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DIPLOMARBEIT

Titel der Diplomarbeit

„The Occurrence of Zearalenone, α-Zearalenol, β-Zearalenol and Ochratoxin A in processed meat products as a result from maize feeding to pigs in Austria and their impact on human"

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Knowledge is of two kinds. We know a subject ourselves, or we know where we can find information upon it.

(Johnson)

The categories of human thought are never fixed in any one definite form; they are made, unmade and remade incessantly: they change with places and times.
(Durkheim)

Whatever is known has always seemed systematic, proven, applicable and evident to the knower. Every alien system of knowledge has likewise seemed contradictory, unproven, inapplicable, fanciful or mystical.

(Fleck)

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AGES	Agentur für Gesundheit und Ernährungssicherheit (Austrian Agency for Health and Food Safety)
ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
AST	Aspartate Amino-Transferase
ATA	Alimentary Toxic Aleukia
aw	active waterlevel
ELISA	Enzyme linked Immunosorbent Assay
FLD	Fluorescence Detection
GC	Gas Chromatography
GGT	Gamma-Glutamyltransferase
HPLC	High Pressure Liquid Chromatography
IAC	Immune Affinity Column
LC-MS	Liquid Chromatography-mass spectrometry
LD	Actatedehydrogenase
LD	Lethal dose
LOD	Level of Detection
LOEL	Lowest Observed Effect Level
LOQ	Level of Quantification
MCV	Mean Corpuscular Volume
MERCOSUR	Mercado Común del Sur (Southern Common Market)

MS	Mass Spectrometry
MW	Molecular Weight
nm	Nano Meter
NOAEL	No Observed Adverse Effect Level
NOEL	No Observed Effect Level
ppb	parts per billion (µg/kg, µg/l, ng/g, ng/ml)
ppm	part per million
ppt	parts per trillion
PTDI	Provisional Tolerable Daily Intake
PTWI	Provisional Tolerable Weekly Intake
SPE	Solid Phase Extraction
TDI	Tolerable Daily Intake
TLC	Thin layer chromatography
UV	Ultraviolet

1 Introduction and questions

There is animmense variety of fungi that grow on almost every kind of nourishment depending on climate distinctions. The infection of cereal and maize products with mycotoxins produces by fungicauses agricultural problems worldwide. Additional, it influences livestock and in a following step the health of humans. Mycotoxins are thermally stable and persists normally in processed food products. So as a fact it comes to mycotoxicoses. The total impact of these naturally occurring contaminants on human health cannot be estimated, due to insufficient toxicological information even for the most well documented toxins. The tolerable daily intake established by international working groups remains temporary or provisional [THUVANDER A. et al, 2001].

In relation to food and animal feed, next to aflatoxin B1 and M1,deoxynivalenol, fumonisins and ochratoxin A, zearalenone is one of the most important mycotoxins. However wheat, oats, as well as soybean products have been found to be contaminated occasionally with zearalenone, valid data in Europe indicate that maize is the most prominent cereal of high levels of contamination with zearalenone [EC, 2004]. Depending on climate, harvest, and storage conditions, maize and maize products show levels of zearalenone between 1 and 2900 µg/kg [KUIPER-GOODMAN T.; et al., 1987].

The presence of ochratoxin A in human blood has been reported for many countries.Levels greater than 0.1 µg/kg in more than 90 % of human and swine blood samplesin Europe have been detected[PETZINGER E.; WEIDENBACH A. 2002]. The toxin exposure is based on the ingestion of contaminated food and animal feed and the ochratoxin Acontamination of cereal is most prominent, even though contaminations are influenced by storage conditions post harvest. Products from animals can contribute to the ochratoxin A intake of humans. Especially pig is known to be sensitive to the toxin, with a tissue distribution following the pattern kidney > liver> muscle > fat [CHIAVARO E. et al. 2002].

In the past years a few studies have been carried out on the occurrence of Ochratoxin A in pork and sausages [JØRGENSEN K. 1998, GAREIS M. and SCHEUER R. 2000], but only limited data is available on the presence of ochratoxin A in ham.Information about zearalenone in processed products from pig israre. Ochratoxin A carryover from feed to meat has been shown experimentally [MADSEN et al. 1982]. The kidney is highly contaminated followed by liver, muscle and fat. Former studies found ochratoxin A levels of 4.6 and 3.2 µg/kg in blood- and liver type sausages. Humans may not be free of toxins because they are ingested with almost every meal. Research on the occurrence of mycotoxins and toxic fungi in Austria started in the mid seventies[ÖHLINGER R. et al. 2004].

A number of Aspergillus and Penicillium species producing ochratoxin A and growing on cereals and other plant substrates. It is very toxic to several animal species, in which the kidney being the main affected organ. Ochratoxin A inhibits protein synthesis [GAUTIER J.C. et al., 2001]. These mechanisms may generate neurotoxic, nephrotoxic and immunotoxic effects [MUNOZ et al. 2006]. The immune system appears to be the most sensitive among other sensitive organs and can be affected by very low concentrations of ochratoxin A in the ng/ml range.

Zearalenone and metabolites act as estrogens and can lead to hyperestrogenism and severe reproductive and infertility problems in swine [ROSENBERG E. et al 1998]. Zearalenone biotransforms in animals in two metabolites α-zearalenol and β-zearalenol.Furthermore, Zearalenone showed to be haematotoxic, hepatotoxic, immunotoxic and genotoxic, though the exact mechanism of Zearalenone toxicity is not completely established [ZINEDINE A. et al. 2007]. From bioassays a carcinogenic activity of zearalenone has been indicated. Furthermore the upcoming of public awareness of substances that mimic or interfere with the activity of natural hormones like zearalenone give cause to greater study of mycotoxins with estrogenic potential.

In conclusion, the exposure to zearalenone, α-zearalenol, β-zearalenol and ochratoxin A in Austria did not give rise to any major health concerns, but the

intake of several mycotoxins through the food chain emphasizes the need for preventive measures and improved control of toxin levels in further food items. Through the wide field of fungus kingdom and thereby emerging mycotoxins some efforts have already been started to develop multi-mycotoxin-detection for prospective food analyses (in Austria, IFA Tulln (Krska), CC Cluster Chemie Linz AGES GmbH (W. Brodacz)). This is quite a young field of research which is important to continue, to detect faster and more efficiently the presence for example of zearalenone, α-zearalenol, β-zearalenol, ochratoxin A and further mycotoxins in the food chain. Through globalization and open trade markets a peck of different mycotoxins in human nutrition will influence human health and measurements should be enforced to keep down contaminated food to lowest levels.

Questions:

- **The aim of this study is to assess zearalenone, α-zearalenol, β-zearalenol and Ochratoxin A concentrations in processed food products from pigs in Austria.**

- **Show the differences between field fungi (zearalenone) and storage fungi (ochratoxin A) and their effects in agriculture and livestock.**

- **Effects of zearalenone, α-zearalenol, β-zearalenol and ochratoxin A exposure to human health**

- **Novel strategies on mycotoxin prevention**

2 Review

2.1 Mycotoxins

2.1.1 Historical background

Mycotoxins are small (MW ~700 Dalton) toxic compounds that are formed as secondary metabolites by fungal species. These fungal species can colonies crops and contaminate them with toxins in the field or post harvest [TURNER et al., 2009]. Mycotoxins are capable of having acute toxic, mutagenic, carcinogenic, teratogenic, immunotoxic and oestrogenic effects in human and animals. Syndromes resulting from intake of mycotoxins by humans and animals are known as “mycotoxicoses”[VAN EGMOND et al., 2007].

Chinese used ergot alkaloids from fungi as medicinal preparations over 500 years ago where at the Middle Ages in Europe St. Antony’s Fire was mentioned after human consumption of ergot contaminated grain. During the late 19th and early 20th century the ability of fungi to carry out fermentations for food preparation were recognized. This development led to the interest in the toxicity of these products [RICHARD 2007], but the true chemical nature of the secondary metabolites from fungal was not known until recent times.

In 1922, the systematic study of fungal secondary metabolites began under the leadership of Harold Raistrick. He characterized more than 200 mould metabolites. Diseases like Alimentary Toxic Aleukia, caused by T-2 toxins from *Fusarium sporotrichioides*growing on harvested cerealsin Russia during the Second World War[RICHARD 2003] or similar diseases occurring in the USA in the 1960s, led scientists began a systemic approach to the study of mycotoxicoses. [RICHARD 2007].

In 1960, the sudden appearance of turkey-X disease that resulted in the death of more than 100.000 turkeys in the United Kingdom led to the discovery of aflatoxins [BLOUNT 1961]. At that time, mycotoxins were considered as a storage phenomenonbecausecereals become moldy during storage. These

secondary metabolites were proven to be toxic to humans and animals. Later on, aflatoxins and other mycotoxins were found to be formed also during development of crop plants in the field[RICHARD 2007].

Regulations gradually developed for mycotoxin in food and feed, since the discovery of the aflatoxins in 1960. They were established by industrialized countries.These limits often had an advisory or guideline character [VAN EGMOND et al.,2007]. Due to the fact that human food could be contaminated with mycotoxins led to more research efforts into all areas of mycotoxicology.Especially into their implications for human health and therefore analytical methods for the chemical determination of these new identified compounds [SHEPHARD 2008].

Moreover the mycotoxin problem became a multidisciplinary issue. Analytical chemists, microbiologist, agronomists, agricultural engineers, entomologists, plant pathologists, crop breeders, geneticists, medical and veterinary practitioners and producerswere involved.Among the mycotoxins of severe health concerns are the aflatoxins, deoxynivalenol, fumonisins, zearalenone, T-2 toxin, ochratoxins and certain ergot alkaloids. [RICHARD 2007].Therefore was a need for specialist laboratories due to the variety of structures of these toxins. For high sensitive analyses it was impossible to use one standard technique and these new settings created challenges for routine analysis.Until now, there have been several developments in detection of mycotoxins. Particular, the application of mass spectrometry in conjunction with for example HPLC for decreasing limits of detection has been of interest in recent times. Future trends would focus on rapid assays andtools for multiple toxin measurements from a single matrix[TURNER et al., 2009].

Due to the fact that mycotoxins pass through the food chain and are perilous for humans and animals governments had to focus on this problem. Through theincrease of the exchange of goods across national borders, and the lowering of trade barriers, governing bodies had todevelop regulations to ensure competitive trade of domestic food [KENDRA and DYER 2007].To protect the consumer from the harmful effects of these compounds, regulations

formycotoxins have been established in many countries. Until the late 90th mycotoxin regulations were mostly a national affair. Step by step, several economic communities (e.g. EU, MERCOSUR, Australia and New Zealand) harmonized their mycotoxin regulations.Thereby they overruled existing national regulations [VAN EGMOND et al., 2007].

Nowadays more than 100 countries have assumed regulation to limit the exposure of consumers to the adverse health risks from selected mycotoxins[FAO 2004].In 2003, the Council for Agricultural Science and Technology Report on mycotoxin listed the development of uniform standards and regulations for mycotoxin contamination in food and feed as one of the public policy goals for the 21st century [CAST 2003]. The regulations contain limits for aflatoxins (B1, B2, G1, and G2), aflatoxin M1, trichothecenes (deoxynivalenol, diacetoxyscirpenol, T-2 toxin and HT-2 toxin), fumonisins (B1, B2, and B3), agaric acid, ergot alkaloids, ochratoxin A, patulin, phomopsins, sterigmatocystin and zearalenone [VAN EGMOND et al., 2007].

2.1.2 Field fungi vs. Storage fungi – Two different problems

2.1.2.1 Field fungi - zearalenone

The following focuses on two mycotoxins. The first mycotoxin zearalenone belongs to the family of *Fusarium spp.* toxins. Most zearalenone is found in maize, but also found in other crops such as wheat, sorghum, barley, and rye throughout various countries of the world [CAST, 2003]. Generally, *Fusarium* species grow at moist and cool conditions.In wheat, sorghum and maize, zearalenone occurs in preharvest cereals but there is not enough information about the occurrence of zearalenone post-harvest [WHO 2000].Theappearance of zearalenone occurdifferent year by year, in variably cereal cropsas well as geographical areas [RICHARD 2007]. Austria fits to the preferred climate conditions of *Fusarium spp*. Referring to that recent survey on the Austrian mycotoxin situation showed that B-trichothecene, deoxynivalenol and zearalenone are the most important mycotoxins occur in Austria due to a field fungi problem [ÖHLINGER et al., 2004]. As a matter of fact feed for pigs contain mostly maize and other cereals. Moreover swine is the most significantly affected species and is more sensitive to zearalenone than, for example, rodents, cattle and poultry. The most considerable effect of zearalenone is that it causes estrogenic effects like precocious development of mammae in young gilts as well as prepucial enlargement in young barrows [RICHARD 2007].

The “journey” of zearalenone through the food chain starts here on the field and progresses by the biotransformation for zearalenone in animals due to the formation of two metabolites α-zearalenol and β-zearalenol [ZINEDINE et al., 2007]. The Austrian Agency of Health and Food Safety reported in their assessment form 2008 that the content of zearalenone and its metabolites could be determined in urine and liver from swine [AGES 2008]. Determined values of the metabolites α-zearalenol and β-zearalenol could also refer to illegal use of growth promoters (Zeranol; RalgroR).As zearalenone is accumulating in livestock, mycotoxins can reach humans through the food

chain.Depending on absorbed concentrations of this toxin, effects on humans were already noticed.

2.1.2.2 Storage Fungi – Ochratoxin A

The second mycotoxin mentioned in this thesis is ochratoxin A. Ochratoxin Aconcentrations have been found in foods of plant origin, in eatable animal tissues, as well as in human blood sera and tissues [KUIPER-GOODMAN and SCOTT 1989]. Ochratoxin Ais produced by several species of *Aspergillus* and *Penicillium* [PITT 2000]. But in contrast to the *Fusarium* toxins that grow well under Austrian climate conditions,as reported in the former studies, ochratoxin A is a mainly "imported" mycotoxin in Austria [ÖHLINGER et al., 2004]. Therefore it shows that mycotoxin contamination is not a national problem. To underline this, a study from Wu, 2004 indicates that "the United States, China and Argentina export more than 89% of the world's corn."[WU 2004].Under several effects of ochratoxin A mentioned above the most acute toxicity is in the kidney [SIMON 1996]. Furthermore it was tested that ochratoxin A elimination is slower in humans than in all other species[STØRMER 1992].Ochratoxin contamination is widespread in humans. Most people have detectable concentrations of ochratoxin A in their bloodstream, though usually at very low levels.It is imaginable that an amount of these levels is coming by nutrition [BAYMAN and BAKER 2006].

2.1.3 How to deal with mycotoxin contamination

To keep down related hazards, governments and scientists work together and try to find different strategies. On the one hand analytical chemists try to determine very low concentrations down to ppb and even ppt levels and develop more and more methods. Practical methods for high-sensitivity analysis and specialist laboratory settingsare a need for routine analysis

[TURNER2009]. Therefore itincludesadequate HPLC methods with UV or fluorometric detection. These are already used in both, research and legal enforcement of food safety legislation and for regulations in international agricultural trade. Other chromatographic methods employed for the determination of mycotoxins are Thin Layer Chromatography and Gas Chromatography.Furthermore LC-MS methods show new possibilitiesof analytical instruments, especially for multi toxin determination and for confirmation purposes [SHEPHARD 2008]. Most of these methods are expensive and time consuming, but for routine and rapid analysis immunological principles methods, like ELISA or Immunoaffinity Chromatography, have been developed and commercialized.On the other hand current discussions about mycotoxins and agricultural biotechnology reveal differences in risks and benefits. When faced with uncertainty, regulators willing do set limits as low as possible.

2.1.4 Zearalenone and its metabolites

2.1.4.1 Zearalenone

The discovery of estrogenic mycotoxins from the study of estrogenism was first reported by Buxton in 1927 where swelling and eversion of the vagina in young gilts were observed [BUXTON1927]. The major active toxin was named zearalenone by Urry et al. [URRY et al., 1966] who determined the structure in 1966 [KRSKA R. and JOSEPHS R. 2001].Besides estrogenic, alsohepatotoxic, haematotoxic, immunotoxic and genotoxic effects of zearalenone has been shown [ZINEDINE et al., 2007].

Zearalenone($C_{18}H_{22}O_5$) [6-(10-hydrox-6-oxo-trans-1-undecenyl)-β-resorcyclic-acid-lactone]),previously known as F-2 toxin, is a nonsteroidal oestrogenic mycotoxin. It is biosynthesized through a polyketide pathway by *Fusarium* fungi, including *F. graminearum (Gibberella zeae), F. culmorum, F. cerealis, F.*

equiseti, F. crookwellense and F. semitectum. They are common soil fungi and are regular contaminants of several cereals worldwide [BENNETT and KLICH 2003].

Zearalenone (318.36428 g/mol) is a white crystalline compound, which exhibits blue-green fluorescence when excited by long wavelength UV light (360 nm) and a more intense green fluorescence when excited with short wavelength UV light (260 nm). Solubility in water is about 0.002g/100ml. It is slightly soluble in hexane as well as benzene, acetonitrile, methylene chloride, methanol, ethanol and acetone [EMAN 2010].

2.1.4.2 α-zearalenol

α-zearalenol is a metabolite from zearalenone and can also be produced by Fusarium spp. Molecular formula is $C_{18}H_{24}O_5$ [2,4-dihydroxy-6-[6α,10-dihydroxy-trans-1-undecenyl]benzoic acid micro-lactone] and molecular weight 320.38016 g/mol.

2.1.4.3 β-zearalenol

β-zearalenol is a metabolite from zearalenone and can also be produced by Fusarium spp. Molecular formula is $C_{18}H_{24}O_5$ [2,4-dihydroxy-6-[6β,10-dihydroxy-trans-1-undecenyl]benzoic acid micro-lactone] and molecular weight is 320.38016 g/mol.

2.1.4.4 α-zearalanol (zeranol)

α-zearalanol (zeranol) ($C_{18}H_{26}O_5$, 322.39604 g/mol, melting point:178-185°C) is a metabolite of zearalenone as well as a non-steroidal oestrogenic growth promoter which increases live weight gain in food animals. It has been banned within the EU [COUNCIL DIRECTIVE 96/23/EEC, 1996]and Member States are prescribed to monitor foodproducing animals for possible abuse [COUNCIL DIRECTIVE 1996].

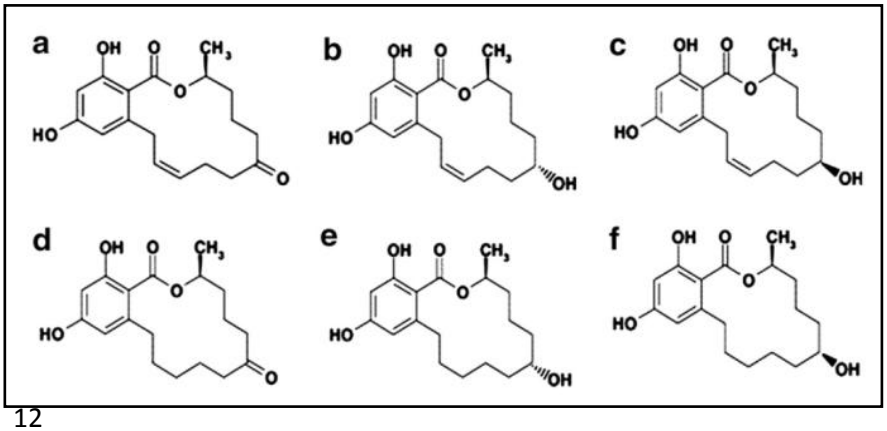
In the USA, zeranol has been widely adopted as a growth stimulant to improve fattening rates of cattle since 1969 [COUNCIL DIRECTIVE 1985]

2.1.4.4.1 Zeranol as growth promoter in livestock

It also has to be mentioned that due to the fact that food safety authorities investigate zeranol as a growth promoter in livestock an epidemiological study from Launay F. M. et al. 2004 show that zeranol is not a preferred growth stimulant.It describes data from involving four European Union control laboratories in which 8008 urine samples from bovine, porcine and caprine origin were screened for the presence of zeranol. Of these samples, 93.6% screened negative for zeranol and only 4 samples could be confirmed as true-positive. In this study it is also mentioned that it is currently impossible to determine whether the presence of zeranol (and/or taleranol) have its source from abuse or from environmental contamination. Zeranol is generally not the anabolic agent of choice for farmers to promote the growth of their animals. Drugs like β-agonists are much more effective growth promoters and have been much more widely abused in the EU and elsewhere [LAUNAY et al., 2004].

