Current trends in rapid tests for mycotoxins

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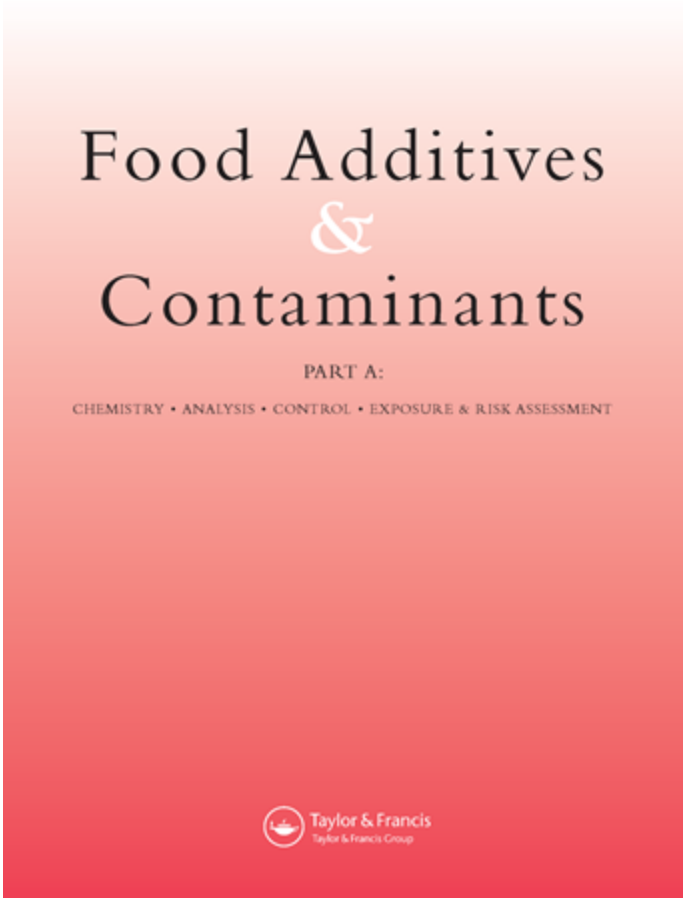

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Current trends in rapid tests for mycotoxins

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Abstract

There is an ample number of commercial testing kits available for mycotoxin analysis on the market today, including enzyme-linked immunosorbent assays, membrane-based immunoassays, fluorescence polarisation immunoassays and fluorometric assays. It can be observed from the literature that not only are developments and improvements ongoing for these assays but there are also novel assays being developed using biosensor technology. This review focuses on both the currently available methods and recent innovative methods for mycotoxin testing. Furthermore, it highlights trends that are influencing assay developments such as multiplexing capabilities and rapid on-site analysis, indicating the possible detection methods that will shape the future market.

Keywords: mycotoxin; immunoassay; biosensor; on-site detection; rapid detection method; multiplex detection

Introduction

Naturally occurring food and feed contaminants are unavoidable making them a major issue in global food safety, particularly those that pose serious health concerns to humans and animals. Mycotoxins are one such example. These are secondary metabolites of low-molecular weight that are produced by various fungi, the genera of which include

Aspergillus, *Penicillium* and *Fusarium* (Marin et al. 2013; Sweeney et al. 1998). Mycotoxins mainly enter the food chain because of fungal colonization either of pre-harvest susceptible crops, or during the time between harvesting and drying, or during storage (Alshannaq et al. 2017). The most agriculturally significant mycotoxins in terms of being a health threat to humans and animals include aflatoxins (AF), fumonisins (FUM), deoxynivalenol (DON) and other trichothecenes (T2 and HT2 toxins), ochratoxin A (OTA) and zearalenone (ZEN) (Raiola et al. 2015) (Table 1). The ergot alkaloids (EA), which are produced by fungi of the genus *Claviceps*, are another class of mycotoxins tested for in food and feed. The three main groups of EA's are the clavine alkaloids, lysergic acids and ergopeptines. Ergotamine and ergovaline are some of the EAs most commonly tested for (Crews 2015). Whilst mycotoxin remediation seems an ideal solution to the problem, many commonly used physical and chemical methods raise issues regarding safety and nutritional loss of food and feed (Ji et al. 2016).

The difficulties associated with remediation combined with the health risks of mycotoxins has provoked other control methods to ensure food safety. This is utilising accurate and reliable food testing methods to prevent food and feed contaminated with high levels of mycotoxins reaching the consumer market. The detection of mycotoxins has been ongoing for approximately 50 years (Wong and Lewis 2017). Maximum levels at which mycotoxins are permitted in food and animal feed have been set in Regulation (EC) 1881/2006 (EC, 2006a) and subsequent amendments under European legislation and implemented by Member States. This has subsequently driven the development and commercialisation of methods for mycotoxin analysis in food and feed.

A myriad of methods for mycotoxin analysis are available and their usage is dependent on a variety of factors. For example, liquid chromatography tandem mass spectrometry (LC−MS/MS) is currently extensively used as a confirmatory reference method as it allows the simultaneous determination of different mycotoxins. Other chromatographic-based methods exist which can be coupled with ultraviolet (UV), diode array (DAD), and fluorescence (FLD) detectors including thin-layer chromatography, or high-performance liquid chromatography (HPLC), the latter of which is more sensitive and reliable, particularly when coupled FLD's and subject to adequate clean-up (De Santis et al. 2017; Matabaro et al. 2017; Man et al. 2017). These methods however are limited to large commercial companies, reference and academic laboratories with skilled technicians and expensive laboratory equipment. Furthermore, they are generally time-consuming and labour-intensive (Ahmed et al. 2017). In this regard rapid commercial test kits for mycotoxin analysis are used as an alternative for more user-friendly, inexpensive, robust detection.

The majority of test kits on the market today are based on an immunoassay format which relies on the ability of an antibody to bind to a specific antigen structure (target molecule). As mycotoxins are relatively small molecules (less than 1 kDa) a 'sandwich' type immunoassay in which multiple antibodies bind to a single toxin is not suitable, rather a competitive assay is generally employed (Li et al. 2014; Maragos 2009). In a competitive immunoassay, the analyte of interest from a sample will affect the detection and measurement of a competitor, meaning the result generated is an indirect measurement of the analyte of interest. The properties of the competitor will vary depending on the assay but regardless, every competitor must be capable of triggering a signal that can be detected and measured by the device/reader in place for that assay. Conventional and currently manufactured immunoassay based test kits for screening of mycotoxins can be used by importers, traders, and food and feed manufacturers. These include Enzyme Linked Immuno-Sorbent Assays (ELISAs), Lateral Flow Immunoassays (LFIA) and fluorometric assays that use two sample clean-up methods; immunoaffinity column (IAC) and solid phase extraction (SPE) column clean-up (Alshannaq et al. 2017; Berthiller et al. 2017; Li et al. 2014; Turner et al. 2015) (Table 2).

