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PCR METHODS IN MEAT SPECIES IDENTIFICATION AS A TOOL FOR THE VERIFICATION OF REGIONAL AND TRADITIONAL MEAT PRODUCTS

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Abstract. In the times of industrial food production, regional and traditional food articles provide an attractive alternative for people looking for unforgettable sensory impressions. Regional or traditional food, commonly recognized as palatable and healthy, is also, for many consumers, a unique, sentimental journey back to tastes from childhood times. A gradual increase of demand for this type of food articles as well as relatively high prices of these products may generate among unscrupulous food manufacturers a number of improper production practices, e.g. replacement of a more expensive meat by a less expensive alternative. Species composition of meat products can be verified using chromatographic, immunological, electrophoretic, or genetic methods.
One of the genetic methods applied in examining the authenticity of food composition, including meat and its products, is the polymerase chain reaction (PCR). This paper presents the most important techniques utilizing this technology to identify the origin of specific meat components constituting part of regional or traditional food articles. It was demonstrated that PCR techniques, in combination with species-specific primers, PCR--RFLP, PCR-SSCP and real-time PCR, allow identification of meat species occurring independently or in mixtures with other meat species as well as meat subjected to thermal treatment or other technological processes in the course of industrial production. The only exception is the PCR-RAPD method that fails to identify meat species in the case of strong DNA degradation or in complex meat mixtures.

Key words: PCR methods, species identification, regional and traditional meat products

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INTRODUCTION

At the present time, steadily growing numbers of consumers appear to appreciate the taste and nutritional values of regional and traditional food products understood as food articles whose quality or uniqueness stem from the application of traditional production methods, which constitute an element of identity of a given region. Regional products are characterised by extraordinary organoleptic traits: exceptional taste, smell and appearance. Curiosity as well as emotions that this kind of food arises cause that regional and traditional products find their buyers despite higher prices in comparison with prices of food manufactured industrially [Gąsiorowski 2006 a].

In 1992 a directive was issued associated with the introduction in the territories of EU member states of legal protection of food products using the following three labels: "Protected Designation of Origin", "Protected Geographical Indications" and "Traditional Speciality Guaranteed" [Council Regulation (EC) no 510/2006, 509/2006 and Commision Regulation (EC) No 1898/2006]. These labels are intended to provide consumers with information about the quality and origin of a given agricultural or food product. If a group of producers secures legal protection for the name of their product, they will automatically be obliged to voluntary control the manufacturing process and to check if it complies with the recipe specification of a given food article [Gąsiorowski 2006 b].

There is a strong tendency in countries that belong to the European Union to emphasise regional affiliations and to cultivate traditions of their ancestors. Regional and traditional food products may be used as assets allowing the promotion of the unique character of rural areas and act as magnets attracting tourists. Both in Poland as well as in many other European states meat products are considered as regional and traditional articles and enjoy considerable popularity. Regional and traditional meat products should be characterised by stable quality and sensory values and this can only be ensured when the same raw material and the same technology are applied [Zin 2005].

The steadily growing demand for regional and traditional products as well as higher prices consumers are ready to pay for them may give rise to temptation to counterfeit this kind of food article [Colombo et al. 2002]. Their falsification, apart from the obvious dishonesty in relation to the consumer, may, in some situations, also result in certain health problems in the case of food allergies to proteins. The requirement for species identification is also associated with the meat trade prohibition of certain animal species which are under protection. To protect consumers' interests and not to lose consumer trust, it appears necessary to introduce an effective control of the manufacturing process and to verify species composition of regional and traditional food articles. Fortunately, it is now possible to identify individual food species, using molecular biology techniques. Some of these methods allow unequivocal species identification only in the case of raw meats, while others can do it also when meats have been subjected to various technological treatments. The methods employed in species identification include: chromatographic, immunological, electrophoretic and genetic. This manuscript only considers methods based on DNA analysis.

SPECIFICITY OF GENETIC METHODS

The comparison of methods of species identification based on protein analysis with those employing DNA analysis appears to indicate that the second group offers unquestionable advantages resulting from the characteristics unique for DNA. It is in DNA that all genetic information of a given organism is gathered and it is independent of the type of tissue it resides in. Therefore, it does not matter whether the sample taken for analyses is collected from muscles, blood or liver. DNA provides far more information than protein and this results from the character of the genetic code, i.e. degeneration of the genetic code [Lockley and Bardsley 2000]. In comparison with protein, DNA is more stable and resistant to such factors as high temperature, pressure, and chemical compounds. The above mentioned properties of nucleic acids allow species identification not only of raw meat products, but also those subjected to thermal treatment or supplemented with various chemical compounds. Moreover, genetic methods make it possible to distinguish meat species of closely related animals, such as, for example, chicken and turkey, or meat that occurs individually or in a complex mixture and, last but not least, they can also identify tissues that belong to genetically modified organisms.

