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INCREASED SENSITIVITY OF LATERAL FLOW IMMUNOASSAY FOR OCHRATOXIN A THROUGH SILVER ENHANCEMENT

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Abstract

Silver nucleation on gold has been exploited for signal amplification and has found application in several qualitative and quantitative bio-sensing techniques, thanks to the simplicity of the method and the high sensitivity achieved. Very recently, this technique has been tentatively applied to improve performance of gold-based immunoassays. In this work, the exploitation of the signal amplification due to silver deposition on gold nanoparticles has been first applied to a competitive lateral flow immunoassay (LFIA). The signal enhancement due to silver allowed us to strongly reduce the amount of the competitor and of specific antibodies employed to build a LF device for measuring ochratoxin A (OTA), thus determining the attainment of the high sensitive assessment of OTA contamination, with a sensitive gain of more than 10-folds compared to the gold-based LFIA that used the same immunoreagents and to all previously reported LFIA for measuring OTA. In addition, a less sensitive "quantitative"-LFIA could be established, by suitably tuning competitor and antibody amounts, which was characterized by reproducible and accurate OTA determinations (RSD% 6-12%, recovery% 82-117%). The quantitative system allowed a reliable OTA quantification in wines and grape musts at the µg/l level requested by the European legislation, as demonstrated by agreeing results obtained through the "quantitative" silver enhanced-LFIA and a reference HPLC-FLD on 30 samples.

Keywords Immunochromatographic assay; silver nanoparticles; mycotoxins; food analysis

Introduction

The last generation of immunoassays developed to assess food safety belongs to rapid methods of analysis and addresses the increasing demand of portable, self-sufficient, easily operated analytical tools to screen large number of samples and to be operated outside the laboratory. Lateral flow immunoassay (LFIA),

known for a long time in clinical diagnostics, combines a series of benefits, including extreme simplicity, rapidity, and cost effectiveness, which makes it ideally suited for the purpose.

A standard LF device comprises: a migration membrane, onto which immunoreagents are immobilized in reactive zones; a reporter probe capable of interacting with the membrane-linked reagents and of providing some detectable response; and additional components to make the system fully self-sufficient. Although several labels have been proposed to serve as reporters in LFIAs, gold nanoparticles (GNPs) have long been used for the purpose, because of their distinguished features (such as: ease of preparation, capability to adsorb proteins, and detectability to the naked eye). Nevertheless, the growth of LFIA applications increasingly demands sensitivity improvement (especially for low-molecular-mass analytes) and the conversion of visually evaluated yes/no assays into non-subjective quantitative tests.

Silver nucleation on gold is a well-known phenomenon, which has been exploited since the past thirty years in bio-sensing for signal amplification thanks to the simplicity of the method and the high sensitivity achieved [1]. The gold silver enhancement has found application in qualitative and quantitative bio-sensing techniques, combined to several detection methods, such as: colorimetric, electrical, electrochemical, and gravimetric methods, chemiluminescence, Raman spectroscopy, and inductively coupled plasma mass spectrometric method [1]. Silver deposition on gold is simply realized by combining a silver salt (nitrate or acetate) with a reducing agent (hydroquinone buffered to acid pH by citric acid-sodium citrate) [2]. The exploitation of the signal enhancement strategy based on silver deposition on gold nanoparticles has been rarely applied to immunoassays, except for the work of Gupta et al., who described a model sandwich immunoassay in which antigens (i.e.: immunoglobulins) were captured by antibodies immobilized onto glass slides and detected by means of a second antibody labeled with gold nanoparticles. After silver deposition, images were observed by an optical microscope and quantified by densitometry [3]. Despite the novelty of the approach, the immunoassay procedure involved several steps due to the need of wash out unreacted reagents after each incubation.

Very recently, this technique has been also tentatively applied to improve performance of gold-based LFIAs. The pioneer work of Yang et al. [4] first reported the profitable use of the deposition of metallic silver onto gold nanoparticles to increment their visibility and, thus sensitivity, in a colloidal gold probe-based LFIA for detecting abrin-a in food samples. The assay was conceived as a sandwich immunoassay, with the capture antibody immobilized onto the migration membrane and the detection antibody labeled with GNPs. After strip development and GNP accumulation in the immunoreactive zones of the membrane, two additional pads were placed above the membrane, which contained $AgNO_3$ and the reducing agent, respectively. The wetting of pads re-solubilized reagents and metallic silver covered GNPs, thus augmenting particle dimensions and turning red lines (the color of GNPs) into sharply contrasting black lines. The gain in term of sensitivity achieved by the silver enhancement technology was impressive (100-fold compared to the gold probe assay), only paid by a 10 minute increase of the assay time. Sensitivity amelioration obtained through

silver enhancement of gold probe-based LFIAs has also been attested by the work of Linares et al. [5], who compared detection limits of most used nanoparticle markers in a streptavidin-biotin model assay. Most convenient signal reporters for LFIAs proved to be black carbon nanoparticles, followed by silver enhancement of GNP, GNPs themselves and latex beads. According to this comparative study, sensitivity could be incremented by a factor of ten by means of the silver enhancement compared to the use of GNPs.

The remarkable improvement of detectability achieved by the silver enhancement technique has been attributed both to the enlargement of nanoparticles that augment visibility of each individual nanoparticle and to the increased contrast of black Ag-GNPs compared to red GNPs on the white background of the migration membrane. This is intuitively true when applied to sandwich-type LFIAs for which no analyte means no visible (detectable) color in the Test line and the limit of detection is determined by the appearance of the lowest color, no matter how pale provided perceptible is (Figure 1a).

In this work, the silver enhancement strategy has been first applied to a LFIA aimed at measuring a hapten. Specifically, silver enhancement was exploited to ameliorate a fully optimized gold-based LFIA for measuring ochratoxin A (OTA) in wine and grape must [6].

