Isotope ratio analysis of glutamic acid using Orbitrap Exploris Isotope Solutions

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Introduction

Amino acids are the building blocks of proteins and hence a crucial part of life and life processes. A central amino acid in metabolism is glutamic acid and its most common form glutamate, which is part of the citric cycle. Glutamate serves as neurotransmitter, undergoes metabolism in the brain and is part of transamination processes for excreting excess nitrogen via urea. Recent publications demonstrate the value of isotope ratio analysis of amino acids using Thermo Scientific™ Orbitrap™ technology.[1-4]

In this technical note we demonstrate how Thermo Scientific™ Orbitrap Exploris™ Isotope Solutions enables carbon, nitrogen, oxygen, and hydrogen isotope analysis of amino acids, using glutamic acid as a model compound. Primarily, this includes simultaneous analysis of ^{13}C, ^{15}N, ^{18}O and ^{2}H carrying isotopocules from intact molecular ions.

In addition, higher-energy collisional dissociation (HCD) can be used to fragment the molecular ion of glutamic acid into its immonium ion. The carboxyl group is lost during this process. Analyzing the heavy isotope substituted isotopocules of the immonium ion in addition to those of the intact glutamic acid molecule subsequently gives access to the position specific isotope ratio of $^{13}C/^{12}C$ in the carboxyl group (C_1-Position). Position specific isotope analysis (PSIA) allows the investigation of biochemically and even artificially induced signatures on specific positions in the molecule as well as clumping effects in parts of the molecule (e.g. $^{13}C^{13}C$ accumulation). This opens new routes into the deconvolution of biochemical pathways, points of origin and authenticity control.

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. Electrospray ionization in positive mode produces intact molecular isotopocule ions ($[M+H]^+$) of glutamic acid. These target ions are separated from most other ions with a < 10 amu mass window using the Advanced Quadrupole Technology (AQT). Any interfering ions within this mass window as well as all relevant target isotopocules are resolved by high resolution accurate mass (HRAM) (Figure 1). Controlled fragmentation of the molecular ions to deduce position-specific isotope information is achieved by HCD in the ion routing multipole. More information on Orbitrap MS methodology can be found at Kantnerová et al.[5-6]

Throughout this and the following paragraphs the heavy isotope substitutions will be used as shortcuts for their corresponding isotopocules, e.g. $^{13}C = {}^{13}C^{12}C_4{}^{1}H_{10}{}^{14}N^{16}O_4$. The unsubstituted isotopocule of glutamic acids molecular ion ($^{12}C_5{}^{1}H_{10}{}^{14}N^{16}O_4$ = M0) and fragmented immonium ion ($^{12}C_4{}^{1}H_6{}^{14}N^{16}O$ = F0) were used as denominators (basepeak) for the isotopocule ratios.

