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DETERMINATION OF *ALTERNARIA* MYCOTOXINS IN TOMATO: METHOD VALIDATION

ODREĐIVANJE *ALTERNARIA* MIKOTOKSINA U PARADAJZU: VALIDACIJA METODE

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ABSTRACT

Alternaria alternata has been identified as the most common fungal species affecting tomatoes. Contamination of tomatoes with mycotoxins produced by Alternaria species poses a significant health risk, as these toxins can accumulate in the fruit and potentially cause adverse effects when consumed by humans. Among the primary mycotoxins produced by this species and found as food contaminants are alternariol (AOH), alternariol monomethyl ether (AME), and tentoxin (TEN). This study presents an analytical method based on liquid chromatography-tandem mass spectrometry (LC-MS/MS), for the simultaneous quantification of AOH, AME, and TEN in tomatoes. The LC-MS/MS method was validated in accordance with the EC/401/2006 and Commission Recommendation EC/2022/553. The detection and determination of alternariol, alternariol monomethyl ether and tentoxin was carried out by the LC-MS/MS after using QuEChERS extraction . Mycotoxins were efficiently extracted from the spiked tomato samples, and the obtained results indicate that the validated method was robust and reliable.

***Key words**: Alternaria mycotoxins; tomato; validation; LC-MS/MS*

REZIME

Alternaria alternata je prepoznata kao najčešći soj plesni koji napada paradajz. Kontaminacija paradajza mikotoksinima produkovanih od roda Alternaria predstavlja ozbiljan zdravstveni rizik, usled njihove sposobnosti akumuliranja u plodovima i negativnog efekta po ljudsko zdravlje usled konzumiranja. Među glavnim mikotoksinima koje ovaj soj proizvodi i koji se nalaze kao kontaminanti u hrani su alternariol (AOH), alternariol monometil etar (AME) i tentoksin (TEN). Za simultano kvantifikovanje AOH, AME i TEN u paradajzu, korišćena je tečna hromatografija sa tandem masenom spektrometrijom (LC-MS/MS). Ova metoda je validovana u skladu sa EC/401/2006 i EC/2022/553. Detekcija i određivanje ispitivanih analita izvršena je primenom LC-MS/MS analize nakon QuEChERS ekstrakcije, . Mikotoksini su efikasno ekstrahovani iz obogaćenih uzoraka paradajza, a dobijeni rezultati ukazuju da je validovana metoda robusna i pouzdana. Regulative Evropske unije niti, kao ni Pravilnik o maksimalnim nivoima određenih kontaminanata u hrani (Sl. gl. RS br. 73/2024, 90/2024, 47/2025 i 61/2025), ne propisuju maksimalne nivoe Alternaria mikotoksina u hrani, Evropska komisija je izdala Preporuku (EU) 2022/553 za praćenje prisutnosti ovih toksina u hrani. Preporuka pruža smernice za nadzor i indikativne nivoe, ali ne precizira standardizovane metode ili obavezne maksimalne koncentracije. U tom kontekstu, razvoj i validacija pouzdanih analitičkih metoda su ključni. Validovana metoda je pokazala visoke odzive za sva tri mikotoksina, odličnu linearost u opsegu od 2–100 $\mu g\ kg^{-1}$ ($R^2>0.99$), granicu kvantifikacije od 4 $\mu g\ kg^{-1}$ i granicu detekcije od 1.33 $\mu g\ kg^{-1}$. Dobijeni rezultati ukazuju na pouzdanu i preciznu metodu za analizu Alternaria toksina u paradajzu.

***Ključne reči**: Alternaria mikotoksini, paradajz, validacija, LC-MS/MS*

INTRODUCTION

Since its introduction from the American continent to Europe, the tomato has become so well adapted to the Balkan Peninsula, due to favourable climatic conditions for its cultivation, that it began to be regarded as if it were an indigenous crop. Nevertheless, the presence of domestic varieties on local markets has been steadily declining, as evidenced by the statistical data collected over a ten-year period, from 2010 to 2019. During this time, in the Republic of Serbia (RS), tomatoes were cultivated on about 9,248.8 ha, with the cultivated area showing a steady annual decrease of 2.04%. During the same period, the tomato production amounted to about 157,000 t, with the yields declining at an average annual rate of 5.7%. Throughout the period, the average yield in RS was 16.9 t/ha, accompanied by a continuous annual decrease of 3.68% (*Ilin, 2021*).

