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Original Article

Determination of T-2 Mycotoxin in *Fusarium* strains by HPLC with fluorescence detector

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Abstract

T-2 toxin is the most poisonous trichothecene produced by *Fusarium* species especially *F.sporotrichioides*. T-2 toxin is a biological contaminant in a number of agricultural commodities that can cause severe diseases among humans and animals and even lead to death. The aim of the current study is the analysis of T-2 mycotoxin in *Fusarium* species by high-performance liquid chromatography (HPLC) combined with florescence detection and derivatization with 1-antroylnitrile (1-AN). Totally, 11*Fusarium*isolates and reference strains were studied. The isolates were tested for the T-2 toxin production after growing on rice substrate followed by using specific "Multisep 225 Trich Clean up columns" purification. In this study, T-2 toxin production was ranged from 197.05ug/kg to 8503.07ug/kg. This is the first study of T-2 toxin analysis by HPLC-F in Iran.

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Introduction

Penicillium, *Aspergillus* and *Fusarium*species are three major mycotoxin producers in the world. Among fumonisins, zearalenone, and trichothecenes as important mycotoxins of *Fusarium* sp., trichothecenes are the most dispersed and the most diverse ones including 200 analogs [1-2]. Based on their chemical characteristics and origination, trichothecenes are classified into A, B, C, and D groups. T-2 toxin, a member of the A-group trichothecenes, is the most poisonous trichothecene (10 folds more poisonous than B-group trichothecenes like deoxynivalenol). Chemically, T-2 toxin is insoluble in water but soluble in acetone, ethyl acetate, chloroform, ethanol, methanol and propylene glycol. The toxin is stable in various environmental conditions especially autoclaving. To inactivate, the toxin should heat to 900 °F for 10 min or 500 °F for 30 min [1, 3-5].

Worldwide, food poisoning of livestock and human with various reported epidermises along with toxin based biological weapons are two major concerns in T-2 detection. Agricultural crop contaminations especially grains (e.g. wheat, maize, barley and etc.) have been repeatedly reported causing thousands of livestock and human deaths [6-12]. In the other hand, misusing toxins as biological weapons in the former Soviet Union (1974-1981) in Laeous, Kambuj and Afghanistan, Vietnam War and probably in Iran-Iraq war (1983-1984) show the great significance of the concern [3, 13]. Amongst, *F. sporotrichioides*, is the major source of T-2 toxin production. Some isolates including *F. armeniacum*, *F. equeiseti*, *F.musarum*and *F. poae* are able to produce T-2 too. Recently, *F. langsethiae* is introduced as an important isolate for T-2 production [14, 15].

Methodologically, T-2 analysis methods are divided into two classes; 1) screening methods such as Thin Layer Chromatography (TLC) & ELISA, and 2) analytical methods like Gas chromatography (GC) and high-performance liquid chromatography (HPLC) methods [3]. HPLC methods in some cases are combined with light detection methods like HPLC-RI (refractive index), UV

detection, and HPLC-F (fluorescence) detection systems to detect T-2 [16-18]. In this study, HPLC-F detection of T-2 toxin from *Fusarium* strains is studied.

Materials and Methods

Chemicals

The reagents were analytical grade of Merck Chemicals Co. Stock solution of T-2 was prepared by dissolving commercial T-2 (Sigma Aldrich) in acetonitrile in 1000 ppm concentration (1µg/µl) and stored at -20. Other solvent including methanol, chloroform, hexane, and etc. were from Merck, Germany Company.

Fungi and Culture preparation

Eleven standard strains, isolated from Iran, were studied (Table 1). A section of pure and proliferated colony on PDA medium were added to sterile rice substrate (50 mg of rice in 50 mL sterile distilled water) and was incubated (27±1 °C for 5 days). Then 3 weeks was incubated at 10±2 °C according to literature [12]. Then growth medium was transferred onto a sterile aluminum foil and then was oven-dried (45°C for 24-48 hours). The dry weight was powdered by electronic grinder. Afterward the extract was prepared and used for further analysis.

