Immunoanalytical methods for ochratoxin A monitoring in wine and must based on innovative immunoreagents

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14 **ABSTRACT**

15 Immunochemical methods are highly deployed in analytical laboratories worldwide for monitoring the
16 incidence of mycotoxins in the food chain. Nevertheless, most conventional immunoassays for ochratoxin A
17 (OTA), including commercial kits, show limitations to robustly determine this mycotoxin in grape-derived
18 products below regulated levels (2 ng/mL). Herein, two rapid tests for sensitive OTA determination in wine
19 and must were developed capitalizing on a collection of bioconjugates from innovative synthetic haptens
20 and monoclonal antibodies with subnanomolar affinity. The ELISA (LOD = 8 pg/mL) showed excellent
21 performance in recovery studies, and it was applied to survey commercial wines and musts for OTA
22 contamination. Concerning LFIA, validation according to the Commission Regulation 519/2014 showed that
23 samples exceeding 2 ng/mL were properly scored as uncompliant. More importantly, illegal samples
24 provided a complete inhibition of the test signal, making this test an easy-to-use, rapid, and convenient
25 screening method for in-house control of OTA in wineries.

26

27 **Keywords**

28 competitive immunoassay, dipstick, food safety, hapten, monoclonal antibody, mycotoxin

29

30 1. Introduction

31 Mycotoxins are secondary metabolites produced by a number of filamentous fungi or molds. Their
32 presence in food and feed may cause health problems in humans and livestock, as well as huge economical
33 losses ([Flores-Flores & González-Peñas, 2017](#); [Yang, Song, & Limb, 2020](#); [Zhou & Tang, 2020](#)). In order to
34 reduce human exposure to these toxins, the European Commission has set legal concentration limits for
35 the presence of several mycotoxins in different foodstuffs ([COMMISSION REGULATION \(EC\) No 1881/2006](#)).
36 Ochratoxin A (OTA) is a harmful mycotoxin produced by *Aspergillus* and *Penicillium* species that can be
37 found in infected groceries, such as cereals (corn, wheat, and barley), coffee, spices, dried fruits, and grape-
38 derived products. OTA is the most relevant member of the ochratoxin family, which also includes
39 ochratoxin B (OTB) and ochratoxin C (OTC) (Fig. S1). In 1993, this mycotoxin was classified by the
40 International Agency for Research on Cancer (IARC) into group 2B, that is, possible carcinogen to humans.
41 Moreover, nephrotoxic, teratogenic, and immunotoxic effects have also been attributed to this toxin ([Tao](#)
42 [et al., 2018](#)). Wine is a main source of OTA intake for consumers in Europe so, in order to protect human
43 health through minimizing public exposure to this mycotoxin, a maximum level of 2 ng/mL was established
44 in the European Union for grape-derived products ([COMMISSION REGULATION \(EC\) No 1881/2006](#)).
45 Because of its social and economic relevance, wine has been deeply surveyed for OTA contamination by
46 instrumental methods ([Bellver Soto, Fernández-Franzón, Ruiz, & Juan-García, 2014](#); [Gentile, La Torre,](#)
47 [Potortì, Saitta, Alfa, & Dugo, 2016](#)). Those studies showed that, although OTA was sometimes detected in
48 100% of the analysed samples ([Remiro, González-Peñas, Lizarraga, & López de Cerain, 2012](#)), values above
49 the demanding European limit are.

50 The European Committee for Standardization recommends high-performance liquid
51 chromatography (HPLC) with fluorescence detection after immunoaffinity column clean-up for OTA
52 quantification ([Quintela, Villarán, López de Armentia, & Elejalde, 2012](#)). Nevertheless, a great variety of
53 instrumental analytical methods have been developed, with HPLC and mass spectrometry detection being
54 more likely the preferred method for OTA determination at trace levels ([Mariño-Repizo, Gargantini,](#)

55 [Manzano, Raba, & Cerutti, 2017; Wei et al., 2018](#)). Although highly reliable, these methods require
56 expensive and complex equipment, and they commonly involve time-consuming procedures, complex
57 clean-up and sample purification steps, as well as highly trained personnel. In contrast, specific receptor-
58 based assays are user-friendly, rapid, and high-throughput analytical methods that may assist analysts in a
59 variety of real situations. Aptamers ([Costantini et al., 2019](#)) and molecularly imprinted polymers ([Baggiani,](#)
60 [Giovannoli, & Anfossi, 2015](#)) have been developed for OTA analysis. Nevertheless, antibody-based assays,
61 like the enzyme-linked immunosorbent assay (ELISA) ([Oplatowska-Stachowiak et al., 2017; Zhang et al.,](#)
62 [2015; Zhang et al., 2017](#)) or the lateral flow immunoassay (LFIA) ([Majdinasab, Zareian, Zhang, & Li, 2019;](#)
63 [Zhang et al., 2018](#)), are the most widespread rapid methods in quality control laboratories and industries
64 worldwide. Nowadays, a high number of immunoassays for OTA determination in several foodstuffs have
65 been described ([Alhamoud et al., 2019; Meulenber, 2012](#)); nonetheless, most conventional
66 immunochemical methods reported so far, as well as commercial kits, show limitations to robustly
67 determine OTA in wines and grape musts at regulated levels. Even though several sensitive and
68 sophisticated approaches have been recently published ([Jiang, Li, Xiong, Pei, Nie, & Xiong, 2017; Lai et al.,](#)
69 [2020; Pei et al., 2018; Xiong, Zhou, Huang, Yu, Lai, & Xiong, 2017](#)), further studies are still required for these
70 innovative technologies to be implemented in routine analysis. Concerning conventional ELISA,
71 Oplatowska-Stachowiak *et al.* recently reported a comprehensive validation of a direct immunoassay in
72 different commodities, including wine and must, according to the guidelines included in Commission
73 Regulation (EU) No 519/2014 ([Oplatowska-Stachowiak et al., 2017](#)). With respect to common LFIA, the
74 most remarkable example is that reported by Anfossi *et al.*, even though the visual detection limit of that
75 assay is above the European limit for grape-derived beverages ([Anfossi, Giovannoli, Giraudi, Biagioli,](#)
76 [Passini, & Baggiani, 2012](#)). To the best of our knowledge, there is no commercial LFIA sensitive enough to
77 enable the visual screening of wines contaminated with OTA at 2 ng/mL.

