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Authentication of meat and meat products vs. detection of animal species in feed – what is the difference?

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Abstract. Authenticity of food is an issue that is growing in awareness and concern. Although food adulteration has been present since antiquity, it has broadened to include entire global populations as modern food supply chains have expanded, enriched and become more complex. Different forms of adulteration influence not only the quality of food products, but also may cause harmful health effects. Meat and meat products are often subjected to counterfeiting, mislabelling and similar fraudulent activities, while substitutions of meat ingredients with other animal species is one among many forms of food fraud. Feed is also subject to testing for the presence of different animal species, but as part of the eradication process of transmissible spongiform encephalopathies (TSE). In both food and feed cases, the final goal is consumer protection, which should be provided by quick, precise and specific tools. Several analytical tests have been employed for such needs. This paper provides an overview of authentication of meat and meat products compared with species identification in feed control, highlighting the most prevalent laboratory methods.

1. Introduction

Adulteration in food has been a concern since the beginning of civilization, as it not only influences the quality of food products but also may cause harmful health effects. Adequate testing of food and adulterant detection in various food products are required for value assessment and to assure consumer protection against fraudulent activities [1]. The food protection concept includes food quality, food safety, food fraud and food defense. Although there are some overlaps between these four elements, in general, they are defined as follows: A food quality risk is an economic threat that is unintentional and influences a product's value to consumers. A food safety risk is unintentional contamination of food that causes adverse health consequences. A food fraud risk is economically motivated and intentional, but is not intended to be a public health food threat. A food defense risk is a public health threat that is intentional, such as malicious tampering or terrorism. It is ideologically motivated and makes the food injurious to health. [2].

Food fraud is a public health food risk that is growing in awareness, concern and danger. It has been present since antiquity, as evidence found in Roman amphorae containing fraudulent olive oil and wine shows. But in ancient times, the scale was limited and it covered a small geographic area. Because modern food supply chains have been expanded, enriched and compounded, the risk of food fraud has broadened to include entire global populations [3]. This collective term is used to cover



deliberate substitution, addition, tampering, or misrepresentation of food, food ingredients, or food packaging; it also includes false or misleading statements made about a product for economic gain [4]. Most food fraud cases are not harmful, but there are exceptions, like melamine in Chinese skimmed milk powder [5], sudan dyes in spices [6], false labeling of puffer fish as monkfish [7] and the plasticizer di(2-ethylhexyl) phthalate (DEHP) being used as a cheaper substitute for clouding agents in food and beverages [8].

The main areas susceptible to fraud in the meat industry are: 1) the origin of meats and the animal feeding regime (as in the case of certificated regional products, for example); 2) substitutions of meat ingredients with other animal species, tissues, fat or proteins; 3) modifications of the processing methods for producing meat products and 4) additions of non-meat components such as water or additives. Recently, greater numbers of people are concerned about the meat they eat, so accurate labelling is important to inform their choice. Detection of animal species in feed occurs from a completely differently motivation, and it recently became mandatory in the eradication process of transmissible spongiform encephalopathies (TSE). In fact, shortly after the escalation of the bovine spongiform encephalopathy (BSE) crisis in Great Britain in 1986, it was determined that the source of infection was contaminated feed, i.e. infectious ruminant protein processed in meat and bone meal (MBM). One of the most important measures was the introduction of legislation which prevented these nutrients from entering the food chain and the establishment of appropriate analytical control of implementation of such regulations [9].

This paper intends to provide an overview on the issue of authentication of meat and meat products in comparison to species identification in feed control, highlighting the most prevalent laboratory methods. The aim is also to emphasize that simple transfer of protocols between these similar, but not the same matrices is not advisable.

2. Detection of animal species

Meats derived from different animal species are priced differently, and consumers want to know what kind of meat they are purchasing with absolute certainty. Adulteration of meat products poses a serious problem, not only for economic reasons, but also for religious and moral reasons, as well as due to allergies to meat derived from individual animal species. Proper labelling is also important to help fair trade [10]. According to the European Council Regulation (EC) No. 178/2002 28 January 28 2002, laying down the general principles and requirements of food law, food adulteration and misleading consumers is illegal. The same rules for food are implemented in Serbia through national regulations, as well as compliant legislation that applies to animal feed [11]. Some examples of undeclared animal species in meat products are given in table 1, adapted from Ballin [12].

Table 1. Examples of undeclared animal species in meat products.

