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Ochratoxin A in Humans: Exposure, Kinetics and Risk Assessment

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presented by
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Wenn sie die Wahrheit
in der Natur gefunden haben,
so schmeissen sie sie wieder in ein Buch,
wo sie noch schlechter aufgehoben ist.

Georg Christoph Lichtenberg (1742-1799)

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Zusammenfassung

Ochratoxin A (OA) ist ein Mycotoxin, das von zwei ubiquitären Schimmelpilzarten (Aspergillus und Penicillium) produziert wird. OA weist eine relativ geringe akute Toxizität auf. Es wurden jedoch grosse Speziesunterschiede beobachtet. Die LD_{50} nach oraler Applikation lag für Mäuse zwischen 46-58mg/kg KG, für Schweine, Katzen, Kaninchen und Hunde jedoch zwischen 0.2-1mg/kg KG. In allen bisher untersuchten Tierarten fand man Nierenschäden nach chronischer Verfütterung von OA. Nephropathien in Schweinen und Geflügel konnten auf kontaminiertes Futter, welches hohe OA-Konzentrationen aufwies, zurückgeführt werden. Im 2-Jahres Versuch an der Ratte erwies sich OA als starkes Nierenkanzerogen. Grosse Unterschiede in der Tumorinzidenz wurden jedoch zwischen den Geschlechtern beobachtet, wobei die männlichen Tiere viel höhere Tumorraten als die Weibchen aufwiesen. Ähnliche Beobachtungen wurde bei Mäusen gemacht, wobei jedoch 20x höhere Dosen notwendig waren, um vergleichbare Schäden zu erzeugen.
Hauptsächlich findet man OA auf Getreide, aber auch in Bier, Kaffee, getrockneten Früchten und sekundär auch im Fleisch von Nutztieren nach Verfütterung verschimmelten Getreides.
Eigene Untersuchungen von 31 Getreideproben des Schweizerischen Detailhandels, zusammen mit 2 anderen Studien, ebenfalls duchgeführt mit Getreideproben vom Schweizer Markt (n=152, OA-Konzentrationen: 0.1-3.8µg/kg), haben gezeigt, dass die OA-Konzentrationen in der Schweiz in demselben Bereich liegen wie in anderen europäischen und nordamerikanischen Ländern. In der vorliegenden Studie waren 7 der 31 Proben positiv und wiesen Konzentrationen zwischen 0.1-1.4µg/kg auf. In der Schweiz wurde vor kurzem ein Toleranzwert von 2µg/kg für Getreide und die daraus hergestellten Produkte festgesetzt. Würde dieser Wert auf die obengenannten Proben angewendet, so hätten lediglich 6 der 183 analysierten Proben zurückgewiesen werden müssen. Auf Grund der vor allem in Deutschland erhobenen Cerealien und Fleischproben wurde eine durchschnittliche tägliche Aufnahme von OA von ca. 80ng/Person berechnet. Dieser Wert kann jedoch je nach Essgewohnheiten auch erheblich höher liegen.

Das Vorkommen von OA in grünem Kaffee ist schon öfter beschrieben worden. Über den Einfluss des Röstens von Kaffee auf den OA-Gehalt wurden hingegen sehr unterschiedliche Daten publiziert. In der vorliegenden Studie wurde in 13 von 25 grünen Kaffee-Proben OA nachgewiesen, was gut mit den Daten aus der Literatur übereinstimmt. Mit einer verbesserten Analysenmethode konnte aber gezeigt werden, dass das OA beim Rösten nicht zerstört wird und, dass auch bei der Zubereitung des Kaffeegetränks das OA vom Pulver in das Gebräu übergeht. In 40 Kaffeegetränkeproben, die aus Mustern des schweizerischen Detailhandels hergestellt wurden, wiesen 18 nachweisbare Mengen an OA auf (0.4-7.8µg OA/kg Kaffeebohnen). Wird daraus die täglich via Kaffee aufgenommene Menge OA berechnet, so ergibt dies ca. 25ng/Person. Daraus wurde der Schluss gezogen, dass regelmässiger Kaffeekonsum wesentlich zur Belastung des Menschen mit OA beitragen könnte.

Auf Grund der grossen Häufigkeit kontaminierter Esswaren wurde OA auch in menschlichen Blutproben untersucht. Grosse Studien wurden zunächst im Balkangebiet durchgeführt, da man einen Zusammenhang zwischen OA und der endemischen Balkannephropathie vermutete. Später wurde jedoch OA auch in verschiedenen anderen europäischen Ländern in Humanblutproben nachgewiesen. Durch verbesserte Analysenmethoden konnte die Nachweisgrenze ständig gesenkt werden, was dazu führte, dass in den neuesten Studien in sämtlichen analysierten Blutproben OA nachgewiesen werden konnte. Die Konzentrationen lagen zwischen 0.06-14.0ng/ml, wobei die Mittelwerte zwischen 0.2-0.6ng/ml lagen.

In der vorliegenden Arbeit interessierte vor allem der Verlauf des Plasmaspiegels in einzelnen Personen über längere Zeit. Bei 8 Personen wurde über 2 Monate hinweg regelmässig Blut untersucht. Die OA-Konzentrationen im Plasma lagen zwischen 0.2-0.9ng/ml. Der Verlauf der Plasmaspiegel war bei den jeweiligen Personen stark unterschiedlich. Bei einzelnen ergaben sich praktisch keine Unterschiede zwischen den Messpunkten, während bei anderen riesige Schwankungen auftraten.

Nicht nur bei der akuten Toxizität, auch bei den pharmakokinetischen Parametern existieren grosse Speziesunterschiede. Die Halbwertszeiten variieren von 0.7h beim Fisch bis zu 510h beim Affen. Da auf Grund dieser Daten eine Voraussage bezüglich der OA Kinetik im Menschen schwierig war, wurde von einem freiwilligen männlichen Probenden 395ng

radiomarkiertes OA eingenommen. Die Eliminationskurve konnte am besten durch ein offenes 2-Kompartimente Modell beschrieben werden. Die Halbwertszeit wurde auf 35.5 Tage berechnet. Im Gegensatz zur Anfangsphase, in der nur ein Teil des OA im Urin wiedergefunden wurde (was auf eine Verteilung im Körper hindeutet), wurde während der Eliminationsphase praktisch das gesamte Ochratoxin und dessen Metaboliten via der Nieren ausgeschieden. Auf Grund der langen Halbwertszeit wurde nur eine sehr kleine renale Clearance gefunden (ca. 0.05ml/Min.). Basierend auf diesen kinetischen Daten und einer mittleren Plasma Konzentrationen wurde die tägliche Aufnahme von OA auf ca. 85ng/Person geschätzt. Dieser Wert korreliert gut mit der geschätzten täglichen Aufnahme, die mittels OA-Konzentrationen in Lebensmitteln berechnet wurde.

Eine präzise Risikovoraussage für den Menschen, basierend auf den Tierdaten und den hier präsentierten Daten vom Menschen ist dennoch sehr schwierig zu machen, da der Kanzerogenitätsmechanismus nach wie vor unbekannt ist.

Summary

Ochratoxin A (OA) is a mycotoxin which is produced by two ubiquitous fungal species (Aspergillus and Penicillium). Ochratoxin A has a relatively low acute toxicity, however large species differences were observed. After oral application the LD_{50} was found to be in the range of 46-58mg/kg bw in mice and between 0.2-1mg/kg bw in pigs, cats, rabbits and canines, respectively. It was found to be nephrotoxic in all species tested so far. Nephropathies in swine and poultry were found to be induced by feed containing high concentrations of OA. In a 2-year-study with rats OA was shown to be a potent kidney carcinogen. However, large sex differences in the tumor incidence were observed, with the males being much more sensitive to OA than the females. Similar observations were made in mice but a 20-fold higher dose was needed to induce similar damage.

OA is a frequent feed and food contaminant, i.e. is found predominantly in cereals including corn, but also in beer, coffee and dried fruit. The contamination of OA found in meat is due to indirect transmission of contaminated feed into the tissue of cattle.

One of the goals of this study was to look at the degree of OA contamination in cereals of the Swiss retail market. Our results of 31 analysed cereal samples together with the results of two other studies with totally 152 samples (OA-levels: 0.1-3.8µg/kg) from the Swiss retail market revealed that the levels measured were in the same range as they were found in other European and Northern American countries. In our study 7 positive samples were found containing OA in the range of 0.1-1.4µg OA/kg. Switzerland only recently established a tolerance level for OA contamination in cereals and its products of 2.0µg/kg. Applying this tolerance level to the 183 samples analysed from the Swiss retail market only 6 would have had to be rejected. Based on a large data base of cereal and meat samples especially from Germany it was calculated that the average daily intake via food is around 80ng/person, but depending on nutritional habits it may also be considerably higher.

The frequent contamination of green coffee beans with OA was also reported several times. Inconsistent results, however, have been published regarding the influence of roasting on the OA content in roasted beans and the transfer into the coffee brew. In our investigations we detected OA in

13 of 25 green coffee samples, which is in line with the results of the literature. With an improved method of analysis it could be shown, that OA is not destroyed by the roasting process and that it is eluted from the powder into the brew. It was further found, that of 40 coffee brews prepared from coffee samples from the Swiss retail market, 18 contained OA in the range of 0.4-7.8µg OA/kg. Applying these results for calculating the daily intake of OA it was found that around 25ng OA/person and day are taken up via coffee. This indicates that regular coffee consumption may contribute significantly to human OA exposure.

As a result of the frequent contamination of OA in food items OA was also analysed in human blood samples. Large studies with human blood samples were carried out mainly in the Balkan area where a link between the Balkan endemic nephropathy and OA was suggested. But OA was also found in many other European countries in human blood samples. With improved analytical methods the detection limit could be lowered and as a result the percentage of blood samples, in which OA could be detected, increased. Already several groups from different European countries reported that 100% of the analysed samples were positive with levels ranging from 0.06-14.0ng OA/ml plasma. The mean concentrations were usually around 0.2-0.6ng OA/ml.

In order to get more distinct informations about the variation of the plasma levels the following trial was carried out: the intraindividual variations of OA were measured in plasma levels in 8 volunteers over a period of two months. The concentrations detected in the 8 persons ranged from 0.2-0.9ng OA/ml. The levels in some individuals remained nearly constant, whereas others varied considerably during the observation period.

