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Risk evaluation of the *Alternaria* mycotoxin tenuazonic acid in foods for adults and infants and subsequent risk management

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ABSTRACT

Since the first risk evaluation of *Alternaria* toxins by the European Food Safety Authority (EFSA) in 2011, new stable isotope dilution assays for tenuazonic acid (TA) broadened and deepened the data for exposure assessment. Moreover, recently unraveled high contents of TA in infant food based on sorghum/millet required an updated risk assessment. With the new data, the risk evaluation still revealed a low risk for adults as the maximum upper-bound data of the 95th percentile dietary exposure of TA can be estimated to be below the threshold of toxicological concern of 1500 ng/kg$_{b.w.}$ d. However, the evaluation of infant cereals based on sorghum/millet revealed a risk for the health of children consuming these foods, when two complementary approaches were applied. On the one hand, the maximum value of 1200 μg/kg TA in sorghum/millet-based infant food results in an intake of 3670 ng TA/kg $_{b.w.}$ d, which exceeds the threshold of toxicological concern by a factor of 2.4. On the other hand, this intake only leaves a margin of safety of 340 to the lowest dose showing subacute adverse effects in animals (1.25 mg/kg$_{b.w.}$ d), However, a safety factor of at least 1000 would be required. Therefore, infant cereals based on sorghum/millet with high content of TA cannot be regarded as safe. Based on toxicological considerations a limit of 500 μg TA per kg of sorghum/millet-based infant food is suggested. In these foods, other relevant *Alternaria* toxins such as alternariol or its methyl ether were not detected and obviously do not impose a risk for the consumers.

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1. Introduction

Tenuazonic acid (TA, Fig. 1) is one of the predominant mycotoxins produced by *Alternaria* spp. (Bottalico & Logrieco, 1998), which are also capable to synthesize other toxic metabolites such as alternariol, alternariol monomethyl ether, altertoxins and tentoxin [Lee, Patriarca, & Magan, 2015]. TA also has been reported to be biosynthesized by *Pyricularia oryzae* (Umetsu, Kaji, & Tamari, 1972, 1974) and *Phoma sorghina* (Steyn & Rabie, 1976). A variety of biological activities has been observed including antiviral (Miller et al. 1963), antitumor, antibacterial, cytotoxic (Gitterman, 1965) and phytotoxic properties (Lebrun et al. 1988). Considering its oral LD_{50}-values of 182 or 225 mg kg^{-1} body weight (b.w.) for male mice (Miller et al. 1963; Smith, Fredrickson, & Hadidian, 1968) and 81 mg kg^{-1} b.w. for female mice (Miller et al. 1963), TA is regarded as the *Alternaria* mycotoxin with the highest acute toxicity (Bottalico & Logrieco, 1998). Referring to human toxicoses, TA has been made responsible for the outbreak of “onyalai”, a human haematologic disorder disease occurring in Africa (Steyn & Rabie, 1976). However, further toxicological evidence is lacking.

Reports on the occurrence of TA in foods point to a nearly ubiquitous existence including contamination of cereals, vegetables, beverages and spices (Bottalico & Logrieco, 1998).

A first comprehensive risk evaluation of *Alternaria* toxins including TA was undertaken by the European Food Safety Authority (EFSA) in 2011 [Alexander et al., 2011]. In the latter assessment, for the genotoxic compounds alternariol and alternariolmonomethyl ether, the mean chronic dietary exposures of European consumers were found to exceed across most dietary surveys their respective threshold of toxicological concern (TTC) of 2.5 ng/kg$_{b.w.}$ d. A recent evaluation for Germany underlined these findings by estimating the chronic exposition to alternariol based on mean consumption data up to 1400% of the TTC [Hickert, Bergmann, Ersen, Cramer, & Humpf, 2016]. For TA, the EFSA evaluation deduced a minor risk for adults as the 95th percentile dietary exposure (in maximum 362 ng/kg$_{b.w.}$ d) was well below the

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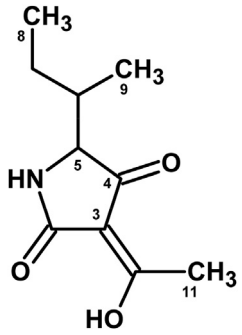

Fig. 1. Chemical structure of tenuazonic acid.

TTC of 1500 ng/kg$_{b.w.}$ d assumed for TA. However, in particular the high occurrence in sorghum/millet-based infant food were not known at that date.

The goal of the study presented here was, therefore, to present actual exposition data of TA and to correlate them with the actual knowledge on toxicology. Moreover, it was intended to update the risk evaluation for consumers based on this correlation.

2. Results and discussion

2.1. Analytical methods

An important prerequisite for an adequate risk assessment are accurate analytical methods in the first place to enable collecting reliable exposure data. As for other *alternaria* toxins (Asam & Rychlik, 2015), accurate stable isotope dilution assays (SIDAs) have been developed for TA since stable isotopologic standards have been available. The first stable isotope labeled TA was synthesized from [$^{13}C_6^{15}N$]-methyl isoleucinate to give sevenfold labeled [$^{13}C_6^{15}N$]-tenuazonic acid [Asam, Liu, Konitzer, & Rychlik, 2011]. The subsequently developed SIDA was based on derivatization of TA with 2,4-dinitrophenylhydrazine during extraction, solid phase clean up using C_{18} material and liquid chromatography before mass spectrometric detection. With this approach, not only accuracy but also sensitivity was increased as limits of quantitation (LOQ) of 0.3 μg/kg e.g. in tomato products were achieved.

