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Comparative Study of the Performance of Two Different Luciferases for the Analysis of Fumonisin B_1 in Wheat Samples

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In memory of Maria Moreno-Bondi, who passed away in June 2022

The development of two different immunoassays for the determination of fumonisin B_1 in wheat samples is reported. A previously described mimopeptide for fumonisin B_1 (FB_1) was used to produce fusion proteins in combination with two different luciferases: *Gaussia* luciferase (GLuc) and NanoLuc luciferase (NLuc). The production, expression and the development of two immunoassays based on these fusion proteins (A2-GLuc and A2-NLuc) is detailed. The assay showing the best performance, A2-NLuc, with a limit of detection of 0.61 $ng\,mL^{-1}$ and a dynamic range from 1.9 to 95 $ng\,mL^{-1}$, was employed for the analysis of spiked wheat samples, a reference matrix material, as well as naturally contaminated wheat samples. The recoveries obtained in the spiked samples were acceptable, between 81.5 and 109%, with relative standard deviations lower than 14%. The analysis of naturally contaminated wheat was validated by a liquid chromatography coupled to tandem mass detection method.

Introduction

Enzymes and fluorescent proteins are considered of great importance for many optical applications, including fluorescent imaging and immunoassay development. Despite the great applicability of fluorescent proteins in current research, enzymes are considered the most prevalent in sensing applications.[1,2] High sensitivities can be obtained with their use as it has been estimated that, under optimal pH, ionic strength and temperature conditions, a single enzyme molecule is able to convert up to 10^7 molecules of its corresponding substrate per minute.[3]

Luciferases are a specific type of enzymes that have been considered of utmost relevance over the last years. They are able to catalyze reactions that emit light, and are currently applied in highly relevant fields, such as food testing, diagnostics or drug screening.[4,5] Two of the smallest luciferases currently known are *Gaussia* luciferase (GLuc) and NanoLuc luciferase (NLuc), with a size of only 19.9 kDa and 19 kDa, respectively. These two luciferases offer an exceptional performance in protein–protein interactions or bioluminescence resonance energy transfer (BRET) immunoassays, as they are considered two of the brightest luciferases yet discovered.[6–8]

Fumonisins are mycotoxins produced as secondary metabolites by several *Fusarium* species, such as *F. verticillioides* and *F. proliferatum*, two common pathogens found in some cereals, maize, wheat, and sorghum.[9,10] Fumonisins were reported and characterized back in 1988, and among the different metabolites found in this group, fumonisin B_1 (FB_1) is the most prevalent one.[11,12] Fumonisins pose a dangerous health risk for both humans and animals, as they have been reported to cause equine leukoencephalomalacia,[13] porcine pulmonary edema,[14] and in humans, fumonisins have been associated with esophageal cancer, neural tube defects and nephrotoxicity.[10,15,16] Due to their high risk, the detection of fumonisins in foodstuff is of great importance. In fact, some of the most eminent international authorities regarding food safety, such as the European Commission and the US Food and Drug Administration (FDA),

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have set maximum limits of these mycotoxins in a variety of foodstuff.[17,18]

Traditional chromatographic methods, especially those based on mass spectrometry detection (LC-MS/MS), have been extensively applied to the determination of fumonisins in a wide variety of matrixes.[19–25] Despite the fact that these methods offer sensitive determinations (μg Kg^{-1} level using triple quadrupole analyzers)[26] in a wide variety of matrixes, in many cases they suffer from matrix effects, requiring extensive sample preparation and chromatographic optimization to reduce them to a minimum.[26]

As an alternative, fast screening methods, such as immunoassays, have proven to give reliable results providing high sensitivity (limits of detection in the microgram per kilogram detection levels) in cereal samples (Table S1),[26,27] at a substantially reduced cost and in a lesser time. Epitope-mimicking peptides, also known as mimopeptides or mimotopes, have become a successful tool to avoid the main limitations of tedious antigen conjugations and its potential toxicity to the user. Mimopeptides have the intriguing ability to bind to the same antibody paratope as the corresponding antigen and establish a competition for its binding sites.[28] A broad variety of mimopeptides have been obtained by phage display technology.[29] Among them, mimopeptides have been obtained for some of the most ubiquitous mycotoxins, such as aflatoxin, fumonisin B_1, ochratoxin A, or deoxynivalenol.[30–33] The potential of bioconjugating mimopeptides to optical labels such as the aforementioned luciferases, GLuc and NLuc, has opened a great scope of sensing possibilities.

This work aims for a closer comparison of these two fascinating luciferases in the development of a bioluminescent immunoassay for the detection of FB_1 in wheat samples. For this aim, the construction of two different fusion proteins, A2-GLuc and A2-NLuc, in combination with a previously reported mimopeptide for FB_1 is detailed. Then, the analytical characteristics of both fusion proteins is tested and compared. Finally, the best bioluminescent system was chosen for the analysis of fumonisins in wheat samples, validating the results with a chromatographic-based method.

