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A LATERAL FLOW IMMUNOASSAY FOR THE RAPID DETECTION OF OCHRATOXIN A IN WINE AND GRAPE MUST

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Abstract

A one-step lateral flow immunoassay was developed for semi-quantitatively detecting ochratoxin A (OTA) in wines and grape musts. Matrix-matched calibration curves carried out in blank wines showed a detection limit of $1 \mu\text{g l}^{-1}$ and IC_{50} of $3.2 \mu\text{g l}^{-1}$. Relative standard deviations for intra- and inter-day precision were in the 20 - 40% range. A simple treatment of samples, which only included dilution with sodium bicarbonate and polyethylene glycol (4% w/v) for red and white wines and the further addition of ethanol (12% v/v) for grape musts, was established. The developed assay allowed OTA detection in 5 minutes and proved to be accurate and sensitive enough to allow the correct attribution of samples as compliant or non-compliant according to EU legislation. Agreeing results to those of a reference chromatographic method were obtained on 38 wines and 16 musts. Although some lateral flow devices aimed at detecting OTA have been previously described, this is the first assay capable to measure the toxin in wine and grape must, which represent a major source of OTA dietary intake. Analytical performances of the method are comparable or better than previously reported assays showed. In addition, the assay, including sample treatments, is extremely simple and rapid, and can be effectively regarded as a one-step assay virtually usable anywhere.

Keywords Immunochromatographic assay, gold-colloid, natural toxins, matrix interference

Introduction

Ochratoxin A (OTA) belongs to the group of toxic compounds produced as secondary metabolites by several *Aspergillus* and *Penicillium* fungal species. Ochratoxin-producing fungi can contaminate several crops, thus toxic metabolites may be present in many raw agricultural materials, commodities and beverages, including cereals, beans, nuts, spices, dried fruits, coffee, cocoa, beer, and wine. Indeed, the ubiquitous presence of OTA has been confirmed in several surveys of raw and processed agricultural and derived products [1-3]. OTA is teratogen, mutagen, hepatotoxic, nephrotoxic and immunosuppressive to different animal species, and the International Agency for Research on Cancer has classified it as a possible carcinogen to humans (group 2B) [4-5]. Therefore, regulations about maximum admissible levels of OTA in food and feed have been set worldwide [6-9]. 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 [10]); therefore poses a serious risk for human health. Hence, a maximum admissible level as low as 2 $\mu\text{g kg}^{-1}$ has been established by European Union for OTA in wine and grape must [6]. The same value has also been set by Canada [11], though specific legislation on this subject is missing in most extra-European countries.

Continuous monitoring of wine for the presence of OTA would be advisable to reduce risks to human health. Available analytical methods includes the mouse bioassay, thin-layer chromatography, liquid chromatography combined to fluorescence or mass spectrometric detection and various immunochemical methods, generally after extraction of the target compound [12-14]. Rapid and on-site screening methods have also been developed for the detection of such a contaminant in food and beverages, including wine [15-18]. Among rapid methods aimed at the first-level screening of food contaminants, the immunochromatographic or

