

## A high-throughput MALDI-MS workflow for accelerated critical quality-attribute monitoring of therapeutic proteins

Leveraging the speed of MALDI-MS in combination with automated sample preparation, accurate-mass measurement on timsTOF flex and a streamlined data analysis workflow offers great potential for enhancing the efficiency in biologics development.

### Abstract

A high-throughput (HT) MALDI-MS workflow has been developed enabling rapid monitoring of critical quality attributes (cQA) of therapeutic proteins during biologics development. The method is based on automated sample preparation and MALDI spotting using a liquid handling device in combination with fast acquisition of accurate-mass MALDI-MS data on a timsTOF flex instrument followed by batchwise data analysis using a tailored BioPharma Compass® (BPC) software workflow.

When compared to LC-MS, the new HT-MALDI workflow enables more than a 3-fold reduction in time to result. cQA monitoring of a batch of 96 antibody samples was accomplished within five hours from start of sample preparation to final reporting of results.

### Introduction

During biologics development, several screening steps are performed during optimization of down- and upstream processes, resulting in several hundred samples that need to be analyzed within a short period of time.

Keywords:  
MALDI, timsTOF flex, cQA monitoring, biotherapeutics, biologics, proteins, antibodies, glycoproteins, high throughput, automation, sample preparation, data analysis, software, Biopharma Compass

Analysis methods based on liquid chromatography coupled to mass spectrometric detection (LC-MS) are well established for monitoring of critical quality attributes (cQA) of biologics and provide enhanced analytical depth and detail when compared to optical detection methods. However, LC-MS is limited in data acquisition speed, requires rather complex instrumental setups, and inherently has to rely on sophisticated data analysis pipelines for extraction of information from raw data, which can become a limiting factor regarding sample throughput and time to result. Therefore, fast direct MS approaches represent a highly attractive alternative.

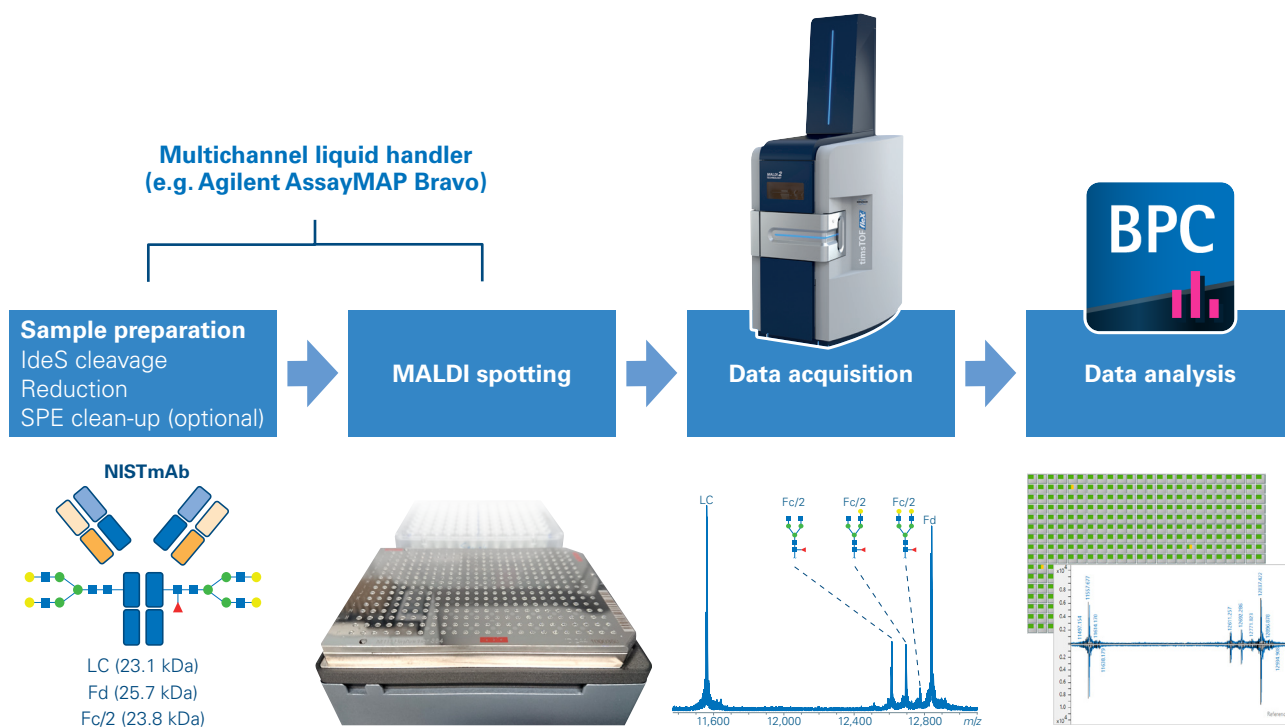
Here, we describe a MALDI-MS based workflow for high-throughput cQA monitoring of therapeutic proteins that has the potential to significantly accelerate biopharmaceutical development. The method is based on automated sample preparation in 96-well plate format and MALDI spotting using a liquid handler, fast acquisition of accurate-mass MALDI-MS data on a timsTOF fleX instrument, and batchwise data analysis using a tailored multi-attribute monitoring (MAM) workflow in BioPharma Compass (BPC) software.

