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Identification of Animal Muscle Proteins by 2D-Electrophoresis and Time-of-Flight Mass Spectrometry

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Abstract: A method for determining the quantitative content of meat proteins by two-dimensional electrophoresis (2D). The method is designed to quantify muscle protein in raw meat samples through proteomic technology complex comprising a two-dimensional polyacrylamide gel electrophoresis and densitometry obtained electrophoregrams computer.

Keywords: Proteomics, Two-Dimensional Electrophoresis, Biomarkers, Mass Spectrometry

1. Introduction

Proteomic technologies have proven very effective for the detection in meat products of biochemical changes, such as changes in heat resistant and species-specific proteins that could be relevant biomarkers [1, 2]. In agricultural sciences as well as in all other life sciences, the introduction of proteomics and other post-genomic tools - an important step toward understanding the biochemical processes occurring in complex biological matrices [3, 4].

It is now established that all the qualitative characteristics of the muscle tissue has a rather complicated and complex nature, despite the fact that many of them are intensively studied, the molecular mechanisms of these indicators remain unclear to this day. Consequently, there is a need for innovative approaches in order to clarify the formation of a more complete picture of quality indicators of muscle tissue. Using proteomic strategies in the study of molecular mechanisms of quality indicators of muscle tissue of meat raw materials is an important step towards the production of high quality animal products and more stable production of its [5-10].

The aim of the work was to create a common methodological approach to the determination of the amount of muscle protein in unstructured boiled products by 2D electrophoresis for identification time-of-flight mass spectrometry (MALDI-TOF MS / MS) confirming the protein markers.

The sequence of the research included:

- selection of the sample (cells, tissue, body fluid),
- preparing the sample, cell lysis, protein extraction,
- isoelectric focusing, electrophoresis in the first direction,
- electrophoresis in second direction polyacrylamide gel with sodium dodecyl sulfate,
- display of protein spots on the gel,
- analysis of two-dimensional electrophoregrams (number of spots, their location),
- allocation of gel regions containing individual protein spots,
- splitting of individual proteins with trypsin directly in the gel,
- mass spectrometric analysis:
  - determination of the amino acid sequences of the individual protein fragments,
  - the identification of each protein and measuring its concentration, documenting, processing results,
  - interpretation of the data using bioinformatics, database analysis, as a result, obtaining the differential profile of the proteins.

Schematically it can be represented as follows (Figure 1, 2):

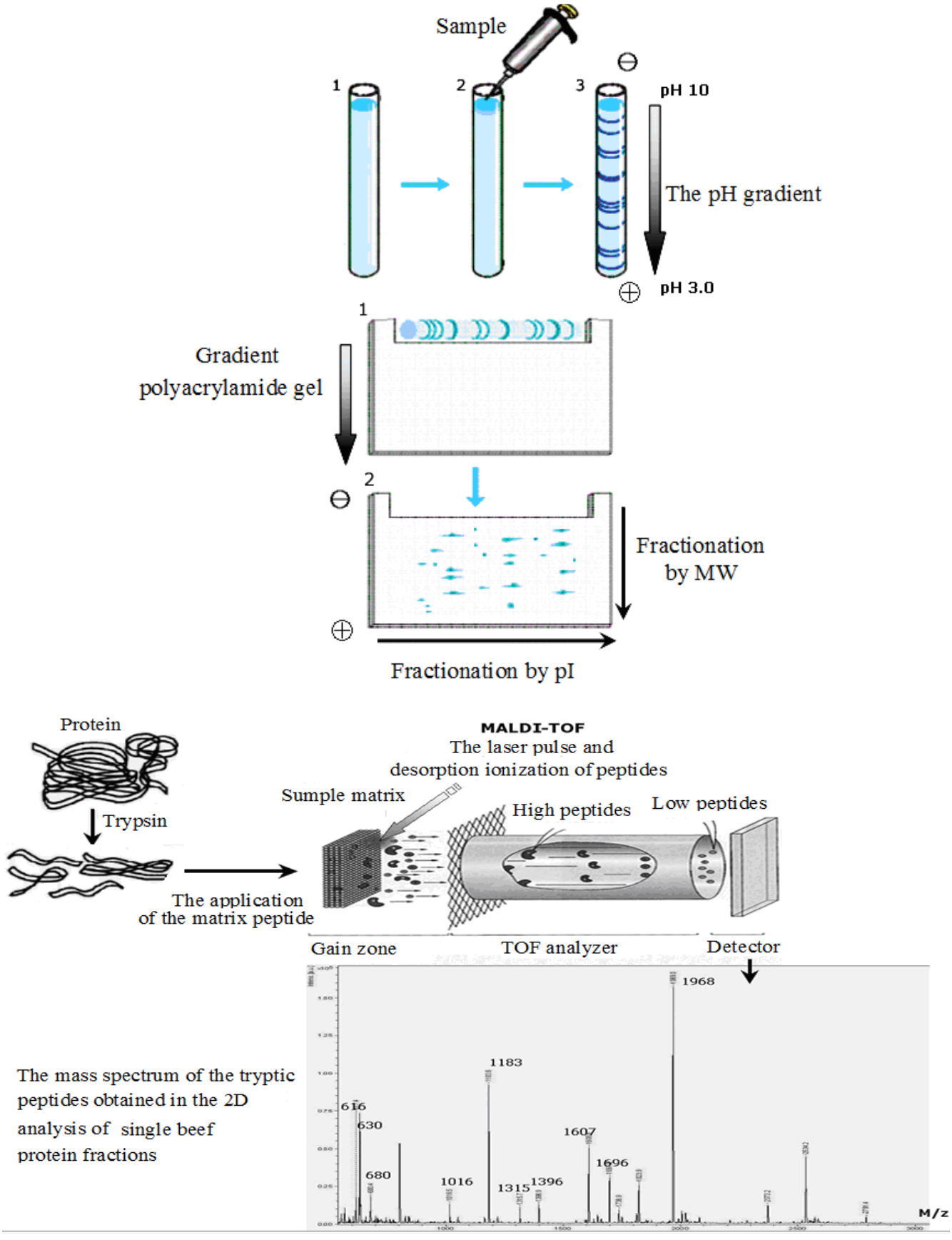


Figure 1. *Scheme of 2-D electrophoresis followed by identification of the spectra of proteins using the method of time-of-flight mass spectrometry (MALDI-TOF).*

