



Application Note # MT-112

## Precise MW Determination of Intact Proteins by Multiple Charge State Analysis of MALDI Generated Ions

### Abstract

The use of DHAP as MALDI matrix enhances the formation of multiple charged protein ions. These ions can be well calibrated by protein standards such as myoglobin or cytochrome c in the 8-20 kDa  $m/z$  range.

The ultrafleXtreme with FlashDetector in combination with 4GS/s digitization rate can turn externally calibrated high quality data obtained from DHAP preparations into intact protein molecular weight (MW) information at hitherto unknown accuracy levels in the low ppm range.

Accurate intact molecular weight analysis adds an additional characterization tool to the already considerable range of biopharmaceutical characterization workflows which are possible with Bruker MALDI-TOFs.

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MALDI-TOF	microflex
High Accuracy	

## Introduction

The utility of MALDI-TOF for the determination of protein molecular weights (MWs) with the typical  $MH^+$  ions has been restricted by the availability of well characterized and highly homogeneous mass calibrants at larger MWs. In addition, extensive adduct formation made large MW determinations difficult and inaccurate resulting in relatively large mass errors, e.g., 200-300 Da for 67 kDa BSA. Reflector mode analyses with the typically used protein matrices (DHB, SA) induced strong metastable decay disabling the analysis of proteins with MWs  $\sim >15$  kDa. We used the MALDI matrix 2,5-dihydroxyacetophenone/diammonium hydrogen citrate (DHAP/DAHC), well-known for its suitability for the analysis of labile and large proteins [1,2] in a new way to overcome these limitations. DHAP generates multiple charged ions at utmost resolution which are ideally suited for precise and accurate MW determinations.

We describe here a new approach in which the well-established protein mass calibrants cytochrome c and myoglobin (both equine) are used in their singly and doubly protonated forms to calibrate the  $m/z$  range 8-20 kDa. Proteins much larger than 20 kDa produce multiple charged ions that fall into the calibrated 8-20 kDa analysis window and only these ions are taken for MW determination. The new MALDI-TOF-DHAP method produces isotope resolved ions for up to 30 kDa proteins in reflector mode and mass accuracies in the 15-30 ppm range even for BSA. In linear mode 1500 resolution is observed in the entire protein mass range and achievable mass accuracies are improved 10-100-fold compared to previous capabilities.

## Experimental

### Materials

All standard proteins and BioXtra diammonium hydrogen citrate (DAHC) were from Sigma. MOPC21 was from Sigma and TCEP reduced for the analysis of the light chain (MOPC21-LC) without any further purification.

The matrices sinapinic acid (SA) and 2,5-dihydroxyacetophenone (DHAP) were from Bruker, all solvents were HPLC grade: water (Millipore), ethanol (Fluka), acetonitrile (Sigma-Aldrich) and trifluoroacetic acid (TFA, Merck).

### Sample Preparation

Protein stock solutions (100 pmol/ $\mu$ l in 0.1 % TFA) were diluted 1-10 fold prior to sample preparation. MALDI matrices (Bruker) were prepared exactly as described in the product information. In brief, the DHAP solution was prepared from 7.6 mg of DHAP in 375  $\mu$ l ethanol and 125  $\mu$ l of aqueous DAHC solution (18 mg/ml). One part sample solution was mixed with one part of 2 % TFA and one part of DHAP solution and mixed using a pipette tip. The solution was ready for spotting when the crystallization started and the formerly clear mixture turned opaque. One to 2  $\mu$ l were spotted on a stainless steel MALDI target and dried at ambient air.

### MALDI-TOF MS Data Acquisition and Processing

All spectra were acquired on the new ultrafleXtreme MALDI-TOF/TOF mass spectrometer equipped with 2 kHz Bruker smartbeam-II solid state laser and FlashDetector using Compass 1.4 control and processing software. For linear mode acquisition  $U_{IS1}$ ,  $U_{IS2}$ ,  $U_{Lens}$  and  $PIE_{Delay}$  were optimized for high resolution in the 5-20 kDa  $m/z$  range using the LP 5-20 kDa parameter file. Sample rate was 0.4 ns.

