Application Note

Application of ACQUITY TQD for the Analysis of Mycotoxin Contaminants in Pistachio, Almond, and Cashew Nuts

James Morphet, Peter Hancock, Andre de Kok, Peter Rensen, Jos Scholten, Martien Spanjer

Waters Corporation, Food and Consumer Product Safety Authority

Abstract

This application note will evaluate the suitability of Waters ACQUITY TQ detector (TQD) for tandem quadrupole based analysis of mycotoxins using multiple reaction monitoring (MRM) experiments. Pistachio, almond, and cashew nut matrices will be used for this analysis.

Benefits

- Determination of multiple contaminants per sample which enables a complete picture to be obtained of exposure to these compounds from the human diet
- Increased speed whilst further reducing solvent usage and therefore the costs of solvents and solvent disposal

Introduction

Many agricultural crops are susceptible to colonization by molds and fungi. Stress during plant growth or poor post-harvest storage conditions allow fungal species to infect a variety of commodities, often leading to unacceptable taste, odor or appearance. It is also possible for some fungal infestations to produce toxic secondary metabolites that have the potential to contaminate both animal feed and food intended for human consumption. These secondary metabolites are known generally as mycotoxins[1]. The Food and Agriculture Organization of the United Nations (FAO) estimates world losses of foodstuffs due to mycotoxins are in the range of 1000 million tons per year[2].

Aflatoxins form part of the mycotoxin family and have been associated with acute liver damage, liver cirrhosis, induction of tumors and teratogenic and other genetic effects[3].

From a regulatory standpoint, aflatoxins are considered unavoidable contaminants in foods since they cannot be prevented or eliminated by current good agricultural practices[2]. The four major naturally produced aflatoxins are B1, B2, G1 and G2 ('B' and 'G' referring to the blue and green fluorescent colors produced by these compounds under UV light on TLC plates).[3] This group of compounds is governed by EU legislation "Commission Regulation (EC) No 1881/2006," which states that the sum of the concentrations of the four aflatoxins in the edible part of nuts may not exceed 4 µg/kg. The concentration of aflatoxin B1 alone may not exceed 2 µg/kg.

Due to the 16x dilution factor in the extraction procedure, the concentration level of detection that the ACQUITY TQD must achieve for the EU legislation is 0.0625 ng/mL or lower. Similar legislation is enforced in Japan[3] and the USA[4] with limits of 10 μg/kg and 20 μg/kg for the sum of the four aflatoxins applying, respectively.

The aflatoxins, ochratoxin A, the fumonisins, and tricothecenes such as deoxynivalenol are legislated against in many countries. Rapid, sensitive and accurate analysis may be carried out for these compounds using immunoaffinity test kits. Immunoaffinity sample preparation is also appropriate for chromatography based analysis where the maximum sensitivity and selectivity is required[5]. In addition, a single analytical method able to target a variety of mycotoxin classes in a range of agricultural produce is desirable in order to obtain more comprehensive information on the range of contaminants that are present in human food. Such a multimycotoxin method is appropriate for laboratories testing food for consumption in the European Union, where the range of contaminants legislated against is the most extensive in the world.

The introduction of the ACQUITY TQD detector (Figure 1) allows scientists to perform mycotoxin analysis while harnessing all the benefits that this new instrument brings to the laboratory. The latest IntelliStart Technology in this instrument is designed to reduce the burden of complicated operation, time-intensive troubleshooting, and upkeep. Its small footprint will give any laboratory an advantage as this powerful tool removes the need for larger instrumentation.

This note describes an extended multi-mycotoxin method, for 25 contaminants in 3 nut matrices, which is able to meet the requirements for the analysis of regulated compounds and also includes a range of the other compounds of concern.

Figure 1. Waters ACQUITY TQD.

Experimental

The sample extraction method has been previously reported and remains unchanged.[1,6,7,8] Mycotoxin extracts in pistachio, almond and cashew nut matrices were provided along with solvent standards of the compounds by the Food and Consumer Product Safety Authority (VWA), Amsterdam, NL. The extracts were analyzed on the ACQUITY TQD.