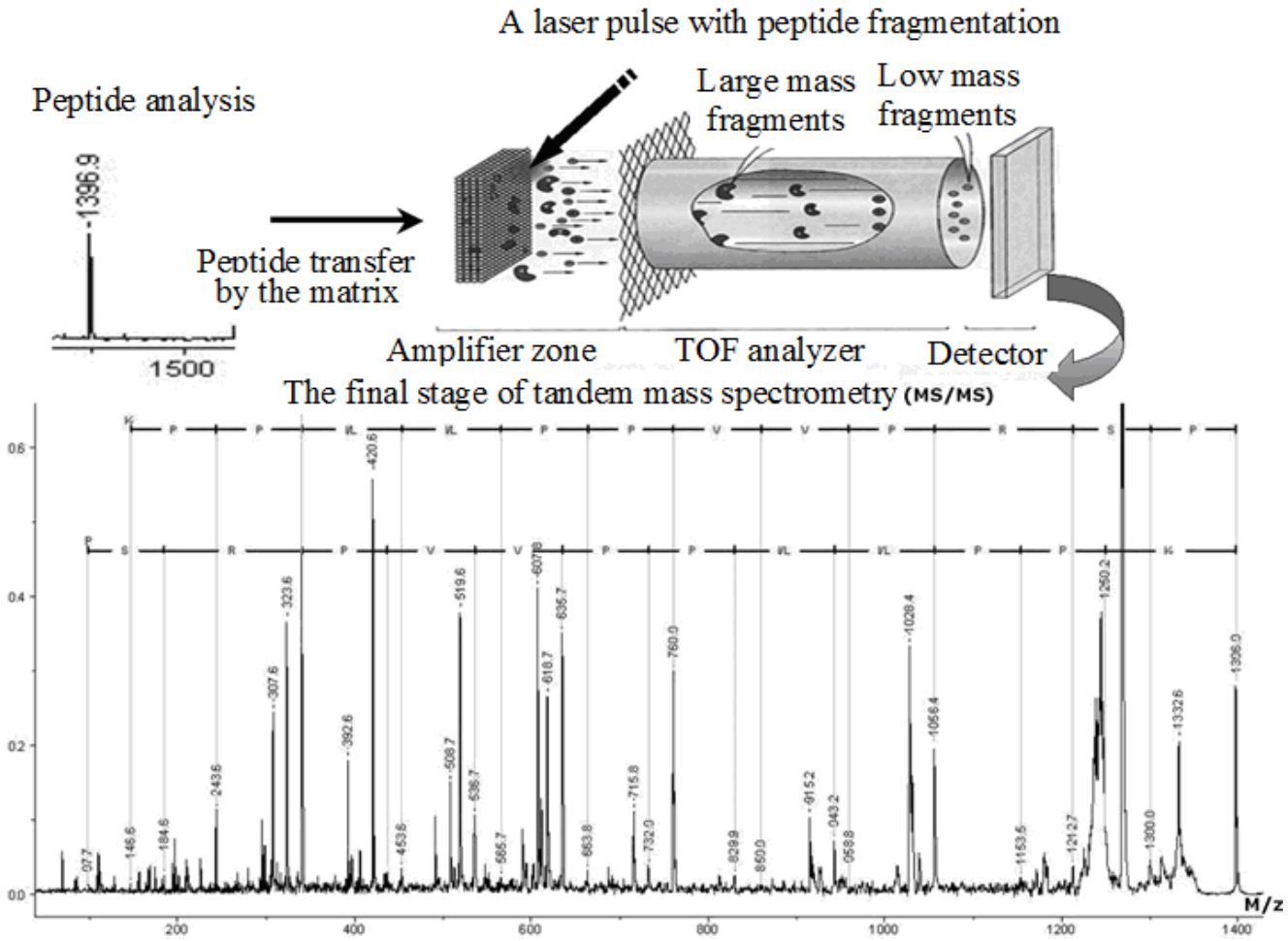


Figure 2. *Mass spectrometric identification of the selected peptide (a marker / title).*

2. Experimental

In the following methods were used:

- 2D protein fractionation on O'Farrell using isoelectric focusing in pH gradient. The main technology used proteomic own two-dimensional electrophoresis for modification of O'Farrell, in which, in particular, isoelectric focusing was performed using a pH gradient amfaliny (IEF-PAGE) and focusing in a nonequilibrium pH gradient (NEPHGE).
- The fractionation of proteins by two-dimensional electrophoresis O'Farrell using isoelectric focusing in pH gradient (IPG- PAGE). When IPG-PAGE using a modification of the two-dimensional electrophoretic fractionation in a first direction (isoelectric focusing) was performed in so called strips - polyacrylamide strips made industrially conjugated immobilin that provide a gradient in pH range 3-10 (13 cm ImmobilineTM DryStrip pH 3-10, firm «GE Healthcare») on the Ettan IPGphor 3 device [4, 8].
- Mass spectrometry identification of proteins. Protein fractions were chosen for identification, excised from the gel plate obtained by two-dimensional electrophoresis. Land comminuted gels contained protein digested with trypsin and the tryptic peptides was extracted for identification by TOF mass spectrometry on a matrix (MALDI-TOF). When MS / MS analysis of the mass spectra of the fragments were recorded on a mass spectrometer Bruker Ultraflex in tandem (TOF-TOF) mode with positive ion detection. Identification of proteins was performed using Mascot software option Peptide Fingerprint ("Matrix Science", USA), with an accuracy of determining the mass of MH^+ equal to 0.01% (allowing the possibility of modification of cysteines by acrylamide and methionine oxidation), and US Biotechnology Information database of the National Center Database (NCBI, address: http://www.ncbi.nlm.nih.gov) [1, 8].

