundance from Human and Faunal Bone Collagen Using Liquid Chromatography/isotope Ratio Mass Spectrometry. *Rapid Commun. Mass Spectrom.* **2006**, *20* (18), 2761–2768.

(29) 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.* **2018**, *32* (12), 992–1000.

(30) Elsner, M.; Jochmann, M. A.; Hofstetter, T. B.; Hunkeler, D.; Bernstein, A.; Schmidt, T. C.; Schimmelmann, A. Current Challenges in Compound-Specific Stable Isotope Analysis of Environmental Organic Contaminants. *Anal. Bioanal. Chem.* **2012**, *403* (9), 2471–2491.

(31) Jézéquel, T.; Joubert, V.; Giraudeau, P.; Remaud, G. S.; Akoka, S. The New Face of Isotopic NMR at Natural Abundance. *Magn. Reson. Chem.* **2017**, *55* (2), 77–90.

(32) Fenner, K.; Canonica, S.; Wackett, L. P.; Elsner, M. Evaluating Pesticide Degradation in the Environment: Blind Spots and Emerging Opportunities. *Science* **2013**, *341* (6147), 752–758.

(33) Elsner, M.; Imfeld, G. Compound-Specific Isotope Analysis (CSIA) of Micropollutants in the Environment - Current Developments and Future Challenges. *Curr. Opin. Biotechnol.* **2016**, *41*, 60–72.

(34) Ivdra, N.; Fischer, A.; Herrero-Martin, S.; Giunta, T.; Bonifacie, M.; Richnow, H.-H. Carbon, Hydrogen and Chlorine Stable Isotope Fingerprinting for Forensic Investigations of Hexachlorocyclohexanes. *Environ. Sci. Technol.* **2017**, *51* (1), 446–454.

(35) Elsner, M.; Zwank, L.; Hunkeler, D.; Schwarzenbach, R. P. A New Concept Linking Observable Stable Isotope Fractionation to Transformation Pathways of Organic Pollutants. *Environ. Sci. Technol.* **2005**, *39* (18), 6896–6916.

(36) Bakkour, R.; Bolotin, J.; Sellergren, B.; Hofstetter, T. B. Molecularly Imprinted Polymers for Compound-Specific Isotope Analysis of Polar Organic Micropollutants in Aquatic Environments. *Anal. Chem.* **2018**, *90* (12), 7292–7301.

(37) Torrentó, C.; Bakkour, R.; Glauser, G.; Melsbach, A.; Ponsin, V.; Hofstetter, T. B.; Elsner, M.; Hunkeler, D. Solid-Phase Extraction Method for Stable Isotope Analysis of Pesticides from Large Volume Environmental Water Samples. *The Analyst*. 2019, pp 2898–2908.

(38) Schreglmann, K.; Hoeche, M.; Steinbeiss, S.; Reinnicke, S.; Elsner, M. Carbon and Nitrogen Isotope Analysis of Atrazine and Desethylatrazine at Sub-Microgram per Liter Concentrations in Groundwater. *Anal. Bioanal. Chem.* **2013**, *405* (9), 2857–2867.

(39) Brailsford, A. D.; Gavrilović, I.; Ansell, R. J.; Cowan, D. A.; Kicman, A. T. Two-Dimensional Gas Chromatography with Heart-Cutting for Isotope Ratio Mass Spectrometry Analysis of Steroids in Doping Control. *Drug Test. Anal.* **2012**, *4* (12), 962–969.

(40) Gueneli, N.; McKenna, A. C.; Ohkouchi; Boreham; Beghin, J.; Javaux, E.; Brocks, J. 1.1 Billion Years Old Porphyrins and Their Isotopic Composition Establish a Marine Ecosystem Dominated by Bacterial Primary Producers. *Proc. Natl. Acad. Sci. U. S. A.* **2018**.

(41) Hattori, S.; Iizuka, Y.; Alexander, B.; Ishino, S.; Fujita, K.; Zhai, S.; Sherwen, T.; Oshima, N.; Uemura, R.; Yamada, A.; Suzuki, N.; Matoba, S.; Tsuruta, A.; Savarino, J.; Yoshida, N. Isotopic Evidence for Acidity-Driven Enhancement of Sulfate Formation after SO_2 Emission Control. *Sci Adv* **2021**, *7* (19).

(42) Burke, A.; Moore, K. A.; Sigl, M.; Nita, D. C.; McConnell, J. R.; Adkins, J. F. Stratospheric Eruptions from Tropical and Extra-Tropical Volcanoes Constrained Using High-Resolution Sulfur Isotopes in Ice Cores. *Earth Planet. Sci. Lett.* **2019**, *521*, 113–119.

