Technical note | 002570

Isotope ratio MS

Isotope ratio analysis of acetate using Orbitrap Exploris Isotope Solutions

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Introduction

Acetate is an important organic molecule in environmental processes, serving as a key intermediate in the degradation of organic matter (plants, cells, etc.) to greenhouse gases. It is rapidly cycled by microbial communities in the environment, resulting in low concentrations that make it challenging to investigate its flux in biogeochemical cycles. This can be addressed using naturally occurring stable isotopes as a tool to quantify acetate cycling. The carbon and hydrogen isotope fingerprint of acetate can constrain its metabolic sources and the mechanisms of organic remineralization in anoxic settings.[1-3]

In this technical note we demonstrate how Thermo Scientific™ Orbitrap Exploris™ Isotope Solutions enable carbon and hydrogen isotope analysis of acetate dissolved in complex environmental matrices. This includes molecular-average carbon and methyl-specific H-isotope compositions of acetate with nanomole sensitivity. Further comprehensive information about methodology tests and evaluation of acetate isotope analysis using Thermo Scientific™ Orbitrap™ technology is described by Mueller at al. (2022).[2]

Isotope ratios by Orbitrap MS technology

Orbitrap Exploris Isotope Solutions enable measurement and calculation of isotope ratios directly from the relative abundances of a compounds isotopocules. Intact molecular ions are produced by electrospray ionization (ESI) and delivered to the Orbitrap analyzer. In addition, controlled fragmentation of the molecular ions can be used to deduce site-specific isotope compositions of some organic compounds.[4] Isotope ratios of samples are analyzed in comparison to a reference with known isotope ratios, which allow reporting of results relative to international standards.

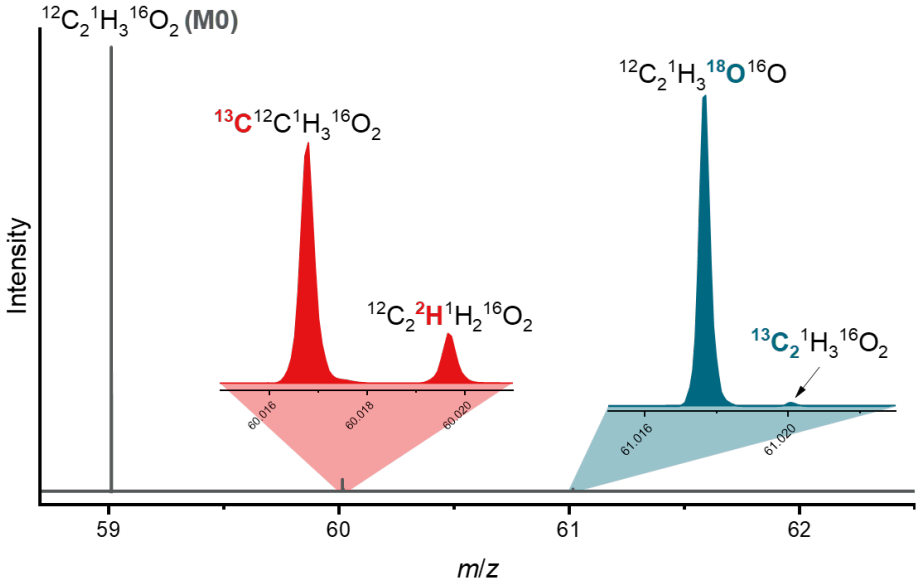

Figure 1. Mass spectrum acquired with acetate 'M0' methodology.

Using ESI-Orbitrap technology, intact molecular isotopocule ions are produced and differentiated using high resolution accurate mass (HRAM). Figure 1 shows a mass spectrum acquired with acetate 'M0' methodology (including $^{12}C_2{}^1H_3{}^{16}O_2$; M0) displaying the isotopocules detected. The isotopic composition is therefore determined as ratios of a heavy isotope substituted over the unsubstituted isotopocule (e.g. $^{13}C^{12}C^1H_3{}^{16}O_2/^{12}C_2{}^1H_3{}^{16}O_2$).

