

**SPECIES SPECIFIC DETECTION OF MEAT
BY POLYMERASE CHAIN REACTION
TECHNIQUES**

PhD thesis


Anna Jánosi

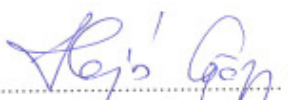
Budapest
2006

PhD School/Program

Name: PhD School of Food Science
Field: Food Science
Head: Prof. András Fekete
CORVINUS UNIVERSITY OF BUDAPEST
Supervisor: Prof. Gyöngyi Hajós
CENTRAL FOOD RESEARCH INSTITUTE

The applicant met the requirement of the PhD regulations of the Corvinus University of Budapest and the thesis is accepted for the defence process.


.....
Head of PhD School
Prof. András Fekete


.....
Supervisor
Prof. Gyöngyi Hajós

1. RESEARCH BACKGROUND

Authenticity testing of the animal species present in food is important for economic, safety, legal, religious and health reasons. Food labelling regulations require that the species of meat in food products are accurately declared to the consumer. Food labelling regulations (Hungarian Food Law LXXXII, 2003; 197/2004 (II.26) FVM-ESZCSM-GKM regulation, *Codex Alimentarius Hungaricus* 2-13, 1-3/13-1) require that the species of meat in food products are accurately declared to the consumer. This requires reliable and specific methods of meat species determination in a variety of products where meat may be comminuted, mixed with other ingredients and processed. A variety of analytical approaches for species identification have been described in the literature, which can be based either on protein or DNA detection methods. Isoelectric focussing (IEF) and immunoassays (ELISA, immunostrip) are the most commonly used protein-based techniques nowadays. Isoelectric focussing is based on the separation of proteins on polyacrilamid gel by the use of a pH-gradient and on the subsequent staining of the proteins by using *Comassie Blue*, silver or pseudoperoxidase staining methods. It has been reported that IEF is not suitable for the processed meat products, because most soluble proteins degrade very rapidly under such conditions. Certain immunoassays have been shown that they are suitable for the detection of heat-treated proteins. The disadvantage of immunoassays is the restricted availability of antibodies free from many cross-reactions to the related organism. Furthermore the presence of proteins is always a function of age, race, maturity and type of tissue in which they are expressed.

For the identification of species, it is preferable to detect DNA. It is identical in all cell types of an organism, therefore it is not essential whether the DNA is extracted from the muscle, fat or offal. DNA hybridisation and polymerase chain reaction (PCR) offer analytical approaches based on nucleic acid to identify species specificity. PCR restriction fragment length polymorphism (PCR-RFLP) is nowadays the most widely used method of identifying different fish and meat species. Most of these methods use the highly conserved regions of cytochrome b gen as a target sequence.

I have adopted and developed assays based upon PCR and PCR-RFLP amplification of genomic and mitochondrial genes for species-specific detection of bovine, porcine, chicken from processed meat products and game meat (wild boar, moufflon, red deer, roe deer) from raw meats.

2. AIM OF RESEARCH

- Adaptation and selection of DNA-based analytical methods for selective, species-specific identification of livestock animal (beef, pork, chicken).
- Development of meat model systems for determination of DNA isolation methods efficiency and detection limit of PCR methods.
- Examination of PCR and PCR-RFLP techniques applied for the species-specific detection of livestock animal (beef, pork, chicken) by sausage-type models.
- Examination of PCR and PCR-RFLP techniques applied for the species-specific detection of livestock animal (beef, pork, chicken) by can-type models.
- Species-specific random screening of commercial meat (sausages, cans and liver pie) products.
- Adaptation and selection of simplex PCR and PCR-RFLP methods for selective, species-specific identification of game animal (wild boar, red deer, roe deer, moufflon) meats.

3. MATERIALS AND METHODS

Samples of fresh raw meats (beef, pork, chicken, turkey) and frozen game meat (wild boar, moufflon, roe and red deer) were obtained from slaughterhouses (Bicske, Vecsés, forestry of Pilis), processed meat were purchased from local supermarkets. For the testing of the PCR and PCR methods a sausage-type model matrix (30% meat) and a can-type model matrix (60% meat) were used, that contained water, fat, soy protein and salt. The sausage-type models were heat-treated at 78°C for 45 min and the can-type models were sterilised at 121°C for 50 min.

DNAs from beef, pork, chicken, turkey and game meat species were obtained by extraction using the Wizard DNA Clean up purification kit (Promega) and modified CTAB methods. Isolations of DNA from heat-treated model matrixes (beef-pork and chicken-pork meat contained matrix-mixtures), commercial meat products (ham, sausage, liver pie) were performed using Wizard DNA Clean up purification system. This method was used to purify DNA from sterilized models and luncheon meat after the samples were concentrated by Amicon ultra filtrate system (Millipore). Extracted DNA was stored in Tris-EDTA buffer and quantified spectrophotometrically. The average size of DNA fragments was analysed using 2 % agarose-gel electrophoresis.

The primers were designed from the growth hormone gene of beef (Ca03-Ca04 primers, 130 bp fragment) and pork (Sw01-Sw02 primers, 108 bp fragment). In case of chicken species-specific detection 359 bp PCR-RFLP method was used, based on amplification and *Rsa*I (5'..GT↓AC..3') digestion of mitochondrial gene fragment (Cytb1-Cytb2 primers).

Three of PCR and PCR-RFLP assays were used to find the differences between game and domesticated animal meat samples. Three primer pairs amplify 175 bp (Scytb1-Scytb2 primers), 194 bp (RD1-RD2 primers) and 359 bp (Cytb1-Cytb2 primers) length fragments from the mitochondrial gene. The PCR products were digested with restriction enzymes *Alu*I (5'.. AG↓CT..3') and *Hin*FI (5'..G↓ANTC..3'). Amplifications were performed in a final volume of 50 µl in thin walled PCR tube containing 1x Sigma Ready mix, 0,5 mM primer.

