Qualitative detection of species adulteration in commercial meat products using a validated ELISA method

B. Solomun Kolanović, I. Varga, Đ. Božić Luburić, I. Varenina, N. Bilandžić and M. Đokić*

Abstract

The aim of this study was to develop and validate an ELISA method for determining the type of meat in meat and meat products and its application in the detection of species adulteration in commercial meat products from retail chains in the Republic of Croatia. The developed method showed 100% specificity towards cattle, pig, sheep and poultry proteins and no cross-reaction with non-target food type antibodies, and was able to rapidly and reliably detect a given meat species in cooked meat samples at a 2% level of contamination. The analysis of meat products collected from various retail chains and butchers in the period from 2012 to 2022 showed satisfactory agreement with the declared species in 95.7% of cases. Canned pork products contained the least amount of potentially mis-declared products. Further, 35% of sudjuk samples, contained pork in addition to declared beef, while 26.7% of chicken sausages contained undeclared pork. Given that deviations of meat products from the declaration were determined in 4.3% of the total analysed samples, the importance of systematic prevention and detection of food fraud is emphasised through the establishment of a regular and effective system of supervision and control.

Key words: *meat; meat products; food safety; adulteration; ELISA method*

Introduction

During recent years, adulteration of meat and meat products has become a global threat to food safety due to possible harmful health effects. Although there is no harmonised definition of food adulteration in the European Union, the term fraud in the meat industry most often refers to substitutions with cheaper meat of lower quality, addition of undeclared ingredients to mask organoleptic defects, or false declaration of the production process or geographical region of production, all for the purpose of economic gain (Fikselova et al., 2020; Robson et al., 2021). This type of unfair practice in the food industry can cause a loss of consumer confidence,

Božica SOLOMUN KOLANOVIĆ*, PhD, Expert Advisor in Science, (Corresponding author: e-mail: solomun@veinst.hr), Ines VARGA, Mag. Chem, Professional Associate in Science, Đurđica BOŽIĆ LUBURIĆ, PhD, Senior Associate in Science, Ivana VARENINA, PhD, Research Associate, Nina BILANDŽIĆ, PhD, Scientific Advisor in Tenure, Maja ĐOKIĆ, PhD, Research Associate, Croatian Veterinary Institute, Zagreb, Croatia

economic losses, and can lead to health risks in sensitive individuals if allergic reactions occur, which is why it is extremely important to determine the authenticity of meat and establish regular controls. Provisions that mandate accurate food labelling and prevent fraudulent practices are established by Regulation (EC) No. 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety and Regulation (EU) No. 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers (EC, 2002; EC, 2011). Deviation of the composition of food from the declaration is considered non-compliance with food regulations, and in the case of proven intent, it is considered fraud for the purpose of achieving financial gain. In European Union Member States, the Rapid Alert System for Food and Feed (RASFF) has been established for the rapid exchange of information on health risks related to food among the competent authorities of the Member States.

Some cases of meat adulteration have caused great concern globally, such as the 2013 horsemeat scandal in Great Britain, where several regular monitoring activities detected the presence of horsemeat in beef products (hamburgers, lasagne, meatballs, etc.) (Abbots and Coles, 2013; Brooks et al., 2017). Another example occurred the same year in Ireland in frozen beef hamburgers, in which horse meat was found, and 85 percent of all samples contained pork (O'Mahony, 2013). Some recent reports indicate that food fraud remains widespread, as in the case in Colombia in 2022 where donkey and horse meat were adulterated with chemicals and sold in schools as beef (Anonymous, 2022). Recently, a long-standing halal beef fraud was also reported in Malaysia, where kangaroo and horse meat were mixed and sold as halal beef (Anonymous, 2020). In the case of persons with religious restrictions, this kind of adulteration of meat is a serious offense due to its impact on the religious or cultural identity. From the above, it is clear how important it is to implement the food declaration regulations and to develop a reliable analytical method for verifying the authenticity of meat-based food.

