

Structural modification and digestibility change of β -lactoglobulin modified by methylglyoxal with the simulated reheating of dairy products

Lu Dong^a, Yuekun Wu^a, Wenxiang Wang^a, Yajing Wu^a, Yan Zhang^{b,*}, Shuo Wang^{a,c,*}

^a State Key Laboratory of Food Nutrition and Safety, Key Laboratory of Food Nutrition and Safety, Ministry of Education of China, Tianjin University of Science and Technology, Tianjin 300457, China

^b Tianjin Key Laboratory of Food Science and Health, School of Medicine, Nankai University, Tianjin 300071, China

^c Beijing Advanced Innovation Center for Food Nutrition and Human Health, Beijing Technology & Business University (BTBU), Beijing 100048, China

ARTICLE INFO

Keywords:

Methylglyoxal-modified β -lactoglobulin
Advanced glycation end products
Modification sites
Modification types
Digestibility
Reheating

ABSTRACT

A methylglyoxal (MG)- β -lactoglobulin (bLG) model was established to simulate reheating conditions (60–100 °C) to investigate the modification effect that α -dicarbonyl compounds had on protein structure and on the digestibility of milk protein. The results showed that bLG can be modified by MG, and the modification degree increased with the increase in reheating temperature. The reacted lysine and arginine as well as the generated protein-bound N^ε-carboxymethyllysine and N^ε-carboxyethyllysine in the modified bLG also increased with temperature. The high-resolution mass spectrometry results revealed that the modification site is at the lysine and arginine residue of bLG. Additionally, nine types of modifications were detected, and N^ε-carboxyethyllysine was the dominant modification product. The in vitro digestibility of MG-modified bLG clearly decreased with the increase in reheating temperature. This result was consistent with the degree of structural modification and could be explained by the specific action sites (lysine and arginine) of the digestive enzyme, which were modified by MG.

1. Introduction

Heat processing is a classic and widely used food processing method, however, heating can lead to complex reactions within the food matrix, including Maillard reaction, lipid oxidation, protein oxidation, etc., which would result in the production of certain toxic substances (Birlouez-Aragon, Morales, Fogliano, & Pain, 2010). Protein oxidation has the potential to influence protein structure, which may alter the utilization of dietary proteins (Öste, Miller, Sjöström, & Norén, 1987; Trnková, Dršata, & Boušková, 2015). As one type of protein oxidation, reducing sugar-induced protein glycation can lead to the formation of advanced glycation end products (AGEs), which is considered as the product of Maillard reaction as well.

AGEs are thought to be formed between the carbonyl groups of reducing sugars (or its glycolytic derivatives) and the amino groups of free amino acids or amino acid residues of proteins during the Maillard reaction (Arena, Renzone, D'Ambrosio, Salzano, & Scaloni, 2017). AGEs are detected in foods rich in sugar and protein. N^ε-carboxymethyllysine (CML), N^ε-carboxyethyllysine (CEL), methylglyoxal (MG)-derived hydroimidazolones (MG-H1) and pyrrole have been detected in the

hydrolysates of heated milk products (Ahmed et al., 2005; Hegele, Buetler, & Delatour, 2008). The content of AGEs in spray-dried infant formula is 670 times that of breast milk (Arena et al., 2017). Study has reported that food-derived AGEs significantly increase AGE levels in the body (Uribarri et al., 2005), which may induce the development of glycation and inflammatory associated diseases, such as Alzheimer and diabetes (Borg et al., 2018; Estévez & Luna, 2016; Nowotny, Schröter, Schreiner, & Grune, 2018).

α -Dicarbonyl compounds (α DCs), such as 3-deoxyglucosone, MG, and glyoxal, are intermediates derived from sugar fragmentation by retro-aldolization and the degradation of reducing sugars during the Maillard reaction, both in vivo and in vitro (Arena, Salzano, Renzone, D'Ambrosio, & Scaloni, 2014). α DCs can react with free amino acids to form low molecular weight AGEs, they can also react with the amino-groups in the amino acid residues or the N-terminus of protein to form linear or cross-linked protein-bound AGEs, which vary according to the different substrates, such as CML, CEL and allysine formed by glyoxal or MG modified lysine (Lys); dihydroxyimidazolines, hemiaminals and hydroimidazolones formed by MG, glyoxal or 3-deoxyglucosone modified arginine (Arg); α -amino adipic- δ -semialdehyde residue formed by

* Corresponding authors at: State Key Laboratory of Food Nutrition and Safety, Key Laboratory of Food Nutrition and Safety, Ministry of Education of China, Tianjin University of Science and Technology, Tianjin 300457, China (S. Wang).

E-mail addresses: y Zhang@nankai.edu.cn (Y. Zhang), wangshuo@nankai.edu.cn (S. Wang).

<https://doi.org/10.1016/j.foodchem.2019.03.021>

Received 19 November 2018; Received in revised form 5 March 2019; Accepted 7 March 2019

Available online 09 March 2019

0308-8146/ © 2019 Elsevier Ltd. All rights reserved.

MG or 3-deoxyglucosone modified Lys; lysine dimers and cross-linked imidazolium formed by MG, glyoxal or 3-deoxyglucosone modified Lys (Akagawa, Sasaki, & Suyama, 2010; Arena et al., 2014; Henle, 2005; Luna & Estévez, 2019; Mittelman & Pischetsrieder, 2011). The activity of α DCs is much greater than that of glucose, therefore, α DCs can rapidly react with the amino groups of protein amino acid residues (Thornalley, 2005). An inspection showed that the dietary intake of MG was up to 20 mg/day (Degen, Hellwig, & Henle, 2012), which represents a potential risk for human health.

Studies have revealed protein modifications by MG under simulated physiological conditions. One study reported that 94% of the target amino acids were lost after incubating with MG at 37 °C for 12 h, which was a 7.2-fold increase compared to glucose (13%) (Mittelman & Pischetsrieder, 2011). Glutathione peroxidase was found to be modified by MG at Arg 184 and Arg185, leading to the inactivation of the peroxidase and an increase in intracellular peroxides (Park et al., 2003). The antimicrobial and chemotactic functions of β -defensin-2, a skin antimicrobial peptide, were significantly reduced due to modifications by α DCs (Kiseler et al., 2015).

Several kinds of α DCs have been found in dairy products, including levels up to dozens of milligrams per liter of α DCs in milk (Hellwig, Degen, & Henle, 2010). Some kinds of dairy products possibly need to be reheated, especially milk powder, which must be dissolved in hot water before ingestion. Due to the high reactivity of α DCs, reactions between α DCs and proteins may occur in the reheating process. Study has shown that glucose as a precursor of α DCs can promote protein glycation and tryptophan loss (Luna & Estévez, 2018). Glyoxal and MG can induce the formation of allysine in β -lactoglobulin (bLG) and myofibrillar proteins, and reduce the water-holding capacity and trypsin-chymotrypsin digestibility during incubation at 80 °C for 48 h (Luna & Estévez, 2019). In addition, the in vitro digestion experiment demonstrated that the modification occurred between α DCs and β -casein, which significantly altered the release of peptides from gastrointestinal digested protein (Zhao, Le, Larsen, et al., 2017). Thus, it can be seen that protein glycation is closely related to its processing characteristics and bioavailability.

In this study, MG and bLG were used as the representatives of α DCs and milk proteins, respectively, to create a simulated chemical model under reheating conditions to investigate the effect of α DCs on the alteration of bLG structure, including changes in the molecular weight, UV-spectrum, intrinsic fluorescence, and secondary structure. Modification types (MTs) and modification sites (MSs) were particularly investigated by high-resolution mass spectrometry, and the corresponding in vitro digestibility of the bLGs modified by MG (MG-bLGs) were also analyzed. These results will provide fundamental information on the quality and safety of milk proteins that undergo reheating processes to avoid inappropriate reheating, thus benefiting human health.

2. Materials and methods

2.1. Chemicals and materials

bLG ($\geq 90\%$, identified by SDS-PAGE), MG (40% aqueous solution, w/w), pepsin (3800 units/mg protein), trypsin (10,000 units/mg protein) and chymotrypsin (40 units/mg protein) were purchased from Sigma-Aldrich (St. Louis, MO, USA). CML and CEL were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). All other reagents were of analytical reagent grade. Milli-Q water was used in the experiments.

2.2. Chemical model to simulate reheating conditions

bLG (100 μ M) was incubated with MG (2 mM) in 0.05 M phosphate buffer (PB, pH 7.0) at different reheating temperature (60, 70, 80, 90 and 100 °C) for 1 h to prepare MG-bLGs, and untreated bLG (ubLG) served as a control. Ultrafiltration was performed immediately

following the reaction to remove excess MG. The solution containing MG-bLGs was lyophilized for subsequent study.

2.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

ubLG and MG-bLGs were subjected to SDS-PAGE with a 12% separating gel and 3% stacking gel. A protein molecular weight marker (14.4–116 kDa) was used. Electrophoresis was performed at 80 V. The gel was stained with Coomassie blue R-250.

2.4. Analysis of hyperchromicity of MG-bLGs

Solutions of 1 mg/mL ubLG and 1 mg/mL MG-bLGs (both in 0.05 MPB (pH 7.0)) were measured. The absorption profiles of ubLG and MG-bLGs were recorded using a spectrophotometer in the wavelength range of 250–400 nm.

2.5. Analysis of intrinsic fluorescence of MG-bLGs

The fluorescence profiles of the 1 mg/mL ubLG and MG-bLGs solutions (0.05 MPB (pH 7.0)) were recorded by a spectrofluorophotometer, with an excitation wavelength of 295 nm and an emission spectra range of 300–400 nm.

2.6. Analysis of secondary structure of MG-bLGs

The 1 mg/mL ubLG and MG-bLGs solutions (0.05 MPB (pH 7.0)) were measured on a Jasco-815 Chirascan Circular Dichroism Spectrometer (JASCO, Tokyo, Japan) using a 1 mm path length quartz cuvette. Spectra were recorded in the far UV regions of 190–250 nm. The data were evaluated by the Pro Data Chirascan program. The percentage of secondary structural content was calculated by using the program CDNN.

2.7. Quantitation of reacted Lys and reacted Arg in MG-bLGs

A fluorescamine assay was applied to determine the free Lys content of the protein, as previously described (Ashraf et al., 2015). Fluorescence was measured at the excitation/emission wavelengths of 390/490 nm on a spectrofluorophotometer. $N\alpha$ -acetyllysine (0–2 mM) was used as the standard to determine the free Lys content of the MG-bLGs. ubLG was used as a control.

Examination of the free Arg content of protein with a 9,10-phenanthrenequinone assay was performed, as previously described (Ashraf et al., 2015). Fluorescence was measured at the excitation/emission wavelengths of 312/395 nm on a spectrofluorophotometer. $N\alpha$ -acetylarginine at concentrations of 0–0.5 mM was used as a standard to measure the free Arg content. ubLG was used as a control. The ratio of reacted Lys (Arg) of MG-bLGs were calculated as follows:

$$\begin{aligned} &\text{reacted ratio (\%)} \\ &= \frac{\text{content of Lys (Arg) of ubLG} - \text{content of Lys (Arg) of MG-bLGs}}{\text{content of Lys (Arg) of ubLG}} \\ &\quad \times 100 \end{aligned}$$

2.8. Quantitation of protein-bound CML and CEL by HPLC-MS/MS

The amount of protein-bound CML and CEL in MG-bLGs was determined according to the reported internal standard method (Teerlink, Barto, Brink, & Schalkwijk, 2004). Briefly, MG-bLGs were reduced by sodium borate buffer (0.5 M, pH 9.2) and sodium borohydride (2 M) overnight at 4 °C, followed by acid hydrolysis with 12 M HCl at 110 °C for 24 h. The hydrolysates were then extracted and purified using a PCX C18 cartridge (200 mg/3 mL, Agela Technologies, Tianjin, China).

Eluates containing CML and CEL were evaporated to dryness under a stream of nitrogen gas, and the residue was reconstituted in Milli-Q water-methanol (80:20, v/v), followed by filtering through a 0.22 µm organic filter. The analysis was performed using an Agilent 1200 LC system coupled to an Agilent 6410 triple-quadrupole MS system. The purified sample was separated on a Waters HILICA C18 column (2 × 150 mm, 4 µm particle size) in a column oven at 35 °C operated in isocratic elution for 6 min. Solvent A was Milli-Q water with 0.1% (v/v) formic acid, and solvent B was chromatographic grade methanol. The injection volume was 2 µL, with flow rate of 0.2 mL/min. MS analysis was operated in positive electrospray ionization mode using the multiple reaction monitoring mode. The ion-spray voltage was set at 4500 V. The product ions at *m/z* 83.9 and 129.8 (CML) and at *m/z* 84.3 and 130.2 (CEL) were used for quantification and confirmation, respectively.

2.9. Analysis of structural modification of MG-bLGs by high-resolution mass spectrometry

The MG-bLGs protein bands of interest were cut from the SDS-PAGE gel and subjected to in-gel trypsin digestion. Briefly, gel particles of target bands were decolorized to a colorless and transparent state. The proteins were reduced with dithiothreitol, followed by alkylation with iodoacetamide to reduce disulfide bridges. Subsequently, the samples were subjected to proteolysis overnight at 37 °C by trypsin, with an enzyme to protein ratio of 1:50 (w/w) at 37 °C for 12 h. The enzymatic peptide solutions were desalted using C18 tips, and the eluates were collected for further study.

The peptides were separated using an EASY-nLC 1000 instrument (Thermo Fisher, San Jose, USA). A trap C18 column (5 µm pore size, 150 µm i.d. 3 cm length, 120 Å) and a separating C18 column (3 µm pore size, 75 mm i.d. 15 cm length, 100 Å) were used to separate the peptides, and a linear gradient was applied. Enzymatic peptide eluates from the LC were directly nanosprayed into the Orbitrap Fusion Lumos Mass Spectrometer (Thermo Fisher, San Jose, USA). All mass spectra were operated in data-dependent mode. MS1 and MS2 spectra were acquired in the positive ion mode with a full scan MS. The MS1 resolution was 120,000 (at *m/z* 200), and the MS2 resolution was 30,000 (at *m/z* 200). A parent ion scan range of *m/z* 350–1550 and daughter ion scans were acquired with a starting mass of *m/z* 120, followed by selecting the twenty most intense ions (top 20) for MS2 fragmentation using a precursor isolation width window of 1.6 *m/z*. Electron-transfer/higher-energy collision dissociation fragmentation was performed with a normalized collision energy of 25 at parent ion valence ≥ 4; higher-energy collision dissociation fragmentation was performed with a normalized collision energy of 32 at a parent ion valence of 2 or 3. The dynamic exclusion time was 10 s. The same amount of iRT (Biognosys) peptides was added to each sample during MS data acquisition.

The DDA raw files were searched against the UniProt protein database of *Bos taurus* (downloaded on 2017.3.6, 31,867 entries) using Proteome Discoverer 2.1 (Thermo Fisher, San Jose, USA). The protein sequence was appended with the iRT fusion protein sequence. The Sequest HT search engine was used with the following search parameters: semi-specific enzymolysis, mass accuracy values of 18 ppm and 2 ppm for MS1 and MS2, respectively, and variable modifications, including oxidation of methionine and deamidation of asparagine, glutamine, MG and dehydrated MG modifications for Arg and Lys amino groups were set. In addition, all detected MS2 spectra for each site of modification were manually verified. The data were retrieved by PMI-Byonic software, and the protein modification was analyzed. PMI-Byologic software was used to collect the various peak areas of each of the five samples. The Percolator algorithm was used to keep the false discovery ratio of peptide less than 1%, and the *q*-value used for protein identification was 0.01. For quantitation of each specific MSs modified by MG in each sample, peptides containing each specific MSs area were extracted and further normalized according to the iRT abundance in

each sample.

2.10. In vitro gastrointestinal digestion

In vitro simulation of gastrointestinal digestion was performed as previously described, with some modifications (Zhao, Li, Le, et al., 2017). Solutions of ubLG and MG-bLGs at 1% (w/v) were prepared with 0.6 M NaCl, warmed in a 37 °C water bath for 10 min and adjusted to pH 3 with 1 M HCl. Pepsin was added to each MG-bLGs solution at a final concentration of 200 U/mg of protein, and the digestion and hydrolyzation was performed at 37 °C for 1.5 h. A 0.1 mL aliquot of 1 M NaHCO₃ was added to inactivate the pepsin. The sample solutions were adjusted with 1 M NaOH to pH 7, and then chymotrypsin and trypsin were added to each sample at a final concentration of 0.5 and 35 U/mg of protein, respectively. The digestion was carried out at 37 °C for 2 h, and the enzymes were then inactivated in a boiling water bath for 5 min. Trichloroacetic acid was used to precipitate the protein, followed by a 4000 r/min centrifugation for 10 min. The nitrogen content of the test samples and its supernatants were determined by the Kjeldahl method. Protein digestibility was calculated as follows:

$$\text{Protein digestibility (\%)} = \frac{\text{nitrogen content of supernatant}}{\text{nitrogen content of test samples}} \times 100$$

3. Results and discussion

MG and bLG were used as the representatives of αDCs and milk proteins, respectively, to simulate the effects of different reheating temperature to prepare the MG-bLGs. The molecular weight, UV-spectra, intrinsic fluorescence, secondary structure, MTs, MSs, and in vitro digestibility of the MG-bLGs were analyzed to investigate the modification effect of αDCs on the nature of milk proteins.

3.1. The modification degree of bLG by MG

The SDS-PAGE profiles of the MG-bLGs (Fig. 1A) reflected the size change of the bLG peptide chain. Compared to ubLG (band 6), the MG-bLGs bands (bands 1–5) were slightly broadened, indicating the existence of bLG-related proteins with different molecular weights. Extra bands appeared at approximately twice the molecular weight of bLG, indicating the formation of bLG aggregates. The results revealed that reactions occurred between molecules of bLG and MG-bLGs were modified by MG to a certain degree, thus leading to the formation of bLG-related proteins with diverse molecular weights. Meanwhile, the two carbonyl groups of MG induced covalent interactions between bLG molecules, leading to the formation of bLG-MG-bLG molecules. In vivo experiments indicated similar result that αDCs induced the covalent cross-linking between histone H2A molecules, with the formation of histone H2A dimer. (Mir, Uddin, Alam, & Ali, 2014).

The chromophoric groups may be exposed or generated during the modification process caused by structural changes, thus showing a change of absorbance at 280 nm of proteins (Ashraf et al., 2015). In comparison with ubLG, MG-bLGs exhibited a gradual increase in the absorbance density at 280 nm (Fig. 1B). The hyperchromicity of MG-bLGs (at 60, 70, 80, 90 and 100 °C) increased approximately 20.8%, 40.6%, 50.9%, 62.1% and 63.0%, respectively, which further proves that the reactions occurred between molecules of bLG and MG, and the extent of the interactions increased with the temperature rise. A slight increase in absorbance at wavelengths of 300–350 nm with elevating temperature was observed, which may be attributed to the formation of AGEs in the MG-bLGs (Mir et al., 2014).

Glycation of proteins can promote tryptophan loss (Luna & Estévez, 2018). The fluorescence spectra of MG-bLGs are shown in Fig. 1C. The wavelength of maximum fluorescence emission intensity of ubLG was approximately 331 nm, characteristic of tryptophan emission, with an

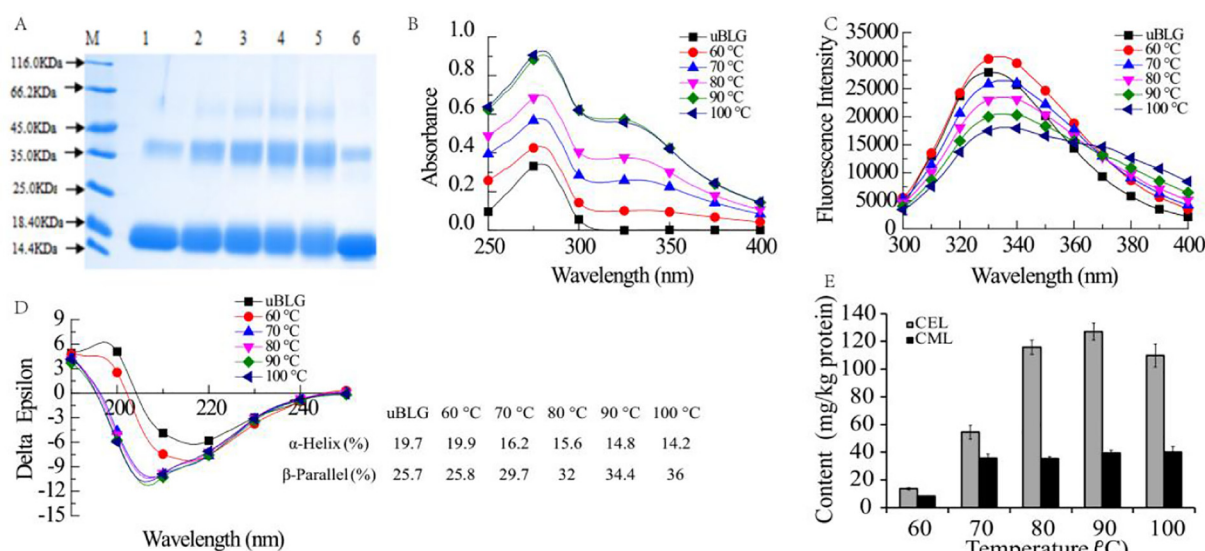


Fig. 1. A: SDS-PAGE profile of ubLG and MG-bLGs. M: Marker proteins, Lane 1–5: MG-bLG 60–100 °C, Lane 6: ubLG treated at 100 °C. B: UV profile of ubLG and MG-bLGs. C: Fluorescence profile of ubLG and MG-bLGs. D: CD spectra of ubLG and MG-bLGs in far UV region (190–250 nm). E: The generated CML and CEL in MG-bLGs (reheating at 60–100 °C).

excitation wavelength of 295 nm. Except for MG-bLG at 60 °C, the fluorescence emission intensity of MG-bLGs (70–100 °C) was weaker than ubLG, and the fluorescence emission intensity of MG-bLGs decreased gradually with the increase of reheating temperature, which indicates that the αDC modified protein may cause loss of tryptophan. Changes in protein fluorescence intensity may also be caused by changes in the polarity of the environment around the tryptophan residues (Mir et al., 2014), hence, the different fluorescence intensities between the MG-bLGs reflected the conformational changes of the MG-bLGs. The decrease of fluorescence intensity may also have been caused by the formation of aggregates or crosslinked protein molecules, resulting in changes in the tryptophan residue microenvironment, or by the embedding of tryptophan residues, thereby weakening the MG-bLG fluorescence. MG-bLGs also appeared to have a redshift of approximately 4 nm with the increasing temperature. A red shift in the maximum fluorescence emission intensity suggests the exposure of tryptophan residues to a more polar environment (Chaudhuri, Halder, & Chattopadhyay, 2010). Study has found phenomenon similar to that of the tryptophan fluorescence in histone H2A were modified by αDCs in vivo (Mir et al., 2014).

3.2. Secondary structural changes in MG-bLGs

The secondary structural alteration of MG-bLGs was studied by far-UV CD spectroscopy (Fig. 1D). The CD spectra showed obvious conformational changes in the MG-bLGs. Negative ellipticity within the range of 208–222 nm is related to the secondary structure of proteins. The ratios of ubLG α-helices and β-sheets were 19.7% and 25.7%, respectively. Compared with ubLG, the α-helix ratio of MG-bLGs gradually decreased from 18.9% to 14.2%, in contrast, the β-sheet content of the MG-bLGs gradually increased from 25.8% to 36.0% with an increase in the reheating temperature. The β-sheet structure is more likely to promote intermolecular interactions between proteins to form aggregates (Bakhti, Habibi-Rezaei, Moosavi-Movahedi, & Khazaei, 2007), hence, the increased β-sheet content in MG-bLGs reflected an increased aggregation degree of MG-bLGs, also indicating the modification degree of bLG by MG.

The above results prove that bLG was modified by MG upon reheating, and the modification degree increased with an increase in the reheating temperature.

3.3. Formation of protein-bound AGEs in MG-bLGs

Basic amino acids are likely to react with active carbonyl groups, because their side chains contain amino groups that can be protonated. Studies have shown that up to 50% of Lys residues are modified in skim milk powder (Henle, Walter, Krause, & Klostermeyer, 1991). Morgan et al. found that the free amino groups content of bLG was decreased during the incubation of reducing sugar and bLG (Morgan et al., 1998). In the reheating milk model, the reacted Lys and Arg residues were detected (Table 1). The percentage of reacted Lys and Arg residues in MG-bLGs appeared as an upward trend along with the increase of reheating temperature ($P < 0.05$). The maximum percentage of reacted Lys (32.5%) and Arg (66.7%) was obtained at 100 °C. Therefore, the reaction products, protein-bound CML and CEL in MG-bLGs, were detected using HPLC-MS/MS. As can be seen in Fig. 1E, the bound CEL increased from 13.6 to 109.7 mg/kg protein with reheating temperature increasing from 60 to 100 °C. The bound CML content also showed a similar upward trend from 8.2 to 40.2 mg/kg protein. The contents of bound CML and CEL generated at 100 °C were, respectively, approximately 8 and 5 times higher than those at 60 °C.

Lys is an essential amino acid, but studies have found that Lys is readily modified at a given temperature by sugars or by αDCs, which contain active carbonyls. The modification of proteins could lead to the loss of amino acids and the decrease of nutritional value in dairy products (Arena et al., 2014; Heilmann et al., 2014). The bioavailability of proteins will decrease due to the formation of AGEs (Wada & Lönnnerdal, 2014). Therefore, temperature should be minimized during the reheating of dairy products to reduce the loss of nutritional value and inhibit the generation of AGEs. This would protect people from an excessive ingestion of AGEs, which will benefit human health.

3.4. MSs and MTs of MG-bLGs

Studies have shown that reducing sugar can modify proteins and the

Table 1
The percentage of reacted Lys and Arg of MG-bLGs.

	60 °C	70 °C	80 °C	90 °C	100 °C
Ratio of reacted Lys (%)	23.7%	25.0%	28.7%	31.2%	32.5%
Ratio of reacted Arg (%)	33.3%	36.7%	43.7%	53.3%	66.7%

MSs were detected through liquid chromatography/electrospray ionization mass spectrometry or matrix-assisted laser desorption/ionization time-of-flight mass spectrometry to understand the impact on protein structure and properties (Chevalier, Chobert, Mollé, & Haertlé, 2001; Fenaille, Morgan, Parisod, Tabet, & Guy, 2004; Morgan et al., 1998). So, it is essential to reveal the specific changes in bLG structure, and analyze the MSs and the MTs of the MG-bLGs by high-resolution mass spectrometry after reheating.

The results showed that the bLG used in the experiment was sp. P02754 | LACB_BOVIN Beta-lactoglobulin (OS = Bos Taurus, GN = LGB, PE = 1, SV = 3; UniProt protein database), with a peptide amino acid sequence of LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGLLEILLQKWEDECAQKKIAEKTKIPAVFKIDALNENKVLVDLDYKYYLLFCMENSAPESQSLVCQLVTRTEVDDEALEKFDKALKALPMHIRLSFNPTQLEEQCHI (1–168), containing 15 Lys (K) residues and 3 Arg (R) residues.

The variable modifications were set as follows: CEL (+58 Da), CML (+72 Da), pyrroline (+108 Da), pyrroline-carboxymethyllysine (Pyr-CML, +40 Da), dihydropyrimidine (DHP, +126 Da), tetrahydropyrimidine (THP, +144 Da), arg-pyrimidine (+108 Da), MG-derived dihydroxyimidazolines (MG-DH, +72 Da), and MG-H1 (+54 Da) (Arenas et al., 2014; Henle, 2005; Mittelmayer & Pischetsrieder, 2011).

First, the MTs were obtained from peptide precursor fragmentation, followed by b and y ion analysis. Fig. 2 lists the example peptides of KIDALNENK, with different MTs (CML (A) and CEL (B)), IRLSFNPTQLEEQCHI, with an MT of MG-H1 (C), and ALKALPMHIRL, with different MTs of CML and MG-H1 (D). Based on an analysis of all mass spectrometry peptide information, the Lys residues were modified in 4 types, including CEL, CML, pyrroline and Pyr-CML, and Arg residues were modified in 5 types, including DHP, THP, arg-pyrimidine, MG-DH and MG-H1. All secondary mass spectra (Figs. 1–165) will be shown in the supplemental material.

When the MTs were analyzed by mass spectrometry, the MSs can also be obtained. As shown in Table 2, CEL was the main MTs of Lys in MG-bLGs, which was detected at K14, K47, K69, K83, K91, K100, K135 and K141 in all MG-bLGs. CML was detected less often compared with

CEL, which was consistent with the protein-bound CML and CEL content. Pyr-CML was detected at K8, K14, K47, K69 and K91 in MG-bLG at 60 °C. In addition, Pyr-CML was also detected at K14 (70 °C) and K69 (80 °C and 90 °C). Pyrroline was only detected at 60 °C at K8, K83 and K91. MG-H1 and MG-DH were detected at all Arg MSs in all MG-bLGs. Arg-pyrimidine was also detected at all Arg MSs in MG-bLGs, except at R40 in MG-bLG at 100 °C. THP was detected at R124 for all temperatures. DHP was also detected at different MSs for different temperature. The effect of modifications on the structure and function of a protein may depend on the MSs (Yeboah et al., 2004). Some reports have shown that modifications can elevate steric hindrance and electrostatic interactions and change the structure of a protein (Liu & Zhong, 2012; Pinto et al., 2011). Therefore, the diverse locations of the main MSs appearing in the MG-bLGs are a possible explanation for MG-bLGs with different structures, which was reflected by the SDS-PAGE profiles, UV spectra fluorescence spectra and CD spectra.

The quantitative analysis results showed that the main MSs were different between the MG-bLGs. As shown in Fig. 3, K69, K70, K101, and K141 were detected to have the highest modification ratios in MG-bLG at 100 °C. K8, K14, K60, K77, K91, K100, K135, and K138 were detected to have the highest modification ratios in MG-bLG at 60 °C. K63 was detected to have the highest modification ratio at 70 °C. K91 and K93 were detected to have the highest modification ratios at 80 °C. The modification ratio of R124 is greater than 97% at all temperatures. The modification ratio of R148 increases with an increase in reheating temperature. Except for at 100 °C, the modification ratio of K138 was greater than 50%. The different modification ratios of each MSs at different temperatures may be due to changes in the structure. All modification ratio of different MTs at each MSs at all reheating temperatures were shown in the supplemental material (Table 1).

