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Detection of the Species of Origin for Pork, Chicken and Beef in Meat Food Products by Real-Time PCR

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Abstract: Processed food products of animal origin raise questions related to industrial safety and human health protection. This paper aimed to optimize and validate a real-time, sensitive, and accurate PCR method for the detection and quantification of meat species in selected processed meat products: chicken sausages, beef bologna, and pork bologna. A common detection limit of 8 DNA copies was established for each sample, corresponding to 0.1% for beef and pork and 0.2% for chicken. For the limit of quantification, dilutions of 20 copies of DNA for the bovine and pig species and 50 copies of DNA for the chicken species were performed. Specificity and selectivity tests in six replicates each showed no extraneous meat species, in line with the label. Repeatability was assessed in six replicates, both quantitatively and qualitatively, by the same analyst, on the same day, and with the same equipment. The results showed that beef bologna contained 84.49% beef meat, pork bologna 92.8% pork meat, and chicken sausages 95.14% chicken meat. The reproducibility results obtained by two analysts, on different days, for each sample were very similar. The real-time PCR technique can be used as a tool in internal and public safety control to improve industrial safety and human health protection.

Keywords: public safety; human health; food quality; meat analysis; species identification; molecular biology; DNA extraction

1. Introduction

Meat fraud in food products of animal origin raises questions related to industrial and human health safety, besides economic concerns. The identification of meat species in fresh or processed products has been performed according to techniques based on the analysis of specific meat proteins or DNA in these samples [1]. The analysis of specific meat proteins is effective for fresh meat samples but has limits for processed meat due to heat denaturation of meat proteins during product preparation [2]. For products undergoing thermal processing [3], analysis of peptide biomarkers is performed by HPLC [4–6]. The most common methods used for the determination of meat species are based on DNA analysis, such as polymerase chain reaction (PCR) [7]. In order to identify chicken, turkey, duck, and goose species present in the animal feed, PCR methods based on variations of the nucleotide sequences of the mitochondrial genes coding for *cytB*, *D-loop*, and rRNA 12S have been used [8]. The PCR method is also used for the identification of species in cooked food [9–11].

The most used PCR technique is real-time PCR due to its high sensitivity and specificity, low contamination risk, and robustness for the identification and quantification of DNA. The real-time quantitative PCR technique (qPCR) allows the direct assessment of results based on a fluorescence signal associated with the amplified target gene. The related PCR correlation methods employ interleaved fluorescent dyes such as SYBR Green [12]. The substitution of meat species [13] is a critical issue because it impacts consumers' health and products' trade and imports, as it is easy to conceal [11].

The labeling of food products is intended to identify the real meat species contained in the products. However, the identification of meat species in a food product may be challenging for food inspectors and analysis. Consumers need the correct labeling of food products of animal origin. There are methods for the analysis of protein and DNA, such as electrophoretic [14], chromatography [15], and immunologic techniques.

The PCR technique in the analysis of food is largely used due to its simplicity, speed, and specificity [16,17]. In addition to PCR, the most used methods in the identification of meat species are random amplification of polymorphic DNA PCR (RAPD-PCR) [18], PCR restriction fragment length polymorphism (PCR–RFLP) [19–21], and real-time PCR [20]. The analytical methods used are as diverse as the authentication issues that may be encountered and employ various types of equipment and techniques [22]. PCR is used in many research fields—biological, clinical, forensic, and diagnostic—and has revolutionized research in life sciences [23–29].

PCR has become one of the most frequently used techniques in molecular biology and it is used in applications from basic research to high-performance screening. Besides being a powerful technique, it is relatively simple and low-cost, which has allowed its universal adoption and diversity of application. The technique is used to amplify specific target DNA fragments present in small quantities of DNA or RNA (the latter after a reverse-transcription step to generate complementary DNA (cDNA)). A major advantage of PCR is that sequence targets can be amplified from a single copy of raw material, even when the sample is degraded and contaminated with inhibitors [23].

This paper is aimed at the optimization and validation of a real-time, sensitive, and accurate PCR method for the detection and quantification of meat species in selected processed meat products, precisely, chicken sausages and beef and pork bologna, in order to authenticate them, confirming the absence or presence of extraneous meat species. Food product authentication is an important issue for public and industrial safety and human health protection. This research focuses on the importance of quality control and inspection programs in the meat industry and emphasiszes the need to check labeling statements.

2. Materials and Methods

Chicken sausages, beef bologna, and pork bologna purchased in supermarkets and prepared by various romanian local producers (Caroli Foods [Caroli], Bucharest, Romania; CRISTIM 2 PRODCOM [Cris-Tim, Matache Macelaru], Bucharest, Romania) were subject to analysis in order to check the authenticity of the products and labels. Samples were taken from single chicken, beef, and pork products and were used directly for the extraction of DNA, using a DNA extraction kit (SureFood®PREP Advanced, manufactured by Congen, Berlin, Germany) according to the manufacturer's instructions. The DNA concentration was measured using a spectrophotometer (Eppendorf, Hamburg, Germany).

In order to validate the method, the following performance parameters were validated in-house: detection limit, quantification limit, selectivity and specificity limit, reproducibility, and measurement uncertainty. Specific commercial kits were used for the three types of sample, depending on the species existing in the product: SureFood® ANIMAL QUANT Beef for beef bologna, SureFood® ANIMAL QUANT Pork for pork bologna, SureFood® ANIMAL QUANT Chicken for chicken sausages (manufactured by Congen, Berlin, Germany), according to the instructions and the work protocol provided by the manufacturer (Art. No. S1014, 2 × 50 rxn). Amplification and detection of the species were performed using real-time PCR (GeneAmp PCR System 9700, Applied Biosystems).