Another stable isotopologue, [$^{13}C_2$]-tenuazonic acid was elaborated in a three-step procedure starting from unlabeled Boc-protected isoleucine (Lohrey, Marschik, Cramer, & Humpf, 2013). Using the [$^{13}C_2$]-TA isotopologue a SIDA was developed for tomato and pepper products based a QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) sample preparation. No derivatization was applied and, therefore, the method was less sensitive (LOQ 2.89 μg/kg).

2.2. Occurrence of TA in foods

TA is almost ubiquitous in foods derived from plants and contamination also may occur in foods from animal origin via spices being often high in TA contents. This result is underpinned by a human biokinetic study in which a blank urine hardly was obtainable from the volunteers (Asam, Habler, & Rychlik, 2013). Solely a diet based on milk products and non-spiced meat could generate blank urines. This is partly reflected in the literature, as TA regularly was found in food commodities, especially in cereals (Azcarate, Patriarca, Terminiello, & Pinto, 2008; Li & Yoshizawa, 2000; Patriarca, Azcarate, Terminiello, & Pinto, 2007; Siegel, Merkel, Koch, & Nehls, 2010; Webley, Jackson, Mullins, Hocking, & Pitt, 1997) and in tomatoes and their respective processing products (Asam et al. 2011; Da Motta and Soares 2001; Mislivec, Bruce, Stack, & Bandler, 1987; Scott & Kanhere, 1980; Stack, Mislivec, Roach, & Pohland, 1985; Terminiello, Patriarca, Pose, & Pinto, 2006;), beer (Siegel et al. 2010), beverages (Asam et al. 2011), spices (Asam, Lichtenegger, Liu, & Rychlik, 2012) and even infant food (Asam & Rychlik, 2013).

When comparing the recent results for TA in food samples from the EU with the data on which the aforementioned EFSA evaluation was based, the latter has to be considered as partly outdated. Firstly, exposure assessment was mainly based on so-called left-censored data (LC, i.e. below limit of quantitation or detection) as many methods to that date were not sensitive enough. This is also the reason for the wide EFSA dietary exposure ranges, e.g. between 49 (lower bound, LB) and 97 (upper bound, UB) ng/kg$_{b.w.}$ d for the median of the mean dietary exposure across the EU member states. In this respect, the lower bound was assessed assigning a value of zero to left-censored results and the upper bound assigning the value of LOD or LOQ to results below the LOD and LOQ, respectively. Secondly, some recent results based on SIDA technology resulted in completely different evaluations. According to a recent Belgian study (Van de Pirre et al., 2014), tomatoes and products derived thereof revealed maximum contents of 4800 μg/kg TA, which resulted in a mean dietary exposure of 2900 ng/kg$_{b.w.}$ d in contrast to the mean EFSA exposure range for Belgium of 41 (LB) and 94 (UB) ng/kg$_{b.w.}$ d. In contrast to the results from Belgium, analyses from the German market revealed TA values between 15 and 195 μg/kg (tomato ketchup, n = 9), 363–909 μg/kg (tomato paste, n = 2) and 8–247 μg/kg (pureed tomatoes and comparable products, n = 5) (Asam et al., 2011) and, in another survey, 26 tomato samples and 4 bell pepper samples, TA was found in each sample with levels ranging from 3 to 2330 μg/kg (Lohrey et al., 2013). The third reason to question the EFSA assessment are the data for cereals. With the new SIDAs in place, 92% of cereal and 87% of spice samples in a German survey were found TA positive [Asam et al. 2012], which could significantly alter the exposure calculation by the EFSA based on only 10% samples lying above the limit of detection or quantitation. In another Belgian LC-MS/MS study also using [$^{13}C_6^{15}N$]-TA as the internal standard, a total of 71% of the rice samples, and 31% of the oat flake samples were found to be contaminated with concentrations in the range of 1.9–113 μg/kg and 2.1–39 μg/kg, respectively (Walravens et al., 2014).

Last but not least, when regarding infant food, exposure data were completely missing in the EFSA assessment. A recent investigation, however, revealed a median content of TA in infant tea infusions (n = 12) of 2 μg/L, but values up to 20 μg/L were found in fennel tea infusions. In puree infant food in jars (n = 12), the median content of TA was 7 μg/kg, but higher values were detected in products containing tomato (25 μg/kg), banana and cherry (80 μg/kg) and sorghum/millet (20 μg/kg). Infant cereals on the basis of wheat and/or oats, rice, spelt and barley (n = 4) did not contain TA in values higher than 30 μg/kg, but if sorghum/millet was the major ingredient (n = 12) the mean content of TA was 550 μg/kg and the maximum level was 1200 μg/kg.

In order to clarify the cause of the contamination, sorghum/millet grains of different origin (n = 20) were analyzed for their content of TA. Obviously, sorghum/millet grains grown in countries of the moderate climate zone like Germany and Austria (mean value: 380 μg/kg TA) were higher contaminated than sorghum/millet grains from countries with warmer or drier climate such as North Africa, Ethiopia, India, Hungary and Ukraine (mean value: 75 μg/kg TA). Interestingly, sorghum/millet grains of Chinese or Ethiopian origin did not contain tenuazonic acid in amounts larger than 20 μg/kg (mean value: 12 μg/kg TA). Although we could not clarify the sorghum/millet species by molecular biological methods, we have several indications (phenotyping of the grains

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