APPLICATION NOTE

Liquid Chromatography

Authors

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Determination of mycotoxins in food by LC and LC/MS

Introduction

Mycotoxins are secondary metabolites produced by certain types of mould. These molecules are highly toxic to all animal organisms, which have harmful effects even at very low doses (neurotoxic, nephrotoxic, hepatotoxic, enterotoxic, immunosuppressive, and teratogenic action). The contamination of food (especially those of vegetable origin) by mycotoxigenic fungi can cause an accumulation of these metabolites in feed or food, jeopardizing the safety and wholesomeness of use.

The contamination can occur in the field, but also during the subsequent phases of transportation, storage and/or processing, when the environmental conditions of temperature and humidity able to develop fungal spores naturally present in the environment, resulting in the production of mycotoxins.

Mould	Development Conditions
Aspergillus spp.	After collection Field
Penicillium spp.	After collection Field
Fusarium graminearum, F. culmorum, F. sportrichioides	Field
F. verticilioides, F. proliferatum	Field

Tab 1.

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Since these substances may be present not only in food but also in animal feed, human exposure to contamination may occur not only through the direct consumption of contaminated food, but also consuming animal products, such as milk, in case of presence of mycotoxins in the animal feed used.

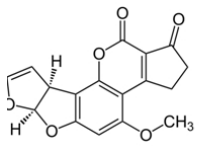
Mycotoxigenic mould belong to Penicillium, Aspergillus and Fusarium species and the type of toxins synthesized varies depending on the fungal genus and species considered.

The high toxicity of mycotoxins, even in very low concentrations (in the order of ppb), makes them essential to be controlled in those foods where it is most likely the development of toxigenic mould (cereals, nuts, milk, and coffee). The classes of mycotoxins that frequently are found in foods are aflatoxins, ochratoxins, fumonisins, trichothecenes and zearalenone.

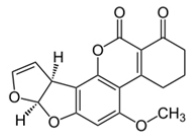
Funghi produttori e substrati potenzialmente contaminati dalle principali micotossine

Micotossina	Funghi produttori	Prodotto contaminato
Aflatossine B_1, B_2, G_1, G_2	*Aspergillus flavus, A.parasiticus*	Mais, frutta secca
Aflatossina M_1		Latte
Ocratossina A	*Aspergillus ochraceus, Penicillium verrucosum*	Cereali, caffè, uva, frutta secca
Patulina	*Penicillium spp., Aspergillus spp.*	Succhi di mela
Tricoteceni	*Fusarium graminearum, F.culmorum, F.sporotrichioides*	Cereali
Zearalenone	*Fusarium graminearum, F.culmorum, F.sporotrichioides*	Cereali
Fumonisine	*F.verticilioides, F.proliferatum*	Mais

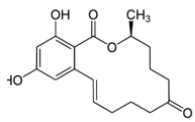
Tab 2.



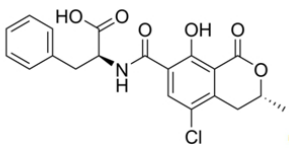
Aflatoxin B1



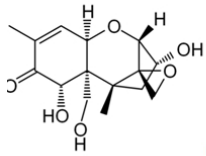
Aflatoxin G1



Zearalenone



Ochratoxin A



Deoxynivalenol

Regulations

Mycotoxin control in foods is regulated at Community level by Reg. (EC) No. 1881/2006 (as amended) concerning the presence of contaminants in food.

The regulation sets the maximum contaminant limits (expressed in µg/kg), which vary depending on the quality of toxin and food at issue.

The table below shows the minimum allowed by law (Reg. EC 1881/2006) for the presence of mycotoxins in different food matrices with the exception of foods consumed by infants and children.

