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Chapter 9

PCR: A Powerful Method in Food Safety Field

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Additional information is available at the end of the chapter

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Abstract

In this chapter, application of the polymerase chain reaction (PCR) technique in food safety, considering all the branches of this concept, is presented. The area of interest contains important analysis for both human health and the identification of food adulteration. PCR techniques used for detection of genetically modified organisms (GMO) in different matrices, identification of different animal species in meat and dairy products, as well as the detection of food infection with food-borne pathogens and toxicogenic fungi are described. The working methods and result analysis are exemplified, starting with DNA isolation adjusted to different matrices, detection of target genes, and validation for all of these methods. Techniques of simplex PCR, primer multiplexing, primer design, validation of the laboratory methods, optimization of the PCR results, and result interpretation through the analysis of the electrophoresis gels and sequencing data are studied. At the same time, the obtained results, the obstacles encountered, and how they were overcome could be an example for specific analysis developed with less resources and also for adapting the existent validated methods to the new laboratory conditions. The practical applicability and the consumer's demands are of great importance and always must be considered in developing and validating those methods.

Keywords: PCR application, food safety, method validation, species identification, GMO detection

1. Introduction

Food safety concept has been emerged in the last decades as a scientific discipline concerning the handling, processing, storage, and packaging of food in order to prevent food-born illness. This concept has been developed and extended arriving to include practice considerations of food hygiene, labeling, additives, and other exogenous chemical residues but also

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the biotechnology products. The primary categories of food-borne pathogenic organisms are fungi, bacteria, viruses, and parasites. Intrinsic food components include nutritional factors and thousands of contaminant compounds naturally present in foods. Hazardous chemicals in foods include naturally occurring toxicants, agro-industrial contaminants, and food additives. Naturally occurring toxicants are chemicals from the natural environment that occur in foods and animal feeds, such as mycotoxins, aquatic biotoxins and phytoalexins, intrinsic components of plants, bacterial toxins, cyanobacterial toxins, and food decomposition components. Worldwide, if the system of safety risk management is not fully functional, food contaminated with pathogens or their toxins could be sent to the consumers. Besides, because of a low economic efficiency, the producers could appeal to food adulteration. Whatever problem occurred, the specialists in food control have to be prepared to apply the most appropriate analysis methods. The huge progress registered in the last period in the area of nucleic acid research determined the development of analysis methods with direct applicability for different practically aims. The multitude of resources used in the DNA analysis is available all over the world leading to the development and improvement of laboratory techniques [1]. The common food markets, the regulations in this area, and the increased demanding of the consumers determine the manufacturers to send to consume products appreciated due to their organoleptic qualities, origin and composition, and last but not least their safety. Illnesses associated with foods are not very abundant. Nevertheless, when they occur, the adverse effect on human society and the food supply availability has been proved to be significant. Therefore, studies developed in research laboratories play an important role in identifying the sources of food-borne health risks, in developing procedures and products that reduce the effects of health and economic hazards. These studies must provide the assurance of a safe, wholesome food supply. The way of choosing a food may reflect aspects of lifestyle (vegetarianism and a diet based on organic food), religion (the absence of pork in some diets), diet, and health issues (e.g., the presence of the allergens). In addition, accurate labeling is important to support fair trade. Supplementary information can be added on a descriptive label as a consequence of branding and of regulations for the marketing of products. While regulations enshrined in national and international law are the underlying of mandatory label information, unfortunately, those regulations are not sufficient to prevent food fraud. Most often manufacturers choose to mislead the consumer by adding or substituting ingredients in food with cheaper ones for a higher economic gain [2] which leads to an impaired overall quality of the food. To prevent these frauds, there are a multitude of options available to identify species whose products constitute ingredients in food such as meat or dairy products. First, there are physical identification performed by monitoring the labeling and the microscopic analysis and the identification of lipid, volatile organic compounds and proteins, and nucleic acid analysis [3]. Initial methods for identifying food composition were based on morphological characteristics such as flavor, color, shape, or taste [4]. European countries are still using microscopic methods for detecting animal or plant material in food and feed which in most cases is limited [3]. The authenticity of products of animal origin and traceability issues are increasingly important in modern society, as can be inferred, for example, from the relative recent events regarding adulteration of meat products, with species that are not declared, such as horsemeat [5], adulteration of products of sea food

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type, the deliberate introduction of animal proteins in feed ingredients, or fraudulent mislabeled or label unlisted foods are a serious problem affecting the end consumer in many ways . Today, many consumers are concerned about animal products they consume, and accurate labeling is important for consumer information in the choices they make.

2. Design of a qualitative PCR investigation

A qualitative polymerase chain reaction (PCR) investigation has as starting points the materials/matrices which will be analyzed and the specific DNA sequence which will be amplified. Considering the types of matrices and the primers used for amplification, the factors influencing to analyze uncertainty in laboratory should be considered. The sample homogeneity is an important factor, in a direct relation with the matrix type.

2.1. DNA extraction and reference sample preparation

Seeds (wheat, soybean, and maize), forages, and food products as substitutes for meat based on soy protein (granules, textured, and schnitzel) have to be grinded at the same speed of the mill, and particles with the same size are selected. The minimum quantity is 1000 grains (approximate 200 g for soybean and 300 g for maize) when seeds are analyzed. For the mentioned food products, all the package content is grinding. Meat, usually sampled from fresh material, could be used directly, but we recommend its dehydration. Thus it will be easier to manipulate, the long-term storage of reference sample will be facilitated in terms of time and space, and the samples used for DNA extraction will be homogeneous. The samples can be freeze-dried (the best option), but air drying in special condition can be also used. Considering that air drying takes a long time (several weeks for best results), a free contaminant environment, a constant air flow, and a temperature below 24 °C have to be ensured to avoid DNA degradation. Before drying the samples, they need to be chopped in small pieces, and when the process is finalized, the material is ground to obtain homogeneous samples for DNA extraction. The same preparation method could be used when different meet products are analyzed (salami, sausages, etc.) [6, 7].

Food products as milk, soybean milk, yogurt, cheese, tofu, and pate need to be shaken/mixed. From the homogenized material, the samples are collected. From grinded/mixed material, a 1 g test sample is collected. From the test sample, two analytical samples (100 mg) are taken according to the quartering method for DNA extraction. Besides, a quantity of 50 g is stored as a reference, if the analysis fails or if it needs to be repeated. Other factors that must be followed are DNA quantity and quality. To meet these requirements, all the DNA samples which will be compared are extracted based on the same DNA extraction method, selected according to the matrix specificity. Each DNA extraction procedure is based on the elimination of all the materials present besides DNA in a specific matrix. Therefore different matrix compositions require different physical and/or chemical treatments to extract DNA.

Nowadays a large variety of DNA extraction kits was developed, but using conventional, standardized methods gives a much greater flexibility.

