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Biosensors and multiple mycotoxin analysis

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Abstract

An immunochemical biosensor assay for the detection of multiple mycotoxins in a sample is described.

The inhibition assay is designed to measure four different mycotoxins in a single measurement, following extraction, sample clean-up and incubation with an appropriate cocktail of anti-mycotoxin antibodies.

The different mycotoxins could be detected simultaneously in relevant concentrations within a time frame of 25 minutes, including the time needed for sensor regeneration.

The application of the developed assay on presently available miniature SPR devices allows the development for low-cost instruments, which can be used in field measurements.

1. Introduction

Mycotoxins are toxic fungal metabolites that can occur in primary food products such as nuts, cereals and fruits as a result of mould growth. Some mycotoxins have been proved strong carcinogenic agents like aflatoxin B1; others are under suspicion to have carcinogenic effects. Currently a few hundred mycotoxins are known which are often produced by the genera *Aspergillus*, *Penicillium* and *Fusarium*. The most prominent toxins are aflatoxins, deoxynivalenol (DON), zearalenone, ochratoxin, fumonisin and patulin.

There is an increasing awareness of the hazards imposed on both human and animal health by mycotoxins present in food and feed. Legislation regarding the allowed levels of mycotoxins present in food and feed products and in raw materials is presented by the FAO in 1995 (FAO, 1995). Therefore, a need exists for a reliable, economical and easy to use assay for the measurement of the mycotoxin contents, especially in the raw materials for food and feed production.

Common methods for mycotoxin determination include TLC, ELISA and HPLC. Several methods using one of these detection techniques have AOAC approval. Especially the HPLC method is a very sensitive method, which however is also the most costly and time consuming due to extraction and cleanup procedures necessary. The assay that is presented here is designed as an inhibition assay, in which the principle of detection is based on surface plasmon resonance (SPR). In principle SPR is a measurement of mass concentration changes that occur at the sensor surface due to binding of molecules. At a specific wavelength and angle of incidence, light will be absorbed by free electrons in the thin metal film of the sensor surface and the intensity of the reflected light will decrease. The angle where this phenomenon takes place, changes as the mass concentration on the sensor surface changes. The changes in mass concentration thus measured by the biosensor are those due to binding and dissociation of interacting molecules, in this case between the immobilised antigen (mycotoxin) and the specific antibody added to the sample. For a more detailed description of the phenomenon and technique used (see Johnsson, Löfås, & Lindquist, 1991). An example of an aflatoxin B1 assay based on this technique has been described by Daly et al. (2000).

With the SPR equipment used, the BIACORE 2000 (see Fig. 1), the measurements are limited to molecules larger than 10–15 kD.

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Fig. 1. BIACORE equipment.

Therefore the assay method presented here is designed as an inhibition assay in which a fixed concentration of mycotoxin specific antibodies is mixed with a sample containing an unknown concentration of mycotoxin. The mixture is then passed over a sensor surface on which a mycotoxin is immobilised. Non-complexes antibodies are then measured as they bind to the mycotoxin on the sensor surface. The sensor surface contains four serial connected flow cells, making it possible to detect four mycotoxins in a single measurement and will contribute to an efficient monitoring and control of mycotoxins in the food and feed chain.

2. The assay

2.1. Materials

Aflatoxin B1, zearalenone, ochratoxin A, DON and fumunosin B1 were purchased at Sigma. The aflatoxin B1 and zearalenone antibodies were from Sigma. The anti-ochratoxin A antibody is produced by TNO and the anti-fumonisin B1 and DON antibodies were a gift from Dr. Chris Maragos, USDA. All antibodies were monoclonals of murine origin.

Carboxymethoxylamine, *N*-hydroxysuccinimide (NHS), hexanediamine, 2-[*N*-morpholino]ethanesulfonic acid and guanidine chloride were from Sigma. Dimethyl sulphoxide (DMSO), pyridine, ethanolamine and acetonitrile were from Baker. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was purchased at Fluka. Boric acid and glycine were from Merck. MycoSep 224 cleanup columns were purchased at Romer Laboratories.