UPLC Conditions

LC system:	ACQUITY UPLC System
Column:	ACQUITY UPLC BEH C_{18} Column 2.1 x 100 mm, 1.7 µm
Column temp.:	40 ˚C
Flow rate:	400 µL/minute
Mobile phase A:	Water + 0.1% formic acid
Mobile phase B:	Acetonitrile + 0.1% formic acid
Total run time:	15 minutes
Injection volume:	50 µL

Gradient

Time (min)	%A
0.0	90
3.0	90

Time (min)	%A
10.0	30
10.1	10
12.0	10
12.1	90

MS Conditions

MS System:	Waters ACQUITY TQ detector
Ionization mode:	ESI positive
Capillary voltage:	4 kV
Coen voltage:	Various
Desolvation gas:	Nitrogen, 800 L/Hr, 450 ˚C
Cone gas:	Nitrogen, 5 L/Hr
Source temp.:	120 ˚C
Acquisition:	Multiple Reaction Monitoring (MRM)
Collision Gas:	Argon at 3.5×10^{-3} mBar

The ACQUITY TQD was tuned so that the precursor and product ions were resolved with a peak width at half height of less than 0.7 Da. The list of mycotoxin residues and the MRM transitions, along with the dwell times, cone voltages, and collision energies for the method are listed in Appendix 1.

Acquisition and Processing Methods

Waters MassLynx Software version 4.1 was used for acquisition and its TargetLynx Application Manager was used for data processing.

Results and Discussion

All 25 compounds were separated successfully. Figure 2 shows the total ion chromatogram for all compounds. Varying dwell times (listed in Appendix 1) and time windows were employed to achieve an average of 12 data points across each peak with Nivalenol eluting first at 1.16 min and Cyclopiazonic acid eluting last at 9.52 minutes.

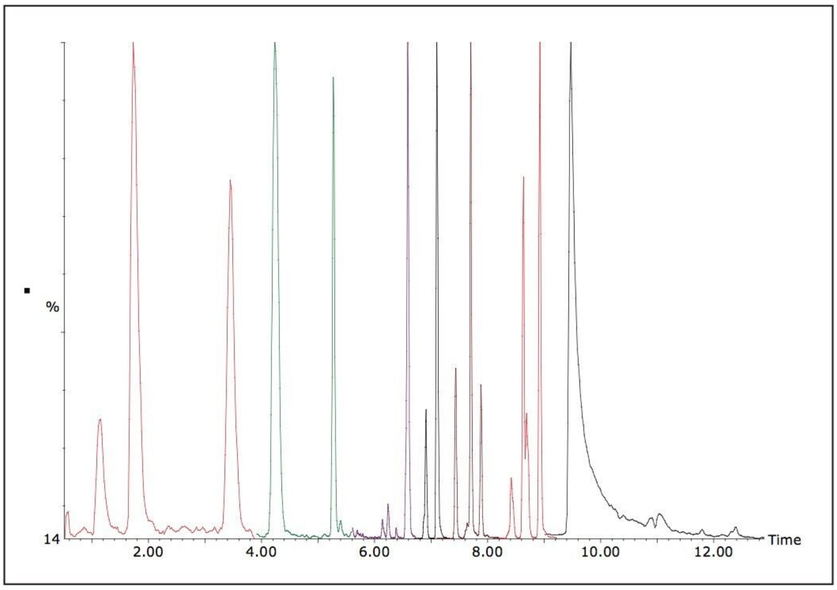


Figure 2. TIC showing 25 mycotoxins in almond nut matrix. Compounds injected range between 1 to 50 ng/mL, (corresponding with 16 to 800 µg/kg equivalent sample concentration).

Figure 3 shows the four main aflatoxins in pistachio nut matrix. When lower limits (such as baby foods) have to be achieved, a sample clean up step via SPE[9] can be utilized to help reduce matrix effects and concentrate the sample to improve sensitivity.