3.5. In vitro digestibility of MG-bLGs

The mass spectrometry study showed that the modification of bLG by MG mainly occurred at the Lys and Arg sites, and the enzymatic site of gastrointestinal digestive enzymes is also at Lys and Arg residues. To verify whether the modification of these amino acid sites would affect

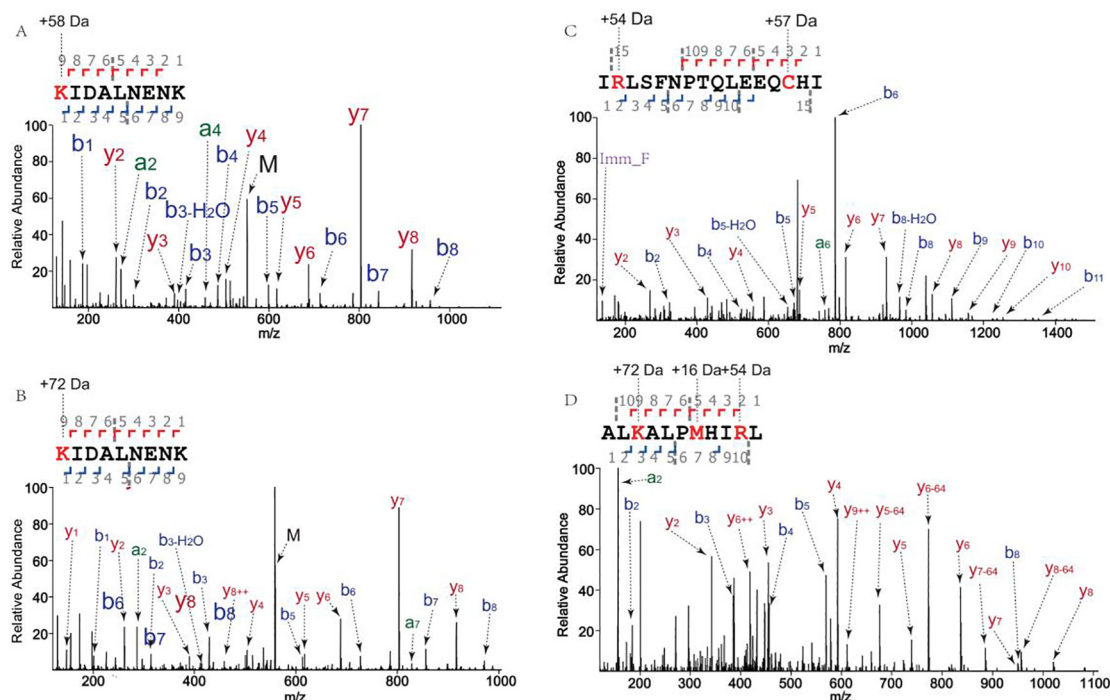


Fig. 2. Mass spectrometry results. Four MS2 for peptide KIDALNENK with different MTs, CML (A) and CEL (B). Peptide IRLSFNPTQLEEQCHI with an MT of MG-H1 (C). Peptide ALKALPMHIRL with MTs of CML and MG-H1 (D).

Table 2
The MSs and MTs of MG-BLGs.

MTs of K	Pyr-CML	CML	CEL	Pyrraline	
K8	T1,	T1,	T1, T2, T4, T5	T1	
K14	T1, T2	T1, T2, T3, T5	T1, T2, T3, T4, T5		
K47	T1,	T4	T1, T2, T3, T4, T5		
K60	T3, T4	T1, T 4, T5	T1, T2, T3, T4		
K69		T1, T2, T3, T4, T5	T1, T2, T3, T4, T5		
K70			T1, T2, T4, T5		
K75			T1, T3, T5		
K77			T1, T2, T3, T4		
K83		T1, T 4, T5	T1, T2, T3, T4, T5	T1	
K91	T1	T1	T1, T2, T3, T4, T5	T1	
K100		T1, T2, T4, T5	T1, T2, T3, T4, T5		
K101			T5		
K135		T1	T1, T2, T3, T4, T5		
K138		T1	T1, T2, T5		
K141			T1, T2, T3, T4, T5		
MTs of R	MG-H1	MG-DH	Arg-pyrimidine	THP	DHP
R40	T1, T2, T3, T4, T5	T1, T2, T3, T4, T5	T1, T2, T3, T4	T1, T2, T3, T4	T1, T2, T3, T4
R124	T1, T2, T3, T4, T5	T1, T2, T3, T4, T5	T1, T2, T3, T4, T5	T1, T2, T3, T4, T5	T3, T4
R148	T1, T2, T3, T4, T5	T1, T2, T3, T4, T5	T1, T2, T3, T4, T5	T1	T1, T5

T1, T2, T3, T4, and T5 refer to 60, 70, 80, 90, and 100 °C.

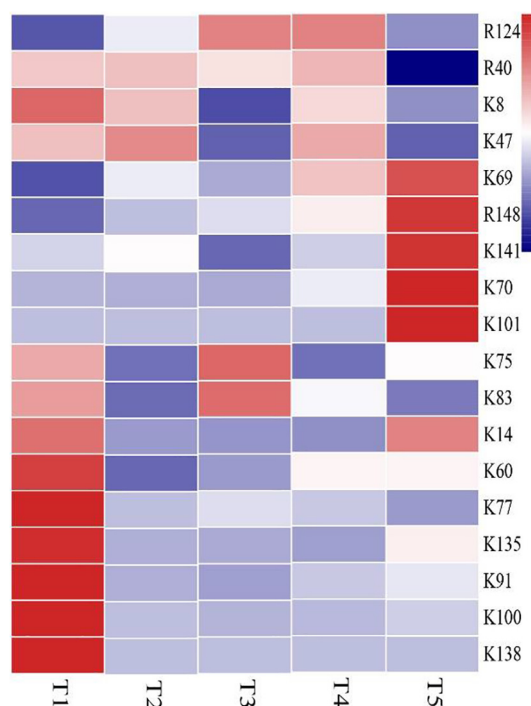


Fig. 3. Quantitative heat maps for modification ratios at each site at different temperatures. T1–T5 was 60–100 °C.

the enzyme hydrolysis, the in vitro digestibility of MG-bLGs was investigated. The results showed that the simulated gastrointestinal digestibility of the MG-bLGs decreased significantly compared with ubLG and differed between the MG-bLGs at different reheating temperatures. The digestibility values of ubLG and MG-bLGs were 84.67%, 77.11%, 70.89%, 57.78%, 43.67% and 38.89%. Compared with ubLG, the digestibility of MG-bLGs significantly decreased as the reheating temperature increased (Fig. 4).

Pepsin has a certain amino acid sequence specificity when hydrolyzing proteins or peptides, and it tends to hydrolyze peptide bonds at aromatic residues, such as phenylalanine, tryptophan and tyrosine (Fruton, 1974), which were not the modification sites of MG-modified by bLG. However, trypsin cleaves peptides on the C-terminal side of Lys

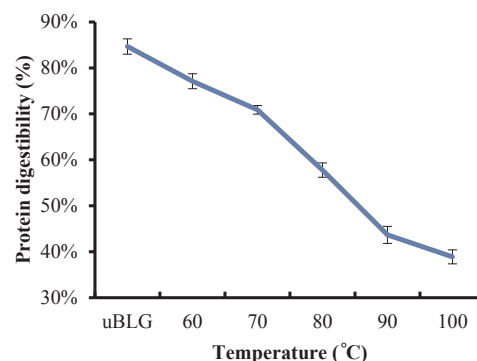


Fig. 4. In vitro digestibility of ubLG and MG-bLGs (reheating at 60–100 °C).

and Arg residues (Wada & Lönnerdal, 2014), which are the main MSs of MG-bLGs. Previous study demonstrated that some of the released peptides disappeared after the protein was modified by αDCs, mainly because the modified structure massively hindered the action of trypsin. (Zhao, Le, Larsen, et al., 2017). Other study has also found that the digestibility of proteins reduced after modified by αDCs (Luna & Estévez, 2019). Therefore, the decrease of digestibility of the MG-bLGs was inferred to be the result of the structural changes of the MG-bLGs. Some reports have shown that glycation can increase steric hindrance and electrostatic interactions (Liu & Zhong, 2012; Pinto et al., 2011), which may suppress the interaction between bLG and enzymes, explaining the decreased digestibility of MG-bLGs. A decrease in protein digestibility means an increase in non-absorbability, resulting in a loss of protein nutrition. The nonabsorbable fraction, including amino acids and peptides, is utilized by intestinal flora, generating a series of substances harmful to the body (Kasubuchi, Hasegawa, Hiramatsu, Ichimura, & Kimura, 2015; Windey, Preter, & Verbeke, 2012).

4. Conclusions

During reheating, MG can modify bLG to form MG-bLG, which changed the molecular mass and secondary structure of the protein. Lys and Arg residues, with four and five MTs, respectively, have been identified as major MSs, and CEL and CML were the two most dominant MTs. With increasing of reheating temperature, Lys and Arg residues were modified rate increased. The digestibility of MG-bLG decreased as the reheating temperature increased. In summary, MG modified protein

causes essential amino acid loss, protein digestibility reduced, which gives rise to potential risks for human health. Therefore, we should appropriately reduce the reheating temperature to avoid negative reactions.

Declaration of interests

None.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (No. 31430068), the National Key R&D Program of China (project No. 2018YFC1604203) and the 111 Program of the Ministry of Education, China (T2017002).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2019.03.021>.

References

- Ahmed, N., Mirshekar-Syahkal, B., Kennish, L., Karachalias, N., Babaei-Jadidi, R., & Thornalley, P. J. (2005). Assay of advanced glycation endproducts in selected beverages and food by liquid chromatography with tandem mass spectrometric detection. *Molecular Nutrition and Food Research*, 49(7), 691–699. <https://doi.org/10.1002/mnfr.200500008>.
- Akagawa, M., Sasaki, T., & Suyama, K. (2010). Oxidative deamination of Lys residue in plasma protein of diabetic rats: Novel mechanism via the maillard reaction. *FEBS Journal*, 269(22), 5451–5458. <https://doi.org/10.1046/j.1432-1033.2002.03243.x>.
- Arena, S., Renzone, G., D'Ambrosio, C., Salzano, A. M., & Scaloni, A. (2017). Dairy products and the Maillard reaction: A promising future for extensive food characterization by integrated proteomics studies. *Food Chemistry*, 219, 477–489. <https://doi.org/10.1016/j.foodchem.2016.09.165>.
- Arena, S., Salzano, A. M., Renzone, G., D'Ambrosio, C., & Scaloni, A. (2014). Non-enzymatic glycation and glycoxidation protein products in foods and diseases: An interconnected, complex scenario fully open to innovative proteomic studies. *Mass Spectrometry Reviews*, 33(1), 49–77. <https://doi.org/10.1002/mas.21378>.
- Ashraf, J. M., Rabbani, G., Ahmad, S., Hasan, Q., Khan, R. H., Alam, K., & Choi, I. (2015). Glycation of H1 histone by 3-deoxyglucosone: Effects on protein structure and generation of different advanced glycation end products. *PLoS One*, 10(6), e0130630. <https://doi.org/10.1371/journal.pone.0130630>.
- Bakhti, M., Habibi-Rezaei, M., Moosavi-Movahedi, A. A., & Khazaei, M. R. (2007). Consequential alterations in haemoglobin structure upon glycation with fructose: Prevention by acetylsalicylic acid. *Journal of Biochemistry*, 141(6), 827–833. <https://doi.org/10.1093/jb/mvm096>.
- Birlouez-Aragon, I., Morales, F., Fogliano, V., & Pain, J. P. (2010). The health and technological implications of a better control of neoformed contaminants by the food industry. *Pathologie Biologie*, 58(3), 232–238. <https://doi.org/10.1016/j.patbio.2009.09.015>.
- Borg, D. J., Yap, F. Y. T., Keshvari, S., Simmons, D. G., Gallo, L. A., Fotheringham, A. K., ... Forbes, J. M. (2018). Perinatal exposure to high dietary advanced glycation end products in transgenic nod8.3 mice leads to pancreatic beta cell dysfunction. *Islets*, 10(1), 10–24. <https://doi.org/10.1080/19382014.2017.1405189>.
- Chaudhuri, A., Haldar, S., & Chattopadhyay, A. (2010). Organization and dynamics of tryptophans in the molten globule state of bovine α -lactalbumin utilizing wavelength-selective fluorescence approach: Comparisons with native and denatured states. *Biochemical and Biophysical Research Communications*, 394(4), 1082–1086. <https://doi.org/10.1016/j.bbrc.2010.03.130>.
- Chevalier, F., Chobert, J. M., Mollé, D., & Haertlé, T. (2001). Maillard glycation of beta-lactoglobulin with several sugars: Comparative study of the properties of the obtained polymers and of the substituted sites. *Dairy Science and Technology*, 81(C7), 651–666. <https://doi.org/10.1051/lait:2001155>.
- Degen, J., Hellwig, M., & Henle, T. (2012). 1, 2-Dicarbonyl compounds in commonly consumed foods. *Journal of Agricultural and Food Chemistry*, 60(28), 7071–7079. <https://doi.org/10.1021/jf301306g>.
- Estévez, M., & Luna, C. (2016). Dietary protein oxidation: A silent threat to human health? *Critical Reviews in Food Science and Nutrition*, 57(17), 3781–3793. <https://doi.org/10.1080/10408398.2016.1165182>.
- Fenaile, F., Morgan, F., Parisod, V., Tabet, J. C., & Guy, P. A. (2004). Solid-state glycation of β -lactoglobulin by lactose and galactose: Localization of the modified amino acids using mass spectrometric techniques. *Journal of Mass Spectrometry*, 39(1), 16–28. <https://doi.org/10.1002/jms.539>.
- Fruton, J. S. (1974). Active site of pepsin. *Accounts of Chemical Research*, 7(8), 241–246. <https://doi.org/10.1021/ar50080a001>.
- Hegele, J., Buetler, T., & Delatour, T. (2008). Comparative LC–MS/MS profiling of free and protein-bound early and advanced glycation-induced lysine modifications in dairy products. *Analytica Chimica Acta*, 617(1–2), 85–96. <https://doi.org/10.1016/j.aca.2007.12.027>.
- Heilmann, M., Wellner, A., Gadermaier, G., Ilchmann, A., Briza, P., Krause, M., ... Toda, M. (2014). Ovalbumin modified with pyralline, a Maillard reaction product, shows enhanced T-cell immunogenicity. *The Journal of Biological Chemistry*, 289, 7919–7928. <https://doi.org/10.1074/jbc.M113.523621>.
- Hellwig, M., Degen, J., & Henle, T. (2010). 3-Deoxygalactosone, a “new” 1,2-dicarbonyl compound in milk products. *Journal of Agricultural and Food Chemistry*, 58(19), 10752–10760. <https://doi.org/10.1021/jf102388v>.
- Henle, T. (2005). Protein-bound advanced glycation endproducts (AGEs) as bioactive amino acid derivatives in foods. *Amino Acids*, 29(4), 313–322. <https://doi.org/10.1007/s00726-005-0200-2>.
- Henle, T., Walter, H., Krause, I., & Klostermeyer, H. (1991). Efficient determination of individual Maillard compounds in heat-treated milk products by amino acid analysis. *International Dairy Journal*, 1(2), 125–135. [https://doi.org/10.1016/0958-6946\(91\)90004-R](https://doi.org/10.1016/0958-6946(91)90004-R).
- Kasubuchi, M., Hasegawa, S., Hiramatsu, T., Ichimura, A., & Kimura, I. (2015). Dietary gut microbial metabolites, short-chain fatty acids, and host metabolic regulation. *Nutrients*, 7(4), 2839–2849. <https://doi.org/10.3390/nu7042839>.
- Kiseler, J. G., Wang, X., Dubyak, G. R., Sanadi, E. C., Ghosh, S. K., Lundberg, K., & Williams, W. M. (2015). Modification of β -defensin-2 by dicarbonyls methylglyoxal and glyoxal inhibits antibacterial and chemotactic function in vitro. *PLoS One*, 10(8), e0130533. <https://doi.org/10.1371/journal.pone.0130533>.
- Liu, G., & Zhong, Q. (2012). Glycation of whey protein to provide steric hindrance against thermal aggregation. *Journal of Agricultural and Food Chemistry*, 60(38), 9754–9762. <https://doi.org/10.1021/jf302883b>.
- Luna, C., & Estévez, M. (2018). Oxidative damage to food and human serum proteins: Radical-mediated oxidation vs. glyco-oxidation. *Food Chemistry*, 267, 111–118. <https://doi.org/10.1016/j.foodchem.2017.06.154>.
- Luna, C., & Estévez, M. (2019). Formation of allysine β -lactoglobulin and myofibrillar proteins by glyoxal and methylglyoxal: Impact on water-holding capacity and in vitro digestibility. *Food Chemistry*, 271, 87–93. <https://doi.org/10.1016/j.foodchem.2018.07.167>.
- Mir, A. R., Uddin, M., Alam, K., & Ali, A. (2014). Methylglyoxal mediated conformational changes in histone H2A—generation of carboxyethylated advanced glycation end products. *International Journal of Biological Macromolecules*, 69, 260–266. <https://doi.org/10.1016/j.ijbiomac.2014.05.057>.
- Mittelmair, S., & Pischetsrieder, M. (2011). Multistep ultrahigh performance liquid chromatography/tandem mass spectrometry analysis for untargeted quantification of glycation activity and identification of most relevant glycation products. *Analytical Chemistry*, 83(24), 9660–9668. <https://doi.org/10.1021/ac2025706>.
- Morgan, F., Bouhallab, S., Mollé, D., Henry, G., Maubois, J. L., & Léonil, J. (1998). Lactation of β -lactoglobulin monitored by electrospray ionization mass spectrometry. *International Dairy Journal*, 8(2), 95–98. [https://doi.org/10.1016/s0958-6946\(98\)00025-9](https://doi.org/10.1016/s0958-6946(98)00025-9).
- Nowotny, K., Schröter, D., Schreiner, M., & Grune, T. (2018). Dietary advanced glycation end products and their relevance for human health. *Ageing Research Reviews*, 47, 55–66. <https://doi.org/10.1016/j.arr.2018.06.005>.
- Öste, R. E., Miller, R., Sjöström, H., & Norén, O. (1987). Effect of maillard reaction-products on protein digestion – studies on pure compounds. *Journal of Agricultural and Food Chemistry*, 35(6), 938–942. <https://doi.org/10.1021/jf00078a020>.
- Park, Y. S., Koh, Y. H., Takahashi, M., Miyamoto, Y., Suzuki, K., Dohmae, N., ... Taniguchi, N. (2003). Identification of the binding site of methylglyoxal on glutathione peroxidase: Methylglyoxal inhibits glutathione peroxidase activity via binding to glutathione binding sites Arg 184 and 185. *Free Radical Research*, 37(2), 205–211. <https://doi.org/10.1080/1071576021000041005>.
- Pinto, M. S., Bouhallab, S., Carvalho, A. F., Henry, G., Putaux, J. L., & Leonil, J. (2011). Glucose slows down the heat-induced aggregation of β -lactoglobulin at neutral pH. *Journal of Agricultural and Food Chemistry*, 60(1), 214–219. <https://doi.org/10.1021/jf2037664>.
- Teerlink, T., Barto, R., Brink, H. J. T., & Schalkwijk, C. G. (2004). Measurement of N ϵ -(carboxymethyl) lysine and N ϵ -(carboxyethyl) lysine in human plasma protein by stable-isotope-dilution tandem mass spectrometry. *Clinical Chemistry*, 50(7), 1222–1228. <https://doi.org/10.1373/clinchem.2004.031286>.
- Thornalley, P. J. (2005). Dicarbonyl intermediates in the Maillard reaction. *Annals of the New York Academy of Sciences*, 1043(1), 111–117. <https://doi.org/10.1196/annals.1333.014>.
- Trnková, L., Dršata, J., & Boušová, I. (2015). Oxidation as an important factor of protein damage: Implications for maillard reaction. *Journal of Biosciences*, 40(2), 419–439. <https://doi.org/10.1007/s12038-015-9523-7>.
- Uribarri, J., Cai, W. J., Sandu, O., Peppas, M., Goldberg, T., & Vlassara, H. (2005). Diet derived advanced glycation end products are major contributors to the body's AGE pool and induce inflammation in healthy subjects. *Annals of the New York Academy of Sciences*, 1043(1), 461–466. <https://doi.org/10.1196/annals.1333.052>.
- Wada, Y., & Lönnérdal, B. (2014). Effects of different industrial heating processes of milk on site-specific protein modifications and their relationship to in vitro and in vivo digestibility. *Journal of Agricultural and Food Chemistry*, 62(18), 4175–4185. <https://doi.org/10.1021/jf501617s>.
- Windey, K., Preter, V., & Verbeke, K. (2012). Relevance of protein fermentation to gut health. *Molecular Nutrition and Food Research*, 56(1), 184–196. <https://doi.org/10.1002/mnfr.201100542>.
- Yeboah, F. K., Alli, I., Yaylayan, V. A., Yasuo, K., Chowdhury, S. F., & Purisima, E. O. (2004). Effect of limited solid-state glycation on the conformation of lysozyme by ESI-MSMS peptide mapping and molecular modeling. *Bioconjugate Chemistry*, 15(1), 27–34. <https://doi.org/10.1021/bc034083v>.
- Zhao, D., Le, T. T., Larsen, L. B., Li, L., Qin, D., Su, G., & Li, B. (2017). Effect of glycation derived from α -dicarbonyl compounds on the in vitro digestibility of β -casein and β -lactoglobulin: A model study with glyoxal, methylglyoxal and butanedione. *Food Research International*, 102, 313–322. <https://doi.org/10.1016/j.foodres.2017.10.002>.
- Zhao, D., Li, L., Le, T. T., Larsen, L. B., Su, G., Liang, Y., & Li, B. (2017). Digestibility of glyoxal-glycated β -casein and β -lactoglobulin and distribution of peptide-bound advanced glycation end products in gastrointestinal digests. *Journal of Agricultural and Food Chemistry*, 65(28), 5778–5788. <https://doi.org/10.1021/acs.jafc.7b0195>.

Supplementary material

Figure 1-14. The secondary mass spectrum of the CEL modification of the peptides at 60 °C.

Figure 15-24. The secondary mass spectrum of the CML modification of the peptides at 60 °C.

Figure 25-37. The secondary mass spectrum of the Pyrroline modification of the peptide at 60 °C.

Figure 28-31. The secondary mass spectrum of the Pyr-CML modification of the peptide at 60 °C.

Figure 32-34. The secondary mass spectrum of the Arg-Pyrimidine modification of the peptide at 60 °C.

Figure 35, 36. The secondary mass spectrum of the DHP modification of the peptide at 60 °C.

Figure 37-39. The secondary mass spectrum of the MG-DH modification of the peptide at 60 °C.

Figure 40-42. The secondary mass spectrum of the MG-H1 modification of the peptide at 60 °C.

Figure 42-45. The secondary mass spectrum of the THP modification of the peptide at 60 °C.

Figure 46-59, 75. The secondary mass spectrum of the CEL modification of the peptides at 70 °C.

Figure 58-61. The secondary mass spectrum of the CML modification of the peptides at 70 °C.

Figure 62. The secondary mass spectrum of the Pyr-CML modification of the peptide at 70 °C.

Figure 63-65. The secondary mass spectrum of the Arg-Pyrimidine modification of the peptide at 70 °C.

Figure 66-68. The secondary mass spectrum of the DHP modification of the peptide at 70 °C.

Figure 69-71. The secondary mass spectrum of the MG-DH modification of the peptide at 70 °C.

Figure 48, 72-74. The secondary mass spectrum of the MG-H1 modification of the peptide at 70 °C.

Figure 75, 76. The secondary mass spectrum of the THP modification of the peptide at 70 °C.

Figure 77-88. The secondary mass spectrum of the CEL modification of the peptides at 80 °C.

Figure 86-89. The secondary mass spectrum of the CML modification of the peptides at 80 °C.

Figure 90. The secondary mass spectrum of the Pyr-CML modification of the peptide at 80 °C.

Figure 91-93. The secondary mass spectrum of the Arg-Pyrimidine modification of the peptide at 80 °C.

Figure 94, 95. The secondary mass spectrum of the DHP modification of the peptide at 80 °C.

Figure 96-98. The secondary mass spectrum of the MG-DH modification of the peptide at 80 °C.

Figure 86, 87, 90, 99-101. The secondary mass spectrum of the MG-H1 modification of the peptide at 80 °C.

Figure 102, 103. The secondary mass spectrum of the THP modification of the peptide at 80 °C.

Figure 104-115. The secondary mass spectrum of the CEL modification of the peptides at 90 °C.

Figure 115-120. The secondary mass spectrum of the CML modification of the peptides at 90 °C.

Figure 121. The secondary mass spectrum of the Pyr-CML modification of the peptide at 90 °C.

Figure 114, 122-124. The secondary mass spectrum of the Arg-Pyrimidine modification of the peptide at 90 °C.

Figure 125, 126. The secondary mass spectrum of the DHP modification of the peptide at 90 °C.

Figure 127-129. The secondary mass spectrum of the MG-DH modification of the peptide at 90 °C.

Figure 107, 130-132. The secondary mass spectrum of the MG-H1 modification of the peptide at 90 °C.

Figure 133, 134. The secondary mass spectrum of the THP modification of the peptide at 90 °C.

Figure 145-147, 163. The secondary mass spectrum of the CEL modification of the peptides at 100 °C.

Figure 146-153. The secondary mass spectrum of the CML modification of the peptides at 100 °C.

Figure 154, 155. The secondary mass spectrum of the Arg-Pyrimidine modification of the peptide at 100 °C.

Figure 156. The secondary mass spectrum of the DHP modification of the peptide at 100 °C.

Figure 157-160. The secondary mass spectrum of the MG-DH modification of the peptide at 100 °C.

Figure 161-163. The secondary mass spectrum of the MG-H1 modification of the peptide at 100 °C.

Figure 164, 165. The secondary mass spectrum of the THP modification of the peptide at 100 °C.

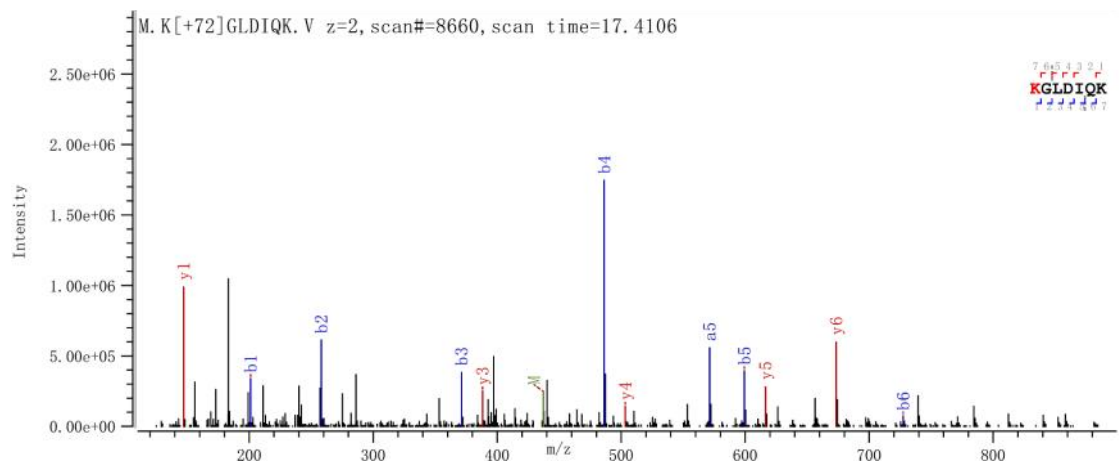


Figure 1.

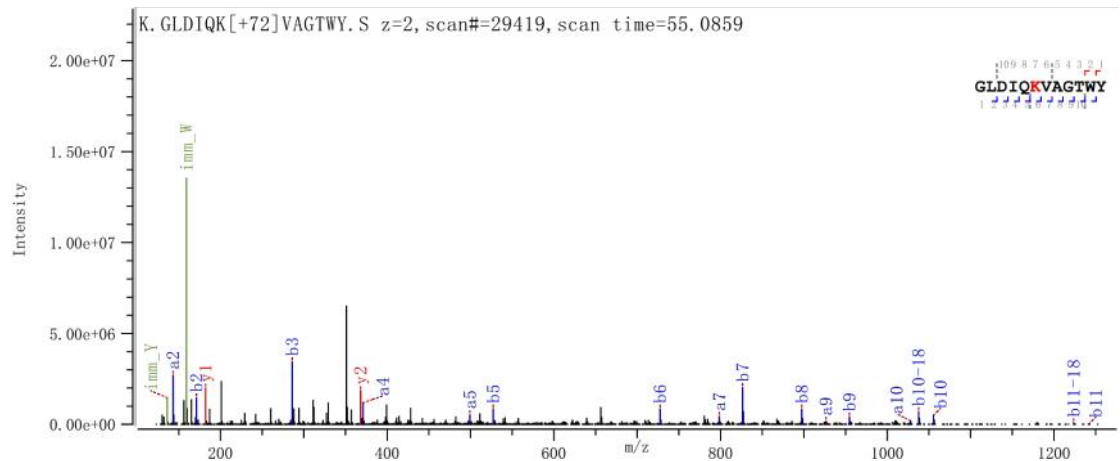


Figure 2.

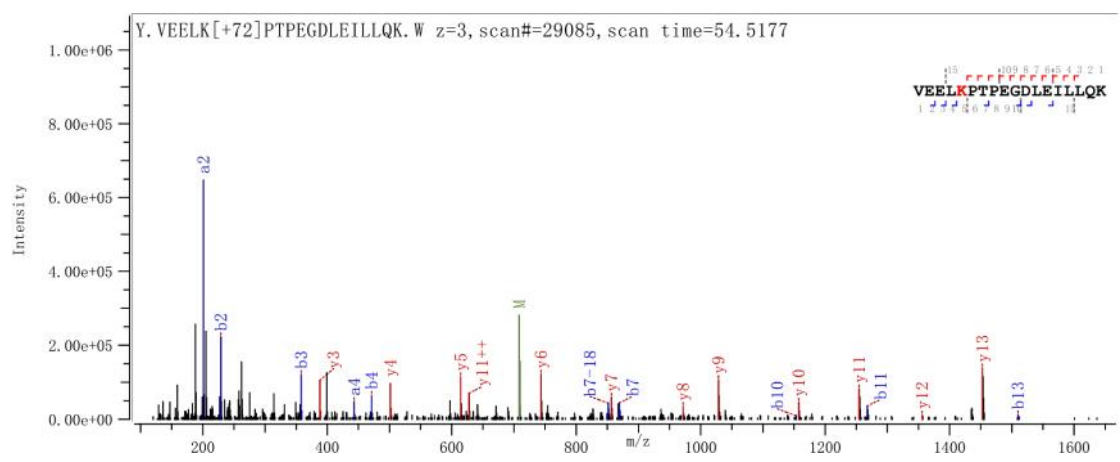


Figure 3.

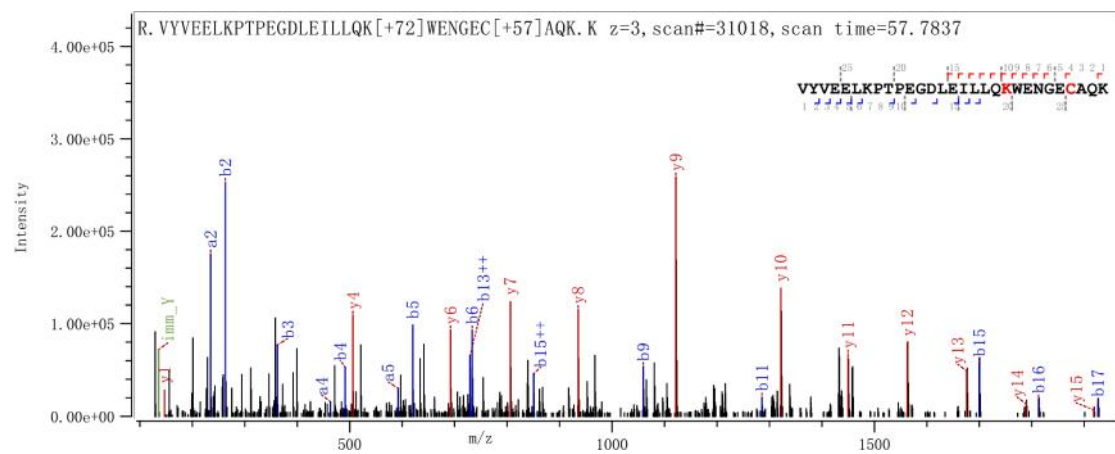


Figure 4.

