# Electrochemical biosensors for *Salmonella*: State of the art and challenges in food safety assessment

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A B S T R A C T

According to the recent statistics, *Salmonella* is still an important public health issue in the whole world. Legislated reference methods, based on counting plate methods, are sensitive enough but are inadequate as an effective emergency response tool, and are far from a rapid device, simple to use out of lab. An overview of the commercially available rapid methods for *Salmonella* detection is provided along with a critical discussion of their limitations, benefits and potential use in a real context. The distinguished potentialities of electrochemical biosensors for the development of rapid devices are highlighted. The state-of-art and the newest technologic approaches in electrochemical biosensors for *Salmonella* detection are presented and a critical analysis of the literature is made in an attempt to identify the current challenges towards a complete solution for *Salmonella* detection in microbial food control based on electrochemical biosensors.

## 1. Introduction

Foodborne diseases are caused by ingestion of water or food contaminated by pathogenic microorganisms, like bacteria and virus, pesticides residues or other toxins(Xihong Zhao et al., 2014). Despite the legislation and control methods developed to preserve food nutritional quality and prevent contamination, a significant increase in foodborne diseases has been observed since 1980 and it continues to be an emerging public health theme in whole world(2009; Brandão et al., 2015; Thakur and Ragavan, 2013). According to World Health Organization (WHO) the consumption of food and water contaminated by pathogenic microorganisms causes 1.8 millions of deaths per year worldwide (Shen et al., 2014), and the various *Salmonella* serotypes are the more predominant cause of alimentary infection (Dong et al., 2013; Lee et al., 2015).

In Europe, as reported in the Rapid Alert System for Food and Feed (RASFF) in 2013, the priority vehicles of contaminations were animal products (meet, eggs, milk, and sea products), vegetables and water. *Salmonella* is one of the most common pathogens in meat (Chemburu et al., 2005; Farabullini et al., 2007; Lee et al., 2015). *Salmonella* is a Gram-negative bacterium, from *Enterobacteriaceae* family. *S. (Salmonella) enterica* and *S. bongori* are the species that can cause illness in humans producing numerous symptoms like diarrhea, vomiting, gastroenteritis, severe dehydrating (Bula-Rudas et al., 2015; Dong et al., 2013; Yang et al., 2009) and other sickness stages as typhoid fever. These two species were divided into 2500 known serotypes based on the Kaufmann-White typing scheme (Brenner et al., 2000; Bula-Rudas et al., 2015). The *S. enterica* serotype *typhi* is the bacteria responsible for most of the foodborne diseases and along with serotype *paratyphi,* it can be found only in humans. The *S. paratyphi* causes typhoid salmonellosis, which according to the Food and Drug Administration (FDA), if not treated can result in a mortality rate of 10%. In this case, the infection dose is 1000 Colony Forming Unit (CFU), which is much higher than the infection dose required to occur the symptoms associated with a non-typhoid salmonellosis - which are as low as 1 CFU - although the dangerousness of the side-effects is higher for typhoid salmonellosis (Administration, 2012; Dong et al., 2013; Dungchai et al., 2008).

Due to the extremely low infection limits, 1 CFU, the associated side effects and the high *Salmonella* susceptibility for dissemination in perishable and semi-perishable products, the limits imposed by law have been tightened over the years. In the European Commission (EC) regulation No 2073/2005 on microbiological criteria for foodstuffs, the *Salmonella spp*. are considered a group of pathogens which its presence by itself in ready-to-eat food (portion of 25 g), is enough to be considered a risk factor for human health. Consequently, if this pathogen is detected the food product is classified as unsatisfactory. The absence of *Salmonella* spp. is a figurative quantification, since

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"zero" in analytical measures is unreal, because each method has a limit of detection and there are always errors associated. Even the conventional culture methods recommended by International Organization for Standardization (ISO) (ISO standard 6579:2002) due to their exceptional sensitivity (Melo et al., 2016) are only capable to detect 1 CFU/ 25 g of foodstuffs.

These regulations are compatible with the Hazard Analysis and Critical Control Point (HACCP) approach, which are used in most of the countries, including the European Union (EU) and United States of America (USA), to establish adequate controls for the identification of *Salmonella* in ready-to-eat foods to assure that it is absent when it is taken by the consumers(Lawley, 2012). Additionally, some countries have specific rules for products like eggs and fresh daily products. For instance, the FDA has a specific rule to prevent S. *enteritis* in eggs, because it is one of the largest contamination vehicles for infection dissemination in the country. This rule is a set of measures which are implemented in the production (for example, the pasteurization implementation), storage and transportation of shell eggs(Lawley, 2012). The effect of more control and the sanctions for non-compliant producers has recently shown positive effects in the statistics of salmonellosis outbreaks in EU. Indeed, between 2004 and 2009 the human cases reduced almost for one-half (EFSA 2014). Counterbalancing these encouraging statistics from the European Food Safety Authority (EFSA), in the USA it was estimated from 2 to 4 million cases of salmonellosis annually, being already considered one of the major causes of hospitalization and dead (Elaine et al., 2011; Oliver et al., 2005; Xihong Zhao et al., 2014).

Because of these alarming statistics, it is still necessary to develop new simple methods and technologies for *Salmonella spp*. detection with the ability to provide valid results at the time of consumption of perishable foods, thus avoiding mass contaminations. Nowadays there are several methods purposely designed to accelerate the pathogen detection but most of them have difficulties to get validated and enter to the market, because they have a high probability of false negative results, sometimes are restricted to a specific type of food or considered expensive by the food industries (Valderrama et al., 2016). In the future, the best approaches for rapid *Salmonella* detection in food control will be designed for application outwards the laboratory and may involve disruptive innovations to minimize the pre-enrichment and sample preparation steps.

The purpose of this review is to give an overview of current methods for *Salmonella* detection in microbial food control and to present the authors view about the most promising route to develop new rapid methods. A critical survey of rapid commercial methods is presented aiming to identify current needs for further development in rapid practical food control. Among several existing methods, which have already been recently reviewed (Lee et al., 2015; Rahman et al., 2016; Su et al., 2011; Valderrama et al., 2016), the biosensors were chonse as an emerging tool for *Salmonella spp*. control due to the increasing interest in the scientific community, as shown by the increasing number of publications using this technology, and their characteristics, namely the operational simplicity, sensitivity, readiness and real-time analysis potential. Among all existing biosensors for *Salmonella spp*., the electrochemical biosensors are reviewed because they show distinguished advantages like the low cost of the equipment, miniaturization capacity and inherent sustainability, due to the use of a few solvents and low sample volumes, both in its development and application. Considering that the acceptance by the industry for novel rapid methods depends not only on speed but also on the initial investment, cost, technical support, and ease of use, electrochemical biosensors are specially well suited to fulfill these requirements[1].

[1] This review is not intended to endorse or recommend any commercial product, and any omission of a commercial product is not intentional.

## 2. Commercial rapid methods for *Salmonella spp*. detection in food products

Conventional methods for bacteria detection rely on standard culture methods that involve the use of different enrichment and selective broths for the isolation of each bacteria, in which large amounts of sample are used in a complex sequencing of steps (Lee et al., 2015). Beyond their sensitivity and high accuracy, the conventional methods require at least 1 week for trusted results (2–3 days for results and 7–10 days for confirmation)(Farabullini et al., 2007; Yang et al., 2009). Besides these time consuming methods recommended by ISO, it is already possible to obtain similar results in 24–48 h using nucleic acid-based assays or even in less than 24 h with some immunologically-based methods like Enzyme Linked Immunosorbent Assay (ELISA), which together with the biosensors belongs to the rapid methods for pathogen detection in food samples (Valderrama et al., 2016).

In the last years, various devices for rapid detection of *Salmonella spp*. were developed, tested and commercialized (Brandão et al., 2015; Law et al., 2015; Lee et al., 2015; Melo et al., 2016; Valderrama et al., 2016). According to current regulation for food control parameters, commercial methods should accomplish several requirements: the devices have to be able to detect a single *Salmonella* CFU in 25 g of food; they must have a sensitivity and specificity of at least 99%; and operational personnel ideally must need no special skills to perform the analysis (Eijkelkamp et al., 2009). Besides these general requirements, the analysis time of rapid methods preferably must be in the range of hours to a limit of 24 h (Valderrama et al., 2016).

Commercial rapid detection methods should be validated by the competent authorities for example the HACCP, the FDA and the Association of Official Analytical Chemists (AOAC) in the United States of America, and the European Certification Organization (ECO) for the validation and approval of alternative methods for the microbiological analysis of food and beverages (MicroVal) in the EU. The validity of a method depends upon its sensitivity and specificity. Sensitivity is the probability of the test to detect a true positive, while specificity is the probability of the test to detect a true negative. A schematic overview of current rapid methods for salmonella detection in food products is provided in Fig. 1. They can be divided into several categories including miniaturized culture assays - modified or adapted from conventional procedures, but using new selective culture media - immunologically-based assays, nucleic acid-based assays and biosensors. It is difficult to make an accurate comparative analysis about the performance of commercial rapid methods because it depends on several experimental factors, such as sampling, sample matrix, enrichment processes and it lacks normalization of the evaluation schemes (Lee et al., 2015). Comparative studies for the test kits should be set up under identical test conditions to better compare and evaluate the test results from different laboratories. Information about the performance (sensitivity, analysis time, advantages and limitations) of validated commercial methods were obtained from the producer's brochures and websites, or scientific papers (Barthelmebs et al., 2010; Cheung et al., 2007; Eijkelkamp et al., 2009; Oxoid Limited; RomerLabs 2013b; SM, 2004/, 2005a) and it is organized in Tables 1–4, according to their methodology.

### 2.1. Immunologically based methods

The immunologically based methods for *Salmonella* spp. detection explore the specificity of the antibodies (monoclonal or polyclonal) for specific antigens, normally located at *Salmonella* cellular membrane surface. There are several formats for these assays but the commercially available methods are mainly based on agglutination, immunoprecipitation, immunodiffusion and enzyme immunoassay (EIA) / ELISA, which includes several lateral flow devices.

