One-and two-photon induced fluorescence spectroscopy enabling the detection of localized aflatoxin contamination in individual maize kernels

L. Smeesters a, *, W. Meulebroeck a, S. Raeymaekers b, H. Thienpont a

a Vrije Universiteit Brussel, Faculty of Engineering, Dept. of Applied Physics and Photonics (TONA), Brussels Photonics Team (B-PHOT), Pleinlaan 2, B-1050 Brussel, Belgium
b Tomra Sorting Solutions Food, Research Park Haasrode 1622, Romeinse straat 20, B-3001 Leuven, Belgium

ABSTRACT

The presence of carcinogenic aflatoxins in food and feed products is a major worldwide problem. To date, the aflatoxin contamination can only be detected by the use of destructive sample-based chemical analyses. Therefore, we developed an optical setup able to detect the localized aflatoxin contamination in individual maize kernels, on the basis of one- and two-photon induced fluorescence spectroscopy. Our developed optical configuration comprises a tunable titanium-sapphire laser (710nm-830nm) in combination with second harmonic wavelength generation (355nm-415nm), enabling the measurement of both one- and two-photon induced fluorescence spectra. Moreover, an accurate scanning of the kernel’s surface was induced by the use of automated translation stages, allowing to study the localized maize contamination. First, the operation of the setup is validated by the characterization of pure aflatoxin B1 powder. Second, the fluorescence spectra of healthy (<1ppb aflatoxin B1) and contaminated maize kernels (>70ppb aflatoxin B1) were measured, after excitation with 365nm, 730nm, 750nm and 780nm. For both the one- and two-photon induced fluorescence processes, the presence of the aflatoxin inside the contaminated maize kernels influenced the intrinsic fluorescence signals. Based on the fluorescence spectrum between 400nm and 550nm, we defined a detection criterion to identify the contaminated maize kernels. Furthermore, we demonstrate the sensing of the localized contamination level, indicating both contaminated maize kernels with a high contamination level in a limited surface area (as small as 1mm²) as with a lower contamination spread over a large surface area (up to 20mm²). As a result, our developed measurement methodology allows the identification of the localized aflatoxin contamination, paving the way to the non-destructive, real-time and high-sensitive industrial scanning-based detection of aflatoxins in food products.

Keywords: spectroscopy, fluorescence, two-photon induced fluorescence, multiphoton processes, optical sensing, aflatoxin, spectrum analyses, scanning process

1. INTRODUCTION

Fluorescence spectroscopy has already been integrated in an extensive range of applications in the biochemical, medical and petrochemical industry. In biology and chemistry, it facilitates the study of proteins and the tracking of bio-chemical reactions. In the medical industry, it is used to follow smart drug delivery systems that map the drug interaction with diseased tissue, while in the petroleum industry it enables to characterize crude oils during refining processes.

Considering food applications, fluorescence spectroscopy has been demonstrated for the screening of products on basis of their chlorophyll concentration. Particularly, the detection of foreign objects in food streams by the monitoring of their chlorophyll fluorescence has been presented. For example, when considering a mixture of green glasses and peas, a distinction can be made on basis of the chlorophyll fluorescence intensity, since the peas emit strong fluorescence signals...
while the glasses emit no fluorescence. In addition, also a distinction between peas, garden beans and sprouts has been demonstrated by evaluating the shape of the chlorophyll fluorescence spectrum. However, toxic contaminants in food and feed products can hardly be optically detected, due to the presence of large background fluorescent signals emitted by natural food substituents, like proteins, and the localized presence of the toxins. The current published fluorescence measurements only allow the identification of mycotoxins in homogeneous liquids, like beer or wine, in which no or very low background fluorescent elements are present. Therefore, we investigate the use of fluorescence spectroscopy for the detection of toxins in unprocessed, solid food products that show high intrinsic fluorescence signals.

