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Food safety hazard of Ochratoxin A and its occurrence in Norway

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Content

1. Introduction

Ochratoxin A (OTA) is a mycotoxin produced by secondary metabolism of many filamentous fungi species belonging to the genera *Aspergillus* and *Penicillium* (Magan and Aldred, 2005). They can produce this toxin under favourable conditions. OTA was first discovered and isolated in 1965 from a culture of *Aspergillus ochraceaus* (IARC, 1993). And from then on it has been detected worldwide in various food and feed sources.

OTA is one of the most important mycotoxins of concern of human health (Magan and Aldred, 2005). It can reach humans trough the food chain and can then be excreted in biological fluids, including human milk. It can therefore be transmitted from mother to child during breast-feeding (Khoury et al. 2010). Cereals are regarded as the major source of OTA on a worldwide basis (CVUAS, 2013). OTA has also been deteced in blood samples from swine at the slaughterhouse (IARC, 1993), and it may form residues in edible offal after contamination of animal feedstuff. So OTA exposure to humans may occur directly via contaminated food, or indirectly via animals ingesting contaminated feedstuff (EFSA, 2006). Exposure can also occur via inhalation of OTA in airborn dust and fungal conidia (Skaug et al., 2001).

Studies show that this molecule can have several toxicological effects. It has been linked to kidney problems in both livestock and human populations. It has also carcinogenic, genotoxic and immunotoxic properties (André el Khoury et al. 2010, Meulenberg, 2012). The intake of high concentration of OTA may lead to acute toxicity. Low concentration of OTA is also harmful and provides a risk. The constant exposure of low OTA concentrations might lead to immunological impairments and possible cancer (Segvic Klaric 2012). However, the extent of the toxic effects and risks of OTA are debated. There are many theories about OTA beeing a part of the aetiology of Balkan Endemic Nephropathy (BEN), which is a chronic wasting kidney disease occuring in Croatia, Serbia, Bulgaria and Romania (Bui-Klimke and Wu, 2014, Petkova-Bocharova and Castegnaro, 1985, Khoury et al. 2010, Krogh, 1976, Faucet et al., 2004).

The aim of this study was to investigate possible risk of both animal and human health by this toxin, and the food safety issue concerning this.

2. Mycotoxins

Mycotoxins are secondary metabolites that are produced by certain fungi species of *Aspergillus*, *Penicillium* and *Fusarium*. These fungi will proliferate, and may produce mycotoxins under favourable environmental conditions. These toxic metabolites are not necessary for normal growth or development of the fungi (Fox and Howlett, 2008). The mycotoxins can enter the food chain through foodstuff that are contaminated directly as a result of manufacturing, processing, preparation, treatment, packing, transport, holding or from environmental contamination (European Mycotoxins Awareness Network (EMAN), 2012). They can also enter the food chain indirect via different food products from animals fed mycotoxin-contaminated feedstuffs. Inhalation of mycotoxins and skin contact with substrates infected by mould are also important sources of infection, but in a lesser extent than through different foodstuff (J. W. Bennet and M. Klich, 2013).

Mycotoxins may lead to acute disease when a high amount of the toxin is ingested. But it is more common that animals and humans will ingest a low amount of it, during long periods. And this may lead to gradually developement of metabolic, physiologic and immunologic disturbances (Sorrenti et al. 2013). Mycotoxins is a very important chronic dietary risk factor. The severity of mycotoxicosis depend on many different factors, like the chemical structure of the toxin (J. Fink-Gremmels, 1999), the amount of toxin and duration of exposure, interactions with other toxins, age, health status and sex of the exposed individual (J. W. Bennet and M. Klich, 2013). There is still much research to do on this field, as other factors may play a role as well. Individuals that suffer of mycotoxicosis may be more exposed to secondary bacterial infections and other health disturbances due to the reduced resistance of the host (J. W. Bennet and M. Klich, 2013). These toxins occur worldwide in agricultural products. Foods that are typically contaminated with mycotoxins include cereal products, nuts, spices, coffee, dried fruit, wine and grape juice (CVUA, 2013). One quarter of the world's crops is estimated to be contaminated with mycotoxins to some extent (J. Fink-Gremmels, 1999). Well-known mycotoxins include aflatoxins, patulin, trichothecenes, citrinin, ergot alkaloids, fumonisins, zearalenone and ochratoxins.

3. OTA

3.1. Taxonomy

The only reported species capable of producing OTA belong to the genera *Aspergillus* and *Penicillium* (Khoury, et al. 2010).

Kingdom: Fungi

Division: Deuteromycota

Phylum: Ascomycota

Class: Euascomycetes

Order: Eurotiales

Family: Trichocomaceae

Genus: Penicillium and Aspergillus

Penicillium and *Aspergillus* make up the majority of the species included in the Trichocomaceae (Pitt et al., 2000).

3.1.1. OTA producing *Aspergillus* species

Aspergillus species able of producing OTA can be divided into two distict groups: *Circumdati* and *Nigri.* Fungi species that belongs to the *Circumdati* group produce conidia that have a golden brown colour. The *Nigri* group includes species producing black conidia. *A.ochraceus* and its two segregates, *A. westerdijkiae* and *A.steynii,* are important species in the *Circumdati* section. *A.carbonarius* is the major OTA producer that belongs to the *Nigri* section. *A.niger* is also included in this group, however only a few strains of it can produce OTA. Several other Aspergilli species like *A.lacticoffeatus, A.sclerotioniger, A.cretensis, A.flocculosus, A.pseudoelegans, A.roseoglobulosus, A.sclerotiorum, A.suplhureu,* can produce OTA, but they are relatively rare (JECFA, 2008).

A.ochraceus is xerophilic and will grow on grains with a moisture content of 9-16%. The optimal temperature for OTA production is between 25 and 30 °C (Segvic Klaric et al., 2013), and is important in the warmer climatic regions of the world. It can infect cereals, like barley and maize, coffee, nuts and cocoa (Magan and Aldred, 2005).

Members of the *Nigri* section*,* especially *A.carbonarius* has been found responsible for OTA presence in grapes and wine from winegrowing areas with warm and dry conditions (Mangan and Aldred 2005). It has also been detected in coffee beans (Khoury et al., 2010), and in

tropical and dried fruits (Segvic Klaric et al., 2013). *A.carbonarius, A.westerdijkiae* and *A.steynii* have been reported in many food products, especially dried and stored food. *A.niger* is xerophilic, and has an optimal temperature around 35-37 °C (JECFA, 2008).

3.1.2. OTA producing *Penicillium* species

P.verrucosum and *P.nordicum* are classified in *Penicillium* subgenus *Penicillium. P. verrucosum* and *P.nordicum* have common morphological characteristics such as very similar colony diameters on many culture media (Segvic Klaric et al., 2013), and they are closely related in physiology (JECFA, 2008). These are slow growing species of the subgenus *Penicillium.*

Penicillum verrucosum is the major producer of OTA in cereals such as wheat, barley, oats and rye. It grows only at lower temperatures, so it is distributed in temperate and cold climates. The optimal temperature for OTA production is 25 °C. *P.verrucosum* can grow on grains with a moisture content of 10-20% (Segvic Klaric et al., 2013). This species is the main source of OTA contamination in cereals associated with the procine and avian nephropathy detected in temperate and cold contries such as Denmark, Sweden, Canada and the Unied States (Khoury et al., 2010). Its habitat ranges across northern and central Europe and Canada (JECFA, 2008).

Penicillium nordicum is normally present in the air, and on the surface of hams and sausages. (Khoury et al., 2010). Most of the reported cases of *P.nordicum* occurence have been in products kept under refrigeration under manufacture. It is expected to have similar physiology as *P.verrucosum* (JECFA, 2008).

It is important to understand the physiology and ecology of these species, and the varieties among them, in order to reduce the contamination and OTA production (JECFA, 2008).

3.2. Chemical properties

Ochratoxin have several different variants of secondary metabolites. Ochratoxin B is a non-chlorinated form of OTA, while ochratoxin C is an ethyl ester form of OTA (Bullerman and Bianchini, 2011), ochratoxin α which is the isocoumarine derivative of OTA, and its dechloro analog ochratoxin β (Khoury and Atouli, 2010). They are often co-produced (Reddy and Bhoola, 2010).
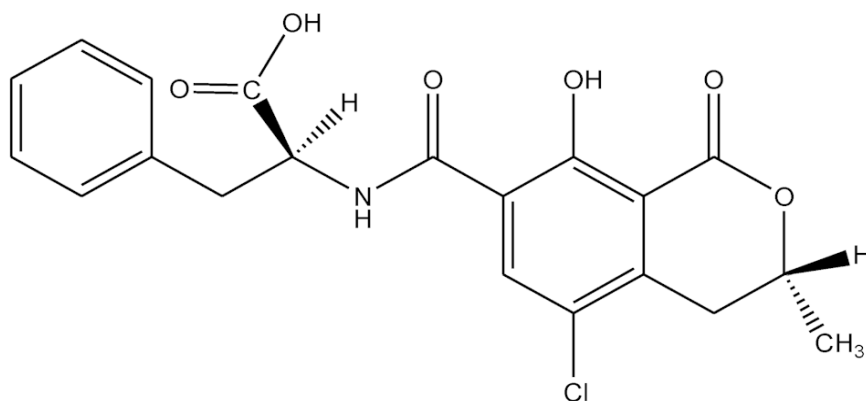
Figure 1. Chemical structure of ochratoxin A (Khoury and Atouli, 2010).

It is a pentaketide derived from the dihydrocoumarins family coupled to β-phenylalanine. The chemical abstract name is L-Phenylalanine, *N*-[(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1*H*-2-benzopyrane-7-yl)-carbonyl] -(R)-isocoumarine (Khoury and Atouli, 2010). OTA is a weak organic acid with pKa value of 7,1. It has a melting point of 169 °C (IARC, 1993). OTA is a colourless crystalline compound (JECFA, 2008). The crystals show an intense green fluorescence in acid solutions under ultraviolet light. In alkaline solutions it has blue fluorescence (IARC, 1993). The OTA has a high stability, and possesses a resistance to acidity and high temperatures. So once foodstuffs are contaminated, it is very difficult to totally remove this molecule (Khoury et al., 2010).