In our work the method described by SR EN ISO 21571 was suitable for a very wide range of matrices, from seeds, flour, or food to meat, dairy, or meat products. The method comprises a step of thermal lysis, in the presence of cetyltrimethylammonium bromide (CTAB), followed by several extraction steps for the removal of protein or polysaccharide compounds. For some matrices different enzymatic steps are necessary. For example, the samples containing high quantity of starch have to be treated with α-amylase; for the meat-derived products, a longer treatment (up to 3 h) with proteinase-K will remove proteins and will facilitate the tissue dissociation, and the RNA can be removed by RNase treatment. One of the most important concerns during the DNA extraction procedure is cross contamination, which can be monitored through the following control samples: environment control (EC), a nuclease-free water (100 μl) sample, present in an open tube during the DNA extraction process; extraction blank (EB), obtained by performing all stages of standard DNA extraction procedure, except the introduction of biological sample; positive control for target DNA (PDT), extracted from positive samples, namely, certified reference or validated in-house materials; and negative control for target DNA (NDT), extracted from negative samples, namely, certified reference or validated in-house materials.

To evaluate the DNA yield and purity, the absorbance method was applied. Therefore a spectrophotometer is used to measure the absorbance at 260 nm for DNA concentration and also the ratios $A_{260/280}$ and $A_{260/230}$ for DNA purity. It is known that the aromatic amino acids from proteins absorb at 280 nm and other contaminants as organic compounds or chaotropic salts absorb at 230 nm. $A_{260}/A_{280} = 1.7–2$ and $A_{260/230} > 1.5$ for a good-quality DNA [8]. If the quality was not appropriate, the DNA samples were subjected to an advanced purification through sodium acetate ($C_2H_3O_2Na$) precipitation. All the DNA samples used in an experiment are diluted at the same concentration (25–100 ng/μl) and stored at −20 °C, until use. The concentration of the DNA samples originated from unprocessed products as seeds or meats are usually higher and their quality meets the purity requirements. But the DNA quality is not optimal if highly processed materials are analyzed, mainly due to the DNA degradation during the mechanical and thermal treatments. But, considering that PCR technique amplifies short DNA fragments (few hundreds base pairs), it is assumed that at least few copies of that fragments are intact in the DNA samples.

Considering that the DNA samples are further on evaluated by PCR, it is necessary to determine if they are amplifiable, namely, inhibitors do not interfere with the polymerase activity, and the DNA fragments have suitable length. Therefore, the first step in each PCR experiment is an amplification reaction with species-specific primers: lectin for soybean, zein for maize, etc.

Another fundamental factor is the calibration with certified materials or validated in-house materials.

For genetically modified organism (GMO) analysis, the certified reference materials (CRM) provided by the Institute for Reference Materials and Measurements (IRMM) were used. For soybean analysis the materials with certified GM content have the following concentrations: <0.3, 1.0, 5.0, 10.0, 20.0, and 50.0 g/kg.

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In most cases the animal reference material can be purchased from entities that are developing [9], but it may be found that some of them are missing from the market and also, in some cases, are expensive. For research purposes we found that it is better to prepare own reference material followed by in-house validation, respecting all the good laboratory practice (GPL) regulations. The best reference material is prepared from the muscular tissue, but in its absence, the organ tissue can be used as well. Samples of the blood can be also collected, but this is not the best option because in this case, the blood-free circulating DNA (even remaining from other species tissue intake) is extracted together the species DNA.

In a preliminary phase of a method validation, it is indicated to isolate and purify the DNA from a fresh raw material, and thus the obtained DNA is of best amplifiable quality. In our experiments the reference materials for animal species identification were validated in-house. The target animal species were cattle, swine, poultry, and fish. Two different types of materials were obtained, according to the analyzed samples origin—meat or milk. To obtain reference materials from meat, fresh muscle was collected from the already mentioned animal species. They were prepared as previous described, starting from 200 g of fresh tissue. The DNA samples were amplified with the species-specific primers to validate the DNA identity. These DNA samples were further on used as reference materials, with a content of 100 % validated animal species.

Milk samples from different animal species were collected for reference materials used for cheese analysis, namely, cow, sheep, and goat. Further on, cheeses were manufactured in our laboratory from fresh milk by a traditional recipe for the preparation of fermented cheese: milk was filtered, stirred, and heated at 30 °C for 1 h and allowed to clot for 2 days (free of added rennet). After 2 days it was heated again, followed by the whey separation for 12 h. Therefore, the reference materials were cow's milk cheese (100 %), goat milk cheese (100 %), and sheep milk cheese (100 %) which were further validated with species-specific primers.

The reference materials used for *Fusarium* species identification strains were validated in-house. For the species with the highest frequency in our country, the fungal strains were isolated and identified according to their morphological description and microscopic analysis (shape of the macroconidia, the presence or absence of chlamydospores). Their identity was confirmed by DNA sequencing of the gene encoding the elongation factor 1 alpha (TEF) and comparison with the available databases.

Other factor involved in analysis uncertainty is the amplification reaction. All the DNA samples used in an analysis series are diluted at the same concentration, to provide similar number copies of the target sequences. All the reactions follow the same amplification conditions programed in a calibrated thermal cycler. The NTC- non-template control has to be used besides the already mentioned controls. This consists of an amplification mixture where the DNA sample is replaced with sterile distilled water.

Considering that the qualitative PCR needs the amplification product analysis, the electrophoresis and the gel evaluation are the last step.

The regular amplification products are few hundred base pair length; therefore, they can be separated by agarose gel. The gel concentration is correlated with the DNA fragment size,

being ranged between 1.5 and 2.5 %. To monitor the precision, accuracy, and the lack of cross contamination, the following samples are analyzed on each gel: analyzed samples (usually two repetitions for each), **EC**, environment; **EB**, extraction blank; **NDT**, negative DNA template; **PDT**, positive DNA template; and **NTC**, non-template controls.

The specificity of the amplified fragments is determined through similarity with the positive DNA template controls and comparing with a molecular weight marker. To evaluate the amplified fragment size, visual observation or the use of specific software could be done. Therefore, to validate a result obtained by qualitative PCR, which is a positive/negative result, few conditions need to be met: same length for the amplified fragments of unknown sample and positive DNA control; environment, extraction blank, negative DNA template, and non-template controls need to be negatives. If all controls are not showing the expected results, the qualitative PCR test is not validated [10, 11].

To obtain reliable results in PCR experiments, it is necessary to meet the good laboratory practice requirements. Therefore, to avoid the cross contamination risks, all the working steps have to be developed in different rooms. A DNA-free area has to be organized where the pre-PCR is performed, namely, the preparation of the PCR mixtures to avoid the original reagent contamination. After amplification the copy number of the interest sequences is very high; therefore, the post-PCR step, namely, electrophoresis, needs to be done in a special area, which is not connected with the rest of laboratory.

