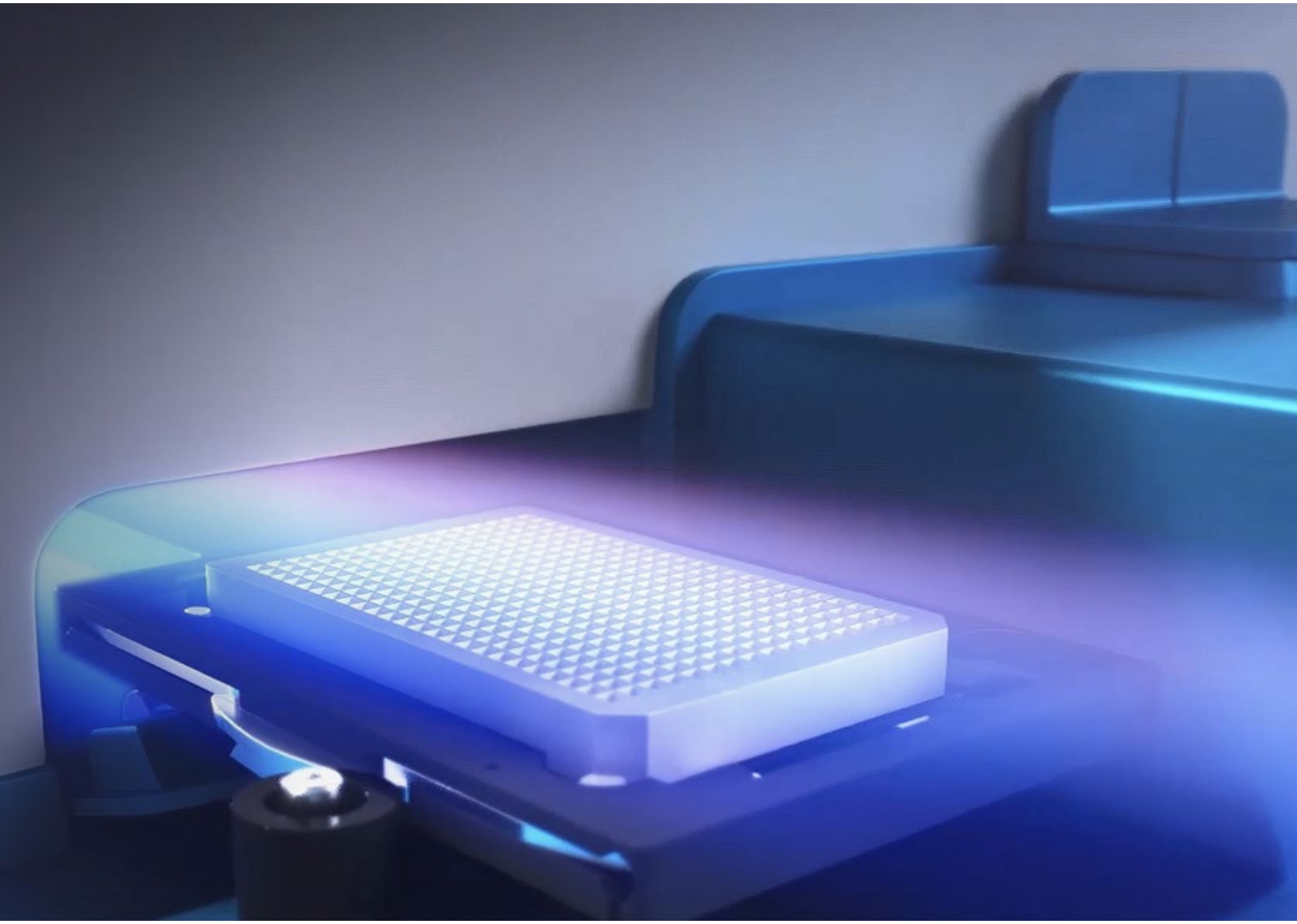
2024 生物製藥應用文集

高通量分析 (High-throughput analysis)



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目錄

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

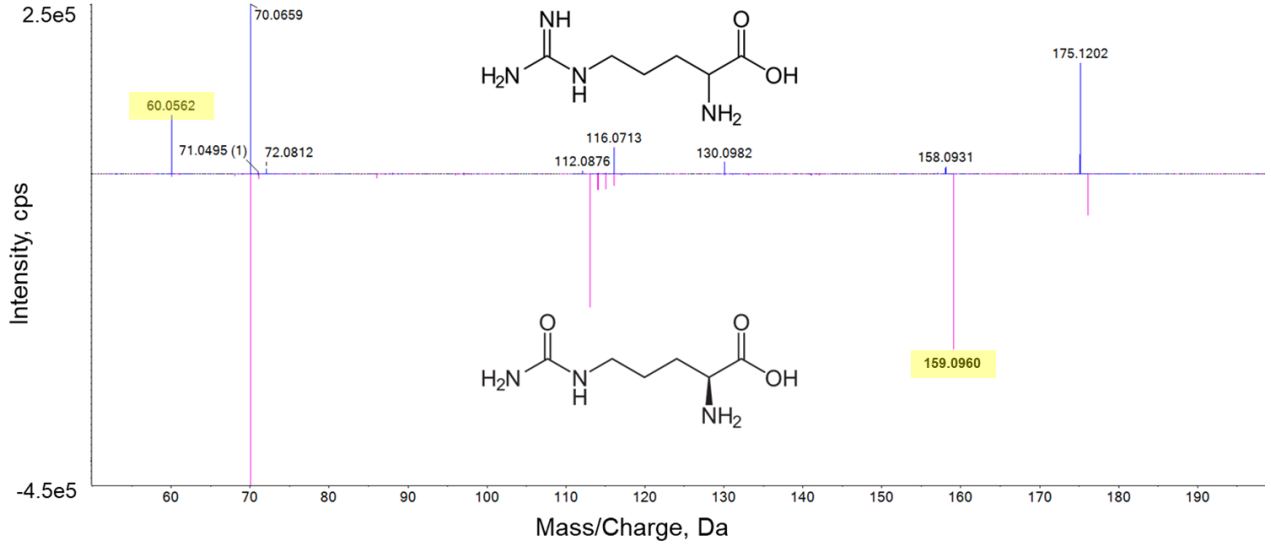

Figure 1. Arginine and citrulline product ions. The product ions for arginine (blue trace) and citrulline (pink trace, inverted) were simultaneously obtained from a 5-second Zeno MRM^{HR} scan of a sample containing arginine and citrulline. The yellow highlighting indicates the unique product ions that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

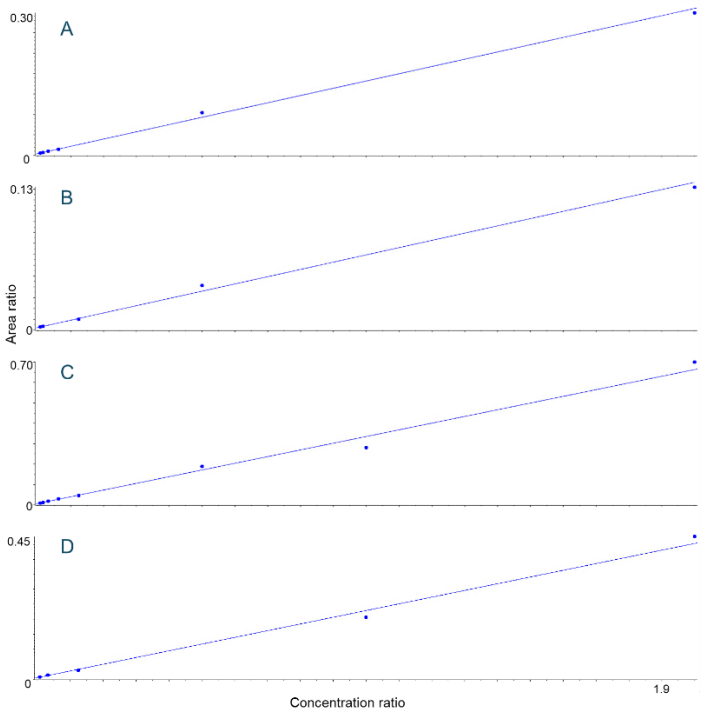

Figure 3. Calibration curves for the analysis of arginine and citrulline. Calibration curves for arginine based on a common (A) or unique product ion (B) are shown, in addition to calibration curves for citrulline based on a common (C) or unique product ion (D). The area ratio was calculated by dividing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

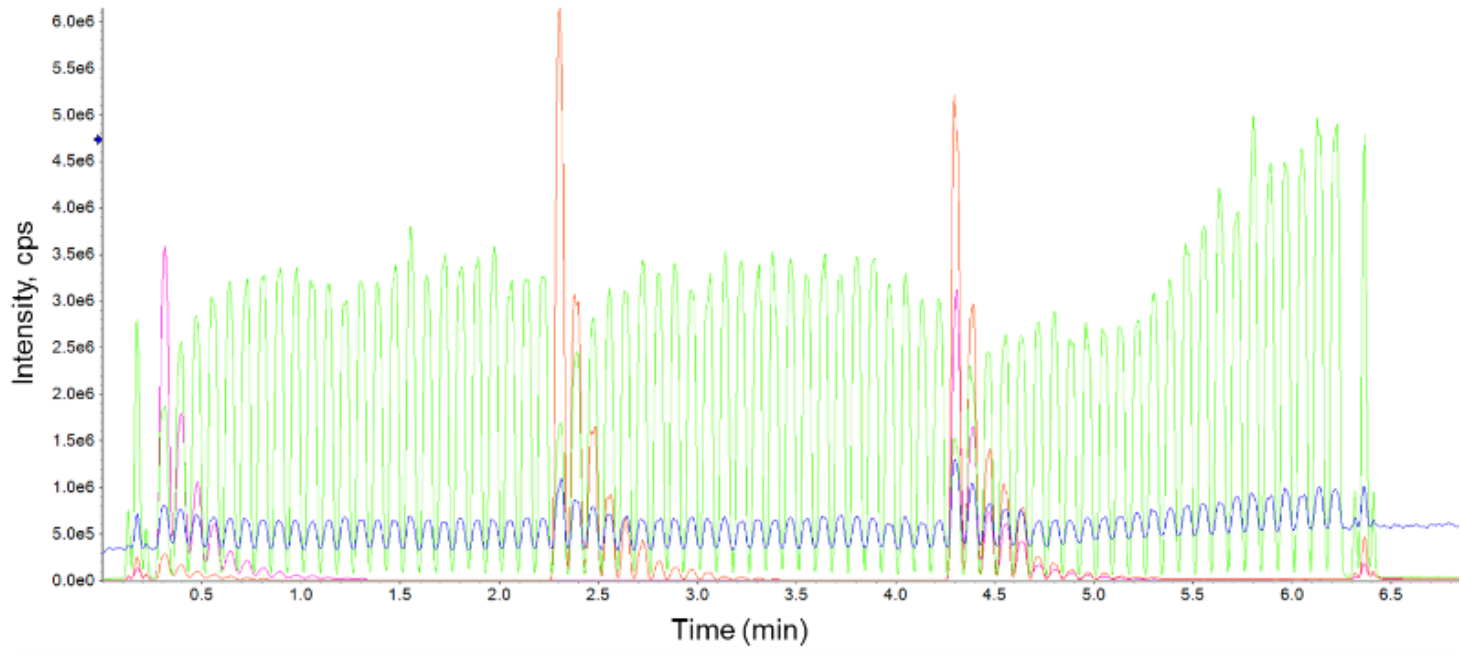

Figure 2. Rapid, quantitative analysis of arginine, citrulline and phenylalanine using the Echo® MS+ system with ZenoTOF 7600 system. The sample was analyzed in triplicate. Arginine is shown in pink and citrulline is shown in orange. Phenylalanine was used as an IS and is shown in green. The TOF MS scan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

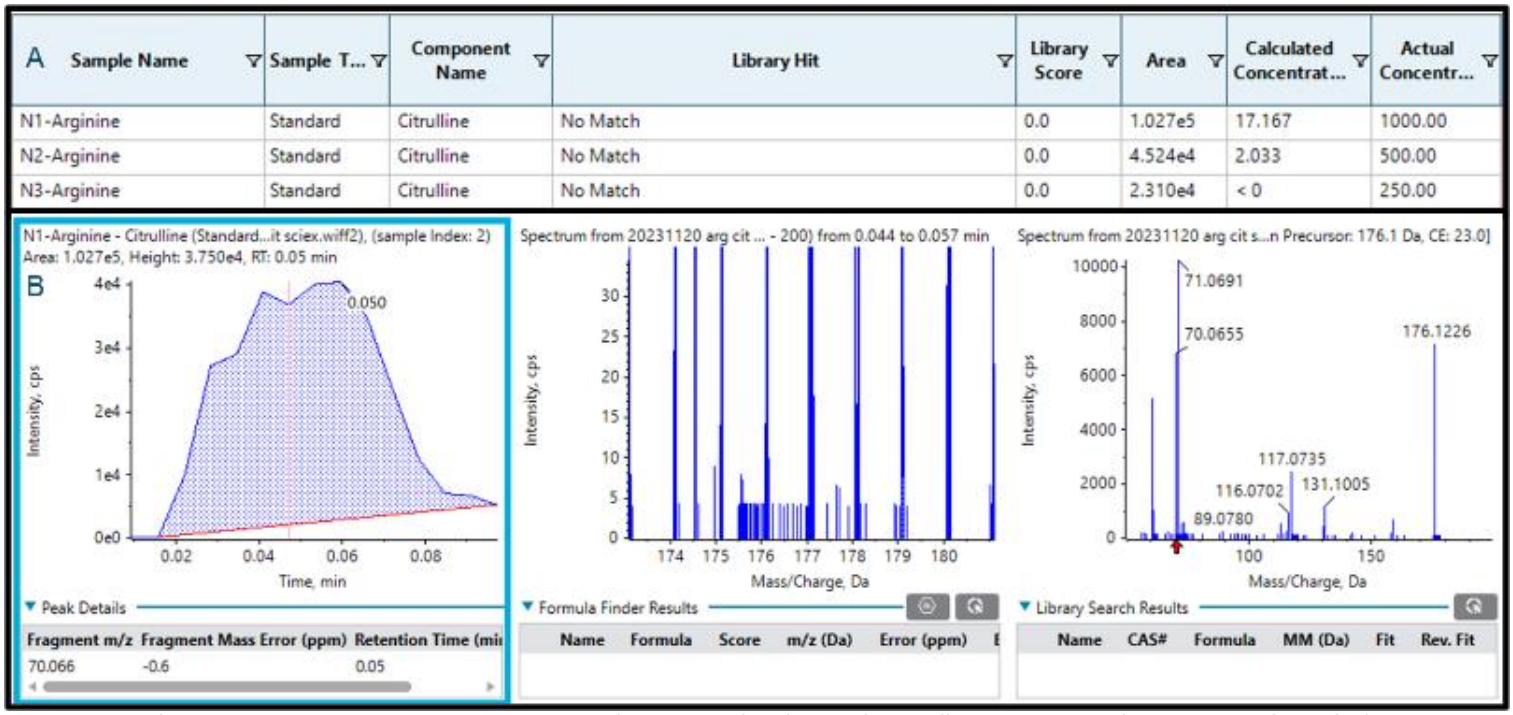

Figure 4. Samples containing arginine at 1000µM, 500µM and 250µM analyzed using the citrulline common product ion MRM channel. This analysis registered 17.17µM, 2.033µM and < 0µM of mass interference, respectively, indicated in the "calculated concentration" column (A). The extracted ion chromatogram 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.