Assay requirements are evolving which has an impact on the performance of the current above mentioned immunological test kits. The main current trends to be met include portability and multi-toxin detection. The reason for portability becoming increasingly important lies in the growing demand for on-site testing. On-site testing can occur at points in the food production process. For example, upon reception of raw materials to a feed mill, analysis of the material can be conducted in a low technology environment using a portable test kit. Results can be obtained rapidly as samples are not required to be sent off to reference laboratories for analysis. This also prevents the food production process slowing down. On-site testing can therefore be considered a time-efficient and cost-effective choice. Multi-toxin testing eliminates the need to purchase and run multiple single-toxin tests for one sample batch. Therefore, portable multi-toxin testing devices suitable for onsite testing would give added cost and time-saving benefits. Of course, another factor in producing a cost-effective assay lies in the actual fabrication and overall cost of the test itself. Furthermore, the length of time it takes to generate a result is another important factor in time-efficient assays.

This review will give a description of commercially available immunological test kits for mycotoxin analysis as well as highlighting advantages and disadvantages of the methodology. Following this, any progression in immunological test kits in regard to current trends in mycotoxin analysis will be discussed as well as any limitations of the assay hindering progression. Moreover, there are also advancements in the development of

biosensor platforms offering to meet current trends in mycotoxin analysis which will also be discussed in this review.

Commercially available Test Kits

As is evident from Table 2, the market today for mycotoxin test kits is already very competitive. Some recent developments include the lateral flow devices being sold by R-Biopharm that utilise a mobile application on a smartphone to analyse colour signal in lieu of a reader, specifically for AFs, T2/HT2, ZEN and FUM. Another advance in that market from Randox is the development of Biochip Array Technology (BAT), a chemiluminescent based assay capable of multiplex analysis. The chips, which are specifically available for mycotoxin testing, include the Myco10, 7, 5 and flex arrays. The Randox device is laboratory based and whilst there is a portable device on offer, it is not currently specified to be used with the mycotoxin specific arrays. Of the companies in Table 2, 67% offer ELISAs, 75% offer lateral flow devices and 25% offer fluorometric assays. These assays along with some others will be discussed in more detail below.

Enzyme-linked Immunosorbent Assay

ELISAs are traditional for mycotoxin detection. Most commercial ELISAs employ a direct competitive immunoassay format (*Figure 1***(a)**), using an antibody coated 96 well microtiter plate (Li et al. 2014). Sample/standard is added into the appropriate wells of the plate followed by an enzyme-coupled mycotoxin conjugate solution. During an incubation period, competition occurs between any mycotoxin present in the sample/standard and the enzyme-conjugated mycotoxin for a limited number of antibody binding sites. After incubation the plate is washed to remove any unbound mycotoxin and an enzyme substrate is added. This results in a colour reaction occurring between the enzyme substrate and any enzyme-coupled mycotoxin bound to the antibodies immobilised onto the microtiter plate. The intensity of the colour is therefore inversely proportional to the mycotoxin concentration in the standard/sample extract. After another short incubation period with the enzyme substrate for colour development, the reaction is halted by adding a stop solution. The plate is then placed immediately into a colorimetric reader for optical density measurement.

Commercial ELISA kits are high throughput, selective, sensitive assays that require little sample preparation (Rahmani et al. 2009; Zheng et al. 2006). Assays have also become more rapid, for example Romer labs offers ELISAs for DON, OTA, AF's, T2, ZEN and FUM with incubation periods of 15 min. On the other hand, most are between 1 and 2 hours. In some cases, overestimation of results, due to cross-reactivity of antibodies (Zachariasova et al. 2014), and false positive results due to the matrix effect, can interfere with ELISA

readings. To eliminate these effects, most kits specify the matrices to which the ELISA kit can be applied. However, this can also be considered a disadvantage of the product.

In terms of meeting current trends, multiplexing abilities of ELISAs have been demonstrated by Urusov et al. (2015). The group immobilised AFB1, OTA and ZEA in different wells of a single microplate. The study used a competitive format based on *Figure 2***(b)**, were analytes in the sample and biotin conjugated antibodies were added to antigen-coated wells followed by a streptavidin–polyperoxidase conjugate, which through reaction with bound biotin-antibody produced a high detectable signal. The developed method was successfully validated using poultry processing products and corn samples spiked with known quantities of mycotoxins. The LODs for AFB1, OTA and ZEA in these matrices were 0.24, 1.2 and 3 ng/g, respectively. Furthermore, with the advancements in nanotechnology, McNamee et al. (2017) demonstrated a multiplex nanoarray based on ELISA. ZEA, T2 and FUMB1 conjugates were nano-spotted into single wells of a microplate. The sensitivity of the assay was determined by the IC50 values which were 197.4, 0.7 and 216.7 µg/kg in wheat and 43.6, 0.5 and 25.9 µg/kg in maize for ZEA, T2 and FUMB1 respectively. The group highlighted the assays comparison to an ELISA protocol, making it easily adaptable by end-users accustomed to running ELISAs, whilst providing higher sample throughput with high sensitivity and accuracy.

Developments in the ELISA technique are also being made with the use of nanomaterials (Liang et al. 2016; Pei et al. 2018; Xiong et al. 2018). For example, Hendrickson et al. (2018) used magnetic nanoparticles (MNP) suspended in the reaction medium as a solid support for antibody binding. This pseudo-homogeneous regime as the group named it, results in the antibody covering a greater surface area and being more evenly distributed in the medium which increases antibody-antigen diffusion processes, reducing the time of the assay. With the use of a magnet, the assay also allows for rapid and simple separation of the MNP-antibody-antigen complexes. The group referred to this as a pre-concentration step, which when combined with chemiluminescence detection, achieved ZEN control in wine with detection limit of 0.03–0.05 ng/g.

