Ph.D. THESIS

Analytical assessment of endocrine disruptor xenobiotics in food - influence of matrix and packaging materials

(SUMMARY OF THE DOCTORAL THESIS)

Ph.D. **Diana TOMA**

Scientific coordinator **Prof. dr. Adela PINTEA, Ph.D.**

Introduction

Endocrine-disrupting compounds have gathered serious attention in the last few years due to their involvement in human health. As hormones act, endocrine-disrupting compounds can generate effects even at very low concentrations. Doing so can cause significant disruption at a living organism's biological and developmental levels. Therefore, scientists around the world conducted studies, both in vitro and in vivo, in order to confirm the implication of endocrine-disrupting compounds in pathologies such as diabetes and associate diseases, obesity, infertility, cancer and thyroid dysfunction respectively.

Unfortunately, endocrine-disrupting compounds, such as bisphenol A and some mycotoxins among which are aflatoxins, deoxynivalenol, and T-2 toxin, are widely present, worldwide people being exposed massively to these compounds through diet.

The aims of this thesis are fulfilled by achieving the following objectives:

- ✓ Development of an extraction and an immunoaffinity-based clean-up method for determination of total aflatoxins and aflatoxin B1 from different types of nuts, dried fruits and mixes available on Romanian market.
- ✓ Determination of total aflatoxins and aflatoxin B1 from purified extracts obtained from nuts using ELISA kits based on antigen-antibody reaction.
- ✓ Application of advanced statistical tests to differentiate the levels of total aflatoxins and aflatoxin B1 in food samples in relation to the packaging materials.
- ✓ Extraction and purification of thirteen type A and B trichothecenes from bakery products, pasta, and wheat samples from Romanian market using different solvent mixtures and solid-phase extraction.
- ✓ Analysis of trichothecenes by gas chromatography coupled with mass spectrometry.
- ✓ Development of a HPLC-FLD method for quantification of bisphenol A in biological samples and application on a cell culture model (RPE cells).
- ✓ Evaluation of the cytotoxicity of bisphenol A and its effect on the antioxidant status of RPE cells, in the presence or in the absence of zeaxanthin.

The first three chapters of the thesis contain the current state of knowledge, the aims and the objectives. The results obtained in this work are presented in chapters 4, 5, and 6 as original research. They are meant to bring significant contribution to existing data regarding two major and problematic types of food contaminants: mycotoxins and bisphenol A.

1st Study – The Occurrence of Aflatoxins in Nuts and Dry Nuts Packed in Four Different Plastic Packaging from the Romanian Market

1.Introduction and aims

Mycotoxins are secondary metabolites with low molecular weight, produced by various fungi that are able to grow on different agricultural commodities. Consumption of contaminated food with mycotoxins leads to adverse effects on human health, such as carcinogenic, estrogenic, neurotoxic, hepatotoxic, teratogenic, and even immunosuppressive effects, further causing acute or chronic diseases (Diaz, 2005; Agriopoulou et al., 2020).

Given that nuts in general and dried fruits are consumed in large quantities by the worldwide population, herein we studied the incidence of mycotoxins in these types of products. We also investigated the effect of plastic packaging materials on the level of total aflatoxins and aflatoxin B1, well known as favorable factors in the occurrence of hepatocellular carcinoma in humans.

2. Materials and methods

Food matrices were represented by 64 samples of different types of nuts and mixes (ex. pistachio, peanut, dry fruits, hazelnuts, walnuts) packed in PET (polyethylene terephthalate), PP (polypropylene), LDPE (low-density polyethylene), and PE (polyethylene) bags from the Romanian market. Samples were divided into 4 experimental groups based on their type of packaging. All samples were subjected to methanol extraction followed by cleanup step using immunoaffinity columns. By cleaning up the extract, the specificity and sensitivity were enhanced, resulting in improved accuracy and precision. For the quantitative determinations of total aflatoxins and AfB1, we utilized the laboratory kits RIDASCREEN FAST Aflatoxin and RIDASCREEN FAST Aflatoxin B1, respectively, based on enzyme-linked immune sorbent assay (ELISA).