Results and Discussion

A2-GLuc and A2-NLuc expression and purification

The construction of the recombinant fusion proteins was based on the bioluminescent GLuc and NLuc proteins in combination with a fumonisin B_1 mimopeptide, A2, previously obtained by phage display.[31] To carry out the expression of the fusion proteins (A2-GLuc and A2-NLuc), the plasmids were transformed into different *E. coli* cells. In the case of the A2-GLuc fusion protein, Shuffle cells were utilized because the GLuc contains disulfide bonds that ought to be formed in an oxidizing environment. An especially important indication to point out in the expression of the A2-GLuc fusion protein is that this expression was carried out at 15 °C. Low temperature was critical due to the fact that higher temperatures led to the protein aggregation and consequent denaturation, obtaining dissatisfactory results. On the other hand, the A2-NLuc needed no formation of disulfide bonds, and therefore BL21 cells were used. In this case, the expression was successfully conducted at 37 °C for 4 h. Once both fusion proteins were purified, the correct expression was confirmed by SDS-PAGE (Figure S1). As can be seen, the molecular mass obtained on both cases was in accordance with the theoretical values of 63748 g mol^{-1} for A2-GLuc, due to the presence of the maltose binding protein (MBP) and 22982 g mol^{-1} for A2-NLuc since the fusion protein was not expressed in combination with the MBP.

Bioluminescent characterization and kinetics of the fusion proteins

The bioluminescent nature of the fusion proteins was confirmed after the addition of the respective substrates, coelenterazine for A2-GLuc and furimazine or NanoGLO for A2-NLuc. As can be observed in Figure 1, the kinetics observed for each of the luciferases vary significantly. On the one hand, the A2-GLuc fusion protein experiences a remarkably fast kinetics, decreasing the bioluminescence signal by a third of the original value in just one minute. On the opposite site, the A2-NLuc presents a much slower reaction kinetics, with a similar decrease of the signal in more than five minutes after the bare substrate (furimazine in PBS) was added. Moreover, the NanoGLO™ Luciferase Assay Reagent provided a glow-like kinetics, avoiding any flash consumption of the reagent and increasing the time of the bioluminescent signal more than two times

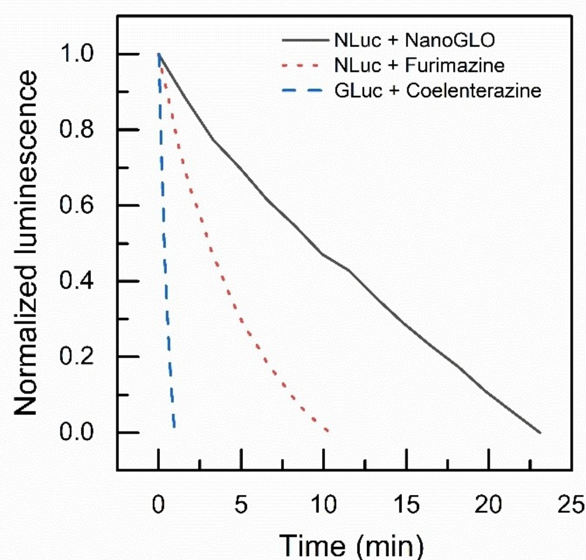

Figure 1. Kinetic curves for A2-GLuc (blue dashed line), A2-NLuc with furimazine (red dotted line) and A2-NLuc with NanoGLO reagent (black solid line). To obtain these curves, 50 μL of each substrate (coelenterazine for A2-GLuc and furimazine or NanoGLO for A2-NLuc) were added to a solution of 50 μL of each fusion protein. Different concentrations of the fusion proteins were tested in the experiment, obtaining similar results for all of them. The graphs represent the normalized bioluminescence of three replicates.

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with respect to the bare substrate. Taking these results into account, the assay using the A2-GLuc fusion protein was carried out with the help of a controlled injection of the substrate inside the microplate reader for a higher reproducibility and sensitivity of the assay. However, this was not necessary in the case of the A2-NLuc when the Nano-GLO substrate was used.

Assay optimizations

It has been previously reported that the immunoassay sensitivity can be improved if the antibody is immobilized on the surface of magnetic beads, instead of coating it directly to a well plate.[34] This was the main reason why the assay was developed using protein G-coupled magnetic beads to immobilize the antibody. Prior to that, the competition step was established in solution between the free FB_1 and the corresponding fusion protein for the binding sites of the antibody (Figure 2).

A checkerboard titration was conducted for the A2-GLuc fusion protein varying the antibody and fusion protein concentration, for a fixed magnetic bead concentration of 0.1 $mg\,mL^{-1}$, in the absence and presence of free FB_1. In order to obtain the optimal concentrations for the immunoassay, the maximum signals obtained in the absence of free FB_1 were compared to those signals obtained in the presence of 10 $\mu g\,mL^{-1}$ free FB_1. The best maximum/minimum signal ratio was chosen for the development of the immunoassay. The best ratio was obtained for a total antibody amount of 100 ng per well and a concentration of 3 $\mu g\,mL^{-1}$ fusion protein (Figure S2).

