REVIEW

Recombinant antibodies and their use for food immunoanalysis

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Abstract

Antibodies are widely employed as biorecognition elements for the detection of a plethora of compounds including food and environmental contaminants, biomarkers, or illicit drugs. They are also applied in therapeutics for the treatment of several disorders. Recent recommendations from the EU on animal protection and the replacement of animal-derived antibodies by non-animal-derived ones have raised a great controversy in the scientific community. The application of recombinant antibodies is expected to achieve a high growth rate in the years to come thanks to their versatility and beneficial characteristics in comparison to monoclonal and polyclonal antibodies, such as stability in harsh conditions, small size, relatively low production costs, and batch-to-batch reproducibility. This review describes the characteristics, advantages, and disadvantages of recombinant antibodies including antigen-binding fragments (Fab), single-chain fragment variable (scFv), and single-domain antibodies (V_{HH}) and their application in food analysis with especial emphasis on the analysis of biotoxins, antibiotics, pesticides, and foodborne pathogens. Although the wide application of recombinant antibodies has been hampered by a number of challenges, this review demonstrates their potential for the sensitive, selective, and rapid detection of food contaminants.

Keywords Recombinant antibody · Antibody fragment · Food analysis · Biosensing · Immunoassay

Introduction

Antibodies play a pivotal role in biomedical research, diagnostics, therapeutics, and virtually every branch of biotechnology. These fascinating proteins are characterized by their exceptional ability to identify and bind to specific targets of interest, even in a complex environment. The exquisite specificity of antibodies has also paved the way for their ubiquitous use as biorecognition elements in biosensing applications. Antibody-based sensors and assays—immunosensors and immunoassays—have revolutionized the analysis of a plethora of analytes, including biomarkers as well as food and environmental contaminants. Consequently, there is a continuous demand for antibodies or antibody mimics in research and development.

Formerly, immunoassays were mainly based on polyclonal antibodies (pAb) from the sera of immunized mice or rabbits, or monoclonal antibodies (mAb) developed by the hybridoma technology, which by all means revolutionized the face of biomedicine and overcame many drawbacks related to the use of pAbs. Nowadays, monoclonal antibodies continue to be the predominant immunoreagent although also polyclonal antibodies still retain their share of the applications and market [1]. While some modifications and improvements in the preparation of monoclonal and polyclonal antibodies, such as various immunization strategies [2], have been introduced over the years, the methods for generating these animal-derived antibodies have not significantly improved in the past 40 years [3]. Moreover, validation of monoclonal and polyclonal antibodies is often lacking, which can be seen as a major limitation considering the quality and consistency of antibody-based technologies [4].

On the other hand, the emergence of recombinant antibody technologies over the last decades has enabled the production of antibodies without the need for animal immunizations. Such non-animal-derived antibodies are produced from

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synthetic or naïve (non-immunized) antibody repertoires, which do not require animal immunization at any stage of the library construction or antibody production. Various display methods, most notable one being phage display which was awarded with the Nobel Prize in Chemistry in 2018 to George P. Smith and Sir Gregory P. Winter [5], provide a straightforward methodology for guided selection of appropriate binders. Antibody production by phage display employs the same mechanistic principles as the immune system and results in antibodies that are functionally indistinguishable from those produced in vivo. Non-animal-derived antibodies that encompass various formats, including antibody fragments, such as fragment antigen-binding (Fab), single-chain fragment variable (scFv), and full-length antibody or other engineered variants, are now emerging as credible alternatives for monoclonal antibodies [6]. Even though antibodies originating from phage-displayed repertoires are still far behind monoclonals, it is noteworthy that in 2020 already more than 70 phage-derived mAbs have entered clinical studies, and 14 of them have been approved [7].

Even though synthetic and naïve antibody repertoires are now capable of outweighing the obsolescent animal immunization protocols, the majority of antibodies used in research are still made by immunizing animals, most frequently rabbits or mice. Antibody production requires the use of a considerable number of animals with substantial animal welfare consequences. It has been estimated that almost 1 million animals are used annually for antibody production in the EU alone [3]. In accordance with the EU directive 2010/63/EU on protecting animals used for scientific purposes [8], the EU Reference Laboratory for alternatives to animal testing (EURL ECVAM) published new recommendations on non-animal-derived antibodies in 2020 [3]. The recommendation clearly states that "animals should no longer be used for the development and production of antibodies for research, regulatory, diagnostic and therapeutic applications" [3]. In particular, antibody production using the ascites method should not be acceptable under any circumstances. Accordingly, the manufacturers should replace animal-derived antibodies in their selection and label unambiguously whether the antibodies in their catalogues are animal-derived or not.

Despite this clear missive from the EU experts, many scientists are reluctant to give up on the use of animal-derived antibodies, and to a large extent, the EURL ECVAM recommendation has been plagued with criticism. There is an ongoing debate in the scientific community on the use of animal-derived antibodies and the readiness of the technologies to produce non-animal-derived antibodies [9–11]. While some are posing non-animal-derived antibodies to supersede monoclonal and polyclonal antibodies, others argue that the required technology is not quite there yet and non-animal-derived antibodies should complement rather than replace monoclonal antibodies. In this review, we discuss the potential of recombinant antibodies for their application in rapid screening immunoassays and biosensors for the analysis of food samples with special emphasis on the detection of biotoxins, antibiotics, pesticides, and foodborne pathogens.

Conventional antibodies

Antibody structure

Antibodies are produced by the immune system of vertebrates as a defense mechanism against foreign or harmful agents, such as pathogenic bacteria, viruses, toxins, and the like. In the body, antibodies recognize, bind, and mark their target antigens for removal, for example, by neutralization or via phagocytosis. Antibodies, also referred to as immunoglobulins, are classified into five isotypes, IgA, IgD, IgE, IgG, and IgM, based on their heavy chain constant domains [12]. IgG is the most commonly used isotype and the most prevalent in human, comprising 80% of serum antibodies. These antibodies are very stable and easily purified by affinity chromatography with protein A or protein G, making IgGs the most important antibody isotype for biotechnological and medical applications [12, 13].

In terms of structure, antibodies have been among the most studied of all proteins [14]. The Y-shaped structure of the IgG molecule, with a molecular weight of about 150 kDa, consists of two identical heavy chains and two identical light chains, both of which have variable and constant domains (Fig. 1a). The highly conserved stem of the Y, known as the Fc region (for fragment crystallizable), is responsible for interacting with cells and effector molecules [12]. Within each variable domain, the complementary determining regions (CDRs), or hypervariable loops, form the antigen-binding site. This region is responsible for the vast recognition variety and exquisite specificity of the IgGs, which is achieved by a high sequence variation through combinatorial assembly of the gene segments. During B-cell development, these genes are irreversibly joined by DNA recombination to form a complete variable region with enormous diversity potential. Furthermore, in the process of joining the gene segments, additional junctional diversity is formed by adding or subtracting nucleotides. The complete antigen-binding site is finally created by pairing the variable regions of both heavy and light chains, each composed of distinct gene sets, and as a result, antibodies gain the apparently infinite variety of subtly different forms that allow them to recognize specifically a huge number of different antigens [12, 16].

Polyclonal and monoclonal antibodies

Polyclonal antibodies are produced by injecting the antigen of interest into an animal and collecting the serum as a source of antibodies some weeks after the immunization. Most
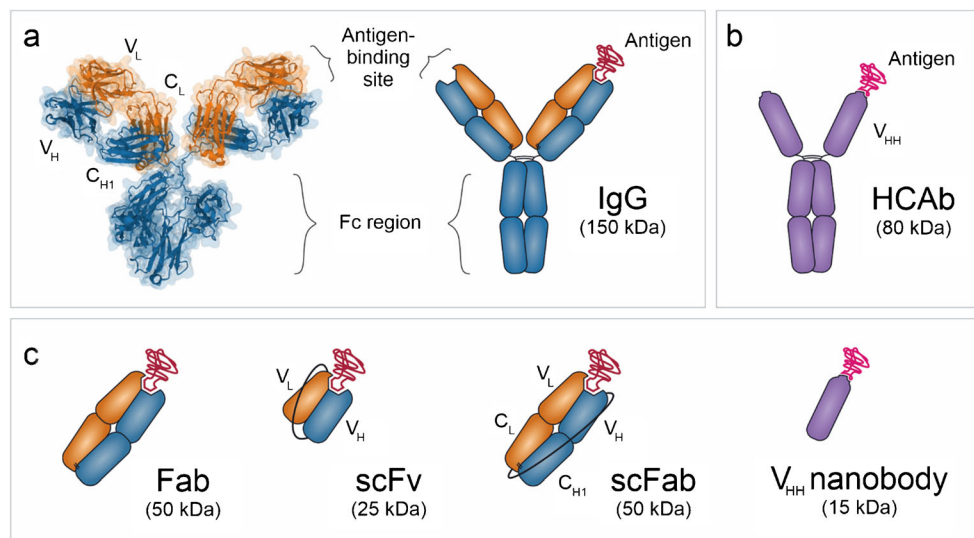
Fig. 1 **a** Structure of a typical IgG antibody depicted as a molecular model (PDB ID 1IGY; Mol* [15] RCSB PDB, https://www.rcsb.org) and as a schematic representation. The whole IgG antibody of about 150 kDa is made up of four peptide chains, two identical heavy chains (blue) and two light chains (orange). The constant Fc-region forms the stem of the antibody molecule whereas the antigen-binding sites are formed by the two variable domains (V_L and V_H). **b** Structure of the heavy chain antibody (HCAb) which naturally is devoid of light chains and is capable of antigen recognition solely by one single domain, the variable heavy domain (V_{HH}). The HCAb depicted in the figure with two constant domains is derived from Camelidae family (shark-derived HCAbs are constituted by 5 constant domains). **c** Antibody fragments Fab (for fragment antigen-binding), scFv (for single-chain variable fragment), and scFab (for single-chain Fab) retain the antigen-binding site of the intact IgG but lack some or all of the constant domains making them significantly smaller in size. The single-domain antibody derived from HCAb, known as the V_{HH} or nanobody, represents the smallest available antibody fragment with functional antigen binding.

commonly used animal species include rabbit, mouse, rat, hamster, and guinea pig. When larger amounts of antibodies are required, larger farm animals, such as sheep or goats, are often used [17]. The blood of the immunized animal is collected to monitor the antibody response and obtain the polyclonal sera, and finally, exsanguination is performed under general anesthesia, usually by heart puncture, which results in the death of the animal. Despite their wide and continuing use, polyclonal antibodies have several limitations. Polyclonal antibodies provide a finite supply of antibodies, and each batch is unique because immunizing an animal never results in an identical mixture of antibodies [18]. Yet, polyclonal antibodies are relatively inexpensive and quick to produce without the need of specialized skill or equipment, and they can function as excellent recognition elements because they usually have a high overall affinity against their target. For some targets, polyclonal antibodies are the only available antibody type, and they are widely used, for example, as secondary detection antibodies in numerous applications [19]. Nevertheless, this kind of antibodies always represents a mixture of different antibodies that recognize several epitopes of the target as well as unknown targets. In fact, according to some reports, only around 0.5–5% of antibodies in the serum sample of an immunized animal bind to the intended target [20]. This can result in high background signals or cross-reactivity in immunological methods, if the antibodies are not affinity purified against the target they recognize. Moreover, production of polyclonal antibodies is inherently

dependent on the use of animals for immunization and source of antibodies.