3.3. Biosynthesis

The isocoumarine group of OTA is thought to be formed from acetate and malonate via a polyketide synthesis pathway. The polyketid synthase (PKS) is considered a key enzyme in this process (Gallo et al., 2014, Khoury and Atoui, 2010).
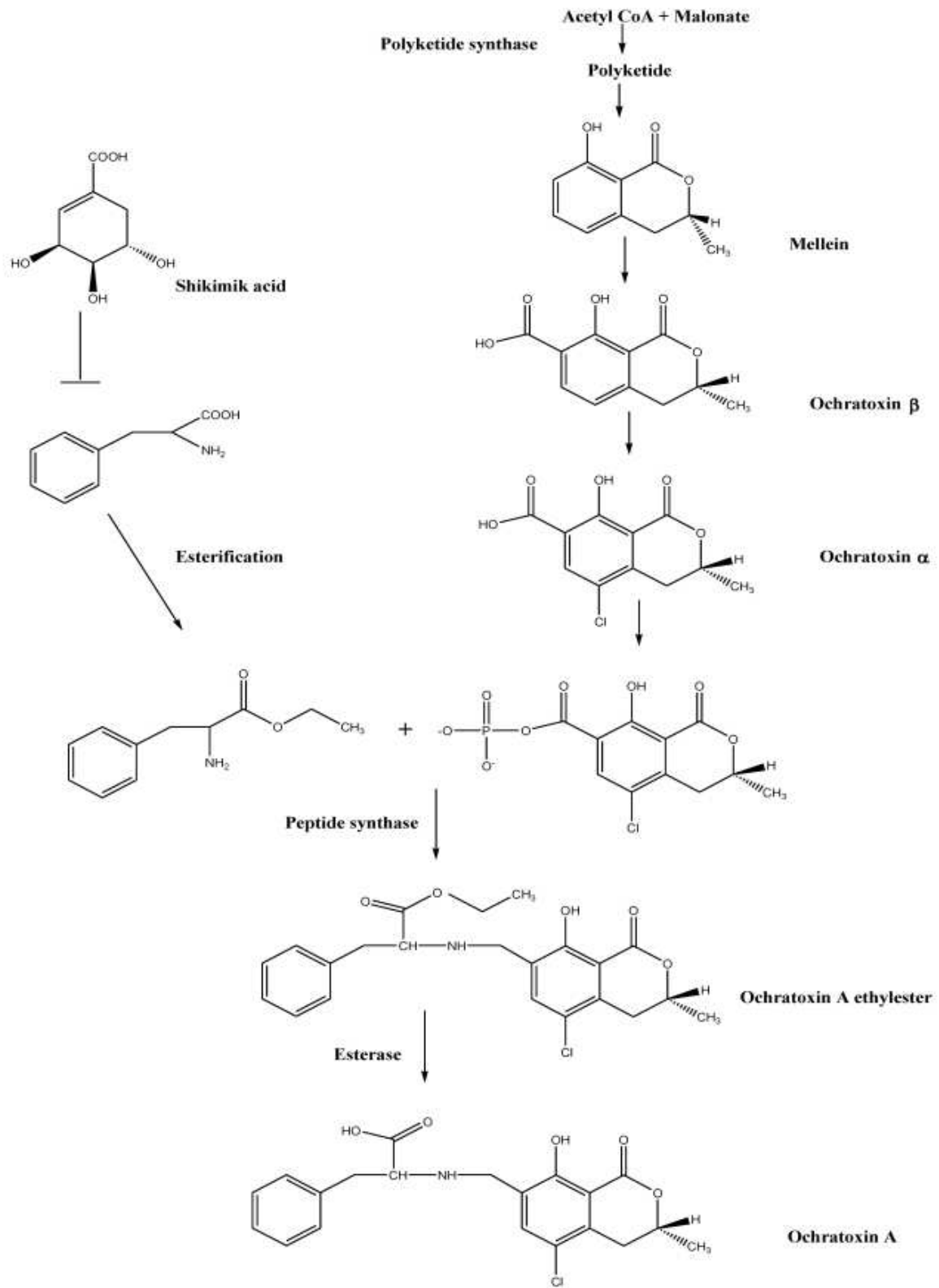
Figure 2. A hypotetical biosynthetic pathway of OTA (Huff and Hamilton, 1979)

However, the biosynthetic pathway of OTA is relative unknown compared to other important mycotoxins. Harris and Mantle questioned the intermediate role of ester ochratoxin C and the mellein as a precursor. But they agreed with Huff and Hamilton that the biosynthetic pathway involves ochratoxin β → α → A. Based on several experiments they state that chlorination of both ochratoxin β and OTB can give rise to OTA (Harris and Mantle, 2001).

3.4. Pharma- and Toxicokinetics

There are differences between the species regarding the pharmacokinetics of OTA, including the biological half-life of OTA. This is thought to play a great role for the actual toxic effects that occur in the different species (Galtier et al.,1981). OTA is absorbed from the gastrointestinal tract, mainly in the small intestine. Then it reaches the bloodstream and is distributed further to the kidneys, and some toxins also reach the liver, muscle and fat (Sorrenti et al., 2013). Studies have showed that there is a high correlation of OTA in milk and in plasma, which suggests that OTA passes from the bloodstream and into the milk by passive diffusion (Gianni Battacone et al., 2010). OTA is excreted by urine and faeces, the extent of these two routes of eliminaion is depending on the enterohepatic circulation and the binding to serum protein (JECFA, 2008). There has been performed many studies on different animals to obtain information of OTA's toxicokinetic profile (Hagelberg and Hult, 1989, Fuchs et al., 1986, Castegnaro et al., 1989).

Studer-Rohr found that the elimination half-life of OTA in humans is approximately 36 days (Studer-Rohr et al., 2000). This is much longer compared to other mammals which have been studied; it is reported to be 55hours in rats (Galtier et al., 1979). OTA was excreted via urine and feces from the rats, some in the toxic form and some in the hydrolyzed ochratoxin alpha form (Galtier et al., 1979). The serum half-life is 1-1,5 days in mice, 3-5 days in pigs, and around 20 days in macaque and vervet monkeys (JECFA, 2008). The great difference between plasma half-life of the toxin should be taken into consideration when rat cancer studies regarding OTA are compared to humans.

Hagelberg and Hult did a study to evaluate the elimination patterns of OTA in fish, quail, mouse, rat and monkey. They administered 50 ng/g body weight OTA either orally or intravenously. The elimination half-life was only 0,68 h in fish after oral administration. On the contrary, it was up to 840 h in monkey after intravenous administration. The distribution

volume was detected to be 57 ml/kg in fish (i.v. admin.), while it was 1500 ml/kg in quail (p.o. admin.). The mouse had a rapid plasma clearance of 72 ml/kg∗h. In monkey it was only 0,17 ml/kg∗h. The lowest bioavailability was detected in fish and the highest in mouse, 1,6% and 97% respectively. The binding capacity of the toxin to macromolecules, like serum albumin, is believed to be a factor affecting these interspecies differences. Hagelberg and Hult found that their experimental animals elimited just a small part of the toxin via clearance by filtration, due to restriction by protein-binding, leading to elimination by other routes for almost all species. In rat they saw that ochratoxin B had similar kinetic profile as OTA, but it was not bound as tightly to plasma proteins and had a 15 times greater clearance value than OTA (Hagelberg and Hult, 1989). In another study of OTA in rainbow trout, whole-body autoradiography was used. It was detected that the concentration of the toxin dropped remarkably in the blood, but it remained much higher in kidney, urine and bile. So this suggests that the fish can exclude OTA by bile excretion through hydrolysis or conjugation, and elimination (Fuchs et al., 1986, Hagelberg and Hult, 1989). After oral administration of OTA in the monkey, it showed a slow absorption from the gastrointestinal tract. And it took as long as 24 h before it reached the maximum concentration. Due to its low clearence level, the OTA plasma concentration was measured to be over 100 ng/ml even six weeks after the single dose of 50 ng/g b.w. In studies of humans the OTA was observed to have high persistence in the blood (Hagelberg and Hult, 1989). After oral ingestion the OTA has a half life of 35 days in humans. It may be due to reabsorption during the enterohepatic circulation or reabsorption from the urine after tubular secretion (Reddy and Bhoola, 2010). It is also suggested to be in accordance with the strong binding capacity to serum proteins (Reddy and Bhoola, 2010, Hagelberg and Hult, 1989). This similarity of OTA behaviour in human and monkey suggest that monkey is a good model for humans. The fish and quail had the highest clearance value, and at the same time they have the lowest fraction of elimination by glomerular filtration. On the contrary, the monkey had the lowest clearance value, and the glomerular filtration seemed to be its dominant route of excretion (Hagelberg and Hult, 1989).

These great varieties of the kinetics in different species naturally indicate that the toxicity of OTA also differs between species. Not only there are interspecies differences, but also intraspecies differences. Castegnaro et al. found that the OTA metabolism was different in rats, depending on their genetic strains. Lewis rat strains have showed a higher hepatic and renal OTA 4-hydroxylase activity *in vitro,* compared to Dark agouti strains. An *in vivo* study of OTA administration to these two types of rats was performed. The result reveiled that the

metabolic ratio of OTA:4-hydroxy-OTA was two to five times greater in Dark agouti rats than in Lewis rats. This indicates that genes play an important role in regulation of OTA metabolism (Castegnaro et al., 1989). It is of course a positive trait to be able to metabolise more of OTA into other compounds, but studies show that the 4-hydroxy-OTA is an effective immunosuppressor. Creppy et al. investigated this matter by administration of 1 migrogram of (4R)-4-hydroxy-OTA per kg to mice. This was almost as effective as OTA on their immunesystem. Cells producing immunoglobulin M were reduced by 80%, while OTA caused an reduction of 90%. Cells producing immunoglobulin G were reduced by 93%, OTA reduced them by 92%. Immunosuppressive properties have not been detected by Ochratoxin α (Creppy et al., 1983). Zepnik et al. studies the toxicokinetcs of OTA in rats, and found varoius results depending on the sex of the rat. Male rats had higher concentration of OTA in their kidneys than the females. This indicates that it has sex-specific toxicity (Zepnik et al., 2003).