2.1. Reagents

We used the following reagents: urea, acrylamide, methylene bisacrylamide, agarose, Tris, glycine, sodium dodecyl sulfate, ammonium persulfate, Triton X-100, Amberlite MB-1, 2-mercaptoethanol, bovine serum albumin, pH 3-10, 5-7, 5-8 ampholins, company «Sigma» (USA); immobilin strips pH 3-10 and a pH of 9-11, 13 cm «GE Healthcare» (Sweden), detergents, CHAPS, CHAPSO, nitrocellulose filters (0.45 microns) «Bio-Rad» (USA),

Coomassie brilliant blue R-250, Coomassie brilliant blue G-250, 4-chloro-1-naphthol "Serva" (Germany), Tween-20, «Pancreac» (Spain). The molecular weight (MW) of the protein fractions was determined using multiple sets of highly purified recombinant proteins with molecular weights: 10-170 kDa «PageRuler ™ Prestained Protein Ladder» (10 protein); 40-300 kDa «SpectraTM Multicolor»; 10-200 kDa «Fermentas». Other reagents were domestically produced brands o.s.ch. or chemically pure.

2.2. Preparation of Samples for Analysis

To prepare protein extracts of muscle tissue samples were homogenized (strictly, 200 mg) in 4.0 ml of lysis solution containing denaturants following: 9 M urea, 5% mercaptoethanol, 2% Triton X-100, 2% ampholine pH 3.5 10. Homogenates were clarified by centrifugation (7000 rpm, 5 min). The resulting protein extracts were used in further work with the application on the IEF tube gel in an amount of 50 or 75 microliters.

2.3. Fractionation of Proteins by Two-Dimensional Electrophoresis O'Farrell Using Isoelectric Focusing in pH Gradient Amfolins

Proteins examined bioobjects embodiment fractionated by O'Farrell two-dimensional electrophoresis, isoelectric focusing was carried out in which with IEF-PAGE using a pH gradient, as described in detail previously [4, 5].

The features of the modified technique is: the use of IEF mixture ampholine pH of 3.5-10.0 and pH 5-8 (company «Sigma», USA) in a ratio of 1: 4; application of samples with "sour" edge polyacrylamide column and fractionation conditions - up to 4000 volts set-hours (IEF for tubes 2.4 mm inner diameter columns and 13 cm length of the gel).

Fractionation of proteins in the second direction by the method of Laemmli conducted in the presence of sodium dodecyl sulfate (SDS), in two variants: gradient polyacrylamide gel (PAGE) and 7-20% in 15% PAGE. Each column PAGE obtained after fractionation in a first direction (IEF), SDS laid on the end plate and filled with a solution of 1% agarose in electrode buffer. At the end of each parallel plate laid next to column IEF shaped hole for applying the protein sample containing proteins of molecular mass markers (Mm). As MW-marker proteins using the corresponding «Fermentas» company sets (PageRulerTM Unstained Protein Ladder №SM0671).

Subsequent electrophoresis was performed in devices for vertical electrophoresis firm "Helicon" (Russia) for the night under the following conditions: 0.5 h - 30mA / gel; 16h -4 mA / gel. The next morning, set the finishing options - 30 mA / gel (250V, 13W) and ends fractionation, when the marker dye (bromphenol blue) reaches the bottom edge of the gel plates.

2.4. Detection of Proteins on the Gel Plates

Densitometry and computer analysis of results. Staining with Coomassie R-proteins was carried out in 250 solution of the following composition: 10% acetic acid, 25% isopropanol, 0.05% Coomassie R-250. Staining was performed in a water bath for 15 min followed by washing unbound dye acetic acid 10% [3, 12].

Staining with silver nitrate was carried out by the method of Blum H. et al. [2] with some modifications. Modifications: all processes under silvering carried out on a shaker and plastic containers. Gels for 1 minute washed in hypo Na (0.2 g / 1 liter with addition of 4 ml of 10% acetic acid). After three washes with distilled water by incubation in the silver nitrate solution (0.4 g / 200ml distilled water with the addition of 150 ul of formalin) for 10 min. Next, the gel was washed with distilled water (three times for 20 seconds) and incubated with 0.2 liter of developing solution (8 g - sodium carbonate, and 100 ul of formalin, 4 ml solution of hypophosphite). Staining was stopped by washing with plenty of distilled water.

For the two-dimensional computer densitometry electrophoregrams their full digital images obtained by scanning in the wet state (Epson Expression 1680 scanner).

The resulting digital image editing in a graphics editor and cheated quantitative protein content using the ImageMaster 2D Platinum software package version 7 («GE Healthcare», Switzerland).

Gels were dried for long term storage. For this purpose, they were dehydrated in a solution containing 3% glycerol and 50% ethanol for 30 minutes, then tightly fixed between two layers of cellophane and dried under tension at ambient temperature.

2.5. Mass Spectrometric Identification of Proteins

Protein fractions were chosen for identification, excised from the gel plate obtained by two-dimensional electrophoresis. Land comminuted gels contained protein digested with trypsin and the tryptic peptides was extracted for identification by TOF mass spectrometry on a matrix (MALDI-TOF) according to previously published protocols that [7, 11] were slightly modified in the future.

The analyzed sample (0.5 mkl) was mixed on the target with the same volume of 20% solution acetonitrile containing 0.1% trifluoroacetic acid and 20 mg/ml 2,5-dihydroxybenzoic acid ("Sigma", USA), and dried on air.

