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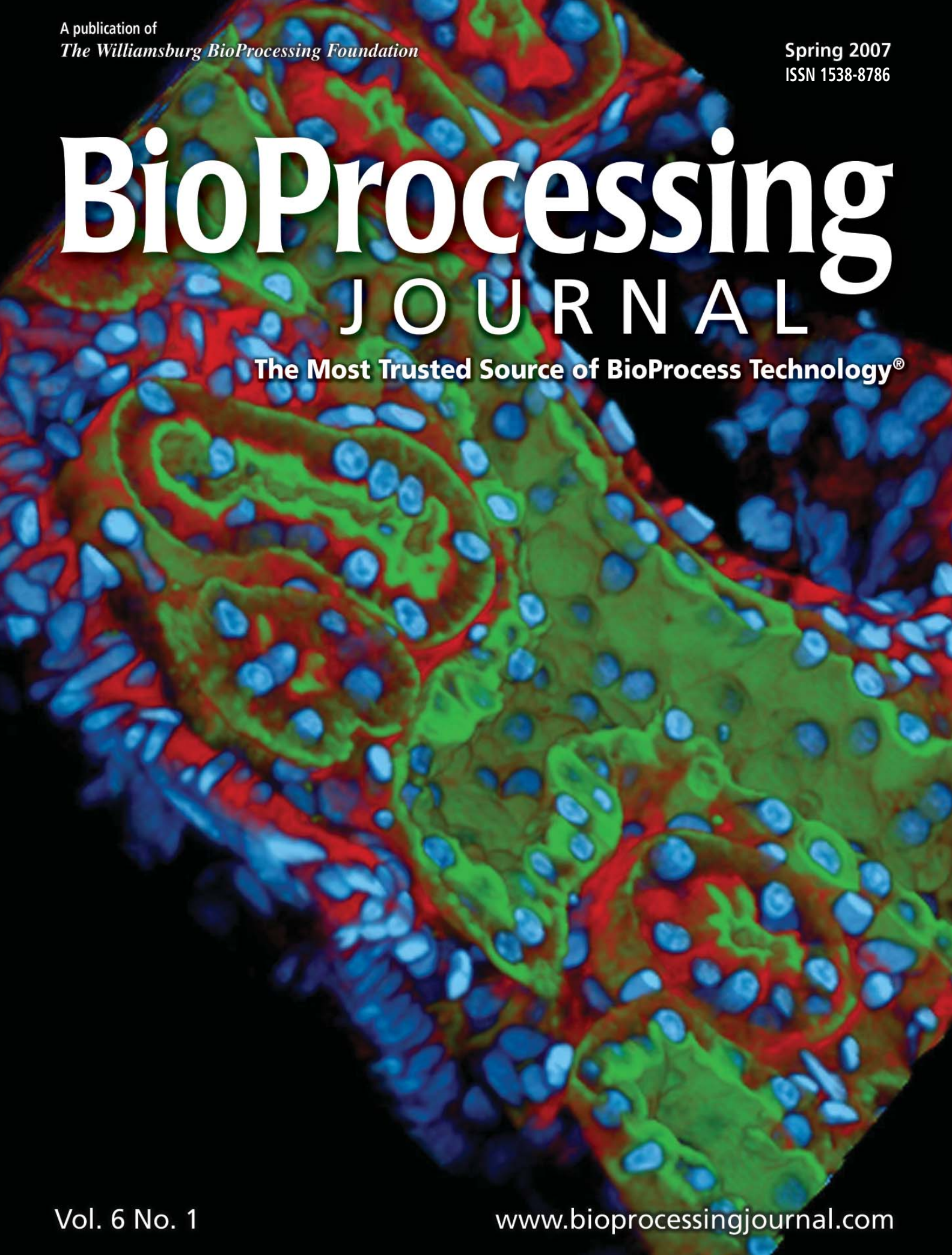

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Fast On-Line Desalting of Proteins for Determination of Structural Variation Using Exact Mass Spectroscopy

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Analytical tools for the characterization of protein identity and structure are fundamental to many fields of biochemical research. For the development of protein biopharmaceuticals, it is particularly important to measure modifications of the structure that may affect safety and efficacy. This application requires the analysis of large numbers of samples during process development. Small quantities of modifications must be detected in samples that are in the presence of more abundant native protein. Many kinds of analytical techniques are applied to this problem including peptide mapping, bioassays, liquid chromatography, spectroscopy, and so on.