3.4.1. Monogastrics vs. ruminants

Cattle have a lower risk of intake of OTA, compared to pigs and poultry species, because their feeding is mostly based on forages and only partially on cereals (Battacone, 2010). As described earlier the cereals have a high risk of OTA contamination. In addition to that, ruminants are able do degrade OTA due to their microbial flora in the rumen. The protozoa will cause hydrolysis of OTA, and will result in formation of the less toxic metabolite; ochratoxin α. Due to this degradation, ochratoxin α is the main fraction that is excreted in the milk of ruminants. In humans and monogastric animals on the other hand, OTA is excreted in the milk (VKM, 2013).

3.5. Toxicology

On a cellular level, the toxic effects of OTA are as following; inhibition of protein and DNA synthesis, lipid peroxidation, competition with phenylalanine and inhibition of Phe-enzymes, oxidative stress, DNA damage (Gianni Battacone et al., 2010, Segvic Klaric 2012) and inhibition of mitochondrial respiration (Sorrenti, 2013). Several studies have showed that antioxidants have a preventive effect on OTA toxicity, and this provide further evidence that this toxin can cause oxidative stress (JECFA, 2008). It also affects blood coagulation (Segvic Klaric 2012, Rjau and Devegowda, 2000). This can lead to several serious lesions listed below. The effect of the toxin naturally depends on the concentration of OTA and the period of exposure. These factors will also affect the disorders caused by OTA, wether it is acute

toxicity or chronic disorders (Segvic Klaric, 2012). The acute toxicity after high OTA intake may lead to death. A lower OTA intake that leads to subacute or chronic toxicity may not directly causes the death of the host, but it will graduall weakens and lower the general health status of the host (Bullerman and Bianchini, 2011).

3.5.1. Nephrotoxicity

The OTA are known to act their toxic effects primarily on the renal system, and its major toxic effect is nephropathy (Bui-Klimke and Wu, a; 2014). It is potentially nephrotoxic in all non-ruminant mammals (Khoury et al., 2010). OTA has been proved to cause karyomegaly in target renal tubular cells (Taniai et al., 2014). Renal damage has been induced by OTA in experiments with monogastric animals, as well as in young ruminants before the proper development and funtion of their forestomach (Krogh, 1992). Krogh et al. did a survey on the long term ingestion of OTA in swine. About 1 mg/kg concentration of crystalline OTA was administered into the feed of nine pigs. Within a few weeks they could observe decreased ability to concentrate urine and an increased urinary excretion of glucose. Progressive changes occured during longer time of exposure also. The autopsy revealed that the renal structure was changed by degeneration and atrophy of proximal tubules, interstitial fibrosis and hyalinization of glomeruli. After three months they could detect 3 to 27 microgram OTA/kg in the kidney, liver, musles and adipose tissue of the swine (Krogh et al., 1979). A study for nephrotoxicity by OTA in chickens was done by Elling et al. They collected 14 chickens at a slaughterhouse that had macroscopic changes in their kidneys. OTA residues ranging from 4.3 to 29.2 µg/kg was detected in the muscular tissues of 5 birds. In 4 out of these 5 birds they found toxic nephropathy, including atrophy and degeneration of proximal and distal tubules and interstitial fibrosis (Elling et al., 1975). Based on their study, O'Brien et al. suggested that relative low plasma concentrations of OTA in humans could cause a toxic response in primary renal epithelial cells, but not in mesenchymal cells. This was also suggested as valid for pigs. They concluded that their findings supported the possible nephrotoxic activity caused by OTA in humans and pigs (O'Brien et al., 2001).

3.5.2. Neurotoxicity

It has been shown that the administration of OTA at gestation period in rats induced many malformations in the central nervous system. (Khoury et al., 2010). Microcephaly has been reported in mice offsprings after prenatal exposure to OTA (Miki et al., 1994). Zhang et al. found that OTA was able to induce apoptosis in neuronal cells *in vitro*. They concluded that

this toxin may be an contributor to the development of degenerative diseases in which apoptotic processes are highly involved (Zhang et al., 2009). However, sufficient information regarding neurotoxic effect of OTA is lacking, and more research should be done.

3.5.3. Teratogenicity

OTA has been reported to be able to cross the placenta and cause prenatal dysmorphogenesis in laboratory animals like rats, mice, hamsters and chick embryos (Khoury et al., 2010). Oral administration of OTA to pregnant rats has been reported to cause increased incidence of resorption and lower weight of fetuses, as well as deformities and anomalies of fetuses (Brown et al., 1976). This has also been reported in case of subcutaneous administration of OTA to pregnant rats (Maruya et al., 1982). At low doses OTA was teratogenic. In high doses OTA had embryocidal effect (Brown et al., 1976). Wangikar et al. studied the possible teratogenic effect of OTA in rabbits. OTA was administered to rabbits by gastric intubation on days 6-18 of gestetion. They had one control group, and groups receiving dose levels of 0,025, 0,050 and 0,100 mg/kg body weight. The number of live fetuses was significantly less in the 0,100 mg/kg group compared to those of the 0,025 mg/kg group, and also the mean weight of the fetuses was reduced. The group that was exposed for highest OTA levels suffered from several fetal abnormalities. Malformations such as wrist drop, rudimentary tail, knuckling of fetlock and agenesis of tail was observed. They detected skeletal anomalies such as agenesis of caudal vertebrae, incomplete ossification of skull bones and wavy ribs. Also soft tissue anomalies as internal hydrocephalus, microphthalmia and agenesis of kidneys were observed. The two figures below show some of the findings in the group receiving 0,100 mg OTA/kg b.w. compared to the control group.

Figure 3. Rabbit fetus from control group show proper development of tail and legs. The fetus on the upside show agenesis of tail, and knuckling of fetlock joints. (Wangikar et al., 2004)

Figure 4. Rabbit fetal skeleton from control group show a normal skeleton. The fetus on the upside show wavy ribs and incomplete ossification of skull bones. (Wangikar et al., 2004)

The incidence of gross anomalies in the control group was measured to be 4,16%. It was significantly increased by the administration of OTA (0,100 mg/kg b.w.) to measure 31,25% gross anomoalies (Wangikar et al., 2004). Similar fetal malformations and teratogenic effects have also been found in mice (Hayes et al., 1974) and in chick embryos (Gilani et al., 1978).

3.5.4. Immunotoxicity

The OTA has caused lymphopenia, regression of the thymus, and supression of the immunity response when administered to several animal species (Khoury et al. 2010). The reduction of thymus, spleen and lymphnodes, together with the depression of antibody responses is typical effects on the immunesystem. Modulation of cytokine production and changes in functions of immune cells also characterize the immunosuppressive activity of OTA. This is thought to be caused by cell death and degenerative changes induced by OTA (Al-Anati and E. Petzinger, 2006). It is also suggested that the inhibition of protein synthesis is a major factor resulting in immunosuppression (Szeleszczuk et al., 2007). It has been found that OTA delays humoral and cellular immune responses to some antigens, and cause a reduction of immunoglobulin concentration in serum (Kozaczynski, 1994, Dwivedi and Burns, 1985, Szeleszczuk et al., 2007) Lymphoid depletion and atrophic changes in the bursa of Fabricius of chickens has

been reported (Kozaczynski, 1994, Szeleszczuk et al., 2007). In turkeys the same result was obtained: depletion of lymphoid cells in thymus, bursa of Fabricius, spleen, caecal tonsils and Peyer's patches was the main effects (Dwivedi and Burns, 1985). Bone marrow depression and hypocellularity, including decreased number of pluripotent stem cells, granulocyte-macrophage progenitors and decrease in erythropoiesis were observed in mice after intraperitoneal administration of OTA (Boorman et al., 1984).

The OTA exposure of human lymphocyte populations *in vitro* showed inhibitory effect on antibody production. IL-2 production and IL-2 receptor expression of activated T lymphocytes was impaired after OTA exposure. It was also observed that the B lymphocytes did not respond to polyclonal activators (Lea et al., 1989). This implies that a host suffering from immunesuppression caused by OTA, are more susceptible to secondary bacterial infection and viruses. However, Harvey et al. found no difference in immune response to antigens by chicks hatched from OTA-treated eggs compared to the control group. But the OTA concentration was low in this experiment: 2.5 micrograms (Harvey et al., 1987).

3.5.5. Carcinogenesis

Several experiments with the administration of OTA in the diet of rats and mice has induced renal cell tumors and hepatocellular tumors. As mentioned earlier it is also believed that the urothelial urinary tract tumors of Balkan Endemic Nephropathy were affected by the OTA. However, there is still no adequate studies of the relationship between exposure to OTA and human cancer reported (Khoury et al. 2010). Oral administration of OTA to mice caused hepatocellular adenoma and/or carcinoma in both sexes and renal-cell adenoma and carcinoma in male mice (NTP, 2014). Huff found that OTA induced atypical hyperplasia, cystadenomas and carcinomas of the renal tubular cells, as well as neoplastic nodules and hepatocyte tumorurs of the liver in male mice. He also did experiments on rats, and found that OTA induced degeneration, karyomegaly, proliferation, cytoplasmic alteration, hyperplasia, and adenomas and carcinomas with metastasisin the kidneys of both sexes. In female rats he observed that the incidence of fibroadenomas of the mammary glands was increased (Huff, 1991).

