APPLICATION NOTE

Liquid Chromatography/ Mass Spectrometry

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Simple and Sensitive Method for Determination of Ochratoxin A in Wine by LC/MS Using QSight Technology

Introduction

Ochratoxin A (OTA) is a secondary metabolite produced by *Penicillium* and *Aspergillus* fungi species found in soil and organic matter, and is one of the most widely reported of the nearly 400 recognized mycotoxin contaminants of agricultural products.[2] OTA (Figure 1) is a derivate of phenylalanine, which is amide-linked to a dihydro-coumarin group linked to a para-chlorophenolic moiety (IUPAC: (2S)-2-[[(3R)-5-chloro-8-hydroxy-3-methyl-1-oxo-3,4-dihydroisochromene-7-carbonyl] amino]-3-phenylpropanoic acid).[3] This mycotoxin poses toxicity concerns to the renal, hepatic, cellular and immune systems, and has been classified by the International Agency for Research on Cancer as a possible human carcinogen (group 2B).[4] OTA can enter the human food chain through contaminated feed, primarily cereal-based, that is ingested by animals that are farmed for human consumption.[5,6,7] The presence of OTA in wine is mainly a result of contamination of the grapes by some species of *Aspergillus*, in particular, the strains of *A. carbonarius* and *A. niger*.[8,9]

Current analytical methods for the sensitive determination of OTA in wine use commercially available immunoaffinity columns (IACs) as a sample clean-up tool. IACs allow a highly selective isolation of the analyte from a complex matrix, however, they suffer from some disadvantages associated with their relatively high cost and their limited storage time. However, compared to traditional liquid–liquid extraction, this technique is significantly less time-consuming and less error-prone.[9]

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In this work, a rapid and sensitive analytical method for the determination of the extent to which OTA residues contaminate wine is presented.

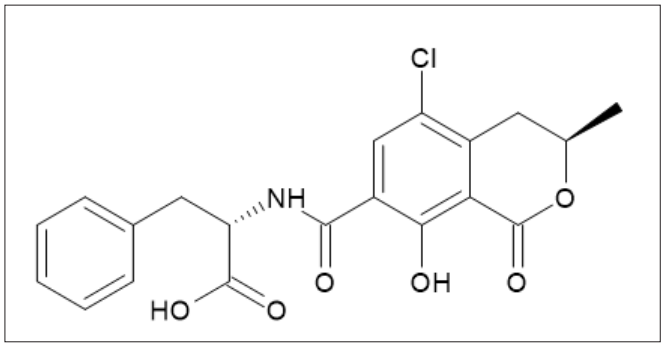

Figure 1. Ochratoxin A (OTA), IUPAC name : (2S)-2-[[(3R)-5-chloro-8-hydroxy-3-methyl-1-oxo-3,4-dihydroisochromene-7-carbonyl]amino]-3-phenylpropanoic acid.

Experimental

Hardware and Software

Chromatographic separation was performed in reversed phase condition using the PerkinElmer QSight® LX-50 ultra high performance liquid chromatography (UHPLC) system. Subsequent detection was achieved with the QSight 220 tandem mass spectrometer (MS), equipped with both ESI and APCI ionization sources. All instrument control, analysis and data processing were performed using the Simplicity™ 3Q software platform.

Standard Solutions

A Stock solution at 0.20 mg/mL was prepared by dissolving 5 mg of OTA in 25 mL of methanol. The standard working solutions were prepared by serial dilution, and the resulting calibration levels were: 0.1 – 0.5 – 1.0 – 5.0 - 10 µg/L in methanol.

Sample Preparation

Ochratoxin A extraction from wines was performed as follows:

1. 0.5 gr of NaCl was added to 5 mL of wine and shaken for 10 seconds.
2. 25 mL of ethyl acetate was added and mixed on a shaker for 15 minutes.
3. The extract was centrifuged for two minutes at 3000 rpm.
4. 5 mL of ethyl acetate extract was evaporated to dryness on a rotary evaporator.
5. The extract was reconstituted with 1 mL of methanol for LC/MS analysis.

Method Parameters

The LC method and MS parameters are presented in Table 1 and 2, respectively. Table 2 is further divided into Table 2a (MRM transitions with their respective optimized voltages) and Table 2b (MS source parameters). The MRM transitions, collision energies (CE), entrance voltages (EV) and collision cell lens 2 (CCL2) for OTA were detected and optimized by direct infusion of the standards. MS source conditions such as drying and nebulizer gas flow and temperature settings were optimized by a flow injection analysis (FIA) method.

