High-resolution, specific analysis of arginine and citrulline on the Echo® MS+ system with ZenoTOF 7600+ system

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This technical note demonstrates a high-throughput, high-resolution method for differentiating amino acids that differ by only 1 Dalton. Distinguishing 2 compounds that differ by 1 Dalton can be challenging on a low-resolution instrument, as mass interference is likely to occur and the quantitative values obtained from these instruments will not be trustworthy. Arginine and citrulline are amino acids that differ in molecular weight by 1 Dalton. Differentiating between these amino acids is critical for identifying them as biomarkers or enzyme activators. Chromatographic methods used to separate these compounds can be complex and time consuming. In this work, the Echo® MS+ system with ZenoTOF 7600+ system was used to resolve arginine from citrulline in a high-throughput manner at a rate of 5 seconds per sample. A Zeno MRM^{HR} scan was employed to generate high-resolution product ions for arginine and citrulline simultaneously (Figure 1). Data review was conducted using the SCIEX OS software with library searching for added confidence in identifying arginine and citrulline mixed in solution.

Key features of the rapid, high-resolution analysis of arginine and citrulline

- **Accurate and specific quantitation of arginine (175.1 Da/60.0562 Da) and citrulline (176.1 Da/159.0960 Da):** Quantify compounds that are 1 Dalton apart with unique product ions and eliminate issues from mass interference
- **Rapid analysis:** Process Zeno MRM^{HR} data performed at 5 seconds per sample to achieve ample scans for quantitation
- **Linear and accurate calibration curves:** Quantify values with a wide dynamic range, spanning 3 orders of magnitude from 1.95µM to 1000µM with r values >0.99, and accuracies within ±20% of their stated concentrations
- **Full scan and targeted MS/MS data:** TOF and Zeno MRM^{HR} data can be collected within the same full scan MS experiment with targeted MS/MS of precursor masses

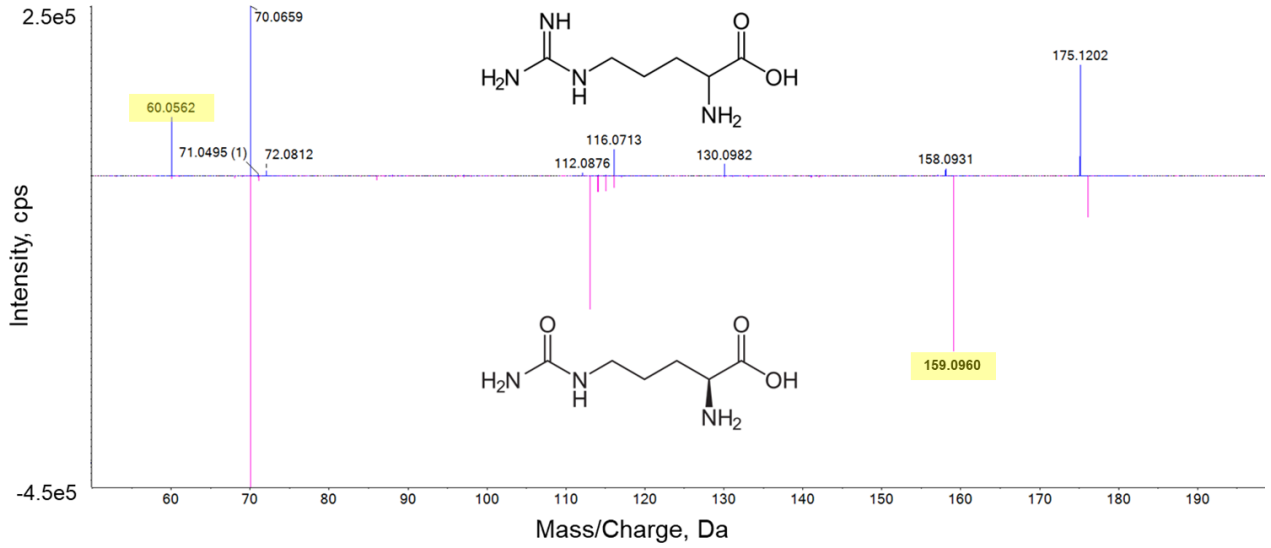

Figure 1. Arginine and citrulline product ions. The product ions for arginine (blue trace) and citrulline (pink trace, inverted) were simultaneously obtained from a 5-second Zeno MRM^{HR} scan of a sample containing arginine and citrulline. The yellow highlighting indicates the unique product ions that occurred at 60.0562 Da for arginine and 159.0960 Da for citrulline.