3. Detection of genetically modified organisms (GMO) in agriculture products, forage, and food products based on PCR

Nowadays, according to the European Union regulations, all the food or forage products containing GMO (genetically modified plants) in a concentration higher than 0.9 % need to be labeled. It is known that the GM product with the higher prevalence on the European market is GM soybean [12]. Even if it is not approved for cultivation in EU, it is imported in very large quantities and represents the most important ingredient of forages used for livestock [13]. Therefore, the implementation of the detection methods for this product is of great importance. They were developed starting from the molecular sequence specific for the construct used for genetic transformation [14]. This sequence includes the promoter responsible for the transcription of the whole genome of a *Cauliflower mosaic virus* named CaMV 35S promoter or 35S promoter; the region CTP4, encoding a transit polypeptide involved in protein transport to chloroplast originating from *Petunia* hybrid; and the gene CP4 EPSPS (5-enolpyruvylshikimate-3-phosphate synthase), a mutant gene originated from *Agrobacterium* sp., which is insensitive to glifosat action and the terminal sequence nopaline synthase (nos) [15].

For the primary screening, the detection of the 35S promoter was used as a target sequence, using the primers p35S-cf3 and p35S-cr, or the terminal sequence nos, using the primers HA-nos118-f şi HA-nos118-r. The expected amplicon was a fragment of 123 bp length when the primers are placed in the promoter sequence or 118 bp if the primers were specific for the nos terminal sequence [6, 16, 17]. Considering that the analyzed DNA is extracted from different

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processed matrices, the controlling of its quality is necessary. First the absorbance measurements are done by spectrophotometer to evaluate the DNA concentration and quality followed by PCR amplification with primers specific for the analyzed species (lectin) to determine if the DNA samples are amplifiable (**Table 1**).

Organism/target gene	Primer sequences 5'…3'	Annealing temperature (°C)	Amplicon size (bp)	Reference
GMO3/GMO4 *Lectin* gene	GCCCTCTACTCCACCCCCATCC GCCCATCTGCAAGCCTTTTTGG	63	118	[18]
p-35S-cf3/p-35S -cr4, 35S promoter	CCACGTCTTCAAAGCAAGTGG TCCTCTCCAAATGAAATGAACT TCC	62	123	[18]
zein3-zein4 Zein gene	AGTGCGACCCATATTCCA GACATTGTGGCATCATCATT	60	277	[18]
mg1–mg2	TATCTCCACTGACGTAAGGGATGAC TGCCCTATAACACCAACATGTGCTT	61	430	[18]
HA-nos 118-f/HA-nos118-r, nos gene	GCATGACGTTATTTATGAGATGGG GACACCGCGCGCGATAATTTATCC	62	118	[18]
CW/CX Rubisco gene	CGTAGCTTCCGGTGGTATCCACGT GGGGCAGGTAAGAAAGGGTTTCGTA	63	150	[26]
Fusarium ssp./ region TEF	ATGGGTAAGGAGGACAAGAC GGAAGTACCAGTGATCATGTT	54	760	[30]
Fusarium graminearum	GTTGATGGGTAAAAGTGTG CTCTCATATACCCTCCG	53	500	[46]
Fusarium proliferatum	CGGCCACCAGAGGATGTG CAACACGAATCGCTTCCTGAC	65	230	[46]
Fusarium verticillioides	CGCACGTATAGATGGACAAG CACCCGCAGAATCCATCCATCAG5	65	700	[46]
ef1/ef2	ATGGGTAAGGAGGACAAGAC GGAAGTACCAGTGATCATGTT	53	700	[47]
ef1/ef22	ATGGGTAAGGAGGACAAGAC AGGAACCCTTACCGAGCTC	53	450	[47]
Cattle/16S rRNA	TAA GAG GGC CGG TAA AAC TC GTG GGG TAT CTA ATC CCA G	60	104	[42]
Swine/12S rRNA-tRNA	CTA CAT AAG AAT ATC CAC CAC A ACA TTG TGG GAT CTT CTA GGT	60	290	[42]
Poultry/12S rRNA	TGA GAA CTA CGA GCA CAA AC GGG CTA TTG AGC TCA CTG TT	60	183	[42]
Fish/12S rRNA	TAA GAG GGC CGG TAA AAC TC GTG GGG TAT CTA ATC CCA G	60	224	[42]
Horse/mtDNA	CCATCCCTACGCTCCATTCCC TGTTTTCGATGGTGCTTGCG	60	280	Unpublished own research
Salmonella typhimurium/alfa gene	CCT TTC TCC ATC GTC CTG AA TGG TGT TAT CTG CCT GAC CA	56	120	[36]

Organism/target gene	Primer sequences 5'…3'	Annealing temperature (°C)	Amplicon size (bp)	Reference
Escherichia coli/ fimA	GCT GGG CAG CAA ACT GAT AAC TCT C CAT CAA GCT GTT TGT TCG TCC GCC G	56	750	[36]
Bos taurus 12S rRNA-16S rRNA	GTACTACTAGCAACAGCTTA GCTTGATTCTCTTGGTGTAGAG	55	256	[45]
Capra hircus/12S rRNA	CGCCCTCCAAATCAATAAG AGTGTATCAGCTGCCAGTAGGGTT	55	326	[45]
Ovis aries/12S rRNA	ATATCAACCACACGAGAGGAGAC TAAACTGGAGAGTGGGAGAT	55	172	[45]

Table 1. The sequences of the primers used in described experiments.

Our aim was to implement in our laboratory the standardized methods used for DNA isolation from different matrices containing soybean [18] and the qualitative PCR used for GM detection [19].

The first step was the method validation, considering that it was a standardized method used for the proper aim, started from the mentioned types of matrix, developed in our laboratory conditions. Therefore it was not necessary to evaluate the specificity, but the limit of detection (LOD) and the repeatability were determined. According to the standardized method, the absolute limit of detection was not determined, but it was demonstrated that it can detect 50 copies of the DNA originated from Soybean 40-3-2 GTS. In our experiments the samples extracted from the certified reference materials (CRM) were analyzed, each amplification reaction containing 50 ng of DNA (2 μl DNA, concentration 25 ng/μl). For all the reference materials, the expected amplicon (118 bp) was visible. According to the literature data, it is known that there is only one copy of the transgene in the haploid genome [20] and the soybean haploid genome is 1.13×10^9 bp, equivalent with 1.13 pg DNA (a fragment with a 1000 bp length is equivalent with 10^6 pg DNA).

Consequently a quantity of 50 ng of DNA contains approximate 40×10^3 soybean genomes. The transgene copy numbers depending of GM concentration in 50 ng of DNA are as follows: 10 % = 4000, 5 % = 2000, 1 % = 400, 0.5 % = 200, and 0.1 % = 40 copies.

