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PROCEDURES FOR THE IDENTIFICATION AND DETECTION OF ADULTERATION OF FISH AND MEAT PRODUCTS

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ABSTRACT

The addition or exchange of cheaper fish species instead of more expensive fish species is a known form of fraud in the food industry. This can take place accidentally due to the lack of expertise or act as a fraud. The interest in detecting animal species in meat products is based on religious demands (halal and kosher) as well as on product adulterations. Authentication of fish and meat products is critical in the food industry. Meat and fish adulteration, mainly for economic pursuit, is widespread and leads to serious public health risks, religious violations, and moral loss. Economically motivated adulteration of food is estimated to create damage of around € 8 to 12 billion per year. Rapid, effective, accurate, and reliable detection technologies are keys to effectively supervising meat and fish adulteration. Various analytical methods often based on protein or DNA measurements are utilized to identify fish and meat species. Although many strategies have been adopted to assure the authenticity of fish and meat and meat a fish products, such as the protected designation of origin, protected geographical indication, certificate of specific characteristics, and so on, the coverage is too small, and it is unrealistic to certify all meat products for protection from adulteration. Therefore, effective supervision is very important for ensuring the suitable development of the meat industry, and rapid, effective, accurate, and reliable detection technologies are fundamental technical support for this goal. Recently, several methods, including DNA analysis, protein analysis, and fat-based analysis, have been effectively employed for the identification of meat and fish species.

Keywords: food fraud; adulteration; detection method; protein technologies; DNA technologies

INTRODUCTION

At present, there is no harmonized definition of food fraud in the European Union (EU) 2017. However, it is commonly accepted that the term 'food fraud' covers any violation of food law that is an intentional and deceptive misrepresentation of food for financial gain **(van Ruth et al., 2017; EC, 2019.** Food fraud is about "any suspected intentional action by businesses or individuals to deceive purchasers and gain undue advantage therefrom. **Spink and Moyer (2011)** have elaborated on this definition and describe seven types of food fraud: adulteration, tampering, over-run, theft, diversion, simulation, and counterfeit. These intentional infringements to the EU agri-food chain legislation may hinder the proper functioning of the internal market and may also constitute a risk to humans. However, existing databases that monitor food fraud Such as the Rapid Alert System for Food and Feed (RASFF) and HorizonScan have their categorizations **(Bouzembrak et al., 2018)**. RASFF has six categorizes for fraud (Improper, fraudulent, missing or absent health certificates; illegal importation; tampering; improper, expired, fraudulent or missing common entry documents or import declarations; expiration date; mislabeling) as does HorizonScan (adulteration/substitution, fraudulent health certificate/documentation, produced without an inspection, unapproved premises, expiry date changes). Four key operative criteria are referred to for distinguishing whether a case should be considered as fraud or as non-compliance: if a case matches all four criteria, then it could be considered a suspicion of fraud: violation of EU rules, deception of customers, undue advantage and intention. Meat and fish are food categories that are highly vulnerable to adulteration. Although there are various national and international laws for supervising the quality and safety of fish, meat, and meat and fish products, meat adulteration is still widespread. Most meat adulteration is economically motivated, such as the low-cost addition of duck meat and fish to mutton **(Wang et al., 2019a),** which causes consumers to suffer economic losses. Meat and fish adulteration may lead to serious public health risks, such as exposure to toxins, pathogens, or allergens in these products **(Magiati et al., 2019; Spink and Moyer, 2011)**.

MEAT AND FISH ADULTERATIONS

The demand for meat and fish products is high and as a result, meat is one of the most highly-priced food commodities; therefore, a prime target for food fraud **(Cawthorn et al., 2013)**. The examples of adulteration are presented in Table 1.

Table 1 Scholarly reports on fish and meat ingredient fraud and analytical methods for detection.

