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**Editorial Office:**
Federal State Budgetary Scientific Institution
"V.M. Gorbatov Federal Research Center for Food Systems of Russian Academy of Sciences"
Talalikhina str. 26, Moscow, Russia, 109316
Tel.: +7–495–676–95–11 extension 300
e–mail: a.zakharov@fncps.ru

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# TECHNOLOGICAL APPROACHES TO THE EXTRACTION AND PURIFICATION BY ULTRAFILTRATION TECHNIQUES OF TARGET PROTEIN MOLECULES FROM ANIMAL TISSUES: A REVIEW

**Elena A. Kotenkova*, Ekaterina K. Polishchuk**
V. M. Gorbatov Federal Research Center for Food Systems, Moscow, Russia

**Keywords:** *protein stability and solubility, kosmotrope, chaotrope, amino acid, sugar, polyhydric alcohol, detergent, osmolyte*

***Abstract***

*Effective isolation and purification of protein is a great challenge nowadays. The key aspect is protein stability and solubility, which primarily depend on protein structure and its amino acid sequence. Manipulations with pH and ionic strength are the first attempts to increase protein stability and solubility. Different additives that are allowed or prohibited in the food industry are applied for overcoming protein aggregation. Sugars, polyhydric alcohols and amino acids are the most attractive among them. Trehalose, glycerol, arginine, glycine and proline demonstrated outstanding properties that make them perspective for application during isolation and purification of proteins singly or in combination with each other or other compounds. However, the algorithm of effective isolation and purification of protein could be significantly varied depending on its structure.*

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## Introduction

Specific proteins are in great demand in laboratory practice, pharmacy and food industry. Most protein therapeutics currently on the market are recombinant and developed to treat a wide variety of clinical indications, including cancers, autoimmunity/inflammation, exposure to infectious agents, genetic disorders and other diseases [1,2]. The standard proteins, enzymes, antibodies, etc. are amongst the most widely used research reagents but often their quality is inadequate and can result in poor data reproducibility, including due to non-sufficient purification or loss of structure or activity during these processes [3]. Plant and animal (dairy, egg, and meat) proteins are widely used in the food industry [4], including therapeutic food additives based on tissue-specific proteins [5].

Proteins are polypeptide structures consisted of unique sequences of amino acids. Side amino acid chains could be positively or negatively charged, form four different levels of complexity (primary, secondary, tertiary, and quaternary structure) by hydrogen, ionic and hydrophobic bonds or disulfide bridges, which also contribute to stabilization of protein structure [6, 7, 8]. Amino acids could be hydrophobic or polar, basic or acidic, forming net charge and solubility of protein. The acid/base properties of proteins are essential in biochemistry [9], as well as isoelectric point value prediction [10]. Various methods of isolation exist and have been developed for certain purposes based on the unique characteristics of each protein, such as the amino acid composition, sequence, subunit structures, size, shape, net charge, isoelectric point, solubility, heat stability and hydrophobicity [11]. The aim of the article is to review the technological approaches to the extraction and purification by membrane techniques of protein molecules from animal tissues.

## Isolation of target protein molecules from animal tissues

Tissue homogenization is a key step for molecular biology studies [12], where chemical or mechanical approaches are chosen depending on a purpose or type of a target biomolecule. Mechanical/physical methods for disrupting samples include grinding, shearing, beating, and shocking, which could be combined with chemicals for process intensification [13]. A wide range of laboratory, semi- and industrial equipment is successfully used, but the final approach is based on the properties and further use of a target biomolecule. Most proteins are sensitive to high temperature and aggressive chemicals. Moreover, for some purposes it is necessary to obtain proteins with the preserved biological activity, and a lot of chemicals are not permitted in pharmaceuticals or food additives.

Innovative techniques for protein extraction are intensively developing, including the aqueous two-phase system, subcritical water extraction, enzyme-, microwave- and ultrasound-assisted extraction, pulsed electric field and high voltage electrical discharge extraction, high hydrostatic pressure-assisted extraction, and supercritical carbon dioxide techniques [14]. However, water-based extraction remains the cheapest one, where the most important is knowledge of the value of the isoelectric point (pI) of target proteins. The pI is the pH of a solution, at which the net charge of a protein becomes zero, the negative and positive charges are balanced, reducing repulsive electrostatic forces, and the attraction forces predominate, causing aggregation and precipitation [15]. Modification of pH by alkali or acid leads to proteins become negatively or positively charged, resulting in electrostatic repulsions between molecules and hydration of charged residues, contributing to the solubility of proteins [16]. Moreover, salts could also stabilize protein molecules [17]. Based on the known pI value of a target protein group, as well as on predominance of acidic or basic amino acid residues in protein structure, it is possible to predict the advisable pH and ionic strength of solution for intensification of the extraction process. Summarizing, if pI > buffer pH, lower the pH by 1 unit, if pI < buffer pH, raise the pH by 1 unit, if pI=buffer pH, try both ways [18].

**Purification of animal proteins by membrane techniques**

Membrane technologies represent an efficient and environmentally friendly option for the separation, fractionation, and purification of bioactive compounds from different animal tissues [19]. The most widespread use of membrane technologies is in the dairy industry [20]. Membrane processes are extremely diverse; various types of filtration processes, membranes, polymers for membrane manufacture are used depending on purposes. The ultrafiltration is a commonly used approach for protein separation, fractionation, and purification. However, adsorption, molecule aggregation, and denaturation are the main problems that a scientist faces during the ultrafiltration process of proteins in the native form [21,22]. Aggregation is a general term that encompasses several types of interactions or characteristics. Protein aggregates can be a result of various mechanisms and can be classified in several ways, including soluble/insoluble, covalent/noncovalent, reversible/irreversible, and native/denatured. For protein solutions, the presence of aggregates of any type is usually considered undesirable for the reason that aggregates can reduce the efficiency of purification and separation of protein-peptide mixtures or protein solutions [23]. Aggregation, as well as formation of a highly concentrated layer at the border of the filtration membrane or adsorption to it significantly interferes with filtration (Figure 1).

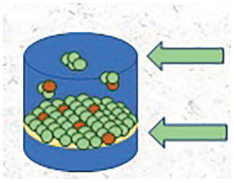


**Figure 1.** The main challenges during protein ultrafiltration

The easiest way to prevent aggregation and adsorption during ultrafiltration is the dilution, followed by implementation of diafiltration (feeding solution with the same pH and ionic strength) in order to maintain constant pH and ionic strength [24–26]. Cromwell et al. noted that the selection of the membrane material, the optimal pore size (throughput) and the rate of transmission of the protein solution through it are important aspects in preventing adsorption of aggregates on the membrane surface and affect the efficiency of purification or separation of target proteins [23].

Another way is to use agents that may promote protein solubility, such as kosmotropes, weak kosmotropes, chaotropes, amino acids, sugars and polyhydric alcohols, detergents [27].

Kosmotropic salts have a higher salting-out effect according to the Hofmeister series. They act as a protein stabilizer (usually small ions, low polarizability), and as polar water-structure makers [28]. For weak kosmotropic salts, such as NaCl and KCl, the recommended initial concentration is 300mM and 200mM, respectively, the recommended concentration range is 0–1M [27,28]. Strong kosmotropic salts are $MgSO_4$, $(NH_4)_2SO_4$, $Na_2SO_4$, $Cs_2SO_4$, with the recommended concentration range from 0–0.2M to 0–0.4M [27, 28]. It was also reported that potassium citrate in a concentration of 0.1M was effective in solubilizing four proteins, such as proline-rich antigen 2, C2 domain-containing protein (in combination with mannitol), unnamed apical complex protein, which previously appeared totally insoluble [29].

Chaotropic salts have a higher "salting-in" effect according to the Hofmeister series, but they can reduce protein–protein interactions by shielding charges and by preventing the stabilization of salt bridges [28]. The recommended concentration range for such salts as $CaCl_2$, $MgCl_2$, LiCl, RbCl, NaSCN, NaI, $NaClO_4$ and NaBr varies from 0–0.2 M to 0–0.8 M [27], the recommended initial concentration for $CaCl_2$ and $MgCl_2$ is 10–50 mM, while for NaI — 0.2 M [28]. Urea, guanidine HCl, N-Methylurea, N-Ethylurea and N-Methylformamide belong to mild chaotropes. Guanidine HCl and urea are the most common denaturing agents for protein denaturation and then renaturalize the protein to its active form [30]. To form β-sheets, the protein–protein interaction must be larger than the hydrogen bond interaction formed between urea and protein, which slows down the aggregation process in urea. The two different behaviors of urea indicate that it can affect the aggregation in a nonmonotonic way [31]. Therefore, the low concentration of urea in many cases has also been used to

solubilize inclusion body aggregates, while the use of the high concentration of chaotropes like urea and guanidine hydrochloride results in complete denaturation of these existing secondary structures and often leads to aggregation of protein molecules during the refolding process [32]. The recommended initial concentration for urea and guanidine HCl is 0.5M, the recommended concentration range is 0–2M [28]. However, urea or guanidine HCl addition is a hard denaturation step [33] that is usually applied in proteomic studies, such as electrophoresis [34].

Non-ionic (triton X-100, tween 80 or 20, n-dodecyl β-d-maltoside (DDT), polyoxyethylene cetyl ether (Brij 56), n-octyl-β-d-glucoside (OG)), ionic (cetyltrimethylammonium bromide (CTAB), sodium lauroyl sarcosinate (Sarkosyl), sodium dodecyl sulfate) and zwitterionic (non-detergent sulfo betaine (NDSB), 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), zwittergent 3–14, lauryldimethylamine N-oxide (LDAO)) detergents are also used for prevention of protein aggregation [28]. The use of non-ionic or zwitterionic detergents at low concentrations (no more than 1%, usually recommended 0.1%) helps solubilize protein aggregates without denaturing the proteins [27,28,35].

Trimethylamine N-oxide (TMAO) forms direct attractive interactions with polypeptides, stabilizes collapsed conformations via a mechanism that is distinct from glycine and betaine. It was also proposed that TMAO stabilizes proteins by acting as a surfactant for the heterogeneous surfaces of folded proteins [36]. The recommended initial concentration for TMAO is 0.5M, the recommended concentration range is 0–1M [28]. Glycine betaine (GB) is a naturally occurring osmolyte that has been widely recognized as a protein protectant preventing protein aggregation, but it may have opposite effects on protein stability [37–39]. 1M glycine betaine was effective in solubilizing four proteins, for e. g. lanosterol 14-alpha demethylase [29].

Sugars and polyhydric alcohols are also widely used for prevention of protein aggregation, including glucose, sucrose, trehalose, lactose, glycerol, sorbitol, mannitol, xylitol, inositol [27,28]. Polyol and sugar osmolytes can perturb protein h-bonds to affect protein function [40] and stabilize the lattice structure of water, thus increasing surface tension and viscosity. They stabilize hydration shells and protect against aggregation by increasing the molecular density of the solution without changing the dielectric constant, the usually recommended amount varies from 10 to 40% [41].

A number of articles report on the stabilization of various biomolecules by trehalose (and in some cases, sucrose) [42]. Trehalose inhibits aggregation of lysozyme, insulin [43], as well as 0.75 M trehalose was effective in solubilizing 21 proteins, such as 14-alpha sterol demethylase Cyp51B, sensory transduction histidine kinase, putative (in combination with mannitol), sensor proteins, metallopeptidase domain protein, cytochrome P450 51, proline-rich antigen 5, etc. [29]. It is hypothesized that trehalose prevents the inactivation and aggregation of proteins at lower temperature and stabilizes the cell membrane by delaying the onset of phase shift from liquid crystal to gel state [42]. Both trehalose and sucrose induce a well-defined protein–protein distance, which could explain why these inhibit protein–protein interactions and associated protein aggregation, but superior anti-aggregation effect of trehalose could be also explained by the fact that local solvent structures are highly important for explaining the protein stabilization mechanism [44]. The structure-stabilizing effect of sucrose is conferred on the protein by the increase in the solvent cohesive force when sucrose is added to water in the solvent system [45]. Sucrose has been shown to inhibit IL-1ra dimer formation [46]. The recommended initial concentration for trehalose and sucrose is 0.5M, the recommended concentration range is 0–1M; for glucose and lactose, the recommended concentration range is 0–2M and 0.1–0.2M, respectively [27,28].

It was reported that glycerol prevented protein aggregation by inhibiting protein unfolding and by stabilizing aggregation-prone intermediates through preferential interactions with hydrophobic surface regions that favor amphiphilic interface orientations of glycerol [47]. It was also found that the preferential hydration of proteins in glycerol-water mixture minimized the surface of contact between proteins and glycerol to stabilize those native structures [48]. The recommended initial concentration for glycerol is 10%, the recommended concentration range is 5–40% [27,28]. Sorbitol in a concentration of 0.5M-2M was shown to demonstrate a negative influence on the unfolded form of lysozyme, thereby, stabilizing the native form [49], and 0.3 M sorbitol increased recombinant bovine sex determining region Y protein solubility [50]. Sorbitol has also been shown to reduce aggregation of nucleocapsid protein of rhabdovirus after its expression in *Escherichia coli*, which is likely due to exert its effect on folding by altering the structure and properties of water around the folding protein molecule [51]. It is also commonly used as an additive to promote refolding of solubilized proteins [32]. The recommended initial concentration for sorbitol is 0.5M, the recommended concentration range is 0.2–1M or 0–40%w/v [27,28]. The addition of 10% (w/v) mannitol to the buffer matrix resulted in a 4.2-fold decrease in the IgG4-N1 aggregation rate constant compared to that for the control condition [52]. Mannitol in a concentration of 0.5M could increase the solubility of seven proteins, such as sensory transduction histidine kinase, putative (in combination with trehalose), Hsp20/alpha crystallin domain-containing protein and unnamed apical complex protein (as component of complex buffer), etc. [29]. The recommended initial concentration for sorbitol is 2%, the recommended concentration range is 0–15%w/v [27,28]. Xylitol in a concentration of 0.1M could increase the solubility of metalloprotease 1 and lanosterol 14-alpha-demethylas [29]. The recommended initial concentration for xylitol is 0.5M, the recommended concentration range

is 0.2–1M or 0–30%w/v [27,28]; while for inositol, the recommended concentration range is 0–10% w/v [27].

The application of amino acids as anti-aggregation agents is in demand in the food industry and bioactive additives production. It has also been reported that after a compound is combined with an amino acid, the pharmacological activity of the compound is enhanced, water solubility is improved, and cytotoxicity is reduced [53]. Amino acids and derivatives thereof increase the surface tension of water in a concentration of 20–500 mM [41]. The summarizing information about amino acid application is presented in Table 1.

**Table 1. Amino acids used to stabilize proteins and to prevent aggregation [27,28]**

| Amino acid and derivatives thereof | Recommended initial concentration | Recommended concentration range |
|---|---|---|
| Glycine | 250 mM | 0.5–2 M/0.5–2% |
| Arginine L-HCl | 125 mM | 0–2 M |
| Arginine ethylester | 250 mM | 0–500 mM |
| Proline | 250 mM | 0–1 M |
| Potassium glutamate | 250 mM | 0–500 mM |
| Arginine L | 50 mM | 0–5M |

Among 15 amino acids tested, arginine exhibited the best results in preventing the formation of aggregates [54]. The hydrophobic surfaces present on the proteins interact with the hydrophobic surface presented by the arginine clusters. The masking of hydrophobic surface inhibits protein-protein aggregation [55]. Arginine in a concentration of 0.1 to 1 M is customarily included in solvents used for refolding the proteins by dialysis or dilution. In addition, arginine at higher concentrations, e. g., 0.5–2 M, can be used to extract active, folded proteins from insoluble pellets obtained after lysing *Escherichia coli* cells. It was shown that interactions between the guanidinium group of arginine and tryptophan side chains may be responsible for suppression of protein aggregation by arginine [56]. In general, arginine is found to interact with the aromatic and charged side chains of surface residues. In particular, arginine interacts with aromatic and charged residues due to the cation–π interaction and salt-bridge formation, respectively, to stabilize the partially unfolded intermediates. The self-interaction of arginine leads to the formation of clusters which, due to their size, crowd out the protein–protein interaction [57]. Arginine is also shown to form stacking and T-shaped structures with aromatic amino acids, the types of cation–p and N–H...p interactions, respectively, known to be important contributors to protein stability. The analysis also shows that arginine-arginine interactions lead to stable clusters, with the stability of the clusters arising from the stacking of the guanidinium part of arginine. The results show that the unique ability of arginine to form clusters with itself makes it an effective aggregation suppressant [58]. Arginine in a concentration of 10–500 mM demonstrated the inhibitory effects on the initial aggregation kinetics of bovine insulin [59], in a concentration of 0.75 M used for renaturation of lysozyme from hen egg white and the prevention of aggregation resulted in 94% recovery yield [43]. L-Arginine in a concentration of 0.375 M increased the solubility of six proteins, such as pentapeptide repeat family protein, LPPN Rv2270, membrane skeletal protein IMC1, TgDCX, unnamed apical complex protein in mixture with trehalose or as a component of the complex buffer [29]; in a concentration of 400 mM, it was the key player in the refolding of human glucose 6-phosphate dehydrogenase, preventing the aggregation of folding intermediate [60]. Arginine (in the form of hydrochloride salt Arg·HCl) is often used in formulations exhibiting high RSA (reversible self-association) and a propensity for aggregation; glutamate salt of arginine (Arg·Glu) was able to decrease the propensity of the mAbs (monoclonal antibodies) to aggregate, particularly at pH values closer to their pI [61]. It was also demonstrated that addition of L-Arg and L-Glu at 50 mM to the buffer could dramatically increase the maximum achievable concentration of soluble protein, preventing protein aggregation and precipitation, increasing the long-term stability and protecting from proteolytic degradation [62]. It was found that the protein solubility enhancement is related to the relative increase in the number of arginine and glutamic acid molecules around the protein in the equimolar mixtures due to additional hydrogen bonding interactions between the excipients on the surface of the protein when both excipients are present. The presence of these additional molecules around the protein leads to enhanced crowding, which suppresses the protein association [63]. It was also proposed that below 100 mM arginine acts like glycine, above 100 mM it shows destabilizing effects similar to guanidinium hydrochloride [64].

Glycine alone demonstrates two stages of stabilization. The first effect (at concentrations below 100 mM) is protein specific and is probably due to multiple direct interactions with the polar or charged side chains and the partial charges on the peptide backbone of the protein. The second stage (at concentrations above 100 mM) is similar to high charge density anions where it was ascribed to competition for water between the unfolding protein and the cosolute [64]. Glycine in a concentration of 100 mM is often used for preparing elution buffers [65–67]. Glycine stabilizes collapsed conformations of hydrophobic elastin-like polypeptides via a classical preferential depletion mechanism [36].

Glycine and proline showed a certain ability to stabilize hemoglobin [68]. It was proposed that proline with a concentration of >3 M behaves as an enzyme stabilizer as well as a protein solubilizing solute and forms an amphipathic supramolecular assembly and successfully thwarts the aggregation associated with the refolding of bovine carbonic anhydrase [43]. Experimental evidence suggests that proline inhibits protein aggregation by binding to folding intermediate(s) and trapping the folding intermediate(s) into enzymatically inactive, "aggregation-insensitive"

state(s) [69]. Proline contains a closed ring structure in its side chain which has a hydrophobic surface, which enables it to interact with proteins through hydrophobic interactions [43]. It has been suggested that multimeric forms proline may be responsible for its aggregation inhibitory effects [55]. Proline in a concentration of 0.5 M increases the solubility of four proteins, such as lanosterol 14-alpha demethylase, TgDCX, unnamed apical complex protein as a component of the complex buffer [29], it effectively inhibits protein aggregation during the refolding of bovine carbonic anhydrase [70]. Proline in a concentration of 0.2 M inhibits aggregation of Alzheimer's amyloid beta 1–42 (Aβ1–42) peptide; the effect of 0.5 M and 1.0 M concentrations was also studied [55]. Besides arginine, a positively charged amino acid (such as histidine and lysine) can inhibit aggregation [71], and arginine stabilized all three domains of IgG [72].

Many researchers use a comprehensive approach, preparing complex buffers with compounds that serve as a ligand to allow the protein to remain in a soluble conformation (metal or an amino acid); additives that reduce protein-protein interactions (chaotropic agents) or stabilize intra-molecular bonds (kosmotropic agents); compounds known to affect protein stability (charged amino acids, reducing agents, polyols and sugars); and, additives that significantly altered buffer or salt conditions [29].

## Conclusion

Proteins are complex biomolecules, each certain protein is a unique sequence of amino acids. During isolation and purification of proteins, its structure should be taken into account. When facing challenges accompanied isolation and purification of proteins, the first steps are changing the pH of the solution, the salt concentration or the salt. The easiest way to prevent protein aggregation and adsorption during ultrafiltration is the dilution, followed by implementation of diafiltration. However, it is often not enough. Another way is to use agents that may promote protein solubility, such as kosmotropes, weak kosmotropes, chaotropes, amino acids, sugars and polyhydric alcohols, detergents, as well as use combinations of these compounds.

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## AUTHOR INFORMATION

**Elena A. Kotenkova,** Candidate of Technical Sciences, Research Scientist, Experimental Clinic — Research Laboratory of Biologically Active Substances of an Animal Origin, V. M. Gorbatov Federal Research Center for Food Systems. 26, Talalikhina str., 109316, Moscow, Russia. Tel.: +7–495–676–92–11, E-mail: lazovlena92@yandex.ru
ORCID: https://orcid.org/0000–0003–1864–8115
* corresponding author

**Ekaterina K. Polishchuk,** Research Engineer, Research Scientist, Experimental Clinic — Research Laboratory of Biologically Active Substances of an Animal Origin, V. M. Gorbatov Federal Research Center for Food Systems. 26, Talalikhina str., 109316, Moscow, Russia. Tel.: +7–495–676–92–11, E-mail: e.politchuk@fncps.ru
ORCID: https://orcid.org/0000–0003–2719–9649

All authors bear responsibility for the work and presented data.

All authors made an equal contribution to the work.

The authors were equally involved in writing the manuscript and bear the equal responsibility for plagiarism.

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# INFLUENCE OF PEPTIDES FROM THE BURSA OF FABRICIUS IN BROILER CHICKENS ON THE FUNCTIONAL ACTIVITY OF LYMPHOCYTE SUBPOPULATIONS IN IMMUNODEPRESSIVE MICE

**Natal'ya A. Kolberg[1], Sergey L. Tikhonov[1],* Natal'ya V. Tikhonova[1], Leonid S. Kudryashov[2]**
[1] Ural state economic University, Ekaterinburg, Russia
[2] V. M. Gorbatov Federal Research Center for Food Systems, Moscow, Russia

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***Abstract***
*It is known that peptides inhibit the enzymes of viruses and are able to penetrate into cells by their embedding in the cell membrane, as a result of which the penetration of viruses into the host cell is blocked, which makes it possible to consider peptides as an alternative to antiviral drugs. In this regard, the demand for immune-boosting nutraceuticals and functional foods containing biologically active peptides is growing. The immunomodulating effect of the peptides were studied on the mice of the BALb/c line that suffered from experimentally induced immunodeficiency; the mice got injections of peptides isolated from the bursa of Fabricius (bursal sac) of broiler chickens. 5 groups of BALb/c mice were formed. The animals of the 1st group (control one) received physiological saline per os as a placebo, animals of the 2nd group got bursal peptides per os at a dose of 0.02 mg/kg per body weight, the mice of 3rd group (immunosuppressed) got saline per os as a placebo, the 4th group (immunosuppressed) was administered the bursal peptides per os at a dose of 0.02 mg/kg of body weight, the 5th group was held as the control one (immunosuppressed group). Blood for tests was taken on days 1, 7 and 14 of the experiment. The functional activity of neutrophils was determined by the method of spontaneous and induced chemiluminescence. Among the immudepressive animals (the 3rd group) on the 7th day the researchers observed a decrease in CD3+ by 55.3%, CD22+ by 83.7%, CD3+CD4+ by 51.9% and CD3+CD8+ by 54.6% in comparison with the intact (the 1st group). Administration of peptides to immunosuppressed mice (the 4th group) increases the number of subpopulations of CD3+ lymphocytes by 126.6%, CD22+ by 381.6%, CD3+CD4+ by 8.9% and CD3+CD8+ by 81.8% compared to immunosuppressed animals, receiving saline per os as a placebo (group 3). Similar results were obtained on the 14th day of the experiment. On the basis of the performed studies, it can be argued that the immunocompetent organs of broiler chickens (bursa of Fabricius) are a promising source of immunotropic peptides.).*

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## Introduction

Biologically active peptides (BAP) are considered to be the main products of protein hydrolysis. The activity of BAP depends on the sequence of amino acid, pf molecular weight and chain length, type and charge of the amino acid at the N-terminus and C-terminus, hydrophobicity and hydrophilicity of their spatial structure. They provide a positive effect on many systems of the human body, including the blood circulatory system, nervous, immune, gastrointestinal system and others. According to [1], the curative effect of bioactive peptides is achieved due to their antioxidant, antihypertensive, antithrombotic, immunomodulatory, antimicrobial, antiallergic, anti-inflammatory and other propeties.

Currently there is a growing demand for immune-boosting nutraceuticals and functional foods that contain immunomodulatory proteins of animal origin, in particular, BAP [2].

However, it is necessary to take into account the lack of correlation between the results obtained *in vitro* and the peptides functions observed *in vivo* due to their low bioavailability. Once ingested, the peptides must resist the action of digestive enzymes during their passage through the gastrointestinal tract and have to cross the intestinal epithelial barrier to reach target organs in an intact and active form. Thus, in order to understand better the physiological effects of bioactive peptides *in vivo*, more extensive studies of their stability and transport in the gastrointestinal tract, as well as the study of the mechanism of action, turned out to be necessary [3].

Peptides act as signal substances containing fundamental molecular information, peptides are able to penetrate cell membranes or reach intracellular targets [4].

The immunomodulatory effect of peptides allows them to be involved in cancer therapy. However, cancer immunotherapy so far could not improve the outcomes for most cases of "cold tumors", which tumors are characterized by low immune cell infiltration and an inner immunosuppressive tumor microenvironment. Increasing the sensitivity

of cold tumors to cancer immunotherapy by stimulating components of the tumor microenvironment is a strategy being pursued in the last decade. Currently, most of the drugs, used to modify the tumor microenvironment are the small molecules or antibodies. Small molecules exhibit low affinity and specificity in relation to the target tissue, and antibodies have certain disadvantages such as poor tissue penetration and high cost of production. Peptides do not have those disadvantages and, therefore, can serve as a promising material for immunomodulatory agents [5].

Particular attention should be given to immunomodulators based on peptides isolated from meat raw materials, tissues of immunocompetent organs of animals and poultry, in particular, glucosaminylmuramyl dipeptide (GMDP), registered under the name Likopid® [6], and also obtained from biological fluids, for example, from cow colostrum.

Biologically active peptides are found in raw meat, and for their greater accumulation the starter cultures of microorganisms and proteolytic enzymes are used. Using T-RFLP analysis, it was found that dry-cured and raw-smoked sausages made from horse meat using starter cultures contain short peptides of horse myoglobin, troponin-T, and muscle creatine kinase [7].

The immunocompetent organ in a chicken is the bursa of Fabricius (bursa of Fabricius, sac of Fabricius, or bursa), where plasma cells are formed, that synthesize antibodies. Removal of the bursa in poultry leads to inhibition of antibody biosynthesis. 20 peptides with immunomodulating properties have been isolated from the bursa [8]. The peptides isolated from the bursa inhibit the growth of cancer cells by discontinuance of the cell life cycle in the G1 phase, i. e. the peptides stop the process of formation of a new structure formed by the centriole of the mother cell — the primary cilium, which protrudes from the cell surface — this process is not initiated [9].

The source of peptides possessing antiviral and antitumor properties is cow colostrum, in particular, proline — this is polypeptide (PRP), or a transfer factor — a stimulator of lymphocytes (T-killers) proliferation, which cells feature cytotoxicity against tumor cells and virus infected cells. The daily intake of dry colostrum by healthy people for two weeks at a dose of 400 mg stimulates humoral and cellular immunity [10].