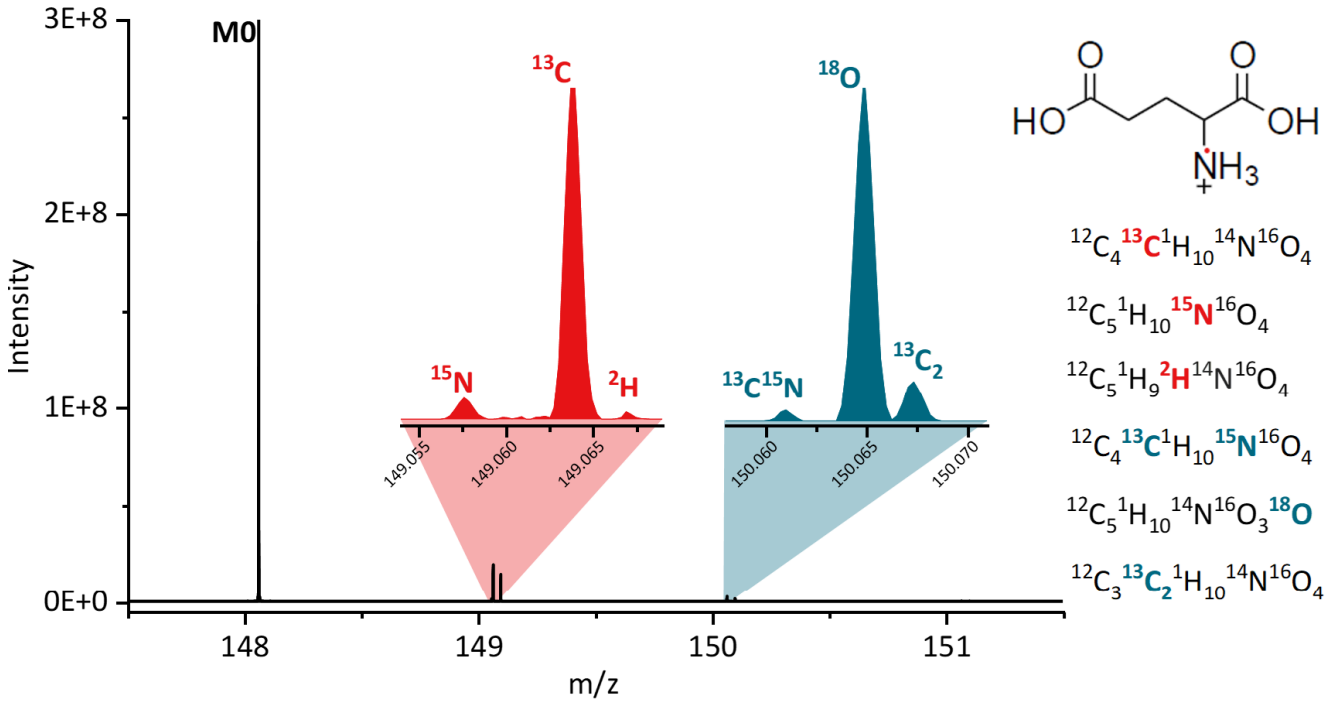

Figure 1. Mass spectrum of glutamic acid [M+H]+ ion at 120,000 Orbitrap resolution.

Equipment and methodology

For isotopic analysis, the glutamic acid samples were dissolved in water at 10 mM concentration. These stock solutions are diluted (1:1000, *v/v*) in methanol to a concentration of 10 µM with 0.1 % formic acid for Orbitrap Exploris Isotope Solutions analysis. The stock solutions were kept at 72 °C for 2 hours to ensure complete dissolution. During flow injection analysis the 10 µM solutions were placed in polypropylene vials (PN: 6ESV9-1PP) in the autosampler of the Thermo Scientific™ Vanquish™ Neo UHPLC System at <10 °C.

The Orbitrap Exploris Isotope Solutions presented here includes the Thermo Scientific™ Orbitrap Exploris™ 240 MS and data evaluation package for isotope ratio analysis. For sample introduction and sample/standard comparison an automated in-flow injection approach utilizing the Vanquish Neo UHPLC System coupled to the Orbitrap Exploris MS was used. The UHPLC pump module delivered a constant flow of 90% methanol/water (LC/MS-grade) at a flow rate of 4 µL/min. The autosampler is equipped with a 100 µL nominal volume sample loop. For each injection 40 µL of glutamic acid solution was injected resulting in 10 min wide plateau peaks. Each injection included a 10 min wash out time resulting in a total run time of 20 min and a sample consumption of 59 ng.

Orbitrap Exploris MS settings

Electrospray ion source parameters and settings used for isotope ratio analysis are listed in Table 1.

MS settings used for the analysis of isotopocule ratio of the intact glutamic acid molecule (Bulk/with M0) and of the immonium ion (Fragmentation) are listed in Table 2.

