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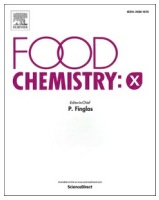

Development and application of an efficient, accurate, and environmentally friendly liquid chromatography–tandem mass spectrometry method for the determination of five Alternaria toxins in wheat

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ABSTRACT

The contamination of Alternaria toxins poses a potential risk to human health. This study developed a rapid, efficient, and environmentally friendly method for the simultaneous determination of five types of Alternaria toxins in wheat using high-precision and stable isotope liquid chromatography tandem mass spectrometry. The comparison between dilution method and solid-phase extraction method shows that the former achieves satisfactory results with a simple and convenient sample purification method. The quantitative limit range is 0.88 to 1.68 μg/kg. The recoveries are between 81.40% and 102.68%, with RSD less than 11.95%. The method was used to analyze 60 samples from the main wheat producing areas in China. The results showed that Tenuzonic acid had the highest detection rate (100%), followed by Tentoxin (95%), Alternariol (66.67%), and Alternariol monomethyl ether (53.33%). There is a certain pollution risk that needs to be taken seriously and monitoring should be strengthened.

1. Introduction

Alternaria toxins (ATs) are a group of secondary metabolites produced by the Alternaria species, and they are commonly found in various foodstuffs such as wheat, maize, rice, barley, olive oil, sunflower seed oil, tomatoes, and fruits (Goncalves et al., 2022; Ji et al., 2022; Lin et al., 2022; Puntscher et al., 2018; Su et al., 2020). To date, more than 70 ATs have been identified, and including tenuazonic acid (TeA), alternariol (AOH), alternariol monomethyl ether (AME), tentoxin (TEN), and altenuene (ALT) (Fig. S1),(Ji, Deng, Xiao, Jin, Lyu, Wang, & Yang, 2023). Among these, Altenuene (ALT) shows the highest acute toxicity among the toxins covered by this study with a LD50 value of 50 mg/kg b.w. (mice) (Pero et al., 1973).TeA is considered the most toxic and has been shown to exhibit acute toxicity in animals (e.g., mice, chickens, and dogs) (Zwickel et al., 2016). while AOH and AME are known for their genotoxic, mutagenic, and cytotoxic properties (Liu & Rychlik, 2013; Nagda & Meena, 2024). It has also been reported that ATs may also be implicated in the increasing incidence of esophageal cancer in humans (Dall'Asta et al., 2014). In 2011, a risk assessment of AOH, AME, TeA, and TEN was performed by the European Food Safety Authority (EFSA). Thus, given the potential toxicity and chronic dietary exposure risks posed by ATs, EFSA recommends strengthening both contamination monitoring and the necessary risk assessments. Wheat, which is one of the top three staple crops worldwide, plays a crucial role in providing protein and calories to the human diet, and is highly susceptible to ATs contamination (Janić Hajnal et al., 2019). Countries such as Germany (Mueller & Korn, 2013), Italy, Austria (Puntscher et al., 2019), and Argentina (Azcarate et al., 2008) have reported wheat contamination, with TeA being the most frequently detected. Considering that China is the world's largest producer and consumer of wheat (Ji et al., 2024), However, there are relatively few studies on the ATs contamination in wheat in China. It is imperative to intensify the monitoring and assessment of ATs contamination in wheat.

The efficient and accurate detection of ATs is crucial for monitoring and assessing their contamination levels in wheat. Currently, the primary detection methods include high-performance liquid

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chromatography (HPLC) (Fente et al., 1998; Xu & Zhai, 2020), liquid chromatography–tandem mass spectrometry (HPLC-MS/MS) (Nguyen et al., 2018), and gas chromatography–tandem mass spectrometry (GC–MS/MS) (Chakraborty et al., 2023). However, HPLC suffers from poor interference resistance, requires high-purity purification materials during preprocessing, and exhibits a low sensitivity (Wang et al., 2023). In addition, GC–MS/MS requires expensive derivatization processes, and involves complex operational steps (Font et al., 2013). Consequently, ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) has emerged as the preferred method for detecting multiple ATs owing to its high throughput, high sensitivity, simplicity for pretreatmen, and strong resistance to interference. Given the complexity of the wheat matrix, sample preparation is of particular importance prior to analysis. Currently, solid-phase extraction (SPE) and the QuEChERS extraction method are employed as the primary purification techniques. More specifically, SPE involves activation, sample addition, washing, elution, and nitrogen blowing, which are cumbersome and consume large amounts of organic solvents, Furthermore, improper nitrogen blowing can lead to the loss of target analytes. In a previous study, Four ATs were detected in 15 food samples using SPE combined with UPLC-MS/MS, with the recoveries ranging from 72.1 to 113.6% (Zhang et al., 2024). Additionally, the QuEChERS method, which is commonly applied in the purification of high-pigment matrices, such as fruits, vegetables, herbal medicines, and seafood, involves salting and purification (Ji, Deng, Xiao, Jin, Lyu, Wang, & Yang, 2023; Ji, Deng, Xiao, Jin, Lyu, Wu, & Yang, 2023; Xing et al., 2020). In recent years, dilution method has gained popularity for the extraction of mycotoxin because of its simplicity, rapid, and environmental friendliness (Greer et al., 2021). Independent of the method selected, during sample preparation it is essential to minimize labor-intensive or high-cost steps, whilst also enhancing the sample throughput to meet the demands of large-scale detection.

Considering the above factors, the objective of this study was to develop a rapid, efficient, and environmentally friendly LC-MS/MS method for the simultaneous determination of five ATs in wheat. Following systematic optimization of the preprocessing method using negative samples and naturally contaminated wheat quality control samples, two purification methods were compared, namely dilution method and SPE method, The performance of the LC-MS/MS method was also validated in terms of its linearity, specificity, accuracy, limit of detection (LOD), limit of quantification (LOQ), and intra-and inter-day variabilities. Finally, the developed method was applied in the analyses of 60 wheat samples obtained from major wheat-producing regions in China in 2023 to provide a preliminary assessment of ATs contamination.

