A multi-screening method based on ultra-high-performance liquid chromatography tandem high-resolution mass spectrometry (UHPLC-HRMS) to detect both regulated and emerging mycotoxins in different feed matrices

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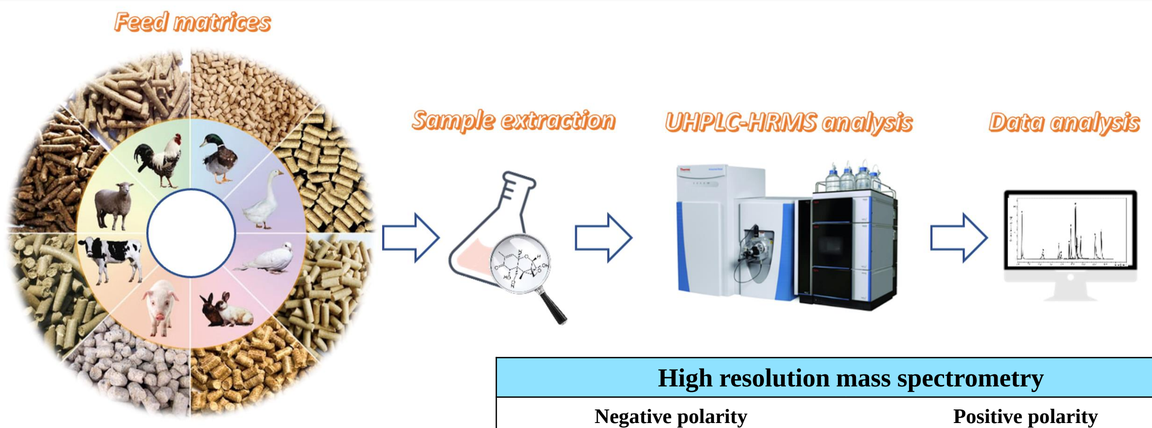
Abstract

Mycotoxins are secondary metabolites produced by filamentous fungi belonging to different genera, such as *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria*. These compounds can cause several health damages for animals, thus determining reduced animal performance and profitability. In this work, we optimized a multi-screening method to detect both UE-regulated and emerging mycotoxins through UHPLC-HRMS Orbitrap platform, considering a resolution of 70,000 FWHM at 200 m/z. To test the overall performance, different feed samples of interest in dairy cow nutrition including corn silage, cereal silage, hay, corn, soy, rapeseed, sunflower, wheat bran, rice husk and corn germ collected from farms located in Po Valley (Italy) were considered. We identified and accurately quantified several mycotoxins, including fumonisins (R^2:0.99, 10-100 µg/kg), trichothecenes (R^2: 0.99, 25-1000 µg/kg), zearalenones (R^2:0.99, 1-100 µg/kg), enniatins (R^2:0.99, 1-1000 µg/kg), mycophenolic acid (R^2:0.99, 1-1000 µg/kg), beauvericin (R^2:0.99, 1-1000 µg/kg), fusaric acid (R^2:0.99, 1-1000 µg/kg), roquefortine c (R^2:0.99, 1-1000 µg/kg), 3-nitropropionic acid (R^2:0.99, 100-1000 µg/kg), moniliformin (R^2:0.99, 10-1000 µg/kg), tenuazonic acid (R^2:0.99, 10-250 µg/kg) and alternariol derivatives (R^2:0.99, 1-100 µg/kg). Overall, fumonisin B1 was the most abundant mycotoxin detected in both corn silage and corn germ (5,67-6,38 and 3,02-3,22 mg/kg, respectively) while hay, soy and rapeseed were mainly characterized by trichothecenes being DON in the range 0.30-32,2 mg/kg. The developed method will be tested in future applications to monitor the presence of emerging mycotoxins.

In the last few years, research on mycotoxins is moved fast towards the utilization of targeted LC-MSMS and LC-HRMS multiscreening approaches to determine multiple mycotoxins.

Therefore, the so-called multi-screening methods (for both qualitative and quantitative purposes) started to be rapidly developed. One of the major advantages of using HRMS over targeted MS/MS techniques is represented by the possibility to perform both untargeted and retrospective data analysis, with the latter allowing the reconsideration of analytical results from stored data and already analyzed samples.

Particularly, these methods are based on the measurement of accurate MS and MS/MS spectra with a mass resolution lower than 5 ppm. These conditions usually allow the detection of several compounds, together with the possibility to performing a structural elucidation of unknowns. All these aspects represent a valuable tool because of the lack of analytical standards for several known mycotoxins.



