Simultaneous and Sensitive Determination of Multiple Mycotoxins in Wines by UHPLC Triple Quadrupole Mass Spectrometry

Application Note

Food

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Abstract

A method for simultaneous determination of eight mycotoxins in wines, including aflatoxins B1, B2, G1, and G2, patulin, deoxynivalenol, ochratoxin A, and zearalanone was developed. The samples were initially extracted using acetonitrile in the presence of sodium chloride and magnesium sulfate. The resultant extracts were then subjected to concentration by rotary evaporation and redissolution followed by ultra-high performance liquid chromatography (UHPLC) coupled with JetStream electrospray (JetStream ESI) triple quadrupole mass spectrometric detection under the multiple reaction monitoring mode. It has demonstrated that the limits of detection (LOD) for the eight mycotoxins in red and white wines ranged from 0.050 to 3.3 µg/L and 0.030 to 8.0 µg/L respectively, and the limits of quantitation (LOQs) ranged from 0.15 to 12.5 µg/L and 0.10 to 25 µg/L respectively. Linear responses were obtained for all eight mycotoxins in two orders of magnitude of the examined matrix-matched calibration ranges with linear regression coefficients of 0.995 or above. At the two spiking levels examined, the recoveries were all within 59.6–132.4% with RSD between 1.2–21.1% (n = 6). Among them, the majority of the spiked samples had recoveries within the range of 70%–120% with the RSDs below 10%. The developed method is rapid, accurate, and sensitive, and thus can be applied for high throughput routine screening of multiple mycotoxins in wine and may potentially extend to other fermented alcoholic beverages.

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Introduction

Mycotoxins are a group of secondary metabolites produced naturally by a variety of fungi, which reproduce themselves under favorable climate conditions. More than 300 mycotoxins have been reported. Research has demonstrated that a number of mycotoxins can lead to various adverse health effects including suppression of immune system, induction of cancers and abnormality, interruption of growth and development, and so forth [1,2]. Agricultural products, such as grapes, for producing wine are susceptible to contamination by mycotoxins during growth, storage, and processing due to infection of fungi.

Currently, ochratoxin A (OTA) is the most common mycotoxin found in wine [3-5]. The European Union (EU) has regulated the maximum limit of OTA in wine at 2 µg/L. In fact, many other mycotoxins can potentially be present in the finished wines, such as aflatoxins B1, B2, G1, and G2, deoxynivalenol, zearalanone, patulin, and so forth [6,7]. These mycotoxins have been regulated in most raw agricultural products by a number of countries and international organizations. However, these mycotoxins have not been paid much attention in wines so far.

Unlike other food commodities, wines are a special beverage with the major component of alcohol. Intake of mycotoxins through drinking wine may complicate the health effects to humans compared to those induced by mycotoxins themselves. A recent report has shown that co-exposure to mice of aflatoxin B1, one carcinogenic mycotoxin, with ethanol displayed additive effects in mice by inducing severe oxidative damage to their livers [8]. Compared to other food products, humans may tolerate a lower level of certain mycotoxins through the intake of wines. Therefore, a sensitive and reliable method is required for routine screening of the potential mycotoxins in wine to ensure safe consumption.

High performance liquid chromatography coupled with electrospray tandem mass spectrometry (HPLC-ESI-MS/MS) has been combined with various extraction techniques for detection of mycotoxins in maize, feedstuff, milk matrix, and herb medicines [9-14]. However, very few studies focused on multiple mycotoxins in wines [15]. This application note demonstrates a high throughput method for rapid and efficient detection of eight mycotoxins potentially in wine based on a UHPLC-ESI-MS/MS technique.

Experimental

Reagents, chemicals, and samples

Eight mycotoxins including patulin (PAT), deoxynivalenol (DON), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2), aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), ochratoxin (OTA), and zearalanone (ZEN) (purity ≥ 99%) were purchased from Romer Labs (Austria). Formic acid and ammonium acetate were purchased from Tedia and Fisher Scientific respectively. Both red and white wines were imported wines, randomly selected from local importers.

The standard solutions of mycotoxin compounds were prepared using pure methanol with stock concentrations of 10 µg/mL, and stored at –18 °C. They were diluted to the appropriate concentration using methanol/water solution (20:80, v/v) for calibration.