OTA is labeled as a potent renal carcinogen, but its mode of action is very unclear and is yet to be discovered (Kuiper-Goodman et al., 2010). In the 13th edition of Report on Carcinogens it says that *"Ochratoxin A is reasonably anticipated to be a human carcinogen based on*

sufficient evidence of carcinogenicity from studies in experimental animals." (NTP, 2014). The International Agency for Research on Cancer (IARC) also states that the evidence for OTA intake related to carcinogenicity in humans is inadequate, but that there is sufficient evidence in experimental animals for the carcinogenicity of OTA. The overall evaluation from IARC is that "OTA is possibly carcinogenic to humans", which place it into group 2B (IARC 1993).

3.5.6. Correlation with Balkan Endemic Nephropathy

It is suspected to be the cause of the human fatal disease known as Balkan Endemic Nephropathy (BEN), an interstitial chronic disease affecting the south-eastern population of Europe (Croatia, Bosnia, Serbia, Bulgaria and Romania) (Khoury et al., 2010). It is a progressive wasting disease of the kidneys that is irreversible, and with no treatment available. The disease is manifested in people typically between 50 and 60 years of ages. It was first described in the 1950's in Bulgaria (Bui-Klimke and Wu, a; 2014). There has been many studies to find a possible connection between BEN and OTA exposure. Krogh found that there was similarities between the epidemiology of mycotoxic porcine nephropathy and the epidemiology of the endemic Balkan nephropathy (Krogh, 1976). He also found similarities of their histopathology (Bui-Klimke and Wu, a; 2014). Faucet et al. did a research on DNA adduction in kidney following chronic OTA exposure to rat and subacute exposure of OTA to pig. They found that the OTA reacted with DNA, resulting in the formation of covalent DNA adducts. This strengthens the theory of the connection between OTA exposure and BEN (Faucet et al., 2004). Many of the food serveys performed in Bulgaria in the 1980's and 1990's discovered that staple foods in BEN-endemic areas contained more OTA compared to non-endemic areas. BEN-patients had more OTA positive blood samples than unaffected people from endemic and control areas, and the contamination level of OTA was generally higher in BEN-patients (Petkova-Bocharova et al. 1988, Petkova-Bocharova and Castegnaro, 1991, Bui-Klimke and Wu, a; 2014). However, serum OTA have shown a great variability when relation between serum/plasma concentrations to dietary intake has been analyzed. There are several factors that affect the concentration of OTA measured in serum/plasma of healthy humans, e.g. geography, season of the year, intrapersonal variation etc. (Scott, 2005). Petkova-Bocharova and Castegnaro did a study of OTA contamination in cereals from areas in Bulgaria where it was prevalence of endemic nephropathy and urinary tumours, and from non-endemic areas. They analyzed 130 samples of beans, maize and wheat flour (65 in each area). There were no visible signs of mould on the samples. In the bean

samples from endemic areas the OTA levels were 16,7% (25-27µg/kg) and 7,1% (25-50 µg/kg) in samples from non-endemic area. Levels were 27,3% (25-35 µg/kg) and 9,0% (10-25 µg/kg) respectively in maize samples. So a larger proportion of the samples from areas of high incidence of BEN were contaminated with OTA compared to cereals in non-endemic areas in Bulgaria. But the mean values of OTA levels did not show a significant difference. The samples for wheat flour came out negative; they did not contain measurable amounts of OTA (Petkova-Bocharova and Castegnaro, 1985).

On the other hand, a recent study states that there is little statistically significant evidence for human health risks associated with OTA exposure. (Bui-Klimke and Wu, b; 2014). One should also keep in mind that OTA exposure is only one of many hypotheses concerning an environmental aetiology of BEN. Bui-Klimke and Wu did an evaluation of possible risk factors and hypothesis regarding the aetiology of BEN. By doing a systematic review of available information of different hypothesis, they incorporated the pro and cons in a system called the Bradford Hill criteria (BHC). They conluded that OTA could play a contributory role in BEN, with the help of other risk factor(s). Aristolochic acid scored highest in their BHC evaluation (Bui-Klimke and Wu, a; 2014), and is regarded as a possible aetiologic factor responsible for BEN (Schmeiser et al., 2012). The EFSA Journal of 2006 clearly states that the previous epidemiological studies and available information concerning BEN are incomplete, and do not justify the classification of OTA as a human renal carcinogen. But they do inform that OTA has been found to be a potent renal toxin in all animal species tested.

Some also consider OTA to be the major cause of the Tunisian Nephropathy (TCIN) affecting the population in Tunisia (Khoury et al. 2010). High concentrations of OTA were detected in food, and in urine and blood samples from human in endemic nephropathy areas. Also, OTA-DNA adducts in kidneys and associated urinary tract tumors found in human with endemic nephropathy, suggest that OTA may be the main aetiological factor (Segvic Klaric et al., 2013).

4. Occurance and contamination of food

OTA was first discovered and isolated in 1965 from a culture of *Aspergillus ochraceaus* (IARC, 1993). The occurence of moulds producing OTA is influenced by many factors, like temperature, water activity, mould physiology, substrate composition, and interactions with

other microbes, which all are affected by the climate (Segvic Klaric, 2012). On a worldwide basis, cereals are considered as being the major source of OTA contamination. Cereals may be contaminated in the field and/or during storage. Growth of toxigenic filamentous fungi is most favoured in countries with hot and wet climatic conditions, leading to contamination of foods by aflatoxins and ochratoxins. But Penicillium species are able to grow and to produce OTA at low temperatures, so countries of Northern Europe are especially contaminated by these. Wine is also an important source of OTA human consumption. Fungi are unable to grow in fluids that contain more than 2% volume alcohol, because the alcohol prevents the conidia from germinating. The problem starts earlier. Grapes are contaminated in the vineyard by various mould species that produce the toxic metabolite (CVUAS, 2013). The production of OTA rapidly increases with the maturation stages of the grapes, so the date of harvesting will have an important effect on the OTA content in grape and its derived products (Khoury et al., 2010). It is also claimed that mould infestation, and hence OTA production, usually occurs when the grapes are damaged. It is possible for mould to grow in wooden barrels with wine and in hoses as well, but contamination of grapes is the most likely source of OTA in wine (CVUAS, 2013). There has also been detection of the toxin in licorice flavuoring, candy and spices in many countries. In tropical areas where cocoa is produced, OTA contamination has been observed. OTA has been found in both raw cocoa beans, chocolate and chocolate cream (NTP, 2014).

A survey performed by Mounjouenpou et al. studied the OTA contamination of 216 samples of cocoa and cocoa derived products, with the help of HPLC with fluorimetric detection. They collected samples from products derived from different processing steps: fermented sun-dried cocoa, roasted cocoa, nibs, cocoa butter, cocoa powder and chocolate. *Aspergillus carbonarius* contamination was found mainly in damaged cocoa pods and in those with a delayed pod-opening. They found that the shelling of the cocoa beans in a mortar and roasting by using fire wood was useless to reduce the OTA contamination significantly since the problem was not superficial. Cocoa butter was the only product that seemed to be resistant to the contamination. 67% of the analysed chocolate spread and coca samples were contaminated with the toxin, and 50% contained levels that exceeded the limit set by the European Union (Mounjouenpou et al., 2012). When it comes to beer, the malting process will degrade OTA if barley is moderately contaminated by the toxin. But in case of heavily infected barley, 2-7% of OTA remained in the final product (NTP, 2014).

The OTA can infect humans directly by these feedstuffs, but we can also be exposed to the toxin indirectly, via animals. The OTA contamination of animal feedstuff may cause formation of residues in edible offal and blood serum (EFSA, 2006). Several blood samples from swine at slaughtering have revealed a high frequency of OTA occurance. This indicates that pork products can be an important source of OTA consumption for humans (IARC, 1993). Due to the prolonged plasma half-life of OTA, it can occur in blood-containing tissues (NSCFS, 2013). The EFSA Journal from 2006 states that the OTA contamination in meat, milk and eggs is negligible. However, several studies have stated the opposite. Skaug has done several surveys on the possible OTA contamination of both human and cow's milk, suggesting that OTA residues in milk may be a potential health problem for humans (Skaug et al. 1998, Skaug 1999, Skaug et al. 2001).

In 2001 Skaug presented a survey that analysed possible OTA contamination in dust and fungal conidia. OTA was found in 6 out of 14 dust samples (0,2-0,70 µg/kg) collected from three cowsheds in Norway. Even though the inhalation of OTA makes a smaller source of exposure to OTA than food, it should be taken into consideration. Due to fast absorbtion from the lung, the inhalation of the toxin may contribute significantly to the total intake (Skaug et al., 2001).

5. Regulation and legislation

Most countries have established regulations to control the mycotoxin contaminants in foodstuff to protect human health (EMAN, 2012), this is also true for OTA. The aim is to estimate and to minimize the OTA contamination of various foods. A good agricultural practice and decontaminating the end product are important aspects to reduce the contamination. OTA has been evaluated by the Scientific Committee for Food (SCF) and the Joint Food and Agricultural Organization (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA), and established limits for the OTA intake (André el Khoury et al. 2010). In 1991 the Joint FAO/WHO Expert Committee on Food Additives (JECFA) evaluated OTA and a provisional tolerable weekly intake (PTWI) of 112 ng/kg body weight was set, based on data of nephropathies in pigs. They recommended that more studies concerning the toxicological effect of OTA should be done. In 1995 OTA was re-evaluated by the JECFA, and rounding the PTWI to 100 ng/kg b.w, and they requested further studies on OTA (JECFA, 2008). More and more countries introduce guidelines and set limits for OTA in foods, especially cereals (Food and Agriculture Organization, 2004).

The European Commission Regulation (EC) No. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs established a tolerable weekly intake (TWI) of 120 ng/kg body weight regarding OTA. This regulation also states that it is essential, in order to protect public health, to keep contaminants at levels which are toxicologically acceptable. In 2010 EC included maximum levels for OTA in spices and liquorice: The European Commission Regulation (EC) No. 105/2012 of 5 February 2010.

