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09 April 2024

A FLUORESCENT IMMUNOCHROMATOGRAPHIC STRIP TEST USING QUANTUM DOTS FOR FUMONISINS DETECTION

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Highlights

- A QD-based strip test for fumonisin B1 qualitative and quantitative detection
- Reagents synthesis, preparation, optimization and performing of assay is described
- Direct comparison of QD, gold nanoparticles and chemiluminescent as ICST labels
- The test allowed ultrasensitive fumonisins detection in maize flour samples
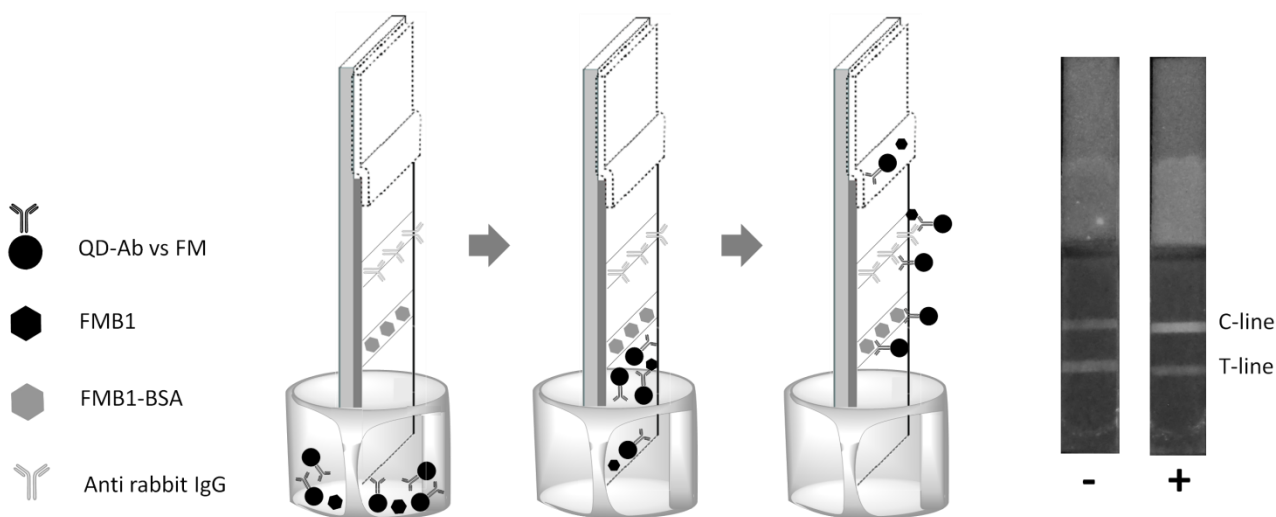
Graphical abstract. The fluorescent immunochromatographic strip test based on the use of Quantum Dots as labels for the sensitive and rapid detection of fumonisins (FMs) in maize flour. The test allows the rapid and simple visual detection of FM contaminations and the quantitative determination of FMs at levels of regulatory interest.

Abstract

A fluorescent immunochromatographic strip test (ICST) based on the use of quantum dots (QD) was developed and applied to detect fumonisins in maize samples. A limit of detection for fumonisin B1 of 2.8 µg*L^{-1} was achieved, with an analytical working range of 3–350 µg*L^{-1}, corresponding to 30–3500 µg*kg^{-1} in maize flour samples, according with the extraction procedure. The time required to perform the analysis was 22 min, including sample preparation. Recovery values in the range from 91.4 to 105.4 % with coefficients of variation not exceeding 5 % were obtained for fortified and naturally contaminated maize flour samples. To evaluate the possible improvements due to the use of QD for ICST technology, we performed a direct comparison of the proposed QD-ICST to a gold nanoparticles- and a chemiluminescent-ICST previously developed for fumonisins detection, in which the same immunoreagents were employed.

Keywords: Fumonisin B1, immunoassay**,** immunochromatography, quantum dot, maize

Introduction

Rapid diagnostic assays have been in use for decades in the clinical and medical area. Nowadays rapid immunoassay-based tests are widely applied in clinical, drug, food [1-3], forensic [4] and environmental [5] analysis.