The agglutination and immunoprecipitation methods use particles

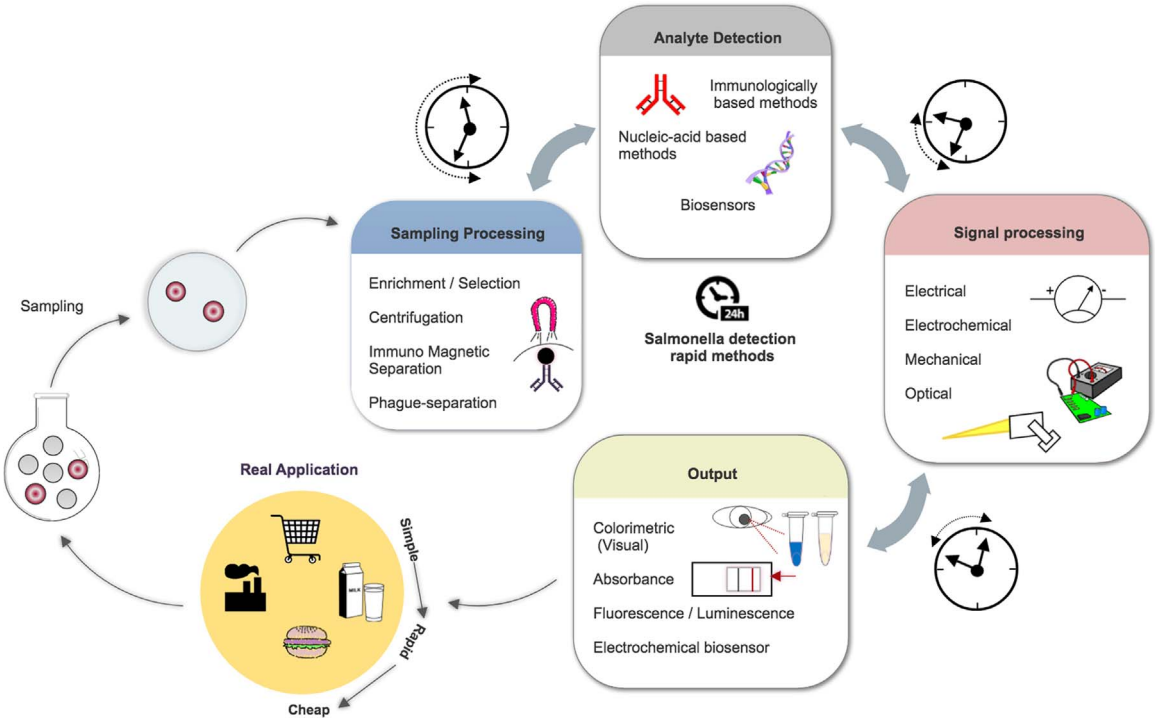


**Fig. 1.** Schematic overview of current rapid methods for Salmonella spp detection in food products.

coated with antibodies that react with antigens and form clusters visible to the naked eye. This approach gives simplicity and rapidity to the analysis but the methods show a high limit of detection (LOD) that is incompatible with the regulated limits. It is worth to note that all the analyzed commercial methods (Table 1) need an enrichment step prior to the test. For this reason, these tests are frequently used as a confirmatory analysis technique or for serotyping previous positive findings. Among the latex agglutination tests, the Spectate test from May & Baker diagnostics Ltd. and the color *Salmonella* from Wellcolex allow simultaneously the *Salmonella* spp. detection and serotyping. From the user point of view, it is very simple to make and to interpret the analysis, because the positive presumptive result is visually identified by the clusters formed in the presence of the antigen or by a color change attributed according to the serotype, making them attractive devices to the food industry despite the impossibility of quantification.

The EIA/ELISA are the most promising methods for rapid detection, because they combine the specificity of the antibodies with the sensitivity of the enzymatic assays by coupling easily assayed enzymes to antibodies or antigens. Usually, they are more selective and sensitive than agglutination or immunoprecipitation assays but need a longer time to obtain quantitative results, although still shorter than most of the nucleic acid-based methods.

There are three ELISA formats schematized in Fig. 2: the direct, indirect and sandwich or capture assay. In the direct approach, it is used an enzyme labeled antibody that recognizes the antigen which is previously bound to a solid matrix. In the indirect formats two antibodies are used, a primary non-labeled antibody that recognizes the immobilized antigen and a secondary enzyme labeled antibody that binds to the primary antibody. In the sandwich formats, a capture antibody immobilized on a solid matrix binds to the antigen and then an enzyme labeled antibody also binds to the captured antigen. The sandwich ELISA formats benefit in terms of selectivity due the use of a second labeled antibody after the capture of the antigen and attain low detection limits due to enzymatic amplification. For these reasons, it is the most used formats in immunologically-based methods. Apart of the formats, the ELISA assays can be competitive or non-competitive, if the measured signal is inversely or directly proportional to the amount of antigen present in the sample.

Almost all ELISA commercial rapid methods (TRANSIA® Plate *Salmonella* Gold, the 3M™ Tecra™ *Salmonella* VIA, Ridascreen® *Salmonella* R-Biopharm, assurance GOLD and Assurance *Salmonella* from Biocontrol, *Salmonella* Tek from Organon TeKnika, BacTrace from KPL and BioLine from HardyDiagnostics) uses a sandwich format, where a peroxidase enzyme like Horseradish Peroxidase (HRP) was used as enzymatic label. This design is very attractive both from the analytical and practical application point of view, due to the capacity of these enzymes to catalyze chromogenic substrates like tetramethylbenzidine (TMB) in the presence of a substrate solution ($H_2O_2$ or urea and $H_2O_2$). After adding a stop solution, a color change is observed and the results can be read visually or more precisely with an automatic micro plate reader.

The ELISA methods are in a rather advanced development stage and has already application in the food industry (Lee et al., 2015; Valderrama et al., 2016). The major challenge for these methods is to achieve good sensitivity and specificity values in complex matrixes containing inhibitory substances, like fats or proteins present in food, and background microflora, because these substances inhibit the immunological response and mask the presence of target bacteria, increasing the probability of false negatives (Valderrama et al., 2016). The detection limit of the ELISA methods under optimized conditions is typically in the range $10^4$ to $10^5$ CFU $mL^{-1}$ (Lee et al., 2015; López-Campos et al., 2012). Considering the regulated limit all ELISA methods for food control may involve selection/enrichment steps to attain the required limits of detection and to minimize sample matrix interference. In fact, a brief analysis of data in Table 1 shows that enrichment/selection step is necessary for most of the commercial ELISA based methods, thus increasing the total analysis time, typically between 18 and 48 h for presumptive results, despite of the short analysis time for the ELISA process, which usually is complete in less than 2 h. Furthermore, most of the commercial devices can only be used in specific food types, like for example the Assurance test for *Salmonella* from Biocontrol, or need extra time for sample pre-enrichment/selection proportional to the matrix complexity. Indeed, the Assurance Gold

**Table 1**
Immunologically-based commercial methods for Salmonella detection.

| Method | Assay/Manufacturer | Analysis time | Sensitivity | Advantages | Disadvantages |
|---|---|---|---|---|---|
| **Latex Agglutination** | Spectate (May & Baker Diagnostics Ltd.) | 3-5 min. for test only (after enrichment) | NR | - Specific and simple;<br>- Used as a confirmatory analysis technique; | - Allows the detection and the serotyping/ grouping |
| | Wellcolex color *Salmonella* (Wellcolex) | 3 min. for test only (after enrichment) | NR | - High positive and negative predictive values (PPV > 98.7%);<br>- Easy interpretation. | - Only for screening proposes (presence/ absence);<br>- Need storage at 2 - 8° C;<br>- Allows the detection and the serotyping/ grouping. |
| | Salmonella Latex test (Oxoid) | 3-5 min. for only test (after enrichment) Total time 24 h | NR | - Easy interpretation;<br>-Ssensitivity of 100% and a specificity of 98.7% (Oxoid Limited). | - Effective only in some *Salmonella* serotypes;<br>- Not validated for non-motile specie;<br>- Store all reagents at 2 -8° C. |
| | Bactigen (Wampole Laboratories) | 3-5 min for test only (after enrichment) | NR | | |
| | Slidex (biomerieux) | NR | NR | - Reliable results;<br>- Easy interpretation. | - Only applicable to pure culture or animal Specimens. |
| **Immunomagnetic Precipitation** | VIP for *Salmonella* (BioControl) | Total 24 h | NR | - Room Temperature storage;<br>- Suitable for testing all food products<br>- lateral flow assay | - Only positive or negative result;<br>- Need confirmative tests for quantification;<br>- 81.9% and 98.8% (relative sensitivity to reference method OMA, depending the contamination level of poultry). (Eijkelkamp et al. 2009). |
| | *Salmonella* enteritidis | Total analysis time 22h | NR | - Can be integated in analytical detection procedure;<br>- latex agglutination for positive samples. | - Relatively expensive cost;<br>- Need confirmative tests. |
| **ELISA** | TRANSIA® Plate *Salmonella* Gold (Raiso Diagnostics Ltd.) | Enrichment/Selection 36 to 46 h.<br>ELISA assay – 1.5 h | 1 CFU/25 g (Eijkelkamp et al. 2009) | - Easy interpretation: based on a simple color change;<br>- Results in 24h with TAG 24 supplement. | - High LOD;<br>- Long analysis time.<br>- The Transia Card is less selective in food samples; |
| | TRANSIA® Card (Raiso Diagnostics) | Enrichment/Selection<br>18 to 24h. ELISA assay –10 min. | Transia Card:<br>$10^5$-$10^6$ cells/mL | - Simplicity;<br>- Shorter enrichment and detection time. | - High LOD. |
| | 3M™ Tecra™ *Salmonella* VIA (Tecra) | Enrichment/Selection 18 to 24h.<br>ELISA assay –less than 2 h. | 1-5 CFU/25 g | - Good sensitivity;<br>- Simultaneous detection of various pathogens in a single analysis. | - Long analysis time. |
| | 3M™ Tecra™ *Salmonella* Unique Plus™ (Tecra) | Results in < 22h | 1-5 CFU/25 g | - Convenient in medium and small scale samples;<br>- Simultaneous analysis of different<br>- Simple results interpretation;<br>- All food application;<br>- Satisfactory sensitivity;<br>- Automation | - Need of pre-enrichment;<br>- Validated for: Salmonella spp. in food and environmental samples;<br>- Relatively expensive cost. |
| | Ridascreen® *Salmonell*a (R-Biopharm) | Presumptive results in less than 23h | 1 cell/25 g<br>≈ $10^4$ cells/mL after enrichment | - Approved for AFNOR EN/ISO16140, FDA and for ISO EN/ISO 16140;<br>-Simplicity in results analysis, based in a simple color changes;<br>- Good sensitivity;<br>- Approved for food, feed and | - Long analysis time.<br>- Laborious;<br>- Only screening result (presumptive presence /absence). |

