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Evaluation of a fluorometric–enzymatic method based on 3α-hydroxysteroid dehydrogenase for the mycotoxin zearalenone determination in corn

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Abstract

A new method for the fluorometric determination of zearalenone (ZEN) based on its reaction with βNADH in the presence of the enzyme 3α-hydroxysteroid dehydrogenase (3α-HSD) is described. The procedure is based on the change in fluorescence intensity that takes place during the enzymatic reaction (excitation at 340 nm and emission at 454 nm). The optimum reaction conditions and the analytical characteristics were studied; linear response range (1–10 mg l^{-1}) and reproducibility (8 mg l^{-1}, 2.7%, $n = 7$). Moreover, a mathematical model explaining the analytical signal is proposed. The method has been applied to zearalenone determination in a spiked corn sample.

Keywords: Zearalenone; Fluorometry; Enzymatic determination; Corn

1. Introduction

Zearalenone (ZEN) (6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcyclic acid lactone) (Fig. 1) is an important mycotoxin found in temperate and warm regions. It is produced by fungi of the genus *Fusarium*. This toxin is common in corn but it also occurs in small grain like barley, wheat, sorghum, millet and rice. Studies with many species have shown that ZEN and its metabolites present estrogenic activity because they are able to adopt similar conformations to natural estrogens [1,2].

This is why several methods have been studied and developed for the determination of ZEN in foods, feeds, animal tissues, blood and urine. Although TLC [3,4] and GC, especially GC–MS [4–6], methods are available, LC by reversed phase with direct fluorescence detection is considered as the usual choice for the determination of ZEN and its metabolites mainly in corn and other cereals [7–10]. A recent alternative is based on immunoaffinity columns (IACs) and quantification by fluorescence detection after reaction with aluminium chloride [11]. Increasingly, enzyme-linked immunosorbent assays (ELISAs) are used for the determination of ZEN in food and feeds. One ELISA [12] has been tested collaboratively and approved by the Association of Official Analytical Chemists (AOAC) as a first-action screening method for ZEN concentrations higher than 800 μg kg^{-1}. The success of immunoassay methods and IACs is due to their greater simplicity in extraction and clean up steps than in other more conventional methods. However, these systems have interferences of ZEN metabolites, which have similar reaction capabilities [11,13].

In the present paper, a new alternative method is proposed for the determination of ZEN based on the fluorescence changes produced during the reaction with an enzyme. ZEN is not known as the natural substrate of any enzyme but it is able to act as a competitive substrate for hydroxysteroid dehydrogenase enzymes. These compounds react with estrogens regulating occupancy and activation of steroid hormone receptors [14]. ZEN reacts with these enzymes

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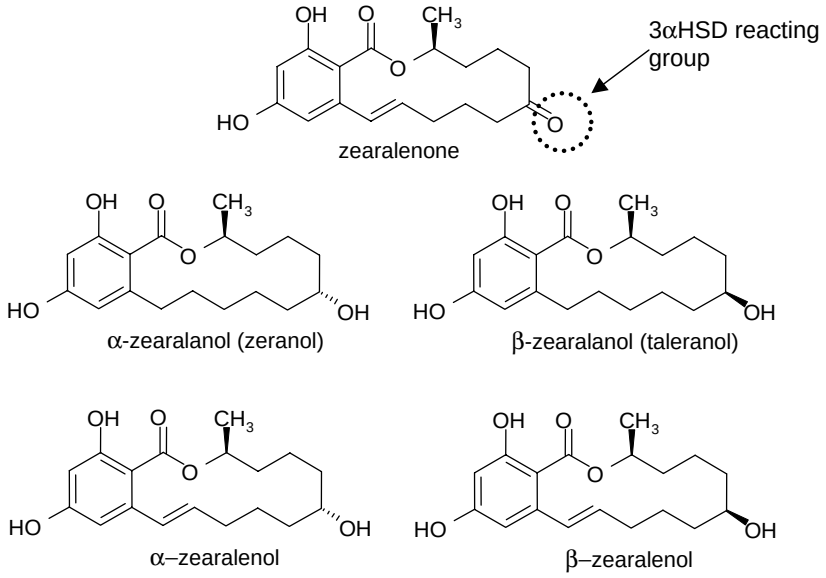