This fit-for-purpose performance of the automated MALDI workflow is demonstrated here on the well characterized NIST reference monoclonal antibody, giving significantly reduced turnaround time. Compared to LC-MS, the MALDI-MS method was more than 3 times faster and accomplished the analysis of a batch of 96 samples within five hours from start of sample preparation to final reporting of results.

## Experimental

NISTmAb 8671 humanized IgG1k monoclonal antibody reference material was used here as a therapeutic protein for workflow demonstration and evaluation.

The high-throughput MALDI-MS workflow scheme is shown in Figure 1.



**Figure 1.** Scheme of the high-throughput MALDI-MS workflow for cQA monitoring of therapeutic proteins.

### Automated sample preparation and MALDI spotting

All sample preparation steps including MALDI spotting were performed automatically using an Agilent AssayMAP Bravo liquid handler equipped with 96-channel pipetting head.

NISTmAb stock solution (10 mg/mL in histidine buffer) was diluted in water to a final protein concentration of 1 mg/mL. 96 aliquots of the 1 mg/mL NISTmAb solution were placed into a 96-well plate. Samples were cleaved into subunits using IdeS (FabRICATOR, Genovis) and reduced using dithiothreitol (Merck). The sample preparation method developed on the AssayMAP Bravo incorporated an optional automated clean-up step using solid phase extraction (SPE) tips packed with C<sub>18</sub> reversed-phase resin. For the NISTmAb samples used here, a purification step prior to MALDI spotting was not required.

IdeS digested and reduced NISTmAb samples were automatically spotted in 4 replicates on a Bruker MTP BigAnchor 384 BC target plate using 2,5-dihydroxyacetophenone (DHAP) as a MALDI matrix and diammonium citrate as an additive.

### Data acquisition and batch-processing

MALDI-MS spectra were acquired as an automation sequence on a Bruker timsTOF fleX instrument equipped with a dual ESI/MALDI ion source and 10 kHz smartbeam 3D laser. All spectra were recorded in positive ion mode. To obtain the highest sensitivity for ions in the high  $m/z$  range, the TIMS in pressure was lowered and the timsControl acquisition method was tuned for optimum ion transmission in the high  $m/z$  range as described previously [1].

Data files comprising MALDI-MS spectra from all 384 MALDI plate positions were batch-processed in DataAnalysis 6.2 applying smoothing, baseline correction, peak finding, and final conversion of spectra into \*.jpeaks format. Two alternative processing methods were applied for comparison: Processing method A aimed for monoisotopic peak detection applying a narrow smoothing width (Savitzky Golay, 0.1  $m/z$ , 1 cycle) in combination with SNAP2 as a peak finder. Processing method B aimed for detection of average  $m/z$  features and, therefore, applied a significantly wider smoothing width (Savitzky Golay, 2  $m/z$ , 2 cycles) in combination with the Apex peak finder.