Table 1. Ion source settings for Orbitrap Exploris 240 MS, *Arb = Arbitrary units

Sheath gas (Arb)*	4
Auxiliary gas (Arb) *	6
Sweep gas (Arb) *	0
Positive ion spray voltage (V)	2800
Spray current (observed)	< 0.2 µA
Ion transfer tube temp (˚C)	320

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

Bulk (with M0, Full Scan)		Fragmentation (MS/MS)	
Scan type	Full scan	Scan type	MS2
Scan ranges (*m/z*)	136–158	Center mass (*m/z*)	149
Orbitrap resolution	120,000	Isolation window (*m/z*)	5
Polarity	Positive	Normalized collision energy (%)	70
Microscans	10	Scan range (*m/z*)	50–150
AGC target	Custom	Orbitrap resolution	90,000
Normalized AGC target (%)	30	Polarity	Positive
Maximum injection time (ms)	1,000	Microscans	10
RF lens (%)	100	AGC target	Custom
		Normalized AGC target (%)	30
		Maximum injection time (ms)	1,000
		RF lens (%)	100

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.

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

Standardization

Calculation of final δ-values was performed using Microsoft™ Excel™. Presented sample (Sam) data were measured against a solution of in-house working standard ("Lab", Std) with known molecular average values on ^{15}N and ^{13}C determined by Thermo Scientific™ EA IsoLink™ IRMS System (EA-IRMS). To compare the bulk isotope ratios analyzed by EA-IRMS with isotopocule ratios analyzed with Orbitrap Exploris MS, expected isotopocule ratios were calculated based on the results of EA-IRMS. For this calculation we assumed that the probability for a heavy isotope substitution of every atom of each element was equal to the value expected from EA-IRMS. Without position specific isotope enrichment/depletion the Orbitrap isotopocule ratios and the expected isotopocule ratios are equivalent.

A "Lab" standard of glutamic acid was used for sample/standard comparison (1-point calibration) with $\delta^{13}C_{Std/VPDB}$ = -29.3 ‰ and $\delta^{15}N_{Std/Air}$ = -3.1 ‰ determined by EA-IRMS. The Lab Standard is injected between all sample acquisitions. Standardization is achieved using standard/sample/standard bracketing, which consequently uses the average ratio (here R_{Std}) of the preceding and the following standard injections for calculation.

Formula (1) shows the calculation of δ-values against the standard for isotope ratios of $^{13}C/M0$ as an example:

$$(1) \qquad \delta^{13}C_{Sam/Std}\,[‰] = \left(\frac{R(^{13}C/M0)_{Sam}}{R(^{13}C/M0)_{Std}} - 1 \right) * 1000$$

To calculate the δ-values against international primary reference materials, the δ-values of the "Lab" standard against the primary reference materials ($\delta^{13}C_{Std/VPDB}$) determined by EA-IRMS and the measured sample values ($\delta^{13}C_{Sam/Std}$) were evaluated using equation (2).

$$(2)\ \delta^{13}C_{Sam/VPDB}\,[‰] = \delta^{13}C_{Sam/Std} + \delta^{13}C_{Std/VPDB} + (\delta^{13}C_{Sam/Std} \cdot \delta^{13}S_{Std/VPDB})/1000$$

Results

Molecular bulk glutamic acid isotope analysis

The Full MS experiment at 120,000 resolution allows the characterization of the intact glutamic acid molecule by 6 isotopocule ratios (Figure 1, Table 3). For verification purposes three different glutamic acid materials USGS 40, USGS 41 and SA (Sigma-Aldrich) were isotopically characterized with the Orbitrap MS using in-flow injections (OT). All materials were additionally characterized or confirmed by EA-IRMS (Bulk).

Results are listed in Table 3 with the standard deviation (σ) of five (OT) replicate injections as error. The average values of $\delta^{13}C_{VPDB,OT}$ and $\delta^{15}N_{Air,OT}$ are close to the values of $\delta^{13}C_{VPDB,Bulk}$ and $\delta^{15}N_{Air,Bulk}$ within 2σ.

Table 3. Results of isotopocule ratio analysis of the six most abundant isotopocules of the glutamic acid $[M+H]^+$ ion. δ-values were calculated against international reference materials or a "Lab" standard (Std).