OTA is a major mycotoxin, produced by several species of fungi [7], which can affect a variety of crops, including: cereals, coffee, the, grapes. Due to OTA persistence and resistance to heat and acidity, it has been found in stored and processed food, where the producing fungi had been disappeared, as well. Its chronic toxic effects have been widely demonstrated [8-9]; therefore maximum admissible levels in several commodities have been set all over the world [10-13]. Among all commodities involved in OTA contamination, wine is the second major source of OTA dietary intake by the EU population, following cereals (EU SCOOP project, Scientific Cooperation Task 3.2.7 [14]); therefore a maximum admissible level as low as 2 μg/l has been established by European Union for OTA in wine and grape must [11]. The same value has been also set by Canada [15], although a specific legislation on the subject is missing in most extra-European Countries.

Methods of analysis for OTA based on liquid chromatography combined to fluorescence or mass spectrometric detection have been described and represent the methods of election for confirmatory purposes [16-19]. However, immunochemical analyses are largely employed as screening methods, thanks to their simplicity, rapidity and cost effectiveness [16, 20-22]. LFIAs aimed at measuring OTA in food, beverages and feed have been reported, as well [23-27].

In a preceding work, our group demonstrated the feasibility of using a gold-based LFIA to semi-quantify OTA contamination in wines and grape musts [6]. The assay format was based on the competition between the analyte and a conjugate of OTA immobilized onto the migration membrane (at the Test line) for the binding of antibodies directed toward OTA and labeled with GNPs. The color intensity of the Test line inversely correlated with OTA concentration and a limit of detection of 1 μg/l was achieved when color intensity was instrumentally determined by scanning images of the strips. Despite a poor reproducibility,

the system was sensitive enough to allow the correct attributing of samples as positive or negative according to the European legislation in force. Nevertheless, a reliable OTA quantification could not be achieved. Furthermore, the gold-based assay exhibited a major limitation in the fact that matrix-matched calibration was needed (indeed, a fortified white wine was used to calibrate the assay).

Therefore, the silver enhancement technique was explored as a mean to improve sensitivity and precision of the gold-based LFIA with the final aim of attain the affordable quantitation of ochratoxin A in wines and grape musts at levels of legal concerns.

Materials and methods

Materials

Gold (III) chloride trihydrate (ACS reagent), ochratoxin A (Oekanal standard solution), bovine serum albumin (BSA), ovalbumin (OVA), and polyethylene glycol (PEG, average mw 10 kDa) were purchase from Sigma-Aldrich (St. Louis, MO, USA). Tween 20, absolute ethanol, silver nitrate, hydroquinone and all other chemicals were obtained from VWR International (Milano, Italy).

The anti-OTA antibody (the immunoglobulin fraction of a polyclonal rabbit antiserum) and OTA-BSA conjugate were kindly provided by Euroclone Spa (Milano, Italy). The goat anti-rabbit antibody was purchase from AbCam (Cambridge, MA, USA). 20 mM Borate Buffer pH 8 (BB) was prepared from sodium borate and boric acid. OTA standard solutions were obtained by properly diluting OTA stock solution in 12% aqueous ethanol.

Sample and adsorbent pads were cellulose fiber, release pads were glass fiber, membranes were nitrocellulose (Hi-Flow Plus 180 membrane cards, 60x300 mm); all these materials were purchased from Millipore (Billerica, MA, USA). Release pads and the membranes had spots traced upon them by means of an XYZ3050 platform (BioDot, Irvine, CA, USA), equipped with three BioJet Quanti™ 3000 Line Dispenser for non-contact dispensing. Membranes were cut into strips by a CM4000 guillotine (BioDot, Irvine, CA, USA). Plastic cassettes were from Dima Diagnostics (Goettingen, Germany).

Stock solutions of silver nitrate (0.3% w/v in water) and hydroquinone (3% w/v in 0.5 M citrate buffer pH 4.0) were stored at room temperature in the dark for no more than a month and the enhancing solution was freshly prepared at the time of use by 1:1 mixing stock solutions.

Preparation of the lateral flow device

Strips were prepared as previously reported [6], except for the concentration of the OTA-BSA conjugate applied to build the T-line.

Gold nanoparticles with an adsorption maximum of 522 nm and OD 0.9 were prepared using the sodium citrate method [28].

A goat polyclonal antibody towards rabbit immunoglobulins (Ab_{rab}) was used to functionalize GNPs (Ab_{rab} : GNPs : BB = 4 µg : 1 ml : 0.1 ml). After 30' incubation at room temperature, uncovered GNP surface was blocked by adding 0.1% BSA and incubating for 10 min at 37°C. The resulting conjugate (GNP- Ab_{rab}) was collected by centrifugation, re-suspended in BB supplemented with 0.1% ovalbumin (BB-OVA) and used to label the anti-OTA antibodies as follows. 1 ml of GNP- Ab_{rab} (OD =1) was reacted with 10 µl of the specific antibody (0.1 mg/ml) for 1h at room temperature to yield the anti-OTA antibodies labeled with GNPs as the final result (GNP-Ab_{rab}-Ab_{OTA}). GNPs conjugated with both antibodies were recovered by centrifugation, washed twice with the BB-OVA and finally diluted in the same buffer to which 0.25% Tween 20, 2% sucrose, and 0.02% sodium azide were added. Release pads were previously treated with the same supplemented buffer, then GNP-Ab_{rab}-Ab_{OTA} (OD = 4) were dispensed at 5 µl/cm on treated release pads and these were dried at room temperature for 2 hours.

Lateral Flow Immunoassay

The test was carried out by adding 100 µl of OTA standards (treated as wine samples), diluted wine or must into the sample well. After 10 minutes of incubation at 37°C, 50 µl of the enhancing solution was added directly into the reading window of the cassette (Figure 2) and developed further for 10 minutes at 37°C in the dark. Line intensities were recorded by scanning images (OpticSlim 550 scanner, Plustek Technology GmbH, Norderstedt, Germany). The Scannex 3.0 software (Skannex AS, Hoenefoss, Norway) was used to acquire and process images, as reported [6].