With reference to the A2-NLuc fusion protein, a much higher concentration of the fusion protein was needed to obtain measurable bioluminescence signals. Taking into account the lower molecular weight of the A2-NLuc fusion protein in comparison to the A2-GLuc, it would be expected that a lower concentration of the protein would provide similar results. However, based on the experimental trials, it was determined that the molar concentration of the A2-NLuc fusion protein needed to be almost 10 times higher than that of the A2-GLuc. In this case, once the fusion protein concentration was optimized, a checkerboard titration was carried out varying the antibody and magnetic bead concentration, for a concentration of 8.5 $\mu g\,mL^{-1}$ A2-NLuc. In this case, the best ratio was observed for a total antibody amount of 100 ng per well, similar to the A2-GLuc assay, and a bead concentration of 12.5 $\mu g\,mL^{-1}$ (Figure S3).

Bioluminescent-based immunoassays

Once the conditions for each of the fusion proteins were optimized, the bioluminescent-based immunoassays were conducted for both of them. Figure 3 shows the calibration curves

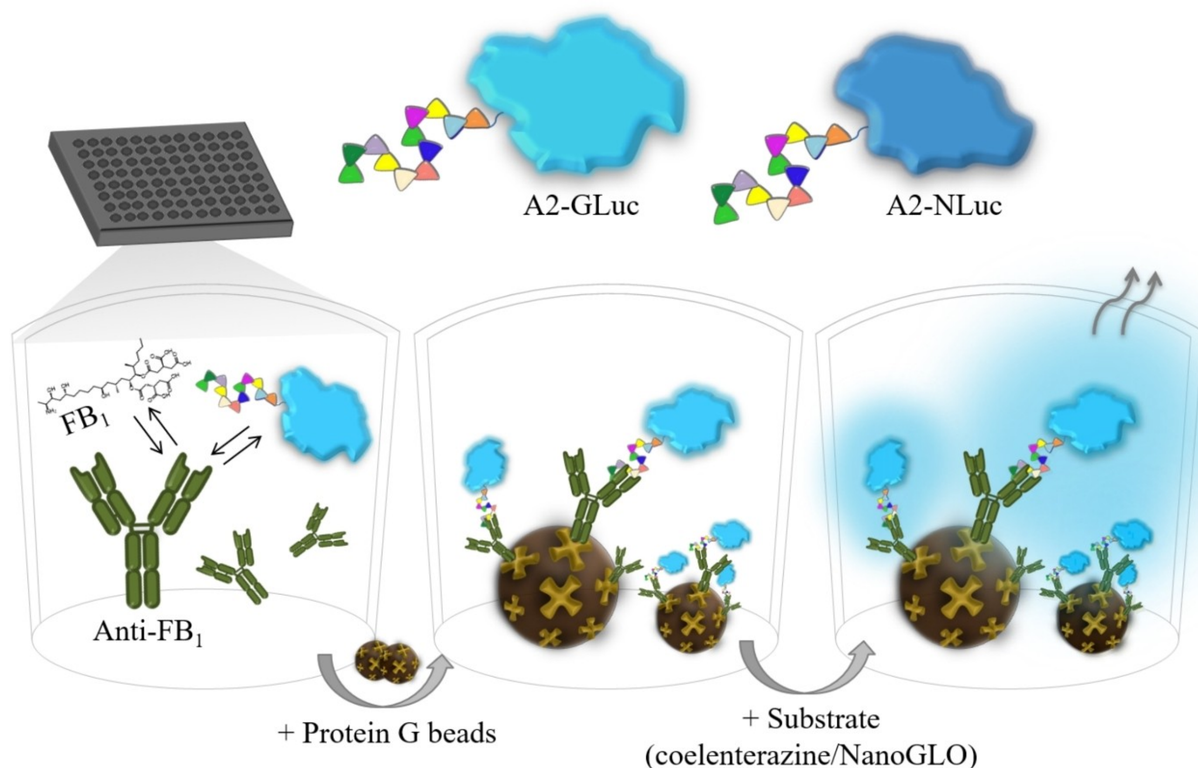

Figure 2. Scheme of the bioluminescent immunoassay. On black microtiter wells, competition between free FB_1 and the fusion protein (either A2-GLuc or A2-NLuc) for the binding sites of the anti-FB_1 antibody is established in solution. Then, protein G-coated magnetic beads are added to capture the formed immunocomplex. After a washing step using a magnetic support to retain the beads, the corresponding substrate is added (coelenterazine for GLuc and furimazine for NLuc) and the bioluminescence is measured on a microplate reader.