lateral flow immunoassay (LFIA) technology has increased scientific and industrial interest in the last few years and its exploitation has rapidly spread, particularly for mycotoxin detection [19-22]. For ochratoxin A, the first published LFIA dated on 2005 and described the approach for developing a visual device [23]. Following works demonstrated the applicability of the LF technology to OTA detection in various cereals [24-27] and in coffee [25,28]. Wang et al firstly applied their visual LFIA to also detect OTA in beverages (grape juices and beer) declaring high sensitivity (Visual Detection Limit, VDL, $1 \mu\text{g l}^{-1}$) and rapidness (the assay could be completed in 10 minutes and no sample treatment was needed except from dilution with PBS), nevertheless results on grape juice did not correlate at all with the reference ELISA and with the amount of OTA added to fortify samples. In fact, grape juices contaminated at 2.5, 5 and $10 \mu\text{g kg}^{-1}$ were all measured as negative (OTA content lower than the VDL, $1 \mu\text{g l}^{-1}$) which meant the assay completely failed in detecting OTA in such a beverage [25]. Undoubtedly, very colored matrices such as grape juice, red wine and grape must pose serious concerns in the reliable application of LFIA, because the method is exactly based on the observation of intensity of colored lines. In a typical LFIA for OTA detection, specific antibodies are labeled with colored particles and flow across a membrane onto which partner reagents (a protein conjugate of OTA and anti-species IgG antibodies) have been coated in spatially confined zones, indicated as “lines”. In the absence of the target toxin, labeled antibodies bind to the first line (consisting of the OTA-protein conjugate and named Test line, T) and a visible color appears as the effect of focusing of colored particles used to label antibodies. Any excess of labeled antibodies passes over and reaches a second line (so-called Control line, C) where is captured by the anti-species IgG antibodies and is focused to form a second colored line. If some OTA is present in the sample at a level above the detection limit, it occupies the sites of labeled antibodies and inhibits their binding to the Test line. As a result, only one line (C) appears on the membrane. In short, the result of the test is interpreted as positive (contaminated sample)

when observing the appearance of the sole Control line, negative (non contaminated sample) when two colored lines become visible, and invalid if there is no C line, independently from the presence of any T line. Quantification is also possible by photometrically measuring color intensity of T line and correlating it to the concentration of the target [24,27,29]. Intensely colored matrices always determine high background and poor detectability in LFIA; moreover, red-purple matrices (such as grape juice and red wines) exasperate matrix interference because the most popular probes used in lateral flow devices are gold nanoparticles (GNPs), which give rise to exactly red-purple colored lines. Therefore, this work was aimed at identifying a feasible sample treatment and at adapting a LFIA previously developed for OTA detection in cereals [24] for measuring OTA in wine and grape must. The method should permit the reliable quantification of the toxin at a level of regulatory concern and, in the meantime, should preserve characteristics of rapidity, simplicity, cost-effectiveness and on-site applicability of the lateral flow technology to be employed as a first-level screening analytical tool.

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, acetonitrile (HPLC grade), methanol (HPLC grade) and all other chemicals were obtained from VWR International (Milano, Italy). OchraTest immunoaffinity columns were supplied by Vicam (Watertown, MA, USA). 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).

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).

The HPLC apparatus was a Hitachi LaChrom Elite (VWR International, Milano, Italy) consisting of a L-2130 quaternary pump, a L-2480 fluorescence detector, and a L-2200 Autosampler. Data acquisition and peak integration were carried out by EZChrom Elite 3.1.7 software (Scientific Software, Pleasanton, CA, USA). The analytical column was an Onyx Monolithic reversed-phase C18 (100 × 4.6 mm) from Phenomenex (Bologna, Italy).

Preparation of the strips

Strips were prepared as previously reported [24], with minor modifications. Briefly, a BSA conjugate of OTA (0.2 mg/ml) and a goat anti-rabbit IgG (0.5 mg/ml) both diluted in PBS were applied to the nitrocellulose membrane at a flow rate of 1 µl/cm to form the Test and Control lines, respectively. The membranes were dried at 37°C under vacuum for 60 min and used without further treatments.

Gold colloids with an adsorption maximum of 525 nm were prepared using the sodium citrate method [29]. An excess of the polyclonal antibody towards ochratoxin A was used for the conjugation to a colloidal gold solution, which pH had been adjusted to 8.5. After 30' incubation at room temperature, free GNP surface was blocked by means of incubating BSA (1% w/v in borate buffer) for 10 min at 37°C. The GNP-labeled antibodies (GNP-Ab) were collected by centrifugation, washed twice with borate buffer, and re-suspended in borate buffer supplemented with 1%

ovalbumin, 0.25% Tween 20, 2% sucrose, and 0.02% sodium azide and stored at 4°C until use. Release pads were previously treated with the same supplemented buffer, then GNP-Ab (optical density 4) were dispensed at 5 µl/cm on treated release pads and these were dried at room temperature for 2 hours.