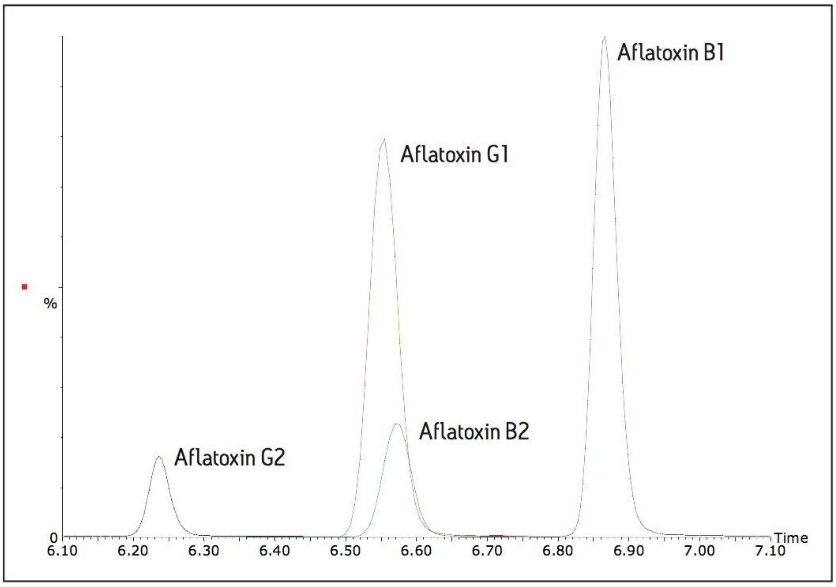


Figure 3. Chromatogram showing the four aflotoxins in pistachio nut matrix at 1ng/mL, corresponding with 16 µg/kg equivalent sample concentration.

Figure 4 shows the four legislated aflatoxin standards in solvent at 0.0625 ng/mL, corresponding to 1 µg/kg equivalent sample concentration, which is two times below the limit set for aflatoxin B1 in the EU legislation 1881/2006.

The signal to noise ratio (S:N) for the smallest peak, aflatoxin G2 is 5:1 (peak to peak). Thus the TQD can detect all four aflatoxins at concentration levels corresponding with the EU limits.

The aflatoxin solvent standards supplied for this analysis ranged between 0.0625–1.0 ng/mL. Due to the sample extraction method which incorporates a 16x dilution factor, these values are corresponding with an equivalent sample concentration range of 1-16 µg/kg when referring to the edible part of the nut.

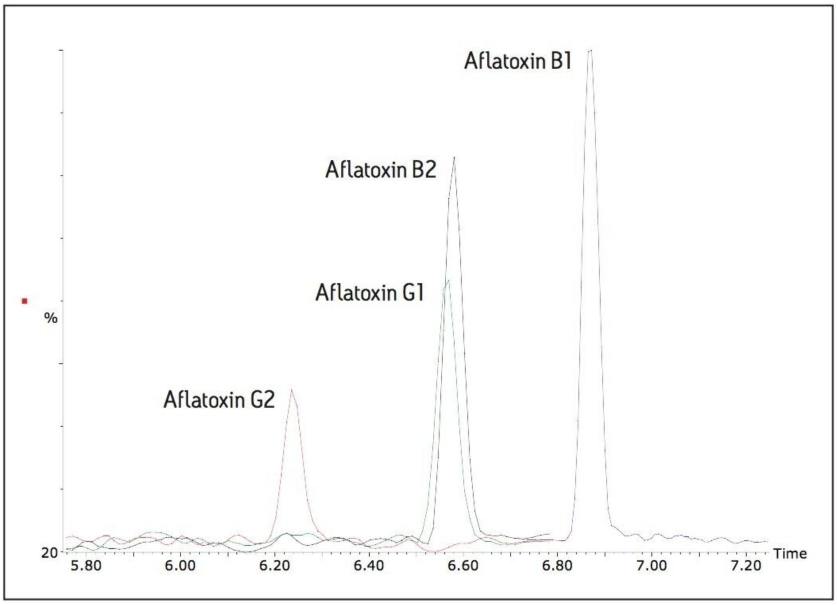
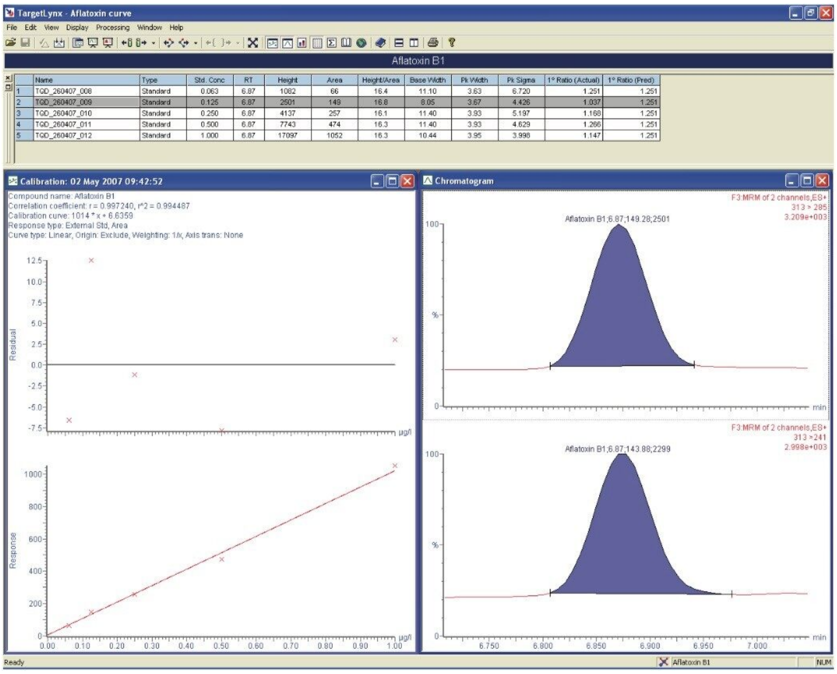


