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Determination of the *Alternaria* mycotoxin tenuazonic acid in cereals by high-performance liquid chromatography–electrospray ionization ion-trap multistage mass spectrometry after derivatization with 2,4-dinitrophenylhydrazine

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ABSTRACT

Tenuazonic acid (TA) is a major *Alternaria* mycotoxin. In the present work a novel approach for the detection of TA in cereals by liquid chromatography–ion-trap multistage mass spectrometry after derivatization with 2,4-dinitrophenylhydrazine is described. The product of the derivatization reaction and its major MS^2 fragments were characterised by Fourier transform-ion cyclotron resonance tandem mass spectrometry. Without preconcentration, the established method features a limit of detection of 10 μg/kg using 2 g of sample in a rapid workup procedure. Accuracy, precision and linearity were evaluated in the working range of 50–5000 μg/kg. TA was detected in 13 and quantified in 3 out of 27 cereal samples obtained from a local supermarket, the average content being 49 μg/kg (highest incidence: 851 ± 41 μg/kg).

1. Introduction

Tenuazonic acid [TA, (5S,8S)-3-acetyl-5-*sec*.-butyltetramic acid, **1**] is a toxic metabolite produced by *Alternaria* spp., *Phoma sorghina* and *Pyricularia oryzae* [1–4]. It is considered to be of the highest toxicity amongst the *Alternaria* mycotoxins (Fig. 1) [5]. TA inhibits protein biosynthesis [6] and is biologically active, exerting antitumor, antiviral, and antibiotic activities [5,7,8]. It was furthermore associated with the mycotoxicosis Onyalai, a hematologic disorder observed in Africa [1,9].

Alternaria spp. are commonly infesting a broad range of agricultural products, including various grains [5]. Generally, a moist environment (water activity $a_w = 0.98$) is favourable for *Alternaria* mycotoxin production [10]. Besides TA, the dibenzo-α-pyrone derivatives alternariol (AOH), alternariol monomethyl ether (AME) and altenuene (ALT) as well as the 4,9-dihydroxyperylene-3,10-quinone Altertoxin I (ATX-I) are well-known toxic *Alternaria* metabolites.

In a recent article, Azcarate et al. [11] reported high *Alternaria* mycotoxin levels in Argentinian wheat of the 2004/2005 harvest, with a mean of 2.313 mg/kg for TA in the positive samples. Li and Yoshizawa [12] detected similar quantities in weathered wheat from China. Patriarca et al. [13] reported that out of 123 *Alternaria* strains isolated from Argentinian wheat, 72% produced TA with TA being the *Alternaria* mycotoxin produced in the highest concentrations.

To date, the before mentioned *Alternaria* mycotoxins have been quantified by high-performance liquid chromatography (HPLC) coupled with UV (for TA), fluorescence (for AOH, AME, ALT and ATX-I) [12,14–21] or electrochemical detection (for AOH, AME and ATX-I) [21–23], thin-layer chromatography [21,24,25] as well as by gas chromatographic methods [21,25–27].

For AOH, AME, ALT and ATX-I, HPLC–tandem mass spectrometry (MS^2) methods have been published as well [21,28–30]. However, apart from a poster presentation [31], there are to our knowledge no publications on LC–MS methods available for TA. This is most likely due to the low molecular weight, relatively high acidity ($pK_a = 3.5$) [7] and metal chelating properties of this compound. In order to obtain acceptable peak shapes in HPLC, ionization on column has to be suppressed (low pH conditions) or ion-pairing techniques have to be used. The latter have been applied widely with $Zn(II)SO_4$ as an eluent modifier [12,14,16–20], however, $Zn(II)SO_4$ is not compatible with common MS ion sources, while other ion-pairing systems are not likely to show sufficient retention [18]. At low pH conditions (ionization suppression chromatography), on the other hand, unfavourable ion-yields are expected when using MS detectors.

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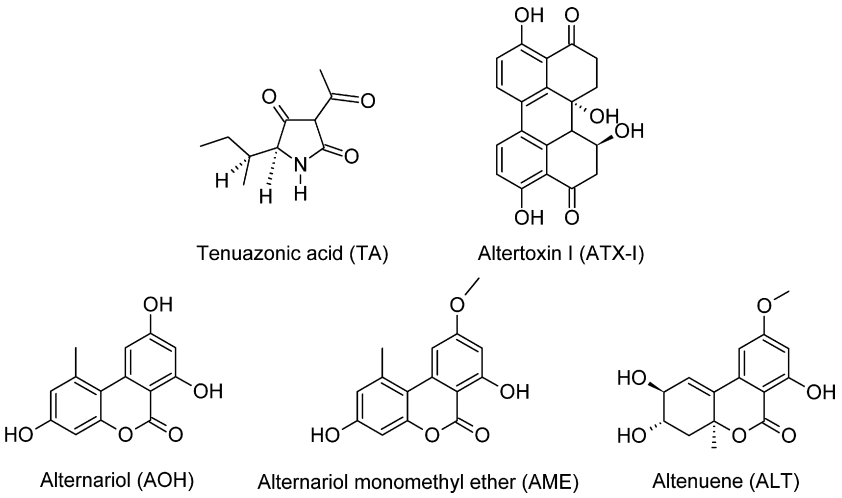

Fig. 1. Structures of common *Alternaria* mycotoxins.

There is, to our knowledge, currently only one LC–MS method available for TA quantification, using low-retention ion-pairing with ammonium hydrogencarbonate and detection by tandem MS [31].