Introduction

Arginine and citrulline are amino acids which only differ in molecular weight by 1 Dalton. Many analytical methods require these compounds to be derivatized for their analysis due to the lack of a natural chromophore in their structure.[1] Derivatization adds time to sample preparation and subsequent liquid chromatography methods can take minutes to complete.[2] These amino acids can be used as biomarkers and enzyme activators but their proximity in molecular weight makes them difficult to be accurately quantified even if derivatization is performed. Here, we present a rapid, high-resolution solution to identify and quantify arginine and citrulline.

Methods

Sample preparation: Arginine, citrulline and a combination of equimolar arginine and citrulline were serially diluted in water to a concentration range of 1.95µM to 1000µM. All samples contained 250µM of phenylalanine as an internal standard (IS).

Acoustic ejection: A total of 70 nL of sample was ejected in 5 second intervals at 10 Hz in wide peak mode. Methanol with 0.1% formic acid was the carrier solvent and a flow rate of 400 µL/min was used.

Mass spectrometry: A Zeno MRM^{HR} method was used to quantify arginine and citrulline using both a common product ion and a unique product ion. The data from the common and unique product ions were then compared and the amount of mass interference was calculated. A single unique product ion for the IS was analyzed (Tables 1-5).

Data processing: The Analytics and Explorer modules of SCIEX OS software were used for data processing. The NIST library was used to ensure that the analyte identification was correct for arginine and citrulline. The amount of mass interference was expressed in percent and was calculated using the following equation:

$$Mass\ interference\ (\%) = \left(\frac{A}{B}\right) \times 100$$

where A = the calculated concentration of the interfering analyte and B = the actual concentration of the targeted analyte. Both concentrations were expressed in µM.

Table 1. Source parameters and values.

Parameter	Value
Polarity	Positive
Spray voltage (V)	5500
Curtain gas (psi)	35
CAD gas (psi)	11
Ion source gas 1 (psi)	90
Ion source gas 2 (psi)	75
Temperature (°C)	400

Table 2. TOF MS parameters and values.

Parameter	Value
Scan type	Zeno MRMHR
TOFMS start mass (m/z)	50
TOFMS stop mass (m/z)	200
Accumulation time (s)	0.1
Declustering potential (V)	60
Time bins to sum	4

Table 3. TOF MS/MS parameters and values.

Parameter	Value
Q1 resolution	Unit
Zeno pulsing	On
Zeno threshold (cps)	2000

Table 4. Zeno MRM^{HR} transitions selected for quantitation.

Analyte	Common product ion (m/z)	Unique product ion (m/z)
Arginine	175.1256/70.0659	175.1256/60.0562
Citrulline	176.1094/70.0659	176.1094/159.0960

Table 5. Zeno MRM^{HR} parameters and values.

Compound ID	Precursor ion	TOF start (m/z)	TOF stop (m/z)	Accumulation time (s)	Declustering potential (V)	Collision energy (V)	CE spread (V)	Time bins to sum
Arginine	175.1	50	200	0.07	50	23	10	4
Citrulline	176.1	50	200	0.07	50	23	10	4
Phenylalanine (IS)	166.2	50	200	0.07	50	21	10	4

High-resolution product ions

The Zeno MRMHR scan provides a TOF MS scan in addition to performing an MRM scan for precursor masses. The user enters a precursor ion for each analyte or IS to be measured and then the user can choose the TOF MS/MS mass range to be scanned. The collision energy can be set to a fixed number or spread around a central collision energy value.

When combined with wide peak mode ejections, the resulting scans include data-rich product ions that were individually selected for analyte and IS processing with a library search using the Analytics module in SCIEX OS software (Figure 1). Wide peak mode with a 5 second sample ejection interval was chosen to ensure that 12 scans were obtained for each sample.