Thereby the investigations performed in our laboratory allowed the emphasizing of 40 transgene copies (50 ng DNA 0.1 % MG), lower than the value mentioned in the standard.

To verify the detection limit, soybean samples with different GM concentrations were prepared in the lab, because products with similar GM concentrations were not available on the market. As started materials flour was obtained from conventional and 100 % GM soybean and also from substitute of meat by grinding. First we prepared a 1 % GM soybean flour (1 g MG and 99 g conventional soybean flour) which was used for 0.1 % MG (90 g conventional and 10 g 1 % MG soybean flour) and 0.1 % textured—substitute of meat (90 g substitute of meat and 10 g 1 % MG soybean flour). Two samples from each experimental variant were analyzed, starting from the first step. The DNA was extracted from the samples in five experimental series, one

series per week. All the DNAs were evaluated spectrophotometrically, diluted at 25 ng/μl, and amplified with the lectin and nos primers.

All the samples with a 0.1 % GM concentration (40 transgene copies) were positive each time, pointing out 100 % repeatability.

In conclusion it was demonstrated that the detection limit for the GM soybean detection is 40 transgene copies or material with 0. 1% GM content in condition of repeatability.

Experiments with lower GM concentrations were not performed because the European legislation requires product labeling only if the GM concentration is higher than 0.9 %; therefore, the detection of concentrations lower than 0.1 % is not necessary.

Analysis is performed in condition of repeatability. The repeatability conditions are considered when the results of the independent tests are obtained based on the same method, in the same laboratory, and with the same equipment in short interval of time. Therefore in our experiments, five identical series were performed in the conditions previous described. The materials used for analysis were based on soybean as follow: meat substitute granules and schnitzel, pate, pate with pepper, mayonnaise sauce, vanilla dessert, yogurt chocolate drink, tofu, forage, and laboratory-prepared flour 100 % and 1 % MG.

The DNA was extracted from each sample in two repetitions, in five experimental series, one series/week. First time the quality, integrity, and concentration of the extracted DNAs were determined. The quality of the DNA extracted from the food samples was not optimum (the values for OD280/260 and OD 230/230 were less than the recommended ones) because they were highly processed. Therefore the amplifications with species-specific primers (lectin) were performed to demonstrate that the DNA is amplifiable by PCR reaction. Previously all the samples were diluted to 25 ng/μl. All the analyzed samples were positive with lectin primers, pointing out that DNA samples can be used in amplification reactions. The positive and negative controls were as expected, and the length of the resulted amplicon was 118 bp, same size for both analyzed samples and certified reference material.

Further on, the DNA samples were amplified with *nos*-specific primers. The analyzed samples had similar results for all fifth experimental series. The size of the resulted amplicon and the positive and negative controls were, as expected, identical with the certified reference material.

The gel analysis pointed out that all of the nine types of food products (textured, pate, milk, cheese, yogurt, mayonnaise, and desert drink) were negative. The chicken forage was positive, as a result of MG soybean, but it was properly labeled. Positive results were determined for flour samples 100 % MG and 1 % MG prepared in our laboratory; the differences between band intensities for 100 and 1 % GM flour were visible for all the fifth experimental series [21, 22]. We can conclude that the results were similar for all the fifth experimental series in terms of repeatability conditions, with DNA extracted from different matrices: agricultural products, food, and forages.

When the detection method was implemented and validated in our laboratory, different samples containing soybean were analyzed. We analyzed samples of food products derived from soybean traded on Romanian market. Thus 50 unlabeled food products were analyzed

such as baby food and dietary products, soy beverages and desserts, tofu, yogurt, and substitutes for meat based on soy protein (grains, textured, and schnitzel). None of the analyzed samples was positive for the GM transgene, pointing out the correct labeling of the food products based on soybean.

Besides, some products like salami and sausages were tested for the soybean and GM content. It turned out that all the processed meat products contained soybean, because their DNA was amplified with the lectin primers. For three samples the results were positive also for the GM gene (nos gene). These results opened other questions—were the transgenic fragments emphasized in highly processed meat products originated from added soybean, or were they already present in the meat? As it was previously described, for GM detection a very short DNA fragment (118 bp) is identified; therefore, its transfer from forage to animal tissue is possible [23]. This assumption is supported by the literature data, which mentioned that transgenic DNA can be detected in different animal tissues, probably transported by body fluids in the same way as other nutrients as exogenous DNA which does not interfere with any metabolic pathway [24, 25].

However the traceability of the transgene sequences from forage to the final processed product was not studied before. In our studies we used as biological materials samples of the liver, muscle, and stomach collected from five pigs, normally fed with GM soybean in a local farm. For comparison a forage sample used for animal fed was analyzed.

The DNA was extracted from both fresh animal tissues and processed ones based on CTAB method [19]. Samples from each organ/animal were chopped, boiled under high pressure (121 °C, for 30 min), dried, ground, and homogenized. Therefore the steps usually used in food industry were simulated.

First, the DNA extracted from fresh material was subjected to absorbance evaluation to determine the concentration and its quality. Further on, the primers species-specific for pork were used for PCR, to determine if the DNA samples are amplifiable. It turned out that all the analyzed samples were positive, excepting those originated from forage.

To emphasize the presence of plant DNA in the analyzed samples, the chloroplast ribulose-bisphosphate carboxylase-1.5 (RuBisCo) gene was amplified [26]. All the analyzed samples were positive, with pointing out the transfer of the plant DNA to the animal tissue.

For soybean detection the primers designed for the lectin gene were used, and for transgene fragment, the nos sequence was amplified. Considering that the chloroplast genes are present in many copies in each cell in contrast to lectin gene which is a single-copy gene, the PCR reactions needed to be suitable adapted.

In these conditions the specific 118 bp fragment for lectin gene was identified in all the analyzed samples, with differences related to the organ of origin. Therefore the highest number of residual lectin sequences was detected in the liver tissue, followed by the muscle tissue and slightly observable in the stomach tissue. The samples originated from the same organs but from different animals had homogenous intensities, indicating a specific mechanism of transfer.

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Finding that fragments from the soybean genome could be identified in tissues collected from the animals fed with this forage, the next goal was to determine if the transgene sequence could be detected by a regular PCR screening. Our results showed that the presence of the nos target gene was not similar in the same organ originated from different animals suggesting that its transfer can be possible but randomly. It was appreciated that the lower content of GM sequences may be caused by the complex composition of forage which can contain a mixture of conventional and GM soybean. Even if the transgene sequences are in lower concentration than those originated from conventional ones, they are still detectable.