Calibration curves were carried out by plotting the ratio between the intensity of the Test and the Control lines (T/C) for OTA standards (0-1-2-4-8-20 µg/l) versus the log of OTA concentration [29]**.** The calibration curve was determined by a nonlinear regression analysis of the data using the four-parameter logistic equation.

LFIA optimization

Inhibition curves carried out by properly diluting OTA in 12% aqueous ethanol and analyzing by various batches of LF strips were compared on the basis of the obtained IC_{50}, as an indicator of both assay sensitivity and dynamic range. Data were normalized by dividing the signal measured at a certain OTA concentration (B) by the signal of the blank (B_0). Investigated batches of strips varied because of: the Ab_{OTA}: GNP ratio (batch 1), the amount of OTA-BSA dispensed to form the Test line (batch 2), and the hapten-to-protein ratio (conjugation rate, cr) of the OTA-BSA used to form the Test line (batch 3). To assess performances of batch 1, the OTA-BSA with cr 11.7 was dispensed at 0.1 mg/ml to form the Test line. To investigate batch 2, the OTA-BSA with cr 11.7 was used, together with Ab_{OTA}-GNPs in which the Ab_{OTA} was kept at 1 µg per ml of GNPs. To evaluate batch 3, OTA-BSA were dispensed at different amounts (0.2, 0.1,

and 0.05 µg/l for cr 5.6, 8.6, and 11.7, respectively) to achieve a similar signal of the zero standard and Ab_{OTA}-GNPs was prepared by using 1 µg Ab_{OTA} per ml of GNPs.

Silver enhancement optimization

Two standard solutions (0-3 µg/l OTA) were run in triplicate in the LFIA strips and various enhancement solutions ($AgNO_3$/hydroquinone 0.15%/1.5%, 0.3%/3%, 0.6%/6%, and $AgCH_3COO$/hydroquinone 0.3%/3%) were applied and incubated for 5-10-15 minutes. T/C values were recorded and the optimal silver enhancement solution was established to be the one assuring the larger difference in signals for the two standard solutions of OTA.

Samples and sample treatments

Wines and grape must samples were treated as reported [6] that is diluted 1:2:2 with $NaHCO_3$ (0.15M, pH 9.0) and PEG (4% w/v). Grapes musts were also supplemented with 12% (v/v) of absolute ethanol.

Results and discussion

Optimization of the Silver enhanced Lateral flow immunoassay for OTA

High sensitivity in indirect lateral flow immunoassays for haptens is promoted from having the opportunity of tuning some key-factors, such as the amount of specific antibodies and of immobilized antigens in the Test line [30-34]. Specifically, the lower the amount of one or both of those components, the higher the sensitivity reached. Nevertheless, bringing down the amount of labeled antibodies and/or the amount of the capturing reagent would imply missing some signals. Therefore, a compromise should be pursued between decreasing the amount of immunoreagents and obtaining a tolerably detectable signal. This is especially valid for gold-based LFIA, in which the signal should be evaluated by the naked eye or through some kinds of color reader. The exploitation of the silver enhancement technique would allow immunoreagent optimization to be conducted in a wider range of concentrations (especially in the direction of the lower levels), to pursue high sensitivity (Figure 1b).

The effectiveness of this approach has been investigated in the re-optimization of a former gold-based LFIA, by evaluating the sensitivity gain which could be reached through reducing: (i) the amount of the specific antibody (Ab_{OTA}), (ii) the amount of the OTA conjugate used to form the Test line (OTA-BSA), and (iii) the hapten density of the OTA-BSA (i.e.: the molar conjugation rate, cr, between OTA and BSA of the OTA-BSA conjugate). All those factors were demonstrated to play some role on determining the sensitivity of the assay in our previous observations [30] and according to several other authors [31-34].

All investigated strategies influenced assay sensitivity and comparably when lowering the specific antibody or the competitor (OTA) in the Test line (Figure 3a, 3B and 3c).

Antibody reduction could be evaluated in a wide interval (0.2 - 5.0 µg Ab_{OTA} per ml of GNPs) by using a double coating strategy: GNPs were first covered with secondary anti-species antibodies (Ab_{rab}) to prevent aggregation by following conventional protocols for GNP-antibodies conjugation. Then, variable amounts of the specific anti-OTA antibodies were added to obtain Ab_{OTA}-GNP preparations characterized by varying Ab_{OTA}-to-GNP rates. It has been noted that the amount of secondary antibodies used to coat nanoparticles should be maintained below the amount needed to saturate GNPs to prevent precipitation of nanoparticles during the following reaction with the Ab_{OTA}. The amount of secondary antibodies needed to saturate GNP surface was experimentally determined through incubating a fixed volume of the GNP preparation with increasing quantity of Ab_{rab} and observing aggregation when 10% NaCl was added (the aggregation determined a color shift of GNPs from brilliant red to purple-blue). The saturating condition was judged as the lower antibody amount that prevented aggregation [35]. The amount of Ab_{rab} able to completely cover GNP surface was also theoretically calculated as the ratio between the total surface area of GNPs and the size of an antibody molecule [30]. The experimental and the calculated values were in agreement (exp = 4.5 µg antibody per ml of GNP preparation, calc = 6.0 µg antibody per ml of GNP preparation). Nevertheless, as above mentioned, the quantity of Ab_{rab} used to coat GNPs should be kept lower (3-4 µg antibody per ml of GNP preparation) to avoid GNP aggregation and precipitation during the following binding with anti-OTA antibodies. Likely, in the excess of secondary antibodies, Ab_{rab}-GNPs could form sandwiches between two functionalized particles and a molecule of Ab_{OTA} in solution, thus determining aggregation among several nanoparticles [36]. Provided that, the use of a double layer of antibodies allowed us to easily investigate very differing Ab_{OTA}:GNP preparations in which the only variable was effectively the amount of the specific antibody. In the investigated range, the lower the Ab_{OTA}:GNP, the highest the sensitivity (Fig. 3a).

