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ORIGINAL ARTICLE

A Rapid Method with UPLC for the Determination of Fusaric Acid in *Fusarium* Strains and Commercial Food and Feed Products

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Abstract A rapid, sensitive and validated method for the determination of fusaric acid (FA) in several *Fusarium* strains and different commercial food and feed products is reported based on ultra-performance liquid chromatography. This method requires only crude sample by a simple extraction with methanol, and requires a very short time of 8 min for completion. Separation of FA was performed at injection volume of 1 µl with a 20:80 (v/v) water/acetonitrile mobile phase containing 0.1 % formic acid at a flow rate of 0.05 ml/min and detected with UV at 220 nm. Nice linearity and good correlation coefficient ($R^2 > 0.99$) were obtained in the concentration range of 1–200 µg/ml. Validation was demonstrated using blank samples spiked at three different concentrations with standard solution, and the method yielded more than 98.2 % recovery efficiencies and below 2.56 % R.S.D. when applied in the analysis of FA produced by *Fusarium verticillioides* and a set of transgenic strains of this fungus. Satisfactory recoveries in the range of 79.1–105.8 % and R.S.D lower than 10 % were also obtained for the tested commercial food and feed products. The concentration FA detection in the transgenic strains ranged from 9.65 to 135 µg/kg (0.29–4.05 µg per gram of biomass). However, FA was not detected in most of the commercial products with the exception of niblet, oatmeal, red kidney bean and soybean, for which the concentrations of FA ranged from 2.5 to 18 µg/kg (below the permitted maximum). These results show that the proposed method has a great potential application to analyze FA from different sources rapidly.

Keywords Mycotoxin · Fusaric acid (FA) · UPLC · Food and feed products · *Fusarium*

Introduction

Fusaric acid (5-butylpicolinic acid, FA) is a broad-spectrum mycotoxin produced by various *Fusarium* species, including *F. heterosporum, F. verticillioides* and *F. oxysporum* [1, 2]. This compound is thought to be directly related to the pathogenesis of vascular wilt, damping off, and root rot diseases of numerous vegetable crops [3–8]. In addition to the suggested role in plant pathogenesis, FA is also a potential health hazard as it easily contaminates agricultural commodities [9–11]. Consumption of FA-contaminated feedstuffs and food may cause severe disorders in animals and humans, including impairment in nerve [12–14], cardiovascular [15] and immune systems [16, 17], as well as some mammalian tumor cell lines [16]. Even though FA exhibits low acute toxicity in animals, synergism with certain mycotoxins combined with FA can enhance toxicity [13, 18].

Several methods are in use for the detection of FA in agricultural commodities and cell culture, involving high-performance liquid chromatography (HPLC) coupled with UV or ESI–MS/MS [19–21]. In addition, GC–MS methods and several qualitative methods such as the use of luminescent bacterium assay have been developed [22]. However, these methods involving HPLC in FA analysis require

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at least 20 min for completion [21], and it is also less sensitive than UPLC (requires about 20 µl injection volume while UPLC only needs 1 µl). The GC–MS method has almost never been used since it was reported in 1995, and the major disadvantage is its complex extraction and analysis process [14]. What's more, FA at a low level usually cannot be detected, because of poor recovery during sample treatment or a relatively low instrumental sensitivity. Therefore, it is necessary to develop an efficient, simple and reproducible method for detection of FA produced by the pathogen. More recently, the introduction of UPLC has attracted interest in the determination of mycotoxins in foodstuffs as it exhibits advantages of fast analysis, greater resolution, higher peak capacity, and sensitivity compared to HPLC. Using a proper solid stationary phase, UPLC can analyze multiple samples in a shorter time period compared to HPLC and has employed in determination of a variety of mycotoxins, including aflatoxins [23], ochratoxin [24] and deoxynivalenol [25]. We devised and optimised an UPLC protocol for the determination of FA in cell cultures and commercial food and feed stocks.

