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# ASSESSMENT OF THE ALTERNARIA MYCOTOXIN TENUAZONIC ACID IN FRUIT JUICE SAMPLES

*Vahid Safavizadeh*[1,2], *Ali Shayanfar*[3], *Masoud Ansarin*[4] , *Mahboob Nemati*[5]

***Address(es):***
[1] Food and Drug Safety Research Center, Health Management and Safety Promotion Research Institute, Tabriz University of Medical Sciences, Tabriz, Iran.
[2] Department of Pharmaceutical and Food Control, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran.
[3] Pharmaceutical Analysis Research Center and Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran.
[4] Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.
[5] Halal Research Center of IRI, FDA, Tehran, Iran.

*Corresponding author: nematim@tbzmed.ac.ir

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**ABSTRACT**

Tenuazonic acid (TeA) is a secondary toxic metabolite that is produced by some *Alternaria* species. The aim of this study was to determine the presence of TeA in fruit juice samples. A total of 50 (40 Grape; 5 Apple; 5 Orange) fruit juice samples were collected from Tabriz, Iran local market and were analyzed for TeA contamination via HPLC-UV. Analyte extraction was done by acetonitrile/water/formic acid (84/16/1 v/v/v). Lower limit of quantitation and upper limit of quantification for the developed method were 10 µg/L and 4000 µg/L respectively. Recovery ranged was between 96 to 108 %. The results showed 42.5% of grape juice samples were contaminated with TeA and the average concentration of TeA was 139.2±115.5 µg/L. However, it was not detected in apple and orange juice samples. This is the first study on the presence of TeA in Iranian food samples and showed that the necessity of more supervision on the production of grape juice.

**Keywords:** Mycotoxins; Alternaria; Fruit Juices; HPLC; Tenuazonic Acid; Iran

## INTRODUCTION

Some microfungi produce toxic secondary metabolites called mycotoxins **(Amirkhizi, Arefhosseini, Ansarin, & Nemati, 2015; Cunha, Sá, & Fernandes, 2018; Fernández-Cruz, Mansilla, & Tadeo, 2010; Pizzutti et al., 2014; Walravens et al., 2014)**. Mycotoxins are one of the most important contamination factors in plants and cause the destruction of crops **(da Motta & Valente Soares, 2000)**. Some of the most important types of fungi that produce mycotoxins are *Aspergillus* spp, *Penicillium* spp, *Fusarium* spp and *Alternaria* spp **(Prelle, Spadaro, Garibaldi, & Gullino, 2013; Walravens et al., 2014)**. *Alternaria* fungi are pathogens and saprophytic species that have the ability to grow at low temperature **(De Berardis et al., 2018; Myresiotis, Testempasis, Vryzas, Karaoglanidis, & Papadopoulou-Mourkidou, 2015)**. This mycotoxin could be found in grains, fruits, vegetables and oilseeds **(Asam, Liu, Konitzer, & Rychlik, 2011; Broggi et al., 2013; De Berardis et al., 2018)**. Generally, a wet environment (water activity aw = 0.98) is suitable for the growth of this fungus **(Siegel, Merkel, Koch, & Nehls, 2010; Siegel, Rasenko, Koch, & Nehls, 2009)** therefore prevention of growth of mycotoxigenic fungi is the most important way to control its presence in the foodstuff **(Prendes et al., 2018)**. Having melanize walls in the spores of the *Alternaria*, makes it possible, to protect themselves from the ultraviolet (UV) radiation and desiccation **(Panel on Contam in the Food Ch, 2011)**. *Alternaria* species can produce more than 70 secondary toxic metabolites but a few of them are structurally identified and called mycotoxin **(Siciliano et al., 2018; Wei et al., 2017; Zhao, Shao, Yang, & Li, 2014)**. Concerns about public health increased, when many of articles reported the presence of *Alternaria* mycotoxins in foodstuff **(Tralamazza, Piacentini, Iwase, & Rocha, 2018; Zwickel, Klaffke, Richards, & Rychlik, 2016)**. The major *Alternaria* mycotoxins have three structural classes, tenuazonic acid (TeA) derived from tetramic acid, alternariol (AOH) and alternariol monomethyl ether (AME) and altenuene (ALT) are the dibenzopyrone derivatives and also perylene derivatives the alter toxins (ATX-I, ATX-II,ATX-III ) **(da Cruz Cabral, Terminiello, Pinto, Nielsen, & Patriarca, 2016; Müller & Korn, 2013; Patriarca, Azcarate, Terminiello, & Pinto, 2007; Zwickel et al., 2016)**. Their acute toxicity is followed as ALT > TeA > AME and AOH. However, the study in this field is not enough and this data needs more research **(Panel on Contam in the Food Ch, 2011)**.