Figure 4. Chromatogram showing the four aflatoxin standards in solvent at 0.0625 ng/mL, corresponding with 1 µg/kg equivalent sample concentration.

Figure 5, a screenshot of the Targetlynx application manager, shows how linearity was achieved over the concentration range with all standards lying within 15% of their nominal value.

The upper chromatogram, the primary transition, is used for quantification whereas the secondary transition (lower chromatogram) is used for confirmation via the ion ratio. The ion ratio of the sample extract must lie within a 20% tolerance range of the corresponding calibration standard injections' ion ratio for positive confirmation



	Name	Type	Std. Conc	RT	Height	Area	Height/Area	Base Width	Pk Width	Pk Sigma	1° Ratio (Actual)	1° Ratio (Pred)
1	TQD_260407_008	Standard	0.063	6.87	1082	66	16.4	11.10	3.63	6.720	1.251	1.251
2	TQD_260407_009	Standard	0.125	6.87	2501	149	16.8	8.05	3.67	4.426	1.037	1.251
3	TQD_260407_010	Standard	0.250	6.87	4137	257	16.1	11.40	3.93	5.197	1.168	1.251
4	TQD_260407_011	Standard	0.500	6.87	7743	474	16.3	11.40	3.93	4.629	1.266	1.251
5	TQD_260407_012	Standard	1.000	6.87	17097	1052	16.3	10.44	3.95	3.998	1.147	1.251

Compound name: Aflatoxin B1
Correlation coefficient: r = 0.997240, r^2 = 0.994487
Calibration curve: 1014 * x + 6.6359
Response type: External Std, Area
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

Figure 5. Targetlynx view showing aflatoxin B1 calibration curve. The extracted ion chromatograms for aflatoxin B1 were obtained at an equivalent sample concentration of 2 µg/kg.

Due to limiting sample extract volume, a run of 15 injections in cashew nut matrix was performed to evaluate the robustness of the confirmatory ion ratio. Figure 6 shows the changes in ion ratio over the course of the run. All injections were within the 20% tolerance set with the average difference shown in the header of the graph for each compound.

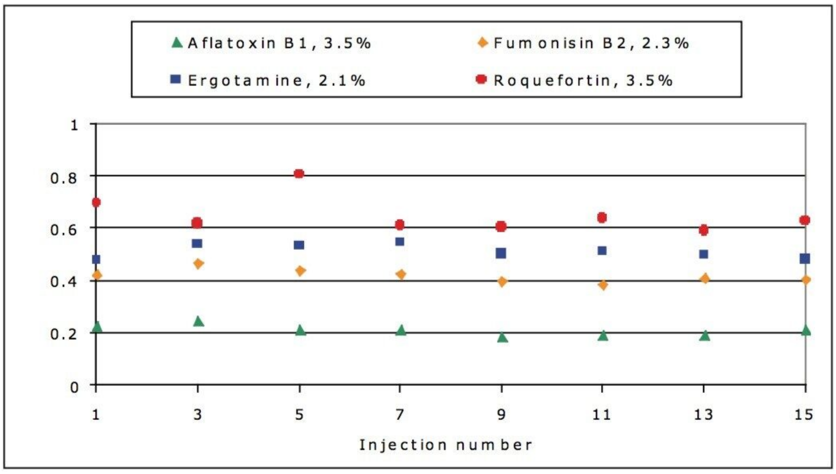


Figure 6. Plot showing confirmatory ion ratios of four mycotoxins over 15 injections of cashew nut matrix extracts. Extracts were injected at concentrations of 0.125 ng/mL for aflatoxin B1, 5 ng/mL for ergotamine and 2.5 ng/mL for fumosin B2 and Roquefortin (alternate injections were matrix blanks).