78 Antibodies are at the core of any immunoanalytical method; their affinity and specificity to the
79 target analyte will severely determine the analytical performance of the test. In order to obtain antibodies
80 for small organic compounds, the target molecule, or an analogue, needs to be covalently coupled to an

immunogenic macromolecule, like a protein (Cavalera et al., 2020; Quiñones-Reyes, Agulló, Mercader, Abad-Somovilla, & Abad-Fuentes, 2019; Zhou, Xu, Kuang, Xiao, & Xu, 2020). In the case of OTA, this conjugation has been always carried out using the carboxylic group that is present in the structure of the mycotoxin, which can be readily activated for conjugation with the ϵ -amine groups of the lysine residues of the carrier proteins (Liu, Xu, Suryoprabowo, Song, & Kuang, 2017). However, this straightforward strategy is probably not the most convenient from an immunological point of view, as it was recently demonstrated by our group (López-Puertollano, Mercader, Agulló, Abad-Somovilla, & Abad-Fuentes, 2018). The native carboxylic group of OTA represents an important antigenic determinant for shaping the antigen-binding site during the immune response. Moreover, earlier theoretical calculations, as well as crystallography and NMR studies, support the idea about the great significance of that carboxylic acid moiety in the conformational arrangement of OTA in solution (Bredenkamp, Steyn, Dillen, & van Rooyen, 1989; Dais, Stefanaki, Fragaki & Mikros, 2005). Therefore, OTA derivatives with a functionalized linker at alternative tethering sites may better display the relevant and characteristic moieties of the molecular structure of this compound, while maximizing the similarity of its conformation and electronic distribution to those of the parent molecule. Following this reasoning, a large collection of mAbs from a series of conjugates was generated by our group. Up to six OTA haptens with different linkers and tethering sites (Fig. 1) were employed for the preparation of those conjugates and the generation of monoclonal antibodies (mAbs). By taking advantage of this panel of immunoreagents, the objective of the present study was the development of novel and highly sensitive competitive ELISA and immunocromatographic strips for the rapid determination of OTA in wine and must samples below the legal concentration limit established by the European legislation. Every antibody was evaluated in combination with homologous and heterologous conjugates by using two alternative competitive ELISA formats, *i.e.*, the conjugate-coated indirect and the antibody-coated direct format (icELISA and dcELISA, respectively). The analytical performance of the optimized immunoassay was evaluated with OTA fortified samples and with a certified reference material. Furthermore, the experimental procedure described in Commission Regulation (EU) No 519/2014 for

validation of semi-quantitative screening methods for mycotoxin analysis was applied to the developed immunostrips.

Fig. 1. Structure of the different OTA haptens used in this study to prepare bioconjugates and to generate monoclonal antibodies.

2. Materials and methods

2.1. Reagents and instrumentation

Analytical standard of OTA (CAS number 303-47-9, Mw 403.8 g/mol) was purchased from Merck (Madrid, Spain), and those of OTB (CAS number 4825-86-9, Mw 369.4 g/mol), and OTC (CAS number 4865-85-4, Mw 431.9 g/mol) were from Toronto Research Chemicals (Ontario, Canada). *o*-Phenylenediamine (OPD), Tween-20, H₂O₂ 30% (v/v), and poly(vinylpyrrolidone) (PVPP; 110 μm particle size) were provided by Merck (Madrid, Spain). 3,3',5,5'-Tetramethylbenzidine (TMB) was purchased from Zeulab (Zaragoza, Spain). Goat anti-mouse immunoglobulins polyclonal antibodies (GAM) were obtained from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA). As secondary antibody, RAM-HRP conjugate (enzyme labelled rabbit anti-mouse immunoglobulins polyclonal antibody) from Dako (Glostrup, Denmark) was used. The certified wine sample naturally contaminated with OTA was acquired from the Federal Institute for Materials Research and Testing (BAM, Berlin, Germany).

123 Costar flat-bottom high-binding 96-well polystyrene ELISA plates from Corning (Corning, NY, USA)
124 were employed. Absorbances from ELISA microplates were read with a PowerWave HT from BioTek
125 Instruments (Winooski, VT, USA). Microwells were washed with an ELx405 microplate washer also from
126 BioTek Instruments. Nitrocellulose membranes (15 μ m pore size) were purchased from MDI Membrane
127 Technologies (Ambala, India), and backing cards were purchased from Kenosha (Amstelveen, The
128 Netherlands). Cellulose sample pad and adsorbent pad were obtained from Merck-Millipore (Billerica, MA,
129 USA) and Ahlstrom-Munksjö (Manchester, UK), respectively. GAM-modified 40-nm gold nanoparticles (OD
130 = 10) were purchased from BBI solutions (Crumlin, UK). A ZX1010 system from Biodot (West Sussex, UK)
131 was used for dispensing immunoreagents onto the nitrocellulose membrane. Immunostrips were manually
132 assembled, and they were cut using a CM5000 guillotine also from Biodot. For signal reading, immunostrips
133 were scanned using an EPSON V39 scanner, and RGB signals were processed using ImageJ (version 1.52a)
134 free software. Data processing and graphical representation were carried out using SigmaPlot 14.0
135 software from SPSS Inc (Chicago, IL, USA).

136 *2.2. Preparation of the LFIA immunoconjugate*

137 Gold nanoparticles (GNPs) were diluted 10 times in 10 mM Tris-HCl buffer, pH 7.4. Anti-OTA mAb was
138 added to the desired concentration from a 100 μ g/mL stock solution in 10 mM Tris-HCl buffer, pH 7.4, and
139 the mixture was incubated for 1 h at room temperature with gentle stirring. Afterwards, Tween-20 was
140 added to a final concentration of 0.5% (v/v).

141 *2.3. Assembly of the immunostrips.* GAM or BSA–haptens solutions (1 mg/mL) in 100 mM phosphate buffer,
142 pH 7.4, with 150 mM NaCl, were dispensed onto the nitrocellulose membrane (300×25 mm) at a flow rate
143 of 0.5 μ L/cm using a BioDot dispensing system. BSA–haptens for the test line and GAM for the control line
144 were dispensed at 10 mm and 15 mm distance from the base of the membrane, respectively. Membranes
145 were dried at room temperature for 1 h and were stuck on a backing card (300×78 mm) separated 14 mm
146 from the base. Nitrocellulose sample pad (300×17 mm) and adsorbent pad (300×43 mm) were manually

147 assembled overlapping the membrane 3 mm and 4 mm, respectively. The assembled components were cut
148 using a Biodot guillotine to give 4×78 mm immunostrips.