Sample preparation

Each wine sample (2.5 mL each) was mixed with 20 mL of pure acetonitrile (ACN) in a centrifuge tube. Sodium chloride (2 g) and magnesium sulfate (0.25 g) were added to the centrifuge tube. The resultant mixture was vortexed for 3 minutes, followed by centrifugation at 8,000 rpm for 10 minutes. 80% of the original volume for the supernatant solutions was then transferred to the new flask for drying using rotary evaporation at 40 °C. The nearly dry residue was dissolved in 2 mL of methanol/water (20/80, v/v) and subjected to filtration using a 0.22-µm membrane before analysis by UHPLC-ESI-MS/MS.

To examine if further cleaning was required, C18, PSA, and graphitized carbon black (GCB) were applied for cleanup. The supernatant solution resulting from the acetonitrile extraction above was split into three equal quotas and mixed with C18, PSA, and GCB in the centrifuge tubes with constant shaking for 10 minutes respectively. The mixtures were then centrifuged at 8,000 rpm for 10 minutes, and the resulting supernatant solutions were further concentrated by rotatory evaporation. The obtained residues were dissolved in 2 mL of methanol/water (20/80, v/v) solution, filtrated through a 0.22-µm membrane, and analyzed by UHPLC-ESI-MS/MS. Recovery of the mycotoxins was used to evaluate the cleanup efficiency.

Matrix effect

To evaluate if the matrix from red or white wines could affect the accuracy of these mycotoxins detection, wines spiked with certain amount of mycotoxins were prepared according to the sample preparation procedure. The resultant samples were analyzed by UHPLC-ESI-MS/MS. The peak area obtained for each analyte was compared with that obtained from analysis of the same level of mycotoxins in the standard solution. The percentage of the peak area obtained from spiked matrices decreasing or increasing from those obtained from standard solution represented the degree of matrix suppression or enhancement respectively.

Matrix-matched linear calibration, LOD, and LOQ

To evaluate the linearity and detection sensitivity, blank matrices previously tested with undetected target analytes were selected as the blank matrices. The blank matrices were subjected to extraction and evaporation same as samples. The resultant residues were dissolved using a series of mycotoxin standard solutions prepared in methanol/water (20/80, v/v). The matrix-matched standard solutions were filtered through the 0.22-µm membrane and analyzed using a UHPLC-ESI-MS/MS method. The obtained peak area of each analyte was correlated with their concentration to examine the linearity. The S/N from the lowest concentration of matrix-matched calibration standard solution was used to calculate the LOD (S/N = 3) and LOQ (S/N = 10) of the developed method.

Accuracy and precision

To evaluate the accuracy and precision of the method, spiked samples in the blank matrices with two spiking levels for each mycotoxin were determined with six replicates. Since the regulated level of each mycotoxin varies dramatically in wines, grapes or other referable agricultural raw materials, and the MS sensitivity for each mycotoxin varies greatly, the spiked levels were then set based on the available maximum limit levels of China regulations and one quarter of these values for individual analyte. The spiked samples were then followed the sample preparation process and analyzed by UHPLC-ESI-MS/MS. The average recoveries and the relative standard deviations for the six replicates were used to evaluate the accuracy and precision of the method.

UHPLC-MS/MS conditions

An Agilent 1290 Infinity UHPLC System was used throughout the study. It consisted of a 1290 Infinity binary pump, an autosampler, and a temperature column compartment. An Agilent ZORBAX Eclipse Plus C18 column (100 mm × 2.1 mm, 1.8 µm) was used for separation of the eight mycotoxins. The mobile phase was an on-line mixture of solvent A and solvent B where solvent A was pure water and solvent B was methanol containing 5 mmol/L ammonium acetate. The gradient started at 10% B, and linearly increased to 42% B within 0–2.4 minutes, it continuously increased to 51% B within 2.4–6 minutes. Then the % B was rapidly increased to 90% within 6.0–6.2 minutes, and maintained at 90% for 2 minutes to ensure all the analytes and interferences eluted out of column. The flow rate was set at 0.4 mL/min with the injection volume of 5 µL and the column temperature of 40 °C. The column equilibrium time between two consecutive runs was set at 1 minute.

The eluent from UHPLC system was directed to an Agilent 6460 Triple Quadrupole LC/MS System using a JetStream electrospray ionization (ESI) source and detected at multiple reactions monitoring mode (MRM). The general source parameter settings in the positive (pos) and negative (neg) modes were shown as follows: capillary voltage, 3,500 V pos and 2,000 V neg; nozzle voltage, 500 V pos and 1,900 V neg; drying gas temperature, 325 °C; drying gas flow rate, 6 L/min; nebulizer gas pressure, 45 psi; sheath gas temperature, 350 °C; sheath gas flow rate, 11 L/min. The fragmentor voltage and collision energy for each compound were optimized individually.