Table 1. HPLC conditions.

Step	Time (min)	Flow Rate (mL/min)	%A	%B	Curve
1	Initial	0.3	95	5	
2	0.2	0.3	95	5	Linear
3	10.0	0.3	0	100	Linear
4	12.5	0.3	0	100	Linear
5	12.6	0.3	95	5	Linear
6	15.0	0.3	95	5	Linear
Mobile Phase A	0.1 % Formic Acid in Water				
Mobile Phase B	0.1 % Formic Acid in Methanol				
Column Oven Temperature	40 °C				
Autosampler Temperature	30 °C				
Injection Volume	10 µL				

Table 2. MS conditions.

Parameter	Setting Value
Ionization Mode	ESI Negative
Drying Gas Setting	100
HSID Temperature (°C)	275
Nebulizer Gas Setting	300
Electrospray Voltage (V)	-5000 V
Source Temperature (°C)	300

Table 3. MRM conditions.

Compound Name	Polarity	Compound Name	Polarity	CE (V)	EV (V)	CCL2 (V)
OTA	Negative	402.1	167	50	6	-44
OTA	Negative	402.1	358	26	18	-36
OTA	Negative	402.1	211	35	10	-40

Results and Discussion

Calibration

The optimized method was used to quantify OTA spiked into various matrices. A typical OTA chromatogram obtained from a 2 µg/L standard solution injection is shown in Figure 3.

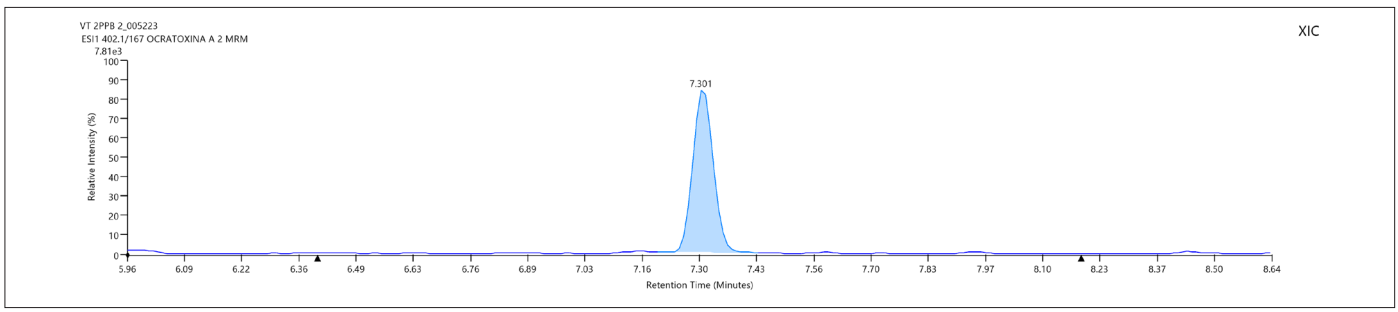

Figure 3. Typical Chromatogram of OTA at 2.0 µg/L.

Calibration curves were made with concentrations ranging between 0.1 µg/L to 10.0 µg/L. Standard solutions were made by serial dilutions in methanol. During the study, no matrix effect was observed between the standard solutions and the spiked wine samples.

A linear regression equation and r^2 values for the curves are given in Tables 4, 5 and 6. For each curve, using a linear fitting and a weighting factor of 1/x, a correlation coefficient r^2 over 0.99 was obtained.

Table 4. Calibration Table for Day 1.

Inj#	Sample	Sample Type	Know Conc. (ppb)	Peak Area	Conc. By Area (ppb)	Sample Accuracy % (Area)	Ion Ratio 1 (358/167) Area	Ion Ratio 2 (211/167) Area	Retention Time (min)
1	0.1 ppb_141226	Std	0.1	1480	0.083	82.74	0.39	0.48	7.311
2	0.5 ppb_141226	Std	0.5	6033	0.554	110.81	0.38	0.48	7.301
3	1.0 ppb_141226	Std	1.0	11185	1.087	108.74	0.41	0.49	7.311
4	5.0 ppb_141226	Std	5.0	47963	4.895	97.89	0.39	0.48	7.301
5	10.0 ppb_141226	Std	10.0	91097	9.981	99.81	0.40	0.49	7.301

Table 5. Calibration Table for Day 2.

