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# PHD THESIS

## TOXIC POTENTIAL OF PLANT SUBSTRATES USED IN HUMAN AND ANIMAL FOOD EVALUATED BY MYCOLOGICAL AND MYCOTOXINIC INVESTIGATIONS

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# SUMMARY

**Kej words**: micromycetes, mycotoxins, nutrients, risk, control

The PhD thesis entitled **„Toxic potential of vegetal substrates used in human and animal food evaluated through mycological and mycotoxinic investigation**" fills in a number of 272 pages and is structured, according to the current regulations, in two main parts. The first part entitled: "**The current stage of knowledge**" is elaborated on 69 pages, containing 7 tables and 15 figures. In what concerns the second part, entitled of "**Personal research**", it is spread on 194 pages, containing 28 tables and 64 figures, in order to offer a synthetic and representative display of the obtained results.

In the first chapter, entitled **"Biological characterization of the main types of fungal contamination of substrates involved in plant contamination**", an overview from speciality upon data from speciality literature are presented, with general considerations concerning the micromycetes, with a review underlining the dominant types of toxigenic micromycetes that frequently contaminates the food substrates, and especially the biological characterization of some species from the Aspergillus, Penicillium and Fusarium types, and the importance of the diseases they determine.

The second chapter entitled "**Mycotoxin contamination of plant substrates evaluated in terms of maximum allowable**" is structured in two parts that present data from the speciality literature, referring to the incidence of mycotoxins in the vegetal substrates and in animal products, briefly describing the mycotoxins involved more frequently in human and animal pathology, and reviewing the legislation concerning the control of residuals from mycotoxins.

Mycotoxins, are major risk factors for human and animal health and they are spread everywhere. They were observed in cereal grains (corn, wheat, barley, oatmeal, rice, sorghum, rye) and in leguminous (peanut, soy, beans, peas), in all their products and plant-derivate (bread, dough, cakes, corn bread), in oil seeds (sun flower, pumpkin, walnut), in fresh or preserved vegetables and fruits (carrot, pepper, parsley, apple, pear, quince, citrus, figs, kiwi, peaches, apricots), in already made products (compote, jam, jelly) and natural juice, in beer, grape must, wine and cider, in spices (green, black and white pepper, paprika, curry, oregano, thyme) and in herbs (St John's Wort, plantain).

In the final chapter of this part - entitled "**Current analytical methods of isolation, identification and assessment of mycotoxins"** are described the methods used to determine the

residuals from mycotoxins and the analytical procedures available in this field. Since the discovery of mycotoxins, a lot of determination methodologies for them were developed.

The currently used methods for highlighting and quantitative determination of mycotoxins are based on the principle of chromatographic techniques, more important among them being the thin layer chromatography (CSS), the high performance liquid chromatography (HPLC) combined with different types of detectors (UV, DAD, MF) or mass spectrometry (MS), the gas-chromatography (GC), combined with electron capture detectors (ECD), with flame ionization (FID) or detectors of MS type.

In recent years, the liquid chromatography combined with mass spectrometry (LC-MS) gained a dominant status because of the advantages it presents, firstly because of the possibility of simultaneous determination of different categories of mycotoxins (Krska and col., 2008, Rahmani and Soleimany, 2009, Maragos and Busman, 2010)

The second part of the PhD thesis represents almost 70% from its total volume. It is structured in 10 chapters that include the aim and objectives of the undertaken investigations, the working materials and working methods, the institutional frame that allowed the research, as well as the research results and their interpretation in conformity with the consulted references.

The undertaken research had a stage-focused development, following with strictness the individual working plan, in which the results of a stage constituted the objectives of the following stage.

The quality of vegetal substrates used in human and animal food were appreciated through mycological and mycotoxinic examinations.

For this purpose, a number of 159 of samples were taken, among which cereal grains (corn, wheat, rye, barley, oatmeal), oil seeds (soy, sun flower, peanut), coffee grains, chickpeas, beans, pistachio, sunflower and rapeseed meals.

The degree of fungal contamination of the 87 samples representing vegetal substrata, that usually enter in human food, was very high, even if the number of units forming colonies (UFC/g) varies within very large limits, with minimal values that confer them the status of "micromycetes free", and with maximal values of 1450 x $10^3$ UFC/g.

Corn and wheat fodder grains, but above all compound feed have constantly exceeded the accepted maximum limit of 50x$10^3$UFC/g.

The fungal flora that dominates food products usually entering animal food is represented by species belonging to Penicillium (73.56%), Aspergillus (52.87%), Cladosporium (41.38%) and Fusarium (34.48%) types. The micromycetes isolated and identified from fodder had extremely large variation limits, and the species belonging to the types Aspergillus (58.33), Penicillium (43.06)%, Fusarium (36.11%) and Cladosporium (15.89%) proved to be dominant.

The species *Cunninghamella CN012*, as well as the toxigenic strain *Penicillium purpurogenum* were isolated and identified for the first time in our country.

We presented them under the form of synoptic tables, the macro and microscopic morphological characters of species differentiation, for those species taxonomically framed within the types of Penicillium, Aspergillus and Fusarium.

The examinations undertaken in a dark field with UV light, for the evaluation of toxic food and fodder potential allowed the identification of 11 micromycetes strains. They proved to be fluorescent in UV light, with α=366nm, and five of them proved to be capable of synthesizing mycotoxins of the aflatoxins B1 and B2, ochratoxin A and sterigmatocistine type. These toxigenic strains isolated from food substrates have proved that the food and fodder infested with toxigenic fungi may become harmful for human and animal health. In order to highlight and quantify the existing mycotoxins in various food and fodder we used relatively simple techniques of screening (the thin layer chromatography or the competitive type ELISA technique), but also reference methods (HPLC).

