Fighting Counterfeits for Biopharmaceuticals with Accurate Mass Spectrometry

Featuring the SCIEX X500B or TripleTOF® 6600 LC-MS/MS System with BPV Flex 2.0 Software

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Online shopping has become a convenient way of buying necessities, often for lower prices than in regular stores. This includes prescription medicine, such as biopharmaceuticals. However, the convenience brings certain risks. Unfortunately, counterfeits are becoming more of a problem within the (bio)pharmaceutical industry. Biopharmaceuticals, with their highly complex development and manufacturing process, and therefore associated high costs, have become a lucrative target for fraud. The American Food and Drug Administration (FDA) is warning about counterfeit medicine, as it is not only illegal but could be harmful to one's health.[1]

Different things can threaten the health of a patient when they receive a counterfeit instead of the actual biopharmaceutical:

- Wrong dosage of the correct product—either below an effective dose for treatment or overly potent
- Other ingredients that can be harmful—toxic ingredients or impurities that can provoke an immune reaction
- No active pharmaceutical ingredient

Key Features of Counterfeit Analysis with SCIEX Accurate Mass Spectrometry

- High quality data with SCIEX accurate mass systems: X500B or TripleTOF 6600 systems
- Simple, standard methods for intact, subunit and peptide analysis, suitable for different proteins without the need for extensive optimization
- Ease-of-use of data processing with BPV Flex 2.0 Software for straight-forward intact, subunit and peptide analysis

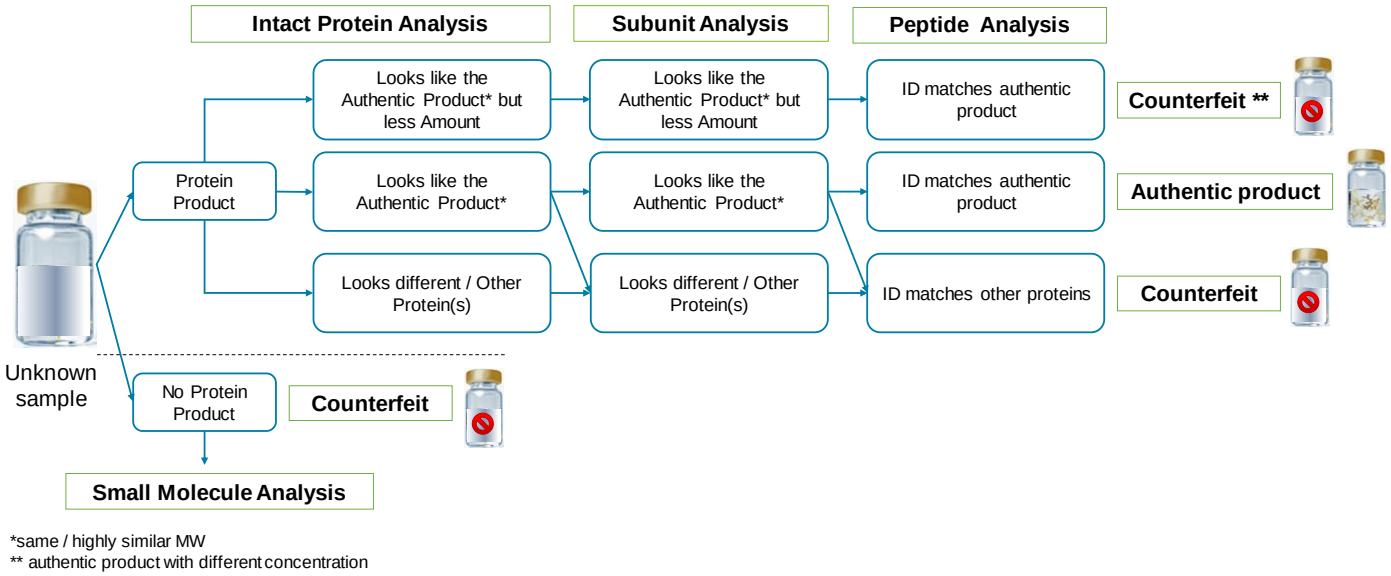

Figure 1: Overview of the SCIEX Workflow for Protein Counterfeit Analysis via LC-MS.