Extract preparation

The grinded powder was mixed well. To each 25 gr of the powder was added 100 mL of acetonitrile solution and water in 84:16. Then, for 12 hours was shacked vigorously in >210 rpm. The achieved extract was fined twice by 9cm No.120 paper and then whatman paper 12.5cm No.1. The fat of the extract was removed by hexane by adding 2:1 ratio of hexane to extract and shacked for 5-10 min. Subsequently, the upper phase which included fat was removed. The remained extract was used in rotary evaporator to dry it. Finally the powder was solved in 3mL of acetonitrile and water in 84:16 ratios.

Cleaning up

In order to cleaning up the extra contaminations of the extract, special clean up columns (Multisep 225 Trich, Romer Company, USA) were used. After cleaning up, the solution was divided into 3 microtubes and then dried under the slow flow of N2 gas. Afterwards, 0.5 mL of acetonitrile solvent and ethanol (3:1 ratio) was added and mixed. Solvent was vaporized again and 0.5 mL chloroform and methanol (9:1 ratio) was added, mixed and washed twice. Then 20 mL of solvent was used for rotary evaporator to concentrate it in 2 mL of dichloromethane. Then, the residue was dried under slow flow of N_2 gas [18, 19].

Derivatization and HPLC determination

4-dimethyl amino pyridine (DMAP) (Sigma Aldrich) and 1-anthroylnitrile (1-anthroylcyanide: 1-AN) (Wako, Japan) powders were dissolved in toluene in 0.325 µg/µl and µg/µl 0.3 concentration, respectively. The prepared solutions were preserved at -20. DMAP (50 µl) was added to the residue and then derivatization reagent 1-AN was added (50 µl) [17], mixed and heated to 50 °C for 15 min. After the residue was cooled on ice for 15 min and then was dried under N_2 slow circulation. Then residue was solved in 100 µl acetonitrile and water (80:20ratio). 5 µl of the achieved solution was used for HPLC analysis.

HPLC apparatus and analysis

The HPLC system (Waters, USA, No. 2695) was connected to fluorescence scanning system of 474 integrated with EM Power software. Reverse phase column was used for isolation and the analytical column 18 reverse (200×4.6 mm, 5µm particles) (Waters, USA) combined by a guard filter (0.5µm, Merck, Germany). The mobile phase (acetonitrile-water in 65:35 v/v) had the flow rate of 2.5 ml/min. Excitation and emission wavelengths of fluorescence were 381 nm and 470 nm, respectively.

Table 1. T-2 toxin production in standard strains and isolates of studied strains.

Fusarium Species	T-2 Production amount (ppb)	Recovery (%)
F. sporotrichioides(VTT)	798.17	
F. sporotrichioides (BBA)	8503.07	
F. sporotrichioides (MCR4333)	6370	
F. sporotrichioides (MCR0043)	513.24	
F. poae(MCR8485)	0	
F. poae (MCR8486)	197.05	
Fusariumcflangsethiae	5133.17	
F. tricinctum(isolated)	0	
F. oxysporum (isolated)	0	
F. camptoceras (isolated)	0	
F. compactum (isolated)	0	
Spike1 (100ppb)	80.41	80.4
Spike2 (3000ppb)	1921.5	64

After setting up HPLC apparatus with standard toxin, sample toxin was injected each step to monitor the constant and accuracy of the system. Quantitative analysis of the T-2 toxin in samples was performed by comparing standard and sample peaks. For standard curve, four concentrations of 10, 50, 100 and 500 ppm of standard stock and pure residue of samples were dissolved in acetonitrile. Injection to HPLC performed after derivatization with 1-AN dissolved in acetonitrile-water (80:20 v/v ratio). Derivatization reagents, DMAP and 1-AN, were injected to the system too.

Statistical analysis

Percent of recovery was estimated as accuracy index. Percent of recovery was prepared for two spiking samples. For two samples including 5 gm of rice were added 0.5 µg/gr of standard toxins 100bbp and 3000bbp, respectively. After one hour, extractions was collected and analyzed. Also, rice substrate was used as control sample for toxin analysis.

