EuroProxima

VALIDATION REPORT

Ochratoxin A ELISA

(According to the Commission Regulation (EU) No 519/2014)

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1. Introduction

Ochratoxin A is a nephrotoxic and nephrocarcinogenic mycotoxin produced by *Penicillium verrucosum* and *Penicillium viridicatum* in temperate and cold climates and by a number of *Aspergillus* species such as *A. ochraceus* in warmer and tropical areas of the world [1]. Ochratoxin A has been shown to occur in various cereals and other plant products, coffee beans and coffee products, wine, and feed. In the European Union maximum levels (MLs) for ochratoxin A have been set for different food commodities. The MLs vary from 0.5 to 20 µg/kg (ppb) depending on the food type [2]. The guidance limits for feed are 250 µg/kg for cereals and cereal products and 50 and 100 µg/kg for complete and complementary feedingstuffs for pigs and poultry, respectively [3].

Maximum limits for ochratoxin A in food in the EU:

Commodity	ML [ppb]	Commodity	ML [ppb]
Unprocessed cereals	5	Wine and grape juice	2
Processed cereals	3	Baby food and dietary food	0.5
Dried vine fruit	10	Pepper, nutmeg, ginger, turmeric	15
Roasted coffee	5	Chilli, cayenne, paprika	20
Instant coffee	10	Liquorice	20

A new ELISA for the detection of ochratoxin A was developed and validated. A new validation approach was used that complies with the Commission Regulation 519/2014 [4] for the validation of screening methods for mycotoxins. This new Regulation is specifically intended for bioanalytical methods such as ELISA and LFD. The concept of screening target concentration (STC) is used. STC is the concentration of interest for detection of the mycotoxin. For the purpose of this validation the STC was set to 2 ppb in cereals, 0.4 ppb in wine and must and 2.5 ppb in coffee and cocoa. The aim of the validation was to demonstrate the fitness of purpose of the OTA ELISA as a screening method for the detection of OTA at the set STC level and higher in different commodities.

The Regulation requires to determine 2 parameters: cut-off and false suspect rate. Cut-off is the concentration measured in a samples above which the sample is classified as “suspect”, meaning it may contain mycotoxin at a level higher than STC. Cut-off is calculated from the results obtained for 20 samples spiked at STC by using equation given in the Regulation. Another parameter that needs to be determined in the validation study is a false suspect rate. It is calculated based on the results obtained for 20 blank samples and 20 samples spiked at STC. The false suspect rate gives an estimation on how often the method will generate false suspect result. A 5% false suspect rate means that 1 sample in 20 can be wrongly classified as suspect. The lower the false suspect rate the better, as it means that fewer false suspect samples will require analysis by confirmatory method. As also advised by the Regulation, the samples should be spiked and analysed at other levels in order to determine how the method can distinguish between different mycotoxin concentrations.

For the purpose of this validation the STC was set at **2 ppb in cereals, 0.4 ppb in wine and must and 2.5 ppb in coffee and cocoa.** Cut-off and false suspect rate were determined. The samples were also spiked with other concentrations of ochratoxin A and analysed to determine the method recovery at different levels. When available, also quality control samples and reference sample were analyzed. Additionally (not required by the Commission Regulation 519/2014), detection limit (LOD) was calculated based on 20 blank samples for each matrix tested.

2. Kit characteristics: see manual
The reactivity pattern of the antibody.
Cross- reactions:
Ochratoxin A 100%
Ochratoxin B 18%

3. Scope of validation
The validation study was carried out with the Ochratoxin A ELISA (5121OTA). The study included:
- Determination of a limit of detection (LOD) for ochratoxin A toxin in cereals (wheat and corn), wine: red and white, must, coffee: roasted, instant and green, and cocoa.
- Determination of cut-off and false suspect rate at screening target concentration (STC) 2 ppb for cereals, 0.4 ppb for wine and must, and 2.5 ppb for coffee and cocoa.
- Determination of the % recovery in cereals, wine, must, coffee and cocoa.
- Determination of inter- and intra-assay coefficients of variation.

4. Sample material
The validation study included four to five different samples of the following non-contaminated matrices: wheat, corn, red wine, white wine, must, ground roasted coffee, instant coffee, green coffee, and cocoa.