Strips were composed as follows: from the top; the adsorbent pad, the nitrocellulose membrane, the release pad and the sample pad were pasted in sequence, with 1-2 mm of overlap. The assembled membrane was cut into strips of 5 mm, which were inserted into rigid plastic cassettes (Dima Diagnostics, Goettingen, Germany), each one with a sample well, a reading window and a barcode for strip identification. Cassettes were stored in plastic bags containing silica at room temperature until use.

Lateral Flow Immunoassay

The test was carried out by adding 100 µl of diluted wine or must into the sample well. After 5 minutes of incubation at 37°C, the cassette was placed above a mobile scanner (OpticSlim 500, Plustek Technology GmbH, Norderstedt, Germany) connected to a laptop. The Scannex 3.0 software (Skannex AS, Hoenefoss, Norway) was used to acquire and process images. The program recognizes a barcode printed on the cassette containing the strip and converts the ratio between line intensities into a concentration value according to a calibration curve, which had been stored in the barcode itself. Matrix-matched calibration curves were obtained by fortifying a blank white wine with the OTA standard solution and by plotting the ratio between the intensity of the Test and the Control line (T/C) for each fortification level against 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.

Samples and sample treatments

31 wine samples (9 white and 22 red wines) and 15 grape musts (7 fermentable and 8 mute musts) were obtained from various wineries of the northern Italy. Positive samples (4 red wines, 3 white wines and 1 mute must) were generated by fortification of some samples with the OTA standard solution.

For LFIA analysis, wines were diluted 1:2:2 with NaHCO_3 (0.15M, pH 9.0) and a water solution of PEG (4% w/v) and pipetted into the sample well. Grapes musts were treated in the same way, except from the fact that 12% (v/v) of absolute ethanol was added before dilution.

For chromatographic analysis, samples were extracted as previously reported [30]: 10 ml of wine or grape must was diluted with 10 ml of NaHCO_3 (5% w/v) with 1% (w/v) PEG added, incubated at 4 °C overnight, centrifuged at 8000 rpm for 15 min and filtered on 0.2 μm cellulose membranes. 10 ml of the filtrate was applied to the immunoaffinity extraction column, previously equilibrated with 3 \times 1 ml of sodium chloride / sodium hydrogen carbonate solution (2.5 % w/v NaCl and 0.5% w/v NaHCO_3). After sample loading, the column was washed with 5 \times 1 ml of sodium chloride / sodium hydrogen carbonate solution and 5 \times 1 ml of water. OTA was eluted with 2 \times 1 ml of methanol–acetic acid (49:1, v/v). The eluate was evaporated under a stream of nitrogen and re-dissolved in 250 μl of mobile phase.

LC-FLD analysis

Isocratic RP-HPLC with fluorescence detection (λ_{ex} 333 nm, λ_{em} 460 nm) was used to measure OTA, according to the literature and as described in detail in a previous paper of our group [30]. Briefly, the mobile phase consisted of acetonitrile–water–acetic acid (55:44:1, v/v/v) and flow rate was set to 1.0 ml min⁻¹. Reference standard solutions of OTA were analyzed in triplicate and peak areas were plotted against OTA concentration for calibration. Data fit was obtained by a weighted linear

regression (weight = 1/conc). The reference method has a limit of quantification of $0.10 \mu\text{g l}^{-1}$ and a mean RSD% of 21%.

Results and discussion

Lateral flow immunoassay for OTA detection

As reported in a previously published paper of the group, a sensitive LFIA was developed which enables OTA quantification in cereal samples [24]. Strips were composed of a cellulose sample pad, a releasing pad onto which the GNP-labeled antibodies directed towards OTA were pre-adsorbed, a nitrocellulose (NC) membrane and an adsorbent pad. The Test line traced upon the NC membrane consisted of a BSA conjugate of OTA, which competed with the toxin present in the sample for binding to labeled antibodies. As expected for a competitive immunoassay, the signal of the Test line decreased for increasing concentrations of the target. A Control line was also deposited onto the NC membrane and its color intensity was exploited to normalize strip-to-strip variation [29]. Strips were included into plastic cassettes, whose role was the compression of zones where pads and membranes overlapped each other so that a reproducible flow was assured.