2.1. Sampling for the Performance of Molecular Biology Tests

The term "sample" indicates any product or material intended for laboratory examination. Regardless of the nature of the samples subjected to analysis by molecular biology techniques, sampling was performed according to the following rules: the sampled sticks or bars were sectioned longitudinally for the performance of the organoleptic test; the obtained two halves were the sample

and the counter-sample; from one of the halves (middle and ends), 300–600 g were taken and sent to the laboratory in ice at 0–4 °C where they were analyzed within 36 h.

2.2. Extraction of Specific DNA Species from the Meat Products

2.2.1. Preparation of Reagents

The pre-washing buffer, which was used to eliminate salts, proteins, lipid membranes, RNA, and other residues resulting from cellular lysis, was mixed with 30 mL ethanol 70% concentration, and the mixture was kept in a tightly closed tube; then, was 42 mL ethanol 100% concentration was added, and the mixture was stirred again; finally proteinase K that eliminates proteins from the DNA, was added with 1 mL of water for PCR, and the mixtire was stirred and kept at −20 °C if not used immediately.

2.2.2. Extraction Protocol

After preheating, an aliquot of the elution buffer was introduced in the extraction tube to detach the DNA from the column and elute it in the tube located below the column, and the thermoblock was preheated at 65 °C.

Calculation of the quantity of elution buffer for 10 samples:

$$10 \times 100\ \mu L \text{ elution buffer} + 100\ \mu L \text{ excess volume} = 1100\ \mu L \text{ elution buffer}$$

For the lysis process, ach sample was homogenized with a mixer and weighed 0.050 g ± 0.001 g in a storage tube (for samples with high moisture content, the sample quantity was 0.100 g ± 0.001 g). Then, 400 µL lysis buffer was added (for breaking the cell wall or cell membrane and release DNA from the cell nucleus) together with 20 µL Proteinase K, followed by vortexing and incubation at 65 °C for 30 min while stirring at 1000 rpm. For pre-filtering and setting of the optimal binding conditions, the lysed sample was spun for 1 min at 12,000 rpm, then the supernatant was transferred directly through the spin filter. The test tube with the spin filter was spun for 1 min at 12,000 rpm. After spinning, the spin filter was removed. Then, the DNA was bound to the spin filter by adding 200 µL of binding buffer on top of the filtered solution and vortexing. A binding spin filter was fastened to a 2.0 mL extraction test tube, and the complete solution was passed through the filter and incubated at room temperature for 1 min followed by spinning at 12,000 rpm for 1 min. The filtrate was removed, and the filter was fastened back to the test tube. For purification of the bound DNA, 550 µL of pre-washing buffer was added through the spin filter, followed by spinning for 1 min at 12,000 rpm. The filtrate was removed, and the filter was fastened back to the test tube. Then, 550 µL of pre-washing buffer was added through the spin filter, followed by spinning for 1 min at 12,000 rpm. The filtrate was then removed, and the filter was put back in the test tube. In order to dry the spin filter, residual ethanol was removed by spinning for 2 min at 12,000 rpm. During DNA elution through the spin filter, the spin filter was fastened to a 1.5 mL storage tube, and 100 µL of elution buffer preheated at 65 °C was added directly on the filter, followed by incubation at 65 °C for 3 min without stirring and then spinning for 1 min at 10,000 rpm. After spinning, the filter was disposed of. The extracted DNA was kept at 4 °C if analyzed within 24 h or at −20 °C if analyzed after 24 h.

2.3. Amplification and Detection by Real Time-PCR to Determine Meat Percentagse

The detection limit certified in the kit was ≤5 DNA copies (0.1% for chicken and 0.04% for beef and pork).

2.3.1. Preparation of the Reaction Mix (Reaction Mix Ref/Reaction Mix Bos, Sus, or Gallus)

In order to prepare the reaction mix, the calculation of the total number of reactions (samples and control samples) was necessary. The control samples included negative control, positive control,

and extraction control. It was recommended to prepare 10% more mix than necessary. Each mix was slightly spun before use. Table 1 lists the components and quantities necessary to prepare the reaction master-mix.

Table 1. Components of the reaction mix, depending on the examined species, and quantities necessary for the preparation of the mix for 10 Real-Time PCR tests.

Mix Components	Quantity/Reaction (µL)	10 Reactions (10% Excess)
Reaction mix Ref/Reaction mix Bos	18.0	198.0
Taq Polymerase	0.1	1.1
Total volume	18.1	199.1
Reaction mix Ref/Reaction mix Sus	18.0	198.0
Taq Polymerase	0.1	1.1
Total volume	18.1	199.1
Reaction mix Ref/Reaction mix Gallus	19.9	218.9
Taq Polymerase	0.1	1.1
Total volume	20	220.0

2.3.2. Preparation of DNA Standards

In order to prepare the DNA standards, dilution of the DNA standard (serial dilutions of 1:10), to obtain 5 different DNA concentrations was necessary, all illustrated in Tables 2 and 3. For dilutions, the dilution buffer was used.

Table 2. DNA dilutions for beef and pork.

Standard	Dilutions	No. of Copies/µL	No. of Copies/Reaction (for the Volume of 2 µL Standard DNA Used in the Reaction)
S1	45 µL buffer TE + 5 µL Standard DNA	100,000 copies	200,000 copies
S2	45 µL buffer TE + 5 µL S1	10,000 copies	20,000 copies
S3	45 µL buffer TE + 5 µL S2	1000 copies	2000 copies
S4	45 µL buffer TE + 5 µL S3	100 copies	200 copies
S5	45 µL buffer TE + 5 µL S4	10 copies	20 copies

Table 3. DNA dilutions for chicken.

Standard	Dilutions	No. Copies/µL	No. of Copies/Reaction (for the Volume of 5 µL Standard DNA Used in the Reaction)
S1	45 µL buffer TE + 5 µL Standard DNA	100,000 copies	500,000 copies
S2	45 µL buffer TE + 5 µL S1	10,000 copies	50,000 copies
S3	45 µL buffer TE + 5 µL S2	1000 copies	5000 copies
S4	45 µL buffer TE + 5 µL S3	100 copies	500 copies
S5	45 µL buffer TE + 5 µL S4	10 copies	50 copies

Unless used immediately, standards were stored at −20 °C. Dilutions were stable for 2 months. In the case of chicken analyses, DNA extracted fro the samples was diluted 1:10 using the dilution buffer to obtain optimal fluorescence and detection within the measurement range.

