INTRODUCTION

The Russian Federation strategy to improve the quality of food products until 2030 prioritizes research in the field of quality management.

Today, the problem of food adulteration is of particular concern. Food manufacturers are increasingly replacing expensive raw materials, such as good quality beef, with cheaper poultry. According to the public report “Consumer Protection in the Russian Federation in 2017”, Rosvoorubezhdor (Russian Federal Service for Surveillance on Consumer Rights Protection and Human Wellbeing) detected 3410 adulterated products out of 310 000 inspected food samples [1]. In 2018, the volumes of rejected meat, poultry, and their products doubled compared to 2017. In particular, Rospotrebnadzor rejected 519 batches of meat and meat products weighing 3509 kg (compared to 459 batches of 1685 kg in 2017) and 168 batches of poultry, eggs, and their products weighing 1951 kg (compared to 159 batches of 975 kg in 2017).
Species identification of meat and meat products is becoming more important due to increased international trade and labeling rules introduced in many countries. Morphological and anatomical characteristics are used to identify fresh and unprocessed meat. However, processed meat loses its characteristic morphological features, which creates favorable conditions for adulteration, namely for replacing one type of meat with another, less valuable type. Poultry—a cheaper raw material compared to pork, beef or other meats—is often used to adulterate products, both semi-finished and finished. Especially difficult is species identification of multicomponent products containing several types of meat, egg impurities, various food additives, enzyme preparations, as well as products subjected to rigorous mechanical or thermal processing, such as canned foods and pastes [2–7]. According to Rospotrebnadzor, most violations of the technical standards in 2018 were detected in canned meat and sausages [1].

At the moment, the Russian Federation has no method for quantifying the content of chicken and/or egg melange in food products and isolating possible contamination on the production line.

Scientific literature reports numerous methods for qualitative identification of meat species [8–11]. A group of scientists from Gorbatov’s Federal Scientific Center for Food Systems and the National Center for Fishing Products Safety attempted to identify egg melange at the 30th PCR cycle [12, 13]. However, there were no data on the quantitative identification of impurities [14, 15]. Therefore, we need to develop a quantitative method for identifying ingredients in the analyzed products to prevent producers from replacing a specified content of meat with cheaper raw materials and to distinguish between adulteration and inevitable contamination in production [16–20].

The highly sensitive PCR method can reveal even trace amounts of meat ingredients, which are essentially technical impurities. However, in order to distinguish a minor technical impurity from intentional adulteration, we need a methodology for a quantitative or semi-quantitative evaluation of meat, for example, chicken, in food products [20–38].

Therefore, we aimed to develop an effective method for identification and quantification of chicken meat and egg products in multicomponent meat systems using the real-time PCR.

**STUDY OBJECTS AND METHODS**

Our objects of study included native animal tissue purchased in retail chain stores (chicken, pork, beef, turkey, quail, duck, horse meat, rabbit, sheep, and goat) or obtained at the Russian State Center for Animal Feed and Drug Standardization and Quality, Moscow (mink, cat, and dog). Pure fresh chicken muscle tissue was used as standard samples. The species identity of all the materials was confirmed by the Sanger DNA sequencing method based on the standard CytB gene [3]. In addition, we used raw, boiled, and powdered chicken eggs.

We used only certified equipment, materials, reagents, and utensils.

The tests were conducted using the following methods:

- taking laboratory samples from different product groups (State Standard 31904-2012[III]);
- adsorption DNA extraction based on silicon dioxide (State Standard R 56140-2014[IV];
- guanidine-chloroform-based DNA extraction (State Standard R ISO 21571-2014[V]). This method can purify DNA from fatty and protein impurities, reduce the inhibition of the reaction, and eliminate the influence of food additives on the final result (it also works well with egg impurities);
- real-time polymerase chain reaction with hybridization-fluorescence detection (State Standard ISO 22119-2013[VI]);
- evaluation of metrological characteristics of measurement procedures (RIS 61-2010[VII]);
- certification of measurement procedures (State Standard R 8.563-2009[VIII]).

When sampling and preparing test samples, we took measures to prevent the seeding of environmental objects in line with State Standard 8756.0-70[VIII] and State Standard 31719-2012[IX]. The samples were homogenized and 0.05 g weighed, placed in a 1.5 cm Eppendorf type disposable microcentrifuge tube, labeled, and used to isolate DNA.