Investigated product	Country of investigation	% of mislabelling cases (No of analysed samples)
Hamburgers	Mexico	39% (23)
Sausages	Mexico	29% (17)
Meat products	USA	15.9% raw; 22.9% cooked (902)
Meat products	Turkey	22% (100)

Identifying authenticity of meat and meat products is an important issue in food regulatory control for determining fraudulent actions and for assuring accurate food labelling, while detection of animal species in feed belongs to the group of systemic measures for the eradication and prevention of TSEs, so it is a part of the activities within the food safety chain [13]. According to feed legislation [14], feed must be in accordance with the following principles: no use of ruminant processed animal protein (PAP), no use of any PAP in feed intended for ruminants and herbivores, and no intra-species

recycling (commonly termed cannibalism). So, as indicated in the TSE Road Map 2 [15], the goal of the European Commission is to continue the review of the measures following a stepwise approach supported by a solid scientific basis while maintaining a high level of food safety, so there must be adequate analytical methods to ensure absolute reliability.

In both food and feed cases, the final goal is consumer protection, which should be provided by quick, precise and specific food and/or feed control and proper identification of animal species. Several analytical methodologies have been employed for such needs, based on anatomical, histological, microscopic, organoleptic, chemical, electrophoretic, chromatographic, or immunological principles. However, due to specificities of their distinctive limitations, many techniques have been surpassed by the DNA-based molecular techniques or determination of proteins/peptides. In recent decades, most of the methods for identifying the species origin in meat and meat products, and especially in feed, are based on polymerase chain reaction (PCR) due to their high specificity and sensitivity and rapid processing time [16].

The authenticity of meat and meat products, as well as feed, includes various aspects and involves a wide range of analytes. Meat substitution is among the most frequent fraudulent and/or accidental situations, and refers to both species and tissue adulteration. Unfortunately, there is no perfect analytical tool that can provide solutions for all the problems in controlling the composition of food and feed. Still, many complementary, different approaches do exist. Techniques based on spectroscopy would be appropriate screening methods for meat and meat products, while more reliable and unambiguous results are achievable by immunoassays, molecular or mass spectrometry-based analysis [17]. Nevertheless, the most common methodologies in species determinations are based on DNA and immunological principles. Genetic methods are the most specific and sensitive for this purpose. However, they require expensive laboratory equipment and a certain degree of expertise. As an alternative, immunological assays can be used to reduce the test time and cost. Among these, ELISA (Enzyme-Linked ImmunoSorbent Assay) has been the most widely used technique in detecting food authenticity because of its specificity, simplicity and sensitivity. Regarding feed control, for all EU Member States and candidate countries, the only officially allowed method, is to implement SOPs and protocols published on the EU Reference Laboratory for Animal Proteins in Feedingstuffs (EURL-AP) website (<http://eurl.craw.eu>).

3. Enzyme-Linked ImmunoSorbent Assay (ELISA)

ELISA is an immunological technique in which an enzyme, a protein that catalyzes a biochemical reaction, is utilized to detect the presence of an antibody or an antigen in a sample. Two types, the indirect and the sandwich ELISA, are the most commonly used for food authentication. The indirect ELISA uses two antibodies, one specific to the antigen and the other coupled to an enzyme. This second “enzyme-linked” antibody gives the assay its name, and causes a chromogenic or fluorogenic substrate to produce a signal. Sometimes, this second antibody can be linked to a protein such as avidin or streptavidin if the primary antibody is biotin labeled. In the sandwich ELISA, the antigen is bound between two antibodies: the capture antibody and the detection antibody. The detection antibody can be tied to an enzyme or can bind the conjugate (enzyme-linked antibody) that produces the biochemical reaction. This method produces qualitative or quantitative results. Qualitative ELISA detects the presence or absence of an analyte in the sample. The cutoff between positive and negative result is determined by the analyst and is statistically based. In quantitative ELISA, used to detect amounts of the analyte, the optical density or fluorescent units of the sample is interpolated into a standard curve which is typically a serial dilution of the target [18].

Both polyclonal and monoclonal antibodies against muscle and serum animal proteins are used in the method variants for identification of different animal species. Until recently, the great limitation of ELISA methodology has been that the target proteins are sometimes denatured during processing. This has been particularly important for feed control in which the animal proteins are treated by sterilization: by steam pressure of 3 bar, at a temperature of 133°C for 20 minutes, and therefore, the target protein epitope cannot be present in the condition detectable by the antibodies. This limitation

has been mainly solved because of the development of antibodies against thermostable proteins [19]. In recent years, advances in immunoassay technology have led to development of lateral flow tests (or dipsticks), which employ the same principles as the ELISA tests, but coat the antibodies and other reagents on a nitrocellulose membrane rather than the inside of test wells or paddles, and they use colloidal gold, dye, or latex bead conjugates to generate a visible signal. The simplicity of both type of tests and the short time required for the analysis make them suitable for screening of a large number of food samples [20].