Fig. 1. Structures of ZEN and its metabolites.

because of its capability to mimic estrogen conformation [13]. This reaction is selective for ZEN with respect to its metabolites because the reaction happens at a carbonyl group that differentiates ZEN from its metabolites (Fig. 1). We have found [15] that ZEN reacts with β-NADH in the presence of 3α-hydroxysteroid dehydrogenase (3α-HSD) producing changes in β-NADH fluorescence. These changes can be related to ZEN concentration.

2. Experimental

2.1. Apparatus

All fluorescence measurements were carried out in a PTI Time Master (TM) luminometer TM-2/2003 (Photon Technology International, Lawrenceville, NJ, USA). A triangular Hellma 101.061-QS quartz cuvette was used. Slit widths of 1 and 6 nm were applied for excitation and emission, respectively.

Molecular absorption measurements were performed with a Perkin-Elmer Lambda 5 Spectrophotometer (1 cm path length conventional quartz cuvettes were used).

A Mettler Toledo AG245 (±0.01 mg precision) balance was also used.

2.2. Reagents and solutions

Buffer phosphate (0.1 M) solution of pH 6.5 (from solid KH_2PO_4 and solid Na_2HPO_4).

ZEN stock solution was prepared by dissolving 25 mg of zearalenone (Sigma Z-2125) in 3 ml of 0.1 M NaOH solution. This solution was divided into fractions of 200 μl, which were immediately frozen. When ZEN was required, one fraction was defrosted and the appropriate dilution was carried out with doubly distilled water.

βNADH solution was prepared by dissolving 1.2 mg of βNADH (Sigma N-8129) in 10 ml of phosphate buffer. This solution was prepared daily.

3α-Hydroxysteroid dehydrogenase was taken from *Pseudomonas testosteroni*, EC 1.1.1.50 (Sigma H-1506). Solutions were prepared by dissolving 5 IU in 5 ml of buffer solution. This solution was prepared immediately before use and it was kept in an ice bath (about 6 °C) during the work session.

2.3. Procedure

The fluorescence intensity was measured with the instrumental conditions previously described, using 340 nm as λ_{exc} and 454 nm as λ_{em}. By using the multiple addition method (see Section 3.2), 800 μl of buffer solution, 20 μl of βNADH solution and 30 μl of 3α-HSD solution were added to the measuring cuvette, the solution was vigorously stirred and the variation of intensity measured. Subsequently, 50 μl of ZEN solution (giving a ZEN concentration ranging from 1 to 10 mg l^{-1} in the cuvette) was spiked and a gradual decrease in fluorescence with time was observed. For ZEN determination in corn, 900 μl of a buffered sample solution (containing from 1 to 10 μg ZEN), 20 μl of βNADH solution and 30 μl of 3α-HSD solution were added to the measuring cuvette.

For ZEN determination in corn, 2 g of NaCl was added to 20 g ground corn. The mixture was homogenized and extracted in methanol (50 ml) for 2 min under vigorous stirring. The sample was then filtered in a porous plate (porosity ranging from 10 to 16 μm). The unfiltered fraction was cleaned five times using 1 ml of methanol. The filtered

fraction was then dried with steam of nitrogen. The residue was dissolved in 2 ml of doubly distilled water and filtered with 0.2 μm nylon membrane with polypropylene housing filters.

The I_t/I_0 ratio was used as the measurement parameter, I_0 and I_t being the fluorescent intensity at 0 and t minutes after starting the reaction, respectively; most studies were carried out when $t = 16$ min.