Regarding the toxicokinetic profile of OA large species differences were observed. Half-lifes varied from 0.7 hours in fish up to 510h in monkeys. Based on these results a prediction of the human kinetic parameters seemed to be difficult. The half-life of OA in human and the clearance was therefore studied by giving 395ng radio labelled OA to one male volunteer. The elimination was best described by a two compartment open model. The elimination half-life of the plasma was calculated to be 35.5 days. In contrary to the first phase, where only part of the OA was excreted via urine (which could be a result of concomitant distribution of OA within the body), in the elimination phase most of the OA and its metabolites were

found to be excreted via urine. The renal clearance was therefore very low (around 0.05ml/min). Based on these kinetic data and a mean plasma level a daily uptake of 85ng OA/person was calculated, which compares well with the daily uptake calculated from intake of contaminated food.
A precise risk prediction for humans, based on the data of the animal assays and the known human data, is however difficult to achieve, as the mechanism of carcinogenesis is still unknown.

1. Introduction

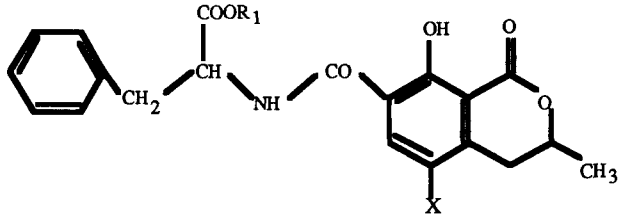
1.1. Mycotoxins

The occurrence of mycotoxins in food and feed is a problem of major concern. Various genera of fungi can produce mycotoxins whenever appropriate conditions of temperature, humidity and a suitable substrate prevail in a given area. Many mycotoxins can cause diverse adverse effects: some are primarily acute toxic (e.g. trichothecenes), while others can induce chronic diseases or cancer. Common mycotoxins, found frequently in food are aflatoxin, ochratoxin, sterigmatocystin, patulin, trichothecene mycotoxins and fumonisines (Chu, 1991; Zoller et al., 1994). Historically, the aflatoxins have received greater attention than any of the other mycotoxins because of their demonstrated carcinogenic effects already at low doses and its frequent occurrence in foods and feeds. Clear evidence for carcinogenic properties were also found for sterigmatocystin (Chu, 1991). Unknown is the carcinogenic potential of patulin and penicillic acid. Nevertheless, both toxins are hepatotoxins (Chu, 1991). Trichothecene mycotoxins, a group of toxins produced by very common moulds (*Fusarium*), cause significant tissue damages and immunosuppression. *In vitro* studies for the detection of mutagenicity were negative for these mycotoxins with one exception: the T-2 toxin showed a weak mutagenic effect (Chu, 1991). No mutagenic potential was seen for fumonisines, toxins produced by *Fusarium moniliforme*, but it was carcinogenic in rats (50ppm/kg diet over 26 month) with the liver being the target organ (Gelderblom et al., 1991), most probably by interfering with cell-cell communication. Furthermore, fusarin C, produced by the same mould is a potent mutagen (Chu, 1991). Ochratoxin A has only recently been considered a highly carcinogenic substance (Boorman, 1989). Nevertheless, only little is known about occurrence, toxicity, kinetic and possible carcinogenic mechanisms. It is therefore subject of the present investigations.

1.2. Ochratoxins

1.2.1. Physical and chemical properties

Ochratoxins are metabolites of fungi, which constitute a group of closely related derivatives of isocumarin linked to L-phenylalanine (Fig. 1)
The first compound discovered, ochratoxin A (OA) was isolated from a culture of *Aspergillus ochraceus*, hence the name (van der Merwe et al., 1965a).



Ochratoxin A: X = Cl; R_1 = H
Ochratoxin B: X = H; R_1 = H
Ochratoxin C: X = Cl; R_1 = CH_3

Fig. 1. Chemical structure of ochratoxin A, B and C

Subsequent investigations have revealed, that a variety of fungal organisms included in the genera Aspergillus and Penicillium are able to produce ochratoxins (DFG, 1990; IPCS, 1990). OA and very rarely ochratoxin B are the only ochratoxin species found as natural contaminants in plant material and most of the information available concerns OA (IPCS, 1990). Pure OA is a colourless, crystalline compound which is soluble in polar organic solvents, slightly soluble in water and soluble in dilute aqueous bicarbonate (DFG, 1990; IPCS, 1990). The pK_a-value of OA is 7.1 (Pitout, 1969) and it has a melting point in the range of 168-173°C (IPCS, 1990).
OA is stable in alcoholic solutions but is destroyed by UV-radiation and strong acids (van der Merwe et al., 1965b; Harwig et al., 1983). Enzymes

(α- chymotrypsine, carboxipeptidase A) hydrolyse OA to Oα (Fig. 2) and phenylalanine (Pitout, 1969; Neely and West, 1972). The toxin is heat stable in neutral solutions but is hydrolysed in alkaline media under heat (Müller, 1983). Naturally contaminated wheat did not loose OA when heated up to 200°C. A reduction of 32% was found when wheat was heated for 40 minutes at 250° and 300°C (Harwig et al., 1983). OA was stable even upon heating also in pig meat and beans (Kuiper-Goodman and Scott, 1989). About 20-30% of OA in naturally contaminated pig products was lost during frying at 150-160°C up to 12 minutes. During canning procedures of contaminated mouldy white beans only 11% of the total OA content was lost because of the influence of heating. (Harwig et al., 1983). Contradictory results concerning the heat stability of OA were published for coffee beans. In 4 different studies (Levi et al., 1974; Gallaz and Stadler, 1976; Cantafora et al., 1983; Micco et al., 1989) it was found that between 80 and 100% of the OA was destroyed during roasting. Tsubouchi et al. (1987), however, found only a destruction between 0 and 12%. (For more details and discussion see chapter 3). Despite of these contradictory results obtained with coffee beans it can be concluded that OA is only slightly reduced during heating of food items.

1.2.2. Toxic effects and mechanisms

1.2.2.1. Mechanisms

The primary effect of OA is the inhibition of protein synthesis (Creppy et al., 1979a; Creppy et al., 1980a; Creppy et al., 1980b; Creppy et al., 1983a; Creppy et al., 1984). As a secondary effect to this, RNA synthesis may be inhibited. The inhibition of protein synthesis is specific and occurs at the post-transcriptional level, with OA having a direct effect on the translation step in protein synthesis. OA competes with the phenylalanine for the enzyme phenylalanine t-RNA^{Phe}-synthetase (Röschenthaler et al., 1984), which results in a competitive inhibition of the enzyme. In this reaction OA may be regarded as an analogue of phenylalanine and in cell cultures the competitive inhibition could be reversed by an increase in phenylalanine concentration (Creppy et al., 1979b). *In vivo* there was a dose related inhibition of protein synthesis in mice given OA i.p. at doses between

1-15mg/kg bw. These doses can already produce acute toxic effects since the LD_{50} of mice by i.p. injection is 22mg/kg bw. The degree of inhibition of protein synthesis at the lowest dose of 1mg/kg bw after 5h of administration for liver, kidney and spleen was 26, 68 and 75% compared to that of controls (Creppy et al., 1984). Phenylalanine (100mg/kg) injected together with OA prevented the inhibition of protein synthesis by a dose of 10mg OA/kg bw in all of these organs. In yeast the 4(R)-hydroxyochratoxin A (Fig. 2) had an effect similar to that of OA on protein synthesis. Oα (Fig. 2), lacking the phenylalanine moiety, as well as ochratoxin B (Fig. 1) had no such effect (Creppy et al., 1983b).

An other enzyme, inhibited by OA is the renal phosphoenolpyruvate carboxykinase. The activity of this key enzyme of the gluconeogenic pathway was lowered by 50% in kidney slices from rats fed 2.5mg OA/kg bw for two days (Meisner and Krogh, 1986).

Rahimtula et al. (1988) demonstrated that OA enhanced lipid peroxidation when added to liver or kidney microsomes or when administered to rats *in vivo*. However, the doses used were usually high, i.e. for the *in vitro* experiments concentrations of 50µg/ml were used, for the *in vivo* experiment 6mg/kg bw were administered which corresponds to about 25% of the LD_{50}.

A third mechanism of OA toxicity is its effect on mitochondrial ATP production. In *in vitro* studies a decreased respiration rate of whole mitochondria was observed at 485µg/litre medium (Wei et al., 1985). A 50% inhibition of the intra-mitochondrial phosphate transport was seen at OA concentrations of 94µg/mg protein. (Meisner, 1976). After *in vivo* administration of 5 or 15mg/kg bw and day for 3 days, an altered mitochondrial morphology in the proximal convoluted tubules of the kidneys was observed (Suzuki et al., 1975). Also in these three studies the OA concentrations applied were very high.

Recently Gekle et al. (1993) discovered in an *in vitro* study with Madin-Darby canine kidney cells that OA induces a reduction of plasma membrane anion conductance and possibly blocks the Cl^- channels. OA seems to be very potent as channel blocker and an IC_{50} value of 30nmol OA/l medium was measured.

However, at present, it is not known how or whether all these different effects are also related to the carcinogenic action of OA (see 1.2.2.3.).

1.2.2.2. Acute toxicity

The susceptibility to acute OA poisoning varies considerably among different species. The LD_{50} values by oral administered OA range from 0.2 mg/kg body weight for dogs to 58.3mg OA/kg bw for mice. (Harwig et al., 1983; Krogh, 1987; Kuiper-Goodman and Scott, 1989). The acute effect can be blocked by administration of high doses of phenylalanine. When mice received an LD_{100}-dose of OA simultaneously with phenylalanine in concentrations, which are 1.2 times higher than those of OA, all mice survived (Creppy et al., 1979c). The acute toxicity of OA can also be diminished in mice which are pre-treated with phenobarbital or 3-methylcholanthrene (Moroi et al., 1985), indicating that an increased metabolism of the parent compound is associated with lower acute toxicity.

1.2.2.3. Effects of prolonged exposure

The acute toxicity of OA is negligible with regard to chronic exposure of animals and humans to only low doses (no cases of acute poisoning with OA are known). The main target organ for the effects of chronically ingested OA is the kidney. OA has proved to be nephrotoxic already at low doses to all species tested so far. Histopathologically, degenerative changes in the proximal tubules and in advanced stages interstitial fibrosis were seen (DFG, 1990). Dose related changes have been found in rats (morphological kidney changes) and in pigs (functional kidney changes) after daily administration of 0.015 and 0.005mg OA/kg body weight for 90 days (Kuiper-Goodman and Scott, 1989).

Controversial results have been published regarding the genotoxicity of OA. A number of tests for the determination of the genotoxic properties of OA were carried out (for overview see IARC, 1993). The majority of the tests showed negative results. However, single strand breaks and more recently DNA adducts were found in small numbers (Pfohl-Leszkowicz et al., 1991; IARC, 1993; Pfohl-Leszkowicz et al., 1993a; Pfohl-Leszkowicz et al., 1993b).