(43) Eiler, J. M. The Isotopic Anatomies of Molecules and Minerals. *Annu. Rev. Earth Planet. Sci.* **2013**, *41* (1), 411–441.

(44) Thevis, M.; Kuuranne, T.; Geyer, H. Annual Banned-Substance Review - Analytical Approaches in Human Sports Drug Testing. *Drug Test. Anal.* **2020**, *12* (1), 7–26.

(45) 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.* **2021**, *413* (22), 5655–5667.

(46) Tobias, H. J.; Brenna, J. T. Cryofocus Fast Gas Chromatography Combustion Isotope Ratio Mass Spectrometry for Rapid Detection of Synthetic Steroid Use in Sport Doping. *Analyst* **2018**, *143* (5), 1124–1132.

(47) McLuckey, S. A.; Wells, J. M. Mass Analysis at the Advent of the 21st Century. *Chem. Rev.* **2001**, *101* (2), 571–606.

(48) Hoegg, E. D.; Barinaga, C. J.; Hager, G. J.; Hart, G. L.; Koppenaal, D. W.; Marcus, R. K. Preliminary Figures of Merit for Isotope Ratio Measurements: The Liquid Sampling-Atmospheric Pressure Glow Discharge Microplasma Ionization Source Coupled to an Orbitrap Mass Analyzer. *J. Am. Soc. Mass Spectrom.* **2016**, *27* (8), 1393–1403.

(49) Pappireddi, N.; Martin, L.; Wühr, M. A Review on Quantitative Multiplexed Proteomics. *Chembiochem* **2019**, *20* (10), 1210–1224.

(50) Knolhoff, A. M.; Callahan, J. H.; Croley, T. R. Mass Accuracy and Isotopic Abundance Measurements for HR-MS Instrumentation: Capabilities for Non-Targeted Analyses. *J. Am. Soc. Mass Spectrom.* **2014**, *25* (7), 1285–1294.

(51) Gu, H.; Zhao, Y.; DeMichele, M.; Zheng, N.; Zhang, Y. J.; Pillutla, R.; Zeng, J. Eliminating Preparation of Multisample External Calibration Curves and Dilution of Study Samples Using the Multiple Isotopologue Reaction Monitoring (MIRM) Technique in Quantitative LC-MS/MS Bioanalysis. *Anal. Chem.* **2019**, *91* (13), 8652–8659.

(52) Herath, K. B.; Zhong, W.; Yang, J.; Mahsut, A.; Rohm, R. J.; Shah, V.; Castro-Perez, J.; Zhou, H.; Attygalle, A. B.; Kang, L.; Others. Determination of Low Levels of ^{2}H-Labeling Using High-Resolution Mass Spectrometry: Application in Studies of Lipid Flux and beyond. *Rapid Commun. Mass Spectrom.* **2014**, *28* (3), 239–244.

(53) Zubarev, R. A.; Makarov, A. Orbitrap Mass Spectrometry. *Anal. Chem.* **2013**, *85* (11), 5288–5296.

(54) Taubert, M.; Baumann, S.; von Bergen, M.; Seifert, J. Exploring the Limits of Robust Detection of Incorporation of ^{13}C by Mass Spectrometry in Protein-Based Stable Isotope Probing (protein-SIP). *Anal. Bioanal. Chem.* **2011**, *401* (6), 1975–1982.

(55) Sachsenberg, T.; Herbst, F.-A.; Taubert, M.; Kermer, R.; Jehmlich, N.; von Bergen, M.; Seifert, J.; Kohlbacher, O. MetaProSIP: Automated Inference of Stable Isotope Incorporation Rates in Proteins for Functional Metaproteomics. *J. Proteome Res.* **2015**, *14* (2), 619–627.

(56) Snijders, A. P. L.; de Koning, B.; Wright, P. C. Perturbation and Interpretation of Nitrogen Isotope Distribution Patterns in Proteomics. *J. Proteome Res.* **2005**, *4* (6), 2185–2191.

(57) Fischer, C. R.; Bowen, B. P.; Pan, C.; Northen, T. R.; Banfield, J. F. Stable-Isotope Probing Reveals That Hydrogen Isotope Fractionation in Proteins and Lipids in a Microbial Community Are Different and Species-Specific. *ACS Chem. Biol.* **2013**, *8* (8), 1755–1763.