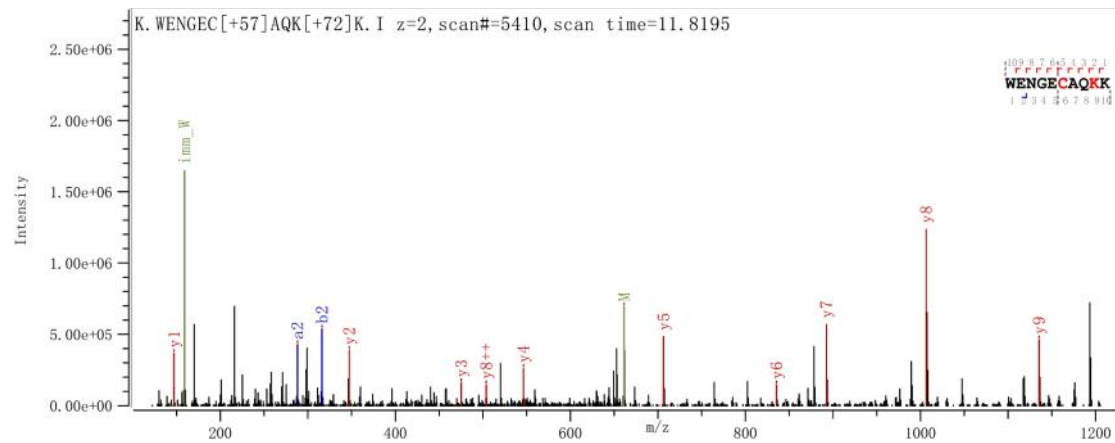


Figure 5.

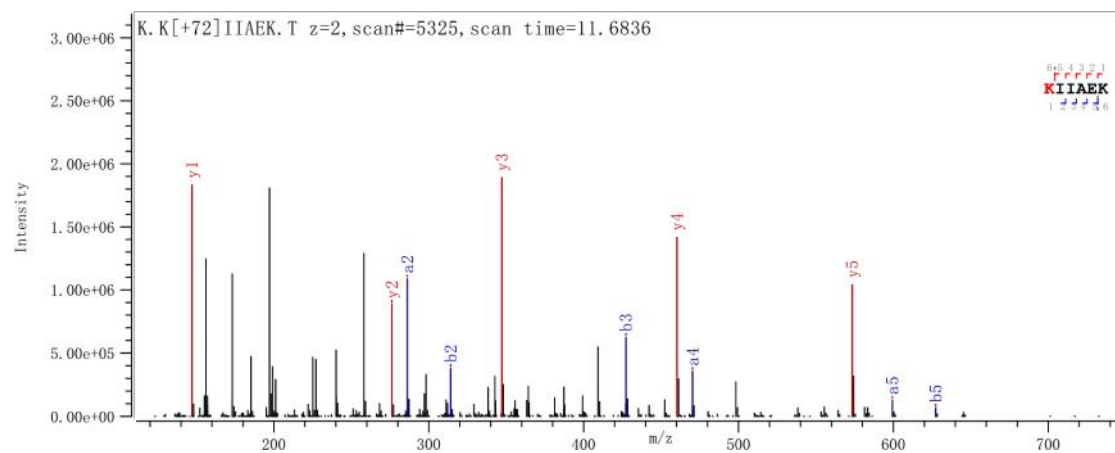


Figure 6.

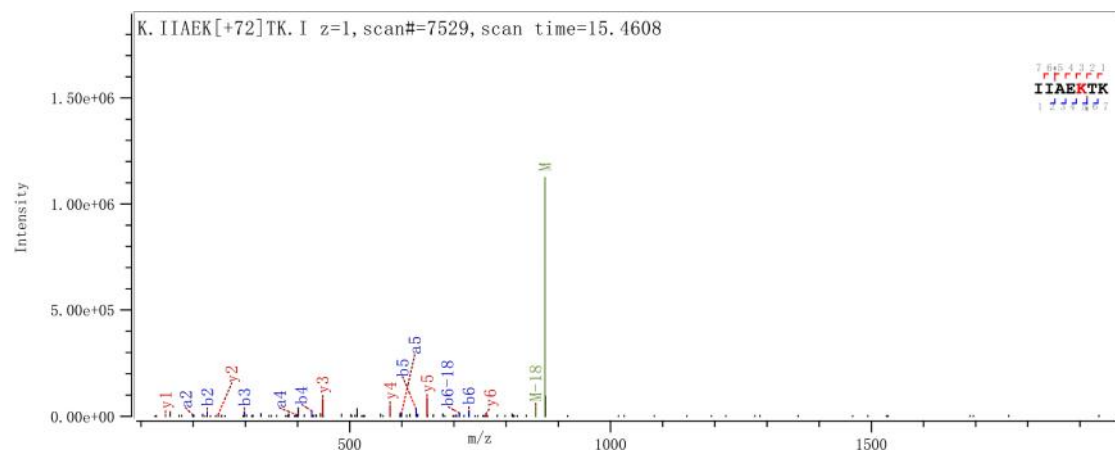


Figure 7.

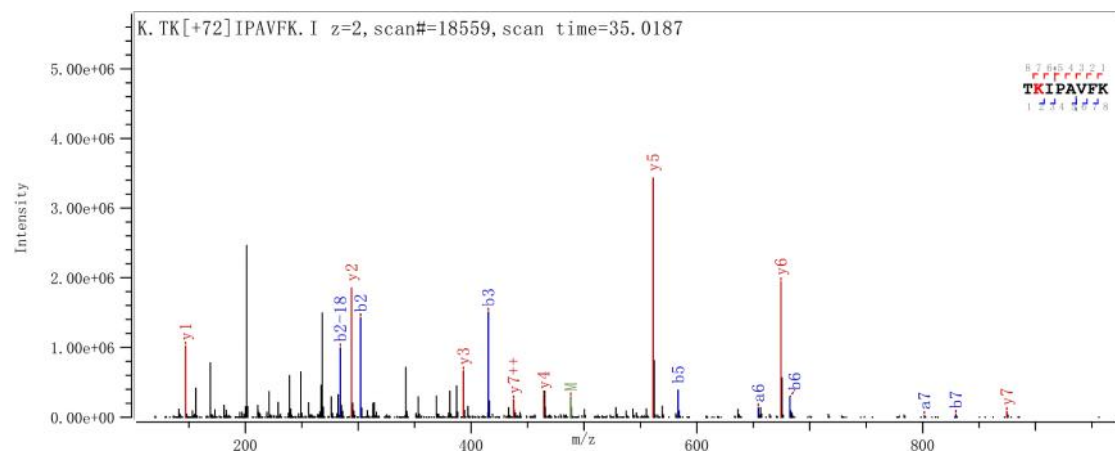


Figure 8.

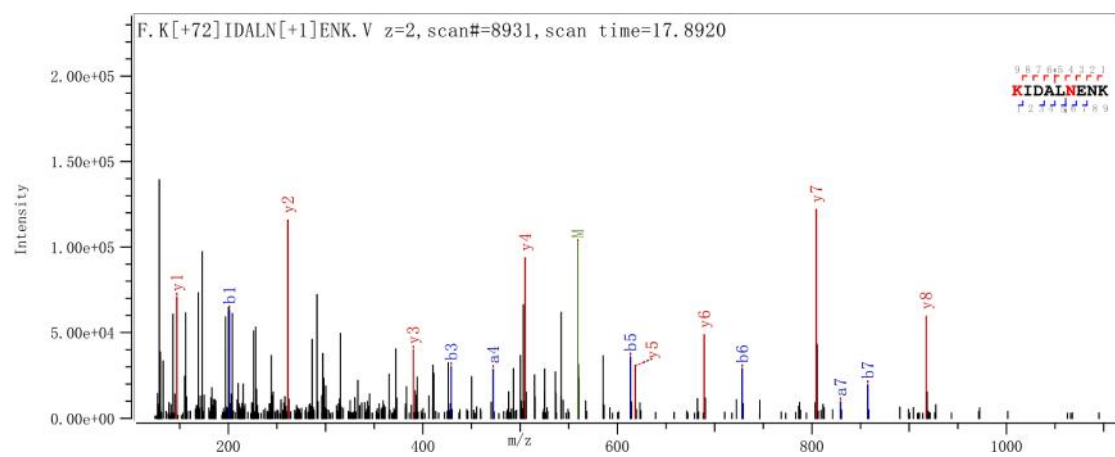


Figure 9.

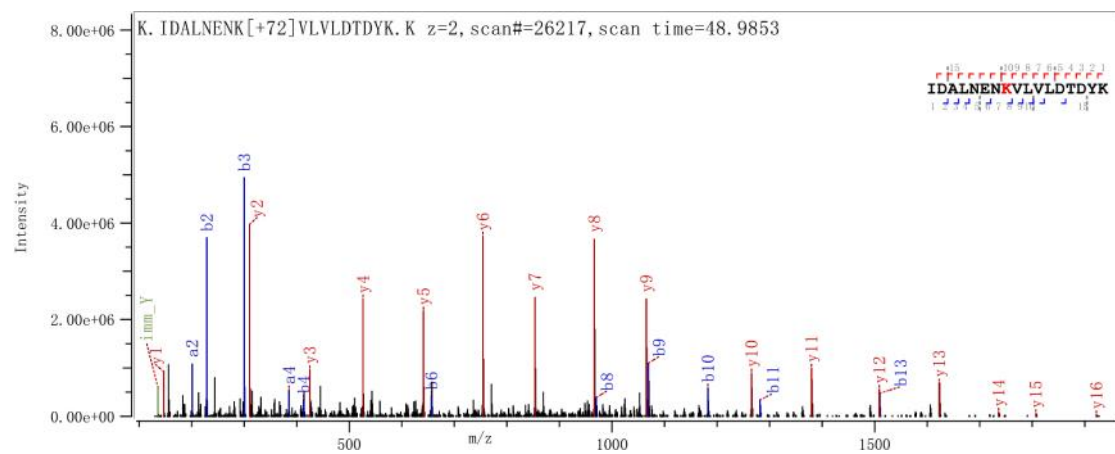


Figure 10.

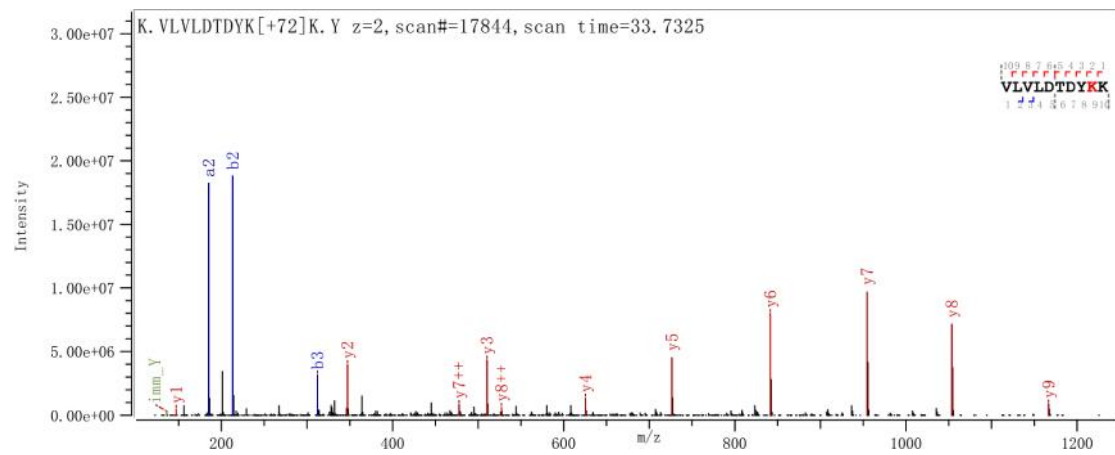


Figure 11.

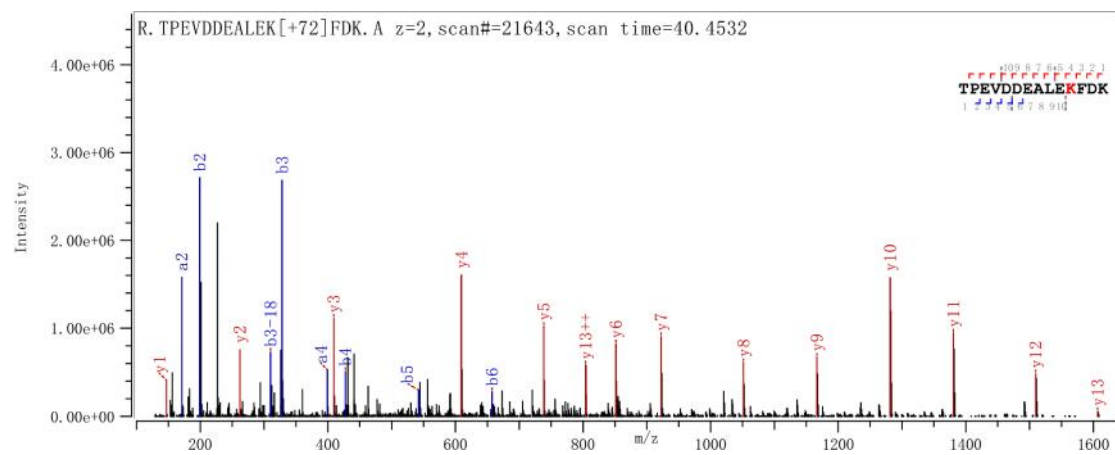


Figure 12.

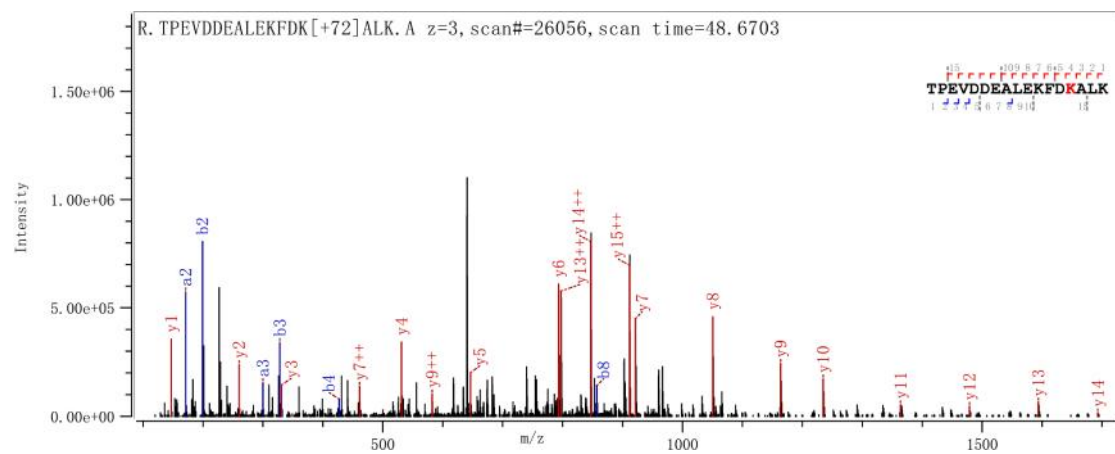


Figure 13.

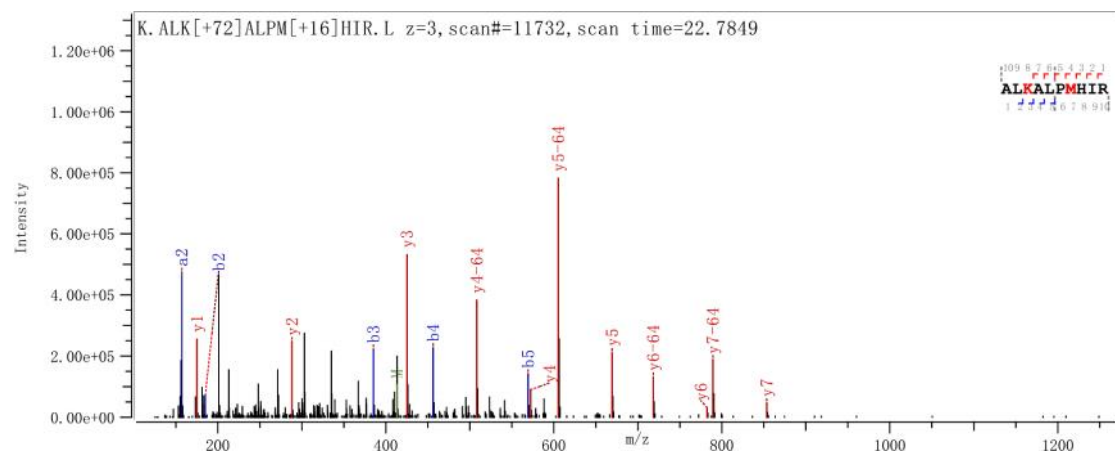


Figure 14.

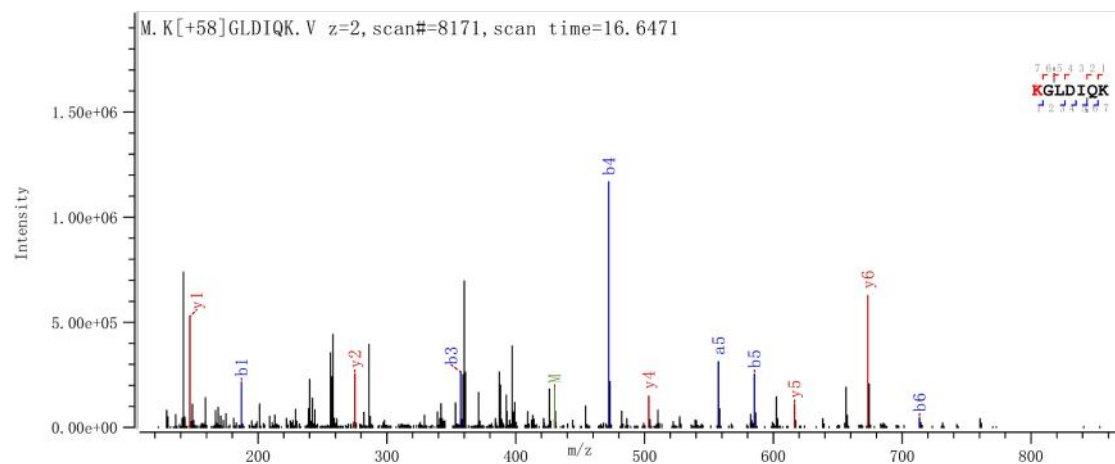


Figure 15.

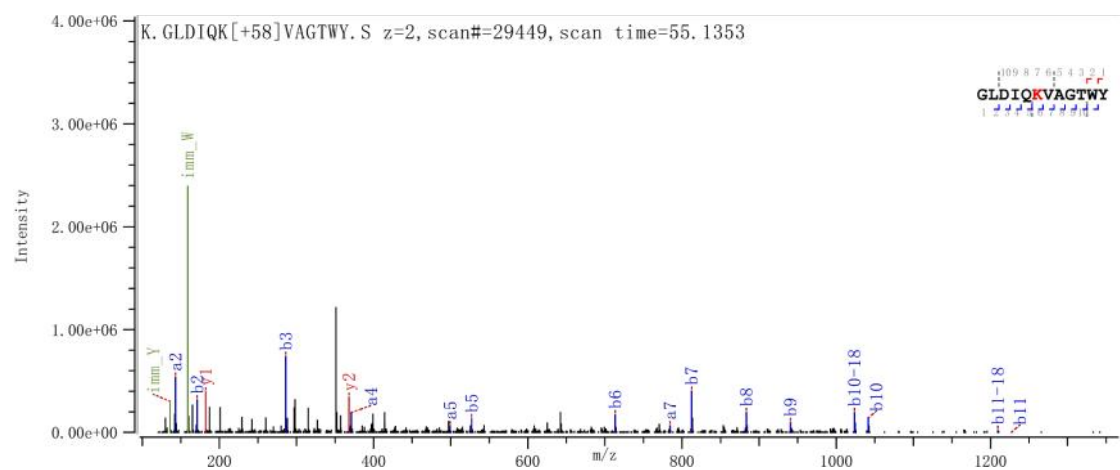


Figure 16.

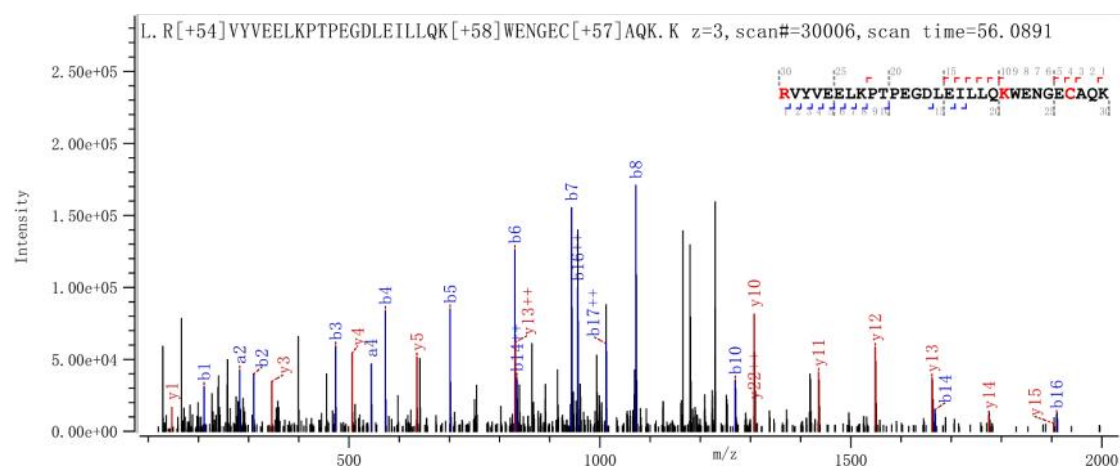


Figure 17.

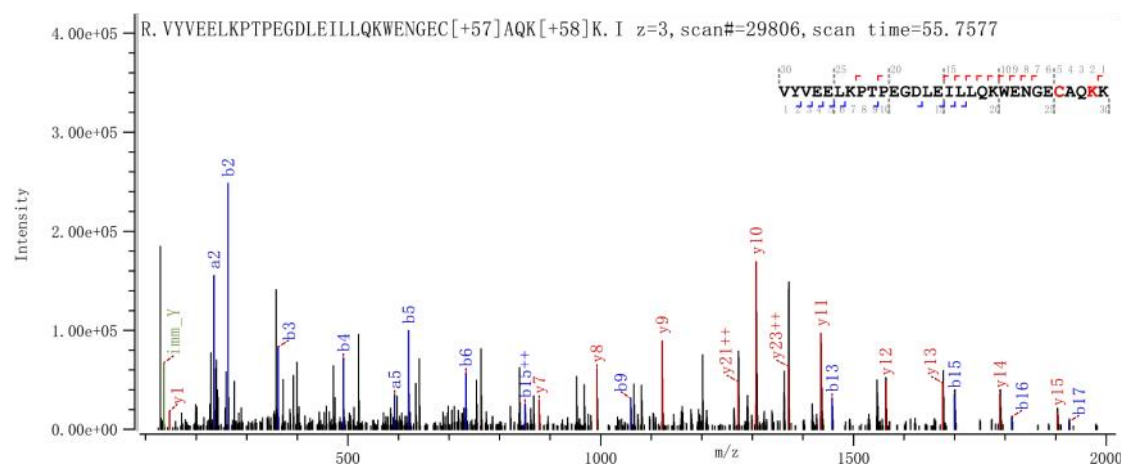


Figure 18.

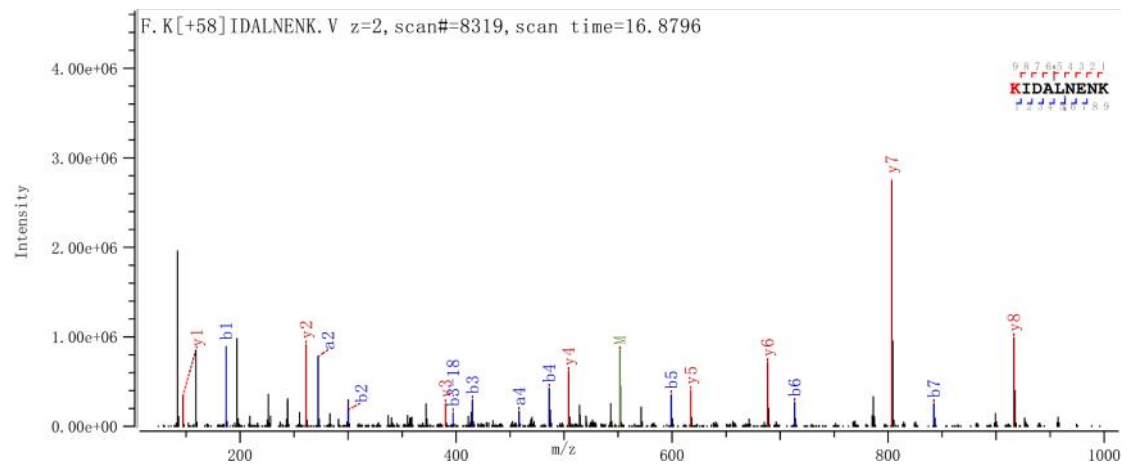


Figure 19.

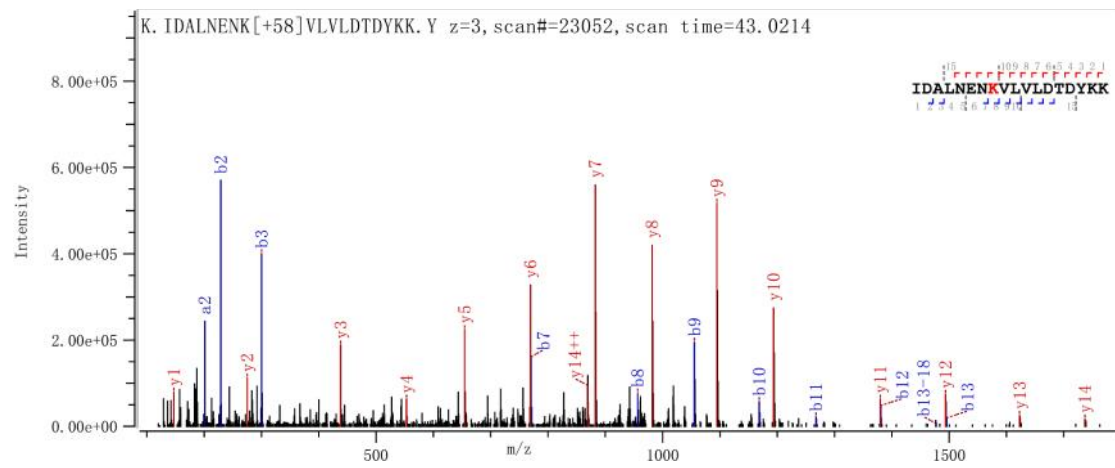


Figure 20.

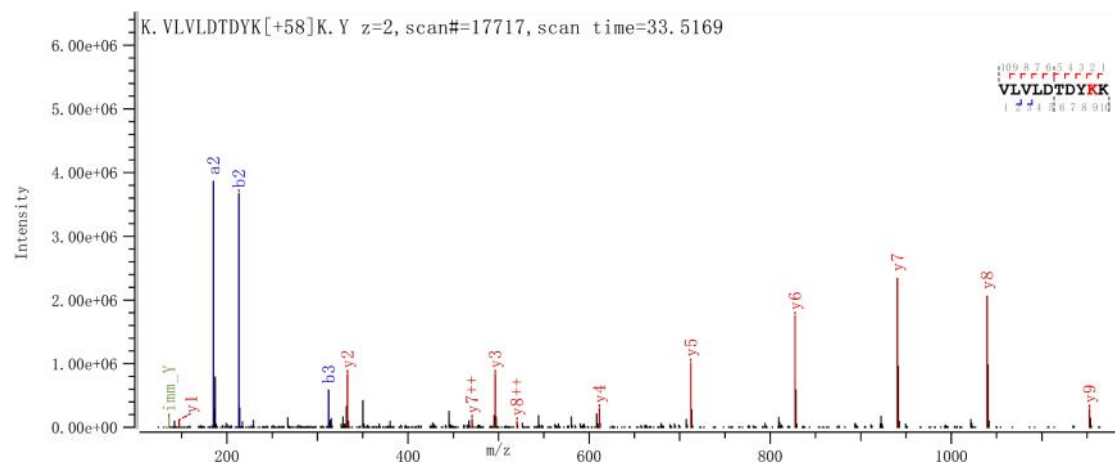


Figure 21.

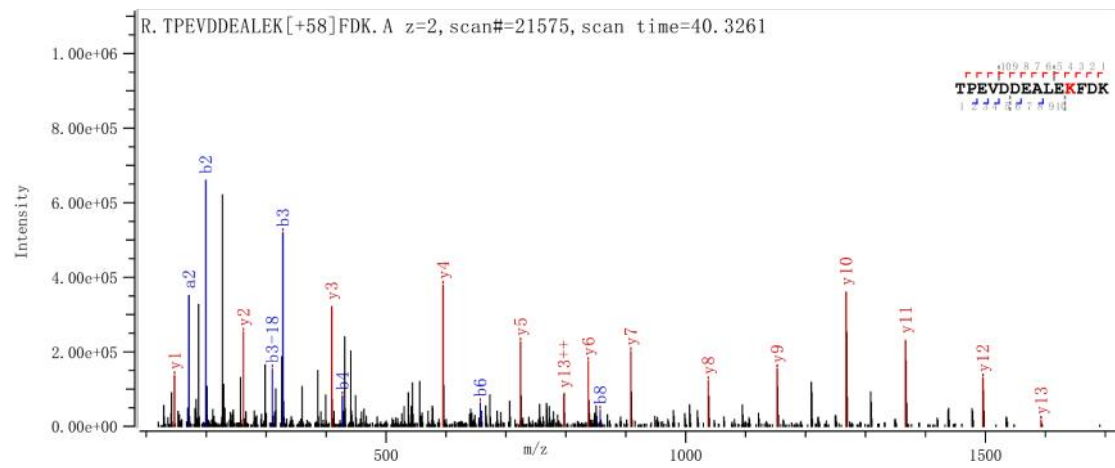


Figure 22.

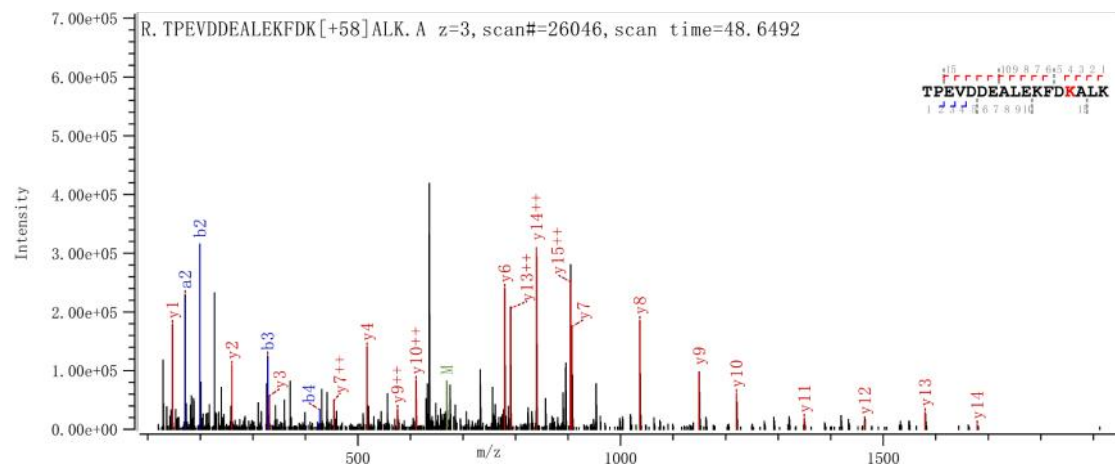


Figure 23.