The aim of the research was to study the effect of immunotropic peptides isolated from the broiler chicken bursa of Fabricius on the decrease in the functional activity of lymphocyte subpopulations.

**Materials and methods**

For the experiment peptides were isolated from the broiler chicken bursa of Fabricius (Pervouralskaya poultry farm) using the following technology: the raw material was washed with running water, ground in a laboratory mill (OLIS, Russia), and a solution was prepared with a hydromodulus 1:3 (crushed bursa of Fabricius: distilled water), papain enzyme was introduced (Enzyme BioScience (P) Ltd, India) (0.15% from the weight of the raw material) in a phosphate buffer solution prepared by us (disodium hydrogen phosphate dodecahydrate (Rosspolymer, Russia), citric acid monohydrate (Alkhimpro, Russia) at pH 6; the obtaid mixture was exposed to hydrolysis for 12 hours at a temperature of 36 °C; after that the temperature was increased up to 75 °C to inactivate the enzyme. Moisture was removed from the enzymatic hydrolyzate of the bursa of Fabricius by freeze drying (Institute of Biological Instrumentation, Russian Academy of Sciences, Russia) at a temperature of minus 40 °C till achieving moisture content of 6%.

The molecular weight distribution of the peptides was implemented by mass spectrometry and was identified by MALDI-TOF and MS/MS mass spectrometry on the device Ultraflex MALDI time-of-flight mass spectrometer (Bruker, Germany). Mass spectra analysis was run with the help of Mascot software, Peptide Fingerprint option (Matrix Science, USA), using the Protein NCBI database.

The peptides effects on the immune system were studied on BALb/c mice with induced experimental immunodeficiency. In order to induce the immunosuppression, the day before the experiment the exposed animals got one intraperitoneal injection with the cytostatic Cyclophosphan (Lens-Pharm, Russia) at a dose of 100 mg/kg. The number of surviving animals was equal to 100%. Usually three doses every 72 hours are given. The immunomodulatory effects were studies on the examples of the mice that got *per os* the peptides isolated from the bursa of Fabricius of broiler chickens. The BALb/c mice were sorted into 5 groups, including 3 groups of mice with experimental immunodeficiency. Animals of the first (control) group received saline *per os* as a placebo, animals of the second group received bursal peptides *per os* at a dose of 0.02 mg/kg of body weight for 7 days (the amount of peptides was determined by the protein content by the Keldahl method on the automatic protein analyzer (ERKAYA, Turkey), the third group (immunosuppressed) got saline solution *per os* as a placebo, the fourth group (immunosuppressed) was injected with bursal peptides, the fifth control group was a control group (immunosuppressed mice). The amount of peptides for feeding the laboratory animals was calculated according to the recommendations [11].

On the 1st, 7th, and 14th days of the experiment, blood was taken into BD Microtainer® tubes with EDTA anticoagulant (Beckman Coulter, USA) by cutting off a 1 mm tail tip. Blood samples from experimental and control groups of mice were stained with monoclonal antibodies CD22-PE-CY5, CD3-FITC, CD4-APC, CD8-PE (BDPharmingen™, USA) according to the manufacturer's instructions. For blood staining an appropriate volume of fluorochrome-conjugated monoclonal antibodies was added to 100 µl of whole blood in a 12 x 75 mm polystyrene tube with a cap closure; the contents of the test tubes were shaken and incubated for 20 minutes in the dark at a tem-

perature of 23–25 °C; 2 ml of non-concentrated BD FACS lysing solution (Becton, Dickinson and Company BD Biosciences, USA) was added to each tube and the contents of the tubes were shaken, incubated for 10 minutes in the dark at room temperature; centrifuged (ELMI, Latvia) for 5 minutes at 3,000 rpm. The supernatant was taken and resuspended in a buffer solution (Becton, Dickinson and Company BD Biosciences, USA); 0.5 ml of fixing agent Perfix-ne was added (Beckman Coulter Life Sciences, USA); the contents of the tube were mixed, incubated at 6–8 °C for 30 minutes. The obtained substance was studied 2 hours after staining of blood cells. To calculate the absolute number of lymphocytes in the blood, BD Trucount Tubes cytometric tubes (Becton Dickinson and Company, USA) were used. The number of lymphocyte populations with surface markers was determined on a FACSCalibur cytofluorimeter (Beckton and Dickenson, USA) using the CellQuestPro software. The effect of drugs on the functional state of lymphocytes was assessed by changing the number of lymphocytes populations CD3+ (T-lymphocytes), CD22+ (mature B-lymphocytes), CD3+CD4+ (T-helpers), CD3+CD8+ (cytotoxic T-lymphocytes) in the whole blood samples [12].

The functional activity of neutrophils was determined by the method of spontaneous and induced chemiluminescence (CL). As objects of study neutrophils of the peritoneal exudate of mice were chosen. To obtain peritoneal exudate, animals were euthanized using cervical dislocation. The surgical area was treated with a 70% ethanol solution, then 10 ml of the RPMI-1640 nutrient medium (M. P. Chumakov Plant for bacterial and viral preparations production, Russia) was injected into the abdominal cavity, massaged, and in 5 minutes the abdominal cavity was opened, 10 ml of liquid was taken with a syringe and the liquid centrifuged for 10 minutes at 3,000 rpm, the supernatant was drained off.

To activate phagocytes the opsonized zymosan (manufactured by Merck KGaA, Germany) was used as phagocytosis inducer. Neutrophils of the peritoneal exudate of intact mice (group 1) were taken as a control reference according to the above-described method.

During the experiments the phagocytes suspension was added to each well of a 96-well plate for scintillation counting. so that the final concentration was 0.5x106 cells/ml, which was counted in a Goryaev counting chamber, then a suspension of zymosan was added at a concentration of 2 mg/ml, 0.02 ml per each well and 5.6 × 10–4 96% solution of luminol was added (Panreac / AppliChem, USA) in amount of 0.02 ml each well. The plates were placed in a universal thermostatted plate scanner Victor for detection of fluorescent signals (manufactured by PerkinElmer, USA) and chemiluminescence was measured at 37 °C at 0.1-minute intervals for 100 minutes long. The chemiluminescence level was assessed by kinetic curve change in the registered pulses, recorded by the Victor fluorescent signal scanner hardware-software complex (manufactured by PerkinElmer, USA). The device registered the signal amplitude at its maximum and registered the light sum.

The functional activity of phagocytes was analyzed on the basis of assessment of chemiluminescent activity of peritoneal neutrophils in healthy and in immunodeficient mice at different times after the completion of the course of taking the peptides. The intensity of the chemiluminescent response was estimated from the maximum value of neutrophil impulses on the kinetic curve. The mean chemiluminescence intensity was determined from three identical measurements using a Victor fluorescent signal scanner. The results were evaluated by the number of pulses for minute per 1 μl of peritoneal exudate per the number of neutrophils in 1 μl.

Data was analyzed in the STATISTICA 9.0 statistical software package. Data are presented as arithmetic mean (M) + standard error of the mean (m). To test the hypothesis about the homogeneity of two independent samples, the nonparametric Mann-Whitney Utest was used. The discrepancies between the obtained values were considered significant when $p < 0,05$.

**Results and discussion**

We have conducted studies on the study of the immunomodulatory effect of peptides isolated from the bursa of Fabricius in broiler chickens. The absolute content of lymphocytes subpopulations in intact mice blood is presented below in the Table 1.

Among the immunosuppressed mice at various times of the study there was a decrease in number of analyzed lymphocytes subpopulations (CD3+, CD22+, CD3+CD8+) in comparison with the intact group ($P \leq 0.05$). Among the animals that were injected with CF (the 5$^{th}$ group) on the 7$^{th}$ day there was a decrease in CD3+ by 55.3%, CD22+ by 83.7%, CD3+CD4+ by 51.9% and CD3+CD8+ by 54.6% in relation to the intact (the 1st group). The introduction of bursal peptides to the animals (the 2$^{nd}$ group) contributed to a significant ($P \leq 0.05$) increase from 0.637 to 1.044 of cells number per liter, ×109 on the 7$^{th}$ day CD22+ or by 61.0% in relation to the intact (the 1$^{st}$ group). When peptides were used in immunosuppressed mice (the 4$^{th}$ group) there was an increase in subpopulations of CD3+ lymphocytes from 0.873 to 1.976 cells per liter, ×109 or by 126.4%, CD22 from 0.112 to 0.429 cells per liter, ×109 or by 283.0 .6%, CD3+CD4+ from 0.936 to 1.326 cells per liter, ×109 or 41.7% and CD3+CD8+ from 0.175 to 0.318 cells per liter, ×109 or 81.7% in comparison with immunosuppressed animals treated *per os* with saline as a placebo (group 3) ($P \leq 0.05$).

Similar results were obtained on the 14$^{th}$ day of the experiment. Animals treated with cyclophosphan (group 5) showed a decrease in CD3+ by 60.0%, CD22+ by 26.1%, CD3+CD4+ by 32.7% and CD3+CD8+ by 22.6% in relation to the intact group (the 1$^{st}$ group) ($P \leq 0.05$). The inclusion of bursal peptides in the diet of animals (the 2$^{nd}$ group) significantly increased ($P \leq 0.05$) the number of

**Table 1. Absolute content of lymphocyte subpopulations in whole blood of mice in the control and the experimental groups**

| Number of cells per one liter, ×10⁹ | | | | |
|---|---|---|---|---|
| 1st day | | | | |
| Group | CD3 + (T-lymphocytes) | CD22 + (mature B-lymphocytes) | CD3 + CD4 + (T-helpers) | CD3 + CD8 + (cytotoxic T-lymphocytes) |
| 1 (intact group / control) | 2.413 ± 0.045 | 0.659 ± 0.021 | 1.723 ± 0.031 | 0.471 ± 0.031 |
| 2 (bursal peptides) | 2.203 ± 0.034* | 0.632 ± 0.013* | 1.727 ± 0.032 | 0.394 ± 0.037* |
| 3 (immunosuppressed + per os saline as placebo) | 1.292 ± 0.023 | 0.130 ± 0.0282 | 1.057 ± 0.024 | 0.199 ± 0.031 |
| 4 (immunosuppressed + bursal peptides) | 1.432 ± 0.035** | 0.437 ± 0.026** | 1.521 ± 0.027** | 0.342 ± 0.026** |
| 5 (Cyclophosphan) | 1.294 ± 0.027*** | 0.128 ± 0.022*** | 1.052 ± 0.032*** | 0.196 ± 0.019*** |
| 7th day | | | | |
| 1 (intact group / control) | 2.425 ± 0.042 | 0.637 ± 0.024 | 1.618 ± 0.042 | 0.467 ± 0.033 |
| 2 (bursal peptides) | 2.034 ± 0.031 | 1.044 ± 0.027* | 1.559 ± 0.030 | 0.453 ± 0.039 |
| 3 (immunosuppressed + per os saline as placebo) | 0.873 ± 0.025 | 0.112 ± 0.027 | 0.936 ± 0.026 | 0.175 ± 0.021 |
| 4 (immunosuppressed + bursal peptides) | 1.976 ± 0.025** | 0.429 ± 0.029** | 1.326 ± 0.026** | 0.318 ± 0.031** |
| 5 (Cyclophosphan) | 1.085 ± 0.025*** | 0.104 ± 0.024*** | 0.778 ± 0.022*** | 0.212 ± 0.025*** |
| 14th day | | | | |
| 1 (intact group / control) | 2.398 ± 0.037 | 0.639 ± 0.021 | 1.625 ± 0.035 | 0.452 ± 0.039 |
| 2 (bursal peptides) | 2.244 ± 0.036 | 1.051 ± 0.034* | 1.722 ± 0.036 | 0.366 ± 0.034 |
| 3 (immunosuppressed + per os saline as placebo) | 0.827 ± 0.022 | 0.098 ± 0.025 | 0.875 ± 0.023 | 0.307 ± 0.028 |
| 4 (immunosuppressed + bursal peptides) | 1.521 ± 0.027** | 0.426 ± 0.024** | 0.953 ± 0.028** | 0.296 ± 0.025 |
| 5 (Cyclophosphan) | 1.056 ± 0.024*** | 0.472 ± 0.029*** | 1.093 ± 0.023*** | 0.350 ± 0.028*** |

* — differences from the 1st group of animals (intact control group) are significant (P ≤ 0.05);
** — differences from the 3rd group of animals (immunosuppressed + per os saline as placebo) are significant (P ≤ 0.05);
*** — differences with the 1st group 1 of animals (control) are significant (P ≤ 0.05)

subpopulations of CD22+ lymphocytes from 0.827 to 1.521 cells per liter, ×109 or by 83.9%, in relation to the intact (the 1st group) (P ≤ 0.05). It was shown administration of bursal peptides to immunosuppressed mice (the 4th group) on 14th day leads to an increase in subpopulations of CD3+ lymphocytes from 0.827 to 1.521 cells per liter, ×109 or by 83.3%, CD22+ from 0.098 to 0.426 cells per liter, ×109 or by 334.7% and CD3+CD4+ from 0.875 to 0.923 cells per liter, ×109 or by 5.5% compared with immunosuppressed animals treated *per os* with saline as placebo (group 3) (P ≤ 0.05).

Figure 1 shows the data of induced chemiluminescence of mice neutrophils on the 1st day of the experiment.

From the presented kinetics of neutrophils chemiluminescence it follows that under the influence of the immunosuppressor cyclophosphan, there is a slight decrease in the level of induced CL (the 5th group). The highest level of CL is observed when it was exposed to bursal peptides (the 2nd group) — it was significantly higher than the control group by 112 imp/sec (P ≤ 0.05) or 108% (P ≤ 0.05) at the 70th minute. Under the influence of bursal peptides in immunosuppressed mice (the 4th group) there is a well pronounced increase in chemiluminescence — an increase in the level of CL in comparison with the 3rd group (immunosuppressed + *per os* saline as a placebo) by 90 imp/sec or 84.4% (P ≤ 0.001) and by

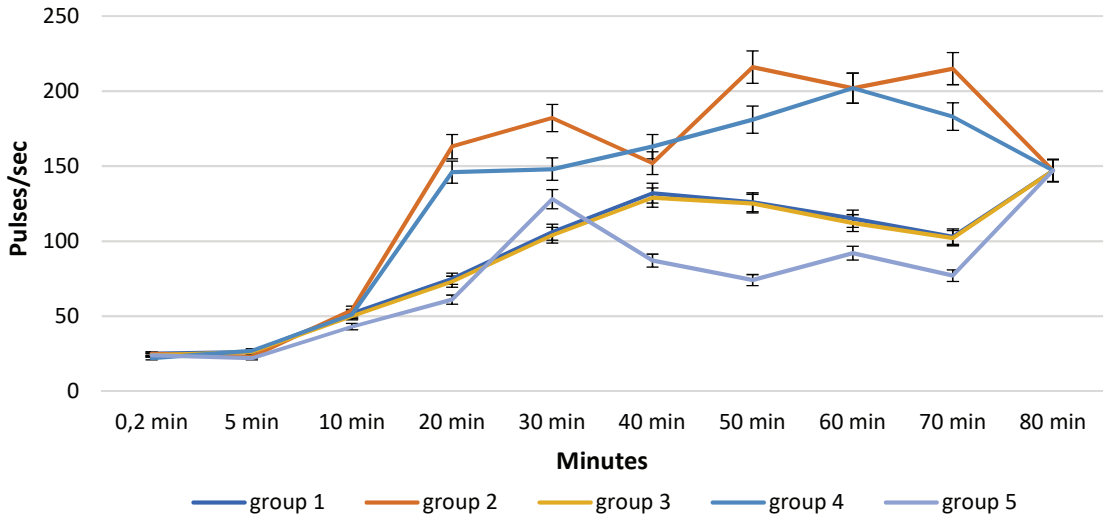


**Figure 1.** Induced neutrophils chemiluminescence in laboratory animals (n = 3), measurement inaccuracy per each group is no more than 5%

81 pulses/sec or 79.4% ($P \leq 0.001$) at the 60th and 70th minutes after induction, respectively.

The Figure 2 below shows the data of induced chemiluminescence of mice neutrophils on the 7th day of the experiment.

From the data presented, it follows that the level of neutrophil CL obtained from mice treated with bursal peptides (group 2) is higher than the neutrophil CL level of intact animals (group 1). So the number of neutrophil pulses at the 60th, 70th and 80th minutes of induced chemiluminescence against the background of the bursal peptides introduction is higher in comparison with the 1st group by 71 pulses/sec or 63.4% ($P \leq 0.001$), by 107 pulses/sec or 146.6% ($P \leq 0.001$) and by 81 pulses/sec or 103.8% ($P \leq 0.001$), respectively.

The use of bursal peptides among the immunosuppressed mice (group 4) increases the number of neutrophil pulses at 60, 70 and 80 minutes of induced chemiluminescence in comparison with immunosuppressed mice treated per os with saline as a placebo (group 3) by 44 pulses/sec or by 40.0% ($P \leq 0.05$), by 91 imp/sec or by 116.1% ($P \leq 0.001$) and by 39 imp/sec or 51.3% ($P \leq 0.05$), respectively.

The Figure 3 shows the data of induced chemiluminescence of mice neutrophils on the 14th day.

In all groups of animals treated with peptides, the level of neutrophil CL exceeded that of intact animals (group 1). Thus, the induced neutrophils chemiluminescence in the second group was at the 60th, 70th and 80th minutes higher by 8 pulses/sec or 21.1%, by 21 pulses/sec or 47.7% and by 17 pulses/sec or 42.5% ($P \leq 0.05$) in comparison with the first group. In immunosuppressed animals treated with bursal peptides (group 4), neutrophil chemiluminescence induced at the 60th, 70th and 80th minutes was higher by

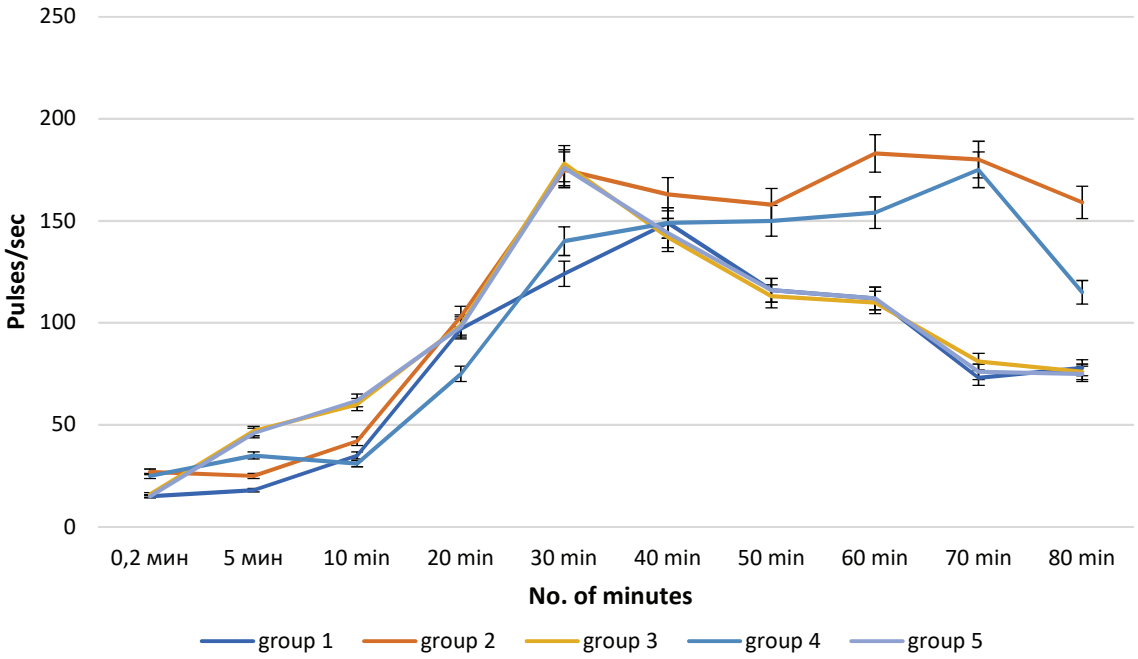


**Figure 2.** Induced chemiluminescence of neutrophils of laboratory mice (n = 3), measurement error for each group is not more than 5%

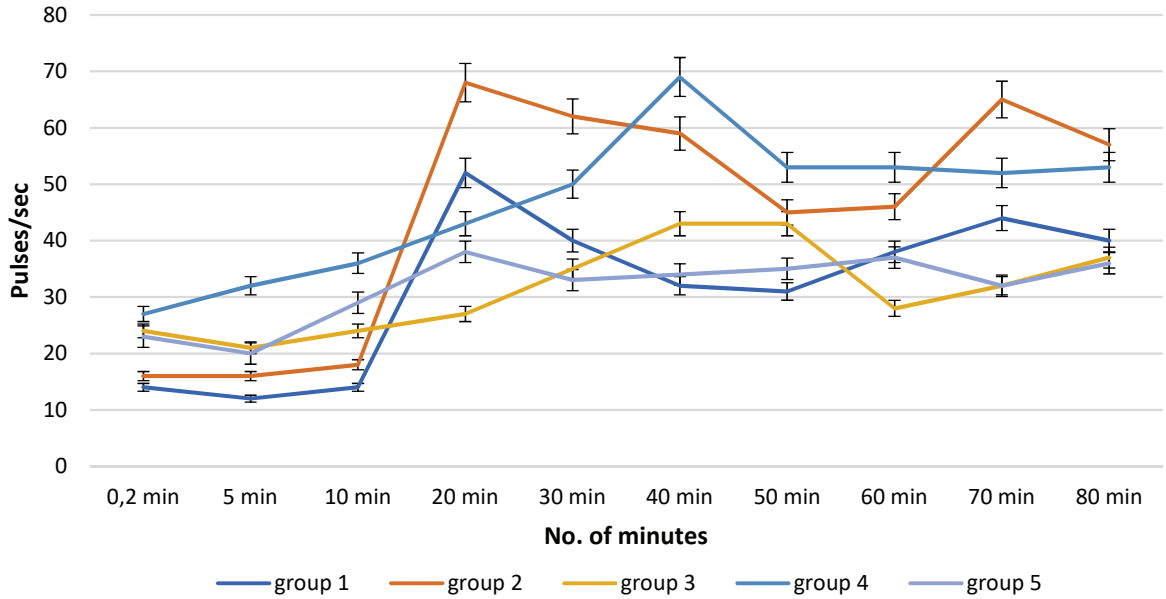


**Figure 3.** Induced neutrophils chemiluminescence in laboratory mice on the 14th day of the experiment (n = 3), measurement inaccuracy for each group is not more than 5%

25 pulses/sec or 89.3% ($P \leq 0.01$), by 20 pulses/sec or 62.5 ($P \leq 0.05$) and by 16 pulses/sec or 43.2% ($P \leq 0.05$) in comparison with the group of mice treated with saline *per os* as a placebo (the 3rd group). The increase in the amount of induced neutrophils CL in mice treated with cyclophosphan is probably caused by the fact that after a short suppression of immune responses the compensatory immune stimulation response is observed in the body.

The results of the studies indicate that a day before the experiment, a single administration of cyclophosphan to mice leads to a persistent immune disorder. The characteristic signs of immunosuppression were a significant decrease in the number of subpopulations of CD3+, CD22+, CD3+CD8+ lymphocytes in the blood in animals of the third, fourth and fifth groups against the background of the use of cytostatics. The data obtained are consistent with the studies of the authors [13], which showed that immunosuppression in mice can be induced by a single intraperitoneal injection of cyclophosphan at a dose of 100 mg/kg, and in another study of the authors [13], immunosuppression was induced using an intraperitoneal injection of 50 mg/kg of cyclophosphan twice with a break of three days or cyclophosphan at a dose of 125 μg/mouse intraperitoneally one time.

Similar decrease in number of lymphocytes subpopulations in the blood in immunodeficient mice were obtained in the other studies [14].

According to researchers [15,16], biologically active peptides with a medicinal effect, including an immunomodulatory effect, can be obtained by enzymatic and subsequent technological processing of raw materials of animal origin, which is consistent with the technology for obtaining peptides, studied in our experiment.

Peptide-based immunomodulators isolated from the tissues of the immunocompetent organs of animals and poultry, in particular, glucosaminylmuramyl dipeptide (GMDP), registered under the name Likopid® [6], deserve special attention. It was found that the introduction of bursal peptides into immunosuppressed mice allows maintaining the homeostasis of subpopulations of T- lymphocytes and B-lymphocytes, and on the 7th day there is a trend to increasing the number of studied immune cells. The obtained data are consistent with the statement of the authors [17], who ran an experiment on immunization of chickens with bursal pentapeptide-II (BPP-II) and a vaccine against avian influenza virus (AIV) with the determination of antibodies and interleukin-4 production. The results showed that BPP-II plays a strong inducing role in humoral immune responses. To examine gene expression at the transcriptional level, avian B-lymphocyte DT40 cells were treated with BPP-II and analyzed by gene microarray. The obtained results proved that the administration of BPP-II regulates 11 ways in which the homologous recombination is a vital mechanism involved in conversion and diversification of immunoglobulin antibody genes during development of B-cells. These results suggested that the biologically active BPP-II peptide derived from the bursa may be involved in antibody production and development of B -cells.

The obtained data on the activation of T- lymphocytes and B-lymphocytes are consistent with the results of studies [18] where it was found that the bursal peptide BSP-II induces strong production of AIV-specific HI antibodies in immunized chickens and increases the viability of avian pre-B-lymphocytes DT40 cells.

Studies have found that the introduction of peptides into laboratory animals leads to a weakening of the effect of cytostatics and contributes to the activation of the immune system. The data obtained are consistent with the results of studies by the authors [9], who state that bursal peptides have immunosuppressed and antioxidant activity, in particular, the BP5 peptide isolated from the bursa of Fabricius significantly stimulates the expression of the p53 protein in HCT116 colon cancer cells. BP5 has a strong inhibitory effect on cell growth and induces apoptosis in HCT116 cells. Mechanically, BP5 stops the cell cycle in the G1 phase by increasing the expression of p53 and p21 and decreasing the expression of cyclin E1-CDK2 complex. Introduction of BP5 dramatically activates the stress-mediated endoplasmic reticulum (ER) apoptotic pathway, as evidenced by a significant increase of expression of unfolded protein response sensors (IRE1a, ATF6, PERK) as well as downstream signaling molecules (XBP-1s, eIF2a, ATF4, and CHOP) and a significant change in the phenotypic changes induced by BP5 in IRE1, ATF6 and PERK knockout cells.

Our study on assessment of immunomodulating effect of bursal peptides is consistent with the results of studies [19] where the effect of bursopentin (BP5) on the protection of dendritic cells from oxidative stress during immunosuppression was studied. BP5 has shown potent protective effects against lipopolysaccharide (LPS)-induced oxidative stress in dendritic cells, including nitric oxide, reactive oxygen species, and lipid peroxidation. In addition, BP5 increased cellular reduction status by increasing reduced glutathione (GSH) and the GSH/GSSG ratio. Along with this, the activity of some antioxidant redox enzymes, including glutathione peroxidase, catalase and superoxide dismutase, was clearly increased. BP5 also suppressed submucosal maturation of dendritic cells in the LPS-stimulated intestinal epithelial cell co-culture system. As a result, it was found that under the influence of BP5 the concentration of heme oxygenase 1 in LPS-induced dendritic cells significantly increases and plays an important role in suppressing oxidative stress and maturation of dendritic cells. These results indicated that BP5 could protect dendritic cells from LPS-induced oxidative stress and would have potential applications in management of associated inflammatory responses.

We have found that bursal peptides enhance the immune response in the form of a change in the number of subpopulations of B-cells and / or T-cells. In a healthy

body, CD4+ helper T-cells enhance the immune response regulated by CD8+ T-cells, while activated CD8+ T-cells release inflammatory cytokines, leading to death of infected cells. In immunosuppressed patients, CD8+ T-cells are not properly activated by mitogenic stimuli [20]. Therefore, activation of CD8+ lymphocytes by bursal peptides can relieve immunosuppression.

