Chemiluminescent and bioluminescent assays as innovative prospects for mycotoxin determination in food and feed

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ABSTRACT: Mycotoxin contamination of food and feedstuffs is among the top priorities for human and animal safety. The currently used techniques for mycotoxin determination, either chromatography or ELISA, are unsuitable for routine in-field assessment. There is an urgent need for other accurate, simple and cost-effective techniques that can be used as a screening tool for a rapid estimation of mycotoxin contamination in commodity lots. This paper reviews the literature on the use of chemiluminescence (CL) and bioluminescence (BL) assays for direct or indirect mycotoxin assessment. The chemiluminescence immunoassays, adenosine triphosphate (ATP) bioluminescence and bioassays are reviewed and their advantages and limitations discussed. These techniques used in food testing and the pharmaceutical industry offer promise as rapid techniques for mycotoxin determination. Chemiluminescence and bioluminescence bioassays are the most innovative alternatives to the conventional techniques used for mycotoxin determination in food and feed. Copyright © 2004 John Wiley & Sons, Ltd.

KEY WORDS: mycotoxin, bioluminescence, chemiluminescence, bioassay, food testing

INTRODUCTION

Mycotoxin contamination of food and feedstuffs is among the top priorities for human and animal safety. Consumers are more and more concerned by public health-related issues and show high preoccupation about the risks associated with human exposure to mycotoxins. Indeed, mycotoxins are fungal metabolites that have been shown to be carcinogenic, mutagenic, teratogenic and immunosuppressive (1). Furthermore, mycotoxins have attracted worldwide attention because of the significant economic losses associated with their impact on human health, animal productivity and both domestic and international trade (2, 3).

The main mycotoxins currently considered of importance include the aflatoxins, the tricothecenes, zearalenone, the fumonisins and ochratoxin A. They can occur in various commodities, such as cereal grains, pulses, nuts, milk and dairy products, coffee and wines (4, 5). The control of mycotoxins is currently pursued through quality and regulatory procedures. Limits of these substances in traded goods are becoming more and more restrictive. The techniques currently most used for mycotoxin determination are: (a) chromatography, including high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC) and gas chromatography–mass spectrometry (GC–MS); and (b) ELISA techniques. Considering the limitations of these techniques (see next section), there is still an urgent need for other accurate, simple and cost-effective techniques that could be used as a screening tool for a rapid estimation of mycotoxin contamination in commodity bulks. Luminescence assays currently used in food testing and the pharmaceutical industry (6) could be promising as rapid and sensitive techniques.

This article reviews the bioluminescence (BL) and chemiluminescence (CL) methods that have been investigated for mycotoxin analysis and discusses their advantages and limitations for the determination of mycotoxins in food and feed.

STATE OF THE ART ON ANALYTICAL METHODS FOR MYCOTOXIN DETERMINATION

The assessment of contamination is based on the analytical determination of mycotoxins. Conventional methods include: high-performance liquid chromatography (HPLC), with either fluorescence or diode array detection; thin-layer chromatography (TLC); and gas chromatography coupled to mass spectrometry (GC–MS) or electron capture detection (GC–ECD). These methods are used for determination of different types of mycotoxins, such as aflatoxins, ochratoxin A, fumonisin and deoxynivalenol (DON) in different commodities, such as coffee (7–9), cereal and legume grains (10, 11),

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black pepper (12), wine and beer (5, 13, 14) and cheese (15). However, these methods need sophisticated equipment and trained analysts, and the procedure for mycotoxin extraction from the sample must be adapted according to the type of mycotoxin and food matrix to be tested. Progressively, improvements were made to the chromatographic methods through the introduction of clean-up procedures by use of immunoaffinity columns and post-column derivation, but this has increased the cost of analysis. Traditional methods involve lengthy extraction procedures, expensive chemical clean-up and require the handling of hazardous materials, all factors which make such tests unsuitable for routine use in the food industry or in a farm setting (16).

The availability of rapid diagnostics for detection of toxins in the food chain is an area which has developed rapidly in the last 10 years, predominantly in response to legislative requirements (17). This orientated analytical mycotoxin assessment towards the application of enzyme-linked immunosorbent assays (ELISA). Most commercial ELISAs for mycotoxins rely on competition between the toxin from the sample with a labelled toxin (such as a toxin–enzyme conjugate) for a limited number of antibody-binding sites. The greater the amount of toxin present in the sample, the lower the binding of the labelled toxin and the lower the signal generated by the assay (18–20).