LC conditions	
Coloumn	Thermo Scientific™ Hypersil GOLD™ aQ, 100 x 2.1mm (1.9 µm)
Coloumn working T°	35 °C
Mobile phase A	Water + 2% Methanol + 5 mM Ammonium Formate + 0.1% Formic Acid + 0.1% Acetic Acid
Mobile phase B	Methanol + 2% Water + 5 mM Ammonium Formate + 0.1% Formic Acid + 0.1% Acetic Acid
LC gradient	At a flow rate of 0.3 mL/min start at 0% B and hold for 0.5 minutes, switch to 30% B and start a linear gradient to 100% B for 8 minutes, hold for one minute, drop to 0% B and equilibrate for three minutes for a total run time of 15 minutes
Volume of injection	3 µl

High resolution mass spectrometry							
Negative polarity				Positive polarity			
Scan parameters		MS settings		Scan parameters		MS settings	
Scan Type	Full MS	Sheath gas flow rate	35	Scan Type	Full MS	Sheath gas flow rate	40
Scan range	90.0 to 500.00 m/z	Aux gas flow rate	15	Scan range	90.0 to 850.00 m/z	Aux gas flow rate	20
Fragmentation	None	Spray voltage (|KV|)	2.80	Fragmentation	None	Spray voltage (|KV|)	3.50
Resolution	70000	Capillary temp (°C)	320	Resolution	70000	Capillary temp (°C)	320
AGC target	1e6	S-lens RF level	50.0	AGC target	1e6	S-lens RF level	50.0
Maximum inject time	100	Aux gas heater temp (°C)	50	Maximum inject time	100	Aux gas heater temp (°C)	50

Regulated mycotoxins

	AFB1	AFB2	AFG1	AFG2	FB1	T-2	HT-2	DON	NIV	OTA	ZEN
Corn silage	< LOD	< LOD	< LOD	< LOD	6025,04 ± 352.5^{c}	< LOD	< LOD	< LOD	< LOD	< LOD	23.33 ± 7.37^{a}
Cereal silage	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Hay	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	32196.10 ± 5580.16^{c}	< LOD	< LOD	< LOD
Corn	< LOD	< LOD	< LOD	< LOD	382.03 ± 200.95^{a}	< LOD	< LOD	619.42 ± 54.96^{a}	< LOD	< LOD	< LOD
Soy	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	2033.75 ± 160.49^{ab}	< LOD	< LOD	< LOD
Rapeseed	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	4120.72 ± 412.75^{b}	< LOD	< LOD	< LOD
Sunflower	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	298.29 ± 53.19^{a}	< LOD	< LOD	65.05 ± 6.60^{b}
Wheat bran	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	765.39 ± 31.13^{a}	< LOD	< LOD	141.59 ± 57.83^{c}
Rice husk	< LOD	< LOD	< LOD	< LOD	224.54 ± 17.06^{a}	< LOD	< LOD	947.04 ± 93.42^{a}	< LOD	< LOD	n.d.
Corn germ	< LOD	< LOD	< LOD	< LOD	3116.00 ± 100.91^{b}	< LOD	< LOD	< LOD	< LOD	< LOD	164.55 ± 40.68^{c}

Levels of contamination express in µg/kg according to the UE Regulation*

*Directive 2002/32/EC of the European Parliament and of the council of 7 May 2002 on undesirable substances in animal feed
*Commission Recommendation 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
*Commission Recommendation (EU) 2016/1319 of 29 July 2016 amending Recommendation 2006/576/EC as regards deoxynivalenol, zearalenone and ochratoxin A in pet food

Emerging mycotoxins

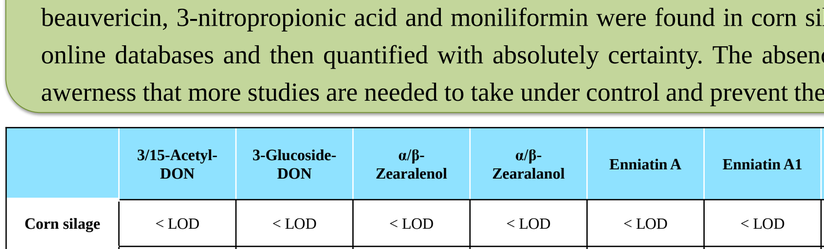
Focusing our attention on emerging mycotoxins we observed that various of these new and more unknown contaminants characterized feed samples analyzed for this trial. Especially, high levels of mycophenolic acid, beauvericin, 3-nitropropionic acid and moniliformin were found in corn silage and cereal silage samples. Through UHPLC-HRMS analysis we were able to recognize their different m/z ratio that were compared with online databases and then quantified with absolutely certainty. The absence of regulation and specific information related to this molecules and thier potential damages on animal health and productivity lead to the awerness that more studies are needed to take under control and prevent the increasing of emerging mycotoxins in field and throughout the feed chain production.