Methods of analysis

HPLC with Conventional Detector

Some mycotoxins are naturally fluorescent (B2, G2, M1,...) or can be easily made through such a chemical derivatization

	Toxin	Lowest conc.	LC Detectror
Aflatoxins (Aspergillus spp.)	B1	2 ppb for B1	FL + deriv.
	B2	4 ppb for (B1+B2+G1+G2)	FL
	G1		FL + deriv.
	G2		FL
	M1	0,05 ppb	FL
Aspergillus and Penicil-lium spp.	Ocratoxin A	2 ppb	FL
	Patuline	10 ppb	UVVIS
Fusarium spp.	Deoxynivalenol	200 ppb	UVVIS
	Zearalenone	75 ppb	FL/UVVIS
	B1+B2 Fumonisins	800 ppb	FL + deriv.
	T2+HT2 Toxin		FL + deriv.

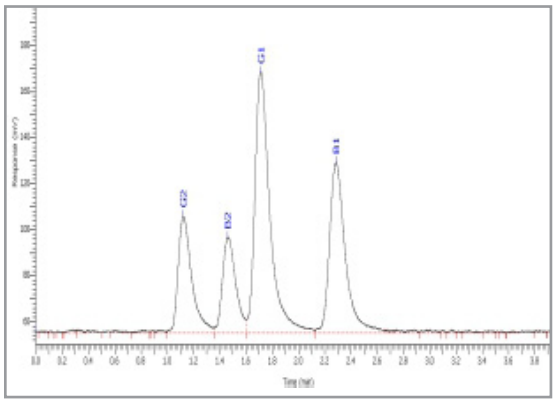
Tab 3. (*) The limit for T2 and HT2 Toxins is not shown in the table as it has not been introduced yet a limit by Law

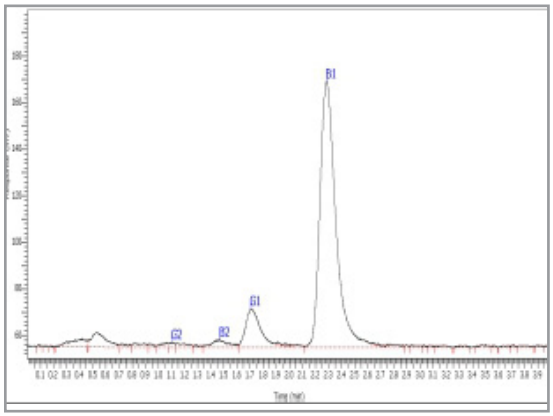
(Fumonisins) or by UV radiation. For these molecules is normally used a fluorescence detector. In other cases, mycotoxins can be detected by a common UV-VIS detector. Using a fluorimetric detector has the indubitable advantage of high sensitivity but it is sometimes necessary to perform a pre- or post-column derivatization process.

A typical pre-column derivatization is that which is obtained through the use of TFA while the most widespread systems of post-column derivatization are: Pickering, KobraCell or UV photo-derivatization devices.

In some cases, the addition of derivatization reagents is directly performed in the mobile phase, making it difficult to use a gradient HPLC system when a final determination of more toxins in the same chromatographic run is needed.

As a result, in order to quantify mycotoxins within the detection limits specified in Table 3, it is necessary an HPLC system equipped with UV-VIS and Fluorimetric Detectors as well as derivatization system.





Instrumental Configuration:
UHPLC Flexar FX-10
Flexar Autosampler
FL Flexar Detector
Chromera CDS

Flexar FX 10

Kobra Cell™:	100 µA
Analytical Column:	Brownlee Pinnacle DB C18 HPLC Column, 1.9 µm, 50 mm × 2.1 mm i.d.
Mobile phase:	Water / Acn / MeOh 75-10-15 + 119mg KBr + 350µl HNO3 4M
Flow:	0,7 ml/min; 6000 psi
Fluorescence Detector:	Excitation 362 nm
	Emission 435 nm
	Emission Bandwidth: Wide
Injection Volume:	2 µl
Total Run Time:	3 min.

HPLC with MS Detector

In order to deal with a complete analysis of mycotoxins reported in Table 3 by means of an HPLC system equipped with a conventional detector, it is necessary an instrumental configuration consisting of several detectors. Similarly, it is occasionally necessary to operate with different methods depending on the toxin to be determined in a specific matrix.

