High Resolution Intact Glycoprotein Analysis by CESI-MS

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Introduction

The analysis of intact proteins by Electrospray Ionization - Mass Spectrometry (ESI-MS) provides the most direct route for the identification and characterization of these highly complex molecules. With the potential presence of multiple isoforms, it is also widely accepted that the presence of a liquid-phase separation technique upstream from the MS detection step would greatly contribute to a complete deciphering of the structure of these molecular entities. Capillary electrophoresis (CE), compatible with the preservation of delicate protein structure, post-translational and other modifications, while simplifying sample complexity through high resolution separation capabilities, appears as a very valuable alternative prior to introduction into the mass spectrometer. To achieve the greatest advantage of coupling CE with ESI-MS for intact protein analysis, we are currently developing a novel technology called CESI that integrates CE with ESI into a single dynamic process. The CESI technique has been successfully implemented for the analysis of a model glycoprotein, Ribonuclease B (RNase B). The efficient coupling between these liquid and gas phase separation systems allowed the rapid, sensitive and high resolution detection of the five known Ribonuclease B glycoforms. Further deconvolution of the resulting Time of Flight (ToF) mass spectra by the Maximum Entropy Algorithm identified differences in the attached high mannose structure as the source of the electrophoretic heterogeneity. Produced on multiple sheathless sprayers, the data confirm that these separations are highly repeatable and reproducible.

Materials and Methods

Instrumentation

All CE-MS separations were performed on a prototype CESI 8000 High Performance Separation - ESI Module*. Separations were performed on OptiMS 100 cm long 150 µm o.d. x 30 µm i.d. Surface+ coated capillaries. Instrument temperature settings were 10 ºC for the sample storage module and the OptiMS capillary was maintained at 20ºC with a rapidly circulating liquid coolant.

MS detection was performed using a Waters Xevo[Ŧ] QToF set with a scanning range between 800 – 3000 m/z and a sampling rate of 1 Hz. Electrospray ionization was achieved at 1.4 – 1.6 kV with the CESI 8000 OptiMS sprayer needle filled with background electrolyte (BGE).

200 mM Ammonium Acetate pH 3.6 BGE Solution

A 200 mM acetic acid solution was produced by diluting 5.7 mL of 17.4 M glacial acetic acid (EMD P/N UN 2789) in 494.3 mL of 16 MΩ water using a 500 mL volumetric flask. The 200 mM acetic acid solution was titrated to a pH of 3.6 with a 200 mM ammonium acetate solution produced by diluting 2.7 mL of 7.5 M ammonium acetate (Sigma P/N A2706-1L) in 97.3 mL of 16 MΩ water using a 100 mL volumetric flask.

Capillary Coating Procedure

The reagents used are as follows: Optima[Ŧ] LC/MS grade methanol (MeOH) (Fisher Scientific P/N 456-1), 0.1 M sodium hydroxide (NaOH) (Beckman Coulter P/N 391988), 0.1M hydrochloric acid (HCl) (Beckman Coulter P/N 391646).

Prior to introducing the Surface+ Capillary Coating Reagent (Beckman Coulter P/N B08746), the capillary was conditioned by performing a series of conditioning step washes.

Reagent	Pressure	Direction	Duration
MeOH	50 psi	Forward	20 min.
16 MΩ Water	50 psi	Forward	10 min.
0.1 M NaOH	50 psi	Forward	20 min.
0.1 M HCl	50 psi	Forward	10 min.
16 MΩ Water	50 psi	Forward	10 min.
MeOH	50 psi	Forward	20 min.
Air	50 psi	Forward	20 min.

Following the conditioning step, a 20% mixture of Surface+ Coating Reagent in MeOH was introduced into the capillary at 50 psi for 85 min. The coating reagent was left to incubate in the capillary overnight to allow for the covalent attachment between the capillary wall and the cationic polymer in the Surface+ coating reagent. After overnight incubation, the system was purged of the Surface+ coating reagent using a series of purge steps.

Reagent	Pressure	Direction	Duration
MeOH	50 psi	Forward	60 min.
MeOH	50 psi	Reverse	60 min.
Air	50 psi	Forward	10 min.
Air	50 psi	Reverse	10 min.