Research was initially performed on experimental matrices, such as mycotoxin standards, urine or blood serum samples, or extracts from pure mould cultures. Later on, ELISA techniques were adapted for mycotoxin determination (21–25). Many commercial kits are now available, such as Ridascreen kits for aflatoxin, fumonisins, DON and zearalenone (R Biopharm Ltd), Aflatox Cup (International Diagnostic System) and Aflatoxin B test (Cite Probe). They are simple and easy to use, with a moderate to high cost, according to the context of use. However, ELISA techniques have been shown to be less accurate and sensitive than conventional chromatographic assays. Very few correlations were found between the two types of techniques. In addition, false positive or false negative results often occurred with ELISA because of cross-reactions between molecules or interferences with the antibody reagents. They are thus considered to be suitable for qualitative assessment or for sample pre-screening but not for quantitative determination. It is also recommended to use the ELISA techniques for the foods they were developed for. It should be pointed out that the ELISA techniques are nowadays mostly used by industries for rapid monitoring, whereas chromatographic methods are still used in laboratories for investigation purposes.

Fluorescence polarization immunoassays (FP) have been developed in a laboratory context for aflatoxin (26) and DON (27) determination in grains. These assays are based upon the competition between free aflatoxin and an aflatoxin–fluorescein tracer for an aflatoxin-specific monoclonal antibody in solution. FP assays have two important differences from ELISA: the detection does not involve an enzymatic reaction, and separation of the bound and free label is not required. As a result, FP assays do not require the wash step essential to many ELISAs, and they do not require waiting for an enzyme to produce a coloured product. Following the same approach, the enhancement of luminescent capacity of mycotoxins through immunoenzymatic assays could be used as an indicator for contamination assessment.

Biomolecular techniques, such as polymerase chain reaction (PCR), are emerging as promising tools for mould detection, rather than mycotoxin determination (28). However, these techniques require expensive investment and trained analysts and can only serve for detecting the responsible coding genes of mycotoxin-producing moulds but not for routine in-field assessment of contamination, especially in developing countries.

**CL IMMUNOASSAYS**

Chemiluminescence is based on a chemical reaction that can be described as follows:

\[ A + B = C^* + D = C + \text{light} \]  

(29), where * indicates an electronically excited state.

In aqueous solution, the most frequently used CL compound is luminol (5-amino-2,3-dihydrophtalazine-1,4-dione or its derivatives, such as isoluminol). Luminol reacts with \( \text{H}_2\text{O}_2 \) in the presence of a catalyst (metal or metal-containing compound or enzyme) in alkaline solution to yield 3-aminophthalate in an excited electronic state, which returns to the ground state with the production of light. However, the intensity of the light signal is low and of short duration. To amplify and prolong the signal, a compound known as ‘enhancer’ (e.g. 4-iodophenol) is added to the reaction medium (‘enhanced chemiluminescence’). The enhanced CL reaction is one of the most sensitive and rapid detection methods in medical and analytical biochemistry (30, 31).

This luminescent reaction can be used for the detection of antigen–antibody binding at the final stage of an immunoenzymatic assay. Such a solid-phase chemiluminescent immunoassay (CIA) is described below (Fig. 1). This technique was used to analyse ochratoxin A and the results were compared to a conventional ELISA test [OcA–HRP (horseradish peroxidase) conjugate] (32). The CL compound used was ABEI (N-4-aminobutyl-N-ethyl-isoluminol) and this was conjugated to the mycotoxin antigen (OcA–ABEI).

As shown in Fig. 1, the assay protocol is based on the following steps:
Figure 1. Solid-phase chemiluminescent immunoassay, as described by Kim et al. (32) for ochratoxin A (OcA). ABEI, N-4-aminobutyl-N-ethyl-isoluminol.