2.1.4.5 β-zearalanol (taleranol)

β-zearalanol, also named taleranol, is a mycotoxin and likewise a metabolite of zeranol. Molecular formula is $C_{18}H_{24}O_5$ [3,4,5,6,7,8,9,10,11,12-decahydro-7b,14,16-trihydroxy-3-methyl-1H-2-benzoxacyclotetradecin-1-one] and molecular weight is 322,39604 g/mol.



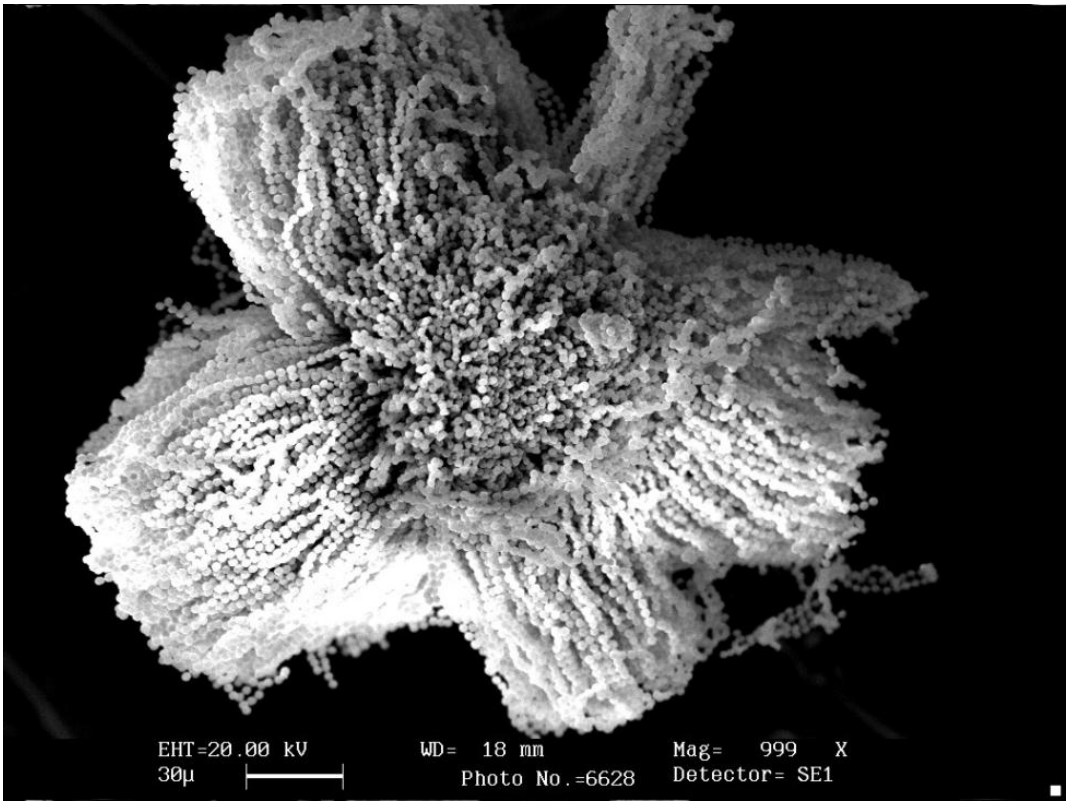
Source: ZINEDINE A. et al., 2007

Fig.1 Chemical structures of Zearalenone and its derivatives: (a) zearalenone, (b) α-zearalenol, (c) β-zearalenol, (d)

zearalanone, (e) α-zearalanol and (f) β-zearalanol

2.1.5 Ochratoxin A

First isolation of ochratoxin A was from *Aspergillus ochraceus* in South Africain 1965 [VAN DER MERWE et al., 1965]. This fungal secondary metabolitecan be produced by *Aspergillius ochraceus, Aspergillus niger,Aspergillus carbonarius, Penicillium verrucosum*, etc., which are known as “storage” fungi [BLUMENTHAL 2004].



Source: http://www.schimmel-schimmelpilze.de/images/aspergillus-rem-1000.jpg; Date: 6.12.2010

Fig.2 Aspergillus sp.; scanning electron microscope – Magnification 1000 Times

Ochratoxin A ($C_{20}H_{18}ClNO_6$, molecular weight: 403.82 g/mol) is a colorless, crystalline compound and its chemical name is L-phenylalanine N-[5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H2-benzopyran-7yl]carbonyl-(R)-isocoumarin [KOS and KRSKA, 2010]. The sodium salt is soluble in water. As the acid, it is moderately soluble in polar organic solvents like chloroform, methanol and acetonitrile and dissolves in dilute aqueous sodium bicarbonate.Ochratoxins consists of an isocoumarin and a phenylalanine

partlinked by an amide bond.The melting point is at 168°C. [STØRMER 1992].Ochratoxin A is chlorinated, which is unusual for natural products.

The ultraviolet absorption spectrum, which varies with pH and solvent polarity, shows maxima at 213 nm and 332 nm in ethanol. A maximum in the fluorescence emission spectrum can be observed at 428 nm[KOS and KRSKA 2010].The extinction coefficient in methanol and ethanol at 332 nm is in between 5500 and 6640 M^{-1} cm^{-1} [VALENTA 1998].

2.1.6 Mycotoxins in agriculture by using the example of maize

As we know now, a wide range of mycotoxins start their “journey” through the food chain by growth of crops all over the world. Placinta et al.stated: “It is possible that from a global perspective, *Fusarium* mycotoxins may be spread from one country to another with the increase in global grain trade.”[PLACINTA et al., 1999].

Although zearalenone is more a field mycotoxin than a storage toxin some reports show that high levels of zearalenone occurring naturally in some samples of maize based feedstuff. This could result from improper storage rather than development in the field [KUIPER-GOODMAN et al., 1987]. Additional forochratoxin A itis directly influenced by storage conditions after harvest.Especially in times of globalization, it means that preventing strategies against mycotoxin contaminationsuch as storage, transport, and processing conditions for cereal crops are very important. Badly treated maize crops that contain toxins, can be transmitted from moldy feed to swine from which a carryover to humans has been shown [PETZINGER and WEIDENBACH 2002].

Plenty of ochratoxin Acontaminated food was found in Europe. About 68.6% of 2374 food samples were examined to contain Ochratoxin A above the detection limit of 0.01 µg/kg [WOLFF 2000].

In the mid seventies due to the occurrence of mycotoxins and toxigenic fungi in Austria researches started on the estrogenic mycotoxin zearalenone as a feed contaminant that causes non specific sterilities in cattle in the province of Upper Austria [LENGAUER 1977]. In the winter of 1977/78 *Fusarium* toxins affected large stretches of Southeast Austria's maize growing areas. Massive feeding problems in swine caused by these toxins led to further surveys [LEW et al., 2001]. Due to early frost in September, heavy infestation of the ears of maize with *Fusarium graminearum* occurred, resulting in high deoxynivalenol concentrations, up to 20 ppm, in harvested maize.The consequence was that in numerous farms the pigs either refused intake of the ensiled maize kernel feed or throw up the feedthat they had reluctantly eaten [LEW et al., 1979]. About 20% of Austrian's overall arable land is involved. Mycotoxin research on maize plant in Austria increased because of the great importance of maize for feeding stuff. The influence of *Fusarium* species and their toxins on selection of varieties, fertilization, time of harvest and pest infestation being the main points of interest. From the report from Lew et al. it is noticed that: "it can be safely assumed that deoxynivalenol and its derivatives as well as zearalenone, moniliformin, beauvericin and nivalenol are the most common toxins contaminating local cereals"[LEW et al., 2001].

2.1.6.1 Mycotoxins on the field

Due to higher technology standards and better knowhow, storage fungi are not common in Middle Europe. To deal with field fungi like *Fusarium spp.* has more priority for prevention due to the fact that it is much more work to harvest less contaminated crops.

InAustria,cereal crops are harvested with 16% of moisture content inthe kernels. Field fungi need over 20% of moisture contents to cultivate. In contrast, Scandinavia cereal crops are harvested between 20 – 30% moisture content. Afterwardstheyare dried to 18 – 20% before storage [JOHNSSON and PETTERSON 1990].In Austria, the cereals stay until theyare ripe and dry at the

field. In this last vulnerable stage, the kernels can easily infected by*Fusarium*species. In Austria it is not common to find a corncob with massive *Aspergillus* infection.*Aspergillus* and *Penicillium*fungi are more typical Austrian storage fungi. They can occur when the crops are stored after harvesting, because of their moisture content or when they get moist afterwards.If cereals are storedbelow 13% moisture content (aw - Water activity: 0.65) after harvest, there is no subsequent fungi proliferation. Even at aw under 0.83 there is no significantly fungal growth and storagemycotoxins like ochratoxin A should not be of any concern.[LEW 1995]. The water activity means theratio of water vapor pressure above the product to the water vapor pressure over pure water at a certain temperature. This value is an important measure respective to the stability of nourishment and the influence of the occurrence of micro organisms, who demand different free water concentrations for their development.

Source: http://www.biologie.uni-hamburg.de/bzf/mppg/pat.htm

Fig.3 Infection of maize ears with F. graminearum; c: uninfected plant; wt: inoculation with wild-type conidia

Very high toxin concentrations are found in maize on the parts of the maize cob where the plant anatomy produces an incubator effect. Nevertheless only a few maize cobs are severely affected with *Fusarium*, mostly with *Fusarium sacchari var. subglutinans* and *Fusarium graminearum*(see Tab.1 and Tab.2) [LEW 1995]. Besides monoculture, moist weather and the cultivation of “lateripe” or vulnerable strains promotes pest infestations and hence *Fusarium* contamination. A study from Lew H. et al. shows that more than 80% of the maize cobs that were infected with *Fusarium sacchari var. subglutinans*were damaged by the European corn borer(*Ostrinia nubilalis*). The caterpillar drills into the maize cob and benefits fungi infection, so that mould could emerge in the area of the borehole.Only 15% were infected with Fusarium graminearum after damages by the corn borer [LEW et al., 1991].

An additional factorcausing*Fusarium*toxin contamination is climate changes.In the last years hotter, drier summers and milder, moist winters support fungal cultivation.*Fusarium spp.*often colonizes maize during heyday and produces deoxynivalenol and zearalenone. The fungi grow through the maize cob and furthermore into the kernels. Hence the toxin concentration of the inner part ismany times higher as in the kernels [LEW 2001].

	Ø Deoxynivalenol (mg/kg)		**Ø Zearalenone (mg/kg)**	
n	Kernels	Cobs	Kernels	Cobs
12	50	185	6	140

Source: LEW H. 2001

Tab. 1Concentration of toxins in kernels and cobs of maize ears visibly infected with fusaria

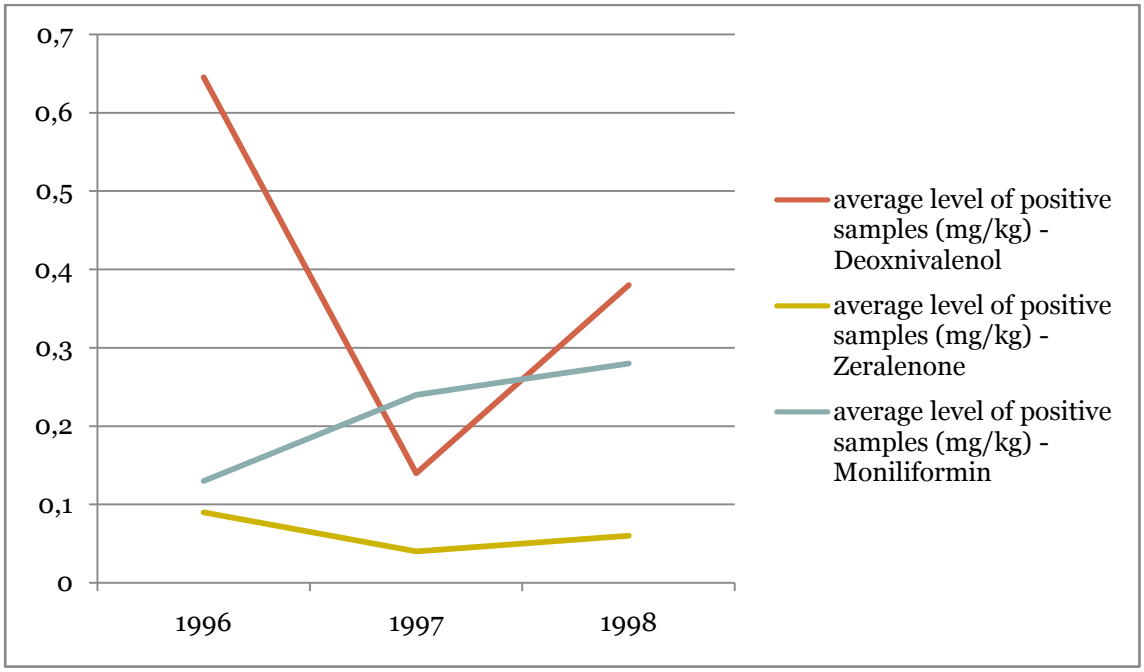
Furthermore the harvest time is a main factor for the farmer to lower the toxin concentration in his crops. The sooner the maize is harvested, the lesser is the hazard of moldy crops and mycotoxins.

Crop year	1996	1997	1998
Infected cobs	482	289	438
Analyzed cobs	343	228	316
***Fusarium* species**			
F. graminearum	47	16	31
F. subglutinans	29	32	33
F. avenaceum	14	23	17
F. poae	2	17	4
F. proliferatum	2	5	7
F. equiseti	1	2	2
Other *Fusaruim* spp.	5	7	6

Source based on:http://www.boku.ac.at/diebodenkultur/volltexte/band-52/heft-3/lew.pdf

Tab. 2Frequency distribution of Fusarium species on maize ears.

The three investigated growing periods 1996, 1997, 1998, show significant differences of the species assemblage and therefore resulting toxin values concerning the climate conditions (see Tab.3).



Source: http://www.boku.ac.at/diebodenkultur/volltexte/band-52/heft-3/lew.pdf

Tab. 3 Diagram based on information of:Fusarium toxins in maize grain samples of the 1996, 1997, 1998 crop

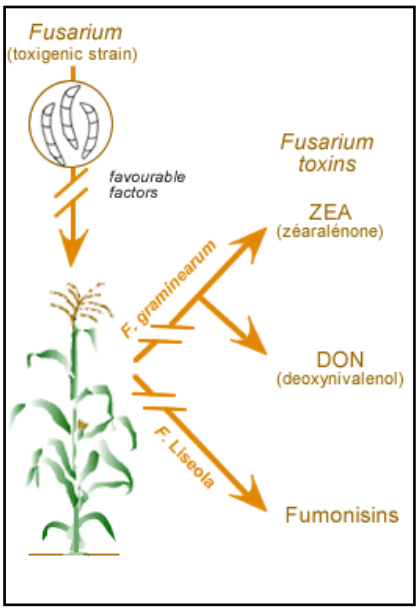
2.1.6.2 Fungicides usage and cultivation

Detailed preventing strategies against fungi contamination on the field on dependent weather conditions e. g. temperatures over 18°C and rain in blooming period are necessary particularly for high sensitivevarieties. For this the usage of fungicides could not be avoided. The operational application of the fungicides has to be in the development at stage of the blossom. Ideal timeframe is about two to four days after infection by fungi. Unfortunately the timeframe of the blossom varies from plant to plant inside a field and the ideal usage of the fungicides is easy to define.

Most fungicides eliminate the competitive flora of *Fusarium* species. Due to this missing of competitors it led to the promotion ofthe growth of *Fusarium*itself [LENGAUER 1977].At the same time,increasedfertilization, particularly high nitrogen doses, increase the sensitivity of maize varieties towards *Fusarium spp*.Leaves and stalks can also contain high concentrations of deoxynivalenol and zearalenone, because after infection for example with

Fusariumgraminearum through moist weather conditions (like in summer and autumn 1996 in Austria) leaves can die off after a few days and stalks get fragile[LEW H. 1995].

Maize cultivation produces more harvest residuals than any other crop. At the end of the vegetation period stalks are highly infected with *F. graminearum* (see Fig.4). The fungi overwinter in and on the maize residuals and there they develop their fruiting bodies (perithecien). At favorable weather conditions the asci, the sexual spore-bearing cell produced in the perithecien, could easily attack the ear of the crop (see Fig. 5) [MEINERT 2003].



Source: http://upload.wikimedia.org/wikipedia/commons/6/6a/F.graminearum.jpg

Fig.4Fusarium graminearum

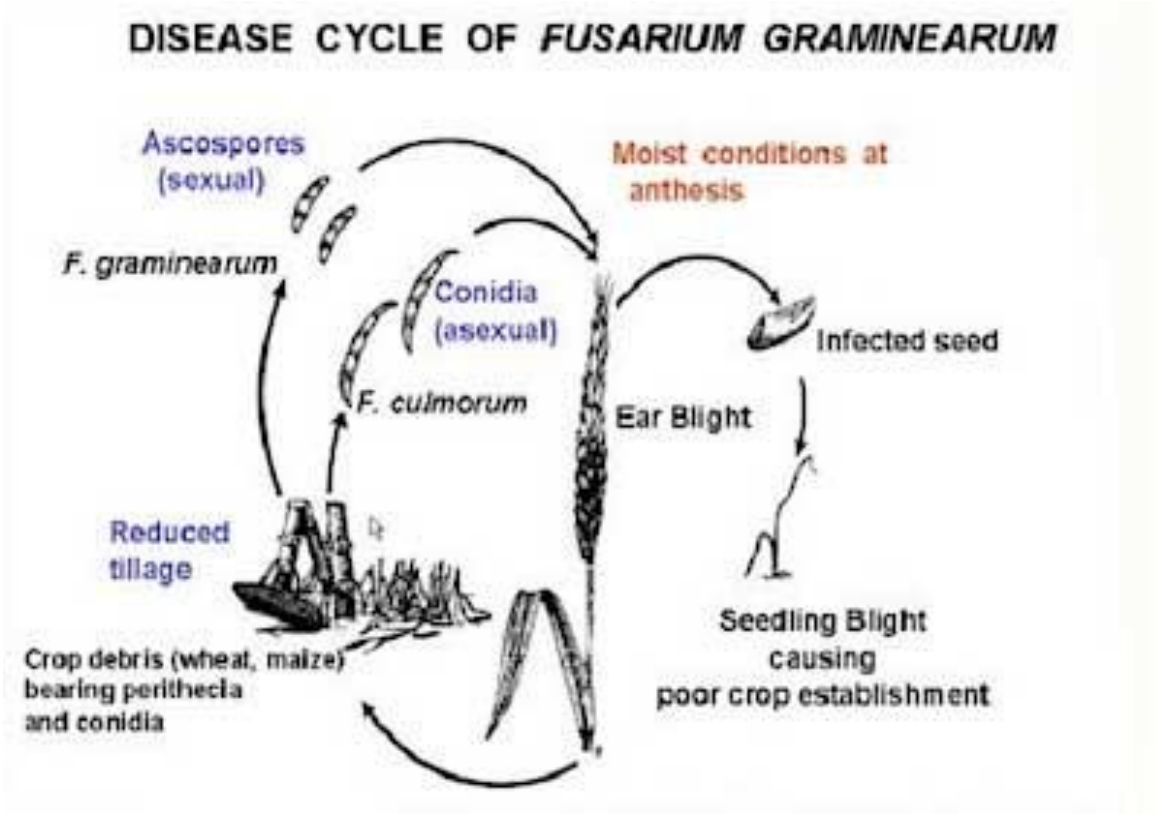
Source: http://www.maisadour-semences.fr/maitriser-les-fusarium-gestion-du-risque-mycotoxines/en-fusarium-mais.php?menu=introduction&page=lexique&page2=fusarium

Fig.5The main mycotoxins are zearalenone and deoxynivalenol produced by F. graminearum

The cultivation of the soil can have significantly influence on fungi exposure. In some areas plant residuals stay on the surface of the soil after harvesting instead of plowing them into the ground. This helps against water erosion near hillsides. Furthermore it is a method to avoid that plant protection products

reach the surface water.The plant residuals which remain on the surface provide an infection potential for the whole vegetation period. In contrast to this, plowing could reduce the proliferation of *Fusarium spp*. over winter by forwarded degradation of infected material in deeper bottomset beds, especially at preceding crops with high hazard potential like wheat and maize.

