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A validated multi-residue ultra-high-performance liquid chromatography-high-resolution mass spectrometry method for emerging mycotoxins in pork, liver and pig feed

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Abstract
Emerging mycotoxins produced by *Fusarium* and *Alternaria* species, such as enniatins (ENN), beauvericin (BEA), alternariol (AOH), and alternariol monomethyl ether (AME), are increasingly detected along the feed–food chain and raise growing concerns for animal health and food safety. Despite their widespread occurrence, regulatory limits are currently lacking, mainly due to insufficient occurrence and exposure data, highlighting the need for robust analytical methods applicable to both feed and edible animal tissues. In this study, a multi-residue ultra-high-performance liquid chromatography-high-resolution mass spectrometry (UHPLC-HRMS) method was developed and validated for the simultaneous determination of seven emerging mycotoxins (AOH, AME, BEA, ENA, ENA1, ENB, and ENB1) in pork, liver, and pig feed. Sample preparation was based on solvent extraction followed by a defatting step, while chromatographic separation was achieved using a reversed-phase column coupled to Orbitrap high-resolution mass spectrometry operating in full-scan and product ion scan modes. Method validation was performed according to Commission Implementing Regulation (EU) 2023/2782, evaluating selectivity, linearity, precision, recovery, and sensitivity. Excellent linearity was obtained for all analytes in all matrices, with coefficients of determination >0.99. Recoveries ranged from 70 to 91% in liver, from 72 to 95% in muscle and from 73 to 120% in feed, while intra- and inter-day precision values were consistently below 15%. The limit of quantification was established at 1 ng g^{-1} for all compounds. Overall, the validated UHPLC-HRMS method proved to be sensitive, selective, and reproducible, providing a reliable analytical tool for monitoring emerging mycotoxins in swine production and associated feedstuffs and supporting future occurrence studies, exposure assessments, and risk evaluation activities along the agri-food chain.

Introduction
Mycotoxins are naturally occurring fungal secondary metabolites that can contaminate raw agricultural commodities and derived food and feed products throughout cultivation, storage and processing, representing a persistent concern for animal health, food safety and public health (Bennett and Klich, 2003; Marin *et al*., 2013). In the European Union, maximum levels are currently established for several well-known mycotoxins, including aflatoxins, ochratoxin A, patulin, deoxynivalenol, zearalenone, fumonisins, citrinin, ergot sclerotia and ergot alkaloids, T-2 and HT-2 toxins in specific commodities, under Commission Regulation (EU) 2023/915 (European Commission, 2023a). Alongside these regulated contaminants, increasing attention has been directed toward so-called emerging mycotoxins, *i.e*., fungal metabolites that are frequently detected in food and feed, but for which occurrence data, toxicological characterization and harmonized regulatory limits remain limited (Jestoi, 2008; Gruber-Dorninger *et al*., 2019).
Among these compounds, the analytes considered in the present study belong to two distinct fungal, chemical and toxicological groups. The first group includes enniatins (ENNs) [enniatin A (ENNA), enniatin A1 (ENNA1), enniatin B (ENNB), enniatin B1 (ENNB1)] and beauvericin (BEA), which are mainly produced by *Fusarium* spp. and are structurally classified as cyclic hexadepsipeptides. Due to their ionophoric properties, ENNs and BEA can disrupt membrane permeability and cellular ion homeostasis, and they have been mainly associated with cytotoxic, pro-apoptotic and oxidative stress-related effects demonstrated in vitro, whereas in vivo evidence is still comparatively limited (EFSA CONTAM Panel, 2014; Juan-García *et al*., 2015; Manyes *et al*., 2018; De Felice *et al*., 2023; Behr *et al*., 2025a;). The second group includes the *Alternaria* toxins alternariol (AOH) and alternariol monomethyl ether (AME), two dibenzo-α-pyrone derivatives mainly produced by *Alternaria* spp. and commonly reported in plant-derived commodities. In contrast with ENNs and BEA, AOH and AME have been primarily associated with genotoxic and mutagenic activity, mainly demonstrated in bacterial and mammalian in vitro systems, while in vivo evidence remains limited (EFSA CONTAM Panel, 2011; Fraeyman *et al*., 2017; Solfrizzo, 2017; Crudo *et al*., 2023). This distinction is relevant because these two groups differ not only in fungal origin and chemical structure, but also in their toxicological profiles and analytical behavior.