Mass spectra were obtained on a MALDI-TOF-mass spectrometer Reflex III ("Bruker", USA) with a UV laser (336 nm) in the positive ion mode, mass range 500-8000. And their calibrated using known internal standards. When MS / MS analysis of the mass spectra of the fragments were recorded in positive ion detection mode. Fragmentation of ions induced supply of helium to the initial section of the trajectory of the free ion drift (an inert gas pressure of $2 \cdot 10^{-7}$ Pa). Measuring the mass of fragments error does not exceed 0.05%. In the mass spectrum of the signals present only C-terminal peptides fragments have undergone gap peptide bond (y-ions).

3. Results and Discussion

3.1. The Results of Two-Dimensional Electrophoresis Fractionation of Proteins Extracted From Samples of Sausages

Fractionation of two-dimensional electrophoresis of protein extracts of the example samples "Doctor's" sausage yielded about a hundred protein fractions, stained with Coomassie R-250. These fractions were placed in a wide range - MW and isoelectric points (pI). In particular, some fractions possessed MW with values more than 150kDa, and others – about 10 kDa. pI values ranged detected fractions of 4.5 to 9.0 pH. Below in Figure 3, show an example of a typical two-dimensional electrophoregrams (DE) of proteins from samples of "Doctor" sausage sumple DK1.

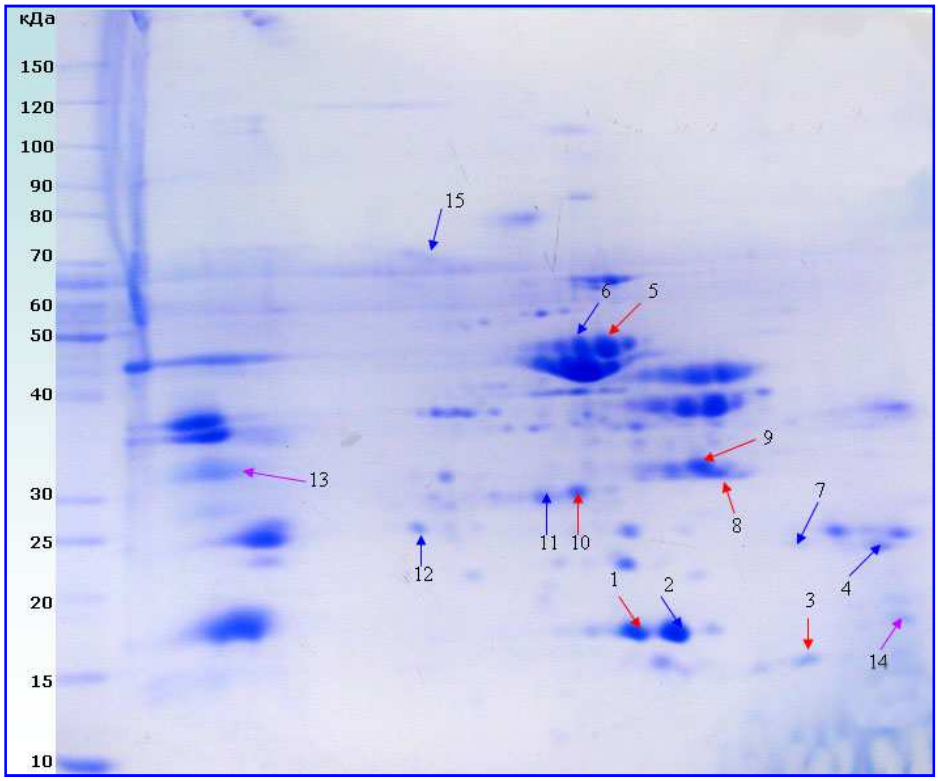


Figure 3. *Typical DE obtained in the analysis of protein extract from the sample DK1 (Doctor's sausage) Colouring with Coomassie R-250. The right side shows mm (kDa) protein markers, the distribution of which is available as a special track"lanes, which are located on the right edge of the DOE. Bottom DE shows pH.*

The arrows and the corresponding numbers marked protein fraction, identified by mass spectrometry (see. Table 1).

Table 1. *Mass spectrometric identification of protein fractions identified in DOE extracts sausage Doctor"*

No	Name	No in Protein NCBI	Identifi-cation*	Probability (score),%	MW/pI experiment	Mw/plcalculation
1	Myoglobin (Sus scrofa)	47523546	157/13	77	17.0/7.75	17.1/6.76
2	Myoglobin (Bos taurus)	27806939	146/13	75	17.0/6.90	17.7/6.90
3	Alpha haemoglobin (Sus scrofa)	229626	91/9	58	15.0/8.70	15.0/8.73
4	Troponin I, Type 2muscle fast (Bos taurus)	300797481	59/10	39	22.0/8.90	21.4/8.88
5	Beta enolase (Sus scrofa)	113205498	343/30	66	46.5/6.80	47.1/8.05
6	Beta enolase (Bos taurus)	77736349	260/31	61	46.5/6.70	47.1/7.60
7	Troponin I (Bos taurus)	300797481	172/21	57	22.0/8.80	21.4/8.88
8	Phosphoglycerate mutase 2 (Sus scrofa)	201066358	284/28	74	30.0/7.60	28.7/8.86
9	Karoangidraza 3 (Sus scrofa)	56711366	153/11	47	30.5/7.55	29.4/7.72
10	Triose phosphate isomerase 1 (Sus scrofa)	262263205	388/24	94	27.0/6.50	26.7/6.45
11	Triose phosphate isomerase 1 (Bos taurus)	61888856	246/18	69	26.5/6.40	26.7/6.45
12	Myosin light chain 6B (Bos taurus)	115496556	148/13	61	24.0/5.40	23.4/5.40
13	CaseinCSN2 (Bos taurus)	83406093	97/9	29	33.0/4.80	25.2/5.53
14	Kappa Casein A (Bos taurus)	229416	94/6	77	14.0/8.75	12.3/8.92
15	Albumin (Bos taurus)	30794280	140/21	34	67.0/5.60	67.0/5.71
16**	Glycinin (Glycine max) [24-322]	254029113	93/16	47	35.0/4.90	43.5/5.51

* Probabilistic reliability coefficient / number of identified peptides masses
** Soy protein.