3. Results and discussion

3.1. Origin of the signal and general mathematical model

The structural rigidity and aromatic nature of ZEN suggest its intrinsic fluorescence (excitation maxima at 250 and 315 nm, emission maximum at 410 nm). Thus, the enzymatic reaction could be followed by a decrease in either ZEN or βNADH fluorescence. Following reasons discourage the use of the intrinsic fluorescence of ZEN: (a) as can be seen, its excitation and emission maximum are located at lower wavelengths than βNADH, so ZEN fluorescence is more affected by spectral interferences, (b) because of the high molar absorptivity of ZEN, its fluorescence shows a strong primary inner filter effect (it can be observed in Fig. 2) and (c) the fluorescence quantum yield of βNADH is higher than for ZEN (as can be observed by comparing the fluorescence intensity obtained for isolated ZEN and βNADH solutions containing similar concentrations of both).

Fig. 3 shows the changes in fluorescence intensity of βNADH ($\lambda_{exc} = 340$ nm and $\lambda_{em} = 454$ nm) when consecutive ZEN additions are made to an enzyme and cofactor solution. First, doubly distilled water (50 μl) is added (marked with an arrow and number 1 in the Fig. 3) after which three ZEN additions (50 μl of 50 mg l^{-1} each) are made (numbers 2, 3 and 4). A comparison of Figs. 2 and 3 shows that for the experimental conditions used and ZEN concentration involved, the ZEN fluorescence is negligible in relation to βNADH fluorescence.

A simplified kinetic model for dehydrogenases is applied to the enzymatic reaction in order to develop a mathematical model explaining the analytical signal obtained:

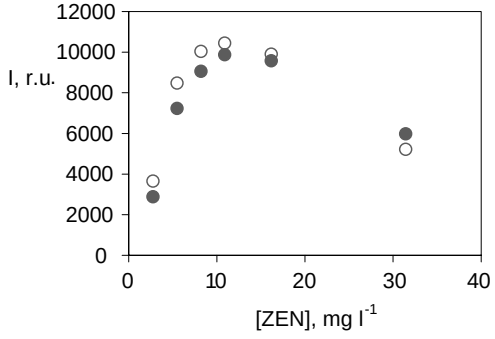

Fig. 2. Fluorescence intensity at different ZEN concentrations for two excitation wavelengths ($\lambda_{em} = 410$ nm, pH = 9.5): (●) $\lambda_{exc} = 315$ nm and (○) $\lambda_{exc} = 250$ nm.

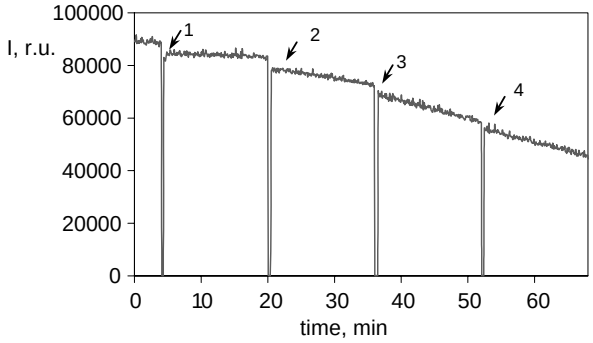

Fig. 3. Fluorescence intensity variation with time after blank (marked as 1) and three consecutive ZEN additions (marked as 2, 3 and 4, giving ZEN concentrations of 2.5, 5.0 and 7.5 mg l^{-1}, respectively) to a cofactor and enzyme solution (experimental conditions as indicated in Section 2.3).

$$\beta\text{NADH} + 3\alpha\text{-HSD} \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} \beta\text{NADH} \cdot 3\alpha\text{-HSD}$$

$$\beta\text{NADH} \cdot 3\alpha\text{-HSD} + \text{ZEN} \xrightarrow{k_2} \beta\text{NAD} + \text{zearalenol} + 3\alpha\text{-HSD}$$

Applying a mass balance for enzyme and the stationary state condition for βNADH·3α-HSD:

$$-\frac{\mathrm{d}[\beta\text{NADH}]}{\mathrm{d}t} = k_1[\beta\text{NADH}]_t[3\alpha\text{-HSD}]_0 \times \left(\frac{k_2[\text{ZEN}]_t}{k_{-1} + k_1[\beta\text{NADH}]_t + k_2[\text{ZEN}]_t}\right) \quad (1)$$