Despite various attempts made in the original work to level results obtained when different cereals and particularly cereals and calibrators prepared in buffers were analyzed, matrix-matched calibration was needed to achieve the reliable quantification of OTA in real samples. Therefore, the adaptation of the previously developed assay to OTA detection in wine and grape must required to set up a devoted calibration and to re-evaluate the analytical performance of the assay. Optimal concentration of reagents for the Test and Control lines and of GNP-Ab for detection was redefined in checkerboard titrations to achieve the best sensitivity, as well.

For the construction of the standard curve a blank white wine that did not show any detectable residues of the target toxin when analyzed by the reference LC-FLD analysis was fortified with increasing amounts of the standard OTA solution (0, 1, 2, 4, 8, 16 $\mu\text{g l}^{-1}$) and the T/C value for each level of fortification was measured in triplicate (Figure 1). The limit of detection of the assay, calculated as the mean of the blank minus three standard deviation of the mean, and the IC_{50} of the curve were 1.0 $\mu\text{g l}^{-1}$ and 3.2 $\mu\text{g l}^{-1}$, respectively. Relative standard deviation of six replicates in the same day and of five replicates on consecutive days for a blank and two naturally contaminated samples (OTA 1.1 and 2.6 $\mu\text{g l}^{-1}$) were calculated for determining intra- and inter-day precision, respectively. Values were high for all samples and comprised between 20 and 40%.

Sample preparation for LFIA analysis

As above described, one of the major concern in the application of a LFIA to wine analysis is represented by the color background, which is particularly detrimental for red wines. In addition, the acidic pH, the alcoholic content and the high ionic strength of wines determine interferences in the signal intensity, flow rate and gold colloid stability. Therefore, several conditions aimed at controlling the above-mentioned parameters were evaluated and subsequently optimized in experiments carried out according to a checkerboard scheme. Optimized conditions were chosen as the best compromise which assured lower color background on the NC membrane, lower GNP precipitation rate (evaluated by color changes of GNP-Ab preparation when mixed with a red wine diluted in different ways), and best similarity between signals obtained for red and white wines. Factors, values investigated in this study, and conditions chosen as optimal for OTA detection in wine by means of the developed LFIA are summarized in Table 1.

Factors that mostly demonstrated to influence the assay were pH and ethanol content. An adequate pH control could be achieved by increasing: buffer pH, buffer concentration, or sample

dilution (with a buffer). Nevertheless, we observed that a high buffer concentration or a limited sample dilution determined GNP colloid instability; therefore, the minimum sample dilution was firstly established as 1:4 – 1:5, which allowed us to minimize ethanol interference on GNP-Ab stability and on flow through the membrane (Figure 2). Accordingly, the effective neutralization of even the most acidic white wines was achieved by means of a decidedly basic buffer (pH 9), fairly concentrated (0.15 M). As regards buffering salts, their effect was substantially irrelevant; the same was assessed for the addition of Tween 20 aimed at reducing non-specific interaction with the NC membrane. In addition and contrarily to what observed in the development of the device aimed at measuring OTA in cereals, the saturation of the membrane with proteins and/or surfactants was completely ineffective in reducing background and leveling signals obtained for red and white wines. Thus, strips were prepared with untreated NC membranes.