Ingredient Category	Ingredient	Adulterant	Type of fraud	Publictation year	Reported detection method and reference
Meats	Chicken meat (cornfed)	Chicken meat fromnon cornfed chickens	Replacement	2010	IRMS (13C/12C) on extracted protein and lipid fractions of meat **(Rhodes et al., 2010)**
Meats	Meat products	Chickpea flour	Replacement	2009	HPLC for isoflavones, phytic acid, and galactooligosaccharides (adulterant markers) **(Vanha et al., 2009)**
Meats	Meat products	Pea flour	Replacement	2009	HPLC for isoflavones, phytic acid, and galactooligosaccharides (adulterant markers) **(Vanha a et al., 2009)**
Meats	Meat products	Rice flour	Replacement	2009	HPLC for isoflavones, phytic acid, and galactooligosaccharides (adulterant markers) **(Vanha et al., 2009)**
Meats	Meat products	Soy flour	Replacement	2009	HPLC for isoflavones, phytic acid, and galactooligosaccharides (adulterant markers) **(Vanha et al., 2009)**
Meats	Minced meat (beef)	Ox offal tissue (kidney or liver)	Replacement	1999	MIR with chemometrics **(Al-Jowder et al., 1999)**
Meats	Minced meat (chicken, pork, or turkey)	Meat from non-authentic species	Replacement	1999	MIR with chemometrics **(Al-Jowder et al., 1999)**
Meats	Processed meat product	Soybean protein	Replacement	2005	Perfusion reversed phase chromatography with UV detection on extracted protein for adulterant marker detection **(Castro- Rubio et al., 2005)**
Seafood	Anglerfish	Anglerfish of non-authentic species	Replacement	2008	Review of methods: HPLC-MS/MS, ELISA, dients ed compounds extractive electrospray ionization timeofflight MS, and GC-MS **(Tittlemier, 2010)**
Seafood	Canned tuna	Bonito (*Euthynnus affinis*)	Replacement	1996	Sequence and restriction site analysis of PCR mitochondrial DNA **(Ram, Ram and Baidoun, 1996)**
Seafood	Canned tuna	Frigate mackerel (*Auxis thazard*)	Replacement	1996	Sequence and restriction site analysis of PCR mitochondrial DNA **(Ram, Ram and Baidoun, 1996)**

Table 1 Scholarly reports on fish and meat ingredient fraud and analytical methods for detection. Continue.

Ingredient Category	Ingredient	Adulterant	Type of fraud	Publictation year	Reported detection method and reference
Seafood	Crab (species specific)	Crustacean of non- authentic species	Replacement	2007	UV-Vis spectrometry with chemometrics **(Gayo and Hale, 2007)**
Seafood	Crab meat	Surimi-based artificial crab meat	Replacement	2006	UV-Vis spectrometry with chemometrics **(Gayo and Hale, 2006)**
Seafood	Eel	Fish of non-authentic species	Replacement	2008	DNA based method using fluorogenic ribonuclease protection assay to detect single nucleotide polymorphisms **(Kitaoka et al., 2008)**
Seafood	Fish	Melamine	Replacement	1982	Wet-chemical method with UV detection **(Cattaneo and Cantoni, 1982)**
Seafood	Fish	Non-authentic species	Replacement	2001	Isoelectric focusing electrophoresis for protein fingerprinting **(Etienne et al., 2001)**
Seafood	Grouper (*Epinephelus guaza*)	Wreck fish (*Polyprion americanus*) or Nile rRNA gene by PCR followed by single perch (Lates strand conformational polymorphism niloticus)	Replacement	2001	DNA analysis using mitochondrial 12S rRNA gene by PCR followed by single strand conformational polymorphism analysis **(Asensio et al., 2001a)**
Seafood	Prawns	Crustacean of non-authentic species	Replacement	2008	PCR **(Pascoal et al., 2008)**
Seafood	Scampi (*Neplirops norvegicus*)	Crustacean of non-authentic species	Replacement	1995	SDS electrophoresis on protein extract **(Craig, Ritchie and Mackie, 1995)**

Uncovering of adulterated meat products is important for several reasons. Allergic individuals and those who hold religious beliefs that specify allowable intake of certain species have a special interest in proper labeling. Proper labeling is also important to help fair-trade. The need for analytical species-specific methods is clearly illustrated by the following examples: **Hsieh, Chai and Hwang (2007)** found, with the use of immunoassays, meat from undeclared animal species in 15.9% of cases in raw products and 22.9% of cases in cooked products analyzing a total of 902 meat products. In a more recent investigation performed on 100 meat products, also with the use of immunoassays, meat from undeclared species was found in 22.0% of cases, primarily with poultry substituting beef **(Ayaz et al., 2006)**. The provenance of food, especially meat products, is a sensitive topic but there are tools available to support producers in demonstrating compliance with legislators and other authorities. Since the level of awareness about food quality and safety has recently increased, food fraud has become a major global issue. Hence, the identification of meat and fish products adulteration with unfavorable and inappropriate animal species is important from health, economic, and religious points of view **(Mousavi et al., 2015)**. Currently, the protein-based techniques (e.g. electrophoresis, isoelectric focusing, ELISA, and chromatography) have been utilized for meat and fish adulteration. These methods are laborious, expensive, and sophisticated instrumentation

with great technical proficiency **(Calvo et al., 2002, von Bargen et al., 2014)**.

Numerous analytical techniques which rely on protein analysis have been developed for fish species identification: electrophoretic techniques such as isoelectric focusing or SDS-PAGE **(Ataman, Celik and Rehbein, 2006, Mackie et al., 2000)**; chromatographic techniques **(Horstkotte and Rehbein, 2003, Knuutinen and Harjula, 1998)** and immunological techniques such as immunodiffusion and ELISA **(Fernández et al., 2002a, Ochiai et al., 2001)**. Therefore, the development of advanced detection methods constitutes an important first line of defense for both detecting and deterring food fraud **(Moore, Spink and Lipp, 2012)**. Although most of these methods are of considerable value in certain instances, they are not suitable for routine sample analysis because proteins lose their biological activity after animal death, and their presence and characteristics depend on the cell types. Furthermore, most of them are heat-labile. Thus, for fish species identification in heat-processed matrices, a DNA method rather than protein analysis is preferable **(Lockley and Bardsley, 2000)**.