Against the background of immunomodulators application, the immunological studies pay special attention to the study of the chemiluminescence kinetics of neutrophils, as they are the most common leukocytes in the blood circulation and the first cells recruited to infection lesions or inflammation foci. Neutrophils in the blood circulation system are considered to be short-life cells that undergo constitutive apoptosis after 24 hours only. The migration of neutrophils from the blood circulation system into tissues is a multistage process that includes their passage along the vascular endothelium, adhesion to endothelial cells, extravasation through the vascular endothelium, and migration to inflammatory foci [21]. Neutrophilic clearance of microbes occurs by several processes, including phagocytosis, degranulation reactions, generation of reactive oxygen species (ROS), and formation of extracellular traps (networks) of neutrophils [22].

The granules are essential for neutrophils to implement their role in innate immunity. Upon activation of the neutrophils, the granules can release their contents into the immediate microenvironment. There are three types of granules in neutrophils: 1. Azurophilic granules, which are reservoirs of antimicrobial compounds, including myeloperoxidase (MPO), defensins, lysozyme, bactericidal-penetrating protein, neutrophil elastase (NE), and cathepsin. 2. Secondary granules, which are characterized by the glycoprotein lactoferrin, including NGAL and hCAP-18 [23]. 3. Gelatinase granules, which are thought to be the storages for metalloproteases such as gelatinase and leucolysin [23]. In response to infections, neutrophils can lead to pathogens destruction by releasing reactive oxygen species ((ROS) and MPO and NADPH oxidase activity) and reactive nitrogen species ((RNS) and nitric oxide synthase (NOS)) [23]. Despite their beneficial role against pathogens, chronic or uncontrolled production of ROS can contribute to damage of lipid membrane, DNA damage, and genetic instability. Extracellular neutrophil traps (networks), which are generated by activated neutrophils, play a crucial role in the immune system [23]. The networks are made up of cell-free DNA, histones, antimicrobial proteins, dangerous molecules and autoimmune antigens and play a vital role in fighting against bacterial, viral, fungal and parasitic infections. Since the increase in neutrophil activity is an important direction of therapy in immunosuppression according to studies [24], we determined the number of neutrophil pulses during the induced chemiluminescence. The obtained results of neutrophil chemiluminescence reflect the status of the immune defense of laboratory animals. Thus, our functional analysis of the measurement of neutrophil luminescence allows us to confirm that the administration of bursal peptides to immunosuppressed mice contributes to weakening of the cytostatic effect, which is represented by a greater number of neutrophil pulses at the $50^{th}$ and $70^{th}$ minutes of induced chemiluminescence in comparison with immunosuppressed mice that got saline *per os* as a placebo. The data obtained are consistent with the results of studies [25] where an increase in neutrophils activity under the action of animal origin peptides was established.

**Conclusion**

It follows from the analysis of scientific knowledge and the results of our own research in the field of biologically active peptides, obtained from raw materials of animal origin, that the mechanism of peptides action in various pathologies is currently being intensively studied by domestic and foreign scientists.

On the basis of the performed researches it can be assumed that the immunocompetent organs of broiler chickens (bursa of Fabricius) are a promising source of immunotropic peptides. As a result of studies on immunosuppressed mice it was found that intake of peptides isolated from bursa of Fabricius of the broiler chicken into animals increases the functional activity of lymphocyte subpopulations (CD3+, CD22+, CD3+CD4+, CD3+CD8+), which increase evidences an immunotropic effect.

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## AUTHOR INFORMATION

**Natalia A. Kolberg,** Candidate of Veterinary Sciences, Docent, Docent, Department of Food Engineering, Ural state University of Economics. 62/8 March str., 620144, Yekaterinburg, Russia. Tel.: +7–343–257–02–27, E-mail: innomed13@mail.ru
ORCID: https://orcid.org/0000–0002–6548–5104

**Sergey L. Tikhonov,** Doctor of Technical Sciences, Professor, Head of the Department of food engineering, Ural State University of Economics. 62/8 March str., 620144, Yekaterinburg, Russia. Tel.: +7–912–276–98–95, E-mail: tihonov75@bk.ru
ORCID: https://orcid.org/0000–0003–4863–9834
* corresponding author

**Natal'ya V. Tikhonova,** Doctor of Technical Sciences, Professor, Professor, Department of food engineering, 620144, Ural state University of Economics. 62/8 March str., 620144, Yekaterinburg, Russia. Tel.: +7–912–276–98–95, E-mail: tihonov75@bk.ru
ORCID: https://orcid.org/0000–0001–5841–1791

**Leonid S. Kudryashov,** Doctor of Technical Sciences, Professor, Chief Researcher, V. M. Gorbatov Federal Research Center for Food Systems. 26, Talalikhina str., 109316, Moscow, Russia. Tel.: +7–903–627–33–06, E-mail: lskudryashov@yandex.ru
ORCID https://orcid.org/0000–0002–6597–0492

All authors bear responsibility for the work and presented data.

All authors made an equal contribution to the work.

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# N-ETHYLMALEIMIDE INFLUENCED THE EVALUATION OF DISULFIDE CROSS-LINKS IN THE OXIDIZED MYOFIBRILLAR PROTEINS USING THE NON-REDUCING SDS-PAGE

**Wenhui Wang[1], Shouyin Wang[1], Shengjie Li [1,2]***
[1] School of Food Science and Technology, Dalian Polytechnic University, Dalian, Liaoning, China
[2] National Engineering Research Center of Seafood, Dalian, Liaoning, China

**Keywords:** *protein oxidation, disulfide bond, N-Ethylmaleimide, SDS-PAGE*

***Abstract***
*The present study aimed to investigate the effect of N-Ethylmaleimide (NEM) on the evaluation of disulfide formation in the oxidized myofibrillar proteins during the sample preparation of the non-reducing SDS-PAGE procedure. For this purpose, extracted myofibrillar proteins were oxidized firstly via a Fenton oxidation reaction, and non-oxidized proteins were used as a control. Before running SDS-PAGE, in the sample preparation, these oxidized and non-oxidized proteins were prepared according to the three different sample preparation methods with or without the presence of N-Ethylmaleimide or β-mercaptoethanol. Results showed that oxidized proteins treated with NEM regardless of sample preparation methods presented attenuated bands of myosin heavy chain monomer in the non-reducing SDS-PAGE gels, suggesting that the disulfide bonds formed as a result of protein oxidation could be preserved by NEM during sample preparation. Meanwhile, a possible mechanism for the effect of NEM was proposed.*

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## Introduction

Protein oxidation frequently occur during processing and storage of meat and meat products, and leads to impaired meat quality and recued nutritional values [1–3]. Particularly, along with microbial spoilage and lipid oxidation, protein oxidation has been proposed as one of the main reasons for quality deterioration of meat and meat products during processing and storage [4]. However, compared to the other two factors, protein oxidation is easily ignored due to its subtle effect on food flavor or appearance. Until recent decades, increasing attention from meat scientist has been focused on protein oxidation and its consequences on meat quality. Protein oxidation is manifested by modifications of amino acid side chains, peptide fragmentation, and formation of covalent protein cross-linking [5]. Furthermore, protein cross-linking is one of the most important consequences of protein oxidation, which might have important impact on meat quality, for instances, meat tenderness [6–8], water-holding capacity [9] and protein functionalities [10]. Particularly, the disulfide cross-linking has attracted lots of attention, and it has been proposed as the major form of protein cross-links when meat proteins are oxidized [11]. The possible reason could be ascribed to the fact that cysteine is the most susceptible amino acid residue and is usually one of the first to be oxidized, thus leading to the formation of disulfide bonds.

As a routine method, the non-reducing and reducing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) has been commonly used to determine the protein covalent links [11], where protein samples were dissolved in the SDS-PAGE sample buffer with or without 5% of β-mercaptoethanol (MCE) and boiled for several minutes. The presence of MCE in the reducing SDS-PAGE serves as a disulfide bond breaking agent, leading to breakage of all the formed disulfide bonds present in the proteins. On the other hand, these disulfide bonds would remain intact in the absence of MCE. As revealed in the stained SDS-PAGE gels, for samples treated without MCE, protein cross-linking leads to the loss of myosin heavy chain (MHC) and the appearance of cross-linked products with larger molecular weights, whereas the presence of MCE recovers the lost proteins cross-linked via disulfide bonds in the identical samples treated with MCE. Based on these observations, formation of disulfide protein cross-linking as the consequences of treatment related to protein oxidation could be drawn and the role of disulfide cross-linking in affecting meat quality could be further elucidated. However, artifacts might be introduced during the boiling process in the

non-reducing SDS-PAGE since sulfhydryl group is highly susceptible to form disulfide [11]. Therefore, these artifacts formed during sample preparation would cause the overestimation of disulfide bonds, and thus the exaggerate the role of disulfide cross-linking of proteins in affecting meat quality. N-ethylmaleimide (NEM) has been reported to be a specific sulfhydryl blocking agent, and therefore, theoretically be able to prevent disulfide artifacts during sample preparation. Therefore, to solve the aforementioned problem, in the early studies [11–13], NEM was commonly used to prevent the formation of disulfide bonds during sample preparation for SDS-PAGE. However, searching throughout the published literatures, NEM treatment is often ignored in the sample preparation process in many other studies [10,14]. Moreover, it remains unknow whether NEM treatment in the sample preparation can influence the evaluation of the disulfide formation by non-reducing SDS-PAGE analysis, and the inhibition degree of NEM remains unclear.

The present study was designed to examine whether boiling of the protein samples in the non-reducing SDS-PAGE analysis could cause disulfide artifacts of protein cross-links, and whether NEM could inhibit the occurrence of these artifacts. Unexpectedly, the opposite effect of NEM was observed in this study, and the effect of NEM in the sample preparation for the non-reducing SDS-PAGE on the evaluation of disulfide formation in oxidized myofibrillar proteins was further studied.

## Materials and methods

*Materials*

Pig loins were purchased from a local slaughter house 24 h postmortem, and the visible fat and connective tissues were removed. Afterwards, the muscles were cut into small pieces, and stored at minus 40 °C until use. All chemicals used were of reagent grade.

*Extraction of myofibrillar proteins*

Myofibrillar proteins were extracted according to the method as reported by Takahashi et al. [15]. In short, 5 g of minced meat were washed twice with 5 volumes of 0.1 M NaCl, 20 mM Tris-HCl (pH 7.5), followed by the homogenization in 5 volumes of the same buffer using an Ultra-Turrax T25 homogenizer (IKA Labortechnik, Staufen, Germany), and the homogenate was centrifuged at 3000 ×g for 5 min (4 °C). The obtained pellets washed three times with the above solution, and the final pellet was dissolved and regarded as myofibrillar protein (MFP) suspensions. Finally, to the MFP suspension, a solution of 3 M NaCl, 20 mM Tris-HCl (pH 7.5) was added to give a final NaCl concentration of 0.5 M NaCl. Protein concentrations of the MFP suspensions were measured and then adjusted to the same level of 4 mg/ml.

*Protein oxidation*

Myofibrillar proteins were oxidized in a Fenton protein oxidation system [16]. 10 mL of MFP suspension (4 mg/mL) were oxidized for 12 h at 4 °C with 10 μmol/L $FeCl_3$, 0.1 mM ascorbic acid, and 1 mmol/L $H_2O_2$. Oxidation was terminated by the addition of a mixture of oxidation terminators, that is, 1 mM propyl gallate, 1 mM trolox C, and 1 mM EDTA. The non-oxidized group was treated with these terminators only and incubated for 12 h at 4 °C.

*Sample preparation for SDS-PAGE*

Samples were prepared according to one of the following three different methods.

Traditional method: 0.5 mL of MFP suspensions were mixed thoroughly with 0.5 mL of one of the following different loading buffers: (1) NEM loading buffer: 0.1 M Tris, 4% SDS, 20% glycerol, 4 mg N-ethylmaleimide, 0.2% bromophenol blue, pH 6.8; (2) MCE loading buffer: 0.1 M Tris, 4% SDS, 20% glycerol, 5% 2-mercaptoethanol, 0.2% bromophenol blue, pH 6.8; (3) CON loading buffer: 0.1 M Tris, 4% SDS, 20% glycerol, 0.2% bromophenol blue, pH 6.8. Afterwards, the mixtures were incubated for 2 h at room temperature, and boiled for 5 min, followed by centrifugation at 10,000 g for 5 min.

Un-heating method: 0.5 mL of MFP suspensions were mixed thoroughly with 0.5 mL of one of the following different loading buffers: (1) NEM loading buffer: 0.5 M Tris,16 M Urea, 4% SDS, 4 mg N-ethylmaleimide, 0.4% bromophenol blue, pH 7.5; (2) MCE loading buffer: 0.5 M Tris, 16 M Urea, 4% SDS, 10% 2-mercaptoethanol, 0.4% bromophenol blue, pH 7.5; (3) CON loading buffer: 0.5 M Tris,16 M Urea, 4% SDS, 0.4% bromophenol blue, pH 7.5. Afterwards, the mixtures were incubated for 24 h at room temperature, and centrifuged at 10,000 g for 5 min.

TCA method: For the NEM sample, 1 mL of MFP suspension was added with 4 mg NEM, and incubated for 2 h at room temperature. For the MCE and CON samples, the MFP suspensions were added without NEM, and incubated at the same situation. After incubation, 100 μL of 100% trichloroacetic acid (TCA) was added, and incubated in ice for 10 min, followed by centrifugation at 1200 g for 10min. Subsequently, 2 mL of acetone was added to the pellet and centrifuged at 1200 g for 10min. The obtained pellets were dried in the air, and dissolved in 30 μL 0.2 M NaOH. The dissolved samples were then mixed with the loading buffer of 0.25 M Tris, 8 M Urea, 2% SDS, pH 7.5 for NEM and CON treatment, and with the loading buffer of 0.25 M Tris, 8 M Urea, 2% SDS, 5% 2-mercaptoethanol, pH 7.5 for MCE treatment. Afterwards, the mixtures were incubated for 24 h at room temperature, and centrifuged at 10,000 g for 5 min.

*SDS-PAGE*

10 μL of supernatants were loaded for SDS-PAGE analysis with an electrophoresis system consisting of a continuous 4–20% polyacrylamide gel. Gels were stained with 0.1% coomassie brilliant blue R250 in 45% methanol and 10% acetic acid and then destained with 10% methanol and 10% acetic acid. The gels were captured with ChemiDocTM Imaging System (Biorad, Hercules, CACalifornia, USA).

**Results and Discussion**

Disulfide protein cross-links of myosin heavy chain (MHC) has been considered as one of the major protein oxidation consequences during meat processing and storage, which show the most important implications in meat quality. The non-reducing and reducing SDS-PAGE have been commonly applied to examine the disulfide protein cross-links [11]. In this technique, extracted myofibrillar proteins are treated with or without MCE, respectively. As a result, the presence of MCE would break the disulfide bond of the oxidized proteins, mainly MHC, whereas the protein samples without MCE treatment would show attenuated MHC bands. However, boiling process is usually used in the sample preparation of SDS-PAGE, and high temperature might introduce disulfide formation and the results would be interfered. Therefore, NEM has been introduced to block the thiol group and prevent the formation of disulfide bonds [11,12,17]. On the other hand, NEM treatment is often ignored in the sample preparation process in many other studies. In the present study, the effect of NEM treatment on the prevention of disulfide formation during the boiling process in the SDS-PAGE procedure was firstly studied. It is expected that NEM could prevent the formation of protein cross-linking, but it did not. Subsequently, different alternative methods for sample preparation were compared to minimize the formation of disulfide artifacts formation.

In this study, both the oxidized myofibrillar proteins via the Fenton oxidation and the non-oxidized ones were used for the SDS-PAGE analysis. These two different protein solutions were boiled in the presence of either NEM, MCE or none during sample preparation. Compared to the non-oxidized myofibrillar proteins, the oxidized proteins showed an attenuated MHC band along with the appearance of a dimer MHC product in the CON sample where NEM and MCE were not present (Figure1), suggesting that covalent cross-linking of MHC due to the oxidation incubation process or, at least partially due to the boiling process in the sample preparation. Furthermore, when the oxidized proteins were treated with MCE, these reduced MHC band was almost recovered, indicating these covalent protein cross-links were mainly attributed to the disulfide formation. This traditional sample method for SDS-PAGE has been commonly applied to detect the disulfide cross-linking during protein oxidation. However, in this traditional method, 5-min of boiling process was applied, which might cause the oxidation of thiol group and thus lead to the formation of disulfide cross-links [11], thus contributing to the attenuated MHC band in the CON sample. Therefore, this artifact might lead to the overestimation of the disulfide formation in the process of protein oxidation. Theoretically, NEM could inhibit the disulfide formation due to its ability to block the thiol group, and thus it was used to treat the samples before non-reducing SDS-PAGE analysis in the present study. It was expected that NEM could prevent the formation of disulfide artifact. However, as revealed in Figure 1, it was shown that the NEM treatment led to more attenuated MHC bands than the CON treatment present in the gels. Meanwhile, in the non-oxidized proteins, slight increase in the density of dimer MHC band in the NEM ample was also observed compared to that of the CON sample. Though the results were unexpected, we then speculated that the high temperature and excess of NEM or the oxidation terminators might be the reasons for the increased cross-linking of MHC in NEM treated oxidized myofibrillar proteins. In this sense, these speculations were testified in the following experiments.

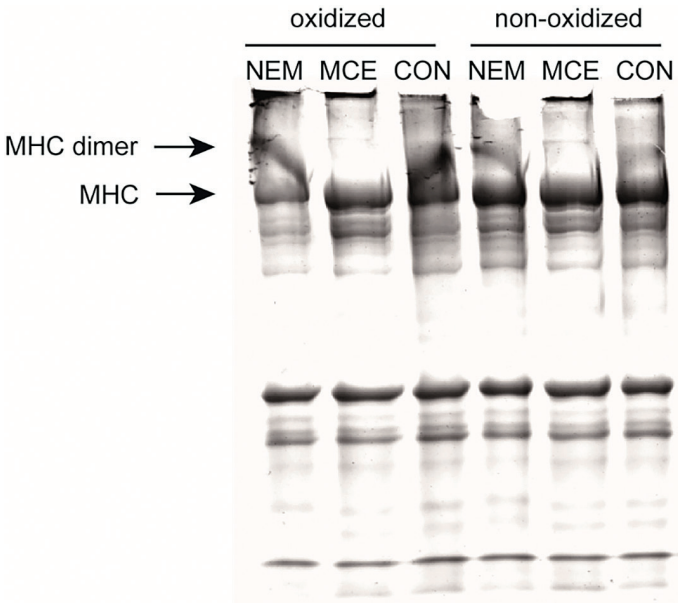


**Figure 1.** SDS-PAGE patterns of non-oxidized and oxidized myofibrillar proteins prepared by the traditional method. Note: NEM, in the presence of N-ethylmaleimide during sample preparation; MCE, in the presence of β-mercaptoethanol during sample preparation; CON, neither N-ethylmaleimide nor β-mercaptoethanol were present during sample preparation

Base on the previous trial, the enhanced MHC covalent cross-links might be attributed to some unknown cross-linking reactions induced by high temperature of the boiling process. Therefore, a non-heating sample preparation process was examined where Urea was included in the sample buffer to denature proteins at room temperature. As shown in Figure 2, similar to the traditional method, a weaker MHC band was observed in CON sample compared to the MCE sample for oxidized myofibrillar proteins, while no such observations were found for non-oxidized proteins. However, the NEM treated oxidized proteins presented even weaker bands than the CON treatment of the oxidized proteins, suggesting more covalent bonds were still formed due to the presence of NEM. Therefore, high temperature in the traditional method seems not to be reason for the increased cross-linking of MHC in oxidized myofibrillar proteins during sample preparation.

In the next trial, to exclude the interference of excess NEM or the oxidation terminators on the formation of MHC cross-linking during sample preparation process, TCA was used to precipitate the proteins after the NEM treatment. As shown in Figure 3, though the NEM sample

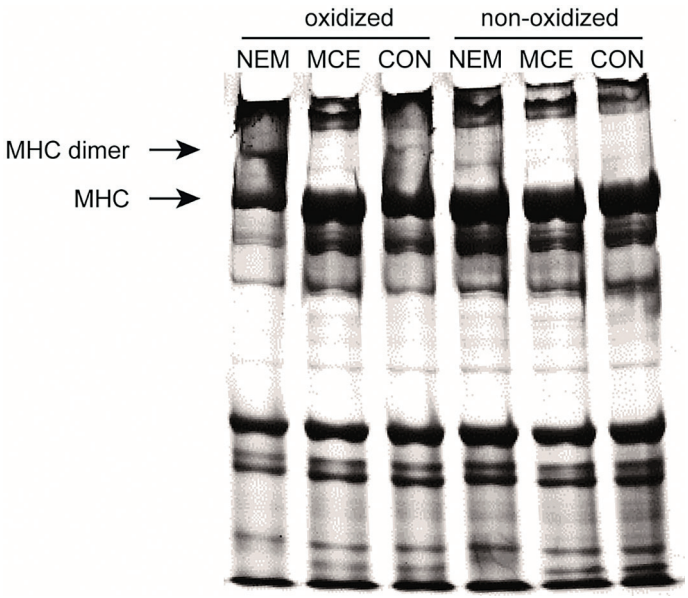


**Figure 2.** SDS-PAGE patterns of non-oxidized and oxidized myofibrillar proteins prepared by non-heating method. Note: NEM, in the presence of N-ethylmaleimide during sample preparation; MCE, in the presence of β-mercaptoethanol during sample preparation; CON, neither N-ethylmaleimide nor β-mercaptoethanol were present during sample preparation

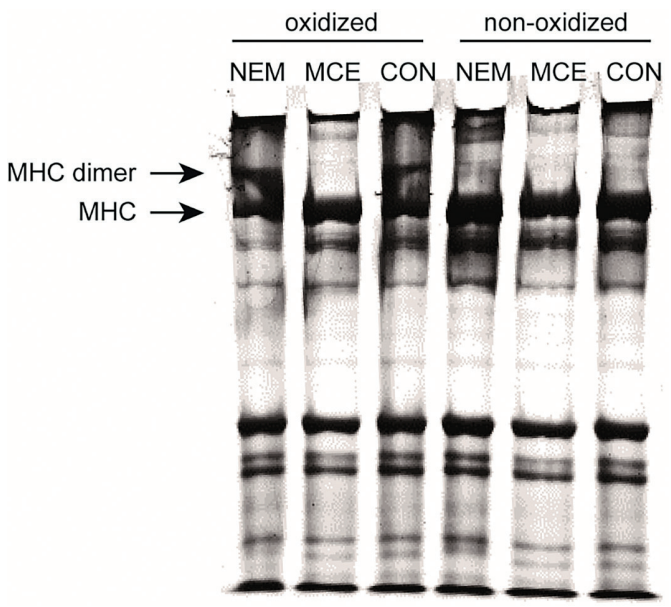


**Figure 3.** SDS-PAGE patterns of non-oxidized and oxidized myofibrillar proteins prepared by TCA method. Note: NEM, in the presence of N-ethylmaleimide during sample preparation; MCE, in the presence of β-mercaptoethanol during sample preparation; CON, neither N-ethylmaleimide nor β-mercaptoethanol were present during sample preparation

had similar intensity of MHC band to that of the CON sample, but a more stronger dimer MHC was shown, suggesting increased formation of MHC cross-linking occurred in samples treated with NEM. Therefore, this finding indicated that the excess of NEM or the oxidation terminators were not neither the reason for the diminished MHC bands in the non-reducing SDS-PAGE gels of NEM treated samples. Interestingly, a previous study has reported that whey protein exhibited an increase in the disulfide crosslinking in the SDS-PAGE gels after incubation with NEM for a period of time, and the authors has pointed out that NEM could somehow promote protein cross-linking though it is most extensively used to block the thiol groups and thus to prevent the disulfide formation [18]. Furthermore, a possible explanation was proposed that the formation of covalent crosslinks through non-natural amino acids due to the presence of NEM, as in lysinoalanine [19]. Such cross-links cannot be cleaved with MCE, and therefore should be presented as dimer MHC in reducing SDS-PAGE gels. However, as illustrated in Figure 4, the formed dimer MHC band (lane "NEM") as a result of NEM treatment disappeared (lane "M+N") when MCE was included at the same time in the sample buffer, indicating that the formation of dimer MHC band due to NEM treatment could be attributed to the disulfide type of protein cross-linking.

Covalent disulfide cross-linking of protein molecules can be brought by sulfhydryl oxidation into disulfide bonds and/or sulfhydryl group/disulfide bond interchange reaction [20]. In a previous study, the disulfide cross-link between two polypeptides of rhodopsin was broken in preparation for non-reducing SDS-PAGE and a new disulfide bond was re-formed in one of the subunits of rhodopsin via the sulfhydryl group/disulfide bond interchange reaction,

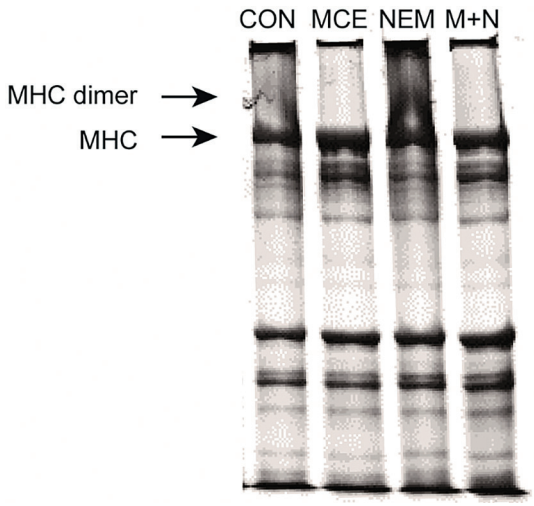


**Figure 4.** SDS-PAGE patterns of oxidized myofibrillar proteins prepared by the TCA method. Note: CON, neither N-ethylmaleimide nor β-mercaptoethanol were present during sample preparation; MCE, in the presence of β-mercaptoethanol during sample preparation; NEM, in the presence of N-ethylmaleimide during sample preparation; M+N, in the presence of both β-mercaptoethanol and N-ethylmaleimide

however, the presence of NEM could prevent the occurrence of these events due to its blocking of sulfhydryl group of an adjacent cysteine residue in this subunit [21]. Along with this observation, a possible mechanism was proposed to explain why oxidized myofibrillar proteins treated with NEM exhibited higher degree of disulfide cross-linking of MHC in SDS-PAGE gels under non-reducing conditions, as observed in the present study. (a): In oxidized myofibrillar proteins, an MHC dimer is formed through disulfide cross-linking between two MHC monomers during Fenton oxidation reaction; (b): In the sample preparation for SDS-PAGE, upon protein denaturation either by

the boiling treatment or the non-heating Urea treatment, a sulfhydryl group of an adjacent cysteine residue in one of the MHC monomer reacts with the disulfide bond, and thus a sulfhydryl group/disulfide bond interchange reaction occurs, leading to the broken of the already formed disulfide bond and the formation of a new intramolecular disulfide bond. Consequently, more MHC monomers and less MHC dimers will appear in the non-reducing SDS-PAGE gels; (c): In contrast, when the oxidized proteins are treated with NEM during sample preparation, the sulfhydryl group of that adjacent cysteine is modified by NEM. Therefore, the sulfhydryl group/disulfide bond interchange reaction described in (b) will not happen, and the disulfide bond is preserved. As a result, more MHC dimers will be observed in the non-reducing SDS-PAGE gels accompanied by less amounts of MHC monomers.

