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# Rapid Surface Plasmon Resonance-Based Inhibition Assay of Deoxynivalenol

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Deoxynivalenol belongs to a group of highly toxic fungal metabolites produced by *Fusarium* species that may contaminate food and animal feed, mostly grains. Three different monoclonal mouse anti-deoxynivalenol antibodies were compared for the development of a surface plasmon resonance (SPR)-based immunoassay for the selective and quantitative determination of deoxynivalenol in naturally contaminated matrices. A conjugate of deoxynivalenol with the protein casein was prepared and immobilized on the sensor chip surface. An excess of antibody was added to each test solution before the measurement. The assay was based on the competition for antibody binding between the immobilized deoxynivalenol conjugate on the sensor and the free deoxynivalenol molecules in the test solution. The deoxynivalenol−casein sensor could be reused more than 500 times without significant loss of activity using 6 M guanidine chloride solution for regeneration. The cross-reactivity of the three antibodies in the SPR assay was tested with other trichothecene mycotoxins (3-acetyl-deoxynivalenol, 15-acetyl-deoxynivalenol, nivalenol, HT2-toxin, and T2-toxin). The only sample preparation was extraction with max 80 vol % acetonitrile and 10-fold dilution with the running buffer. The assay had an optimal range between 2.5 and 30 ng/mL deoxynivalenol in the test solution. Most results of the SPR-based assay were in agreement with liquid chromatography/tandem mass spectrometry measurements of naturally contaminated wheat samples.

**KEYWORDS: Deoxynivalenol; DON; surface plasmon resonance; inhibition immunoassay; wheat**

## INTRODUCTION

The adverse health effects of the fungal metabolites, called mycotoxins, were discovered in the 10th century when a chronicler described the disease of "St. Anthony's fire" caused by toxic products of molds growing on rye (*1*). At present, various mold genera are known to produce mycotoxins; most relevant are *Aspergillus*, *Penicillium*, and *Fusarium*. Adverse health effects of mycotoxins can range from nausea, visual disturbances, and vertigo, to carcinogenic and even genotoxic effects. *Fusarium* fungi are prevalent toxin-producing fungi in the moderate climate areas of America, Europe, and Asia, producing the mycotoxins zearalenone, fumonisin, and the trichothecene mycotoxins including deoxynivalenol (**Figure 1**).

Deoxynivalenol is the most predominantly occurring trichothecene on grains such as wheat, corn, rye, rice, and barley and is sometimes found in food products, leading to product recalls. Deoxynivalenol has been implicated in two outbreaks of gastrointestinal illness in China and India (*2*). In animals, deoxynivalenol causes feed refusal and at higher levels growth depression (*3*). When administered for a longer period in animal studies, deoxynivalenol has also been shown to suppress immunoresponse (*4*, *5*), to inhibit protein synthesis, and to affect blood levels of proteins (*6*, *7*).

Because of an increasing awareness of the hazards imposed on human and animal health by mycotoxins, North American and European food authorities have set maximum levels for various mycotoxins (e.g. aflatoxin B1, ochratoxin A, and fumonisin B1). Limits for deoxynivalenol and other *Fusarium* toxins are under investigation (*8*, *9*). The European Scientific Committee on Food proposed a tolerable daily intake of 0.5 $\mu$g/kg bodyweight per day, which corresponds to levels in wheat products in the range of 100−500 $\mu$g/kg (*10*). To enforce legislation, reliable, economical, and easy-to-use assays are required for the quantitative determination of mycotoxins in raw materials and in various food and feed matrices.

Current screening methods for deoxynivalenol include thin-layer chromatography (*11*), colorimetric techniques (*12*), and enzyme-linked immunosorbent assay (ELISA) tests (*13*). For quantitative determination, separation-based analysis methods are used, e.g., gas chromatography (GC) (*14*−*16*) and liquid chromatography (LC) with electron capture, UV/vis (*17*), MS (*18*), or electrochemical detection (*19*). Often, extensive sample cleanup with solid phase extraction cartridges (*20*), commercial cleanup columns (*21*, *22*), or immunoaffinity colums is neces-