Capillary Maintenance Procedures

Newly coated capillaries were cleaned, and used capillaries were prepared for storage following the completion of an analytical cycle by first purging the separation capillary and conductive solution capillary for 5 min. at 50 psi with MeOH, then electrokinetically cleaned at 20 kV in reverse polarity. After cleaning the capillaries, both capillaries were air-dried using pressure rinses with empty vials for 5 min. at 50 psi.

Protein Analysis Procedures

Prior to starting an analysis cycle, the OptiMS sprayer needle was filled with conductive by applying 50 psi on a vial in the buffer outlet tray containing BGE for 1 min. The cationic coating was conditioned by applying 50 psi on a vial in the buffer inlet for 10 min., then at 20 kV in reverse polarity for another 10 min.

Protein samples were hydrodynamically injected into the separation capillary using 5 psi for 5 sec. for a total plug volume of approximately 7 nL and separated using a 20 kV potential in reverse polarity. A separate BGE vial was used to rinse the capillary for 6 min. at 50 psi following the completion of the separation.

References

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OptiMS CESI Interface

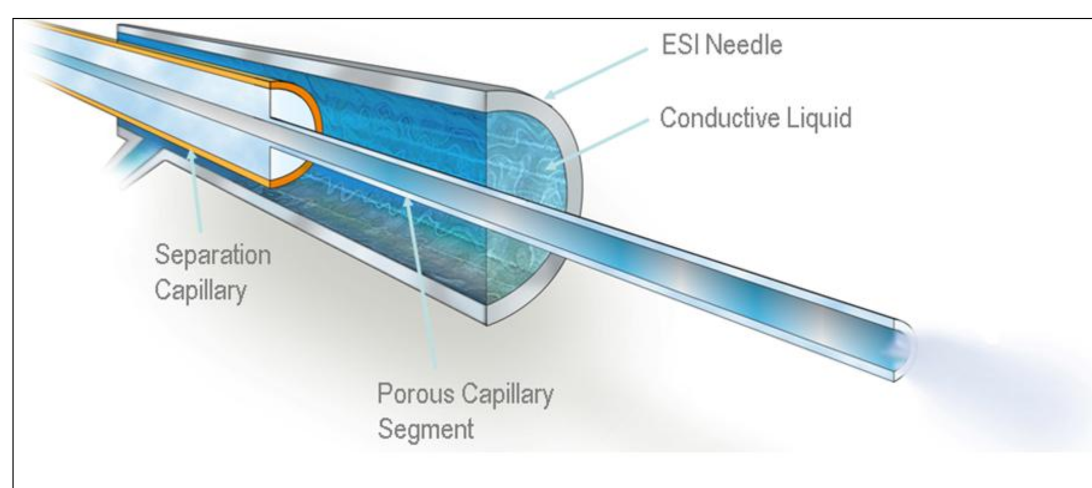


Figure 1. The terminal portion of the OptiMS capillary has been made porous to ion flow, providing an electrical contact between the BGE inside the separation capillary and a conductive liquid filling a metal ESI needle. This porous glass junction provides the ground for the CE circuit and high voltage contact for the ESI circuit .