The CM5 sensor surfaces, HEPES buffered saline (HBS) and the BIACORE 2000 used for these analysis were provided by Biacore AB.

2.2. Methods

In order to be able to immobilise the aflatoxin and zearalenone on the sensor chip surface, the mycotoxins were modified as described by Thouvenot and Morfin (1983). The aflatoxin B1 and zearalenone were carboxylated using carboxymethoxylamine (10 mg per 5 mg of mycotoxin) in pyridine (7.5 ml). This mixture was shaken overnight at 200 rpm protected from light. The pyridine was evaporated to dryness and the mycotoxins were redissolved in DMSO.

Immobilisation on the CM5 sensor surface took place using a modification of the standard EDC–NHS reaction as described by Jönsson et al. (1991). Every flow cell of a docked sensor chip was immobilised separately. A diamine spacer was coupled to a EDC–NHS activated CM5-sensorchip after which EDC–NHS activated aflatoxin or zearalenone were injected. For blocking of non-reacted groups an ethanolamine injection was used. For coupling fumonisin, aminocaproic acid was used as a spacer. For the immobilization of DON, the method described by Xiao, Clarke, Marquardt, and Frohlich (1995) was used using BSA as a carrier protein.

Before use, each flow cell is regenerated 20 times with a 6 M guanidine chloride solution in 50 mM glycine pH 2.0.

Samples were extracted with acetonitrile–water. About 10 g sample is mixed with 40 ml 90% acetonitrile and blended at high speed for 2 min. The resulting mixture is allowed to precipitate and a 5 ml aliquot was removed for further clean up on a Mycosep 224 MFC column. The resulting filtrate was diluted 10 times with HBS and used for analysis. Standards for calibration were prepared in 8.4% acetonitrile–water. The concentration of mycotoxins in the samples were determined by HPLC and GC. Calibration and sample concentration determinations on the BIACORE 2000 equipment were done according to a standard method, using a buffer flow of 20 μl/min and an injection of 70 μl the appropriately diluted sample, mixed 1:1 with an antibody solution-mix consisting of antibodies against the four mycotoxins that were determined. This injection was followed by a regeneration, which consisted of an injection of 6.0 M guanidine chloride solution in 50 mM glycine pH 2.0 during 1 min.

2.3. Results

Fig. 2a–d show the calibration curves for four mycotoxins that were determined simultaneously. These calibration curves are the result of multiple separate measurements. The calibration curve for ochratoxin A is not shown but the performance of the assay, detection limit and the reproducibility, is pointed out in Table 1.

Fig. 3 shows the correlation of the multi-analyte SPR sensor, and the GC-HPLC mycotoxin determinations.