These emerging mycotoxins have been increasingly reported in cereals and compound feed and, in some cases, also in products of animal origin, raising concern about their carry over along the food chain and their potential implications for consumer exposure (Gruber-Dorninger *et al*., 2019; Křížová *et al*., 2021; Serra *et al*., 2021; Lorusso *et al*., 2025). For *Fusarium* emerging mycotoxins Human toxicological data are scarce, but biomonitoring has detected ENNB in human serum, indicating dietary exposure (EFSA CONTAM Panel, 2014; Warensjö Lemming *et al*., 2020; Behr *et al*., 2025;). With respect to *Alternaria* metabolites, the present study focused on AOH and AME because they are among the best investigated representatives of the dibenzo-α-pyrone group and can be included in a common multiresidue LC-based workflow together with ENNs and BEA (EFSA CONTAM Panel, 2011; Solfrizzo, 2017).

From an analytical point of view, the determination of these compounds has been mainly addressed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) or high-resolution mass spectrometry (LC-HRMS), owing to their high sensitivity, selectivity and specificity for multiresidue analysis in complex matrices (Jensen *et al*., 2019 Gámiz-Gracia *et al*., 2020). Conventional chromatographic approaches based on optical detection (*e.g*., UV or fluorescence) are inherently limited by selectivity issues, matrix interferences, and a reduced capacity to comprehensively cover structurally diverse mycotoxins (Janik *et al.*, 2021). Similarly, immunochemical methods, while valuable for rapid screening purposes, lack the robustness required for the simultaneous and unequivocal determination of multiple analytes across different chemical classes (Li *et al*., 2021). In this context, HRMS offers the additional advantage of accurate-mass measurement and retrospective data analysis, which may be particularly valuable in exploratory and multi-class contamination studies (Jensen *et al*., 2019).

Swine-related matrices are of particular interest in this field because pigs are highly exposed to cereal-based feed and represent an important link between feed contamination and food of animal origin. Moreover, feed is the primary exposure matrix, while liver and muscle are relevant edible tissues for investigating possible residue occurrence. The liver is especially informative because it is the major organ involved in xenobiotic metabolism and may represent a preferential site for residue detection following dietary exposure (Křížová *et al*., 2021).

Based on these considerations, the aim of the present study was to develop and validate an ultra-performance liquid chromatography–high-resolution mass spectrometry (UPLC-HRMS) method for the simultaneous determination of seven emerging mycotoxins, namely AOH, AME, BEA, ENNA, ENNA1, ENNB and ENNB1, in pork liver, muscle and feed. The method was validated in terms of sensitivity, linearity, accuracy, precision and recovery, with the overall objective of providing a robust analytical tool for monitoring selected emerging *Fusarium* and *Alternaria* mycotoxins in matrices relevant to the swine production chain.

Materials and Methods

Chemical and reagents

All solvents and reagents were of HPLC or analytical grade and were purchased from Merck (Darmstadt, Germany). Deionized water (<18 MΩ cm resistivity) was obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA).

All standard (AOH, AME, BEA, ENNA, ENNA1, ENNB and ENNB1) were purchased from Merck, instead Ochratoxin A-d5, used as an internal standard, was provided by LGC Standards (Wesel, Germany). Individual stock solutions of AOH, AME, BEA, ENNA, ENNA1, ENNB, ENNB1 and OTA-d5, with concentrations of 1 mg mL^{-1} were prepared in acetonitrile. The solutions were stored at −20°C in glass-stoppered bottles, protected from light. These stock solutions were then diluted with acetonitrile to obtain the appropriate working solutions (ranging from 100 ng mL^{-1} to 10000 ng mL^{-1}) and were stored in darkness at -20°C.

Samples

Five samples for each type of matrix (liver, muscle, finisher pig feed) were collected from local retailers. Each of the five samples collected for each matrix was processed and analyzed individually. The five finisher pig feed samples shared the same declared composition, although they originated from different brands and, therefore, from different batches. The finisher feed was formulated with maize, barley, wheat middlings, wheat, dehulled soybean meal, dehulled sunflower meal, wheat bran, sugar cane molasses, calcium carbonate, animal fat, sodium chloride, and sodium bicarbonate.
Samples were transported under refrigerated conditions to the Food Safety Unit laboratory of the Department of Veterinary Medicine of the University of Bari Aldo Moro and stored at -80°C until analysis.