Results and Discussion

Selection of ionization modes and MRM parameters

For the eight mycotoxins (Figure 1) studied, PAT, DON, OTA, and ZEN contain hydroxyls, phenol hydroxyls, or carbonyl ligands, having the potential to lose protons during the ESI process, and were thus analyzed under negative ionization mode to achieve high sensitivity. In contrast, aflatoxins contain carbonyl and methoxy ligands, tending to obtain protons during ESI process, were then ionized under positive ionization mode for high sensitivity.

Under the selected ionization mode, an individual mycotoxin standard compound prepared in 20% methanol water solution was introduced into the 6460 Triple Quadrupole LC/MS System. Both the fragmentor voltage and collision energy of each MRM transition were optimized either manually or using optimizer software. The fragmentor voltage was scanned from 70 V to 200 V, and the voltage that provided the highest MS response was selected as the optimal value and used for further collision energy selection. The analyte was then fragmented under a range of collision energies (CE, from 5 V to 50 V). The CEs that provided the most intensive and the second most intensive fragments were selected as the respective optimal energies for both quantitative and qualitative MRM transitions. The optimized parameters are listed in Table 1.

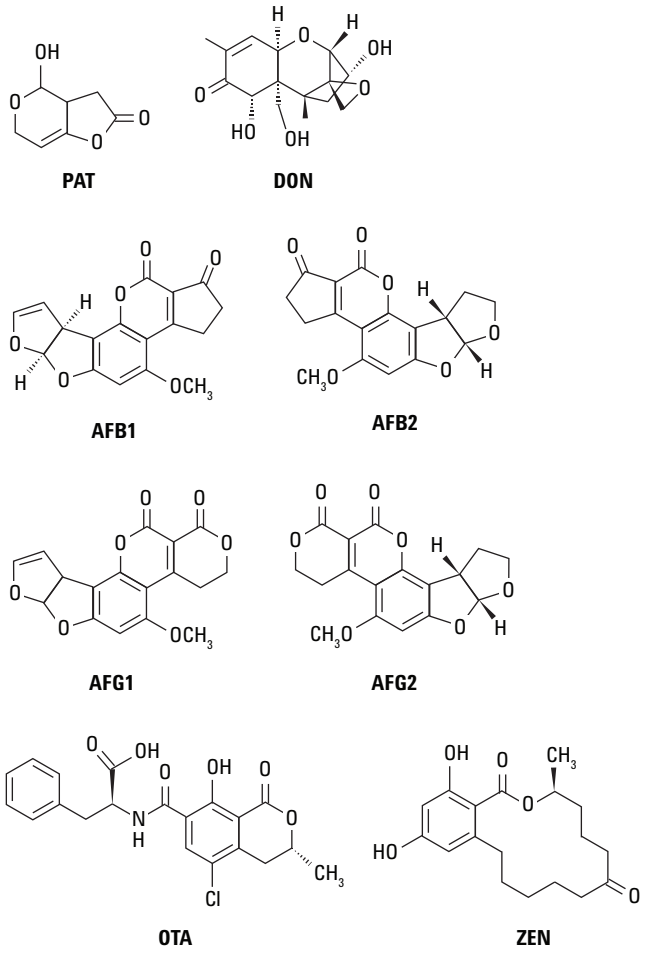

Figure 1. Chemical structures of eight mycotoxins studied.

Table 1. Optimized MRM Parameters for Detection of Mycotoxins

Time segment	Analyte	Precursor ion	Frag. vol (V)	Dwell time (ms)	Quant. ion (CE, V)	Qual. ion (CE,V)	Polarity
1	PAT	153.0	80	150	109.1(3)	81.2(7)	Neg
1	DON	295.2	90	200	265.2 (4)	138.1 (10)	Neg
2	AFG1	329.2	150	120	311.1(20)	243.1(25)	Pos
2	AFG2	331.2	160	120	313.1(23)	245.1(30)	Pos
2	AFB1	313.0	160	120	285.1(24)	241.1(36)	Pos
2	AFB2	315.0	160	120	287.1(27)	259.1(30)	Pos
3	OTA	402.1	120	240	358(12)	211(22)	Neg
3	ZEN	317.1	190	120	175(25)	130.8(33)	Neg

