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# Novel multiplex fluorescent immunoassays based on quantum dot nanolabels for mycotoxins determination

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***Abstract***

The aim of this manuscript was the development of easy-to-operate quantum dots (QDs)-based immunochemical techniques for simultaneous screening of several mycotoxins in cereals. Two different approaches for multiplex fluorescent immunosorbent assay (FLISA) were employed. In the first approach a multiwell plate in which the different wells express a different mycotoxin (deoxynivalenol, zearalenone, aflatoxin B1, T2-toxin and fumonisin B1) was considered as a multiplex because each sample was pretreated once and then will be distributed over a series of wells within the same plate (single-analyte multiplex, SAM). The entire assay allows the simultaneous determination of all compounds. For the double-analyte multiplex (DAM) two different specific antibodies were co-immobilized in one single well. Zearalenone and aflatoxin B1 were

simultaneously determined, provided their conjugates are labeled with QDs which are fluorescent in different parts of the spectrum, by scanning the assay outcome at two different wavelengths. The limits of detection (LOD) for the simultaneous determination of deoxynivalenol, zearalenone, aflatoxin B1, T2-toxin and fumonisin B1 by SAM FLISA were 3.2, 0.6, 0.2, 10 and 0.4 μg $kg^{-1}$, respectively, while for the DAM FLISA they were 1.8 and 1 μg $kg^{-1}$ for zearalenone and aflatoxin B1, respectively. SAM FLISA principle was also presented in a qualitative on-site format and tested for on-site multiplex determination of four mycotoxins in cereals. The achieved cut-off values of 500, 100, 2 and 100 μg $kg^{-1}$ for deoxynivalenol, zearalenone, aflatoxin B1 and T2-toxin respectively. For simplification of multiassay results' evaluation the conjugates with QDs of different colors were used.

***Keywords:*** Multiassay; immunoassay; quantum dots; mycotoxin; cereals; rapid tests; multiplex screening.

***Introduction***

Nowadays the amount of publications devoted to the simultaneous determination of several analytes is constantly rising. Together with chromatography, screening methods which are cost-effective, simple to perform and not laboratory-based became increasingly popular. Most of these multiplex techniques are immunochemical such as ELISA (Qiagen®, PathScan®), fluorescent polarization immunoassay (Mi et al., 2013), a bead-based multianalyte immunoassay (Elshal and McCoy, 2006), lateral flow dipsticks (Kolosova et al., 2007), immunochips (Wang et al., 2012), microarrays (Hu et al., 2013, Oswald et al., 2013), electrochemical immunosensors (Vidal et al., 2013), immunoblot (Abouzied and Pestka, 2004), membrane (Njumbe Ediage et al., 2012, Saha et al., 2007) and column flow-through (Basova et al., 2010, Beloglazova et al., 2010, Goryacheva et al., 2007) tests. As a rule, these techniques do not require complicated sample pretreatment except for one-step extraction or/and dilution. Although on-site tests are less precise and specific than chromatography, the best examples allow to operate in the pg $mL^{-1}$- ng $mL^{-1}$ concentration range and they are suitable for preliminary on-site screening of large amounts of samples.

Most of earlier described immunoassays for simultaneous detection of multiple analytes (multiplex immunoassays) were developed by placing immunoreagents, specific towards different analytes, on separate spots (or test-zones, or tubes, or wells) within one test system. This approach allows single pretreatment of each sample followed by its distribution over a series of wells. This

multiplex approach was widely common because the signals provided by two enzymes cannot be separated. Separation of the signals provided by two organic dyes requires statistical methods due to their asymmetrical unsharpened emission peaks which are broadened by a red-tail (Chan et al., 2002). Multiplex based on the simultaneous detection of multiple analytes on a single spot is exclusively possible through detection with quantum dots (QDs) due to QDs are characterized by narrow symmetrical photoluminescent peaks (Alivisatos et al., 2005, Chan et al., 2002, Goryacheva et al., 2013, Jaiswal et al., 2003, Medintz et al., 2005). Multiplex systems based on QDs which emit in different parts of spectrum without any mathematical or statistical processing of the obtained results, were already successfully described for multicolour bioimaging (Gao et al., 2007; Shi et al., 2008), multiplex electrochemiluminescence immunoassay (Guo et al., 2013), multiplex luminescent microarrays (Nichkova et al., 2007).