It may be noted the characteristic distribution of a number of "major" sarcomeric proteins, which are located in the lower left corner of the DE (green dotted ovals shown in Figure 3) such as actin, tropomyosin and myosin light chain. Use of other, more sensitive methods to detect protein DE, particularly silver nitrate staining leads to a certain increase in the amount of detectable fraction (250).

Thus, it can be assumed that in the processes of preparing raw meat and then cooking sausages, apparently losses occur significant numbers of muscle proteins (yielding, typically in the representation of "buoyant" sarcomeric proteins). To test this assumption, it seemed appropriate to conduct a comparative proteomic analysis of the main types of raw meat (pork and beef samples) used in the preparation of sausages. The corresponding results are shown below.

3.2. Comparative Proteomic Analysis of Samples of Beef and Pork Used in the Preparation of the Sausage "Doctor"

DE protein extracts prepared from beef and pork samples (Figure 4) revealed a rather similar distribution of fractions, and is quite comparable with the results of analysis of protein extracts from samples of sausages "Doctor" (Figure 3). In this regard, for identifying various species-specific has been used analytical technique called koelektroforez in which simultaneously two compared fractionated sample introduced into the sample for a total of research in the ratio 1:1.

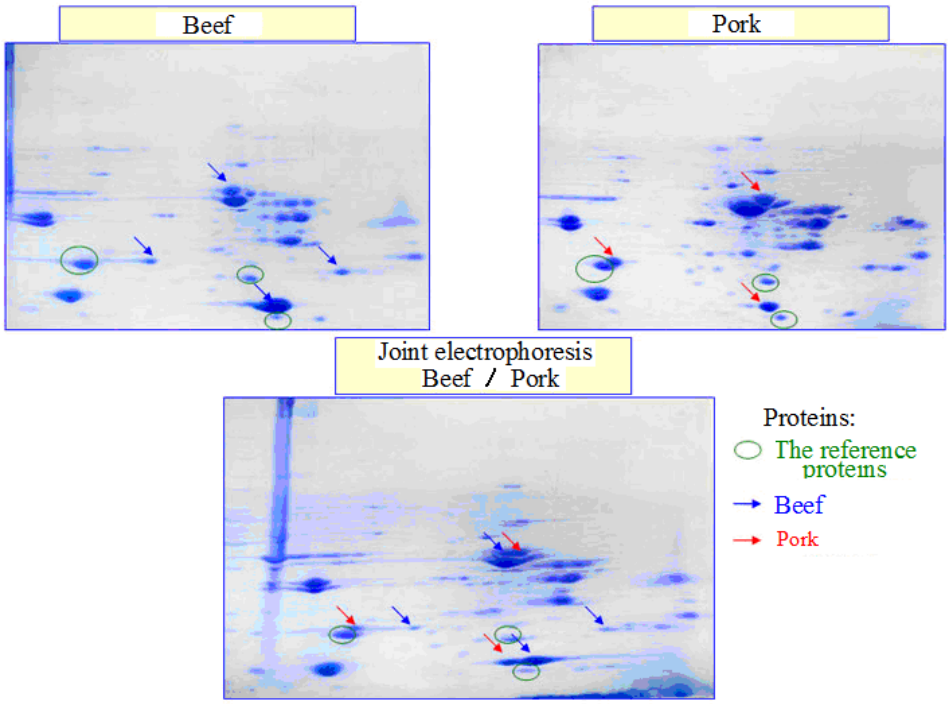


Figure 4. *The results of the comparative proteomic analysis of samples of beef and pork used in the preparation of the sausage "Doctor".*

As can be seen from the results shown in Figure 4, with the samples koelektroforeze beef and pork to JA failed to demonstrate total availability of species-specific differences in the electrophoretic properties of proteins mioglobin Y and Y isoforms beta and enolase isoforms of myosin light chains (as noted above, in Table 1). Especially clearly these differences are detected in mioglobinovyh proteins, since the positions of the factions can be compared with reference ones located near two fractions (shown in Figure 4 green ovals. The remaining symbols are the same as in Figure 3).

Moreover, the results of mass spectrometry and bioinformatics materials available (on the basis of data and program UniProt Compute pI / MW online Proteomic ExPASy Bioinformatics Resource Portal) is fully consistent with the data obtained with respect to species differences myoglobins pigs and cows. Since it was found that these proteins have similar molecular weights but differ in pI: 16956/16946 and 6.83/6.97, respectively. An important part of strategy to proteomic studies considered holding different samples of protein fractions of detectable identification identification of protein fractions with the proteomic analysis of samples "Doctor" sausage performed by mass spectrometry (MALDI-TOF), part of this work are presented below.

3.3. The Results of the Mass Spectrometric Identification of Protein Fractions

Summary data on mass-spectrometric identification of 15 protein fractions, identified by fractionation of extracts of "Doctor" sausage are summarized in the Table 1. These fractions are shown by arrows with the corresponding numbers in Figure 3, wherein the red marked proteins identified as pig (Sus scrofa), cow blue origin proteins (Bos Taurus), and violet - milk caseins. In addition, this table

includes the data obtained from the identification one protein fraction which was selected among other drug on DE soy protein that can be used as additives in the production of meat products.

The result of the mass spectrometric identification of protein molecules is a list of potential protein candidates, ranked in accordance with the index value Score (measure of conformity or "scorecard"), calculated for each potential candidate (Table 1). The result is considered valid if the value of this index exceeds 200. Also, for each of the candidates, listed species, that could be decisive in the interpretation of, and links to personal pages (outcome), containing detailed information on the potential protein (the value of its molecular weight and isoelectric point, tryptic peptide sequence decoding, the number of coincidences% cover the entire amino acid sequence of the protein identified peptides, etc.).