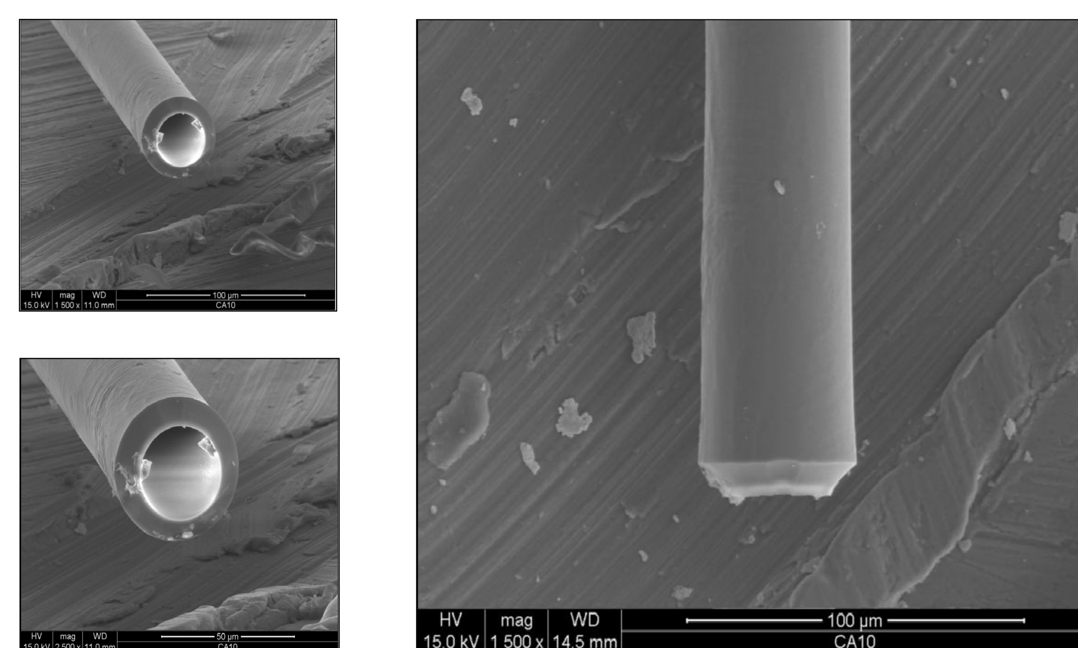


Figure 2. SEM imaging of the OptiMS Capillary emitter tip shows that the inner lumen of the capillary is preserved and the rim is highly sharpened to reduce the outer diameter radius. The shape is ideal for the ESI at low flow and electrospray voltages.