Colloidal semiconductor nanocrystal QDs are characterized by unique size-tunable optical properties which favor their use for biomedical diagnostics (Chen et al., 2012, Yezhelyev et al., 2007), molecular imaging (Liu et al., 2008, Ruan et al., 2007) and chemical analysis (Gill et al., 2008, Pinwattana et al., 2010, Vinayaka et al., 2009, Wang et al., 2011). They possess a broad absorbance band and a narrow size-dependent symmetrical sharply defined emission peak (Alivisatos et al., 2005, Chan et al., 2002, Goryacheva et al., 2013, Jaiswal et al., 2003, Medintz et al., 2005). They have higher brightness and a higher signal to noise ratio compared with traditional organic fluorophores due to the combined effect of high absorption across a wide spectral range ($0.5\text{-}5*10^6$ $M^{-1}cm^{-1}$) and good photoluminescence quantum yield (PLQY). QDs with different colors of emission (e.g. with different core size) can be simultaneously excited with a single wavelength light source which dramatically simplifies the realization of multicomponent analysis. It has already been shown that QDs have several advantages compared to other routine fluorescent labels (Beloglazova et al., 2011, Beloglazova et al., 2012, Maestro et al., 2012). Therefore, their use in multiplex analysis is obvious and prospective.

This article is the first to describe the immobilization of two antibodies specific to different analytes into the same well for simultaneous fluorescent detection of two analytes: aflatoxin B1 (AfB1) and zearalenone (ZEN), so called double-analyte multiplex (DAM). This approach was well investigated and compared with the single-analyte multiplex (SAM) FLISA where the specific antibodies were immobilized into separate wells. Simultaneous detection of five mycotoxins (deoxynivalenol (DON), ZEN, AfB1, T2-toxin (T2) and fumonisin B1 (FB1)) in one plate (quantitative format) and simultaneous determination of four analytes (DON, ZEN, AfB1, T2) in one test column (qualitative format) were realized by the SAM FLISA. To the best of our knowledge, this is the first

time that on-site QDs-based multiplex was developed for simultaneous determination of four analytes.

**Materials and methods**

*Reagents and materials*

AfB1, aflatoxin M1 (AfM1), T2, HT2-toxin (HT2), ZEN, DON, FB1, fumonisin B2, nivalenol were purchased from Sigma-Aldrich (Bornem, Belgium). Jeffamine M1000 (1000 g/mol) was kindly provided by Huntsman (Belgium). A full reagent list is presented in the Supporting Information (**SI1**).

The anti-ZEN monoclonal antibody (1 mg $mL^{-1}$) was produced from a stable hybridoma cell line (Burmistrova et al., 2009). The antibody was characterized by a high ZEN (100%) and α-zearalenol (69%) recognition (cross-reactivities for α-zearalanol, zearalanone, β-zearalenol and β-zearalanol were 42%, 22%, <1% and <1%, respectively (Beloglazova et al., 2013). The production of the anti-FB1 antibody (6.4 mg $mL^{-1}$) was done by Sheng et al. (Sheng et al., 2012), the anti-T2 antibody (1.89 mg $mL^{-1}$) was described by Li et al. (Li et al., 2012). According to our data the specific anti-T2 toxin showed 110% cross-reactivity for HT2; the anti-FB1 antibody was characterized by high cross-reactivity for fumonisin B2 (around 150%). For detection of AfB1 specific antibody produced against AfM1 was used. It was found that this antibody had a 120% cross-reaction with AfB1. Monoclonal anti-DON antibody (clone 4, developed at USDA–ARS–NGAUR, Peoria, IL, USA) was kindly provided by Dr. Chris Maragos. The applied anti-DON antibody was characterized by ~435% cross-reaction with 15-acetyldeoxynivalenol and less than 1% with 3-acetyl-DON and nivalenol.

CdSe-based QDs with orange, red and green emission were prepared in organic solution and transferred to aqueous medium by polymer encapsulation, as described by Speranskaya et al. (Speranskaya et al., 2014). The details of the syntheses as well as the methods of QDs characterization are described in the Supporting Information (**SI2**).

The protocols of fluorescent based immunosorbent assay (FLISA) and multiplex gel-based immunoassay procedure (GBI) are presented in the Supporting Information (**SI3** and **SI4**).

