Direct electrochemical detection of trichothecenes in wheat samples using a 96-well electrochemical plate coupled with microwave hydrolysis

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Abstract

The use of a 96-well electrochemical plate for the fast and sensitive detection of deoxynivalenol and nivalenol in wheat samples is shown. Deoxynivalenol and nivalenol are hydrolysed using a microwave hydrolysis procedure (2 min) which leads to the production of electroactive compounds that can be sensitively detected by the use of cheap screen-printed electrodes. A procedure of extraction with aqueous acetonitrile and a clean-up step was demonstrated to be suitable for the application with wheat samples providing suitable detection limit (LOD=1.1 µg/g) and working range (2-20 µg/g) for the determination of deoxynivalenol in cereals for feed consumption.

Keywords: deoxynivalenol, determination, rapid methods

1. Introduction

Mycotoxins are low-molecular-weight natural products produced as secondary metabolites by filamentous fungi which cause food- and feed-borne intoxication and carcinogenic effects in humans and farm animals (Krska et al., 2001). The Fusarium genus is the most frequent fungal contaminant of field maize (Zea mays L.) and represents one of the first causes of mycotoxin production (Bottalico, 1998; Creppy, 2002; Kuiper-Goodman, 1995). Trichothecenes are commonly found in cereals and for this reason represent a serious threat for the safety of cereal-based food and feedstuffs. They are classified into Group A and Group B compounds depending on their structure. The most important trichothecenes are T-2 and HT-2 within group A, and deoxynivalenol (DON) and nivalenol (NIV) within group B. Trichothecenes B lead to feed refusal, vomiting, anaemia, haemorrhage, and immunosuppression (D’Mello et al., 1999) and their concentration in cereal-based food is strictly regulated by the European Commission. The level of concern for DON is 1.75 µg/g in unprocessed durum wheat and oats for human consumption (EC, 2006a) and 8.0 µg/g for cereal and cereal products used as feed materials (EC, 2006b). Most of the trichothecene contamination reports are from wheat and wheat-based products with DON as the predominant toxin among the different trichothecenes (Lincy et al., 2008). Commonly used methods for trichothecene detection include separation techniques coupled to different detectors such as GC/FID, GC/ECD, GC/MS, HPLC/UV, HPLC/FL, HPLC/MS (Anklam et al., 2002; Klotzel et al., 2006; Laganà et al., 2003; Schothorst and Jekel, 2001; Stroka et al., 2001) but, although very sensitive and precise, they require specialised personnel and are generally expensive. For this reason, much effort has been focused on the development of ‘rapid tests’ for mycotoxin analysis during the last decade (Krska et al., 2007). ELISA kits for DON detection are commercially available as rapid screening tests to check DON content in grains (Haouet and Altissimi, 2003). One drawback is the occurrence of both false positive and false negative results, which necessitate confirmation by HPLC-based procedures of doubtful and/or positive ELISA results (Anklam et al., 2002).
The availability of sensitive and fast methods of analysis that can be used in situ or for decentralised tests is highly desirable. From this perspective, electrochemical methods have shown important advantages compared to traditional methods currently in use because of cost effectiveness, ease of handling and sensitivity (Draisci et al., 2000; Valentini et al., 2003). Moreover, the introduction of screen-printed electrochemical probes as complete electrochemical cells has led to further advantages in terms of production cost and suitability of mass production. However, the electrochemical approach is not feasible for detection of trichothecenes, which are not electroactive and only a few examples of electrochemical detection of trichothecenes have been reported (Hsueh et al., 1999; Palmisano et al., 1981; Visconti et al., 1984). One of these is based on the disclosure that after a hydrolysis step performed in basic solution (Hsueh et al., 1999), group B trichothecenes give rise to electroactive compounds. This, however, is the only example of this approach and to our knowledge there are no other examples in the literature. The objective of this work is to adapt and improve this approach by using both screen-printed electrodes (SPEs) and a new 96-well electrochemical plate. SPEs offer several advantages compared to the classic electrode surfaces usually adopted in analytical chemistry applications. They are in fact inexpensive, easy to produce and very reproducible. This allows to use these sensors as disposables and avoids any problems which usually arise from electrode fouling. The use of SPE also allows for the handling of a very limited sample volume which is always desirable when extraction procedures are employed for sample treatment.