It appears quite probable that methods of species identification based on the analysis of nucleic acids will be employed more and more frequently to control the composition of ready-to-use food products. The wide spectrum of potentials offered by genetic methods, namely: possibilities of meat species identification occurring in mixtures and of meats subjected to technological treatments as well as the rapidity and ease of carrying out analyses causes that genetic methods have unquestionable advantages over the other ones.

Many techniques based on DNA analysis were adopted for the needs of the food industry and allow carrying out investigations aiming at species identification, but the polymerase chain reaction (PCR) method deserves special attention because it is characterised by high sensitivity and specificity as well as relatively short period of time necessary to perform the analysis. From among the methods based on the PCR technique, the most frequent ones employed to check food falsifications include: PCR with species-specific primers, as well as PCR-RFLP (Restriction Fragments Length Polymorphism), PCR-RAPD (Randomly Amplified Polymorphic DNA), PCR-SSCP (Single Strand Conformation Polymorphism), and real-time PCR as a method which allows quantitative contamination assessment.

POLYMERASE CHAIN REACTION (PCR)

Polymerase chain reaction allows amplification of a definite DNA fragment or RNA (RNA is first transcribed onto cDNA in a reverse transcription reaction) in *in vitro* conditions. The amplification of a selected DNA sequence is possible to the appropriate selection of single-stranded DNA molecules. Primers are obtained by means of chemical synthesis and, usually, are made up of about 20 nucleotides. Each cycle of synthesis of a DNA fragment consists of three stages performed at different temperatures: 1) denaturation, temperature over 90°C; 2) primer addition to the matrix, temperature 40-60°C; 3) elongation, temperature about 70°C. Most frequently, each PCR reaction comprises from 20 to 40 cycles of DNA synthesis and in each consecutive cycle,

the quantity of the definite matrix fragment is doubled and becomes a matrix for the synthesis of a new strand [Słomski et al. 2004].

POLYMERASE CHAIN REACTION (PCR) WITH THE APPLICATION OF SPECIES-SPECIFIC PRIMERS

At present, the PCR technique utilizing species-specific primers is one of genetic methods allowing novel identification of different meat species, both raw meats as well as meats subjected to thermal treatments. In this method, the necessary requirement is to know the nucleotide sequence of the gene on the basis of which the species identification will be performed, in other words primers will be designed. PCR with species-specific primers requires the application of appropriate control samples which will rule out possibilities of obtaining false positive or negative results.

Dishonest manufacturers of meat products frequently replace more expensive meat with cheaper one. This type of practice takes place, among others, in the case of an Italian traditional article namely 'Mortara' salami in which goose meat is replaced by the meat of other poultry, e.g. turkey or duck [Colombo et al. 2002]. This kind of salami is manufactured, according to the old tradition, in two versions: the first one "ecumenico", i.e. salami manufactured exclusively from goose meat and salami prepared from swine and goose meats mixed at the proportion of 2:1. In experiments conducted by Colombo and co-workers [2002] species-specific primers were designed on the basis of the cytochrome *b* mitochondrial DNA which allowed the identification of goose (*Anser anser*) meat in this product.

Cytochrome *b* gene is located on the mitochondrial genome which is used very often in studies concerning meat species identification and therefore sequence data of many verebrate and nonvertebrate species are available. Mitochondrial DNA owns several advantages over nuclear DNA. Mitochondrial DNA is presented in thousands of copies per cell and possesses many point of mutations allowing the discrimination of even closely – related species. Mitochondrial DNA is maternal inheritance and therefore is free of heterocygosity [Lockley and Bardsley 2000, Unseld et al. 1995].

Within the framework of the performed investigations, DNA was isolated from two kinds of salami, i.e. with and without the addition of pork meat and, in addition, from pork meat and duck meat. Following the analysis using PCR and then electrophoresis, one characteristic band exclusively for geese was obtained with no bands for the remaining species. The results obtained in these studies appear to indicate that the developed primers can find application for goose meat identification.

Increasing interest in food articles containing low fat levels make consumers look for meats derived from game animals, such as: red deer (*Cervus elaphus*), fallow deer (*Dama dama*) and roe-deer (*Capreolus capreolus*). To protect consumers' interests against improper or dishonest labelling of these very attractive culinary meat species, researchers developed on the basis of gene 12S rRNA of the mitochondrial DNA primers which allowed unequivocal identification of meat of the above-mentioned species [Fajardo et al. 2007]. Identical reverse primers and species-specific forward primers were applied to meat from red deer, fallow deer and roe-deer. Selective amplification of the three pairs of primers was confirmed by the performed PCR reaction not only with the DNA of the three above-mentioned species of game animals but also with that de-

rived from: chamois, mouflon, pyrenean ibex, cattle, sheep, goat, swine, horse, rabbit, duck, turkey, chicken and geese. The performed experiments yielded amplicons of desirable size for deer – 175 bp, fallow deer – 169 bp and for roe-deer – 175 bp at a simultaneous absence of products for the remaining analysed species. In order to verify practical usefulness of the developed method for the identification of the above-mentioned species of game animals, meat products prepared experimentally on their basis were also analysed, i.e. products subjected to pasteurisation (72°C for 30 min) and sterilisation (121°C for 20 min) as well as articles manufactured on industrial scale (dry cured and cooked products). Satisfactory results were obtained also in these cases confirming the possibility of DNA amplification which underwent strong damage (only relatively short DNA segments, i.e. 175 bp were amplified). The obtained results confirmed species specificity of the designed primers indicating that they will find application in routine investigations [Fajardo et al. 2007].