2. Materials and methods

2.1. Chemicals and reagents

Standards of tenuazonic acid (TeA, CAS: 610–88-8) (100 μg/mL), alternariol (AOH, CAS: 641–38-3) (100 μg/mL), alternariol monomethyl ether (AME, CAS: 26894–49-5) (100 μg/mL), tentoxin (TEN, CAS: 28540–82-1) (100 μg/mL), and altenuene (ALT, CAS: 29752–43-0) (100 μg/mL), as well as isotopically labelled internal standards including $^{13}C_{10}$-TeA (25 μg/mL), $^{13}C_{14}$-AOH (10 μg/mL), $^{13}C_{15}$-AME (10 μg/mL),$^{13}C_{15}$-ALT (25 μg/mL), and $^{13}C_{22}$-TEN (5 μg/mL), were bought from Pribolab (Singapore). LC-MS grade methanol (MeOH), acetonitrile (ACN), formic acid (FA), acetic acid (HAC), and ammonium bicarbonate (NH_4HCO_3) were obtained from Fisher Scientific (Waltham, MA, USA). Laboratory water was prepared with a Milli-Q water purification system (Massachusetts, USA). One step SPE purification method (adsorb impurities, 60 mg, 3 cc) were obtained from the Academy of National Food and Strategic Reserves Administration (Beijing, China), and traditional SPE method (adsorb target toxins, 60 mg, 3 cc) were purchased from Waters(Massachusetts, USA). The 0.2 μm polytetrafluoroethylene (PTFE) syringe filters were purchased from PALL Corporation (PALL, NewYork, USA).

2.2. Samples collection

The blank sample and naturally contaminated samples were obtained from the Academy of National Food and Strategic Reserves Administration (Beijing, China). A total of 60 wheat samples were randomly collected in the provinces of major production regions in China (i.e., the Anhui, Henan, Hebei, Hubei, Shandong, and Jiangsu provinces). All samples were ground into powder using a laboratory grinder to ensure thorough homogenization and were stored 4 °C in the dark until analysis.

2.3. Sample preparation

2.3.1. Sample extraction

5.00 ± 0.01 g of wheat samples were added to 50 mL Teflon centrifuge tube, followed by the addition of 20 mL of extraction solvent (acetonitrile/water/acetic acid 85:14:1, *v*/v/v), the mixture was shortly vortexed for 20 min and centrifuged for 5 min at 7000 r/min. The supernatant was collected for the next purification step. Finally, the sample was filtered through a 0.2 μm filter, 20 μL of internal standard mixture and 180 μL of sample filtrate were absorbed into 400 μL of internal intubation for ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) analysis.

2.3.2. Sample purification

2.3.2.1. Dilution method. 0.5 mL of the supernatant and 0.5 mL of water were mixed, then centrifuged at 12,000 rpm and 4 °C for 10 min.

2.3.2.2. Solid phase extraction (SPE)

2.3.2.2.1. One step SPE purification method. 1.5 mL of the supernatant was transfered into One step SPE purification column, 0.5 mL of the column liquor and 0.5 mL of water were mixed, centrifuged at 12,000 rpm for 10 min at 4 °C.

2.3.2.2.2. Traditional SPE purification method. The traditional SPE purification column was activated with 5 mL MeOH and 5 mL H_2O, respectively, and the supernatant (diluted 1: 4) was transferred to the column. Control the drop rate of the sample solution at 1–2 drops per second. Subsequently, 5 mL of 20% MeOH was added into the column for washing. The column was drained using a vacuum pump, and 5 mL MeOH and 5 mL ACN successively were added for elution. At 40 °C, the eluent was slowly blowed to near-dry by nitrogen. Samples were reconstituted in acetonitrile: water: formic acid (42.5:7:0.5, v:v:v) with mixing.

2.4. UPLC-MS/MS analysis

Optimized LC-MS/MS method was applied for determining Alternaria toxins in wheats. The UPLC system was coupled to a tandem mass spectrometry QTRAP 6500 (Sciex Pte. Ltd., Massachusetts) with electrospray ionization sources (ESI). Acquity UPLC HSS T3 (1.8 μm, 2.1 mm × 100 mm, Waters Corp.) analytical column was used for the chromatographic separation of the five Alternaria toxins. The mobile phase included of 0.5 mmol/L ammonium hydrogen carbonate (A) and MeOH (B) The gradient elution program is detailed in Table 1. The flow rate was 0.2 mL/min, and the injection was 2 μL. The column temperature was maintained at 40 °C. Multiple reaction monitoring (MRM) with negative ion modes was used. MS detection conditions included a curtain gas (35 psi), nebulizer gas (60 psi), and auxiliary gas (60 psi); the ion spray voltage was−4500 V (negative mode), and the source temperature was 500 °C. The optimized LC-MS/MS acquisition parameters for Alternaria toxins are provided in Table 2. Analyst software (Version

Table 1
Gradient elution program for the UPLC-MS/MS method.

Time(min)	flow rate(mL/min)	A (%)	B (%)
1.0	0.2	95.0	5.0
2.0	0.2	95.0	5.0
3.0	0.2	25.0	75.0
4.0	0.2	10.0	90.0
6.0	0.2	5.0	95.0
7.0	0.2	5.0	95.0
9.0	0.2	95.0	5.0
11.0	0.2	95.0	5.0

Table 2
The optimized LC-MS/MS acquisition parameters for five Alternaria toxins.