Recently, the use of SPE has also been associated with a new measuring platform which is very similar to the classic 96-well plate used for the spectrophotometric ELISA assay (Delibato et al., 2006; Piermarini et al., 2007). However, in this case the bottom of each well is an SPE which could be connected to an instrument capable of measuring 96 wells in a very limited time. The electrochemical procedure adopted with this format is called Intermittent Pulsed Amperometry (IPA) and consists of the use of small amplitude potential pulses and the measuring of the resulting current at the end of each pulse. The 96-well electrochemical plate combines the advantages already discussed for SPEs (low cost, low sample volume needed, etc.) with the well-known positive characteristics of the 96-well plate, which include the possibility of measuring a wide number of samples in a short time with the possibility of many replicates in the same plate. Moreover, the present paper describes the possibility of adopting a new hydrolysis procedure, which will be based on the microwave technique thus allowing a reduction in the overall analysis time. Results obtained with this new approach will be presented together with preliminary results on wheat samples.

2. Materials and methods

Apparatus

Amperometric measurements were carried out using single SPEs or a 96-well screen-printed microplates obtained from Alderon Bioscience Incorporated (Durham, NC, USA) (Delibato et al., 2006; Piermarini et al., 2007). In this plate, working graphite electrodes (Ø 3 mm) with silver reference electrode, screen-printed on a 0.5 mm plastic substrate, formed the two-electrode system used (Figure 1A). The plate is connected to the electrochemical reader through a 56 dual positions card edge connector. The electrochemical 96-well microplate reader (AndCare 9600) operates using IPA. IPA measurement on the AndCare 9600 sensor reader (Figure 1B) instrument involves a series of millisecond
pulses of the same potential applied individually to each of the 96 sensing electrodes.

Single SPEs were home produced with a 245 DEK (Weymouth, United Kingdom) screen printing machine. Graphite-based ink (Elettrodag 421) from Acheson (Milan, Italy) was used to print the working and counter electrode (Ricci et al., 2003 and references therein). The substrate was a flexible polyester film (Autostat HT5) obtained from Autotype Italia (Milan, Italy). The electrodes were produced in foils of 20. The diameter of the working electrode was 0.2 cm resulting in an apparent geometric area of 0.03 cm². The silver ink was used to print the reference electrode. The single SPEs were used in conjunction with a portable electrochemical instrument (Palmsens handheld, Palmsens, the Netherlands).

The microwave digestion system was an MDS 2000 (CEM, Matthews, NC, USA). This microwave oven has a maximum power of 630±50 Watt and a magnetron frequency of 2,435 MHz. The system was equipped with probes to detect and control the pressure and temperature inside the sealed vessel. The sample (3 ml), was placed in a Teflon PFA digestion vessel (capacity volume 50 ml).

The efficiency of microwaves is strictly related to the chemical-physical characteristics of samples, and to allow uniform microwave distribution and absorption, each cycle of hydrolysis (4 containers) was carried out on samples of the same typology. The vessel cup was screwed manually; the pressure and fibre optic probes were connected to the vessel with the triple ported cap. After the irradiation cycles the vessels were cooled and samples analysed.

The operative conditions used for the microwave hydrolysis of DON and NIV standards are listed in Table 1.

The HPLC analytical system was from Dionex Corporation (Sunnyvale, CA, USA) and employed a Prodigy C18 column ODS3 100Å (5 µm, 250×4.60 mm) from Phenomenex (Phenomenex srl, Castel Maggiore, BO, Italy) and a Rheodyne injection valve (Rheodyne, Cotati, CA, USA) with a 20 µl injection loop. The mobile phase was a mixture of water:acetoneitrile (87:13) and the flow rate was 1 ml/min. A UV detector (Dionex Corporation, Sunnyvale, CA, USA) (Model UV-D170U) was used at λ = 225 nm.

Reagents

All chemicals from commercial sources were of analytical grade. The standard stock solutions of DON and NIV were prepared by dissolving 2 mg of pure toxin in 1 ml of an acetonitrile/water mixture (84:16; v/v) obtaining a final concentration of 2.0 mg/ml. For the construction of the calibration curves DON and NIV standard solutions were prepared diluting the stock standard solution in NaOH 0.1 M + KCl 0.1 M and successively hydrolysed.