PCR makes it possible to distinguish meat species derived from closely related animals whose nucleotide sequences exhibit considerable degree of homology, e.g. chickens and turkeys. Hird et al. [2003] developed a method that combines PCR with visualisation using vistra green allowing significant shortening of the time of analysis. The two pairs of primers developed on the basis of the cytochrome *b* mitochondrial DNA yielded amplification product of 120 bp for the chicken and 101 bp for the turkey both in the case of raw as cooked and autoclaved meats allowing identification of these species. The detection of amplicons of the above-mentioned animal species was carried out also by measurements of fluorescence emission which was possible because the vistra green dye reacted with PCR products.

One of the critical points in meat species identification not only in the case of methods based on protein analyses, but also DNA studies is thermal denaturation which takes place during heating in the case of manufacture of certain food articles. Hopwood et al. [1999] reported the identification of chicken meat heated to the temperature of 120°C for 30 min, whereas Matsunaga et al. [1999] present a rapid and simple method of qualitative identification of six meat species (meats of cattle, swine, chicken, sheep, goat and horse), both raw and heated to the temperature of 100°C and 120°C for 30 min. The proposed method involves mixing in appropriate proportions of seven primers, i.e. one forward primer designed for the conservative sequence of the cytochrome *b* mitochondrial DNA and six reverse primers specific for each of the analysed species which makes it possible to identify six meat species simultaneously. Only horse meat, in the case of heating to the temperature of 120°C for 30 min, failed to be identified using the method presented in this study. The possible explanation of this situation can be the fact that in the case of DNA derived from horse meat, the determined amplification product was longer (439 bp) than for the remaining examined species. Primers for horse meat should be designed in such a way as to amplify shorter DNA fragments.

Arslan et al. [2006] reported considerable resistance to high temperature of DNA derived from beef meat during their experiments in which they tested the impact of different methods of meat thermal treatment, namely: cooking (at 97.5°C for 140 min, 200 min, 230 min), roasting (at 200°C for 80 min, 120 min, 150 min), autoclaving (at 120°C for 30 min, 60 min, 90 min) and frying (temperature of fat 173°C for 45 min, 80 min) on the PCR technique accuracy. Melted fat and beef extract were collected for species analyses. The primers developed on the basis of the mitochondrial DNA amplified very well the 271 bp long fragment from all samples taken for analyses with the exception of meat fried for 80 minutes.

Schwägele et al. [2007] based on the mitochondrial *cytochrome b* gene a series of species specific primer systems adapted and developed that allow a strictly specific detection of chicken, turkey, duck, goose, pheasant, quail and guinea fowl in meat and processed meat products. Detection of PCR-products was accomplished using gel electrophoretic separation on polyacrylamide gels with subsequent visualization with ethidium bromide. To verify the specificity the primer systems were tested against domestic poultry species as well as against beef, bison, sheep, goat, horse, kangaroo, and ostrich. There was no cross-similarity detectable regarding the analyzed domestic poultry species. According to the authors, the system was created a tool to deal with possible adulteration using authentication with respect to food labeling as well as compliance with ingredient composition covering the range of all common domestic poultry species in the European market.

Taking on assumption that the gene is in all species but the sequence is specific to each species, PCR was also used to distinguish between species on the basis of the sequence coding the PEVK region of titin [Spychaj et al. – in press]. The PEVK region of titin is coding by nuclear DNA and attracts the most attention while this protein is very large and has many introns of different size in nuclear DNA, which can be used for species identification similar to the studies exploited mitochondrial DNA. The differences in electrophoretic pattern of titin between rats and rabbits were indicated by Warren et al. [2003]. Studies of Tanabe et al. [1997] revealed the differences in peptide mapping in proteins derived from chicken, pig and cattle muscle, and also the differences in aminoacid sequence of Ig and FN3 domain, among the same animal species. Greaser et al. [2005] observed age-dependent titin polymorphism in N2BA domain of the PEVK region. Using primers designed on the basis of the sequence coding the titin PEVK region for chicken DNA [Spychaj et al. – in press], it was possible to detect even 1% chicken meat in the mixture consisting of chicken and beef and chicken and pork. In case of beef primers on DNA isolated from the mixture consisted of bovine and porcine meat, it was possible to detect as little as 1% beef in meat mixtures. Beef primers were also tested on DNA, which was isolated from meat mixture consisted of chicken and bovine meat. In this case the detection of as little as 5% beef in the meat mixtures and the amplified product matched the predicted size and sequence.