Figure 7 shows the peak area stability over the same 15 injections. The RSD for the compounds is below 9% in all cases.

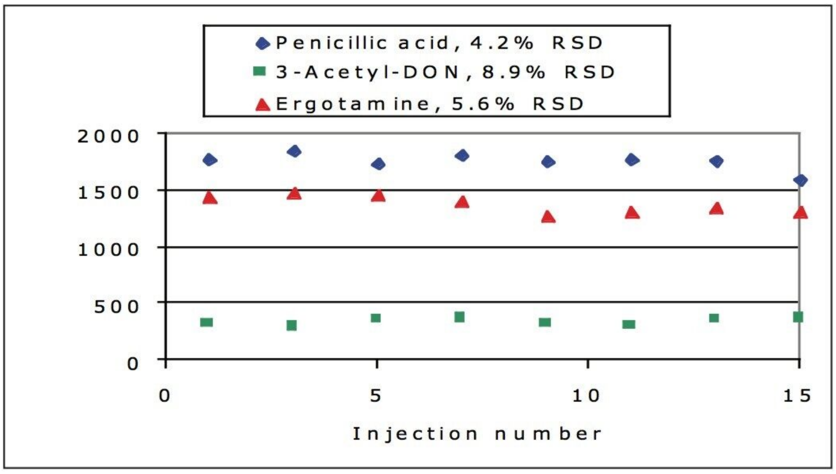


Figure 7. Plot showing peak areas of 3 mycotoxins over 15 injections of cashew nut matrix extracts. Extracts were injected at concentrations of 2.5 ng/mL for penicillic acid, 6.25 ng/mL for 3-acetyl-DON and 5 ng/mL for ergotamine (alternate injections were matrix blanks).

Conclusion

The contaminants that cause serious harm to human health, such as aflatoxins, ochratoxin A, and trichothecenes, can be quantified using the method described at levels corresponding with the EU legislation limits on ACQUITY TQD.

Ion ratios for confirmation using two MRM transitions were shown to be stable, which is important for quantification and confirmation.

The method is also applicable to the monitoring of various mycotoxin contaminants of emerging concern.

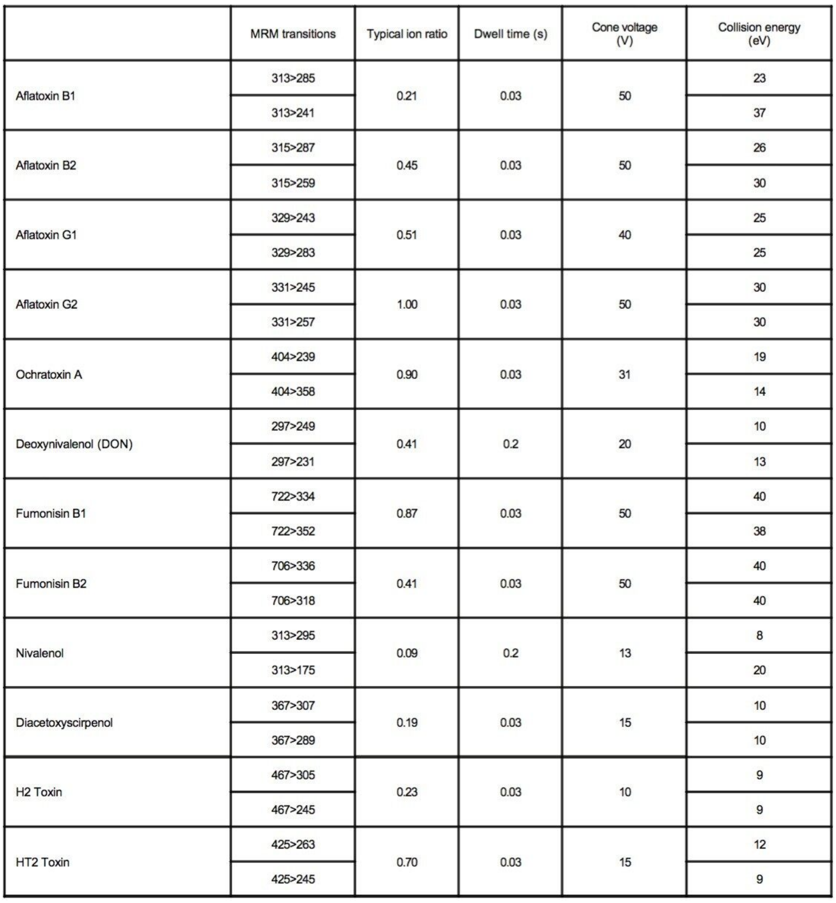
This method allows the determination of multiple contaminants per sample which enables a complete picture to be obtained of exposure to these compounds from the human diet.