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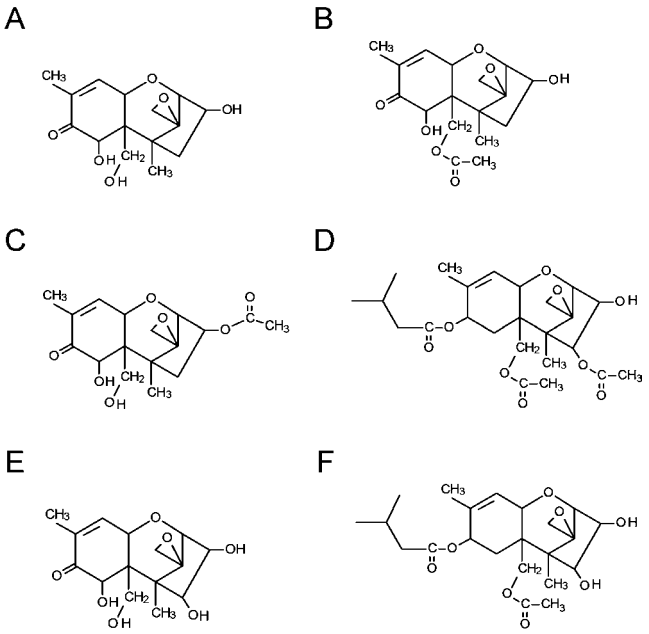


**Figure 1.** Molecular formula of trichothecene mycotoxins (A) deoxynivalenol, (B) 15-O-acetyl-4-deoxynivalenol, (C) 3-acetyldeoxynivalenol, (D) T2-toxin, (E) nivalenol, and (F) HT2-toxin.

sary (*23*). As described in a recent review, the results of laboratory intercomparison studies prove the necessity of further improvements of the analysis methods for trichocetene mycotoxins (*24*), especially where methods are used for the quantification of mycotoxins with analytical performance strongly dependent on the cleanup procedures. The use of an affinity-based surface plasmon resonance (SPR) sensor system, with less demanding sample cleanup, might be a useful alternative for screening and cost effective quantitative determination.

Assays based on SPR detection rely in principle on the measurement of mass concentration changes at the sensor surface; hence, no labeling of analytes is required. SPR is an optical technique based on the reflected light intensity inside a prism or optical fiber coated with a thin metal film. Light enters the prism at an angle guaranteed to produce total internal reflection and will be partly absorbed by free electrons in the thin metal film on the sensor surface; hence, the intensity of the reflected light will decrease. The so-called critical angle where this phenomenon takes place changes with the optical density, i.e., with the refractive index and the mass concentration at the sensor surface. Minute changes in mass concentration can thus be measured by the sensor due to binding and dissociation of interacting molecules, for example, between an immobilized antigen and a specific antibody in the test solution. For a more detailed description of SPR as an analytical technique, see ref *25*. Various suppliers produce SPR-based instruments (*26*); generally, direct detection is limited to molecules larger than approximately 10 kDa or to substances with a high refractive index (*27*). Applications in food testing range from the quantitative determination of antibiotics (*28*, *29*) and hormone residues (*30*) through screening of microbial contaminations (*31*) to allergens (*32*, *33*) and other compounds (*34*). SPR-based immunoassays have been developed for fumonisins (*35*) and aflatoxin B1 (*36*); however, no examples have so far been reported for the determination of mycotoxins in naturally contaminated samples.

In the present study, an inhibition assay was developed and optimized for deoxynivalenol in food and feed matrices. The cross-reactivity of three antibody preparations in the SPR assay was tested with other trichothecene mycotoxins. The results of the SPR-based assay were compared with LC/MS measurement data.

## MATERIALS AND METHODS

**Chemicals.** The mouse anti-deoxynivalenol monoclonal antibodies (clone nos. 1, 4, and 22) used throughout this study were kindly provided by Dr. C. Maragos (*37*). The mycotoxins deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, T2-toxin, HT2-toxin, and nivalenol, as well as N-hydroxysuccinimide (NHS), guanidine chloride, N-ethyl-N-(dimethylaminopropyl)carbodiimide hydrochloride (EDC), carbonyldiimidazole, picrylsulfonic acid, and casein, were purchased from Sigma (Zwijndrecht, Netherlands). Acetic acid, ethanolamine, and acetonitrile were obtained from Merck (Darmstadt, Germany). The CM5 sensor chips, the HEPES-buffered saline (HBS), and the Biacore-Q apparatus were from Biacore AB (Uppsala, Sweden).

