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Development of an electrochemical immunosensor for aflatoxin M_1 in milk with focus on matrix interference

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ABSTRACT

A simple sensor method was developed for aflatoxin M_1 analysis to be applied directly with milk by using antibody modified screen-printed carbon working electrode with carbon counter and silver–silver chloride pseudo-reference electrode. A competitive ELISA assay format was constructed on the surface of the working electrode using 3,3,5′,5′-tetramethylbenzidine dihyrochloride (TMB)/H_2O_2 electrochemical detection scheme with horseradish peroxidase (HRP) as the enzyme label. The performance of the assay and the sensor was optimised and characterised in pure buffer conditions before applying to milk samples. Extensive interference to the electroanalytical signal was observed upon the analysis of milk. Through a series of chemical fractionations of the milk, and testing the electrochemical properties of the fractions, the interference was attributed to whey proteins with focus towards α-lactalbumin. A simple pre-treatment technique of incorporating 18 mM calcium chloride, in the form of Dulbucco's PBS, in a 1:1 ratio to the milk sample or standards and also to the washing buffer stabilised the whey proteins in solution and eliminate the interfering signal. The resulting immunosensor was interference free and achieved a limit of detection of 39 ng l^{-1} with a linear dynamic detection range up to 1000 ng l^{-1}. The developed immunosensor method was compared to a commercial ELISA kit and an in-house HPLC method. The immunsensor was comparable, in term of sensitivity, but vastly superior in term of portability and cost therefore a key instrument for the detection of aflatoxin M_1 at the source of the contamination.

1. Introduction

Although the first reported cases of mycotoxicoses was in 1722, not until 1960 was there significant research into the causes of mycotoxicoses with the onset of 'turkey X' disease (Farrer, 1987). At that time the mould *Aspergillus flavus* was isolated and correlated with aflatoxin production. Although *A. flavus* can grow in range of temperatures (10–45 °C), the optimum temperature is 30 °C. Additionally a relative humidity of 80% is required; hence aflatoxin contamination is more of a concern in humid tropics regions (Moreau, 1979). It was also recognised that ruminants upon the consumption of aflatoxin B_1 contaminated feed would excrete aflatoxin M_1 through milk (Sargeant et al., 1961; Holzapfel and Steyn, 1966). Subsequently it has been shown that alfatoxin B_1 can also be produced to a lesser extent by *A. parasiticus*. It has been postulated that aflatoxin M_1 is a detoxification product of aflatoxin B_1 since the carcinogenicity of aflatoxin M_1 is lower than aflatoxin B_1 (Neal et al., 1998). However, aflatoxin M_1 is still regarded as carcinogenic, genotoxic, teratogenic and immunosuppressive compound. Reports have hypothesised that the excretion of aflatoxin M_1 is between 1 and 4% of the amount of ingested aflatoxin B_1 for cows milk (van Egmond, 1983).

Alfatoxin M_1 can be found in dairy based products such as cheese, yogurt and infant formulae (van Egmond, 1983; Sharman et al., 1989; Martins and Martins, 2004), and also in human breast milk and acts as a good biomarker (El-Nezami et al., 1995). Due to the fact that milk intake in infants is high and when young they are vulnerable to toxins, the European Commission regulation 472/2002 imposes maximum permissible levels of aflatoxin M_1 in milk of 50 and 25 ng l^{-1} for infant formulae (Henry et al., 2001; Gilbert and Vargas, 2003). Austria and Switzerland have imposed stricter limits of 10 ng l^{-1}, whereas the USA have higher regulatory of 500 ng l^{-1}. Although most concerning is many underdeveloped countries do not impose aflatoxin M_1 restrictions.