149 2.4. Immunoassays

150 2.4.1. *Antibody-coated direct competitive ELISA*. Microplates were coated by overnight incubation with 100
151 µL per well of mAb solution in coating buffer (50 mM carbonate–bicarbonate buffer, pH 9.6) at 4 °C. Plates
152 were washed four times with washing solution (150 mM NaCl and 0.05% (v/v) Tween-20). The competitive
153 reaction was carried out by mixing 50 µL per well of OTA solution in PBS or Milli-Q water and 50 µL per well
154 of HRP–hapten tracer solution in PBS-T (PBS containing 0.05% (v/v) Tween-20) or 2×PBS-T (20 mM
155 phosphate buffer, pH 7.4, containing 280 mM NaCl and 0.05% (v/v) Tween-20), and incubating at room
156 temperature for 1 h. After washing the plates as before, the signal was generated by adding 100 µL per well
157 of 2 mg/mL *o*-phenyldiamine solution in substrate buffer (25 mM citrate and 62 mM sodium phosphate
158 buffer, pH 5.4, containing 0.012% (v/v) H₂O₂), and it was stopped after 10 min at room temperature with
159 100 µL per well of stop solution (1 M H₂SO₄). The absorbance was read at 492 nm with a reference
160 wavelength at 650 nm.

161 2.4.2. *Conjugate-coated indirect competitive ELISA*. Plates were coated by overnight incubation at room
162 temperature with 100 µL per well of OVA conjugate solution in coating buffer. Microwells were washed
163 four times with washing solution after each incubation step. The competitive immunochemical reaction
164 was performed by mixing 50 µL per well of OTA solution in PBS or Milli-Q water and 50 µL per well of mAb
165 solution in PBS-T or 2×PBS-T, and incubating for 1 h at room temperature. The bound mAb was indirectly
166 detected with 100 µL per well of RAM–HRP conjugate (1/2000 dilution in PBS-T), and incubation at room
167 temperature for 1 h. Finally, signal were obtained and processed as described above for the direct assay
168 format.

169 2.4.3. *Competitive LFIA*. Dipstick lateral flow immunoassays were carried out in 300-µL wells by mixing 20
170 µL of gold immunoconjugate colloidal solution with 80 µL of OTA standard solution or sample in 100 mM
171 Tris-HCl buffer, pH 8.0, containing 30 mM NaCl. The mixtures were incubated 5 min at room temperature,

172 and then the immunostrips were dipped into the well. After running the test in vertical position for 10 min,
173 sample pads were removed and the strips were dried for 3 min under a cold airflow and scanned with a
174 desktop scanner (1200 ppp).

175 2.5. Optimized immunoassay procedures

176 2.5.1. *Direct competitive ELISA*. Sample treatment consisted of a 1/10 dilution of samples in 75 mM
177 phosphate buffer, pH 7.4, and a clean-up step with 1% or 4% PVPP (w/v) for wine and grape must samples,
178 respectively. Samples were centrifuged (4800×g, 10 min) and supernatants were assayed. Microplates were
179 coated by overnight incubation at 4 °C with 100 µL per well of a 300 ng/mL solution of mAb f#223 in
180 coating buffer. After washing the plates four times with washing solution, 50 µL of OTA standard solution or
181 sample in 75 mM phosphate buffer, pH 7.4, was added to each well and incubated for 30 min at room
182 temperature. Afterwards, 50 µL per well of a 60 ng/mL solution of HRP-**b** tracer in 75 mM phosphate
183 buffer, containing 0.05% (v/v) Tween-20, were added, and the mixtures were incubated during 30 min at
184 room temperature. After washing the plates as described previously, the signals were obtained by adding
185 100 µL per well of ready-to-use TMB solution as enzyme substrate. The signal was developed for 15 min at
186 room temperature and the reaction was stopped using 100 µL per well of stop solution. Absorbance was
187 read at 450 nm using 650 nm as reference wavelength.

188 2.5.2. *Competitive LFIA*. A mixture containing 900 µL of sample, 900 µL of CH₂Cl₂, and 90 µL of 6 M H₃PO₄
189 was vigorously shaken in a head-over-head rotator for 15 min. After centrifugation (4800×g, 3 min), 600 µL
190 of the organic phase were extracted with 300 µL of 100 mM Tris-HCl buffer, pH 8.0, containing 30 mM NaCl.
191 Assays were carried out by mixing 20 µL of GNP@mAb **b**#311 immunoconjugate (prepared with 3 µg of
192 mAb per millilitre of nanoparticle colloidal solution, OD = 1), in 10 mM Tris-HCl buffer, pH 8.0, containing
193 0.5% (v/v) Tween-20, with 80 µL of sample extract, and the mixture was incubated for 5 min at room
194 temperature. Immunostrips, prepared with BSA-**e** conjugate for the test line, were dipped into the reaction
195 mixture and the tests were run vertically during 10 min at room temperature. Next, sample pads were
196 removed and the membranes were dried under a cold airflow for 3 min. Immunostrips were digitalised

197 using a desktop scanner and RGB signals of the test and control lines were acquired using ImageJ free
198 software.

199 2.6. Data analysis

200 Immunoassay signal values from OTA standards were fitted to a four-parameter logistic equation.
201 $A_{\max}$ and $(T/C)_{\max}$ are the signals obtained in the absence of analyte in competitive ELISA and LFIA,
202 respectively. The OTA concentration affording a 50% reduction (IC_{50}) of $A_{\max}$ or $(T/C)_{\max}$ was considered as
203 the reference value for comparing antibody affinity and immunoassay sensitivity. The LOD was defined as
204 the OTA concentration that reduced $A_{\max}$ or $(T/C)_{\max}$ by 10% (IC_{10}).

205 2.7. LFIA validation

206 The guidance for validation of semi-quantitative screening methods for mycotoxins included in the
207 Commission Regulation (EU) 519/2014 was followed. Sets of 4 samples were spiked at the screening target
208 concentration (STC) and at $1/4 \times STC$ and analysed by the immunochromatographic assay. The study was
209 performed under intermediate precision conditions by spreading the analysis of these samples over 5
210 different days to obtain a total of 20 measurements. The obtained results were then used to determine the
211 cut-off value and the false-suspect rates. The cut-off value of a competitive test represents the assay signal
212 value below which a sample is classified as “suspect” of containing OTA above the STC, and it was
213 calculated, assuming a 5% rate of false negative results, using equation (1) for methods with inversely
214 proportional response:

$$215 \text{Cut-off} = R_{STC} + t_{\text{value}0.05} \times SD_{STC} \quad (1)$$

216 where R_{STC} is the mean concentration of the positive control samples at the STC; $t_{\text{value}0.05}$ is the one tailed
217 t_{value} for a rate of false negative results of 5%, which is 1.729 for 19 degrees of freedom (20 sample set); and
218 SD_{STC} represents the standard deviation at the STC. The false-suspect rate is obtained from a t -distribution
219 table using the t_{value} calculated in equation (2) for inversely proportional methods. The $\text{mean}_{\text{blank}}$ value is the
220 mean concentration of 20 blank samples and SD_{blank} the standard deviation of the blank.