In this paper, we focus on the detection of aflatoxin B1 in maize kernels. Aflatoxins are a group of toxic metabolites (mycotoxins) that are produced by the fungi Aspergillus flavus and Aspergillus parasiticus on a wide range of food products, like nuts, maize, cheese, pistachios and peanuts. Aflatoxins exist in more than ten varieties of which aflatoxin B1 is the most dominant and toxic one. Generally, they are associated with serious health risks, like liver diseases and cancer, in both humans and animals. Aflatoxins can contaminate the food both before and after harvest and cannot be destroyed by any form of food processing. Therefore, its presence in food and feed is regulated in over hundred nations. The European Commission has stated the maximal allowed total aflatoxin concentration in maize to be 10ppb. The USA food safety regulations included a maximum contamination limit of 20ppb of total aflatoxins, for all food products. To fulfill these limitations, aflatoxins are nowadays detected by using time-consuming, sample-based chemical analyses, like liquid chromatography – dual mass spectrometry (LC-MS/MS). However, due to the localized presence of the toxin in the food products and crops, these analyses often give a limited view on the degree of contamination, inducing a large amount of food waste, without entirely preventing the toxins to enter the food chain. Consequently, to increase food safety, the development of a non-destructive detection methodology, able to identify the localized contamination on the food products is indispensable. We especially focus on the detection of aflatoxins in maize, because maize is the staple food in many countries and cultivates in climates that show an extensive presence of the fungi, giving rise to permanent high aflatoxin contamination levels.

We investigate the use of fluorescence spectroscopy, including one-and two-photon induced fluorescence (OPIF and TPIF) spectroscopy, as a non-destructive, optical detection technique for the identification of aflatoxins in individual maize kernels. OPIF and TPIF are two types of fluorescent processes, both resulting in the emission of a fluorescent photon during the relaxation process of an excited electron, but with a different excitation process. To generate an OPIF photon, only one single excitation photon needs to be absorbed, while during the generation of a TPIF photon two excitation photons need to be absorbed simultaneously to excite the electron and generate the fluorescence signal (Fig. 1). In the case of OPIF, the energy of the incident photon equals the energy difference between the electronic states, such that the electron obtains sufficient energy to bridge the energy gap between the ground state and the excited state (Fig. 1a). Considering TPIF, the excitation requires two incident photons, of which the total energy equals the bandgap energy (Fig. 1b). The first incident photon excites the electron to a virtual state, which does not need to correspond to any electronic or vibrational energy eigenstate. The second incident photon excites the molecule from the virtual state to its higher-energy excited state. Because for both OPIF and TPIF the electron is excited to the same energetic state, they give rise to the same fluorescence wavelengths, but with another excitation wavelength. Generally, OPIF requires ultraviolet (UV) light for the excitation of the electrons, while TPIF uses near-infrared (NIR) light. If the energy of the incident photons does not match with the bandgap, no fluorescence emission will occur. As a result, because the bandgap is a molecular specific property, fluorescence is known to be a selective process. In addition, because TPIF is a non-linear process that requires the absorption of two photons instead of one, it is considered to be more selective than OPIF.

![Figure 1: Jablonski diagram for (a) OPIF, in which the electron is excited by the absorption of a single incident photon and (b) TPIF, in which the electron is excited by the simultaneous absorption of two incident photons.](http://proceedings.spiedigitallibrary.org/)

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OPIF and TPIF spectroscopy are both considered as promising optical detection techniques. OPIF gives stronger fluorescence signals than TPIF, which can result in a strong aflatoxin signal of the solid food products and therefore the detection of low contamination levels\textsuperscript{10}. The TPIF process features a more selective excitation of the aflatoxins, which can minimize the influence of the natural background fluorescent signals. Moreover, TPIF is obtained after excitation with NIR laser light, which is more widely commercially available than the UV laserlines that are used during OPIF. In addition, compact NIR lasers generally feature higher output powers than UV lasers, enhancing the fluorescence signal. Besides, when using UV light in an optical setup, the optical mirrors and lenses need to be made from or coated with fused silica, resulting in a more expensive setup than for NIR wavelengths.

In this paper, we demonstrate the use of OPIF and TPIF for the localized detection of aflatoxin in maize kernels. We first discuss our measurement methodology, including the measurement challenges and the selection of the excitation wavelengths. Second, we present our measurement configuration to study both OPIF and TPIF, which we validated by the characterization of pure aflatoxin B1 powder. Following, we focus on the detection of aflatoxin B1 in solid maize kernels. We characterize the OPIF and TPIF spectra, examine the development of a detection criterion and study the contaminated areas on the samples surfaces. Finally, we evaluate and compare the optical performance of OPIF and TPIF.

2. METHODOLOGY AND MEASUREMENT SETUP

We pursue a fluorescence measurement setup suitable for both OPIF and TPIF fluorescence measurements. However, to obtain reliable measurement data, some challenges need to be tackled. First, an optimal excitation wavelength is of major importance, to maximize the aflatoxin fluorescence and to minimize the influence of the intrinsic fluorescence signal. Second, the excitation spot size and excitation laser power should be optimized to enable the measurement of the weak aflatoxin fluorescence without damaging the kernels. Third, the natural variation within food products should be monitored, to assure we are measuring the influence of the toxin instead of the optical contrast between different product batches. Finally, a careful selection of the maize kernels and the illumination positions on their surfaces is indispensable to overcome the in inhomogeneous presence of the toxin.