Analytical protocol

5 g of homogenized liver, muscle and feed were spiked with internal standard at 20 ng g^{-1} followed by the addition of 10 mL of acetonitrile/water/acetic acid 79:20:1, v/v/v. After 1 min of vortex, 30 min of sonication and centrifugation (3700 g, 4°C, 10 min), 1 mL of supernatant was transferred in a tube where 1mL of hexane was added to perform the extract defatting. Subsequently, the solution was centrifugated at 3700 g, 4°C, 10 min and 200 µL of the intermediate phase were recovered and placed in a vial. Prior to fortification, non-spiked samples of each matrix were also analyzed to confirm the absence of detectable target analytes.
The analysis was performed by an UPLC-HRMS system composed of a Vanquish device (Thermo Fisher Scientific, Waltham, MA, USA) coupled to a Thermo Orbitrap™ Exploris 120 (Thermo Fisher Scientific, Waltham, MA, USA), equipped with a heated electrospray ionization (HESI) source. A Raptor ARC-18 5 µm, 150 × 2.1 mm column (Restek, Bellefonte, PA, USA) maintained at 40 °C was used for the separation of the analytes. The elution gradient was formed by mixing mobile phases A (5 mM aqueous ammonium formate with 0.1% formic acid) and B (methanol). It started at 2% of B for 2 min, followed by a linear increase to 50% B over the next 3 min, and then another linear increase to 100% B over the next 9 min. This was followed by a 4-minute hold at 100% B, and after 0.5 minutes, a re-equilibration at 2% B until the 23rd minute. The flow was set at 0.3 mL min^{-1}. The injection volume was 10 µL
With regard to the detector, the ion transfer tube and vaporizer temperatures were set at 330 and 280°C, respectively, the sheath and auxiliary gas at 35 and 15 arbitrary units (AU) and the electrospray voltage at 3.50 kV for both positive and negative mode. The full-scan (FS) acquisition was combined with a product ion scan mode for the confirmatory response based on an inclusion list. The FS worked with a resolution of 60,000 FWHM, a scan range of 200–810 *m/z*, a standard automatic gain control (AGC), an RF lens % of 70 and an automatic maximum injection time. The product ion scan acquisition operated at 15,000 FWHM, with a standard AGC target, an automatic maximum injection time and scan range mode and an isolation window of 1 *m/z*. Fragmentation of the precursors was optimized with a two-step normalized collision energy (20 and 60 eV). Xcalibur™ 4.5 (Thermo Fisher Scientific, Waltham, MA, USA) was the software used. Figure 1 shows the chromatograms of the parent ions extracted from full-scan acquisition and the corresponding fragmentation mass spectra of the seven mycotoxins and the internal standard in a liver sample, as an example.

Validation design

For the validation study, the method's performance was assessed through several parameters according to the Guidelines Commission Implementing Regulation (EU) 2023/2782 (European Commission, 2023) selectivity/specificity, linearity (R^2), precision in terms of repeatability (RSD) intra and inter-day, recovery and sensitivity. Briefly, the selectivity of the method was evaluated by injecting extracted blank of the different matrices , and the lack of signal near the expected mycotoxin retention time with a signal-to-noise ratio (S/N) < 3 indicated the absence of interference. The linearity was evaluated by 5-point matrix-matched calibration curves in duplicate at LOQ, 2, 3, 5, 10 ng/g, expressed as coefficient of determination (R^2).

The intra-day and inter-day precision, expressed as CV% (coefficient of variation), were assessed at LOQ by 5 replicated on the same day and by 5 replicates in 3 distinct days, respectively. Recovery was evaluated at two spiking levels (1 and 10 ng/g) by comparing analyte concentrations in samples spiked before extraction with those measured after the entire extraction procedure.
The limits of quantification (LOQ) were determined experimentally, based on signal-to-noise (S/N) ratios of 10.

