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METHODOLOGY FOR THE IDENTIFICATION OF BIOACTIVE AND MARKER PEPTIDES IN THE ORGANS OF CATTLE AND PIGS

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Keywords: *bioactive peptides, biomarkers, aortas, hearts, proteomics, LC–MS/MS*

Abstract

The development of general conception methodology for the meat-based functional food compositions is especially relevant today due to the growing consumers' interest and attention to their health. This category of these food-products is intended for personalized nutrition of various age groups in the population, taking into account fortification of the food with nutraceuticals and with functional and metabolically active ingredients obtained from animal and vegetable source. Therefore, it was necessary to develop a certain tool for reliable identification of free peptides from the offals (by-products like hearts and aorta from Sus scrofa and Bos taurus) and from the ready-to-consume meat food (canned food) based on the free peptides, which food is potentially targeted to help with some issues in the human body. The authors proposed the methodology for identification of peptides weighing less than 5 kDa. This methodology has a row of significant advantages, such as a short time of analysis (90 minutes) and the possibility to prepare a large number of samples simultaneously (n = 16). Analysis of bioactive peptides (BAPs) was performed by liquid chromatography combined with time-of-flight mass spectrometry (Agilent 6545XT AdvanceBio LC/Q-TOF). The marker peptides were detected by a triple quadrupole mass spectrometer (Agilent 6410 Triple Quadrupole LC/MS). All peptide sequences were defined with the help of mass spectrometric data processing databases like PepBank, BioPep, AHTPDB. In this work from 39 to 269 peculiar soluble peptides were found, with an extraction level of 0.17–0.23%. The main fraction consisted of short peptides less than 1000 Da (71.0–98.0%). In experimental samples of pork hearts and arteries 7 peculiar marker peptides were identified. FFESFGDLSNADAVMGNPK peptide obtained from the β-hemoglobin protein is of a special interest, as this peptide showed the maximum intensity of a signal. Presumably, this peptide can serve as an indicator of the blood presence in the finished food product. So it can serve as an assessment tool of bleeding degree of meat raw. For pork aortas a specific peptide TVLGNFAAFVQK was isolated from serum albumin, which turned out to be stable during heat treatment. This is also important for assessment of meat food that are subjected to high thermal exposure.

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Introduction

The development of new technologies and recipes for meat food that have targeted positive or corrective effect for human body are quite relevant today. The bioactive peptides (BAP), found in the food, feature these useful properties. BAPs are natural or derived synthetic food components. In addition to their nutritional value, they also provide physiological effect on the human body [1]. The length of BAPs varies within the range of 2–20 amino acid residues. Some of BAPs contain more than 20 amino acids in their composition. BAPs are inactive within the sequence of the parent protein. Only low molecular weight peptides provide a positive effect on the human body. BAPs are formed in the human body when these proteins are exposed to digestive enzymes [2], to plant and bacterial proteases [3–6]. BAP can cause spot and physiological effects in the digestive tract by their absorption through the intestines, where they subsequently enter the blood-circulatory system, being unchanged [7]. BAPs have been identified in a wide row of foods, including plant, dairy, and meat products. For example, muscle peptides of cattle, pigs, horses, poultry (chicken, turkey), some fish species, etc. were identified. It was shown that dietary BAPs have a wide range of physiological functions, including antihypertensive [8], immunomodulatory, antimicrobial, antithrombotic antioxidant effects (milk [9], wheat [4], potatoes [10], and mushrooms [11]), opioid and hypocholesterolemic effects [12]. Peptides with similar properties were also found in hydrolysates of meat and fish proteins [13, 14]. The isolation of BAPs after bacterial fermentation of milk proteins

was described [15]. However, microbial fermentation of meat proteins proved to be less efficient [16].

