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Invited Review Article

Authentication of muscle foods: A proteomic and metabolomic insight

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Abstract

Economically motivated adulteration can be a serious issue for food safety; besides it plays a major role in negatively impacting consumers' trust. Muscle foods are increasingly associated with malpractices globally and hence their authentication is gradually becoming the focus of food safety and quality. Various analytical techniques including biochemical, immunological, mass spectrometry based proteomic and metabolomic approaches have addressed the issues associated with authentication of foods. Mass spectrometry-based approaches are robust, reliable and sensitive and are amenable to all kinds of raw and processed foods. The current review describes different protein fractionation techniques including novel approaches like OFFGEL and GELFrEE fractionation, two-dimensional gel electrophoresis, use of various mass spectrometers in authentication, application of lateral flow assay techniques as point-of-care tools and detection of other food frauds like geographical origin, additives, processing methods etc.

Key words: Authentication, Mass spectrometry, Metabolomics, Muscle foods, Proteomics

Highlights

- Muscle food adulteration is widespread leading to public health risks.
- Proteomics and metabolomics play major role in the authentication.
- Non-destructive technologies based on spectroscopy were discussed.
- Lateral flow assay for point-of-care monitoring is a future trend.

Introduction

Consumers need precise and accurate information to make informed choices about their food habits. This choice not only reflects lifestyle or economic status but also the religious practices of our multicultural society. Therefore, the labelling of food must be done honestly, especially where the food has been processed and the ability to distinguish one ingredient from another is lost. In order to protect consumer interests, and to combat the food fraud issues, such as adulteration, mislabelling or counterfeit problems, scientific expertise and advanced technologies to test the authenticity of different food products are being developed. Furthermore, depending on the nature of adulterants, undeclared ingredient can also represent a health risk for the consumer. The most common type of food fraud, reported in 95% of publications, is substitution (Stamatis *et al*., 2015) of an original ingredient with a cheaper one, which becomes difficult to detect by conventional analytical techniques. There are several ways in which food can be mis-described, some examples of which are listed in Table 1.

The use of rapid, effective, and reliable analytical techniques represents a valuable tool to establish control over food products in value

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Table 1. Selected examples of misdescription of muscle foods

1. Substitution of one ingredient by a similar but cheaper one	• Using whiting or pollack in place of cod • Substituting bonito for tuna or sea trout for salmon
2. Extending or adulterating food with a cheaper or base material	• Adding water to increase weight of chicken breasts
3. Presence of undeclared ingredients	• Offal in processed meat products • Mechanically separated meat (MSM) in processed meat products
4. Non-declaration or false declaration of processes	• Labelling poultry as fresh even though it has been previously frozen
5. Over-declaration of a quantitative ingredient	• Including hydrolysed protein as part of the meat content
6. False claims regarding geographical orproduction origin	• Labelling South American beef as British beef • Declaring farmed fish as 'wild' • Labelling conventionally produced meat as organic

Source: Modified from Primrose *et al*. (2010)

chain. In the last few decades, several authentication technologies for muscle foods have been established. Proteomics and metabolomics have been projected as robust and sensitive techniques for food authenticity assessment. Proteomics is the large-scale analysis of proteins in a particular biological system at a specific time (Pandey and Mann, 2000). Proteins can act as suitable markers for many meat quality characteristics from farm to fork. Proteomics-based tools can be applied for the discovery of new protein markers, such as species-specific peptides and then developing and validating analytical assays targeting these markers for qualitative and quantitative assessment of adulteration and deceptive practices (Gallardo *et al*., 2013). Metabolomics, a novel approach, focuses on relative quantification of all or as many as possible metabolites in a biological sample followed by application of chemometric methods for selection of the characteristic compounds of the sample. Two complementary approaches are followed for metabolomic investigations (Dettmer *et al*., 2007); metabolite profiling, which analyse a group of metabolites, and metabolic fingerprinting, which is used to compare changes in patterns or "fingerprints" of metabolites in response to disease, environmental or genetic variations.

The current review will address different proteomic and metabolomic approaches for the detection of food fraud and authentication in muscle foods.