(continued on next page)

**Table 1** *(continued)*

| Method | Assay/Manufacturer | Analysis time | Sensitivity | Advantages | Disadvantages |
|---|---|---|---|---|---|
| | | | | environmental samples | |
| | Assurance EIA GOLD *Salmonella* (BioControl) | Total time Analysis:<br>- Processed food (24h)<br>- Raw foods (28h); ELISA - 2 hours | | - High throughput efficiency;<br>- AOAC Official Method 999.08;<br>- All food approval | - Need storage 2 - 8° C |
| | Assurance Salmonella) (BioControl | Total analysis time 48h | | - Affordable cost; | - Need storage 2 - 8° C<br>- Only applicable to some foods<br>- Several equipment requirements: microplate washer, microplate reader; |
| | *Salmonella* Tek (Organon Teknika) | Total analysis time 48 h | 1-5 CFU/25 g<br>≈10000 -50000 cells/mL | - Simplicity;<br>- Easy interpretation. | - Only presumptive results; |
| | BacTrace (KPL, Inc.) | Total 42–52 h | | - Easy interpretation | . - Long analysis time |
| | BioLine (Hardy Diagnostics) | Total 42–52 h | 1 CFU/25 g | - Approved for HACCP and AOAC<br>- Already tested by some authors in alfalfa sprouts and chicken meat | - Long analysis time |
| | Assurance Enzyme Immunoassay (EIA) | | | | |
| | MicroELISA (Dynatech Lab.) | Total 48h | NR | - Semi-automation | - Need to buy equipment relatively expensive; |
| **Enzyme-linked Fluorescence assay** | EIA Foss (Foss Electric) | Total time Analysis<br>< 24 h | NR | - Automated process;<br>- Applicable to all foods and feedsproducts;<br>- Immunomagnetic separation of the target<br>- Give next day results | - Relatively expensive cost |
| | VIDAS SLM + ICS (bioMerieux Vitek) | Total time Analysis:<br>24 h (IMS) - 48 h | 1 CFU/ 25 g (producer)<br>5–50 CFU/25 g (Eijkelkamp et al. 2009; Uyttendaele et al. 2003) | - Allows to perform 30 tests simultaneously;<br>- AOAC official method no. 2001.09;<br>- All foods application; | - Long time to results;<br>- Sensitivity 93% and specificity 96% (relative reference method DIASALM) (Torlak et al. 2012) |
| Lateral Flow | RapidChek® Select™ (Romer Labs®) | Total: 22-30h | 1 CFU/25 g (Torlak et al. 2012) | - Simple use;<br>- Validated by AFNOR, AOAC and FDA;<br>- High selectivity and selectivity | - Not applicable to all foodstuffs;<br>- Only for Salmonella Enteritidis specie;<br>- Available two different modules: for presumptive detection and identification(extra 24h) |
| | Reveal® 2.0 for *Salmonella* (Neogen) | Total time Analysis:<24h | 1-10 CFU \25 g | - Provide next day results;<br>- Simplicity;<br>- IMS sample isolation;<br>- Good sensibility for emergency applications. | - Not validated for all foods;<br>- Sensitivity of 52–71% and specificity of 58–78% relative to reference method (SM 2004/2005). |

NR- Not reported.

**Table 2**
Nucleic acid-based commercial methods for Salmonella detection.

| Method | Assay/Manufacturer | Analysis time | Sensitivity | Advantages | Disadvantages |
|---|---|---|---|---|---|
| **PCR** | 7500 Fast system (Applied Biosystems) | Less than 30min | 1 cell/20 µL<br>99,7% sensitivity | - Very Good sensitivity;<br>- Fast in PCR Analysis | - Need of sample preparation;<br>- Long period to achieve a quantitative result. |
| | BAXR System Real-Time | Enrichment/Selection: | $10^4$ CFU/mL | - High specificity | - Less sensitivity relatively with others PCR techniques; |
| | PCR Assay for *Salmonella* (Dupont) | 10 to 24h; PCR 70 min<br>10 to 24h; PCR 70 min | after enrichment | - Automated procedure;<br>- Sensitivity 98%; | - Long analysis time;<br>- High limit of detection |
| | TAQMAN (PE Applied Biosystems) | Enrichment/Selection: 16 h. PCR - 4 hours | 1-10 copies of the target DNA per reaction;1 CFU/ 25 g of food. | -100% inclusivity for 51 strains of *Salmonella* enterica – and 100%. exclusivity for 24 other non-Salmonella strains;<br>- Certified by AOAC and AFNOR;<br>- Simple, reliable, and rapid procedure. | - Long analysis time;<br>- Can be only used for screening proposes. |
| **DNA Hybridization** | Gene – Trak® (Neogen) | Total assay: 27 – 48 h | 1-5 CFU/25 g<br>≈$10^4$ cells/mL | -Simultaneous detection of various food pathogens in a single analysis;<br>- Good sensitivity 99,2% and specificity 98.5–99.75% (Eijkelkamp et al. 2009) | - Long analysis time;<br>- Expensive |

**Table 3**
Commercial miniaturized biochemical tests for Salmonella detection.

| Method | Assay/Manufacturer | Analysis time | Sensitivity | Advantages | Disadvantages |
|---|---|---|---|---|---|
| **Miniaturized Biochemical tests** | API 20E (BioMérieux sa.) | Enrichment/Selection; 18 to 24h PCR – 70 min. | 100% True Positive | - Good sensitivity;<br>- High confidence test: 99,9% correlation with conventional tests results. | - Expensive, relatively with other biochemical test;<br>- Can be necessary more 24h of incubation for confirmation;<br>- Matrixes: only pure cultures. |
| | 1-2 Test kit (BioControl) Motility/Immunodiffusion | 36-58h (14 hours after enrichment); | NR | - Single-use test;<br>- Easy of use;<br>- High specificity;<br>- Low cross-reaction.<br>- Suitable for testing all food products<br>- No equipment is required to read results | - Relatively expensive cost.<br>- Need storage at 2 - 8° C |
| | *Salmonella* Rapid Test (Oxoid) Motility/Selective and indicator media | 42 h | NR | - Sensitivity: 96.8%;<br>- Approved by FDA and HACCP authorities; | - Application in all food materials and finished food products;<br>- Only presumptive detection.<br>- Relatively expensive cost. |
| | S.P.R.I.N.T. *Salmonella* (Oxoid) Enrichment Selective and Identification | Enrichment/Selection: < 24 h | NR | - Approved by FDA and HACCP authorities; | - Only for screening; |
| | *Salmonella* Rapid Test (Unipath) Motility, Enrichment/biochemical detection | 42 h | NR | - Detects motile and non-motile *Salmonella*;<br>- Saves on unnecessary confirmations<br>- Low false positive rate (<0.3% for all matrices) | - Relatively expensive cost |

NR- Not reported.

**Table 4**
Commercial biosensors for Salmonella detection.

| Method | Assay/Manufacturer | Analysis time | Sensitivity | Advantages | Disadvantages |
|---|---|---|---|---|---|
| **Biosensors** | RBD 3000 Micro PRO™ (AATI)<br>Flow cytometry method | Enrichment/Selection: 18 to 24h<br>Measurement 3-5 min | $10^1$-$10^6$ CFU/mL | - Simultaneous detection of various food pathogens in a single analysis;<br>- Reduce the human errors;<br>- Good sensitivity<br>- Simplicity;<br>- Allows to choose for a qualitative or quantitative analysis;<br>- Similar results compared to counting plate methods; | - Long total analysis time |
| | RAPID-B ™ Vivione (Biosciences Company)<br>Flow cytometry method | | 1 CFU/25 g | - Able to provide living bacteria counts within 15 min;<br>- Can be coupled with a bacterial destroy system; | - Quantitative data |

version for *Salmonella* from the same company distinguishes the total time necessary to perform the analysis in processed or raw food, and the last type of food needs extra 4 h, perhaps due the presence of bacteria from their normal microflora. In a rapid approach, these ELISA give only a presumptive result and need confirmatory tests. Both presented fluorescence ELISA assays (EIA from Foss Electric and VIDAS SLM plus ICS from bioMérieux Vitek), involve immuno-separation and probably for this reason are classified as "all food application" without significant change in total time of the test.

Most of the lateral flow assays is an adaptation of ELISA method involving more simple procedures, although present higher rate of false positives (FP) due to matrix effects comparatively to other methods. Therefore, testing for each foodstuff is necessary before utilization. The lateral flow device Singlepath® *Salmonella* from Merck Millipore (Table 1) shows a considerable FP rate of 7.3%. On the other hand, the Reveal system from Neogen fulfills the time and LOD requirements for rapid tests but lacks full validation and application to all food samples because presents low values of sensibility and specificity when compared with reference methods (SM, 2004/, 2005b). The RapidChek® Select™ from Romer Labs® bypassed this lack introducing a patented phage-based enrichment step that increases the sensitivity and selectivity of the method to 100%, receiving the validation from FDA and AOAC(RomerLabs 2013).

### 2.2. Nucleic acid-based assays

Nucleic acid-based assays detect a specific nucleic acid sequence within the target organism. Several PCR methods have already been validated and standardized by ISO to be used in the industry and in a screening context (Valderrama et al., 2016). There are many nucleic acid-based assay formats for foodborne pathogens but direct hybridization (DNA probe) and nucleic acid amplification techniques as polymerase chain reaction (PCR) are the most popular and have already been developed commercially. The performance characteristics of some representative nucleic acid-based commercial methods for *Salmonella* control in food is summarized in Table 2.

