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Alternaria mycotoxins in fruits and vegetable samples in the Republic of Serbia – is there an impact on human health?

Alternaria mikotoksini u uzorcima voća i povrća u Republici Srbiji – postoji li uticaj na zdravlje ljudi?

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ABSTRACT

The *Alternaria* fungi are capable of producing more than 70 distinct mycotoxins, widespread across numerous food and feed matrices, including cereals, beverages (e.g. wine), vegetables and fruit, making human dietary and environmental exposure highly probable. Among the *Alternaria* mycotoxins, alternariol (AOH) and alternariol monomethyl ether (AME) are considered relevant for human and animal health due to their documented carcinogenic, mutagenic and genotoxic properties. In contrast, tentoxin (TEN) primarily affects plants. This study aimed to determine levels of AOH, AME, and TEN in vegetables and fruit samples purchased from the market of the Republic of Serbia and to estimate human exposure. None of the three *Alternaria* mycotoxins examined in this study was detected in any of the tested matrices. Concurrently, the importance of AOH and AME was highlighted by the European Commission, which has recommended the enhanced monitoring of the two compounds in foodstuffs, since chronic dietary exposure may exceed the relevant threshold of toxicological concern. Further monitoring in the Republic of Serbia should be extended, aggregate exposure taken into account, and the risk for human health estimated because of toxicological concerns related to their carcinogenic, mutagenic and genotoxic properties.

Keywords: alternariol, alternariol monomethyl ether, tentoxin, exposure assessment, LC-MS/MS, risk assessment

APSTRAKT

Gljive roda *Alternaria* mogu da proizvedu više od 70 različitih mikotoksina, koji se detektuju u rezličitim namirnicama i hrani za životinje, poput žitarica, pića (npr. vino), povrća i voća, čineći izloženost ljudi ovim toksinima mogućom, pre svega preko hrane i iz životne sredine. *Alternaria* mikotoksinima koji narušavaju zdravlje ljudi, smatraju se alternariol (AOH) i alternariol monometil etar (AME), posebno zbog svojstava karcinogenosti, mutagenosti i genotoksičnosti dok tentoksin (TEN) pre svega utiče na biljke. Cilj studije je bio da se detektuju nivoi AOH, AME i TEN u uzorcima voća i povrća sakupljenim na teritoriji Republike Srbije i da se proceni izloženost ljudi ovim mikotoksinima usled potencijalnog rizika po zdravlje. Rezultati pokazuju da u ispitivanim uzorcima nije detektovan ni jedan od tri ispitivana toksina. Istovremeno, Evropska Komisija apostrofira značaj monitoringa AOH i AME zbog mogućnosti da hroničan unos putem hrane prevaziđe granične vrednosti koje su od toksikološkog značaja. Stoga bi dalji monitoring u Republici Srbiji trebalo da bude proširen u obimu, kao i da se za procenu izloženosti primeni agregativni pristup zbog navedenih opasnosti po zdravlje ljudi, posebno mutagenih, karcinogenih i genotoksičnih.

Ključne reči: alternariol, alternariol monometil etar, tentoksin, procena izloženosti, LC-MS/MS, procena rizika

INTRODUCTION

Mycotoxins are bioactive secondary metabolites synthesized by certain fungi and are regarded as important contaminants of food and feed with significant implications for public health. Under favorable environmental conditions, especially within specific ranges of temperature and humidity, or when crops are exposed to biotic stress, toxigenic fungi can proliferate. As a result, they may produce mycotoxins that contaminate agricultural commodities at different stages, including cultivation, harvesting, storage and processing. These compounds are notably stable to thermal processing, allowing them to persist in the finished food products even when the fungal source has been inactivated or removed. Dietary exposure to mycotoxin-contaminated food can elicit a broad spectrum of adverse health effects, including organ-specific toxicity, carcinogenicity, genotoxicity and immunosuppression (Milićević et al., 2021).