Proteomic and metabolomic approaches

Although many strategies have been implemented to verify the authenticity of muscle foods, rapid, effective, accurate, and reliable detection methods are the fundamental prerequisites to achieve this goal. The most commonly employed proteomic and metabolomic methods for the evaluation of authenticity issues of meat, fish and seafoods are depicted in Fig. 1.

Electrophoresis: The electrophoretic methods are based on the separation of proteins in the electric field following their extraction from the source. The electrophoretic separation is conducted on polyacrylamide gels (PAGE) containing sodium dodecyl sulfate (SDS), a denaturing agent or by isoelectric focusing (IEF) on the agar or polyacrylamide gel (PAGIF). In IEF method, separation of proteins on immobilized pH gradient (IPG) gel works on

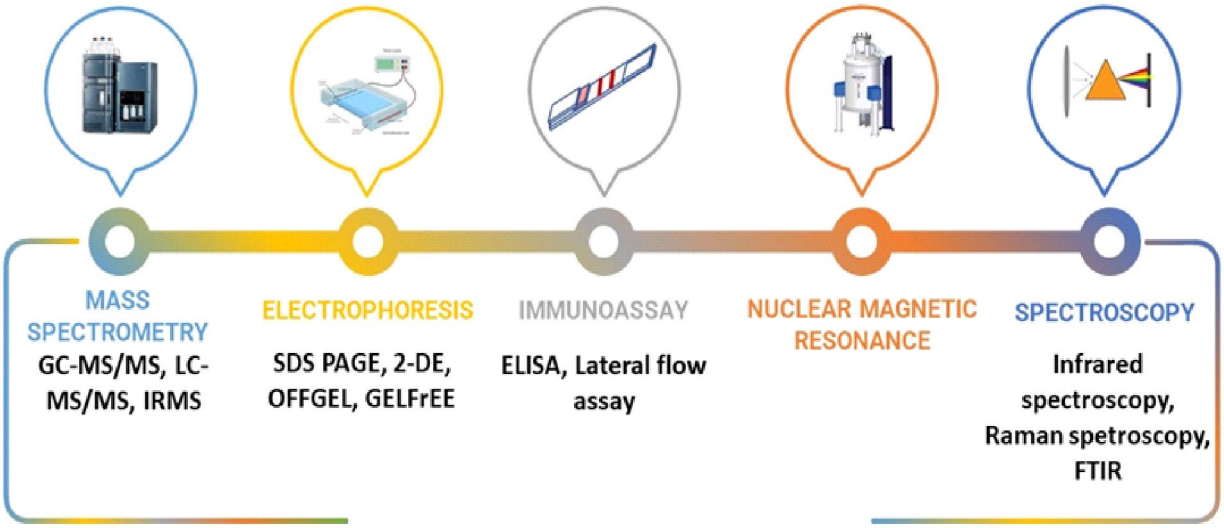

Fig. 1. Commonly employed proteomic and metabolomic techniques in authentication of muscle foods

the principle of pH gradient, which results from the addition of ampholyte. Individual proteins move towards the pH value corresponding to their isoelectric point (pI).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE): The single most widely used protein separation method according to their size or molecular weight is sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), often referred to as one-dimensional electrophoresis (1-DE) (Laemmli, 1970). When an electric field is applied, proteins migrate on polyacrylamide gel; the rate/velocity of which depends on the ratio between the protein's charge and mass. The complex mixture of proteins can be resolved into multiples of bands on a gel. However, the resolution of the gels is very low; multiple proteins with similar molecular weight may appear in a single protein band.

2-Dimensional Gel Electrophoresis (2-DE): Thousands of proteins can be simultaneously separated on a single gel using two-dimensional electrophoresis. In the first dimension, proteins are separated with the assistance of iso-electric focusing depending on their isoelectric point (pI) while in the second dimension, SDS-PAGE is used to separate proteins depending on their size or molecular weight (MW). The combination of the above-mentioned techniques results in the development of 2-D maps with uniform distribution of protein spots visualized across the gel. Up to 10,000 proteins can be simultaneously separated by this technique and the spots containing less than 1 ng proteins can be identified and determined qualitatively and quantitatively as well (López, 2007). Inspite of its certain limitations, this versatile technique is most commonly used in proteome analysis.