$$221 t_{\text{value}} = (\text{mean}_{\text{blank}} - \text{cut-off})/SD_{\text{blank}} \quad (2)$$

222

223 3. Results and discussion

224 3.1. Characterization of the collection of immunoreagents

225 Eighteen protein conjugates prepared from 6 haptens were employed in the present study (Fig. S2).
226 Haptens **a** and **b** bore the spacer arm at the C-4 position of the phenyl group of the OTA phenylalanine
227 moiety, whereas haptens **c** and **d** held the linker at the C-3 position of the methyl group of the
228 dihydroisocoumarin moiety. In the case of haptens **e** and **f**, the native carboxylic group of OTA was used for
229 tethering of a spacer arm at this position or for direct conjugation, respectively. All haptens were covalently
230 conjugated to bovine serum albumin (BSA), ovalbumin (OVA), and horseradish peroxidase (HRP). Haptens
231 **b**, **d**, **e**, and **f** were conjugated by the active ester method through the formation of an amide bond
232 between the NHS-activated carboxylic group that was present at the end of the spacer arm and the ϵ -
233 amino groups of the lysine residues of the proteins. In the case of haptens **a** and **c**, conjugation was carried
234 out by CuAAC chemistry using proteins whose lysine residues had been previously modified to introduce
235 alkyne groups (López-Puertollano, Agulló, Mercader, Abad-Somovilla, & Abad-Fuentes, 2020).

236 A collection of 22 anti-OTA mAbs was generated by hybridoma technology using mice immunized
237 with BSA–hapten conjugates. The whole antibody collection was evaluated by checkerboard competitive
238 ELISA using homologous and heterologous conjugates in the dcELISA and the icELISA formats (Table 1). The
239 highest number of antibodies were generated from conjugates with haptens linked through the
240 dihydroisocoumarin moiety (7 mAbs from BSA–**d** and 5 mAbs from BSA–**c**); concerning conjugates coupled
241 through the phenylalanine moiety, 4 mAbs derived from the conjugate BSA–**b** and 3 mAbs from BSA–**a**
242 were produced; lately, 3 antibodies were obtained from conjugates with haptens tethered through the
243 native carboxylic acid of OTA, 2 mAbs from the conjugate BSA–**e** and 1 mAb from BSA–**f**.

244 Very low signals were obtained with all of the mAbs when the enzyme tracer HRP–**f** was employed
245 as competitor in the dcELISA format, even under homologous conditions with mAb f#223. This result was
246 not unexpected because enzyme tracers with short or no spacer arms, as HRP–**f**, usually are poorly bound

247 by immobilized antibodies, as our group and others have previously observed for other analytes ([Galvidis,](#)
248 [Wang, Nuriev, & Burkin, 2018;](#) [Suárez-Pantaleón, Mercader, Agulló, Abad-Somovilla, & Abad-Fuentes,](#)
249 [2011](#)). Antibodies obtained from the conjugates BSA-**a** and BSA-**b** (same linker tethering site but different
250 linker structure) recognised the reciprocal competitor conjugates in both assay formats. The same mutual
251 recognition was found between antibodies raised from conjugates BSA-**c** and BSA-**d**, and between
252 antibodies from BSA-**e** and BSA-**f**. On a general basis, antibodies generated from haptens **c** and **d** were not
253 capable of recognising conjugates prepared with haptens **e** or **f** – conjugates prepared through the
254 carboxylic group present in the OTA structure – in any of the two studied ELISA formats, a finding that is
255 probably pointing out the importance of that moiety in the antibody-OTA interaction.

256 Concerning OTA recognition in solution, several immunoreagent combinations exhibiting IC₅₀ values
257 (analyte concentration affording 50% reduction of the maximum signal) below 0.5 nM in either or both
258 formats were found.

259 After affinity assessment, the specificity of the antibody panel was evaluated by icELISA using the
260 homologous coating conjugate. Standard solutions of OTB and OTC were evaluated as competitors, and the
261 obtained IC₅₀ values were compared with those for OTA under the same conditions ([Table S1](#)). Antibodies
262 generated from haptens **a** and **b** were quite specific of OTA, as judged by the low cross-reactivity values to
263 OTB and OTC. The specificity of antibodies from haptens **c** and **d** was more varied, and some of them were
264 able to clearly recognize OTB (mAb **d**#111 and mAb **d**#115), even though they mostly did not bind OTC.
265 Antibodies from hapten **e** showed high cross-reactivity with OTC, in particular mAb **e**#13, a result in line
266 with the high similarity between this toxin and hapten **e**; in fact, the structure of this hapten was
267 theoretically best suited for the generation of anti-OTC antibodies than to anti-OTA antibodies. Finally,
268 antibody **f**#223 displayed moderate cross-reactivity with both toxins.

269

270

Table 1
IC₅₀ values (nM) obtained by checkerboard assay with anti-OTA mAbs using the icELISA and the dcELISA formats (n=3).^a

mAb	Hapten in the assay conjugate										
	a		b		c		d		e		f
	icELISA	dcELISA	icELISA	dcELISA	icELISA	dcELISA	icELISA	dcELISA	icELISA	dcELISA	icELISA
a#118	6.36	3.93	6.73	10.8	7.49	4.27	14.9				
a#122	4.05	5.09	3.34	1.53					2.01		5.99
a#125	3.93	1.99	5.74	2.02	3.29	1.70	2.92				
b#39	0.95	0.53	0.90	0.32	2.09	0.28	1.12	0.26			
b#41	1.69	1.11	2.23	1.25	3.57	1.52	2.28	1.57			
b#310	0.79	0.30	0.39	0.10	0.23		0.21		0.09		0.28
b#311	0.69	0.12	0.25	0.11	0.19		0.11		0.15		0.14
c#12					0.39	0.18	1.51	0.37			
c#29					0.69	0.43	1.84	0.48			
c#116	0.38	0.24	0.53	0.53	0.58	0.28	0.54	1.73			
c#117	0.10		0.07		0.58	0.20	0.30	0.25			
c#134					0.93	0.52	0.79	0.52			
d#16	0.47				0.27	0.67	0.21	0.29			
d#21	0.18		0.13		0.44	0.53	0.27	0.37			
d#27	1.16		1.19		0.96	0.87	1.15	0.56			
d#111	0.16		0.45		0.29	0.52	0.20	0.54			
d#114	0.46		0.59		0.85	0.84	0.39	0.24			
d#115					0.60	0.26	0.56	0.17			
d#118	0.62		0.45		1.09	0.33	0.46	0.26			
e#13	2.05	0.80	1.80	0.64	2.51				1.40	1.31	1.87
e#115	0.45		0.28						0.23	0.27	0.39
f#223	0.63	0.17	0.16	0.08					0.34	0.14	0.19

^a Maximum signal was between 0.6 and 1.4 absorbance units. Empty cells indicate that the conjugate was poorly or not recognized.