In reflector mode, in addition, the parameters  $U_{Ref1}$  and  $U_{Ref2}$  were optimized for isotopic resolution of myoglobin using the RP 5-20 kDa parameter file. Sampling rate was 0.25 ns equivalent to a 4 GS/s digitization rate. Five thousand-10,000 shots were accumulated in "random walk" and with "large" smartbeam laser focus. Spectra were processed in Compass using "centroid" peak picking (80%).

Prior to calibration, the spectra were processed with Compass 1.4 using smoothing and baseline subtraction for reproducible peak annotation on non-resolved isotope distributions: centroid 80 %, 5 cycles, 0.7 Da.

For external quadratic calibration, the Bruker "Protein Calibration Standard 1" was used with the myoglobin and cytochrome c  $[M+H]^+$  and  $[M+2H]^{2+}$  ions - from now on referred to as 1+ and 2+ ions - with the following average  $m/z$  values:

Protein	Element composition	$m/z$ 1+	$m/z$ 2+
CYC_HORSE	C560H874N148O156S4Fe1	12360.97	6180.99
MYG_HORSE	C769H1212N210O218S2	16952.31	8476.66

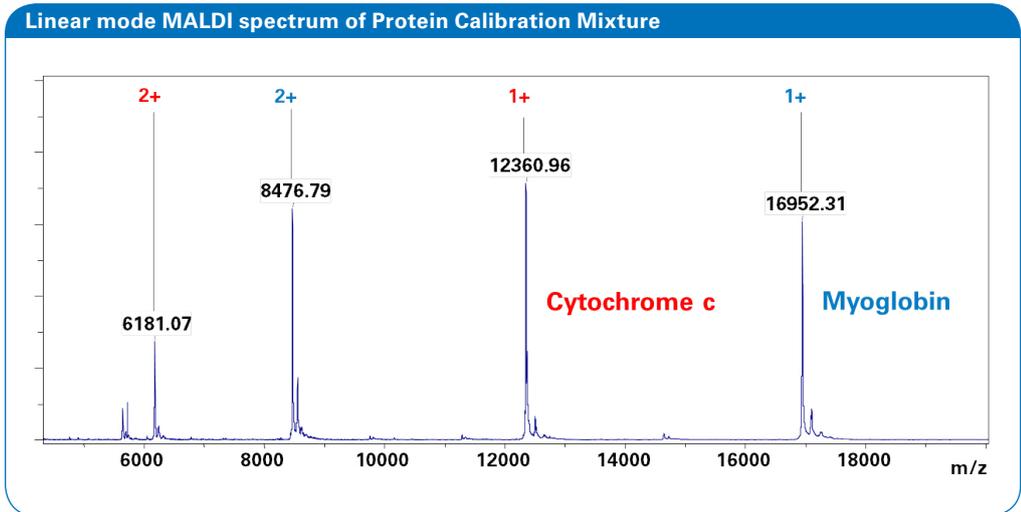


Figure 1: Linear mode MALDI spectrum of Protein Calibration Standard 1 in the m/z range < 20 kDa. The spectrum was acquired from a DHAP/DAHC preparation. Resolution achieved is typically > 1500 and the maximal mass deviation 0.2 Da.