Different techniques for investigating meat composition are described in the literature, from immunoelectrophoresis (Necidova et al., 2002), spectroscopic methods (Al-Jowder et al. 2002; Alamprese et al., 2013), immunochemical methods (Asensio et al., 2008; Thienes et al., 2019a), molecular biology methods (Krčmař and Renčova, 2001; Aida et al., 2005; Ren et al., 2017) and mass spectrometry (Li et al., 2018; Prandi et al., 2019; Pu et al., 2023), of which enzyme-immunoassay (ELISA) and PCR methods are most often used. The ELISA method is widely used due to its fast and simple sample preparation, satisfactory sensitivity and relatively low cost; however, it is not reliable for species identification in highly processed food due to protein denaturation (Giovannacci et al., 2004; Asensio et al., 2008). Polymerase chain reaction (PCR) is a highly specific and sensitive method that, due to the stability of DNA at high temperatures, is also very robust and overcomes the limitations of the ELISA method. The disadvantage of this method is the reduced ability to distinguish tissues of the same species and reduced selectivity in the presence of plant ingredients in processed food (Cammà et al., 2012; Piskata et al., 2019). During the last decade, sophisticated -omics (genomic, proteomic and transcriptomic anal-

yses) and droplet digital PCR methods have become more interesting, not only for the identification of multiple species but also for the quantification of adulterated meat, though they are still not widely used due to the high costs of analysis (Flaudrops et al., 2015; Cai et al., 2017). Recently, biosensor methods have been developed which, due to the low cost and simplicity of the procedure, provide an attractive alternative to instrumental methods (Ali et al., 2014; Xu et al., 2018).

This paper presents the results of validation of a qualitative ELISA test for determining the presence of beef, sheep, pork and poultry proteins in meat, as well as the results of checking the conformity of canned meat products with the manufacturer's declaration.

Materials and methods

Sampling and sample preparation

Raw poultry, pork, beef and sheep meat were purchased from a retail chain in the city of Zagreb and used to prepare reference tissue samples for method validation. Reference samples were prepared by contamination of samples, for which two types of meat were used: meat of the tested species and background meat. By adding the appropriate amount of the tested species to the background meat and ensuring complete homogenisation, samples of known mass proportions, from 1% to 25% w/w protein originating from the tested species, were obtained. For example, a reference sample of 2% (w/w) pork was prepared by adding 2 g pork to 98 g background beef. Connective tissue and visible fat were removed from each sample, and homogenisation was performed carefully avoiding cross-contamination. Homogenised samples were stored at -18°C until analysis.

In addition, a total of 704 commercial meat products collected in the period from 2012 to 2022 from various retail chains, farms and butchers were analysed. Products belonged to various categories including dry-cured products and cooked sausage meats, minced meat products, pates and spreads and products from chopped meat (luncheon meats). The contents of these products consisted of pork, beef, chicken or turkey as the only meat ingredients, or a combination of two or more types of meat.

Reagents and standards

A double sandwich ELISA kit ELISA-TEK Cooked meat species kits (cat. no. 510604) manufactured by ELISA Technologies (Florida, USA) was used to determine the type of protein in cooked and preserved meat. The package contains a microtiter plate with 96 wells, positive control for each species, biotinylated antibody for each species, streptavidin peroxidase conjugate, ABTS substrate, peroxide citrate buffer, concentrated washing solution and stop solution.

Sodium chloride, sodium hydroxide and hydrochloric acid from Sigma-Aldrich (St. Louis, Missouri, USA) and Whatman® filter paper from Merck Millipore (Darmstadt, Germany) were used in sample preparation. Ultrapure water was prepared using a Direct Q 5UV system (Merck Millipore, Darmstadt, Germany).

Instrumentation

The following instruments were used in sample preparation: precision balance model Pioneer™ PA413C, Ohaus (New York, USA), homogeniser Waring 7011HS (Hartford, USA), water bath GFL 1083, GFL mbH (Burgwedel, Germany), Benchmixer™ mixer, Benchmark Scientific (New York, USA), centrifuge model Rotanta 460R, Hettich Zentrifugen (Tuttlingen, Germany) and mixer MS2 Minishaker

IKA®-WERKE (GmbH & Co. KG, Staufen, Germany). The absorbance was measured using an absorbance microreader Tecan model Sunrise (Salzburg, Austria).

Sample preparation

To start, 10 mL sodium chloride solution (0.9% (0.15M)) was added to 5 g homogenised sample (meat, meat product) and mixed by hand with a mixer for 1 minute. After standing for 1 hour at room temperature, the aqueous layer was centrifuged for 10 minutes at 3000 xg and filtered. After adjusting the extract pH to 6.0–8.0 by adding sodium hydroxide or hydrochloric acid, the obtained extract was used in the test. If the sample was not previously heat-treated, the sample was heated in a water bath for 15 minutes at 95-100°C before vortexing and centrifugation.

