Sensing and Bio-Sensing Research 29 (2020) 100339

Contents lists available at ScienceDirect

Sensing and Bio-Sensing Research

journal homepage: www.elsevier.com/locate/sbsr

Genipin cross-linked chitosan for signal enhancement in the colorimetric detection of aflatoxin B1 on 3MM chromatography paper

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ARTICLE INFO

Keywords:
paper
chitosan
cross-linking
aflatoxin B1
colorimetric detection

ABSTRACT

Detection of mycotoxins by conventional methods such as ELISA or LC-MS can be expensive and time-consuming. Therefore, paper-based biosensors can be effectively used for on-site analysis, due to their low cost and easy detection procedures. Nevertheless, even when the application of colorimetric methods on paper enhance the simplicity and affordability of multiple determinations, the signal intensity and final readout can be affected by a limited color uniformity. In this work, Ellman's method for the quantification of aflatoxin B1 was utilized as a model colorimetric assay on paper, in which the test zones were modified with chitosan-immobilized enzyme (AChE). A comparison of the cross-linking effect of genipin on two chitosans of varying molar mass and degree of acetylation, exhibited a greater signal enhancement from the sample with a higher degree of acetylation and molecular weight.

1. Introduction

From the different metabolites affecting crops and human health, mycotoxins represent a big concern when ingested through different food products. Among the diverse group of mycotoxins, aflatoxins have been widely studied and controlled as they represent a main issue in food safety and agricultural economy [1]. Aflatoxin B1, commonlly produced by *Aspergillus flavus* and *Aspergillus parasiticus*, has been widely explored because of its high toxicity and carcinogenic effects [2], caused by both long term and acute exposure [3]. As an example of its impact, it has been estimated that aflatoxin contamination can cause a loss of 52.1 million to 1.68 billion dollars per year in the USA [4].

Conventional analysis methods for aflatoxin B1 include ELISA or LC-MS; however, despite their high sensitivity, they normally require long detection times and complicated procedures [5]. One of the main advantages of paper-based biosensors is the flow of samples promoted by capillary forces, which can be translated to reductions on the analysis time and the application of specialized instruments [6]. To control the diffusing behavior of varied solutions, a hydropobic detection area can be delimited on the paper-based biosensor through painted, stamped and printed wax [7–9] or laser cutting [10]. Alternatively, permanent markers are a low cost and affordable option for designing hydrophobic barriers on paper [11].

Moreover, one of the main challenges to overcome in paper-based sensors is the enhancement of the final readout signal, which is commonly addressed by the use of nanomaterials, comprising metallic nanoparticles, fluorescent, electrochemical and colorimetric particles, along with the utilization of nucleic acids for signal amplification [12]. Because of its simple operation, colorimetric assays are more feasible for remote applications and on-site determinations. However, color heterogeneity, mostly produced by a washing effect from the sample front, has been one of the main challenges in colorimetric techniques, which could be prevented with the impregnation of the detection zones with polymeric materials such as chitosan [13].

As a result of its biocompatibility, chitosan has been incorporated for antibody immobilization on paper-based electrodes and microfluidic paper-based analytical devices (μPADs) [14,15], as well as enzyme immobilization on electrodes [16]. Nothwisthanding the successful application of polymeric compounds for signal improvement, its effectivenes might depend on the properties of the selected polymer, as well as the application method on the porous support. In this work, the application of two types of chitosan was evaluated on the colorimetric detection of aflatoxin B1 (AFB_1) by Ellman's colorimetric method. The enzymatic immobilization with cross-linked chitosan demonstrated a better performance by the application of the sample with high molecular weight and a high degree of acetylation.

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https://doi.org/10.1016/j.sbsr.2020.100339
Received 31 October 2019; Received in revised form 24 March 2020; Accepted 3 April 2020

