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Abstract

The exposure to mycotoxins, which may contaminate food and feed commodities, represents a serious health risk for consumers. Ochratoxin A (OTA) is one of the most abundant and toxic mycotoxins, thus specific regulations for fixing its maximum admissible levels in foodstuff have been established. Laboratory-based analysis methods for detection and quantification of OTA (e.g., TLC, HPLC, MS and immunochemical methods) play a key role in ensuring food safety. However, to avoid OTA contamination along the whole food chain, rapid and easy-to-use diagnostic tools suitable for performing the analysis on-site are required. To this end, the use of lateral flow immunoassay (LFIA)-based devices has been proposed. In this context, we develop a portable and user-friendly smartphone-based biosensor for the detection and quantification of OTA in wine and instant coffee. The proposed biosensor combines the LFIA approach with chemiluminescence (CL) detection, providing adequate performance and compact analytical instrumentation. Indeed, the device consists in a smartphone-integrated self-standing device and employs low-cost, disposable analytical cartridges containing all the reagents required for the execution of the analysis. The cartridge can be used through simple manual operations and the analysis can be carried out at the point of need by non-specialized operators using the smartphone camera as light detector. The biosensor allows to quantify OTA in wine and coffee samples with limits of detection of 0.3 – 0.1 μg L^{-1}, respectively, which allow detection of samples with OTA content over the law-fixed limits. The method also exhibits a wide dynamic range extended up to 25 μg L^{-1}. These results demonstrated that the developed device can be used for routine monitoring of OTA contamination in wine and coffee enabling rapid and reliable identification of positive samples requiring confirmatory analysis.

Keywords: lateral flow immunoassay, ochratoxin A, chemiluminescence, smartphone, food safety, mycotoxins, point of need

Introduction

Food safety is a global health priority and a key factor to safeguarding the well-being of people, pursuing food security, and fostering economic development. The World Health Organization (WHO) has estimated that 600 million cases of foodborne illnesses and 420.000 related deaths occur annually [1]. Among strategies to overcome foodborne diseases WHO has emphasized the importance of providing consumers with tools to make safe food choices. In addition, food contamination has a relevant economic impact in terms of both market loss and public health impact. For example, it has been estimated that the Serbian farm-level dairy sector suffered a loss of more than 90 million euros from the aflatoxins outbreak in 2013 [2]. Thus, the availability of reliable, sensitive, and fast portable analytical devices will also contribute to reduce social and financial burden of food contamination.

Smartphone-based biosensors based on optical detection principles have recently emerged as powerful tools with the potential to revolutionize food testing by engaging farmers or consumers in their own food safety analysis. Indeed, smartphones combine pervasive distribution with rapidly developing technologies for connectivity, image acquisition and processing, and customizable applications into multifunctional, pocket-sized devices and thus represent an ideal facilitator for point-of-need devices [3-7]. Integration with paper-based assay technology allows development of simple, cheap, and portable analytical devices that meet the needs of commercial applications in outbreak control, food chain monitoring and regulatory inspection [5-9]. In this context, consumer-friendly smartphone-based biosensors have been already developed for the detection of food allergens [7, 10], pathogens [11-16] and chemical contaminants [17-22].

Mycotoxins are fungal secondary metabolites highly toxic to humans and cattle. The Food and Agriculture Organization (FAO) of the United Nations has estimated that mycotoxins

contaminate 25% of the world's food crops [23]. Filamentous fungi proliferate in food commodity in environmental conditions (i.e., temperature, humidity, and sunlight) typical of tropical and subtropical countries. Food production globalization (involving long storage and transportation times) and climate changes (causing temperature rise in cool or temperate countries) further increases the risk of mycotoxins widespread contamination [6, 24, 25]. This points out the need of portable measurement devices for rapid mycotoxin detection along the whole food supply chain and at the consumer endpoint [26, 27]. However, the potential of smartphone-based optical biosensors for mycotoxins monitoring is still underexplored and only few examples have been reported in literature [14, 28-30].

In this context, we propose a portable and user-friendly smartphone-based biosensor for the detection of Ochratoxin A (OTA), a mycotoxin with cytotoxic, carcinogenic, mutagenic, and immunosuppressive activity, in wine and instant coffee [31, 32]. Wine and coffee represent important sources of OTA dietary intake for the EU population, posing a serious risk for human health. Hence, maximum admissible levels as low as 2 μg kg^{-1} and 10 μg kg^{-1} have been established by the European Union in wine and instant coffee, respectively (the limit decreases to 5 μg kg^{-1} for roasted coffee beans and ground roasted coffee) [33]. The same values have also been set by Canada [34], but specific legislation on this topic is missing in most extra-European countries.