According to data from the World Integrated Trade (WIT), by the end of 2023, RS was ranked 49th among tomato exporters. However, information available on the website Divercitytimes, reports that in 2022 Serbia's tomato imports amounted to 28.59 million kg, valued at $36.09 million. This presents a decrease of 20.52% compared with 2021, when imports totalled 35.97 million kg worth $33.68 million. The net change from 2021 to 2022 corresponds to a reduction of 7.38 million kg and a decline of $2.41 million in value (Figure 1).

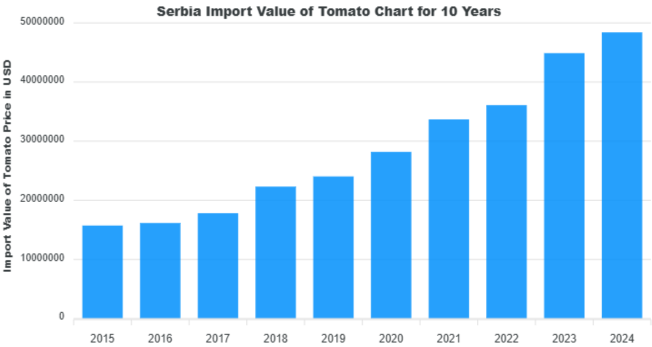
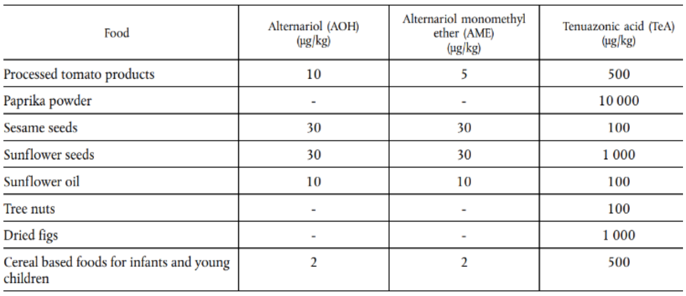


Fig. 1. Value of tomato imports to Serbia (https://divercitytimes.com/agriculture/trade/serbia-import-of-tomato)

The contamination of food with mycotoxins is recognised as a global issue, with recent reports indicating their worldwide occurrence in 60–80% of cases (*Yilmaz, 2025*). In contrast, Eskola et al. (2019) highlighted that, back in 1985, the Food and Agriculture Organization (FAO) estimated that only 25% of global food crops were contaminated with mycotoxins. Species of the genus *Alternaria* are capable of producing over 70 secondary metabolites (including mycotoxins), under specific temperature and humidity conditions (*Vuković et al., 2019*). Several studies have assessed the presence of tenuazonic acid (TeA), alternariol (AOH), and alternariol monomethyl ether (AME) in different food commodities such as wheat, soybeans, sunflower seeds, tomatoes, nuts, oranges, and apples (*Nagda and Meena, 2024*). Although most *Alternaria* toxins show relatively low acute toxicity, AOH, AME and tentoxin (TEN) are of greater concern due to their genotoxic, cytotoxic, carcinogenic, and mutagenic potential, as demonstrated *in vitro* in both bacterial and mammalian cells (*Daichi et al., 2025*).

Despite the widespread occurrence of *Alternaria* species and their mycotoxins in crops and food commodities, there are currently no legally binding limits for their presence in food and feed. The only exception is the European Union (EU), which has issued recommendations on indicative levels of AOH, AME and TeA for certain food products (*EC/2022/553*), based on data provided by the European Food Safety Authority (EFSA, 2016) (Figure 2).