OFFGEL electrophoresis: The OFFGEL electrophoresis allows fractionation of proteins or peptides according to their pI in a two-phase system followed by their direct recovery in solution. The upper liquid phase is divided in compartments and the lower phase is a conventional rehydrated IPG strip. The sample is diluted in the focusing buffer followed by loading into 12 or 24 well frames. As there is no fluidic connection between the wells, the proteins are focused through the IPG strip and migrate from well to well under the applied electric field until it is positioned at the right well with pH value corresponding to its pI and

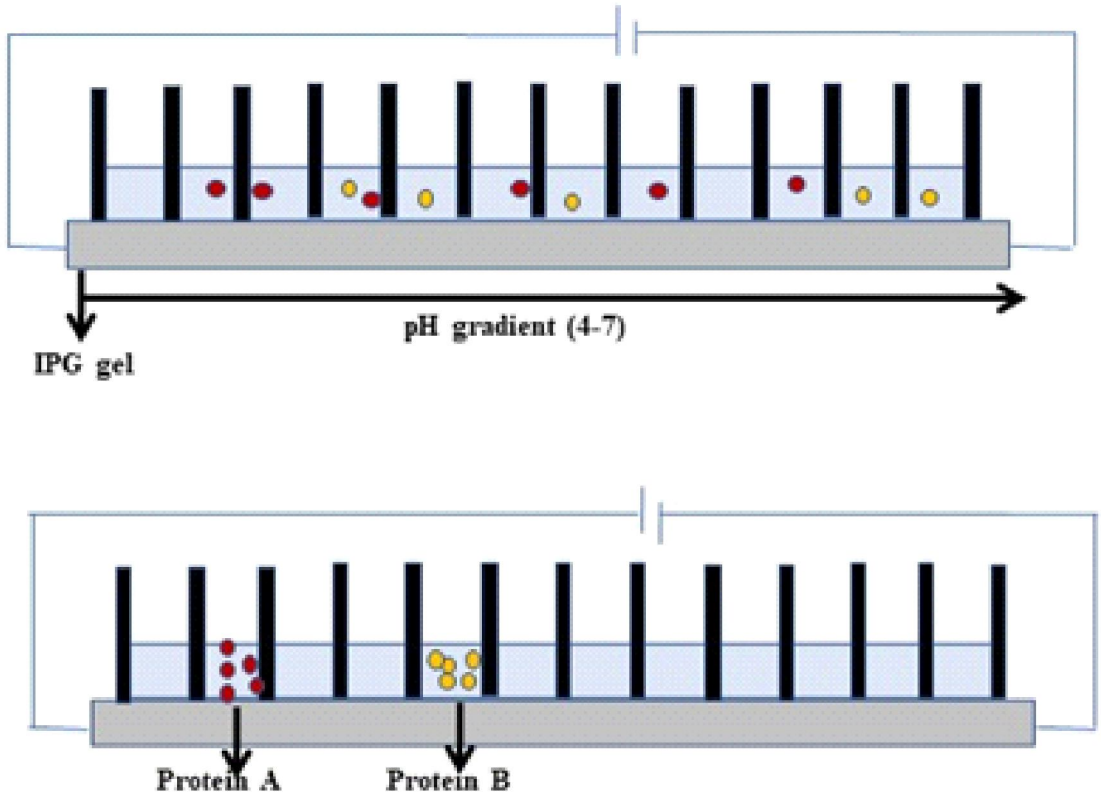

Fig. 2. Principle of OFFGEL electrophoresis

permeates into the solution-filled compartment above the gel strip. High resolution, high sample loading, well-defined pH range, and the flexibility of choosing the desired pH gradient and fractionation of multiple samples in the same run are some of the noted advantages of OFFGEL electrophoresis (Manadas *et al*., 2010). The time required for the fractionation varies between 24-32 hrs depending on sample composition.