DON and NIV pure standards were obtained from BioPure, Biopure Referenzsubstanzen GmbH (Tulln, Austria). Mycosep 227 Trich columns, for trichothecenes of type-A and B clean-up, was from Romer Labs (Romer Labs Diagnostic GmbH, Austria).

Sample treatment

The following sample treatment procedure was adopted when wheat samples were used. (1) 25 g of sample (blank certified sample wheat flour) were added to 100 ml of acetonitrile:water (84:16) and left stirring for 1 hour at room temperature. (2) The sample was then filtered using Whatman no. 4 filters. (3) 8 ml of filtered sample were then refined by passing them over a Mycosep cartridge. For the analysis of the sample it is necessary to remove the acetonitrile by means of a nitrogen flow. To do this 3 ml of the refined sample were dried under nitrogen flow at 60 °C. The dried samples were subsequently re-dissolved with 0.75 ml (minimum volume to perform microwave hydrolysis) of NaOH 0.1 M + KCl 0.1 M (preferred solvent for electrochemical measurements and for the hydrolysis step).

The procedure was designed so as to have a concentration in the final hydrolysis solution equal to the initial concentration in the sample. For evaluation of recovery, samples were spiked before the addition of the solvent with known amounts of toxins while for evaluation of matrix effect, samples were spiked with known amounts of toxins before the drying step.

Table 1. Operative conditions for microwave hydrolysis of DON and NIV standards.

<table>
<thead>
<tr>
<th></th>
<th>First cycle</th>
<th>Second cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Power (% 630 Watts)</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Pressure (psi)</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Run time (min)</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Time at pressure (min)</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>80</td>
<td>80</td>
</tr>
</tbody>
</table>

Sample measurement

Soon after redissolving the sample in NaOH 0.1 M + KCl 0.1 M, the solution containing the toxin was subjected to the hydrolysis step. In this work both the classic hydrolysis procedure (80 °C for 1 h) and the microwave procedure (detailed above) were adopted.

After the hydrolysis step an aliquot of the solution (ca. 80 µl) was pipetted on to the surface of the single SPE
or on the well of the 96-electrochemical plate. In case of the single SPE a Differential Pulse Voltammetry (DPV) technique was used, while for the 96-well electrochemical plate, Intermittent Pulse Amperometry was applied. Both these techniques allow the application of a controlled potential to the working electrode. At a certain applied potential the species deriving from the hydrolysis of type-B trichothecenes are able to undergo a redox reaction thus giving a measurable current signal proportional to their concentration and thus to the original concentration of the trichothecenes.

3. Results and discussion

An alternative approach to circumvent drawbacks related to the use of classic time-consuming and complicate analysis procedures (such as HPLC and GC) would be the use of electrochemical methods which are known to be easy to perform, cost-effective and very fast. It has been reported that after a hydrolysis step in basic solution, group B trichothecenes give rise to electroactive compounds (Hsueh et al., 1999). This means that the hydrolysis of type-B trichothecenes will lead to compounds which can directly be measured with voltammetric techniques without the need for expensive and time-consuming antibody-based methods. This has been exploited to obtain an electrochemical method for type-B trichothecenes. In this paper we took advantage of this disclosure and we employ an advancement based on the use of single disposable SPEs or a 96-well electrochemical plate and of a new hydrolysis procedure. First, the effect of the classic hydrolysis step (performed in basic solution at high temperature) was evaluated in respect of electrochemical signal towards DON and NIV, the most important trichothecenes of the type-B class. The results obtained following a 1 h hydrolysis at 80 °C show a well defined voltammetric peak due to the oxidation of the hydrolysis product of DON and NIV. The signal is readily observable with DPV using a single disposable graphite SPE and shows a peak at around 0.35 V vs. Ag/AgCl consistent with what already previously observed using classic electrodes (Hsueh et al., 1999) (Figure 2A). Moreover, as expected, the signals for DON and NIV are comparable being the hydrolysis product the same for both toxins. Both the medium and pH employed for the hydrolysis step are well suited for the voltammetric measurement and for this reason, and also considering the easiness of operation, they were not changed during subsequent experiments.