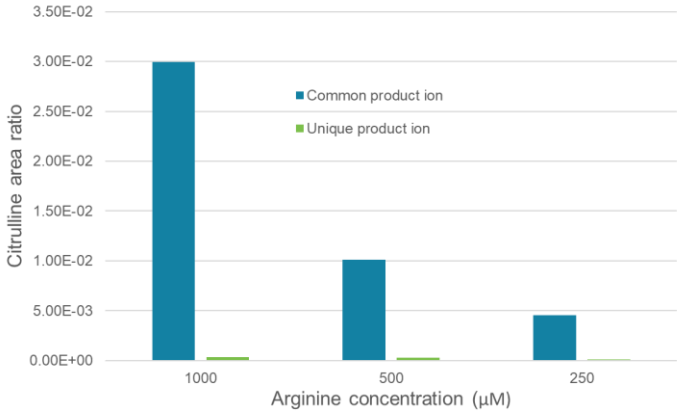

Figure 5. Observed arginine peak area ratios determined by the citrulline common and unique product ions. The peak area ratios were determined at concentrations of 1000µM, 500µM and 250µM.

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

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2. Perez-Neri, E. Castro, S. Montes, M-C. Boll, J. Barges-Coll, JL, Soto-Hernandez, C. Rios. Arginine, Citrulline and Nitrate Concentrations in the Cerebrospinal Fluid from Patients with Acute Hydrocephalus. Journal of Chromatography B. 2007, 851;250-6. https://www.sciencedirect.com/science/article/abs/pii/S1570023206008658?via%3Dihub

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Rapid and accurate bioanalysis for drug screening

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Sensitivity, specificity and throughput are vital factors in bioanalytical laboratories. Most laboratories aim to achieve low levels of detection while using less sample and processing many samples per minute. These priorities can help increase efficiency and productivity in a therapeutic laboratory. Plate readers have commonly been used for drug screening but developing these methods can be time consuming. Furthermore, achieving high specificity with plate readers can be challenging, as can achieving high throughput with liquid chromatography methods. This technical note demonstrates a highly sensitive, highly specific, high-throughput quantitative solution for drug screening using the Zeno trap on the Echo® MS+ system with ZenoTOF 7600 system.

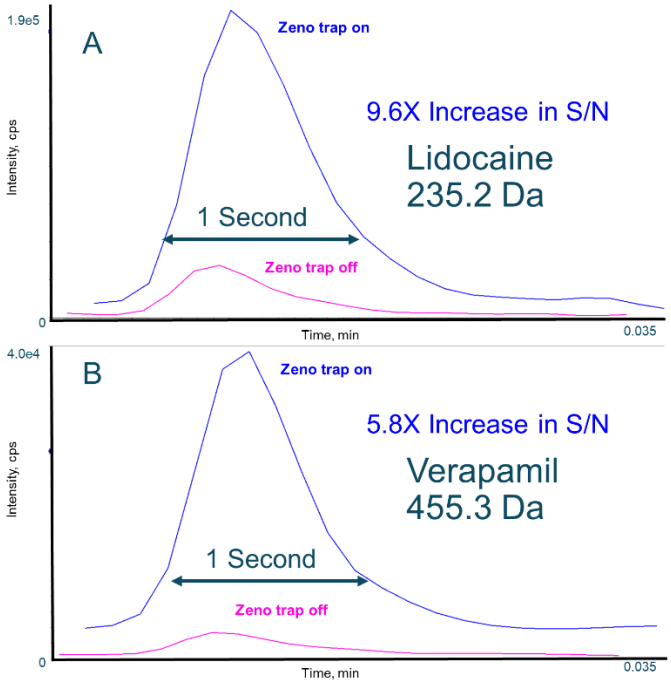

Figure 1. Peaks observed with and without the Zeno trap enabled. Samples of 7.813 ng/mL lidocaine (A) and verapamil (B) were analyzed.

Key benefits of the Zeno MRM^{HR} scan with and without application of the Zeno trap

- **Enhanced signal response:** Achieve approximately 7x more signal for lidocaine and verapamil with the Zeno trap enabled compared to analysis without the Zeno trap
- **Low detection limits:** Detection of lidocaine and verapamil at 7.813 ng/mL was achieved with the Zeno trap
- **Increased specificity:** The Zeno MRM^{HR} scan provided a full product ion scan from a targeted precursor analyte for a 1-second peak width and could be used for library searching to aid with compound confirmation[1,2]
- **High throughput:** Rapid batch analysis of 63 samples in 2.6 minutes is possible
- **Zeno MRM^{HR} method customization:** Analyze a fixed fragment ion or scan a wide range of fragment ions

Introduction

High-throughput screening labs commonly seek instruments with high sensitivity, high specificity and high throughput.[3] Plate readers are often employed for early phase drug screening but development for these types of assays can be time consuming.[4] Additionally, the endpoint of a plate reader assay is based on a reaction that generates an absorption or emission signal that is not caused by the compound itself, which can reduce the specificity of the assay.[4]

The Echo® MS+ system with ZenoTOF 7600 system provides high-resolution mass spectral data, enhanced sensitivity with the application of the Zeno trap, high specificity and rapid throughput with sample ejection rates up to 1 sample/second.[5] With the Zeno MRM^{HR} scan selected, the Echo® MS+ system with ZenoTOF 7600 system can generate a single TOF MS spectrum and specific MS/MS product ion scans from targeted compounds with defined precursor masses. The user can opt to analyze a single product ion or opt to analyze a wide range of product ions at collision energies and collision energy spreads (a range of energies within a TOF MS/MS accumulation).

In this study, we demonstrated the ability of the Echo® MS+ system with ZenoTOF 7600 system to produce full-range product ion scans in 1 second for 2 commonly used small molecule probes, lidocaine and verapamil. Nine different concentrations ranging from 7.813 ng/mL to 2000 ng/mL were analyzed using Zeno MRM^{HR}.

Methods

Sample preparation: Concentration curves for lidocaine and verapamil were prepared in an Echo MS® qualified 384-well plate using the serial dilution function of the INTEGRA (Hudson, NH) 8-channel pipettes. The samples contained lidocaine and verapamil at 2000 ng/mL, 1000 ng/mL, 500 ng/mL, 250.0 ng/mL, 125.0 ng/mL, 62.50 ng/mL, 31.30 ng/mL, 15.60 ng/mL and 7.813 ng/mL concentrations.

Acoustic ejection: A total of 30 nL of the sample was ejected at an interval of 2 seconds per ejection using standard peak mode (400 Hz repetition rate). The carrier solvent consisted of a mix of 70:30 (v/v), water/acetonitrile and 0.1% (v/v) formic acid. A flow rate of 400 µL/min was used.

Mass spectrometry: A Zeno MRM^{HR} method with a wide range of fragment ions was optimized and the "Zeno trapping" checkbox of the optimized method was then toggled "on" and "off" as the sample batches were run. The Zeno MRM^{HR} method was optimized by directly infusing the 125 ng/mL standard of each compound via the Echo® MS+ system (Tables 1-4).

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 MRM^{HR}*
TOF MS start mass (m/z)	*50*
TOF MS stop mass (m/z)	*600*
Accumulation time (s)	*0.02*
Declustering potential (V)	*30*
Time bins to sum	*4*

Table 3. TOF MS/MS parameters and values.

Parameter	Value
Q1 resolution	*Unit*
Zeno trapping	*On and off*
Zeno threshold (cps)	*20,000*

Table 4. Zeno MRM^{HR} parameters and values.

Compound	Precursor ion (m/z)	TOF start (m/z)	TOF stop (m/z)	Accumulation time (s)	Declustering potential (V)	Collision energy (V)	Collision energy spread (V)	Time bins to sum
Lidocaine	235.1790	50	200	0.04	30	40	10	4
Verapamil	455.2878	50	500	0.04	30	41	5	4

Data processing: Data were processed using the Analytics and Explorer modules of SCIEX OS software.

Increased peak areas observed at all tested concentrations with the Zeno trap enabled

On average, with the Zeno trap enabled, 9.6-fold and 5.3-fold increases were observed for the signal-to-noise ratios for lidocaine and verapamil, respectively. These results were achieved when the Zeno MRM^{HR} scan was performed with a wide range of fragment ions (Figure 1).

The average area values for lidocaine and verapamil both increased at different magnitudes when the Zeno trap was enabled. These results are key at low concentrations, as the Zeno trap can therefore be used to enhance sensitivity (Figure 2).

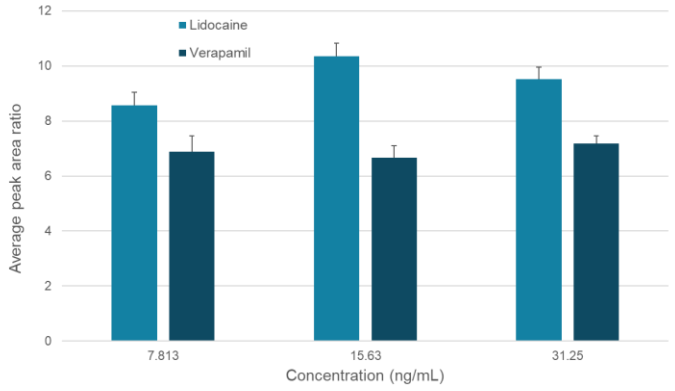

Figure 2. Average peak area ratios for lidocaine and verapamil at low concentrations. The peak area ratio was determined by dividing the peak area observed with the Zeno trap enabled by the peak area observed without the Zeno trap. The ratio was calculated for lidocaine and verapamil at 3 low concentrations using a Zeno MRM^{HR} method with a wide range of fragment ions.

The calibration curves for both lidocaine and verapamil showed an increased slope with the Zeno trap enabled, indicating increased sensitivity for both analytes when using the Zeno trap (Figure 3). The peak area observed with the Zeno trap was significantly ($p < 0.05$) better than that without the Zeno trap (Table 5).

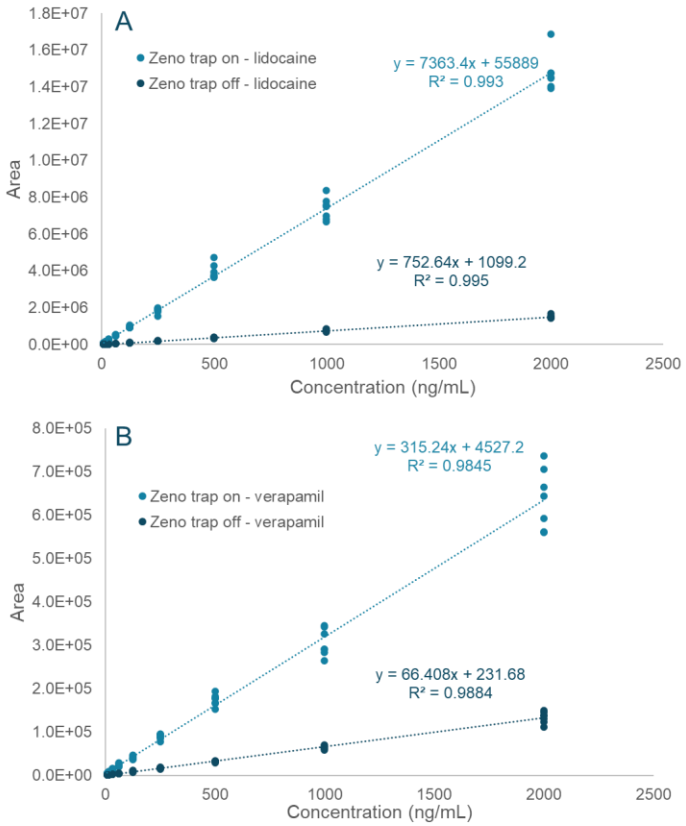

Figure 3. Calibrations curves for lidocaine (A) and verapamil (B). The calibration curves were constructed with and without use of the Zeno trap.

Accuracy and percent coefficient of variation (%CV) did not change significantly with and without the Zeno trap. However, no %CV or average accuracy values were reported for lidocaine at 7.813 ng/mL without the Zeno trap due to the lack of sensitivity. Analysis of 7.813 ng/mL lidocaine with the Zeno trap yielded a %CV of 6.7% and an average accuracy of 86.97% (Table 6).

Table 5. Calculated p values from the difference in peak areas with and without the Zeno trap enabled at 9 different concentrations. Seven replicates were performed at each concentration.

Concentration (ng/mL)	Lidocaine	Verapamil
2000	*2.27E-08*	*2.24E-06*
1000	*9.65E-08*	*8.70E-07*
500	*3.40E-07*	*6.43E-08*
250	*1.67E-07*	*1.73E-07*
125	*3.45E-08*	*1.80E-06*
62.5	*2.07E-08*	*3.26E-06*
31.25	*1.40E-07*	*1.04E-06*
15.63	*1.18E-06*	*7.03E-08*
7.813	*1.00E-06*	*6.96E-06*

Table 6. Average percent accuracy and %CV across all replicates of lidocaine and verapamil. Analysis was performed with and without the Zeno trap enabled at 9 different concentrations.

	Zeno trap enabled				Zeno trap disabled			
	Lidocaine		Verapamil		Lidocaine		Verapamil	
Concentration (ng/mL)	% CV	Average accuracy (%)	% CV	Average accuracy (%)	% CV	Average accuracy (%)	% CV	Average accuracy (%)
7.813	*6.7*	*87*	*11.7*	*94.9*	*N/A*	*N/A*	*7.3*	*94*
15.63	*11.6*	*101.6*	*10.5*	*98*	*11.6*	*95.1*	*9.1*	*104.3*
31.25	*9.5*	*102.6*	*8.9*	*99.8*	*8.4*	*100.5*	*6.6*	*107.8*
62.5	*6.6*	*103.1*	*8*	*97.8*	*6.4*	*109.2*	*6.8*	*90.6*
125	*6.8*	*100.2*	*11.3*	*94.9*	*9.4*	*97.7*	*7.7*	*97.1*
250	*8.3*	*98.5*	*7.6*	*108.4*	*10.4*	*97.9*	*7*	*101.8*
500	*6.2*	*103*	*7.6*	*107.5*	*12.5*	*102.8*	*6.6*	*108*
1000	*8.3*	*99.5*	*10.4*	*95.4*	*8.1*	*98.1*	*5.3*	*96.7*
2000	*6.7*	*99.7*	*10.9*	*99.8*	*10.7*	*100.5*	*4.6*	*99.8*

Discussion

When scanning a range of product ions, it is possible to quantify more than 1 product ion. By inputting 1 Zeno MRM^{HR} channel in the "MS Method" of SCIEX OS software, the user can select multiple fragment ions to quantify in the Analytics module (Figure 4). This experiment can be performed efficiently at rates as fast as 1 sample/second and is compatible with batch processing.