TeA (5S)-3-acetyl-5[(2S)-butan-2-yl]-4-hydroxy-1,5-dihydro-1H-pyrrol-2-one, (Fig. 1) is a toxic metabolite, which is produced by *Alternaria* spp., *Phoma sorghina* and *Pyricularia oryzae* **(Asam et al., 2013; Chen & Qiang, 2017; Liu, Ge, Peng, & Pan, 2017; Oliveira et al., 2017)**

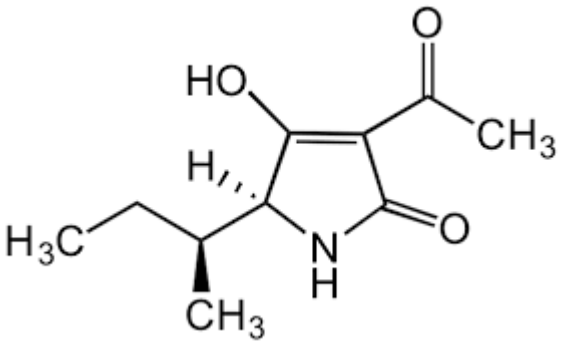



**Figure 1** Chemical structures of TeA

TeA was found in agricultural products such as olives, cotton (seeds and bolls), sunflower seeds, peppers, tobacco seeds, sorghum kernels, rice,wheat, barley and oats as well as some fruits including apples, tomatoes, blueberries, lemons, oranges and wine and beer **(Chen & Qiang, 2017; De Berardis et al., 2018; Walravens et al., 2014).** It is the cause of the human haematologic disorder called “onyalai” in central southern Africa **(Fan, Cao, Liu, & Wang, 2016)**. In addition, It has been suggested that in certain areas of China, the presence of Alternaria toxins in grains may cause oesophageal cancer **(Panel on Contam in the Food Ch, 2011; Prelle et al., 2013)** but due to the presence of other mycotoxins, so this conclusion needs more research **(Panel on Contam in the Food Ch, 2011)**. The oral $LD_{50}$ of TeA has been found to be 182 or 225 mg/kg body weight (BW) in male mice and 81 mg/kg body weight (BW) for female mice **(Asam et al., 2013)**. Also, in Macaca fascicularis this limitation is 100-150 mg/kg body weight (BW) **(Liu et al., 2017)**. TeA inhibits protein biosynthesis and is biologically active, exerting antitumor, antiviral and antibiotic activities, together with cytotoxic and phytotoxic properties **(Rychlik, Lepper, Weidner, & Asam, 2016; Siegel et al., 2010)**. The European Food Safety Authority

(EFSA) has set threshold of toxicological concern (TTC) value of 1.5 mg TeA/kg BW per day for TeA **(Panel on Contam in the Food Ch, 2011; Tralamazza et al., 2018)**. Many studies have already been done on mycotoxin in Iran and the detection of aflatoxin in foodstuff is one of the most commonly reported cases **(A. Cheraghali et al., 2007; Dini et al., 2013; Fallah, Jafari, Fallah, & Rahnama, 2009; Hashemi, 2016; Mazaheri, 2009; Nemati, Mehran, Hamed, & Masoud, 2010; Rahimi, Bonyadian, Rafei, & Kazemeini, 2010; Sani, Nikpooyan, & Moshiri, 2010)**. In addition, the most studies about Fruit Juices have focused on the detection of Patulin (PAT)**(A. M. Cheraghali et al., 2005; Khorasgani, Jalali, Hossieni, & Gudarzi, 2010).** A study in Argentina (2016) showed the presence of TeA in 57% of wine grapes **(Fontana, Prendes, Morata, & Bottini, 2016)**. After reporting this research, it seems necessary to do a similar study in Iran to compare the presence of TeA in Iran with other countries. For the first time, in this study, TeA was analyzed in fruit juice samples of Iranian market by HPLC-UV because of fruit Juices is one of the most important beverage industry in the world **(Asadpoor, Ansarin, & Nemati, 2014)**.