	$\delta^{15}N_{Air, OT}$ ‰	$\delta^{13}C_{VPDB, OT}$ ‰	$\delta^{2}H_{Std, OT}$ ‰	$\delta^{18}O_{Std, OT}$ ‰	$\delta^{15}N^{13}C_{Std,OT}$ ‰	$\delta^{13}C^{13}C_{Std, OT}$ ‰	$\delta^{15}N_{Air, Bulk}$ ‰	$\delta^{13}C_{VPDB, Bulk}$ ‰
SA	-1.0±1.6	-27.6±1.0	23.5±3.7	1.0±1.0	5.8±6.6	6.5±2.6	-2.6	-27.8
USGS 40	-7.5±2.9	-26.0±1.7	14.9±8.8	-1.6±1.9	4.7±8.9	4.6±3.5	-4.52	-26.1
USGS 41	53.2±2.9	39.0±0.7	10.6±7.1	8.0±1.7	98.9±9.3	158.1±7.0	49.5*	37.0*

*Calculated isotopocule ratios based on Qi et al.[7]

USGS 40 and USGS 41 are international reference materials, which have been set up for bulk isotope analysis, e.g. EA-IRMS.[7] While USGS 40 is an industrial product and can be assumed to be close to stochastic isotope distribution, USGS 41 is a mixture of USGS 40 with small amounts of 1-^{13}C enriched glutamic acid and ^{15}N enriched glutamic acid as described by Qi et al.[7] Hence, parts of USGS 41 are not stochastically distributed. This leads to differences between the bulk isotope ratios and the expected ratios for ^{13}C and ^{15}N bearing isotopocules. Therefore the expected $\delta^{15}N_{Air,Bulk}$ and $\delta^{13}C_{VPDB,Bulk}$ for USGS 41 are slightly different to the isotope ratios determined by EA-IRMS. Further effects on the isotopocule ratios of USGS 41 are described during the discussion of the fragmentation experiments.

Fragmentation and position specific information

One of the features of the Orbitrap Exploris MS is the fragmentation of the target molecule *via* HCD, which gives access to parts of the molecule and consequently to PSIA.[8] For this MS2 experiment the *m/z* range of the glutamic acid molecular ion and its isotopocules is isolated by the quadruple (*m/z* 149 ± 5 amu) and ions are collected in the ion routing multipole. Here the ions are fragmented under controlled energy conditions (NCE 70 %) by collisions with small amounts of nitrogen gas. By HCD the molecular ion of glutamic acid is fragmented mainly into its immonium ion at *m/z* 84 losing the carboxylic group in C_1-position (Figure 2). Due to the lower *m/z* of the immonium ion, resulting in a larger relative mass difference between its isotopocules, 90,000 resolution resolves the ^{2}H peak from ^{13}C peak with an increased scan rate of ~5 scans/s compared to ~4 scans/s at 120,000 (Figure 3).