One of the most attractive tools is mass spectrometry, since essentially any change in the molecule is accompanied by a change in molecular weight. With the recent advent of readily accessible mass spectrometers capable of routine exact mass measurement, it is appropriate to consider the detailed requirements for this application.

First, of course, a mass spectrometer is required. That instrument must be capable of measuring the molecular weight of the protein with an accuracy and precision compatible with discriminating changes corresponding to the chemical modifications being measured. These range from an increase of 1 Da for a deamidation, to 16 Da for an oxidation, to over 100 Da for a change in amino acid sequence or a modification of glycosylation state. Second, software must be available to analyze the multiply-charged spectrum, extracting the true molecular weight while preserving information that can be used for relative quantitation. Third, this deconvolution must reveal the presence of low-concentration contaminant proteins in the presence of much larger amounts of native protein. Finally, this must be accomplished with minimal prior knowledge of the sample, and without human intervention. Fortunately, the instrumental requirements can be met using electrospray/time-of-flight mass spectrometers like the LCT Premier™ and Q-Tof micro™ used in these experiments. Appropriate spectral processing can be achieved using the MaxEnt™ 1 algorithm. However, satisfactory results can only be achieved with high signal-to-noise mass spectra that are free of artifacts. Such results are challenging with real biopharmaceutical samples.

The protein samples encountered in the development of biotherapeutics are dissolved in buffers, often with additional salts. Other contaminants may be present as components of the production and purification. These sample constituents interfere with the ionization of the protein molecule in the electrospray source. They also form adducts and other artifactual mass signals that do not reflect the state of the protein in solution. A variety of off-line and on-line techniques have been developed to prepare proteins for electrospray ioniza-

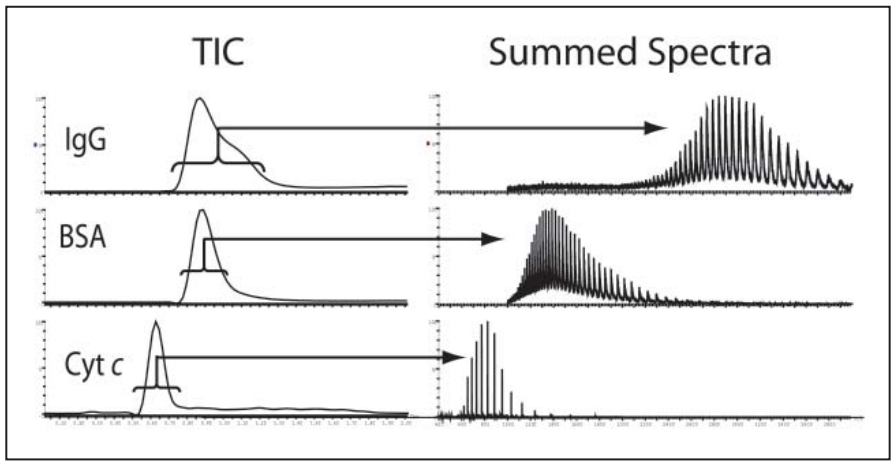

Figure 1. The chromatographic elution profile is shown for three test proteins (left). Mass specta were summed across the chromatographic peaks (right). These experiments show that the cartridge can be used over a wide range of protein types.

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tion/mass spectroscopy (ESI)/(MS). We have developed a device and method that is shown to work for a wide range of protein samples. The MassPREP™ on-line desalting cartridge is a 2.1 x 10 mm cartridge packed with a polymeric material. It has a long life and high capacity. There is neither detectable carryover nor background bleed of MS interferences. We have used this approach to monitor oxidation in a forced degradation experiment and to assess the glycosylation state of a therapeutic antibody.