It is estimated that the *Alternaria* fungi are capable of producing more than 70 distinct mycotoxins, widespread across numerous food and feed matrices, including cereals, beverages (e.g. wine), vegetables and fruit, making human dietary and environmental exposure highly probable. Among the *Alternaria* mycotoxins, alternariol (AOH) and alternariol monomethyl ether (AME) are considered relevant for human and animal health due to their documented carcinogenic, mutagenic and genotoxic properties. In contrast, tentoxin primarily affects plants by disrupting chloroplast biogenesis and inducing chlorosis. AOH exerts its genotoxic action partly through interactions with the DNA (deoxyribonucleic acid) topoisomerases, which can promote the formation of reactive oxygen species (ROS) and lead to both single- and double-strand DNA breaks. This DNA damage can trigger G2-phase (second gap phase) cell-cycle arrest, likely reflecting activated repair pathways and reduced cellular proliferation. Furthermore, AOH has been shown to inhibit macrophage differentiation in THP-1 (human monocytic leukemia cell line) monocytes and to suppress TNF-α (cytokine, a signaling protein) secretion. It also interferes with steroidogenesis and elicits estrogen-like activities comparable to those of estradiol. Both AME and AOH have been reported to reduce progesterone synthesis by downregulating key steroidogenic enzymes (Puvača et al., 2022).

AOH is also characterized by its ability to inhibit cholinesterase activity, specifically targeting acetylcholinesterase (AChE). By suppressing AChE-mediated hydrolysis of acetylcholine, AOH leads to its accumulation at cholinergic synapses and prolonged stimulation of both muscarinic and nicotinic receptors. Such dysregulation of cholinergic signaling may manifest as excessive salivation and lacrimation, muscle fasciculation, respiratory impairment, bradycardia, hypotension and, in severe cases, convulsive episodes associated with cholinergic crisis. AOH undergoes microsomal biotransformation predominantly through aromatic hydroxylation in the liver, generating catechol- and hydroquinone-type metabolites capable of engaging in redox cycling. Reported exposure routes include oral, dermal, parenteral and inhalation pathways. Although the data on chronic toxicity remain limited, regulatory evaluations, such as those from EFSA (European Food Safety Authority), recognize AOH as a contaminant of food and feed and confirm its genotoxic potential, highlighting its relevance for public health risk assessment (NCBI, 2025b).

In addition to AOH, PubChem (NCBI, 2025c) highlights that its monomethylated analogue AME undergoes extensive biotransformation in the liver. Microsomal enzymes predominantly catalyze aromatic hydroxylation, yielding catechol- or hydroquinone-like metabolites capable of entering redox cycling, and AME may also be demethylated back to AOH as a part of this metabolic process. As an altertoxin produced by *Alternaria* sp., AME is regarded as a contaminant of various plant-derived foods, which is a classification consistent with its presence in fruits, vegetables and cereals. Toxicological summaries describe AME as a compound with pronounced DNA-reactive properties, including induction of strand breaks, intercalation, formation of cross-links, as well as the inhibition of the replication and repair pathways. These effects are associated with its strong

affinity for the DNA minor groove, through which it can interfere with the activity of the DNA-modifying enzymes (e.g. topoisomerases). While AME is not currently listed as carcinogenic by the IARC (International Agency for Research on Cancer), PubChem reports associations between altertoxins and oesophageal cancer in humans. The documented exposure routes for AME include oral, dermal, inhalation and parenteral pathways. EFSA evaluations confirm its genotoxic potential and recognize it as a relevant contaminant in food and feed within the broader group of *Alternaria* toxins (NCBI, 2025c).

