SERVIÇO PÚBLICO FEDERAL
UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE BIOTECNOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

BIOSSENSORES ELETROQUÍMICOS PARA DETECÇÃO DE ADULTERAÇÃO EM CARNE BOVINA

Aluno: José Manuel Rodrigueiro Flauzino

Orientador: Profª. Drª. Ana Graci Brito Madurro

Co-orientador: Prof. Dr. João Marcos Madurro

Uberlândia – MG

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Tese apresentada à Universidade Federal de Uberlândia como parte dos requisitos para obtenção do Título de Doutor em Genética e Bioquímica (Área: Bioquímica)

Uberlândia – MG

2021

UNIVERSIDADE FEDERAL DE UBERLÂNDIA
Coordenação do Programa de Pós-Graduação em Genética e Bioquímica
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ATA DE DEFESA - PÓS-GRADUAÇÃO

Programa de Pós-Graduação em:	Genética e Bioquímica				
Defesa de:	Doutorado Acadêmico - nº 06/2021 - PPGGB.				
Data:	Vinte e seis de novembro de dois mil e vinte e um	Hora de início:	14:00h	Hora de encerramento:	20:30h
Matrícula do Discente:	11723GBI004				
Nome do Discente:	José Manuel Rodrigueiro Flauzino				
Título do Trabalho:	Biossensores eletroquímicos para detecção de adulteração em carne bovina.				
Área de concentração:	Bioquímica				
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Aos vinte e seis dias do mês de novembro de dois mil e vinte e um, às 14:00 horas, reuniu-se via web conferência pela Plataforma *Microsoft Teams*, em conformidade com a Portaria nº 36, de 19 de março de 2020 da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES e Resolução de nº 06/2020 do Conselho de Pesquisa e Pós-graduação pela Universidade Federal de Uberlândia, a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em Genética e Bioquímica, assim composta: Profª. Drª. Cecilia de Carvalho Castro e Silva, Prof. Dr. Frank Nelson Crespilho, Profª. Drª. Djenaine de Souza, Prof. Dr. André Luiz dos Santos e Profª. Drª. Ana Graci Brito Madurro, orientador (a) do (a) candidato (a) e demais convidados presentes conforme lista de presença. Iniciando os trabalhos o (a) presidente da mesa, Profª. Drª. Ana Graci Brito Madurro, apresentou a Comissão Examinadora e o (a) candidato (a), agradeceu a presença do público, e concedeu o (à) Discente a palavra para a exposição do seu trabalho. A duração da apresentação do (a) Discente e o tempo de arguição e resposta foram conforme as normas do Programa de Pós-graduação em Genética e Bioquímica. A seguir o (a) senhor (a) presidente concedeu a palavra, pela ordem sucessivamente, aos examinadores, que passaram a arguir o (a) candidato (a). Ultimada a arguição, que se desenvolveu dentro dos termos regimentais, a Banca, em sessão secreta, atribuiu os conceitos finais. Em face do resultado obtido, a Banca Examinadora considerou o candidato (a):

APROVADO.

Esta defesa de Tese de Doutorado é parte dos requisitos necessários à obtenção do título de Doutor. O competente diploma será expedido após cumprimento dos demais requisitos, conforme as normas do Programa, a legislação pertinente e a regulamentação interna da UFU. Nada mais havendo a tratar foram

encerrados os trabalhos. Foi lavrada a presente ata que após lida e achada conforme foi assinada pela Banca Examinadora.

Documento assinado eletronicamente por **Ana Graci Brito Madurro**, **Professor(a) do Magistério Superior**, em 26/11/2021, às 21:13, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

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Comissão Examinadora

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Examinadores: Profª. Drª. Cecília de Carvalho Castro e Silva
Prof. Dr. Frank Nelson Crespilho
Profª. Drª. Djenaine de Souza
Prof. Dr. André Luiz dos Santos

Data da Defesa: 26/11/2021

As sugestões da Comissão Examinadora e as Normas do PPGGB para o formato da tese foram contempladas

Ana Graci Brito Madurro

Profª. Drª. Ana Graci Brito Madurro

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Agradeço primeiramente à minha família, pelo amor incondicional e pelo apoio em toda minha jornada durante meu período de graduação e pós-graduação.

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E agradeço a você, leitor, por demonstrar interesse na leitura deste trabalho!

Sumário

Apresentação

Desde o primeiro biossensor proposto por Clark e Lyons em 1962 para detecção de glicose, uma infinidade de novos biossensores foi desenvolvida para a detecção de inúmeros analitos. Suas aplicações em diversas áreas como saúde, alimentos, meio-ambiente, indústria, segurança, criminalística, dentre outras, aliado às várias vantagens frente às técnicas de detecção convencionais, consagraram esses dispositivos fazendo com que o seu mercado mundial só tenda a crescer nos próximos anos.

No entanto, o desenvolvimento de novos biossensores não é trivial, visto que há uma série de importantes fatores a serem considerados: a amostra, o tipo de analito, o transdutor, a biomolécula de reconhecimento, a plataforma e o método de imobilização, a sensibilidade, seletividade e estabilidade do biossensor, dentre outros. Todas essas variáveis devem ser estudadas e levadas em consideração. Dentre as várias possíveis metodologias de detecção, as técnicas eletroquímicas são amplamente utilizadas em sensores e biossensores por serem de simples execução, baixo custo, fácil miniaturização e possibilidade de serem aplicadas em dispositivos *point-of-need.*

Um mercado multibilionário pouco explorado pelos biossensores é o de detecção de adulteração em carnes, possuindo grande potencial no Brasil visto que nosso país é o maior exportador mundial de carne bovina. Recentes operações de fiscalização colocaram a qualidade da carne bovina brasileira em dúvida, e aliando-se isso a restrições de ingestão de carne suína por fatores religiosos e alérgicos, o campo para desenvolvimento de novos métodos de detecção de diferentes espécies em misturas de carnes é muito propício para a aplicação dos biossensores.

Desta maneira, a presente tese de doutorado teve como objetivo o desenvolvimento de biossensores eletroquímicos para detecção de adulteração de carne bovina por carne suína. Com o objetivo de obter um maior impacto na literatura científica, este trabalho é composto de uma coletânea de artigos de revisão e de pesquisa originais redigidos em inglês, sendo alguns já publicados, de acordo com as normas de confecção de tese do Programa de Pós-graduação em Genética e Bioquímica do Instituto de Biotecnologia da Universidade Federal de Uberlândia.

O Capítulo 1 apresenta uma revisão bibliográfica do tema na literatura atual, englobando os trabalhos já publicados que desenvolveram biossensores para detecção de diferentes espécies animais em carnes e os desafios na área. O capítulo 2 é referente a um genosensor eletroquímico para detecção do DNA mitocondrial bovino desenvolvido em eletrodos de grafite modificados com um nanocompósito. Já o capítulo 3 apresenta uma plataforma miniaturizada e descartável baseado num eletrodo impresso modificado com um nanomaterial derivado do grafeno, para detecção do DNA mitocondrial suíno em amostras de carne sem necessidade de marcação ou pares redox.

Capítulo 1: *Application of biosensors for detection of meat species: a review*

O capítulo 1 corresponde a um manuscrito de artigo de revisão redigido nas normas do periódico *Food Control* (ISSN: 0956-7135).

Application of biosensors for detection of meat species: a review

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Resumo

Os biossensores são ferramentas fáceis de manusear que tiveram muitos avanços nos últimos anos. Na indústria da carne, a maioria desses dispositivos são aplicados na detecção de patógenos ou análise de frescor. No entanto, eles também são aplicáveis na investigação de adulteração em carnes por misturas de diferentes espécies, uma vez que a autenticidade da composição de alimentos é um problema complexo que envolve questões de saúde, econômicas, jurídicas e religiosas. Esta revisão tem como objetivo sumarizar os biossensores desenvolvidos para esse fim e como é realizada a detecção de espécies de carne em misturas complexas.

Palavras-chave: adulteração, fraude, carne bovina, carne suína, halal, kosher, genossensor, imunossensor

Abstract

Biosensors are easy-to-use tools that have made many advances in recent years. In the meat industry, most of these devices are applied in pathogen detection or freshness analysis. However, they are also applicable in the investigation of adulteration in meat by mixtures of different animal species, since the authenticity of food components is a serious problem involving health, economic, legal and religious concerns. This review aims to summarize the biosensors developed for this purpose and how they manage to detect different meat species in complex mixtures.