In the 1970s, the hybridoma technology created by Köhler and Milstein [21] revolutionized the way of making antibodies. Monoclonal antibodies are made by fusing a normal short-lived antibody-producing B cell with a cancer cell line. Production of antigen-specific B cells requires also immunization of the animal, most often mouse, rat, or hamster, with the antigen under study. The B-cells are harvested from the spleen by sacrificing the animal generally after several booster immunizations. Antibody-producing B cells from the spleen are then fused with non-secreting myeloma cells (hybridoma cells), and the product is cloned and tested for antigen specificity [2, 17]. The resulting hybridomas are, in theory, capable of producing only one identical antibody clone, i.e., a monoclone, which is a significant advantage over polyclonal antibodies. Moreover, monoclonal antibodies are capable of recognizing very specific and unique epitopes with high affinity and reduced probability of cross-reactivity. In addition, hybridoma cell lines have the capacity of producing large quantities of the identical antibody without batch-to-batch variations, thus overcoming some of the major limitations of polyclonal antibodies. However, also monoclonal antibodies can be contaminated with additional unwanted antibody chains, and even hybridoma cell lines are not infinite; they can die off or lose their antibody genes [19, 20]. Monoclonal antibodies are also significantly costlier to produce than polyclonals. Their production can be not only tedious and time-consuming but also require considerable skills

and training. Moreover, despite their superior specificity over polyclonal antibodies, numerous reports have also shown the lack of specificity of monoclonal antibodies. Even many commercial monoclonal antibodies can react with more than one target as well, which often arises from an additional antibody chain in the hybridoma cell [22].

Polyclonal and monoclonal antibodies have been the cornerstone of immunological methods for decades, yet they pose several intrinsic limitations. Sometimes, whole antibodies result in practical drawbacks, such as slow and costly production, low stability, slow kinetics, or weak tissue penetration. Indeed, although highly effective, monoclonal and polyclonal antibodies are laborious and expensive to produce, they require the use of animals, and they result in non-human and potentially immunogenic antibodies, which is a major concern depending on the final application (e.g., for antibody therapeutics). In addition, the natural immune system imposes restrictions complicating the production of antibodies against unstable antigens, highly conserved proteins between species, or toxic compounds. Although researchers have tried to overcome these limitations by different strategies, including the generation of human antibodies in transgenic mice designed to produce a repertoire of human immunoglobulins followed by hybridoma, some of these caveats still persist [23]. The advantages, limitations, and key differences of different antibodies are presented in Table 1.

Recombinant antibodies

Origin and characteristics

Recombinant antibodies (rAb) are generated by recombinant DNA technologies, and they can offer several advantages over polyclonal and monoclonal antibodies (Table 1). In contrast to glycosylated whole antibodies, recombinant antibody fragments can be produced in bacterial cells [25]. Since recombinant antibodies are based on a known and defined DNA sequence, their production is highly reproducible and low cost. Moreover, recombinant antibodies can be engineered further, for example, with different affinity tags to facilitate their purification or immobilization for the downstream applications [26]. Alternatively, recombinant binders can be multimerized to increase their avidity.

Recombinant antibodies can originate from various sources; and thus, they are not always truly animal-free. For example, a known sequence of the monoclonal antibody can be used to construct recombinant antibody fragments by direct cloning of the gene fragments from the hybridoma cell lines. Despite the fact that at that point, such antibody fragments can be readily produced for example in *Escherichia coli* without the use of animals, the generation of the parental monoclonal antibody still requires animal immunization; and thus, such recombinant antibodies are considered animal-derived. In some cases, isolation of functional recombinant antibody fragments from hybridoma cell lines can be challenging, and the fragments might have lower affinity than their parental monoclonal antibody [27, 28].

Alternatively, phage-displayed antibody repertoires (either as naïve or engineered libraries) provide an actual alternative to circumvent some of these caveats, especially regarding animal immunization. In vitro selection methods by means of phage display can be used to get antibodies against almost any target in a high-throughput or even automated manner [29]. In addition, in vitro selection can offer a more precise control over the selection of antibodies against toxic targets or unstable proteins, or regarding their affinity maturation, if needed [30, 31].

Phage display technology was originally developed by George P. Smith in 1985 [32], and since then it has become the most widely used selection method for human antibodies thanks to the breakthrough achievements made by Sir Gregory P. Winter [5]. This technology is used to generate

Table 1 Comparison of different antibody types and their advantages and limitations. Adapted from [24]

	Polyclonal (pAb)	Monoclonal (mAb)	Recombinant (rAb)
Production			
- Time	Short (3–4 months)	Long (up to a year)	Short to moderately long
- Cost	Low	Moderate to high	Low to high
- Ease	Very easy	Difficult	Easy
Targets	Immunogenic targets	Immunogenic targets	No limitations
Stability	High	Moderate	Depends on the format
Reproducibility	Limited	Virtually reproducible	Fully reproducible
Specificity	Moderate	High	High
Sensitivity	Variable	Moderate to high	High
Engineering	Not possible	Only after converting to rAb	Possible
Availability	Commercially available	Commercially available	Limited commercial availability

and/or affinity maturate antibodies for research and medical use [33, 34]. In phage display, antibody pools displayed on phages (synthetic or constructed from human or immunized or naïve animals) are subjected to in vitro selection with the immobilized target of interest on plates or in solution. Specific clones bound to the target are then amplified after infection of bacterial cells and directly used for subsequent rounds of selection. Finally, after three or four rounds of selection, antigen-binding clones are sequenced to identify the DNA sequences of the antibodies displayed on the surface of the isolated phages. *E. coli* phages are the most extensively used owing to their ease of culture and rapid amplification, making their production fast and economical. This technology is now the most widely used method for generation of antibodies in vitro without the need for animal immunization. One key advantage of phage display consists of the easy manipulation, intriguing flexibility, and the potential of reformatting antibodies in order to optimize their binding properties for their targets. The selection conditions can be freely chosen, and the properties of in vitro selected antibodies can be further improved by affinity maturation after the initial selection. Moreover, selections from phage-displayed antibody libraries can be performed within a few weeks, whereas generation of the hybridoma cell lines for monoclonal antibody production inevitably takes several months. These properties together with the easy integration of conjugated or engineered phages into transduction devices have boosted their use on multiple bioassays and biosensors [35].

A plethora of antibody libraries developed in recent years has provided rapid alternatives for the antibody generation process. Immune- or non-immune natural B-cells are the usual sources to achieve the diversity of antibody libraries. In addition, the actual knowledge regarding the antibody structure has also permitted increasing the antibody diversity using synthetic DNA variety repertoire using degenerated CDRs of antibodies on a predefined antibody framework. Thus, the immunogenicity can be minimized and the need for further steps of antibody humanization can be avoided. The use of synthetic libraries allows strict control over the library design and selection procedure facilitating the identification of antibodies with preferred binding specificities. Moreover, synthetic antibody libraries can serve as the origin for truly non-animal-derived antibodies.

Antibody fragments

The best-known antibody fragments are the antigen-binding fragment (Fab) and the even smaller single-chain fragment variable (scFv) (Fig. 1c). The Fab fragment consists of the region of an antibody that actually binds to their target antigen. Fab is composed of the C_{H1} and V_H domains of the heavy chain and the constant C_L and the variable V_L domains of the light chain. Conventionally, Fab fragments ($F(ab)_2$ and Fab′)

have been generated by proteolytic digestion of animal-derived monoclonal antibodies [25], but recombinant DNA technologies and protein engineering have made it possible to modify the antibody structure and develop various recombinant antibodies. The scFv format consists of the variable region of the heavy chain (V_H) joined to the variable region of the light chain (V_L) with a peptide linker between 10 and 25 amino acids in length. The average molecular weight of scFv is about 25 kDa; and thus, scFv can be easily produced in *E. coli.*

One advantage of using scFvs, or other recombinant antibody fragments, for biosensor development is that they can be engineered in different ways, for example, to facilitate their immobilization on the transducer surface. Several approaches have been described to this aim [36]: (1) introduction of a peptide linker containing histidines or cysteines for their immobilization on metallic surfaces, or adding positively charged amino acids, such as arginine, for derivatization of transducer surfaces negatively charged upon modification with self-assembled monolayers (SAMs); (2) introduction of a His-tag at the N- or C-terminus of the protein for immobilization on surfaces functionalized with Ni-iminoacetic acid (Ni-IDA), Ni-nitroloacetic acid (Ni-NTA), or Co-carboxymethylaspartate (Co-CMA); (3) conjugation of biotin to the free amines of the scFv for immobilization on streptavidin-modified surfaces; (4) genetic modification of the linker region of the scFv to contain lysines for covalent coupling to the transducer surface using carbodiimide chemistry.

Antibody fragments, such as Fab and scFv, can be used to construct phage-displayed antibody libraries. These antibody fragments usually retain the binding properties of the parental antibody while enabling high-yield production in different expression systems. In this sense, although these antibody fragments have been mostly expressed at high yield in prokaryotic expression systems, depending on the required characteristics and subsequent use of the recombinant antibody fragments, they can be expressed also in yeast and mammalian and insect cells, or in plant-based systems to increase their production while reducing costs as molecular plant farming [24, 37]. The construction of Fab phage libraries requires the cloning of the light and heavy chain domains of antibodies rendering a molecular weight of about 50 kDa. Therefore, obtaining high-quality libraries with a high diversity is usually challenging. Furthermore, working with Fab phage display libraries can be challenging in comparison to smaller antibody fragments isolated from phage display libraries. Fab libraries have been extensively used in research pursuing mostly clinical applications [36, 38]. The easy display of scFv on phages in comparison to Fab and their straightforward in vitro engineering for maturation have boosted the construction of phage-displayed scFv antibody libraries for biomedical and clinical applications, and their use in biosensors [36, 39–41].

Single-domain antibodies

The overall structure of antibodies is well conserved among vertebrates. Nonetheless, some deviations from the classical structure have been described consisting of only the heavy chain. Initially, heavy chain antibodies were described in the context of a pathological disorder, known as the heavy chain disease [42]. These truncated antibodies lacked various parts of the antibody, including the V_L and part of the V_H domain, making them incapable of target recognition. Later on, however, completely functional heavy chain antibodies which devoid of light chains were found in the sera of camel and other species in the *Camelidae* family (Fig. 1b) [43] and certain cartilaginous fishes [44], such as sharks [45]. These unique classes of heavy chain antibodies (HCAbs) are capable of target recognition solely by one single domain, i.e., the variable heavy domain abbreviated as V_{HH}. This domain, also referred to as nanobody or single-domain antibody, represents the smallest available antibody fragment with functional antigen binding (Fig. 1c). Single-domain antibodies are characterized by their small molecular weight (12–15 kDa), high penetration in tissues, and high solubility and stability in harsh environments, such as high temperatures or denaturing conditions, as well as easy production in bacteria. Specifically, their high stability in thermally and chemically stringent conditions together with their special characteristics to form unusual paratopes and recognize native epitopes (mostly conformational epitopes, small cavities, and active sites of enzymes), which are rare for classical antibodies, has increased their use for development of new immunobiotechnological methods [46–48]. Also domain antibodies consisting of a single-variable domain have been displayed on phages [49, 50], and in fact, highly functional phage-displayed antibody libraries can be constructed using any of these smaller antibody fragments [51, 52].

In addition to the advantage of small size, antibody fragments are simple to produce by various expression systems, such as *E. coli* where production of antibody fragments is simple and economic. However, the use of single-domain antibodies has shown some limitations for the analysis of low molecular weight analytes, such as mycotoxins, pesticides, or antibiotics, which have been attributed to the lack of a light chain that is present in conventional and other recombinant antibodies. More efforts are needed to investigate the structure of the nanobody and the recognition mechanism of small haptens using, for example, nuclear magnetic resonance (NMR) and crystallization techniques. The knowledge of such interaction patterns would allow a more effective design of nanobodies applying antibody maturation techniques, as well as of the immunogen applied for antibody generation [53].

Other synthetic antibody mimics

The unique properties of antibodies have provided inspiration for active research in antibody engineering trying to downsize the antibody molecule, even further than single-domain antibodies or nanobodies. In this sense, synthetic antibody mimics generated entirely in vitro, and thus completely eliminating animals from the production process, have been the focus of many research groups in recent years. Such antibody mimics include cyclic peptides joint to a scaffold mimicking the antibody paratope (peptidomimetics), nucleic acid aptamers, molecularly imprinted polymers, non-immunoglobulin protein scaffolds, or even in silico designed recognition elements that have been applied to the development of biosensors showing good selectivity and sensitivity for the analysis of different targets in various fields [54–59]. As a consequence of their in vitro production, the antigen recognition site can be theoretically engineered to recognize any desired target.

