



Application Note # LCMS-78

Automated Analysis of Protein Sequence Variants by LC-MS Under the Control of BioPharma Compass

Abstract

Here we describe an automated UPLC-MS based method for the analysis and quality confirmation of the intact mass for protein sequence variants. The automated workflow is implemented using Bruker Daltonics' Biopharma Compass™ software which allows for data acquisition, processing and analysis to occur with minimal user intervention.

Data acquisition is set up through Biopharma Compass ensuring all acquisition parameters are traceably linked to the samples analysed and any future processing that may occur. Post-acquisition samples are automatically processed to determine the intact mass of the protein sequence variant. The difference in measured mass from the theoretical mass is calculated indicating whether the measured mass is within the specified acceptance criteria. The intensity fraction of the major species is also displayed as a percentage, providing information on sample homogeneity.

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Once processing is complete, data is presented in a graphical interface which allows rapid identification of samples that have passed or failed the set acceptance criteria and also highlights any samples which require further user investigation.

Ultimately the various automation steps allow intact mass screening for numerous protein variants while saving significant resources and ensuring a high quality for the generation and interpretation of the data.

Introduction

The beneficial application of protein therapeutics with suitable efficacy is often limited by short protein half-life and poor bioavailability. Rapid drug clearance from the body due to short protein half-life can result in higher and more frequent dosing, causing peaks and troughs of drug concentration in the patient. The circulatory half-life of the target protein can be extended by applying albumin fusion technology, resulting in less frequent administration and reduced side effects.

Albumin naturally has a long half-life of 19 days, and fusion of therapeutic proteins to the albumin molecule has been shown to extend drug half-life in humans. The extended half-life of albumin, like IgG, is controlled by its pH-dependent interaction with the major histocompatibility complex (MHC) class I-related neonatal receptor (FcRn). Both albumin and IgG are protected from intracellular degradation through binding to FcRn in acidic endosomes. The half-life of IgG-based therapeutics has been shown to be correlated to their affinity for FcRn. Furthermore, albumin can be re-engineered to modulate binding affinity to FcRn.

Once these re-engineered albumin variants have been produced and purified, it is essential to check that the measured intact mass corresponds to the theoretical mass of the variant. The analytical technique for intact mass analysis is UPLC-MS, combined with Bruker Daltonics' software, Biopharma Compass.

Biopharma Compass software allows fast, reliable, and quantitative characterization of biopharmaceuticals in a high throughput, automated manner. In addition, data is captured and stored via fully auditable and traceable processes. Methods can be optimized for each type of analysis so as to highlight abnormal or unexpected results.

Automated, visual reports are generated for each individual analysis and also for the batch of samples so that product purity and identity can be observed at a glance.

Experimental

UPLC-MS

The intact masses of albumin sequence variants were determined by UPLC-MS. LC separation was carried out using a Waters Acquity on a BEH 50 × 2.1 mm ACQUITY BEH 1.7 μm C4 column and a 5 min analytical gradient (Table 1).

Table 1: Experimental conditions for UHPLC.

HPLC	Waters Acquity UHPLC
Column	BEH 50 x 2.1 mm Acquity BEH 1.7 μm C4 column
Column Oven	50°C
Flow Rate	0.4 mL/min
Solvent A	0.1% FA in H2O
Solvent B	0.1% FA in ACN
Run Time	0.5 min 5% B within 2.5 min increase to 70% B within 2.6 min increase to 80% B Hold for 3.2 min within 3.3 min increase to 5% B equilibrate for 5 min
Mass Spectrometer	Bruker micrOTOF

Eluted proteins were directly introduced to a Bruker MicroTOF II mass spectrometer via an ESI source. All instrument control and sample tables were controlled using BioPharma Compass which ensures all samples, parameters and results are traceably linked with a full audit trail. The complete workflow is summarized in Figure 1, from the design of albumin variant molecules through to the interpreted analytical results. Key variable workflow parameters, such as sample name and injection volume, are submitted to BioPharma Compass as part of an Excel spreadsheet for each experiment (Figure 2).