ELISA test procedure

The test was carried out according to the manufacturer's instructions with absorbance measurement at a wavelength of 414 nm and a reference wavelength of 492 nm. Negative and positive controls are provided with the test, which serve to assess the validity of the test and interpret the results, in accordance with the manufacturer's protocol.

Method validation

The following parameters were determined in method validation: specificity, detection limit, cut off level and sensitivity of the method.

The specificity towards meat proteins is determined by analysing samples of different types of meat and of different origins, as well as by analysing samples that, due to their protein content, could cause a cross-reaction with antibodies (milk powder, eggs, wheat flour, peanuts, soy flour or fish). For each of the examined animal species, 20 samples without contamination and 20 samples with a mass fraction of 2% of the contamination species were analysed.

The limit of detection was determined on samples that did not contain proteins of interest in 20 repetitions and calculated by adding the mean value of blank samples concentrations and three standard deviations of the samples.

The sensitivity to specific proportions of the protein of interest was determined by mixing reference meat samples to mass proportions of 1%, 2%, 5%, 10% and 25% w/w protein of the tested species, in six repetitions, while calculating the repeatability of the results expressed as the coefficient of variation of results (CV %).

The threshold value indicating that the sample contains proteins above the selected target level is called the cut-off or resolution level (Fm), and is determined by analysing 20 meat samples that do not contain proteins of interest and 20 meat samples with a mass fraction of 2% of the species of interest. From the obtained data, the lower limit of positivity T and the cut-off Fm value are calculated according to the following formulas:

$T = B + 1.64 \times SD$ and

$Fm = M - 1.64 \times SD$,

where B is the mean value of blank samples response, M is the mean value of positive samples response and SD is the standard deviation of the response of negative and positive samples.

The method is capable of detecting the proteins of tested species at the selected level if the difference between the blank and positive samples is successfully achieved, and at most one sample of the total number of tested samples has an absorbance lower than the calculated cut-off value.

Control of analytical results

In each analysis, samples with known mass fractions of the species of interest

and negative controls were also tested. In addition, the quality control of the results is ensured by participation in Proficiency testing organised by FAPAS, Fera Science Ltd (February 2021).

Results and discussion

The validation procedure of the qualitative ELISA test for species identification included determination of specificity, detection limit, sensitivity and cut-off level, as the level of decision on sample conformity (Table 1). Reference samples for validation of known mass fractions of 1% to 25% (w/w) protein of the tested species were prepared by combining previously weighed target species and background meat and performing the extraction according to the manufacturer's instructions, with pre-heat treatment. Detection limits were calculated in the range from the lowest 0.07 for poultry to the highest 0.27 for cattle, expressed as optical density (OD). The ability of the method to detect a certain type of meat in the presence of a non-target meat type was determined by testing the cross-reaction against antibodies and shown in Table 2. As can be seen, the ELISA test showed 100% specificity for each animal species.

In all samples contaminated with 1% poultry, pork, beef or sheep, the target proteins were found to be present, however this level proved to be too low for identification of pork proteins as it gave more than 5% false negative results. Validation was therefore continued at a contamination level of 2% for all animal species, still low enough to estimate exceedance rates in routine analysis. At the selected level for each animal species, the cut off value was calculated as the limit for the assessment of sample conformity (Table 1).

According to Liu et al. (2006), assay responses to pork proteins were lower when prepared in a binary mixture with beef compared to pork in poultry, which was probably the case in this study. Testing the sensitivity of the method at lower pork levels using poultry as the background meat will be the scope of future research. Figure 1 shows a clear difference in the distribution of OD values for blank samples and samples contaminated with 2% protein of the tested species, indicating a reliable detection of protein species up to a mass fraction of 2%.