Calibration curves

Calibration curves with common and unique product ions were constructed from the samples containing a combination of arginine and citrulline at concentrations ranging from 1.95µM to 1000µM (Figure 3).

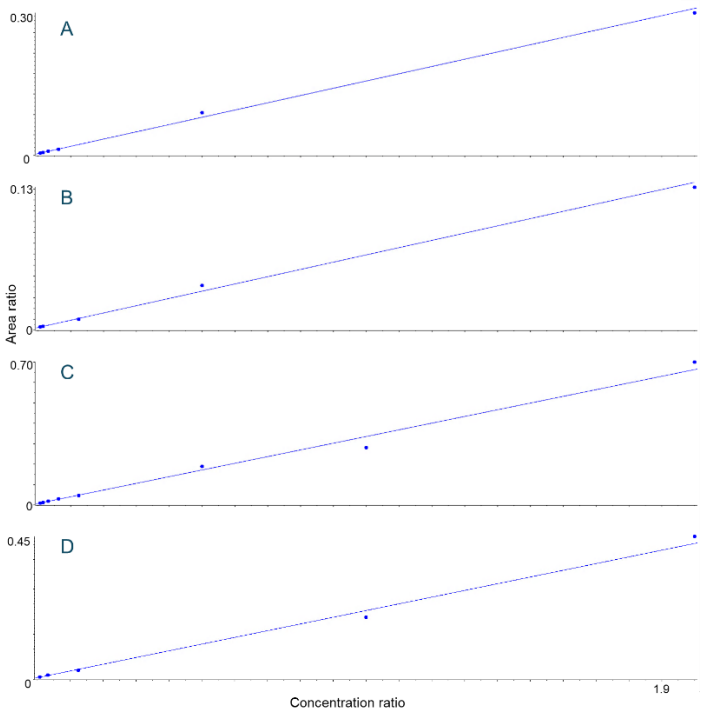

Figure 3. Calibration curves for the analysis of arginine and citrulline. Calibration curves for arginine based on a common (A) or unique product ion (B) are shown, in addition to calibration curves for citrulline based on a common (C) or unique product ion (D). The area ratio was calculated by dividing the analyte peak area by the IS peak area. The concentration ratio was calculated by dividing the analyte concentration by the IS concentration.