Food	Alternariol (AOH) (μg/kg)	Alternariol monomethyl ether (AME) (μg/kg)	Tenuazonic acid (TeA) (μg/kg)
Processed tomato products	10	5	500
Paprika powder	-	-	10 000
Sesame seeds	30	30	100
Sunflower seeds	30	30	1 000
Sunflower oil	10	10	100
Tree nuts	-	-	100
Dried figs	-	-	1 000
Cereal based foods for infants and young children	2	2	500

Fig. 2. Indicative levels of AOH, AME and TeA according to the EC/2022/553

The present study was therefore designed to develop and validate a reliable LC-MS/MS (liquid chromatography-tandem mass spectrometry) method for the simultaneous determination of selected *Alternaria* mycotoxins belonging to the different structural groups, in compliance with the Commission Regulation (EC) No 401/2006 and Commission Recommendation EC/2022/553.

MATERIAL AND METHOD

Chemical and reagents

Acetonitrile (MeCN) and methanol (MeOH), both of HPLC ultra-gradient grade, were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Ammonium formate of analytical grade was purchased from Merck (Darmstadt, Germany). Analytical standards of AOH, AME, and TEN (100 µg mL^{-1} each) were supplied by Biopure (Romer Labs Diagnostic GmbH, Austria). Working standard solutions of AOH, AME, and TEN were prepared in MeCN at final concentrations of 1.0 and 0.1 µg mL^{-1}, and were used for spiking blank samples during method validation. Dispersive solid phase extraction (dSPE) 15 mL tubes for fatty samples (EN) and the QuEChERS extraction kit (original method) were obtained from Agilent Technologies (Santa Clara, CA, USA). Deionized water was produced using a Milli-Q purification system (Thermo Scientific™ Barnstead™ GenPure™ Pro Water Purification System).

Spiking blank tomato samples and extraction

A matrix-matched calibration curve was prepared with 6 concentration levels, i.e., at 2, 4, 10, 20, 40 and 100 µg kg^{-1}. Spiking experiments were performed to determine the recovery of the target analytes. Blank samples of tomato (10 g) were spiked at three different concentrations (4, 10 and 40 µg kg^{-1}) using a working standard solutions of 1 and 10 µg mL^{-1}. After spiking, the solution was left in contact with the matrix at room temperature in the dark for 15 min.

The QuEChERS method was shown on Figure 3.

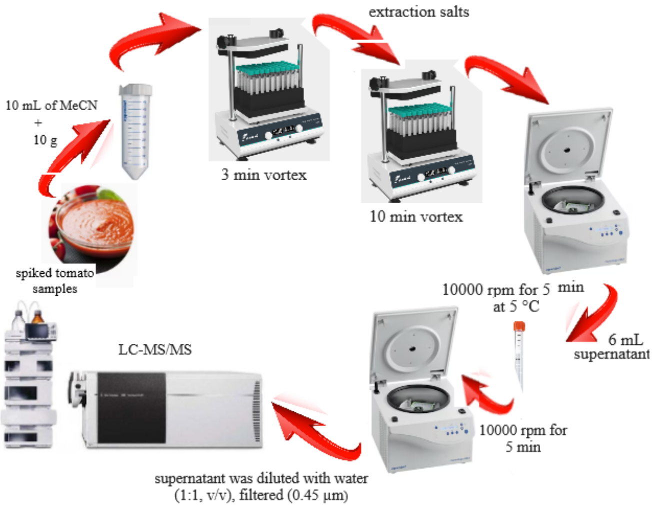


Fig. 3. Extraction procedure of spiking tomato samples

Instrumentation

The analysis of AOH, AME, and TEN was performed using an Agilent 1290 Infinity II high-performance liquid chromatography (HPLC) system equipped with a quaternary pump, multisampler, and column compartment thermostat. The HPLC system was coupled to an Agilent 6470B LC/TQ triple quadrupole mass spectrometer fitted with an Agilent Jet Stream electrospray ionization (AJS ESI) source. Chromatographic separation was achieved on an Agilent Zorbax Eclipse Plus C18 column maintained at 35 °C, with an injection volume of 20 µL. The mobile phase consisted of water (A) and acetonitrile (B), both containing 10 mM ammonium formate (pH 4), and was delivered in gradient mode at a flow rate of 0.3 mL min^{-1}. The gradient program started at 10% B for 1 min, increased to 50% B at 3 min, and further to 95% B at 8.6 min, which was maintained for 3 min. The mobile phase was then returned to the initial composition within 1 min, followed by a 5 min re-equilibration period, resulting in a total run time of 10 min. The ESI source parameters were as follows: drying gas (nitrogen) temperature 220 °C, flow rate 10 L min^{-1}, nebulizer pressure 40 psi, sheath gas temperature 250 °C, sheath gas flow 12 L min^{-1}, and capillary voltage 4000 V. Detection was conducted in dynamic multiple reaction monitoring (dMRM) mode. Instrument control, data acquisition, optimization, and quantification were carried out using Agilent MassHunter software (version 10.0; Agilent Technologies, Santa Clara, CA, USA, 2006–2018).