272

273 3.2. Development of the competitive ELISA

274 Two combinations of immunoreagents displaying the lowest IC₅₀ values and moderate slopes of the
275 inhibition curve were selected for competitive ELISA development. For the indirect assay, mAb **c#117** was
276 chosen in combination with the site-heterologous coating conjugate OVA-**b**, and for the direct format, mAb
277 **f#223** was selected in combination with the site-heterologous enzyme tracer HRP-**b**.

278 The influence of pH and ionic strength over the A_{max} (maximum signal in the absence of analyte)
279 and the IC₅₀ values of the selected competitive ELISAs was studied ([Fig. S3](#)) in order to figure out whether

280 conditions different to that of PBS may result in better detectability. The assay parameters under each of
281 the examined conditions were compared to those obtained under standard conditions with PBS. Overall,
282 pH and ionic strength values differing from physiological conditions did not give rise to a significant
283 improvement in immunoassay performance, so a PBS-like buffer with a higher buffering capacity was
284 selected for further work (75 mM sodium phosphate buffer, pH 7.4, $I = 167$ mM).

285 As these ELISAs were intended for the determination of OTA in wine, the influence of ethanol on
286 the parameters of the standard curve was also assessed. For both the indirect and the direct immunoassay,
287 increasing concentrations of the solvent led to higher IC_{50} values and to lower A_{max} values (Fig. S4). These
288 results evidenced that, for reliable immunoanalysis, it is advisable to keep the ethanol content as low as
289 possible.

290 Finally, the matrix effect of red wine over the standard curve of both immunoassays was studied.
291 With this purpose, wine samples were serially diluted using 75 mM phosphate buffer, pH 7.4, and OTA
292 standard solutions were prepared in those wine-diluted solutions. The resulting inhibition curves were
293 compared with the curve obtained in buffer, which was run in parallel. This experiment showed that
294 standard curves prepared in 100-fold diluted wine, or higher, perfectly overlap with the curve run in buffer.
295 Although this simple procedure allowed to completely remove matrix effects, the required dilution reduces
296 the detectability of the method to such an extent that it was not suitable for determining OTA at the level
297 regulated in the EU (2 ng/mL). At this point, we decided to test an alternative protocol consisting in treating
298 the wine sample with PVPP (poly(vinylpolypyrrolidone)), a polymer that is commonly employed for
299 removing tannins and polyphenols from wine samples without affecting OTA contents (Gil et al., 2017;
300 Robinson, Lee, & Ryu 2017). Accordingly, OTA standard solutions were prepared in 10-fold diluted wine and
301 further cleaned-up with different amounts of PVPP (Fig. S5). After comparing the so-prepared inhibition
302 curves with the curve run in buffer, it was observed that this approach was unsuitable for minimizing matrix
303 effects with the indirect ELISA. However, in the case of the direct ELISA, adding 1% (w/v) PVPP to a 10-fold
304 diluted wine sample looked promising (Fig. S6). Further equivalent studies with grape musts raised similar

305 results, although with this sort of samples a 4% (w/v) PVPP was advisable. Attending to these findings,
306 additional work was just carried out with the direct ELISA format.

307 3.3. Application of the direct competitive ELISA to sample analysis

308 At this point of the ELISA development, OPD was replaced by TMB as the HRP substrate in order to
309 attain higher signals. Fig. 2A summarizes the main analytical parameters of the final ELISA. Remarkably, the
310 assay displayed IC₅₀ and LOD values in the low picogram per millilitre range (0.036 and 0.008 ng/mL,
311 respectively).

312 The analytical performance of the ELISA was first evaluated with fortified wine and must samples.
313 Six wines (3 red and 3 white) and 4 musts (2 red and 2 white) were fortified with OTA at six levels (from 0.5
314 to 20 ng/mL). Samples were 10-fold diluted in 75 mM phosphate buffer, pH 7.4, and 1-mL aliquots were
315 cleaned-up with 1% or 4% PVPP (w/v) for wines or musts, respectively. After centrifugation, the
316 supernatants were analysed by ELISA in microtiter plates wherein 7 OTA standard solutions in buffer plus a
317 blank were concurrently run. An additional 1/10 dilution in the same buffer was applied to those samples
318 with OTA concentrations above 2 ng/mL. As shown in Table 2, excellent recovery values were obtained, and
319 coefficients of variation were mostly below 20%. This study revealed that the developed direct ELISA
320 enables the simple determination of OTA, with minimum sample clean-up, not only at the challenging level
321 demanded by the EU (2 ng/mL), but also at a 4-fold lower concentration (0.5 ng/mL), at least for wines –
322 must samples showed a slight tendency to overestimate at 0.5 ng/mL. In addition, this immunoassay
323 exhibited a good linearity, allowing the detection of OTA at higher concentrations by applying an additional
324 dilution in buffer of the PVPP-treated sample. To further prove the practical suitability of the method, a
325 certified reference material (ERM-BD476) consisting in a cloudy red wine containing 0.52 ± 0.11 ng/mL of
326 the mycotoxin was analysed, and a value of 0.50 ± 0.05 ng/mL (n=4) was obtained.

327

328

Table 2

Recovery values (%) obtained by the direct competitive ELISA with red and white wine and grape must samples fortified with OTA (n = 4).

Sample	[OTA] ^a	Wine 1	Wine 2	Wine 3	Must 1	Must 2
Red	0.5	107 ± 6	102 ± 4	98 ± 15	121 ± 11	127 ± 12
	1	98 ± 9	100 ± 7	94 ± 12	106 ± 5	119 ± 7
	2	102 ± 14	94 ± 8	102 ± 8	118 ± 9	122 ± 2
	5	95 ± 20	98 ± 9	112 ± 11	107 ± 13	82 ± 6
	10	95 ± 13	101 ± 2	102 ± 3	93 ± 4	82 ± 7
	20	93 ± 6	95 ± 3	96 ± 6	95 ± 7	102 ± 1
	[OTA]	Wine 4	Wine 5	Wine 6	Must 3	Must 4
White	0.5	101 ± 12	94 ± 12	92 ± 24	116 ± 3	130 ± 5
	1	89 ± 10	94 ± 11	107 ± 13	98 ± 6	109 ± 8
	2	90 ± 8	94 ± 12	119 ± 17	106 ± 13	111 ± 2
	5	103 ± 14	94 ± 5	111 ± 2	101 ± 14	83 ± 1
	10	110 ± 4	104 ± 7	109 ± 8	86 ± 11	83 ± 10
	20	101 ± 6	91 ± 5	97 ± 3	86 ± 6	87 ± 7

^a Values are in ng/mL.

