UNIVERSITA' DEGLI STUDI DI NAPOLI "FEDERICO II"

FACOLTA' DI AGRARIA

Dottorato in Scienze e Tecnologie delle Produzioni Agro-alimentari

XXIV ciclo

Tesi di dottorato

Risk analysis of the main mycotoxins from food in children

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ABSTRACT

Mycotoxins are secondary metabolites produced by fungi that may contaminate all stages of the food chain and that are toxic or carcinogenic to animals and humans. In this PhD thesis, several aspects of risk analysis of mycotoxins are treated. In particular the studied mycotoxins included: Patulin (PAT), Deoxynivalenol (DON), Ochratoxin A (OTA), Aflatoxin B_1 (AFB_1), Aflatoxin M_1 (AFM_1), and the attention is focused on hazard for infants and children that represent more sensitive and vulnerable categories respect to adults.
The first study case (chapter 3) treats with risk management to control blue mould on apples whose products of industrial transformation are widely consumed by whole population, above all children. The aim of the study was to test compatibility of the biocontrol yeasts (*Rhodosporidium kratochvilovae* LS11 and *Cryptococcus laurentii* LS28) with the recently developed fungicides boscalid (BOSC), cyprodinil (CYPR) and fenhexamid (FENH). The fungicide thiabendazole (TBZ) was used as the control. After 7 days of storage, the integrated treatment based on biocontrol agents (BCAs) with BOSC or CYPR resulted in a significant rot reduction (as much as 98%). Integrated treatments (BCAs + BOSC or CYPR) resulted in lower fungicide residues and PAT contamination in apples.
During apple products manufacturing, the formation of undesirable products like 5-hydroxymethylfurfural (HMF) that is a marker of thermal processing can occur. In the second topic (chapter 4) apple juice and puree were prepared, artificially contaminated with PAT and submitted to a heat treatment to evaluate PAT's reduction. HMF's level was also detected in both juice and puree. Then, uncontaminated apple products samples (n=7) included juices, nectars and purees belonging to different commercial brands were collected, artificially contaminated with PAT at 50µg/l (limit established for PAT in juices) and 25 µg/kg (limit established for PAT in purees), digested with an *in vitro* gastrointestinal digestion protocol in children and bioaccessibility values (%) were calculated. After heat treatment, the evidenced PAT's loss was of 1.41 ± 0.52% in purees whereas it was of 62.62 ± 2.53% in the juices. HMF's level was 0.045±0.002µg/ml in puree and 8.3 ± 0.06 µg/ml in the juices. These differences can be related to the major protection of mycotoxin in semi-solid matrix, characterized by a major viscosity. Relatively to results of bioaccessibility, juices with pulp showed values of 70.89 ± 4.93% and 55.69 ± 4.73%, purees showed levels of 67.30 ± 10.76% and 58.15 ± 5.50% whereas nectar and clarified juices showed percentages of 38.88 ± 2.42%, 28.59 ± 0.46% and 25.28 ± 0.61% respectively. The physical structure of clarified products could have favored the action of digestive enzymes.
In the third topic (chapter 5) twenty-seven samples of dried pasta characterized by size, packaging and marketing intended for children consumption were collected and analyzed for OTA, AFB_1 and DON determination. The samples that showed the highest amounts of one of the mycotoxins were cooked, digested with an *in vitro* gastrointestinal protocol and bioaccessibility values were calculated. Seven of the twenty-seven samples exceeded from 120% to 225% the legal limit of 200 µg/kg for DON fixed for processed cereal-based baby foods by an European Regulation; all the collected samples were under the OTA legal limit (0.05 µg/kg) fixed by the European Regulation and none of the samples was contaminated by AFB_1 over the instrumental limit of detection of 0.10 µg/kg. The mean value of gastric bioaccessibility verified for the DON resulted of 23.1%, whereas mean duodenal bioaccessibility was 12.1%. Considering only the samples treated with the child digestion the mean DON duodenal bioaccessibility data was of 9.7%. Therefore, the DON could interact with the intestinal epithelium cells at concentrations of 3.4-18.9 µg/kg and these levels are cytotoxic on several cell lines.
The fourth topic (chapter 6) treats with the exposure assessment to AFM_1 by breast milk collected from 50 lactating mothers in three senatorial districts of Ogun State, Nigeria. The relationship with dietary AFB_1 exposure was also studied. AFM_1 was detected in 41 (82%) of breast milk samples ranging from below limit of detection (<LOD) to 92.14 ng/l (mean = 15.9 ng/l). Nine (18%) of the breast milk samples had AFM_1 below the detectable limit while eight (16%) of the samples had levels exceeding the European standard limit. The highest AFM_1 risk was found in Ogun Central senatorial district. The level of AFB_1 contamination in the foods consumed by the mothers were generally low with foodstuffs from Ogun Central senatorial district having a significantly higher ($p<0.05$) mean (0.33 µg/kg) than those collected from Ogun East senatorial district (0.18 µg/kg) and Ogun West (0.16 µg/kg). There was a significantly ($p<0.05$) positive correlation between the AFB_1 consumed in foodstuffs by the lactating mothers and the AFM_1 detected in their breast milk.

Le micotossine sono metaboliti secondari prodotti da funghi che possono contaminare tutti gli stadi della catena alimentare, tossici o cancerogeni per uomini e animali. In questo lavoro di tesi sono stati presi in esame vari aspetti dell'analisi del rischio delle micotossine. In particolare i metaboliti esaminati comprendono: Patulina (PAT), Deossinivalenolo (DON), Ocratossina A (OTA), Aflatossina B_1 (AFB_1),

Aflatossina M_1 (AFM_1), focalizzando l'attenzione sul rischio diretto a neonati e bambini, categorie più sensibili e vulnerabili rispetto agli adulti.
Il primo caso studio (capitolo 3) riguarda la gestione del rischio per il controllo della muffa blu sulle mele i cui prodotti di trasformazione sono ampiamente consumati dalla popolazione generale, soprattutto i bambini. Lo scopo dello studio è stato quello di testare la compatibilità dei lieviti di biocontrollo (*Rhodosporidium kratochvilovae* LS11 e *Cryptococcus laurentii* LS28) con i fungicidi di recente sviluppo boscalid (BOSC), ciprodinil (CYPR) e fenhexamid (FENH). Il fungicida tiabendazolo (TBZ) è stato usato come controllo. Dopo 7 giorni di conservazione, il trattamento integrato basato sull'utilizzo di agenti di biocontrollo con BOSC o CYPR ha comportato una riduzione significativa della decomposizione delle mele (fino al 98%). I trattamenti integrati (BCA + BOSC o CYPR) hanno comportato la minore presenza di residui di fungicidi e PAT. Durante la trasformazione delle mele si verifica la formazione di prodotti indesiderati come il 5-idrossimetilfurfurale (HMF) che è impiegato come marker di trattamento termico. A tal riguardo nel secondo caso studio (capitolo 4) sono stati preparati succo e purea di mele, successivamente contaminati artificialmente con PAT e sottoposti a un trattamento termico per valutare la riduzione di PAT. Anche il livello di HMF prodotto a seguito del trattamento termico è stato quantificato nel succo e nella purea. Successivamente campioni commerciali (n = 7) risultati negativi per la presenza di PAT sono stati artificialmente contaminati a livelli di 50 µg/l (limite stabilito per PAT in succhi) e 25 mg/kg (limite stabilito per PAT in purea), digeriti secondo un protocollo di digestione in vitro gastrointestinale simulante il sistema enzimatico dei bambini e si sono stimati i valori bioaccessibilità (%). Dopo il trattamento termico, la perdita di PAT nella purea è stata dell'1.41 ± 0.52% mentre di 62.62 ± 2.53% nei succhi. Il livello di HMF dopo il trattamento termico è risultato nella purea di 0.045 ± 0.002 µg/ml e 8.3 ± 0,06 µg/ml nel succo. Queste differenze possono essere legate alla protezione della micotossina nella matrice semi-solida della purea, caratterizzata da maggiore viscosità. Relativamente ai risultati di bioaccessibilità, i succhi con polpa hanno mostrato valori di 70.89 ± 4.93% e 55.69 ± 4.73%, le puree hanno mostrato livelli di 67.30 ± 10.76% e 58.15 ± 5.50% mentre il nettare e i due prodotti chiarificati hanno mostrato percentuali di bioaccessibilità pari a 38.88 ± 2.42 %, 28.59 ± 0.46% e 25.28 ± 0.61% rispettivamente. Pertanto la struttura fisica di prodotti chiarificati avrebbe potuto favorire l'azione degli enzimi digestivi.
Nel terzo caso studio, 27 campioni di pasta secca caratterizzata da dimensioni, confezionamento e marketing destinati all'alimentazione dei bambini sono stati raccolti e analizzati per la presenza di OTA, AFB_1 e DON. I campioni che hanno mostrato la più alta quantità di micotossine sono stati cotti, digeriti in vitro secondo le condizioni gastrointestinali dei bambini e si sono stimati i livelli di bioaccessibilità. Sette dei 27 campioni superava dal 120% al 225% il limite di 200 µg/kg fissato dal regolamento europeo per il DON nei prodotti trasformati a base di cereali per i bambini; tutti i campioni si sono rilevati sotto il limite legale di OTA (0.05 µg / kg) e nessun campione è risultato contaminato da AFB_1 oltre il limite di detezione (LOD) strumentale, pari a 0.10 µg / kg. La bioaccessibilità duodenale media per il DON è risultata del 9.7%. Pertanto, il DON potrebbe interagire con le cellule dell'epitelio intestinale a concentrazioni di 3.4-18.9 µg / kg e questi livelli sono considerati citotossici su diverse linee cellulari.
Il quarto caso studio (capitolo 6) mira alla valutazione dell'esposizione da parte di neonati all'AFM_1 da latte materno raccolto da 50 madri in tre distretti senatoriali dello Stato di Ogun in Nigeria. E' stata studiata la correlazione con l'esposizione dietetica da parte delle madri all'AFB_1. L'AFM_1 è stata rilevata in 41 (82%) campioni di latte materno che variano da livelli inferiori al limite di rilevabilità (<LOD) a 92.14 ng/l (media = 15.9 ng/l). Nove (18%) campioni di latte materno contenevano AFM_1 al di sotto del limite rilevabile mentre otto (16%) campioni presentavano livelli superiori al limite standard europeo. Il più alto rischio AFM_1 è stato trovato nel distretto centrale Ogun senatoriale. Il livello di contaminazione da AFB_1 negli alimenti consumati dalle madri erano generalmente bassi e i prodotti alimentari provenienti dal distretto senatoriale di Ogun centrale erano caratterizzati da un valore medio significativamente ($p < 0.05$) più elevato (0.33 µg/kg) rispetto a quelli raccolti dal distretto senatoriale Ogun Est (0.18 µg/kg) e Ogun Ovest (0.16 µg/kg). Una correlazione positiva ($p < 0.05$) è stata rivelata tra la presenza di AFB_1 nei prodotti alimentari consumati dalle madri in allattamento e la presenza di AFM_1 nel latte materno.

Chapter 0 - PREFACE

0.1 Mycotoxins : the importance of risk analysis

Mycotoxins are secondary metabolites produced by fungi that may contaminate all stages of the food chain and that are toxic or carcinogenic to animals and humans. While a low level of mycotoxins in food is generally regarded as safe and in any case unavoidable, conditions such as unusual weather, insect pest damage, improper breeding and harvesting, or poor storage can lead to high levels of mycotoxins in crops that can cause severe disease outbreaks. Despite efforts to control fungal contamination, toxigenic fungi are ubiquitous in nature and occur regularly in worldwide food supplies due to mold infestation of susceptible agricultural products. It has been estimated that 25% of the world's crops are affected by mould or fungal growth (Dowling, 1997).

Exposure to mycotoxins is mostly by ingestion; however, other routes such as inhalation, contact, and passive exposure resulting from a mycotic infection by a toxigenic fungus have been recognized (CAST, 2003).

Today, the globalization of the food trade has contributed to losses due to mycotoxins in the developing world in two important ways. First, stringent mycotoxin standards on exported foods mean that developing nations are likely to export their best-quality foods while keeping contaminated foods domestically, which inadvertently results in higher risk of mycotoxin exposure in those nations (Cardwell et al, 2004).

Since the discovery of the aflatoxins in 1960 and subsequent recognition that mycotoxins are of significant health concern to both humans and animals, regulations gradually developed for mycotoxins in food and feed. Over the years, the number of countries with known specific mycotoxin regulations has increased from 33 in 1981 (Schuller et al, 1983) to 56 in 1987 (Van Egmond, 1989), 77 in 1995 (FAO 1997), and 100 in 2003 (FAO, 2004).

In the context of food safety, risk is defined as an estimate of the likelihood/probability of the occurrence of an adverse health effect in humans, weighted for its severity that may result from exposure to a biological, chemical or physical agent in food. Risk analysis must be the foundation upon which food safety policy and consumer protection measures are based (FAO, 2004). Risk analysis is a scientific tool applied to food safety concerns is made up of three separate parts:

- **Risk assessment:** scientific evaluation of the probability of occurrence of known or potential adverse health effects resulting from human exposure to food-borne hazards.
- **Risk management:** process of weighing policy alternatives in light of risk assessment and if required, selecting and implementing appropriate control options and regulatory measures.
- **Risk communication:** exchange of information and opinions concerning risk and risk management options and actions among risk assessors, risk managers, consumers and other interested parties.

Figure 1 shows the main elements of a process of risk assessment and risk management that are the basis for promulgation of regulations. Risk assessment of human health hazards associated with mycotoxins must rely on extrapolation from toxicity data obtained in animal models and human exposure assessments.

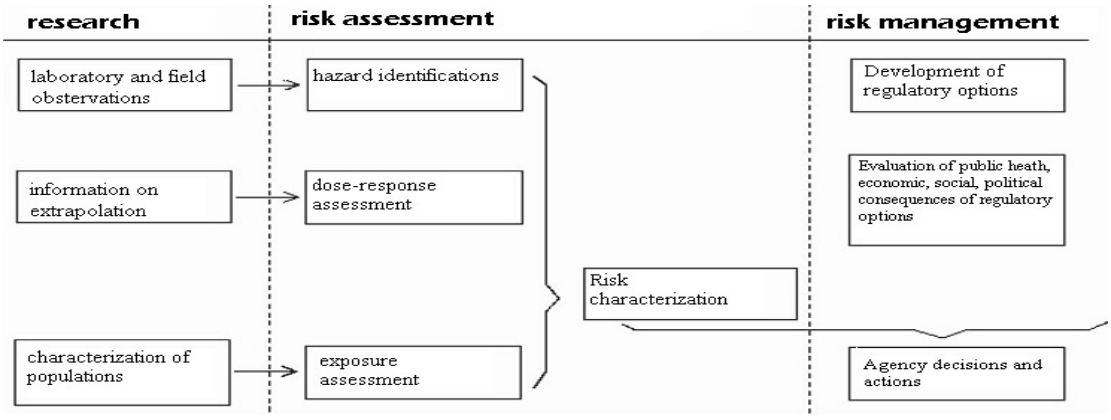


Figure 1. Diagram of elements of risk assessment and risk management (WHO, 2002)

Figure 2 represents the general factors involved in a mycotoxicosis. Exposure assessments are quite sketchy at best, given that there is no publicly accessible ongoing systematic surveillance for human mycotoxin

exposures. There are some interesting recent examples of attempts to perform such extrapolations and comparisons. Advances in the statistical estimation of uncertainty make extrapolations increasingly relevant.

Generally speaking, the term hazard refers to an intrinsic property of a chemical and to the occurrence of a toxic effect in a particular species with a certain degree of exposure.

In order to evaluate the impact on human health due to the ingestion of mycotoxins, it is essential to develop an appropriate methodology for the assessment of the risk associated with these toxins (Miraglia et al, 1996).

Risk assessment consists of some or all of the following four steps:

- hazard identification;
- analysis of effects;
- exposure assessment;
- risk characterization (Bryden, 2007).

Differences in environmental conditions in the crop-growing regions of the world, as well as differences in control methods used to prevent or decontaminate mycotoxins, lead to vastly different levels of mycotoxin exposure and consequent economic and health risks. The insidious nature of many mycotoxicoses make it difficult to estimate incidence and cost (CAST, 1989). In addition to crop losses and reduced animal productivity, costs are derived from the efforts made by producers and distributors to counteract their initial loss, the cost of improved technologies for production, storage and transport, the cost of analytical testing, especially as detection or regulations become more stringent and the development of sampling plans, (Whitaker, 1995). Regulations are primarily based on known toxic effects. For the mycotoxins currently considered most significant (aflatoxins B_1, B_2, G_1 and G_2; aflatoxin M_1; ochratoxin A; patulin; fumonisins B_1, B_2 and B_3; zearalenone; T-2 and HT-2 toxins; and deoxynivalenol) the Joint Expert Committee Committee on Food Additives (JECFA—a scientific advisory body of the World Health Organization (WHO) and the Food and Agriculture Organization (FAO)) has evaluated their hazard in several sessions (WHO, 1990-WHO, 2002).

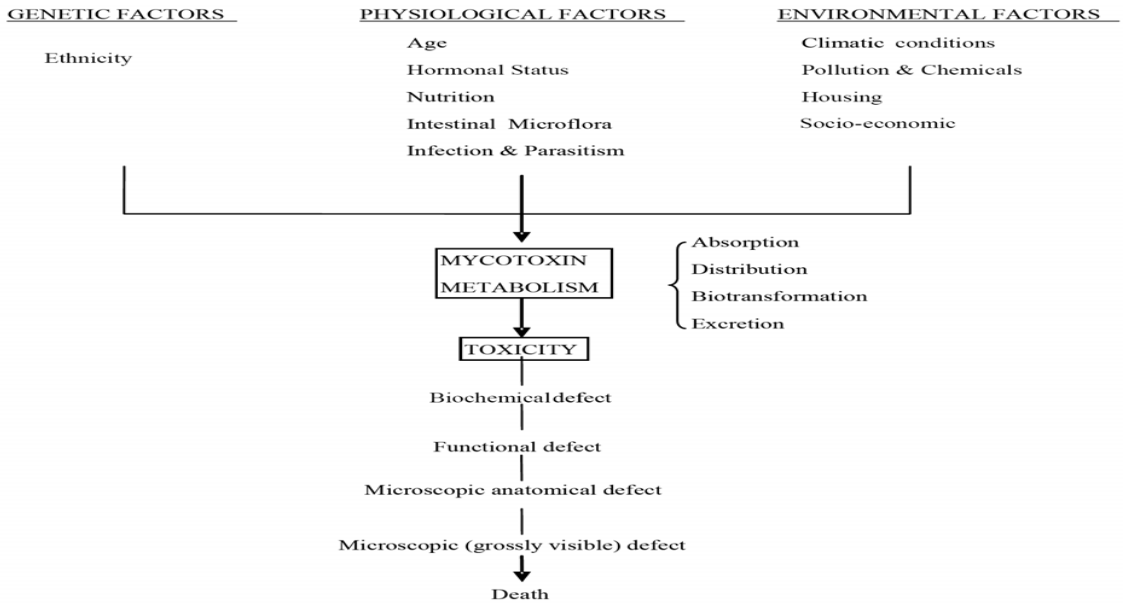


Figure 2. A simplified representation of some general relationships in a mycotoxicosis. Adapted from Bryden (2007)

As in the case of many other types of toxins which may be present in food, the risk assessment is strictly related to an accurate evaluation of dietary intake. This is also essential in order to establish a possible correlation between food-borne exposure and disease occurrence.

In the field of mycotoxins, like for other food contaminants, the most used methodology for dietary intake evaluation is based on occurrence data in food products combined with consumption data.

With regard to occurrence data, it has to be stated that in the past three decades, hundreds of papers have been published both on methods of analysis and on the incidence of mycotoxins in food, but unfortunately not all results may be considered reliable for an accurate information on the occurrence of mycotoxins in food (Thuvander et al, 2001).

In the study of mycotoxins three major parameters contribute to the reliability of occurrence data:

• quality assurance of analytical data
• sampling procedures
• monitoring programs (Miraglia et al, 1996).

Quality assurance of the data is not always satisfactory, especially for old data; furthermore in the evaluation of the level of mycotoxins in food a crucial point is represented by the sampling procedures adopted, since these toxins are far from being distributed in a homogeneous way in the commodities. Moreover the problem of obtaining a representative sample has not yet been definitively solved and sampling procedures are sometimes particularly time-consuming and difficult to apply. Unreliability of occurrence data can also be attributable to inadequately developed monitoring programs. An additional source of error in the evaluation of dietary intake is also to be attributed to the uncertainty of available food consumption data.
More data, both on levels in food and on the intake of these foods in various population groups, are needed to perform reliable exposure analysis.

0.1.1. Approaching risk assessment for children

It has been recognized that children may be a potentially vulnerable subgroup respect to assumption of mycotoxins. Children consume more food and water compared to adults when expressed per kg body weight (bw) (Health Council of the Netherlands, 2004; SCF, 1998), resulting in relatively higher exposures to compounds. Also specific dietary patterns of children may contribute to a higher exposure to mycotoxins present in food.
Because children have more future years of life than do most adults, they have more time to develop chronic diseases that may be triggered by early environmental exposures. That overpopulation is more a feature of developing countries; millions of children are thus prone to be exposed to these toxins in developing communities (FAO, 2005).
Apart from having a higher exposure per kg bw, children have a different physiology from that of adults. This has been thoroughly described in a recent report published by the Dutch Food and Consumer Product Safety Authority (VWA) (VWA, 2008). In a report (Boon et al, 2009) the development of children in relation to possible toxic effects of food contaminants was discussed. It was concluded that children are more sensitive and vulnerable respect to adults. Especially young children under the age of 1 may be more vulnerable, since their enzymatic activity and therefore their ability to break down chemical compounds is low compared to adults. They may therefore be longer exposed to compounds than adults (VWA, 2008). Due to significant postnatal development of different organ systems during childhood, children may be more sensitive to neurotoxic, endocrine and immunological toxic effects up to 4 years of age. This is also true for children aged 5 to 12 years, however to a lesser extent for immunological toxic effects (VWA, 2008). Due to these differences between adults and children regarding exposure and physiology, it is important to address children as a separate subgroup in risk assessments.

References

Boon P.E., Bakker M.I., van Klaveren J.D., van Rossum C.T.M. (2009). Risk assessment of the dietary exposure to contaminants and pesticide residues in young children in the Netherlands RIVM report 350070002/2009

Bryden W.L. (2007). Mycotoxins in the food chain: human health implications. Asia Pac J Clin Nutr; 16 (Suppl 1):95-101

Cardwell, K. F., Desjardins, A., Henry, S. H.; Munkvold, G.; Robens, J. (2004). Mycotoxins: The Cost of Achieving Food Security and Food Quality. www.apsnet.org/online/feature/mycotoxin/ top.html

CAST. (1989) Mycotoxins: Economic and Health Risks. Report n. 116. Council for Agricultural Science and Technology.

CAST, (2003). Mycotoxins: risks in plant, animal and human systems. Task Force Report n. 139. Council for Agricultural Science and Technology, Ames, IA, 1–191.

Dowling, T.S. (1997). Fumonisins and its toxic effects. Cereal Food World 42: 13-15.

Food and Agricultural Organization, World Health Organization , Evaluation of Certain Food Additives and Contaminants, (1995). 44th report of the joint FAO/WHO Expert Committee on Food Additives, Tech. Report Series 859, WHO.

Food and Agriculture Organization (1997). Worldwide regulations for mycotoxins. A compendium. FAO Food and Nutrition Paper 64.

Food and Agriculture Organization (2004). Worldwide regulations for mycotoxins in food and feed in 2003. FAO Food and Nutrition Paper 81.

Food and Agriculture Organization (2005). The State of Food Insecurity in the World 2005. Eradicating world hunger key to achieving the millennium development goals. http://www.fao.org/icatalog/inter-e.htm

Health Council of the Netherlands (2004). Pesticides in food: assessing the risk to children (publication no. 2004/11E). The Hague: Health Council of the Netherlands. Available at <www.gr.nl>.

Miraglia M., Brera C., Colatosti M. (1996). Application of Biomarkers to Assessment of Risk to Human Health from Exposure to Mycotoxins. Microchemical Journal, 5:472–477

Schuller P.L., van Egmond H.P., Stoloff L. (1983). Limits and regulations on mycotoxins. In: Naguib K, Naguib MM, Park DL, Pohland AE (eds) Proc Int Symp on Mycotoxins, Cairo, pp 111–129

SCF (1998). Further advice on the opinion of the Scientific Committee for Food expressed on the 19th September 1997 on a Maximum Residue Limit (MRL) of 0.01 mg/kg for pesticides in foods intended for infants and young children.

Thuvander A., Möller T., Enghardt Barbieri H., Jansson A., Salomonsson A.C., Olsen M. (2000). Dietary intake of some important mycotoxins by the Swedish population. Food Additives and Contaminants, 18 (8): 696-706

Van Egmond HP (1989) Food Addit Contam 6:139–188

VWA (2008). Exceedances of health based limit values for chemicals present in food for children). Den Haag: Voedsel en Waren Autoriteit, Bureau Risicobeoordeling. Available at <www.vwa.nl>

Whitaker, T.B., Springer, J., Defize, P., de Koe, W.J., Coker, R. (1995). Evaluation of sampling plans used in the United States, United Kingdom and The Netherlands to test raw shelled peanuts for aflatoxin. J. Assoc. Off. Anal. Chem. Intl., 78: 1010-1018

World Health Organization (1990) Toxicological evaluation of certain food additives and contaminants. Chapter Patulin. The 35th meeting of the Joint FAO/WHO Expert Committee on Food Additives, Series 26: 143–165

World Health Organization (2002) Evaluation of certain mycotoxins in food. Fifty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives, WHO Technical Report, Series 906: 70

Chapter 1 : STATE OF THE ART

1. Risk analysis for mycotoxins: deoxynivalenol, patulin, aflatoxins, ochratoxins

1.1. Deoxynivalenol

The trichothecenes comprise a unique family of over 200 structurally related secondary metabolites that are produced by the fungi *Fusarium*, *Stachybotrys,* and *Myrothecium* during growth in food and the environment (Grove 2000). These low molecular weight (200–500D) sesquiterpenoids contain both a common 9, 10 double bond and 12, 13 epoxide group and varied substituent groups that significantly contribute to their toxic potential.
Edwards (2004) reviewed the environmental conditions that favour deoxynivaleol (DON) (Fig.1) accumulation in food crops. Minimal tillage, nitrogen fertilizers, application of azoxystrobin (fungicide) or glyphosate (herbicide), and production of grains where maize had been grown the previous year were the main risk factors associated with increased DON accumulation.
Discovered in the early 1970s, the trichothecene DON is elaborated during growth of *Fusarium graminearum* on cereal staples such as wheat, barley, and corn (Yoshizawa and Morooka 1973). Colloquially known as "vomitoxin" because of emetic effects in pigs, DON is not only the most commonly detected trichothecene in cereal grains but is also found at the highest concentrations (Canady et al. 2001).
Chemically, 4-Deoxynivalenol (DON) is known as 12,13-epoxy-3, 4,15-trihydroxytrichotec-9-en-8-one, ($C_{15}H_{20}O_6$, MW: 296,32). DON occurs predominantly in grains such as wheat, barley, and maize and less often in oats, rice, rye, sorghum and triticale. The occurrence of DON is associated primarily with *Fusarium graminearum* (*Gibberella zeae*) and *F. culmorum*, both of which are important plant pathogens, causing *Fusarium* head blight in wheat. (Rotter et al., 1996). The incidence of *Fusarium* head blight is most affected by the timing of rainfall rather than by the amount at the time of flowering (Creepy, 2002).

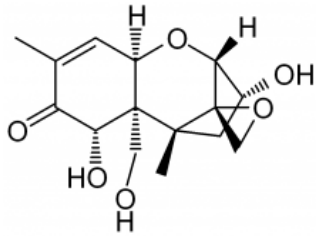


Fig.1: Chemical structure of Deoxynivalenol (DON)

1.1.1.Toxicity

Cronic effects: The general strategy for evaluation of toxicity is to take the highest dose that has no effect in the most sensitive species and divide it by uncertainty factors that reflect interspecies differences and interindividual variability to yield a tolerable human daily intake (TDI). Canadian researchers conducted an early risk assessment for DON in 1983 and proposed provisional TDIs of 1.5 and 3.0 µg/kg bw for children and adults, respectively (Kuiper-Goodman 1994). The IARC classified DON in 1993 in Category 3, i.e., not classifiable as to its carcinogenicity to humans; however, at that time the negative chronic study in mice (Iverson et al., 1995) was not available. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) performed another detailed risk assessment in 2001 (Canady et al. 2001). A provisional maximum tolerable daily intake (PMTDI) of 1 µg/kg bw was proposed based on potential effects of DON on the growth, immune function, and reproduction.
After subchronic oral exposure of various species (mouse, rat and pigs) several effects were found i.e. reduced feed intake, reduced weight gain, and changed levels in some blood parameters including serum immunoglobulins (Baars et al., 1999).
Acute effects: Consumption of a single meal containing a high concentration of DON could cause gastroenteritis with attendant vomiting in humans (Canady et al. 2001).
High dose, acute exposure of pigs and other sensitive animal species to DON elicits abdominal distress, increased salivation, malaise, diarrhea, and emesis. Although DON is one of the least lethal trichothecenes, its emetic effects are equivalent or exceed those described for other more potent trichothecenes (Rotter et al.

1996). Experimentally, low to moderate dose acute oral exposure to DON cause vomiting and gastroenteritis, whereas higher doses cause severe damage to the lymphoid and epithelial cells of the gastrointestinal mucosa resulting in hemorrhage, endotoxemia and shock (Ueno, 1984).
Swine consuming 2 and 4 ppm of DON exhibit a dose-related decrease in weight gain within the first 8 weeks of feeding with 4 ppm causing decreased feed intake, weight gain, and efficiency of feed utilization throughout the experiment (Bergsjo et al. 1992).
Increased incidence of upper respiratory tract infections was reported in children who had consumed the wheat bread for more than a week. Illnesses subsided when consumption of the bread ceased. (Hopton et al. 2010).
These results provide solid evidence for the potential health implications of fairly low exposure to the toxin and emphasize the need for more work to define human risk from this common foodborne contaminant. Investigators have raised questions about exposures to specific population groups, notably children and vegetarians, who might consume DON at purportedly unsafe levels.

1.1.2. Control of DON in field and processings

Field: *Fusarium* head blight of wheat (*Triticum aestivum* L.) continues to be a concern throughout the world, causing substantial grain yield and quality losses almost every year in some major wheat-growing region.
Efforts to minimize the impact of this disease and DON have been based on the use of management strategies such as host resistance, crop rotation, tillage, and fungicide application. However, none of these methods used alone has been fully effective. Of the fungicides most widely tested triazole chemistry (e.g., tebuconazole) have been the most effective, giving the most consistent results (Hershman et al, 2004). Besides tebuconazole, other triazole-based fungicides have been tested against *Fusarium* head blight and DON, with some showing numerically, but not always statistically, superior efficacy relative to tebuconazole in individual studies (Paul et al, 2005). Recent evaluations of integrated approaches for managing *Fusarium* head blight and DON showed that percent control increased substantially more when fungicide application was combined with residue management and cultivar resistance (McMullen, 2007) than when any of these strategies were used alone. Another strategy to control field infection of wheat and barley by *Fusarium* was the employment of biological agents to protect flowering heads from infection.
In wheat, seed treatment with several bacteria, including fluorescent pseudomonas, *Pantoea* sp., *Bacillus cereus*, and the fungus *Trichoderma harzianum*, has shown promise for control of *Fusarium* seedling blight (Bello et al, 2002). Strains of *Pantoea* sp. antagonize by means of chitinolytic enzymes, while strains of some *Bacillus* spp. have the ability to secrete chitinolytic enzymes and to induce systemic resistance in the host plant (Tsai et al, 2002). *Trichoderma* spp. have been reported to compete for nutrients, secrete antifungal compounds, parasitize fungal pathogens, and induce systemic resistance in the host plant (Whipps et al, 2001).
Food processings: While DON levels were unaffected by heat treatment of 100-120°C at pH 4.0 and 7.0, heat treatments of 120°C for 30 min or 170°C for 15 min led to complete degradation of DON at pH 10 (Wolf et al, 1999). The conditions used for baking bread and other leavened products, *e.g.*, cakes and biscuits, vary and variables such as fermentation conditions, dough additives, and the length and temperature of the baking process can all affect the amount of deoxynivalenol lost during the process. An important level of DON reduction was observed during each of the processing steps from uncleaned durum wheat to cooked spaghetti. The average levels of DON were 77% in cleaned wheat, 37% in semolina, 33% in spaghetti and 20% in cooked spaghetti respect to uncleande wheat. (Visconti et al, 2004). In parboiled rice, DON contaminated 45% of the samples evaluated at levels between 180-400 ng/g. The shortest soaking time (4 hours) and the lowest level of DON contamination (720 ng/g) led to the highest level of migration of DON into the endosperm starch. A six hour soak resulted in the least migration of DON into the endosperm starch, (Dors et al, 2009). The pearling process efficiently reduces deoxynivalenol contamination and also may reduce or prevent recontamination of the starchy endosperm (Rios et al., 2009). Baking reduces DON by 24-71% in bread and 35% in biscuits (El-Banna et al, 1983).
The thermal decomposition products of DON are unknown, and there are no data on their toxicity, so there is currently no evidence that DON's thermal instability leads to the detoxification of human foods or animal feeds.

1.1.3. Exposure to DON by the general population and by children

JECFA estimated human dietary intake of DON using food consumption data and dietary intake estimates (Canady et al. 2001). DON concentrations were highest in wheat, rice and maize samples. DON content in specific food commodities were multiplied by the amount of each corresponding commodity consumed in

several regional diets. Using the JECFA TDI and European consumption data the EU established regulations in 2006 for DON in unprocessed durum wheat and oats (1750 ppb), other unprocessed cereals (1250 ppb), cereal flour and pasta (750 ppb) and processed infant foods (200 ppb) (EC Commission regulation n. 1881/2006) (Table 1).

Table 1: Maximum level of DON in foostuffs (Reg CE 1881/2006)

Deoxynivalenol	
Unprocessed cereals other than durum wheat, oats and maize	1 250
Unprocessed durum wheat and oats	1 750
Unprocessed maize	1 750
Cereals intended for direct human consumption, cereal flour (including maize flour, maize meal and maize grits ([21])), bran as end product marketed for direct human consumption and germ, with the exception of foodstuffs listed in 2.4.7	750
Pasta (dry) ([22])	750
Bread (including small bakery wares), pastries, biscuits, cereal snacks and breakfast cereals	500
Processed cereal-based foods and baby foods for infants and young children ([3]) ([7])	200

Report from SCOOP task (2003) reported occurrence data from 12 European countries. A total of 11022 samples were analysed for DON, with 57% of positive samples. For each Member State, the best estimate of total dietary intake (ng/kg bw/day) for the whole population and/or for specific groups of the population was calculated. The percentage of cereal samples with a DON level of 750 µg/kg or higher was 7%. The percentage of cereal samples with a DON level of 500 µg/kg or higher was 6%. In consideration of the EU draft recommendations on DON in cereals and cereal products (DG-SANCO, 2000), the provided occurrence data showed very few levels of contamination higher than the proposed limits of 500 µg/kg and 750 µg/kg. From the reported data, it was clear that wheat and wheat containing products (like bread and pasta) represent the major source of intake for DON. Considering DON mean intake, most of the intakes for the population groups were far below the TDI of 1µg/kg bw. Considering DON high level intake, especially for the young children groups, the TDI is exceeded.. For the group of 13–18 years adolescents the intake is close to the TDI. However, there is a significant lack of consumption data in some countries, such as Italy. In particular, information on consumption of baby food is in general not available. In children, ingestion of heavily DON-contaminated food results in vomiting within hours (Pestka et al., 2004). In 1987, nearly 100 persons in India became ill after they consumed wheat products from which vomitoxin and other trichothecene mycotoxins were recovered (Bhat et al., 1989). In 1997 and 1998, approximately 1700 school children in the United States developed vomiting, nausea, headache and abdominal cramps after eating burritos. Vomitoxin was identified as a contaminant in the burritos and might have caused the outbreaks, which subsided within 24 h of onset (CDC, 1999).
The dietary intake of DON in the Netherlands exceeded the provisional TDI of 1 µg/kg bw, especially in children, (Kistemaker et al., 1998). Eighty percent of the one-year-olds had a DON intake above the provisional TDI and 20% of this age group was exposed to DON levels exceeding twice the provisional TDI. At the age of 4, these percentages are 50 and 5, respectively. The monitoring data showed that the average DON concentration in wheat was 446 µg/kg (n = 219). Table 2 shows the relative contribution of different food categories to the total DON intake. Bread/biscuits/crackers are the main source for DON exposure. Porridge made of wheat and other grains is categorized in baby/toddler food and forms only a relevant source of DON for the very young.

Table 2. Relative contribution of food categories to the total DON intake (Pieters et al., 2001)

Food category	age (yr.)				
	1	2-4	5-10	11-20	>20
Bread/biscuits/crackers	53	64	71	69	65
Baby/toddler food	22	6	0.8	0.6	0.6
Cookies/cakes/pastry	9	11	11	12	11
Paste	3	4	4	5	5
Composites	2	3	3	5	7
Barley (mainly beer)*	0	0	0	2	4
Other	11	12	10	6	7

1.2. Patulin

Patulin (4-hydroxy-4H-furo [3,2c] pyran-2[6H]-one) (PAT) (Fig.2) is a secondary metabolite naturally produced by a variety of fungi like *Aspergillus*, *Penicillium* and *Byssochlamys*. It is a colourless crystal with a molecular weight of 154 Daltons and a melting point of 111°C, and it is stable to heat processing at pH<6. (Trucksess and Tang, 1999). PAT is a water-soluble lactone isolated as an antibiotic during the 1940s (Stott and Bullerman 1975). It was later found to inhibit more than 75 different bacterial species including both Gram-positive and Gram-negative bacteria (Ciegler et al, 1971). Various studies suggested PAT to be not only toxic to fungi and bacteria but also to animals and higher plants, including cucumber, wheat, peas, corn, and flax (Berestets'kyi and Synyts'kyi 1973).

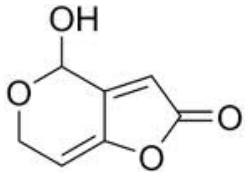


Figure 2: Chemical structure of Patulin (PAT)

1.2.1 Toxicity

The basis for various toxic effects appears to be mixed. On a cellular level, PAT has been shown to have effects including plasma membrane disruption (Mahfoud et al, 2002), inhibition of protein synthesis (Arafat and Musa 1995), disruption of transcription and translation (Lee and Roschenthaler 1987), inhibition of DNA synthesis (Cooray et al, 1982), and inhibition of interferon-γ-producing T-helper type 1 cells (Wichmann et al, 2002).

At relatively high doses, PAT has immunosuppressive properties, although a study in mice based on realistic human exposures failed to demonstrate any immunotoxicity. Based on available experimental results, it was concluded that PAT is genotoxic, but that no adequate evidence existed for carcinogenicity in experimental animals (FAO, 1995). No evaluation could be made of the carcinogenicity of PAT to humans and there is inadequate evidence in experimental animals (IARC 1986). PAT was reviewed by JECFA in 1995. Since it became apparent that PAT was administered only three times per week during 24 months, the NOEL in this study was calculated to be 43 µg/kg bw/day. As PAT doesn't accumulate in the body and in the light of consumption pattern, the PTWI was changed to a provisional maximum tolerable daily intake (PMTDI). Based on a NOEL of 43 µg/kg bw/day and a safety factor of 100, a PMTDI of 0.4 µg/kg bw was established (JECFA, 1995). Assessment of the health risks posed by PAT to humans is based upon a wide number of studies during the past 50-plus years that implicate a number of acute, chronic, and cellular level health effects. Results of these studies suggest that acute symptoms of PAT consumption can include agitation, convulsions, dysponea, pulmonary congestion, edema, ulceration, hyperemia, GI tract distension, intestinal hemorrhage, epithelial cell degeneration, intestinal inflammation, vomiting, and other gastrointestinal and kidney damage (McKinley and others 1982; Mahfoud et al, 2002). Chronic health risks of PAT consumption can include neurotoxic, immunotoxic, immunosuppressive, genotoxic, teratogenic, and carcinogenic effects

(Wichmann et al, 2002). Reg CE 1881/2006 (Table 3) established limits of 50 µg/kg and 10 µg/kg for apple products intended for adults and infants and young children respectively.

Table 3: Maximum levels of PAT in foodstuffs (Reg CE 1881/2006)

Patulin	
Fruit juices, concentrated fruit juices as reconstituted and fruit nectars (14)	50
Spirit drinks (15), cider and other fermented drinks derived from apples or containing apple juice	50
Solid apple products, including apple compote, apple puree intended for direct consumption with the exception of food-stuffs listed in 2.3.4 and 2.3.5	25
Apple juice and solid apple products, including apple compote and apple puree, for infants and young children (16) and labelled and sold as such (4)	10,0
Baby foods other than processed cereal-based foods for infants and young children (3) (4)	10,0

1.2.2. Control of PAT during harvest, post-harvest and processings

Harvest/postharvest: The quality of fruit resulting from harvesting is the 1st step in controlling PAT levels. Bruises, skin breaks, and other physical damage within these apples provide a perfect entry for *P. expansum* and other PAT-producing species into the fruit. Various treatments have been investigated for the ability to reduce the apple rot and PAT production seen within the harvest, processing, and storage steps. Postharvest treatment of apples with benzimidazole fungicides was used from the 1970s through the early 1990s but has since been largely abandoned due to fungal resistance (Rosenberger 2003). Combinations of heat treatment, calcium infiltration, and biological control with an antagonistic bacteria, *Pseudomonas syringae,* were investigated alone or in combination to reduce incidence of postharvest apple decay. Apples inoculated with *P. expansum* and heated at 38°C for 4 days showed no decay lesions after storage at 1°C for 6 months. (Conway et al, 1999). Finally, other methods used for the control of human pathogens like *Escherichia coli*, such as washing with solutions of peroxyacetic acid, chlorine dioxide, and chlorine (Annous et al, 2001), may also provide some benefit toward preventing postharvest apple decay.
The accumulation of PAT is independent of fungicide applications during pre-storage with the temperature prior to refrigeration, probably the most important variable in toxin production. As refrigerated storage in a controlled atmosphere does not suffice to prevent fungal growth and PAT production, additional treatments also have been evaluated (Morales et al., 2007).
Processings: Immersion in 2-5% acetic acid (Sholberg, 1996) suffices to inhibit the growth of *P. expansum* and the production of PAT. For industrial applications, guaranteed contact time is important, and apples are either dipped in a 2% solution or sprayed with a 5% solution of acetic acid. Washing with water under high pressure can reduce PAT levels by 10-100% depending on the initial amount of the mycotoxin present (Acar *et al.*, 1998).
PAT can be removed from fruit juice with granular activated carbon. Passage of apple juice through activated charcoal (40-60 mesh) reduces the levels PAT by > 98% in apple juice (Kadakal et al., 2002). Clarification of apple juice with activated carbon can reduce both color and phenol content while also reducing the PAT level by > 40% (Gökmen et al, 2001). Studies of pasteurization on the levels of PAT in apple juice have not produced consistent results. Juice contaminated with PAT (1500 µg/l) and heated to 90°C for 2 minutes, followed by mixing with boiling water for 2 minutes and then cooled to room temperature reduces PAT levels by 60% (Taniwaki et al, 1989). In another study (Kadakal & Nas, 2003) treatments at 90°C and 100°C for 20 min reduced PAT content by 19% and 26%, respectively. In Turkey, a commercial process of 90°C for 10 seconds can reduce PAT contamination by an average of 13% in apple juice (Kadakal et al, 2002). These data suggest that if the initial contamination level is high, that it is impossible to achieve an economically significant reduction of PAT in the final product. PAT levels also may be affected by chemical additions to juices and nectars. PAT disappears rapidly at 25°C following the addition of 2% ascorbic acid or ascorbate to phosphate buffer containing the mycotoxin (Brackett and Marth, 1979). PAT decomposes due to free radicals generated from the oxidation of dehydroascorbic acid, so, when

all of the ascorbic acid is oxidized, no further degradation of the PAT occurs. Given the low levels of oxygen in the headspace of juice packs, the addition of ascorbic acid prior to packaging is not a good decontamination strategy. SO_2 at 100 ppm reduces PAT by 50%. The presence of PAT is an indicator of the use of low quality raw materials. Only through interactions between the producers, fruit processors and companies can a safe product for consumers be obtained (Yazici and Velioglu, 2002).

1.2.3. Exposure to PAT by the general population and children

The Scoop Task 3.2.8 "Assessment of dietary intake of patulin by the population of EU Member States" conducted in 2002 reported incidence of PAT contamination in different food matrices in European countries. The provided information on the PAT occurrence depicted a sufficient overall scenario of PAT contamination, to be used as the benchmark for European legislation. The analysed products included: juices (especially apple juices), apple juice concentrate, cider, pear juice, grape juice and other juices, baby foods. In consideration of the hypothetical level of 50 µg/kg of JECFA, the provided occurrence data for apple juice and products showed very few higher levels of contamination, leading to the general conclusion that products circulating in EU are of good sanitary quality with respect to PAT contamination.
Austria, Belgium, France, Germany, Italy, Norway, Portugal, Sweden, Spain, and United Kingdom have provided not exhaustive information about the issues indicated. An extract of contribution to dietary intake from each group of commodities in some participating Member States is presented in Table 2. For Italy three total dietary intakes are reported: one for fresh fruit, one for juices and purees, and one for baby foods. This because in the opinion of the Italian representative, the possibility to find PAT also in fresh fruit is considered noteworthy.
In year 2008-09 a study was carried out in Italy about assessment of dietary intake of PAT from baby foods (Bonerba et al, 2010). A total of 120 homogenized baby foods were analyzed to evaluate the exposure of baby and children to PAT through the consumption of these products. None of the 120 fruit-homogenized samples examined showed a toxin concentration above the limit imposed by the law (10 µg/kg). The PAT concentration found in all samples was included in the range 3 to 9 µg/kg with an average value equal to 6.28 µg/kg. Paradoxically, they are the products that consumers pay more on the market and buy because they are considered more safe because of the hypothetical absence of chemical contaminants, since by nonutilizing antifungal agents in organic farming products, it is likely that fungi develop at the risk of producing secondary metabolite like PAT. This suggests that, although there is certainly a particular attention by baby food producers, sometimes they select raw materials of poor quality, which increases the value of PAT just below the specified limits.