Inj#	Sample	Sample Type	Know Conc. (ppb)	Peak Area	Conc. By Area (ppb)	Sample Accuracy % (Area)	Ion Ratio 1 (358/167) Area	Ion Ratio 2 (211/167) Area	Retention Time (min)
1	0.1 ppb_201828	Std	0.1	559	0.098	97.552	0.39	0.54	7.311
2	0.5 ppb_203341	Std	0.5	2194	0.475	95.029	0.38	0.43	7.301
3	1.0 ppb_204854	Std	1.0	4933	1.108	110.77	0.41	0.44	7.311
4	5.0 ppb_210407	Std	5.0	20684	4.745	94.906	0.39	0.39	7.311
5	10.0 ppb_211920	Std	10.0	44192	10.174	101.743	0.40	0.41	7.311

Table 6. Calibration Table for Day 2.

Inj#	Sample	Sample Type	Know Conc. (ppb)	Peak Area	Conc. By Area (ppb)	Sample Accuracy % (Area)	Ion Ratio 1 (358/167) Area	Ion Ratio 2 (211/167) Area	Retention Time (min)
1	0.1 ppb_074314	Std	0.1	1294	0.099	99.007	0.32	0.49	7.301
2	0.5 ppb_075817	Std	0.5	5965	0.491	98.193	0.42	0.46	7.301
3	1.0 ppb_081341	Std	1.0	12498	1.039	103.917	0.41	0.47	7.301
4	5.0 ppb_082853	Std	5.0	58716	4.917	98.349	0.42	0.49	7.301
5	10.0 ppb_084407	Std	10.0	119921	10.053	100.534	0.42	0.50	7.301

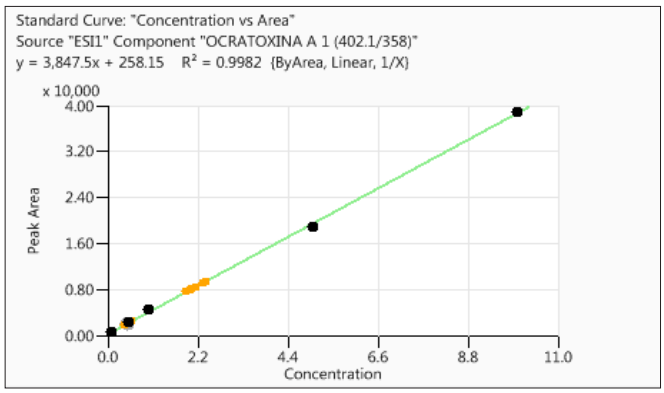

Figure 3. Calibration curve for Day 1.

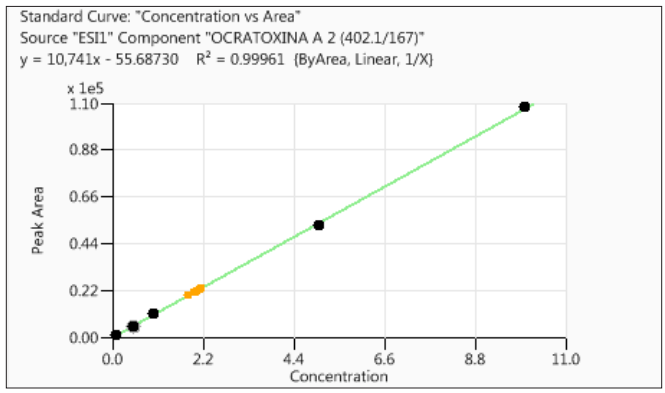

Figure 4. Calibration curve for Day 2.

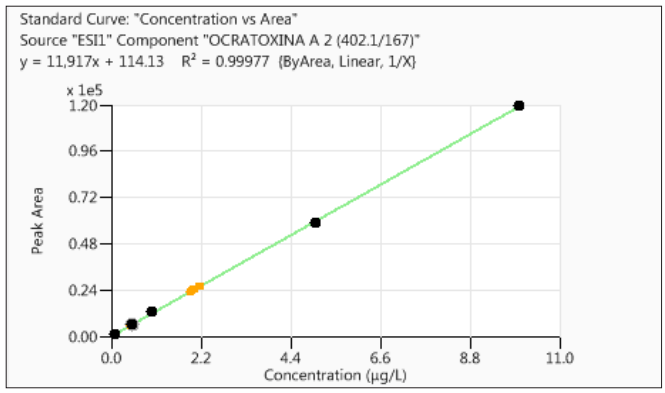

Figure 5. Calibration curve for Day 3.