### Data analysis

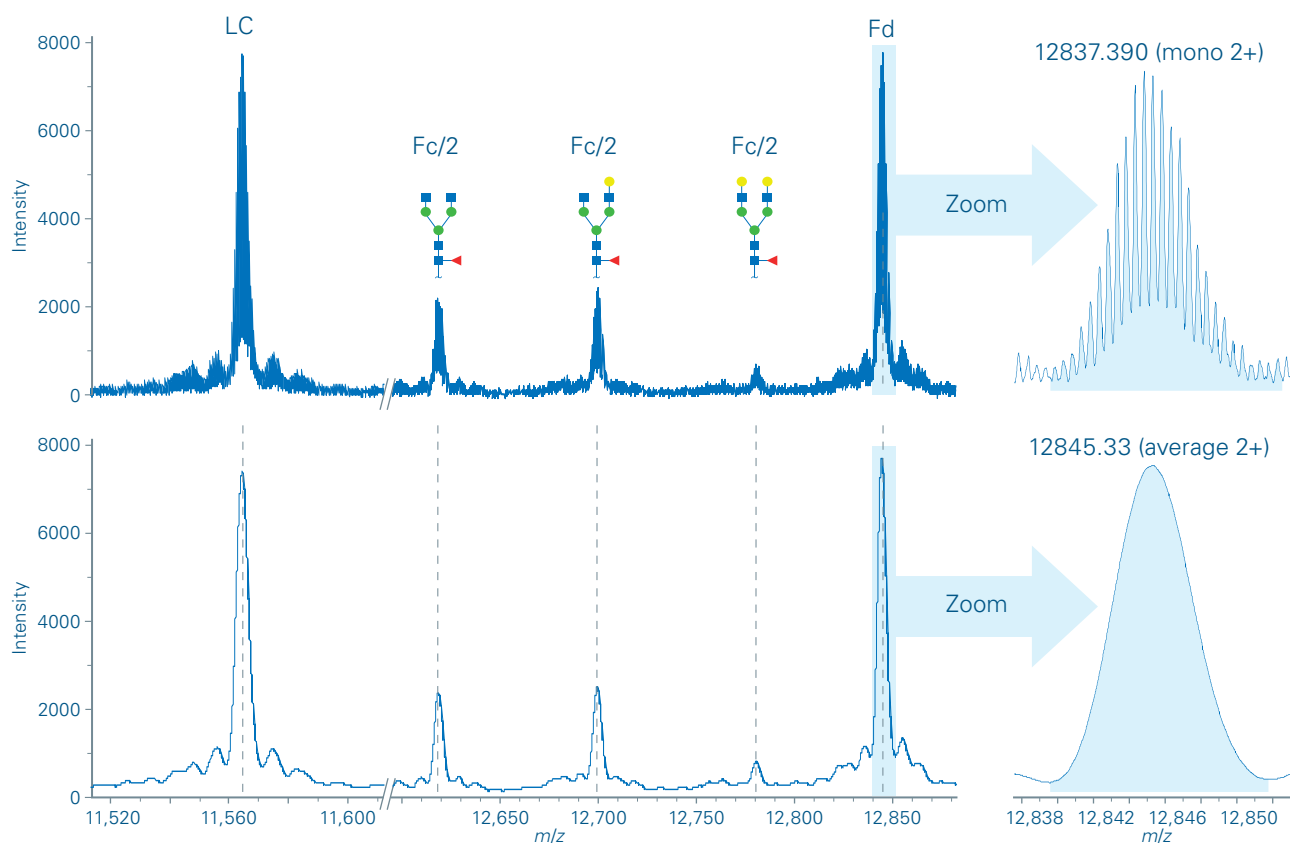
Preprocessed \*.jpeaks formatted MALDI spectra were uploaded to Bruker BPC 2025b software and analyzed as a batch, applying the BPC Multi Target Screening MALDI workflow. Two spectral attributes, i.e. matching quality against a predefined reference profile and mass accuracy, were selected for cQA monitoring. The spectrum match quality attribute, reported by BPC software as cosine similarity score, was set up to account for the relative intensities of doubly charged ion signals representing NISTmAb subunits and fragments light chain (LC), Fd and Fc/2, for the latter considering glycoforms G0F, G1F and G2F.