Table 2: Dietary intake of PAT in Member States.

	apple juice	pear juice	grape juice	fruit juice	cider	puree	baby food	others	fresh fruit	total dietary intake
France (Children: P)[7]	3,00				0,10	1,70				**4,90**
France (Children: C)[7]	18,80				5,40	6,80				**12,90**
France (Children: 3-5 y, P)[7]	4,90				0,10	3,10				**8,10**
France (Children: 6-8 y, P)[7]	4,50				0,10	2,20				**6,90**
France (Children: 9-11 y, P)[7]	1,60				0,10	1,00				**2,70**
France (Children: 12-14 y, P)[7]	1,20				0,06	0,60				**1,80**
France (Adults: P)[7]	0,30				0,30	0,60				**1,23**
France (Adults: C)[7]	8,90				5,60	3,30				**4,90**
France (Adults: males, P)[7]	0,36				0,43	0,40				**1,19**
France (Adults: females, P)[7]	0,25				0,22	0,78				**1,26**
Germany (Children, girls, 1 y, P)							25,00			**25,00**
Germany (Children, girls, 1,5 y, P)							36,00			**36,00**
Germany (Children, girls, 2 y, P)							27,00			**27,00**
Germany (Children, girls, 3 y, P)							41,00			**41,00**
Germany (Children, boys, 1 y, P)							22,00			**22,00**
Germany (Children, boys, 1,5 y, P)							49,00			**49,00**
Germany (Children, boys, 2 y, P)							25,00			**25,00**
Germany (Children, boys, 3 y, P)							51,00			**51,00**
Germany (Children, girls, 4-6 y, C)	47,00	6,40	11,00			1,60		0,60		**66,60**
Germany (Children, girls, 4-6 y, P)	43,00	0,30	0,60			0,02		0,10		**44,02**
Germany (Children, 6-14 y, C)	30,00	5,40	7,70			1,20		0,40		**44,70**
Germany (Children, 6-14 y, P)	9,50	0,05	0,16			0,01		0,04		***9,76***
Germany (Adults, >14 y, C)	11,00	2,30	3,40			0,60		0,20		**17,50**
Germany (Adults, >14 y, P)	1,90	0,02	0,06			0,01		0,02		**2,01**
Italy (only juice and puree, P)[5]				9,56		0,03				***9,59***
Italy (only juice and puree, C)[5]				51,97		1,12				**53,09**
Italy (only fresh fruit, P)[6]									83,05	**83,05**
Italy (only fresh fruit, C)[6]									140,36	**140,36**
Italy (fresh fruit, juice and puree, P)										***92,64***
Italy (fresh fruit, juice and puree, C)										***193,45***
Italy (Toddlers, P)							0,02			**0,02**
Italy (Toddlers, C)							5,91			**5,91**

P All population
C Consumers
5 For the summary of daily intake the occurrence data of apple (with and without peel, peach and pear) are not considered.
6 For the summary of daily intake the occurrence data of apple without peel are not considered.
7 Instead of summing up the daily intakes of each commodity the French representative proposed to do the calculation of the daily intake of patulin with the real disaggregated data on individuals from all the commodities

1.3. Aflatoxins

The aflatoxins, are difuranocoumarin derivatives produced by a polyketide pathway by many strains of *A. flavus*, *A. parasiticus* and *A. nomius*. It is possible that further species of fungi will be added to this list, indeed, a new species of *Aspergillus*, *A. ochraceoroseus* isolated from the Tai rain forest, has been reported to produce aflatoxins (Goto et al., 1996). Although 20 AFs have been identified, only four of them, that are the AFs B_1, B_2, G_1 and G_2 (AFB_1, AFB_2, AFG_1 and AFG_2), (Fig. 3) occur naturally and are significant contaminants of a wide variety of foods and feeds. AFB_1 is generally the most common and it is the most toxic in terms of both acute and chronic toxicity.
The minimum temperature range for *A. parasiticus* growth is 6-8°C, the maximum is 44-46°C, and the optimum is 25-35°C. *A. flavus* can produce aflatoxin at temperatures of 12-42°C, but the optimum is 28-30°C. The spores of *A. flavus* and *A. parasiticus* can germinate on the stigma surfaces of plants, such as peanuts and maize, the germ tube then penetrating to the developing embryo in a manner mimicking pollen germ tubes. If the plant is at all stressed, and the most usual stress is drought, then significant levels of AFs may be produced in the plant tissue during growth in the field. Under these circumstances food commodities may already be contaminated at harvest (Hill et al., 1985) and, although the concentrations are never as high as those formed in stored commodities, they can be economically significant.

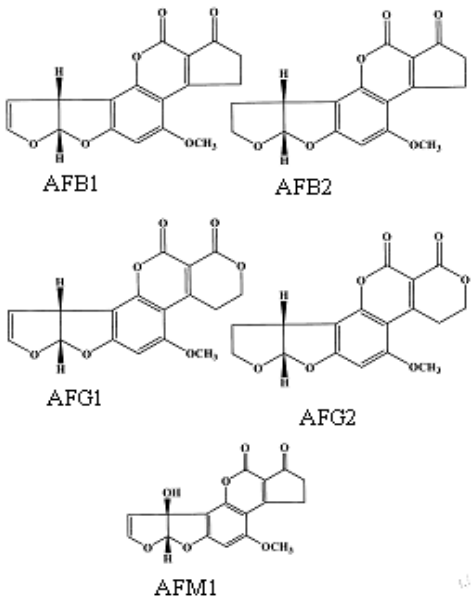


Figure 3: Chemical structures of Aflatoxins B_1; B_2;G_1;G_2 and M_1

1.3.1. Toxicity

The liver is the main target organ for AFs toxicity and carcinogenicity (Abdel-Wahhab et al., 2007). Early symptoms of hepatotoxicity from aflatoxicosis are protean and may masquerade many other forms of toxaemias. It can manifest as anorexia, malaise, and low-grade fever. Aflatoxicosis can progress with vomiting, abdominal pain, hepatitis, and eventually death (Etzel, 2002). Clearly cases of acute poisoning are rare and exceptional.
Chronic toxicity is of serious concern and, although this may include both immunosuppressive activity and liver cirrhosis, it is the carcinogenicity of AFB_1 which drives international concern about the occurrence of AFs in food.

At the molecular level it is clear that the tumour-suppressor gene p53 may be involved for, not only are mutations in this gene the most frequent changes found in human tumours, but it plays an important role as a transcription and translation regulator of a number of other genes involved in the control of cell division and differentiation (Bourdon et al., 1997). AFB_1 is metabolised in the animal liver to a number of products. An important metabolite is the 8,9-epoxide (Fig. 4) which is known, not only to react with DNA, but to do so at the guanine residues of specific sites, one of these being the third base position of codon 249 of the p53 gene (Hsu et al., 1991). The International Agency for Research on Cancer (IARC) classified AFB_1 as a group 1 carcinogen (i.e. carcinogenic to humans) in 1987 and exposure to hepatitis B virus as a group 1 carcinogenic agent in 1993 (Castegnaro and McGregor, 1998).
Mammals that ingest AFB_1-contaminated diets excrete amounts of the principal 4-hydroxylated (AFM_1) into milk. A tolerable daily intake of 0.2 ng/kg b.w. for AFM_1 was calculated by Kuiper-Goodman (1990) and this toxin has been categorized by the International Agency for Research on Cancer (IARC) as a class 2B toxin, a possible human carcinogen. In the assessment of cancer risk, the infants are more exposed to the risk because the milk is a major constituent of their diet. It must be also considered that young animals have been found to be more susceptible to AFB_1 (and so probably AFM_1) than adults. It is hypothesized that early and repeated exposures to AFs in-utero and through childhood might predispose to cancer liver later in life. AFs thus, may increase child susceptibility to infections and may cause failure of immunizations (Hendrickse, 1997). Table 4 reports AFs limits in foodstuffs fixed by Reg. CE 1881/2006.

1.3.2. Control during harvest and processings

Harvest: In order to prevent losses of yield and hazards for human and animal health, it is necessary to take care of coltural phases that can represent a critical point for fungal growth and AFs production in organic, low and high-input farming system (e.g. excessive nitrogen fertilization in high-input farming system, sod seeding or minimum tillage in organic and low-input farming system). To prevent future AFs outbreaks it is needed to communicate about the potential risk deriving from unsuitable farming managements that could lead to the development of contaminated feeds and foods (Prandini et al., 2009). Palumbo et al. (2006) reported that a number of *Bacillus*, *Pseudomonas*, *Ralstonia* and *Burkholderia* strains could completely inhibit *A. flavus* growth, while *B. subtilis* and *P. solanacearum* strains were also able to inhibit AFs accumulation. Shetty et al. (2007) found that the ability of *S. cerevisiae* to bind AFB_1 was strain specific with 7 strains binding 10-20%, 8 strains binding 20-40% and 3 strains binding more than 40% of the added AFB_1.
Processings: AFB_1 may be reduced by up to 92% during preparation of the Mexican drink atole by simple heating at 94°C for 10 minutes and then drying at 40°C for 48 hours. In the process of making corn flakes with cooked oatmeal and no sugar that has been coarsely ground, AFs are reduced by 64-67%, while after roasting both with and without sugar the reduction is between 78-85%. Extrusion, a process frequently used to produce breakfast cereals and snacks, uses processing temperatures > 150°C. (Lu et al, 1997).

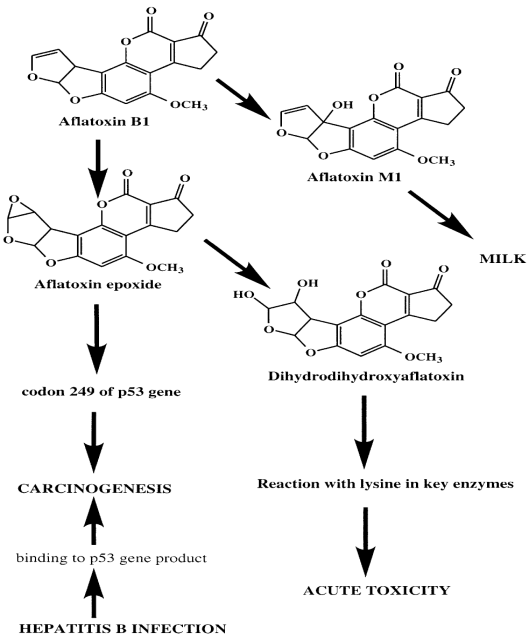


Figure 4: Metabolites of AFB_1 involved in toxicosis

Fungi that produce AFs also can colonize rice. If rice is cooked with normal or excess water, then up to 89% of the lactone rings in the AFs molecules can be hydrolyzed to the corresponding carboxylic acid. If cooked under pressure at 0.10 MPa then up to 88% of the AFs in a sample of naturally contaminated rice were reducted, compared to 34% degradation in unpressurized boiling water (Park et al, 2006).
The presence of AFs in matrices such as coffee and peanuts requires evaluating the roasting process as a part of the potential detoxification process. Public reports of AFs reductions between 45% and 83% in these substrates are available, (Lee et al, 1969). In order to face the problem of AFM_1 in milk and dairy products, it is necessary to focus the attention on the most sensitive steps of feedstuff production for lactating cows. Pasteurization of milk at 62°C for 30 minutes reduced AFM_1 contamination by 32% (Purchase et al., 1972). This result is in concordance with a study carried out in year 1977 by Kiermeier reporting a decrease of AFM_1 in milk from 12% to 35%.

Table 4: Maximum levels of AFs in foodstuffs (Reg. CE 1881/2006)

Aflatoxins	B_1	Sum of B_1, B_2, G_1 and G_2	M_1
Groundnuts to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs	8,0	15,0	—
Nuts to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs	5,0	10,0	—
Groundnuts and nuts and processed products thereof, intended for direct human consumption or use as an ingredient in foodstuffs	2,0	4,0	—
Dried fruit to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs	5,0	10,0	—
Dried fruit and processed products thereof, intended for direct human consumption or use as an ingredient in foodstuffs	2,0	4,0	—
All cereals and all products derived from cereals, including processed cereal products, with the exception of foodstuffs listed in 2.1.7, 2.1.10 and 2.1.12	2,0	4,0	—
Maize to be subjected to sorting or other physical treatment before human consumption or use as an ingredient in foodstuffs	5,0	10,0	—
Raw milk (6), heat-treated milk and milk for the manufacture of milk-based products	—	—	0,050
Following species of spices: *Capsicum* spp. (dried fruits thereof, whole or ground, including chillies, chilli powder, cayenne and paprika) *Piper* spp. (fruits thereof, including white and black pepper) *Myristica fragrans* (nutmeg) *Zingiber officinale* (ginger) *Curcuma longa* (turmeric)	5,0	10,0	—
Processed cereal-based foods and baby foods for infants and young children	0,10	—	—
Infant formulae and follow-on formulae, including infant milk and follow-on milk	—	—	0,025
Dietary foods for special medical purposes , intended specifically for infants	0,10	—	0,025

1.3.3. Exposure to AFs by the general population and children

FAO reported data submitted by 33 countries for AFB_1 and 48 countries for total AFs (B_1, B_2, G_1 and G_2) used to estimate median levels of 4 and 8 µg/kg, respectively, in foodstuffs. The range of levels reported for AFB_1 was from 0 to 30 µg/kg and for total AFs from 0 to 50 µg/kg (FAO, 1997). SCOOP concluded that AFs were found in a broader range of foods than had been previously assumed, but that most samples did not contain any detectable aflatoxin.
Information on the occurrence of AFs in imported spices in the European Union (EU) is given in Table 3. Among the total of 3098 spice samples including nutmeg, pepper, chilli and paprika, 183 samples (5.9%) contained more than 10 µg/kg AFs (European Commission, 1997). Data on AFM_1 in milk samples collected

by EFSA and originating from different EU Member States show that the prevalence of AFM_1 contaminated samples seems to be very low (Table 4).
It may be interesting to note, considering a TDI of 0.2 ng/kg b.w. (14 ng/person, for a mean weight of 70 kg), that an intake of 15 ng/person per day estimated in the European regional diet could represent a significant dose (JECFA, 2001).

Table 3: Occurrence of AFs in imported spices in the European Union (EU), (European Commission, 1997)

Product	Detected/ total samples	Aflatoxin B_1 (µg/kg)	
		> 2	> 10
Nutmeg	333/546	25%	8%
Pepper	282/828	7%	1%
Chilli and chilli powder	148/509	28%	9%
Paprika powder	195/1215	21%	7%
Total spices	958/3098	> 1 µg/kg	
	591/3098	> 2 µg/kg	
	183/3098	> 10 µg/kg	

In exposed communities, AFs have been detected in breast milk. Estimated carryover of AFs from dietary intake to milk in animals is around 1%, and similar estimates were made from studies measuring intakes versus excretion in individual women in The Gambia (Zarba et al., 1992). Recently, Polychronaki et al. (2008) reported that breast milk samples obtained from 388 Egyptian women were shown to have detectable AFM_1 with levels in nearly 36% of samples.
AFs have been identified as a risk factor in newborn jaundice (NNJ). Many recent epidemiologic studies show inconclusive evidence of the association between NNJ and exposure to AFs (Galal et al., 2006). A probable consequence of early exposure to AFs is growth faltering. Epidemiological evidence derived from cross-sectional studies comprising West African young children < 5 yr, where a dose response relation between aflatoxin exposure and the degree of stunting and underweight has been observed (Gong et al., 2003). Kwashiorkor, a disease of children in Northern Africa and elsewhere in undernourished populations, which is usually attributed to nutritional deficiencies, may also be related to AFs intake based on observational studies (Golden and Ramadath, 1987).

Table 4: Daily intake of milk and consequent quantity of AFM_1 ingested in the five regional diets (JECFA, 2001)

Diet	Milk intake (kg/day)	Weighted mean		
		Aflatoxin M1 in milk (µg/kg)	Aflatoxin M1 intake	
			µg/person per day	µg/kg b.w. per day
European	0.29	0.02	6.80	0.11
Latin American	0.16	0.02	3.50	0.06
Far Eastern	0.03	0.36	12.00	0.20
Middle Eastern	0.12	0.01	0.60	0.10
African	0.04	~0.00	0.10	~0.00

1.4. Ochratoxins

Ochratoxins (OTs) are a group of structurally related secondary metabolites, produced by some toxic fungi such as *Penicillium verrucosum* and by *Aspergillus ochraceus*; occasionally also isolates of the common species *Aspergillus niger* can produce Ochratoxin A (OTA) (Fig.5) the main mycotoxin in the group of OTs and the only one of toxicological significance. OTA contains an isocoumarin moiety linked by a peptide bond to phenylalanine and it is generally found in cereals, oleaginous seeds, green coffee, pulses, wine, and poultry meat. OTA production depends on both environmental and processing conditions (climatic conditions, abnormally long storage, transportation, wet or dry milling , roasting procedures, fermentation etc.).

The minimum aw values for OTA production are 0.83-0.87. At 24°C, optimum aw values for *A. ochraceus* are 0.99 and for both *Penicillium* fungi are 0.95-0.99, (Pitt, 2000).

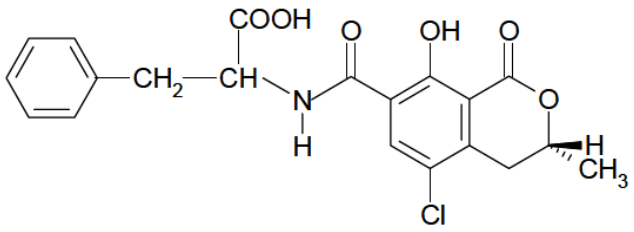


Figure 5: chemical structure of OTA

1.4.1. Toxicity

OTA has been shown to be nephrotoxic, hepatotoxic, teratogenic and immunotoxic to several species of animals and to cause kidney and liver tumours in mice and rats. As far as humans are concerned, the IARC (International Agency for Research on Cancer) classified OTA as a possible carcinogen to humans (Group 2B), (IARC, 1993). With regard to nephrotoxicity, OTA is considered to be involved in the Balkan Endemic Nephropaty (BEN), a severe kidney pathology, generally occurring in some areas of South-Eastern Europe and linked to urinary tracts tumours (Castegnaro et al., 1991). As regards to the toxicity of OTA, it was observed that its biotransformation is cytochrome P450 dependent in animals and humans, and it results in the formation of metabolic intermediates active in the carcinogenic and other toxic activities. The cytochrome stimulates the OTA induced lipids peroxidation, this process involves moreover some enzymes, present in the cell, and leads to the formation of toxic metabolites (Fink et al, 1995).
As regards oral LD_{50} values, they are 20 mg/kg and 3.6 mg/kg in young rats and in day-old chicks, respectively; OTA is also lethal to mice, trout, dogs and pigs (Pitt et al, 2000).
The complex toxic activity of OTA is multifaceted in relation to the role of one of its structural components, L-phenylalanine, which is involved in the inhibition of numerous reactions where it is known to function. The covalent bonds of chemical substances, or their metabolites, to DNA are considered a key step in the processes which induces to carcinogenesis. Acute lethal doses of OTA produce widespread multifocal haemorrhages, intravascular coagulation and necrosis of liver, kidney and lymphoid organs. OTA is a potent renal toxicant in all animal species tested. In the absence of evidence that the genotoxicity of OTA *in vitro* and *in vivo* was mediated by formation of a reactive intermediate of OTA or by direct interaction with DNA it has been speculated that OTA may act as a genotoxin by generating reactive oxygen species or other, indirect epigenetic mechanisms (Walker & Larsen, 2005). The Joint FAO/WHO Expert Committee on Food Additives (JEFCA), on the basis of the nephrotoxicity of OTA, proposed a provisional tolerable weekly intake (PTWI) for OTA of 0.1 µg/kg body mass (equivalent to 14 ng/kg body mass/day) (WHO, 1996).
Experimental in-utero transfer of OTA has been documented in mice and rats (Abdel-Wahhab et al., 1999). In humans, OTA levels were twice as high in umbilical cord blood as in maternal blood at the time of delivery suggesting that active placental transfer may be occurring in humans (Jonsyn et al., 1995). OTA in breast milk was found to affect the kidney function and the development of urinary tumors in infants and young children (Skaug, 1999). Table 5 shows limits fixed for OTA in foodstuffs (Reg CE 1881/2006).

Table 5: Maximum levels of OTA in foodstuffs (Reg CE 1881/2006).

Ochratoxin A	
Unprocessed cereals	5,0
All products derived from unprocessed cereals, including processed cereal products and cereals intended for direct human consumption	3,0
Dried vine fruit (currants, raisins and sultanas)	10,0
Roasted coffee beans and ground roasted coffee, excluding soluble coffee	5,0
Soluble coffee (instant coffee)	10,0
Wine (including sparkling wine, excluding liqueur wine and wine with an alcoholic strength of not less than 15 % vol) and fruit wine	2,0
Aromatised wine, aromatised wine-based drinks and aromatised wine-product cocktails	2,0
Grape juice, concentrated grape juice as reconstituted, grape nectar, grape must and concentrated grape must as reconstituted, intended for direct human consumption	2,0
Processed cereal-based foods and baby foods for infants and young children	0,50
Dietary foods for special medical purposes intended specifically for infants	0,50
Green coffee, dried fruit other than dried vine fruit, beer, cocoa and cocoa products, liqueur wines, meat products, spices and liquorice	—

1.4.2. Control during harvest and processings

Harvest: As mycotoxin producing molds can usually only colonize damaged parts of plants, crops must be protected against damage caused by either mechanical processes or insects.

For lowering pre-harvest contamination, treatment of field crops with fungicides is the traditional technique (Aldred, 2004). The organophosphate fungicide, dichlorvos, was found to inhibit OTA production of *A. sulphureus*, *P. verrucosum,* and *A. ochraceus* (Varga, 2004). Another fungicide, iprodione, has successfully been used in agricultural commodities to prevent the growth of various fungal species, including OTA producers (Kabak, 2006).

Several bacterial and fungal strains belonging to *Streptococcus, Bifidobacterium, Lactobacillus, Pleurotus, Saccharomyces,* genera, are able to degrade OTA *in vitro* up to more than 95% (Abunrosa L. et al., 2006).

Fuchs et al. in year 2008 screened 30 different lactic bacteria strains for their ability to remove OTA from solution, and they reported 95% removal of OTA in liquid media by *L. acidophilus.*

It was suggested that the end product of degradation of OTA by *A. calcoaceticus* is a less toxic compound, ochratoxin α (OTα) (Halasz et al., 2009). Experimental results showed that yeast strains are able to degrade OTA more than 80% during their growth under *in vitro* conditions (Patharajan et al, 2010).

Processings: OTA's insensitivity to thermal processes enables it to persist through the processing of products such as coffee, cocoa, wine, beer, and cereals. Roasting coffee at 200°C for periods of 10-20 minutes reduces the amount of OTA present between 12 and 100% (Roman *et al*., 2003). Cooking rice in excess water reduces OTA levels by 86%. In the production of beer OTA is sensitive to hydrolysis during the malting, boiling and fermentation steps with minimal losses of 12%, 20% and 20% of the OTA present, respectively (Chu et al, 1975).

OTA is stable during the baking of bread, but during biscuit preparation two-thirds of the OTA is either destroyed or immobilized. OTA also is reduced during the production of cereals, due to extrusion cooking, by a maximum level of 40% (Castells et al, 2005). γ radiation reduces OTA production by *Aspergillus ochraceus* (Refai *et al.*, 1996). In poultry feed, fungal growth ceases at 4 kGy of radiation. OTA production ceases at 3 kGy, and is reduced from 60 to 1.9 ng/g after exposure to 2 kGy. γ radiation in doses of 15 and 20 kGy, respectively, completely destroy OTA in yellow corn and soybeans. In poultry layer's and broiler's concentrates and cotton-seed cake however, the detoxification rates are 40%, 47% and 36%, at radiation

dose of 20 kGy (Refai et al, 1996). The lack of detection of OTA need not imply a reduction in potential toxicity, because the breakdown by-products can be as toxic as OTA itself. (Suárez et al, 2005).

1.4.3. Exposure to OTA by general population and children

OTA is found in wheat, corn, and oats having fungal infection and in cheese and meat products of animals consuming ochratoxin-contaminated grains (Aish and others 2004). *A. ochraceus* is found on dry foods such as dried and smoked fish, soybeans, garbanzo beans, nuts, and dried fruit. *A. carbonarius* is the major pathogen in grapes and grape products including raisins, wines, and wine vinegars. Although reported to occur in foods around the world, the main regions of concern are Europe and, for some foods, Africa. JEFCA and WHO presented data indicating that cereals, wine, grape juice, coffee, and pork are the major sources of human OTA exposure, at levels of 58%, 21%, 7%, 5%, and 3% of total OTA intake, respectively (JECFA 2001). Levels reported range from 100 to 700 ng/kg in cereals, 30 to 9000 ng/l in European wines, 170 to 1300 ng/kg in coffee, and 150 to 2900 ng/kg in pork.
In a recent submission of the Swedish Food Administration (Möller, 2005, personal communication) 192 analytical results on the occurrence of OTA in cereals and cereal products are presented, with an average level of 0.12 µg/kg. This level is about one half of that reported by JECFA and SCOOP. For wine, 5 surveys were published including about 550 analyses (Pietri et al., 2001; Stefanaki et al., 2003; Soufleros et al., 2003; Shephard et al., 2003; Blesa et al., 2004). Average contamination ranged between 0.2 and 0.4 µg/L and the highest values reported were below 4 µg/L. No systematic differences between countries could be found. It should be noted that the average concentration was derived from a large number of samples of food available on the entire EU market. This implies that in certain countries or regions due to climate or storage conditions the mean value for food contamination could be higher.
The contribution to the dietary exposure for each category was estimated using the mean concentrations of OTA in the individual food categories from the most recent SCOOP report, and the mean and high consumption data from the draft EFSA food consumption database (table 6).
In breast milk samples, the highest concentrations of OTA have been found in Sierra Leone, where 35% of the samples contained OTA at levels from 200 to 337 ng/ml (Jonsyn et al., 1995). The highest concentration in breast milk was 1.890 ng/l in Egypt (Hassan et al., 2006). Total daily intake of OTA varies among countries, depending on food-handling methods, and has been estimated based on total diets or on consumption of specific contaminated foods or beverages. The highest estimated daily intake was 1.21 µg for adults with Balkan Endemic Nephropathy in Bulgaria (Clark and Snedeker 2006), and the highest estimated daily intake for children was 3.6 ng/kg of body weight for Swiss children who consumed grape juice.

Table 6: Dietary exposure to OTA in mean consumers (average consumption in the total population) and high consumers (97.5th percentile consumption in consumers only) from the relevant food categories using the draft EFSA concise database.

	EFSA Code	Concentration OTA	Average dietary exposure			97.5th percentile exposure		
	(a)	(b)	France	Italy	Sweden	France	Italy	Sweden
		µg/kg	(ng/day/person)			(ng/day/person)		
Cereals and cereal products	1	0.29	63	78	85	145	140	165
Sugar and confectionery (c)	2	0.24	9	5	7	28	17	22
Hot beverages (c)	8	0.72 (c)	11	5	24	39	15	52
Beer	9A	0.028	1	1	4	22	14	20
Wine	9B	0.36	39	33	14	235	191	93
Edible offal	10A	0.2	1	1	1	9	15	9
Fruit juices (c)	7A	0.55	25	9	48	173	119	267

a) EFSA opinion on exposure assessment (EFSA, 2005)
b) Mean concentrations from SCOOP 3.2.7 (EC, 2002)
c) Consumption of fruit juices, hot beverages and confectionery was matched in the applied model calculations with at the OTA concentrations in grape juice, coffee and chocolate products. This may lead to

an overestimate of the number of consumers per category and to an overestimate of average levels of exposure in the total population. However, it probably does not lead to an overestimate of the rate of exposure of high consumers, since in most cases high consumers of a single food commodity are not high consumers of other food commodities within the same category. For example, the high consumers of grape juice do not consume large quantities of other fruit juices at the same time.

References

Abdel-Wahhab, M.A., Abdel-Galil, M.M., Hassan, A.M., Hassan, N.H. , Nada, S.A., Saeed, A., El-Sayed, M.M., (2007). Zizyphus spina-christi extract protects against aflatoxin B1-intitiated hepatic carcinogenicity. Afr.J.Trad. CAM 4: 248–256.

Abdel-Wahhab M.A., Nada, S.A., Arbid, M.S., (1999). Ochratoxicosis: prevention of developmental toxicity by L-methionine intherats. J.Appl.Toxicol. 19:7–12.

Abunrosa L, Santos L, Venancio A. Degradation of ochratoxin-A by proteases and crude enzyme extract of *Aspergillus niger* (2006). Food Biotechnol. 20: 231-236.

Acar, J., Gökmen, V., and Taydas, E.E. (1998). The effects of processing technology on the patulin content of juice during commercial apple juice concentrate production. European Food Research and Technology 207: 328-331.

Aish J.L., Rippon E.H., Barlow T., Hattersley S.J. (2004). Ochratoxin A. In: Magan N, Olsen M, Eds. Mycotoxins in food: detection and control. Boca Raton, Fla. 2004, pp 307–38.

Aldred, D.; Magan, N. (2004). The use of HACCP in the control of mycotoxins: the case of cereals. In *Mycotoxins in Food: Detection and Control*; Magan, N., Olsen, M., Eds.; CRC Press: Boca Raton, FL, USA 2004, pp 139–173.

Annous B.A., Sapers G.M., Mattrazzo A.M., Riordan D.C. (2001). Efficacy of washing with a commercial flatbed brush washer, using conventional and experimental washing agents, in reducing populations of Escherichia coli on artificially inoculated apples. J Food Prot 64:159–63.

Arafat W., Musa M.N. (1995). Patulin-induced inhibition of protein synthesis in hepatoma tissue culture. Res Commun Mol Pathol Pharmacol 87(2):177–86.

Baars AJ, Van Apeldoorn M, Wouters M, (1999). Deoxynivalenol. Derivation of concentration limits in wheat and wheat containing products. RIVM report 388802008.

Bello, G. M. D., Monaco, C. I., and Simon, M. R. (2002). Biological control of seedling blight of wheat caused by *Fusarium graminearum* with beneficial rhizosphere microorganisms. World J. Microbiol. Biotechnol. 18:627-636.

Berestets'kyi OO, Synyts'kyi. (1973). Phytotoxic activity in different strains of *Penicillium urticae* banier. Mikrobiol Zh 35:349.

Bergsjo B, Matre T, Nafstad I (1992) Effects of diets with graded levels of deoxynivalenol on performance in growing pigs. Zentralbl Veterinarmed[A] 39:752–758

Bhat R.V, Beedu S.R., Ramakrishna Y., Munshi K.L. (1989). Outbreak of trichothecene mycotoxicosis associated with consumption of mould-damaged wheat products in Kashmir Valley, India. Lancet 1(8628): 35–37.

Blesa, J., Soriano, J.M., Molto, J.C. and Manes, J. (2004). Concentration of ochratoxin A in wines from supermarkets and stores of Valencian Community (Spain). J Chromatogr A 1054(1-2): 397-401.

Bonerba E., Conte R., Ceci E., Tantillo G., (2010). Assessment of Dietary Intake of Patulin from Baby Foods. Journal of Food Science 75 (7): 123–125

Bourdon, J.-C., Deguin-Chambon, V., Lelong, J.-C., Dessen, P., May, P., Debuire, B., May, E., (1997). Further characterisation of the p53 responsive element—identi5cation of new candidate genes for trans-activation by p53. Oncogene 14:85–94.

Brackett, R.E., and Marth, E.H. (1979). Ascorbic acid and ascorbate cause disappearance of patulin from buffer solutions and apple juice. Journal of Food Protection, 42:864-866.

Canady RA, Coker RD, Rgan SK, Krska R, Kuiper-Goodman T, Olsen M, Pestka JJ, Resnik S, Schlatter J (2001). Deoxynivalenol. Safety evaluation of certain mycotoxins in food. Fifty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives. WHO Food Additives Series 47. International Programme on Chemical Safety, 420–555

Castegnaro M., Plestina R., Dirheimer G., Chernozemisky I.N., Bartsch H. (Eds.), (1991). Mycotoxins, Endemic Nephropaty and Urinary Tracts Tumours, IARC Scientific Publication No. 115, International Agency for Research on cancer, Lyon.

Castegnaro, M., McGregor, D., 1998. Carcinogenic risk assessment of mycotoxins. Revue Médecine Vétérinaire 149: 671–678.

Castells M., Marìn S., Sanchis V.,. Ramos A. J. (2005) Fate of mycotoxins in cereals during extrusion cooking: A review. Food Additives & Contaminants: Part A: Chemistry, Analysis, Control, Exposure & Risk Assessment. 22 (2): 150 - 157

CDC (Centers for Disease Control and Prevention), 1999. Outbreaks of gastrointestinal illness of unknown etiology associated with eating burritos –. Morb. Mortal. Wkly. Rep. 48:210–213.

Chu, F.S., Chang, C.C., Ashoor, S.H., Prentice, N., (1975). Stability of aflatoxin B_1 and ochratoxin A in brewing. Applied Microbiology 29 (3): 313–316.

Ciegler A, Detroy RW, Lillehoj EB. (1971). Patulin, penicillic acid, and other carcinogenic lactones. In: Ciegler A, Kadis S, Ajl SJ, editors. Microbial toxins. Vol 6. New York and London: Academic Press. 1971, p 409.

Clark H.A., Snedeker SM. (2006). Ochratoxin A: Its cancer risk and potential for exposure. J Toxicol Environ Health B Crit Rev 9(3): 265-296.

Commission Regulation (EC) No. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Official Journal of the European Union, 364: 5–24

Conway WS, Janisiewicz WJ, Klein JD, Sams CE. (1999). Strategy for combining heat treatment, calcium infiltration, and biological control to reduce postharvest decay of 'Gala' apples. Hort Sci 34(4):700–704.

Cooray R, Kiessling K-H, Lindahl-Kiessling K. (1982). The effects of patulin and patulin- cysteine mixtures on DNA synthesis and the frequency of sister-chromatid exchanges in human lymphocytes. Food Chem Toxicol 20:893–8.

Creepy E.E. (2002). Update of survey, regulation and toxic effects of mycotoxins in Europe. Toxicology Letters, 127: 19–28.

Dors, G.C., Almeida, L.A., Badiale-Furlong, E. (2009). Migration of mycotoxins into rice starchy endosperm during parboiling process. Food Science and Technology 42: 433-437.

DG-SANCO, Draft Commission Recommendation of July 2000 on the reduction of the presence of deoxynivalenol in cereals and cereal products. SANCO/1925/00-rev-1, 5

Edwards SG. (2004). Influence of agricultural practices on Fusarium infection of cereals and subsequent contamination of grain by trichothecene mycotoxins. Toxicol Lett, 153:29–35.

EFSA (European Food Safety Authority), (2005). Opinion of the Scientific Committee on a request from EFSA related to Exposure Assessments. Adopted on 22 June 2005. The EFSA Journal (2005) 249: 1-26.

El-Banna, A.A., Lau, P.-Y., Scott, P.M., (1983). Fate of mycotoxins during processing of foodstuffs. II-Deoxynivalenol (vomitoxin) during making of Egyptian bread. Journal of Food Protection 46: 484–486

Etzel, R.A., (2002). Mycotoxins. J. Am. Med. Assoc. 287: 425–427.

European Commission (1997) Coordinated Programme for the Official Control of Foodstuff, Luxembourg

European Commission (2002). Assessment of dietary intake of ochratoxin A by the population of EU Member States. Report of the Scientific Cooperation, Task 3.2.7. Directorate-General Health and Consumer Protection, European Commission.

Fink-Gremmels J., Blom M.J., Woutersen van Nijnantem F.M.A., Jahn A., de Groen E.M., (1997) Biotransformation process in the etiology of ochratoxicosis. In: Molecular Approaches to Food Safety: Issues Involving Toxic Microorganisms. M. Eklund, J.L. Richard, K. Mise (Eds.). Alaken, Inc., Ft. Collins 1997, pp 107-121.

Food and Agriculture Organization (1997) Worldwide regulations for mycotoxins. A compendium. FAO Food and Nutrition Paper 64.

Food and Agriculture Organization (2005). The State of Food Insecurity in the World 2005. Eradicating world hunger key to achieving the millennium development goals. http://www.fao.org/icatalog/inter-e.htm

Frohlich A.A., Marquardt R.R., Ominsky K.H., Ochratoxin A as a contaminant in the human fod chain: a Canadian perspective (1991). In: Mycotoxins, Endemic Nephropaty and Urinary Tracts Tumours, M. Castegnaro, R. Plestina, G. Dirheimer, I.N. Chernozemisky, H. Bartsch (Eds.), IARC Scientific Publication No. 115, Oxford University Press, UK. 1991, pp 139-143.

Galal, N., Gamal El-Din, R.M., Arafa, A., Mohamad, I.M., Abdel-Wahhab, M.A., (2006). Aflatoxin levels in Egyptian neonates with unexplained neonatal jaundice: morbidity and correlation to maternal assays in breast milk. Egypt. J. Neonatol. 7 (3), 161–169.

Golden, M.H., Ramadath, D., (1987). Free radicals in the pathogenesis of Kwashiorkor. Proc. Nutr. Soc. 46: 53–68.

Gong, Y., Cardwell, K., Hounsa, A., Egal, S., Turner, P.C., Hall, A.J., Wild, C.P., (2002). Dietary aflatoxin exposure and impaired growth in young children from Benin and Togo: cross sectional study. BMJ 325 (7354), 20–21.

Gökmen, V., Artik, N., Acar. J., Kaharaman, N. & Poyrazoglu, E. (2001). Effects of various clarification treatments on patulin, phemolic compound and organic acid composition of apple juice, European Food Research Technology, 213: 194-199

Goto, T., Wicklow, D.T., Ito, Y., (1996). Aflatoxin and cyclopiazonic acid production by a sclerotium-producing Aspergillus tamarii strain. Applied and Environmental Microbiology 62: 4036–4038.

Grove JF (2000). Non-macrocyclic trichothecenes. Part 2. Prog Chem Org Nat Prod 6: 1–70

Halasz A, Lasztity R, Abony T, Bata A. Decontamination of mycotoxin-contaminating food and feed by biodegradation. (2009). Food Rev Int. 25:284-298.

Hassan A.M., Sheashaa H.A., Fattah M.F.A, Ibrahim A.Z., Gaber O.A., Sobh M.A. (2006). Study of ochratoxin A as an environmental risk that causes renal injury in breast-fed Egyptian infants. Pediatr Nephrol 21(1): 102-105.

Hendrickse, R.G., (1997). Of sick Turkeys, Kwashiorkor, malaria, perinatal mortality, Herion addicts and food poisoning: research on influence of aflatoxins on child health in the tropics. J. Trop. Med. Parasitol. 91: 87–93.

Hershman, D., and Draper, M. (2004). Analysis of uniform fungicide trials across locations and wheat classes. in: Proc. 2nd International Symposium on Fusarium Head Blight 2001, pp 318-319

Hill, R.A., Wilson, D.M., McMillan, W.W., Widstrom, N.W., Cole, R.J., Sanders, T.H., Blankenship, P.D., (1985). Ecology of the *Aspergillus flavus* group and aflatoxin formation in maize and groundnuts. In: Lacey, J. (Ed.), Trichothecenes and other mycotoxins. 1985, pp 79–95.

Hopton R.P., Turner E., Burley V.J., Turner P.C., Fisher J. (2010). Urine metabolite analysis as a function of deoxynivalenol exposure: an NMR-based metabolomics investigation. Food Addit Conta. Part A Chem Anal Control Expo Risk Assess 27: 255–261

IARC (1986). IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans, 40.

IARC (1993). Ochratoxin A. In Some Naturally Occurring Substances: Food Items And Constituents, Heterocyclic Aromatic Amines and Mycotoxins. IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans, 56: 489-521.

Hsu, I.C., Metcalf, A., Sun, T., Welsh, J.A., Wang, N.J., Harris, C.C., (1991). Mutational hotspot in the p53 gene in human hepatocellular carcinomas. Nature 350: 427–428.

JECFA (1995). Evaluation of certain food additives and contaminants. WHO Technical Report Series n.859.

JECFA (2001). Ochratoxin A. Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives. Available from: http://www.inchem.org/documents/jecfa/jecmono/v47je04.htm.

Jonsyn, F.E., Maxwell, S.M., Hendrickse, R.G., (1995). Human fetal exposure to ochratoxin A and aflatoxins. Ann. Trop. Paediatr. 15 (1): 3–9.

Kabak, B.; Dobson, A.D.W.; Var, I. (2006). Strategies to prevent mycotoxin contamination of food and animal feed: a review.Crit. Rev. Food Sci. Nutr. 46: 593–619.

Kadakal C., Sebahattin, N., and Poyrazoglu, E.S. (2002). Effect of commercial processing stages of apple juice on patulin, fumaric acid and hydroxymetilfurfural (HMF) levels. Journal of Food Quality 25: 359-368.

Kadakal. C., and Nas S. (2003) Effect of heat treatment and evaporation on patulin and some other properties of apple juice. Journal of the Science of Food and Agriculture 83: 987-990.

Kiermeier F. and Mashaley R., (1977). Influence of raw milk processing on the aflatoxin M content of milk products [author's translation] Z Lebensm Unters Forsch 164:183–187

Kistemaker, C., Bouman, M. and Hulshof, K.F.A.M. (1998). Consumption of separate products by Dutch population groups - Dutch National Food Consumption Survey 1997 – 1998 (in Dutch). Zeist, TNO-Nutrition and Food Research Institute, TNO-report V98.812

Kuiper-Goodman, T., (1990). Uncertainties in the risk assessment of three mycotoxins: aflatoxin, ochratoxin and zearalenone. Can. J. Physiol. Pharmacol. 68: 1017–1024.

Kuiper-Goodman T. (1994). Prevention of mycotoxicoses through risk management and risk assessment. In: Miller JD, Trenholm HL (eds) Mycotoxins in grain. Eagan Press, St.Paul 1994, pp 439–469

Lee L.S., Cucullu A.F., Franz A.O. and Pons W.A., (1969). Destruction of aflatoxins in peanuts during dry and oil roasting. J Agric Food Chem 17:451–453

Lee K-S, Röschenthaler RJ. (1986). DNA-damaging activity of patulin in Escherichia coli. Appl Environ Microbiol 52(5):1046–54.

Lewis, L., Onsongo, M., Njapau, H., Schurz-Rogers, H., Luber, G., Kieszak, S., Nyamongo, J., Backer, L., Dahiye, A.M., Misore, A., Decoct, K., Rubin, C. (2005). The Kenya Aflatoxicosis investigation group. Aflatoxin contamination of commercial maize products during an outbreak of acute aflatoxicosis in Eastern and Central Kenya. Environ. Health Perspect. 113: 1763–1767.

Lu, Z., Dantzer, W.R., Hopmans, E.C., Prisk, V., Cunnick, J.E., Murphy, P.A., Hendrich, S., (1997). Reaction with fructose detoxifies fumonisin B_1 while stimulating liver associated natural killer cell activity in rats. Journal of Agricultural and Food Chemistry 45: 803–809.

Mahfoud R, Maresca M, Garmy N, Fantini J. (2002). The mycotoxin patulin alters the barrier function of the intestinal epithelium: mechanism of action of the toxin and protective effects of glutathione. Toxicol Appl Pharmacol 18:1209–18.

McMullen, M. (2007). Experiences in reducing disease and DON through components of FHB management. in: Proc. 2007 Natl. Fusarium Head Blight Forum. Michigan State University, East Lansing.2007, pp 102-103

McKinley E.R., Carlton W.W., Boon G.D. (1982). Patulin mycotoxicosis in the rat: toxicology, pathology, and clinical pathology. Food Chem Toxicol 20:289–300.

Morales, H., Marìn, S., Rovira, A., Ramos, A.J., and Sanchis, V. (2007) Patulin accumulation in apples by *Penicillium expansum* during postharvest stages. Letters in Applied Microbiology 44: 30-35.

Palumbo J.D., Baker J.L., Mahoney N.E. (2006). Isolation of bacterial antagonists of *Aspergillus flavus* from almonds. Microb Ecol. 52:45-52

Park JW and Kim YB, Effect of pressure cooking on aflatoxin B_1 in rice. (2006). J Agric Food Chem 54:2431–2435.

Patharajan S., Reddy K.R.N., Karthikeyan V., Spadaro D., Gullino M.L., Garibaldi A. Potential of yeast antagonists on *in vitro* biodegradation of ochratoxin A. Food Control. 2010:DOI: 10.1007/s12550-010-0064-5

Paul, P. A., Lipps, P. E., Hershman, D. E., McMullen, M. P., Draper, M. A., and Madden, L. V. (2007). A quantitative review of tebuconazole effect on Fusarium head blight and deoxynivalenol content in wheat. Phytopathology 97:211-220.

Pestka J.J, Zhou H.R, Moon Y., Chung Y.J. (2004). Cellular and molecular mechanisms for immune modulation by deoxynivalenol and other trichothecenes: unraveling a paradox. Toxicol Lett 153:61–73

Pieters M.N., Fiolet D.C.M., Baars A.J. (1999). Deoxynivalenol. Derivation of concentration limits in wheat and wheat containing products. RIVM report 388802018.

Pieters M.N., Freijer J., Baars A.J., Slob W., (2001) Risk Assessment of Deoxynivalenol in Food An assessment of exposure and effects in the Netherlands. RIVM report 388802 022

Pietri, A., Bertuzzi, T., Pallaroni, L. and Piva, G. (2001). Occurrence of ochratoxin A in Italian wines. Food Addit Contam 18(7): 647-654.

