Accurate Mass Measurement for Intact Proteins using ESI-oa-TOF

Application Note

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Introduction

Mass spectrometry (MS) has become a core technology for identification of primary protein structure, and many combinations of ionization mechanism and mass analyzer have been applied to protein research. The pairing of electrospray ionization (ESI) with time-of-flight mass spectrometry combines the advantages of online LC separation and multiply charged protein ions with extremely accurate mass measurements. This application note explores the usefulness of ESI with an orthogonal-acceleration reflectron time-of-flight mass spectrometer (oa-TOF MS) for the identification of intact proteins. It demonstrates the ability of an ESI-oa-TOF MS to deal with challenges such as detection of small variations in intact proteins, compatibility with fast chromatography, and identification of low-abundance proteins in the presence of much higher-abundance proteins; challenges encountered daily in many laboratories.

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Experimental

Sample

Protein standards were purchased from Sigma and the stock solutions (1 nM) were prepared in 0.01% TFA in water. The solutions were stored at –4°C and freshly diluted prior to analysis. PPAR gamma, a recombinant protein (MW=34763.2), was obtained in native buffer solution (0.106 mM) from GSK. The sample was diluted to 10.6 µM, 2.12 µM, 212 nM and 21.2 nM with 50% acetonitrile/ 0.1% formic acid.

Instrumentation

All experiments were performed using an electrospray ionization (ESI) interface on an Agilent LC/MSD TOF system (G3250AA) coupled to an Agilent 1100 Series LC system. The LC system consisted of a binary pump, well-plate autosampler with cooler, thermostatted column compartment and diode-array detector. Complete system control was accomplished using Agilent LC/MSD TOF software.

The TOF system used a combination of a dual-sprayer (dual-nebulizer) ESI source and a automated calibrant delivery system (CDS) to continuously introduce low-level reference masses. The TOF software included real-time reference mass correction software which automatically corrected mass assignments before the mass spectra were written to disk, thus simplifying and speeding later data processing. Deconvolution of the protein spectra was done using Agilent TOF Protein Confirmation software.

LC Conditions

Column:	Poroshell 300SB-C18, 1x75 mm, 5 µm
Flow rate:	0.6 mL/min (1.5 mL/min for the fast chromatography method)
Injection volume:	1–3 µL
Mobile phase:	A = 0.1% formic acid in water B = 0.1% formic acid in acetonitrile
Gradient:	20% B at 0 min 100% B at 5.5 min (1.0 min for the fast chromatography method)
Diode-array detector:	Signal 220, 4 nm Reference 360, 100 nm

MS Conditions

Ionization mode:	Positive ESI
Drying gas flow:	13 L/min
Nebulizer:	45 psig
Drying gas temp.:	350°C
Vcap:	4000 V
Fragmentor:	225 V
Skimmer:	60 V
OCT RFV:	250 V
PMT:	700 V
Scan range:	*m/z* 300–2000

Results and Discussion

The high sensitivity of the LC/MSD TOF proved very useful in the analysis of low-level intact proteins. As shown in Figure 1, both recombinant PPAR gamma (21.2 fmol) and BSA (100 fmol) were easily analyzed at low-to mid-femtomole levels.

The resolution of a mass spectrometer is very important for protein identification and characterization as well as for the determination of minor variants of recombinant proteins. Due to the high mass resolution of the LC/MSD TOF, a minor variant of recombinant PPAR gamma was detected. The signal at *m/z* 892.3683 (Figure 2) represents the +39 charge state of a protein with a molecular weight of 34763.4 u, and the signal at *m/z* 892.8388 represents the +39 charge state of a

