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Assessment of Inhibitory Effects of Citrus Flavanones on Deoxynivalenol Production Using Response Surface Methodology

M.P. Salas[1,2], P.S. Pok[1], S.L. Resnik[1,3], A.M. Pacin[4*], M.S. Munitz[5]

1. *Organic Chemistry and Industry Department, Faculty of Natural Sciences, University of Buenos Aires, Intendente Güiraldes 2160, C1428EGA, Buenos Aires, Argentina*
2. *Faculty of Engineering, University of Buenos Aires, Intendente Güiraldes 2160, C1428EGA, Buenos Aires, Argentina*
3. *Scientific Research Commission of the Province of Buenos Aires, Calle 526 entre 10 y 11, B1897, La Plata, Buenos Aires, Argentina*
4. *Scientific Research Foundation Teresa Benedicta de la Cruz, Dorronzoro 141, B6700, Luján, Buenos Aires, Argentina*
5. *Food Science Faculty, Entre Ríos National University, Monsenor Tavella 1450, Concordia, Argentina*

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Abstract

Background: Deoxynivalenol (DON) is a mycotoxin produced mainly by *Fusarium graminearum* in grains such as wheat and maize. The aim of this study was to evaluate the inhibitory effects of citrus flavanones including, naringin (NAR), hesperidin (HES), and neohesperidin (NEO) on deoxynivalenol production using Response Surface Methodology (RSM).

Methods: The studied flavanones were extracted from residues of citric industries and assayed in rice media inoculated with *F. graminearum*. After Gas Chromatography (GC) analysis, RSM was applied to find the optimal flavanones concentrations that would lead to total inhibition of DON production. The four levels studied were 0, 0.11, 0.21, and 0.42 mmol/kg rice in dry basis, for each flavanone. Statistical analysis was performed using the statgraphics centurion XV package, version 15.2.6 (StatPoint Technologies, USA). Experimental design consisted in ten factorial points was evaluated in triplicate and used in the model.

Results: All flavanones and their mixtures significantly decreased the accumulation of DON in rice media respect to control (p<0.05). NEO, when applied alone, was the only flavanone that could reach 100% inhibition of DON accumulation in all concentrations tested, followed by HES and NAR that could only reach total inhibition of DON at 0.21 and 0.42 mmol/kg, respectively. Only the mixtures NAR-HES 0.11-0.11 mmol/kg and NEO-HES 0.11-0.11mmol/kg could not completely inhibit DON accumulation in comparison with other mixtures. The obtained optimal combinations were HES-NAR 0.050-0.236, HES-NEO 0.046-0.217, and NAR-NEO 0.034-0.193 mmol/kg rice in dry basis.

Conclusion: Using RSM, the citric flavanones studied in this work, was proved to be effective for total inhibition of DON accumulation.

Introduction

Deoxynivalenol (DON), known as vomitoxin, belongs to a group of structurally related mycotoxins called trichothecenes with high toxicity on health of animals

*Corresponding author
E-mail: benedicta@ictbdelacruz.org.ar

and humans. It is may interrupt protein synthesis, food intake, as well as immune function (Martins et al., 2008). This secondary metabolite is produced by certain strains of *Fusarium* genus and is the most frequently trichothecene found in cereals, such as wheat, barley, oat, rye, and maize (Karlovsky, 2011). The occurrence of DON is associated primarily with *F. graminearum* and *F. culmorum*, both important plant pathogens that cause *Fusarium* head blight in wheat and *Fusarium* ear blight in maize. It has been established that DON contamination of wheat is in direct relationship with the incidence of *Fusarium* head blight (Gautam and Dill-Macky, 2011).