The proposed biosensor is based on Lateral Flow Immunoassay technique coupled with chemiluminescence detection (CL-LFIA), which provides enhanced analytical performance with respect to visual detection (e.g., exploiting gold nanoparticles as labels), often employed for readout of LFIA assays. In addition, it could be recommendable to develop a method characterized by a dynamic range suitable for application for analysis of both wine and instant coffee despite the different regulatory limits. With this respect, CL is an ideal detection principle since it combines wide dynamic range, simplicity of signal measurement, and amenability to miniaturization [35-37]. The biosensor consists in a smartphone-

integrated self-standing device comprising a low-cost and disposable analytical cartridge containing all the reagents required for the execution of the analysis. The cartridge can be used through simple manual operations and the entire analysis can therefore be carried out at the point of need by non-specialized operators using the smartphone CMOS (Complementary Metal-Oxide Semiconductor) camera as light detector. Analysis relies on a competitive immunoassay, in which OTA in the sample and a horseradish peroxidase OTA conjugate (HRP-OTA) compete for a limited amount of an anti-OTA antibody immobilized on the LFIA nitrocellulose strip. The HRP-OTA bound to immobilized antibody is then detected by CL upon addition of an HRP CL substrate based on luminol/H_2O_2 and enhancers. According to the competitive format, the amount of HRP-OTA bound, and thus the intensity of the CL signal, are inversely related to the amount of OTA in the sample. The proposed system allows the reliable quantification of OTA as required by the current regulations and could thus be used as first-level screening analytical tool for detecting potentially contaminated samples to be subjected to confirmatory analysis with reference instrumental analytical methods.

2. Materials and Methods

2.1 Reagents

The polyclonal anti-OTA antibody produced in rabbit and the HRP-OTA conjugate were kindly provided by Euroclone (Milan, Italy). Polyclonal anti-HRP antibody produced in rabbit, ovalbumin (OVA), Tween-20, and polyethylene glycol 10000 were obtained from Sigma-Aldrich Co (St. Louis, MO). The CL HRP detection substrate Supersignal ELISA Femto was purchased from Thermo Scientific Inc. (Rockford, IL). The other reagents were of analytical grade and were employed as received. Phosphate buffered saline (PBS) contained 10 mmol

L^{-1} Na_2HPO_4, 2 mmol L^{-1} KH_2PO_4, 137 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl, with pH adjusted to 7.4. The LFIA strips were produced using Whatman Standard 14 glass fibre sample pad (GE Healthcare Lifescience, Chalfont St. Giles, UK), Hi-flow plus 180 nitrocellulose membrane cards (Merck Millipore, Billerica, MA), and cellulose adsorbent pads (Merck Millipore).

2.2 Preparation of the LFIA strips

Anti-OTA antibody 1:50 (v/v) and anti-HRP antibody 1:500 (v/v) solutions in 20 mM phosphate buffer at pH 7.4 (PB) were applied onto the nitrocellulose (NC) membrane cards to form the Test line (T-line) and the Control line (C-line), respectively, by a XYZ3050 dispenser platform (Biodot, Irvine, CA) The solutions were non-contact dispensed at 1 µL cm^{-1} keeping a distance of 5 mm between the two lines. The NC card were dried at 37°C for 45 minutes under vacuum, saturated with 1% (w/v) OVA in PB, washed with PB supplemented with 0.05% (v/v) Tween-20, and finally dried under vacuum at 37°C for 90 min. The sample pads were saturated with PB supplemented with 3% (w/v) OVA and 0.1% (v/v) Tween-20, then dried for 90 min at 40°C. The cards were laminated with the sample and absorbent pads, then cut by a CM400 guillotine (Biodot) to obtain 5-mm width LFIA strips. The strips were sealed in plastic bags containing a silica desiccant and stored at room temperature in the dark until use.

2.3 Analytical device

The analytical device includes two components, a disposable analytical cartridge and a mini dark box with a smartphone holder which hosts the cartridge during the measurement of the CL signal.

The disposable analytical cartridge (Figures 1a and 1c) consists of a plastic holder (size 110 × 90 × 4 mm) containing a fluidic element that houses the LFIA strip, the reagents necessary for the analysis and the fluidic system for metering the correct amount of sample and transferring sample and reagents to the LFIA strip.

The fluidic element is composed of two transparent polypropylene layers (thickness 200 µm). The upper layer has embossed elements (e.g., chambers and channels) obtained by vacuum thermoforming while the bottom one is flat and adhesive to allow assembly of the fluidic element. In more detail, the upper layer includes:

- a PEEK tube for connecting a unidirectional sample injection valve;
- a 30-µL sample metering chamber;
- a sample overflow chamber;
- a LFIA strip chamber;
- three reservoirs for the pouches containing the OTA-HRP conjugate and the two components (luminol/enhancer and oxidant) of the SuperSignal ELISA Femto HRP CL substrate;
- a rectangular cavity for an additional large adsorbent pad to further promote flow of sample and reagents along the LFIA strip;
- fluidic channels connecting the sample injection valve, the sample metering and overflow chambers, the reagents reservoirs, and the LFIA strip chamber;
- two manually actuated valves for controlling sample and reagents flow.

The plastic holder protects the fluidic element and supports the unidirectional valve for sample injection, thus acting as an interface towards the sampling equipment.