Equipment and methodology

The Orbitrap Exploris Isotope Solution presented here includes the Thermo Scientific™ Orbitrap Exploris™ 240 MS and data evaluation package for isotope ratio MS. Two sample introduction methods developed for sample/reference comparison are available:

1. Dual Syringe Inlet system based on a syringe pump and a diverter valve

2. An automated In-flow Injection approach utilizing the Thermo Scientific™ Vanquish™ Neo UHPLC System

In this study a flow rate of 5 µL/min was used. Table 1 shows instrument settings of the Orbitrap Exploris 240 MS used for the isotope ratio analysis of low molecular weight organic acids. Ion source parameters should be checked/tuned daily. Typical settings provided in Table 1 serve as starting point and guidance for this.

Data acquisition and evaluation

Thermo Scientific™ Xcalibur™ Software is used for instrument setup and data acquisition. Every measurement generates a RAW file that is processed by Thermo Scientific™ IsoX™ Software to extract all relevant parameters for the calculation of isotope ratios. The resulting IsoX Software output files, including all the data and parameters needed for the further evaluation steps, are simple tab-delimited files and can be opened as spreadsheets. For the processing of multiple RAW files, a combined IsoX Software output file can be created.

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

Table 1. Ion source settings for Orbitrap Exploris 240 MS

Ion source		Define scan	
Sheath gas (Arb.)	5 (Typical range 1-10)	**Scan type**	Full scan
Aux gas (Arb.)	1 (Typical range 1-10)	**Orbitrap resolution**	60,000
Sweep gas (Arb.)	1 or 0	**Polarity**	Negative
Neg ion spray voltage	3000 V (Typical range 2800 – 3200 V)	**Microscans**	1
Spray current (observed)	<0.3 µA	**Maximum injection time (ms)**	1000
Ion transfer tube temp (˚C)	320	**Scan range (m/z)**	57-62 *m/z*
		RF lens (%)	50
		AGC target	Standard (equals 1E6 absolute AGC target)

Standardization

Standardization for acetate measurements uses three external standards known as AcSt, AcA and AcB, which have been measured for their carbon and hydrogen isotope compositions via combustion and thermal conversion elemental analysis isotope ratio mass spectrometry.[2] For the following calculations we assume a similar degree of clumping (i.e. multiple rare isotope substitutions) between sample and standard, which results in an approximation where the isotopocule sample/standard ratios are equivalent to the ratios from classical molecular average isotope measurements.

Standard solutions are infused/injected between sample replicates. Standardization is achieved using standard/sample bracketing according to the following equation.

$$\delta^{13}\mathrm{C} = \left[\frac{{}^{13}R_{SA}}{({}^{13}R_{ST} / {}^{13}R_{ST,acc}) \times {}^{13}R_{VPDB}} - 1 \right] \times 1000\ ‰$$

Where ${}^{13}R_{SA}$ is the Orbitrap-measured value of the isotope ratio (${}^{13}C/{}^{12}C$) of the sample, ${}^{13}R_{ST}$ is the average Orbitrap-measured isotope ratio of the two adjacent standard replicates, ${}^{13}R_{ST,acc}$ is the accepted isotope ratio of the standard and ${}^{13}R_{VPDB}$ is the isotope ratio of the Vienna Pee Dee Belemnite standard (0.01118 and 0.00015576 for ${}^{13}C/{}^{12}C$ and ${}^{2}H/{}^{1}H$ isotope ratios (${}^{2}R$), respectively). This standardization procedure yields accurate $\delta^{13}C$ and $\delta^{2}H$ of acetate from AcA and AcB.[2]

Sample preparation

Certain analytes (e.g. nitrate) can be directly measured in complex matrices through the dilute-and-shoot method, but only when the ratio of analyte to other major anions (e.g. chloride) is high enough (~1:100). However, acetate is even more sensitive to ionization suppression by other anions. Standard solutions (50 µM) mixed with various chloride salts to a ratio of 1:20 acetate:chloride did not exhibit any acetate ionization.[2] In environmental samples of acetate, the ratio is even lower. In salty brines, acetate can accumulate to millimolar concentrations, chloride concentrations are >3 mol/kg, leading to a ratio of <1:1000. In marine sediment with 20 µM acetate and 500 mM chloride, the ratio is 1:25000. Thus, acetate from environmental samples often requires purification before analysis.