The results of method sensitivity and precision on different levels of proteins of interest are shown in Table 3. Variability was lower at higher protein levels, with an overall CV of the assay of 9.77%. Repeatability was also determined by analysing the control proficiency sample organised by FAPAS Fera Science Ltd in six replicates. The laboratory successfully detected all animal species making up the tested

Table 1. Validation parameters of ELISA method for determining the type of protein in cooked meat samples

Animal species	Level of contamination (% (w/w))	Lower limit of positivity T (OD)	Cut-off value (OD)	Limit of detection (OD)	False negative rate (%)
Poultry	2	0.10	0.30	0.07	0
Pork	2	0.15	0.63	0.21	0
Beef	2	0.13	1.16	0.27	0
Sheep	2	0.14	0.55	0.19	0

Table 2. Cross-reactivity of the ELISA method

Sample	Absorbance at 414-492 nm[a]			
	Poultry	Pork	Beef	Sheep
Pork	0.092 ± 0.005	2.649 ± 0.087	0.039 ± 0.004	0.045 ± 0.004
Beef	0.047 ± 0.014	0.090 ± 0.007	1.482 ± 0.077	0.132 ± 0.017
Poultry	2.582 ± 0.154	0.101 ± 0.003	0.043 ± 0.003	0.047 ± 0.004
Sheep	0.044 ± 0.002	0.157 ± 0.049	0.092 ± 0.005	1.738 ± 0.123
Milk powder	0.048 ± 0.002	0.075 ± 0.001	0.274 ± 0.002	0.076 ± 0.004
Eggs	0.084 ± 0.005	0.102 ± 0.046	0.035 ± 0.001	0.090 ± 0.007
Wheat flour	0.051 ± 0.004	0.081 ± 0.009	0.042 ± 0.002	0.094 ± 0.009
Sea fish	0.062 ± 0.007	0.062 ± 0.014	0.034 ± 0.002	0.127 ± 0.014
Soya flour	0.054 ± 0.010	0.039 ± 0.002	0.037 ± 0.001	0.119 ± 0.005
Peanut	0.053 ± 0.005	0.046 ± 0.004	0.033 ± 0.001	0.111 ± 0.017

[a]mean of 5 replicates ± SD

Table 3. Method sensitivity and reproducibility at different protein levels

Protein species	Reference samples	$OD_{414\ i\ 492\ nm}$ [a]	SD	CV (%)
Beef	2% beef in pork	1.40	0.14	10.4
	5% beef in pork	1.55	0.07	4.74
	10% beef in pork	1.61	0.09	5.32
	25% beef in pork	1.65	0.06	3.53
Sheep	2% sheep in beef	0.78	0.14	18.1
	5% sheep in beef	0.83	0.06	7.36
	10% sheep in beef	0.95	0.05	4.91
	25% sheep in beef	1.23	0.04	3.48
Pork	2% pork in beef	0.73	0.06	8.20
	5% pork in beef	1.09	0.06	5.32
	10% pork in beef	1.32	0.10	7.73
	25% pork in beef	2.25	0.05	2.44
Poultry	2% poultry in beef	0.48	0.11	23.5
	5% poultry in beef	0.59	0.14	24.1
	10% poultry in beef	0.64	0.16	24.1
	25% poultry in beef	0.69	0.02	3.04