Results

In this study a fine peek of T-2 toxin indicating its presence underutilized analytical method was achieved. This peak was clearly defined and distinguishable from the other peaks (Fig. 1). In this study a good linear response in

T-2 toxin derivatives (in the range of 10 to 500 ppm) were also observed (Fig. 2). During the study, there was a significant linear peak on other studied strains, too. For instance, the graph of peak T-2 toxin, produced by *F. sporotrichioides* (BBA 10329), and atypical strains of *F. langsethiae* isolated from Iran [20] is shown in Fig. 3. Among the toxic strains, the highest T-2 toxin production (8503/70 ppb) and lowest (197/05 ppb) was related to*F. sporotrichioides* (BBA 10329) and *F. poae* (MCR8486), respectively (Table. 1).

In this study, *F. poae* (MCR8485), was not T-2 producing along with negative control strains; *F. tricinctum, F. oxysporum, F. camptoceras and F. compactum*.

Discussion

In the present study, different methods were evaluated, including the use of handheld two-step purification columns according to Lauren *et al*. [21], and using different solvents and substrates (corn instead of rice) at 15°C. Eventually realized that, using a clever combination of two methods [17, 18], the current protocol can be introduced as a new method of analysis.

Method of derivatives and wavelengths was according to Pascale *et al* [17]. Somewhat, extraction methods, type of solvents and fluorescence probe was based on the protocol of Mateo *et al* [18]. The type of filter used was different from two mentioned studies. The culture conditions and incubation of different strains was based on Mateo *et al*.

Results indicate that rice substrate functions better than maize. Mateo *et al* have been reported the highest and lowest rate of the type-A trichothecenes production in rice and maize, respectively [18]. However, it is likely that there is no difference in the rate of toxin production in both types of substrate. Based on reports, T-2 toxin in corn production can be more [22]. Even so, the filter column used in this study may not be able to do well on corn than rice. Therefore, corn pigments may have not been refined well in column and reduced efficiency of the T-2 toxin. Thus one of the possible reasons for the T-2 separation failure by Milanez and Valete-Soares on 8o corn samples could be in result of Mycosep columns [23]. Other reports also exist in using Mycosep TM # 225 purification Columns [24]. In the present study, it was better to use specific immune-affinity column purification of T-2. But because of unavailability of these columns, the MultisepTM # 225 columns were used, instead.

In the current study, in order to analyze T-2 toxin production by *Fusarium* sp. temperature of 10±2°C was used. The optimal temperature for T-2 toxin production in *F. sporotrichioides* was reported in low temperatures [18, 25].