## Results and discussion

### Accurate mass data delivered at MALDI speed enabling high-throughput analysis with confidence

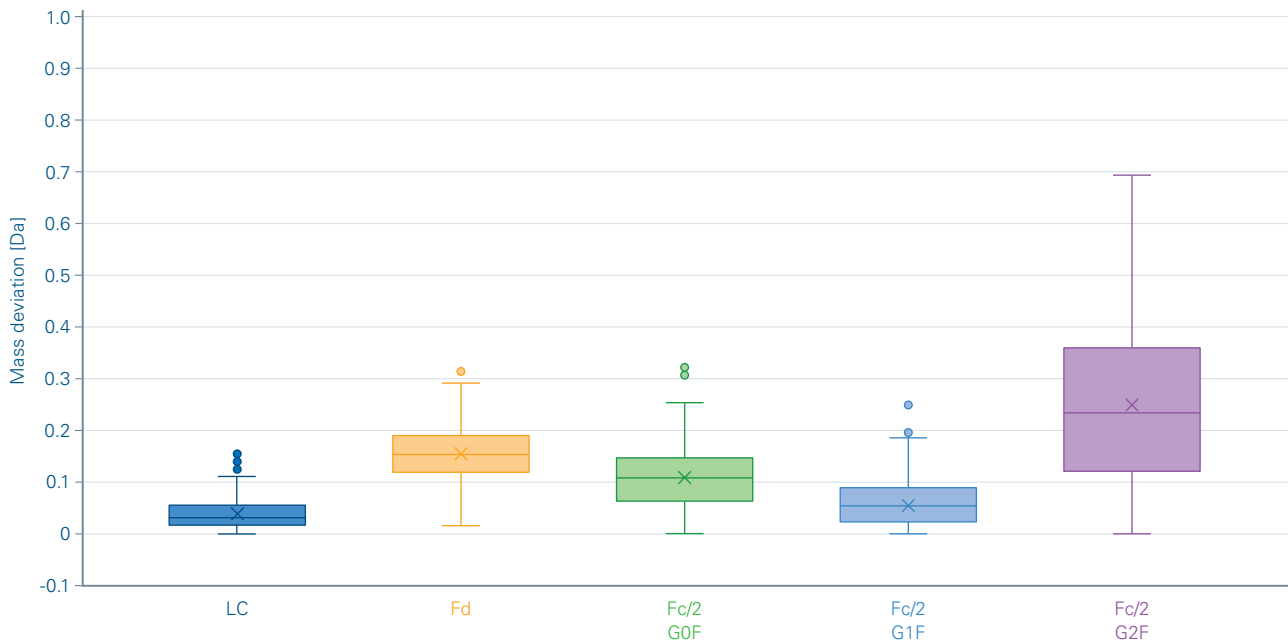
The automated MALDI method described here can generate accurate mass data at high acquisition speed and sample throughput. Figure 2 depicts a representative MALDI-MS spectrum obtained from IdeS digested and reduced NISTmAb applying the newly developed workflow.

For cQA monitoring of NISTmAb subunits, doubly charged ion signals were taken into account. The timsTOF fleX MALDI-MS data provided an outstanding level of mass accuracy and resolution allowing for reliable detection of even minor proteoforms and discrimination of modifications inducing only small mass distances, such as oxidation. However, detection of average  $m/z$  features using the Apex peak finder (processing method B) yielded optimum sensitivity and robustness in the detection of glycoforms such as Fc/2\_G2F. Accordingly, data processed with processing method B (i.e. detection of average  $m/z$  features) was used as input data for cQA monitoring in Biopharma Compass software. Figure 3 provides a box plot evaluating the mass accuracy of average-mass processed MALDI-MS data acquired from 384 MALDI spots of NISTmAb subunits (96 samples, 4 replicate spots each). Mean mass deviations were in the low ppm range ( $\leq 10$  ppm / 0.25 Da) at 100% detection success rate (384 out of 384 spots) even for Fc/2 glycoform G2F.