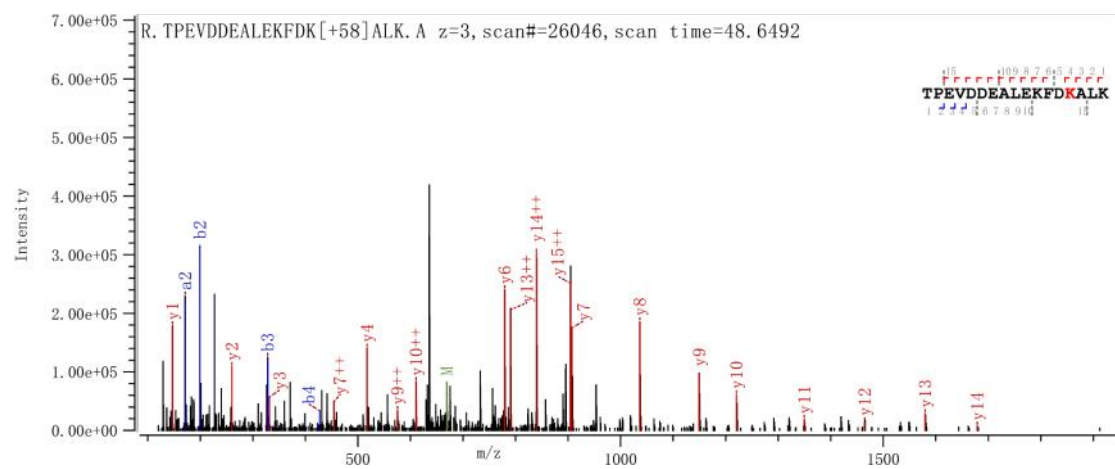


Figure 24.

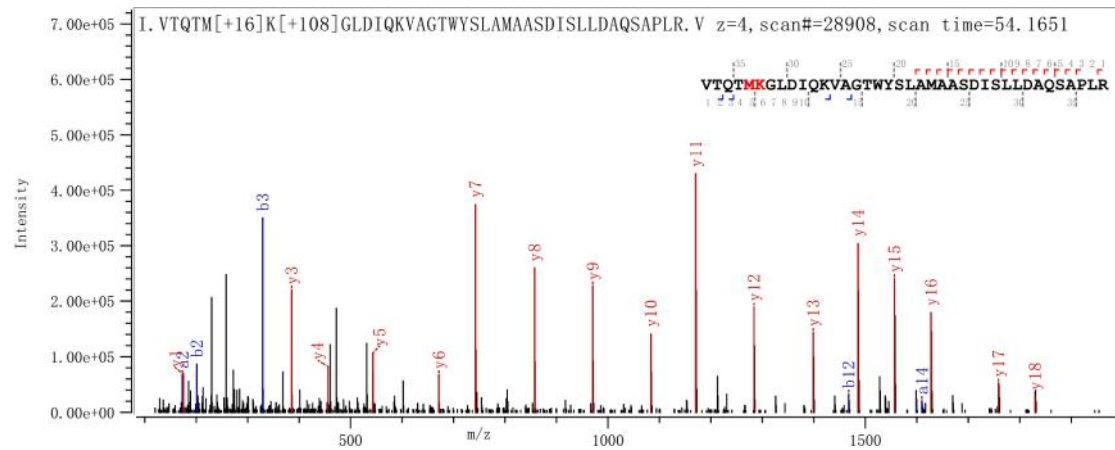


Figure 25.

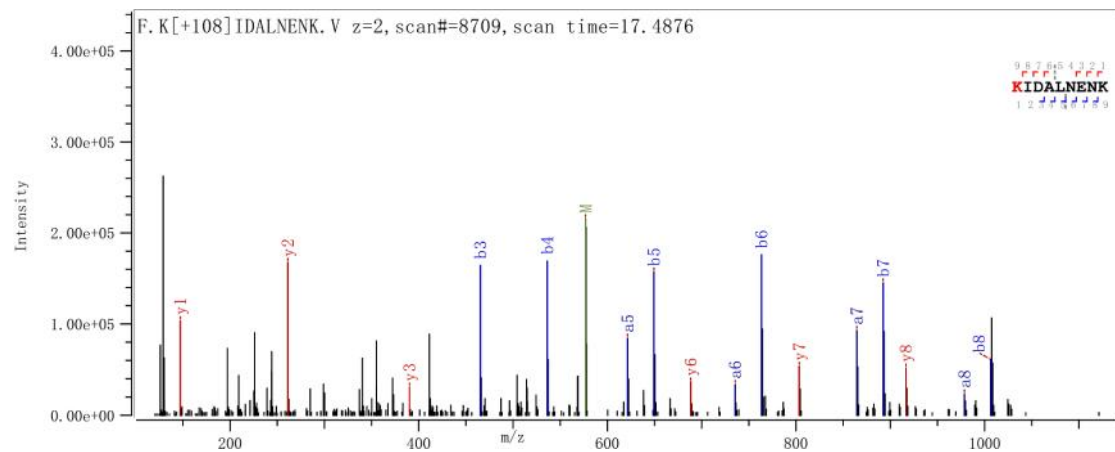


Figure 26.

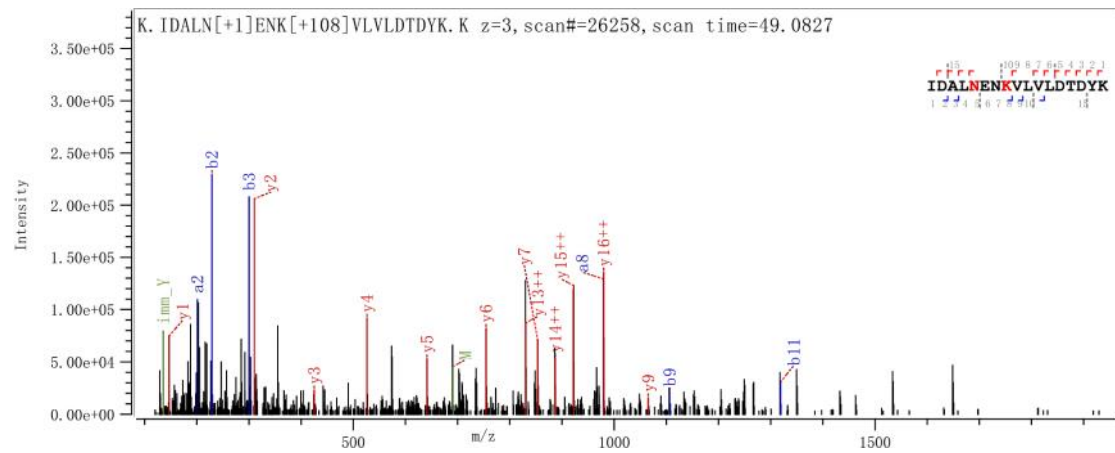


Figure 27.

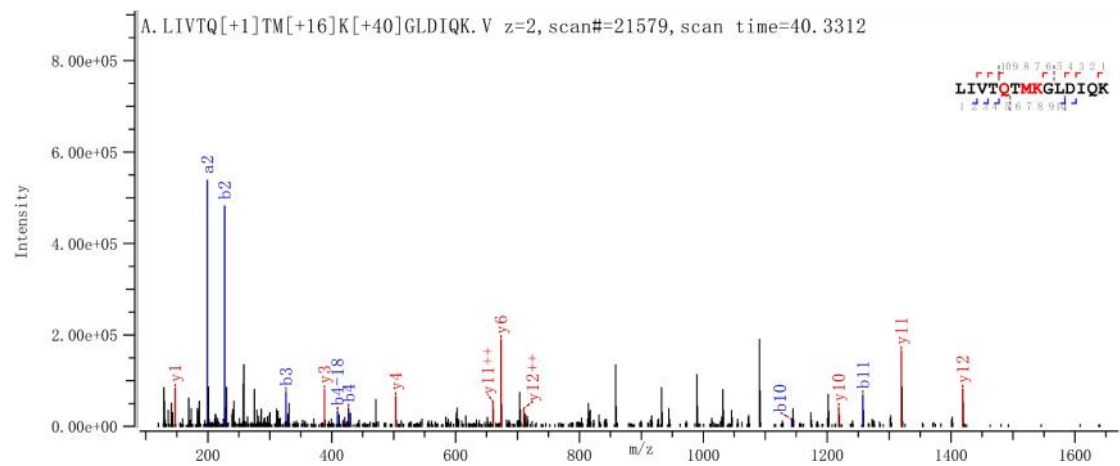


Figure 28.

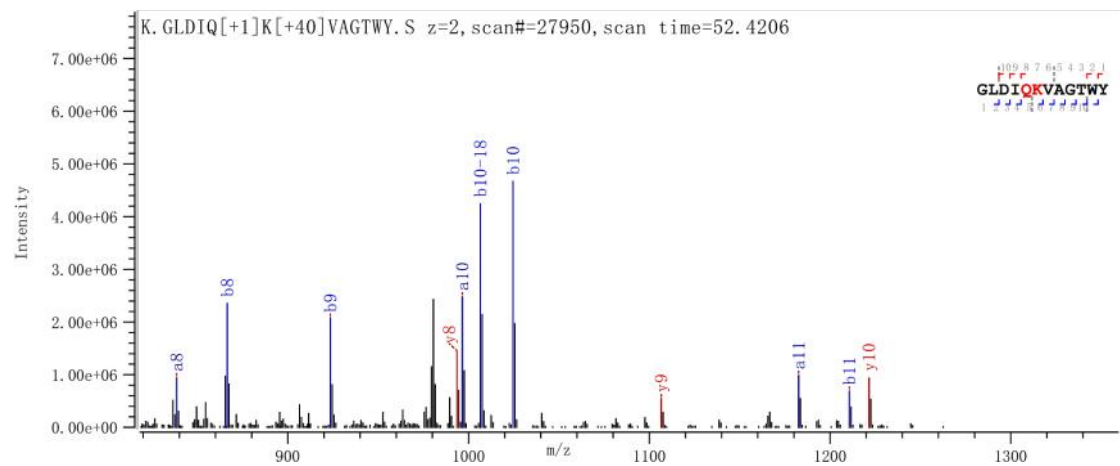


Figure 29.

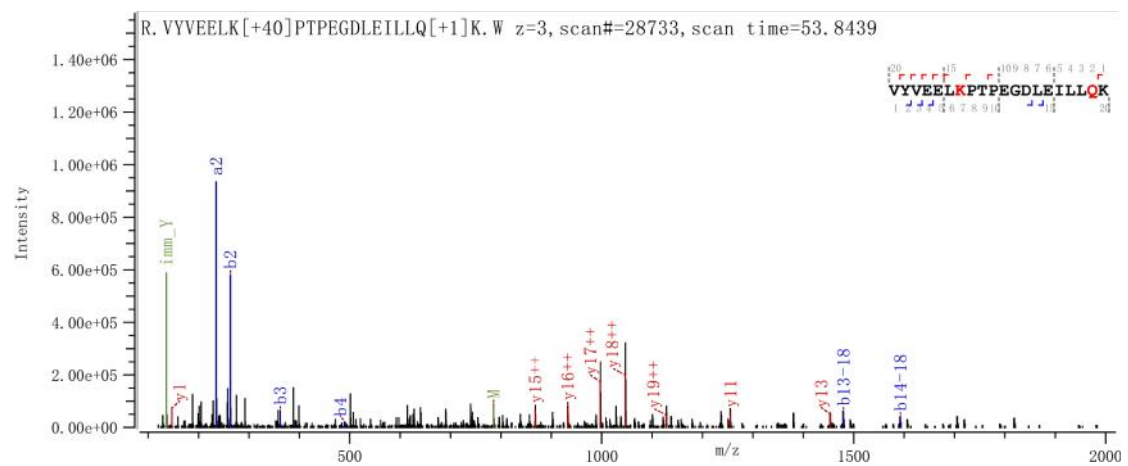


Figure 30.

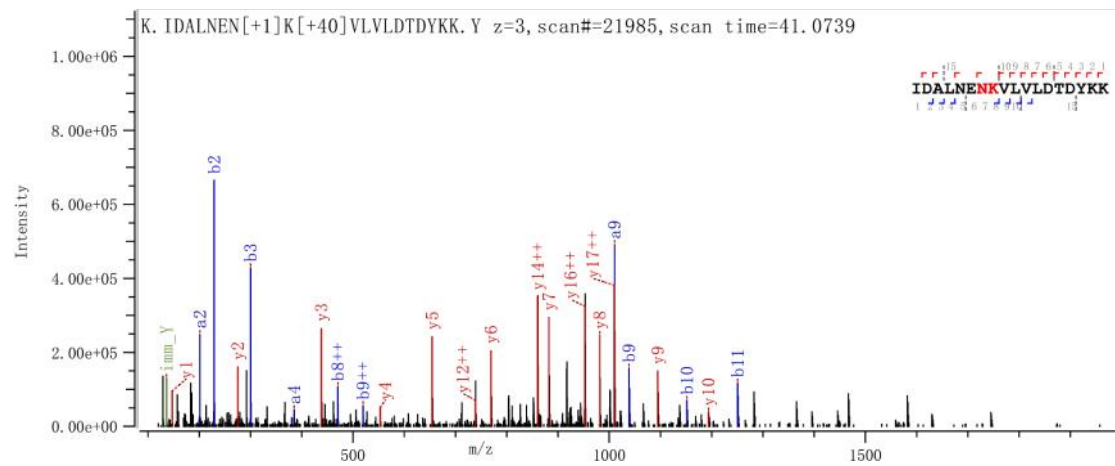


Figure 31.

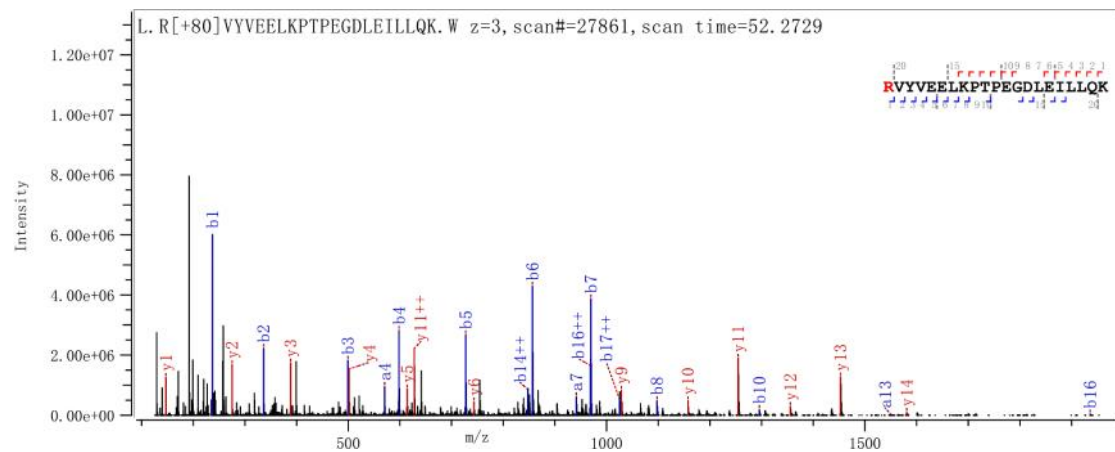


Figure 32.

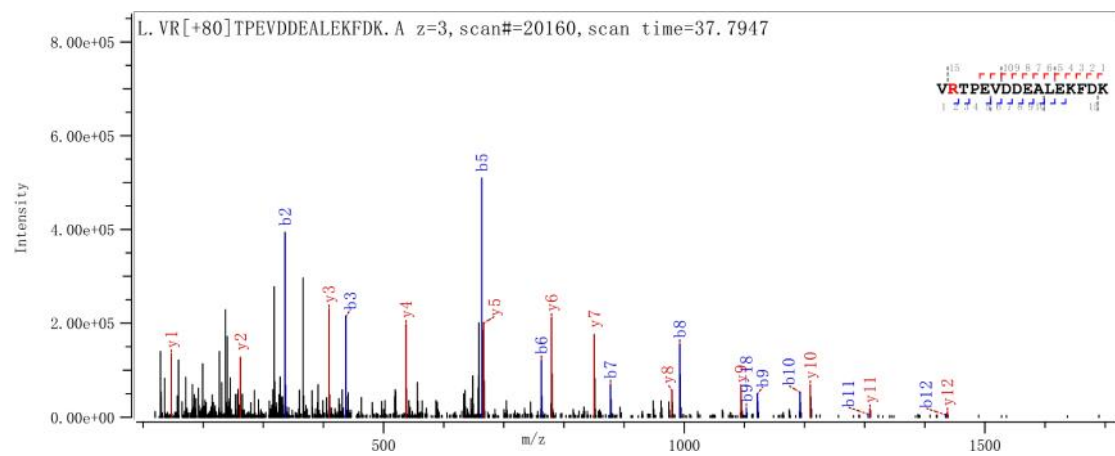


Figure 33.



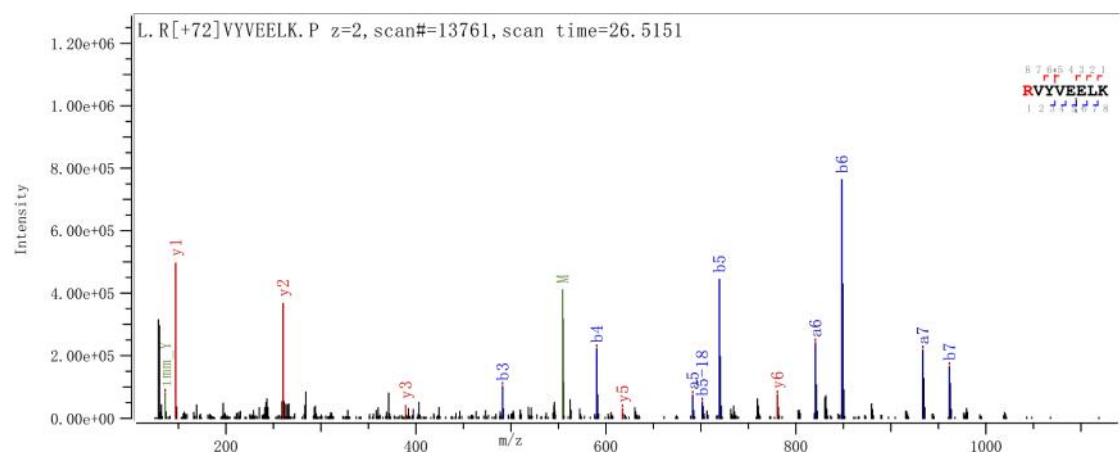


Figure 37.

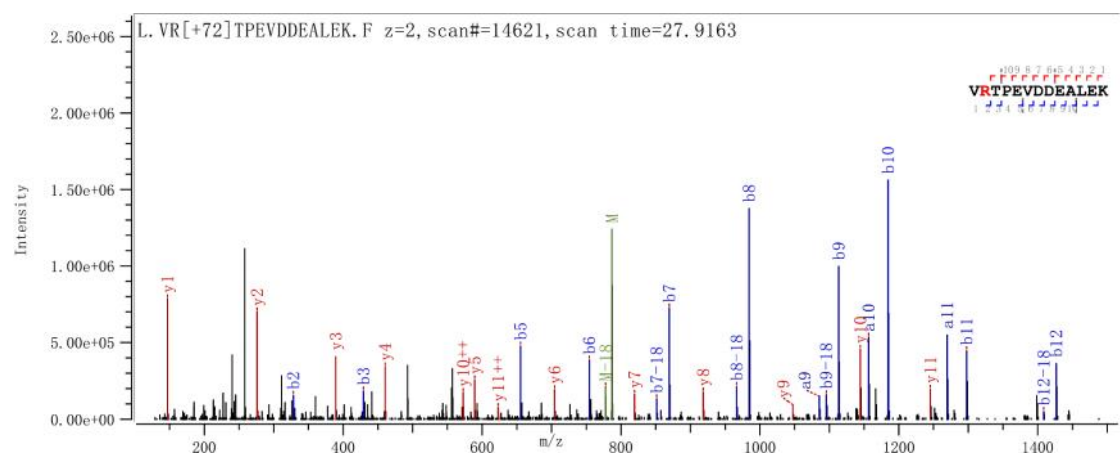
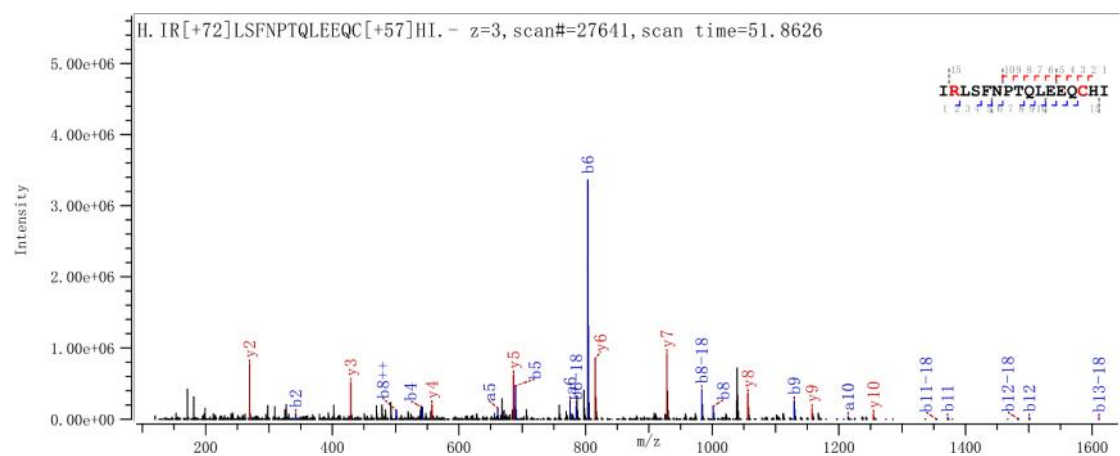


Figure 38.



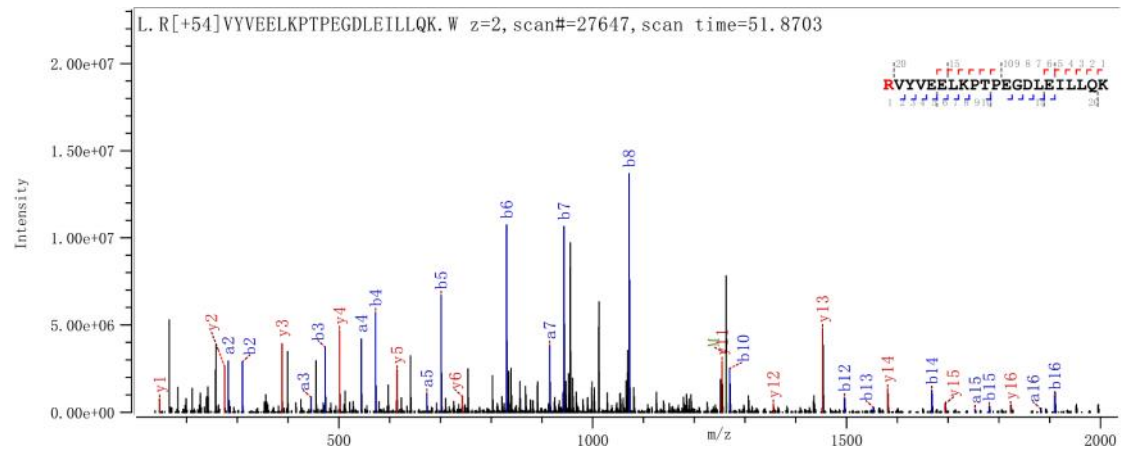


Figure 40.

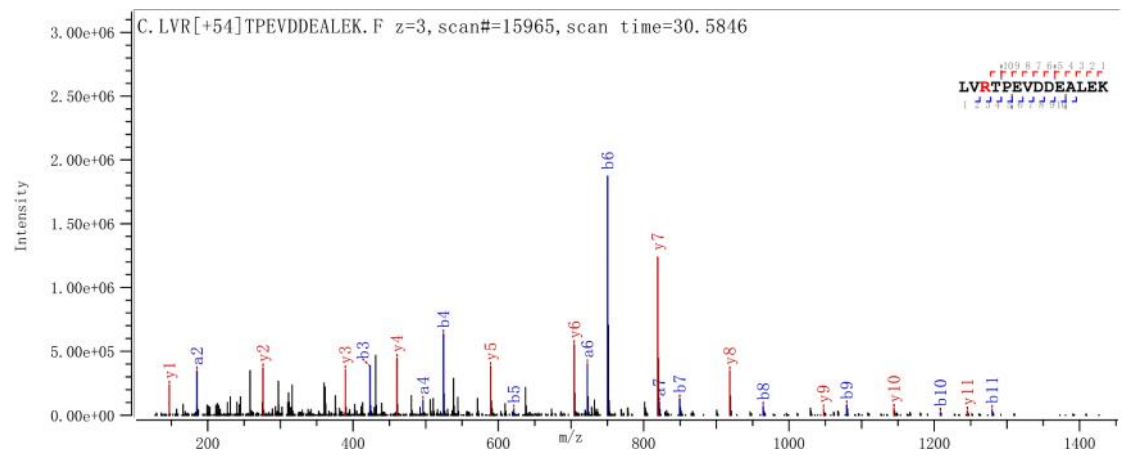


Figure 41.

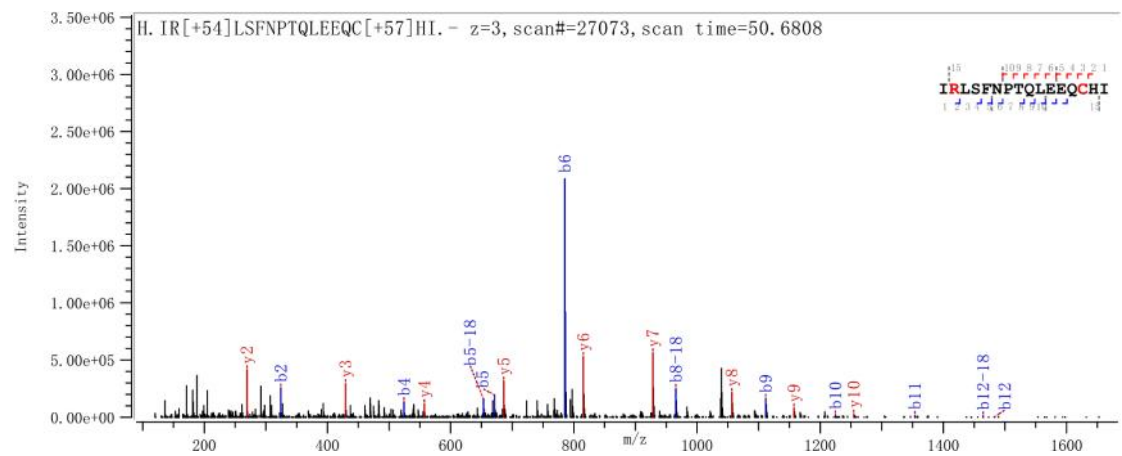


Figure 42.

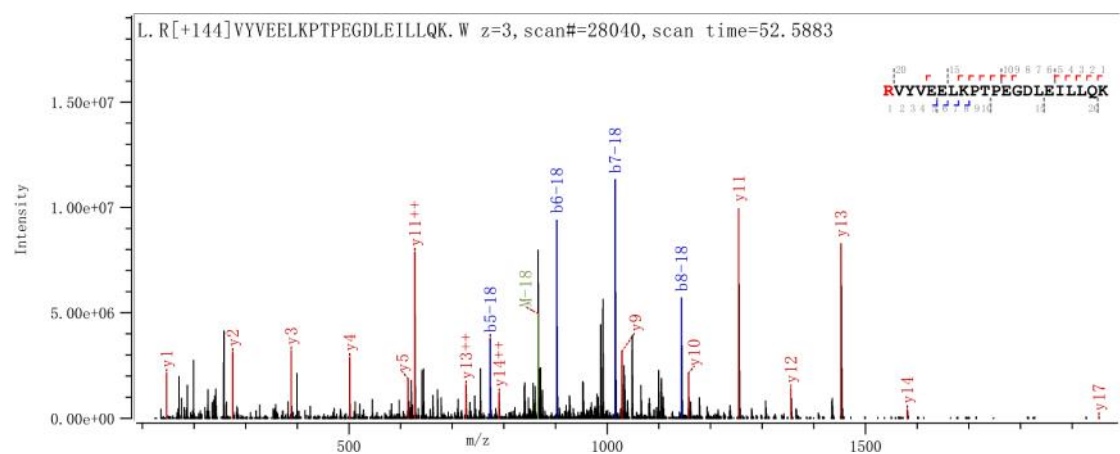


Figure 43.

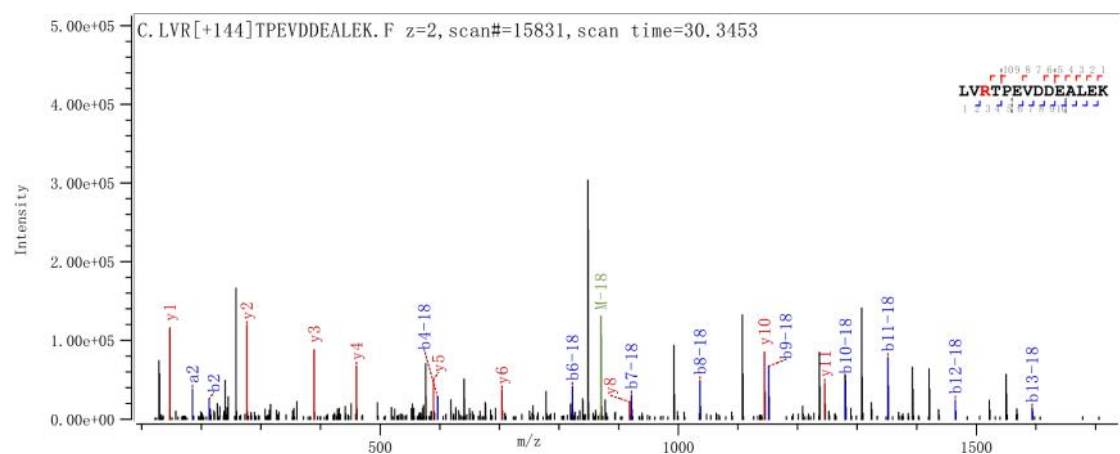


Figure 44.

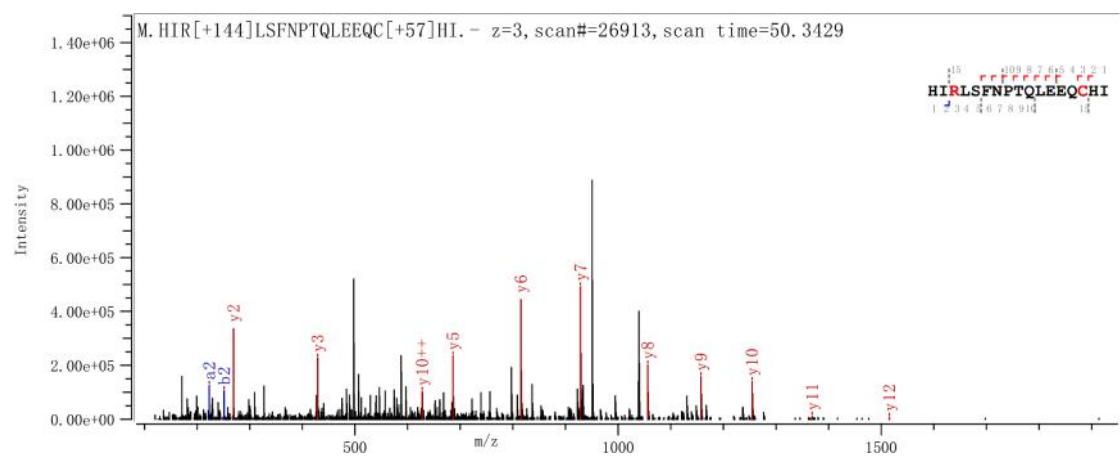


Figure 45.