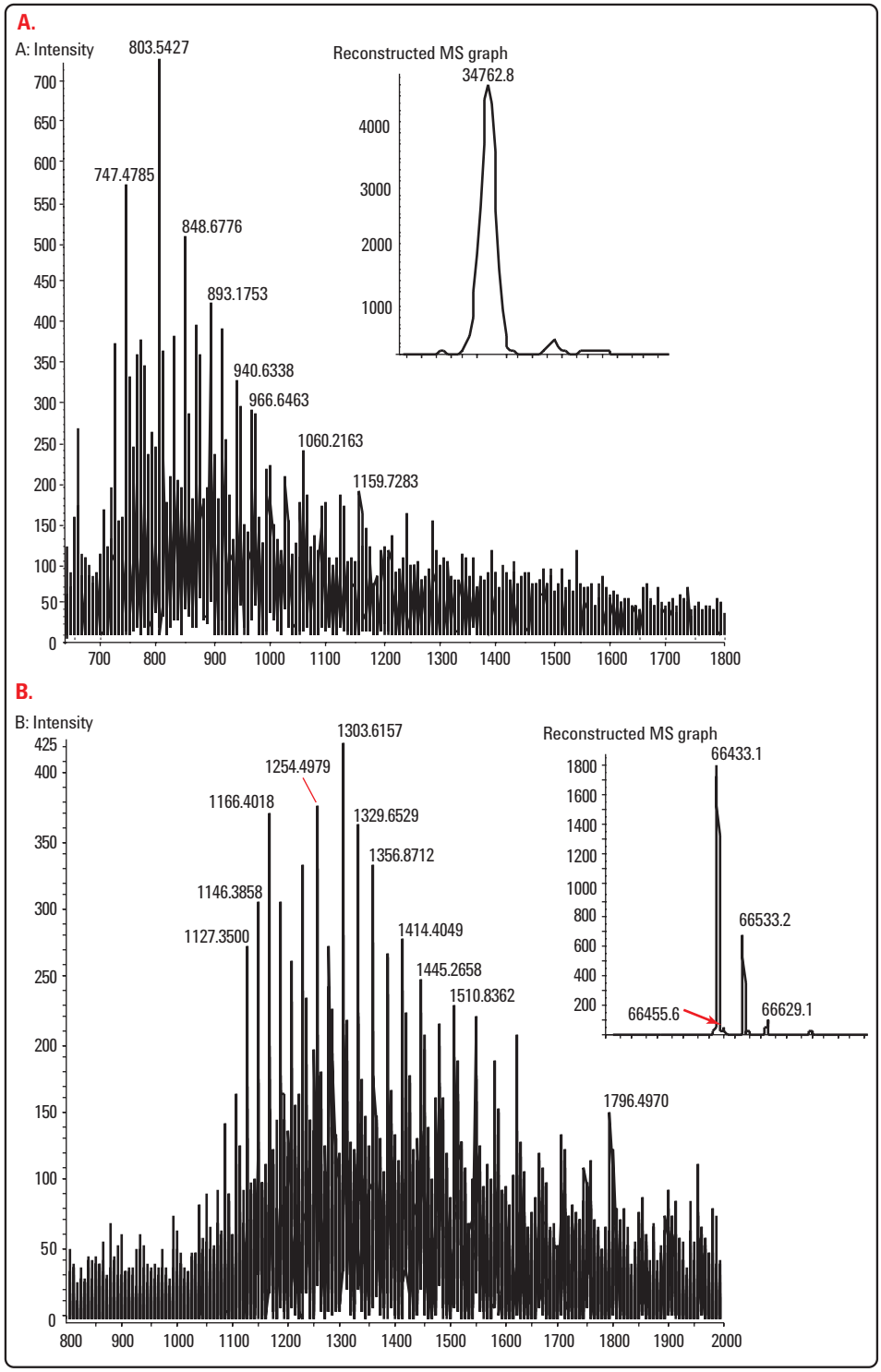

Figure 1A. Mass spectrum and deconvoluted spectrum of PPAR gamma (21.2 fmol on column)

Figure 1B. Mass spectrum and deconvoluted spectrum of BSA (100 fmol on column)