3. Results and discussions

From the total of 64 samples analyzed for total aflatoxin content, 60 (93.75%) were positive and 4 (6.25%) were below the detection limit (<1 μg/kg) for aflatoxins. Out of the positive samples, 9 (14.06%) exceeded the maximum level admitted by regulation CE Reg. No. 1881/2006 and Commission Regulation (EC) No 165/2010 (EU Commission, 2010). The samples that exceeded the tolerated values of total aflatoxins were: 3 samples of fruits and nuts mix with the highest value of 12.46 μg/kg from the LDPE group, 1 sample of roasted corn with 7.81 μg/kg from the LDPE group, 1 sample of raw hazelnuts with 12.15 μg/kg from the LDPE group, 1 sample of apricot kernels with 10.67 μg/kg from the LDPE group, 1 sample of raw peanuts with 8.22 μg/kg from the LDPE group, 1 sample of raw peanuts with 4.4 μg/kg from the PE group, and 1 sample of roasted corn with 5.04 μg/kg from the PP group. The PP group had a maximum value of 7.36 μg/kg, and for the PE group was 4.4 μg/kg. The highest value from

the PET group for total aflatoxins was obtained from a sample of raw almonds, i.e., 2.13 μg/kg. The results for total aflatoxins are displayed in Fig.1.

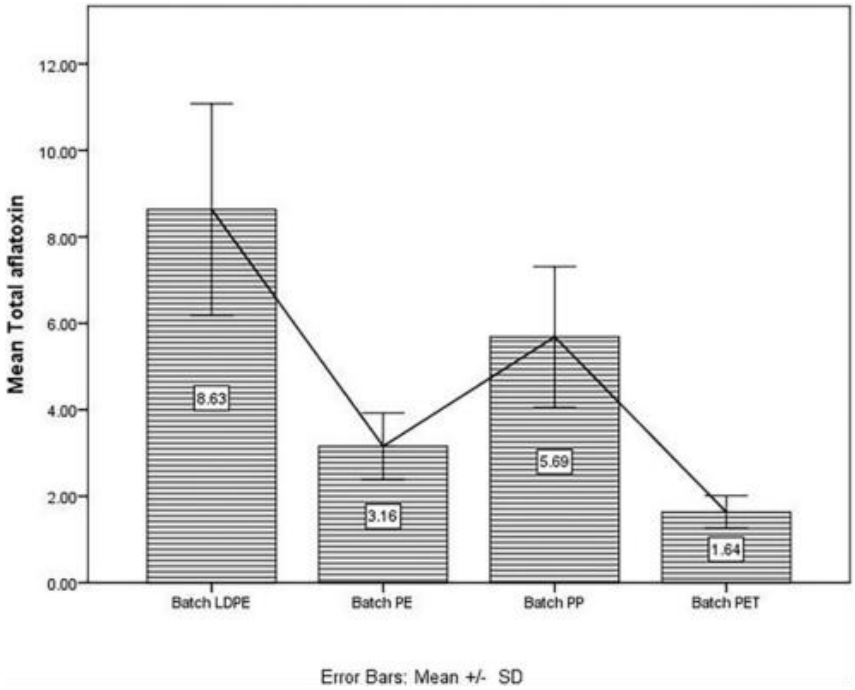

Figure 1. Mean concentration of total aflatoxins (μg/kg) and calculated standard deviation corresponding to each batch of screened samples.

All data from each group were included in statistical analysis, except 4 samples from PET group, which were below the detection limit (<1 μg/kg) for total aflatoxins. Following the descriptive statistics, the highest mean value for total aflatoxins came from the LDPE group samples (Table 1).

Table 1. Descriptive statistics for total aflatoxins (μg/kg).