5. Sample treatment
Sample preparation was performed according the manual 5121OTA[1]03.17, procedures 8.1-8.5.

6. Results
For determination of LOD, cut-off and recovery samples were spiked with ochratoxin A purchased from LGC, product number B-MYC0490-5.

Table 1. LOD determined for different matrices.

Matrices	Wheat	Corn	Red wine	White wine	Must	Roasted coffee	Instant coffee	Green coffee	Cocoa
LOD [ppb]	1.7	1.4	0.3	0.3	0.3	1.9	1.8	1.2	1.7

LOD: mean of 20 blanks + 3 x SD

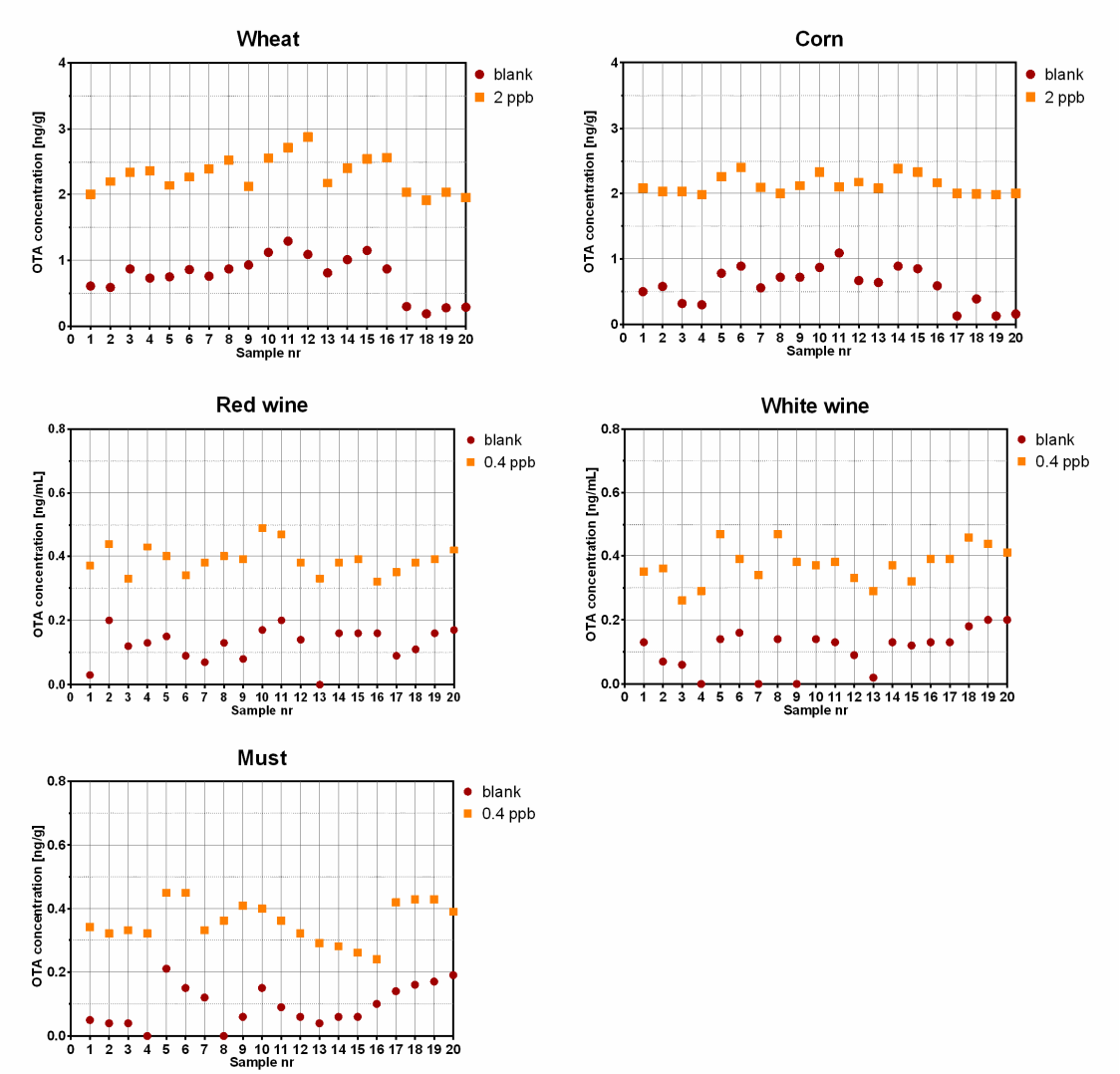

Fig. 1. Results obtained for 20 blank samples and 20 samples spiked at standard screening concentration (STC) 2 ppb for cereals and 0.4 ppb for wine and must.

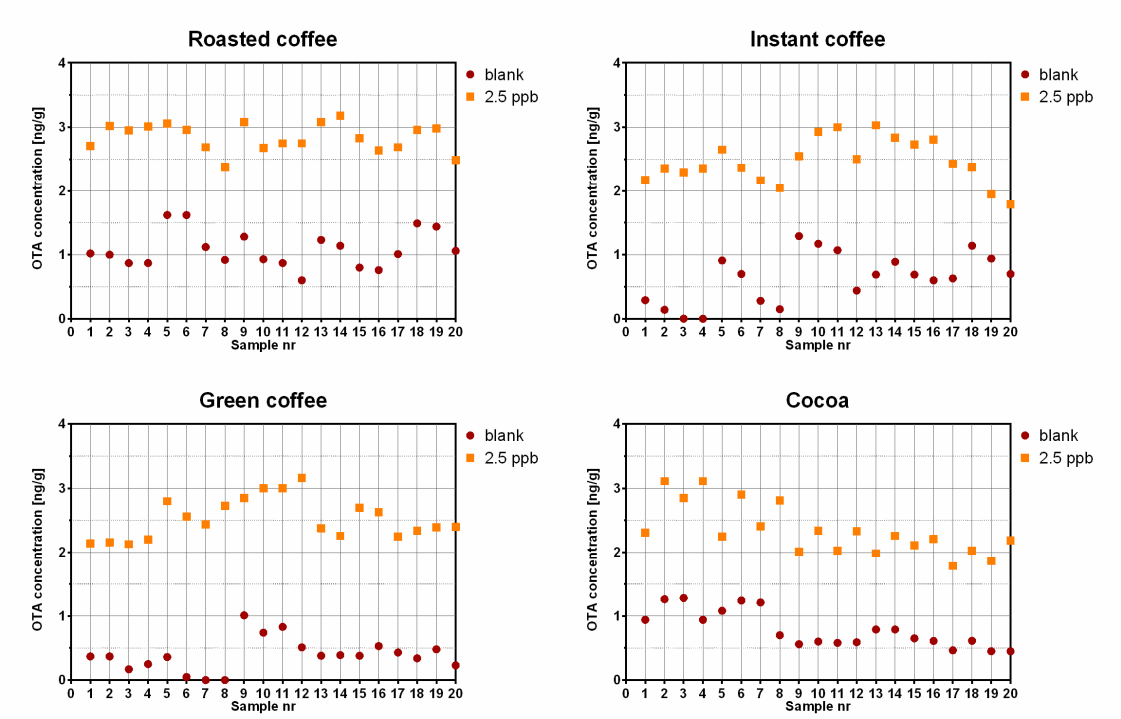

Fig. 2. Results obtained for 20 blank samples and 20 samples spiked at standard screening concentration (STC) 2.5 ppb for coffee and cocoa.

Table 2. Results of the validation study according to the Commission Regulation (EU) No 519/2014.

Matrices	Wheat	Corn	Red wine	White wine	Must	Roasted coffee	Instant coffee	Green coffee	Cocoa
STC [ppb]	2	2	0.4	0.4	0.4	2.5	2.5	2.5	2.5
Cut-off [ppb] (n=20)	1.8	1.9	0.3	0.3	0.2	2.5	1.9	2	1.7
False suspect rate [%]	<1	<1	<1	1	1	<1	<1	<1	<1

STC: screening target concentration, the concentration of interest for the detection of ochratoxin A

Cut-off: the concentration above which samples is classified as suspect, cut-off = mean of spiked samples – 1.729 x SD (at STC)

False suspect rate: probability of false suspect samples for a calculated one-tailed t-value: t-value = (cut-off - $mean_{blank}$)/SD_{blank}

Table 3. Recovery [%] of ochratoxin A in cereals.