In this regard, the use of aptamers for food analysis has been extensively explored in the literature [59]. Some of the advantages of aptamers over antibodies include high stability under a wide pH range and temperature, long shelf-life and ease of transportation, ease of chemical modification, production reproducibility, simple regeneration after denaturalization, and access to protein epitopes of limited access by antibodies. However, the affinity of aptamers for certain type of molecules, especially those containing hydrophobic and negatively charged parts, is not always high and cross-reactivity issues are a limitation in some cases.

Application of recombinant antibodies in food analysis

Mycotoxins

Mycotoxins are toxic compounds produced naturally by some genera of fungi, which can grow in the field or during harvesting, drying, or improper storage of certain crops, including cereals, fruits, seeds, or nuts [60]. They can cause acute or chronic diseases and disorders, known as mycotoxicosis, in humans and animals, as well as significant economic losses. Nowadays, around 300−500 different mycotoxins have been characterized, although, according to current research, the real number of these compounds can be significantly higher [61]. Several national and international authorities, including the EU and the US Department of Agriculture, have set regulatory and legislative limits for the main mycotoxins in different food and feed commodities [62]. However, the fulfilment of these limits is problematic in developing countries where contamination is endemic, and the population is exposed to mycotoxin concentrations in food that would not be acceptable in developed countries [61]. In this context, there is a great interest of

developing simple, fast, and robust analytical methods that can be applied for multi-mycotoxin and/or in-field analysis at low cost. In this regard, antibody-based techniques are especially suitable for point-of-use analysis, and although pAbs and mAbs continue to be the most commonly used antibodies [59, 63], the search of new and sensitive rAbs for mycotoxin analysis has experienced a great focus of attention in the past decade [64].

Table 2 collects the characteristics of selected rAbs developed for the analysis of mycotoxins in food since 2015. Most of them describe the application of novel rAbs to the development of ELISA-based methods or homogeneous assays [27, 53, 67, 75] with good sensitivities and selectivities in comparison to the application of animal-derived antibodies [53, 89, 90]. In fact, rAbs have shown to be especially suited for mycotoxin analysis because the small size and toxicity of these molecules sometimes make it difficult to generate natural antibodies against them [19]. Lateral flow assays [68, 70] and immunosensors [87] with rAbs have been described as well; however, applications in these areas are still scarce. In fact, test kits using rAbs have not been commercialized so far for food analysis [19]. Therefore, more efforts should be focused on the generation of new immunodetection formats to broaden the applicability of rAbs for mycotoxin detection.

As shown in Table 2, different anti-idiotypic (anti-ID) antibodies have been developed for their use as antigen surrogates, in replacement of mycotoxin-carrier proteins or toxin-conjugated enzymes in immunoassays [71, 78–80, 83]. Anti-ID antibodies, also known as Ab2, bind to the CDR region (idiotype) of the primary anti-target Ab (named as Ab1) [19, 91]. Such antibodies thus mimic the epitope of the target antigen and can replace the hapten-protein conjugates conventionally used in competitive immunoassays and improve the assay performance. Phage-displayed antibody libraries have been used to select various anti-ID antibodies for the detection of contaminants in food [73, 85–87]. For mycotoxin detection, several anti-ID-V_{HH} antibodies have been reported. Some of the advantages of anti-ID-V_{HH} for these applications include [68] (a) easy engineering due to their small size; (b) higher solubility and chemical stability than animal-derived antibodies, which helps minimize the matrix effects; (c) interactions mainly based on main-chain rather than on side-chain contacts; and (d) easy large-scale production. In comparison with traditional ELISAs, the sensitivity of these assays is usually significantly better [71, 78, 80].

For example, the use of a phage-displayed anti-ID-V_{HH} isolated from a naïve alpaca V_{HH} library, with great affinity to an anti-zearalenone (ZON) mAb, provided a 26-fold improvement in the sensitivity in comparison with a competitive ELISA using bovine serum albumin (BSA)-conjugated ZON [83]. In order to decrease the detection limit, the phage particles bound to the 96-well microtiter plates were eluted after

the assay and applied as templates for immunopolymerase chain reaction (IPCR). In comparison with the V_{HH} phage-based ELISA, the IPCR approach allowed a 12-fold improvement in the limit of detection (6.5 pg/mL vs 0.08 ng/mL) and a wider linear range; however, the reproducibility of the assay decreased (RSD: 5.5–28.1%). High sensitivities (9-fold improvement) were also achieved for the analysis of ochratoxin A (OTA) using phage-displayed anti-ID-V_{HH} and IPCR (LOD 4.17 pg/mL) [73] in comparison with conventional phage-based ELISA, improving the performance of other rAbs for OTA detection [75–77]. This amplification technique has also been applied in combination with phage-displayed V_{HH} for the detection of different mycotoxins [53].

In another example, Tang et al. [68] reported the application of a time-resolved fluorescence immunochromatographic assay (TRFICA) using Eu/Tb(III) nanospheres, labelling either an anti-toxin mAb (mAb-TRFICA) or the anti-ID-V_{HH} (anti-ID-V_{HH}-TRFICA), for the simultaneous analysis of ZON and aflatoxin B_1 (AFB_1). The anti-ID-V_{HH}-TRFICA showed an 18.3 and a 20.3 higher sensitivity, for AFB_1 and ZON, respectively, than the mAb-TRFICA approach. This behavior was attributed to the longer interaction between the mycotoxin and the corresponding mAb before competition with the anti-ID-V_{HH} immobilized on test line of the immunochromatographic strip. In the mAb-TRFICA approach, competition between the labelled antigen and the free toxins for the binding sites in the mAb took place on the test line resulting in lower sensitivities. The method was successfully applied to the analysis of both toxins in naturally contaminated maize samples and validated by LC-MS/MS.

Further improvements in the sensitivity of competitive assays for the detection of mycotoxins have been proposed using site-directed mutagenesis of antigen-mimicking nanobodies. Deoxynivalenol (DON) has been detected using phage-displayed anti-ID-V_{HH} mutants [79] obtained upon site saturated mutagenesis of five amino acids identified at the binding site of a DON-mimicking V_{HH} selected by phage display [78]. Competitive ELISAs using the mutants were up to 3.2 times more sensitive than those using the wild type. Nevertheless, it was necessary to apply a large-scale screening process to obtain high-affinity mutants; thus, an alternative strategy to improve the sensitivity of the competitive immunoassays would be in search of lower-affinity nanobodies. Moreover, the selection of the positions for mutagenesis will significantly affect the results and sometimes the mutation of the amino acids close to the key ones allows very effective modifications.

Several studies have shown that the use of alkaline phosphatase (AP) fusion proteins (scFv-AP, V_{HH}-AP) in replacement of free scFv or V_{HH} fragments usually results in competitive ELISAs with better detection limits, shorter

Table 2 Summary of recombinant antibodies applied to the detection of mycotoxins in food

Analyte	Antibody format	Antibody source library	Assay format	Ab affinity (K_D) (M)	Analytical characteristics	Detection	Sample	Ref
AFB$_1$	scFv	Synthetic (yeast display)	icELISA: immobilized AFB$_1$-BSA + soluble scFv	82.7×10^{-8}	*n.d.*	SPR	–	[65]
AFB$_1$	scFv	Naïve (mouse)	icELISA: immobilized AFB$_1$-BSA + scFv-AP, phage scFv	*n.d.*	*n.d.*	Abs	Corn, groundnut, and certified reference materials	[66]
AFB$_1$	scFv	V$_H$-shuffled scFv library	icELISA: immobilized AFB$_1$-BSA + soluble scFv, protein L-HRP/His probe-HRP	$2.29 \times 10^{-9} - 5.22 \times 10^{-8}$	•IC$_{50}$: 0.02–0.06 μg/mL	Abs	–	[67]
AFB$_1$	AI-V$_{HH}$	Immunized (alpaca)	Immobilized AI-V$_{HH}$ + anti-AFB$_1$ mAb labelled with Eu/Tb NPs	*n.d.*	•IC$_{50}$: 0.53 ng/mL •LOD: 0.05 ng/mL •DR: 0.13–4.54 ng/mL	TR-FIC	Corn	[68]
FB$_1$	scFv	Derived from anti-FB$_1$ mAbs	Immobilized scFv + FB$_1$-biotin	1.20×10^{-9}	•IC$_{50}$: 79.40 ng/mL (0.11 μmol/L)	Abs	Maize, rice	[27]
FB$_1$	scFv	Immunized (mouse)	icELISA: immobilized FB$_1$-BSA + soluble scFv, anti-His Ab + anti-mouse IgG-AP	1.89×10^{-7}	*n.d.*	SPR	–	[69]
FB$_1$	scFv-Au	Derived from anti-FB$_1$ mAbs	Immobilized FB$_1$-BSA + scFv-Ag	*n.d.*	•LOD: 2.5 ng/mL	Visual	Maize	[70]
FB$_1$	Ab2β V$_{HH}$	Naïve (alpaca)	Immobilized Ab2β V$_{HH}$ + anti-FB$_1$ mAb, anti-mouse-HRP Ab	164.6×10^{-9}	•IC$_{50}$: 0.95 ng/mL •DR: 0.27–5.92 ng/mL •LOD: 0.15 ng/mL	Abs	Corn, rice, and feedstuff	[71]
FB$_1$	AI-V$_{HH}$-phage Ab2α, AI-V$_{HH}$ Ab2β, anti-FB$_1$ mAb	Naïve (llama)	Immobilized anti-FB$_1$ mAb + V$_{HH}$-phage Ab2α + V$_{HH}$ Ab2β, anti M13 Mab-HRP	*n.d.*	•IC$_{50}$: 0.19 ng/mL •IC$_{50}$: 0.68 ± 0.03 ng/mL	Abs	Corn, wheat, rice	[72]
OTA	AI-V$_{HH}$-phage	Naïve (alpaca)	Immobilized anti-OTA mAb + AI-V$_{HH}$-phage, anti-M13 mAb-HRP	*n.d.*	•LOD: 0.004 ng/mL •DR: 0.01–10 ng/mL	Fluor	Rice, corn, wheat, feedstuff	[73]
OTA	Fab	Multi-immunized (mouse)	icELISA: immobilized OTA-HSA + soluble Fab, anti-Ig-AP	34×10^{-9}	•IC$_{50}$ < 40.38 ng/mL (100 nmol/L)	Abs	*n.d.*	[74]
OTA	V$_{HH}$-AP	Immunized (alpaca)	icELISA: immobilized OTA-OVA + soluble V$_{HH}$-AP	*n.d.*	•IC$_{50}$: 0.13 ng/mL •DR: 0.06–0.43 ng/mL; •LOD: 0.04 ng/mL (Fluor)	Abs Fluor	Rice, oat, barley	[75]
OTA	V$_{HH}$	Immunized (alpaca)	icELISA: immobilized OTA-OVA + V$_{HH}$, anti-His Ab-HRP	$0.039–7.327 \times 10^{-9}$ (SPR)	•IC$_{50}$: 0.64 ng/mL •DR: 0.27–1.47 ng/mL •LOD: 0.16 ng/mL	Abs	Rice, oats, barley	[76]
OTA	V$_{HH}$	Immunized (alpaca)	icELISA Immobilized OTA-BSA + V$_H$H, anti-His Ab-HRP	*n.d.*	•LOD: 0.625 ng/mL	Visual, densitometry	Rice, oats, barley	[77]
DON	AI-V$_{HH}$	Naïve	Immobilized V$_{HH}$, anti-DON mAb + DON	*n.d.*	•IC$_{50}$: 8.77 ng/mL •LOD: 1.16 ng/mL •DR: 2.18–62.25 ng/mL	Abs	Corn, feed, and wheat	[78]
DON	V$_{HH}$	Site-directed mutagenesis AI-V$_{HH}$ clone [78]	Immobilized anti-DON Mab + phage-V$_{HH}$, anti M13 Mab-HRP	*n.d.*	•IC$_{50}$: 24.49 ng/mL •DR: 9.51–180.15 ng/mL	Abs	Corn, wheat, feedstuff	[79]
CIT	AI-V$_{HH}$	Naïve (alpaca)	Immobilized anti-CIT mAb, phage V$_{HH}$ + CIT, anti M13 mAb-HRP	*n.d.*	•IC$_{50}$: 10.9 μg/kg	Abs	Rice, corn, feed, and wheat	[80]
CIT	scFv				•DR: 2.5–100 μg/kg	Abs	–	[81]