PCR-RADP (RANDOMLY AMPLIFIED POLYMORPHIC DNA)

The PCR-RADP method is a variant of the PCR technique in which a single arbitrary primer is employed that means the same primer initiates the elongation reaction of both strands of the amplified fragment. Arbitrary primers generate species-specific 'fingerprints' whose visualisation occurs after performing electrophoresis. This solution eliminates the need to employ sequencing, hybridisation or the use of restriction enzymes during the consecutive stages. The main advantage of the PCR-RADP method is the fact that it is relatively cheap and simple to perform and, what is even more important, it makes it possible to reveal genetic variability without prior knowledge of the sequence of the examined DNA. Unfortunately, this method has also its shortcomings including: inability of species identification without simultaneous possession of a known standard and uselessness of the method when investigating the composition of meat mixtures or severely (autoclaved) heat treated meat. Additionally, as pointed

out by some researchers [Wolko et al. 2004], the method is characterised by poor repeatability of the obtained results that is associated with its sensitivity to changes in amplification conditions.

The PCR-RAPD method allowed, among others, simultaneous meat identification of five animal species (pork, beef, lamb, chicken and turkey) in experiments carried out by Saez et al. [2004] where DNA derived from raw meats, from meat products such as: hamburgers, raw sausages, dry fermented sausage, and cooked meat products. The obtained DNA profile showed a repeatable number of bands separated from one another for the analysed species allowing their identification.

PCR-RADP was also used to identify raw meats of: a wild boar, a pig, a horse, a bison, a cow, a dog, a cat, a rabbit and a kangaroo. In the investigations, a commercially available set of primers was used to obtain characteristic electrophoretic patterns allowing the identification of the different meat species [Koh et al. 1998].

PCR-RADP was also employed to identify fish species as exemplified by the application of arbitrary primers to distinguish fish meats from the Salmonidae family, namely the following three species: Manchurian trout (*Brachymystax lenok*), rainbow trout (*Oncorhynchus mykiss*) and amago salmon (*Oncorhynchus masou ishikawai*) [Jin et al. 2006].

PCR-RFLP (RESTRICTION FRAGMENT LENGTH POLYMORPHISM)

The PCR-RFLP technique involves amplification of a DNA fragment followed by its digestion with an appropriately selected restriction enzyme. The application of the PCR-RFLP technique allows the identification of various meat species which derive from mammals, poultry or fish. The disadvantage of the PCR-RFLP technique is the possibility of occurrence of incomplete digestion of the restriction site or occurrence of intraspecific differences which may result in development or removal of restriction sites [Gil 2007].

Girish et al. [2007] conducted an experiment with the aim to identify various types of poultry on the basis of the 12S rRNA gene occurring in the mitochondrial DNA. The researchers examined meats of five poultry species: chicken (*Gallus gallus*), duck (*Anas platyrhynchos*), turkey (*Meleagris gallopavo*), guinea fowl (*Numida meleagris*) and quail (*Coturnix japonica*). The meats of all five poultry species were analysed in raw form and in the case of the chicken – also in processed form, namely as: 1) pâté (about 70 g) – heated for 15 min at the temperature of 120°C followed by another 10 min with the temperature of 72°C inside the product; 2) meat block (about 600 g) – steam-cooked for 30 min at the temperature of 90°C; 3) meat block (about 600 g) – autoclaved for 30 min at the temperature of 120°C; 4) croquette (about 15 g) deep fried in fat at the temperature of 180°C for 5 min. The mitochondrial 12S rRNA gene was amplified employing universal primers and polymerase chain reaction. The 456 bp long PCR product was subjected to digestion with selected restriction enzymes (*Hinf*I, *Mph*1103I, *Mva*I, *Eco*47I). The analysis of the electrophoretic separations obtained using the PCR-RFLP technique identified all the poultry species in raw meats. Chicken meat detection was also possible in heated products, although in the case of autoclaved meat, the obtained band was barely visible.

The PCR-RFLP technique allowed simultaneous recognition of three mammal species, namely: cattle, sheep and swine. The method employed the analysis of the mito-

chondrial gene fragment of the eight synthase ATP subunit (ATP8). The obtained PCR product (176 bp) was then subjected to digestion with restriction enzymes *Mse*I and *Sau3A*I generating electrophoretic bands making it possible to distinguish different species [Natonek-Wiśniewska et al. 2007].

