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Technical note

Fumonisin-*ortho*-phthalaldehyde derivative is stabilized at low temperature

Lonnie D. Williams [a,b], Filmore I. Meredith [b], Ronald T. Riley [a,b,*]

[a] *College of Agriculture and Environmental Sciences, University of Georgia, Athens, GA, USA*
[b] *Toxicology and Mycotoxin Research Unit, USDA, ARS, RRC, 950 College Station Road, Athens, GA 30605, USA*

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Abstract

Fumonisins are water soluble mycotoxins produced by the fungus *Fusarium verticillioides* (formerly *F. moniliforme*). Fumonisin B_1 (FB_1) is a diester of propane-1,2,3-tricarboxylic acid and 2-amino-12, 16-dimethyl-3,5,10,14,15-pentahydroxyeicosane, and is the most abundant of the naturally occurring fumonisins. Upon removal of the two tricarballylic acid side chains, the structure is referred to as hydrolyzed FB_1 (HFB_1). FB_1 and HFB_1 are structurally similar to sphinganine, a sphingoid base. The fumonisins do not absorb UV light or fluoresce; therefore, derivatizing reagents are used for detection when separation is by high performance liquid chromatography (HPLC). The standard derivatizing reagent used for HPLC is *ortho*-phthalaldehyde (OPA) plus 2-mercaptoethanol (ME) reaction partner, however, the OPA-FB_1 derivative is not stable at room temperature. The objectives of this study were to: (1) determine the effect of temperature on the stability of the OPA-FB_1 derivative and (2) determine which structural characteristics of FB_1 contribute to the instability of the OPA-FB_1 derivative. The results indicate that OPA-FB_1, OPA-FB_3 and OPA-HFB_1 derivatives are unstable at 24 °C but that their stability improves significantly at 4 °C. The OPA-sphinganine derivative is stable for at least 24 h at 24 °C. Thus, the instability of the OPA-FB_1 derivative may be attributed to its lack of a hydroxyl group at the carbon 1 position.

Keywords: Derivatization, LC; Fumonisin

1. Introduction

Fumonisins are mycotoxins produced by the fungus *Fusarium verticillioides* (formerly *F. moniliforme*). At present, at least 28 different fumonisins have been reported, although some of them do not occur naturally [1]. Fumonisin B_1 (FB_1) (Fig. 1) is the most abundant of the naturally occurring fumonisins [1]. The pure substances are amphipathic zwitterions, which are water soluble, heat, and light stable [2–4].

Fumonisins are areas of concern for corn producers, processors, consumers, and regulators. The occurrence and contamination of corn is worldwide and can have health effects in animals and possibly humans [5]. There are currently several methods used to measure fumonisins in various matrices [6]. One of the most common methods for quantitative analysis in corn is solvent extraction, solid-phase clean-up and *ortho*-phthalaldehyde (OPA) plus 2-mercaptoethanol (ME) derivatization followed by high performance liquid chromatography (HPLC) separation and quantification of the fluorescent OPA-FB_1 derivative [7]. The HPLC method for the OPA-FB_1 derivative is accepted as an official method for the analysis of corn by the Association of Official Analytical Chemists International [8]. One problem with the method is that the fluorescent intensity of the OPA-FB_1 derivative decreases rapidly after derivatization. It has been reported [7,9] that the OPA-FB_1 derivative is stable at room temperature for a period of 4 min after preparation, however, after 8 min a 5% decrease was seen and after 64 min a 52% decrease was seen. At the time of this report, the only solution to this problem was to inject the derivatized samples within 4 min of preparation, which some believe limits the use of auto sampling for maximizing throughput [10]. Improved stability has been attained using reaction partners other than 2-mercaptoethanol [11]. However, even the most stable OPA-FB derivatives that are suitable for

Abbreviations: FB_1, Fumonisin B_1; OPA, *ortho*-Phthalaldehyde

* Corresponding author. Tel.: +1-706-546-3377; fax: +1-706-546-3116.
E-mail address: rriley@saa.ars.usda.gov (R.T. Riley).

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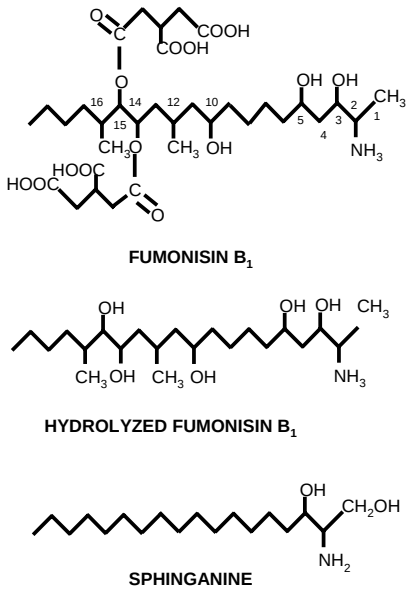

Fig. 1. Chemical structure of FB_1, HFB_1, and sphinganine.