Results

The analytical method developed for the simultaneous determination of seven emerging mycotoxins, ENNA, ENNA1, ENNB, ENNB1, BEA, AME, and AOH, was successfully validated in liver, muscle, and feed matrices.
Linearity was excellent across all compounds, with coefficients of determination (R^2) consistently higher than 0.99 in every matrix (Tables 1 and 2). Specifically, R^2 values ranged from 0.9900 (AOH, feed) to 0.9993 (ENNB1, liver and muscle), confirming a strong correlation between instrumental response and analyte concentration.
Recoveries were within acceptable ranges in all tested matrices. In liver, recovery rates varied between 70-91 %, in muscle between 72-95 %, and in feed between 73-120 %, depending on the toxin and concentration level. The slightly higher recoveries observed in feed samples (up to 120 % for ENNB1) are consistent with matrix complexity and possible co-extractive effects but remained within the acceptance range of 70-120 % established by Commission Implementing Regulation (EU) 2023/2782 and SANTE/11312/2021 (Pihlström *et al.*, 2021).
Intra-day precision (CV %) ranged from 5 % to 8 % for liver and muscle and from 6 % to 13 % for feed, while inter-day CV % values were between 5 % and 14 %, always below the 15 % threshold recommended for quantitative methods. These results indicate good repeatability and intermediate precision across all matrices.
The limit of quantification (LOQ) was established at 1 ng g^{-1} for all analytes, ensuring a signal-to-noise ratio ≥ 10 and a CV % below 10 %. This low LOQ demonstrates that the method is sensitive enough to detect trace levels of emerging mycotoxins in both animal tissues and feed, meeting the requirements for high-resolution monitoring along the food chain.
Overall, the method exhibited excellent linearity, accuracy, and precision across all tested matrices, confirming its reliability for the simultaneous determination of ENNs, BEA, AME, and AOH in liver, muscle, and feed matrices.

Discussion

The validation data obtained confirm that the UHPLC-HRMS method developed in this study is a reliable and versatile tool for the simultaneous determination of seven emerging mycotoxins, ENNA, ENNA1, ENNB, ENNB1, BEA, AME, and AOH, in complex matrices such as liver, muscle, and feed.
The method showed excellent linearity across all analytes (R^2>0.99) over the tested concentration range of 1-10 ng/g, demonstrating a consistent correlation between instrumental response and concentration. Similar linear performance has been reported for enniatins and BEA in broiler chicken liver, and animal feed (Fraeyman *et al.*, 2018; Tolosa, Rodríguez-Carrasco *et al*., 2019), although direct comparison of determination coefficients across studies should be interpreted with caution due to differences in calibration range and matrix composition. Overall, the validation results indicate that the UHPLC-HRMS method provides adequate analytical performance for trace-level quantification in heterogeneous matrices.
From a sample-preparation perspective, the proposed procedure is simpler than several previously published methods for the same or closely related analytes. For example, ENNs and BEA in animal feed have been determined using acetonitrile-based extraction followed by QuEChERS/dSPE clean-up and LC-QTRAP/MS/MS detection (Tolosa, Rodríguez-Carrasco *et al*., 2019), whereas multi-mycotoxin methods including ENNs, BEA, AOH and AME have relied on buffered

acetonitrile/methanol/water extraction combined with Oasis HLB SPE prior to UHPLC-MS/MS analysis (Sun *et al*., 2019). In tissue-oriented applications, more elaborate clean-up strategies such as solid–liquid extraction followed by DLLME have also been proposed (Castell *et al*., 2024), while dedicated methods for Alternaria toxins have employed DLLME coupled to LC-ESI-MS/MS (Rodríguez-Carrasco *et al*., 2016). In comparison, the extraction and defatting strategy adopted in the present study allowed acceptable recoveries and precision in feed, liver and muscle without sorbent-based purification, supporting its suitability as a streamlined multi-matrix workflow for the selected analytes, with the additional advantages of reduced sample preparation time and lower analytical costs.

From an instrumental standpoint, most previously published quantitative methods for these mycotoxins have been based on triple-quadrupole MS/MS platforms (Jensen *et al*., 2019; Sun *et al*., 2019; Tolosa, Barba *et al*., 2019; Tolosa, Rodríguez-Carrasco *et al.*, 2019). In the present study, the use of UHPLC-HRMS provided accurate-mass selectivity while maintaining satisfactory sensitivity and precision at low ng/g levels. This is particularly advantageous in heterogeneous matrices and may also facilitate future extension of the method toward retrospective screening of additional contaminants.

The recovery values obtained (70-91 % in liver, 72-95 % in muscle, and 73-120 % in feed) fall within the acceptance ranges established by the EU guidelines (SANTE/11312/2021; European Commission, 2023). The slightly higher recoveries observed in feed, particularly for ENNB1, may be attributed to matrix-related ion enhancement effects, which are common when analyzing complex cereal-based matrices under electrospray ionization conditions (Kaufmann, 2012). Nevertheless, all recoveries remained within the tolerance limits for quantitative methods, confirming the robustness of the extraction and clean-up procedure across different sample types (European Commission, 2023; SANTE/11312/2021).

Precision in terms of repeatability intra and inter-day were satisfactory in all cases, with CV% consistently below 15 %. These values indicate a high degree of reproducibility, which is essential for the reliable monitoring of mycotoxins in routine control laboratories. The overall accuracy of the method is in line with reports in previous validated multi-analyte studies of emerging mycotoxins in feed and food (Fraeyman *et al*., 2017; Sun *et al*., 2019; Tolosa, Barba *et al.*, 2019; Tolosa, Rodríguez-Carrasco *et al*., 2019).