Keywords: meat adulteration, meat fraud, beef; pork; halal; kosher; genosensor; immunosensor.

1 Introduction

The value of the global meat sector was estimated at US$ 830 billion in 2020, with potential to surpass 1 trillion dollars by 2025, as meat consumption is increasing in developing countries (Food and Agriculture Organization of the United Nations, 2020). To ensure the supply of a quality product to this growing population, all types of meat must undergo rigorous analysis of composition and quality, especially in the exportation process. The mixture of different species of animals in meat is a major problem in the guarantee of a pure product for the final consumer (Kane & Hellberg, 2015). For example, in Muslim and Jewish societies, the consumption of pork is forbidden, so beef must be free from traces of pig contents to meet Halal and Kosher standards (Hossain *et al.*, 2020). Allergies to pork proteins are frequent in some populations as well (Wilson & Platts-Mills, 2018). In addition, horsemeat is avoided in many cultures (Smith & McElwee, 2020).

The mixing of different meat species, especially in processed products such as hamburgers, sausages and meatballs, is impossible to be detected by naked eye. This type of adulteration can be unintentional, as cross-contamination may occurs due to poor cleaning of industrial equipment, processing, packaging, storage, and transport (Supian, 2018); or intentional, with the objective of adding cheaper meats to a final product aiming the reducing of costs (Lianou *et al.*, 2021). Meat frauds result in the loss of confidence and trust among the consumers, health organizations, and food safety regulators (Martins *et al.,* 2021). Hence, detections methods to investigate the species within raw meat products are of major importance, both for food inspectors and for the final consumer.

Numerous methods are used to detect specie adulteration in meat: physical, sensory, anatomical and histological analysis of meat cuts (Singh & Neelam, 2010), biochemical techniques like chromatography, electrophoresis and immunodiffusion (Rahmati *et al.*, 2016) and also spectral techniques like Fourier-transform infrared, ultraviolet-visual, and Raman spectroscopy (Kumar & Chandrakant, 2017). However, all the above-mentioned methods require appropriate laboratory equipment, qualified personnel and are time-consuming. Faster and reliable molecular methods such as polymerase chain reaction (PCR) can also be utilized (Doosti *et al.*, 2014), but require the use of expensive

reagents, laboratory facilities and skilled staff. Therefore, the search for new methods to determine the origin of meat that are fast, cheap and accurate is very attractive. Biosensors enter in this scenario, with the proposal to develop new point-of-need, that is, easy-to-handle portable devices that can be used at any stage of the industrial process or even by final consumers in any situation where the analysis is required.

Figure 1 shows a general scheme of the components of a biosensor. They are analytical devices that incorporate some biological element, capable of producing measurable signals that relate to the concentration of the analyte of interest (Turner, 2013). Biosensors combine a biochemical recognition system with a transducer, which converts this recognition event into a measurable signal. (Bhalla *et al.*, 2020). The successful association between detection techniques, specific biomolecules and new materials enabled the development of sensitive and selective molecular biosensing technologies, with applications that include not only the food industry, but also clinical analysis (Haleem *et al.*, 2021), environment (Bose *et al.*, 2021), defense (Yahaya *et al.*, 2021), forensic (Selli *et al.*, 2022), among others. Several types of biomolecules can be incorporated into biosensors, such as antibodies, antigens, enzymes and nucleic acids. In addition, the availability of several types of transducers, such as electrochemical, optical and piezoelectrical, create a variety of combinations that result in countless variations of biosensors.

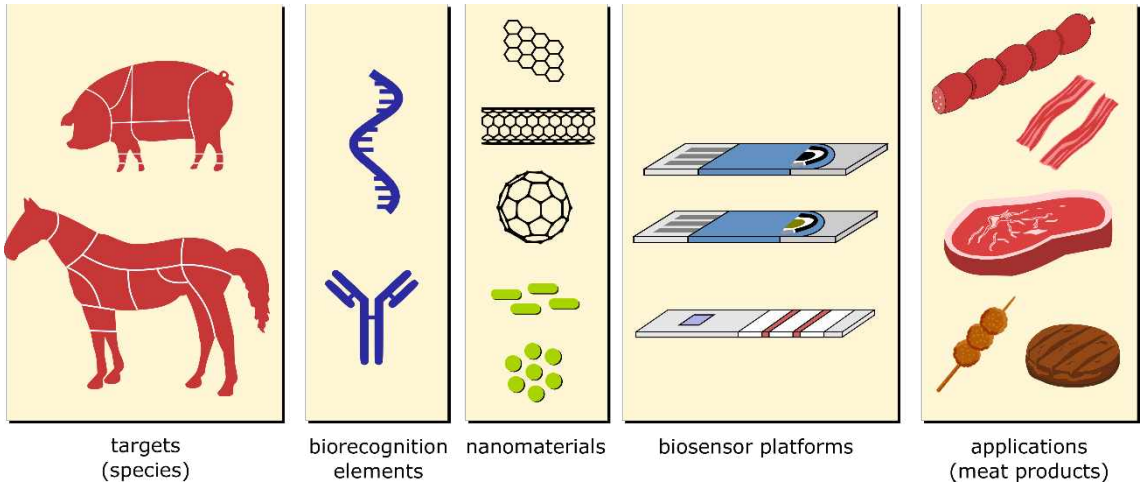

Figure 1 – Scheme of the constituent parts of a biosensor applied for detection of meat species. For a selected target, which can be proteins or DNA of a particular specie, a specific biorecognition element is used. To enhance the biosensor performance, nanomaterials are

usually employed in the biosensing platform, which can be an electrode or a sample pad strip. The samples can be different types of processed meat, though its preparation steps are crucial for the success of the analysis.

To improve the analytical performance of biosensors and the biorecognition molecule immobilization, the transducers are usually modified with nanomaterials (Kucherenko *et al.*, 2019). Nanomaterials are materials of which a single unit small sized (in at least one dimension) is between 1 and 100 nm (Kreyling *et al*., 2010). Nanomaterials may have different peculiar properties compared to bulk material, such as high surface area and high conductivity, as in graphene and derivatives, or present plasmonic resonance, as in metallic nanoparticles. These new properties are very useful in biosensors, increasing the sensitivity, selectivity and stability of these devices (Naresh & Lee, 2021). In recent years, many approaches have been described for this purpose, such as the use of polymeric films, graphene derivatives, nanoparticles and others (Weiss *et al*., 2020).

In the meat industry, biosensors are mainly employed to detect freshness, based on the detection of spoilage biomarkers such as biogenic amines (Vasconcelos *et al.*, 2021), or the presence of pathogens such as *Escherichia coli* (Hassan *et al.*, 2015), *Listeria monocytogenes* and *Staphylococcus aureus* (Primiceri *et al.*, 2016). To access meat species, genosensors (biosensors whose recognition molecules are nucleic acids) can be used since each specie has genetic markers that allow its differentiation (Quinto *et al.*, 2016). The stability of DNA against temperature and meat processing, compared to enzymes and other proteins, highlights genosensors for this type of analysis (Hellberg *et al.*, 2017). However, with few exceptions, these devices require cell lysis, genetic material purification and amplification protocols. In the other hand are the immunosensors, which can specify recognize proteins biomarkers in food samples (Kim *et al.*, 2017). They may not require complex sample preparation steps, but protein structure is easily denatured with temperature, pH, or salinity. Thus, the type of biosensor directly affects the sample preparation.

In addition to the type of biomolecule, the transducer plays a key role in the biosensor. For biosensors in meat samples, there are mainly two types of platforms: electrochemical and optical, which will be explained later in this article.

Consequently, combining different possible analytes, recognition biomolecules and transducers, numerous biosensors were developed. Therefore, the purpose of this review was to make a summary of published works showcasing biosensors applied in the detection of different species in meat samples.

2 Articles search

The terms "biosensor", "meat", "adulteration" and "species detection" were searched in the databases Science Direct, PubMed and Google Scholar. For this review, only works involving meat adulteration by species of mammal and/or bird meat were considered. Thus, works that involved detection of adulteration by physical components, traceability, fish meat, detection of contamination by bacteria, heavy metals, antibiotics or others, were not considered. A total of 37 works were found, consisting of 12 articles related with electrochemical biosensors and 25 with optical biosensors (Figure 2). In this review, the developed works were divided according to their transduction element. Optical biosensors are more common probably due to the development of more portable and fast assays such as lateral-flow strips.