Compound	Molecular Ion	Retention Time/min	Precursor ion *m/z*	Product ion m/z	DP/V	CE/V
TeA	$[M-H]^-$	4.50	196.2	139.0*	60	32
				112.2	60	26
AOH	$[M-H]^-$	5.32	257.0	213.0*	40	34
				147.0	40	42
ALT	$[M-H]^-$	5.36	290.9	185.9*	40	34
				214.1	40	28
TEN	$[M-H]^-$	5.66	413.2	141.0*	40	23
				271.1	40	17
AME	$[M-H]^-$	6.12	271.1	256.0*	40	31
				228.0	40	38
$^{13}C_{10}$-TeA	$[M-H]^-$	4.50	206.0	145.0	60	27
$^{13}C_{14}$-AOH	$[M-H]^-$	5.32	271.0	226.1	40	22
ALT-d_3	$[M-H]^-$	5.36	293.2	221.1	25	23
$^{13}C_{22}$-TEN	$[M-H]^-$	5.66	435.2	147.2	40	23
$^{13}C_{15}$-AME	$[M-H]^-$	6.12	285.9	270.0	40	21

* Representative quantitative ion.

1.6.1, AB Sciex) was used for instrument control and data analysis.

2.5. Method validation

In this study, the analysis method for five ATs in wheat was validated using spiked blank samples. The UPLC-MS/MS method was evaluated in terms of linearity, method limits of detection (LODs), method limits of quantification (LOQs), matrix effects, accuracy and precision (intra-and inter-day).

2.5.1. Linearity

The linearity was evaluated by preparing different solvent standard solution, with concentration levels at 0.1, 0.2, 0.5, 1, 5, 10, 50 and 100 ng/mL. Linearity was evaluated using standard calibration curves constructed for each mycotoxin by plotting the signal intensity against the analyte concentration, with the area ratios (analyte area/internal standard area) used to obtain the calibration. The calibration curves were derived from the peak area ratio of each analyte to the internal standard. The analyte concentrations in wheat samples were subsequently determined using the corresponding response function. Sensitivity was evaluated by limits of detection (LODs) and limits of quantification (LOQs) derived from signal-to-noise ratio (S/N) of 3:1 and 10:1, respectively.

2.5.2. Matrix effects

At the same concentration, the matrix effect is calculated using the matrix standard solution and the solvent standard solution. The calculation formula is as follows: ME(%) = (the slope of the matrix calibration curve − the slope of the solvent calibration curve) × 100%/the slope of the solvent calibration curve (Braun et al., 2018). A positive value indicates matrix enhancement, while a negative value indicates matrix suppression. When the average matrix effect (enhancement or suppression) exceeds 20%, it is considered to have a significant impact on quantitative detection and cannot be ignored.

2.5.3. Recovery and precision

Recoveries were assessed by blank samples at three spiked concentration levels. To ensure the reliability of the results, each spiking level was tested in triplicate, and the relative standard deviation (RSD) was obtained. Additionally, spiked samples were analyzed over three consecutive days to evaluate intra-day and inter-day precision, with three repeated measurements conducted each day.

2.6. Contamination assessment

In this study, 60 commercially available wheat samples from different provinces in 2023 (Anhui, Henan, Hebei, Hubei, Shandong, and Jiangsu) were analyzed for five ATs using the established UPLC-MS/MS method. During the sample analysis, spiked negative samples were used as quality control to ensure the accuracy and reliability of the results.

2.7. Statistics and analysis

Data were accurately collected and analyzed using the dedicated software for the AB SCIEX TripleQuad 6500+ and Origin 8.5 comes from OriginLab Corporation, (Northampton, Massachusetta, USA). Average recoveries of five ATs in wheat samples were analyzed as triplicate. Standard deviations (SD) and relative standard deviation (RSD) of five ATs were calculated.

3. Results and discussion

3.1. Optimization of the mass spectral parameters

To obtain the best sensitivity and selectivity for the MS conditions, data acquisition was carried out using multiple reaction monitoring (MRM). For every target analyte, two transitions from precursor to product ions were identified. An increase in the declustering potential (DP) can be used to prevent the aggregation of target compounds during ionization, thereby enhancing their responses. However, an excessively high DP may lead to in-source fragmentation of the target compounds (Wu et al., 2018). The collision energies (CE) were optimized for the current MS process by adjusting its magnitude to enhance the ionization efficiencies of specific compounds, thereby boosting their signal intensities and improving their detection sensitivities (Bustamante et al., 2024). The optimized MS parameters for these target toxins are shown in Table 2. TeA, AOH, ALT, TEN, and AME exhibited higher abundance in the ESI^- mode than in the ESI^+ mode. Each compound had one precursor ion and two product ions. The most sensitive transition was selected for quantification, while the others were used for confirmation. Relevant parameters, including declustering potential (DP) and collision energy (CE), were optimized accordingly.

3.2. Selection of the chromatographic columns

Among the five ATs, AOH and ALT exhibited closely overlapping retention times of 5.32 and 5.37 s, respectively, which resulted in a merged peak in the total ion chromatogram. However, extraction of the fragment ions for each compound confirmed that this overlap did not affect quantification. To select the most appropriate chromatographic columns for analysis of the five compounds, Acquity UPLC HSS T3 (1.8 μm, 2.1 mm × 100 mm) and Kinetex C18 (1.7 μm, 2.1 mm × 100 mm) columns were assessed (Fig. 1). It was found that the HSS T3 column demonstrated a superior separation resolution for the various ATs. More specifically, a sharper TeA peak was observed, indicating that the HSS T3 column exhibits and improved retention and separation of polar compounds during reversed-phase chromatography. Conversely, the experimental results showed that when C18 column was used, the