The analytical cartridge is prepared in advance prior to the analysis. First, the reagent vacuum thermoformed polypropylene pouches are filled with the reagents, namely 60 µL of

OTA-HRP conjugate diluted 1:2500 (v/v) in PBS supplemented with 3% (w/v) OVA as a saturating agent to reduce nonspecific binding and 40 µL of the two components of the CL HRP detection substrate, and sealed with adhesive tape. Then, the fluidic element is assembled after inserting the reagent pouches, the LFIA strip and the additional adsorbent pad, connected to the sample injection valve and glued between the two halves of the plastic holder.

For performing CL measurement, the analytical cartridge is inserted in a mini dark box to avoid ambient light interference (Figures 1b and 1d). The mini dark box is equipped with an adapter for the OnePlus 6 smartphone (OnePlus, Shenzen, China) and a plano-convex lens (diameter 6 mm, focal length 9 mm, Edmund Optics, York, UK) that allows correct imaging of the LFIA strip by the smartphone camera.

The fluidic element plastic holder and the mini dark box have been designed using the browser-based 3D design platform Tinkercad (Autodesk, San Rafael, CA) and produced in black acrylonitrile-butadiene-styrene (ABS) copolymer using a Makerbot Replicator 2X printer (Makerbot Industries, New York, NY) exploiting Fused Deposition Modelling (FDM) 3D printing technology.

2.4 Assay procedure

The analysis procedure is shown in Figure 2. While keeping the flow control valves in the “sample inject” position, the sample was injected using a syringe into the sample injection valve until the sample metering chamber is filled and excess sample arrived at the sample overflow chamber (Figure 2b). Then, the flow control valves were shifted to the “analysis” position and the sample and the HRP-OTA conjugate solution were both transferred to the sample pad of the LFIA strip by pushing the HRP-OTA chamber, thus squeezing the reagent pouch inside (Figure 2c). The mixed solutions began flowing across the membrane where

the immunoreactions took place. Upon complete migration across the LFIA strip (30 min), the HRP CL substrate was added to the strip by simultaneously pushing the other two chambers (Figure 2d). After 15 minutes the analytical cartridge was inserted into the mini dark box connected to the smartphone and the CL signal was measured using a 4-sec exposure time.

To obtain quantitative information on the OTA content of samples, CL images were analysed by the freely available image analysis software ImageJ (v. 1.53c, National Institutes of Health, Bethesda, MD). For each sample, the photon emissions corresponding to the C-line and the T-line of the LFIA strip were calculated by integrating the CL signal in the line areas and subtracting the background signal obtained by averaging the CL signals measured in two areas just below and above each line. Then, the T-line/C-line CL signal ratio was calculated and the concentration of OTA was determined by interpolation on calibration curves generated by analysing matrix-matched standard OTA solutions (concentration range 0 - 25 µg L^{-1}) in wine or coffee matrices, plotting the corresponding T-line/C-line CL signal ratios against the analyte concentration in logarithmic scale and fitting the resulting sigmoidal curve with a four-parameter logistic equation. Data graphing and fitting were performed using the Prism data graphing and analysis software (v. 8.0.3, GraphPad Software, San Diego, CA).

2.5 Analysis of real samples

Red or white wine and instant coffee samples were obtained directly from local stores. Instant coffee was dissolved in hot water (80°C) at a concentration of 50 g solid L^{-1}, then let to cool to room temperature. To remove substances that could interfere with the CL detection (e.g., polyphenols) samples were subjected to a pre-analytical procedure developed for wine matrices by Anfossi et al. [38], with slight modifications. Briefly, sample

solutions were thoroughly mixed with 0,15 M $NaHCO_3$ (pH 9.0) and 4% (w/v) PEG 10000 water solutions in the 1:2:2 (v/v) ratio and let to react for a few minutes before analysis (matrix-matched standard OTA solutions used for the generation of calibration curves were prepared in wine or coffee blank matrices subjected to the same pre-analytical treatment).

For evaluation of assay performance, the OTA content of the samples was also determined by a previously described HPLC-FLD reference method [39]. The chromatographic separation was carried out on a C_{18} RP column operating in an isocratic mode with an acetonitrile–water–acetic acid 55:44:1 (v/v/v) mobile phase at a flow rate of 1.0 mL min^{-1}. The target analyte was detected using a fluorescence detector (λ_{ex} = 333 nm, λ_{em} = 460 nm) and its concentration was determined by interpolating peak areas on a calibration curve generated by analysing standard OTA solutions, plotting the peak areas against OTA concentration and fitting the data using a weighted linear regression model (weight = 1/x). The HPLC-FLD reference method has a limit of quantification of 0.10 µg L^{-1} OTA and a mean relative standard deviation (RSD) of 21% in the concentration working range (0,1-10µg L^{-1}).

3. Results and discussion

After designing and producing the device, in the first phase of the work experimental conditions was optimised for quantifying the target analyte in the two selected matrices with high sensitivity. Then an analytical cartridge and accessories were designed to allow performing the entire analysis on site by non-specialized operators and employing a smartphone for measuring CL emission, still maintaining high analytical performance. The sample pre-treatment procedure to remove interfering substances that could affect the development of the CL signal in the detection step was also defined and tested.