Table 2 (continued)

Analyte	Antibody format	Antibody source library	Assay format	Ab affinity (K_D) (M)	Analytical characteristics	Detection	Sample	Ref
		Ribosome display library	Immobilized CIT-BSA, scFv + CIT, anti-His tag Ab and anti-IgG-HRP	7.644×10^4 M^{-1}, 2.013×10^5 M^{-1}, 9.049×10^4 M^{-1}	•IC$_{50}$: 3.271, 1.242, and 2.763 µg/mL •LOD: 0.011, 0.003, and 0.006 µg/mL			
CIT	AI-V$_{HH}$ Ab2β	Naïve (alpaca)	Immobilized AI-V$_{HH}$ + anti-CIT mAb + CIT, anti-mAb-HRP	160×10^{-9}	•IC$_{50}$: 44.6 ng/mL •DR: 5.0 – 300.0 ng/mL	Abs	Wheat, rice	[82]
ZON	Phage-AI-V$_{HH}$	Naïve (alpaca)	Immobilized anti-ZON mAb, phage-AI-V$_{HH}$ + ZON, anti-phage-HRP	n.d.	•IC$_{50}$: 0.25 ± 0.02 ng/mL •DR: 0.11–0.55 ng/mL •LOD: 0.08 ng/mL •LOD: 0.0065 ng/mL and LR: 0.01–100 ng/mL (with PCR)	Abs, Fluor	Corn, wheat, and rice	[83]
ZON	scFv scFv-AP	Non-immunized (human) and immunized (rabbit)	iCELISAs: immobilized ZON-OVA + scFv with His-probe-HRP or scFv-AP	n.d.	•IC$_{50}$: 90 ng/mL (scFv) and 14 ng/mL (scFv-AP) •LOD: 20 ng/mL (scFv) and 2 ng/mL (scFv-AP)	Abs	Corn, wheat	[84]
ZON	AI-V$_{HH}$	Immunized (alpaca)	Immobilized AI-V$_{HH}$ + anti-ZON mAb labelled with Eu/Tb NPs	n.d.	•IC$_{50}$: 0.52 ng/mL •LOD: 0.07 ng/mL •DR: 0.20–2.77 ng/mL	TR-FIC	Corn	[68]
HT2	Anti HT2-Fab-Eu and anti-HT-2-IC scFv-AF	Fab: immunized (mouse) scFv: naïve (human)	Soluble anti HT2-Fab-Eu + anti-HT2-IC scFv-AF	n.d.	•IC$_{50}$: 134 ng/mL (SPR, competitive assay) vIC$_{50}$: 9.6 ng/mL (TR-FRET) •LOD: 19 µg/kg; LR: 25–400 µg/kg in wheat (TR-FRET)	Fluor SPR	Wheat	[85]
HT2	Anti HT2-Fab and anti-HT-2-IC scFv-AP	Fab: immunized (mouse) scFv: naïve (human)	Immobilized anti-HT2 Fab + anti-HT2-IC scFv-AP	n.d.	•IC$_{50}$: 5 ng/mL (non-competitive assay) •LOD: 13 µg/kg wheat; 4 µg/kg barley and 16 µg/kg oat	Abs	Wheat, barley, and oats	[86]
HT2	Anti HT2-Fab and anti-HT-2-IC scFv-AP	Fab: immunized (mouse) scFv: naïve (human)	Anti-HT2 Fab immobilized on the microelectrode surface + anti-HT2-IC scFv-AP	n.d.	•LOD: 1.6 ng/mL	n.d.	–	[87]
TeA	V$_{HH}$-NLuc	Immunized (camel)	BLEIA: Immobilized TeA-CMO-BSA + V$_{HH}$-NLuc, CTZ/ CLEIA: Immobilized TeA-CMO-BSA + V$_{HH}$-NLuc, HRP-anti-HA-Tag	n.d.	•IC$_{50}$: 9.3 ng/mL; LOD: 1.1 ng/mL (BLEIA) •IC$_{50}$: 8.6 ng/mL; LOD: 0.3 ng/mL (CLEIA)	BL	Rice, flour, apple juice	[88]

Abbreviations: *Ab*, antibody; *Abs*, absorbance; *AFB$_1$*, aflatoxin B$_1$; *AF*, Alexa Fluor 647; *AI-V$_{HH}$*, anti-idiotypic V$_{HH}$; *AP*, alkaline phosphatase; *BSA*, bovine serum albumin; *BL*, bioluminescence; *BLEIA*, bioluminescent enzyme immunoassay; *CLEIA*, chemiluminescent enzyme immunoassay; *CIT*, citrinin; *CTZ*, coelenterazine substrate; *DON*, deoxynivalenol; *DPV*, differential pulse voltammetry; *DR*, dynamic range; *Fab*, antigen binding fragment; *Fluor*, fluorescence; *FB$_1$*, fumonisin B$_1$; *HSA*, human serum albumin; *HRP*, horseradish peroxidase; *IC*, immunocomplex; *iceELISA*, indirect competitive ELISA; *LOD*, limit of detection; *mAb*, monoclonal antibody; *NLuc*, nanoluciferase; *n.d.*, not determined; *OVA*, ovalbumin; *OTA*, ochratoxin A; *IC$_{50}$*, 50% maximal signal; *scFv*, single-chain variable fragment; *SPR*, surface plasmon resonance; *TeA*, tenuazonic acid; *TeA-CMO*, tenuazonic acid coupled with carboxymethoxylamine hemihydrochloride; *TR-FRET*, time-resolved fluorescence resonance energy transfer; *TR-FIC*, time-resolved fluorescent immunochromatography; *V$_{HH}$*, variable region of the heavy (H) chain of a camelid antibody; *ZON*, zearalenone

analysis times, and reduced variability, due to the elimination of one assay step when compared with an indirect ELISA [75, 84]. This effect is attributed to an avidity effect since AP exists as a dimer in solution; therefore, the antibody fragments would also bind effectively as dimers [40].

Furthermore, the use of recombinant antibody techniques has enabled the development of antibodies that can be applied in non-competitive assays for the detection of the target compound. Usually, non-competitive immunoassays can provide better analytical characteristic in terms of sensitivity, dynamic range, and selectivity than competitive assays. This approach has been applied, for example, to the detection of fumonisin B_1 (FB_1) using two types of anti-ID nanobodies isolated from a naïve phage display library [72]. One of them, Ab2α, recognized an epitope far from the binding site of the toxin and the other, Ab2β, competed with FB_1 for the antibody binding. In the presence of the target, the phage-displayed Ab2α does not bind to Ab2β–Ab1 complex due to steric hindrance. The detection was carried out using an HRP-conjugated anti-phage antibody. This approach has allowed an over 17-fold improvement in the LOD in comparison with a competitive ELISA (0.19 ng/mL vs 3.41 ng/mL) with a good selectivity in the presence of other toxins different from fumonisins and other primary antibodies.

Another alternative for developing non-competitive immunoassay for low molecular weight targets is the use of anti-metatype antibodies, i.e., anti-immune complex (anti-IC) antibodies that bind to the primary antibody only when it is bound to the antigen. Arola et al. [85–87] demonstrated the applicability of an anti-IC-scFv against HT-2 toxin and a recombinant anti-HT-2 Fab, isolated from naïve scFv and immunized phage libraries, respectively, for the analysis of the toxin in cereal samples. One advantage of this approach was that although the primary anti-HT-2 Fab cross-reacted with T-2, a toxin from the same family, the anti-IC-scFv allowed the development of selective non-competitive assays for HT-2 toxin. As shown in Fig. 2a, the antibody fragments were applied in homogeneous time-resolved Förster resonance energy transfer (TR-FRET) assays by labelling the primary antibody, anti-HT-2 Fab, with europium as the donor and the anti-IC-Fab with Alexa Fluor 647 as the acceptor. Alternatively [86], the detection was based on a heterogeneous ELISA using a biotinylated anti-HT-2 Fab immobilized on streptavidin-coated microwells and a scFv-AP fusion specific for the IC (Fig. 2b). The LOD of both assays was similar (0.38 and 0.3 ng/mL for the FRET and enzyme-based assays, respectively), although the first approach had the advantage that the hands-on time for the user was minimized as it did not require immobilization, washing, and separation steps. The FRET-based assay was 13 times, while the AP-based was 10.8 times more sensitive than a competitive ELISA demonstrating the usefulness of anti-IC Abs for this application.

Foodborne pathogens and other biotoxins

The main pathogens that contaminate foodstuff include bacteria, viruses, parasites, fungi, and prions. They are responsible of adverse health effects ranging from mild (e.g., diarrhea or vomiting) to severe (e.g., septicemia, liver or kidney damage, hemorrhagic colitis), or even life-threatening. Some of these microorganisms, such as bacteria and fungi, can release toxic compounds into the food that are resistant to inactivation during food processing, leading also to food poisoning [92]. The applications of rAbs for mycotoxins analyses have been reviewed in the previous section, and in this section, we will focus on the detection of pathogens and other toxic targets. The most common bacteria responsible for foodborne illnesses include *Salmonella* spp., *Campylobacter spp.*, *Listeria monocytogenes*, *Staphylococcus aureus*, and the diarrheic *E. coli* pathotypes, known as shiga-toxin producing *E. coli*. Parasites such as *Cryptosporidium* spp. and *Cyclospora* spp. and viruses (e.g., hepatitis A and norovirus) are also responsible for foodborne diseases [19, 93]. Contamination by microorganisms may occur during crop growth, harvesting, inappropriate storage, transport, post-processing adulteration or distribution, and their growth and survival are affected by climatic factors, with an increment in the replication cycles with increasing temperatures.

According to the World Health Organization [93], the most commonly reported zoonosis since 2005 in the EU is campylobacteriosis followed by salmonellosis. In fact, the past two decades have witnessed an increment of bacterial infections due to the increased consumption of fresh products, following the recommendations for a healthy diet, in Europe and North America [94]. The most widely applied techniques for microbial detection still involve culture and microscopy [95, 96], but they are time-consuming and require skilled personal and substantial efforts. Alternative approaches, such as PCR, immunological or proteomic-based methods, and biosensors, have also been reported in an effort to improve pathogen detection and reduce the analysis time. The use of bacteriophages as recognition elements for bacterial detection has received an important attention in the last decade [35, 97], but not many rAbs have been reported for this application. However, as described below, there are many examples of their use in the implementation of assays for the analysis of bacterial, aquatic, and marine toxins. Table 3 shows the characteristics of selected rAbs developed for the analysis of these contaminants in food since 2015.