As far as the conjugate used to form the Test line, according to previous observations [31-32], both the amount of OTA-BSA sprayed onto the membrane and the hapten density of the OTA-BSA itself were varied. Unlike expected, the hapten density that was accounted for the strongest influence on assay sensitivity for a gold-based LFIA system previously optimized by our group [30], played a minor role compared to the effects due to lowering the anti-OTA antibody or the sprayed conjugate (Fig 3 and Table 1). However, the lower the OTA quantity in the Test line (because of a lower OTA-BSA amount was sprayed or because less OTA was present in the OTA-BSA conjugate) the highest the sensitivity.

The immunoreagent combination that determined the lowest detection limits was: Ab_{OTA}-GNPs prepared by adding 0.2 µg Ab_{OTA} to 1 ml of GNPs to be used as the signal reporter and BSA-OTA with cr 11.6 sprayed at 0.1mg/ml to form the Test line. LF strips prepared as above combined to the use of the silver enhancement technique yielded to a 10-folds gain in terms of sensitivity compared to the previous optimized gold-based LFIA. A typical calibration curve obtained from the “ultra-sensitive” silver-enhanced LFIA and the optimal curve obtained from the gold-based LFIA are depicted in Figure 4 (open squares and open circles,

respectively). The IC_{50} for the silver enhanced system was calculated to be 0.8 $\pm$ 0.1 µg/l whereas for the gold-based system, which used the same immunoreagents, was 11 $\pm$ 4 µg/l.

Nevertheless, as for the case of the gold based-LFIA, going into the direction of pursuing the lowest detection limit determined imprecise and poorly accurate determinations. Therefore, taking into account that the sensitivity attained by the silver-enhanced LFIA exceeded detectability request imposed by European legislation, a second batches of LF strips was prepared employing an immunoreagent combination which assured the highest slope of the curve in the region encompassing the maximum admissible level for OTA (2 µg/l). These conditions (reported in detail in the experimental section) modified the shape of the curve in the direction of narrowing and shifting the dynamic range however increasing the slope (Figure 4, solid circles). As a consequence, a strong improvement of both precision and accuracy was reached compared to the standard curve obtained from the gold-based LFIA in the same OTA concentration range. The more accurate and precise system was named "quantitative" silver enhanced-LFIA and showed an IC_{50} = 2.8 $\pm$ 0.2 µg/l and a LOD of 0.9 µg/l.

The silver enhanced-LFIA demonstrated to be less prone to matrix interference compared to the gold-based assay and allowed us to exploit standard solutions of ochratoxin A diluted in 12% ethanol as calibrators instead of having to recourse to fortification of real samples, as requested by the gold-based LFIA for matching matrix composition. This behavior was attributed partially to the increased signal-to-noise ratio and to the lengthening of the overall reaction time, which permitted whatever samples to complete the run and to fully develop line color.

Despite its many advantages, the silver enhanced-LFIA took significantly much time to be completed than the gold-based assay (20 minutes rather than 5 minutes); therefore the enhancement step was studied to possibly reduce the time of analysis. Several combinations of amounts of reagents, dispensed volume, and time of reaction were investigated. The one which assured the best yield were confirmed to be: dispensing 50 µl of a 1+1 mixture of 0.3% aqueous $AgNO_3$ and 3% hydroquinone dissolved in citrate buffer and let to react 10 minutes (Figure 5).

OTA determination in wines and grape musts by the silver enhanced-LFIA

The developed "quantitative" silver enhanced-LFIA was applied to determine OTA contents in 35 real samples: 21 red wines, 8 white wines, and 9 grape musts. According to the reference HPLC-FLD method [6] 26 samples were contaminated at a level below the limit of detection of the newly developed LFIA and were thus analyzed to establish the LOD in matrix, which was interpolated on the calibration curve as the OTA concentration corresponding to the mean signal of those samples minus three standard deviations, and turned out to be 1.2 µg/l.

The accuracy of the developed assay was evaluated by testing 6 samples naturally contaminated at detectable levels and further 18 samples fortified at 4 µg/l, and was comprised between 82 and 117%

(Table 2). RSD% was in the range 6-12%. These figure of merits allowed the "quantitative" silver enhanced-LFIA affordably quantifying OTA in wines and grape musts, with performances comparable to other screening methods, such as for example ELISAs. Unalike, the former gold-based LFIA could only achieve a semi-quantitative evaluation of OTA contamination levels [6] due to its limited precision, and also entailed a certain degree of uncorrected attributions of samples as compliant or non-complaint to the European legislation in force. To further confirm advantages of the new approach, also the qualitative evaluation on compliancy to the legislative requirements was carried out by re-evaluating results obtained from the "quantitative" silver-enhanced LFIA as follows. The cut-off level was established at the legal limit (2 μg/l); therefore samples whit OTA content less than 2 μg/l were attributed as negatives, while samples contaminated at or above 2 μg/l were judged as positives. The evaluation was extended to 34 negative samples and 19 positive samples (naturally contaminated above the limit or contaminated through fortification). According to those definitions, 32/34 samples were classified as negatives and 21/19 as positives, that is two negative samples were incorrectly attributed as positives. It should be noted, however, that the two false positives were two wines naturally contaminated at levels close to the cut-off, as measured by the reference method (1.9 and 1.6 μg/l, respectively).

Therefore, sensitivity, defined as the rate of truly positive and selectivity, defined as the rate of truly negative results were calculated as 100% and 94.1%, respectively. The qualitative gold-based LFIA had proven to be slightly more selective (100%) and noticeably less sensitive (77.8%) [6].