The low limit of quantification (1 ng g^{-1}) achieved for all analytes across the tested matrices represents a significant analytical advantage, enabling direct comparison of contamination levels among feed and food. The method's sensitivity is sufficient to detect trace concentrations that may arise from dietary exposure or bioaccumulation phenomena. In this respect, the inclusion of liver and muscle as validation matrices is particularly relevant, as these tissues are primary targets for the absorption, metabolism, and potential accumulation of lipophilic mycotoxins such as enniatins and BEA (Dolenšek *et al*., 2021; Křížová *et al*., 2021; Hasuda *et al*., 2023; Behr *et al*., 2025;).

The availability of a single validated multi-residue method applicable to feed and animal tissues provides an important analytical tool to trace the fate of emerging mycotoxins along the food chain. Such harmonized analytical approaches are consistent with EFSA's current framework for exposure assessment and data collection, supporting the generation of robust occurrence data required for future risk evaluations of emerging mycotoxins (EFSA, 2016; EFSA, 2019; Bampidis *et al*., 2024).

In conclusion, the method developed and validated in this study meets international performance criteria in terms of linearity, accuracy, precision, and sensitivity. Its robustness and low quantification limits make it particularly suitable for routine monitoring and research purposes, providing a solid basis for future studies on the bioaccumulation and carry-over of emerging mycotoxins in food-producing animals.

Conclusions

The UHPLC-HRMS method developed and validated in this study provides a sensitive, selective and reproducible tool for the simultaneous determination of seven emerging *Fusarium* and *Alternaria*-

related mycotoxins (BEA, ENNA, ENNA1, ENNB, ENNB1, AOH, AME) in pork liver, muscle and pig feed.

Compared with previously published approaches, the present method offers the advantage of combining, within a single validated workflow, the determination of both *Fusarium*-derived enniatins/ BEA and *Alternaria* toxins across three matrices directly relevant to the swine production chain. Previous methods have generally focused on ENNs and BEA in feed, on individual tissues, or on analytical approaches not specifically developed for the integrated assessment of feed and edible pig tissues. In addition, the use of UHPLC-HRMS provides accurate-mass confirmation and the possibility of retrospective data evaluation, which may represent a practical advantage for future multi-contaminant surveillance applications.

These analytical features make the method applicable to: (i) occurrence and biomonitoring studies aimed at characterising contamination along the feed–food chain; (ii) carry-over investigations in livestock; and (iii) support to exposure assessment frameworks and analytical surveillance activities.

Notwithstanding its strengths, the method would benefit from further work before routine regulatory implementation, including: i) extension of validation to a larger and more diverse set of real samples, to determine whether geographical and seasonal variability may affect analyte occurrence and method applicability; ii) assessment of performance in additional food matrices; iii) stability studies on real feed samples to evaluate whether storage under actual farm conditions may influence the occurrence and/or degradation of the investigated molecules; iv) inter-laboratory ring trials to confirm transferability and robustness under routine laboratory conditions; and v) expansion of the method to include additional regulated and emerging mycotoxins, thus providing business operators with a rapid and cost-effective tool for the simultaneous control of known and emerging mycotoxins.

In conclusion, the validated UHPLC-HRMS method represents a robust analytical platform for the integrated surveillance of selected emerging mycotoxins in pig feed and edible tissues, providing a useful basis for future occurrence studies aimed at characterizing the risk along this production chain and supporting the definition of management and mitigation measures to protect animal and human health.

References

Bampidis V, Azimonti G, Bastos MdL, Christensen H, Durjava M, Dusemund B, Kouba M, López-Alonso M, López Puente S, Marcon F, Mayo B, Pechová A, Petkova M, Ramos F, Villa RE, Woutersen R, Gropp J, Hoogenboom L, Richen G, Innocenti ML, Kujawa M, Rovesti E, 2024. Animal dietary exposure in the risk assessment of contaminants in feed. EFSA J 22:8858.