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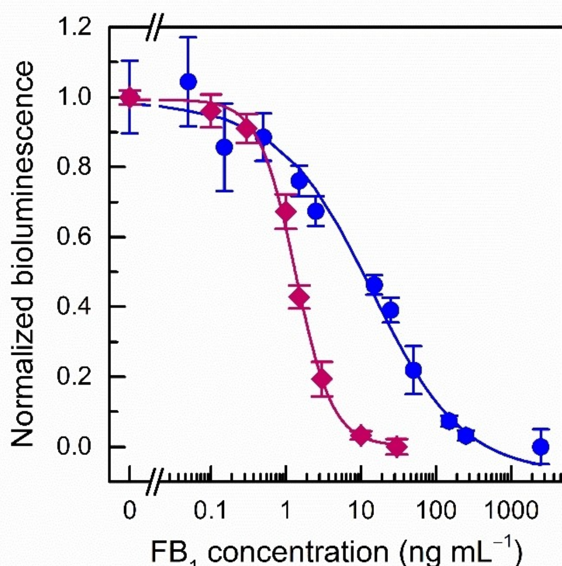

Figure 3. Bead-based bioluminescent immunoassays for the determination of FB_1 in assay buffer using A2-GLuc (purple diamonds) and A2-NLuc (blue circles) fusion protein. In both cases, a competition was established between FB_1 and the fusion protein containing the FB_1 mimopeptide for the binding sites of the antibody. Then, magnetic beads were added to collect the antibody in solution. The bioluminescence (total luminescence for A2-GLuc, $\lambda_{em} = 470 \pm 40$ nm for A2-NLuc) was measured after the addition of each substrate. The bioluminescence values were normalized to the maximum and minimum values. The results show the mean values ± the standard error of the mean (*n* = 3) adjusted to a logistic fit using OriginPro 2019 software.

for both fusion proteins in the presence of increasing concentrations of FB_1. As can be observed, both the A2-GLuc and A2-NLuc provide a great analytical performance, but their behavior was not exactly the same.

Table 1 provides a comparison of the main analytical characteristics of the two different assays described for the determination of FB_1. The A2-GLuc assay provided an IC_{50} of 1.34 ng mL^{-1} and a limit of detection (LOD), taken as the 10% inhibition,[35] of 0.38 ng mL^{-1}. The dynamic range, calculated as the 20–80% inhibition,[36] for the bead-based approach for A2-GLuc was between 0.6 and 2.9 ng mL^{-1}. However, the results of the bead-based approach utilizing the A2-NLuc fusion protein proved a much wider dynamic range, of almost two orders of magnitude, with a relatively similar LOD as the A2-GLuc approach. The A2-GLuc presents a good reproducibility, with relative standard deviations (RSD) of 7.4% in average (*n* = 3) for intra-day determinations and 15.5% for the inter-day assays on 3 different days. The A2-NLuc provides an average intra-day

Table 1. Analytical characteristics of the immunoassays for FB_1.

Fusion protein	IC_{50} [ng mL^{-1}]	LOD[a] [ng mL^{-1}]	DR[b] [ng mL^{-1}]
A2-GLuc	1.3 ± 0.1	0.38 ± 0.09	0.61–2.9
A2-NLuc	13.5 ± 1.7	0.61 ± 0.18	1.91–95.2

[a] Limit of Detection (taken as 10% inhibition) [b] Dynamic range (taken as 20–80% inhibition).

RSD of 9.0% (*n* = 3) and 15.9% for the inter-day assays on 3 different days. The implementation of magnetic beads allowed a competition between the free FB_1 and the fusion protein for the binding sites of the antibody, for a later capture of the antibody with the magnetic beads. This approximation reduces the number of washing steps in comparison to any other plate-based method. When comparing these results to other methods described in the bibliography, the bioluminescent-based immunoassays described here present higher sensitivities than most of the immunoassays previously described, and are within the most sensitive methods applied to the analysis of real samples (Table S1).

Cross reactivity evaluation

The specificity of each of the bead-based immunoassays for the determination of FB_1 was assessed with several mycotoxins that could be potentially present in the same food samples. The response curves are represented in Figure 4, and the results can be observed in Table S2. It is noted that for both cases, the FB_2 presents a cross reactivity of circa 100%, with the remaining toxins evaluated proving a negligible cross reactivity in the immunoassay. The high cross reactivity of FB_2 is associated to the extremely similar structure between both fumonisins. The antibody used in this case could not differentiate between them. Therefore, the assays could be implemented for the determination of the total amount of fumonisin in the analyzed samples.

Sample analysis

Matrix effect

In order to implement the immunoassays to the analysis of wheat samples, the matrix effect was tested with different percentages of wheat extract in the assay. To carry out these experiments, a non-contaminated wheat sample was utilized. The extraction procedure was conducted according to a previously reported method, in which a mixture of acetonitrile/water/acetic acid (79/20/1, v/v) was used.[37] As can be observed, only low percentages of the extract, 5% in both cases, could give a similar response in the immunoassay with negligible interferences (Figure 5). Higher concentrations of the extract severely interfered with the immunoassay, mainly because of the high percentage of acetonitrile. Due to the similarities in the cross reactivity and matrix effect, the immunoassay with the A2-NLuc fusion protein was chosen for the analysis of real samples due to the wider dynamic range and similar LOD in comparison with the A2-GLuc fusion protein. The LOD calculated for the analysis of FB_1 in wheat samples was 80 µg kg^{-1}, whereas the LOQ was 276 µg kg^{-1}.