Three sets of samples were prepared in duplicate. The first set was not subjected to heat treatment. The samples of the second set were mixed with 100 mm³

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[1]: MU A 1/022 Sekvenirovanie fragmentov mitokhondrial'nogo genoma zhivotnykh i ryb dlya opredeleniya vidovoy prinadлежnosti myasa v odnokomponentnykh produktakh [MU A 1/022 Sequencing fragments of the mitochondrial genome of animals and fish to determine meat species in mono-component products].

[2]: State Standard 31904-2012. Food products. Methods of sampling for microbiological analyses. Moscow: Standartinform; 2014. 8 p.

[3]: State Standard R 56140-2014. Medicine biological remedies for veterinary use. Polymerase chain reaction for the *Mycoplasma* DNA detection. Moscow: Standartinform; 2015. 12 p.

[4]: State Standard R ISO 21571-2014. Foodstuffs. Methods of analysis for the detection of genetically modified organisms and derived products. Nucleic acid extraction. Moscow: Standartinform; 2016. 46 p.

[5]: State Standard ISO 22119-2013. Microbiology of food and animal feeding stuffs. Real-time polymerase chain reaction (PCR) for the detection of food-borne pathogens. General requirements and definitions. Moscow: Standartinform; 2014. 15 p.

[6]: RIS 61-2010. State system for ensuring the uniformity of measurements. Accuracy, trueness and precision measures of the procedures for quantitative chemical analysis. Methods of evaluation. Moscow: Standartinform; 2013. 62 p.

[7]: State Standard R 8.563-2009. State system for ensuring the uniformity of measurements. Procedures of measurements. Moscow: Standartinform; 2011. 20 p.

[8]: State Standard 8756.0-70. Canned food products. Sampling and preparation of samples for test. Moscow: Standartinform; 2010. 8 p.

[9]: State Standard 31719-2012. Foodstuffs and feed. Rapid method of identification of raw composition (molecular). Moscow: Standartinform; 2014. 24 p.
of water and heated at 99°C on a Termite solid-state thermostat (DNA-Technology, Russia) for 30 min. The third set was sampled in quadruplicate and autoclaved at 110°C and 0.5 atm. for 30 min and an hour, respectively. For the purity of the experiment, we used chicken muscle tissue (breast fillet and drumstick), parenchymal and hollow internal organs (kidney, heart, liver), skin and cartilage, as well as minced pork meat containing 1% and 10% chicken.

Since chicken eggs are widely used in the food industry, we had to determine their effect on the PCR results. For this, we analyzed raw, boiled and powdered eggs, as well as pancake flour. In addition, we investigated 20% egg in minced pork, 10% raw egg in water, and 10% egg in minced chicken. A model panel was made from the above samples.

To eliminate the likelihood of PCR inhibition, we used an internal control sample (ICS) which was added to each test sample starting from the DNA extraction stage.

DNA was extracted by the sorbent method recommended by State Standard R 52723-2007, using a standard set of DNA-Sorb-S reagents (Central Research Institute of Epidemiology, Russia). A number of experiments performed with the extracted DNA showed that a 100% chicken content (whether fillet, hollow and parenchymal internal organs or connective tissue) produced a threshold cycle ($C_t$) ≤ 15, whereas 10% and 1% chicken contents in minced meat produced $C_t$ ≥ 18 and $C_t$ ≤ 21, respectively. There is a correlation with the ICS detection. When egg is present, the values decrease to $C_t$ ≥ 23 and the ICS also drops to $C_t$ ≥ 28 due to inhibition ($C_t$ ≥ 24 with no inhibitors). DNA is obviously less degraded in a pure product (raw and boiled egg) than in egg powder, but $C_t$ is inversely related: $C_t$ ≥ 27 and $C_t$ ≥ 20 for the egg powder sample and the ICS, respectively; $C_t$ ≥ 30 and $C_t$ ≥ 28 for the raw and boiled egg sample and the ICS, respectively.

