

Review

Nuclear Magnetic Resonance-Based Approaches for the Structural and Quantitative Analysis of Mycotoxins

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Abstract: Mycotoxins are toxic secondary metabolites produced by various fungal species, posing significant food safety concerns due to their health impacts and economic burden. Accurate structural elucidation and quantitative analysis are essential for effective risk assessment and regulatory control. This review highlights recent advances in the application of nuclear magnetic resonance (NMR) spectroscopy for the structural and quantitative analysis of major mycotoxins, including aflatoxins, ochratoxins, fumonisins, trichothecenes, and zearalenone. One- and two-dimensional NMR techniques enable precise molecular characterization, positional isomer identification, including modified forms such as masked or conjugated mycotoxins, and toxicity-related molecular interaction investigation. NMR spectroscopy offers superior structural resolution, high reproducibility, and nondestructive analysis, making it invaluable in mycotoxin research. Quantitative NMR spectroscopy has emerged as a robust and accurate method for determining the absolute concentration and purity of mycotoxins, without requiring analyte-specific reference standards, an advantage particularly important for modified toxins lacking commercially available standards. The integration of NMR-based approaches strengthens analytical reliability, supports reference material development, and contributes to enhanced food safety assessment. This review also discusses ongoing analytical challenges and future directions, including the application of artificial intelligence to improve the automation and interpretation of NMR data in mycotoxin research.

Keywords: mycotoxin; nuclear magnetic resonance; modified forms; structure; quantification



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1. Introduction

Mycotoxins are a group of diverse low-molecular-weight toxic compounds that are mainly produced as secondary metabolites by several filamentous fungi belonging to the genera *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria*. These toxic compounds frequently contaminate agricultural products such as grains, nuts, fruits, and spices either before harvest or during storage, posing significant health risks to both humans and animals [1–3]. Adverse effects associated with mycotoxins include hepatotoxicity, nephrotoxicity, immunosuppression, carcinogenicity, and teratogenicity. Regulatory efforts have mainly focused on major mycotoxins that are toxicologically significant and frequently detected in the food supply chain, such as aflatoxins, trichothecenes, fumonisins, ochratoxins, and zearalenone [3].

Research has increasingly expanded to encompass modified mycotoxins, which arise from metabolic conjugation by plants, microbial biotransformation, or food processing

methods such as fermentation and heat treatment [4]. These structurally altered forms—often termed masked or conjugated mycotoxins—may escape detection by conventional analytical techniques but can still release their toxic parent compounds during gastrointestinal digestion. In addition, certain modified mycotoxins exhibit altered toxicological properties, bioavailability, or synergistic effects, making their identification and quantification increasingly relevant in food safety evaluations. Therefore, accurate structural elucidation and quantitative analysis of both parent and modified mycotoxins are essential for comprehensive risk assessment and effective food safety management [5].

Various analytical techniques—including gas chromatography (GC), high-performance liquid chromatography (HPLC), and liquid chromatography–mass spectrometry (LC-MS)—have been widely employed for mycotoxin analysis [6]. However, these conventional methods often fall short in delivering comprehensive structural information, particularly in distinguishing positional isomers, stereoisomers, and specific conjugation sites [7–10]. Moreover, they typically require authentic reference standards, which are often unavailable or unstable for newly discovered or modified mycotoxins. This dependency limits their utility in the untargeted screening or evaluation of compounds lacking commercial standards.

To overcome these limitations, nuclear magnetic resonance (NMR) spectroscopy has gained attention as an effective, complementary tool for both the structural characterization and quantitative analysis of mycotoxins [7]. NMR spectroscopy is nondestructive, highly reproducible, and capable of providing detailed molecular structure information. In particular, two-dimensional (2D) NMR methods such as correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), heteronuclear multiple-bond correlation (HMBC), total correlation spectroscopy (TOCSY), and nuclear Overhauser effect spectroscopy (NOESY) are now crucial for elucidating complex structural characteristics of mycotoxins, including the identification of linkage positions, functional group connectivity, and stereochemical configurations. These capabilities are especially critical for modified mycotoxins, whose altered structures may hinder MS-based identification [11].

In addition to its role in structural analysis, quantitative NMR (qNMR) spectroscopy has emerged as a reliable approach for the absolute quantification of mycotoxins. qNMR offers several advantages over traditional chromatographic techniques, including direct quantification via signal integration, independence from compound-specific reference standards, and suitability for multicomponent systems. Moreover, qNMR plays a key role in the development of certified reference materials (CRMs) for regulatory and research purposes [12].

This review discusses the recent research trends in the application of NMR spectroscopy to mycotoxin analysis, with an emphasis on two key aspects: (i) the structural elucidation of mycotoxins and their molecular interactions and (ii) quantitative analysis using qNMR techniques. The discussion encompasses methodological advances, current challenges, and future directions, underscoring the increasing significance of NMR-based approaches in promoting food safety and enhancing analytical reliability in mycotoxin research (Figure 1).

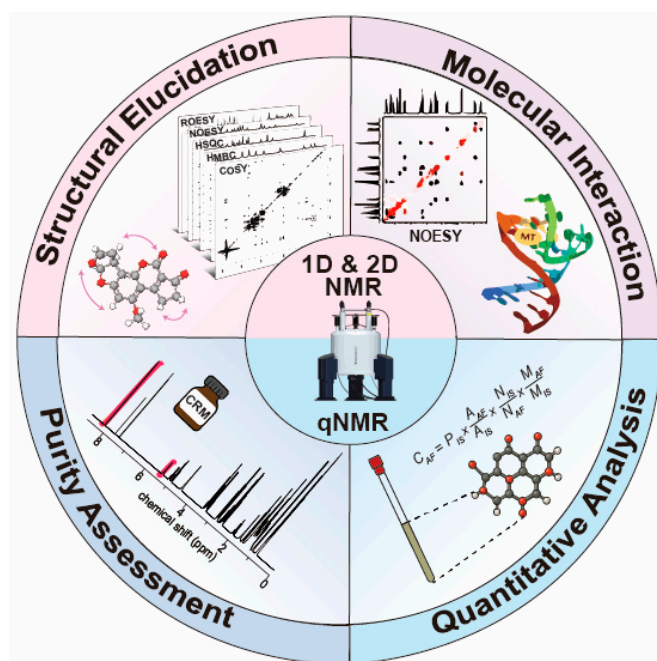


Figure 1. Overview of recent nuclear magnetic resonance (NMR) applications discussed in the review, emphasizing its utility in both the structural analysis and quantitative evaluation of mycotoxins. One-dimensional (1D); two-dimensional (2D); correlation spectroscopy (COSY); heteronuclear single quantum coherence (HSQC); heteronuclear multiple-bond correlation (HMBC); total correlation spectroscopy (TOCSY); nuclear Overhauser effect spectroscopy (NOESY); certified reference material (CRM).