**Preparation of a Deoxynivalenol−Casein Conjugate.** A deoxynivalenol stock solution was prepared by adding 1 mL of acetonitrile to 5 mg of deoxynivalenol. To 8.8 mg of carbonyldiimidazole, 200 $\mu$L of the deoxynivalenol stock solution was added, mixed thoroughly, and allowed to react in the dark for 1 h. A solution of 30 mg of casein in 0.5 mL of 100 mM sodium carbonate buffer (pH 8.5) was slowly added to the reaction mixture under continuous mixing and was allowed to react overnight in the refrigerator (4 °C). The protein content of the solution was determined as described by Bradford (*38*). The total amount of amine functions was determined using picrylsulfonic acid with glycine as standard according to the manufacturer's protocol (TNBS(A), Sigma).

**Immobilization of the Deoxynivalenol−Casein Conjugate on the CM5 Sensor Chip.** Immobilization on the CM5 sensor surface was carried out using a modification of the standard EDC−NHS reaction as described by Jönson (*25*). First, the surface was activated with a mixture of 200 mM EDC and 50 mM NHS in 10 mM MES (4-morpholineethanesulfonic acid monohydrate) buffer, pH 6.0. After activation, 0.05 mg/mL conjugate in acetate buffer (10 mM, pH 3.7) was injected. To block unreacted activated carboxyl functions, a solution containing 1 M ethanolamine in 50 mM borate buffer (pH 9.0) was injected. Before use, each sensor surface was regenerated 20 times with a 6 M guanidine chloride solution in 50 mM glycine (pH 2.0).

**Deoxynivalenol Calibration Curves and Measurement of Cross-Reactivity.** Calibration curves were obtained using a Biacore-Q system (Biacore) with a range of solutions of the mycotoxins deoxynivalenol, HT2-toxin, T2-toxin, 3-acetyl-deoxynivalenol, and 15-acetyl-deoxynivalenol made in HBS buffer (pH 7.4). Measurements were performed using a standard method consisting of a 7 min injection step of the appropriately diluted sample, mixed 1:1 with the antibody solution in HBS buffer (antibodies from clone nos. 1 and 4 were diluted 5000-fold; those from clone no. 22 were used in 10 000-fold dilution). The buffer flow during the measurements was 5 $\mu$L/min. When the buffer contained 8.4 vol % acetonitrile, the antibody−sample mixture was allowed to incubate for 30 min. The injection was followed by a 1 min regeneration step with 6 M guanidine hydrochloride solution in 50 mM glycine (pH 2.0).

**Sample Preparation and Measurement.** *Method 1, Wet-Ground Samples.* Naturally contaminated wheat samples were ground with water (1:1.5 m/m), and 50 g of slurry was extracted with 60 mL of acetonitrile. The resulting mixture was filtered (602H 1/2, Schleicher & Schuell, Feldbach, Switzerland) and diluted 10-fold with the running buffer.

*Method 2, Dry Samples.* Twenty-five grams of naturally contaminated dry wheat powder samples was extracted with 100 mL of 80% acetonitrile/water (v/v); the resulting solution was filtered and diluted 10-fold with the running buffer.

The solutions were filtered (0.22 $\mu$m) and were injected into the Biacore-Q system under the same measurement conditions as the calibration solutions.

The Alliance LC/MS/MS apparatus (Waters, Etten-Leur, NL) was equipped with a 150 mm × 3.2 mm i.d., 4 $\mu$m Altima RP-18 column, a 10 mm × 3.2 mm i.d. precolumn (Alltech, Berda, NL), and a Quattro Ultima detector (Micromass, Almere, NL). The LC system was used in the gradient elution mode, with an injection volume of 20 $\mu$L and a flow rate of 0.3 mL/min (*39*). Identification of deoxynivalenol was