Materials and Methods

The Waters Alliance® 2796 separations module was used for automated sample introduction and for eluting the protein from the cartridge. A Waters two-position, six-port switching valve was used to divert the flow-through salt to waste before eluting the protein into the mass spectrometer. Protein spectra were collected on either a Waters Micromass® Q-Tof micro or LCT Premier. Spectra were processed with MaxEnt 1.

Bovine serum albumin (BSA), cytochrome *c*, a monoclonal IgG, ribonuclease and enolase were obtained from Sigma (Sigma-Aldrich, Inc., St. Louis, Missouri, USA). All proteins were dissolved in phosphate-buffered saline (PBS) at a concentration of 100-1,000 ng/µL as noted. A therapeutic antibody was kindly provided by Pierre Fabre Médicaments–CIPFI. Where noted, samples were oxidized by exposure to hydrogen peroxide, as described in the figure legends.

The MassPREP on-line desalting cartridge was used as received. It was equilibrated in 95% 0.1% formic acid (FA) in water: 5% 0.1% FA in acetonitrile. Upon sample injection, flow was diverted to waste for 0.5 min. The protein was then eluted into the mass spectrometer with a gradient to 80% 0.1% FA in acetonitrile. All steps were at 0.4 mL/min.

Results and Discussion

The functionality and compatibility of the desalting technique for proteins of different size and properties was tested. Figure 1 shows the elution profile and resultant spectra for IgG, BSA, and cytochrome *c*.

These three proteins represent a small basic protein (~12 kDa), a medium size acidic protein (~62 kDa), and a large globular protein (150 kDa). While there were some differences in the elution profiles, there was little separation on such a small cartridge with such a steep gradient. High quality spectra were obtained for all three proteins. This technique can, therefore, be used across a wide range of proteins.

The utility of the cartridge in a high sample load environment depends on its lifetime and whether it exhibits any sample carryover. The test protocol included a series of 100 injections in 25 sets of four repetitions. Each set of four injections included a blank, a sample of BSA, a sample of IgG, and a blank. The results of the 25th cycle, injections 97 through 100, are shown in Figure 2. Both the BSA and IgG yielded clean spectra with no overlap or cross-talk. There was no evidence of protein spectra or background bleed from the cartridge in the blanks around these injections. Under these operating conditions, the performance did not degrade over at least 100 injections. There was no detectable carryover from one sample into the next. This technique can be used to measure molecular weights over large sample sets.

The loading capacity of the cartridge must be appropriate for the dynamic range required by the analytical problem and compatible with the dynamic range of the detector. Several loading studies were performed. The results of loading increasing amounts of IgG are shown in Figure 3. With 0.1 µg applied to the cartridge, the eluted protein spectrum was detectable but near the intensity limits for good quantitation. With increasing amounts up to 10 µg of IgG, the same elution pattern was observed, and the intensity of the MS signal increased appropriately. There was no breakthrough of protein. The early eluting material was salt and other small molecule sample contaminants.

When the same loading study was repeated for other proteins, the range of utility was shifted in proportion to the molecular weight of the protein. That is, the cartridge gave good results from 0.1–10 µg of IgG (150 kDa) and 0.01–1 µg of cytochrome *c* (12.5 kD) (data not shown). The loading ranges were fairly conservative. In particular, there was no chromatographic evidence that the