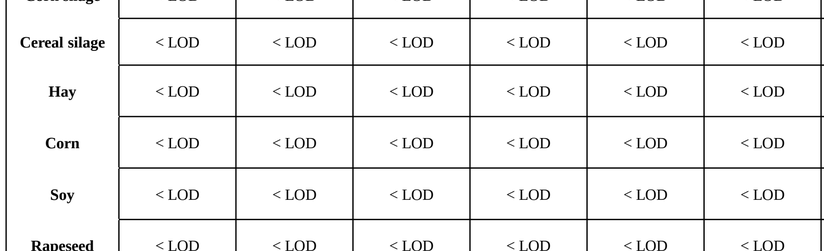
	3/15-Acetyl-DON	3-Glucoside-DON	α/β-Zearalenol	α/β-Zearalanol	Enniatin A	Enniatin A1	Enniatin B	Enniatin B1	Mycophenolic acid	Beauvericin	Gliotoxin	Fusaric acid	Roquefortine C	3-Nitropropionic acid	Moniliformin	Tenuazonic acid	Alternariol	Alternariol monomethyl ether	Tentoxin
Corn silage	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	1.67 ± 0.39^{a}	1.06 ± 0.10^{a}	969.62 ± 1.47^{g}	365 ± 27.75^{e}	< LOD	1167.76 ± 68.61^{d}	< LOD	1735195.57 ± 76707.43^{c}	34317.4 ± 6139.47^{b}	< LOD	< LOD	< LOD	< LOD
Cereal silage	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	41.36 ± 6.74^{c}	14.83 ± 1.22^{b}	2716.99 ± 10.67^{h}	244.45 ± 1.48^{d}	< LOD	< LOD	< LOD	7325360 ± 638482.85^{d}	76934 ± 14.313.67^{c}	< LOD	< LOD	< LOD	< LOD
Hay	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	1.18 ± 0.82^{a}	0.27 ± 0.04^{a}	8.59 ± 0.022^{a}	0.74 ± 0.0013^{a}	< LOD	< LOD	< LOD	881.9 ± 24.05^{a}	9541.8 ± 4200.87^{a}	0.91 ± 0.14^{a}	< LOD	< LOD	< LOD
Corn	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	380.13 ± 01.03^{e}	36.50 ± 1.47^{b}	< LOD	118 ± 16.5^{b}	< LOD	2930.25 ± 679.08^{a}	911.88 ± 209.8^{a}	4.14 ± 2.98^{a}	< LOD	< LOD	< LOD
Soy	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	1.46 ± 0.41^{a}	0.76 ± 0.10^{a}	380.08 ± 5.84^{e}	50.44 ± 1.73^{b}	< LOD	128.23 ± 4.01^{b}	< LOD	12496.19 ± 901.93^{a}	798.23 ± 260.81^{a}	8.27 ± 6.37^{a}	< LOD	< LOD	< LOD
Rapeseed	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	21.40 ± 2.69^{b}	7.52 ± 0.45^{ab}	371.42 ± 0.42^{d}	35.35 ± 0.23^{b}	< LOD	< LOD	102 ± 0.08^{b}	7734.15 ± 115.65^{a}	6562.94 ± 1009.93^{a}	11.17 ± 4.3^{a}	< LOD	< LOD	8.24 ± 0.62^{c}
Sunflower	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	5.33 ± 0.6^{ab}	3.32 ± 0.53^{a}	387.16 ± 0.88^{f}	37.52 ± 0.4^{b}	< LOD	< LOD	102 ± 0.2^{b}	9984.53 ± 134.74^{a}	547.15 ± 109.75^{a}	407.31 ± 29.54^{b}	24.94 ± 1.21^{d}	5.49 ± 0.21d	123.19 ± 37.07^{b}
Wheat bran	< LOD	< LOD	< LOD	< LOD	54.91 ± 16.83^{b}	< LOD	240.65 ± 29.52^{d}	85.89 ± 19.47^{c}	319.2 ± 0.72^{b}	33.41 ± 0.85^{b}	< LOD	< LOD	< LOD	4637.32 ± 515.45^{a}	5635.10 ± 1499.61^{a}	22.79 ± 1.75^{a}	3.83 ± 0.34^{b}	< LOD	18.04 ± 1.34^{ab}
Rice husk	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	1.96 ± 0.52^{a}	0.64 ± 0.20^{a}	371.94 ± 0.58^{d}	177.47 ± 38.9^{c}	< LOD	100.92 ± 6.84^{b}	< LOD	3072.43 ± 170.52^{a}	1469.19 ± 1219.77^{a}	379.35 ± 90.97^{b}	17.22 ± 2.31^{c}	4.77 ± 0.64^{c}	36 ± 8.05^{b}
Corn germ	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	0.68 ± 0.15^{a}	1.45 ± 1.68^{a}	349.5 ± 0.78^{c}	193.5 ± 24.46^{c}	< LOD	799.70 ± 38.22^{c}	< LOD	452727.09 ± 20378.52^{b}	27785.26 ± 7304.39^{b}	< LOD	< LOD	1.29 ± 0.11^{b}	< LOD