Keeping these challenges in mind, we will now first explain the selection of the optimal excitation wavelengths, after which we present and validate our fluorescence measurement configuration.

2.1. Selection of the excitation wavelengths

To maximize the aflatoxin fluorescence intensity, we select an excitation wavelength that is strongly absorbed by the aflatoxin, since an increasing amount of absorbed photons gives rise to an increasing amount of excited electrons and thus also to an enlarged number of fluorescent photons. Furthermore, by using an excitation wavelength with a strong aflatoxin absorbance, the influence of the aflatoxin fluorescence emission onto the natural fluorescence spectrum of the food products can be maximized. Considering the absorption spectrum of aflatoxin B1, the strongest absorbance can be observed in the range between 200nm and 275nm and around 365nm\textsuperscript{9} (Fig. 2).

![Absorbance and fluorescence spectrum of aflatoxin B1](image-url)

Figure 2: Absorbance and fluorescence spectrum of aflatoxin B1, indicating the optimal excitation wavelengths between 200nm-275nm and around 365nm.
Next to the absorbance of the aflatoxin, also the intrinsic fluorescence of the food products needs to be taken into account. Maize contains several fluorescent proteins, like Tryptophan (Trp), Tyrosine (Tyr) and Phenylalanine (Phe), which all show a strong absorbance in the 200nm-300nm range\(^1\) (Fig. 3). If we would excite our maize kernels with a wavelength between 200nm and 275nm, the fluorescence of these proteins would disturb our measurements. The proteins show no fluorescence for excitation with 300nm-400nm light, since they only show a weak absorbance from 300nm onwards.

![Absorbance spectrum of different fluorescent proteins in maize: Phenylalanine (Phe), Tryptophan (Trp) and Tyrosine (Tyr).](image)

Finally, when selecting the excitation wavelengths, we keep in mind the commercial available laserlines. We prefer using commercially available wavelengths, to enable the integration of our developed optical detection criterion into industrial scanning-based sorting devices.

To study the OPIF spectrum of aflatoxin B1, we use an excitation wavelength of 365nm, because of the high aflatoxin absorbance, low proteins absorbance and commercial availability of this wavelength. Consequently, to study the TPIF spectrum, excitation wavelengths close to 730nm are required. In addition, when studying the maize kernels, we also investigated the fluorescence spectrum after excitation with 750nm and 780nm, to study the influence of the matrix constituents, the natural occurring fluorescent proteins, onto the fluorescence spectrum.

### 2.2. OPIF and TPIF measurement setup

To measure both OPIF and TPIF spectra, we pursue the generation of UV and NIR illumination wavelengths, in combination with an accurate detection of the fluorescence signal while scanning the sample’s surface. Generally, our measurement setup can be divided into different building blocks, comprising an illumination laser system, the sample objective and the detecting spectrum analyser (Fig. 4). A frequency doubled Nd:YAG pump laser (Spectra-Physics Millennia Prime high-power continuous wave 532nm laser) pumps the tunable titanium-sapphire laser (Spectra-Physics Tsunami laser). The wavelength of the titanium-sapphire laser can be tuned from 710nm to 835nm by the use of an internal slit, which selects the preferred wavelength after the dispersion of the generated laser light by internal prisms. Its maximum output power ranges between 1.20W and 1.50W, depending on the selected wavelength. The laser light of the titanium-sapphire laser can follow one out of two optical paths, depending on whether OPIF or TPIF measurements are performed (Fig. 4a). For TPIF, the selected laser light is immediately directed towards the sample. In the case of OPIF, the fundamental laser light of the titanium-sapphire laser is directed towards a second-harmonic generating crystal, to generate the necessary UV excitation wavelengths (Fig. 4b). The second-harmonic generating crystal is able to generate laser light with wavelengths between 355nm and 417nm, with a maximal output power between 200mW and 450mW. During both OPIF and TPIF measurements, the sample is illuminated with a circular beam, with a spot diameter of 951µm and 231µm respectively. To maximize the TPIF irradiance, the illumination laser beam was minimized by the use of an additional focusing lens positioned in front of the sample (Fig. 4d).