Table 1. Maximum levels for OTA (The European Commission Regulation (EC) No. 1881/2006 of 19 December 2006)

2.2	Ochratoxin A	Maximum levels (μg/kg)
2.2.1	Unprocessed cereals	5,0
2.2.2	All products derived from unprocessed cereals	3,0
2.2.3	Dried vine fruit	10,0
2.2.4	Roasted coffee beans	5,0
2.2.5	Soluble coffee	10,0
2.2.6	Wine and fruit wine	2,0
2.2.7	Aromatised wine	2,0
2.2.8	Grape juice, grape nectar, grape must	2,0
2.2.9	Processed cereal-based foods and baby foods for infants and young children	0,50
2.2.10	Dietary foods for special medical purposes intended specifically for infants	0,50
'2.2.11.	Spices including chilli, chilli powder, cayenne, paprika, pepper, nutmeg, ginger, turmeric, or any mixtures containing one or more of these spices	15,0
2.2.12.1	Liquorice root	20,0

2.2.12.2	Liquorice extract	80,0

According to this regulation it is totally forbidden to mix foodstuffs complying with the maximum levels together with foodstuffs which exceed these maximum levels, in order to reduce the contamination. Also, foodstuffs containing OTA should not be detoxified by chemical treatments (The European Commission Regulation (EC) No. 1881/2006 of 19 December 2006). Norway take reference to the EU legislation. Denmark, Hungary, Italy and Germany have established additional maximum limits for OTA in their national legislation. USA, Canada, Australia, New Zealand, Japan, Mexico and South Africa have no special limits set regarding OTA in foodstuffs (EMAN, 2012). In Denmark they have special guidelines at the slaughterhouse concerning OTA contamination of swine. Porcine kidneys that show macroscopic lesions of mycotoxic nephropathy are analysed chemically for OTA. If the concentration of OTA exceeds 25 µg/kg, the whole carcass is condemned, because its reason to believe that the meat is highly contaminated. Levels between 10-25 ppb suggest the rejection of edible offals. If OTA content is below 10 ppb, only the kidneys will be discarded (Food and Agriculture Organization, 2004). The Danish monitoring system has included surveillance of OTA in cereals since 1986 (Jørgensen et al., 1996).

6. OTA occurence in Norway

In the Norwegian climate it is mainly *Penicillium verrucosum* that will infect cereals if the storing procedures are inadequate. The intake of cereals that has been stored in a moist environment increase the exposure of OTA. In Norway the cultivated cereals have low water content, hence the problem with OTA is rather limited. Samples of coffee that was investigated by FSA in the period 2003-2005 gave low values of OTA, but samples of dried fruit had a high frequency for OTA contamination. A survey was conducted to check the occurence of OTA in blood samples from 200 random blood donors in Oslo. The intake was also calculated on the basis of a questionnaire about their diet. Based on the blood concentration and cost calculations the average daily intake were 0.24 and 3.1 ng/kg b.w. respectively. The upper 95 percentage was 0.38 and 6,5 ng/kg b.w. per day (FHI, 2008). The estimated daily intake of OTA in Europe is 2-8 ng/kg b.w. (EFSA, 2006). These estimates indicate that the intake in Europe and Norway is well below the EFSA's TWI of 120 ng/kg b.w. The Norwegian Institute of Public Health (Folkehelseinstituttet, FHI) states that the incidence of ochratoxicosis in humans is unknown and has not been reported as a problem in

Norway. But they inform that people suffering of kidney disease are a sensitive group regarding OTA. There is no special treatment listed, but they point out the importance of prevention. Mouldy food should not be consumed, especially not fruit, berries, jam and juice. One should also not make juice from fruit that has fallen down from the trees, due to possible damages and fungi proliferation (FHI, 2010).

6.1. Control of OTA in Norway

It is important with proper control by monitoring programs in order to detect possible OTA contamination. In Norway it is regulated by The Norwegian Food Safety Authority (FSA). They perform import control, and analysis of animal and human feedstuff. The Norwegian Veterinary Institute is the National Reference Laboratory for mycotoxins in Norway. They receive samples from the national monitoring programs for mycotoxins in feed and food, organized by the Norwegian FSA.

6.1.1. Cereals

OTA has been analysed in both Norwegian and imported crude cereal grains. Their typical limit of detection (LOD) for OTA in grains is 0,015 µg/kg. Wheat samples were analysed for OTA in 1990 to 1998, except in 1991 and 1992. The results are listed in the table below (VKM, 2013).

Table 2. OTA in wheat grain in Norway (VKM, 2013)

Year	Mean concentration of OTA (µg/kg)	No of samples	No of positive samples
1990	0,2	165	5
1993	0,7	18	8
1994	0,4	33	5
1995	0,3	45	6
1996	0,4	42	2
1997	0,2	38	26
1998	0,5	59	3

The table shows that levels of OTA concentration and number of positive samples in wheat are low. This indicates that the procedures of drying and storage limit the toxin effectively. In

the years 1991, 92, and 1999-2011 OTA was not analysed in wheat, due to the low levels previously detected (VKM, 2013).

Table 3. OTA in oat grains in Norway (VKM, 2013).

Year	Mean concentration of OTA (µg/kg)	No of samples	No of positive samples
1990	0,4	20	3
1993	0,2	3	1
1994	3,5	3	1
1995	0,3	20	1
1996	0,2	14	-
1997	0,1	11	7
1998	0,1	22	8
2005	0,2	17	9
2006	0,2	15	6
2007	0,1	17	7
2008	0,1	17	2
2009	0,8	12	3

Table 4. OTA in barley and rye in Norway, combined results for 1990-2011 (VKM, 2013)

Species	Mean concentration of OTA (µg/kg)	No of samples	No of positive samples
Barley	1,01	82	27
Rye	0,3	36	6

In 2012 OTA was analysed in one sample of corn and one from corn gluten imported from USA. They detected only traces of OTA (0,21 and 0,08 µg/kg respectively). In a sample of corn imported from Russia, no OTA could be detected (Norwegian Veterinary Institute, 2013).

6.1.2. Aquafeeds

The Norwegian aquaculture and farming of fish, especially Atlantic salmon (*Salmo salar*), has become a large scale industry by its exponential growth. Fish feed formulations are under constant development. Norwegian produced salmonid feeds contains imported vegetable feed ingredients, and do not include Norwegian grown cereals. On behalf of the Norwegian FSA the Norwegian Veterinary Institute analysed a random selection of the major vegetable feed ingredients used at nine Norwegian commercial fish feed production sites. They also did the same selection and analysis from feed used at two large scale experimental sites. Maize and wheat are the main cereals used in salmon feeds. The main oilseed feed ingredient is soybean products, followed by rapeseed and sunflower. They also analysed samples of marine feed ingredients such as fish meal and ensilage (VKM, 2013).

Table 5. OTA in feed ingredients used in Norwegian produced aquafeeds 2005-2009 (VKM, 2013)

Feed ingredient	Mean concentration of OTA (µg/kg)	No of samples
Ensilage	0,05	3
Fish meal	0,05	49
Horsebeans	0,03	2
Wheat products	0,3	28
Maize and maize gluten	1,3	16
Rapeseed products	0,04	6
Sunflower products	3,9	7
Soya products	0,06	21

OTA was the only mycotoxin analysed in ensilage and fish meal, due to its typical role as a storage toxin. OTA values exceeded the LOD in only 6 out of the 49 samples of fish meal.

6.1.3. Infant porridge

OTA together with other mycotoxins were analysed in various types of infant porridges in 1999, 2000 and 2008. The porridges were sampled in the shops (VKM, 2013).

Table 6. Combined results of OTA in infant porridge in Norway in 1999, 2000 and 2008 (VKM, 2013).

Type of infant porridge	Mean concentration of OTA (µg/kg)	No of samples	No of positive samples
Wheat	0,2	45	43
Oat	0,1	26	22
Spelt	0,04	2	2
Maize	0,01	26	6
Mixed grains	0,45	9	9

A high number of the samples contained OTA, but at low concentrations. Other mycotoxins, such as trichothecenes are often prioritized compared to OTA in Norway, because they usually have a higher concentration and frequency of contamination.

6.1.4 Other foodstuff

On behalf of the Norwegian FSA, the National Veterinary Institute took samples from various foods sold on the Norwegian market in 2012. They analyzed them for possible mycotoxins. In earlier years, the main focus has been on *Fusarium*-toxins in corn. But in 2012 they focused on a wider spectrum of mycotoxins, and of sampling from various foods. They wanted to get an idea of the possible presence of OTA in raisins, cocoa, coffee, spices and grape juice on the Norwegian market. The goal of the FSA was to verify compliance, and randomly check foods that have a high risk of contamination of different mycotoxins. If the mycotoxin concentration exceeds the given limit in foodstuffs, the product must be withdrawn from the market. FSA will follow up the pruducer/buisness and may inform consumers if necessary.

The sampling and methods of detection was carried out according to the guidelines prepared by the Norwegian FSA, which are based on the EU guidelines. They used a method based on immunoaffinity column and quantitative determination using HPLC with fluoresence marker for OTA detection. The method of measurement has an uncertainty of 25%. The limit values for mycotoxins are found in the regulation from 27th Sept. 2002 concerning certain contaminants in foodstuffs. This regulation refers to several EU directives, which are

implemented in Norway (see figure 5 for maximum levels of OTA). The maximum limit of cocoa is not determined, see coffee for guideline value.