Membrane-based Immunoassays

Lateral Flow Immunoassay

Lateral flow immunoassays (LFA) also known as lateral flow tests, immunochromatographic tests or strip tests are typically a competitive format where a labelled antibody is used as a signal reagent (*Figure 2***(b)**). The label can vary from quantum dots (QDs) to luminescent nanoparticles to amorphous carbon nanoparticles (Zhang et al. 2017). Traditionally it is a gold nanoparticle (Krska and Molinelli 2009). The strip itself consists of four parts, the

sample pad, conjugate pad, porous membrane and final absorbent pad. If the strip is freestanding, the labelled antibody is mixed with the sample extract in a microwell before application to the sample pad. If the strip is enclosed in plastic housing, the sample is directly added to the sample pad; it then migrates to the conjugate pad, binding to already immobilized labelled antibody here. Regardless of application, the sample and labelled antibodies will migrate to the nitrocellulose membrane, which contains a test zone and a control zone. In a competitive assay, mycotoxin–protein conjugate is coated on the test zone and the control zone consists of a 2nd antibody. The control zone will always be visible regardless of the presence or absence of mycotoxin because the 2nd antibody always captures an anti-2nd labelled antibody. The anti-2nd labelled antibody resides on the conjugate pad and flows with the sample and labelled antibodies over the test and control zones. This is an important part of the assay as it ensures that the sample has in fact flowed through the device. In a negative sample, the free labelled antibody binds to the mycotoxin–protein conjugate on the test zone, forming a visible line. In a positive sample, the labelled antibody will not bind to the mycotoxin-protein conjugate on the test zone as the binding sites of the antibody will be saturated from the toxin in the contaminated sample. As a result, no visible line will form (Maragos and Busman, 2010). Line visibility at the test zone is dependent on the degree of sample contamination. LFAs will have a cut-off level which is the point of discrimination between positive and negative samples. A positive sample with a mycotoxin concentration equal to or beyond the assay cut-off level will therefore result in no visible line in the test zone. This level must meet the regulatory requirements for the maximum permissible level of contamination.

LFAs are strong competitors on the market for mycotoxin detection (Tripathi et al. 2018). The method can give qualitative and/or semi-quantitative results, is simple, and capable of generating results within minutes (for example Afla-V by Vicam, 4 min). Furthermore, LFAs and their readers are easily portable, making them ideal for on-site analysis. In recent years, LFAs have been demonstrated as capable of multiplex mycotoxin determination. Song et al. (2014) demonstrated this in a recent paper where an LFA was developed for qualitative and/or semi-quantitative determination of AFB1, ZEN, DON and their analogues (AFs, ZENs, DONs) in cereal samples. The LFA device had multiple test lines, each with a different mycotoxin-conjugate (DON-BSA, ZEN-BSA and AFB1-BSA). The monoclonal antibodies adopted were class specific, so the LFA strip could simultaneously detect three groups of mycotoxins in a single assay. The assay was rapid (15 min) and both visual LODs and calculated LODs (0.05, 1, and 3 μg/kg, respectively) were lower than the EU maximum levels. Recoveries also ranged from 80% to 122%. Foubert et al. (2017) used green, orange, and red epoxy-functionalized silica-coated QDs as a signal reagent, rather than gold

nanoparticles, conjugated to anti-ZEN, anti-DON, and anti-T2 mAb, respectively, for ZEN, DON and T2 toxin detection. The LFA also gave a fast result (15 min) with a low false-negative rate (<5%). Results were easy to interpret visually by having a different colour correspond to a different toxin. Validation studies on multiplex lateral flow devices have also been conducted by Lattanzio et al (2013). The study demonstrated the ruggedness of the test and showed a false positive rate lower than 6 %. Furthermore, the group considered the test to have significant economic benefits when using it under real-world conditions.

Flow-Through Immunoassay

Flow-Through tests are in principle based on the competitive format commonly used in ELISAs. However, rather than anti-mycotoxin antibody being bound to a microwell, it is bound to a membrane upon filter paper (*Figure 1***(a)**), (Trucksess et al. 1994). The test relies on the sample extract flowing through filter paper and any mycotoxin present binding to the antibody on the membrane. Along with the sample extract, enzyme-conjugated mycotoxin is added, and competition between it and any mycotoxin in the sample occurs for free antibody binding sites on the membrane. Following this an enzyme substrate solution is added. If the sample is negative, then the antibody binding sites become saturated with the enzyme-conjugated mycotoxin and a colour reaction will occur between the enzyme and substrate. However, if a sample is positive, the mycotoxin will bind to the antibody binding sites and no colour reaction will take place upon addition of the substrate. Therefore, the colour intensity is inversely proportional to the amount of toxin present in the sample.

Flow-Through immunoassays can give qualitative and/or semi-quantitative results, are simple and capable of generating results within minutes (for example OCHRACARD by R-Biopharm, 5 min). In terms of limitations of the test, membrane saturation and high cut-off values can lead to inaccurate interpretation of results (Beloglazova et al. 2017). Membrane saturation can occur when the volume of sample used exceeds that which can be absorbed by the system. This can affect colour development and subsequently the final readings. However, the addition of more absorption layers can reduce potential membrane saturation. Furthermore, recent studies have also aimed to optimise cut-off values. From 2000-2010 there have been developments in Flow-Through tests from single to two-analyte analysis (Paepens et al. 2004, Saha et al. 2007; Sibanda et al. 2000). More recently, multiplexing has been shown possible with the flow-through approach. (Burmistrova et al. 2014; Ediage et al. 2013). developed a flow-through test for multiplex screening of ZEN, DON, AFB1 and OTA. The group tested cereal-based feed ingredients and compound feeds (wheat, barley, soybean, wheat bran, rice, rice bran, maize, rapeseed meal, sunflower meal) and various types of complete feed (duckling feed, swine feed, broiler feed, piglet feed).

The developed assay revealed cut-off levels for ZEN, DON, AFB1 and OTA that were 50, 200, 1, and 10 µg/kg, respectively, which comply with European regulations No 401/2006 (EC 2006b) and 519/2014 (EC 2014). Sample pre-treatment involved extraction, dilution and solid-phase extraction by addition of C18 sorbent followed by final filtration of the supernatant. The numbers of false-positive and false-negative outcomes were <5%, which is consistent with the Commission Decision 2002/657/EC (EC 2002). Furthermore, according to Burmistrova *et al* (2014) and Beloglazova *et al* (2017), it is expected that separate test zones in a multiplex Flow-Through assay minimise non-specific interactions between immunoreagents compared to the test zones in a multiplex lateral flow assay. This is due to the sample solution simultaneously contacting the separate immunoreagent test zones in a Flow-Through assay, compared to a lateral flow, where cross-influence of immunoreagents can be affected by the liquid running from the front of the system and passing all test zones (Beloglazova et al. 2017). Furthermore, Flow-Through assays and their readers are easily portable, making them ideal for on-site analysis.