RESULTS AND DISCUSSION

In a previous study (*Puvača et al., 2022*), the optimization of MS/MS parameters was carried out using a liquid chromatography system equipped with an electrospray ionization (ESI) interface operating in positive ion mode and a standard mixture of mycotoxins. The precursor ions for each mycotoxin were identified in product ion scan mode. As presented in Table 2, the protonated molecule ($[M+H]^+$) was observed as the base peak ion in the mass spectra of AOH, AME, and TEN. Optimization of the product ions (Q3), collision energy (CE), and fragmentation energy (Frag) was performed under product ion scan conditions. The final selection of multiple reaction monitoring (MRM) transitions in positive ion mode for each analyte is summarized in Table 1.

Table 1. Acquisition parameters

Mycotoxin	Molecular mass	Precursor ion (m z-1)	Product ion (m z-1)	Frag (V)	CE (eV)
AOH	256	257	215.2	170	23
			213		23
TEN	412.3	413.3	271.2	170	17
			141		23
AME	270	271	256.2	135	23
			227		30

The blank sample was analyzed and the absence of matrix effects (ME) was confirmed for AOH (Parent Mass (m/z): 257.0, Product Ions (m/z): 213.0 and 215.2), TEN (Parent Mass (m/z): 413.3, Product Ions (m/z): 141.0 and 271.2), and AME (Parent Mass (m/z): 271.0, Product Ions (m/z): 256.2 and 227.0).

Table 2. Recovery of Alternaria mycotoxins

Mycotoxin	Recovery and (%RSD), n=6							
	4 µg kg^{-1}		10 µg kg^{-1}		40 µg kg^{-1}		**Average**	
	Rec1(%)	%RSD$_1$	Rec2(%)	%RSD$_2$	Rec3(%)	%RSD$_3$	Rec(%)#	%RSD$_r$##
AOH	96.5	3.36	87.5	2.05	99.1	2.98	94.4	4.93
TEN	96.3	4.21	99.4	3.10	99.9	2.37	98.5	5.74
AME	96.1	2.91	95.6	1.69	102.8	1.50	98.2	3.68

The recovery criterion was adopted according to Commission Regulation (EC) No 401/2006 (70-110%)

Predicted relative standard deviation, PRSD$_r$% <20%

Accuracy was evaluated through recovery studies using spiked blank samples at concentration levels of 4, 10 and 40 µg kg^{-1}. Recovery studies were performed in six replicates (Table 2).