2. NMR Techniques for Mycotoxin and Modified Mycotoxin Analysis

NMR spectroscopy is an effective and nondestructive analytical tool widely utilized for both the structural and quantitative analysis of organic compounds, including mycotoxins. It is based on the interaction of atomic nuclei with an external magnetic field and radiofrequency radiation, producing characteristic resonance signals that reflect the chemical environment of each nucleus. In mycotoxin analysis, NMR spectroscopy offers high-resolution structural elucidation and accurate quantitative information without requiring derivatization or extensive sample preparation.

2.1. One-Dimensional (1D) NMR Techniques

One-dimensional ^1H NMR spectroscopy is frequently used as a basic analytical tool for characterizing mycotoxins. It provides information on proton chemical shifts, coupling constants, and signal multiplicities, which are valuable for initial structural identification. For example, ^1H NMR spectroscopy has been used to identify specific proton environments in mycotoxins, helping differentiate isomeric forms and identify degradation products. Although ^{13}C NMR spectroscopy is less sensitive, it offers complementary information on the carbon framework of the molecule and has proven useful for confirming the backbone structures of mycotoxins and their derivatives.

2.2. Two-Dimensional (2D) NMR Techniques

Two-dimensional NMR techniques such as COSY, HSQC, HMBC, TOCSY, and NOESY are widely used in mycotoxin analysis to resolve spectral overlap and obtain detailed structural information. Among these, TOCSY is particularly useful for assigning spin systems in complex or overlapping proton spectra, as demonstrated in the analysis of trichothecene conjugates. The HMBC method, on the other hand, allows the analysis of long-range ^1H – ^{13}C correlations that are essential for determining the attachment sites

of functional groups in modified mycotoxins, while NOESY provides spatial proximity information, aiding the elucidation of molecular conformations and the distinction of epimeric forms. Together, these techniques enhance the accuracy of structural elucidation in mycotoxin research, especially for novel or structurally modified analogues.

2.3. Quantitative NMR (qNMR)

qNMR is a highly reproducible and accurate technique for determining the absolute concentration or purity of compounds based on the proportionality between signal integrals and the number of nuclei. Unlike chromatographic methods, qNMR does not require calibration curves or analyte-specific standards; instead, quantification is achieved by comparing the signal of the analyte to that of a known internal standard. This is especially advantageous in mycotoxin research, where pure reference materials for certain toxins or their modified forms are often scarce or unstable. qNMR has been widely used to assess the purity of CRMs, including aflatoxin B₁, fumonisin B₁, and deoxynivalenol, and is particularly useful for analytes with weak ultraviolet (UV) absorbance or low chromatographic detectability. Accurate quantification requires the careful selection of an internal standard—commonly benzoic acid, maleic acid, or dimethyl sulphone—that is chemically stable, well-resolved, and free of overlap with analyte signals. In qNMR, the analyte concentration is calculated by comparing the integrated area of its NMR signal to that of a known amount of an internal standard.

To ensure accurate and traceable measurements, internationally recognized guidelines have been established for qNMR. ISO 24583:2022 [13] specifies the general requirements for ¹H quantitative NMR using the internal standard method, particularly for determining the purity of organic compounds in food and related products. The standard outlines key elements including sample preparation, acquisition parameters, uncertainty estimation, and metrological traceability. In parallel, the IUPAC Technical Report [14] provides a comprehensive framework for SI-traceable purity assessment, identifying qNMR as a primary method owing to its high precision and direct link to mass fraction purity. Together, these guidelines reinforce the reliability of qNMR in the certification of organic reference materials and establish its value as a metrologically traceable technique in both regulatory and analytical applications.

2.4. Advantages of NMR in Mycotoxin Analysis

In the context of mycotoxin analysis, NMR spectroscopy offers several distinct advantages. It enables highly reproducible and precise measurements, critical for both the qualitative and quantitative assessment of structurally diverse toxins. Unlike many chromatographic or mass spectrometric techniques, NMR spectroscopy allows for direct and nondestructive analysis without requiring derivatization or compound-specific calibration, thereby minimizing sample preparation and avoiding analyte degradation. Furthermore, its unique ability to simultaneously provide detailed structural information and accurate quantitative data makes it especially valuable for the characterization of modified mycotoxins, unknown conjugates, and metabolites that may be challenging to detect or interpret using conventional methods. These strengths position NMR spectroscopy as an effective complementary tool for enhancing analytical reliability and advancing food safety research in the field of mycotoxins.

3. Applications of NMR in Structural Analysis of Mycotoxins

Mycotoxins are commonly classified on the basis of their chemical structures, biological effects, fungal origins, and mechanisms of action. They can also be categorized by their chemical constituents, including polyketides, lactones, alkaloids, terpenes, and other

organic compounds. For instance, aflatoxins constitute a distinct group of mycotoxins characterized by a bisdihydrofuran ring structure. In this review, mycotoxins are further classified into five major groups—aflatoxins, ochratoxins, fumonisins, trichothecenes, and zearalenone—on the basis of their target organs and toxicological mechanisms. The representative chemical structures of each group are illustrated in Figure 2.