As described in the literature, immunodetection of *S. aureus* has been hampered by the unspecific and undesirable interaction between staphylococcal protein A (SpA), an immunoglobulin-binding protein of the surface of the bacteria, and the Fc region of most mammalians immunoglobulins that results in false positive signals [99, 103]. The use of nanobodies has been proposed as an alternative to avoid such
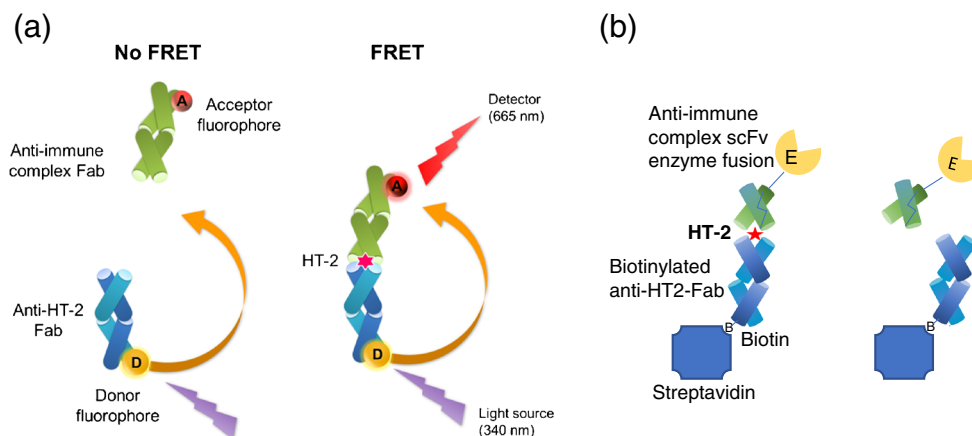
Fig. 2 Principle of the non-competitive immune complex ELISAs for the detection of HT-2. **a** Homogeneous immunoassay based on FRET between the anti-HT-2 Fab labelled with the donor fluorophore and the anti-immune complex Fab labelled with the acceptor in the presence of the toxin. Reprinted with permission from reference [60]. **b** Heterogeneous ELISA where the anti-immune complex single-chain variable fragment alkaline phosphatase fusion (scFv-AP) binds to the immobilized anti-HT-2 Fab in the presence of HT-2 toxin. Adapted from reference [86]

interaction with the advantages of high sensitivity, stability in extreme conditions, and easy genetic manipulation, for example, for further tagging. Hu et al. [99] reported the optimization of a sandwich ELISA for the analysis of *S. aureus* in milk using His-tagged V_{HH} and biotinylated V_{HH} as capture and detection antibodies, respectively. The antibodies were selected from a library of nanobodies generated by immunizing an alpaca with the inactivated bacteria. The assay showed a high selectivity to *S. aureus* and an LOD of 1×10^4 CFU/mL in milk that was improved to 6 CFU/mL after 10 h of incubation. The application of a pre-enrichment step has also allowed the detection of *Salmonella enteritidis* in milk at the same detection limit using a sandwich assay with a polyclonal anti-*Salmonella* antibody as the capture agent and the selected V_{HH} fragments as detection antibodies [101]. The detection limit of these assays could be improved using fluorescent or chemi-/bioluminescent detection, and they could be easily applied for biosensor development.

A combination of three phage display techniques has been reported for the discovery and characterization of novel anti-*Listeria* recombinant antibodies against a selective bacterial biomarker, pyruvate dehydrogenase complex–enzyme 2 (PDCE2) [113]. Two naïve human antibody libraries were used for the isolation of scFv fragments against *Listeria* protein fractions and transformed into the scFv-Fc format (with mouse IgG2a-type Fc). The interaction between the antibodies and *Listeria* spp. cells was evaluated by ELISA. ORFeome phage display, a technique that allows functional screening of proteomes and metagenomes for identifying immunogenic proteins, and immunomagnetic separation–mass spectrometry were applied, with similar results, for target identification confirming the selection of PDCE2 as a new target for bacterial detection. Finally, single-gene display allowed epitope characterization. This combined approach provides an alternative tool for the identification of novel biomarkers together with the production of selective antibodies for bacterial detection that can be of interest for commercial applications.

Staphylococcal enterotoxin B (SEB) is the most potent toxin produced by *S. aureus* that shows high stability and high toxicity even at very low concentrations. Several analytical techniques have been implemented for the analysis of SEB in food, including commercial ELISA kits which are commonly applied in routine analysis [103]. However, as discussed previously, the presence of SpA in the sample may result in false positives that will have a negative impact on the analysis of SEB. The use of nanobodies specific to SEB has allowed overcoming this limitation using a sandwich assay with anti-SEB nanobody as capture antibody and a phage-displayed anti-SEB nanobody as the detection element. The assay provided an LOD approximately one-half lower than the conventional mAb-based ELISA (0.3 vs 0.69 ng/mL) and a much wider dynamic range (1−512 ng/mL vs 2−64 ng/mL), due to the signal amplification resulting from the use of HRP-labelled anti-phage antibody for detection. The assay showed a low cross-reactivity to staphylococcal enterotoxin C (SEC) but none to staphylococcal enterotoxin A (SEA) or to SpA, avoiding the false positive problems described previously. The substitution of the capture nanobody by a mAb and of the detection phage-nanobody by an anti-SEB nanobody-AP fusion and chemiluminescent detection did not improve either the LOD (1.44 ng/mL) or the dynamic range of the assay (3.12–50 ng/mL), although the cross-reactivity in the presence of SEA was also negligible [104]. The production of single-domain antibody fragments has also been reported for the analysis of the biomarker OmpU a major protein of the membrane of *V. parahaemolyticus*, a pathogenic bacterium that can be present in raw and undercooked seafood, although the assays were not applied to food analysis [114].

Table 3 Summary of recombinant antibodies applied to the detection of foodborne pathogens and toxins in food

Target	Antibody format	Antibody source library	Assay format	Ab affinity (K_D) (M)	Analytical characteristics	Detection	Sample	Ref
S. aureus	scFv	Immunized (chicken)	icELISA: immobilized S. aureus + scFv-His, anti-His IgG + HRP-anti-IgG	n.d.	n.d.	n.d.	–	[98]
S. aureus	V_{HH}	Immunized (alpaca)	Anti-Staphylococcus V_{HH}-His (immobilized) and V_{HH}-biotin, HRP-SA	n.d.	•LOD: 1.4×10^5 CFU/mL in milk (10 CFU/mL after 8-h enrichment step)	Abs	Milk	[99]
L. monocytogenes	V_{HH}	Naïve (alpaca)	Anti-Listeria mAb and V_{HH}-HRP-anti-His	n.d.	•LOD: 10^4 CFU/mL	Abs	Milk	[100]
S. enteritidis	V_{HH}	Immunized (camelid)	Anti-Salmonella pAb (immobilized) and V_{HH}, HRP-anti-HA tag	n.d.	•LOD: 1.4×10^5 CFU/mL; 6 CFU/mL after 8-h enrichment step in milk	Abs	Milk	[101]
A. flavus; A. parasiticus	V_{HH}	Immunized (alpaca)	Anti-Aspergillus V_{HH} (immobilized) and pAb, HRP-anti-rabbit	n.d.	•LOD: 1 µg/mL (buffer) •LOD: 2 µg/mg (sample)	Abs	Peanut, corn	[102]
SEB	V_{HH}	Immunized (camelid)	Anti-SEB V_{HH} (immobilized) and phage-V_{HH}, anti-M13-HRP	7.95×10^{-9}, 26×10^{-9}, 4.58×10^{-9}	•LOD: 0.3 ng/mL •DR: 1–512 ng/mL	Abs	Milk, cheese, milk powder, beef	[103]
SEB	V_{HH}-AP	Naïve	Anti-SEB mAb (immobilized) and V_{HH}-AP, AP substrate	n.d.	•LOD: 1.44 ng/mL •DR: 3.12–50 ng/mL •IC_{50}: 8.59 ± 0.37 ng/mL	CL	Milk, drinking water	[104]
Cry1C	Two V_{HH}s	Immunized (camelid)	V_{HH} (1) immobilized on a GCE + V_{HH} (2)@GO-Th	n.d.	•LOD: 3.2 pg/mL •DR: 0.01–100 ng/mL	SWV	Corn	[105]
Cry1F	scFv	Immunized (rabbit)	icELISA: immobilized Cry1F + scFv, anti His-Tag-HRP mAb	n.d.	•LOD: 0.18 ng/mL •DR: 11.56 ng/mL	Abs	Rice, wheat, corn, and soil samples	[106]
Cry1Ab	Anti-idiotypic V_{HH}	Naïve (camelid)	Immobilized anti-Cry1Ab mAbs + phage-V_{HH} anti-idiotypic Ab, anti-phage-HRP	n.d.	•IC_{50}: 42.68 ± 2.54 ng/mL •DR: 10.49–307.1 ng/mL	CL	Rice, wheat, and corn	[107]
Cry1Ab, Cry1Ac, Cry1B, Cry1C and Cry1F	scFv	Naïve (human)	icELISA: immobilized Cry1Ac toxin + scFv anti-Cry toxin, anti His-Tag-HRP Mab	n.d.	•LOD: Cry1Ab (5.3 ng/mL), Cry1Ac (4.8 ng/mL), Cry1B (69.7 ng/mL), Cry1C (5.5 ng/mL), Cry1F (35.2 ng/mL) •DR: 0.20–2.22 µg/mL	Abs	Rice	[108]
CP4-EPSPS	V_{HH}	Immunized (camelid)	V_{HH} immobilized of a GCE coated with OMC-AgNPs + Th	n.d.	•DR: 0.001 to 100 ng/mL •LOD: 0.72 pg/mL	DPV	Soybean	[109]
MC-LR	V_{HH}	Naïve (alpaca)	icELISA: immobilized MC-LR-BSA + V_{HH} anti-MC-LR, anti His-Tag-HRP mAb	n.d.	• LOD: 0.06 ng/mL • IC_{50}: 0.87 ng/mL	Abs	Water	[110]
MC-LR	V_{HH}	Immunized (llama)	icELISA: immobilized MC-LR-BSA + biotin-V_{HH} anti-MC-LR, SA-HRP	n.d.	• IC_{50}: 0.28 ± 0.021 ng/mL • LOD: 0.05 ng/mL	Abs	Water	[111]
	scFv-AP (anti-IC)	Synthetic		n.d.	•LOD (MC-LR): ~0.026 ng/mL (1 h) •IC_{50} (MC-LR): 1.2 ng/mL (1 h)	TRF	Water	[112]

Table 3 (continued)

Target	Antibody format	Antibody source	Assay format	Ab affinity (K_D) (M)	Analytical characteristics	Detection	Sample	Ref
MC-LR, -dmlR, -RR, -dmRR, -LA, -LY, -LF, -LW, -YR, -WR, and Nod-R		library	Immobilized anti-Adda mAb + toxin + anti-IC scFv-AP, Eu(III) labelled anti-AP pAb					

Abbreviations: Abs, absorbance; Adda-group, (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(E),6(E)-dienoic acid); AP, alkaline phosphatase; Anti-IC, anti-immune complex; BSA, bovine serum albumin; CFU, colony forming units; CL, chemiluminescence; CP4-EPSPS, CP4 5-enolpyruvylshikimate-3-phosphate synthase; DA, domoic acid; DPV, differential pulse voltammetry; Fluor, fluorescence; GCE, glassy carbon electrode; HKC, heat-killed cells; His, histidine tag; iceLISA, indirect competitive ELISA; mAb, monoclonal antibody; MC-LR, microcystin-LR; OMC-AuNPs, ordered mesoporous carbon modified with gold nanoparticles; pAb, polyclonal antibody; n.d., not determined; Nod, nodularin; STX, saxitoxin; SA, streptavidin; SA-PE, streptavidin–R phycoerythrin; SEB, staphylococcal enterotoxin B; SWV, square-wave voltammetry; TRF, time-resolved fluorescence; V_{HH} (2)@GO-Th, nanobody (2) immobilized on a π–π stacked graphene oxide/thionine assembly

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Bacillus thuringiensis (Bt) is a gram-positive soil bacterium that synthesizes a variety of toxins with different chemical structures, mechanisms of action, and biological targets. The main ones include crystal (Cry and Cyt) and vegetative (secretable) insecticidal proteins (Vip) that are broadly applied in insect pest control. These toxins are regarded as safe for humans and animals; however, there is an increasing concern about the potential toxicity and health effects of Bt toxins expressed in genetically engineered plants [115]. Several reports describe the generation and application of nanobodies selective to Bt toxins [105–108]. For example, a novel electrochemical biosensor was reported for the selective analysis of Cry1C using an immunized Bactrian camel library to isolate nanobodies that bind to two different epitopes of the toxin [105]. For the preparation of the immunosensor, one of the selected nanobodies was coated on the modified surface of a glassy carbon electrode. Following incubation with the sample, a dispersion containing the second nanobody immobilized on a π–π stacked graphene oxide/thionine assembly (Nb@GO-Th) was dropped on the surface of the electrode as an electroactive probe for the sandwich immunoassay. The immunoreaction was quantified by square wave voltammetry. The LOD (3.2 pg/mL) was significantly lower than the one reported for commercial kits and ELISAs, and the sensor did not show crossreactivity with other toxins from the same group, such as Cry1F, Cry1B, and CryAa.