When a single SPE was used, DPV shows high sensitivity and a detection limit of 1 µg/ml (defined as the concentration of toxin giving a signal 3 times higher than the mean background signal) with a linear range from 2 µg/ml to 27 µg/ml was observed (Figure 2B). In order to obtain a more feasible method for the detection of trichothecenes in a high number of samples, the use of a 96-well electrochemical plate, comprised of 96 screen-printed sensors (Figure 1) was proposed. This would allow for the measurement of a high number of samples in a limited time. In this case the only voltammetric method applicable is the IPA, which consists of the application of milliseconds pulses of a potential value followed by the measurement of the current produced. First, the best applied potential was selected considering both the sensitivity towards DON and NIV and the possible effect of electrochemical interferences. At the chosen applied potential (0.6 V vs. Ag/AgCl), as expected, for both DON and NIV a sensitivity similar to that obtained with single SPE and DPV technique was observed. A detection limit of 1 µg/ml and a linearity up to 20 µg/ml for both compounds and sensitivity of 408 and 382 nA ml/µg/cm² for DON and NIV respectively was recorded (Table 2).
Optimisation of the microwave hydrolysis step

The use of SPEs and a 96-well electrochemical plate is very suitable for a fast and low-cost approach for type-B trichothecenes detection. However, these experiments were performed using a hydrolysis step based on a high temperature (80 °C) and a relatively long duration (1 h) which severely affects the overall analysis time. For this reason, the possibility of using an alternative hydrolysis step based on the use of microwave, in order to reduce the overall time of analysis, was studied. The rapid heating, obtained with this approach, allows the sample to digest or dissolve in a short time.

The MDS-2000 microwave sample preparation system is operated under full microprocessor control with constant monitoring of the door safety interlocks, temperature, and, if required, pressure, within the digestion vessel. An exhaust tube to vent fumes is permanently connected to an adjacent fume cupboard. As stated in the experimental part, the hydrolysis was performed in 0.75 ml re-dissolved samples. Different times and temperatures were tested and finally a satisfying result was observed with 2 minutes of hydrolysis at 80 °C (the same temperature used with the classic procedure). Our results confirm that the use of microwave leads to a complete hydrolysis thus demonstrating the suitability of this method for this type of application. This has been observed with HPLC instrumentation where the peak due to DON (standard solution) completely disappears after the microwave hydrolysis step (Figure 3). A confirmation result was also obtained with direct electrochemical assay as shown in Figure 4 where the calibration curves obtained with standard solutions of DON hydrolysed following the classic time-consuming hydrolysis procedure and the novel microwave hydrolysis are shown and appear comparable. Results shown in Figure 4 were obtained with the IPA method and comparable results were also obtained with DPV and single SPEs.

Measurement of DON in wheat samples

By using the new optimised hydrolysis technique, wheat samples spiked with known amounts of toxins were used to evaluate the analytical performances of the method. First, the matrix effect was evaluated using blank wheat samples spiked with DON after the sample treatment. Many reports have been published concerning DON extraction procedures from cereals. Nowadays, acetonitrile:water (84:16; v/v), a ratio which was introduced by Chang et al. (1984), is a common solvent used in combination with a clean-up step for the extraction of type-B trichothecenes (Klotzel et al., 2005, 2006). We first evaluated the possibility of avoiding the clean-up step. Soon after extraction, the solvent was evaporated under nitrogen flow and the dried extract resuspended in NaOH (as hydrolysis solvent). Before the hydrolysis step, the extract solution was spiked with different amounts of DON and the signal was evaluated and compared with that observed with DON standard solution. The need for a drying step is due to the fact that the presence of a high amount of organic solvent negatively affects the response of the SPEs and, because of the detection limit achieved with this method (1 µg/g), it is not possible to perform any dilution of the extraction solvent. The results

<table>
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<tr>
<th>Analyte</th>
<th>Detection limit (µg/ml)</th>
<th>Working range (µg/ml)</th>
<th>Sensitivity (nA.ml/µg/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DON</td>
<td>1.1±0.1</td>
<td>2-20</td>
<td>408±25</td>
</tr>
<tr>
<td>NIV</td>
<td>1.0±0.1</td>
<td>2-20</td>
<td>382±20</td>
</tr>
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</table>

Table 2. Analytical parameters obtained for DON and NIV detection using IPA (with a 96-well electrochemical plate).

Figure 3. HPLC confirms the success of the microwave hydrolysis. Shown are chromatograms of DON standard (25 and 50 µg/g) before (A, B) and after (C, D) the alkaline microwave hydrolysis procedure.

Figure 4. The time-effective microwave hydrolysis procedure leads to sensitivity comparable to that obtained with the traditional procedure. The graph shows the curves obtained with DON hydrolysed using a traditional procedure (80 °C for 1 h) and the microwave hydrolysis (3 min, 80 °C) with the IPA method.
obtained with wheat extract solution (without clean-up step) showed an average recovery (calculated comparing the results in the extract solution and that in standard DON solution) of less than 50%, thus accounting for the possible presence of electrochemical interfering species in the extract solution.

This result demonstrates the need for a purification step in the extraction procedure. In this case the use of Mycosep was proposed as the best compromise for a rapid and effective clean-up of crude extracts for the analysis of trichothecenes. The results obtained are extremely satisfactory for the clean-up approach chosen showing a similar slope for standard solutions and fortified samples (Figure 5) and thus demonstrating the low matrix effect. However, the two curves showed a slightly different y-intercept demonstrating the presence of possible endogenous electrochemical interference.

After this study the recovery of the developed method was evaluated by spiking blank wheat samples before the sample treatment. Results obtained with 3 different DON levels were satisfactory in terms of mean recovery and demonstrated the applicability of the method for real samples measurements (Table 3).

![Graph of graph of recoveries](image)

Figure 5. The clean-up approach chosen showed a similar slope for standard solutions and fortified samples thus demonstrating a low matrix effect.

Table 3. Recovery values obtained with certified blank wheat samples spiked at different concentrations of DON (n=4).

<table>
<thead>
<tr>
<th>DON expected (µg/g)</th>
<th>DON found (µg/g)</th>
<th>Recovery %</th>
</tr>
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<tbody>
<tr>
<td>2.0</td>
<td>2.1±0.1</td>
<td>105±5</td>
</tr>
<tr>
<td>4.0</td>
<td>4.1±0.1</td>
<td>102±3</td>
</tr>
<tr>
<td>8.0</td>
<td>6.6±0.1</td>
<td>83±2</td>
</tr>
</tbody>
</table>

4. Conclusions

The present study shows for the first time the use of a 96-well electrochemical plate for the determination of DON and NIV in wheat samples. The use of a microwave hydrolysis procedure for the production of electroactive compounds from DON and NIV was proposed coupled with SPEs for the sensitive and fast detection of type-B trichothecenes.

An extraction procedure with aqueous acetonitrile and a clean-up step was demonstrated to be suitable for the application with wheat samples providing good sensitivity and an optimal working range for the determination of DON and NIV in cereals for feed consumption. However, it should be pointed out that the sensitivity of the present method does not allow its use with samples for human consumption according to EC Regulation No 1881/2006 that lays down levels from 200 µg/kg (processed cereal-based foods and baby foods) to 1,750 µg/kg (unprocessed durum wheat and oat) (EC, 2006a). Moreover, the method is not able to distinguish between DON and NIV, which could be a further problem. Further optimisation of the electrochemical approach and of the sensitivity of this method is presently under investigation for the application with other cereal-based samples for human consumption.

Although the sensitivity of the proposed method still requires an optimisation for its practical application with cereal-based food for human consumption and the presence of other drawbacks (i.e. selectivity), its possible use as a screening tool for feed samples presents several advantages over other screening approaches. A direct electrochemical measurement which does not involve the use of any biological recognition element (antibody) seems particularly suitable for a portable, easy-to-handle and fast tool not affected by stability problems which sometimes afflict immunoassays. Also, the use of an immunosensor, even if it is often accompanied by a good sensitivity, is usually accompanied by several drawbacks. A calibration curve is always needed to obtain a final result and the measurement procedure is complicated by several immunoassay steps (Ricci et al., 2007). Usually, in fact, depending on the immunoassay format employed (sandwich or competitive) two to three incubation steps are required leading to an overall time-consuming procedure. The use of a straightforward electrochemical platform and the coupling of a 96-well electrochemical plate allows the processing of a high number of samples in a limited time thus overcoming these drawbacks. Moreover, the optimisation of a fast microwave hydrolysis procedure is a further element which makes this approach particularly advantageous. As a rapid and reliable method which can give a yes/no answer or semi-quantitative results, the proposed method represents a valuable alternative to classic immuno-based approaches with promising features for...
practical application. Further optimisation of this approach is under investigation for its application with cereal-based products for human consumption.

Acknowledgements

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References