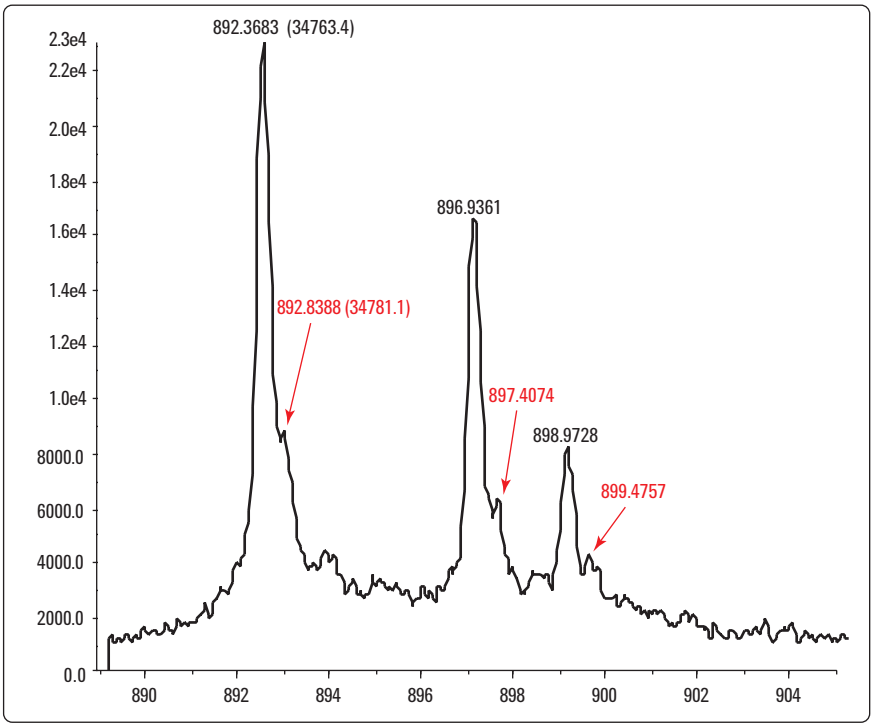

Figure 2. Mass spectra of recombinant protein PPAR gamma (2 pmol on-column). The shoulder peak at *m/z* 892.8388 represents the protein with a molecular weight of 34781.1 u, which is 18 u higher than the main component, PPAR gamma.

protein with a molecular weight of 34781.1 u. The 18 u mass difference between the two proteins suggests a DNA point mutation resulting from the substitution of a methionine for a leucine or isoleucine, although the presence of a spurious ammonium adduct cannot be ruled out.

In addition to high mass resolution, the LC/MSD TOF is capable of rapid scanning which makes it compatible with faster chromatography. A mixture of six proteins (ribonuclease A (R), cytochrome C (C), myoglobin (M), BSA (B), beta-lactoglobulin (Be) and PPAR gamma (P)) was analyzed by the LC/MSD TOF system. Figure 3 shows the total ion chromatograms (TIC) and selected spectra acquired using the regular method (Figure 3A) and a fast chromatographic method (Figure 3B). The six proteins were clearly separated with the regular method, but were not completely resolved with the fast chromatographic method. The spectrum from the unresolved components in Figure 3B was the same as for the spectrum from the resolved components in Figure 3A. The three proteins were correctly identified and the same molecular weights were determined using both the regular and fast chromatographic methods. This demonstrates that even when the proteins are not chromatographically resolved, the components can be correctly identified by the deconvolution process.

Biological samples are frequently very complex, and protein levels can vary greatly. A wide dynamic range is essential for the detection of lower-abundance proteins, such as post-translationally modified proteins, in the presence of higher-abundance proteins. The dynamic range of the LC/MSD TOF was evaluated by analyzing protein mixtures containing a fixed, low level of PPAR gamma and widely differing levels of BSA. Both of the proteins were identified in all of the samples and the deconvoluted molecular weights for the two proteins are listed in Table 1. The mean molecular weight for BSA was 66431.4 u (standard deviation = 0.5126) and for PPAR gamma was 36743.2 u (standard deviation = 0.1112). Figure 4 shows spectra from both proteins acquired when the BSA amount was approximately 500-fold higher than PPAR gamma amount.