1. Addition of the sample or standard solution to the antibody-coated tube.
2. Addition of the tracer OcA–ABEI for the CIA assay.
3. The mixture is incubated at 37°C/2 h.
4. Removal of the solution and 3 times washing of the antibody-bound fraction with phosphate buffer.
5. Addition of NaOH (5 mol/L) and incubation at 60°C/1 h.
6. Injection of the microperoxidase solution and diluted H₂O₂ to the assay tube in the luminometer.
7. Integration of the signal for 4 s.

There was an excellent correlation ($r = 0.996$) obtained between the results of ELISA (OcA–HRP) and those of the CIA (OcA–ABEI) and this shows that the CL technique could replace conventional ELISA. The detection threshold for the CL immunoassay was 20 pg ochratoxin A/tube. This sensitivity is similar to that obtained with ELISA for ochratoxin A analysis in wheat (33). Known quantities of ochratoxin A were added to corn samples which were analysed before and after this step. In these conditions, less than 70% of the added material was recovered. This loss may probably arise from the extraction procedure, which constitutes a major problem for mycotoxin food determination. According to this observation, this CL immunoenzymatic assay cannot yet be used in routine testing for agricultural commodities.

A similar limitation has been observed by Wittmann and Schreiter (34), who tested a CL ELISA to quantify a herbicide, terbutylazine (s-triazines class) in soil samples. This interesting study used a peroxidase label, and the label was detected using the enhanced CL reaction (detection reagents: luminol + p-iodophenol + hydrogen peroxide in NaOH/borate buffer, pH 8.5).

The thresholds of detection obtained by the various methods were as follows:

- ELISA (HRP label): 0.3–3 µg/L.
- ELISA (alkaline phosphatase label) detected by reflectance: 3–300 µg/L.
- ELISA (with HRP label) detected by CL: 0.05–10 µg/L, showing a lower detection limit and a larger range of measurement.

The ELISA with luminescence detection showed a superior measuring range but failed in the measurement of the soil samples collected from the environment. Indeed, the authors analysed samples from the environment according to three methods: (a) gas chromatography; (b) reflectance immunoassay (alkaline phosphatase); and (c) CL immunoassay (HRP). The first two techniques gave similar results, whereas the CL ELISA type using an HRP label revealed several false positive results.

It can be presumed that either a matrix effect, an interference affecting the detection method or antibody cross-reactivity could be the potential source of the overestimation. However, the source of the discrepancy was not determined. The CL immunoassay format must therefore be improved before it can be used as a routine field testing method.
In the context of enhanced CL assays, it is important to note that buffers are not inert and should be considered as a crucial fourth component of the HRP-enhanced CL light-generating system (35). Cercek et al. (36) shows that, at the same pH and HRP concentration, the magnitude of the light signal (RLU) is significantly influenced by the reagent buffer composition, and could increase up to 10-fold, e.g. at pH 7.3 and HRP concentration 4 fmol/40 µL assay, the glycine buffer signal is 10 times higher than that obtained with tricine buffer. At pH 8.5 and 1.6 fmol HRP/40 µL assay, the borax buffer gives the highest magnitude, which is 10-fold higher than with glycylglycine buffer.

The CIA method has been used by Kang et al. (37) to determine the production, under artificial conditions, of ochratoxin A by several fungi strains (from the genera Aspergillus, Penicillium, Paecilomyces) isolated from Korean traditional fermented soybean foodstuffs (Maeju, Dwangjang and Kangjang). The method was accurate in the 20 pg/assay range with a 90% recovery in laboratory cultures, but determination of mycotoxin directly from the fermented soybean foodstuff was not indicated.

**ATP BIOLUMINESCENCE**

Another alternative to the direct determination of mycotoxins in food is the detection of fungi in the sample. We are aware that the presence of fungi is not a direct determination of the presence of mycotoxins but it could serve as a screening test to distinguish potential mycotoxin-producing food lots. ATP bioluminescence has been mostly used for determining yeast or bacterial contamination in food (38–44). It is a rapid technique, based on the reaction between microbial ATP and firefly luciferase to produce luminescence. The microbial ATP is extracted with acid, organic solvent or detergent after the removal of somatic ATP (from non-microbial cells, if any) together with any free ATP using ATPase. Luciferase is then added and the emitted light is measured using a luminometer.