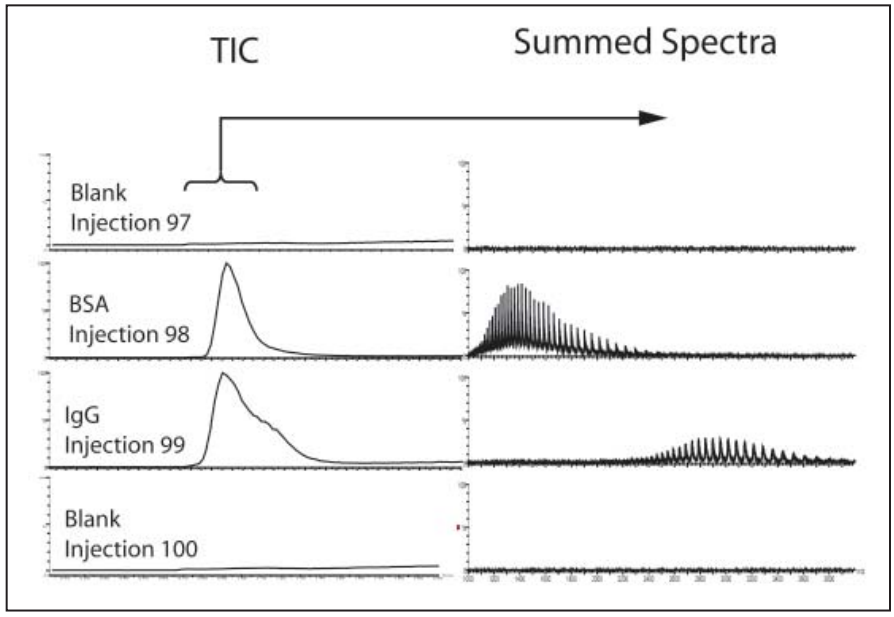

Figure 2. A series of injections consisting of a blank, 1 µg BSA, 1 µg monoclonal IgG, and blank was repeated 25 times for a total of 100 injections. The performance of the cartridge is unaltered over this series. There is no evidence of carryover or background interference in the final blank injection.

cartridge was overloaded at these concentrations, so higher loads should be possible for many samples.

The above-mentioned experiments demonstrate that the basic properties of the MassPREP on-line desalting cartridge are appropriate for preparing protein samples for ESI/MS. While this function is important in many applications, it should prove particularly valuable in the development of protein-based biopharmaceuticals. This developmental process includes stages like optimizing tissue culture conditions, protein purification, developing formulations, stability testing, and so on. In every step, it is necessary to monitor the protein for identity, purity, and structural integrity. On-line desalting directly into the ESI/MS can provide a solution to these analytical problems. We have tested and illustrated these ideas.

Stability testing often begins with a forced degradation experiment to ensure that the analytical method can be used to detect all the modifications. In the case of proteins, oxidation of methionine represents an important degradative pathway that can be forced with exposure to hydrogen peroxide.

Ribonuclease was dissolved in phosphate-buffered saline at a concentration of 100 ng/mL. It was incubated for 24 hours at 37°C, and then desalted directly into the LCT Premier. The deconvolution of the resultant data is shown in Figure 4. When MaxEnt 1 was applied to the control and oxidized ribonuclease spectra, the complexity of the samples was apparent. Both the heterogeneity of the starting material and the effect of oxidation were readily apparent.

It was interesting to note that the sample of ribonuclease contained a mixture of native and doubly-oxidized material after 24 hours. No other oxidation states were apparent. Since this sample has four possible oxidation sites, it appears that they were not all equally reactive. It was not possible to discern, from this forced degradation experiment, whether there were two rapidly oxidized sites or only a single one that has been forced to the sulfone by the extreme conditions.

Enolase was dissolved in PBS at a concentration of 100 ng/mL. It was incubated for 24 hours at 37°C and then desalted directly into the LCT Premier. As in the ribonuclease experiment, the product reached a steady state. To determine whether the analytical technique could discriminate intermediate stages in the oxidation process, samples were incubated with different concentrations of peroxide and evaluated after four hours of incubation, as shown in Figure 5. Under these conditions, the oxidation intermediates can be discriminated. The results are consistent with two labile methionine residues of the five present