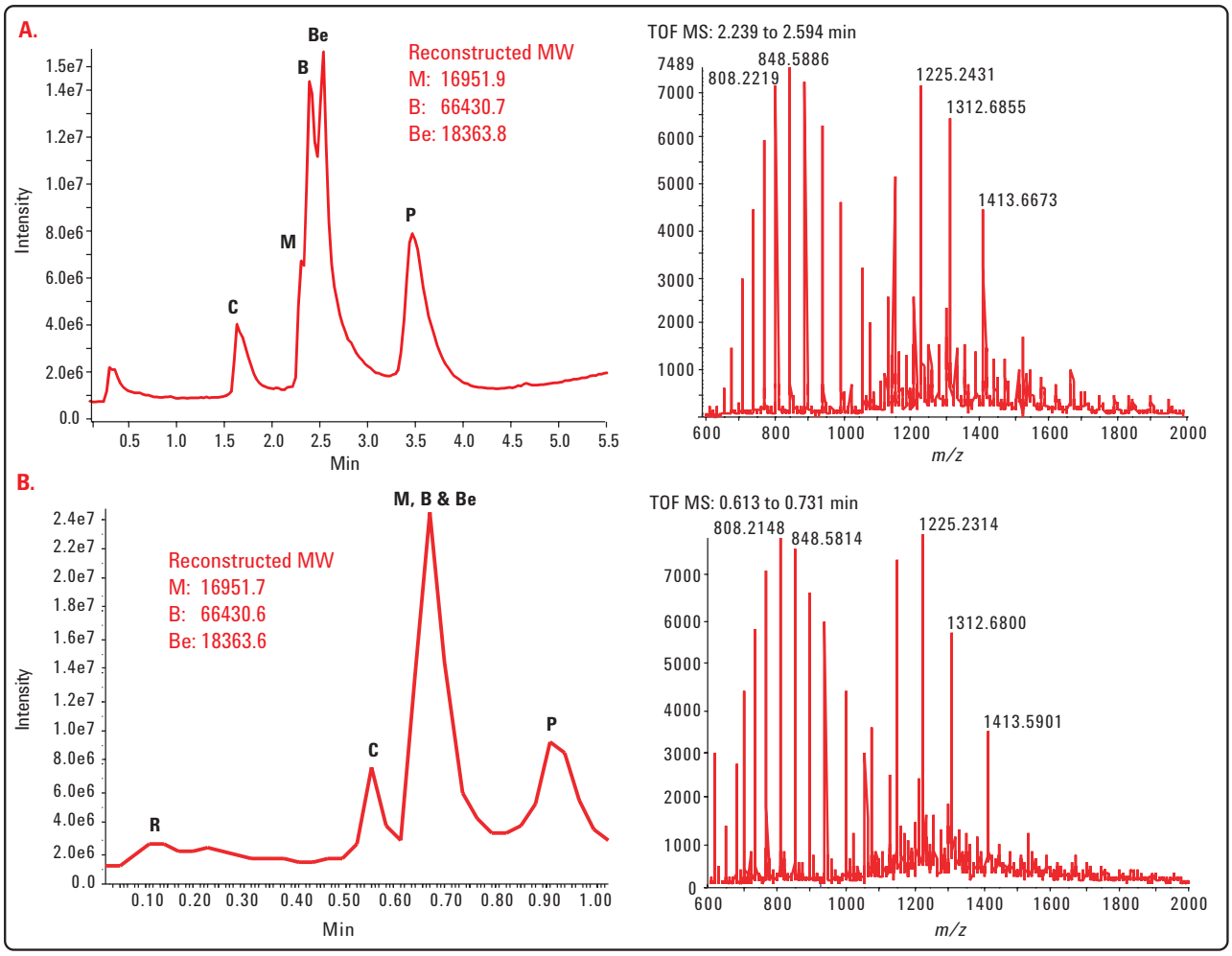

Figure 3. TIC and selected spectra from the analysis of a mixture (1 pmol on-column of each protein) of six proteins (ribonuclease A (R), cytochrome C (C), myoglobin (M), BSA (B), beta-lactoglobulin (Be) and PPAR gamma (P)) using a regular chromatographic method (3A) and a fast chromatographic method (3B).