Understandably, both governments and the pharmaceutical companies that produce the products are very interested in stopping this fraud and helping to ensure safe and effective medicine for patients. Mass spectrometry can help to better characterize the counterfeit and provide information to help identify the source of the fraud.

Methods

Sample Preparation:

For this study, trastuzumab was used as a model biotherapeutic monoclonal antibody (mAb). Another sample was treated as a potential counterfeit (unknown sample). The workflow shown can be also used for other protein-based biotherapeutics.

For intact protein analysis, samples were diluted in a 5% acetonitrile/1% formic acid (*v*:*v*).

Subunit analysis was performed after reduction using tris(2-carboxyethyl)phosphine (TCEP) with a final concentration of 50 mM at room temperature for 30 min followed by a dilution in 5% acetonitrile/1% formic acid (*v*:*v*).

For peptide analysis, the samples were denatured with 7 M guanidine HCl/100 mM Tris (pH = 8.3±0.1). Reduction was performed at 56 °C for 30 min using TCEP at a final concentration of 10 mM. Samples were alkylated for 20 min in the dark using 20 mM sodium iodoacetate. The excess of alkylation agent was quenched by adding additional TCEP. Samples were digested using sequencing grade trypsin 37 °C for 30 min. Digestion was quenched by adding trifluoro acid 1% (*v*:*v*). samples were diluted with 5% acetonitrile/1% formic acid (*v*:*v*) prior to analysis.

Chromatography:

For intact and subunit analysis a C4 column (2.1×50 mm, 300, 1.7 um) was used at a flow rate of 250 µl/min. The column oven temperature was set to 80 °C. The chromatographic information for intact protein analysis is summarized in Table 1, for subunit protein analysis in Table 2.

Table 1. Chromatography for Intact Protein Analysis.

Time [min]	Mobile Phase A [%]	Mobile Phase B [%]
Initial	*90*	*10*
2.0	*90*	*10*
7.0	*40*	*60*
7.1	*20*	*80*
8.0	*20*	*80*
8.1	*90*	*10*
10.0	*90*	*10*

Peptide analysis was performed using a Phenomenex bioZen Peptide-XB-C18 column (2.1×100 mm, 2.6 µm). The flow rate was kept at 250 µL/min and the oven temperature was set to 40 °C. Chromatographic information for the analysis of peptides can be found in Table 3.

Table 2. Chromatography for Subunit Protein Analysis.

Time [min]	Mobile Phase A [%]	Mobile Phase B [%]
Initial	*85*	*15*
2.0	*85*	*15*
12.0	*40*	*60*
12.1	*20*	*80*
13.0	*20*	*80*
13.1	*85*	*15*
15.0	*85*	*15*

Table 3. Chromatography for Peptide Analysis.

Time [min]	Mobile Phase A [%]	Mobile Phase B [%]
Initial	*95*	*2*
2.0	*95*	*2*
55.0	*60*	*40*
55.1	*20*	*80*
57.0	*20*	*80*
57.1	*95*	*2*
60.0	*95*	*2*

Mass Spectrometry:

For intact and subunit analysis one method containing different subsections (experiments) with different settings was run (Table 4).

Table 4. MS Parameters for Intact and Subunit Data Acquisition.

Parameter	Experiment 1	Experiment 2	Experiment 3
Scan Mode		*TOF-MS positive*	
Gas 1		*50 psi*	
Gas 2		*50 psi*	
Curtain Gas		*35 psi*	
Source Temperature		*300 °C*	
Ion Spray Voltage		*5500 V*	
Start m/z	*400*	*650*	*650*
Stop m/z	*4,000*	*4,000*	*4,000*
Declustering Potential	*125 V*	*125 V*	*250 V*
Collision Energy	*10 eV*	*10 eV*	*10 eV*
Time bins to sum	*4*	*80*	*80*

For peptide analysis, a Top10 data-dependent acquisition (information dependent acquisition or IDA) with dynamic background subtraction was used. Detailed settings are listed in Table 5.

Table 5. MS Parameters for Peptide Data Acquisition.