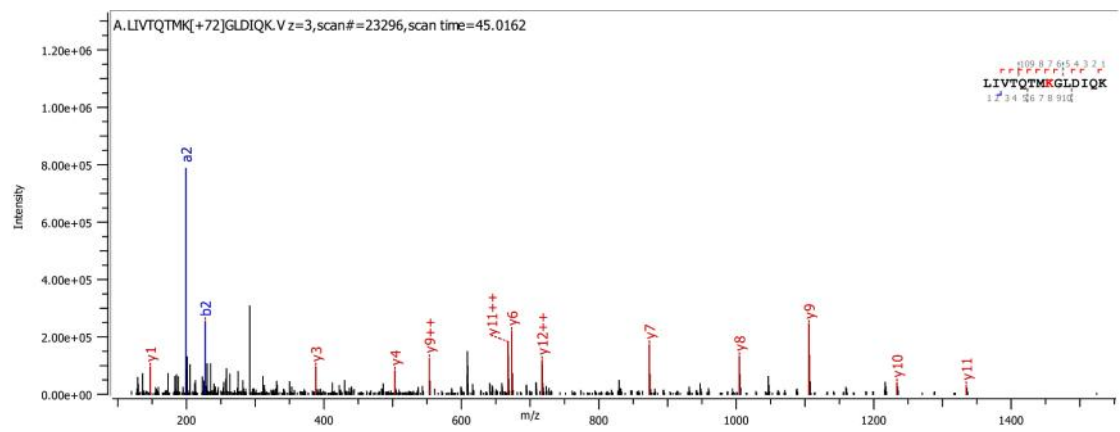


Figure 46.

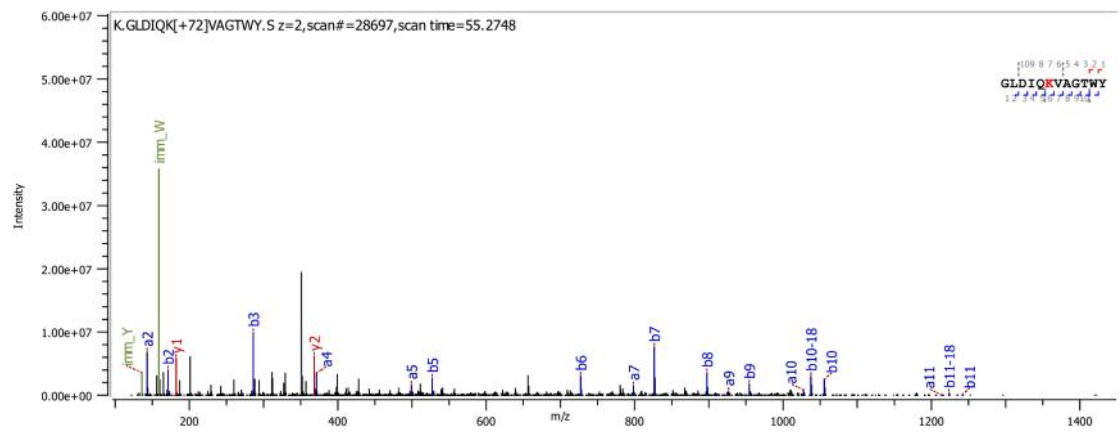


Figure 47.

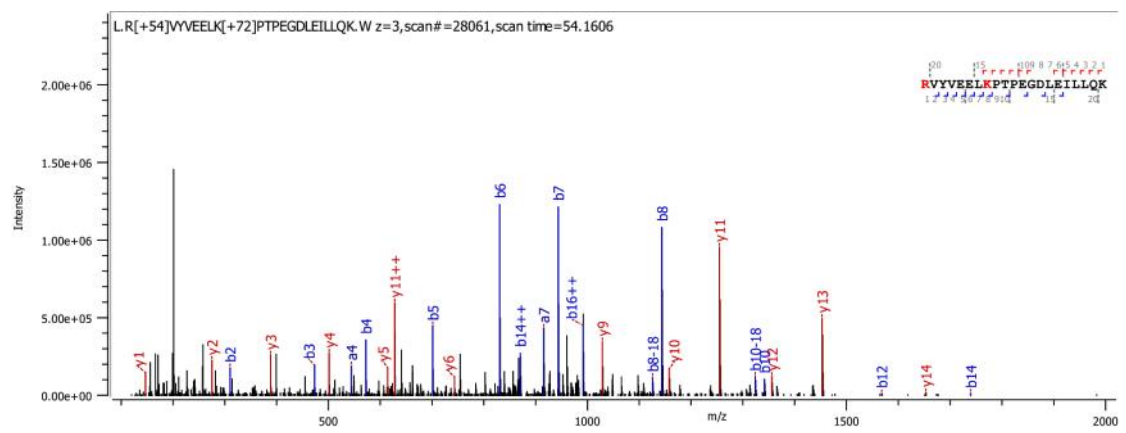


Figure 48.

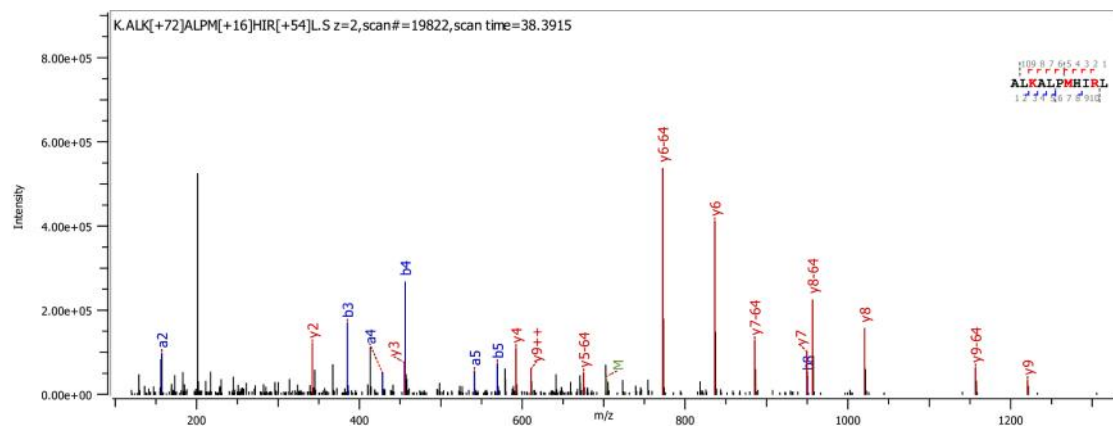


Figure 49.

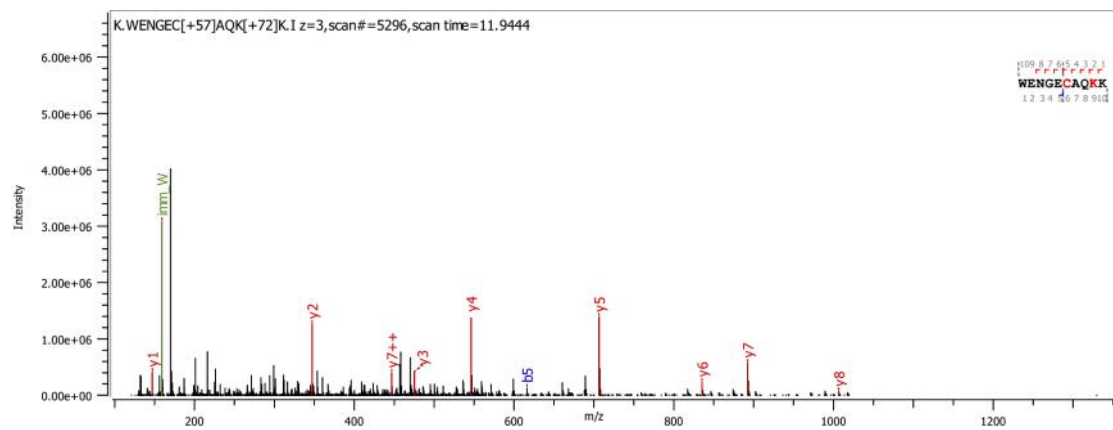


Figure 50.

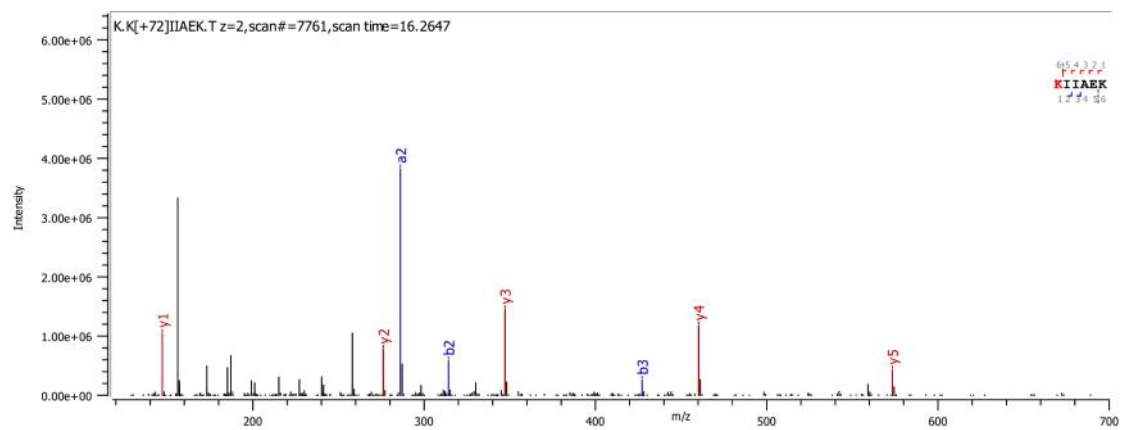


Figure 51.

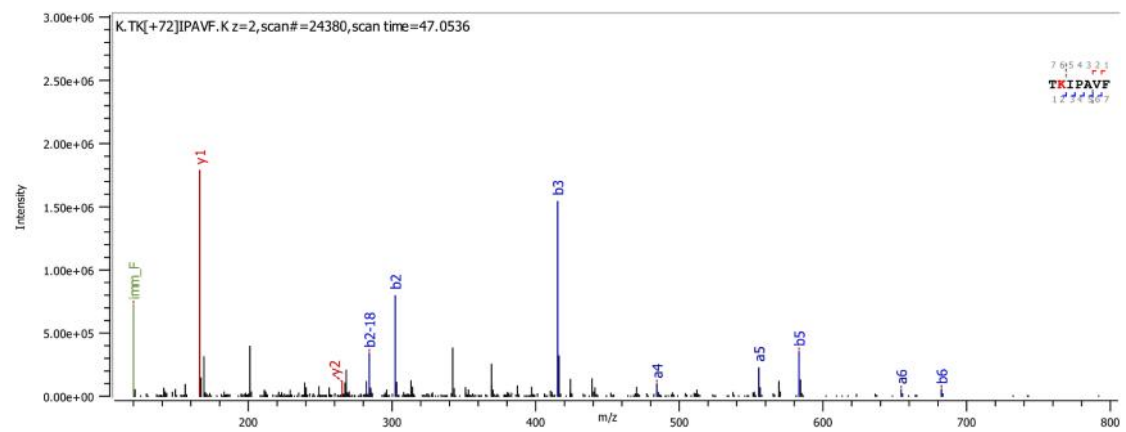


Figure 52.

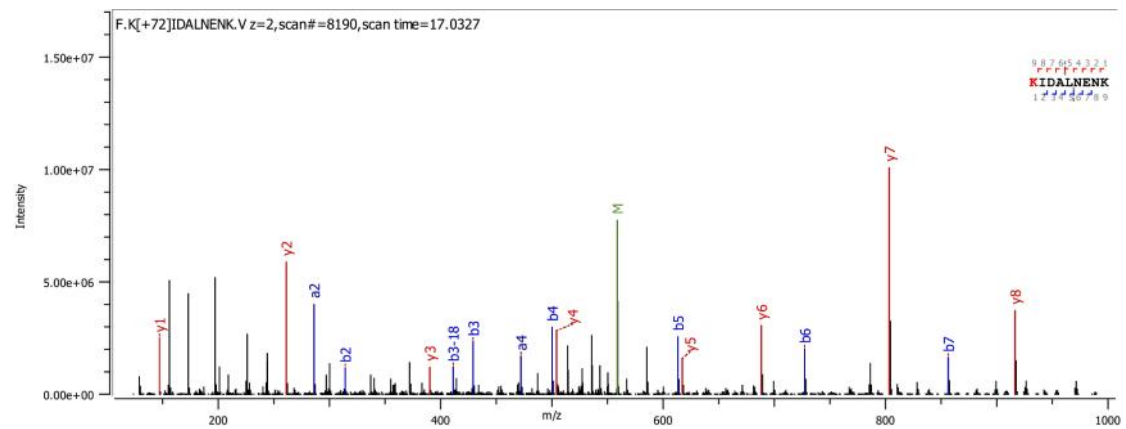


Figure 53.

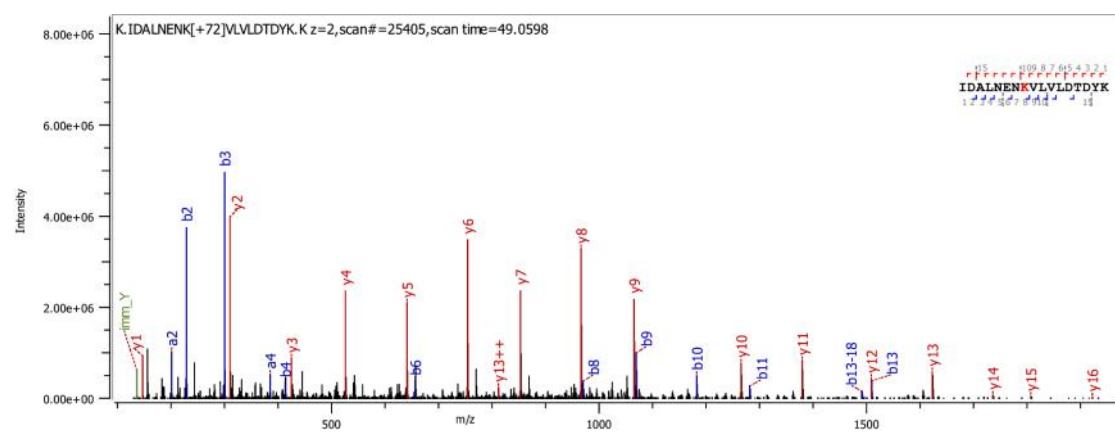


Figure 54.

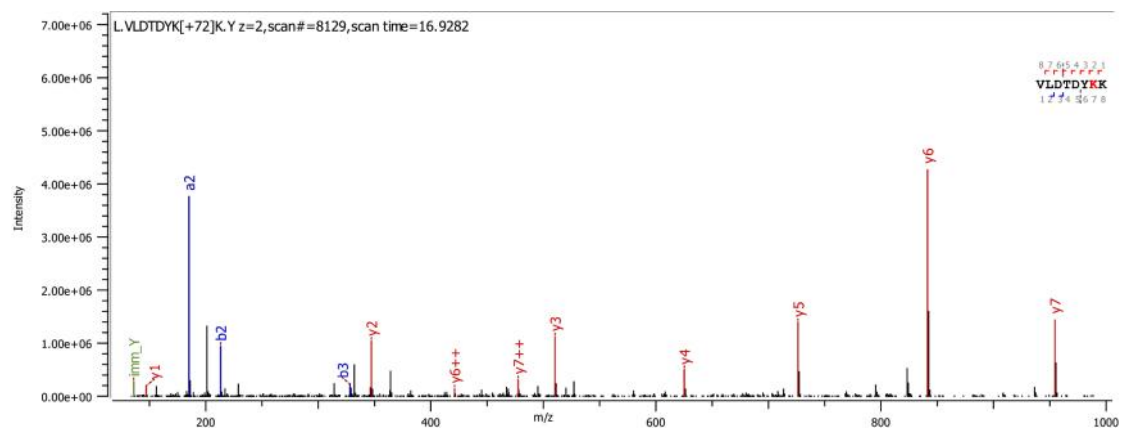


Figure 55.

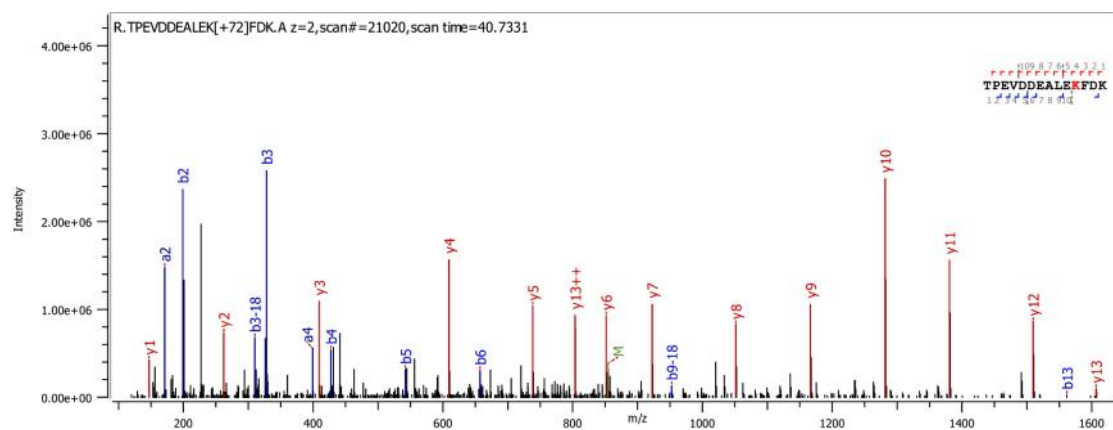


Figure 56.

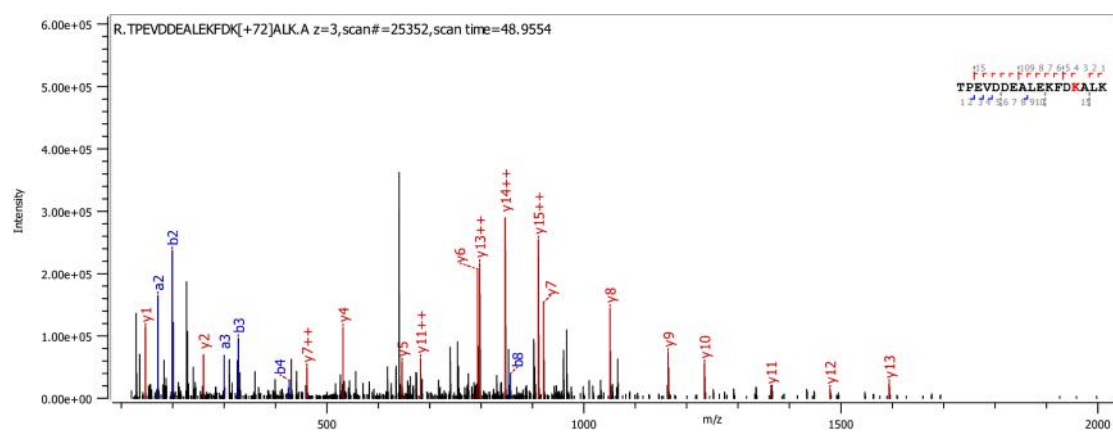


Figure 57.

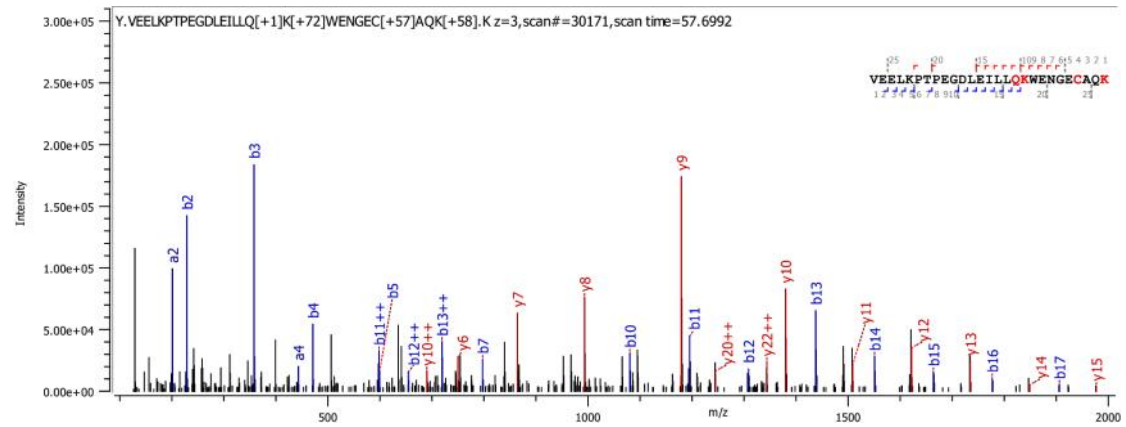


Figure 58.

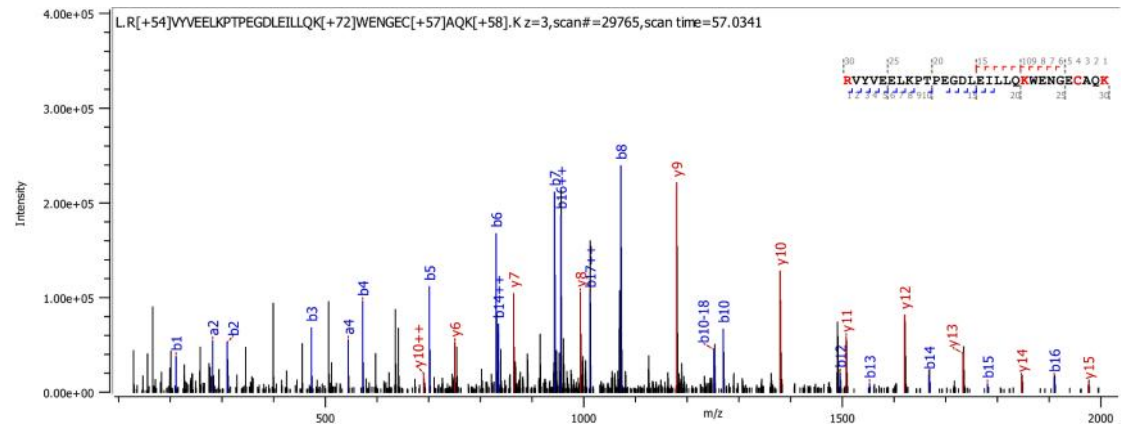


Figure 59.

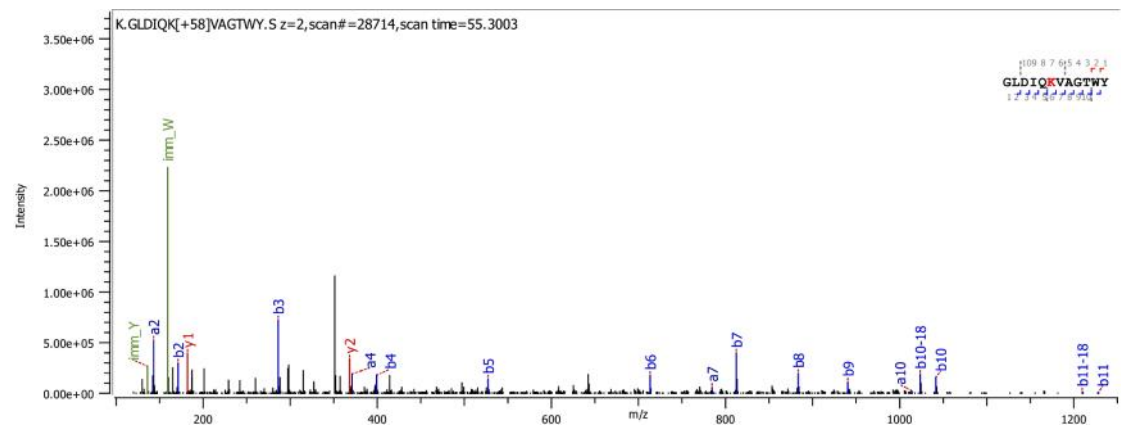


Figure 60.

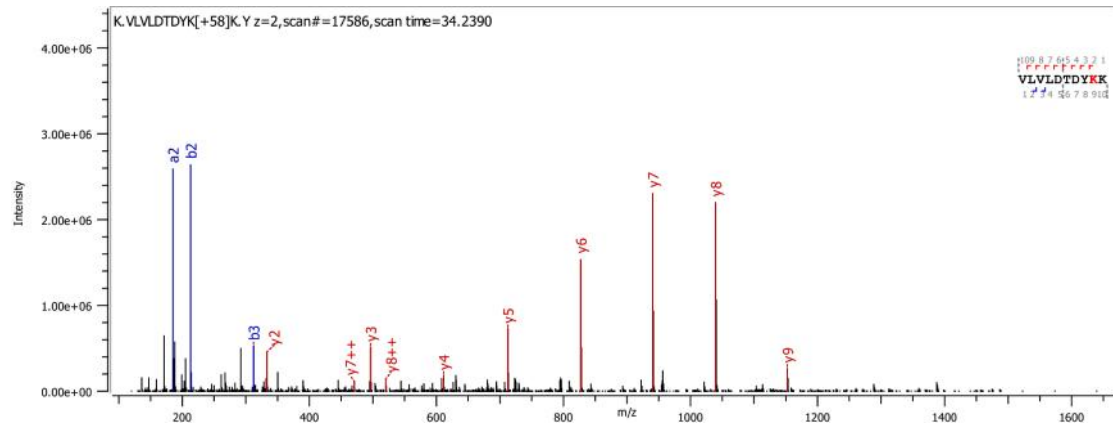


Figure 61.

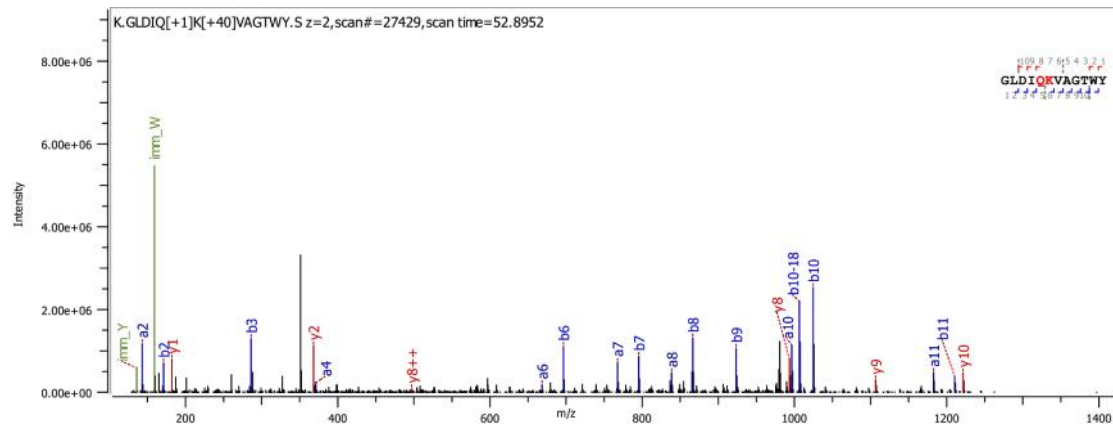


Figure 62.

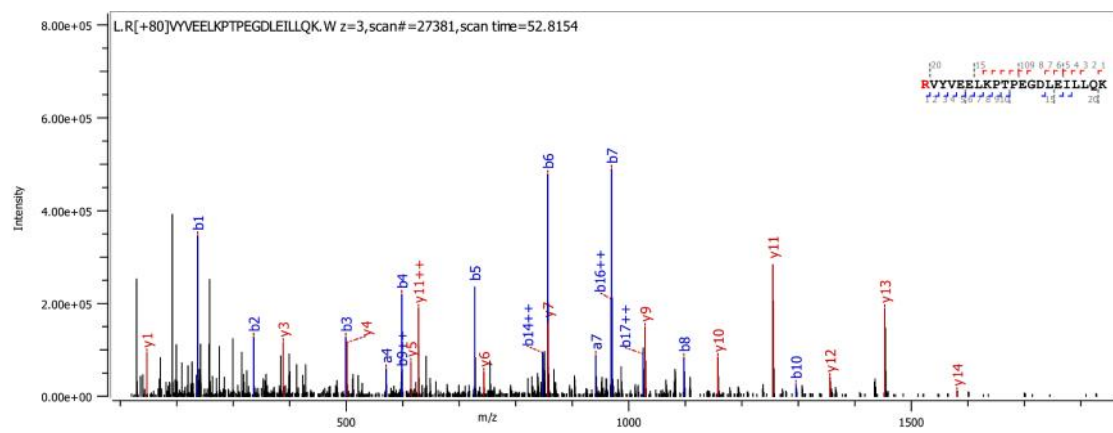


Figure 63.

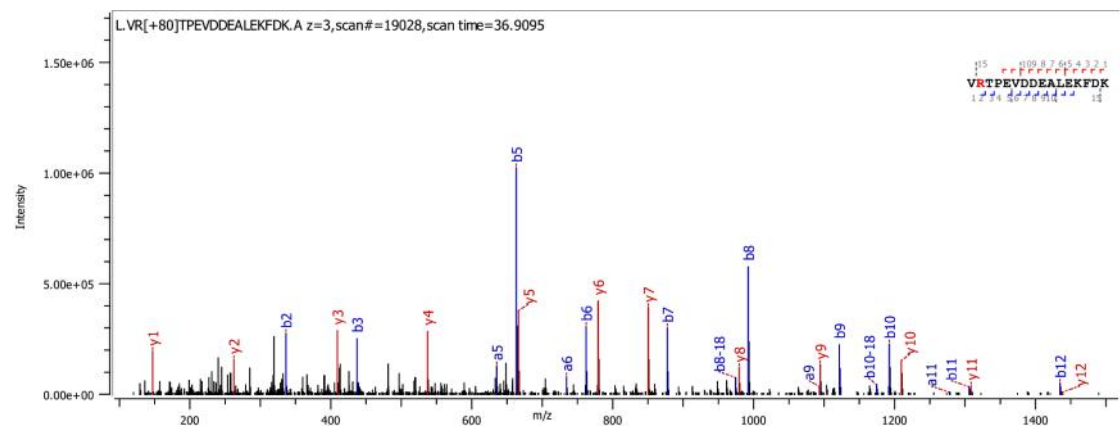


Figure 64.

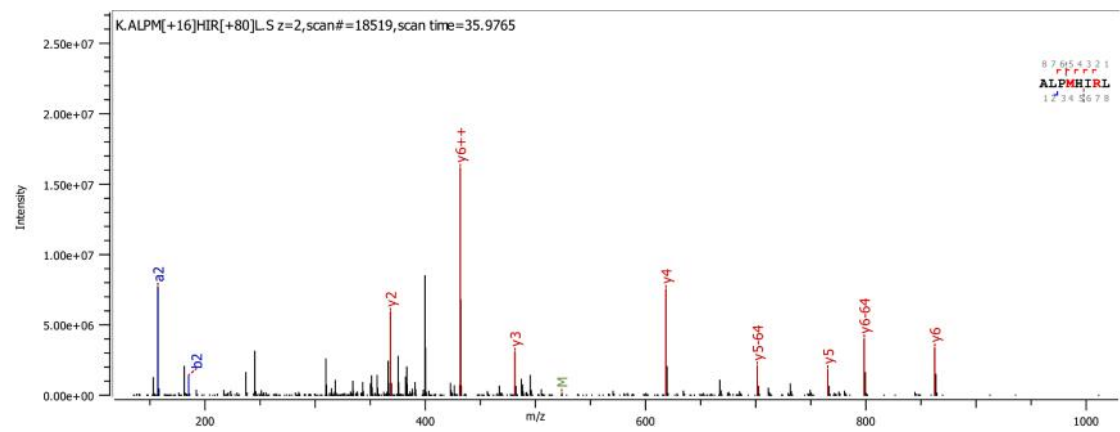


Figure 65.