The official methods of analysis for aflatoxin M_1 rely upon high performance liquid chromatography (HPLC) or thin layer chromatography (TLC) (Sydenham and Dhephard, 1996) with sample extraction and clean up conducted before the analysis. Immunochemical techniques are becoming very popular for mycotoxins analysis with many literature reporting the use of either a commercially developed enzyme linked immunosorbant assay (ELISA) or self-developed immunoassays (El-Nezami et al., 1995; Thirumala-Devi et al., 2002; Lopez et al., 2003; Rodriguez Velasco et al., 2003; Rastogi et al., 2004; Sarimehmetoglu et al., 2004; Logrieco

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et al., 2005). Additionally liquid chromatography–mass spectrometry (LC–MS) (Sørensen and Elbæk, 2005) has also been employed. All of these methods are slow and most are performed in laboratory settings and by qualified personnel. Unfortunately the regions of the world which are most affected by aflatoxin contamination tends to be poorer areas with minimal laboratory facilities. In India, for example, a recent survey found that 87.3% of the milk-based samples analysed were contaminated, of these 99% were outside European limits. This is a major concern considering that India is the largest producer of milk in the world (Rastogi et al., 2004). Therefore as stipulated by the united nations '*there is an urgent need for simple, robust, low-cost analysis methods, for the major mycotoxins, which can be used in developing countries laboratories*' (Proctor, 1994). Furthermore the United Nations are quoted saying that '*the systematic and complete monitoring of aflatoxin is a major challenge for the future, as food production increases*' (Stroka and Anklam, 2002).

In this paper we present a cost effective, disposable immunosensor for the detection of aflatoxin M_1 which can be preformed in the field to meet the detection requirements set out by the European Union and fulfilling the requirements quoted by the United Nations. Primarily, the two main enzyme substrates used for immunosensors are alkaline phosphatase and horseradish peroxidase. Volpe et al. (1998) reported that using 3,3′,5,5′-tetramethylbenzidine (TMB) as an enzyme substrate for horseradish peroxidase yields greater sensitivity than substrates for alkaline phosphatase. Furthermore with the designed immunosensor to be used in raw milk, naturally present alkaline phosphatase potentially may cause interference. Using TMB as a substrate is re-enforced by Fanjul-Bolado et al. (2005) who reported that TMB out performs 2,2′-azino-bis-(3-ethyl-benzthiazoline-6-sulfonic acid) (ABTS) and *o*-phenylenediamine (OPD), furthermore OPD and ABTS have shown to be mutagenic and carcinogenic (Voogd et al., 1980).

The oxidation of TMB is a two-step reaction. Firstly the addition of hydrogen peroxide to heme group containing HRP enzyme, reduces the HRP to form an intermediate (compound 1), involving a 2-electron process, by changing the heme (Fe^{3+}) group into a ferryl oxo iron (Fe^{4+}=O) and a porphyrin (P) cation radical. Upon the addition of TMB, 2 molecules of TMB are oxidised by compound 1 to form a blue coloured electrochemical product. Upon the release of H_2O the peroxidase returns to the native state via a further intermediate, leaving the TMB in an oxidised state. Commonly sulphuric acid is added to the oxidised TMB to develop a stable yellow diiamine product that is measured at 450 nm and can be measured by differential pulsed voltammetry (Josephy et al., 1982; Ruzgas et al., 1996; Frey et al., 2000; Tanaka et al., 2003).

In this work we report the development of a screen-printed electrode immunosensor, based on a competitive reaction between the free aflatoxin M_1 in the sample and an aflatoxin M_1–horseradish peroxidase conjugate, for an immobilised monoclonal antibody for aflatoxin M_1. Using chronoamperometry, the signal generated by the use of TMB/H_2O_2 was monitored to ascertain the concentration of HRP on the sensor and consequently the concentration of aflatoxin M_1 in the sample. The immunosensor was optimised with regard interferences from the milk matrix. The simple method of milk sample pre-treatment which was developed in this work and combined with the optimised sensor is novel and being reported for the first time in this application.