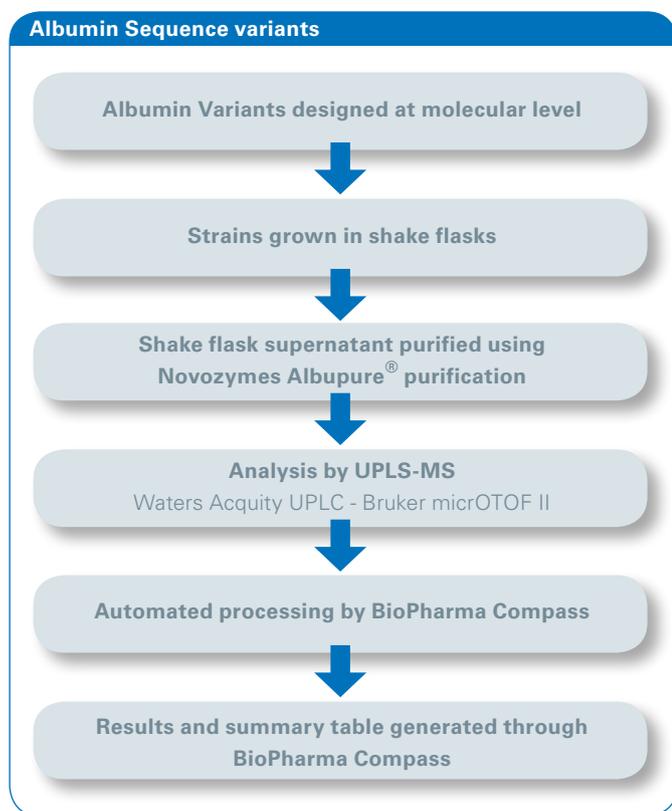


Figure 1: Typical workflow for the creation and validation of albumin sequence variants.

Data Analysis

All data was processed in an automated manner via the application of a specific processing script which runs after each sample is analyzed, which for example may or may not include recalibration of the acquired data, in accordance with the parameters which were submitted by the user (Figure 2). In this example recalibration was not applied. In the same Excel table the user supplies information regarding the retention time of the peaks of interest in order to direct the automated deconvolution of the raw data by the Maximum Entropy algorithm. The reference molecular weight is also provided for each sample in addition to a window of tolerance which is used to judge the success of the experiment.

Results

Processed data is traceably linked to the appropriate raw data and all analysis and processing information. Once samples have been analyzed and processed by Biopharma Compass, data is displayed initially in two forms; the tray view and results table.

The tray view (shown in Figure 3) summarizes the results of the UPLC-MS analysis in a simple format. Each well represents a processed sample and corresponds to the position in the queue. Color schemes can then be applied

Key workflow parameters

Sample	Sample Description	Position	Injection Volume [µL]	Automatic Calibration	Relevant RT Range Start	Relevant RT Range End	Mutant Name	Reference Mr	Accepted Mass Deviation in Da	Report Layout
1	Variant 1	A1	5	false	1	5	Mutein A	66439	5	micrOTOF\Novozymes_Mutant.layout
2	Variant 2	A2	5	false	1	5	Mutein B	66416	5	micrOTOF\Novozymes_Mutant.layout
3	Variant 3	A3	5	false	1	5	Mutein C	66435	5	micrOTOF\Novozymes_Mutant.layout
4	Variant 4	A4	5	false	1	5	Mutein D	66572	5	micrOTOF\Novozymes_Mutant.layout
5	Variant 5	A5	5	false	1	5	Mutein E	66434	5	micrOTOF\Novozymes_Mutant.layout
6	Variant 6	A6	5	false	1	5	Mutein F	66383	5	micrOTOF\Novozymes_Mutant.layout
7	Variant 7	A7	5	false	1	5	Mutein G	66439	5	micrOTOF\Novozymes_Mutant.layout
8	Variant 8	A8	5	false	1	5	Mutein H	66395	5	micrOTOF\Novozymes_Mutant.layout
9	Variant 9	B1	5	false	1	5	Mutein I	66459	5	micrOTOF\Novozymes_Mutant.layout
10	Variant 10	B2	5	false	1	5	Mutein J	66337	5	micrOTOF\Novozymes_Mutant.layout
11	Variant 11	B3	5	false	1	5	Mutein K	66421	5	micrOTOF\Novozymes_Mutant.layout
12	Variant 12	B4	5	false	1	5	Mutein L	66394	5	micrOTOF\Novozymes_Mutant.layout
13	Variant 13	B5	5	false	1	5	Mutein M	66367	5	micrOTOF\Novozymes_Mutant.layout
14	Variant 14	B6	5	false	1	5	Mutein N	66408	5	micrOTOF\Novozymes_Mutant.layout
15	Variant 15	B7	5	false	1	5	Mutein O	66436	5	micrOTOF\Novozymes_Mutant.layout
16	Variant 16	B8	5	false	1	5	Mutein P	66453	5	micrOTOF\Novozymes_Mutant.layout
17	Variant 17	C1	5	false	1	5	Mutein Q	66454	5	micrOTOF\Novozymes_Mutant.layout
18	Variant 18	C2	5	false	1	5	Mutein R	66422	5	micrOTOF\Novozymes_Mutant.layout
19	Variant 19	C3	5	false	1	5	Mutein S	66538	5	micrOTOF\Novozymes_Mutant.layout
20	Variant 20	C4	5	false	1	5	Mutein Q	66452	5	micrOTOF\Novozymes_Mutant.layout