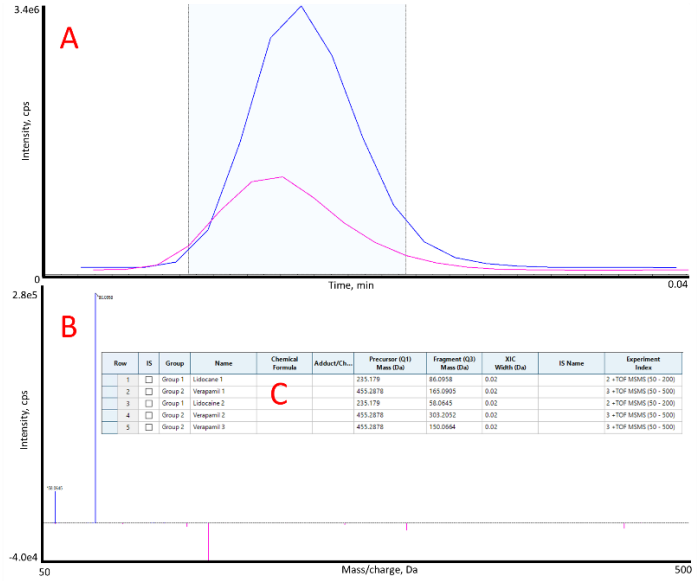

Figure 4. Processing results in SCIEX OS software for lidocaine and verapamil. A) Traces are shown for 250 ng/mL samples of lidocaine (blue) and verapamil (pink) based on a wide range of product ions from the MRM^{HR} scan. Peaks span 1 second of data. B) Product ion scans are shown for lidocaine and verapamil. C) The quantitation table showing multiple product ions, derived from the Zeno MRM^{HR} scan, for both lidocaine and verapamil.

The area values significantly increased when the Zeno trap feature was applied to the Zeno MRM^{HR} scan. The Zeno trap routinely improved lower limits of quantitation by approximately 7-fold. The amount of increase appeared to be analyte-dependent, related to the m/z of the fragments.

Conclusions

- Use of the Zeno trap yielded approximate 6- and 9-fold increases in signal for verapamil and lidocaine, respectively
- Linearity was observed from 7.813 ng/mL to 2000 ng/mL when using the Zeno trap
- Analysis of 63 samples was completed in 2.6 minutes using the Zeno MRM^{HR} scan
- A full TOF MS scan was performed and a wide range of product ions were analyzed for verapamil and lidocaine using the Zeno MRM^{HR} scan

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4. Rapid MS/MS analysis with Acoustic Ejection Mass Spectrometry (AEMS). SCIEX technical note, RUO-MKT-02-11385-A.
5. Highly sensitive quantification and selective identification of pesticides in food with Zeno MRMHR. SCIEX technical note, RUO-MKT-02-13902-C.

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Rapid and accurate quantitation of thyroglobulin biomarkers using the Echo® MS+ system with ZenoTOF 7600 system

Aaron Stella, Jacob W. McCabe and Anuja Bhalkikar
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Thyroglobulin is a protein biomarker used to monitor thyroid cancer treatment and is also responsible for synthesizing thyroid hormones. In this technical note, we describe a rapid Zeno MRM^{HR} method capable of detecting and quantifying singly and doubly charged thyroglobulin peptides using the Echo® MS+ system with ZenoTOF 7600 system.

Serum thyroglobulin is a tumor marker used in managing patients diagnosed with differentiated thyroid carcinoma.[1] It also plays a role in synthesizing the thyroid hormones triiodothyronine (T3) and thyroxine (T4).[2] Many LC-MS/MS methods for quantifying thyroglobulin peptides have been established but runtimes take several minutes.[3]

Here, we developed a Zeno MRM^{HR} method to quantify 2 peptides rapidly and accurately without sample preparation. The 2 peptides derived from the thyroglobulin protein, VIFDANAPVAVR and FSPDDSAGASALLR, were serially diluted to create a calibration curve with values ranging from 3.91 ng/mL to 250 ng/mL in a solvent. We achieved the same sensitivity level as polyclonal antibody enrichment but at a significantly faster rate using the Echo® MS+ system with ZenoTOF 7600 system.[4]

The Bio Tool Kit micro-application in SCIEX OS software was used to select the peptide fragments for the quantitation assay. After peptide fragment selection, the singly and doubly charged peptides were quantified using the Analytics module of SCIEX OS software. Each sample containing the 2 peptides and 2 isotopically labeled peptide analogs was analyzed at a rate of 5 seconds per sample to achieve high-quality Zeno MRM^{HR} data.

Key features of the quantitation of thyroglobulin peptides

- **Low-ng/mL level quantitation of singly and doubly charged peptides:** A lower limit of quantitation (LLOQ) of 3.91 ng/mL was achieved for thyroglobulin peptides VIFDANAPVAVR and FSPDDSAGASALLR
- **Rapid sample acquisition:** Samples were analyzed at a rate of 5 seconds per sample using the wide peak mode for Zeno MRM^{HR} quantitation

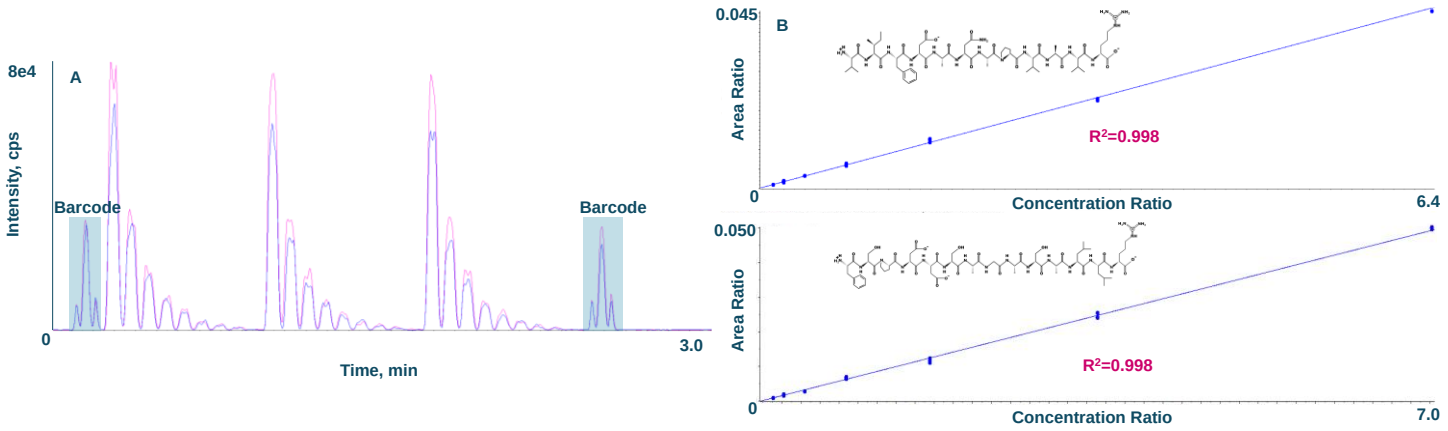

Figure 1. Representative peak ejections and calibration curves from the Zeno MRM^{HR} analysis of the thyroglobulin peptides, VIFDANAPVAVR and FSPDDSAGASALLR. A) Rapid, 5-second-wide peak ejections from the triplicate analysis of VIFDANAPVAVR (blue) and FSPDDSAGASALLR (pink). Start and finish barcodes are shown. B) Calibration curves are shown alongside the structure of the 2 peptides derived from the thyroglobulin protein. VIFDANAPVAVR and FSPDDSAGASALLR are shown at the top and bottom, respectively. Linearity was achieved between 3.91 ng/mL and 250 ng/mL, with an r^2 of 0.998. Each concentration level was run in triplicate.

- **Fast fragment selection using the Bio Tool Kit micro-application:** Fragment ions were rapidly identified, which facilitated product ion selection for the quantitation assay
- **Fast analysis with negligible carryover:** Peptide quantitation analysis was rapidly performed with negligible carryover
- **Streamlined data management:** Data acquisition and processing are integrated into SCIEX OS software

Methods

Sample preparation: Thyroglobulin peptides VIFDANAPVAVR and FSPDDSAGASALLR were diluted to 1000 ng/mL in 35:65 (v/v), water/acetonitrile. A concentration curve was prepared by serial dilution using 35:65 (v/v), water/acetonitrile covering concentrations ranging from 3.91 ng/mL to 250 ng/mL of each peptide. Isotopically labeled analogs of VIFDANAPVAVR and FSPDDSAGASALLR were analyzed as internal standards at 39.3 ng/mL and 35.6 ng/mL, respectively.

Acoustic ejection: The carrier solvent was methanol with 0.1% formic acid and its flow rate was set to 400 µL/min. A total of 70 nL of the sample was ejected over 5 seconds in wide peak mode using the SP fluid class. Each sample was ejected in triplicate.

Mass spectrometry: A Zeno MRM^{HR} method using the "peptides" workflow was developed to quantify the 2 thyroglobulin peptides and their labeled internal standards. The conditions used for this method are outlined in Tables 1–4.

Table 1. Source parameters and values.

Parameter	Value
Polarity	*Positive*
Spray voltage (V)	*5500*
Curtain gas (psi)	*25*
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 MRM^{HR}*
TOF MS start mass (m/z)	*100*
TOF MS stop mass (m/z)	*1000*
Accumulation time (s)	*0.05*
Declustering potential (V)	*40*
Time bins to sum	*8*

Table 3. TOF MS/MS parameters and values.

Parameter	Value
Q1 resolution	*Unit*
Zeno pulsing	*On*
Zeno threshold (cps)	*1000*

Data processing: Data were processed in the Analytics module and the Bio Tool Kit micro-application within SCIEX OS software. The product ions used for quantitation were m/z 213.1620 and m/z 586.8049 for VIFDANAPVAVR and FSPDDSAGASALLR, respectively.

Table 4. Zeno MRM^{HR} parameters and values.

Compound ID	Precursor ion (m/z)	TOF start (m/z)	TOF stop (m/z)	Accumulation time (s)	Declustering potential (V)	Collision energy (V)	Collision energy spread (V)	Time bins to sum
VIFDANAPVAVR	*636.36*	*100*	*1000*	*0.05*	*40*	*32*	*10*	*8*
FSPDDSAGASALLR	*703.85*	*100*	*1000*	*0.05*	*40*	*32*	*10*	*8*
VIFDANAPVAVR IS	*647.39*	*100*	*1000*	*0.05*	*40*	*32*	*10*	*8*
FSPDDSAGASALLR IS	*715.38*	*100*	*1000*	*0.05*	*40*	*32*	*10*	*8*

Rapid analysis

The analysis time for the batch of calibrators ejected in triplicate was 3.2 minutes, including the initial and final mandatory barcodes (Figure 1A).

Linearity was achieved from 3.91 ng/mL to 250 ng/mL with r^2 values of 0.9976 and 0.9978 for the VIFDANAPVAVR and FSPDDSAGASALLR peptides, respectively (Figure 1B). A summary of the quantitative performance can be found in Table 5.

Quantitation

Specific product ions were selected for the quantitation of each peptide. The Zeno MRM^{HR} scan performed a product ion scan from m/z 100–1000 (Figure 2) and the Bio Tool Kit micro-application was employed to facilitate product ion selection for the quantitation assay in SCIEX OS software (Figure 3). Together, this approach provided a fast identification of peptide fragments in the MS/MS spectrum.

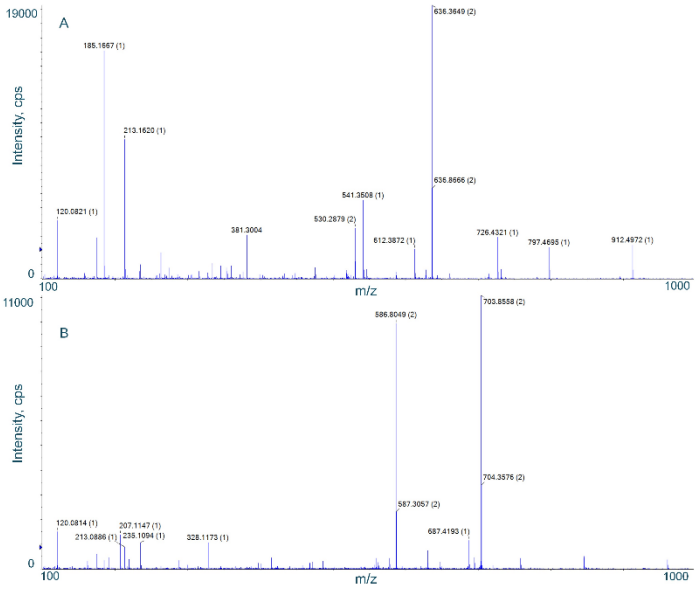

Figure 2. MS/MS spectra obtained from the Zeno MRM^{HR} scan. MS/MS spectra are shown for 250 ng/mL of VIFDANAPVAVR (A) and 250 ng/mL of FSPDDSAGASALLR (B).

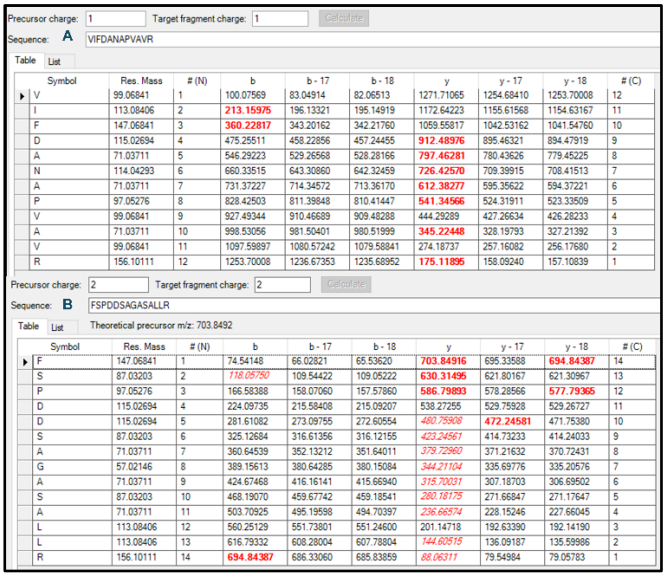

Figure 3. Peptide identification using the Bio Tool Kit micro-application in SCIEX OS software. Results are shown for VIFDANAPVAVR (A) and FSPDDSAGASALLR (B). Bold, red font indicates that the corresponding fragment was identified in the MS/MS spectrum as shown in Figure 2. Red italic numbers indicate a match to a fragment in a different charge state than what has been indicated.[5]

Table 5. Quantitative performance of the thyroglobulin peptide standards, VIFDANAPVAVR (A) and FSPDDSAGASALLR (B).

A

Actual concentration (ng/mL)	Mean (ng/mL)	Percent CV	Average accuracy across replicates (%)
3.91	*3.46*	*17.7*	*88.5*
7.81	*8.53*	*0.65*	*109*
15.6	*15.7*	*6.33*	*100*
31.3	*33.7*	*7.59*	*108*
62.5	*61.9*	*1.74*	*99.1*
125	*116*	*3.45*	*92.5*
250	*257*	*3.66*	*103*

B

Actual concentration (ng/mL)	Mean (ng/mL)	Percent CV	Average accuracy across replicates (%)
3.91	*3.81*	*13.5*	*97.6*
7.81	*8.41*	*10.9*	*108*
15.6	*15.0*	*11.9*	*95.9*
31.3	*33.3*	*4.55*	*107*
62.5	*56.9*	*1.75*	*91.1*
125	*124*	*3.25*	*99.5*
250	*254*	*0.770*	*102*

Conclusions

- Data acquisition at rates as fast as 5 seconds per sample was achieved using Zeno MRM^{HR} mode
- Linearity was achieved from 3.91 to 250 ng/mL for 2 different thyroglobulin peptides
- The Bio Tool Kit micro-application in SCIEX OS software facilitated fast product identification and selection
- No carryover was observed due to constant carrier solvent flow in the Acoustic Ejection Mass Spectrometry method

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High-throughput protein analysis using acoustic ejection mass spectrometry

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This application note demonstrates the ultra-high throughput analysis of intact proteins in addition to 3 different types of proteoforms: point mutations, phosphorylation and cleavage of a peptide using the Echo® MS+ system with ZenoTOF 7600 system. Proteoforms have historically served as crucial biomarkers for both health and disease in medicine.[1] The variation in the structure of a protein contributes to the biological complexity observed in living organisms. The analytical approaches used to analyze proteoforms have evolved considerably over the years.[2] Traditional analysis of the intact protein and proteoforms using mass spectrometry requires microliters of sample and analysis time upwards of minutes per sample. However, reducing turnaround time is crucial in a high-throughput environment to make critical decisions faster.