3.1 Design of the fluidic cartridge

The fluidic cartridge has been designed taking into account two main prerequisites: it must contain all the reagents required for performing the assay and the analytical system had to be portable and easy-to-use, still assuring the correct and reproducible handling of sample and reagents.

All elements of the fluidics (i.e., the reagents chambers and the sample metering chamber) were dimensioned taking into account the channels' dead volume to deliver the desired volumes of sample and reagents to the LFIA strip (the volumes required to wet the sample pad and achieve complete migration along the LFIA strip were preliminary established by dispensing the solutions directly on the sample pad of the strip).

During the analysis, the flows of sample and reagents are driven by capillary force (along the LFIA strip) or by finger pressure on the reagent chambers. Two valves, each of them constituted by a cavity containing a rectangular-shaped PDMS mobile element, were developed for control the direction of flow. The inner PDMS element of the valve is manually shifted between the two sides of the cavity to open/close the desired flow channels. In more detail, the first valve (located after the sample injection unidirectional valve) had the task of control the inlet (sample or OTA-HRP conjugate) of the sample metering chamber. The second valve, located just after the sample metering chamber, controls the outlet of the chamber by driving the fluid to the sample overfull chamber or to the LFIA strip. During the analysis, the mobile elements of the valves were shifted from the left side ("sample inject" position) to the right side ("analysis" position) of the cavities.

An important issue in the analytical procedure, which could affect assay performance, is the mixing of the sample and the HRP-OTA conjugate before delivering to the LFIA strip. Indeed, according to the design of the analytical cartridge, the flow of the HRP-OTA conjugate

pushed the sample from the sample metering chamber to the LFIA strip. Therefore, since mixing of the two solutions inside the sample metering chamber is expected to be not efficient, at least part of the sample arrived to the LFIA strip before the HRP-OTA conjugate (differently, the two components of the CL substrate are completely mixed in the serpentine channel before arriving to the LFIA strip). We investigated this phenomenon by analysing OTA standard solutions prepared in OTA-free wine matrix using two slightly different protocols. In the first one (which represented the ideal analytical procedure) the OTA and HRP-OTA conjugate solutions were mixed before dispensing on the sample pad of the LFIA strip. In the second protocol (which simulated the real analytical procedure) the OTA and HRP-OTA conjugate solutions were sequentially dispensed on the sample pad. In comparison to the ideal analytical procedure, the sequential addition protocol produced higher decreases of the T-line/C-line CL signal ratios (for example, for the 5 μg L^{-1} OTA standard solution the T-line/C-line CL signal ratio was 0.34 instead of 0.32). This behaviour could be ascribed to the fact that in the sequential addition protocol the first aliquot of solution flowing along the LFIA strip was enriched in sample, so that OTA in the sample was favoured in the competition for binding the immunoreagents immobilized in the T-line (the CL signal of the C-line is not affected because the binding of the excess HRP-OTA conjugate is not a competitive process). Nevertheless, the differences in the T-line/C-line CL signal ratio were small, thus it was concluded that the sequential addition of the sample and the HRP-OTA did not negatively affect assay performance.

We also observed a relatively slow flow along the LFIA strip in the cartridge, which was attributed to the absence of evaporation that could accelerate the process. Since complete migration of sample and HRP-OTA conjugate solutions toward the adsorbent should took place before delivering the CL substrate to the LFIA strip, the analytical protocol provided a 30-minutes interval between the delivering of sample and HRP-OTA solutions and of CL substrate to the LFIA strip. An appropriate time interval (i.e., 15 minutes) was also provided

between delivering of the CL substrate to the LFIA strip and CL measurement, since the CL substrate had the dual function of developing the CL signal and washing the membrane from unbound species (thus reducing the CL background signal).

3.2 Optimization of experimental parameters

Assay parameters were optimised to achieve limits of detection (LODs) and dynamic ranges useful for detecting OTA in wine and instant coffee according to the current regulatory limits (i.e., 2 µg kg^{-1} and 10 µg kg^{-1} for wine and instant coffee, respectively). As target OTA concentrations corresponding to the regulatory limits we considered 2 µg L^{-1} for wine (assuming a density of 1 kg L^{-1}) and 0.5 µg L^{-1} for instant coffee (taking into account the overall solid concentration in the instant coffee solution). Different HRP-OTA conjugate dilutions, namely 1:1000, 1:2500 and 1:5000 (v/v), were evaluated by comparing the CL signals obtained analysing OTA-free wine and instant coffee samples before and after spiking with known amounts of OTA, employing LFIA strips in which the anti-OTA antibody was immobilized on the T-line at 1:50 and 1:100 (v/v) dilutions. Based on results shown in Figure 3, the 1: 2500 (v/v) HRP-OTA conjugate dilution and the 1:50 (v/v) anti-OTA antibody dilution immobilized on the T-line were selected as the most suitable for the OTA detection at regulatory limits. Indeed, they provided the most intense CL signals, thus facilitating CL measurement with the smartphone camera, and also allowed clear discrimination of wine and instant coffee samples containing 2 µg L^{-1} and 0.5 µg L^{-1} OTA, respectively, from blanks and samples at higher OTA concentration.