Conclusions

In this work, the technique of enhancing detectability of gold nanoparticles by silver deposition has been first applied to a lateral flow immunoassay in the competitive format. Although the strategy had been widely employed to improve detectability of several gold-based bio-sensors, the application to lateral flow immunoassays had not been investigated, except from the recent work of Yang et al [4], in which a huge sensitivity increment due to the use of the silver enhancement strategy was demonstrated in a sandwich lateral flow immunoassay. In this assay format, the limit of detection is established by the appearance of any colors at least distinguishable from the background (by the naked eye or by some kinds of instrumental color readers) in the zone of the Test line. Deposition of silver on gold nanoparticles after usual strip development and gold nanoparticle focalization in the reactive lines allowed Authors to achieve improved detectability both because of the increment of the dimension of each single nanoparticle (the bigger the particle, the more visible) and because silver turned particle color from red to black, which showed up on the white background of the strip. In competitive lateral flow immunoassays the limit of detection is determined by a clearly perceptible (or detectable with a certain degree of precision) decrease of color intensity of the Test line compared to the absence of the analyte. Therefore, a sensitivity improvement could only be achieved by exploiting the increment of nanoparticle detectability due to silver depostion to

reduce immunoreagent amounts and, thus, to favor the competition (Figure 1b). Effective advantages in terms of gain on sensitivity were demonstrated on a competitive LFIA for measuring ochratoxin A in wine and grape must, previously developed on a gold-based model. The IC_{50} for the "ultrasensitive" silver enhanced-LFIA was more than 10-folds lower compared to the one of the gold-based LFIA, and, based on our knowledge, the developed method is the most sensitive LFIA for measuring ochratoxin A [23-27]. This assay exceeded requirements for measuring OTA in beverages according to the legislation in force. Therefore, the use of the enhancing strategy allowed us to opt for a different combination of immunoreagents, which assured accurate and precise quantification in the interval of OTA concentrations of legal relevance. The optimized quantitative assay was applied to determine OTA content in 30 samples of wine and grape musts giving quantitative results that agree well with those obtained through a HPLC-FLD reference method (*$y=1.00x+0.02$, $r^2=0.960$*, Figure 6).

Acknowledgments

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Tables

Table 1. Effect of varying the Ab_{OTA} amount used to coat GNPs and the competitor to be used in the Test line of the LF device.

Ab_{OTA}		Amount of OTA-BSA in the Test line		Hapten density of the OTA-BSA	
µg /ml GNPs	$IC_{50} \pm SD$	mg/ml	$IC_{50} \pm SD$	cr[a]	$IC_{50} \pm SD$
0.2	0.8 ± 0.09	0.025	2.2 ± 0.3	5.6	1.9 ± 0.2
0.4	1.7 ± 0.4	0.1	5 ± 1	8.6	2.8 ± 0.1
1.0	2.5 ± 0.9	0.2	8 ± 2	11.7	4.3 ± 0.3
2.0	3.6 ± 0.8				
5.0	8 ± 3				

[a] conjugation rate: moles of hapten/moles of protein

Table 2. Recovery of OTA determination from naturally and artificially contaminated wines and grape musts samples as determined by the developed "quantitative" silver enhanced-LFIA.

Sample	OTA content[a] (µg/l)	OTA estimated by "quantitative" silver-enhanced LFIA (µg/l)	Recovery[b] (%)
White wines	1.9	2.2	115
	4.0	3.3	82
	4.6	4.6	99
	4.8	5.2	108
	5.9	6.2	105
Red wines	1.2	1.3	108
	1.6	1.9	119
	2.5	2.8	112
	4.0	3.6	96
	4.1	3.8	92
	4.2	3.9	93
	4.2	4.2	100
	4.2	3.9	92

	4.4	3.7	84
	4.4	3.9	89
	4.5	5.3	117
	4.8	5.3	111
	4.9	4.3	88
Grape musts	1.2	1.0	83
	1.3	1.5	115
	4.2	3.7	88
	5.1	5.6	110
	5.2	5.8	112
	5.3	5.9	111

[a] established as the sum of the OTA measured by the reference HPLC-FLD method [5] and the fortification level

[b] calculated as OTA estimated by the silver-enhanced LFIA / OTA content *100

Figure captions

Figure 1. Expected effects on the LFIA sensitivity due to the silver enhancement technique for: (a) sandwich-type assays, and (b) competitive assays. For sandwich LFIAs the increase of detectability of each single nanoparticle allows a visible (detectable) signal to appear for lower analyte amounts. For competitive assays, the gain in nanoparticle detectability could allow to reduce immunoreagents and, thus to increment the sensitivity.

Figure 2. Schematic draw of the silver enhanced lateral flow immunoassay

Figure 3. Normalized inhibition curves obtained from the silver enhanced-LFIA with variable amounts of: (a) the anti-OTA antibody (▲0.2, ● 0.4, ■ 1.0, ▼2.0, and ◆ 5.0 µg/ml GNPs); (b) the OTA-BSA conjugate used to form the Test line (▲0.025, ● 0.1, ■ 0.2 mg/ml); (c) the OTA in the OTA-BSA conjugate used to form the Test line (▲5.6, ● 8.6, ■ 11.7)

Figure 4. Calibration curves for OTA obtained from: the gold-based LFIA (○) the "ultrasensitive" silver enhanced- LFIA (□), the "quantitative" silver enhanced- LFIA (●). Bars represent standard deviations of four replicates.

Figure 5. Measured signals (T/C) for two OTA standard solutions at 0 (●) and 3 µg/l (▲) as a function of: (a) the time of incubation of the enhancing solution (0.3% $AgNO_3$/3% hydroquinone) and (b) the amount of reagents (the rate between $AgNO_3$ and hydroquinone was maintained at 1:10)

Figure 6. Correlation of results obtained by both "quantitative" silver enhanced-LFIA and reference HPLC-FLD method for ochratoxin A detection on wines and grape musts. The linear regression analysis yielded a good correlation between methods ($y = 1.00\ x - 0.02$, $r^2 = 0.960$, $n=30$)