Herein, the Echo® MS+ system with ZenoTOF 7600 system was used to analyze 3 protein sets across multiple concentrations in a single 384-well plate. The analysis utilized nanoliters of sample, a single MS method regardless of the protein, and the increased versatility of wide peak mode.

Key features of high-throughput intact protein analysis using the Echo® MS+ system with ZenoTOF 7600 system

- **High-throughput proteoform analysis:** Accurately determine the difference between the intact mass of 2 samples at 2.5 seconds per sample
- **Automated sample preparation:** Easily perform sample preparation using the Biomek i7 Automated Workstation
- **Increased versatility:** Varied rate of ejection allows for 4.8x more analytical data points across the peak at 10 Hz than in standard mode
- **Streamlined data management:** Utilize the mass reconstruction workflow to automate the results review process using the SCIEX OS software

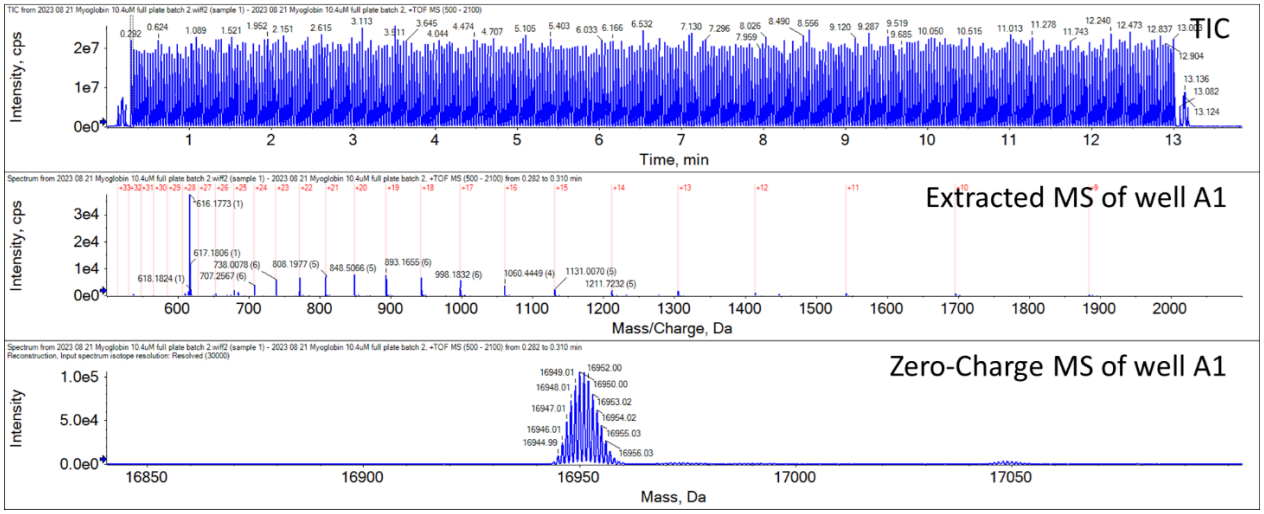

Figure 1: Intact protein analysis using the Echo® MS+ system with ZenoTOF 7600 system. The high-throughput nature of this instrument allows for rapid characterization of 384 samples in under 13 minutes. The top panel depicts the total ion chromatogram (TIC) for 384 different wells of intact myoglobin in a single microtiter plate with barcodes for data processing at the beginning and end of the acquisition. The extracted mass spectrum from one well (A1) with the respective deconvoluted mass spectrum of 10µM myoglobulin is depicted in the middle and bottom panels, respectively.

Introduction

Proteins play a key role in almost every biochemical process. Analyzing intact protein molecules via traditional mass spectrometry allows for characterizing these proteins. For example, accurate molecular weight determination is crucial for assessing protein expression and detecting unexpected modifications during development. The need for high-throughput intact protein analysis has increased in the pharmaceutical and biopharmaceutical industries.[3] The most common way to analyze these proteins is by peptide mapping, or a "bottom-up" experiment, in which a protein is digested with an enzyme (such as trypsin) and the resulting peptides are analyzed by liquid chromatography-mass spectrometry (LC-MS) to confirm the sequence and determine site-specific post-translational modifications. Throughput currently limits peptide mapping LC-MS analysis, as minutes or hours are needed to achieve the necessary characterization and extensive sample preparation before analysis by bottom-up applications. An alternative approach is to analyze the intact mass of the protein first and compare the data against a known sequence, allowing faster screening and shorter analysis time while preserving high sensitivity and mass accuracy.

Methods

Sample preparation: Protein standards for myoglobulin, β-lactoglobulin A and B (BLG A and BLG B, respectively), α and β casein, chymotrypsin, α-chymotrypsinogen A and bovine serum albumin were purchased from Sigma Aldrich. Proteins were diluted at 1 mg/mL concentration in 70:30 (v/v), ACN/H_2O with 0.1% formic acid. Protein stocks were diluted to 20µM before further dilution with the Biomek i7 liquid handler. Assay plates were prepared using the Biomek i7 Automated Workstation from Beckman Coulter® Life Sciences. Using selective tip pipetting, a single row of 12 tips was loaded on the 96-multichannel pipetting head to execute a 2-fold serial dilution vertically down the plate to obtain an 8-point concentration. The samples were then stamped into each quadrant of a 384-well plate (Figure 2B). Before the analysis on the prototype system, 50 µL of the sample was transferred to a well of an Echo® MS+ system-qualified 384-well polypropylene (PP) microtiter plate. The plate was centrifuged at 1,530 x *g* for 2 minutes.

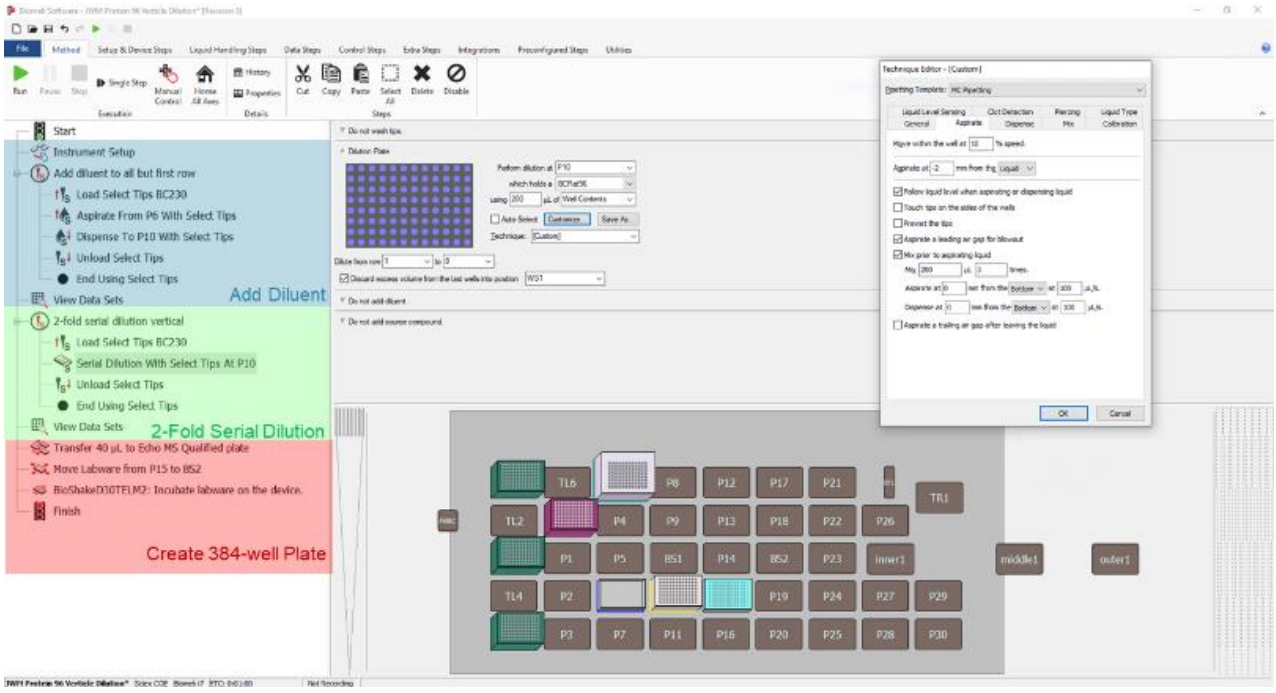

Figure 2: **Sample preparation for replicate analysis is done using the Biomek i7 automated workstation.** Briefly, protein samples were 2-fold serially diluted vertically down the plate.

Acoustic Ejection Method:

Parameter	Value
Carrier solvent	70:30 ACN/H_2O with 0.1% formic acid
Carrier solvent flow rate	400 µL/min
Fluid class	AQ
Ejection volume	100 nL
Rep rate	varied
Interval	varied

Mass spectrometry: The ZenoTOF 7600 system was operated using the TOF MS scan with the following parameters.

Parameter	Value
Polarity	Positive
Ion source gas 1	90 psi
Ion source gas 2	40 psi
Curtain gas	35 psi
Source temperature	300°C
Ion spray voltage	5000 V
CAD gas	10
MS method	TOF MS
Time bins to sum	40

Data processing: The SCIEX OS software was used to process the data qualitatively and quantitatively. The mass reconstruction workflow within the SCIEX OS software was used for peak area determination of proteins.

Peak area reproducibility

The speed of the Echo® MS+ system with ZenoTOF 7600 system allows for 384 samples completed in as little as 8 minutes. Data is collected in a single data file and automatically split during post-processing, with the well position indicated in the split data file. Due to the low volume of the acoustic ejections in the nanoliter range, a significant concern is the reproducibility of the ejections across an entire 384-well plate. An overlay of 384 split ejections of 10.4µM intact myoglobin is shown in Figure 3 with a calculated %CV of peak area under 10%.

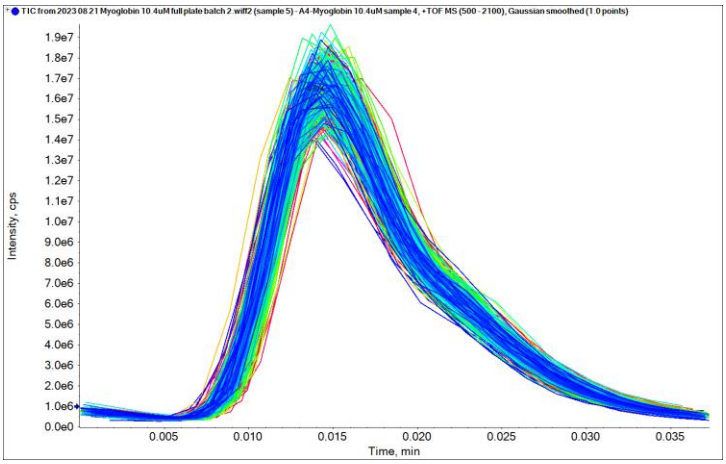

Figure 3: Overlay of the TIC for 384 ejections of intact myoglobin. Overall, the calculated %CV of the peak area was <10%.

Data processing using the SCIEX OS software

The SCIEX OS software enables targeted analysis and deconvolution of large intact molecules on a per-well basis, providing TIC, extracted MS for a single well, and reconstructed mass. The ejection status, time, and volume can be observed on a per-well basis to check the data quality. The split data file can be processed for the target protein mass at a reconstruction resolution of 5000. The reconstruction mass output range was set between 15 kDa and 20 kDa. The reconstructed masses were found at 16,951 Da, matching literature values (Figure 4).[4] Once a specific processing method is determined, the results file can be generated through batch submission after acquisition. The reviewed data in SCIEX OS can be directly integrated with a laboratory information management system (LIMS) for key information storage.

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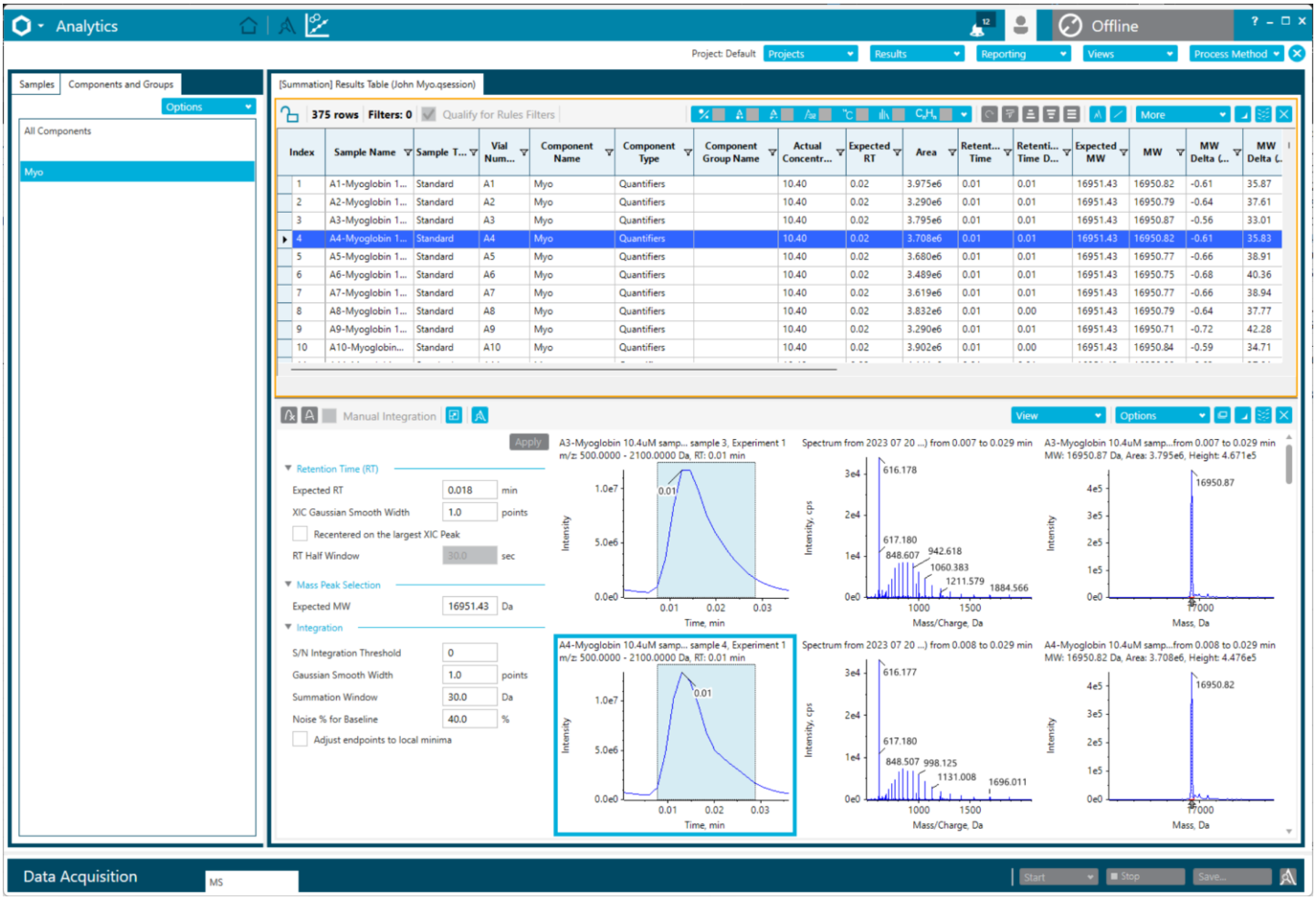

Figure 4: The data from Figure 6 can be processed in an automated, high-throughput fashion. A defined target of multiply charged ions can be deconvoluted, and a results table can be used to review data before report generation.