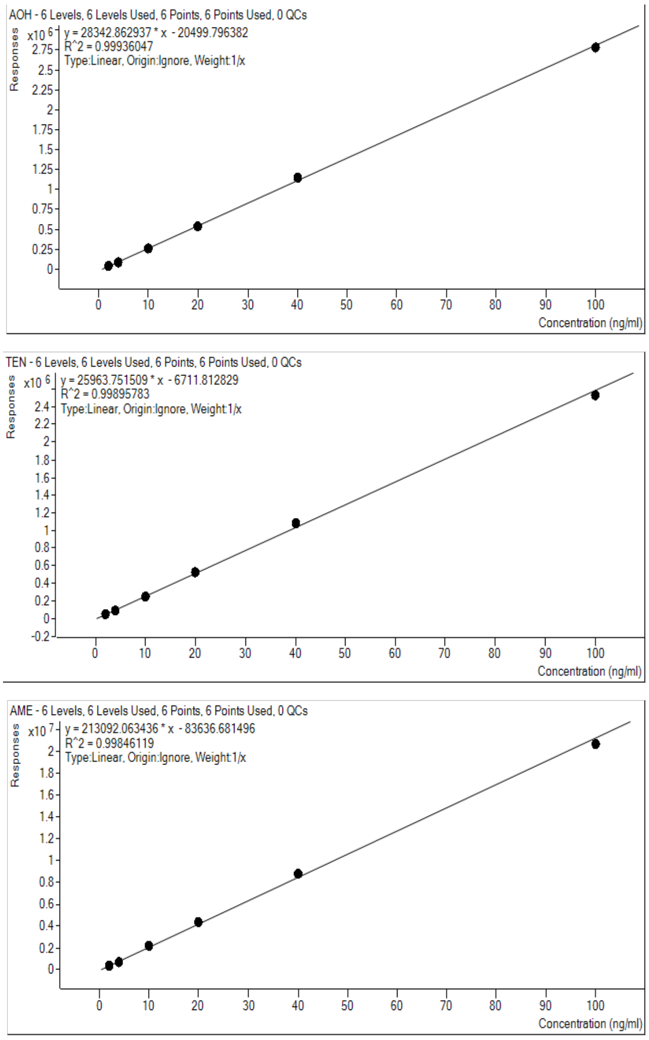


Fig. 4. Calibration curves for determination of AOH, TEN and AME

Good linearity was achieved for all *Alternaria* mycotoxins in the range from 2 to 100 µg kg^{-1}, with the coefficients of determination higher than 0.99. Linearity of AOH, TEN and AME are shown in Figure 4.

The limit of quantification (LOQ) for all the investigated *Alternaria* mycotoxins was set at 4 µg kg^{-1}, while the calculated limit of detection (LOD) was 1.33 µg kg^{-1}. It is important that the LOQ is lower than the referent value from the Regulation. The overlapped MRM chromatogram of AOH, TEN and AME are presented in Figure 5.

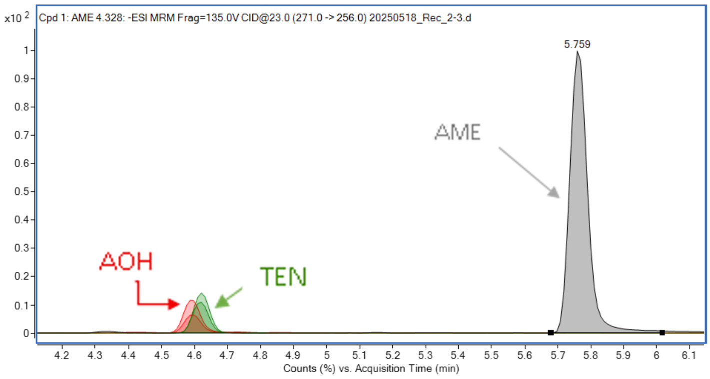


Fig. 5. Overlapped MRM chromatograms for AOH, TEN and AME

The repeatability parameter (RSD) for all investigated mycotoxins was evaluated in accordance with the EC/401/2006, and the obtained values are presented in Table 3.

Table 3. Validation parameters

Validation parameters	Mycotoxin		
	AOH	**TEN**	**AME**
Linearity, R^2	0.9994	0.9989	0.9985
Average Recovery (%)	94.40	98.56	98.17
Relative standard deviation, RSDr%	4.93	5.74	3.68
Predicted relative standard deviation, PRSD$_r$%	20	20	20
Referent value from Reg.	10	-	5
LOQ (µg kg^{-1})	4	4	4
LOD= 3/10 LOQ (µg kg^{-1})	1.33	1.33	1.33
Rt (min)	4.59	4.62	5.76

According to the obtained data, all the repeatability and LOQ criteria were met.

Previous studies have suggested that pressurised liquid extraction (PLE), followed by molecularly imprinted solid-phase extraction (MISPE) and high-performance liquid chromatography with fluorescence detection (HPLC-FLD), can be applied for the determination of AOH and AME in tomato (Rico-Yuste et al., 2018). Oliveira Gonçalves et al. (2022) employed solid-phase extraction (SPE) to isolate *Alternaria* mycotoxins from tomato, with chromatographic separation carried out using LC-MS/MS. Ji et al. (2023) reported the successful development, validation, and application of a QuEChERS extraction method coupled with LC–MS/MS for the simultaneous analysis of AME, AOH, and TEN in a survey of 194 tomato and tomato-based products from Chinese markets.