[a]absorbance was measured at 414 nm with a 492 nm reference filter

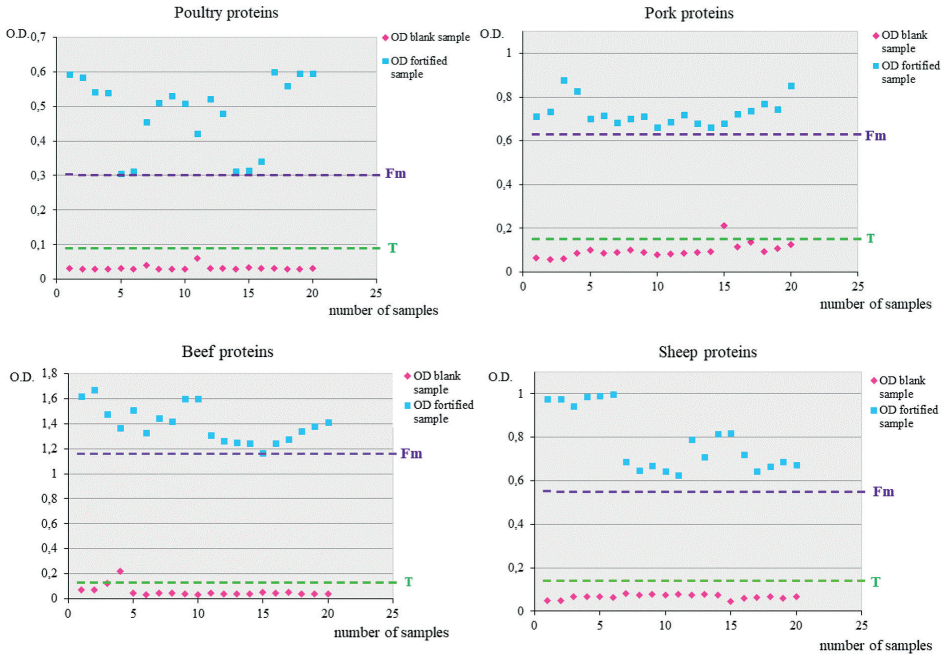

Figure 1. Distribution of optical densities (OD) of blank samples and samples contaminated with 2% of the tested species

material (92% bovine protein and 4% each sheep and pork protein), thus confirming the acceptability of the method.

In addition to the cross-reactivity of the ELISA test with other types of meat, the reactivity to proteins from milk, eggs, wheat flour, peanuts, soy flour and fish was tested following the same analytical protocol. As seen in Table 2, the test did not cross-react with the tested proteins, which is particularly important due to the frequent use of proteins as additives in the production of meat products. However, the test detected beef in milk powder samples around the detection limit of the method, though lower than the cut-off value. According to Thienes et al. (2019b), products containing cow's milk can affect the results of the ELISA test, although most processed foods contain insufficient quantities of dairy products to interfere with interpretation of the test.

Detection of meat species in commercial meat products

The ability of the ELISA method to detect pork, beef and poultry proteins in commercial samples was tested on a variety of processed meat products, including various dry-cured products, cooked sausage meats, minced meat products, pates and canned meat. The results in Table 4 show that 7 of 28 dry-cured products (25.0%), 16 of 129 cooked sausage meats (12.4%), 4 of 135 canned meat (3.0%) and 3 of 15 raw meat products (20.0%) did not comply with the specified product declaration. Seven sudjuk samples declared as beef contained a mixture of beef and pork.

All dry-cured pork products, cooked pork sausages, and pates corresponded

Table 4. Results of meat type identification in commercial meat products

Type of product	Number of samples with declared meat ingredient	Identified type of protein	Non-compliant declaration	Non-compliant declaration (%)
Dry-cured products (kulen, tea sausage, winter salami, sudjuk)	8 pork	8 pork	0	25.0
	20 beef	13 beef, 7 beef and pork	7	
Semi-permanent sausages (Tyrolean, Zagorje, Carniola, Posebna Cooked Pork, Parisian, safalada, hot dogs, wrapped ham, ham sausage)	66 pork	66 pork	0	12.4
	2 beef	2 beef	0	
	46 poultry	32 poultry 14 poultry and pork	14	
	6 pork and beef	4 pork and beef, 2 pork, beef and poultry	2	
	9 pork and poultry	9 pork and poultry	0	
Canned meat	103 pork	103 pork	0	3.0
	16 beef	12 beef 4 beef and pork	4	
	14 pork and beef	14 pork and beef	0	
	1 pork and poultry	1 pork and poultry	0	
	1 pork, beef and poultry	1 pork, beef and poultry	0	
Pate (liver, tea, with vegetables)	309 pork	309 pork	0	0
	85 poultry	85 poultry	0	
	3 beef	3 beef	0	
Raw meat products (minced meat, burgers, čevapi, hamburger)	5 pork	4 pork 1 pork and beef	1	20.0
	2 poultry	2 poultry	0	
	7 beef	5 beef 2 beef and pork	2	
	1 beef and poultry	1 beef and poultry	0	

to the protein composition on the product declaration. Among the hot dog samples, 8 of 30 declared as chicken contained poultry and pork. Analysis of turkey salami also showed unsatisfactory results in 6 of 10 samples due to pork content. In addition to declared beef, one Zagorje sausage and one Tyrolean sausage contained pork and poultry. All luncheon meats and cold cuts showed compliance with the declaration, except for four beef cold cuts that contained a mixture of beef and pork.