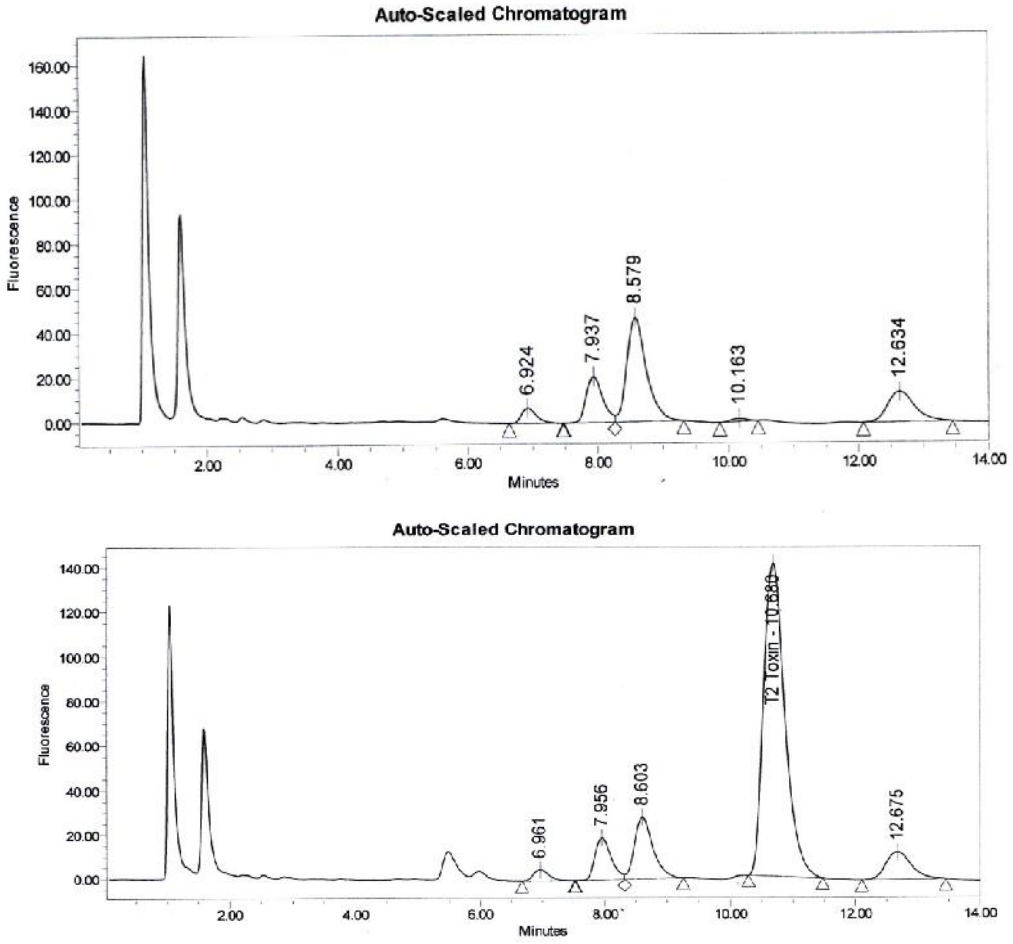

Figure 1. Chromatograms show mixture of derivatization reagents,
DMAP and 1-AN, as control reaction (up) and chromatogram of derivatization reaction for T-2 toxin 50 ppm (down).

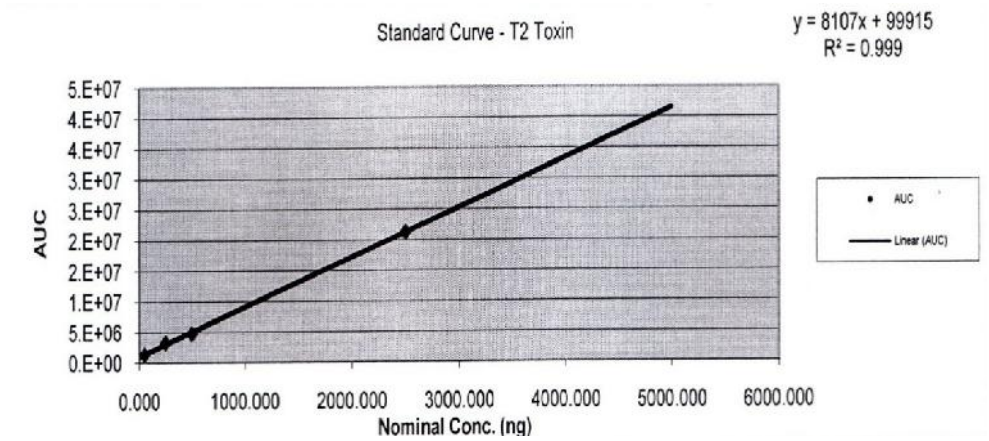

Figure 2. Standard curve of the T-2 toxin at 10-500 ppm concentrations.