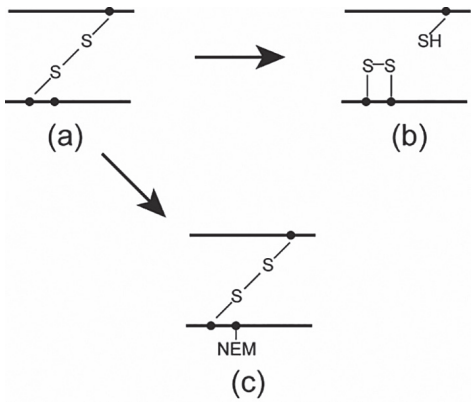


**Figure 5.** Possible role of NEM in the prevention of cleavage of disulfide bonds in the dimer MHC during sample preparation for SDS-PAGE. Notes: (a), MHC dimers formed as a result of protein oxidation; (b), broken of disulfide bonds due to protein denaturation in the sample preparation for SDS-PAGE; (c), MHC dimers after sample preparation in the presence of NEM; a dot indicates a cysteine residue; a line indicate a monomer MHC

## Conclusion

In conclusion, the present study was designed to investigate the effect of NEM on the evaluation of disulfide formation in oxidized myofibrillar proteins during the sample preparation of the non-reducing SDS-PAGE procedure. The results demonstrated that inclusion of NEM could lead to more cross-linked MHC in the non-reducing SDS-PAGE gels, indicating that the disulfide bonds formed as a result of protein oxidation could be preserved during sample preparation. On the other hand, it is speculated that when treated without NEM, these formed intermolecular disulfide bonds might be broken, which can lead to underestimation of protein cross-linking via disulfide formation. Therefore, it is suggested to include NEM in the sample preparation for SDS-PAGE analysis in the studies that examine the formation of disulfide protein cross-linking in myofibrillar proteins and its consequence in meat quality. In addition, whether the boiling process in sample preparation for non-reducing SDS-PAGE could introduce disulfide artifacts were not testified in this study. However, considering the presence of these oxidation terminators, the oxidation of sulfhydryl groups into disulfide bonds might not occur or develop to a great extent.

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## AUTHOR INFORMATION

**Wenhui Wang,** Master student, School of Food Science and Technology, Dalian Polytechnic University, Ganjingzi #1, 116304, Dalian, Liaoning, China. Tel.: +86–0411–86–31–86–75, E-mail: fumei1998@126.com
ORCID: https://orcid.org/0000–0001–6247–3557

**Shouyin Wang,** Master student, School of Food Science and Technology, Dalian Polytechnic University, Ganjingzi #1, 116304, Dalian, Liaoning, China. Tel.: +86–0411–86–31–86–75, E-mail: shouyin_wang@126.com
ORCID: https://orcid.org/0000–0003–4865–4831

**Shengjie Li,** PhD, Associate Professor, School of Food Science and Technology, Dalian Polytechnic University, Ganjingzi #1, 116304, Dalian, Liaoning, China, National Engineering Research Center of Seafood, Ganjingzi #1, 116304, Dalian, Liaoning, China. Tel.: +86–0411–86–31–86–75, E-mail: lishj@dlpu.edu.cn
ORCID: https://orcid.org/0000–0001–6940–6066
* corresponding author

All authors bear responsibility for the work and presented data.

All authors made an equal contribution to the work.

The authors were equally involved in writing the manuscript and bear the equal responsibility for plagiarism.

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# RAMAN SPECTROSCOPIC TECHNIQUES FOR MEAT ANALYSIS: A REVIEW

**Viktoriya A. Pchelkina*, Irina M. Chernukha, Liliya V. Fedulova, Nikolai A. Ilyin**
V. M. Gorbatov Federal Research Center for Food Systems, Moscow, Russia

**Keywords:** *Raman spectroscopy, nondestructive analysis, meat quality, meat products*

***Abstract***

*Raman spectroscopy (vibrational spectroscopy) proved to be an effective analytical approach in the field of geology, semiconductors, materials and polymers. Over the past decade, Raman spectroscopy has attracted the attention of researchers as a non-destructive, highly sensitive, fast and eco-friendly method and has demonstrated the unique capabilities of food analysis. The use of Raman spectroscopic methods (RSMs) to assess the quality of meat and finished products is rapidly expanding. From the analysis of one sample, you can get a large amount of information about the structure of proteins, the composition of fatty acids, organoleptic parameters, autolysis and spoilage indicators, authentication of raw materials, technological properties. An important advantage of the method is the comparability of the results obtained with the data of traditional analytical methods. Traditional methods of determining the quality of meat are often time-consuming, expensive and lead to irreversible damage to a sample. It is difficult to use them in production conditions directly on the meat processing lines. Technological advances have made it possible to develop portable Raman spectroscopes to use directly in production. The article presents the basic principles of Raman spectroscopy, systematizes the results of the use of RSMs for the analysis of meat quality from different types of slaughter animals and provides tools for analyzing the data of the obtained spectra. Raman spectra have many dependent variables, so chemometric assays are used to work with them. Literature analysis has shown that currently there is no unified database of meat spectra in the world, standardized protocols for conducting research and processing the obtained results. In Russia, the use of RSMs is a new, promising and relevant area of research in the field of meat quality.*

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## Introduction

With the growth in global import and export of agri-food products, the questions of food safety have received increased attention worldwide.

Meat is the main protein source and has a great physiological value for humans; its consumption is growing every year [1]. In 2020, the global meat consumption was 324 million metric tons, which is three times higher than 50 years ago [2]. In Russia, per capita consumption of meat was about 76–77 kg in 2021; a slight increase is possible in 2022 [3]. With a growth in meat consumption, its quality is becoming an increasingly important factor influencing a consumers' decision [4].

For meat quality assessment, two main approaches are used: subjective and objective. Subjective methods include sensory evaluation, which involves visual and eating experiences [5]. Their disadvantage resides in poor repeatability, dependence on taster's experience and difficulties in quantitative interpretation of results. Objective methods include various laboratory tests that evaluate physical and chemical properties of meat, including electrophoresis [7], enzyme-linked immunosorbent assay (ELISA) [8], mass-spectrometric methods [9], gas chromatography-mass spectrometry [10], high performance liquid chromatography (HPLC) [11,12] and methods based on the polymerase chain reaction (PCR) [13,14,15]. Although PCR and ELISA are the most specific and sensitive methods, they require expensive equipment and highly qualified specialists, which restricts their use. Chromatographic methods usually have low repeatability. These methods give accurate results, but a sample is damaged or destroyed, and the procedure, especially sample preparation, requires, as a rule, large amounts of time and resources. This hinders significantly their use for automated analysis directly in production [16].

Therefore, the development of rapid and non-destructive detection methods is necessary to ensure the population health, analysis of meat quality and safety.

Over the last decade, many complex studies associated with quantitative assessment of characteristics of carcasses and meat of slaughter animals were carried out using methods of imaging and spectroscopy [17,18], as well as tools for assessment and analysis of images and new algorithms for effective prediction of quality indicators in meat raw materials [19,20].

Much attention is given to the spectroscopic methods in visible and near-infrared range (VIS–NIRS and NIRS), hyperspectral imaging (HSI) and Raman spectroscopy. In this paper, we consider a possibility of using Raman spectroscopy for analysis of quality indicators of meat raw materials from different species of slaughter animals.

Raman spectroscopy is spectroscopy that allows identification of vibrational modes of molecules and is a non-destructive method of analysis. When photons collide with molecules, three different types of scattering occur: Rayleigh scattering, anti-Stokes Raman scattering and Stokes Raman scattering. Raman scattering is caused by the fact that photons give energy to molecules (Stokes scattering) or receive energy from molecules (anti-Stokes scattering) [21]. Due to this exchange of energy, shifts between the energetic levels in molecules are caused. Raman scattering (RS) spectra represent both structural and qualitative information about a substance [22,23].

Raman spectroscopic methods (RSMs) demonstrated a significant potential in analysis of various indicators in agricultural products such as milk, eggs, nuts, vegetable oils, fruit and vegetables, grain (Figure 1) [24].

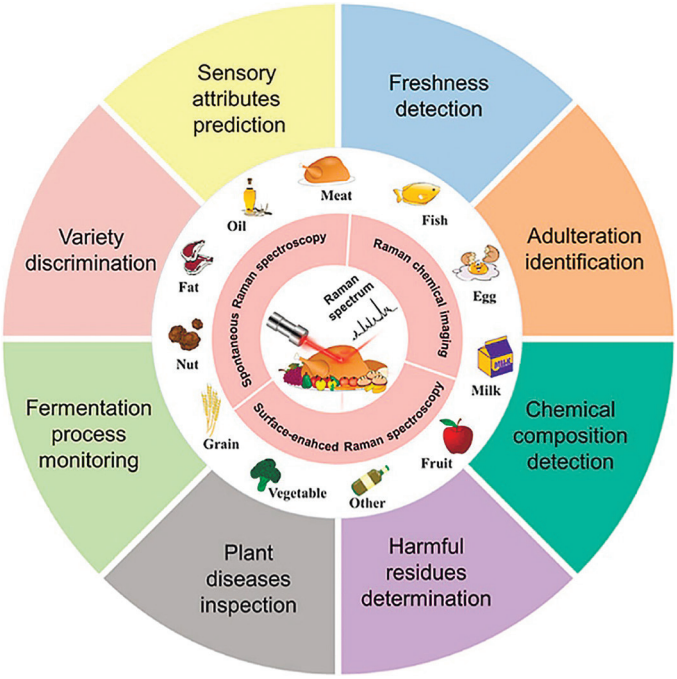


**Figure 1.** Application of Raman spectroscopy in analysis of agricultural products [24]

Vibrational spectroscopy attracts attention as an alternative to traditional methods for assessment of meat quality indicators [5,25,26,27]. Its advantages include minimal sample preparation, fingerprint spectrum (unique spectra of molecules of different substances), high sensitivity, rapid acquisition of data, non-destructive control, environmental friendliness.

Contrary to infrared spectroscopic methods, the Raman effect is observed in the scattered light from a sample and not in a spectrum of light absorption by a sample. With that, heavy molecules, such as water molecules, scatter Raman radiation worse, which makes Raman spectroscopy less sensitive to the moisture content both in a sample and in the environment, in which the measurement is performed. This fact is extremely important in analysis of food and, first of all, meat, dairy and fish products.

The paper presents a review of the potential of using Raman spectroscopy in tandem with approaches of chemometric modeling in analysis of meat raw materials and finished products.

**Objects and methods**

Design of the study:

The systematic review was carried out according to PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) [28].

The strategy for searching publications is presented in Figure 2.

Inclusion criteria were as follows:

1. Correspondence to the theme of the systematic review by one of three modalities: Raman spectroscopy, non-destructive analysis of meat products, use of Raman spectroscopy for meat quality analysis.
2. Original research published in a peer-reviewed journal.
3. Presentation of data about methods of statistical and/or chemometric analysis
4. Publication is dedicated to the application of Raman spectroscopy for analysis of meat raw materials and finished meat products including detection of structural changes in proteins, intramuscular fat, pH, drip losses during storage, detection of fatty acids, raw material falsification.

Exclusion criteria were as follows:

1. Studies that envisage the use of alternative types of spectroscopy for analysis of meat samples (for example, IR-spectroscopy, UV-visible spectroscopy).
2. Studies that envisage the use of RSMs to determine microelement composition in samples.
3. Studies that envisage the use of RSMs to study food products of the agro-industrial complex not relevant to meat raw materials.
4. Studies that envisage the use of RSMs to study inorganic materials.
5. Studies that envisage the use of RSMs to study cells and tissues (of animals and humans), as well as microorganisms.

A search of relevant scientific publications was carried out in Russian and foreign electronic databases (Web of Science, U. S. National Library of Medicine (pubmed.ncbi.nlm.nih.gov), Russian Scientific Electronic Library (elibrary.ru), Russian National Public Library for Science and Technology) in Russian and English for a period of 2007 to 2022. A special attention was paid to publications issued over the last five years.

During the last 15 years, 401 studies dedicated to various investigations of meat raw materials by the spectroscopic methods were published.

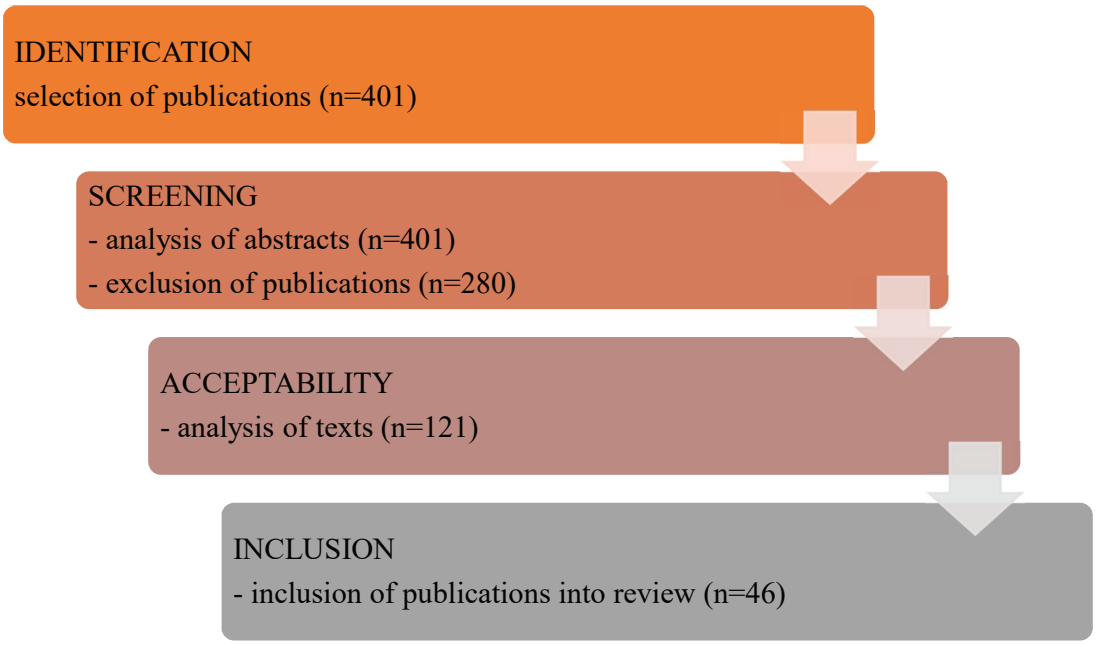


**Figure 2.** The strategy for selection and inclusion of publications into the systemic review

At the first stage, the titles of the papers obtained as a result of the search were analyzed. Part of publications was excluded as not corresponding to the inclusion criteria. Then, analysis of abstracts of the selected papers was carried out, on which basis the second exclusion was performed. At the next stage, the following information was taken from each publication included in the review: author(s), publication year, country; aim and methods of investigation; testing of the statistical hypothesis; description of the methodology of the experiment; the obtained results. Detailed analysis of each publication included into the review was conducted based on the specific elements of investigation questions and the aim of the review by double data extraction (two independent researchers worked on the review).

All obtained data were used for analysis and systematization of the results.

**History of discovering Raman spectroscopy**

Inelastic scattering of light was predicted by A. Smekal as far back as 1923. He assumed that light has the quantum structure and that, after scattering, monochromatic light would have both the original frequency and frequencies of higher and lower wavelengths [29]. However, in practice, inelastic scattering was not observed until 1928. The Raman effect is called in honor of one of its discoverers, the Indian scientist C. V. Raman, who together with K. S. Krishnan observed this effect in organic liquids in 1928 (Figure 3). In 1930, C. V. Raman was awarded a Nobel Prize in physics for this invention. Russian scientists G. Landsberg and L. Mandelstam observed the similar effect in inorganic crystals independently of them. Raman spectra in gases were observed for the first time by F. Rasetti in 1929 [30].

Between 1930 and 1934, the American physicist of the Czechoslovak origin G. Placzek developed theoretically the "effect of Raman scattering". The experiments were

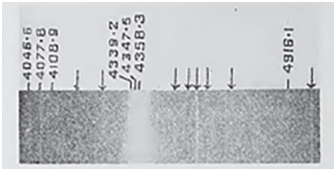


**Figure 3.** The first Raman spectrum of benzol published by C. V. Raman and K. S. Krishnan in 1928 [31]

carried out using the mercury arc as the main light source with photodetectors that were replaced with spectrophotometric detectors. During the years after its discovery, Raman spectroscopy was used for creation of the first catalog of molecular vibrational fingerprints. However, enormous effects were needed to obtain the Raman spectrum because of the essentially weak sensitivity of the method. Thus, the use of Raman spectroscopy decreased, in particular, after the development of commercial IR spectrophotometers in the 1940s. Raman spectroscopy again attracted the attention in 1960, when laser appeared. This source of monochromatic light simplified the detection tool and increased the sensitivity of the method. The use of laser as a source of monochromatic light stimulated the development of Raman spectroscopy as a valuable analytical method [32].

**Principle of Raman spectroscopy**

The principle of the Raman effect is based on the inelastic process of light scattering between the incident light and a substance under irradiation. During the interaction between light and a sample, the incident light interacts with the molecules and distorts the electron cloud forming a "virtual level". The "virtual level" is instable; thus, photons are scattered immediately to a relatively stable state. When photons return to the initial level of energy (ground level), there is no energy transfer between the incident light and scattered light, and photon frequency and wavelength are not changed (Figure 4). This elastic collision process is referred to as Rayleigh scattering. On the other hand, when

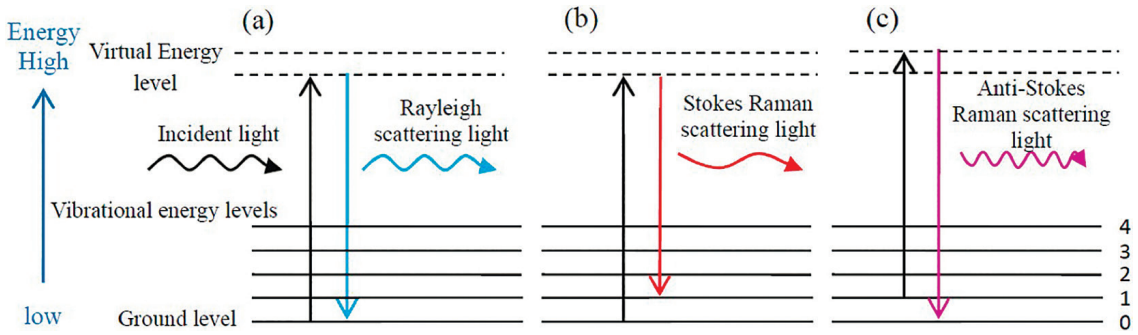


**Figure 4.** Diagram of the Rayleigh and Raman scattering processes:
(a) Rayleigh scattering, (b) Stokes Raman scattering and (c) anti-Stokes Raman scattering [33]

photons move to a new energy level that is different from the initial one, the energy transfer occurs (that is, a photon loses or acquires a certain amount of energy), which leads to a downward or upward shift in the energy of laser photons. This provides information about a substance under investigation [33].

Raman scattering can be divided into two types: Stokes Raman scattering and anti-Stokes Raman scattering. In Stokes Raman scattering, photons are excited from the initial energy level and move to a higher energy level. As a result, the scattered light has a lower frequency than the incident light. In anti-Stokes Raman scattering, photons are excited from the initial energy level and move to a lower energy level. In this case, the scattered light has a higher frequency compared to the incident light [33].

Transitions that have large Raman intensities often have weak IR intensities and vice versa. When a bond is strongly polarized, a small alteration in its length, which occurs, for instance, during vibration, will have only a small effect on polarization. Thus, vibrations that are associated with polar bonds (for example, C-O, N-O, O-H) are relatively weak Raman scatterers. However, such polarized bonds carry their electrical charges during the vibrational motion (unless neutralized by symmetry factors), which leads to a larger alteration in the net dipole moment during vibration, generating a strong IR absorption band. On the contrary, comparatively neutral bonds (for example, C–C, C-H, C=C) undergo large changes in polarizability during vibration. Nevertheless, there is no similar effect on the dipole moment; therefore, vibrations that involve mainly this type of bonds are strong Raman scatterers, but they are weak in the IR range [34].

Raman shifts are usually expressed in wavenumbers that have the inverse length since this value is directly related to energy. For conversion of a spectral wavelength into wavenumbers of a shift in the Raman spectrum, the following equation can be used:

$$\Delta\tilde{\nu} = \left(\frac{1}{\lambda_0} - \frac{1}{\lambda_1}\right), \qquad (1)$$

where

$\Delta\tilde{\nu}$ is the Raman shift expressed in a wavenumber;

$\lambda_0$ is the excitation wavelength;

$\lambda_1$ is the Raman spectrum wavelength.

An inverse centimeter ($cm^{-1}$) is the most frequently used measurement unit for expression of a wavenumber in Raman spectra. As a wavelength is often expressed nanometers (nm), the equation given above can be scaled for this conversion of units:

$$\Delta\tilde{\nu}(cm^{-1}) = \left(\frac{1}{\lambda_0(nm)} - \frac{1}{\lambda_1(nm)}\right) \times \frac{(10^7\,nm)}{(nm)}. \qquad (2)$$

Modern Raman spectroscopy almost always envisages the use of lasers as a source of light excitation. As lasers became available only more than three decades after the discovery of the effect, C. V. Raman and K. S. Krishnan used a mercury lamp and photographic plates for spectra recording [31]. It required hours or even weeks to obtain early spectra because of weak light sources, low sensitivity of detectors, as well as weak Raman scattering cross sections of most materials. To choose certain regions of wavelengths for excitation and detection, different color filters and chemical solutions were used. Nevertheless, a wide central line that corresponded to Rayleigh scattering of the excitation source still dominated in the photographic spectra [35].

Usually, Raman scattering is very weak, which was a problem for spectra collection for a long time. There was a need for methods that could separate weak inelastic scattering from intensive Rayleigh scattering. To this end, holographic gratings and dispersion came into use. Initially, photomultipliers were used as detectors; however, this method of collection was time consuming [36].

Technological achievements appeared in the 1980s made Raman spectroscopy much more sensitive. This was facilitated by the development and invention of modern radiation detectors such as charge-coupled devices — CCD detectors. The development of the method was also strongly affected by the appearance of reliable, stable, low-cost lasers [37].

The principle scheme of the modern Raman spectroscope is presented in Figure 5.

**Modifications of Raman spectroscopy**

The term Raman spectroscopy usually refers to vibrational Raman radiation with the use of laser wavelengths that are not absorbed by a sample. No less than 25 modifications of Raman spectroscopy were developed including surface-enhanced Raman spectroscopy, resonance Raman

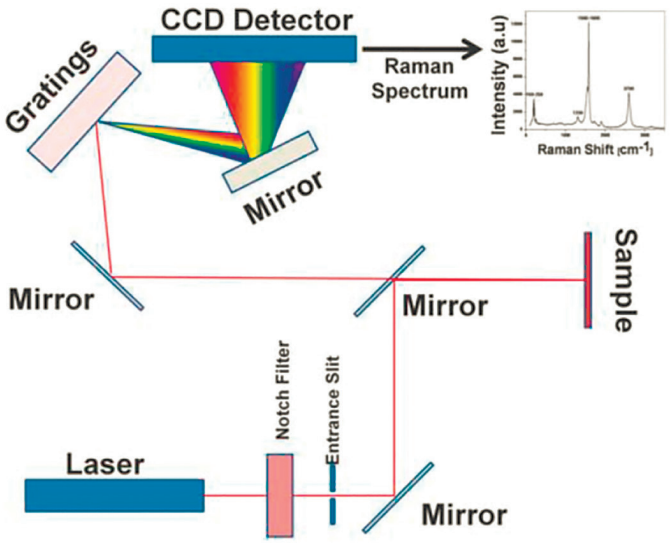


**Figure 5.** Principle scheme of the modern Raman spectroscope [36]

spectroscopy, polarized Raman spectroscopy, stimulated Raman spectroscopy, transmission Raman spectroscopy, spatially offset Raman spectroscopy and hyper-Raman spectroscopy [30,36]. As a rule, the aim is an increase in sensitivity (for example, surface-enhanced Raman spectroscopy), improvement of spatial resolution (Raman microscopy) or acquisition of a very specific information (resonance Raman scattering).

**Spontaneous (or far-field) Raman spectroscopy**

Spontaneous Raman spectroscopy or normal Raman spectroscopy includes Raman spectroscopic methods based on Raman scattering with the use of normal far-field optics. There are several variants of normal Raman spectroscopy regarding the excitation-detection geometry, combination with other methods, application of specific (polarization) optics and specific selection of excitation wavelengths to enhance resonance:

- Correlative Raman imaging [38]
- Resonance Raman spectroscopy (RRS) [39]
- Angle-resolved Raman spectroscopy [40]
- Optical tweezers Raman spectroscopy (OTRS) [41]
- Spatially offset Raman spectroscopy (SORS) [42]
- Raman optical activity (ROA) [43]
- Transmission Raman spectroscopy [44]
- Micro-cavity substrates [45]
- Remote Raman spectroscopy [46]
- X-ray Raman scattering [47]

**Enhanced (or near-field) Raman spectroscopy**

In enhanced Raman spectroscopy, the enhancement of Raman scattering is attained due to the enhancement of the local electric field by the optical near-field effect (for instance, localized surface plasmons). The examples include:

- Surface-enhanced Raman spectroscopy (SERS) [48, 49]
- Surface enhanced resonance Raman scattering (SERRS) [50]
- Tip-enhanced Raman spectroscopy (TERS) [51,52]
- Surface plasmon polariton enhanced Raman scattering (SPPERS) [53]

**Non-linear Raman spectroscopy**

In non-linear Raman spectroscopy, the enhancement of the Raman signal is attained due to the non-linear optical effects achieved, as a rule, by mixing two or more wavelengths that are emitted by spatially and temporally synchronized pulsed lasers. The examples include:

- Hyper Raman spectroscopy [54]
- Stimulated Raman spectroscopy (SRS) [55]
- Inverse Raman spectroscopy [56]
- Coherent anti-Stokes Raman spectroscopy (CARS) [57]

There is also morphologically directed Raman spectroscopy (MDRS), which combines the methods of automated particle imaging and Raman microspectroscopy into an integrated platform that allows detecting the chemical and morphological characteristics of individual components in a multi-component sample [58].

The main Raman spectroscopic methods used in analysis of biological objects are presented in Table 1 [21].