Pitt J.I., (2000). Toxigenis fungi and mycotoxins, Br. Med. Bull., 56 (1): 184-192

Polychronaki, N., Wild, C.P., Mykkänen, H., Amra, H., Abdel-Wahhab, M.A., Sylla, A., Diallo, M., El-Nezami, H., Turner, C.P., (2008). Urinary biomarkers of aflatoxin exposure in young children from Egypt and Guinea. Food Chem. Toxicol. 46 (2): 519–526.

Prandini A., Tansini G., Sigolo S., Filippi L., Laporta M., Piva G., (2009). On the occurrence of aflatoxin M_1 in milk and dairy products. Food and Chemical Toxicology 47: 984–991

Purchase I.F.H., Steyn M., Rinsma R. and Tusin R.C., (1972). Reduction of the aflatoxin M content of milk by processing. Food Cosmet Technol 10:382–387

Refai, M.K., Aziz, N.H., El-Far, F., and Hassan, A.A. (1996). Detection of ochratoxin produced by *A. ochraceus* in feedstuffs and its control by γ radiation. Applied Radiation and Isotopes 7: 617-621.

Report of experts participating in Task 3.2.8 (2002). Assessment of dietary intake of Patulin by the population of EU Member States Directorate-General Health and Consumer Protection

Rìos G., Pinson-Gadais L., Abecassis J., Zakhia-Rozis N., Lullien-Pellerin V. (2009). Assessment of dehulling efficiency to reduce deoxynivalenol and *Fusarium* level in durum wheat grains. Journal of Cereal Science 49: 387-392.

Roman S., Pinnavia, G.G., and Rosa, M.D. (2003). Influence of roasting levels on ochratoxin A content in coffee. Journal of Agricultural and Food Chemistry 51: 5168-5171.

Rosenberger D.A., (2003). Control of *Penicillium expansum* during apple harvest and storage. In: Patulin technical symposium. Kissimmee, Fla.; National Center for Food safety and Technology; Summit, Ill.

Rotter B.A., Prelusky D.B., Pestka J.J. (1996). Toxicology of deoxynivalenol (vomitoxin). J Toxicol Environ Health 48:1–34

SCOOP (2003). Collection of occurrence data of Fusarium toxins in food and assessment of dietary intake by the population of EU member states. Directorate-general health and consumer protection.

Shephard, G.S., Fabiani, A., Stockenstrom, S., Mshicileli, N. and Sewram, V. (2003). Quantitation of ochratoxin A in South African wines. J Agric and Food Chem 51(4): 1102-1106.

Shetty P.H., Hald B., Jespersen L. (2007). Surface binding of aflatoxin B1 by *Saccharomyces cerevisiae* strains with potential decontaminating abilities in indigenous fermented foods. Int J Food Microbiol. 113:41-46.

Sholberg P.L., Gaunce A. P. (1996). Fumigation of stonefruit with acetic acid to control postharvest decay Crop Protection, 15(8): 681-686

Soufleros, E.H., Tricard, C. and Bouloumpasi, E.C. (2003). Occurrence of ochratoxin A in Greek wines. J Sci Food and Agriculture 83(3): 173-179.

Skaug M.A., (1999). Analysis of Norwegian milk and infant formulas for ochratoxin A. Food Addit. Contamin. 16: 75–78.

Stefanaki I., Foufa E., Tsatsou-Dritsa A., Dais P., (2003). Ochratoxin A concentrations in Greek domestic wines and dried vine fruits. Food Additives and Contaminants 20(1): 74-83

Stott W.T, Bullerman LB. (1975). Patulin: a mycotoxin of potential concern in foods. J Milk Food Technol 38 (11):695–705.

Suàrez-Quiroz, M., De Louise, B., Gonzalez-Rios, O., Barel, M., Guyot, B., and Schorr-Galindo, S. (2005). The impact of roasting on the ochratoxin A content in coffee. International Journal of Food Science and Technology 40:605-611

Taniwaki, M. H., Bleinroth, E. W., & De Martin, Z. J. (1989). Bolores produtores de patulina em maçã e suco industrializado. Coletânea do Instituto de Tecnologia de Alimentos, 19, 42–49.

Trucksess M.W. and Tang Y. (1999), Journal of AOAC International, 82: 1109-1113

Tsai, G. J., Su, W. H., Chen, H. C., and Pan, C. L. (2002). Antimicrobial activity of shrimp chitin and chitosan from different treatments and applications of fish preservation. Fish. Sci. 68:170-177.

Ueno, Y., 1984. Toxicological features of T-2 toxin and related trichothecenes. Fundam. Appl. Toxicol. 4:S124–S132.

Walker R. and Larsen C.J. & Larsen J. C. (2005). Ochratoxin A: Previous risk assessments and issues arising. Food Additives and Contaminants, Supplement 1 2005: 6–9

Whipps, J. M., and Lumsden, R. D. (2001). Commercial use of fungi as plant disease biological control agents: Status and prospects. In: Fungi as Biocontrol Agents: Progress, Problems and Potential. T. M. Butt, C. Jackson, and N. Magan, eds. CABI Publishing, Wallingford, UK 2001 , pp 9-22.

Wichmann G, Herbarth O, Lehmann I. (2002). The mycotoxins citrinin, gliotoxin, and patulin affect interferon-γ rather than interleukin-4 production in human blood cells. Environ Toxicol 17(3):211–8.

Wolf-Hall, C.E., Hanna, M.A., and Bullerman L.B. (1999). Stability of deoxynivalenol in heat treated foods. Journal of Food Protection 62: 962-964.

World Health Organization (1996) Ochratoxin A- Toxicological Evaluation of Certain Food Additives and Contaminants, WHO Food Additives series 35, (WHO), 1996, pp 363-376.

Yazici S., Velioglu S. (2002). Effect of thiamine hydrochloride , pyridoxine hydrochloride and calcium-d-pantothenate on the patulin content of apple juice concentrate. Nahrung/Food, 46:256-257

Yoshizawa T., Morooka N. (1973). Deoxynivalenol and its monoacetate: new mycotoxins from Fusaruium roseum and moldy barley. Agric Biol Chem 37: 2933–2934

Van Egmond, H. P. (2002). Worldwide regulations for mycotoxins. Adv. Exp. Med. Biol. 504: 257-269.

Varga, J. University of Szeged, Hungary. (2004).Unpublished work.

Visconti, A., Haidukowski, E.M., Pascale, M., and Silvestri, M. (2004). Reduction of deoxynivalenol during durum wheat processing and spaghetti cooking. Toxicology Letters 153: 181-189.

Zarba, A., Wild ,C.P., Hall, A.J., Montesano, R., Hudson, G.J., Groopman, J.D., (1992). Aflatoxin M_1 in human breast milk from the Gambia,West Africa, quantified by combined monoclonal antibody immunoaffinity chromatography and HPLC.Carcinogenesis 13: 891–894.

Chapter 2: AIMS OF THE STUDY

In this PhD thesis several aspects of risk analysis of mycotoxins are treated. In particular the studied mycotoxins included: PAT, DON, AFLA B_1, AFLA M_1, OTA and the attention is focused on hazard for infants and children that represent more sensitive and vulnerable categories respect to adults (FAO, 2005; Boon et al, 2009). In literature, few studies have dealt the risk associated to mycotoxins in food for this target. So this thesis can represent an original contribution to the mycotoxins risk management and risk assessment.

The main objectives of the topics are:

- **Risk management to control blue mould on apples**: evaluation of the compatibility of the biocontrol agents (BCAs) *Rhodosporidium kratochvilovae* LS11 and *Cryptococcus laurentii* LS28 with recently developed fungicides in order to create a new integrated approach to control *P. expansum* on apples and reduce: a) PAT contamination; b) fungicide residue; c) risks of the onset of *P. expansum* fungicide-resistant strains.

- **PAT risk management and exposure assessment in apple products:** evaluation of the PAT's thermal resistance in apple juices and puree and the production of HMF through a simulation of heat treatment and study of PAT's bioaccessibility in apple juices, nectars and purees in a procedure of *in vitro* digestion reproducing the physiological condition of the children that represent a great slice of consumers of apple products.

- **DON, OTA, AFLA B_1 exposure assessment by pasta:** surveillance for DON, OTA and AFB_1 on Italian pasta intended for children and study of the bioaccessibility of DON in a model system that simulates the digestion steps in children. This mycotoxin was chosen for bioaccessibility evaluation because several studies reported it is the main metabolite contaminating durum wheat products.

- **AFM_1 exposure assessment by breast milk:** determination of the levels of AFM_1 in breast milk from lactating mothers in Ogun State (Nigeria) and their relationship with dietary exposure of mothers to AFLA B_1.

References

Boon P.E., Bakker M.I., van Klaveren J.D., van Rossum C.T.M. (2009). Risk assessment of the dietary exposure to contaminants and pesticide residues in young children in the Netherlands
RIVM report 350070002/2009

Food and Agriculture Organization (2005). The State of Food Insecurity in the World 2005. Eradicating world hunger key to achieving the millennium development goals. http://www.fao.org/icatalog/inter-e.htm

Chapter 3: INTEGRATED CONTROL OF BLUE MOULD USING NEW FUNGICIDES AND BIOCONTROL YEASTS LOWERS LEVELS OF FUNGICIDE RESIDUES AND PATULIN CONTAMINATION IN APPLES

3.1 Introduction

Apples are grown in many countries and consumed worldwide. Because of their high content in bioactive molecules (e.g., antioxidants and vitamins), such fruit can exert beneficial effects in a balanced diet (Boyer and Liu, 2004; Gallus et al., 2005). The storability and nutritional value of fresh fruit can be heavily affected by postharvest fungal diseases (Snowdon, 1990) and chemical contaminants (Castoria and Logrieco, 2007; WHO, 2008). Blue mould caused by the fungus *Penicillium expansum* is one of the most important postharvest rots of pome fruit (Rosenberger, 1990). This pathogen is also a major producer of patulin (PAT), a mycotoxin which can reach concentrations of mg/kg in infected apples and pears (Battilani et al., 2008) and is known to have cytotoxic, genotoxic and immunosuppressive activity (Wouters and Speijers, 1996). As a consequence, it is a major health hazard for children, who consume great quantities of fruit juices and/or baby food produced from pome fruit (Beretta et al., 2000). Despite the wide-spread use of modern storage facilities and techniques, synthetic fungicides are still frequently used immediately before or after harvest to control postharvest deterioration of fruit. However, chemical control is being increasingly limited because of environmental and toxicological risks as well as the onset of fungicide-resistant strains of fungal pathogens. Moreover, the legal limits of chemical residues left by pesticides in imported fruit are much lower in some countries, thus discouraging the use of chemical products. In the absence of fully effective postharvest fungicides, alternative or integrative measures are becoming increasingly important in controlling postharvest fungal disease and in maintaining a high level of quality. Biocontrol by antagonistic microorganisms, including yeasts, yeast-like fungi and bacteria, appears to be a promising tool for preventing postharvest fungal rots and minimizing the use of fungicides (Janisiewicz and Korsten, 2002; Ippolito et al., 2004). However, biocontrol agents (BCAs) are sometimes not sufficient to control fungal decays satisfactorily when applied alone under practical conditions. On the other hand, fungicide-resistant strains of *P. expansum* and other fungal pathogens in packinghouses are constantly increasing as an consequence of prolonged fungicide treatments (Janisiewicz and Korsten, 2002). This is the case with thiabendazole (TBZ), one of the very few synthetic fungicides that is still allowed in many countries for postharvest treatment of pome fruit. Therefore, integrated approaches based on the combination of BCAs and fungicides or alternative means have been suggested to prevent a resistance increase in the pathogen population and limit risks due to intensive use of chemicals (Lima et al., 2005, 2008; Droby et al., 2009). Several papers have shown the protective activity of selected BCAs from *P. expansum* rot on apples. Surprisingly, very little research has taken into account the influence of BCAs on the accumulation of mycotoxins in fruit. Recently, some studies found that biocontrol yeasts can lower PAT as well as ochratoxin accumulation in apples and wine grapes, respectively, by preventing attacks by *P. expansum* and *Aspergillus carbonarius*, and these BCAs can detoxify these mycotoxins *in vitro* by transforming them into less toxic compounds (Castoria et al., 2005, 2007; De Felice et al., 2008). On the other hand, no information is available on the effects of BCAs combined with fungicides on the accumulation of mycotoxins in general, and PAT in particular, and on the persistence of fungicide residues in stored apples. The protective activity of the two selected antagonist yeasts, *Rhodosporidium kratochvilovae* LS11 and *Cryptococcus laurentii* LS28, is enhanced by combining them with a low dosage of fungicides and/or natural adjuvants, and that such strategies can control both resistant and sensitive strains of fungal pathogens (Lima et al., 2005, 2008). Therefore, selected biocontrol yeasts are very interesting candidates for their utilisation in integrated control strategies aimed at reducing the use of fungicides and the contamination by chemical residues and mycotoxins (Castoria et al., 2008; Lima et al., 2008).
This work was aimed at evaluating the compatibility of the BCAs *R. kratochvilovae* LS11 and *C. laurentii* LS28 with recently developed fungicides in order to create a new, efficient, integrated approach to control *P. expansum* on apples and reduce (i) PAT contamination, (ii) fungicide residue and (iii) risks of the onset of *P. expansum* fungicide-resistant strains.

3.2. Materials and methods

3.2.1 Biocontrol agents (BCAs)

Rhodosporidium kratochvilovae strain LS11 and *Cryptococcus laurentii* strain LS28, isolated from olives and apples, respectively, were the BCAs used in this study; these antagonists had previously been characterized for antagonistic activity (Lima et al., 1998, 1999) and mechanisms of action (Castoria et al., 1997, 2003).

3.2.2 Fungicides

In the experiments *in vitro* and/or *in vivo*, the following fungicides were used: boscalid (Chemical group anilides; trade formulate Cantus, 50%, w/w a.i., Basf, Milan, Italy); cyprodinil (Chemical group anilinopyrimidines; trade formulate Chorus®, 50% a.i., w/w, Syngenta Crop Protection, Milan, Italy); fenhexamid (Chemical group hydroxyanilides; trade formulate Teldor® , 50% a.i., w/w, Bayer Crop Science, Milan, Italy); thiabendazole (Chemical group benzimidazoles; trade formulate Decco T® , 50%, w/v a.i., Cerexagri, Cesena, Italy).

3.2.3 Fungal cultures

Isolates of *P. expansum* used in experiments on apples (isolate FS7) and for the assessment of in vitro resistance to fungicides (isolates FQ42, FQ44, FQ45, FQ46, FQ47, FQ48) were obtained from decaying apples. Moreover, isolates P32-R and LB8/99-S, from decaying pears, supplied by CRIOF (Centro per la Protezione e Conservazione dei Prodotti Ortofrutticoli), University of Bologna, Italy, were used as reference isolates for their known high resistance or low sensitivity to benzimidazoles, respectively (Baraldi et al., 2003).

In order to obtain conidial suspensions for fruit inoculation the pathogen (isolate FS7) was grown on potato dextrose agar (PDA) under fluorescent light for 5–7 days at 21°C. Five milliliter of sterile distilled water containing 0.05% Tween 20 were poured into Petri dishes, and conidia were scraped from the agar by using a sterile loop. The suspension obtained was filtered through 4 layers of cheesecloth. The inoculum concentration was adjusted by an haemocytometer to 2×10^4 conidia/mL.

3.2.4 Compatibility of BCAs with TBZ and more recent fungicide

The BCAs LS11 and LS28 were tested *in vitro* for their sensitivity to commercial formulates of thiabendazole (TBZ) as well as the more recent developed fungicides boscalid (BOSC), cyprodinil (CYPR) and fenhexamid (FENH). The assays were performed on basal yeast agar (BYA: 10 g bacteriological peptone, 1 g yeast extract, 20 g dextrose, 18 g agar, 1 L distilled water). Briefly, each fungicide was suspended in distilled water and mixed with the medium at 45°C and, according to the full dose suggested by the manufacturers for pre and/or postharvest application on fruit, the following concentrations of fungicide active ingredient (a.i.) were tested: BOSC 375 µg/mL (75 g h/L of commercial product), 187.5 µg/mL (50% of the full suggested dose), 93.8 µg/mL (25% of the full suggested dose); CYPR 150 µg/ mL (30 g h/L of commercial product), 75 g/mL (50% of the full suggested dose), 37.5 µg/mL (25% of the full suggested dose); FENH 600 µg/mL (120 g h/L of commercial product), 300 µg/mL (50% of the full suggested dose), 150 µg/mL (25% of the full suggested dose); TBZ 418 µg/mL (100 g h/L of commercial product), 209 µg/mL (50% of the full suggested dose), 104.5 µg/mL (25% of the full suggested dose). Each plate (4 replications per treatment) was poured with 100 µL of yeast suspension containing about 100 cells and incubated for 7 days at 23°C. In each plate the growing yeast colonies were counted and minimum inhibitory concentration (MIC, i.e. the lowest concentration of fungicide inhibiting the growth of yeast colonies) was assessed.

3.2.5 Sensitivity of *P. expansum* isolates to TBZ and more recent fungicides

The resistance of isolates of *P. expansum* to commercial formulates of TBZ, BOSC and CYPR was assessed *in vitro*. The fungicide FENH was not used in this assay because of its incompatibility with (i.e. high inhibiting activity to) both biocontrol yeasts. The following concentrations of a.i. of each fungicide were tested: BOSC 375 µg/mL (75 g h/L of commercial product), 187.5 µg/mL (50% of the full suggested dose), 93.8 µg/mL (25% of the full suggested dose), 37.5 µg/mL (10% of the full suggested dose); CYPR 150 µg/mL (30 g h/L of commercial product), 75 µg/mL (50% of the full suggested dose), 37.5 µg/mL (25% of the full suggested dose), and 15 µg/mL (10% of the full suggested dose); TBZ 418 µg/mL (100 g h/L of commercial product), 209 µg/mL (50% of the full suggested dose), 104.5 µg/mL (25% of the full suggested dose), 41.8 µg/mL (10% of the full suggested dose), 20.9 µg/mL (5% of the full suggested dose), and 4.2 µg/mL (1% of the full suggested dose). Each fungicide, was mixed with PDA at 43°C, and the mixture was immediately poured into Petri dishes (100 mm diameter). The plates were left to cool and mycelial plugs (6 mm diameter) of each fungal strain were withdrawn from actively growing colonies (4–5 days PDA dark-grown cultures at 23° C), and placed in the centre of the Petri dishes containing the fungicide. Four replicates for each fungicide concentration were prepared. Cultures were incubated in the dark at 21°C and colony diameters were measured on a daily basis until they reached their highest value in fungicide-free control dishes (after 7–10 days, depending on the fungal strain). The percentage of inhibition of radial mycelial growth on amended PDA was calculated and data obtained were subjected to Probit Analysis to determine the EC50 value (i.e. concentration of each fungicide causing 50% reduction in mycelial growth).

3.2.6 Integration of BCA and fungicide for the control of *P. expansum* on apples

Apples cv Golden delicious, from an orchard conducted by the integrated crop management method, were purchased from a local supplier and kept at 3°C, and 95–98% RH prior to use. The fruit was then removed from cold storage 24 h before performing each experiment. During storage (from 5 to 20 days) average values of fruit firmness and soluble solid concentration were 4.5 kg and 11.5° Brix, respectively, with a low variability throughout the time of the experiments. Apples were superficially disinfected by dipping them in a sodium hypochlorite solution (2% active chlorine) for 1 min; the fruit were then rinsed twice with sterile distilled water and dried at room temperature. Four wounds (5 mm wide by 3 mm deep) on each fruit were made around the blossom end. For biocontrol treatments, 30 µL of yeast cell suspension in distilled water at 5×10^6 cells/mL were placed in each wound. The combined treatments (BCA + fungicide) were performed by applying 30 µL of yeast at 5×10^6 cells/mL suspended in low dosage (25% of the full suggested dose) of each fungicide (TBZ, BOSC or CYPR) in each wound.

In each experiment, controls were represented by fruit in which wounds were treated with 30 µL of sterile distilled water alone, 30 µL of fungicide alone at low dosage or 30 µL of fungicide applied alone at full dose. After 2 h at room temperature, each wound was inoculated with 15 µL of a conidial suspension of *P. expansum* at 2×10^4 conidia/mL

The apples were incubated in the dark at 21°C for 7 days at 95–98% RH. Each treatment included 3 replicates and each replicate consisted of 8 fruit. The number of wounds showing rot symptoms as well as the diameter of rotting lesions in each wound were assessed on a daily basis.

3.2.7. Analysis of fungicide residues and patulin contamination in apples

3.2.7.1 Preparation of samples

After 7 days at 21°C, apples were sampled to determine residues of fungicides (CYPR and BOSC) and PAT accumulation. Each fruit was cut in half at the equatorial line, and infected or uninfected wounds from the wounded half were withdrawn with a cork borer (15 mm diameter). For each replicate (8 apples), withdrawn wounds were pooled, and each sample (each consisting of 32 wounds) was stored at −20° C. Extraction and analysis of fungicide residues and PAT were performed as described below.

For the analysis of fungicides 10 g of each sample were homogenized using a Ultra-Turrax T25 (IKA-WERKE, Germany) and homogeneously mixed with 10 g of fine diatomaceous earth at pH 10. The mixture was then placed onto a SPE polypropylene tube and the sample eluted with 100 mL of a dichloromethane/ethyl acetate (80:20, v/v) solution. The extract was completely dried and then dissolved in 10 mL of a methanol/water (70:30, v/v) solution, centrifuged at 10,000 rpm for 3 min and used for LC/MS/MS analysis of fungicides. The standard working solutions of fungicides were prepared by appropriate dilutions with the same methanol/water (70:30, v/v) solution.

For the analysis of PAT, 10 g of sample were transferred into a falcon tube containing 15 g of Na_2SO_4, 2 g of $NaHCO_3$ and 10 mL of extraction solution (ethyl acetate/hexane 60:40, v/v). The tube was shaken for 3.5 min on a mechanical shaker and then centrifuged at 2000 rpm for 1 min to force separation of layers. Since PAT is not stable at alkaline pH, this step was carried out as quickly as possible. The centrifuged extract (2.5 mL) was immediately placed onto an unconditioned Strata C18-E solid phase extraction column (Phenomenex, USA) (parameter: Surface Area 500 m^2/g; pore size 70 ˚A; pore volume 0.88 mL/g ; average particle size 58 µm) which was washed with 3 mL of extraction solution. The eluate was collected in a test-tube containing 50 µL of acetic acid (Merck, Darmstadt-Germany), and the flow was adjusted to 1 drop per second by using slight air pressure. The solvent was evaporated at max 40° C for about 35 min in a vacuum centrifuge Thermo Savant (SVPT Srl-JOUAN) and 1 mL of acidulated water (pH 4) was added to the dried sample. This solution was vortexed in order to fully dissolve PAT and then transferred into a vial before injection.

3.2.7.2 LC/MS/MS analysis of fungicides

Chromatographic separation was performed using an HPLC apparatus equipped with two micropumps Series 200 (Perkin Elmer, Canada, USA) and a Gemini 5 µm C18, 110 ˚A column (150 mm × 2 mm) (Phenomenex, CA, USA). The eluents were: (A) water 0.1% formic acid; (B) acetonitrile. The gradient program was as follows: 35–50% B (5 min), 50–95% B (7 min), 95% B (2 min), 95–35% B (2 min), at a constant flow of 0.2 mL/min. Injection volume was 20 µL. MS/MS analyses of BOSC and CYPR were performed on an API 2000 triple quadrupole mass spectrometer (Applied Biosystems, Canada) equipped with a TurboIonspray source. Analyses were performed in the positive ion mode in MRM (multiple reaction monitoring). The calibration curve displayed good linearity in the range 5–500 ng/mL. All chromatographic points of the calibration curve were run in triplicate, and the standard deviation was lower than 0.05. The limits of detection (LOD, with a signal to noise ratio of 3) for CYPR and BOSC were 0.5 and 2.5 ng/g respectively, limits of quantification (LOQ, with a signal to noise ratio of 6) were 2 ng/g for CYPR and 5 ng/g for BOSC.

3.2.7.3 HPLC analysis of patulin

Patulin (4-Hydroxy-4H-furo[3,2-c]pyran-2(6H)-one) contamination in apples was assessed by HPLC according to Arranz et al. (2005) and Katerere et al. (2007) with slight modifications as described below. Analyses were carried out by using an HPLC apparatus (Shimadzu-Japan) equipped with an autosampler SIL-20A, two pumps LC-20AD and a UV/vis detector SPD-20A set at 276 nm wavelength. The column was a Gemini 5 µ C18, 110 ˚A (150 mm × 2 mm) (Phenomenex, CA, USA). The mobile phase was isocratic:(A) (95%) was water containing 1% acetic acid and (B) (5%) was methanol. The flow rate was set at 1 mL/min.
In these chromatographic conditions, the retention time for PAT was 14.0 ± 0.1 min. The quantification was carried out by including signal area values into a linear calibration curve, in the range of 5–1000 µg/kg, and by correcting errors imputable to variable sensitivity of instrument.

3.2.7.4 Data analysis

The percentages of apple wounds infected with *P. expansum* were converted to Bliss angular values (arcsine square root) before analysis of variance. Each experiment was performed at least twice. Homogeneity of variance for repetitions of each experiment was evaluated and data from separate experiments with homogeneous variances were pooled. Data were subjected to one-way analysis of variance (ANOVA univariate, SPSS release 15.0 for windows; SPSS Inc., Chicago, IL) and the means were compared by using Tukey's multiple range test. In sensitivity fungicide assays, the EC50 values were determined by subjecting to Regression Probit Analysis the results of fungal growth inhibition by using the software SPSS release 11.0 for windows. In order to compare the integrated treatments with those applied separately, the type of effect (additive, synergistic or antagonistic) was evaluated. For this purpose, percentage values of infected wounds were transformed into percentages of protection efficacy (PE) as follows: $PE = [(C - T)/C] \times 100$, where C is the number of infected wounds in the control (water + pathogen) and T is the number of infected wounds in the examined treatment (BCA alone or BCA + fungicide). Values ranged from 0 (no PE), to 100 (maximum PE). The synergy factor (SF) was calculated according to Abbott's formula (Levy et al., 1986), as follows: SF = E(obs.)/E(exp.), where E(obs.) and E(exp.) are observed and expected PE of the mixture (BCA + fungicide), respectively. E(exp.) was calculated as follows: $a + b - a \times b/100$, where a = PE of the factor a (BCA) applied alone; b = PE of the factor b (fungicide) applied alone. If SF = 1, the interaction between BCA and fungicide is additive; if SF < 1, the interaction between BCA and fungicide is antagonistic; if SF > 1, the interaction between BCA and fungicide is synergistic.

3.3 Results

3.3.1 Compatibility of BCAs with TBZ and more recent fungicides

In vitro, both the tested biocontrol yeasts LS11 (*R. kratochvilovae*) and LS28 (*C. laurentii*) were highly resistant to the fungicides BOSC (MIC > 375 µg/mL) and CYPR (MIC > 150 µg/mL), whereas they were strongly inhibited by FENH (no growth observed at lower concentration tested; MIC < 150 µg/mL); isolate LS28 also showed a good compatibility with TBZ (MIC > 418 µg/mL), while LS11 was very sensitive to this fungicide (MIC < 104.5 µg/mL) (Table 1). On the basis of these results BOSC and CYPR were chosen for the following *in vitro* and *in vivo* assays.

3.3.2 Sensitivity of *P. expansum* isolates to TBZ and more recent fungicides

The effects of the fungicides TBZ, BOSC and CYPR on mycelia growth of isolates of *P. expansum* from pome fruit were tested on PDA medium amended with different concentrations of fungicide.
The results of these experiments are reported in Table 2. Toward TBZ, isolates FQ44, FQ45, FQ48, and LB8/99-S, reporting EC50 values below 4.2 µg/mL of a.i. were sensitive, whereas isolates FQ42, FS7, FQ46, FQ47 and P32-R with EC50 values ranging from 160 µg/mL to higher than 418 µg/mL of a.i. were resistant.
As regards the more recently developed fungicides, all the tested isolates of *P. expansum* were strongly inhibited by CYPR (EC50 < 15 µg/mL of a.i.) and BOSC (EC50 < 37.5 µg/mL of a.i.).

3.3.3 Integration of BCAs and fungicides for the control of *P. expansum* on apples

The results of the experiments carried out on wounded apples with the biocontrol yeasts *R. kratochvilovae* LS11 and *C. laurentii* LS28, applied alone or in combination with low rates of TBZ, BOSC or CYPR for the control *P. expansum* are reported in Fig. 1. After 4 days of storage, 100% of the wounds were infected with the pathogen in control apples (water + *P. expansum*). TBZ, either at full or at low dosage, was ineffective against the fungus (90% and 100% of infected wounds, respectively). Conversely, BOSC at full and low dosage (72% and 67% reduction of infections as compared to the control, respectively) and, particularly, CYPR at full and low dosage (100% less infections at any dose), were highly effective against the pathogen. The BCAs LS11 and LS28 alone also appreciably reduced the level of infection with respect to the control

(by 35% and 52%, respectively), although they were applied at a relatively low concentration of cells (5×10^6 cfu/mL). When combined with TBZ, BOSC or CYPR at low dose, both the BCAs yielded significant reductions of infected wounds. In particular, LS11 plus BOSC and LS28 plus BOSC or CYPR were the most effective treatments against the pathogen (100% reduction of infected wounds).
After 7 days of storage, diameters of rotting lesions consistently increased on control fruit, which had already reached 100% of infected wounds at 4 days (see above). The protection of the BCAs and the fungicides BOSC and TBZ applied alone decreased consistently or was completely lost. Only CYPR applied alone at full dosage was still effective (0% infected wounds). As regards the integrated treatments, the combination of the BCAs with TBZ proved to be ineffective. Conversely, the treatments based on the combination of the BCAs with a low dosage of BOSC or CYPR were very effective against the pathogen. In particular, the best results were obtained with LS11 plus BOSC (50% reduction of infected wounds) and with LS28 plus BOSC or CYPR (75% and 98% of reduction, respectively).
In both assessments (4 and 7 days), the diameters of rotting lesions on apples seem in substantial agreement with the respective percentages of infected wounds (Fig. 1A and B).
At the end of the experiments (after 7 days of storage), the synergy factor (SF) was calculated in order to ascertain whether the different BCA-fungicide combinations were synergistic, additive or even antagonistic (Table 3). The integration of both BCAs with low rate of TBZ evidenced a clear antagonistic effect, with SF values < 1 (0.0 and 0.2 for LS11 and LS28, respectively). The integrated treatments LS11 + BOSC, LS28 + BOSC and LS28 + CYPR were clearly synergistic, with SF average values markedly > 1 (6.7, 3.2 and 2.4, respectively). Lastly, the combination of LS11 and CYPR, which resulted in a SF value around 1, can be considered as additive.

3.3.4 Fungicide residues and patulin contamination in apples
After 7 days, both fungicide residues and PAT contamination were determined in apple subjected to different treatments. The results of these analyses are reported in Fig. 2.
In apples treated with BOSC, an average of 1103 and 521 µg/kg of residual fungicide were recorded for full dosage (FD) or low dosage (LD) treatments, respectively. When a low dosage of BOSC was used in the integrated treatments with LS11 or LS28, fungicide residues were 269 and 338 µg/kg, respectively (Fig. 2A).
In apples treated with full and low dosages of CYPR, fungicide residues were 488 and 282 µg/kg, respectively. In the integrated treatments with the two doses of CYPR plus LS11 or LS28, the residual concentrations of fungicide on apples were 91 and 240 µg/kg respectively (Fig. 2B).
As regards contamination of apples with the mycotoxin PAT, significant differences were found in the various treatments (Fig. 2C).
In particular, the highest level of PAT (24437 µg/kg) was recorded in the control apples (water + *P. expansum*), followed by BOSC at full dose (22579 µg/kg) and TBZ at full dose (18078 µg/kg). A significantly lower level of PAT was detected in the following treatments: CYPR at low dose (2080 µg/kg), CYPR at full dose (45 µg/kg), LS11 plus BOSC at low dose (556 µg/kg), LS28 plus BOSC at low dose (338 µg/kg) and LS28 plus CYPR at low dose (13 µg/kg). The other treatments (i.e. BCAs alone, TBZ at low dose, BOSC at low dose, LS11 plus TBZ at low dose, LS28 plus TBZ at low dose and LS11 plus CYPR at low dose, resulted in intermediate levels of PAT, and were not statistically different from the control. In general, apples with higher amount of PAT accumulation (Fig. 2C) also had larger diameters of rotting lesions (Fig. 1A and B).

3.4 Discussion and conclusions
An integrated strategy based on the combination of BCAs with natural compounds or reduced dosage of fungicides appears to be one of the most reliable options for large-scale utilisation of microbial antagonists in the control of postharvest fungal rots of fruit and vegetables (Lima et al., 2008; Droby et al., 2009). Therefore, the optimisation of biocontrol efficacy also depends on survival and colonisation of BCAs in wounded and unwounded fruit surfaces in the presence of low quantities of fungicides applied separately or in combination with these microbial antagonists. Moreover, the diffusion of fungal pathogen isolates that have become resistant to fungicides used for a long time in the field and/or in packinghouses (e.g. benzimidazoles), has led to the need of assessing the compatibility and efficacy of BCAs with new and recently developed fungicides.
In the present study, the two biocontrol yeasts *R. kratochvilovae* isolate LS11, and *C. laurentii* isolate LS28, known for their wide spectrum of activity against major postharvest fungal pathogens on various crops (Lima et al., 1998, 1999) were tested in combination with low rates of the recently developed fungicides BOSC and CYPR in comparison with TBZ, which has been on the market for many years.
CYPR is a broad-spectrum fungicide belonging to the chemical class of anilinopyrimidines. In the fungal cell, this compound inhibits the biosynthesis of methionine and other aminoacids and inhibits the secretion of

hydrolytic enzymes associated with pathogenesis, such as pectinases, cellulases and proteases (Leroux, 1996).
Recently, CYPR has also been shown to be effective in the control of both benzimidazole-resistant and -sensitive isolates of *P. expansum* (Errampalli and Brubacher, 2006).
BOSC, a chemical compound belonging to the anilide class, is a fungicide showing a good effectiveness against several plant fungal pathogens. Its mechanism of action differs from that of previous similar fungicides (e.g. strobilurines) and is based on the inhibition of the enzyme succinate ubiquinone reductase (complex II) in the mitochondrial electron transport chain (Stammler et al., 2007). Both fungicides, CYPR and BOSC, are good candidates to replace TBZ and/or other old fungicides in the control of pre and/or postharvest fungal rots of fruit, because they from toxicological and environmental points of view are also considered as low-risk fungicides (Adaskaveg et al., 2006).
The biocontrol yeast isolates LS11 and LS28 exhibited good compatibility in vitro with BOSC and CYPR. These fungicides also showed clear and strong inhibitory activity on different isolates of *P. expansum*, whereas TBZ was less effective or ineffective. For these reasons, BOSC and CYPR were subsequently used in experiments of integrated control of blue mould on apples. This is the first study showing the compatibility of selected biocontrol yeasts with the fungicides BOSC or CYPR. Only two previous studies showed a good level of compatibility of CYPR with biocontrol bacteria (*Pseudomonas syringae*, isolate MA-4 or ESC-10) that provided a more efficient control of *P. expansum* in integrated application on apples (Zhou et al., 2002; Errampalli and Brubacher, 2006).
The fungicide TBZ was highly toxic toward the BCA *R. kratochvilovae* LS11, whereas it appeared to be compatible with *C. laurentii* LS28. This result is in full agreement with previous research in which isolates LS11 and LS28 were tested for their compatibility with benzimidazoles (Lima et al., 2006). *Penicillium expansum* FS7, the challenging isolate of the pathogen used in this study, as compared with *P. expansum* reference strains (P32-R, resistant, and LB8/99-S, sensitive) and also with other isolates collected from decaying fruit, was highly resistant (EC50 > 418 µg mL−1 of a.i.) to TBZ in vitro. In other studies (Viñas et al., 1991; Errampalli et al., 2006) it was found that a majority of *P. expansum* isolates collected from packinghouses and tested in vitro toward fungicides was resistant to TBZ because of their ability to grow on PDA amended with 5 or 40 µg/mL of a.i. Accordingly, the majority of isolates we tested in this work can be considered highly resistant to TBZ since their EC50 values resulted higher than 160 µg/mL of a.i.
Conversely, in vitro assays of this study showed high antifungal activity of the recent developed fungicides BOSC and CYPR against all tested isolates of *P. expansum*, yielding EC50 values below 37.5 and 15 µg/mL of a.i., respectively, which correspond to 10% of the full dose suggested by the manufacturers for these fungicides. The aim of this work was to assess the antifungal activity of these fungicides at a concentration corresponding to 25% of the full dosage (to be used in BCA + fungicide integrated combinations); therefore, in this work it did not test concentrations of BOSC and CYPR lower than 10% of the full dosage. However, other research has shown that the EC50 toward fungal pathogens is below 5 µg/mL for BOSC (Avenot et al., 2008) and 1 µg/mL for CYPR (Sholberg et al., 2005).
In the assays on apples, the application of the BCAs LS11 and LS28 in combination with small doses of BOSC or CYPR consistently enhanced the efficacy and persistence of the control of *P. expansum* rot with respect to the treatments (biological and chemical) applied separately; the BCAs combined with BOSC or CYPR were highly effective against *P. expansum* after 4 days and even at the end of the experiments (7 days of apple storage) when most of the treatments (BCAs or fungicides) applied individually at the same doses yielded unsatisfactory control. After 7 days, however, only reduced and full doses of CYPR still showed a significant reduction of infected wounds among the individual treatments, thus confirming the efficacy of this fungicide against *P. expansum* on apples as previously reported by Errampalli and Brubacher (2006). Interestingly, the combination of LS11 with BOSC, and LS28 with either BOSC or CYPR, also yielded a synergistic effect on the protective activity. This result is in agreement with previous integrated control experiments, in which combined applications of BCAs with fungicides (Lima et al., 2006) or other compounds (Lima et al., 2005) displayed a comparable synergistic improvement of protective activity. At the end of the experiments, the combined application of the BCA LS11 with TBZ proved to be ineffective against *P. expansum*. As observed in vitro assays, this result is most likely due to the high resistance of *P. expansum* isolate FS7 to TBZ as well as the strong sensitivity of LS11 to TBZ. Actually, TBZ is so toxic to LS11 that previous assays (Lima et al., 2006) show that *in vitro* the growth of this BCA was inhibited even at 1.2 µg/mL of a.i. Although isolate LS28 was compatible with TBZ *in vitro*, this BCA fungicide combination also failed to control *P. expansum* on apples. Lima et al. in year 2006 reported that this BCA-fungicide integration, with respect to treatments applied separately, provided a more efficient control of *B. cinerea* on apples. The worse performance of isolate LS28 combined with TBZ recorded in this study can be explained by both the higher virulence of *P. expansum* on apples with respect to *B. cinerea* (Jones and Aldwinckle, 1990) and by the high degree of resistance to TBZ of isolate FS7 of *P. expansum*, the challenging pathogen used in our experiments. Chemical analyses of apples at the end of the experiments

showed that the most effective integrated treatments (i.e. BCA s plus BOSC or CYPR) achieved in fruit a level of fungicide residues consistently lower (about 3–4 times lower) with respect to residues recovered in fruit treated with CYPR or BOSC at full dosage (Fig. 2A and B). With the exception of BOSC applied at full dosage, fungicide residues were abundantly below the maximum residue limit (MRL) established for CYPR and BOSC (MRL = 1000 µg/kg for both fungicides). Interestingly, the integrated treatment based on the BCA LS11 resulted in lower levels of fungicide residues, as compared with residues found in control apples treated with the fungicides alone at low dosage. Castoria et al., in 2005 and 2007 found that isolate LS11 can resist in the presence of a toxic compound such as patulin, and this resistance could rely on the yeast ability to degrade this mycotoxin.

Integrated treatments with BOSC and CYPR also achieved significant lower levels of PAT contamination as compared to the untreated control (Fig. 2C). For apple-derived products, the European Community (EC) has set the highest tolerable levels of PAT in the range from 10 to 50 µg/kg, depending on foodstuffs (solid apple products, fruit juices, fermented drinks, etc.) and population group (adults, babies or infants) (EC Reg., 1881/2006). In our research, values of PAT in untreated control apples (water + *P. expansum*) and other ineffective treatments (i.e. the fungicides TBZ and BOSC alone) were consistently higher (around from 18000 to 24000 µg/kg) and considerably above the maximum tolerable level. These very high levels of PAT are due to the drastic experimental conditions used in this work, i.e. artificial pathogen inoculation, wide size of fruit wounds and fruit keeping at room temperature, all of which are very conducive to high levels of infection and PAT contamination (Morales et al., 2010). The high level of PAT in apples treated with TBZ and BOSC individually applied is in agreement with the lower activity against blue mould displayed by these fungicides with respect to the most effective fungicide CYPR.

These results partially disagree with those reported by Morales et al. (2007). Although a significant reduction of blue mould rot was recorded, they found that the application of some fungicides on PAT accumulation in apples had no effect. On the other hand, these results showed that CYPR not only was highly effective against *Penicillium* rot, but also kept PAT accumulation low in fruit. Nevertheless, even under the drastic conditions used in our experiments, the best integrated treatments (i.e. LS11 with BOSC, and LS28 with either BOSC or CYPR) were highly effective against *P. expansum*, since they led to a dramatic reduction of both rot incidence and PAT contamination; in particular, in apples subjected to integrated treatments with CYPR and BOSC, PAT contamination was from 78% to 100% lower than in those of the untreated control.

In previous reports Castoria et al., (2005) showed that the biocontrol yeast LS11 determined a significant reduction of PAT accumulation in apples pretreated with this BCA and inoculated with *P. expansum.* Conversely, in this work, the utilization of isolate LS11 as a stand-alone treatment did not achieve a significant reduction of PAT contamination on apples. This may be due to the lower number of cells of the biocontrol yeasts used in this experiments (5×10^6 cfu/mL vs 10^8 cfu/mL) and/or to the different apple cultivars that were used in the two studies.

Recently, reduced sensitivity to CYPR and BOSC in the population of some fungal pathogens on crops treated with these fungicides has been reported (Babij et al., 2000; Avenot et al., 2008).

This study shows that the integrated strategy based on BCAs combined with BOSC or CYPR can control isolates of *P. expansum* that are sensitive or resistant to TBZ more efficiently with respect to the chemical control applied alone. At the same time, this type of integrated strategy may also reduce the risk of the onset of pathogen strains toward more recent fungicides such as BOSC or CYPR, since control also relies on the different and multifaceted modes of action that are involved in the protective activity of biocontrol yeasts such as LS11 and LS28: competition for space and nutrients and wound competence based on resistance to oxidative stress caused by reactive oxygen species generated in fruit wounds (Castoria et al., 2003) as well as production of lytic enzymes (Castoria et al., 1997). These mechanisms are complex and make it unlikely for a fungal pathogen to develop resistance.

In conclusion, the integration of the selected biocontrol yeasts isolates *R. kratochvilovae* LS11 and *C. laurentii* LS28 with low dosages of the recently developed fungicides BOSC and CYPR appears to be a very promising method to control blue mould and keep low both fungicide residues and PAT accumulation in apples.

Table 1. Minimum inhibitory concentration (MIC: µg/ml of active ingredient) of the fungicides boscalid, cyprodinil, fenhexamid and thiabendazole towards the *in vitro* growth of the biocontrol yeasts *R. kratochvilovae* LS11 and *C. laurentii* LS28.

Fungicide[a]	**Yeast isolate**	
	LS11	LS28
Boscalid (Cantus®, 50)[b]	>375	>375
Cyprodinil (Chorus®, 50)	>150	>150
Fenhexamid (Teldor®, 50)	<150	<150
Thiabendazole (Decco T®, 50)	< 104.5	>418

[a] For each fungicide concentrations of 25%, 50% and 100% of the full label dose of the respective trade product were tested.
[b] In brackets, the trade product used and its percentage (w/v) of active ingredient are reported.

Table 2 Sensitivity of some isolates of *P. expansum* toward the fungicides thiabendazole, boscalid and cyprodinil, expressed as EC50 (µg/mL of active ingredient reducing by 50% the growth in vitro of the fungal mycelium).

Isolate of *P.expansum*	**Source**	**Fungicide**		
		Thiabenzadole (Decco, 50)	**Boscalid (Cantus,50)**	**Cypronidil (Chrus,50)**
		EC_{50}	EC_{50}	EC_{50}
FQ42	Apple	160.0	<37.5	<15
FS7	Apple	>418	<37.5	<15
FQ44	Apple	<4.2	<37.5	<15
FQ45	Apple	<4.2	<37.5	<15
FQ46	Apple	>418	<37.5	<15
FQ47	Apple	250.0	<37.5	<15
FQ48	Apple	<4.2	<37.5	<15
P32-R	Pear	194.5	<37.5	<15
LB8/99-S	Pear	<4.2	<37.5	<15

[a] **FS7**: isolate of the pathogen used for artificial inoculations in experiments on apples; **P32-R** and **LB8/99-S**: reference isolates known for high resistance or high sensitivity to benzimidazoles, respectively.
[b] In brackets: name of the commercial product and percentage (w/v) of active ingredients.
[C] In the presence of exact values of EC50, numbers in parenthesis indicate 95% confidence limits as determined by probit analysis.

Table 3: Values of the synergy factor (SF)a for the activity of the biocontrol yeasts *R. kratochvilovae* LS11 and *C. laurentii* LS28 combined with a low dose of commercial formulates (25% of the full dose suggested by the manufacturers) of the fungicides thiabendazole, boscalid or cyprodinil against *P. expansum* on apples kept for 7 days at 21° C. Average values±standard deviation are reported.

Fungicide	Biocontrol agent	
	LS11	LS28
Thiabendazole	0.0±0.0	0.2±0.4
Boscalid	6.7±2.0	3.2±0.4
Cyprodinil	1.1±0.8	2.4±0.5

[a] according to the Abbott's formula (Levy *et al*., 1986), for SF values: = 1, the interaction is additive; < 1, the interaction is antagonistic; > 1, the interaction is synergistic.
[b] Average values ± standard deviation from the means o three replications are presented.