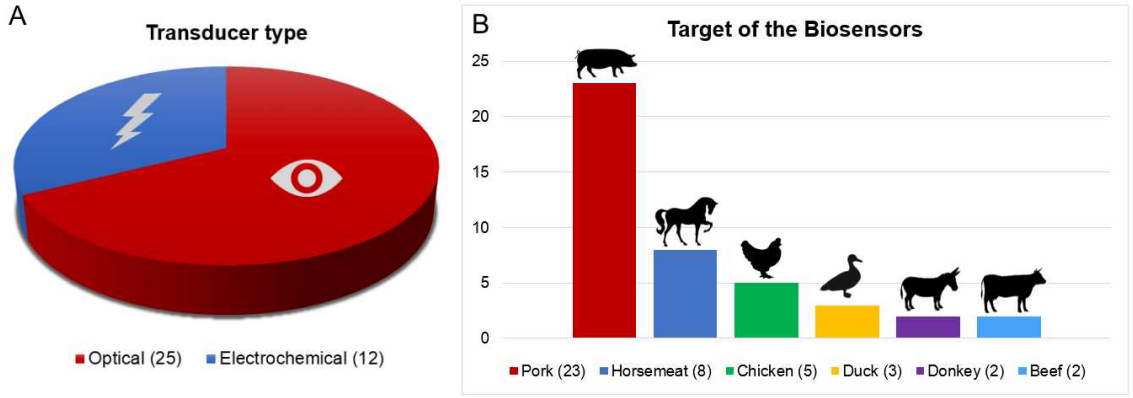

Figure 2 – (A) Pie chart the distribution of the type of transducer present in the listed biosensors. (B) Bar graph of meat types targeted by the listed biosensors. Some articles report more than one target.

Pork was the detection target in most works, appearing in 23 articles, highlighting its importance as an adulterant mainly due to religious and allergic issues. The vast majority of works reported biosensors requiring full purification and/or amplification of the sample, a process found in 30 papers. In comparison

with other areas of application of biosensors in the food industry and in biomedicine, we noticed that there are not many works involving these devices to investigate meat adulteration. This fact may be due to the complexity of the meat sample, in which interfering agents can affect the performance of the biosensor, requiring a very delicate experimental design to obtain a satisfactory response. Thus, we were not surprised to find that majority of works required long sample purification steps, but this becomes unfeasible in a point-of-need device.

Only 5 works reported some study of the stability of the platform in function of time. This is an important parameter to verify the shelf-life and future commercialization of these devices, but in general they are not studied in biosensors papers. Additionally, the given assay times only encompassed the technique time and not the sample preparation, thus hiding the total test time. As the industry aims for fast processes, a short sample preparation time is also needed in those devices.

3 Electrochemical biosensors

Electrochemical biosensors are based on the consumption and/or generation of electroactive species in the biochemical recognition events onto the biosensor, as well the monitoring of electrochemical labels or redox pairs before and/or after biological interactions (Koyappayil & Lee, 2020). In this process the transducer, usually an electrode connected to a potentiostat or galvanostat, measures the electrochemical signal produced by this interaction (Merhvar & Abdi, 2004) and convert it in an readable signal. Figure 3 shows a scheme of an electrochemical genosensor.

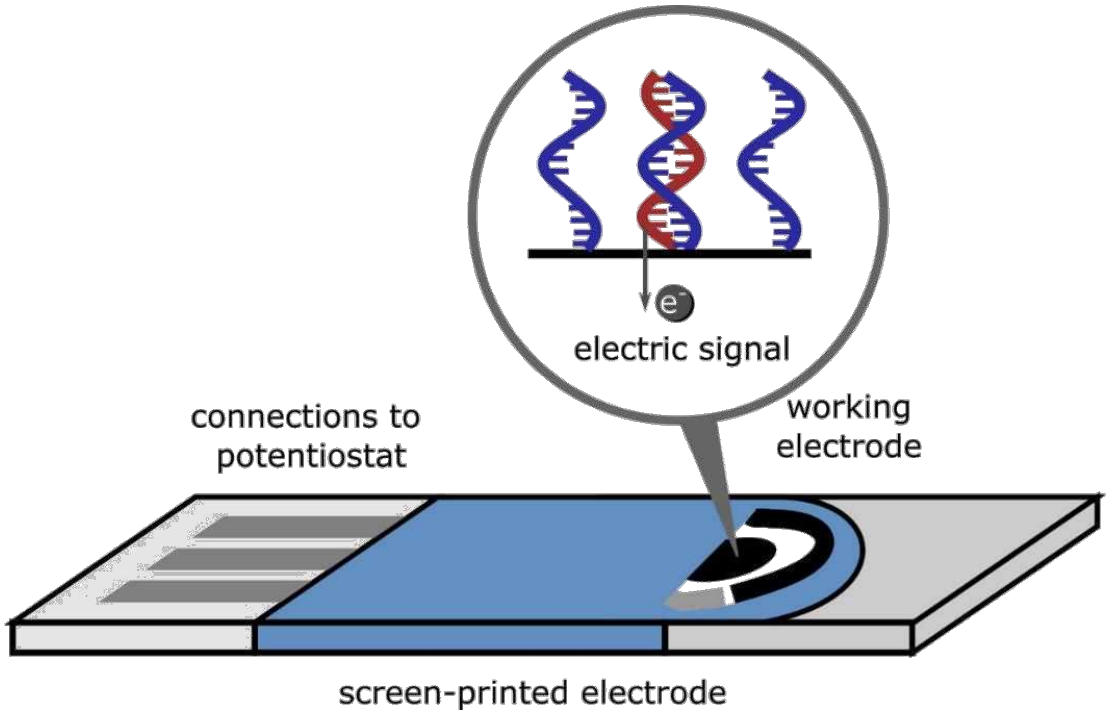

Figure 3 – Scheme of an electrochemical genosensor: nucleic acids (blue) are immobilized onto a screen-printed electrode, which recognize a complementary target (red) in the sample. The electrical signal is then measured and converted in a readable signal by the transducer.

Frequently measured variables in this type of sensor are current, potential, conductivity and impedance (Bansod *et al.*, 2017). For analyte detection, voltammetric techniques such as Differential Pulse Voltammetry (DPV) and Square Wave Voltammetry (SWV) are usually applied as they decrease the residual capacitive current in the electrodes, producing a current signal proportional to the analyte concentration (Menon *et al*., 2020). Amperometry is also widely used in food analysis, a technique in which a fixed potential is applied between the electrodes in contact with the sample and the resulting current is measured (Chillawar *et al*., 2015). Another electrochemical technique utilized in biosensors is the Electrochemical Impedance Spectroscopy (EIS), which provides information of the species adhered to the electrode surface, for example by calculating the charge transfer resistance, which is correlated with the amount of adhered analyte on the surface of the biosensor (Bertok *et al.,* 2019).

These techniques have been used as useful tools for traditional analytical methods and have several advantages compared to other methods, such as high sensitivity, selectivity, reliability, stability, short response time, cost-effectiveness, and ease of miniaturization (Ronkainen *et al.*, 2010). Due to their advantages,

electrochemical biosensors have been widely applied in several areas such as medical, environmental, forensics and food, including food adulteration detection (Ferreira *et al.*, 2019; Hasan *et al.*, 2021; Kurbanoglu *et al.*, 2020; Riu & Giussani, 2020; Uniyal & Sharma, 2018; Ye *et al.*, 2020).

Several strategies have been used for the electrochemical detection of meat species (Table 1). Most of the described electrochemical biosensors in the literature are based on DNA-hybridization or protein detection. Despite most of the electrochemical biosensors being very sensitive, most of them requires sample purification and/or amplification. On the other hand, some works proposes use of less purified samples and have lower detection limits. In any case, long-term stability studies of these platforms are scarce, an important parameter aiming future commercialization of the biosensor. The detection limits are generally given in two forms: weight by weight percentage (% w/w), which means the lower percentage of weight of target that could be detect in the same weight unit of the whole sample; or in concentration units of detected nucleic acid such as mol L^{-1} and g L^{-1}.