Levels of contamination express in µg/kg

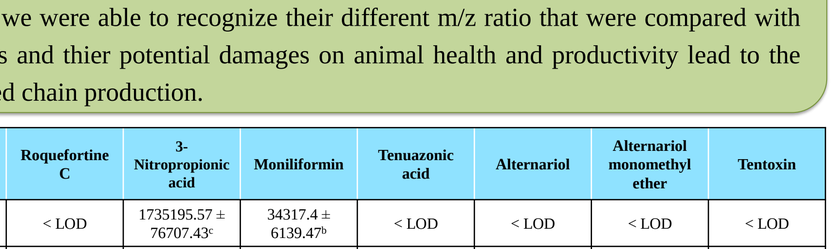
Statistical analysis

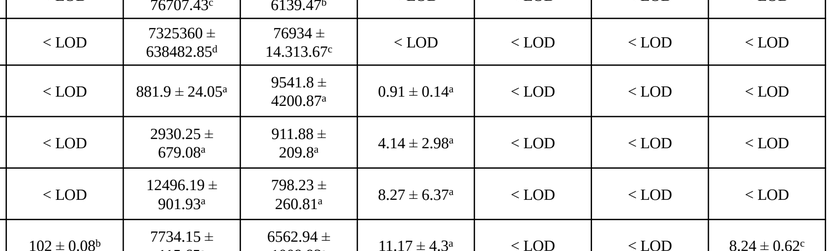
A one-way analysis of variance (ANOVA) and a post-hoc test (Duncan test) was conducted to determine the significance of the data with a confidence level of 95% ($P < 0.05$). The results obtained from these statistical procedures revealed significative differences between each average level of mycotoxin contamination related to each analyzed matrix. According with this method, it is possible to cluster samples in different sub-groups for each contamination level. The statistical analysis confirmed significant differences in the contamination of different feed samples, highlighting a strong matrix effect.





Emerging mycotoxin	Formula	Mass (m/z)	Species	R.T. (min)	Ionization	Fragment ion
Moniliformin (MON)	C_4HO_3	96.99312	-	0.85	ESI -	78.95
3-nitropropionic acid (3-NP)	$C_3H_5NO_4$	137.05568	+ NH_4	5.39	ESI +	91.05
Mycophenolic acid (MPA)	$C_{17}H_{20}O_6$	321.13326	+ H	8.43	ESI +	207.06
Beauvericin (BEA)	$C_{45}H_{57}N_3O_9$	801.44331	+ NH_4	10.83	ESI +	244.13-262.14





Conclusions

LC–HRMS is a rising approach, and it is today highly suitable for detecting multiple mycotoxins and modified mycotoxins in feed samples, although the proper quantification and detection of masked/hidden mycotoxins in complex matrixes is still one of the major obstacles. Additionally, the implementation of regulations for the so-called emerging mycotoxins is still challenging. HRMS combined with an omics-approach has the potential soon to be of great help to regulatory bodies to limit the presence of mycotoxins feed products. This developed multi-screening method allows us to identify and quantify 37 mycotoxins, both EU-regulated and not regulated, but our aim for the future is to enhance this screening method with other emerging mycotoxins of interest in animal feeding and related with health and performance problems.

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