At present, due to the complex composition of food products, it is quite difficult to guarantee their personalization. Due to the use of a wide range of additives, the importance of monitoring indicators of identification characteristics is increasing. Especially for food product composition authentication. Such identification is relevant for identifying undeclared or rarely used types of meat raw materials in product formulations. Such raw materials include, for example, pork heart and aorta. To identify such raw materials, various methods are used, including new approaches in proteomics, metabolomics, peptidomics, which are now considered to be one of the most efficient methods. The aim of the research was to develop a fast and reproducible method for the isolation of free peptides from meat raw materials and finished food products with their separation by the LC-QTOF/MS method, identification of the peptide profile below 5 kDa. Using bioinformatics databases, to determine BAPs, potentially able to provide a positive effect on a human body. Also, part of the work in this publication is directed to the search for marker peptides, using previously developed searching protocols [17], in order to evaluate the other types of tissues (offals), which are deliberately used to deacon the food, in particular the canned food.

Objects and methods

The objects of research were: beef and pork aortas and hearts, which, after trimming and grinding in a cutter (50 liters; *KG Wetter 258/1336*), were transferred to 10 ml falcons and stored in a freezer at a temperature of minus 43 °C until the beginning of the analysis. The samples were frozen to preserve protein fraction. For analysis the innovative meat canned product "Healthy heart" (HH) was used; this canned food contains tissues of a pork myocardium (heart) and aorta, processed as described below [18] (can No. 1 weighing 100 g, ingredients: pork hearts 61.9 g, pork aorta 20.6 g, potato starch 3.66 g, table salt 0.37 g); canned meat "Pork heart in own meat juice", produced in accordance with GOST R55477–2013[1] (can No. 8 weighing 325 g, ingredients: fresh pork heart 260 g, beef fat 37 g, wheat flour 9.4 g, dried carrots — 5 g, dried onion — 5 g, salt, black pepper).

Extraction of free peptides from analytes.

On the day of analysis, 500 µl of bicarbonate buffer was added to 100 mg of the taken sample, and the mixture transferred to special tubes filled with plastic balls (300 mg). The mixture was homogenized on a MagNA Lyser device (Roche, Switzerland) for 30 s at 7,000 rpm without overheating the samples. Due to the presence of the plastic balls, tissue cells were destroyed almost instantly, thus releasing peptides and proteins suitable for subsequent purification, extraction and analysis. The mixture was centrifuged at 13,500 rpm for 10 minutes at 4 °C, and 400 µl of the supernatant was collected into Amicon filtration tubes (Merck KGaA, Darmstadt, Germany) with a cutoff by mass of 10 kDa in order to purify peptides from large proteins. The obtained mass was filtered in a centrifuge (13,500 rpm, 10 minutes at 4 °C) and the filtrate was collected in a volume of 300 µl. Further, in the obtained peptide fraction, the total protein was measured using a fluorimeter Qubit (Thermo Fisher Scientific, USA), following the manufacturer's protocol. Next, 5 µl of 20% formic acid was added to the samples to stabilize the protein components and improve ionization during chromatographic analysis. 150 µl of the samples were transferred into special vials with inserts and placed in a chromatograph for identification of peptide sequences (Figure 1).