Materials and Methods

Reagents, Strains and Culture Conditions

FA standard substance (purity 99.4 %) was purchased from Sigma-Aldrich (St. Louis, MO, USA) in 1-mg package. Analytic-grade methanol (MeOH) for FA extraction was supplied by Sangon Biotech Co., Ltd (Shanghai, China). UPLC-grade acetonitrile (MeCN) and trifluoroacetic acid (TFA) for mobile phase were bought from Sigma-Aldrich (St. Louis, MO, USA). UPLC-grade water was obtained from a Milli-Q Gradient water system (Bedford, MA, USA). FA standard stock solution (1 mg/ml) was prepared by dissolving accurately weighed portion of the standard in MeOH. Then, the standard stock solution was further diluted by MeOH to obtain the standard working solution (50 µg/ml). For calibration curve, the standard stock solution was sequentially diluted with MeOH to prepare a series of standard solutions with concentrations of 1, 10, 20, 50, 100, 150 and 200 µg/ml. All of the prepared standard solutions were stored in brown vials at 4 °C. Commercial food and feed including peanut, two rice samples (sticky and black rice), three maize samples (maize, corn flakes and niblet), two wheat samples (oatmeal and millfeed), and seven bean samples (red kidney bean, red bean, pinto bean, black soybean, white bean, mung bean and soybean) were purchased from a local supermarket in Fuzhou (China).

Fusarium verticillioides A0149 (FGSC number 7600), the wild-type strain, was kindly donated by Shandong University. Seven transgenic strains of *F. verticillioides* A0149 were obtained in our laboratory by random insertions of plasmid PII99 into the wide type. The wild-type strain was maintained on potato dextrose agar (PDA) plates containing 50 % (v/v) sea water, and the transgenic strains were maintained on the same plates adding with 50 µg/ml geneticin.

Fusaric Acid Extraction

To extract FA from *F. verticillioides*, the solid cultures were chopped into small pieces and then soaked in MeOH three times under continuous ultrasonication for 4 h. The supernatants were centrifuged at 6000 rpm for 5 min and stored at 4 °C for UPLC analysis. The extraction of FA from fifteen agricultural products was conducted based on the method described by Noser [26] with some modifications (Fig. 1). In brief, all samples were first homogenised with a Retsch Grindomix GM 200 (Jin Tan Tong electronic, LTD, China). Then an aliquot of 20 g sample was weighed and transferred to centrifuge tubes (100 ml). Samples were extracted by soaking with 25 ml MeOH under continuous ultrasonication. After 4 h, another 25 ml MeOH was added and the samples were kept to be extracted overnight. Afterwards, the mixture was centrifuged at 6000 rpm for 5 min, and the supernatant was transferred into an intubation tube for UPLC–MS/MS analysis. In all cases, the extracts were filtered through a 0.22 µm syringe filter before using for analysis.

UPLC Analyses

A waters acquity UPLC system (Waters, Millford, MA, USA) consisting of a vacuum degasser, an auto-sampler and a binary pump, a BEH reverse-phase C18 column (10 cm × 2.1 mm, 1.7 µm) were used for the analysis of FA. The column temperature was maintained at 25 °C with the detection wavelength set at 220 nm [27]. The mobile phase "A" was water/TFA (1000:1, v/v) and the phase "B" was acetonitrile containing TFA (1000:1, v/v). To optimize the UPLC condition to obtain the best chromatogram with the lowest noise over signal ratio and shorter running time, the following UPLC conditions were experimented: (1) Mobile phase composition (A to B) varied at 10:90, 20:80, and 30:70 were tested with injection volumes and flow rate kept constant at 1 µl and 0.05 ml/min, respectively. (2) Flow rate was varied (0.02, 0.05 and 0.07 ml/min) with injection volumes and mobile phase composition maintained at 1 µl and 20:80, respectively.(3) different injection volumes (1, 2 and 3 µl)was performed keeping the composition of mobile phase 20:80 and flow rate of 0.05 ml/min fixed. The identification and quantification of FA were performed by comparing the retention