*Fluorescence measurements*

For fluorescence measuring the Envision 2104 Multilabel Plate Reader (PerkinElmer, USA) and the Infinite Tecan Plate Reader (Tecan, Switzerland) were used by changing of emission wavelength depending on the QDs fluorescence peak position: 540, 594 and 642 nm for green, orange and red QDs, respectively.

*Syntheses of QDs-labeled antigens*

For the synthesis of QDs-labeled conjugates a two steps protocol based on the EDC/sulfo-NHS activation of carboxyl groups were applied. This technique was already described for the production of ZEN-QDs (Beloglazova et al., 2012) and DON-QDs (Speranskaya et al., 2014). The preparation of AfB1-QDs, FB1-QDs and T2-QDs is presented in the Supporting Information (**SI5**). All conjugates were kept at 4 °C within 6 months.

*Sample preparation*

For the preparation of wheat and maize samples, a portion of ground cereal (5 g) was extracted with 20 mL of methanol/water (80/20, v/v) for 15 min on a horizontal shaker at RT. Afterwards the supernatant was five times diluted with PBS and submitted to analysis.

**Results and discussion**

*Synthesis of the Cd-based core/shell quantum dotswith different emission*

CdSe quantum dots were prepared by a rapid hot-injection method adopted from (Jasieniak et al, 2005; Capek et al, 2010). To improve photoluminescence quantum yield (PLQY) and stability of CdSe QDs they were covered with the inorganic shell of the wider band-gap semiconductor (Talapin et al, 2004, Reiss et al, 2009). The most suitable shell for CdSe cores is zinc sulfide due to its largest band gap between Cd-Zn chalcogenides. But ZnS can efficiently cover only small CdSe cores due to a high difference in lattice parameters (Hines et al, 1996). To cover the bigger CdSe cores in the most cases an additional layer of semiconductor with intermediate parameters is grown between CdSe core and ZnS outer shell to reduce a strain inside nanocrystals (Reiss et al, 2009). In this work these two approaches to produce core-shell QDs were used: the small CdSe cores with initial d~2.6 nm (with an the first exciton peak at 508 nm) were employed to obtain both core-shell structures and the bigger CdSe cores (d~4.6 nm, with the first exciton peak at 610 nm) were used for shelling only by the second approach (Fig. 1). The formation of the CdS shell around CdSe cores results in a sufficient red shift of both the first absorption maximum and fluorescence due to the band gap of CdS is not large enough to provide the potential barrier necessary to block both electrons and holes inside CdSe core. Growing the shells of ZnS directly on CdSe cores results in a small (~ 6 nm) red shift of absorption and fluorescence spectra (Fig. 1). The fluorescence maximums of the obtained core-shell

nanocrystals were 525, 592 and 638 nm for green, orange and red QDs, respectively; the full width at half maximum (FWHM) for the CdSe cores was 40 nm, for the core-shell QDs the FWHM did not exceed 35 nm. The relative PLQY of the QDs were determined by direct comparison with coumarin 2 (for CdSe and CdSe/ZnS QDs) and with Rhodamine 6G (for CdSe/CdS/ZnS QDs). In both cases the PLQY was sufficiently improved after coating with wider band gap semiconductor layer: ~1.5% (CdSe), ~35% (CdSe/ZnS), ~40% (CdSe/CdS/ZnS). TEM images of initial CdSe cores and core-shell QDs are presented in Fig. 2: the nanocrystals are quite spherical with a good uniformity in size and shape.

To transfer the obtained QDs into aqueous solutions a polymer encapsulation approach was used: the synthesized PMAO-Jeffamine M1000 amphihilic polymer contains nonpolar chains which can interact with hydrophobic ligands covered QDs surface and also has hydrophilic polyethers fragments providing water-solubility. It's interesting to note that the fluorescence peak of CdSe/CdS/ZnS QDs only slightly shifted (~2-4 nm) after transfer to water solution, but the peak of CdSe/ZnS QDs always shifted to longer wavelength region by ~15 nm. The fluorescence peak positions and the QY of the obtained hydrophilic QDs were 540 nm (QY 30%), 594 (QY 33%) and 642 (QY 37%) for green, orange and red QDs, respectively.