The next objective was to determine whether the plant DNA sequences could be also detected in the processed materials. The PCR results obtained through lectin and nos primer amplifications emphasized that the DNA sequences present in fresh tissues were not totally degraded during the chopping, heating, and grinding. We can conclude that DNA sequences transferred from feed to animal are still detectable, even after mechanical and thermal treatments. Considering that according to EU legislation the food products containing more than 0.9 % GMO have to be labeled but the meat of animals fed with GM forage does not require labeling, it is of great importance to follow the transgene traceability along the food chain. If the transgenes are detected in a food product, they can be originated either from meat or from added soybean. Knowing the origin of transgenic sequences absolves the manufacturer of mislabeling.

4. Detection of *Fusarium* infection in agricultural, forage, and food products based on PCR

A complex widespread disease of the small grains (wheat, maize, or barley) is *Fusarium* head blight (FHB) produced by the *Fusarium* sp. infection. Besides the considerable loss of yield, it has the ability to produce mycotoxins which are harmful to human and animal consumer. For this reason it is of great importance to identify *Fusarium* infections before the toxins are synthesized and in the same time to determine its toxicogenic potential [27].The first aim of our research was to evaluate the specificity of the primers mentioned in the literature for the detection of the *Fusarium* strains present in our country. For this, 60 *Fusarium* strains were isolated from wheat samples, collected from different Romanian locations. As a result of the microbiological evaluation and microscopic analysis of the colonies, it was pointed out that *Fusarium graminearum* had the highest incidence (70.68 %), followed by *Gibberella fujikuroi* complex (13.79 %). Within *G. fujikuroi* complex, some small differences were observed, indicating the affiliation to *Fusarium proliferatum* and *Fusarium verticillioides*, but an accurate determination at this level was impossible. Consequently all the isolates were generally classified as belonging to *G. fujikuroi* complex (complex species *G. fujikuroi*, GFSC) [28]. Besides, for three other strains, the morphological identification was impossible because of the spores' absence; therefore, they were collectively classified as *Fusarium* sp. (5.17 %). Three other strains were classified as *Fusarium equiseti* considered to be a secondary invader. It was also identified one strain of *Fusarium avenaceum* (1.72 %), one *Fusarium solani* (1.72 %), and another one *Fusarium cerealis* (1.72 %) which is considered to be pathogenic for humans. Considering that

the simple observation of the phenotypical symptoms produced by *Fusarium* sp. and the microbiological evaluations are time consuming and uncertain, with low precision the new methods based on DNA sequence for pathogen monitoring were applied, using the primers mentioned in the literature [29] (**Table 1**).

The DNA was extracted from the collected seeds, based on CTAB method (SR EN ISO 21571). It was considered that this method allows the isolation of DNA from both wheat and infected microorganisms. For all of the strains considered to be *F. graminearum*, the specific primers were used for amplification. The preparation and identification of the samples used as reference materials were previously described. From these, positive results were obtained for 39 strains, whereas three samples were not amplified. The absence of amplification indicated either the lack of primer specificity due to DNA mutations or the fact that the isolates are not belonging to *F. graminearum* species. The amplification based on *F. proliferatum* and *F. verticillioides* primers of the corresponding strains was positive, pointing out the species identity. To overcome these uncertainties, the PCR followed by DNA sequencing was performed for all of the other strains whose identity was not confirmed by regular amplification.

4.1. PCR followed by DNA sequencing

During the last years, the DNA sequencing techniques had a very important development; therefore, the availability and costs make them accessible for each laboratory. They can be applied in many domains, including the establishment of the identity for the fungi belonging to *Fusarium* genus. In this respect genes as TEF, the gene encoding the elongation factor 1 alpha, were found to be an excellent tool. TEF gene has a special phylogenetic utility because (i) it is very specific for *Fusarium* genus, (ii) non-orthologous copies of the gene were not detected, and (iii) universal primers were designed, appropriated for phylogenetic studies [30]. Besides this gene is always present as a single copy and has a very high degree of polymorphism. Since September 2003, a database containing TEF sequences for hundreds of strains of the genus *Fusarium* was created—Fusarium-ID publicly available database which contain data regarding 78 species and almost 6000 sequences.

The molecular identification based on DNA sequencing involves the following steps: initiation of a pure culture, fungal DNA isolation, PCR amplification for the TEF gene, purification for the amplification product, and fragment DNA sequencing. The primers used for TEF gene amplification were ef1, ef2, and ef22 primers (**Table 1**). These primers amplify a region of the TEF gene delimited by three introns and four exons. In some laboratories the pair of primers ef1 and ef2 is used, generating an amplicon of 700 bp which provides enough information for an accurate species identification. As an alternative the pair of primers ef1 and ef22 could be used, with an amplicon of 450 bp. This is part of the previous fragment, generating also a good identification process.

In our research the pair of primers ef1/ef2 was used for the amplification of 18 isolates with uncertain identity. Previously, fungal pure cultures were obtained starting from single colony, and the DNA was extracted from the mycelium for all the 18 strains with uncertain identity. First, the eight isolates classified as *G. fujikuroi* complex were analyzed. After sequencing, five strains were identified as *F. proliferatum*, with 98–99 % of identity in Fusarium-ID database.

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Besides, one strain from each of the following species was identified: *F. verticillioides* (99.5 % identity Fusarium-ID), *Fusarium subglutinans* (91 % identity NCBI), and *Fusarium andyiaze* (99 % identity Fusarium-ID). The three samples classified as *Fusarium* sp. because of lack of spores were confirmed to be *F. graminearum*, with percent of 99 % identity in both Fusarium-ID and NCBI databases. The same results were emphasized for the isolates characterized as *F. graminearum* by phenotypical and microbiological observations, but were not amplified by the specific primers recommended by the literature for *F. graminearum*. In other situations the morphological data were confirmed by sequencing; therefore, three isolates were confirmed to be *F. equiseti* and the other one as *F. avenaceum*. Our results pointed out that screening with specific primers recommended in the literature had a 93 % percent of success, but the PCR amplification followed by DNA sequencing can provide the most reliable and accurate method for *Fusarium* species identification. Therefore, if we are interested in a general screening of the agricultural products, the regular PCR amplification can be used. Further different samples collected both from fields and Romanian market were analyzed.

Biological material: eight samples of commercial feeds (labeled A, B, C, D, E, F, G, and H) were subjected to screening for pathogenic fungal species. First, eight samples of commercial complex forages were subjected to screening for the most common pathogenic fungal species, namely, *F. graminearum* and *F. proliferatum*. As reference materials the DNA extracted from fungal strains with identity confirmed by sequencing were used. To evaluate the amplifiable quality of DNA samples, the (RuBisCo) gene was amplified, considering that the forage samples had a complex composition based on vegetal products. All the samples had positive results pointing out the lack of inhibitors. Besides, all of them were positive for both *Fusarium* species, underlining their fungal contamination [31]. Next, samples were collected from seven locations, where GM corn (MON 810) was cultivated in 2010. From each location both GM and correspondent conventional corn samples were analyzed. After DNA extraction from all the collected samples, the identity and in the same time the amplifiable quality were evaluated using the zein primer amplifications. To confirm the GM identity, the 35S promoter was used as target gene, with mg1 and mg2 primers. All the results were as expected; thus, the amplifications with primers specific for the most common *Fusarium* species were performed. *F. graminearum* and *Fusarium culmorum* were identified in both conventional and GM corn, with a higher prevalence in the first type. *F. proliferatum* was identified only in conventional corn. The results were predictable considering that GM corn lacks the European borer attack, making more difficult for fungal infection [32].