Figure 2: Key workflow parameters as submitted via an Excel spreadsheet for the automated acquisition, processing, evaluating and reporting of data under the control of BioPharma Compass.

to display a summarized output of the results for the desired type of information. In this example, the left half indicates whether the sample is within the acceptance criteria of the expected mass (Green <2Da; Orange 2-5Da; Red >5Da). The right half indicates intensity fraction of the major peak in relation to other peaks present as an indicator of sample purity (Green = >75%; Orange = 50%-75%; Red = <50%). A quick observation allows the user to determine if the variants are of the correct mass in addition to indicating the approximate level of homogeneity in the samples. In this experiment, samples 1 and 7 are of questionable purity (50-75%), whilst samples 9 and 19 have been flagged for being outside of the expected mass by >5 and 2-5 Da respectively. All other samples had acceptable mass accuracy and purity of the major peak.

A results summary table is also generated in both PDF and Excel format along with the HTML version. This table can be tailored by the user to display parameters of interest according to the purpose of the experiment. For this experiment the table includes the sample name, the expected mass, the measured mass, the difference in mass and whether or not the sample passes the acceptance criteria set by the user (Figure 4). Acceptance limits and such like can be defined for the various parameters depending on which are considered most critical for the experiment.

Consequently this enables samples of the expected mass to be easily identified and also highlights results that will require further user investigation. In this instance differences of more than 5 Da and 2-5 Da in measured mass compared to expected mass prevented samples 9 and 19 from passing the QC test, whereas even though samples 1 and 7 passed the QC test, the user's attention was brought to the finding that the main peak was not as pure for these two samples as it was for the others.

A report summarizing the mass spectra and showing the deconvoluted mass of the intact protein for each sample is automatically generated as a result of the processing script attached to the method (Figure 5). The report also contains a table which provides detailed information on other masses detected in the sample and assigns an intensity fraction percentage for each mass. This data is used to assess homogeneity of the sample in greater detail and can also be used to monitor ratios of modifications, for example oxidation.

The PDF reports are linked to the samples in the tray view, allowing unexpected results highlighted in the graphical interface to be quickly checked to decide whether the raw data needs to be further interrogated.