The results of the mycotoxinic examination of the 87 food samples examined through chromatographic screening (CSS) revealed that 29.8% samples were contaminated with aflatoxin, ochratoxin, zearalenone and sterigmatocistine. From the 72 analyzed fodder samples, 48.61% were contaminated with mycotoxins (aflatoxin, ochratoxin A, zearalenone and sterigmatocistine), the most affected among them being the corn grains and the compound fodder.

For greater sensitivity analysis, high performance techniques are recommended, as HPLC-FLD and derivatization (for aflatoxins).

We analyzed the toxic effects of mycotoxins through the experimental reproduction of the broiler chickens, poisoned with deoxynivalenol, ochratoxin A and aflatoxin B1. The effects induced by the poison were reflected in the heterogeneity of the chickens from each lot and in the fact that they were daily losing weight.

The organs involved in the direct mycotoxinic metabolization were taken and analyzed through ELISA and HPLC-FLD, in order to rediscover the ingested mycotoxins.

Thus, if the initial average body weight of chickens included in the group that ingested OTA was 76.5 g and the control group to 75.8 g, after 7 days the difference between chickens was that of 85.83 g in favor of the control group. After another 21 days, period in which they received about 371.68 mg OTA / kg b.w the chicken from the control group had an average weight of 420.1 g, while those taking part of the intoxicated group with. 226g,with a difference of 194.1 g per chicken. The same dynamic was also found disproportionate to those groups of chickens that received aflatoxin B1, more precisely DON.

The toxic potential of the organs and tissues taken from the experimentally poisoned chickens with deoxynivalenol (DON) was evaluated through ELISA technique.

The working technique was improved by the introduction of a degreasing stage with petroleum ether of the liver and kidneys samples taken from the experimentally poisoned chickens. The results clearly revealed the presence of DON in the tissue structures of the poisoned organisms, but with sensitivity deficiencies depending on the extraction variant used in the process.

The extracts of samples examined through ELISA, using an improved technique with a supplementary degreasing stage, highlighted a greater sensitivity, in the sense that the samples were sensitive 100%, with detectable concentrations of DON both in the liver, where these oscillated between 28.28 ppb and 38.61 ppb, and in the kidneys, where the variation limits ranged between 22.14 and 48.40 ppb.

We have determined the tissue residuals (from liver and kidneys) of ochratoxin A, using the commercial pack RIDASCREEN OTA and the HPLC method with fluorescence detector. The obtained results analyzed comparatively highlight significant differences regarding the degree of contamination with OTA of the same substrates. Thus, in the kidneys, OTA was quantified in all of the 10 analyzed samples, but through the imunoenzymatic test the quantities are much greater, oscillating between 1.760 and 5.122 ng/g, than the ones detected through HPLC-FLD, where the variation limits ranged between 0.496 ng/g and 1.465 ng/g. In the liver the differences are even more striking because the HPLC method has eliminated 6 OTA positive samples. In four liver samples the detectible octatoxin residuals ranged between 0.430 and 0.937ng/g.

For the determination of mycotoxin residuals from the liver and kidneys samples of the experimentally poisoned chickens, we have used commercial packs RIDASCREEN® AFLATOXIN $B_{1.}$

The obtained results indicated a maximum level of $AFB_{1,}$ of 464.92 ± 6.2 ng / mL, after 21 days of acute intoxication, in the chickens liver from group E1 and only of 15.59 ± 6.1 ng / mL in the chickens' liver from group E2, at the end of the experiment. In the kidneys we have detected 500ng/g mycotoxin at the chickens poisoned for 21 days.

During a comprehensive analysis they were shown the advantages and disadvantages presented by different analytical methods (TLC, GC, ELISA, HPLC, LC-MS) and other less invasive methods. From the bibliography I have analised they tend to abandon higher risk pollutant techniques such as CSS, in favour of the reference ones.

The thin layer chromatography may serve both as a purifying step and of quantification one, but many factors may affect in a negative way the separation and quantification CSS. The

CSS technique suggested by Coman and col, 1978, for the rapid screening of a big number of samples, assures a good fluorescence to mycotoxins, and the present study comes to complete it from the point of view of the approximate quantitative estimation.

According with the speciality literature, a quantity of approximately 0.5 ng⁄µL can be detected in a spot with a minimum of fluorescence, be it visually or instrumentally (AOAC, FAO).

For the determination of the detection limit of the technique and the achievement of the standard curve for the ochratoxin A and aflatoxin B1 to the starting line in the middle of each migration lane, we spotted in duplicate known quantities of standard mycotoxin.

We have elaborated and improved the classical technique of chromatography, known as CSS, of the fluorodensitometric type that allows the semi quantitative evaluation of AFB1 up to 5 ng/spot, and of OTA up to10 ng/spot, with limits of detection ranging between 0.025 and 0.050 µg/kg.

Extent of mycotoxin contamination of vegetable substrates in the biosphere is so intense that one can say that mycotoxins have become a planetary pollutant, since they were found all over the world, both in the cold and wet lands of Russia (Siberia) and in the archipelago of Japan, Australia and New Zealand, the United Kingdom to South Africa's arid areas.

These bold assertions are supported by rigorous scientific research, which unfortunately does not have the strength to reveal but only the top of the iceberg, because only a few countries apply a strategic mycotoxicological preventive control.