DNA TECHNOLOGIES

As a prerequisite for accurate species quantification, DNA has to comply with minimum requirements about yield, purity, and integrity. Yield is an important parameter since food DNA has to be in a sufficient amount to allow the reliable and repeatable downstream analysis of meat species **(Heydt et al., 2014)**. The concentration and purity of DNA extracts are critical factors dominating the results of real-time PCR. DNA quantification is typically measured by either spectrophotometric or fluorometric methods, with the former representing the most commonly used technique **(Costa et al., 2017)**. DNA integrity determines the fraction of DNA that can be amplified by PCR **(Gilbert et al., 2007)** and it can be evaluated based on the average size distribution of fragmented DNA. Although often underestimated, DNA isolation is a crucial step for molecular analysis of food due to its heterogeneity in terms of composition and processing. The presence of chemical inhibitors, proteins, and/or damaged DNA are common situations in meat food analyses. Moreover, the extraction methods themselves can further influence the yield, purity, and integrity of DNA depending on the type of food matrix **(Şakalar et al., 2012)**. The final consequence is that the amount of species DNA determined in the product would not reflect the real amount in the source material, impairing quantitative measurements **(Primrose et al., 2010)**. DNA exists in all tissues of individual animals and is more conserved than proteins **(Kumar et al., 2015; Xiang et al., 2017)**. More importantly, DNA fragments have shown better thermal stability than that of proteins in processed meat, so they could be chosen as markers for authenticity determination in processed meat **(Kaltenbrunner, Hochegger and Cichna-Markl, 2018; Kang and Tanaka, 2018; Kumar et al., 2015; Ruiz-Valdepeñas Montiel et al., 2017; Xu et al., 2018)**. Of the different DNA markers used for fish species identification, mitochondrial DNA (mtDNA) possesses several advantages over nuclear DNA for studies of speciation in fish products. It is relatively more abundant in total nucleic acid preparations than nuclear DNA, with the copy number of the mitochondrial genome exceeding that of the nuclear genome several folds **(Alberts et al., 1994)**. Research on fish mitochondrial DNA (mtDNA, mitogenome) has led to substantial advances in the fields of species authentication and population biology **(Miya et al., 2001)**. Mitochondrial DNA tends to be maternally inherited so that individuals normally possess only one allele and thus sequence ambiguities from heterozygous genotypes are generally avoided. The relatively high mutation rate compared to nuclear genes has tended to result in the accumulation of enough point mutations to allow the discrimination of even closely related species. It should however be noted that mitochondrial DNA also exhibits a degree of intraspecific variability and so care has to be taken when studying differences between organisms based on single base polymorphisms **(Chow and Inogue, 1993)**. However, the use of nuclear markers may be useful for fish species discrimination because of the existence of introns of different sizes which allow sometimes the amplification of species-specific DNA fragments **(Ferguson et al., 1995)**. The comparative analysis of the commonly applied meat adulteration DNA techniques is present in Table 2.

Polymerase chain reaction-restriction fragment length polymorphism

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) is a technique for variation analysis by using restriction endonuclease digestion to identify specific sequences of conserved regions of DNA amplified by using PCR. PCR-RFLP is a sensitive, accurate, and versatile method for meat authenticity verification **(Hsieh, Chai and Hwang, 2007; Rashid et al., 2015)**, and more simple and time-saving than real-time PCR **(Ali et al., 2018)**. The result is that each meat species displays its typical restriction profile **(Fajardo et al., 2006)**. Several studies have demonstrated that LAMP might be a fast, efficient, and economical method for meat adulteration detection **(Azam et al., 2018; Cho et al., 2014; Deb et al., 2016; Ran et al., 2016; Sul, Kim and Kim, 2019; Wang et al., 2019b; Xu et al., 2017; Zhang et al., 2019)**. Using LAMP combined with colorimetric detection technology for the COI gene, 0.1% of horse meat could be detected from processed meats **(Wang et al., 2019a)**.

Loop-mediated isothermal amplification

Loop-mediated isothermal amplification (LAMP) is a newly developed meat adulteration identification technology based on DNA markers in recent years **(Lee et al., 2016; Zhang, Lowe and Gooding et al., 2014)**. LAMP is simple and easy to perform once the appropriate primers are prepared, requiring only four primers, a DNA polymerase, and a regular laboratory water bath or heat block for reaction **(Notomi et al., 2000)**.