PCR products were identified by separation on 10 % polyacrylamide gel in TBE running buffer, for 1 h at 200 V and ethidium bromide was used for the visualisation. The gels were documented with Kodak EDAS 290 system.

4. RESULTS

4.1. Detection of pork, beef and chicken meat in meat-model matrixes and food products by PCR and RFLP-PCR methods

Simplex PCR and PCR-RFLP methods were applied for species-specific identification of livestock animal meat (beef, pork, chicken). Developments of methods are based on sausage- and can-type meat model matrix.

Appropriate quantity (8,59 µg DNA/ 100 mg sample) and high-quality DNA could be extracted by using the Wizard DNA Clean-Up System in case of the raw meat samples although using of the CTAB method the meat R-value was under 1.7. The samples contains protein contamination below the R value of 1.7 and RNA contamination above R value of 2.0. For this reason the Wizard technique was used for the isolation of DNA in models and foodstuffs.

The pork and beef PCR assays demonstrated to be highly specific for porcine/ beef DNA, producing an amplification product of the expected size of 108 and 130 bp. No amplification product was obtained from chicken and poultry DNA. With 35 amplification cycles, using ethidium-bromide-based detection the minimum detection level was 0,5 % for the raw and the heat-treated model mixtures using pork, beef or chicken specific PCR assays respectively. The primers did not have any cross-reactivity with plant-derived soy or wheat DNA. The 359 bp fragment of cytochrome b gene could be amplified successfully from pork, beef and poultry DNA. RsaI (5'..GT↓AC..3') restriction sites were found in cytb amplicon from DNA of chicken and turkey origin. Whereas the RsaI fragments of chicken were 210 bp and 149 bp long and the poultry amplicon yielded RsaI fragments 149 bp, 109 bp and 101 bp long.

The amount of extracted DNA ranged from 24 to 29 µg / 300 mg raw model mixtures and 7 to 9 µg / 300 mg autoclaved model mixtures. Applying Amicon ultra filtrating system combined with 1,5 g starting sample mass, the amount of extractable DNA could be raised up to 20-37 µg in the case of autoclaved models. The yields of DNA ranged from 8 to 45 µg / 300 mg for the heat-treated meat products and 127-320 µg / 1500 mg for the autoclaved food samples.

In the case of sterilized matrix a filter concentrator was used to raise the recoverable DNA content. Using this concentration step the detection limit could be lowered up to 10% from sterilized models.

40 commercially purchased heat-treated meat products were tested using these PCR and PCR-RFLP assays and the results were compared to the labelling. Depending on the product type, 6-23 % of the samples were false labelled.

4.2. Detection of game meat species

The polymerase chain reaction-restriction length polymorphism was applied to species identification of raw meat from wild animal (wild boar, red deer, roe deer, moufflon). The sequences selected for amplification were a 359, 194 and a 175 base pair fragment of the mitochondrial cythochrome b gene as a part of the template DNA. A 194 bp signal was obtained from game samples, but some bands were detected in the case of beef and pork meats. The 359 bp and the 175 bp fragment was amplified as a product of PCR from each species, but in the AluI and Hinf I restriction fragment pattern we could find enough differences for the identification of the individual species. In contradiction to special literature the restriction point was not found in the 359 bp length product of the wild boar. It was the first investigation when the analysis of 175 bp products was applied in case of the wild animals.

5. NEW SCIENTIFIC RESULTS

I. Species-specific identification of livestock animal meat in model mixtures and food samples using simplex PCR and PCR-RFLP techniques

- 1) New, complex meat model system was introduced for adaptation of simplex-PCR and PCR-RFLP methods to meat-based food product analysis.
- 2) Amicon Ultra filter system based concentration step was successfully applied for increasing of quantity and quality of fragmented DNS in the case of can type meat model matrix.

II. Monitoring of commercial food product

- 1) New data was summarized about the labelled and measured meat species content of inland-made meat product

III. Selective identification of game meat species

- 1) A simplex-PCR techniques was adapted to selective, species-specific differentiation of game meat from livestock animal meat. This PCR technique based on the amplification of a 194 bp length fragment from mitochondrial, cytochrome b gene using RD1-RD2 primerpair.
- 2) By the PCR-RFLP of 359 bp fragment of mitochondrial, cytochrome b gene with HinfI restriction analysis a new wild boar population (Vecsés, 4 animal samples) was find, that DNS sample does not contain a restriction site in PCR product. In the future it can be a basis of a geographical origin research project.
- 3) 175 bp cytochrome b fragment amplification (Scytb1-Scytb2 primerpair) based PCR-RFLP was proved to be a new method for differentiation of frozen moufflon, wild boar and red deer meats.

6. CONCLUSION

The PCR and PCR-RFLP methods combined with model-matrix systems and DNA concentration step developed by me is a useful analytical method for the identification of different animal species in raw materials and processed foods.

In the future I will pay a great attention to the investigation of non-meat origin type ingredients in the meat products such as fat, gelatine, milk powder and egg. It would be interesting to learn, if they are present in the products and in what extent they can give overlapping results in the PCR system. I would suggest the refinement and standardisation of the sampling methods in the development of such chain reaction systems, which are highly sensitive and could be applied for quantitative analysis as well. In addition I would propose the application of the mitochondrial DNA based methods for the selection of the gene sequences which must be multiplied for the analysis of the strongly heat-treated or fried products because of their higher copy number presented in the cells.

7. LIST OF PUBLICATIONS

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