Treatment	LDPE Group	PE Group	PP Group	PET Group
Observations (n)	15	15	15	15
Mean ± std dev.	8.63 ± 2.44	3.16 ± 0.77	5.69 ± 1.62	1.64 ± 0.37
Min-Max	3.45–12.46	2.15–4.40	2.79–7.63	1.14–2.13

(n)-number of samples from each group; std dev-standard deviation; Min-minimum; Max-maximum; LDPE-low-density polyethylene; PE-polyethylene; PP-polypropylene; PET-polyethylene terephthalate.

The concentrations of AfB1 from all screened samples had lower values than the maximum limits imposed by legislation, except for one sample of raw hazelnut from the LDPE group, which had a value of 5.78 μg/kg. The PE group presented the lowest mean values. The data from PET group were excluded from statistical analysis due to the fact that most of the concentrations were below the detection limit of 1 μg/kg.

2nd Study – Occurrence of Types A and B Trichothecenes in Cereal Products Sold in Romanian Markets

1. Introduction and aims

Monitoring exposure to various mycotoxins has become a key part in order to ensure food safety. The main objective of the present study was to evaluate the occurrence of trichothecenes mycotoxins from several staple foods consumed in Romania, such as bakery products, pasta, and wheat. Therefore, the GC-MS method was used in order to simultaneously determine the presence of 13 trichothecenes DON, NIV, SCIRP, T2 TETRAOL, FUS-X, MAS, 15-ADON, 3-ADON, T2 TRIOL, NEO, DAS, HT-2, T-2 in the above mentioned samples.

2. Materials and methods

A total of 121 samples of wheat, bakery products, and pasta were collected randomly from the Romanian market. All collected samples were divided into 5 groups: group A (white bread), group B (half-brown bread), group C (brown bread), group D (pasta), group E (wheat from Timis, Alba, and Cluj counties). All samples were subjected to extraction with solvent mixtures followed by a solid-phase extraction cleanup step and further analyzed by GC-MS for the trichothecenes determination.

3. Results and discussions

The GC-MS method was applied to determine de presence of thirteen type A and type B trichothecenes in wheat, bread, and bakery products and pasta commercialized in Romania. A summary of the contamination in analyzed samples is presented below (Table 1).

As regards the bakery products, the highest level of DON was found in group B (192 μg/kg) and the lowest value in group D (21 μg/kg). As concerns the wheat samples, the highest level of DON was found in subgroup E3 (509.50 μg/kg). The lowest level was found in subgroup E1 (205.50 μg/kg). Subgroup E2 registered a value of 502.50 μg/kg.

From all analyzed samples, HT-2 occurred in 21 wheat samples, from all three counties, and in one sample (bio bread) from group A where the concentration was 3 μg/kg. None of the samples from groups B, C, and D were contaminated with HT-2.

From the total of 121 analyzed samples, 90.08% (109) were contaminated with one (77.06%), two (11%), three (10.09%), or four trichothecenes (1.83%). From all samples subjected to our study, 12 (9.92%) were free of contamination with trichothecenes.

Table 1. Summary of the occurrence of trichothecenes type A and Type B in analyzed samples.

Sample type	Toxin detected	No. of pozitive samples	Range (µg/kg)
Group A	DON	29	15-352
	HT-2	1	3
	T-2	1	5
Group B	DON	13	50-346
Group C	DON	14	15-326
Group D	DON	15	15-35
Group E			
	DON	12	70-1346
Subgroup E_1	15-DON	2	6-9
	HT-2	7	3-7
	DON	8	21-3395
Subgroup E_2	15-DON	5	6-99
	DAS	1	19
	HT-2	4	5-8
	T-2	1	7
	DON	18	41-2048
Subgroup E_3	15-DON	8	8-52
	HT-2	10	3-18
	NIV	1	30