PCR

The direct PCR method has the characteristics of high sensitivity, high resolution, and specificity, so it is commonly used in meat authenticity and origin traceability **(Bhat et al., 2016; Ha et al., 2017)**. **Ha et al. (2017)** developed species-specific PCR methods of the mitochondrial D-loop to detect pork adulteration in

commercial beef and/or chicken products, and the methods were able to detect as little as 1% pork in heat-treated pork-beef-chicken mixtures. However, the conventional single-species PCR method could only detect one specific species of adulterant in products **(Kumar et al., 2015)**, which is of low commercial value because there might be many other adulterants in the products. This method provides very accurate and reproducible quantitation of gene copies. Unlike other quantitative PCR methods, real-time PCR does not require post-PCR sample handling, preventing potential PCR product carry-over contamination and resulting in much faster and higher throughput assays **(Heid et al., 1996)**. Multiplex PCR assays with multiple species-specific primers have been greatly developed since they offer multiple target detection in a single reaction **(Ali et al., 2015; Böhme et al., 2019; Dai et al., 2015; Hou et al., 2015)**. PCR-SSCP has proved successful for the identification of fishery products such as salmon, trout, eel, and sturgeon **(Rehbein et al., 1997)**, canned tuna species **(Rehbein et al., 1999, Weder et al., 2004)**, flatfish species **(Céspedes et al., 1999)**, grouper, Nile perch and wreckfish fillets **(Asensio et al., 2001b)**, clam species **(Fernández et al., 2002b)** and codfish **(Comi et al, 2005)**, among others.

PCR-RFLP

In PCR-RFLP, a conserved region of the DNA sequence is amplified using PCR, followed by digestion with restriction enzymes, which can reveal genetic variation between species **(Partis et al., 2000)**. In a search for fast and simple genetic techniques, PCR-RFLP has gained acceptance among fish species identification methods, since it is much easier to perform and less costly than conventional DNA sequencing and nucleotide sequence analysis **(Meyer et al., 1995)**. This method has been used for the discrimination of mackerel species **(Arahishi, 2005)**, commercial canned tuna species **(Lin and Hwang, 2007, Pardo and Pérez-Villarea, 2004)**, eel species **(Rehbein et al., 2002)**, flatfish species **(Céspedes et al., 1998, Comesaña et al., 2003)**, cephalopod mollusks **(Colombo et al., 2002)**, or different processed fish products **(Akasaki et al., 2006, Chakraborty et al., 2007, Hsieh, Chai and Hwang, 2007)**.

Real-time PCR

Real-time PCR is performed by monitoring the fluorescence signal, which allows for deducing the initial quantity of the target genes without additional steps **(Xu et al., 2018)**. The real-time PCR method has a very large dynamic range of starting target molecule determination (at least five orders of magnitude). Real-time quantitative PCR is extremely accurate and less labor-intensive than current quantitative PCR methods **(Heid et al., 1996)**. SYBR Green and TaqMan technology are commonly used in quantitative methods (the working principle is outlined in the review of **Kumar et al., 2015**). SYBR Green technology can only detect a single species, but the detection cost was lower than that of TaqMan technology. **Li et al. (2019)** developed a novel reference primer-based mitochondrial 12S rRNA for the quantitative determination of goat meat adulterated with pork by using real-time PCR. The method showed high specificity and sensitivity for goat meat mixed with pork within the 10% to 100% mixture-level range. TaqMan technology has higher specificity and sensitivity than those of SYBR Green technology. More importantly, it can be used for multispecies detection **(Xu et al., 2018)**.

Droplet digital PCR

Droplet digital PCR (ddPCR) is a new method for nucleic acid detection and quantification. The principle of this method is to perform independent PCR on a large number of small reactors in the form of droplets that contain or do not contain one copy of the target molecule template in each reactor, to achieve "single-molecule template PCR amplification" **(Cai et al., 2017; Li et al., 2018a; Pohl and Shih Ie, 2004)**. After amplification, the number of copies of the target sequence can be counted by the number of positive reactors based on the fluorescence signal.

RAPD

The RAPD technique involves PCR amplification with a single primer to generate a collection of DNA fragments or fingerprint, which is expected to be consistent for the same primer, DNA, and conditions used **(Williams et al., 1990)**. This technique has been used for the discrimination of populations of Hilsa shad **(Dahle et al., 1997)**, species of Anguilla **(Takagi and Taniguchi, 1995)**, tilapia fish species and subspecies **(Bardakci and Skibinski, 1994)**, species of the genus Barbus **(Callejas and Ochando, 2001)**, grouper, Nile perch and wreckfish **(Asensio et al., 2002)**, salmonids **(Jin et al., 2006, Yamazaki et al., 2005)**, among others **(Dinesh et al., 1993, Partis and Wells, 1996)**. The main advantages of RAPD are (i) it does not require previous knowledge of DNA sequences of the species under study and (ii) it targets many sequences in the DNA of the sample, producing DNA patterns that allow comparison of many loci simultaneously. However, RAPD analysis presents some disadvantages: (i) it may not be practical to identify the species of origin in products containing mixtures of species **(Martínez and Malmheden Yman, 1998)** and (ii) it does not seem to be adequate for analysis of severely degraded material, as in autoclaved samples **(Martínez and Malmheden Yman, 1998)**.