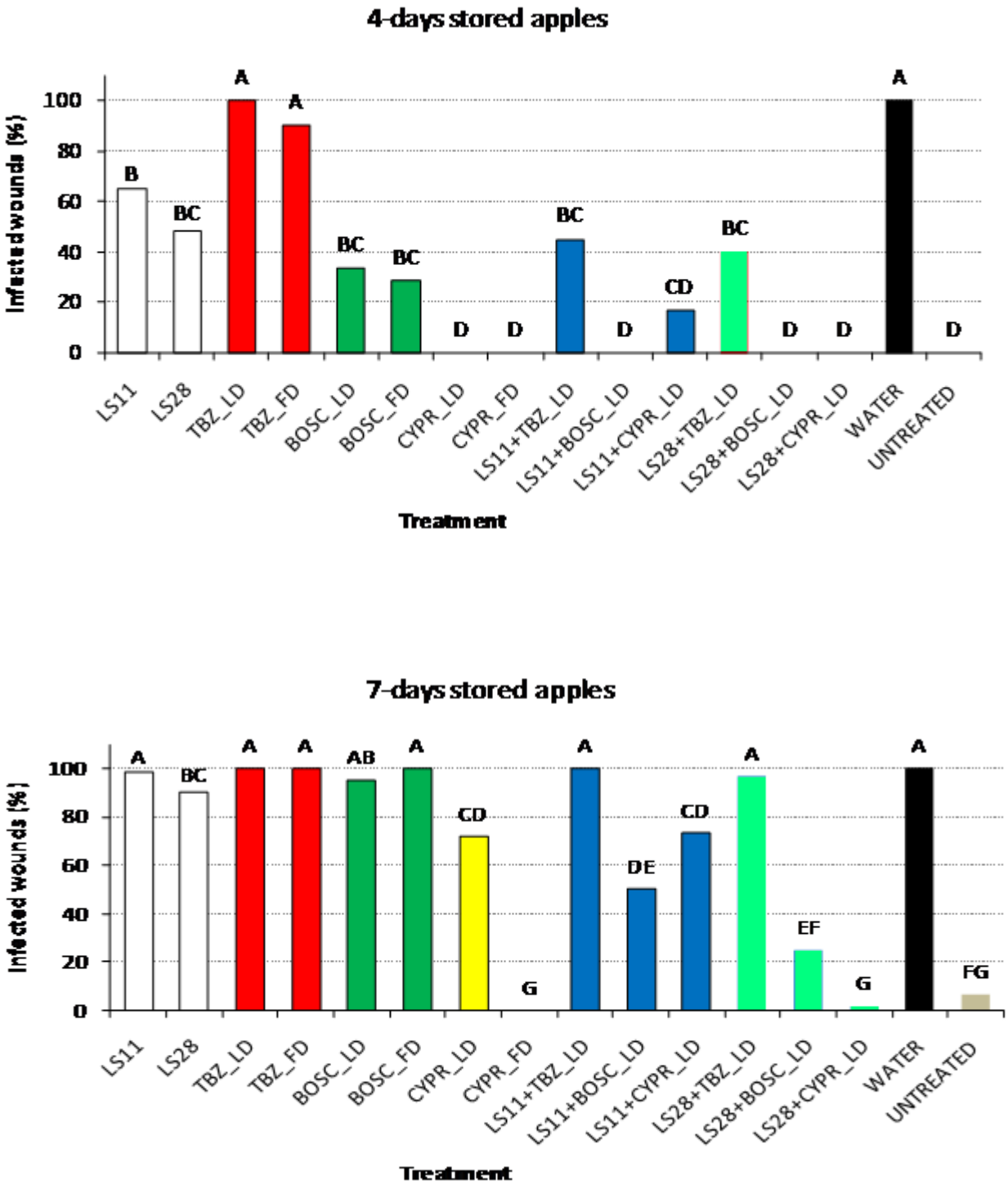


Fig. 1. Combined activity of the biocontrol yeasts *R. glutinis* LS11 and *C. laurentii* LS28 with a low dosage of the fungicide thiabendazole (TBZ), boscalid (BOSC) or cyprodinil (CYPR) against *P. expansum* on apples kept at 21°C for 4 days (A) and 7 days (B). Values marked by common letters are not statistically different at P= 0.01 (Tukey's test). LD and FD are low and full dosage of fungicide, respectively. For each fungicide, the LD used was 25% of the label FD. The biocontrol yeast LS11 and LS28 were applied at low concentration of cells ($5x10^6$ cfu/ml). Except for the untreated control, all the apples were artificially inoculated with *P. expansum* ($2x10^4$ conidia/ml).

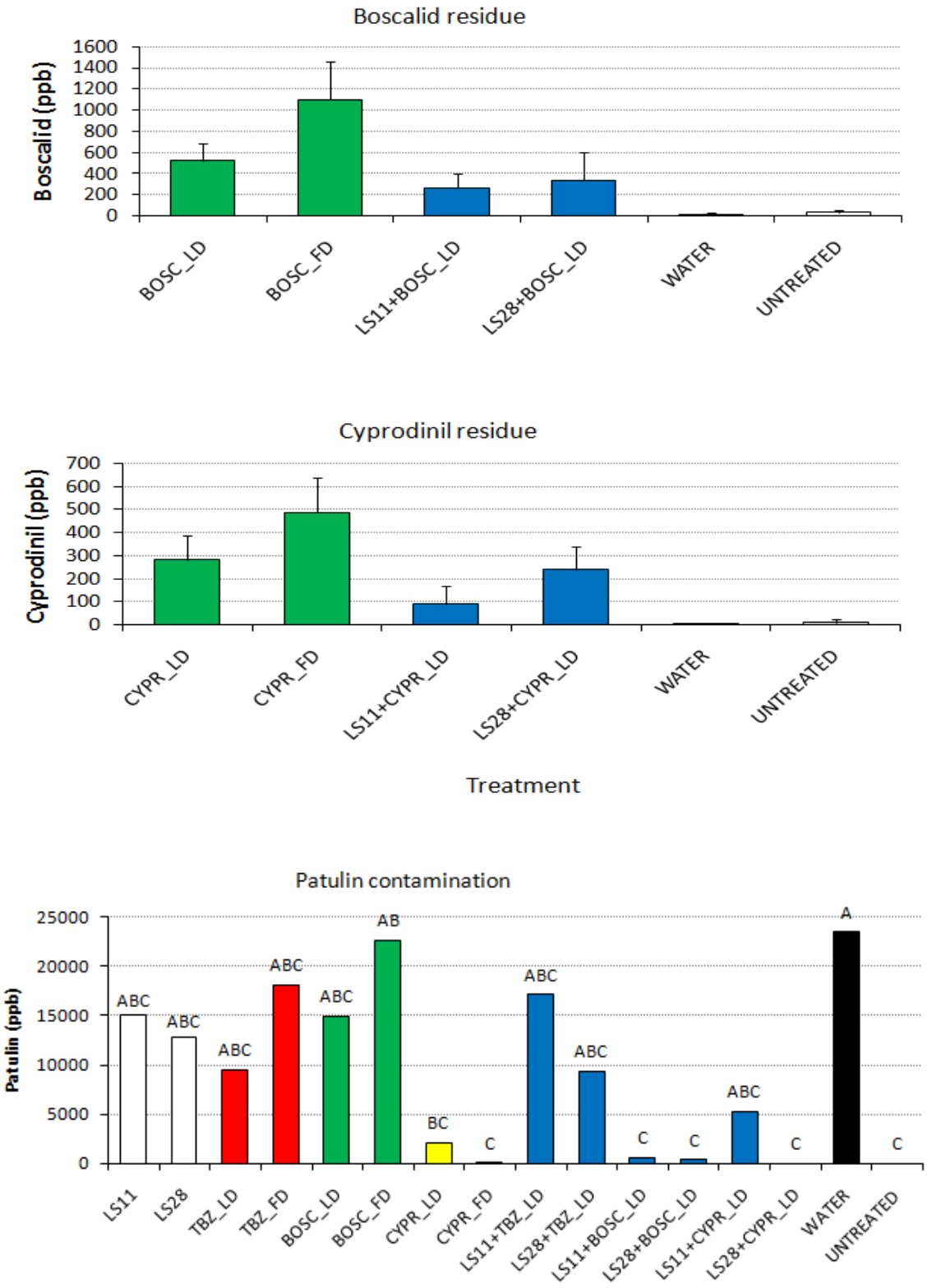


Fig. 2. Fungicide residues of boscalid and cyprodinil and level of contamination by the mycotoxin patulin in apples after 7 days of storage at 21°C. Fruit were subjected to different treatment as in figure 1. Values marked by common letters are not statistically different at P= 0.01 (Tukey's test) and bars on histograms represent the SD from the mean.

References

Adaskaveg, J.E., Förster, H., Gubler, W.D., Teviotdale, B.L., Thompson, D.F., (2006). Reduced-risk fungicides help manage brown rot and other fungal diseases of stone fruit. Calif. Agric. 59: 109–114.

Arranz, I., Derbyshire, M., Kroeger, K., Mischke, C., Stroka, J., Anklam, E., (2005). Liquid chromatographic method for quantitation of patulin at 10 ng/mL in applebased products intended for infants: interlaboratory study. J. AOAC Int. 88: 518–525.

Avenot, H., Morgan, D.P., Michailides, T.J., (2008). Resistance to pyraclostrobin, boscalid and multiple resistance to Pristine (pyraclostrobin + boscalid) fungicide in Alternaria alternata causing alternaria late blight of pistachios in California. Plant Pathol. 57:135–140.

Babij, J., Zhu, Q., Brain, P., Hollomon, D.W., (2000). Resistance risk assessment of cereal eyespot, Tapesia yallundae and Tapesia acuformis, to the anilinopyrimidine fungicide, cyprodinil. Eur. J. Plant Pathol. 106: 895–905.

Baraldi, E., Mari, M., Chierici, E., Pondrelli, M., Bertolini, P., Pratella, G.C., (2003). Studies on thiabendazole resistance of Penicillium expansum of pears: pathogenic fitness and genetic characterization. Plant Pathol. 52: 362–370.

Battilani, P., Barbano, C., Logrieco, A., (2008). Risk assessment and safety evaluation of mycotoxins in fruits. In: Barkai-Golan, R., Paster, N. (Eds.), Mycotoxins in Fruits and Vegetables. Academic Press in an imprint of Elsevier, USA, 2008, pp 1–26.

Beretta, B., Gaiaschi, A., Galli, C.L., Restani, P., (2000). Patulin in apple-based foods: occurrence and safety evaluation. Food Addit. Contam. 17: 399–406.

Boyer, J., Liu, R.H., (2004). Apple phytochemicals and their health benefits. Nutr. J. 3:1–17.

Castoria, R., Logrieco, A., (2007). Mycotoxins in fruits and major fruit-derived products — an overview. In: Ward, O.P., Ray, R.C. (Eds.), Microbial Biotechnology in Horticulture, vol. II. Science Publishers, New Hampshire, USA. 2007, pp 305–344.

Castoria, R., De Curtis, F., Lima, G., De Cicco, V., (1997). ß-1,3-glucanase activity of two saprophytic yeasts and possible mode of action involved as biocontrol agents against postharvest diseases. Postharvest Biol. Technol. 12, 293–300.

Castoria, R., Caputo, L., De Curtis, F., De Cicco, V., (2003). Resistance of postharvest biocontrol yeasts to oxidative stress: a possible new mechanism of action. Phytopathology 93: 564–572.

Castoria, R., Morena, V., Caputo, L., Panfili, G., De Curtis, F., De Cicco, V., (2005). Effect of the biocontrol yeast Rhodotorula glutinis strain LS11 on patulin accumulation in stored apples. Phytopathology 95: 1271–1278.

Castoria, R., Mannina, L., Maiuro, L., Sobolev, A., Ritieni, A., Ferracane, R., Spina, A., (2007). Nuovi dati sul decremento di patulina causato da un lievito di biocontrollo in mele infettate da Penicillium expansum. Rapporti ISTISAN 07/37, 147–151. ISSN:1123–3117, www.iss.it/publ/rapp/index.php?anno=2007&lang=1&tipo=5.

Castoria, R., Wright, S.A.I., Droby, S., (2008). Biological control of mycotoxigenic fungi in fruits. In: Barkai-Golan, R., Paster, N. (Eds.), Mycotoxins in Fruits and Vegetables. Elsevier, San Diego, USA. 2008, pp 311–333.

Commission Regulation (EC) No. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Official Journal of the European Union, 364: 5–24

De Felice, D.V., Solfrizzo, M., De Curtis, F., Lima, G., Visconti, A., Castoria, R., (2008). Strains of Aureobasidium pullulans can lower ochratoxin A contamination in wine grapes. Phytopathology 98:1261–1270.

Droby, S., Wisniewski, M., Macarisin, D., Wilson, C., (2009). Twenty years of postharvest biocontrol research: is it time for a new paradigm? Postharvest Biol. Technol. 52: 137–145.

Errampalli, D., Brubacher, N.R., (2006). Biological and integrated control of postharvest blue mold (Penicillum expansum) of apples by Pseudomonas syringae and cyprodinil. Biol. Control 36: 49–56.

Errampalli, D., Brubacher, N.R., DeEll, J.R., (2006). Sensitivity of Penicillium expansum to diphenylamine and thiabendazole and postharvest control of blue mold with fludioxonil in 'McIntosh' apples. Postharvest Biol. Technol. 39: 101–107.

Gallus, S., Talamini, R., Giacosa, A., Montella, M., Ramazzotti, V., Franceschi, S., Negri, E., La Vecchia, C., (2005). Does an apple a day keep the oncologist away? Ann. Oncol. 16:1841–1844.

Ippolito, A., Nigro, F., Schena, L., (2004). Control of postharvest diseases of fresh fruits and vegetables by preharvest application of antagonistic microorganisms. In: Dris, R., Niskanen, R., Jain, S.M. (Eds.), Crop Management and Postharvest Handling of Horticultural Products. Disease and Disorders of Fruits and Vegetables, 4. Science Publisher Inc., Enfield, NH, USA, 1–30.

Janisiewicz, W.J., Korsten, L., (2002). Biological control of postharvest diseases of fruits. Annu. Rev. Phytopathol. 40: 411–441.

Jones, A.L., Aldwinckle, H.S. (Eds.), (1990). Compendium of Apple and Pear Diseases. American Phytopathological Society, St. Paul, MN, p. 100.

Katerere, D.R., Stockenstrom, S., Balducci, G., Shephard, G.S., (2007). Determination of patulin in apple juice: comparative evaluation of four analytical methods. J. AOAC Int. 90:162–166.

Leroux, P., 1996. Recent developments in the mode of action of fungicides. Pesticide Sci. 47, 191–197.

Levy, Y., Benderly, M., Cohen, Y., Gisi, U., Bassand, D., (1986). The joint action of fungicides in mixture; comparison of two methods for synergy calculation. EPPO Bull. 16: 651–657.

Lima, G., De Curtis, F., Castoria, R., De Cicco, V., (1998). Activity of the yeasts Cryptococcus laurentii and Rhodotorula glutinis against postharvest rots on different fruits. Biocontrol Sci. Technol. 8 : 257–267.

Lima, G., Arru, S., De Curtis, F., Arras, G., (1999). Influence of antagonist, host fruit and pathogen on the biological control of postharvest fungal diseases by yeasts. J. Ind. Microbiol. Biotechnol. 23: 223–229.

Lima, G., Spina, A.M., Castoria, R., De Curtis, F., De Cicco, V., (2005). Integration of biocontrol agents and food-grade additives for enhancing protection of apples from Penicillium expansum during storage. J. Food Protect. 68: 2100–2106.

Lima, G., De Curtis, F., Piedimonte, D., Spina, A.M., De Cicco, V., (2006). Integration of biocontrol yeast and thiabendazole protects stored apples from fungicide sensitive and resistant isolates of Botrytis cinerea. Postharvest Biol. Technol. 40: 301–307.

Lima, G., De Curtis, F., De Cicco, V., (2008). Interaction of microbial biocontrol agents and fungicides in the control of postharvest diseases. Stewart Postharvest Rev. 1: 1–7.

Morales, H., Sanchis, V., Rovira, A., Ramos, A.J., Marin, S., (2007). Patulin accumulation in apples during post-harvest: effect of controlled atmosphere and fungicide treatments. Food Control 11:1443–1448.

Morales, H., Marín, S., Rovira, A., Ramos, A.J., Sanchis, V., (2010). Influence of post-harvest technologies applied during cold storage of apples in Penicillium expansum growth and patulin accumulation: a review. Food Control 21: 953–962.

Rosenberger, D.A., (1990). Blue mold. In: Jones, A.L., Aldwinckle, H.S. (Eds.), Compendium of Apple and Pear Diseases. American Phytopathological Society, St. Paul, MN, 1990, pp. 54–55.

Sholberg, P.L., Bedford, K., Stokes, S., (2005). Sensitivity of Penicillium spp. and Botrytis cinerea to pyrimethanil and its control of blue and gray mold of stored apples. Crop Prot. 24: 127–134.

Snowdon, A.L., (1990). A Color Atlas of Post-harvest Diseases and Disorders of Fruits and Vegetables. General Introduction and Fruits, 1. CRC Press, Boca Raton, FL, USA, pp 302

Stammler, G., Brix, H.D., Glattli, A., Semar, M., Schoefl, U., (2007). Biological properties of the carboxamide boscalid including recent studies on its mode of action. In: Proc. 16th Int. Plant Protection Congress, Glasgow, UK, 2007. BCPC, Alton, UK, pp. 16–21.

Viñas, I., Usall, J., Sanchis, V., (1991). Tolerance of Penicillium expansum to postharvest fungicide treatment in apple packinghouses in Lleida (SP). Mycopathologia 113: 15–18.

WHO (World Health Organization), (2008). Dietary exposure assessment of chemicals in food. Report of a Joint FAO/WHO Consultation Annapolis, Maryland, USA, 2–6 May 2005. Home page: http://www.whqlibdoc.who.int/publications/2008/9789241597470 eng.pdf.

Wouters, M.F.A., Speijers, G.J.A., (1996). Toxicological evaluations of certain food additives and contaminants: patulin. WHO Food Addit. Ser. 35:377–402.

Zhou, T., Northover, J., Schneider, K.E., Lu, X., (2002). Interactions between Pseudomonas syringae MA-4 and cyprodinil in the control of blue mold and gray mold of apple. Can. J. Plant Pathol. 24:154–161.

Chapter 4: STUDY OF PATULIN THERMAL RESISTANCE AND BIOACCESSIBILITY *IN VITRO* FROM ARTIFICIALLY CONTAMINATED APPLE PRODUCTS

4.1. Introduction

Consumption of fruit juice helps fulfill the recommendation to eat more fruits and vegetables, with fruit juice accounting for 50% of all fruit servings consumed by children aged two through 18 years and 1/3 of all fruits and vegetables consumed by preschoolers. According to fruit products manufacturers, children under 5 years of age consume, on average, 3.2 fl oz (fluid ounce equal to 28.4 ml)/day of fruit juice until to 6 fl oz (Dennison et al, 1997). Eleven percent of healthy preschoolers consumed ≥ 12 fl oz/day of fuit juice (Barbara and Dennison, 1996, US).

Consumption of fruit juices in EU member states varies considerably depending on climatic conditions and habits of consumption. Consumption of fruit juices and nectars in the EU in 2007 amounted in average, to 11.2 billion gallons (3.9 fl oz/day) (Štrbac, 2010).

Several studies were carried out in various countries around the world about PAT presence in apple and its products. Martins, et al. (2002) reported the occurrence of PAT in portuguese apples with small rotten spots and the maximum detected level exceeded 80 mg/kg. A study carried out by Ritieni (2003) in Italy reported mean levels of PAT equal to 24.8 µg/kg in conventional apple juices and 28.3 µg/kg in organic apple juices. Piemontese et al., (2005) reported levels of 3.1 and 7.1 µg/kg for conventional and organic Italian apple juices respectively, in accordance with Versari et al., (2007) that reported a PAT content below the limit of 50 µg/l for the analyzed samples. Barreira et al., (2010) studied the occurrence of PAT in apple-based-foods in Portugal, and was detected in 23% of samples with values ranging from 1.2 µg/kg to 42 µg/kg, similar to levels found in Belgium, equal to 10.2 and 43.1 µg/kg for conventional and organic juices respectively (Baert et al., 2006).

These data indicate there is a risk associated to the PAT intake, through the consumption of purees and apple juices.

To achieve any effects in a specific tissue or organ, the mycotoxins must be available, which refers to the compound´s tendency to be extracted from the food matrix, and they must then be absorbed from the gut via the intestinal cells. Bioaccessibility has been defined as the fraction of a compound that is released from its matrix in the gastrointestinal tract and thus becomes available for intestinal absorption (ie, enters the blood stream) (Benito and Miller, 1998).

In human health risk assessment, ingestion of food is considered a major route of exposure to many contaminants either caused by industrial or environmental contamination or as result of production processes. The total amount of an ingested contaminant (intake) does not always reflect the amount that is available to the body. Only a certain amount of the contaminant will be bioavailable (Versantvoort et al., 2005). Bioavailability is a term used to describe the proportion of the ingested contaminant in food that reaches the systemic circulation (Fernández-García et al., 2009).

Authors like Avantaggiato et al. (2003) and Avantaggiato et al. (2004) studied the intestinal absorption of several mycotoxins (zearalenone (ZEA), fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), ochratoxin A (OTA), deoxinivalenol (DON) and aflatoxin B_1 (AFB_1). During apple products manufacturing, the formation of undesirable products like 5-hydroxymethylfurfural (5-HMF) (figure 1) can occur. This compound, a marker of thermal processing, is naturally formed as a Maillard reaction (MR) product (MRP) (Ames, 1998), and from dehydration reaction of hexoses in mild acidic conditions (Kroh, 1994). Maximum mean values for HMF of 8.91 mg/l and 10.14 mg/l were obtained for treatment at 90 °C and 100 °C for 20 min (Kadakal and Nas, 2003). Scientific panel on food additives, flavourings, processing aids and materials in contact with food (AFC) has estimated for this compound, an intake of 1.6 mg/day/person (EFSA 2005a). The International Federation of Fruit Juice Processors (IFFJP) recommends a maximum concentration of 5-10 mg/l HMF in fruit juices and 25 mg/l in concentrates juices (Wagner and Beil-Seidler, 2006).

The aims of this work were: a) to evaluate the differences of PAT's thermal resistance in apple juices and purees and the production of HMF through a simulation of heat treatment; b) to study PAT's bioaccessibility in apple juices, nectars and purees in a procedure of *in vitro* digestion reproducing the physiological condition of the child.

This study can represent an original contribution to the mycotoxin risk assessment in so far treats for the first time both the control of processing and the bioaccessibility of PAT in fruit products, that represent a category of beverages widely consumed by children.

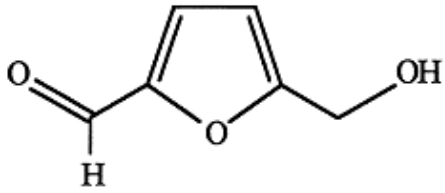


Figure 1. 5-HMF chemical structure

4.2 Materials and methods

4.2.1 Materials

Potassium chloride (KCl), potassium thiocyanate (KSCN), monosodium phosphate (NaH_2PO_4), sodium sulphate ($NaSO_4$), sodium chloride (NaCl), sodium bicarbonate ($NaHCO_3$), urea, α-amilase, hydrochloric acid (HCl), pepsin, pancreatin, bile salts were obtained from Sigma-Aldrich (Madrid, Spain). Solvents (ethyl acetate, hexane, acetonitrile, methanol, water) were purchased from Merck (Milan, Italy). Deionized water (<18MΩ cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Chromatographic solvents and water were degassed for 20 min using a Branson 5200 (Branson Ultrasonic Corp., CT, USA) ultrasonic bath. PAT and HMF standards were purchased from Sigma-Aldrich (Milan, Italy).

4.2.2 Sampling

Fresh apples used for thermal treatment tests belonged to variety Golden Delicious and were acquired from a market in the city of Naples (Italy).
Apple products samples (n=7) used for test of PAT's bioaccessibility included apple juices, nectars and purees belonging to different brands and were collected from supermarkets located in Naples.

4.2.3 Heat treatment tests

One kilogram of skimmed apples were homogenized using a blender (Bosch 600 W; Milan, Italy) at 10000 rpm during 3 min to obtain apple puree; then the product was centrifuged at 15000 rpm during 3 min to separate the juice from the solid fraction.
Twenty five grams of uncontaminated juice and puree were artificially contaminated at level of 50µg/l and 50µg/kg respectively. Successively, the apple products were treated with process simulating pasteurization constituted by 3 steps: a) first pasteurization at 80°C during 20 minutes; b) cooling at 4°C in 30 min.; c) second pasteurization at 80°C during 20 min. Each product (juice and puree) was treated in triplicate and a control test was carried out (product not thermally treated).

4.2.4 Preparation of working standard solutions

One milligram of PAT standard (4-Hydroxy-4H-furo[3,2-c]pyran-2(6H)-one Sigma Chemicals , St. Louis, MO) was weighed and diluted in 10 mL of methanol (Merck, Darmstadt-Germany) to obtain a stock solution of 1000 mg/l. The working standard solutions were obtained by diluitions of the stock solution. The solutions were stored in the dark at 4°C before using.

4.2.5 PAT extraction

For PAT extraction the method of Arranz et al (2005) with some modifications was applied.
In particular 10 g of sample were transferred into 50 mL plastic tube containing 15 g of Na_2SO_4, 2 g of $NaHCO_3$ and 10 mL of extraction solution (ethyl acetate/hexane 60:40, v/v) were introduced. The tube was shaken for 3.5 min on a mechanical shaker (Intercontinental equipment, Hidalgo, TX) and then centrifuged at 2000 rpm for 1 min at 4°C. The supernatant (2.5 mL) was immediately placed onto an unconditioned Strata C18-E solid phase extraction column (Phenomenex, USA) (parameter: Surface Area 500 m^2/g; pore size 70 ˚A; pore volume 0.88 mL/g; average particle size 58 µm) which was washed with 3 mL of the extract solution. The eluate was collected in a 10 mL plastic tube test-tube containing 50 µL of acetic acid (Merck, Darmstadt-Germany), and the flow was adjusted to 1 drop per second by using slight air pressure. The solvent was evaporated at max 40° C for about 35 min in a vacuum centrifuge Thermo Savant (Speed Vacuum Thermo Electron Corporation Milford, MA, USA) and 1 mL of acidulated water (pH 4) was added to redissolve the sample. The solution was mixed with a vortex (Biosan MSV-3500, Lietsa, Finland) filtered with 0.22µm filters (Phenomenex, Palo Alto, CA) and injected in the LC apparatus.

4.2.6 PAT LC analysis

PAT determination was carried out according to Arranz et al. (2005). Analyses were carried out by using an LC apparatus (Shimadzu-Japan) equipped with an autosampler SIL-20A, two pumps LC-20AD and a UV/vis detector SPD-20A set at 276 nm wavelength. The column was a Gemini 5 µ C18, 110 ˚A (150 mm × 2 mm) (Phenomenex, CA, USA). The mobile phases were water containing 1% of acetic acid (A) and methanol (B) in isocratic conditions 95/5 (v/v). The flow rate was of 1 mL/min. In these chromatographic conditions, the retention time for PAT was 14.0 ± 0.1 min.

4.2.7 HMF extraction

HMF extraction from juice and puree was carried out according the method of Zappalà et al. (2005) with slight modifications. In a falcon tube 1 mL of sample was diluted with 5 mL of deionized water, filtered on 0.45 µm filter (Phenomenex, Palo Alto, CA) and immediately injected in LC apparatus.

4.2.8 HMF LC analysis

HMF determination was carried out according to Zappalà et al. (2005) with slight modifications. Analyses were carried out by using an LC apparatus (Shimadzu-Japan) equipped with an autosampler SIL-20A, two pumps LC-20AD and a UV/vis detector SPD-20A set at 280 nm wavelength. The HPLC conditions were the following: isocratic mobile phase, 90% H_2O at 1% of CH_3COOH and 10% CH_3OH at a flow rate of 1 mL/min under isocratic conditions. The column was Sphereclone (Phenomenex) (size 250 x 4.60 mm, pore size: 5 µm).

4.2.9 *In vitro* digestion model

Commercial samples including apple nectars and juices were artificially contaminated with PAT at the level of 50 µg/l (legislative limit fixed for PAT in juices) and purees at the level of 25 µg/kg (legislative limit fixed for PAT in solid products) to carry out bioaccessibility tests in children.

The procedure of *in vitro* digestion used in this study was adapted from the method outlined by Gil-Izquierdo, Zafrilla, Tomás-Barberá, (2002), and Jovanì et al (2004) with slight modifications. The method consists of three sequential steps; an initial saliva/pepsin/HCl digestion for 2 h at 37º C, to simulate the mouth and the gastric conditions, followed by a digestion with pancreatic juice for 2 h at 37 ºC to simulate duodenal digestion.

For the saliva/pepsin/HCl digestion, the sample (10 g) was mixed with 80 mL of distilled water and 6 mL of artificial saliva composed by: KCl (89.6 g/l), KSCN (20 g/l), NaH_2PO_4 (88.8 g/l), $NaSO_4$ (57 g/l), NaCl (175.3 g/l), $NaHCO_3$ (84.7 g/l), urea (25 g/l) and 290 mg of α-amilase. The pH of the solution was adjusted at 6.8 with HCl 0.1 N.

Immediately, 0.02 g of pepsin dissolved in HCl 0.1 N were added, and the pH was adjusted at a value of 3 with HCl 6 N, and then incubated at 37 ºC in an orbital shaker (250 rpm) (Infors AG CH-4103, Bottmingen, Switzerland) for 2 h.

After the gastric digestion, the pancreatic digestion was simulated. The pH was increased to 6.5 with $NaHCO_3$ 0.5 N and then 0.0005 g of pancreatin and 0.03 g of bile salts dissolved in 20 mL of water were added and incubated at 37ºC in an orbital shaker (250 rpm) for 2 h.

After pancreatic digestion, 30 mL of the mixture were centrifuged at 4.000 rpm and 4ºC during 1h. To determine the PAT concentration in studied samples, surnatant obtained from the intestinal phases (pancreatin–bile digestion) were analyzed according to the method described in par. 4.2.6.

4.3 Results and discussion

4.3.1 Analytical performance

For recovery studies of PAT, 10 mL (gr) of uncontaminated apple juice (puree) were spiked at levels of 10, 50 and 100 µg/kg with a PAT's standard dissolved in methanol. Biological fluids obtained from digestion of uncontaminated apple products were also spiked with PAT at the same concentrations to develop recovery tests. For each level of contamination the test was performed in triplicate. The samples and digested fluids were let overnight at room temperature and then extracted according to the method explained in par. 4.2.5 with blank samples.

For recovery study of HMF, 1 mL (gr) of uncontaminated apple juice (puree) was spiked at levels of 0.05, 5 and 10 µg/ml with a HMF's standard dissolved in water.

The mean PAT's recoveries were independent of spiking levels assayed ranged from 78.1 ± 5.4% in puree (digested and not digested) to 96.4±6.3% in juice (digested and not digested) respectively, with relative standard deviation (RSD) below 15%. The limit of detection (LOD) of the PAT's method was 5 µg/kg, while limit of quantification (LOQ) was 15 µg/kg. The quantification was carried out by including signal area

values into a linear calibration curve, within the range of 5–1000 µg/kg, and by correcting errors imputable to variable sensitivity of instrument.
The mean HMF's recoveries were independent of spiking levels assayed ranged from 96.3% ± 4.2% in puree to 101.4% ± 3.5% in juice. The LOD of the HMF's method was 0.010 mg/L, while LOQ was equal to 0.030 mg/L. HMF was quantified using the external standard method within the range 0.025-75 mg/L and by correcting errors imputable to variable sensitivity of instrument.

4.3.2 Statistical analysis
Data of bioaccessibility were subjected to analysis of variance (ANOVA). Duncan Multiple Range Test was used to separate the means of values of bioaccessibility in different apple products (fig. 6) and it was determined at the 5% probability level.

4.3.3 PAT loss
The effects of the heat treatment on reduction of PAT level in apple juice and puree artificially contaminated at the level of 50 µg/l are showed in Figures 3,4,5. In purees samples, the evidenced reduction was of 1.41±0.52%, whereas in the juices was of 62.62±2.53%. These differences can be related to the major protection of mycotoxin in semi-solid matrix that was characterized by major viscosity. Temperature had a smaller effect on viscosity of cloudy juices than of clear juices. The apparent viscosity of fruit purees (pseudoplastic fluids) decreased slightly at higher temperatures. The activation energy for flow increased with the juice concentration and decreased with the presence of suspended particles in the fruit product. (Saravacos, 1970). The lowest efficacy of heat treatment about PAT degradation in puree can explain the major incidence of the mycotoxin in apple puree respect to apple juice as reported in literature as well the process of depectinization, clarification and filtration applied for juice production, that can reduce the levels of PAT between 20.5 and 39.1% (Acar et al., (1998), Bissessur, et al., (2001)). Funes and Resnik (2009) determined PAT in solid and semisolid apple and pear products marketed in Argentina. The results showed that 21.6% of products were contaminated (average of positive samples 61.7µg/kg), but the highest levels were found in apple puree with 50% contaminated samples (average of positive samples 123 µg/kg).
Several studies were carried out about the PAT reduction during industrial manufacturing. In particular, Taniwaki et al. (1989) treated the apple juice contaminated with PAT (1500 µg/l) with a heat treatment at 90°C for 2 min, followed by hot-filling and a final 5 min heat treatment in boiling water followed by a cooling at room temperature, observing the 60% of PAT reduction.
Kadakal et al. (2002), studied the mean reduction of PAT content in the apple juice after heat treatment (90°C for 10 s) in a Turkish factory evidencing a reduction of 13.4%
Kadakal and Nas, (2003) evaluated the PAT degradation during the apple juice production treated at 90 °C and 100 °C for 20 min, evidenced a mean reduction of the bioactive compound employed of 18.81 and 25.99 % respectively.

4.3.4 HMF production
HMF levels in juices and purees treated thermically are showed in Figure 6.
The results show that HMF was partially produced during the juice preparation with a value of 0.035±0.002 µg/ml due to heating for mechanical friction in centrifuge before heat treatment, whereas after thermal treatment the evidenced data was of 8.3 ± 0.06 µg/mL. The evidenced HMF level in apple puree was equal to 0.014±0.001µg/ml, whereas after the heat treatment the evidenced data was of 0.045±0.002µg/ml. These data are explaining according the study cited above (Saravacos, 1970).
In the literature is not reported HMF levels produced during preliminary phases of juice and puree manufacturing, but only at the end of production and during various steps of storage. However our results obtained after heat treatment are comparable with data reported by Kim & Richardson (1992) that reported HMF concentrations in a variety of aseptically processed juice drinks from 2.0 to 19.4 ppm. Yan & Chen in 1998 reported levels of HMF contamination between 4.2 and 21.9 mg/l; in particular in apple juice detected value amounted to 19.0 mg/l.
Kadakal et al. (2003) evidenced levels of 5-HMF in a range between 2.07 mg/L and 10.14 mg/L after heat treatment and evaporation on apple juices. Burdurlu et al., (2003) reported HMF concentration, in Golden Delicious apple juice concentrates between 0.52 and 963 mg/kg, and between 0.52 and 190 mg/kg in Amasya apple juice concentrates.
Kus et al. (2005) analyzed HMF content for several food products; in particular they found levels between 0.4-4.5 mg/l for fruit concentrates, 12.8-3500 mg/l for boiled juices.
Vorlovà et al. (2006) determined HMF contents in juices at levels between 0.0–27.3 mg/kg while in fruit baby foods the content of HMF ranged from to 2.1 to 9.8 mg.kg.

4.3.5 PAT content in duodenal fluids

Bioaccessibility percentages of PAT in 7 artificially contaminated apple products after the process of digestion *in vitro* are represented in Figure 7. Samples of apple juice with pulp showed values equal to 70.89± 4.93% and 55.69±4.73% respectively while samples of puree showed levels of 67.30±10.76% and 58.15±5.50% respectively. The categories characterized by the lowest levels of bioaccessibility were represented by nectar and two clarified juices equal to 38.88±2.42%, 28.59±0.46% and 25.28±0.61% respectively. Mean values of bioaccessibility from clarified and containing pulp products were 30.92±7.09% and 63.01±7.25% respectively. A sample of juice with pulp and a sample of puree showed values statistically comparable (p=0.05) as well as two samples of puree and two clarified juices.

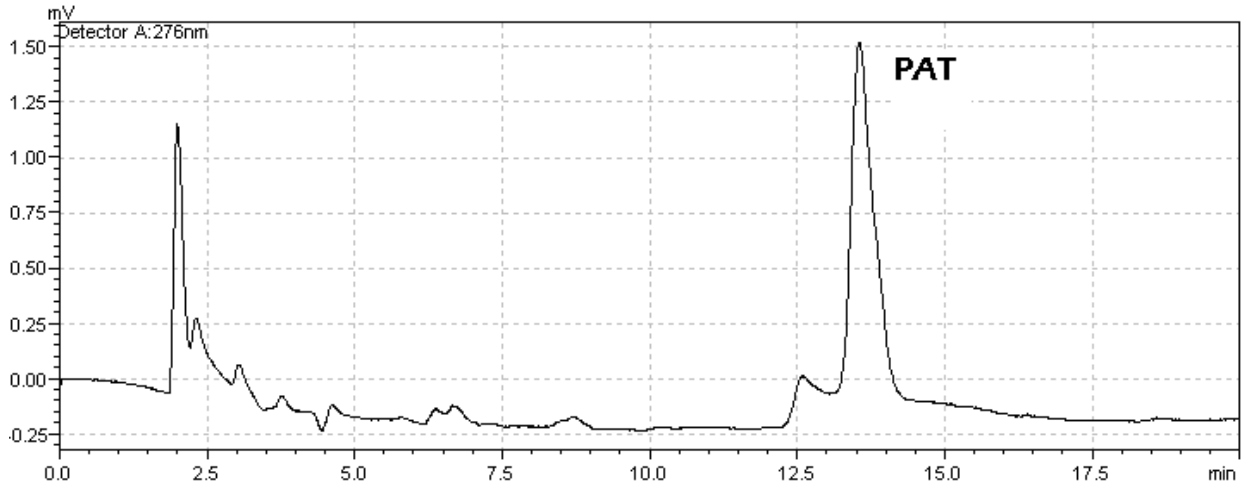
These results suggested that different matrixes have influenced the mobilization of the mycotoxin.

Generally, absorption and metabolism depend more on the compound specific properties and physiology and, therefore, the matrix is expected to have less influence on these processes. However, in some cases, the ingested matrix has been shown to affect the transport of the contaminant across the intestinal epithelium (Brandon et al., 2006, Wienk et al., 1999). The hyphothesis is that the physical structure of clarified products could have favored the action of digestive enzyme, while in the presence of fruit pulp, this effect could be disadvantaged.

Considering the PAT initial concentration, 15.5 µg/l and 24.4 µg/kg in clarified juices and products with pulp respectively could interact, in the worst case, with the intestinal epithelium cells in children over 3 years old. In this conditions the levels of bioaccessible fraction could exceed of 55% and 144% respectively the limit established in fruit products for infant and young children (10µg/l).

An insufficient knowledge on the bioavailability may hamper an accurate risk assessment of ingested contaminants in humans. Actually few studies around bioaccessibility of mycotoxins have been carried out. Versantvoort et al. (2005) studied the bioaccessibility of AFB_1 from peanut slurry and OTA from buckwheat. Both mycotoxins were almost completely mobilised from the peanut slurries during digestion, at levels of 94% and 100%, respectively for AFB_1 and OTA. Simla et al., (2009) carried out an *in vitro* digestion experiment to analyze the bioaccessibility of AFB_1 in grinded corn and peanut. Results indicated values equal to 95% and 94%, respectively. Dall'Asta et al., (2010) studied an in vitro digestion model applied to raw maize to evaluate the possible release of hidden fumonisins under gastrointestinal conditions. After digestion, an increased amount of total detectable fumonisins was observed in comparison with the analysis on the not digested matrix. Data found in this study can be really interesting for the evaluation on risk assessment in children that represent a category of market that usually consume juices and puree.

a)



b)

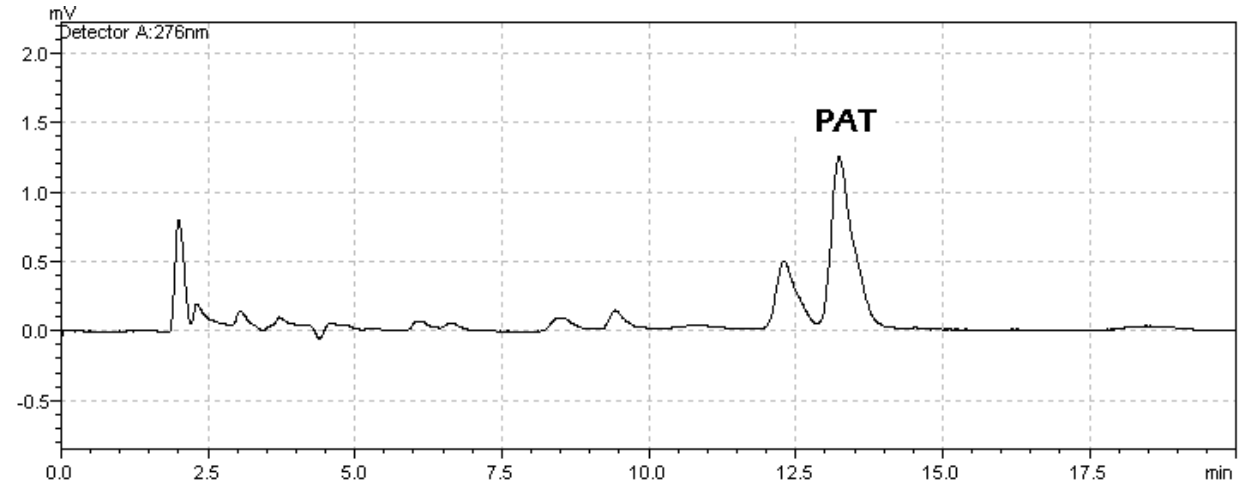
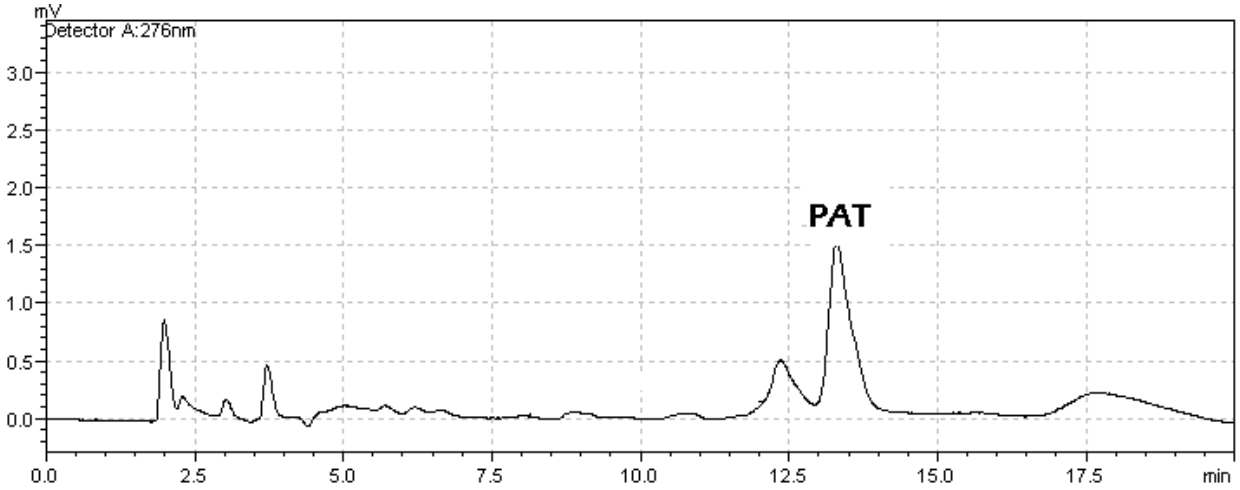


Figure 3: Ultraviolet chromatogram of PAT in:
a) apple mousse artificially contaminated at 50 µg/kg before heat treatment
b) apple mousse artificially contaminated at 50 µg/kg after heat treatment

a)



b)

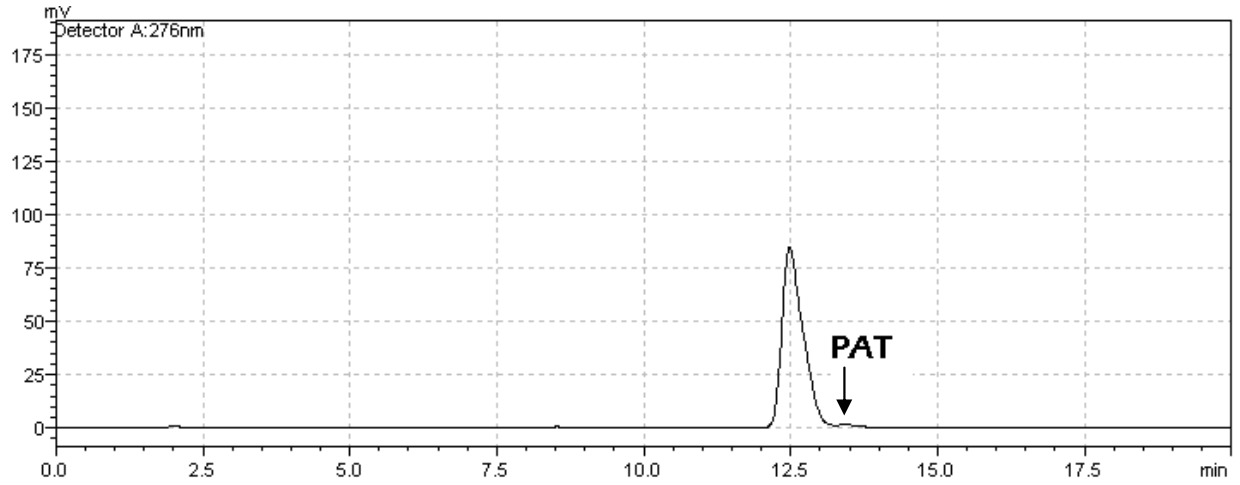


Figure 4: Ultraviolet chromatogram of PAT in:
c) apple juice artificially contaminated at 50 µg/kg before heat treatment
d) apple juice artificially contaminated at 50 µg/kg after heat treatment

Figure 5. Thermal degradation of PAT in apple puree and juice artificially contaminated at 50 µg/l.