Furthermore, Cry1F has been analyzed in food and environmental samples with an indirect ELISA using scFv antibody fragments selected from a Cry1F-immunized rabbit phage display library [106]. The assay showed low crossreactivities (< 2.8%) for Cry1Ab, Cry1Ac, Cry1B, Cry1C, Cry1Ie, and Cry2A and an LOD of 0.18 ng/mL for the target Bt toxin. As an alternative approach, site-directed mutagenesis has been proposed to improve the generic specificity of the scFv [108]. The mutant antibody was applied to the development of rAbs with a broad specificity for the recognition of Cry1Ab, Cry1Ac, Cry1B, Cry1C, and Cry1F. This method provided worse sensitivities, but a broader selectivity, than previous assays allowing the detection of five Cry toxins in food samples.

The group of Hammock proposed a different approach for the analysis of the Cry1Ab toxin using anti-ID camel nanobodies specific for anti-Cry1Ab mAb [107]. The assay provided an LOD of 6.45 ng/mL that was higher than other reported assays based on monoclonal or polyclonal antibodies. However, the application of the nanobody-based competitive assay exhibited lower blank signals and higher specificity, requiring shorter analysis times than conventional ELISAs. Moreover, the nanobodies could be easily produced at a large scale. The authors found that the replacement of a phage-displayed nanobody by a soluble V_{HH} did not improve the sensitivity and in fact decreased the response range of the assay. This finding has been reported previously in the literature, and

the authors propose that it could be avoided using eukaryotic rather than prokaryotic expression systems for nanobody production.

The naturally occurring *Agrobacterium* spp. strain CP4 encodes the CP4, 4 5-enolpyruvylshikimate-3-phosphate synthase (CP4-EPSPS) gene that confers tolerance to glyphosate. An electrochemical sensor has been developed by immobilizing the nanobody specific for CP4-EPSPS protein on the surface of a glassy carbon electrode coated with ordered mesoporous carbon modified with gold nanoparticles (OMC-AuNPs) [109]. Thionine was added as the electrochemical probe for the analysis of the biomarker in soybean samples by differential pulse voltammetry (DPV) with a much better sensitivity (LOD 0.72 pg/mL) than previous reports or commercial kits using mAbs.

Microcystins (MCs) and nodularins (Nods) are low molecular weight peptides produced by several bloom-forming cyanobacteria that can be found in freshwaters (e.g., rivers, lakes, reservoirs) after bacterial cell lysis. They can bioaccumulate in several organisms used as food sources, e.g., bivalves, crustaceans, fish, plants, or for example in algal dietary supplements and their concentration must be monitored at very low concentrations [116]. As described previously, the application of anti-IC antibodies allows the implementation of non-competitive assays for the analysis of low molecular weight targets, such as MCs and Nods. These toxins have been analyzed by time-resolved immunofluorometry [112] using a generic anti-IC-scFv, selected by phage display and fused to alkaline phosphatase (scFv-AP) to recognize the complex between MC or Nod and a biotinylated mAb that binds to the Adda-group (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(E),6(E)-dienoic acid) which is present in both toxins. A secondary anti-AP polyclonal antibody labelled with europium was used as a tracer to detect the interaction. The assay required a single incubation and washing step before signal generation allowing the detection of 11 toxins (MC-LR, -dmlR, -RR, -dmRR, -LA, -LY, -LF, -LW, -YR, -WR, and Nod-R) with a LOD of ~0.1 μg/L within 10 min. Similar detection limits and sensitivities have been also reported using a competitive immunoassay and a MC-LR nanobody selected from an alpaca phage display library [110]. However, this assay provided lower cross-reactivities with other toxins different from MC-LR (between 82.7 and 116.9% only for MC-RR, MC-YR, and MC-WR). The group of González-Sapienza [111] has demonstrated the importance of the panning strategy for the selection of highly sensitive nanobodies with different cross-reactivities for the analysis of MCs. Highest affinities for the immobilized hapten were reported by selecting the phages expressing the V_{HH}s with the slowest k_{off} values (IC$_{50}$ 0.28 μg/L).

The sequence of the variable regions of a monoclonal antibody selective to [Arg4]-MCs (MC10E7) determined by peptide mass-assisted cloning was expressed as scFv in yeast and as chimeric full-size antibody in yeast and tobacco leaves of *Nicotiana tabacum* and *Nicotiana benthamiana* [117]. The scFv fragment provided approximately16-fold lower IC$_{50}$ than the full-sized mAb in the ELISA. However, the LOD obtained with the chimeric antibody, in an indirect competitive ELISA (12.5 ng/L), was only slightly lower than that of the MC10E7 antibody (10.9 ng/L), while the IC$_{50}$ were 50.0 ± 3 ng/L and 44.0 ± 4 ng/L for the rAb and mAb, respectively. The cross-reactivity of both antibodies was very similar (MC10E7: 58% for MCRR; plant-derived MC10E7: 62% for MC-RR; < 0.1% for MC-LW and MC-LF for both antibodies). The chimeric antibody was applied to the development of a lateral flow immunoassay for the detection of [Arg4]-MCs in the range of 100–300 ng/L in freshwater samples. In a different approach, the plant-derived antibody encapsulated in a sol-gel matrix was applied to the removal of MC-LR from freshwater samples in lab-scale experiments [118]. The authors point out that plant-based and microbial expression systems are attractive alternatives for the production of recombinant antibodies at large quantities and low cost.

Recombinant scFvs against two marine toxins, saxitoxin and domoic acid, have been produced using the sequence information of the corresponding mAbs [119]. The assays were implemented in a microsphere-based competitive immunoassay, and the detection limits were similar to those reported with other antibodies, although the method requires further implementation. First attempts to carry out multiplex detection were reported in food samples using a flow cytometer. Nevertheless, more work is required to avoid the cross-reactivity issues found for both assays that were attributed to the non-specific binding of the scFv to the microspheres.

Antibiotics

The discovery of the first antibiotic in the mid-twentieth century changed modern medicine and treatment of most bacterial infections in both humans and animals. Today, the wide spread of drug-resistant bacteria [120] is one of the most severe threats to public health. Multidrug-resistant bacteria cause 33,000 deaths per year in Europe due to hospital infections, and additional healthcare costs of about 1.5 billion euros [121]. The inappropriate and indiscriminate use of these drugs, in both humans and farm animals, is one of the main factors behind the observed increment in the number of antibiotic-resistant bacteria. It has a significant clinical, epidemiological, and microbiological impact. Moreover, it is a global problem that affects various productive sectors, such as livestock, agriculture, the environment, trade, and, therefore, the world economy. Thus, sensitive, selective, rapid, low-cost, and easy-to-use analytical methods are required to reduce the emergence and spread of antibiotic resistance worldwide. This interest is evidenced by efforts focused on the implementation of screening methods for antibiotic control

in the last 30 years, with immunoanalytical techniques showing the greatest impact on the market.

However, due to the small size of these drugs, there are a series of limitations for the production of polyclonal and monoclonal antibodies that limits the availability of selective and sensitive immunoassays towards these substances. In this regard, the use of recombinant antibodies can be an excellent alternative to improve the sensitivity and selectivity of the immunoassay-based methods available for antibiotic detection. The number of publications found in the last 5 years focusing on the analysis of antibiotics in food using immunochemical technologies is enormous. However, less than 7% of them utilize recombinant antibodies and in most cases engineered antibodies are used in ELISA-based methods. Table 4 collects the characteristics of selected rAbs developed for the analysis of antibiotics in foodstuffs since 2015.

As commented before, recombinant antibodies have several advantages over conventional antibodies [19]; however, some specific drawbacks have been pointed out for the production of antibiotic-specific rAbs. For example, Du et al. [127] reported a detailed comparative study of the detection of chloramphenicol (CAP) using a purified anti-CAP scFv-AP fusion protein and its parenteral mouse mAb. Two ELISA approaches, one based on the engineered Ab and the other on the mAb, were implemented in parallel, and the analytical characteristics were compared in buffer and real samples. The sensitivity of the mAb-based ELISA was approximately 9 times better than the scFv-AP-based ELISA while the cross-reactivity was similar in both cases. However, the CV% obtained in the analysis of real samples with the mAb was higher than those obtained with the scFv-AP, indicating that the rAb could cope better with matrix effects. On the other hand, they observed the existence of many aberrant mRNAs produced by the single clonal hybridoma cell [22], approximately 32%, that prevented the production of suitable mAb-derived single-change antibody fragments. Some alternatives have been suggested to overcome this problem, like the ribozyme cleavage of aberrant sequences [129], RNaseH digestion of aberrant mRNA-DNA hybrids [130], or mRNA sequencing. Among these methods, screening of functional scFv antibodies using transcription-translation systems, such as phage display, is nowadays the preferred strategy of choice.

Li et al. [122] reported the application of a hyperimmunized chicken phage display library to screen specific binders against gentamicin (GENT). Compared with other phage display libraries, the authors proposed that the avian model allowed a simplification of rAb generation. Chicken antibodies have only one isotype light chain (lambda); and thus, chicken-derived scFvs are easier to make [131]. After the panning rounds, two anti-GENT binders (S-1, S-5) were isolated, and after the expression and purification of the scFv antibodies, indirect competitive ELISAs (icELISA) were conducted with both scFv variants. The sensitivity obtained with the best antibody (S-1)

(IC_{50} 12.4 ng/mL and LOD 0.15 ng/mL) was suitable for GENT detection in food samples, such as milk, pork, beef, and chicken meat with recoveries ranging from 61 to 118% (RSD < 11%).

In addition to display technologies and parenteral mAb gene sequencing, some authors have described further binding improvements between rAbs and their corresponding targets through homology modelling and molecular docking [27]. As shown in Table 4, specific and internal changes to the DNA sequence of pristine scFv have been performed by either site-directed mutagenesis or codon optimization [124, 125]. For example, He et al. [124] presented an anti-amoxicillin (AMOX) scFv with improved recognition performance towards AMOX and 10 more penicillins. Directed mutagenesis of a pair of amino acid residues localized in the binding site pocket was changed to produce two scFv variants, scFv-mutant 1 (V_L CDR3 Ser95 → Glu) and scFv-mutant 2 (V_L CDR3 Ser95 → Glu and V_H CDR1 Tyr160 → Arg) which were evaluated by icELISA to compare their analytical characteristics. The results indicated that both mutants preserved the recognition towards the 11 penicillins with the IC_{50} values between 3.1 and 16.3 ng/mL. However, mutant 1 showed highly improved sensitivity of 2–6-fold in terms of the IC_{50} compared with native scFv and was used to analyze milk samples with excellent results.

Although technologies to generate recombinant antibodies against any desired target are well established, the development of immunosensors and similar platforms for the analysis of antibiotics is still limited. In this regard, Yu et al. [123] reported the development of a biosensor based on bioluminescence resonance energy transfer (BRET) as the basis for enrofloxacin (ENR) analysis using a homogeneous wash-free immunosensing approach. In this work, *Renilla* luciferase (Rluc) was genetically fused to an anti-fluoroquinolone scFv, which acted as the energy donor and quantum dot-conjugated norfloxacin (QD-NOR) which functioned as an energy acceptor. As shown in Fig. 3, when the donor and the acceptor are in close proximity with each other, there is an energy transfer from the Rluc to the QDs via BRET. In the presence of the analyte, the Förster distance increases and energy transfer decreases. ENR was detected with an LOD of 2.54 ng/L and the dynamic range covered 4 orders of magnitude (from 0.023 to 25.6 ng/mL). Finally, the BRET assay was applied to analyze milk samples, and five fluoroquinolones (ciprofloxacin, norfloxacin, danofloxacin, marbofloxacin, and flumequine) were successfully determined.