2.3.3. Preparation of the Mix for Beef and Pork Real Time-PCR

The preparation of the negative control Ref/*Bos*/*Sus* involved dropping 18 µL of reaction mix Ref/*Bos*/*Sus* in the reaction tube (capillary, plate), which was closed immediately. For preparing the

negative extraction control, 18 µL of reaction mix Ref was dropped in the reaction tube with 2 µL of negative extraction control, and the tube was closed immediately. The preparation of samples and standards Ref/*Bos*/*Sus* involved dropping 18 µL of reaction mix Ref/*Bos*/*Sus* + 2 µL DNA extract of samples/standards. For preparing the positive control Ref/*Bos*/*Sus*, 18 µL of reaction mix Ref/*Bos*/*Sus* were dropped in the reaction tube with 2 µL of DNA positive control. All tubes were slightly spun (up to 4000 rpm) and placed in the real time-PCR machine.

2.3.4. Preparation of the Mix for Chicken Real Time-PCR

The preparation of the negative control Ref/*Gallus* involved dropping 20 µL of reaction mix Ref/*Gallus* in a reaction tube (capillary, plate), which was closed the tube immediately. The preparation of the negative extraction control involved 20 µL of reaction mix Ref in the reaction tube with 5 µL of DNA (diluted 01:10), and the tube was closed immediately. The preparation of samples and standards Ref/*Gallus* involved 20 µL reaction mix Ref/*Gallus* + 5 µL of DNA extract of samples (diluted 1:10)/standards, while the preparation of the positive control Ref/*Gallus* involved 20 µL reaction mix Ref/*Gallus* in the reaction tube wtih 5 µL of DNA positive control. All the tubes were slightly spun (up to 4000 rpm) and placed in the real time-PCR machine. Table 4 reports the real-time PCR amplification protocol for the detection of the beef and pork species, and Table 5 that for the detection of the chicken species in food products of animal origin (chicken sausages, pork bologna, beef bologna).

Table 4. Real-time PCR protocol for the beef and pork species with fluorescence reporter dye (FAM) and fluorescence quencher dye (TAMRA).

Settings	Blockcycler	Rotorcycler	Lightcycler
Initial denaturation (HOLD)	5 min, 95 °C	1 min, 95 °C	1 min, 95 °C
Cycles	45	45	45
Denaturation	10 s, 95 °C	5 s, 95 °C	5 s, 95 °C
Annealing/Extension (CYCLE)	15 s, 62 °C 30 s, 65 °C	10 s, 62 °C 15 s, 65 °C	10 s, 62 °C 15 s, 65 °C
Temperature of the transition/time ratio	Maximum	Maximum	Maximum
Fluorescence detector	Detection: End of extension phase Reporter: FAM Quencher: TAMRA Passive reference; none	Reporter: FAM	Channel 530/610 or F1/F2 Acquisition mode: single in extension phase

Table 5. Real Time-PCR protocol for the identification of chicken meat.

Settings	Blockcycler	Lightcycler/Rotorcycler
Initial denaturation (HOLD)	5 min, 95 °C	1 min, 95 °C
Cycles	45	45
Denaturation	15 s, 95 °C	10 s, 95 °C
Annealing/Extension (CYCLE)	30 s, 60 °C	15 s, 60 °C
Temperature of the transition/time ratio	Maximum	20 °C/s
Fluorescence detector	Detection: End of the extension phase Reporter: FAM Quencher: TAMRA	Channel 530/610 or F1/F2 Acquisition mode: single in the extension phase

2.4. Expression of the Results

The sample was considered positive if the DNA in the sample included a fluorescent amplification signal. The sample was considered negative if the DNA in the sample did not include a fluorescent amplification signal. A separate calculation was performed for the total percentage of meat (Ref) and species (*Bos*, *Sus*, or *Gallus*). The cycle threshold (CT) values for standards must have a constant ΔCT;

ΔCT must be approximately 3.2 up to 3.6 cycles (±0.2) between two dilutions. If the standard curve indicates different values for ΔCT, a correct evaluation cannot be performed. The calculation method for the expression of the chicken, beef, or pork percentages used the values obtained for the positive control (PC) Ref and the positive control *Bos*/*Sus*/*Gallus* to calculate the recovery percentage and the correction factor (k):

$$\text{PCRef} = 503.9 \text{ and } \text{PC}\textit{Gallus} = 505.1$$

$$\text{R\%} = \text{CP}\textit{Gallus} \times 100/\text{CPRef} = 505.1 \times 100/503.9 = 100\%$$

$$\text{K} = \text{theoretical CP value/measured CP value} = 100/100 = 1$$

The value obtained for the samples was 49,950 copies of *Gallus* DNA and 50,810 copies of Ref DNA. The chicken percentage in the sample was 49,950 × 100/50,810 = 98.31%. The obtained result was multiplied by the correction factor: in the given example k = 1, therefore the final result was 98.31% × 1 = 98.31% chicken. The result was expressed as percentage for the samples with meat contents higher than the quantification limit, below the quantification limit for samples with meat contents between the detection limit and the quantification limit, and non-detectable for samples with meat content below the detection limit.

Internal Control of Result Quality

In order to control contamination during extraction, a negative extraction control was performed. For amplification and detection, a negative reaction control and a positive reaction control were prepared, and the samples were processed in duplicate.

3. Results and Discussions

3.1. Selectivity and Specificity

In order to control for false negative or positive signals, tests were performed on the three samples of chicken sausage, beef bologna, and pork bologna, and their responses were tracked, using SureFood ANIMAL QUANT Beef, SureFood ANIMAL QUANT Pork, and SureFood ANIMAL QUANT Chicken. The obtained results indicated positive or negative confirmation for the presence of the three meat types in the tested samples and are given in Table 6, below.