The picture below showing the vicious cycle indicates that crops are infected with perithecien of *Fusarium graminearum* season by season.



Source:http://www.bayercropscience.co.uk/content.output/554/556/Crop%20Centre/Cereal%20Fungicide/Ear%20Disease%20Complex.mspx

Fig.6Fusarium species are a component in stem base disease and this provides the spores for infection of the ears

2.1.6.3 Mycotoxins and storage

Primarygoal of prevention strategies is to bring uncontaminated crops into storage facilities and try to prevent further mycotoxins development through appropriate storage conditions.

In Austria massive *Aspergillus* contaminated maize cobs are rare. *Aspergillus* and *Penicillium* are typical storage fungi who ordinarily appear after harvesting moist crops [LEW 1995].In northern parts of Europe where crops are harvested by moisture content between 20 – 30% and not properly dried before storage, especially *Penicillium verrucosum* can proliferate and produce ochratoxin A. But also in south eastern parts of Europe ochratoxin A can occur due to improper storage conditions [LEW 2001].

On the one hand “field hygiene” is important to prevent overwinter of fungi, on the other hand measurements should be used to bring in clean crops in storage facilities. Plant residuals that are left on the surface can provide different kinds of diseases for further crop generations (see Fig 7).

Source: http://www.organicfarmermagazine.org/wp-content/uploads/2010/09/P1010331.jpg

Fig.7Crop residuals – maize

On the following pictures different storage possibilities are shown. Discriminative practices of crops like open storage or closed facilities, stored cobs, kernels or ground kernel,result in different amount of work and also hazards of mycotoxin contamination.

Source: http://lh6.ggpht.com/_5oS_IghpzYw/SvVl5gee_VI/AAAAAAAAAys/Eq-nMohorjs/Biketour+KA-ULM+%28Sep-Oct+%2709%29+016+%5B1024x768%5D.jpg

Fig.8Open Storage facility

Source: http://www.farmersguardian.com/Pictures/inline/s/i/j/Crops_grain.jpg

Fig.9Maize Storage

Source:http://www.siskiyou-county-online.com/Agriculture/IMAG018.JPG

Fig.10 Maize Storage Facility

If the operating possibilities are limited, e.g. ventilation systems with more than 65% moisture content, preservatives are required. For this purpose organic acids like propionic-, formic- and sorbic acid, urea and maybe sodium hydroxide solution can be used.Due to the fact that mycotoxins are robust compounds, heating is not an effective measurement. Mycotoxins remain stable and the only effect is that the germs content is decreased and the shelf life is improved [COENEN 2003]. After proper ensilage fungi cannot proliferate because of rapidly decreasing oxygen shortage and increasing CO_2concentrations. Therefore no further mycotoxin production occurs, but already produced toxins persist. [LEW 2001]

2.1.6.4 Impact of mycotoxins in feed for swine

In recent years, increasing attention has been paid to the riskof chemical contaminates or residues in animal feed, according to the appearance of various cases of eggs, milk, or animal products. Compounds like hormones, antibiotics, dioxins, etc. have been found in feed either deliberately, from malpracticeor sloppy manufacturing practice. Particular attention should be paid on highly lipophilic compounds such as mycotoxins. The toxins can accumulate in organisms in which the transfer factor depends on the lipophilicity of the compound, the potential accumulation of the compound in animal matrices, and/or the feeding level and feeding period.The highest transfer factor determined in the order to fat>edible offal>meat>eggs>whole milk [LEEMAN et al., 2007].

A major factor of hazard potential to health and performance in livestock is the influence of the quality of the feed which is significantly affected by microbial contamination, thereby causing feed spoilage including mycotoxins. Therefore best conditions of all factors should be warranted, especially husbandry, feeding and disease prevention[HÖRÜGEL et al., 2003].

In 1996, massive contamination of maize and cereal with *Fusarium* fungi has been determined in different parts of middle Europe [HÖRÜGEL et al., 2003]. Between 2004 and 2006 zearalenone was detected around 90% in unprocessed maize kernels samples. Over 200 µg/kg of zearalenone concentration could be determined in 25% of the samples. Guideline value for feed is 100 µg/kgand so only 50% of the unprocessed maize kernels could be used [ÖHLINGER 2008].However, ochratoxin A were found very rarely in Austrian cereals (median <0,0002 mg/kg) [ÖHLINGER et al., 2004].

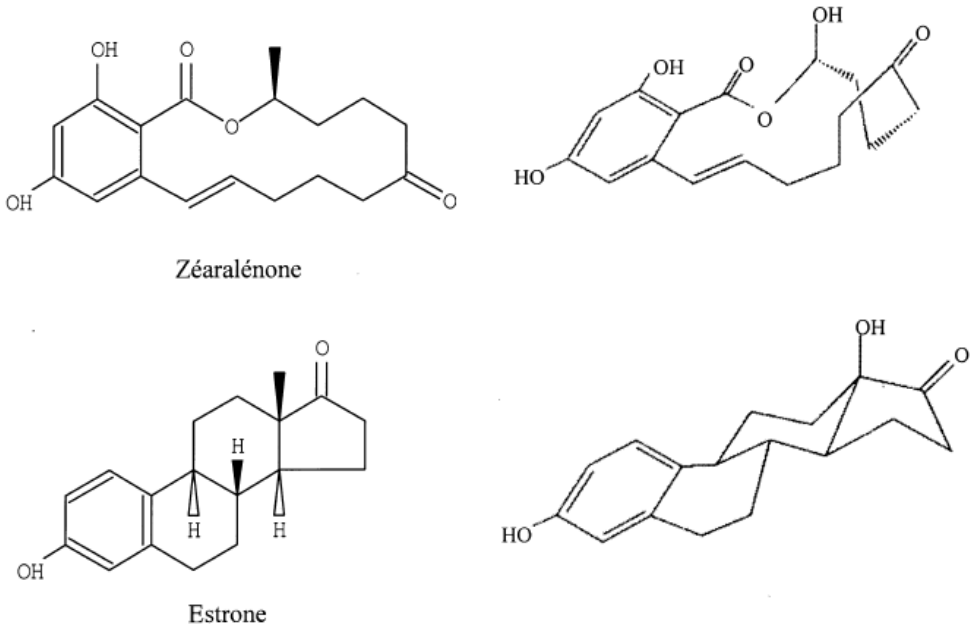
2.1.7 Livestock exposure

2.1.7.1 Effects on Livestock

To perceive the effects of mycotoxins in feed given to swine are not easy because typical diseases are not often triggered. Monogastric animals like pig or chicken are more vulnerable than ruminants due to the inactivation of mycotoxins in the rumen. Furthermore cases of loss are often caused by multitoxin attacks with unspecific health and performance depression. Feeding experiments show decreasing littering rates after giving *Fusarium* toxin contaminated dried feed over the whole pregnancy to sows. Significantly lower weight of the piglets and less vitality of the newborns were proven. Moreover the used feed is often not available anymore and retrospective investigations of mycotoxin content cannot be carried out.Experiments with 2000 sows that got fusariumtoxin contaminated feed after a strong *Fusarium* infestation on the field in this year, led to fertility- and litter rates by young and older sows. It was slowly improved after given unsuspicious feed from a new harvest of the following year [HÖRÜGEL et al., 2003].

2.1.7.2 Effects of Zearalenone on animals

Zearalenone can cause severe infertility and reproductive problems in animals [HÖRÜGEL et al., 2003].The structure of Zearalenone is similar to estrogens (see Fig. 11). The mycotoxin can act like estrogens because they can adopt a conformation which sufficiently resembles 17β-estradiol and other natural estrogens able to bind to the estrogen receptor [KRSKA and JOSEPHS 2001].



Source: http://www.revmedvet.com/2001/RMV152_219_234.pdf

Fig.11Structures of Zearalenone and Estrone

To affirm the fact that feed from the silo could also contain mycotoxins, it has been shown that green maize with high fusariumtoxin concentration after harvest, was still containing the toxin after some month.The silo feed was given to porker and approximately after four weeks female animals show typical hyperestrogen syndromes, particularly swollen vulva (see Fig.12) and mating behavior, which continued until the slaughtering over several months. The uteri of the slaughtered animals were significantly bigger and edematised. Additionally, high concentrations of zearalenone and derivates could be detected in the bile[HÖRÜGEL et al., 2003].

Source: http://www.apsnet.org/edcenter/intropp/topics/Mycotoxins/Pages/Zearalenone.aspx,

Date: 06.12.2010

Fig.12Zearalenone may cause vulvovaginitis in young pigs

2.1.7.3 Further toxin effects of mycotoxins for domestic animals

In the following table the reproductive dysfunction due to zearalenone in pigs can be seen as reported by Gaumy et al. [GAUMY et al., 2001].

premature gilts	1 – 5 ppm	vulva swelling, vulvovaginitis, nipple enlargement
mature non pregnant females	6 – 9 ppm	Anestrus and maintained corpora lutea, Infertility, pseudo-pregnancy
	20 ppm	Ovarian and mammary anomalies
Pregnant primiparous	>30 ppm	Inhibition of fetal development, embryonic mortality
Reproductive sows	>30 ppm	Infertility, return into oestrus, pseudo-pregnancy
Premature males	30- 40 ppm	Delayed puberty, depression of spermatogenesis and libido
Boars	200 ppm	No anomaly

Source: GAUMY et al., 2001

Tab. 4Reproductive dysfunction due to zearalenone in pigs

2.1.7.4 Effects of Ochratoxin A on animals

Ochratoxin producingfungal species occur worldwide.It is a contaminant of agricultural commodities, especially in cereals, but can also affect a variety of other food commodities.Contamination is often a result of poor storage or poor agricultural practice during drying procedures.The toxicological profile of ochratoxin A includes teratogenesis, nephrotoxicity and immunotoxicity and causes mycotoxicoses in animals, particularly in swine [KOTOWSKI et al. 1993]. It is also rated as being carcinogenic in animals and the International Agency for Research on Cancer classified these toxins as a possible human carcinogen (group 2B) [WALKER 2002].The high affinity of ochratoxin A to proteins, especially to serum albumin, allows its transport in the organs of animals [HAGELBERG et al. 1989] In fact, animal products and tissues for human consumption may present ochratoxin A residues, even if the animal has been nourished with low contaminated feed. In many studies, ochratoxin A has been detected in different parts from pigs, such as blood, kidney, liver muscle and adipose tissue [MILICEVIC et al., 2008].Ochratoxin A was reported as a strong nephrotoxin for all kinds of mammals. ALowest Observed Effect Level of ochratoxin A about 8 µg/kg body weight per day could be derived from short term feeding studies in pigs [JECFA 2001]. At these levels, changes in nephritic specific enzymes and changes in elimination functions of the kidney occurred [ELLING 1979].

2.1.7.5 Effects of mycotoxins to the immune system in animals

Mycotoxins can have different effects on the immune system, like inflammation, humoral immune response and cellular immune response. These effects on animal health could be increasing vulnerabilityfor infections. Reactivation of sub-clinical infections, decreasing vaccine efficacy and decreasing drug efficacy can be further consequences.

In a study form Stoev et al., experimental trails have been done to show that ochratoxicosis can have increasing effects on bacterial infection in pigs [STOEV et al., 2000].

Type of bacterial infection	Control	OTA 1 ppm	OTA 3 ppm
Salmonella cholerasuis (day 13)	0/6	2/6	6/6
Serpulina hyodissenteria and campylobacter coli (day 47)	0/6	-	2/6

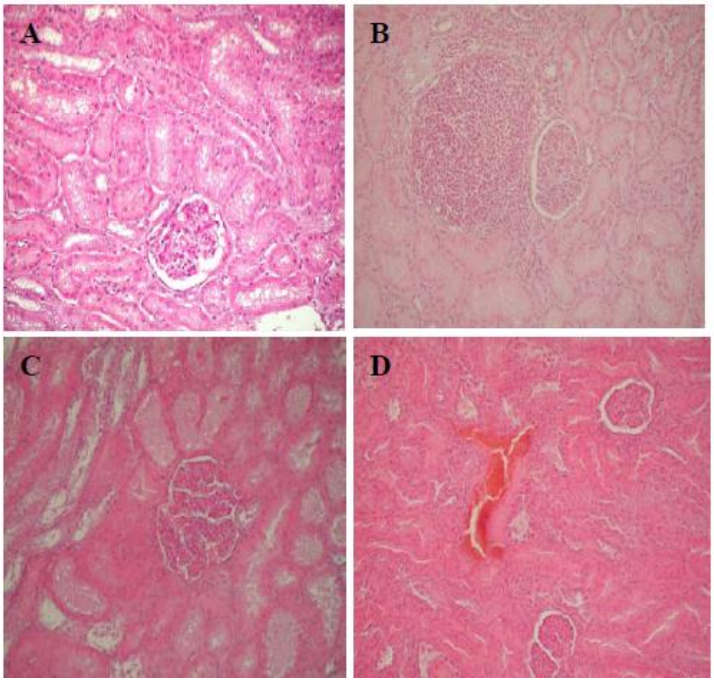
Source: STOEV et al., 2000

Tab. 5Ochratoxicosis effects of bacterial infection in pigs

2.1.7.6 Mycotoxin effects on animal organs

In a study from Milicevic D. et al. the occurrence of ochratoxin A and porcine nephropathy in Serbia was investigated. 90 samples of blood, kidney and liver were randomly selected from slaughtered pigs and analyzed for ochratoxin A by HPLC with FLD and MS/MS detection. 26.6% of the liver samples contained ochratoxin A in the range of 0.22-14.5 ng/g, the serum samples showed maximum concentration of 220.8 ng/ml and 52.5 ng/g in kidney samples were found.

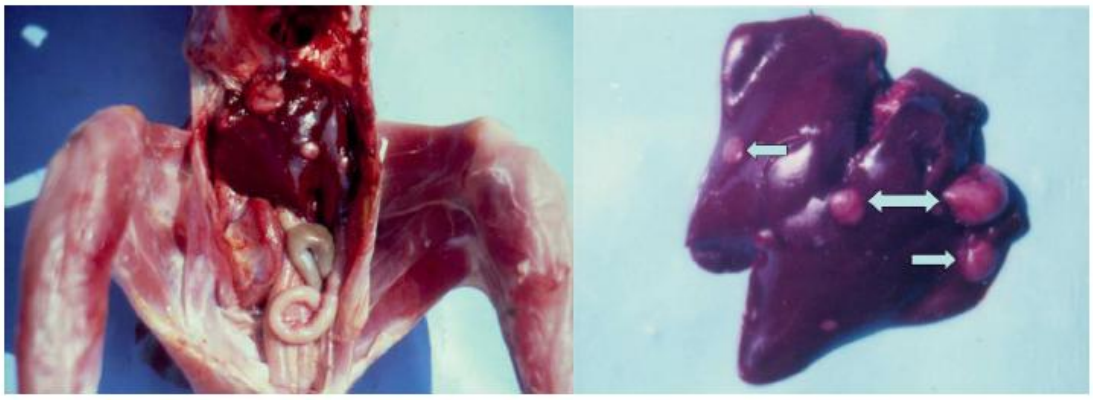
As the pictures below (Fig.13) demonstrate, histopathological examination of kidney confirmed tubulopathies with edema and cell vacuolization. In addition, hemorrhages and necrosis of proximal kidney tubules cells were found [MILICEVIC et al., 2008].



Source: MILICEVIC et al., 2008

Fig.13Dystrophy and vacuolar degeneration in the epithelium of proximal tubules' cells (A), focal interstitial fibrosis (B), necrosis of proximal tubules' cells (C) and hemorrhages in cortex (D)

A recent study from Stoev S.on the carcinogenic and toxic effects of ochratoxin A in chicks show adenocarcinoma in the liver of male chicken after feeding 5 ppm ochratoxin A via feed. The chicken died after the end of the 10 months experiment and also showed large grey white neoplastic foci in the liver which protruded significantly above its surface (see Fig. 14) [STOEV 2010].



Source: STOEV 2010

Fig.14Adenocarcinoma in the liver of male chicken

2.1.7.7 Mycotoxin effects on animal blood

A study form EasternEurope, estimated the occurrence of ochratoxin A in animal feed and metabolite residues in porcine blood serum in Poland. 40 and 45 samples of feed and porcine blood serum were analyzed for ochratoxin A andin 25% of the samples ochratoxin A was present in both types of investigated material.Metabolites were found in 27.5% of the blood samples and 7.5% of the feed samples. Furthermore, both sample matrices showed higher contamination in the winter season than in the spring season, maybe corresponding that kernels being stored afterexposed to spring thaw.Moreover increasing temperatures resulting in higher air humidity and as a consequence, in higher moisture content in stored cereals and feeds [KOTOWSKI et al., 2000].

2.1.7.8 Mycotoxin effects on animal muscle compartment

An Austrian study from Zöllner P. et al. reported that in all muscle tissue samples from pigs, zeranol was detected with concentrations of up to 13.3 µg/kg along with α-zearalenol and traces of zearalenone and taleranol, with LC-MS/MS detection.A possible explanation for this might be the fact that muscle tissue form the back is better supplied with blood, which can be considered as the most important transport system for the analytes in the animal body [ZÖLLNER et al., 2002].

2.1.8 Human exposure

2.1.8.1 Health Risk

Mycotoxins are a diverse group of chemical compounds. If the levels in the diet are high enough, it can cause acute and/or chronic adverse health effects in humans. Mycotoxins can affect organs and systems in the body, notably the liver, the kidney, the nervous, endocrine and immune systems [KUIPER-GOODMAN 1999]. Although secondary metabolites from fungi can never be completely removed from the food supply, risk analysis based on scientific knowledge makes it possible to define the levels in food (tolerances, guideline levels and maximum residue levels) that are unlikely to be harmful to health.