3.3 Calibration curves

Matrix-matched calibration curves were obtained in the optimized experimental conditions by analysing OTA-free wine and instant coffee samples spiked with known amounts of OTA,

up to 25 µg L^{-1}. Being the assay a competitive one, the CL intensity of the T-line decreased as the concentration of the analyte in the sample increased and the T-line disappeared for the highest OTA concentrations (Figure 4a). In this context, it should be noted that the calibration curve was generated considering the T-line/C-line CL signal ratio as the analytical signal instead of the CL signal of the T-line alone. This improved assay accuracy by ruling out factors, such as environmental temperature, that globally influenced the rate of the HRP-catalysed CL reaction and thus the intensity of the CL emission.

As shown in Figure 4b, the calibration curves obtained in the two matrices are very similar each other, suggesting that the pre-treatment procedure efficiently removed any sample component that might interfere with the assay. The LODs of the assay, calculated as the OTA concentration giving a T-line/C-line CL signal ratio corresponding to that of the blank minus three times its standard deviation, were 0.3 µg L^{-1} and 0.1 µg L^{-1} in wine and instant coffee matrices, respectively, while dynamic ranges extended up to the maximum OTA concentration (25 µg L^{-1}). The LOD values obtained are comparable to those reported in the literature for other OTA LFIA-based assays, as well as to those of commercial LFIA assay kits (Table 1). The assay also showed a good reproducibility, with RSD associated to the points of the calibration curve always below 12% (wine matrix) and 7% (instant coffee matrix).

Table 1. Comparison of LODs of literature and commercial LFIA-based OTA assays.

Sample	Detection principle	LOD	Reference or assay trade name/producer
Wine Grape must	Colorimetry (instrumental detection)	1 µg L^{-1}	[38]
Wine Grape must	Colorimetry (instrumental detection)	0.9 µg L^{-1}	[40]

Wine Grape juice	Fluorescence (instrumental detection)	0.06 μg L^{-1}	[41]
Wine	Colorimetry (visual detection)	10 μg L^{-1}	[42]
Coffee	Fluorescence (instrumental detection)	0.88 μg L^{-1}	[43]
Coffee	Colorimetry (visual detection)	5 μg L^{-1}	[44]
Wine	Colorimetry (visual or instrumental detection)	1 μg L^{-1} (visual detection) 0.18 μg L^{-1} (instrumental detection)	[45]
Wine	Fluorescence (visual or instrumental detection)	5 μg L^{-1} (visual detection) 1.9 μg L^{-1} (instrumental detection)	[46]
Wine Grape juice	Colorimetry (instrumental detection)	1 μg L^{-1}	OCHRAQ-W Test (Charm Sciences Inc., Lawrence, MA)
Coffee	Colorimetry (instrumental detection)	1.1 μg L^{-1}	Reveal® Q+ MAX for Ochratoxin (NEOGEN, Lansing, MI)
Wine	Colorimetry (instrumental detection)	0.4 μg L^{-1}	QuickTox for QuickScan Ochratoxin (EnviroLogix Inc., Portland, MA)

3.4 Sample pre-treatment

Wine and coffee contain, among others, antioxidant substances that can interfere with the luminol oxidation reaction catalysed by HRP, thus not allowing the production of the CL signal. It is therefore necessary to remove these substances prior to analysis. Since the proposed biosensor was designed to be employed directly on site the sample pre-treatment

must also consider this need. In this view, the wine sample pre-treatment procedure described by Anfossi et al. [38] was simplified by eliminating the filtration step and directly analyzing the sample solution. Indeed, the comparison of the CL signals obtained by analyzing wine or instant coffee samples subjected or not to filtration showed no significant differences in the intensity of the CL signals in the test and control lines. We assumed that the sample pad was able to retain precipitated impurities avoiding their migration along the LFIA membrane.

3.5 Assay performance

To evaluate assay performance as requested for the initial in-house validation of newly developed screening methods [47], in accordance with current EU regulations [48, 49] and opinions of EU reference laboratories [50], the following procedure was adopted. We tested 20 blank samples of wine and 20 blank samples of instant coffee before and after spiking with OTA at the maximum admissible levels according to the current regulatory limits. The obtained results are reported in Table 2.

Table 2: OTA concentrations measured for blank wine and instant coffee samples before and after spiking with OTA at the maximum admissible levels to the current regulatory limits.

Wine			Instant coffee		
Sample no.	Before spiking (μg L^{-1})	After spiking with 2 μg L^{-1} OTA (μg L^{-1})	Sample no.	Before spiking (μg L^{-1})	After spiking with 0,5 μg L^{-1} OTA (μg L^{-1})
1	0.00	2.15	1	0.12	0.43
2	0.05	2.52	2	0.09	0.54
3	0.09	1.90	3	0.07	0.47
4	0.07	1.75	4	0	0.55
5	0.10	2.52	5	0.05	0.42