From literature, the precipitation of tannins responsible of most analytical interference of red wines could be attained by diluting the wine in concentrated bicarbonate and by adding PEG [31]. Therefore, bicarbonate was arbitrarily established as the buffering salt and its effectiveness in lowering tannin interference in combination with varying amounts of PEG was evaluated. It should be noted that protocols reported in the literature for wine treatment are definitely not applicable for preparing samples to be analyzed by LFIA, because those protocols include time-consuming operations and the use of laboratory equipment; indeed, a typical protocol prescribes to dilute sample, let it to stand overnight, and centrifuge. The last two steps are incompatible with rapidity and simplicity of a so-defined one-step and in situ applicable analytical method. Nevertheless, the use of a concentrated PEG solution and the filtering action of the cellulose pad used as the sample pad permitted us to reduce the interference of colored components of red wines to a level compatible with reliable measurements (Figure 2). Despite the signal-to-noise ratio of lines remained lower for red wines in comparison to that of white wines (Figure 3), interference equally

affected the T and C lines. Therefore, the applied normalization of the data (correction of T line by dividing by the C line intensity) was also effective to allow matching of measurements obtained for red wines to those obtained for white wines (Figure 1).

Grape must roughly differs from wine for sugar and ethanol contents; sugars are more concentrated in the must, whereas ethanol is absent. Moreover, a common practice adopted by winemakers to control fermentation is the addition of bisulfite, most frequently as the sodium salt and sometimes by bubbling sulfur dioxide into the must. Highly concentrated bisulfite ($0.15\text{--}0.2\text{ g l}^{-1}$) completely inhibits any fermentation. Winemakers call these treated musts “mute musts” and exploit them for long-term storage; at the opportunity, mute musts are re-inoculated or mixed with fermentable musts, and a new fermentation starts. When fermentable and mute grape musts were analyzed by the developed LFIA and by applying the sample treatment optimized for wines, results were always strongly overestimated, determining a false positive response for the totality of analyzed samples (8 mute and 7 fermentable musts). A new checkerboard optimization was conducted, by varying the same parameters considered in the study of wines; however, none of them significantly modified the response of the LFIA for mute and for fermentable musts. Likewise, attempts aimed at eliminating sugars by means of affinity resins or at compensating for their presence by pre-treating pads and the membrane with concentrated sucrose were completely ineffective. Conversely, the addition of a small amount of ethanol (12%) to resemble wine alcoholic content, straightforwardly resolved the point. Apparently, the slightest amount of ethanol that remained after 1:5 dilution (2.5%), still affected the test significantly.

Wine and grape must analysis by the LFIA

A total of 31 wines and 15 grape musts naturally contaminated were treated as described and analyzed by the developed LFIA and by a reference LC-LFD method. The chromatographic method

had a LOQ of $0.10 \mu\text{g l}^{-1}$ and a mean relative standard deviation of the method (RSD %) of 21%. Quantitative results obtained from both analytical methods are shown in Table 2 and demonstrate that the applied treatments of samples allowed the determination of the toxin in red wines and grape musts as well as in white wines, notwithstanding the calibration has been carried out by fortifying a white wine. The same results are also reported in a semi-quantitative form to allow judgment of compliance according to EU legislation. The indicator range was established at $2 \pm 0.5 \mu\text{g l}^{-1}$ that is the maximum admissible level $\pm 25\%$ to take into account the high imprecision of the LFIA (RSD 30-40%) and the imprecision of the LC-FLD method (21%). Besides precision of analyses, the definition of an individual cut-off has been primarily questioned by several authors because of the finite slope of calibration curves, which suggested defining a concentration range among which the concentration of the target toxin gradually decreases rather than a fixed concentration value at which the toxin switches from presence to absence [21, 32-33]. Therefore samples with OTA content lower than $1.5 \mu\text{g l}^{-1}$ were assigned as negatives (compliant to legal requirements according to EU legislation [6]), samples with OTA content higher than $2.5 \mu\text{g l}^{-1}$ were assigned as positives (non-compliant to legal requirements) and samples with an OTA content comprised between 1.5 and $2.5 \mu\text{g l}^{-1}$ were assigned as uncertain (non attributable).

Only one of the 46 analyzed samples was contaminated at a level above the EU legal limit (one grape must which shows an OTA content of $2.54 \mu\text{g l}^{-1}$). The low OTA contamination level of Italian wines registered in this study was in agreement with data reported for larger surveys conducted in the recent past by other authors on the same subject [see for example ref 34-35] and confirmed a recent trend of low occurrence of this toxin in wines [36].