Membrane based immunoassays (lateral flow and Flow-Through) are progressing to meet current trends as both have shown the possibility of multiplex testing and both are very suitable for on-site testing. Furthermore the tests are very quick, generating results within minutes.

Fluorescence Polarization Immunoassay

The fluorescent polarization immunoassay (FPIA) is based on the principle that when a fluorescent molecule in solution is exposed to polarized light at its excitation wavelength the resulting emission is depolarized. The polarization is a measure of the orientation of the fluorescence emission from both horizontal and vertical directions. Small fluorescent molecules have higher rates of rotation and lower polarization than larger molecules. The interaction of a fluorophore with a relatively large molecule such as an antibody reduces the rate of the rotation motion of the fluorophore, resulting in an increase in observed polarization. This polarisation increase can be detected and measured, making this phenomenon suitable for the development of a competitive immunoassay for mycotoxin detection (Smith and Eremin, 2009). In a competitive format the mycotoxin in the sample competes with a tracer (mycotoxin-fluorophore tracer) for binding sites on a mycotoxin-specific antibody (*Figure 1***(b)**). In a negative sample, binding of the antibody to the tracer increases polarization whereas in a positive sample, lesser antibody is bound to the tracer, reducing polarization. The polarization value is thus inversely proportional to mycotoxin concentration.

FPIA has a homologous format conducted in solution phase, which unlike a heterogeneous immunoassay such as ELISA does not require the separation of the free and bound tracer nor the use of an enzymatic reaction. This is beneficial, as no separation and washing steps are required which reduces assay time, increases throughput and eliminates incubation steps for colour development and overall simplifies the method for ease-of-use. On the other hand, matrix effects can cause interference in reading of results if samples with coloured compounds are not correctly pre-treated (Valenzano et al. 2014). Rapid FPIA procedures have been developed since the early 2000's for the determination of many mycotoxins including DON, ZEN, FUMs, AFs and OTA, a review of which was done by Maragos (2009). More recently Bondarenko and Eremin (2012) compared the effect that various fluorescein-based tracers had on the sensitivity of detection of ZEN and OTA. The study found that not only were the best tracers ethylenediamine thiocarbamoylfluorescein (EDF) and aminomethylfluorescein (AMF) but that using OTA-AMF gave a lower detection limit of 1.5 ng/ml compared to OTA-EDF, which was 6 ng/ml in a model system. When carried out using spiked grain samples, LODs for ZEN and OTA with their respective best tracers were 15 and 10 µg/kg, respectively. In 2014, Sheng et al. tested an antiAFB1 mAb with the aim of developing a total AF assay. The antibody had high cross reactivities of 100%, 65.7%, 143%, 111.4% to AFB1, AFB2, AFG1 and AFM1, respectively, the LOD was 13.12 ng/mL and total analysis time was 5 minutes. Li et al. (2016) developed a multi-wavelength fluorescence polarization immunoassay (MWFPIA) for multiplexed detection of DON, T-2 toxin and FUMB1 in maize which were labelled with different dyes for discrimination during detection. Under optimal conditions, the LODs using MWFPIA were 242.0 µg/kg for DON, 17.8 µg/kg for T-2 toxin and 331.5 µg/kg for FUMB1, providing sufficient sensitivity to meet the action levels of these three contaminants in maize as set by the EU. Twenty naturally contaminated maize samples were tested using MWFPIA and HPLC–MS/MS, with correlation coefficients (R^2) of 0.97 for DON and 0.99 for FUMB1. FPIA requires sample pre-treatment to decrease the matrix effects and obtain accurate polarisation readings, otherwise there is a risk of overestimation of results. Furthermore, sample pre-treatment increases total assay time (30 min in total for Li et al 2016). Nevertheless, the application is still suitable for onsite analysis provided reliable sample pre-treatment is in place.

Fluorometric Assay

In a fluorometric assay, after a preliminary solvent extraction from the solid matrix, typically with a mixture of acetonitrile or methanol and water, the mycotoxin extract is commonly cleaned up/enriched by SPE or IAC. The purified sample is then put into a fluorometer for

analysis. Extract purification is required for eliminating matrix effects and potential fluorescent compounds that could generate false-positive results. Although analysis time and cost are increased in this case, the analytical method benefits from increased sensitivity and robustness (Huertas-Pérez et al. 2016; Nilüfer and Boyacıoğlu, 2002). There are many commercially available SPE and IAC products available for on-site mycotoxin analysis (Table 2). Şenyuva et al (2010) have also reviewed these products extensively. IAC and SPE coupled with fluorometric meters give semi-quantitative and results within minutes (NeoColumn for Aflatoxin <10 min) and do not require expensive laboratory equipment.

Principle of Immunoaffinity and Solid Phase Extraction clean up columns

IACs work by immobilizing anti-mycotoxin to a solid support contained within a small plastic column. The solid support is commonly an inexpensive material such as agarose gel or cellulose, or synthetic organic supports including acrylamide polymers, copolymers or derivatives, polymethacrylate derivatives and polyethersulfone matrixes. When a sample extract is passed through the column, any mycotoxin present will selectively bind to the immobilized antibody and what remains of the extract is flushed out through the column in a washing step, typically done with water. A solvent is then passed through the column removing the mycotoxin from the antibody and eluting it from the column. In the now purified sample extract, the solvent can be evaporated off, leaving a concentrated mycotoxin sample. An important additional step to this assay is the fluorescent derivatization step to either enhance the fluorescence of a mycotoxin or render the mycotoxin fluorescent before measuring in a fluorometer. The natural fluorescence of mycotoxins such as the aflatoxins may be chemically enhanced via reaction with trifluoroacetic acid, bromine, or iodine (Wacoo et al. 2014). Other mycotoxins such as DON that are not naturally fluorescent must be derivatized with zirconyl nitrate and ethylenediamine in methanol for example (Malone et al. 1998).

SPE makes use of a sorbent agent, known as the ‘stationary phase’, loaded inside a plastic column (like that of IAC) or supported on a disc. The sample passes through the stationary phase and depending on the type of sorbent material being used, either the analyte of interest will be captured and retained inside the column or disc while impurities are filtered out, or it will capture matrix contaminants leaving the analyte of interest left in the eluted sample (Huertas-Pérez et al. 2016). The latter is known as a one-step SPE column, packed with a porous frit at the top of the column packing, in a durable plastic tube with plastic caps at both ends. Sample extract is added to the sample reservoir and a rubber syringe plunger, or a similar device, is used to push the sample extract through the one-step SPE column. The purified extract collected at either the lower or upper end of the tube

contains the mycotoxin, which can immediately be derivatized and placed in a fluorometer for analysis.