Thus, we can conclude that raw and boiled eggs contain PCR-inhibiting substances. The presence of 10% raw eggs in minced chicken leads to ICS $C_t$ ≥ 27 versus ICS $C_t$ ≤ 21 for 100% minced chicken. It is impossible to evaluate the results when the reaction is so strongly inhibited. Therefore, we chose a different DNA extraction method described by Minaev et al. [2]. For this, we used a SORB-GMO-B kit (Syntol, Russia) in accordance with the manufacturer's recommendations. The PCR results are shown in Table 1. As we can see, the ICS threshold cycle values indicate insignificant inhibition of the reaction, confirming the right choice of the DNA isolation method.

We selected those primers and probes that fluoresce to the target DNA of chicken and the ICS in the Green and Yellow channels. The solutions of direct and reverse PCR primers and a probe at a known molar concentration were diluted to a working molar concentration of 6 μmol/dm$^3$ and 3 μmol/dm$^3$, respectively. For PCR, we used a dNTF solution (Syntol, Russia), a PCR buffer-Flu and TaqF DNA polymerase (Central Research Institute of Epidemiology, Russia).

The DNA extracted from each test sample was analyzed in at least two replicates. For amplification control reactions, we used recombinant plasmids based on the pAL-2 vector (solutions of plasmid DNA at a concentration of 0.01 mg/dm$^3$) as positive reaction controls. They were a plasmid containing a chicken DNA fragment (pCh) and a plasmid of the internal control sample (pICS).

For real-time PCR, we used Rotor-Gene Q amplifiers (QIAGEN, Germany) and Rotor-Gene 6000 amplifiers (Corbett Research Pty Ltd., Australia). We programmed the device according to the operating instructions and optimized the PCR-RT conditions for the duplex format. The primer annealing temperature was 60°C, with a PCR total temperature profile of 40 cycles.

RESULTS AND DISCUSSION

The PCR results for the model meat systems before and after heat treatment (at various temperatures) are presented in Table 1. The Background Threshold was set at 15% and the Threshold was 0.05. We interpreted the results based on the presence (or absence) of the intersection between the fluorescence curve and a threshold line set at an appropriate level. The conditions for analysis were as follows: for a positive PCR control, the threshold cycle values of $C_t < 26$ were present in the Green and Yellow channels; for a negative extraction control and a negative PCR control, the threshold cycle values were absent in all the channels; the threshold cycle value for the ICS was not lower than $C_t ≤ 24$ for qualitative determination, since higher values indicate PCR inhibition.

As we can see in Table 1, all the raw samples containing meat or offal (including extremely low concentrations) were identified at no later than the 19th cycle; egg impurities, no earlier than the 25th cycle; and egg powder and pancake flour, at the 29–30th cycle. Interestingly, pure chicken meat, whether fillet or offal, was identified at no later than the 14th cycle, while connective tissue, no later than the 17th cycle. The chicken contents of 10% and 1% produced $C_t$ ≤ 15 and $C_t$ ≤ 19, respectively. These results allowed us to conclude that:

- $C_t < 15$ indicated over 10% chicken in the test sample;
- $C_t < 19$ indicated over 1% chicken or high concentrations of connective tissue in the test sample. This conclusion makes it impossible to quantify the chicken content at this stage of the study. However, it leaves a possibility of a semi-quantitative analysis, whose result can be expressed as “chicken content at least N%”.

5 State Standard R 52723-2007. Foodstuffs and feeds. Rapid method of identification of raw composition (molecular). Moscow: Standartinform; 2007. 22 p.
The heat-treated samples containing meat or offal (including extremely low concentrations, up to 1%) were identified at no later than the 21st cycle and egg impurities, no earlier than the 21st cycle. A 10% chicken content in minced meat produced \( C_t \leq 17 \), whereas 1% chicken showed \( C_t \leq 21 \). From these results, we concluded that \( C_t < 21 \) indicated more than 1% chicken in the test sample.

The autoclaved samples containing chicken meat or offal were identified at no later than the 17th cycle, whereas the samples with extremely low concentrations of chicken meat (up to 1%) and egg impurities, no later than the 26th cycle. The chicken contents of 10% and 1% resulted in \( C_t \leq 21 \) and \( C_t \leq 25 \), respectively. Thus, the detection of \( C_t < 25 \) indicated over 1% chicken in the test sample.