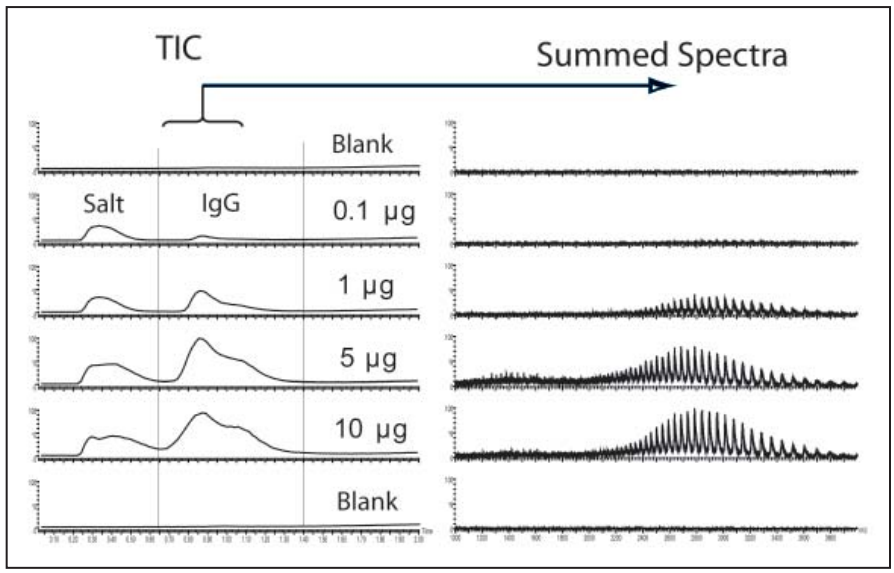

Figure 3. Total ion chromatograms and summed spectra for indicated region demonstrate loading capacity and carryover of monoclonal IgG1. Blanks were injected between increasing loads of IgG1 to measure carryover (data not shown). The amount of sample can be increased to 10 µg in 10 µL of PBS without breakthrough and with no detectable carryover in the blank following a 10 µg injection.

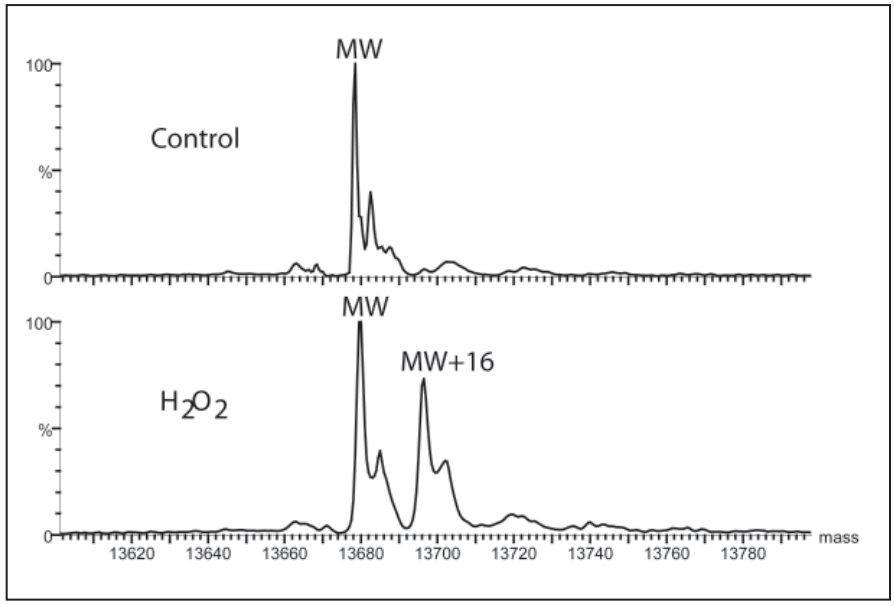

Figure 4. MaxEnt 1 is applied to the ribonuclease spectra acquired after desalting. The complexity of the samples is apparent. Both the heterogeneity of the starting material and the effect of oxidation are readily seen. It is interesting to note that the sample of ribonuclease contains a mixture of native and doubly-oxidized material after 24 hours. No other oxidation states are apparent.