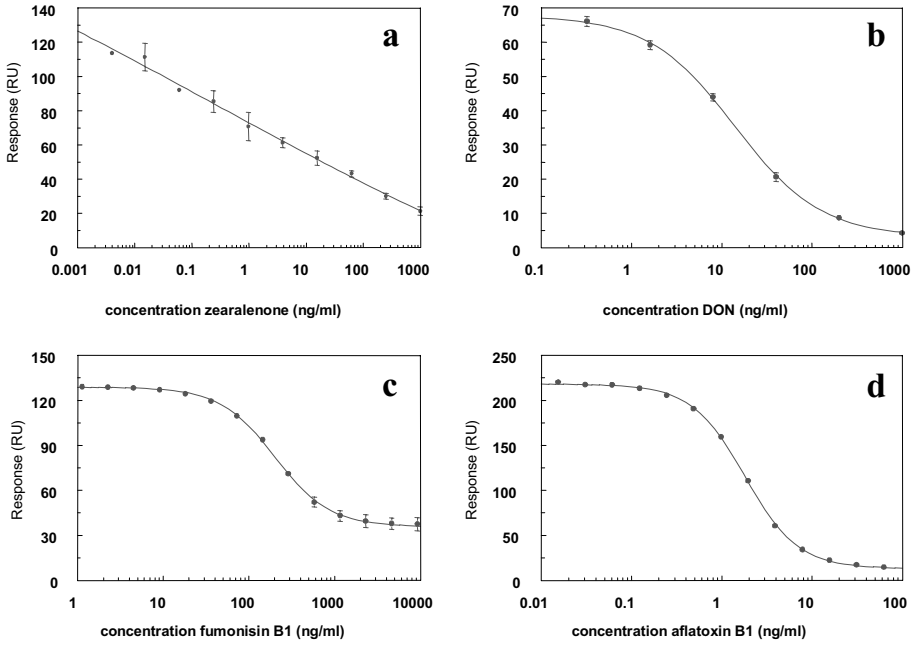

Fig. 2. Calibration curves of zearalenone, DON, fumonisin B1 and aflatoxin B1 in 4.2% acetonitril.

Table 1
Legal or advisory levels, detection limits and the reproducibility of the multi analyte assay

Mycotoxin	Levels (ng/g)	Detection limits (ng/g)	RSD (%)
Aflatoxin B1	2	0.2	2–10
Zearalenone	200	0.01	16–19
Ochratoxin A	3	0.1	4–9
Fumonisin B1	1000	50	2–8
DON	500	0.5	2–5

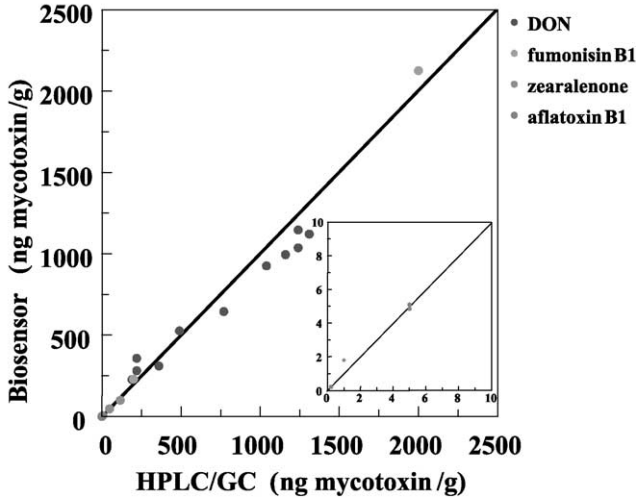

Fig. 3. Correlation between the biosensor and standard HPLC–GC analyses for several mycotoxins.

3. Conclusions

A biosensor for the simultaneous detection of four mycotoxins has been developed. Extraction and clean-up of the sample require approximately 15 min. Additionally, the measurement takes 10 min, including regeneration of the sensor chip surface, making a total of approximately 25 min for the simultaneous determination of four mycotoxins in a single sample.

The *Fusarium* and *Aspergillus* toxins tested can all be detected in the relevant concentrations (see Table 1).

Applying commercially available SPR devices like the SPREETA (see Figs. 4 and 5) allows the development of low-cost equipment which can be used in the field and/or at intake and/or import facilities.

Fig. 4. SPREETA device from Texas instruments.

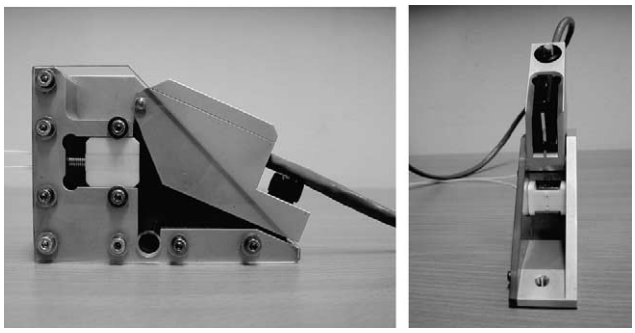

Fig. 5. TNO's flow cell system for the SPREETA.

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