To verify accuracy of the developed method at levels of contamination above the European legal limit, additional positive samples were generated through fortification of some of the analyzed samples (Table 2). No false negatives, summing truly positive and truly uncertain samples (0/12),

occurred. Similarly, no false positives (intended as truly negative or truly uncertain samples incorrectly assigned as positive) were observed (0/47). Nevertheless, two truly negative samples were incorrectly attributed as uncertain (2/42). Seven samples were not attributable (two false and five truly uncertain samples), which meant that 13% samples would have needed further investigation before making a decision on them. Or else, the lack of attribution would prudentially have led to discard these uncertain samples as potentially non-compliant. In both cases, the result of uncertainty would have determined an economic damage. However, the establishment of the cut-off level at exactly the maximum admissible level ($2 \mu\text{g l}^{-1}$) produced two false negatives (Table 2). Sensitivity and selectivity of the developed assay at this cut-off level were also calculated [32, 37]. Sensitivity defined as the rate of truly positive results and calculated as the number of truly positive tests divided by the sum of known positive samples, was 77.8%. Selectivity defined as the rate of truly negative results and calculated as the number of truly negative tests divided by the sum of known negative samples, was 100%. Similarly, the false compliant rate (calculated as the number of false negative tests divided by the sum of known negative samples) and the false non-compliant rate (calculated as the number of false positive tests divided by the sum of known positive samples) [32,37] were determined to be 4.4% and 0%, respectively.

Despite the developed sample treatment and the LFIA were shown to be imprecise to some extent, they proved to be sensitive and accurate enough to allow the correct attribution of samples (regardless being white or red wines, and mute or fermentable grape musts) as compliant or non-compliant according to EU legislation and therefore to be proposed for the reliable first level monitoring of OTA in wines and grape musts.

Although some lateral flow devices aimed at detecting OTA have been previously developed [23-28], to our knowledge, this is the first assay capable to effectively detect the toxin in wine and grape must, which represent a major source of OTA dietary intake. Analytical performances of the

developed assay are comparable or better than those previously described (Table 3). In addition, the method is very rapid (all reagents are included in the device itself, sample needs only dilution before analysis, and strip development is completed in 5 minutes), extremely simple, and can be effectively regarded as a one-step assay virtually usable anywhere.

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TABLES

Table 1 Parameters investigated with the aim of: (i) lowering of background color on the NC membrane; (ii) reducing of GNP precipitation, and (iii) leveling response obtained from red and white wines. The optimal value for each parameter is underlined.

Parameter	Tested values						
pH	6	8	7	<u>9</u>			
Buffer salt	<u>Bicarbonate</u>	Borate	Citrate	Phosphate			
Buffer concentration (M)	0.05	0.1	<u>0.15</u>	0.2	0.3		
PEG concentration (w/v %)	1	2	<u>4</u>				
Dilution factor (wine:buffer:PEG)	1:0:4	1:1:1	1:1:2	<u>1:2:2</u>	1:2:1	1:4:0	1:4:4

Table 2 Comparison on results obtained by the developed LFIA and a reference LC-FLD method on wine and grape must samples. Semi-quantitative attribution was done according to following

criteria: negative sample (-), samples with OTA content lower than $1.5 \mu\text{g l}^{-1}$ OTA; positive sample (+) >, samples with OTA content higher than $2.5 \mu\text{g l}^{-1}$; uncertain sample (\pm), OTA content comprised between 1.5 and $2.5 \mu\text{g l}^{-1}$. In addition, uncertain samples were also classified as positive (+) / negative (-) according to the establishment of the individual cut-off level at $2 \mu\text{g l}^{-1}$ and this attribution is provided in brackets.