This multiple mycotoxin method obsoletes the use of several single mycotoxin methods where repeat analysis is required. The benefits of UPLC for a revenue conscious laboratory are shown with increased speed whilst further reducing solvent usage and therefore the costs of solvents and solvent disposal.

Acknowledgements

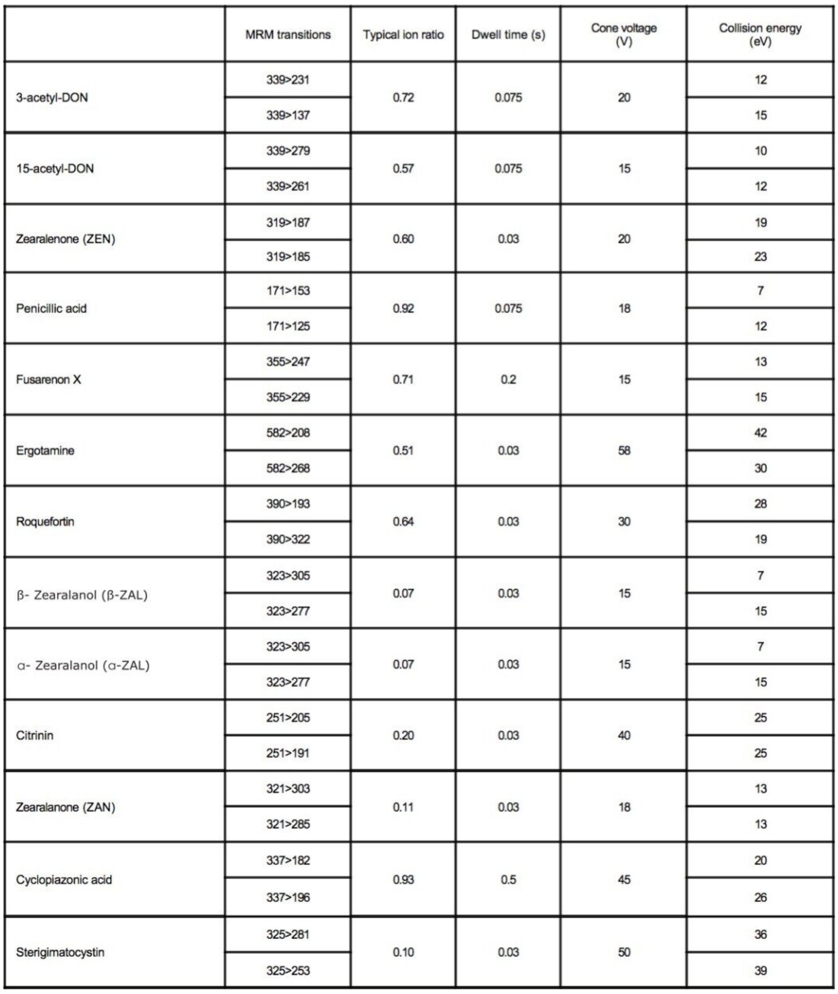
The authors would like to thank the Food and Consumer Product Safety Authority (VWA), Amsterdam, Netherlands, for kindly supplying all the standard solutions, and Elma Bobeldijk in particular, for preparing all the sample extracts that were analyzed in this project.

Appendix 1. ACQUITY TQD MRM parameters.