Behr AC, Fæste CK, Azqueta A, Tavares AM, Spyropoulou A, Solhaug A, Olsen AK, Vettorazzi A, Mertens B, Zegura B, Streel C, Ndiaye D, Spilioti E, Dubreil E, Buratti FM, Crudo F, Eriksen GS, Snapkow I, Teixeira JP, Rasinger JD, Sanders J, Machera K, Ivanova L, Gaté L, Le Hegarat L, Novak M, Smith NM, Tait S, Fraga S, Hager S, Marko D, Braeuning A, Louro H, Silva MJ, Dirven H, Dietrich J, 2025. Hazard characterization of the mycotoxins enniatins and beauvericin to identify data gaps and improve risk assessment for human health. Arch Toxicol 99:1791-841.

Bennett JW, Klich M, 2003. Mycotoxins. Clin Microbiol Rev 16:497-516.

Castell A, Arroyo-Manzanares N, Palma-Manrique R, Campillo N, Torres C, Fenoll J, Viñas P, 2024. Evaluation of distribution of emerging mycotoxins in human tissues: applications of dispersive liquid–liquid microextraction and liquid chromatography-mass spectrometry. Anal Bioanal Chem 416:449-59.

Crudo F, Hong C, Varga E, Del Favero G, Marko D, 2023. Genotoxic and mutagenic effects of the alternaria mycotoxin alternariol in combination with the process contaminant acrylamide. Toxins 15:670.

De Felice B, Spicer LJ, Caloni F, 2023. Enniatin B1: emerging mycotoxin and emerging issues. Toxins 15:383.

Dolenšek T, Švara T, Knific T, Gombač M, Luzar B, Jakovac-Strajn B, 2021. The influence of fusarium mycotoxins on the liver of gilts and their suckling piglets. Animals 11:2534.

EFSA, 2016. Dietary exposure assessment to Alternaria toxins in the European population. EFSA J 14:e04654.
EFSA, 2019. Animal dietary exposure: overview of current approaches used at EFSA. EFSA J 17:e05896.
EFSA CONTAM Panel, 2011 Scientific Opinion on the risks for animal and public health related to the presence of Alternaria toxins in feed and food. EFSA J 9:2407.
EFSA CONTAM Panel, 2014. Scientific opinion on the risks to human and animal health related to the presence of beauvericin and enniatins in food and feed. EFSA J 12:3802.
European Commission, 2023a. Regulation of the European Commission of 25 April 2023 on maximum levels for certain contaminants in food and repealing Regulation (EC) No 1881/2006, 915/2023/EU. In: Official Journal, L 119/103, 08/10/2025.
European Commission, 2023b. Commission Implementing Regulation (EU) 2023/2728 of 14 December 2023 laying down the methods of sampling and analysis for the control of the levels of mycotoxins in food and repealing Regulation (EC) No 401/2006. In: Official Journal, L 2782, 15/12/2023.
Fraeyman S, Croubels S, Devreese M, Antonissen G, 2017. Emerging fusarium and alternaria mycotoxins: occurrence, toxicity and toxicokinetics. Toxins 9:228.
Fraeyman S, Croubels S, Devreese M, Ducatelle R, Rychlik M, Antonissen G, 2018. Chronic dietary intake of enniatin b in broiler chickens has low impact on intestinal morphometry and hepatic histology, and shows limited transfer to liver tissue. Toxins 10:45.
Gámiz-Gracia L, García-Campaña AM, Arroyo-Manzanares N, 2020. Application of LC-MS/MS in the mycotoxins studies. Toxins 12:272.
Gruber-Dorninger C, Jenkins T, Schatzmayr G, 2019. Global mycotoxin occurrence in feed: a ten-year survey. Toxins 11:375.
Hasuda AL, Person E, Khoshal A, Bruel S, Puel S, Oswald IP, Bracarense APFRL, Pinton P, 2023. Emerging mycotoxins induce hepatotoxicity in pigs' precision-cut liver slices and HepG2 cells. Toxicon 231:107195.
Janik E, Niemcewicz M, Podogrocki M, Ceremuga M, Gorniak L, Stela M, Bijak M, 2021. The existing methods and novel approaches in mycotoxins' detection. Molecules 26:3981.
Jensen T, De Boevre M, Preußke N, De Saeger S, Birr T, Verreet JA, Sönnichsen FD, 2019. Evaluation of high-resolution mass spectrometry for the quantitative analysis of mycotoxins in complex feed matrices. Toxins 11:531.
Jestoi M, 2008. Emerging fusarium-mycotoxins fusaproliferin, beauvericin, enniatins, and moniliformin: a review. Crit Rev Food Sci Nutr 48:21-49.
Juan-García A, Ruiz MJ, Font G, Manyes L, 2015. Enniatin A1, enniatin B1 and beauvericin on HepG2: evaluation of toxic effects. Food Chem Toxicol 84:188-96.
Kaufmann A, 2012. The current role of high-resolution mass spectrometry in food analysis. Anal Bioanal Chem 403:1233-49.
Křížová L, Dadáková K, Dvořáčková M, Kašparovský T, 2021. Feedborne mycotoxins beauvericin and enniatins and livestock animals. Toxins 13:32.
Li R, Wen Y, Wang F, He P, 2021. Recent advances in immunoassays and biosensors for mycotoxins detection in feedstuffs and foods. J Anim Sci Biotechnol 12:108.
Lorusso P, Rusco G, Manfredi A, Iaffaldano N, Di Pinto A, Bonerba E, 2025. Emerging mycotoxins in aquaculture: current insights on toxicity, biocontrol strategies, and occurrence in aquafeed and fish. Toxins 17:356.
Manyes L, Escrivá L, Ruiz MJ, Juan-García A, 2018. Beauvericin and enniatin B effects on a human lymphoblastoid Jurkat T-cell model. Food Chem Toxicol 115:127-35.
Marin S, Ramos AJ, Cano-Sancho G, Sanchis V, 2013. Mycotoxins: occurrence, toxicology, and exposure assessment. Food Chem Toxicol 60:218-37.
Pihlström T, Fernández-Alba AR, Ferrer Amate C, Erecius Poulsen M, Lippold R, Carrasco Cabrera L, Pelosi P, Valverde A, Unterluggauer H, Mol H, Jezussek M, Malato O, Štěpán R, Lambert M,