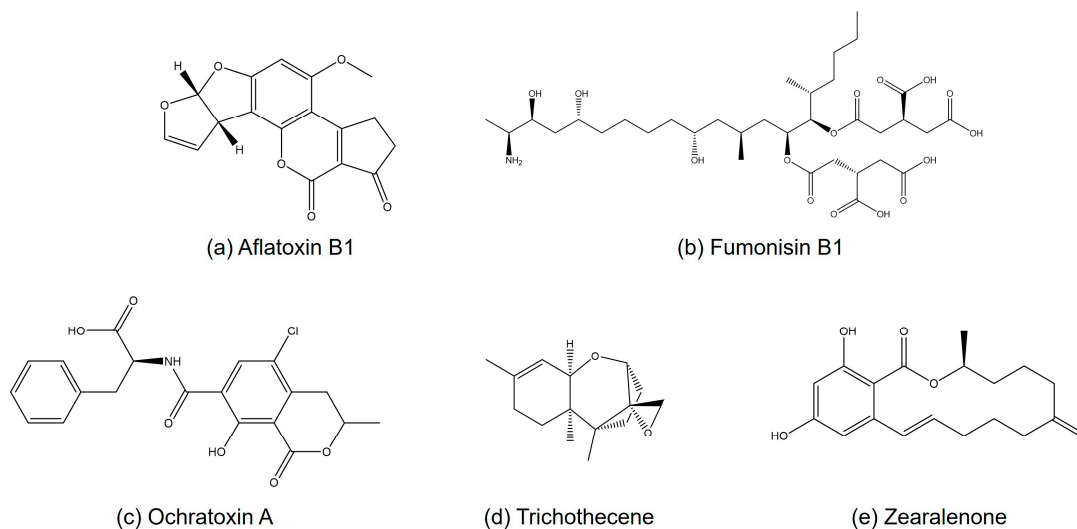


Figure 2. Representative structures of major mycotoxins described in the text.

This classification offers valuable insights into the health impacts of mycotoxins and deepens our understanding of their biological significance. Because mycotoxin toxicity is closely linked to the molecular structure, this classification also contributes to interpreting their structural and functional properties [15]. As summarized in Table 1, this section explores the role of NMR spectroscopy in the structural characterization of mycotoxins, highlighting key developments from foundational studies to recent advances.

Table 1. Representative studies using NMR spectroscopy for structural analysis of mycotoxins and their modified forms.

Toxin Type	NMR Method	Information	Reference
Aflatoxins	^1H NMR, ^{13}C NMR, COSY, HMBC, NOESY	Structural elucidation of UV-degraded products of AFB ₁ using MS and 2D NMR	[16]
	^1H NMR, TOCSY, DQF-COSY, NOESY, HMBC, ^{31}P - ^1H COSY, SOFAST-HMQC	Structure determination of AFB ₁ -AF26 aptamer complex using 2D NMR, revealing binding pocket and recognition mechanism	[17]
	^1H NMR, ^{13}C NMR, COSY, HSQC, HMBC, ROESY	Structural elucidation of AFB ₁ degradation products (aflatoxicol and epi-aflatoxicol) using isotope tracing and NMR	[7]
	^1H NMR, ^{13}C NMR, COSY, HMBC	Identification of two stereoisomers of AFQ ₁ (oxidized products of AFB ₁) using 1D and 2D NMR	[18]

Table 1. Cont.

Toxin Type		NMR Method	Information	Reference
Fumonisin	FB ₁	¹ H NMR	Structural analysis of degraded products of FB ₁	[19]
	FB ₁ derivatives	¹ H NMR, ¹³ C NMR, HSQC-CLIP-COSY, HMBC	Structural elucidation of site-specific acylated FB ₁ derivatives using 2D NMR and LC-HRMS	[20]
Ochratoxins	Ochratoxin A (OTA)–DNA aptamer	¹ H NMR, 2D NOESY, TOCSY, DQF-COSY, JR-HMBC, ³¹ P– ¹ H COSY, HSQC	Structural studies of OTA-bound DNA aptamer using 2D NMR; binding mechanism via halogen bonding, π stacking, and hydrophobic interactions	[11]
	OTA, OTA methyl ester, OTB, ochratoxin β	¹ H NMR, ¹³ C NMR, COSY, HSQC, HMBC, NOESY	Structural elucidation of ochratoxins, including ochratoxin β , using 1D and 2D NMR techniques	[21]
	OTB-GSH, OTB-NAC	¹ H NMR, ¹³ C NMR, COSY, HSQC, HMBC	Structural confirmation of synthesized OTB-GSH and OTB-NAC using 2D NMR with HPLC-MS/MS	[22]
Trichothecenes	EPT skeleton (e.g., DON, T-2 toxin)	¹ H NMR, ¹³ C NMR, HSQC, HMBC	Elucidation of the biosynthetic mechanism of the EPT tricyclic core, identifying Tri3 (acetyltransferase) and Tri14 (cyclase) as essential enzymes	[23]
	Trichodermarins G–N (1–8), Trichoderm	¹ H NMR, ¹³ C NMR, COSY, HSQC, HMBC, NOESY	Structural elucidation of 8 new trichothecenes and 2 cuparene derivatives from marine <i>Trichoderma</i> using 2D NMR	[24]
	Trichobreols A, D, E	¹ H NMR, ¹³ C NMR, COSY, HMBC, NOESY	Elucidation of stereochemistry and structural variations of the isolated trichobreol D and E from marine <i>Trichoderma</i> using 2D NMR	[25]
	Trichothecrotocins M–S (1–7)	¹ H NMR, ¹³ C NMR, COSY, HMBC, HSQC, ROESY	Structural elucidation of 7 new trichothecenes from <i>Trichothecium crocinigenum</i> and cytotoxicity evaluation using 2D NMR	[26]
Zearalenone	ZEN glycosides (Z14G, Z16G)	¹ H NMR, ¹³ C NMR, DEPT, COSY, HSQC, HMBC	Structure confirmation of Z14G and Z16G using 1D and 2D NMR after biotransformation using <i>Cunninghamella</i> strains	[27]
	ZEN	¹ H NMR, ¹³ C NMR, COSY, HSQC	Elucidating the degradation product of ZEN under alkali treatment using 1D and 2D NMR, confirming ring opening and decarboxylation	[28]
	ZEN-diglucoside	¹ H NMR, ¹³ C NMR	Structural characterization of ZEN-14,16-diglucoside using NMR analysis	[29]

3.1. Aflatoxins

Aflatoxins are toxic secondary metabolites produced by fungi *Aspergillus flavus* and *A. parasiticus*. These mycotoxins frequently contaminate food and feed, posing serious health risks. Among them, aflatoxin B₁ (AFB₁) is the most potent and has been classified as a Group I carcinogen by the International Agency for Research on Cancer [30].