In the table below you can see how Kuiper-Goodmann interpreted the health risk from contaminants in food:

Rating health risks from foods

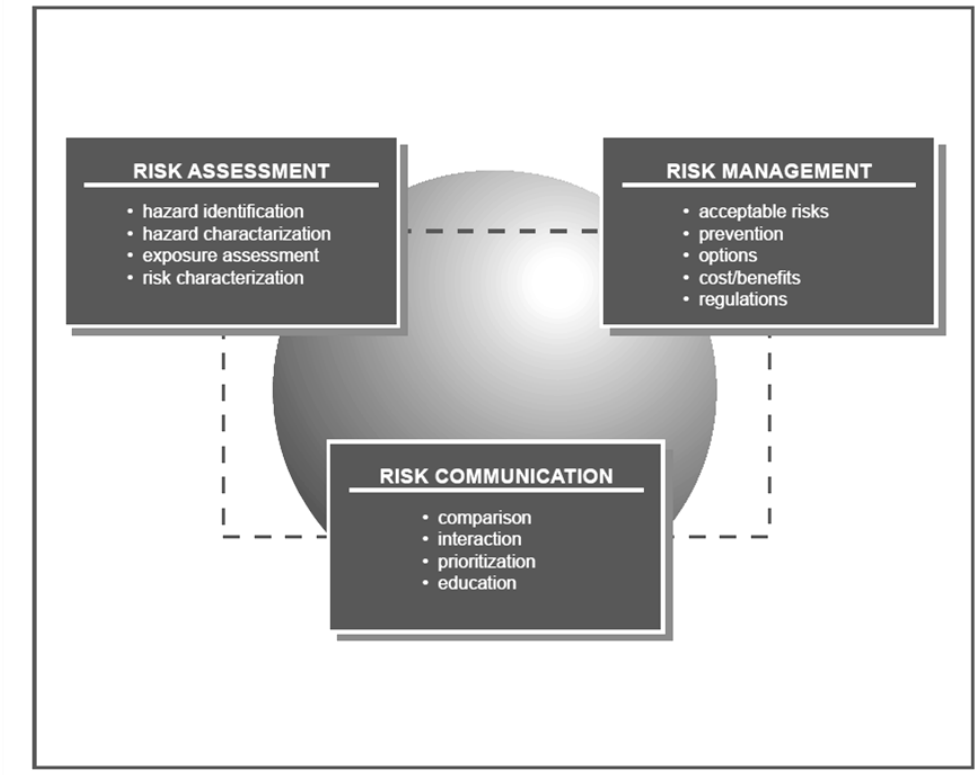
Acute	Chronic
High	
Microbiological	Mycotoxins
Phycotoxins	Anthropogenic contaminants
Some phytotoxins	Some phytotoxins
Mycotoxins	Unbalanced diet
Anthropogenic contaminants	Phycotoxins
Food additives	Microbiological
Pesticide residues	Food additives
	Pesticide residues
Low	

Source: KUIPER-GOODMAN, 1999

Tab. 6 Rating health risks from foods

The lack of harmonization among countries resulted in a wide variety of guidelines and regulations regarding mycotoxins. A leading role to approach risk analysis for all the compounds that may cause concern for food safetylike mycotoxins, had several international organizations such as WHO and FAO (especially through the Codex Alimentarius Commission, the Joint Expert Committee on Food Additives (JECFA), the International Agricultural Research

Centers, the Organization for Economic Co-operation and Development (OECD) and the International Life Science Institute (ILSI) as well as several national agencies. Therefore scientific evaluations became the basis for recommendations regarded by the Codex Committee on Food Additives and Contaminants and the European Union, in terms of international regulation for mycotoxins such as aflatoxins, ochratoxins, patulin and zearalenone. According to Kuiper-Goodmann “Risk is defined as the estimated likelihood of an adverse health effect, weighed for its severity, occurring in humans as a result of exposure to a biosocial, chemical or physical agent in food. A risk assessment involves a complete toxicological assessment, an epidemiological assessment, an exposure assessment and a risk characterization.”[KUIPER-GOODMAN 1999]



Source: KUIPER-GOODMAN 1999

Fig.15Risk analysis framework for food safety

2.1.8.2 Hazard characterization

Kuiper-Goodman stated that: “Hazard characterization is the extrapolation phase of risk assessment. Its aim is to make a predictive characterization of the hazard to humans, based on animal studies under low exposure conditions. The endpoint of hazard characterization is the estimation of a “safe dose” such as a provisional tolerable daily intake.”[KUIPER-GOODMAN 1999].

If there is probably a threshold of knowledge in the relationship between cause and effect of the mechanism and mode of action of a substance a TDI should be determined.Common practice to estimate a TDI for humans is to divide the No Observed Adverse Effect Level by a safety factor of 100 when extrapolating from animals to humans. This includes a factor of 10 for interspecies varieties and another factor of 10 for intraspecies differences.Furthermore it is important to take account to the exposure variety according to age, for instance that young childrenbeing exposed at a much higher rate in terms of body weight. Sometimes exposure assessment can be based on measurements of biomarkers in humans, for example for ochratoxins. Then the intake can be estimated on the basis of pharmacokinetic relationships[KUIPER-GOODMAN 1999].

2.1.8.3 Risk characterization

The severity and expected occurrence or absence of substance and theknowledge of the potential adverse health effects on an exposed population characterizes the risk and should be estimated qualitative and/or quantitative, including collateral uncertainties. Risk characterizations can for example being established for daily levels of exposure at which the risk is significant over a lifetime [KUIPER-GOODMAN 1999].

2.1.8.4 Risk assessments

For risk assessment evaluations have to be reexamined time by time. New information on both exposure and basic toxicology should be taken into account as well as improved understanding of mechanisms of action in the body. Detailed risk assessments have been done only for a few mycotoxins such as aflatoxins, deoxynivalenol, ochratoxin A, zearalenone and fumonisins [KUIPER-GOODMAN 1999].

Thuvander et al. stated: “Even for the most well documented toxins, the tolerable daily intakes established by international working groups remain temporary or provisional due to insufficient toxicological information. A second problem in assessing the possible health risks due to exposure to mycotoxin is the lack of information on exposure” [THUVANDER et al., 2001].

2.1.8.5 Human exposure – Zearalenone

Food safety authoritiesand scientistsall over Europe investigate the hazard of mycotoxin in their countries. Demonstrating that zearalenone are found all over the food chain a lot of surveys have been done.To describe the contamination in field crops Placinta et al. reported levels from few µg/kg up to 8 mg/kg of zearalenonein wheat, barley, oat, rye and feedsamples from Bulgaria, Germany, Finland, Netherlands, Norway and Poland [PLACINTA et al., 1999].In former Yugoslavia, zearalenone was found at high levels up to 10 mg/kg in maize [BALZER et al., 1977].Surveys from Hungary showed contamination of moldy and stored maize with zearalenone from 0.01 to 11.8 mg/kg. [FAZEKAS et al., 1996].Contamination of 14% of maize samples with a levels from 0.2 mg/kg to 6.5 mg/kg was reported from Italy [SCOOP 2003].In the United Kingdom, zearalenone was detected in maize and ingredients of feed[SCUDAMORE et al., 1998] and in Scotlandhigh contamination of zearalenone in stored barley was detected at levels between 2.1 and 26.5 mg/kg. [GROSS and ROBB 1975].Concerning on human exposure with

mycotoxins in Europe4918 cereal samples were analyzed for zearalenone.The occurrence of the toxin was reported in 32% of the samples in nine European countries. The distribution demonstrates that much of this contamination was in maize and wheat. Therefore raw maize was the nourishment with the highest level of zearalenone[ZINEDINE et al., 2007].

Zearalenone is found all over European crops which also reaches animals and humans after they consume these contaminated corps. In the following chapter results from animal testing is used to show the effects of zearalenone on animals.Furthermore, the impact of the toxin on humansisreported.

2.1.8.5.1 Toxicity of zearalenone

Fusarium species have been the reason for several human outbreaks of mycotoxicoses [HUSSEIN and BRASEL 2001]. Both deoxynivalenol and zearalenone have been linked to moldy grain toxicoses in the USA, China, Japan, and Australia, including symptoms such as nausea, vomiting, and diarrhea [BILGRAMI and CHOUDHARY 1998].In respect to acute toxicity, LD 50 values from zearalenone are about 2.000-20.000 mg/kg b.w. A relative low acute toxicity was reported after several trails of oral administration in mice, rats and guinea pigs [FLANNIGEN 1991]. Subacute and subchronic toxicity identified a NOEL in pigs of about 40 µg/kg body weight/daycompared to NOEL indentified by Kuiper-Goodman et al. and JECFA of 100 µg/kg b.w. in rats.Moreover it should be mentioned that the International Agency for Research on Cancer classified zearalenone and its derivatives in group 3 [IARC 1999] [ZINEDINE A. et al. 2007].

In a survey from Videmann B. et al. the metabolism and transfer of mycotoxin zearalenone was investigated in human intestinal Caco-2 cells using them as a model of intestinal epithelial barrier. As a result after given 10 to 200 µM zearalenone the survey shows on the one hand that the cell apical exposure to

zearalenone and α-zearalenol was predominantly found at the basal side. On the other handwere β-zearalenol and both glucuronides metabolitesespecially excreted at the apical side.Due to the fact that the strongest estrogenic activity results from α-zearalenol, the preferential production and the transfer of this metabolite to the basal side indicate that intestinal cells may contribute to the adverse health effects of zearalenone [VIDEMANN B. et al., 2008].

2.1.8.5.2 Zearalenone exposure in humans

A surveys with 49 women of zearalenone in endometrial tissue show 27 endometrial adenocarcinomas, 11 endometria hyperplasis and 11 normal proliferative endometrial. Zearalenone concentration of 47.8±6.5, 167.0±17.7 ng/ml and below the limit of detection were found. Zearalenone was not detected in 8 cases of hyperplastic and 5 cases of neoplastic endometrial tissue [TOMASZEWSKI et al., 1998].From the southeast region of Hungary, zearalenone concentrations were found from 18.9 to 103.5 µg/ml in serum samples. Early telearche has been reported from the patients and zearalenone was also present in samples of food collected from the patients [SZUETZ et al., 1997]. Nevertheless zearalenone can be found in every kind of nourishment and a potential danger of health could only be reached after absorption of high amounts or by exposure over a long period [ZINEDINE et al., 2007]

2.1.8.5.3 Tolerable Daily Intake of zearalenone

The Scientific Committee on Food has accepted a provisional TDI of 0.2 µg/kg body weight for zearalenone.

[http://europa.eu/legislation_summaries/consumers/product_labelling_and_packaging/l21101a_en.htm]

2.1.8.6 Human exposure – Ochratoxin A

2.1.8.6.1 Ochratoxin A exposure in food

Plenty of ochratoxin Acontaminated food is found in Europe. 68.6% of 2374 food samples were examined containingochratoxin A above the limit of detectionof 0.01 µg/kg [WOLFF 2000]. A large database, over 20,000 data points, of ochratoxin A levels exists. Subdivided into seven food categories (cereal and cereal products, wine, beer, grape juice, brewed coffee, cocoa and cocoa products, pork which contribute most to the ochratoxin A exposure [JECFA 2001],[EFSA 2005].

A special problem presents ochratoxin A in meat and meat products, as ochratoxincarry over from feed to meat has been experimentally established [MADSEN et al., 1982].Highly contaminated are the kidney followed by liver, muscle and fat. Noticeable amount of ochratoxin A have been found in blood. The occurrence of ochratoxin A in blood sausage from swine being 77.2%, livertype sausage (67.9%) and raw sausages (46.7%)[MORTENSEN et al., 1983]. Maximum ochratoxin A levels of 4.6 and 3.2 µg/kg in blood and liver type sausages could be determined [PETZINGER and WEIDENBACH 2002].

The AGES reported ochratoxin A concentrations from a trial on 870 groceries samples. For examples in 257 samples of cereals and cereal products an average content of 0.26 µg/kg ochratoxin A was detected. In 131 meat samples the average value was below 0.1 µg/kg. Referring to this average daily intakes in Austria were between 0.09 – 0.16 µg. For high consumers the values increased up to 0.3 – 0.8 µg/day [RAUSCHER-GABERNIG and GROSSGUT 2007].

Anaverage contamination of 0.11 µg/kg in 76 pork samples was found [JØRGENSEN 1998], while another survey showed an average ochratoxin A contamination of 0.14 µg/kg in 58 pork samples [GAREIS and SCHEUER 2000].

A dietary exposure assessment by EFSA 2006,indicates that average adult consumers (60kg) have an ochratoxin intake of 15 to 20 ng/kg b.w. per week.High consumers (97.5th percentile) have about 40 to 60 ng ochratoxin A/kg b.w. per week [EFSA 2006].

Frying or boiling could decrease the content of ochratoxin A in meat, but ripening processes have been proved to be ineffective for ochratoxin A reduction in meat products[TOSCANI T. et al., 2007].

2.1.8.6.2 Ochratoxin A exposure in humans

South America studies reported the occurrence of ochratoxin A in human plasma, because of relatively low control by food safety authorities as well as bad manufacturing practice on the field and in storage facilities. A survey from Munoz K. et al. analyzed 88 blood samples from healthy donors from two different Chilean agricultural zones. 91% of samples from San Vicente de Tagua-Tagua and 54 % of samples from Colbún were positive to ochratoxin A. Detected levels were between 0.07 – 2.75 µg/kg and 2.12 µg/kg [MUNOZ et al., 2006]. In another report from South America199 plasma samples, from blood donors in Mar del Plata and 236 from General Rodríguez, Argentina, were analyzed.63.8% of human plasma from Mar del Plata and 62.3% from General Rodríguez were positive for Ochratoxin A. Values could be determined between 0.15 and 0.43 ng/ml [PACIN et al., 2008].

A relation in between dietary intake and the presence of ochratoxin A in human milk was explored by a study from Skaug M.A. et al. wherehuman milk samples were collected from 80 Norwegian women.A quantitative food frequency questionnaire was used to record the usual food intake during the last year. From the 80 human milk samples 17 have been contaminated with ochratoxin A in the range of 10 to 182 ng/l. The women with a high dietary intake of liver paste, liverwurst,cakes, cookies, fruitcakes, chocolate cakes, etc. were more likely to have ochratoxin Acontaminated milk. Also the risk of ochratoxin

Acontamination increased by the intake of all kinds of juice. Moreover, the resultsnotify that breakfast cereals, processed meat productsas well as cheese could be important contributors to dietary ochratoxin A intake [SKAUG et al., 2001]. Additional to that, the ochratoxin A concentration in foetal serum is reported to be twice the maternal one. This indicates an active transfer of the toxin across the placenta [ZIMMERLI and DICK1995].

On the other hand a Scandinavian survey from Sweden tried to estimate the intake of some mycotoxins from food, in which 600 samples were collected and analyzed for ochratoxin A among other mycotoxins. Intakes were calculated for average and high consumers in adults and children and compared with the tolerable daily intake of 5 ng/kg body weight. They state that the exposure to mycotoxin in Sweden did not give rise to any major health concerns in the present, due to the fact that the intake of ochratoxin A was below the temporary TDI values from international expert groups[THUVANDER et al., 2001].

An average daily intake of ochratoxin A was calculatedon the basis of blood plasma concentrations to be 0.35ng/kg b.w./dayin Norway and 2.34 ng/kg b.w./dayin Spain. For babies daily ochratoxin A intake via breast milk is ranged from 1.0 ng/kg b.w./day in Norwayto 24.0 µg/kg b.w./day in Italy [DUNKELBERG et al. 2007].

2.1.8.6.3 Toxicity of Ochratoxin A

Information about the effect of ochratoxin A after a single exposition is limited to one case report from a farmer couple. After working (screen, sift) in a granary for eight hours the women got acute kidney failure with tubular necrosis. The wheat contained an *Aspergillus species* that produced ochratoxin A.A study from Germany investigated 6476 groceries and 927 serum samples from healthy donorsbetween 1995 to 1998. 98% of the blood samples dedicated positive, between 0.11 and 0.5 ng/ml. Comparable results reported from between 1977-1994 within the EU (0.45 ng/ml) and from a recent study from

several EU member countries (0.35 ng/ml). Even in breast milk ochratoxin A could be detected at a level of 0.18 µg/L[DUNKELBERG H. et al., 2007].

A lot of surveys have been done to investigate the correlation between ochratoxin A contamination in food and feed and the probability to cause Balkan Endemic Nephropathy [CASTEGNARO et al., 1987]. Balkan Endemic Nephropathy is a chronically progression renal tubulointerstitial disease. This disease appears in north-western Bulgaria, south-eastern Romania, and the former Yugoslavia (Serbia, Croatia, Bosnia and Herzegovina, Slovenia, and the Former Yugoslav Republic of Macedonia). Compared with other studies individuals with Balkan Endemic Nephropathy have higher ochratoxin A concentrations in blood and urine as healthy ones. In this study from Abouzied M.M. et al. they investigated ochratoxin A contamination in 165 samples of homeproduced food and feed from households in villages from the Balkan Endemic Nephropathy region of north-western Bulgaria.Households were Balkan Endemic Nephropathy occurs had more ochratoxin Apositive samples then the other households.Average per capita monthly consumption of basic food in rural Bulgaria, showed the highest ochratoxin A intake in Balkan Endemic Nephropathy households of 1.21 µg/day.In conclusion it is reported that the results indicate that ochratoxin A may not alone cause Balkan Endemic Nephropathy, but only synergistically with other environmental toxicants and/or predisposing genotypes [ABOUZIED et al., 2002].

In human ochratoxin Ashow unusual toxicokinetics. The toxin has a half life in blood of 840 h after oral ingestion.This long time of excretion of ochratoxin A in human can relate to the reabsorption during an enterohepatic circulation as well as extensive protein binding[SCHLATTER et al., 1996]. The elimination of ochratoxin A in the liver is maintained by protein carriers. The toxin is shuffled from its proteinbound form in blood into the hepatocyte. There it is secreted into bile. A further carrier system is involved in the uptake of ochratoxin A by proximal tubule cells, which secret the toxin into urine [TSUDA et al., 1999].

2.1.8.6.4 Ochratoxin A effects on the immune system

Toxic effects in the immune system resulting from very low concentrations of ochratoxin A in the ng/ml range, which is by far the most sensitive among all other sensitive organs.Ochratoxin A is an immunosuppressive agent[MÜLLER et al., 1999] and a concentration of 5 ng ochratoxin A per kg b.w. suppresses the immune responses in mice[HAUBECK et al., 1981].On one hand concerning the cellular immunity ochratoxin A inhibits the immune responses transmitted by B- and T-lymphocytes and on the other hand in question of the humoral immunity ochratoxin A induces a regression of IgGs, IgAs, and IgMs [PETZINGER E. and WEIDENBACH A. 2002].

2.1.8.6.5 Tolerable Daily Intake of Ochratoxin A

In the year 2001 the WHO set a provisional tolerable weekly intake of 100 ng ochratoxin A/kg b.w. per week. The intake quantity relies on the NOAELfor nephrotoxic effects of ochratoxin A in male rats with a safety factor of 1500. Inside the EU daily intake differ in different countries due to varying food pattern and different ochratoxin A contamination of food from 0.53 in Great Britain to 2.31 ng/kg b.w./day in France. The WHO established a PTWIof 100 ng/kg b.w. per week[DUNKELBERG H. et al. 2007].

The following table shows LD50 values of ochratoxin A (mg/kg body weight) for the mouse, rat, dog, pig and chicken.

Species	Oral	i.p.	i.v.
Mouse	46 – 58.3	22 – 40.1	25.7 – 33.8
Rat	20 – 30.3	12.6	12.7
Rat neonate	3.9		
Dog	0.2		
Pig	1		
Chicken	3.3		

Source: Based on literature compilations in Harwig et al., (1983) and NIOSH (1986)

Tab. 7: LD50 (mg/kg b.w.) values for ochratoxin A in various species

3 Materials and Methods

3.1 Sampling

The sample pool consists of 44 samples from main grocery stores in Austria, traditional butchers and discounters. Excluded were farmer markets, gastronomy, catering, suppliers on the “road” like gas station shops, airplane and train food down to school buffets and fast food suppliers. To include all of the distributers would go beyond the scope of this work and could be interesting for further studies.

The idea behind the sampling was first of all to monitor the general concentration of mycotoxins coming through the food chain. The literature part above provides information from numerous studies on mycotoxin concentrations in animal body compartments. Less information is available about the concentration of the mycotoxins in processed products from pig. As a matter of fact pigs are very sensible organisms to mycotoxins and are one of the main sources for processed meat products in Austria. Furthermore the interest in mycotoxin-accumulation in different parts of the pig, which are separately used for different kinds of processed meat products, could also give indication of vulnerable organs in humans, exposed to mycotoxinsfrom contaminated food. The tissue distribution follows the pattern kidney>liver>muscle>fat [GALTIER P et al., 1981]. So as these parts are the main target for mycotoxin accumulation, the sampling focused on processed meat products containing these tissues. As a matter of fact kidneys are not often used in processed products.So more focus was put on mycotoxin research in the blood of swine, because former studies claim that in central European ochratoxin A is probably the most ubiquitous carcinogenic mycotoxin, which can be detected at levels higher than 0.1 µg/kg in more than 90% of human and swine blood samples [RICHARD et al., 1999]. Additionally, the concentration of mycotoxins in blood led to the idea that the steady circulation of mycotoxins in blood plasma across the organism can have effects on blood compartments and further metabolic influences [PETZINGER E. and WEIDENBACH A. 2002].

The table below shows the different products from various distributers containing the selected body parts (liver, muscle, blood and fat).