2021. Analytical quality control and method validation procedures for pesticide residues analysis in food and feed SANTE 11312/2021.
Rodríguez-Carrasco Y, Mañes J, Berrada H, Juan C, 2016. Development and validation of a LC-ESI-MS/MS method for the determination of alternaria toxins alternariol, alternariol methyl-ether and tentoxin in tomato and tomato-based products. Toxins 8:328.
Serra V, Salvatori G, Pastorelli G, 2021. Pilot study: does contamination with enniatin B and beauvericin affect the antioxidant capacity of cereals commonly used in animal feeding? Plants 10:1835.
Solfrizzo M, 2017. Recent advances on Alternaria mycotoxins. Curr Opin Food Sci 17:57-61.
Sun D, Qiu N, Zhou S, Lyu B, Zhang S, Li J, Zhao Y, Wu Y, 2019. Development of sensitive and reliable UPLC-MS/MS methods for food analysis of emerging mycotoxins in China total diet study. Toxins 11:166.
Tolosa J, Barba FJ, Font G, Ferrer E, 2019. Mycotoxin incidence in some fish products: QuEChERS methodology and liquid chromatography linear ion trap tandem mass spectrometry approach. Molecules 24:527.
Tolosa J, Rodríguez-Carrasco Y, Ferrer E, Mañes J, 2019. Identification and quantification of enniatins and beauvericin in animal feeds and their ingredients by LC-QTRAP/MS/MS. Metabolites 9:33.
Warensjö Lemming E, Montano Montes A, Schmidt J, Cramer B, Humpf HU, Moraeus L, Olsen M, 2020. Mycotoxins in blood and urine of Swedish adolescents—possible associations to food intake and other background characteristics. Mycotoxin Res 36:193-206.

Table 1. Validation parameters obtained for all mycotoxins in liver and meat.

	LIVER					
	Recovery %		Linearity	Precision		LOQ
	1 ng g^{-1}	10 ng g^{-1}	R^2	CV % intra-day	CV % inter-day	ng g^{-1}
ENA	75	70	0.9983	6	5	1
ENA1	74	86	0.9959	5	7	1
ENB	79	88	0.9989	7	10	1
ENB1	72	87	0.9993	7	14	1
BEA	78	87	0.9903	8	7	1
AME	75	87	0.9958	5	7	1
AOH	72	91	0.9909	6	12	1
	Muscle					
	Recovery %		Linearity	Precision		LOQ
	1 ng g^{-1}	10 ng g^{-1}	R^2	CV % intra-day	CV % inter-day	ng g^{-1}
ENA	78	72	0.9983	5	6	1
ENA1	76	90	0.9959	6	6	1
ENB	81	91	0.9989	6	9	1
ENB1	75	88	0.9993	6	13	1
BEA	79	86	0.9903	7	6	1
AME	76	85	0.9958	6	6	1
AOH	76	95	0.9909	5	11	1

Table 2. Validation parameters obtained for all mycotoxins in feed.