*Single-analyte multiplex FLISA (SAM FLISA)*

The first QDs-based multiplex approach is a multiwell plate in which the different wells express a different mycotoxin (single-analyte multiplex, SAM). This format can be considered as a multiplex procedure because each sample was pretreated just once and then it was distributed over a series of wells within the same plate. Thereby the entire assay allowed the simultaneous determination of all compounds. The principle of FLISA was analogous to the ELISA principle but the FLISA protocol included less assay steps (luminescence can be immediately observed after the washing step which removed unbound conjugate molecules, no addition of chromogenic substrate and incubation steps), therefore analysis time was shorter.

Analytical characteristics of SAM FLISA and SAM ELISA are given in Table 1, while the calibration curves are included in the Supporting Information (**Fig. SI-2**). It was found that application of QDs as a label in competitive immunosorbent assay led to a significant increase of method sensitivity: $IC_{50}$ values for FLISA were in the range of 0.11-11 ng $mL^{-1}$, whereas those for ELISA were 1.5-62 ng $mL^{-1}$ (~3-12 times decrease depending on the mycotoxin) (Table **SI-2**). Decreasing of LODs is a big advantage for real samples analysis. It is known that immunosorbent assays are prone to the appearance of false results related to the high sensitivity of antibodies and proteins towards matrix constituents and environmental conditions. Therefore, higher sample dilution is very important in

order to correct this effect. Lower LOD and linear range allow to increase dilution factor of sample and decrease the chance to obtain false results.

*Double-analyte multiplex FLISA (DAM FLISA)*

But if SAM FLISA only demonstrated an increased sensitivity as a benefit over the traditional immunochemical multiplexes, DAM FLISA resulted in a substantial simplification of the analysis procedure. Since two specific antibodies (anti-ZEN and anti-AfB1) were immobilized into the same well, the DAM format allowed addition of a mixture of AfB1-QDs and ZEN-QDs (1/1 v/v, in appropriate dilutions). This is in contrast with SAM FLISA, where different labeled conjugates were added into different wells separately. The conjugates were labeled with QDs which luminescent in different parts of the spectrum: ZEN was labeled with green-emitted QDs, and AfB1-with orange-emitted QDs. Since the spectra of QDs slightly overlapped (a wavelength of maximum of emission peak of green QDs was at 540 nm, whilst orange QDs emitted with the maximum at 594 nm, **Fig. SI-3**), the analytical signal could be detected for both analytes separately without any statistical tools by double scanning of the plate with different emission wavelengths.

Because the mixture of labeled conjugates was added into the same well, the number of washing steps after the addition of conjugate increased to four instead of three to obtain complete removal of unbound QDs-conjugates. This could lead to a decrease in analytical signal intensity and subsequently to an ambiguous interpretation of the results. Therefore, concentrations of the labeled conjugates were increased in comparison to SAM FLISA in order to enhance the analytical signal (**Table SI-1**). This resulted in a lower DAM FLISA sensitivity compared to SAM FLISA: $IC_{50}$ values of 13 μg $kg^{-1}$ and 10 μg $kg^{-1}$ for DAM multiplex instead of 2.6 μg $kg^{-1}$ and 2.2 μg $kg^{-1}$ for SAM multiplex for ZEN and AfB1 respectively. Calibration curves for mycotoxin detection by DAM FLISA are presented in Fig. 3. It should be emphasized that an influence between the labeled conjugates could not be detected/established.

The simultaneous detection of two analytes can cause false results related to a slight overlap of the fluorescence spectra of the used QDs (= cross-reactivity between analytes, CR). To evaluate this CR, the solutions containing either ZEN or AfB1 were analyzed at both wavelengths. As expected, an analytical response was observed for the corresponding QDs and detected solely at the corresponding wavelength. Simultaneous addition of ZEN and AfB1 resulted in the simultaneous decrease of fluorescence intensity at both wavelengths, and the intensities were similar to the generated signal when only the analyte was present. These experiments indicated that the CR

between the targets was negligible and simultaneous multianalyte determination could be achieved using the proposed technique.

*Validation of the developed multiplex FLISAs*

Validation of the developed multiplex immunoassays was performed using blank wheat and maize samples, spiked with set of the mycotoxins at different concentrations. As a sample pretreatment, extraction with methanol/water (80/20 v/v) and further fivefold dilution of the extract was chosen. This sample pretreatment was already tested for ZEN determination in cereals and cereal-based products by ELISA (Burmistrova et al., 2009, Beloglazova et al., 2013) and enzyme-labeled GBI (Beloglazova et al., 2013), and for ZEN and DON determination by QDs-labeled FLISA and QDs-GBI (Beloglazova et al., 2012, Speranskaya et al., 2014) and it was proved that this the easiest procedure is enough for a sufficient decrease of matrix effect. After extraction, the extract was usually standing for some minutes for sedimentation of particulate matter and afterwards the supernatant was collected and diluted with PBS.