Proteoform analysis

Six protein standards in sequential wells were analyzed using the Echo® MS+ system with the ZenoTOF 7600 system. This is an example of protein modification analysis in a high-throughput screening environment. Bovine β-lactoglobulin (BLG) has 2 genetic variants, BLG-A and BLG-B, which differ in 2 out of 162 residues.[5] In the case of sequence mutations, differences in the intact mass can rapidly reveal variances in the primary sequences by comparing the 2 data files (Figure 5A). The α and β-casein proteins from bovine milk were analyzed in a subsequent well to evaluate phosphorylation differences (Figure 5B). Phosphorylation is essential for precisely controlling cellular processes, allowing cells to respond and adapt to various signals and environmental conditions. It is a versatile and dynamic mechanism for regulating protein function, signal transduction, enzyme activity, cell cycle progression and membrane transport.[6,7] Chymotrypsinogen is an inactive precursor of chymotrypsin, an enzyme that breaks down proteins.[8] Chymotrypsinogen remains inactive until it reaches the digestive tract to prevent undesired cleavage. Activation occurs when trypsin cleaves a specific peptide bond on α-chymotrypsinogen (pink trace of Figure 5C) to form active chymotrypsin (blue trace of Figure 5C). This leads to the formation of π-chymotrypsin, which further reacts with other molecules to produce α-chymotrypsin. The yield of α-chymotrypsin can be influenced by factors such as inhibitors, pH, temperature and calcium chloride.

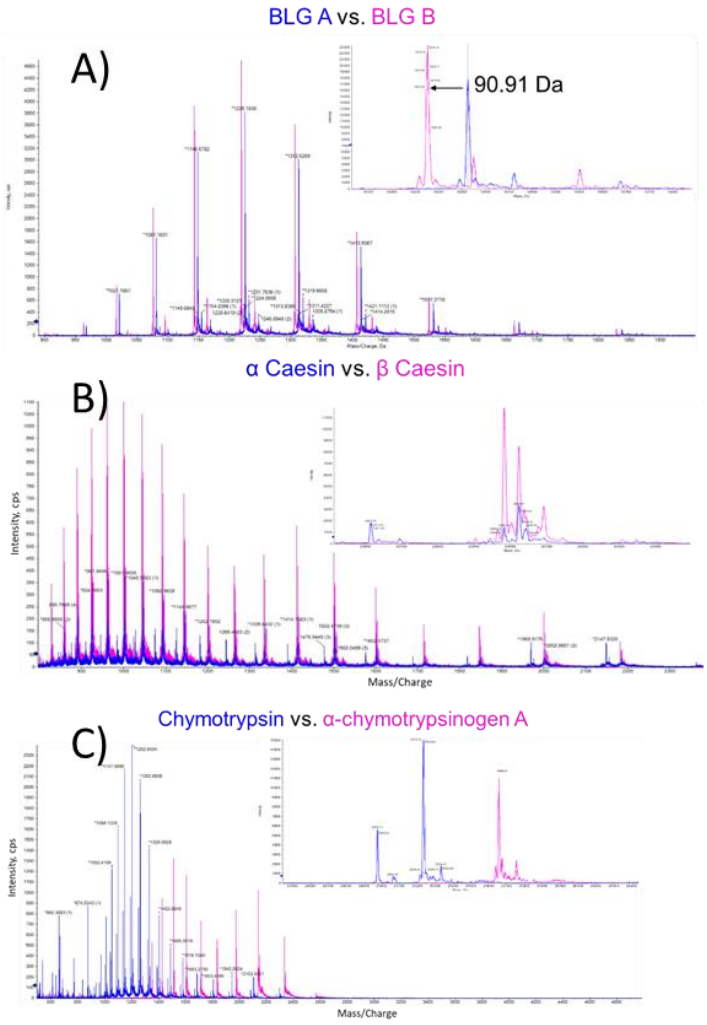

Figure 5: Intact analysis of 3 sets of 10µM proteins. A) BLG A (blue) vs. BLG B (pink), B) α and β-casein (blue vs. pink, respectively) and C) chymotrypsin and α-chymotrypsinogen A (blue vs. pink, respectively).

Conclusions

- Rapid proteoform differentiation can be achieved effectively via the Echo® MS+ system with the ZenoTOF 7600 system
- Increased versatility of the acoustic method enables additional analytical time per sample when needed
- Low consumption of 100 nL per ejection enables sample analysis with a limited amount
- A platform MS method can be applied for a wide range of intact protein analysis
- SCIEX OS software enables fast mass reconstruction and quantitation

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Lightning capillary electrophoresis sodium dodecyl sulfate (CE-SDS) workflow for high-throughput analysis of biotherapeutics

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SCIEX, USA
Biopharma

Introduction

There are hundreds to thousands of drug candidates to screen during early drug development, posing a considerable bottleneck in the biopharma industry. High-throughput analytical platforms and fast analysis methods are attractive approaches to help solve the high sample volume issue. CE-SDS is the gold-standard method widely used for drug purity, integrity and stability analysis. However, this need is not met with the current single-capillary system. In this technical note, we propose the lightning CE-SDS workflow, which, together with a multi-capillary system, this workflow speeds up separation to 1.5 x faster than SCIEX gold standard CE-SDS workflow (Figure 1). This method can analyze 192 reduced samples in 14 hours (4.3 min/sample) with high data quality. The relative standard deviation (RSD) % of relative migration time (RMT) and corrected peak area (CPA) % across 192 injections are below 1% and 3%, respectively. Through a systematic evaluation of multiple variables in only 6days, we proved the high accuracy and separation efficiency with no carryover.

During early drug development, screening a large number of clones for lead clone selection is a critical step in cell line development. This process can be time-consuming and a labor-intensive process without high-throughput methodology.[1]

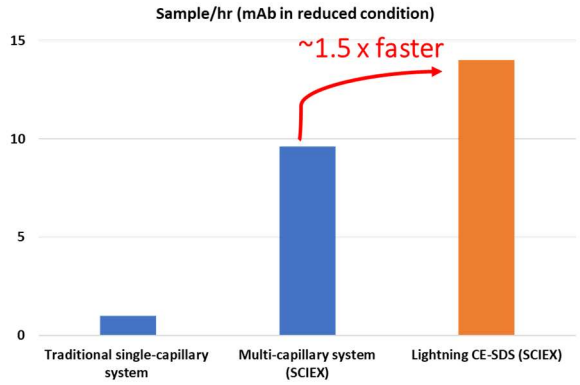

Figure 1. Throughput capability achieved by lightning CE-SDS compared to validated gold-standard workflows.

Meanwhile, developability assessment studies, as a screening strategy to identify process development issues associated with product stability, purity and integrity, require robust and fast analytical approaches to redirect resources to more promising products.[2] To this point, CE-SDS is widely used in biotherapeutics analyses for lot release, stability testing, formulation-buffer screening, process development, cell line development and product characterization. CE-SDS is an automation-friendly application that when combined with a robust multi-capillary electrophoresis system that offers high-throughput, becomes a powerful analytical tool for product characterization during process and cell line development.[3] In this technical note, we increased the throughput on the CE-SDS workflow, enabling the analysis of 192 injections (2 full 96-well plates worth of samples) of IgG standard under reduced (R) and non-reduced (NR) conditions in 14 and 18 hours, respectively, with remarkable reproducibility. This study demonstrates that lightning CE-SDS is accurate and precise as the original workflow with no carryover or loss of separation efficiency.

Key features

- The lightning CE-SDS workflow is up to 1.5 x faster than SCIEX original CE-SDS workflow enabling the analysis of 192 injections of IgG standard in reduced and non-reduced condition within 14 and 18 hours, respectively. That equals to 4.3 min and 5.5 min per injection.
- Exceptional repeatability over 192 injections was achieved with < 1% RSD% and < 3% RSD of relative MT and CPA%, respectively, for all major peaks of IgG standard
- Highly robust workflow with excellent intermediate precision, accuracy and no carryover or loss of separation efficiency

Materials and methods

Chemicals: IgG control standard (PN: 391734) and CE-SDS Protein Analysis Kit (PN: C30085) and the IgG control standard (PN 391734) were from SCIEX, (Framingham, MA). The NIST mAb (RM 8671) reference material 8671 was from NIST (Gaithersburg, MD). The iodoacetamide (PN: I6125-5G) and the 2-mercaptoethanol (PN: M3148-25ML) were from Sigma Aldrich (St. Louis, MO). Chromeo P503 dye (PN: 15106) was from Active Motif, Inc (Carlsbad, CA).

***Materials and instruments*:** BioPhase 8800 system (PN: 5083590F) equipped with UV absorbance detection at 220 nm and LIF detector with 488 nm excitation and 600 nm emission. BioPhase BFS capillary cartridge - 8 x 30 cm (PN: 5080121) and Sample and Reagent Plates (PN: 5080311) were from SCIEX (Framingham, MA). Multi-Therm shaker incubator (Part # H5000-H) was from Benchmark Scientific (Sayreville, NJ). 600 nm/80 nm bandpass filter FWHM 12.5 mm (PN 65736) was from Edmond Optics Worldwide (Barrington, NJ).

Sample preparation for CE-SDS analysis using UV detection: The IgG control standard was prepared by adding 16 µL of 10 kDa Internal Standard and 40 µL of 250 mM iodoacetamide (IAM) for non-reduced sample or β-mercaptoethanol (β-ME) for the reduced sample to 760 µL of the IgG control standard solution. The sample mixture was vortexed, centrifugedand then heat denatured at 70°C for 10 min. The sample was then cooled to room temperature and 100 µL aliquots were transferred to the 8 wells of the sample plate for CE-SDS analysis. The NIST reference standard was prepared by adding 995 µL SDS-MW sample buffer, followed by adding of 25 µL of 10kD, 60 µL of 250 mM IAM for non-reduced sample or β-ME for reduced sample to 120 µL of 10mg/mL NIST. The sample mixture was vortexed, centrifuged and then heat denatured at 70°C for 10 min. The final concentration of NIST reference standard was 1 mg/mL.

Sample preparation for CE-SDS analysis using LIF detection: 12 µL of 10 mg/mL NIST mAb was added to 1128 µL SDS-MW sample buffer, followed by either 60 µL of 250 mM IAM for non-reduced sample or β-ME for reduced sample. The sample mixture was vortexed, centrifuged and then heat denatured at 70°C for 10 min. After cooling down to room temperature, 4 µL Chromeo P503 dye (1 mg/mL) was added to the sample. The sample mixture was vortexed, centrifuged and then heated at 70°C for 10 min for fluorescent labeling. The final concentration of NIST mAb for CE-SDS-LIF analysis is 0.1 mg/mL.

50 µL of treated NIST mAb was transferred to the sample plate for CE-SDS analysis (UV and LIF). Figure 2 shows the sample plate layout used in the systematic study.

CE methods: Figures 3, 4 and 5 show the cartridge conditioning, original sample separation and the lightning CE-SDS separation methods used in this work.

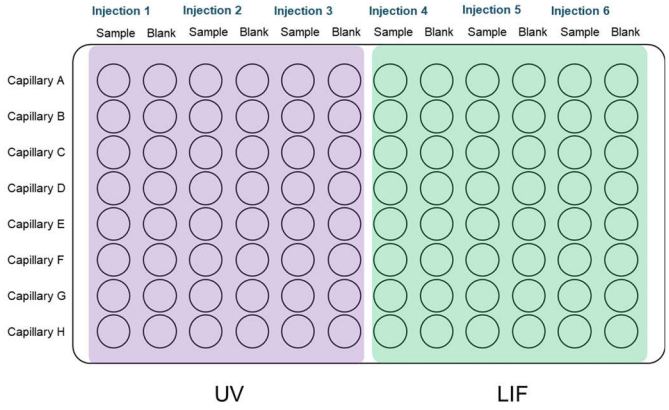

Figure 2. Layout of the sample plate for 1 analysis run of the systematic study.

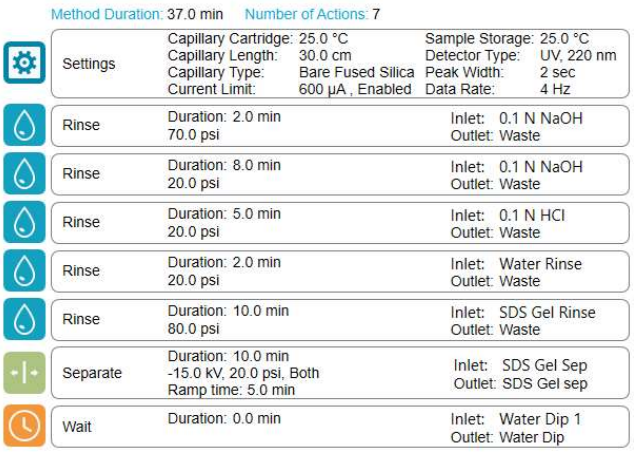

Figure 3. Screenshot of cartridge conditioning method.

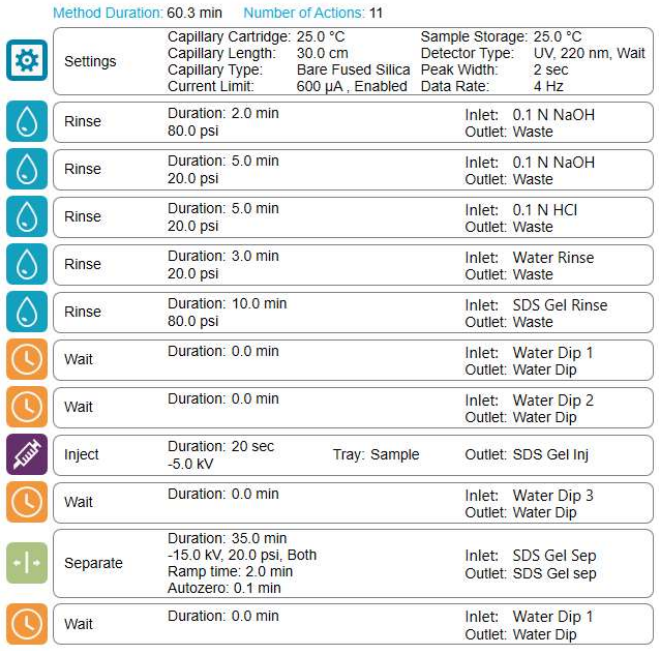

Figure 4. Screenshot of CE-SDS original separation method (for non-reduced antibody analysis condition). Separation time is set to 25 min for reduced antibody analysis.

Figure 5. Screenshot of lightning CE-SDS separation method (for non-reduced antibody analysis condition). Separation time is set to 25 min for reduced antibody analysis.

The rinse cycles in the lightning CE-SDS method has been reduced to only 9 min while maintaining all the rinsing reagents. That reduced the total separation time by 64% compared to the original workflow.