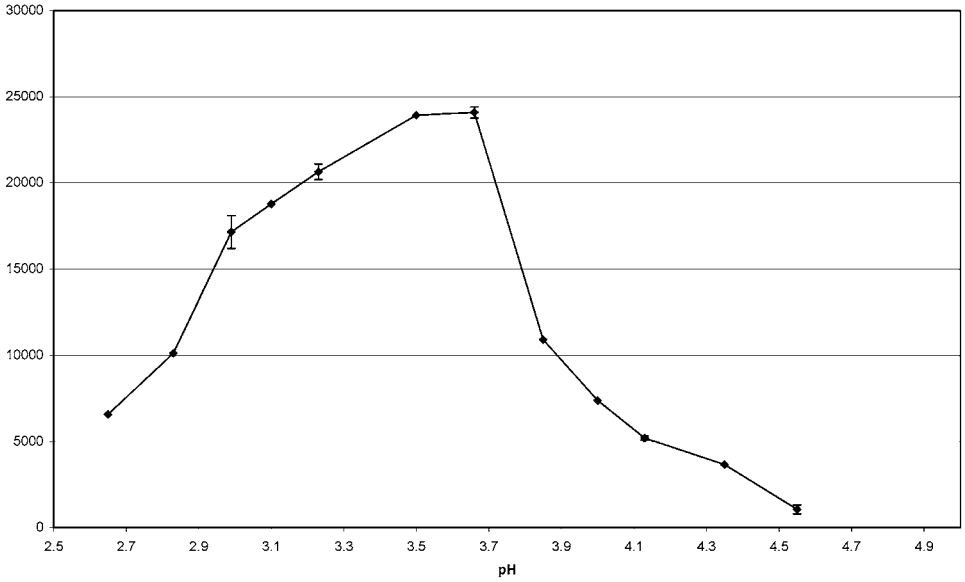


**Figure 2.** Effect of the pH on the interaction of the deoxynivalenol–casein conjugate with the CM 5-dextran sensor chip.

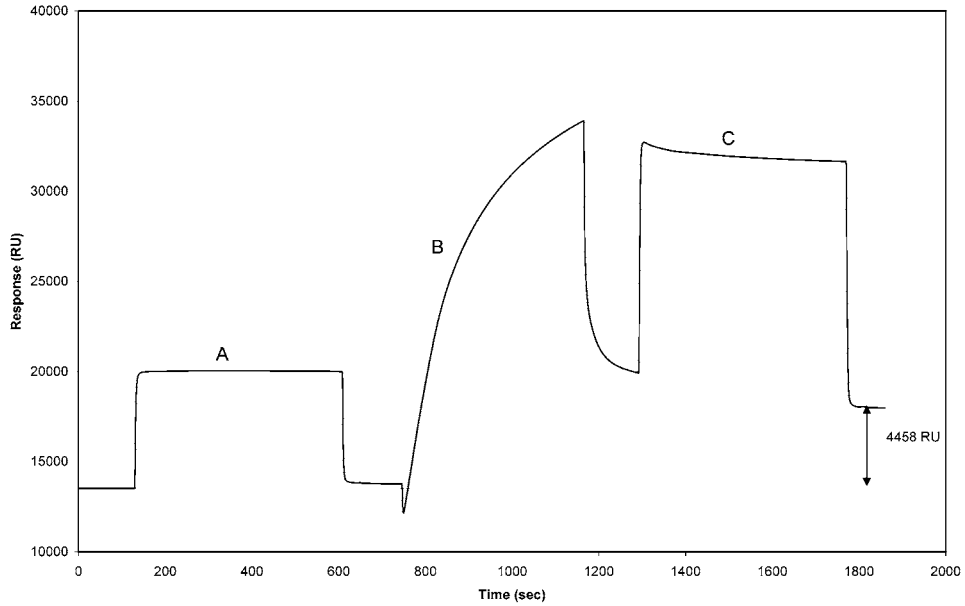


**Figure 3.** Typical sensorgram of the immobilization of the deoxynivalenol–casein conjugate. (A) Surface activation with EDC and NHS, (B) immobilization of deoxynivalenol–caseinate, and (C) inactivation of the sensor surface with ethanolamine. The surface coverage calculated between the signals before surface activation and after inactivation is 4458 RU, corresponding to 4.5 ng/mm$^2$.

carried out in the detector at *m*/*z* 297 in electrospray positive ionization mode and at *m*/*z* 249, 231, 203, and 175 of the fragmentation products in MS/MS mode. Quantification was based on the product ion signal at *m*/*z* 249, where all four characteristic signals were present at S/N > 6.