The βNADH concentration is very low so it can be assumed that $k_1[\beta\text{NADH}] \ll k_{-1}$. Taking into account that in enzymatic reactions usually $k_{-1} \gg k_2$, the following simplification can be applied:

$$k_{-1} + k_1[\beta\text{NADH}]_t + k_2[\text{ZEN}]_t \cong k_{-1} \quad (2)$$

Fig. 3 suggests that the reaction rate is low, so the βNADH consumption during a moderately long time (i.e. 15 min) is low; additionally taking into account that $[\text{ZEN}]_0 > [\beta\text{NADH}]_0$, the following approximation can be made:

$$[\text{ZEN}]_t \cong [\text{ZEN}]_0 \quad (3)$$

Combining Eqs. (1)–(3):

$$-\frac{\mathrm{d}[\beta\text{NADH}]}{\mathrm{d}t} = \frac{k_1k_2}{k_{-1}}[\beta\text{NADH}]_t[3\alpha\text{-HSD}]_0[\text{ZEN}]_0 \quad (4)$$

After integrating and rearranging, the following expression is obtained:

$$\frac{[\beta\text{NADH}]_t}{[\beta\text{NADH}]_0} = \mathrm{e}^{-(k_1k_2/k_{-1})t[3\alpha\text{-HSD}]_0[\text{ZEN}]_0} \quad (5)$$

For low $[\text{ZEN}]_0$ and $[3\alpha\text{-HSD}]_0$ values, the mathematical approximation $\mathrm{e}^{-x} \approx 1 - x$ yields

$$\frac{[\beta\text{NADH}]_t}{[\beta\text{NADH}]_0} = 1 - \frac{k_1k_2}{k_{-1}}t[3\alpha\text{-HSD}]_0[\text{ZEN}]_0 \quad (6)$$

Finally, as the NADH fluorescence is measured along the reaction (K_{fl} being the fluorescence proportionality constant)

$$I_t = K_{fl}[\beta\text{NADH}]_t$$

it can be concluded

$$\frac{I_t}{I_0} = 1 - \frac{k_1 k_2}{k_{-1}} t[3\alpha\text{-HSD}]_0[\text{ZEN}]_0 \quad (7)$$

I_0 and I_t being the fluorescence intensities at 0 and t minutes after the start of the reaction, respectively. Eq. (7) establishes a linear relation between ZEN concentration and (I_t/I_0). It should be remarked that this expression does not depend on βNADH concentration.

3.2. Multiple addition model

The ZEN concentration does not change during the reaction time considered. This statement is based on two facts: (1) according to Fig. 3, the reaction is not extensive and (2) the initial ZEN concentration is higher than the cofactor concentration. As the enzyme is not consumed and the analytical parameter is not dependent on the βNADH concentration, consecutive ZEN additions to the same enzyme and cofactor solution are possible, resulting in an easier and cheaper calibration method. Considering 16 min as the reaction time (after each ZEN aliquot addition) and f as enzyme dilution factor, the Eq. (7) is easily modified to

$$\frac{I_{16}}{I_0} = 1 - \frac{k_1 k_2}{k_{-1}} 16 f[3\alpha\text{-HSD}]_0[\text{ZEN}]_0 \quad (8)$$

I_0 and I_{16} being the fluorescence intensity values at the beginning and end of each ZEN addition, and $[\text{ZEN}]_0$ being the accumulative concentration after each analyte addition. This expression can be used when the extension of the reaction is short, so that the ZEN consumption is not significant.

Eq. (8) indicates that if the product of measuring time and enzyme concentration is constant, the same results should be obtained. This has been experimentally proved by two multiple addition experiments. Firstly, the same experiment as shown in Fig. 3 was carried out, that is different additions were made to cofactor and enzyme solution ($[3\alpha\text{-HSD}]_0$) in 16 min steps. Secondly, the experiment was carried out using two times $[3\alpha\text{-HSD}]_0$ enzyme concentration in 8 min steps, so that the product of measuring time and enzyme concentration was same in both the experiments. A linear fitting of this results gives the following equations ([ZEN] in mg l^{-1}):

$$\frac{I_{16}}{I_0} = 0.985 - 0.0225 f[\text{ZEN}]_0, \qquad r = 0.995 \quad (9a)$$

$$\frac{I_8}{I_0} = 992 - 0.0222 f[\text{ZEN}]_0, \qquad r = 0.993 \quad (9b)$$

These results are in agreement with the mathematical model proposed and show the possibility of decreasing the measuring time using a higher enzyme concentration to produce the same results.