DNA barcoding and next-generation sequencing

The above reviewed DNA-based technologies are mainly targeted detection methods, but in meat adulteration detections, many unknown meat species should be identified **(Cottenet et al., 2020)**. Following this need, an untargeted detection technology named DNA barcoding had been developed **(Cavin et al., 2018; Hebert et al., 2003)**. DNA barcoding is particularly successful when applied to seafood because of several reasons:

i) in comparison to other animal sources (e.g. cattle, sheep, goat, horse) the number of species is higher, so the effectiveness of the technique is enhanced;

Table 2 Comparative analysis of the commonly applied meat adulteration DNA techniques.

Techniques	Specificity	Sample preparation	Detection time	Multispecies detection	Operator requirements	Detection costs	Commercial availability	Application locations
Direct PCR	High but vulnerable	Sampling→smashing or ground→DNA extraction→ purification→ quantification	Time-consuming	Yes	Professional	High	Commercial kits available	Lab
Real-time PCR	High	Sampling→smashing or ground→DNA extraction→ purification→ quantification	Time-consuming	Yes	Professional	High	Commercial kits available	Lab
PCR-RFLP	High	Sampling→smashing or ground→DNA extraction→ purification→ quantification	Time-consuming	Yes	Professional	High	Commercial kits available	Lab
LAMP	High	Sampling→smashing or ground→DNA extraction→ purification→ quantification	Less time-consuming	Yes	Professional	High	Commercial kits available	Lab or onsite
Protein mass	High	Sample ground→ protein extraction→ purification→ digestion	Time-consuming	Yes	Professional	High	No	Lab
ddPCR	High	Sampling→smashing or ground→DNA extraction→ purification→ quantification	Less time-consuming	Yes	Professional	High	No	Lab
A barcoding	High	Sampling→smashing or ground→DNA extraction→ purification→ quantification	Less time-consuming	Yes	Professional	High	Public databases available (BOLD)	Lab
ELISA	High	Sample ground→protein extraction→ quantification	Less time-consuming	No	Simple training	Low	Commercial kits available	Lab or onsite
Protein immunosensor	High	Sample ground→protein extraction→ quantification	Less time-consuming	No	Simple training	Low	No	Lab or onsite

ii) classical identification approaches are not useful in many cases (following industrial processing, morphological characteristics are often lost and classical identification processes are no longer effective) and

iii) identification can often proceed beyond species level, allowing the identification of local varieties and hence the origin of the product. Through PCR amplification and sequencing of specific gene fragments, and then search it in the Barcode of Life Data (BOLD) system and the U.S. National Center for Biotechnology Information database, the adulterated meat species could be identified **(Fiorino et al., 2018)**. The early DNA barcoding technology mainly relied on Sanger DNA sequencing for an approximately 650 bp region of COI and the CtyB gene of the animal species **(Böhme et al., 2019)**. DNA Barcoding application can be applied to authenticate labeling and certification labels. This technique has aided several researchers in discovering mislabeled/substitution incidences, for example, **Filonzi, et al., (2010)** found halibut were substituted with pangasius However, when there are multiple adulterated ingredients in meat products, the traditional Sanger sequencing will generate multiple or overlaying sequencing peaks, resulting in false sequence information. Therefore, a DNA metabarcoding method had been constructed to implement multispecies identification in complex samples using next-generation sequencing (NGS) technology. Furthermore, for processed meat products, DNA can be degraded to small fragments (<200 bp) depending on the treatment **(Cavin et al., 2018)**. Thus, a mini-barcoding method, which focuses on shorter DNA fragments (100 to 200 bp), had been developed by using NGS technology **(Böhme et al., 2019; Hu et al., 2018)**. Compared to the early DNA barcoding technology, mini-barcoding has the advantages of higher throughput and higher sensitivity **(Böhme et al., 2019; Hu et al., 2019)**. Also, it is applicable for meat identification even on highly processed meat products when targeting small fragments **(Cottenet et al., 2020).** Recently, **Cottenet et al. (2020)** successfully applied a commercial NGS Food Authenticity Workflow to identify untargeted meat species, 46 pure and mixture meat species were successfully tested, including some close-related species, such as bison versus beef and red deer versus reindeer. Furthermore, the method was also suitable for processed (grounded, cooked, and canned) samples identification. However, DNA barcoding technology also has some disadvantages, such as expensive sequencing costs, time-, and sample-consuming **(Fiorino et al., 2018)**.