In the past decade, numerous analytical methods have been developed for the determination of *Alternaria* toxins in various food matrices, including tomatoes, tomato-based products, wheat, and sunflower seeds. Rodríguez Carrasco et al. (2016) developed an LC–ESI–MS/MS methodology for the simultaneous determination of AOH, AME, and TEN in tomatoes and tomato-derived products. The LOD and LOQ were approximately 0.7 ng g^{-1} and 3.5 ng g^{-1}, respectively. Recoveries exceeded 80%, while intra-day precision (RSDr) was ≤ 9% and inter-day precision (RSDR) ≤ 15% at concentrations of 25 and 50 ng g^{-1}. The method was based on dispersive liquid–liquid microextraction (DLLME), allowing efficient and rapid toxin extraction from complex matrices.

A similar approach was applied in a 2018 study by Puntsher and associates, which described an LC–MS/MS method for the determination of both free and modified *Alternaria* toxins in various food products, including tomato sauce, sunflower seed oil and wheat flour. LOD values ranged from 0.03 ng g^{-1} for AME to 7 ng g^{-1} foraltenusin (ALS), while LOQ values ranged from 0.06 ng g^{-1} to approximately 19 ng g^{-1}. Recoveries and precision were reported as satisfactory for almost all analytes and matrices. RSDr and RSDR also demonstrated reliable performance, confirming the method's applicability for diverse sample types, including modified toxins.

The most comprehensive inter-laboratory study, conducted in 2021/22 (*Gonçalves et al., 2022*), focused on validating an SPE–LC–MS/MS method for the simultaneous determination of ALT (altenuene), AOH, AME, TeA, and TEN in tomato puree, wheat, and sunflower seeds. The method enabled detection and quantification of toxins at or near 1 µg/kg. Average recoveries were high: ALT ~97%, AOH ~95%, AME ~98%, TeA ~97%, TEN ~102%, while RSDr and RSDR values indicated satisfactory precision (RSDr: 4.2–9.2%; RSDR: 9.3–23%). Twenty-three laboratories participated, providing key data for the standardization of Alternaria toxin determination across different matrices.

Collectively, these studies demonstrate that LC–MS/MS approaches, combined with appropriate extraction techniques (DLLME or SPE), enable highly sensitive and precise determination of major *Alternaria* toxins in complex food matrices. Comparisons indicate continuous improvement in reducing LOD/LOQ values, increasing recoveries, and enhancing precision, as well as extending the methods to modified toxins and diverse sample types. These advances are critical for food safety monitoring and the standardization of analytical procedures.

CONCLUSION

Although neither European Union regulations nor national legislation in Serbia (e.g., the Rulebook on Maximum Levels of Certain Contaminants in Food and Feed, Official Gazette of RS Nos. 73/2024, 90/2024, 47/2025 and 61/2025) establish legally binding maximum levels for *Alternaria* mycotoxins, the European Commission has issued Recommendation (EU) 2022/553 on monitoring the presence of these toxins in food. This recommendation provides guidance for surveillance and indicative levels but does not specify standardized methods or mandatory maximum concentrations.

In this context, the development and validation of reliable analytical methods are essential to support monitoring efforts. In the present study, a validated LC–MS/MS approach, following Commission Regulation (EC) No 401/2006 and Commission Recommendation (EU) 2022/553, was applied for the simultaneous determination of Alternaria mycotoxins AOH, AME, and TEN in tomato matrices. The method demonstrated high recoveries for all three mycotoxins, excellent linearity over the range of 2–100 µg kg⁻¹ ($R^2 > 0.99$), a limit of quantification of 4 µg kg⁻¹, and a limit of detection of 1.33 µg kg⁻¹. These results indicate that the method is both reliable and precise for the analysis of *Alternaria* toxins in tomato.

Taken together, these findings highlight the importance of validated analytical methodologies in the absence of legally established maximum levels. The validated method presented here provides a robust tool for future monitoring and risk assessment of *Alternaria* mycotoxins in food matrices, supporting both regulatory surveillance and food safety initiatives.

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