Protein Analysis by CESI 8000

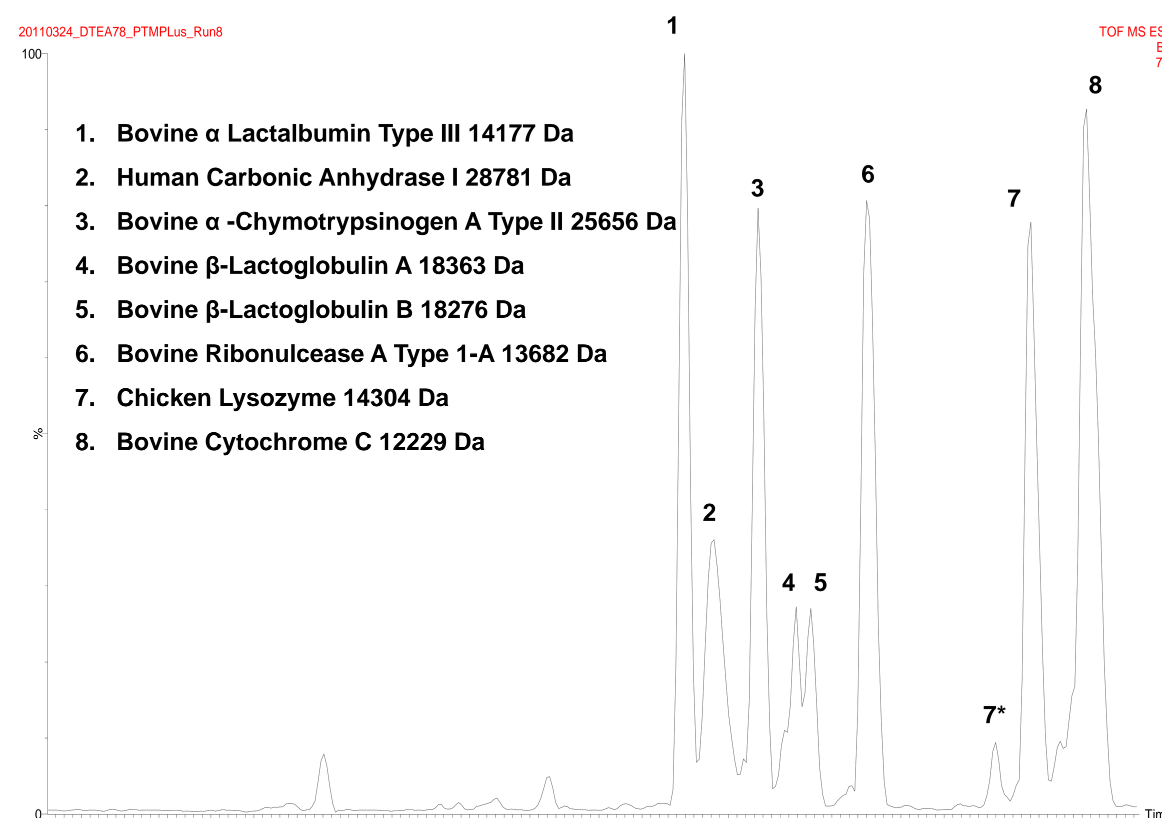


Figure 3. Eight proteins (50 µg/mL each) of various intact masses and isoelectric points are well separated using a 100 mM ammonium acetate pH 3.1 buffer at 20 kV reverse polarity. Partial separation between the two forms of Bovine β-Lactoglobulin show the high resolution of the system. The two peaks attributed to Lysozyme are not differentiated by the Mass Spectrometer indicated by the same intact mass, but can be easily separated by the CE, indicating a difference between the two components.

Glycoprotein Analysis by CESI 8000

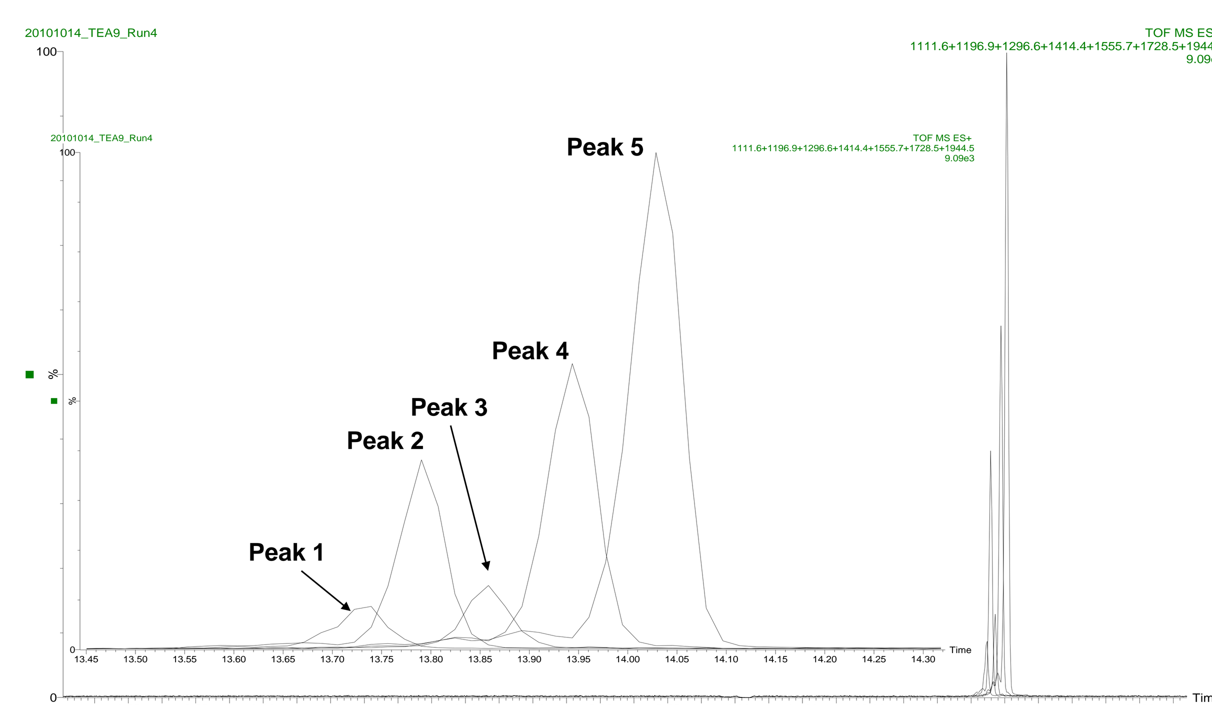


Figure 5. Separation of (200 µg/mL) Bovine Ribonuclease B at 20 kV reverse polarity in 200 mM ammonium acetate pH 3.6 buffer resulted in the separation of five distinct components.

Figure 5a.