Flavonoids are the most widespread groups of secondary plant metabolites. Their backbone structure is composed of two aromatic rings, which are connected through a pyrone or hydropyrone ring (Gattuso et al., 2007). They are classified in groups according to the level of oxidation of its central nucleus. Four types of flavonoids occur in *Citrus* sp. and can be classified into the groups of flavanones, flavones, flavanols as well as anthocyanins (Benavente-Garcia and Castillo, 2008). In particular, flavanones are found in citrus as glycosides. The most common citrus flavanone glycosides are hesperidin (HES) or 3',5,7-trihydroxi-4'-methoxyflavanone-7-6-O-α-L-rhamnopyranosyl-D-glucopyranoside, which is mainly found in oranges, lemons, and the other citrus fruits; naringin (NAR) or 4',5,7-trihydroxyflavanone-7-β-L-rhamnoglucoside-(1,2)-α-D-glucopyranoside in the grapefruits and sour oranges and neohesperidin (NEO) or (S) 4'-methoxy-3′,5,7-trihydroxyflavanone-7-[2-O-(α-L-rhamnopyranosyl)-β-D-glucopyranoside in sour oranges.

The increasing concern of the consumers towards food safety has pushed the industries to the elimination of synthetic additives. Their replacement by the natural additives is seen as a benefit from the point of view of quality as well as food safety (Viuda-Martos et al., 2008). Consequently, several investigations are focused in the use of natural compounds as food preservatives (Moradi et al., 2014; Singh et al., 2010; Velluti et al., 2003). Some flavonoids have been obtained as by-products of the citrus industry at low cost, particularly flavanones that could present antifungal and antitoxigenic properties (Salas et al., 2012). Several workers demonstrated that extracts of some *Citrus* sp., that could have some of the flavanones studied in this work, had inhibitory activity against mycotoxin producing fungi. For example, Bejarano Rodriguez and Centeno Briceno (2009) proved that 20 µl/g of *Citrus limon* extract in poultry feedstuffs initially contaminated with 45 µg/kg aflatoxin, achieved almost 74% reduction after 1 h of treatment. Also, Oliveira and Furlong (2008) found that the phenolic extracts from orange peel inhibited the production of aflatoxin B_1 and B_2 at a concentration of 250 µg/ml. Essential oils from *Citrus reticulata* and *Cymbopogon citratus* completely inhibited aflatoxin B_1 production at 750 and 500 ppm, respectively.

Factorial designs are efficient methodologies to study the joint effect of two or more factors on a response (Grum and Slabe, 2004). Response Surface Methodology (RSM) is very useful for the modeling and analysis of situations in which a response of interest is influenced by several variables (Ibrahim and Elkhidir, 2011) with much less effort than the classical approaches, resulting in less laborious and time-consuming assays (Esbensen et al., 2002).

The aim of this study was to analyze the utilization of three flavanones obtained from the citrus industry, NAR, HES as well as NEO applying RSM to find the optimal concentrations to inhibit the production of DON by *F. graminearum.*

Materials and methods

Reagents and chemicals

All solvents were LC grade including, methanol (Sintorgan, Buenos Aires, Argentina), toluene (U.V.E. Dorwill S.A., Buenos Aires, Argentina), ethyl acetate (Ultimar V553, Mallinckrodt, Paris, KY, USA), and also acetonitrile (Ultimar H454, Mallinckrodt). DON standard was prepared from Biopure Referenzsubtanzen, Austria (GmbH, Tulln, 10.1 mg/ml). Internal standards were 2-amino-5-chloro-benzophenone (ACBP) in toluene (A-4632, Sigma, St Louis, MO, USA) and deepoxy-deoxynivalenol (E-DON) in acetonitrile (Biopure Referenzsubtanzen GmbH, Tulln, Austria). Stock solutions were ACBP 365 mg/ml toluene as well as E-DON 50.6 mg/ml acetonitrile. Heptafluorobutyric anhydride (HFBA; Fluka, Sigma-Aldrich, Busch, Switzerland); sodium bicarbonate 5% (w/v) aqueous solution; catalyst solution 2 mg/ml 4-(N, N-dimethylamino)-pyridine (4-DMAP) (D-5640; Sigma) in toluene/acetonitrile (80: 20, v/v).