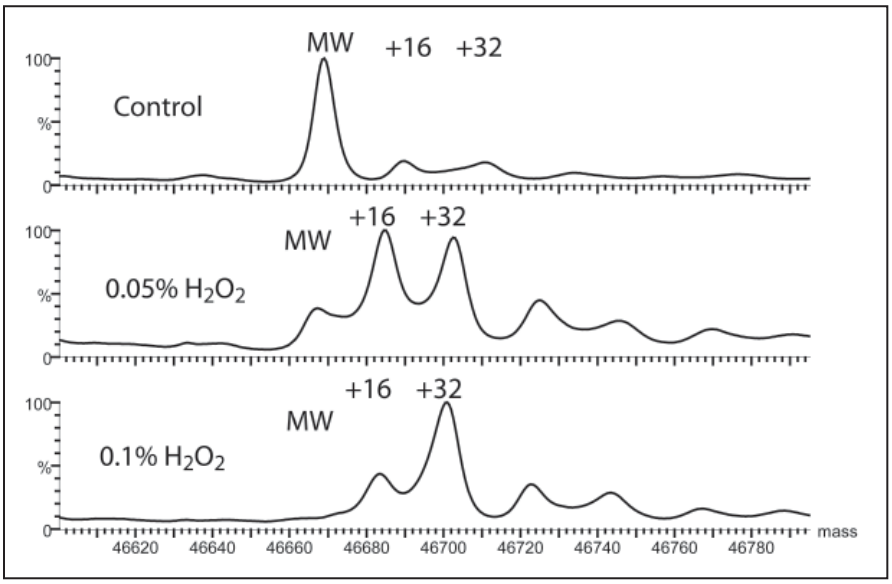

Figure 5. Samples of enolase incubated at two different concentrations of hydrogen peroxide for four hours. Under these conditions, the oxidation intermediates can be discriminated. The results are consistent with two labile methionines of the five total.

in this molecule.

The development of biopharmaceuticals includes ensuring that the molecule has been correctly synthesized and folded. For example, changes in culture media or conditions can affect the pattern of glycosylation in monoclonal antibodies. This is often assessed through enzymatic release of the glycans and oligosaccharide mapping using high-performance anion exchange or capillary electrophoresis. Direct measurement of the glycoforms would answer many of the analytical questions. For these experiments, the MassPREP on-line desalting cartridge was mounted directly on the diverter valve of the mass spectrometer, and the procedure was executed manually using a syringe. First, in load position, the cartridge was washed with about 1 mL of 5% acetonitrile, 95% water and 0.1%