The immunochromatographic strip test (ICST), also known as lateral flow immunoassay, offers several advantages when compared to other immunoassay methods, such as an easy-to-operate format, rapid detection, no requirements of technical expertise and relatively low cost. To perform a test with the ICST technique, no instrumentation is needed. It is thereby considered as particularly feasible for using outside the laboratory. An ICST device is based on immunoassays in which the sample flows by capillary forces along an analytical membrane that contains immobilized immunoreagents. Traditional ICSTs employ colloidal gold to generate visual signals and usually provide a binary yes/no answer that corresponds to the presence of the analyte over or under a certain concentration in accordance with a specific or mandatory cut-off level. To reach a better sensitivity and an easier objective interpretation, however, new labels need to be explored.

The use of fluorescent labels instead of colorimetric labels leads to a significant lowering of the detection limit in different analytical methods [6,7]. Fluorescent labels, moreover, have found widespread use in biosensing applications including immunoassays, nucleic acid detection, resonance energy transfer studies, diagnostic assays, cellular labeling and others [8-13].

Fluorescent materials could represent, therefore, an obvious choice also for the ICST development. However, many of the organic dye- and protein-based fluorophores currently in use suffer from serious chemical and photophysical liabilities, such as the pH dependence, the self-quenching at high concentrations, the susceptibility to photo-bleaching, the short-term stability in aqueous media, and the short lifetimes of the excited state . These limitations have been partially solved by the development of the fluorescent semiconductor nanocrystals (Quantum Dots, QDs) [14].

Since their first description in a biological context [15,16], QDs have attracted the interest of the biosensing community due to their unique luminescent properties. These inorganic compounds are mainly composed of elements of the II–VI or III–V groups (e.g., CdSe, CdTe, CdS, ZnSe, or core/shell systems such as CdSe/ZnS and CdSe/ZnSe, among others) with sizes comprise between 2-10 nm. The QDs' shape is generally spherical, and they contain hundreds or thousands of atoms, depending on their final size. Compared to conventional fluorophores, QDs have excellent fluorescent properties, such as high quantum yields, size-tunable fluorescence and broad absorption spectra, narrow and symmetric emission spectra, large molar extinction coefficients, strong fluorescence emission, and high resistance to photobleaching. The special properties of these nanomaterials are explained by the strong confinement of electrons when the radius of the particle is smaller than the Bohr exciton radius, which results in a quantization of the electronic energy levels. The band gap of QDs increases as their size decreases. The distance between the discrete energy levels, which defines the absorption and photoluminescence characteristic, can be tuned through the choice of the material and the particle size [17].

Since high-fluorescent QDs are usually synthesized at high temperature in organic solvents, they are only soluble in non-polar organic solvents, which are not suitable for biological applications. For water compatibility and bioconjugation, QDs are covered with hydrophilic shells.

Among the different fluorescent labels for ICST, QDs are the most promising. Recently, several non-competitive QD-based ICSTs have been developed, which aimed at detecting *Staphylococcus aureus* in food [18], alpha fetoprotein and carcinoembryonic antigen simultaneously [19], alpha fetoprotein [20], foodborne pathogens [21], syphilis [22], and *Mycobacterium* species [23]. Nevertheless, very limited research on competitive QD-based ICSTs [24-26] has been reported in the literature.

In the present study, we described the use of QDs for the development of a fluorescent competitive ICST for fumonisins (FMs) detection in maize flour.

FMs are mycotoxins mainly produced by *Fusarium* species growing on agricultural commodities in the field, at the harvest or during the storage [27]. There are different forms of fumonisins and among these, Fumonisin B1 (FMB1) is the most common naturally occurring form, followed by

FMB2 and FMB3. FMB1, mainly found in maize and maize-derived food, is a hepatocarcinogen in rodents and a kidney carcinogen in rats [28]. It has been classified as a possible human carcinogen (Group 2B) by the International Agency for Research on Cancer (IARC) [29]. Due to adverse effects in animals and humans and to their incidence in food, FMs are regulated by European Union [30] and their monitoring is mandatory for food safety assessment.

Moreover, the use of FMB1 as a model analyte for the development of a QD-based ICST allowed us to make a well-founded evaluation of the potential of QDs as new labels for ICST, thanks to the comparison with more traditional probes (gold nanoparticles, GNP-ICST) and an ultrasensitive chemiluminescent-based ICST (CL-ICST). These assays were previously developed by our group and employed exactly the same analytical antibody (Ab vs FM, raised against the FMB1) and reagents deposited on the nitrocellulose membrane [31,32].