PROTEIN TECHNOLOGIES

Meat adulteration detection by using PCR methods is usually affected by many factors, such as poor trace quantitative analysis, sampling pollution, and DNA degradation in meat processing **(Di Pinto et al., 2015; Li et al., 2018a; Naveena et al., 2017)**. Moreover, DNA extraction is time-consuming and must be optimized for each particular case to ensure that enough DNA was obtained for the analysis **(Song et al., 2017)**. Protein is the main component of meat. The specific protein composition and three-dimensional structure of specific proteins have certain conservation and specificity between species, which is suitable for meat adulteration detection. Moreover, some protein molecules are tissue-specific and can be used for the identification of less valuable additives, such as connective tissue, blood plasma, or milk preparations **(Jiang et al., 2018; Montowska and Spychaj, 2018; Ofori and Hsieh, 2015)**. The comparative analysis of the commonly applied meat adulteration protein techniques is present in Table 3.

Enzyme-linked immunosorbent assay

EIA/ELISA uses the basic immunology concept of an antigen-binding to its specific antibody, which allows detection of very small quantities of antigens such as proteins, peptides, hormones, or antibodies in a fluid sample. There are two kinds of immunoassay techniques used in meat adulteration detection: enzyme-linked immunosorbent assay (ELISA) and immunosensors. ELISA is the most widely applied immunoassay method of meat adulteration detection **(Thienes et al., 2018)**. The commonly used ELISA methods for meat adulteration detection are direct ELISA **(Mandli et al., 2018; Seddaoui and Amine, 2020)**, sandwich ELISA **(Ayaz et al., 2006; Hsieh and Ofori, 2014; Thienes et al., 2018; Zvereva et al., 2015)**, and indirect competitive ELISA **(Hsieh and Ofori, 2014; Jiang et al., 2018; Mandli et al., 2018)**. Compared to DNA-based detection technologies, ELISA methods show the simplicity of sample preparation, low cost, and less time consumption. Also, ELISA detection does not require complex equipment and is easily feasible for onsite monitoring **(Mandli et al., 2018; Thienes et al., 2019)**.

Immunosensors

However, immune techniques are characterized by their simplicity of sample preparation, absence of the need for complex equipment and qualified personnel, and high productivity of serial testing. As well, for food authentication, electrochemical immunosensors are an alternative detection tool and are highly feasible for on-site usage; therefore, there is only one previously reported immunosensor for meat authentication **(Lim and Ahmed, 2016)**. The principle of immunosensor methods is similar to that of ELISA methods, but the former uses a biosensor to transmit and amplify the optical, electrical, or other signals of the immune response to a detectable signal, so the sensitivity of the method is better than that of ELISA. The immunosensor technique has been widely used in food allergy, pesticide residue, and milk adulteration analyses, among others. However, only a few reports have utilized immunosensing for meat adulteration detection **(Kuswandi et al., 2017; Lim and Ahmed, 2016; Mandli et al., 2018; Masiri et al., 2016)**.

Protein mass spectrometry analysis

Modern mass spectrometers can accurately measure thousands of compounds in complex mixtures over a given liquid chromatography method, depending on the desired outcome and method duration. This stream of analytical chemistry has wide-ranging applications across food, pharma, environmental, forensics, clinical, and research **(Broadbent et al., 2020)**. Recently, mass spectrometry technologies based on protein and peptide analysis have rapidly evolved and have been increasingly applied for meat species identification.

Table 3 Comparative analysis of the commonly applied meat adulteration protein techniques.