Flavanones

The flavanones including NAR, HES, and NEO were obtained from residues of citric industries in the "Instituto de Investigaciones para la Industria Química" (Universidad Nacional de Salta, Salta, Argentina). Briefly, the flavanones obtaining procedure consisted of grinding the discarded fruits, or the residues of juice processing, to an average size of 2 mm in diameter and then performing an extraction in fixed bed column. To obtain NAR, the solvent used was distilled water at 80 °C; for HES an aqueous solution, pH 10.0-10.5 at 70 °C, was used; and NEO was extracted with ethanol/water (50:50, v/v) at 25 °C. In all three processes, the obtained extract

was cooled, leading to crystallization of the flavanones. The precipitate was then filtered and washed. Finally, the solid was dried in an oven at 50 °C. This process was considered simple and inexpensive.

Fungal strains

F. graminearum CIM 30425 was isolated from blueberries (Munitz et al., 2013; Munitz et al., 2014) and kept in Type Culture Collection of the Natural Science Faculty, University of Buenos Aires, Argentina. This mould was previously cultivated in slant tubes containing Malt Extract Agar (MEA) during 7 days. After that, 10 ml Tween 80 (0.02%) were added and the tubes were shaken for 1 min in a vortex to separate the conidia from the rest of the medium. The concentration of conidia in suspension was 1.6×10^8 conidia/ml, determined by a Neubauer counting chamber.

Preparation of flavanone solutions

In this step, test concentrations were selected according to previous work (Salas et al., 2016). NAR, HES, and NEO were dissolved in ethanol/water (5:95, v/v) at 3 different concentrations separately (0.15; 0.30; 0.60 mM) and in different binary mixtures: NAR-HES, NAR-NEO, and HES-NEO at the concentrations of 0.15-0.15; 0.30-0.30; 0.60-0.60 mM. Besides, 50 µl of the conidia suspension of *F. graminearum* were inoculated in erlenmeyers containing 25 g rice as a substrate sterilized at 40% humidity. Then, 15 ml of the different flavanones solutions were added and thoroughly shaken, being the final concentrations of flavanones in the medium 0.11, 0.21, and 0.42 mmol/kg rice in dry basis, for NAR, NEO, and HES; and 0.11-0.11, 0.21-0.21, and 0.42-0.42 mmol/kg rice in dry basis for the mixtures. The erlenmeyers were incubated at 25 °C, during 30 days.

Extraction and Gas Chromatography (GC) procedures

Extraction was performed by blending during 3 min the entire content of the erlenmeyer (substrate with the mycelia growth) with 105 ml of acetonitrile. Then, 8 ml of the supernatant were passed through an extraction column (TC-M Puritox 220, Trilogy Lab, USA) as it was previously described by Garrido et al. (2013). One ml of the filtrate was evaporated to dryness and suspended in 200 µl acetonitrile/methanol (19:1, v/v) and 150 µl of this solution was again evaporated to dryness under a nitrogen stream. The solid residue was derivatized using solution of toluene/acetonitrile (80:20, v/v) which contained 2 mg/ml DMAP, shaked for 15 s, and then added 50 µl HFBA, and shaked again for another 15 s (Croteau et al., 1994). The tubes were put in a sand bath at 60-65 °C during 30 min. After that, 1.2 ml $NaHCO_3$ (5%) and 400 µl toluene were added, shaked for 30 s and centrifuged at 2000 rpm during 2 min. An aliquot of 300 µl organic phase was separated in a GC vial. The volume injected was 2 µl in GC equipment (Agilent Technology 7890A) with µECD (micro electron capture detector). The GC column used was HP-5, 30 mm, 0.32 mm, 0.25 µm (Agilent Technology, USA), the gas was nitrogen and the working temperature was 300 °C. Retention time of DON was about 22.2 min. Limits of Detection (LOD) and Limits of Quantification (LOQ) were 4.0 as well as 10 µg/kg, respectively according to the study of Garrido et al. (2013).