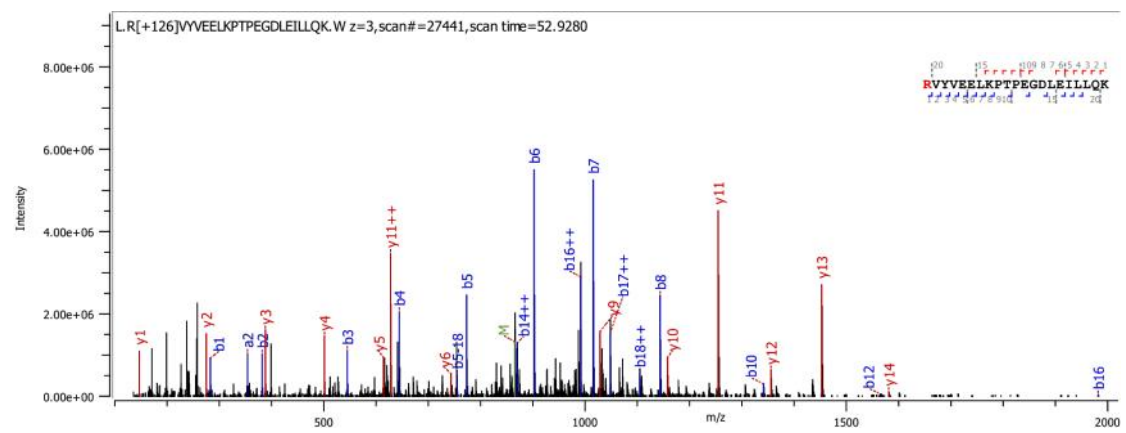


Figure 66.

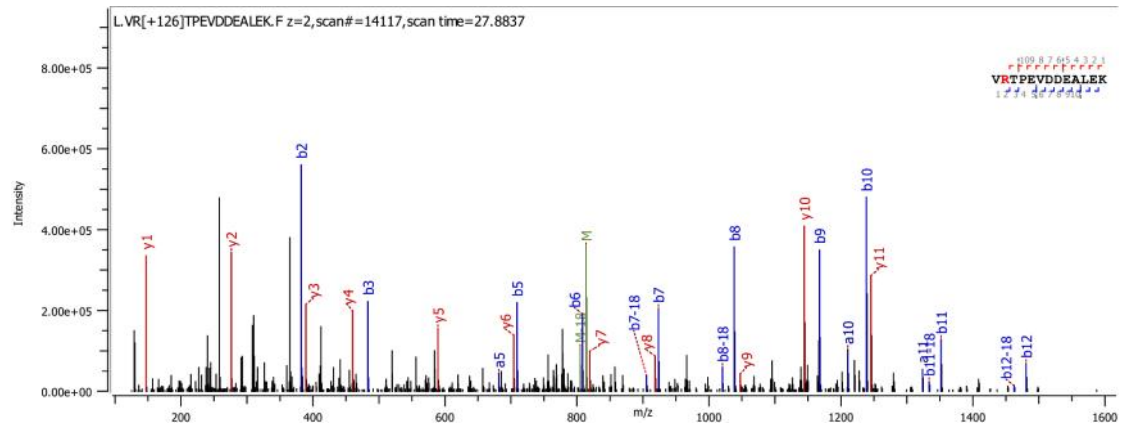


Figure 67.

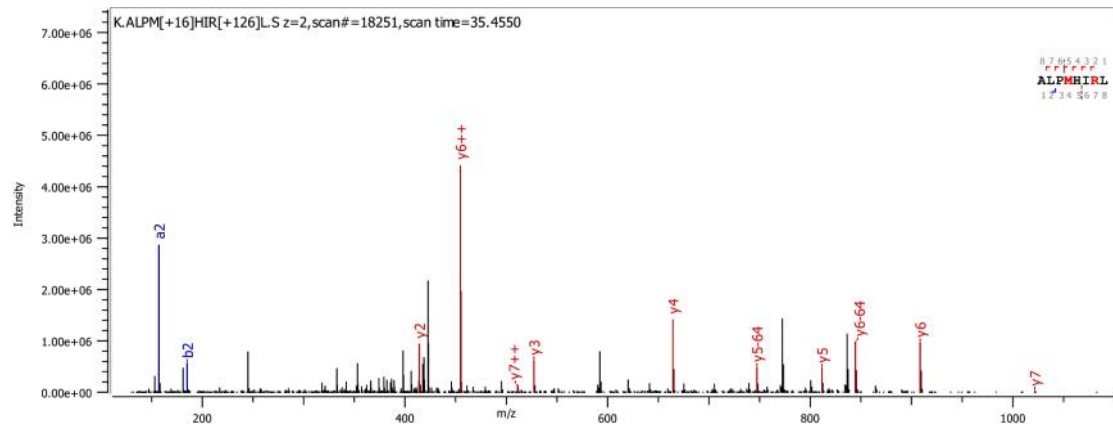


Figure 68.

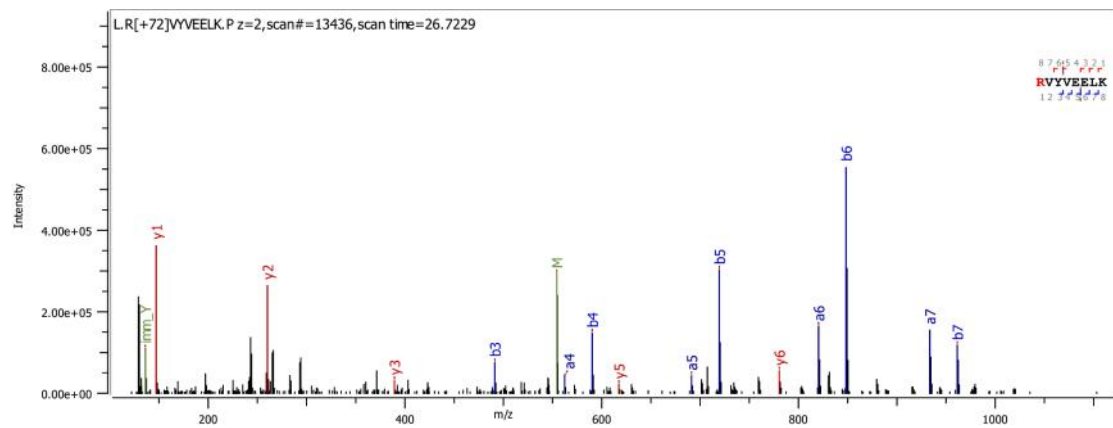
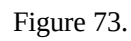


Figure 69.





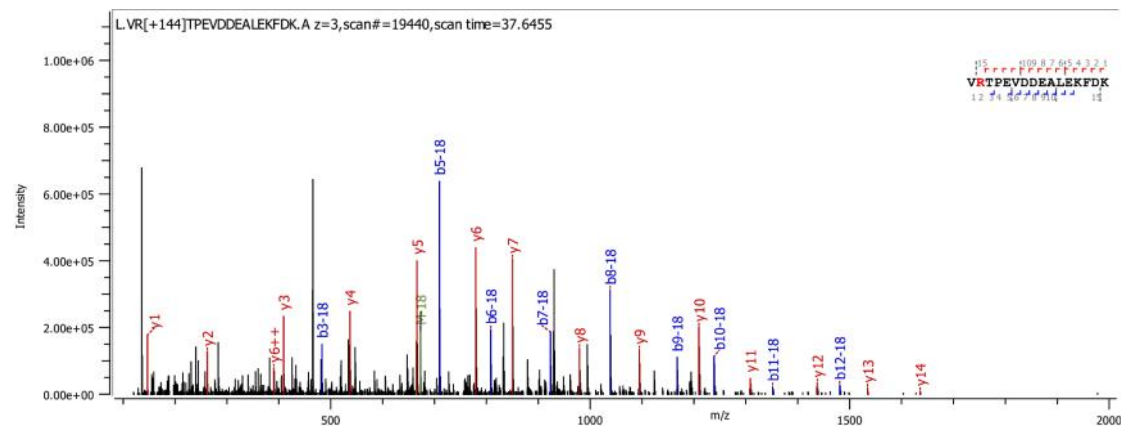


Figure 76.

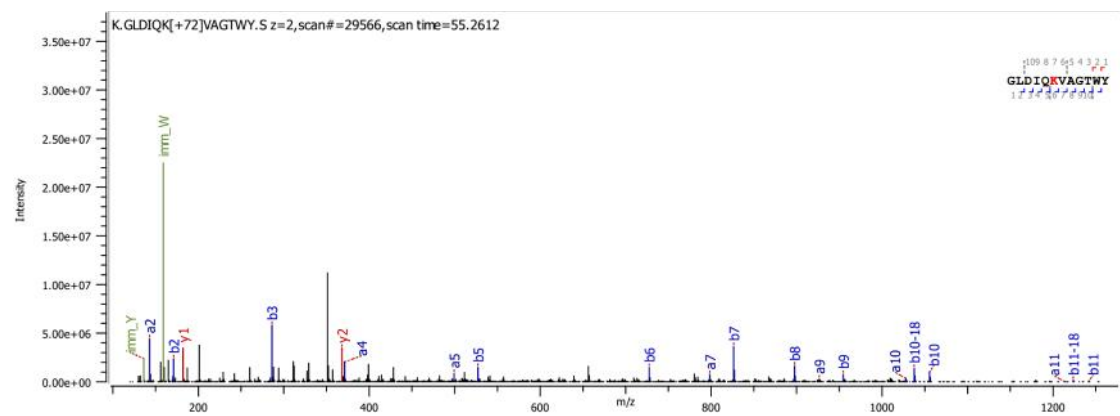


Figure 77.

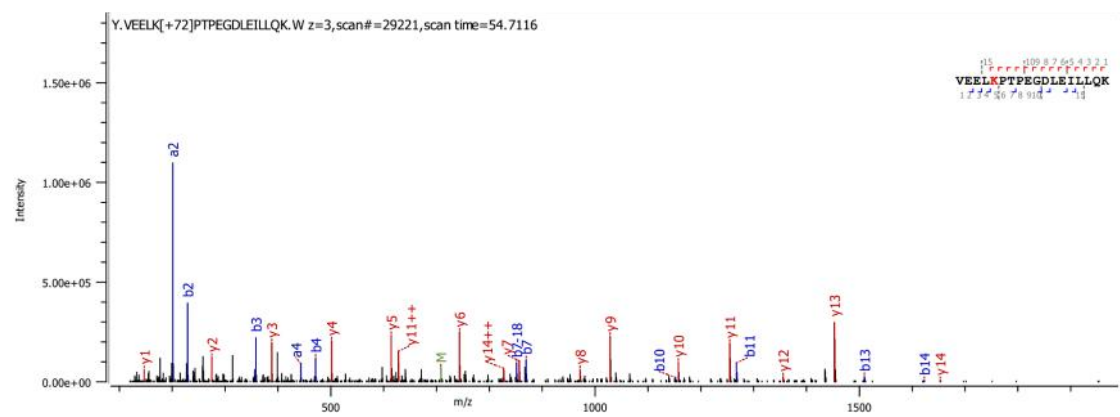


Figure 78.

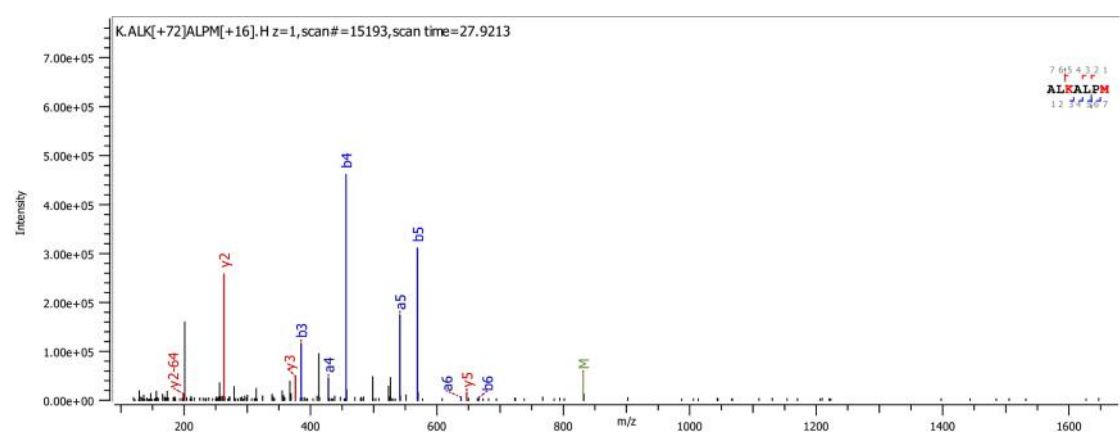


Figure 79

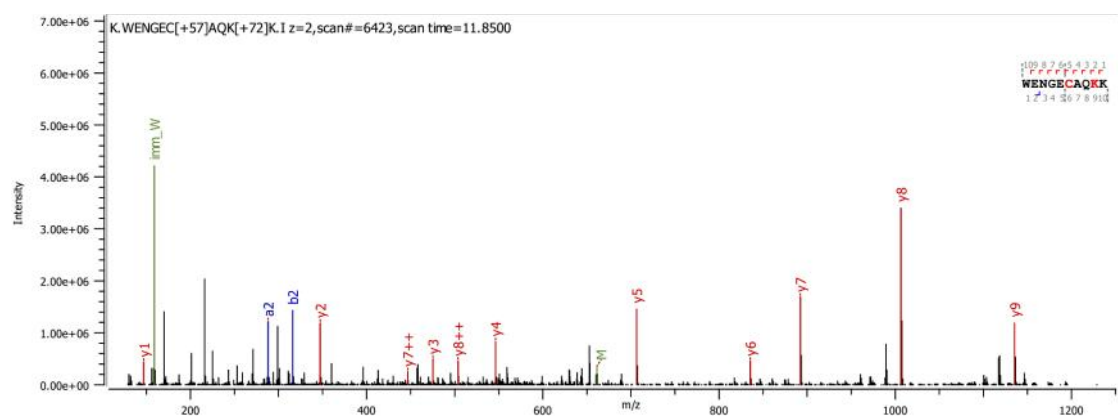


Figure 80.

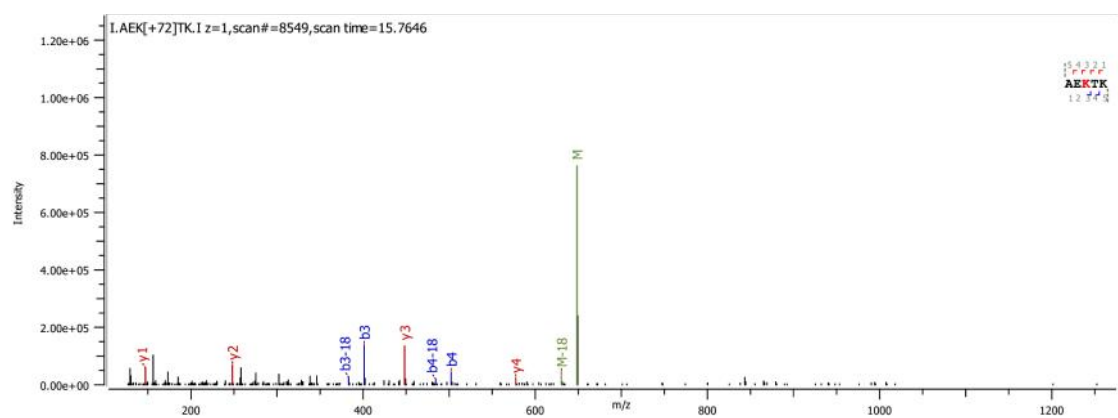


Figure 81.

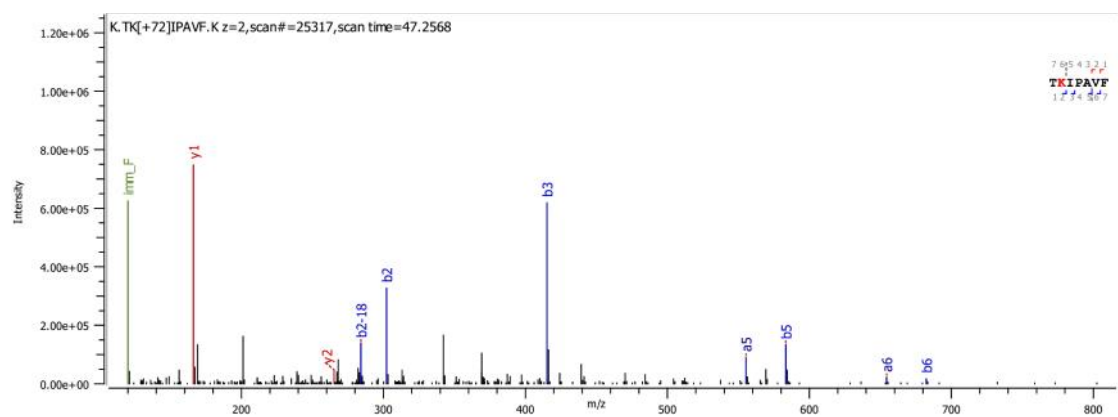


Figure 82.

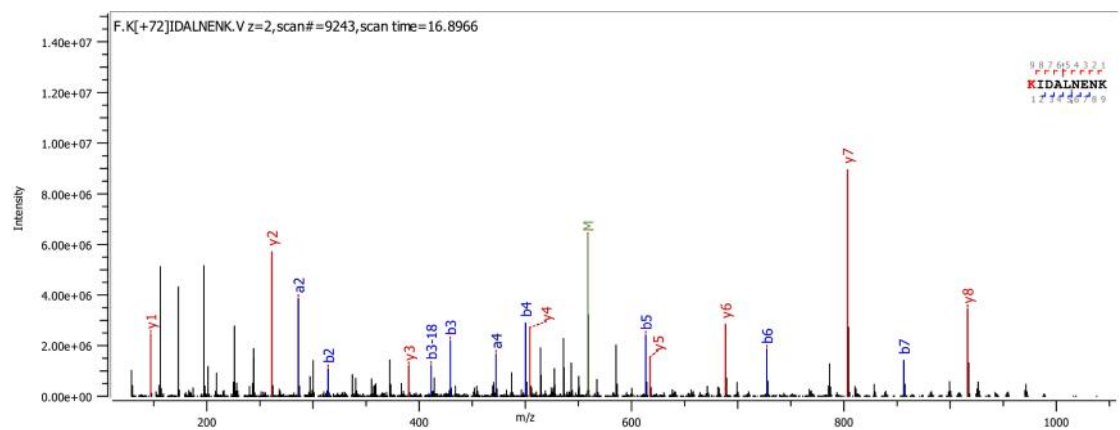


Figure 83.

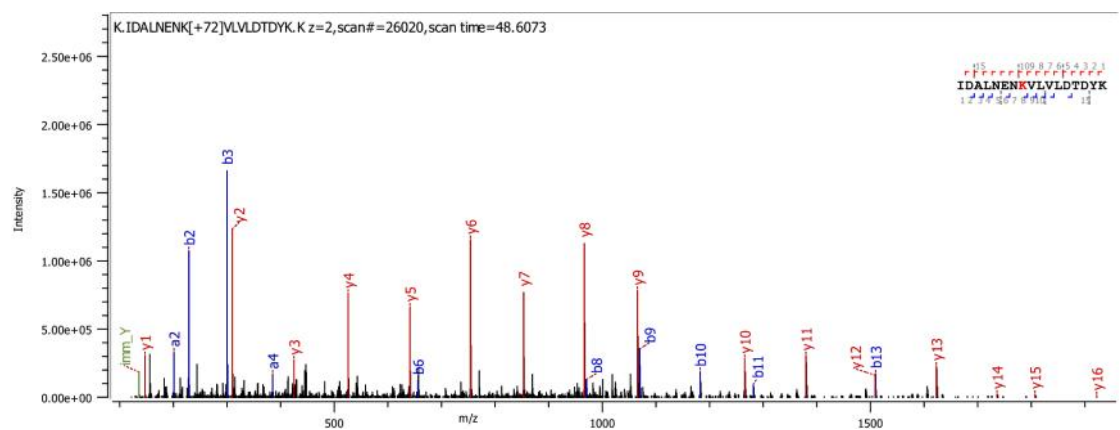


Figure 84.

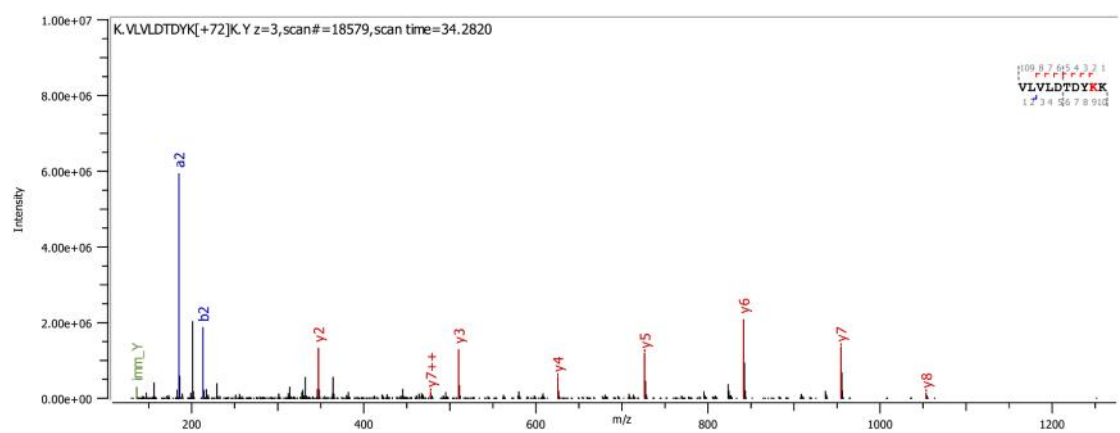


Figure 85.

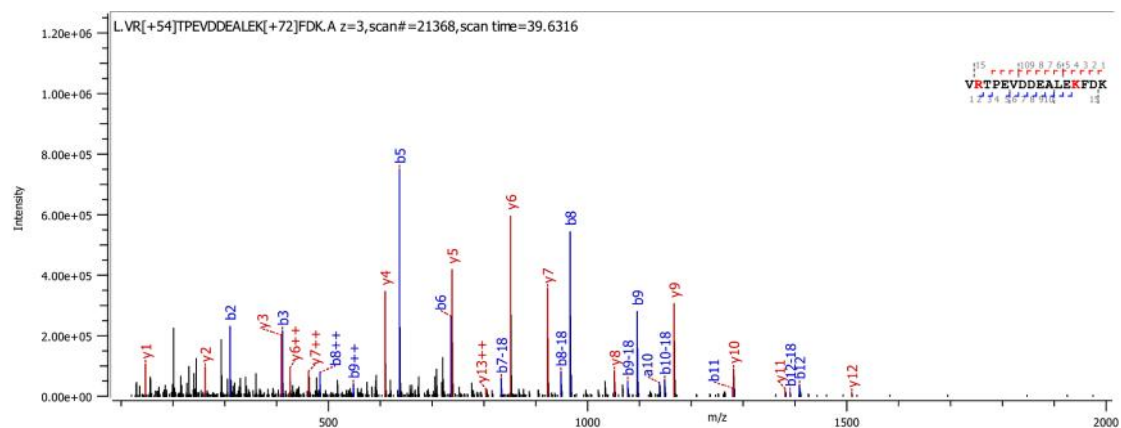


Figure 86.

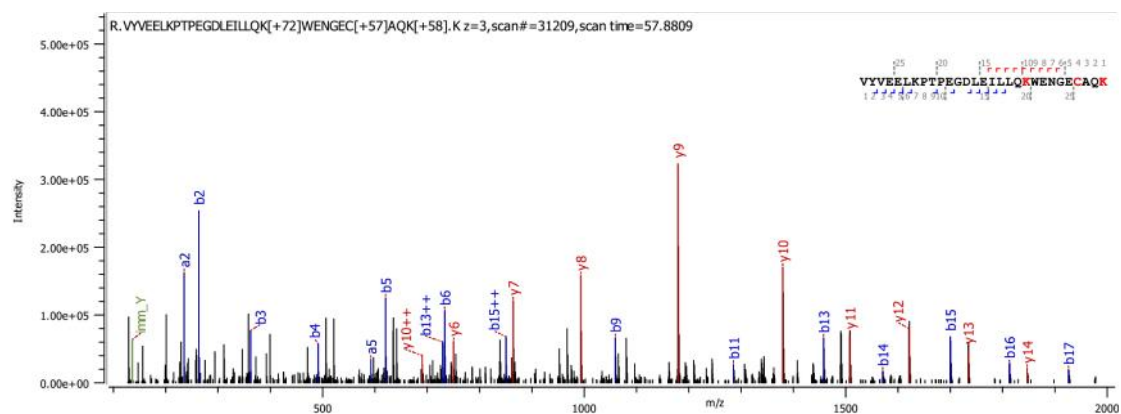


Figure 87.

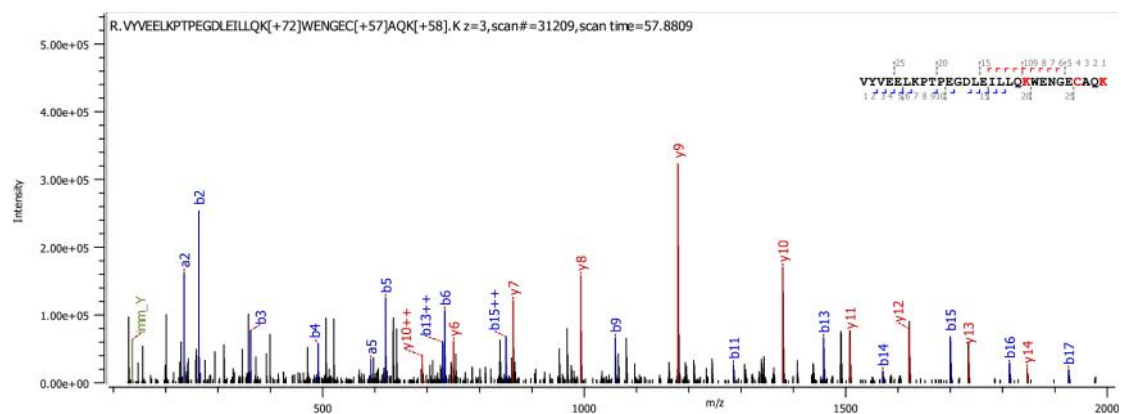


Figure 88

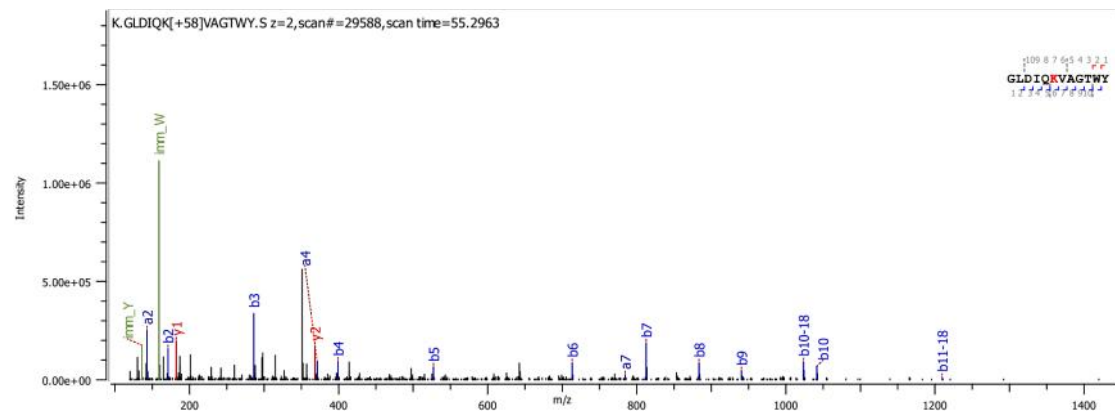


Figure 89.

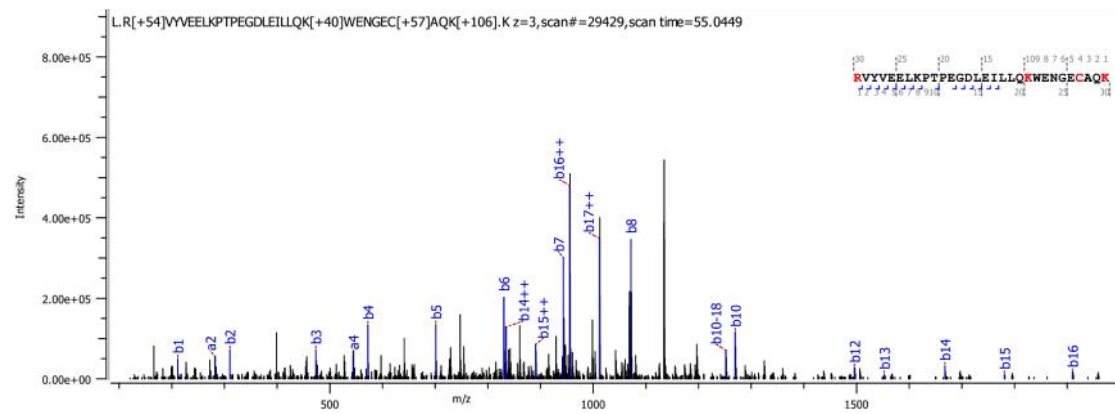


Figure 90.

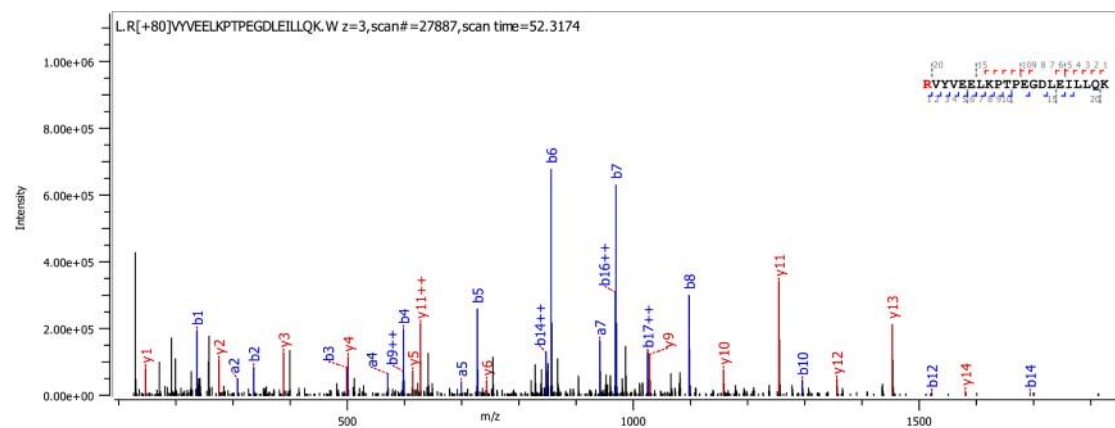


Figure 91.

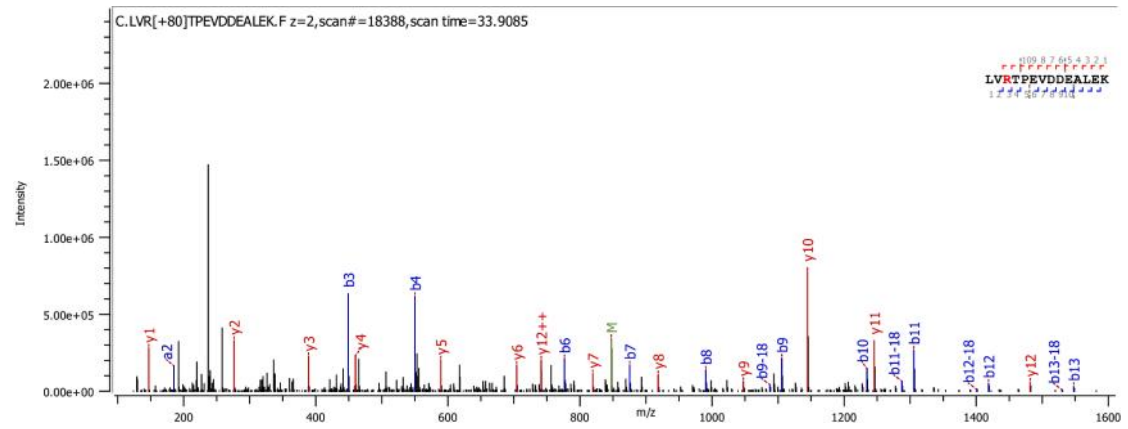


Figure 92.