Next, we proceeded to the development of a semi-quantitative method for determining chicken meat in food products, since a quantitative method was not possible due to the equality of cycles for the 10% minced chicken samples and the connective tissue samples.

As adulterating a product with less than 1% meat (1 g chicken meat per 1 kg of product) seems impractical, we decided that the methodology should allow us to determine the content of chicken in the product in relation to several threshold values of calibration samples, namely:

- “at least 1%” if \( C_t \leq C_{10\%} \leq C_t \leq C_{1\%} \);
- “at least 10%” if \( C_t \leq C_{10\%} \leq C_t \leq C_{10\%} \);
- “high content” if the sample’s \( C_t > C_{1\%} \).

Further, we evaluated the following criteria: sensitivity and specificity of the primers, detection limits, and a range of values for calibration samples, and internal control samples. Each experiment was performed by two different researchers, at different times, with reagents of different series, on different amplifiers of the same type. Each sample was tested in duplicate.

To assess the specificity of PCR, we created a panel of DNA samples isolated from chicken, pork, beef,
Table 2 Specificity assessment of the duplex PCR system for chicken identification

| Expected amplification result | Actual amplification result, threshold cycle values, $C_t \pm SD$ | Name of sample |
|-------------------------------|---------------------------------------------------------------|----------------|
|                               | Replicate № 1                                      | Replicate № 2 |
| FAM, ICS Yellow, chicken      | $21.66 \pm 0.05$                                     | $21.61 \pm 0.10$ |
|                               | $12.76 \pm 0.18$                                     | $13.46 \pm 0.01$ |
| +                             | +                                             | +               |
| +                             | –                                             | –               |
| +                             | +                                             | +               |
| +                             | –                                             | –               |
| +                             | +                                             | +               |
| +                             | –                                             | –               |
| +                             | +                                             | +               |
| +                             | –                                             | –               |
| +                             | +                                             | +               |
| +                             | –                                             | –               |
| +                             | +                                             | +               |
| +                             | –                                             | –               |
| –                             | –                                             | –               |

* $Ci$ – isolation control (shows the absence of inhibition at the stage of DNA isolation)
** – $C$ – negative PCR control (shows the purity of the reaction, mixes, and the laminar, as well as the absence of contamination)

Table 3 Sensitivity of the duplex PCR system (initial concentration of plasmid DNA – 4 ng/μL)

| Number of genomic copies in the reaction | $C_t \pm SD$, Yellow (chicken) | $C_t \pm SD$, Green (ICS) |
|------------------------------------------|---------------------------------|--------------------------|
| 20 000                                   | $23.16 \pm 0.10$               | $24.41 \pm 0.15$         |
| 2 000                                    | $26.87 \pm 0.10$               | $28.19 \pm 0.05$         |
| 200                                      | $30.58 \pm 0.56$               | $32.01 \pm 0.18$         |
| 20                                       | $34.00 \pm 0.79$               | $35.29 \pm 1.07$         |
| 2                                        | –                              | –                        |

turkey, quail, duck, horse, mink, rabbit, cat, dog, goat, and sheep. The results are shown in Fig. 1 and Table 2.

Within the proposed panel, the chicken DNA identification methodology showed 100% specificity: we observed the ICS amplification only on the Green channel and the target chicken DNA on the Yellow channel.

The assessment of the control panel for validation confirmed a 100% convergence of the results.

To determine the analytical sensitivity of the primers, we isolated DNA from a sample of 100% chicken meat and prepared a series of 10-fold dilutions. The maximum dilution was determined which allowed reproducible (in duplicate) detection of DNA.

In addition, we used plasmid DNA solutions at a specified concentration containing a cloned chicken gene fragment and a ICS fragment. Two series of ten-fold dilutions were prepared in a TE buffer with various concentrations: series № 1 – pICS plasmid DNA solution; series № 2 – pCh plasmid DNA solution. The initial concentration of plasmid DNA in each series was 4 ng/μL, which corresponds to ~ 20 000 genomic copies in PCR (5 μL of a DNA solution for a 25 μL reaction). The results are presented in Table 3.