**Table 1. Raman spectroscopic methods used in analysis of biological objects [21]**

| Method | Characteristics | Advantages | Drawbacks | Application |
|---|---|---|---|---|
| Coherent anti-Stokes Raman spectroscopy (CARS) | Non-linear approach using multiple laser frequencies; generated strong anti-Stokes signal reveals vibrational coherence | Increased signal ($10^3$–$10^6$); high sensitivity; 3D imaging | Non-resonant background can dominate over weak resonant signals | Imaging of cells and tissues; diagnosis of cancer |
| Confocal Raman microspectroscopy | Adding a confocal microscope allows tissue depth measurement. A pinhole is used in the spectrometer for stray light rejection | High sensitivity; high lateral and depth resolution; 3D imaging | Diffraction-limited resolution | Imaging of cells and tissues; diagnosis of cancer |
| Drop coating deposition Raman spectroscopy (DCDRS) | Small volume of a liquid sample is dropped onto the flat substrate and dried | Small volumes (2–10 μl) of liquids are needed | Not fully free from the "coffee ring" effect | Analysis of biofluids; quantification of protein |
| FT-Raman spectroscopy | System that uses Fourier transformation and the Michelson interferometer | High throughput; high resolution; fluorescence-free | Low scattering intensity; limited to IR measurements; detector noise limited | Plant materials |

*Table 1. End*

| Method | Characteristics | Advantages | Drawbacks | Application |
|---|---|---|---|---|
| Kerr-gated Raman spectroscopy | Linear method that uses the repeated laser pulses and the Kerr gate (capture Raman light temporally — up to 3 picoseconds) | Depth measurement up to several millimeters; fluorescence rejection; high sensitivity | Not fully fluorescence-free; better performance in combination with shifted excitation | Depth profiling of human tissue |
| Polarized Raman spectroscopy (PRS) | Polarized light with the specific electric field vector obtains spectral information only from specific vibrational modes according to their orientation in reference to the incident beam | Information about the molecular structure and orientation | Inapplicable to the majority of samples; loss of the spectral information; time consuming | Orientation of collagen structures; plant photosystems |
| Raman Optical Activity (ROA) | Use of right- and left-circularly polarized incident light, which allows detecting the optical activity of discrete molecular vibrations. | Structural information from specific conformations of chiral molecules | Circular intensity differences are very small; vibrational coupling in signals can hamper accurate band assignment | Analysis of biopolymers |
| Resonance Raman spectroscopy (RRS) | Uses the "resonance effect" when the laser frequency coincides with (or is close to) the frequency of the electronic transition of a sample or compound under investigation | An increase in a signal of up to 6 orders of magnitude | Susceptible to fluorescence interference | Photosystems of plants; Analysis of human tissues |
| Shifted excitation Raman difference spectroscopy (SERDS) | Non-linear approach, in which two spectra at slightly different laser frequencies are obtained and a difference spectrum is created by subtracting the two; hence, eliminating background fluorescence | Fluorescence rejection; increased sensitivity | Difference spectra are reconstructed with the use of peak fitting; error-prone | Living cells and tissues of animals and humans |
| Spatially offset Raman spectroscopy (SORS) | For illumination of the the sample surface, continuous low intensity laser beams are used. Spectra are then derived at different distances from the surface. A scaled subtraction between these spectra shows changes indicative of the underlying layers | Depth measurements up to several millimeters | Comparatively weak signal | Diagnosis of cancer Chemical analysis upon physical impacts |
| Surface enhanced spatially offset Raman spectroscopy (SESORS) | SERS and SORS approaches are combined, enabling detection of SERS nanoparticles added to turbid samples | Detection of the SERS signals up to 50 mm below the sample surface | Addition of nanoparticles is needed | Depth measurements of samples |
| Stimulated Raman scattering (SRS) | Non-linear approach with the use of wave pumping and scattered Stokes radiation, which are tuned to a specific frequency representative of molecular vibrations. The transmitted intensity is proportional to the biochemical components | Not susceptible to the effect of fluorescence and the nonresonant background; high sensitivity (1 in $10^6$ photons); high spatial resolution | Proneness to interference from strong Raman scatterers; restricted to measuring one Raman peak per acquisition | Imaging of cells and tissues |
| Surface enhanced Raman scattering (SERS) | Surface plasmon resonance of the metal surface with nanoscale roughness is used, which significantly increases the electric field upon excitation by a laser. Upon adsorption on a biomolecule, nanoparticles lead to a significant enhancement of Raman scattering | Enhanced signal ($10^3$–$10^{10}$); resolution is lower than the diffraction limit; fluorescence quenching; low limit of detection; molecular labeling | Lack of reproducibility; band intensity of high frequency modes can be reduced; molecular selectivity to nanoparticle adherence | Detection of single molecules; analysis of living cells; diagnosis of cancer; identification of bacteria; plant materials |
| Surface enhanced resonance Raman scattering (SERRS) | RRS and SERS approaches are combined with the use of the laser frequency in resonance with a biomolecule in question and introduction of the SERS active substrate | Enhancement up to $10^{15}$; incremental benefits of both SERS and RRS | Increased complexity of the experiment | Detection of biomolecules Analysis of protein |
| Tip enhanced Raman spectroscopy (TERS) | Tip of the atomic force microscope, which is coated with SERS active metal, is used. Upon placement in close proximity to a sample, it leads to enhanced scattering | Tip-dependent spatial resolution; low limit of detection; fluorescence quenching; resolution is lower than the diffraction limit | Increased complexity of the experiment; sample is heated at the tip apex | Microbiology; biochemical imaging |
| Total internal reflection Raman spectroscopy | Sample is placed in contact with a reflective prism, through which a laser beam is reflected, generating an evanescent wave that penetrates the sample below | Specified penetration depth | Surface sensitivity is reduced | Plant materials |
| Transmission Raman | Raman scattered light is collected on the opposite side of laser illumination | Depth measurements up to 30 mm; appropriate for non-transparent materials | Interference from surface molecules | Diagnosis of cancer |

**Application of Raman spectroscopy in the meat industry**

Recently, Raman spectroscopy has received much attention. Many authors confirm that Raman spectroscopy is of great interest in assessment of meat composition and quality [59,60,61]. However, it has to be taken into account that Raman spectra have many dependent variables; therefore, it is necessary to use methods of multivariate analysis. The most often used method of multivariate analysis for this technique is partial least-squares regression (PLSR) analysis [62]. Several authors additionally use chemometrics to extract representative information from Raman spectra of meat and analyze the relation between the molecular structure and different radical groups to determine and assess meat quality (Figure 6) [63,64].

Data of Raman spectroscopy correlate with results obtained using the traditional control methods (water binding capacity, detection of texture, content of dimethylamine, peroxide value and fatty acid composition) and can be used for meat quality assessment. Raman spectroscopic methods (RSMs) give structural information about changes in meat proteins and lipids occurring during storage [65].

Raman spectroscopy is an effective and non-invasive method for studying alterations in the protein secondary structure, analysis of amide I (1650–1680 $cm^{-1}$) and amide III (1200–1350 $cm^{-1}$) regions, C–C groups (940 $cm^{-1}$) and modifications of local muscle proteins (tryptophan residues, bands of aliphatic amino acids) [22]. Herrero [22] used Raman spectroscopy to reveal structural changes in isolated myofibrillar and connective tissue proteins due to the addition of various compounds and an effect of freezing and storage in the frozen state. It was found that RSMs are a tool for in situ monitoring of protein structural changes in meat during storage in the frozen state and prediction of functional and organoleptic properties of raw materials [22].

Chemical substances, such as glycogen, glucose, lactate and cortisol, are predictors of meat quality; however, their detection on the meat surface by conventional Raman spectroscopy is restricted due to a low concentration. Ostovar Pour et al. [66] used spatially offset Raman spectroscopy (SORS) to detect spectral bands of glycogen, lactate, glucose and cortisol in beef muscle tissue (5 mm below the surface). The chemometric analysis performed by the authors revealed clearly the separation of peaks of metabolites into four groups under investigation [66].

Later on, Ostovar Pour et al. [67] studied the potential of spatially offset Raman spectroscopy (SORS) in discrimination between beef cuts (rump, Scotch fillet, round, chuck, tenderloin, and T-bone). The obtained results showed differences in the structure-sensitive bands from the amide I and III regions, cysteine, glutamic acid, and phenylalanine [67].

Cama-Moncunill et al. [68] investigated the potential of RSMs with subsequent chemometrics to predict Warner-Bratzler shear force (WBSF), intramuscular fat (IMF), pH, drip losses and cooking losses. Regression models PLS were developed based on the spectra recorded in thawed *longissimus thoracis et lumborum* muscle frozen 2 days after slaughter. Except pH, models demonstrated pronounced performance in calibration (coefficient of determination $R^2$ was in a range of 0.5 to 0.9) and promising predictive capability: WBSF (root-mean square error of prediction (RMSEP) was in a range of 4.6 to 9 N,) IMF (RMSEP from 0.9 to 1.1%), drip losses (RMSEP from 1 to 1.3%) and cooking losses (RMSEP from 1.5 to 2.9%).

Yang et al. [69] studied pH, meat color and microbial counts in beef steaks stored at 4 °C for 21 days using two different packaging methods: vacuum packaging (VP) and modified atmosphere packaging (MAP). The PLSR models demonstrated that Raman spectroscopy was able to predict total viable counts (TVC) and lactic acid bacteria (LAB) counts that were measured 21 days after slaughter (TVC in VP: $R^2_{cv} = 0.99$, RMSEP = 0.61; TVC in MAP: $R^2_{cv} = 0.90$, RMSEP = 0.38; LAB in VP: $R^2_{cv} = 0.99$, RMSEP = 0.54; LAB in MAP: $R^2_{cv} = 0.75$, RMSEP = 0.60). The obtained results showed a possibility of using Raman spectroscopy to rapidly detect meat spoilage.

Combination of Raman spectroscopy with the chemometric method for quantification of myoglobin proportions (deoxymyoglobin and oxymyoglobin) is presented in [70]. The optimal results were obtained with the prediction model "random frog-partial least squares (projection into latent structure)" (RF-PLS) for both

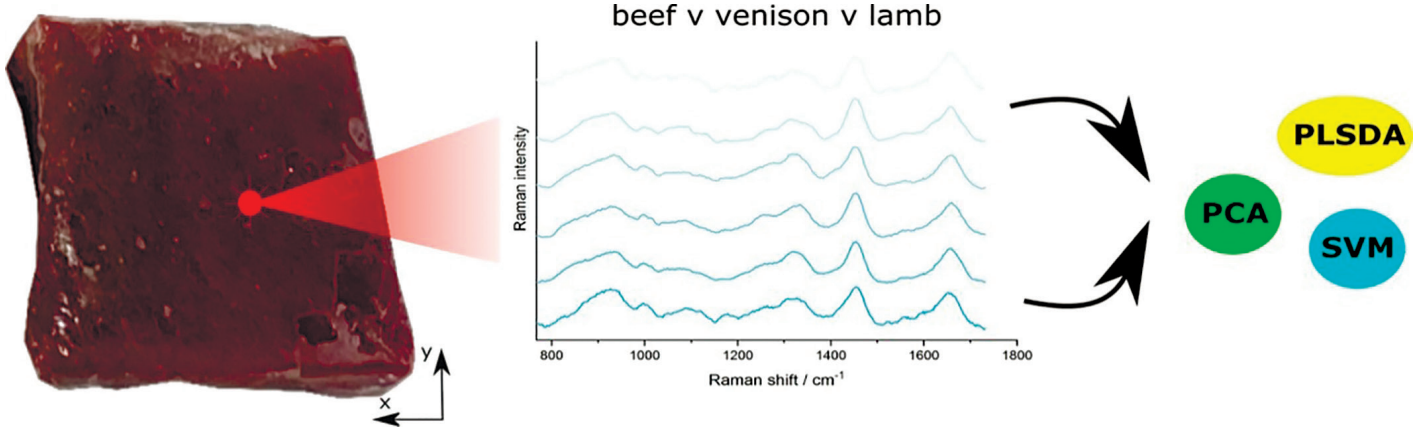


**Figure 6.** Scheme of Raman spectroscopy and analysis of the obtained results [64]

deoxymyoglobin (Rp = 0.8936; RMSEP = 2.91) and oxymyoglobin (Rp = 0.9762; RMSEP = 1.23).

Boyacı et al. [71] used the Raman spectroscopic method coupled with chemometrics to detect beef falsification with horsemeat (n = 49). Processing of data from the collected Raman spectra was carried out using principal component analysis (PCA). All meat samples were correctly classified by their origin. In addition, different concentrations (25%, 50%, 75%, w/w) of horsemeat in the beef samples were also determined using the created model system.

The results of the studies demonstrate that Raman spectroscopy in combination with the chemometric method of data processing can be used to determine an origin of meat from different species of slaughter animals over a very short time of analysis (30s) without a need for sophisticated chromatography, immunological or genetic methods of analysis [72,73].

The potential of Raman spectroscopy combined with three chemometric methods for differentiation of red meat samples (beef, lamb and venison; n = 90) is shown in [64] (Figure 7). PLSDA (partial least squares discriminant analysis) and SVM (support vector machines) classifications were used for creation of classification models, while PCA was used for the exploratory research (Figure 8). The results obtained with the linear and non-linear kernel SVM models demonstrated sensitivity of more than 87% and 90%, respectively. The PLSDA model showed accuracy of 92% and 81% in determining lamb and 88% and 79% in determining beef for both the training and test sets, respectively.

Zhao et al. [74] used RSMs to predict organoleptic properties of beef samples (n = 72) (Figure 9). The best results of prediction were achieved when a Raman frequency range of 1300–2800 $cm^{-1}$ was used. The prediction performance of the PLSR models was moderate to high for all organoleptic indicators ($R_{CV}^2$ = 0.50–0.84; RMSECV = 1.31–9.07) and especially high for flavor characteristics ($R_{CVS}^2$ = 0.80–0.84, RMSECVs = 4.21–4.65).

Raman spectroscopy is widely used in studying quality of meat from different species of slaughter animals, as well as chicken, including analysis of raw fat characteristics [75,76], detection of boar taint in pork [77], determination of organoleptic properties of raw materials [78], pH [79], spoilage [80] and identification of meat from different animal species [72,81] (Table 2).

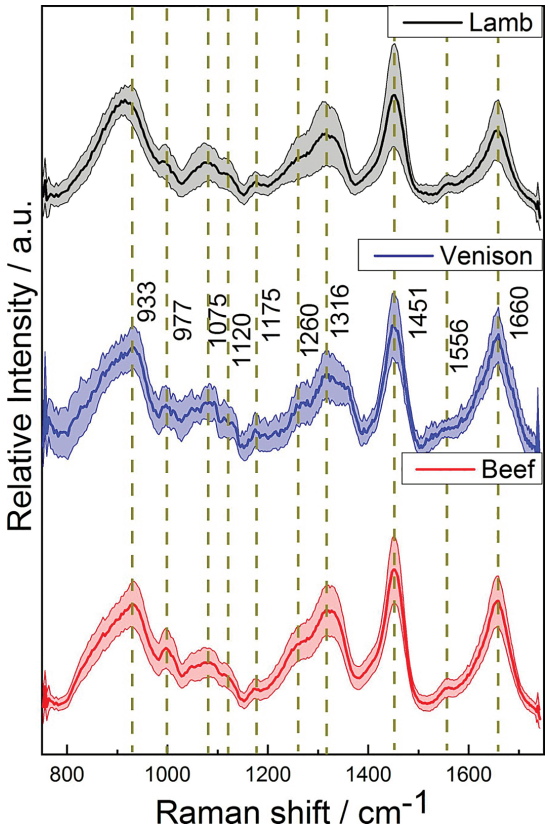


**Figure 7.** Mean Raman spectra of beef, venison and lamb [64]

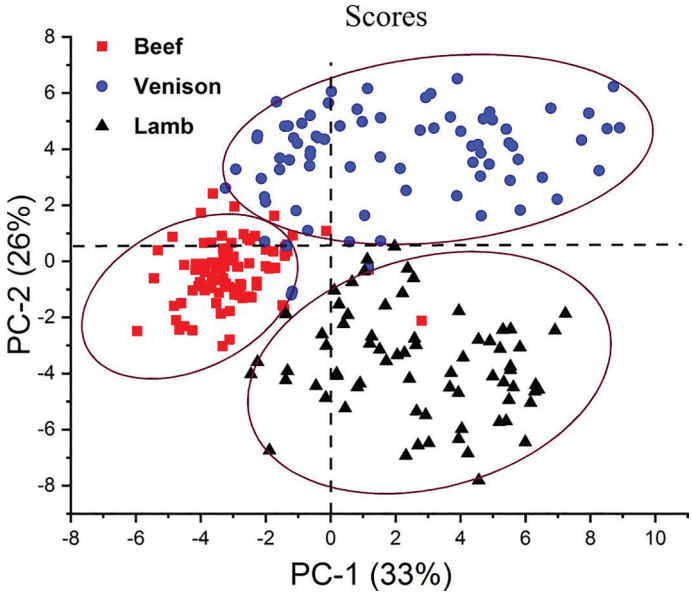


**Figure 8.** Separation of beef, venison and lamb samples using PCA [64]

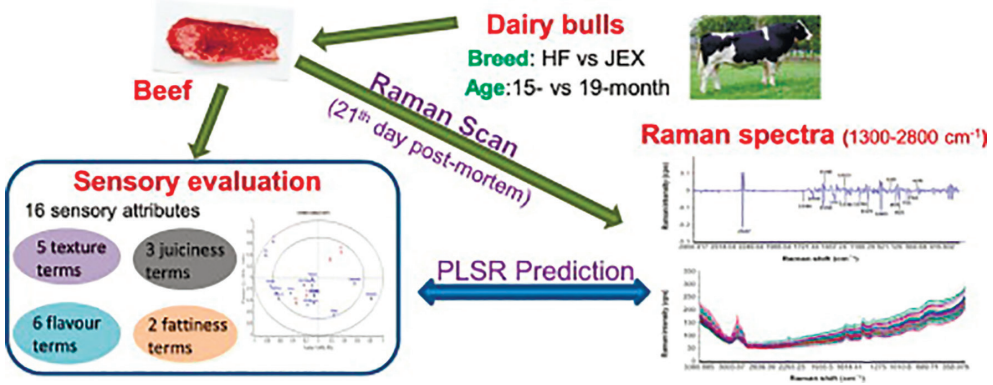


**Figure 9.** Scheme of research using RSMs and PLSR [74]

Table 2. Use of Raman spectroscopy for meat raw material analysis

| Sample | Indicator | Algorithm of data analysis | Results | Source |
|---|---|---|---|---|
| Chicken | Protein structure | one-way ANOVA* | Upon addition of sodium bicarbonate, an increase in hydrophobic interactions as a result of protein unfolding and exposure of aliphatic residues was established. It was concluded that sodium bicarbonate can be used for reduction of the sodium chloride content. | [82] |
| Pork backfat | Fatty acids | PLS* | Correlation of spectra with parameters of the total fatty acid composition and most of the individual fatty acids ($R_{CV}^2$ = 0.78–0.90)* | [83] |
| Pork after heat treatment | Temperature control during heat treatment | PLS-DA* PCA | Detection of processing temperature of below or above 65 °C (accuracy of 97.87% and 97.62%, respectively) | [84] |
| Pork | pH | PLS-DA*, PCA | It is possible to predict pH values by spectra, (RMSECV = 0.13 for pH after 45 min. and RMSECV = 0.21 for pH after 24 hours) | [79] |
| Pork (longissimus lumborum) | Drip losses and pH | PLSR* | It is possible to use Raman spectroscopy for rough screening of drip losses and pH ($R_{CV}^2$ = 0.75 for drip losses and $R_{CV}^2$ = 0.72 for pH)* | [85] |
| Beef | Tenderness | PLSR* | Tough and tender samples can be identified with the accuracy of 70–88% | [25] |
| Beef | Falsification | PLSDA* | Detection of falsification with the efficiency rate of 86.6% and 79.8% for the training and test sets, respectively | [86] |
| Beef | Texture | PCA*, PLSR* | Prediction of tenderness, chewiness and firmness with $R^2$ = 0.81, 0.80 and 0.81*, respectively | [87] |
| Beef | Organoleptic characteristics | PLSR | $R^2$ = 0.63–0.89* for the same breed and 0.52–0.89 for the same age | [74] |
| Beef | Saturated fatty acids | PCA* | Differences between Australian grass-fed and grain-fed beef by average spectra of carcasses indicating different fatty acid content | [88] |
| Beef | Physico-chemical indicators | PLS-DA*, PLSR | All samples were correctly classified using PLS-DA*; with that, correct identification was achieved for 86.7% of samples from different muscles. The PLSR* models that used Raman spectra of the 3rd day after slaughter had better prediction performance compared to the models that used Raman spectra of the 7th and 14th days | [89] |
| Beef | Organoleptic indicators (juiciness and tenderness) | PLSR* | Correlation between predicted and observed values of juiciness and tenderness of 0.42 and 0.47, respectively | [90] |
| Beef, venison and lamb | Identification of meat from different animal species | PCA*, PLS-DA*, SVM* | Models providing accuracy of more than 80% (PLSDA*) and 92% (SVM*) for identification of unknown meat samples (test set) | [64] |
| Beef tallow, pork lard, chicken fat, duck oil | Fatty acid analysis: unsaturated fatty acids and total fatty acids | Linear correlation | Fat classification using Raman peak ratio. An indicator "oil gauge (OG)" was proposed as a standard trait for fat classification | [91] |
| Lamb (*m. Longissimus lumborum*) | Intramuscular fat content and major fatty acid groups | PLSR* and linear regression | Prediction of PUFA ($R^2$ = 0.93)* and MUFA ($R^2$ = 0.54)*, as well as SFA levels adjusted with regard to the IMF content ($R^2$ = 0.54)* | [92] |
| Lamb | Technological properties (Warner-Bratzler shear force, cooking losses) | PLSR* | For shear force $R^2$ = 0.79* and $R^2$ = 0.86*, for cooking losses $R^2$ = 0.79* and $R^2$ = 0.83* for two models between observed and predicted values | [93] |
| Lamb (*m. Longissimus lumborum*) | Technological properties | PLSR* | $R_{CV}^2$ = 0.06* between observed and cross validated predicted values | [26] |
| Lamb (*m. Semimembranosus*) | Technological properties | PLSR* | $R_{CV}^2$ = 0.27* between observed and cross validated predicted values | [94] |
| Lamb (*m. Semimembranosus*) | Indicators of meat freshness during storage and after freezing/thawing | PLSR* | $R_{CV}^2$ from 0.33 to 0.59 for various indicators between observed and predicted values. Possibility to identify carcasses with deviations during autolysis | [95] |

* PLS — partial least squares (projection into latent structure); PLSR — partial least squares regression (PLS-regression); ANOVA — analysis of variance; PLS-DA — partial least squares discriminant analysis; PCA — principal component analysis; SVM — support vector machines; $R^2$ — coefficient of determination; $R_{CV}^2$ — coefficient of determination in cross-validation

Andersen et al. [96] compared the results of Raman, near infrared (NIR) and fluorescence spectroscopy for analysis of pH and porcine intramuscular fat (*m. Longissimus lumborum*) (n = 112) 4–5 days after slaughter. The results of Raman spectroscopy showed $R_{CV}^2$ in a range of 0.49 to 0.73 for all examined indicators (upon PLSR). Near infrared and fluorescence spectroscopy demonstrated limited possibilities for quality analysis ($R_{CV}^2$ was in a range from 0.06 to 0.57 and from 0.04 to 0.18, respectively).

Later on, Andersen et al. [85] carried out research on prediction of drip losses using RSMs. Their results revealed that Raman spectroscopy can be used for rough screening of drip losses and pH in pork and that a sampling site is important for successful predictions. PLSR models were created using spectra from each of two samples of *m. Longissimus lumborum* (ventral and dorsal) individually or averaged spectra from both samples. The best results were observed for the models that used the sample from the ventral part of the muscle: $R_{CV}^2 = 0.75$, RMSECV = 1.27%, the ratio of prediction to deviation (RPD) = 2.0 for drip losses measured by the method EZ-DripLoss, and $R_{CV}^2 = 0.72$, RMSECV = 0.05 and RPD = 2.0 for pH.

Using a Raman spectrometer, samples of dry-cured ham from Iberian pigs were analyzed (n = 110). Four commercial categories were used in the study: pure-bred Iberian acorn-fed pigs, crossbred Iberian acorn-fed pigs, crossbred free-range feed-fed Iberian pigs and crossbred Iberian feed-fed pigs [97]. The results presented by the authors demonstrate that RSMs can be used as a rapid screening tool for quality verification of commercial dry-cured Iberian ham. LDA (linear discriminant analysis) chemometric models obtained using a Raman signal enabled classifying pigs according to the breed and feeding regime.

Tomasevic et al. [27] studied a possibility of using Raman spectroscopy for species identification of beef and pork in frankfurters. To this end, five different sausage recipes that included beef and pork were investigated. Linear discrimination analysis in combination with PCA and PLS was used for data analysis. The results showed high sensitivity of the models for beef sausages: 91.67% and 100%. The authors concluded that Raman spectroscopy can be used as a non-invasive method for rapid authentication of frankfurters.

Beattie et al. [98] used a combination of the Raman method with the method of multivariate analysis and neural networks achieving accuracy of 96.7% (with PCLDA) to 99.6% (with PLSDA) in classification of chicken, beef, lamb and pork fat. This possibility of identification was confirmed by others scientists worked with RSMs and used principal component analysis for successful classification of fat samples [81] (Figure 10 and Figure 11). The authors proposed RSMs as a useful tool for detecting falsifications in the meat industry, which will facilitate alleviation of consumers' concerns about meat they eat [98].

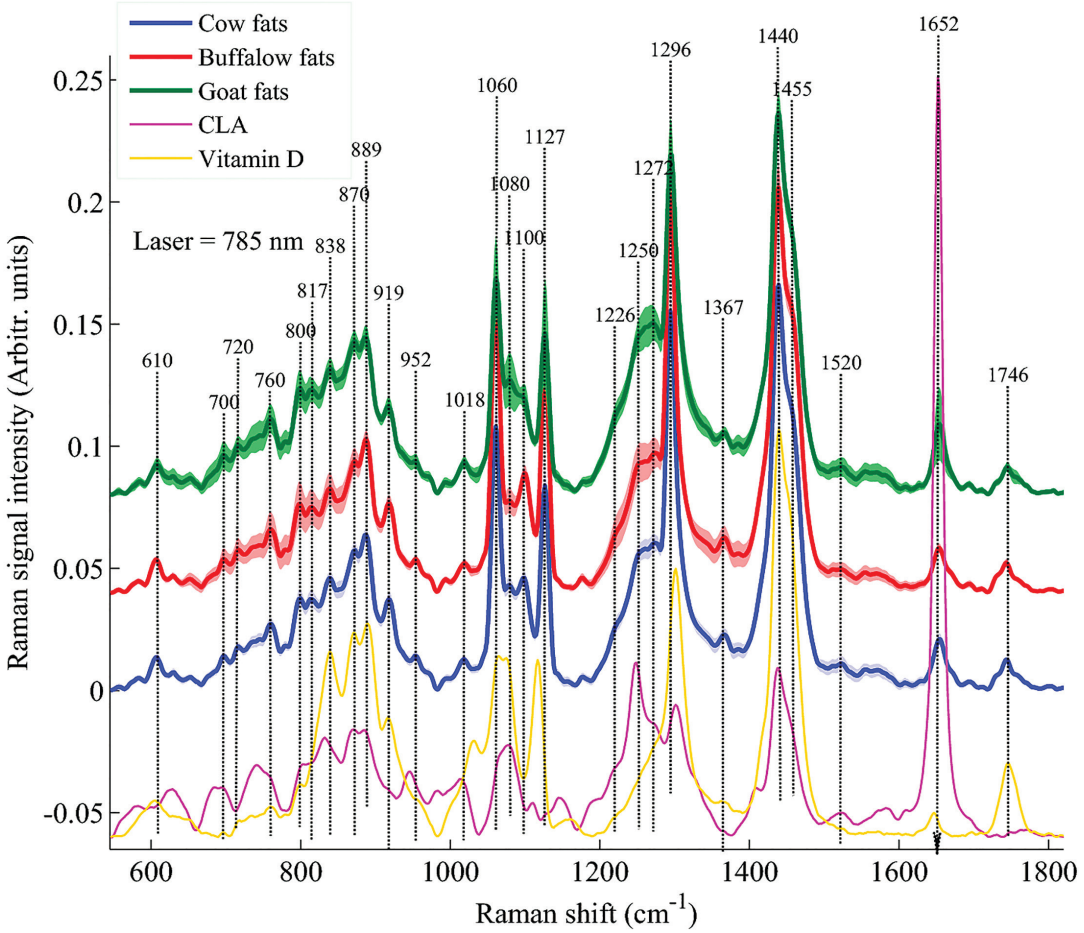


**Figure 10.** Raman spectra of 13 samples of beef fat, 18 samples of buffalo fat and 16 samples of goat fat, vitamin D and CLA (conjugated linoleic acid) obtained using laser with a wavelength of 785 nm [81]

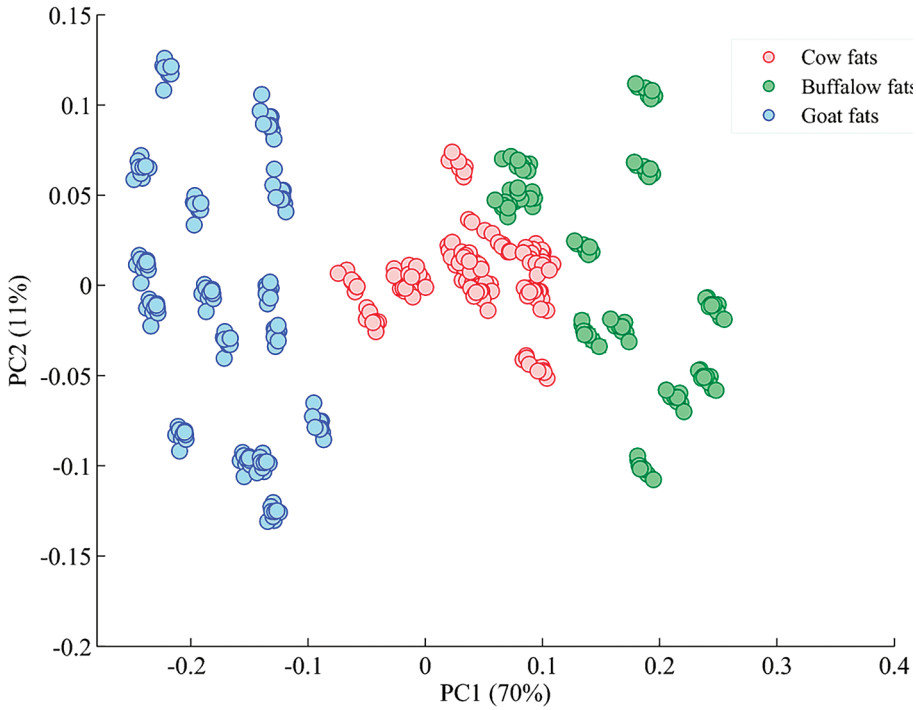


**Figure 11.** PCA analysis of Raman spectra of goat, beef and buffalo fat samples obtained using laser with a wavelength of 785 nm [81]

The prediction results for several technological characteristics of lamb quality such as Warner-Bratzler shear force (WBSF), color, cooking losses and pH using the method of Raman scattering are presented in [26,93,94,95]. The most interesting results were obtained when studying shear force. Prediction of this indicator in the samples of *m. Longissimus thoracis et lumborum* showed low accuracy ($R_{CV}^2$ = 0.06 between observed and cross validated predicted values) in [26], in contrast to the study by Schmidt H. et al. [93], where coefficients of determination of 0,79 and 0.86 were obtained for this characteristic (measurement in two muscle sites after freezing and thawing). When predicting shear force in *m. Semimembranosus*, a reduction in root mean square error by 12.9% and 7.6% was observed during aging for one and five days, correspondingly [94]. Raman spectroscopy was not able to predict this indicator in analysis of the *m. Semimembranosus* samples after freezing and thawing [95].

