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VERIFICATION OF ANIMAL SPECIES IN HAM AND SALAMI BY DNA MICROARRAY AND REAL TIME PCR METHODS

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ABSTRACT

Consumer protection and detecting of adulteration is very important and has a wide societal impact in the economic sphere. Detection of animal species in meat products and the use of combining different methods is one of the means to achieve relevant product status. The aim of this study was to reveal whether or not the products label clearly meets the content declared by producer. In our study, 29 samples of meat products such as salami and ham obtained from stores and supermarkets in Slovakia were analyzed to detect the existing animal species according to the product label the use of Chipron LCD Array Analysis System, Meat 5.0. Products in which the presence of non-declared animal species has been detected were subjected to testing by the innuDETECT PCR Real-Time Kit, repeatedly. The results showed that 20 (68.96%) samples were improperly labeled. From in total 14 tested ham samples 11 (78.57%) products exhibited non-conformity with declared composition. Tested salami samples (15) revealed 9 (60%) incorrectly labelled products. The results obtained by DNA Microarray and Real Time PCR methods were identical, and both methods should be extensively promoted for the detection of animal species in the meat and meat products.

Keywords: Ham; Salami; DNA Microarray; RT-PCR; meat

INTRODUCTION

Consumers require clear and accurate information to make the decision in personal diet. Consumer choice might reflect lifestyle, religious concerns, or health status. Therefore, the description and labelling of food must be based on the true. The information that must be given is definied by the current legislation of developed countries; the food must be authentic and not misdescribed **(Woolfe and Primrose, 2004)**.

The adulteration of food is associated with food quality. Verification of genuineness of certain products is a necessary part of a comprehensive examination of quality with regard to consumer protection **(Maršálková et al., 2014)**. Considering the recent cases of meat adulteration and fraud, efficient and accurate analytical methods are essential for identification of meat species as a key importance to maintain consumer trust and to comply with labelling legislations **(Cottenet, 2016)**.

Meat products usually contain meats of various origin, this should meets the producer declaration posted on the product label **(Mašlej, Golian and Maršalková, 2014)**. In this way, meat authenticity not only relates to industrial economic profit resulting from illegal trading, handling or substitution of species, but also to public health risks such as zoonoses or allergenicity to specific meat protein. In this context, wild game meats may originate from farms having regulated hygienic standards and fair commercial practices **(Hoffman and Wiklund, 2006; Fajardo et al., 2010)**. Following the horse meat crisis which spread throughout Europe in 2013, food fraud and adulteration are identified as a top priority addressed by authorities, regulators and food industries **(Elliott, 2014, Cottenet et al., 2016)**. Economically motivated adulteration presents many challenges because perpetrators are specifically seeking to avoid detection and circumvent existing regulatory systems or testing methodologies **(Everstine, Spink and Kennedy, 2013; Cottenet et al., 2016)**.

The application of quality assurance systems through the food chain requires the development of reliable and simple tools, which facilitate routine control assessments. The detection of meat species in various food products deserves special attention due to the recent crisis in the meat sector **(Brodmann and Moor, 2003; Saez, Sanz and Toldrá 2004)**. As a consequence of the tremendous profit that results from selling cheaper meat as meat from more profitable and desirable species, fraudulent misdescription of game meat products is becoming a common practice among unscrupulous processors who apply deceptive practices on their products **(Brodmann et al., 2001; Fajardo et al., 2010)**.

In the last years, the attention has been paied towards implementation of molecular genetic approaches for meat species identification due to their high sensitivity and

specificity, as well as rapid processing time and low costs **(Fajardo et al., 2010)**.

Furthermore, DNA analysis presents an attractive strategy for meat species identification. In comparison with protein detection, DNA is stable against technological treatments and independent of the considered tissue **(Martinez and Yman, 1998; Wolf, Rentsch and Hübner 1999; Saez, Sanz and Toldrá 2004)**.

Real-Time PCR and DNA chip technique in detection of animal species are well suited for rapid screening of meat products in a routine analytical laboratory. However, the DNA Chip offers additional advantage, undeclared and unknown animal species presented in meat products, resulting from contamination or deliberate adulteration, can be detected **(Iwobi et al., 2011)**.

In our study, 29 samples of meat products (salami and ham) reached from stores and supermarkets in Slovakia were analyzed to detect animal species according to product label by using Chipron LCD Array Analysis System, Meat 5.0. Those products where the presence of unlabel animal species was detected have been subjected to innuDETECT PCR Real-Time test.

In recent time, numbers of food products have been revield as fraud products, where their label is not follow the statement provided by the producer. Real Time PCR and Microarray technics presents a usefull tool in the elimination of deep-laid business practices. Their reliability and above mentioned theory have been confirmed by our study on tested products.