Various physicochemical analytical techniques have been utilized to investigate the structural changes in AFB₁. NMR spectroscopy has been particularly useful because it allows for the analysis of planar structure, stereochemistry, and isomer differentiation. In one study, AFB₁ was exposed to ultraviolet light to induce degradation, resulting in 17 degradation products, 7 of which were purified. Furthermore, 1D and 2D NMR techniques (¹H, ¹³C, COSY, HMBC, and NOESY) were applied to conduct structural analysis, and they played critical roles in distinguishing four (stereo)isomers with the same mass (*m/z* 345), a task impossible by MS/MS alone. This study demonstrated that an integrated approach combining UPLC-Q-TOF-MS/MS and NMR spectroscopy was effective in elucidating the degradation mechanism of AFB₁ and assessing toxicity reduction [16].

Xu et al. [17] reported the first high-resolution solution NMR structure of the AFB₁–DNA aptamer (AF26) complex, revealing a novel induced-fit recognition mechanism. Upon AFB₁ binding, the unstructured loop region of the aptamer undergoes conformational folding to form a compact binding pocket stabilized by G·C base pairs, a G·G·C triple, and five thymine residues that wrap around the toxin. Extensive 2D NMR analyses, including NOESY, COSY, TOCSY, HMBC, and ³¹P–¹H COSY, enabled full resonance assignment and identification of intermolecular interactions (Figure 3).

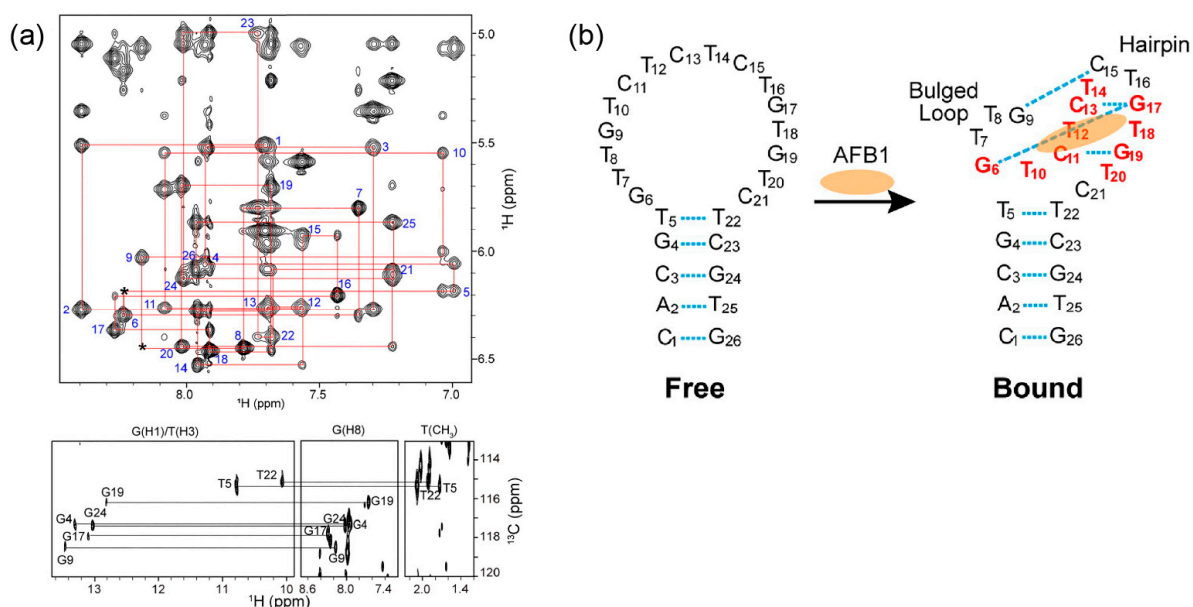


Figure 3. Three-dimensional binding structure of AFB₁ within the loop and stem region of the DNA aptamer (AF26) determined using NMR-based structural analysis: (a) NOESY (top) and HMBC (bottom) spectra of the complex. (b) Secondary structure of the free and bound AF26 aptamers. AFB₁ is shown as an orange ellipse. Reprinted from Ref. [17].

In another case, the biological degradation of AFB₁ by the microorganism *Meyerozyma guilliermondii* was studied. Because conventional methods could not distinguish true degradation from adsorption when both occurred simultaneously, the researchers used isotope-labelled ¹³C₁₇-AFB₁ to trace the formation of degradation products. Through NMR analysis, aflatoxicol and epi-aflatoxicol were identified as the main products. Structural analysis included ¹H NMR, ¹³C NMR, COSY, HSQC, HMBC, and ROESY, and the ability

to differentiate isomers enabled the identification of the degradation pathway. This study clearly showed that the biological control strain could convert AFB₁ to less toxic structures and emphasized the role of NMR spectroscopy in not only identification but also structure-based toxicity prediction and evaluation [7]. Therefore, NMR analysis has become an indispensable tool for identifying the structures of AFB₁ degradation products.

NMR spectroscopy was also instrumental in monitoring the Rh(II)-catalyzed synthesis of aflatoxin B₂, in which it facilitated the characterization of both intermediates and the final product [31]. ¹H and ¹³C NMR data enabled the precise assignment of regio- and stereochemistry, especially in validating the formation of the tricyclic ABC ring system characteristic of aflatoxins. This underscores the importance of NMR spectroscopy in synthetic studies involving structurally complex natural products.

These studies collectively highlight the expanding role of NMR spectroscopy in aflatoxin research, encompassing the structural elucidation of native toxins, degradation products, synthetic intermediates, and toxin–biomolecule complexes. With its ability to resolve isomeric and stereochemical detail, distinguish binding conformations, and quantify degradation pathways, NMR spectroscopy has become an indispensable analytical tool in mechanistic toxicology and applied food safety evaluation.

3.2. Ochratoxins

Ochratoxin A (OTA) is a representative mycotoxin produced by the fungi of the genera *Aspergillus* and *Penicillium*. It exhibits various toxic effects, including nephrotoxicity and carcinogenicity, and is widely detected in both food and feed [32].