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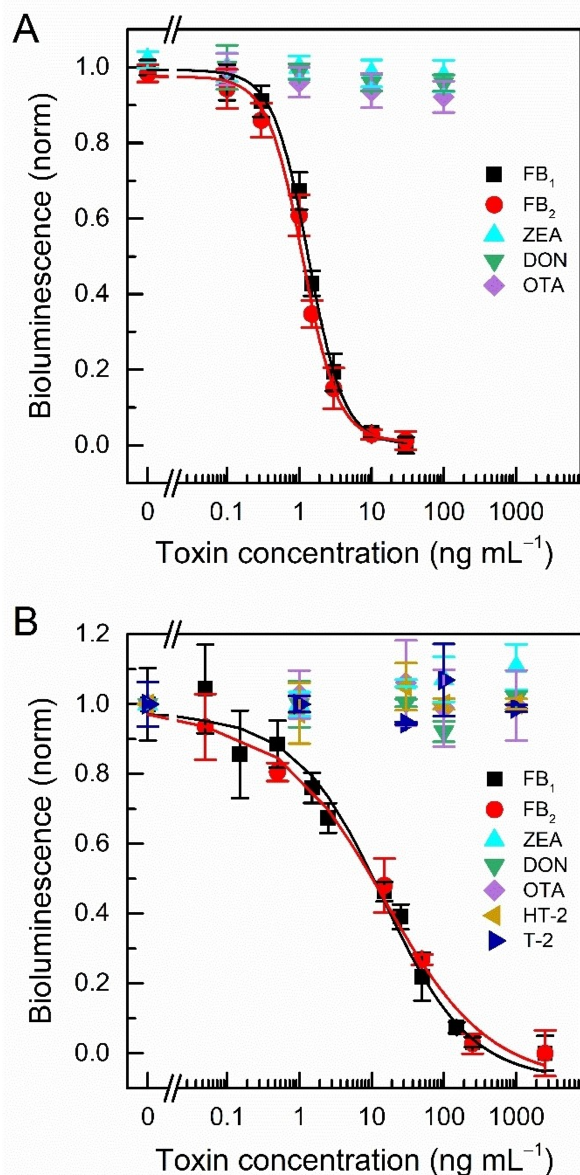

Figure 4. Cross reactivity evaluation for the bead-based immunoassay with (A) A2-GLuc and (B) A2-NLuc. Some of the most abundant mycotoxins which can be found in combination with fumonisin were tested in the immunoassay under identical experimental conditions. The results were normalized to the maximum and minimum values. The results show the average values ($n=3$)±the standard error of the mean and they were adjusted to a logistic fit using OriginPro 2019.

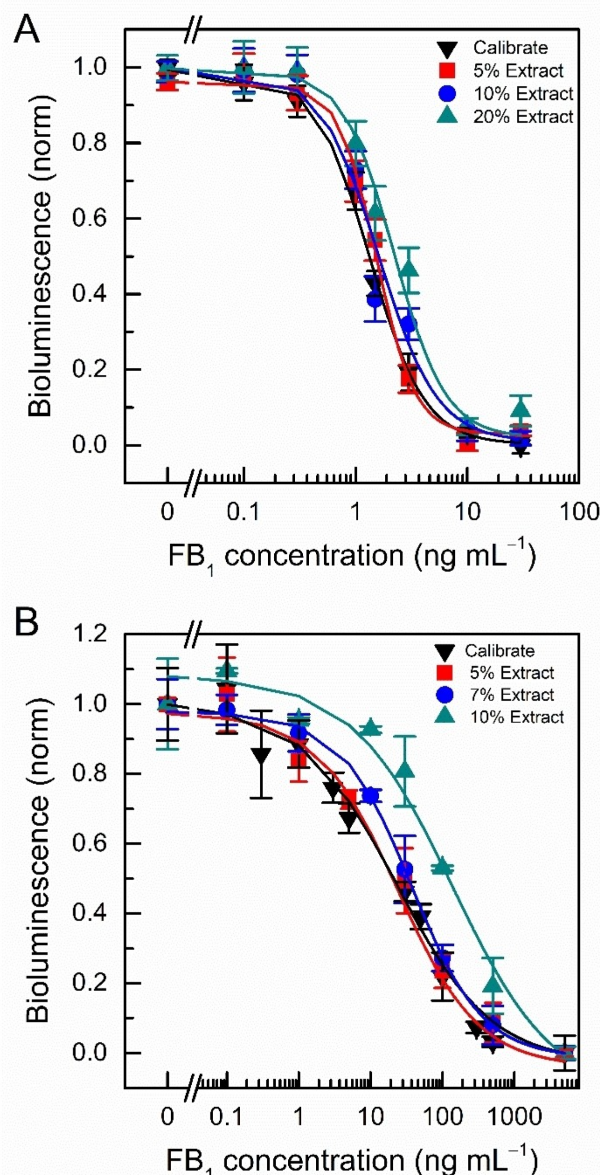

Figure 5. Matrix effect evaluation for the bead-based immunoassay with (A) A2-GLuc and (B) A2-NLuc. Different calibrate curves in the presence of a certain percentage of wheat extract were compared to a calibrate in assay buffer. The bioluminescence results were normalized to the maximum and minimum signals. The results are shown as the mean bioluminescence±the standard error of the mean ($n=3$). The calibrate curves were adjusted to a sigmoidal fit with OriginPro 2019.