Sample	Wheat	Corn
Spiked at 2 ppb*	115.4 ± 13.6	106.5 ± 7.3
Spiked at 3 ppb**	98.7 ± 6.5	104.9 ± 5.2
Spiked at 6 ppb**	95.7 ± 4.2	95.0 ± 1.4
Reference wheat sample 3.3 ± 0.7 ppb**	101.8 ± 3.2	
Reference wheat sample 9.4 ± 1.4 ppb**	95.8 ± 2.6	
Reference corn sample 2.9 ± 0.4 ppb**		102.3 ± 4.2
Reference corn sample 12.3 ± 1.3 ppb**		92.0 ± 6.1

* n=20
** n=6

Table 4. Recovery [%] of ochratoxin A in wine and must

Sample	Red wine	White wine	Must
Spiked at 0.4 ppb*	97.1 ± 11.2	93.0 ± 14.6	89.0 ± 15.7
Spiked at 1 ppb**	97.0 ± 15.2	104.0 ± 7.6	67.6 ± 8.5
Spiked at 2 ppb**	94.4 ± 5.0	94.4 ± 7.5	93.9 ± 8.6
Spiked at 4 ppb**	87.7 ± 10.6	94.0 ± 7.3	89.5 ± 7.6
QC FAPAS white wine sample 2.34 ppb**		92.2 ± 3.0	
QC FAPAS white wine sample 0.91 ppb**		113.4 ± 11.1	
QC FAPAS white wine sample 1.23 ppb**		75.7 ± 12.0	
QC FAPAS white wine sample 1.02 ppb**		87.6 ± 7.0	

* n=20
** n=6

Table 5. Recovery [%] of ochratoxin A in coffee and cocoa.

Sample	Roasted coffee	Instant coffee	Green coffee	Cocoa
Spiked at 2.5 ppb*	113.7 ± 8.6	98.7 ± 14.0	101.0 ± 12.7	93.8 ± 16.1
Spiked at 5 ppb**	112.3 ± 2.6	106.2 ± 2.6	104.0 ± 2.0	75.5 ± 2.8
Spiked at 10 ppb**	102.6 ± 2.5	96.0 ± 1.7	100.5 ± 3.2	78.7 ± 2.8
QC FAPAS roasted coffee 4.87 ppb**	134.0 ± 3.2			
QC FAPAS roasted coffee 3.29 ppb**	121.3 ± 6.3			
QC FAPAS green coffee 7.53 ppb**			119.6 ± 4.5	
QC FAPAS green coffee 6.24 ppb**			97.1 ± 2.0	

* n=20
** n=6

Table 6. Inter-assay coefficient of variation for 0.125 ng/ml ochratoxin A standard.

Sample	Mean OD ± SD* at 450nm	CV (%)
0.125 ng/mL standard	1.256 ± 0.135	10.8

*Mean of 10 results in duplicate

Table 7. Intra-assay coefficient of variation for buffer spiked at 0.125 ng/mL with ochratoxin A.

Sample	Mean OD ± SD* at 450nm	CV (%)
Buffer spiked at 0.125 ng/mL	1.035 ± 0.084	8.1

*Mean of 40 samples

7. Literature

1. Kuiper-Goodman. Risk assessment of the mycotoxin Ochratoxin A. *Biomedical and Environmental Sciences* 1989, **2**, 179-248.
2. Commission Regulation (EC) 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Official Journal of the European Union* **L364**, 1-26.
3. Commission Recommendation 2006/576/EC of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding. *Official Journal of the European Union* **L229**, 7-9.
4. Commission Regulation (EU) No 519/2014 of 16 May 2014 amending Regulation (EC) No 401/2006 as regards methods of sampling of large lots, spices and food supplements, performance criteria for T-2, HT-2 toxin and citrinin and screening methods of analysis. *Official Journal of the European Union* **L147**, 29-43.