329

330 Finally, the immunoassay was applied to the determination of OTA in bottled wines and musts
 331 bought at local supermarkets. The survey included 50 red wines, 20 white wines, 9 red musts, and 8 white
 332 musts (Table S2), all of them of Spanish origin. We found that all samples were compliant with the EU
 333 legislation. Six red wines contained OTA at detectable levels, three of which above the limit of
 334 quantification (RW-15, RW-28, and RW-31), whereas the mycotoxin was undetectable in the white wines.
 335 Concerning grape musts, most of them contained OTA at quantifiable concentrations, although clearly
 336 below 2 ng/mL. The highest OTA level was found in a red must sample (RM-2, 1.38 ± 0.17 ng/mL), whereas
 337 the red wine sample with the highest OTA content was RW-28 (0.56 ± 0.04 ng/mL). These two samples
 338 were independently analysed by an accredited laboratory. The determinations by HPLC-MS/MS confirmed
 339 the presence of OTA in both samples at levels of 1.2 ± 0.3 ng/mL for the must sample and below the
 340 quantification limit for the wine sample.

341 3.4. Development of chromatographic immunostrips

342 Six mAbs and four BSA conjugates were selected for evaluation by competitive
 343 immunochromatography in order to find the immunoreagent combination affording the highest sensitivity.
 344 The test line was drawn with the bioconjugates BSA-b, BSA-d, BSA-e or BSA-f, and the control line consisted

345 of GAM. Antibodies **b#311**, **d#21**, **d#111**, **d#115**, **f#223**, and **c#117** were coupled to GNPs at 1 µg of mAb
346 per millilitre of colloidal gold at OD=1. This screening assay was carried out with three standard solutions of
347 OTA (0, 2, and 10 ng/mL). The assay consisted in mixing the solution containing the GNP@mAb with the
348 sample, incubating at room temperature for 5 min, and then dipping the immunostrip. After running the
349 test in vertical position for 10 min, the immunostrips were air-dried and lines were scanned. The output of
350 the assay was the quotient between the signal at the test line and the signal at the control line (T/C). In the
351 absence of OTA, the nanoparticles will preferentially bind to the test line, resulting in the maximum
352 accumulation of GNPs at this area of the membrane and affording (T/C)_{max}. On the contrary, as the
353 concentration of OTA increases, less nanoparticles will interact with the conjugate spotted at the test line,
354 so colour intensity at this line fades until complete disappearance. From this study, mAb **b#311** together
355 with the heterologous conjugate BSA-e was selected for further immunostrip development. We first
356 optimized the volume of nanoparticles and the amount of antibody used in the preparation of the
357 GNP@mAb **b#311**. As shown in [Fig. S7](#), superior test line signals and inhibition rates were found when the
358 antibody concentration was set at 3 µg per millilitre of GNPs, and the volume of nanoparticle suspension
359 per assay was set at 20 µL.

360 Thereafter, the influence of pH and ionic strength, as well as the effect of ethanol, over the assay
361 signal and the detectability of the assay was assessed ([Fig. S8](#)). For these experiments, OTA standard
362 solutions were prepared in buffers with different pH, ionic strength, or ethanol concentration, and the
363 obtained T/C rates were compared to that obtained under standard conditions. Interestingly, the
364 immunostrip was able to tolerate even an ethanol concentration of 10%. Concerning pH and ionic strength,
365 the best signal and inhibition rates were obtained with 100 mM Tris-HCl buffer, pH 8.0, with 30 mM NaCl.

366 The characteristics and main analytical parameters of the final immunochromatographic assay are
367 shown in [Fig. 2B](#). The slope of the LFIA was much higher than that of the ELISA, and the IC₅₀ value was
368 about seven times higher. The immunostrip showed a LOD of 0.1 ng/mL, a remarkable detectability for an
369 assay that just takes 15 min.

370

371 **Fig. 2.** Analytical parameters of the developed direct ELISA (A) and the immunochromatographic assay (B). Data are the
 372 mean of four independent experiments. The LOD was calculated from the signal at 10% inhibition. The working range was
 373 calculated from the signals at 20% and 80% inhibition.

374

375 *3.5. Application of the immunostrips to sample analysis.*

376 The same sample treatment employed with the ELISA (1/10 sample dilution plus PVPP) was
 377 deemed unsuitable for LFIA analysis of OTA at regulated levels because of the lower sensitivity of the
 378 immunostrip over that of ELISA. Accordingly, we decided to evaluate a completely different procedure that
 379 takes advantage of the acid-base character of the mycotoxin. Samples were first extracted using acidified

380 dichloromethane and then re-extracted using a slightly basic buffer (100 mM Tris-HCl, pH 8.0, with 30 mM
 381 NaCl). The so-obtained solution was directly analysed by the developed immunochromatographic assay.
 382 Although maximum signals in the absence of OTA slightly differ among the commodity under consideration
 383 (Fig. 3), reproducible inhibition values of around 85% were observed at 0.5 ng/mL OTA for all sample types.
 384 More importantly, total signal inhibition at the test line was found at OTA concentrations of 1 and 2 ng/mL,
 385 which demonstrates the suitability of the developed competitive immunostrips for the rapid and visual
 386 detection of this mycotoxin in wines and musts.

388 **Fig. 3.** Inhibition pattern obtained for four different matrices with the developed competitive LFIA.

389
 390 Because of the increasing acceptance of rapid screening methods, the EU published a specific
 391 regulation setting up the procedure for the validation of semi-quantitative methods intended for mycotoxin
 392 determination (COMMISSION REGULATION (EU) No 519/2014). This procedure requires to first determining
 393 the cut-off value of the method, *i.e.*, the T/C value of the immunostrip below which a sample is deemed

394 suspect of containing the mycotoxin above the so-called STC ([Lattanzio, 2016](#)). With this aim, four samples
395 of wines and musts with no OTA, both red and white, were analysed during five consecutive days along
396 with the same samples spiked with OTA at 2 ng/mL, the concentration chosen as the STC in this study. As
397 can be observed in [Table 3](#), the output from samples spiked at the STC (mean T/C = 0.02) strongly differs
398 from that of unspiked samples (mean T/C = 0.68). From these results, the cut-off value and the false-
399 suspect rate were calculated at the 95% confidence interval. As summarized in [Table 3](#), the cut-off values
400 for the different types of food matrices were between 0.02 and 0.04 at the STC, whereas the highest false-
401 suspect rate was 0.13% for the white musts. This result means that, on average, only 1.3 negative samples
402 every 1000 analyses would be incorrectly classified as containing OTA over the STC. Even assuming a false
403 negative rate of 1% (99% confidence interval), the highest false-suspect rate was 0.14%, again for the white
404 grape musts. For the other food samples, the false-suspect rates were even lower or negligible.