Analysis of wheat samples

The bioluminescent assay using the A2-NLuc fusion protein was applied to the analysis of spiked wheat samples, a wheat reference material and naturally contaminated wheat samples. First, the blank wheat quality control material extracts were spiked with ranging concentrations of FB_1 from 200 to 1800 ng mL^{-1} (corresponding to 1000 to 8000 μg kg^{-1}). These spiked samples were analyzed according to the developed immunoassay and the results are shown in Table 2. As can be seen, mean recoveries of 81–109% were obtained for the

Table 2. Analysis of spiked wheat samples with FB_1.

Spiked [ng mL^{-1}]	Measured [ng mL^{-1}]	Recovery [%]	RSD[a] [%]
1600	1482	92.6	8.0
1200	1056	88.0	4.6
1000	815	81.5	9.8
400	430	107	14
200	217	109	3.2

[a] Relative Standard Deviation.

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different spiked samples analyzed, with the corresponding RSD ranging from 3 % to 14 %. The assay was applied to the analysis of a wheat reference material with a total fumonisin certified value of 86431 ± 25900 μg kg^{-1}, and the results confirmed that there were not significant differences at a 95 % confidence level with the optimised method, obtaining a total fumonisin value of 80000 ± 20000 μg kg^{-1} (n = 3).

Finally, eight wheat samples were analyzed, following the same extraction protocol, and the results were validated by an HPLC-MS/MS reference method. The results appear on Table 3. It can be seen that the majority of the wheat samples analyzed contained a very low total fumonisin concentration which could not be quantified by the immunoassay. However, some of the samples contained enough analyte to be detected by the immunoassay, proving that it presented enough sensitivity to detect fumonisin in wheat samples at levels well below the regulatory limits for fumonisin in maize (1000 μg kg^{-1} for direct human consumption), since it has not been established yet maximum levels for wheat samples.[38] All these results demonstrate that the bioluminescent method can be efficiently applied to the analysis of contaminated wheat samples.

Conclusions

This work reports the development of competitive bioluminescent immunoassays for the determination of fumonisin in wheat samples. Two different bioluminescent proteins, GLuc and NLuc, were expressed in combination with the FB_1 mimopeptide previously obtained. The recombinant nature of both fusion proteins can guarantee an unlimited source as well as a fixed stoichiometry of the components of the fusion protein, greatly reducing batch-to-batch variations. It was proven that both luciferases presented different kinetics when the corresponding substrate was added. On the one hand, the GLuc fusion protein presented a flash kinetics, whereas the NLuc showed a more glow-type kinetics, especially if the NanoGLO substrate was added instead of the bare substrate diluted in PBS. The glow-type luminescence allows an extended luminescence and permits a longer measuring time than a flash-type luminescence.

Table 3. Analysis of real samples and validation by HPLC-MS/MS reference method.

Sample	Fumonisin concentration (sd)[a] [μg kg^{-1}] Immunoassay	HPLC-MS/MS
1	< LOD	< LOQ
2	< LOD	42.6 (1.4)
3	< LOQ	82.7 (1.3)
4	493 (32)	526 (36)
5	< LOD	< LOQ
6	409 (11)	402 (28)
7	< LOQ	291 (5.6)
8	1475 (53)	1512 (2.6)

[a] Standard Deviation.

A bead-based immunoassay was conducted for both fusion proteins, A2-GLuc and A2-NLuc. The implementation of a bead-based immunoassay offers faster analysis in comparison to plate-based methods in which the antibody needs to be immobilized onto the well plates. Both fusion proteins provided a reproducible, selective and sensitive detection of fumonisin at very low concentrations. However, the dynamic range obtained using the A2-GLuc was much narrower than that of the A2-NLuc, possibly due to the lower amount of fusion protein added to the immunoassay. Once it was proven that the matrix effect was negligible at 5 % extract concentration, the A2-NLuc fusion protein was used for the analysis of wheat samples, obtaining satisfactory results. Therefore, it can be concluded that the favorable analytical performance obtained makes the developed immunoassay a very useful tool for a fast and sensitive analysis of fumonisin in wheat samples. Further work will be aimed at the implementation of bioluminescent immunoassays in different food matrixes and for a wider scope of analytes for a development of multiplexing analysis.