Flauzino *et al.* (2021 & 2022), and Hartati *et al.* (2020) described the development of genosensors for identification of pork in meat using graphene-modified electrodes. The target used in the development of the genosensors was the cytochrome b gene, present in mitochondrial DNA. In his works, Flauzino and co-workers used square wave voltammetry, faradaic and non-faradaic electrochemical impedance spectroscopy techniques to monitor probe-target DNA hybridization, with simple and fast sample preparation steps, not requiring amplification. Hartati and co-workers used differential pulse voltammetry based on the target's guanine oxidation signal and obtained a low detection limit, however with additional steps of DNA extraction and application.

Table 1 – Summary of electrochemical biosensor for detection of species in meat.

Electrochemical Technique	Biorecognition molecule	Sample	Target	LOD	Sample preparation and assay time	Stability	Ref
SWV/ Faradaic EIS	DNA	Beef	Pork	1% w/w	60 min	6 weeks	(Flauzino *et al.*, 2021)
Non-faradaic EIS	DNA	Beef	Pork	9% w/w	45 min	4 weeks	(Flauzino *et al.*, 2022)
DPV	DNA	Pork, chicken and beef	Pork	1.76 μg mL^{-1}	n.a.	n.a.	(Hartati *et al.*, 2020)
LSV	DNA	Raw meat/ processed foods	Pork, chicken and bovine	~20.33 ng μL^{-1} (pork), ~78.68 pg μL^{-1} (chicken) and ~23.63 pg μL^{-1} (beef)	65 min	n.a.	(Ahmed *et al.*, 2010)
DPV	DNA	Pork, chicken and beef	Pork	10^{-13} μmol L^{-1}	n.a.	n.a.	(Halid *et al.*, 2014)
LSV/SWV/DPV/ EIS	DNA	Cooked sausages	Donkey	148 pmol L^{-1}	n.a.	n.a.	(Mansouri, Khalilzadeh, *et al.*, 2020)
Amperometry	RNA	Beef	Horse	0.12 pmol L^{-1}	75 min	23 days	(Montiel *et al.*, 2017)
SWV/ chronocoulometric	DNA	Processed foods (Chicken, beef, mutton, pork)	Chicken/ Pork	1 pg mL^{-1} (chicken) / 100 pg mL^{-1} (pork)	n.a.	n.a.	(Roy *et al.*, 2016)
DPV	DNA	13 species of meat	Beef	8.2 fmol L^{-1}	60 min	n.a.	(Zhang *et al.*, 2020)
SWV/DPV	Porcine albumin antibody	Pork, chicken and beef	Pork	0.5 pg mL^{-1}	n.a.	n.a.	(Lim & Ahmed, 2016)
Chronoamperometry	IgG antibodies	Beef	Pork	0.1 w/w (direct) and 0.01% (competitive)	2 h (direct) and 20 min (competitive)	n.a.	(Mandli *et al.*, 2017)
EIS	IgG antibodies	Horsemeat, pork and beef	Horse	0.004% w/w	72 minutes	n.a.	(Faria *et al.*, 2018)

n. a.: not available; SWV: Square Wave Voltammetry; EIS: Electrochemical Impedance Spectroscopy; LSV: Linear Sweep Voltammetry; CV: Cyclic Voltammetry; DPV: Differential Pulse Voltammetry.

Also using graphene-modified electrodes, Roy and co-wrokers developed an approach to detection of isothermal amplicons of meat species using square wave voltammetry and chronocoulometric analysis of hexamine ruthenium (Roy *et al.* 2016). Ahmed and co-workers also determined species-specificity using a combination of loop mediated isothermal amplicons for DNA amplification followed by their electrochemical detection on disposable electrochemical printed chips (Ahmed *et al.* 2010). The interaction between target DNA amplicons and the DNA minor-groove binder molecule Hoechst 33258 was used to detect target DNA, by measuring the changes in the oxidation peak current of Hoechst 33258 using linear sweep voltammetry. The device was able to distinguish species-specificity in control and processed pork, chicken and bovine meats.

Halid and co-workers developed an electrochemical DNA biosensor for detection of porcine oligonucleotides using ruthenium (II) as label redox complex (Halid *et al.* 2016) The system was based on the immobilization of porcine DNA probe on screen-printed carbon electrodes containing gold nanoparticles and poly(n-butylacrylate-N-acryloxysuccinimide) microspheres, utilizing differential pulse voltammetry for detection. The results showed that the platform was efficient to detect porcine DNA at low concentrations, but it was not selective.

Zhang and co-workers proposed an electrochemical biosensor for detection of specie-specific DNA sequences from meat products based on toehold-mediated strand displacement as enzyme free isothermal strand displacement amplification strategy (Zhang *et al*., 2020). Thirteen different species meats (cattle, sheep, pig, horse, donkey, dog, fox, rabbit, mouse, rat, chicken, duck and goose) were tested by the developed method to evaluate the analytical capacity in animal species identification. Although it was sensitive, it requires DNA purification steps, and the authors did not study the stability of the platform.

Mansouri and co-workers described a genosensor based on the immobilization of specific LNA (Locked Nucleic Acid) on gold electrodes to detection of donkey meat adulteration in consumable beef sausage preparations. LNA are modified RNA strands with increased stability against enzymatic degradation. The developed device was successfully applied for detection of donkey adulteration in cooked sausages and the obtained results were compared with qRT-PCR as standard method.

An electrochemical sensor to detect horsemeat adulteration was developed using magnetic capture on screen-printed carbon electrodes (Montiel *et al.* 2017). The proposed assay involved direct hybridization of the target mitochondrial DNA fragment with a specific RNA capture probe immobilized onto streptavidin-functionalized magnetic microcarriers, recognition of the captured DNA/RNA heteroduplexes with a commercial antibody and labeling with a bacterial protein conjugated with a horseradish peroxidase homopolymer. Another electrochemical approach has been described for horsemeat adulteration screening (Faria *et al.* 2018). The methodology is based on the immobilization of anti-horse Immunoglobulin G on screen-printed carbon electrodes containing conductive polyaniline and uses electrochemical impedance spectroscopy technique for detection.

Other electrochemical immunosensors for detection of species-specific proteins using IgG and porcine albumin antibodies as biorecognition molecule have been described in the literature. Mandli and co-workers proposed two formats of electrochemical immunosensors (direct and competitive) to detect low levels of pork adulteration in beef meat (Mandli *et al.*, 2018). The detection platform was based on a detection of pig-IgG on electropolymerized polypyrrole modified graphite paste electrodes. The capture of pig-IgG was performed using peroxidase conjugated anti-pig IgG polyclonal antibody and the detection was monitored by chronoamperometry.

Lim and a co-worker reported a label free electrochemical immunosensor for to detect pork adulteration in raw meat samples (beef, chicken and pork) based on the immobilization of porcine serum albumin antibody on carbon nanofiber-modified screen-printed carbon electrodes functionalized with 4-carboxyphenyl (Lim & Ahmed, 2016). The detection of porcine serum albumin was then carried out in potassium ferrocyanide/ferricyanide solution, employing the fact that porcine serum albumin has strong affinity for anions while the large surface area of carbon nanofiber increased the antibody immobilization capacity and electronic conductivity.

3 Optical biosensors

Optical biosensors are based on the measure of optical properties changes due to analyte recognition by biological molecules, including nucleic acids and antibodies (Chen & Wang, 2020; Khalil *et al.*, 2021). These optical properties include colors detected by naked eye (colorimetry), as well as more advanced techniques, such as surface-enhanced Raman scattering (SERS), surface plasmon resonance (SRP) and fluorescence spectroscopy (FS) (Chen & Wang, 2020; Khalil *et al.*, 2020, 2021; Liu *et al.*, 2021; Mansouri, Fathi, *et al.*, 2020). Various strategies have been used for the optical detection of meat species (Table 2), mostly based in DNA or antibody detection. Figure 4 shows a scheme of a colorimetric immunosensor.