Method Validation

To evaluate the method, a method validation protocol described in the document EUR 24105 EN, which was designed for food contact materials, was applied.

Method validation was conducted at two concentration levels, 0.5 and 2 µg/L, by characterizing linearity, selectivity, accuracy (recovery), intermediate precision and uncertainty. The validation was carried out along three sequences of analysis between different days (4 sample preparations per day and for each level), obtaining a total of 12 results per level. White and red wines were validated separately. Each sequence of validation of wines were carried out using different denomination of origin wines (D.O. Rioja, D.O. Ribera del Duero and D.O. La Mancha for red wines, and D.O. Montilla-Moriles, D.O. Rias Baixas and D.O. Jerez for white wines).

Each sample has been quantified using the calibration table, and minimal matrix effect was observed during the study results. Results are shown in Tables 7 and 8, and in Figures 6 and 7.

Table 7. Summary of Data Resulted by Validation of the Method with Red and White Wines.

	Red Wine				White Wine			
	0.5 µg/L	Rec. (%)	2.0 µg/L	Rec. (%)	0.5 µg/L	Rec. (%)	2.0 µg/L	Rec. (%)
Day 1	0.585	117.0	2.015	100.8	0.453	90.6	1.997	99.9
	0.460	92.0	2.135	106.8	0.474	94.8	1.900	95.0
	0.456	91.2	2.400	120.0	0.454	90.8	2.057	102.9
	0.468	93.6	2.146	107.3	0.465	93.0	2.042	102.1
Day 2	0.504	100.8	2.184	109.2	0.486	97.2	1.889	94.5
	0.472	94.4	2.060	103.0	0.53	106.0	1.845	92.3
	0.470	94.0	2.225	111.3	0.524	104.8	1.964	98.2
	0.476	95.2	2.043	102.2	0.446	89.2	1.968	98.4
Day 3	0.579	115.8	2.078	103.9	0.507	101.4	1.973	98.7
	0.442	88.4	1.966	98.3	0.553	110.6	1.903	95.2
	0.511	102.2	2.189	109.5	0.518	103.6	2.009	100.5
	0.513	102.6	1.954	97.7	0.511	102.2	2.181	109.1

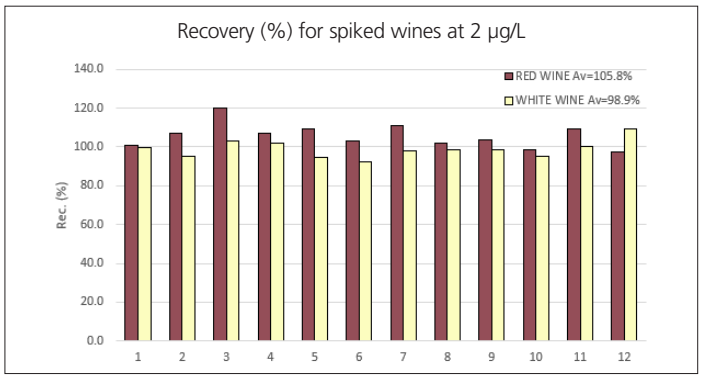

Figure 6. Recovery results for spiked wines at 2 µg/L .

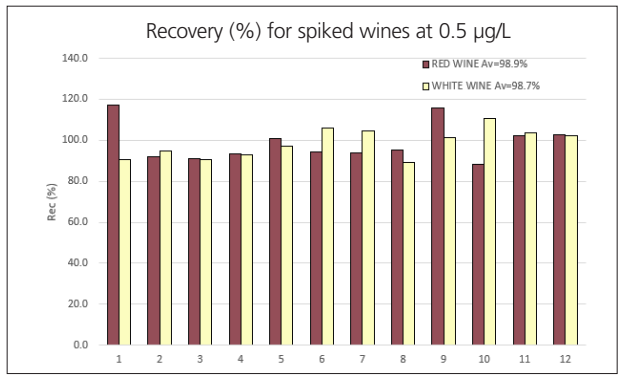

Figure 7. Recovery results for spiked wines at 0.5 µg/L.

Table 8. Method Validation Results Table.