Factorial designs and RSM

The behavior of the system was explained by the following second-degree polynomial equation:

$$Y = a_0 + \sum_{i=1}^{n} a_i x_i + \sum_{i=1}^{n} a_{ii} x_i^2 + \sum_{i<j=2}^{n} a_{ij} x_i x_j$$

In this equation, Y represents the response function, in this case being the accumulation of DON in rice media, a_0 is a constant term and a_i and a_{ii} are the coefficients of the linear and quadratic terms, respectively. Term a_{ij} represents the interaction between factors. Accordingly X_i and X_j represent the coded independent variables (Gonzalez-Gomez et al., 2014; Li et al., 2007). Statistical analysis was performed using the statgraphics centurion XV package, version 15.2.6 (StatPoint Technologies, USA). The codifications of the independent variables applied in RSM were X1: flavanone 1, and X2: flavanone 2. The four levels studied were 0, 0.11, 0.21, and 0.42 mmol/kg rice in dry basis, for each flavanone. The experimental design consisted in ten factorial points evaluated by triplicate. The mean values and the standard deviation of those triplicates was calculated and used in the model.

Results

A preliminary study was carried out to evaluate DON accumulation in the rice media with the addition of different flavanones, respect to DON accumulation without flavanones (control). It can be seen in Table 1 that all flavanones and their mixtures significantly decreased the accumulation of DON in rice media respect to control (p<0.05). NEO, when applied alone, was the only flavanone that could reach 100% inhibition of DON accumulation in all concentrations tested, followed by HES and NAR that could only reach total inhibition of DON at 0.21 and 0.42 mmol/kg, respectively. The most consistent results were obtained with the mixtures of the studied flavanones, where at higher concentrations (0.21 and 0.42 mmol/kg), total inhibition of DON production could be observed. Only the mixtures NAR-HES 0.11-

0.11 mmol/kg and NEO-HES 0.11-0.11mmol/kg could not completely inhibit DON accumulation as all other mixtures did (p<0.05) showing a dose-response effect between flavanone mixtures levels as well as DON accumulation in rice media.

To find the most suitable mixture with minimal flavanones concentrations that would lead to total inhibition of DON accumulation, RSM methodology was applied as described in materials and methods section. Fig. 1 shows estimated response surfaces obtained for flavanones and their mutual interaction on the DON accumulation.

The response surfaces allowed obtaining a second degree equation to calculate the concentration of DON for each mixture of flavanones and to optimize accumulation of these mycotoxins in order to reach 100% inhibition. The equations for DON concentrations (µg/kg) were the following, in which NAR, NEO, and HES are the concentration levels of the flavanones in mmol/kg rice in dry basis:

HES-NAR

$DON=1077.8–5417.8Nar–5277.68Hes +6563.38Nar^2 + 7238.4NarHes+6031.37Hes^2$

Mixture concentration to minimize content of DON was HES 0.050–NAR 0.236 mmol/kg rice dry basis.

HES-NEO

$DON=1048.82–4402.85Hes–6001.44Neo+4003.57Hes^2 +7383.05HesNeo+7957.26Neo^2$

Mixture concentration to minimize the accumulation of DON was HES 0.046–NEO 0.217 mmol/kg rice dry basis.