Direct hybridization assay use a labeled DNA probe with an oligonucleotide sequence highly complementary to the target sequence of a DNA or RNA molecule present in *Salmonella*, with the intention of using the hybridization phenomenon between them as a mediator to DNA quantification, by correlation of labels or labeled substrates or, sub-products correlation (Lee et al., 2015; Mozola, 2006). The analytical detection technique used depends on the DNA labels characteristics (enzymatic, radioisotope, fluorescence, etc.), but the colorimetric assays are the most common. The simple concept of the probe methods can hide the complexity of the steps necessary to perform before the DNA probe test: lysis of *Salmonella* cells, DNA probes purification, DNA labelling and several washing steps made to reject unbound DNA probes (Lee et al., 2015). A key factor for the success of the hybridization-based tests is the amount of DNA present in the final culture used for detection, which can extend the time of pre-enrichment of the samples depending on their complexity. In fact, the GeneTrack ® from Neogen presents a higher analysis time comparing to the PCR methods presented in Table 2, where the DNA amplification is performed, although it can be an interesting device to make an intensive and detailed analysis of several serotypes or pathogens in the same sample, due its multiplexing detection potential.

The conventional PCR methods are based on the isolation, amplification and quantification of a small portion of DNA genetic material of the bacteria under study. Therefore, have a unique pathogen detection potential (Lazcka et al., 2007; Pestana et al., 2010). The real-time PCR (rt-PCR) has an added value because it amplifies, detects and quantifies the target labeled-DNA sequence in the sample after each PCR cycle in “real-time” exploring the correlation of the label signal intensity with the number or DNA copies (amplicons)(Pestana et al., 2010).

Several commercial kits based on real-time PCR technique are already available in the market for the detection and characterization of foodborne pathogens (Table 2). They are faster than colony counting methods, with LOD typically of $10^4$ CFU $mL^{-1}$ and involve analysis time comparable to the ELISA kits (Cheung and Kam, 2012; López-Campos

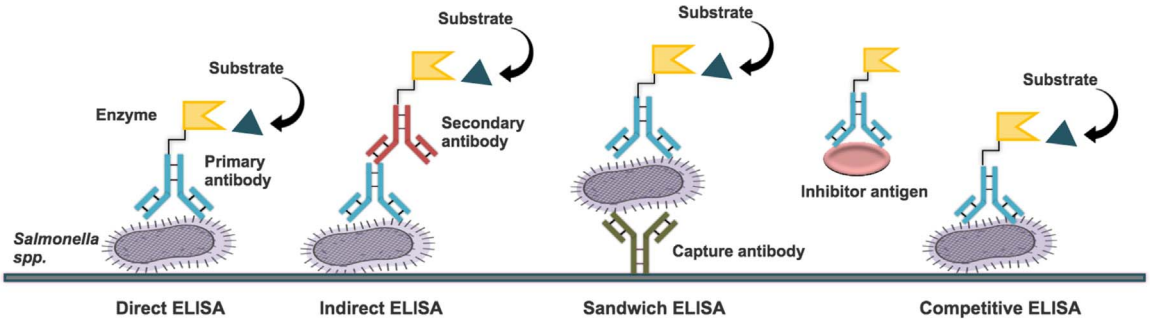


**Fig. 2.** Schematic representation of the ELISA formats.

et al., 2012). On the other hand, as they are based on DNA detection it is impossible to distinguishing between viable and non-viable cells as in the immunologically-based methods. In a brief analysis it can be clearly observed that rt-PCR kits have been developed in an attempt to eliminate the need or to decrease the time necessary for the preprocessing steps (Wilson and Gifford, 2005), but have not yet achieved the level of sensitivity required for a quantitative method with validation for foodstuffs. Therefore, most rt-PCR kits are used as qualitative methods (López-Campos et al., 2012). Among the rapid nucleic acid-based methods (Table 2), only the TAQMAN and the BAX® System Real time PCR assay for *Salmonella* are capable to give results in less than 24 h. Although, only the TAQMAN achieved to a LOD of 1 CFU per 25 g with the disadvantage that it can only be used for screening purposes and for the detection of only one of *Salmonella* species (*S. enterica*). Other limitations of commercial devices with PCR technology is that normally involve the acquisition of expensive equipments in addition to the detection kits. As output, experimental results are not of simple visual interpretation (fluorescence and absorbance signals) as immunological and need trained specialists to conduct the analysis and treat the data. These requirements limit the possibilities to use nucleic acid-based methods to achieve the lab-on-chip reality or a cheap method to implement in mass scale by the food industries or services.

### 2.3. Miniaturized culture assays

The miniaturized culture assays come from conventional counting plate methods showing 90–99% accuracy in comparison with those, but with higher sample throughput. This is achieved by the reduction of the plaques to reduced vessels, that need less amounts of reagents and sample volumes, resulting in an economical saving, especially if automatic methods are used (Lee et al., 2015; Lindström and Andersson-Svahn, 2011). These miniaturized tests for *Salmonella* consist mainly in devices containing 15–30 media or substrates selected specifically to identify a target serotype or a *Salmonell*a species. The selection of the media is based on the identification of compositional or metabolic intrinsic properties of the target *Salmonella* serotype or of the entire specie. The detection is made using chromogenic reagents in the substrate in which the color change can be directly correlated with the sample colony density, by a simple visual chromogenic evaluation, after an incubation period that normally is in the range of 18–24 h (Feng, 2001). In immunodiffusion techniques, like the 1–2 Test kit from Biocontrol, a positive result is identified visually by the appearance of a track line indicative of immunocomplex conjugation.

Various miniaturized kits for rapid biochemical characterization of *Salmonella* are commercially available (Table 3), including the *Salmonella* Rapid test (Oxoid), S.P.R.I.N.T. *Salmonella* (Oxoid) and *Salmonella* Rapid Test (Unipath), that are all validated by both FDA and HACCP authorities. These tests have a maintained interest in routine lab because they can be used in all food materials, have similar sensitivity to reference methods, can distinguish motile and non-motile *Salmonella* and can be performed in a large-scale sampling cases, still they show a relatively high cost and slow analysis. API 20E (bioMerieux sa.), apply a rt-PCR technique to perform the final detection, achieving an excellent sensitivity (100% true positives) with improved time of analysis, although limiting the test applicability only for pure cultures and the presumed positive results need confirmation tests. The other commercial kits have superior total time analysis, even less than conventional methods, but generally can be applied to all food materials and avoid unnecessary confirmative tests.

### 2.4. Biosensors

A biosensor is an analytical device able to perform chemical or biological analysis theoretically with no considerable sample preprocessing. A biosensor comprises a bio-receptor integrated with a signal transducer. The different types of biosensors can be classified according with the mechanism that confers biological specificity, the type of signal transduction or combining both criteria (Thévenot et al., 2001). In the first case, biosensors can be classified by their bioreceptor (which recognizes the target analyte) as bio-catalytic or bioaffinity biosensors. The catalytic biosensors are based on macromolecules that catalyze reactions, in which the biological components can be enzymes (the most widely used), whole cells, particles (microorganisms, bacteria) or portions of animal or vegetal tissues (Thévenot et al., 2001). The bio-affinity biosensors explore the binding events through specific proteins, like membrane receptors, antibodies or their fragments, nucleic acids or related substances with bio-molecular recognition capacity. Biosensors that use antibodies as bio-receptor, are denominated immunosensors (Piro et al., 2016). They use the same concept of the immunoassays and the high specificity of the antibody-antigen complex, with the advantage that normally biosensors are portable, need a reduced sample volume per analysis and have an elevated potential for automation (Afonso, 2012). Transducers have the capability to translate the biological reaction detected by de bioreceptor, in a measurable signal, proportional to the target analyte concentration (Alonso-Lomillo et al., 2010; Sharma and Mutharasan, 2013; Su et al., 2011). Relatively to the transducing methods, most of the biosensors can be classified as electrochemical, optical or piezoelectric/mass sensors (Leonard et al., 2003). Electrochemical transducing seems to be the most promising in terms of autonomy, applicability and output read by the ordinary user, both in screening as quantitative goals.

From all commercial devices analyzed, about 55% allow visual identification of a positive result to *Salmonella,* showing the significance of this design in the food control specific market. Although, the most rapid ones only can be used as screening of contaminated samples and need extra confirmative tests. The remaining are mainly represented by nucleic acid-based methods and enzyme linked fluorescence assays, that contrarious to the methods presented before comprise the quantification of the target at low levels of contamination, but a more laborious interpretation of results and more equipment resources are necessary. In this point of view, the biosensors are the ones capable to comprise high sensibility, real-time analysis and lab-on-a-chip concept, in a friendly physical support capable to give an intuitive user interface and easy interpretation of the results.

Many commercial devices for rapid foodborne pathogen detection were developed recently (Bahadır and Sezgintürk, 2015; Barthelmebs et al., 2010; Law et al., 2015; Lee et al., 2015; Melo et al., 2016; Pashazadeh et al., 2017; Valderrama et al., 2016) but, to the best of our knowledge, the examples of commercial biosensors currently available in the market are limited. The RBD 3000 Micro PRO™ and the Rapid B (Valderrama et al., 2016) biosensors requires flow cytometry equipment and trained personal to perform the analysis and treat the output data. Therefore, the commercially available biosensors for *Salmonella* detection are still far from a simple to use out of lab device. In this context, it is worth to analyze the literature in the field of electrochemical biosensors for *Salmonella* detection to identify the current challenges towards a complete solution for rapid detection of this pathogen in microbial food control.