Pesticides

Pesticides are used to keep crops healthy and avoid devastation by diseases and plagues. They include herbicides, fungicides, insecticides, acaricides, among others. Pesticide residues pose a risk to public health; and thus, the EU and other countries in the

Table 4 Summary of recombinant antibodies applied to the detection of antibiotics in food

Analyte	Antibody format	Antibody source	Assay format	Analytical characteristics	Detection	Sample	Ref
GENT	scFv	Hyperimmunized library (chicken)	icELISA: immobilized Gent-OVA + soluble scFv + anti-His Ab-HRP	•IC_{50}: 12.42 ng/mL •LOD: 0.15 ng/mL •No CR with kanamycin, amikacin	Abs	Pork, beef, chicken, milk	[122]
ENR	scFv-RLuc	mRNA (V_H,V_L) isolated from hybridoma cell C49H1 producing anti-FQ mAb	Immobilized NOR onto QD + soluble scFv-RLuc	•IC_{50}: 1 ng/mL •DR: 0.023−25.6 ng/mL •LOD: 2.54 ng/L (milk after sample treatment) •CR > 60% for up to 10 FQs	Fluor BRET	Milk	[123]
AMOX	scFv	Directional mutagenesis of parental scFv	icELISA: immobilized Amox-OVA + His tagged soluble scFv + anti-His Ab-HRP	•LOD: 0.2–3.0 ng/mL •IC_{50}: 3.5–15 ng/mL •CR%: 31–132% for up to 11 PCs	Abs	Milk	[124]
SARA	scFv	Directional mutagenesis of parenteral scFv	icELISA: immobilized Sara-OVA + His tagged soluble scFv + anti-His Ab-HRP	•IC_{50}: 3.2–63 ng/mL (12 FQs) •LOD: 0.3–8.0 ng/mL (12 FQs)	Abs	Milk	[125]
AMP	scFv-AP	mRNA (V_H,V_L) isolated from hybridoma cell 9B4C7E producing anti-AMP MAb	icCLEIA Immobilized Amp-OVA + soluble scFv-AP	•IC_{50}: 0.75 ± 0.04 ng/mL •LOD: 0.110–0.004 ng/mL •CR% (analogues): < 4%	CL	Milk	[126]
CAP	scFv-AP	mRNA (V_H,V_L) isolated from hybridoma cell producing anti-CAP MAb	Immobilized CAP-OVA + soluble scFv-AP	•IC_{50} (scFv-AP): 6.92 ± 0.24 ng/mL •IC_{50} (mAb): 0.74 ± 0.03 ng/mL Less CR% (MAb) vs CR% (scFv-AP)	Abs	Shrimp + codfish	[127]
NOR	scFv	mRNA (V_H,V_L) isolated from hybridoma cell producing anti-NOR MAb	–	–	–	–	[128]

Abbreviations: *Ab*, antibody; *Abs*, absorbance; *AMOX*, amoxicillin; *AMP*, ampicillin; *AP*, alkaline phosphatase; *BRET*, bioluminescence resonance energy transfer; *BSA*, bovine serum albumin; *CAP*, chloramphenicol; *CL*, chemiluminescence; *CLEIA*, chemiluminescent enzyme immunoassay; *CR*, cross-reactivity; *DR*, dynamic range; *ENR*, enrofloxacin; *Fab*, antigen-binding fragment; *FQ*, fluoroquinolone; *GENT*, gentamicin; *HRP*, horseradish peroxidase; *icELISA*, indirect competitive ELISA; *LOD*, limit of detection; *mAb*, monoclonal antibody; *NOR*, norfloxacin; *n.d.*, not determined; *OVA*, ovalbumin; *PCs*, penicillins; *QD*, quantum dot; *RLuc*, *Renilla* luciferase; *Sara*, sarafloxacin; *scFv*, single-chain variable fragment; V_H,V_L, heavy and light variable regions of Ab

world have established a legislative framework to control these chemicals in foodstuffs [132], and sensitive analytical methods are needed to control their presence in both raw materials and processed foods. The number of papers dealing with the analysis of pesticides in food is extensive, and immunochemical techniques have been regularly applied to their control during decades [133–135].

As is the case of mycotoxins and antibiotics, pesticides are also haptens with low molecular weights; and thus, the use of the recombinant antibody technology would also be beneficial for the development of advanced immunoassay methodologies. Selected examples of the application of recombinant antibodies in this area are discussed below, and Table 5 collects the characteristics of selected rAb-based immunoassays reported for the analysis of pesticides in food since 2015.

Carbamates and organophosphorus (OP) pesticides are most widely applied in agro-industrial processes, and many analytical methods have been reported for their analysis. Immunoassay applications for the analysis of these pesticides in food are based, in many cases, on the application of

Fig. 3 Schematic of QD-BRET based on scFv-Rluc and QDs for the detection of several FQs. **A** QD-NOR binds to scFv-Rluc in the absence of the antibiotic and the energy released from the substrate is transferred to the QDs (620 nm) via BRET. **B** In the presence of the target antibiotic, scFv-Rluc binds to the FQ increasing the distance between the Rluc and QDs; thus, no energy transfer is observed. Reprinted (adapted) with permission from [123]. Copyright (2016) American Chemical Society
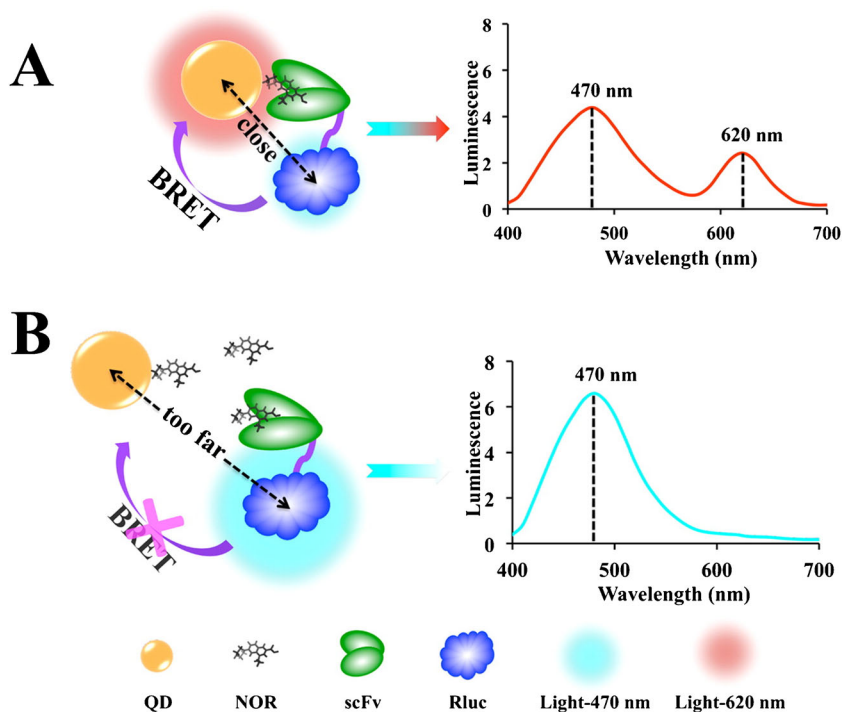
engineered antibodies generated in vitro using phage display libraries. As shown in Table 5, these libraries generally consist of antibodies from a variety of species (chicken, mice, camel-id, and alpaca) from different origins (naïve or immunized) and in different formats, in terms of fragment antibody type (V_H, V_{HH}, scFv, Fab). As discussed previously, it is usually challenging to isolate a highly sensitive target-specific rAb from a naïve phage display library [106]; therefore, some authors have reported the construction of personalized immunized antibody libraries that allow the isolation of antibodies with high affinity and selectivity for the target pesticide [137, 139, 142].

Several immunized chicken and alpaca phage display antibody libraries have been prepared for the detection of carbaryl in foodstuff. For example, two scFv mutants (X1 and X2) against the insecticide were generated from a chicken immunized with carbaryl-related hapten conjugated to keyhole limpet hemocyanin (KLH) [137]. The scFv antibodies were genetically fused to AP to develop a one-step ELISA method. The binding affinity of scFv-AP to carbaryl was evaluated by icELISA and compared with the native scFv. The assays showed 2 or 3 times better sensitivities than the classical ELISA based on the hapten-BSA conjugates and colorimetric detection. The authors suggested that the improvement in sensitivity could be related to the prevention of aggregates and dimerizations that usually happens during the expression of scFv [145, 146]. In fact, the scFv-AP (X2) fragment showed a better sensitivity to carbaryl than parental scFv featuring an LOD of 1.6 ng/mL (IC$_{50}$ 15 ng/mL). No cross-reactivity was observed with other pesticides, and the

assay was successfully applied to the analysis of fruit and soil samples with recoveries in the range of 90–114%, with CVs of 6–11%. The same authors [140] applied also V_{HH} antibodies isolated from an immunized alpaca library to the detection of carbaryl in cereal samples. The assay was linear in the range of 0.8–38 ng/mL with an IC$_{50}$ of 5.4 ng/mL and a LOD of 0.3 ng/mL. Thus, the sensitivity was better than with the scFv-AP approach, although worse than other methods using conventional antibodies. The authors claimed that the V_{HH} would be more suitable for binding to larger molecules than to small ones because the CDR3 moiety of heavy-chain antibody domain fragments is long and fits better in the concave binding sites and cryptic pockets of protein antigens [147]. The selectivity for other tested carbamates, such as methylcarb, pirimicarb, propoxur, or carbosulfan, was lower than 8%. Recoveries of carbaryl and reproducibility of the method when analyzing spiked rice, maize, and wheat samples were satisfactory (recovery 81–113%, CV 2.9–9.7%).

In a similar approach, but using a V_{HH}-AP fusion, Zhang et al. [142] developed a one-step ELISA to detect parathion in cabbage, cucumber, and lettuce samples. The authors suggested that the simplicity of the assay favored the enhanced sensitivity compared with a direct competitive ELISA or an icELISA. In this case, the use of a fluorogenic substrate resulted in 3–4-fold improvement in the sensitivity (IC$_{50}$ 1.6 ng/mL and LOD 0.2 ng/mL) achieved with the assay, showing the importance of selecting the appropriate detection method [148].

Ideally, recombinant antibody fragments might exhibit high affinity, simple heterogeneous expression in bacteria,

Table 5 Summary of recombinant antibodies applied to the detection of pesticides in food

Analyte	Antibody format	Antibody source	Assay format	Ab affinity (K_D)	Analytical characteristics	Detection	Sample	Ref
ICP	Fab-R6G	mRNA (V_H,V_L) from mAb-producing hybridoma cell	Homogeneous assay Soluble Fab-R6G	12.7×10^{-9} M	•IC_{50}: 174 ng/mL •LOD: 10 ng/mL •CR%: 3.2% (ACP), 1.7% (TCP)	FQ	River water, agricultural crop	[136]
CBR	scFv-AP	Immunized library (chicken)	icELISA: immobilized carbaryl C1-BSA + soluble scFv-AP	n.d.	•IC_{50}: 15 ng/mL •DR: 3.5–76 ng/mL •LOD: 1.6 ng/mL •CR% < 1% except 1-naphthol (5%)	Abs	Apple, pear, soil	[137]
PM	Biotinylated scFv-BAD	Immunized library (mouse)	icELISA: immobilized PM-BSA + soluble biotinylated scFv-BAD + SA-HRP	n.d.	•IC_{50}: 14.5 ng/mL •LOD: 0.9 ng/mL •CR% 7% (parathion), 5.7% (fenitrothion), 4.5% (paraoxon-methyl)	Abs	-	[138]
Triazophos	V_{HH}-AP	Immunized library (camelid)	icELISA: immobilized TR1-BSA + soluble V_{HH}-AP	n.d.	•IC_{50}: 9.3 ng/mL •DR: 20 – 800 ng/mL •LOD (apple): 12 ng/mL •CR% nule with other OPs	Abs	Apple	[139]
CBR	V_{HH}	Immunized library (alpaca)	icELISA: immobilized CBR-BSA + soluble His-tagged V_{HH} + anti-His Ab-HRP	n.d.	•IC_{50}: 5.4 ng/mL •LOD: 0.3 ng/mL •CR%: < 0.8% (other carbamates)	Abs	Maize, rice, wheat, grain	[140]
OPs	scFv-BAD	Immunized library (mouse)	icELISA: immobilized OP-BSA + soluble biotinylated scFv-BAD + SA-HRP	n.d.	•IC_{50}: 19.4 ± 515.2 ng/mL vLOD: 4.1 – 230.7 ng/mL •CR% (30 OPs): 12.9 – 343%	Abs	Cucumbers, lettuce, tomatoes	[141]
Parathion	V_{HH}-AP	Immunized library (camelid)	icELISA: immobilized carbaryl H1-OVA + soluble scFv-AP	n.d.	•IC_{50}: 1.6 ng/mL •LOD: 0.2 ng/mL •CR: < 8% (11 parathion analogues)	Fluor	Cabbage, cucumber, lettuce	[142]
DPPs	scFab ([1]κ-LK-Fd or [2]Fd-LK-κ)	mRNA Fab isolated from mAb-producing hybridoma cell	icELISA: immobilized DPP-OVA + soluble [1,2]scFab + anti-Fab mouse Ab-HRP	n.d.	•IC_{50} ([1]scFab): 1.5 ng/mL (coumaphos), 3.1 ng/mL (parathion); IC_{50} ([2]scFab): 3.7 ng/mL (coumaphos), 8.5 ng/mL (parathion)	Abs	Cabbage, cucumber, lettuce	[143]
CYP, β-CYP, FEN	Phage-V_H anti-BPA	Naïve library (human)	icPhage-ELISA: immobilized PBA-OVA + soluble phage-V_H + anti-M13 Ab-HRP	n.d.	•IC_{50}: 2.59 μg/mL (CYP), 1.81 μg/mL (β-CYP), 2.25 μg/mL (FEN) •LOD: 0.4 μg/mL (CYP), 0.37 μg/mL (β-CYP), 0.43 μg/mL (FEN)	Abs	Chinese cabbage	[144]