## RESULTS AND DISCUSSION

**Deoxynivalenol−Casein Conjugate Immobilization, Antibody Binding, and Sensor Regeneration.** Analysis of the deoxynivalenol−casein conjugate with picrylsulfonic acid showed that on average 1.8 ± 0.1 amine groups per casein molecule reacted with deoxynivalenol molecules. The remaining approximately 12 free amine groups of the casein allowed immobilization of the conjugate on the CM5 dextran chip. To find the optimal immobilization conditions, the casein−deoxynivalenol conjugate was diluted to a concentration of 50 μg/mL in 10 mM acetate buffers with pH values ranging from 3.5 to 5.0. Every solution was separately injected during 5 min at a flow rate of 10 μL/min; the results are shown in **Figure 2**. The pH chosen for immobilization was pH 3.7, resulting in an optimal combination of net response and reproducibility.

**Figure 3** shows the sensorgram of the in situ immobilization of a deoxynivalenol−casein conjugate, after activation and blocking, yielding on the sensor approximately 4.5 ng/mm$^2$ surface coverage (4458 response units (RU); 1 RU corresponds to 1 pg/mm$^2$) (*40*). **Figure 4** shows sensorgrams of full measurement cycles with antibody from clone no. 22 at three different concentrations of deoxynivalenol. The signal is inversely proportional to the deoxynivalenol concentration. The bound antibody could be effectively removed using a solution of 6 M guanidine hydrochloride in 50 mM glycine buffer, pH 2.0, with 1 min of contact time without damaging the surface.

**Table 1** shows results for three sensors tested for a period of up to 2 months. No significant loss of the surface activity was observed after more than 500 injection and regeneration cycles for the sensors tested. During this period, the maximal signal

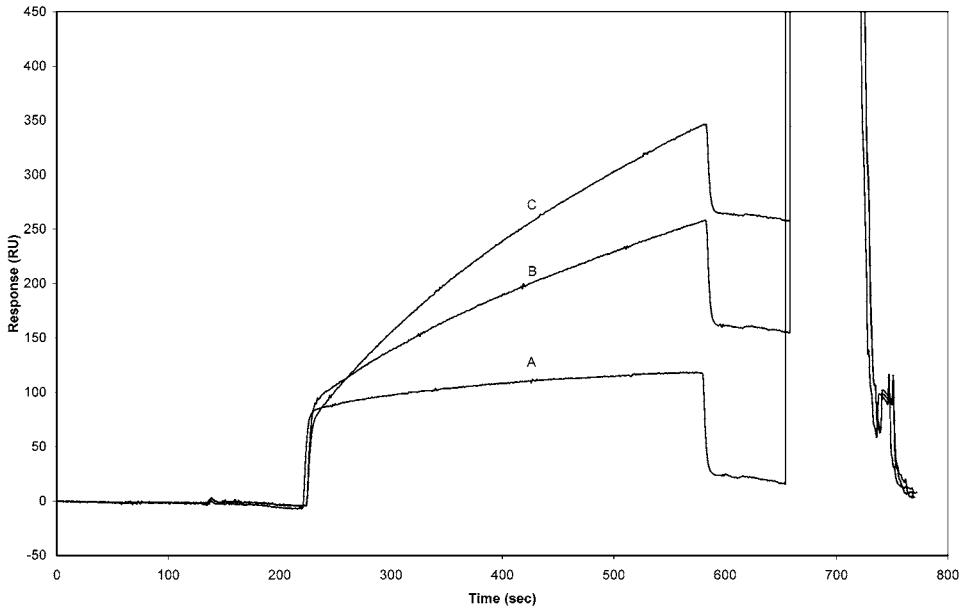


**Figure 4.** Sensorgrams showing an analysis cycle with deoxynivalenol concentrations of (A) 333, (B) 12.3, and (C) 0.15 ng/mL. Mouse anti-deoxynivalenol antibody clone no. 22 was used. Injection: 30 $\mu$L at 5 $\mu$L/min in HBS buffer.