Table 6. Selectivity and specificity results.

PRODUCT	DATE					
	13.03.2018	**14.03.2018**	**28.03.2018**	**29.03.2018**	**23.06.2018**	**27.06.2018**
	Chicken Detection	**Chicken Detection**	**Beef Detection**	**Beef Detection**	**Pork Detection**	**Pork Detection**
Chicken sausage	positive	positive	negative	negative	negative	negative
Chicken sausage	positive	positive	negative	negative	negative	negative
Chicken sausage	positive	positive	negative	negative	negative	negative
Beef bologna	negative	negative	positive	positive	negative	negative
Beef bologna	negative	negative	positive	positive	negative	negative
Beef bologna	negative	negative	positive	positive	negative	negative
Pork bologna	negative	negative	negative	negative	positive	positive
Pork bologna	negative	negative	negative	negative	positive	positive
Pork bologna	negative	negative	negative	negative	positive	positive

The following equations were used:

$$\text{Specificity}: \ \text{SE\%} = \frac{PC}{PC + NF} \times 100 = \frac{18}{18 + 0} \times 100 = 100$$

$$\text{Specificity}: \ \text{SP\%} = \frac{NC}{PF + NC} \times 100 = \frac{36}{0 + 36} \times 100 = 100$$

$$\text{Accuracy}: \ \text{AC\%} = \frac{PC + NC}{PC + PF + NC + NF} = \frac{18 + 36}{18 + 0 + 36 + 0} \times 100 = 100$$

where:

PC = number of positive results
NF = number of negative false results
NC = number of negative results
PF = number of positive false results

The results did not indicate the presence of extraneous meat species in the three products, confirming that each product contained the species marked on the label. The used primers confirmed that all samples subject to examination included enhanceable DNA. Heat-processed pork, beef, and chicken products were used to validate the PCR tests specific to each meat species. The results indicated high sensitivity for the test, which allowed the detection of 0.04% of beef and pork and 0.1% of chicken. An extensive search within the literature demonstrated that the obtained sensitivity was considerably higher than that acheived in other studies, which obtained 0.1% (weight/weight) for pork in binary mixtures of raw or heat-treated meat [2,9,11,30–32], 0.05% for pork in mutton. and 1% for pork in meat mixtures [2,33] using pork-specific PCR tests targeting various genes, such as the *cytb*, *rRNA 12S*, *a-actin*, *ATPase8/ATPase6*, *D-loop*, *COI*, and pituitary porcine growth hormone (*PGH*).

3.2. Establishment of the Limit of Detection (LOD) and of the Limit of Quantification (LOQ)

The LOD declared in the SureFood ANIMAL QUANT kit is 5 DNA copies corresponding to the concentrations of 0.1% for chicken and 0.04% for beef and pork. The analysis was performed using the species DNA extracts provided in the kit (according to Table 7), from which dilutions were prepared according to the instructions of the kit).

Table 7. Work dilutions for DNA standards depending on the analyzed species.

Standard	No. of Copies Certified DNA	Dilution Factor	The Determined Number of Copies	
			Total Meat	Species
S5 beef	20	1:4	6.53	5.42
S5 chicken	50	1:10	5.08	4.28
S5 pork	20	1:4	8.10	8.08

Common LOD was established for eight DNA copies (0.2% chicken and 0.1% pork and beef). LOQ = 50 DNA copies for chicken, corresponding to a percentage of 1% chicken, and 20 DNA copies for beef and pork, corresponding to a percentage of 0.2% pork and beef. The calibration curves registered by the real-time PCR equipment for beef and chicken DNA and the readings for the detection limit were determined in the repeatability study illustrated in Figure 1a,b. Figure 1c shows the calibration curve and the detection limit for pork DNA.

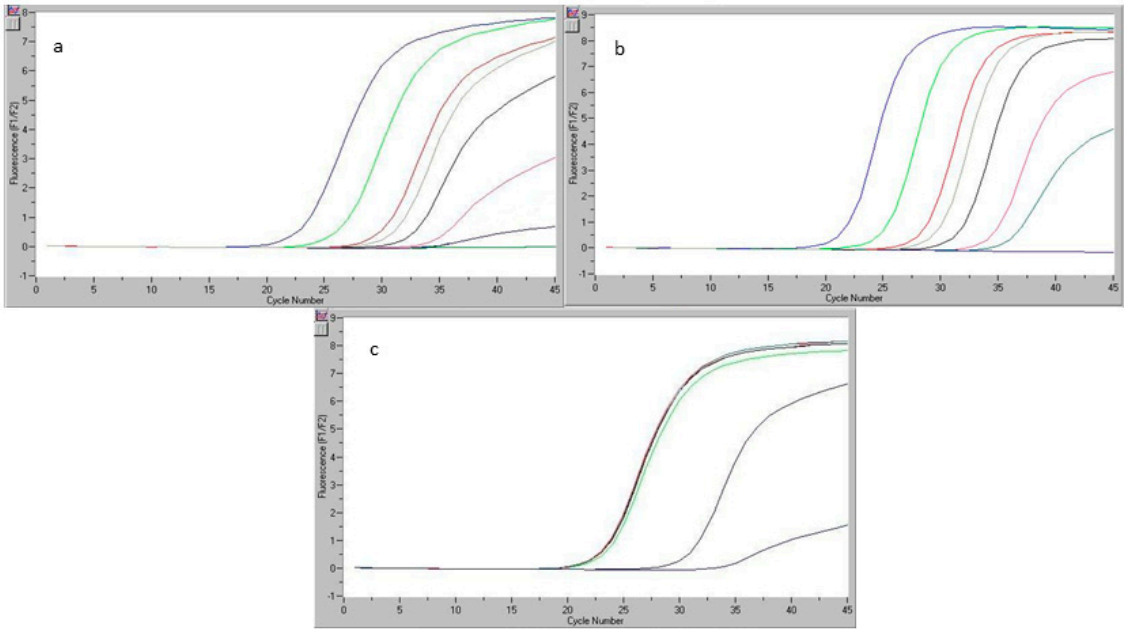

Figure 1. Limit of detection (LOD) and five-point calibration curve for beef DNA (**a**), chicken DNA (**b**), pork DNA (**c**) (detection limit 10^{-3}).