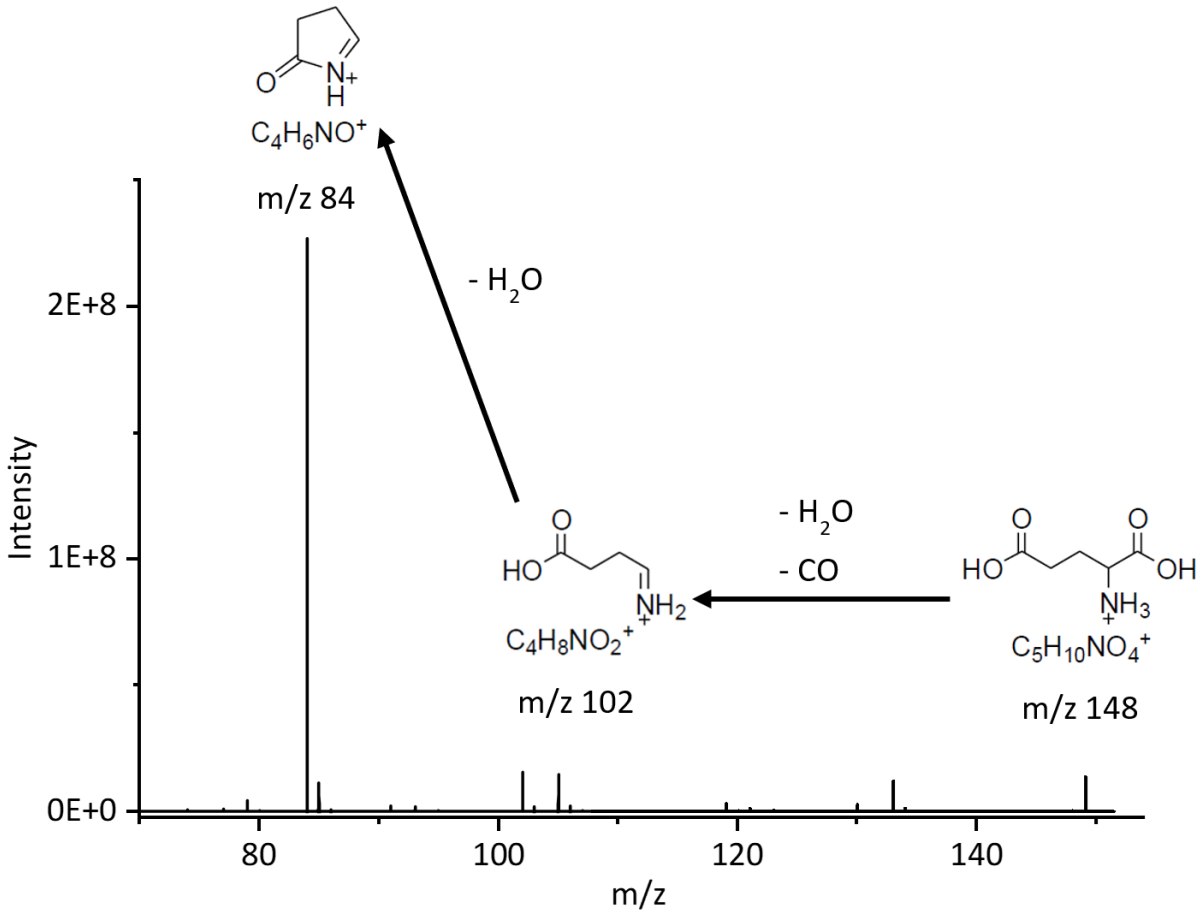

Figure 2. MS2 mass spectrum with 70% NCE acquired with glutamic acid.