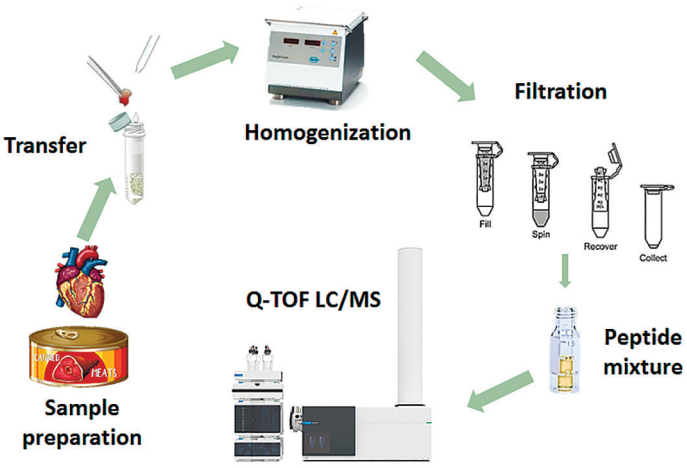


Figure 1. Protocol of samples preparation

Obtaining a profile of peptide fractions using LC/Q-TOF MS

Peptide analysis was performed on the Agilent 1260 Infinity system (Agilent Technologies, USA) coupled to a time-of-flight mass spectrometric detector Agilent 6545XTAdvanceBio LC/Q-TOF (Agilent Technologies, USA). A Poroshell 120 EC–C18, the reversed-phase analytical column (2.1*100 mm, 1.8 µm) and a ZORBAX Extend-C18 Analytical Guard Column (4.6*12.5 mm, 5 µm) were used. The column temperature was maintained at 50 °C. The mobile phase, H_2O (A) and ACN (B), that contained 0.1% (v/v) formic acid (Sigma Aldrich, USA), was pumped at a rate of 0.4 ml/min, and the injection volume was 5 µl. Chromatography was performed in a linear gradient: 1% of solvent B over 3 min, 1% to 35% of solvent B over 19 min, 35% to 50% of solvent B over 4 min, 50% to 98% and then return to 1% of solvent B for 3 min. The analysis was performed in MS mode with full scanning within the range from 100 to 1700 m/z. For the collision cell, a software algorithm for obtaining MS2 ions was chosen (Table 1), based on the collision energy increase dependence on the ion mass and charge. This algorithm made it possible to obtain the maximum number of *y* and *b* ions within the spectra, according to which the amino acid sequence of

[1] GOST R55477–2013 «Meat cans from by-products. Specifications». Moscow: Statdartinform, 2014. — 19 p.

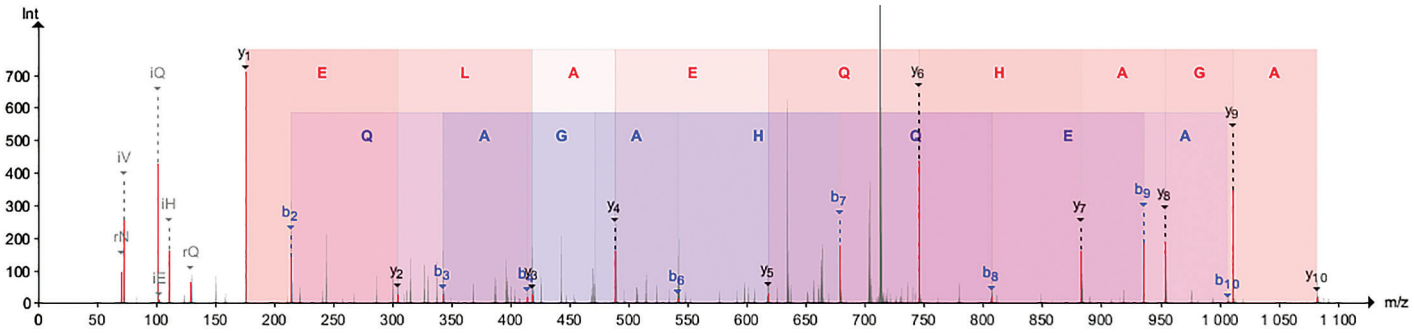


Figure 2. Mass spectrum identification of AGAHGEALE peptide by y-ions

BAPs was ranged and identified. It was decided to conduct a full analysis of 30 minute long. This duration provided for the maximum separation of the protein components on the column.

Table 1. Software algorithm used for the collision cell in QTOF 6545XT

Charge	Slope	Offset
1	3.8	3
2	3.1	1
3	3.6	–4.8
>3	3.6	–4.8

In this work we used the following materials and substances: acetonitrile for chromatography (≥99.9% Sigma Aldrich, USA); water for chromatography, deionized (18Ω), obtained using a Milli-Q Merck water purification system (Millipore); methanol for chromatography (chromatographic purity, LC–MS); formic acid (≥95%, Sigma Aldrich, USA); bicarbonate buffer (50 mM, pH = 8.2). MassHunter Workstation software from Agilent (version B.08.01) was used for primary processing of all samples. Processing of chromatograms for peptide identification was performed using the DeNovoGUI program [19]. This program provides a user-friendly open source GUI to run Novor, DirecTag, PepNovo+, and pNovo+ (beta) *de novo* sequencing algorithms on Windows. In each sample, free peptides were identified with the help of the Novor algorithm (Figure 2). The resulting amino acid sequences of the BAPs samples were compared with the PepBank [20], BioPep [21], and AHTPDB [22] electronic databases. In the work only those peptides were demonstrated that featured a homologous similarity with known biologically active peptides.