The amount of light may be directly related to the microbial number (45, 46) but both difficulties of extracting ATP from moulds and distinguishing it from the bacterial ATP could explain why this method is not recommended for moulds (47). Kaspersson et al. (48) measured the total ATP contents by BL in several foodstuffs during storage and correlated it to the count of bacteria, yeasts and moulds. They showed that the best correlation was obtained with bacteria and the worst on food contaminated by moulds.

**BIOASSAYS**

Tests using bacteria as indicators are widely used to identify the toxicity of chemicals or commercial products, and environmental pollution (49, 50). Both CL and BL have been investigated to assess the effects of mycotoxins on biological systems. We review the principles of these different CL- and BL-based methods to show how they could be innovative for toxicological assays applied to mycotoxins.

Nishimoto (51) tested the toxicity of toxins from *Fusarium* species on viable human cells (HuH-6KK). This CL assay was not intended to determine mycotoxin concentrations, but instead to evaluate their toxic effect on cellular cultures. The cells (HuH-6KK, NIH3T3, PC-3) were trypsinized and used to prepare $2 \times 10^4$ cells/well (500 µL) in 24-well tissue culture plate and the cells mixed with the *Fusarium* toxins (NIV, DON, FX) and incubated for 48 h (Fig. 2). The viability was then

![Figure 2. Mechanism of the production of H$_2$O$_2$ catalysed by exogenous menadione in cell culture (51).](image-url)
assessed by the CL technique, which consists of adding a solution of menadione/ethanol into the medium for incubation at 37°C for 10 min. After that, 50 µL CL reagent [10 mg bis(2-(3,6,9-trioxadecanyloxycarbonyl-4-nitrophenyl) oxalate + 1 mg pyrene in 10 mL acetonitrile] was added and the total emitted light measured by integration. The results show that the production of H2O2 through the mechanism described above is proportional to the cellular enumeration. Based on these findings, human cell cultures (HuH-6KK) were monitored in presence of various Fusarium mycotoxins—nivalenol (NIV), deoxynivalenol (DON) and fusarenon-X (4-acetylnivalenol, FX). The cellular activity was measured by CL after 24 h of incubation and was compared to the conventional methods: (a) neutral red inclusion assay; and (b) MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] reduction assay. The viability curves showed a perfect correlation between these three methods. Indeed, the CL assay was superior in speed and sensitivity for the detection of cytotoxicity, the intensity of which was observed in the following order: FX > DON > NIV. This assay makes possible the assessment of the cytotoxic effect of Fusarium toxins on human cellular cultures (HuH-6KK). Similar results were obtained on mouse fibroblasts (NIH3T3) and human prostate cancer cells (PC-3).

Another toxicity test is based on the natural genetic BL potential of bacteria due to specific genes called ‘lux genes’. The bacterial luminescence reaction (Fig. 3) is catalysed by luciferase and can be summarized as follows (52):

\[
\text{RCHO} + \text{FMNH}_2 + \text{O}_2 = \text{RCOOH} + \text{FMN} + \text{H}_2\text{O} + \text{hv}
\]

Luciferase is an enzyme composed of two subunits, \( \alpha \) and \( \beta \), encoded by genes \( \text{luxA} \) and \( \text{luxB} \), respectively (53). These \( \text{lux} \) strains belong to genera such as Vibrio, Photobacterium, Xenorhabdus and Alteromonas. Two species, Vibrio fisheri and V. harveyi, have been investigated intensively. In both, \( \text{lux} \) genes are organized in an operon together with other genes involved in the BL reaction. Expression of the \( \text{lux} \) operon in both strains undergoes a specific regulation, called ‘quorum sensing’ (54). In the presence of inhibiting substances such as antibiotics, other antimicrobial agents, bacteriophages, or toxic substances such as mycotoxin, the reduced numbers of bacteria can be detected by BL through the determination of effective concentration \( \text{EC}_{50} \), which corresponds to the mycotoxin concentration that causes a 50% reduction of bioluminescence.

The use of bacterial BL as a toxicological assay for mycotoxins was investigated with V. fisheri (formerly Photobacterium phosphoreum). The protocol is described by Bulich and Isenberg (55). It uses the Microtox™ method (registered trademark of Beckman Instruments) with a freeze-dried luminescent bacterium, V. fisheri. The sample is adjusted to 2% NaCl and then diluted using the diluent provided (osmotic protection to the marine microorganism). The luminescent bacteria are hydrated with the reconstitution solution provided, cooled to 3°C, and aliquots are transferred to cuvettes containing 0.5 mL diluent equilibrated to 15°C. Initial light measurement is made for each cell suspension. Sample dilutions and the control are then added and the light is again measured after 5 min for each cuvette. Using this method, Yates and Porter (56) studied the effect of different mycotoxins (patulin, penicillic acid, citrinin, zearalenone, ochratoxin A, aflatoxin B1, rubratoxin B). Bioluminescence determinations were made with the Microtox® analyser at 5, 10, 15 and 20 min after addition of the toxin to the microbial suspension. All these mycotoxins induced an \( \text{EC}_{50} \) (µg/mL) after 5 min of incubation ranging from 7.51 µg/mL for patulin to 31.79 µg/mL for rubratoxin B. The inhibitory action of all mycotoxins approached the maximum effect by 15 min. These assays depend much on the nature of the mycotoxin, the pH, the temperature,

\[\text{Figure 3.} \quad \text{Mechanism of bacterial bioluminescence: FMN, flavin mononucleotide; RCHO, long-chain aldehyde; RCOOH, long chain fatty acid (52).}\]
the age of the bacteria and the time exposure. With the exception of zearalenone, the longer the incubation time, the less the concentration of toxins required to induce a EC_{50} of *P. phosphoreum*.

When increasing the pH of the assay suspension from 6.0 to 8.0 (testing at pH 6.0, 6.5, 7.0, 7.5 and 8.0), the toxicity of zearalenone and penicillic acid decreased and, inversely, the toxicity of patulin increased. Aflatoxin B1 demonstrated its greatest toxicity at pH 7.5. Each mycotoxin showed its greatest toxicity at a different pH and temperature combination: patulin, pH 8.0; 30°C; penicillic acid, pH 6.5/25–30°C; zearalenone, pH 6.0/10–15°C, and aflatoxin B1, pH 7.0–7.5/20–30°C. This study showed that optimizing pH and temperature for each mycotoxin enhanced the sensitivity of the bacterial BL assay (57).

These results demonstrated a reliable short-term method for assessing the toxicity of mycotoxins. The advantage of this system is its sensitivity and simplicity. Such a procedure may serve to discriminate food samples, but it should be emphasized that the food sample extract (corn, maize or wheat) might contain other fungal metabolites or pesticide residues accumulated in the grains and which could also have an inhibitory effect on the bacterial activity.

Krataisky et al. (58) developed a luciferase-based BL test for monitoring wheat grain (*Triticum vulgare*, type IV) infection with *Fusarium*. The test kit ‘NADH-KRAB’ contains: (a) NADH-reagent (lyophilized preparation of luciferase and NADH;FMN oxidoreductase); (b) 0.2% alcohol solution of tetradecanal; and (c) FMN and NADH. The results show that infected grain extract inhibits bioluminescence, and that the inhibition increases when the content of infected wheat grains increases in the mixture sample (consisting of intact and infected grains). The maximum inhibitory effect was observed when the content of infected grains was 0.5–1.5%, which corresponds to a DON (the main mycotoxin produced by *Fusarium*) concentration of 0.5–1.5 mg/kg. The authors proposed that this method measures a total toxicity rather than just the mycotoxin effect. They proposed to use it as a rapid screening method for rejecting infected grain batches in production.

**CONCLUSION**

We should emphasize that mycotoxin management is more and more switching from an expensive wasteful end-point testing/segregation towards an integrated, systematic and cost-effective approach throughout the whole agrifood chain. This increases the need for analytical tools that are accurate, cost-effective and suitable for *in situ* routine control. While a range of kits exist today, there are still limitations to their use *in situ* and the economic costs can still deter use, mainly in developing countries, even when they are heavy exporters of commodities. This article reviews the literature on BL and CL techniques for mycotoxin determination in food and feed. The CL immunoassay and BL-based bioassays showed good sensitivity and accuracy in laboratory studies. These methods constitute an innovative alternative to conventional techniques for mycotoxin analysis. However, they need to be improved before they can be applied in large-scale food production testing.

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