2. Materials and methods

2.1. Materials

Chromatography paper grade 3MM was acquired from Whatman™ (UK). Permanent marker (Medium Point 1.0 mm Write-4-All Pen Permanent – Black) and stamp pad blue ink without oil were purchased from Stabilo (UK) and Pelikan® (Germany), respectively. Chitosan A (D.A. 17%, M.W. 28 000; HMC 70/5 batch number 212-170614-01) was purchased from Heppe Medical Chitosan GmbH (Hale, Germany), and Chitosan B (D.A. 28.8%, M.W. 1,460,000; batch number: SPchan-08127) was obtained in our laboratory from previously isolated squid pen β-chitin. Aflatoxin B1 from *Aspergillus flavus* (A6636), ochratoxin A (32937), fumonisin B1 (F1147), acetylcholinesterase from *electrophorus electricus* (electric eel) (AChE, C2888), and acetylthiocholine iodide (ATCh, A5751) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dithiobis (2-nitrobenzoic acid) (DTNB, 22582) was purchased from Thermo Fisher Scientific (USA). Tris HCl Buffer (UltraPure™ 1 M pH 7.5, 15,567,027) was obtained from Invitrogen™(USA). Corn was brought from local Sainsbury's supermarket. Methanol (10675112) was obtained from Fisher Scientific (UK).

2.2. Methods

2.2.1. Construction of μPADs

Cellulose 3MM chromatography paper previously characterised in its imbibition and diffusion properties using ink models [17], was cut into squares (4.5 cm × 4.5 cm), and the hydrophobic boundaries were drawn with a permanent marker using either circular spots or a 'flower' shape arrangement with a 3-D printed template, as shown in Fig. S1.

2.2.2. Preparation of solutions

Solutions containing 0.2% of either chitosan A or B powders were prepared by overnight stirring in 85 mM NaCl solution, stoichiometricaly acidified with acetic acid. Genipin was dissolved in 100% ethanol. AChE was dissolved in Tris HCl Buffer (pH 7.5 20 mM). DTNB and ATCh were dissolved in Tris HCl Buffer (pH 7.5 100 mM). Standards of AFB_1 were dissolved in Tris HCl Buffer (pH 7.5 50 mM). Unless mentioned, other solutions were dissolved in Milli-Q water. Note: After preparation, DTNB solution should be immediately put on ice and store in the dark.

2.2.3. Performance of cross-linked chitosan on μPADs

A visual assessment on the effect of cross-linked chitosan was conducted under the presence of chitosan (0.2% w/w), blue ink, and genipin (59.25 μM), combined at percentage ratios (by vol.) of 92/2.3/5.7 for chitosan A, and 97.3/2.5/0.2 for chitosan B. To this purpose, different volumes (0-6 μL) of each mixture were applied on half of the detection areas (flower-shaped μPAD), while the other areas were added with a mixture of chitosan solution (0.2% w/w), ink, and water, at the same ratios. After the application step, the paper μPADs were incubated at either 25 or 37 °C for 1 h, followed by image scanning for subsequent visual comparison.

2.2.4. Color intensity of μPADs modified with cross-linked chitosan.

Mixtures were prepared and applied as previously specified in Section 2.2.3, followed by incubation at 25 °C for 1 h. The incubated μPADs were scanned and the color intensity was obtained in Image J for the 6 μL samples.

2.2.5. Detection zones preparation with cross-linked chitosan immobilized AChE

AChE (final concentration 50 U/mL) was mixed with 398 μL of a 0.2% chitosan solution (w/w) for 3 minutes (final volume 500 μL), followed by the addition of 16.2 μL and 13.8 μL genipin solution (59.25 mM) for Chitosan A and B respectively. After 3 minutes, the detection zones were modified with 1.6 μL of chitosan/genipin/AChE mixture or a 50 U/mL AChE solution. The treated papers were stored for 1.5 h at 25 °C, to allow the cross-linking reaction to proceed.

2.2.6. Colorimetric detection on μPADs (8 circular spots)

For the colorimetric assay, 3.2 μL of AFB_1 solution at different concentrations (0 to 100 μM) and 1.6 μL of DTNB (final concentration: 500 μM) were subsequently added to each testing spot. After incubating for 3 min, 1.6 μL of ATCh solution (final concentration: 300 μM) was added and incubated for 5 min. All paper biosensors were scanned for further analysis in image J.

2.2.7. Colorimetric detection of standard solutions ('flower' shape μPAD)

For the 'flower' shaped assay, 2.5 μL of AChE or AChE/Genipin/Chitosan, were added to the edges of the μPAD, and the cross-linking reaction was carried at 25 °C for 2 h. Then 40 μL of AFB_1 solution with different concentration (0 to 60 μM) were added at the center (loading zone), followed by 20 μL of DTNB solution. After 3-min incubation, 20 μL of ATCh solution was also applied at the center, and incubated for 5 min. All the paper biosensors were scanned for further analysis in image J.