Table 1. Deconvoluted molecular weights of BSA and PPAR gamma

BSA Conc.	0.1	1	5	10	20	50	100
PPAR 212 fmol/µL	34763.4	34763.3	34763.3	34763.2	34763.2	34763.1	34763.1
BSA	66430.4	66432.1	66431.4	66431.6	66431.3	66431.5	66431.2

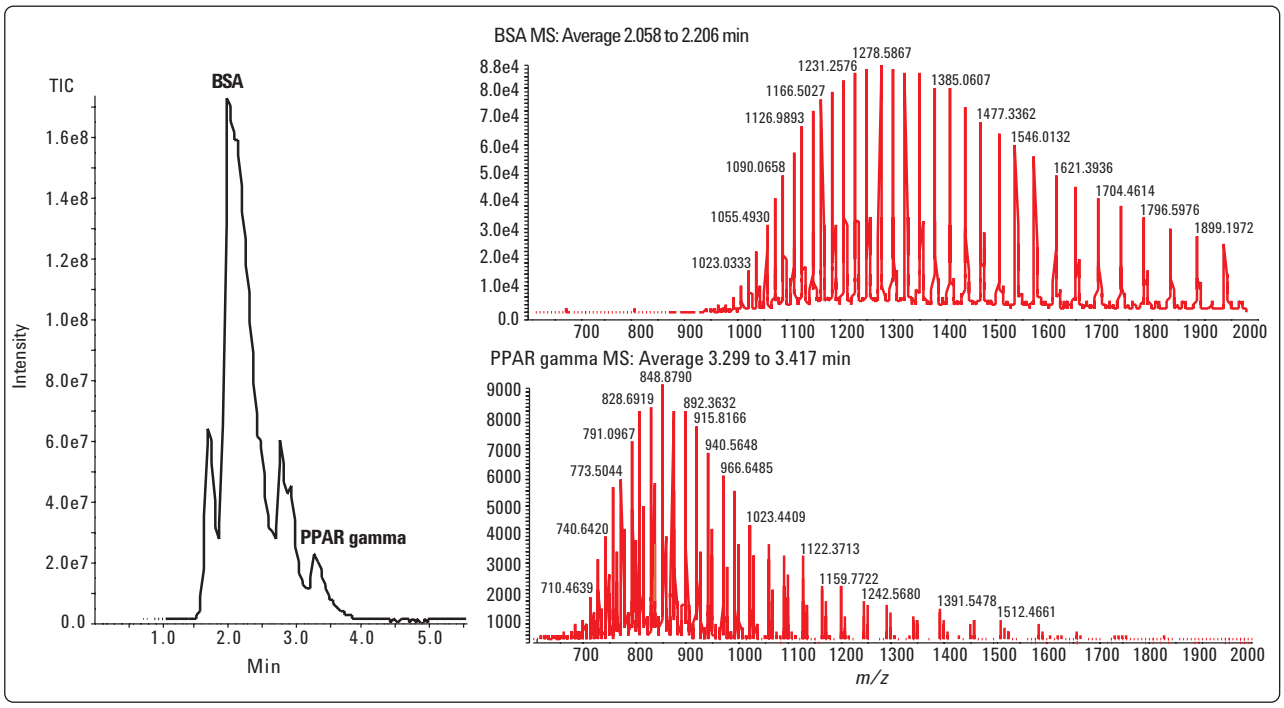

Figure 4. TIC and mass spectra from a mixture of BSA (300 pmol) and PPAR gamma (636 fmol)

When sample amount is limited, and additional information is needed, it is possible to split the LC flow prior to mass spectral analysis; analyzing a portion of the sample and collecting the remaining sample for further analysis. To determine the impact of flow splitting on ESI-oa-TOF MS sensitivity, myoglobin (200 fmol) was injected on column without split (Figure 5A) and split 10:1 prior to TOF MS analysis (Figure 5B). The results show that the sensitivity was decreased by only 20–30% when only 10% of the sample was consumed. The remaining 90% of the sample could be collected for proteolysis and peptide sequencing using other techniques.