Selection of marker peptides with the help of LC–MS/MS

Proteins were extracted from the samples and digested with trypsin based on previously published studies [17]. Peptide mixtures were analyzed by LC–MS with a three-quadrupole mass spectrometer (6410, Agilent Technologies, Santa Clara, California, USA) [23]. To select potential markers of meat raw materials, the peptides were selected which length exceeded six amino acids. These peptides were presented in the review [24]. Using bio-modeling in the Skyline software, theoretical protein digestion was performed and SRMs list per each peptide was obtained [25].

Statistical analysis

STATISTICA 10.0 software was used for statistical analysis. Data were extracted from bioprograms in Microsoft Excel (USA).

Results and discussion

Analysis of the peptide fraction in the samples

The peptides were totally extracted within the range of 0.17–0.23% of the total sample. The highest content of free peptides was noted in the HH product (0.23%), which is explained by the partial destruction of proteins and the formation of new peptide sequences due to the conditions of the canning food technology (the cans are sterilized at a temperature of 115 °C — as described [17]). The rate of peptides extraction from aortas without heat treatment was equal to 0.17–0.18%. These data are lower by 0.01–0.03% than for the heart muscle of the same animal species. The rate of extraction (recoverability) of free peptides from all samples during heat treatment was significantly higher ($p<0.05$-), due to the partial denaturation of proteins being exposed to positive temperatures. As a result, from 39 to 269 free peptides were identified in the studied samples (Figure 3). In raw tissues a fairly low amount of peptides was isolated. Their least amount was contained in the beef heart, i. e. only 39 peptides were extracted. Thermal processing of the food products led to an increase in the total number of protein molecules by an average of 180%, which increases the chance of finding the BAPs.

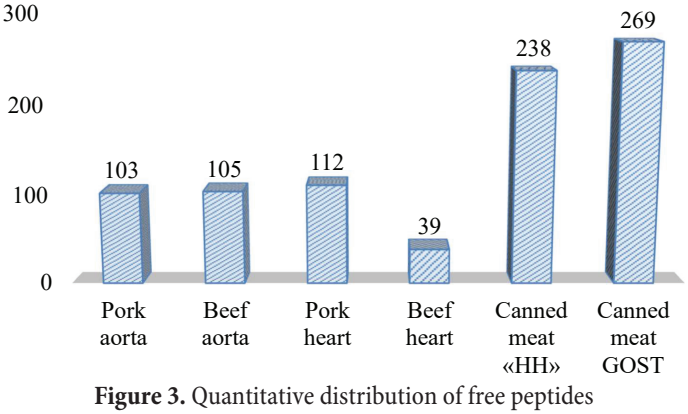
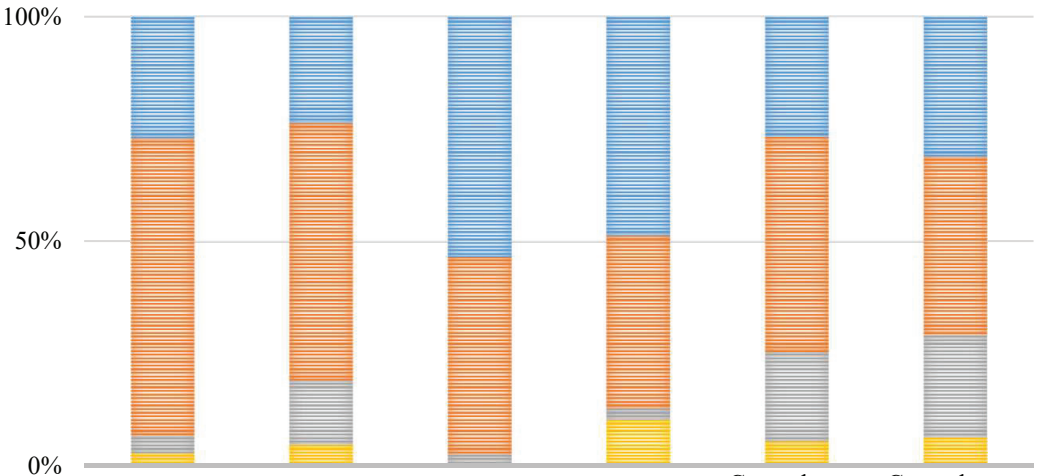