From all 13 trichothecenes investigated in the analyzed samples, SCIRP, T2-TETRAOL, FUS-X, MAS, 3-ADON, T2-TRIOL, and NEO were not detected in any group of samples. DAS was found only in one wheat sample coming from Alba county (subgroup E2) with a concentration of 19 µg/kg. NIV occurred only in one sample of wheat originating from Cluj county, in a concentration of 30 µg /kg. As regards T-2 mycotoxin, this was identified in two samples, one bio bread (group A) (5 µg /kg), and one wheat sample from Alba county (7 µg /kg). The same bio bread sample where T-2 mycotoxin was detected, was contaminated with both DON and HT-2, in concentrations of 48 µg /kg and 3 µg /kg, respectively.

3rd Study- Bisphenol A effect on human retinal cell line D407

1.Introduction and aims

Present study aims to explore the cytotoxicity of bisphenol A on human retinal pigment epithelial cells (RPE), in an attempt to bring new information regarding the harmful effect of BPA on the retina. From what we acknowledge, there is a lack of information regarding the antioxidant activity of zeaxanthin in the presence of bisphenol A as inductor of oxidative stress. Therefore, we intended to study the possible protective effect of zeaxanthin against oxidative activity of bisphenol A on the same cell line. Moreover, we developed an HPLC-FLD method for the quantification of bisphenol A, which was further used to demonstrate that BPA can be uptaken by RPE cells.

2. Materials and methods

The chosen *in vitro* model was the human retinal pigment epithelium (RPE) D407 cell line. Cells were treated with different concentrations (5-100 μM) of BPA (Sigma Aldrich, Germany) to observe the cytotoxic effect of BPA on D407 cells, and also to note the protective effect of zeaxanthin (20 μM). This comparative experiment involved pretreatment of RPE cells for 24h with zeaxanthin, prior to BPA administration. Cell viability was assessed using the MTT assay. The number of apoptotic and necrotic cells was measured by flow-cytometry using the Annexin V-FITC and propidium iodide (PI) staining kit. The assessment of ROS generation was performed through a semiquantitative DCF-DA (2', 7'-dichlorofluorescein diacetate) fluorescence assay after pretreatment with zeaxanthin and treatment with BPA. An HPLC-FLD method using C18 column and gradient elution was developed to assess the BPA internalization in D407 cells.

3. Results and discussions

The cytotoxicity of BPA against D407 cells was evaluated after the cells were treated with the compound at different concentrations and various treatment durations. Our results display a difference in terms of viability of the cells as regards the influence of zeaxanthin in cellular medium. Also, can be observed that BPA treatment reduced D407 cell viability in a dose-dependent manner. After 24h exposure to BPA, the lowest viability was observed in cells treated with 100 μM of BPA, for both groups, pretreated and untreated with zeaxanthin, where survival rate was 69% and 57%, respectively. The only study which examined the cytotoxicity of BPA in RPE cells revealed that for 10 μM BPA the viability decreased significantly (to 80%) only after longer exposure times (8, 24h) (Chiang et al., 2021).

The number of apoptotic and necrotic cells was measured by using the Annexin V-FITC and propidium iodide (PI) staining kit. After exposure to 25, 50 and 100 μM of BPA, the rates of apoptosis (Q_4) were 5.2%, 4.8%, and 6.8%, respectively. As regards the group which received pretreatment with zeaxanthin, the apoptosis rates (Q_4) were 4.4%, 4.1%, and 6.4% for the same BPA concentrations.

ROS levels are higher in cells treated with BPA only, and lower in cells which received zeaxanthin. This underlines the protective potential of zeaxanthin against oxidative action of BPA on D407 cells. When HCT116 cells were exposed to BPA, it was observed the influence on intracellular ROS generation, proliferation and apoptosis of the cells (Qu et al., 2018).