2. Materials and methods

2.1. Reagents and solutions

Aflatoxin M_1 was purchased from Axxora UK Limited (Nottingham, UK), Anti-aflatoxin M_1 antibody (raised from rat) from Abcam Limited, (Cambridge, UK), Aflatoxin M_1–HRP conjugate from a RIDASCREEN® kit from R-Biopharm (Glasgow, UK) as well as Alfaprep® M immunoaffinity columns. 3,3′,5,5′-Tetramethylbenzidine dihydrochloride, hydrogen peroxide, fish skin gelatine, polyvinyl alcohol, polyvinylpyrrolidone and Tween 20 purchased from Sigma–Aldrich (Poole, UK). Anti-rat immunopure antibody (raised in goat with affinity for the Fc fragment only) was from Perbio Science (Cramlington, UK). Milinex sheets from Cadillac plastics (Swindon, UK), Electrodag 423-SS graphite ink, Electrodag 6038-SS Ag/AgCl from Acheson industries (Plymouth, UK), Blue epoxy insulating ink 242-SB, from ESL electroscience products (Reading, UK), Milk and dried milk samples were obtained from the local supermarket.

2.2. Electrodes fabrication

Screen-printed electrodes (SPEs) were fabricated in-house by a multistage deposition process using a DEK 248-screen printer and stencils (DEK, Weymouth, UK) (Kadara and Tothill, 2004). The electrodes were printed using 250 μm thick polyester Melinex sheets. The print parameters were set so that the squeegee pressure was 4 psi, a carriage speed of 50 mm s^{-1} and a print gap of 2.5 mm. For the fabrication, the basal tracks for the three-electrode system were printed first using Electrodag 423-SS graphite ink. The reference electrode was printed on one of the basal tracks using Electrodag 6038-SS silver–sliver chloride ink and left to dry. The two other tracks (graphite-carbon working electrode with a 5 mm diameter giving a 19.6 mm^2 planar area and a graphite carbon counter electrode (1.3 mm^2 planar area). The blue epoxy insulating layer was printed last using 242-SB protective polymer. Between each layer the sheets were allowed to dry for 2 h at 60 °C and then after the insulating layer the sheets were cured at 120 °C for 2 h. The different inks used and the polyester sheet used in the sensor fabrications are stable at this temperature.

2.3. Procedures

2.3.1. Electrochemical measurements

For the electrochemical procedures a computer controlled four-channel Autolab electrochemical analyser multipotentiostat (Eco Chemie, Utrecht, The Netherlands) was used throughout which allows the simultaneous detection of four sensors. Data capture was through the supplied GPES version 4.9 software installed onto a PC. The screen-printed electrodes were connected to the Autolab, using an in-house fabricated connector from a PCB edged IDC socket, aluminum instrument box, ribbon cable and 4 mm cable sockets. The individual components were purchased from Maplin Electronics (Milton Keynes, UK). For the C.V. scans a 100 μl of sample drop was placed onto the electrode and was disposed of after each scan. The scanning range was from −1 to +1 V at a rate of 99.78 mV/s with steps of 2.74 mV. Studies into the suppression effects of milk used samples of milk with different pre-treatments mixed with 5 mM potassium hexacyanoferrate (III) in 0.1 M KCl.

2.3.2. Immunoassay developments

For the sensor construction, 8 μl of 0.12 mg ml^{-1} anti-primary antibody in 0.1 M carbonate buffer pH 9.6 was placed onto the working graphite electrode, placed into a humid environment (stored overnight at 4 °C), to allow passive adsorption of the antibody onto the carbon surface. The sensor was then washed with 0.05% Tween 20 in 10 mM PBS buffer and 18.0 MΩ water. The electrodes were then shaken to remove most of the surplus water and anti-aflatoxin M_1 monoclonal antibody at 0.04 mg ml^{-1} (8 μl) in 10 mM PBS buffer was added and incubated for 2 h at 37 °C, in a humid environment. The surface of the sensor was then blocked by immersed in 1% PVA in PBS to cover the working, reference and auxiliary electrodes for 2 h at 37 °C. The sensor was then washed and stored at 4 °C until used.