	FEED					
	Recovery %		Linearity	Precision		LOQ
	1 ng g^{-1}	10 ng g^{-1}	R^2	CV % intra-day	CV % inter-day	ng g^{-1}
ENA	89	104	0.9964	9	10	1
ENA1	85	77	0.9903	9	11	1
ENB	103	75	0.9928	9	14	1
ENB1	120	80	0.9912	6	9	1
BEA	108	73	0.9930	13	13	1
AME	81	76	0.9970	13	15	1
AOH	103	88	0.9900	12	12	1

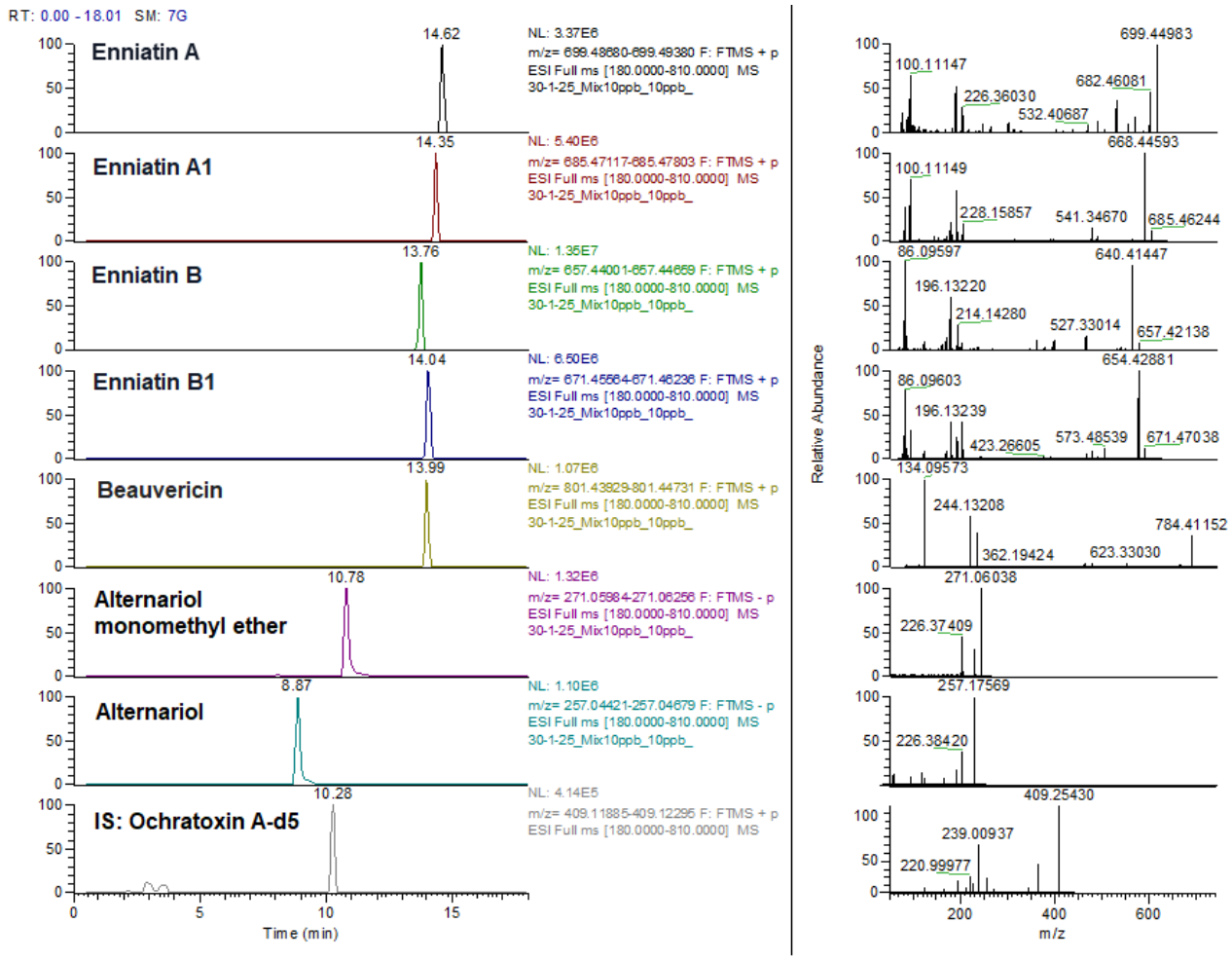

Figure 1. Extracted parent ion chromatograms from FS and relative fragmentation mass spectra of the 7 mycotoxins and IS in a liver sample as an example