(58) Fu, X.; Deja, S.; Fletcher, J. A.; Anderson, N. N.; Mizerska, M.; Vale, G.; Browning, J. D.; Horton, J. D.; McDonald, J. G.; Mitsche, M. A.; Burgess, S. C. Measurement of Lipogenic Flux by Deuterium Resolved Mass Spectrometry. *Nat. Commun.* **2021**, *12* (1), 3756.

(59) Eiler, J.; Cesar, J.; Chimiak, L.; Dallas, B.; Grice, K.; Griep-Raming, J.; Juchelka, D.; Kitchen, N.; Lloyd, M.; Makarov, A.; Robins, R.; Schwieters, J. Analysis of Molecular Isotopic Structures at High Precision and Accuracy by Orbitrap Mass Spectrometry. *Int. J. Mass Spectrom.* **2017**, *422*, 126–142.

(60) Neubauer, C.; Sweredoski, M. J.; Moradian, A.; Newman, D. K.; Robins, R. J.; Eiler, J. M. Scanning the Isotopic Structure of Molecules by Tandem Mass Spectrometry. *Int. J. Mass Spectrom.* **2018**, *434*, 276–286.

(61) Zark, M.; Christoffers, J.; Dittmar, T. Molecular Properties of Deep-Sea Dissolved Organic Matter Are Predictable by the Central Limit Theorem: Evidence from Tandem FT-ICR-MS. *Mar. Chem.* **2017**, *191*, 9–15.

(62) Merder, J.; Freund, J. A.; Feudel, U.; Niggemann, J.; Singer, G.; Dittmar, T. Improved Mass Accuracy and Isotope Confirmation through Alignment of Ultrahigh-Resolution Mass Spectra of Complex Natural Mixtures. *Anal. Chem.* **2020**, *92* (3), 2558–2565.

(63) Su, X.; Lu, W.; Rabinowitz, J. D. Metabolite Spectral Accuracy on Orbitraps. *Anal. Chem.* **2017**, *89* (11), 5940–5948.

(64) Makarov, A.; Denisov, E. Dynamics of Ions of Intact Proteins in the Orbitrap Mass Analyzer. *J. Am. Soc. Mass Spectrom.* **2009**, *20* (8), 1486–1495.

(65) Neubauer, C.; Crémière, A.; Wang, X. T.; Thiagarajan, N.; Sessions, A. L.; Adkins, J. F.; Dalleska, N. F.; Turchyn, A. V.; Clegg, J. A.; Moradian, A.; Others. Stable Isotope Analysis of Intact Oxyanions Using Electrospray Quadrupole-Orbitrap Mass Spectrometry. *Anal. Chem.* **2020**, *92* (4), 3077–3085.

(66) Mueller, E. P.; Sessions, A. L.; Sauer, P. E.; Weiss, G. M.; Eiler, J. M. Simultaneous, High-Precision Measurements of δ^{2}H and δ^{13}C in Nanomole Quantities of Acetate Using Electrospray Ionization-Quadrupole-Orbitrap Mass Spectrometry. *Anal. Chem.* **2022**, *94* (2), 1092–1100.

(67) Rockwood, A. L.; Kushnir, M. M.; Nelson, G. J. Dissociation of Individual Isotopic Peaks: Predicting Isotopic Distributions of Product Ions in MSn. *J. Am. Soc. Mass Spectrom.* **2003**, *14* (4), 311–322.

(68) Larsen, T.; Fernandes, R.; Wang, Y. V.; Roberts, P. Reconstructing Hominin Diets with Stable Isotope Analysis of Amino Acids: New Perspectives and Future Directions. *Bioscience* **2022**, *72* (7), 618–637.

(69) Zeng, X.; Xing, X.; Gupta, M.; Keber, F. C.; Lopez, J. G.; Lee, Y.-C. J.; Roichman, A.; Wang, L.; Neinast, M. D.; Donia, M. S.; Wühr, M.; Jang, C.; Rabinowitz, J. D. Gut Bacterial Nutrient Preferences Quantified in Vivo. *Cell* **2022**, *185* (18), 3441–3456.e19.

(70) Tea, I.; De Luca, A.; Schiphorst, A.-M.; Grand, M.; Barillé-Nion, S.; Mirallié, E.; Drui, D.; Krempf, M.; Hankard, R.; Tcherkez, G. Stable Isotope Abundance and Fractionation in Human Diseases. *Metabolites* **2021**, *11* (6).

(71) Claesen, J.; Rockwood, A.; Gorshkov, M.; Valkenborg, D. The Isotope Distribution: A Rose with Thorns. *Mass Spectrom. Rev.* **2023**. https://doi.org/10.1002/mas.21820.