405 Finally, the immunostrips were evaluated under stricter criteria than those recommended by the
406 European Regulation. To this purpose, samples were spiked with OTA at 0.5 ng/mL (1/4×STC) and analysed
407 as described above. In this case, the cut-off values ranged between 0.13 (red grape must) and 0.24 (white
408 wine), while the false-suspect rate ranged from negligible to 0.97 (white grape must). These results provide
409 compelling evidence of the suitability of the developed immunochromatographic assay for adequately
410 screening grape-derived products for OTA not only at 2 ng/mL, but even at 0.5 ng/mL. To the best of our
411 knowledge, this is the first time that validation of a LFIA for OTA according to the European Regulation (EU)
412 No 519/2014 has been reported.

413

414

415

416

417

Table 3

LFIA validation study according to European Regulation (EU) No 519/2014.

440 **4. Conclusions**

441 Two immunoassays enabling the determination of OTA with LOD values at or below 0.1 ng/mL are
442 herein reported. This outstanding detectability was achieved by taking advantage of a collection of
443 innovative bioconjugates and of a panel of monoclonal antibodies exhibiting subnanomolar affinity to OTA
444 by competitive ELISA. Remarkably, some of these antibodies are very specific to OTA, with negligible
445 recognition of OTB and OTC. Following a simple treatment with PVPP of wine and must samples, the
446 cdELISA is able to accurately and precisely analyze OTA in those complex matrices at concentration values
447 clearly below the maximum limit established by the European Commission (2 ng/mL). Additionally, a highly
448 sensitive LFIA was developed in which an 80% reduction and complete inhibition of the signal at the test
449 line was observed at 0.5 and 2 ng/mL, respectively, for the studied wine and must samples. The developed
450 LFIA meets the level of rigorousness for semi-quantitative screening methods for mycotoxin analysis
451 demanded by Commission Regulation (EU) 519/2014. To the best of our knowledge, this is the first time
452 that an immunochromatographic assay has been validated according to these well-defined criteria. While
453 the ELISA is ideally suited for the simultaneous analysis of dozens of samples in analytical laboratories, the
454 LFIA can be easily implemented in wineries for monitoring potential OTA contamination of grape-derived
455 products by a simple, affordable, and visual approach.

456 **Declaration of Competing Interest**

457 None.

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463 The technical assistance by Paula Peña Murgui is greatly appreciated. Samples of the immunoreagents to
464 OTA herein reported are available upon request from the corresponding author.

465 **Appendix A. Supplementary material**

466 Supplementary material to this article can be found online at

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588 FIGURE CAPTIONS

589 Fig. 1. Structure of the different OTA haptens used in this study to prepare bioconjugates and to generate
590 monoclonal antibodies.

591 Fig. 2. Analytical parameters of the developed direct ELISA (A) and the immunochromatographic assay (B).
592 Data are the mean of four independent experiments. The LOD was calculated from the signal at 10%
593 inhibition. The working range was calculated from the signals at 20% and 80% inhibition.

594 Fig. 3. Inhibition pattern obtained for four different matrices with the developed competitive LFIA.

595

Table 1

IC₅₀ values (nM) obtained by checkerboard assay with anti-OTA mAbs using the icELISA and the dcELISA formats (n=3).^a

mAb	Hapten in the assay conjugate										
	a		b		c		d		e		f
	icELISA	dcELISA	icELISA	dcELISA	icELISA	dcELISA	icELISA	dcELISA	icELISA	dcELISA	icELISA
a#118	6.36	3.93	6.73	10.8	7.49	4.27	14.9				
a#122	4.05	5.09	3.34	1.53					2.01		5.99
a#125	3.93	1.99	5.74	2.02	3.29	1.70	2.92				
b#39	0.95	0.53	0.90	0.32	2.09	0.28	1.12	0.26			
b#41	1.69	1.11	2.23	1.25	3.57	1.52	2.28	1.57			
b#310	0.79	0.30	0.39	0.10	0.23		0.21		0.09		0.28
b#311	0.69	0.12	0.25	0.11	0.19		0.11		0.15		0.14
c#12					0.39	0.18	1.51	0.37			
c#29					0.69	0.43	1.84	0.48			
c#116	0.38	0.24	0.53	0.53	0.58	0.28	0.54	1.73			
c#117	0.10		0.07		0.58	0.20	0.30	0.25			
c#134					0.93	0.52	0.79	0.52			
d#16	0.47				0.27	0.67	0.21	0.29			
d#21	0.18		0.13		0.44	0.53	0.27	0.37			
d#27	1.16		1.19		0.96	0.87	1.15	0.56			
d#111	0.16		0.45		0.29	0.52	0.20	0.54			
d#114	0.46		0.59		0.85	0.84	0.39	0.24			
d#115					0.60	0.26	0.56	0.17			
d#118	0.62		0.45		1.09	0.33	0.46	0.26			
e#13	2.05	0.80	1.80	0.64	2.51				1.40	1.31	1.87
e#115	0.45		0.28						0.23	0.27	0.39
f#223	0.63	0.17	0.16	0.08					0.34	0.14	0.19

^a Maximum signal was between 0.6 and 1.4 absorbance units. Empty cells indicate that the conjugate was poorly or not recognized.

Table 2

Recovery values (%) obtained by the direct competitive ELISA with red and white wine and grape must samples fortified with OTA (n = 4).

Sample	[OTA]^a	Wine 1	Wine 2	Wine 3	Must 1	Must 2
Red	0.5	107 ± 6	102 ± 4	98 ± 15	121 ± 11	127 ± 12
	1	98 ± 9	100 ± 7	94 ± 12	106 ± 5	119 ± 7
	2	102 ± 14	94 ± 8	102 ± 8	118 ± 9	122 ± 2
	5	95 ± 20	98 ± 9	112 ± 11	107 ± 13	82 ± 6
	10	95 ± 13	101 ± 2	102 ± 3	93 ± 4	82 ± 7
	20	93 ± 6	95 ± 3	96 ± 6	95 ± 7	102 ± 1
	[OTA]	Wine 4	Wine 5	Wine 6	Must 3	Must 4
White	0.5	101 ± 12	94 ± 12	92 ± 24	116 ± 3	130 ± 5
	1	89 ± 10	94 ± 11	107 ± 13	98 ± 6	109 ± 8
	2	90 ± 8	94 ± 12	119 ± 17	106 ± 13	111 ± 2
	5	103 ± 14	94 ± 5	111 ± 2	101 ± 14	83 ± 1
	10	110 ± 4	104 ± 7	109 ± 8	86 ± 11	83 ± 10
	20	101 ± 6	91 ± 5	97 ± 3	86 ± 6	87 ± 7

^a Values are in ng/mL.