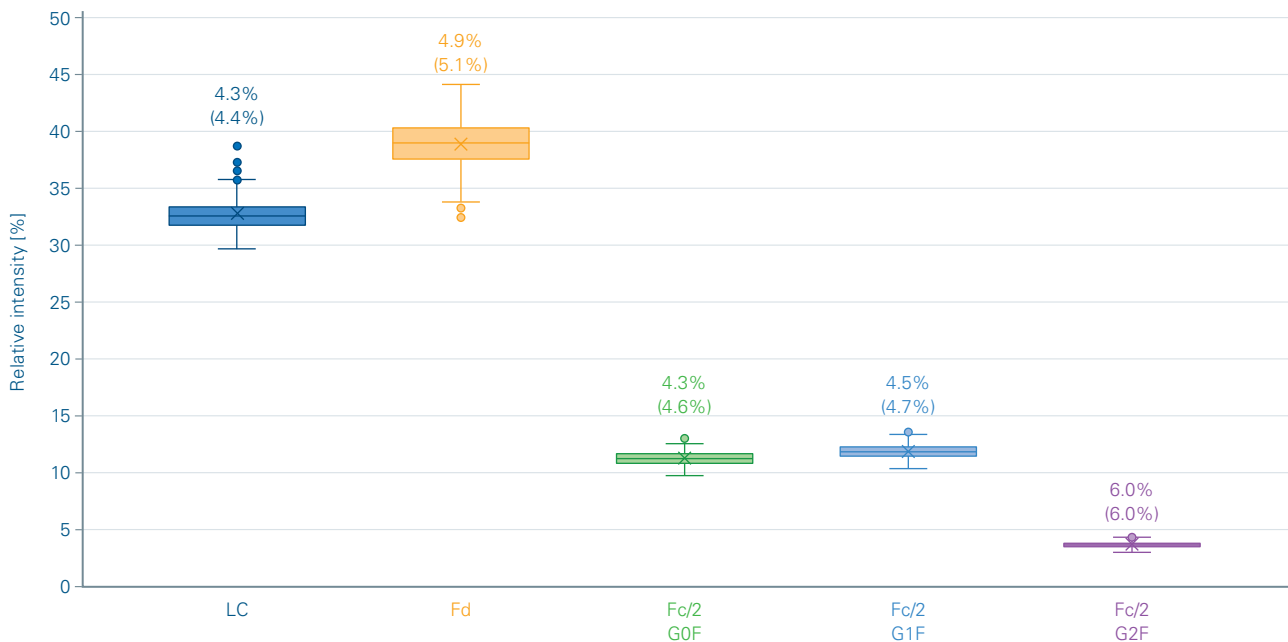


**Figure 2.** MALDI-MS spectrum obtained from IdeS digested, reduced NISTmAb applying the automated MALDI based cQA monitoring workflow. **Top:** Spectrum processed with processing method A aiming for monoisotopic peak detection. **Bottom:** The same spectrum processed with processing method B aiming for detection of average  $m/z$  peaks.

The outstanding quality of timsTOF MALDI-MS data is of particular benefit to the level of confidence achievable in typical cQA monitoring tasks. Figure 4 provides a box plot evaluating the reproducibility of the method taking into account 96 IdeS digested and reduced sample replicates. For all subunits, standard deviations were between 4 and 6% indicating outstanding robustness and reliability of the MALDI based high-throughput cQA monitoring workflow.



**Figure 3.** Mass accuracy of timsTOF fleX MALDI-MS data acquired from 384 sample spots of IdeS digested and reduced NISTmAb (96 samples, 4 replicate spots each).