In other experimental series, ecological products from specialized market, labeled as "bio," were analyzed: wheat, barley, rye, durum wheat pasta, biscuits, and sunflower seeds, in comparison with conventional products as corn flour, wheat flour, and integral cereals. Taking in account that all the samples were originated from plants, the RuBisCo gene was used to evaluate the amplifiable quality of DNA. Then, the fungal amplifications were performed with *F. graminearum* and *F. culmorum* primers. A specific amplification for *F. graminearum* infection was emphasized for bio wheat and barley samples. Besides, many other unspecific bands were visualized for the other bio samples, making the result interpretation more difficult. For the conventional products, the corn and wheat flour were positive for *F. graminearum*, but both

specific and unspecific bands were visualized. The other species, *F. culmorum*, was not identified in any of the analyzed samples [33]. These results showed that both ecological or "bio" products and conventional ones can be infected with fungal strains; therefore, they are not always a solution for healthy food. The obtained results enable us to conclude that the fungal screening based on PCR-specific primers can be applied when agricultural products as grains or forages collected from fields or silages are analyzed. For processed materials the identification is arbitrary and difficult, probably due to the fungal DNA degradation. Besides, when DNA is extracted from vegetal samples that are possibly infected, it is assumed that the fungal DNA is in very low quantities compared to the plant DNA. If more precise results are necessary, the inoculation is recommended, followed by morphological and molecular analysis: PCR with specific primers or PCR followed by sequencing.

5. Detection of food-borne pathogens in forage and food products based on PCR

Assessment of the food quality and safety has a great importance for human health. Some of the most common pathogens that are found in foods and can be easily transmitted from animals to humans are bacterial strains *Salmonella* and *Escherichia coli* [34]. They also produce significant damage in livestock, since infections lead to high disease and death rates. Traditional methods for identifying pathogens in food poisoning, which causes disease in humans, usually include multiple subcultures and biotype or serotype-identification steps. They are not effective in terms of time and cost, being also very laborious [35]. In most of the cases, a rapid identification of the agent that was causing food poisoning is essential for a pertinent intervention in order to overcome the pathogenic effects. Methods based on molecular techniques for identifying bacterial DNA have proved to be efficient for the detection of pathogens in feed and forage product. Considering this frame of work, the aim of our studies is to develop and validate a qualitative PCR-based method for the rapid identification of *Salmonella* and *E. coli* pathogens. The primers that were used are designed for the identification of specific *Salmonella* spp. and pathogenic *E. coli* genes: fimA and afa, respectively, being described to provide highly specific, rapid, sensitive, and reliable results [36].

Bacterial strains of *Salmonella* ssp. and pathogenic *E. coli* O157:H7 were provided on the courtesy of Microbiological Department of our institution, as free of culture media cells, in safe condition, the DNA being extracted immediately without other preparations. The working DNA solution was established at 5 ng/μl for the reference sample. In order to validate a method of detection, the usual steps were followed.

First, the primer specificity was evaluated. The *Salmonella* and *E. coli* primers were challenged in simplex PCR with nontarget species and different types of muscular tissue of fish, ruminant, pork, and poultry; maize grains, two samples of commercial forage and *Fusarium* ssp. along with the negative controls in order to detect possible cross-reactions; and unspecific amplifications products. As positive control for primer specificity, DNA extracted from bacterial cells was analyzed. In no case a cross-reaction neither unspecific amplicons was observed. For the

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positive controls, the primers generated specific fragments of appreciatively 750 bp for *E. coli* strain and 120 bp for *Salmonella* strains. To determine the assay sensitivity for specific DNA quantity, DNA extracted from bacterial cells was prepared in five serial DNA dilutions: 5, 2.5, 1.5, 1, and 0.5 ng/μl, respectively. The dilutions were used as DNA template for PCR amplification with pathogen-specific primers. The assay sensitivity was evaluated according to PCR product intensity, as it can be visualized in agarose gel. The intensity of amplicons was found to decrease gradually from 2.5 ng/μl concentration continuing till the 0.5 ng/μl limit, where the amplicons are still perceptible. For *E. coli* targets, this concentration can be considered a lower threshold for the PCR assay sensitivity, but for *Salmonella* targets, it could be established even at a low concentration. To establish the limit of detection (LOD) expressed in percent, the bacterial genomic DNA was progressively diluted in vegetal genomic DNA suspension with the same concentration. Starting from 50 % bacterial DNA template, five serial DNA dilutions were obtained: 25, 10, 5, 2.5, and 1 %, respectively. For these samples the amplifications with specific primers were performed along with the reference sample. No differences were noticed among the first three dilutions in both cases. In this assay it was found that for 1 % concentration of pathogen, the amplicon can be easily detected suggesting that the method can be very sensitive and accurate.

Applicability on food and feed matrices was representing the last step of in-house validation process. In this experiment we submitted two commercial feed samples, two meat food products, two food products containing eggs, and two samples of cheese. Following the PCR detection, we had no positive results except the reference samples. Therefore, it will be necessary for the correct validation of the method to develop an experiment with the legal control agencies to provide contaminated food samples. The PCR method proposed in this study can be used as an initial screening in detecting food pathogens. In several hours accurate results can be at the disposal of control organisms, thus facilitating the further analysis, and even will indicate the test that should be applied.