2.2.8. Extraction and detection of AFB_1 in corn samples

Corn (7 g) was spiked with 100 μL of AFB_1 solution (161 μM), by mixing for 3 min in a centrifuge tube. The spiked sample extracted with 320 μL of 5% methanol (manual shaking). The extract achieved an expected concentration equivalent to 50 μM AFB_1. The detection procedure was performed as stated in Section 2.2.7, on a 'flower' shape μPAD.

2.2.9. Measurement of color intensity of paper biosensors in ImageJ

The color intensity of scanned detection zones was obtained in ImageJ software, where the image was inverted so the white color was transformed to black for the lowest color intensity (0). Correspondingly, the black color was inverted to white to observe the highest color intensity (256) [18].

2.2.10. Degree of inhibition

The degree of inhibition from AFB_1 was calculated with a modified version of a reported determination [19], in which the intensity of color was integrated to the calculation.

$$\%I = \frac{(IC0 - ICi)}{IC0} \times 100 \quad (1)$$

Where, *%I* is the degree of inhibition, *IC0* is the color intensity without AFB_1, and *ICi* is the color intensity with AFB_1.

3. Results and discussion

3.1. Performance of cross-linked chitosan on μPADs

Representative images of the scanned μPADs after incubation are displayed in Fig. 1. As it can be noted, both chitosan A and B exhibited a homogenous surface when the sample was fixed through cross-linking with genipin, compared to the treated areas without cross-linker. Chitosan (polycationic) and cellulose (anionic) possess structural similarities, which allow binding between amino (chitosan) and aldehyde/carboxyl groups (paper), resulting in electrostatic adsorption [20,21]. Chitosan not only supports electrostatic interactions by protonation of its primary amine groups, but their acetyl functions also favor hydrophobic interactions, while hydrogen bonds can also be formed via hydroxyl groups [22]. Nonetheless, the sole application of ink by mixing with chitosan and water led to sample accumulation on specific areas of the test zone; thus suggesting that this coating method is not the most suitable for immobilization on 3MM chromatography paper, unlike its adequate deposition behavior seen on metallic

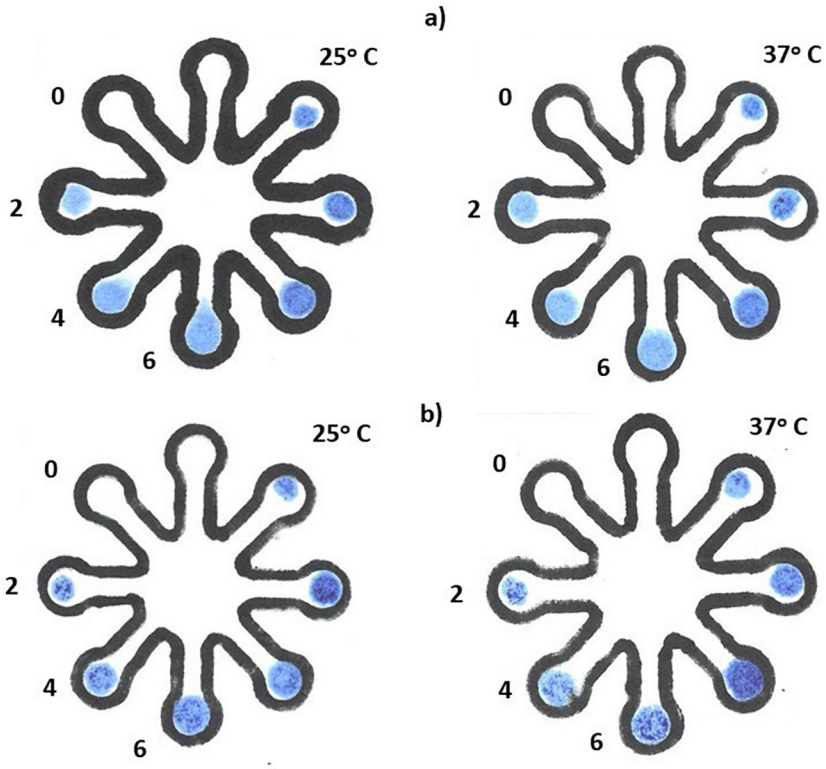

Fig. 1. Symmetrical application of ink on the detection zones, with cross-linked (numbered areas) and mixed (no numbers) solutions of chitosan A (a) and chitosan B (b) at two incubation temperatures. Each number refers to the applied volume (μL) of sample.

electrodes [16].