Abbreviations: Ab, antibody; *Abs*, absorbance; *ACP*, acetamiprid; *AP*, alkaline phosphatase; *BAD*, biotin acceptor domain; *BSA*, bovine serum albumin; *CBR*, carbaryl; *CYP*, cypermethrin; *β-CYP*, β-cypermethrin; *CR*, cross-reactivity; *DR*, dynamic range; *DPPs*, *O,O*-diethyl organophosphorus pesticides; *Fab*, antigen-binding fragment; *Fd*, antibody heavy chain; *FEN*, fenvalerate; *Fluor*, fluorescence; *FluorQ*, fluorescence quenching; *HRP*, horseradish peroxidase; *icELISA*, indirect competitive ELISA; *ICP*, imidacloprid; *κ*, antibody κ light chain; *LOD*, limit of detection; *LK*, (Gly₄Ser)₃ linker; *mAb*, monoclonal antibody; *n.d.*, not determined; *PBA*, phenoxybenzoic acid; *PM*, parathion-methyl; *OPs*, organophosphorus pesticides; *OVA*, ovalbumin; *scFv*, single-chain variable fragment; *scFab*, single-chain antigen-binding fragment; *R6G*, rhodamine 6G; *SA*, streptavidin; *TCP*, thiacloprid; *V_{HH}*, antibody consisting of the variable region of the heavy (H) chain of a camelid antibody; *V_H*, *V_L*, heavy and light variable regions of Ab

and long-term stability. However, the widely used scFv and Fab forms do not meet all these requirements, hindering their broad commercialization as immunoanalytical regents. In this regard, Wang et al. [29] proposed a novel rAb format, a single-chain Fab fragment (scFab; Fig. 1c), obtained by genetic manipulation of the target Fab gene to analyze O,O-diethyl organophosphorus pesticides (DPPs) in food samples. The advantages of scFab are well described [149], highlighting the combined stability and assay performance of Fab fragments with the high level of bacterial expression as a soluble antibody, similar to scFvs. In the study, the authors tested the impact of two different orientations for the Fd and κ fragments to form scFab anti-DPPs with improved production and functionality. The best scFab format was selected by evaluating the expression levels in *E. coli* and its analytical characteristics (sensitivity and selectivity) compared to the respective scFv and Fab analogues. Better sensitivities were achieved using the κ-linker-Fd scFab and the original Fab in comparison with the scFv fragment (IC_{50} for DPP parathion using Fab forms was 3.1 ng/mL vs 6.2 ng/mL for scFv). In addition, the expression extract scFab showed a similar activity than the scFv variant and higher than the Fab fragment, confirming the drawbacks of soluble Fab production in bacteria. Finally, the optimal recombinant scFab was used for the analysis of DPPs-spiked cabbage, cucumber, and lettuce samples by icELISA. The results revealed good sensitivity and reproducibility for the target pesticides with recoveries in the range of 70–121% and CVs of 4–20%.

As reported previously for other small molecule targets, immunosensing approaches using rAbs to analyze pesticides are also scarce. An interesting approach was reported by Zhao et al. using a quenchbody (Q-body) for the analysis of imidacloprid (ICP) [136]. Q-bodies were made by conjugating fluorescent probes to Fab fragments, and they allowed the fast and sensitive analysis of the pesticide without the need for additional reagents [150]. The measuring principle was based on the antigen-dependent removal of the quenching effect on rhodamine 6G of the tryptophan residues in the variable region of the Fab by via photoinduced electron transfer (PET). The homogenous assay exhibited high sensitivity to imidacloprid (10 ng/mL) and low cross-reactivity with other insecticides, such as acetamiprid (CR% 3.2%) and thiacloprid (CR% 1.7%).

Future perspectives

The use of antibodies as selective recognition elements is essential for basic research, diagnostics, and therapeutics applications. According to a recent report published by MarketsandMarkets™, the global market is projected to reach USD 652 million by 2026 from USD 393 million in 2021, at a compound annual growth rate (CAGR) of 10.6% during the

forecast period [151]. The report includes monoclonal, polyclonal, and recombinant antibodies, and rAbs are expected to achieve the highest growth rate during the analyzed period.

Moreover, the COVID-19 pandemic has had an enormous and positive impact on the global custom antibody market. The production of recombinant SARS-CoV-2 binders in just a few weeks from naïve antibody libraries by a host of academic and industrial groups have demonstrated the potential of this technology to develop treatments, vaccines, and diagnostic kits [9]. New funding opportunities in this area are expected to be an incentive for the production and application of non-animal-derived products.

As discussed previously, the production of recombinant antibodies in vitro is especially based on the application of phage and yeast display techniques. Phage display continues to be the most common technique and it allows the isolation of recombinant antibodies from different species (human, camel, llama, shark, macaque, rabbit, or mice). Depending on the source, antibody libraries can be classified either in naïve, immunized, semisynthetic, or synthetic repertoires [152]. The construction of immunized libraries requires the availability of patients or immunized/infected animals to isolate affinity matured antibodies, and a new library must be produced for every target of interest. Such libraries are widely applied not only in medical research, but also for the isolation of highly sensitive antibodies for food analysis [75, 101–103]. On the other hand, the use of naïve libraries allows circumventing the ethical problems and limitations associated to the use of animal-derived antibodies. Unlike immunized libraries, they can be applied for the generation of antibodies against a wide variety of compounds including non-immunogenic, pathogenic, conserved, or self-targets, which is of interest in food analysis. Nowadays, the libraries are rather large (approximately ten billion antibodies) in comparison to the initial developments, which increases the possibilities of isolating high-affinity antibodies if good selection strategies are applied. Semi-synthetic libraries include a combination of CDRs from natural sources and in silico design of defined parts, whereas synthetic libraries are completely based on in silico design and gene synthesis resulting in a well-defined and controlled composition of the CDRs. The application of in silico techniques for the design of recognition molecules has been identified as an exciting area of research for the next decade [153].

At present, a major bottleneck that requires attention is the characterization of the isolated antibodies (e.g., western blots, immunofluorescence, immunohistochemistry, flow cytometry) prior to commercialization [11]. Companies should not only produce the antibodies but also characterize them and guarantee batch-to-batch fidelity. In fact, there is an increasing concern about reproducibility issues due to the application of poorly validated animal-derived antibodies with the corresponding waste of time, money, and human resources.

Recombinant antibodies are versatile and their selectivity can be finely tuned by mutation strategies and controlling the selection criteria during panning. More importantly, the reproducibility between batches, specificity, and long-term supply of recombinant antibodies in combination with other advantages, such as the rapid generation or wider range of target compounds, may represent an economic advantage for companies using this technology and provide higher quality antibodies.

The application of nanobodies for analysis purposes is especially attractive as they can stand harsh measuring conditions, such as extreme temperatures or, for example, the presence of organic solvents that would denature classical antibodies and are usually applied for the extraction of food contaminants. On the other hand, their small size allows the preparation of high-density sensing layers with low non-specific adsorption of interfering species with the corresponding improvement in the assay sensitivity. Moreover, nanobodies as well as other recombinant antibody fragments can be engineered to fit the needs of the final application, for example by a genetic fusion to affinity tags for purification purposes or for their oriented immobilization on sensing platforms [154]. These characteristics together with the robustness, relatively low production costs, and large-scale production make nanobodies especially attractive for the development of biosensing devices and diagnostics kits. They can also broaden the application of affinity reagents for in vivo diagnostics due to their ideal biodistribution properties and fast elimination via kidneys [154].

According to the EURL ECVAM, broad application of non-animal-derived antibodies has been hindered by several challenges; first of all, there is a big tendency to avoid the use of new methods; second, recent advances in the non-animal-derived technology are unknown in many areas of biological research; third, legal and economic constraints have prevented some companies from entering this market; and, fourth, there is limited access to non-animal-derived antibody sources both from academic and commercial sources [3]. In any case, the development of non-animal-derived antibody techniques has increased notably in the last decade and companies have started to include in vitro generated antibodies in their catalogues. However, although some companies have already included a good number of recombinant antibodies in their catalogues for therapeuthic applications [9], at present the available technology and infrastructure is not developed enough for upscaling and the prices for custom-made services are expensive [155]. In addition, only a few companies, such as Creative-Biolabs [156] that include recombinant antibodies for OTA and ZON in their portfolio, focus on food applications.

Nowadays, there is an opportunity for companies and users operating under the EU regulation to replace animal-derived antibodies by the non-animal antibody technology that should be exploited. A broader question, however, is whether non-animal-derived antibodies are capable of replacing monoclonal and polyclonal antibodies that have ruled the field for decades. In recent years, there is a continuous trend to try to replace animal-derived antibodies for recombinant constructs from naïve or degenerated libraries and the use of synthetic constructs, and the literature revised in this review indeed shows the advent of recombinant alternatives for food analysis. While at present complete replacement of monoclonal and polyclonal antibodies by non-animal-derived ones is unlikely, recombinant antibodies should be considered a valuable complementary or even alternative option, especially in certain applications where their adaptability is a true advantage.

Conclusions

Antibody-based technologies have revolutionized the rapid detection of many common contaminants in foodstuffs, including toxins, pesticides, antibiotics, and foodborne pathogens. The limitations associated with conventionally used monoclonal and polyclonal antibodies, as well as concerns of unnecessary use of animals for antibody production, have also spurred efforts to explore recombinant antibodies for food safety applications. Several examples presented in this review show the potential of recombinant antibodies for highly sensitive and specific detection of food contaminants. While not all applications with recombinant antibodies are capable of competing with monoclonal antibodies in terms of sensitivity, they might bring other advantages to the system, including small size, stability in harsh conditions, flexibility, and potential to genetically engineer the binder, as well as simple and low-cost animal-free production. Yet, in spite of the benefits of recombinant antibodies, their application in the field of immunoassays and especially biosensors for food analysis is still not widely exploited. However, monoclonal and polyclonal antibodies have some 40 years of head start, and it remains to be seen how far recombinant antibodies can go once they become more widely available. Some commercial providers have already included a number of recombinant antibodies in their selection, which can be expected to boost their widespread use. Moreover, academic consortia bringing together experts from different fields can serve as an important manner to promote the use of recombinant antibodies and establish new innovative antibody-based technologies for safer food.

Data availability Not applicable.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare no conflict of interest.

M. C. Moreno-Bondi is Editor of *Analytical and Bioanalytical Chemistry* and was not involved in peer reviewing of this manuscript

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