6	0.00	2.04	6	0.09	0.48
7	0.12	2.31	7	0.06	0.48
8	0.09	2.14	8	0.05	0.51
9	0.07	2.16	9	0	0.49
10	0.15	1.87	10	0.15	0.52
11	0.00	1.99	11	0	0.55
12	0.25	2.31	12	0.15	0.49
13	0.06	2.43	13	0.17	0.49
14	0.00	1.81	14	0	0.47
15	0.05	1.72	15	0	0.57
16	0.17	2.03	16	0.09	0.51
17	0.00	2.16	17	0.12	0.51
18	0.00	2.47	18	0.07	0.46
19	0.09	2.23	19	0.09	0.56
20	0.12	2.33	20	0.08	0.5

The highest measured OTA concentrations for blank samples were 0.25 μg L^{-1} for wine and 0.17 μg L^{-1} for instant coffee, while in spiked samples the lowest measured OTA concentrations were 1.72 μg L^{-1} for wine and 0,42 μg L^{-1} for instant coffee. Therefore, the responses of the spiked samples do not overlap with the range of responses of the blanks. The detection capability (CCβ) of the assay was assessed from the threshold (T) and cut-off (F_m) factors for wine and instant coffee, calculated according to the mean response and standard deviation of blanks (B, SD_B) and samples spiked at the maximum admissible levels (M, SD_M) by according to the following equations:

$$T = B + 1.64\ SD_B$$

$$F_m = M - 1.64\ SD_M$$

Since F_m > T (T_{wine}=0.18, $F_{m,\ wine}$=1.73; $T_{instant\ coffee}$=0.16, $F_{m,\ instant\ coffee}$=0.42), it could be concluded that the CCβ of the assay (i.e., the smallest content of the analyte that can be detected in a sample with a chance of 5% of a false negative decision) is lower than the maximum admissible levels for both wine and instant coffee matrices, and therefore the method demonstrated an adequate level of sensitivity for a screening method.

3.6 Analysis of unknown samples

Finally, samples of wine (14), grape must (5) and instant coffee (6) with unknown OTA content were analysed using the smartphone CL-LFIA device and the results were compared with those obtained with the reference HPLC-FLD method (Figure 5). For all samples, the OTA concentration was below the regulatory limits (for three instant coffee samples the OTA content measured by HPLC-FLD was also below the LOD of the CL-LFIA assay, i.e., 0.1 μg L^{-1}). Recoveries of the optical biosensor ranged from 81 to 123% and variation coefficients were lower than 15%. The equation of the correlation curve is y = 0.961x + 0.0397 with r^2 = 0.993 (where y and x are the OTA concentrations in μg L^{-1} measured with the CL-LFIA assay and the HPLC-FLD method, respectively), thus demonstrating a good concordance between the two analytical approaches.

The specificity of the smartphone CL-LFIA device was tested, and no cross-reactivity was detected against other relevant mycotoxins, specifically aflatoxins, fumonisins and zearalenone (data not shown).

Conclusions

Moving from the urgent need for user-friendly, rapid and affordable assays for a promptly and on-site mycotoxins detection, we propose a portable biosensor suitable for quantifying

OTA in wine and instant coffee samples. Applying smartphone-based technologies to traditional LFIA technique, it has been developed a detecting platform laboratory-independent which can be used by non-skilled personnel realizing a real-time and on-site analysis, thus reducing time and costs. The combination with CL detection principle allowed also to obtain an ultrasensitive detection which assured accurate and precise quantification in the interval of OTA concentrations of legal relevance. Furthermore, one step forward is represented by the development of a pocket-sized and disposable microfluidic cartridge to further enhance the automation of analytical processing and reduce the consumption of samples and reagents.

Therefore, this biosensor can be proposed for a reliable first level monitoring of OTA in wines and instant coffee providing a cost-effective and easy-to-use smartphone-based device. In the future, the development of a specific smartphone-App for data analysis and for the traceability of food batches during transportation, would allow a prompt action of isolation of suspect batches with the possibility of tracing the origin of the contamination. This will contribute to the implementation of the whole food supply chain surveillance, and hence significantly improve the current food safety control system.

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Figure 1. Schematic drawings and photographs of the disposable analytical cartridge (a, c) and the mini dark box with smartphone adapter (b, d).

Figure 2. Schematic representation of the assay procedure steps: (a) cartridge ready for analysis, (b) sample injection, (c) transfer of the sample and the HRP-OTA conjugate solution to the LFIA strip, (d) transfer of the HRP CL substrate to the LFIA strip.

Figure 3. Chemiluminescence signals obtained in correspondence of the T-line employing LFIA strips in which the immobilized anti-OTA antibody was dispensed at (a, c) 1:50 and (b, d) 1:100 (v/v) dilutions. Assays were performed in OTA-free wine (a, b) and instant coffee matrices (c, d), before and after spiking with OTA at the maximum admissible levels (2 µg L^{-1} and 0.5 µg L^{-1} for wine and instant coffee matrices, respectively) and at high concentration (25 µg L^{-1}).

Figure 4. (a) CL images obtained by analysing OTA-free wine and instant coffee samples spiked with known amounts of OTA. (b) Areas on the LFIA strip used for the measurement of the CL signals. (c) Representative calibration curves obtained in wine and instant coffee matrices (each standard solution was analyzed in triplicate).