Clear evidence for a carcinogenic potential of OA was determined in two 2 year carcinogenicity studies with rats (Boorman, 1989) and mice (Bendele et al., 1985). In the latter study with mice, kidney adenoma (26/50) and carcinoma (14/50) were found at doses of 4.8mg/kg bw but only in male mice. No kidney adenoma and carcinoma were found at this dose level in

female mice. The results of the rat study are shown in Table 1. Besides the high tumour incidence in male rats already at much lower doses (compared to mice) and the dose related increase in the incidence of kidney tumours in both sexes, again a difference in the sensitivity between male and female rats was observed. In contrast to female mice, adenoma and carcinoma were also found in female rats. Recent investigations in rats, regarding the site of OA toxicity have shown, that predominantly the pars recta paranoia of the proximal tubules are affected (Rásonyi et al., personal communications).

	Kidney-Adenoma				Kidney-Carcinoma			
	(µg OA/kg body weight)				(µg OA/kg body weight)			
Dose	0	21	70	210	0	21	70	210
Male	1/51	1/51	6/51	**10/50**	0/50	0/50	16/50	**30/50**
Female	0/50	0/50	1/51	**5/50**	0/50	0/50	1/51	**3/50**

(Boorman, NTP 1989)

Table 1:Kidney tumors induced by OA (adenomas and carcinomas) in a 2 year bioassay with F344 rats

Increased cell exfoliation and proliferation was observed in male and female rats treated with 210µg and 1000µg/kg bw for 4 weeks. The predominant form of cell death appeared to be apoptosis rather than necrosis. The increased cell damage and subsequent regenerative cell proliferation could explain the development of neoplastic lesions. However, no sex differences regarding toxicity, location of effects and regenerative cell proliferation were seen (Dietrich and Rásonyi, 1995). A possible explanation for the sex differences seen in renal tumour incidence could be the induction of α-2µ-globulin droplets in kidney cells. α-2µ-globulin is a protein which is only found in male rats. However, recent investigation demonstrated that α-2µ-globulin accumulation is not

involved in the enhanced formation of tumors in male rats (Rásonyi et al., 1993; Dietrich and Rásonyi, 1995). Due to the discrepancy found between the weak genotoxic potential of OA and the tumor incidence the observed renal tumors cannot merely be explained by genotoxicity. It is therefore assumed that epigenetic mechanisms must play the major role in the aetiology of OA induced renal tumors (Dietrich and Rásonyi, 1995). However, very little is known about the carcinogenic mechanism of OA. In view of the fact that explanations for the different sensitivities of species and sex are lacking, it is not surprising that the International Agency for Research on Cancer and the World Health Organization (93) classified ochratoxin A as *possibly carcinogenic to humans.*

1.2.2.4. Teratogenic and immunotoxic effects

A teratogenic effect of OA was found in mice, rats, hamsters and chickens already at single doses of 1mg/kg bw (Kuiper-Goodman and Scott, 1989; IARC, 1993). On the other hand no teratogenic effect was observed in a study with two pigs (doses: 0.9 and 2.7mg/kg bw) (Shreeve et al., 1977).
Immunotoxic effects of OA were found in experiments carried out in chickens, mice and rats (Kuiper-Goodman and Scott, 1989). Already at low doses (one intraperitoneal injection of 1µg/kg bw) suppressed both the IgG and the IgM response in BALB/c mice (DFG, 1990). It is possible that the immunosuppressive effect is related to OA's inhibiting effect on protein synthesis (DFG, 1990).

1.2.3. Kinetics

1.2.3.1. Absorption

In monogastric animals most of the oral administered OA is absorbed from the gastrointestinal tract (Galtier, 1978; Hagelberg et al., 1989). In experiments carried out with rats (Galtier, 1978) and mice (Roth et al., 1988) it was found that most of the OA is absorbed from the stomach, aided by the acidic properties of the mycotoxin (pK_a=7.1). The overall percentage of OA absorbed is 66, 56, 56 and 40% for the pig, rat, rabbit and chicken, respectively (Suzuki et al., 1977; Galtier et al., 1981). Ruminants were not considered to absorb much OA because it is rapidly

detoxified in the ruminants stomach due to the presence of protozoa which efficiently hydrolyse OA to Oα (Hult et al., 1976; Pettersson et al., 1982; Kiessling et al., 1984).
Ochratoxin A, that has been transferred across the intestinal mucosa reaches the liver (Kumagai and Aibara, 1982; Støren et al., 1982). As soon as OA reaches the circulatory system, the toxin is bound to serum albumin (Galtier et al., 1980) and to an as yet unidentified macromolecule (Hult and Fuchs, 1986). The fraction of free toxin in serum in most animal species is lower than 0.2% (Hagelberg et al., 1989).

1.2.3.2. Biotransformation
The first step of biotransformation of OA in the organism is the hydrolysation to the non-toxic Oα and phenylalanine (Suzuki et al., 1977; Galtier et al., 1979; Støren et al., 1982; Kiessling et al., 1984; Sreemannarayana et al., 1988; Kuiper-Goodman and Scott, 1989; Xiao et al., 1991). This reaction is catalysed by the enzymes carboxipeptidase and chymotrypsine (Pitout, 1969a; Pitout, 1969b; Pitout and Nel, 1969c). Studies in rats have shown that a major pathway in metabolising OA is lacking in most tissues other than in the intestine and the pancreas (Suzuki et al., 1977; Galtier et al., 1979). It has been suggested from studies conducted in mice, that OA is secreted into bile and is hydrolysed to Oα in the intestine (Moroi et al., 1985). Evidence for an enterohepatic circulation in mice was also found by Roth et al. (1988).
Other metabolites found in *in vivo* and *in vitro* studies are the 4(R)- and the 4(S)-hydroxyochratoxin A, 10-hydroxyochratoxin A (Fig.2), ochratoxin C (Fig. 1) and other unidentified OA-conjugates and decomposition products (Galtier et al., 1979; Elling et al., 1985; Fink-Gremmels et al., 1993). The 4-OH (4R and 4S) epimers are produced in rat and rabbit liver and rat kidney under the influence of cytochrome P450 (Størmer et al., 1981; Størmer et al., 1983). The 4R-OH-OA epimer which is considered less toxic than OA is the predominant of the two OA metabolites formed in human and rat liver microsomal systems (Størmer et al., 1981), whereas the 4S-OH-OA epimer is more prevalent with pig liver

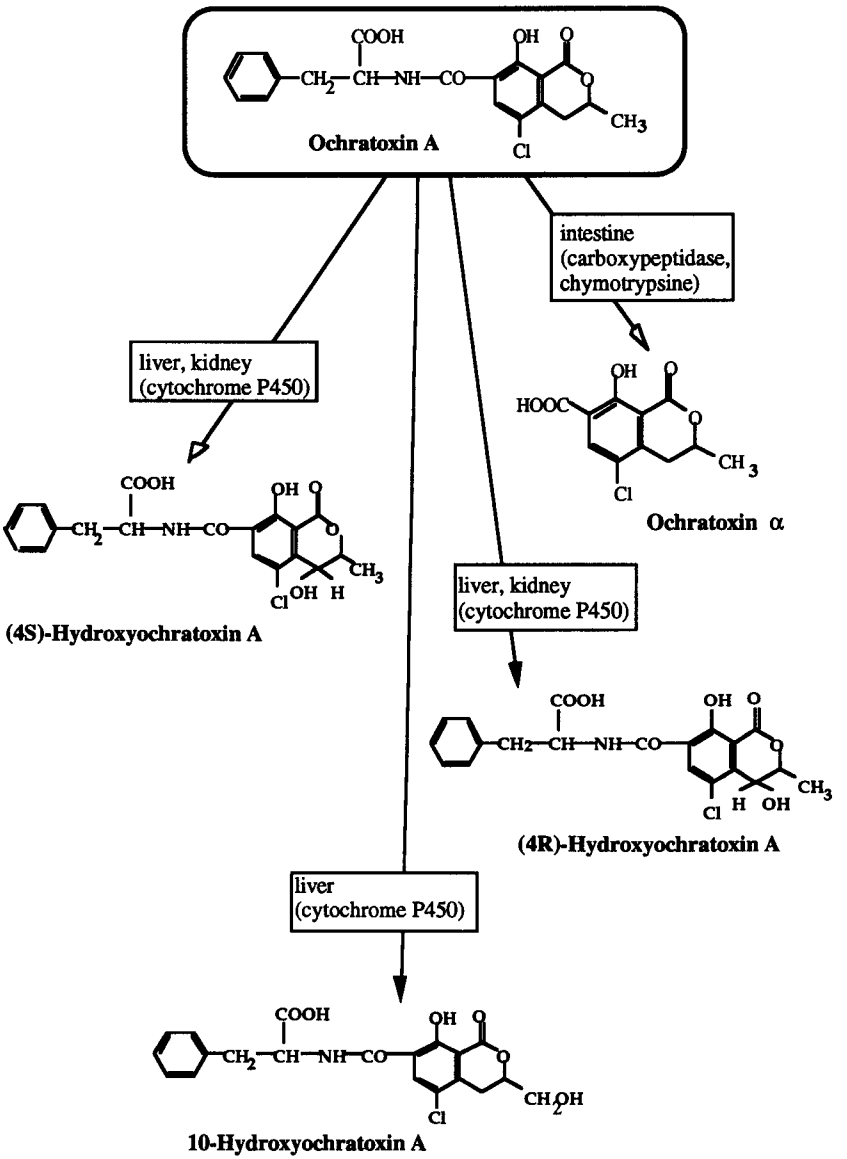


Fig. 2: Ochratoxin and its metabolites

microsomes. No data are available on the toxicity of the 4S-OH-OA epimer (Moroi et al., 1985). Besides the two 4-OH-OA epimers also the 10-OH-OA derivative (Fig. 2) was formed in a rabbit liver microsomal system (Størmer et al., 1983). Ochratoxin C was detected following *in vitro* incubation of OA with rumen fluids (Galtier and Alvinerie, 1976). It was found to be equally toxic as OA, but no indications were given how this was determined (cited by Galtier et al., 1981).