6. Detection of different animal species in meat and dairy products based on PCR

Nowadays, the incidence of food fraud has a wide prevalence all over the world, but sometimes its identification is difficult. To ensure compliance with regulations and to implement punitive measures, when necessary, robust analytical tests are needed. Food adulteration industry could be systematized into four main subsections: (A) the origin of animal products and feeding regime of animals from which they originated (such as certified regional products); (B) replacement of the ingredients in the recipe with other animal species, tissue, fat, or protein of other origin; (C) changes in food processing methods thus altering the original recipe; and (D) additions of nonself components, such as water or additives and flavor enhancers [37]. Therefore, molecular analytical methods have been developed based on the analysis of lipids [38], protein [39], and, most recently, nucleic acids [40]. However, methods that rely on lipids and proteins are limited because protein biomarkers are easily denatured and the amount of protein can be significantly altered by food processing so attention turns to techniques based

on the analysis of nucleic acid. They have superior stability and universal traceability in all the organism cells; thus, DNA-based techniques are reliable, robust, accurate, and fast. Simultaneous detection of several animal species has been extensively developed by PCR techniques, the research being adapted to the producers' requirements to authenticate products [41]. For this type of analysis, both nuclear DNA and mitochondrial DNA were used, but the systems that promise to work in the case of all living animal species—the mitochondrial D-loop region, cytochrome b, cytochrome oxidase subunit I (COI), 16s rRNA, 12S rRNA, and NADH dehydrogenase subunit 5/6 genes—are used intensely nowadays for DNA barcoding. According to the literature data, a lot of DNA markers were developed to evaluate the food/feed compositions. But each experiment has specific particularities; therefore, there are important issues that have to be taken in consideration. First, the matrix of the selected product has to be considered in order to adjust the DNA extraction protocol as described above. The amount of starting material/product will be established in accordance with the degree of processing and the matrix nature and also the animal species that can be identified. The next step is the selection of the biomarkers which will be evaluated. A biomarker needs to meet some requirements to be suitable for detection of animal species in a product, as follow: to be highly specific for the species of interest and to be abundant in its genome. In this respect the most used are the mitochondrial genes 16s rRNA and 12s rRNA that has been proved to be successfully applied for some animal species [42]. There are many primer pairs developed on the basis of these biomarkers, which can be acquired and validated in any laboratory, but usually the best way is to design own primer pairs according to the experiment requirements. For this purpose, the most used DNA sequence is cytochrome C oxidase I (COI), which is specific for species from insects, fish, shellfish, birds, to farm animals and primates. Research focused on very different animal species has shown that sequence variation in the 5' region of COI allows the identification of 98 % of them [43]. The design of animal species-specific primers involves the amplification of the 700 bp fragment of the COI gene biomarker and selection of the sequences which are most suitable to be used for an amplification reaction. The specific primer pairs can be easily designed using dedicated softwares, in order to fulfill all the requirements of the experiment. That softwares can be acquired and used off- and online; however, it is necessarily to confirm the specificity of the newly designed primers by aligning them with sequences from the suitable data basis. In our experiments reliable results have been obtained using NCBI database. The most important issue for primers designed to be used in multiplex PCR reaction is their expected amplicon size. The differences between the amplicon lengths generated with different pairs of primers, each of them specific for an animal species, have to be easily discriminated in electrophoresis gel. The fragments overlapping seriously affect the result of the analysis. Besides, a special attention must be directed into selection and preparing the reference materials, as already described. In our laboratory we have experienced the validation and application for analysis of several methods used for identification of food and feed component species. Our first experiments were focused on the development and implementation of an endpoint simplex PCR for detection of fish or fish products in feedstuffs. This is of great interest for farmers because of the European legislation [44]. The factors followed up for in-house validation of the detection method were specificity, sensitivity, and limit of detection, using an appropriate reference material, prepared in laboratory from fresh muscular

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tissue of common regional fish species as previous described. The detection of possible cross-reactions and unspecific amplification products was followed to evaluate the primer specificity. For this aim the fish primers were used in simplex amplification reactions with different types of templates, namely, DNAs extracted from samples: (a) containing target sequence, muscular tissue of fish and forage containing 2 % fish material, and (b) nontarget sequence—muscular tissue of ruminant, pork and poultry, and soybean and maize. Besides, all the already mentioned positive and negative controls were analyzed. It was demonstrated that only the samples containing fish material were positives, with amplified fragments of 230 bp length as expected. No amplification was visible neither for the other samples nor for the negative controls, pointing out the reaction specificity. To evaluate the assay sensitivity, templates with different content of fish DNA were analyzed. First, the sample of DNA extracted from fish raw muscular tissue was precisely quantified by spectrophotometric method. Then, seven serial DNA dilutions—100, 50, 10, 1, 0.1, 0.01, and 0.001 ng/μl—were prepared and used as templates in amplification reactions. The assay sensitivity was evaluated according to the PCR product intensity as it can be visualized in agarose gel. The highest concentrations of 100 and 50 ng/μl generated very strong bands, but the intensity of amplicons decreased gradually at lower concentrations, starting from 10 ng/μl and continuing to 0.001 ng/μl. At the last concentration, the band was still perceptible; therefore, it was considered that the lower threshold for the PCR assay sensitivity in our laboratory was 0.001 ng/μl. The next goal for method validation was to establish the limit of detection (LOD) expressed in percent of fish material in a mixture. Thus, pure fish genomic DNA with known concentration was progressively diluted in vegetal genomic DNA suspension, resulting five serial DNA dilutions: 1, 0.5, 0.05, 0.005, and 0.001 %, respectively. All of them were used as DNA template for PCR amplification. As positive control, the 100 % fish genomic DNA, considered as reference material, was used. It was shown that it is nearly impossible to discriminate among concentrations of fish material above 0.5 %, but the intensity decreases dramatically in the case of 0.001 % fish DNA template. Considering that for the last concentration the PCR products were still detectable, the detection threshold in our laboratory was established to 0.001 % fish material in a mixture. Further on, the method repeatability was evaluated to complete the validation process. Therefore the already described steps were repeated three times, one time/week with similar results. Applicability on food and feed matrices represents the last step of the process. In our research we analyzed four commercial feed samples, three of them being labeled by producers as non-contaminated with fish material. The PCR detection of fish material was performed for all of them. Positive results were obtained for two of the labeled samples as non-contaminated. Evaluating the intensity of used positive control (0.05 % fish material), it was emphasized that the fish material contamination was below the mentioned percent, probably being due to the manufacturer's faulty procedures, and this might not exceed the limits of regulation. It was demonstrated that the PCR method proposed in this study can be considered as a further improvement of conventional assays. The test could be useful in the control of different food and feed products, to verify the origin of the raw materials, especially in products submitted to denaturing technologies, for which other methods cannot be applied. The next experiments were focused on development and implementation of Multiplex PCR for simultaneous detection of different animal species. We considered the animal groups that are commonly used in European