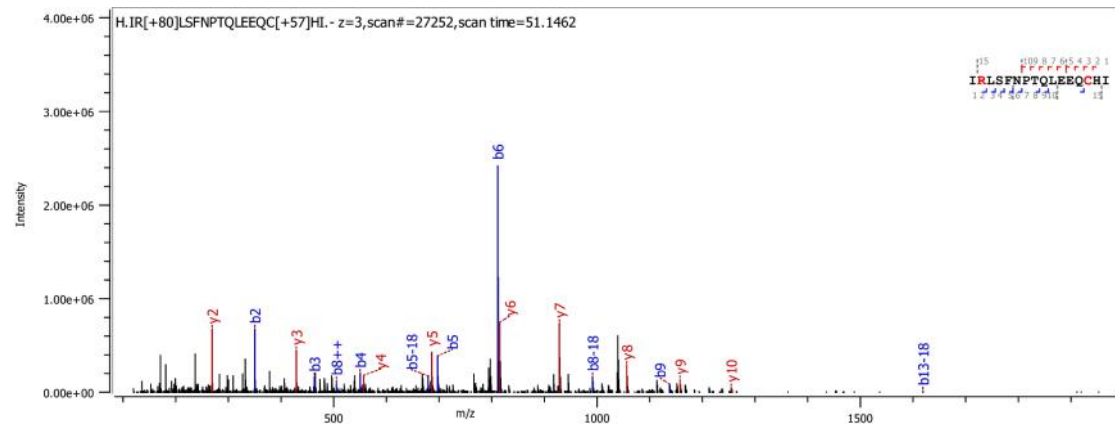


Figure 93.

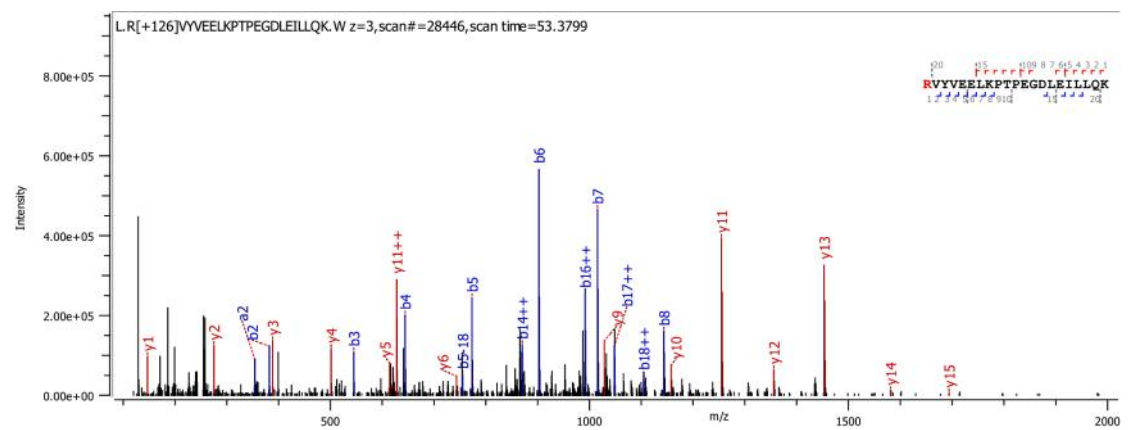
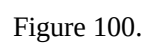
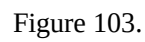
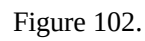
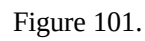


Figure 94.







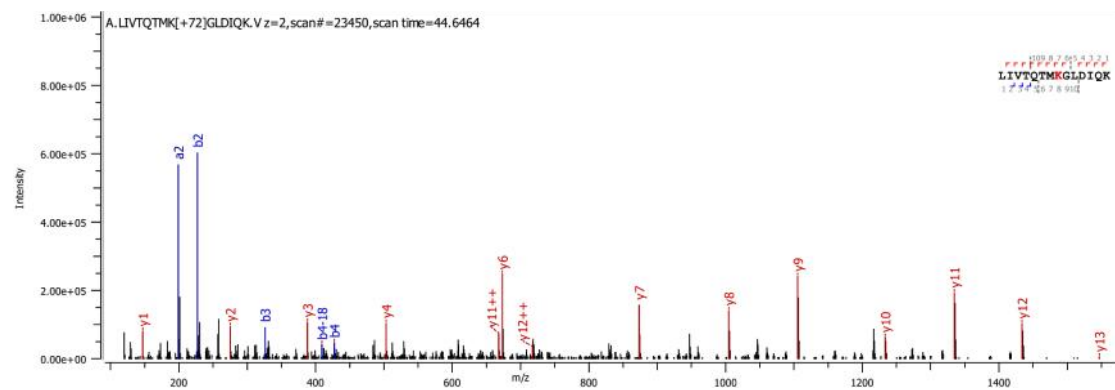
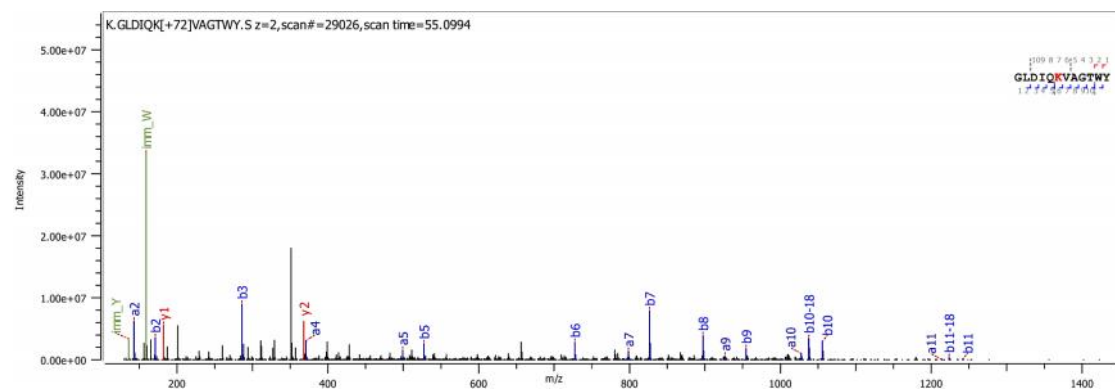


Figure 104.



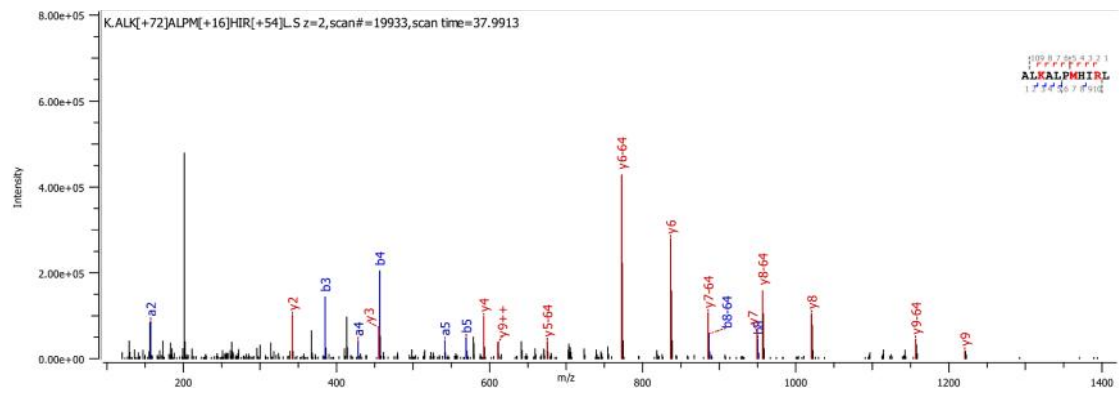


Figure 107.

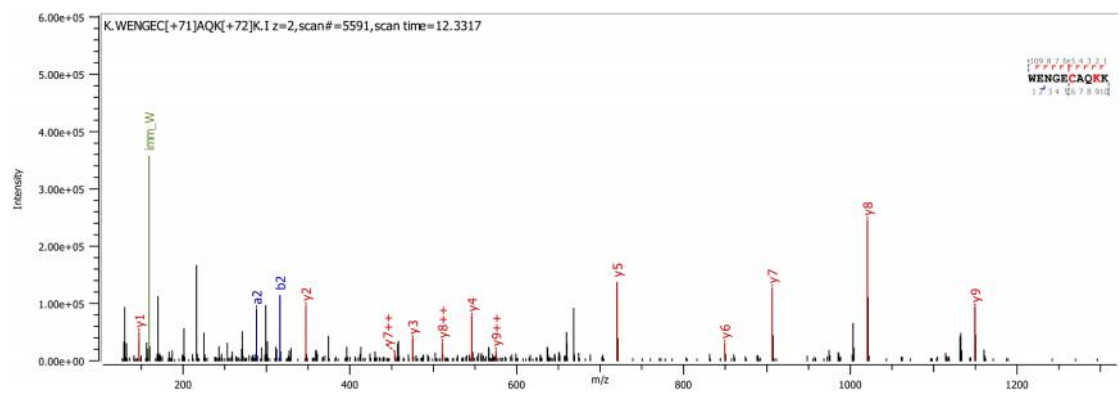


Figure 108.

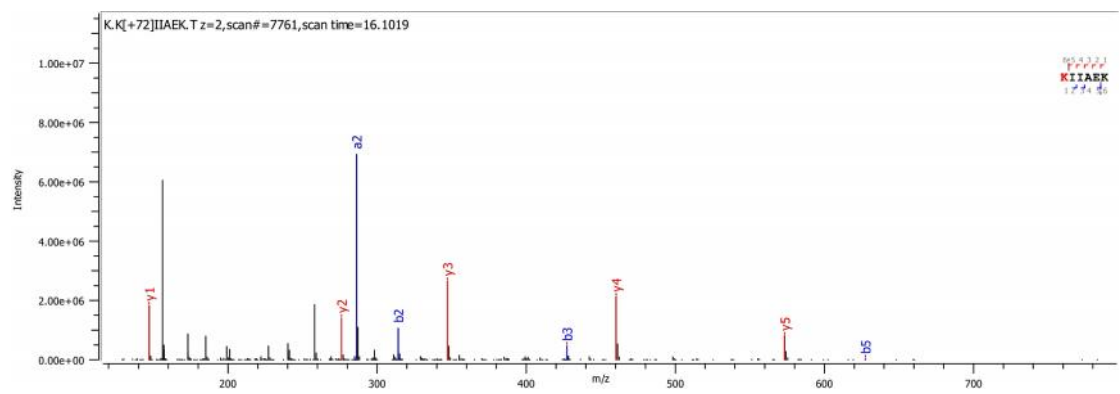


Figure 109.

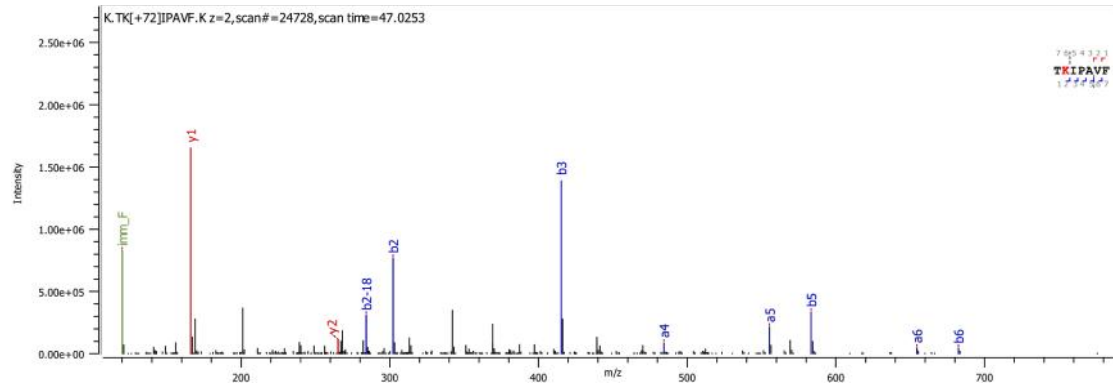


Figure 110.

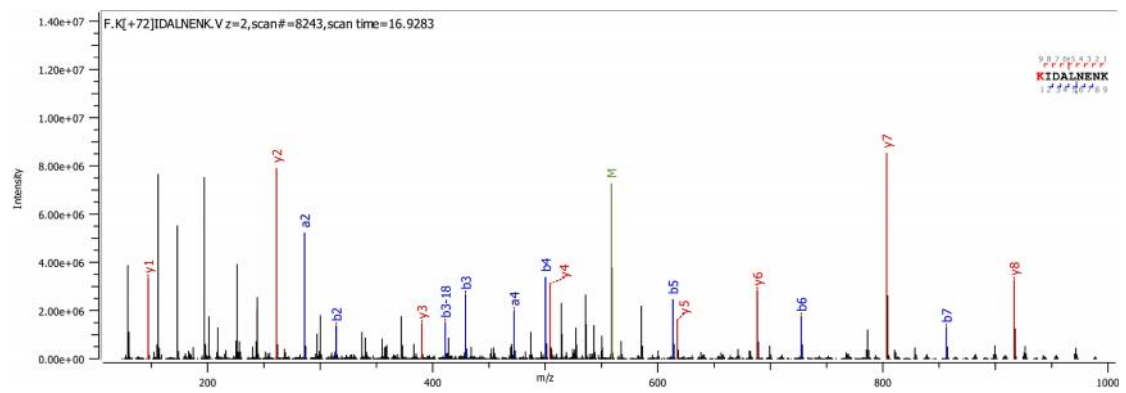


Figure 111.

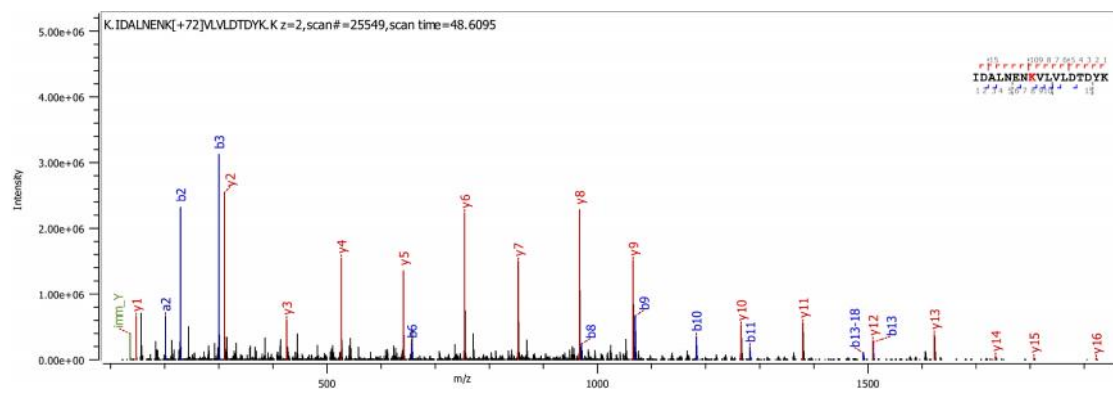
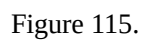
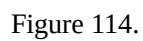
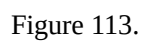


Figure 112.



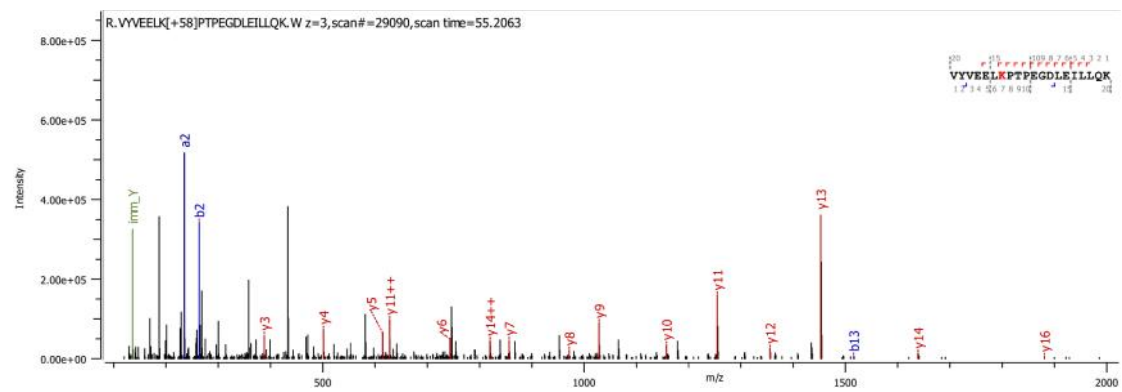


Figure 116.

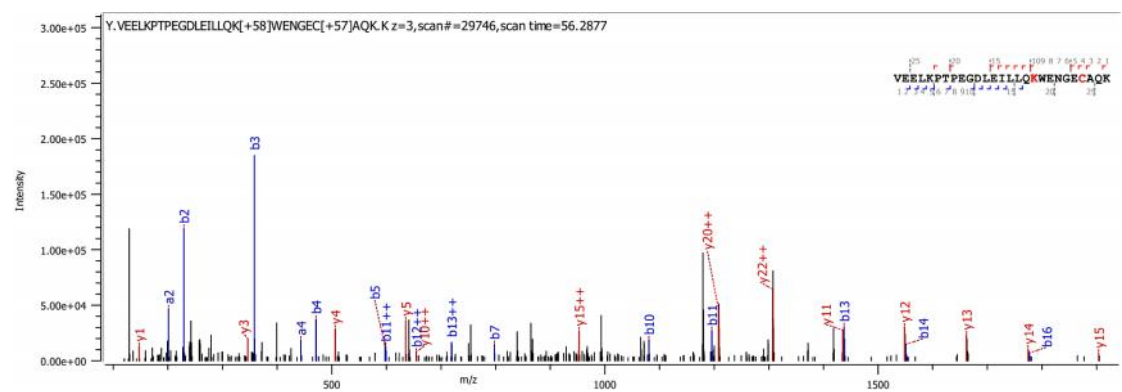


Figure 117.

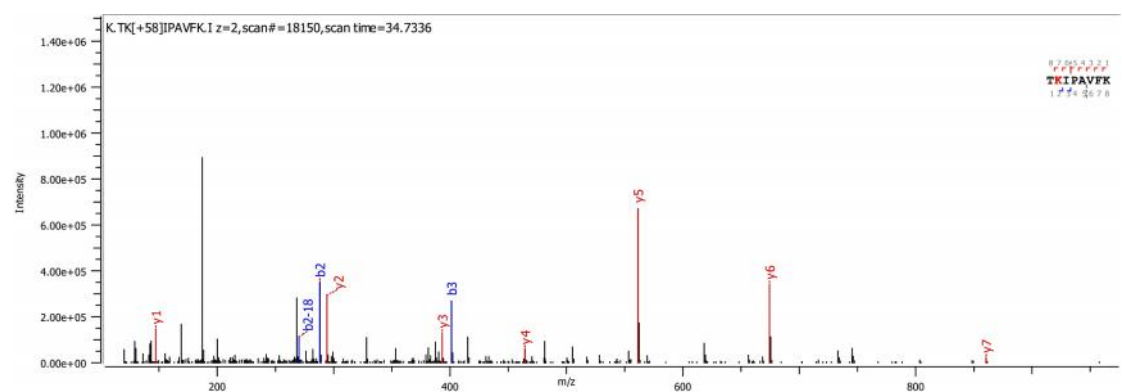


Figure 118.

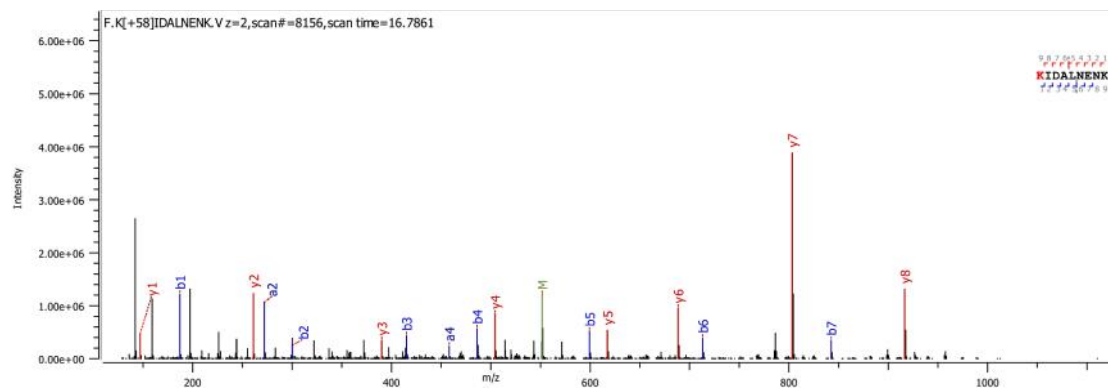


Figure 119.

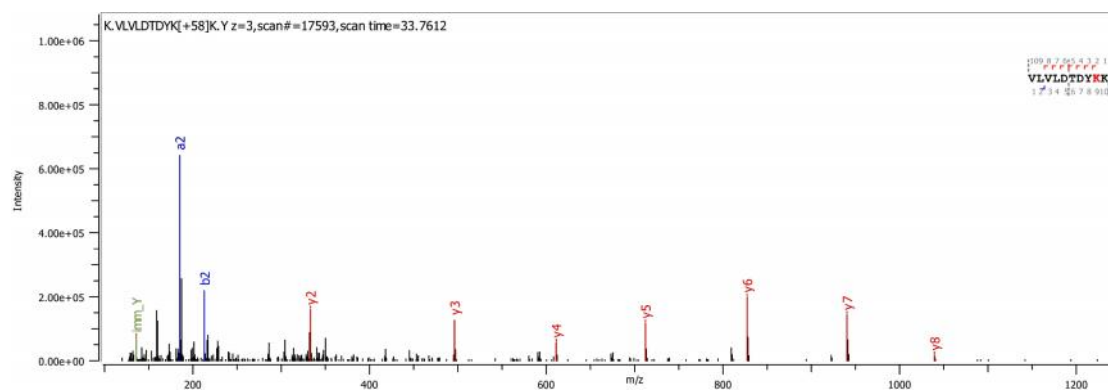


Figure 120.

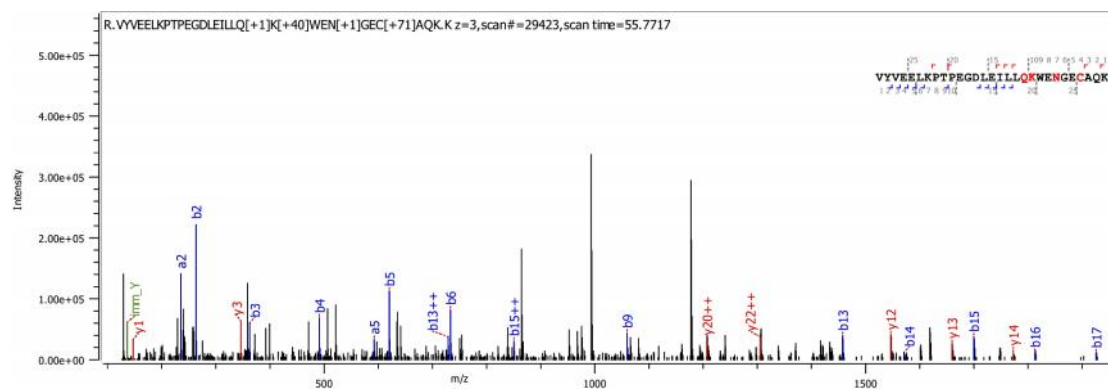
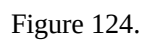
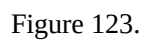
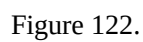


Figure 121



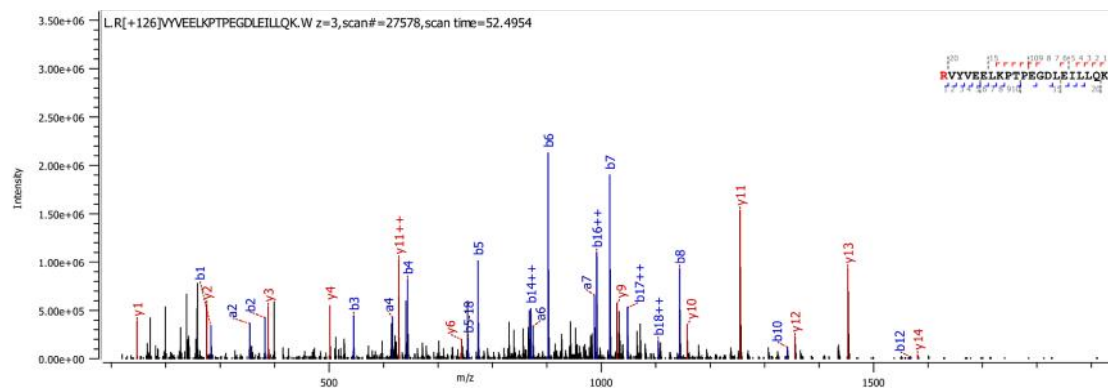


Figure 125

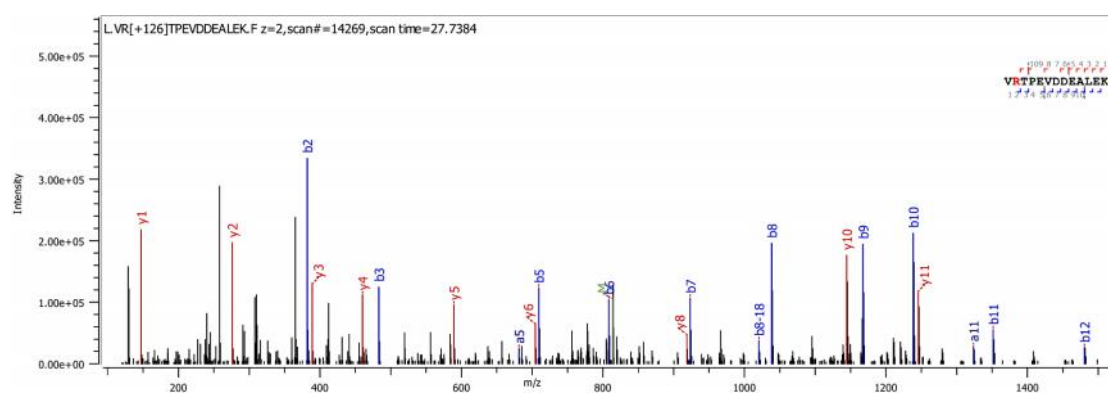


Figure 126.

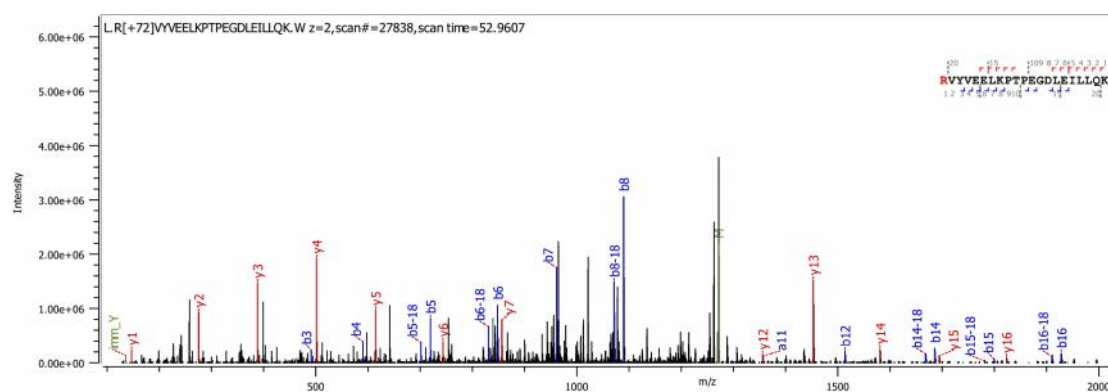
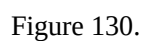


Figure 127.



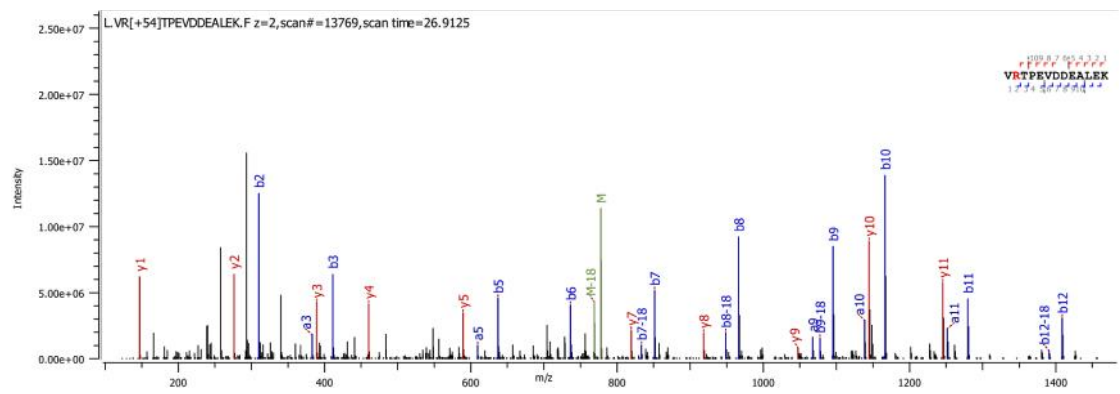


Figure 131.

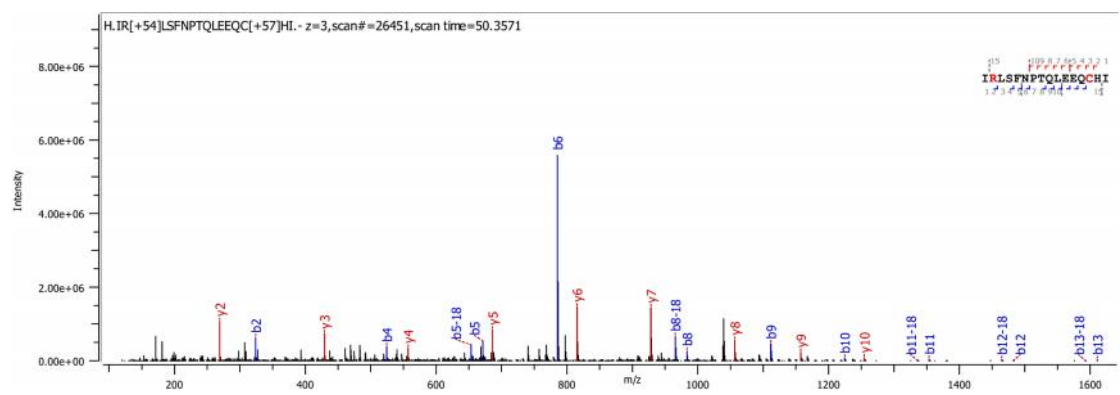


Figure 132.