HPLC analysis showed 41 to 67% decay of fluorescence after 2.5 h [11]. Thus, the time between mixing the OPA and the FB_1 and injection on the column must be carefully controlled or the quantification can be quite variable.

The structure of FB_1 consists of a long hydrocarbon chain (20 carbons), with methyl groups on carbons-1 and 12, an amino group on carbon-2, hydroxyl groups on carbons-3,5, and 10, as well as tricarballylic acid side chains on carbons 14 and 15. Fumonisins are sometimes referred to as "Sphinganine Analog Mycotoxins" (SAMs). Sphinganine is a sphingoid base which serves as a precursor to complex sphingolipids [12]. Sphinganine is structurally similar to FB_1, it has an 18 carbon chain, and like FB_1 it has an amino group on carbon-2 and a hydroxyl group on carbon-3. Sphinganine differs from FB_1 in that it has a hydroxyl group on carbon-1. Hydrolyzed FB_1 (HFB_1), refers to FB_1 that lacks the tricarballylic acid side chains at carbons-14 and 15, which are replaced by hydroxyl groups [13] (Fig. 1). To determine which chemical characteristics of FB_1 contribute to the instability of the OPA-FB_1 derivative, the stability of OPA derivatized FB_1 was compared to the stability of OPA derivatized sphinganine and OPA derivatized HFB_1. The present study was conducted to determine if reduced temperature increases the stability of the OPA-FB_1 derivative and to determine which chemical characteristics of FB_1 contribute to the instability of the OPA-FB_1 derivative.

2. Experimental

2.1. HPLC method

The analytical standard of FB_1 was prepared by the method of Meredith et al. [14] and the purity (>96%) was determined by the procedure of Plattner and Branham [15]. The HFB_1 was prepared by the method of Poling and Plattner [16] and mass spectral data were used to verify the purity of FB_1 and HFB_1 standards [14]. FB_3 was a gift from Ronald Plattner (USDA-ARS Peoria, IL). The sphinganine was purchased from the Sigma (St. Louis, MO). Samples of FB_1 and HFB_1 standard (200 ng/100 μl H_2O) were combined with 500 μl OPA derivatizing reagent (Sigma, St. Louis, MO) and 500 μl of acetonitrile:water (1:1). The OPA reagent (utilizing ME as the reaction partner) was prepared by the method of Riley et al. [17]. Samples of sphinganine standard (18 pg/60 μl H_2O) were combined with 50 μl OPA derivatizing reagent and 490 μl of methanol:water (80:20). Samples of FB_1 and HFB_1 as well as reagents were maintained at constant temperature and mixing and derivatization was accomplished using a Shimadzu model SIL-9A programmable auto-injector. Sphinganine samples were manually mixed and derivatized for 120 min at room temperature to allow for maximal fluorescence [17]. The derivatized samples (50 μl) were injected at various times after mixing with the OPA reagent, and separation was accomplished using a Microsorb^{TM} C18 column (3 μm particle size, 4.6 mm ID × 5 cm L Rainin Instrument Company, Woburn, MA), maintained at 27 °C with a mobile phase for FB_1 and HFB_1 of methanol:1% phosphoric acid in water (66:34) and for sphinganine the mobile phase was methanol:water (85:15) and the flow rate was 0.8 ml/min. OPA-positive substances were detected using a Shimadzu RF-551 spectrofluorometric detector at 335 nm excitation and emission cutoff filter at 440 nm. A complete description of the HPLC system can be found in Riley et al. [17].

To determine the stability of the OPA-FB_1 derivative, a time-course experiment was conducted with samples derivatized as previously described and placed into the auto injector at 4 and 24 °C and analyzed by HPLC at 45 min, 1, 2, 3, 4, 9, 13, 24, and 48 h after derivatization. To determine the statistical significance and verify the finding of the time-course experiment, samples were analyzed in triplicate at 5 min and 24 h after derivatization at 4 and 24 °C. The stability of the OPA-FB_1 derivative was also determined at 4, 10, 24, and 37 °C, and the percent change in fluorescence were compared to determine the effect of temperature on the stability of the OPA-FB_1 derivative. To compare the stability of the OPA-FB_1 and OPA-HFB_1 derivatives, samples derivatized as previously described were analyzed by HPLC 5 min and 24 h after derivatization at 24 °C. Sphinganine samples derivatized as previously described were analyzed by HPLC 125 min and 24 h after derivatization at 24 °C and compared to the OPA derivatized fumonisin samples.

2.2. Statistical analysis

Statistical analysis was done using Sigma Stat software (Jandel Scientific, San Rafael, CA). One way analysis of variance (ANOVA) was used followed by tests for post hoc multiple comparisons where appropriate. All data were ex-

pressed as mean ± S.D., and differences among means were considered significant if the probability (P) was <0.05. For the time course study the data were analyzed by non-linear regression analysis and ANOVA.