We accomplished organic acid purifications using ion chromatography, an approach which is also directly applicable to oxyanions such as nitrate, sulfate and phosphate. To purify organic acids for isotope analysis, they are separated from other ions in three stages. First, chloride and sulfate are precipitated as silver chloride and barium sulfate from solution using flow-through cartridges. Next, undiluted sample is injected onto a high performance IC. To ensure sufficient chromatographic separation, a pre-concentrator column is placed on the front end of the analytical column. The pre-concentrator traps all of the anions as a small plug. The various components of the sample anion matrix (chloride, phosphate, nitrate, organic acids) then elute off the pre-concentrator onto the analytical column as the eluent hydroxide concentration increases. This keeps the analytical column from becoming overloaded with analytes while enabling injections of any volume. The organic acids are fraction collected as a single, co-eluting peak. After titrating to circumneutral pH with degassed NaOH, the eluent is dried down under nitrogen headspace at room temperature. Finally, the sample is dissolved in methanol before Orbitrap analysis.

Results

Isotope ratio linearity

For high precision and accuracy isotope ratio measurements using classical sectorfield mass spectrometers, the isotopic composition of samples is analyzed relative to a known standard. The same principle is also used to enhance accuracy and precision for Orbitrap based IRMS measurements. To achieve the best results from sample standard comparison the concept of identical treatment is applied, meaning that sample and standard are analyzed under conditions that are as identical as possible.

In pure solution, acetate selected ion current (SIC) increases with concentration non-linearly (Figure 2, a). This should be taken into consideration when attempting to match the SIC of sample or when quantifying the concentration of acetate in a sample based on its SIC intensity.

Due to varying analyte concentration in unknown samples, perfect matching of the SIC intensities is not always feasible. In mass spectrometric measurements of isotope ratios, differences in ion current intensity can be a source of isotope ratio inaccuracy if they are not corrected.

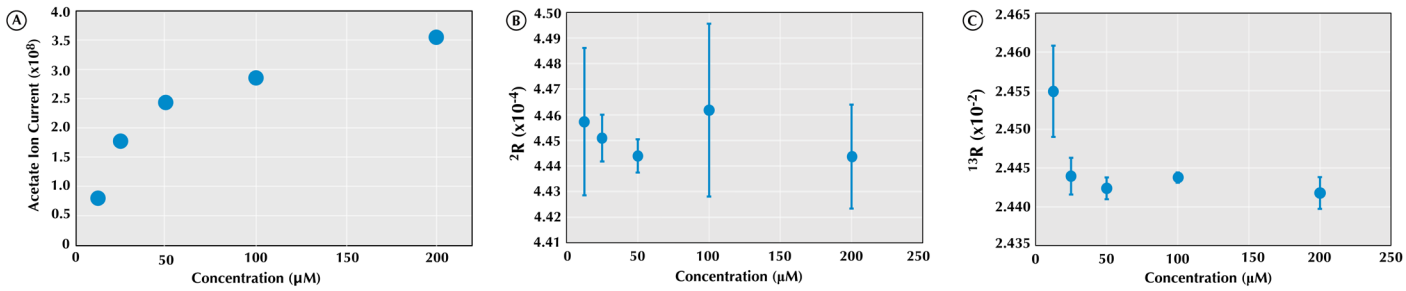

Figure 2: Acetate SIC and isotope ratio non linearity. A) Non-linear SIC response to acetate concentration between 12.5 and 200 µM solution concentration. B) and C) show good linearity with respect to isotope ratios, save ^{13}R at 12.5 µM, which is attributed to blank effects.

However, for some analytes matching sample/standard SIC is not imperative. Certain molecules (e.g. nitrate in Hilkert et al., 2021[5]) show no change in measured isotope ratio over a wide concentration range. The same is true for acetate (Figure 2, b and c). The SIC does not influence acetate's isotope ratios until the SIC reaches below 1e8 at 12.5 µM at which point there is a 5 ± 2 ‰ increase in the carbon isotope ratio. The inaccuracy at low concentrations is likely due to blank contributions, as has been suggested in previous studies of nitrate isotope ratio measurements by ESI-Orbitrap MS[5]. As such, we do not believe the 1e8 SIC threshold will be consistent across all instrument platforms or measurement conditions, since these will have specific ionization efficiencies and blank contributions. These linearity tests should instead be performed for individual instruments and measurement conditions.