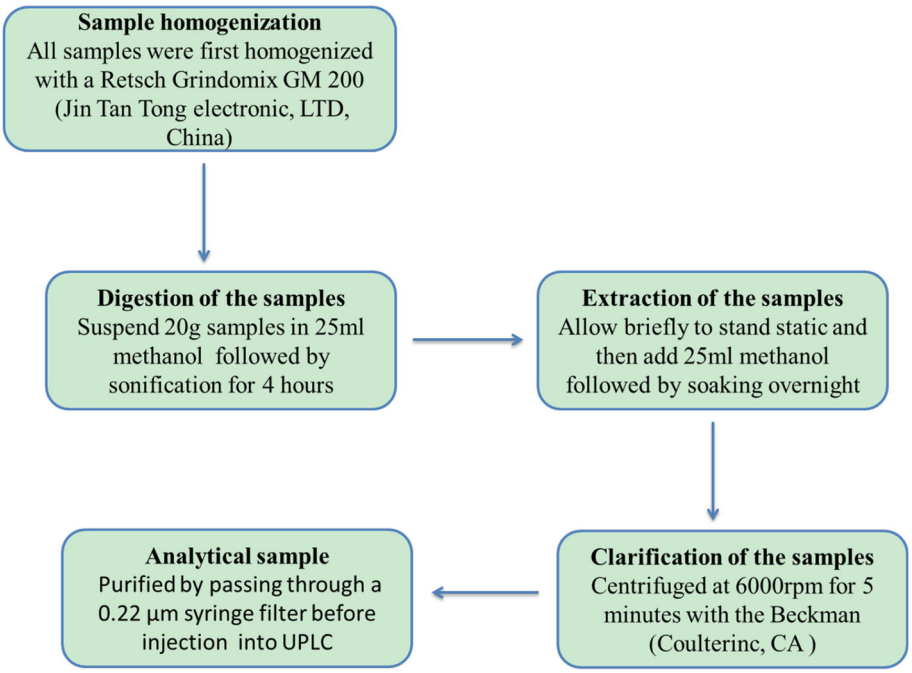

Fig. 1 The protocol for FA extraction in commercial food and feed products

times and peak areas of tested samples with the calibration curve prepared with authentic standard.

Determination of FA in Different Strains of *Fusarium verticillioides* and in Fifteen Food and Feed Products

The optimised protocol was applied to FA analysis in cell cultures of *F. verticillioides* and in fifteen food and feed products purchased from the local market in order to investigate its practical applicability. The wild type strain of *F. verticillioides* and seven transgenic strains (named M1–M7) were cultured on PDA plates containing 50 % sea water (containing both 50 µg/ml geneticin for transgenic strains) at 28 °C for 9 days. Afterward, the strains were scraped out from the surface of the medium and the solid medium was extracted with MeoH for UPLC analysis. Determination of FA in food and feed products was conducted based on the modified protocol as mentioned above.

Accuracy and Precision

Intra- and inter-day variations were chosen to determine the accuracy and precision of the developed assay according to recovery experiments by spiking blank samples at three different concentrations as follows. Solutions with low (10 µg/ml), middle (100 µg/ml) and high (200 µg/ml) concentrations of the calibration curve were prepared by spiking cell cultures of wild type strain of *F. verticillioides* or extracts of niblet, oatmeal, red kidney bean and soybean with appropriately diluted standard solution of FA (1 mg/ml). The relative standard deviation (R.S.D.) was taken as a measure of precision and the accuracy was calculated based on the ratio of FA amount measured actually. For intra-day precision, each low, middle, and high quality control sample was analyzed thrice within 1 day while for inter-day test, this experiment was performed once each day, continuously 3 days.

Results

Optimization of UPLC Conditions

Effect of Mobile Phase Composition

To optimize UPLC condition for FA determination, a set of gradient elution were designed. The effect of differing mobile phase compositions (mobile phase A to B at 10:90, 20:80, and 30:70 v/v levels) with an injection volumes of 1 µl and flow rate of 0.05 ml/min (with column pressure of 4400 psi) was shown in Fig. 2. Based on the results, we can see that the 20:80 of mobile phase A to B has the minimum retention time about 6.61 min and the narrowest peak width of 14.8 s at half height in comparison to the other two ratios of mobile phase A and B tested.

Effect of Mobile Phase Flow Rate

Different flow rates were evaluated in terms of retention time, peak shape and peak width at half height (Fig. 2).