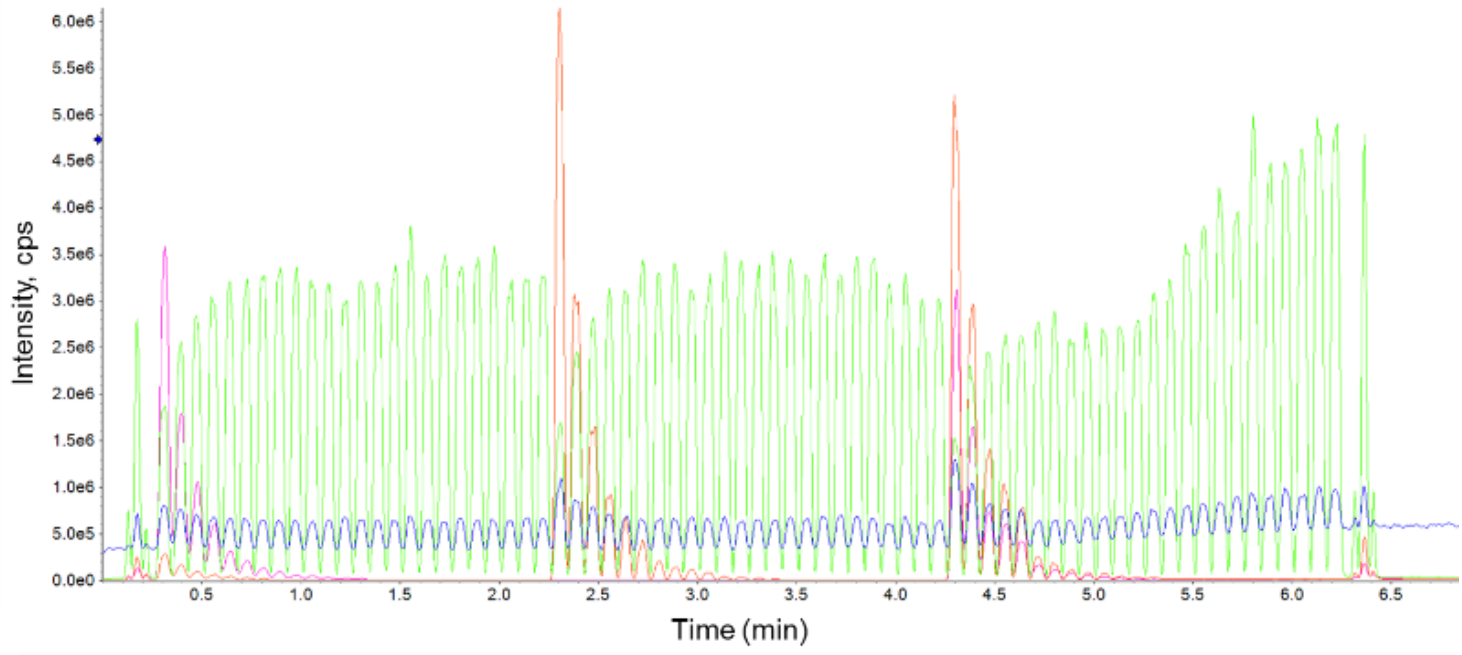

Figure 2. Rapid, quantitative analysis of arginine, citrulline and phenylalanine using the Echo® MS+ system with ZenoTOF 7600+ system. The sample was analyzed in triplicate. Arginine is shown in pink and citrulline is shown in orange. Phenylalanine was used as an IS and is shown in green. The TOF MS scan is shown in blue.