Accurate acetate isotope ratio measurement in organic acid mixtures

Since acetate coelutes from the ion exchange column with the other organic acids, they are all infused into the ESI-Orbitrap simultaneously. This can suppress the ionization of acetate relative to a pure solution. It is important to know whether this suppression causes isotope fractionations. The SIC of acetate was measured in various mixtures of acetate, propionate, and butyrate to assess the ion suppression effects associated with matrix contamination by other organic acids (Figure 3). SIC of acetate was linearly correlated with its relative abundance in the organic acid mixture, causing up to three-fold differences in ion current between sample and pure acetate standard. In all samples and standards, acetate was held at 50 µM. Despite the differences in SIC, the measured $\delta^{13}C$ and $\delta^{2}H$ of acetate was accurate (within 2σ of true value) in every mixture. No inaccuracy (outside 2σ) associated with ion suppression or different ion current magnitudes was observed. This supports the strategy of isolating organic acids as a single, coeluting peak.

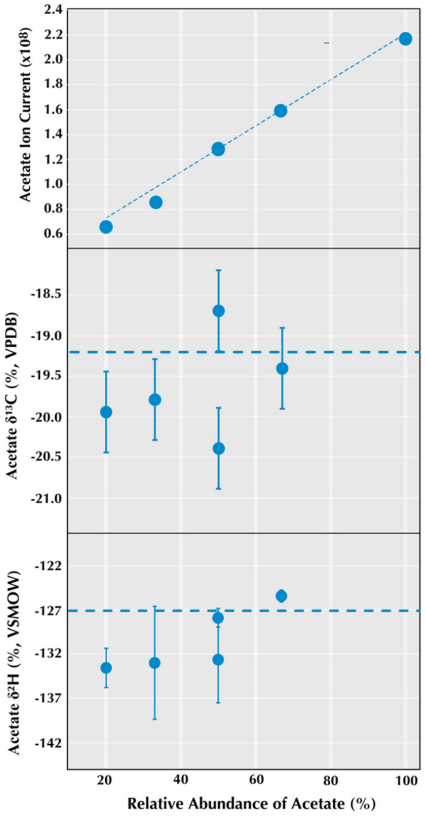

Figure 3: Five mixtures of organic acids containing different proportions of acetate, propionate, and butyrate with known $\delta^{13}C$ and $\delta^{2}H$ values were measured against a pure acetate standard. All mixtures had an absolute acetate concentration of 50 µM, yet acetate's SIC changed dramatically.

Removing artifacts caused by contaminants in the Mass Spectra

Isotope effects of carbonate contamination

Contaminants and interferences can be found in the mass spectra of target analytes. If a contaminant is allowed to pass through the quadrupole mass window, it can cause inaccurate results due to FT-interference or space-charge-effects within the Orbitrap mass analyzer, namely coalescence, the tendency of peaks to merge or distort above a certain ion threshold.[6-8] Depending on the type of contaminant and interference, an increase in Orbitrap resolution or lowering of the AGC target can eliminate the interference.[5]

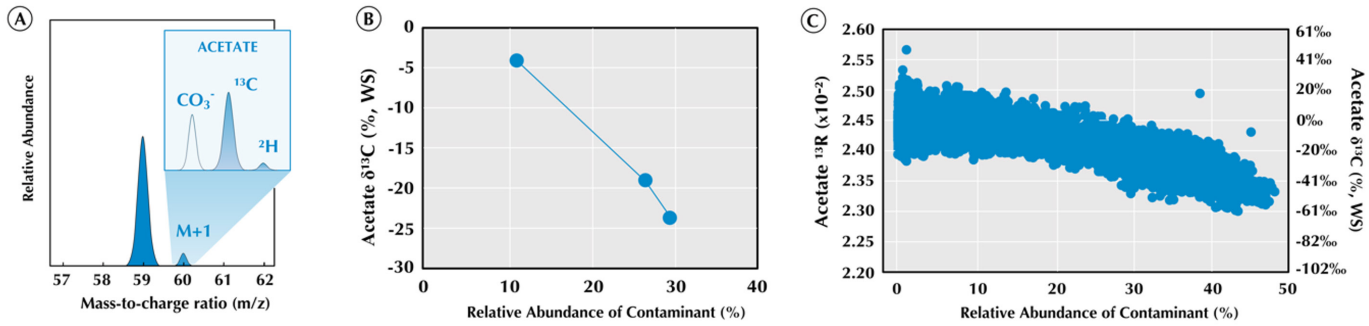

Figure 4: The carbon isotope ratio of acetate is suppressed by mass-resolved contaminants, including the carbonate anion (A). This leads to (B) progressive increases in the abundance of the carbonate ion contaminant, across three acquisitions results in lower ^{13}R measured. (C) Collated scans across the three acquisitions highlight this correlation further, demonstrating that it does not have consistent isotope ratios unless the carbonate ion is <10% of the SIC value. This trend was not observed for hydrogen isotope ratios.