Materials and methods

Reagents

Fumonisin B1, zearalenone, aflatoxin B1, deoxynivalenol (Oekanal standard solutions), polyethylene glycol (PEG, average mw 10 kDa), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), cadmium oxide (CdO, 99,99%), selenium powder (Se, 99,99%), sulfur powder (S, 99%), zinc acetate (Zn(OAc)$_2$, 99,99%), oleic acid (90%), 1-octadecene (90%), oleylamine (70%), octadecylamine (90%), and trioctylphosphine, poly(maleic anhydride-alt-1-octadecene) (PMAO, M ~ 30 000-50 000) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Tween 20, Triton X-100 and other chemicals were purchased from Merck-VWR (Milan, Italy).

Polyclonal antibodies towards Fumonisin B1 Ab vs FM developed in rabbit and BSA-FMB1 were kindly provided by Generon srl (Modena, Italy). The γ-globulin fraction was isolated by ammonium sulfate precipitation and used without any additional treatments. Jeffamine M1000 (1000 g/mol) was kindly provided by Huntsman (Belgium).

Millipore High Flow (HF) 240, 180, 120 and 90, and cellulose adsorbent pad were obtained from Merck Millipore (Billerica, MA, USA).

Buffers and solutions used: sodium hydrogen phosphate-sodium dihydrogen phosphate 20 mM pH 7.4 (phosphate buffer), sodium hydrogen phosphate-sodium dihydrogen phosphate 100 mM pH 7.4, sodium hydrogen phosphate-citric acid 20 mM pH 6.5,), tris(hydroxymethyl)aminomethane hydrochloride (Tris) 20 mM pH 7.4, (Tris) 20 mM pH 8.5, sodium borate-boric acid, 20mM pH 8.5 (borate).

QD synthesis

Hydrophilic core/shell/shell CdSe/CdS/ZnS QDs with emission at 603 nm and photoluminescence quantum yield of ~30 % were synthesized as previously described [33]. Briefly, CdSe QDs were prepared using a hot-injection method, and then they were covered with three layers of CdS and two layers of ZnS by the successive ionic layer adsorption and reaction method.

The hydrophobic QDs were transferred to aqueous solutions by covering with an amphiphilic polymer. The polymer was synthesized from the PMAO and the Jeffamine M-1000 polyetheramines (molar ratio was 1:1). Purified QDs and the amphiphilic polymer were mixed in chloroform and stirred overnight at room temperature (mass ratio of the QD:PMAO was ~1:7). An equal volume of NaHCO$_3$ solution (0.1 M) was added to the QDs-polymer chloroform solution. Afterwards, the chloroform was slowly evaporated with a Bunsen's water-air-jet pump, and a clear fluorescent solution was obtained. Finally, to remove the Jeffamine M1000 excess, we performed three rounds of ultrafiltration with Amicon centrifuge filters (100kD MWCO).

Preparation of QD-Ab vs FM conjugates

The QD-antibody conjugates (QD-Ab) were prepared using the activated ester approach, in which the carboxyl groups of the polymer coating QD surface were activated with EDC. The activated carboxyl groups were subsequently reacted with amine groups of the antibodies.

Operatively, 0.1 ml of QDs diluted in 1 mL of 20mM phosphate buffer, pH 7.4, was pre-activated with 0.05 ml of a freshly prepared aqueous solution of EDC (10 mg*mL^{-1}) for 20 min at room temperature. Then, 0.15 mg of Ab vs FM was added to the pre-activated QDs and incubated for 30 min at room temperature. A second aliquot of EDC was added and the tube was transferred to 4°C for a further overnight incubation. The molar ratio of QD:Ab:EDC in the conjugate synthesis was 1:10:4000. QD-Ab conjugates were centrifuged for four times at 14000 rpm to remove any unbound antibodies. After the addition of 10 % Triton X-100, the conjugates were stored at 4°C, in the dark, until use.

Preparation of test strips

Assay strips were prepared from nitrocellulose membranes (Hi-flow plus 180) employing an XYZ3050 platform (Biodot, Irvine, CA, USA), equipped with two BioJet Quanti™ 3000 Line Dispenser for non-contact dispensing. In particular, from bottom to top of the strip, the FmB1-BSA conjugate (0.2 mg*mL^{-1}) and the goat anti-rabbit IgG antibodies (1 mg*mL^{-1}) diluted in phosphate buffer were dispensed to form the T-line and the C-line, respectively. Reagents were deposited at 1 µL*cm^{-1}, keeping a distance of 4 mm between the lines.