Following the proposed HPLC-FLD method, we successfully applied it to verify the internalization of BPA, in concentration of 50 μM, in D407 cells. After a proper extraction, the samples of medium and cells preliminary treated with bisphenol A, were analyzed. In the samples untreated with zeaxanthin, it was quantified a concentration of 0.0149 ng BPA/mg protein from the medium, and 0.050 ng BPA/mg protein in cells, respectively.

7. General conclusions and recommendations

The aims of this thesis can be divided into three major perspectives. First, we aimed to investigate the occurrence of total aflatoxins and aflatoxin B1, a causal factor for human hepatocellular carcinoma, in several types of nuts as well as the impact of plastic packaging type on the level of contamination with aflatoxins. Secondly, we considered opportune to assess the presence of thirteen trichothecenes in staple foods consumed by Romanian population, such as bread and bakery products, since some of these mycotoxins are not only highly toxic, but poses endocrine disrupting activity in living organisms. Last, but not least, we wanted to address some attention on another important subject, bisphenol A toxicity on human retinal cells.

Herein, we conclude the following:

In chapter four (Study 1), given that nuts and dried fruits are generally highly consumed, we investigated the contamination with total aflatoxins and aflatoxin B1 of 64 different types of samples from this food category, using the ELISA method. Among the analyzed food matrices, we had pistachio, raw hazelnuts, Brazil nuts, walnuts, fruits and nuts mix, peanuts, corn, and dehydrated fruits. Samples were divided into four experimental groups according to their packaging type: LDPE, PE, PP, and PET to investigate the effect of plastic material on the level of contamination with mycotoxins. The results indicate that the method we used to extract, purify, and quantify the mycotoxins is fast, sensitive, and reliable. Contamination with total aflatoxins was observed in 93.75% of the analyzed samples. Aflatoxin B1 was observed in 75% of the total screened samples. In general, corn, pistachio, peanuts, and dried fruits were more prone to contamination. Based on the obtained results we conclude that samples packed in PET significantly yielded the lowest AfB1 concentrations, followed by PE, PP, and LDPE. Likewise, was the case for total aflatoxins content in all analyzed samples.

In chapter five (Study 2) the objective was to evaluate the incidence of trichothecenes mycotoxins from several staple foods consumed in Romania, such as bakery products, pasta, and wheat. Following the GC-MS method, we simultaneously determined the presence of 13 trichothecenes of type A and type B: DON, NIV, SCIRP, T2 TETRAOL, FUS-X, MAS, 15-ADON, 3-ADON, T2 TRIOL, NEO, DAS, HT-2, T-2 in white bread, half-brown bread and brown bread (to cover all consumers' preferences), pasta, and wheat samples. From all investigated mycotoxins, six were identified in analyzed samples: DON, HT-2, T-2, 15-ADON, DAS, and NIV. By far, the predominant mycotoxin was DON, and occurred in 90.62% of white flour-made bakery products, in 92.85% whole wheat flour bakery products, 100% of bakery products which contain rye flour, as well as in 78.94% of pasta samples, and in 90.47% of wheat samples. 15-ADON mycotoxin was present only in two samples of wheat and in low concentrations. HT-2 occurred in wheat samples, from all three counties and only in one bread sample. DAS and NIV occurred each only in one wheat sample, while T-2 toxin was identified in one sample of bio bread and in one wheat sample. From all samples subjected to our study, only 12 (9.92%) were free of contamination with trichothecenes, mostly bakery products. These results point out the necessity of consistent control in order to prevent mycotoxin intake by the Romanian population through cereal-based foods.

In chapter five (Study 3) we came with the idea of investigating the toxicity of bisphenol A on human retinal cells D407 and also to verify if zeaxanthin could offer an antioxidant protection to the cells against bisphenol A activity. We observed that cells

proliferation is influenced by BPA concentration, as well as the apoptosis, and in cells pretreated with zeaxanthin, the viability is positively influenced by this antioxidant. The intracellular ROS generation was observed and is increasing in a dose-dependent manner. By using HPLC-FLD method, successfully was evaluated the internalization of BPA in D407 cells.