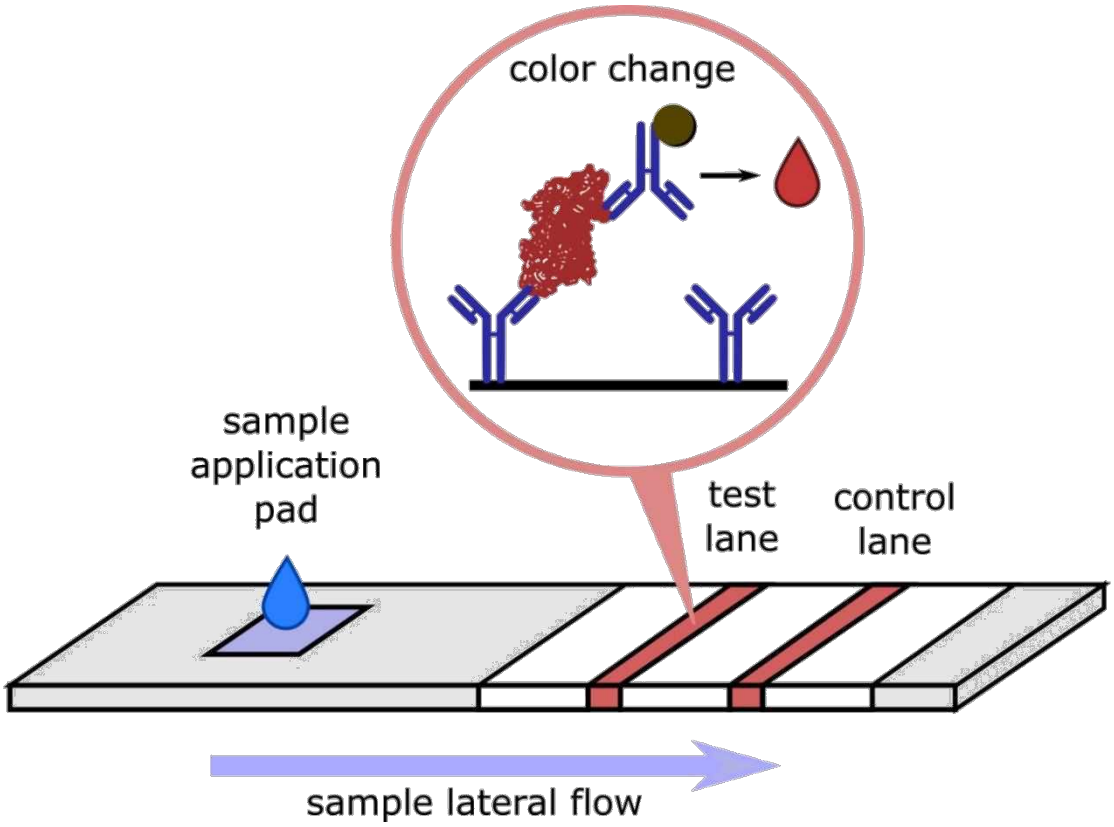

Figure 4 – Scheme of a colorimetric immunosensor: the biological recognition event generates a change in color in the test line, which can be read by naked eye.

Table 2 – Summary of optical biosensor for detection of species in meat

Optical technique	Biorecognition molecule	Sample	Target	LOD	Assay time	Stability	Ref
NE	DNA	Several retail samples	Deer, rabbit, duck, chicken, beef, horse, sheep, pork.	0.5 pg (deer or beef), 0.001% (w/w, deer/beef)	30 min	n.a.	(Wang *et al.*, 2015)
NE	DNA	Several processed camel meat products	Camel	0.1% w/w	n.a.	n.a.	(Zhao *et al.*, 2020)
SPR	DNA	Cooked beef sausage	Donkey	n.a.	n.a.	n.a.	(Mansouri, Fathi, *et al.*, 2020)
SERS/NE	DNA	Beef, lamb roll, pork, mutton, steak	Duck	0.05% w/w (SERS), 0.1% w/w (NE)	n.a.	24h / 48h, 4ºC (liposomes)	(Liu *et al.*, 2021)
NE	DNA	Duck, beef, sheep, chicken and pork meat mixtures	Duck, beef, sheep, chicken, pork	20 pg μL^{-1} (genomic DNA, average)	20 min	n.a.	(Lin *et al.*, 2021)
NE	DNA	Meat samples of 15 animal species	Horse	0.1% w/w	1 h	n.a.	(Wang *et al.*, 2019)
NE	DNA	Meat mixtures and commercial samples from horse and several species	Horse	0.01% w/w	2-3 min	n.a.	(Chen *et al.*, 2021)
NE	DNA	Horse, donkey, duck and chicken meat mixtures	Horse, donkey	40 pg	40 min	n.a.	(Zhang *et al.*, 2019)
NE	DNA	Horse-beef and pork-sheep DNA mixtures	Horse, pork	0.01% w/w (horse); 0.02% w/w (pork)	10-15 min	n.a.	(Magiati *et al.*, 2019)
NE	DNA	Several meat mixtures and processed foods	Mammalian	10 pg	n.a.	n.a.	(Xu *et al.*, 2017)
FE	DNA	Pork-beef meatballs	Pork	1% w/w	n.a.	n.a.	(Ali *et al.*, 2014)
FS	DNA	Autoclaved pork-beef binary mixture	Pork	1% w/w	n.a.	n.a.	(Ali, Hashim, Kashif, *et al.*, 2012)
FS	DNA	Autoclaved pork-beef meat mixtures	Pork	58.6 pM (syntetic target), 230 µg L^{-1} (total DNA).	n.a.	n.a.	(Ali, Hashim, Mustafa, Che Man, Yusop, Kashif, *et al.*, 2011)

NE	DNA	Beef	Pork	0.1% w/w	30 min	n.a.	(El Sheikha, 2019)
NE	DNA	Pork, mutton, beef, chicken, goose, duck, horse and rabbit meat	Pork	10 fg	3 min	n.a.	(Yin *et al.*, 2020)
NE/AS	DNA	Pork-venison, pork-shad, shad-venison mixtures	Pork	6 μg mL^{-1}	< 10 min	n.a.	(Ali, Hashim, Mustafa, Che Man, Yusop, Bari, *et al.*, 2011)
NE/AS	DNA	Pork, beef and chicken meat mixtures	Pork	4 μg mL^{-1}	< 10 min	n.a.	(Ali, Hashim, Mustafa, *et al.*, 2012)
SERS	DNA	Pork DNA	Pork	1 fmol L^{-1}	n.a.	n.a.	(Khalil *et al.*, 2020)
NE	Antibody	Beef and chicken mixture	Beef	25 ng/mL (TnI), 1% (w/w, beef/chicken)	15 min	n.a.	(Zvereva *et al.*, 2020)
NE	Antibody	Raw and cooked mixtures of chicken with pork, beef, lamb and rabbit	Chicken	0.063% (w/w)	20 min	n.a.	(Hendrickson, Zvereva, Vostrikova, *et al.*, 2021)
NE	Antibody	Beeef, pork, sheep, goat, chicken, turkey meat mixtures	Horse	0.01% (raw) and 1.0% (cooked meat)	35 min	n.a.	(Jongkit Masiri *et al.*, 2017)
NE	Antibody	Cooked beef meatballs	Pork	0.1% (w/w)	5 min	n.a.	(Kuswandi *et al.*, 2017)
NE	Antibody	Raw, cooked meat and gellatin from pork, beef, sheep, goat, chicken, turkey and horse	Pork	0.01% (raw), 1.0% (cooked meat), and 2.5% (gelatin)	35 min	n.a.	(Masiri *et al.*, 2016)
NE	Antibody	Mixtures of beef, chicken, rabbit, turkey and lamb with pork	Pork	0.063% (w/w)	35 min	n.a.	(Hendrickson, Zvereva, Dzantiev, *et al.*, 2021)
NE	Antibody	Beef, lamb, rabbit, chicken, turkey, pigeon and duck meat	Pork	0.01% (w/w)	30 min	7 days (37 ºC)	(Seddaoui & Amine, 2021)

NE: naked eye; SPR: Surface plasmon resonance; SERS: Surface-enhanced Raman scattering, AS: Absorption spectroscopy, FS: Fluorescence spectroscopy, n.a.: not available.