NMR analysis plays a key role in elucidating structural differences and metabolic pathways associated with OTA and its derivatives (e.g., OTB and OTα). Various 2D NMR techniques enable the precise identification of functional group composition, stereostructure, interactions with biomolecules, and transformation pathways [11,33]. In one example, the OTA metabolite OTB-NAC (an *N*-acetyl-L-cysteine conjugate) was structurally confirmed through NMR analysis after synthesis. As this metabolite was detected for the first time in human urine, it was proposed as a potential biomarker of OTA exposure [22].

In a separate study, the binding structure between OTA and a DNA aptamer was analyzed using various 2D NMR techniques—including 2D TOCSY, DQF-COSY, NOESY, JR-HMBC, ³¹P–¹H COSY, and HSQC. The analysis revealed that the structural difference between OTA and OTB, particularly the presence or absence of a chlorine atom in the isocoumarin ring, plays a crucial role in binding specificity. In particular, the study confirmed that the halogen bond (X-bond) formed between the chlorine atom of OTA and the C5 base of the aptamer acts as a key interaction driving highly selective recognition (Figure 4) [11].

These studies emphasize the versatility of NMR spectroscopy in ochratoxin research—from determining molecular binding mechanisms to characterizing modified metabolites arising from both chemical synthesis and biological processes. Such insights are crucial for understanding OTA toxicodynamics, evaluating food safety risks, and developing biomonitoring strategies based on structurally confirmed metabolites and conjugates.

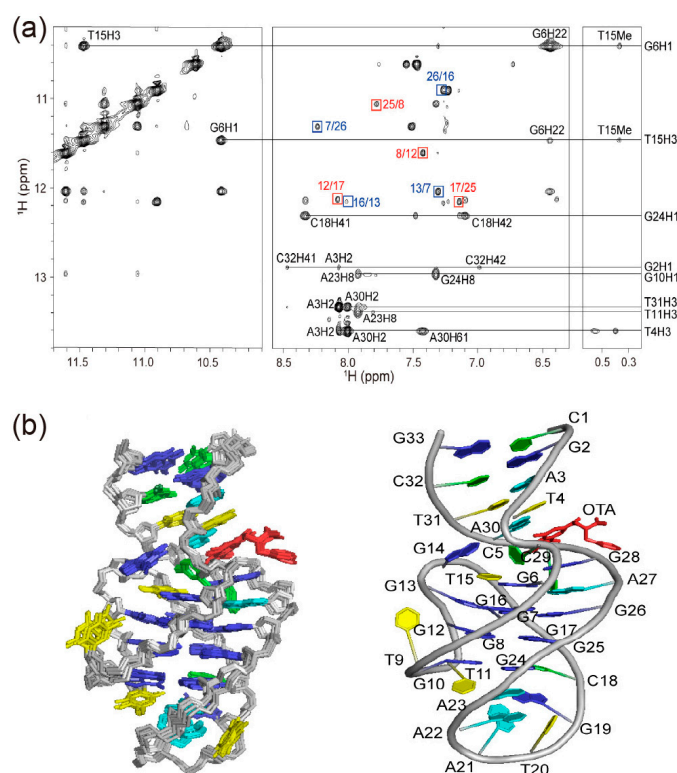


Figure 4. NMR characterization of the ochratoxin A (OTA)–aptamer complex: (a) 2D NOESY NMR spectrum showing intermolecular nuclear Overhauser effects (NOEs) that define the spatial orientation of OTA within the binding site; (b) 3D-NMR-derived structure illustrating how OTA is stabilized in the major groove by π – π stacking and hydrogen bonding. Reprinted with permission from Ref. [11]. Copyright 2022 American Chemical Society.

3.3. Fumonisin

Fumonisin are sphingolipid-like mycotoxins that are mainly produced by *Fusarium* species and are commonly found in corn. Among them, fumonisin B₁ (FB₁) is the most prevalent and toxic, accounting for approximately 70% of total fumonisin contamination. Structurally, FB₁ features an aminopolyol backbone esterified with two tricarballic acid (TCA) moieties. These ester linkages are susceptible to enzymatic or chemical hydrolysis, resulting in structurally modified or degraded derivatives [19].

In a study investigating the enzymatic degradation of FB₁, two major intermediates—pHFB1_6 and pHFB1_7—were isolated and structurally characterized through NMR spectroscopy. Based on 500 MHz ¹H NMR data measured in MeOH-*d*₄, spin–spin coupling constants and chemical shift values were precisely analyzed for each structure. Notably, pHFB1_7 exhibited a characteristic multiplet pattern indicating specific hydrogen bonding and stereochemical arrangements. Compared with FB₁ and the fully hydrolyzed derivative HFB₁, each compound displayed distinct structural characteristics, highlighting the important role of NMR in understanding degradation pathways and determining enzyme substrate specificity [19].

To investigate the structural diversity of FB₁ and its potential hidden derivatives in food, a series of acylated analogues—including 3-O-, 5-O-, and *N*-palmitoyl-FB₁—were synthesized and analyzed. Structural characterization was performed through ¹H and ¹³C NMR along with 2D techniques such as HSQC-CLIP-COSY and HMBC, as well as liquid chromatography–high-resolution mass spectrometry (LC-HRMS) [20]. A comparative analysis of the chemical shifts corresponding to each acylation site revealed a selective introduction of the palmitoyl group at the 3- or 5-hydroxyl positions or the amino group. The exact substitution sites were unambiguously confirmed through HMBC analysis by

quantifying three-bond heteronuclear correlations between the carbonyl carbon of the palmitoyl group and the protons on the fumonisin backbone.

These results demonstrate the indispensable role of NMR in identifying positional isomers of fumonisin derivatives and verifying their structural integrity, particularly in the presence of isobaric or overlapping mass spectral signals. Furthermore, NMR provides critical insights into the metabolic fate and chemical behaviour of fumonisins, thereby expanding its application scope to food safety evaluation and the authentication of structurally modified mycotoxins.

3.4. *Trichothecenes*

Trichothecenes are mycotoxins characterized by a tricyclic structure containing a 12,13-epoxytrichothec-9-ene (EPT) skeleton and exhibit various biological activities such as antifungal, nematocidal, antiviral, cytotoxic, and antitumor effects. To date, more than 200 trichothecenes have been identified [26]. Among them, deoxynivalenol (DON) is one of the most significant, as it is one of the most commonly detected toxins in grain-based food and feed products worldwide. Notably, DON-3-esters and DON-15-esters can be clearly distinguished through ^1H NMR and ^{13}C NMR, whereas the use of conventional HPLC has presented challenges [34].