## 3. Electrochemical biosensors in *Salmonella* control in food products

Electrochemical transducers stand out because the electroanalytical techniques incorporate essential proper characteristics for biosensors analytical applications such as high versatility, sensitivity, instrumental simplicity and miniaturization potential. The analysis of the literature (Table 5) shows that the best electrochemical biosensors developed for *Salmonella* detection incorporated nanomaterials in the biosensor architecture. These materials are employed in attempt to improve detection limits. As the LOD are just very good, the trend is maintain-

**Table 5**
Electrochemical biosensors for Salmonella detection.

| Serotype | Bioreceptor /Design | Transducer | Nanomaterial /Label | Detected analyte | Detection Technique | Working Range | LOD | Analysis Time | Sample | Refs. |
|---|---|---|---|---|---|---|---|---|---|---|
| *S. entérica* (no serotype) | Immunosensor (Sandwich) | SPE | HRP | $H_2O_2$ TMB | A | $5 \times 10^6$ to $5 \times 10^8$ CFU/mL | $2x10^6$ CFU/Ml in Buffer | 3h | PBS | Delibato et al. (2006) |
| *S. pullorum & S. gallinarum* | Immunosensor (Sandwich) | MSPE | rGO HRP | $H_2O_2$ | CV | $10^1$ - $10^9$ CFU/mL | $1.61 \times 10^1$ CFU/mL | | Buffer | Wang et al. (2014) |
| *S. typhi* | Immunosensor (Sandwich) | GCE | copper-enhanced Au@NP's | $Cu^{2+}$ | ASV | 130 – 2600 CFU/mL in PBS 260-2600 CFU/mL in human serum | 98.9 CFU/mL in PBS | About 6.5h | PBS Human serum | Dungchai et al. (2008) |
| *S. typhi* | Immunoassay (Sandwich) | SPCE | Au@NP's, MB's | NA | DPV | $10^3$ - $10^6$ cells/mL in PBS 143 cells/mL in PBS | $1.5 \times 10^3$ cells/mL in milk | 1.5h | PBS Milk | Afonso et al. (2013) |
| *S. spp* (no serotype) | Immunoassay (Sandwich) | MSPE | CuS NC | $Cu^{2+}$ | SWASV | $1 \times 10^3$-$5 \times 10^5$ cells /mL in buffer | 400 cells /mL in buffer | About. 63 min | Tris-HCl and Milk | Viswanathan et al. (2012) |
| *S. typhi* | Immunoassay (Sandwich) | SPCE | $Fe_3O_4$ NP's | $Fe^{3+}$ | ASV | $10^3$-$10^8$ CFU/mL of bacteria and Antigen | 8.18 and 1.51 CFU/ml of bacteria and antigen respectively | | Acidic solution | Brainina et al. (2010) |
| *S. pullorum & S. gallinarum* | Immunosensor (Sandwich) | SPCE | Au@NP's HRP | Thionine (red) | CV | $10^4$ - $10^9$ CFU/mL | $3.0 \times 10^3$ CFU/ mL in PBS for both species | About 24h | PBS Eggs Chicken meat | (Fei et al. 2015) |
| *S. typhi* | Immunosensor (Sandwich) | SPE | Au@NP's CdTe QD | $Cd^{2+}$ Gold Ions | ASV | 1 ng - 625 ng of Vi antigen | | | Buffer Solutions | Pandey et al. (2015) |
| *S. spp.* (no serotype) | Immunosensor (Sandwich) | m-GEC | HRP | $H_2O_2$ | A | 10 - $10 \times 10^7$ CFU/mL | $5 \times 10^3$ / $7.5 \times 10^3$ CFU/ mL in LB /milk LB | About 50 min. | LB Milk | Liébana et al. (2009b) |
| *S. typhi* | Immunoassay (Label – free) | Double Walled Electrode | CNT's | NA | CA CFU/mL | $10^2$ - $10^7$ | 8.9 CFU/mL | Total ≈6h | PBS | Punbusayakul et al. (2013) |
| *S. typhi* | Immunosensor (Sandwich) | SP-IDME | Glucose oxidase Streptavidin- Biotin | Gluconic acid | I | $10^2$ - $10^6$ CFU/mL chicken /pure culture | $1.04x10^3$/$10^2$ CFU/ml in | <2 h water | Pure culture Chicken rinse | Xu et al. (2016) |
| *S. spp. (no serotype)* | Immunosensor (Direct) | GCE | Au@NP's | NA | EIS | $1.0 \times 10^2$ - $1.0 \times 10^5$ CFU/mL in PBS | $1.0 \times 10^2$ CFU/ mL | 40 min. | PBS Pork meat | Yang et al. (2009) |
| *S. typhi* | Immunosensor (Label – free) | GCE | MSNT's | NA | I | $10^3$ – $10^7$ CFU/mL | $5x10^2$ CFU in PBS | 30 min. | PBS | Nguyen et al. (2014) |
| *S. typhi* | Immunosensor (Label – free) | GE | | NA | EIS | | 500 CFU/mL | Total of 6 min | PBS | Nandakumar et al. (2008) |
| *S. typhi* | Immunoassay (Sandwich) | $Cd^{2+}$ ISE | $Fe_3O_4$@Au CdS NC | $Cd^{2+}$ | P | 10 - $10^8$ CFU/mL | 20 cells / mL in PBS | 75 min | PBS Milk | Silva et al. (2015) |
| *S. typhi* | Immunoassay (Indirect) | SPE's | Alkaline phosphatase | 1-naphthol | A | $2.5 - 25.0 \times 10^{-6}$ M | | 1h 15min | Human Serum | Rao et al. (2005) |
| *S. typhi* | Immunoassay (Sandwich) | SPGE | HRP | $H_2O_2$ | CA | 10 - $10^7$ CFU/mL | 20 cells/mL | | Buffer solutions | Salam and Tothill (2009) |
| *S. typhi* | Immunosensor (Label – free) | GCE | Au@NP's MWCNT's | NA | I | $10^3$ - $10^7$ CFU/mL in PBS PBS/milk | 500/1000 CFU/mL in | About 1h | PBS Milk | Dong et al. (2013) |
| *S. typhi* | Genosensor | MSPE | Au@NP's methylene blue | NA | DPV | 0.01 - 5 nM in PBS | 0.05 nM in PBS | About 2h (2 min. for Blood Serum) | PBS Human | Das et al. (2014) |
| *S. typhi* | Genosensor | (ITO)GPE | Graphene oxide (GO) methylene blue | NA | CV/DPV | $10x\ 10^{-6}$ – 50 nM in PBS 100 fM in serum Samples | $10x\ 10^{-6}$ nM in PBS | Preparation ≈20 h; 60 s for hybridization | PBS Human Blood Serum | Singh et al. (2013) |
| *S. enteritidis* | Genosensor | SPCE | Au@NP's, PbS CdS NC | $Pb^2$+, $Cd^{2+}$ | SWASV | 50pg/mL – 50ng/mL | 0.5 ng/mL | Assay (≈2,5h) 10 min. for detection | PBS | Zhang et al. (2010) |
| *S. typhi* (Label – free | Genosensor | GCE | SWCNT's | NA | EIS | | 1 nM | Total of ≈26h | Phosphate buffer | Weber et al. (2011) |
| *S. typhi* | Aptasensor (Label – free) | ISE | SWCNT | NA | P | 0.2 to $10^6$ CFU /mL | 6 /26 CFU/ mL in PBS/ apple Juice | 60 s after inoculation | PBS Milk Fruit juice | Zelada-Guillén et al. (2013) |

(continued on next page)

**Table 5** *(continued)*

| Serotype | Bioreceptor /Design | Transducer | Nanomaterial /Label | Detected analyte | Detection Technique | Working Range | LOD | Analysis Time | Sample | Refs. |
|---|---|---|---|---|---|---|---|---|---|---|
| *S. typhi* | Genosensor | m-GEC | HRP | $H_2O_2$ | A | 1 CFU/mL | | 3.5 h Milk | PBS | Liébana et al. (2009a) |
| *S. typhi* | Immnunosensor (Label Free) | SPE | | EIS | $10^3$ to $10^8$ CFU /mL | $10^3$ CFU /mL | Total ≈16.5h Analysis: 20 min | PBS Milk | | Farka et al. (2016) |
| *S. typhi* | Aptasensor (Label Free) | GE | | EIS (non-faradic) | $10^2$ to $10^8$ CFU /mL | 3 CFU /mL | 45 min. | Apple Juice | | Sheikhzadeh et al. (2016) |
| *S. spp* (no serotype) | Immnunosensor (Sandwich) | GE | Magnetic Beads Alkaline phosphatase | L-Ascorbic Acid | A | | 7.6 x $10^2$ CFU /mL in PBS 6 x $10^2$ CFU /mL agricultural water | 3h | PBS Agricultural water | Wang et al. (2016) |
| *S. pullorum* | Immunoassay (Sandwich) | 4-SPCE | MBeads rGox AuNP' s | AuNP's | DPV | $10^2$ to $10^6$ CFU /mL | 89 CFU /mL | 80 min. preparation 120 s analysis | PBS Chicken liver | Fei et al. (2016) |
| *S.* ATCC 50761 | Aptasensor (Label Free) | GCE | rGO MWCNT's | | I | 75 to 7.5 x$10^5$ CFU /mL | 25 CFU /mL | 60 min. Chicken | Physiological saline | Jia et al. (2016) |
| *S. typhi* | Immunosensor (Sandwich) | GCE | Au@NP's HRP | $H_2O_2$ | CV EIS | 10 to $10^5$ CFU /mL | 25 CFU /mL | Total 4h Tap water Milk | PBS | Xiang et al. (2015) |
| *S. spp* (no serotype) | Genosensor (Label free) | GCE | Nanoporous glassy carbon | | DPV EIS | 10 to 400 pM in DVP 1 to 400 pM in EIS | 2.1 pM in DVP 0.15 pM in EIS | Total ≈4.2h | PBS | Amouzadeh Tabrizi and Shamsipur (2015) |
| *S. typhi* | Genosensor | GC | | | DPV EIS | $10^{-10}$ - $10^{-15}$ M 1000 copies of *Salmonella* in genomic DNA extracts | 0.97 fm in reaction mixture | Total time >56h | DNA extracts | Yan et al. (2016) |

Anodic Stripping Voltammetry ASV; Carbon Nanotubes (CN); Chronoamperometry (CA); Differential Pulse Voltammetry (DPV); Glass Carbon Electrode (GCE); horseradish peroxidase(HRP); Impedimetry (I); Indium Tin Oxide Glass Plate Electrode (ITO-GPE); Lysogeny broth (LB); Magnetic Bed's (MB's); magnetic silica nanotubes (MSNTs); magneto-graphite-epoxy composite (m-GEC); Modified Screen Printed Electrode (MSPE); Multi- walled carbon nanotubes; Nanocristal (NC); Phosphate Buffer Solution(PBS); Potentiometry (P);Quantum Dots (QD); reduced Graphene oxide (rGO); Screen-Printed Carbon Electrode (SPCE); Screen-printed gold electrode (SPGE); Screen-Printed Interdigitated Microelectrode (SP-IDME); Single Walled Carbon nanotubes (SWCNT's);Square Wave Anodic Stripping Voltammetry (SWASV); graphite-epoxy composite magneto electrode(m-GEC).