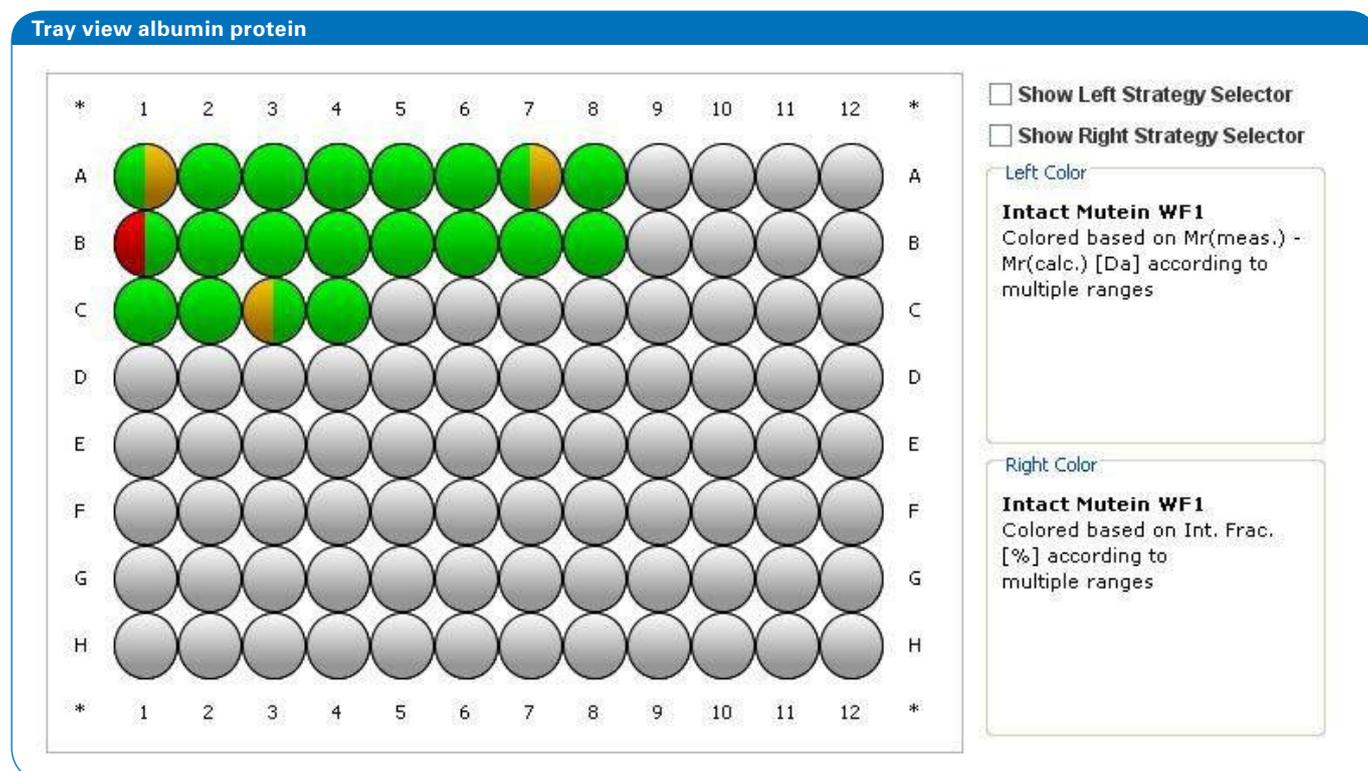


Figure 3: The tray view showing the results for 20 sequence variants of the albumin protein. A different color scheme has been used for each half of the wells in order to visually summarize the results of the experiment. The left half indicates whether the sample is within the acceptance criteria of the expected mass (Green <2Da; Orange 2-5Da; Red >5Da). The right half indicates intensity fraction of the major peak in relation to other peaks present as an indicator of sample purity (Green >75%; Orange 50%-75%; Red <50%).

Conclusion

Biopharma Compass software allows for fully automated analysis and data processing, summarizing results in a simple format for high-throughput screening in a manner that can be tailored specifically for various types of analysis.

In this particular workflow, Biopharma Compass provided a very simple framework for comparing measured intact mass to the theoretical mass, and for assessing the homogeneity of each sample.

Establishing Biopharma Compass methods for routine analysis resulted in a much higher throughput workflow, together with reduced user intervention and generation of consistent and reliable data processing.

Result summary table

Sample	Sample Description	Method	Reference Mr	Name	Mr (meas.) [Da]	Mr(meas.) - Mr(calc.) [Da]	Int. Frac. [%]	QC Test Passed
1	Variant 1	Intact Mutein WF1	66439	Mutein A	66440.0	1.0	70.08	1
2	Variant 2	Intact Mutein WF1	66416	Mutein B	66417.3	1.3	86.83	1
3	Variant 3	Intact Mutein WF1	66435	Mutein C	66436.6	1.6	87.48	1
4	Variant 4	Intact Mutein WF1	66410	Mutein D	66411.5	1.5	87.23	1
5	Variant 5	Intact Mutein WF1	66434	Mutein E	66435.1	1.1	86.66	1
6	Variant 6	Intact Mutein WF1	66383	Mutein F	66384.2	1.2	85.17	1
7	Variant 7	Intact Mutein WF1	66439	Mutein G	66441.0	2.0	60.96	1
8	Variant 8	Intact Mutein WF1	66395	Mutein H	66396.4	1.4	81.78	1
9	Variant 9	Intact Mutein WF1	66418	Mutein I	66428.7	10.7	81.7	0
10	Variant 10	Intact Mutein WF1	66337	Mutein J	66338.0	1.0	85.69	1
11	Variant 11	Intact Mutein WF1	66420	Mutein K	66418.0	-2.0	83.9	1
12	Variant 12	Intact Mutein WF1	66394	Mutein L	66395.1	1.1	91.71	1
13	Variant 13	Intact Mutein WF1	66367	Mutein M	66368.0	1.0	91.97	1
14	Variant 14	Intact Mutein WF1	66408	Mutein N	66409.2	1.2	83.53	1
15	Variant 15	Intact Mutein WF1	66436	Mutein O	66437.3	1.3	82.13	1
16	Variant 16	Intact Mutein WF1	66453	Mutein P	66454.5	1.5	92.74	1
17	Variant 17	Intact Mutein WF1	66454	Mutein Q	66455.6	1.6	86	1
18	Variant 18	Intact Mutein WF1	66422	Mutein R	66423.5	1.5	89.6	1
19	Variant 19	Intact Mutein WF1	66429	Mutein S	66424.4	-4.6	89.37	0
20	Variant 20	Intact Mutein WF1	66452	Mutein T	66453.7	1.7	92.31	1

Figure 4: Results summary table for 20 sequence variants of the albumin protein. This table displays parameters of interest according to the purpose of the experiment, with acceptance limits being defined for the various parameters depending on which are considered most critical for the experiment. In this case differences of more than 2 Da in measured mass compared to expected mass resulted failure of the QC test.