In contrast to AOH and AME, which show well-established DNA-reactive properties, TEN (tentoxin) displays a markedly different toxicological profile. The aggregated data from the ECHA (European Chemicals Agency) classification reports suggest that TEN has not been assigned to any GHS (Globally Harmonized System of Classification and Labeling of Chemicals) hazard category, with the available notifications consistently listing it as "not classified". Likewise, the regulatory summaries describe TEN under the EFSA legal framework, but do not attribute the specific acute or chronic toxicity classes to it. EFSA risk assessment provides a threshold of toxicological concern (TTC) value corresponding to Cramer Class III (15 µg/kg bw per day for adults), which positions TEN within a group of substances requiring conservative exposure limits despite the limited toxicological evidence. Notably, the EFSA genotoxicity evaluations classify TEN as negative for genotoxicity, distinguishing it from the other *Alternaria* metabolites. While toxicological data remain comparatively sparse, the available assessments emphasize its relevance primarily as a plant-associated metabolite rather than as a compound with demonstrated human toxicity. Accordingly, TEN appears in regulatory monitoring alongside AOH and AME, but the current PubChem entries portray it as a substance of lower toxicological concern relative to the other major *Alternaria* mycotoxins (NCBI, 2025a). This study aimed to determine levels of AOH, AME and TEN in vegetables and fruit samples purchased from the market of the Republic of Serbia. Results were intended for use in human exposure estimation and impact on human health.

A study by Marin et al. (2024) examined the effects of AOH and AME, individually and in combination, on porcine intestinal epithelial cells. Both toxins caused a dose-dependent reduction in cell viability, with their mixture producing significantly stronger cytotoxicity than either of the compounds alone, showing additive to clearly synergistic interactions across increasing inhibition levels. In addition, exposure to AOH, AME, or their mixture elevated the intracellular ROS (reactive oxygen species) levels and induced oxidative damage to lipids, proteins and DNA, with the combined treatment exerting the strongest effects. The toxins also reduced the activities of the antioxidant enzymes (catalase and superoxide dismutase), although no synergistic or additive interaction was detected for these enzymatic endpoints. Overall, the findings indicate that the co-exposure to AOH and AME enhances the cytotoxicity and oxidative stress in porcine intestinal cells compared to the single-toxin exposure.

The study by Borsos et al. (2024) provides the key toxicokinetic data relevant to the exposure assessment by examining the phase I hepatic metabolism of AOH and AME. Using the liver microsomes from humans, pigs and rats, the authors demonstrated clear interspecies differences in metabolic turnover, showing that the two toxins are biotransformed at different rates depending on the species. These findings highlight that metabolic processing of AOH and AME varies substantially across the biological systems, meaning that toxicokinetic data generated in animal models cannot be directly extrapolated to humans without accounting for these differences. The study also reports intrinsic clearance parameters that can serve as a basis for physiologically based toxicokinetic modelling, which is essential for improving estimates of the internal exposure following the dietary intake of these mycotoxins.

A recent *in vivo* toxicokinetic study in pigs demonstrated that both AOH and AME have low oral bioavailability, with only about 15 and 9% of the administered dose reaching systemic circulation. The authors showed that this limited systemic exposure is largely due to poor

absorption and extensive first-pass metabolism in the liver, predominantly into phase II metabolites. Following intravenous administration, both toxins exhibited high total body clearance and large volumes of distribution, together with very short elimination half-lives. These findings provide the essential ADME parameters (absorption, distribution, metabolism and excretion) that can support more accurate risk assessment and help improve the estimates of the internal exposure to AOH and AME following dietary intake (den Hollander et al., 2025).

MATERIALS AND METHODS

Chemicals and reagents

The reference standards of AOH, AME and TEN were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). The individual stock solutions were obtained by dissolving in methanol (1 mL) to a concentration of 0.1 mg/mL. The working mixtures of AOH, AME and TEN were then prepared by diluting the stock solutions with acetonitrile to 10 and 1 µg/mL, and afterwards stored at 4 °C in the dark. Acetonitrile and methanol (HPLC Ultra Gradient grade) were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands), while ammonium formate (analytical grade) was purchased from Merck (Darmstadt, Germany). The products Dispersive SPE 15 mL, fatty samples (EN) (part no.5982-51565), and QuEChERS extraction kit original method (part no. 5982-7550) were purchased from Agilent Technologies (Santa Clara, CA, United States) (Puvača et al., 2022).