Sample	Quantitative measurement ($\mu\text{g l}^{-1}$)		Semi-quantitative evaluation		Sample	Quantitative measurement ($\mu\text{g l}^{-1}$)		Semi-quantitative evaluation	
	LC-FLD	LFIA	LC-FLD	LFIA		LC-FLD	LFIA	LC-FLD	LFIA
Red	1.9	1.8	\pm (-)	\pm (-)	White	nd	nd	-	-
wines	0.2	nd	-	-	wines	2.0^a	1.6	\pm (+)	\pm (-)
	0.15	nd	-	-		4.0^a	3.6	+	+
	nd	nd	-	-		0.6	nd	-	-
	0.2	nd	-	-		0.8	nd	-	-
	3.2^a	2.5	+	+		2.8^a	2.6	+	+
	nd	nd	-	-		1.6	1.7	\pm (-)	\pm (-)
	0.3	nd	-	-		0.4	nd	-	-
	nd	nd	-	-		0.5	nd	-	-
	3.0^a	2.8	+	+		0.2	nd	-	-
	nd	nd	-	-		0.4	nd	-	-
	nd	nd	-	-		0.3	0.8	-	-
	nd	nd	-	-	Mute	0.5	nd	-	-

2.0 ^a	1.8	± (+)	± (-)	grape musts	0.6	nd	-	-
4.0 ^a	4.6	+	+		0.6	nd	-	-
0.12	nd	-	-		0.4	0.6	-	-
0.8	nd	-	-		0.9	1.4	-	-
1.12	nd	-	-		1.2	1.9	-	± (-)
0.8	1.4	-	-		0.2	0.6	-	-
0.5	nd	-	-		1.3	1.3	-	-
1.0	nd	-	-		4.3 ^a	4.5	+	+
0.4	nd	-	-	Grape	1.7	1.8	± (-)	± (-)
1.0	nd	-	-	musts	0.6	nd	-	-
0.2	nd	-	-		1.1	1.1	-	-
0.2	nd	-	-		nd	1.8	-	± (-)
0.4	nd	-	-		2.5	2.6	+	+
					0.8	nd	-	-
					0.5	nd	-	-

^a fortified sample, obtained by adding OTA to the sample that immediately precedes in the list.

nd: not detectable

Table 3 Overview of the literature concerning lateral flow immunoassays developed for measuring ochratoxin A compared to analytical performance and characteristics of the method developed in this work.

Ref	Detection	LOD (standard)	Commodities	Time for LFIA (+	Dilution due to	LOD (food samples)
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		ng ml ⁻¹		sample preparation) min	extraction	µg kg ⁻¹
23	Visual	500	/	10	/	/
24	Instrumental	0.15	Corn, wheat, durum wheat	8 (+ 12)	1:10	1.5
25	Visual	1.0	Barley, oat, wheat, rice, corn, raisins, coffee	10 (+ 30)	1:2	2.0
25			Beer	10 (+ 0)	1:2	2.0
			Grape juice	10 (+ 0)	1:2	> 10
26	Visual	10	Corn, wheat	10 (+ 5)	1:4	40
27	Instrumental	5	Corn, barley	10		
28	Visual	5	Coffee	10 (+ 10)	1:20	100
This work	Instrumental	/	White wine, red wine, grape must	5 (+ 0)	1:5	1.0

FIGURE CAPTIONS

Figure 1. Example of a calibration curve obtained by measuring ochratoxin A in a fortified white wine.

Responses of the developed method for a fortified red wine (0, 4, and 10 $\mu\text{g l}^{-1}$) are also shown.

Figure 2. Response of the developed LFIA for a red wine sample which showed OTA level beyond the limit of quantification of the reference method. The wine sample was applied to the strip as it (a), after 1:5 dilution with water (b), and after 1:2:2 dilution with NaHCO_3 and PEG (c).

Figure 3. Image of lateral flow devices used for measuring OTA in a white (WW) and a red wine (RW), which showed OTA level beyond the limit of quantification of the reference method. Samples were fortified with increasing amount of the target toxin, from left to right: 0, 4, and 10 $\mu\text{g l}^{-1}$ and diluted 1:2:2 with NaHCO_3 and PEG before applying them to the strips.