As seen from Table 1, the identification was characterized by high rates of reliability (score) and the large amount of overlap of complete amino acid sequences of these proteins tryptic peptides derived from the protein fractions analyzed (from 29% to 94%) in good accordance of the experimentally determined values MW/pI and settlement terms. Thus, these data allow (for example, sausages) make the conclusion about the prospects of using proteomic methods to evaluate the protein composition of meat products, including the identification of the origin of individual proteins. It is obvious that in order to achieve these objectives are also needed and proteomic studies of raw meat, and possible protein supplements.

3.4. Quantitative Analysis of Individual Protein Fractions

For quantitative analysis of individual protein fractions in the first DOE to create a complete digital image selected for the comparison of two-dimensional electrophoregrams (or their fragments) with the help of the scanner Epson Expression 1680 (or Perfection 2450 Photo). Scanning was conducted in the wet state. Further, digital images edited in the graphic editor of the specialized Melanie ImageMaster software package, version 6 and 7 («Genebio», Switzerland).

The further procedure took place in several stages.

In the first stage, carried out an automatic software analysis of gels in which the program finds the spot and attaches an outline for their colored area. The intermediate result of such computer analysis is shown in Figure 5.

Since protein spots fractions have different degrees of coloration, and may merge with the background program set 3 parameters («Smooth», «Saliency», «Min Area») for detecting («identification») protein fractions («spots»). You can set up automatic detection of spots and an additional window «cursor information» are shown in Figure 6.

Parameter «Smooth» (Figure 6), using the diffuse anti-aliasing algorithm that provides identification of the most strongly marked spots, as well as clarity of definition of the spot edges. In order to separate the weak from the colored spots and background noise parameter varies «Saliency» (Figure 6).

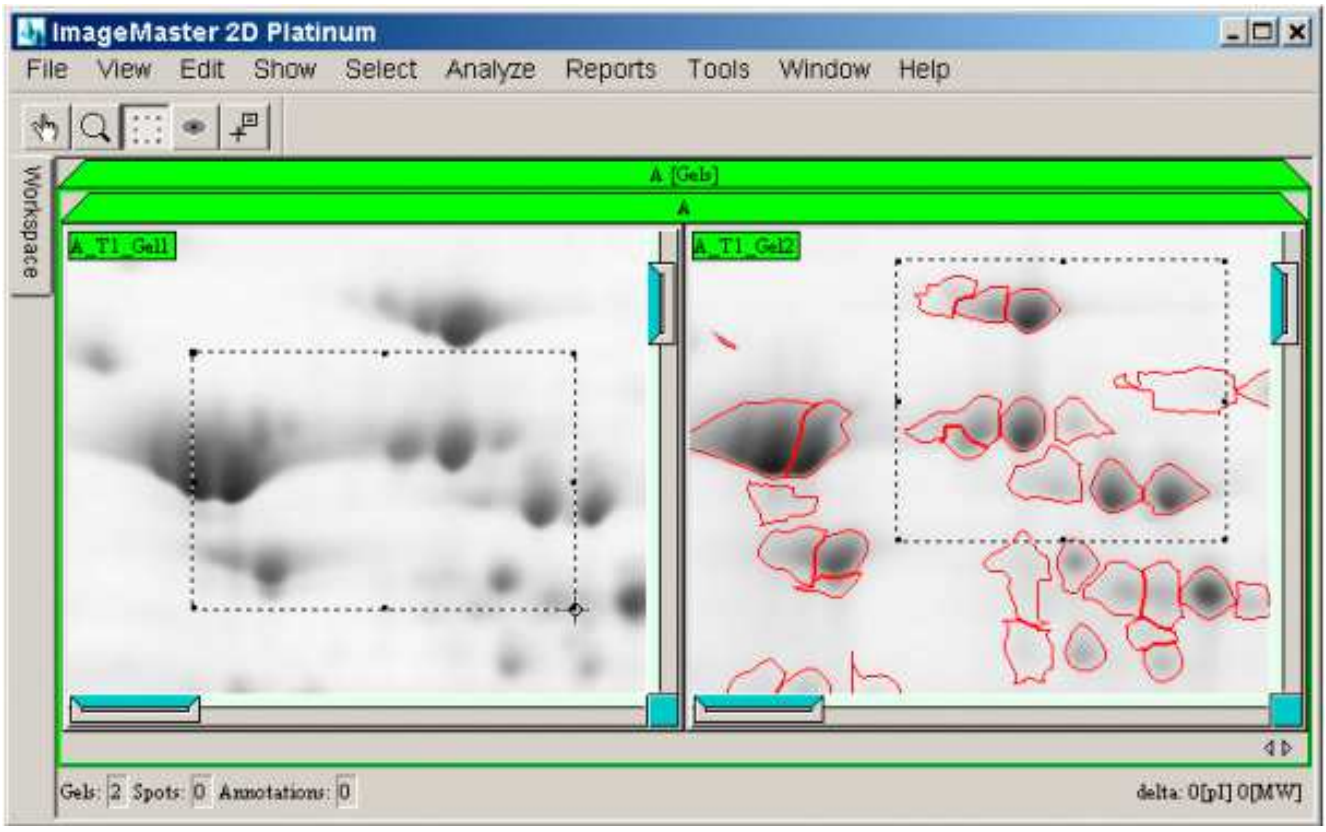


Figure 5. *Automatic detection of spots on the gel.*

By changing this setting, it is possible to provide filtering on the color intensity of spots. To accurately determine the intensity necessary to trigger additional window cursor information, and then hover the mouse pointer on the light-colored spot lights and its numerical measurement that shows the difference between the background and intensely colored spots. Thus, given an intermediate value of intensity spots (between gray background and black spots) through which will take into account all the spots whose intensity is above the threshold.