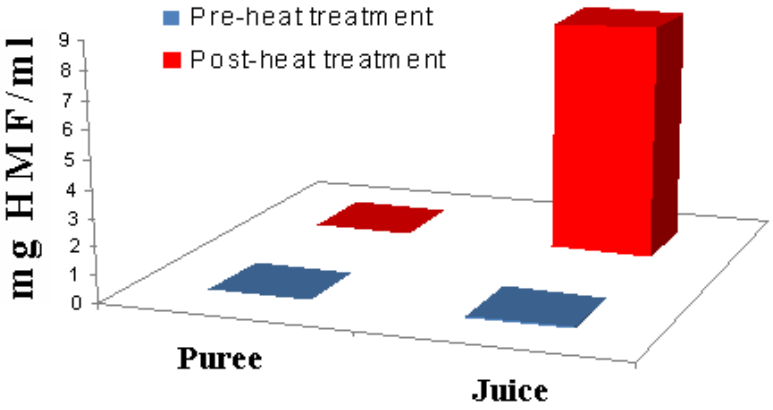


Figure 6. Production of HMF due to thermal treatment in apple juice and puree.

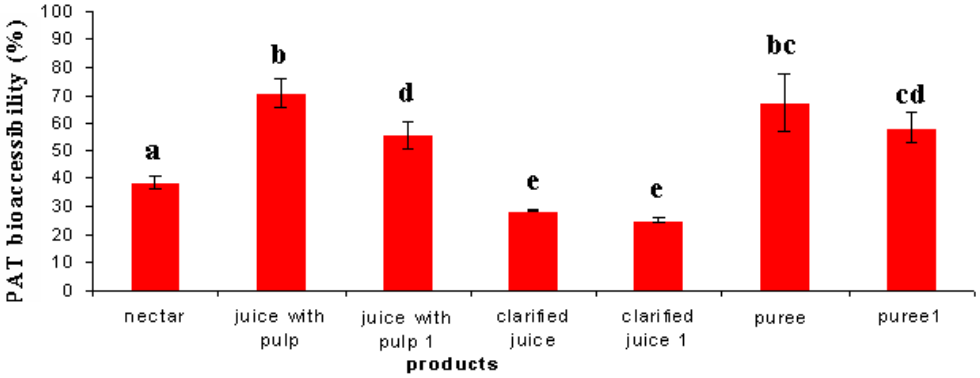


Figure 7. PAT's bioaccessibility in vitro in 7 selected apple products artificially spiked:
a) nectar, juices with pulp, clarified juices at 50 µg/kg
b) purees at 25 µg/kg.
Mean values with the same letter are not significantly different (p=0.05) (Duncan's test)

References

Acar, J., Gökmen, V., & Taydas, E. E. (1998). The effects of processing technology on the patulin content of juice during commercial apple juice concentrate production. European Food Research and Technology, 207: 328–331.

Ames J.M., (1998). Applications of the Maillard reaction in the food industry. Food Chemistry 62, (4): 431-439

Anderson de Souza Sant'Ana, Amauri Rosenthal, Pilar Rodriguez de Massaguer , (2008). The fate of patulin in apple juice processins: a review. Food Research International 41: 441-453.

Arranz, I., Derbyshire, M., Kroeger, K., Mischke, C., Stroka, J., Anklam, E., (2005). Liquid chromatographic method for quantitation of patulin at 10 ng/mL in apple based products intended for infants: inter-laboratory study. J. AOAC Int. 88: 518–525.

Avantaggiato, G., Havenaar, R., & Visconti, A. (2003). Assessing the zearalenone-binding activity of adsorbent materials during passage through a dynamic in vitro gastrointestinal model. Food Chem. Toxicol. 41: 1283-1290.

Avantaggiato, G., Havenaar, R., & Visconti, A. (2004). Evaluation of the intestinal absorption of deoxynivalenol and nivalenol by an in vitro gastrointestinal model, and the binding efficacy of activated carbon and other adsorbent materials. Food Chem. Toxicol. 42: 817-824.

Baert, K., B. De Meulenaer, A. Kamala, C. Kasase and F. Devlieghere, (2006). Occurrence of patulin in organic, conventional and handcrafted apple juices marketed in Belgium. J. Food prot., 6: 1371-1378.

Barbara A., Dennison M.D. (1996). Fruit Juice Consumption by Infant and Children : a review. Journal of the American College of Nutrition 15 (5): 4S-11S

Barreira M.J., Alvito P.C., Almeida C. M.M., (2010). Occurrence of patulin in apple-based-foods in Portugal. Food Chemistry, 121 (3): 653-665

Benito, P., Miller, D., (1998). Iron absorption and bioavailability: an updated review. Nutr. Res. 18: 581-603.

Bissessur, J., Permaul, K., & Odhav, B. (2001). Reduction of patulin during apple juice clarification. Journal of Food Protection, 64:1216–1219

Brandon E.F.S., Oomen A.G., Rompelberg C.J.M., Versantvoort C.H.H., van Engelen J.C.M., Sips A.J.A.M. (2006). Consumer product in vitro digestion model: Bioaccessibility of contaminants and its application in risk assessment. Regulatory Toxicology and Pharmacology 44,:161–171

Burdurlu H.S., Karadeniz F. (2003). Effect of storage on nonenzymatic browning of apple juice concentrates. Food Chemistry 80:91–97

Burkhardt, B., Pfeiffer, E., & Metzler, M. (2009). Absorption and metabolism of the mycotoxins alternariol and alternariol-9-methyl ether in Caco-2 cells in vitro. Mycotoxin Res. 25:149-157.

Dall'Asta C., Mangia M., Berthiller F., Molinelli A., Sulyok M., Schuhmacher R., Krska R., Galaverna G., Dossena A., and Marchelli R., (2009). Difficulties in fumonisin determination: the issue of hidden fumonisins. Analytical and Bioanalytical Chemistry. 395 (5): 1335-1345

Dennison B.A., Rockwell H.L., Baker S.L. (1996). Excess fruit juice consumption by preschool-aged children is associated with short stature and obesity. Pediatrics, 99 (1): 15-22

EFSA (2005a). Opinion of the scientific panel on food additives, flavourings, processing aids and materials in contact with food (AFC) on a request from the commission related to flavouring group evaluation 13: furfuryl and furan derivatives with and without additional side-chain substituents and heteroatoms from chemical group 14. EFSA Journal, 215: 1-73.

Fernández-García, E., Carvajal-Lérida, I., Pérez-Gálvez, A., (2009). *In vitro* bioaccessibility assessment as a prediction tool of nutritional efficiency. Nutr Res. 29: 751-760.

Funes G.J., Resnik S.L., (2009). Determination of patulin in solid and semisolid apple and pear products marketed in Argentina. Food Control 20 (3): 277-280

Gil-Izquierdo, A., Zafrilla, P., Tomás-Barberá, F.A., (2002). An in vitro method to simulate phenolic compound release from the food matrix in the gastrointestinal tract. Eur. Food Res. Technol. 214:155-159.

Jian-Ping Yuan and Feng Chen (1998) Separation and Identification of Furanic Compounds in Fruit Juices and Drinks by High-Performance Liquid Chromatography Photodiode Array Detection J. Agric. Food Chem. 1998, 46: 1286−129

Jovaní, M., Viadel, B., Laparra, M., Barberá, R., Farré, R., (2004). Improvement of Analytical Conditions of Mineral Caco-2 Cell uptake Assays. Food Sci Tech Int. 10:197-201.

Kadakal, C., Sebahattin, N., & Poyrazoglu, E. S. (2002). Effect of commercial processing stages of apple juice on patulin, fumaric acid and hydroxymetilfurfural (HMF) levels. Journal of Food Quality, 25, 359–368.

Kadakal, C., Nas, S. (2003). Effect of heat treatment and evaporation on patulin and some other properties of apple juice. Journal of the Science of Food and Agriculture, 83: 987–990.

Kim H-J. and Richardson M., (1992). Determination of 5-hydroxymethylfurfural by ion-exclusion chromatography with UV detection Journal of Chromatography . 593 :153-156

Kryger, R. A. (2001). Volatility of patulin in apple juice. Journal of Agricultural and Food Chemistry, 49: 4141–4143.

Kroh L.W., (1994) Caramelisation in food and beverages. Food Chemistry, 51 (4), 373-379

Kus S., Gogus F., Eren S., (2005). Hydroxymethyl Furfural Content of Concentrated Food Products. International Journal of Food Properties.8 (2): 367 - 375

Martins, M., Gimeno, A., Martins, H., & Bernardo, F. (2002). Co-occurrence of patulin and citrinin in Portuguese apples with rotten spots. Food Additives and Contaminants, 19(6): 568–574.

Piemontese, L., M. Solfrizzo and A. Visconti, (2005). Occurrence of patulin in conventional and organic fruit products in Italy and subsequent exposure assessment. Food Additives and Contaminants, 5: 437-442

Reports on tasks for scientific cooperation Assessment of dietary intake of Patulin by the population of EU Member States Task 3.2.8, March 2002

Ritieni, A., (2003). Patulin in Italian commercial apple products. J. Agric. Chem., 51: 6086-6090

Saravacos G.D. (1970). Effect of temperature on viscosity of fruit juices and purees. Food Science. 35 (2): 122–125

Simla W., Porasuphatana S., Songsermsakul P., (2009). Bioaccessibility of Aflatoxin B_1 in Grinded Corn and Peanut by in vitro Digestion Model. Journal of Pharmaceutical Sciences. 5 (2):108-113

Criteria for the segmentation of the market in the European Union, Economics of Agriculture, Belgrade, LVII,1, 1-153, 79-90

Taniwaki, M. H., Bleinroth, E. W., & De Martin, Z. J. (1989). Bolores produtores de patulina em maçã e suco industrializado. Coletânea do Instituto de Tecnologia de Alimentos, 19: 42–49.

Versantvoort C.H.M., Oomen A.G., Van de Kamp E., Rompelberg C.J.M.,. Sips A. J.A.M (2005). Applicability of an in vitro digestion model in assessing the bioaccessibility of mycotoxins from food. Food and Chemical Toxicology 43: 31–40

Versari A., Parpinello G. P., Mattioli A. U. (2007) Survey of patulin contamination in italian apple juices from organic and conventional agricolture. Journal of Food Technology 5, (2): 143-146

Videmann, B., Mazallon, M., Tep, J., & Lecoeur, S. (2008). Metabolism and transfer of the mycotoxin zearalenone in human intestinal Caco-2 cells. Food Chem. Toxicol. 46: 3279-3286.

Vorlovà L., Borkovcovà I., Kalàbovà K., Vladimìr V., (2006). Hydroxymethylfurfural contents in foodstuffs determined by HPLC method Journal of Food and Nutrition Research. 45 (1): 34-38

Wagner, B. and Beil-Seidler, S. (2006). Furfural determining agents comprises an 4-amino-phenazone derivative and a barbituric acid and/or thiobarbituric acid derivative in an acidic medium. WO2006042600-A1: MERCK Patent GMBH (MERE) Wagner B (Wagn-Individual) Beil-Seidler S (Beil-Individual).

Wienk, K.J.H., Marx, J.J.M., Beynen, A.C., (1999). The concept of iron bioavailability and its assessment. Eur. J. Nutr. 38: 51–75.

Witchada Simla, Supatra Porasuphatana, Piyada Songsermsakul. Bioaccessibility of Aflatoxin B_1 in Grinded Corn and Peanut by *in vitro* Digestion Model International Journal of Plant Sciences. Vol. 5, No.2, 2009

Zappalà M., Fallico B., Arena E., Verzera A. (2005). Methods for the determination of HMF in honey: a comparison. Food Control 16: 273–277

Chapter 5: BIOACCESSIBILITY OF DEOXYNIVALENOL AND ITS NATURAL CO-OCCURRENCE WITH OCHRATOXIN A AND AFLATOXIN B_1 IN ITALIAN COMMERCIAL PASTA

5.1 Introduction

The Mediterranean diet is an independent protective factor for several affections in children, and has been consistently shown to be associated with favourable health outcomes and a better quality of life (Castro-Rodriguez et al, 2008; Sofi, 2009). In Italy, this diet is characterized by higher intake of complex carbohydrates, mostly due to pasta consumption (Trichopoulou et al, 2000). Dry pasta is made from semolina milled from durum wheat, which could be contaminated simultaneously by DON, OTA and AFB_1.
The main aims of this study are to carry out surveillance for DON, OTA and AFB_1 on pasta characterized by marketing for children and to ascertain the bioaccessibility of DON in a model system that simulates the digestion steps in children. This mycotoxin was chosen for bioaccessibility evaluation because its level in several pasta samples exceeded the legislative limits fixed for babies and infants.

5.2. Materials and methods

5.2.1 Materials
Potassium chloride (KCl), potassium thiocyanate (KSCN), monosodium phosphate (NaH_2PO_4), sodium sulphate ($NaSO_4$), sodium chloride (NaCl), sodium bicarbonate ($NaHCO_3$), urea, α-amilase, hydrochloric acid (HCl), pepsin, pancreatin, bile salts were obtained from Sigma-Aldrich (Madrid, Spain). Acetonitrile, methanol and ethyl acetate were purchased from Fisher Scientific (Madrid, Spain). Deionized water (<18MΩ cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Chromatographic solvents and water were degassed for 20 min using a Branson 5200 (Branson Ultrasonic Corp., CT, USA) ultrasonic bath. DON, OTA, AFB_1 standards were purchased from Sigma-Aldrich (Milan, Italy) and stored at 4°C in the dark. They were dissolved in methanol at 1 mg/mL concentration (stock solution). Working standard solution of DON was prepared by diluting standard stock solution with a suitable solvent mixture (CH_3OH:H_2O, 70:30, v/v), while working standard solutions of OTA and AFB_1 were obtained by diluting stock solutions with methanol.

5.2.2 Sampling
Dry pasta samples of different brands (n=27), characterized by little size, marketing and packaging referring to young children, were collected from several supermarkets located in the city of Naples (Italy). The selected samples included pastas of several forms such as penne, starlets, cut noodles, short pasta mixed, macaroni, bow pasta, spiral shaped pasta. Samples were milled with a knife mill (Grindomix GM 200, Retsch, Haan, Germany) and then divided with a subsample divider (PT 100 Retsch, Haan, Germany). For every sample, a 200 g-subsample was collected in a plastic bag and kept at -20°C until mycotoxin analysis.

5.2.3 DON extraction
The extraction of DON was carried out according to the method of Santini et al. (2009). Briefly, ten grams of finely grounded pasta were mixed with 50 ml acetonitrile:water (84:16; v/v), and extracted by agitation on a mechanical shaker (Intercontinental equipment, Hidalgo, TX) during 1h. The mixture was filtered using a paper filter (LABOR, Microglass Heim SRC, Naples, IT) and 5 mL of the filtered extract was completely evaporated using a centrifugal evaporator (Speed Vacuum Thermo Electron Corporation Milford, MA, USA). The extract was then dissolved in 1 mL of methanol and finally filtered through a 0.22 µm cellulose filter (Chemtek Analytica, Bologna, IT) before liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Each sample was analyzed in triplicate.

5.2.4 OTA extraction
OTA extraction was performed according to Winnie et al., (2009) with slight modifications. 25 g of finely grounded pasta together with 100 mL of acetonitrile:water (60:40, v/v) mixture were homogenized utilizing an Ultra-Turrax (T 25 basic, IKA-Werke, Germany) at 2,000 rpm during 2 min. The samples were centrifuged (Thermo-scientific Centrifuge IEC CL30R, UK) at 4,000 rpm during 5 min, 10 mL of the supernatant were collected and diluted with 40 mL of PBS solution. The sample was then purified by an immunoaffinity column (ICs) (Ochratest, VICAM, Watertown, MA, USA). In details, the ICs were first washed with 10 mL of water, the sample extract was charged on the column and eluted at 1-2 drops/second, then the column was washed with 20 mL of water and eluted with 4 mL of methanol.

The methanolic extract was dried with a centrifugal evaporator, resuspended in 1 mL of methanol, filtered with a 0.22 µm filter (Phenomenex, Torrance, CA) and injected in the LC apparatus. Each sample was analyzed in triplicate.

5.2.5 AFB_1 extraction

For the extraction of AFB_1 the method reported by Herrera et al. (2009) was applied with slight modifications. In details, 25 g of grounded sample were mixed with 2.5 g of NaCl and 50 mL of a mixture methanol:water (80:20, v/v). The sample was homogenized using an Ultra-Turrax (T 25 basic, IKA-Werke, Germany) during 2 min at 2,000 rpm and centrifuged (Thermo-scientific Centrifuge IEC CL30R, UK) during 5 min at 4,000 rpm. 10 mL of the supernatant was collected and diluted with 40 mL of PBS solution before the purification step.

Sample was purified by ICs Aflatest (VICAM, Watertown, MA, USA). ICs were washed with 10 mL of water, the sample extract was charged on the column and eluted at 1-2 drops/second. After washing with 20 mL of water, the column was eluted with 4 mL of methanol. The methanolic extract was dried with a centrifugal evaporator, resuspended in 1 mL of methanol, filtered with a 0.22 µm filter (Phenomenex, Torrance, CA) and injected in the LC apparatus. For every sample, a triplicate analysis was performed.

5.2.6 LC-MS/MS analysis of DON

LC analysis for DON was carried out using a system consisting of two micropumps (Series 200, PerkinElmer, Waltham, MA, USA). A Gemini column (C_{18} 5 µm particle size, 150 x 4.60 mm, pore size 110 Å, Phenomenex, USA) heated at 50°C was used; the flow rate was set to 0.8 mL/min, and the injection volume was 20 µL. Mobile phase A was a H_2O:CH_3OH mixture (90:10, v/v) containing 5 mmol/L ammonium acetate, whereas mobile phase B consisted of CH_3OH:H_2O (90:10, v/v) mixture also containing 5 mmol/L ammonium acetate. The following binary gradient was applied: initial condition 10% B; 0-7 min, 35% B; 7-9 min, 80% B; 9-13 min constant at 80% B; 13-15 min 100% B, finally returning to the initial conditions in 3 min.

MS/MS data were acquired using an API 3000 triple-quadrupole mass spectrometer (Applied Biosystems, Ontario, Canada). All analyses were performed with an atmospheric pressure chemical ionization (APCI) interface with the following settings: probe temperature 450°C, corona current (NC) ±2 µA (depending on use in positive or negative mode). The declustering potential (DP) and collision energy (CE) were optimized for each compound by direct infusion of standard solution (10 µg/mL) into the mass spectrometer at a flow rate of 8µL/min, using a Model 11 syringe pump (Harvard Apparatus, Holliston, MA, USA) and a solution of 5 mmol/l ammonium acetate in CH_3OH:H_2O (90:10, v/v) mixture as liquid carrier at a flow rate of 200 µL/min. The acquisition was carried out by selected reaction monitoring (SRM) both in the negative and positive ion mode (Blesa et al., 2010).

5.2.7 LC-FLD analysis of OTA and AFB_1

OTA and AFB_1 analysis were performed using an LC (Shimadzu-Japan) equipped with an autosampler SIL-20A, two pumps LC-20AD and a fluorimetric detection RF-20AXL (OTA: λ_{ex}: 360 nm; λ_{em}: 460 nm; AFB_1: λ_{ex}: 360 nm, λ_{em}: 440 nm). For AFB_1 analysis, a Gemini column was used, whereas for OTA analysis, an Onyx Monolithic column (3 µm particle size C_{18}, 100 x 3.0 mm) (Phenomenex, USA) was employed. Mobile phase for OTA analysis was used in isocratic conditions: 65% A (1% CH_3COOH in H_2O), 35% B (1% CH_3COOH in CH_3CN) at the flow rate of 1 mL/min (Winnie et al, 2009). In the case of AFB_1, the mobile phase was used in isocratic conditions: 95% A (H_2O), 5% B (CH_3CN:CH_3OH, 50:50, v/v) and the flow rate setted was of 1 mL/min (Giray et al, 2007).

5.2.8 *In vitro* digestion model

The procedure used in this study was adapted from the method outlined by Gil-Izquierdo, et al. (2002), slightly modified. The method consists of an initial saliva/pepsin/HCl digestion for 2 h at 37ºC, to simulate the mouth and the gastric conditions, followed by a digestion with pancreatic juice for 2 h at 37 ºC to simulate duodenal digestion.

For the saliva/pepsin/HCl digestion, the sample (3 g) of cooked pasta (10 g of dry pasta were cooked in 500 mL of hot water during 10 min) were mixed with 6 mL of artificial saliva composed by: KCl (89.6 g/L), KSCN (20 g/L), NaH_2PO_4 (88.8 g/L), $NaSO_4$ (57 g/L), NaCl (175.3 g/L), $NaHCO_3$ (84.7 g/L), urea (25 g/L) and 290 mg of α-amilase. The pH of the solution was adjusted at 6.8 with HCl 0.1 N. The mixture was introduced in a plastic bag, containing 40 mL of water and was homogenised in a Stomacher IUL Instruments (Barcelona, Spain) during 3 minutes.

Immediately, 0.5 g of pepsin (14,800 U) dissolved in HCl 0.1 N was added, the pH was adjusted at a value of 2 with HCl 6 N, and then incubated at 37 ºC in an orbital shaker (250 rpm) (Infors AG CH-4103,

Bottmingen, Switzerland) for 2 h. After the gastric digestion, the pancreatic digestion was simulated as follows: The pH was increased to 6.5 with $NaHCO_3$ 0.5 N and then 5 mL of a mixture pancreatin (8 mg/mL) and bile salts (50 mg/mL) (1:1; v/v), dissolved in 20 mL of water, were added and incubated at 37ºC in an orbital shaker (250 rpm) for 2 h.
After each step of digestion, 30 mL of the obtained extract were centrifuged at 4.000 rpm and 4ºC during 1h. To determine the DON concentration, 10 mL of the supernantant of the gastric and the intestinal phases (saliva/pepsin/HCl and pancreatin–bile digestions) were analysed by LC-MS/MS according to the method described in par. 5.2.6.
The simulated digestion gastrointestinal model, applied in order to reproduce the physiological condition of the child, was basically the same of the one employed for adults with slight modifications. In particular, the pH of the stomach was fixed at 3.0, the quantity of pepsin used for the gastric digestion was reduced to 0.02 g, and in the intestinal digestion the amount of pancreatin and bile salts were reduced to 0.0005 and 0.03 g, respectively (Jovaní et al., 2004). The schematic representation of the *in vitro* digestion model is represented in the Figure 1.

5.3 Results and discussion

5.3.1 Analytical performance

During the optimisation of the extraction procedures, a representative sample of pasta was used. For every different extraction, blank and fortified pasta (30, 100 and 250 µg/kg of DON and 0.1, 10, and 50 µg/kg of OTA and AFB_1) were used. Biological fluids obtained from digestion of uncontaminated pasta were also spiked with DON at the same concentrations above reported to develop recovery tests.
According to Santini et al. (2009) and Gonzalez-Osnaya et al. (2010) the mixture acetronitrile:water (84:16; v/v) was suitable and gave the highest recoveries. For the recovery analysis, five replicates of the samples (10 g for pasta and 10 mL for biological fluid) spiked with three investigated mycotoxins were prepared with blank pasta, and processed as previously described. Calibration curves were obtained by injecting in duplicate standard solutions at different concentrations for each mycotoxin; the areas obtained from integration of the peaks were correlated linearly with the concentrations. For the determination of DON, standard solutions of 1, 10, 20, 50, 100, 250, and 500 ng/mL were prepared by diluting the stock solution (1,000 µg/mL) with a CH_3OH:H_2O mixture (70:30, v/v). For the determination of OTA, standard solutions of 0.05, 0.1, 0.5, 1, 10, and 50 ng/mL were prepared with CH_3OH, whereas for the determination of AFB_1, standard solutions of 0.1, 0.5, 1, 5, 10, 50 ng/mL were prepared with CH_3OH.
Mycotoxin identification was performed by comparing the retention times of the peaks present in the samples with pure standards. Quantification of mycotoxins was carried out by comparing peak areas of investigated samples with the calibration curve of standards. The mean DON recovery, was independent of spiking levels assayed, was of 89.5 % with a relative standard deviation (RSD) of 3.5 %. For AFB_1 and OTA, the mean recoveries obtained were 76.01% (RSD = 4.24 %) and 77.50% (RSD = 9.19 %), respectively (Table 1).
The detection limits (LODs) obtained were 1.0 µg/kg for DON, 0.05 µg/kg for OTA and 0.1 µg/kg for AFB_1, whereas the limits of quantification (LOQs) were 2.0 µg/kg for DON, 0.15 µg/kg for OTA (Figure 4) and 0.3 µg/kg for AFB_1. Fluorescence chromatograms of AFB_1 and OTA can be seen in Figure 2 and 3, respectively. In Figure 4 is reported the total ion chromatogram (TIC) of DON.

5.3.2 Occurrence of DON, OTA and AFB_1 in the analyzed samples

The occurrence of the three mycotoxins analyzed in this study is reported in Tables 2 and 3. In particular, DON was detected in 81.4 % of the 27 samples finding the highest level of contamination (450.0 µg/kg) in sample 22, whereas the lowest was evidenced in sample 13 where the DON concentration was 35.1 µg/kg.
Seven samples (25%) exceeded the maximum permitted level of DON (200 µg/kg) by the EU for processed cereal-based foods destined to babies and infants. Eleven samples (40.7 %) resulted contaminated with values below 100 µg/kg, while four of the analyzed samples (14.81%) evidenced a contamination range variable from 100 to 200 µg/kg. Five of the samples (18.52 %) were under instrumental LOD for DON. The mean DON content was 162.14 µg/kg. All the samples show DON levels below the maximum permitted in pasta established by the EU for adults (750 µg/kg).
Schollenberger et al (2005) calculated the intake for trichothecene toxins for adults, children and babies, in an area of southwest Germany, in 1998 and 1999. The mean DON content in pasta reported was 149 µg/kg in 1998 and 395 µg/kg in 1999. No exceeding of the tolerable daily intake (TDI) of DON, NIV and the sum of HT-2 and T-2, as stated by the EU, was found for adults (70 kg body weight (BW)) and babies (10 kg BW), independently of the year and level of consumption. For children (20 kg BW) the intake of DON exceeded the TDI in both years.

Cano-Sancho et al. (2010) determined HT-2 toxin (HT2) and T-2 toxin (T2) from type A and DON from type B in 479 cereal-based food products (breakfast cereals, snacks and pasta) from Spain. Their results showed that DON was the main trichothecene present with percentages of positive samples ranging from 1.4 to 100.0%. However, despite the high incidence of DON, only five samples were above EU limits.
González-Osnaya et al (2010) studied the occurrence of DON and T-2 toxin in pasta commercialised in Spain. An incidence of these mycotoxins, varying from 9.3 to 62.7% was reported by these authors. The mean value of DON was 137.1 µg/kg. Regarding to the OTA analysis, twenty six samples (96.3 %) resulted contaminated by OTA over its LOD (0.05 µg/kg). The highest level of contamination reported was 0.52 µg/kg for the sample 5 while the average content of OTA was 0.3 µg/kg. Only one of the samples exceeded (3.7 %) the maximum level permitted (0.50 µg/kg) in baby food established by the EU for these products. Fourteen samples (51.85%) contained OTA levels below 0.2 µg/kg, whereas eleven samples (40.74%) evidenced a contamination range variable from 0.2 to 0.5 µg/kg.
Winnie et al. (2009) determined the occurrence of OTA in 274 samples of dry pasta of Canadian origin between 2004 and 2006. Incidence of contamination above 0.5 µg of OTA per kg was 21, 18, and 66% in the years 2004, 2005, and 2006 respectively. Mean levels of OTA in these 3 years were, respectively, 0.3, 0.2, and 0.7 µg/kg, and maximum levels were, respectively, 1.8, 1.4, and 3.3 µg/kg. A mean OTA incidence of 16.7 % in infant cereals of Turkish origin was reported by Kabak, 2009a, with a maximum level of 0.3 µg/kg. All pasta samples analyzed resulted under the instrumental LOD for AFB_1 (0.1µg/Kg). Zinedine et al. (2007) determined the occurrence of aflatoxins in cereals from Rabat (Morocco), finding contamination levels between 0.03 and 0.15 µg of AFB_1/kg in wheat. Moreover, Zinedine et al., (2010) studied the occurrence of OTA in 68 samples of breakfast and infants cereals from Morocco and showed that all analyzed infant cereals were OTA free; however, four samples of breakfast cereals destined to general population were contaminated with OTA at levels ranged from 5.1 to 224.6 µg/kg.
Villa and Markaki, (2009) evaluated the occurrence and the risk assessment of AFB_1 and OTA in breakfast cereals from Athens market. The presence of AFB_1 in 56.3% of the samples (mean 1.42 µg/kg AFB_1) was detected. Seven samples were found contaminated at levels higher than the EU limit, while OTA was detected in 60% of the samples (mean 0.18 µg/kg). The results of our study were comparable with the data reported by Herrera et al (2009), who compared DON, OTA and AFB_1 levels in conventional and organic durum semolina. The AFB_1 was no detected in any of durum semolina samples. The percentage of durum semolina samples positive for the presence of OTA was 8.3 % in conventional and 20 % in organic samples. The occurrence of samples that resulted positive for DON was of 16.7 % in conventional products and 20% in organic products, whereas DON mean levels resulted lowest in conventional samples (77 µg/Kg) respect to the organic samples (89 µg/Kg).
In Algerian wheat and derived products, Riba et al. (2010) investigated the presence of AFs. AFB_1 was detected in 56.6 % of the wheat samples and derived products (flour, semolina and bran), with contamination levels ranging from 0.1 to 37.4 µg/Kg. In year 2009, Soubra et al. assessed the occurrence of aflatoxins, OTA and DON in some foodstuffs available in the Lebanese market and evaluated the potential risk to the health of children and teenagers in Beirut from dietary exposure to these mycotoxins. The calculated intake for aflatoxins exceeded its respective PTDI in all groups by a factor ranging from 3 to 7. The intakes of OTA and DON were found to be below the threshold of toxicological concern established for these mycotoxins by international expert groups, although the intake of DON in children at the highest percentile (P95) was close to its PTDI.
The Italian population consumes 76.7 g per day of pasta (28 kg/persona/year) (ISMEA-AC NIELSEN, 2003), which would correspond to 12.4 µg/day of DON, taking into account the mean occurrence evaluated in this study. Children consume about 45 g per day of pasta which correspond to 7.3 µg DON/day.
The EFSA estimates 1.4 µg/kg b.w. per day the DON intake for European consumers, in particular for an adult (70 kg body weight) the Tolerable Daily Intake of DON (TDI) is 70 µg per day and the pasta intake corresponding to 17.7 % of TDI in adult and 62.1 % for a children between 3 and 5 years of age, with 20 kg body weight (Cole et al, 2000).
The value for DON intake assessed in this study is less than the TDI values for adults, but the subgroup of children can be considered a critical group because the cereal intake can be markedly higher than in adults and in many cases children can have a diet not so varied as the adults.

5.3.3 DON contents in the gastric and duodenal fluids (Bioaccessibility)

Mean recoveries were performed on the fortified intestinal fluid (free from the contamination of DON) (n=5) at levels of DON (0.1-500 µg/g). The DON recoveries obtained in this study varied from 80.2 ± 2.3 % to the 92.5 ± 3.1 %. The values obtained for recoveries and relative standard deviations of the method used are in agreement with those of the Commission Regulation (EC) No 401/2006 for methods of analysis of

mycotoxins in foodstuffs (European Commission, 2006). Intra-day (n=5) and inter-day (5 different days) variation values ranged between 1.5 and 2.1 respectively for DON. These values are below 15% which is the maximum variation for certification exercises for several mycotoxins. In the risk assessment related to the mycotoxins exposure, it needs to be remembered that all the bioactive compounds must be bioaccessible to act on the different organs or tissues of the human body. The bioaccessibility is defined as the fraction of a compound present in a food that pass unmodified the complex of the enzymatic reactions of the gastrointestinal digestion and that is potentially accessible to the absorption by the cells of the intestinal epithelium (Benito and Miller, 1998).

To evaluate the real exposure to DON in adults and children, its bioaccessibility was evaluated with a simulated gastrointestinal digestion optimized for both groups of population. Six pasta samples were selected among 27 analysed samples to be used in the study. Firstly, to verify if the pasta cooking, necessary for the bioaccessibility study, had any effect on the DON concentration present in the samples, each pasta sample was analyzed for the DON content using the method of Santini et al. (2009) before the application of the simulated gastrointestinal digestion model. As it can be shown in the Table 4, the DON content in the analyzed samples, after the boiling, ranged from 35.20 µg/kg of the sample 12I to195.52 µg/kg of the sample 2I evidencing a mean percentage of DON equal to 49.40% compared with the content in raw pasta. Visconti et al (2004) investigated the reduction of DON during durum wheat processing and spaghetti cooking. Compared to the uncleaned wheat, the levels of DON were 33% in spaghetti and 20% in cooked spaghetti.
In the Table 4 there are reported the results regarding the gastric and duodenal bioaccessibility expressed as DON concentration and as % respect to the initial DON concentration in pasta. The sample 12 has been digested with adult model system, whereas the rest have been treated with the alternative digestive process for children. The DON percentages in the gastric fluid ranged from 2.12 to 41.5% with a mean value for this gastrointestinal model of 23.1 %.
High values of gastric bioaccessibility were also evidenced in the sample 12I (child digestion) and 5I (child digestion) with 38.4 and 20.4 % respectively. DON was only detected after adult gastric digestion in the sample 12.
The mean DON bioaccessibility value after duodenal process was of 12.1%, with values ranging from 1.1 to 24.1 %. The highest DON duodenal bioaccessibility value was reported by the sample 12 (adult digestion) where the mean data was of 24.1±1.7%, whereas the lowest was detected in the sample 2 (child digestion), with 1.1±0.01%.
Considering only the samples treated with the child digestion the mean DON duodenal bioaccessibility data was of 9.7%. This data is very interesting by the toxicological point of view, because proved that a 9.7% of the DON present in pasta samples, did not interact with the enzymes of the digestion system and arrived unmodified to the intestinal lumen, available for the absorption by the cells of the intestinal epithelium. Therefore, the DON could interact with the intestinal epithelium cells at concentrations of 3.4-18.9 µg/kg and these level are cytotoxic on several cell lines as demonstrated by Dessing et al. (2001) and Daeniche et al. (2011). Moreover, considering the small dimension of the child intestinal epithelium, the DON concentrations present in the intestinal fluid could probably produce more damage to the intestinal enterocytes respect to that in an adult.
In literature, there are few studies on the mycotoxins bioaccessibility, which are very interesting because try to correlate the bioactive compounds presence in food with the real exposure risks.
In particular, Avantaggiato et al. (2003), studied the intestinal absorption of ZEA by using a laboratory model that mimics the metabolic processes of the gastrointestinal tract of healthy pigs. Approximately 32% of ZEA intake (247 mg) was released from the food matrix to the bioaccessible fraction during 6 h of digestion and was rapidly absorbed at intestinal level.
Carolien et al. (2005) described the applicability of an *in vitro* digestion model allowing the measurement of the bioaccessibility of ingested mycotoxins from food as an indicator of oral bioavailability. Bioaccessibility of AFB_1 from peanut slurry and OTA from buckwheat was 94% and 100%, respectively.
Recently, Kabak et al. (2009) determined the bioaccessibility of the AFB_1 (90 %), and OTA (30 %) in pistachio nuts, buckwheat, and infant foods using an *in vitro* model under fed condition.
It has been also demonstrated that mycotoxin bioaccessibility performances depends on several factors, such as food product, contamination level, compound and type of contamination (spiked versus naturally contaminated) (Kabak et al, 2009). Our *in vitro* study has demonstrated differences in levels of DON bioaccessibility during the child digestion processes, attributable to different typologies of pasta and initial contamination level.
The combination of surveillance data and bioaccessibility provided in this study suggested that DON exposure risk should be reconsidered by JEFCA Commission and that more studies must be done in this topic.

Considering that the legal limit for baby food protects until 2 years old, while in Italy and most of Mediterranean countries pasta consumption starts at age of 2-3, a new more restrictive limit for child than for adult (750 µg/Kg) should be auspicable by the European Commission. Alternatively, the general limit could be decreased to reduce the exposure of the young population, and consequently the adult population.

Table 1. Mean recoveries, LOD and LOQ for the investigated mycotoxins in dry pasta

Mycotoxin	Mean Recovery ± RSD (%)	LOD (µg/Kg)	LOQ (µg/Kg)
OTA	77.50 ± 9.19	0.05	0.15
AFB_1	76.01 ± 4.24	0.10	0.30
DON	89.50 ± 3.54	1.00	2.00

Table 2. Occurrence of DON and OTA in samples of analyzed pasta

Pastas	Level DON*(µg/Kg)	Level OTA(µg/Kg)
Sample 1	350.00 ± 2.00	0.12±0.04
Sample 2	387.00 ± 1.55	0.11±0.03
Sample 3	371.00 ± 2.54	0.20±0.03
Sample 4	41.30 ± 1.70	0.25±0.02
Sample 5	134.00 ± 3.75	0.52±0.01
Sample 6	270.00 ± 4.20	0.09±0.01
Sample 7	79.90 ± 1.50	0.32±0.04
Sample 8	121.00 ± 2.74	0.12±0.09
Sample 9	61.20 ± 3.84	0.17±0.04
Sample 10	62.00 ± 1.57	0.32±0.05
Sample 11	51.70 ± 4.21	0.24±0.01
Sample 12	73.80 ± 2.80	0.35±0.02
Sample 13	35.10 ± 1.64	0.14±0.08
Sample 14	58.50 ± 1.52	0.14±0.06
Sample 15	61.80 ± 2,13	0.21±0.01
Sample 16	299.00 ± 4.89	0.25±0.09
Sample 17	39.80 ± 2.92	0.10±0.01
Sample 18	106.00 ± 4.93	0.18±0.04
Sample 19	176.00 ± 2.12	0.23±0.09
Sample 20	98.00 ± 1.97	0.37±0.07
Sample 21	240.00 ± 2.29	0.14±0.02
Sample 22	450.00 ± 3.20	< 0.05
Sample 23	< 1	0.15±0.02
Sample 24	< 1	0.13±0.05
Sample 25	< 1	0.12±0.01
Sample 26	< 1	0.21±0.06
Sample 27	< 1	0.18±0.03

*Average values ± standard deviation from the means of three replications are presented.

Table 3. Incidence and levels of DON and OTA in pasta samples

Mycotoxin	Positive sample /frequency (%)	Mean (µg/Kg)	Max. level (µg/Kg)	<LOD	Frequency distribution n (%)		
DON	22 (81.48%)	162.14	450.00	5 (18.52%)	**<100 µg/Kg**	**100-200 µg/Kg**	**> 200 µg/Kg**
					11 (40.74%)	4 (14.81%)	7 (25.93%)
OTA	26 (96.30%)	0.21	0.52	1 (3.70%)	**<0.2 µg/Kg**	**0.2-0.5 µg/Kg**	**>0.5 µg/Kg**
					14 (51.85%)	11 (40.74%)	1 (3.70%)

Table 4. Bioaccessibility of DON for pasta samples after gastric and duodenal digestion

Pasta	DON µg/kg	DON µg/kg cooked pasta	Gastric digestion	Duodenal digestion
			Gastric bioacc. (%)	**Duodenal bioacc. (%)**
Sample 1I[a]	350.00±2.00	170.57±1.1	19.72±1.73	8.40±1.12
Sample 2I	387.00±1.55	195.52±3.1	2.12±0.11	1.11±0.01
Sample 3I	371.00±2.54	183.56±1.9	16.76±2.11	10.71±0.25
Sample 5I	134.00±3.75	63.86±0.5	20.40±1.80	10.42±0.14
Sample 12A[b]	73.80±2.80	37.80±0.3	41.49±2.42	24.13±1.71
Sample 12I	73.80±2.80	35.20±0.1	38.41±2.95	17.91±0.80

Average values ± standard deviation from the means of three replications are presented.
[a] I:infant [b] A:adult

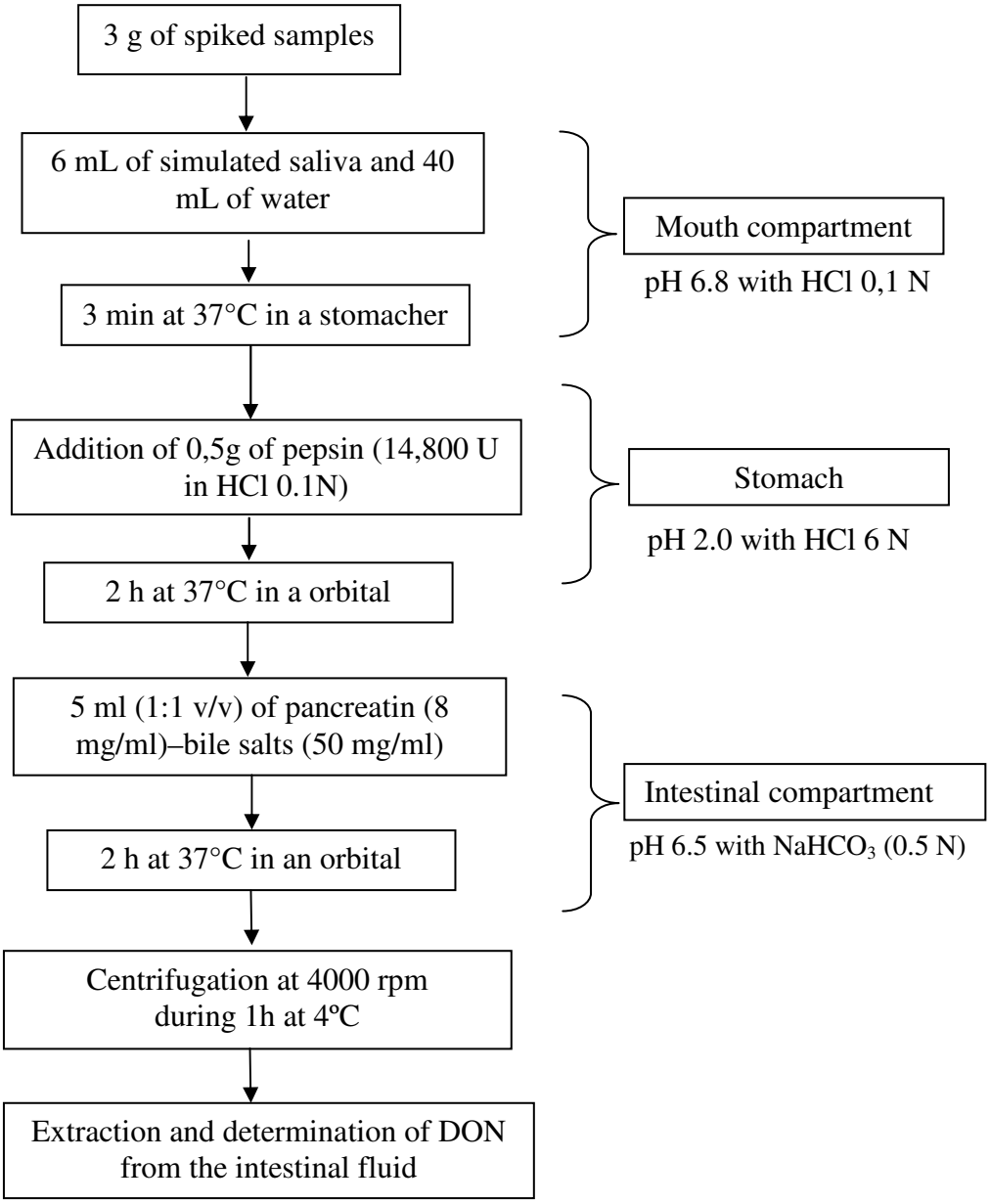


Figure 1. **Schematic representation of the *in vitro* digestion model**

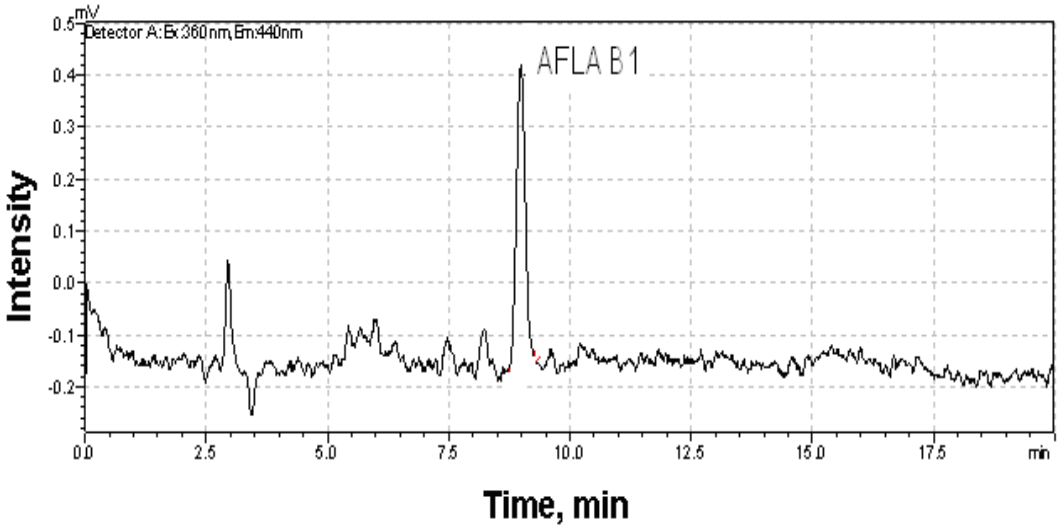


Figure 2. Fluorescence chromatogram of a AFB_1 standard at 1 µg/kg.