4.1 DNA-based optical biosensors

Most of the optical biosensors reported for meat authentication were based on DNA as biorecognition element (Ali *et al.*, 2014; Liu *et al.*, 2021; Mansouri, Fathi, *et al.*, 2020; Xu *et al.*, 2017). Many of them used gold nanoparticles as the detection strategy, since their accumulation or aggregation produces readily visible color changes (Ali *et al.*, 2014; Khalil *et al.*, 2020; Magiati *et al.*, 2019; Zvereva *et al.*, 2020). As an example, Ali and co-workers reported a simple assay in which 40 nm gold nanoparticles and oligonucleotide probes were applied without any modifications for the detection of swine PCR products and genomic DNA in mixed samples, achieving a limit of detection of 6 μg mL^{-1} (Ali, Hashim, Mustafa, Che Man, Yusop, Bari, *et al.*, 2011). The detection involved visual observation of the colloidal solution color shift, due to the salt-induced aggregation of nanoparticles, which could be prevented by the presence of single-stranded DNA (the probe only). When the DNA target was applied, the hybridization formed double-stranded DNA, which did not prevent nanoparticles aggregation, changing the initial bright red color to a pinkish-red. This strategy was validated with absorption spectroscopy and electron microscopy, it was sensible to mismatches and the response time was less than 10 minutes. The same group reported a similar approach with 20 nm gold nanoparticles and an oligonucleotide probe for swine cytochrome b gene detection in beef and chicken meatballs, achieving 4 μg mL^{-1} as limit of detection (Ali, Hashim, Mustafa, *et al.*, 2012).

Moreover, the same group also reported optical biosensors with a combination of modified nanoparticles and oligonucleotide probes for fluorescence spectroscopy-based detection. One of such biosensors consisted of single-stranded DNA probes functionalized with gold nanoparticles in one end and a tetramethyl-rhodamine (TMR) fluorophore in the other end. When the positive samples were applied, probe-target hybridization triggered an increase in the physical distance between the fluorophore and the nanoparticle, allowing fluorescence emission (Ali, Hashim, Mustafa, Che Man, Yusop, Kashif, *et al.*, 2011). This approach was applied to detect pork in autoclaved pork-beef meat mixtures in a single step, achieving a limit of detection of 58.6 pmol L^{-1} (synthetic target) or 230 μg L^{-1} (total DNA). Similar approaches were applied to detect 1% (w/w) pork adulteration in autoclaved pork-

beef mixtures (Ali, Hashim, Kashif, *et al.*, 2012) and 1% (w/w) pork adulteration in raw and cooked meatball formulation (Ali *et al.*, 2014).

Khalil and co-workers developed a sandwich biosensor based on a dual platform consisting of a DNA signal probe with a Raman tag (ATTO Rho6G) and thiol-linked to gold nanoparticles, and a DNA capture probe thiol-linked to graphene oxide-gold nanorods (Khalil *et al.*, 2020). In the presence of the DNA target, its hybridization with both the signal and capture probes joined the two platforms together, enhancing the SERS signal. The biosensor was applied to the detection of DNA extracted from pork samples, achieving a limit of detection of 1 fM.

Some of the reported optical biosensors were based on PCR and the follow-up detection of the amplification products, such as the platform developed by Wang and co-workers for visual detection of meat species using a silicon-based thin film chip (Wang *et al.*, 2015). DNA targets, including mitochondrial *D-loop* gene (duck) or cytochrome *b* gene (deer, rabbit, horse, sheep and pork), were amplified by PCR using modified primers for the addition of biotin to the DNA products. Aldehyde-modified DNA probes were immobilized onto the film surface to hybridize with the PCR products and lead to the interaction with anti-biotin-conjugated peroxidase, triggering a color reaction that could be detected by naked eye. This strategy achieved an absolute limit of detection of 0.5 pg of DNA and a practical limit of detection 0.001% (w/w, deer/beef mixtures).

Some studies combined PCR and gold nanoparticles for visual detection of meat authentication. For example, Magiati and co-workers developed a lateral flow strip test in which the detection of PCR products, marked with a poly(dA) tag and biotin, allowed the hybridization with an immobilized probe and the interaction with gold nanoparticles, respectively (Magiati *et al.*, 2019). The detection was based on the visible color change and the test was applied to horse-beef and pork-sheep mixtures of DNA extracted from blood and meat samples, achieving the detection of 0.01% of horse DNA and 0.02% of pork DNA within 25-30 min after PCR amplification. Another PCR-based biosensor that used nanoparticles aimed the detection of camel mitochondrial *coi* gene, yielding amplification products marked with biotin and FITC, which were captured by gold nanoparticles-labeled antibodies and generated a red color (Zhao *et al.*, 2020). The test showed a limit of detection of 0.1% (w/w) for the detection of camel meat in beef, being successfully applied to

raw and cooked meat samples. A similar strategy used gold nanoparticles conjugated to streptavidin and specific antibodies to detected biotin and fam-labeled amplicons on a nitrocellulose membrane (Yin *et al.*, 2020). The primers targeted pig cytochrome b mitochondrial gene and the method achieved a limit of detection of 10 fg of target DNA, allowing the detection of 0.01% pork-adulterated beef by naked eye within 3 min. The same group reported a similar biosensor targeting the horse cytochrome b gene, accomplishing naked eye detection within 2-3 min of as little as 0.01% horse meat in artificially adulterated mixtures (Chen *et al.*, 2021).

Some studies applied the loop-mediated isothermal amplification (LAMP) of DNA targets to construct biosensors for meat adulteration (Aartse *et al.*, 2017; J. Wang *et al.*, 2019; Zahradnik *et al.*, 2015). LAMP is an alternative DNA amplification method, which can be held in isothermal conditions, with mesophilic DNA polymerase and analyzed without gel electrophoresis, allowing on-site DNA detection (Notomi *et al.*, 2000). Xu and co-workers reported a portable sealed paper-based biosensor for the detection of mammal glucagon gene, using a strategy that combined LAMP with visual detection due to gold nanoparticles accumulation (Xu *et al.*, 2017). LAMP products were labeled with both fluorescein isothiocyanate (FICT) and biotin, being captured by anti-FITC antibodies in the test lane of the strip. Then, biotinylated DNA products interacted with functionalized gold nanoparticles, producing a red color for positive samples. The test was applied to detect mammal DNA in complex and processed foods such as meat products and meat mixtures, showing detection limit of 10 pg for genomic DNA.

Another LAMP-based colorimetric method targeted horse DNA amplification using hydroxy-naphthol blue and neutral red as indicators, achieving the detection within 1 h of as low as 0.1% (w/w) horse meat in horse-cattle meat mixtures (Wang *et al.*, 2019). LAMP strategy was also used in a trident-like lateral flow assay, which achieved multiplexing by bundling three parallel arrays (Zhang *et al.*, 2019). The test yielded double marked amplification products that were captured by specific antibodies in the lateral flow assay and by gold nanoparticle-tagged antibodies for the visual detection. The test achieved horse and donkey detection within 40 min in meat mixtures containing horse, donkey, duck and chicken, with a limit of detection of 40 pg.

Recombinase polymerase amplification (RPA), which is another form of isothermal amplification, was also applied to produce a biosensor for the detection of beef, sheep, pork, duck and chicken in boiled, microwaved, pressure-cooked and fried meat samples (Lin *et al.*, 2021). The strategy was also based on visual detection due to gold nanoparticles accumulation, showed an average detection limit of 20 pg μL^{-1} for genomic DNA and the detection was completed within 20 min.

Other innovative approaches were adopted in the development of optical DNA-based biosensors for food authentication, such as a standalone strip test for visual detection containing a barrel for food matrix processing and a reaction tube for on-site DNA amplification (El Sheikha, 2019). In the presence of positive DNA sample, its hybridization with the complementary target coupled to micro-sized beads led to a visual color reaction, being applied to the detection of pork contamination in beef samples and achieving a limit of detection of 0.01 % (w/w) within 30 minutes of assay. Moreover, Mansouri and co-workers developed an SPR-based biosensor consisting of gold sensor chips and thiol-labeled DNA capture probes for the target DNA (Mansouri, Fathi, *et al.*, 2020). A sandwich format comprised the hybridization of target DNA with both the capture probe and a biotinylated probe plus streptavidin-functionalized gold nanostars, greatly enhancing SPR signal. The limit of quantification for the target oligonucleotide achieved 2 nM for the simple format and 1 nM for the sandwich format, being able to detect 1% of donkey meat in cooked donkey-beef mixtures.