Usage and developments in IAC and SPE

MycoSep® columns (Romer Labs) are an example of a commercial one step SPE column clean-up which has been coupled with fluorometry analysis (Malone et al. 1998; Malone et al. 2000). Companies typically recommend fluorometric readers too, for example, Romer Labs offer a FQ-Reader with their FluoroQuant Aflatoxin test kits which are based on either immunoaffinity column clean-up or solid phase extraction. VICAM also offer IAC kits with their Series-4EX Fluorometer specifically to be used with the following IACs: AflaTest, AflaB, AflaM1 FL+, FumoniTest, OchraTest and ZearalaTest.

In a study by Longobardi et al. (2013) the group used a commercial IAC coupled to SPE for extraction of OTA from red wine samples before direct fluorometric measurement with a spectrofluorometer. The LOD in spiked red wine samples was 0.2 ng/ml, recoveries ranged from 94.5-105.4% and the total analysis time was 30 min. A good correlation (r2 = 0.9765) was observed between OTA levels obtained with the fluorometer and HPLC, showing the results are reliable, with the added advantage of using a simple benchtop fluorometer which evidently reduces the cost and time of analysis. Commercial IACs have also been used in evaluation studies such as that by Li et al. (2014) to evaluate the safety of food in the Yangtze Delta region of China. The group tested for ZEN, OTA, AFs and AFB1 using four different IACs for each toxin (or group of toxins) and four different preliminary extraction techniques. As no IAC coupled with fluorometry was available for DON, HPLC-UV was used. Whilst the study allowed for a reliable assessment of mycotoxins in the Yangtze Delta region, it exemplifies how a high-throughput, affordable, multiplex method of detection would have enabled a more cost-effective and time-efficient sample analysis for the group.

Developments in SPE have branched to usage of engineered receptors. For example, in a study by Ali et al. (2010) the group successfully used molecularly imprinted polymer (MIP) as a selective sorbent for the retention of OTA from cereal extracts. The study compared capacity of the MIP-SPE method with IAC. The capacity in this case was the maximum amount of compound that was retained by the sorbent. For MIP-SPE the recoveries of the extraction were linear up to 5000 ng/g^{-1}, whereas for IAC, when percolating an extract containing 900-4300 ng/g^{-1} of OTA, only 650ng were detected in the elute. These results indicate a decrease of recoveries in IAC caused by overloading of the capacity. In a study by Sergeyeva et al (2017) SPE with an MIP membrane as a stationary phase was developed and used with a fluorescent sensor system (spectrofluorometer from Perkin-Elmer, UK) capable of detecting AFB1 within the range 14–500ng/mL^{-1}. In this paper, the

group aimed to highlight the potential in synthesis of nanostructured polymers (1–10 nm artificial receptor sites) capable of selective recognition of mycotoxins for usage as novel selective layers in portable biosensor technology. Furthermore, MIP membranes would also improve the storage stability of the biosensor as biological reagents such as antibodies and antigens most commonly used are evidently less stable than MIPs.

Today, most recent developments seen across literature in IAC or SPE are coupled with LC-MS/MS or HPLC analysis with fluorometry rather than developments in the traditional fluorometric kits (Wang et al. 2016; Anene et al. 2016). This is likely because LC-MS/MS and HPLC analysis are the current reference methods of choice capable of multiplex analysis. In fact, there are even commercial IACs for multiple mycotoxin clean up (Vicam) that can be used prior to LCMS/MS. In terms of commercial, portable methods, perhaps SPE integrated with MIP coupled with fluorometric biosensors is the future progression in SPE technology.

Biosensor Development

A biosensor device typically has three components (1) the detection layer (2) the transducer and (3) the output system. The detection layer can either consist of biological material (e.g. tissue, microorganisms, organelles, cell receptors, enzymes, antibodies, nucleic acids, natural products etc.), a biologically derived material (e.g. recombinant antibodies, engineered proteins, aptamers etc.) or a biomimic (e.g. synthetic receptors, biomimetic catalysts, combinatorial ligands, imprinted polymers) (Chauhan et al. 2016). The detection layer is integrated within the transducer so that upon interaction with the sample, a binding event or reaction occurring at the detection layer will generate an electronic signal via the transducer that is measured by the output system.

Biosensors today are advancing enormously as analytical devices in food safety with the integration of many modern developing technologies including but not limited to signal transduction technology (magneto, piezo, optical and direct electrochemical techniques and micro-electro-mechanical systems), microfluidics (droplet or digital) and immobilization technologies (Lin and Guo, 2016). The integration of aptamers in particular with biosensing technology (aptasensor) is also becoming increasingly prominent. Aptamers are composed of single stranded oligonucleotides (DNA or RNA), capable of interacting with analytes. There are many reasons for aptasensor progression in recent years, a comprehensive review of which was done by Rhouati et al (2017). The review points out how the chemical production of aptamers is cheaper than that of antibodies. Furthermore, aptamers are easily labelled (with fluorescent dyes, enzymes) and unlike antibodies, can be regenerated and reused for other analyses.

Some examples of biosensors that have been applied to mycotoxin analysis are listed below and in Table 3. The potential barriers to commercialisation are also discussed.

Optical Sensors

Surface Plasmon Resonance

Since the early 2000's, commercially-available laboratory-based systems founded on the principle of surface plasmon resonance have been effectively used for the detection of mycotoxins in a variety of matrices including DON in wheat (Meneely et al. 2010), AFM1 in milk (Wang et al. 2009), OTA in cereals and beverages (Yuan et al. 2009), T2/HT2 in cereals (Meneely et al. 2012) and ZEN in sorghum (Edupuganti et al. 2013). SPR is an optical phenomenon that occurs when polarised light hits a metal film between two media of different refractive index. Biosensors based on SPR typically use a thin gold metal film at the interface of a glass prism and a sample solution (Meneely and Elliott 2014). Typically, light coming from the side of higher refractive index (glass prism), is partly reflected and partly refracted. However, above a critical angle of incidence, total internal reflection will occur (Ying et al. 2012). Under these conditions, an electromagnetic field component of the light, the evanescent wave, will penetrate the lower refractive index medium (the sample solution). When there is a thin metal film at the interface between the high and low refractive mediums, the evanescent wave interacts with the free oscillating electrons of the thin metal layer resulting in excitation of surface plasmons. Energy from the incident light dissipates in the metal film, resulting in a decrease in the intensity of the reflected light. This is the phenomenon of surface plasmon resonance (Hodnik et al. 2009). In SPR biosensing, the gold thin film acts as a sensing surface and is modified with biorecognition probes (antigen-conjugate). Antibody is mixed with the sample solution prior to running it over the sensor surface so that any free mycotoxin in the sample will bind to the mixed antibody resulting in no antibody binding to the probes on the sensor surface (*Figure 2***(a)**). However, in a negative sample, the free antibody will bind to the probes on the sensor surface. A binding event changes the resonance frequency of the surface plasmons since the refractive index of the medium at the interface is changing. This change in resonance, changes the intensity of the reflected light which is detected by the biosensor device as a binding event and is expressed in arbitrary units known as resonance units. These binding events provide a relative response (relative to the baseline) and are measured against a calibration curve, allowing determination of the sample concentration. Each cycle produces a plot known as a sensorgram, a plot of response against time, detailing binding events in real time which is a key advantage of the technique (Xu et al. 2016). SPR based biosensors are also reliable,