1.2.3.3. Excretion

The plasma clearance of OA follows the biliary as well as the renal excretion route. The amount of OA and Oα excreted in faeces and urine vary widely depending on the form and dose of administration (Suzuki et al., 1977; Galtier et al., 1979; Støren et al., 1982). The relative contribution of each excretory route varies from species to species and is also influenced by the degree of serum macromolecule binding (Hagelberg et al., 1989) and distinct amounts of enterohepatic recycling of OA. In rats the major excretory products were Oα, OA and the 4R-OH-OA epimer. In the urine these metabolites represented 11.6-27,2%, 6.9-11.65 and 1.5% of the orally administered dose, respectively (Suzuki et al., 1977; Støren et al., 1982). In the faeces Suzuki et al. (1977) found that 22.9% of the administered dose were excreted as Oα and 11.4% as OA, whereas Støren et al. (1982) could only detect traces of Oα and OA in the faeces.
Biliary excretion of OA was increased and urinary excretion of OA and Oα decreased in mice pretreated with phenobarbital (Moroi et al., 1985).
In preruminant and ruminant calves 85-90% of the administered OA was excreted as Oα, most of it in the urine (Sreemannarayana et al., 1988).

1.2.3.4. Half-lives of ochratoxin A in various species

There are wide spread species differences in the serum half-life of OA (Table 2). The serum half-life was longer after intravenous than after oral administration of OA (Hagelberg et al., 1989). Differences in half-life could be related primarily to differences in the degree of binding to serum macromolecules including albumin. Binding constants of OA to plasma proteins vary up to a factor of ≈ 200 among the species (Hagelberg et al., 1989). Other factors influencing the half-life could be differences in absorption (Galtier et al., 1981), dose (in pigs, serum disappearance of OA

on a high dose is faster than from a low dose) (Hult et al., 1979; Mortensen et al., 1983a), and the use of naturally contaminated grain versus crystalline OA (serum levels in pigs are about 5 times higher with naturally contaminated grain (Madsen et al., 1982; Mortensen et al., 1983a).

Animal	Half-life (hours)	References
chicken	4.1	(Galtier et al., 1981)
quail	6.7	(Hagelberg et al., 1989)
rabbit	8.2	(Galtier et al., 1981)
mouse	24-39	(Fukui et al., 1987; Hagelberg et al., 1989)
rat	55-120	(Galtier et al., 1979; Ballinger et al., 1986; Hagelberg et al., 1989)
preruminant calve	77	(Sreemannarayana et al., 1988)
pig	72-120	(Galtier et al., 1981; Mortensen et al., 1983b)
monkey *(Macaca mulata)*	510	(Hagelberg et al., 1989)

Table 2: Serum half-lives of ochratoxin A of different species after oral administration

As already mentioned earlier, OA binds to albumin and to an as yet unidentified serum macromolecule. The affinity of OA to this unknown molecule is higher than to the albumin. Saturation of this specific binding macromolecule occurs at 10-20ng OA/ml serum. The toxin binds to serum albumin only at higher concentrations in the serum i.e. above several hundred microgram OA/ml serum are needed to reach saturation levels (Stojkovic et al., 1984; Hult and Fuchs, 1986). Consequently, the amount of free toxin is very low. Only around 0.1% of the total OA are free if sera concentrations are below 20ng/ml (Hult and Fuchs, 1986). It is unknown whether the free or any of the bound forms of OA is the important factor for chronic toxicity. The OA fraction bound to serum macromolecules may

form a mobile reservoir of toxin which can release OA for a long time period (Galtier, 1978; Hult et al., 1982).
Very few data are available on the metabolic disposition of OA in humans. It has been suggested that ochratoxin A has a long half-life in humans based on the strong binding of OA to human serum macromolecules (Bauer and Gareis, 1987; Hagelberg et al., 1989).

1.2.4. Occurrence of OA in food

1.2.4.1. Conditions of OA formation
It is evident that toxin production by fungi can occur on most plant products and that the production is determined by two factors, namely temperature and water activity (a_w) of the substrate. Minimal a_w conditions for OA production by *A. ochraceus* are in the range of 0.83-0.87. The temperature range for OA production is between 12-37°C for *A. ochraceus* and between 4-31°C for *P. verrucosum.* (Krogh, 1987).

1.2.4.2. Natural occurrence in plant products
OA is primarily found in northern, temperate areas. Natural occurrence of OA in many commodities, e.g. cereals, vegetables, dried fruit, beer and coffee beans have been reported in a large number of publications (for review see Kuiper-Goodman and Scott, 1989; IARC, 1993). The far most analysed food items are cereals and cereal products. As far as it is known today, OA is not formed in the fields, but rather upon transport and storage (Shotwell and Hesseltine, 1983). As OA is heat stable it is also found in cooked food. The concentrations of OA found in food items range from 0.1ppb to 5000ppb (Kuiper-Goodman and Scott, 1989). However, most samples contain OA in the range of 0.1-50ppb.The contamination frequencies varied between a few and 40% (depending on the food items and the detection limit) and the mean of OA found in the contaminated samples was between 1 and 12ppb (DFG, 1990).

1.2.4.3. Natural occurrence in animal products
OA can occur in meat, meat products and dairy products such as cheese as a result of direct mould contamination, but this seems not to be a problem

of major concern (Kuiper-Goodman and Scott, 1989). More serious is the indirect transmission of OA into meat and meat products from animals exposed to mouldy feed. This concerns mainly pigs, chicken and preruminant calves with not yet fully developed gastrointestinal flora. Several studies were carried out with emphasis on the analysis of pig kidneys, pig blood and a variety of sausages. Highest OA levels found in kidney were around 200ppb, in blood 520ppb and in sausages 3.4ppb (Kuiper-Goodman and Scott, 1989).
The occurrence of OA in cow's milk is reported by Breitholz-Emanuelsson et al. (1993). OA concentrations between 0.01-0.04ng/ml were detected in 5 out of 36 samples.

1.2.4.4. Daily intake of OA in humans

Based on the OA contamination found in plant and animal products a daily intake for humans between 80 and 160ng was calculated, depending on nutritional habits (DFG, 1990; Rühl et al., 1992). The contribution of meat is hereby assumed to be around 1-2%. The daily intake of OA can vary widely from day to day as the occurrence of OA within the food commodities is often only punctual due to the inhomogenous distribution of OA contamination.
This estimated daily intake has to be compared with the different levels of a tolerable daily intake (TDI) which have been suggested. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) (WHO, 1991) proposed a provisional tolerable weekly intake of 112ng/kg bw (16ng/kg bw and day), based on kidney damages in pigs. Based on the carcinogenicity data of Boorman (1989), Kuiper-Goodman and Scott (1989) calculated a TDI of 4.2 or 0.2ng/kg bw. The TDI of 4.2ng/kg bw was calculated by dividing the no observed effect level (NOEL) by a safety factor of 5000, whereas the linear extrapolation of the NOEL to a "virtually safe dose" (VSD = one additional tumour/10^6 lives) resulted in a TDI of 0.2ng/kg bw.
The currently estimated daily intake of 80-160ng (≈1-2.5ng/kg bw) is located in the range of the latter two TDIs.

1.2.5. Occurrence of OA in humans

OA is frequently found in human blood and was also detected in human milk. Due to improved analytical methods with lower detection limits the percentage of OA positive blood samples increased constantly. In the early 80ies several studies with human blood were carried out in the Balkan area as it was suggested, that OA could be a potential factor in inducing Balkan endemic nephropathy. The levels found in the Balkan area ranged from 1-100ng OA/ml blood (Hult et al., 1982; Fuchs et al., 1991; Petkova-Bocharova and Castegnaro, 1991). However, findings of OA in human blood were not limited to that region, findings were also reported from Canada, Czechoslovakia, Denmark, Germany, Italy, Poland Sweden and Switzerland (Bauer and Gareis, 1987; Fukal and Reisnerova, 1990; Breitholz-Emanuelsson et al., 1991; Frohlich et al., 1991; Golinski et al., 1991; Hald, 1991; Breitholz-Emanuelsson et al., 1993; BAG, 1994; Breitholz-Emanuelsson et al., 1994). In the latter countries OA levels found in human plasma ranged from 0.06-35.33ng/ml serum with incidences of positive samples ranging between 7 and 100%. The mean concentration of all samples measured in the latter studies ranged from 0.1-0.45ng OA/ml serum. In two of these studies (Frohlich et al., 1991; Breitholz-Emanuelsson et al., 1994) levels of people with different kidney disorders were also included. No differences were seen between healthy individuals and people with renal impairment with one exception (Frohlich et al., 1991; Breitholz-Emanuelsson et al., 1994): Breitholz-Emanuelsson et al. (1994) found significantly higher OA serum levels in dialysis patients than in healthy individuals .

The occurrence of OA in human milk seems not to be as frequent as the occurrence in blood. In Germany OA was found in 4 out of 36 samples, the concentrations ranging between of 0.017-0.03ng/ml (Bauer and Gareis, 1987). In Sweden the relationship between OA in human blood and in breast milk was investigated (Breitholz-Emanuelsson et al., 1993), demonstrating that while OA was present in all blood samples (n=39, 0.09-0.94ng/ml blood), only 22 milk samples (n=39) were positive for OA and in none of these samples concentrations above 0.04ng/ml (detection limit: 0.01ng/ml) were present. In Italy nine of 50 milk samples contained OA, with levels between 1.2 and 6.6ng/ml (Micco et al., 1991). However, these

levels seemed very high when compared to the results of the former two studies.

1.3. Aims of the present study

The aim of the present study was to increase the basic knowledge on OA contamination of food items and the heat stability of OA. Together with the investigation of OA kinetic data in humans we wanted to provide a better data base for human risk assessment of OA.
To reach this goal several studies were performed. Their specific objectives were:

1. Food items from the Swiss retail market were analysed in order to be able to estimate the daily intake and to be able to compare the contamination levels with those found in other countries.

2. A sensitive analytical method for green and roasted coffee beans and the brew had to be established. With this method the determination of the heat stability of OA during the roasting process and the carry over of OA contamination from roasted coffee into the brew was analysed. Additionally a small number of coffee samples from the Swiss retail market was analysed in order to make a preliminary estimation of the contribution of coffee consumption to the daily intake of OA.

3. The intraindividual variation of OA blood levels in humans over a period of 8 weeks was measured.

4. The plasma half-life and renal clearance of OA in humans was determined and the daily intake calculated by applying these kinetic parameters.