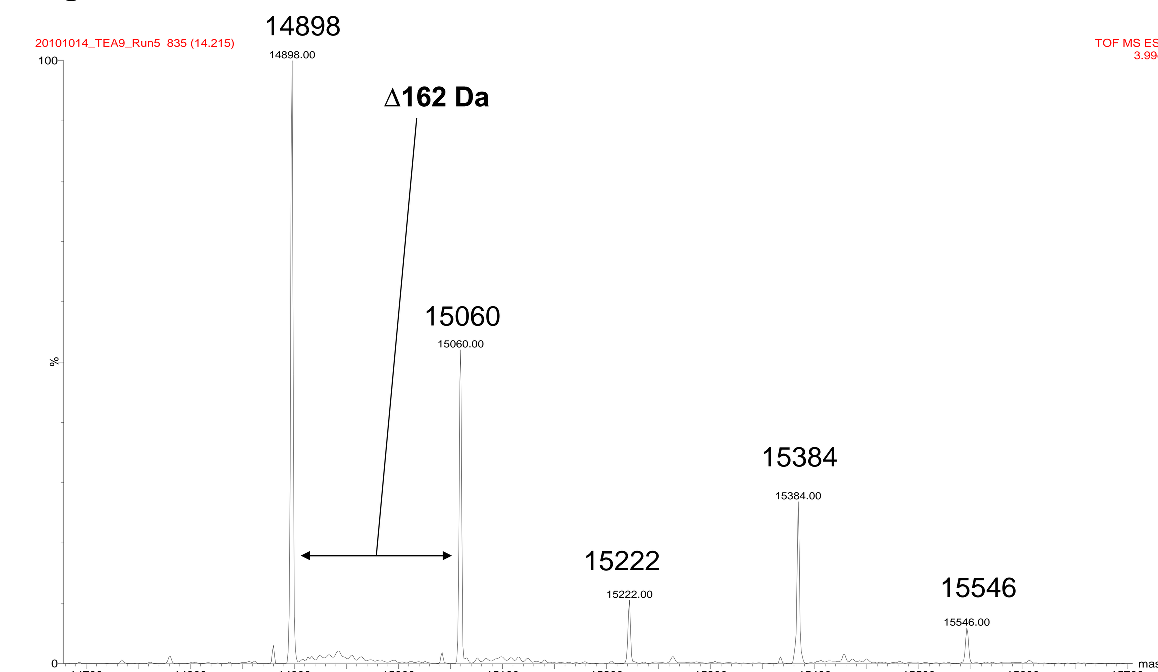
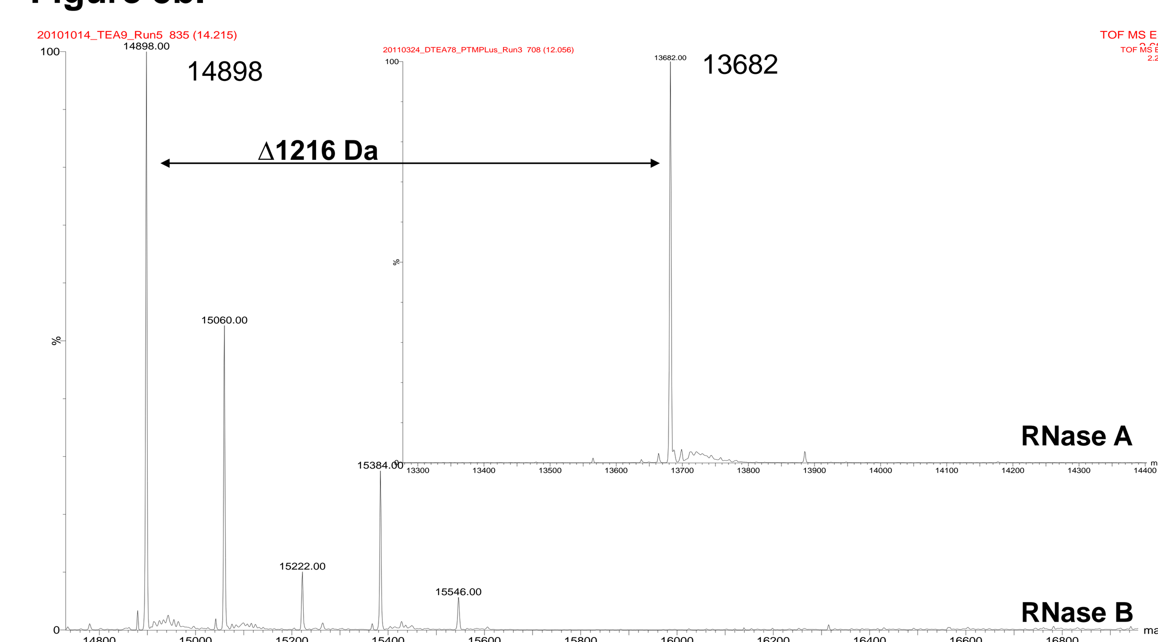


Figure 5b.