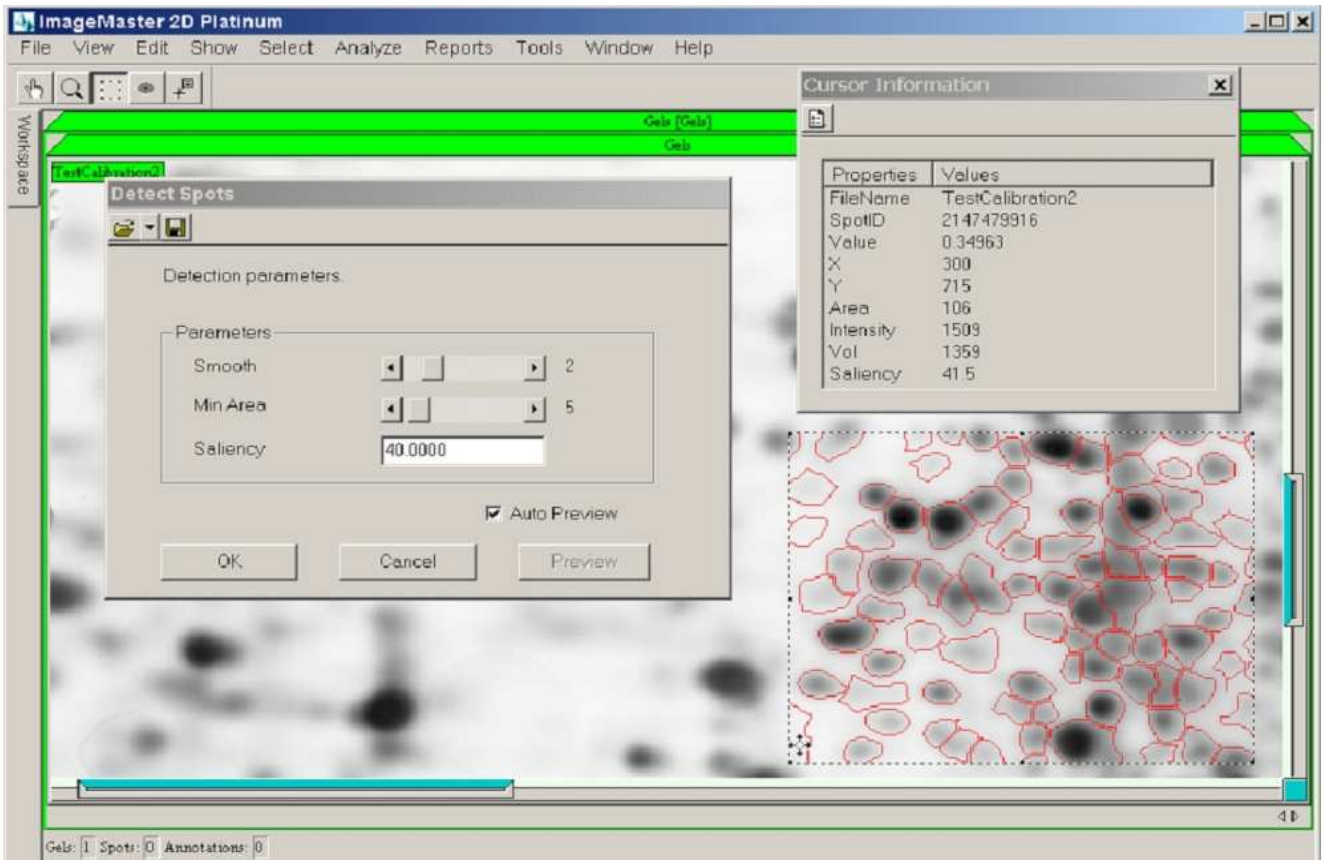


Figure 6. *You can set up automatic detection of spots and an additional window.*

«cursor information»

The third parameter «Min Area» sets the minimum spot area, all the spots having an area below this value will not be identified. Although carried out pre-processing of images, the program sometimes in separate areas of the image gave a not entirely accurate outlines of spots. To eliminate these drawbacks the form of individual spots additionally corrected manually.

Using the additional manual processing of individual sections of the image led to the fact that the construction of a common synthetic images (maps) greatly increased the size of the final that had to be taken into account in the subsequent analysis.

Then in the second stage of image processing is carried out to collect information about the spots and build three-dimensional models based on them. Analysis was performed more spot on three main parameters: the intensity, area and volume of spots.

Intensity - spot indicates the degree of staining compared to the background; at the same time it takes the value of the most highly colored pixels of the spot and the lightest area nearest the background surrounding the spot.

The area of the spot - this option calculates the average value of the staining intensity. Since pinpoint spot boundaries automatically is often difficult, the number of spots is assigned a large area, and the index value will be taken at the rate of 75% from the spot rate. Area expressed in mm^2.

The volume of spot - the indicator is calculated from the spot area, calculated strictly on the stroke line stain. The final three-dimensional model are sets of peaks, thus, the higher the intensity, the higher the peak, and the greater the protein concentration in this fraction. The peak height is taken for 75% of the stain intensity. The basic steps in solving this problem is shown in Figure 7.