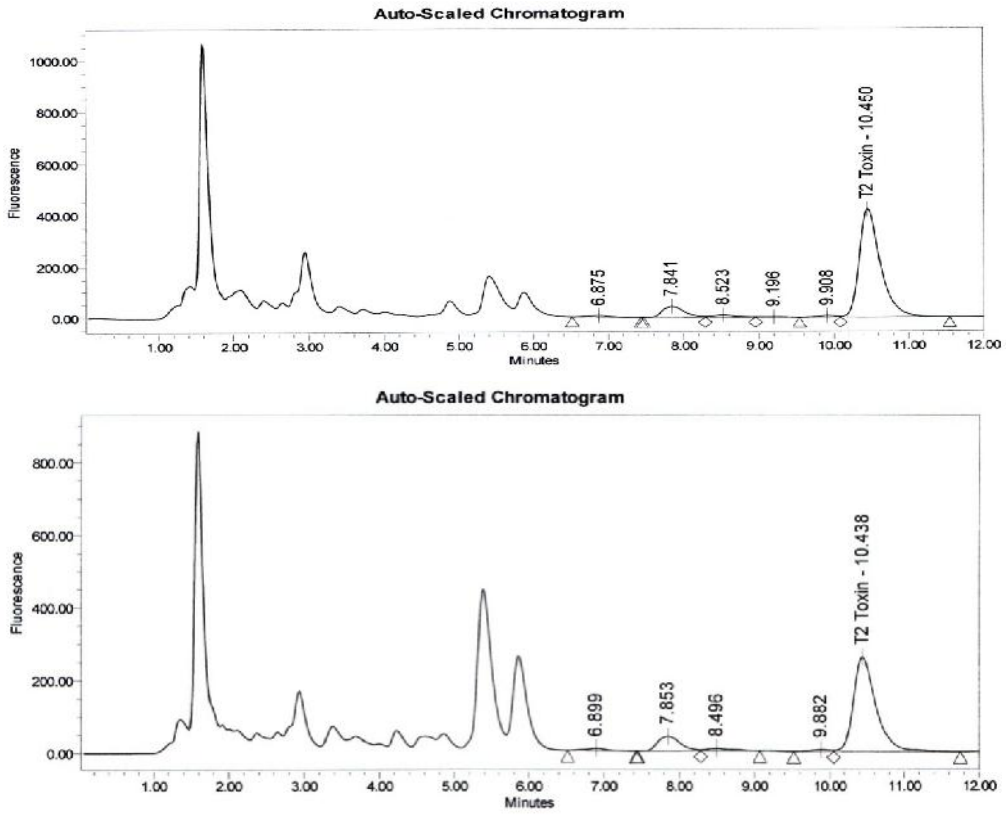

Figure 3. T-2 toxin production of standard *F. sporotrichioides* BBA 10329 (up) and an atypic isolate of *F. langsethiae* isolated from Iran (down).

Mateo *et al* in the study of temperature effect on type-A Trichothecenes of five *F. sporotrichioides* strains, had indicated the 20°C as optimal temperature against 26°C and 33°C degrees [18]. Also, T-2 toxin production is reported winter-passed grains (covered by snow during the winter) or deposited grains [26].For the first time, Pascale *et al* in 2003 used antroylnitrile (1-AN) as a derivative substance for the T-2 toxin derivatization. Subsequently, Visconti andLippolisetalin2005andin 2008also used this compound, which brought good results [17, 18, 27, 28].

T-2 Toxin, because of lacking active carbonyl group in the carbon-8, is not quantifiable via UV absorption and HPLC-UV detection system. So, there are many strong reports on HPLC-F measurement of T-2 toxin [17, 18, 27, 28]. Also for the first time in Iran, T-2 toxin was measured by this system. Previously, using GC-MS, relatively high levels of the T-2 toxin production (µg/gr 600-200) was reported (24). The highest production of T-2 toxin in rice also has been reported about 1ppm [18]. In this study, the T-2 toxin production by studied isolates, found to range from 197 ppb to 8.5 ppm.

Limited number of studies on grains shows that T-2 toxin production in Iran is below the allowed criteria. In studies on 23 samples of wheat-derived products from the shops, the T-2 toxin contamination was below the criteria [29]. Also by using ELISA kits, T-2 toxin in rice consumption, is reported lower than the limit (11.2±2.3 and 13±2.7 µg/kg) in Iran [30].