Gel-eluted liquid fraction entrapment electrophoresis (GELFrEE): GELFrEE is a size-based protein separation platform similar to SDS-PAGE to isolate distinct molecular weight fractions with a high degree of resolution and high sample recovery. The sole difference with SDS-PAGE is that at the end of the run, proteins can be recovered in the solution phase for downstream analysis. The GELFrEE 8100 fractionation system consists of a benchtop instrument including cathode chamber, sample loading chamber, specially designed single-use 8 channels, fraction collection chamber and anode chamber. The channels consist of a precision-cast gel column which measures 2.5 cm the length (including stacking and resolving gel). Upon application of an electric current, charged protein molecules start migrating in the stacking gel and separate according to their MW in the resolving gel. Smaller proteins move faster and elute into the fractionation collection chamber, while the larger proteins end up in the later fractions. The three different proprietary Cartridge Kits (5%, 8% and 12%) from Expendeon Corporation employ large, medium, and short resolving gels to afford partitioning protein complex across the mass range of 3.5 to 500 kDa, 3.5 to 150 kDa, and 3.5 to 50 kDa respectively. Moreover, particular fractions may be targeted, relating the time of collection to a particular mass range of the sample (Banerjee *et al.*, 2021; Maheswarappa *et al*., 2021).

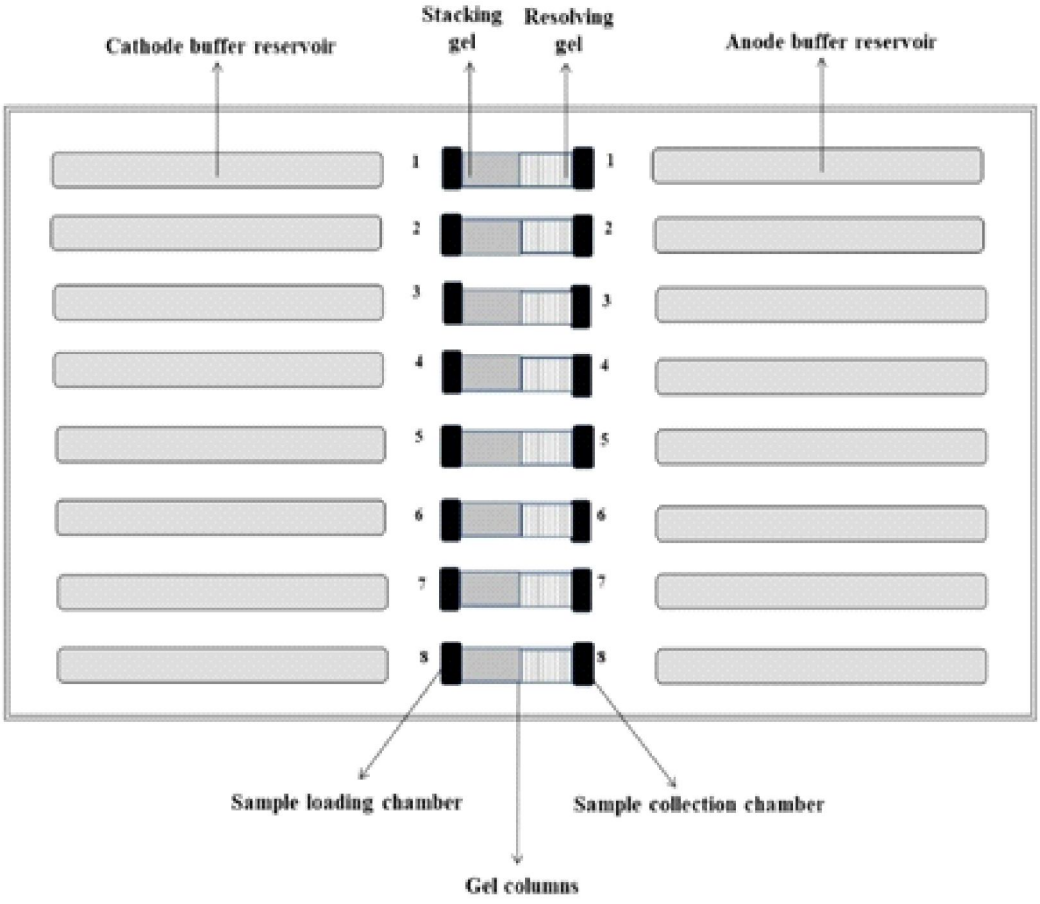

Fig. 3. Schematic representation of GELFrEE cartridge

Immunoassay: Immunoassays based on antigen-antibody reactions are widely been exploited for food authentication. Among different immunoassay formats, the selection of methods depends upon the target molecule and application. The most commonly used methodologies used for food authentication are enzyme-linked immunosorbent assay (ELISA) and lateral flow assay (LFA).