## MATERIAL AND METHODS

### Chemicals

Acetonitrile and methanol, both HPLC gradient grade were supplied by DUKSAN (Gyeonggi-do, South Korea). Formic acid (≥ 99%) and sodium phosphate monobasic ($NaH_2PO_4$) were purchased from Merck (Darmstadt, Germany) and also phosphoric acid was obtained from Kimia Tehran acid co (Tehran, Iran). TeA was procured from Cayman Chemical Company (Ann Arbor, Michigan, United States) and the standard solution was prepared with methanol. Deionized water was prepared using a Mili- Q System(Tehran Absaz co, Iran).

### HPLC conditions

The chromatographic system was a KNAUER HPLC instrument (Knauer, Berlin, Germany) consisting of a Detector S2500 Knauer equipped with a Biotech 2003 degasser (United State), K-1000 Knauer controller Quaternary pump and Rheodyne sample valve fitted with a 20 µl loop (United State). The analytical column was SCIEX AAA C18 column 150 × 4.6 mm, 5 µm (Foster City, USA). The mobile phase was prepared freshly every day by a mixture of MeOH: 0.1 M $NaH_2PO_4$ (2:1 v/v), adjusted to pH 3.2 with phosphoric acid. The eluent flow rate was 1.5 ml/min. The wavelength for recording chromatograms was 279 nm (Fontana et al., 2016).

### Samples

A total of 50 homogenized juice samples (40 Grape; 5 Apple; 5 Orange) that made by Iranian and non-Iranian (Thailand, Belgium, South Korea) companies also homemade samples were analyzed. Samples were purchased in March-April 2018 and were randomly selected to quantification of TeA from retail stores.

### Sample preparation

Sample preparation was preformed based on the reported method by Lopez and coworkers (38). The samples were shaken for homogeneity. Then, 2.5 mL of juice transferred to a 15 mL centrifuge tube and was mixed with 10 mL of acetonitrile/water/formic acid (84/16/1 V/ V/ V). The mixture was manually shaken for 5 min. After centrifugation at 4000 rpm for 5 min, an aliquot of 0.5 mL of the supernatant was taken and filtered. Subsequently, 20µL of the solution was injected directly into the HPLC-UV system.

### Method validation parameters

The HPLC-UV method for the determination of TeA in juices was validated for linearity, accuracy, precision. Calibration curve was prepared by spiking six concentrations (10, 50, 125, 250, 500,1000 µg/L) of TeA in a blank grape juice. The linearity was calculated using these six concentrations in triplicate also linearity requirements were fulfilled when the correlation coefficient was greater than 0.99. The calibration range included concentrations from the lower limit of quantification (LLOQ) to the upper limit of quantification (ULOQ). The LLOQ is defined as the lowest concentration of TeA can be determined with acceptable precision and accuracy as well as the highest amount of TeA that can be quantitatively determined with precision and accuracy is ULOQ **(Ershadi & Shayanfar, 2018; Kollipara, Bende, Agarwal, Varshney, & Paliwal, 2011)**. Recovery and precision were evaluated over three consecutive days at three nominal TeA concentrations (80, 200 and 400 µg/L) by spiking an uncontaminated matrix.

## RESULTS AND DISCUSSION

### Method validation

For the developed analysis methods, coefficients of determination ($R^2$) above 0.99 show an acceptable linear relationship between concentration and response. In this research, $R^2$ is obtained 0.999. The sensitivity parameters, LLOQ and ULOQ were 10µg/L and 1000µg/L respectively. The validity of method was checked by three different concentrations of TeA in fruit sample. Details of the method validation for the developed analysis method for quantification of TeA in fruit samples have been listed in Table 1. The relative standard deviation (RSD) was from 1.5 to 2.8 % for inter-day (n = 3) and 2.9 to 6.6 % for intra-days (n = 3, three days) analysis. The recovery of the developed method for quantification of TeA was between 96 to 108 %. The acceptable range for RSD is ≤ 20% and accuracy is 70%-120% **(Fontana et al., 2016)**.