Figures

Figure 1

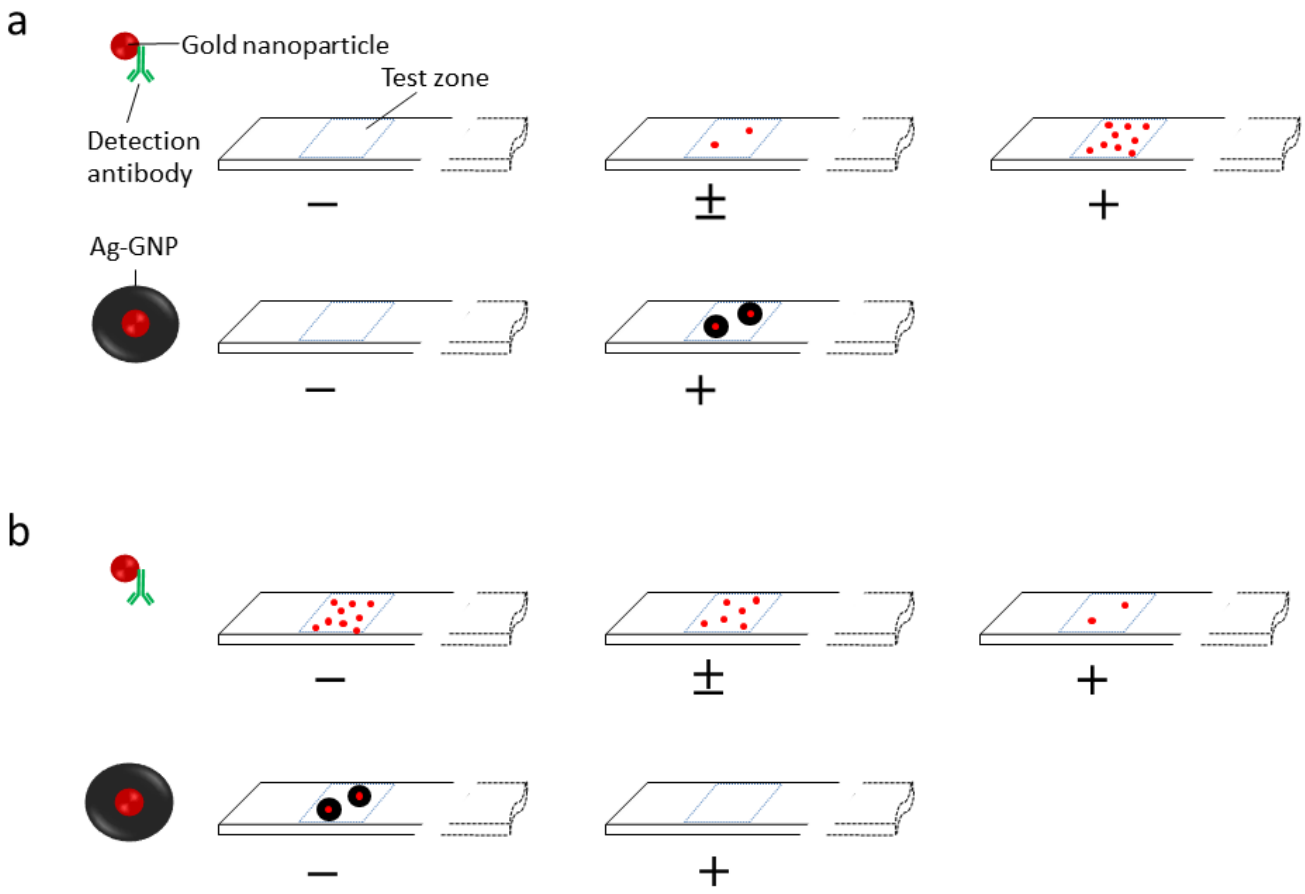


Figure 2

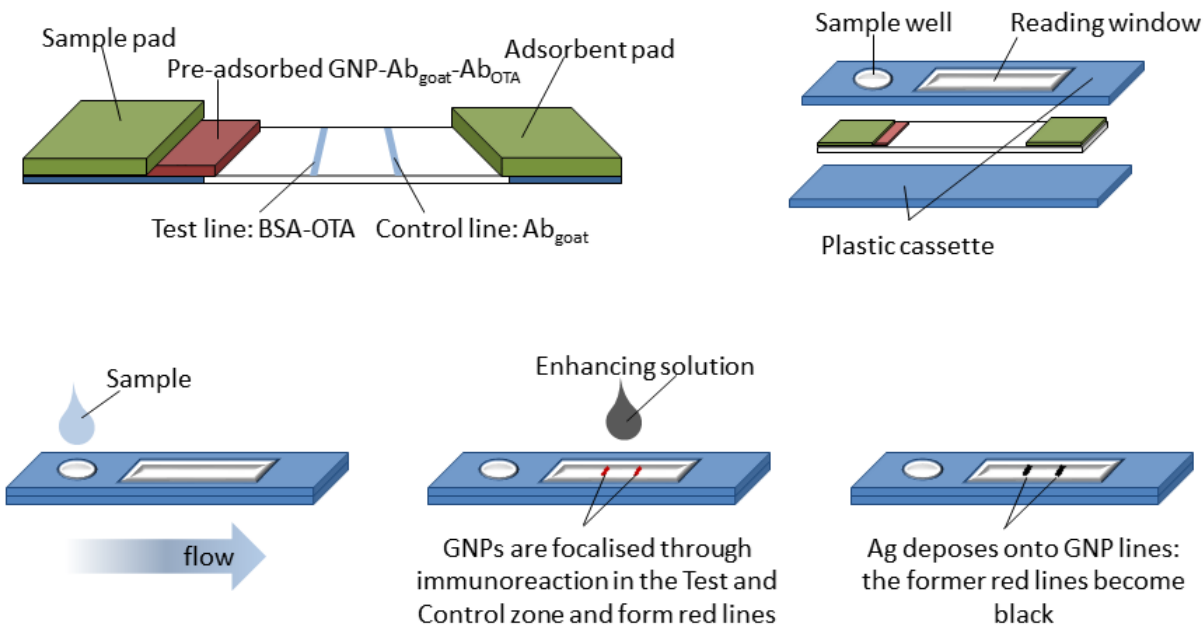
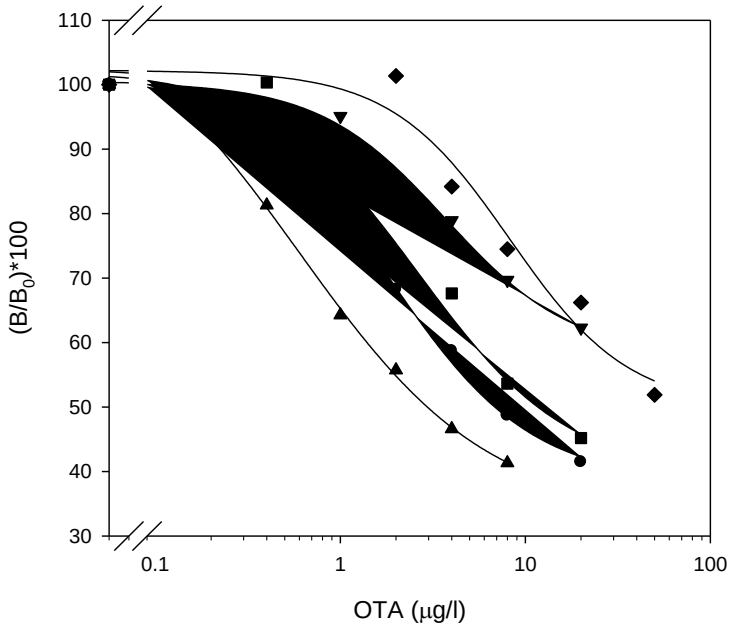
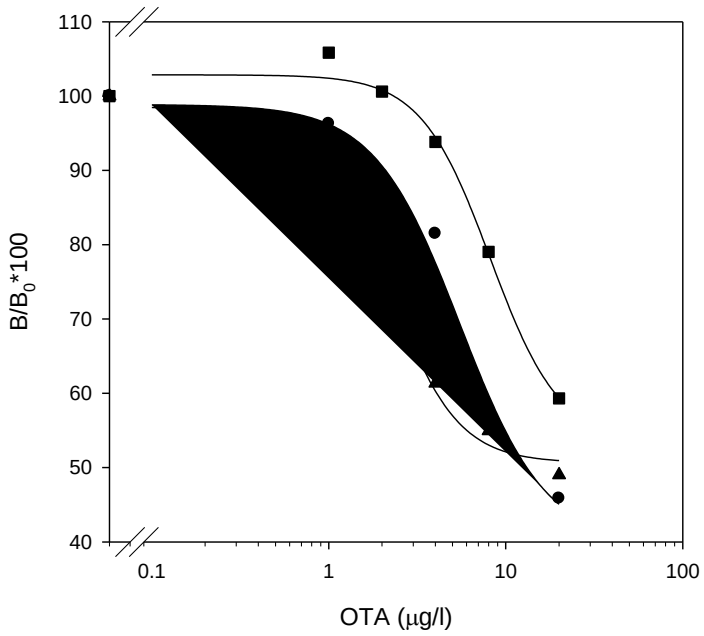

Sample pad
Pre-adsorbed GNP-Ab$_{goat}$-Ab$_{OTA}$
Adsorbent pad
Test line: BSA-OTA
Control line: Ab$_{goat}$
Sample well
Reading window
Plastic cassette
Sample
flow
Enhancing solution
GNPs are focalised through immunoreaction in the Test and Control zone and form red lines
Ag deposes onto GNP lines: the former red lines become black

Figure 3

a


110
100
90
80
70
60
50
40
30
(B/B0)*100
0.1
1
10
100
OTA (µg/l)

b


110
100
90
80
70
60
50
40
B/B0 *100
0.1
1
10
100
OTA (µg/l)