Instrumentation

An Agilent 1290 Infinity II HPLC (High-Performance Liquid Chromatography) system (Santa Clara, CA, United States), equipped with a quaternary pump, multisampler, as well as a thermostated column compartment, was employed for the determination of AOH, AME and TEN. The Chromatograph was coupled to an Agilent 6495 triple quadrupole mass spectrometer (LC/TQ), fitted with an Agilent Jet Stream Technology Ion Source (AJS ESI). The chromatographic separation was achieved on a Zorbax Eclipse Plus C18 Rapid Resolution HD Column (50 x 2.1 mm, 1.8 µm). The column temperature was maintained at 35 °C, with an injection volume of 2 µL. The mobile phase consisted of water (A) and acetonitrile (B), both supplemented with 10 mM ammonium formate, delivered in gradient mode at a flow rate of 0.3 mL/min. The gradient was programmed as follows: 5% B (1 min), linear increase to 40% B (7 min), further to 90% B (8 min) with a 2 min hold, then returned to the initial conditions within 1 min and re-equilibrated for 2 min. The total run time was 11 min. The ESI parameters were: drying gas (nitrogen) at 200 °C and 16 L/min, nebulizer pressure 30 psi, sheath gas 300 °C at 12 L/min, and capillary voltage 3000 V. The detection was carried out in Dynamic Multiple Reaction Monitoring (dMRM) mode. The method optimization and quantification were performed using Agilent MassHunter software (v.B.10.0 SR1, Agilent Technologies, 2006-2019, Santa Clara, United States) (Puvača et al., 2022).

Sample collection and preparation

The fruit and vegetable samples were collected from the organic and conventional production systems over two consecutive growing seasons (2024 and 2025). Sour cherries, grapes, strawberries, tomatoes and carrots were collected from multiple locations across the Republic of Serbia (Belgrade, Novi Sad, Opovo, Aleksa Šantić, Goričani, Kraljevo, Vajska, Čačak, Futog and Inđija). For each matrix, the individual subsamples were taken from several points within the respective agricultural plots to ensure the representativeness of the composite sample, with a minimum total mass of 2 kg. Each sample was placed in a clean, inert container that provided adequate protection against contamination and damage during transport. All necessary precautions were applied to prevent any alteration of the sample composition during transportation to the laboratory, where the samples were homogenized and stored at -20 °C until analysis. The sampling was performed following the principles of the Commission Implementing Regulation (EU) 2023/2782.

The 10 g of homogenized samples were placed into 50 mL PT cuvettes, mixed with 10 mL of acetonitrile (with 1% formic acid), followed by mixing on a shaker at 2,500 rpm for 10 min. Then, the citrate salt mixture for the extraction was added, followed by mixing for 5 min on a shaker at 2,500 rpm, after which they were centrifuged at 6,000 rpm for 5 min. An aliquot of the supernatant (6 mL) was transferred into a cuvette (15 mL) containing adsorbents for the sample clean-up, mixed on a shaker at 2,500 rpm for 1 min and then centrifuged at 6,000 rpm for 5 min. The obtained extract was filtered through a 0.45 µm filter and analyzed by LC-MS/MS (Liquid Chromatography-Tandem Mass Spectrometry).

Validation parameters

AOH, AME and TEN were quantified using electrospray ionization in positive mode (ESI+), applying dMRM. The fragmentation of the protonated *Alternaria* mycotoxins generated two product ions for each of the compounds. 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).

The method validation was performed according to the European Commission 2002/657/EC guidelines, which include the assessment of linearity, specificity, sensitivity, recovery, precision, and matrix effects (Zhang et al, 2025). The accuracy was evaluated through recovery studies using spiked blank samples for each matrix individually at the concentration levels of 4, 10 and 40 µg/kg, while the matrix-matched calibration curve was prepared with 6 concentration levels, i.e. 2, 4, 10, 20, 40 and 100 µg/kg. The validation parameters for all the investigated matrices are presented in Table 1.