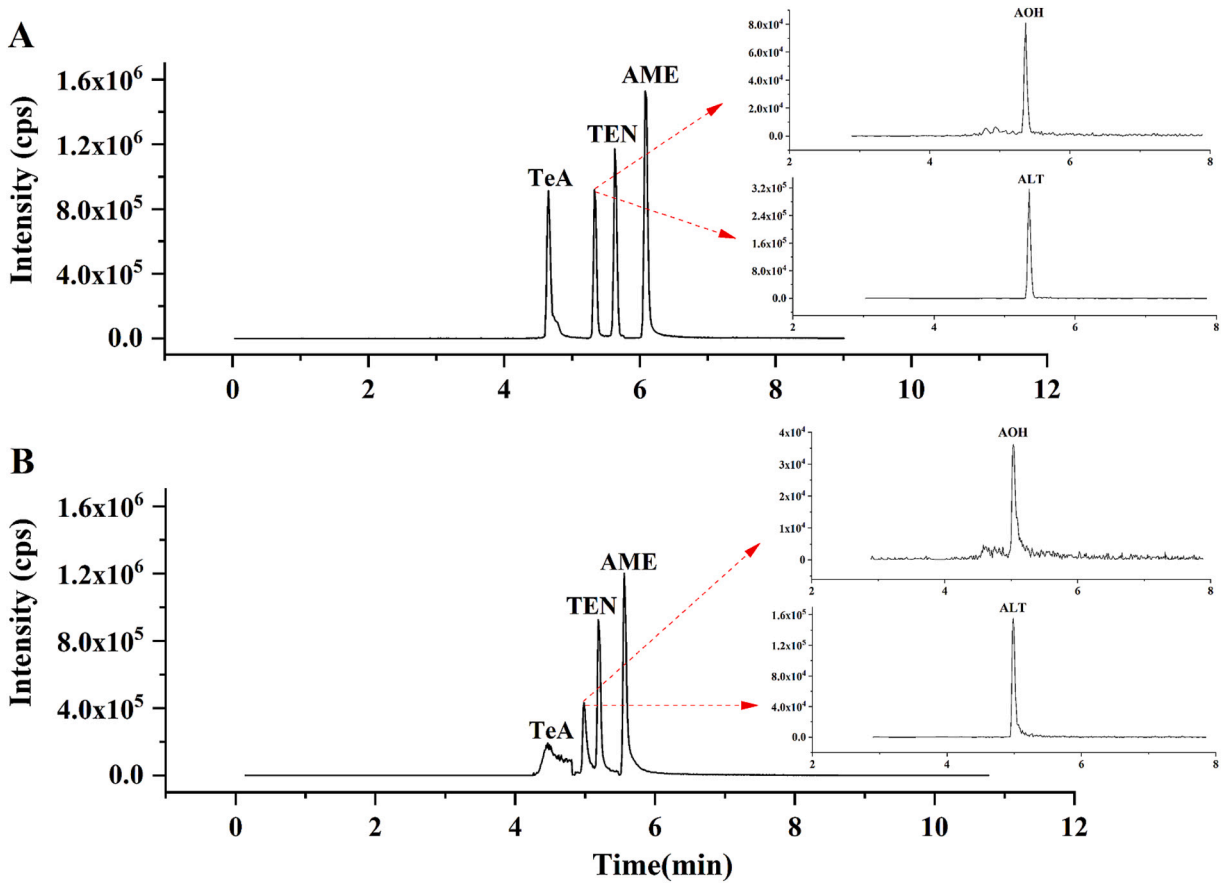

Fig. 1. chromatograms of five Alternaria toxins. ACQUITY UPLC HSS T3 column (A), Kinetex C18 column (B).

elution ability of TeA was weak and the peak tailed. The sensitivity was low (similar situation for other conventional C18 columns). In terms of the peak response, the HSS T3 column demonstrated higher response values than the C18 column for five toxins: TeA (9.0×10^5), ALT and AOH (8.9×10^5), TEN (1.1×10^6), and AME (1.5×10^6). Based on these findings, the HSS T3 column was selected for chromatographic separation of the ATs (Laika et al., 2024).

3.3. Optimization of the mobile phase

The effect of the ammonium hydrogen carbonate (NH_4HCO_3) concentration (0.5, 1.0, and 2.0 mM) was evaluated in terms of the separation efficiency, peak shape, and response intensity. As shown in Fig. 2, the five target compounds were effectively separated at all three concentrations. In terms of the response, the 0.5 mM NH_4HCO_3 mobile phase gave response values of 1.23×10^6, 1.17×10^6, 1.17×10^6, 1.26×10^6, and 1.68×10^6 for TeA, ALT and AOH, TEN, and AME, respectively. These values were higher than those achieved using the other two mobile phase concentrations and so a NH_4HCO_3 concentration of 0.5 mM was selected for further experiments.

3.4. Selection of the gradient elution program

Due to the significant polarity differences among the five ATs, four gradient elution programs (Table S1) were tested using 0.5 mM NH_4HCO_3 as the mobile phase and methanol as the organic phase, with a flow rate of 0.2 mL/min and a column temperature of 40 °C. TeA exhibited a sharper peak under both gradient one and gradient two conditions(Fig. S3), while a broader peak was observed under gradient three and four conditions. Comparing the peak response intensities of the five ATs under gradient one and two conditions, all toxins except TeA showed higher responses under gradient two conditions. However, considering both the peak shapes and the response intensities obtained for all five toxins, gradient one was ultimately selected as the optimal UPLC separation and elution program for simultaneous analysis of the five ATs.