3.3. Repeatability

Tests were performed using samples of chicken sausages, beef bologna, and pork sausages; six determinations were carried out for each product, in reproducibility conditions (same analyst, same equipment, same method, same day). The results obtained and the statistical calculations are given in the tables and figures below.

3.3.1. Detection of the Bovine Species (Quality/Quantity)

The study considered a sample labeled as beef bologna. The sample was selected considering the poor quality of such products with regard to the quantity of meat present; we determined meat content and species of origin. From this sample, six DNA aliquots were extracted and subsequently analyzed by real-time PCR. Figure 2a shows the results of the real-time PCR reaction for the six genomic DNA replicates, work standards included in the kit (S1–S5), and one genomic DNA sample specific to the gallinacean (chicken) species. The readings registered by the real-time PCR equipment for the simultaneous analysis of a set of samples of beef DNA extract in the kit (dilution S5) are given in Figure 2b.

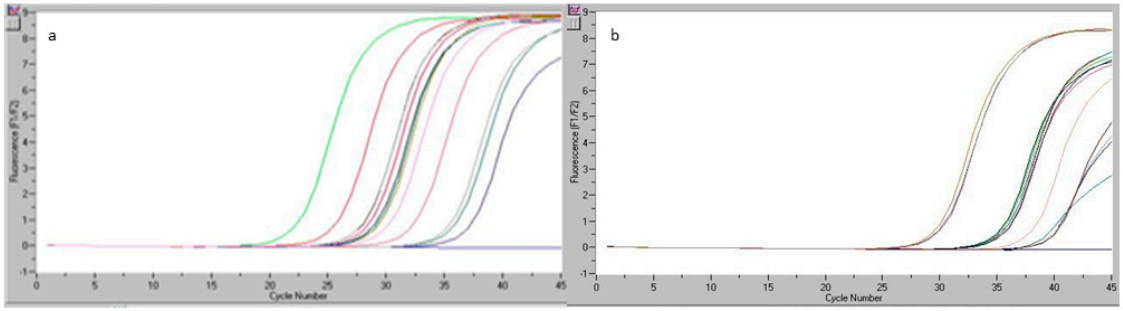

Figure 2. Readings registered by real-time PCR: for the beef bologna sample analyzed during the reproducibility test (**a**), for the beef DNA extract from the kit (dilution S5) within the reproducibility study (**b**).

According to the results, it can be concluded that the analyzed product contained beef and chicken. We then assessed the beef content of the product considered in this study; thus, specific calculations were performed to determine total proteins and specific bovine proteins, and the results are shown in Tables 8 and 9.

Table 8. Results of the reproducibility study for the beef bologna sample.

Product	No. of DNA Copies		Beef%
	Total Protein (Ref)	Beef Species (Bos)	
Beef bologna	4430	3053	81.32
	4473	2890	76.24
	2323	1545	78.48
	2201	1804	96.72
	5580	3982	84.21
	5577	3995	84.53
Positive control	1028	868.6	R% = 84.49, K = 1.18

Table 9. Statistical calculation for the establishment of standard deviation (s_r), relative standard deviation (RSD_r), and reproducibility limit (r).

Reproducibility from Serial Analysis		Replicates						Statistical Calculation			
Matrix	Data	1	2	3	4	5	6	average	s_r	RSD_r	r
beef bologna	06.04.2018	81.32	76.24	78.48	96.72	84.21	84.53	83.583	7.197	0.086	20.150

The process included the simultaneous analysis of a set of samples of beef DNA extract provided in the kit (dilution S5). The results obtained are shown in Table 10.

Table 10. Results obtained for the beef DNA extract provided in the kit (dilution S5).

Reproducibility from Serial Analysis			Replicates (Beef No. of Copies)								
Matrix	Data	Conc. level	1	2	3	4	5	M	Std dev	RSD_r	r
S5	05.04.2018	20 copies	15.98	22.75	15.60	16.01	13.57	16.78	3.485	0.208	9.757

The same protocol also included the analysis of DNA samples generated using minced pork and minced chicken matrix. The analysis of the data shown in Figure 2 indicated no value for the CT for the matrix represented by pork and chicken, thus confirming that the kit used for the determination of the bovine species was specific and did not misidentify other species (pork, chicken).

3.3.2. Detection of the Porcine Species (Quality/Quantity)

The performed study included the analysis of six DNA replicates originating from a pork sausage sample, as shown in Figure 3a, and the test reproducibility, using standard S5 (kit content), with a concentration level of 20 copies of pork genomic DNA, as shown in Figure 3b. The study monitored the specificity of the test and the origination of statistical analysis elements, which may outline the quality of the obtained results.

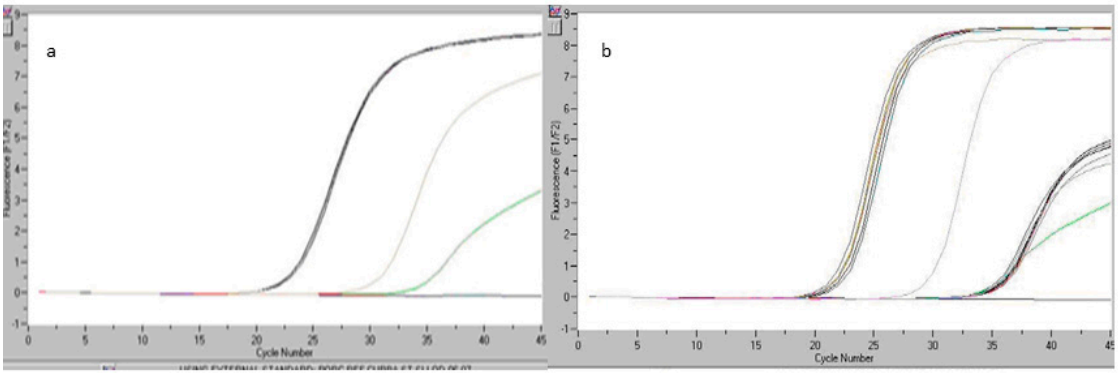

Figure 3. Readings registered by real-time PCR: for the pork sausage sample analyzed during the reproducibility test (**a**), for the analyzed pork sausage sample and ST5 (20 copies of pork genomic DNA) within the reproducibility study (**b**).