Otherwise, contaminant ion intensities can be matched in standards or contaminants can be removed from samples to avoid inaccurate isotopic measurements of the sample.[9] In the case of acetate, contaminant peaks can appear within the quadrupole window (57-62 *m/z*), even after preparative isolation. A common contaminant peak is the signal of a singly charged carbonate anion at 59.98 *m/z* with relative abundances of up to 30 % in samples. This ion appears even in pure standards, but at much lower relative abundance (<0.1 %). While the carbonate ion and acetate ^{13}C-isotopocule (60.01 *m/z*) ion are mass resolved and visually separated in the spectra (Figure 4A), coalescence of their ion rings can cause attenuation, resulting in decreased intensity of the acetate ^{13}C-isotopocule.

We observed this directly during three separate acquisitions of the same environmental sample. These acquisitions were taken from the same solution of acetate in methanol after preparative isolation during a dual inlet run. Across three acquisition blocks, the carbonate ion increased with time despite no changes in spray or mass spectrometer parameters. This contaminant did not appear in the standard. The average ^{13}R of the three acquisitions had a strong correlation with the abundance of this contaminant relative to the base peak (Figure 4B). To investigate this correlation in more detail, we collated the scans from the three acquisition blocks and took a moving average (n=10) of the carbon isotope ratios (Figure 4C). We found a clear trend between the contaminant abundance in individual scans and the isotope ratio, with isotope effects of >50 ‰, well above the 0.5 ‰ precision of typical acetate ^{13}R measurements. Consistent isotope ratios only appeared when the contaminant was <10 % of the base peak. These data demonstrate that contaminants that are within the quadrupole window, but mass resolved from the isotopocules of interest, can influence the measured isotope ratio due to space-charge effects.

The coalescence isotope effects that we have observed for carbon isotopocules of acetate do not extend to hydrogen isotopocules. There is no correlation between the hydrogen isotope ratio of acetate and the abundance of the carbonate anion, even when the latter is 50 % of the TIC. This could be because the errors on hydrogen isotope ratios (~3 ‰ SEM) are too large to capture these effects. Coalescence or ion cloud coupling occurs on two or more ionic species with high abundance/ ion counts in the analyzer and low difference (few mmu) in *m/z*.[2,7,8] The carbonate contamination and the ^{13}C isotopocule are the highest abundance ion signals and closest in *m/z* in the M+1 region. This suggests a stronger effect on ion with high intensities compared to those with lower abundances, like ^2H-isotopocule. However, confirming accuracy with external standards for both isotopocules before analyzing samples is recommended.

Removing carbonate ion contaminants

The physical source of the carbonate contamination has yet to be determined, but there are methods to remove it from the ion current during electrospray ionization. The most efficient strategy is to limit the observable concentration by using the sweep gas flow of the ESI. However, this also lowers the SIC by almost two-fold. Alternatively, the carbonate ion can be suppressed by lowering the spray voltage. High voltages over 3500 V tends to increase the carbonate ion intensity, but voltages near or below 3000 V suppresses its ionization. With these two strategies, carbonate ions can be suppressed to <0.1 % of the SIC, which does not influence the carbon or hydrogen isotope ratios of acetate. Generally, lowering the AGC target to the range of 1E5-1E3 can reduce or eliminate artifacts caused by space-charge-effects.[10]

Isolation Proof-of-Concept: Acetate from brines

The preceding discussion suggests a three-pronged approach to precise and accurate acetate measurements of environmental samples by Orbitrap MS methodology. 1.) Separate organic acids from inorganic anions in the sample matrix. 2.) While there is margin for error, matching SIC to the intensity of the acetate basepeak ion ensures accurate results even with ion suppression from other organic acids. 3.) Remove contaminants from the mass spectra within the quadrupole window (e.g. carbonate ions). By combining these three approaches, we can confidently measure acetate from complex matrices. To demonstrate this, we purified acetate from brine solutions (Table 2) containing a chloride-to-acetate ratio of either 3500:1 (1 mM Acetate) or 1750:1 (2 mM Acetate). The acetate standard, AcSt, that was spiked into these solutions has a known isotope composition. We found that purified solutions matched this composition for both carbon and hydrogen isotopocule measurements (Figure 5).