Table 7. OTA in different foodstuff in Norway in 2012 (Norwegian Veterinary Institute, 2013)

Foodstuff	Limit of detection	No of samples	No of positive samples	Mean concentration of OTA
Spices	0,1 µg/kg	13	11	4,6 µg/kg
Raisins	0,003 µg/kg	10	10	0,60 µg/kg
Coffee	0,030 µg/kg	10	7	0,028 µg/kg
Cocoa	0,015 µg/kg	19	15	0,60 µg/kg
Grape concentrate	0,003 µg/kg	6	6	0,51 µg/kg

None of the OTA concentrations in raisins, coffee, cocoa or grape concentrate exceeded the maximum limit. Two samples of spices had values higher than the limit (15 µg/kg). The highest level detected in spices was 25,2 µg/kg. But taking into account the expanded measurement uncertainty of the method, and by the deduction of this, these two samples also came under the limit. Several samples of spices has been reported to exceed the limit of OTA in 2011 to 2012 by RASFF (Rapid Alert System for Food and Feed). Therefore it is natural to increase the control of spices (Norwegian Veterinary Institute, 2013).

6.2. Possible connection with Radesyken

In 2004 Størmer presented a theory in the Journal of Norwegian Medical Association that mycotoxins could have been a contributing causative of the disease "Radesyken". This disease occured in the 1700s in Norway, and affected many people, especially on the south-west part of the country. Due to the mild climate in this region, and the high intake of cereals at that time, he suggested OTA as a possible factor (Størmer, 2004).

7. Detection

It is important that the methods used for detection are sensitive and accurate, in order to obtain the correct level of contamination and to reduce this (Fujii et al., 2007). The detection

and quantification are needed to ensure a good quality control of food and feed (Meulenberg, 2012). There are several methods that can be used for the detection of OTA. A number of enzyme-linked immunosorbent assay (ELISA) methods can be used for screening, several high-performance liquid chromatographic (HPLC) methods are useful, thin layer chromatography (TLC) method, and immunoaffinity column (IAC) may also detect OTA (IARC, 1993). HPLC is the most common method (Fujii et al., 2007). Most HPLC methods use a reversed-phase column and an acidic mobile phase, so the carboxyl group of OTA is in the undissociated form. TLC is also quite common to use, and it has low cost and adaptability (JEFCA, 2008). It consists of visual detection of OTA by its greenish fluorescence under long wave ultraviolet light. After spraying the chromatographical plate with methanolic sodium bicarbonate solution, or exposing it to ammonia fumes, it will change to a blue fluorescence. Then the scanning densitometric analysis can proceed. The use of immuno-affinity column clean up is considered to be a relatively simple process. This gives sample extracts that are generally free of interferences. Roasted coffe has been reported to be problematic to analyze for OTA (Khoury and Atoui, 2010). Fujii et al. compared the HPLC and ELISA methods for OTA detection in green, roasted and instant coffee. They found that Mab-based indirect competetive ELISA was a highly sensitive and specific screening tool for OTA detection (Fujii et al., 2007). There are several benefits by using ELISA. It is rapid, and can process a large number of samples at the same time. It does not require any clean-up procedure either (Khoury and Atoui, 2010). The disadvantage with this method is possible cross-reactions leading to false-positive and false-negative results (JEFCA, 2008).

7.1. Biomarkers

Blood is considered to be a good biomarker for OTA exposure and detection in humans. Analysis of blood has been widely used in epidemiological studies (JEFCA, 2008). Several studies has suggested the urine as a good biomarker for OTA analysis (Muñoz et al., 2014, Gilbert et al., 2001). Gilbert et al. found a significant correlation between OTA intake and urine OTA concentration expressed as the total amount excreted in humans (Gilbert et al., 2001). A wide range of OTA concentrations has been detected in human milk (JEFCA, 2008). The concentration of OTA in human milk is assumed to reflect its concentration in blood, but the transport mechanism of OTA into milk and distribution ratio between blood and milk is not known (Skaug et al., 2001).

7.2. Occurance of OTA contamination

7.2.1. Mycotoxin interactions

Food is often contaminated with more than one mycotoxin, as many of the fungi species producing OTA are also able to produce other mycotoxins. However, most studies of the toxic effect of these metabolites are limited to one single mycotoxin (Segvic Klaric, 2012). Citrinin, zearalenone, penicillic acid and aflatoxin B1 are the most common mycotoxins occuring together with OTA (G.J.A. Speijers and M.H.M. Speijers, 2004). Raju and Devegowda did a reasearch on broilers to detect the individual and combined effects of OTA, aflatoxin B1 and T-2 toxin. They found that by adding 2 toxins with the same effect (e.g. reduce body weight, decrease food intake, increase liver weight), they would interact and cause a greater effect. However, this additive effect was not seen when they included even one more mycotoxin yielding a total of 3 toxins (Rjau and Devegowda, 2000). Many surveys on the interaction between mycotoxins have revealed the common occurance of OTA together with aflatoxins and fusarium toxins in cereals. In meat, the combination of OTA together with citrinin and penicillic acid is more common (Segvic Klaric et al., 2013). Studies of the nephrotoxic effect of OTA and citrinin, both separately and in combination found that they were synergistic and enhancing each others toxicity (Segvic Klaric, 2012). Surveys that studied the correlation of OTA in combination with other mycotoxin suggested that they are involved in the occurence of endemic nephropathy. They found that endemic nephropathy areas had a higher co-contamination with OTA and citrinin, or OTA and fumonisin B1 in their cereals, compared to non-endemic nephropathy regions (Pepeljnjak, 2010, Segvic Klaric, 2012, Segvic Klaric, 2013). The combination of OTA and fumonisin B_1 contamination has been detected in maize in Croatia. Fumonisin B_1 has also nephrotoxic properties like OTA, and the additive effect of these two mycotoxins can be a health hazard (Segvic Klaric, 2012). *A.niger* are able to produce both OTA and fumonisin B_1. As mentioned before, the *A.niger* can contaminate dried fruit, grapes and hence wine. So according to this we can expect the co-occurance of both OTA and fumonisin B_1 in wine, which may impose a greater risk compared to a single mycotoxin contamination (Segvic Klaric, 2012).

7.2.2. Comparison of milk sample-assays

The use of milk as a biomarker for OTA exposure is questioned. However, it may be important in case of infants that are exposed to OTA contaminated milk from the mother (Scott, 2005). A low biotransfer of OTA into the cow's milk was shown during feeding experimens with OTA contaminated feed to milking cows (Hult et al., 1976). These aspects

indicate that human consumption of cow's milk should not induce a high risk of OTA intake. However, several analysis of cow's milk samples showed results of OTA contamination (Breitholtz-Emanuelsson et al., 1993, Skaug, 1999). It is also of great importance to evaluate the risk assessment of infant exposure to OTA via mother's breast milk. In an experiment were lactating rabbit does ate an OTA contaminated feed, a linear relationship was found between OTA concentration in their milk and in the plasma of the sucklings. It indicates an effective transfer of the toxin from the mother to the sucklings. If this is true for humans, this should be a great concern regarding human health (Ferrufino-Guardia et al., 2000).

OTA has been detected in milk from healthy mothers in several countries, including Norway, Sweden, Hungary, Germany, Italy, Australia and Sierra Leone (Scott, 2005). Jonsyn et al. collected 113 breast milk samples from donor mothers in Sierra Leone. They tested them for mycotoxins in general, and found that only 10 samples were free from mycotoxins. 35% of the samples contained OTA. They ranged from 200 to 337 000 ng/l (detection limit was set to 200 ng/l) OTA. This is the highest OTA concentration detected in human milk samples (Jonsyn et al., 1995, Skaug et al., 1998). In Italy, 111 milk samples were collected from mothers. After analysis they could detect 22 samples contaminated of OTA. The OTA concentration was high and ranged from 0.1 to 12 µg/kg (Micco et. al, 1995). OTA has a high prevalence in Hungary. Kovács et al. did a survey to check the occurence of contamination of the toxin in blood and milk. The samples were collected from different parts in the country. OTA was detected in 52 of the 100 blood samples obtained. The contamination ranged from 0.2 to 12.9 ng/ml. OTA in the range of 0.2-7.3 ng/ml was found in 38 of the 92 milk samples. The milk was collected in the first 24 hour post partum period (Kovács et al., 1995). Breitholtz-Emanuelsson et al. analysed cow's milk, human's milk and their correspnding blood samples, for possible OTA contamination in Sweden. OTA was reported in 5 of 36 cow's milk samples within the range of 10-40 ng/ml. 23 of 40 human milk samples had OTA concentrations within the range of 10-40 ng/l. Blood samples from the mothers were collected, and 39 of these were analysed. All blood samples had OTA values exceeding the quantitation limit of 60 ng/l blood. The OTA levels in the blood samples ranged from 90 to 940 ng/l (Breitholtz-Emanuelsson et al.,1993). A survey was performed in Chile where they tested mother-child pairs. They analyzed the maternal blood, milk and urine samples from the infants. This study lasted for 6 months. Almost all the blood plasma samples from the mothers were contaminated with OTA. The concentrations ranged between 72 and 639 ng/L. On average, the OTA levels in milk were one quarter of those measured in plasma. They also

found that a higher fraction of circulating OTA was excreted in colostrum than compared with mature milk. By using a formula they calculated the infant's daily intake of OTA. This resulted in an average intake of 12,7 ± 9,1 ng/kg b.w. during their first 6 days of life. The daily intake of Chilean infants when they calculated with the values from the mature milk was close to 5,0 ng/kg b.w. per day (Muñoz et al., 2014).