An enzyme conjugate is prepared for ELISA by using a known antigen or antibody adsorbed on the surface of the solid phase, and the enzymes conjugate specifically bind to samples containing antibodies or antigens. The addition of an enzyme substrate produces a visible signal or color, which is measured with a spectrophotometer. The development of color is positively correlated with the amount of the antibody or antigen in the samples. The commonly used ELISA methods for authenticity detection are direct ELISA, sandwich ELISA and indirect competitive ELISA. High specificity, sensitivity, easy sample preparation, low cost, and less time consumption are some of the advantages of ELISA. However, this technique shows some limitations due to false positives caused by cross-reactivity in closely related species and proteolysis caused by severe heat treatment. Researchers are therefore dedicated to develop more sensitive, point-of-care (PoC) protein-based methods for food authentication.

Lateral flow assay (LFA) is a very common technique that has been used to develop PoC devices for application in various fields including food safety and food authenticity (Sajid *et al*., 2015). The simple, rapid, user-friendly, and cost-effective characteristics have

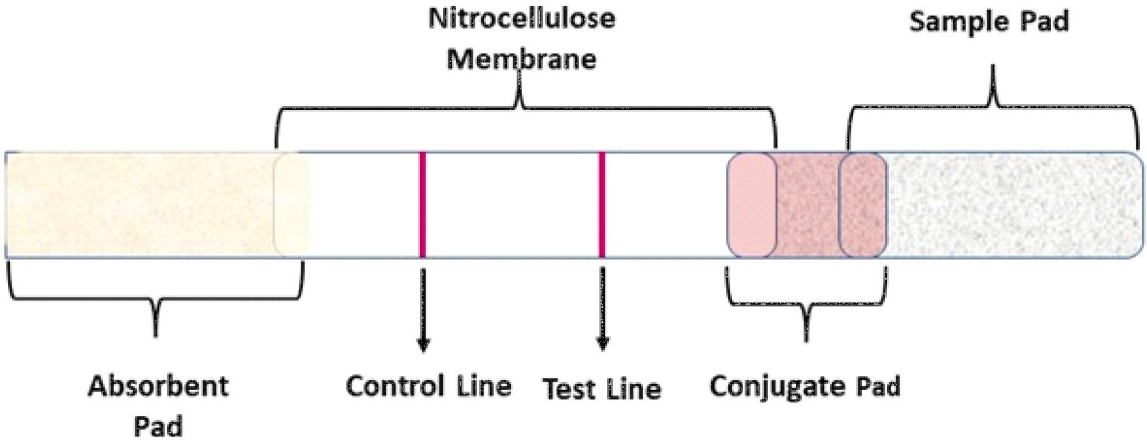

Fig. 4. Components of lateral flow assay strip

made them one of the most widespread point-of-care (PoC) sensors (Bahadýr and Sezgintürk, 2016). The LFAs consist of a sample pad, nitrocellulose membrane containing the test and control zones, and conjugate and absorbent pads. These components are fitted on a backing card or a housing chamber. Sample or analyte encounters the sample pad, migrates through the membrane (Ahmed *et al*., 2016) by the action of capillary force and reaches the conjugate pad. The conjugate pad releases the labelled detection bioreceptor (e.g., an antibody, often conjugated to a nanoparticle for detection) and allows for first analyte-bioreceptor interaction. Analyte-bioreceptor complex continues to migrate and is captured by biorecognition reagents at the test line (T-line) and control line (C-line). The appearance of visible color at T- and C-lines can be detected by naked eyes. The signal at C-line represents the correctness of the test.