NAR-NEO

$DON=989.174–4838.6Nar–6297.06Neo+5707.19Nar^2 +6631.11NarNeo+9128.88Neo^2$

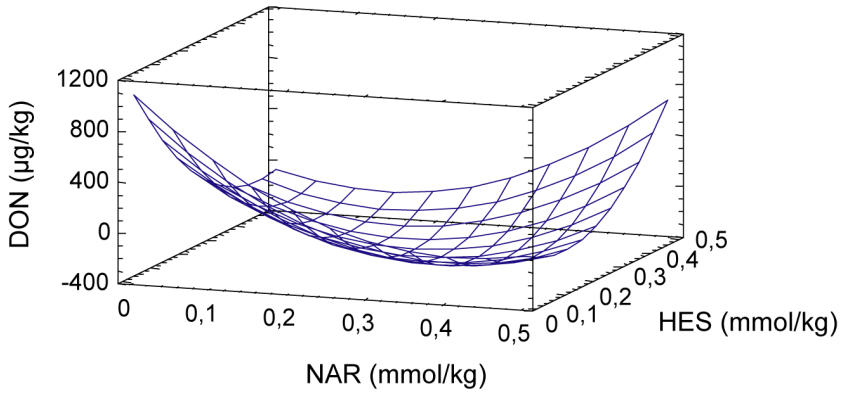
Mixture concentration to minimize the accumulation of DON was NAR 0.034–NEO 0.193 mmol/kg rice dry basis.

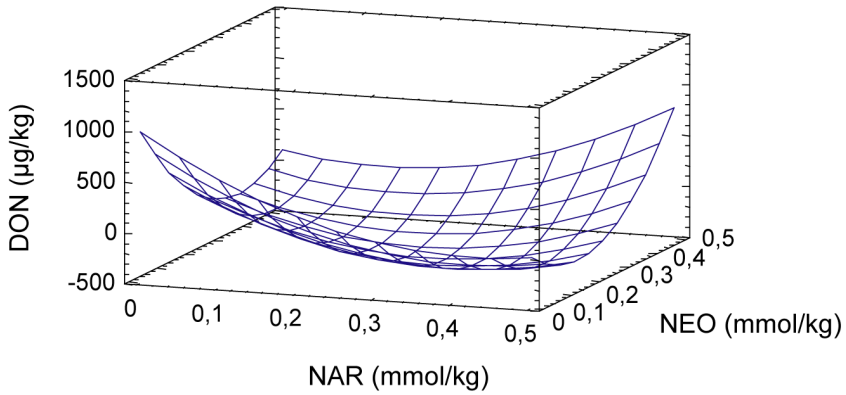
Using the procedure described previously, the mixtures calculated as the optimal by RSM were prepared for verification of the results. It was found that all flavanones mixtures suggested by RSM reduced 100% DON accumulation. In general, HES and NEO were more effective against DON and were used in a lower concentration than NAR in these mixtures.

Table 1: DON accumulations and their percentage inhibition using different flavanones

Flavanones concentration (mmol/kg)	DON accumulation mean±SD (µg/kg)	DON inhibition mean (%)
Control	1597±150	0
NAR 0.11	47±29	97
NAR 0.21	211±130	87
NAR 0.42	ND	100
HES 0.11	337±207	79
HES 0.21	ND	100
HES 0.42	166±102	90
NEO 0.11	ND	100
NEO 0.21	ND	100
NEO 0.42	ND	100
NAR-HES 0.11	300±61	94
NAR-HES 0.21	ND	100
NAR-HES 0.42	ND	100
NAR-NEO 0.11	ND	100
NAR-NEO 0.21	ND	100
NAR-NEO 0.42	ND	100
NEO-HES 0.11	438±270	73
NEO-HES 0.21	ND	100
NEO-HES 0.42	ND	100