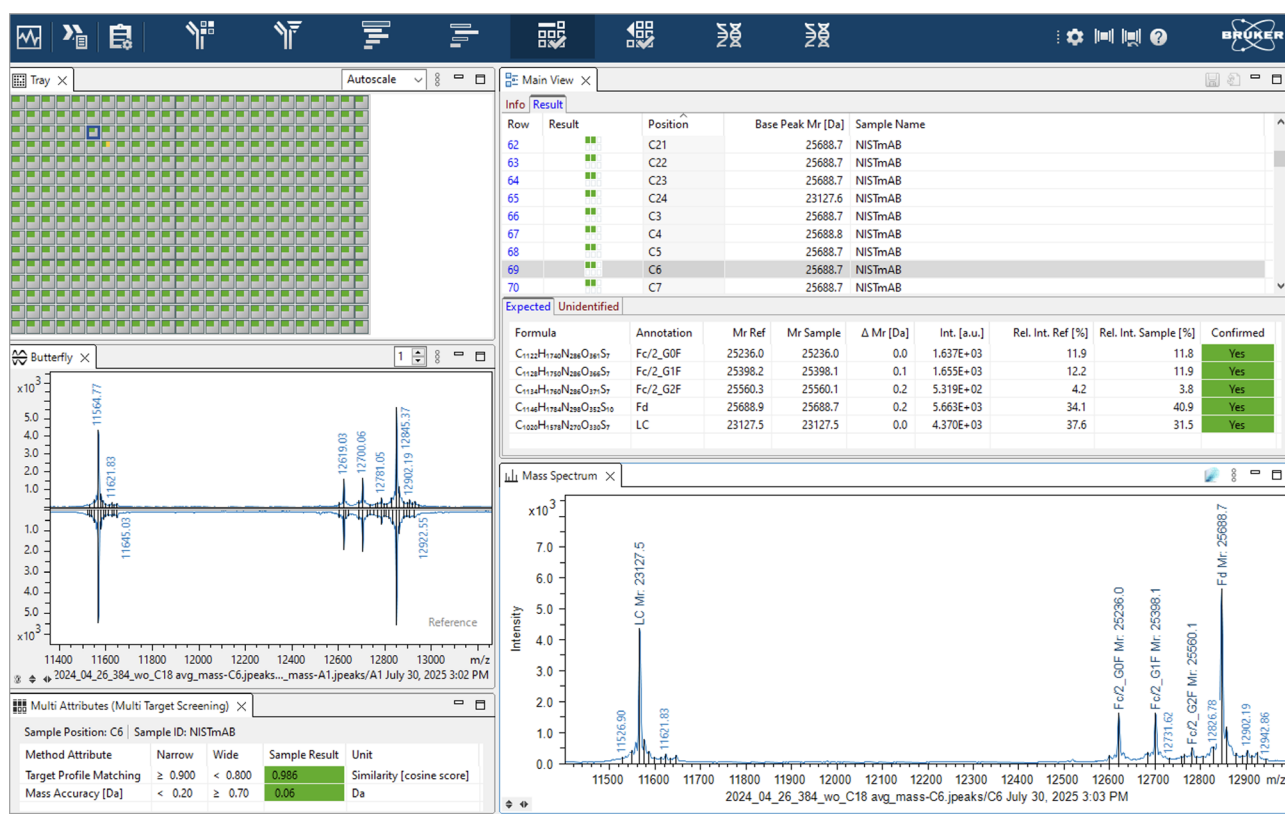


**Figure 4.** Reproducibility of timsTOF fleX MALDI-MS data acquired from 96 IdeS digested, reduced NISTmAb samples (4 MALDI spot replicates each) applying the automated MALDI cQA monitoring workflow.

Percent values indicate the RSD across 96 NISTmAb sample replicates. Percent values in parentheses indicate the RSD across 384 MALDI spot measurements (i.e. 96 NISTmAb sample replicates, 4 MALDI spot replicates each).

## Tailored data analysis workflow in BioPharma Compass software

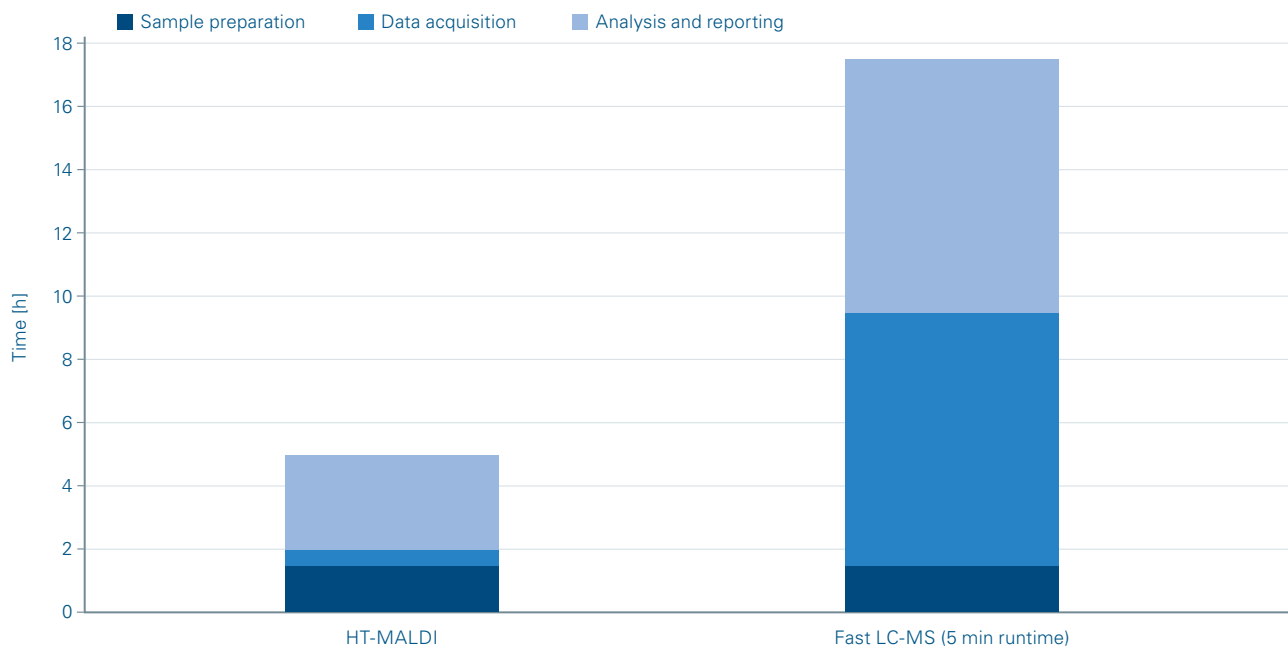
BPC software allows for streamlined analysis of even large batches of MALDI-MS spectra featuring a tailored “Multi Target Screening MALDI” workflow. Figure 5 depicts the BPC graphical user interface when executing this workflow on a batch of 384 MALDI-MS spectra acquired from IdeS digested, reduced NISTmAb. The workflow allows for monitoring of up to 6 attributes, such as match quality against a reference target profile (expressed as cosine score), mass accuracy or intensity coverage, and reports back monitoring results in a traffic-light format customizable by user-defined thresholds. All sample information required for cQA monitoring, i.e. assignment of MALDI spots to individual sample IDs and reference target profile for similarity matching, can be imported with minimal effort as \*.csv files. Execution of the workflow on a batch of 384 MALDI-MS spectra took less than 15 seconds. A batch report is generated automatically by the software and is available for download from the BPC server at any time. Additionally, the intuitive user interface allows for interactive in-depth verification of monitoring results. Using the MALDI plate view for navigation, result details of individual samples are accessible with a single mouse click.