label-free, sensitive and have the added advantage of reusability with regeneration of the biosensor chip surface.

There have been variations to the immunoassay format of SPR to include the combination of SPR with enzyme-derivatised sensors and MIPs. Fluorescence spectroscopy and the use of gold nanoparticles have also been coupled with SPR for signal enhancement. Mennely et al. (2014) extensively reviewed SPR methods and highlighted the need for development and manufacture of portable SPR instruments demonstrating multiplexing capabilities at lower costs. Since then many studies have been focusing on these aspects of SPR development. Nevertheless, in a later review by Dahlin, (2015) it was highlighted that no published studies developing portable or multiplex SPR devices were including a fair benchmark test against state-of-the-art SPR. A year later, a publication by Joshi et al. (2016) aimed to address all of the above mentioned issues by developing a 6-plex competitive inhibition immunoassay for mycotoxins in barley on a prototype portable nanostructured iSPR instrument which has a nanostructured gold sensor surface, eliminating the need for a prism. The group first developed a double 3-plex assay, which involved using a well-established benchtop SPR instrument and two biosensor chips. One chip was used for the detection of DON, ZEN, T-2 toxin and a second chip for the detection of OTA, FUMB1 and AFB1. The ovalbumin (OVA) conjugates of mycotoxins were immobilized on the chips via amine coupling. Upon injection of mixed antibodies at a fixed concentration, with sample or standard, over a chip with the immobilized mycotoxin–OVA conjugates, the SPR response was recorded. The chips could be used for up to 60 cycles after regeneration with 10 mM HCl and 20 mM NaOH after each run. The LODs in barley (in μg kg^{-1}) were 26 for DON, 6 for ZEN, 0.6 for T-2, 3 for OTA, 2 for FUMB1 and 0.6 for AFB1. In accordance with the EU regulatory limits, results could be validated for DON, T-2, ZEN and FUMB1 while for OTA and AFB1 sensitivities should be improved. With these point-of-reference results the group transferred the assay to a 6-plex format (one chip with all six toxins), using the same bio-reagents, in the nanoplasmonics instrument and compared the two assays. The 6-plex portable iSPR assay allowed detection of DON (64 μg/kg), T-2 (26 μg/kg), ZEN (96 μg kg) and FUMB1 (13 μg/kg) at relevant EU levels, although it was less sensitive. The prototype iSPR was therefore shown to have potential for future development for application in rapid in-field and at-line screening of multiple mycotoxins.

From the literature, SPR biosensors from Biacore AB are demonstrated as applicable for mycotoxin testing. Whilst SPR biosensors are widely studied in academic research, their commercialisation for mycotoxin analysis is limited by several factors. For example, the technology and data analysis are not currently usable by non-experts and miniaturisation needs to be optimised to maintain high sensitivity, both of which limit on-site testing.

Furthermore, labelling of reagents to achieve higher sensitivities adds to the overall cost of the potential commercial product. These issues would need to be addressed to achieve an SPR biosensor equivalent test kit.

Mass-Sensitive Sensors

Mass-sensitive sensors are based on electroacoustic technology and are often referred to within the scientific literature as electroacoustic sensors. Electroacoustic technology relies on the transformation of acoustic energy into electric energy or vice versa. This transformation can occur in piezoelectric material "sandwiched" between two electrodes, one of which is functionalised (typically coated with antigen-conjugate) to sense the analyte of interest (*Figure 2***(a)**). When a piezoelectric material is exposed to a force and is mechanically deformed it will generate an electric dipole and an electric voltage. This effect can work in reverse, meaning that if an alternating voltage is applied to a piezoelectric material, it will oscillate and this is known as the piezoelectric effect (Ferreira et al. 2009). Oscillation of the material at its resonant frequency generates an acoustic wave which propagates through the bulk of the material, in a direction perpendicular to the surface. When a sample meets the coated electrode, any binding event will result in slowing of oscillation on the piezoelectric materials surface, thus change the property of the acoustic wave. The wave alteration is detected as a frequency shift from the resonant frequency. This is directly proportional to the mass bound on the electrode, the relationship between which was described by Sauerbrey (1959). Better mass sensitivities are achievable by operating at higher frequencies.

Quartz Crystal Microbalance

The development of quartz crystal microbalances (QCM) for bio-sensing relies on the piezoelectric effect exhibited by quartz crystals (Vashist and Vashist, 2011). A QCM transducer consists of a thin quartz crystal disk in between two gold electrodes, one of which is functionalized to sense the analyte of interest. Like SPR, the sensor is typically coated with antigen-conjugate. QCM has been investigated as a promising transducer for mycotoxin detection providing real-time analysis and high sensitivity. The technology is also simple to operate and offers portability due to the small size of the transducer. Similar to SPR, the functionalised surface of a QCM chip can be regenerated for multiple biosensing runs (Vashist and Vashist 2011). However, QCM assays have suffered as often insufficient signal is generated (frequency change) that is needed for detection in the ng-pg range. In a study by Karczmarczyk et al. (2017) using a QCM based sensing device to detect OTA in red wine, the group aimed to overcome this issue. To reach a lower limit of detection, the signal was amplified using secondary antibody conjugated to gold nanoparticles (AuNPs). Initially, due

to the very low concentration of the primary antibody needed to allow inhibition, the recorded signal for frequency change was weak (Δf =17.97 Hz). Hence, an additional high mass provided by Ab2-AuNPs injection was applied resulting in a signal enhancement (Δf=52.53 Hz) and consequently an improvement of immunoassay sensitivity. This resulted in a linear detection range of 0.2–40 ng mL^{-1} with an LOD of 0.16 ng mL^{-1}, which is one order of magnitude lower than LOD specified by EU legislation concerning the limit of OTA in food. However, the downside to this is that the assay is not label-free and perhaps more time consuming compared to other biosensor techniques on the market for mycotoxin analysis such as SPR. Other methods of enhancing signal response have been explored including the coating process of the QCM transducer. In a study by Chauhan et al. (2016), the group found that in buffer conditions an LOD of 0.008 ng/ml could be achieved with a label-free non-competitive assay by using an electrochemical quartz crystal microbalance (EQCM) based immunosensor coated with self-assembled monolayers of hexandithiol (HDT) and gold nanoparticles (AuNPs) followed by aflatoxin B1 antibody.