Membranes were dried at 37°C for 60 minutes under vacuum and then assembled with a cellulose pad as the adsorbent pad, with 1-2 mm overlap on the nitrocellulose. In the adopted strip configuration, we did not use sample and conjugate pads. Assembled membranes were cut into strips (5 mm width) by means of a CM4000 guillotine (Biodot, Irvine, CA, USA) and stored in a desiccator at room temperature.

Analysis of maize flour samples

Maize flour samples were obtained directly from producers or mills and were stored at -20°C. Their content in fumonisins (FMB1 + FMB2) was determined by the LC-MS/MS, as previously described [31]. An aqueous solution with 2% (w/v) PEG and 1% (w/v) sodium citrate was used to extract maize flour samples (10 ml of extracting solution per 1 g of sample). After a 2 min manual shaking, the suspension was allowed to settle for 5 min and the supernatant was filtered through a 0.45 μm cellulose syringe filter and analyzed or used to prepare FMB1 standards. Standards of FMB1 were prepared daily by diluting the reference solution in the extract of blank maize flour (samples which FMs content resulted undetectable according with the LC-MS/MS measurement).

Assay procedure

The fluorescent ICST in the dipstick format was carried out at room temperature. 95 μl of sample extracts (or FMB1 standards prepared as described above) were transferred into wells of a 96-well black microtiter plate (VWR International, Milan, Italy) and we added 5 μl of QD-Ab conjugate. To start the capillary migration process, the strip was dipped in the well after homogenization of the solution by gently pipetting (Figure 1).

After 10 min, the strip was removed and dried for 5 min at 37°C before observing the fluorescence signal. The test results were both qualitatively estimated by the naked-eye under a UV light at 365 nm (Camag, Berlin, Germany) in a dark room, and quantitatively evaluated using a super charge-coupled device camera (Fujifilm, Tokyo, Japan) and the digital processing of images with QuantiScan 3.0 software (Biosoft, Cambridge, UK). Before data processing, we applied a red filter on the images, in order to subtract the signal on the lines due to the fluorescence of proteins. The quantitative information was obtained processing the negative of the Tagged Image File Format. To evaluate assay results, the signals from T-line and control line (C-line) for each test were processed. Standard calibration curves were obtained by plotting the T-line/C-line ratio versus FMB1 standard concentrations and fitting data with a four-parameter logistic equation.

Analytical performances of QD-ICST

The accuracy of the proposed fluorescent-ICST was evaluated by analyzing recovery values of fortified and naturally contaminated maize flour samples (samples fortified at 200 and 1000 µg*kg^{-1} and eleven samples naturally contaminated). The precision was evaluated by analyzing coefficients of variation of replicate measures of maize flour samples fortified at 200 and 1000 µg*kg^{-1}. The two levels were analyzed in six replicates in the same day for the within-day precision and in duplicate on four days for the between-day precision evaluation. The limit of detection (LOD) was evaluated as the concentration corresponding to the T-line/C-line ratio value of the blank minus three times its standard deviation.

Results and discussion

In the proposed fluorescent ICST, free antigen in solution and the antigen deposited on the T-line compete for binding to the QD-Ab. In the presence of FMs in the sample, its will bind QD-Ab and the complex will flow through the membrane. The fluorescence emission of the T-line will decrease compared to that observed for a blank sample, there all QD-Ab will be bound to the antigen deposited on the T-line, and its decrease can be correlated with FMs concentration. The more FMs present in the sample extracts, the lower the fluorescence emitted by the T-line. Anti-rabbit antibodies deposited on the C-line, instead, would capture any excess of QD-Ab (bound or unbound to FMs), ensuring the validity of the test.

For quantitative analysis, the T-line signal was normalized with respect to C-line signal of the same strip. T-line/C-line ratio was used to offset the inherent heterogeneity of the strips and to take into account environmental and matrix factors that might affect the intensity of fluorescence signals.

Optimization of experimental parameters

Generally, the analytical performances of an ICST can be affected by the properties of the materials used to build the device and, particularly, the nitrocellulose membrane. We tested Millipore High Flow (HF) 240, 180, 120 and 90 nitrocellulose membranes, characterize by different pores sizes and flow rates. None of the tested materials had shown background fluorescence under UV light (365 nm). The HF 180 proved to be the best choice in terms of low background noise, high fluorescence signal intensity, high sensitivity and test rapidity. In addition, using the HF 180, the deposition of the FMB1-BSA to form the T-line showed a narrow binding zone, while with more rapid membranes (HF 90 and HF 120) showed a diffuse binding zone, which determined widened and pale signals.