**Figure 5.** Graphical user interface of BioPharma Compass software when executing batchwise cQA monitoring analysis of 384 MALDI-MS spectra of IdeS digested and reduced NISTmAb.

### Enhancing the efficiency in biologics development

Due to its unparalleled analysis speed, the MALDI based cQA monitoring workflow offers true high-throughput capability. Furthermore, it generates minimal amounts of easy-to-analyze data (1 sample = 1 spectrum; detection of low charge state eliminates the need for a charge deconvolution step). Altogether, this results in minimum time to result for even large sample cohorts. The high-throughput MALDI workflow has, therefore, the potential to significantly enhance the efficiency in biologics development by monitoring various critical attributes such as assembly of multi-chain proteins, glycosylation profile and modifications, f.e. oxidation. This is illustrated in Figure 6. When compared to a short-gradient LC-MS method (considering 5 minutes run time), high-throughput MALDI yielded a more than 3 times shorter turnaround time. Batch analysis of 96 mAb samples by means of the newly developed MALDI workflow was accomplished within five hours from start of sample preparation to final reporting of results.



**Figure 6.** Comparison of the HT-MALDI cQA monitoring workflow with an LC-MS based reference workflow regarding turnaround time required for 96 mAb samples from start of sample preparation to final reporting of results.

## Conclusions

- A new MALDI-MS workflow has been developed for high-throughput cQA monitoring of therapeutic proteins offering great potential for accelerated biopharmaceuticals development.
- The workflow is based on automated sample preparation, including MALDI spotting using a 96-channel liquid handler, such as the Agilent AssayMAP Bravo.
- The method benefits from fast MALDI analysis speed in combination with outstanding quality of accurate mass MALDI data delivered by the timsTOF fleX instrument.
- A tailored multi-attribute monitoring workflow featured in BPC software allows for time-efficient data analysis of even large cohorts of MALDI-MS spectra.
- Overall, the newly developed HT-MALDI workflow achieved a 3-fold reduction of turnaround time over a short-gradient LC-MS reference method accomplishing cQA monitoring of 96 mAb samples within 5 hours from start of sample preparation to final reporting of results.

## References

- [1] Asperger, N. Goedecke, *Developing a timsTOF fleX method for enhanced sensitivity in higher m/z MALDI-MS measurements*, Bruker Technical Note TN-57, [https://www.bruker.com/en/products-and-solutions/mass-spectrometry/timstof/timstof-flex/\\_jcr\\_content/root/sections/more\\_information/sectionpar/search\\_copy\\_copy\\_cop.download-asset.pdf/7d08703f-a00c-4179-b48b-e6f3d03671e8/1904383-tn-57-timstof-flex-high-maldi-ms-mode-ebook.pdf](https://www.bruker.com/en/products-and-solutions/mass-spectrometry/timstof/timstof-flex/_jcr_content/root/sections/more_information/sectionpar/search_copy_copy_cop.download-asset.pdf/7d08703f-a00c-4179-b48b-e6f3d03671e8/1904383-tn-57-timstof-flex-high-maldi-ms-mode-ebook.pdf)

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