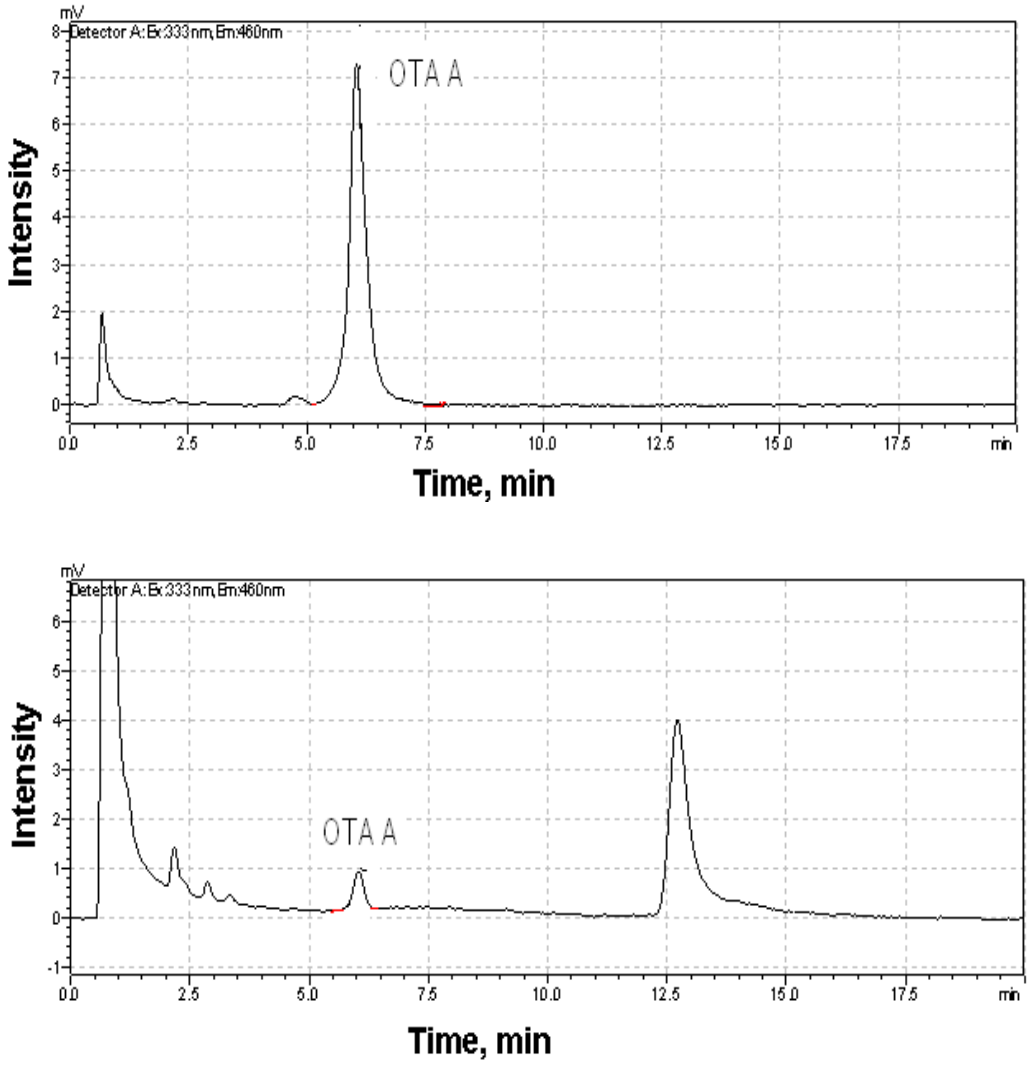


Figure 3. Fluorescence chromatogram of: a) OTA standard at 5 µg/kg; b) sample naturally contaminated with OTA.

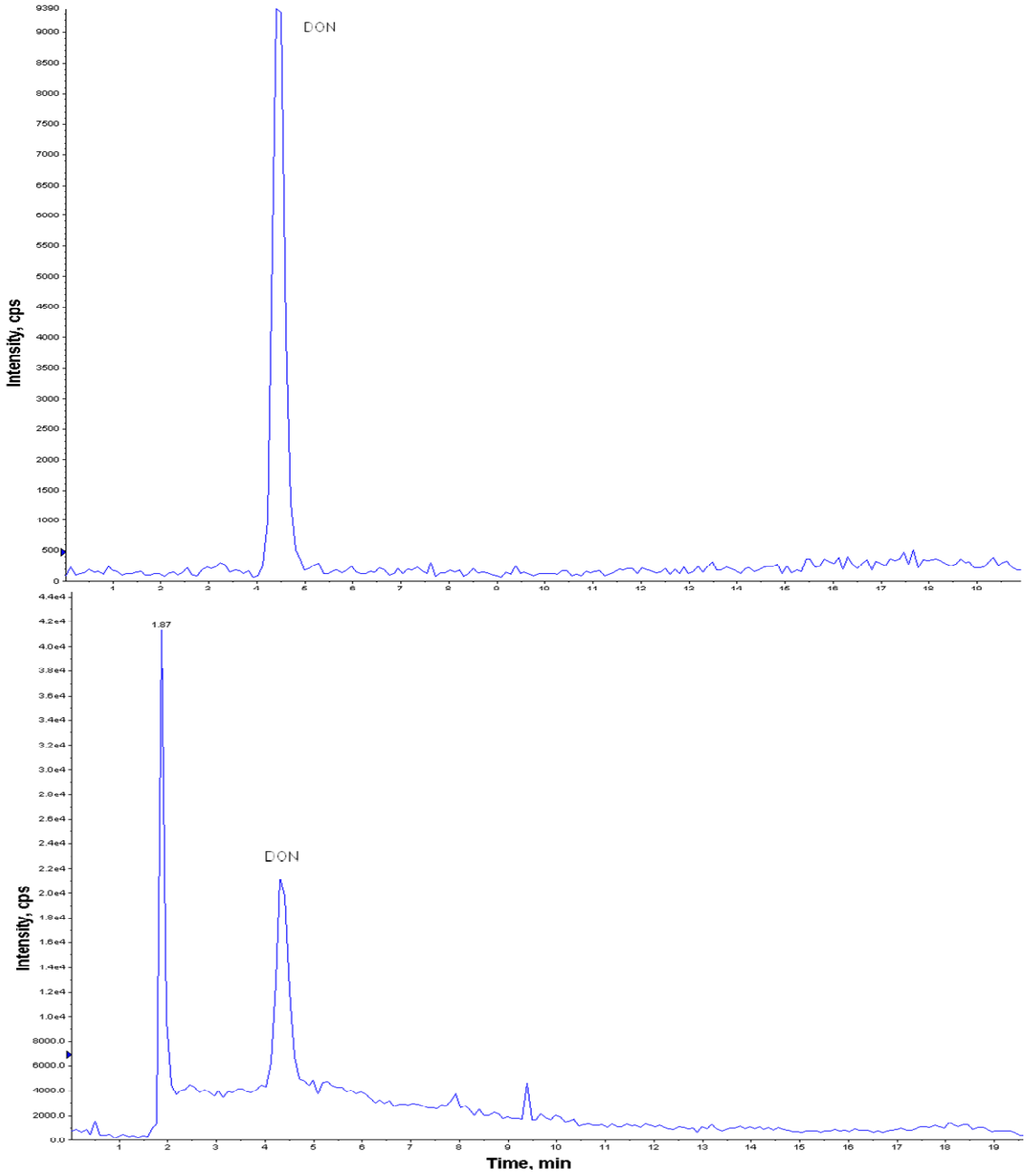


Figure 4. Total ion chromatogram (TIC) of: a) DON standard at 100 µg/kg; b) pasta sample naturally contaminated with DON

References

Avantaggiato, G., Havenaar, R., Visconti, A., (2003). Assessing the zearalenone-binding activity of adsorbent materials during passage through a dynamic in vitro gastrointestinal model. Food Chem. Toxicol. 41: 1283-1290.

Avantaggiato, G., Havenaar, R., Visconti, A., (2004). Evaluation of the intestinal absorption of deoxynivalenol and nivalenol by an in vitro gastrointestinal model, and the binding efficacy of activated carbon and other adsorbent materials. Food Chem. Toxicol. 42:817-824.

Cano-Sancho, G., Valle-Algarra, F.M., Jiménez, M., Burdaspal, P., Legarda, T.M., Ramos, A.J., Sanchis, V., Marín, S., (2010). Presence of trichothecenes and co-occurrence in cereal-based food from Catalonia (Spain). Food Control, 22: 490-495.

Carolien, H.M., Versantvoort, H., Oomen, A.G., Van de Kamp, E., Rompelberg, C.J.M., Adrienne, J.A.M., (2005). Sips Applicability of an in vitro digestion model in assessing the bioaccessibility of mycotoxins from food. Food. Chem. Toxicol. 43: 31-40.

Castro-Rodriguez, J.A., Garcia-Marcos, L., Alfonseda-Rojas, J.D., Valverde-Molina, J., Sanchez-Solis, M., (2008). Mediterranean Diet as a Protective Factor for Wheezing in Preschool Children. J. Pediatrics. 152: 823-828.

Cole T.J., Bellizzi M.C., Flegal K.M., Dietz W. H. (2000). Establishing a standard definition for child overweight and obesity worldwide: international survey BMJ. 320: 1–6

Commission Regulation (EC) No. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Official Journal of the European Union, 364: 5–24.

Commission Regulation (EC) No. 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. Official Journal of the European Union, L 70/12

Daenicke, S., Keese, C., Goyarts, T., Döll, S., (2011). Effects of deoxynivalenol (DON) and related compounds on bovine peripheral blood mononuclear cells (PBMC) in vitro and in vivo. Mycotox Res. 27: 49-55.

Diesing, A.K., Nossol, C., Panther, P., Walk, N., Post, A., Kluess, J., Kreutzmann, P**,** Dänicke, S., Rothkötter, H.J., Kahlert, S., (2011). Mycotoxin deoxynivalenol (DON) mediates biphasic cellular response in intestinal porcine epithelial cell lines IPEC-1 and IPEC-J2. Toxicology Lett. 200, 8-18.

Gil-Izquierdo, A., Zafrilla, P., Tomás-Barberá, F.A., (2002). An in vitro method to simulate phenolic compound release from the food matrix in the gastrointestinal tract. Eur. Food Res. Technol. 214:155-159.

Giray, B., Girgin, G., Engin, A.B., Aydın, S., Sahin, G., (2007). Aflatoxin levels in wheat samples consumed in some regions of Turkey. Food Control. 18: 23-29

González-Osnaya, L., Cortés, C., Soriano, J.M., Moltó, J.C., Mañes, J., (2010). Occurrence of deoxynivalenol and T-2 toxin in bread and pasta commercialised in Spain. Food Chem. 124: 156-161.

Herrera, M., Juan, T., Estopañan, G., Ariño, A., (2009). Comparison of deoxynivalenol, ochratoxin A and aflatoxin B1 levels in conventional and organic durum semolina and the effect of milling. J. Food Nutr. Res. 48: 92-99.

ISMEA-AC NIELSEN, CIRM (ricerca per World Pasta Day 2002 e 2003)

Jovaní, M., Viadel, B., Laparra, M., Barberá, R., Farré, R., (2004). Improvement of Analytical Conditions of Mineral Caco-2 Cell uptake Assays. Food Sci. Tech. Int. 10: 197-201.

Kabak, B., Brandon, E.F.A., Var, I., Blokland, M., Sips, A.J.A.M., (2009). Effects of probiotic bacteria on the bioaccessibility of aflatoxin b1 and ochratoxin a using an in vitro digestión model under fed conditions. J. Environ. Sci. Health. 44: 472-480.

Kabak, B., (2009a). Ochratoxin A in cereal-derived products in Turkey: occurrence and exposure assessment. Food Chem. Toxicol. 47: 348-352.

Kuiper-Goodman T., (1996). Risk assessment of ochratoxin A: An update. Food Add. and Contam. 13: 53-57.

Riba, A., Bouras, N., Mokrane, S., Mathieu, F., Lebrihi, A., Sabaou, N., (2010). Aspergillus section Flavi and aflatoxins in Algerian wheat and derived products Food and Chem. Toxicol. 48: 2772-2777.

Santini, A., Ferracane, R., Aragòn, A., Ritieni, A., (2009). Multitoxin extraction and detection of trichothecenes in cereals; an improbe LC-MS/MS approach. J. Sc. Food Agric. 89: 1145-115.

Sofi, F., (2009). The Mediterranean diet revisited: evidence of its effectiveness grows. Curr. Opin. Cardiol. 24: 442-446

Soubra, L., Sarkis, D., Hilan, C., Verger, P., (2009). Occurrence of total aflatoxins, ochratoxin A and deoxynivalenol in foodstuffs available on the Lebanese market and their impact on dietary exposure of children and teenagers in Beirut. Food Add. and Contam. 26: 189-200.

Trichopoulou, A., Lagiou, P., Kuper, H., Trichopoulos, D., (2000). Cancer and Mediterranean dietary traditions. Cancer Epidem. Biomar. Prev. 9: 869-873.

Villa P., Markaki P., (2009). Aflatoxin B_1 and ochratoxin A in breakfast cereals from athens market: Occurrence and risk assessment. Food Control. 20: 455-461.

Visconti, A., Haidukowski, E.M., Pascale, M., Silvestri, M., (2004). Reduction of deoxynivalenol during durum wheat processing and spaghetti cooking. Toxicol Lett. 153: 181-189.

Winnie, N.G., Mankotia, M., Pantazopoulos, P., Neil, R.J., Scott P.M., Ben Lau P.Y., (2009). Survey of dry pasta for Ochratoxin A in Canada. J. Food Prot. 72: 890-893.

Zinedine, A., Juan, C., Soriano, J.M., Moltó, J.C., Idrissi, L., Mañes, J., (2007). Limited survey for the occurrence of aflatoxins in cereals and poultry feeds from Rabat, Morocco. Int. J. Food Microbiol. 115: 124-127.

Zinedine, A., Blesa, J., Mahnine, N., El Abidi, A., Montesano, D., Mañes, J., (2010). Pressurized liquid extraction coupled to liquid chromatography for the analysis of ochratoxin A in breakfast and infants cereals from Morocco. Food Control. 21: 132-135.

Chapter 6: CORRELATION BETWEEN DIETARY AFLATOXINS AND AFLATOXIN M_1 IN HUMAN MILK IN LACTATING MOTHERS IN OGUN STATE, NIGERIA

6.1 Introduction

Milk is the lacteal secretion of animals that suckle their young (Galvano et al., 1996). It is a good source of protein that has high biological value in promoting the growth of children. Breast milk is considered to be the ideal food for infants and the importance of breast feeding for normal growth and development has been recognised as it provides the nutritional and immunological needs of infants (Picciano, 2001). However, breast milk may be contaminated with trace amounts of contaminants such as aflatoxins.

Aflatoxin M_1 (AFM_1), a hydroxylated metabolite of AFB_1, is an important toxin present in the milk of lactating animals and nursing mothers fed with AFB_1-contaminated feeds or foodstuffs. AFM_1 have been reported in milk of lactating animals and in humans (Atanda et al., 2007; Polychronaki et al., 2007; Dashti et al., 2009; Sadeghi et al., 2009; Gürbay et al., 2010).

Although AFM_1, a genotoxic carcinogen, is less toxic than AFB_1 (Creppy, 2002), exposure of infants to AFM_1 is worrisome since they are considered more susceptible to its adverse effect than adults. This is due to their low body weights, high metabolic rate, lower ability to detoxify toxins and incomplete development of some vital organs and tissues, especially the central nervous system (Galvano et al., 1996).

There is limited information on exposure to AFM_1 contamination in milk and milk products in Nigeria. However, Opadokun et al. (1979) found no detectable level of AF M_1 in 92 samples of milk in a dairy farm in Kano State while Ogunbanwo (2005) conducted a nationwide survey and found AFM_1 concentration in the range of 0.15 - 0.17 μg/l in yoghurt and ice cream. Similarly, Atanda et al. (2007) found AFM_1 contamination in the range of 2.04 - 4.0 μg/l in yoghurt and ice cream in Abeokuta and Odeda local governments of Ogun State.

Since AFM_1 is present in the milk of some lactating mothers in Ogun State, there is need to investigate the possibility of a correlation between the mother's dietary exposure to AFB_1 and AFM_1 contamination herein investigated and reported.

6.2 Materials and methods

6.2.1. Study area

The study was conducted in Ogun State, South Western Nigeria which is divided into three senatorial districts (Ogun Central, Ogun East and Ogun West). The state is situated between latitude 6.2 ^{o}N and 7.8 ^{o}N and longitude 3.0 ^{o}E and 5.0 ^{o}E and the mean annual rainfall varies from 128 cm (southern part) to 105 cm (northern part) while the average monthly temperature ranges from 23^{o}C to 32^{o}C.

6.2.2. Subject recruitment

Fifty lactating mothers that attended maternity centres in Ogun Central (20), Ogun East (15) and Ogun West (10) senatorial districts that agreed to participate in the study were recruited and ethical approval was given by the Ogun State Ministry of Health, Abeokuta, Ogun State, Nigeria.

6.2.3 Collection of Samples

Donors that agreed to participate in the study were recruited from the maternity centres in each senatorial district and were educated on food sampling procedures. A structured food frequency questionnaire (FFQ) was then used to obtain information on the food consumption pattern of the lactating mothers over the last month preceding the collection of samples after which 40kg of the most frequently consumed foods were collected randomly from the three senatorial districts from June to October, 2010. Food samples collected were: rice, beans, cassava flour, semovita, yam flour, wheat flour, whole wheat, maize and "gari". A total of 82 food samples were collected thus: Ogun Central (30), Ogun East (25) and Ogun West (27) and immediately stored at 4°C until extraction and analysis.

Data was also collected on the socioeconomic status (work status, education levels, age and residential location) of the subjects. Twenty millilitres of breast milk was obtained from each lactating mother that agreed to participate in the study and was transported to the laboratory in ice-packed coolers and immediately stored at -20^{o}C until extraction and analysis. In total, 15 samples were collected from Ogun Central, 18 from Ogun East and 17 from Ogun West senatorial districts respectively.

6.2.4. Chemicals and reagents

HPLC-grade solvents were from Merck (Darmstadt, Germany). Water, for the HPLC mobile phase was produced in a Milli-Q system (Millipore, Bedford, MA, USA). Organic solvents used for extraction phases and the phase chromatography were purchased from Merck (Germany) and AFM_1 and AFB_1 standards were from Sigma Chemical Company (St. Louis, MO, USA).

6.2.5. Preparation of standard solutions of AFB_1 and AFM_1

The stock solution of AFB_1 and AFM_1 were prepared in methanol at concentration of 0.5 µg/ml and kept at −20°C. Working solutions of AFM_1 and AFB_1 used for calibrating the HPLC and obtaining the calibration curves were prepared by making appropriate dilutions of the stock solutions in methanol and were also kept at −20°C.

6.2.6. Extraction of AFB_1 and AFM_1

Food samples were extracted according to the method described by Göbel and Lusky (2004) with slight modifications. Two and half grams NaCl was added to twenty five grams of food in a blender followed by the addition of 50 ml of methanol: water (80: 20 v/v). The mixture was blended at high speed for 2 min and filtered through a fluted filter paper; the resultant filtrate was collected and ten millilitres of it diluted with 40 ml purified water in a stopered glass tube. The tube was mixed gently by hand inversion for 1 min. The solution was filtered through a filter in regenerated cellulose (RC) 0.20 µm into a 100 ml beaker and 10 ml of the filtrate passed through an immunoaffinity column (Aflatest) at a flow rate of 1-2 drops/sec. The column was washed with 10 ml of purified water and the bound aflatoxin eluted by passing 3 ml of methanol through the column at 1-2 drops/sec into a glass vial. The eluate was then dried in a centrifugal evaporator (Savant) vacuum centrifuge, dissolved in 200 µl of methanol and 20 µl portion injected into the HPLC for analysis. Breast milk samples were extracted according to the modified method of International Standard Organization (1998) with slight modifications. Briefly, 40 ml of purified water was added to 10 ml of breast milk in a 100 ml beaker, followed by the addition of 0.25g NaCl and the solution properly mixed. The mixture was then centrifuged at 4000 rpm for 10 min. After separation, the skim portion (bottom layer) was filtered through a glass microfiber filter and 8 ml of the filtrate passed through an immunoaffinity column at a steady slow flow rate of 1-2 drops/sec. Eight millilitres of methanol: water (10: 90) were used to wash the column and the bound aflatoxin eluted by passing 3 ml of methanol through the column at 1-2 drops/sec into a glass vial. The eluate was dried in vacuum centrifuge, dissolved in 200 µl of methanol and 50 µl portion injected into the HPLC for analysis.

6.2.7. Determination of AFB_1 and AFM_1 concentrations

Analysis of AFB_1 and AFM_1 were made by HPLC system (Shimadzu, Japan) configured with LC-10AD pumps, coupled with a fluorescence detector RF-10Axl and an injector fitted with a 20 µl loop for AFB_1 and 50 µl loop for AFM_1 respectively. Excitation and emission wavelengths were set at 360 and 440 nm respectively. The stationary phase was 3 µm (150 x 460 mm) column (Gemini C18 110A) (Phenomenex, USA). The mobile phase was isocratic, mixture of methanol/acetonitrile/ water (25:25:50 v/v/v), with a flow rate of 1 ml/min and chromatographic run time of 10 min. Two different calibration curves were determined for both aflatoxins using a series of dilutions containing different levels of each toxin separately, with an average of 10 consecutive injections of standard solutions of AFB_1 and AFM_1. The linearity of the curve was 50 to 0.1µg/kg for AFB_1 and 50 µg/kg to 5 ng/kg for AFM_1. The retention time was 9 min for AFB_1 and 5.8 min for AFM_1. The recovery experiments for AFB_1 were conducted by spiking 25 g of known blank samples with a standard concentration of 5 µg/kg. Artificial contamination was done after establishing that the blanks had AFB_1 which was less than the limit of detection (0.1 µg/kg). The recovery of AFB_1 was found to be between 57 and 100% respectively with a mean of 85.5%. The calibration curve had a linear equation of $y = 5758x – 332.3$ and a correlation coefficient of 0.999.

Standard concentrations of 0.08, 0.8 and 8 µg/kg were used for recovery experiments of AFM_1. Five millilitres of breast milk were contaminated with 125 µl of standard AFM_1 (0.08 µg/kg) for those contaminated up to 0.002 µg/kg and 0.8 µg/kg and the mean recovery was 100%. The equation of the calibration curve was $y = 63116x – 331.9$ and a correlation coefficient of 1.0.

6.2.8. Statistical analyses

The concentrations of AFB_1 and AFM_1 in the samples were reported as means of three replicates and subjected to analysis of variance (ANOVA) using SPSS 16.0 to determine whether there was significance at 5% probability level. Duncan Multiple Range test was used to detect the level of significance. In addition, the Spearman Correlation test was used to correlate the level of AFM_1 in breast milk with of AFB_1 and the socioeconomic status of the respondents.

6.3 Results

AFB_1 was detected in the foods frequently consumed by the lactating mothers with a total occurrence of 79.3% (Table 1). Figure 1 shows the chromatograms of some of the naturally-contaminated foods that were positive for AFB_1. The occurrence of AFB_1 was highest in maize (100%), followed by rice (90.5%) and beans (88.2%) while semovita had the least occurrence of 33.3% (Table 1). Rice was found to be the most frequently consumed food with about 84% of the lactating mothers consuming it daily (Fig 3).

The level of contamination of AFB_1 (Table 1) in the foods was generally low. "Gari" had the highest AFB_1 contamination (0.25 µg/kg) while wheat flour had the lowest AFB_1 (0.04 µg/kg). The level of AFB_1 contamination of foods in Ogun Central senatorial district (0.33 µg/kg) was significantly higher ($p<0.05$) than those from Ogun East (0.18 µg/kg) and Ogun West (0.16 µg/kg) senatorial districts respectively (Table 2). Conversely, the occurrence of AF B_1 was highest in Ogun East (94.4%), followed by Ogun West (94.1%) and Ogun Central (93.8%) senatorial districts respectively.

The highest academic qualification obtained by the lactating mothers was a Bachelor's degree (14%) while 2% of them had no formal education. In addition, 40% of the women were petty traders that lived in urban centres while 50% were in the age group of 20-29 years (Table 3).

Figure 2 shows the chromatogram of breast milk naturally contaminated with AFM_1. AFM_1 contamination was detected in (82%) of the milk samples (Table 4) and the highest AFM_1 risk was found in Ogun Central (100%) followed by Ogun West (82.4%) and Ogun East (66.7%). Furthermore, the level of AFM_1 contamination of Ogun Central senatorial district was significantly higher ($p<0.05$) than those of Ogun West and Ogun East senatorial districts (Table 4).

Generally, there was a significant ($p<0.05$) positive correlation ($r = 0.33$) between dietary AFB_1 and AFM_1 contamination of lactating mothers in Ogun State (Table 5) especially in Ogun central senatorial district ($r = 0.56$) (Table 6). Furthermore, consumption of rice significantly ($p<0.05$) affected the dietary exposure of lactating mother to AFB_1 whereas its consumption had no significant relationship with work status, age, education level and residential location (Table 5). In addition AFM_1 concentration of breast milk showed a significant ($p<0.05$) relationship with educational level ($r = -0.35$), gari and semovita consumption ($r = 0.33$, 0.29) and the senatorial districts ($r = -0.62$, $p<0.01$).

6.4 Discussion

Aflatoxin contamination occurred in different amounts in the food samples. This is in agreement with previous studies of aflatoxin contamination in maize - 22 µg/kg (Bankole and Mabekoje, 2004), rice - 37.2 µg/kg (Makun et al., 2007), beans - 59.29 µg/kg (Makun et al., 2010), "gari" (total aflatoxin) - 5.71 µg/kg (Ogiehor et al., 2007), wheat - 32.33 µg/kg (Makun 2011) yam flour -32.33 µg/kg (Jonathan et al., 2011) and animal feeds (Dashti et al., 2009). However, the concentration of AFB_1 observed in the current study was much lower than those previously reported. This could be due to the several environmental and climatic conditions that affect aflatoxin production (Cotty and Jaime-Garcia, 2007) as fungal growth and mycotoxin contamination are dependent on climate and storage conditions and therefore vary with locations, hot and humid climate, poor storage conditions, poor agricultural and manufacturing practices (Ominski et al., 1994). It may also be due to uneven distribution of mycotoxins in the food matrices. None of the food samples had aflatoxin levels that were more than the European Union limits of 2 µg/kg for cereals intended for direct human consumption and the National Agency for Food, Drug Administration and Control (NAFDAC) recommended value of 10µg/kg for unprocessed foods in Nigeria. This does not guarantee the safety of infants fed with such low level aflatoxins as it has been reported that maternal consumption of AF-contaminated foods during breastfeeding can result in the accumulation of aflatoxins and their toxic metabolites in breast milk (Polychronaki et al., 2006, Gurbay et al., 2010). Besides the joint FAO/WHO Expert Commitee on Food additives (JEFCA) has not established a tolerable daily intake (TDI) for aflatoxins, but strongly recommends that the level of aflatoxins be low as possible (Polychronaki et al., 2006). Consumption of rice, a major staple food of most Nigerians, like "gari", substantially contributed to the dietary exposure of the lactating mothers to aflatoxins and is therefore a source of concern.

The incidence of AFM_1 contamination (82%) of breast milk was lower than the 99.5% recorded in the United Arab Emirate (Abdulrazzaq et al., 2003) and 98.1% in Iran (Sadeghi et al., 2009) but higher than the 31% in Sierra Leone (Jonsyn et al., 1995), 4.8% in Cameroun (Tchana et al. 2010) and 56% in Egypt (Polychronaki et al., 2007). Furthermore Gurbay et al. (2010) reported the contamination of breast milk by AF M_1 (60.90–299.99 ng/l) in Turkey. The low level of AFM_1 contamination observed by us could be as a result of the low level of AF B_1 consumed by the lactating mothers. Eighteen percent of the breast milk samples collected in this study had no detectable AFM_1, unlike 50% of Kuwaiti mothers reported by Dashti et al. (2009) while only 6% exceeded the maximum limit of 50 ng/l set by the Codex Alimentarius Commission (CODEX, 2004)

6.5 Conclusion

This study has shown that there is a significant positive correlation between dietary AFB_1 and AFM_1 contamination of breast milk of lactating mothers in Ogun State, Nigeria. Even though AFM_1 was found in the breast milk of the lactating mothers there is still need to encourage breastfeeding of infants since breast milk is still the best feeding option for infants. Apart from human breast milk, infants are also at risk of exposure to AFM_1 from meat, eggs, milk and other edible products from animals that consume AF-contaminated feeds.

Table 1: Distribution of Aflatoxin B_1 in foodstuffs consumed by lactating mothers

Foodstuff	Occurrence[x]	Mean[y] (µg/kg)	Range (µg/kg)
Rice	90.5% (19/21)	0.14[a]	nd – 0.71
Beans	88.2% (15/17)	0.15[a]	nd – 1.02
Cassava Flour	75.0% (3/4)	0.05[a]	nd – 0.07
Semovita	33.3% (2/6)	0.09[a]	nd – 0.17
Yam	85.7% (6/7)	0.14[a]	nd – 0.27
Wheat flour	66.6% (2/3)	0.04[a]	nd – 0.06
Whole Wheat	66.6% (2/3)	0.05[a]	nd – 0.07
Maize	100% (3/3)	0.16[a]	0.11 – 0.20
Gari	72.2% (13/18)	0.25[a]	nd – 0.69
Total	79.3% (65/82)	0.11	nd – 1.02

[x] – Positive/Total Samples; [y] – Mean of Positive samples; nd – not detected;
[a] – Mean values within a column with the same letter are not significantly different (p>0.05)

Table 2: Occurrence and levels of Aflatoxin B_1 contamination in foodstuff consumed by lactating mothers in 3 senatorial districts of Ogun State

Senatorial District	Occurrence[x]	Contamination	
		Mean[y] (µg/kg)	Range (µg/kg)
Ogun Central	93.7% (15/16)	0.33[a]	nd - 1.02
Ogun East	94.4% (17/18)	0.18[b]	nd – 0.71
Ogun West	94.1% (16/17)	0.16[b]	nd – 0.69

[x] – Positive/Total Samples; [y] – Mean of Positive samples
nd – not detected; [a,b] - Mean values within a column with the same letter are not significantly different (p>0.05)

Table 3: **Socioeconomic data of the respondents**

Work Status	**Frequency (Percentage)**
Teacher	5 (10)
Banker	1 (2)
Civil Servant	4 (8)
Trader	20 (40)
Hairdresser	4 (8)
Nurse	1 (2)
Fashion Designer	3 (6)
Photographer	1 (2)
Food Seller	1 (2)
Farmer	1 (2)
Unemployed	7 (14)
Business	1 (2)
Store Manager	1 (2)
Education Level	
Primary School Certificate	16 (32)
Secondary School Certificate	14 (28)
National Certificate of Education	3 (6)
Ordinary National Diploma	4 (8)
Higher National Diploma	4 (8)
Bachelors (B.Sc)	7 (14)
Postgraduate Diploma	1 (2)
No Education	1 (2)
Age Group	
20-29	25 (50)
30-39	23 (46)
40-49	2 (4)
Residential Location	
Urban	46 (92)
Rural	4 (8)

Table 4: Occurrence and levels of aflatoxin M_1 contamination of breast milk from lactating mothers in 3 senatorial districts of Ogun State

Senatorial District	Occurrence[x]	Frequency Distribution (ng/l)			Contamination (ng/l)	
		1 – 10	11 – 50	> 50	Mean[y]	Range (ng/l)
Ogun Central	100% (15/15)	1	11	3	35.00[a]	4.65 – 92.14
Ogun East	66.7% (12/18)	12	-	-	6.51[b]	nd – 18.58
Ogun West	82.3% (14/17)	14	-	-	3.49[b]	nd – 5.40

[x] – Positive/Total Samples; [y] – Mean of Positive samples
nd – not detected; [a,b] - Mean values within a column with the same letter are not significantly different ($p>0.05$)

Table 5: Correlation between Aflatoxin B_1 and aflatoxin M_1 with socioeconomic parameters and foods consumed by lactating mothers in Ogun State

	AFB_1 Concentration	AFM_1 Concentration
AFM_1 Concentration	0.33*	1^{NS}
AFB_1 Concentration	1^{NS}	0.33*
Work Status	0.06^{NS}	-0.27^{NS}
Age Group	0.1^{NS}	0.26^{NS}
Residence Location	0.05^{NS}	-0.11^{NS}
Education Level	0.06^{NS}	-0.35*
Senatorial District	-0.23^{NS}	-0.62**
Gari	0.18^{NS}	0.33*
Cassava Flour	0.21^{NS}	-0.1^{NS}
Yam	-0.24^{NS}	-0.25^{NS}
Rice	0.28*	0.2^{NS}
Beans	0.15^{NS}	-0.17^{NS}
Semovita	0.21^{NS}	0.29*
Maize	-0.2^{NS}	-0.03^{NS}
Wheat	0.16^{NS}	-0.21^{NS}

* Correlation is significant at $p<0.05$
** Correlation is significant at $p<0.01$
^{NS} not significant

Table 6: Correlation between Aflatoxin B_1 in foodstuffs and Aflatoxin M_1 in breast milk of lactating mothers in 3 senatorial districts of Ogun State

Senatorial District	AF B_1 in Ogun Central	AF B_1 in Ogun East	AF B_1 in Ogun West
AF M_1 in Ogun Central	0.56*	-0.36^{NS}	0.08^{NS}
AF M_1 in Ogun East	-0.37^{NS}	-0.23^{NS}	-0.07^{NS}
AF M_1 in Ogun West	-0.05^{NS}	0.32^{NS}	0.02^{NS}

*. Correlation is significant at the 0.05 level
NS – Not significant

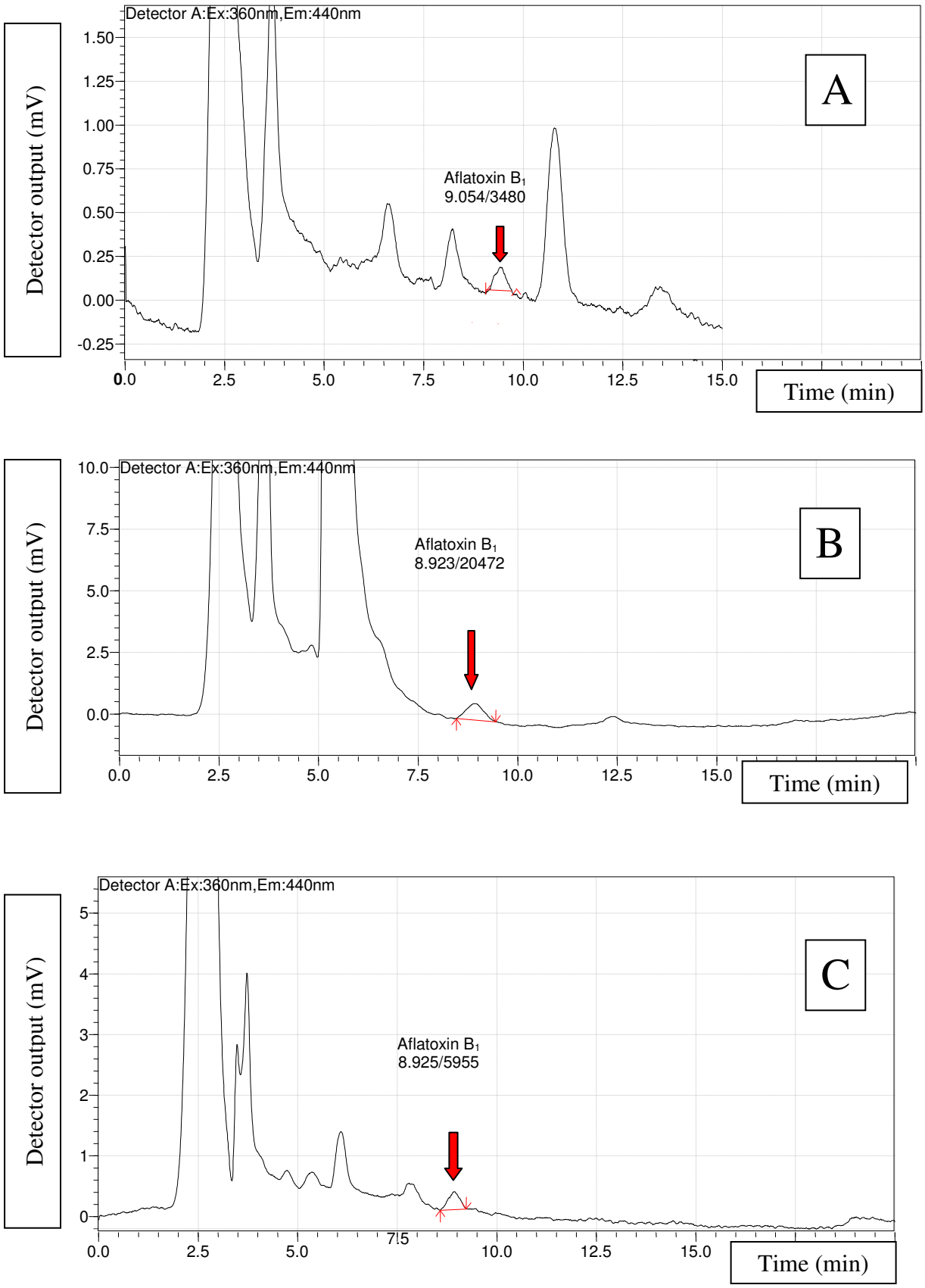


Figure 1: **Chromatograms of naturally contaminated samples positive for Aflatoxin B_1 (A) Maize; (B) Gari; (C) Rice**

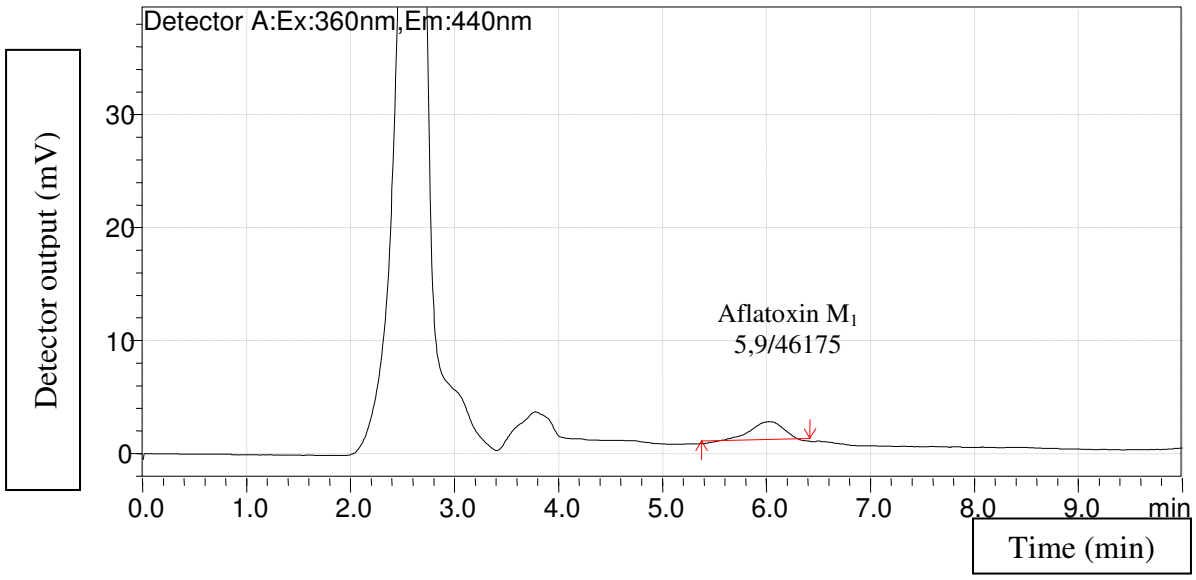


Figure 2: Chromatogram of human breast milk sample naturally contaminated with AFM_1

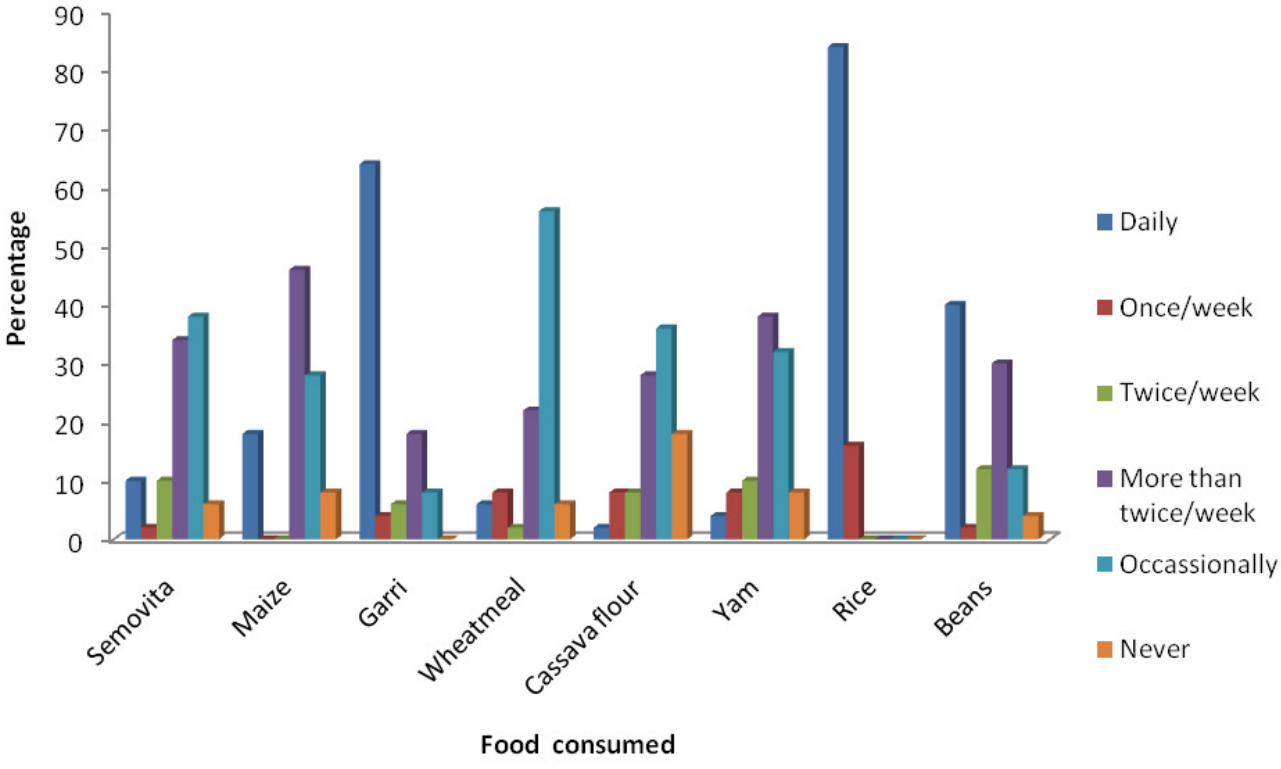


Figure 3: Food consumption pattern by lactating mothers in Ogun State

References

Abdulrazzaq, Y.M., Osman, N., Yousif, Z.M., Al-Falahi, S., (2003). Aflatoxin M_1 in breast-milk of UAE women. Ann. Trop. Paediatr. 23:173–179.

Atanda, O., Oguntubo, A., Adejumo, O. , Ikeorah, J., Akpan, I., (2007). Aflatoxin M_1 contamination of milk and ice cream in Abeokuta and Odeda local governments of Ogun State, Nigeria. Chemosphere 68: 1455–1458.

Bankole, S. A., Mabekoje, O. O., (2004). Mycoflora and occurrence of aflatoxin B_1 in dried yam chips from markets in Ogun and Oyo States, Nigeria. Mycologia 157:111-115.

Codex Alimentarius Commissions, (2004). Discussion and possible opinion on a draft commission regulation amending regulation (EC) No. 466/ 2001 as regards aflatoxins and ochratoxin A in foods for infant and young children (SanCO/0983/2002 REV.6)

Cotty, P.J., Jaime-Garcia, R. (2007). Influences of climate on aflatoxin producing fungi and aflatoxin contamination. International Journal of Food Microbiology 119:109–115.

Creppy, E. E., (2002). Update of survey, regulation and toxic effects of mycotoxins in Europe. Toxicology Letters 127: 19-28.

Dashti, B., Al-Hamli, S., Alomirah, H., Al-Zenki, S., Abbas, A.B., Sawaya , W. (2009). Levels of aflatoxin M_1 in milk, cheese consumed in Kuwait and occurrence of total aflatoxin in local and imported animal feed. Food Control 20:686–690.

Galvano, F., Galofaro,V., Galvano, G., (1996). Occurrence and stability of aflatoxin M_1 in milk and milk products: A worldwide review. J. Food Prot. 59:1079–1090.

Göbel, R., Lusky, K., (2004). Simultaneous determination of aflatoxins, ochratoxin A, and zearalenone in grains by new immunoaffinity column. Journal of AOAC International, 87(2): 411–416.

Gurbay, A., Sabuncuoglu, S.A., Girgin, G., Sahin, G., Yigit, S., Yurdakok , M., Tekinalp, G., (2010). Exposure of newborns to aflatoxin M_1 and B_1 from mothers' breast milk in Ankara, Turkey. Food and Chemical Toxicology 48: 314–319.

International Standard Organization., (1998). Milk and milk powder: Determination of aflatoxin M_1 content – clean up by immunoaffinity chromatography and determination by high performance liquid chromatography. Standard No.14501. Geneva, Switzerland: International Standard Organization (ISO).