Following the assessment of the obtained results, it can be concluded that the analyzed product contained pork, and the results obtained for the six DNA replicates were comparable. We then determined the contents of pork in the product considered in this study, using statistical analysis. The results are shwn in Tables 11 and 12.

Table 11. Statistical calculation for the establishment of s_r, RSD_r, and r.

Reproducibility From Serial Analysis		**Replicates**						**Statistical Calculation**			
Matrix	Data	1	2	3	4	5	6	average	s_r	RSD_r	r
pork sausages	02.06.2018	23.36	21.44	21.74	23.51	23.30	23.30	22,775	3443	0.114	7242

Table 12. Results obtained for the pork DNA extract in the kit (dilution S5).

Reproducibility from Serial Analysis			**Replicates (Beef No. of Copies)**						**Statistical Calculation**			
Matrix	Data	Conc. level	1	2	3	4	5	6	M	Std dev	RSD_r	r
S5	02.06.2018	20 copies	102.8	95.68	92.04	77.85	80.40	100.9	91,628	10.44	0.114	29.24

We also focused on test reproducibility, using standard S5 (kit content), with a concentration level of 20 copies of pork genomic DNA. The obtained results and the application of the calculation formula provided the following concentration levels (in number of copies) for the quantity of total proteins and specific pork proteins, shiwn in Table 13.

Table 13. Results of the reproducibility study for the pork sausage sample.

Product	**No. of DNA Copies**		**Pork (%)**
	Total Protein (Ref)	**Pork Species (Sus)**	
Pork sausages	142,700	135,900	102.85
	172,700	153,000	95.68
	139,400	118,800	92.04
	117,700	84,840	77.85
	140,500	104,600	80.40
	139,500	130,400	100.95
Positive control	563.6	523	R% = 92.80; K = 1.08

3.3.3. Detection of the Gallinacean Species (Quality/Quantity)

On the basis of the same analysis principle, an experiment was optimized for a chicken matrix. Chicken sausage was selected as the matrix, as it may be easily adulterated and contain only a

small quantity of this species and many other ingredients. This can make the meat qualitative and quantitative analysis difficult. The process included the simultaneous analysis of a set of samples represented by the chicken DNA extract in the kit (S5, represented by 50 copies). Table 14 shows the results obtained for six DNA replicates generated by extraction from the chicken sausage matrix and the five standards contained in the kit. Figure 4 illustrates the readings registered by real-time PCR.

Table 14. Statistical calculation for the establishment of s_r, RSD_r, and r.

Reproducibility from Serial Analysis		Replicates						Statistical Calculation			
Matrix	Data	1	2	3	4	5	6	average	s_r	RSD_r	r
Chicken sausage	3 July 2018	99.0	97.4	90.7	99.3	100	96.8	97.3	3.556	0.037	9.95

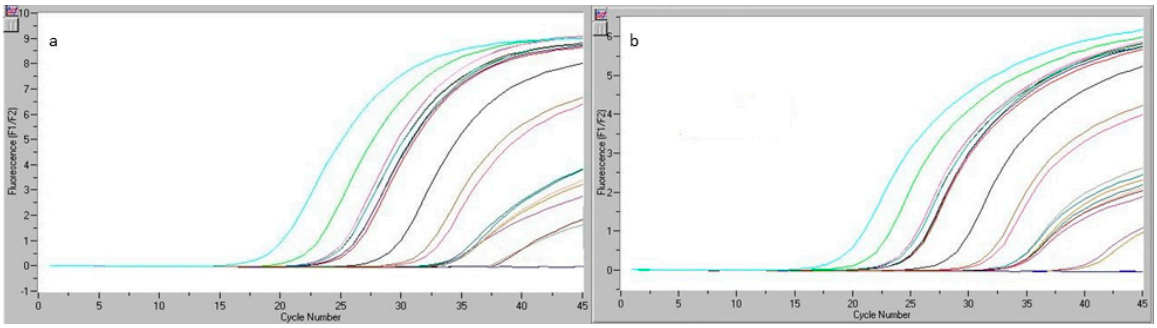

Figure 4. Readings registered by real-time PCR: for the chicken sausage sample analyzed during the reproducibility test (**a**), for the analyzed chicken sausage sample and ST5 (50 copies of chicken genomic DNA) in the reproducibility study (**b**).

Following the assessment of the obtained results, it was concluded that the analyzed product contained chicken, and the results obtained for the six DNA replicates were comparable. We then determined the content of chicken in the product considered in this study, using statistical analysis and the calculation formulae. The results are shown in Table 14.

The process included the simultaneous analysis of a set of samples represented by the chicken DNA extract in the kit (S5, represented by 50 copies). The results obtained are shown in Table 15.

Table 15. Results obtained for the chicken DNA extract in the kit (S5 represented by 50 copies aviary genomic DNA).

Reproducibility from Serial Analysis			Replicates (Aviary Genomic DNA, No. of Copies)						Statistical Calculation			
Matrix	Data	Conc. level	1	2	3	4	5	6	average	sr	RSD_r	r
S5	03 July 2018	50 copies	58.1	54.2	65.13	66.6	57.78	73.3	62.563	7.102	0.114	19.886

The obtained results and the application of the calculation formula provided the following concentration levels (in number of copies) for the quantity of total proteins and chicken proteins (Table 16).