Detection items	Detection technology	Immunogen and antibody	Method sensitivity(limit of detection)	References
Pork adulteration in beef	Direct ELISA	Porcine immunoglobulins G (IgG) and polyclonal antibodies	0.01% (w/w) of pork in beef	**Seddaoui and Amine (2020)**
Pork adulteration in meat	Indirect competitive ELISA	Porcine IgG and polyclonal antibodies	0.1% of pork adulteration	**Mandli et al. (2018)**
Porcine hemoglobin in meat products	Indirect competitive ELISA	Mammalian hemoglobin 13F7 and monoclonal antibodies (MAbs 13F7)	0.5 ppm of P_{Hb}	**Jiang et al., (2018)**
Pork fat protein in other animal meats	Indirect ELISA	Thermal stable-soluble protein (TSSP) and monoclonal antibodies (MAbs PF 2B8-31)	1% (w/w) of pork fat adulteration	**Kim et al. (2017)**
Fat adulteration in cooked and noncooked of pork, beef, and chicken	Indirect ELISA	Skeletal muscle troponin I (smTnI) and monoclonal antibodies (commercial ab97427)	ND	**Park et al. (2015)**
Cooked wild rat meat in pork, beef, and chicken	Sandwich ELISA	Rat heat-resistant proteins and polyclonal antibodies	0.01 μg/L based OD values	**Chen et al., (2020)**
Heated mammalian meats adulterated in poultry meats	Sandwich ELISA	Mammalian skeletal troponin and monoclonal antibodies (MAbs 6G1 and 8F10)	1% (g/g) of heated meats adulterated in poultry meats	**Jiang et al., (2020)**
Cooked beef in the pork, horse, chicken, goat, and sheep meat	Sandwich ELISA	ND	0.1% (w/w) of the cooked products	**Thienes et al., (2019)**
Cooked chicken/turkey in pork, horse, goat, or sheep meat	Sandwich ELISA	ND	0.1% (w/w) of the cooked products	**Thienes et al., (2019)**
Pork is cooked horse, beef, chicken, goat, and lamb meats	Sandwich ELISA	ND	0.1% (w/w) for cooked samples	**Thienes et al. (2018)**
Wheat protein in ground chilled pork and beef mixture	Sandwich ELISA	Gliadin and monoclonal antibodies	1% (w/w) for spiked samples	**Petrášová et al. (2017)**
Soybean proteins in surimi products	Sandwich ELISA	Soybean trypsin inhibitor (STI) and monoclonal antibodies	13.6 mg/kg samples	**Jiang et al. (2015)**
Mammalian muscle tissues in raw meat and meat products	Sandwich ELISA	Skeletal muscle protein troponin I (TnI) and monoclonal antibodies	4.8 ng/mL of bovine TnI	**Zvereva et al. (2015)**
Pork adulteration in beef meatballs	Electrochemical immunosensor	Porcine IgG and polyclonal antibodies	0.01% of pork adulteration	**Mandli et al. (2018)**
Pork adulteration in cooked meatballs	Lateral flow immunosensor	Porcine IgG and polyclonal antibodies	0.1% (w/w) for pork in beef meatballs	**Kuswandi et al. (2017)**
Horse meat adulteration in meat products	Lateral flow immunosensor	Horse serum albumin (HSA) and polyclonal antibodies	0.01% and 1.0% adulteration for raw and cooked horse meat	**Masiri et al. (2017)**
Pork adulteration in raw meat	Label-free electrochemical immunosensor	Porcine serum albumin (PSA) and polyclonal antibodies	0.5 pg/mL PSA in buffer solution	**Lim and Ahmed (2016)**

Table 3 Comparative analysis of the commonly applied meat adulteration protein techniques. Continue.

Detection items	Detection technology	Immunogen and antibody	Method sensitivity(limit of detection)	References
Bovine adipose tissue in meat products	Label-free electrochemical immunosensor	Ruminant-specific muscle protein and polyclonal antibodies	2% bovine fat-in-pork fat 1% bovine fat-in-porcine meat-and-bone meal 0.5% bovine fat-in-soy meal mixtures	**Hsieh and Gajewski (2015)**
Duck, goose, and chicken in processed meat products	LC–ESI–QTOF–MS LC–ESI–QQQ–MS/MS	Hemoglobin alpha for duck: FMCAVGAVLTAK Hemoglobin beta for goose: FFSSFGNLSSPTAILGNPMVR Myosin-binding protein C for chicken: LDVPISGEPAPTVTWK	ND	**Fornal and Montowska (2019)**
Grain proteins adulteration into meat products	HPLC-MS/MS	Barley: IETPGPPYLAK, Oat: DFPITWPWK, Rice: ELGAPDVGHPMSEVFR, Rye: TPFASTVAGIGGQ, Wheat: SVAVSQVAR	Oats and rye: 5 mg/kg meat product; barley and wheat: 10 mg/kg meat product	**Jira and Munch (2019)**
Porcine blood plasma to emulsion-type pork sausages	UHPLC-MS/MS	Plasma peptide marker of ISEPLATETVR GSLDEFFHR, ISPLPDITPADFK, DPFPDFFSPVLK	0.7% (w/w) meat substitution by porcine plasma	**Stader et al., (2019)**
Shrimp species in seafood	SWATH-MS	Myosin heavy chain type a for *Marsupenaeus japonicas*: AAVELDDLHASAER Arginine kinase for *Fenneropenaeus Chinensis*: GTYYPLTGMGK Sarco/endoplasmic reticulum Ca^{2+}-ATPase for *Litopenaeus vannamei*: IGVFGENEETAGK	ND	**Hu et al. (2018)**
Pork, beef, lamb, chicken, duck, soy, peanut, and pea adulteration in meat products	UPLC-MS/MS	Conglutin/Ara h 6 for peanut: EIMNIPQQCNFR, Alpha subunit of beta conglycinin for soy: ESYFVDAQPK, P54 protein for pea: GIIGLVAEDR, Myoglobin for duck: HGVTVLTQLGK, Creatine kinase M-type for chicken: DLFDPVIQDR, Hemoglobin subunit beta for sheep: VDEVGAEALGR, Carbonic anhydrase 3 for beef: LVNELTEFAK, Hemoglobin subunit beta for pig: VNVDEVGGEALGR	0.5% adulterations of any of the eight species	**Li et al. (2018b)**
Horse, pork, and beef meat in smoked sausages	Infusion MS	Myosin-1 for pork: SALAHAVQSSR, Myoglobin for beef: HPSDFGADAQAAMSK, Myoglobin for horse: VEADIAGHGQEVLIR	5% (w/w) for pork and beef in the three-component matrix and 1% (w/w) for horse meat	**Montowska and Spychaj (2018)**

Table 3 Comparative analysis of the commonly applied meat adulteration protein techniques. Continue.