Liver	Muscle	Blood	Fat
Chef Menu Leberspätzle	Natur Pur Bio Knacker	Blutwurst Trünkel	Speck Bauer
Chef MenuMini Leberknödel	TANN Extra	Blutwurst Schreiber	Speck Mader
Leberstreichwurst Stastnik	Reiter Mettwurst	Blutwurst Mader	Speck Trünkel
Leberstreichwurst Landhof	TANN Fränkfurter	Blutwurst Bauer	LOIDL Haussalami
Leberstreichwurst Jauntaler Billa Feinkost	HAM Frühstücksfleisch		
Pluma Feine Leberpate	TANN Pressschinken		
Inzersdorfer Leberaufstrich	Iglo Fleischknödel (frozen)		
HAM Leberbrot			
Pate Grand Mere Brüsseler Art			
Reiter Röstzwiebel Streichwurst			
Radatz Leberstreichwurst			
Trünkel Feine Leberpastete			
Ablinger Streichwurst			
Trünkel Gutsleber Streichwurst			
Clever Streichwurst			
Gourmet Sahnestreich			
Primana Leberbrotaufstrich			
Knorr Leberknödelsuppe (dried)			
MHW Leberwurst			
Knorr Leberknödelsuppe (can)			

Jensen's Luxus Leber Pete
Tann Leberknödel Feinkost
TANN Streichwurst fein
Lutz Leberwurst
Dulano Röstzwiebel Leberwurst
Natur Pur Bio Streichwurst
Dorfgold Sahnestreich
Twinner Leberwurst
Iglo Leberknödel (frozen)

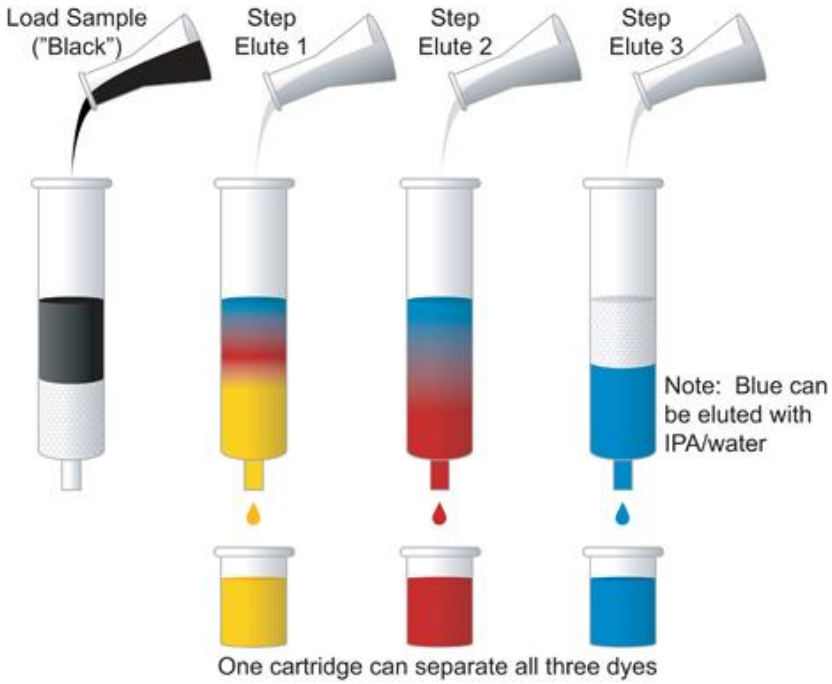
Tab. 8Products from various distributers containing the different body parts (liver, muscle, blood and fat)

3.2 Methods – Theoreticalbackground

3.2.1 Extraction

3.2.1.1 Solid phase extraction

Solid phase extraction columns were invented in the mid 1970s [THURMAN and MILLS 1998].In 1978, Waters promoted the Sep-Pak cartridges, asconvenient and dry packed silica based disposable columns [MCDONALD and BOUVIER 1995]. The versatility of the SPE allows it to be used for a large number of functions, such as an effective method of sample purification, or for compound isolation and removal of reagents [WACHOB 1991]. It is effective because it is a quick procedure for separation and can enriches the minor components of a sample. A great number of quantitative techniques (HPLC, GC, UV) use the benefit of the SPE as a first step for analyzing methods [RUIZ-GUTIERREZ and PEREZ-CAMINO 2000].



Source:
http://www.waters.com/webassets/cms/category/media/other_images/primer_d_%20solidphase.jpg

Fig.16 Principle - Solid Phase Extraction

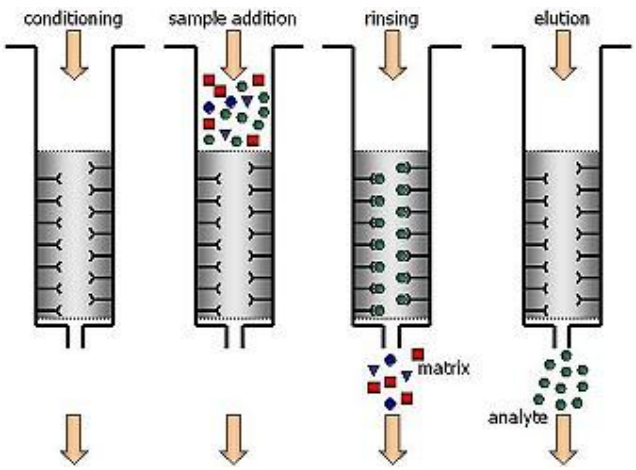
SPE based on the principle of liquid chromatography.In these columns, strong but reversible interactions between the analyte and the stationary phase occur. Typical interactions arenonpolar interactions between the C-H links of the analyte and the C-H links of the absorbent so called Van der Waals forces. The most common support for non-polar interactions is C_{18} cartridges.Nevertheless,C_2 and C_8 columns are also used for lipid separations. Polar interactions create links by hydrogen bonding (dipole-dipole forces, etc.).These interactions are typical of all the cyano (CN), amino (NH_2), diol or silica (Si) supports. Furthermore, ion-exchange interaction occurs in a phase such as the one with quaternary amines, benzenesulfonic acids or propylsulfonic acids and take placewhen the analytes have negative or positive ionic charges [MCDONALD and BOUVIER 1995] [RUIZ-GUTIERREZ and PEREZ-CAMINO 2000].

3.2.1.2 Immunoaffinity chromatography

Immunoaffinity purification is a highly specific andreversible technique, used for the one-step isolation of an analyte from many complex matrices and simplifies sample analysis relative to traditional clean-up techniques. The purification effect is based on the specific identification of a protein by an antibody or by enzymes using the specific affinity of an enzyme to inhibitors, substrate or co-factors. The stationary phase is linked with a proper antibody, which is capable to specificallybind the analyte. Attention should be paid to the affinity of binding the analyte being not too high, because elution thereby can be more difficult. Application of an immunoaffinity column to isolate and concentrate an analyte decreases the amount of solvent used. Also decreases the number of purification steps and shortens analysis time. [SHELVER 2004].

For analyzes of Ochratoxin A respectively for the clean-up step, Ochraprep® immunoaffinity columns were used. The Ochraprep® procedure is based on monoclonalantibody technology. The column contains a gel suspension of monoclonal antibody specific for ochratoxin A, covalently attached to a solid

support. To remove unbound material the column can be washed withphosphate buffered saline. The bound toxin is released from the antibody following elution from the column with methanol:acetic acid (98:2 v/v) [R-BIOPHARM RHÔNE LTD].



Source: http://services.leatherheadfood.com/mycotoxins/wp2training/immunoaffinity_principle.jpg
Fig.17Principle – Immunoaffinity Columns

3.2.2 High Pressure Liquid Chromatography

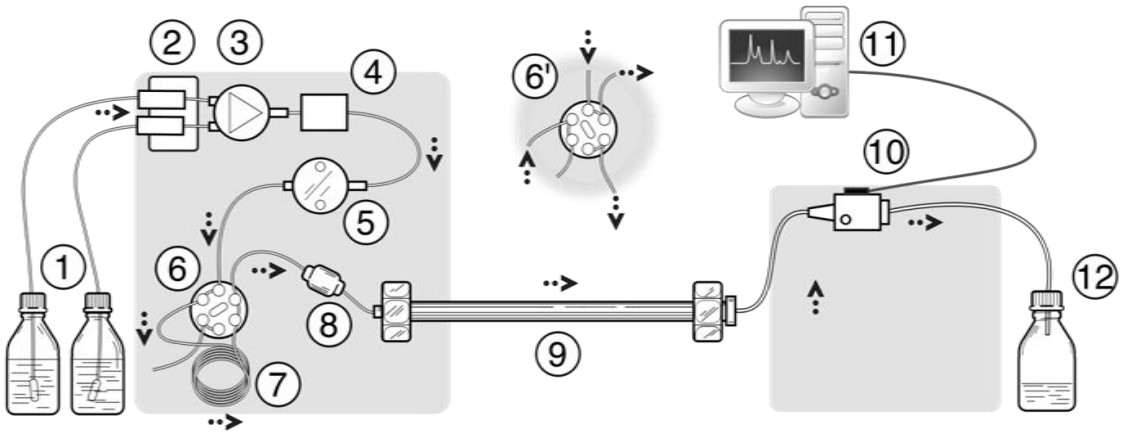
The theory behind chromatography is the separation of two sample components based on their different distribution between two non-miscible phases. The one, the stationary phase, a liquid or solid, is fixed in the system. The other, mobile phase, a fluid, is streaming through the chromatographic system. This is the principle of chromatographic separation: Different distribution of the analytes between mobile and stationary phase results in different migration velocities [KENNDLER 2004].

High Pressure Liquid Chromatography is a separation process, where samples are flushed over a stationary phase with the help of eluent (liquid phase) under high pressure. Main characteristics are: [BERGER 1991]

- Particles of the stationary phase are very small (1-10 µm diameter) which implicate a high active surface and so a huge separating capacity.
- Column inner diameter is 2 – 4 mm

- There must be pressure to put the mobile phase through the column

Schematic representation of High Pressure Liquid Chromatography:



Source: Veronika Meyer, 4th edition, John Wiley and Sons, 2004, ISBN 0470093781, p. 7. andchromatography-online.org. Used file:Computer n screen.svg (Crystal SVG icons). (Date 15.1.2009) http://en.wikipedia.org/wiki/File:HPLC_apparatus.svg

Fig.18Practical High-performance Liquid Chromatography

(1) Solvent reservoirs, (2) Solvent degasser, (3) Gradient valve, (4) Mixing vessel for delivery of the mobile phase, (5) High-pressure pump, (6) Switching valve in "inject position", (6') Switching valve in "load position", (7) Sample injection loop, (8) Pre-column, (9) Analytical column, (10) Detector (i.e. IR, UV), (11) Data acquisition, (12) Waste or fraction collector.

The sample solution is injected without pressure, in general over a sample loop. Most of the HPLC instruments have an autosampler to automatize the injections and it can be programmed over computer software. The solvents in the solvent reservoirs can be degassed in an ultrasonic bath. The gradient valve mixes the solvents in appropriate ratio. The HPLC procedure works here with two different methods:

- isocratic: composition and flow are constant over the whole separation process
- gradient elution: eluent composition and/or flow modified time dependent

Over the switching valve the solvents are added with the sample and transferred on the column where the separation takes place. Routinely a precolumn is used to protect the column from impurity.

For the different liquid chromatographic separation problems variously filling materials for the column are available. The method used here was reversed phase chromatography. A non-polar stationary phase (octylsilica, octadecylsilica (C-18), hydrophobic polymere (polystyrol, divinylbenzol, polyacrylate)) and polar mobile phase (main component: water up to dioxane; modifier: methanol, ethanol, acetonitrile, tetrahydrofuran).

At the end of the column the separated compounds are eluted. This is called retention time and it is the time at which a specific analyte elutes; the retention time under particular conditions is considered a reasonably unique identifying characteristic of a given analyte.

Then the analytes are transferred to a detector like UV/Vis-, IR-, Fluorescence detector or Mass spectrometer. The output of the results is given in form of a chromatogram, where you can see the amount and the retention time of the analyte.

3.2.3 Detectors

3.2.3.1 Fluorescence detector

Fluorescence detector using a beam of light, usually ultraviolet light, that excites the electrons in molecules of certain compounds and causes them to emit light of a lower energy which intensity can be measured. Various light sources may be used as excitation sources, including lasers, photodiodes, and lamps; xenon arcs and mercury-vapour lamps in particular (wave length 200 – 800 nm). Limit of detection is in the ppb range. High selectivity due to the fact that only compounds can be detected that can be excited by fluorescence or which are modified to fluorescent derivates.

3.2.3.2 Mass spectrometry

Mass spectrometry is an analytic technique that measures charged particles in the mass-to-charge ratio. The principle comprehends of ionizing chemical compounds to generate charged molecules of molecule fragments and measurement of their mass-to-charge ratio [SPARKMAN 2000]. Samples are transferred to the mass spectrometer instrument and undergo vaporization. Components of the sample are ionized by for example impacting them with an electron beam. This results in the formation of charged particles so called ions. The ions are separated according to their mass-to-charge ratio, appearing in an electromagnetic field (analyzer). Quantitative methods are used to detect the ions and the signalsare converted into mass spectra.

3.2.3.2.1 Mass analyzer

The ions produced in the ion source are getting accelerated over electric collimator into the analyzer. A negative potential at the blend accelerates positive charged ions (positive ion mode) and vice versa for negative charged ions (negative ion mode).The analyzer arranges the accelerated ions according to their mass-to-charge ratio (m/z). The velocity of the ions depends on charge, mass and acceleration voltage. The ions are spacial or temporal divided before they get to the detector. After appropriate calibration it is possible to determine exactly the mass-to charge ratio.

$eU = \frac{mv^2}{2}$ e: ionic (electric) charge [Coulomb, C]; 1 e_0=1.60 x 10^{-27} kg

m: mass in atomic mass unit (u); 1u = 1/12 of carbon-12 atom

1u is exactly 1.66 x 10^{-27}kg

$V = \sqrt{\frac{2eU}{m}}$ v: velocity [m s^{-1}]

U: acceleration voltage [V]

There are many types of mass analyzers, using either static or dynamic fields, or magnetic or electric fields. There is also the possibility to use two or more mass analyzers for tandem mass spectrometry (MS/MS or MS^n).

Quadrupole mass filter: Quadrupole acts as a mass-selective filter. The quadrupole field is created by four parallel rods where a direct current voltage is overlaid by a high frequency alternating voltage. Only ions with certain m/z ratio have stable (oscillatory) trajectory in the quadruple field, all the other ones touch

the field and discharge itself. Which m/z ratio runs on stable trajectories and hit the detector depends on the applied current voltage [U] and the amplitude of the alternating voltage [*V*]. A triple quad instrument is a combination of two quadrupole mass filters (tandem mass spectrometry), which are detached through collision cell. As a first step a precursor ion with a specific m/z ration is selected (Q1-cell). Next the Q2-cell is filled with nitrogen or argon and there the collision induced dissociation appears. The occurred fragment, called product ion, are sorted towards m/z ration in Q3-cell and finally scanned by a detector [HOPFGARTNER 2007].

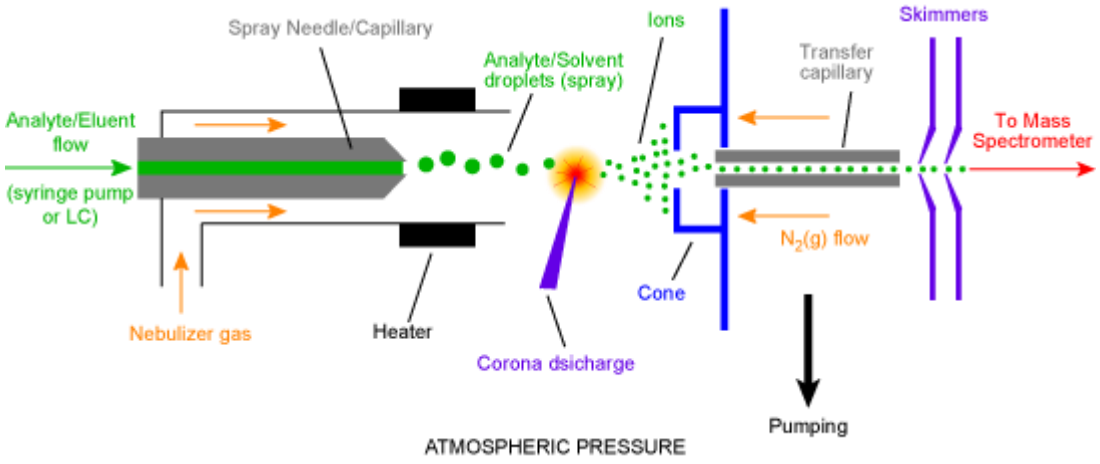
3.2.3.2.2 Detectors

At the end of the mass spectrometer is the detector. The ions pass by or hit on the surface and either the charge or the current is detected. Common types of use are photomultiplier, electron multiplier and faraday cups. The amount of ions leaving the analyzer is small, so amplification is often necessary to get a signal.

3.2.4 Ion source:

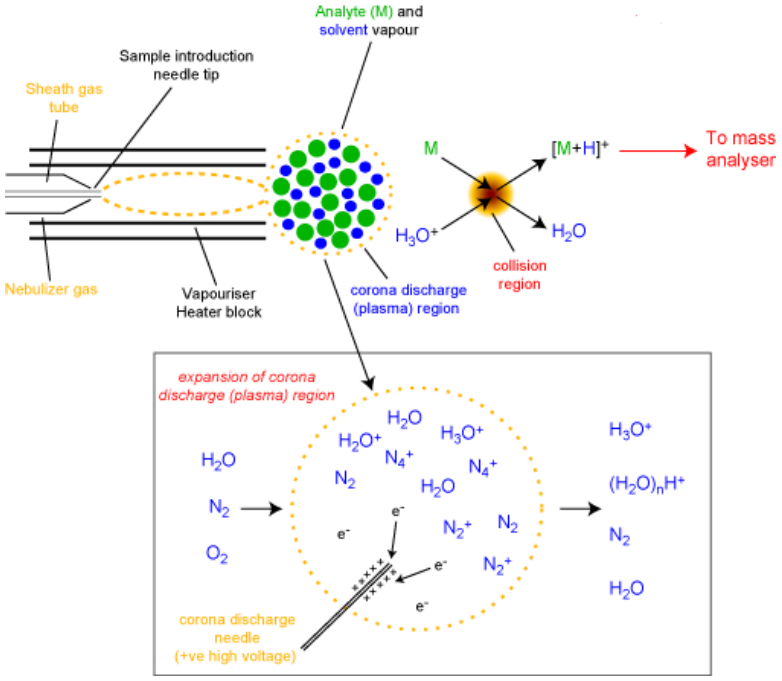
Atmospheric Pressure Chemical Ionization:After chromatographic separation the effluent enters the atmospheric pressure chamber, transported through a specially designed nebulizer needle. (See Fig. 19)The vaporizer is heating up to temperature of 400° C or 350° C when introducing a drying gas of nitrogen. This enhancesthe evaporation of the solvent. The corona needle is kept at high potential, resulting in a corona current. This supports ionization of the introduced molecules by help of solvent molecules. The molecular and cluster ions are deflected and enter the first vacuum stage through a metal plated fused-silica capillary. The role of this capillary is first to create a molecular leak between the API chamber and the first vacuum stage. Then initiate declustering

and partial fragmentation in the collision-induced dissociation region.This is the region between the end of the transfer capillary and the first skimmer. Here a voltage in the range of typically 10 to 100 V is applied. The ions are focused through an octapole mass filer, increasing the transmission of ions, when enter the high vacuum of the mass analyzer through a second skimmer [ROSENBERG et al., 1998].



Source: http://www.bris.ac.uk/nerclsmsf/techniques/hplcms.html

Fig.19A schematic of the components of an APCI source



Source: http://www.bris.ac.uk/nerclsmsf/techniques/hplcms.html

Fig.20A more detailed view of the mechanism of APCI

3.3 Materials

3.3.1 Chemicals and Solvents of Ochratoxin A analysis

Chemicals:

Water deionised, Acetonitrile, Methanol, Phosphate buffer tablets for PBS-buffer, Acetic acid 100% (glacial acetic acid), Sodium hydrogen carbonate, Tween 20, Diethylene glycol

Gas:

Nitrogen 5.0, Helium 4.6

Reference standard:

Ochratoxin A, 50µg/ml in Benzene/glacial acetic acid (99/1), Ampul 1 ml

Solutions:

Stock solution of Ochratoxin A (2mg/l):1ml of Ochratoxin A Standard (50µg/ml) pipettes to 25 ml volumetric flask and evaporate under nitrogen flow at room temperature. Dissolve Ochratoxin A with methanol and fill up to the mark.

External Standard 1 (200µg/l):1 ml of Stock solution pipettes to 10 ml volumetric flask and fill up with methanol to the mark.