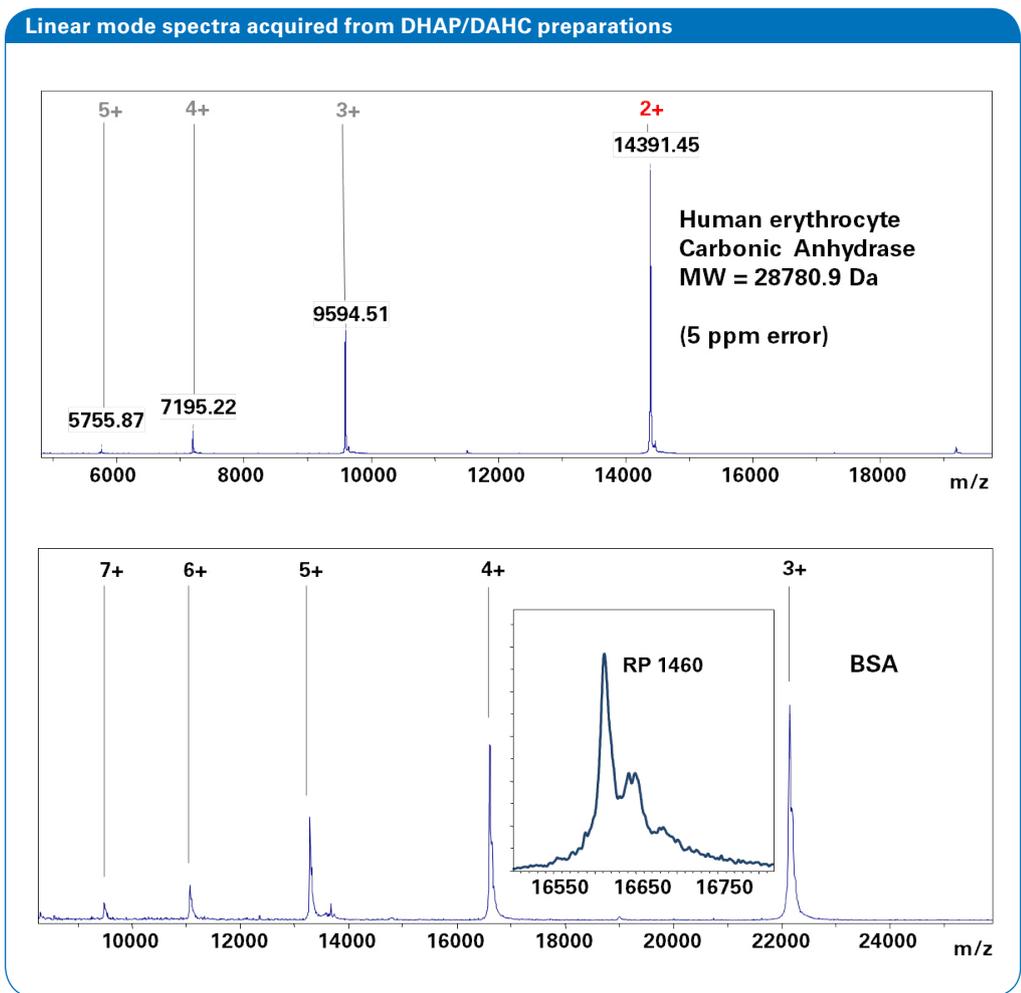


Figure 2: Linear mode spectra acquired from DHAP/DAHC preparations in the m/z range < 22 kDa. (Top) human erythrocyte carbonic anhydrase, the 2+ state was used for MW determination; resolution 1600. (Bottom) Bovine serum albumin provided a resolution of 1460 and typically the 4+ ion was used for MW determination.

## Results

The linear mode spectrum of Protein Calibration Standard 1 in DHAP/DAHC in the mass range 5-20 kDa clearly shows the 1+ and 2+ ions of cytochrome c and myoglobin with high resolution of the 1+ ions of 1502 and 1610, respectively (Fig. 1). The calibration spectrum represents the true MWs of the calibrant proteins with a mass accuracy of better than 0.2 Da. The calibration coefficients were used for external calibration of the analyte spectra, no spiking of internal calibrants is necessary! It is advantageous to use only the highest m/z ion for MW analysis that falls into the ~10-20 kDa analysis window and this is recommended for the analysis of unknowns. Under these conditions the charge multiplier z is as low as possible thus causing a minimal effect during MW calculation from multiple charge states.

The externally calibrated spectrum of carbonic anhydrase ( $MW_{\text{theoretical}}=28780.75$  Da) thus provided the MW from the 2+ ion as 28780.9 Da (Fig. 2, top), which represents a mass error of 0.15 Da or 5 ppm! This is the performance level typical for high quality proteins. BSA on the other hand is known for its large molecular heterogeneity and thus is

absolutely not suitable as calibrant for larger MW proteins. Even under the dramatically increased resolution of DHAP preparations using the new ultrafleXtreme (~1500 resolution were obtained compared to less than 100, which was previously achievable), the molecular heterogeneity is still not entirely resolved (Fig. 2, bottom) leading to mass errors of approx. 15-20 Da (vs. previously obtained mass errors of 200-300 Da) in sinapinic acid preparations and 1+ analysis.