QCM biosensors are the most well-established acoustic wave type sensors and commercially available QSense biosensors by the company Biolin Scientific, Sweden can be used for mycotoxin detection. However, commercialisation of a QCM biosensor test kit equivalent is limited by several factors. For example, whilst quartz plates are 330-55 μm thick with a frequency of 5-20 MHz, it is possible to achieve higher mass sensitivities with thinner materials. However, attempting to make quartz crystals thinner is an expensive process. Higher frequency (1-5 GHz) resonators have been developed by thin film technology, through the emergence of Film Bulk Acoustic Resonators (Webner et al. 2006; García-Farrera et al. 2017).

Film Bulk Acoustic Resonators

Whilst biosensors based on thin film bulk acoustic resonators (FBAR) have not yet been used in mycotoxin analysis, literature highlights the potential of this technology as a highly sensitive technique that could be exploited for the detection of low molecular weight compounds like mycotoxins. An FBAR transducer is based on a similar principle to QCM, however, unlike QCM, the piezoelectric material used in FBAR transducers is generally a thin film (0.5-3μm thick) of aluminium nitride (AlN) or zinc oxide (ZnO) (Chen et al. 2015; Rughoobur et al. 2018). AlN is a popular choice due to its high values of chemical inertness, acoustic longitudinal wave velocity, electrical resistivity and its piezoelectric behaviour (García-Farrera et al. 2017). With these thin piezoelectric films, FBAR biosensors can ultimately provide better mass sensitivities than QCM as the FBAR transducer operates at higher frequencies. This nano-manufacturing process is known as the ‘bottom-up’ approach,

building materials up from atomic- and molecular-scale components. QCM however uses the 'top-down' fabrication process, reducing the size of large quartz crystals. Due to the small size of the FBAR transducers, many of them can be integrated within a small area which gives an added advantage of high throughput and cost-effective analysis, with the possibility of developing a hand held portable device (Zhang et al 2018).

Conclusion and Future Outlook

The market today for mycotoxin test kits is already a competitive one with multiple companies selling variations of similar products. However, judging from the literature it is likely that within the next decade, the market will consist of test kits that are hand-held digital biosensors capable of performing multiplex analysis. One contrasting assay development, for mainly biosensors, is that which regards either the use of label-free detection or the addition of labels such as nanoparticles, to increase sensitivity. Here, a trade-off between sensitivity and cost can be made. There is also a chance that using aptamers or MIPs in commercially available assays will become more popular, however, immunoassays are still the current detection method of choice.

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Table 1. Health risks to humans associated with mycotoxins (information gathered from Alshannaq & Yu 2017)

Mycotoxin	Major Species of Producing Fungi	Toxic Effect[i]
Aflatoxins B1, B2, G1, G2	*Aspergillus flavus* and *Aspergillus parasiticus*	Carcinogenic, teratogenic, hepatotoxic, mutagenic, and immunosuppressive effects
Aflatoxin M1	Metabolite of aflatoxin B1	
Deoxynivalenol	*Fusarium graminearum* and *Fusarium culmorum*	Causes nausea, vomiting, diarrhea, abdominal pain, headache, dizziness, and fever
Fumonisin B1, B2, B3	*Fusarium verticillioides*, *Fusarium proliferatum*	Neurotoxic, hepatotoxic, and nephrotoxic effects in animals, and possible carcinogen to humans
Ochratoxin A	*Aspergillus ochraceus*, *Penicillium verrucosum*, and other *Penicillium* species	Acutely nephrotoxic and hepatotoxic. Causes immunotoxicity, genotoxicity, neurotoxicity, teratogenicity, and embryotoxicity
T2/HT2	*Fusarium langsethiae*, *Fusarium poae*, and *Fusarium sporotrichioides*	Toxic effects include growth retardation, myelotoxicity, hematotoxicity, and necrotic lesions on contact sites
Zearalenone	*Fusarium graminearum* and *Fusarium semitectum*	Strong estrogenic activity resulting in changes.
Ergot alkaloids	*Claviceps* spp.	Vasoconstriction, delirium, hallucinations, muscle spasms, diarrhoea and convulsions

[i] A provisional maximum tolerable daily intake (PMTDI) or a Tolerable Daily Intake (TDI) has been established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) for each mycotoxin. For all AF's Intake should be reduced to levels as low as reasonably possible as it is a genotoxic carcinogen. DON PMTDI is 1 µg/kg bw/day. FUM PMTDI is 2 µg/kg bw/day for FB1, FB2, and FB3 alone or in combination. OTA PMTDI is 0.112µg/kg bw/week. T2 and HT2 Toxins PMTDI is 0.06 µg /kg bw/day. ZEA PMTDI is 0.5 µg/kg bw/day. PATULIN PMTDI 0.4 µg/kg bw/day. Ergot alkaloids TDI 0.06 µg /kg bw (JECFA 2017).

Table 2. Companies that sell commercial test kit products for mycotoxins

		Mycotoxin					
Company	**Product Type**	AF	DON	T2/HT2	ZEN	OTA	FUM
Elbascience	ELISA	✓	✓	✓	✓	✓	✓
	LFA	✓	✓	-	✓	-	-
Envirologics	ELISA	-	-	-	-	-	-
	LFA	✓	✓	✓	✓	✓	✓
Europroxima	ELISA	✓	✓	✓	✓	✓	✓
	LFA	-	-	-	-	-	-
Helica	ELISA	✓	✓	-	✓	✓	✓
	LFA	-	-	-	-	-	-
Neogen[i]	ELISA	✓	✓	✓	✓	✓	✓
	LFA	✓	✓	✓	✓	✓	✓
R-Biopharm	ELISA	✓	✓	✓	✓	✓	✓
	LFA	✓	✓	✓	✓	✓	✓
Randox[ii]	ELISA	✓	-	-	-	-	-
	LFA	-	-	-	-	-	-
Romer Labs[iii]	ELISA	✓	✓	✓	✓	✓	✓
	LFA	✓	✓	-	✓	-	✓
Tecna Diagnostics	ELISA	✓	✓	✓	✓	✓	✓
	LFA	✓	✓	-	✓	-	✓
Vicam[iv]	ELISA	-	-	-	-	-	-
	LFA	✓	✓	-	✓	✓	✓
Unisensor	ELISA	-	-	-	-	-	-
	LFA	✓	✓	✓	✓	✓	✓

[i] Neogen offer a fluorometric assay for AF's
[ii] Randox offer an ELISA for testing Ergot Alkaloids.
[iii] Romer Labs offer a fluorometric assay for AF's
[iv] Vicam offer fluorometric assay's for AF's, ZEN, OTA and FUM.