**Table 1** Recovery and precision (as RSD) of the developed analysis method for quantification of TeA

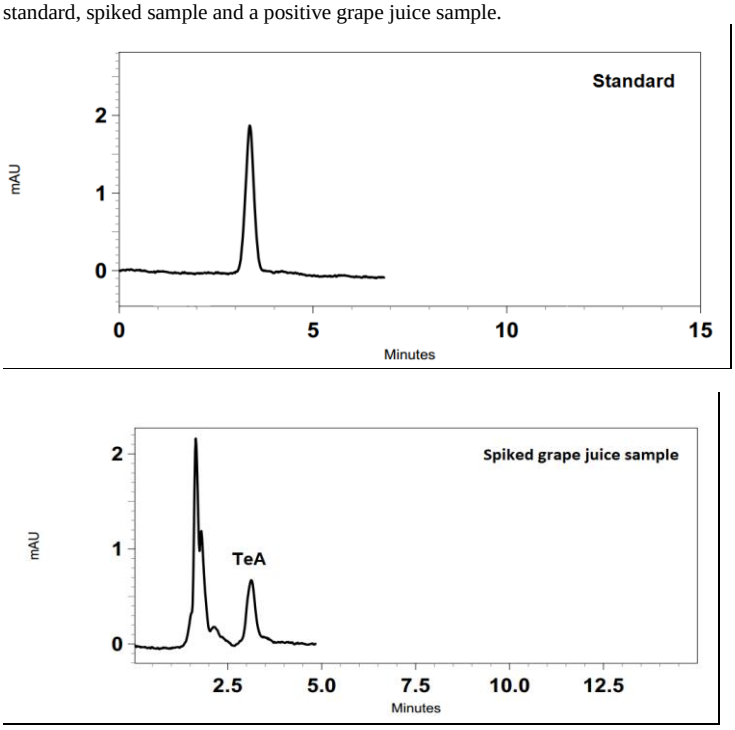
| TeA spiked ($\mu g. L^{-1}$) | TeA found ($\mu g. L^{-1}$) | Recovery (%) | RSD% Inter-day | RSD% Intra-day |
|---|---|---|---|---|
| 80 | 76.8 | 96% | 2.8 | 6.6 |
| 200 | 202 | 101% | 1.8 | 5.1 |
| 400 | 432 | 108% | 1.5 | 2.9 |

### Comparison with HPLC–MS

HPLC-UV and HPLC–MS are commonly used systems for TeA analysis. Each of these systems has advantages and disadvantages. However, HPLC–MS has more ability than HPLC-UV for detecting analytes but HPLC-UV was able to detect TeA sufficiently and had shown good efficiency. The reason for choosing HPLC-UV in this study is its low cost and high availability. A few studies have been conducted on the presence of tenuazonic in foodstuffs throughout the world. More studies should be done especially in developing and least developed countries. In this study, we have tried to use methods that allow researchers around the world to analyze TeA in fruit juices by low cost and fast.

**Survey of grape juice samples from the Iranian market**

The above validated method was finally evaluated on the real fruit juice samples. Fig. 2 shows the HPLC analysis of TeA in fruit juice samples including a standard, spiked sample and a positive grape juice sample.



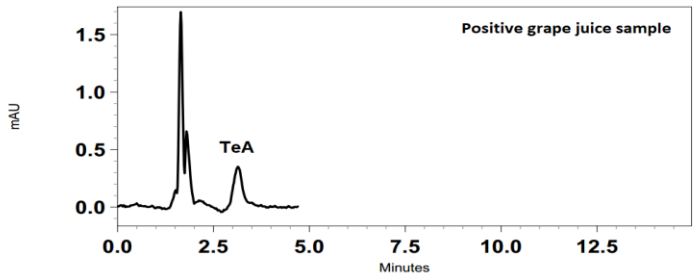



**Figure 2** Chromatogram of (a) TeA in aqeous solution, (b) Fruit samples spiked with TeA (c) and a real fruit samples contaminated with TeA (positive sample)

The results of TeA concentration in positive samples were reported in Table 2. TeA was detected in 14 grape juice samples, respectively. In this study, samples from 4 countries were used and the concentration range from 212 to 702 µg/L was reported. As a result, TeA was present in over 32.5% of the grape juice samples. On the other hand one sample of grape juice, which expired 9 months ago was tested and the highest amount of TeA was observed in it. The maximum and minimum TeA concentrations was 212_702 µg/L, respectively. However, TeA was not found in orange juice and apple juice samples.