Data analysis: The BioPhase analysis software package version 1.0 was used to create methods and sequences followed by data acquisition (not data analysis) and data processing.

Results and discussions:

The high-throughput capabilities of the lightning CE-SDS workflow: To increase throughput by reducing the cycle time of CE-SDS analysis while maintaining separation efficiency and workflow robustness is critical to retaining high data quality. The rinsing time in the SCIEX original workflow takes up to 50% of the total cycle time. One strategy to reduce the cycle time is by reducing the rinsing time. However, for a thorough capillary surface cleaning and conditioning, we only reduced the duration of the rinse steps while keeping all necessary reagents used in the current method. The combination of high pressure (Figure 5) allows for effective capillary surface treatment while reducing the overall rinsing time to only 9 minutes, or 64% compared to the original workflow. As a result, the separation efficiency and migration time requirements remain the same as the original CE-SDS workflow. Table 1 summarizes the throughput results for the lightning and the original CE-SDS workflows. The lightning CE-SDS workflow took only 6.9 and 8.8 hours to complete one 96-well plate for reduced and non-reduced IgG control standards, translating into an average of 14 reduced and 11 non-reduced samples/hr.

Table 1. Throughput result of lightning and original CE-SDS workflow.

Condition	Workflow	Cycle time	Min/Sample*	Hrs/Plate	Samples/hr
Reduced	*Lightning*	*34 min*	*4.3*	*6.9*	*14*
	Original	*50 min*	*6.25*	*10*	*9*
Nonreduced	*Lightning*	*44 min*	*5.5*	*8.8*	*11*
	Original	*60 min*	*7.5*	*12*	*8*

*min/sample acquired by cycle time divided by 8

To determine the reproducibility of the lightning CE-SDS workflow, we performed 24 consecutive injections of IgG control standard from 1 column of sample plate, that is 8 samples injected 24 times for a total of 192 injections, under reduced and non-reduced conditions. Figure 6 highlights the separation consistency of the lightning CE-SDS workflow. The separation profiles between the first and 24th injection were comparable, indicating that the reduced rinsing conditions did not compromise the separation efficiency.

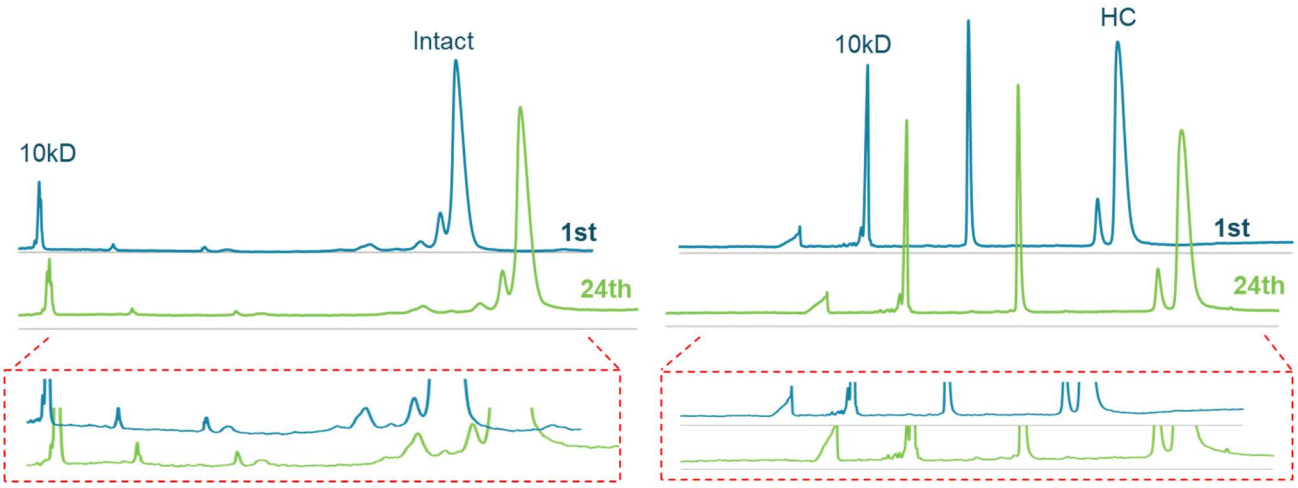

Figure 6. The separation profile comparison between 1st and 24th injection of IgG control standard under non-reduced (left panel) and reduced (right panel) conditions. The figures inside the red blocks showcase the consistency of the profiles at the baseline level for each separation.

To quantify the consistency of the lightning CE-SDS workflow, we calculated the RSD% (N=192) of relative migration time (RMT) and corrected peak area (CPA) % of the major peaks

such as, light chain (LC), heavy chain (HC), non-glycosylated heavy chain (NG-H) of the reduced IgG and HC:HC:LC (HHL) and intact IgG peak of non-reduced IgG. As shown in Figure 7, the RSD% for RMT was below 0.5% for all major peaks. The

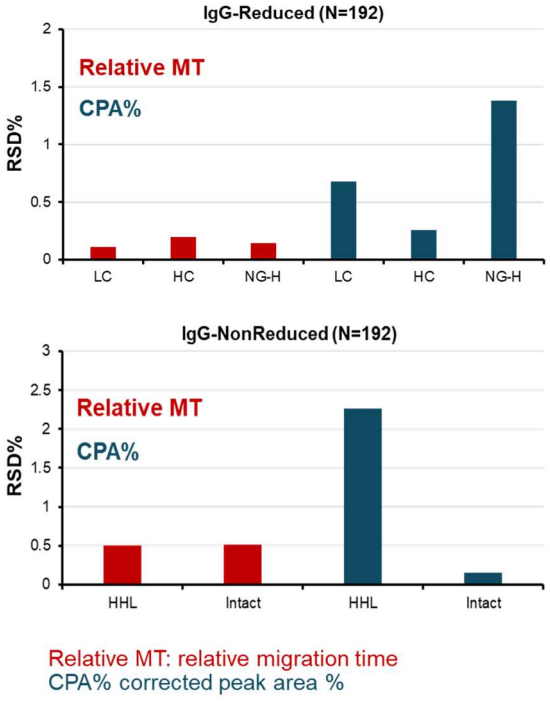

Figure 7. RSD% of lightning CE-SDS workflow for the major peaks of reduced and non-reduce IgG control standard.

RSD% for CPA% was < 1.5% for reduced IgG control peaks and < 2.5% for non-reduced IgG control peaks. The low RSD% of both figures of merit indicates the high reproducibility of the workflow. Most notably, the minor species such as, NG-H and HHL achieved RSD% of less than 3% over 192 replicates for CPA%.

Validation of the Lightning CE-SDS workflow by multiple factorial design using NIST reference standard mAb:
Because of the multi-capillary environment of the BioPhase 8800 system, to better understand the study design and the data output this platform can generate in 1 sequence, the terms used in this work are defined as follows. Data point refers to one separation from 1 well using 1 capillary. Each run refers to 8 data points. The sample plate layout (Figure 2) used in this work comprises 3 columns (or 8 samples) for UV detection and 3 columns of samples for LIF detection. Between each column of samples, a column of sample buffer is used as blanks to assess carryover. One sequence is defined as a single separation of 1 sample (NIST antibody) plate with the layout on Figure 2, generating 48 datapoints. Table 2 illustrates the experimental design to evaluate the robustness of the lighting CE-SDS workflow. The study required each of the 3 analysts to prepare 1 sample plate daily as described in Figure 2. Each analyst ran different instruments using 3 different cartridges (triplicate runs per plate/day) with 9 runs, generating 432 data points for reduced and non-reduced samples. This study provided insights into variation potentially caused by instruments, analyst operation, cartridges, capillaries and different injections. These multiple factors were effectively tested in triplicates to highlight their impact in the overall results directly. Most notably, the multi

capillary environment of the BioPhase 8800 system allowed to execute this study in only 6 days.

Table 2. Systematical study plan of NIST under reduced or non-reduced conditions.

# of Runs	Instrument	Person	Cartridge	Day
1	1	Analyst-1	3	1
2	2	Analyst-2	2	
3	3	Analyst-3	1	
4	1	Analyst-2	1	2
5	2	Analyst-3	3	
6	3	Analyst-1	2	
7	1	Analyst-3	2	3
8	2	Analyst-1	1	
9	3	Analyst-2	3	

Additionally, we used the original CE-SDS workflow as a control experiment but a reduced statistical sampling. In summary, 2 sequence analyses were performed using the same sample plate layout as Figure 2 for reduced and non-reduced NIST antibodies. Therefore, our control runs generated 48 data points for reduced or non-reduced conditions.

This comprehensive study systematically assessed factors that may impact MT, resolution and CPA% of major peaks of the NIST mAb.

Each bar graph shown in Figure 8 represents the average value of 24 data points collected with the original workflow or 216 data points (UV or LIF) collected by the lightning CE-SDS workflow with standard deviations as error bars. The average MT, CPA% and resolution values were very close between the original CE-SDS and the lightning CE-SDS workflow for all major peaks observed under reduced and non-reduced conditions. Most notably, the low CV% found for raw MT, CPA% and resolution was overall below 5%, indicating the robustness and reproducibility of the lightning workflow compared to the original for both detection modes. Additionally, the data also suggested that the duration of the separation is the same between the 2 workflows, facilitating method adoption. Similarly, the resolution between NG-H, HC peaks, HHL and the intact IgG peaks indicated the separation efficiency observed in the lightning CE-SDS workflow was not only maintained across the 216 data points but was equivalent to the original CE-SDS workflow.

Assessment of carryover: To check for any carryover issues due to the shortening of the rinsing steps specially when using LIF was also evaluated. We incorporated 6 blank injections in the plate layout where the separation of a blank sample always followed each sample separation. A closer look at the data from blank injections in Figure 9 revealed no carryover detected in the lightning CE-SDS workflow for both UV and LIF detection schemes.

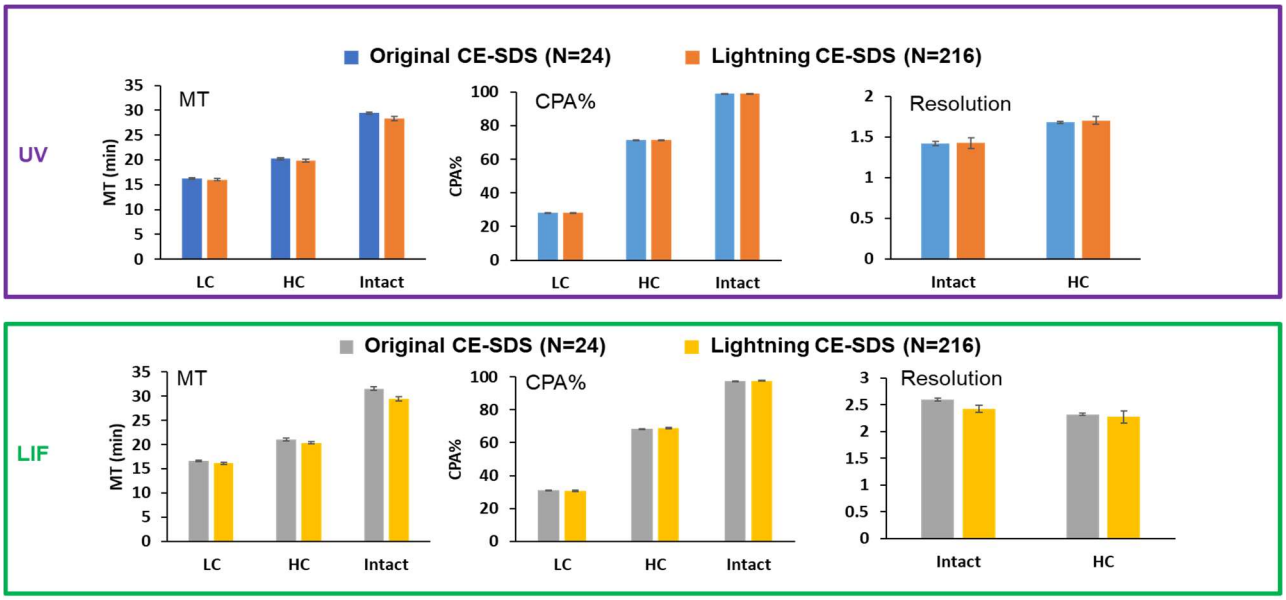

Figure 8. Attributes comparison (MT, CPA% and resolution) between original and lightning CE-SDS workflow (for both UV and LIF). The numbers in parentheses are the number of replicates. The error bars indicate the standard deviation.

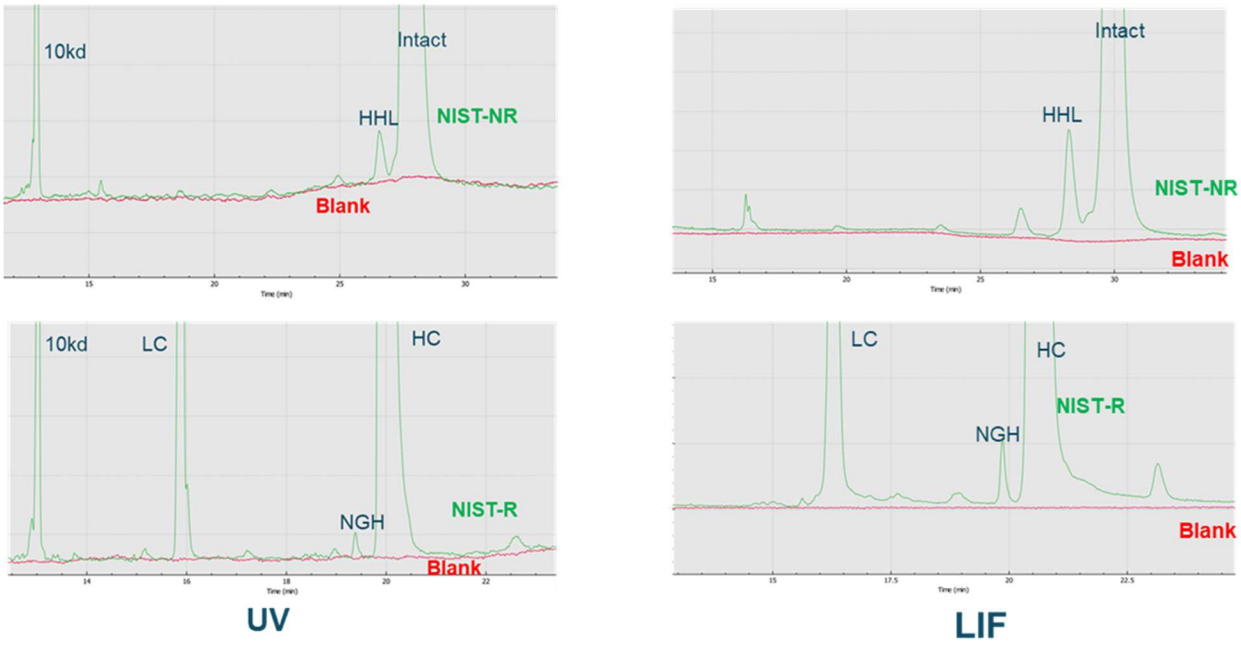

Figure 9. Electropherograms of non-reduced and reduced NIST mAb between sample and blank. The green trace was from sample at 1 mg/mL (UV) and 0.1 mg/mL (LIF). The red trace was from blank (SDS-MW sample buffer). Upper panels show results of non-reduced NIST mAb. Lower panels show results of reduced NIST mAb.