Jonathan, G., Ajayi, I., Omitade, Y., (2011). Nutritional compositions, fungi and aflatoxins detection in stored 'gbodo' (fermented *Dioscorea rotundata*) and 'elubo ogede' (fermented *Musa parasidiaca*) from South western Nigeria. African Journal of Food Science 5(2): 105 – 110.

Jonsyn, F.E., Maxwell, S.M., Hendrickse, R.G., (1995). Ochratoxin A and aflatoxins in breast milk samples from Sierra Leone. Mycopathologia 131:121–126.

Makun, H. A., Gbodi, T. A., Akanya, H. O., Salako, E. A. and Ogbadu, G. H., (2007). Fungi and some mycotoxins contaminating rice (*Oryza sativa*) in Niger State, Nigeria. African Journal of Biotechnology 6(2): 99-108.

Makun, H. A., Anjorin, S. T., Moronfoye, B., Adejo, F. O., Afolabi, O. A., Fagbayibo, G., Balogun, B.O., Surajudeen, A.A., (2010). Fungal and aflatoxin contamination of some human food commodities in Nigeria. African Journal of Food Science 4(4): 127-135.

Makun, H.A., Dutton, M.F., Njobeh, P.B., Mwanza, M., Kabiru, A.Y., 2011. Natural multi-occurrence of mycotoxins in rice from Niger State, Nigeria. Mycotox Res. 27, 97–104.

Ogiehor I.S, Ikenebomeh, M.J., Ekundayo, A.O., (2007). The bioload and aflatoxin content of market garri from some selected states in southern Nigeria: public health significance. African Health Sciences 7(4), 223-227.

Ogunbanwo, B.F., (2005). Incidence of mycotoxins in local and processed foods marketed in Nigeria. Laboratory training on mycotoxin contamination of agricultural commodities and processed foods. National Agency for Food and Drug Administration and Control (NAFDAC)/International Atomic Energy Agency (IAEA) Regional Workshop. Lagos, Nigeria.

Ominsk K., Marquardi R.R., Sinha R.N. Abramson D. (1994). Ecological aspects of growth and mycotoxin production by storage fungi. In: Miler J.D. and Trenholm H.L. Mycotoxins in grains: Compounds other than aflatoxins. Eagan Press, St. Paul Minnesota, USA (1994), pp 287-314.

Opadokun, J.S., Okoye, W.I., Kazaure, I., (1979). The aflatoxin contents of locally consumed foodstuffs, Part V. Milk Report. Nigerian Stored Product Research Institute Rep No. 11, pp 87-90.

Picciano, M.F., (2001). Nutrient Composition of Human Milk. Paediatric Clinics of North America 48 (1): 53-67.

Polychronaki, N.C., Turner, P., Mykkanen, H., Gong, Y., Amra, H., Abdel-Wahhab, M., El-Nezami, H., (2006). Determinants of aflatoxin M_1 in breast milk in a selected group of Egyptian mothers. Food Addit. Contam. 23:700–708.

Polychronaki, N., West, R.M., Turner, P.C., Amra, H., Abdel-Wahhab, M., Mykkanen, H., El-Nezami, H., (2007). A longitudinal assessment of aflatoxin M_1 excretion in breast milk of selected Egyptian mothers. Food Chem. Toxicol. 45: 1210– 1215.

Sadeghi, N., Oveisi, M.R., Jannat, B., Hajimahmoodi, M., Bonyan, H., Jannat, F., (2009). Incidence of aflatoxin M_1 in human breast milk in Tehran, Iran. Food Control 20: 75–78.

Tchana, A. N., Moundipa, P. F., Tchouanguep, F. M., (2010). Aflatoxin contamination in food and body fluids in relation to malnutrition and cancer status in Cameroon. International Journal of Environmental Research and Public Health 7,s 178-188.

GENERAL CONCLUSIONS

The integrated strategy based on BCAs combined with BOSC or CYPR can control isolates of *P. expansum* that are sensitive or resistant to TBZ more efficiently with respect to the chemical control applied alone. At the same time, this type of integrated strategy may also reduce the risk of the onset of pathogen strains toward more recent fungicides such as BOSC or CYPR, since control also relies on the different and multifaceted modes of action that are involved in the protective activity of biocontrol yeasts such as LS11 and LS28. The integration of the selected biocontrol yeasts isolates R. kratochvilovae LS11 and C. laurentii LS28 with low dosages of the recently developed fungicides BOSC and CYPR appears to be a very promising method to control blue mould and keep low both fungicide residues and patulin accumulation in apples (chapter 3).
The lowest efficacy of heat treatment about PAT degradation in puree can explain the major incidence of the mycotoxin in apple puree respect to apple juice as reported in literature. The different behaviour of matrixes in exam to heat treatment can also explain the higher HMF production in juice respect to puree.
In the case of a PAT contamination at level of setted limit for consumers over 3 years old, the values of bioaccessible fraction equal to 15.5 µg/l and 24.4 µg/kg in clarified juices and products with pulp exceeded of 55% and 144% respectively the limit established in fruit products for infant and young children under 3 years old (10µg/l) (chapter 4).
The value for DON intake from pasta assessed in Italy is less than the TDI values for adults, but the subgroup of children can be considered a critical group because in many cases children can have a diet not so varied as the adults. Considering that the legal limit for baby food protects until 3 years old, while in Italy and most of Mediterranean countries pasta consumption starts at age of 2-3, a new more restrictive limit for child than for adult should be auspicable by the European Commission. Alternatively, the general limit could be decreased to reduce the exposure of the young population, and consequently the adult population (chapter 5).
Lactating mothers in Nigeria were exposed to low levels of AFB_1 in foods with the highest contamination being less than 1 µg/kg. None of the food analysed had aflatoxin level exceeding the European standard limits of 2 µg/kg for cereals intended for direct human consumption. AFM_1 contamination was detected in 41 (82%) of the human breast milk samples with only 3 (6%) samples above 50 ng/l. Infants in Ogun State, Nigeria are at risk of exposure to AFM_1 in breast milk and this may have detrimental effects on their health. Though AFM_1 was found in human breast milk, there is still a need to encourage breastfeeding of infants since breast milk is still the best feeding option for infants (chapter 6).

GLOSSARY

AFB_1: Aflatoxin B_1

AFM_1: Aflatoxin M_1

BCA: Biocontrol Agent

BOSC: Boscalid

CYPR: Cypronidil

DON: Deoxynivalenol

FENH: Fenhexamid

HMF: Hydroxymethylfurfural

OTA: Ochratoxin A

PAT: Patulin

APPENDIX 1: ON THE NATURAL OCCURRENCE OF AFLATOXIN M_1 IN RAW, UHT MILK AND DAIRY PRODUCTS FROM SEVERAL PROVINCES OF SICILY (ITALY)

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(submitted)

7.1. Introduction

Carry-over of aflatoxin M_1 (AFM_1) into the milk is generally influenced by the species, the stage of lactation, the moment of milking, the productive level. Carry over rate has been experimentally calculated between 1-5% (Spahr *et al.*, 1999, Veldman *et al.*, 1992, Battacone *et al.* (2002, 2005); in cow it is between 0.35% and 3%, while in sheep the levels were between 0.08% and 0.33% (Battacone et al., 2005).
Yousef and Marth, (1989) and JECFA, (2001) indicated that treatments like pasteurization and Ultra High Temperature techniques (UHT), do not cause an appreciable change in the amount of AFM_1 in these products (Galvano, Galorafo, & Galvano, 1996). In reverse, Purchase *et al.* (1972), found that pasteurisation of milk reduces the AFM_1 content by 32%.
Because of relative stability of AFM_1 in milk and dairy products, if raw milk contains AFM_1, cheese made from such milk will also contain AFM_1 (Lopez, et al., 2001; Galvano *et al.*, 1996). Applebaum *et al.*, 1982 reported that the affinity of AFM_1 for casein can increase AFM_1 concentration in cheese about four times higher than the milk. Lòpez et al., (2001) studied the distribution of aflatoxin M1 in cheese obtained from milk artificially contaminated. The greatest proportion, 60%, was detected in whey while 40% AFM_1 remained in cheese. Kamkar et al., (2008) determined the AFM_1 concentration in curd and whey of Iranian white Cheese derived from milk samples artificially contaminated with AFM_1. Obtained level in curd and cheese was 3.12 and 3.65 fold more than that in whey, that shows the affinity of AFM_1 to the protein fraction of milk.
However, during cheese manufacturing, the distribution of AFM_1 between curd and whey changes with the type of cheese, the particular cheese-making process applied, the type and degree of milk contamination, and the analytical method employed (Blanco *et al.*, 1988). The possible presence of AFM_1 in milk and dairy products is a worldwide concern since these products are largely consumed by children who are considered more susceptible to the adverse effects of mycotoxins than adults, (A. Zinedine et al., 2007).
The aim of this study was to evaluate the level of AFM_1 contamination in milk produced during 6 months of 2010 in several provinces of Sicily (Italy) by different animal species (cattle, buffalo, sheep, goat) at various levels of processing (raw, pasteurized, UHT). Then, transformation products (acidified milk with cream, cheese and other dairy products) were also analyzed to evaluate the effect of industrial technologies.

7.2 Material and methods

7.2.1 Sampling

A total of 96 samples of milk and dairy products were analysed for AFM_1 from January to June 2010 in provinces of Sicily (Italy), including Palermo, Messina, Agrigento, Ragusa, Catania, Enna. In particular samples included raw milk (n=50), mass milk (n=10), UHT milk (n=12), milk cream and coagulated (n= 7) and cheese (n=17). Raw milk samples were from different species: buffalo (n=1), sheep (n=34), goat (n=3), cattle (n=12). Immediately samples were transported to the laboratory in an electric coolbox (EZetil®E26) and then stored at −20 °C until analysis for AFM_1.

7.2.2 Chemicals

Acetonitril and water for chromatography were acquired from Merck (Darmstadt, Germany) and were degassed for 20 min using a Branson 5200 (Branson Ultrasonic Corp., CT, USA) ultrasonic bath. AFM_1 standard was acquired from Sigma-Aldrich (Steinheim, Germany).

7.2.3 Milk preparation for HPLC analysis

The method derived from UNI and was accredited by SINAL (National System for Accreditation of Laboratories; certificate no. 0655, 3 March 2006).
Milk samples (150 mL) were homogenized and heated to 37°C in a thermostatic bath, then centrifuged at 3500 g min^{-1} for 30 min. The milk was left to return to room temperature and the upper phase of fat was eliminated. Then the sample was purified by an immunoaffinity column (Aflatest, VICAM): 50 mL of sample were eluted with a low flow of the vacuum system (one drop per second). After that the column was washed twice with 10 mL distilled water, the AFM_1 was dissociated with methanol (1 +1.25 mL) and water (0.75 mL); the eluate was collected and filtered with filters RC 0,45µm, (Phenomenex) for HPLC injection.

7.2.4 Dairy products preparation for HPLC analysis

This phase was carried out in concordance with Colak et al., (2006) with slight modifications. 10 g dairy products samples were extracted with 50 ml of dichlormethane/acetone mixture (1:1, v/v) by Ultra Turrax (Ultraturrax, IKA-Werke, Staufen, Germany) at 16000 rpm for 2 minutes (Janke & Kunkel) with addition of sodium chloride (10 g). After centrifugation (3000 × g for 10 min), 10 ml of the organic extract were evaporated to dryness under a stream of nitrogen. The residue was dissolved in methanol (0.5 ml) and 0.01 mol/l sodium phosphate-buffered saline (PBS), pH 7.2–7.4 (20 ml) and n-hexane (10 ml) were added. After shaking, the lower layer was quantitatively collected and cleaned up on immunoaffinity column Afla test (VICAM) as described in par.7.2.3. Then, AFM_1 eluate was evaporated under a stream of nitrogen and the residue dissolved in methanol (200 µl). An aliquot (10 µl) of the AFM_1 extract was analysed by HPLC.

7.2.5 AFM_1 determination

The AFM_1 contamination was identified and quantified by HPLC Agilent Tecnologies (Palo Alto, CA) Series 120 equipped with an autosampler and a fluorescence detector (excitation at 365nm, and emission at 435 nm). A column Zorbax Eclipse XDB-C18 (Agilent,USA), 4,6 x 150 mm, ID 5 µm was used. The isocratic mobile phase consisted of a specific solution (acetonitril/water (25/75,v/v), with a flow rate of 1.0 ml/min. In these conditions retention time for AFM_1 was 6.34 min. The AFM_1 calibration curve was prepared using standard solutions of AFM_1 in methanol at concentrations of 5, 20, 50, 60 and 100 ng L^{-1} The limit of determination (LOD) for AFM_1 was 3 ng L^{-1}. Recovery test were performed by spiking aflatoxin-free milk and dairy products samples with known amounts of AFM_1 (10, 20 and 50 ng/L).

7.3 Results and discussion

7.3.1 Recovery results

Recoveries were 90.3%, 95.7%, 98.9% and 89.4%, 90.2% and 93.1% in levels of 10, 20, 50 ng/L for milk and dairy products. The survey results were not corrected for recovery.

7.3.2. AFM_1 occurrence

Table 1 reports the results obtained from complessive analyzed samples of milk and dairy products. Among 72 analyzed milk samples, 48.61% resulted positive for AFM_1 in the range of 0–16 ng/L; 96% of the positive samples were contaminated at level lower than 0.5 ng/L and 4% at level between 10 and 20 ng/L. Finoli and Vecchio (2003) investigated AFM_1 occurrence in samples of sheep milk and cheese collected in Western Sicily to evaluate, from a toxicological point of view, the quality of some feedstuffs and reported levels of AFM_1 between 4 and 23 ng/L. Incidence of AFM_1 in 24 samples of dairy products was 42% with a range of 0-0.75 ng/Kg. These levels were lower than values reported by Minervini et al. (2001), who, on analyzing 94 samples of sheep cheese in Southern Italy, found 17% of the samples positive in the range of 50-210 ng/Kg. Table 2 reports the levels of contamination at different steps of processing. The highest average level was found in raw milk with a range of 0-16 ng/Kg and an incidence of 54%, followed by cheese, with an incidence of 41.18% and a range of 0-0.75 ng/kg. Bakirci (2001) investigated the levels of AFM_1 in raw milk and reported AFM_1 in 79% of 90 examined milk samples. 35 (44.30%) of the positive samples were higher than the maximum tolerance limit (0.05 ng/ml).

The lowest incidence was found in mass milk (30%) with a range of 0-0.30 ng/L. These data show that the production processes can affect the levels of contamination but they aren't able to degrade totally AFM_1. In particular 12 samples treated by UHT process show an incidence equal to 42% and a range of 0-0.50 ng/L. These values are lower than levels reported by Nachtmann et al. (2007) during years 2003-2005 in Piedmont, a specific North West Italian region from milk collected during the commercial phase (2-27 ng/L). Zinedine et al., (2006) surveyed fifty four samples of pasteurized milk produced by five different dairies from Morocco were for the presence of aflatoxin M_1 (AFM_1); the levels ranged from 1 to 117 ng/L and the mean value was 18.6 ng/l. Unusan, (2006) determined the levels of AFM_1 in 129 UHT milk samples in Central Anatolia, Turkey and found a mean value of 108.17 ng/L and an incidence of positive sample equal to 58%. Fallah (2006) determined the occurrence of AFM_1 in commercial UHT milk samples from popular markets in central part of Iran. AFM_1 was detected in 62.3% samples with a mean of 46.4 ng/L.

17 samples of analyzed cheeses reported an incidence of positive samples equal to 41% and the range was 0-0.75 ng/kg and 43% of milk cream and coagulated resulted contaminated at level between 0-0.30 ng/kg.

S. Virdis et al. (2008) reported the results of a two year survey on AFM_1 contamination in goat milk and hard goat cheese produced in Sardinia (Italy). AFM_1 was detected in 4 (9.8%) out of 41 samples of ripened goat cheese at levels of between 79.5 ng/kg and 389 ng/kg. Kaan et al., (2006) analyzed 100 cream cheese samples for AFM_1 from retail outlets in Turkey. 99% of the cream cheese samples contained AFM_1; the incidence in cream cheese samples ranged from 0 to 4100 ng/kg. 18% of the cream cheese samples exceeded the maximum tolerable limit of the Turkish Food Codex and this results showed there is a human health risk in Turkey. Fallah (2009) determined 210 cheese samples composed of white cheese (116 samples) and cream

cheese (94 samples) purchased from popular markets in central part of Iran (Esfahan and Yazd provinces). AFM_1 at measurable level (50 ng/kg) was detected in 161 (76.6%) samples, consisting of 93 (80.1%) white and 68 (72.3%) cream cheese samples. The concentration of AFM_1 in the samples ranged from 52.1 to 785.4 ng/kg

Table 3 reports the distribution of AFM_1 in raw milk from different animal species. The highest incidence was showed in sheep milk (59%), with a range of 0-16 ng/L; AFM_1 in cattle milk showed an incidence of 50% with a range of 0-10 ng/L, while incidence in goat samples was equal to 33% in a range of 0-0.10, where 100% of buffalo products were negative. These dates were in concordance with a study of year 2008 (Montagna et al.). They carried out a screening survey of the presence of aflatoxin M_1 (AFM_1) on 265 samples of cheese made from cow, buffalo, goat, sheep, sheep-goat milk collected in the Apulia region (Southern Italy). AFM_1 was found in 16.6% of the analyzed samples. The highest positive incidence was for medium and long-term ripened cheeses, especially those made from sheep-goat milk, while buffalo cheeses tested consistently negative.

AFM_1 levels in milk are influenced by feeding practices like demonstred by Mahmoud et al., (2009). They collected milk from buffalo, cow, goat and camel species in Egypt. Cows and buffaloes are fed prepared rations and had highest incidence of AFM_1. Camels forage freely on available pasture and had lowest AFM_1 in their milk. Goats are fed a combination of prepared ration as a supplement to pasture grazing. Most milks (80%, 74%, 66% and 52% of the camel, goat, cow and buffalo milks, respectively) were below the European Union maximum of AFM_1 and all milk samples were <500 ng/L. For all analysed samples in this study concentrations did not exceed the maximum limits established by the EU.

AFM_1 exposure

Human exposure to AFM_1 is due to the consumption of contaminated milk and dairy products; its daily intake could be

highly variable in the world. Infants represent the most exposed population due to their high consumption either of bovine milk. The dietary intake of AFM_1 was estimated in general population from data on the concentration of AFM_1 in milk reported by many countries and established by JECFA (2001). Intake of milk in European diet is estimated of 0.29 kg/day. The highest AFM_1 content in milk intended for direct consumption measured in this study was 0.50 ng /kg and so the daily intake is calculated equal to 0.145 ng/day. This value is 46 times lower than the dietary intake

value estimated by JECFA for the European diet (6.8 ng/person/day).

Conclusion

Based on the milk and dairy products samples taken from provinces of Sicily, the occurrence of AFM_1 does not appear to be a serious public health hazard, according to european legislation. However, aflatoxins are recurrent.

The data of this study showed that Good Agricultural Practice, Good Storage Practice and HACCP plans are efficiently carried out in provinces of Sicily and are able to control the risk of toxigenic fungi contamination all along the feed supply chain.

Table 1: Presence of AFM_1 in total samples of milk and dairy products

Product	Sample n.	Incidence of AFM_1 (%)	Range (ng/l)	Distribution *n* (%)		
				≤0,5 ng/l	1-10 ng/l	10-20 ng/l
Milk	72	49 (48.61)	0-16	96 (95.83)	-	4 (4.17)
Dairy products	24	42 (41.67)	0-0.75	93 (93.06)	6 (5.56)	1 (1.39)

Table 2: Presence of AFM_1 in milk and dairy products divided for industrial processings

Product	Sample n.	Incidence of AFM_1 (%)	Range (ng/l)	Distribution *n* (%)		
				≤0,5 ng/l	1-10 ng/l	10-20 ng/l
Raw milk	50	54 (54.12)	0-16	90.00 (90)	8.00 (8)	2.00 (2)
Mass milk	10	30 (30.03)	0-0.30	100.00 (100)	0	0
UHT milk	12	42 (41.67)	0-0.50	100.00 (100)	0	0
Milk cream and coagulated	7	43 (42.86)	0-0.30	100.00 (100)	0	0
Cheese	17	41 (41.18)	0-0.75	94.12 (94)	0	6 (5.88)

Table 3: Presence of AFM_1 in raw milk divided for animal species

Species	Sample n.	Incidence of AFM_1 (%)	Range (ng/l)	Distribution *n* (%)		
				≤0,5 ng/l	1-10 ng/l	10-20 ng/l
Buffalo	1	0	-	100.00 (100)	0	0
Sheep	34	59 (58,82)	0-16	88 (88.24)	9 (8.82)	3 (2.94)
Goat	3	33 (33,33)	0-0.10	100.00 (100)	0	0
Cattle	12	50 (50)	0-10	92 (91.67)	8 (8.33)	0

References

Applebaum, R.S., Brackett, R.E., Wiseman, D.W. and Marth, E.H. (1982). Aflatoxins: toxicity to dairy cattle and occurrence in milk and milk products. Journal of Food Protection 45: 752-777

Bakirci I. (2001) A study on the occurrence of aflatoxin M_1 in milk and milk products produced in Van province of Turkey. Food Control 12, 1:47-51

Battacone G., Nudda A., Palomba M., Pulina G. (2002). Trasferimento di aflatossina dalla razione al latte ovino e alla cagliata. Sci. Tecn. Latt.-Cas. 53: 283-293.

Battacone G., Nudda A., Palomba M., Pascale M., Nicolussi P., Pulina G. (2005). Transfer of aflatoxin B_1 from feed to milk and from milk to curd and whey in dairy sheep fed artificially contaminated concentrates. Journal of Dairy Science, 88, 9:3063-3069.

Blanco J.L., Domingues L., Gomez-Lucia E., Garayzabal J.F.F., Goyache J., Suarez, G. (1988). Behavior of aflatoxin during the manufacture, ripening and storage of Manchego-type cheese. Journal of Food Science 53: 1373-1376.

Colak H., Hampikyan H., Ulusoy B., Ergun O. (2006) Comparison of a competitive ELISA with an HPLC method for the determination of aflatoxin M1 in Turkish White, Kasar and Tulum cheeses. Eur Food Res Technol (2006) 223: 719–723

Dashti B., Al-Hamli S., Alomirah H., Al-Zenki S., Abbas A.B., Sawaya W. (2009). Levels of aflatoxin M1 in milk, cheese consumed in Kuwait and occurrence of total aflatoxin in local and imported animal feed. Food Control, 20:7,686–690

European Commission, Commission Regulation (EC) 1881. Off. J. Eur. Communities 2006, L364, 5-24.

Fallah A.A. (2010) Assessment of aflatoxin M1 contamination in pasteurized and UHT milk marketed in central part of Iran. Food and Chemical Toxicology 48, 3, 988–991.

Fallah A.A., Jafari T., Fallah A., Rahnama M. (2009). Determination of aflatoxin M_1 levels in Iranian white and cream cheese. Food and Chemical Toxicology 47,1872–1875

Galvano F., Galofaro, V. and Galvano, G., (1996). Occurence and stability of aflatoxin M_1 in milk and milk products: a wordwide review. Journal of Food Protection 59: 1079-1090.

JECFA, 2001. Safety Evaluation f Certain Mycotoxins in Food – Prepared by the 56th meeting of the JECFA (Joint FAO/WHO Expert Committee on Food Additives) – FAO Food and Nutrition Paper 74/WHO Foods Additives Series 47

Lopez, C., Ramas, L., Ramadan, S., Bulacio, L.S. and Perez, J. (2001). Distribution of aflatoxin M_1 in cheese obtained from milk artificially contaminated. International Journal of Food Microbiology 64: 211-215.

Montagna M.T., Napoli C. De Giglio O., Iatta R., Barbuti G., (2008). Occurrence of Aflatoxin M1 in dairy products in Southern Italy Int. J. Mol. Sci., 9, 2614-2621

Kamkar A., Karim G., Aliabadi S.F., Khaksar R. (2008). Fate of aflatoxin M_1 in Iranian white cheese processing. Food and Chemical Toxicology 46, 2236–2238

Sengun I.Y., Yaman D.B and Gonul S.A. (2008). Mycotoxins and mould contamination in cheese: a review World Mycotoxin Journal,1(3): 291-298

Spahr, U.,Walther, B., Sieber, R. (1999). Vorkommen von Mycotoxinen in Futtermitteln und Carry-over in die Milch: Eine Übersicht. Mitt. Lebensm. Hyg. 0: 575-609

Nachtmann C., Gallina S., Rastelli M., Ferro G., Decastelli L. (2007). Regional monitoring plan regarding the presence of aflatoxin M1 in pasteurized and UHT milk in Italy. Food Control 18:623–629

Minervini F., Visconti A., Bottalico A., Montagna M.T. (2001). Presenza di aflatossina M1 in formaggi dell'Italia Meridionale. Ind. Alim. 40: 513-516.
Motawee, M. M., Bauer J., McMahon D. J. Survey of Aflatoxin M1 in Cow, Goat, Buffalo and Camel milks in Ismailia-Egypt. Bull Environ Contam Toxicol (2009) 83:766–769

Purchase, I.F.H., Steyn, M., Rinsma, R. and Tustin, R.C. (1972). Reduction of aflatoxin M_1 in milk processing. Food Cosmetics Toxicology 10: 383-387.

Tekinşen K., Uçar G., (2007). Aflatoxin M1 levels in butter and cream cheese consumed in Turkey. Food Control, 19(1), 27-30

UNI (Ente Nazionale Italiano di Unificazione), UNI EN ISO 14501 : 1999, Milk and milk powder – determination of aflatoxin M1 content – clean-up by immunoaffinity chromatography and determination by high-performance liquid chromatography.

Veldman, A., Mejis, J.A.C., Borgrevve, G.J., Heeres an der Tol J.J.(1992). Carry over of aflatoxin from cows' food to milk. Anim. Prod. 55: 163-168

Virdis S., Corgiolu G., Scarano C., Pilo A.L., De Santis E.P.L. (2008). Occurrence of Aflatoxin M1 in tank bulk goat milk and ripened goat cheese Food Control 19:44–49

Yousef, A.E., Marth, E.H., 1989. Stability and degradation of aflatoxin M_1. In: Van Egmond, H.P. (Ed.), Mycotoxins in dairy products. Elsevier Applied Science. 127–161.

Zinedine, L. González-Osnaya, J.M. Soriano, J.C. Moltó, L. Idrissi, J. Mañes (2007) Presence of aflatoxin M1 in pasteurized milk from Morocco International Journal of Food Microbiology 114: 25–29

APPENDIX 2: GOOD FOOD AND FEED PROCESSING TECHNIQUES IN WHEAT

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(Chapter in press)

Abstract

The purpose of this chapter is to highlight the main industrial detoxification processes of mycotoxins in various foodstuff, with particular attention to the row of wheat. After harvest, wheat is subjected to many technological processes in which temperature is the main parameter. Various temperature and moisture conditions are used in food processing and cooking. The main mycotoxin present in wheat are represented by aflatoxin, ochratoxin, deoxynivalenol, and zearalenone. However in literature there are various studies about the presence of fumonisins and moniliformin, but there are few data on the ability to detoxify them.

Introduction

Mycotoxins are secondary metabolites produced by fungi under specific humidity and temperature conditions. The most important mycotoxin producing fungi belong to the fungal genera *Fusarium*, *Penicillium* and *Aspergillus*. The United Nations Food and Agricultural Organization (FAO) estimates that up to 25% of world's grain crops are significantly contaminated by mycotoxins (Dowling, 1997) The most important mycotoxin families are aflatoxins, ochratoxins, fumonisins, trichothecenes, and zearalenone, with lesser important mycotoxins including patulin, ergot alkaloids, beauvericin, citrinin, and moniliformin also important in some cases. Mycotoxins commonly are produced and accumulate in the field and during the post-harvest portion of the food chain. Post-harvest contamination depends on storage conditions, as even excellent quality field material may be contaminated with mycotoxins if it is stored improperly. The thermal processes used in cooking have significantly increased food safety, in general. Unfortunately, most mycotoxins are resistant to heat treatments of 80-120°C, and can persist through the heat treatments commonly associated with most cooking processes. Other factors that influence the stability of mycotoxins include pH, ionic content, water content, temperature, heating time and heating rate.

The remainder of the chapter is organized by the mycotoxin of interest, with relevant processing techniques included under more than one mycotoxin heading, if appropriate.

Aflatoxins

Members of the aflatoxin family are very thermostable. The temperature must reach at least 237°C for them to decompose completely although decomposition may begin at temperatures as low as 150°C (Roustom, 1997)

Wheat: Aflatoxin-fungi are ubiquitous and may grow in cereals as wheat. Reduction of aflatoxin B_1 (AFB_1) toxicity in wheat is directly proportional to washing time, but the concentration of AFB_1 is reduced more by heating than washing treatment. The level of AFB_1 in dried wheat decreases to 50% and 90% by heating at 150° and 200°C, respectively. (Hwang et al, 2006). The reduction of AFB_1 was increased by 8% and 23% in 10% water-added US wheat (soft red white wheat) and Korean wheat (Anbaekmil) compared to dried US and Korean wheat, respectively, through heat treatment. Traditional processing used in Korean foods such as Sujebi (a soup with wheat flakes) and steamed bread caused 71% and 43% decrease in aflatoxin B1 content (Hwang et al, 2006). In the process of baking a temperature of approximately 100°C is reached in the center of the bread. Table 1 shows the fate of AFB_1 in preparing whole wheat bread. The results indicate that in baking the remaining aflatoxin content is not significantly reduced. The low baking temperature as well as the obviously low amounts of volatile acids in the bread itself do not degrade the AFB_1 to any mentionable extent. (Reiss et al, 1978)

Table 1: Fate of aflatoxin B_1 in preparing whole wheat bread.

Experiment No.	Whole-meal Wheat		Dough		Bread
	Aflatoxin B_1 ($\mu g/g$)	Toxin Recovered (%)	Aflatoxin B_1 ($\mu g/g$)	Toxin Recovered (%)	Toxin Recovered (%)
1	20	66	10.8	20	20
2	20	50	10.8	20	20
3	20	45	10.8	20	20
4	10	40	5.4	10	10
5	10	40	5.4	15	15

Maize: aflatoxin may be reduced by up to 92% during preparation of the Mexican drink atole by simple heating at 94°C for 10 minutes and then drying at 40°C for 48 hours. In the process of making corn flakes aflatoxins are reduced by 64-67%, (Lu et al, 1997).
Rice: If rice is cooked with normal or excess water, then up to 89% of the lactone rings in the aflatoxin molecules can be hydrolyzed to the corresponding carboxylic acid (Simionato et al., 2004).
Nuts.: Roasting pistachios at 90°C, 120°C and 150°C for 30-60 and 120 minutes, reduced the aflatoxin contamination by 17% to 63% (Yazdanpanah H. et al, 2005). The presence of NaCl at 50 g/kg during the pistachio roasting process reduces the aflatoxin level by 48% (Ozkarsli et al., 2003). The available moisture (5.6%) in powdered pine nuts generally is low for the temperature used during the roasting . Natural aflatoxin contamination of 120-575 ng/g can be reduced by up to 81% during processing Ozkarsli et al., 2003).
Beer. Aflatoxin in beer is sensitive to the step in the process at which the malt protein is hydrolyzed (27% reduction), boiling (30% reduction) and fermentation (30% reduction) (Chu et al, 1975).
In conclusion, the aflatoxin family of mycotoxins is very stable to heat treatment and technology. The reduction in the level of AFB_1 is related directly to the temperature or duration of the treatment of the contaminated food. The presence of a basic environment favors increased hydrolysis of aflatoxin, especially if the moisture level of the product is not particularly low.

Ochratoxin A
The ochratoxin family includes numerous molecules with various levels of toxigenicity. The most important member of this family is ochratoxin A, which has a melting point of 169°C. Ochratoxin A's (OTA) insensitivity to thermal processes enables it to persist through the processing of products such as coffee, cocoa, wine, beer, and cereals. Roasting coffee at 200°C for periods of 10-20 minutes reduces the amount of OTA present between 12 and 100% (Suarez-Quiroz *et al.*, 2005). OTA in wheat at 100°C dry heat is not degraded after 40-160 minutes of treatment, but > 50% of the toxin was destroyed after 120 minutes of treatment if wet heat was used instead of dry heat (Boudra *et al.*, 1995). Moreover, pressure-cooking the beans variety "Carioca" in water results in > 84% loss of OTA, (Milanez et al, 1996). Cooking rice in excess water reduces OTA levels by 86%, while normal cooking results in a loss of 83% (Simionato et al, 2004). OTA is stable during the baking of bread, but during biscuit preparation two-thirds of the OTA is either destroyed or immobilized (Subirade et al., 1996). Autoclaving oatmeal with 50% of the normal amount of water reduces the OTA present by 74%, while autoclaving oatmeal or rice to dry in a loss of 86-88% of the ochratoxin A present (Tren et al, 1971). OTA also is reduced during the production of cereals, due to extrusion cooking, by a maximum level of 40% (Castells et al, 2005).

The loss of OTA during extrusion is directly proportional to the temperature and the humidity (Scudamore et al. 2004). When the moisture content is 30% and temperature is between 116-120°C the OTA reduction is 12% reduction while at a temperature of 113-136°C the reduction in OTA level is 24%. With the moisture level at 17.5% and a temperature of 157-164°C the average loss of OTA is ~13%, and increasing the temperature to 191-196°C increases the average loss to ~31%. Increasing the residence time in the processing equipment also increase the proportion of the toxin that is lost (Scudamore et al. 2004).
γ radiation also reduces OTA production by *Aspergillus ochraceus* (Refai et al., 1996). In poultry feed, fungal growth ceases at 4 kGy of radiation. OTA production ceases at 3 kGy, and is reduced from 60 to 1.9 ng/g after exposure to 2 kGy. γ radiation in doses of 15 and 20 kGy, respectively, completely destroy ochratoxin A in yellow corn and soybeans. In poultry layer's and broiler's concentrates and cotton-seed cake however, the detoxification rates are 40%, 47% and 36%, at radiation dose of 20 kGy (Refai et al, 1996).
The lack of detection of OTA need not imply a reduction in potential toxicity, because the breakdown by-products can be as toxic as the ochratoxin A itself (Suárez et al, 2005).

Trichothecenes
The trichothecene mycotoxins produced by *Fusarium* spp., *e.g.*, deoxynivalenol, nivalenol, diacetoxyscirpenol and T-2 toxin, are found commonly in cereals *e.g.*, wheat, corn, barley, oats, and rye, which are used as raw materials for food and feed production world wide. Consumer exposure to trichothecenes results from consuming contaminated food products.

Deoxynivalenol
Deoxynivalenol is stable at 120°C, moderately stable at 180°C, and partially stable at 210°C in a weakly acidic environment, but is unstable under alkaline conditions (WHO, 2001). While DON levels were unaffected by heat treatment of 100-120°C at pH 4.0 and 7.0, heat treatments of 120°C for 30 min or 170°C for 15 min led to complete degradation of DON at pH 10 (Wolf and Bullerman, 1998).
The conditions used for baking bread and other leavened products, *e.g.*, cakes and biscuits, vary and variables such as fermentation conditions, dough additives, and the length and temperature of the baking process can all affect the amount of deoxynivalenol lost during the process. In some cases deoxynivalenol may not be detected after baking, but an isomer of deoxynivalenol of unknown toxicity may remain (Kushiro, 2008). Widely varying effects of deoxynivalenol on yeast have been reported. Deoxynivalenol levels increased

during the production of leavened products, which is attributed to the enzymatic conversion of its precursors (Young *et al.*, 1984). Some studies on the effects of additives in the bread on the levels of deoxynivalenol after cooking showed that adding potassium bromate-ascorbic acid or L ascorbic acid did not reduce deoxynivalenol levels, while the addition of sodium bisulfite, L-cysteine or ammonium phosphate could reduce deoxynivalenol levels by > 40% (Hazel et al, 2004). . An important level of DON reduction was observed during each of the processing steps from uncleaned durum wheat to cooked spaghetti. The average levels of DON were 77% in cleaned wheat, 37% in semolina, 33% in spaghetti and 20% in cooked spaghetti respect to uncleande wheat. (Visconti et al, 2004).

Pearling durum wheat reduces the level of deoxynivalenol contamination (Rios *et al.*, 2009). Approximately 45% of the deoxynivalenol is lost with the first 10% of the tissue removed, with an additional 25% of the deoxynivalenol (total reduction of 70%) removed with the removal of an additional 35% of the tissue. Thus, the pearling process efficiently reduces deoxynivalenol contamination and also may reduce or prevent recontamination of the starchy endosperm. In laboratory tests that simulate noodle production from deoxynivalenol-contaminated wheat with extrusion at 42°C and 80-100 bar, followed by drying and boiling for 7 minutes had an average decrease of 20% in deoxynivalenol concentration (Visconti *et al.* (2004). A limit of 0.75 µg/g of deoxynivalenol for the pulp is not guaranteed if the unprocessed wheat is contaminated at 2.0 µg/g. Baking reduces deoxynivalenol by 24-71% in bread and 35% in biscuits (El-Banna et al, 1983).
Loss of > 95% of the deoxynivalenol in maize may occur following extrusion at 150-180°C.
During the preparation of tortillas from contaminated maize, 72-88% of the deoxynivalenol is lost. Wolf Hall *et al.* (1999) reported stable levels of deoxynivalenol in maize flour and extruded pet food and that the deoxynivalenol is not degraded at the temperatures and pressures used in these processes. Soaking wheat in the presence of sodium bisulfite reduces detectable deoxynivalenol through the formation of sulfonate salts. These salts are stable under acidic conditions but may break down resulting in free deoxynivalenol under alkaline conditions.

In corn meal experimentally contaminated with deoxynivalenol at 5 mg/kg, moisture, temperature, and the addition of sodium bisulfite all affect the reduction of deoxynivalenol (Cazzaniga et al, 2001) .
In parboiled rice, deoxynivalenol contaminated 45% of the samples evaluated at between 180-400 ng/g (Dors, 2006). In a second study (Dors et al, 2009) the shortest soaking time (4 hours) and the lowest level of deoxynivalenol contamination (720 ng/g) led to the highest level of migration of deoxynivalenol into the endosperm starch. A six hour soak resulted in the least migration of deoxynivalenol into the endosperm starch.

The loss of deoxynivalenol from naturally and artificially contaminated Empanada clad coatings during preparation in corn oil were evaluated. (Samar et al, 2007). In this study deoxynivalenol naturally contaminated flour (1200 µg/kg) and fortified flour artificially contaminated (260µg/kg) were used to prepare turnover pie dough covers. Deoxynivalenol reduction was greater in the artificially contaminated samples (>66% at 169°C, 43% at 205°C and 38% at 243°C). For the level of 1200 µg/kg, the average percentage of reduction were 28%, 21% and 20% at frying temperature of 169°C, 205°C and 243°C respectively. The thermal decomposition products of deoxynivalenol are unknown, and there are no data on their toxicity, so there is currently no evidence that deoxynivalenol's thermal instability leads to the detoxification of human foods or animal feeds.

Zearalenone
Zearalenone is a chemically stable compound with a melting point of 164-165°C. The compound is stable at 120°C for 4 hours, but complete reduction occurs in buffer solution (Teorell and Stenhagen's citrate-phosphate-borate buffer adjusted to the three pH levels of 4.0, 7.0, and 10.0). within 30 minutes at 225°C (Ryu et al, 2003). During bread baking, the estrogenic activity of potential degradation products of zearalenone may be reduced (Scott et al, 1984). The extrusion of corn grits reduces zearalenone levels by 77-83% at 120°C, by 74-83% at 140°C, and by 66-77% at 160°C (Ryu et al, 1999). Of 32 samples of parboiled rice, 47% were contaminated with ZEA at levels between 317-396 ng/g (Dors, 2006). The best conditions for parboiling rice to reduce the migration of zearalenone into the endosperm include a soaking time of 4 hours (Dors *et al.*, 2009).

Fumonisins
The fumonisin mycotoxins have been shown to occur worldwide in maize and maize-based products, but the recently published European Commission report on tasks for scientific cooperation contains some occurrence data for fumonisins in wheat and wheat flour from France and Italy (EC, 2003).
Fumonisins are stable at 100-120°C. Boiling cultures of *Fusarium verticillioides* for 30 minutes does not reduce the levels of fumonisin B_1 present (Alberts et al, 1990).
When processed for 10-60 minutes at 100-235°C, FB_1 and FB_2 in aqueous buffers are most stable at pH 7 and progressively less stable at pHs 10 and 4 Jackson et al, 1996). Fumonisin decomposition begins at 150 °C and at temperatures > 175°C > 90% of the fumonisin B_1 and the fumonisin B_2 are degraded within 60 minutes, regardless of pH. Furthermore, the fumonisin B_1 hydrolysis products are toxic, although their mode

of action is not yet known. A survey of fumonisin contamination in cereals conducted in Spain reported FB1 in 8 of 17 wheat samples in the range of 0.2-8.8 mg/kg (mean) 2.9 mg/kg) and FB2 in one sample (0.2 mg/kg). (Castella et al, 1999). Although 19 Italian wheat, 11 durum wheat paste, and 27 wheat semolina samples were not contaminated, 87 of 91 wheat samples from France were reported to contain fumonisins at levels up to 1044 µg/kg. Of the 42 white wheat flour samples analyzed in Italy, 5 were contaminated at levels below 100 µg/kg; of the 214 white wheat flour samples from France, 76 were reported to be contaminated, mostly (93%) at levels below 100 µg/kg (EC, 2003). However data about the effects of processing on detoxification of fumonisins in wheat are not reportedm while several studies are carried out about corn processing.

Heating wet and dry corn flour at 190°C for 60 minutes reduces fumonisin levels 60-80%, while almost 100% of the available toxin is degraded after baking at 220 °C for 25 min (Scott and Lawrence, 1987). Corn muffins made from maize meal contaminated with 5 mg/kg of fumonisin B_1 and baked at 175 or 200°C lose 16% and 28% of the fumonisins, respectively, while a tortilla fried at 190°C for 15 min lost 67% of the fumonisins initially present (Jackson et al, 1997). If corn flour, either artificially or naturally contaminated with 5 µg/g of fumonisin B_1, was roasted at 218°C for 15 minutes, then no fumonisins could be detected (Castelo *et al.*, 1998). During the processing and packaging of fumonisin-contaminated products, fumonisins can bind to various components of the matrix and/or react with ingredients such as reducing sugars. For example, if fumonisin B_1 is incubated with D-glucose, then N-carboxymethyl-fumonisin B_1 results. Milling also differentially distributes fumonisins in the resulting products. In particular, fumonisin levels are lowest in the flour, and markedly higher in the bran and germ used for animal feed and/or oil extraction. During wet milling of corn, fumonisins may be dissolved in the process water or distributed amongst the by-products, but not destroyed. Fumonisins also may be transferred from contaminated grain to beer during the malting process, and then degraded by 3-28% during the fermentation (Scott *et al.*, 1987). Artificially contaminated samples of roasted polenta (5 µg/g of fumonisin B_1) and muffins made with naturally contaminated flour, did not lose a significant amount of fumonisins during their preparation (Castelo *et al.*, 1998). In general, fumonisins are more sensitive to dry heat than to moist heat.

The toxin level in cooked products may be underestimated due to the formation of fumonisin derivatives with unknown biological activity, *i.e.*, "hidden fumonisins", as in the case of corn flakes (De Girolamo *et al.*, 2001). In this process, fumonisin levels are reduced by 60-70%, with 30% of the losses attributable to extrusion at temperatures between 70°C and 170°C for 2-5 min. In other studies (Meister, 2001) extrusion and gelatinization reduce fumonisin levels by 30-55%, while toasting the flakes reduces the contamination an additional 6-35%. Producing corn flakes without sugar reduces the fumonisin B_1 present by 49%, while production in the presence of glucose results in an 89% reduction. If sugars other than glucose are present, then the reductions are similar to those observed for the process without sugar (Castelo, 1999). When maize is extruded at different temperatures, using a twin screw extruder at different temperatures (140, 160, 180 and 200°C) and different screw speeds (40, 80, 120 and 160 rpm), then the loss of fumonisin B_1 losses increase as temperature goes up and as screw speed goes down. In a study the pasteurizing milk contaminated with 50 ng/ml fumonisin B_1 and fumonisin B_2 at 62°C for 30 minutes did not significantly reduce the fumonisin contamination (Maragos and Richard, 1994).

Moniliformin

The heat stability of moniliformin is similar to that of other *Fusarium* mycotoxins such as deoxynivalenol and fumonisin B_1. Stability studies of maize and wheat that contain moniliformin at a level of 1 µg/g were held at 50, 100 and 150°C for 0.5-2 hours with a concomitant mean reduction in moniliformin of 55% (Scott et al, 1994). The reduction is correlated with increases in temperature and pH, with a reduction of 99% following heating at 175°C for 60 minutes at pH 10. Complete loss of moniliformin occurs when naturally contaminated maize containing 1.4 µg/g of MON is cooked in an alkaline medium as part of a tortilla preparation process (Pineda et al, 2002). The stability of moniliformin is comparable to or greater than that of other *Fusarium* mycotoxins when the raw materials were baked, fried, roasted or extruded (Pineda-Valdes *et al.*, 2003).

Discussion

Food processing has an impact on mycotoxins in wheat Cleaning removes broken and moldy grain kernels. The milling processes dilute and distribute mycotoxins High temperature processes cause varying degrees of reduction of mycotoxin levels, but most mycotoxins are moderately stable in most food processing systems. Aqueous cooking, steeping, roasting at high temperatures (above 150°C) and extrusion cooking reduce mycotoxins concentrations. However in the case of fumonisin, the fate of the toxin is unclear since studies in literature only regarding maize chain. Then, fumonisins can may be modified or matrix bound and be non-recoverable, but retain toxicity. The discrepancies between the findings reported in cited different studies may be due in large part to the analytical methods employed and to differences in laboratory equipment or experimental conditions employed, the concentration of toxin added, variation in sample source and/or

environmental factors. However the amount of reduction is highly dependent on cooking conditions, such as temperature, time, water and PH , as well as the type of mycotoxin.