Optimization of UHPLC elution conditions

During UHPLC separation, both the mobile phase components and the concentration could affect separation efficiency, peak shape, and detection sensitivity. In this application note, methanol/water was selected as the binary mobile phase, while modifiers including formic acid and ammonium acetate were examined. With a fixed gradient elution profile, it was found that including 0.1% formic acid in the mobile phase showed high sensitivity for aflatoxins, but both OTA and ZEN coeluted with very low response. In comparison, 5 mmol/L ammonium acetate in the methanol phase showed high sensitivity for OTA and ZEN, with an only slight decrease in the MS response to aflatoxins compared to the mobile phase containing 0.1% formic acid. In addition, the concentration of ammonium acetate did not affect PAT and DON clearly. Therefore, 5 mmol/L ammonium acetate was selected as the modifier in the methanol phase to achieve reasonable sensitivity for all eight mycotoixins.

With the selection of mobile phase, the gradient elution profile was further optimized to ensure all eight mycotoxins separated by baseline with reasonable analysis time. Time segment was applied for switching between negative and positive ionization modes to ensure each compound detected with high sensitivity. In this manner, the eight mycotoxins can be simultaneously analyzed in one HPLC run with high sensitivity rapidly (Figure 2).

Optimization of extraction and cleanup conditions

The volume ratio of extraction solvent over wine can affect the extraction efficiency dramatically. In this application note, we spiked mycotoxins into red wine and extracted the mycotoxins using ACN with the volume ratio of ACN over wine at 2, 3, 4, 5, and 8. The resultant samples were extracted and concentrated for analysis. As shown in Figure 3, with a volume ratio of ACN over wine at 2, the recovery for eight mycotoxins ranged from 30.8 to 57.8%. With the increase of ACN volume, the recovery increased gradually. When the ratio reached 8, the recovery for all mycotoxins ranged from 61.6 to 74.7%. Ratios higher than 8 did not show clear increase in the recovery, and lengthened the concentration procedure. Hence, for every 2.5 mL of sample, 20 mL of acetonitrile was applied for extraction.

The major component in wine is water. The presence of water can affect the distribution of mycotoxins between ACN and water phases. Based on the QuEChERs extraction and cleanup technique [16], NaCl and $MgSO_4$ were added to the ACN-wine mixture (volume ratio of 8) to improve the transfer of mycotoxins from aqueous phase to ACN phase. Improved recoveries were obtained by addition of 2 g of NaCl and 0.25 g $MgSO_4$ into the ACN-wine mixture. Hence, for extraction of mycotoxins from every 2.5 mL wine, 20 mL of ACN containing 2 g NaCl and 0.25 g $MgSO_4$ were applied.

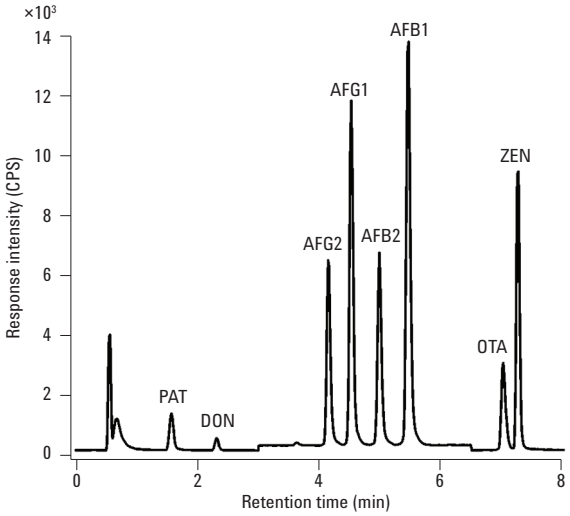

Figure 2. Typical total ion chromatogram showing the baseline separation of eight mycotoxins under the optimal conditions.

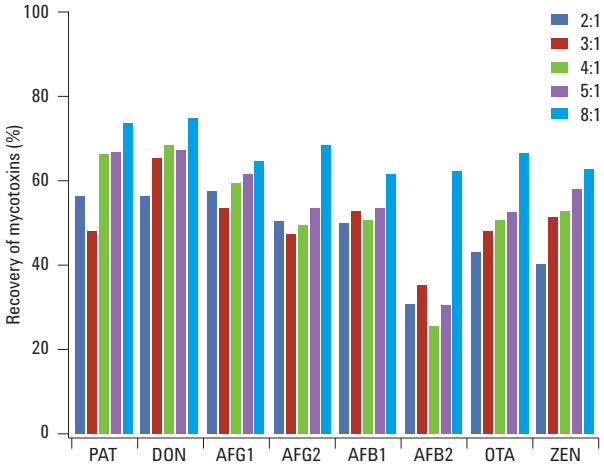

Figure 3. Recovery of mycotoxins with different volume ratio (extraction solvent/sample) without cleanup.