Table 16. Results obtained in the reproducibility study for the chicken sausage sample.

Product	No. of DNA Copies		Chicken%
	Total Protein (Ref)	Chicken Species (Gallus)	
Chicken sausage	117,400	110,700	99.01
	119,200	110,600	97.42
	61,250	52,930	90.74
	61,330	58,020	99.33
	99,540	95,650	100.90
	102,500	94,550	96.86
Positive control	569.9	542.2	R% = 95.14 K = 1.05

It can be concluded that real-time PCR can determine the meat quantity and species contained in a food product of animal origin.

3.4. Reproducibility

In order to make sure that the results obtained by the application of the analysis methods satisfied Regulation (EC) no. 1493/1999 [34], it was necessary to demonstrate that the results were reproducible. We thus performed laboratory analyses to this aim. The analysis included sample sets represented by chicken sausage, beef bologna, and pork bologna and was performed in two different days, using the same equipment, by two analysts (analyst 1 and analyst 2). The results obtained and the statistical calculations are shown in the tables below and their corresponding figures, as follows: for beef bologna sample Table 17, Table 18 and Figure 5; for pork bologna sample Table 19, Table 20 and Figure 6; for chicken sausage sample Table 21, Table 22 and Figure 7).

Table 17. Results obtained in the reproducibility study for the beef bologna sample.

Analyst/Date	Product	No. of DNA Copies		Beef%
		Total Protein (Ref)	Beef Species (Bos)	
Analyst 1/28.04.2018	Beef bologna	4430	3053	81.32
		4473	2890	76.24
		2323	1545	78.48
		2201	1804	96.72
		5580	3982	84.21
		5577	3995	84.53
	Positive control	1028	868.6	R% = 84.49 K = 1.18
Analyst 2/06.04.2018	Beef bologna	3558	2519	82.83
		3624	2730	88.13
		1724	1421	96.43
		1833	1490	95.10
		4985	3415	80.15
		5200	3542	79.69
	Positive control	887.4	754	R% = 84.96 K = 1.17

Table 18. Statistical calculation for the establishment of s_r, RSD_r, and r.

Analyst	Data	Beef%
Analyst 1	28/03/2018	81.32
		76.24
		78.48
		96.72
		84.21
		84.53
Analyst 2	06.04.2018	82.83
		88.13
		96.43
		95.10
		80.15
		79.69
Calculations	Average	85,319
	S_r	7.190
	RSD_r	0.084
	r	20,132

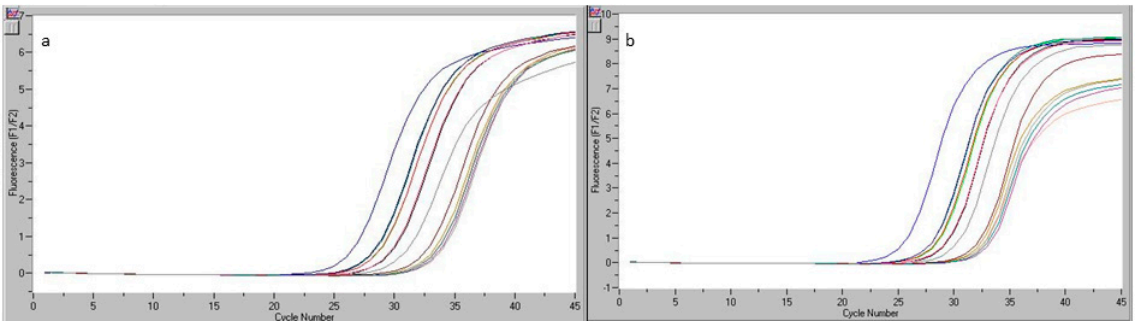

Figure 5. Readings of real-time PCR for the beef bologna sample analyzed during the reproducibility study (Ref and *Bos*): results obtained by analyst 1 (**a**) and analyst 2 (**b**).

Table 19. Results obtained in the reproducibility study for the pork bologna sample.

Analyst/Date	Product	No. of DNA Copies		Pork%
		Total Protein (Ref)	Pork Species (Sus)	
Analyst 1/02.11.2018	Pork bologna	142,700	135,900	102.85
		172,700	153,000	95.68
		139,400	118,800	92.04
		117,700	84,840	77.85
		140,500	104,600	80.40
		139,500	130,400	100.95
	Positive control	563.6	523.0	R% = 92.80 K = 1.08
Analyst 2/06.11.2018	Pork bologna	141,500	139,000	99.22
		162,100	166,700	103.87
		163,100	171,500	106.20
		167,600	170,000	102.45
		146,500	158,600	109.34
	Positive control	567.7	561.0	R% = 98.82 K = 1.01

Table 20. Statistical calculation for the establishment of s_r, RSD_r, and r.

Analyst	Data	Pork Species (%)
Analyst 1	02/11/2018	102.85 95.68 78.48 77.85 80.40 100.95
Analyst 2	06/11/2018	99.22 103.87 106.20 102.45 109.34
Calculations	Average S_r RSD_r r	96,117 11,606 0.121 32,497

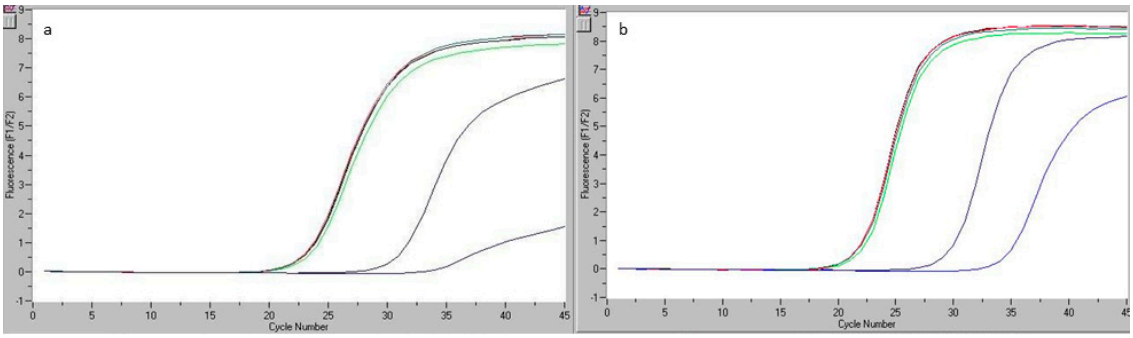

Figure 6. Readings of real-time PCR for the pork bologna sample analyzed in the reproducibility study (Ref and *Sus*): determinations made by analyst 1 (**a**) and analyst 2 (**b**).