(72) Silverman, S. N.; Phillips, A. A.; Weiss, G. M.; Wilkes, E. B.; Eiler, J. M.; Sessions, A. L. Practical Considerations for Amino Acid Isotope Analysis. *Org. Geochem.* **2022**, *164*, 104345.

(73) Hofmann, A. E.; Chimiak, L.; Dallas, B.; Griep-Raming, J.; Juchelka, D.; Makarov, A.; Schwieters, J.; Eiler, J. M. Using Orbitrap Mass Spectrometry to Assess the Isotopic Compositions of Individual Compounds in Mixtures. *Int. J. Mass Spectrom.* **2020**, *457*, 116410.

(74) Neubauer, C.; Sessions, A. L.; Booth, I. R.; Bowen, B. P.; Kopf, S. H.; Newman, D. K.; Dalleska, N. F. Towards Measuring Growth Rates of Pathogens during Infections by D_2O-Labeling Lipidomics. *Rapid Commun. Mass Spectrom.* **2018**, *32* (24), 2129–2140.

(75) Derrick, P. J. Isotope Effects in Fragmentation. *Mass Spectrom. Rev.* **1983**, *2* (2), 285–298.

(76) Zamboni, N.; Fendt, S.-M.; Rühl, M.; Sauer, U. ^{13}C-Based Metabolic Flux Analysis. *Nat. Protoc.* **2009**, *4* (6), 878–892.

(77) Apostel, C.; Dippold, M.; Glaser, B.; Kuzyakov, Y. Biochemical Pathways of Amino Acids in Soil: Assessment by Position-Specific Labeling and ^{13}C-PLFA Analysis. *Soil Biol. Biochem.* **2013**, *67*, 31–40.

(78) Dippold, M. A.; Kuzyakov, Y. Biogeochemical Transformations of Amino Acids in Soil Assessed by Position-Specific Labelling. *Plant Soil* **2013**, *373* (1-2), 385–401.

(79) Aepfler, R. F.; Bühring, S. I.; Elvert, M. Substrate Characteristic Bacterial Fatty Acid Production Based on Amino Acid Assimilation and Transformation in Marine Sediments. *FEMS Microbiol. Ecol.* **2019**, *95* (10).

(80) Long, C. P.; Antoniewicz, M. R. Metabolic Flux Responses to Deletion of 20 Core Enzymes Reveal Flexibility and Limits of *E. Coli* Metabolism. *Metab. Eng.* **2019**, *55*, 249–257.

(81) Wegener, G.; Kellermann, M. Y.; Elvert, M. Tracking Activity and Function of Microorganisms by Stable Isotope Probing of Membrane Lipids. *Curr. Opin. Biotechnol.* **2016**, *41*, 43–52.

(82) Marks, R. G. H.; Jochmann, M. A.; Brand, W. A.; Schmidt, T. C. How to Couple LC-IRMS with HRMS—A Proof-of-Concept Study. *Anal. Chem.* **2022**, *94* (6), 2981–2987.

(83) 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.* **2019**, *91* (15), 9732–9740.

(84) Hellerstein, M. K.; Angel, T. E.; Makarov, A. A. Measurement of Protein Molecular Flux Rates by Quantifying Isotopologue Abundances in Immonium Ion Using High Resolution Mass Spectrometry, US Patent App. 15/758,268, 2020, 2020.

(85) Gharibi, H.; Chernobrovkin, A. L.; Saei, A. A.; Zhang, X.; Gaetani, M.; Makarov, A. A.; Zubarev, R. A. Proteomics-Compatible Fourier Transform Isotopic Ratio Mass Spectrometry of Polypeptides. *Anal. Chem.* **2022**, *94* (43), 15048–15056.

– For Table of Contents Use Only –

Discovering Nature’s Fingerprints: Isotope Ratio Analysis on Bioanalytical Mass Spectrometers

A Perspective written by participants of the workshop “Nature's Fingerprints”, which was held in November 2021 at the Hanse Institute for Advanced Study, Delmenhorst, Germany

Cajetan Neubauer, Kristýna Kantnerová, Alexis Lamothe, Joel Savarino, Andreas Hilkert, Dieter Juchelka, Kai-Uwe Hinrichs, Marcus Elvert, Verena Heuer, Martin Elsner, Rani Bakkour, Maxime Julien, Merve Öztoprak, Stefan Schouten, Shohei Hattori, Thorsten Dittmar

A brief synopsis: Isotope fingerprints are useful tools in a fascinating range of research topics. Larger and smaller areas of isotope research are shown as islands in an archipelago.