**Comparison with results of other studies**

There are not many studies about the presence of TeA acid in foodstuff. In Table 3, the most relevant reported studies for TeA level in other research studies have been compared. In a study in Italy **(Prelle et al., 2013)**, TeA was detected in apple juice. Ten apple juice samples were analyzed and TeA was presented in two samples, but it was not found in Beers, Tomato products, Olive and Dried basil samples. In another study in Argentina **(Fontana et al., 2016)** on wine grapes, the presence of TeA in 57% of the samples was showed and the maximum contamination level of samples was 595 µg/g. A survey in the Netherlands **(López et al., 2016)** showed that all Dried figs, sunflower seeds and tomato sauces were contaminated with TeA, and also in three samples of wine and one olive sample, TeA was detected. There was no TeA in the fresh citrus or apple juice samples.

**Table 2** Occurrence of TeA in real grape Juice samples (TeA was detected in 32.5% of grape juice samples). Average concentrations (µg $L^{-1}$) with their standard deviations, n = 3 replicates

| Sample code | Level found |
|---|---|
| 1 | 234±7 |
| 2 | 353±3 |
| 3 | 597±2 |
| 4 | 375±4 |
| 5 | 608±6 |
| 6 | 702±11 |
| 7 | 334±6 |
| 8 | 358±3 |
| 9 | 393±3 |
| 10 | 289±3 |
| 11 | 428±2 |
| 12 | 212±4 |
| 13 | 254±3 |

An article from Germany about the occurrence of TeA in beers **(Siegel et al., 2010)** has been published that showed in 38 cases of 43 samples, TeA was detected and the highest average of contamination TeA was in bock beer. Another study from Germany **(Zwickel et al., 2016)** presented that all of red wines and 72% of white wines were contaminated with TeA and 62% of all juice samples containing TeA. The best result from the assessment of TeA was a Canadian survey **(Abramson, Delaquis, & Smith, 2007)**, which TeA was not found in any sample of ice-wine. This result may be due to the limit of detection of 70 µg/L. The results of a Chinese article **(Fan et al., 2016)**, indicated that TeA had been found in 6 samples of apple juice and one case of walnut wine. According to these results, TeA can occur in a wide range of foodstuffs at various levels.

**Table 3** Comparison of TeA presence in various samples with other studies

| Sample | NO. of samples | Occurrence | Range | Country | Ref. |
|---|---|---|---|---|---|
| Apple Juice | 10 | 20% | 45.3 - 24.3 (ng $g^{-1}$ ) | Italy | **(Prelle et al. 2013)** |
| Wine grape | 14 | 57% | 0.595 - 0.057 (µg $g^{-1}$) | Argentina | **(Fontana et al. 2016)** |
| Wine | 5 | 60% | 5.0 – 46 (mg $kg^{-1}$) | Netherlands | **(López et al. 2016b)** |
| Beer | 43 | 88% | 174.6 - 8.7 (µg $kg^{-1}$) | Germany | **(Siegel et al. 2010b)** |
| Citrus juice and wine | 103 | 62% juice<br>100% red wine<br>72% white wine | 1.10 - 60.0 (µg $L^{-1}$) | Germany | **(Zwickel et al. 2016)** |
| Ice wine | 26 | Not quantified | Not quantified | Canada | **(Abramson et al. 2007)** |
| Wine and apple juice | 27 | 8.3% wine<br>40% apple juice | 1.75-49.61 (µg $L^{-1}$) | China | **(Fan et al. 2016)** |
| Grape juices | 40 | 32.5 % grape juices | 212_702 (µg $L^{-1}$) | Iran | This work |

## CONCLUSION

The results of this study showed HPLC–UV method could applied successfully for the quantification of the TeA in fruit juices. The data from this survey illustrated that TeA occurs at high levels, up to a maximum of 702 µg/L in grape juices. The method features a LLOQ of 10 µg/L, good selectivity and a rapid sample preparation and analysis procedure.

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