Based on Fig. 1, it can be observed that, at both incubation temperatures, chitosan A had a more uniformly colored surface than does chitosan B. Such behavior could be explained as a result of the higher degree of acetylation of chitosan B, which has been correlated with greater hydrophobicity, rigidity and steric effects [23]. The adsorption of chitosan on cellulose has been found to improve at pH values below its solubility limit (pH = 6.0 ± 0.1 [24]), in which more electrostatic interactions will be promoted [25]. In this case, the pH values for the solutions of chitosan A and B were 5.03 and 5.44 respectively, which also corresponds to a more consistent surface on the test zones treated with chitosan A. It is worth mentioning that, as incubation at 37 °C resulted in more heterogeneity, all the subsequent experiments were carried at 25 °C to avoid water evaporation promoting drier test zones.

As previously mentioned, cross-linked chitosan conferred a better performance on the μPADs. Cross-linking of chitosan has been previously carried with glutaraldehyde, for the immobilization of capture antibodies on Whatman #1 paper [14], however, genipin has been proposed as natural alternative for the formation of the chitosan network at room temperature, due to its lower cytotoxicity [26]. Higher color intensities were achieved with cross-linked chitosan, as denoted in Fig. 2. When genipin was not included, the differences between both types of chitosan was rather well known, as chitosan B produced a greater intensity value. Such divergence relied on the image analysis method, in which an average of all the intensities was calculated for a region of interest, with greater measured intensities for a heterogeneous test zone. Yet the same color intensity was achieved when both chitosan were genipin cross-linked, which indicated a reduction on the heterogeneity of the testing areas (Fig. 2).

The cross-linking mechanism of chitosan and genipin is known to occur in two stages. An initial fast reaction develops between the C3 carbon atom in genipin and a primary amine group from chitosan, resulting in a heterocyclic compound. A second, slower reaction, takes place by a nucleophilic replacement of the ester group in genipin and

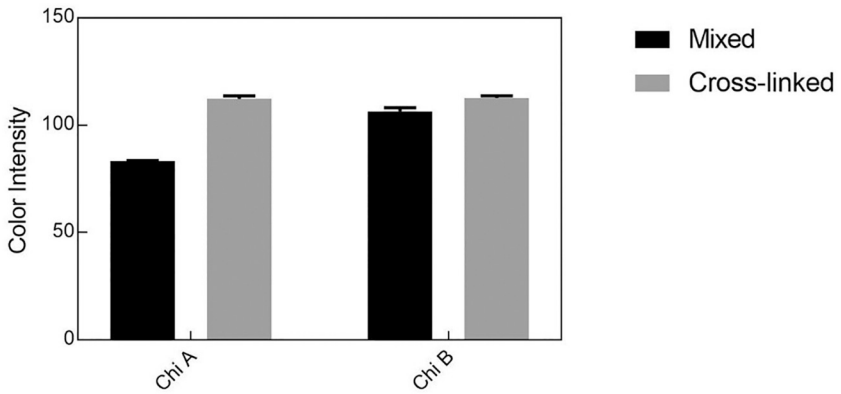

Fig. 2. Color intensity of the applied ink in chitosan and water (mixed) and chitosan with genipin (cross-linked) after incubation (25 °C, 1 h, 6 μL, n = 3).