Parameter	MS	MS-MS
Scan Mode	*TOF-MS with IDA positive*	
Gas 1	*50 psi*	
Gas 2	*50 psi*	
Curtain Gas	*35 psi*	
Source Temperature	*450 °C*	
Ion Spray Voltage	*5500 V*	
Declustering Potential	*80 V*	
Collision Energy	*10 eV*	*Rolling*
Start m/z	*300*	*150*
Stop m/z	*1,800*	*1,800*
Accumulation time	*250 ms*	*40 ms*
Time bins to sum	*4*	*8*

Data Processing:

Data were processed using BPV Flex 2.0 software.

The intact and subunit data obtained were processed without defining the sequence or modifications of the target molecule. Automatic, chromatographic peak detection and a large output mass range for reconstructed masses ranging from 10,000 to 200,000 Da were used to ensure detection of different protein sizes which could be present in the unknown sample.

For the analysis of the digests, a peptide mapping search was performed, using peptides from variable region of trastuzumab, including modifications such as oxidation and deamidation when applicable, 5 ppm matching tolerance and an MS/MS score for auto-validation of 3.

Workflow Overview

Many different scenarios are possible when analyzing a potential counterfeit medicine. Some common cases are listed in Figure 1, for which LC-MS analysis can be helpful to understand the content of the unknown sample. It is important to note that other characterization techniques, such as physico-chemical techniques, can provide a more complete picture, however these approaches will not be discussed here.

Here, an example of a counterfeit containing proteins was selected to demonstrate a subset of the workflows which can be performed using the SCIEX accurate mass systems together with BPV Flex 2.0 software.

Intact Analysis

The trastuzumab sample and the unknown sample were measured using the same generic conditions (see Table 1 and Table 4) without the optimization of LC, nor MS parameters for the specific analytes. Three different sets of MS settings were run within one single injection in order to cover settings suitable for different protein sizes which could be present in the unknown sample (Table 4), thus reducing the need for reruns.

The comparison of the data of the unknown sample with the biotherapeutic shows a shift towards a later retention time for the unknown sample indicating a more hydrophobic nature of the unknown sample (Figure 2A). A zoom into the raw data reveals a very similar pattern for both samples (Figure 2B), so does the reconstructed data (Figure 2C). However, a slight mass shift and differences in ratio of the glycosylation profile were observed (Figure 2C). With the hypermass feature, the reconstructed data can be easily compared to the raw data ensuring that the mass/molecular weight (MW) being looked at is correct (Figure 3). Especially, when it is not known what to look out for, a large output mass range for reconstruction in combination with the hypermass feature are helpful for interpreting the results in a straight forward manner.

The pattern and the MW of the reconstructed data indicates that the unknown sample consists of a monoclonal antibody with glycosylations, however, it is not entirely clear if it is the same antibody as the model biotherapeutic which might have undergone modifications leading to a change in hydrophobicity and mass shifts or if is a different protein which happens to look similar.