3. Results and discussion

The emitted fluorescence of the OPA-FB_1 derivative decreased linearly with time and increasing temperature. After 48 h at 4 and 24 °C the decrease in fluorescence of the OPA-FB_1 derivative was 40 and 90%, respectively (Fig. 2A). Triplicate samples analyzed at 5 min and 24 h showed that the change in emitted fluorescence of the OPA-FB_1 derivative at 4 °C was not statistically significant ($P > 0.05$, $n = 3$), whereas, the 60% decrease at 24 °C was significant ($P < 0.05$, $n = 3$). The relationship between emitted fluorescence at 24 h and temperature (4–37 °C) was best described by a linear decay process; $\Delta F = 1.007–0.0276\ (T)$ where ΔF is the fraction of the maximal fluorescence at 5 min and T is the temperature centigrade ($r^2 = 0.95$, $P < 0.0001$, 11 degrees of freedom).

After 24 h at 24 °C, the mean decreases in fluorescence of the OPA-FB_1 derivative, OPA-HFB_1 derivative, and the OPA-sphinganine derivative were 83, 67, and 15%, respectively (Fig. 2B). The decreases in the OPA-FB_1 and OPA-HFB_1 fluorescence were not significantly different ($P > 0.05$).

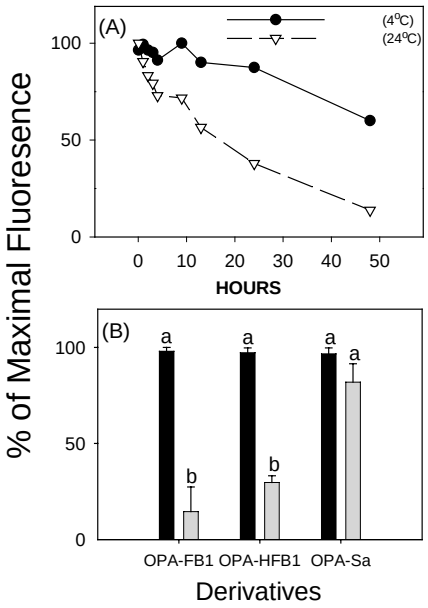

Fig. 2. (A) Fluorescence emitted by the OPA-FB_1 derivative separated by HPLC at the times (hours) and temperatures indicated (4 or 24 °C); (B) Fluorescence emitted by the OPA-FB_1, OPA-HFB_1, and OPA-sphinganine (OPA-Sa) derivatives separated by HPLC approximately 5 min (for FB_1 and HFB_1) and 120 min after derivatization (for sphinganine) and 24 h after derivatization at 24 °C. The change in fluorescence is used as an indicator of the stability of the OPA-derivatized samples. Values are expressed as a percentage of the maximal fluorescence and in (B) values with different superscripts are significantly different ($P < 0.05$, $n = 3$).

The comparable decrease in fluorescence of the OPA-FB_1 and OPA-HFB_1 derivatives, suggests that the tricarballylic acid side chains do not contribute to the instability of the OPA-FB_1 derivative because HFB_1 lacks these side chains and is not stable. Analysis of the OPA-FB_1 derivative and the OPA-sphinganine derivative suggests that the hydroxyl group on carbon-3 of FB_1 does not contribute to the instability because they are present on both FB_1 and sphinganine. Nonetheless, derivatized FB_1 is much less stable at 24 °C than derivatized sphinganine. One difference that may explain the increased stability of the OPA-sphinganine derivative is the hydroxyl group on carbon-1 which is not present in FB_1 or HFB_1 or in any of the other fumonisins. The additional hydroxyl group on FB_1 and HFB_1 at the carbon-5 position does not contribute to the instability of the OPA-FB_1 derivative since the OPA-FB_3 derivative, which lacks the hydroxyl at the carbon-5 position [1], was equally unstable (data not shown). It is possible that the presence of a hydroxyl at carbon-1 stabilizes the ME reaction partner interaction with OPA in the fluorescent derivative. Regardless, maintaining the OPA derivatized samples at 4 °C significantly increased the stability of the OPA-fumonisin derivative.

These results show that if the derivative is refrigerated at 4 °C, it is much more stable, which allows for a more consistent, reproducible experimental method. Normally, due to the instability of the derivative, fumonisin samples would be bracketed by standards before and after every three samples, which is a very time and resource consuming process. Also, if there are unexpected technical problems with analysis, samples can not be re-analyzed without repeating the derivatization. These results are important because they help conserve time by alleviating the need to bracket samples and by allowing samples to be re-analyzed, if necessary, within 24 h without significant loss of fluorescent intensity. These findings also have an economic impact because fewer resources (reagents, solvents, standards, etc.) are used. This may be especially beneficial in developing countries where fumonisin in maize can be a serious problem [4] and where the electricity supply is unpredictable, chemical resources are scarce, and derivatization is often done manually. The ability to derivatize and then refrigerate or cool samples on ice until the analyses have been completed will save time, energy and money.

4. Safety

Fumonisin B_1 is a known liver and kidney carcinogen in rodents; therefore, it should be handled using proper precautionary measures.

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