Spectroscopy: Spectroscopic analysis of meat is based on the principle that moisture, proteins or lipids, present in meat will produce different spectra at different wavelengths (Wang *et al*., 2018a). Due to its non-destructive nature, spectral technologies are being explored in food quality and safety in recent years. Other advantages associated with spectroscopic techniques are simple sample preparation and lack of need for sample pre-treatment (Wang *et al*., 2018a). In Raman spectroscopy, the composition of a substance and its structure are analysed based on the Raman scattering effect (Hu *et al*., 2019). This non-invasive technique does not require sample preparation or large sample volume and is also suitable for opaque samples (Lee *et al*., 2017; Hu *et al*., 2019). The nature of the molecules and their concentration can be analysed by the fingerprints of vibrational frequency and intensity obtained in Raman spectroscopy (Lee *et al*., 2017).

Nuclear magnetic resonance (NMR): In metabolomics studies, NMR is recognised as one of the most important analytical techniques. The advantages of this method are simple sample preparation steps and determination of different chemical species that can be performed in a single experiment. NMR-based chemo-metric approaches have been used for food authentication (Marcone *et al*., 2013). The major challenges are obtaining a good spectrum in terms of quality and resolution, its low sensitivity and the resonance assignment procedure which aims to associate each nuclear spin of the sample molecule with a chemical shift. The later can be solved partially by confirming the proposed assignment with the addition of internal compounds.

Mass spectrometry: Mass spectrometry technologies have rapidly evolved and have been increasingly applied for food authentication. Mass spectrometry allows the

determination of the exact molecular mass of charged molecules. A mass spectrometer is composed of an ionization source that converts analyte molecules into gas-phase ions, a mass analyzer for the separation of the ionized analyte according to their mass-to-charge ratio and a detector for the precise quantification of each species coming out the analyzer. Mass spectrometers are either one-stage instruments (MS) if they are composed of only one analyzer, or multi-stage instruments (MS^n) if they combine several analyzers in tandem (for instance Q3) or in hybrid (Q-TOF) configurations. Advanced progress in the analysis of protein at the sequence/peptide level using mass spectrometry technologies became possible due to the simultaneous advancement in liquid chromatography (LC) and capillary electrophoresis for in-line separation. These new technological advances in mass spectrometry were able to identify peptides with femtomole ($I0^{-15}$ mol) to attomole (10^{-18} mol) sensitivity in complex biological samples (Mallick and Kuster, 2010). MS technologies coupled to chromatographic techniques such as GC and LC are exploited for the analysis of metabolites in animal tissues. Gas chromatography (GC) or LC separates the individual components of a mixture and MS characterises each of the components. In this way, numerous compounds can be analysed both qualitatively and quantitatively. In GC–MS analytes must be sufficiently volatile and thermally stable. GC–MS allows obtaining a characteristic spectrum called the "signature" or "spectral fingerprint" by the analysis of the sample's volatile and semi-volatile fraction, which can be used for the classification of samples for food authentication. Up to date there are not so many metabolomic studies using LC-MS applied to food authentication. They are generally very sensitive techniques but rather expensive and the sample preparation and chromatographic running times are often time-consuming.

Authentication of muscle foods

Authenticity was investigated comprehensively using 2-DE and advance-ments in mass spectrometry. Berrini *et al*. (2006) distinguished four freshwater fish species which are sold as fillets under the generic label of ''perch'' using IEF and 2-DE approach. López *et al*. (2002) analyzed 2-DE protein profiles of two marine mussel species. A total of 37 differentially expressed protein spots were identified. Interspecies differences in 2-DE protein patterns of raw and processed meat of cattle, pig, chicken, turkey, duck and goose were reported by Montowska and Pospiec (2013). Proteins which were found to be stable during meat aging and resistant to thermal processing were proposed as suitable markers. Eighteen unique heat-stable peptide biomarkers were identified by Wang *et al*. (2018b) by LC-MS in cooked pork, chicken, duck, beef and sheep.