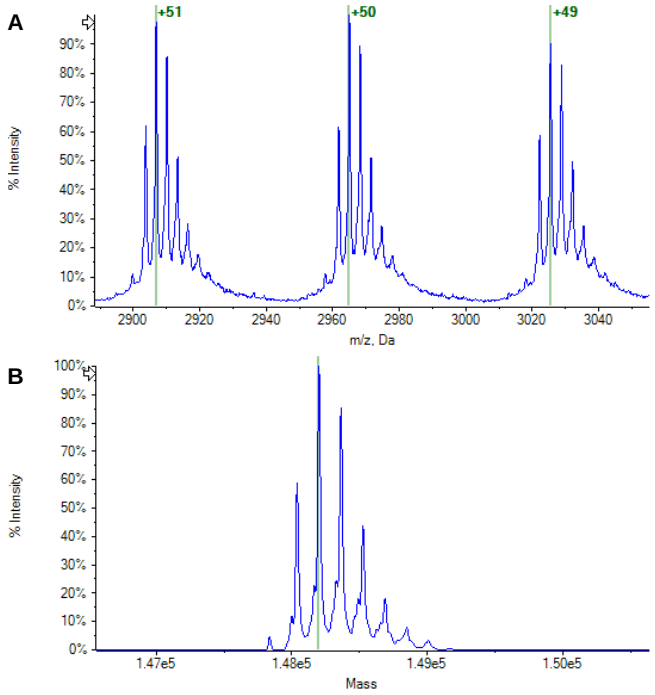

Figure 3. Hypermass Feature for Data of the Unknown Sample. A: TOF MS raw data with theoretically calculated charge states based on chosen reconstructed mass (hypermass feature). B: Reconstructed mass data with one peak being chosen as an example for calculation of charge states in raw data.

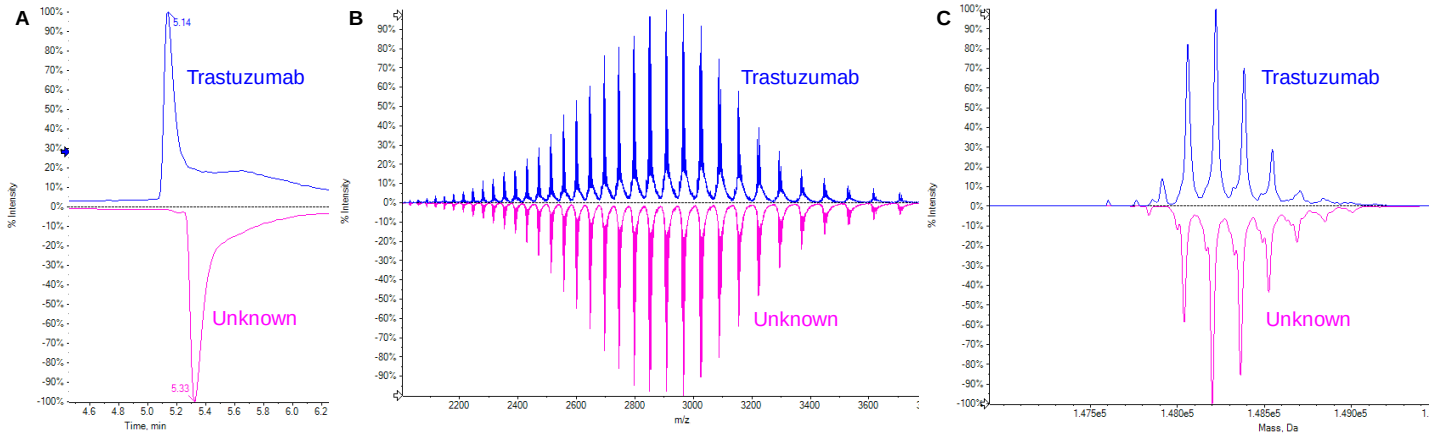

Figure 2: Intact Protein Analysis of Trastuzumab and the Unknown Sample as Mirror Plots. Blue trace: trastuzumab; pink trace: unknown sample. A: Total Ion Chromatogram (TIC). B: TOF-MS raw data zoom-into subset of measured mass range. C: Reconstructed data; zoom-in to subset of reconstruction range.

Subunit Analysis

In order to have better understanding of the unknown sample, a subunit analysis was performed. There are many different approaches for sample preparation of antibody subunits. In this case a simple, nevertheless very versatile, version was used which is suitable for a variety of protein samples containing subunits being covalently linked via disulfide bonds. A slightly longer chromatographic separation was used, whereas data was acquired using the same MS method parameters as for intact analysis (see Table 2 and 4). Since the initial MS method was set up to cover a large range of protein sizes, there was no need for adapting the method parameters for subunit analysis. For processing the samples in BPV Flex 2.0 software, the same processing method as for intact analysis was being used without any optimization of processing parameters; reducing the work load further. The automatic peak detection of the software enabled the reconstruction of both chromatographic peaks for the heavy chain (HC) and the light chain (LC) separately in one method. The raw data for both samples showed a similar pattern, further confirming that the sample contained an antibody which was reduced into heavy and light chains (Figure 4).