NMR spectroscopy also plays a central role in the discovery of new trichothecene derivatives. The structures of eight new derivatives (trichodermarin G–N) isolated from the *Trichoderma brevicompactum* A-DL-9-2 strain were determined through ^1H NMR, ^{13}C NMR, DEPT, COSY, HSQC, HMBC, and NOESY analyses [24].

Additionally, NMR spectroscopy enabled the differentiation of trichobreol D and trichobreol E, which belong to trichothecene sesquiterpenes. Trichobreol D contains five sp^2 methine groups, one sp^2 quaternary carbon, and one carbonyl group, with hydroxyl groups confirmed via infrared (IR) spectroscopy. Based on the chemical shifts and coupling constant differences in the C-2'–C-6' double bond chain, this compound was classified as a (2'E,4'E) isomer similar to trichobreol A. In contrast, trichobreol E showed an NMR spectrum similar to that of trichobreol D but featured a hydroxyl-substituted unsaturated acyl chain and had a molecular formula of $\text{C}_{15}\text{H}_{22}\text{O}_4$, which was 94 Da ($\text{C}_6\text{H}_6\text{O}$) lower than that of trichobreol D. NOESY analysis confirmed that the relative stereochemistry of the two compounds was similar to that of trichobreol A [25].

NMR spectroscopy revealed the presence of characteristic trichothecene features, including an epoxide moiety and a hydroxylated tricyclic sesquiterpene core. HMBC and NOESY analyses, in particular, provided key insights into the substitution pattern and relative stereochemistry of the toxins. These findings supported the identification of four previously unreported trichothecene analogues with distinct oxygenation profiles. Although numerous novel derivatives have been reported, the NMR-based structural characterization of modified trichothecenes, including conjugated forms, remains insufficient [35].

3.5. *Zearalenone*

Zearalenone (ZEN) is a mycotoxin produced by *Fusarium* species and is most commonly detected in grains such as corn, wheat, and barley. It exhibits strong estrogenic activity, which can affect the reproductive and endocrine systems of both animals and humans, posing serious health risks. Due to its structural similarity to natural estrogens, ZEN can bind to receptors and cause hormonal imbalances. Accordingly, regulatory agencies such as the EU strictly control the permissible levels of ZEN in food and feed [36–38].

In vivo, ZEN is metabolized into various derivatives, including α -zearalenol (α -ZOL), β -zearalenol (β -ZOL), and zearalanone (ZAN), along with conjugated forms such as

zearalenone-14-glucoside (ZEN-14-G) and zearalenone-14-sulphate (ZEN-14-S). Among these, α -ZOL, in particular, exhibits significantly higher estrogenic activity than the parent compound, ZEN [29]. Although conjugated forms generally have reduced acute toxicity, they can be hydrolyzed during gastrointestinal digestion, potentially releasing active ZEN and thereby increasing the effective toxicity. This necessitates the precise structural identification of ZEN derivatives. NMR spectroscopy plays a crucial role in the structural elucidation of such metabolites, especially ZEN glycosides such as ZEN-14-G and ZEN-16-G. Although ZEN-14-G has a ^{13}C NMR spectrum similar to that of ZEN, additional glucose-related peaks are observed between 100 and 60 ppm, and HMBC analysis confirms coupling between C14 and C19, validating its structure. In contrast, ZEN-16-G displays distinct chemical shifts for alkenyl protons, with two characteristic peaks appearing near 6.2 ppm, differing from the patterns seen in ZEN and ZEN-14-G [27].

NMR spectroscopy has also proven critical in structurally elucidating the degradation mechanism of ZEN [28]. Under high-temperature alkaline refining conditions in corn oil, ^1H NMR analysis revealed the disappearance of lactone ring proton signals (4.7–4.9 ppm) and the emergence of alcoholic proton signals (3.3–3.5 ppm), indicating lactone ring opening and decarboxylation. These findings suggest that ZEN is irreversibly converted to a nontoxin structure, and NMR analysis contributes significantly to identifying the structure of the degradation product and interpreting its toxicological transformation.

4. Quantitative Analysis of Mycotoxins Using NMR

The previous section addressed the use of NMR spectroscopy in the structural elucidation of mycotoxins. This section focuses on its applications in quantitative analysis. Mycotoxins can cause severe physiological effects such as hepatotoxicity, nephrotoxicity, and carcinogenicity; therefore, international regulations strictly control their permissible levels in food and feed products [39]. To meet these standards and ensure food safety, analytical methods with high accuracy and reliability are required. Selected applications of qNMR in mycotoxin analysis are summarized in Table 2.

Table 2. Selected applications of qNMR for analysis of mycotoxins and their modified forms.

Toxin	NMR Method	Information	Internal Standard	References	
Aflatoxin	AFB ₁ , AFG ₁	¹ H qNMR	Quantification of AFB ₁ -Lys and AFG ₁ -Lys by qNMR using maleic acid as the internal standard	Maleic acid	[40]
	AFB ₁	¹ H NMR, ¹³ C NMR, ¹ H qNMR	Purity quantification of AFB ₁ using ¹ H qNMR and uncertainty evaluation procedure	Benzoic acid	[30]
Fumonisin	FB ₁	¹ H qNMR	Optimized qNMR method for FB ₁ using benzoic acid (internal standard) and DMSO-d ₆ /TFA-d (solvent)	Benzoic acid	[41]
	FB ₁	¹ H qNMR	Reliable purity assessment of FB ₁ using qNMR without interference from impurities	Benzoic acid	[12]
	FB ₁	¹ H qNMR	Determination of the exact concentrations of FB ₁ using qNMR	Thymol	[42]

Table 2. Cont.