Figure 3. Quantitative distribution of free peptides in the analyzed samples



	Pork aorta	Beef aorta	Pork heart	Beef heart	Canned meat «HH»	Canned meat GOST
100-500	27%	24%	54%	49%	27%	31%
500-1000	66%	57%	44%	38%	48%	40%
1000-1500	4%	14%	3%	3%	20%	23%
≥ 1500	3%	5%	0%	10%	5%	6%
peptides, %	0,18%	0,17%	0,21%	0,19%	0,23%	0,23%

Figure 4. Mass distribution of peptides in the samples, expressed in% of the total value

The diagram of mass distribution (Figure 4) shows that in the analyzed samples predominantly short peptides were detected — less than 1,000 Da (71–93%). At the same time, 7, 3, and 5 peptides above 1,000 Da were found in beef and pork hearts, as well as in pork aortas; but in beef aortas their number increased to 20. Moreover, a total of 206 peptides with molecular weights less than 1,000 Da were found, but only 119 peptides were isolated in beef. The obtained data confirm the correctness of the previously made choice of pork raw materials as a potential source of functional peptides. Heat treatment led to the following change in the peptide profile in the ready-to-consume food products. In a sample of canned meat, 16, 62, 108, 83 peptides were found within the range of molecular weights above 1,500; 1,000–1,500; 500–1,000; 100–500 Da, respectively. The peptide profile of the innovative product looks as follows: 12, 48, 114, 64 peptides.

Analysis of bioactive peptides

In beef heart, the GVKYTA**QG**VAAGGMAVRR peptide attracted attention. Previously, it was suggested that **QG** amino acid residues may be a factor contributing to radicals absorption [26]. Antioxidants are known to be beneficial to human health because the antioxidants can protect the body from molecules known as reactive oxygen intermediates (ROIs), which can attack membrane lipids, protein, and DNA. Lipid oxidation can cause deterioration in food quality and shorten the shelf life of foods, while the consumption of foods containing lipid oxidation products is associated with various diseases, including cancer, Alzheimer's disease, diabetes, and cardiovascular disease [27]. No peptides with such residues were found in other samples. One field of particular interest for protein-membrane interactions is the binding of proteins to membrane-bound cholesterol. The binding led to the way of its recognition by cholesterol /peptide interaction (RCPI) [28]. Cholesterol interacting peptides are arranged by the general formula -L/V–X1–5-Y-X1–5-R/K, where X1–5 can be any amino acid sequence of one to five residues long. For example, a leukotoxin produced by the pathogenic bacterium *Aggregatibacter actinomycetemcomitans* shows cholesterol-specific binding and contains two sequences, **L**EEYS**K**RFKK and **V**DYLK**K** [29]. The peptide **V**PPKG**R** was found in the beef aorta, and **L**YPP**K** was found in the pork aorta. Analysis of the Pepbank database revealed the sequence of the EMVLGPP**V**PP**K**RGTVV peptide associated with cardiovascular disease. Peptides **L**LL**R**, LLLRLL**K** and **L**RRLA**K** were found only in the pork heart. This amino acid sequence was found in the R**L**LLRRL**R**R peptide, which is 98% associated with carcinogenic and 68% cardiovascular diseases. The peptide V**L**VYPCY**R**APK was found in pork heart. In beef aorta two peptides were identified: **V**GCYS**R**, V**L**PPYVY**R**, which feature cholesterol-binding properties. Dairy peptides IPP and VPP, which are angiotensin-converting enzyme (ACE) inhibitors, are described in the scientific literature [30]. No such peptides were found for cardiac muscle. In the pork aorta, the VPPDK peptide was found, which may have the same properties as the VPP tripeptide. Previously, pork hearts and aortas were found to possess lipid-lowering and anti-inflammatory effects [31, 32] and cholesterol-binding activity [33]. The studies carried out here confirm the presence of peptides associated with such properties. In the new product, HH pate, 6 peptides were identified that potentially have cholesterol-binding properties. These are **L**CDFYN**K**, **L**GADYT**K**, VPYH**L**AAA**R**, **L**EYFSQ**K**, LLAYTT**K**KK, LFDNYNT**LK**. Only one peptide, VP**V**VYG**K,** was found in the control sample of the meat product.