For both protein subunits of the unknown sample a mass shift compared to trastuzumab could be observed. Interestingly, the mass shift of the smaller subunit of the unknown sample is towards smaller masses whereas the mass shift of the larger subunit is towards higher masses compared to trastuzumab. Both subunits showed a mass shift of a few hundred Daltons. Biotherapeutics such as trastuzumab are extensively optimized to show limited susceptibility to modifications affecting their safety and efficacy. Furthermore, it is unlikely that modifications could have led to positive mass shifts on one subunit and negative mass shift on the other subunit. Therefore it is unlikely that the unknown sample has the same amino acid sequence as trastuzumab.

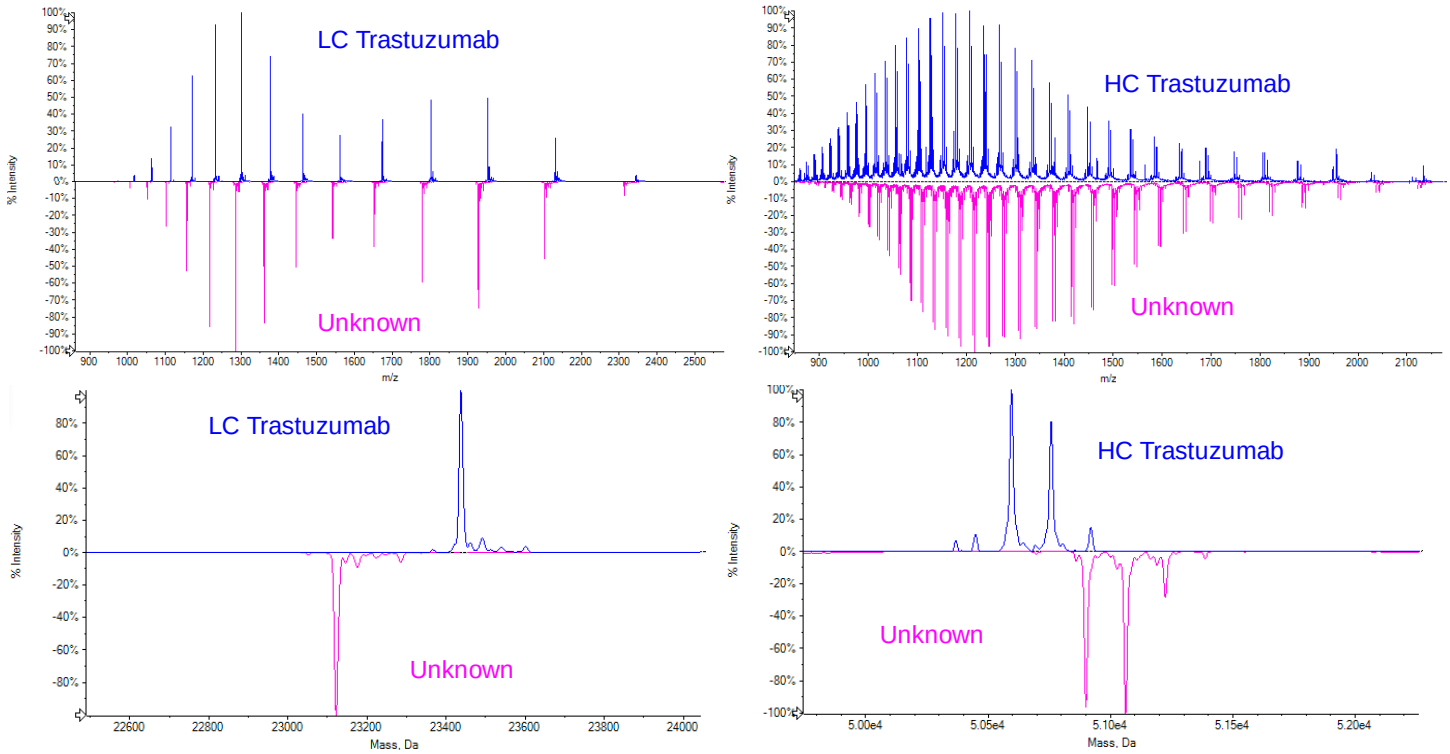

Figure 4: Subunit Protein Analysis of Trastuzumab and the Unknown Sample as Mirror Plots. Top: Raw data of first chromatographic peak (left) and second chromatographic peak (right) with trastuzumab (blue) and the unknown sample (pink). Bottom: Zoom into reconstructed data derived from the raw data. Left: Reconstruction of data from the top left, trastuzumab LC in blue and the unknown sample in pink. Right: Reconstruction of data from the top left, trastuzumab HC in blue and the unknown sample in pink.