To reach an acceptable mobility of the QD-Ab across the nitrocellulose strips we did not use sample and conjugate pads. It was possible to avoid using these pads simply introducing a filtration of the extracts.

As noticed by Berlina, *et al.* [24], the absence of detergent in the running solution does not allow the complete migration of QDs along the nitrocellulose membrane, so we tried the addition of different percentages (2.5, 5 and 10 %) of Tween 20 and Triton X-100. For our assay, we observed that Triton X-100 assured better QDs migration and provided a complete absence of nitrocellulose coloration outside the Test and Control lines (data not shown). In the preliminary tests, we added different percentages of the detergent in the maize flour extract. At a later stage, to remove this additional step from the analytical protocol, we decided to add the surfactant directly to the stock QD-Ab solution. We observed a complete migration of QDs using 10 % Triton X-100.

To achieve the best sensitivity and good fluorescence emission on both lines, we evaluated following parameters: the buffer composition (phosphate, citrate, borate and Tris), the pH (6.5, 7.4 and 8.5) and the ionic strength (20 and 100 mM) for the QD-Ab conjugation and the QD-Ab amount in the running solution. The phosphate buffer 20 mM, pH 7.4 was chosen as the diluent for the QD-Ab synthesis and running. A QD-Ab 1:20 dilution in the running solution was chosen as the best compromise between high sensitivity and the presence of visible signals.

10 min of reaction time was sufficient to obtain a complete migration and stable fluorescence signals. However, on the wet nitrocellulose, fluorescence signals were weak, so it was necessary to dry the strip before proceeding to evaluate Test and Control lines signals. 5 min at 37°C was the minimum drying necessary to obtain clearly visible signals.

Qualitative evaluation of FMs by the QD-ICST

A calibration curve was obtained in the optimal conditions by using standard solution of FMB1 prepared in the extract of blank maize flour in the range between $1 - 500$ µg*L^{-1} (Figure 2). The visual limit of detection (vLOD) of the assay was defined as the lowest FMB1 concentration resulting in a T-line colour significantly weaker than that of the C-line.

Intensities of Test and Control lines were observed under an ultraviolet light source (365 nm), in a dark room, by five operators. All subjects reported a T-line signal significantly weaker than that of the C-line for the extract fortified at 20 µg*L^{-1}. Thus, a FMB1 concentration of 20 µg*L^{-1} in maize flour extract (corresponding to 200 µg*kg^{-1} in the sample) was considered as the vLOD of the system, which equal to the lowest regulatory limit for FMs set by European Union [29].

Quantitative determination of FMs by the QD-ICST

To obtained quantitative results, we processed the recorded images as previously described. The obtained LOD value was 2.8 µg*L^{-1} (28 µg*kg^{-1} in maize flour). The dynamic range of the method extended from 3 to 350 µg*L^{-1} (30 to 3500 µg*kg^{-1} in maize flour), with an IC$_{50}$ of 23 µg L^{-1}.

The assay accuracy and precision (within- and between-day) were evaluated on fortified samples, obtained by adding known amounts of FMB1 to the extract of a blank maize flour sample. We used two levels of fortification, corresponding to two regulatory limits: for processed maize-based foods and baby foods for infants and young children (200 µg*kg^{-1}), and for maize and maize-based foods intended for direct human consumption (1000 µg*kg^{-1}), respectively [30]. Both levels were quantified in six replicates on the same day (within-day) and on four different days for the between-day tests (Table 1). The recovery ranged from 95.5 to 97.5 %, while coefficients of variation of 4 % (for within- and between day) were obtained.

The selectivity of the QD-ICST was evaluated by analyzing the extract of the blank maize flour sample fortified with zearalenone (500 µg*L^{-1}), aflatoxin B1 (50 µg*L^{-1}) and deoxynivalenol (500 µg*L^{-1}) separately. No significant cross-reactivity was observed.

Analysis of maize flour samples

To evaluate further the performances of the developed QD-ICST, we analyzed 11 naturally contaminated maize flour samples obtained from producers or mills.