In reflector mode measurements the mass accuracy can even be further improved, provided the protein does not suffer extensive metastable decay. The calibration spectrum with cytochrome c and myoglobin provided better than 0.01 Da consistency. Based on the calibrated 2+ ion of carbonic anhydrase its MW was determined as 28780.82 Da with a mass error of 0.07 Da or 2.4 ppm (Fig.3).

If the peak resolution is approximately higher than the protein MW smoothing needs to be applied. The resolution of the cytochrome c and myoglobin ions approximates the theoretical envelope of 1766 and 2119 after such smoothing, respectively and the method is applicable for proteins up to

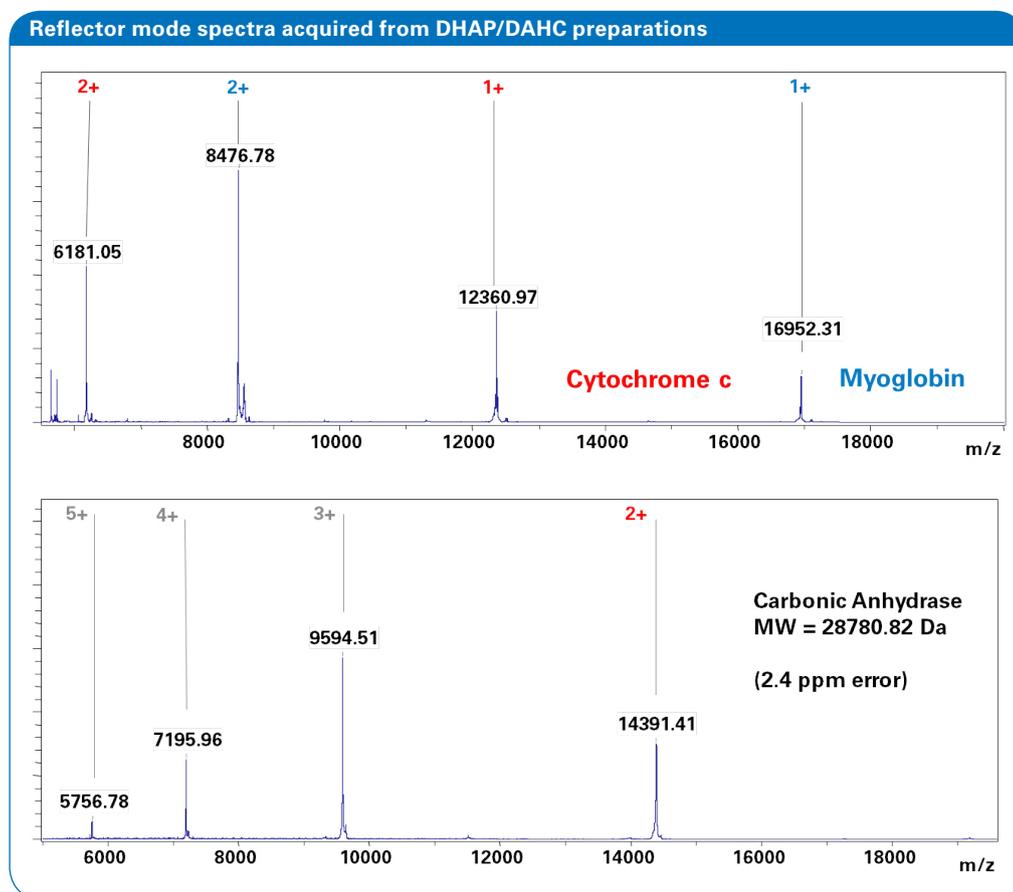


Figure 3: Reflector mode spectra acquired from DHAP/DAHC preparations in the m/z range < 20 kDa. (Top) Protein Calibration Standard 1 provided 1800 and 2150 resolution for cytochrome c and myoglobin, respectively. (Bottom) Carbonic anhydrase provided a resolution of 2880 and the 2+ ion was used for MW determination.

~ 30 kDa. This smoothing strategy facilitates the analysis as all isotopic information is removed and all involved protein ions display well represented isotopic envelopes that can easily be peak picked with a centroid method. This smoothing is, e.g., beneficial for the 23 kDa antibody light chain (LC) analysis and was applied to the MOPC21-LC (Fig. 4) yielding a MW of 23637.06 Da ( $MW_{\text{theoretical}}=23636.96$  Da), which is 0.10 Da or 4.2 ppm higher than the theoretical MW. Peak resolution after smoothing was 2480, closely matching the expected envelope resolution of MOPC21-LC of 2488.