c

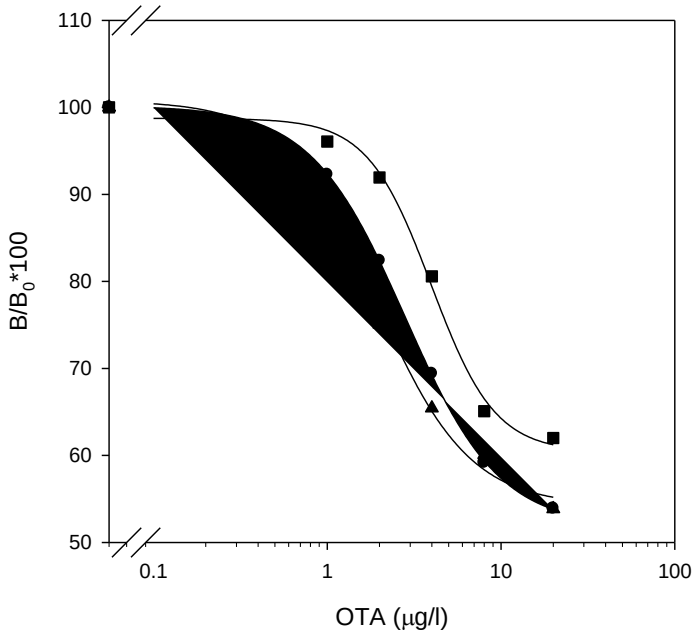


Figure 4

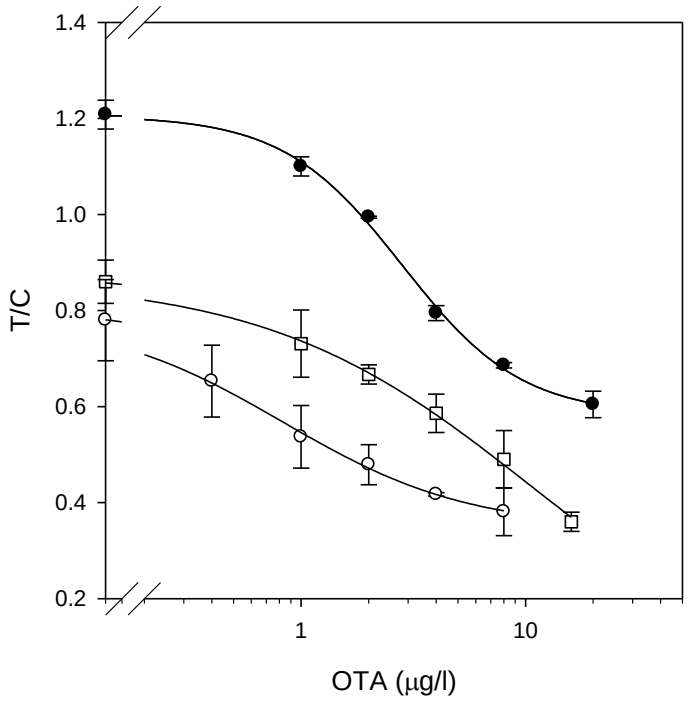
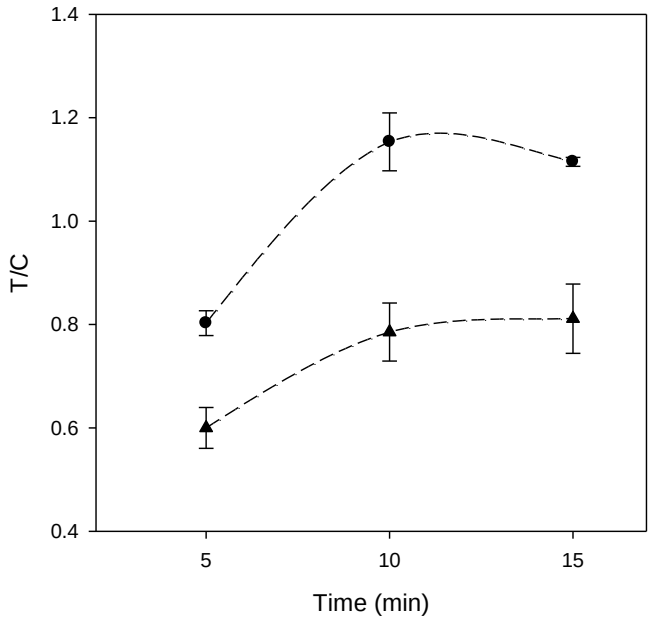


Figure 5

a


1.4
1.2
1.0
0.8
0.6
0.4
T/C
5
10
15
Time (min)

b

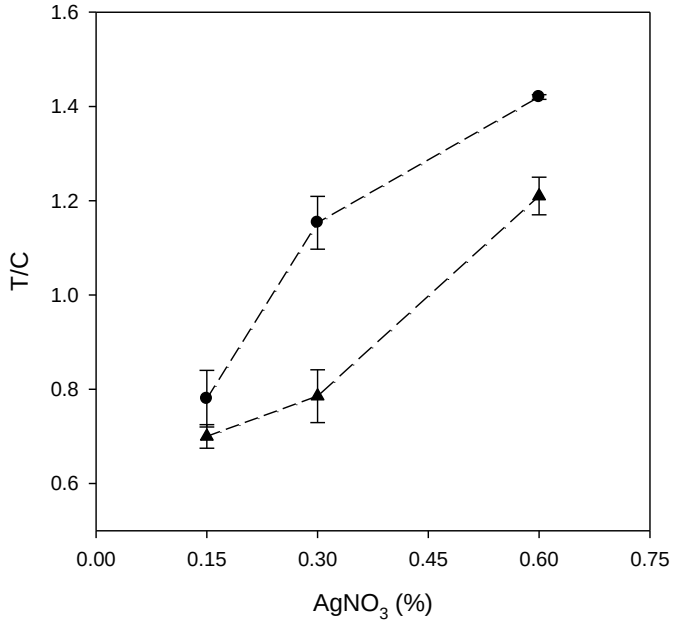
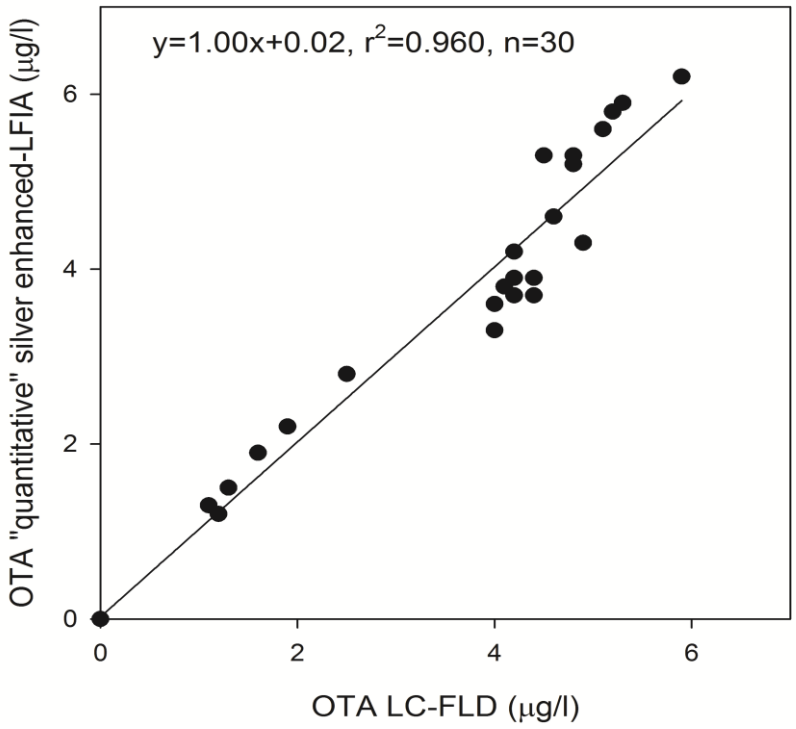

1.6
1.4
1.2
1.0
0.8
0.6
T/C
0.00
0.15
0.30
0.45
0.60
0.75
AgNO3 (%)

Figure 6


y=1.00x+0.02, r2=0.960, n=30
OTA "quantitative" silver enhanced-LFIA (µg/l)
OTA LC-FLD (µg/l)
0
2
4
6