	MRM transitions	Typical ion ratio	Dwell time (s)	Cone voltage (V)	Collision energy (eV)
Aflatoxin B1	313>285	0.21	0.03	50	23
	313>241				37
Aflatoxin B2	315>287	0.45	0.03	50	26
	315>259				30
Aflatoxin G1	329>243	0.51	0.03	40	25
	329>283				25
Aflatoxin G2	331>245	1.00	0.03	50	30
	331>257				30
Ochratoxin A	404>239	0.90	0.03	31	19
	404>358				14
Deoxynivalenol (DON)	297>249	0.41	0.2	20	10
	297>231				13
Fumonisin B1	722>334	0.87	0.03	50	40
	722>352				38
Fumonisin B2	706>336	0.41	0.03	50	40
	706>318				40
Nivalenol	313>295	0.09	0.2	13	8
	313>175				20
Diacetoxyscirpenol	367>307	0.19	0.03	15	10
	367>289				10
H2 Toxin	467>305	0.23	0.03	10	9
	467>245				9
HT2 Toxin	425>263	0.70	0.03	15	12
	425>245				9

Appendix 1. ACQUITY TQD MRM parameters (continued).



	MRM transitions	Typical ion ratio	Dwell time (s)	Cone voltage (V)	Collision energy (eV)
3-acetyl-DON	339>231	0.72	0.075	20	12
	339>137				15
15-acetyl-DON	339>279	0.57	0.075	15	10
	339>261				12
Zearalenone (ZEN)	319>187	0.60	0.03	20	19
	319>185				23
Penicillic acid	171>153	0.92	0.075	18	7
	171>125				12
Fusarenon X	355>247	0.71	0.2	15	13
	355>229				15
Ergotamine	582>208	0.51	0.03	58	42
	582>268				30
Roquefortin	390>193	0.64	0.03	30	28
	390>322				19
β- Zearalanol (β-ZAL)	323>305	0.07	0.03	15	7
	323>277				15
α- Zearalanol (α-ZAL)	323>305	0.07	0.03	15	7
	323>277				15
Citrinin	251>205	0.20	0.03	40	25
	251>191				25
Zearalanone (ZAN)	321>303	0.11	0.03	18	13
	321>285				13
Cyclopiazonic acid	337>182	0.93	0.5	45	20
	337>196				26
Sterigimatocystin	325>281	0.10	0.03	50	36
	325>253				39

References

1. A de Kok, M. Spanjer, J Scholten, P Rensen, and G Kearney, Rapid Multi- Mycotoxin Analysis using ACQUITY UPLC and Quattro Premier XE, Waters application note no. 720001996EN < https://www.waters.com/webassets/cms/library/docs/720001996en.pdf> .
2. Food And Agriculture Organization of the United Nations web site: http://www.fao.org/
3. Central Science Laboratory web site : http://mycotoxins.csl.gov.uk
4. US Food and Drug Administration (FDA) web site: http://www.cfsan.fda.gov/

5. VICAM web site: http://www.vicam.com/products/mycotoxin.html

6. ACQUITY UPLC-MS/MS for Multi-Mycotoxin analysis, Waters application poster no. 720002137EN < https://www.waters.com/webassets/cms/library/docs/720002137en.pdf> .

7. M Spanjer, P Rensen, and J Scholten, Multimycotoxin analysis: The LC-MS approach, in: The Mycotoxin Factbook, Food & Feed Topics. Edited by D Barug, D Bhatnagar, H van Egmond, J van der Kamp, W van Osenbruggen, and A Visconti, Wageningen Academic Publishers, ISBN 90-8686-006-0, p. 249-269, 2006. http://www.wageningenacademic. com/books/factbook_contents.pdf.

8. M Spanjer, P Rensen and J Scholten, LC-MS/MS Multimethod for Determination of 33 Mycotoxins After a Single Extraction and Validation Data for Peanut, Wheat, Cornflakes, Raisin and Fig, accepted for publication in Food Additives and Contaminants, 2007.

9. M Ventura *et al*: Ultra-performance Liquid Chromatography/Tandem Mass Spectrometry for the Simultaneous Analysis of Aflatoxins B1, G1, B2, G2 and Ochratoxin A in Beer. Mass Spectrom. 20: 3199–3204, 2006. Waters, ACQUITY UPLC, UltraPerformance LC and UPLC are registered trademarks of Waters Corporation. IntelliStart, MassLynx, TargetLynx, and The Science of What's Possible are trademarks of Waters Corporation. All other trademarks are property of their respective owners. ©2007.

Featured Products

ACQUITY UPLC System <https://www.waters.com/514207>

MassLynx MS Software <https://www.waters.com/513662>

TargetLynx <https://www.waters.com/513791>

720002244, July 2007