Table 21. Results obtained in the reproducibility study for the chicken sausage sample.

Analyst/Date	Product	No. of DNA Copies		Chicken%
		Total Protein (Ref)	Chicken Species (Gallus)	
Analyst 1/03.07.2018	Chicken sausage	117,400 119,200 61,250 61,330 99,540 102,500	110,700 110,600 52,930 58,020 95,650 94,550	99.01 97.42 90.74 99.33 100.90 96.86
	Positive control	569.9	542.2	R% = 95.14 K = 1.05
Analyst 2/04.07.2018	Chicken sausage	11,990 13,910 4778 5296 9293 8909	11,670 13,170 4092 5149 9087 8867	102.20 99.41 89.92 102.09 102.67 104.50
	Positive control	689.0	656.8	R% = 95.33 K = 1.05

Table 22. Statistical calculation for the establishment of s_r, RSD_r, and r.

Analyst	Data	Chicken Species (%)
Analyst 1	03/07/2018	99.01 97.42 78.48 99.33 100.90 96.86
Analyst 2	04/07/2018	102.20 99.41 96.43 102.09 102.67 104.50
Calculations	Average S_r RSD_r r	98.28 6.714 0.068 18,799

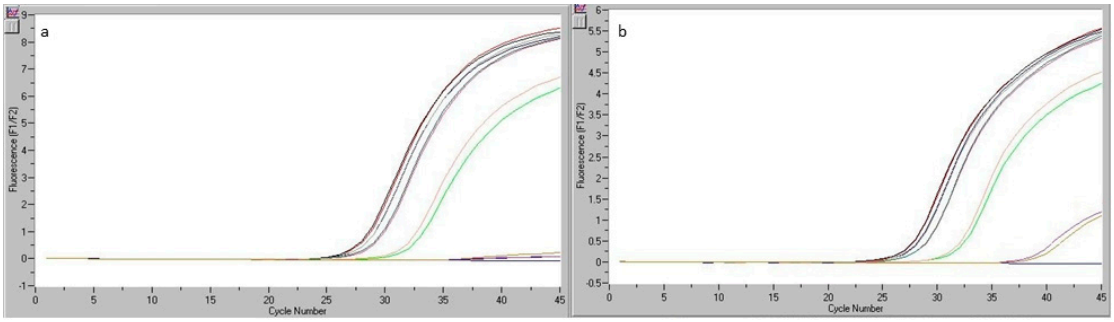

Figure 7. Readings of real-time PCR for the chicken sausage sample analyzed during the reproducibility study (Ref and *Gallus*): determinations made by analyst 1 (**a**) and analyst 2 (**b**).

Table 23 includes a brief description of the performance parameter results for the method used to detect the species of origin (pork, chicken, and beef).

Table 23. Performance parameters of the detection method of meat origin species.

Performance Parameters	Unit of Measure (UM)	Performance Criterion	Value Obtained Following Validation		
			Beef%	**Pork%**	**Chicken%**
Detection limit	%	LOD declared in the SureFood ANIMAL QUANT kit is of 5 DNA copies corresponding to a concentration of 0.1% for chicken and 0.04% for beef and pork.	A common LOD of 8 DNA copies was established		
			0.1%	0.1%	0.2%
Quantification limit	%		20 DNA copies 0.2%	20 DNA copies 0.2%	50 DNA copies 1%
Selectivity/specificity	%	100		100	
Repeatability	%	$RSD_r \leq 25$ $R = 2.8 \times s_r$	8.6 20.15	11.4 29.24	3.70 9.96
Reproducibility	%	$RSD_R \leq 35$ $R = 2.8 \times s_r$	8.4 20.13	12.1 32.49	6.8 18.80
Measurement uncertainty	%	Calculated for a confidence range of 95%, k = 2	17	24.28	13.83

4. Conclusions

A real-time PCR analysis was successfully optimized and validated for processed meat products, allowing the detection and quantification of levels of meat up to 0.1% for pork and beef and 0.02% for chicken. The method was validated effectively using products with low meat content (chicken sausages, pork bologna, and beef bologna) confirming its correctness, accuracy, and repeatability. Following the validation of the performance parameters of the method, the three examined samples showed 100% specificity; repeatability was the highest for pork meat, followed by beef and chicken. Reproducibility was also the highest for pork, followed by beef and chicken. The quantification limit of 20 DNA copies for pork and beef corresponded to 0.2%, while that for chicken, of 50 copies, was 1%. Measurement uncertainty also was the highest for pork, followed by beef and chicken.

In all the tested samples (chicken sausages, pork bologna, and beef bologna), meat of the corresponding animal species was found, in agreement with what reported in the label, and the results obtained for the six DNA replicates were comparable. Using the real-time PCR technique, the determination of the quantity and species contained in processed food of animal origin was obtained. Real-time PCR is a fast and simple method of identifying meat species, it is very sensitive, and has an accuracy of detection of 0.1% DNA in the analyzed products. Considering that the presence of extraneous meat species in food products is one of the authenticity problems that worry the consumers, this paper demonstrates the importance of quality control and inspection programs in the meat industry, with the final aim of improving public and industrial safety and protecting human health.

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