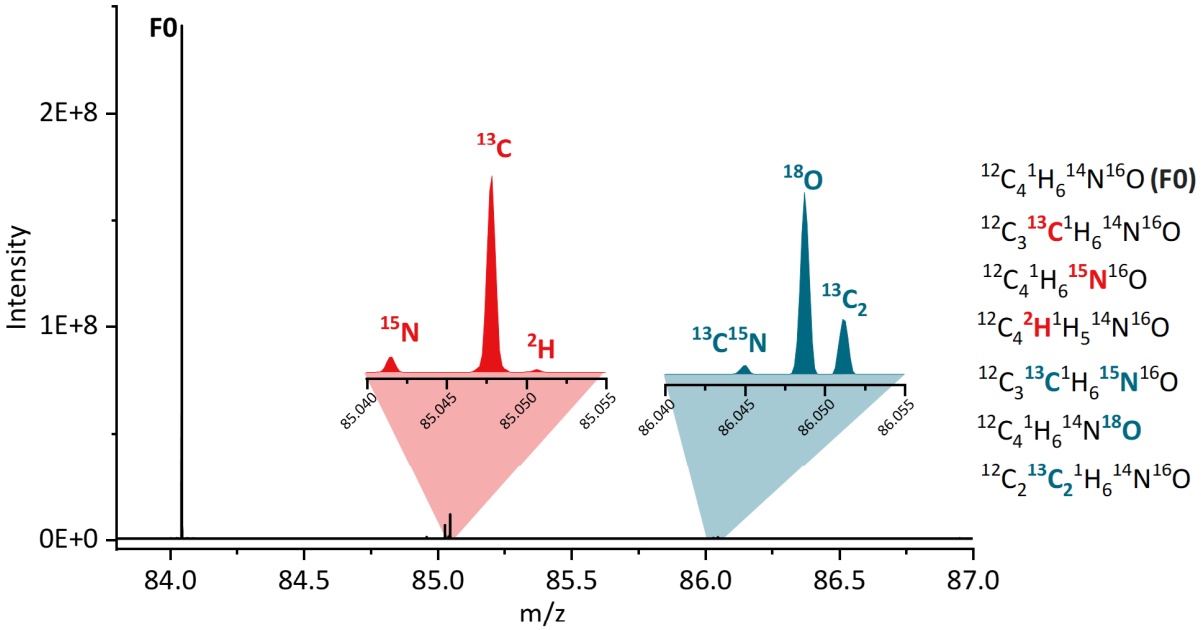

Figure 3. Isotopic pattern of fragment at m/z 84 at 90,000 resolution.

Table 5. Results of isotopocule ratio analysis of the six most abundant isotopocules of the glutamic acid fragment ion at *m/z* 84. *δ*-values were calculated against international reference materials or a "Lab" standard (Std). The $\delta^{13}C_{VPDB,C1}$ was calculated based on the $\delta^{13}C_{VPDB,OT}$ of the glutamic acid molecular ion and the $\delta^{13}C_{VPDB,C2\text{-}C5}$ of the immonium fragment ion.

	$\delta^{15}N_{Air}$ ‰	$\delta^{13}C_{VPDB,C2-C5}$ ‰	$\delta^{2}H_{Std}$ ‰	$\delta^{18}O_{Std}$ ‰	$\delta^{15}N^{13}C_{Std}$ ‰	$\delta^{13}C^{13}C_{Std,C2-C5}$ ‰	$\delta^{13}C_{VPDB}$ ‰	$\delta^{15}N_{Air,C2-C5}$ ‰	$\delta^{13}C_{VPDB,C2-C5}$
SA	-2.6±4.8	-28.1±0.4	26.8±3.4	2.0±4.8	15.1±6.1	1.0±8.8	-25.6±1.1	-2.6	-27.8
USGS 40	-6.1±3.0	-26.9±1.0	15.2±7.6	-1.6±5.7	-7.1±7.9	6.2±2.6	-22.4±2.0	-4.52	-26.1
USGS 41	50.5±3.9	-21.0±0.8**	22.3±3.9	13.6±3.1	46.2±11.0	17.1±12.4	279.0±1.1	49.5*	37.0*

* Calculated isotopocule ratios based on Boehlke et al.[6]
** Loss of 1-^{13}C-label

The $\delta^{13}C$ value of the immonium ion represents the isotope ratios of the C_2-C_5 carbons ($\delta^{13}C_{VPDB,C2-C5}$). The $\delta^{2}H_{Std}$ value gives insight to the isotope ratios of the hydrogens bound to the according carbon atoms and the amino group. The $\delta^{18}O_{Std}$ value determined for the immonium ion reflects the position specific isotope ratio of one O from the terminal carboxylic function and the $\delta^{15}N_{Air}$ remains unchanged compared to the molecular glutamic acid ion. $\delta^{15}N_{Air}$, $\delta^{18}O_{Std}$ and $\delta^{2}H_{Std}$ show the same results as the analysis of the molecular ion of glutamic acid. Therefore, the isotopic compositions of the oxygen and hydrogen sites that left the molecule during the fragmentation are similar to the average of the atoms remaining in the immonium ion. No position specific enrichment or depletion of ^{2}H and ^{18}O in these positions were detected for the three analyzed samples.

The $\delta^{13}C_{VPDB,C2-C5}$ of samples SA and USGS 40 reveal the values of the whole molecule (bulk). The $\delta^{13}C$ differences between the molecular ion and the immonium ion allow the calculation of the $\delta^{13}C$ value of the carboxylic function in the C_1-position ($\delta^{13}C_{VPDB,C1}$). For SA and USGS 40 the $\delta^{13}C_{VPDB,C1}$ of the carboxylic function appears slightly more positive, which might be plausible due to the chemistry applied during industrial production. Overall, this suggests that these two materials have no site-specific differences in the $^{13}C/^{12}C$ isotope ratios at positions C_2-C_5 with a potentially slight enrichment of <5 ‰ in the C_1-position.