In the experiment carried out by Wolf et al. [1999], the PCR-RFLP method allowed recognition of 25 animal species in samples of frozen meat or freeze-dried protein extract. The molecular target in the described investigations was the specific region of the mitochondrial genome ($tRNA^{Glu}$/cytochrome *b*). The DNA fragment (464 bp) obtained following amplification was treated with 11 different restriction endonucleases. The selection of specific enzymes was based on their relative low price and market availability as well as relative activity in the PCR product and possibility of the best identification of the analysed species. The authors suggest that the application of two restriction enzymes was sufficient to distinguish all the 25 analysed animal species. The PCR-RFLP technique also make it possible to identify meat species in two- (roe--deer and cattle) or three- (roe-deer, blesbok and sable antelope) meat component mixtures using *Hinf*I and *Mse*I enzymes.

Pascoal et al. [2004] reported possibilities of identification of meat mixtures proving that PCR-RFLP allows species identification in commercial meat products earlier subjected to technological processes. The authors suggest that the majority of the performed experiments using the PCR-RFLP technique were based on examining experimentally prepared mixtures and not commercial products when various technological processes and additives were applied. They analysed the total of 50 meat articles manufactured commercially: nine raw or cured products and 41 products subjected to various technological processes, including: preliminary cooking, freezing, cooking and smoking, dehydration and sterilisation. Meat mixtures made up 20 of all the 50 examined products. Employing universal primers, the researchers amplified the mitochondrial DNA region coding cytochrome *b*. Species identification was achieved with the assistance of the following four enzymes: *Pal*I, *Mbo*I, *Hinf*I and *Alu*I. It was found that out of 30 products declared as containing only one species of meat, ten articles contained additions of turkey or pork meats, which means that more expensive meats (chicken, quail, beef) were replaced by cheaper turkey or pork meats. In the case of 20 products, labelling the presence of at least two meat species, five labels provided erroneous information. In each of the five incorrectly designated products, one of the declared meat constituents was missing; in three cases high market value meat (roe-deer or beef) was not included, while in the two remaining cases – no chicken or turkey meats were detected. The current investigations proved that the PCR-RFLP technique is an appropriate method that can be employed to identify meat species in commercial products subjected to various technological treatments, and consist of up to three different meat species.

Partis et al. [2000] offer a slightly different opinion about possibilities of meat identification using the PCR-RFLP technique. They agree that the PCR-RFLP can be applied to analyse both raw and cooked meat species but they do not recommend this method to examine species composition of meat mixtures. The researchers tested possibilities of meat identification of 22 animal species using two restriction enzymes: *Hae*III and *Hinf*I. Their experimental samples comprised: raw meat, meat cooked in microwave for 30 seconds as well as meat mixtures. Two primer pairs were used in the experiment: CYT b1 and CYT b2 as well as C1 and C2 primers which amplify the gene coding cy-

tochrome *b* and yield, products of 359 bp and 464 bp. The 'fingerprints' obtained on the polyacrylamide gel as a result of the application of restriction enzymes were identical both for raw and cooked meats and allowed identification of all analysed species with the exception of the kangaroo and buffalo.

Investigations were also conducted on two-component meat mixtures of significant commercial importance, such as: beef, pork, lamb and chicken where the proportion of individual meats ranged from 1 to 99%. Employing CYT b1 and CYT b2 primers and restriction enzymes, pork could be detected at the level of 1% and, frequently, it seemed to be the dominant mixture constituent. Beef meat could not be identified at the level of 80% in the mixture with pork meat; it was detected at the level of 50% in the mixture with poultry meat, while in the mixture with lamb – at the level of 5%. The same mixtures were also tested using the other set of primers: C1 and C2 and, in this case, beef was easily identified in mixtures even at the level of 1%. The second set of primers helped detect beef in mixtures but hampered the identification of chicken and pork meats. The above remarks indicate that the PCR-RFLP method can sometimes be inappropriate for meat mixture analyses because, frequently, the obtained results are qualitative and they do not reflect the true composition of the examined products [Partis et al. 2000].

The PCR-RFLP method is very useful in the case of identification of a wide range of fish species. Carrera et al. [1999 a, b] reported that the PCR-RFLP technique allowed the identification of raw and smoked meats of two fish species belonging to the Salmonidae family, namely: the Atlantic salmon (*Salmo salar*) and the rainbow trout (*Oncorhynchus mykiss*). The molecular target employed in this type of analyses can be a fragment of the conservative gene 16S rRNA [Carrera et al. 1999 b] or the mitochondrial cytochrome oxidase subunit II (COII) gene) [Carrera et al. 1999 a]. Following the application of appropriately selected endonucleases onto the DNA amplification product, a band allowing the distinction of salmon and trout meats on the electrophoretic gel was obtained.

Also experiments carried out by Russell et al. [2000] confirmed the possibility of identification of salmon and trout meats. They reported that it was possible to identify 10 species of salmonid fish using the PCR-RFLP method and cytochrome *b* as a molecular target. The experimental material comprised both raw as well as cooked meat (in water for 15 min). Unequivocal identification of some fish species, e.g. Chum salmon (*Oncorhynchus keta*), silver salmon (*Oncorhynchus kisutch*), red salmon (*Oncorhynchus nerka*), Chinook salmon (*Oncorhynchus tschawytscha*) was possible following the application of one restriction enzyme. However, in the case of the remaining six species, combination of several restriction enzymes was necessary to determine the species.