The sample is positioned on two automated translation stages (Newport 850G linear actuators) enabling to accurately scan the product in both X and Y direction (Fig. 4c). For each X and Y position, the incident laser beam illuminates different parts of the product, allowing to study the localized contamination of the sample. Both automated translation stages have
a travel range of maximum 5 cm, allowing to maximally scan a surface area of 25 cm². The translation stages are driven by a motion controller (Newport ESP300), enabling a high movement speed while assuring a high movement accuracy of 1 μm. During the scanning measurements, we used movement steps between 0.5 mm and 1.0 mm, as a trade-off between the resolution and illumination time of the sample. To avoid damage to the sample, the illumination time needed to be minimized, limiting the scanning resolution of the surface measurements.

After the excitation of the sample, the fluorescent signals are captured by a collimating lens, coupled into a broadband optical fiber (UV1R600 fiber of Avantes, transmitting light between 250 nm and 2500 nm) and guided towards the spectrum analyser (Fig. 4d). We use a collimating lens in combination with a large fiber core diameter (600 μm) to obtain a total acceptance angle of 4.1° that allows capturing the weak fluorescence signals of a surface area of 39 mm². In front of the detecting fiber, we additionally mounted an interference-based filter to suppress the excitation light. During the OPIF measurements, we implement a long-wave pass filter transmitting from 405 nm (Semrock 405 nm EdgeBasic filter BLP01-405R-25) onwards, while during the TPIF measurements a short-wave pass filter with a cut-on wavelength of 650 nm ± 5 nm is used (Newport 105SWF-650-B). Without the use of an optical filter, the excitation signals would saturate the measured fluorescence spectra, leading to incorrect measurement data. Furthermore, we make use of the AvaSpec2048 spectrum analyser, which has a wide entrance slit of 200 μm that allows capturing the weak fluorescence signals and is able to measure the spectrum between 300 nm and 1100 nm with a resolution of 8 nm. The measured spectra are corrected by a transfer function to account for the wavelength dependent transmittance of the optical fiber and the sensitivity of the detector inside the spectrum analyser.

During the fluorescence characterization measurements, a study of the fluorescence intensity as function of the excitation power is essential. Therefore, the excitation laser power can be tuned by mounting attenuation filters behind the laser output, in front of the tilted mirror (mirror indicated in Fig. 4d; the attenuation filters are not presented in this picture).

2.3 Validation of the measurement configuration

To validate the operation of our fluorescence measurement setup, we measured the OPIF and TPIF spectra of pure aflatoxin B1 powder (98% or better purity, produced by the A. flavus fungi), which we purchased from Sigma-Aldrich (Fig. 5). The OPIF spectrum is obtained after excitation with 365 nm, with an excitation power density of 42 mW/mm². The TPIF spectrum is measured after excitation with 730 nm, with an excitation power density of 14.3 W/mm². Both fluorescence spectra show their maximal fluorescence intensity at 428 nm, which corresponds with the expected aflatoxin B1
fluorescence maximum (Fig. 2). The OPIF spectrum shows higher fluorescence intensities than the TPIF spectrum. The maximal OPIF intensity, at 428nm, is equal to 1.68±0.93µW/cm², while we observe a maximal TPIF intensity of 0.20±0.09µW/cm². The measured TPIF intensity is hence 10 times weaker than the OPIF intensity. The shape of the TPIF spectrum shows a narrower peak than the OPIF spectrum, due to the more selective excitation during two-photon absorption than during one-photon absorption. Furthermore, in the TPIF spectrum at 365nm, we observe a second harmonic generation signal. This peak is created by the recombination of two illumination photons to a new photon with the double energy. Around 400nm, the OPIF spectrum shows a steeper increase than the TPIF spectrum, because of the presence of a long-wave pass filter suppressing light below 405 nm during the OPIF measurements.

Figure 5: OPIF and TPIF spectra of pure aflatoxin B1 powder, after excitation with 365nm and 730nm respectively.