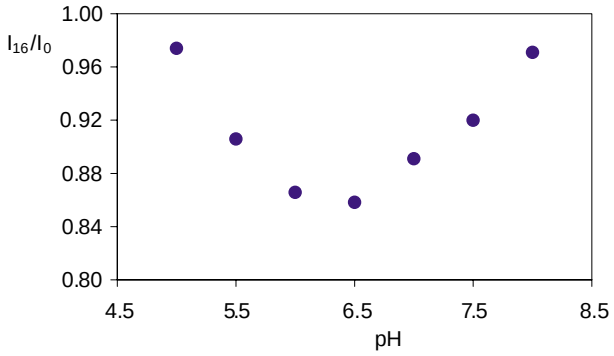

Fig. 4. pH effect on analytical parameter ($[\text{ZEN}]_0 = 8\,\text{mg}\,l^{-1}$, $[3\alpha\text{-HSD}]_0 = 0.35\,\text{units}\,\text{ml}^{-1}$, $[\beta\text{NADH}]_0 = 6.2\times10^{-6}$ M, $\lambda_{exc} = 340$ nm and $\lambda_{em} = 454$ nm).

3.3. Optimisation of parameters

3.3.1. pH optimisation

The pH not only affects the reaction kinetics through the enzyme, but also affects βNADH and 3α-HSD stability. The pH effect has therefore been studied between 3 and 11, however, reaction only occurs from pH 5 to 8. Fig. 4 shows the pH effect on the analytical parameter. The optimal response is obtained at pH 6.5.

3.3.2. Enzyme concentration

The effect of the 3α-HSD concentration on the signal can be seen in Fig. 5. The higher the enzyme concentration, the lower the analytical parameter value because the reaction is more extensive for the same reaction time. However, an extended reaction is not desired for the multiple addition procedure and, taking into account of the measurements costs, 0.35 units ml^{-1} was chosen as the optimal enzyme concentration. This concentration allows the use of multiple addition procedure with appropriate fluorescence changes over a not too long reaction time.

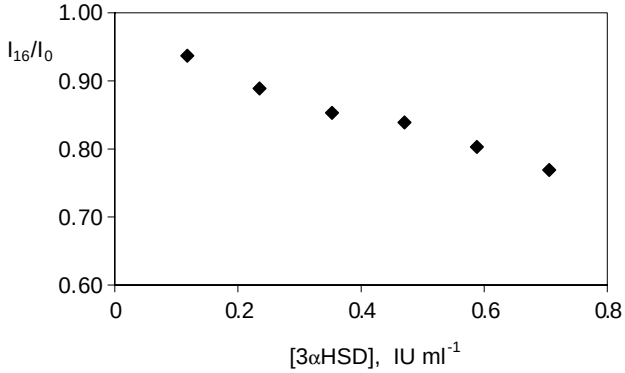

Fig. 5. Enzyme concentration effect on analytical parameter ($[\text{ZEN}]_0 = 8\,\text{mg}\,l^{-1}$, $[3\alpha\text{-HSD}]_0 = 0.35\,\text{units}\,\text{ml}^{-1}$, $[\beta\text{NADH}]_0 = 6.2\times10^{-6}$ M, pH = 6.5, $\lambda_{exc} = 340$ nm and $\lambda_{em} = 454$ nm).

Table 1
Effect of the cofactor concentration on the analytical parameter

$[\beta NADH]_0$ (M)	I_{16}/I_0
2.48×10^{-5}	0.8641
1.24×10^{-5}	0.8411
6.2×10^{-6}	0.8500

[ZEN] = 8 mg l^{-1}, $[3\alpha\text{-HSD}]_0 = 0.35$ units ml^{-1}, pH = 6.5, λ_{exc} = 340 nm and λ_{em} = 454 nm.