countries as ingredients of meat-derived and dairy products. The validation of two endpoint multiplex PCR methods for detecting the main used animal species in meat and dairy products was performed following the same pattern. Three sets of primer designed for different regions of mitochondrial DNA—12S rRNA, tRNA Val, and 16S rRNA—of cattle, swine, poultry, and fish [42] were used to evaluate the meat product adulteration. When adulteration of dairy products was studied, the detection of cow, goat, and sheep milk was aimed, using the specific primers [45]. For in-house validation, the same factors were followed up—specificity, sensitivity, and limit of detection—using appropriate reference materials, prepared in laboratory from fresh muscular tissue collected from cattle, swine, poultry, and fish and from cow, goat, and sheep milk, as previously described. From each material the DNA was extracted based on the described methods. To evaluate primer specificity, two different mixtures of DNA were prepared: one from cattle, swine, poultry, and fish DNA, named bulk A, and the other from cow, goat, and sheep, named bulk B. Bulk A was template for different amplification reactions with each pair of primers separately. Besides, the same bulk DNA was amplified with the mixture of the four pairs of primers. The primers in the simplex had the same concentration as in multiplex reaction. A single, visible fragment was shown for each simplex reaction, with the expected length, similar with the reference materials, pointing out the primer specificity. The multiplex amplification generated four bands, visible, perfectly separated with the length specific for each animal species targeted. To get these results, the electrophoretic analysis must be conducted in optimal condition to permit the correct separation of the multiplex PCR products; otherwise amplicons overlapping would seriously affect the analysis result. The same results were obtained when dairy products (bulk B) were analyzed with specific pair of primers, emphasizing the primer specificity. The next step was determination of the assay sensitivity and establishing the limit of detection (LOD); the assay sensitivity and the limit of detection were evaluated according to PCR product intensity as it can be visualized in agarose gel. The bulk DNA sample composed as a mix of the animal species DNA (equal amounts of 200 ng/μl DNA solution of each species) was diluted in maize DNA to obtain templates with known percent concentrations of animal material. Starting from the considered 100 % DNA bulk, seven serial DNA dilutions were performed—10, 1, 0.1, 0.01, 0.005, 0.002, and 0.001 %, respectively—all of them were used as DNA template for multiplex PCR amplification, with the four pairs of primers simultaneously. Discrimination between samples with DNA concentrations higher than 0.1 % was not possible, but the band intensity decreases gradually at lower concentrations, being faint but detectable in the case of 0.001 % concentration. Considering the results it was concluded that a concentration of 0.002 % represents the lowest concentration that gives reliable results. Therefore 0.002 % was considered the threshold for the sensitivity and the limit of detection (LOD) for the described multiplex PCR assays. Further on, different processed meat and dairy product matrices were analyzed, to evidence the multiplex method applicability. First, the DNA extracted from commercial product matrices were submitted to specific simplex PCR assay using the different primer systems in separate PCR amplifications. The obtained results were compared with those obtained in similar multiplex PCR amplification. A total concordance between the results can be considered successful completion of the multiplex PCR in-house validation process. For detection of animal species in dairy products, a number of 20 milk-derived products were analyzed. Pure goat and sheep milk cheeses and

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yogurt but in most cases products obtained from the mixtures of the three types of milk were purchased from the local market according to the labeled composition. The food and feed samples analyzed for detection of different types of meat could be classified in three main categories: forage samples—commercial products developed for farm animals, pet food either dried or canned, and a large variety of meat-derived products such as hamburgers, sausages, salami, and other products that are obtained from animal tissue flour, all of them being purchased from the local market stores. Using the described methods allowed, it was possible to detect all the specific ingredients from each product and to identify incorrect labeling of several processed meat products and fraudulent addition of cow milk in dairy products that were labeled as pure goat or sheep milk-containing dairy products.

New experiments were focused on horse meat detection. In the context of the big issue of fraudulent addition of horse material in alimentary products that were labeled as containing beef meat, we were endorsed to develop a time- and cost-efficient PCR-based method for horse DNA detection, following the same pattern for method validation. The main goal of this experiment was to design a pair of primers for horse DNA detection which can be successfully used in duplex PCR experiments along with cattle-specific primers that were already validated in our laboratory. For this purpose, we designed specific primers that amplify a 280 bp fragment from cytochrome b mitochondrial gene (GenBank code *JQ340166.1*). Considering that our aim was to use both pairs of primers, horse and cattle in the same amplification reaction, a high difference between the generated DNA fragments was necessary (290 bp for horse and 104 for cattle). Genomic DNA extracted from horse (*Equus caballus*) fresh muscle tissue was used as reference material for in-house validation process. First step was to confirm the species identity; therefore, the fragment generated by the newly designed primers was sequenced and compared with the specialized databases. Thus, the specificity for horse species was confirmed, allowing the primers used in future investigations. The next step was to evaluate primer specificity when different templates are amplified. For these aim different ten types of DNA extracted from raw materials—horse, bovine, sheep, goat, swine, chicken, alfalfa, soybean, and *F. graminearum*—and a DNA mixture of 5 % horse and 95 % beef were amplified with the horse-specific primers. The results were as expected, only the last samples being positive. The assay sensitivity was achieved by amplification of serial dilution from the same horse DNA matrix: 100, 50, 10, 1, 0.1, 0.05, 0.01, 0.005, and 0.001 ng/μl. Slightly visible positive results were recorded even in the case of the lowest concentration; however, the detection in this case may become hazardous; therefore, the threshold for the sensitivity was set at 0.005 ng/μl. In order to determine the detection limit, horse DNA was diluted in vegetal (alfalfa) DNA suspension reaching to the following concentrations: 100, 20, 5, 1, 0.5, 0.1, 0.05, 0.005, and 0.001 %. The band intensity decreases gradually at lower concentrations, being detectable in the case of 0.005 % concentration. This concentration was considered as detection limit for the horse DNA detection. In this experiment a limit of detection for mixtures of heat treated and minced horse and beef meat in different percentages was also established. The mixtures were prepared with a content of 50, 20, 5, 1, 0.5, 0.1, 0.05, and 0.005 % horse and beef meat mixture. In this situation a limit of detection at 0.1 % of horse material was established.

Our experiments focused on identification of the animal species in different matrices enabled us to develop and validate a series of methods as follow: detection of fish meal in complex forages, a multiplex PCR method for identifying the animal species (pork, ruminants, poultry, and fish) in food products, detection by multiplex PCR of cow milk in products made from other species (sheep, goat) milk, and detection of horse meat in food products. The in-house validation process was very laborious but still not very difficult. Even if previously described methods are applied, they have to be adapted to own laboratory conditions (equipments, reagents, manipulators). Particularly, in the case of multiplex PCR, the molar concentrations of the primers have to be adjusted so that their competition must not interfere with the final result.

7. Conclusions

The requirements for food safety are determined by the universal demand of the consumer that all the food products are in conformity with the demands concerning health, diet, lifestyle, culture, and religion. Therefore concerns in this area have exceeded the boundaries of safety for the health of the consumer, getting to heed religious prohibitions, food-borne allergies, and food fraud of animal-derived products. PCR-based applications have seen a significant development in recent decades, many techniques being developed for detection of any potential risk that could arise from food intake. These methods are powerful tools especially when they have immediate practical applications being developed in concordance with current society demands. This chapter is describing the in-house validation processes of few PCR-based methods that were successfully applied in our laboratory and that were chosen according to the present requirements of consumers and food safety authorities.

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