Species identification was also carried out on fishes from the flounder-shaped order, namely on the sola (*Solea solea*) and Greenland halibut (*Reinhardtius hippoglossides*). The identification of these two fish species was conducted on the basis of the amplification of the mitochondrial 12S rRNA gene and the application of restriction enzymes *Aci*I and *Mwo*I [Céspedes et al. 2000].

As in the case of mammal and poultry meats subjected to high temperature treatment, e.g. sterilisation, also in the case of fish meats the PCR-RFLP technique allows their species identification. Experiments carried out by Quinterio et al. [1998] confirmed possibilities of identification of six species of tuna fish whose DNA underwent strong degradation during the process of sterilisation (115°C for 55 min).

PCR-SSCP (SINGLE STRAND CONFORMATION POLYMORPHISM)

The PCR-SSCP technique allows detection of mutations as well as polymorphisms occurring in DNA. A dsDNA fragment amplified with the aid of the PCR technique prior to electrophoresis is subjected to denaturation (most frequently, thermal) in the presence of a denaturation agent (formamide or sodium hydroxide) yielding ssDNA. It can also be obtained following an asymmetric PCR or dsDNA selective enzymatic digestion [Rehbein et al. 1998]. In conditions of native electrophoresis, ssDNA creates internal pairing leading to the development of a specific conformation. DNA fragments containing mutations or polymorphisms create different conformations resulting in the ssDNA molecule mobility change as well as the development of different patterns on the polyacrylamide gel.

Depending on the conditions of PCR, denaturation and electrophoresis, more than one ssDNA conformation state can occur. However, if we have reference samples – which were subjected to amplification – denaturation and electrophoresis in the identical conditions as the analysed samples, species identification is possible [Rehbein et al. 1999].

The quality and effectiveness of conformer creation in the PCR-SSCP method is influenced, among others, by temperature. Excessive temperatures reduce the effectiveness of conformer production, lead to the development of diffused bands making the interpretation of the obtained results more difficult. The PCR-SSCP technique is applied for short DNA fragments (95% detectability of changes for 100-300 bp fragments [Napierała et al. 2004]) making it useful in the case of DNA analysis derived from biological material heated to high temperatures (pasteurization, sterilisation).

The PCR-SSCP method was also employed to identify fish species from the *Scombrinea* sub-family, with special consideration paid to tuna fish [Rehbein et al. 1999]. The analysis was conducted on the 123 bp long amplicon obtained as a result of multiplication with the assistance of PCR of a gene fragment coding cytochrome *b*. Experiments were carried out on seven fish meat samples, including two samples that were two-component mixtures. The species identification was performed on products subjected to thermal treatment during the sterilisation process which posed a considerable challenge because such products contain highly degraded protein and nucleic acids. The reliability of the PCR-SSCP method, as a tool for species identification, was assessed by carrying identical analyses by eight European laboratories.

The investigated fish (eight species) were identified morphologically and then eviscerated and cooked on steam for 90 min at the temperature of 102-103°C (the temperature inside fish amounted to about 65°C). After cooling down, the experimental fish were filleted, the skin removed and placed in tins adding 2 g of sodium chloride and oil in the amount which allowed complete covering of the meat. Next, the tins were sealed and sterilised at the temperature of 115°C for 60 min. Coded fish samples were sent to each participant of the experiment together with the appropriate reference material of known origin.

The obtained research results showed 90.3% traceability of the analysed fish species. Two of the eight laboratories participating in the experiment obtained totally different band patterns on the electrophoretic gel. The arrangement of bands from the remaining laboratories placed them between the separations obtained from the two above-mentioned laboratories. The reason of the differences could be connected with

the condition of PCR, denaturation and electrophoresis which may cause more than on conformation state of ssDNA. (Interaction between residual PCR primers and ssDNAs) [Rehbein et al. 1999].

However, irrespective of differences of the obtained patterns, the results allowed species identification. The only exception was northern bluefin tuna (*Thunnus thynnus*) as well as yellowfin tuna (*Thunnus albacares*). Both fish possess the same DNA sequence expaining the identical arrangement of bands on the gel. In addition, the performed investigations revealed intraspecific variations of electrophoretic patterns in the case of two tuna fish species, namely for the skipjack tuna (*Katsuwonus pelamis*) and Atlantic bonito (*Sarda sarda*) depending on the geographic occurrence of this fish.