In more general terms, it is the capability to generate high fidelity isotope envelopes - rather than isotopic resolution as such - that allows to obtain precise results even if protein MWs vary largely. These envelope centroids are even reliably obtained from automatic scripts.

## Conclusions

The combination of the new ultrafleXtreme with Bruker's DHAP matrix provides very high accuracies for the MW determination of proteins in the 8-70 kDa range in a unique new concept of taking advantage of highly resolved and multiple charged ions in a precisely calibrated  $m/z$  range. Higher MW proteins are also much better analyzed using this approach but the difficulty for the analysis of large proteins is that their heterogeneity increases with molecular weight. The use of protein calibrants in the 10-20 kDa range ensures a proper calibration by accurately known reference standards even for much larger proteins. Similar to ESI spectra, the multiple charged ions of such larger proteins fall into the well calibrated mass range resulting in mass accuracies in the 5 ppm range never described for linear and reflector mode axial MALDI-TOF spectra before.

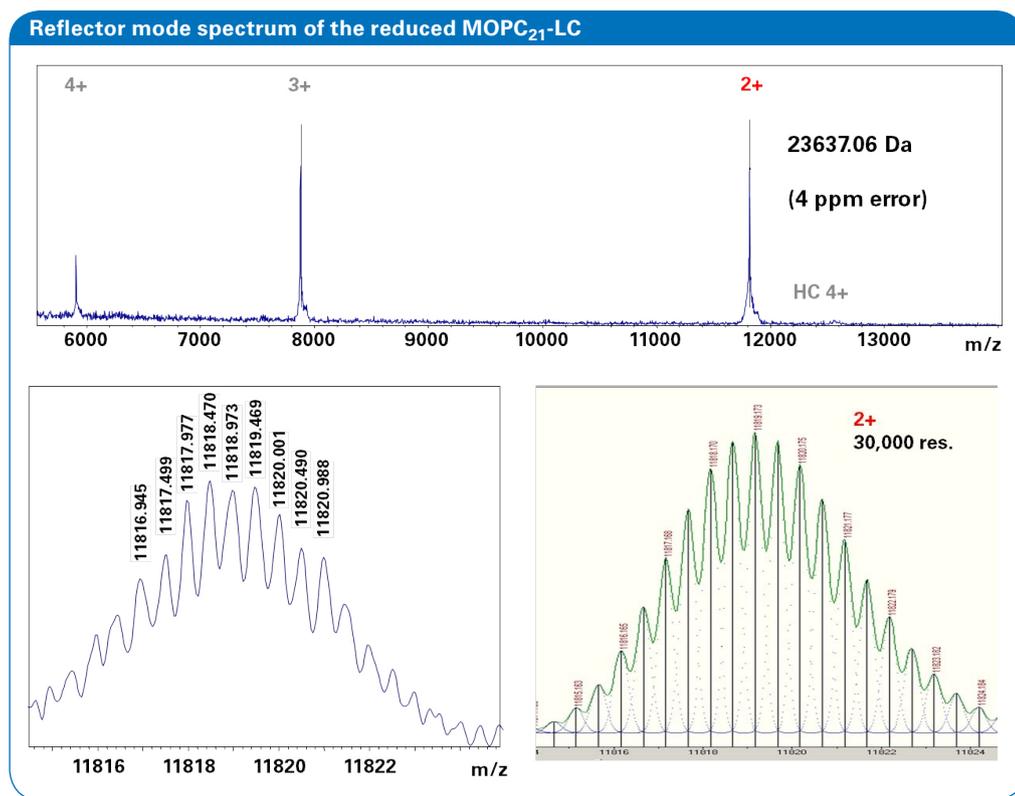


Figure 4: Reflector mode spectrum of the reduced MOPC21-LC acquired from a DHAP/DAHc preparation in the  $m/z$  range < 15 kDa. Only the light chain (LC) is visible, the heavy chain (HC) is only weakly visible as 4+. (Bottom) The 2+ ion is still isotopically resolved with ~30,000 resolution. The spectra was smoothed to isotope envelope resolution (2488 resolution) prior to MW analysis for best results.

## References

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