The use of a universal detector such as the MS detectors allow developing a single analytical method without resorting to any system of derivatization. The MS detector identifies molecules exploiting the ions generated by them when subjected to a process of ionization.

This results, generally, in the determination of their molecular ions or adducts dependent on their chemical nature and the composition of the mobile phase.

Each toxin is analyzed in the most appropriate ionization method: ESI + o ESI -

Sample Preparation

In the case of analysis of mycotoxins in foodstuffs, sampling plays a crucial role and is the subject of a Special Regulation (Reg (EC 401/2006). In the process of sampling, it is necessary to give particular attention due to the imperfect homogeneity of the sample.

Once collected, the sample is subjected to the analytical process that meets the criteria set by Reg. (EC) 401/2006 and Reg. (EC) 882/2004 Annex III sets out the criteria for evaluating the method used.

Assuming that the sampling is done properly, it is necessary to proceed with the preparation of the sample. In the case of mycotoxins, it can be usually followed two ways:

- Use of immunoaffinity columns (IAC)
- Use of SPE columns

Immunoaffinity Columns: IACs are based on the use of mono- or polyclonal antibodies suitably immobilized on a solid phase. These are very selective and allow a fast extraction of mycotoxins from usually complex food matrices. Normally the sample, prior to being loaded on the immunoaffinity column is

homogenized, extracted (e.g. using methanol/water) and then diluted. Before eluting analytes with a suitable solvent (methanol), the column is washed with suitable buffers. The methanol phase can be generally further concentrated to improve its detection limits. IAC columns can be used only once.

SPE (Solid Phase Extraction): SPE columns represent an alternative to those of immunoaffinity. These are generally cheaper but at the same time less specific and selective. Their use provides an activation phase with methanol before loading the sample. Subsequent washing and elution release toxins adsorbed.

Analytical Method

Instrumentation Used:
UHPLC Flexar FX-15 PerkinElmer
Flexar Autosampler
Degassing System
Flexar Peltier Column Oven
SQ 300 MS with ESI
Chromera CDS

Flexar FX 15

Chromatographic and MS methods

The chromatographic method provides a unique analytical run able to separate and determine all mycotoxins indicated in Table 3 operating both in positive and negative ionization (with the exception of the patulin because of its poor ionizability can not be quantified within the limits indicated by law using this method, which requires a dedicated sample preparation).

UHPLC	MS Detector
Analytic Column: HRes DB AQ C18 (1.9 um,100 mm, 2.1 mm id – p/n N9303919)	Cylinder Lens: - 4000V
Mobile phase	End Plate.-5000V
A. Ultrapure water for LCMS + 0.1% v/v HCOOH	Capillary entrance: -6000 V
B. Acetonitrile + 0.1% v/v HCOOH	End Plate Temp.: High
Linear Gradient	Drying gas Temp.: 350°C
Step 0: Equilibration time 4’ – 90%A – 10%B	Drying gas Temp.: 12 L/min
Step 1: Run Time 12’ – 38%A – 62%B	Nebulizer gas pressure: 80 psi
Step 2: Run Time 4’ – 38%A – 62%B	
Flow: 0.65 mL/min	
Column temperature: 50°C	
Injection volume: 5 uL	

Tab 4.

Toxin	Ionization Mode	Capillary Exit (V)	Ion		Dwell Time	Tr (min)
B1	ESI +	120	313.0	(M+H)+	100	6.43
B2	ESI +	120	315.0	(M+H)+	100	6.05
G1	ESI +	120	329.1	(M+H)+	100	5.62
G2	ESI +	120	331.0	(M+H)+	100	5.28
M1	ESI +	120	329.1	(M+H)+	100	4.92
Ochratoxin A	ESI-	-90	402.2	(M-H)-	100	11.91
Deoxynivalenol	ESI-	-60	341.0	(M+HCOO)-	100	1.51
Zearalenone	ESI-	-90	317.2	(M-H)-	100	11.74
FB1+FB2	ESI +	120	FB1= 722.4 FB2= 706.3	(M+H)+ (M+H)+	200 200	11.22 14.73
T2+HT2 Toxins	ESI +	120	T2= 447.0 HT2=489.0	(M+Na)+ (M+Na)+	100 100	8.25 6.80

Tab 5.