Rehbein et al. [1997] applied the PCR-SSCP method in experiments aiming at the identification of raw meat of closely related fish species from the families of: Anguillidae (*Anguilla anguilla*, *Anguilla japonia*, *Anguilla rostrata*, *Anguilla australis*), Salmonidae (*Salvelinus fontinalis*, *Salmo trutta*, *Oncorhynchus mykiss*, *Salmon salar*), thermally treated tinned fish belonging to the families of: Clupeidae (*Sardina pilchardus*, *Clupea harengus*), Gadidae (*Gadus morhua*) and Scombridae (*Scomber scombrus*, *Thunnus thynnus*, *Thunnus albacares*, *Thunnus obesus*, *Thunnus alalunga*, *Euthynnus alleteratus*, *Katsuwonus pelamis*, *Auxis thazard*, *Sarda sarda*) as well as fish products not made up of muscle tissue, i.e. caviar obtained from the family of Acipenseridae fish (*Huso huso*, *Acipenser gueldenstaedti*, *Acipenser stellatus*). In this experiment, short segments, i.e. 123 bp, 148 bp and 358 bp of the gene coding cytochrome *b* were amplified. Both in the case of raw fish as well as tinned fish the identification with the assistance of the PCR-SSCP technique was successful. Each of the analysed fish species yielded characteristic, species-specific patterns of bands on the electrophoretic gel. Moreover, also the identification of caviar obtained from the family of Acipenseridae fish ended successfully.

In Scandinavian countries, e.g. Norway or Island as well as in Greenland or Canada or Japan meat of sea mammals constitutes a traditional diet component. Consumers value highly meat obtained from these animals but it is not very uncommon that seal meat is sold as whale meat or that dishonest salesmen sell meat derived from endangered animal species. Martinez and Daníelsdóttir [2000] carried out investigations using two techniques: SSCP and RAPD with the aim to identify meat species of sea mammals and indicate which of the two was better. The experimental material comprised reference samples: meat of harp seal (*Phoca groenlandica*), sei whale (*Balaenoptera borealis*), minke whale (*Balaenoptera acutorostrata*) and fin whale (*Balaenoptera physalus*) as well as samples intended for species identification declared as the meats of Greenland seal, minke whale and fin whale as well as species belonging to cetaceans which were purchased in local shops in Norway and Greenland. The reference meat was either frozen or preserved in ethanol. Samples for identification derived from the Greenland seal (dried, smoked and salted as well as heat smoked), minke whale (dried meat), fin whale (dried meat and qiporaq muscle), generally declared as whale meat (smoked and marinated meat) as well as salted whale oil.

Both for the RAPD and SSCP techniques, the cytochrome *b* fragment of the mitochondrial DNA was amplified. It was indicated [Martinez and Daníelsdóttir 2000] the advantage of the PCR-RAPD method over the PCR-SSCP one. In the case of the PCR--RAPD method, each of the two primers employed in the experiments generated characteristic 'fingerprints' which allowed the identification of sea mammals. The degree and

the type of meat processing of the same fish species did not affect band patterns obtained on the gel after the electrophoresis. In the case of the PCR-SSCP method, initially, two pairs of primers: bL1 – bH and FB 349 – FB 496 were applied which amplified 358 bp and 148 bp products, respectively. However, in the course of experiments the FB 349 – FB 496 primer was dropped because it did not form amplicons with DNA derived from the seal. The researchers [Martinez and Daníelsdóttir 2000] admitted that the conditions in which the PCR-SSCP was conducted were not optimal, but despite it, the identification was successful with the exception of the minke whale.

REAL-TIME PCR

The real-time PCR is a method used for quantitative measurements of gene copies or the level of DNA expression. By measuring the intensity of signals derived from fluorescent dyes, the quantity of the PCR product created in each reaction cycle is monitored. The continuous measurement of fluorescence allows skipping stages which usually must be carried out after performing the PCR reaction, i.e. electrophoresis and gel staining. In addition, in the case of the real-time PCR, potential risks of contamination are reduced considerably as samples with the reaction mixture remained sealed throughout the analysis [Rodríguez et al. 2005].

The real-time PCR was applied to detect the presence of meat of closely related animal species, i.e. a horse and a donkey in commercial articles [Chisholm et al. 2005]. The researchers found it necessary to develop a method allowing detection of trace quantities of donkey and horse meats in commercial articles because, in some countries, there is a strong aversion against meat derived from the above-mentioned animal species (e.g. Great Britain). In the employed method, the developed primers were modified according to Hird et al. [2004] since it increased the specificity of reaction. The designed, species-specific primers amplified successfully the gene coding cytochrome *b* yielding amplicons 69 bp and 119 bp long for the DNA derived from horse and donkey meats, respectively. The detection limit of DNA diluted in water for the horse meat DNA specific primers amounted to 25 pg, while for the donkey meat DNA specific primers – to 1 pg. The primers were also tested successfully on meat mixtures in which the content of horse or donkey meats amounted to 4%, 2% and 1%, also on commercial products.