**Table 1.** Characteristics of Deoxynivalenol–Casein Sensor Chips

| no. | coating thickness (ng/mm$^2$) | no. of days used (days) | no. of analyses | max response (RU) | $IC_{50}$ intraday RSD[a] (%) | $IC_{50}$ day-to-day RSD[a] (%) |
|---|---|---|---|---|---|---|
| 1 | 5.0 | 32 | 561 | 134 | ND | ND |
| 2 | 2.5 | 65 | 533 | 197 | 3.1 | 8.8 |
| 3 | 2.3 | 45 | 717 | 300 | 2.9[b] | 2.7[c] |
| 4 | 4.5 | 59 | 585 | 194 | ND | ND |
| 5 | 3.9 | 26 | 499 | 286 | 2.2 | 2.6 |

[a] The intraday and day-to-day RSDs are calculated at the $IC_{50}$ value. [b] $N = 2$. [c] $N = 5$.

showed approximately a 10% decrease. The day-to-day and intraday variation of the $IC_{50}$ value (the concentration at which half of the maximum response is observed) per channel was well below 10%. In a separate experiment, 60 measurement cycles with monoclonal antibody no. 22 were carried out, yielding a mean maximal response of 178 RU with a relative standard deviation (RSD) of 3.4%.

To investigate the reproducibility of the sensor preparation, two sensors were coated with deoxynivalenol−casein conjugate. The calibration curves constructed with the three available deoxynivalenol antibodies show a good sensor-to-sensor reproducibility as illustrated in **Figure 5**.

**Choice of Antibody. Figure 5** demonstrates the differences among the available deoxynivalenol antibodies in the SPR assay format. Antibodies from clone no. 22 gave the highest average response of 298.3 ± 2.1 RU. The dynamic range for this antibody was between 2 and 100 ng/mL with an $IC_{50}$ value of 19.1 ± 1.6 ng/mL. The dynamic range of antibodies from clone nos. 1 and 4 is practically identical, ranging from approximately 1 to 100 ng/mL; their $IC_{50}$ values were 5.2 ± 1.5 and 4.3 ± 0.4 ng/mL, respectively. A large dynamic range is advantageous for an assay, as well as a low $IC_{50}$ value. The dynamic ranges of all antibodies tested were similar; the lower $IC_{50}$ values of antibodies from clone nos. 1 and 4 provide an indication of the potential for lower detection limits with these antibodies in an assay.

A determining parameter for antibody suitability is the cross-reactivity. If a selective assay is required, cross-reactivity of

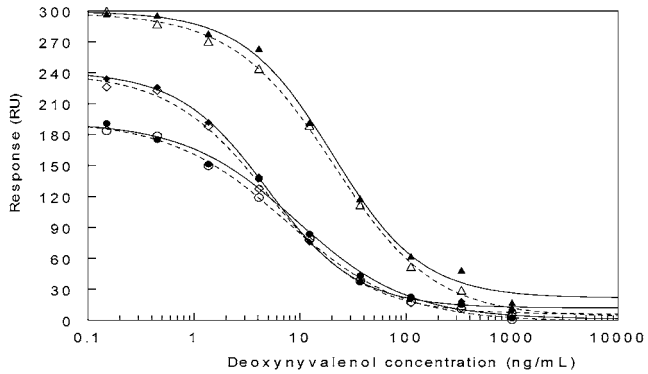


**Figure 5.** Reproducibility of chip preparation and calibration. Channel 1, filled symbols; channel 2, open symbols. Antibody clones no. 22 (▲ and △), no. 4 (◆ and ◇), and clone no. 1 (● and ○).

**Table 2.** Cross-Reactivity of Antibodies from Clone Nos. 1, 4, and 22 toward Trichothecene Mycotoxins Measured in HBS Buffer (% SD in Parentheses)

| | $IC_{50}$ clone no. 1 (ng/mL) | $IC_{50}$ clone no. 4 (ng/mL) | $IC_{50}$ clone no. 22 (ng/mL) |
|---|---|---|---|
| deoxynivalenol | 6.7 (9.5) | 4.1 (3.7) | 19.3 (5.2) |
| 15-Ac-deoxynivalenol | >1000 | >1000 | >1000 |
| 3-Ac-deoxynivalenol | 779 (2.8) | 528 (19) | 4.3 (14) |
| HT-2 toxin | 91 (2.5) | 63 (25) | >1000 |
| T-2 toxin | 141 (57) | 202 (19) | >1000 |
| nivalenol | >1000 | >1000 | >1000 |

the used antibody must be minimal toward all other compounds present in the sample matrix. Therefore, calibration experiments were carried out with five other trichothecene mycotoxins to investigate the cross-reactivity in the SPR assay of the deoxynivalenol antibodies. The results of these tests are summarized in **Table 2**. As also found by Maragos and McCormick (*37*) in an ELISA format with antibodies from clone no. 22, the $IC_{50}$ of 3-acetyl-deoxynivalenol was considerably lower than that of deoxynivalenol. In a quantitative deoxynivalenol assay, this would cause a serious overestimation of the deoxynivalenol concentration. On the basis of the calibration and cross-reactivity results of antibodies from clone nos. 1 and 4, the slightly lower

