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Analytical Methods

Electronic Supplementary Information

A validated multi-channel electrochemical immunoassay for rapid fumonisin B1 determinations in cereal samples

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Antibody immobilization on PrG magnetic beads

PrG functionalized magnetic beads (MBs- PrG) have a binding capacity of approximately 8 µg human IgG per mg of MBs. A dispersion of 10.4 µL of MBs-PrG (30 mg mL^{-1}) was transferred into a 1.5 ml *eppendorf* tube, washed twice with 500 µL of *buffer A* solution to remove the NaN_3 preservative, and re-suspended in 500 µL of *buffer B* containing 8 µg mL^{-1} of the FB1 monoclonal antibody (mAb-FB1). Monoclonal mAb-FB1 was captured via their Fc region after 10 min. incubation at room temperature under continuous stirring (1400 *rpm*). The antibody modified MBs (MBs-mAb-FB1) were washed twice with 500 µL of *buffer A* for 2 min., and the supernatants removed under magnetic separation. The MBs-FB1 were re-suspended in 3120 µL of *buffer B* and stored at 4 °C until use. Properly stored, the MBs-mAb-FB1 were stable for at least 4 weeks.

HPLC-FLD method for validating FB1 content in maize samples

The AOAC official HPLC-FLD method 2001.04 for FB1 detection in maize samples was used for validating the proposed method [19]. In brief: 2 g. of ground sample was extracted with 5 mL of methanol:acetonitrile:water (25:25:50) (v/v/v) for 20 min. and centrifuged for 10 min. at 1600g, filtering supernatants through flutted filter paper. The extract (1 mL) was diluted with 4 mL PBS and

filtered through a microfiber filter, then passed 1 mL through the IAC (inmunoaffinity column) (Fumonitest, from Vicam), and washed by passing 10 mL PBS. Fumonisins were eluted by passing 1 mL HPLC grade methanol followed by 1 mL water elution through the column. After drying down methanol eluate, the extract was re-dissolved in 200 µL acetonitrile:water (50:50), mixed 50 µL of this with 50 µL OPA reagent and after exactly 3 min. injected 20 µL into the chromatograph. HPLC conditions were: C18 reversed-phase column (waters Nova Pack C18, 3.95 mm x 150 mm, 4 µm particles); mobile phase: methanol:NaH_2PO_4 (0.1 M.) 77:23 (v/v), pH=3.3, eluted at a flow-rate of 0.8 mL min^{-1}. Fluorescence detection was done at an excitation wavelength of 335 nm and an emission wavelength of 440 nm.

Analytical performance of the multi-channel electrochemical immunoassay: additional considerations.

Samples for immunoassays are usually run in batches and interpolated with freshly-prepared calibration standard curves performed on the same day, due to the involved parameters can vary widely from day-to-day. We studied (ANOVA, analysis of variance) the influence of the following factors in the measured $EC_{50:}$ *i*) different CH8 electrode arrays; *ii*) calibrations performed on different days; *iii*) stirring or not while incubations, and *iv*) 3PL vs 4PL logistic parameter non-linear regression calculations. In all of these particular studies, the same MBs-mAb-FB1 and FB1-HRP solutions were used. However, the results should be taken with caution, since a significant loss of antibody affinity or the HRP enzymatic activity over time might occur. The affinity of the mAb-FB1 bounded to the MBs does not significantly change approximately for 25 days when stored properly at 4 °C.

In the first test (*i*), 4 independent calibrations on each of four different CH8 SPCEs arrays were carried out in one day. No significant differences in EC_{50} values were obtained (t test, 95% confidence level) for the resulted 16 calibration plots (overall EC_{50}=4.34±0.73, $mean \pm t \cdot s/n^{1/2}$). Nevertheless, in some cases we have found significant differences in other batches of CH8 arrays due to differences in manufacturing. For this reason, we recommend performing the immunoassay calibration and sample measurements with the same CH8 sensor array, which is easily regenerated by simply washing with water giving reproducible results without significant differences in EC_{50} values at the same day (n=4 independent calibrations). But significant differences in EC_{50} values (ANOVA, 95% level of confidence) were found from replicated calibrations per one day (n=4 independent calibrations) made for 5 consecutive days, and using the same CH8 array (*ii*). Yet there were no significant differences using orbital stirring (EC_{50}=4.32±1.44 ng mL^{-1} FB1, n=3) and without using stirring (EC_{50}=4.68±1.66 ng mL^{-1} FB1, n=3) at the incubation step (*iii*). Nevertheless, i_{max} values are slightly higher when the antibody-antigen incubations are carried out with stirring (*i*max: 1.58±0.18 μA, mean±sd) than without it (*i*max: 1.38±0.20 μA, mean±sd), so the orbital stirring during the antigen-antibody incubations is recommendable.

For a practical use, 3PL parameters logistic fittings can be obtained from calibrating plots of n=4 standard points, assuming m=1, instead of 4PL calibrations with n=8 calibration points. These points can be obtained from concentrations corresponding to 0% inhibition (eg. 10^{-3} ng mL^{-1} FB1), 100% inhibition (eg. 10^{+2} ng mL^{-1} FB1) and two intermediate FB1 concentrations (eg. 1 and 10 ng mL^{-1} FB1). A comparison (*iv*) of the fitted EC_{50} from four independent calibrations in a 3PL (4 standard points measured) with four calibrations using 4PL regression (8 standard points measured) the following values ($mean \pm t \cdot s/n^{1/2}$) were obtained respectively: EC_{50}=4.09±0.94 ng mL^{-1} FB1 (n=4 standard points, 3PL) and EC_{50}=4.34±0.15 ng mL^{-1} FB1 (n=8 standard points, 4PL). Consequently, the

same EC_{50} values were obtained in both cases. Calibrations with 4 standard points (3PL nonlinear regressions) are of interest for a practical application in screening analysis, since in a single run experience with the CH8 array, one calibration (n=4 points) and four samples (replicated or not) can be simultaneously measured.

Precision of the reported method: concentrations of FB1 from the same sample and five independent extractions. Each extracted sample was replicated eight times.

The following Table summarize the measured FB1 concentrations by the reported method (extraction of FB1 and determination with the electrochemical immunoassay). Five independent extractions from the same sample (the CRM Trilogy® FC-433, 1500±200 µg kg^{-1} FB1) were performed. Each of the five extracts were simultaneously analysed eight times in a single CH8 sensor array.

All definitions, terms, and calculations are according to Commission Regulation (EC) No 401/2006 *laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs* [41].

	Extraction 1 M1	**Extraction 2 M2**	**Extraction 3 M3**	**Extraction 4 M4**	**Extraction 5 M5**
[a] **Mean (n=8)**	1245.0	1734.2	1234.3	1307.9	1645.7
[b] **s_R (n=8)**	201.9	166.5	151.8	146.5	240.3
[c] **% RSD_r**	16.4	9.6	12.3	11.2	14.6
[d] **% RSD_R**	15.5	14.7	15.5	15.4	14.8
% Relative Error	-17.9	15.6	-17.7	-12.8	9.7
Significance Interval (n=8), 95%	168.5	138.9	126.7	122.2	200.5

[a]FB1 concentration units µg kg^{-1}; [b] Standard deviation calculated from results generated under repeatability conditions; [c] Relative standard deviation, calculated under repeatability conditions; [d] Relative standard deviation under reproducibility conditions (Horwitz equation).