ND: not detected





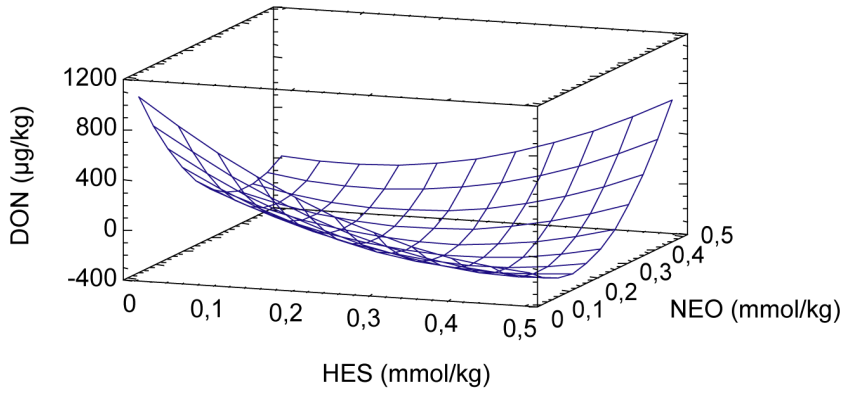


Fig. 1: Estimated response surfaces for flavanones and their mutual interaction on DON accumulation (orderly from up to down: NAR-HES, NAR-NEO, and HES-NEO)

Discussion

Several authors pointed out the diminution of mycotoxins accumulation using essentials oils with phenolic compounds. For example, essential oils extracted from mint and cinnamon, among other plants, achieved 100% DON inhibition at 500 ppm (Sumalan et al., 2013). Boldo, poleo and clove oils affected ochratoxin production by *Aspergillus niger* and *A. carbonarius* (Passone et al., 2012). Also, the plant extracts, rich in phenolic compounds, have been successfully used for preventing mycotoxins accumulation, e.g., peanut skin extracts, obtained as a by-product of the peanut blanching process, decreased fumonisin B_1 production by *F. verticillioides* (Pizzolitto et al., 2013). Also, according to Pagnussatt et al. (2014), methanolic extracts of *Spirulina* sp. achieved 73% reduction of DON and nivalenol production by *F. graminearum*. Also, pure phenolic compounds, such as flavonoids, proved to be effective against fungi and mycotoxin production, namely *Penicillum expansum*, diminishing patulin accumulation on apples (Sanzani et al., 2009) and also production of ochratoxin by *A. carbonarius* (Romero et al., 2009) among others. In addition, previous works carried on by this group pointed out that flavanones could affect the accumulation of mycotoxins. For example, patulin accumulation was reduced by HES, NAR, and NEO in 95, 96, and 99%, respectively (Salas et al., 2012). Although, the results obtained from the previous mentioned studies are somewhat in agreement with our findings, but so far, there was not much exact information related to these particular citrus flavanones and their effect on DON accumulation.

RSM has commonly been used by some researchers to evaluate and optimize several processes. Similar to the present research, Ahmad et al. (2013) used this methodology to study the effect of different nutrients used as media components on the production of aflatoxin B_1 by *A. flavus*. Plackett-Burman experimental as well as Box–Behnken factorial designs were applied. Moreover, Rajkovic et al. (2015) used a Box-Behnken three factors design to assess the inhibitory effect of thyme and cinnamon essential oils on *A. flavus*, and found optimal conditions to achieve maximum inhibition. Regarding microorganisms inactivation, RSM with a central composite design was used by Hossain et al. (2015) to optimize supercritical carbon dioxide sterilization parameters on Gram-positive and Gram-negative pathogenic bacteria in clinical solid waste. However, based on previous experience of our group a modified 4^2 complete factorial design was used (data not shown). The design proposed, permitted studying the joint effect of two flavanones at four concentration levels, determining the optimal mixtures that led to complete inhibition of DON accumulation. Our results demonstrated the possibility to achieve the inhibition of DON production by natural and inexpensively obtained products, such as the flavanones NAR, NEO, and HES. The RSM was successfully employed to optimize the concentration of the flavanones mixtures applied to the medium. The three mixtures proposed completely prevented the accumulation of this toxin, being more effective than the flavanones used alone.

Conclusion

DON production by *F. graminearum* could be prevented by three studied flavanones including NAR, NEO, and HES at the optimal mixtures concentrations predicted using RSM. These citric flavanones could be a natural alternative to synthetic antifungal agents, and could be assayed in different food matrices, applying RSM if any adjustments were needed in order to assess complete inhibition of DON production. Moreover, the studied citric flavanones were efficiently extracted from citric industries residues, in a process that could raise the economic value of those residues.

Conflicts of interest

The authors report no conflicts of interest.

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