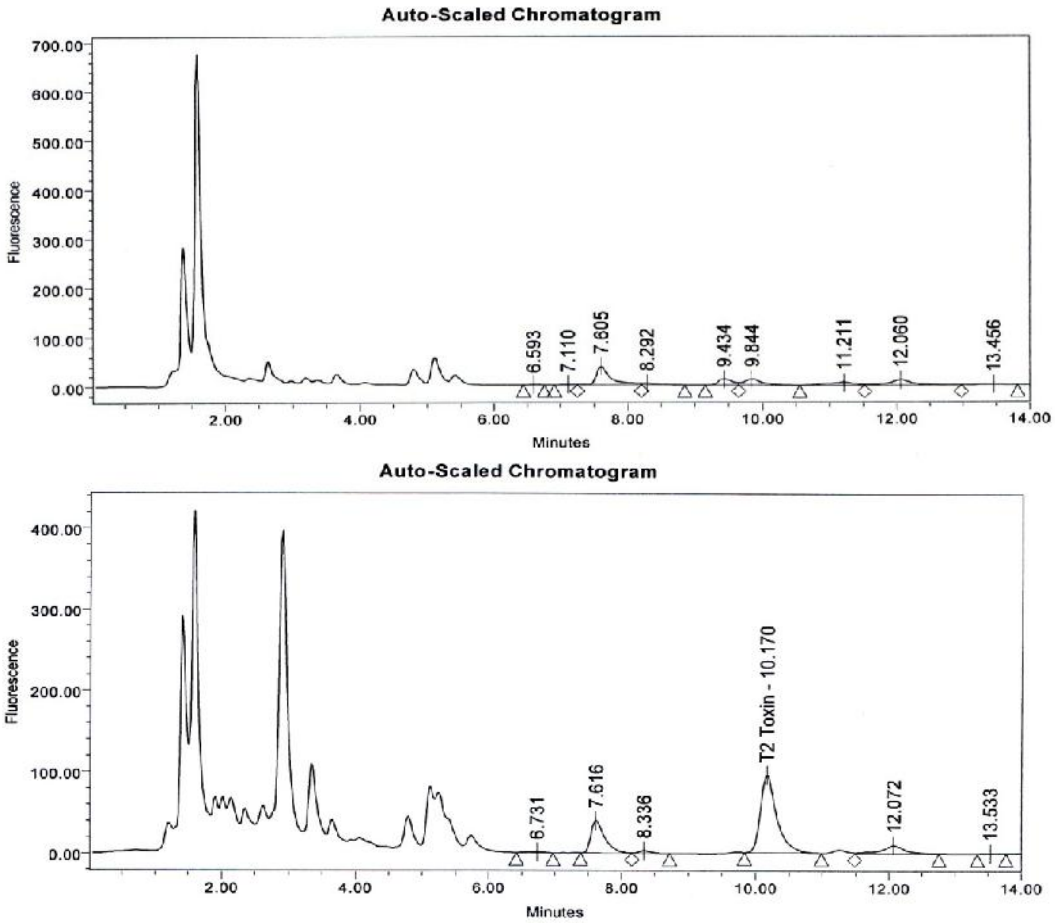

Figure 4. Chromatogram of the rice sample as control (up) and T-2 spiked sample in 3ppm (down).

New data show the common occurrence and breakout of T-2 toxin in cereals in Europe so that all recently isolated *F. langsethiae* strain was reported from Europe [31]. Also in these studies, T-2 toxin production in the atypical strain *F. langsethiae* which was isolated in previous studies of stored wheat is detected [20].The European Committee for Standardization (CEN) has been stated the acceptable T-2 toxin recovery percent of 60-110% for more than 0.25 µg/gr and 60-120% for lower than 0.25 µg/gr [32]. In our study, the recovery of both the concentrations used for each method meet the CEN standards.

Conclusion

T-2 toxin assay protocol using HPLC-F is introduced in this study. This method can be presented as a novel protocol different from the methods presented in literature. Today, T-2-specific immunoaffinity column purification is in the market, which was not available during this study. It's recommended to use these columns for futurestudies. Considering that the optimum temperature of T-2 toxin production is low and in the other hand, *F. sporotrichioides*is a psychrophilic fungus then seems that they are able to grow in environments with 8-20°C even for short periods. Therefore, we suggest storing the grains in higher temperature and low humidity environment. The authors would like to thank the Medicinal and agriculture division of Trabiat Modarres University.

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