3.5. Optimization of the extraction solvent

The addition of acid to organic solvents can increase the extraction recovery by increasing the polarity and enhancing the interaction of organic solvents within the food matrix. Such modifications can also promote bond breakage between the target analyte and the various food components (Wu et al., 2022). All five ATs were found to be readily soluble in a range of organic solvents, with TeA being the most stable under acidic conditions. Thus, to determine the optimal extraction solvent combination, the following systems were evaluated in more detail: acetonitrile/water (70:30, *v*/v/v), acetonitrile/water/formic acid (70:29:1, v/v/v), acetonitrile/water/acetic acid (70:29:1, v/v/v), acetonitrile/water (85:14, v/v/v), acetonitrile/water/formic acid (85:14:1, v/v/v), and acetonitrile/water/acetic acid (85:14:1, v/v/v). As shown in Fig. 2A, the extraction efficiency of TeA was higher in the presence of formic acid (Compared to acetic acid), indicating that formic acid facilitated the extraction of TeA more effectively. In addition, the recovery of ALT reached 79% for the acetonitrile/water/formic acid (70:29:1, v/v/v) solvent system, and increased to 91% for the acetonitrile/water/ formic acid (85:14:1, v/v/v) extraction solvent. Based on the overall recovery rates of the five target compounds, acetonitrile/water/formic acid (85:14:1, v/v/v) was chosen as the extraction solvent.

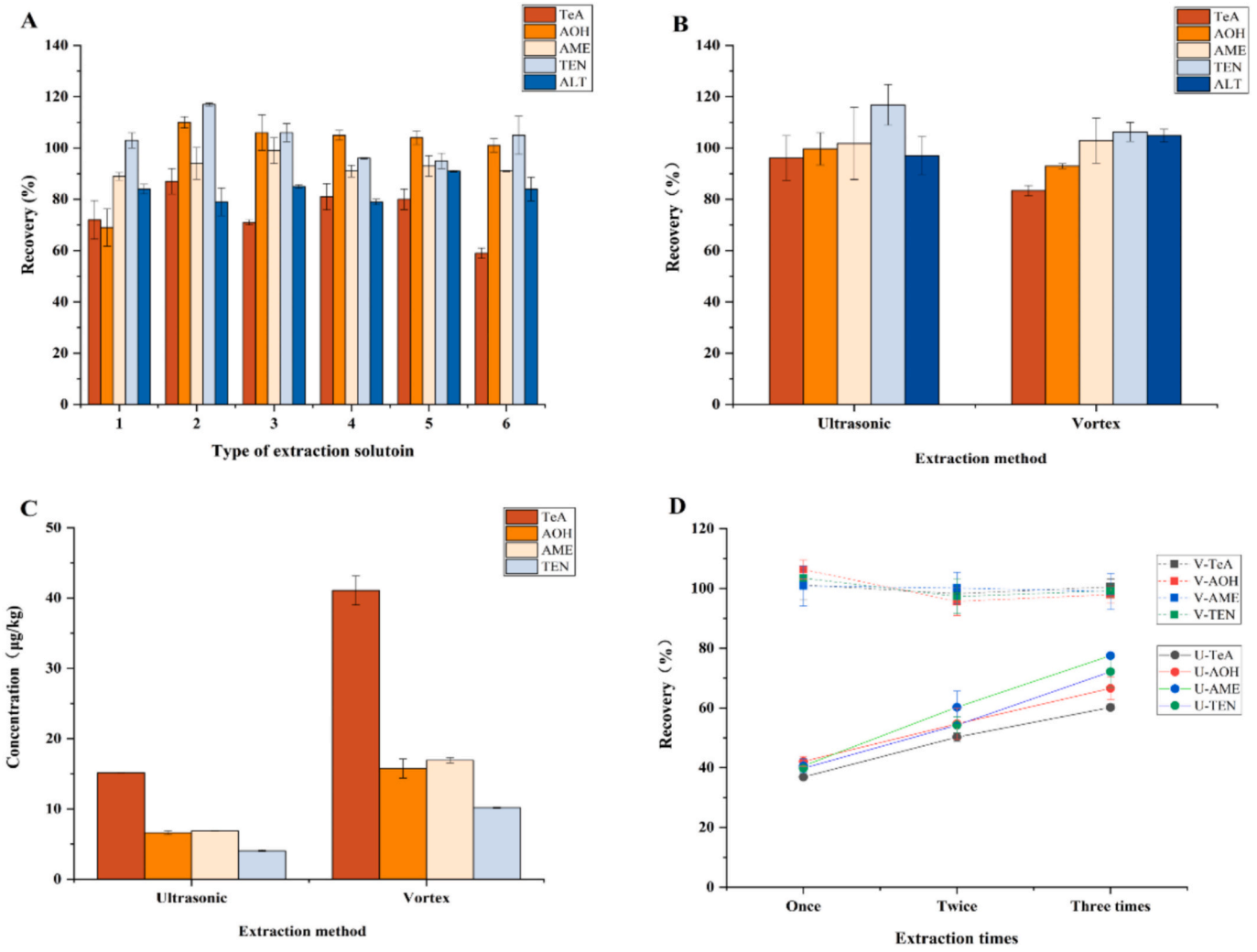

Fig. 2. Effect of extraction solution on the recoveries of five ATs (A), 1: acetonitrile-water (70:30,v:v), 2:acetonitrile-water-formic acid (70:29:1, v:v:v), 3: acetonitrile-water-acetic acid (70:29:1,v:v:v), 4: acetonitrile-water (85:14, v:v), 5: acetonitrile:water:formic acid (85:14:1, v:v:v), 6: acetonitrile-water-acetic acid (85:14:1, v:v:v); different extraction methods on the recoveries of the five ATs (B); Effects of extraction methods on the concentration of naturally contaminated wheat samples (C); The effect of multiple extraction on the recoveries of naturally contaminated wheat samples (D) (solid line circle represents ultrasonic treatment, dotted line square represents vortex treatment);Data are expressed as mean ± SD, $n = 3$.