Table 1. Validation parameters

Matrix	Mycotoxin	R_T, min	LOD, µg/kg	LOQ, µg/kg	R^2	Recovery, % ± RSD, %	Up, %*
Tomato	AOH	4.59	1.4	4.0	0.9994	99.4 ± 2.80	40.80
	TEN	4.63	1.3	4.0	0.9990	98.2 ± 3.23	41.24
	AME	5.76	1.3	4.0	0.9985	98.5 ± 2.03	40.18
Carrot	AOH	4.72	1.4	4.0	0.9981	91.2 ± 5.69	42.40
	TEN	4.75	1.5	4.0	0.9994	93.7 ± 12.40	43.10
	AME	5.89	1.4	4.0	0.9987	90.5 ± 7.10	41.60
Strawberries	AOH	5.17	1.4	4.0	0.9931	89.6 ± 4.32	40.28
	TEN	5.19	1.3	4.0	0.9954	91.7 ± 8.17	39.62
	AME	6.21	1.4	4.0	0.9963	90.1 ± 5.56	40.41
Grape	AOH	5.29	1.4	4.0	0.9987	94.6 ± 9.23	43.82
	TEN	5.32	1.4	4.0	0.9989	98.8 ± 11.71	44.65
	AME	6.47	1.3	4.0	0.9991	101.2 ± 10.28	42.44
Sour cherries	AOH	6.05	1.5	4.0	0.9951	87.6 ± 8.19	39.17
	TEN	6.06	1.3	4.0	0.9917	89.7 ± 10.22	40.87
	AME	7.71	1.4	4.0	0.9928	91.4 ± 9.45	42.15

* Up, % - expanded uncertainty

The obtained data during the LC-MS/MS method validation of AOH, AME and TEN in different matrices are fully compliant with the requirements of the European regulations, in which the identification presents a key component of the confirmatory analysis of mycotoxins in fruits and vegetables. All the relevant criteria outlined in the Commission Implementing Regulations EC/2023/2782 and EC/2023/2783, as well as in the Guidance Document SANTE/11312/2021 V1, were met during the validation.

RESULTS AND DISCUSSION

Although none of the three *Alternaria* mycotoxins examined in this study was detected in any of the tested matrices, the importance of AOH and AME was again confirmed by the European Commission, which has recommended the enhanced monitoring of the two compounds in foodstuffs, following the conclusion by EFSA that the chronic dietary exposure may exceed the relevant threshold of toxicological concern. The Commission Recommendation (EU) 2022/553 specifies that the Member States, in cooperation with the food business operators, should prioritize the analysis of processed tomato products, paprika powder, sesame and sunflower seeds, sunflower oil, tree nuts, dried figs, as well as cereal-based foods for infants and young children. To reduce the current data uncertainty, largely driven by high proportions of the results below the LOQ, it is advised that the analytical methods achieve LOQs not exceeding 2 µg/kg in infant foods, 4 µg/kg in other foods, and 20 µg/kg for all the commodities. Furthermore, when levels surpass the indicative values established for the specific food categories, Member States are encouraged to investigate the underlying factors contributing to elevated contamination, as well as to assess the impact of the food processing. Estimation of the exposure was planned to be calculated using obtained data on toxin concentration in samples, multiply these data by food item intake and divide by the average body weight for males and females. However, exposure assessment could not be interpreted due to concentrations below the LOD/LOQ.

Although the Recommendation focuses specifically on AOH, AME and tenuazonic acid (TeA), the other *Alternaria* secondary metabolites such as TEN are considered relevant in scientific research due to their occurrence in agricultural commodities. However, TEN is currently not included in the Commission Recommendation (EU) 2022/553; it remains of scientific interest due to its toxicological relevance.