In summary, both FLISA formats performed excellent in the analysis of spiked samples, as illustrated by the good correlation between added amounts of mycotoxin and found concentrations (Fig. 4 and 5).

*Analysis of naturally-contaminated cereals by the developed multiplex FLISAs*

Calibration curves for the both immunoassays set up in two different matrices (wheat and maize) led to slight change of the assay sensitivities ($IC_{50}$ values): 14 and 17 ng $mL^{-1}$ for DON, 0.16 and 0.18 ng $mL^{-1}$ for ZEN, 0.14 and 0.18 ng $mL^{-1}$ for AfB1, 11 and 13 ng $mL^{-1}$ for T2 and 0.5 and 0.7 ng $mL^{-1}$ for FB1 by SAM FLISA in wheat and maize, respectively, and 0.69 and 0.74 ng $mL^{-1}$ for ZEN and 0.65 and 0.68 ng $mL^{-1}$ for AfB1 for DAM FLISA in wheat and maize, respectively. Hereupon, determination of mycotoxins in naturally-contaminated samples was done using calibration curves prepared in blank maize extract. A set of 19 wheat and 34 maize samples was used for validation of the developed multiplexes. As a confirmation technique LC-MS/MS was chosen (**SI6**).

The comparison of results obtained by LC-MS/MS and SAM FLISA is shown in Fig. 6. The SAM FLISA allowed AfB1 detection in the concentration range 0.5-18 μg $kg^{-1}$. Among the samples which were negative in LC-MS/MS, 17 samples tested positive for the target mycotoxins (ZEN, AfB1, T2) in a concentration range between the LODs of the SAM FLISA and the LOQs detected by the

chromatographic technique. A good agreement was demonstrated with the samples which were found to be contaminated, as determined with LC-MS/MS ($r^2$=0.981).

For the mycotoxins determination by DAM, an excellent correlation with the data obtained by LC-MS/MS was found: $r^2$=0.983 for ZEN and $r^2$=0.977 for AfB1. Seventeen samples which lacked ZEN and AfB1 according to LC-MS/MS analysis, demonstrated the presence of ZEN and AfB1 in the concentration range ≥LOD (DAM FLISA) and <LOQ(LC-MS/MS).

*Optimization and validation of the multiplex GBI*

This study aimed at the development of a rapid qualitative non-instrumental immunochemical test which can be performed outside the laboratory for the simultaneous screening of four mycotoxins in cereals. The intention was to develop GBI with sensitivities close to the maximum limits of the target mycotoxins, established by the European Commission Regulation as 1250 and 1750 μg $kg^{-1}$ for DON, 100 and 350 μg $kg^{-1}$ for ZEN, 2 and 5 μg $kg^{-1}$ for AfB1, 100 and 200 μg $kg^{-1}$ for the sum of T2 and HT2, 4000 and 4000 μg $kg^{-1}$ for the sum of FB1 and FB2 in unprocessed wheat and unprocessed maize, respectively (EC 2007, EC 20013, EC 2006a). Optimization of the GBI procedure included choice of optimal antibody and QDs-conjugates concentrations, composition and amount of washing buffer. Primary antibodies were placed onto the gel via binding with the already immobilized secondary antibody. This was done to ensure universal distribution of primary antibodies. Thereby, to reach the required sensitivity, three variables: SA-coupled gel dilution, primary antibody concentration and QDs-labeled conjugate dilution were simultaneously evaluated.

First, the column tests were developed and validated for each target mycotoxin (DON, ZEN, AfB1, T2, FB1) separately, afterwards their combination was achieved. A GBI with four test layers was designed. Five layers it was found to be difficult to operate. Application of red-emitted QDs in the GBI was cumbersome. Polyethylene frits as well as sepharose gel showed a weak red color under UV leading to the appearance of false-negative results. So, just one mycotoxin (DON) out of four was labeled with the red QDs. The dilution of the DON-QDs was high (1/15 for the single GBI and 1/12 for the multiplex format) to determine the analytical signal.