By analysing minced meat samples, substitute types of pork meat were identified in 2 of 7 samples declared as beef. One sample of ground pork also contained undeclared beef. The results show that undeclared types of meat were

mostly found in products in which beef was declared as the main raw material (27.1%), followed by poultry products (10.5%) and the least in pork products (0.2%). In the category of meat and meat products, Cubero-Leon et al. (2014) reported minced and homogenised meats as the most frequently adulterated products.

Of the total of 704 totally analysed samples, 30 products listed incorrect data on the declaration, resulting in 4.3% incompliant results. The reasons why the samples may contain proteins of a type not listed on the declaration are of an economic nature in most cases, though unintentional contamination can also occur due to inadequate cleaning of the production line.

In addition to these samples, a large number of beef goulash samples were analysed for beef proteins and gave false negative results, likely as a result of high temperature processing in the production of this type of meat product. The identification of the declared species in the product can also be hindered by the presence of inhibitory substances, moisture or high fat content in the product. For this reason, many authors recommend the PCR method as a more accurate method for protein detection in samples processed under sterilisation conditions.

Conclusions

Validation parameters including specificity, sensitivity, detectability and precision show that the developed ELISA method is suitable for qualitative determination of pork, beef, sheep and poultry in cooked meat samples and processed meat products at the 2% level. The kit showed no cross-reactivity with other types of meat or with common food ingredients, except for a slight reactivity with milk that did not affect the interpretation of the results in the bovine test. Despite the disadvantages in terms of sensitivity and analysis of products processed by the sterilisation process, the ELISA test proved to be rapid, cheap and reliable for determining the type of meat. Various processed meat products from retail chains in the Republic of Croatia were analysed using a validated ELISA method and compared with the species content listed in the declaration. Of the total number of analysed samples, 4.3% of commercial meat products did not meet the requirements of Regulation (EC) No. 178/2002. In conclusion, the current negative practice on the meat market shows that fraud is still a widespread issue, especially in products where beef is declared as the major content, as revealed in our study. In order to ensure consumer protection from unfair practices in the food industry, it is necessary to raise awareness among producers and competent authorities, and to ensure successful implementation of meat quality control programmes.

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Ispitivanje patvorenja komercijalnih mesnih proizvoda validiranom ELISA-metodom

Dr. sc. Božica SOLOMUN KOLANOVIĆ, stručni savjetnik u sustavu znanosti, Ines VARGA, stručni suradnik u sustavu znanosti, Đurđica BOŽIĆ LUBURIĆ, viši stručni suradnik u sustavu znanosti, Ivana VARENINA, znanstveni suradnik, Nina BILANDŽIĆ, znanstvena savjetnica u trajnom zvanju, Maja ĐOKIĆ, Hrvatski veterinarski institut, Zagreb, Hrvatska

Cilj je ovog rada razvoj i validacija ELISA-metode za određivanje vrste mesa u mesu i mesnim proizvodima i njezina primjena u utvrđivanju autentičnosti i patvorenja komercijalnih mesnih proizvoda iz maloprodajnih lanaca u Republici Hrvatskoj. Razvijena metoda je pokazala 100 % specifičnost prema proteinima: goveda, svinja, ovca i peradi, bez unakrsne reakcije s antitijelima neciljnih vrsta hrane. Utvrđeno je da je metoda sposobna brzo i pouzdano detektirati 2 % proteina goveda, ovaca, peradi i svinja u uzorcima kuhanog mesa. Analiza mesnih proizvoda koji su prikupljeni iz različitih trgovačkih lanaca i mesnica u razdoblju od 2012. do 2022. pokazala je zadovoljavajuću podudarnost s deklariranim vrstama u 95,7 % slučajeva. Najmanje potencijalno pogrešno deklariranih proizvoda utvrđeno je kod konzerviranih svinjskih proizvoda. Čak je 35 % sudžuka, osim deklarirane govedine, sadržavalo i svinjetinu, dok je 26,7 % pilećih hrenovki sadržavalo nedeklariranu svinjetinu. S obzirom da su u 4,3 % ukupno analiziranih uzoraka utvrđena odstupanja mesnih proizvoda od navedenog na deklaraciji ističe se važnost sustavne prevencije i otkrivanja prijevara s hranom kroz uspostavu redovitog i učinkovitog sustava nadzora i kontrole.

Ključne riječi: *meso, mesni proizvodi, sigurnost hrane, patvorenje mesnih proizvoda, ELISA metoda*