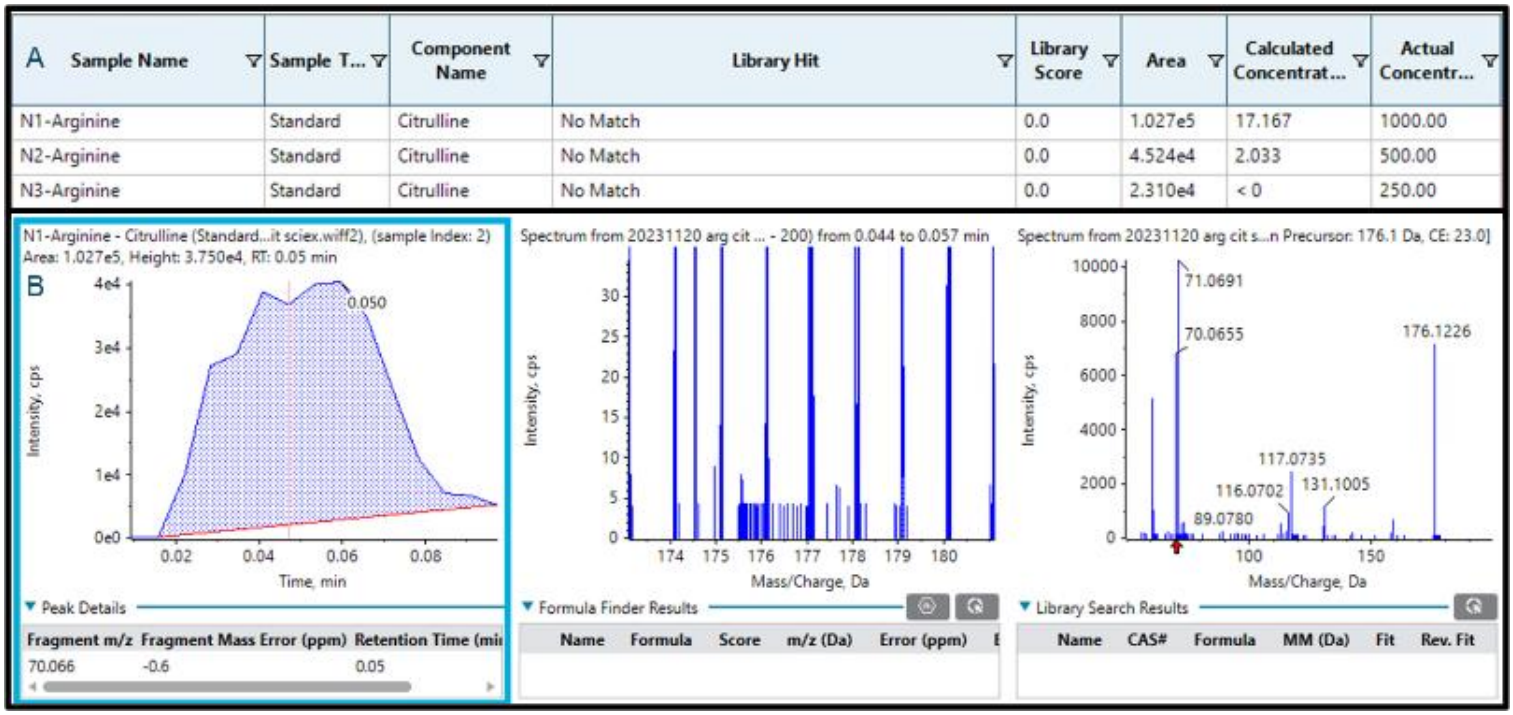

Figure 4. Samples containing arginine at 1000µM, 500µM and 250µM analyzed using the citrulline common product ion MRM channel. This analysis registered 17.17µM, 2.033µM and < 0µM of mass interference, respectively, indicated in the "calculated concentration" column (A). The extracted ion chromatogram peak, TOF MS and TOF MS/MS scan with library search results are shown for the 1000µM arginine sample (B). When quantified with the citrulline common product ion, the results showed 17.17µM mass interference.

The calibration curves were then used to quantify arginine and citrulline and were applied to calculate the amount of mass interference. All calibration point accuracies were within ±20% of their assigned values and all calibration curve r values were >0.994.

Assessment of mass error

When quantifying with unique product ions for arginine and citrulline, no mass interference was detected for either analyte. A small percentage of mass interference was detected when a common product ion was used for quantitation. Only in high concentration arginine samples was arginine mis-identified as citrulline. Less than 2% mass interference was detected in the citrulline common product ion channel when testing a 1000µM and a 500µM arginine sample (Table 6).

No mass interference was detected in samples with concentrations ≤250µM of either analyte, independent of the type of product ion used for quantitation. With regards to the small amount of mass interference observed in the arginine sample when quantifying with the common product ion for citrulline, the library hit yielded "no match," resulting in a library score of 0 (Figure 4).

Table 6. Percent (%) mass interference detected in high concentration samples of arginine and citrulline.

Analyte, concentration	Arginine, common product ion	Arginine, unique product ion	Citrulline, common product ion	Citrulline, unique product ion
Arginine 1000 µM	n/a	n/a	1.72	0
Citrulline 1000 µM	0	0	n/a	n/a
Arginine 500 µM	n/a	n/a	0.203	0
Citrulline 500 µM	0	0	n/a	n/a

The peak area ratios obtained from the citrulline common product ion MRM channel were plotted against the peak area ratios obtained from the citrulline unique product ion MRM channel in samples with arginine concentrations of 1000µM, 500µM and 250µM (Figure 5). The mass interference observed with the common product ion was neutralized when the same 3 samples were analyzed using the citrulline unique product ion.

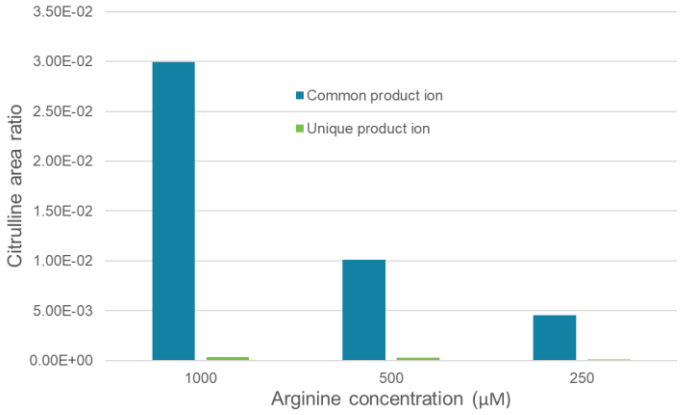

Figure 5. Observed arginine peak area ratios determined by the citrulline common and unique product ions. The peak area ratios were determined at concentrations of 1000µM, 500µM and 250µM.