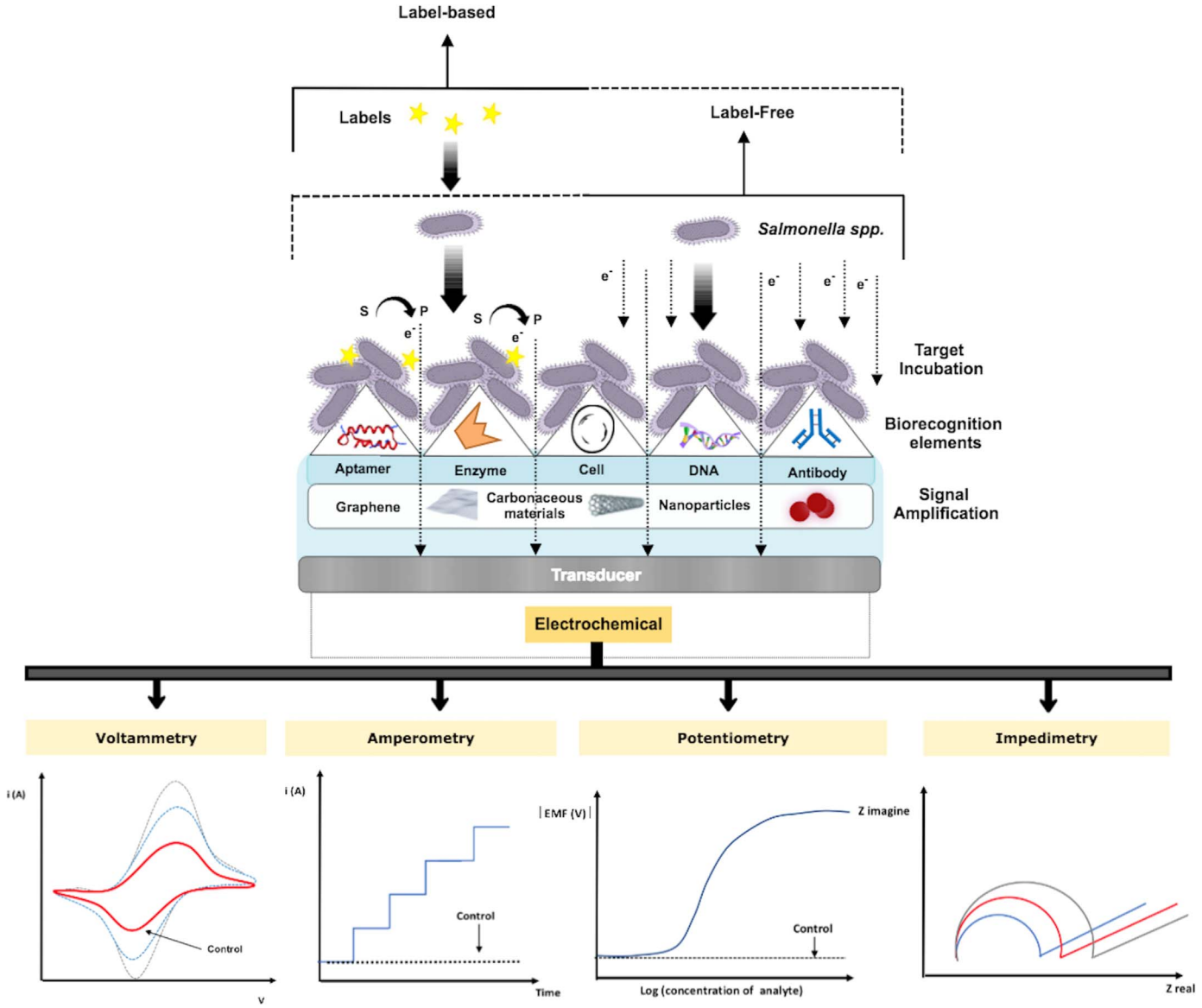


**Fig. 3.** Schematic representation of typical biosensor elements (transducer, amplification layers, bioreceptor), with different detection modes (label-based or label-free) and electrochemical transducing techniques (voltammetry, amperometry, potentiometry and impedimetry).

ing or increasing the sensitivity already achieved in parallel with the development of label-free biosensors or magneto-capture assays adding value to the biosensors in terms of assay simplicity, pre-enrichment step elimination, sample pre-treatment and selectivity. A schematic representation of different types of electrochemical biosensors is presented in Fig. 3.

### *3.1. Bio-receptor immobilization and biosensor design*

Normally the nanoscale materials are used in biosensors design to enhance sensor characteristics like surface reactivity and electrical conductivity, and in some cases, they also add interesting features like paramagnetic and biocompatible properties. So they can be used to play different roles, as is shown in Fig. 3, for example as like support materials for aptamer, DNA, enzyme or antibody immobilization, or as labels for electrochemical signal amplification(Stephen Inbaraj and Chen, 2016). Nanocrystals are the most frequently used for the last purpose because they easily solubilize to ionic species like heavy metals (ex. $Cd^{2+,}$ $Pb^{2+}$), that are unusual and residual in target samples of microbial analysis (food, ambient samples) (Pashazadeh et al., 2017; Stephen Inbaraj and Chen, 2016).

Graphene-based composites are the nanomaterials most chosen in electrochemical biosensors to improve the bio-receptor immobilization and for signal amplification. In 2008, graphene was employed for the first time as an electrode material for electrochemical biosensing(Bo et al., 2017). Since then, its use has been modeled per the type of biomolecule to detect in each application area. Although, according to the literature reviews(Bo et al., 2017; Chen et al., 2011; Kuila et al., 2011; Atta et al., 2015; Park et al., 2016; Zhao, 2015), graphene has been seldom explored in biosensors for food safety. There are only a few studies involving graphene-based electrochemical biosensors for *Salmonella* detection (Fei et al., 2016; Jia et al., 2016; Singh et al., 2013). Considering the current trend of electrochemical biosensors, there is a gap in graphene application in *Salmonella* electrochemical sensing, although sensing of this pathogen using carbonaceous materials like carbon nanotubes conjugated with others materials have been reported since 2004 (Dong et al., 2013; Jia et al., 2016; Nguyen et al., 2014; Punbusayakul et al., 2013; Zelada-Guillén et al., 2013). These materials were mainly used in label-free approaches where the amplification was made by the improving the electric conductance of the biosensor or increasing the active surface area for biomolecules immobilization, which in most of the transducing techniques are

proportional to the signal intensity (Table 5). In labeled approaches, the nanomaterials most chosen are magnetic nanoparticles with a ferrite core – used both to concentrate the sample, decreasing the enrichment times and for bio-receptor immobilization (Brandão et al., 2015).

### 3.2. Label-free electrochemical biosensors

Label-free biosensors use a receptor molecule connected to the biosensor transducer to recognize a specific analyte in a sample. The bio-complex formation is enough to trigger a measurable electrochemical signal correlated in some way with the analyte concentration. These sensors are capable to give a direct and in real-time measurement, with no requirement of labels or intermediaries, making the assay simplest, with less variables to control and resources needs.

Recently this assay format has awakened large attention and there are many just developed label-free biosensors to detect *Salmonella*(Amouzadeh Tabrizi and Shamsipur, 2015; Dhand et al., 2013; Farka et al., 2016; Jia et al., 2016; Nandakumar et al., 2008; Nguyen et al., 2014; Punbusayakul et al., 2013; Sheikhzadeh et al., 2016; Weber et al., 2011; Zelada-Guillén et al., 2013). The most widely used transducing technique in the emerging electrochemical label-free biosensors for detection of bacteria is impedimetry. Using this technique very good results were obtained in complex matrix samples like fruit juice, chicken or milk (Amouzadeh Tabrizi and Shamsipur, 2015; Dhand et al., 2013; Farka et al., 2016; Jia et al., 2016; Nandakumar et al., 2008; Nguyen et al., 2014; Punbusayakul et al., 2013; Sheikhzadeh et al., 2016; Weber et al., 2011; Zelada-Guillén et al., 2013). Recently, Sheikhzadeh et al. (2016), developed an aptasensor capable to detect S. *Typhimurium* selectively in real samples (spiked apple juice) with a limit of quantification (LOQ) of 100 CFU $mL^{-1}$ and a LOD of 3 CFU $mL^{-1}$ in a 45 min assay.

Punbusayakul et al. (2013), developed a double-walled carbon nanotubes (DWCN) electrode to detect *S. typhimurium* in a label-free immunoassay, in which they used chronoamperometry as a transduction technique. They explored the influence of carbon nanotubes architecture in electrochemical signal amplification when they are used as an immunosensor platform. With the conjugation of the nanomaterials and the specificity of antibody anti-*Salmonella* (attached to DWCN), they obtained in about 6 h a very good LOD of 8.9 CFU $mL^{-1}$ in a citrate-phosphate buffer matrix.

### 3.3. ELISA-based electrochemical biosensors

Most of the electrochemical biosensors for *Salmonella* detection are immunosensors based on lock and key binding event between the antibody (usually fixed at the transducer) and the antigen (that are in the sample) which recently have been specifically reviewed(Kokkinos et al., 2016; Melo et al., 2016; Ricci et al., 2007). Antigens usually are peptides, polysaccharides or lipid molecules. The antigens present in microorganism's surface normally are composed by oligosaccharides associated with lipopolysaccharide and flagellar proteins, which are the biomolecules recognized by bio-receptor of the biosensor. Apart from these, the most investigated are DNA-based and enzymatic biosensors, which are characterized by their bio-recognition elements.