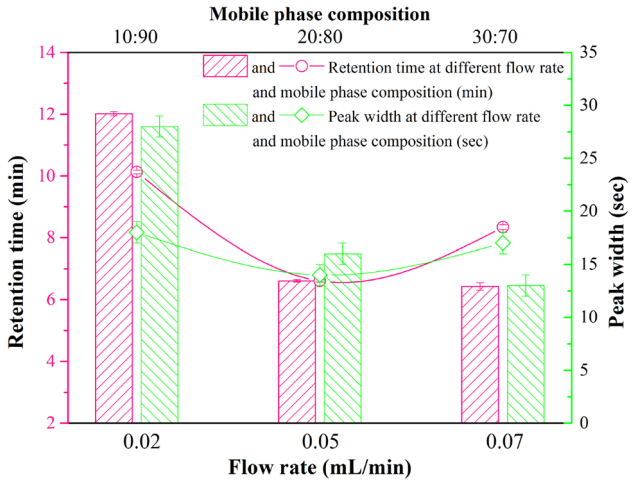

Fig. 2 Effects of mobile phase composition (water: acetonitrile, *line*) and flow rate (*column*) on retention time and peak width at half height

There was no significant difference in retention time, which was at 6.61 min and 6.43 min for 0.05 ml/min flow (column pressure of 4400 psi) and 0.07 ml/min flow (column pressure of 4842 psi), respectively. Good peak symmetry with narrow peaks (16.8 s and 12.3 s respectively) was also achieved. However, an increase in the retention time to 12.21 min with 0.02 ml/min flow (column pressure of 3986 psi), and a broad peak width of 28.8 s were observed under this condition. Figure 3 shows that the flow rate at 0.05 ml/min was better than that at 0.07 ml/min because it had a satisfactory peak shape without tailing.

Effect of Injection Volume

Tests with different injection volumes (1, 2 and 3 µl) indicated that the 1 µl injection yielded the lowest noise signal in the chromatograms and allowed the entire running time to be reduced to 8.0 min (data not shown).

Calibration Curve (Linearity)

The method was validated for linearity, and the calibration curve ($Y = 44681x-99453$) with R^2 value of 0.9992 was obtained. The high correlation coefficient value indicated good linearity between investigated compound concentrations and their peak areas in the studied range of 1–200 µg/ml.

Fast Quantitative Determination of FA in *Fusarium* Strains

This protocol was applied for the analysis of FA in some transgenic strains of *F. verticillioides*. Eight samples were routinely injected and analyzed. The column pressure during the elution was around 4400 psi without big fluctuations. The UPLC chromatographic resolutions of 8 samples are all in 8 min (Fig. 4). The concentration of FA in each sample was calculated by converting the peak area to its molar concentration using the calibration curve, and for per kg of agar culture, the yield of FA ranged from 9.65 to 135 µg. The data could be converted to 0.29–4.05 µg per gram of biomass since an average of 1.5 g mycelium (1/30 time of agar culture, wet weight) was produced on each plate after 9-days cultivation (Fig. 5).

Survey of FA Contaminated Commercial Food and Feed Products

The established method was also used to examine 15 commercial food and feed products collected from the local market. The analytical results of FA in these samples are summarized in Fig. 6. The concentration of FA in each sample was calculated by converting the peak area to its molar concentration according to the calibration curve. FA was detected in 4 out of 15 samples. The highest content of