Traditional methods for determination of fat content and fatty acid composition are based, as a rule, on methods of extraction with a solvent and gas chromatography, and require the use of dangerous chemical solvents and thorough sample preparation, are expensive, labor intensive and result in irreversible damage to a sample. These drawbacks make them unsuitable for using in production conditions of meat plants [99]. Raman spectroscopy showed good results in measuring concentrations of the main fatty acid groups, such as PUFA, MUFA and SFA, as well as intramuscular fat [92]. The use of Raman scattering to assess the fatty acid content has a significant practical advantage as it does not require extraction and purification processes.

Lee, J.Y. et al. [91] classified four animal fats (beef, pork, chicken and duck fat) using Raman spectroscopy in combination with simple calculation of the intensity ratios of the Raman signals at the vibrational modes that corresponded to unsaturated fatty acids and total fatty acids.

When developing spectroscopic equipment for assessment of meat quality and composition, a special attention is being given to RSMs, as these methods do not require long and labor intensive sample preparation, are rapid and easy to perform (analysis can be done within several seconds). A trend towards promotion of the real-time automated control and quality control directly in production is seen worldwide [63]. At present, portable Raman spectroscopes with a robust water-proof casing for sensor protection have been developed for the use in the meat industry [100].

Bauer et al. [25] used a portable Raman system with a wavelength of 671 nm to evaluate tenderness of beef (n = 175) aged at minus 1°C and 7°C for 14 days. The correlation between Raman spectra and Warner-Bratzler shear force with the use of PLS gave cross-validated predictions of WBSF for both storage temperatures with the coefficients of determination $Rcv^2$ = 0.33–0.79. It was found that tough and tender samples could be distinguished with accuracy of 70–88%.

Fowler, S.M. et al. [90] studied sensory characteristics (juiciness and tenderness) of beef loins (*m. Longissimus lumborum*) (n = 45) using a portable Raman spectroscope with a wavelength of 671 nm before and after freezing. It was established that the spectroscopic device could determine juiciness and tenderness with the correlation between the predicted and observed values (ρ) of 0.42 и 0.47, respectively. The main changes were observed in fatty acid concentrations, protein hydrophobicity and collagen orientation.

**Conclusion**

Comprehensive evaluation of meat raw materials by organoleptic characteristics, internal constituents and external factors, as well as the application of the developed high performance quality control systems to real meat processing lines in production are still topical. Researches focus their attention primarily on methods of non-destructive quality control.

The unique analytical possibilities of Raman spectroscopy are demonstrated. The collected data presented in this review show that the use of Raman spectroscopy makes it possible to predict quality indicators of meat raw materials with a high degree of certainty and obtain a large volume of information from an object without its destruction.

The main principles of Raman spectroscopy, used equipment and tools for analysis of obtained spectra are described. RSMs have been successfully used to determine the meat chemical composition, including the content of moisture, fat, fatty acids and protein, pH, indicators of freshness, organoleptic and technological indicators, as well as to reveal raw material falsification.

Raman spectroscopy is an alternative method for rapid identification of the meat chemical composition. Contrary to the traditional methods, it does not require the complex sample preparation, use of chemical reagents or highly qualified personnel. The use of portable spectroscopes allows doing research directly on a technological line.

On the other hand, Raman spectroscopy, like other new technologies, requires further research:

Nowadays, there is no unified database of meat raw material spectra, as well as protocols with optimal conditions (laser wavelength, power, exposure time) of spectra recording; therefore, time is necessary for analysis of obtained results.

Obtained spectra often contain a significant amount of excessive data, which can slow down the speed of their real-time processing.

Developed predictive models are mainly based on a single indicator and, therefore, their use for multivariable prediction is hampered.

The cost of the equipment is very high and it is necessary to develop inexpensive spectroscopes for routine investigations.

Therefore, RSMs can replace several traditional methods for analysis of physico-chemical, biochemical and technological indicators of quality of meat raw materials and products, and a huge work lies ahead for their wide application.

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## AUTHOR INFORMATION

**Viktoriya A. Pchelkina,** Candidate of Technical Sciences, Senior Research Scientist, Experimental Clinic-Laboratory of Biologically Active Substances of Animal Origin, V. M. Gorbatov Federal Research Center for Food Systems. 26, Talalikhina str., 109316, Moscow, Russia.
Tel.: +7–495–676–95–11 (242), E-mail: v.pchelkina@fncps.ru
ORCID: http://orcid.org/0000–0001–8923–8661
* corresponding author

**Irina M. Chernukha,** Doctor of Technical Sciences, Professor, Academician of the Russian Academy of Sciences, Principal Researcher, Experimental Clinic-Laboratory of Biologically Active Substances of Animal Origin, V. M. Gorbatov Federal Research Center for Food Systems. 26, Talalikhina str., 109316, Moscow, Russia.
Tel.: +7–495–676–95–11 (511), E-mail: imcher@inbox.ru
ORCID: http://orcid.org/0000–0003–4298–0927

**Liliya V. Fedulova,** Candidate of Technical Sciences, Head of the Experimental Clinic-Laboratory of Biologically Active Substances of Animal Origin, V. M. Gorbatov Federal Research Center for Food Systems. 26, Talalikhina str., 109316, Moscow, Russia.
Tel.: +7–495–676–95–11 (128), E-mail: l.fedulova@fncps.ru
ORCID: https://orcid.org/0000–0003–3573–930X

**Nikolai A. Ilyin,** Senior Laboratory Assistant, Experimental Clinic-Laboratory of Biologically Active Substances of Animal Origin, V. M. Gorbatov Federal Research Center for Food Systems. 26, Talalikhina str., 109316, Moscow, Russia.
Tel.: +7–495–676–95–11 (128), E-mail: nikolai-1996@mail.ru
ORCID: https://orcid.org/0000–0002–7980–3193

All authors bear responsibility for the work and presented data.

All authors made an equal contribution to the work.

The authors were equally involved in writing the manuscript and bear the equal responsibility for plagiarism.

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# MUTAGENIC AND/OR CARCINOGENIC COMPOUNDS IN MEAT AND MEAT PRODUCTS: HETEROCYCLIC AROMATIC AMINES PERSPECTIVE

**Emel Oz, Fatih Oz***
Ataturk University, Erzurum, Türkiye

**Keywords:** *heterocyclic aromatic amines, cooking, mutagenicity, carcinogenicity, meat, meat products*

***Abstract***

*Meat and meat products, which have a very important place in terms of nutrition, can endanger human health if they are not properly prepared and preserved. Meat and meat products except for products such as sushi, which are deliberately consumed raw, are generally consumed immediately after cooking. Cooking done properly gives meat and meat products their unique taste and aroma, increases their digestibility and makes them microbiologically safe. However, some harmful food toxicants can occur during the cooking process. Heterocyclic aromatic amines can be formed during cooking of the proteinaceous foods such as meat and meat products. Epidemiological studies have proved that heterocyclic aromatic amines are mutagenic and/or carcinogenic compounds. Therefore, having sufficient knowledge about heterocyclic aromatic amines will help to reduce the health risk posed by these compounds. In this context, in the present study, basic information about heterocyclic aromatic amines that can be formed during the heat treatment of meat and meat products was reviewed.*

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## Introduction

Our foods are complex mixtures of micro compounds such as vitamins and minerals and macro compounds such as protein, fat, and carbohydrates. In addition, our foods can contain antioxidants and dietary fiber that have been found to have significant effects on human health. On the other hand, studies have also proven that our food can contain very harmful substances such as microbial contaminants, toxins, or various chemicals that have negative effects on human health. Therefore, our foods, which play a role in the prevention and treatment of some diseases, are also responsible for the emergence of some diseases [1].

Cooking is a heat treatment generally applied immediately before the consumption of foods. This process takes place by heat and mass transfer, and physical, chemical, and biochemical changes occur in meat. As a result, the cooking process increases the flavor of meat, makes a product safe from the microbiological point of view, and increases its digestibility and tenderness [2]. However, if the cooking process is not done properly and carefully, it can cause the formation of some food toxicants such as heterocyclic aromatic amines (HAAs) in meat and meat products [3].

HAAs constitute a group of food toxicants that can be formed during the heat treatment (cooking) of protein-rich foods such as meat and meat products. These food toxicants, first identified in cooked meats by Japanese scientists in 1977, were named heterocyclic aromatic amines due to their chemical structure [4]. Epidemiological studies have proven that HAAs are mutagenic and/or carcinogenic compounds [5,6]. Due to the fact that foods containing these toxicants have been widely consumed in many countries and it is determined by epidemiological studies that the rate of cancer is much higher in countries that consume large amounts of meat, the number of studies done on HAAs has been increasing day by day. Therefore, it is important to have knowledge about these kinds of food toxicants. In this context, in the current study, various information about their structures, mutagenicity and carcinogenicity, formation mechanisms, and reduction of formation of heterocyclic aromatic amines was reviewed.

## Chemical structures, precursors, formation mechanisms, and factors affecting formation of heterocyclic aromatic amines

HAAs are durable solids melting at 200–300 °C with a polycyclic aromatic structure, containing exocyclic amino groups [7]. All of HAAs have the characteristic UV spectrum and high extinction coefficient, while some of them have fluorescence properties. Therefore, they can be analyzed due to these properties. To date, approximately 30 different HAA compounds have been identified [8].

HAAs are divided into two main groups according to their chemical structures. The first group of these compounds is aminoimidazoazoarenes, and the second group is aminocarbolines [9]. Aminoimidazoazoarenes, which form the first group, are also called the IQ-type compounds or thermal HAAs. These compounds are usu-

ally formed at temperatures between 150–300 °C. For this reason, it is stated that these kinds of HAAs can frequently occur in meat and meat products cooked at home, because cooking methods used in the home generally work at these temperatures. These compounds mostly consist of reactions between free amino acids, hexoses, and creatine/creatinine. They commonly contain either a quinoline or a quinoxaline or an imidazo group combined with pyridine. Therefore, aminoimidazoazoarenes include imidazoquinoline, imidazoquinoxaline, and phenylimidazo pyridine compounds [10]. The aminocarbolines, which form the second group, are also called the non-IQ type compounds or pyrolytic HAAs. These compounds are generally formed as a result of the pyrolysis of amino acids and proteins at temperatures above 300 °C. For this reason, it is stated that these kinds of HAAs can frequently occur in barbecued meat and meat products. They contain the 2-aminopyridine moiety as a common structure in their structure. In addition, HAAs can be classified according to their chemical behavior as polar (AIAs, Glu-P-1, and Glu-P-2) and apolar (all others) [11]. On the other hand, there are studies in the literature showing that these compounds can be formed in meat and meat products that are exposed to temperatures lower than these temperatures [12]. In addition, it has been determined that HAAs can occur not only in meat and meat products, but also in other foods such as milk and dairy products, coffee, alcoholic beverages, and tobacco products [13,14,15,16,17]. The chemical name, classification, abbreviation, and chemical structures of the most important HAAs are given in Table 1.

It is known that some precursors must be present in the environment for the formation of HAAs. In this context, it is stated that there are three major classes of the precursors; 1) creatine and/or creatinine, 2) sugars (especially reducing sugars), 3) free amino acids, dipeptides and proteins [18].

It is stated that the Maillard reaction is very important for the formation of HAAs, especially aminoimidazoazoarenes. The Maillard Reaction, which was detected by the French scientist Louis-Camille Maillard in 1912 [1], is still not a fully elucidated reaction, even after 110 years. Some Maillard reaction products are known to be anticarcinogens, but some reaction products have been determined to be mutagens and/or carcinogens. Indeed, it is very well known that as a result of the Maillard reaction, which occurs between reducing sugars and compounds containing free amino groups, taste, aroma, texture and brown pigments are formed; however, various food toxicants with mutagenic and carcinogenic properties can be formed in foods such as meat and cereals prepared for consumption [1].

Creatine, which is only present in foods of animal origin, is a non-protein nitrogenous substance. It loses one molecule of water and turns into creatine when the ambient temperature reaches about 100 °C. Creatine and creatinine are considered as important precursors in the HAA formation. It is thought that creatine forms the aminoimidazo part by cyclization and water elimination. This part is known to provide mutagenicity of aminoimidazoazoarenes and is present in all aminoimidazoazoarenes [19].

**Table 1. Classification, chemical name, abbreviation, and mutagenicities of the most important HAAs**

| Classification and chemical name | Abbreviation | Revertants/μg | |
|---|---|---|---|
| | | TA98 | TA100 |
| *Aminoimidazoazoarenes (IQ-type compounds or thermal HAAs)* | | | |
| 2-amino-3-methylimidazo[4,5-f]quinoline | IQ | 433.000 | 7.000 |
| 2-amino-3-methylimidazo[4,5-f]quinoxaline | IQx | 75.000 | 1.500 |
| 2-amino-3,4-dimethylimidazo[4,5-f]quinoline | MeIQ | 661.000 | 30.000 |
| 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline | MeIQx | 145.000 | 14.000 |
| 2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline | 4,8-DiMeIQx | 183.000 | 8.000 |
| 2-amino-3,7,8-trimethylimidazo[4,5-f]quinoxaline | 7,8-DiMeIQx | 163.000 | 9.900 |
| 2-amino-3,4,7,8-tetramethylimidazo[4,5-f]quinoxaline | 4,7,8-TriMeIQx | na | na |
| 2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine | PhIP | 1.900 | 1.200 |
| *Aminocarbolines (non-IQ type compounds or pyrolytic HAAs)* | | | |
| 2-amino-9H-pyrido[2,3-b]indole | AαC | 300 | 20 |
| 2-amino-3-methyl-9H-pyrido[2,3-b]indole | MeAαC | 200 | 120 |
| 1-methyl-9H-pyrido[4,3-b]indole | Harman | — | — |
| 9H-pyrido[4,3-b]indole | Norharman | — | — |
| 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole | Trp-P-1 | 39.000 | 1.700 |
| 3-amino-1-methyl-5H-pyrido[4,3-b]indole | Trp-P-2 | 104 | 1,8 |
| 2-amino-6-methyldipyrido[1,2-a:3',2'-d]imidazole | Glu-P-1 | 49.000 | 3.200 |
| 2-aminodipyrido[1,2-a:3',2'-d]imidazol | Glu-P-2 | 1.900 | 1.200 |

**na: not available**

The remaining parts of the aminoimidazoazoarenes are thought to be arising from Strecker degradation products (e.g pyridines and pyrazines) formed in the Maillard reaction. Then, an aldol condensation is suggested to link these two parts together via an aldehyde or the related Schiff base [20,21,22]. This hypothesis has been verified using the model systems [23].

The aminocarbolines are thought to be formed via free radicals. Pyrolysis occurs at temperatures above 300 °C and produces many reactive fragments by free radical reactions, and these fragments condense to form new compounds. In addition, it is reported that tryptophan is one of the most important precursors for this kind of heterocyclic amines and aminocarbolines have an indole moiety that comes from tryptophan [10]. On the other hand, there are studies indicating there are other precursors than tryptophan and pyrolysis is not the only way to the formation of aminocarbolines [24,25]. Figure 1 shows the formation mechanisms of IQ-type HAAs, PhIP, and norharman as aminocarboline.

It is stated that types and concentrations of HAAs that can be formed in foods that are subjected to heat treatment using different methods and temperature-time combinations depend on factors such as type of a product being heat treated, the temperature used during cooking, cooking time, cooking conditions, pH, water activity, carbohydrates, free amino acids, creatine, as well as heat and mass transfer, fats, fat oxidation and antioxidants [1,16,26,27].

**Mutagenicity and carcinogenicity of HAAs**

It is stated that meat that has not been subjected to heat treatment does not show the mutagenic activity. However, heat treatment causes the mutagenic activity in meats [28]. Short-term bacterial tests are used for the determination of the mutagenic activities of HAAs [29]. Substances with genotoxic potential are detected by the Ames test [30]. HAAs are very strong mutagens in the Ames test leading to mutations and chromosomal damage in cells of *Salmonella typhimurium* bacteria and carcinogens in animal experiments [31]. It is stated that they are 100 times more muta-

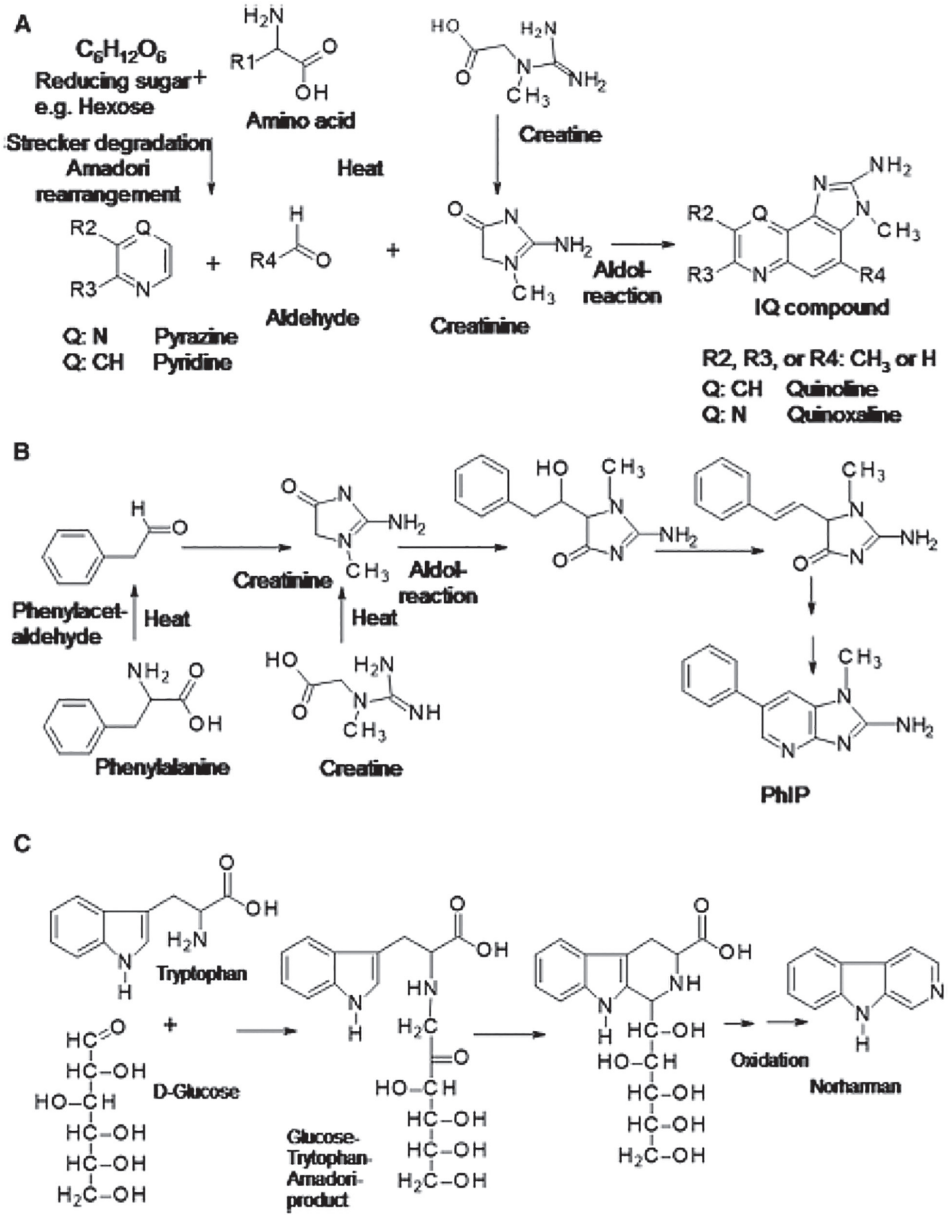


**Figure 1.** Formation mechanisms of IQ-type HAAs, PhIP, and norharman [16]

genic than aflatoxin B1 and 2000 times more mutagenic than benzo[a]pyrene [32]. The aminoimidazoazoarenes are especially highly mutagenic to *Salmonella typhimurium* TA98, ranging from 1.800 to 660.000 revertants/μg [10]. Data on the mutagenicities of the HAAs in *Salmonella typhimurium* histidine auxotroph TA98 and TA 100 are also given in Table 1. On the other hand, it is stated that the harman and norharman compounds in the aminocarbolines class do not show mutagenic activities, but they are co-mutagenic because they increase the mutagenicity and genotoxicity of other HAAs [33].

Epidemiological studies have shown that lifestyle factors, including a diet, have a significant impact on human cancers [34]. F344 rats and $CDF_1$ mice are generally used for the carcinogenicity tests for HAAs [34]. However, further studies are needed to reveal the effect of HAAs on human cancers. Epidemiological studies have proven a positive correlation between high consumption of meat (especially well done) and fish and cancers of the pancreas, breast, colon, colorectal, prostate, and ureter [27]. In addition, the International Agency for Research on Cancer (IARC) has identified HAAs as a probable human carcinogen (IQ, class 2A) and a possible human carcinogen (MeIQ, MeIQx, PhIP, AαC, MeAαC, Trp-P-1, Trp-P- 2 and Glu-P-1, class 2B) [35]. Therefore, it has been suggested that the accepted daily consumption amount should be 0–15 μg/day for a person [36].

**Levels of the HAA formation in meat and meat products**

It is very difficult to predict the exact amount of HAAs that can occur in heat-treated meat and meat products, based on the cooking method, cooking temperature, and cooking time without analysis, because as mentioned before, many factors affect the HAA formation in cooked meat and meat products. However, it can be stated that cooking methods using low cooking temperatures such as boiling, sous-vide cooking, in general, may cause less HAA formation. On the other hand, when eating meat in a restaurant, it can be a boring question to think about what level of HAA compounds was formed in that meat. However, in general, it can be thought of as the more darkened meat surface, the higher internal temperature of the meat, and even the more flavor of the meat, the more HAAs can be formed.

It can be seen from the literature that there are very different results in various studies. For example, several studies that used heat treatment with the same method and almost the same temperature and time did not reveal HAA formation in meat samples, while a very high level of HAA formation was detected in some samples in other studies under similar conditions. In addition, cooking methods were not discussed in detail in some studies, while in others, meat samples were cooked to increase HAA formation generally. It is stated in the studies that the lowest individual HAA amounts were below the detection limits. On the other hand, the maximum amounts of some individual HAAs determined in the meat samples analyzed in some studies were as follows: IQ 303.06 ng/g in beef meatball [37], IQx 3.48 ng/g in beef [38], MeIQ 16.6 ng/g in fish [39], MeIQx 270 ng/g in beef [39], 4,8-DiMeIQx 15 ng/g [39], 7,8-DiMeIQx 5.3 ng/g in fish [39], PhIP 480 ng/g in chicken [39], AαC 106 ng/g in pork [39], MeAαC 3.2 ng/g in pork [39], harman 200 ng/g in pork [39], norharman as 186.1 ng/g in pizza [40].

In the studies carried out, it was determined that the highest individual HAA content (PhIP) in heat-treated meats (cooked chicken) was up to 480 ng/g. This is an important source of risk, because this amount belongs to only one compound in one gram of meat. On the other hand, considering that 100 g of meat is eaten as a portion and approximately 30 different HAA compounds have been isolated and identified from foods until today, and this number may increase, it will be possible to take more HAA compounds into the body than the maximum acceptable daily consumption amount (15 μg). For this reason, studies on reducing the formation levels of these compounds are gradually increasing. However, there is no legal regulation regarding the maximum availability limits of HAAs in foods in any country, which is considered an important disadvantage.

**Methods of reducing HAAs in cooked meat**

It is known that the world population is close to 8 billion as of now [41]. On the other hand, the United Nations has estimated that the world population will be 9.7 billion in 2050 and 11.2 billion in 2100 [42]. As it can be understood from these data, the world population is increasing day by day, despite the recent epidemics (for example, the COVID-19 pandemic). In addition, when the studies in the literature are examined, it is seen that the amount of meat consumption is increasing day by day in parallel with the increase in the world population. For example, per capita meat consumption was 23.1 kg per capita in 1961 [43], reached 42.4 kilograms in 2021 and is expected to increase to 43.7 kilograms by 2030 [44]. Therefore, studies on the complete inhibition or reduction of the formation levels of these kinds of compounds in heat-treated meat and meat products are very valuable, because meat consumption has been increasing day by day and the mutagenicity and carcinogenicity of HAAs have been proven as a result of epidemiological studies. In the studies on the reduction of HAA formation in heat-treated meat and meat products, the following recommendations have been made in general:

- Conventional cooking methods used in the cooking of meat and meat products should be modified and boiling or steaming methods including lower temperature should be preferred instead of frying or grilling;
- Meat and meat products should be pre-cooked for a short time using microwaves, to ensure, if possible, that HAA precursors are removed from meat. After this

short pre-cooking process, it will be healthier to subject meat to the other cooking process;
- Meats such as fish and chicken should be cooked with their skins. By this means, their skin will be a protective shield during the cooking of this kind of meats in terms of the HAA formation. After cooking, the skin should be removed from meat and the meats should be consumed;
- Nowadays, in many steakhouses, waiters ask their customers how they prefer their meat cooked. When faced with such a question, we should prefer medium and well done meats, because rare meats pose a risk in terms of microbiology, while very well done meats pose a risk in terms of heat treatment contaminants such as HAAs;
- If there are blackened/extremely burned parts on the surface of heat-treated meats, meat definitely should not be consumed before these parts are separated from meat;
- If meats are barbecued, meat should be as far away from the heat source as possible and should not come into direct contact with the source if possible;
- It is recommended to consume meat with salad or similar garniture due to the fact that the antioxidant compounds present in these products can interfere with the HAA formation and reduce the amount of meat in the portion. In addition, the use of spices with the antioxidant activity during the cooking of meat and meat products can reduce the HAA formation.

**Conclusion**

Heterocyclic aromatic amines are among the main food mutagens and/or carcinogens found in cooked meat and meat products. In this current study, the basic information about heterocyclic aromatic amines was reviewed. The most important issue about heterocyclic aromatic amines is that these compounds can also occur in normally cooked meat and meat products. Due to their mutagenicities and/or carcinogenicities, their formation in meat and meat products should be reduced. In this context, there is a need to increase the number of studies on reducing the formation levels of these compounds in different meat and meat products.