Experimental Section

Materials

Phusion Hot Start II DNA Polymerase, high-fidelity DNA Polymerase, Blocker™ Casein (in PBS), LB Broth Lennox, Dynabeads Protein G, 96 flat well chimney base black plates and Pierce Centrifuge Columns were from Thermo Fisher Scientific (Waltham, MA, USA). PCR Nucleotide Mix was obtained from Roche Diagnostics (Basel, Switzerland). HisTrap™ FF crude columns, Sephadex™ G-25 M columns and Illustra NAP-5 columns were purchased from Cytiva. (Chicago, IL, USA). Phosphate buffer saline (PBS), pH 7.4, Tween 20, dimethyl sulfoxide (≥ 99.5 %), ampicillin, kanamycin, protease inhibitor cocktail, SDS gel preparation kit, Amicon Ultra 3 K centrifugal filters, isopropyl-β-d-thiogalactopyranoside (IPTG), D-(+)-maltose monohydrate, KOD Xtreme Hot Start Master Mix and the mycotoxin zearalenone were from Sigma-Aldrich (Saint Louis, MO, USA). Fumonisin B_1, fumonisin B_2, HT-2 toxin, T-2 toxin, deoxynivalenol, and ochratoxin A were acquired from Fermentek Ltd. (Jerusalem, Israel). LB Agar and bovine serum albumin were from NZYtech (Lisbon, Portugal). Imidazole was purchased from Alfa Aesar (Maverhill, MA, USA). PCR primers were procured from Integrated DNA Technologies, Inc (San Diego, CA, USA). Shuffle Express Competent and BL21 Competent *E. coli* cells and amylose resin were obtained from New England Biolabs (Ipswich, MA, USA). The anti-fumonisin monoclonal antibody was purchased from BioTez (Berlin, Germany). NanoGLO® Reagent for Immunoassay was from Promega Corporation (Madison, WI, USA), furimazine was purchased from Aobious (Gloucester, MA, USA) and the native coelenterazine was acquired from NanoLight Technology (Pinetop, AZ, USA). The blank wheat quality control material was from Romer Labs (Getzersdorf, Austria) and the wheat reference matrix material was acquired from Aokin (Berlin, Germany).

Construction of the GLuc and NanoLuc fusion proteins

The expression of a previously characterized fumonisin mimopeptide, A2 (VTPNDDTFDPFR) with GLuc bioluminescent protein was carried out through an amplification of the pColdI-GLuc vector,[39] using KOD Xtreme Hot Start Master Mix. Both the DNA encoding for the mimopeptide A2 and a GS-linker were added to the 5′-end of GLuc, and a polyhistidine tag was incorporated to the 3′-end in three

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sequential PCR reactions with the primers that are shown in Table S3. The final PCR product was subcloned at the NdeI and BamHI sites of the pMAL-c5X expression vector.

With reference to the expression of the A2 mimopeptide together with NanoLuc, an amplification of the later one was conducted from the ATG-42 commercial vector,[40] using Phusion Hot Start II DNA Polymerase. Similar to the previous case, the DNA sequence encoding for the fumonisin mimopeptide (VTPNDDTFDPFR) was included in pQE vector in the 5′-end of NanoLuc, whereas a polyhistidine tag was added to the 3′-end using the corresponding primers from Table S2.

Expression and purification of the fusion protein

To express the GLuc-tagged fumonisin mimopeptide (A2-GLuc), the plasmid was initially transformed into Shuffle NEB Express Competent cells. A single colony containing was selected from an LB agar plate supplemented with 100 $\mu g\,mL^{-1}$ ampicillin to inoculate a 5 mL preculture of LB medium with 100 $\mu g\,mL^{-1}$ ampicillin which was previously grown overnight at 37 °C and 250 rpm. The overnight preculture was expanded the following day to a main culture of 180 mL solution of LB with 100 $\mu g\,mL^{-1}$ ampicillin. The main culture was grown at 37 °C and 200 rpm until an optical density at 600 nm (OD_{600}) of 0.7 was achieved. At that point, the protein expression was induced with the addition of IPTG to the main culture at a final concentration of 1 $mmol\,L^{-1}$. The culture was allowed to grow for 4 h at 15 °C and 175 rpm and then, the cells were collected by centrifugation at 5,000 g and 4 °C for 10 min. The pellet was then resuspended in 10 mL lysis buffer (50 $mmol\,L^{-1}$ Tris-HCl pH 8.7, 150 $mmol\,L^{-1}$ NaCl) containing 5% (v/v) protease inhibitor cocktail. The mixture was frozen at −80 °C overnight, and the following day the cells were lysed by sonication on ice during 10 min doing 10 s on/off cycles. The insoluble cell debris was discarded by centrifugation at 12,000 g and 4 °C for 20 min, and the supernatant was filtered through a 0.45 μm filter. Then, the lysate was purified with amylose resin. For this, the clarified lysate was mixed with 1 mL of the amylose resin and incubated 30 min at room temperature and slow shaking. The resin was then collected on a Pierce Centrifuge Column and washed with 10 column volumes of lysis buffer. The purified protein was eluted from the column with lysis buffer supplemented with 10 $mmol\,L^{-1}$ maltose. The protein was stored at 4 °C for several weeks.