The V value of the "capillary exit" has been optimized for each chemical species analyzed in order to allow better qualitative/quantitative analysis using the "RAMP" function. This feature allow finding the optimal value of "capillary exit" verifying as the signal intensity varies with the variation of applied potential.

Standard Solutions

Standard mixtures in contraction varying from 1 to 1000 ppb (1, 10, 100 and 1000 ppb) for each analyte were prepared, starting from a certificate standard (Biopure TM) in a Water/ Acetonitrile (1:1) + 0.1% Formic Acid mixture.

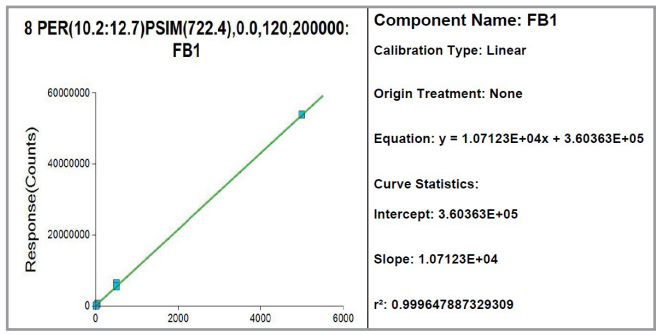
Toxin	Standard 1 (ppb)	Standard 2 (ppb)	Standard 3 (ppb)	Standard 4 (ppb)	Limit (ppb)
B1	//	0.5	5.0	50.0	2
B2	0.05	0.5	5.0	50.0	4 for (B1+B2+G1+G2)
G1	0.05	0.5	5.0	50.0	
G2	0.05	0.5	5.0	50.0	
M1	//	0.5	5.0	50.0	0.05
Ochratoxin A	1.0	10.0	100.0	1000.0	2
Deoxynivalenol	1.0	10.0	100.0	1000.0	200
Zearalenone	1.0	10.0	100.0	1000.0	75
FB1 and FB2	5.0	50.0	500.0	5000	800
T2+HT2 Toxins	5.0	50.0	500.0	5000	

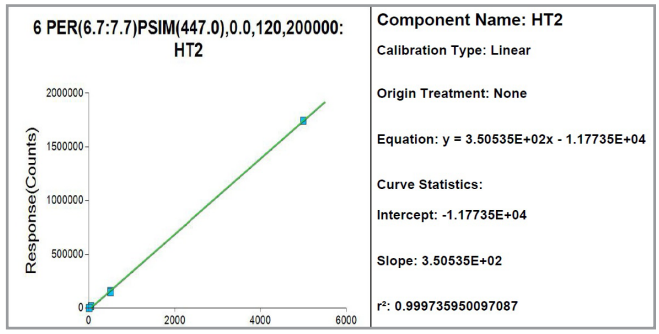
Tab 6.

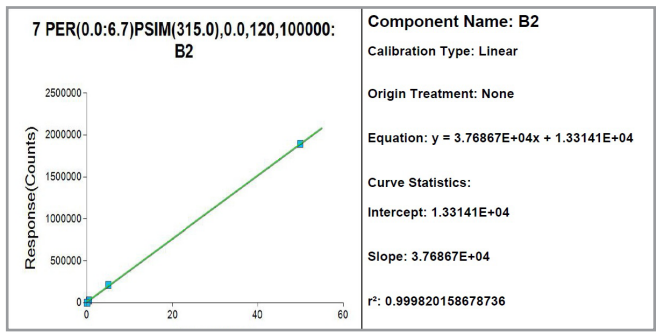
The range of the calibration curves was evaluated according to Table 3 considering the sample preparation.
Calibration curves and the results have been obtained without any pre-concentration performed during the sample preparation. It is therefore possible to reduce approximately ten times the sensitivity limits of the method using sample pre-concentration and possibly increasing the volume of injection.