Skaug performed HPLC analysis for detection of possible OTA contamination in samples of organic cow's milk, conventional cow's milk, and cow's milk-based infant formulas in Norway. The limit of detection was set to 10 ng/l. The results showed that 6 out of 40 conventional milk samples (with a range of 11-58 ng/l), and 5 out of 47 organic milk samples (with a range of 15-28 ng/l) were contaminated with OTA. 20 milk samples of infant formulas, produced by two different manufacturers, was analysed and all came out negative for OTA. These levels of OTA in milk are sufficient to cause a higher intake than the suggested TDI of 5 ng/kg bw/day recommended by The Nordic Working Group on Food Toxicology and Risk Evaluation. Especially small children that drink large quantities of milk are exposed (Skaug, 1999). The cow's milk has previously been more or less neglected as a source of OTA contamination, because of the ruminal degradation of the toxin and the low biotransfer of OTA into the milk (Hult et al., 1976, Skaug, 1999). But raw milk may be contaminated directly by fungi producing OTA. Skrinjar et al. detected growth of toxigenic Penicillium and Aspergillus species in raw milk samples. The hygienic management is important to avoid this (Skrinjar et al., 1995, Skaug, 1999). Other routes than peroral, such as inhalation of fungal spores and dust, may lead to OTA exposure of cows (Skaug, 1999). This may cause a different availability of the toxin uptake in the milk.

Table 8. Occurance of OTA in human blood and milk[*] (IARC, 1993)

| Country	Year	Positive samples/total no.	Content (ng/ml)	Reference
Bulgaria	1984-90	82/576	1-35	Petkova-Bocharove and Castegnaro, 1991
Canada	1988	63/159	0,27-35,3	Frohlich et al, 1991
Denmark	1986-88	78/144	0,1-13,2	Hald, 1991
Germany	1977-85	173/306	0,017-0,03 0,003*	Bauer and Gareis, 1987
Italy	1989-90	9/50	1,7-6,6 1,2*	Micco et al, 1991
Poland	1983-84	77/1065	0,10 (mean)	Golinski et al, 1991
Sweden	1989	38/297	0,3-6,7	Breitholtz et al, 1991
Yugoslavia	1980	42/639	1-40	Hult et al, 1982
Yugoslavia	1981-89	240/17175	5-100	Fuchs et al, 1991

The limits for OTA detection in the studies listed in the table vary, so "content" rather than "no. of positive samples" may be a better indicator of OTA exposure (IARC, 1993).

7.3. Geographical aspects:

Many authors reports of high OTA incidence in the North Eastern European countries, such as Bulgaria, Romania, Serbia, Croatia, Bosnia, Herzegovina, Slovenia, Macedonia, Monte Negro and in Africa, such as Congo, South Africa, Tunisia, Morocco and Egypt (Reddy and Bhoola 2010, Radic et al., 1997, Vrabcheva et al., 2000, Shepard et al., 2003, Romani et al., 2000, Krogh, 1972, Chernozemsky et al., 1977). Skaug et al. analysed several human milk samples from different regions in Norway for containing possible OTA, and detect possible regional differences. OTA was detected in 38 of 115 samples (range 10-130 ng/l). There were characteristic differences depending on the geographical regions. In the east and middle part of Norway 42-58% of the milk samples contained more than 10 ng/l. The incidence and level of OTA was significant lower in the northern part of Norway. These regional varieties may be a result of differences in diet, farming activities, and/or inhalation exposure to fungal spores (Skaug et al, 1998).

CVUAS (Chemisches und Veterinäruntersuchungsamt Stuttgart) did a research on geographical exposure of OTA in wine, in 2013. They collected and analyzed 38 wine samples from Germany and southern Europe for the presence of OTA. Since many OTA-producing moulds grow better in warmer climate, they wanted to check if this correlated with the OTA contamination in wine from different geographical areas. The result showed that countries from southern Europe (France and Italy) had higher levels of OTA contamination in their wine, compared to the wines from Germany (see illustration below). 70% of the wine samples from France was contaminated with OTA, 40% contamination of the Italian wines, and only about 20% of the German wines was detected with OTA. This supports the theory that wines from countries with a warmer climate will have a higher amount of OTA contamination. However, these contaminated samples were still below the valid maximum limit of 2 µg/kg (CVUAS, 2013).
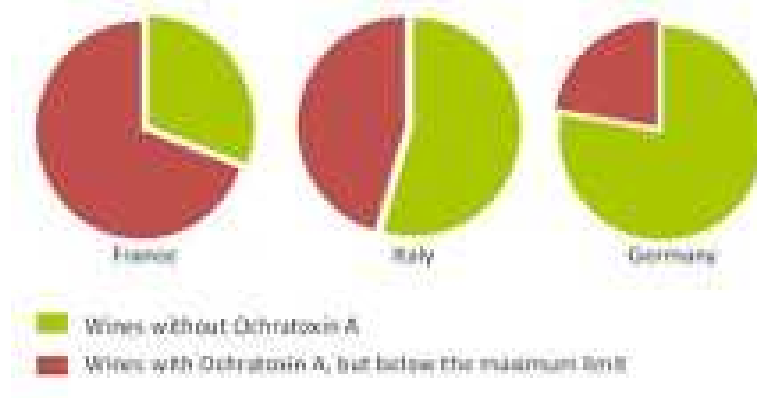
Figure 5. Diagrams with the result of OTA contamination in wine (CVUAS, 2013)

Further investigation should be done, and the increase of number of samples beeing analysed will improve the scientific basis of the result.

7.4. Dietary aspects:

Skaug et. al found that the dietary intake affected the OTA level in women's milk. Milk samples from 80 Norwegian women was obtained and analysed, and 17 of them contained OTA in the range 10-182 ng/l. Their usual food intake had been recorded during the year before the samples were collected. The contamination of OTA in milk was correlated with a high dietary intake of cakes and liver paste. The risk of contamination was increased by intake of juice. They also found that individuals with a diet characterized by high intake of breakfast cereals, cakes, processed meat products and cheese had a high OTA contamination, compared to those who ate less of these products. However, there is only done a few studies concerning the relationship between OTA exposure and dietary habits (Skaug et al., 2001). A study of 111 milk samples from women in Italy showed no correlation between diet and OTA contamination of milk (Micco et al. 1995).

7.5. Seasonal aspects:

In general the growth of mould, and hence production of mycotoxin, is increased in wet summer as these conditions are favourable (Skaug et al.,1998). The weather conditions during growth, harvest and storage play an important role regarding the OTA levels in the crop, which in turn will influence the OTA levels in feedstuff (Jørgensen et. al, 1996). However, a survey was performed in Sierra Leone where they tested OTA concentration of urine samples from children during both dry and wet season. A high occurance of OTA contamination was observed from both seasons. They concluded that the children were exposed to this toxin constantly (Jonsyn-Ellis, 2001). In Turkey they analyzed serum samples from healthy adults in the summer and in the winter. Both genders had significantly hicher OTA concentrations in winter compared with summer. They suggest that there is an continous exposure of OTA, but season can be an effective factor in mycotoxin exposure (Sabuncuoglu et al., 2014).

7.6. Public health aspects:

Both JECFA and EFSA have evaluated the possible hazards of OTA. They concluded that the accumulation of OTA in kidneys is of importance (JECFA, 2008, EFSA, 2006). Infants, children and those who have a high consumption of specific food have a higher risk of OTA exposure. The importance of monitoring programme and continous efforts to prevent the contamination is highlighted in EFSA's report (EFSA, 2006). Based on carcinogenicity studies with OTA on rats, The Nordic Working Group on Food Toxicology and Risk Evaluation suggested a maximum TDI (total daily intake) of 5 ng per kg body weight of OTA in humans (Skaug et al., 2001). O'Brien et al. claim that the average daily intake of OTA for humans are approximately 1,2 ng/kg. Leading to plasma levels of 0,5 ng/kg (O'Brien et al., 2001). This is below the TDI previously mentioned, but the individual dietary habits, geographical aspects, etc. may cause much higher concentration (O'Brien et al. 2001). It is unlikely that acute toxicosis of humans will occur at the OTA levels generally found in foodstuffs, but there may be significant risks by the long-term effects of continous low-dose OTA exposure (Skaug et al., 1998). Transplacental transfer of OTA can cause exposure of the toxin to the fetus. It has also been reported that the OTA concentration in fetal serum is twice the maternal one (Skaug et al., 2001). So even if the TWI of 120 ng/kg body weight of OTA (set by The European Commission Regulation (EC) No. 1881/2006 of 19 December 2006) is in general tolerable for humans, there are certain important aspects that deserves attention. The sensitivity to mycotoxins is inversely related to age (Kovács et al. 1995), hence causing a higher risk for small children and infants when exposed to OTA. In general, infants are more susceptible to exposure of environmental toxins compaired to adults. The continous exposure combined with the long elimination half-life, may cause an accumulation of the toxin over time in infants (Skaug et. al 1998). Muñoz et al. found that Chilean infants are exposed to OTA on an average of 5 ng/kg b.w. daily, which is the same level set as a limit by The Nordic Working Group on Food Toxicology and Risk Evaluation. Since they calculated the average exposure to the infants, probably some had lower concentrations and some had higher concentrations than the mean value. So based on the limit set by The Nordic Working Group on Food Toxicology and Risk Evaluation some of the infants will have a higher risk of the possible toxic effects of OTA (Skaug et al., Muñoz et al., 2014). Skaug emphasize that previosly risk assesments do not differentiate between risk to children and to adults, so they should obtain more information regarding this to be complete (Skaug, 1999). The increased value of OTA can cause production losses, and hence economic losses in food production and livestock breeding (Battacone et al. 2010, Segvic Klaric 2012). A high amount of OTA

contamination will reduce the quality of the food, which then again may affect the whole food chain when entering it.