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2. Ochratoxin A in cereals from the Swiss retail market

2.1. Introduction

Ochratoxin A (OA) has been shown to occur in various food items of plant origin such as grains, cereals, coffee beans, dried fruit and nuts. OA is, in contrast to aflatoxin, predominantly found in countries with temperate climate. OA, a mould product, is most likely produced during storage of the various substrates and not already on the field prior to harvest (Shotwell and Hesseltine, 1983). The conidia of ochratoxin A-producing moulds adhere to grains and germinate under adequate conditions during transport and storage leading to contamination of the external layers of the grain. Longer storage may led to growth and subsequently contamination of not only the pelts but also the grain itself. OA is therefore found not only in the whole grain, but also in the flour. Until today a large number of samples of plant origin has been analysed; most of them were cereal and corn samples as well as their products. Over 7000 cereal samples including whole grain, bran, semolina, flour, bread and pasta have been analysed in North America and in Northern and Western Europe (for review see Kuiper-Goodman and Scott, 1989; IARC, 1993). About 10% of these samples contained detectable amounts of OA in the range of 0.1-5000µg/kg, mainly between 0.1 and 50ppb. For corn and corn products OA was detected in about 4.5% of the nearly 1400 samples with OA levels between 0.2 and 200µg/kg. Based on the analysis of 1100 cereal samples the Deutsche Forschungsgemeinschaft calculated a daily uptake of OA via cereals (DFG, 1990) of ca. 80ng/day and person.

For the present study new data were collected mainly because of two reasons. The data mentioned above are mostly old data and improved analysis methods have now led to lower detection limits. Secondly data specifically from the Swiss market were needed to evaluate the situation in Switzerland.

2.2. Material and Methods

2.2.1. Samples and sample preparation

31 samples, different cereals and corn products, were purchased from the Swiss retail market. All samples except the flours were ground in a laboratory mill before extraction and analysis.

2.2.2. Chemical analysis

The analysis were done according to the method of Baumann and Zimmerli (1988). 40g of finely ground sample material were mixed with 150ml of chloroform and 20ml of 0.1M phosphoric acid for 5 minutes using a Polytron Homogeniser (Polytron PT 3000, Kinematica, Lucerne, Switzerland). The mixture was centrifuged for 20 minutes at 2500g. 100ml of the chloroform extract was poured into an Extrelut® (Merck) column. For this column 7g of Extrelut was mixed with 10ml of a 1% sodium bicarbonate solution. The Extrelut was rinsed with twice 40ml of chloroform and then dried by passing air through. These eluates were discarded. The lower end of the column was closed and 30ml of chloroform mixed with 1ml of 100% formic acid was poured into the column. The Extrelut was stirred carefully and the OA was eluted by opening the lower end of the column. The column was treated with 2x 40ml of chloroform and the combined eluates were evaporated to dryness. The residue was dissolved in 2ml methanol and kept in the dark until HPLC analysis. The HPLC conditions are listed in Table 1.

Instrument:	Kontron HPLC Systems 600
Column:	Spherisorb ODS I 5µm, 250x4mm, Knauer Säulentechnik (Germany)
Precolumn:	Spherisorb ODS II 5µm, 30x4mm, (Knauer Säulentechnik (Germany)
Column-Temp.:	50°C
Injection volume:	20µl
Eluent (Isocratic):	60% methanol, 40% water/acetic acid (375+25)
Flow:	1ml/Min, (for corn flakes 0.5ml/Min.)
Detection:	Fluorescence Detector (Merck F 1000 Fluorescence Spectrophotometer)
Excitation Wavelength:	330nm
Emission Wavelength:	460nm
Detection limit for standards (S/N=3): 10pg	

Table 1: HPLC conditions for OA analysis in cereals

2.3. Results

The recovery of OA from the different matrices was always >85%. With this method it was possible to detect 0.1µg OA/kg sample (S/N=3).
The results of the analyses are listed in Table 2. From the 31 analysed samples, 7 (23%) contained detectable amounts of OA. 5 out of 6 analysed wheat semolina samples contained OA in the range of 0.6-1.4µg/kg. No OA was found in white wheat flour, in wheat bran and bran flakes, whereas one of the three analysed brown wheat samples contained 0.3µg OA/kg. Of the 9 analysed corn semolina samples only one contained a detectable amount of OA (0.1µg/kg). No OA was found in the other corn products (canned sweet corn, corn flakes). The mean concentration of all 31 samples analysed was 0.15µg/kg (for negative samples a zero value was taken).

Product	Composition	No.	µg OA/kg
wheat semolina	wheat	1	<0.1.
wheat semolina	wheat	2	1.4
wheat semolina	wheat	3	0.6
wheat semolina	wheat	4	0.6
wheat semolina	wheat	5	0.9
wheat semolina	wheat	6	0.6
wheat bran	wheat	1-2	<0.1
white flour	wheat	1-2	<0.1
brown flour	wheat	1-2	<0.1
brown flour	wheat	3	0.3
bran buds	wheat bran, malt	1	<0.1
bran flakes	wheat, wheat bran, malt	1	<0.1
corn flakes	corn, malt	1-5	<0.1
corn semolina	corn	1	0.1
corn semolina	corn	2-9	<0.1
sweet corn	corn	1-2	<0.1

Table 2: OA content of the analysed cereal samples

2.4. Discussion

The results of the few samples analysed correspond well with the results published by Baumann and Zimmerli (1988) and the Kantonales Laboratorium Aargau (Anonymous, 1993). The latter two studies also encompassed the analyses of cereals from the Swiss retail market. Baumann and Zimmerli (1988), having a detection limit of 0.1µg OA/kg, analysed 48 cereal samples including grains, bran, flour, semolina, pasta and bread. 71% of these samples contained OA in the range of 0.1-3.5µg/kg. In the other study, with a detection limit of 0.2µg OA/kg, OA was detected in 55% of the 87 analysed cereal samples including flour, dust, semolina, whole grains, shred grain, flakes, bran and pasta. The contamination of OA positive samples ranged from 0.2-3.8µg/kg. The

situation with regard to the corn contamination seems to be more inconsistent. Baumann and Zimmerli (1988) found no positive corn samples (n=6) including corn semolina and pop corn. While the other study reported 5 OA positive out of 11 analysed samples, with OA levels ranging between 0.2 and 2.8µg/kg. This difference could probably be due to the varying origin of the corn, as most of the corn for Switzerland is imported in contrast to wheat.

Obviously these studies, including the presented one, can not give a representative overview of the general OA contamination in cereals in Switzerland. However, compared to other countries in Western and Northern Europe and in North America it can be stated that the contamination situation of OA in cereals, despite the higher percentage of positive samples, is not alarming. Many of samples, mentioned in the introduction, were analysed in the 70ies and 80ies where detection limits were much higher than in the studies presented and discussed here. Thus the higher percentages of positive samples found nowadays can be explained by lower detection limits. Furthermore, a higher percentage of positive samples does not mean that cereals are more frequently contaminated or that the mean level of contamination has risen. Indeed samples with high levels of OA are not found more often nowadays than in the 70's and 80's. These data suggest that the exposure of humans with OA via cereals occurs at low doses continuously and ubiquitously. The low exposure is therefore difficult to eliminate.

Switzerland has recently established a tolerance level for OA contamination in cereals and its products of 2.0µg/kg and in the EU legal limits are also in discussion. The Netherlands have proposed a tolerance level of 3.0µg/kg. Applying the tolerance level of 2.0µg/kg to the Swiss samples, only six out of the 183 samples analysed would have had to be rejected. It can therefore be concluded, that the OA contamination is not alarming. However, steps to diminish the contamination, such as better, e.g. dry transport and storage conditions, should be taken.

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3. The occurrence of ochratoxin A in coffee

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Abbreviations: BEN=Balkan endemic nephropathy; DNA=desoxyribonucleic acid; GC-MS=gas chromatography-mass spectrometry; HPLC=high performance liquid chromatography; IAC=immunoaffinity column; NCI/MID=negative chemical ionisation/multiple ion detection; NMR=nuclear magnetic resonance spectrum; NOEL=no observed effect level; OA=Ochratoxin A; PBS=phosphate buffered saline; PTWI=provisional tolerable weekly intake; VSD=virtually safe dose

3.1. Abstract

Ochratoxin A (OA) is a nephrotoxic and nephrocarcinogenic mycotoxin which is predominantly produced by the two ubiquitous fungal species Aspergillus and Penicillium. OA is found in foodstuff, predominantly in cereals but also in coffee beans. Inconsistent results have been published regarding the influence of roasting on the OA content in roasted beans and the transfer into the coffee brew. In the present study a HPLC-method was used for the detection of OA in green and roasted coffee beans as well as in the coffee brew. For qualitative confirmation and quantification of low OA levels in roasted coffee beans and coffee brew an additional clean up step by immunoaffinity column was applied before HPLC-analysis. In green coffee beans OA was detected in 13 out of 25 analysed commercial samples (detection limit: 0.5µg OA/kg). Roasting (250°C, 150 sec) of naturally contaminated green beans or beans inoculated with A. ochraceus resulted only in a small reduction of the OA level. OA was also found to be eluted into the brew. Of 40 coffee brews, prepared from commercially available samples OA was detected in 18 brews by HPLC and/or additional immunoaffinity column clean up in the range of 0.4-7.8µg OA/kg (referred to ground coffee). Our preliminary results suggest, therefore, that regular coffee consumption may contribute to human OA exposure.

3.2. Introduction

The mycotoxin ochratoxin A (OA) derives its name from Aspergillus ochraceus, the first mould from which it was isolated. It is the main toxic component in cultures of this mould, but it is also produced by other ubiquitously found moulds such as in various other strains of Aspergillus and Penicillium. OA consists of a dihydroisocumarin moiety linked through its 7-carboxyl group by an amid bond to L-phenylalanin (Fig. 1).

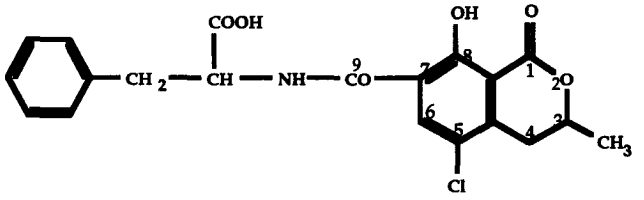

Fig. 1: The chemical structure of ochratoxin A

Special attention is paid to OA since a link between OA and human Balkan endemic nephropathy (BEN) was postulated (IARC, 1993; Radovanovic, 1989) However, this link could not be proven by now. BEN is a chronic kidney disease predominantly of people living in rural areas of the Balkan (Ex-Yugoslavia, Romania, Bulgaria). Elevated amounts of OA were found not only in food, but also in the blood-sera of people living in the endemic area (Petkova-Bocharova *et al.*, 1988). The human pathology of BEN is a diffuse fibrosis subsequently leading to renal failure. A similar pathology was found in pigs following chronic toxicity studies conducted with OA (Radovanovic, 1989). In all species tested so far (pig, chicken, mouse, rat, dog) OA induced nephrotoxic effects although the sensitivity of the different species varied (Kuiper-Goodman and Scott, 1989). The ochratoxigenic nephropathy in pigs is a well known and often observed disease (Krogh *et al.*, 1976). A disease of poultry related to OA contaminated feed has also been reported (Harwig *et al.*, 1983). Outbreaks of a similar disease involving the death of a high number of turkeys have been reported in the USA and were causally related to OA contaminated corn (Harwig *et al.*, 1983).