Hird et al. [2005] also employed cytochrome *b* as the molecular target in their investigations aiming at the species identification of duck meat (*Anas platyrhynchos* and *Cairina moschate*) until recently consumed only on special occasions. However, thanks to the latest advances in breeding techniques, the meat is becoming increasingly popular and hence it is necessary to elaborate a method allowing duck meat detection in meat products. The real-time PCR allowed detection of 0.0001% DNA derived from duck meat and also made it possible to detect this species in commercial articles of different level of processing.

Pork meat identification carried out by Rodríguez et al. [2005] was based on the amplification of the mitochondrial fragment of the 12S rRNA gene. The results obtained on the basis of the performed investigations revealed possibilities of detecting pork meat at the level of 0.5% in a two-component pork/beef mixtures subjected to sterilisation (heating at the temperature of 121°C for 20 min).

Dooley et al. [2004] also reported the possibility of species identification at the level of 0.5%, but in their studies the identification concerned beef, pork, lamb, chicken and turkey meats occurring in mixtures from raw meat where the molecular target was cytochrome *b*. No absolute level of detection was determined in the above-mentioned studies, although theoretically it should be possible to detect beef, lamb and turkey meats at levels less than 0.1%.

CONCLUSIONS

Methods based on the polymerase chain reaction are frequently employed for species identification of meat products and, consequently, can be applied for the verification of regional and traditional meat products in regard to the declarations associated with their composition.

Potential for the exploitation the PCR technique stem from the possibility of identification of meats of different, even closely related animal species. Such identification can be carried out on biological material derived from raw tissue as well as muscles subjected to various technological treatments. Their relative simplicity as well as rapidity of analyses allows assuming that these techniques consisting in DNA analysis will find wide application in future in food quality control. However, despite unquestionable advantages of the techniques based on the PCR method, we should not overlook their shortcomings. Generally speaking, the PCR-RAPD is not recommended for meat species identification in meat mixtures as well as articles subjected to strong heating (sterilisation). According to some researchers also the PCR-RLFP technique is not recommended for the verification of species composition of meat mixtures as the obtained results do not always reflect the true composition of the mixture. In the case of the PCR--RLFP technique, there is a risk of occurrence of the incomplete digestion of the restriction site or intraspecific differences that may contribute to the removal or development of restriction sites. The necessary condition for carrying out species identification with the assistance of the PCR-SSCP and PCR-RAPD is to possess reference samples on the same electrophoretic gel as the analysed samples. The repeatability of the obtained results in the PCR-SSCP method is influenced by conditions in which the analysis is carried out, i.e. temperature and concentration of the applied reagents (e.g. buffers).

It shall be emphasised that the PCR methods are only those which can verify products composition, but complex evaluation of regional and traditional meat products comprises also several other methods, which concurrently can affirm or not the conformity of producer's declaration with that what consumer has obtained.

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METODY PCR W IDENTYFIKACJI GATUNKOWEJ ŻYWNOŚCI JAKO NARZĘDZIE DO WERYFIKACJI ŻYWNOŚCI REGIONALNEJ I TRADYCYJNEJ

Streszczenie. W epoce przemysłowego wytwarzania żywności żywność regionalna i tradycyjna jest alternatywą dla ludzi poszukujących niezapomnianych wrażeń sensorycznych. Żywność regionalna czy tradycyjna, powszechnie postrzegana jako smaczna i zdrowa, dla części konsumentów jest często swoistym i sentymentalnym powrotem do smaków dzieciństwa. Stopniowy wzrost popytu na tego typu żywność, a także stosunkowo wysokie ceny tych produktów wśród nieuczciwych wytwórców żywności mogą generować wiele niewłaściwych praktyk produkcyjnych w wyrobach mięsnych (np. zastępowanie surowca droższego tańszym). W celu weryfikacji składu gatunkowego wyrobów mięsnych stosowane są metody chromatograficzne, immunologiczne, elektroforetyczne oraz genetyczne. Do metod genetycznych wykorzystywanych w badaniach autentyczności składu żywności, w tym mięsa i jego wyrobów, należy łańcuchowa reakcja polimerazy (PCR). W pracy przedstawiono najważniejsze techniki wykorzystujące tę reakcję celem identyfikacji pochodzenia określonych składników żywności wchodzących w skład żywności regionalnej lub tradycyjnej. Wykazano, że techniki PCR wraz ze specyficznymi gatunkowo starterami, PCR-RFLP, PCR-SSCP i real time PCR, pozwalają na identyfikację gatunków mięsa występującego samodzielnie, w mieszaninie z innymi gatunkami, a także poddanego obróbce termicznej czy procesom technologicznym w produkcji przemysłowej. Jedynie metoda PCR-RAPD nie pozwala na identyfikację gatunkową w wypadku silnej degradacji DNA lub w złożonych mieszankach mięsnych.

Słowa kluczowe: metody PCR, identyfikacja gatunkowa, regionalne i tradycyjne wyroby mięsne

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