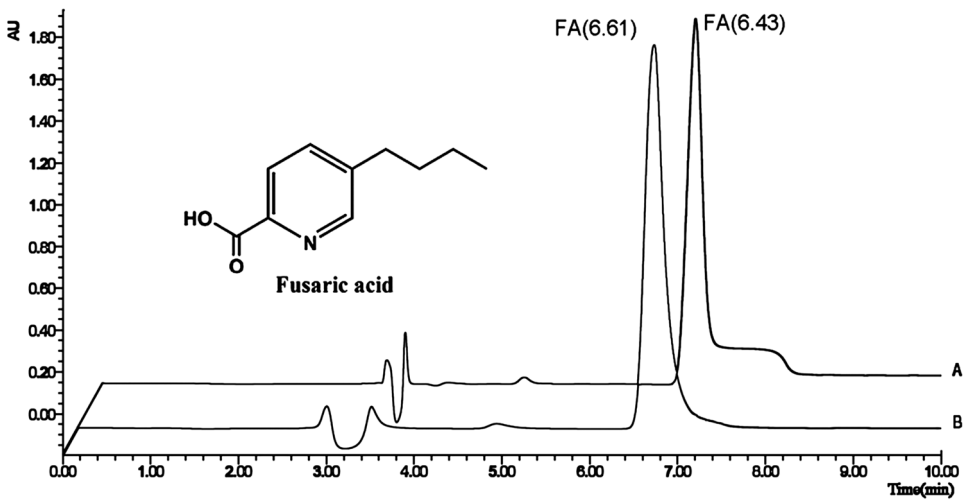

Fig. 3 Chemical structure of FA and different UPLC chromatograms at 0.07 ml/min (*A*) and 0.05 ml/min flow (*B*)

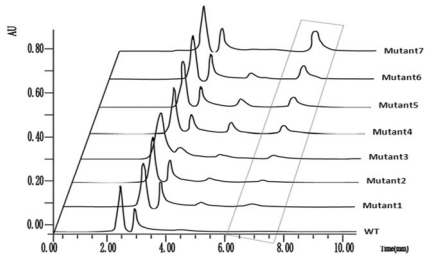

Fig. 4 Chromatograms of the wild type strain of *F. verticillioides* (WT) and its transgenic strains. Transgenic strains of *F. verticillioides* 711 were obtained by random insertions of plasmid PII99 to the wild type (WT) strain

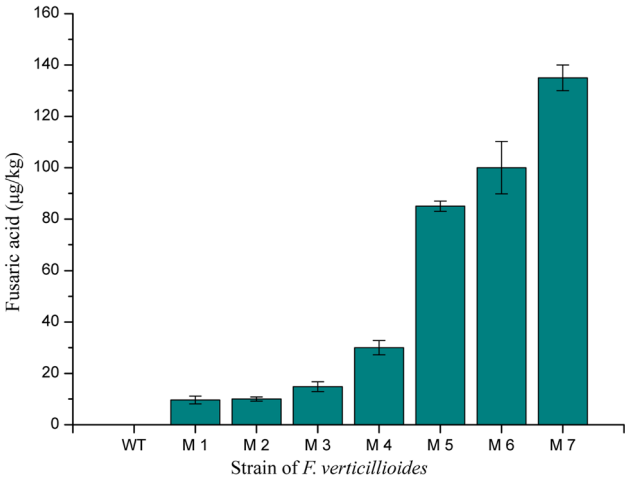

Fig. 5 Concentrations of FA detected in the wild type strain (WT) and transgenic strains (M1–M7) of *F. verticillioides*

FA was determined in a red kidney bean sample (18 μg/kg) and the lowest content of FA was observed in a niblet sample (2.5 μg/kg). The column pressure during the elution was around 4400 psi without big fluctuations.

Precision and Accuracy

The wild type strain of *F. verticillioides* and four commercial products based on analytical results (Fig. 6) were used as blank sample, and recoveries and precision were determined by spiking these blank samples with standard solution in duplicate at the spiking levels indicated above. For the cell culture of *F. verticillioides* strains, the results obtained were satisfactory both in inter-day and intra-day tests. Recovery values varied from 98.2 to 99.6 % demonstrating that there was no interference from endogenous components of cell culture in the procedure used. The RSD ratios of intra- and inter-day studies ranged from 1.21 to 2.48 % indicating that the proposed method was highly precise (Table 1). Satisfactory results were also observed in niblet, oatmeal, red kidney bean and soybean. The average recovery rates were between 70 and 120 %