The main challenge was the simultaneous determination of four mycotoxins at the specified cut-off levels. A mixture of DON-QDs, ZEN-QDs, AfB1-QDs and T2-QDs was used as conjugate, so the dilution of every labeled antigen was elevated in order to decrease their influence on each other and facilitate the signal recognition. Since both wheat and maize were tested, the lowest maximum limit for each toxin was taken as cut-off, while for DON a cut-off level of 500 μg $kg^{-1}$ was chosen. Bearing in

mind the extraction and dilution of sample, a GBI with cut-offs at 500, 100, 2 and 100 µg $kg^{-1}$ for DON, ZEN, AfB1 and T2, respectively, was created. The visual contrast between the results obtained with positive samples and those obtained with negative samples was easily detected.

The developed on-site test was validated using 24 naturally-contaminated cereal samples (12 wheat and 12 maize). Comparison of the GBI and LC-MS/MS results showed good agreement both for positive and negative samples. Analytical characteristics of the test were calculated according to Trullols et al. (2004) and were based on these results and data of an intra-laboratory validation performed with blank cereal extracts artificially spiked with the target mycotoxins at concentrations less, equal and above the corresponding cut-off levels. The rates for false positive and negative results were always below 5% and the specificity and sensitivity rates were > 96 % (**Table SI-3**). All calculated parameters fulfilled the requirements set by the Commission Decision 2002/657/EC (EC 2006b) for a screening method hence makes the multiplex GBI suitable for mycotoxin screening in cereals.

## *Conclusions*

For the first time the immobilization of two different types of antibodies into the same well (double-analyte multiplex, DAM) for simultaneous fluorescent detection of two mycotoxins (zearalenone and aflatoxin B1) was described and compared with single-analyte multiplex (SAM) FLISA. For DAM, QDs which were luminescent in different parts of the spectrum (green and orange-emitted) were chosen for synthesis of the labeled conjugates. The analytical signal was detected for both analytes separately by double scanning of the plate with different emission wavelengths. The limits of detection for the simultaneous determination of zearalenone and aflatoxin B1 by this technique were 1.8 and 1 µg $kg^{-1}$, respectively The simultaneous detection of five mycotoxins (deoxynivalenol, zearalenone, aflatoxin B1, T2-toxin and fumonisin B1) was realized in one microwell plate by attaching specific for each mycotoxin antibodies into separate wells. Separate rows were used for construction of calibration curves and sample screening. For detection, multicolored (red, orange, green) CdSe-based quantum dots were used. Both FLISA formats demonstrated excellent applicability for screening of cereals.

A non-instrumental qualitative QDs-based column immunoassay coupled to a fast and easy sample pretreatment was successfully developed and evaluated for the simultaneous on-site determination of four mycotoxins in wheat and maize.

Thus, the developed multiplex assays capable of screening analytes meet the target requirements providing cheap, simple and reliable analytical techniques.

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**Fig. 1.** Absorbance and fluorescence spectra of core and core-shell QDs obtained from CdSe cores with d~2.6 nm (A) and d~4.6 nm (B).

**Fig. 2.** TEM images of the prepared QDs: CdSe cores with the first exciton peak at 530 nm (A) and corresponding core-shell QDs: CdSe/ZnS (B), CdSe/CdS/ZnS (C); CdSe cores with the first exciton peak at 610 nm (D) and corresponding core-shell CdSe/CdS/ZnS QDs (E) (all in toluene solution). The hydrophilic CdSe/CdS/ZnS QDs (λ(em.)=592 nm).

**Fig. 3.** Calibration curves for the mycotoxins determination by DAM FLISA. $A/A_0$ is a relative luminescence ($\lambda_{ex}$ = 365 nm, $\lambda_{em}$ = 540 nm for green QDs (ZEN determination) , 594 nm for orange QDs (AfB1 determination)). Data indicate averages of threefold determinations.

**Fig. 4.** Linear regression using SAM FLISA data for the mycotoxins screening in artificially spiked wheat and maize samples (data indicate averages of fivefold determinations).

**Fig. 5.** Linear regression equations derived using DAM FLISA data for mycotoxins screening in artificially spiked wheat and maize samples (data indicate averages of fivefold determinations).

**Fig. 6.** Linear regression equations derived using SAM FLISA and LC–MS/MS data for mycotoxins screening in naturally-contaminated cereal samples, found to be positive by both LC-MS/MS and the single-analyte multiplex FLISA (data indicate averages of fivefold determinations).