It is important to note that so far the EU has not established the legally binding maximum levels for any of the *Alternaria* mycotoxins investigated in this study. Only the non-binding indicative values exist for certain food categories, and these serve solely as the triggers for further investigations, rather than as the food safety limits.

A recent analysis of 50 couscous samples marketed in Algeria revealed TEN as the most frequently detected mycotoxin, present in 76% of the tested samples. The study clearly demonstrates a substantial prevalence of co-contamination in this commonly consumed cereal-based product (Mohammedi-Ameur et al., 2025).

Alternaria mycotoxins present in fruits and vegetables pose a direct risk to human health, as they can diffuse into internal plant tissues and cause adverse health effects when consumed, even when no external symptoms of spoilage are visible. In tomatoes, which constitute an important source of dietary exposure, the reported AOH levels ranged from 4.7 to 105.3 µg/kg in tomato-based foods, while products such as tomato sauces (1.1-24.6 µg/kg), ketchup (1.1-27.2 µg/kg), chopped tomatoes (1.7-12.8 µg/kg), tomato hotpot additives (1.1-17.7 µg/kg) and tomato juices (1.9-7.9 µg/kg) consistently contained measurable concentrations of this toxin (Saleh et al., 2024).

In a study evaluating the presence of AOH, AME and TEN in strawberries, the samples were analyzed after storage at either 22 ± 2 or 6 ± 2 °C for up to one month. AOH was the most frequently detected, occurring in 42% of the samples stored at approximately 22 °C and in 37% of the samples stored at 6 °C, with concentrations ranging from 26 to 752 ng/g in fruit kept at ambient temperature. AME was detected less frequently, with levels

between 11 and 137 ng/g after more than 28 days of storage at 6 °C. In contrast, TEN was not detected in any of the analyzed samples, regardless of the storage temperature (Juan et al., 2016).

A study conducted in China analyzed 325 food samples for AOH, AME, TEN and TeA. At least one of the mycotoxins was detected in 53.85% of the samples, with wheat flour showing the highest contamination rate (97.41%). TeA exhibited the highest frequency and concentration, while AOH and AME were detected at lower levels, but still contributed notably to the dietary exposure. The exposure assessment demonstrated that in certain food groups, such as wheat flour and Coix rice, the 95th-percentile exposure estimates for AOH and AME exceeded their respective health-based reference values, indicating a potential health concern, particularly for children aged ≤6 and 7-12 years. In contrast, exposure to TEN remained within the acceptable toxicological threshold. Overall, this study highlights the widespread occurrence of *Alternaria* mycotoxins in commonly consumed foods in this region and identifies children as the most vulnerable subgroup with respect to AOH and AME intake (Lu et al., 2025).

Current toxicological data on *Alternaria* metabolites are still too limited to support a reliable human risk assessment. Considerable gaps remain in the available evidence, and further studies are required even for comparatively better-studied toxins such as AOH and AME in order to properly address the hazard identification and characterization. For several other metabolites, including TEN, the toxicological database is even more restricted. EFSA previously assigned TTC values for these mycotoxins precisely because the available information was insufficient to derive the risk-based guidance levels. For TEN, the lack of data is particularly evident, as no OECD-compliant genotoxicity studies have been identified and only a single experiment reported an absence of significant DNA strand breaks in the exposed cells. Overall, the scarcity of toxicological studies for multiple *Alternaria* metabolites, including AOH, AME and TEN, demonstrates that current evidence remains inadequate for a comprehensive evaluation of human health risks (Louro et al., 2024).

CONCLUSIONS

Even though the results of the study show that none of the examined mycotoxins was determined in the fruit and vegetable samples collected from the market of the Republic of Serbia, monitoring should be enhanced, particularly in terms of exposure assessment and human health risk, because of toxicological concerns related to their carcinogenic, mutagenic and genotoxic properties.

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