As mentioned, ELISA exist in three principal formats in non-competitive version (antibody reacts proportionally to the amount of analyte), that are currently adapted to electrochemical biosensors: sandwich, indirect and direct.

In the various formats, it can be necessary the separation of the bound immunocomplexes from the solution, in solution or in transducer. These assays are known as heterogeneous immunoassays, and normally magnetic beads or nanoparticles are used to enable the pre-concentration or complex separation by simply using a magnet. These procedures normally increase the LOD of the assay or the signal intensity, at same time that reduce the length of the assay.

Sandwich electrochemical immunosensors are the most studied, because they just demonstrated high sensitivity, principally when are developed in simultaneous with new electroactive materials as labels that had reduced costs and greater possibility of reuse compared to the use of enzymatic markers (Piro et al., 2016; Yang et al., 2015). This format give a high efficiency in the antigen capture and at same time allows to use labels in a secondary specific antibody, (but their utilization isn't mandatory). Salam, F. and I.E. Tothill (2009), developed a biosensor for *Salmonella* in sandwich ELISA format, that use a HRP as enzyme and experimented two different antibody immobilization methods on transducer. With a covalent immobilization and amperometry as transducing technique, they achieved to a LOD of 20 cells $mL^{-1}$ in a linear range of $10–10^7$ CFU $mL^{-1}$, by a simple correlation of the enzyme HRP reaction products with the number off cells that are connected with the specific antibody anti *S. typhimurium*.

Wang et al., proposed a voltammetric immunosensor for *S. pullorum & S. gallinarum* with the same enzyme (HRP) based on rGO electrochemical properties to enhance the electric conductivity and a polyvinyl alcohol (PVA)-multilayer polydimethylsiloxane (PDMS) that works as a biocompatibility enhancer for de rGO, increasing the sensitivity of the sensor. The modifying layer showed a good linear response range from $10^1–10^9$ CFU $mL^{-1}$ and LOD of $1.61 \times 10^1$ CFU $mL^{-1}$(Wang et al., 2014). In fact, due the facility of detection of subproducts of HRP, this is one of the enzymes choose for pure ELISA format (Delibato et al., 2006; Fei et al., 2015; Liébana et al., 2009b; Salam and Tothill, 2009; Xiang et al., 2015b).

The direct and indirect formats are very little used in biosensors for *Salmonella* (Table 5), perhaps due to the possible loss of selectivity and sensitivity of the assay, due the connection of only one antibody to the bacteria. In indirect ELISA biosensors, the antigen is immobilized directly in the transducer and use one primary antibody and a secondary one (conjugated with the primary) allowing the detection of the antigen trough a label. In direct format are used only one antibody as recognition and labeled element. The antigen can be immobilized directly on the assay plate or in the form of a capture assay.

### 3.4. Electrochemical detection

In biosensors for microbial detection, the most common transducing methods are the optical and electrochemical. The optical detection uses optical signals like chemiluminescence, color or fluorescence to quantify the concentration of the target compound. The electrochemical biosensors measure de current or/and potential changes that occurred in the interface between de working electrode and the sample matrix (Sharma and Mutharasan, 2013). Normally, in microbial biosensors, this signals translate the interaction of the microorganisms with one specific target (Su et al., 2011).

Recently, some excellent works on microbial detection with optical transduction (Cho et al., 2014; Duan et al., 2015; Duan et al., 2016; Kim et al., 2015; Koba et al., 2016; Rios-Corripio et al., 2016; Wu et al., 2014; Zhang et al., 2016), reporting lower or similar detection limits as compared to most of the electrochemical biosensors, aroused in the literature. Still, in our view electrochemical transducers show significant advantages over their optical counterparts, as they allow developing more versatile detection schemes, their miniaturization is simple and allow for real-time quantification. Moreover, if the aim of the developed application is the food industry and the final consumers, then the lower price and minimal electrochemical equipment requirement also makes them more attractive (Wang et al., 2016).

According to the specific transduction technique employed and type of recorded signal, electrochemical biosensors can be classified into other basic groups as: amperometric (current), potentiometric (potential), voltammetric (current and potential), condutimetric and impedimetric (impedance) biosensors (Su et al., 2011).

### 3.4.1. Amperometric biosensors

Amperometric biosensors operate at a given value of potential difference applied between the working and reference electrodes. This potential difference will trigger an oxidation-reduction reaction on electrode surface involving a metabolic product or an electroactive species in the sample (Luppa et al., 2001) and, consequently, a change in electric current intensity is observed. After current intensity measurement, the values are correlated with concentration of the target analyte (Su et al., 2011).

According to the literature, this type of transduction has been widely explored in biosensors for the detection of proteins but it is not the most widely used transduction technique in microbial biosensors. Liébana et al. (2009b), developed an immunosensor for the detection of *Salmonella* spp. in milk. They used a sandwich format with two polyclonal anti-*Salmonella* antibodies, one was labeled with a HPR enzyme and the other one was used in association with magnetic particles to concentrate and separate the formed immune-complexes from the sample matrix, directly, on a magnetic work sensor. With this methodology they reached to poor limit of detection of $7.5 \times 10^3$ CFU $mL^{-1}$ in 1/10 diluted milk, and a fast response time of 50 min per analysis (Liébana et al., 2009b). In another approach, they used the same enzyme but a different bioreceptor, that was DNA instead antibodies. In this work the specificity of the immunological reaction with the specific antibody against *Salmonella* was used only to capture the bacteria and perform their magnetic immunoseparation from skim milk samples resort to use of magnetic beads with no matrix adulteration. Posteriorly to capture the bacteria, the DNA were amplified by PCR techniques insuring a correct serotype identification (Liébana et al., 2009a). With this labeled design and complementary molecular techniques, in 1.5 h, in PBS, they achieved an incredible LOD of 1 CFU $mL^{-1}$ in milk. The authors proved that immunoseparation can substitute the selective culture media in conventional methods, and the genosensing with electrochemical transducing is a good option to reduce the time to obtain confirmative results (Liébana et al., 2009a, b).

In turn, Punbusayakul et al. (2013), created a label-free immunoassay for *S. thypi*, by covalent immobilization of antibodies onto the double walled carbon nanotubes modified electrode, in which chronoamperometry was used as transducing technique. They achieved a very good LOD of 8.9 CFU $mL^{-1}$, in a linear range from $10^2$–$10^7$ CFU $mL^{-1}$, in a simple assay structure without labels or sample preconcentration.

### 3.4.2. Potentiometric biosensors

Potentiometry consist on measuring the potential difference, between a reference electrode and a working electrode with a current level almost zero. There is a lot of types of potentiometric electrodes, but the most commonly used are the Ion Selective Electrodes (ISE). This type of transducers consists of membranes with selective permeability and high affinity to certain ionic species generated or consumed in a target biological process (Eggins, 2002; Leonard et al., 2003; Luppa et al., 2001).

Potentiometric biosensors show some advantages over other electrochemical transducers due their recognized capacity of miniaturization plus that in comparison with voltammetric techniques, the signal isn't dependent on the electrode surface area. The synergic combination of these features with the notable sensitivity and selectivity levels that ISE can achieve, prompts to think that potentiometric biosensors have a high potential in the field of microbial food and environment control (Hassan et al., 2016). Although this type of biosensors is not the most studied, probably because a lot of work is necessary to optimize the experimental conditions to use the biosensor and the reference electrode stabilization, that according to IUPAC rules must be rigorous (±0,1 mV/min.) poses several challenges specially in miniaturized potentiometric cells.

Despite these limitations, homemade pipette tips electrodes were used in a potentiometric assay, which can detect 20 cells of *Salmonella thyphimurium* in a linear range of $10^1 \times 10^8$ cells $mL^{-1}$, through a capture sandwich assay format, magnetic sample pre-concentration and CdS nanocrystals as labels (Silva et al., 2015). Zelada-Guillén et. al. (2013), developed one aptasensor to detect *Salmonella* using an ISE and a single walled carbon nanotubes (SWCNT). They achieved to a LOD of 6 CFU $mL^{-1}$ and 26 CFU $mL^{-1}$ in PBS and apple juice, respectively.

### 3.4.3. Voltammetric biosensors

Voltammetry is a transducing technique where the current is measured in function to the applied potential, and because of that it is the most versatile electrochemical technique. The position of top of the current peak depends on the chemical species or the target analyte and peak current intensity is proportional to their concentration, allowing in this way the simultaneous detection of multiples analytes (Freitas et al., 2014; Luppa et al., 2001; Su et al., 2011). Among electrochemical techniques, voltammetry is the less to prone to noise, it is the most widely used in microbial analysis by biosensors, and was already applied to all types of bioreceptors (Amouzadeh Tabrizi and Shamsipur, 2015; Das et al., 2014; Freitas et al., 2014; Xu et al., 2016).

Freitas et al. (2014), developed a magnetic immunoassay in a sandwich format for *Salmonella typhimurium*, where Fe@Au nanoparticles was used to increase sample pre-concentration efficiency and, CdS nanocrystals to amplify the obtained electrochemical signal by stripping voltammetry. In this approach, it was possible to obtain the results in 1 h (in PBS matrix) with a LOD of 13 cells $mL^{-1}$ and a linear range of $1 \times 10^1$-$1 \times 10^6$ cells $mL^{-1}$. In a similar approach, Afonso et al. (2013) used a permanent magnet underneath a Screen-Printed Carbon Electrode (SPCE) to do the sample concentration and gold nanoparticles as secondary antibody labels. But in this work the analyze time it is more long (1.3 h), and the LOD higher (143 cells $mL^{-1}$).

Singh et al. (2013), applied for the first time a GO (Graphene Oxide)-Chitosan (CHI) nano-composite in the design of a DNA based electrochemical biosensor for detection for detection of *Salmonella Thypi*. The modification was based on the enhance electrochemical activity and electrons transferring offered by the GO, the bio-affinity of the CHI, and the specificity of 5-amine labeled single stranded (ss) DNA probe. These characteristics united synergistically originate an extremely sensitive biosensor that can detect $10 \times 10^{-15}$M of DNA probe within 60 s hybridization times in a concentration range of $10 \times 10^{-15}$M to $50 \times 10^{-9}$M, able to successfully distinguish between complementary and non-complementary sequences, even in real samples like human serum (Singh et al., 2013).