Toxin		NMR Method	Information	Internal Standard	References
Ochratoxins	OTA	$qH\{^{13}C\}$ NMR	Accurate quantification of OTA using qNMR with ^{13}C decoupling	Maleic acid	[43]
Trichothecenes	DON	1H qNMR, $qH\{^{13}C\}$ NMR, HSQC	Purity determination of DON considering tautomeric and conformational isomers	Dimethyl terephthalate	[43]
	DON	1H qNMR	Determination of the exact concentrations of DON using qNMR	Thymol	[42]
Zearalenone	ZEN	1H qNMR	Purity evaluation of trans-ZEN reference material using qNMR	Benzoic acid	[44]
	ZEN	1H qNMR, $qH\{^{13}C\}$ NMR	Purity determination and detection of low-level impurities using ^{13}C decoupling	Dimethyl terephthalate	[43]

Instrumental analytical methods such as LC-MS/MS, GC-MS, HPLC-UV, and LC-fluorescence detection (FLD) are commonly used for the quantitative analysis of mycotoxins. However, these methods are subject to matrix effects, which can lead to variability in quantification results, and they require complex prerequisites such as calibration curves for standards [45]. Moreover, trace impurities in standard substances can significantly influence analytical outcomes, thereby necessitating more absolute methods for purity evaluation [46].

qNMR is an absolute quantification method based on the principle that the integral value of an NMR signal is proportional to the number of nuclei. It allows the direct determination of the analyte content by comparing the signal area ratio to that of an internal standard [47], bypassing the need for external standards or calibration curves. As a nondestructive technique, it is particularly suitable for CRM purity assessments. Notably, 1H NMR integral values can reveal the presence of unknown impurities, enabling more accurate purity evaluations than LC-based analyses [12].

Due to these advantages, qNMR has recently been actively applied to CRM development and mycotoxin quantification. For example, it has been used to assign certified values to CRMs of various toxins, including OTA, ensuring international traceability of measurements [39,46]. qNMR has also been used to quantify toxins in actual biological and food samples, contributing to toxicity assessments and exposure level estimations. Another study quantitatively analyzed synthesized metabolites such as AFB₁-Lys and AFG₁-Lys using maleic acid as an internal standard and 1H NMR signal integration, thereby providing accurate reference materials for mycotoxin exposure assessment in biological research [40].

For ZEN quantification, a $qH\{^{13}C\}$ NMR method based on 1H - ^{13}C double resonance was introduced to minimize interference from ^{13}C satellite signals. High-precision quantification was achieved by eliminating signal overlap using globally optimized alternating-phase rectangular pulse (GARP) and bi-level adiabatic broadband decoupling techniques, enabling the detection of even hidden tautomer structures (Figure 5) [43].

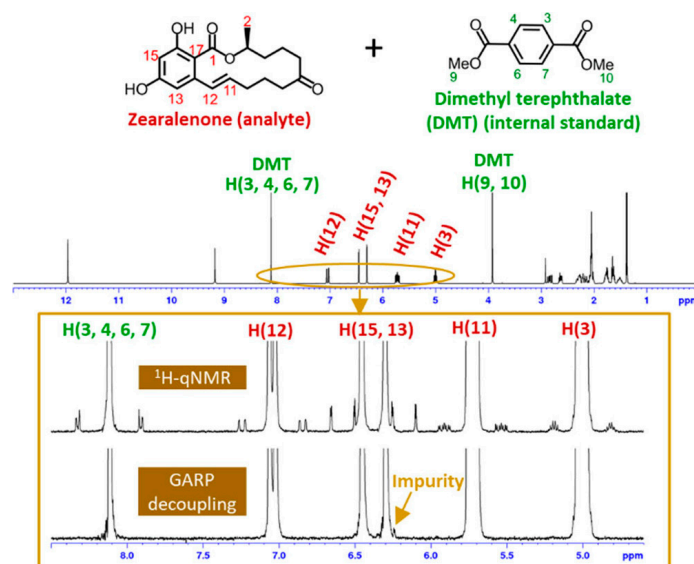


Figure 5. Purity of zearalenone was determined with dimethyl terephthalate as the internal standard at 400 MHz in acetone- d_6 . Globally optimized alternating-phase rectangular pulse (GARP) decoupling revealed a low-level impurity concealed by a ^{13}C satellite in the ^1H -qNMR spectrum. Reprinted with permission from Ref. [43]. Copyright 2020 American Chemical Society.

The purities of biosynthetic derivatives such as ZEN-14-glucoside, ZEN-14-sulphate, and ZEN-16-glucoside were also evaluated through qNMR. The results revealed that ZEN-14-G had a purity of $\geq 82\%$, while that of ZEN-14-S was $\geq 73\%$, indicating their potential use as standard substances for toxicological evaluation [38].

Although FB_1 was previously considered difficult to quantify through qNMR due to its high polarity, a recent study combined qH NMR with a mass balance method to assess the purity of FB_1 and its hydrolyzed derivative (pHFB_1). This study confirmed that a mixture of $\text{DMSO}-d_6$ and trifluoroacetic acid- d provided suitable analytical conditions [41]. The qNMR-based results showed good agreement with the mass balance method, suggesting that qNMR can be applied to highly polar toxins.

In the trichothecene group, qNMR was conducted to determine the precise concentration of synthetically prepared DON-3-glucoside. The resulting data served as calibration references in the development of a stable isotope dilution LC-MS/MS method, which was applied to quantify DON-3-glucoside and DON in beer samples [48].

Beyond the five major mycotoxins typically associated with cereal products, qNMR has also been successfully applied to patulin, a mycotoxin commonly found in fruit-based products. These studies demonstrate that qNMR can be effectively extended to a broader range of mycotoxins and support its utility in the development of CRMs for metrologically traceable analysis [46,49].

In addition to studies on individual mycotoxins, institutional efforts have also played a crucial role in advancing qNMR methodologies. The Bureau International des Poids et Mesures (BIPM) has contributed significantly to the development of metrologically traceable qNMR techniques for mycotoxin analysis. Notably, BIPM has applied qNMR to determine the purity and concentration of key mycotoxins such as aflatoxin B_1 , deoxynivalenol, and patulin. These efforts have been documented through technical reports and emphasize the critical role of qNMR in establishing reference values for CRMs, with particular attention to measurement uncertainty, internal standard validation, and SI traceability [49–51]. Such contributions underscore the value of qNMR as a primary analytical method to ensure global comparability and reliability in mycotoxin quantification.