MATERIAL AND METHODOLOGY

The collected samples, hams and salami were placed in sterile refrigerated container under 8 °C for sample preparation and DNA isolation. The pieces taken by disposable scalpel were placed into Eppendorf tubes. DNA was extracted according to innuPREP DNA Mini Kit (Catalog no: 845-KS-1040250) user guide. The extracted samples of DNA were stored at -18 °C.

The extracted DNA samples were amplified by PCR (Toptical Gradient 96) following the manufacturer requirements of Chipron LCD Array Kit Meat 5.0 (Chipron GmbH, Germany). Since the kit was ready to use, 12.5 µL of Chipron 2x Master mix, 1.5 µL of Primer Mix MEAT and 6 µL of PCR grade water were added into Eppendorf tube. The volume of 25 µL from prepared solution was pipetted to each of the plate well following addition of 5 µl of the DNA template. The plate was closed and installed into the cycler. Thermal processing was setted to 1 cycle at 95 °C for 5 min, then 35 cycles at 94 °C for 30 sec, 57 °C for 45 sec, 72 °C for 45 sec, and finally 72 °C for 2 min **(Chipron, 2014)**.

Twenty two microliter of hybridization buffer and 2 µl of modulator solution were added into Eppendorf tube. This mixture was pipetted in the volume 24 µl to each of the plate well following the addition of 10 µL of PRC product from extracted DNA samples. Chip from the kit was placed in the chip box, incubation of the slide was provided under 35 °C for 30 minutes in humidity chamber. We prepared 3 wash containers filled with 150 ml of washing solution. Slide was incubated and 28 µL from each plate well was pipetted onto the lower left hand corner for each of the eight patterns. Chip box was closed, incubated at 35 °C for 30 min, washed, dried, and then placed in the box again. Putting the dilution solution into the Eppendorf tube, 30 µl of annealing solution was pipetted into each of the patterns of the chip and allowed to standby for 5 min.

After the incubation washing procedure was done, and chip was centrifuged for 15 sec, allowed to dry, and placed in the box again. Twenty seven microliter of dilution buffer, 3 µL modulator and 0.2 µL label were aplicated on eight patterns on the slide and the slide was incubated at room temperature for 5 minutes. Washing buffer was replaced in all containers and washing procedure was repeated. Slide was dried by spinning for 10 seconds in the CHIP Spin FVL2400N (Catalog no: HS-500-01). Twenty eigth microliters of staining solution were added into each of the patterns of the chip, and the chip was allowed to standby for 5 min in room conditions. Following staining procedure, it was kept in washing cointainer for 1 minute, and then centrifuged for 10 sec for drying **(Chipron, 2014)**.

Evaluation of the Results

Chipron LCD Array System can detect cattle, sheep, equine, goat, camels, buffalo, pork, kangaroo, hare, rabbit, reindeer, roe deer, red deer, fallow deer, springbok, dog, cat, chicken, turkey, goose, ostrich, mallard duck, muscovy duck, pheasant in tested sample. The detection in this system is based on specific sites within 16S rRNA mitochondrial locus of all meat species in analyzed food sample. A dark precipitate is formed by the enzyme

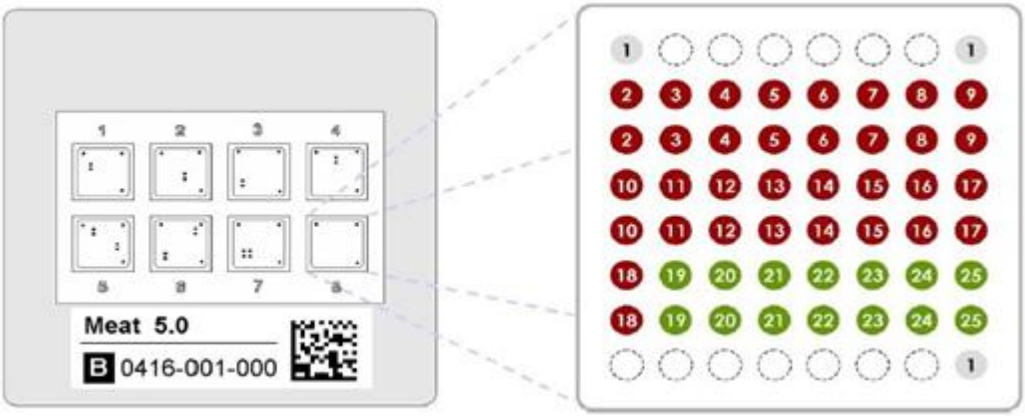

Figure 1. Chipron LCD Array System

Table 1 Capture probes Chipron Meat 5.0 LCD Kit.