The analytical antibody employed in the assay (the polyclonal antibodies directed towards FMB1) showed in previous studies a cross-reactivity for FMB2 of 97 % [31]. Therefore, the assay would provide information about total fumonisins content, as requested by the European Union [30]. The results obtained through the QD-ICST were compared with the total fumonisins concentration (the sum of FMB1 and FMB2) previously obtained by the LC-MS/MS [31]. The samples were both judged qualitatively by the naked eyes by five operators and quantitatively analyzed by recording images of the strips (Table 2). The analysis of maize flour samples naturally contaminated by FMB1 and FMB2 showed a good accuracy, in the range from 91.4 to 105.4 % and a good precision, with CVs not exceeding 5 %.

Comparison of QD-ICST, GNP-ICST and CL-ICST

We could evaluate the possible improvements due to the use of QD in an ICST thanks to the direct comparison with a GNP- and a CL-ICST previously developed by our group the for detection of FMs in maize [31,32]. The analytical antibody and the reagents deposited on the nitrocellulose membrane were the same in the three considered ICSTs. The figures of merit of the assays are schematically reported in Table 3.

The QD-ICST sensitivity is better than the gold-ICST sensitivity and it is very close to that reached with the ultrasensitive CL-ICST. QD- and CL-ICSTs are characterized by a better detectability at the level of the lowest regulatory limit for FMs in maize samples, since the IC_{50} was close to 20 $\mu g*L^{-1}$ (corresponding to 200 $\mu g*kg^{-1}$ in maize samples, according with the extraction procedure).

The GNP-ICST consists in a rapid one-step assay: the sample is directly added into the sample well and signals could be observed and measured after 10 min from sample dispensing. The analytical procedure of the QD-ICST is longer because after 10 min from the beginning of the capillary migration process, it is necessary to dry the strip before proceeding to evaluate Test and Control lines signals. Conversely, the CL-ICST consists in a two-step assay; after 10 min from the addition of the test solution on the sample pad, it is necessary to add the CL-substrate to obtain chemiluminescent signals. For these reasons, the GNP-based ICST is the fastest and the easier-to-operate assay.

The QD-ICST upper limit of quantification is lower than those showed by GNP- and CL-ICSTs. The developed assay cannot be used to analyze unprocessed maize samples, for which the EU regulatory limit, is 4000 $\mu g*kg^{-1}$. Conversely, the QD-ICST shows better accuracy and precision.

Conclusions

In this study, an immunochromatographic strip test employing Quantum Dots as fluorescent probes was developed for fumonisins detection. The analysis required 22 min including the sample preparation and the system is characterized by good recovery. The QD-based ICST is capable to detect a minimum of 28 $\mu g*kg^{-1}$ FMB1 in maize flour samples, according with the extraction procedure. The assay provides both qualitative results for rapid assessment and quantitative results for accurate determination of FMs in maize samples. The use of QDs improved the sensitivity of ICST compared to the GNP-based ICST, according to what observed for zearalenone detection by Duan *et al*. [26]. However, to reach improved sensitivity, Duan *et al.* had to encapsulate the QDs using the microemulsion technique in order to amplify the fluorescence emission of the probes.

The proposed QD-ICST showed sensitivity comparable with that of the ultrasensitive chemiluminescent-based ICST previously developed. The study consolidates QDs as promising label to be applied in ICSTs for small molecule detection reaching high analytical performances.