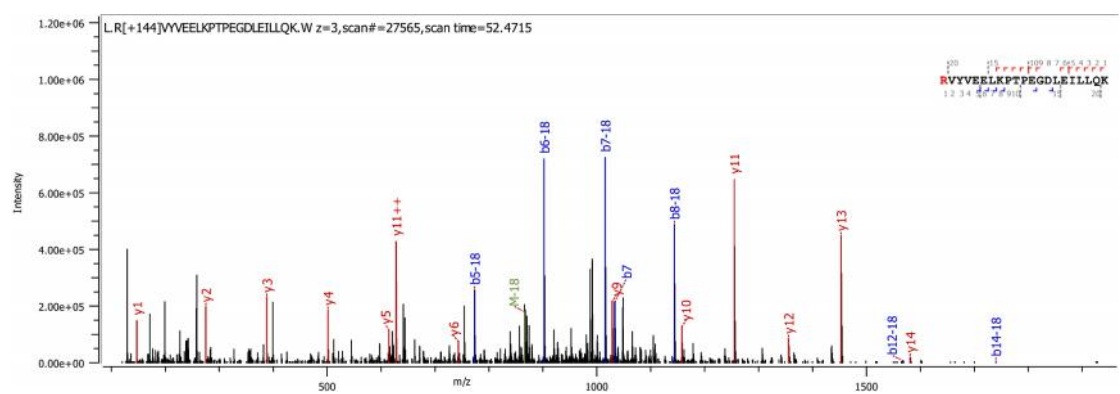


Figure 133.

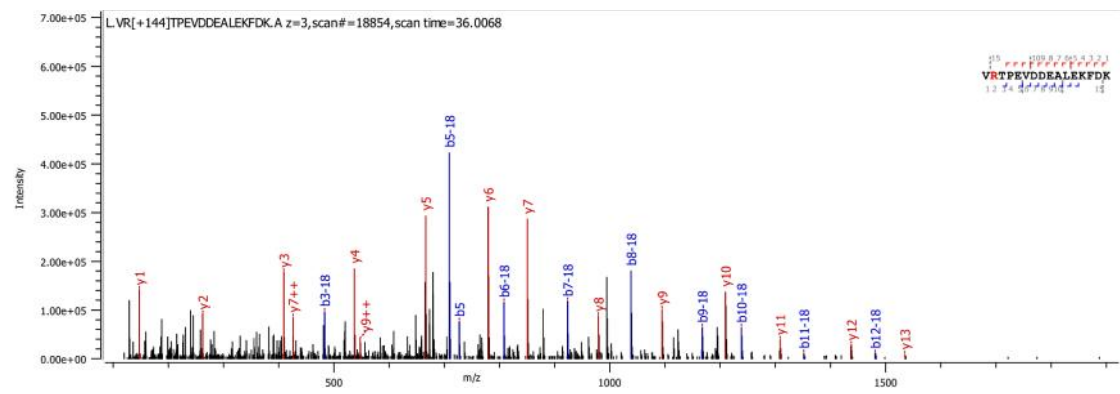


Figure 134.

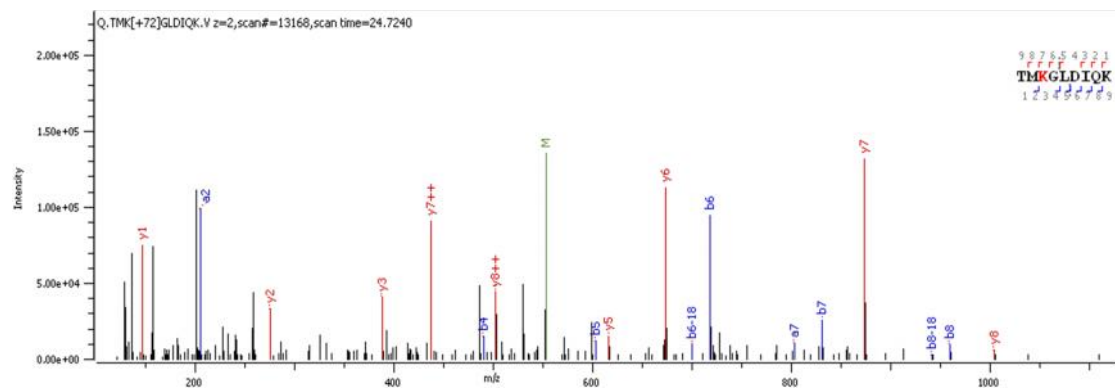
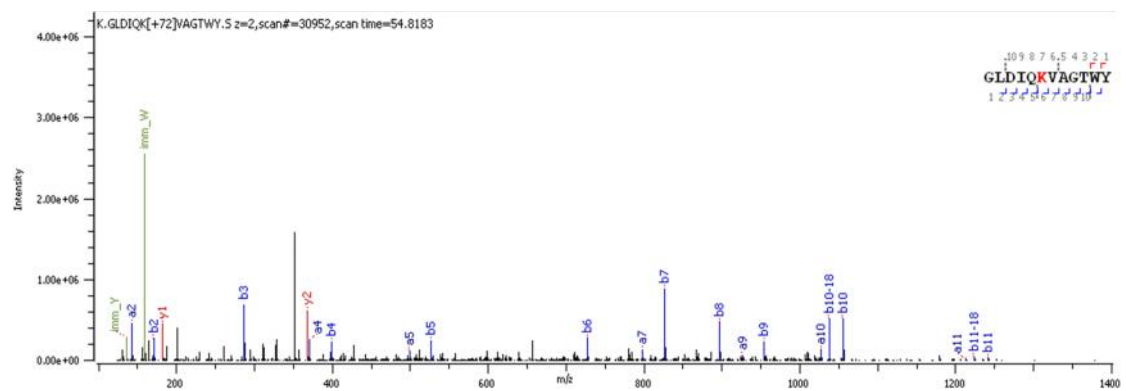


Figure 135.



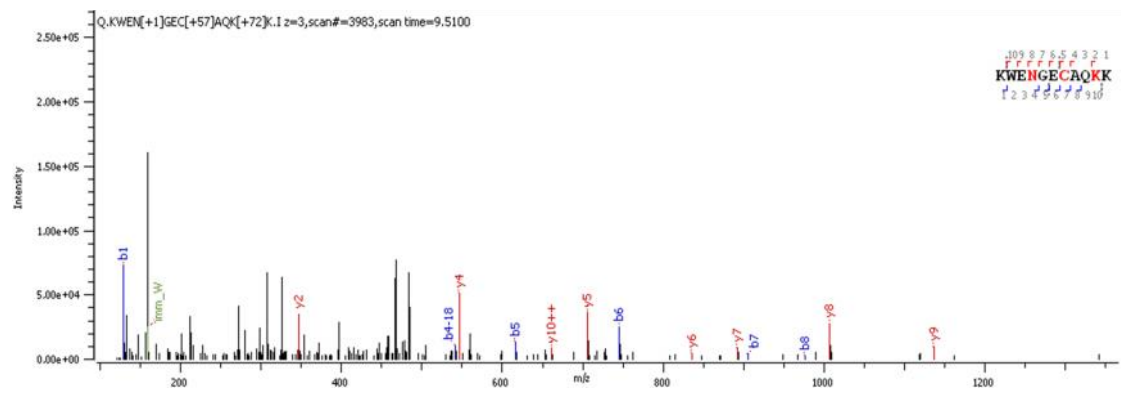


Figure 138.

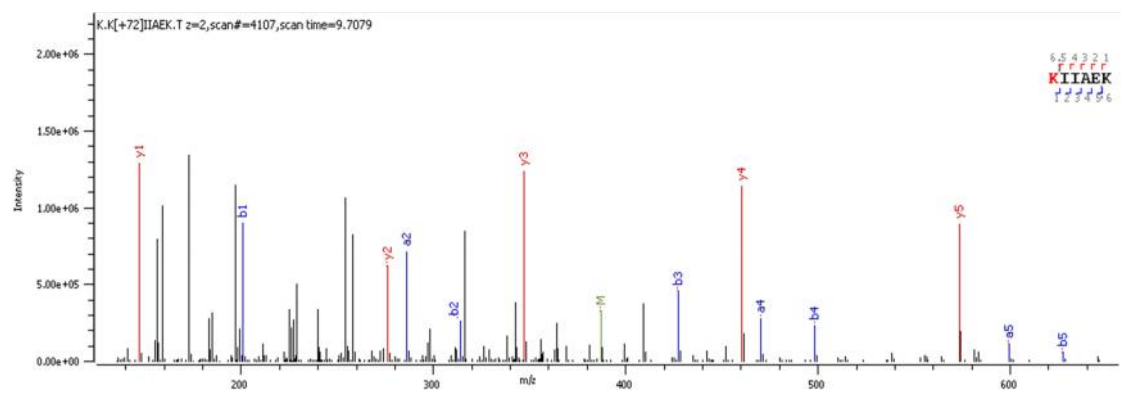


Figure 139.

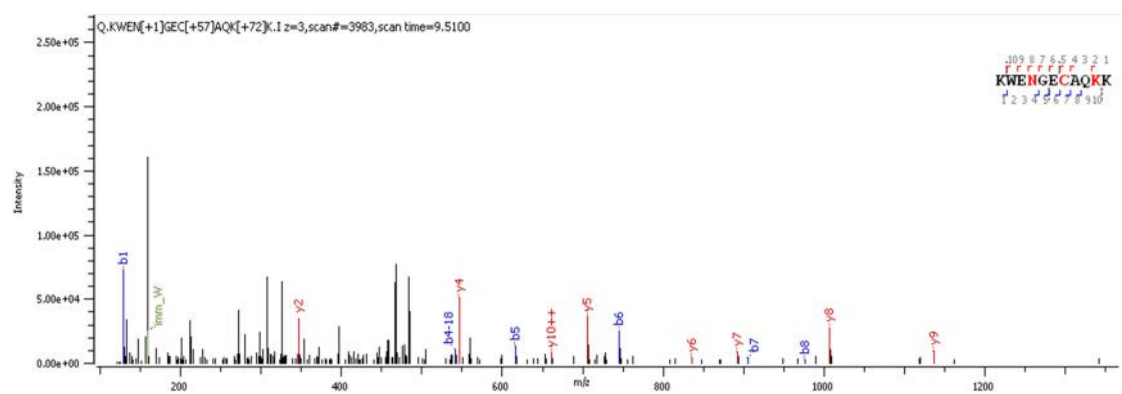
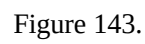
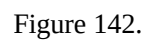
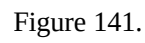
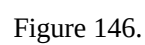
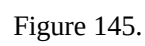
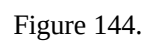
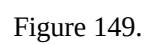
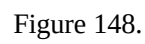
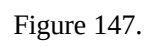


Figure 140.







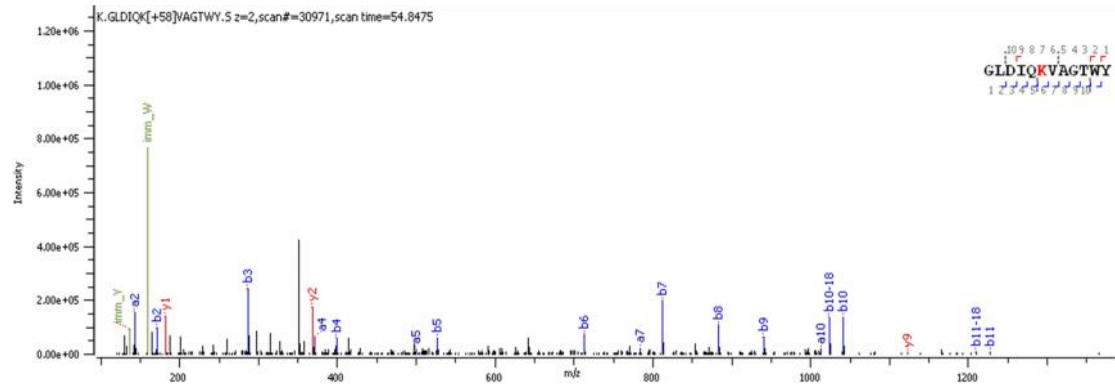


Figure 150.

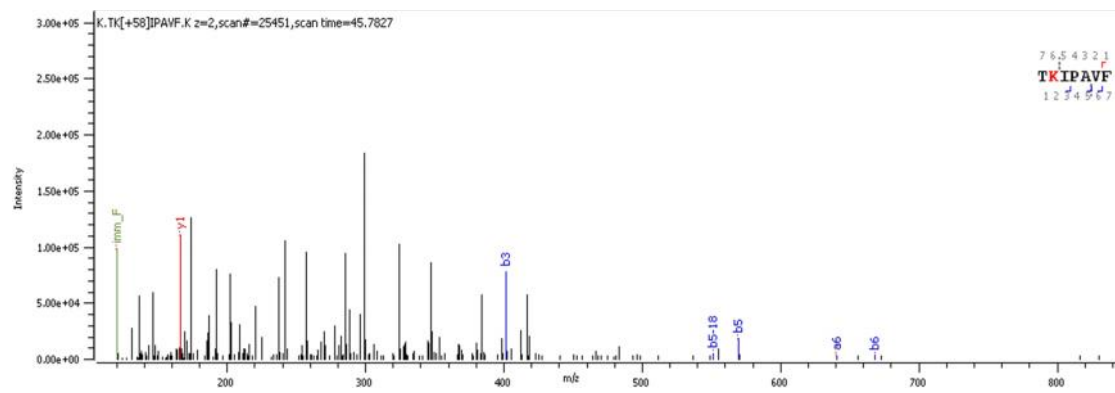


Figure 151

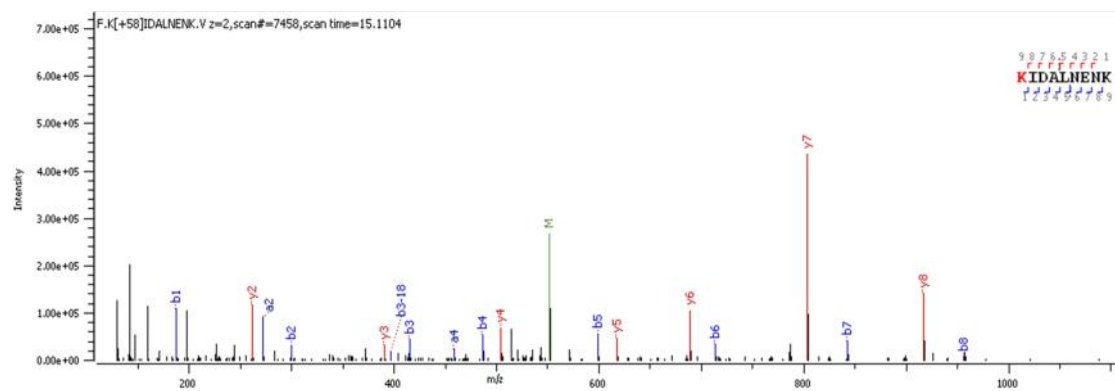


Figure 152.

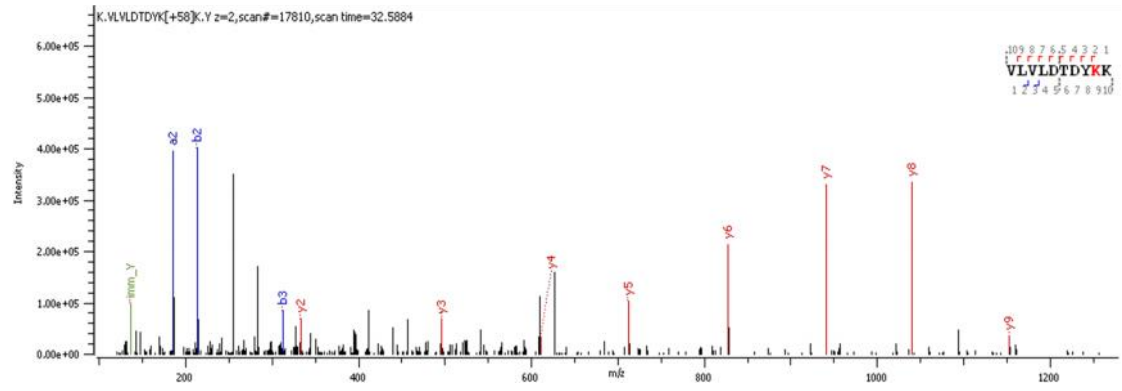


Figure 153.

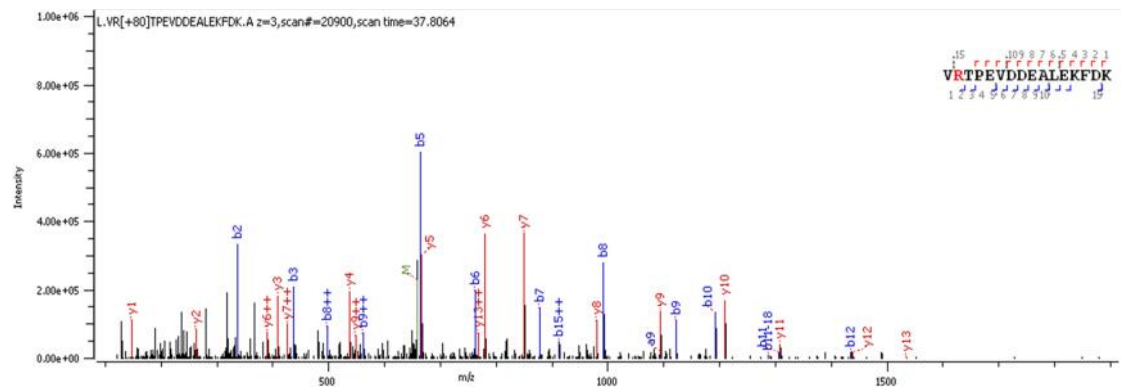


Figure 154.

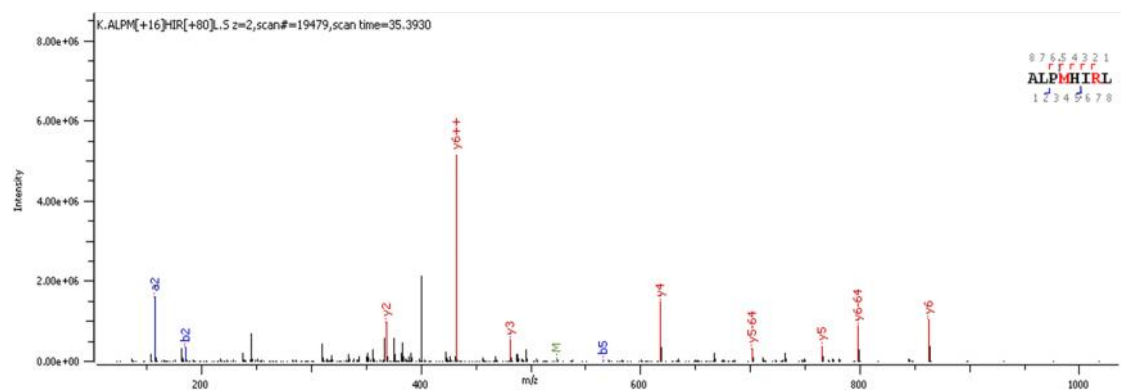


Figure 155.

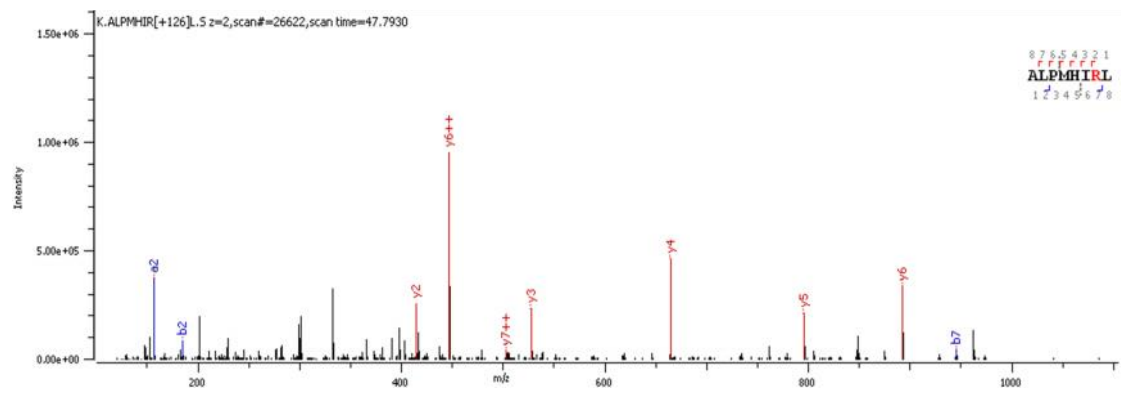


Figure 156.

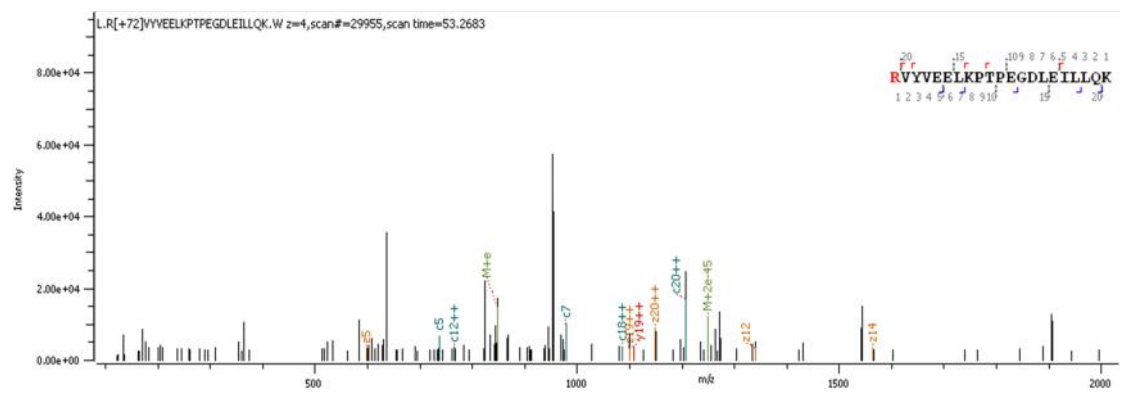


Figure 157.

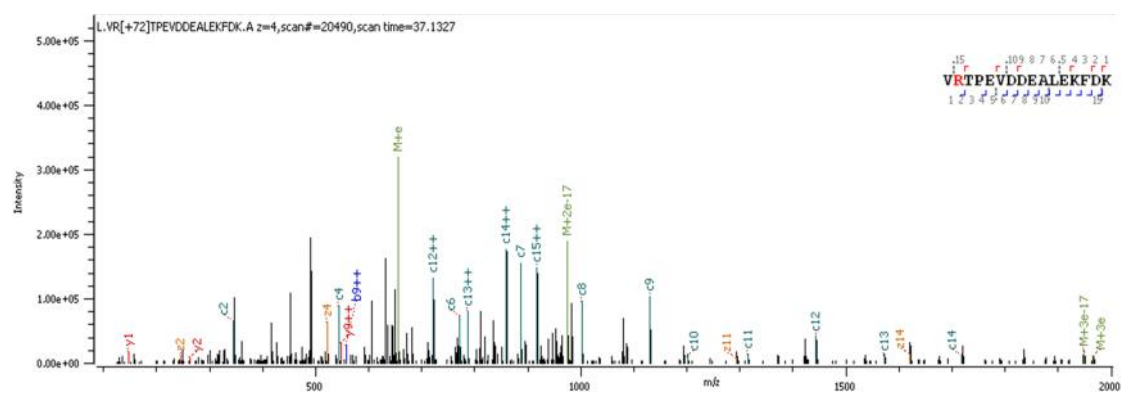
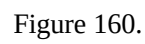
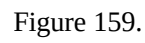
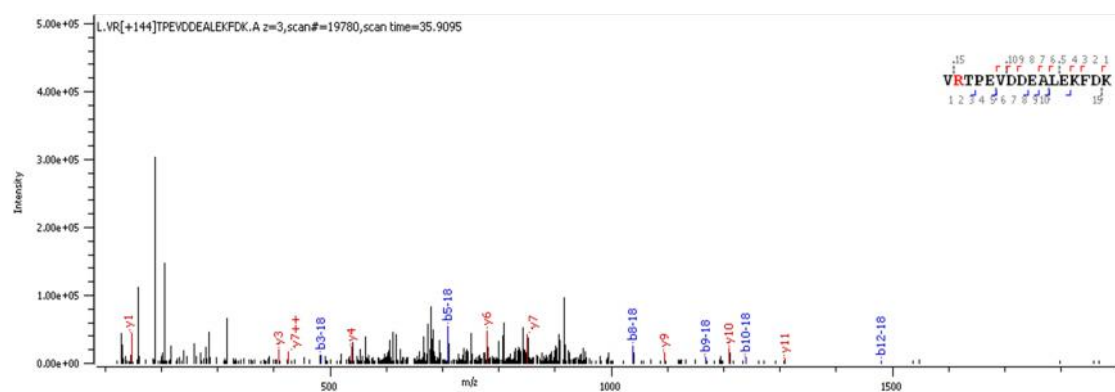
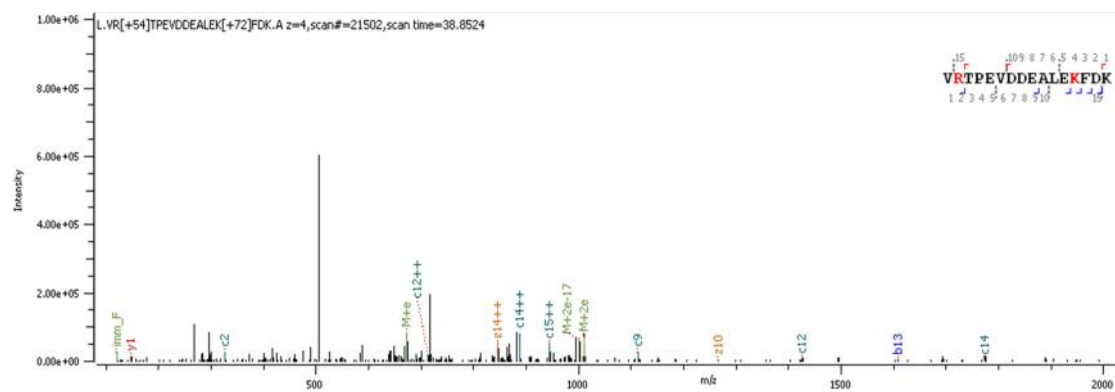
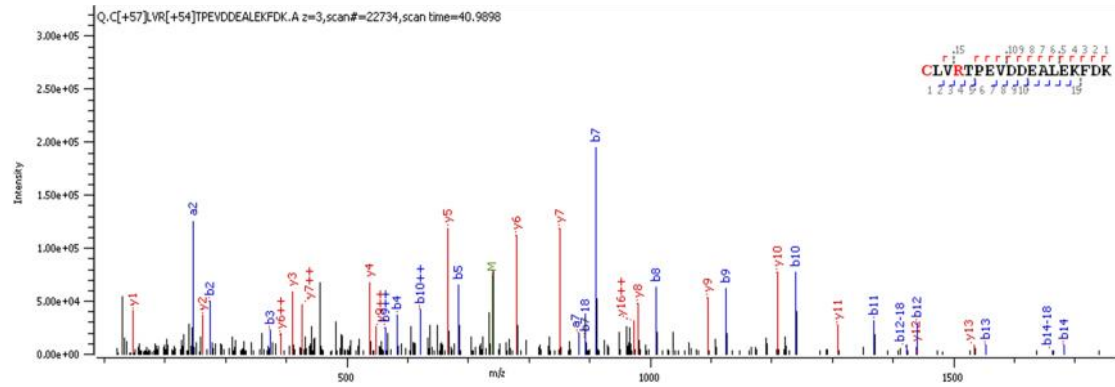


Figure 158.





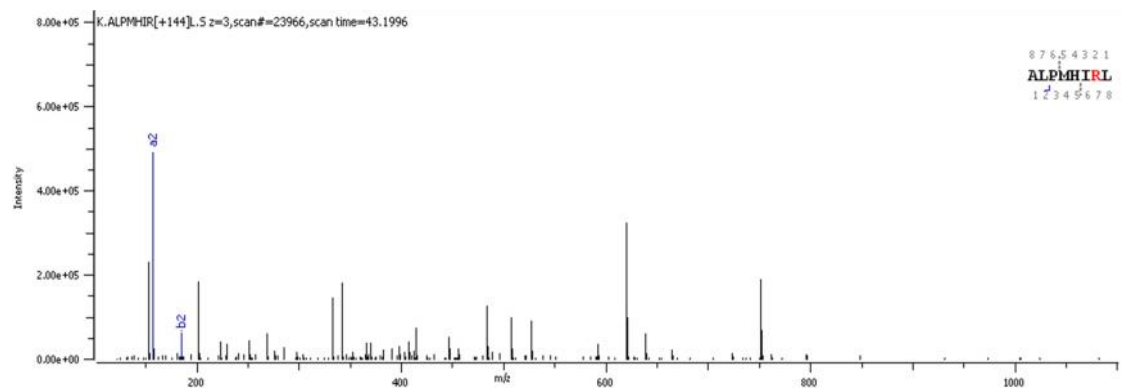


Figure 165.

Table 1. Quantitative heat maps for modification ratios at each site at different temperatures

	MTs of K	KMSs															MTs of R	RMSs		
		K24	K30	K63	K76	K85	K86	K91	K93	K99	K107	K116	K117	K151	K154	K157		R56	R140	R164
60	Pyr_CML	1.63%	3.53%	0.95%						1.07%							MG_H1	31.42%	59.96%	22.32%
	CML	1.18%	6.56%		0.64%	34.78%				2.34%	0.45%	0.17%		0.73%	0.61%		MG_DH	19.69%	27.60%	9.50%
	CEL	1.88%	0.91%	6.09%	0.95%	0.12%	0.97%	34.488%	5.50%	2.68%	3.49%	0.44%		0.87%	0.90%	3.11%	Arg_pyrimidine	2.74%	8.18%	1.29%
	Pyrraline	0.11%								0.44%	0.10%						THP	5.35%	1.31%	0.28%
																	DHP	0.40%		0.84%
70	Pyr_CML		0.38%														MG_H1	23.72%	61.79%	27.03%
	CML		0.11%			39.35%					0.08%						MG_DH	16.30%	21.00%	9.08%
	CEL	3.43%	4.95%	8.71%	1.51%	2.26%	0.64%		3.38%	4.80%	3.50%	0.88%		1.11%	0.23%	43.33%	Arg_pyrimidine	5.30%	10.43%	10.80%
	Pyrraline																THP	13.16%	5.16%	
																	DHP	2.43%		
80	Pyr_CML				0.13%												MG_H1	23.05%	49.25%	30.81%
	CML		0.20%			35.80%											MG_DH	6.34%	18.95%	7.27%
	CEL		3.34%	0.50%	2.13%	2.97%		84.76%	6.63%	9.29%	2.84%	0.26%		0.84%		7.93%	Arg_pyrimidine	5.02%	11.02%	13.79%
	Pyrraline																THP	17.25%	6.10%	
																	DHP	2.87%	14.53%	
90	Pyr_CML				0.05%												MG_H1	23.21%	66.02%	36.25%
	CML			0.30%	2.88%	44.15%			0.32%	0.34%		0.04%					MG_DH	17.19%	18.01%	9.54%
	CEL	2.99%	2.43%	7.36%	1.13%	1.49%	7.43%		4.26%	6.43%	5.13%	0.38%		0.46%		31.68%	Arg_pyrimidine	10.83%	11.53%	13.33%
	Pyrraline																THP	8.92%	3.77%	
																	DHP	2.86%	0.47%	
100	Pyr_CML																MG_H1	6.75%	53.95%	62.13%
	CML		7.31%		4.07%	48.60%	1.06%			0.22%		0.10%					MG_DH	1.92%	28.95%	7.49%
	CEL	0.90%	85.65%	0.50%		2.67%	36.27%	37.39%		4.82%	7.17%	2.88%	1.58%	5.14%	0.22%	97.85%	Arg_pyrimidine		14.34%	19.52%
	Pyrraline																THP		0.34%	
																	DHP			1.17%