	Red Wine		White Wine	
REFERENCE VALUE, OTA in µg/L	0.5	2.0	0.5	2.0
MEAN, µg/L	0.49	2.12	0.49	1.98
RSD, %	9.4	5.9	7.1	4.6
RECOVERY, %	98.9	105.8	98.7	98.9
$U_{unaccuracy}$, µg/L	0.003	0.067	0.004	0.013
$U_{precision}$, µg/L	0.046	0.125	0.035	0.091
U_{total}, µg/L	0.046	0.142	0.035	0.092
$U_{comb, K=2}$, µg/L	0.093	0.284	0.071	0.183
U_{exp}, %	18.8	13.4	14.3	9.3

U_{comb} is the combined uncertainty:

$$U_{comb} = 2\,U_{total} = 2\sqrt{U_{unaccuracy}^2 + U_{precision}^2}$$

U_{exp} is the expanded uncertainty:

$$U_{exp} = 2\,U_{comb} / Mean^{10}$$

Percent recovery was determined by spiking known amounts of OTA in wine samples. OTA percent recoveries obtained were between 88.4 and 120%, which is an acceptable criterion according to ISO CEN/TR 15356 for low levels (<10 ppb). Intermediate precision is expressed with %RSD values that are below 10%, which applies for both types of wine, and for the two spiking levels.

The expanded uncertainty provides an interval within which the value of the measurand is believed to lie with a higher level of confidence. Expanded uncertainties found here are satisfying, and they are below 20% for the four categories of spiked samples.

Based on the OTA peak height and background height (between 7.5 and 8.5 min) measurements, a S/N ratio of 200:1 was obtained for a 2 µg/L spiked red wine sample, according to EU regulation limit.

Chromatogram Examples

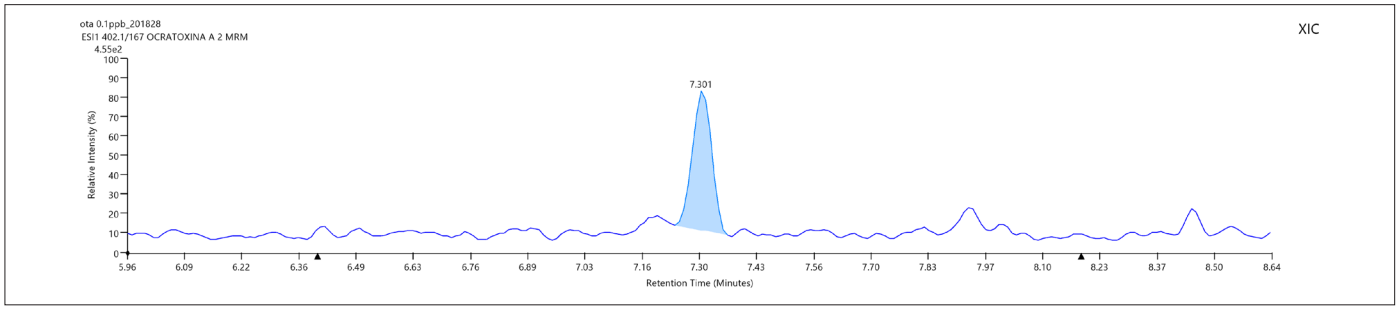

Figure 8. Chromatogram for 0.1 µg/L standard solution.

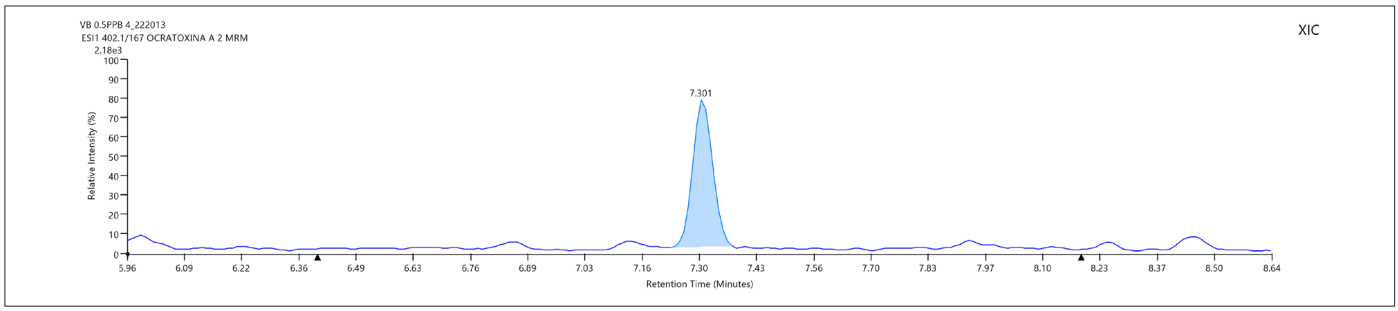

Figure 9. Chromatogram for 0.5 µg/L spiked sample (white wine).