External Standard 2 (20µg/l):1 ml of external standard 1 pipettes to 10 ml volumetric flask and fill up with methanol to the mark.

External Standard 3 (5µg/l):125 µl of external standard 1 pipettes into 5 ml volumetric flask and evaporate under nitrogen flow at 35°C. Dissolve ochratoxin A in reconstitution solution and fill up to the mark.

Extracting agent:710 ml methanol and 290 ml water deionised

Phosphate buffer solution (0.01M, pH 7.4):5 phosphate buffer tablets dissolve in 1000 ml volumetric flask with water deionised and fill up with water to the mark.

Reconstitution solution:25 ml methanol, 75 ml water deionised and 1 ml glacial acetic acid

Mobile phase:149.8g acetonitrile, 204g water deionised and 4 ml glacial acetic acid and degas in ultrasonic bath.

Diethylene glycol solution (10%):1 ml diethylene glycol pipette into 10 ml volumetric flask and fill up with methanol to the mark.

Tween 20-Solution (50%):5 ml Tween 20 pipette into 10 ml volumetric flask and fill up with water deionised to the mark.

Equipment:

Scale, Homogenizer, Ultraturax, Glass funnel, Pleated filter, Vac Elut workstation (ICT AI 600), Immunoaffinity column (Ochrapep®, Rhone-Diagnostics), Plastic syringe, Ultrasonic bath, Volumetric flasks different sizes

Laboratory glassware, Centrifuge tube with screw plug and Teflon seal, Sample vials (brown glass) (Chromacol 2CV (A)), Crimp Caps (Hewlett Packard), Seal pliers for sample vials, Piston stroke pipette, Disposable pipette tip

Instruments:

HP 1090 Liquid Chromatograph with fluorescence detector 1100 series and analytical workstation (Hewlett Packard)

Column: ODS Hypersil, 5µm, 200mm x 2.1mm (Hewlett Packard)

Precolumn: ODS Hypersil, 5µm, 20mm x 2.1mm (Hewlett Packard)

Frit: 0.5µm premium steel frit

3.3.2 Chemicals and Solvents of Zearalenone analysis

The following information for chemicals and solvents of zearalenone analysis is based on the method of MARKER 2009.

Chemicals:

Methanol HPLC Grade, Glacial acetic acid, Sodium acetate, Tert-butyl methyl ether, Acetate buffer (2 mol/l; pH 5.2), n-hexane, beta-glucuronidase-sulfatase, acetone, sodium hydroxide, HPLC water

Gas:

Nitrogen 5.0, Helium 5.0

Solutions:

Reference standards: α-zearalanol (zeranol), β-zearalanol, α-zearalenol, β-zearalenol, zearalanon and zearalenon (Sigma), internal standard β-zearalenol-d4

Stock solution of α-zearalanol, β-zearalanol, α-zearalenol, β-zearalenol, zearalanon and zearalenon (1000 µg/ml) in methanol

Methanol stock solution of beta-zearalenol-d4 (100 µg/ml)

Standard working solution (10 µg/ml):100 µl of all six methanol stock solution (α-zearalanol, β-zearalanol, α-zearalenol, β-zearalenol, zearalanon and zearalenon (1000 µg/ml)) pipette to 10 ml volumetric flask and fill up with methanol to the mark.

Internal Standard (1 µg/ml):100 µl of methanol stock solution of beta-zearalenol-d4 (100 µg/ml) pipette to 10 ml volumetric flask and fill up with methanol to the mark.

Spiking solution (0.1 µg/ml) of stock solution of α-zearalanol, β-zearalanol, α-zearalenol, β-zearalenol, zearalanon and zearalenon and β-zearalenol-d4.

Calibration solution (0 – 50 ng/ml):Spiking solution and methanol-water-solution (v/v%-50/50)

Storage for all solvents at 4°C under light protection.

Concentration of internal standard in all standard solutions amounts 50 ng/ml, corresponding 2.0 µg/kg Liver.

Acetate buffer (2 mol/l; pH 5.2):Dissolve 25.2g acetic acid and 129.5g Sodium acetate in 800 ml water. pHadjusts with acetic acid or sodium acetate to 5.2 and fill up with water deionised to 1000 ml.

Eluent for SPE:Methanol/water (80/20 v/v-%), Acetone/methanol (80/20 v/v-%), Methanol/water (50/50 v/v-%), Methanol/water (40/60 v/v-%) as wash solution

HPLC mobile phase A: methanol, HPLC mobile phase B: water deionised

Equipment:

SPE columns C18, SPE columns NH2, 50 ml plastic tubes, 15 ml glass tubes, Glass-HPLC-vials with 100 µl glass vial inserts with polymer feet, Blue screw caps, PTFE with red silicon septa

Instruments:

HPLC Agilent Technologies 1200 series

Applied Biosystems 4000 Q-Trap LC/MS-MS

Column: ODS Hypersil 5µm, 200 mm x 2.1 mm

Precolumn: ODS Hypersil 5µm, 10 mm x 2.1 mm

3.4 Methods

3.4.1 Sample preparation

The sample preparation occurred in the preparation section of the competence centre of Veterinary Drugs and Hormones. There, samples were homogenised with a small electrical appliance (Moulinette), packed in resealable plastic packaging, labelled and stored at -18° C.

3.4.2 Sample extraction method

For zearalenone the existing method was validated only for liver matrix and so further experiments with other matrices (e.g. liver dumpling, bacon, sausage, blood sausage) had to be realized, because of the possibility of matrix depended interaction of compounds (see below 3.4.4 Validation).

3.4.2.1 Extraction method- Ochratoxin A

Weigh in 15 g homogenized sample (+/- 0.1g accurately) into a 300 ml volumetric flask. Add 100 ml extracting agent and 1.35 g sodium hydrogen carbonate. Homogenize all for 3 min with ultraturax and filter with pleated filter.

Immunoaffinity column clean up:Fill in 5 ml from the eluate with 1 ml Tween 20 solution and 65 ml phosphate buffered saline, merge and fill into plastic reservoirs of the immunoaffinity columns. Apply the sample with a flow rate of 1 drop/s (max. 3 ml/min) to the column. Afterwards wash with 10 ml deionised water and then remove the plastic reservoir.

Elution of immunoaffinity column: Apply a 20 ml centrifuge tube into the Vac Elut workstation. Elute with 4 ml methanol without vacuum. Press the rest of the remaining methanol through the IAC with the help of a plastic syringe, add 100 µl diethylene glycol to the eluate and dry it at 40°C while adding nitrogen gas. Solve it in 1 ml reconstitution solution.

As the distribution of mycotoxins is generally non-homogeneous, samples should be prepared, and especially homogenised, with extreme care.

For the analyzing of mycotoxins, daylight should be excluded as much as possible during the procedure, since some mycotoxins gradually break down under the influence of ultra-violet light[COMMISSION REGULATION No 401/2006].

3.4.2.2 Extraction method – Zearalenone

The following method is based on the studies of Marker C.[MARKER 2009].

Weigh 5 g (+/- 0.01 g) sample into 50 ml plastic tube.

To determine the recovery rate, the current retention time and the ion ratio control sample must be applied. A negative sample is added with 100 µl standard solution(0.1 µg/ml) which is equivalent to 2 µg/kg Liver.

To all sample and control samples 100 µl of the internal standard solution (0.1 µg/ml) is added, which is also equal to 2 µg/kg Liver.

Add 40 µl beta-glucuronidase-sulfatase and 2 ml acetate buffer (2 mol/l; pH 5.2) for deconjugation. Shake it on the Vortex. Control the pH with 1 M acetic acid or 0.1 M sodium hydroxide at pH 5.2. Hereafter the samples are incubated over night by 37°C in a water bath.

On the next day the samples are taken out of the bath and cooled down to room temperature. Thereafter 10 ml tert-Butyl methyl ether (TBME) is added and 10 min. shaken. Then centrifuged at 4000 rpm for 5 minutes.The upper organic (TBME) phase is transferred into 15 ml glass tubule with screw caps and the TBME is vaporized at 50°C under nitrogen flow. The residue is solved in 5 ml methanol/water (v/v-% 50/50) by shaking 30 sec on the vortex.

Next step is the defattening step. 1 ml n-hexane is added, 30 sec rotation on the vortex and centrifuged at 4000 rpm for 5 minutes. Subsequently the upper phase is sucked off. Repeat this step.

For the solid phase extraction with SPE C18, the samples are first added with 2 ml deionised water because of the polarity effectsand besides the SPE columns are preconditioned with first 5 ml methanol and then 5 ml deionised water. The sample extract is applied on the column, observing that the columns do not run dry. Afterwards the washing step follows. 5 ml methanol/water put through the columns and finally the columns are dried with the help of a vacuum.

The elution occurs with 5 ml methanol/water (80/20 v/v-%) also with a vacuum (not under 820 mbar) in 15 ml glass tubule with screw cap. The eluate is dried under nitrogen flow at 50°C.

The dried eluate is dissolved in 5 ml acetone/methanol (80/20 v/v-%) by rotating 30 seconds on the vortex.

For the solid phase extraction with SPE NH_2 columns the tubules are preconditioned with 5 ml acetone/methanol (80/20 v/v-%). The sample is applied on the column and the eluate is caught in 15 ml glass tubule. The eluate is dried again with the help of nitrogen flow at 50°C and afterwards dissolved in 0.5 methanol, shaken for 30 seconds on the vortex, and again vaporized. At the end the residue is solved in 200 µl methanol/water (50/50 v/v-%), shaken on the vortex and filled up in 2 ml glass-HPLC-vials with cartridge.

3.4.3 Analyzing

For analyzing of zearalenone and its metabolites the method of MARKER 2009 was used.

Differences in varying fat amount of the samples, especially high fat concentration in liver paste samples, had the consequence that it was not possible to evaporate to dryness after extracting with TBME.

3.4.3.1 Ochratoxin A – Analysis

Column:	**ODS Hypersil, 5 µm, 200 mm x 2.1 mm**
Precolumn:	ODS Hypersil, 5 µm, 20 mm x 2.1 mm
Frit:	0.5 µm premium steel frit
Mobile phase:	149.8g acetonitrile, 204g water deionised, 4ml glacial acetic acid
Flow:	0.2 ml/min
Temperature:	35°C
Injection volume:	250 µl
Fluorescence detection:	Wavelength excitation: 333 nm Wavelength emission: 460 nm

Tab. 9 Parameters for Ochratoxin A determination

3.4.3.2 Zearalenone – Analysis

Column:	ODS Hypersil, 5 µm, 200 mm x 2.1 mm
Precolumn:	ODS Hypersil, 5 µm, 20 mm x 2.1 mm
Mobile phase:	A: methanol; B: water deionised, degassed
Gradient:	0 min: 50% A, 50% B 20 min: 100% A, 0% B 21 min: 50% A, 50% B 31 min: 50% A, 50% B
Injection volume:	25 µl
Flow:	0.25 ml
Temperature:	250°C

Tab. 10Parameters for the determination of zearalenone by LC-MS/MS-method

Parameters for the MS/MS-method in APCI negative mode:

Substance	Precursor Ion	Product ion I	Product ion II
Zearalanon	319.1	275.0	106.7
Zearalenon	317.1	131.1	174.8
alpha-zearalanol	321.0	276.6	62.9
beta-zearalanol	321.0	276.6	62.9
alpha-zearalenol	319.1	275.0	129.8
beta-zearalenol	319.1	275.0	129.8
Beta-zearalenol-d4 (internal standard)	323.2	159.7	

Tab. 11MS/MS-parameters of zearalenone and its metabolites determination with LC-MS/MS

Below an extracted ion chromatogram of zearalenone and metabolites (standard solutions) determined with APCI-MS/MS, 4000qtrap, in negative mode.

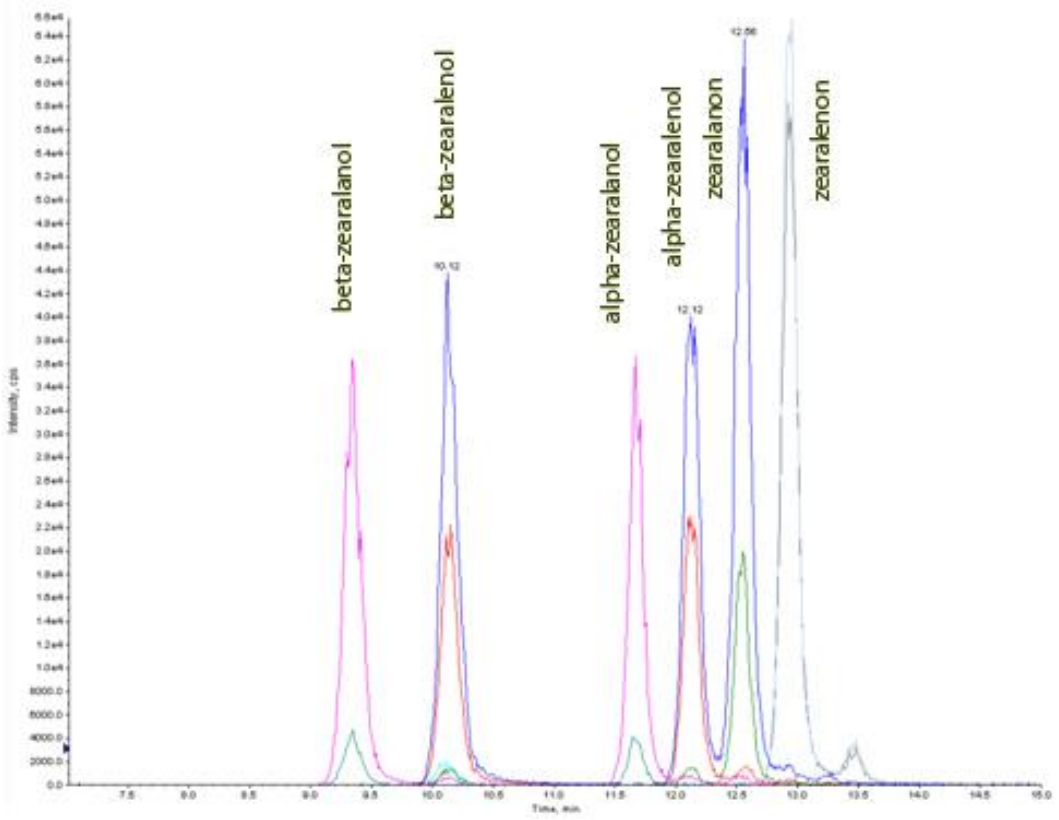


Fig.21Chromatogram standards zearalenone and metabolites, APCI MS/MS, 4000qtrap, negative mode

3.4.4 Validation

The Validation was implemented to determine the Level of quantification (LOQ) and the Level of detection (LOD). In the following it is explained which parameters have to be calculated for confirmation methods according to CD 2002/657/EC. The confirmation criteria are explained for the analysis of zearalenone and its metabolites.

The analysis is based on the method from [MARKER 2009], which was validated by CD 2002/657/EG. The method was validated for liver and urine. To confirm the received values from this survey, an appropriate validation has to be implemented due to the fact that the sample pool mostly consists of liver-matrices.

The table below show LOD (CCα), CCβ, LOQ for zearalenone and metabolites in liver samples from Marker 2009:

Substances	LOD (CCα) (µg/kg)	CCβ (µg/kg)	LOQ (µg/kg)
α-zearalanol	0.38	0.60	0.76
α-zearalenol	0.59	0.93	0.95
β-zearalanol	0.34	0.55	0.78
β-zearalenol	0.31	0.49	0.56
zearalanon	0.44	0.69	0.88
zearalenon	0.47	0.75	0.90

Source: Based on MARKER 2009

Tab. 12 relative CCα, CCβ values and LOQ of zearalenone and its metabolites in liver samples

To get the parameters in accordance with conventional methods it is needed to perform several individual experiments. For multi-analyte methods, several analytes can be analyzed simultaneously, as long as possibly relevant interferences are ruled out.

With the help of the absolute recovery rate it is possible to monitor the amount of the analyte that left through the extraction process. The method validated for liver samples (Marker 2009) showed following recovery rates:

Substance	Recovery rate (absolute)	Recovery rate (related to internal standard)
α-zearalanol	12.6%	100.7%
β-zearalanol	13.2%	105.9%
α-zearalenol	17.1%	121.6%
β-zearalenol	11.8%	88.1%
zearalanon	20.0%	154.1%
zearalenon	13.4%	96.5%

Source: Based on MARKER 2009

Tab. 13 Summary of average values of recovery rates of zearalenone and its metabolites in liver samples

3.4.4.1 Definitions

Decisional limit $CC_{\alpha}$and detection capability CC_{β}: By definition of the Official Journal of the European Union from the 17.08.2002: “Decision limit (CCα) means the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant.”.“The CCβ has been defined in the 2002/657/EC as:“the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β.”[COMMISSION DECISION 2002/657/EC 2002].

Two methodologies for the determination of these critical concentrations (CCα and CCβ) during method validation are defined by the Commission Decision 2002/657/EC.The first method is based on the determination of signal to noise ratio of 3:1 in blank samples and matrix material fortified at the CCα. The second method refers to the international standard ISO 11843-2, in which CCα and CCβ based on a linear regression model analyzing fortified material at different concentration levels. [VAN LOCO et al., 2007].

Limit of quantification: is the lowest concentration of an analyte that can be quantitative estimated with a defined precision. Only quantitative values can be specified that reaches that limit. It is assumed that the LOQ is according to a signal to noise ration of 10:1.

Relative ion intensities:“The relative intensities of the diagnostic ions and/or precursor/product ion pairs have to be identified by comparing spectra or by integrating the signals of the single mass traces. Whenever background correction is applied, this shall be applied uniformly throughout the batch and shall be clearly indicated” stated by the Official Journal of the European Communities. (See Tab.14)[COMMISSION DECISION 2002/657/EC 2002].

Relative ion intensity (% of base peak)	Maximum permitted tolerance
>50 %	± 20 %
>20 % to 50 %	± 25 %
>10 % to 20 %	± 30 %
≤10 %	± 50 %

Source: [COMMISSION DECISION 2002]

Tab. 14Maximum permitted tolerance for relative ion intensities of mass spectrometric techniques.

3.4.5 Performance characteristics

3.4.5.1 Determination of CCα, CCβ and limit of quantification for zearalenone

In the first pre-trial samples of blood sausage (blood), lard (fat), sausage (muscle), liver, liver dumplings, liver paste, pork blood, and bacon were tested at the experimental model: standard series of 0.08, 0.72, 1.36, 2.00, 4.00, 6.00, 10.0µg/kg(methanol stock solution of alpha-zearalanol, beta-zearalanol, alpha-zearalenol, beta-zearalenol, zearalanon and zearalenon (1000 µg/ml) and methanol stock solution of beta-zearalenol-d4 (100 µg/ml)) for calibration. Each sample was analyzed blank and spiked. Additional to that one blank liver sample was analyzed to compare the validated method from Marker C. 2009, and three spiked ones. This was accomplished to verify if this method is applicable to processed products from pig. Hereby it was well spotted that the employment of the method to this samples was possible. By the detection of the same amounts of internal standard in the liver samples and the other processed productsamples, it could be concluded that the method could be used for this task and further validation steps could be followed.

In the following assay steps the samples for the validation were settled to the groups of blood sausages, sausages, liver dumplings, liver paste and bacon for the different matrices blood, muscle, liver, and fat.

According to this validation step each group (blood sausages, sausages, processed liver products and bacon)was spiked with ascendant concentrations of the standards (0.3, 0.6, 0.9, 1.2, 1.5, 1.8 µg/kg) to obtain a calibration curve. The results were evaluated by ISO11843 and DIN 32645. Therefore the “Response” of each concentration was used to determine the CCα, CCβ, the limit of detection and the limit of determination for each ion (precursor and product ion). The Limit of detection was affirmed by a significance level of 99% and 95% for the limit of quantification.

3.4.5.2 Zearalenone limit of detection and limit of quantification for all samples

In the tables below all results from the validation approach for the limit of detection and the limit of quantification are reported. The values for the LOD are evaluated by DIN 32645 and ISO 11843 to the significance level of 99 %. The values for the LOQ are evaluated by DIN 32645 to the significance level of 95%. The value of the less sensitive ion is used for the detection limit and is declared in [µg/kg].