3.6. Optimization of the extraction method

In this study, spiked blank wheat samples were used to evaluate the extraction efficiencies of these two methods(Fig. 2B). The recoveries of all five toxins exceeded 80% for both methods, thereby meeting the detection requirements. Due to the fact that the spiked negative samples cannot accurately simulate the forms of these compounds in real samples, naturally contaminated quality control (QC) samples were used for further validation(Fig. 2C). ALT was not detected in the naturally contaminated QC samples, likely because of its low natural concentration. For the other four toxins, vortex extraction outperformed ultrasonic extraction. More specifically, ultrasonic extraction gave concentrations of 15.15, 6.63, 6.88, and 4.05 μg/kg for TeA, AOH, AME, and TEN, respectively, whilst vortex extraction yielded higher concentrations of 41.09, 15.76, 16.93, and 10.17 μg/kg, respectively. Compared with ultrasonic extraction, vortex extraction significantly increased the interfacial area available for mass transfer, reduced the diffusion distance, and enhanced the extraction efficiency (Psillakis, 2019; Serrano et al., 2013). Subsequent multiple extraction experiments further validated these findings. Fig. 2D, showed the impact of multiple extractions on the recoveries obtained from the naturally contaminated QC samples. Six samples were examined, wherein the first three were subjected to ultrasonic extraction and the remaining three were subjected to vortex extraction (first extraction). All samples were then subjected to the same extraction procedure twice more, representing the second and third extractions. As shown in Fig. 2D, the recoveries of the four ATs increased with successive ultrasonic extractions. However, the first round of vortex extraction gave a recovery of around 100%, with no subsequent increases being observed following the second or third extraction steps. Consequently, vortex extraction was selected as the optimal method for sample preparation.

3.7. Optimization of the purification method

The effects of dilution method, one step SPE purification method, and traditional SPE purification method on the recoveries of toxins were subsequently evaluated, the schematic diagram of the three purification methods and the results are shown in Fig. 3, The absorption of several toxins on the two SPE columns led to low recoveries for some toxins. Finally, the dilution method was selected for further experiment because of its potential for the simultaneous extraction of all targeted toxins, as well as its simpler, faster, and more cost-effective characteristics than the SPE clean-up approach (Wu et al., 2020). In addition, the time taken for the proposed method was about 30 min, including extraction step

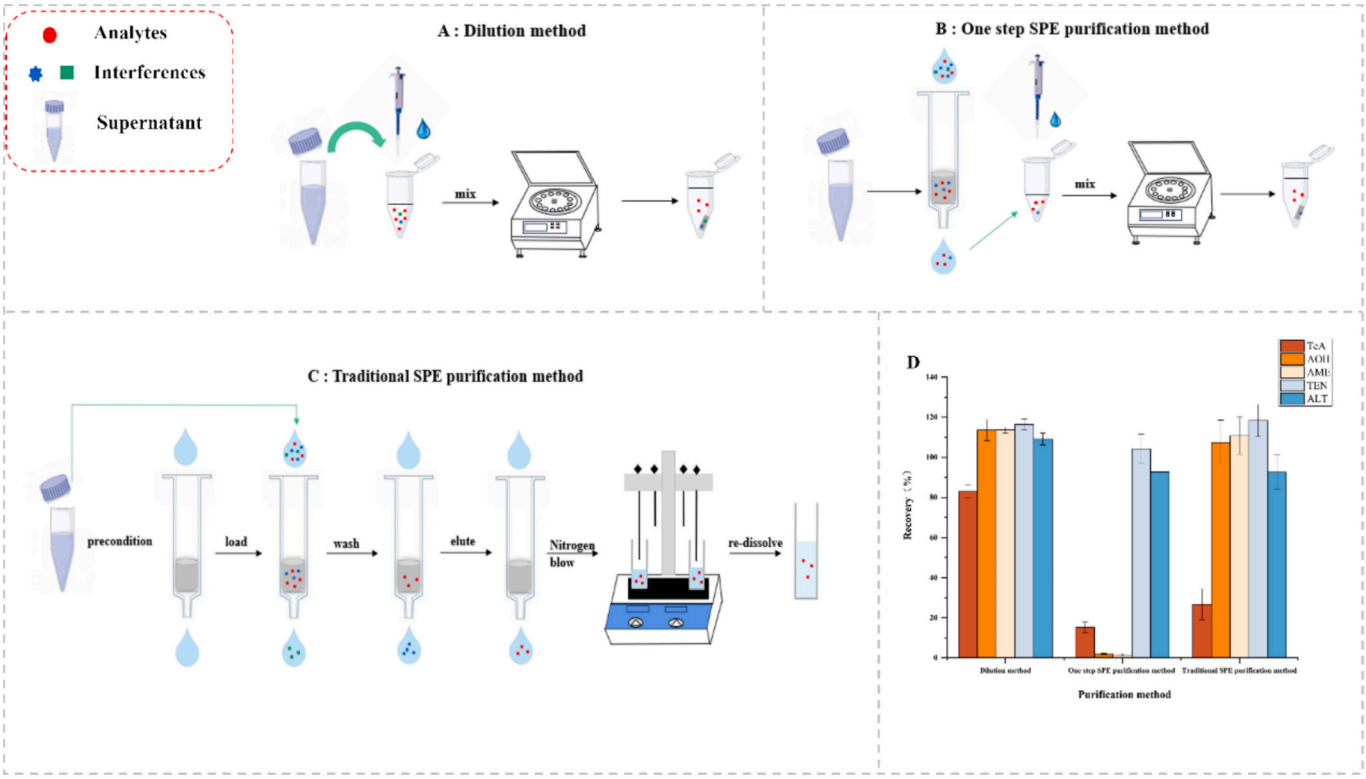

Fig. 3. dilution method schematic diagram (A), One step SPE purification method schematic diagram (B), Tradition SPE purification method schematic diagram, Effects of different purification methods on the recoveries of the five ATs (D).

(~20 min) and purification step (~10 min), and it was 50% of the time taken for the traditional MFC method.The high-throughput advantage of this method is more significant, especially when the sample size is large.