Furthermore, Liu and co-workers developed a dual detection system based on SERS and naked-eye colorimetric detection, using a platform based on clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated nuclease (Cas12a) (Liu *et al.*, 2021). In the presence of target DNA, the CRISPR/Cas12a system was activated and triggered the cleavage of DNA linkers, reducing the capture of signal-loaded liposomes via biotin-streptavidin interaction. Liposomes contained both 4-nitrothiophenol (4-NTP), for SERS detection; and cysteine, which induced gold nanoparticles aggregation and the corresponding color shift. The whole system was applied for the detection of duck DNA in lamb roll, pork, mutton, steak and beef, achieving limits of detection of 0.05% (w/w) for SERS detection and 0.1% (w/w) for naked eye detection. Stability assessments were

restricted to 4-NTP and cysteine-loaded liposomes, which kept stable for 24h and 48h at 4ºC, respectively.

4.2 Antibody-based optical biosensors

Besides DNA targeting, some optical immunoassays were also reported for meat authentication (Hendrickson, Zvereva, Dzantiev, *et al.*, 2021; Hendrickson, Zvereva, Vostrikova, *et al.*, 2021; Kuswandi *et al.*, 2017; Masiri *et al.*, 2016; Masiri *et al.*, 2017; Seddaoui & Amine, 2021; Zvereva *et al.*, 2020). Most of these were lateral flow assays aiming the detection by naked eye of pork in meat samples, using gold nanoparticles conjugates to obtain color reactions (Hendrickson, Zvereva, Dzantiev, *et al.*, 2021; Kuswandi *et al.*, 2017; Masiri *et al.*, 2016). One example focused on the detection of pig serum albumin (PSA) in raw meat and porcine thermal-stable meat protein (P-TSMP) in cooked meat using polyclonal antibodies conjugated to gold nanoparticles (Masiri *et al.*, 2016). Such assay had a time of response of around 35 min and achieved a limit of detection of 0.01% (raw meat), 1.0% (cooked meat), and 2.5% (gelatin), presenting no cross-reactivity with chicken, turkey, horse, beef, lamb or goat samples.

Another example applied an anti-swine polyclonal antibody conjugated to gold nanoparticles to detect pork adulteration in cooked beef meatballs, achieving a detection limit of 0.1% (w/w) and a response time of 5 min, being validated with halal or not halal real samples (Kuswandi *et al.*, 2017). Another group reported a sandwich-based strategy with polyclonal anti-species antibodies (RAPI) that were either immobilized in the test strip or conjugated to gold nanoparticles, interacting with swine immunoglobulins in the positive samples and triggering nanoparticles accumulation (Hendrickson, Zvereva, Dzantiev, *et al.*, 2021). This last immunoassay has a response time of 35 min and a limit of detection of 0.063% (w/w) of pork in raw meat mixtures. Another report of immunoassay-based detection of pork adulteration focused on a competitive immobilization strategy between pig immunoglobulins (target) and polyclonal anti-pig IgG conjugated to peroxidase (Seddaoui & Amine, 2021). The application of the enzymatic substrate tetramethylbenzidine produced a color reaction with proportional intensity to analyte concentration. The strategy was coupled to smartphone signal readout and allowed

the detection of as low as 0.01% (w/w) of pork in pork/beef mixtures within 30 min, with stability of at least 7 days when stored at 37 ºC.

Other reports presented platforms for the detection of other target besides pork (Hendrickson, Zvereva, Vostrikova, *et al.*, 2021; Jongkit Masiri *et al.*, 2017; Zvereva *et al.*, 2020). Gold nanoparticles conjugated to polyclonal antibodies where used in competitive and sandwich assays to detect horse serum albumin (HSA) and horse thermal-stable meat protein (H-TSMP), allowing the detection of horse contamination in both raw (0.01% w/w) and cooked (1.0% w/w) meat samples (beef, pork, sheep, goat, chicken, turkey) within 35 min (Jongkit Masiri *et al.*, 2017). Another platform was based on a sandwich immunoassay for the detection of chicken immunoglobulins through their interaction with polyclonal immunoglobulins G either as capture antibodies or conjugated to peroxidase, thus producing a color reaction for positive samples (Hendrickson, Zvereva, Vostrikova, *et al.*, 2021). The platform detected as low as 0.063% (w/w) raw chicken in mixtures with beef, pork, lamb, rabbit and turkey, within 20 min. Another sandwich-based immunoassay aimed the capture of mammalian thermostable troponin I skeletal isoform (TnI), and its detection via the interaction with antibodies conjugated with gold nanoparticles (Zvereva *et al.*, 2020). The platform could detect concentrations as low as 25 ng mL^{-1} of TnI, as well as 1% of beef in chicken mixtures, with a time response of only 15 min.

5 Perspectives

Works with biosensors in the meat adulteration area are scarce, especially electrochemical ones. The complexity of food samples is one of the great challenges to be overcome in these analytical devices, as the presence of many interferents can reduce the sensitivity of the biosensors. Many reported works solved this problem with extensive purification and amplification protocols. However, they are unfeasible in industrial processes, where a quick response and easy-to-handle device is required. It is also necessary to further study the stability of biosensors over time, to estimate their shelf-life in order to make their commercialization viable. That said, it's not surprising that there are no readily available commercial biosensors applied in the area of meat adulteration.

The offer and need for new biosensors to recognize adulteration in meat tends to increase in the future with greater consumption and concern about fraud. However, these devices, in addition to being sensitive, must be selective, stable, cheap and quick to respond. Uniting all these features in a single flawless platform for the market and industry is not yet a reality, but the development of new materials and detection strategies may bring this technology soon, especially due the extensive discovery of new nanomaterials. Combining fast and effective sample preparation with a sensitive analytical device is a task for different types of professionals, hence the importance of collaboration between different areas of biology, chemistry and engineering.

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Capítulo 2 - *A novel and reusable electrochemical genosensor for detection of beef adulteration*

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A novel and reusable electrochemical genosensor for detection of beef adulteration

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Resumo

Este trabalho descreve a construção de um genossensor baseado em um eletrodo de grafite modificado com um nanocompósito de óxido de grafeno reduzido/poli (ácido 3-hidroxibenzoico) com um oligonucleotídeo de DNA específico para detecção de DNA mitocondrial bovino, a fim de certificar a pureza da carne bovina. Análises eletroquímicas e morfológicas indicam que o genossensor permite a formação de duplex com o DNA na amostra pura de lisado de carne. O genossensor foi seletivo, identificando até 1% (w/w) de carne suína em amostras bovinas, apresentando boa reprodutibilidade e estabilidade em seis semanas de armazenamento, podendo ser reutilizado quatro vezes, sendo uma ótima ferramenta para avaliação da pureza da carne bovina, com aplicação na cadeia de produção e comercialização de carnes.

Palavras-chave: genossensor; adulteração de alimentos; óxido de grafeno reduzido; ácido 3-hidroxibenzóico

Abstract

This work describes the construction of a genosensor based on a graphite electrode modified with an reduced graphene oxide/poly(3-hidroxybenzoic acid) nanocomposite with an specific DNA oligonucleotide for detection of cattle mitochondrial DNA, in order to certify beef purity. Electrochemical and morphological analyses indicate that the genosensor allows duplex formation with the DNA of pure sample of beef lysate. The genosensor was selective, identifying up to 1% (w/w) of pork in beef samples, showing good reproducibility and stability within six weeks of storage, and can be reused four times, being a great tool for the

evaluation of beef purity, with application in the meat production and marketing chain.

Keywords: genosensor; food adulteration; reduced graphene oxide; 3-hydroxybenzoic acid

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A Novel and Reusable Electrochemical Genosensor for Detection of Beef Adulteration

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Abstract: This work describes the construction of a genosensor based on a graphite electrode modified with an reduced graphene oxide/poly(3-hidroxybenzoic acid) nanocomposite with an specific DNA oligonucleotide for detection of cattle mitochondrial DNA, in order to certify beef purity. Electrochemical and morphological analyses indicate that the genosensor allows duplex formation with the DNA of pure sample of beef lysate. The genosensor was selective, identifying up to 1 % (w/w) of pork in beef samples, showing good reproducibility and stability within six weeks of storage, and can be reused four times, being a great tool for the evaluation of beef purity, with application in the meat production and marketing chain.

Keywords: genosensor · food adulteration · reduced graphene oxide · 3-hydroxybenzoic acid

1 Introduction

Beef consumption has been growing worldwide, especially in emerging countries such as China, Brazil, North Africa and West Asia [1]. After the USA, Brazil is the second largest beef producer worldwide, and is the largest exporter. Brazilian beef is present in more than 140 countries, especially in East Asian markets, the European Union and countries with Muslim majorities [2]. Brazilian beef exports totaled more than 6.2 billion dollars in 2017, highlighting the importance of this commodity for the national economy.