Quantitation of arginine and citrulline was accurate and specific based on the calibration curve data. To further ensure accurate identification of arginine and citrulline, we utilized the NIST library. At concentrations as low as 1.95µM, the NIST library accurately identified the correct compounds in the samples (Figure 6). For the sample containing arginine, only arginine and the phenylalanine IS were detected and for the sample containing citrulline, only citrulline and the phenylalanine IS were detected. For the combined arginine and citrulline sample, arginine, citrulline and the phenylalanine IS were all detected. All library scores suggested a good level of confidence, as the lowest library score was 79.9.

Sample Name	Component Name	Fragment Mass	Library Hit	Library Score
N10-Arginine	Arginine	70.066	DL-Arginine (NIST) [Smart Confirmation]	84.9
N10-Arginine	Citrulline	70.066	No Match	0.0
N10-Arginine	Phenylalanine IS	120.082	DL-Phenylalanine (NIST) [Smart Confirmation]	100.0
N10-Arginine	Arginine 2	60.056	DL-Arginine (NIST) [Smart Confirmation]	79.9
N10-Arginine	Citrulline 2	159.096		N/A
O10-Citrulline	Arginine	70.066		N/A
O10-Citrulline	Citrulline	70.066	L-Citrulline (NIST) [Smart Confirmation]	98.4
O10-Citrulline	Phenylalanine IS	120.082	DL-Phenylalanine (NIST) [Smart Confirmation]	100.0
O10-Citrulline	Arginine 2	60.056		N/A
O10-Citrulline	Citrulline 2	159.096	L-Citrulline (NIST) [Smart Confirmation]	98.9
P10-Arginine and Citrulline	Arginine	70.066	L-Arginine (NIST) [Smart Confirmation]	88.5
P10-Arginine and Citrulline	Citrulline	70.066	L-Citrulline (NIST) [Smart Confirmation]	96.5
P10-Arginine and Citrulline	Phenylalanine IS	120.082	DL-Phenylalanine (NIST) [Smart Confirmation]	100.0
P10-Arginine and Citrulline	Arginine 2	60.056	L-Arginine (NIST) [Smart Confirmation]	84.3
P10-Arginine and Citrulline	Citrulline 2	159.096	L-Citrulline (NIST) [Smart Confirmation]	97.6

Figure 6. Library search confirmation performed using the Analytics module in SCIEX OS software. The sample name, MRM component, fragment mass, library hit and library score for samples containing arginine, citrulline and a 1.95µM solution containing arginine and citrulline are shown. Each sample included 250µM phenylalanine as an IS.

Mass interference when quantifying with the common product ion for citrulline was only observed at concentrations >250µM. For quantitation at concentrations <250µM, either the unique or common MRM channel could produce specific results. Ultimately, choosing a unique product ion is preferred, as the likelihood of registering mass interference is much lower, given the product ion is exclusive to the analyte of interest. However, these results show that when unique product ions are not available, the common product ions from the high-resolution, Echo® MS+ system with ZenoTOF 7600+ system could be used for sensitive and specific quantitation if excessively high concentrations of potential mass interfering substances are avoided.

Conclusions

- Achieved specific quantitation of arginine and citrulline, which are 1 Dalton apart in parent mass
- Wide peak mode provided 10 data points per peak with a 3.7 ms ejection of 70 nL of sample
- Rapid, linear quantitation with an IS across 3 orders of magnitude
- Mass interference was significantly reduced by quantifying with a unique product ion in Zeno MRM^{HR} mode
- Mass interference was absent when quantifying with unique product ions
- NIST library search was used to increase our identification confidence

References

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