Well No	Probe	Specifity	Well No	Probe	Specifity
01			Hyb-Ctrl		
02	Cattle	*Bos taurus, Bos bison*	14	*Red deer*	*Cervus elaphus*
03	Sheep	*Ovis aries*	15	*Fallow deer*	*Dama dama*
04	Equine	*Equus caballus, E. asinus*	16	*Springbok*	*Antidorcas marsupialis*
05	Goat	*Capra hircus*	17	*Canine*	*Canis sp.*
06	Camel	*Camelus sp.*	18	*Cat*	*Felis silvestris*
07	Water buffalo	*Bubalus bubalis*	19	*Chicken*	*Gallus gallus*
08	Pork	*Sus scrofa*	20	*Turkey*	*Meleagris gallopavo*
09	Kangoroo	*Macropus rufus / giganteus*	21	*Goose*	*Anser sp.*
10	Hare	*Lepus europaeus*	22	*Ostrich*	*Struthio camelus*
11	Rabbit	*Oryctolagus cuniculus*	23	*Mallard Duck*	*Anas platyrhyncos*
12	Rein deer	*Rangifer tarandus*	24	*Muscovy Duck*	*Cairina moschata*
13	Roe deer	*Capreolus capreolus*	25	*Pheasant*	*Phasianus sp.*

substrate provided in the test kit, and it indicates a positive hybridization reaction. After staining procedure completed chip was read with the scanner, and analysis was done by the software from the "Analysis-Package" provided by Chipron. Three different spots on the chip are called the control points to detect a positive reaction which are located in upper-left, upper right and lower right corners.

If no control spots occur, the test should be repeated. The animal species were identified using Slide Scaner (Catalog no: HS-300-01), Slide Reader Software (Catalog no: HS-200-01) (Table 1).

Samples analysed by DNA Microarray method were verified by Real Time PCR method (innuDETECT Assay).

The DNA previously isolated by using innuPREP DNA Mini Kit (Catalog no: 845-KS-1040250) stored at -18 °C was used. The procedure given by innuDETECT Assay was followed up. Positive and negative controls were run. All solutions and materials in the kit were dissolved before the use. Twenty microliters of PCR master mix including 10 μL of PCR, 2x master mix, 3 μL primer/probe mix, 1 μL internal control and 3 μL PCR-grade water was pipetted into each of the plate well. Three microliters of previously extracted DNA were added onto each.

The tubes were closed tightly and placed in LightCycler 2.0. The thermal processing was designed as one cycle at 95 °C for 120 sec, then 35 cycles at 95 °C for 10 sec and 62 °C for 45 sec. The analysis was done by the LightCycler 2.0 software.

RESULTS AND DISCUSSION

The results obtained by DNA Microarray indicated that 20 (68.96%) samples were improperly labeled. Adulteration was made accoring to the notifications on the label. From tested ham samples 11 (78.57%) products exhibited non-conformity with declared composition of the product from analyzed samples. In the second analyzed category 9 (60%) from 15 of analyzed salami samples were labelled incorrectly (Table 2, Table 3). The presence of several unlabeled species has been identified in the products. The results obtained by DNA Microarray and Real Time PCR methods were identical, both methods should be extensively promoted for the detection of animal species in the meat and meat products, these findings are in accordance with **Özpinar et al. (2013)**.

DNA Microarray indicated that 39 out of 73 samples (53.4%) were labelled incorrectly, and adulteration was made in contrary to the notifications on the label. The adulteration was detected mostly in meat balls (87.5%), ground meat (72.7%), salami (57.1%), sausages (50%) and fermented sausages (30.3%), respectively.

It was mostly seen that meat balls and ground meat have significantly potential risk for adulteration. Following them fermented

The adulteration was detected mostly in meat balls (87.5%), ground meat (72.7%), salami (57.1%), sausages (50%) and fermented sausages (30.3%). It was found that meat balls and ground meat significantly have potential risk for adulteration. Following them fermented sausage

Table 2 Authentication of meat species in ham.

No	Describe of sample	Beef		Pork		Chicken		Turkey	
		Chipron Meat 5.0 LCD Kit	PCR- RT	Chipron Meat 5.0 LCD Kit	PCR- RT	Chipron Meat 5.0 LCD Kit	PCR- RT	Chipron Meat 5.0 LCD Kit	PCR- RT
1	pork 60%	-	-	+	+	-	-	-	-
2	pork 70%	-/+	-/+	+	+	-/+	-/+	-/+	-/+
3	pork 96%	-	-	+	+	-	-	-	-
4	pork 97%	-	-	+	+	-	-	-/+	-/+
5	turkey 31%, chicken 30%	-	-	-/+	-/+	+	+	+	+
6	chicken 63%	-	-	-/+	-/+	+	+	-/+	-/+
7	pork 92%	-	-	+	+	-/+	-/+	-	-
8	pork 51%, pork natural protein	-/+	-/+	+	+	-/+	-/+	-/+	-/+
9	pork 65%, pork natural protein	-	-	+	+	-/+	-/+	-	-
10	pork 90%,	-/+	-/+	+	+	-	-	-	-
11	pork 87%,	-	-	+	+	-/+	-/+	-/+	-/+
12	pork 65%,	-	-	+	+	-/+	-/+	-	-
13	pork 70%, pork natural protein, hemoglobin	-	-	+	+	-	-	-	-
14	turkey 64%	-	-	-/+	-/+	-/+	-/+	+	+