Table 3 Different biosensor techniques developed for mycotoxin analysis

Biosensing Device	Matrix	Analyte	LOD (µg kg−1)	Reference
SPR	Milk	AFM1	0.018	Karczmarczyk et al 2016
iSPR	Beer and barley	DON, OTA	17, 7	Joshi et al 2016 (2)
iSPR	Barley	DON, T-2, ZEA, FB1	64, 26, 96, 13	Joshi et al 2016 (1)
SPR	Wine and peanut oil	OTA	0.005	Zhu et al 2015
QCM	Buffer	OTA	17.2	Pirincci et al 2018
QCM-D[i]	Red wine	OTA	0.16	Karczmarczyk et al 2017
EQCM	Cereal	AFB1	0.008	Chauhan et al 2016

[i] Quartz crystal microbalance with dissipation monitoring.

Figure 1 & *2* Key:

= Analyte in sample/standard

= Antibody

= Labelled analyte

= Labelled Antibody

= Solid support

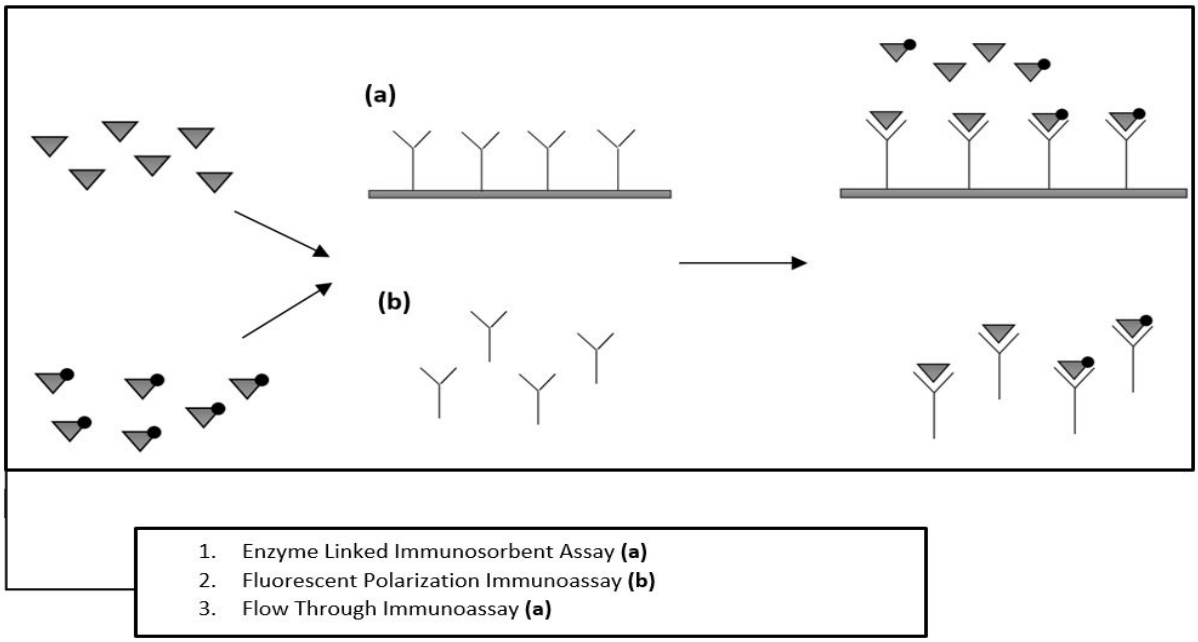

Figure 1. A competitive immunoassay format where the antibody is either **(a)** immobilised on the solid support surface or **(b)** suspended in solution.

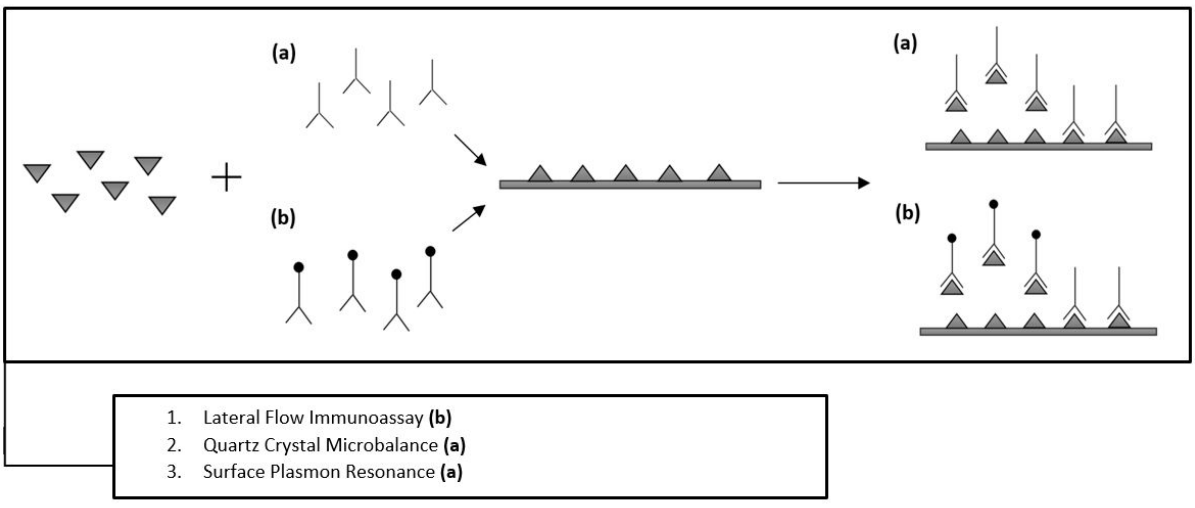

Figure2. A competitive immunoassay format where the antigen is immobilised on the solid support surface and the antibody is either **(a)** not labelled or **(b)** labelled.