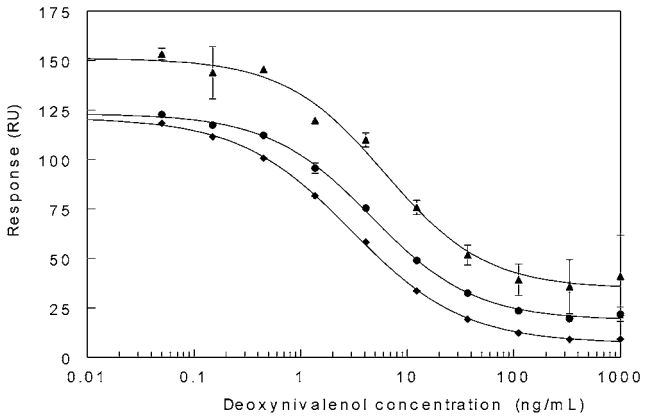


**Figure 6.** Effect of the incubation time in HBS buffer containing 8.4% acetonitrile (v/v) on the response curve of clone no. 4: no incubation time (▲), 30 min of incubation time (●), and 60 min of incubation time (◆).

**Table 3.** Comparison of Sample Measurements Using Biosensor and LC/MS/MS Analysis (SD in Parentheses, $N = 4$)

| sample code | LC/MS/MS ($\mu$g/kg) | biosensor ($\mu$g/kg) | difference (%) |
|---|---|---|---|
| 1 | 870 | 951 (8.0) | 9 |
| 2 | 250 | 331 (4.4) | 28 |
| 3 | 170 | 201 (7.2) | 17 |
| 4 | 760 | 983 (6.1) | 26 |
| 5 | 415 | 297 (7.2) | −33 |
| 6 | 1115 | 1148 (4.3) | 3 |
| 7 | 180 | 165 (1.3) | −9 |
| 8 | 1370 | 1379 (14.1) | 1 |
| 9[a] | 900 | 1359 (14.4) | 41 |
| 10[a] | 595 | 1051 (9.0) | 55 |

[a] Dry-ground samples, different extraction method.

$IC_{50}$ value and higher maximum response in the deoxynivalenol assay, antibodies from clone no. 4 were chosen for further assay development.

Initially, the calibration curves obtained with solutions containing 8.4 vol % acetonitrile showed poor reproducibility (**Figure 6**, uppermost curve). The presence of the organic solvent delayed establishment of equilibrium of the antibodies between the surface-bound and the free deoxynivalenol moieties. As shown (**Figure 6**, lower two curves), the problem could be alleviated by increasing the incubation time. In all further experiments, 30 min of incubation time was allowed to avoid reproducibility problems.

**Determination of Deoxynivalenol in Naturally Contaminated Samples.** The analysis results of the optimized SPR assay and a validated LC/MS/MS method were compared for naturally contaminated wheat samples. In naturally contaminated samples, the SPR assay is expected to yield slightly higher results because of the cross-reactivity of the antibody with other trichothecene mycotoxins. The results, shown in **Table 3**, indicate good agreement between the two methods for wet-ground wheat samples (samples 1−8). Samples 9 and 10 underwent a different sample preparation procedure: the dry powdered samples were extracted with higher concentrations (80 vol %) of acetonitrile. The differences indicate a matrix problem because of excessive uptake of the extraction solution; this problem is currently under investigation in our laboratory.

An easy, rapid, toxin specific biosensor assay was developed based on a simple and general extraction procedure with only an additional dilution step for the determination of deoxynivalenol. The extraction procedure used is suitable for the isolation of toxins from wheat and other food and feed matrices. The assay presented here operates unattended and can be combined with similar affinity-based assays for the determination of multiple mycotoxins. The majority of the assay results is in agreement with LC/MS/MS results. The matrix effects responsible for the higher levels found in two samples are the subject of a follow-up investigation.

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