Figure 5. Correlation between OTA concentrations measured in real samples using the smartphone CL-LFIA device and the reference HPLC-FLD method.

Figure 1

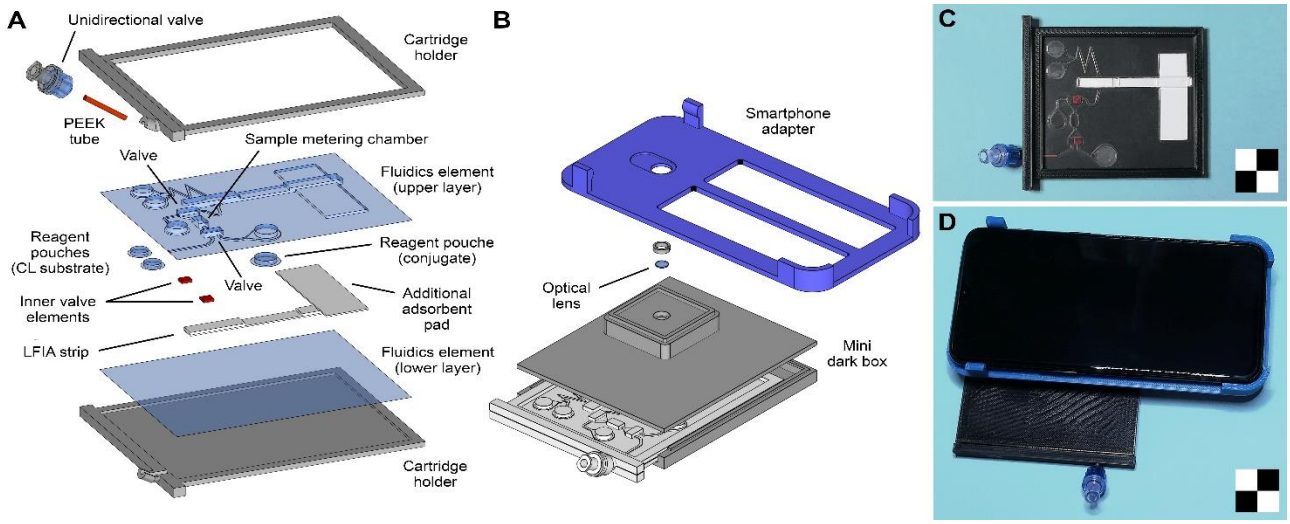

A
Unidirectional valve
Cartridge holder
PEEK tube
Sample metering chamber
Valve
Fluidics element (upper layer)
Reagent pouches (CL substrate)
Reagent pouche (conjugate)
Valve
Inner valve elements
Additional adsorbent pad
LFIA strip
Fluidics element (lower layer)
Cartridge holder
B
Smartphone adapter
Optical lens
Mini dark box
C
D

Figure 2

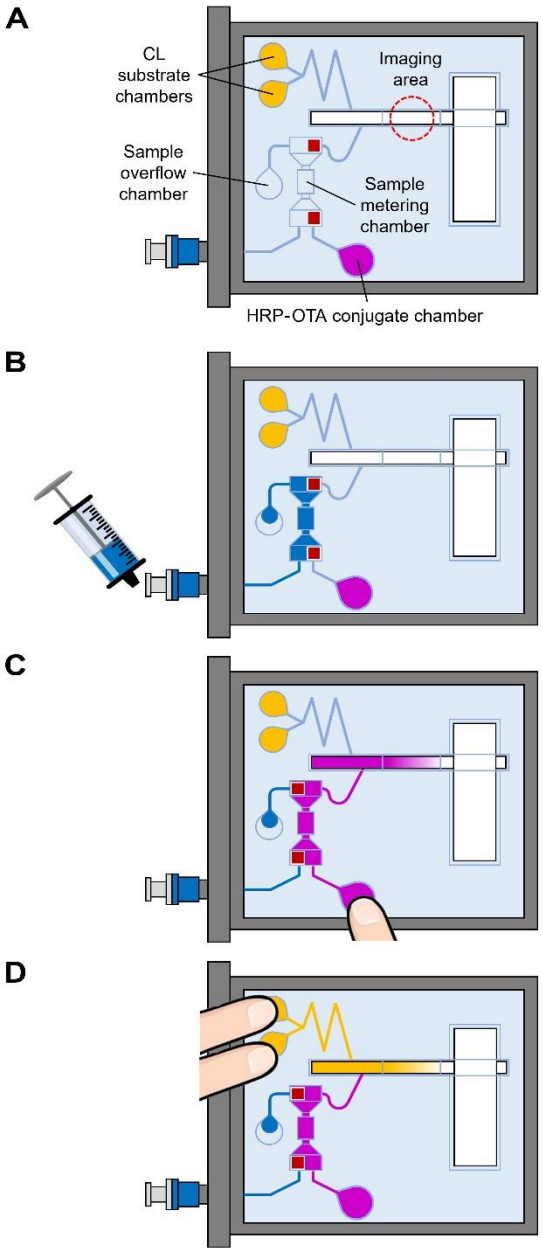

A
CL substrate chambers
Imaging area
Sample overflow chamber
Sample metering chamber
HRP-OTA conjugate chamber
B
C
D