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## AUTHOR INFORMATION

**Emel Oz,** PhD, Associate Professor, Department of Food Engineering, Faculty of Agriculture, Atatürk University, 25240, Erzurum, Türkiye. Tel.: +90–402–231–26–44, E-mail: emel.oz@atauni.edu.tr
ORCID: https://orcid.org/0000–0003–3766–2713

**Fatih Oz,** PhD, Professor, Department of Food Engineering, Faculty of Agriculture, Atatürk University, 25240, Erzurum, Türkiye. Tel.: +90–402–231–26–44, E-mail: fatihoz@atauni.edu.tr
ORCID: https://orcid.org/0000–0002–5300–7519
* Corresponding author

All authors bear responsibility for the work and presented data.

All authors made an equal contribution to the work.

The authors were equally involved in writing the manuscript and bear the equal responsibility for plagiarism.

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

**Daniil V. Khvostov*, Natalya L. Vostrikova, Irina M. Chernukha**
V. M. Gorbatov Federal Research Center for Food Systems, Moscow, Russia

**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).

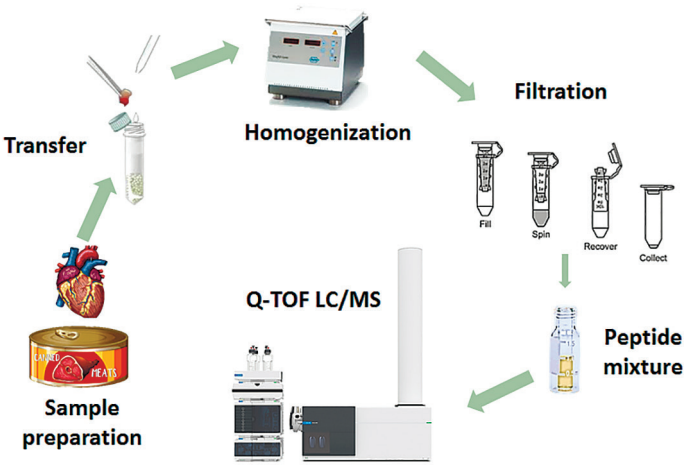



**Figure 1.** Protocol of samples preparation

*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.

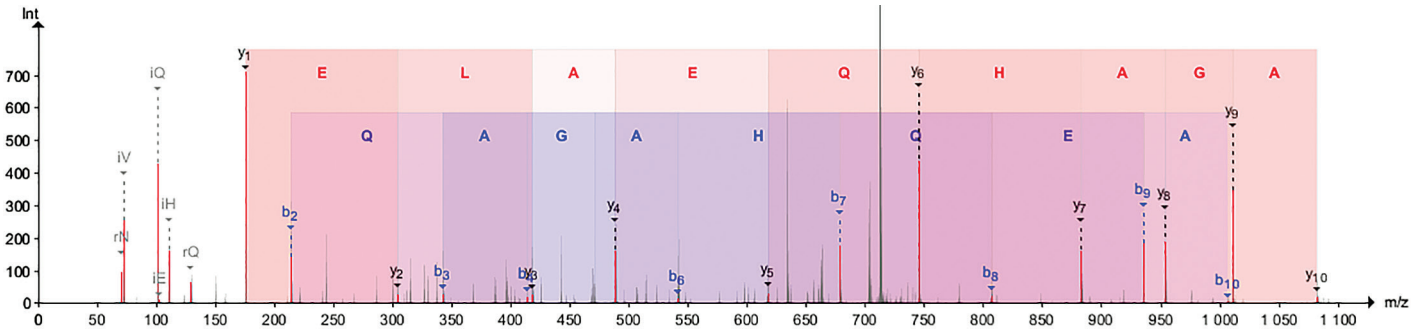



**Figure 2.** Mass spectrum identification of AGAHGEALE peptide by y-ions

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.

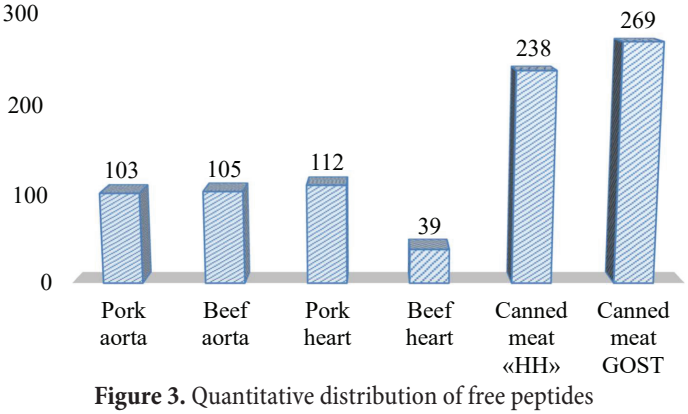
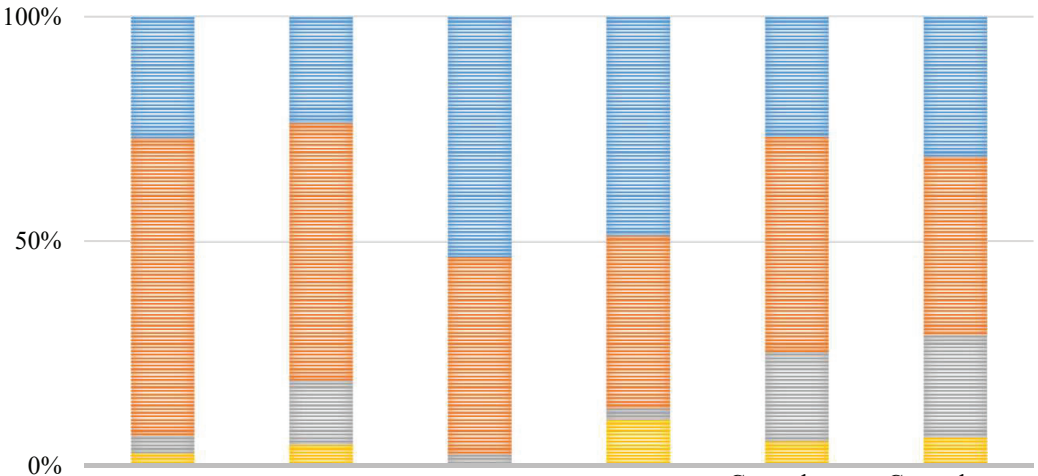



**Figure 3.** Quantitative distribution of free peptides in the analyzed samples




| | 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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## AUTHOR INFORMATION

**Daniil V. Khvostov**, Candidate of Technical Sciences, Researcher, Laboratory "Molecular Biology and Bioinformatics", V. M. Gorbatov Federal Research Center for Food Systems. 26, Talalikhina str., 109316, Moscow, Russia. Tel.: +7–495–676–95–11 (402). E-mail: d.hvostov@fncps.ru
ORCID: https://orcid.org/0000–0002–3445–4559
* corresponding author

**Natalia L. Vostrikova**, Doctor of Technical Sciences, Head of the Research Testing Center, V. M. Gorbatov Federal Research Center for Food Systems. 26, Talalikhina str., 109316, Moscow, Russia. Tel.: +7–495–676–95–11 (413). E-mail: n.vostrikova@fncps.ru
ORCID: https://orcid.org/0000–0002–9395–705X

**Irina M. Chernukha**, Doctor of Technical Sciences, Professor, Academician of RAS, Leading Research Scientist, Experimental Clinic — Laboratory of Biologically Active Substances of Animal Origin, V. M. Gorbatov Federal Research Center for Food Systems. 26, Talalikhina str., 109316, Moscow, Russia. Tel.: +7–495–676–63–21 (109). E-mail: imcher@inbox.ru
ORCID: https://orcid.org/0000–0003–4298–0927

All authors bear responsibility for the work and presented data.

All authors made an equal contribution to the work.

The authors were equally involved in writing the manuscript and bear the equal responsibility for plagiarism.

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# LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) METHOD FOR FAST DETECTION OF *CAMPYLOBACTER* SPP IN MEAT FOOD PRODUCTS AND ENVIRONMENTAL OBJECTS OF A PROCESSING PLANT'S

**Yuliya K. Yushina[1],* Elena V. Zajko[1], Maria A. Grudistova[1], Maksim D. Reshchikov[1], Nasarbay A. Nasyrov[1], Dmitriy V. Nikitchenko[2]**
[1] V. M. Gorbatov Federal Research Center for Food Systems, Moscow, Russia
[2] Peoples' Friendship University of Russia, Moscow, Russia

**Keywords:** *bacteria of the genus Campylobacter, PCR, loop isothermal amplification method (LAMP), poultry and meat processing enterprises*

***Abstract***

*There is constant necessity of developing the accurate and fast methods for detection of foodborne pathogens. Microorganisms of Campylobacter genus are one of the main causes of foodborne diseases worldwide. Fast identification of Campylobacter at all stages of the food life cycle is an efficient strategy to control foodborne campylobacteriosis. This article the authors evaluated a commercial loop-mediated isothermal amplification (LAMP) system with bioluminescence, called as the 3M™ Molecular Detection Analysis (MDA), which was used to find Campylobacter in food products with the help of a certain standard method, which is referred to as the reference method. The results of this study showed that the commercial LAMP-based method is as efficient as the reference method, and features high specificity and minimum determinability (sensitivity). The LAMP-based method has been shown to be a fast and reliable method for detection of Campylobacter spp. scarce presence (10 CFU/25 g) in meat, meat products, as well as carcass swabs and production facilities' environment. The LAMP analysis required about 24–27 hours to achieve a result. However the LAMP-based method will facilitate the detection of Campylobacter, as it provides much easier and faster detection of Campylobacter spp., including Campylobacter jejuni/Campylobacter coli, than standard microbiological methods. The LAMP-based method is an efficient tool to prevent the spreading of Campylobacter spp. contamination in food products.*

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## Introduction

Campylobacteriosis is one of the main causes of bacterial diarrhea all over the world [1,2,3]. Two species of bacteria *Campylobacter jejuni* and *Campylobacter coli* are considered the main causes of campylobacteriosis in human [4]. Poultry products remain the main sources of pathogens which cause this disease. Organisms of the genus *Campylobacter* spp. are not usually pathogenic in adult poultry and are considered to be commensal microorganisms of the poultry's gastrointestinal microbiota. Therefore, campylobacteriosis spreads quickly and asymptomatically among the poultry and is extremely difficult to detect and trace [5]. According to the Center for Active Surveillance for Foodborne Diseases (FoodNet), 14 cases of campylobacteriosis are diagnosed per 100,000 population in the United States (USA) (approximately 1.3 million people) and 71 cases per 100,000 population in the European Union (EU) (approximately 200,000 people) every year [1,6]. Campylobacteriosis is rarely a fatal disease and rare reports of mortality are usually recorded for the old age groups (elderly consumers) and/or immunocompromised patients (WHO).

The main target of activity to reduce the number of infections is the timely identification of *Campylobacter* spp. in food products.

In order to identify this pathogen the standard ISO 10272–1:2017 is used [7]. This standard provides for application of modified charcoal cefoperazone deoxycholate agar (m CCDA). However, it has recently been found that *Escherichia coli* and *Pseudomonas* spp, which are widely distributed in poultry, also actively grow together with *Campylobacter* spp. under the same conditions when found in the same sample [8,9]. This is explained by acquired resistance of *Escherichia coli* and *Pseudomonas* spp. to selective factors of nutrient media recommended in the study [7]. This microorganisms' competition can lead to a significant underestimation of the presence of *Campylobacter* spp. in the tested matrix. Thus, there is a need to improve the methodology for detecting *Campylobacter* spp.

in food products. Traditional methods of microbiological cultivation must constantly evolve by expanding the scope of application of selective media and by optimizing the growth conditions to suppress the growth of associated microflora, including new antibiotics [10].

Although methods for isolating *Campylobacter* spp. continue to improve, problems remain that reduce the efficiency of these methodologies. When exposed to stress factors such as refrigeration and freezing, *Campylobacter* spp. can become viable but not culturable (VBNC), thus making it impossible to detect it by many traditional microbiological methods [11,12,13].

Bacteria in the VBNC state can remain dormant for several years and recover in favorable conditions and subsequently cause infections to human [14]. As a result infections and diseases outbreaks caused by VBNC bacteria are underestimated. It has been proven that 13 out of 20 chicken carcasses are likely to contain VBNC *Campylobacter* [15].

Fast PCR-based methods are to eliminate the problems and issues, associated with the certain difficulties of microbiological approaches, and allow fast assessment of degree of *Campylobacter* spp. incidence in various food matrices. Molecular methods for identifying and detecting foodborne pathogens are more sensitive. One of the most valuable methods, used in the food safety industry and science in general, is the polymerase chain reaction (PCR) [10].

The advanced and potential integrating systems such as LAMP-based microdevices, microchips, and other CD-LAMP-based systems are being developed today. These systems provide high specificity, high speed, multiple detection of foodborne pathogens and maximal minimum determinability (sensitivity). In addition, these LAMP-based systems provide the detection of opportunistic and pathogenic microorganisms [16].

The aim of this study is to evaluate the performance of a loop-mediated isothermal amplification (LAMP) based method with bioluminescence, called the 3M™ Molecular Detection System (MDS), used for fast detection of *Campylobacter* spp. in meat, including poultry meat samples and samples of the environmental objects and facilities.

## Objects and methods

To determine the relative level of bacteria detection, the artificially contaminated samples of minced pork and minced chicken were used. Samples (minced pork and minced chicken) were purchased from a local supermarket in the central region of the Russian Federation in February-March, 2021.

To establish inclusion and exclusion of the pure cultures of 20 strains were used, including *Campylobacter jejuni* subsp. *jejuni* ATCC 8841 and *Campylobacter coli* ATCC 33559, and 18 *Campylobacter* spp. isolates, which were collected from poultry processing plants and previously found and confirmed by tests. On the other hand, exclusion tests were performed with non-target organisms using the 10 strains listed below in the Table 1.

**Table 1. Microorganism strains used to define the specificity of the LAMP method**

| No | Bacterial strains | Source |
|---|---|---|
| 1 | *Bacillus cereus* | ATCC 11778 |
| 2 | *Citrobacter freundii* | ATCC 43864 |
| 3 | *Enterococcus faecalis* | ATCC 29212 |
| 4 | *Escherichia coli* | ATCC 25922 |
| 5 | *Klebsiella pneumonia* | ATCC 13883, NCTC 9633 |
| 6 | *Proteus mirabilis* | ATCC 35659 |
| 7 | *Pseudomonas aeruginosa* | ATCC 27853 |
| 8 | *Salmonella enterica* subsp. *enterica* serovar *Typhimurium* | ATCC 14028 |
| 9 | *Listeria innocua* serotype 4a | ATCC 33090, NCTC 11288 |
| 10 | *Salmonella enterica* subsp. *enterica* serovar *Enteritidis* | 11272 |

Environmental swabs were analyzed to evaluate this method as a tool for monitoring the degree of *Campylobacter* spp. incidence. The swabs were taken at the poultry and meat processing plants in the Central region of Russia.

*Definition of inclusion and exclusion*

*Campylobacter* spp. strains were restored in Bolton broth (Merck, Germany), with further reinoculation on blood agar (OOO Sredoff, Russia) at temperature 41.5 °C for 48 hours under microaerophilic conditions (5% $O_2$, 10% $CO_2$, 85% $N_2$). Microaerophilic conditions were created in the device Anaxomat (Advanced Instruments Inc., USA). Non-target microorganisms were grown on tryptic soy broth (TSB, Merck, Germany) at temperature 37 °C overnight under aerobic conditions, followed by its reinoculation on trypticase soy agar (TSA, Merck, Germany). Each test was performed five repetitions once — i. e. five replications. Then, strains of microorganisms of *Campylobacter* genus were introduced into Bolton broth (Merck, Germany) at a concentration of 100 CFU/225 ml, and non-target strains were inoculated in TSB (Merck, Germany) at a concentration of 1,000 CFU/225 ml. Further studies were carried out according to the method [7] and by the LAMP method, starting from the stage of enrichment.

The relative level of detection of *Campylobacter* spp. the LAMP method used in samples of minced chicken and minced pork was determined using bacterial cells of *Campylobacter jejuni* subsp. *jejuni* ATCC 8841 (from the American Type Culture Collection, Manassas, Virginia, USA). To prove the absence of *Campylobacter* in the food matrix (minced chicken and minced pork) used for further studies, the samples were examined according to ISO 10272–1:2017 [7] and the protocol of the LAMP method. Campylobacter-negative food matrices were used as a negative control sample in the analysis to establish the relative level of bacteria detection.

To prepare the required number of cells, *Campylobacter* spp. genus microorganisms were restored in Bolton

broth (Merck, Germany). Then they were centrifuged in a MiniSpin centrifuge (Eppendorf, Germany) at 3,000 g for 10 min, flushed twice in 0.85% sodium chloride solution (JSC VEKTON, Russia). The obtained cells were diluted by decimal dilutions, and the concentrations of microorganisms of $10^1$ and $10^2$ CFU/ml were obtained. Before inoculation, the amount of the prepared *Campylobacter coli* suspension was counted by spreading a 100 µl aliquot on mCCD agar (Merck, Germany) in duplicate; and the suspension was incubated at 42 °C for 48 hours under microaerophilic conditions to assess the level of inoculum. After confirming the required number of cells, the minced meat samples were inoculated. There were two inoculation levels for the matrices: the high inoculation level of approximately 100 CFU/25 g and the low inoculation level of approximately 10 CFU/25 g. After inoculation the samples were tested by two methods to reveal the presence of *Campylobacter* spp.

*Campylobacter* spp. was detected using a commercial LAMP-based kit (3M Molecular Detection Analysis 2 — Campylobacter; 3M). The strain was detected according to the manufacturer's guidelines. 25 g sample was mixed with 225 ml 3M Enrichment broth (3M, USA). Then 20 µl of 3M Enrichment broth was added to the vial with lysis solution. The mixture was heated in a thermoblock (Germany, IKA) at 100 °C for 15 min followed by its immediate cooling at room temperature in a cooling block (3M, USA) for 10 min. After cooling, 20 µl of the lysate was mixed with the lyophilized mixture in the test vial with the reagent included in the kit. The prepared vial with the reagent was put into a molecular detection system (3M, USA) for the detection of *Campylobacter* spp. cells using isothermal amplification and bioluminescence for 75 min. All analyses included negative controls and reagent controls to test the performance of the molecular detection system.

To test for the presence of *Campylobacter* spp. in accordance with ISO 10272–1:2017 [7], 25 g of the sample was homogenized in 225 ml of Bolton broth (Merck, Germany) and incubated under microaerophilic conditions for 4–6 hours at temperature 37 °C, and then 40–48 hours at temperature 41.5 °C. After the end of incubation the enriched material from the broth was streaked onto mCCD agar (Merck, Germany) and Preston selective agar (Oxoid, United Kingdom) with a 3 mm loop, and incubated under microaerophilic conditions at 41.5 °C for 44±4 hours. The colonies typical of *Campylobacter* spp. were inoculated on blood agar and then confirmed with biochemical tests api® Campy (bioMerieux, France).

## Results and discussion

Microorganisms of the genus *Campylobacter* spp. are responsible for approximately 17% of human diarrhea worldwide, what makes them one of the leading causes of foodborne gastrointestinal infections. Governments and industry in many countries develop strategies to reduce the level of food contamination with *Campylobacter* spp. [17]. There is an urgent need for fast and simple methods capable to detect *Campylobacter* spp. throughout the entire chain of food production. At this stage of the study, the efficiency of the LAMP method (using a commercial kit) was evaluated in comparison with the method according to ISO 10272–1:2017 [7].

The specificity of the method was assessed in terms of inclusion and exclusion. Twenty bacterial strains (*Campylobacter jejuni* subsp. *jejuni* ATCC 8841 and *Campylobacter coli* ATCC 33559, 18 *Campylobacter* spp. isolates) were tested for inclusion. During the LAMP method testing, the positive results were obtained only with samples containing *Campylobacter* spp., including *Campylobacter jejuni* and *Campylobacter coli*, while no amplification was found in experiments with ten non-target strains of microorganisms (Table 2). In a parallel study according to ISO 10272–1:2017 [7] only target strains of microorganisms were identified and validated.

**Table 2. Results of inclusion and exclusion of the LAMP analysis for *Campylobacter* spp.**

| Strains | NO. of tests | | | |
|---|---|---|---|---|
| | LAMP | | ISO 10272–1:2017 | |
| | Posit. | Negat. | Posit. | Negat. |
| **Results of inclusivity** | | | | |
| *Campylobacter jejuni* subsp. *jejuni* ATCC 8841 | 5/5* | 0/5 | 5/5 | 0/5 |
| *Campylobacter coli* ATCC 33559 | 5/5* | 0/5 | 5/5 | 0/5 |
| *Campylobacter* spp. | 18/18 | 0/18 | 18/18 | 0/18 |
| **Results of exclusivity** | | | | |
| Untargeted strains** | 0/10 | 10/10 | 0/10 | 10/10 |

**Notes:**
*** number of the test replications;**
**** referred to in the chapter "Objects".**

The data in the Table 2 above show that the LAMP method was highly specific for *Campylobacter* spp., including *Campylobacter coli* and *Campylobacter jejuni* subsp. *jejuni*. The specificity of the method was 100%. The results obtained are also confirmed by a number of specialists' studies, which proved the high specificity of LAMP amplification, since 4 primers should specifically bind to 6 separate sections of the target DNA [18].

To determine the minimum determinability (sensitivity) of the LAMP method, various concentrations of the target *Campylobacter jejuni* ATCC 8841 were tested in the artificially contaminated samples of minced meat (Table 3).

At the levels of inoculation of 10 CFU/25 g and 100 CFU/25 g, the method could detect *Campylobacter* in all samples (sensitivity amounted to 100%), and the result in all samples was confirmed by the reference method. In a similar study, the detection limit for *Campylobacter* spp. by the LAMP method accounted for 1,000 CFU/g after incubation and 1–5 CFU/sample before enrichment. At the same time, the minimum determinability (sensitivity) of the reference method ISO 10272–1:2017 [7] was lower than

that of the LAMP method, which was consistent with the study by Rajagopal et al. [19].

**Table 3. The results of the test for the minimum determinability (sensitivity) of the LAMP method for *Campylobacter* spp. in the artificially contaminated samples**

| Level of inoculation (CFU/g) | Minced meat samples | ISO 10272–1:2017 / LAMP | | | | Minimum determinability (sensitivity) (%) | Specificity (%) |
|---|---|---|---|---|---|---|---|
| | | $T_{pos}$ | $T_{neg}$ | $F_{pos}$ | $F_{neg}$ | | |
| $10^0$ (not inoculated) | Poultry | 0/5 | 5/5 | 0/5 | 0/5 | 100 | 100 |
| | Pork | 0/5 | 5/5 | 0/5 | 0/5 | 100 | 100 |
| $10^1$ | Poultry | 5/5 | 0/5 | 0/5 | 0/5 | 100 | 100 |
| | Pork | 5/5 | 0/5 | 0/5 | 0/5 | 100 | 100 |
| $10^2$ | Poultry | 5/5 | 0/5 | 0/5 | 0/5 | 100 | 100 |
| | Pork | 5/5 | 0/5 | 0/5 | 0/5 | 100 | 100 |

A significant number of studies have reported that the minimum determinability (sensitivity) of PCR methods is often higher than the one of classical microbiological methods [20, 21]. When examining a non-inoculated sample (the negative control sample), both methods did not detect *Campylobacter* spp. No false negative results were obtained.

To confirm the sensitivity and performance of the LAMP method, the researchers monitored the rate of *Campylobacter* spp. detection in the objects of the production plant's environment (poultry processing plant and meat processing plant). In total 308 samples were collected and tested (Table 4).

**Table 4. Minimum determinability (sensitivity) results of the LAMP method compared to the method ISO 10272–1:2017, used for detection of *Campylobacter* spp. in the samples of native contamination**

| Samples (swabs) | LAMP / ISO 10272–1:2017 | | | | Minimum determinability (sensitivity) (%) | Specificity (%) |
|---|---|---|---|---|---|---|
| | $T_{pos}$ | $T_{neg}$ | $F_{pos}$ | $F_{neg}$ | | |
| Meat processing plant | 14/152 | 138/152 | 14/152 | 138/152 | 100 | 100 |
| Poultry processing plant | 60/156 | 96/156 | 57/156 | 99/156 | 100 | 94 |

**Note:**
**$T_{pos}$, $T_{neg}$ are true-positive and true-negative samples confirmed by both ISO 10272–1:2017 [7] and LAMP methods; $F_{pos}$ and $F_{neg}$ are false-positive and false-negative samples confirmed only by the LAMP method or the ISO 10272–1:2017 method [7], respectively;**
*** after the slash, the number of samples tested at the corresponding level of contamination is shown.**

During the analysis by the LAMP method, 14 positive samples were found of 152 samples of the environment in the meat processing plant; and 60 positive samples were found of 156 samples of the environment in the poultry processing plant. All positive samples, obtained by LAMP method, were confirmed according to the method ISO 10272–1:2017 [7] starting from their reinoculation from enrichment media to solified media. 3 samples (swabs) were taken from a poultry processing plant, that proved to be positive for *Campylobacter* spp. when using the LAMP method, however they gave a negative result when analyzed by traditional methods. The discrepancy in results may have been caused by the low number of *Campylobacter* spp. in the enrichment media, which was insufficient for their detection by the conventional culturing methods. Another reason could be the increased antimicrobial resistance in representatives of *Enterobacteriaceae* in recent years. The problem of *Campylobacter* detection in poultry meat samples is confirmed by a wide number of publications [22, 23, 24, 25]. It was shown that β-lactamase-resistant *Escherichia coli,* that is widespread in poultry, significantly outgrow *Campylobacter* spp. when both organisms are present in the same sample [8]. In addition, not only *E. coli*, but also other bacteria such as *Pseudomonas* spp. were found in large numbers in poultry samples and poultry processing plant's environment, and it may impede the detection of *Campylobacter* spp. [9].

The group of researchers Sabike et al. [26] evaluated the Loop-mediated Isothermal Amplification (LAMP) method, applied for direct screening of naturally contaminated chickens' cloacal swabs for *Campylobacter jejuni/Campylobacter coli* to compare this analysis with conventional quantitative microbiological methods. In the comparative study LAMP analysis of 165 broilers showed a sensitivity of 82.8% (48/58 in conventional culturing), 100% (107/107) specificity, 100% (48/48) positive predictive value, and 91.5%. (107/117) negative predictive value. LAMP analysis took less than 90 minutes from the moment of faecal samples obtaining till getting the final results from the laboratory. This suggests that LAMP analysis would facilitate the identification of *Campylobacter jejuni/Campylobacter coli.*-positive broiler flocks at the farm or at slaughterhouses prior to slaughter, making it an efficient way to prevent the spread of *Campylobacter* infection [26].

In the course of this study, it was noted that the LAMP method showed high specificity with a significantly shorter time of analysis: 24–27 hours in comparison with 5–7 days of analysis by the conventional control method of culturing.

It is reported that the sensitivity of PCR methods is often higher than that of classical microbiological methods [20,21]. In another study, an optimized LAMP analysis was tested on the cells of *Campylobacter jejuni* and *Campylobacter coli* inoculated into the samples of chicken faeces. High sensitivity has also been demonstrated in the LAMP analysis, where LOD of 1 CFU/reaction (corresponding to 50 CFU/mL) was observed for both *Campylobacter jejuni* and *Campylobacter coli* within 30 minutes of amplification [4]. This suggests that LAMP analysis would facilitate the identification of *C* at the farm or at pre-slaughter stage in

the slaughterhouses, thus making it an efficient means of preventing the spread of *Campylobacter* spp.

Detection of foodborne pathogens using traditional culturing methods is a reliable approach, but to obtain the results it is necessary to use the specialized laboratories and wait for several days, so there is no real-time information on pathogens presence or absence [27]. Molecular analysis based on LAMP is used to detect a wide range of pathogens [28,29].