The $\delta^{13}C_{VPDB,C2-C5}$ of USGS 41 of -21.0±0.8 ‰ is ~60 ‰ depleted compared to the $\delta^{13}C_{VPDB,OT}$ of the molecular ion of 39.0±0.7 ‰. Accordingly, the $\delta^{13}C_{VPDB,C1}$ of USGS 41 is 279 ‰. These results align with the information from Qi et al.[7] and proof that USGS 41 includes a substantial amount of 1-^{13}C-labelled glutamic acid.

On USGS 41 the doubly substituted isotopocules of the immonium ion ($\delta^{13}C^{13}C_{Std,C2-C5}$ and $\delta^{15}N^{13}C_{Std,C2-C5}$), both fall back into the expected ranges. The $\delta^{13}C^{13}C_{Std,C2-C5}$ isotopocule of USGS 41 is sightly enriched compared to the two stochastically distributed samples. The $\delta^{15}N^{13}C_{Std,C2-C5}$ isotopocule reflects the $\delta^{15}N$ enrichment of USGS 41 and meets the expected difference of 53 ‰ against USGS 40.

Conclusions

Orbitrap Exploris Isotope Solutions allow the comprehensive isotope ratio analyses of glutamic acid samples within 20 min analysis time and reduced sample size compared to conventional IRMS approaches utilizing fully automated UHPLC in-Flow Injection. Soft ionization by ESI-technology enables the analysis of the intact glutamic acid ions. This allows, for the first time, the simultaneous measurement of $\delta^{15}N$, $\delta^{13}C$, $\delta^{2}H$, $\delta^{18}O$, $\delta^{13}C^{13}C$ and $\delta^{15}N^{13}C$ by a single analysis. Additionally, position specific isotope analysis is enabled by the measurement of fragment ions. Isotopic values of the 1-^{13}C group and the C_2-C_5 backbone of glutamic acid as well as non-stochastic isotopic distributions for the clumping of heavy isotopes ^{15}N and ^{13}C give unique insights into the intramolecular pattern.

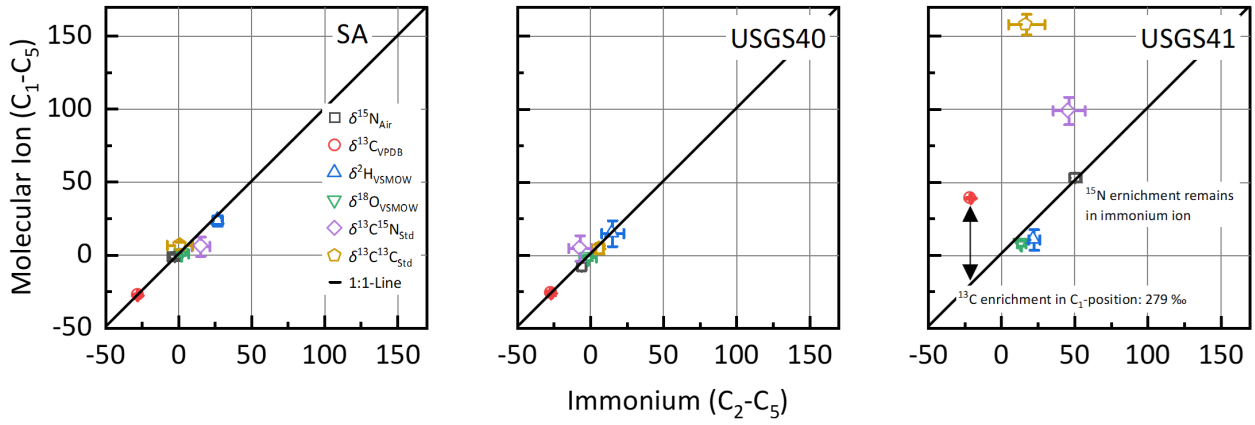

Figure 4. Comparison of *δ*-values of the molecular ions with the *δ*-values of the fragment (Immonium) ions.

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