OA was also found to be carcinogenic. In two 2 year-carcinogenicity studies with mice and rats, OA was shown to induce kidney tumours (Bendele *et al.*, 1985; Boorman, 1989; IARC, 1993; Kuiper-Goodman and Scott, 1989). Because of the low doses of OA required to induce tumours in mice and rats, research on OA gained momentum. No genotoxic effect of OA was observed in several genotoxicity test systems (DFG, 1990). However, preliminary experiments have indicated a metabolic activation of OA by primary cultures of rat hepatocytes resulting in a mutagenic and genotoxic effect (Hennig *et al.*, 1991). Formation of DNA adducts in mouse tissues (kidney, liver, spleen) after administration of a large single oral dose of 2.5mg OA/kg and using the ^{32}P-post-labelling method was also observed (Pfohl-Leszkowicz *et al.*, 1993 a). The highest number of adducts ($103/10^9$ nucleotides) were measured in the kidney. In the liver and the spleen the adducts disappeared 5 days after OA was administered, whereas a small number of adducts ($7.7/10^9$ nucleotides) persisted at least 16 days in the kidney. DNA adducts were also found in tumourous tissues from three kidneys and five bladders of Bulgarian patients in the range of 11-61 adducts/10^9nucleotides (Pfohl-Leszkowicz *et al.*, 1993 b). Of these adducts several showed the same R_F-values as those obtained from mouse kidney after OA treatment. In view of these partially contradictory findings it appeared important to know more about the daily intake of OA in humans.

OA has been detected in a variety of foods and feeds, mostly from countries with a temperate climate. For humans the main sources seems to be maize and cereals (DFG, 1990). In a study carried out in Germany with cereals and cereal products about 40% of the samples contained between 0.1 and 1.5µg/kg (Rühl *et al.*, 1992). In another study from Germany it was found that 6% of the cereal samples contained OA in the range of 0.1-17.7µg/kg (Majerus *et al.*, 1993). In a further study 56 different beers were analysed (El-Dessouki, 1992). 10 samples were positive (> 0.3µg/l). 9 of the positive samples were of the rarely consumed "Starkbier" (strong beer) variety and contained between 0.35 and 1.53µg OA/l.

The daily intake of OA was calculated to be about 100ng (DFG, 1990, Rühl *et al.*, 1992). OA was also found in about 60% of more than 500 human blood sera (mean of positive samples ca. 0.8µg/l, maximum value 14.4µg/l (DFG, 1990)). In a recent study in Sweden 39 human blood

samples were measured. All samples were positive and contained between 0.09 and 0.94µg OA/l blood (mean: 0.17µg/l) (Breitholz-Emanuelsson *et al.*, 1993). These relatively high levels and the high frequency demands further research on OA kinetics in humans and a broader search for other sources of human OA exposure.

OA has also been detected in green and roasted coffee beans (Cantafora *et al.*, 1983; Micco *et al.*, 1989; Tsubouchi *et al.*, 1984; Tsubouchi *et al.*, 1988), cocoa beans, soybeans and visibly mouldy peanuts (Harwig *et al.*, 1983). Moreover, OA was found in hazelnuts and dates (Majerus *et al.*, 1993). While only scattered data exist regarding nuts, dates, soybeans and cocoa beans, the natural occurrence of OA in green and roasted coffee beans has been reported several times. However, inconsistent results have been published with respect to the influence of the roasting process on the OA content (Gallaz and Stadler, 1976; Micco *et al.*, 1989; Tsubouchi, *et al.*, 1987) and the transfer of OA into the brew (Micco *et al.*, 1989; Tsubouchi *et al.*, 1987).

As OA was shown to be a potent carcinogen in animals and as OA could be present in significant amounts in green and roasted coffee as well as in coffee brew a HPLC-method was adjusted in order to detect reliably OA without prior derivatization. In the present study the occurrence of OA in green and roasted coffee beans, its destruction by the roasting process and its presence in the coffee brew was investigated. A preliminary estimation of the daily human OA intake is given and its possible health risk is discussed.

3.3. Materials and Methods

3.3.1. Chemicals

Ochratoxin A and phosphate buffered saline, pH 7.4 were purchased by Sigma LTD (St. Louis, MO, USA), sodiumhydrogencarbonate Art. 6329, methanol p.a. Art. 6009, n-hexane puriss. Art. 4360 and formic acid p.a. Art. 264 were from Merck (Darmstadt, Germany), celite 545 Art. 22140, isooctane purum Art. 59050, chloroform puriss Art. 25690, hydrochloric

acid p.a. Art. 84422 and sodium sulphate anhydrous p.a. Art. 71960 were purchased from Fluka Chemie AG (9470 Buchs, Switzerland). Acetonitrile 190 (far UV) was from Romil Chemicals Limited (Loughborough, Leics UK). Folded filters 595 1/2 were from Schleicher & Schuell (Kassel, Germany). The immunoaffinity columns were obtained from Biocode Limited (York, United Kingdom).

3.3.2. Methods

3.3.2.1. Coffee samples

25 green coffee samples (weighing between 1 and 5kg) of commercial quality were kindly provided by various coffee companies. 5 samples originated from spoiled coffee lots (no. 4, 5, 6, 7, 8) which were expected to contain high amounts of OA and would not have been marketed. 40 samples of roasted coffee were purchased in different Swiss retail markets.

3.3.2.2. Green coffee sample treatment

Each sample of green beans, also to be used for roasting tests, was divided into two parts: 100g to 1kg was ground with a whole-wheat mill. The other part (125g to 500g) was roasted for 100-190sec (depending on the sample size) at 252°C in a roasting machine (kindly supplied by Tchibo GmbH, Hamburg). For samples, which were not roasted 1kg was ground. Roasted coffee was ground with a commercial coffee mill. For OA analysis aliquots of 30g of ground coffee were used.

3.3.2.3. Inoculation of green coffee beans

Two samples of 500g green coffee beans (no. 43 and 44) were heat sterilised and inoculated with a spore suspension of Aspergillus ochraceus (according to the method of Gallaz and Stadler (1976)). The A. ochraceus strain used was IBT/D 738 kindly provided by Dr. Jens Frisvad, Copenhagen, Denmark. The inoculated beans were kept at room temperature for two weeks and then frozen and stored at -20°C. Prior to roasting the coffee beans were dried for two hours at 80°C and then treated as described above.

3.3.2.4. Preparation of the coffee brew

Coffee was brewed according to general Swiss habit: 30g ground coffee was placed into a commercial paper coffee filter and 500ml of boiling water was poured through the filter. After cooling 300ml of the brew was taken for analysis.

3.3.2.5. Chemical analysis

Green and roasted coffee: A modified extraction method from the one originally published by Micco *et al.* (1989) was employed. An aliquot of 30g of green or ground roasted coffee was mixed with 60ml of $NaHCO_3$ (5%) and 130ml of methanol for 5 minutes using a Polytron Homogeniser (Polytron PT 3000, Kinematica, Lucerne, Switzerland). The mixture was centrifuged for 20 minutes at 3000g. 100ml of the liquid phase (or a lower amount if no derivatization or repeated injections were planned) were transferred into a separatory funnel, defatted with isooctane (30ml), acidified with HCl conc. (4ml) and extracted with $CHCl_3$ (1x 50ml, 1x 25ml). The combined chloroform extracts were washed with 3x 10ml of 1.4M HCl and dried over anhydrous sodium sulphate. To this final solution 40ml of n-hexane were added and then cleaned up by pouring it into a bicarbonate-celite column (previously prepared according to the AOAC, 1984). After washing the column with 50ml of $CHCl_3$ the celite was air dried. 31ml chloroform-formic acid (30ml +1ml) was poured into the column, the celite was carefully stirred and the OA was eluted. The column was treated with 2x 35ml chloroform and the combined eluates were evaporated to dryness. The residue was dissolved in 1ml acetonitrile and the OA-amount was determined by HPLC-analysis at least twice. The excitation and emission wavelengths recommended by Bauer *et al.* (1984) were found to be appropriate. The HPLC conditions (System A) are listed in Table 1.1.

The detection limits (observation of a distinct signal above background) for green and roasted coffee were 0.5µg/kg and 1.0µg/kg, respectively.

Brew: In two beakers each 150ml of brew (or a lower amount if less than 1ml of extract was needed), 2ml conc. HCl and 150ml of chloroform were mixed using a Polytron Homogeniser for 5 minutes. The mixtures were centrifuged for 20 minutes at 3000g. The clear chloroform phases (100ml each) were combined and 40ml of n-hexane was added. This solution was

poured into a bicarbonate-celite column and then continued as described above for green and roasted coffee. The detection limit was 1.0µg/kg coffee beans (observation of a distinct signal above background).

3.3.2.6. Confirmation procedures

Methylation: (Method originally published by Uchiyama *et al.* (1985). 200µl of the extract ready for HPLC injection was evaporated to dryness and 2.5ml of methanol and 0.1ml of conc. HCl were added. The solution was left standing overnight at room temperature. Thereafter the methanol was evaporated and the residue taken up in 200µl acetonitrile. The HPLC-analysis was identical to the one described above.

Confirmation via clean up by immunoaffinity columns (IAC): 100µl of extract ready for HPLC, were applied to the IAC under addition of 4x5ml of phosphate buffered saline (PBS), pH 7.4 containing 15%(v/v) of methanol and pushed through the column using low pressure (1-2ml/min). The column was washed with 10ml distilled water and air dried. The PBS/methanol and the water was discarded. OA, now bound in the IAC was then eluted from the column with 3ml of 100% methanol. The OA containing methanol was evaporated in a water bath (40°C) under a nitrogen stream. The residue was taken up in 100µl of methanol and analysed by HPLC (System B, conditions see Table 1.2). 50µl of this immunoaffinity column purified extract were methylated as described above and analysed again via HPLC (System B).