Fresh chicken meat suspected of being contaminated with *Campylobacter jejuni* / *Campylobacter coli* was subjected to analysis. For these samples, analysis takes approximately 24–48 hours from the start of the cumulative phase till the pathogen was finally detected. The sensitivity of the LAMP analysis has been found to be ten times higher than the sensitivity of the equivalent PCR analysis. The increase of LAMP sensitivity can be proven both by turbidimetric analysis and by visual assessment with the naked eye. The LAMP analysis is faster and easier to perform than conventional PCR analyses, and is also more specific and requires only a simple heating block or constant temperature water bath [30]. The development of molecular methods, including the LAMP-based methods, is currently receiving a lot of attention. In the research of Kreitlow et al., the developed *LAMP analysis system based on the rplD and cdtC-gyrA genes* provided a fast, sensitive, and highly specific method for detecting and differentiating *Campylobacter jejuni* and *Campylobacter coli* in meat products [31].

**Conclusion**

This study represents data of validation the performance of a new fast method for detection of *Campylobacter* in poultry carcasses compared to conventional methods of culturing based on the standard ISO 10272–1:2017.

Thus, the present study describes a simple and fast LAMP analysis used for the detection of *Campylobacter jejuni* and *Campylobacter coli* in the analyzed samples. Validation of the LAMP method in comparison with the standard method of mCCDA coating showed that LAMP has a certain limit of detection (LOD) of 10 CFU/25 g. This method features good specificity as all strains of *Campylobacter jejuni* and *Campylobacter coli* were detected without failure, and no false positive results were obtained in analyses of the other tested bacterial control strains. These parameters were determined by analyzing 152 environmental samples obtained from the pork and poultry processing plant, as well as naturally contaminated pork and poultry carcasses. Only 3 samples that were found *Campylobacter* positive by conventional LAMP methods, turned out to be culture negative, ie. false negative. To validate the alternative method, all positive results were validated by the standard method ISO 10272–1:2017.

LAMP method and the reference method showed very good compliance between them. It was evident that the LAMP method was very specific with a much shorter time of analysis: 24–27 hours compared to 5–7 days of the reference method of culturing. The performance of the LAMP method was equivalent to the reference method.

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## AUTHOR INFORMATION

**Yulia K. Yushina,** Candidate of Technical Sciences, Docent, Deputy Head of Laboratory «Center for food and feed testing», V. M. Gorbatov Federal Research Center for Food Systems. 26, Talalikhina str., 109316, Moscow, Russia. Tel.: +7–495–676–91–26, E-mail: yu.yushina@fncps.ru
ORCID: https://orcid.org/0000–0001–9265–5511
* corresponding author

**Elena V. Zayko,** Researcher, Department of Hygiene of Production and Microbiology, V. M. Gorbatov Federal Research Center for Food Systems. 26, Talalikhina str., 109316, Moscow, Russia, Tel.: +7–960–548–71–95, E-mail: el.zaiko@yandex.ru
ORCID: https://orcid.org/0000–0002–5048–9321

**Maria A. Grudistova,** Researcher, Department of Hygiene of Production and Microbiology, V. M. Gorbatov Federal Research Center for Food Systems. 26, Talalikhina str., 109316, Moscow, Russia, Tel.: +7–916–311–64–30, E-mail: m.grudistova@fncps.ru
ORCID: https://orcid.org/0000–0002–8581–2379

**Maksim D. Reshchikov,** Senior Laboratory Assistant, Department of Hygiene of Production and Microbiology, V. M. Gorbatov Federal Research Center for Food Systems. 26, Talalikhina str., 109316, Moscow, Russia, Tel.: +7–962–959–32–22, E-mail: m.reshchikov@fncps.ru
ORCID: https://orcid.org/0000–0002–1344–823X

**Nazarbay A. Nasyrov,** Researcher, Department of Hygiene of Production and Microbiology, V. M. Gorbatov Federal Research Center for Food Systems. 26, Talalikhina str., 109316, Moscow, Russia, Tel.: +7–906–830–17–77, E-mail: n.nasyrov@fncps.ru
ORCID: https://orcid.org/0000–0001–8545–6266

**Dmitriy V. Nikitchenko,** Doctor of Biological Sciences, Docent, Professor, Department of Veterinary Medicine, Peoples' Friendship University of Russia, 6, Miklukho-Maklaya str., 117198, Moscow, Russia, Tel.: +7–910–421–32–58, E-mail: dvnikitchenko@mail.ru
ORCID: https://orcid.org/ 0000–0003–2294–6908

All authors bear responsibility for the work and presented data.

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The authors were equally involved in writing the manuscript and bear the equal responsibility for plagiarism.

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# REVIEW OF NEW TECHNOLOGIES USED FOR MEAT IDENTIFICATION

**Vladimir Yu. Kornienko[1]*, Tatyana A. Fomina[1,2], Mihail Yu. Minaev[1]**
[1] V. M. Gorbatov Federal Research Center for Food Systems, Moscow, Russia
[2] National Centre for Safety of Aquatic Fisheries Products and Aquaculture, Moscow, Russia

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***Abstract***

*The present article represents an analysis of trends in development of test-systems for identification of meat. These test systems are commonly used in food production and research laboratories. The relevance of development of methods for identification of meat kinds is related not only to the food restrictions, which are practiced in some religions and related to consumption of certain types of meat, but also with the hygienic aspects of food production. Also, this research is inspired also by the acute issue of food products adulteration and the replacement of one type of meat with another one. The article considers the trends in the development of microanalysis method that use immunochromatic research, i. e. methods based on molecular biology. Also this article considers the devices that do not use chromatographic methods of analysis. Examples of the development of test systems based on various methods of analysis for the identification of meat are given below. Attention is focused on the prospects of combining these methods, including colorimetric methods for identification of meat. It is also specified that the emergence of new dyes and new enzyme systems, suitable for use in enzyme-immunoassay, can enhance the sensitivity of these test systems. It is also noted that the development of technologies associated with sorbents can contribute to a better separation of the test substrates and this way to increase the sensitivity of the test in case of small amounts of test substrate. It is also noted that the use of various types of isothermal amplification can reduce the analysis time necessary for meat identification. Various schemes of devices for microanalysis are given; their advantages and disadvantages are listed. An example of proteomes application for meat identification is given. It is shown that this method can also be applied in the heat treatment of meat. The prospects for the development of such devices are analyzed. It is concluded that the development of systems for microanalysis in the form of quick tests is quite relevant and promising. It is indicated that theoretically in the future such analytical systems, due to the use of microfluidic technologies, will be able to combine several methods. The authors proposed to use machine-aided cognition methods to analyze data obtained from similar test systems in order to increase their sensitivity.*

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## Introduction

Meat identification is necessary to determine the fact of substitution of one type of meat for another. In some cases, contamination with a foreign type of meat does not occur purposefully during the production process. At the same time, adulteration of semi-finished meat products is widespread both in developed and developing countries [1–7]. One of the important reasons for the relevance of meat identification is certain religious prohibition on consumption of certain types of meat. Currently, identification methods based on the analysis of proteins and genetic sequences are widely used [3,6,8,9,10,11]. These methods include analysis which uses gel electrophoresis, isoelectric focusing, chromatography, and enzyme immunoassay [12–15]. Other methods used for meat identification include PCR (polymerase chain reaction), sequencing, and various types of DNA hybridization [16]. These methods are based on determination of specific nucleic acid sequences, peculiar for the sought-for type of meat. As a rule, during the process of DNA analysis, amplification of certain DNA sectors occurs, which are further investigated [9]. There are also methods that allow amplifying DNA at a constant temperature, without any cyclic change in temperature, peculiar for PCR, for example, such methods include loop-mediated isothermal amplification (LAMP) [8,17]. Other similar methods are cross-priming amplification (CPA), recombinase polymerase amplification (RPA) and SEA amplification (Denaturation Bubble-mediated Strand Exchange Amplification) [18,19,20].

The devices used for quick identification of meat are of particular interest. These test systems and devices are in demand in food production [1].

## Systems of microanalysis

Meat analysis systems with compact dimensions are called microanalysis systems. According to the principle of their operation, they are divided into two types. The first type of devices is aimed to study of the DNA sequence [18]. The second type of devices runs one of the variations of enzyme immunoassay or immunofluorescent analysis [12,13].

Most often they are executed in the form of test strips, i. e. in such a system the sorbent is applied directly on the test-strip, or the strip itself consists of a material that has the sorbent properties used for chromatography. In some cases, before the analysis a certain preparation of the sample is required, for example, extraction of DNA [21]. Some test systems combine both methods based on the analysis of DNA sequences and immunofluorescence analysis.

**Immunochromatographic analysis**

Immunochromatographic analysis implements the mechanisms of thin layer chromatography. The principle of this system is as follows: when a liquid sample is applied on the test strip, the dissolved components migrate along the sorbent and separate due to chromatographic mechanisms. To visualize the substances separated on the chromatographic system, the antibodies are used, labeled with a dye. In this case antibodies can bind in a direct or competitive manner. The direct method implies that the test substrate migrates along the chromatographic strip and binds to antibodies thus forming a sandwich-like structure. A competitive method is implemented when it is necessary to detect low molecular weight compounds. In this case, a system is implemented not only when the analyzed compound is there, but also its analogue is there too. The test compound and its analogue compete for binding with a limited number of specific binding centers located on antibodies.

Usually a strip of thin layer chromatography visualizes two zones arranged as a line, called a test line (T-line) and a control line (C-line). As a sorbent in such test systems they use a nitrocellulose-based material, it is the most popular. However, there are studies demonstrating the possibility of using other materials for immunochromatographic analysis [22,23]. The main problems in creating such test systems are diffusion and loss of selectivity [1, 24]. It is also noted that when developing such a test system, the processes of obtaining, labeling, amplification, and modification are simpler with aptamers compared with antibodies [1].

One of the advantages of portable test systems based on immunochromatographic analysis is the ability to detect test results visually, without the use of special equipment. It is reported that in some cases, an increase in the level of the fluorescent signal from the test and control lines increases the sensitivity of the test system [1,25,26,27]. Nardo et al. developed a two-color (red and blue) detection test system, but with one test line (T-line) [28]. A combination of both immunochromatographic analysis and surface Raman spectrometry (*SERS, Surface enhanced Raman spectroscopy*) has been reported [29,30]. Fu et al. combined the SERS method with a competitive immunochromatographic assay and found that the system was about three orders of magnitude (the authors probably meant three times) more sensitive than a similar commercially available kit [31]. Wang et al. reported that the portable immunochromatographic system in combination with the SERS method is 10,000 times more sensitive in comparison with the aggregation-based colorimetric method [32]. The sensitivity of the test system can also be increased by optimizing of the mathematical methods in data processing. Thus, the application of approaches using artificial neural networks or other machine-aided cognition methods is relevant and promising.

**Methods of molecular biology used to identify meat**

One of the common methods of molecular biology is PCR. This method is known for a long time. It serves as the basis for the methods like sequencing and various types of isothermal reactions. The use of the latter methods in diagnostic systems is particularly promising. Most methods, based on PCR or some type of isothermal amplification, require sample preparation immediately before the analysis (homogenization, nucleic acid isolation). Some test systems use identification of mitochondrial DNA sequence [19,33,34]. So Zhao et al. developed a test system for turkey meat identification in food products [34]. This diagnostic system showed no cross-reactivity with any of 21 other animal meats and plant species (Figure 1).

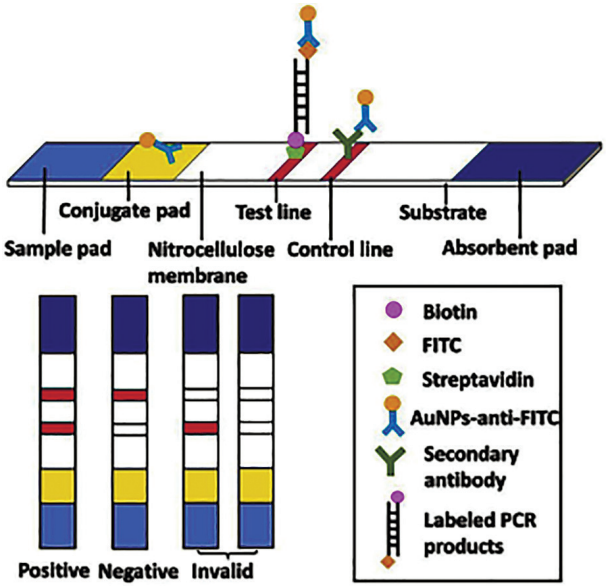


**Figure 1.** The example of a device for portable PCR test system [34]

Yin et al. developed a PCR test system in the form of a chromatographic strip for the quick identification of pork [35]. In the described test system, amplification and hybridization of amplicons with the probe were made outside the test strip. Visualization was achieved by test strips through hybridization of PCR resulted product. This test system detected an admixture of 0.01% pork in the tested product within 3 minutes (apparently, the authors mean the time without performing PCR).

To identify chicken meat in minced meat products, a method for the quick detection of counterfeit was developed [36]. The method was based on PCR combined with the use of a microfluidic chip. It featured a sensitivity of

0.1 pg for chicken DNA, and 0.1% for raw and autoclaved chicken meat in binary meat mix samples. The entire process of obtaining the result took 25 minutes — from sample preparation till getting the results [1,36]. Yin et al. developed a simple and quick test for identification of raw and cooked lamb using an immunochromatographic test strip [37]. After PCR the test mixture was applied to the test strip the test results were available in 5 minutes. This test system had a sensitivity of 0.01 pg for sheep DNA and 0.01% for the detection of adulterated meat. Qin et al. developed a quick and sensitive visual detection method for meat species identification [38]. Their method combined PCR and immunochromatographic analysis to detect duck meat admixtures in samples of beef. The method involved joint PCR amplification from the samples of beef and duck. This method made it possible to detect an admixture of foreign meat in amount of 0.05%. Magiati et al. developed a visual identification method based on immunochromatographic analysis for identification of horse meat and pork, as well as their binary mixtures with beef and lamb [39]. The authors used biotinylated primers for DNA amplification of different animal species' meat. Biotinylated amplicons were subjected to heat treatment to obtain single-stranded DNA. This single stranded DNA was hybridized with a complementary oligonucleotide probe that features a poly-A sequence at one end. The hybridized product was introduced into the conjugation zone of the immunochromatographic test strip. Poly-T sequences, immobilized on the T- and C-zones of DNA and bovine serum albumin, were used as reagents for biological determination of the presence of the sought-for hybridized structure. As a result of this procedure on the poly-A test system, the nucleic acid sections formed stable structures with poly-T sequences, thus making a visible color line. For this method, the analysis took from 25 to 30 minutes: the method had a sensitivity of 0.01% for horse DNA and 0.02% for pork DNA in binary meat mixtures.

LAMP (Loop-Mediated Isothermal Amplification) is a nucleic acid amplification method that does not require a thermal cycler. In this case DNA amplification runs at a constant temperature [1]. This method requires four or six primers to complete. Li et al. developed a test system that combined the LAMP method with immunochromatographic analysis [40]. At the first stage, isothermal amplification was run, and then the results were visualized on the test system in the form of a strip. The method made it possible to detect an admixture of 0.1% beef in a lump of minced meat, while the analysis took 50 minutes. Shi et al. developed a LAMP-based test system for the detection of duck DNA [41]. The method involved DNA amplification at a constant temperature of 65 °C for 30 min. The authors were able to achieve a sensitivity of 3 pg for duck DNA. The authors note that the sensitivity was higher than that of PCR. The authors also combined two varieties of the LAMP method (using two dyes as fluorescent marks).

**Non-genetic devices for meat identification**

Devices based on non-genetic methods of meat identification, as a rule, have simpler preparation of sample, and short time of analysis, but less specificity and lower sensitivity. As a rule, such test systems use the direct introduction of meat extract into a special zone on the test strip for immunochromatographic analysis. Most of these test systems use specific antibodies for meat proteins. Yayla et al. proposed a test system for pork detection in foods [42]. The authors report that their test system does not show cross-reactivity with beef, lamb, horse meat, mice and rabbit meat. When creating this test system, the authors conjugated antibodies with colloidal gold. Kuswandi et al. developed a quick test for detection of pork in ready-cooked meat food [43]. To do this, the developers obtained and conjugated gold nanoparticles with polyclonal immunoglobulin G. This test system detected pork admixture in amount of 0.1%. Masri et al. developed a test system for quick identification of horse meat [44]. In their test system, the authors used antibodies specific to horse serum albumin (HSA) and horse thermostable meat protein (H-TSMP). The method was able to detect 0.01% raw and 1.0% cooked horse meat in xenogeneic meat sources within 35 minutes. The method has demonstrated specificity in regards to serum albumin and meat derived from chicken, turkey, pig, cow, lamb and goat.

**Meat identification methods that do not use chromatographic methods of analysis**

Such test systems can demonstrate color detection of results in microtrays. Or these test systems can be, for example, run with the help of magnetic particles. So Seddaoui and Amine developed this test system [45]. It allowed detection of 0.01% pork admixture and was able to specifically detect pork among other types of meat (lamb, turkey, chicken and beef). Wu et al. developed a colorimetric system for detection of pork in binary meat mixtures [46]. A feature of this test system was that PCR was implemented in a glass capillary, and the results could be observed 20 minutes after the start of the test after hybridization. While the analysis SYTO 9 dye was used to visualize the PCR results. To detect pork in mixtures of beef and chicken meat, Skouridou et al. developed a test system using combined PCR and enzyme-immunoassay (PCR-ELONA test) [47]. As a result of the test, after hybridization of the PCR outcomes, the researchers observed color changes in the cells of the immunological tray, which made it possible to judge on the presence of pork admixtures in the analyzed samples. The sensitivity of the method ranged from 71 to 188 pg of genomic DNA. Lee et al. described a quick identification method using a portable colorimeter [48]. This method was based on the LAMP method and was able to detect 1 pg of pork DNA or 0.1% pork admixture in ground beef within 30 minutes. Wang et al. developed a LAMP-based method to detect trace amounts of horsemeat in foods [49]. This method could detect the presence

of 0.1% foreign meat and showed no cross-reactivity with 14 other animal species. Yan et al. developed a test to identify duck meat admixture [50]. The authors used the SEA method as the base of the test system. This quick method could find 10 pg/μl duck DNA or 0.1% duck meat in binary mixtures. The results of the test were available after 1 hour, and in this test no DNA extraction step was required (but still there was a cell lysis step during the analysis). The authors note that the reading of the results in their method can be implemented without special tools or instruments, and can be done visually (Figure 2).

Wang et al. also developed a test system based on SEA [51]. Their test system completed the analysis in 50 minutes and allowed to detect 1% beef admixture in the beef-duck mixture. A similar system was developed by Liu et al. for pork identification [20]. The idea of the method was to determine the presence of a mitochondrial DNA sequence specific for certain species. The authors note that their method was quick and could detect as little as 30 pg/μl of pork DNA. This method together with a colorimetric (fluorescence) detection method was able to find an admixture of 1% pork in a binary mixture. The proposed method included a protocol of quick DNA isolation and took 1 hour [20]. Montowska et al. analyzed the possibility of using a proteome to identify different types of meat [14]. As a result suitable protein markers were found to identify a particular type of meat before and after heat treatment (Figure 3).

In Figure 3 the identified proteins are marked with colored labels. The proteome gives a specific pattern made of separated proteins. That allows distinguishing one type of meat from another. Figure 3 shows that marker proteins are present on the proteome after heat treatment

**Prospects for further development**

The designing of new antibodies by genetic engineering methods can lead to higher specificity of portable test systems assigned for meat identification. On the other

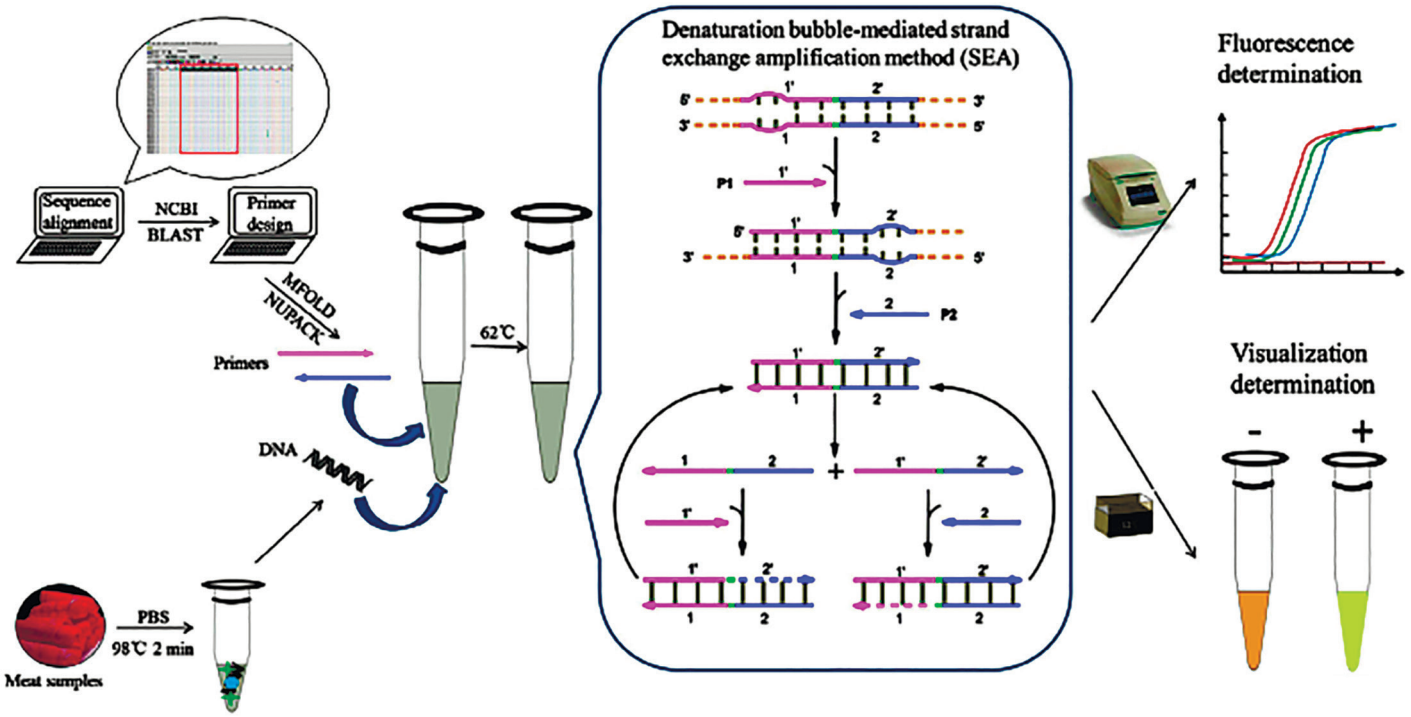


**Figure 2.** The example of a scheme for identifying the presence of foreign admixtures in a semi-finished meat product using SEA [50]

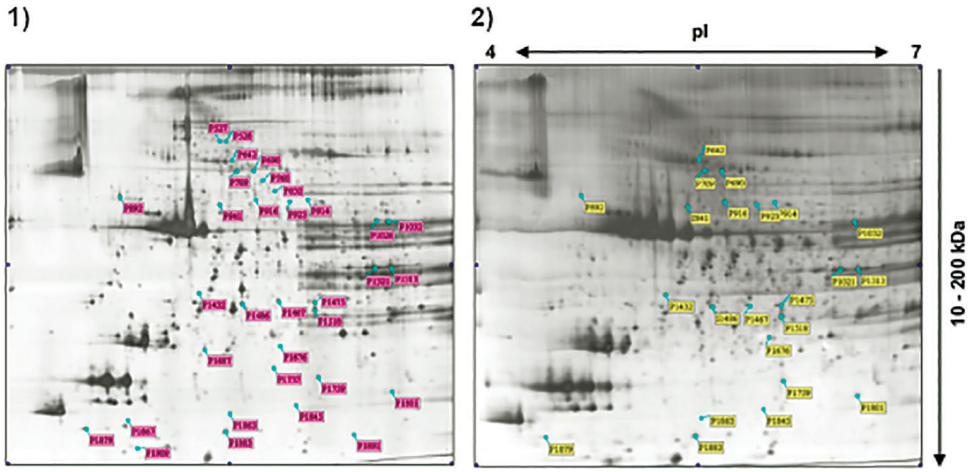


**Figure 3.** The example of a proteome obtained with the help of gel electrophoresis: 1 — raw pork, 2 — heat-treated pork [14]

hand, the emergence of new types of sorbents for thin layer chromatography will also contribute to a better separation of the components being under study, which sorbents will improve the performance of the developed test systems. One of the promising ways to develop test systems based on the study of nucleotide sequences is the use of isothermal amplification. The development of microfluidic technologies can lead to emergence of portable test systems for meat identification that combine several methods, for example: purification of meat extract and subsequent separation of the meat extract components on sorbent bedding.

**Conclusion**

Meat identification often requires bulky laboratory equipment, highly qualified lab-personnel, and a relatively time-consuming process of analysis. Meanwhile the possibility of quick identification without sophisticated molecular biological studies is often important. Therefore, the development of quick testing systems is naturally determined. Typically, quick test systems based on immunochromatographic methods have a shorter analysis time compared to test systems based on DNA sequences, and are more suitable for creating portable quick systems. However, the combination of methods for studying DNA, RNA and methods of enzyme-immunoassay and immunofluorescence analysis with thin layer chromatography makes it possible to create new species identification methods that feature both high speed of analysis and high sensitivity, moreover these new methods are quite compact to do. The development of molecular biology methods contributed to emergence of new methods for identification of meat species, for example, various types of isothermal amplification appeared. It is known that isothermal amplification, for example, LAMP, has a shorter analysis time in comparison with PCR and requires simpler equipment for its implementation; however at the same time it possesses a sensitivity and specificity comparable to PCR.

Thus, we observe a trend towards dimensions reduction of the test systems used for identification of meat species, meanwhile maintaining high sensitivity and high specificity. Probably in the future, isothermal amplification will be introduced even more widely, because it is a sensitive and specific method with a high speed of analysis. At the same time, it is likely that if successful detection systems are created for isothermal amplification, for example, colorimetric ones that do not require additional equipment, then they will probably push out the methods that use thin layer chromatography. In this case the parameters of such test systems are likely to be comparable with PCR-based identification methods. Meanwhile methods based on sequencing of genomic sequences are likely to be used in laboratories, but as the most accurate and non-portable test method that requires a lot of time.

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## AUTHOR INFORMATION

**Vladimir Yu. Kornienko,** Candidate of Biological Sciences, Senior Researcher, Laboratory of Molecular Biology and Bioinformatics, V. M. Gorbatov Federal Research Center for Food Systems. 26, Talalikhina Str., Moscow, 109316, Russia. Tel.: +7–495–676–95–11 (109), E-mail: unipraim@yandex.ru
ORCID: https://orcid.org/0000–0001–9068–9814

**Tatyana A. Fomina,** Candidate of Technical Sciences, Senior Research Scientist, Laboratory of Molecular Biology and Bioinformatics, V. M. Gorbatov Federal Research Center for Food Systems. 26, Talalikhina str., 109316, Moscow, Russia
Head of the Department of Molecular Diagnostic Research at the Testing Reference Laboratory, National Centre for Safety of Aquatic Fisheries Products and Aquaculture
14–1, Grafskiy per., 129626, Moscow, Russia. Tel.: +7–903–732–74–19, E-mail: fomina1032@yandex.ru
ORCID: https://orcid.org/0000–0001–8848–0632

**Mihail Yu. Minaev,** Candidate of Technical Sciences, Head of Laboratory of Molecular Biology and Bioinformatics, V. M. Gorbatov Federal Research Center for Food Systems. 26, Talalikhina Str., Moscow, 109316, Russia. Tel.: +7–495–676–95–11 (109), E-mail: m.minaev@fncps.ru
ORCID: https://orcid.org/0000–0002–0038–9744

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All authors made an equal contribution to the work.

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