Figure 3

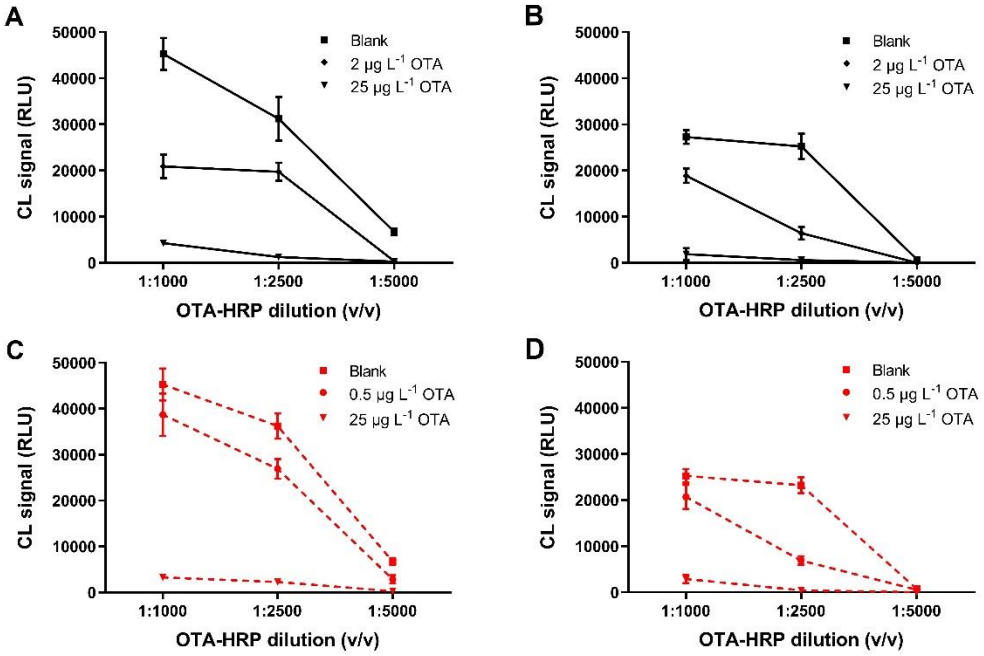


Figure 4

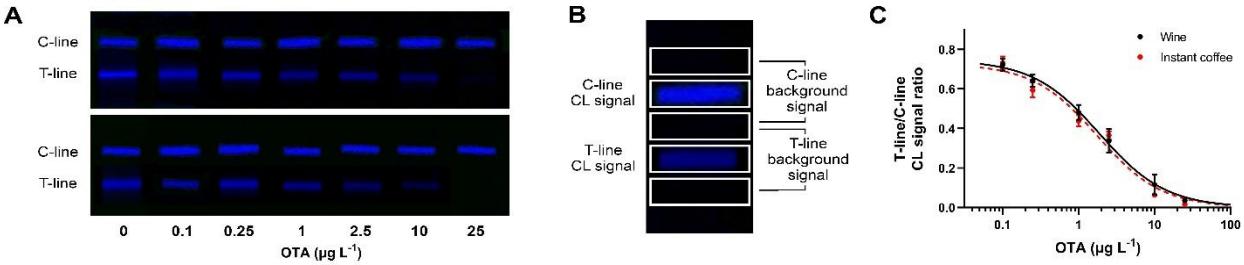
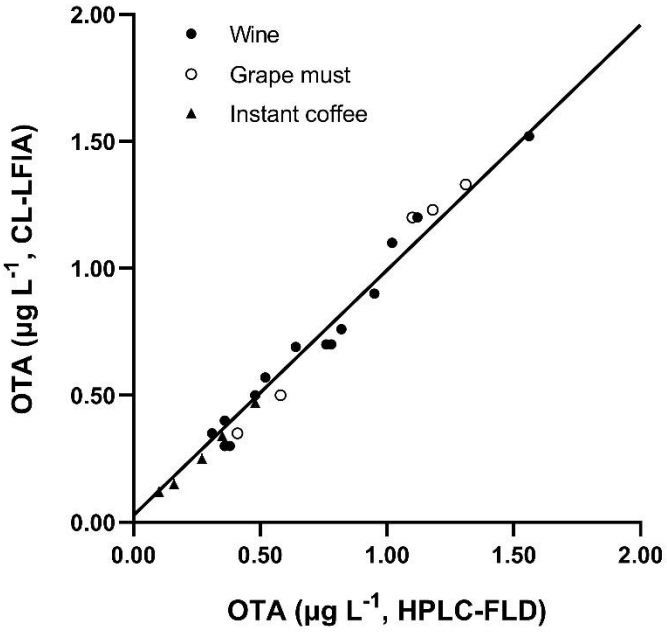


Figure 5


Wine
Grape must
Instant coffee
2.00
1.50
1.00
0.50
0.00
0.00
0.50
1.00
1.50
2.00
OTA (µg L-1, CL-LFIA)
OTA (µg L-1, HPLC-FLD)