Specific culinary practices or sensory characteristics associated with regional products are convincing the consumers to show an increased interest in the provenance of the foods they consume. Moreover, concern about animal welfare is decreasing their confidence in the quality and safety of products produced outside their local region. The four most common elements in living matter are hydrogen, carbon, nitrogen and oxygen. Each of these elements has two stable isotopic forms ($^2H/^1H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{18}O/^{16}O$) whose ratio can be of value in authenticity studies (Primrose *et al*. 2010). Climatic conditions, geographical origin, soil pedology, and geology of the locations of food ingredients origin strongly affect the changes in the ratio of these stable isotopes (Camin *et al*., 2017). This strategy was used by Heaton *et al*. (2008), to distinguish Brazilian from UK and Irish beef. Zhao *et al*. (2013) classified beef coming from different Chinese regions by isotope ratio mass spectrometry (IRMS) to correctly determine the origin of 60–80% of samples. Use of isotopic analysis to determine the authenticity of fish and seafood, was less reported especially when compared to the research on meat or dairy products. Carbon and nitrogen stable isotope ratios were used by

Dempson and Power (2004) to distinguish farmed from wild Atlantic salmon. The researchers reported that aquaculture salmon was more significantly enriched in ^{13}C but depleted in ^{15}N compared to wild fish.

Sentandreu *et al*. (2010) was able to detect the presence of 0.5% w/v contaminating chicken in a mixture with pork meat by OFFGEL isoelectric focusing followed by analysis of species-specific peptide biomarkers by MALDI–TOF MS and LC–ESI–MS/MS. Naveena *et al*. (2017) succeeded in identification of peptide marker (MLC-1/MLC-2) for authenticating contaminating buffalo meat in sheep meat mix as low as 0.5% in both raw and cooked meat mixtures involving OFFGEL fractionation and tandem mass spectrometry. In another study, while comparing both in-gel and OFFGEL-based proteomic approach for authentication of water buffalo, sheep and goat meat (raw and cooked), Naveena *et al*. (2018) reported that 2DE and MS-based in-gel method can detect up to 1.0% substitution, whereas OFFGEL electrophoresis and MALDI-PMF can detect even up to 0.1% substitution of sheep and goat meat in buffalo meat.

Immunosensors for the detection of meat adulteration have been reported by a few researchers (Lim and Ahmed, 2016; Masiri *et al*., 2016; Kuswandi *et al*., 2017; Mandli *et al*., 2018). An electrochemical competitive immunosensor was developed based on antipig IgG polyclonal antibody to identify as low as 0.01% pork adulteration within 20 min. Compared with competitive ELISA methods, which can detect 0.1% pork adulteration within 45 min, the detection limit and detection time were greatly improved by using immunosensor (Mandli *et al*., 2018). Kuswandi *et al*. (2017) developed a LFIA for measurement of pork adulteration in cooked beef meatballs with 0.1% (w/w) LOD, 65 min sample preparation time and 5 min response time for the assay. Recently, ICAR-NRC on Meat, Hyderabad has developed and commercialized an absolute point-of care pork detection kit, which can detect pork components in raw and processed meat within 15 min (unpublished data).

The lipidome could be used to distinguish the species of a meat adulterant as the type and quantity of fatty acids in tissues in each meat species are specific (Ballin, 2010). By using GC-MS and UHPLC-MS with multivariate analysis viz. principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA), Trivedi *et al*. (2016) reported a metabolomics and lipidomics approach to detect pork adulteration in beef. The researchers reported that 23 metabolites were significantly correlated with pork adulteration in beef. Volatile compounds can be targeted as marker for meat adulteration identification as different species of meat have special characteristic flavor. By using an electronic nose and GC-MS, Nurjuliana *et al*. (2011) and Haddi *et al*. (2015) successfully identified meat adulteration.