Blood sausage:

Substance	LOD (CCα) (µg/kg)	CCβ (µg/kg)	LOQ (µg/kg)
a-zearalanol	0.53	0.69	1.11
a-zearalenol	0.49	0.65	1.04
b-zearalanol	0.48	0.63	1.01
b-zearalenol	0.55	0.73	1.17
zearalanon	0.60	0,79	1.27
zearalenon	0,60	0,78	1.26

Tab. 15Limit of detection (CCα), CCβ, Limit of quantification [µg/kg] for zearalenon in blood sausage

Frankfurter (muscle)

Substance	LOD (CCα) (µg/kg)	CCβ (µg/kg)	LOQ (µg/kg)
a-zearalanol	0.32	0.42	0.69
a-zearalenol	0.36	0.48	0.77
b-zearalanol	0.27	0.36	0.59
b-zearalenol	0.31	0.41	0.67
zearalanon	0.57	0.76	0.76
zearalenon	0.35	0.46	0.75

Tab. 16Limit of detection (CCα), CCβ, Limit of quantification [µg/kg] for zearalenon in frankfurter

Processed liver products:

Substance	LOD (CCα) (µg/kg)	CCβ (µg/kg)	LOQ (µg/kg)
a-zearalanol	0.26	0.34	0.56
a-zearalenol	0.44	0.58	0.66
b-zearalanol	0.34	0.45	0.74
b-zearalenol	0.21	0.28	0.48
zearalanon	0.35	0.47	0.76
zearalenon	0.20	0.27	0.46

Tab. 17Limit of detection (CCα), CCβ, Limit of quantification [µg/kg] for zearalenon in liver dumpling

Bacon (fat)

Substance	LOD (CCα) (µg/kg)	CCβ (µg/kg)	LOQ (µg/kg)
a-zearalanol	0.37	0.48	0.79
a-zearalenol	0.57	0.75	0.93
b-zearalanol	0.31	0.41	0.67
b-zearalenol	0.38	0.50	0.82
zearalanon	0.49	0.65	1.04
zearalenon	0.71	0.93	1.52

Tab. 18Limit of detection (CCα), CCβ, Limit of quantification [µg/kg] for zearalenon in bacon

3.4.5.3 Determination of the recovery rate

Each of the 44 samples were analyzed twice, one blank sample and one spiked sample. Additional to that one blank liver sample and one spiked liver sample were investigated to confirm the recovery rates. For the evaluation of the received recovery rates the usage of the method from Marker C. could be confirmed again due to the fact that the results from the different matrices did not show any abundance.

The determination of the recovery rates follows the following calculation model:

$$Recovery\ rate\ (spiked\ sample) = \frac{conc.spiked\ mea.}{conc.exp.} \cdot 100\ [\%]$$

Conc. spiked mea.: measured concentration of the spiked sample [µg/kg]

Conc.exp.: expected concentration of the spiked sample (theoretical) [µg/kg]

With the help of the recovery rate it is possible to monitor the loss of the amount of an analyte during the extraction process.

For the quantification of the samples a correction of recovery rate, called relative recovery rate, referring to the internal standard is necessary. Therefore possible extraction differences can be adjusted, so that variations could be reduced.Internal standardβ-zearalenol-d4 was used.

The variations in the recovery rates are resulting in slightly physical and chemical differences from zearalenone and its metabolites to the internal standardβ-zearalenol-d4. Reasons for this are matrix effects like matrix compounds that influence the ionization process or for example influences of background noise to peak integration.

	α-zearalanol	α-zearalenol	β-zearalanol	β-zearalenol	zearalanon	zearalenon
Processed liver products(liver)	121,1%	84,0%	138,5%	110,6%	62,1%	54,3%
Sausages(muscle)	131,5%	75,1%	147,2%	116,8%	86,8%	44,6%
Blood sausages(blood)	156,6%	92,7%	186,2%	116,2%	128,2%	44,3%
Bacon (fat)	137,3%	85,4%	159,4%	113,2%	77,1%	47,0%

Tab. 19Relative recovery rates of zearalenone determination (LC-MS/MS) for liver, muscle, blood, fat compartments

Absolute recovery rates are showing the actual detected values and give information about losses that occur through the whole extraction process.

	α-zearalanol	α-zearalenol	β-zearalanol	β-zearalenol	zearalanon	zearalenon
Processed liver products(liver)	23,9%	15,1%	26,5%	20,8%	17,5%	8,1%
Sausages(muscle)	22,8%	13,7%	26,8%	21,4%	15,8%	7,5%
Blood sausages(blood)	17,7%	10,4%	17,2%	12,6%	15,7%	5,8%
Bacon (fat)	10,4%	7,8%	13,6%	9,6%	6,6%	4,0%

Tab. 20Absolute recovery rates referred to internal standard of zearalenone determination (LC-MS/MS) for liver, muscle, blood, fat compartments

3.4.5.4 Validationfor Ochratoxin A

The samples of blood sausage, sausages, liver dumpling and liver paste were spiked with 0.5 µg/kgand 1.0 µg/kg and analyzed. (See table 21)

Sample name	spiked with Ochratoxin A[ng/kg]	Ochratoxin A conc. from spiked samples [ng/kg]	Recovery rate [%]
Liver dumpling 1	0	0	0
Liver dumpling 2	500	430	86
Liver dumpling 3	1000	888	89
Blood sausage 1	0	0	0
Blood sausage2	500	433	87
Blood sausage3	1000	1020	102
Frankfurter 1	0	0	0
Frankfurter 2	500	415	82
Frankfurter 3	1000	993	99
Liver Pâté 1	0	90	-
Liver Pâté 2	500	502 (412)	82
Liver Pâté 3	1000	959 (1049)	105
Bacon 1	0	0	0
Bacon 2	500	537	107
Bacon 3	1000	982	98

Tab. 21Validation experiment – Recovery rates for Ochratoxin A

To determine the LOD and the LOQ, a five point linear calibration line was made, at concentrations of 0.01 µg/l, 0.025 µg/l, 0.05 µg/l, 0.1 µg/l and 0.25 µg/l. The values for the LOD are evaluated by DIN 32645 to the significance level of 99 %. The values for the LOQ are evaluated by DIN 32645 to the significance level of 95%.

Substance	LOD [µg/l]	LOQ [µg/l]	LOD [µg/kg]	LOQ [µg/kg]
Ochratoxin A	0.010	0.025	0.013	0.033

The analyses of the contaminated samples were repeated to verify the results. Therefore each contaminated sample was investigated twice, one blank sample and one sample spiked with 0.133 µg/kg ochratoxin A (respectively 0.1 µg/l).

4 Results and Discussion

4.1 Results

4.1.1 Results – Zearalenone and metabolites

From the 44 analyzed samples, two samples show resultsabove the limit of detection. 0.54 µg/kgα-zearalenol (LOD: 0.44 µg/kg; LOQ: 0.66 µg/kg),0.22 µg/kg zearalenon (LOD: 0.20 µg/kg; LOQ: 0.46 µg/kg). One value was above the LOQ, 0.82 µg/kg β-zearalenol (LOD: 0.21 µg/kg; LOQ: 0.48 µg/kg) in a liver dumpling sample (Tann Leberknödel). Another liver dumpling sample (dry product and not ready to consume) contains 0.52 µg/kg zearalenon(LOD: 0.20 µg/kg; LOQ: 0.46 µg/kg) (Knorr Leberknödelsuppe,dried).

The following figure shows the chromatogram of zearalenone and its metabolites in a liver dumpling sample. It was determined with APCI-4000 qtrap in negative mode.

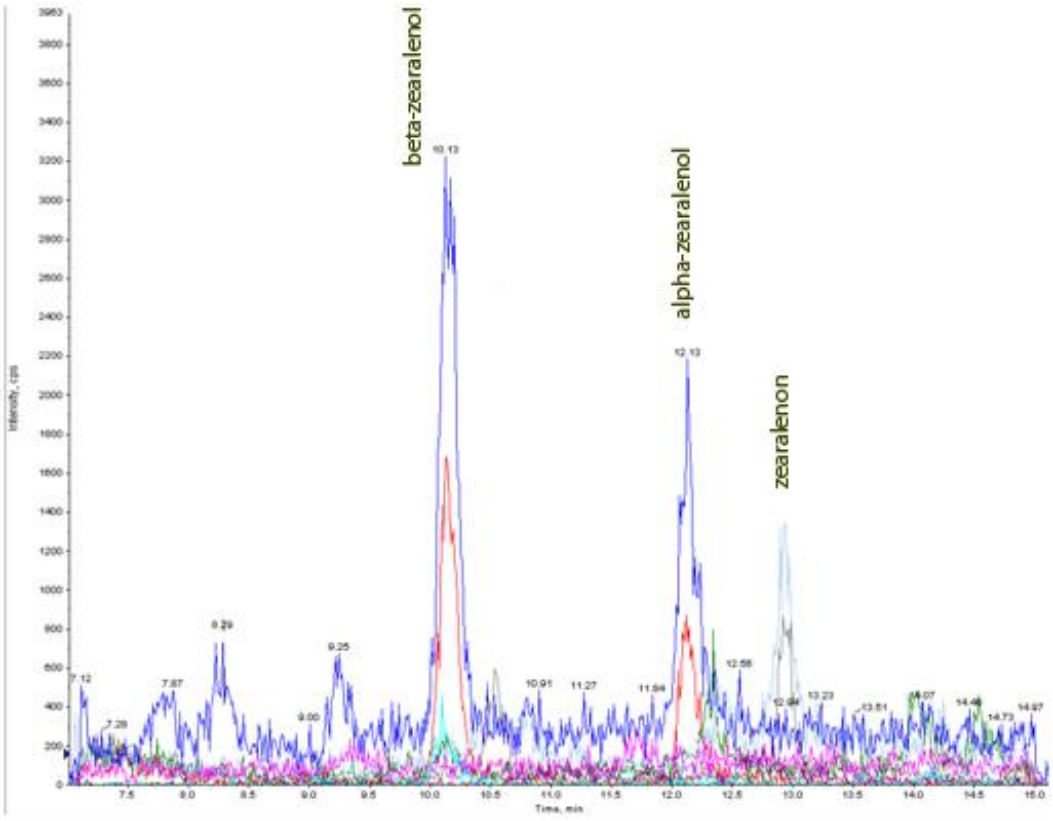


Fig.22Chromatogram Liver dumpling – zearalenone and metabolites, APCI MS/MS, 4000qtrap, negative mode

Furthermore there is evidence that in another processed liver product sample zearalenon could be found close to the limit of detection (LOD: 0.20 µg/kg zearalenon). But concerning the validation of the limit of detection for liver dumplings, this value could not be specified at significance level of 99%. This is also true for a liver pâté sample concerning α-zearalenol (LOD: 0.44 µg/kg).

A mean recovery rate of 121 – 137% (10 – 24%) for α-zearalanol, 75 – 92% (8 – 15%) for α-zearalenone, 138 – 186% (13 – 27%) for β-zearalanol, 110 – 117 % (10 – 21%) for β-zearalenol, 62 – 128% (6 – 17%) for zearalanon and 44 – 54% (4 – 8%) for zearalenon. The results in the brackets are absolute values.

Products with a high muscle percentage and also bacon samples did not show any concentration of zearalenone.

4.1.2 Results – Ochratoxin A

The 44 samples,same as from zearalenone analytical study, where determined also for ochratoxin A.

From these samples 5 products with a main liver component show a significant concentration of ochratoxin A above the limit of quantification (LOQ: 33 ng/kg)(see table 22). One sample show values above the limit of determination (LOD: 13 ng/kg) at the second analysis.

Processed liver products	OTA conc. [ng/kg]	OTA conc. [ng/kg]
	124	86
	155	107
	73	47
	201	131
	34	> LOD

Tab. 22Concentration [ng/kg] of Ochratoxin A in Liver dumpling- and Liver pâté samples

In two blood sausages samples values could be specified above the limit of quantification (LOQ: 33 ng/kg), although one sample show values above the limit of detection at the second analyzing step.

Blood sausage	OTA conc. [ng/kg]	OTA conc. [ng/kg]
	>LOD	42
	93	96

Tab. 23Concentration [ng/kg] of Ochratoxin A blood sausage samples

The chromatogram below shows the determination of ochratoxin A (at 10.385 min) in a liver paste sample with HPLC-FLD detection.

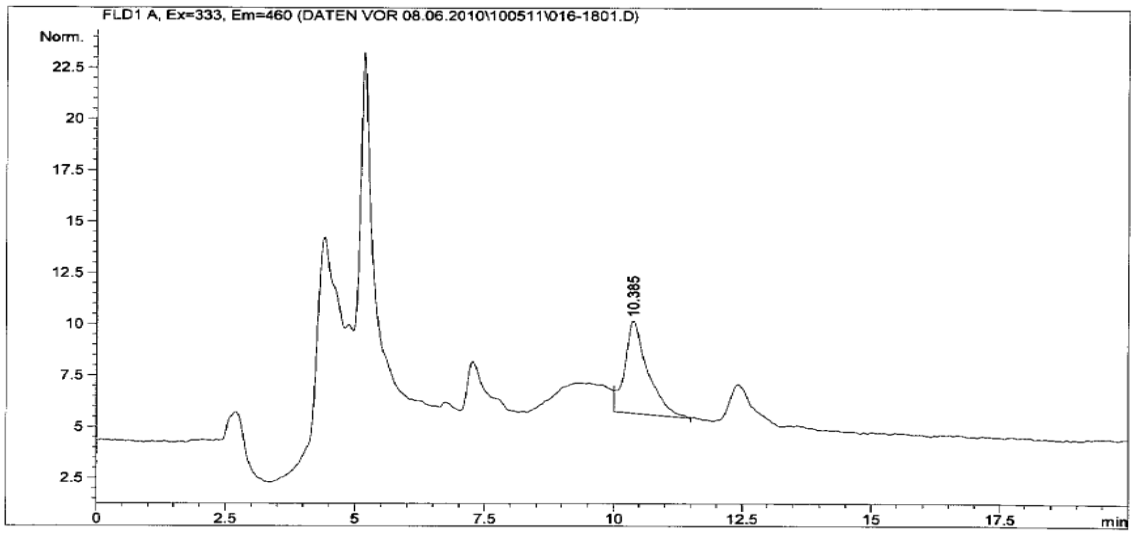


Fig.23 Chromatorgramm of Ochratoxin A in a liver pâté sample.

No significant concentrations are found in products that mainly consist of muscle meat and products with high amount of fat.

4.2 Discussion

The main aim of this work was the determination of zearalenone, a mainly field mycotoxin, and its metabolites and the ubiquitous, mainly storage mycotoxin, ochratoxin A in processed products from pig to clarify the carryoverfrom field, over contaminated feed up to human nourishing medium.

At this moment, neither a survey of the occurrence of zearalenone and its metabolites nor for ochratoxin A contamination in processed products of pig in Austria had been done.

Several national and international studies on the effects of mycotoxins on human suggest different ways of dealing with the problem.Nutrition, being an interdisciplinary field of study, sees many factors playing together when mycotoxins occur in the food chain. Accurate analyzes of environmental toxins are requirement for science basedadvices. Governmental or non-governmental organizations have to handle estimated values and set guidelines. Nutrition and health are topicsthat are deeply involved in every day's life and regulations therefore are influenced by a lot of stakeholders. Detected values of substances in nourishing mediums are only numbers and are meaningless when they do not fit into the right surrounding. The importance of highly developed analysis methods helps to focus where even very low concentrations of highly toxic compounds appear. Information of chemical analysis helps to determine where prevention strategies should or canbe implemented. Moreover some groups of persons are more sensitive to toxic substances and therefore special maximum permitted values should be evaluated. In future there should be more non nutrients diet surveys to estimate the usual intake of food chemicals like mycotoxins [COUNIL E. et al., 2006].

Germany, Switzerland and Sweden for example discuss limit values of 0.01 – 0.03 µg/kg for ochratoxin A in infant food. Even though it was reported that only 3 % of the Swiss population could exceed the proposed "virtually safe dose" of 5 ng/kg b.w. per day, the exposure of the population to ochratoxin A should be low as reasonably achievable [ZIMMERLI B. and DICK R., 1995]. Moreover, the

distribution of mycotoxins into human milk was reported. The exposure to ochratoxin A during the foetal and neonatal period is may be potentially harmful [SKAUG M.A. et al., 2001].

Furthermore, it has been reported that in areas where Balkan Epidemic Nephropathy occurs ochratoxin A was found in human blood. Especially due to bad storage conditions of food at home mycotoxins can enter the human body. The survey from Balkan as well as from Argentina showed that lower socio-economical levels correlated with higher ochratoxin A contamination in blood [PACIN A.M. et al., 2008].

Nevertheless referred to Grossgut, AGES, the average daily intake of zearalenone is about 0.2 µg/kg b. w. This is only a fragment of the currently allowed dose of 1 µg/kg b.w. per day in Austria. Investigated foods in Austria (by AGES) presented a certain contamination with ochratoxin A, which did not refer to a TWI (100 ng/kg body weight per week) by average consumption in any population group. For high consumers or e.g. preschooler the TWI could be reached. 95.5 % of the ochratoxin A concentration of food investigated in Austria lay below maximum values. Summarizing the health risk for the Austrian population from the intake of Ochratoxin A over food in the investigation timeframe from 2003 to 2007 seems to be low [RAUSCHER-GABERNIG E. and GROSSGUT R., 2007].

The following discussion show the analyzed values of zearalenone and metabolites as well as ochratoxin A values found in Austrian processed products from pig to identify hazards coming from this kind of nourishment. Furthermore the discussiondoes involve the advantages of novel analytical methods and further development of faster, less expensive and more accurate analysis. Moreover,nations are working together to deal with hazards like mycotoxins in the food chain and at no time before it was that easy to have international information exchange over the whole planet as nowadays. With the help of information technologies scientists as well stakeholders, NGO’s, governments etc. can handle problems faster and better prepared.

But it is important to understand the reasons for regulations and guidelines. This discussion contains a part of explanations why detection methods should be enhanced. This information should help to understand the occurrence of mycotoxins in human life much better and that it is a global problem affecting us permanently.

Therefore prevention strategies for mycotoxin contamination are a major step. Both substances, zearalenone and ochratoxin A, show that the diversity of their appearance, through for example climate conditions or cultivation, is manifold. Prevention is a first step to avoid the carry over through all kinds of processes of food. For the future all groups of interests have to have a conscientiously look to the possibilities and hazards of new technologies in prevention.

4.2.1 Analysis of food samples

44 products from pig, divided into four different compartments (liver, muscle, fat, and blood), were determined for zearalenone and metabolites as well as for ochratoxin A.

The Results show that only two samples contain values between the LOD and LOQ of zearalenone. One of these samples contains β-zearalenol above the limit of quantification (0.82 µg/kg). In sausage and bacon samples no significant detection of zearalenone and metabolites could be established.

The other liver dumpling sample from a dry product contains 0.52 µg/kg zearalenon (LOD: 0.20 µg/kg; LOQ: 0.46 µg/kg) (Knorr Leberknödelsuppe, dried). This product is going to be boiled with water. Therefore the amount of toxin will be much lower for consumption.

Recovery rates, LOD and LOQ were similar to the method of Marker C. for liver samples. All results were corrected with the estimated relative recovery rates.

Ochratoxin A could only be found in liver and blood samples, where 5 samples with main liver components showed significant concentrations. Values are between 34 and 201 ng/kg (LOD: 13ng/kg; LOQ: 33 ng/kg). In two samples with blood as main component, concentrations of ochratoxin A between 42 and 96 ng/kg, could be determined.

No significantconcentrations are found in products with main amount of muscle meat and products mostly consisting of fat.

A lot of surveys reports contamination of crops with mycotoxins all over the world. Even though not every kind of mycotoxin is able to develop in Austria,a lot of different mycotoxins can reach into the food chain be trading crops from all over the world. Storage fungi produce mycotoxins like ochratoxin A and crops like maize could contain this toxin and travel around the globe. The usage of contaminated crops for feed is still common. To be frank it is impossible to achieve crops free from all environmental toxins, but that is not the point.