Peptide Mapping

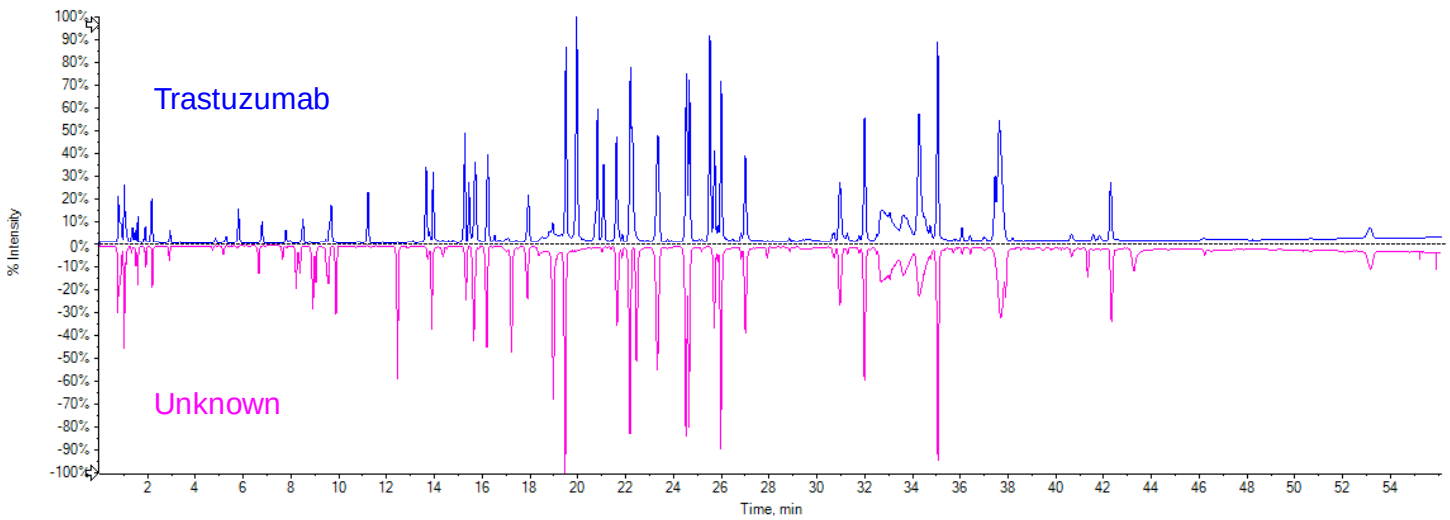

Figure 5: TIC of Tryptic Digest of Trastuzumab (Blue Trace) and Unknown Sample (Pink Trace) as Mirror Plot.

Neither intact, nor subunit protein analysis is sufficient to identify the unknown protein. Therefore, a tryptic digestion was performed using a generic digestion protocol. Both samples showed similarities for many chromatographic peaks, however also some clear differences could be observed (Figure 5). The similarities could be linked to the conserved region in antibodies, whereas the variable region at the N-terminus of antibodies can lead to proteotypic peptides or also called signature peptides only being present in a certain protein.

In order to test this hypothesis, a peptide mapping search was run for both samples focusing on peptides from the complementary determining region (CDR) of the heavy and light chain of trastuzumab. Only the trastuzumab digest resulted in validated matches (Figure 6) for these peptides. There was no evidence for any of the peptides of the CDR from trastuzumab being present in the unknown sample (see an example for one peptide in Figure 7).

Taking all the information from the intact, the subunit and the peptide analysis together, it was proven that the unknown sample contains an antibody of some kind, however, it is not the same as trastuzumab. If that sample was labelled as the biotherapeutic trastuzumab, it would classify as a counterfeit which is likely to be a threat to patient's health.