The evaluation of bisphenol A toxicity using *in vitro* testing could provide a better understanding of the mechanisms of action regarding this compound. Future attention deserve natural antioxidants which have the ability to counteract the negative effects of contaminants found in our day to day food.

A special attention should be given to the detailed effects of the type of plastic packaging on the concentration of mycotoxins in food commodities.

Also, bisphenol A is still under the eye of regulatory organizations due to its toxic effect on human health and to its ubiquitous presence. It can enter in human body also from nondietary sources, therefore the continuous need of investigations for BPA occurrence in tissues and biological samples.

8. Originality and personal contributions

Our findings, presented in this thesis, could add valuable information regarding two major contamination problems: mycotoxins and bisphenol A. Why we have chosen to study these compounds? Well, nowadays people are exposed to many chemical compounds, a cocktail of chemicals we may say, many of them toxic and with long term effects on human organisms, especially if these can affect even the endocrine system. Chemical contamination of food is real and occurs at any stage of the food chain.

Total aflatoxins and aflatoxin B1 can be found in many food items, including different types of nuts and dry fruits, which are highly consumed for their nutritional advantages and are considered snacks between main meals. Although the plastic packaging is nowadays under the scrutiny of scientists and regulatory organizations, very few studies are focusing on the impact of packaging type regarding mycotoxins contamination.

From what we observed, the simultaneous determination of mycotoxins with economic impact, such as type A and type B trichothecenes, in different food matrices was not performed that often before. The GC-MS method applied for selected samples gave excellent results and by analyzing simultaneously 13 trichothecenes, we believe that we bring the advantage of reducing the analysis time. Also, the topic of trichothecenes is still little explored as regards the contamination of bakery products available on the Romanian market.

Although in the last years has been much concern regarding bisphenol A negative impact on human health, there is a lack of evidence as regards *in vitro* testing on human retinal cells. Therefore, we found it appropriate to evaluate apoptosis, viability and ROS generation, which can be influenced by the bisphenol A presence. Also, we addressed the possibility to diminish the negative effects of bisphenol A on human retinal cells by the mean of zeaxanthin, which was less studied in the presence of endocrine disruptors, such as bisphenol A. We aimed to also develop a liquid chromatography method coupled with fluorescence detector to assess the internalization of bisphenol A in cells, method which can be applied to assess the presence of bisphenol A in human tissues or biological samples.

References

1. Agriopoulou, S., Stamatelopoulou, E., & Varzakas, T. (2020). Advances in Occurrence, Importance, and Mycotoxin Control Strategies: Prevention and Detoxification in Foods. Foods, 9, 137.
2. Chiang, Y.W., Su, C.H., Suhn, H.Y., Chen, S.P., Chen, C.J., Chen, W.H., Chang, C.C., Chen, C.M., Kuan, Y.H. (2021). Bisphenol A induced apoptosis via oxidative stress generation involved Nrf2/HO-1 pathway and mitochondrial dependent pathways in human retinal pigment epithelium (ARPE-19) cells. Environmental Toxicology. 2022;37:131–141. DOI: 10.1002/tox.23384.
3. Commission Regulation (EU). 2023/915 of 25 April 2023 on Maximum Levels for Certain Contaminants in Food and Repealing Regulation (EC) No 1881/2006. OJEU 2023, 119, 103–157. Available online: https://eur-lex.europa.eu/legalcontent/EN/TXT/PDF/?uri=CELEX:32023R0915
4. Diaz, D. (2005). The Mycotoxin Blue Book. Nottingham University Press: Nottingham, UK, (p. 187–201).
5. Qu, W., Zhao, Z., Chen, S., Zhang, L., Wu, D., Chen, Z. (2018). Bisphenol A suppresses proliferation and induces apoptosis in colonic epithelial cells through mitochondrial and MAPK/AKT pathways. Lfs., 208,167-174. .doi:10.1016/j.lfs.2018.07.040.