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Development and validation of a liquid chromatography/linear ion trap mass spectrometry method for the quantitative determination of deoxynivalenol-3-glucoside in processed cereal-derived products

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ABSTRACT

Cereal-based food can be frequently contaminated by the presence of mycotoxins derived from *Fusarium* fungus, and, in particular, by deoxynivalenol (DON). Nowadays, analytical strategies for the detection of DON are well developed, but there are gaps for what concerns a correct identification, quantification and toxicological evaluation of the respective metabolites, mainly related to detoxifying actions via plant metabolism or to processing technologies and also referred to as "masked" mycotoxins.

Here, we report the development of a liquid chromatography/linear ion trap mass spectrometry method capable of determining deoxynivalenol-3-glucoside (DON-3G), which is the main known DON metabolite, in different processed cereal-derived products. Samples were extracted with a mixture of methanol/water (80:20; v/v) and cleaned up using immunoaffinity columns. Chromatographic separation was performed using a core–shell C_{18} column with an aqueous acetic acid/methanol mixture as the mobile phase under gradient conditions. The method was in-house validated on a bread matrix as follows: matrix-matched linearity ($r^2 > 0.99$) was established in the range of 10–200 μg kg^{-1}; trueness expressed as recovery was close to 90%; good intermediate precision (overall RSD < 9%) and adequate detection quantitation limits (4 and 11 μg kg^{-1}, respectively) were achieved. Furthermore, applying a metrology approach based on intralaboratory data, the estimated measurement expanded uncertainty was determined to be equal to 29%. The reliability of the method was finally demonstrated in bread, cracker, biscuit and minicake commodities, resulting in relatively low levels of DON-3G, which were not higher than 30 μg kg^{-1}.

1. Introduction

Mycotoxins are low molecular compounds, produced by the secondary metabolism of fungi able to attack a wide group of cereals, such as wheat, maize, barley, oat and rye. They can exert toxic and/or carcinogenetic actions in animals and humans, in some cases even at low concentrations close to μg kg^{-1} levels, (Bennet & Klich, 2003; Hussein & Brasel, 2001; Sudakin, 2003) in food and feed. Weather conditions, agricultural practices, and other environmental and contingent factors can strongly influence their incidence in food crops from year to year.

Deoxynivalenol (DON) is the most important toxin for wheat and wheat-derived products due to its worldwide occurrence and potential toxicological impact on animal and human health through immunomodulation, protein synthesis inhibition, ribosome disaggregation, mitochondrial function compromise, lipid peroxidation, and cellular death (Prelusky, Rotter, & Rotter 1994).

Mycotoxins can be covalently or non-covalently bound to phenolics, proteins, cell wall polysaccharides, lipids and lignin (Lamourex & Rusness, 1986). Cereal crops exposed to deoxynivalenol (DON) infection are capable of detoxifying this mycotoxin through plant metabolism. One of the most important resistance mechanisms is the detoxification of DON into 3-ß-D-glucopyranosyl before compartmentalisation of this metabolite into vacuoles or cell walls (Poppenberger et al., 2003).

Approximately 600 million tons of wheat is produced per year worldwide, and most of the wheat is converted to wheat flour for human consumption and processed into various foods, such as breads, pastas, noodles, and cakes (Pacin, Ciancio Bovier, Cano, Taglieri, & Hernandez Pezzani, 2010).

Fusarium toxins are generally quite stable during commercial food processing (Masayo, 2008) and cannot be completely eliminated, which leads to potential contamination of finished cereal-based foods.

To protect consumers from an unacceptably high dietary intake of trichothecenes, maximum levels have been set in EU legislation (European Commission, 2006). For example, deoxynivalenol

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maximum levels are currently regulated in cereals (1250 μg kg^{-1}), flour (750 μg kg^{-1}) and bread (500 μg kg^{-1}).

There is an increasing focus at the worldwide level in the research field related to the occurrence and modifications of mycotoxins during typical bakery technological processes (Numanoglu, Uygun, Koksel, & Solfrizzo, 2010; Bullerman & Bianchini, 2007).

Many factors (e.g., temperature, pH, enzymatic activities, food macroconstituents and food microconstituents) must be considered during technological processes because complex physicochemical modifications occur during the transformation of raw ingredients into the final product. Mechanical or thermal energy may have an important role in the masking mechanism inducing reactions with macromolecular components, such as sugars, proteins or lipids, as well as releasing native forms.

Investigations on the stability of DON during food processing have been reviewed (Kushiro, 2008), and studies on the stability of DON during the production of flour-based foods have been recently published (Bergamini et al., 2010; Lancova et al., 2008a,b; Voss & Snook, 2010). DON reduction is related to a combination of dilution by recipe ingredients and processing effects; moreover, changes in DON concentration may be associated with the conjugation and/or liberation from masked forms. Assuming a preventive approach, masked mycotoxin toxicity can be reasonably attributed to one of the correspondent native mycotoxin forms because they can potentially release an additional amount of the parent mycotoxin after hydrolysis in the intestinal tract during digestion or because of technological processes during the transformation of raw materials into finished food products. Furthermore, it cannot be excluded that these metabolites exert toxicity themselves and, therefore, more information is needed for the fate of mycotoxins during production to estimate their real dietary intakes.

For the first time, the Joint FAO/WHO Expert Committee on Food Additives expressed its opinion on bound forms of deoxynivalenol in 2010 concluding that 3-acetyl-deoxynivalenol (3-Ac-DON) contributes to the total DON-induced toxicity, while indicating that more data should be collected before the potential future inclusion of DON-3-glucoside in the PMTDI group (1 μg kg-1 bw).

The co-occurrence of DON and DON-3G in cereal products has previously been reported in recent scientific literature showing few conflicting data; reliable analytical methods, standards and data regarding their absolute and relative concentrations are still lacking. Glucoside conjugates occur naturally, and they have been found in wheat at concentrations of 12% (Berthiller et al., 2005), 46% (Berthiller, Schuhmacher, Adam, & Krska, 2009) and slightly more than 100% as compared to concentrations of DON (Sasanya, Hall, & Wolf-Hall, 2008). Other authors have proposed (Liu, Walker, Hoegleninger, & Buchenauer, 2005; Zhou, He, & Schwarz, 2008) a method to determine bound DON in fusarium head blight-infected grains using solvolysis with trichloroacetic or trifluoroacetic acid treatments, observing that in samples which exhibit a significant increase with respect to free DON (analyzed by common extraction methodologies), bound DON represents an additional relevant percentage up to 10–20%; very recently (Tran & Smith, 2011) a similar hydrolysis procedure, applying trifluoromethanesulfonic acid, conduced to obtain an increase of DON determined in wheat comprised between 7% and 75%.

Focusing the attention on the development of methods specifically devoted to quantify DON-3G in cereal-based foods by LC–MS, in the last two years Vendl et al. (Vendl, Berthiller, Crews, & Krska, 2009; Vendl, Crews, McDonald, Krska, & Berthiller 2010) proposed an approach based on a simple extraction procedure combined with a hybrid mass spectrometry instrument (QTrap), and they concluded that the concentration of DON-3G (and other deoxynivalenol-conjugated or zearalenone-conjugated forms) is low and, therefore, not significant in terms of harming consumers: the absence of any cleanup in the sample preparation phase, even if associated with the exploitation of high performance mass spectrometry instruments may introduce some uncertainty about the accurate quantification of the investigated masked forms. After this published way of measuring, in the current year Desmarchelier and Seefelder (Desmarchelier & Seefelder, 2011) applied an extraction procedure based on a QuEChERS-like method (originally developed for the extraction/purification for pesticide analysis), and they found that the proportion of DON-3G in relation to DON concentration is within a range of 6–29% with an average value of 12 ± 7%.

The exploitation of the innovative ultra high performance liquid chromatographic (UHPLC) and high resolution mass spectrometry (TOF-MS) solutions has been recently demonstrated as being effective for the study of DON-3G in cereal matrix products, such as malt and beer (Zachariasova et al., 2010), but there are obvious drawbacks, including the high costs in terms of investments and skilled technicians, especially for industrial needs. Therefore, considering the increasing availability of less expensive instrumentation, strengthening the potential applicability in control laboratories, the aim of the present work was to develop and validate a reliable linear ion trap LC–MS/MS analytical strategy for the determination of DON-3G.

The application of an antibody-based cleanup (IACs) phase for the purification and a core–shell C_{18} column for the chromatographic separation guarantee adequate selectivity/sensitivity to evaluate relatively low levels of this masked form in complex food matrixes.

2. Materials and methods

2.1. Chemicals and reagents

A deoxynivalenol-3-glucoside (DON) standard solution (50 μg L^{-1} in acetonitrile) was purchased from Biopure Referenzsubstanzen GmbH (Tulln, Austria). The stability of DON-3G stock solutions in acetonitrile can be estimated in terms of 6–8 months when stored in the dark at 4 °C or below, the correspondent standard calibration solutions (by evaporation/dilution into acidified water /bread matrix extract solvent) were freshly prepared each week and have demonstrated to be stable 2–3 days at room temperature.

Methanol (HPLC grade) and ammonium acetate (puriss. p.a.) were obtained from Merck (Darmstadt, Germany). Acetic acid (HPLC grade) was obtained from BDH VWR International Ltd. (Poole, England). Phosphate buffered saline (PBS) was purchased from Sigma–Aldrich (Milan, Italy). Immunoaffinity columns (DON Neocolumn 8340) were obtained from Neogen (Lansing, MI, USA). Glass vials with septum screw caps were purchased from Phenomenex (Torrance, CA, USA). Deionised water was used for all procedures. Paper filters (597 ½, code 10311847, diameter 90 mm) and glass microfibre filters (code 1820-030, diameter 90 mm) used during extraction and PBS dilution steps were purchased by Whatman (Whatman, Springfield Mill, UK). Centrifugal filter units (Ultrafree MC 0.22 μm, diameter 10 mm) were obtained from Millipore (Millipore, Billerica, MA USA).

DON analytical measurements have been performed according to a previously published procedure (Suman & Catellani, 2008).

Handling and safety procedures for DON and DON-3G standards and solutions, together with extraction and clean-up steps, prefigure the use of gloves and manipulations under hood; decontamination of all volumetric flasks and vials containing concentrated sample extract or reference standard solutions has been performed by 2% v/v sodium hypochlorite solution treatment (Mycotoxin prevention and control in food grains, FAO, 1990).