To characterize the aflatoxin B1 fluorescence spectrum, we studied the integrated fluorescence intensity as function of the excitation power, while maintaining a constant spot diameter of 951µm during the OPIF measurements and 231µm during the TPIF measurements (Fig. 6). For each excitation power, we integrated the measured mean fluorescence spectrum between 400nm and 600nm to obtain the depicted integrated fluorescence intensity. Studying the integrated OPIF intensity as function of the excitation laser power, we observe a linear relationship, representing the linear one-photon absorption. However, at higher excitation powers, starting from 35mW onwards, the integrated OPIF intensity deviates from the linear relationship and starts saturating. In the saturated region, the maximum fluorescence intensity is reached since then all electrons are excited to the higher energy state. The TPIF intensity shows a quadratic dependence on the excitation laser power, confirming the occurrence of nonlinear two-photon absorption. Moreover, we can observe that TPIF requires much higher excitation powers, starting from 100mW onwards. Both the linear and exponential fits show an adjusted r-square value of 0.98 and 0.97 respectively, ensuring that the fitted function is a good representation of the measured data.

Figure 6: Intensity of the integrated OPIF spectrum increases linearly with the excitation power, while the intensity of the integrated TPIF spectrum shows a quadratic dependence on the excitation power.
TPIF requires a higher excitation power density than OPIF. The minimal excitation power density for OPIF is 5mW/mm², while TPIL requires a minimal power density of 2W/mm². As a result, the measurement constraints are more severe for TPIL than for OPIF. A high excitation power, a small spot size and a sensitive detector are indispensable to measure TPIL signals. However, considering the implementation in practical applications, TPIL uses NIR excitation wavelengths which are more widely commercially available than the required UV excitation wavelengths for OPIF.

To validate the automated screening of the samples, a scanning of the pure aflatoxin B1 powder is performed (Fig. 7). Particularly, we consider a Petri dish on which the aflatoxin B1 powder is disposed in two separate areas, of which we screen an area of 20.0 mm by 25.0 mm, with a resolution of 0.5 mm. By moving the X and Y position of the automated translation stages, while measuring for each position the fluorescence signal, the aflatoxin powder can be identified (indicated by the circles and dotted-circles in Fig. 7). During both the OPIF and TPIL measurements, the aflatoxin fluorescence is detected. The encircled regions indicate the areas from which a minimum fluorescence intensity of 0.8 µW/cm² and 0.1 µW/cm² at 428 nm is detected, during the OPIF and TPIL measurements respectively. The aflatoxin powder consists of granules positioned at two areas on the Petri dish (Fig. 7a). If a small aflatoxin-granule is illuminated, we detect a fluorescence signal. However, we obtain a smoother surface plot for TPIL than for OPIF, due to the larger spot diameter of the illumination beam (951 µm) during the OPIF measurements. During the OPIF measurements, most illumination positions around the powder excite one or multiple aflatoxin granules, since the surface area in-between the granules is smaller than the spot diameter. When using a smaller spot diameter, like during the TPIL measurements (231µm), no fluorescence signal can be captured when the illuminating beam is positioned in-between the aflatoxin granules. Consequently, a more fragmented surface plot is obtained.

Figure 7: Scanning of the aflatoxin B1 powder, visualizing the location of the emitted fluorescence signals: (a) aflatoxin B1 powder; (b) OPIF surface plot; (c) TPIL surface plot.

Generally, our fluorescence measurements of the pure aflatoxin B1 powder correspond with the theoretical characteristics, validating a correct measurement of the fluorescence signals. In addition, the aflatoxin B1 powder could be successfully visualized, indicating a good operation of the automated scanning of the samples. In the next section, we investigate the one- and two-photon induced fluorescence spectra of different maize batches.

3. LOCALIZED DETECTION OF AFLATOXIN CONTAMINATION IN MAIZE KERNELS

As our main goal, we focus on the localized aflatoxin detection in individual maize kernels. We first give an overview of the investigated samples, including both an Italian and Croatian batch to take the natural variation into account. Second, we investigate the one- and two-photon induced fluorescence spectra of healthy and aflatoxin-contaminated maize kernels. Specifically, we study the intrinsic fluorescence intensity, quantify the optical contrast between healthy and contaminated samples and define a detection criterion to optically sense the aflatoxins. Finally, the detection of the localized contamination areas onto the kernel’s surfaces are studied and visualized on surface plots.
3.1 Overview of the investigated samples
We consider two different independent maize batches, each with a healthy and contaminated subsample. One healthy and one contaminated maize batch were harvested in 2012 and provided by an Italian company. The second set of healthy and contaminated maize samples was collected from Croatian farmers, after the harvest in 2013. From each maize batch, a subsample of 25g was drawn for the analytical determination of the aflatoxin contamination level. Each sample was chemically analysed using the ToxiQuant mycotoxin testing system of ToxiMet\(^{19}\). Considering the Italian maize kernels, the contaminated sample shows 72.1ppb aflatoxin B1 and the healthy one 0.0ppb. The maize samples from Croatia show approximately the same aflatoxin B1 contamination level, being 78.9ppb for the contaminated sample and 0.8ppb for the healthy sample. After our measurements were performed, the contamination level of the samples was confirmed by the CODA-CERVA, the Belgian Reference Laboratory for Mycotoxins. Both the Italian and Croatian contaminated batches show a contamination-level exceeding the European limitations that impose a maximum total aflatoxin contamination of 10ppb.