Acknowledgements

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Figure captions

Figure 1. Schematic description of QD-based ICST in dipstick format. The strip is dipped in a well that contains the sample and QD-labeled antibodies. During migration across the membrane, QD-labeled antibodies bind to the analyte or to the anchored antigen (T-line). When reaching the C-line, QD-labeled antibodies are captured by anti-species antibodies. Two fluorescent lines appear, due to the focusing of bound QD-Abs. The T-line intensity is inversely related to the analyte concentration; therefore a positive sample (+), which contains FMB1 above the cut-off level, shows higher fluorescence on the T-line compared to the C-line, while a negative sample (-), which contains FMB1 below the cut-off level, shows less intense fluorescent emission on the Test-line compared to the C-line.
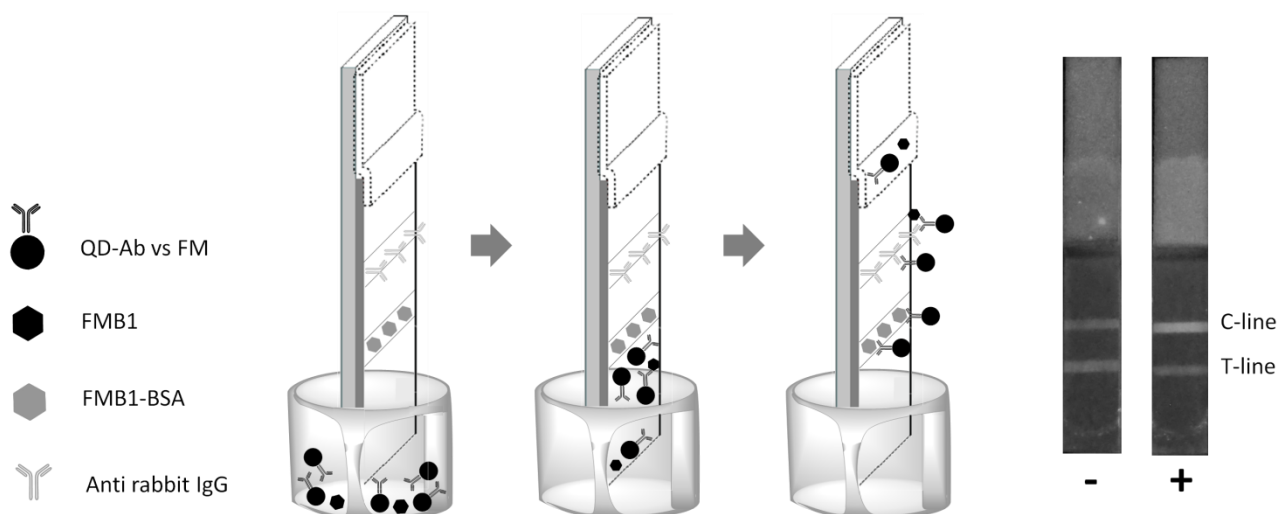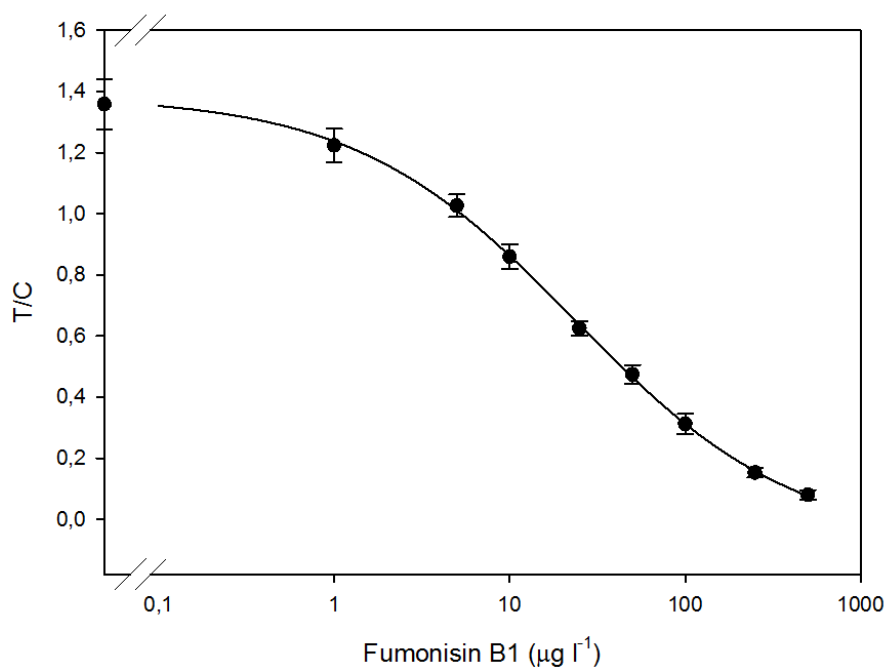
Figure 2. Typical calibration curve carried out by diluting FMB1 in the extract of a blank maize flour sample: (a) for the quantitative analysis, the ratio of T- and C-line fluorescence emission was plotted against the log of FMB1 concentration; (b) images of the strips under UV irradiation.

a

b

TABLES

Table 1. Within- and between-day accuracy and precision of the developed QD-ICST for maize flour samples fortified at two levels (200 and 1000 µg*kg^{-1}).

FMB1 expected concentration (µg*kg^{-1})	Within-day			Between-day		
	Measured concentration (µg*kg^{-1})	CV % (n=6)	Recovery (%)	Measured concentration (µg*kg^{-1})	CV % (n=12)	Recovery (%)
200	191	4	95.5	195	4	97.5
1000	974	4	97.4	971	4	97.1