Millions of year'sspecies of all kinds interact among each other and many mechanisms in organisms are generated to handle hazards from the environment. But especially risk groups like infants, elderly or people who suffer from diseases should be reason for further lower guidelines in food.

It refers to good manufacturing practices to produce high quality food. In the chapter below some prevention strategies will be discussed. For examplemaize harvests contain a few contaminated kernels and after milling the mycotoxins aredistributedall over the product. To handle such problems awareness training should be accessible for all participants through the food chain processes.

From the literature is known that mycotoxins follow different matrices in body compartments. The results led to the conclusion that the distribution of mycotoxins in pigs correlates with the distribution pattern liver>blood>muscle>fat. No determined values from this study show any risk for human health, but the results indicate processed liver and blood products to be of more health concern than for example sausages with high meat content. Moreover the information of the distribution of mycotoxins from this and from former studies refer to consideration that liver and blood in human body are compartments that are more sensitive to mycotoxins. This led to the conclusion that more research can be done on this sector of risk factors.

In the literature it was demonstratedthat values can be determined in human blood and human milk, also to point out that this are risk factors especially for infants and elderly as well as for patient who have problems with theirimmune system. New maximum intake levels will be discussed and also therefore enhanced detection methods described, which will be necessary for further investigations to very low concentration levels.

The increasing costs for highly sensitive analyzing methods have to be justified. New methods therefore have been established and further are coming. The combination of different methods will help to adjust the right demands for analytical questions. Especially immunoaffinity based methods can enhance the quality of determination, but also novel strategies like multi-mycotoxin detection

methods or biotechnology methods will help to increase analytical costs and are less time consuming in the laboratories.

4.2.2 Development of mycotoxin issues

The advantage of the IAC to SPE columns was the decreased amount of solvent used. Due to the less purification steps it simplified the sample analysis and shortened the analysis time [SHELVER 2004].Even though mass spectrometry detection is the current reference method for mycotoxin determination, the high costs and the extensive workflow lead to the conclusion that immunochemical techniques remain the method of choice for screening large numbers of samples [ROSEN S. 2009].

In future mycotoxin detection will move from specialized laboratories to the point of service. New technologies emerge with miniaturization of chromatographic techniques such as laboratory-on-chips or by the replacement of antibodies by DNA aptamers. The goal is to provide instant results from like harvesting machinery or grain escalators where such methods can give immediate decisions. [KARLOVSKY P. 2009]

Further novel strategies for mycotoxin detection are multi mycotoxin detection methods. In Austria only a few scientific groups are searching for an accurate method to combine the highly selective determination of the different kinds of mycotoxins. Worldwide first developed multi mycotoxin methods focused on *Aspergillus*, *Penicillium*, *Alternaria* or *Fusarium* toxins. Further published methods included mycotoxin metabolites, masked mycotoxins and ergot alkaloids. [MONBALIU S. et al., 2010] A first multi mycotoxin method that meet al AOAC Official Method acceptability criteria using multitoxin immunoaffinity column cleanup with liquid chromatography and fluorescence detection for determination of aflatoxins B1, B2, G1, G2 and ochratoxin A in powdered ginger [TRUCKSESS M. W. et al., 2008]. Further 23 mycotoxins (aflatoxin B1, B2, G1, G2, ochratoxin A, deoxynivalenol, zearalenone, fumonisin B1, B2, B3, T2-toxin-

HT2-toxin, nivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, diacetoxyscirpenol, fusarenon-X, neosolaniol, altenuene, alternariol, alternariol methyl ether, roquefortine-C, and sterigmatocystin) in feed could simultaneously detected by LC-MS/MS and validated according to Commission Decision 2002/657/EC and accredited by EN ISO 17025 [MONBALIU S. et al., 2010].

For samples with a content lower than 0.5 µg/kg, interfering associated substances affect the analysis negative due to the fact that at a measuring range of 330/460 nm these substances have a native fluorescence. An improved specific analytic method for ochratoxin A, is combining HPLC separation with enhanced fluorescence detection by post-column addition of ammonia. The quantitation limit for ochratoxin A was improved and estimated at 5 – 10pg/g for human milk and serum. Advantages of this method are for example that the excitation- and emission wave length shift in this range or that at 390/440 nm native fluorescence disturbs much lesser. The sensitivity of the method increases dramatically, because of the resulting derivate having a higher fluorescence intensity than pure ochratoxin A and associated interfering substances get “invisible” due to derivatization [KASTRUP S. and AULWURM U. 2002]

For further perspective it should be mentioned that prevention strategies in agriculture for mycotoxin contamination are manifold. For example plowing could reduce the proliferation of *Fusarium spp*. over winter by forwarded degradation of infected material in deeper bottomset beds, especially at preceding crops with high hazard potential like wheat and maize. Furthermore infected kernels should be eliminated by cleaning procedures from the crops, e.g. for example remove dust, small particles, weed seeds, etc. Then separate lighter kernels from the rest, peel and moist crushing (milling) the crop. In the end the yield could be washed, before dried to low aw and stored. Over 50% of mycotoxins could be eliminated with these measures. Storage fungi demand lower demands than field fungi and therefore they can proliferate at inferior water content [COENEN 2003]. Although interaction between relative humidity, temperature in storage facilities and water content in crops are explored, the

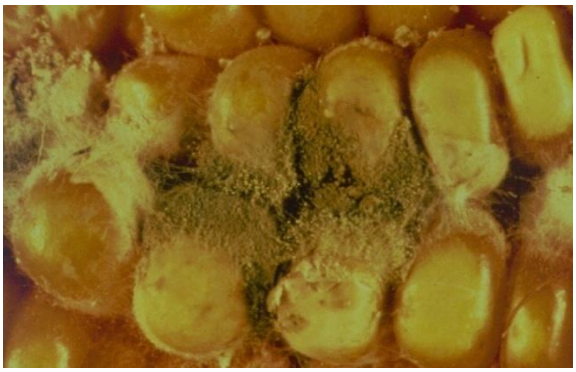
daily practical implementation are not always used and followed. New storage technology for crops is cool storage around 10°C to sustain quality of the goods and minimize the growth of insect pests and mites. It is noticed that bugs and mites could create temperature increase in stored crops.

If it is not possible to cultivate mycotoxin free crops, detoxification of the feed could be a method. These feeds with less mycotoxin content could be given to less sensitive livestock like fattened bull, poultry or hog. In some countries contaminated material could be diluted with non contaminated crops. But this method will not have a promising future because of food safety measures of health consumer protection. It should also be mentioned that chemical treated feed, e.g. urea, sodium-disulfide or sodium hydroxide solution can significantly lower the mycotoxin contamination but can only be given to cattle. Feed treated with mycotoxin binder or by enzymatic cleavage inactivation show no significantly effect in surveys [HÖRÜGEL K. et al., 2003].

Ochratoxin A is thermally stable and can remain stable over years. On the one hand reports mentioned that the toxin is not destroyed by heating, roasting or common food preparation procedures [EFSA 2006]. On the other hand some effects have been shown by extrusion cooking of hulled barley meal. 17 to 86 % of ochratoxin A was destroyed after the process by temperature level of 160° C and 24 to 30 % moisture content for 70 seconds. Further studies can be done to animal feed if after treatment with heat can lower mycotoxin concentrations [CASTELLS et al., 2006].

A lot of mills reject wheat deliveries, when they are suspicious of being contaminated with mycotoxins. They could be detected either by optical check (red kernels, see Fig. 24, 25) or ELISA tests in the incoming control. But it should also be noticed that often the values of contamination are overestimated due to improper sampling. Often the investigation of the contaminated crops should be determined on the field, where they are not rehashed for example by pre-cleaning. This can reduce the zearalenone contamination significantly due to the fact that the majority of the toxin is located in the dust of the cereals and

the external parts of the kernels. Marketable cleaning facilities in mills are able to reduce zearalenone contamination by 50% [BICKERT C. 2003].



Source: http://www.ces.ncsu.edu/depts/pp/notes/Corn/corn001.htm

Fig.24The fungus Aspergillus flavus sporulating on corn.

Fig.25Corn kernels infected with Fusarium moniliforme showing "starburst" symptom

The high usage of fungicides is neither cost effective nor environmental acceptable and residuals on food is not wanted. For pre and post harvest processes effective methods to reduce or eliminate ochratoxin A contamination are needed. An alternative method could be natural products. Substances of piperaceous plants may be effective in reducing ochratoxin A contamination in agricultural commodities. It was noticed that four alkaloids from this plants had effects on ochratoxin A, B and citrinin producing Aspergilla and they were also toxic to grain storage insect. But also reported was the moderately toxicity to mice of piperine and piperlongumine [LEE S.E. et al., 2007].

Biotechnology methods will help to reduce mycotoxins in cereal grains. New information on fungal and plant genomes and gene expression was developed. Complete genomes of *Fusarium graminearum* and *Fusarium verticillioides* and several *Fusarium* gene expression sequence databases can help to identify mycotoxin biosynthetic and regulatory genes [DESJARDINS and PROCTOR 2007]. But controversy discussion rose whether environmental safety, intellectual property rights, consumer choice, ethics, food security, poverty

reduction and environmental conservation can go along with this new technology. Lower levels of mycotoxins as well as reduced needs for herbicides, pesticides, water and tilling are extraordinary effects [KENDRA and DYER 2007]. A study from 2007 reported about a transgenic maize line that can mediate detoxification in kernels. For the elimination of zearalenone *egfp::zhd101* gene (*gfzhd101*) was used, encoding a protein fused to a zearalenone degrading enzyme. Further studies will develop anti-mycotoxin antibodies or mycotoxin-degrading enzymes. Even if the transgenic strategies have not yet achieved a practical level of resistance to fungi on the field, Genetic Modified crops will be part of our future. Effects on nutritional properties and the risk form allergens have to be assessed in the future [IGAWA T. et al., 2007].

From a critical point of view, to show how important it is to coordinate all kinds of transactions to regulate the mycotoxin problem and put efforts to the right place the statement of the former UN Secretary Kofi Annan has to be mentioned. He said that: “A World Bank study has calculated that the European Union regulation on aflatoxins costs Africa $750 million each year in exports of cereals, dried fruit and nuts. And what does it achieve? It may possibly safe the life of one citizen of the European Union every 2 years … Surely a more reasonable balance can be found.” [CARDWELL et al., 2001]. It is clear that by trade barriers global economical problem appear. The statement shows that food compounds like mycotoxins are reaching a wide field in human coexistence.

In the end more personal responsibility must be transferred to the consumer and knowledge must be strengthened so that everyone can do his/her own part to prevent the intake of mycotoxins through the food chain. This must not implicate that everyone has to improve their scientific knowledge but a lot can be done to enhance common sense for daily life problems. Legislations already discuss to implement health lesson in school to enhance the awareness of the pupils to the environment. And further cooperation between agriculture and governments as well as food safety authorities should be extended.

5 Conclusion

This study along with several other studies done before concludes that mycotoxins are still a problem on the field and in livestock breeding. The average intake of Austrian inhabitants of mycotoxins through the food chain however is not perilous. But as we know from animal and genetic studies zearalenone and metabolites as well as ochratoxin A are severe toxins to human exposure and should be kept as low as possible. Especially for risk groups like infants and the elderly, it is important to continue searching for more health risk information and appropriate tolerable intake values. Summarizingthe detected values from this study of zearalenone and metabolites as well as ochratoxin A were not of any health concerns to the Austrian population.

Multi mycotoxin methods, similar to the one used in this study for zearalenone and its metabolites with APCI-HPLC-MS/MS, will help to optimize time and material consuming single methods for analyzing mycotoxins in food matrices. For further investigation, the usage of immunoaffinity methods can be applied to multi-mycotoxin determinations, for efficient removal of interfering substances.

Mycotoxins can “travel” through the food chain and start in growing on the field, developing in storage facilities and accumulation in livestock organisms. To prevent agricultural loss and health risk factors, several prevention strategies are implemented. Soil cultivation, alternative fungicides and biotechnology as well as appropriate storage manufacturing practice, cleaning procedures and screening methods of crops can reduce mycotoxin contamination in the food chain. Even the awareness of feeding uncontaminated feed to livestock before slaughtering show significant reduction of mycotoxins in food.

Through globalization,mycotoxin contamination is not only a homemade problem. Mycotoxins like ochratoxin A remain stable over years and trading with contaminated goods could lead to trading problems and economical losses. Due to enhanced determination methods, toxic substances can be found in every kind of nourishment. Furthermore due to less information from animal and

genetic studiesrisk assessment and regulation limits are often performed basing on worst case scenarios. For most of the contaminants, this will lead to an excessive overestimation of residue levels. Mycotoxin like ochratoxin A or zearalenoneare potential health risk factors and concentrations in the food chain should be kept as low as possible. Even for the most well documented toxins, the tolerable daily intake remains temporary or provisional due to insufficient toxicological information. A further problem to determinethe health risks of mycotoxin is the lack of information on exposure [THUVANDER et al., 2001].Moreover,several secondary fungi metabolites and other environmental toxins are consumed and therefore combined toxicity is very complicated so that the exposure often results in an additive effect, indicating a synergistic interaction [MONBALIU S. et al., 2010]. The chronic effects are more severe than the acute toxicity of mycotoxins so that even with small amounts of the toxin, constant intake can influence human life right at the beginning by accumulation in blood and human milk. Risk assessment for high risk groups like fetus, babies, children, sick persons or elderly should be done and more information about occurrence should be coming from appropriate dietary surveys. These evaluations need to be reexamined from time to time taking into account new information on both exposure and basic toxicology as well as improved understanding of mechanism of action [KUIPER-GOODMAN 1999]. But also personal responsibility and enhanced knowledge on nutrition are very important factors to take care of each individual life.

Recommendations from EFSA 2006 for ochratoxin A prevention could be taken as a guideline for first steps for the next years to overcome the problem. These recommendations can also be used for other mycotoxins.

- Continuing efforts to reduce ochratoxin A contamination in foods. (Monitoring programs targeted to known and emerging sources of exposure)
- More data on relationship between maternal exposure and resulting human milk concentrations.
- Adequate studies on reproductive and developmental toxicity are needed.
- Need for data on species specific accumulation of ochratoxin A in the kidney.

[EFSA 2006]

6 Summary:

Mycotoxins are widespread contaminants of feedand food, meat, cheese, spices and beverages, coming from different kinds of fungi that grow on almost every kind of nourishment. Zearalenone and its metabolites are nonsteroidal estrogenic mycotoxins produced by *Fusarium* species, colonizing maize as a special problem in Austria. Ochratoxin A is mainly produced by the four genera of fungi *Fusarium, Claviceps, Aspergillus* and *Penicillium*.In human blood ochratoxin A has been reported in several studies and the main concerns are the carcinogenic, nephrotoxic potential and alterations of immune responses. The toxins are mainly transmitted to swine by contaminated feed from which carry-over to humans has been demonstrated. An HPLC-APCI-MS/MS method with SPE extraction for the determination of the mycotoxin zearalenone and its metabolites and an HPLC-FLD method with IAC extraction for ochratoxin A detection was used to clarify the mycotoxin content in processed products from pigs in Austria. Both methods were applied to 44 food samples of processed products from pigs with the main focus of containing liver, muscle, fat and blood amount where former studies show a mycotoxin accumulation in these parts of livestock.The concentrations of zearalenone and metabolite in most samples were too low for quantification. Two samples show results above the limit of detection. 0.54 µg/kg α-zearalenol (LOD: 0.44 µg/kg; LOQ: 0.66 µg/kg), 0.22 µg/kg zearalenon (LOD: 0.20 µg/kg; LOQ: 0.46 µg/kg). One value was above the LOQ, 0.82 µg/kg β-zearalenol (LOD: 0.21 µg/kg; LOQ: 0.48 µg/kg) in a liver dumpling sample. Another liver dumpling sample contains 0.52 µg/kg zearalenon (LOD: 0.20 µg/kg; LOQ: 0.46 µg/kg). This product was dried and concentration of the toxin in rehydrated food would be diluted.From the samples 5 products with a main liver component show a significant concentration of ochratoxin A above the limit of quantification (LOQ: 33 ng/kg). In two samples with high blood amount, values could be specified above the limit of quantification (LOQ: 33 ng/kg). A maximum concentration of ochratoxin A was 201 µg/kgfound in a Liver pâté sample. No significant concentrations of zearalenone and metabolites as well as ochratoxin A could be detected in

processed products from pig with a main ingredient of meat or fat. That refers to the distribution pattern liver>blood>muscle>fat of mycotoxins in higher organisms.

The occurrence of zearalenone and metabolites as well as ochratoxin A in processed products from pig should not be a matter of serious concerns. Undertaken measures to prevent mycotoxin contamination in Austria are well developed and knowledge could be spread to other countries. Nevertheless, the exposure of the population with potential human toxins should be as low as reasonably achievable.

7 Zusammenfassung

Mykotoxine sind weitverbreitete Kontaminanten in Futtermittel und Lebensmittel wie Fleisch, Käse, Gewürze, Früchte und Getränken und werden von verschieden Arten von Pilzen produziert. Zearalenon und seine Metaboliten gehören zu den nonsteroidalen östrogenen Mykotoxinen und werden von Fusariumspezies gebildet. Der Befall von Mais ist in Österreich ein spezielles Problem. Ochratoxin A wird hauptsächliche von vier Pilzarten produziert, Fusarium, Claviceps, Aspergillus und Penicillium. Das Vorkommen von Ochratoxin A im menschlichen Blut, die krebserregende und Nieren schädigende Wirkung, sowie Veränderungen im Immunsystem wurden in mehreren Studien aufgezeigt. Die Gifte sind hauptsächlich in Schweinen zu finden, die mit kontaminiertem Futtermittel gefüttert wurden. Eine Übertragung von Schweinefleisch auf den Menschen konnte gezeigt werden. Mittels einer HPLC-APCI-MS/MS Methode mit SPE Extraktion für Zearalenon und seinen Metaboliten und eine HPLC-FLD Methode mit IAC Extraktion für Ochratoxin A, wurde die Gehalte dieser Gifte in verarbeiteten Lebensmitteln vom Schwein aus Österreich bestimmt. 44 Lebensmittelproben aus verarbeiteten Lebensmitteln vom Schwein wurden untersucht. Unterschieden wurden die Proben im Anteil von Leber, Muskel, Fett und Blut, da diese Körperkompartimente in vorigen Studien Akkumulationen dieser Gifte zeigten. Die gefundenen Konzentrationen, von Zearalenon und Metaboliten waren in den meisten Proben unter der Bestimmungsgrenze. Zwei Proben zeigten Werte über der Nachweisgrenze. 0.54 µg/kg α-Zearalenol (NG: 0.44 µg/kg; BG: 0.66 µg/kg) und 0.22 µg/kg Zearalenon (NG: 0.20 µg/kg; BG: 0.46 µg/kg). In einer Leberknödelprobe war der Wertfür β-Zearalenolmit einer Konzentration von 0.82 µg/kg (NG: 0.21 µg/kg; BG: 0.48 µg/kg)über der Bestimmungsgrenze. In einer weiteren Leberknödelprobe konnte eine Konzentration von 0.52 µg/kg Zearalenon (NG: 0.20 µg/kg; BG: 0.46 µg/kg) detektiert werden. Fünf der 44 Proben mit hohem Leberanteil und zwei Blutwurstproben zeigten signifikante Konzentrationen von Ochratoxin A über der Bestimmungsgrenze von 33 ng/kg. Die höchste Menge von 201 µg/kg Ochratoxin A konnte in einer Leberpastetenprobe analysiert

werden. Keine signifikanten Werte konnten in jenen Proben festgestellt werden, die hauptsächlich aus Muskel oder Fett bestanden. Diese Ergebnisse korrelieren auch mit dem Verteilungsmuster der Gifte in höheren Organismen (Leber>Blut>Muskel>Fett).Das Vorkommen von Zearalenon und seinen Metaboliten sowie Ochratoxin A in verarbeiteten Lebensmitteln vom Schwein geben keinen Grund für gesundheitliche Bedenken. Die unternommenen Maßnahmen zur Prävention in Österreich sind gut entwickelt und dieses Wissen kann an andere Länder weitergegeben werden. Dennoch sollte die Exposition mit potentiell giftigen Substanzen für den Menschen so niedrig wie möglich gehalten werden.

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9 Attachment

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