During our fluorescence measurements, we investigate the fluorescence spectra of 45 healthy and contaminated Croatian maize kernels, to obtain a statistic relevant distribution of our measurement data. To observe the influence of the sample type and the harvest environments on the fluorescence spectra, we also measure the fluorescence spectra of 15 healthy and contaminated Italian maize kernels. The Italian company provided only small maize batches, which limits the number of studied maize kernels, but is sufficient to monitor the environmental influences on the spectra. Because aflatoxin is sensitive to light, the samples are permanently stored in a dark enclosure to minimize the environmental influences onto the measurements. In-between the measurements we stored the samples in a fridge to avoid cross-contamination.

3.2 OPIF and TPIF spectra of the maize kernels
To investigate the optical detection of aflatoxin, we study the OPIF and TPIF spectra of healthy and contaminated maize kernels, of both the Croatian and Italian maize batches. We measured the OPIF spectrum after excitation with 365nm, with an excitation power density of 317mW/mm\(^2\) (Fig. 8a). The TPIF spectra are measured after excitation with 730nm, 750nm and 780nm, with an excitation power density of 26.2W/mm\(^2\), 29.1W/mm\(^2\) and 36.0W/mm\(^2\) respectively (Fig. 8b-8d). During the TPIF measurements, we investigate the fluorescence spectrum after excitation with multiple wavelengths to monitor the influence of the illumination wavelength onto the fluorescence of the maize kernels. To increase the signal to noise ratio, we illuminate the maize kernels with the maximal output power of the titanium-sapphire laser and the harmonic generating unit. However, we limited the illumination time to 20ms and 200ms for the OPIF and TPIF measurements respectively, to avoid damage to the sample surface. For every maize batch, the mean fluorescence spectrum is depicted. Both the healthy as the contaminated samples show a fluorescence signal, due to the intrinsic fluorescence of the maize kernels (Fig. 8).

Comparing the different maize batches, the fluorescence spectra of the Croatian and Italian maize correspond well, within the variances of the measurements (Table 1). For all excitation wavelengths, both maize types emit corresponding fluorescence signals, showing a similar spectral shape in the same wavelength regions. The present intensity variances are caused by the large internal variation of the kernels, like their varying surface shape, density and natural composition. In correspondence with the measurements on the pure aflatoxin B1 powder, the fluorescence intensity induced by one-photon excitation is much stronger than intensity induced by two-photon excitation. Specifically, the TPIF signal is approximately 500 times weaker than the OPIF signal.
Comparing the fluorescence spectra of the healthy and contaminated maize kernels, we observe significant differences in intensity and emission wavelength. We do not directly observe the aflatoxin fluorescence, but we measure its influence on the intrinsic fluorescence of the maize. In the contaminated samples, the aflatoxin is bonded to the different natural constituents of the healthy maize, changing the molecular structure of the constituents and therefore influencing the fluorescence intensity and emission wavelengths of the healthy maize. During both OPIF and TPIF measurements, the fluorescence spectrum of the healthy and contaminated maize kernels show a wavelength shift of approximately 50nm (Table 1). Furthermore, we generally observe higher fluorescence intensities for the healthy maize kernels than for the contaminated ones (Table 1). The intensity contrast is the largest for the TPIF spectra, since the different bonds inside the maize are more selectively excited during the TPIF process than during the OPIF one. The largest intensity differences between the healthy and contaminated samples are observed after excitation with 730nm, where the mean fluorescence intensity of the healthy maize is 4 times stronger than the mean fluorescence intensity of the contaminated maize. In contrast, the minimum intensity differences are observed after excitation with 365nm. The observed large intensity variations within the healthy and contaminated samples are caused by both the curved surface of the maize kernels and the non-homogeneous presence of the toxins inside the maize. Besides, the spectroscopic differences between the healthy and contaminated samples can only be observed when illuminating the kernels with an excitation wavelength that is strongly absorbed by the aflatoxin. As is shown in previous published research, no spectroscopic contrast can be observed when illuminating the kernels with 405nm and 830nm laser light.
Table 1: Dominant emission wavelength and its corresponding fluorescence intensity, for both Croatian and Italian maize samples, after excitation with the different wavelengths.