7.7. Possible solutions of prevention and control

The agrotechnological production practices and storage of foods are important critical control points regarding contamination. By the incorrect handling and process of food production, the fungi can establish and produce toxin. So the agrotechnological practices for raw materials and processed products should be performed correctly, in order to reduce the OTA concentration of food (Ravelo Abreu et al., 2011). There are many factors that may affect the OTA contamination of cereals at harvest; the weather before and after harvest, time before drying, efficiency of drying machinery, physical state of grains, fungal competition, temperature at harvest and cleanliness of harvesters and transport. During the storing process factors such as time, temperature, condensation, cleanliness of storage containers and the absence of structural leaks are important to optimize in order to avoid OTA formation. Because of the heat stable properties of OTA, prevention should be the aim rather than treatment (Scudamore, 2005). Since the water availability and moisture content is crucial for the growth of moulds, the moisture content values should be kept at a safe treshold during storage; 14-14,5% for wheat, barley and oats, 14% for maize, 13-14% for rice and 7-8% for rape seed. So the grains should be quickly dried and reach the optimal moisture content (and keep it) during storing, to avoid the contamination and toxin production. One should keep in mind that its a variation in optimum conditions for growth and OTA production when management strategies for prevention is carried out (Magan and Aldred, 2005). In case of wine, the grapes should be protected from infection of pathogens. The harvesting should occur quickly in case of rain damaging the grapes. Bunches of poor quality should be rejected, and there should be a minimal delay between harvest and crushing. Studies have shown that the vinification process, especially pressing and racking, will reduce the OTA contamination of wine. Roasting of coffee beans has also a reducing effect on OTA, if the temperature is high enough (JECFA, 2008). Scudamore et al. found that OTA could be reduced from whole wheat during the bread-making process. Scouring was effective and removed up to 44% of the OTA present. By the combination of cleaning scouring and removal of bran and offal fraction, about 75% of the OTA present in white bread was reduced (Scudamore et al., 2003). Adequate monitoring and control program for the presence of OTA in food and feed should reduce the health risk. In addition to this contaminated products should be disposed (Meulenberg, 2012). There are several possible methods to detoxify the mycotoxin containing

food. Fumigation may be used as a chemical approach to prevent mould growth on cereals, by directly destroying of spores, by inhibiting growth, or by killing insects that damage kernels (JECFA, 2001). But unfortuneately the detoxification will often cause a reduction of nutritional values and loss of palatability (Sorrenti et al., 2013). The usage of chemicals should also be kept at a low level, considering its harmful effects on the environment. It is possible to add nutrients or additives with protective effect to contaminated foodstuffs. Yeasts have a good property, their ability to act as a biosorbant. Studies have demonstrated that yeast can work as a biological removal of OTA. It is thought to be a better solution, compared to many physical and chemical methods, since there are no harmful chemicals and substances being used. The nutritional value and palatability is also maintained (Petruzzi et al., 2014).

Studies have showed that antioxidants can counteract some of the negative effect of OTA contamination. OTA has several cytotoxic properties, and the antioxidants are a natural fighter against oxidative stress. Atroshi et al. performed a study to check the appearance of liver apoptosis after OTA was administered in male mice, and how it was affected by antioxidants. The mice received OTA twice a week, for one or two weeks. Intracellular apoptosis bodies were observed in the mice's liver, two weeks after the toxin administration. Centrilobular necrosis was also detected in the liver. They investigated the ability of the combined antioxidants: Coenzyme Q 10, L-carnitine, Zn, Mg, N-acetyl cysteine, vitamin C, vitamin E and selenium to interact with the apoptosis process of the liver. They found that after the OTA treatment, the tissue reduced glutathione (GSH) level was reduced. GSH is an important factor that protects against toxic liver injury, and inhibit apoptosis. After the antioxidant treatment, the GSH levels were increased. This suggests that antioxidants can inhance the detoxification system of the liver. They also suggest that the antioxidants should posses several qualities, the ability to neutralize oxygen readicals beeing an important trait (Atroshi et al., 2000). Zheng et al. studied the effect of possible protection against OTA toxicity by zinc. The supplementation of zinc was able to reduced the cytotoxicity of OTA, by inhibiting the oxidative damage and DNA damage, and by regulation of the zinc-associated genes. The authors concluded that zinc may be used to reduce the OTA toxicity of contaminated feeds (Zheng et al., 2013). Grosse et al. performed a survey on the effect of several vitamins against OTA. After a single administration of OTA in mice and rats, several DNA adducts which induce hepatocellular adenomas were detected in their kidneys. Retinol (vitamin A), ascorbic acid (vitamin C) and alpha-tocopherol (vitamin E) are important superoxide anion scavengers. These were tested on the OTA genotoxicity. They did a

pretreatment of the mentioned vitamines on mice before the administration of OTA. This pretreatment had a very good effect on reduction of DNA adduct formation in the kidney. Ascorbic acid was the most efficiant one, pretreatment by vitamin C decreased DNA adduct levels by 90% in kidney. Vitamin E decreased DNA adducts by 80% and Vitamin A by 70% in kidney (Grosse et al., 1997). The effect of melatonin on the oxidative stress induced by OTA on rats has also been studied. Rats receiving only OTA (250 μg/kg) had high levels of lipid peroxidation product in serum, liver and kidneys. A reduction of important enzymes was also detected; glutathione, superoxid dismutase, catalase, glutathione peroxidase and glutathione reductase. The group of rats receiving both OTA and melatonin (5 mg/kg body mass) had a much lower level of lipid peroxidation product in the serum, liver and kidneys. In addition to this, they had significantly increased levels of glutathione peroxidase, glutathione reductase and glutathione-S-transferase activity in both liver and kidneys. The authors concluded that clinical application of melatonin may be possible in the therapy of ochratoxicosis (Meki and Hussein, 2001). So according to these studies, a dietary strategy (containing a combination of many antioxidants) may counteract the harmful effects of chronic OTA intake. The EFSA Journal from 2006 states that a certain degree of OTA contamination seems impossible to avoid at the present.

8. Conclusion

By reduction of OTA the food safety can be improved, and economic losses due to different disturbances caused by this toxin can also be reduced. Based on the fact that OTA takes longer time to be metabolized and eliminated in humans, it is reason to believe that the toxic effects are enhanced. The toxin has a longer period to perform its actions, however more studies should be done on this area. It is debated wether OTA is the aetiological factor of BEN or not, and there exist a great variety of opinions between researchers conserning this topic. This shows that the OTA toxicosis is a very complex process, and not easy to evaluate. Even though some scientists are critical and dismiss the carcinogenic property of OTA in humans, there are many studies that confirm its toxicity. Wether it is the causative agent for BEN or not, I think OTA deserve awereness. I think it is important to prevent the growth of moulds; to reduce the OTA contamination of feed by reducing the fungal species producing the toxin. If this is done properly, the toxin will not enter the food chain and affect both animals and humans. The natural growth of moulds should be prevented both on the crops out in the field and after harvesting, to minemize the contamination as much as possible. The kind of food that is contaminated by OTA is consumed on a daily basis, so I believe that the

avoidance of food that can contain OTA is an impossible and a non-preferable solution. It would probably lead to many serious deficiencies in vitamins and minerals if people avoided food that could contain OTA. Therefore, a good choise would be to reduce the contamination before entering the food chain. Antioxidants are well known for their positive effects, and they may also help against OTA consumption as the previously mentioned surveys has confirmed. A varied and healthy diet that include a good amount of antioxidants can be a preventative solution. This may especially be of importance for pregnant women, except from the obvious health benefits, it may prevent high concentrations of OTA in their milk. The shipping, handling and storage practices of different foodproducts should be optimal, to avoid the proliferation of moulds. I also think that control programmes should be carried out carefully, to detect possible high levels of contamination.

The data obtained provide an insight on the possible effects of OTA exposure to animals and humans. From this study it appears that OTA has several toxic effects, but studies are mostly performed on animals. The carcinogenic effect of OTA has been proved in experimental animals, but the mechanism of action and its relation to humans is still a matter of debate. However, I believe that the long plasma half-life of OTA in humans, compared to other mammalian species, is an important factor. A longer period of exposure to this toxin via food etc. has showed to cause serous consequenses, and this could also be related to the prolonged period of high plasma levels of OTA. The great differences found between species, both for pharmacokinetics, toxicity and sensitivity should be considered. Even though the toxic effects in humans are uncertain, and in clearly need of more scientific research, the prevention and reduction of OTA exposure is a goal that should be taken seriously. As this study shows, the prevention of this toxin should occur on several levels. The propagation of OTA producing fungi together with the production of OTA can be avoided by the correct handling and processing of food. If the food is already contaminated, there are many available methods for detoxification, and one can also add different substrates to counteract OTA's effect. But as the EFSA Journal from 2006 states, a certain degree of OTA contamination seems impossible to avoid at the present, as it is a naturally occuring metabolite.

9. Summary

The mycotoxin OTA occurs on a worlwide basis, and has been detected in a great variety of food. Especially cereals are frequent contaminated by this toxin, due to the proliferation of several *Penicillium* and *Aspergillus* species, both out on the field and during storing. Many experiments have been carried out to discover the effect of OTA exposure. OTA possess several toxicological properties, such as immunotox, neurotoxic, nephrotoxic, teratogenic, and carcinogenic properties. This has been proved on experimental animals. How this toxic metabolite affect human health is a rather uncertain topic, but it is regarded as an important chronic dietary risk factor. Experiments have also showed that the toxin has a long plasma half-life in humans, which will lead to a longer time of exposure. Many factors influence the extent of toxicity, such as age, species, health status, possible interactions with other mycotoxins, and duration of exposure. The constant exposure of low OTA contamination may lead to chronic toxicosis. The OTA contamination of food in Norway seems to be at a tolerable level, according to control programs performed by the Norwegian Veterinary Institute and the Norwegian Food Safety Authority. However, it has been detected in human breast milk of Norwegian women at a level that indicates possible harmful effect of infants. Transplacental transfer can also cause exposure of OTA to the fetus. A healthy and varied diet may result in a lower level of OTA contamination. Since cattle are able to degrade OTA to the non-toxic Ochratoxin α and its low biotransfer of OTA into milk, the exposure throgh cow milk has not been regarded as an important source of contamination. However, OTA was detected in several milk samples from cows in Norway, and this could be a risk factor for small children that drink large quantities of milk. Infants, small children and people suffering of kidney disease are believed to be more sensitive towards this toxin. Extra care should be taken regarding these groups. The exposure of this toxin to aniamls and humans seems impossible to avoid completely, but optimal processing methods and management together with control and monitoring programs help to reduce the OTA contamination in the food chain.

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