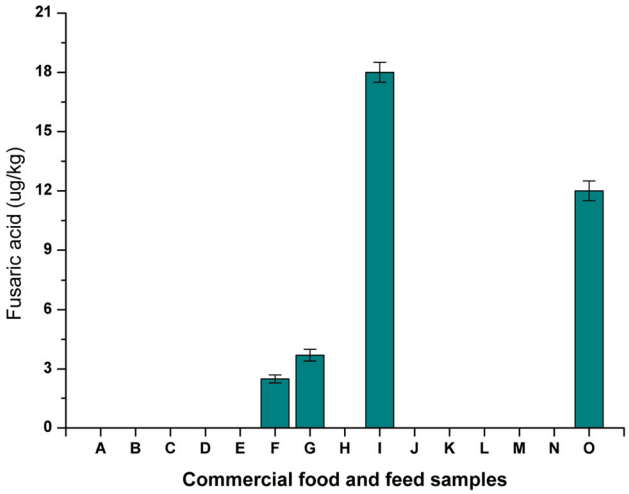

Fig. 6 FA levels in the analyzed samples purchased from the China market. *A–O* were sticky rice, maize, black rice, peanut, corn flakes, niblet, oatmeal, millfeed, red kidney bean, red bean, pinto beans, black soybean, white bean, mung bean and soybean, respectively

and precisions (R.S.D., %) were less than 10 % (Table 2), which meet the regulations of European Food Safety Authority (EFSA) [28].

Discussion

This study has established a useful method using UPLC to simultaneously analyze FA in fungal culture and some food and feed commodities. For the optimization of UPLC, the best chromatograms for FA with the lowest noise were obtained using a mobile phase consist of water and acetonitrile (both containing 0.1 % TFA) of 20–80 volume ratio at a flow rate of 0.05 ml/min with injection volume of 1 μl. Under the designated conditions, FA got eluted at 6.61 min, and all analytes were completely resolved within 8 min, which was much shorter than currently used methods. The method was found to be linear over an analytical range of 1–200 μg/ml with high correlation coefficients ($Y = 44681X-99453$, $R^2 = 0.9992$). It was first achieved in application in analysis of cell cultures of *Fusarium* strains, and perfect results were also observed when it was applied to analyse FA in food and feed products. The method was validated and reasonable recoveries in *Fusarium* strains (ranged from 98.2 to 99.6 %) and in food and feed products (ranged from 81.2 to 105.8 %) were achieved. Satisfactory precisions (R.S.D., %) in *Fusarium* strains and food and feed products less than 3.0 % and 10 % respectively were also obtained. Although there has not been a commission recommendation on the maximum level of FA from authoritative organizations, we find that the FA contents in all the commercial food and feed samples are below to that of the permitted maximum of T-2

Table 1 The results of inter-day (n = 3) and intra-day (n = 3) precision and accuracy in the wild type strain of *F. verticillioides*

Nominal concentration (μg/ml)	Mean concentration found[a] (μg/ml)	Mean accuracy[b] (%)	Precision (R.S.D., %)	Confidence interval (CI)
Inter-day (n = 3)				
10	9.96	99.6	1.21	10 ± 0.14
100	98.42	98.4	2.56	100 ± 3.34
200	198.23	99.1	1.17	200 ± 4.12
Intra-day (n = 3)				
10	9.95	99.5	1.84	10 ± 0.18
100	98.17	98.2	2.48	100 ± 2.13
200	198.01	99.0	1.26	200 ± 3.42

[a] Mean value of three determinations at three concentration levels for inter-day and intra-day respectively

[b] All the mean accuracies were calculated against their nominal concentrations

Table 2 The results of inter-day (n = 3) and intra-day (n = 3) precision and accuracy in commercial food and feed products

Samples	Mean accuracy (%)	Precision (R.S.D., %, Intra-day) (n = 3)	Precision (R.S.D., %, Inter-day) (n = 3)
Niblet	81.2	8.3	8.7
Oatmeal	79.1	6.4	6.9
Red kidney bean	90.3	7.5	8.6
Soybean	105.8	4.2	6.1

toxin, another fusarium-toxin showing stronger toxicity than FA, established by the European Food Safety Authority (EFSA) in the commission recommendation 2013/165/EU [29]. It means that all the tested food and feed products from market in Fuzhou are safe. In conclusion, the method may serve as a reference for developing analytical standards on FA by regulatory agencies conveniently in a short time following simple pretreatment without any kind of pre-concentration.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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