Detection items	Detection technology	Immunogen and antibody	Method sensitivity (limit of detection)	References
Duck, pig, cattle, chicken, and sheep in cooked meats	UPLC-TripleTOF-MS UPLC-MS/MS	M-protein, striated muscle for chicken: FWIQAESLSPNSTYR, Alpha-enolase for duck: LMLDMDGSENK, Trifunctional enzyme subunit alpha (mitochondrial) for pig: FAGGNLDVLK, Stress-induced-phosphoprotein 1 for bovine: ALDLDSNCK, Hemoglobin subunit beta for sheep: FFEHFGDLSNADAVMNNPK	ND	**Wang et al., (2019b)**
Pork gelatin adulteration in meat products	High-resolution MS	Type I collagen: TGETGASGPPGFAGEK, HGNRGEPGPAGSVGPAGAVGPR	0.1% (w/w) of undesired pork gelatin	**Yang et al., (2018)**
Buffalo, sheep, and goat meat in minced meat and meat products	MALDI-TOF MS	Myosin light chain 1 for sheep: EAFLLYDR, Myosin light chain 2 for buffalo: NMWAAFPPDVGGNVDYK, Myosin light chain 1 for goat: EAFLLYDR	1.0% for raw meat and 0.1% cooked samples	**Naveena et al. (2017)**
Chicken blood in sheep whole blood samples	Internal extractive electrospray ionization mass spectrometry (iEESI-MS)	Hemoglobin for blood samples, peptide marker Not determined	2% chicken blood in sheep blood	**Song et al. (2017)**
Water buffalo and sheep meat in raw and cooked ground meat mixtures	MALDI-TOF MS UPLC-QTOF	Myosin light chain 1 for sheep: EAFLLYDRTGDGK, Myosin light chain 2 for sheep: FSQEEIR; Myosin light chain 1 for sheep: EAFLLFDRTGECK, Myosin light chain 2 for sheep: FSKEEIK	0.5% (w/w) of buffalo meat in sheep meat	**Naveena et al. (2017)**
Beef and pork meat is highly processed food matrices	HPLC/ESI-MS/MS	Collagen a2-chain for beef: IGQpGAVGPAGIR, Collagen a2-chain for pork: TGQpGAVGPAGIR	2% pork meat in Bolognese sauce	**Prandi et al. (2017)**
Chicken, duck, and goose meat in processed meat products	Nano-LC-QTOF-MS/MS	Pyruvate kinase for chicken: EPADAMAAGAVEASFK, Alpha-enolase for duck: NYPVVSIEDPFDQDDWGAWK, Hemoglobin alpha-A for the goose: TYFPHFDLQHGSAQIK	1% (w/w) of chicken or pork in chicken, duck, and goose meat mixture, 0.8% (w/w) beef proteins in commercial poultry frankfurters	**Fornal and Montowska (2019)**
Meat adulteration in mammalian meat samples	Q Exactive Orbitrap LC-MS/MS	Myoglobin for pork: HPGDFGADAQGAMSK, Myosin-1 for horse: TLALLFSGPASADAEAGGK, Myosin-2 for beef: TLAFLFSGTPTGDSEASGGTK, β-Hemoglobin for lamb: FFEHFGDLSNADAVMNNPK, β-Hemoglobin for chicken: FFASFGNLSSPTAILGNPMVR	1% (w/w) of pork or horse meat in a mixture before and after cooking	**Orduna et al. (2017)**

Since the amino acid sequence of peptides is more stable than DNA during meat processing, they have an incomparable advantage in meat adulteration identification, especially for highly processed meat products and similar meat species **(Prandi et al., 2017)**.

CONCLUSION

Food adulteration occurs globally and in many facets and affects almost all food commodities. Adulteration not only constitutes a considerable economic problem but also may lead to serious health issues for consumers. Many of the methods for detection of food adulteration require elaborate steps of sample preparation before analysis involving high-end technologies and that makes the whole process difficult to perform and time-consuming. As the methods of adulterating foods have become more sophisticated, very efficient, and reliable techniques for the detection of fraudulent manipulations are required. The analytical techniques commonly used for meat and fish species identification can be broadly divided into protein-based and deoxyribonucleic acid (DNA)-based techniques. The protein-based methods include immunological assays, electrophoretic, and chromatographic techniques. These methods are fast and easy to perform and the investment in equipment is much less compared to DNA-based methods. Food chain transparency and full raw material traceability are primordial for an effective food fraud prevention system.

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