Note: +/- declared, absent; -/+ undeclared, present; + declared, present, - undeclared, absent

samples showed incorrect labelling with the range of 30%. On the other hand, mentioned types of food claimed 100% beef on the labels. Hence, species detected in meat ball, ground meat and fermented sausage samples were presented by chicken, turkey and sheep species. Pig and equine species were not detected in 79 samples.

The fraudulent misdescription of food contents declared product labels is a widespread problem, particularly with value products of premium price. In respect of this detection and quantification of food constituents is required. As they are oftenly biochemically similar to the materials they replace, their identification and measurement is extremely difficult **(Woolfe and Primrose, 2004).**

DNA Microarray and Real Time PCR offer detection of animal species in one reaction. Common similarity between them is the step of DNA isolation. Microarray Analysis enable the detection of more than one species in one reaction whereas Real Time PCR requires specially designed primers and probes needed for amplification of specially selected DNAs regions belonging to different species. This difference means longer time needed for the optimization step of primers and probes **(Myers et al., 2010, Özpinar et al., 2013)**. DNA Microarray can deliver the results faster and more sensitive using amplified DNA in comparison to conventional PCR technique **(Azuky et al., 2011)**. Polymerase chain reaction (PCR) is commonly used technique in many fields of molecular biology due to its sensitivity, specificity and capability to detect even a single copy of DNA sequence from a single cell sample **(Chikuni et al., 1994)**.

DNA Microarray as a method has been widely preferred for understanding mechanisms, detection of foodborne microbial pathogens and food safety studies, nutreaceuticals and functional foods as well as following up the different expression levels of DNA in bacteria, yeasts, plants and human; genetic and mutation analyses; environmental studies; identification of antimicrobial genes, proteomics, protein-nucleic acids, protein-protein interactions, biochemical analysis of protein functions and drug development **(Bottero and Dalmasso, 2010; Kostrzynska and Bachand, 2006)**. A study done in USA indicated that 62% of meat products had only one foreign species, 36% had two, and 2% had three. A similar study in the States also showed that the adulteration ratio has increased up to 46.4% **(Macedo-Siva et al., 2000, Özpinar et al., 2013)**. In Brasil commercial samples of swine hamburgers showed no adulteration with bovine, chicken, swine or horse meats, and expectation of hamburger adulteration was not confirmed **(Özpinar et al., 2013)**.

Table 3 Authentication of the meat in salami.

No	Describe of sample	Beef		Pork		Chicken		Turkey	
		Chipron Meat 5.0 LCD Kit	PCR-RT	Chipron Meat 5.0 LCD Kit	PCR-RT	Chipron Meat 5.0 LCD Kit	PCR-RT	Chipron Meat 5.0 LCD Kit	PCR-RT
1	beef and pork 70%	-	-	+	+	-	-	-	-
2	beef and pork 88%	+	+	+	+	-	-	-/+	**-/+**
3	pork 70%, pork natural protein	-	-	+	+	-	-	-	-
4	pork 89%, hemoglobin	-	-	+	+	-	-	-	-
5	pork 60%, pork natural protein	-	-	+	+	-	-	-	-
6	port and beef 56%, pork natural protein	+	+	+	+	-/+	-/+	-/+	**-/+**
7	pork and beef 65%, pork natural protein	+/-	+/-	+	+	-	-	-	-
8	pork and beef 58%, pork natural protein	+/-	+/-	+	+	-	-	-	-
9	pork 48 %, beef 5 %,	+	+	+	+	-	-	-	-
10	pork and beef 93%, pork hemoglobin	+	+	+	+	-	-	-	-
11	turkey 40%	-	-	-/+	**-/+**	-	-	+	+
12	chicken, pork bacon, hemoglobin	-	-	+	+	+	+	-/+	**-/+**
13	pork 42.3%, beef 25.8%, pork bacon 23.4%	+	+	+	+	-/+	-/+	-	-
14	pork	-/+	**-/+**	+	+	-/+	-/+	-	-
15	pork	-/+	**-/+**	+	+	-/+	-/+	-	-

CONCLUSION

In conclusion, adulteration is a serious global problem in food industry. Regular controls are necessary to ensure food security. It was found that the results obtained by DNA Microarray and Real Time PCR assays were identical with each other, and both methods should extensively be promoted for the detection of animal species in meat products.

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