<table>
<thead>
<tr>
<th>Excitation wavelength</th>
<th>Dominant emission wavelength Croatian maize (nm)</th>
<th>Dominant emission wavelength Italian maize (nm)</th>
<th>Maximum fluorescence intensity Croatian maize (µW/cm²/nm)</th>
<th>Maximum fluorescence intensity Italian maize (µW/cm²/nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>healthy</td>
<td>contaminated</td>
<td>healthy</td>
<td>contaminated</td>
</tr>
<tr>
<td>365nm</td>
<td>443 ± 5</td>
<td>497 ± 15</td>
<td>15 ± 8</td>
<td>17 ± 10</td>
</tr>
<tr>
<td>730nm</td>
<td>441 ± 3</td>
<td>496 ± 20</td>
<td>0.09 ± 0.05</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>750nm</td>
<td>444 ± 7</td>
<td>497 ± 24</td>
<td>0.07 ± 0.03</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>780nm</td>
<td>450 ± 10</td>
<td>503 ± 19</td>
<td>0.06 ± 0.04</td>
<td>0.02 ± 0.01</td>
</tr>
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</table>

To obtain a quantitative comparison between the measured spectra, taking the shape, intensity and maximum emission wavelength of the fluorescence spectra into account, we defined a detection criterion based on the integral of the fluorescence spectra in various wavelength intervals. We obtained an optimal contrast between the healthy and contaminated samples when considering the ratio of the integrated fluorescence intensity from 475nm until 550nm and the integrated fluorescence intensity from 400nm until 475nm. When visualizing the ratio of these integrals, for each maize kernel, a clear distinction between the healthy and contaminated samples is visible (Fig 9). Particularly, the ratios show a minimum contrast of 0.24, 0.13, 0.19, 0.25 and a maximum contrast of 2.15, 1.93, 2.87, 3.39 for excitation with 365nm, 730nm, 750nm and 780nm respectively. The largest difference between the mean ratio of the healthy and contaminated samples is observed for excitation with 780nm. Consequently, the largest contrast can thus be found after excitation with 365nm and 780nm, during the OPIF and TPIF measurements respectively.

The ratios of the integrated fluorescence intensities of the contaminated samples generally show a larger variation than the ratios of the healthy samples, because of the localized presence of the aflatoxin and the variable aflatoxin-concentration in the maize kernels. Depending on the illumination position on the maize kernel’s surface, a different contamination-level may be present, influencing the wavelength and intensity of the fluorescence spectrum. To visualize the localized aflatoxin contamination on the maize kernel’s surfaces, scanning measurements needed to be performed.
3.3 Monitoring of the localized presence of the aflatoxin
To monitor the localized presence of the aflatoxin, we scanned the maize kernels by using the automated translation stages. We investigated the localized aflatoxin contamination when illuminating the samples with 365 nm and 780 nm laser light, since these wavelengths showed the largest spectroscopic differences during the OPIF and TPIF measurements respectively.

During the OPIF measurements, we scanned the maize kernels with a resolution of 0.5 mm. For every sample, we recorded 405 spectra, each of which corresponding with a different illumination position of the laser beam. Considering the fluorescence wavelength showing the maximum fluorescence intensity, different regions in the kernels can be identified (Fig. 12). In correspondence with the previous measurements, the fluorescence spectra of the healthy maize kernels show their maximum fluorescence intensity between 400nm and 450nm (Fig. 12a). The contaminated areas show their maximum fluorescence intensity at wavelengths longer than 450nm. By interpreting the surface plots, various areas containing different contamination levels can be observed on different positions of the kernel’s surface (Fig. 12b-12d). As an example, the contaminated kernels can show a small localized contaminated area, a homogeneous contamination along the maize kernel or a medium to large contamination at the edge of the kernel. When considering the ratio of the integrated...
fluorescence spectra, specifically the ratio of the integrated fluorescence intensity between 475nm and 550nm and the integrated fluorescence intensity between 400nm and 475nm, a similar observation can be made (Fig. 11). In correspondence with Figure 9, the high-contaminated areas show the largest ratios. Furthermore, the gradient of the contamination level is clearly visible. When considering Figure 11b, two highly contaminated areas surrounded by a medium contaminated area can be identified, while Figure 11d shows a high contaminated area at the edge that flows into a healthy area in the center of the kernel. Concluding, localized contaminated areas can be accurately identified and characterized by mapping the integral ratio on the screened kernel’s surfaces.