In recent work, Fei et al., (2015, 2016) showed clearly the effect of nanomaterials as label in sensitivity of voltammetric immunosensors. In a first work, they used a 4-SPCE modified with an ionic liquid, gold nanoparticles and antibody anti-*Salmonella pullorum* to capture de *Salmonella* from one spiked sample, and before in a sandwich format they incubate the immunocomplex with a secondary antibody labeled with HRP, reaching to a LOD of $3 \times 10^3$ CFU $mL^{-1}$(Fei et al., 2015). With the same biosensor design and electrode, using silica modified immunomagnetic beads for capture and reduced graphene oxide coated with gold nanoparticles instead an enzyme as label, they succeeded at amplifying the electrochemical signal and attained a LOD as low as 89 CFU $mL^{-1}$(Fei et al., 2016).

### 3.4.4. Impedimetric biosensors

Impedimmetry is frequently associated with immunosensors. In this technique the changes in an electric field caused by the antibody/antigen interaction and resultant by the change in the electric conductance or capacitance that happens at the electrode surface or in solution in a constant potential (condutimetry) is detected (Eggins, 2002; Jiang et al., 2008).

This technique is the one of the most used in the microbial electrochemical analysis, because it allows the miniaturization and a

fast response; although even combined with nanomaterials, when it is used in samples with low conductance (Su et al., 2011) it can't achieve the other techniques sensitivity. These implies higher LOD's and in last instance a weaker potential use in real samples, demonstrating that it will need more optimizations in future. Despite of these general considerations,(Yang et al., 2009) developed an immunosensor with high sensitivity towards for *Salmonella spp*, based on grafted ethylene diamine and self-assembled gold nanoparticle monolayer. The developed biosensor showed a good LOD of 100 CFU $mL^{-1}$ in a complex matrix (pork meat), in a 40 min analysis time, with a simple and direct detection methodology based in conductance changes on the immunosensor surface, probing that is too a capable technique for pathogens screening, although more complex and laborious than the others electrochemical techniques.

Yan et al. (2016) proposed the coupling of a homogeneous target-initiated transcription amplification (HTITA) method directly into sensing interface without resorting to nanomaterials. Although the simplification of the procedures and resources needs, important towards point-of-care screening, the biosensor developed don't show be appropriate in real contamination scenarios due the long time needed to achieved to results, still its good LOD reached of $9.7 \times 10^{-16}$ M.

### *3.5. Biosensors with developed food application*

To compare the different electrochemical transducers used in the literature on *Salmonella spp* analysis some application in foodstuffs were selected (Table 6) although the complexity of the samples wasn't taken into account. The critical analysis was based on increasing complexity of the biosensor design where the simplest assay is one that doesn't require labels and allows a direct measure, followed by assays that require labels but are made through a direct measure and the most complex design involves using labels and the detection is based on indirect measures. Other parameters considered in the analysis were the LOD; the time spent to perform the analysis and their potential application in real samples considering the EC legislated limits. Taking into account these marks the study by (Delibato et al., 2006) is the worst classified because has a LOD that is far from zero *Salmonella* spp. CFU's (presence or absence). Among the methods (Table 6) the study with best evaluation is terms of LOD is from (Liébana et al., 2009a) and it is the only one with potential for application in real samples because its LOD of 1 CFU/25 g covers the imposed limits in EU regulations for food products. Although the analysis time was increased in 6 h (pre-enrichment) and complementary molecular techniques were used to achieve this detection limit in skimmed milk (dilution factor 1/10).

Beyond this finding almost two thirds of the works can detect infection contamination levels of *Salmonella spp*. Among this group, the study by Sheikhzadeh et al. (2016), stands out because the results can be achieved in less than 1 h and it combines the simplicity of conception with an excellent LOD (3 CFU $mL^{-1}$) and adequate % recoveries obtained in food samples.

This analysis shows that the label-free biosensors, beyond simplifying the procedures of detection, have a high potential for application in real samples, due to their sensitivity and reproducibility. Although an equilibrium between the endeavor to reduce the time of analysis and to improve the simplicity of the assay is a key issue.

## 4. Conclusions and prospects

Many scientific studies on biosensors for *Salmonella* detection are still being carried out, emphasizing the importance of its accurate and rapid detection in foodstuffs, which is reinforced by the recent alarming statistics. At the same time, many commercial rapid methods are just available. In this paper, was presented a perspective in which were highlight the development stage and relative value for food industry of both. Most of the commercial rapid methods derive from technologies already in use in the biochemistry or microbiological labs. The development of methods in microplates allowed performing many tests almost simultaneously whereas most biosensors pose challenges at the base sensors development and most of them incorporate the schemes and knowledge from bioassays.

Sample preparation, enrichment and selection are critical steps in the performance of all detection methods, including the electrochemical biosensors. The ideal methods shall be as simple as possible, rapid, low cost and with minimal sample preparation requirements, applicable to all foodstuffs and materials, especially where the rapid method is developed in a lab-on-chip concept or for out-of-lab usage. Additionally, a correct and specific separation of all *Salmonella* target cells from the samples matrix is a crucial step to improve the sensibility and specificity of methods based on electrochemical detection due to the elimination of inhibitory substances, microflora or physical interferences from the analytical detection.

It is difficult to compare the sensitivity and specificity of different electrochemical biosensors and bioassays in the literature because some of them were only applied on ideal optimized conditions (phosphate buffers), and others were applied to different types of food samples which follow different steps of sample preparation. The validation of the methods sensibility should attend to its detection limit, but also to the probably of heterogeneous distribution of *Salmonella* in foodstuffs when this pathogen is present at low levels. Consequently, the methods used for sampling and the stress and alterations suffered by the bacteria during sample processing

**Table 6**
Comparison of developed electrochemical methods in the literature with food application.

| Serotype | Simplicity | LOD | Sample | Time | in Food Samples | Refs. |
|---|---|---|---|---|---|---|
| *S. spp* (no serotype) | + | 1 CFU/mL in milk | Milk | +++ | Yes | Liébana et al. (2009a) |
| *S. typhimurium* | +++ | 3 CFU/mL | Apple juice | + | infection leve | Sheikhzadeh et al. (2016) |
| *S. typhimurium* | + | 13 cells/mL | PBS | + | infection level | Freitas et al. (2014) |
| *S. typhimurium* | + | 20 cells/mL | PBS/Milk | ++ | infection level | Silva et al. (2015) |
| *S. typhimurium* | + | 25 CFU/mL | Tap water | +++ | infection level | Xiang et al. (2015) |
| *S. typhimurium* | ++ | 26 CFU/mL 1 | Apple juice | + | infection level | Zelada-Guillén et al. (2013) |
| *S. pullorum* | + | 89 CFU/mL | PBS Chicken liver | ++ | infection level | Fei et al. (2016) |
| *S. typhimurium* | ++ | $1.5\times10^3$ and 143 cells/mL | PBS Milk | ++ | infection level | Afonso et al. (2013) |
| S. *spp*(no serotype) | ++ | $1.0\times10^2$ CFU/mL | PBS pork meet | + | infection level | Yang et al. (2009) |
| *S. spp*(no serotype) | + | $4\times10^2$ cells/mL | Buffer | ++ | infection level | Viswanathan et al. (2012) |
| *S. typhimurium* | +++ | $10^3$ CFU/mL | PBS /milk | +++ | No | Farka et al. (2016) |
| *S. typhimurium* | +++ | $10^3$ CFU/mL | Milk | + | No | Dong et al. (2013) |
| *S. typhimurium* | + | $1.04\times10^{3\backslash}$ CFU/mL | Chicken | ++ | No | Xu et al. (2016) |
| *S. pullorum and S. gallinarum* | + | $3.0\times10^3$ CFU/mL PBS for both species | | +++ | No | Fei et al. (2015) |
| *S. spp* (no serotype) | + | $7.5\times10^3$ | Skim milk (1/10) | + | No | Liébana et al. (2009b) |
| *S. enterica* | +++ | $5\times10^7$ CFU/mL | | +++ | No | Delibato et al. (2006) |

Simplicity: +++ no labels and direct measure; ++ label, direct measure; +labels, indirect measure; Analysis time: + ≤ 1 h; ++ ≤ 2 h; +++ > 2 h.

are important parameters to optimize but these issues have been poorly explored in the literature on rapid methods. Therefore, besides ensuring that the analyzed sample is representative of the complete foodstuff it is also necessary to consider the level of pathogenicity for humans according salmonella cells viability. The DNA-based and immnunosensors are unable to discriminate among living or death *Salmonella* cells. This is a key point as bacterial cell pathogenicity is related to their grown potential in foodstuffs and in humans after their ingestion.

Very good results have been accomplished with immnunomagnetic and phage-based separation techniques in both commercial devices and academic works. They are highly effective in accurate selection of the target bacteria and in reducing the time needed for sample preparation, due the elimination of conventional enrichment steps, thus allowing next day results for a better food emergence response preventing spreading of microbial contamination. Their effectiveness is more visible in biosensors that incorporate simultaneously a very sensitive and selective bioreceptor platform, for example antibodies or DNA-based nanomaterials, and a simple and direct transduction technique such as electrochemical techniques. The simplicity and cost efficiency of the biosensors can also be increased, with apparent no loss of accuracy, using label-free electrochemical biosensors.

Despite of the great advances in the technologies and in line with previous studies in the literature, none of the current rapid detection methods for *Salmonella,* both the commercially available or the ones yet at a development stage, do not meet all the requirements for food application, considering the regulation limit. Therefore, further improvements in terms of validation parameters, time of analysis, portability and autonomy are necessary. Furthermore, electrochemical biosensors seem to be the most acceptable and reliable technology to achieve the regulation requirements and overcome industrial implementation barriers, because it is possible to achieve a lab-on-chip device, with the desired analytical properties and adapted to specific industrial needs, at a potentially low cost.

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