3.8. Filter membrane optimization

Spiked negative wheat samples were used to evaluate the efficiencies of four filter membrane brands and materials (i.e., PALL-PTFE, ANPEL-nylon, Jinteng-nylon, and Whatman-PTFE) for the five ATs (Fig. 4A), the PALL-PTFE membrane achieved recoveries of around 100% for all five toxins (Compared to Whatman-PTFE), providing the best overall recovery performance. However, both the nylon filter membranes strongly adsorbed AOH and AME, which resulted in lower recoveries. Therefore, the PALL-PTFE membrane was selected as the optimal filter membrane.

3.9. Method validation

3.9.1. Linearity, limit of detection (LOD), and limit of quantification (LOQ)

The linear range, MEs, method LODs, method LOQs, recovery, and precision of the proposed method were verified (Table 3). The

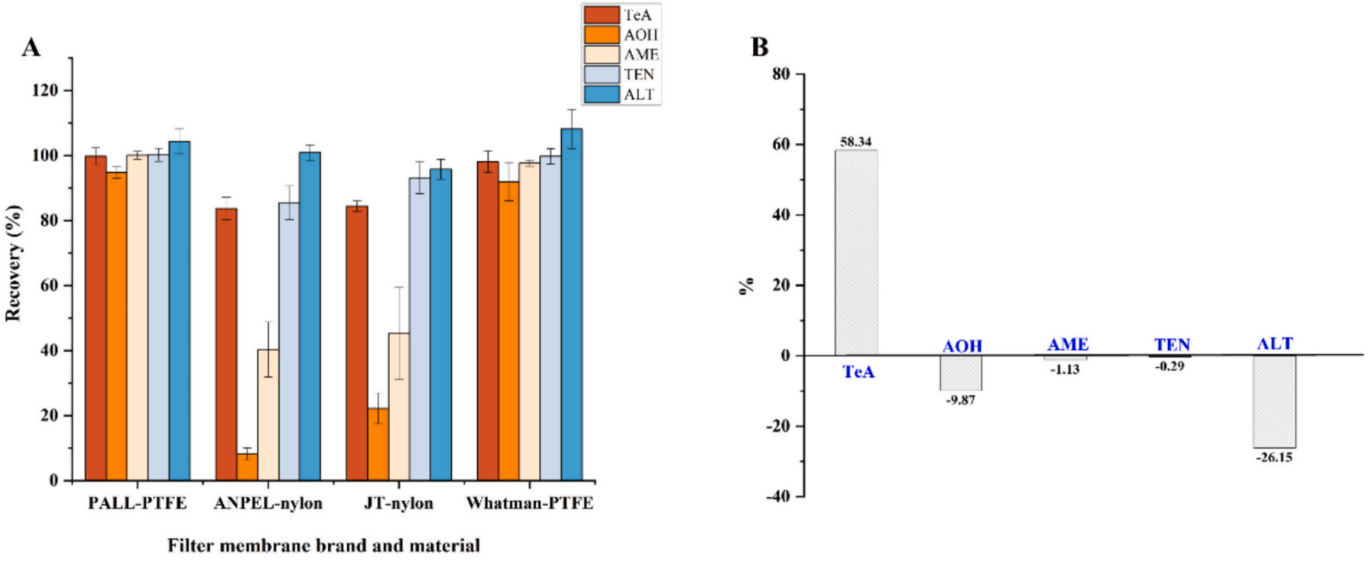

Fig. 4. Effects of different membrane brands and materials on the recoveries of five ATs (A), Matrix effects of five ATs (B).

Table 3
Validation results for linear range, linear equation, R^2, LOD, LOQ, recoveries, intra-day precision (RSD_r), and inter-day precision (RSD_R) (n = 3).

Analyte	Linear Range (ng/mL)	Linear Equation	R^2	LOD (μg/kg)	LOQ (μg/kg)	Intra-day						Inter-day	
						Low Spike Levels		Medium Spike Levels		High Spike Levels		Medium Spike Levels	
						Recovery (%)	RSD_r (%)	Recovery (%)	RSD_r (%)	Recovery (%)	RSD_r (%)	Recovery (%)	RSD_R (%)
TeA	0.10–100	y = 0.1133× + 0.0018	0.9997	0.29	0.96	90.90	3.69	81.40	6.29	87.75	2.37	86.72	5.15
AOH	0.20–100	y = 0.4747× + 0.1969	0.9999	0.42	1.60	93.44	5.67	87.00	3.36	82.82	3.48	86.20	7.38
AME	0.20–100	y = 0.2463× + 0.0204	0.9998	0.50	1.68	87.37	11.95	90.17	3.43	86.94	8.17	97.73	4.00
TEN	0.10–100	y = 1.3289× + 0.4266	0.9998	0.26	0.88	84.74	4.22	97.09	1.95	86.46	2.67	99.79	3.64
ALT	0.10–100	y = 0.0045×-0.2160	0.9998	0.48	1.60	81.83	3.03	102.68	3.20	89.89	4.62	87.00	1.66

established method demonstrated satisfactory linearities for TeA (0.10–100 ng/mL), AME (0.10–100 ng/mL), ALT (0.10–100 ng/mL), AOH (0.20–100 ng/mL), and TEN (0.20–100 ng/mL), with all correlation coefficients (R^2) being >0.999. The LODs and LOQs ranged from 0.26 to 0.50 μg/kg and from 0.88 to 1.68 μg/kg, respectively.

3.9.2. Matrix effects

It has been reported that matrix effects (MEs) are common when analyzing ATs using UPLC-MS/MS. MEs are caused by the influence of co-eluting compounds on the ionization efficiency at the electrospray interface during LC-MS/MS analysis, which manifests as either matrix enhancement or suppression (Rausch et al., 2021). Generally, matrix effects within ±20% are considered acceptable (Zhou et al., 2017). among the five ATs, TeA and ALT were the most susceptible to matrix effects(Fig. 4B). More specifically, TeA exhibited a significant matrix enhancement of 58.34%, while ALT showed a strong matrix suppression of −26.15%. The average matrix effects (either enhancement or suppression) exceeded 20%, thereby rendering them non-negligible. Thus, to minimize the impact of matrix effects, the use of isotope-labelled internal standard calibration is the best option.