Hence, it was our aim to develop an improved HPLC–MS method for the quantification of TA, which ought to be compatible with the electrospray (ESI) ion source and applicable to solid food matrices. Key objectives were the optimization of specificity and chromatographic retention as well as an improved limit of detection (LOD).

For this purpose, 2,4-dinitrophenylhydrazine (DNPH, **2**) was applied as a derivatization reagent. The latter is commonly utilized in environmental analysis [32]. Inter alia was shown to be useful in the LC–MS detection of poorly ionizing carbonyl compounds [33–38]. Recently, the enhancement of the mass spectrometric detection of the β-dicarbonyl houttuynin in blood by derivatization with DNPH has been reported as well [39]. However, we are not aware of any previous applications of DNPH in the field of mycotoxins or cereals.

2. Materials and methods

2.1. Chemicals

The TA copper(II) salt (no purity given), undecylic aldehyde (97%) and Dowex 50WX8-200 cation-exchange resin (H^+ form) were purchased from Sigma–Aldrich (Schnelldorf, Germany). DNPH (analytical-reagent grade), phlegmatized with 33% water, was obtained from Merck, Darmstadt, Germany. Solvents were of HPLC grade, all standard chemicals were of analytical-reagent grade.

2.2. Preparation of derivatization reagent

DNPH hydrochloride was prepared similarly to the procedure suggested by Brady and Elsmie [40]: phlegmatized DNPH (0.3 g, 1.0 mmol) was suspended in 2 M hydrochloric acid (3 mL). Consequently, 2 mL concentrated hydrochloric acid were added and the suspension was ultrasonicated for 2 min. The crystalline DNPH hydrochloride was redissolved in another 60 mL of 2 M hydrochloric acid. The resulting solution was ultrasonicated again until it cleared up and used directly as the derivatization reagent.

2.3. Preparation of TA stock solutions

Dowex 50WX8-200 cation-exchange resin (H^+ form) was left to stand in deionized water for 2 h. A minicolumn (4 cm × 1.5 cm) was packed with the conditioned resin and washed with methanol several times.

Approx. 5 mg of copper(II) tenuazonate were dissolved in 2 mL methanol, put on the column, and eluted with 15 mL methanol. A colourless solution was obtained. It was diluted with methanol to obtain the stock solution.

The actual concentration of the methanolic stock solution was determined by UV spectroscopy (Unicam 5625 UV spectrometer, Unicam, Cambridge, UK) using the extinction coefficient ε_{277} = 12,980 cm mol/L [7]. Units were converted to mg/kg using d(MeOH) = 0.791 kg/L.

The resulting stock solution [$c = m$(TA)/m(methanol) = 133 mg/kg] was further diluted with methanol to obtain three working solutions at concentrations of 1, 0.1 and 0.005 mg/kg. All solutions were stored at −20 °C.

2.4. Synthesis of the DNPH–TA hydrazone (5S)-3-[N-(2,4-dinitrophenyl)ethanehydrazonoyl]-5-[(1S)-1-methylpropyl]pyrrolidine-2,4-dione (**3**) for NMR measurements

TA (10 mg, 51 μmol, 1 equiv.) was prepared from its copper salt as described above and dried in a gentle nitrogen stream. Consequently, the derivatization reagent was added (30 mL, 468 μmol, 9.2 equiv.). After ultrasonication, a yellowish white solid precipitated. The mixture was shaken for further 30 min on a Promax 2020 horizontal shaker (Heidolph, Kelheim, Germany) at 400 rpm. The precipitate was collected, washed with water and recrystallized from ethanol to obtain compound **3** (10 mg, 27 μmol). Yield: 53%.

Compound **3** occurs in two rotameric forms which are differentiable in its ^{1}H NMR spectrum (see Section 3). Their approximate ratio is rotamer A (rot. A):rotamer B (rot. B), 70:30. Affected ^{1}H NMR signals are split in that ratio.

^{1}H NMR {600 MHz, [$^2H_6$]dimethyl sulfoxide (DMSO-d_6)}: δ 0.84 (m, 3H, CH_3), 0.93 (m, 3H, CH_3), 1.19 (m, 1H, CH_2), 1.30 (m, 1H, CH_2), 1.91 (m, 1H, CH), 2.42 (s, 3H, CH_3, rot. A), 2.47 (s, 3H, CH_3, rot. B), 3.64 (d, 1H, CH, rot. A), 3.68 (d, 1H, CH, rot. B), 7.18 (m, 1H, $CH_{arom.}$), 7.75 (s, 1H, lactam-NH, rot. B), 8.02 (s, 1H, lactam-NH, rot. A), 8.40 (m, 1H, $CH_{arom.}$), 8.88 (m, 1H, $CH_{arom.}$), 10.42 (s, 1H, amine-NH), 11.57 (s, 1H, H-bond, rot. A), 11.66 (s, 1H, H-bond, rot. B)

^{13}C NMR (151 MHz, DMSO-d_6): δ 11.9 (CH_3), δ 12.6 (CH_3), 15.8 (CH_3), 23.1 (CH_2), 36.7 (CH), 65.8 (CH), 96.6 ($C_{quart.}$), 115.4 ($CH_{arom.}$), 122.9 ($CH_{arom.}$), 130.4–130.7 ($CH_{arom.}$, $C_{arom.}$), 137.5 ($C_{arom.}$), 147.8 ($C_{arom.}$), 168.1 ($C_{quart.}$), 173.6 ($C_{quart.}$), 196.5 ($C_{quart.}$)