Figure 10: Visualisation of the localized aflatoxin contamination during OPIF, by mapping the dominant fluorescent emission wavelength along the kernel’s surface: (a) healthy kernel; (b) kernel with small localized contamination area; (c) kernel with homogeneous contamination; (d) kernel with a medium to large contamination at the edge.

Figure 11: Visualisation of the localized aflatoxin contamination during OPIF, by mapping fluorescence ratios (integrated fluorescence intensity between 475nm and 550nm divided by the integrated fluorescence intensity between 400nm and 475nm) along the kernel’s surface: (a) healthy kernel; (b) kernel with small localized contamination area; (c) kernel with homogeneous contamination; (d) kernel with a medium to large contamination at the edge.

Considering the TPIF measurements, the maize kernels could only be partially scanned with a lower resolution of 1mm (instead of the 0.5mm resolution used during the OPIF scanning measurements). When accurately scanning a complete maize kernel’s surface during the TPIF measurements, the kernel’s surface would be damaged, due to the required long illumination time and high illumination power density. Specifically, the illumination time is significantly longer during
the TPIF measurements than during the OPIF measurements (approximately 10 times longer), due to the longer measurement time of the spectrum analyser required to capture the weak TPIF signals. Consequently, to avoid measuring the influence of damaging effects, the kernels are measured with a lower accuracy (Fig. 12). Studying the corresponding surface plots, regions with a high and low contamination level can still be observed. Specifically, the regions with a dominant fluorescence wavelength longer than 450nm can be considered as contaminated. Furthermore, the longer the fluorescence wavelength, the higher the contamination level on the kernel’s surface. The maize kernel depicted in Figure 12a shows generally a lower contamination than the one depicted in Figure 12b.

The scanning plots demonstrate a successful monitoring of the localized aflatoxin contamination. In contrast to the destructive chemical analyses that measure the mean contamination of a certain number of maize kernels, we are able to measure the localized contamination in individual food products without pre-processing or grinding of the kernels. In addition, by monitoring the localized contamination-level of the kernels, we allow to sort-out the maize grains that contain a low mean contamination-level but feature a small, localized high-contaminated area, which would not be detected by any type of chemical analyses. As a result, our optical detection methodology contributes to an improved food safety.

4. CONCLUSION

We demonstrated the use of one- and two-photon induced fluorescence spectroscopy for the detection of the localized aflatoxin contamination in solid maize kernels. We first optimized our fluorescence measurement setup, comprising a tunable titanium-sapphire laser and a harmonic generating unit, able to measure both the one- and two-photon induced fluorescence spectra. Following, we characterized the fluorescence spectra of healthy and contaminated (with >70ppb of aflatoxin B1) maize kernels. The largest spectral difference between the healthy and contaminated maize kernels was obtained after excitation with 365nm and 780nm, during one- and two-photon induced fluorescence spectroscopy respectively. Specifically, the contaminated kernels showed a lower fluorescence intensity at a longer emission wavelength than the healthy kernels. Based on the observed spectral differences, we defined a detection criterion by considering the ratio of two integrated fluorescence wavelength regions, from 400nm until 475nm and from 475nm until 550nm. This fluorescence ratio indicated a mean optical contrast of 1.15 for the OPIF spectra, while the TPIF spectra gave rise to a slightly larger mean contrast of 1.66. Besides, two-photon induced fluorescence shows weaker fluorescence intensities and requires a higher excitation power density than OPIF, but is more easily integratable in a commercial scanning-based configuration, due the commercial availability of high-power NIR laserlines. For both one- and two-photon induced fluorescence measurements, we demonstrate the detection of the localized aflatoxin contamination by interpreting surface plots obtained by a scanning of the kernel’s surface. We observed different contamination areas on the contaminated maize kernels: both kernels containing a small area with a high contamination level as kernels containing a large region with a medium contamination level could be identified. As a result, our scanning-based detection methodology enables to identify individual, solid, aflatoxin-contaminated maize kernels, paving the way to an industrial detection of aflatoxins in maize.

Figure 12: Visualisation of the localized aflatoxin contamination during TPIF, by mapping the dominant fluorescent emission wavelength along the kernel’s surface: (a) low-contaminated kernel; (b) high-contaminated kernel.
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REFERENCES AND LINKS