To ensure an export quality product, beef must undergo rigorous composition and quality analysis: even though it can be free of harmful bacteria and unwanted chemicals, it is necessary to verify if the beef is pure. For example, in Muslim and Jewish cultures the consumption of pork is prohibited, according to kosher and halal rules, so beef must be free of traces of pork [3], with an 100 % purity. Several methods are used for the identification of meat species, which include physical [4], sensory [5], anatomical and histological analysis of meat [6], chromatography [7], spectroscopy [8] and enzyme-linked immunosorbent assay [9]. However, these methods are time consuming, require appropriate laboratory equipment and qualified personnel. Faster methods such as polymerase chain reaction [10] and loop-mediated isothermal amplification [11] are also used in certain cases, but require use of expensive reagents, laboratory environment and highly qualified personnel. Thus, the demand for fast, cheap and accurate methods for ascertaining the purity of beef, i.e. the absence of other types of meat, is very attractive for both beef production chain and the final costumers.

Biosensors enter this niche, with the proposal to develop devices that are fast, easy to handle and able to detect numerous analytes [12]. Biosensors are analytical devices that incorporate some biological element, capable of producing measurable signals that relate to the concentration of the analyte of interest by the combination of a biochemical recognition system with a transducer that converts this recognition event into a measurable signal [13].

Nanocomposites can improve the biosensor response, by increasing the surface area, enabling better biomolecule binding [14], and in case of electrochemical biosensors, can improve the electrode conductivity [15]. In this work, reduced graphene oxide was combined with a polymer to improve the properties of the transducer. Polymers has been widely used in bioanalytical applications due to their biocompatibility and electron transport properties, improving the selectivity, stability and sensitivity of these systems [16]. Poly(3-hydroxybenzoic acid) has carboxylic groups located on its extremities which can act as reactive groups for the immobilization of biomolecules, becoming an interesting matrix for biological biosensors, also being easily and cheaply synthesized [17,18].

In the meat industry, research focused on the development of food-applied biosensors has broad application but is still in its early stages [19]. For meat origin investigation, genosensors (biosensors whose recognition molecules are nucleic acids) are the most recommended, since each specie has gene markers that allow its differ-

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Capítulo 3 - *Label-free and reagentless electrochemical genosensor based on graphene acid for meat adulteration detection*

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Label-free and reagentless electrochemical genosensor based on graphene acid for meat adulteration detection

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Highlights

- A new screen-printed genosensor was developed for pork detection in beef.
- Graphene acid was used as an effective matrix for DNA immobilization.
- The impedimetric detection requires only buffer solution.
- The genosensor did not require DNA purification neither amplification.
- The sample preparation and assay time was only 45 min, with a 4-week stability.

Resumo

Com o aumento da demanda por carne bovina nos mercados emergentes, é essencial o desenvolvimento de dispositivos de controle de qualidade que sejam rápidos, baratos e fáceis de manusear. Especialmente em cenários que a carne bovina deve estar livre de resíduos de carne suína, devido a razões religiosas, culturais ou alérgicas, a disponibilidade de tais ferramentas de diagnóstico é crucial. Neste trabalho, um genossensor impedimétrico sem marcadores foi desenvolvido

para a detecção de resíduos de carne de porco em carne bovina, aproveitando as capacidades de biossensoriamento do ácido grafeno - um derivado do grafeno funcionalizado densa e seletivamente. Uma sonda de DNA de fita simples, específica para o genoma mitocondrial de porco, foi imobilizada em eletrodos de carbono modificados com ácido grafeno. Foi demonstrado que o ácido grafeno melhorou as propriedades de transporte de carga do eletrodo, seguindo um protocolo simples e rápido de modificação. Utilizando espectroscopia de impedância eletroquímica não-faradaica, que não requer nenhum indicador eletroquímico ou pares redox, a detecção de resíduos suínos na carne bovina foi alcançada em menos de 45 min (incluindo o tempo de preparação da amostra), com um limite de detecção de 9% w/w de carne suína em amostras de carne bovina. É importante ressaltar que a amostra não precisou ser purificada ou amplificada, e o biossensor manteve suas propriedades de desempenho inalteradas por pelo menos 4 semanas. Este conjunto de características coloca o biossensor desenvolvido entre os mais atrativos para desenvolvimento e comercialização visando detecção DNA de suínos. Além disso, ele abre o caminho para o desenvolvimento de dispositivos *point-of-need* sensíveis e seletivos para o monitoramento rápido, simples e confiável da pureza da carne.

Palavras-chave: adulteração de alimentos; biossensor de DNA; espectroscopia de impedância eletroquímica não-faradaica; carne bovina; carne suína.

Abstract

With the increased demand for beef in emerging markets, the development of quality-control diagnostics that are fast, cheap and easy to handle is essential. Especially where beef must be free from pork residues, due to religious, cultural or allergic reasons, the availability of such diagnostic tools is crucial. In this work, we report a label-free impedimetric genosensor for the sensitive detection of pork residues in meat, by leveraging the biosensing capabilities of graphene acid - a densely and selectively functionalized graphene derivative. A single stranded DNA probe, specific for the pork mitochondrial genome, was immobilized onto carbon screen-printed electrodes modified with graphene acid. It was demonstrated that graphene acid improved the charge transport properties of the electrode, following

a simple and rapid electrode modification and detection protocol. Using non-faradaic electrochemical impedance spectroscopy, which does not require any electrochemical indicators or redox pairs, the detection of pork residues in beef was achieved in less than 45 min (including sample preparation), with a limit of detection of 9% w/w pork content in beef samples. Importantly, the sample did not need to be purified or amplified, and the biosensor retained its performance properties unchanged for at least 4 weeks. This set of features places the present pork DNA sensor among the most attractive for further development and commercialization. Furthermore, it paves the way for the development of sensitive and selective point-of-need sensing devices for label-free, fast, simple and reliable monitoring of meat purity.

Keywords: food adulteration; DNA biosensor; non-faradaic electrochemical impedance spectroscopy; beef; pork

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Label-free and reagentless electrochemical genosensor based on graphene acid for meat adulteration detection

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ABSTRACT

With the increased demand for beef in emerging markets, the development of quality-control diagnostics that are fast, cheap and easy to handle is essential. Especially where beef must be free from pork residues, due to religious, cultural or allergic reasons, the availability of such diagnostic tools is crucial. In this work, we report a label-free impedimetric genosensor for the sensitive detection of pork residues in meat, by leveraging the biosensing capabilities of graphene acid - a densely and selectively functionalized graphene derivative. A single stranded DNA probe, specific for the pork mitochondrial genome, was immobilized onto carbon screen-printed electrodes modified with graphene acid. It was demonstrated that graphene acid improved the charge transport properties of the electrode, following a simple and rapid electrode modification and detection protocol. Using non-faradaic electrochemical impedance spectroscopy, which does not require any electrochemical indicators or redox pairs, the detection of pork residues in beef was achieved in less than 45 min (including sample preparation), with a limit of detection of 9% w/w pork content in beef samples. Importantly, the sample did not need to be purified or amplified, and the biosensor retained its performance properties unchanged for at least 4 weeks. This set of features places the present pork DNA sensor among the most attractive for further development and commercialization. Furthermore, it paves the way for the development of sensitive and selective point-of-need sensing devices for label-free, fast, simple and reliable monitoring of meat purity.

1. Introduction

The beef industry and the food demand are rapidly growing, mainly in developing countries, due to the rising population (Hansen, 2018). Food adulteration in the meat industry is a recurring topic: in pieces of solid meat adulteration is challenging, however in processed meats, such as hamburgers and meatballs, the mixture of different species is imperceptible (Rahmati et al., 2016). Final consumers, especially from groups with dietary restrictions (e. g. Halal and Kosher diet followers), must be sure of the origin of the meat they consume, especially regarding the total absence of pork, as well as there is a potential danger of serious allergic responses or intolerance to proteins derived from specific animals (Wilson and Platts-Mills, 2018).

Meat quality control analysis usually applies physical, sensory, anatomical and histological methods and, in rare cases, molecular techniques such as polymerase chain reaction (Okuma and Hellberg, 2015) and loop-mediated isothermal amplification (Kumar et al., 2017). However, these methods of beef purity analysis are time consuming,

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