References

Alberts JF, GelderblomWCA, Thiel PG, Marasas WFO, Van Schalkwyk DJ and Behrend Y, (1990). Effects of temperature and incubation period on production of fumonisin B1 by *Fusarium moniliforme*. *Appl EnvironMicrobiol* 56:1729–1733

Boudra, H., Le Bars, P., and Le Bars, J. (1995). Thermostability of ochratoxin A in wheat under two moisture conditions. *Applied and Environmental Microbiology* 61: 1156-1158.

Bullerman, L.B, and Bianchini, A. (2007) Stability of mycotoxins during food processing. *International Journal of Food Microbiology* 119:140-146.

Castella, G.; Bragulat, M. R.; Cabanes, F. J. (1999). Surveillance of fumonisins in maize-based feeds and cereals from Spain. J. Agric. Food Chem., 47: 4707-4710

Castells M.; Marìn S. V. Sanchis; A. J. Ramos (2005). Fate of mycotoxins in cereals during extrusion cooking: A review Food Additives & Contaminants: Part A: Chemistry, Analysis, Control, Exposure & Risk Assessment 22 (2): 150 - 157

Castelo, M.M., (1999). Stability of mycotoxins in thermally processed corn products. Section VI. Loss of fumonisin B1 during the corn flake process with and without sugars. PhD Dissertation. University of Nebraska, Lincoln NE.

Castelo, M.M., Sumner, S.S., and Bullerman, L.B. (1998) Stability of fumonisins in thermally processed corn products. *Journal of Food Protection* 61:1030-1033.

Cazzaniga, D., Basílico, J.C., González, R.J., Torres, R.L., de Greef, D.M., (2001). Mycotoxins inactivation by extrusion cooking of corn flour. Letters in Applied Microbiology 33:144–147.

de Girolamo A., Solfrizzo, M., and Visconti, A. (2001) Effect of processing on fumonisin concentration in corn flakes. *Journal of Food Protection* 64: 701-705.

Dors, G.C., Almeida, L.A., Badiale-Furlong, E. (2009) Migration of mycotoxins into rice starchy endosperm during parboiling process. *Food Science and Technology* 42: 433-437.

Dowling, T.S. (1997) Fumonisins and its toxic effects. *Cereal Food World* 42:13-15.

El-Banna, A.A., Lau, P.-Y., Scott, P.M., (1983). Fate of mycotoxins during processing of foodstuffs. II-Deoxynivalenol (vomitoxin) during making of Egyptian bread. Journal of Food Protection 46: 484–486

European Commission (2003).Collection of Occurrence Data of Fusarium Toxins in Food and Assessment of Dietary Intake by the Population of EU Member States; Reports on Tasks for Scientific Cooperation; European Commission: Brussels, Belgium.

Fokunang, C.N., Tembe-Fokunang, E.A., Tomkins, P., Barkwan, S. (2006). Global impact of mycotoxins on human and animal health management Outlook of Agriculture 35 (4): 247-253(7)

Hazel, C.M., and Patel, S. (2004). Influence of processing on trichothecene levels. *Toxicology Letters* 153: 51-59.

Hwang J-H., Lee K.G (2006). Reduction of aflatoxin B_1 contamination in wheat by various cooking treatments. Food Chemistry 98:71–75

Jackson L.S., Hlywka J.J., Senthil K.R., Bullerman L.B. and Musser S.M., (1996).Effects of time, temperature, and pH on the stability of fumonisin B1 in an aqueous model system. *J Agric Food Chem* 44:906–912

Jackson LS, Katta SK, Fingerhut DD, De Vries JW and Bullerman LB, (1997) Effects of baking and frying on the fumonisin B_1 content of cornbased foods. *J Agric Food Chem* 45:4800–4805

Katta, S.K., Jackson, L.S., Sumner, S.S., Hanna, M.A., and Bullerman, L.B. (1999). Effect of temperature and screw speed on stability of fumonisin B_1 in extrusion-cooked corn grits. *Cereal Chemistry* 76:16-20.

Kushiro M., Effects of Milling and Cooking Processes on the Deoxynivalenol content in wheat Int. J. Mol. Sci. 2008, 9: 2127-2145;

Lu, Z., Dantzer, W.R., Hopmans, E.C., Prisk, V., Cunnick, J.E., Murphy, P.A., Hendrich, S.(1997). Reaction with fructose detoxifies fumonisin B_1 while stimulating liver associated natural killer cell activity in rats. Journal of Agricultural and Food Chemistry 45:803–809.

Maragos, C.M., and Richard, J.L. (1994) Quantitation and stability of fumonisins B_1 and B_2 in milk. *Journal of AOC International* 77: 1162-1167.

Meister, U. (2001). Investigations on the change of fumonisin content of maize during hydrothermal treatment of maize.Analysis bymeans of HPLC methods and ELISA. European Food Research and Technology 213: 187–193

Méndez-Albores, F.DeJ.-F., Castañeda-Roldan, E., Aràmbula-Villa, G., and Morteno-Martìnez, E. (2004) . The effect of toasting and boiling on the fate of aflatoxins during pinole preparation. *Journal of Food Engineering* 65: 585-589.

Milanez TV and Leitão MF, (1996). The effect of cooking on ochratoxin A content of beans variety 'Carioca'. *Food Addit Contam* 13:89–93

Ozkarsli M, (2003). Effect of traditional-roasting and microwave-roasting on aflatoxin B_1 in peanuts. MSc Thesis, *Cukurova University Institute of Natural and Applied Sciences*, Adana, Turkey

Pineda-Valdes G, Ryu D, Jackson DS and Bullerman LB, (2002). Reduction of moniliformin during alkaline cooking of corn. *Cereal Chem* 79:779–782

Pineda-Valdes G, Ryu D, Hanna MA and Bullerman LB, (2003). Reduction of moniliformin in corn by heat processing. *Food Microbiol Saf* 68:1031–1035

Pineda-Valdes G, Ryu D, Jackson DS and Bullerman LB, (2002). Reduction of moniliformin during alkaline cooking of corn. *Cereal Chem* 79:779–782

Refai, M.K., Aziz, N.H., El-Far, F., and Hassan, A.A. (1996). Detection of ochratoxin produced by *A. ochraceus* in feedstuffs and its control by γ radiation. *Applied Radiation and Isotopes* 7: 617-621.

Reiss J., (1978) Mycotoxins in foodstuffs. XI Fate of aflatoxin B_1 during preparation and baking of whole-meal wheat bread. Cereal chemistry, 55(4):421-423

Rìos G., Pinson-Gadais L., Abecassis J., Zakhia-Rozis N., Lullien-Pellerin V. (2009). Assessment of dehulling efficiency to reduce deoxynivalenol and *Fusarium* level in durum wheat grains. *Journal of Cereal Science* 49: 387-392.

Romani S., Pinnavia, G.G., and Rosa, M.D. (2003) Influence of roasting levels on ochratoxin A content in coffee. *Journal of Agricultural and Food Chemistry* 51: 5168-5171.

Rustom IYS, (1997) Aflatoxin in food and feed: occurrence, legislation and inactivation by physical methods. Food Chem 59:57–67.

Ryu, D., Hanna, M.A., Bullerman, L.B., (1999). Stability of zearalenone during extrusion of corn grits. Journal of Food Protection 62 (12): 1482–1484.

Ryu D, Hanna MA, Eskridge KM and Bullerman LB, (2003). Heat stability of zearalenone in an aqueous buffered model system. *J Agric Food Chem* 51:1746–1748

Samar, M., Resnik, S.L., Gonzàlez, H.H.L., and Castillo, M.D. (2007) Deoxynivalenol reduction during the frying process of turnover pie covers. *Food Control* 18: 1295-1299.

Simionato EMRS and Sylos CM, (2004). Effect of cooking on the levels of aflatoxin B_1 and ochratoxin A in rice. *Braz J Food Technol* 7:167–171

Scott PM, (1984). Effects of food processing on mycotoxins. *J Food Protect* 47:489–499

Scott, P.M., and Lawrence, G.A. (1987). Liquid chromatographic determination and stability of the *Fusarium* mycotoxin moniliformin in cereal grains. *Journal of the Association of Official Analytical Chemists* 70: 850-853.

Scott PM and Lawrence GA, (1994). Stability and problems in recovery of fumonisins added to corn-based foods. *J AOAC Int* 77:541–545

Scudamore, K.A., Banks, J.N., Guy, R.C.E., (2004). Fate of ochratoxin A in the processing of whole wheat grain during extrusion. Food Additives and Contaminants 21 (5): 488–497.

Simionato EMRS and Sylos CM, (2004). Effect of cooking on the levels of aflatoxin B1 and ochratoxin A in rice. *Braz J Food Technol* 7:167–171

Subirade, I., (1996). Fate of Ochratoxin A during breadmaking. Food Additives and Contaminants 13 (Suppl): 25–26.

Trenk, H.L., Butz, M.E., Chu, F.S., (1971). Production of ochratoxins in different cereal products by Aspergillus ochraceus. Applied Microbiology 21: 1032–1035

Visconti, A., Haidukowski, E.M., Pascale, M., and Silvestri, M. (2004) Reduction of deoxynivalenol during durum wheat processing and spaghetti cooking. *Toxicology Letters* 153: 181-189.

WHO Food Additives Series 47, (2001). Safety Evaluation of Certain Mycotoxins in Food. Deoxynivalenol, pp 419-528

Wolf-Hall, C.E., Hanna, M.A., and Bullerman L.B. (1999) Stability of deoxynivalenol in heat treated foods. *Journal of Food Protection* 62: 962-964.

Yazdanpanah H., Mohammadi T, Abouhossain G, Cheraghali A. M. (2005). Effect of roasting on degradation of Aflatoxins in contaminated pistachio nuts Food and Chemical Toxicology 43: 1135–113

Young, J.C., Fulcher, R.G., Hayhoe, J.H., Scott, P.M., and Dexter, J.E. (1984). Effects of milling and baking on DON content on Canadian wheats. *Journal of Agricultural and Food Chemistry* 32: 659-664.

APPENDIX 3: QUALITY AND ANTIOXIDANT ACTIVITY AFTER *IN VITRO* DIGESTION OF APPLE JUICES AND PUREES

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(submitted)

Introduction

Quality of apple juices and puree can be affected by different factors like cultivar, geographical region, climate, cultivar practices, harvest (Picinelli et al, 1997), storage conditions (Addie A. Van Der Sluis et al., 2005, S. Perales et al, 2008), and processing (Addie A. Van Der Sluis, 2004, Cetin Kadakal, 2003, Valdramidis V., 2010).

The parameters considered relevant to describe the overall quality of fruit juices and their processed derivatives are the amount of 5-hydroxymethylfurfural (5-HMF), the reducing sugars and ascorbic acid content, and the total antioxidant activity.

Dietary recommendations include the consumption of apples and their derivatives mainly for their antioxidant properties, associated to the polyphenols and vitamin C content. These compounds can reduce the risk of coronary heart disease, carcinogenesis, aging processes, and can inhibit human low density lipoprotein oxidation (Boyer, 2004, Pearson et al, 1999, Dembinska-Kiec et al., 2008). Consumer trends show an increasing interest for fruit juices with a high natural antioxidants content, e.g. vitamins and polyphenols. On the other hand, thermal treatment, generally used to extend shelf life of fruit products, can also affect their quality. The most important transformations during the processing concern the loss of antioxidant compounds, non-enzymatic browning reactions (Rattanathanalerk M., et al. 2005), and formation of undesirable products like 5-hydroxymethylfurfural (5-HMF).

Various analytical methods have been reported to evaluate the real amount of antioxidant bio-available compounds in juices and puree, and an *in vitro* digestion procedure is needed.

Few studies have analyzed the antioxidant capacity of fruit juices after they have undergone the digestion process. The first aim of the present study is to investigate selected parameters considered relevant to describe the quality, like 5-HMF, reducent sugars, ascorbic acid, total antioxidant activity, total antioxidant activity in bio accessible fraction after *in vitro* digestion in 36 commercially available fruit derivatives, namely apple juices and apple puree obtained starting from conventional and biological agriculture produced fruits.

Materials and methods

Chemicals

Fehling's reagents A and B, 2,6-dichlorophenolindophenol sodium salt hydrate (DIF), potassium chloride (KCl), potassium thiocyanate (KSCN), monosodium phosphate (NaH_2PO_4), sodium sulphate (Na_2SO_4), sodium chloride (NaCl), sodium acid carbonate ($NaHCO_3$), urea, α-amilase, hydrochloric acid (HCl), pepsin, pancreatin, bile salts, 2,2'-azinobis(3-ethylbenzothialozinesulfonate) diammonium salt (ABTS), potassium persulfate ($K_2S_2O_8$), were obtained from Sigma-Aldrich (Steinheim, Germany). Acetonitrile, water, acetic acid for chromatography were purchased from Merck (Darmstadt, Germany). De-ionized water (<18MΩ cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Chromatographic solvents and water were degassed for 20 min using a Branson 5200 (Branson Ultrasonic Corp., CT, USA) ultrasound device.

Apple juices and puree

The apple juices and puree analyzed include: 16 apple-based nectars (fruit content: 30-60%), 15 apple-based juices (fruit content: 100%) and 5 apple-based puree. All were purchased from the local market (Napoli, Italy).

5-HMF determination

Extraction was carried in an eppendorf tube by adding to 1 mL of juice sample, 1 mL of purified water. The solution was centrifuged at 13000 rpm for 5 minutes and 20 µL of the aqueous phase were analyzed by HPLC.

The mobile phase was a mixture of acetonitrile in water (5 % v/v) at a flow rate of 1 mL/min under isocratic conditions. The column used was a Sphereclone (Phenomenex) (size 250 x 4,60 mm, pore size: 5 µm). The UV detector was set at 280 nm and 5-HMF was quantified using the external 306 standard method within the range 0.025-75 mg/L. The limit of detection (LOD) of the method was 0.010 mg/L. In these conditions the retention time for HMF is about 7.2 minutes (figure 1). Limit of quantification (LOQ) was 0.03 mg/L. All the analyses were performed in triplicate.

Reducing sugars determination

Reducing sugars determination was carried out according to Official method of sugar analysis (ICUMSA, 1994) known as Fehling's method, below described.

Twenty grams of the samples were weighted and transferred in a flask. The volume was adjusted to 150 mL by adding purified water. After a few minutes to allow the sugar dissolution, 10 mL of lead acetate and the minimum amount of sodium oxalate were added. Then, the volume of the resulting solution was adjusted to 200 mL and the solution shacked, filtered and transferred in a burette to carry out the titration.

Five mL of Fehling reagent A, 5 mL of Fehling reagent B and 40 mL of purified water were transferred in a flask. The solution was heated up to boiling point and the solution was added drop by drop till the nearly complete de-coloration of the Fehling reagent. Two drops of methylene blue were added, and the boiling continued for 3 minutes. Meanwhile, solution was added up to the disappear of indicator blue coloration and the toning into a red coloration. Each sample was analyzed in triplicate.

Ascorbic acid determination

Ascorbic acid determination was carried out according to AOAC official method (1990) using a titration with a solution prepared by weighting 50 mg of DIF and dissolving them in 50 mL H_2O added with 42 mg of $NaHCO_3$. The volume was adjusted to 200 mL. DIF was diluted with H_2O (1:30 for samples with poor vitamin C content and 1:5 for samples with higher vitamin C content.

Five mL of an aqueous solution of 10% acetic acid, CH_3COOH, solution were added to 2 mL of sample and this solution was titrated with DIF up to the onset of a permanent and soft pink colour.

***In vitro* digestion procedure**

Eight different products among 36 analyzed beverage were selected for evaluation of antioxidant power after procedure of *in vitro* digestion, including: apple juice (sample 8), apple with pear and banana juice (sample 13), apple and banana juice (sample 16), apple and pear with rice drops nectar (sample 28), green apple with aloe nectar (sample 31), apple and peach with flesh puree (sample 32), apple puree (sample 33), apple and soft fruit high-fibers puree (sample 35).

The *in vitro* digestion model was adapted from Versantvoort et al., 2004 with slightly modifications. It consisted of an initial saliva/pepsin/HCl digestion for 2 h at 37 ºC, to simulate the mouth and the gastric conditions, followed by a digestion with bile salts/pancreatin for 2 h at 37 ºC to simulate duodenal digestion. The samples were mixed with 6 mL of artificial saliva (KCl at a concentration of 89.6 g/L, KSCN, 20 g/L, NaH_2PO_4, 88.8 g/L, $NaSO_4$, 57 g/L, NaCl,175.3 g/L, $NaHCO_3$, 84.7 g/L, urea, 25 g/L, and α-amilase, 290 mg in 80 mL purified water. The pH of the solution was adjusted to 2 with HCl 6 N. Immediately, 0.5 g of pepsin (14,800 U) dissolved in HCl 0.1 N was added, and then incubated at 37 ºC in an orbital shaker (250 rpm) (Infors AG CH-4103, Bottmingen, Switzerland) at 55 rpm for 2 h.

After the gastric digestion, the pancreatic digestion was simulated. The pH of the solution was increased to 6.5 with $NaHCO_3$ 1 N and then 5 mL (1:1; v/v) of pancreatin (concentration 8 mg/mL), bile salts (concentration 50 mg/mL), dissolved in 20 mL of water were added. The solution was incubated at 37 ºC in an orbital shaker (55 rpm) for 2 h. Thirty mL of the mixture were then centrifuged at 4,000 rpm at 4ºC for 1h. The supernatant, constituting the bioaccessible fraction, was collected and the antioxidant activity was immediately evaluated.

Antioxidant activity determination

The procedure for the reagent 2,2'-azino-bis-3-ethylbenzotiazolin-6-sulfonic acid (ABTS) preparation was described by Pellegrini et al., (1999); a concentrate solution of the reagent (stock solution) was prepared dissolving 9.6 mg of ABTS in 2.5 mL of water and adding 44 mL of a solution (37.5 mg potassium persulphate in 1 mL of water). Stock solution was kept in the dark at 4 °C for 8 h before use; work solution was obtained from the stock solution by dilution using a 1:88 (v/v) ratio. Dilution was adjusted depending on the measured absorbance at wavelength 734 nm (A_{734}) in the work solution, until a value between 0.7 and 0.8. Subsequently, 100 µL of sample and 1 mL of work solution were added, and A_{734} was measured exactly after 2 min and 30 sec. Calibration curve for ABTS was obtained using Trolox, a water-soluble analog of α-tocopherol, as standard. Antioxidant activity was expressed as Trolox equivalent mmol.

Results and discussion

Table 1 reports data for the five quality descriptors that have been evaluated in this study, namely: 5-HMF, reducing sugars, ascorbic acid, and anti oxidant activity. The reported values refer to each analyzed category: juices, nectars, and puree. Even if some of the analyzed products were constituted by apple mixed to other fruits or enriched with fibres or fortified, as it can be seen in Table 1, the observed data can indicate the quality descriptors and also suggest possible source of variability in quality parameters.

5-HMF is naturally formed as a Maillard reaction (MR) product (Ames JM, 1998), and from dehydration reaction of hexoses in mild acidic conditions (Kroh, 1994). MR include condensation between reducing

sugars and amino acids, also called “caramelization reaction”, with ascorbic acid and pigment destruction (Cohen et al., 1998, Damasceno et al, 2008). 5-HMF has been considered a heat-induced-marker for a wide range of carbohydrate-containing foods, and is considered a marker for monitoring the heating process during food factory processing. This compound is formed from cyclization and dehydration of 3-deoxyosone, a dicarbonyl intermediate that can be formed by the direct caramelization reaction through the Maillard reaction by 1,2 enolization of the Amadori product.
Results for 5-HMF in analyzed samples show that the highest detected level in apple juices was 18.12 mg/L (sample 8) and the lower value was 0.06 mg/L (sample 2) with an average value of 3.76 mg/L. Two of the 15 analyzed apple juices were characterized by level of 5-HMF below the LOD (sample 11 and 12). The highest detected value in nectars was 28.61 mg/L (sample 30) while the lower was 0.24 mg/L (sample 21) and the average value for this commercial category was 3.47 mg/L. In apple puree samples, one out of five analyzed samples evidenced levels of 5-HMF below the LOD (sample 32). Values were between 0.14 and 0.24 mg/L and the average value was 5.14 mg/L. Three analyzed samples exceeded limit indicated for 5-HMF (samples 8, 30 and 35).
5-HMF content in apple juice concentrate properly produced and stored, was reported to be considerably lower than 10 mg/100 g (Babsky et al., 1986). More recently, Çetin Kadakal et al. (2003), reported levels of 5-HMF in a range between 2.07 mg/L and 10.14 mg/L after heat treatment and evaporation of apple juices.
More in general, and with reference to 5-HMF content in fruit derived foodstuff, Ulbricht, 1984, suggested a value up to 150 mg/day/person, while the Scientific panel on food additives, flavourings, processing aids and materials in contact with food (AFC) estimated an intake of 1.6 mg/day/person (EFSA 2005(a); Capuano & Fogliano, 2011). The International Federation of Fruit Juice Processors (IFFJP) recommended a maximum concentration of 5-10 mg/L 5-HMF in fruit juices and 25 mg/L in concentrates (Wagner, B. & Beil-Seidler, S. (2006).

Relatively to reducing sugars, no significative differences between the three analyzed categories can be observed. The highest level in apple juices was 4.05 g/100 mL detected in sample 28, and the lower value was 0.43 g/100 mL in sample 11. The average value for this category was 2.91 g/100 mL. The levels of reducing sugars in apple nectars, were comprised in a range between 0.25 g/100 mL (sample 28) and 4.05 g/100 mL in sample 30. The average value was 2.81 g/100 mL. For the apple puree, the highest level was 4.74 g/100 mL (sample 36) while the lower value was 0.54 g/100 mL (sample 35) and the average was 3.32 g/100 mL.
The observed data are in agreement with previously reported values of reducing sugar amount in fruit juices. Klockow et al., (1994) measured levels between 2.27 and 2.43 g/100 mL, while Karadeniz F. and Ekşi A. (2002) reported the levels of glucose in apple juices ranged between 0.93 and 3.22 g/100 mL while for fructose values were in a range 6.61-9.60 g/100 mL. Rodriguez et al., (2001) reported values of glucose between 2.78 g/100 mL and 3.18 g/100 mL and more recently Chinnici et al., (2005) reported for glucose values between 2.46 g/100 mL and 6.27 g/100 mL, and, for fructose, values between 2.22 and 7.54 g/100 mL. Eisele and Drake (2005) reported an average value of 2.01 g/100 mL and 5.69 g/100 mL for glucose and fructose, respectively. Our data do not vary significantly between the three analyzed typology of apple derivatives suggesting that the factory processing does not influence significantly the total reducing sugar content.

The antioxidant ability of the ascorbic acid, vitamin C, is very well known, however it is not generally included in foodstuff nutritional labels. As a reference value, it could be observed that Elkins et al. (1997), in a compositional characterization of commercially produced pineapple juice concentrate, reported for citric acid a value of 3%. Chinnici et al., (2005), and amounts varying from 0.52 to 5.61 g/L for ascorbic acid content.
In the analyzed juices, a product containing a mixture of apple and carrot (sample 2), was the richest in vitamin C, with a level of 18.89 mg/100 mL. The lower quantity, 11.92 mg/100 mL, was observed in sample 10. The average value was 15.41 mg/100 mL. In one case, the sample 31, the value reported on the packaging for vitamin C content (24 mg/100 mL) was higher than the measured level (17.96 mg/100 mL). This could be attributed to the storage or transportation of the product: a fraction of the ascorbic acid present in the juice degraded probably due to high temperature exposition. In analyzed nectars, the highest detected level for vitamin C was found in a product that, according to nutritional label, was fortified with vitamins. Referring to only apple nectars, the highest observed value was 13.31 mg/100 mL for sample 22, and the lower value was 11.92 mg/100 mL, sample 36. The average value was 18.01mg/100 mL. In apple puree, the maximum value was 21.22 mg/100 mL, detected in sample 35, a product containing apple and soft fruit enriched with natural fibres; the lower value was 11.92 mg/100 mL, observed in sample 36, and the average value was 15.82 mg/100 mL. In all analysed samples, the ascorbic acid content was quite high compared to reported literature data, and did not significantly vary between the three products typologies analysed, the lower values being observed in apple nectars as in can be seen in Table 1.

Addie A. Van der Sluis et al., (2002) described the effect of producing apples juices on polyphenolic antioxidant content, and activity. Raw juice obtained by pulping and straight pressing or after pulp enzyming had an antioxidant activity that was only 10 and 3%, respectively, compared to the antioxidant activity of the fresh apples. Most of the antioxidants were retained in the pulp rather than being transferred into the juice. In apple juice, 45% of the total measured antioxidant activity could be ascribed to the antioxidants still contained in the juice.

For the analysed samples, the levels of antioxidant activity in juices showed levels in a range between 0.21 (samples 9 and 10), and 5.40 mmol Trolox for a product containing apple and red fruits (sample 3). The average value was 2.16 mmol Trolox. In the case of nectars, values were between 0.37 (sample 31) and 2.21 mmol Trolox in a product containing also fresh apple fruit (sample 18), while the average value was 1.28 mmol Trolox. In apple puree, the levels were highest compared with the other analyzed categories; amounts were between 3.56 (sample 32) and 7.68 mmol Trolox, observed in a product containing apple and fresh fruit and also fibers enriched (sample 35), with an average value of 5.08 mmol Trolox.

The effects of the *in vitro* digestion on the antioxidant activity for 8 selected products are reported in Figure 1. There were considerable differences in the effects of an in vitro digestion procedure on the different juice kind. Five among the artificially digested products showed an increase of the antioxidant activity after the *in vitro* procedure (samples 13, 16, 28, 31, and 33) in a range between 0.05% (sample 16) and 2.04% (sample 31). On the opposite, samples 8, 32, and 35 showed a decrease of antioxidant activity, with values between 0.03 (sample 32) and 0.38% (sample 35).

Observed data, as reported in Figure 1, partially agree with previously reported studies. This allows to speculate on the possibility that exposition to the *in vitro* digestion conditions, cause a part of the active compounds to assume different structure with different chemical properties causing a possible underestimation of the total antioxidant compounds amount after the *in vitro* digestion. Reported studies in fact seem to suggest that it is possible to measure an increase or a decrease of the antioxidant activity.

Perales et al., (2008) observed that the bioaccessible fractions (maximum soluble fraction in simulated gastrointestinal media) of beverages obtained after an in vitro gastrointestinal digestion, had antioxidant activities significantly lower ($p<0.05$) than the original beverages. The loss of antioxidant activity was always lower than 19%, thus indicating the stability of the total antioxidant capacity under the applied conditions.

Recently however Lisa Ryan & Stuart L. Prescott (2010) reported that it is possible to observe an increase in the antioxidant capacity of red fruit juices after in vitro digestion. This could be due to an increase in anthocyanins content. Bermùdez-Soto et al. (2007) found an increase in a number of polyphenols after the gastric phase of the in vitro digestion process, while after the pancreatic digestion phase these antioxidants were degraded by the alkaline value of the pH. McDougall et al. (2005 a,b) found a decrease in antioxidants after in vitro digestion when analysing specific antioxidant compounds, rather than total antioxidant capacity.

Conclusion

The data observed for 5-HMF are in general higher then the suggested values as estimated by the International Federation of Fruit Juice Processors (IFFJP): maximum concentration of 5-10 mg/L 5-HMF in fruit juices and 25 mg/L in concentrates (Wagner, B., & Beil-Seidler, S. (2006). Our values range from 0.06-18.12 mg/L in juices, from 0.24-28.61 mg/L in nectars, and from 0.14-0.24 mg/L in puree, and could be attributed to a strong thermal treatment during the processing and manufacturing of the fresh apple fruits.

Our data for the reducing sugar amount do not vary significantly between the three analyzed typology of apple derivatives suggesting that the factory processing does not influence significantly the total reducing sugar content. In fact a range 0.43-4.05 g/100 mL, 0.25-4.05 g/100 mL, and 0.54-4.74 g/100 mL, for apple juices, nectars and puree, respectively, do not indicate any alteration of the sugar content related to thermal treatment during the processing or to the storage conditions.

In all analysed samples, the ascorbic acid content was quite high compared to reported literature data, and did not significantly vary between the three products typologies analysed, the lower values being observed in apple nectars. Interestingly a value of 11.92 mg/100 mL of vitamin C was measured as the minimum content of this compound in juices, nectars and puree. The higher levels were, 18.89, 13.31, 21.22 mg/100 mL for juices, nectars and puree, respectively.

Measured values for the antioxidant activity after an *in vitro* digestion partially agree with previously reported studies. It is possible to speculate that the *in vitro* digestion conditions, cause the structural change of some of the active compounds in structures with different chemical properties. Reported studies in fact seem to suggest that it is possible to measure an increase or a decrease of the antioxidant activity. The measured values seems to indicate that processing can have a major impact on the bioactivity of products. In fact values in the range 0-21-5.40 mmol of Trolox, 0.37-2.21, and 3.56-7.68 for juices, nectars and puree, respectively, seem to indicate that thermal treatment, more present in puree making process, affects more the anti oxidant activity.

Table 1. Levels of quality parameters: 5-HMF (mg/l), reducing sugars (g/100 mL), ascorbic acid (mg/100 ml), antioxidant activity (mmol Trolox) in 36 analyzed samples.

N.	SAMPLE	HMF (mg/L)	REDUCING SUGARS (g/100mL)	ASCORBIC ACID (mg/100 mL)	ANTIOXIDANT ACTIVITY (mmol Trolox)
	Juice 100%				
1	apple bio (*)	6.47±1.02	3.37±0.73	16.56±3.15	5.08±0.08
2	apple and carrot bio (*)	0.06±0.01	1.52±0.38	18.89±4.07	1.79±0.03
3	apple and red fruits	2.04±1.43	3.05±0.98	15.17±2.98	5.40±0.02
4	apple	1.39±0.31	3.63±0.99	17.02±3.34	4.56±0.02
5	apple and red fruits vitamin enriched	5.49±0.82	2.88±1.23	18.86±2.97	3.60±0.03
6	apple bio (*)	0.11±0.02	3.37±1.32	16.57±3.32	1.99±0.02
7	apple bio (*)	1.83±0.43	3.37±1.42	13.79±2.97	2.23±0.01
8	apple	18.12±2.92	3.37±1.23	18.83±4.33	2.39±0.02
9	apple from Trentino	0.30±0.02	3.37±1.42	14.24±3.41	0.21±0.01
10	apple	0.70±0.31	2.52±0.45	11.92±2.34	0.21±0.02
11	apple with ginger	n.d.	0.43±0.08	14.24±1.18	1.77±0.05
12	apple juice	n.d.	4.05±1.15	14.24±2.34	0.56±0.01
13	apple with pear and banana	1.96±0.23	3.16±1.12	14.23±3.01	1.42±0.03
14	apple	5.17±0.92	3.37±1.40	14.24±2.31	0.68±0.05
15	apple	5.26±0.98	2.23±0.65	12.38±1.89	0.51±0.04
	Nectars				
16	apple and banana	0.36±0.05	2.39±0.45	25.40±2.43	1.84±0.02
17	apple with pulp	1.41±0.63	3.16±0.76	25.87±3.21	2.05±0.07
18	apple with pulp	1.25±0.57	1.81±0.43	15.43±2.91	2.21±0.07
19	apple and banana	0.30±0.01	3.37±0.80	19.82±2.47	1.63±0.02
20	green apple	3.00±1.52	1.85±0.32	16.57±1.94	1.25±0.03
21	apple, carrots and lemon	0.24±0.02	3.61±1.73	18.89±2.33	1.85±0.07
22	apple	1.67±0.04	3.49±1.52	13.31±2.89	2.05±0.08
23	apple bio (*) with pulp	2.27±0.73	2.88±0.85	14.24±2.32	2.09±0.06
24	apple vitamin enriched light	1.16±0.57	2.65±0.97	30.52±3.88	0.85±0.04
25	apple and green fruits	0.88±0.07	2.88±1.42	14.24±3.04	0.46±0.04
26	apple and white fruits	1.32±0.10	2.88±1.20	14.24±2.88	0.95±0.07
27	apple	2.34±0,21	2.88±1.16	11.92±3.43	0.38±0.06
28	apple and pear with rice drops	7.76±1.23	4.05±1.32	15.17±3.50	0.53±0.04
29	apple and kiwi	2.01±0.84	3.37±1.25	16.56±3.45	0.46±0.02
30	apple vitamin enriched	28.61±3.56	0.25±0.02	14.24±3.24	1.55±0.05
31	green apple with aloe	0.95±0.12	3.37±1.14	17.96±2.33	0.37±0.01
	Puree				
32	apple and peach with flesh	n.d.	3.89±1.62	15.17±3.53	3.56±0.06
33	apple	3.37±0.88	4.05±1.76	16.57±4.34	3.68±0.07
34	apple	0.14±0.02	3.40±1.43	14.24±2.80	5.81±0.08
35	apple and soft fruit high-fibers	12.28±1.67	0.54±0.04	21.22±3.69	7.68±0.08
36	apple and soft fruit	4.75±1.71	4.74±1.41	11.92±2.61	4.65±0.06

(*) fruit from biological agriculture.

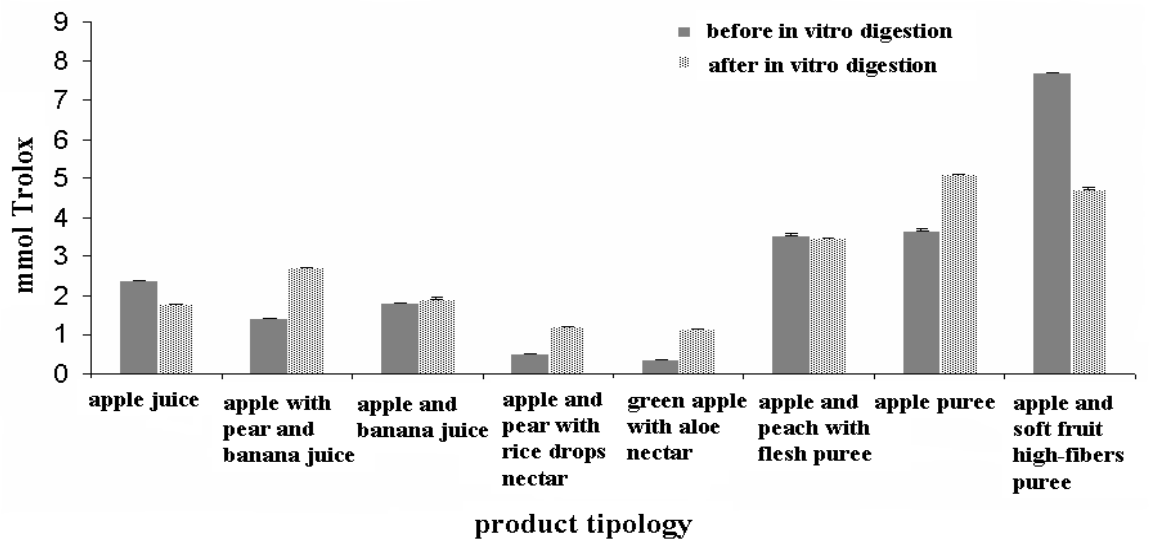

Figure 1. Antioxidant activity after and before *in vitro* digestion for the 8 selected products.

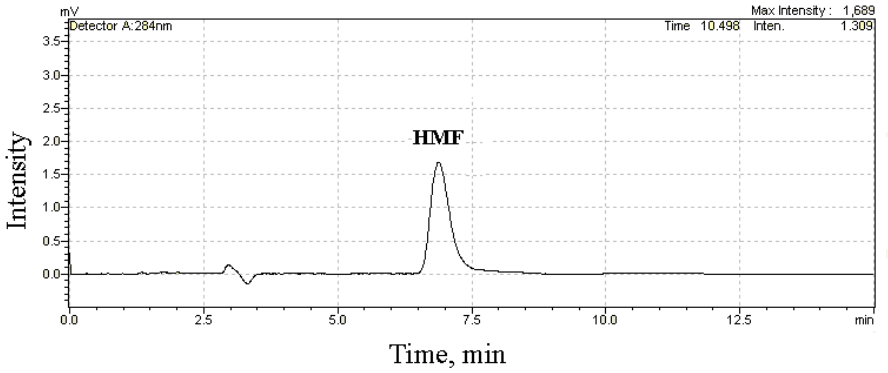

Figure 2: UV chromatogram of HMF standard (0.25 ppm)

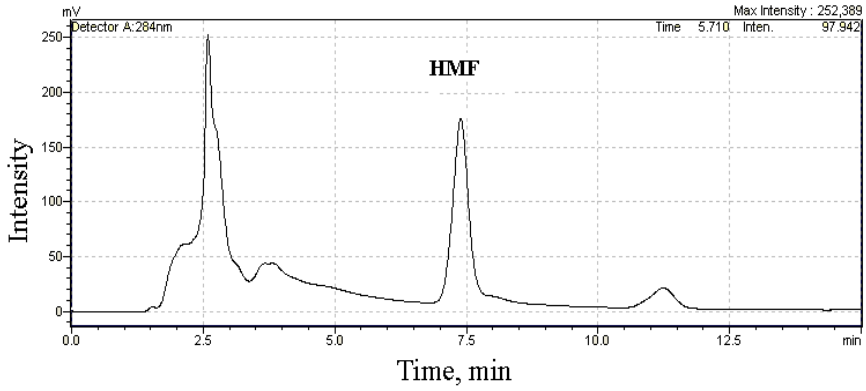

Figure 2: UV chromatogram of HMF in a sample of apple juice

References

Ames JM, (1998). Applications of the Maillard reaction in the food industry. Food Chemistry 62 (4): 431-439

Babsky, N. E., Torbio, J. J. & Lozano, J. E. (1986). Influence of storage on the composition of clarified apple juice concentrate. Journal of Food Science, 51: 564–567

Bermùdez-Soto, M.J., Tomàs-Barberàn, F.A. & Garcìa-Conesa, M.T. (2007). Stability of polyphenols in chokeberry (Aronia melanocarpa) subjected to in vitro gastric and pancreatic digestion. Food Chemistry, 102: 865–874.

Boyer J.& Liu R.H.(2004) Apple phytochemicals and their health benefits. Journal of Nutrition, 3(5) 1-15

Capuano E., Fogliano V.(2011). Acrylamide and 5-hydroxymethylfurfural (HMF): A review on metabolism, toxicity, occurrence in food and mitigation strategies. Food Science and Technology, 44 (4): 793-810.

Chinnici F., Spinabelli U., Riponi C., Amati A. (2005). Optimization of the determination of organic acids and sugars in fruit juices by ion-exclusion liquid chromatography. Journal of Food Composition and Analysis 18 (2-3):121–130

Cohen E., Birk Y., Mannheim C. H, Saguy I. S. (1998). A Rapid Method to Monitor Quality of Apple Juice During Thermal Processing. Lebensmittel-Wissenschaft und-Technologie, 31 (7-8): 612-616

Damasceno L.F, Fernandes F.A.N., Magalhães M.M.A. & Brito E.S. (2008). Non-enzymatic browning in clarified cashew apple juice during thermal treatment: Kinetics and process control, Food Chemistry 106 (1): 172-179.

Dembinska-Kiec A., Mykkänen O., Kiec-Wilk B., Mykkänen H. (2008). Antioxidant phytochemicals against type 2 diabetes. *British Journal of Nutrition*, 99: E-S1

EFSA. (2005a). Opinion of the scientific panel on food additives, flavourings, processing aids and materials in contact with food (AFC) on a request from the commission related to flavouring group evaluation 13: furfuryl and furan derivatives with and without additional side-chain substituents and heteroatoms from chemical group 14. EFSA Journal, 215: 1-73.

Elkins E. R., Loyon Roy, Huang and Matthys Allen (1997). Chacterization of commercially produced pineapple juice concentrate. Journal of food composition and analysis 10: 285-298.

Helrich. K. (1990). AOAC Official Methods of Analysis (15th edn ed.), AOAC, Virginia

International Commission for Uniform Methods of Sugar (ICUMSA) (1994). Report of the proceedings of the twenty first session, Havana, Cuba

Kadakal C. & Nas S. (2003). Effect of heat treatment and evaporation on patulin and some other properties of apple juice. Journal of the Science of Food and Agriculture, 83:987–990

Karadeniz F., and Ekşi A. (2002). Sugar composition of apple juices. European Food Research and Technology, 215 (2): 145-148.

Klockow A., Paulus A., Figueiredo V., Amadò R., Widmer H.M. (1994). Determination of carbohydrates in fruit juices by capillary electrophoresis and high-performance liquid chromatography. Journal of Chromatography A, 680: 187-200

Kroh L.W. (1994). Caramelisation in food and beverages. Food Chemistry, 51 (4): 373-379.

McDougall, G.J., Dobson, P., Smith, P., Blake, A. & Stewart, D. (2005a). Assessing potential bioavailability of raspberry anthocyanins using an in vitro digestion system. Journal of Agriculture and Food Chemistry, 53: 5896–5904.

McDougall, G.J., Fyffe, S., Dobson, P. & Stewart, D. (2005b). Anthocyanins from red wine – their stability under simulated gastrointestinal digestion. Phytochemistry, 66: 2540–2548.

Pearson D.A, Tan C.H, German J.B., Davis P.A., & Gershwin M.E. (1999). Apple juice inhibits human low density lipoprotein oxidation . Life Sciences, 64 (21): 1913-1920

Pellegrini N., Yang M., Rice-Evans C (1999). Screening of dietary carotenoids and carotenoids-rich frits extract for the antioxidant activities applying ABTS radical cation decoloration assay- Methods in Enzymology 299: 379-389.

Perales S., Barberá, R. M. J. Lagarda & Farré R. (2008). Antioxidant capacity of infant fruit beverages; influence of storage and in vitro gastrointestinal digestion. Nutrición Hospitalaria, 23(6): 547-553

Picinelli, A., Suárez, B. & Mangas, J. J. (1997). Analysis of polyphenols in apple products 204: 48 –51.

Rattanathanalerk M., Chiewchan N. and Srichumpoung W. (2005) Effect of thermal processing on the quality loss of pineapple juice. Journal of Food Engineering 66(2): 259-265

Ryan L. & Prescott S.L, (2010). Stability of the antioxidant capacity of twenty-five commercially available fruit juices subjected to an *in vitro* digestion. International Journal of Food Science and Technology, 45: 1191–1197

Rodriguez-Saona L.E., Fry F.S., McLaughlin M.A., Calvey E.M. (2001). Rapid analysis of sugars in fruit juices by FT-NIR. Spectroscopy. Carbohydrate Research 336 (1): 63–74

Ulbricht RJ, Northup SJ, Thomas JA. (1984). A review of 5-hydroxymethylfurfural (HMF) in parenteral solutions. Fundamental and Applied Toxicology, 4 (5): 843-853

Valdramidis V., Cullen P.J., Tiwari B., O'Donnell C. P. (2010). Quantitative modelling approaches for ascorbic acid degradation and non-enzymatic browning of orange juice during ultrasound processing. Journal of Food Engineering, 96 (3): 449-454

van der Sluis A.A., Dekker M., Skrede G., Jongen W.M.F. (2002). Activity and Concentration of Polyphenolic Antioxidants in Apple Juice. 1.Effect of Existing Production Methods Journal of Agricultural and Food Chemistry. 50: 7211-7219

van der Sluis A.A, Dekker M., Skrede G., & Jongen W.M.F. (2004). Activity and Concentration of Polyphenolic Antioxidants in Apple Juice. 2. Effect of Novel Production Methods. Journal of Agricultural Food Chemistry, 52: 2840-2848

van der Sluis A.A., Dekker M., & van Boekel M.A. J. S., (2005). Activity and Concentration of Polyphenolic Antioxidants in Apple Juice. 3. Stability during Storage. Journal of Agricultural. Food Chemistry, 53 (4):1073–1080

Versantvoort C.H.M., Oomen A.G., Kamp E.V., Cathy J.M. Rompelberg, Adriënne J.A.M. Sips (2005). Applicability of an in vitro digestion model in assessing the bioaccessibility of mycotoxins from food. Food and Chemical Toxicology 43: 31–40

Wagner, B., & Seidler, B.S. (2006). Furfural determining agents comprises an 4-amino-phenazone derivative and a barbituric acid and/or thiobarbituric acid derivative in an acidic medium. United States Patent.

Ringraziamenti

A chiusura di questi tre anni sento il dovere di ringraziare tutti coloro che hanno trasformato un ciclo di studi in un percorso di crescita su più fronti. Primo tra tutti il Prof. Alberto Ritieni, sempre pronto col suo sorriso ad incoraggiarmi a dare il meglio; i suoi consigli mi hanno aiutata ad affrontare il tutto con grande serenità. Un caloroso ringraziamento è rivolto a tutto il LABS, che mi ha accolto fin dall'inizio come una seconda famiglia, a partire dai veterani come Lia, Giulia, Monica, Paola, Alberto sempre disponibili a condividere non solo le proprie esperienze scientifiche, ma anche l'umanità e la sensibilità. Ringrazio i più giovani tesisti che mi hanno dato l'opportunità di trasmettere il mio interesse per il mondo scientifico. Un pensiero speciale va naturalmente alla mia famiglia, che da sempre mi sostiene in tutte le mie scelte e agli amici costantemente presenti in questi anni. Infine, ma non meno importanti sono i doverosi riconoscimenti rivolti agli istituti con cui ho collaborato nelle varie attività di ricerca, quali il CNR di Bari, il dipartimento di Scienze Animali, Vegetali e dell'Ambiente dell'Università del Molise, il laboratorio di tossicologia dell'Università di Valencia, il dipartimento di Chimica dell'Università di Catania, il dipartimento di Scienze degli Alimenti dell'Università della Nigeria. Questi istituti hanno permesso di confrontarmi con realtà diverse, un elemento indispensabile per l'approccio con la ricerca. In definitiva quest'esperienza costituisce una tappa fondamentale per la mia maturazione e sicuramente mi aiuterà ad affrontare la carriera futura con un atteggiamento più consapevole.