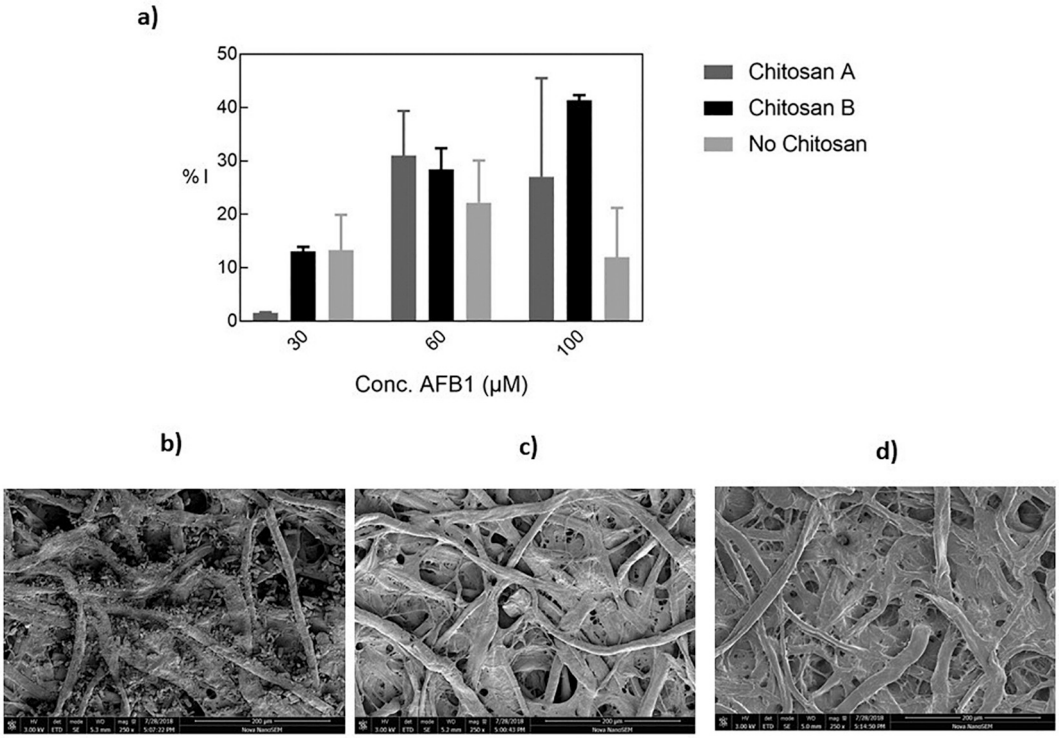

Fig. 3. a) Percentage of inhibition of AFB_1 on the activity of free (no chitosan) and immobilized AChE (50 U/mL) with chitosan A and B. SEM images (200 μm) of the detection zones after the colorimetric determination with b) free, c) chitosan A and d) chitosan B immobilized AChE (50 U/mL) at 25 °C (reaction time: 8 min; n = 3). The displayed "No chitosan" values are an average from the corresponding samples in Fig. S4a and S4b.

the formation of a secondary amide bond with chitosan [27]. As the produced network presented a better performance on μPADs, the consecutive colorimetric assays were carried through enzyme immobilization with cross-linked chitosan at 25 °C.

3.2. Performance of chitosan on the colorimetric detection of AFB_1 on μPADs

The colorimetric determination of aflatoxin B1 was based on Ellman's assay [28], in which AFB_1 acts as an inhibitor of AChE (see Supplementary Information). This effect has been studied on gelatin-immobilized AChE, where neither the immobilization step nor the presence of up to 60% (v/v) methanol generated a negative effect on the enzymatic activity [29]. Preliminary bulk assays confirmed no interference by both chitosan solutions with the final signal, at the concentration of AChE utilized in this work (Fig. S3a); as well as a significant specificity ($p < 0.05$) of this colorimetric assay to AFB_1, when compared with FB1 and ochratoxin A (Fig. S3b). This result confirmed the feasibility of performing the assays in real food sampes (e.g. corn) without the interference of some other maize-related mycotoxins.

The percentages of inhibition displayed in Fig. 3a, were calculated based on the color intensities achieved on each treated test zone at different concentrations of AFB_1 (Fig. S4). Unlike the invariable detection with free enzyme (no chitosan), the chitosan treated zones displayed an increasing inhibition rate when the concentration of AFB_1 was raised. The greatest degrees of inhibition were determined as 31.27 ± 5.96% (chitosan A) and 41.13 ± 0.87% (chitosan B) upon addition of 60 and 100 μM AFB_1, respectively. Thus, chitosan B had the most differentiated effect, as noted in Fig. 3a by the highest gradient in percentage of inhibition. A more intense signal was also visually confirmed on AChE immobilized with cross-linked chitosan (Fig. S5), which can be neglected as an inhibitory effect from chitosan due to its null intervention in the reaction mechanism (Fig. S3a). Furthermore, the interaction of chitosan with cellulose promotes a convenient condition for electron transfer in the enzymatic reaction [13].

The SEM images (Fig. 3b) confirmed a dry, heterogeneous profile in the absence of chitosan, while the addition of the cross-linked polymer resulted in a smooth, homogenous surface, where no particles were observed (Fig. 3c and d). This film-like property of chitosan A (pH = 5.03, $\gamma = 0.07228$ N/m, $\boldsymbol{\mu} = 0.0015$ Pa·s) and chitosan B (pH = 5.44, $\gamma = 0.07306$ N/m, $\boldsymbol{\mu} = 0.01$ Pa·s), could be related to its physical attributes, and possibly due to the cross-linking effect of genipin on preventing the re-crystallization of chitosan acetate.