Table 1.1: HPLC conditions of System A, used for non-immunoaffinity column purified extracts

Instrument:	Kontron HPLC Systems 600
Column:	LiChrosorb RP 18 5µm, 250x4mm, Knauer Säulentechnik (Germany)
Precolumn:	LiChrosorb RP 18 5µm, 30x4mm, Knauer Säulentechnik (Germany)
Column-Temp.	50°C
Injection volume:	20µl
Eluent(isocratic):	
for green samples:	45% Acetonitrile, 55% Water/Acetic Acid (19:1 v/v)
for roasted samples:	35% Acetonitrile, 65% Water/Acetic Acid (19:1 v/v)
for coffee brew:	35% Acetonitrile, 65% Water/Acetic Acid (19:1 v/v)
Flow:	1ml/Min.
Detection:	Fluorescence Detector (Merck F 1000 Fluorescence Spectrophotometer)
Excitation Wavelength:	330nm
Emission Wavelength:	460nm
Detection limit for standards (S/N=2) :	35pg

Table 1.2: HPLC conditions of System B, used for immunoaffinity column purified extracts

Instrument:	Kontron HPLC Systems 600
Column:	Spherisorb ODS I 5µm, 250x4mm, Knauer Säulentechnik (Germany)
Precolumn:	Spherisorb ODS II 5µm, 30x4mm, Knauer Säulentechnik (Germany)
Column-Temp.	50°C
Injection volume:	20µl
Eluent(isocratic):	65% Methanol, 35% Water/Acetic Acid (91:9 v/v)
Post-column alkalisation:	25% Ammonium hydroxide, 0.1ml/Min
Flow:	1.0ml/Min
Detection:	Fluorescence Detector (Merck F 1000 Fluorescence Spectrophotometer)
Excitation Wavelength:	390nm
Emission Wavelength:	440nm
Detection limit for standards (S/N=2):	4pg

Table 1.3: HPLC conditions of System C, used for non-immunoaffinity column purified extracts and immuno-affinity column purified extracts

Instrument:	Hewlett Packard 1090 Liquid Chromatograph		
Column:	Spherisorb ODS II endcapped, 3µm, 150x2mm, Chemie Brunschwig, Germany		
Column-Temp.	40°C		
Injection volume:	5-10µl		
Eluent:	Min:	Acetonitrile:	Water/Acetic Acid (19:1 v/v)
	0-1	30	70
	1-20	80	20
	20-25	80	20
	25-30	30	70
	posttime 10min.	30	70
Flow:	1ml/Min.		
Detection:	Fluorescence Detector (Hewlett Packard 1046 A)		
Excitation Wavelength:	330nm		
Emission Wavelength:	460nm		
Detection limit for standards (S/N=2):	7pg		

The immunoaffinity column was regenerated with 20ml of PBS and was used up to 10 fold without decrease of recovery. The recovery from the column with the extract of roasted coffee was ≥80%. The detection limit was ca. 0.1µg/kg (S/N≅5).

Confirmation of the OA-identity via an additional HPLC-system (System C, conditions see Table 1.3): In order to test the selectivity of the IAC and to confirm the OA-identity, an OA-standard, a coffee extract before and after cleaning by IAC were analysed with a third HPLC-system, similar to system A but with a lower detection limit for OA-standards.

Confirmation by GC-MS: The extract of green coffee, ready for HPLC analysis in system A was methylated with diazomethane. The extracts of the roasted coffee samples had first to be cleaned up with the immunoaffinity column and were then methylated with diazomethane. OA was identified and quantified (comparison to a standard solution of methylated OA) by NCI/MID (negative chemical ionisation/multiple ion detection) technique with a detection limit of ca. 0.1µg/kg (S/N≅5) for OA in green and roasted coffee extracts. The GC and MS conditions were as described in Table 2.

Table 2: GC and MS conditions

	GC conditions:
Instrument:	HRGC 5160 Mega Series, Carlo Erba Instruments
Column:	20mx0.3mm
Stationary phase:	PS 086 (Polymethylphenylsiloxane containing 12% phenylgroups)
Injection volume:	1-5µl on column (solvent: methanol)
Temperature program:	70°C, 1 min. isothermal 20°C/Min. -250°C 5°C/Min. - 320°C
Elution temperature:	307°C
	MS conditions:
Instrument:	Finnigan MAT 4510
Ionisation Mode:	Negative chemical ionisation (NCI)
Temperature of ion source:	190°C
Reagent gas:	Methane at 0.6 Torr
Registration mode:	Multiple ion detection (MID)
m/e detected:	416, 418, 431, 433, cycle time: 1sec.

3.3.2.7. Heat stability experiments

From a standard stock solution of 10µg OA/ml ethanol 500µl were given into 5 small glasses and were kept for 0, 3, 6, 9 and 12 minutes at 270°C. The residues were dissolved in 1ml acetonitrile and quantified by HPLC.

3.3.2.8. Calculation of the results

Comparisons of the OA levels in green and roasted beans were made on the basis of the weight of green beans. For calculation of OA levels in roasted beans an average loss of 13% water was assumed during roasting. This value was found in our experiments and corresponds well with those described in the literature (Smith, 1985).

Statistically significant differences of OA contents between green and roasted beans were calculated with the Mann-Whitney-Test at a $p<0.05$. The maximal percentage of destruction was calculated by a simple regression analysis applying a one sided confidence limit of 95%.

3.4. Results

3.4.1. Analytical methods

The analytical method of Micco *et al.* (1989) was modified in order to be able to measure the OA content in green and in roasted coffee without previous derivatization as it was originally done by Micco *et al.* A typical HPLC chromatogram of a green coffee sample with a low OA content is depicted in Fig. 2A, while a chromatogram of a roasted sample is shown in Fig. 2B. The eluent composition used for green coffee consisting of a 45% acetonitrile and 55% H_2O mix, had to be changed for roasted coffee analysis to a mix of 35% acetonitrile and 65% H_2O.

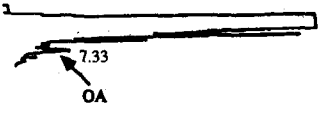

Fig. 2A: HPLC chromatogram (System A) of a green coffee sample with 2.5µg OA/kg

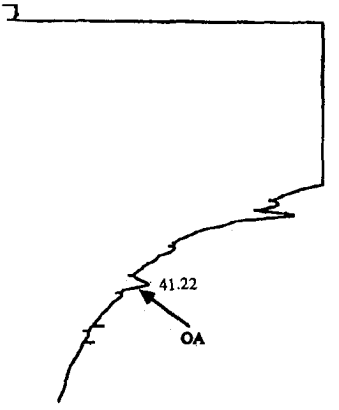

Fig. 2B: HPLC chromatogram (System A) of a roasted not immunopurified coffee sample with 5µg OA/kg

Due to the appearance of peaks interfering with the OA peak, a reduction of the organic component was necessary leading to a change of OA retention time. In green beans the retention time of OA was 7.3 min., in roasted beans OA was detected at 41.2 min. Especially for samples containing only small amounts of OA it was imperative that the HPLC column was in good condition for flawless detection. The condition of the column was checked following 100 injections of roasted coffee or brew samples by injecting the extract of a roasted coffee sample containing a known, small amount (1 or 2µg OA/kg coffee) of OA. Nevertheless, exact quantification of small amounts (≤ 2-5µg OA/kg coffee) was not possible. Thus, the HPLC-method (system A) allowed merely semiquantitative assessment of OA in samples containing OA concentrations near the detection limit.

In order to test the extraction method, three independent recovery experiments per sample type were carried out with spiked samples. OA recovery in green coffee was 97% ± 8% (arithmetic mean ± one standard deviation), for roasted coffee 116% ± 6% and for the brew 87% ± 11%. The relatively low standard deviation indicated a good repeatability of the extraction and analysis method.

Derivatization of OA via methylation of the extracts of several green and roasted coffee samples and brews with subsequent HPLC analysis was used for qualitative confirmation of OA positive and negative samples. With the derivatization method (Uchiyama *et al.*, 1985) used here approximately 60-80% of the OA was methylated. A chromatogram of a low contaminated methylated green sample is shown in Fig. 2C.

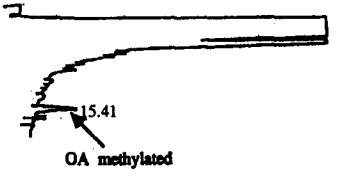

Fig. 2C: HPLC chromatogram (System A) of a green coffee sample with 2µg OA/kg coffee, where the extract was methylated prior to analysis

In higher contaminated samples (no. 6,7,8,43 and 44, Fig. 3) both, the methylated and the unmethylated OA peak could be seen in the chromatograms.
In all the 23 samples used for derivatization the OA results were confirmed. The identity of OA found in green samples was further confirmed by GC-MS (1 mouldy and 5 commercial samples). Fig. 4A shows a total mass spectrum of the methylated standard (10ng which corresponds to about 500µg/kg).

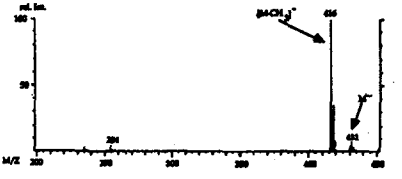

Fig. 4A: NCI Mass spectrum: standard 500µg/kg methylated OA, retention time: 1353'
The mass of 416 represents the mono-methylated OA, the one of 431 the di-methylated OA

OA concentrations above 50µg/kg could easily be identified and quantified via a total mass spectrum (Jiao *et al.*, 1992). A green sample, containing 300µg/kg is shown in Fig. 4B.

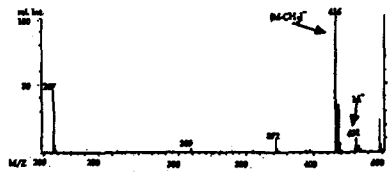

Fig. 4B: Mass spectrum: green coffee with 300µg/kg methylated OA retention time: 1371'

Low concentrations of OA (<20µg/kg) were identified and quantified in a multiple ion detection chromatogram as seen in Fig. 4C (Ions 416, 418 (Cl-Isotope peak) 431, 433 (Cl-Isotope peak)). Furthermore, the identity of OA in commercial roasted coffee was qualitatively confirmed by GC-MS with two samples (containing 1.5 and 2.0 µg/kg) after clean up via immunoaffinity column. Exact quantification of these samples was not possible due to problems with the GC-column.

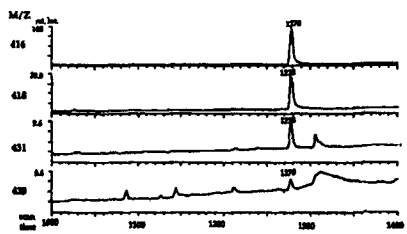

Fig. 4C: MID-mass chromatogram: green coffee with 1.5µg/kg methylated OA, retention time: 1278' ; Ions detected: 416, 418, 431, 433

The derivatization method with diazomethane could not be used for the confirmation by HPLC because of peaks interfering with the OA-peak in the HPLC-chromatogram.