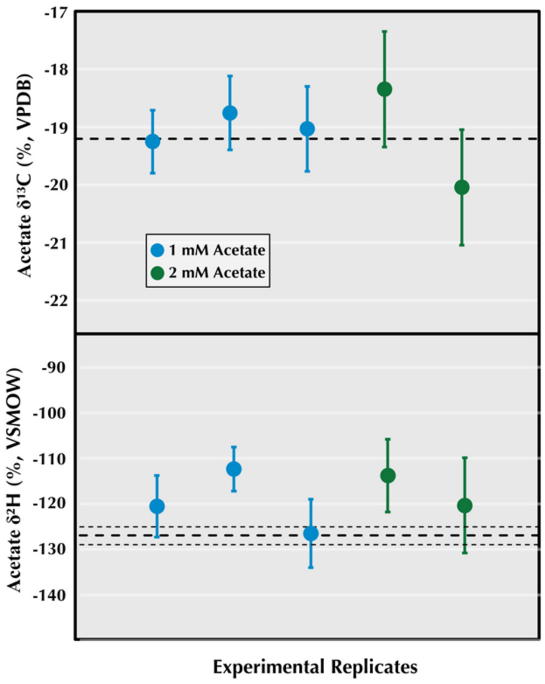

Figure 5: Proof-of-concept experiments in which acetate of known $\delta^{13}C$ and $\delta^{2}H$ was extracted from a synthetic brine. The isolation procedure did not produce measurable isotopic fractionations outside the accuracy of the analytical method (1 ‰ and 10 ‰ for $\delta^{13}C$ and $\delta^{2}H$, respectively).

Table 2. Brine solutions matrix

Component	Concentration (g/L)
$CaCl_2$	144.274
NaCl	36.817
KCl	0.201
$MgCl_2$	8.278
NaBr	2.469
$NaNO_3$	0.017
$NaNO_2$	0.014
KH_2PO_4	0.027
$MgSO_4$	0.072
Na-formate	0.034

Conclusions

One of the major goals of the Orbitrap Exploris Isotope Solutions platform is to equip environmental scientists with novel tools to investigate the natural world. Acetate is an important molecule for biogeochemical cycling of carbon but is often found at too low concentration to allow 'dilute-and-shoot' methods of isotope ratio measurements. This technical report outlines an optimized strategy for isolating and measuring acetate from environmental samples and demonstrates its accuracy and precision.

References

1. Heuer, V. B.; Pohlman, J. W.; Torres, M. E.; Elvert, M.; Hinrichs, K.-U. Geochim. *Cosmochim. Acta* **2009**, 73, 3323–3336.
2. Mueller, E. P.; Sessions, A. L.; Sauer, P. E.; Weiss, G. M.; Eiler, J. M. *Anal. Chem.* **2022**, 94, 1092–1100.
3. Heuer, V. B.; Krüger, M.; Elvert, M.; Hinrichs, K.-U. *Org. Geochem.* **2010**, 41, 22–30.
4. Weiss, G. M.; Sessions, A. L.; Julien, M.; Csernica, T.; Yamada, K.; Gilbert, A.; Freeman, K. H.; Eiler, *J. M. Int. J. Mass Spectrom.* **2023**, 493, 117128.
5. Hilkert, A.; Böhlke, J. K.; Mroczkowski, S. J.; Fort, K.L.; Aizikov, K.; Wang, X.T.; Kopf, S.H.; Neubauer, C. *Anal. Chem.* **2021**, 93, 26, 9139–9148.
6. Rockwood, A. L.; Erve, J. C. L. *J. Am. Soc. Mass Spectrom.* **2014**, 25, 12, 2163–2176.
7. Gorshkov, M. V.; Fornelli, L.; Tsybin, Y. O. *Rapid Commun. Mass Spectrom.* **2012**, 26, 15, 1711–1717.
8. Grinfeld, D.; Stewart, H.; Skoblin, M.; Denisov, E.; Monastyrsky, M.; Makarov, A. *Int. J. Mod. Phys.* A **2019**, 34, 36, 1942007.
9. Hofmann, A. E.; Chimiak, L.; Dallas, B.; Griep-Raming, J.; Juchelka, D.; Makarov, A.; Schwieters, J.; Eiler, J. M. *Int. J. Mass Spectrom.* **2020**,457, 116410.
10. Gorshkov, M. V.; Fornelli, L.; Tsybin, Y. O. *Rapid Commun. Mass Spectrom.* **2021**, 26, 15, 1711-1717.

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