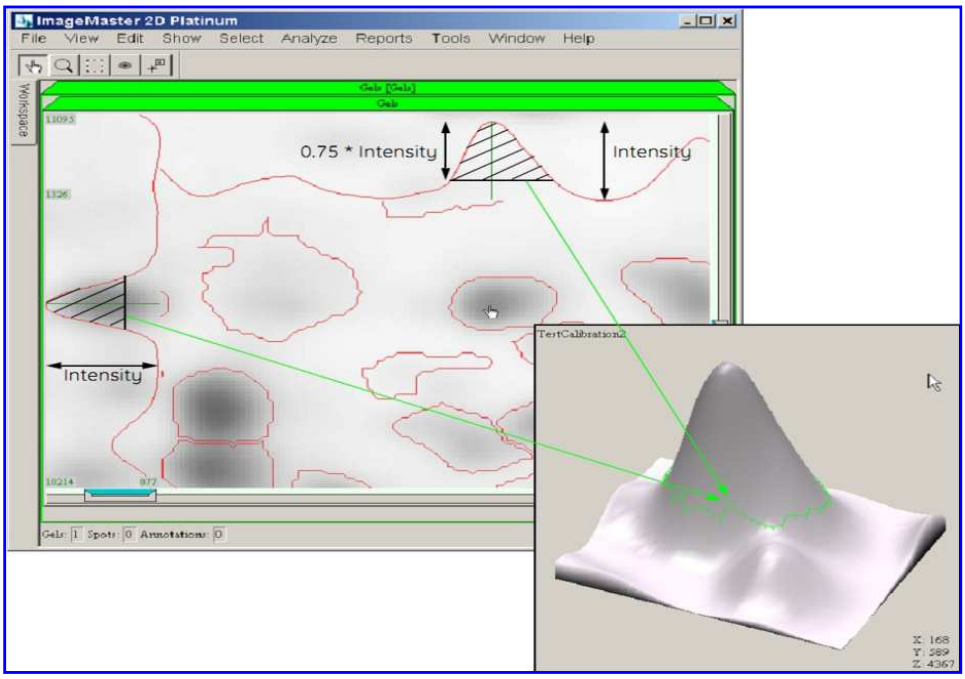
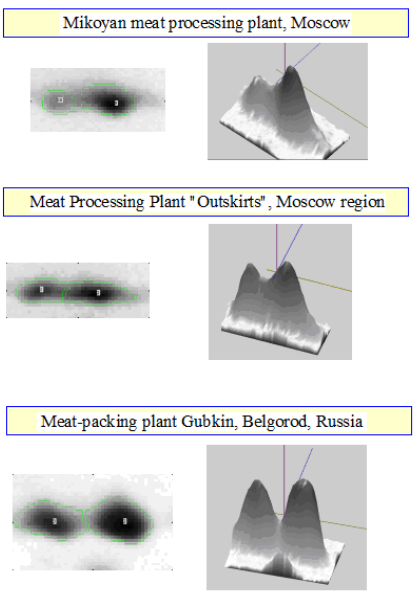


Figure 7. *The basic steps in the task of building a three-dimensional model of the investigated area of the image (indicated by arrows: the difference in the calculation of intensity parameters, area and volume (100 * 75), three-dimensional model of the spot, is presented in the form of a peak, which displays the shape and volume of the spot).*

Thus, in principle, a comprehensive computer densitometry on a number of species-specific protein markers can significantly increase the accuracy of the original species of meat raw material in the final product.

3.5. The Results of the Several Potential Biomarkers in Samples of Sausage Products

As evidenced by the results of the comparative proteomic analysis of sausage samples "Doctor", as well as beef samples and pork used in the preparation of "Doctor" sausage identified mioglobin fraction suitable for use as a biomarker by which to judge the ratio of these types of meat raw material in the final product. Accordingly, this section of the work was an attempt made to enter into the practical implementation of the example of a comparative analysis of three samples of the sausage "Doctor's" made by different manufacturers. For this purpose, a quantitative analysis was conducted mioglobin fractions on DOE samples DK1, DK3 and TPC2. The results are summarized in Figure 8.



The content of raw meat in the samples boiled sausages according to computer densitometry, %

Meat Processing Plant	Pork	Beef
1 Gubkin	46	54
2 Mikoyan	34	66
3 Outskirts	45	55

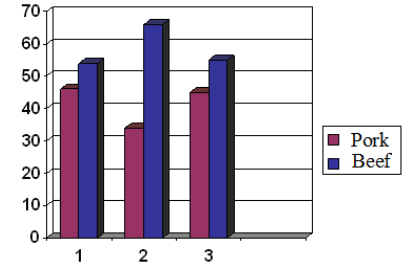


Figure 8. *Summary of the results of quantitative densitometry and subsequent computer analysis mioglobinovyh fractions on DE protein extracts obtained from samples of sausages "Doctor," produced by the following manufacturers: DK1 - meat packing plant Gubkin (Belgorod region.); TPC2 - Mikoyan meat processing plant (Moscow); DK3 - Meat Processing Plant "Outskirts" (Moscow reg.).*

As can be seen from the data value for pork and beef samples myoglobins in DK1 and DK3 were practically the same, whereas in the sample TPC2 this was significantly altered ($p<0,05$; when $n = 5$ to U-Mann-Whitneyratio) [6].

4. Conclusion

Thus, the quantitative analysis of potential biomarkers identified by proteomic technology in the sausage product samples (like mioglobinovymi fractions) is able to provide important information for assessing the quality of these products, including the identification of the presence of non-muscle protein supplements. As a consequence, it is possible to think that the expansion of proteomic studies of meat products and various raw materials used for their production, will allow based on the identified potential biomarkers (such as myoglobin, muscle enolase, troponin I, myosin light chains of type 1, muscle carbonic anhydrase) to create the whole panel of new effective methods of quality control of meat products.

Using proteomic strategies in the study of molecular mechanisms of quality indicators of raw meat is an important step towards the production of high quality animal products and its more stable production.

As a result of this work it was formed by a single methodological approach to the determination of the amount of muscle protein structureless cooked product by two-dimensional electrophoresis followed by the identification of time-of-flight mass spectrometry, confirming marker proteins.

From the research shows that the use of proteomic technologies along with access to new knowledge about the muscle proteins opens the way for the creation of new effective methods of control over the quality of meat products, including, in particular, the definition of the content of the final products of muscle proteins and their species.

The experimental data can be used for the construction of proteomic maps of proteins native meat raw materials used for the manufacture of domestic meat products.

Acknowledgements

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