RT	Theoretical Mono. Mass	Theoretical Mono. m/z	Observed Mono. m/z	Error (ppm)	Score	Charge	XIC Area	Auto-Validated	Use	Sequence
15.26	968.4815	485.2480	485.2487	1.5	12.43	2	1.5534e7	✓		FTISADTSK
21.61	1309.6449	655.8297	655.8304	1.1	14.61	2	5.9532e6	✓		NTAYLQMNSLR
25.51	1880.9956	628.0058	628.0043	-2.4	21.60	3	4.6702e6	✓		EVQLVESGGGLVQPGGSLR
22.27	1877.8789	626.9669	626.9657	-2.0	16.56	3	2.7937e6	✓		DIQMTQSPSSLSASVGDR
34.27	1771.9509	886.9827	886.9823	-0.5	13.72	2	2.3409e6	✓		LLIYSASFLYSGVPSR
21.73	1707.8216	570.2812	570.2813	0.2	18.29	3	2.1129e5	✓		ASQDVNTAVAWYQQK

Figure 6. Validated Matches for CDR Peptides of Trastuzumab for the Trastuzumab Digest in BPV Flex 2.0 Software.

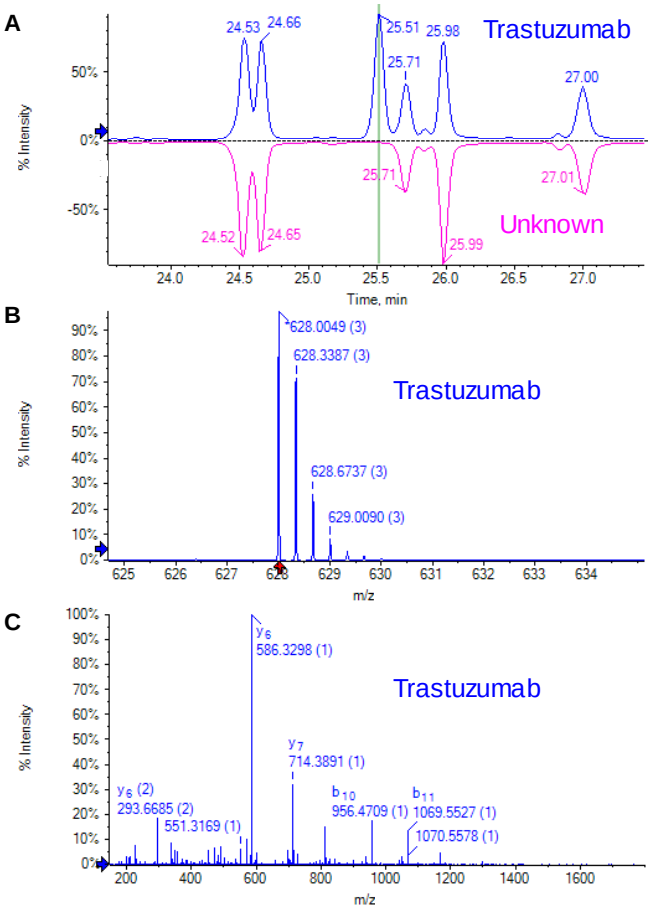

Figure 7. Results of Peptide Analysis for Peptide EVQLVESGGGLVQPGGSLR from the CDR of Trastuzumab. A: TIC mirror plot of both digests with green highlight of retention time of the peptide B: TOF MS raw for peptide in trastuzumab digest. C: Associated MS/MS data for the same peptide in trastuzumab digest.

Conclusions

- SCIEX accurate mass systems with generic methods show great data quality enabling scientists to understand potential protein counterfeits better and faster
- The full workflow of intact, subunit and peptide analysis in combination with BPV Flex software can be used to easily compare unknown protein samples with originator protein therapeutics
- Verification of hypotheses can lead to the identification of proteins in an unknown sample paving the way the way of fighting counterfeit drugs on a scientific level

References

1. FDA at : https://www.fda.gov/drugs/buying-using-medicine-safely/counterfeit-medicine.

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