Finally, we systematically evaluated the impact of multiple factors such as, instrument, analyst, cartridge and capillaries on the attributes of resolution and CPA% of the lightning CE-SDS workflow. We isolated the results of each attribute so we could easily underscore how a factor such as, an instrument to instrument has any impact on the average of CPA% and resolution between NG-H and HC. Figure 10 illustrates the average CPA% and resolution between NG-H and HC peaks when data is organized into instrument (green bars), analysts (blue bars), capillary cartridge (yellow bars), day (gray bars), injection (dark blue bars) and capillaries (orange bars). The error bars indicate the standard deviation of the replicates for each factor. Overall, this study revealed that the lightning CE-SDS

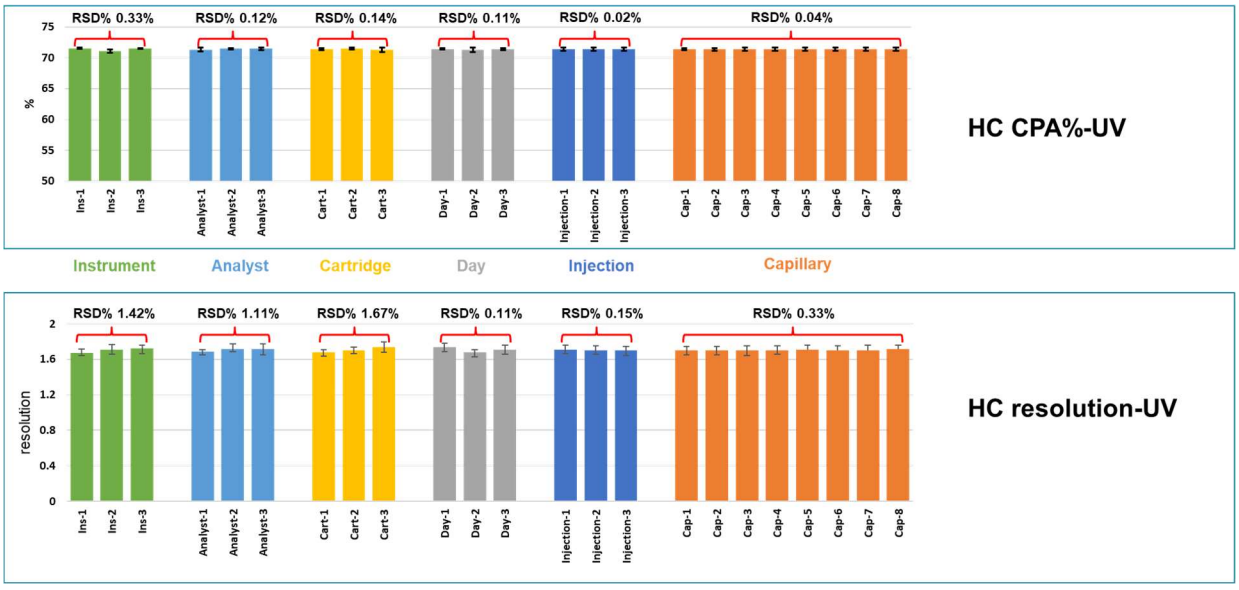

Figure 10. Attributes comparison under different conditions.

generated very reproducible data. For example, when considering instrument to instrument, we observed %RSD as low as 0.4% and below 2% for CPA% and resolution between NH-H and HC peaks, respectively. We also performed a similar analysis on other peaks under reduced and non-reduced conditions (data not shown) and reached the same conclusion.

When considering the 3 analysts as source of variation, our data showed minimum impact on all attributes considered in this study, indicating the robustness of the lightning CE-SDS workflow generating similar results as the SCIEX original CE-SDS workflow.

Conclusions

- The lightning CE-SDS increased the analysis speed up to 1.5 x compared to the original workflow, where 192 injections can be analyzed within 18 hours without the need to change reagent plates
- The reproducibility of the lightning CE-SDS workflow is remarkable with RSD% of < 1% and < 3% for calibrated MT and CPA%, respectively, for all major peaks of IgG standard
- The multi-factorial design of this experiment demonstrated that the lightning CE-SDS workflow is as accurate and precise as the original validated CE-SDS workflow with no carryover and loss of separation efficiency

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Streamlined protein characterization workflows for capillary electrophoresis using automated sample preparation

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This technical note describes a streamlined process for protein characterization work utilizing the Biomek i5 multichannel (MC) workstation for sample and reagent preparation for downstream separation and data analysis using the BioPhase 8800 system.

Biotherapeutics have revolutionized modern medicine and created a multi-billion-dollar business, with hundreds more currently in the development pipeline. Biotherapeutic molecules range from traditional monoclonal antibodies (mAb) and more complex mAb-derived modalities to cell and gene therapy products. In the process development environment of a biotherapeutic lifecycle, thousands of samples must be prepared and analyzed consistently and reliably by chromatographic or electrophoretic means to determine and quantify the purity, charge heterogeneity, and glycan composition.

Key features of the automated CE workflows

High-throughput automated sample preparation: Hands-free sample preparation of an entire 96-well plate in less than 3 hours for capillary electrophoresis sodium dodecyl sulfate (CE-SDS) – Figure 1 and 2.3 hours for capillary isoelectric focusing (cIEF) and glycan analysis

Robust and reproducible analytical performance: Exceptional intra- and inter-capillary performance and reproducibility

Enhanced usability and sample preparation flexibility: The Biomek i5 MC workstation from Beckman Coulter® features flexible configurations that increase user confidence and walk-away time

Streamlined workflow: An integrated assay coupling automated sample preparation, multichannel CE data acquisition and intuitive data analysis software for rapid reporting and decision-making

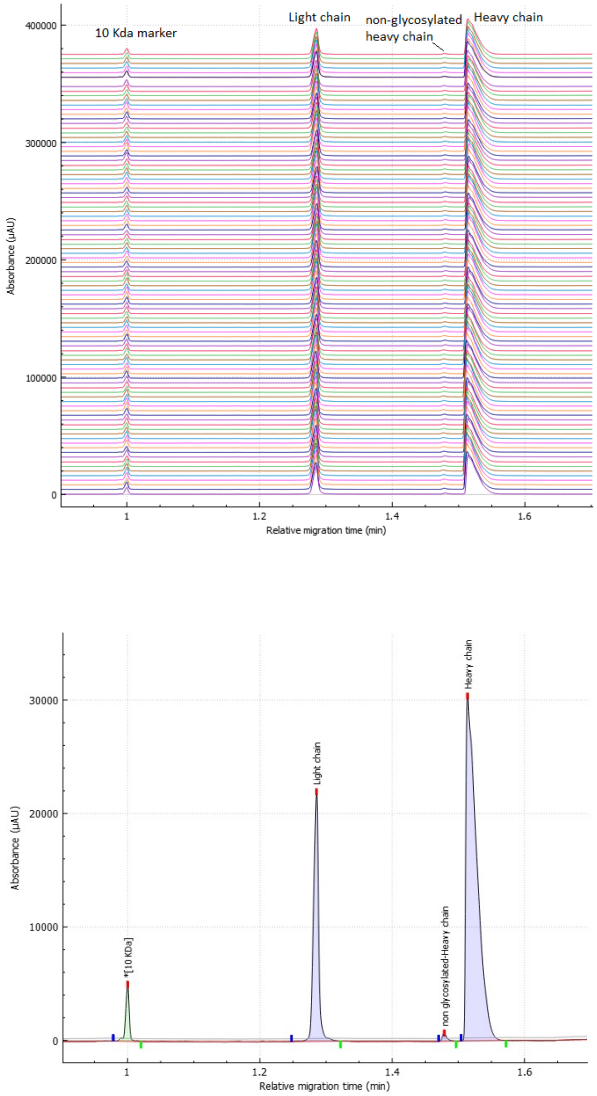

Figure 1:(Top) CE-SDS electropherograms of 96 reduced USP mAb IgG SS RF samples. The different colors represent the different capillaries, with the first run displayed at the top. (Bottom) An electropherogram from a single run shows the 4 peaks (Capillary A).

Introduction

Analyzing thousands of samples in the early development can quickly become a bottleneck for sample testing throughput. Streamlined workflows with automated sample preparation and high-throughput data acquisition and analysis can maximize sample processing time, minimize human intervention, and eventually address the throughput limitation in the analytical characterization of intermediate samples.

This work demonstrates the full automation of 3 key applications in the characterization of biopharmaceuticals using capillary electrophoresis: CE-SDS[1], cIEF[2] and glycan[3] from sample preparation to data analysis.

Methods

The automated CE-SDS preparation method (workflow shown in Figure 2) started with 95 µL of the USP system suitability standard reference standard (USP mAb IgG SS RS) in a 96-well plate. To each sample, 2 µL of 10 kDa internal standard and 5 µL of 2-Mercaptoethanol (β-ME) were added. The plate was then sealed with a film and placed back on the deck. The plate was then heated to 70°C for 10 minutes before 90 µL aliquots of these samples were transferred to the sample inlet tray. The sample inlet tray was centrifuged at 177x g for 20 minutes and then placed into the BioPhase 8800 system for electrophoretic separation. For the reagent inlet plate, all reagents were pipetted column-wise, as described in the application guide.[4]

For the reagent outlet, a single tip was used for each reagent. For the sample outlet tray, a tip was used for each well to be filled, which depended on the number of samples to be analyzed. The reagent inlet and sample outlet trays were prepared with the Biomek i5 MC workstation and then placed directly into the BioPhase 8800 system. The capillary conditioning was performed according to the application guide[4] before the samples were separated.

The automated cIEF sample preparation (workflow in Figure 3) started with 8 µL per well of 5 mg/mL USP mAb IgG SS RS in a deep well plate format. The cIEF master mix was prepared manually in advance, according to the cIEF for the BioPhase 8800 system application guide.[5] The final cIEF master mix contained 4M urea cIEF gel, cathodic stabilizer, anodic stabilizer, Pharmalyte 3-10 and pI 4.1, 5.5 and 10 markers. A 248 µL aliquot of master mix was added to each well and tip-mixed 5 times with the sample. Once mixed, 100 µL aliquots of the prepared samples were transferred to the sample inlet tray. All solutions for the inlet reagent plate were pipetted column-wise, as described in the application guide.[5] For the reagent outlet plate, a single tip was used for each reagent.

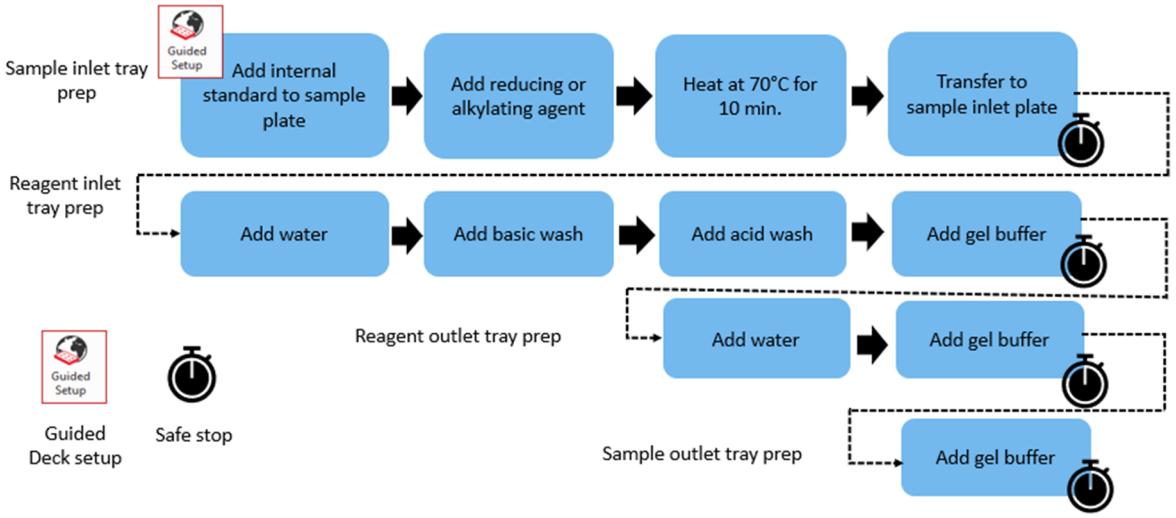

Figure 2. The sample and reagent preparation workflow for automated CE-SDS analysis using the Biomek i5 MC workstation.

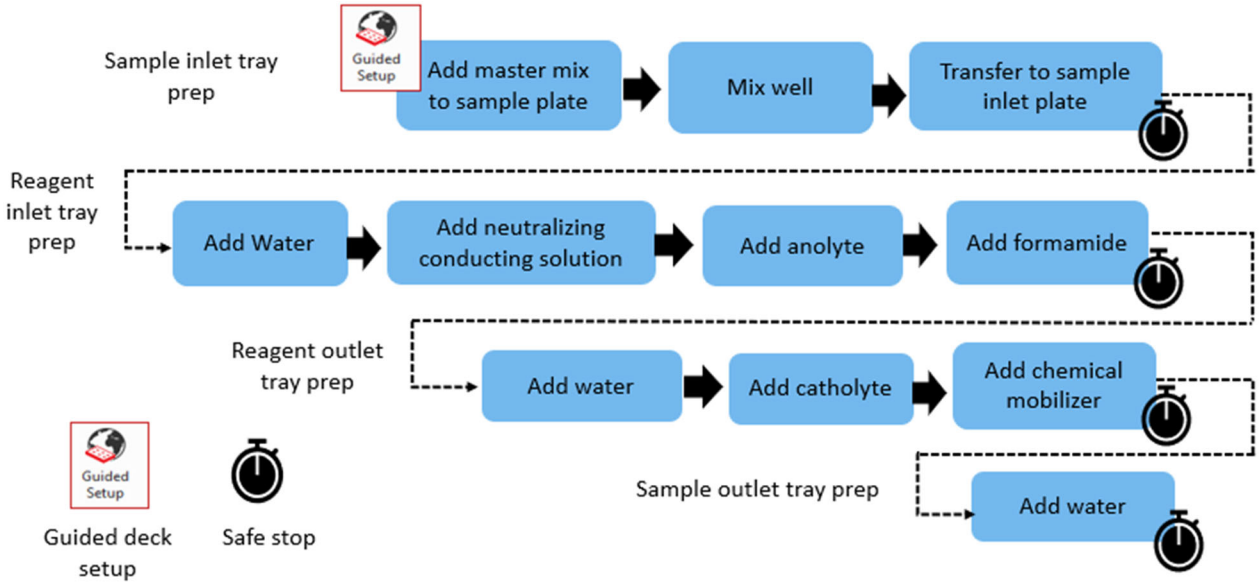

Figure 3. The sample and reagent preparation workflow for automated cIEF analysis using the Biomek i5 MC workstation.

After the Biomek i5 MC workstation completed the method, all 4 trays were manually placed directly into the BioPhase 8800 system.

The automated N-linked glycan sample preparation method (workflow in Figure 4) can be run from start to finish with full walk-away capability. This workflow features a magnetic bead-meditated denaturation, digestion, labeling and clean up. The labeling is performed via reductive amination using 1-aminopyrene-3,6,8-trisulfonic acid (APTS), a highly charged fluorescent tag that allows for high sensitivity and fast separation of N-linked glycans. The denaturing, digestion and labeling solutions were prepared according to the application guide.[6]

Performance of automated sample and reagent preparation of the CE-SDS workflow

CE-SDS analysis: Inter-capillary reproducibility

Figure 1 demonstrates the inter-capillary reproducibility for the analysis of the USP mAb IgG SS RS.