Conclusions

Successful LC/MS analysis of intact proteins requires a number of instrument attributes. These include high sensitivity that is not greatly affected by flow splitting, excellent mass resolution, fast spectral acquisition (scanning), and a wide dynamic range. In experiments with a mixture of intact proteins, the Agilent LC/MSD TOF, an ESI-oa-TOF mass spectrometer, exhibited all of these attributes. It consistently identified proteins at the low- to mid-femtomole level, and that sensitivity decreased only 20-30% even when 90% of the sample was split away prior to analysis. It easily identified minor (18 u) variations in intact proteins. Incompletely resolved chromatographic peaks resulting from fast chromatography did not negatively affect either protein identification or accurate mass assignment. Finally, a wide dynamic range allowed the LC/MSD TOF to identify a protein at the femtomole level in the presence of a second protein whose abundance ranged from the same to 500-fold greater.

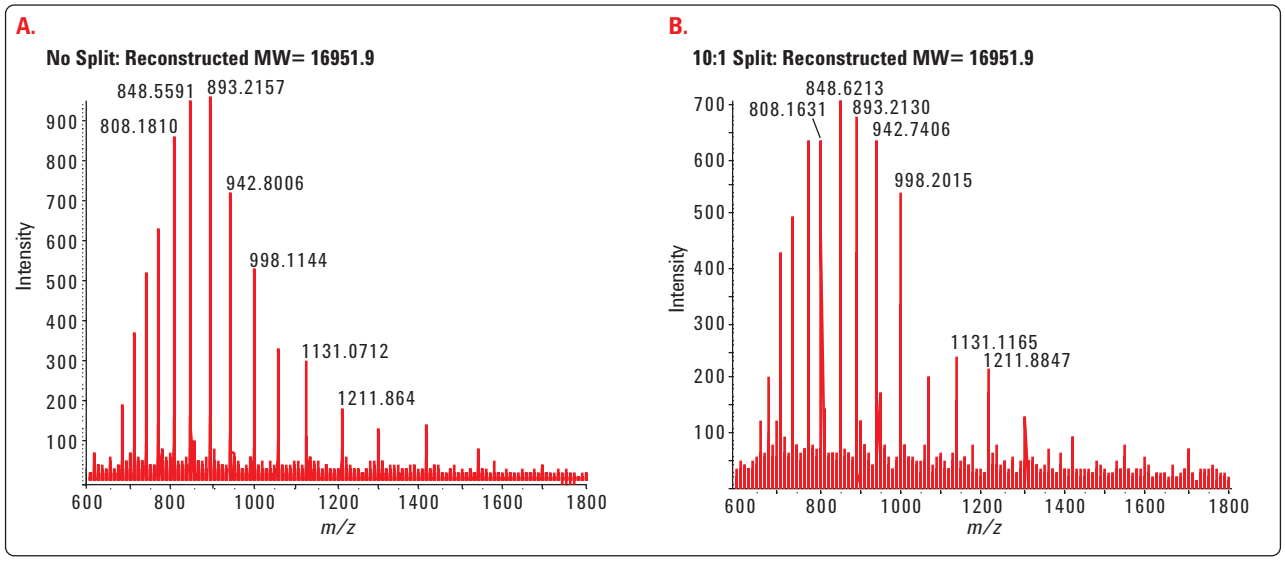

Figure 5. The spectra of myoglobin (200 fmol) without (5A) or with (5B) splitting prior to ESI-oa-TOF MS analysis

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