A similar protocol with some modifications was conducted for the expression of the NLuc-tagged fumonisin mimopeptide (A2-NLuc). Briefly, a plasmid containing the fusion of the NanoLuc and the fumonisin mimopeptide was transformed into *E. coli* BL21 Competent cells. A single colony was picked from LB agar plates containing 50 $\mu g\,mL^{-1}$ kanamycin and was grown overnight on a 15 mL LB preculture supplemented with 50 $\mu g\,mL^{-1}$ kanamycin. The following day, an aliquot of the aforementioned preculture was transferred to a 200 mL LB culture with 50 $\mu g\,mL^{-1}$ kanamycin and grown at 37 °C and 180 rpm until an OD_{600} of 0.6 was reached. To induce the expression, a final concentration of 0.4 $mmol\,L^{-1}$ IPTG was added to the culture, and it was grown for 4 h at 37 °C and 180 rpm. Next, the culture was centrifuged at 5000 g for 10 min at 4 °C to collect the cells, and the pellet was resuspended in NZY Bacterial Cell Lysis supplemented with protease inhibitor cocktail, NZY Lysozyme and DNAse I according to the manufacturer's instructions. The cells were subsequently sonicated on ice for 10 min doing 10 s on/off cycles, and the insoluble cell debris was separated by centrifugation at 15000 g for 15 min at 4 °C. The purification of the cell lysate was carried out with HisTrap™ purification columns and Sephadex™ G-25 M columns were utilized afterwards to change the buffer to PBS. The purified A2-NLuc fusion protein was stored at −20 °C for several weeks. The purity of all the fusion proteins was verified by SDS-PAGE analysis, and the concentration was determined by measuring the absorbance at 280 nm with a Nanodrop spectrophotometer.

Optimization of the assay conditions for each luciferase

Consideration of kinetics

A total of 50 μL of the fusion protein, either A2-GLuc or A2-NLuc, were added to a 96-well black plate. The bioluminescence was measured immediately after the addition of 50 μL of the substrates in PBS buffer, coelenterazine for GLuc and furimazine or NanoGLO for NLuc, on a CLARIOstar microplate reader.

Optimized immunoassays for FB_1

Bead-based assay with immobilized antibody

A checkerboard-type titration was carried out to evaluate the optimal concentrations of the antibody and fusion protein on the immunoassay for A2-GLuc. However, for A2-NLuc, the fusion protein concentration was initially optimized and then a checkerboard-type titration evaluated the optimal concentrations of antibody and magnetic beads.

A2-GLuc

Black microtiter plates were blocked with 200 μL Casein for 1 h at 37 °C and then, rinsed thrice with washing buffer (137 $mmol\,L^{-1}$ NaCl, 2.7 $mmol\,L^{-1}$ KCl, 10 $mmol\,L^{-1}$ Na_2HPO_4, 1.8 $mmol\,L^{-1}$ KH_2PO_4, 0.05% T20, pH 7.4). Varying concentrations of free FB_1, from 0 to 100 $ng\,mL^{-1}$ in assay buffer (PBS supplemented with 0.05% T20 and 0.1% BSA, pH 7.4), were mixed with a solution of 3 $\mu g\,mL^{-1}$ A2-GLuc and 1.66 $\mu g\,mL^{-1}$ of antibody per well. The mixture was incubated for 1 h at room temperature and slow shaking and subsequently 40 μL of a solution of protein G beads were added to the wells for a final concentration of 0.1 $mg\,mL^{-1}$. After incubation for 30 min, the wells were washed thrice with an automatic washer under a magnet. Finally, the bioluminescence was measured immediately after the addition of 50 μL of a solution of 2.0 $\mu g\,mL^{-1}$ coelenterazine in PBS.

A2-NLuc

Black microtiter plates were blocked with 200 μL Casein for 1 h at room temperature. After rinsing the plate three times with washing buffer, varying concentrations of free FB_1, from 0 to 2500 $ng\,mL^{-1}$ in assay buffer were added to the wells containing 8.5 $\mu g\,mL^{-1}$ of A2-NLuc and 1.66 $\mu g\,mL^{-1}$ of antibody per well in a total volume of 60 μL. The mixture was incubated for 1 h at room temperature and slow shaking and then, 40 μL of a solution of 0.31 $mg\,mL^{-1}$ of protein G beads in assay buffer were added to each well and incubated for 30 min at room temperature and slow shaking. The wells were then washed thrice with an automatic washer under a magnet and finally, the bioluminescence was measured after the addition of 60 μL of a solution of NanoGLO reagent. For the cross reactivity tests, the FB_1 concentration was replaced with other mycotoxins at ranging concentrations between 1 and 1000 $ng\,mL^{-1}$.

Sample analysis and spiking

Wheat samples were collected in an Experimental Field and a Flour Company. Fumonisin extraction was carried out following a method previously described by Krska et al.[37] with some modifications. Briefly, 5 mL of a mixture containing acetonitrile/water/acetic acid (79/20/1 v/v/v) were added to 1 g of wheat sample. The samples were gently shaken for 60 min at room temperature and then centrifuged for 15 min at 15000 g. The supernatant was filtered with a 0.22 μm filter and conveniently diluted in assay buffer prior to the analysis.

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For the analysis of spiked samples, different concentrations of FB_1 were added to the wheat extract and conveniently diluted to carry out the analysis.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: bioluminescence · fumonisin B_1 · fusion proteins · immunoassays · luciferases

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