The inter- and intra-capillary values for reproducibility are quantified in Table 1 (n=96). The observed relative standard deviation (RSD) was approximately 1% for migration time (MT) and for the corrected peak area percentage (CPA %) for the 10 kDa marker, the LC and HC peaks and the HC/LC ratio.

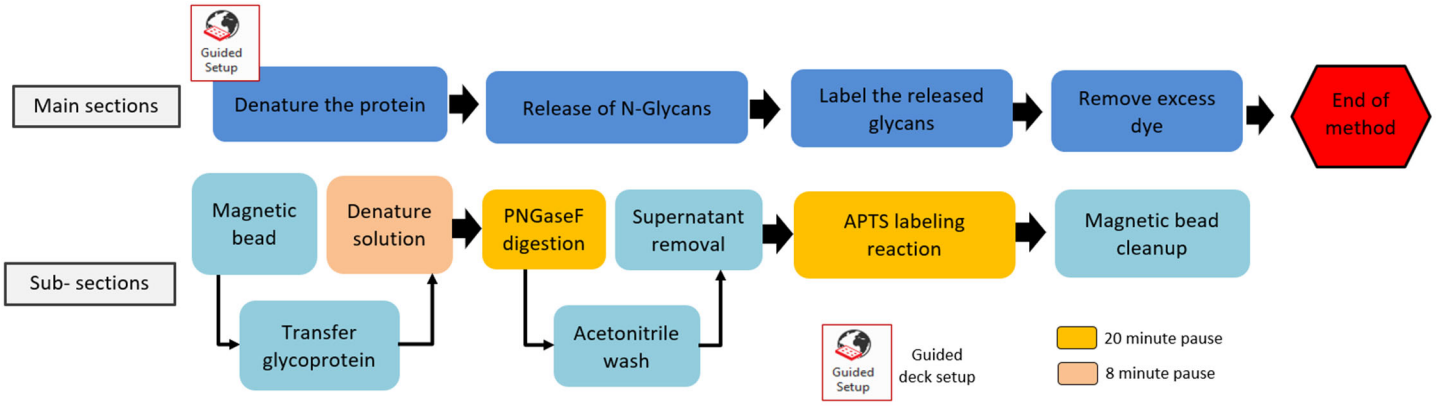

Figure 4. Direct comparison between PA 800 plus and the BioPhase 8800 systems of a mAb sample under non-reduced conditions.

Table 1. Intra- and inter-capillary %RSD for MT, CPA% and HC/LC ratio for the 10 kDa marker and IgG control standard (N=96).

Intra-capillary reproducibility (%RSD)								
		Migration time (min)			Corrected peak area percentage			Ratio
Capillary	10 Kda	LC	ng-HC	HC	LC	ng-HC	HC	HC/LC
A	0.69	0.67	0.66	0.65	0.21	2.15	0.07	0.43
B	0.56	0.52	0.52	0.50	0.23	1.77	0.08	0.34
C	0.55	0.51	0.50	0.48	0.36	2.21	0.13	0.38
D	0.64	0.61	0.60	0.59	0.21	2.71	0.09	0.38
E	0.74	0.73	0.73	0.70	0.34	2.23	0.14	0.34
F	0.76	0.74	0.72	0.71	0.22	2.00	0.08	0.27
G	0.96	0.93	0.93	0.92	0.14	1.77	0.05	0.24
H	1.11	1.10	1.11	1.09	1.12	1.82	1.04	0.44
Inter-capillary	0.83	0.81	0.82	0.80	0.47	2.24	0.38	0.37

Table 2 shows the average values observed, demonstrating the inter- and intra-capillary consistency for MT, CPA% and HC/LC ratio.

Performance of the automated sample and reagent preparation for the cIEF workflow

Figure 5A shows the overlay of 96 runs, demonstrating the reproducibility of both instrument and sample preparation.

Figure 5B shows a typical USP IgG SS RS profile and the integration strategy to report the area percentage composition of the main, basic and acidic variants.

Figure 5C shows the detection times for the main peak, pI markers 4.1, 5.5 and 10. In Figure 5D, each bar represents the average peak area percentage for the basic, main or acidic isoforms, calculated from 96 data points collected on the BioPhase 8800 system.

Table 2: Intra- and inter-capillary average MT, CPA% and HC/LC ratio values for the 10 kDa marker and IgG control standard (N=96).

Intra-capillary average								
		Migration time (min)			Corrected peak area percentage			Ratio
Capillary	10 Kda	LC	ng-HC	HC	LC	ng-HC	HC	HC/LC
A	13.18	16.95	19.5	19.96	28.19	0.75	71.06	2.52
B	13.13	16.89	19.43	19.89	28.14	0.75	71.10	2.53
C	13.13	16.88	19.42	19.88	28.17	0.74	71.08	2.52
D	13.12	16.87	19.4	19.85	28.15	0.74	71.09	2.53
E	13.17	16.94	19.48	19.95	28.2	0.74	71.05	2.52
F	13.16	16.90	19.46	19.92	28.22	0.75	71.02	2.52
G	13.19	16.96	19.51	19.78	28.24	0.75	71.00	2.51
H	13.28	17.08	19.65	20.12	28.09	0.74	70.86	2.52
Inter-capillary	13.17	16.93	19.48	19.92	28.18	0.75	71.03	2.52

Error bars indicate the standard deviation. The RSD values for peak area percentage across all data points were 1.5%, 0.67% and 1.5% for basic, main and acidic isoforms, respectively, indicating robustness across sample preparations.

Excellent intermediate precision was demonstrated for the calculated pI value.

Table 3 shows the reproducibility of cIEF analysis for USP mAb

Table 3 Reproducibility of cIEF analysis for USP mAb IgG SS RS isoforms on a BioPhase 8800 system.

	Intra-capillary average					
Capillary	**Average (n=12)**	**%RSD**	**Average (n=12)**	**%RSD**	**Average (n=12)**	**%RSD**
A	17.72	1.20	60.92	0.35	21.28	0.99
B	17.52	0.84	60.94	0.24	21.51	0.87
C	17.64	1.36	60.76	0.70	21.57	1.52
D	17.70	1.34	60.90	0.96	21.37	2.00
E	17.80	1.63	60.58	0.64	21.59	1.12
F	17.84	1.46	60.65	0.73	21.48	2.01
G	17.81	1.19	60.83	0.76	21.31	1.61
H	17.89	1.59	60.60	0.72	21.46	1.54
Inter-capillary average (n=8)	17.74		60.77		21.45	
% RSD	0.68		0.24		0.53	

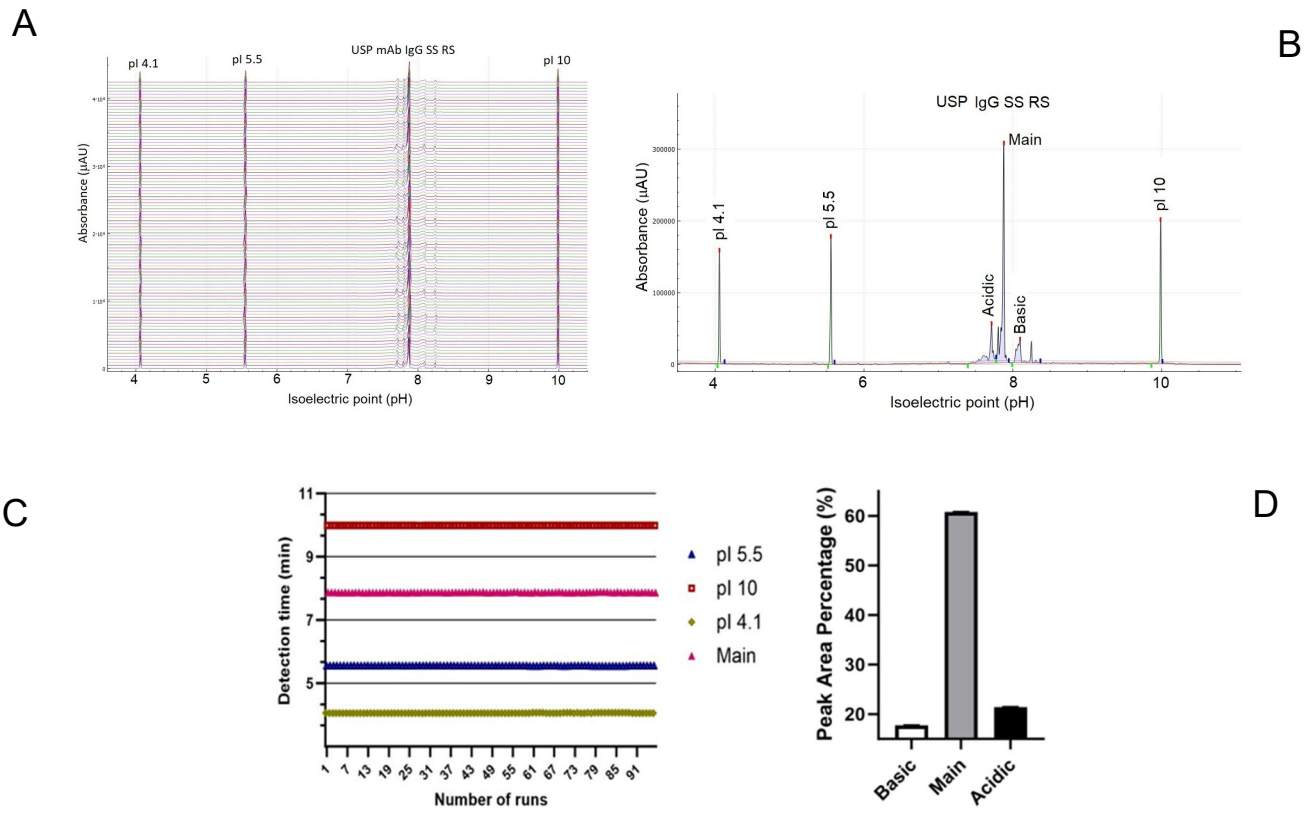

Figure 5. (A) Electropherograms of 96 samples of USP mAb IgG SS RF. Peaks 1, 2 and 7 show the pI 4.1, 5.5 and 10 markers, respectively. Peaks 3, 4 and 5 represent the acidic, main and basic groups, respectively. The color of each trace (A-H) indicates the capillary used for analysis. The first run is shown at the top. (B) A representative electropherogram for USP mAb IgG SS RF with absorbance and pI value indicated on the y- and x-axes, respectively. Each peak is annotated with peak number and name. (C) Plot of detection times observed for the 96 runs for pI 4.1 (olive), pI 5.5 (blue), main group (pink) and pI 10 (maroon). (D) Graph shows assay robustness with a plot of peak area % values for different isoforms of USP mAb IgG SS RS.

IgG SS RS isoforms on a BioPhase 8800 system. The average values and %RSD values were calculated for the peak area percentage from 12 data points per capillary for the basic, main or acidic isoforms. The inter-capillary values were calculated across 8 capillaries.

Performance of the automated sample and reagent preparation for the fast glycan workflow

The performance of the automated sample and reagent preparation workflow for the fast glycan analysis using a human serum IgG sample is demonstrated in Figure 8.

The reproducibility of the automated, fast glycan sample preparation was assessed by evaluating the inter- and intra-capillary %RSD of CPA% (Table 4), relative migration time (RMT GU) and glucose units (GU) for FA2G2S1/M5, FA2, M7[D1]/FA2(6)G1, FA2G2 and FA2(3)G1/FA2B(6)G1 species.

Table 4 demonstrates the excellent reproducibility of automation in complex sample preparation schemes.

Table 4 Inter- and intra-capillary reproducibility for CPA%, RMT GU and GU values.

Inter-capillary reproducibility			
Glycan species	**CPA%**	**RMT GU**	**GU**
FA2(3)G1	9.92	0.06	0.05
M5	1.82	0.08	0.07
FA2	1.78	0.06	0.05
FA2(6)G1	.041	0.05	0.05
FA2G2	3.79	0.05	0.05
Intra-capillary reproducibility			
Glycan species	**CPA%**	**RMT GU**	**GU**
FA2(3)G1	1.10	0.04	0.04
M5	1.73	0.05	0.05
FA2	2.26	0.04	0.03
FA2(6)G1	0.94	0.07	0.06
FA2G2	1.10	0.04	0.04

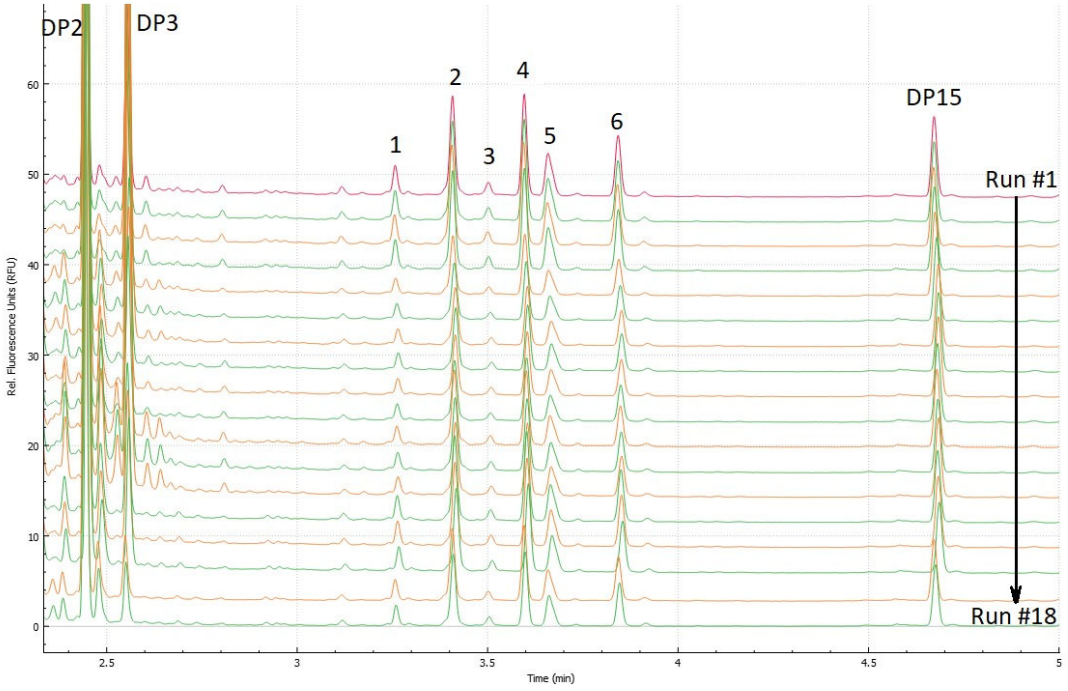

Figure 6. Overlay of 18 capillary electrophoretic separations of released N-linked glycans labeled with APTS from a serum IgG sample. Peak legend: 1) M5, 2) FA2, 3) FA2B, 4) FA2(6)G1, 5) FA2(3)G1 and 6) FA2G2.

Conclusions

- Automated sample and reagent inlet and outlet plate preparation on the Biomek i5 MC workstation eliminated labor-intensive bench work and provided high-throughput with highly consistent results when combined with the BioPhase 8800 system
- Robust, automated sample preparation yielded excellent intra-capillary reproducibility data for migration time, relative migration time and corrected peak area percentage compared to manual preparation
- The streamlined workflow coupling automated sample preparation, high-throughput data acquisition and intuitive data analysis facilitated a rapid question-to-answer process in a fast biopharma environment

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