To test whether further cleanup was required, three absorbants (C18, PSA, and GCB) were examined. It was found that GCB showed the worst recovery of most mycotoxins. The result was consistent with the absorption properties of GCB, which prefers to absorb compounds with planar rings. Both C18 and PSA provided similar recovery for DON and four aflatoxins. However, for PAT, OTA, and ZEN, C18 cleanup was better. Recoveries for OTA and ZEN after C18 cleanup were not as good as those without cleanup as shown in Figure 3. Therefore, the compounds after extraction with salted ACN were directly evaporated and redissolved in an appropriate solution for UHPLC-ESI-MS/MS analysis.

Matrix effects

To further examine if a calibration curve from standard compounds can be applied for quantitation, matrix effects of red and white wines on the detection of eight mycotoxins were investigated. As shown in Figure 5, both red and white wines can suppress the signals of PAT and DON significantly, and the suppression percentage can be as high as 88.4%. For aflatoxins and ZEN, the suppression was lower, with the suppression percentage ranging from 34.9% to 50.6% in red wine and from 11% to 21.1% in white wine. In addition, both red and white wine can enhance the signal for OTA clearly. Therefore, matrix-matched calibration curve should be applied for accurate quantitation of mycotoxins in wines.

Method performances

In order to quantitate analytes accurately, both red and white wines previously tested with mycotoxins under detection limit were used as the blank matrix to obtain matrix-matched calibration curves. As shown in Table 2, excellent linear relationships were obtained for all eight mycotoxins in both red and white wine matrices, with linear regression coefficients of 0.995 or above. The LOD and LOQ for the four aflatoxins, OTA and ZEN, ranged from 0.030–0.30 µg/L and 0.10–1.0 µg/L in red wine and white wine respectively (Table 2). For PAT and DON, the LOQs in both red and white wines ranged from 10.0 to 25.0 µg/L. All LOQs were significantly lower than the available maximum limit regulated in most food matrices. For the spiking samples, the recovery values ranged from 59.6%–132.4%, with RSD (n = 6) within 0.97–21.1% (Table 3). Among them, the majority of recovery values were within 70–120% with the RSD (n = 6) less than 10%. It indicated that the method can meet the requirement for routine screening of mycotoxins in wine matrices.

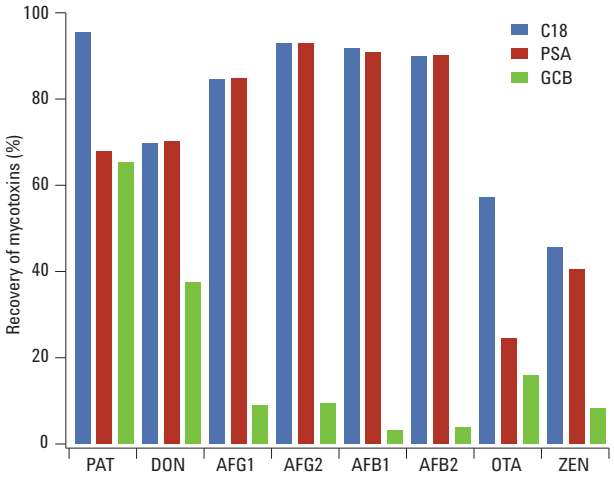

Figure 4. Nonspecific adsorption of mycotoxins to cleanup matrix decreasing the recovery of mycotoxins.

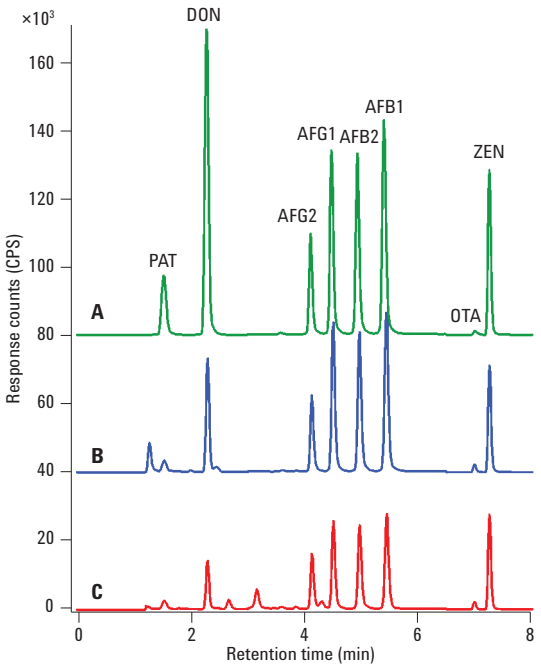

Figure 5. Comparison of the MS response for eight mycotoxins in standard solution and wine matrices under MRM mode.
A) Standard solution; B) White wine matrix; C) Red wine matrix.