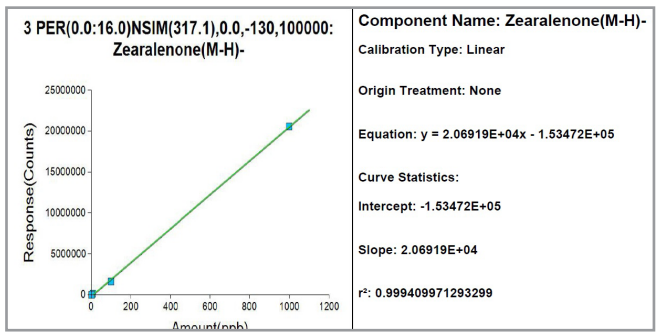
Calibration Curves

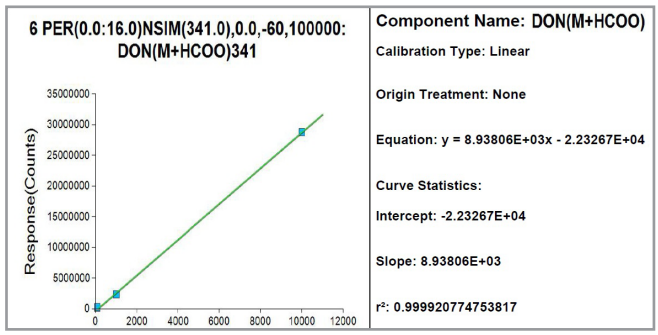
Here below some calibration curves are showed by way of example:





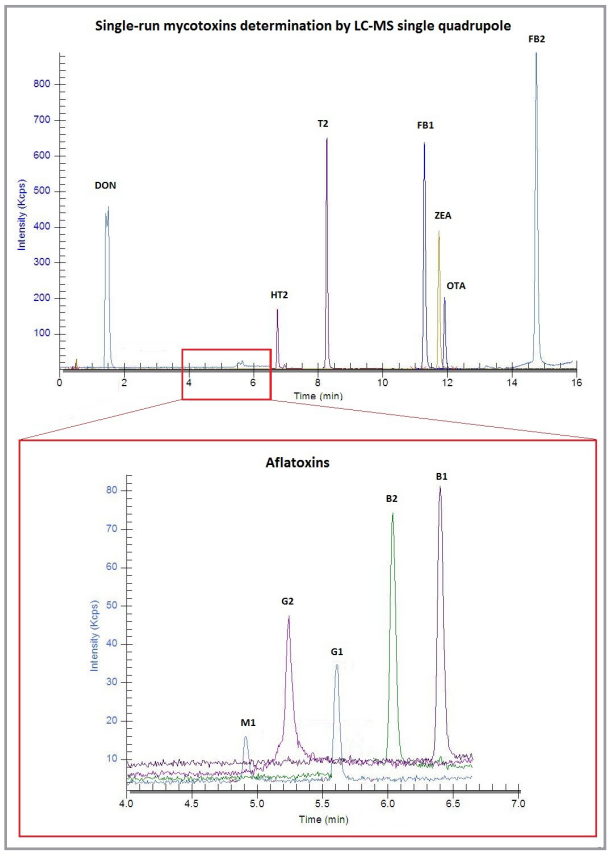


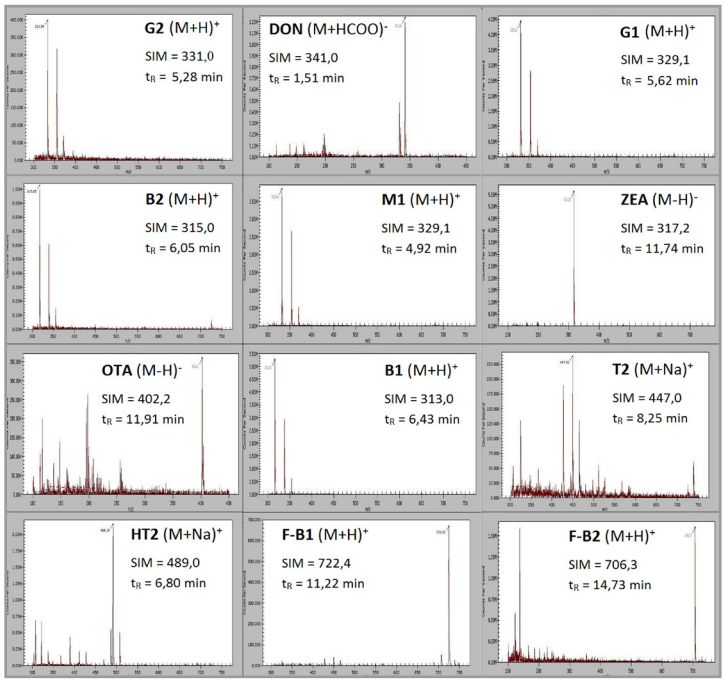




MS Chromatograms and Spectra

This chromatogram shows chromatographic peaks of each mycotoxin analyzed. The response is different due to the different chemical nature; in the case of Aflatoxins (chromatogram in the bottom), the scale has been expanded to better highlight the chromatographic signal.





Sample Analysis

Certified samples were analyzed as follow:
- Peanuts for Aflatoxins
- Corn Flour for DON (Biopure TM)

The samples of peanuts were analyzed with two different methods of preparation: through specific immunoaffinity columns (Romer Labs Diagnostic GmbH) and using SPE columns (Supra-Clean C18 - 500 mg/3 ml p/n N9306438). For corn flour and milk were used IACs.

Procedures specified by the manufacturer have been used with regards to IACs, while SPE columns have been performed following the extraction and purification protocol:

- Place 5 g of sample in a 100 mL beker and add a 100 mL solution containing water/methanol 3:2 and 2 g of NaCl.
- After 45' stirring, leave the sample to precipitate. Centrifugate for 15'.
- Pass 10 mL of the supernatant on SPE column previously activated with passages of methanol (2 x 3 mL) and distilled water (2 x 3 mL) using a suitable vacuum manifold.
- Leave to dry for several minutes under vacuum
- Wash with 3 mL of a water/methanol mixture 9:1.
- Leave to dry for several minutes under vacuum.
- Elute the SPE column in 3 mL of pure methanol.
- Leave to dry the solution thus collected in a nitrogen stream;
- Re-suspend with 0.5 mL of a water/acetonitrile solution 1:1.

The sample is now ready for analysis.

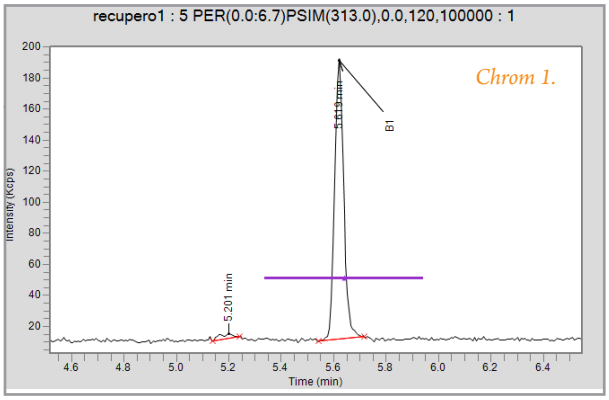
Peanuts:
The sample was found to contain only the B1 Aflatoxin.