Hu *et al*. (2017) detected 10% of animal offal in ground beef with 99% accuracy using Fourier transform infrared spectroscopy (FT-IRS). Recently, Wu *et al*. (2018) developed an FT-IR method combined with PLS-DA to detect Norwegian salmon adulteration with Chinese salmon. By using raman spectroscopy (RS) and the PLS-DA model based on functional groups of amino acids, lipids, and proteins, the nonmeat ingredients (sodium chloride, sodium tripolyphosphate, and carrageenan) were determined in pork products (Nunes *et al.,* 2019). Recently, Lee *et al.* (2018) successfully distinguished beef tallow, pork lard, chicken fat, and duck oil by RS method. Some of the selected authentication studies of muscle foods employing proteomic and metabolomic approaches are enlisted in Table 2.

Conclusion

Muscle foods are one of the most vulnerable targets for food fraud incidents. Authentication is of importance to gain consumers' trust in a specific brand and also to prevent economic losses in an already sensitive sector. A wide variety of techniques are being implemented to safeguard the consumers and screen for adulteration in the meat value chain. Efforts should be directed to standardize the

Table 2. Proteomic and metabolomic approaches adopted to assess authenticity of muscle foods

Food samples	Purpose of analysis	Detection technology	References
Raw and cooked meat and meat mixes from six meat animal species	Species identification	2-DE, MALDI-TOF MS	Montowska and Pospiech, 2013
Chicken in meat mixes	Species identification	OFFGEL isoelectric focusing, LC-MS/MS	Montowska *et al.*, 2015
Characterization of species-specific peptides from different seafood	Species identification	MS/MS ion monitoring	Ortea *et al.*, 2011
Lamb from different regions of South Africa	Geographical origin	Isotope ratio mass spectrometry	Erasmus *et al.*, 2018
Organic and conventional Irish beef	Geographical origin	Isotope ratio mass spectrometry	Bahar *et al.*, 2008
Buffalo, sheep and goat meat mix	Detection of adulteration/ substitution	2-DE and OFFGEL electrophoresis, MALDI-TOF MS	Naveena *et al.*, 2018
Raw and cooked ground sheep meat and buffalo meat up to 0.5% substitution	Detection of adulteration/ substitution	OFFGEL electrophoresis, Quantitative label-free mass spectrometry	Naveena *et al.*, 2017
Commercially available highly processed meat products containing chicken, duck, goose meat, pork, beef and turkey	Detection of adulteration/ substitution	LC–ESI–QTOF MS/MS and LC–ESI–QQQ MS/MS	Fornal and Montowska, 2019
Mechanically recovered meat in food products	Detection of adulteration/ substitution	GC-MS	Surowiec *et al.*, 2011
Porcine (meat and collagen) residues	Detection of adulteration/ substitution	Lateral Flow Assay	Masiri *et al.*, 2016
Pork in beef meatball	Detection of adulteration/ substitution	Fourier transform infrared (FTIR) spectroscopy and partial least square (PLS) calibration	Rohman *et al.*, 2011
Wild and cultured sea bass	Difference in production system	NMR	Mannina *et al.*, 2008

MALDI-TOF MS: Matrix-assisted laser desorption/ionization-Time of flight mass spectrometry; LC–ESI–QTOF MS/MS: Liquid chromatography and electrospray ionization quadrupole time-of-flight mass spectrometry; LC–ESI–QQQ MS/MS: Liquid chromatography and electrospray ionization triple quadrupole mass spectrometry

extraction of analytes in order to increase the sensitivity as well as reproducibility of proteomic or metabolomic techniques. Sample collection should avoid intra-species variability, season changes within a year or developmental status (age) of animals for

studies involving the geographical origin of food products. The development of high-throughput mass spectrometric methods and new bioinformatic tools will definitely play a key role in the authentication studies. New interdisciplinary technologies, such as affordable PoC devices, biochips and biosensors are promising applications for on-site detection of food adulteration. Finally, integration of genomics and transcriptomics with proteomics and metabolomics will definitely add to the quality and reliability of authentication results.

Conflict of interest: Authors have no conflict of interest in this study.

Author's contribution: RB: Involved in conceptualization, investigation, data curation and writing-original draft; NBM: Involved in conceptualization, writing-review and editing; YPG: Engaged for resources and data curation; SB: involved in supervision for the entire work, GP: Involved in visualization and supervision; DB: Involved in visualization and supervision; GM: Engaged for resources and data curation; AKV: Involved in resources and data curation.

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