In summary, recent applications of qNMR in mycotoxin analysis have demonstrated its versatility across a wide range of toxins, including aflatoxins, fumonisins, ochratoxins, trichothecenes, and ZEN. Studies have applied qNMR to determine the purity of CRMs, quantify both protein-bound and free forms in biological matrices, and verify the structural integrity of synthetic or biosynthetic derivatives. Its ability to provide matrix-independent, calibration-free absolute quantification makes it particularly valuable for developing traceable standards and supporting mass spectrometric methods through orthogonal validation. As regulatory demands increase and attention shifts toward modified and low-abundance toxins, the role of qNMR is expected to continue expanding, both in routine monitoring and advanced toxicological research.

5. Challenges and Future Perspectives

NMR spectroscopy offers a robust and reproducible tool for both structural elucidation and quantitative analysis of mycotoxins. However, its relatively low sensitivity compared to techniques such as LC-MS/MS remains a key limitation, particularly for the direct detection of trace-level mycotoxins within complex food matrices. This results in reduced signal-to-noise ratios (SNRs) and diminished analytical reliability, especially when strong matrix effects are present [3].

In actual food matrices, mycotoxins rarely exist in isolation; they are frequently associated with proteins, lipids, polysaccharides, and other matrix components. These interactions can cause significant signal overlap and spectral interference in NMR measurements and may also alter the physicochemical properties of the toxins, complicating their extraction and recovery [52,53]. As a result, most NMR-based studies to date have relied on isolated or purified mycotoxins obtained through labour-intensive and time-consuming sample preparation procedures. While not common, qNMR has been successfully applied to partially purified fungal extracts, provided that spectral resolution is sufficient to distinguish the target signals from matrix interferences [54]. In such cases, complete purification is not necessarily required, particularly when appropriate internal standards are used and high-field NMR instrumentation is available to enhance resolution and sensitivity.

Accordingly, to enhance the applicability of NMR-based mycotoxin analysis in complex food matrices, a thoughtful combination of several complementary strategies may be required. These include combining NMR with other analytical techniques, developing appropriate internal standards to avoid spectral overlap, utilizing 2D NMR methods for improved signal resolution, applying spectral deconvolution techniques to resolve overlapping peaks, and employing high-sensitivity NMR instrumentation to improve detectability. These approaches can help mitigate matrix-related spectral interference and expand the practical utility of NMR in mycotoxin analysis beyond highly purified systems.

In addition to these methodological considerations, further opportunities exist in extending qNMR applications beyond parent toxins to include modified mycotoxins, such as conjugated, acetylated, and glucosylated forms. Although these derivatives have received relatively limited attention in NMR-based studies, their quantification is highly relevant to food safety and regulatory monitoring. Expanding the scope of qNMR to encompass these structurally diverse compounds represents a promising direction for future research.

Recently, artificial intelligence (AI) techniques have been applied to the spectroscopic analysis of mycotoxins. In particular, machine learning (ML) and deep learning (DL) models have been combined with spectroscopic techniques such as near-infrared (NIR), surface-enhanced Raman spectroscopy (SERS), visible/near-IR, and fluorescence spectroscopy to improve the sensitivity, accuracy, and speed of mycotoxin detection [55–58]. Although AI applications in NMR-based mycotoxin analysis remain limited, AI-driven models have

been successfully used in NMR-based food analysis for purposes such as authentication, adulteration detection, and compositional profiling [59,60].

These developments indicate that the integration of AI into NMR spectroscopy could address key challenges in mycotoxin analysis. AI-based modelling approaches can assist in extracting structural information from complex spectral patterns, identifying toxin-specific features in mixtures, and streamlining the quantification process with improved consistency and speed. As illustrated in Figure 6, this integration enables efficient workflows that support the simultaneous evaluation of multiple compounds, facilitate the automated recognition of structural characteristics, and improve the speed and reliability of quantitative analysis. Such an approach has the potential to significantly broaden the utility of NMR spectroscopy in mycotoxin research, particularly in high-throughput and quality-assured analytical settings.

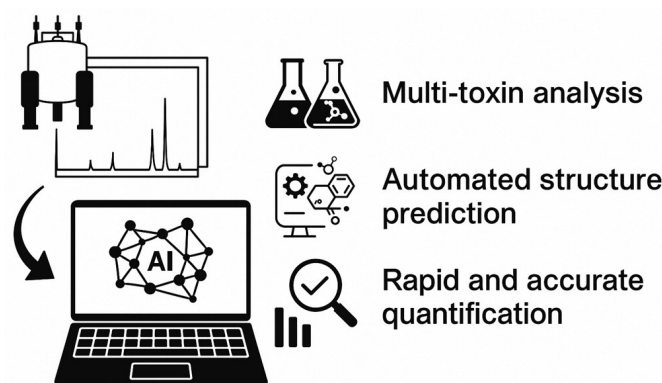


Figure 6. Prospective applications of AI-assisted NMR spectroscopy in advancing mycotoxin analysis.

To further advance this field, future research should focus on developing NMR-AI hybrid models to overcome the limitations of existing analytical techniques and establish new analytical platforms for the rapid and precise detection of mycotoxins in food safety assessments. To achieve this, it will be necessary to accumulate a large amount of NMR spectral data on various mycotoxins and apply optimized AI algorithms to enhance data interpretation and automation models.

6. Conclusions

NMR spectroscopy plays an increasingly important role in mycotoxin research, providing detailed structural elucidation and accurate quantitative analysis. This review examined recent applications of NMR in identifying various mycotoxins, including modified forms and degradation products, as well as its use in quantifying toxins and reference materials through qNMR. Discussions of specific studies highlighted the advantages of NMR in resolving isomeric structures, verifying detoxification pathways, and evaluating purity in both synthetic and biological matrices.

Despite these strengths, challenges remain due to the low concentrations of mycotoxins in complex food matrices and the resulting matrix effects that interfere with both detection sensitivity and quantitative precision. Addressing these challenges requires improvements in sample preparation, calibration strategies, and analytical integration.

Looking ahead, the incorporation of AI into NMR data processing offers promising opportunities to automate spectrum interpretation and enhance analytical efficiency. As NMR continues to evolve, its combined use with data-driven approaches may significantly contribute to advancing food safety and toxicological evaluation in mycotoxin analysis.

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