According to Giovannacci et al. [21], there are limits for species detection in processed meat products, depending on various parameters, such as the fat content, the severity of heat processing, the origin of muscles and the maturation state of meat. Hence, detection limits might be different from one product to another. Although, from a theoretical point of view, ELISA methods, as well DNA methods are quantitative, they mainly give qualitative results. However, Kotoura et al. [22] described a strategy to determine the content of beef meat in different mixed meat products. In contrast to the performance of most ELISA tests for meat authentication, the reliability of immunological determination of ingredients of animal origin in animal feed is still under question [23]. Moreover, some requirements, such as greater sensitivity and better specificity need to be fulfilled [23]. Based on the latest EFSA scientific opinion [24], positive results require confirmation by another method, while false negatives frequently occur.

4. Polymerase Chain Reaction (PCR)

PCR is an efficient way to copy small segments of DNA, to determine animal species. Compared to proteins, DNA has a higher thermal stability, it is present in the majority of cells and potentially enables identical information to be obtained from the same animal, regardless of the tissue of origin. As this technique can theoretically amplify one copy of target DNA, the limit of detection is therefore often lower than observed in protein-based methods. It allows identification of meat species under different processing conditions. However, the variability of DNA at the species and target tissue levels makes DNA-based methods somewhat unsuitable for the quantification of exact percentages of different species in meat and meat products. That is why the majority of published methods are qualitative, although there are some studies that have demonstrated a correlation between meat content (w/w) and signal intensity [25]. Quantitative analysis should be based on real time PCR and results expressed as genome/genome equivalents. The PCR amplified sequence must originate from genomic DNA, while use of mitochondrial DNA is not possible in quantification of species as the amount of mitochondrial DNA in tissue varies. The specific PCR amplified DNA sequence has a large influence on the limit of quantification [26].

Regarding feed control, besides microscopy, the introduction of PCR as an official method and the validation of a PCR assay for the detection of ruminant DNA in feed allowed the re-authorization of non-ruminant PAP in feed for aquaculture animals as of 1 June 2013. The next steps could be the use of poultry PAP for pigs and pig PAP for poultry, but only after validation of adequate analytical tools (e.g. PCR assays) that would allow efficient control. Due to interference of authorized animal ingredients (e.g. fats, blood products, dairy products) with PCR results, additional analytical approaches will probably be needed [27]. In addition to its own research, in 2014, the EURL-AP initiated an international laboratory network to investigate and develop alternative techniques, such as aptamers, mass spectrometry and ELISA. The most promising method is probably mass spectrometry [28]. Recently the identification of proteins and peptide biomarkers allowing the detection of PAPs by mass spectrometry produced very interesting results, but efforts must be continued and the journey to validation and implementation in the control laboratories is long. All official protocols and the versions in force are easily downloadable from the EURL-AP website [29].

Species differentiation of raw materials in industrial food and feed preparation, as well as the detection of animal species in foods and feeds remains a constant challenge. Various PCR protocols have proved adequate for detecting small amounts of DNA in meat samples, by amplifying a target

region of template DNA in a rapid and sensitive manner. Real time PCR seems to be the backbone of the future system of TSE prevention in feed control [30].

5. Conclusion

Among the wide range of analytical methods available for determining food authenticity, ELISA has performed well because of its sensitivity, specificity, promptness, cheapness, ease of performance and the low investment in equipment compared to other techniques. Although ELISA limitations for feed control have been shown, and most of the immunoassays for meat speciation are not applicable to feed detection because protein antigens denature during high-temperature rendering, this methodology could allow specific screening in both the meat and feed industries. On the other hand, PCR-based methods are highly specific and sensitive, but generally are unable to distinguish between different tissues of the same species. Also, variability of DNA content on the species and target tissue levels makes DNA-based methods somewhat unsuitable for quantification of different animal species in foods and feeds. For all these reasons, the proteomic approach, identifying different peptide biomarkers, and usually combined with mass spectrometry, is of importance.

It is important to emphasize that differences in the authentication of meat and meat products in comparison with feed do exist and they are related to the complexity of the matrices and processing treatments. Feed control, as food safety issue, is a very well regulated area, and analytical methods and procedures to detect constituents of animal origin are strictly prescribed. These must be fully respected and completely implemented during official controls. Finally, all analytical methods and control strategies, regardless of sample type, have to enforce national and trans-national laws and regulations and to ensure full consumer protection from all possible viewpoints: food fraud, food quality, food safety and food defense.

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