Marker identification of objects of research

Previously published articles have identified marker peptides for pork hearts and aortas [34] and stable tissue-specific proteins [35]. In this study, thermostable peptides for hearts and aortas were analyzed in the ready-to-consume product: innovated meat canned product "Healthy heart" (HH) and canned meat "Pork heart in own meat juice". The signal-to-noise ratio (S/N) became the selection criterion for the obtained thermostable peptides. It must be greater than 3 units. After bioinformatics processing of the results in mixtures containing pork muscle tissue, 6 various proteins were found (Table 2), where marker peptides showed S/N higher than 3 units at least in one sample.

The foremost represented peptide features the sequence FFESFGDLSNADAVMGNPK [23] (S/N — 11.53±3.36; 9.30±4.16) made up of β-Hemoglobin protein. Serum albumin marker TVLGNFAAFVQK [29] was found in Pate NN, which marker is specific for pork aortas [34]. The S/N index for this marker is 19% lower in the canned meat. The data are confirmed by the fact that in the hearts and aortas there are many proteins associated with the formation of the blood-circulatory system in the animal body. Further, a number of biomarkers can be identified that confirm the presence of pork. Markers were obtained from Myosin 2 and 7, from serum albumin and Trifunctional enzyme subunit. All 7 bi-markers showed the best detection rate and specificity for the analyzed samples. The remaining peptides did not pass the threshold of 3 S/N units and thus were excluded from the Table 2.

Conclusion

The research resulted to development of an express method that allows for the isolation of free peptides from meat raw materials and ready-to-consume food products in 1.5 hours. The developed method includes preparation of up to 16 samples simultaneously. Measurement of total soluble protein showed an extraction level of 0.17–0.23%, which indicates the efficiency of filters application for picking peptides with molecular weights of 1,500 Da and below. In the objects of research from 39 to 269 peptides were found. All peptide sequences with a non-zero score per each sample were established with the help of software, used for processing mass spectrometric data obtained from chromatograms. The application of the above described method allowed identifying 5 peptides with cholesterol-binding potential in the experimental food product, and only one peptide was found in the control sample. For the first time the application of this method of multiple reactions monitoring made it possible to identify up to 7 most appropriate peptides — the biomarkers of pork muscle tissue. The peptide FFESFGDLSNADAVMGNPK (S/N — 11.53±3.36; 9.30±4.16) from the β-Hemoglobin protein was chosen as the best candidate marker for the food products potentially containing hearts. To determine the presence of vascular tissue in the food product, the peptide TVLGNFAAFVQK (S/N — 8.54±0.75; 6.35±0.95), obtained from serum albumin, can serve as a marker. The developed method is universal for comparing peptides and identifying the most appropriate marker peptides specific for tissues of any mammal.

Table 2. Comparison of marker peptides according to the characteristics of two samples of ready-to-consume food products

Protein	Marker peptide sequence	Pate NN, (S/N±SD)	Canned meat, (S/N±SD)
β-Hemoglobin	FFESFGDLSNADAVMGNPK	11.53±3.36	9.30±4.16
Myosin-2	TLAFLFSGAQTGEAEAGGTK	8.73±0.54	7.07±2.15
Serum albumin	TVLGNFAAFVQK	8.54±0.75	6.35±0.95
	EVTEFAK	8.39±0.84	9.80±1.79
Trifunctional enzyme subunit	TVLGAPEVLLGILPGAGGTQR	6.76±1.14	15.63±2.77
Myosin-7	LLSNLFANYAGADTPVEK	3.03±0.61	6.00±0.98
β-Hemoglobin	VNVDEVGGEALGR	2.81±0.29	4.56±0.43

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