3.9.3. Recovery and precision

Using the established method, the recoveries of TeA (20, 200, and 500 μg/kg), AOH (20, 100, and 200 μg/kg), AME (2, 20, and 200 μg/kg), TEN (2, 20, and 200 μg/kg), and ALT (2, 20, and 200 μg/kg) were assessed in triplicate for each concentration (Table 3). The average recoveries for the five ATs ranged from 81.40 to 102.68%, with RSDs of 1.95–11.95%, thereby demonstrating the accuracy of this method. Additionally, the intra- and inter-day precision values were both <7.40%, indicating the excellent stability and reproducibility of the method.

3.10. Contamination assessment

Currently, there is limited research on ATs contamination in wheat in China. and toxicological data are insufficient, making it challenging to conduct food safety risk assessments using traditional methods (Tralamazza et al., 2018). Furthermore, despite China's abundant wheat resources, data on overall contamination levels remain scarce, and relevant maximum limits have yet to be established (Xu et al., 2024).

we conducted a preliminary screening of commercially available wheat samples from Anhui, Henan, Hebei, Hubei, Shandong, and Jiangsu provinces using the developed method (Table 4). TeA was detected in 100% of the wheat samples, with concentrations ranging from 26.66 to 1586.20 μg/kg (average = 220.58 μg/kg, maximum = 1586.20 μg/kg), AOH was detected in 66.67% of the samples, with concentrations ranging from 0.10 to 307.39 μg/kg (average = 36.32 μg/kg, maximum = 307.39 μg/kg). Additionally, AME was found in 53.33% of the samples (1.11–94.95 μg/kg; average = 14.51 μg/kg, maximum = 94.95 μg/kg), and TEN was detected in 95.0% of the samples (1.33–133.70 μg/kg; average = 69.17 μg/kg, maximum = 133.70 μg/kg). In contrast, ALT was not detected in any of the evaluated samples. Overall, ALT was widespread in wheat across the six provinces.The high detection rate in 2023 may be attributed to increased rainfall in these regions, which raised and facilitated the production of ATs. Future monitoring will focus on the levels of ATs contamination in wheat and investigate common causes of contamination.

Table 4
Contamination assessment of five Alternaria toxins in wheat samples across China.

Toxins	TeA	AOH	AME	TEN	ALT
Total number/piece	60	60	60	60	60
Detected quantity/piece	60	40	32	57	N.D.
Detection rate%	100	66.67	53.33	95	–
average value	220.58	36.32	14.51	69.17	–
minimum value	26.66	0.10	1.11	1.33	–
Maximum value	1586.20	307.39	94.95	133.70	–
1/4 median	82.06	2.42	3.44	55.22	–
median	120.37	7.39	7.77	72.73	–
3/4 median	200.03	16.12	10.55	83.60	–

N.D.: Concentration was below detection limits or not detected.

4. Conclusion

A rapid, efficient, and environmentally friendly method was developed for the simultaneous determination of five Alternaria toxins in wheat using a highly precise and stable isotope liquid chromatography–tandem mass spectrometry (LC-MS/MS) approach. A comparison between two purification methods, namely dilution method and SPE methods, revealed that the former achieved sample recoveries between 82.19 and 116.41%, whereas the later produced lower recoveries of TeA, AOH, and AME. Importantly, this method allows the simultaneous processing of batch samples. Optimization of the analytical parameters indicated that the use of a 0.5 mM NH_4HCO_3 mobile phase combined with an ACQUITY UPLC HSS T3 column significantly improved the ionization efficiency of TeA, thereby enhancing the method sensitivity toward the target analytes. The use of stable isotope internal standard quantification effectively mitigated the issue of false-negative results during ATs screening, and reduced the analytical costs by eliminating matrix effects. The established method exhibited satisfactory linearities for TeA (0.10–100 ng/mL), AME (0.10–100 ng/mL), ALT (0.10–100 ng/mL), AOH (0.20–100 ng/mL), and TEN (0.20–100 ng/mL), with all correlation coefficients (R^2) exceeding 0.9990. The limits of detection and limits of quantification fell within the ranges of 0.26–0.50 and 0.88–1.68 μg/kg, respectively. Furthermore, spiked recoveries of

81.40–102.68% were obtained with relative standard deviations of ≤11.95%. The method was applied to preliminary screening of 60 wheat samples from major production regions in China (i.e., the Anhui, Henan, Hebei, Hubei, Shandong, and Jiangsu provinces). The results revealed the presence of four ATs contamination in Wheat (TeA, AOH, AME, and TEN), with TeA having the highest detection rate (100% of samples), followed by TEN, AOH, and AME (95, 66.67, and 53.33% of samples, respectively). Considering that the widespread ATs contamination of wheat necessitates an urgent reinforcement of monitoring effort.

CRediT authorship contribution statement

Huiyuan Lang: Writing – original draft, Validation, Investigation, Formal analysis, Conceptualization. **Zengwang Guo:** Writing – review & editing, Formal analysis. **Yu Wu:** Data curation, Formal analysis, Writing – review & editing. **Li Li:** Writing – review & editing, Methodology, Data curation, Formal analysis. **Hongmei Liu:** Investigation, Methodology, Writing – review & editing. **Lianzhou Jiang:** Supervision, Resources, Methodology, Formal analysis, Conceptualization. **Songxue Wang:** Visualization, Software. **Jin Ye:** Conceptualization, Funding acquisition, Investigation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2025.102167.

Data availability

Data will be made available on request.

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