Table 2. The Linearity of Matrix-Matched Calibration, Limit of Detection (LOD), and Limit of Quantitation (LOQ) of the Method

		Red wine matrix				White wine matrix			
Compound	Spiked level (µg/L)	Linear equations	R^2	LOD (µg/L)	LOQ (µg/L)	Linear equations	R^2	LOD (µg/L)	LOQ (µg/L)
PAT	25–625	Y = 6.223901x + 362.24592	0.9994	3.0	10.0	Y = 23.015212x + 346.197885	0.9954	8.0	25
DON	200–1,600	Y = 7.862917x – 797.4475	0.9984	3.3	12.5	Y = 19.411030x +1727.814871	0.9988	3.0	10.0
AFG1	2.5–62.5	Y = 1242.324154x – 701.701663	0.9994	0.050	0.15	Y = 1872.903941x – 146.795988	0.9999	0.030	0.10
AFG2	2.5–62.5	y = 774.096228x – 349.987693	0.9989	0.060	0.18	Y = 910.241257x – 45.185568	0.9980	0.15	0.40
AFB1	2.5–62.5	Y = 1283.555222x – 846.940492	0.9991	0.11	0.35	Y = 1821.940824x – 523.089751	0.9999	0.030	0.10
AFB2	2.5–62.5	Y = 1251.670189x – 586.826157	0.9991	0.14	0.42	Y = 1708.912301x – 29.263067	0.9999	0.06	0.20
OTA	1.0–25	Y = 379.569893x + 128.103796	0.9986	0.14	0.45	Y = 244.749778x + 120.771668	0.9980	0.15	0.50
ZEN	10–250	Y = 343.77344x – 1218.423002	0.9991	0.23	0.77	Y = 319.982227x – 906.574327	0.9998	0.30	1.0

Table 3. The Recovery and Precision of Mycotoxins in Red/White Wine Matrices

		Red wine matrix (n = 6)		White wine matrix (n = 6)	
Compound	Spiked (µg/L)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
PAT	100	113.0	13.4	103.71	6.1
	25	100.8	17.3	99.5	12.8
DON	800	119.1	4.8	132.4	3.6
	200	115.8	4.9	59.6	1.2
AFG1	10	91.2	7.1	91.4	3.6
	2.5	95.0	5.0	66.7	0.97
AFG2	10	99.5	1.8	96.7	2.1
	2.5	106.9	4.8	101.3	6.7
AFB1	10	73.7	4.3	83.4	4.6
	2.5	107.2	4.6	72.2	1.2
AFB2	10	83.5	3.8	102.6	2.0
	2.5	119.6	1.5	92.3	2.7
OTA	4	63.2	4.4	75.2	3.8
	1.0	103.8	16.2	78.1	11.0
ZEN	40	76.6	7.3	73.9	1.3
	10	84.5	21.1	94.6	4.2

Real sample screening

Eight red wine and seven white wine samples imported into the region were selected randomly and subjected to analysis using the developed method. OTA was detected in four of eight red wines and five of seven white wines, with the amount between LOD and LOQ, significantly lower than the regulated maximum limit by both EU and China.

Conclusion

This application note has developed a sensitive method for simultaneous monitoring of eight mycotoxins in red and white wine matrices. The samples were extracted with eight sample volumes of salted acetonitrile and concentrated for UHPLC-ESI-MS/MS analysis under MRM mode. By removing the cleanup step and employing a time-segment for simultaneous detection of mycotoxins in both positive and negative modes in one single HPLC analysis, the throughput of the method was improved significantly. The method can be used to detect the eight mycotoxins in both red and white wine matrices reliably with satisfactory recovery and precision. It is sensitive and can detect the eight mycotoxins at a much lower level than the current available maximum limit in the finished wines, raw material of wines, or other food products and, hence, can be applied to routine monitoring of mycotoxins in wine products.

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