Despite the reported slight inhibitory effect of genipin (18.18%) on the activity of AChE [30], a constant concentration of this cross-linking agent still revealed a distinctive color development at different AFB_1 concentrations, as indicated in Fig. S6. The equations describing the above-mentioned curves (Fig. S6) are expressed in Table 1, as it can be recognized a linear function described the addition of AFB_1 on the test zones with immobilized chitosan. In contrast with the assays with free enzyme, the addition of chitosan intensified the slope of the plotted curves, which was also supported by the high determination coefficients (r^2), observed especially for chitosan B (0.9911). Based on the assays with ink, the SEM images, the linearity of the resulting color intensities, and the well-defined effect of AFB_1, it can be argued that cross-linked chitosan might result in an enhancement of the enzymatic reaction rate, thus resulting in greater color intensity and overall

Table 1
Mathematical expression of the color intensity as a function of the concentration of AFB_1.

Sample	Equation[a]	r^2
No chitosan	$CI = -0.0706\ [AFB_1] + 55.493$	0.4146
Chitosan A	$CI = -0.1946\ [AFB_1] + 58.617$	0.7302
Chitosan B	$CI = -0.3621\ [AFB_1] + 85.624$	0.9911

[a] CI = Color Intensity, $[AFB_1]$ = Concentration of AFB_1 (μM). Data based on Fig. S6.

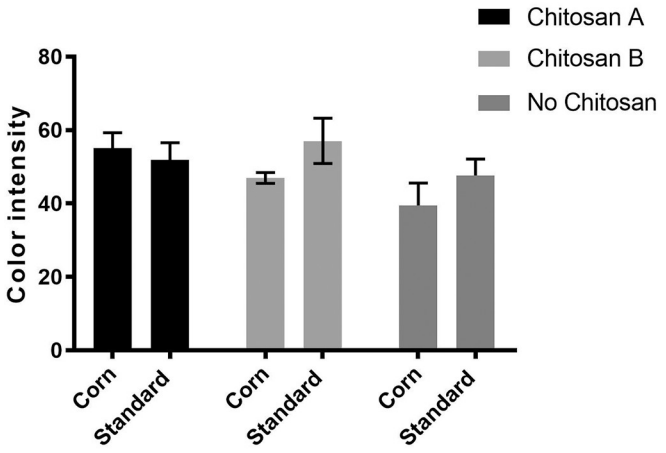

Fig. 4. Comparison between the signals produced by corn samples and AFB_1 standards (50 μM) inhibiting free and immobilized AChE with a) chitosan A and b) chitosan B (25 °C, 8 min reaction time and 50 U/mL AChE, n = 3).

readout resolution of the assay. Similar to non-cross-linked chitosan, genipin cross-linked chitosan has been disclosed as a cytocompatible and biocompatible material, when compared with glutaraldehyde cross-linked chitosan [31].

In addition, genipin cross-linking is also expected to prevent the dissolution of chitosan acetate upon wetting of the test zone. When testing spiked corn samples, a yellow color was observed on the loading zone, which could be identified as zeaxanthin, the principal pigment of yellow corn [32]. Yet, 3MM Chr paper allowed a separation between pigments and AFB_1, as the latest metabolite moved along the channel as part of the sample run (Fig. S7). As a consequence, the intensities reported in Fig. 4 indicated the positive effect of the immobilization step on the accomplishment of similar values to their corresponding standard solutions.

4. Conclusion

A comparison between cross-linked and non-cross-linked chitosan on 3MM chromatography paper by ink immobilization was carried out. The color intensities and visual inspection revealed more homogeneity on the test zones treated with cross-linked chitosan. Cross-linked chitosan also helped immobilize AChE, which simultaneously enhanced the color intensity of the test signal, as confirmed by the high resolution at different concentrations of AFB_1 and the film formation on the detection zones. Chitosan with a high molecular weight and a higher degree of acetylation exhibited a better performance than the lower molecular weight low degree of acetylation polymer for the achievement of a homogeneous colorimetric reaction with a linear dependence to the concentration of AFB_1. The impact of the present findings in the future development of paper-based biosensors for multiple applications is yet to be fully realized.

Acknowledgments

V. Mirón-Mérida received a scholarship from CONACyT (Mexico).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sbsr.2020.100339.

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