To determine the absolute limit of detection (LOD) at which the PCR method is able to detect and quantify chicken genetic material, we performed 10 PCRs, with 5, 10, 20, and 40 genomic copies of chicken DNA in each. Our PCR methodology detected chicken even in the strongest dilution, with only five genomic copies in the PCR.

To determine the limit of detection of chicken and egg products in multicomponent raw and heat-treated products, we used a number of model samples prepared in two replicates and containing 10, 1.0, 0.1, 0.01, and 0.001% chicken in minced pork (isolated DNA). The samples were preliminarily cooked at 99°C for 30 min. To determine the LOD of chicken and egg products in canned foods, the model samples were autoclaved at 110°C and 0.5 atm. The minimum chicken content in minced pork was determined, at which chicken DNA was reproducibly (in duplicate) detected. The results are shown in Table 4.
The limit of detection for chicken DNA ranged from 0.1 to 0.001% of the chicken content in the sample.

The methodology should allow us to assess the content of chicken and egg products in food products relative to several selected threshold values of calibration samples. To prepare calibration samples of various compositions for the semi-quantitative analysis of raw and cooked products, we mixed 100% minced chicken meat with 100% minced pork (1%, 10%, and 50% chicken) and heated at 99°C for 30 min.

We decided to evaluate both cooked and raw products in relation to the values of heat-treated calibrators, since fresh chicken meat was used to prepare model samples of raw products, which cannot be guaranteed by product manufacturers. Moreover, samples for analysis do not always get delivered to the laboratory directly, bypassing the stages of storage or freezing, which increases the likelihood of DNA degradation. The calibration samples for canned products were autoclaved at 110°C and 0.5 atm. The uniformity coefficient of the calibrators was 0.99 (99%).

To confirm the constancy of the calibrators’ $C_T$ ranges, we performed a series of tests. In particular, we studied 15 series of calibration samples prepared on different days, by different people, each in two replicates. For each series, we determined the minimum and maximum values of the threshold cycle on the Yellow-chicken channel, a standard deviation, and a relative standard deviation. The results are presented in Table 5.

As a result, we selected the following threshold cycle values on the “Yellow-Chicken DNA” channel for the calibrators of:

- raw products and those subjected to light heat treatment: $18 \leq C_T < 21$; $14 \leq C_T < 18$; $C_T < 14$;
- autoclaved products (canned food): $25 \leq C_T < 28$; $22 \leq C_T < 25$; $C_T < 22$.

Also, a threshold cycle value of at least $C_T \leq 24$ was chosen as acceptable on the “Green-ICS” channel for the calibrators ($C_Tics 1\%, C_Tics 10\%, C_Tics 50\%) and the negative control sample.

**CONCLUSION**

We developed a method (certified methodology) for a semi-quantitative assessment of chicken content in multicomponent food systems of varying degrees of heat and mechanical treatment: raw, heat-treated, canned, finely ground, and homogenized. Having tested various DNA extraction methods, we concluded that the guanidine-chloroform method reduces the content of PCR-inhibiting substances compared to the sorption method.

Our methodology was tested on model samples, as well as product samples from retail stores, to exclude the possibility of PCR inhibition by food additives, stabilizers, emulsifiers, etc. With PCR, we can distinguish between chicken meat and egg products in raw and cooked products (over 21 cycles), as well as canned foods (over 28 cycles). Our results suggest that this methodology is suitable for analyzing...
multicomponent food products, raw materials, feeds, and feed additives. In addition, it can identify the content of chicken meat at a concentration of up to 1%, as well as detect egg impurities and contamination of various origins.

Taking into account the current need for distinguishing adulteration from the inevitable contamination on the production line, as well as preventing adulteration of expensive raw materials with chicken meat by introducing egg products, we believe that our methodology could make a significant contribution to the production of high-quality foods.

CONTRIBUTION
Each of the authors was directly involved in the development, testing, and validation of the above methodology, as well as in writing this article.

CONFLICT OF INTEREST
The authors state that there is no conflict of interest.

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ORCID IDs
Mariya A. Pleskacheva https://orcid.org/0000-0002-6721-2812
Marina P. Artamonova https://orcid.org/0000-0001-8372-3594
Elena V. Litvinova https://orcid.org/0000-0003-4670-8832
Mariia A. Gergel https://orcid.org/0000-0002-8033-1154