Tray view albumin protein

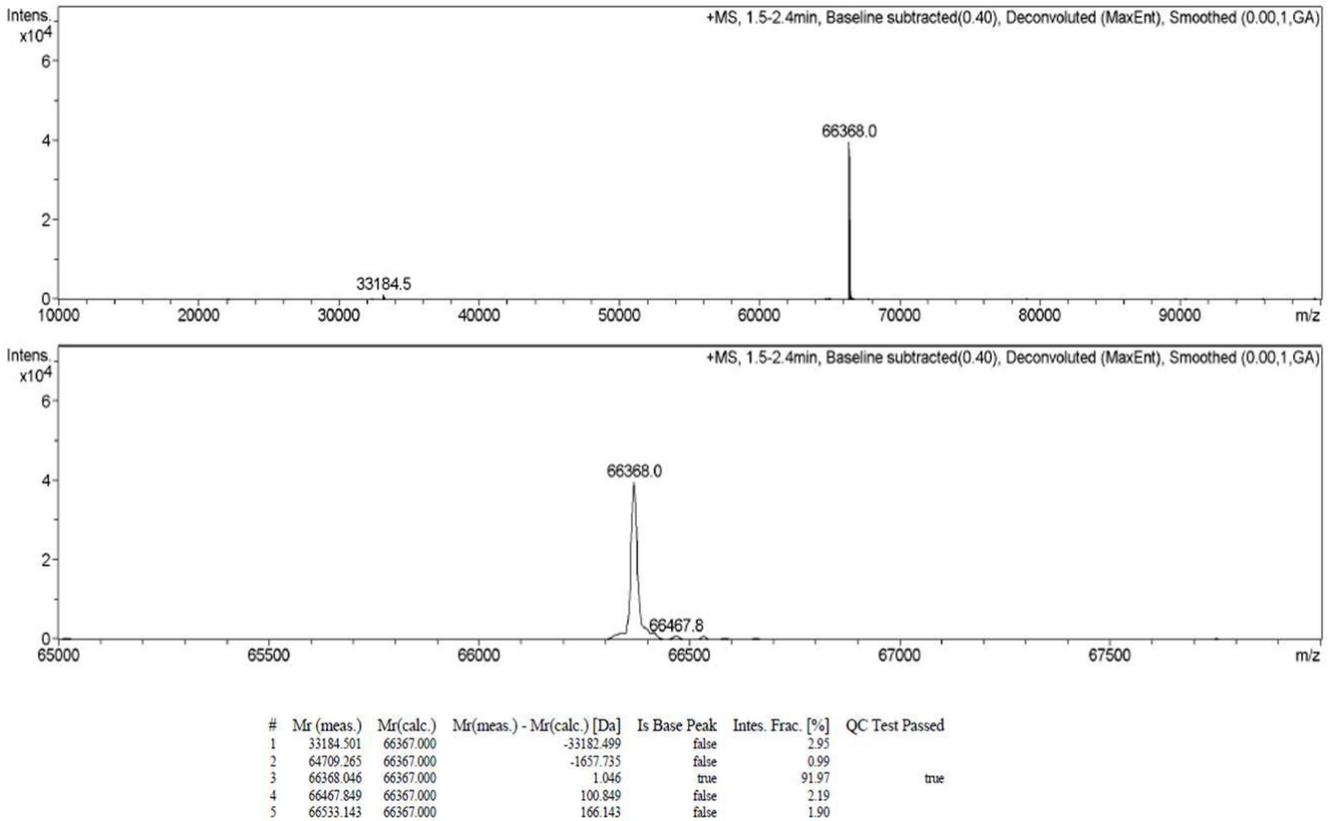


Figure 5: Automatically generated summary PDF report showing the mass spectra and deconvoluted mass of the intact protein for each sample. This also includes a table which provides information regarding sample homogeneity by listing all observed masses detected in the sample.



References

- [1] Bruker Daltonics Application-Note ET-20
BioPharma Compass: A fully Automated Solution for
Characterization and QC of Intact and Digested Proteins

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