3.3.3. βNADH concentration

According to the mathematical model, the analytical parameter is non-dependent on the cofactor concentration. However, the βNADH concentration has two effects. Firstly, the higher the βNADH concentration, the higher the fluorescence intensity. Secondly, the βNADH concentration must be high enough to obtain a fluorescence intensity value suitable for measurement.

Table 1 shows the parameter behaviour at different βNADH concentrations. The lowest studied βNADH concentration was chosen (6.2×10^{-6} M) in order not to increase the extension of the reaction. It is not possible to work with lower concentrations because of the required value of fluorescence intensity.

3.4. Analytical characteristics

Under the optimal conditions, the linear response obtained between the analytical parameter and the ZEN concentration ranged from 1 to 10 mg l^{-1}. Using multiple addition methodology in the conditions described, the experimental results, by using the least squares method, were as shown previously in Eq. (9a). The R.S.D. of the slope of the regression line was 4%.

The mathematical model predicts a *y*-intercept value of 1, although experimentally this value is lower. Because of cofactor photodegradation, the fluorescence intensity decreases during the measurement. The obtained signal is thus due to both reaction and photodegradation. No reaction contribution corresponds to the analytical parameter of the blank assay.

The reproducibility (R.S.D.) was 2.7% for a ZEN concentration of 8 mg l^{-1} ($n = 7$).

4. Analytical application

The possibility of using the recommended method for ZEN determination in corn samples has been studied. Prior to the detection step, extraction and clean-up steps are required. The extraction solution is usually a mixture of methanol or acetonitrile and water and NaCl. Filtration is usually used to separate the extract and particles. Generally, the clean-up may be carried out in three ways: by using solid phase extraction cartridges, by immunoaffinity columns or by liquid–liquid partitioning. The resulting solution is dried and the residue obtained is dissolved in an appropriate solution for the detection step [7,11,13,16,17]. Finally, we used the method described in Section 2.3.

Table 2
Results obtained in the determination of ZEN concentration in multiple ZEN additions on a corn sample

Experiment	Slope/*f*	Intercept	*r*
1	0.0220	0.917	0.9995
2	0.0231	0.929	0.994
3	0.0234	0.933	0.998
4	0.0216	0.917	0.9991
5	0.0217	0.924	0.9990
6	0.0229	0.927	0.998

$[3\alpha\text{-HSD}]_0 = 0.35$ units ml^{-1}, $[\beta NADH]_0 = 6.2 \times 10^{-6}$ M, pH = 6.5, λ_{exc} = 340 nm and λ_{em} = 454 nm.

The sample matrix produces an increase in fluorescence intensity due to light scattering because of the formation of an emulsion. Continuous stirring is therefore necessary to keep the light dispersion constant so as not to affect the ZEN determination.

The experiment described in Fig. 3 was carried out six times, using corn sample in addition to number 1. In each experiment, three successive ZEN additions were made corresponding to 2.9, 5.4 and 7.7 mg l^{-1}, respectively; these ZEN concentrations are in the same range as found in a contaminated corn sample [13]. The corresponding regression lines obtained for the six experiments are indicated in Table 2.

Comparing equations in Table 2 with Eq. (9a), a good concordance is observed in the slope value, but the values of the *y*-intercept are lower due to a non-reaction decrease in the intensity (both cofactor photodegradation and sample behaviour contribute to this). The *y*-intercept value can be obtained by measuring a solution containing only cofactor and sample where enzymatic reaction is not possible.

For each addition of the six experiments previously indicated, the recovery was calculated obtaining values ranging from 100 to 107%. The average ZEN concentration found is related to the ZEN concentration added according to:

$$[ZEN]_{found} = 1.05[ZEN]_{added} - 0.068, \qquad r = 0.997$$

This result shows the possibility of determining ZEN by the method recommended in this work.

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