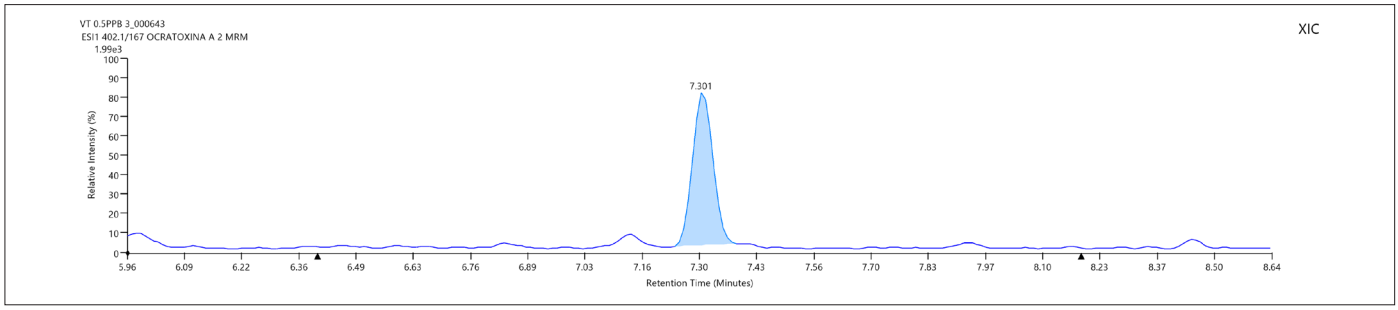

Figure 10. Chromatogram for 0.5 µg/L spiked sample (red wine).

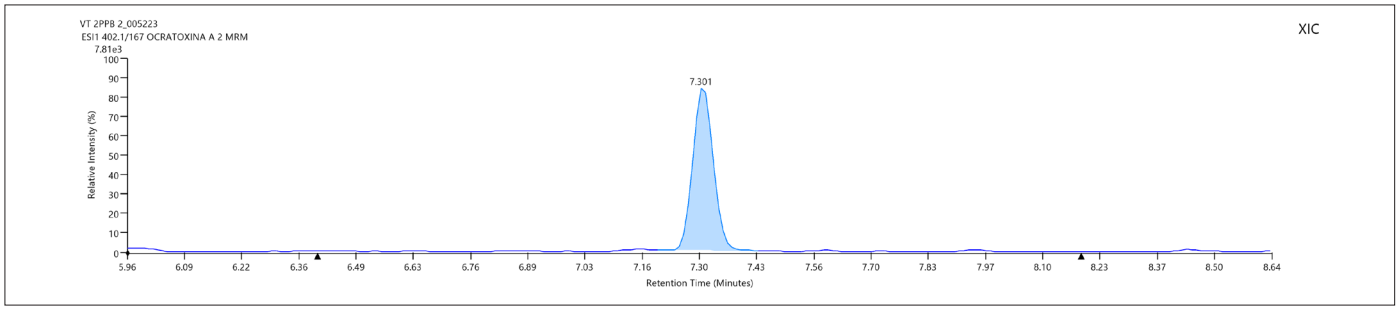

Figure 11. Chromatogram 2 µg/L spiked sample (red wine).

Conclusion

A rapid, rugged and sensitive method for the determination of Ochratoxin A in wine by LC/MS using a PerkinElmer QSight 220 Mass Spectrometer was developed and presented in this work. For the validation of the method presented herein, after an easy extraction, two wine samples were analysed, and various statistics were considered, such as RSDs, recoveries and accuracies. Results of these statistics shows that this method exhibits excellent ruggedness and specificity, specificity, meeting performance criteria and validation procedures of EUR 24105 EN and ISO CEN/TR 15356.

The method presented here allows the detection and quantification of ochratoxin A in wine down to 0.1 µg/L. In addition, the validation carried out on 6 different types of wine confirms that the method can be used for a reliable determination Ochratoxin A in wine at levels well below the limits set by the European Commission[11].

The method is suitable for testing laboratories to determine OTA contamination, as well as wine producers who need to monitor OTA in their wine production.

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