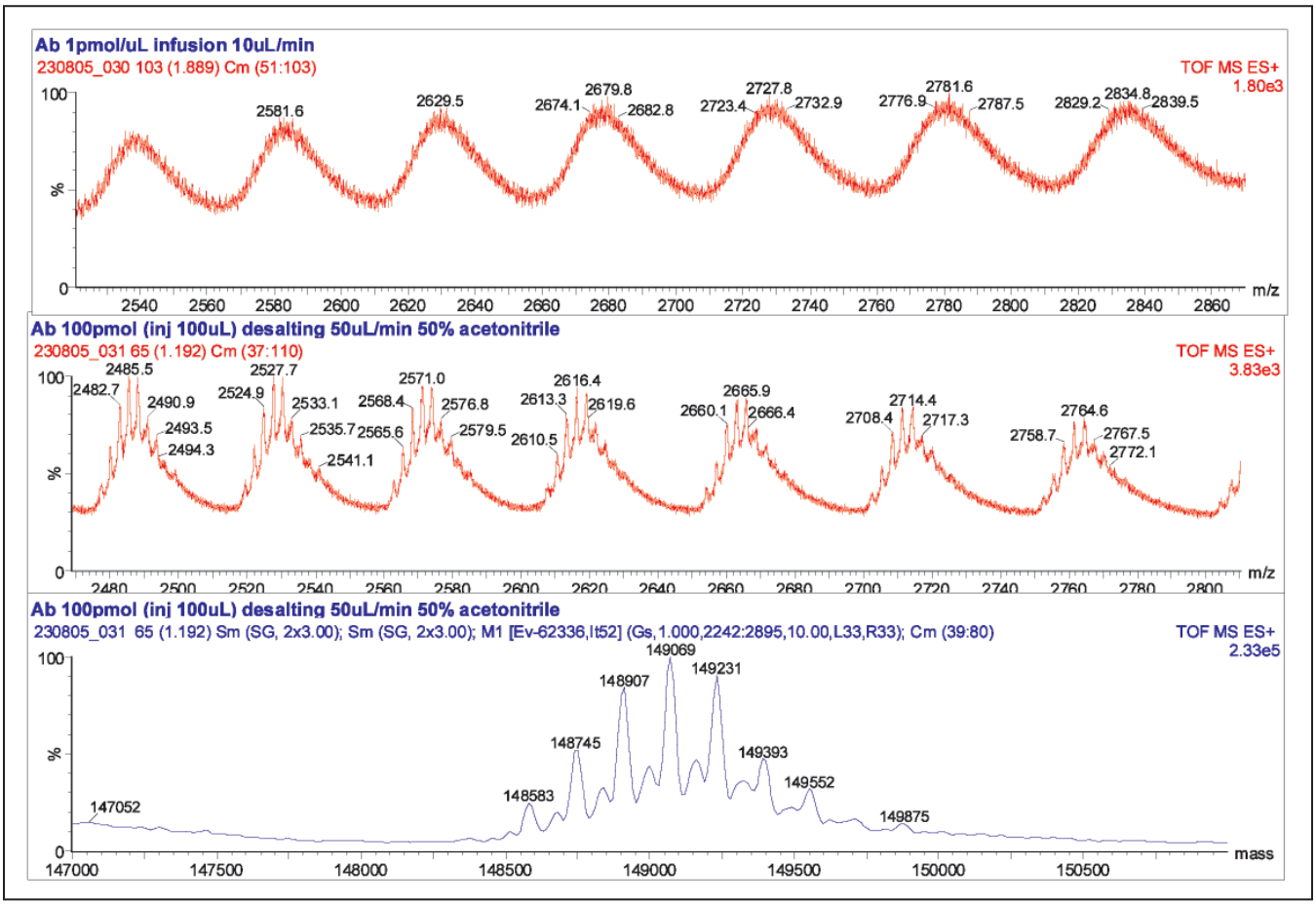

Figure 6. When a therapeutic antibody sample is directly infused (top), the charge state envelope can be discerned. The signal-to-noise of this spectrum is, however, too low for highly discriminatory deconvolution. The spectrum obtained as the antibody elutes from the desalting cartridge (middle) immediately shows the fine structure in the charge state envelope. This indicates that there are multiple molecular species in the sample. When the spectrum of the desalted sample is deconvoluted, the species are apparent. The multiple glycosylation states of the antibody can be distinguished in this way.

FA. Then a sample aliquot (100 µL at 1 µM) was injected. The absorbed sample was desalted with about 1 mL of 5% acetonitrile, 95% water and 0.1% FA.

The valve was switched to the inject position, and the sample was eluted at 50 µL/min with a solution of 50% acetonitrile, 50% water and 0.1% FA. The results are shown in Figure 6. When the antibody sample was directly infused (top), the charge state envelope could be discerned, but the signal-to-noise of this spectrum was too low for highly discriminatory deconvolution. The spectrum obtained as the antibody eluted from the desalting cartridge (middle) immediately showed the fine structure in the charge-state envelope. This indicates that there were multiple molecular species in the sample. When the spectrum of the desalted sample was deconvoluted (bottom), several protein species were apparent. The multiple glycosylation states of the antibody can be distinguished in this way, and good estimates of the relative amounts are possible.

Conclusion

These experiments show the evaluation of the MassPREP on-line desalting cartridge. High-resolution mass spectra can be obtained from protein samples in less than five minutes. The device and method can be applied across a wide range of protein properties. Cartridge life is at least 100 injections and there is no evidence of carryover or bleed across such a series. Loading capacity exceeds two orders of magnitude. The spectra can be deconvoluted with MaxEnt 1 to identify the components of the mixture. This approach is used to monitor oxidation in a forced degradation experiment and proved suitable for detecting variations in the proportion of oxidation as a function of peroxide concentration. The technique yields sufficiently high signal-to-noise spectra for identifying the glycosylation states of a therapeutic antibody.

The examples demonstrate the utility of the MassPREP on-line desalting cartridge across a wide range of applications, important in the development of biopharmaceuticals.