Figures 5 a, b: The calculated intact mass between the peaks in the electropherogram in Figure 4 show a mass shift of 162 Da, this along with the 1216 Da shift between the glycosylated (RNaseB) and non-glycosylated (RNase A) provides strong evidence that the peaks observed in the electropherogram are the result of an attached high mannose structure containing between 5 and 9 mannose subunits.

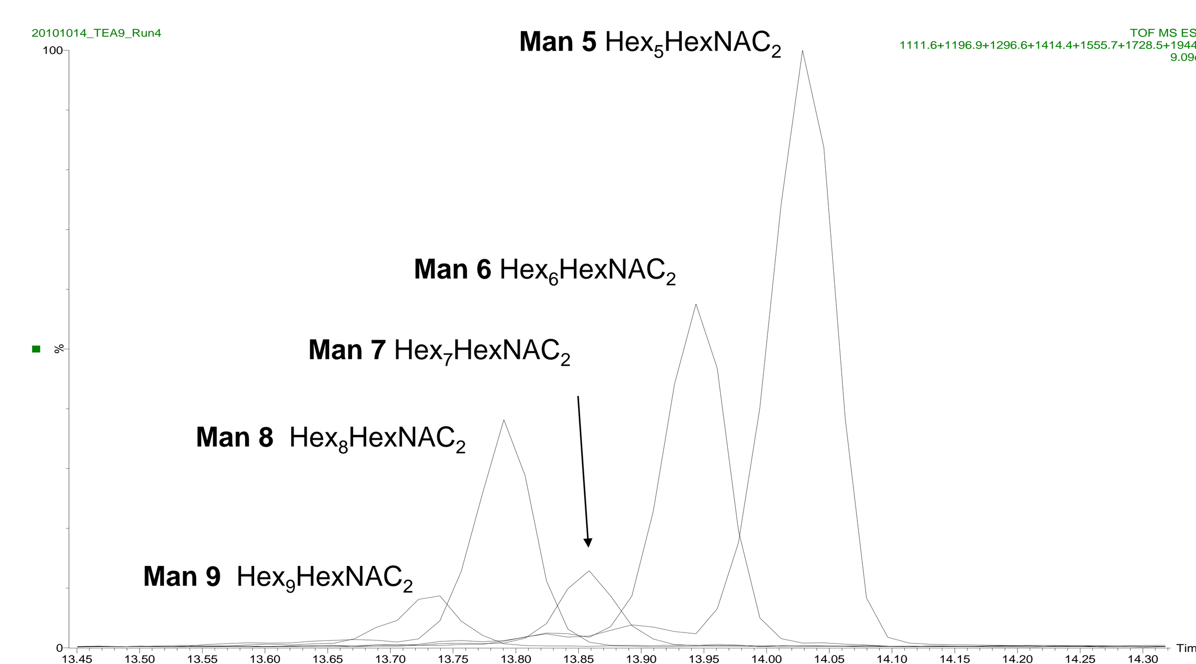


Figure 6. Shows the assignments of the various high mannose structures to the five peaks present in the separation of RNase B. The shift in electrophoretic mobility is also consistent with the addition of mass.

Peak Area Percent

	Avg.	StdDev	RSD
Man 5	41.76%	1.52%	3.65%
Man 6	27.27%	1.07%	3.92%
Man 7	7.97%	0.67%	8.45%
Man 8	16.58%	0.95%	5.74%
Man 9	6.48%	0.42%	6.43%

Table 1. Shows the results of 100 compositional analysis runs of RNase B glycoforms performed on 3 separate capillaries over 5 days. The results show that the analysis method are both highly repeatable and reproducible.

Conclusions

- Surface+ Coated OptiMS capillaries are capable of separating and detecting a broad range of proteins.
- Separations of RNAse B produced five highly resolved peaks which were identified as high mannose glycoforms.
- Highly reproducible assessment of the percent composition were achieved with RSD ranging between 3.65% to 8.45%

* In development.

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