Table 3

LFIA validation study according to European Regulation (EU) No 519/2014.

Red wine				White wine

Spiked [OTA] ^a	False-suspect rate (%)			T/C ± sd	False-suspect rate (%)		
	T/C ± sd	1/4×STC	STC		1/4×STC	STC	
0.0	0.68 ± 0.09	<0.01	<0.01	0.83 ± 0.10	<0.01	<0.01	
0.5	0.15 ± 0.03	-	0.02	0.18 ± 0.03	-	0.03	
2.0	0.02 ± 0.01	-	-	0.02 ± 0.01	-	-	
<i>Cut-off</i>		0.20	0.03		0.24	0.04	
Red must				White must

Spiked [OTA] ^a	False-suspect rate (%)			T/C ± sd	False-suspect rate (%)		
	T/C ± sd	1/4×STC	STC		1/4×STC	STC	
0.0	0.72 ± 0.17	0.13	0.03	0.74 ± 0.20	0.97	0.13	
0.5	0.11 ± 0.01	-	<0.01	0.14 ± 0.04	-	1.09	
2.0	0.01 ± 0.01	-	-	0.02 ± 0.01	-	-	
<i>Cut-off</i>		0.13	0.02		0.22	0.03	

^a Concentration values are expressed in ng/mL.

Figure(s)

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Supplementary Material

Immunoanalytical methods for ochratoxin A monitoring in wine and must based on innovative immunoreagents

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Table S1. Cross-reactivity (%) of anti-OTA monoclonal antibodies to other ochratoxins.

mAb	OTB^a	OTC^b	mAb	OTB	OTC
a#118	4.5	4.8	d#16	1.7	-
a#122	3.1	12.6	d#21	2.2	-
a#125	0.1	- ^c	d#27	-	-
			d#111	87.9	0.6
b#39	-	-	d#114	-	-
b#41	1.0	1.1	d#115	58.7	1.3
b#310	0.7	-	d#118	18.1	-
b#311	1.1	0.3			
			e#13	5.1	404
c#12	1.0	-	e#115	51.3	55.4
c#29	7.5	-			
c#116	2.9	0.2	f#223	26.8	11.8
c#117	4.4	20.8			
c#134	13.2	-			

^a Ochratoxin B. ^b Ochratoxin C. ^c Cross-reactivity was lower than 0.1%.

Tabla S2. OTA contents in commercial bottled wines and grape musts as determined by the developed direct ELISA.^a

Sample ^b	[OTA]	Sample	[OTA]	Sample	[OTA]
RW-1	< LOD ^c	RW-30	< LOD	WW-9	< LOD
RW-2	< LOD	RW-31	0.28 ± 0.03	WW-10	< LOD
RW-3	< LOD	RW-32	< LOD	WW-11	< LOD
RW-4	< LOD	RW-33	< LOD	WW-12	< LOD
RW-5	< LOD	RW-34	< LOD	WW-13	< LOD
RW-6	< LOD	RW-35	< LOD	WW-14	< LOD
RW-7	< LOD	RW-36	< LOQ	WW-15	< LOD
RW-8	< LOD	RW-37	< LOD	WW-16	< LOD
RW-9	< LOD	RW-38	< LOD	WW-17	< LOD
RW-10	< LOD	RW-39	< LOD	WW-18	< LOD
RW-11	< LOD	RW-40	< LOD	WW-19	< LOD
RW-12	< LOD	RW-41	< LOD	WW-20	< LOD
RW-13	< LOD	RW-42	< LOQ	RM-1	0.49 ± 0.15
RW-14	< LOD	RW-43	< LOD	RM-2	1.38 ± 0.17
RW-15	0.34 ± 0.02	RW-44	< LOD	RM-3	0.65 ± 0.03
RW-16	< LOD	RW-45	< LOD	RM-4	0.36 ± 0.12
RW-17	< LOD	RW-46	< LOD	RM-5	0.40 ± 0.02
RW-18	< LOD	RW-47	< LOD	RM-6	0.36 ± 0.05
RW-19	< LOD	RW-48	< LOD	RM-7	< LOQ
RW-20	< LOD	RW-49	< LOD	RM-8	0.49 ± 0.10
RW-21	< LOD	RW-50	< LOD	RM-9	0.66 ± 0.04
RW-22	< LOD	WW-1	< LOD	WM-1	0.43 ± 0.11
RW-23	< LOD	WW-2	< LOD	WM-2	0.33 ± 0.06
RW-24	< LOD	WW-3	< LOD	WM-3	0.63 ± 0.03
RW-25	< LOD	WW-4	< LOD	WM-4	0.36 ± 0.06
RW-26	< LOD	WW-5	< LOD	WM-5	0.65 ± 0.09
RW-27	< LOD	WW-6	< LOD	WM-6	0.49 ± 0.03
RW-28	0.56 ± 0.04	WW-7	< LOD	WM-7	< LOD
RW-29	< LOQ ^d	WW-8	< LOD	WM-8	0.37 ± 0.02

^a Values in ng/mL are the mean of two independent determinations. ^b RW: red wine, WW: white wine, RM: red must, WM: white must. ^c Below the limit of detection of the method (0.16 ng/mL). ^d Below the limit of quantitation of the method (0.28 ng/mL).

Fig. S1. Chemical structures of ochratoxins.

Fig. S2. Chemical approaches for coupling the different haptens to the selected carrier proteins (BSA and OVA) and to HRP. Numbers in the conjugates indicate the hapten-to-protein molar ratios for each protein as determined by MALDI-TOF-MS analysis.

Fig. S3. Influence of pH and ionic strength over the A_{max} and IC_{50} values of the studied immunoassays.

Fig. S4. Influence of ethanol over the A_{max} and IC_{50} values of the studied immunoassays.

Fig. S5. Wine (A and B) and must samples (C and D) after 1/10 dilution in buffer and following treatment with 1% PVPP (wines) and 4% PVPP (grape musts).

Fig. S6. Matrix effect of red wine over the two selected competitive ELISAs after buffer dilution and clean-up with PVPP.

Fig. S7. Dependence of the LFIA sensitivity and maximum signal over the volume of GNP colloid used in the assay (10 µL or 20 µL) and the amount of antibody immobilized on the GNPs (1, 3, or 6 µg/mL). Bars are the mean and standard deviation of two independent experiments.

Fig. S8. Influence of pH, ionic strength, and ethanol over the $(T/C)_{\max}$ and IC_{50} values of the studied immunostrip.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: