

FEASIBILITY STUDY FOR THE DEVELOPMENT OF NEW REFERENCE MATERIALS FOR MYCOTOXIN ANALYSIS IN CEREALS

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Abstract – This paper reports a feasibility study for the development of new Reference Materials (RMs) for mycotoxin analysis in cereals. In particular, different batches were prepared starting from lots of un-contaminated durum wheat grains, durum wheat flour, soft wheat grains, and soft wheat flour. Then, an aliquot of each raw material was artificially inoculated with *Fusarium graminearum*, so as to obtain DON and Ac-DON contamination. By mixing in different ratio the mycotoxin contaminated materials and the un-contaminated materials, test batches of (durum and soft) wheat grains and flour at three different levels of DON and Ac-DON (*zero level, low level, high level*) have been developed. Afterwards the characterization of the materials was carried out and stability and homogeneity studies have been started.

Keywords: Reference Materials, mycotoxins, DON, Ac-DON, wheat

1. INTRODUCTION

Mycotoxin contamination in food and feed represent one of the more relevant food safety issues. Mycotoxins are secondary metabolites produced in food and feed commodities by pathogenic moulds, mainly belonging to the genera *Aspergillus*, *Penicillium* and *Fusarium*. Mycotoxin contamination may occur at all stages of the production chain (cultivation, harvest, storage, transformation). Among crops, cereals are mainly affected by this contamination. Mycotoxins are naturally produced xenobiotics and their occurrence is due to geographical, environmental and climatic factors, as well as to agronomical conditions applied during cultivation, harvesting, storage and transportation.

Other noteworthy mycotoxins are produced by *Fusarium* and they are of great concern for animal and public health: these substances are structurally unrelated compounds produced mainly on grains, in particular corn and wheat. Among the main *Fusarium* metabolites, fumonisins, zearalenone (ZEA) and trichothecenes are the most important for food safety, on account of their toxicity towards animals and humans and of their broad worldwide diffusion. *Fusarium culmorum* and *F. graminearum* are able to colonize grains in field and to produce both trichothecenes and ZEA. In particular, deoxynivalenol (DON) is the most commonly worldwide occurring trichothecene in wheat, barley and corn.

Mycotoxines represent one of the classes of chemical substances on which prior attention was to be focused identified by the WHO. In particular, the following mycotoxines are included in the GEMS¹/Food comprehensive list of priority contaminants (last update, December 2011): aflatoxins - milk and dairy products, corn, cereals, peanuts, nuts, spices, dried figs and total diet; ochratoxin A (OTA) - wheat, cereals, wine; DON - wheat and cereals; patulin - apple juice; fumonisins - corn and wheat. On account of the acute and chronic health risk associated with the intake of all the cited mycotoxins, European Union set the maximum levels of Aflatoxins, OTA, DON, ZEA and fumonisins in foodstuffs [1]. In addition, European Commission established the maximum levels of contaminants, including mycotoxins, permitted in feed [2] and adopted a two-step approach due to the wide year-to-year variation and the limited carry-over from food to feed of *Fusarium* toxins and OTA [3].

The accurate determination of mycotoxins in cereals and derivatives is generally carried out by chromatographic

¹ Global Environmental Monitoring System/Food Europe - http://www.who.int/foodsafety/areas_work/chemical-risks/gems-food/en/

methods. These methods although guarantee high sensitivity and specificity, aren't eligible as early screening methods but they are mainly used as confirmatory methods. In order to satisfy the industry demand of test methods for application on routine scale, researchers and commercial companies, are developing sensors and devices "fit for purpose" based on different techniques (DNA amplification, antibody/aptamers, NIR, electrokinetic separation techniques, etc). These new systems require a large availability of Reference Materials (RMs) so as to ensure metrological comparability and traceability of measurements. In particular, RMs are required for conducting interlaboratory tests and assessment precision of the methods and Certified Reference Materials (CRMs) are necessary for calibration, uncertainty evaluation and assuring metrological traceability.

According to BCR and ISO Guides [4,5], a feasibility study for the preparation of new *Matrix*-RMs for mycotoxin analysis in cereals was conducted. With this purpose, different test batches of durum wheat grains, durum wheat flour, soft wheat grains, and soft wheat flour at different concentration levels of DON and Ac-DON (*zero level, low level, high level*) were prepared. Mycotoxin contamination was obtained by artificial inoculum with *Fusarium graminearum* and the different concentration levels were obtained by different dilutions of the contaminated materials with the un-contaminated ones.

2. MATERIALS AND METHODS

Different batches of candidate RMs were prepared at the *Agrofood RM Plant* located at the ENEA Trisaia Research Centre (in South Italy) starting from lots of about 50 kg of un-contaminated durum wheat grains (DG), durum wheat flour (DF), soft wheat grains (SG), and soft wheat flour (SF) supplied by Barilla G. e R. Fratelli S.p.A in September 2014. Raw materials were homogenized by a Vrieco Nauta ® Conical Mixer (V = 240 l – Fig. 1) equipped with a dosing valve, with the following order of processing: SG, DG, DF, SF.



Fig. 1. Vrieco Nauta ® Conical Mixer (V = 240 l)

After homogenizing each different matrix, the dust remained on the Nauta inner walls was removed by blowing N₂; after processing the DF, a complete washing with deionized water was performed. In all cases, the Nauta was at first rinsed with an aliquot (about 5 kg) of the material to be processed; rinsing was carried out maintaining the Nauta in rotation for 15 minutes and collecting and reloading multiple times the same material. The aliquot employed for cleaning was then put aside. Therefore the Nauta was loaded with all the available material (about 45 kg), leaved in rotation for 15 minutes, and then different aliquots were collected in plastic bags by the dosing valve, always maintaining rotation. For each different matrix 10 aliquots were collected by the following sequence: two aliquots of about 8 kg, five aliquots of about 1 kg, three aliquots of about 8 kg. The aliquots were labelled as follows: RM-SG(1÷8), RM-DG(1÷8), RM-DF(1÷8), RM-SF(1÷8). All bags were tared and gross weights were registered. Bags were sealed under vacuum and sent to the ENEA Casaccia Research Centre (near Rome), where were stored at T = - 80 °C. For each raw material, two bags of 1 kg were sent to UNICATT (Piacenza, Italy) where artificial inoculation with *Fusarium* was performed. At the beginning of the study, the strain *Fusarium graminearum* (ITEM 8585) able to produce DON and Ac-DON, obtained from the culture collection of the CNR Institute of Sciences of Food Production (ISPA-CNR, Bari, Italy), was used. Subsequently, other strains *Fusarium culmorum* of the same collection (ITEM 6221, 6234, 6264) were tested, because they can produce higher amounts of DON and Ac-DON [6]. Toxin production by the *Fusarium* strains was conducted on autoclaved wheat grains. Wheat grains were moistened with sterile distilled water for 4 days at 4°C until thermodynamic water activity was maximal. Then, 100 g of grain was distributed into a 500-ml Erlenmeyer flask and sterilized twice for 25 min at 110°C [7]. Each aliquot was inoculated with a suspension of 1 x 10⁶ conidia/ml and incubated at 25°C for 24 days with daily manual shaking for the first 4 days of inoculation in order to homogenize the inoculum.

Ground wheat and flour contaminated were assayed for DON and Ac-DON concentration by competitive enzyme immunoassay using Ridascreen fast Don kit (R-Biopharm AG, Darmstadt, Germany), with detection limits of < 0.2 mg/kg [8]. After reaching the desired degree of contamination (about 100-200 ppm), a part of the test batches were sealed and sterilized in autoclave (120 °C; 15 min), while a part to be subsequently treated with radiosterilization was only sealed. All samples were sent to the ENEA Casaccia Research Centre, where were stored at T = - 80 °C.

By mixing in different proportions the contaminated aliquots with the un-contaminated ones, test batches of the materials at three different concentration levels (*zero level – L0, low level – L1, high level – L2*) of DON and Ac-DON are being obtained. In particular, starting from DG and DG contaminated with ITEM 6221 at a DON and Ac-DON concentration of 200 ppm, a test batch of homogeneous durum wheat grains (H-DG) and a test batch of homogeneous milled durum wheat grains (M-DG) were prepared, at the three different contamination level. For homogenization, a Vrieco Nauta ® Conical Mixer (V = 10 l) was employed. About 6.5 kg of durum wheat grains picked up from the RM-DG(2) aliquot were homogenized for about

2 h at 35 rpm and then partitioned in 16 aliquots, 8 of which were then partitioned in 8 sub-aliquots of about 50 g (tot. 64 sub-aliquots of homogeneous DG L0). About 6.5 kg of durum wheat grains picked up from the RM-DG(8) aliquot were mixed with about 44 g of DG contaminated with ITEM 6221 for obtaining the L1 batch at a DON and Ac-DON concentration of about 1.35 ppm, while about 6.5 kg of durum wheat grains picked up from the RM-DG(9) aliquot were mixed with about 70 g of DG contaminated with ITEM 6221 for obtaining the L2 batch at a DON and Ac-DON concentration of about 2.16 ppm. Each aliquot was subsequently partitioned in 16 aliquots, 8 of which were partitioned in 8 sub-aliquots of about 50 g (tot. 64 sub-aliquots of homogeneous DG L1 and 64 sub-aliquots of homogeneous DG L2). The homogeneous sub-partitioning was obtained by means of a Fritsch rotary sample divider Laborette27. In all cases, the remaining 8 aliquots were milled at 0.5 mm, grouped together and homogenized again with the Vrieco Nauta® Conical Mixer and then partitioned in 6 aliquots, each of which was the partitioned in 8 sub-aliquots. In this way, 48 sub-aliquots of Milled Durum Grain (MDG) at the three different levels (L0, L1 and L2) were obtained. For milling the durum wheat grains, a Retsch Ultra Centrifugal Mill ZM 200 with a 12 teeth push-fit rotor and 0.50 mm trapezoid holes, at 10000 rpm was employed.

The obtained aliquots (Fig. 2) were stored at $T = -80\text{ }^{\circ}\text{C}$. They are being used for the material characterization and for homogeneity and stability studies.



Fig. 2. Aliquots of Durum Wheat Grains (DG) and Milled Durum Wheat Grains (MDG)

In order to better characterize the DON profile in the contaminated batches of samples, a hyphenated analytical method will be used. By means of an Ultra High Performance Liquid Chromatography associated with an Orbitrap mass spectrometer Q-Exactive, the DON and its two acetylated forms, namely 3-AcDON and 15-AcDON, will be detected and quantified. The sample pre-treatment will be performed by testing a QuECHERS based extraction method and an Immunoaffinity column extraction method (DONPREP, R-Biopharm; cross-reactivity of more than 85% for both metabolites).

Homogeneity studies are underway for the test batches of both DG and MDG at the three different levels of DON and Ac-DON (L0, L1, L2).

Furthermore, effects of radiosterilization are being evaluated by submitting a few aliquots to γ -ray irradiation. For this purpose the ENEA's Calliope Plant equipped with the ^{60}Co γ source) was employed. In particular: 1 aliquot of L0-M-DG, 1 aliquot of L1-M-DG and 2 aliquots of L2-M-DG were irradiated at 10 kGy, so to simulate the maximum

admitted irradiation dose for food [9], while 3 aliquots of L2-H-DG, 2 aliquots of L0-M-DG, 2 aliquots of L1-M-DG and 12 aliquots of L2-M-DG were irradiated at 25 kGy, so to reach the common radiation dose for the obtainment of sterility. Irradiated aliquots will be analysed for DON and Ac-DON contents and for microbiological profiles, in comparison with un-irradiated aliquots of the same test batch, so to evaluate the effects of radiosterilization both on the mycotoxins and on the matrix.

Isochronous stability tests will be carried out under thermal (at different temperatures) and luminous stress on the materials both in grains and milled. The stability of the materials stored in different packaging (glass bottles, plastic bottles and bottles into vacuum bags), autoclaved or not, dehydrated or not, radiosterilized or not will be compared.

3. FURTHER DEVELOPMENTS

This feasibility study is preparatory to the creation of larger batches of RM to be certified for the content of DON and Ac-DON. Furthermore, as further development of this work a new feasibility study for the development of a *driven*-RM will be conducted in the next season, performing the inoculation directly in field.

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