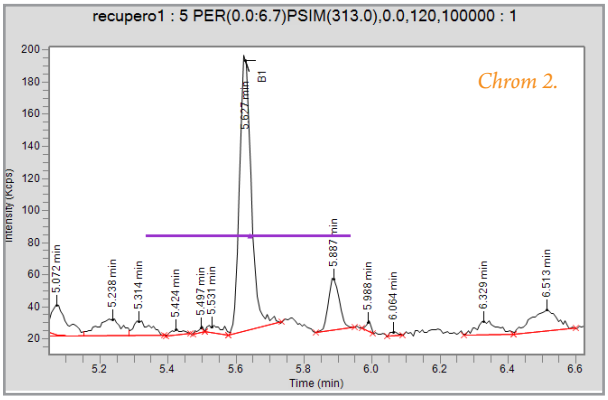
The analysis conducted on the sample of Peanuts showed no significant difference in terms of results even when conducted with the two extraction techniques indicated.

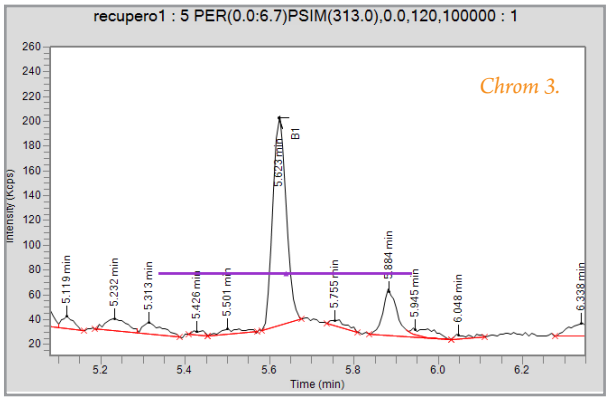
As for the specific case, the results also show that by varying the type of the washing solution do not have a significant change in the measured value.

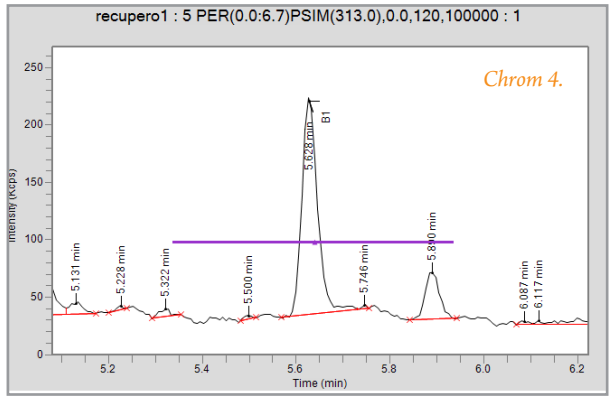
In particular, the following values were obtained:

B1 Aflatoxin	With IAC	With SPE Washing following the procedure	With SPE Without wash	With SPE Washing with water only
	14.2 ppb	13.8 ppb	14.45	12.1
Chromatogram	1	2	3	4



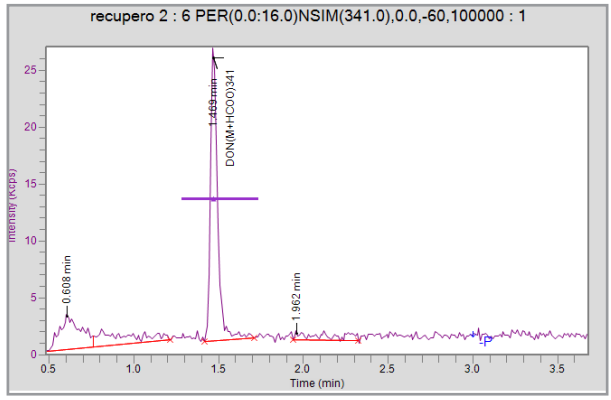






The samples of Corn Flour were analyzed using SPE columns.

One chromatogram is reported below. The sample recovery for DON has been roughly in the range 80-85%.



Conclusion

This method shows how a Single quadrupole can be a valid alternative to a more complex hardware configuration. Furthermore its sensitivity allows to reach the lowest concentration according to the current Regulation (Tab. 3).

The results have been obtained with a standard sample preparation without any sample concentration after the extractions. In some case a sample concentration can be useful in order to achieve better result in terms of LOD and LOQ.

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