In the HPLC chromatograms of highly contaminated roasted samples and the corresponding coffee brews (>100µg OA/kg) as well as of the pure heated OA, a second peak after the OA peak was observed (Fig. 5).
The areas of these peaks ranged between 10 and 30% of the OA-peak area. In chromatograms of green beans no such peak could be seen. The two peaks were separately methylated and analysed by GC-MS. Practically identical mass spectra and retention times were observed. Therefore there is strong evidence that this peak is a diastereomer of ochratoxin A formed at elevated temperature. This was also supported by ^{1}H-NMR spectroscopy, which indicated an inversion at the C(3)-position of the molecule. So far there is no information available regarding the toxicity of the latter diastereomer of ochratoxin A.

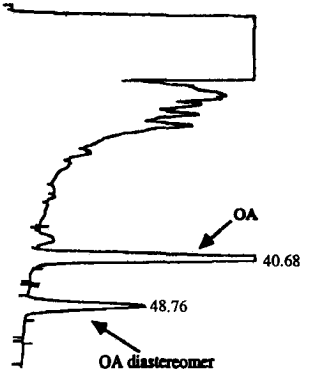

Fig. 5: HPLC chromatogram of a roasted sample with 1500µg OA/kg demonstrating the peak of the diastereomer of ochratoxin A

3.4.2. Analytical results

The wide range of the OA concentrations found in green coffee (Table 3), emphasises the general problem which is encountered when working with mycotoxins. The inhomogeneity of the OA contamination within one sample is enormous, due to the fact that often only a few beans within a bag are infested by the mould.

sample no.	1	2	3	4	5
individual values of single analysis	<0.5	<0.5	64.0	<0.5	9.1
	<0.5	42.0	<0.5	<0.5	<0.5
	44.0	7.0	<0.5	2.7	16.0
	2.0	<0.5	1.0	1.8	1.8
	3.6	<0.5	7.3	2.5	2.1
			0.7		
			<0.5		
			<0.5		
			<0.5		
			<0.5		

Table 3: OA (µg/kg) in five green coffee samples (analysed without immunoaffinity cleaning); the inhomogeneity of low OA contamination whiten samples is shown

This inhomogeneity necessitates an extraordinary high number of analyses of aliquots of the same sample in order to allow to quantify the average degree of OA contamination. In highly contaminated samples the OA contamination seemed to be more homogenous as shown in Fig. 3.
In the naturally contaminated samples (no. 6,7,8, Fig. 3) no statistically significant difference between the OA content of the green and the roasted beans was found. The maximal loss of OA that could not be detected because of the high standard error of the mean (due to the inhomogeneity of OA distribution) was 14% for sample 6, 45% for sample 7 and 62% for sample 8. Among the two artificially inoculated samples (no. 43, 44, Fig. 3) showing a very homogenous contamination no statistically significant OA destruction was seen after roasting in sample no. 44. The maximal not detectable loss of OA because of roasting would be 13%. In the sample no. 43 however, a statistically significant loss was detected. If applying the one sided 95% confidence limit the loss of OA would be between 2% and 29%.
In order to investigate the carry-over of OA from green beans via roasting into the coffee brew (Fig. 6), three selected commercial samples (no. 36, 37, 38) were analysed.

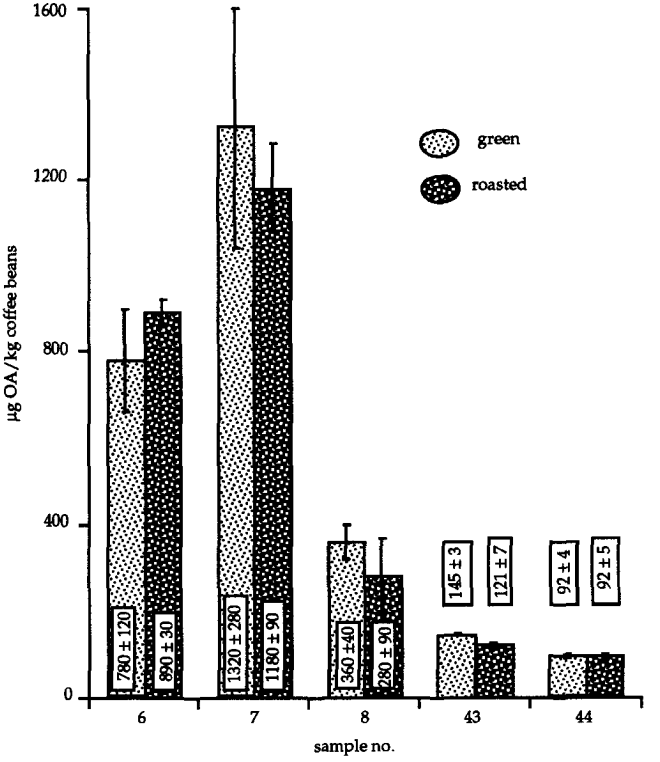

Fig. 3: OA content in naturally contaminated spoiled coffee beans (no. 6,7,8) and in artificially inoculated coffee beans (no. 43 and 44) before (green) and after (roasted) roasting
(the OA amounts are calculated based on green bean weight)
(arithmetic mean of 3-6 samples ± standard error of the mean)

Although the green beans were ground as finely as possible and mixed well before analysis, a certain inhomogeneity remained as it was not possible to grind the green beans finer than semolina. No statistically significant difference between the OA levels of green beans and the coffee brew was detected. The maximal, not detectable loss of OA from green coffee to the brew was 52%, 67% and 74% for sample no. 36, 37 and 38 respectively. Lower amounts of OA were however found in the roasted aliquots than in either the green beans or the brew from the same sample.

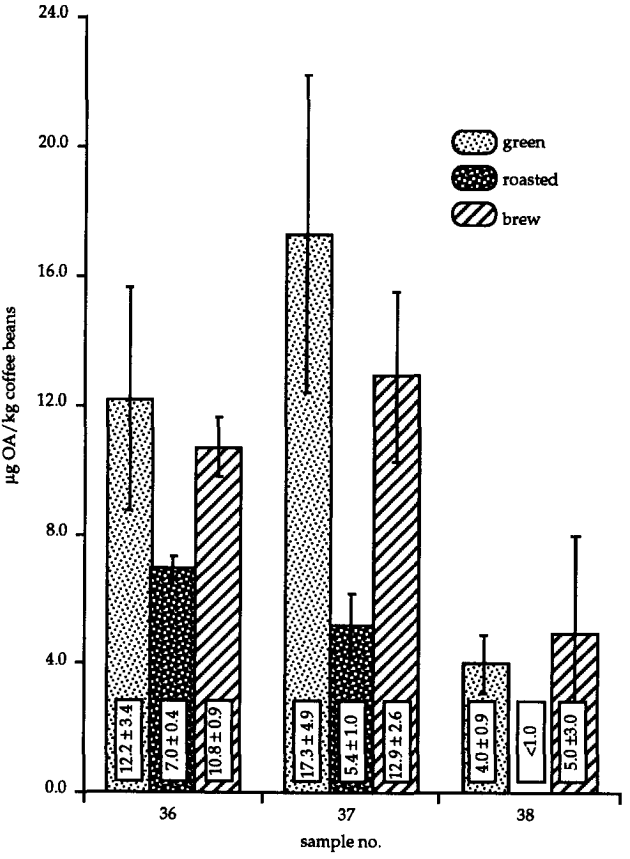

Fig. 6: Carry over of OA from green beans via roasted beans into the brew of 3 commercial coffee samples
(the OA amounts are calculated based on green bean weight)
(arithmetic mean of 4-6 samples ± standard error of the mean)

To investigate the heat stability, pure OA was kept at similar conditions (time and temperature) as used for roasting (normal roasting temperatures for 500g coffee are between 250°C and 270°C for 120-150 sec). This experiment showed clearly, that no OA was destroyed up to 6 minutes of heat exposure (Fig. 7) and a 50% reduction of the OA content, mainly due

to the epimerization as discussed above, was observed only after 12 minutes at 270°C .

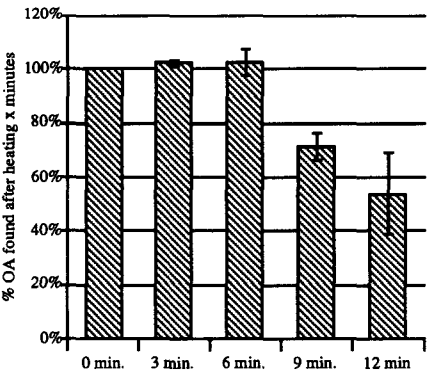

Fig. 7: Heat stability of OA kept for 3, 6, 9 and 12 minutes at 270°C (arithmetic mean of 3 experiments ± standard error of the mean)

The analysis of 25 green coffee samples obtained from various companies revealed that 13 contained detectable amounts of OA (Table 4). The range of contamination was between 1.2 and 56µg/kg. It must be stated that the sample containing 56µg/kg had a rather spoiled appearance.
The results of 40 commercially available roasted samples purchased in various Swiss retail shops are shown in Table 5. In 21 samples both the brew and the ground coffee were analysed, while for 19 samples only the brew was analysed, being the relevant OA source for the consumer. Samples with amounts >2.0µg/kg were analysed at least twice. Of the 21 analysed ground coffee samples 7 were positive, but except two all had amounts near the detection limit (around 1µg/kg).

Origin and/or type of coffee	total samples	samples positive at first analysis (> 0.5µg/kg)	values of positive samples [µg OA/kg]
Columbia	5	3	1.2; 9.9±8.1*†; 9.8±8.4*†
Santos	2	1	7.4±6.3*†
Brasil	3	2	2.0; 4.0±0.9*
Central America	1	0	
Robusta	3	2	2.2; 3.6
Ivory Coast	2	2	9.9; 56.0
Kenia	3	0	
Guatemala	1	0	
Costa Rica	1	0	
Tanzania	1	1	2.2
New Guinea	1	0	
Zaire	1	1	17.3±4.9*
unknown	1	1	11.8±3.3*

* arithmetic mean of 4 to 10 samples ± standard error
† individual values are shown in Table 3 (sample no. 1, 2 and 3)

Table 4: OA amount of 25 commercial green coffee samples received from European suppliers (analysed without immunoaffinity cleaning)

3.4.3. Identification of OA in low contaminated roasted samples

To verify the results of the commercial roasted coffee samples and in order to be able to give a more reliable quantification, all positive extracts with amounts ≤2µg/kg and four negative extracts, listed in Table 5 were cleaned up with the immunoaffinity column (IAC) and again measured by HPLC (System B). OA could be detected in all IAC-cleaned extracts and in addition could be confirmed with methylation (Table 5).
The quantification of the non methylated and the methylated extracts after IAC cleaning was facilitated by better peak separation and is therefore more reliable. HPLC-chromatograms of the same extract of a brew before IAC purification, after IAC purification and the methylated IAC-extract are shown in Fig. 8.

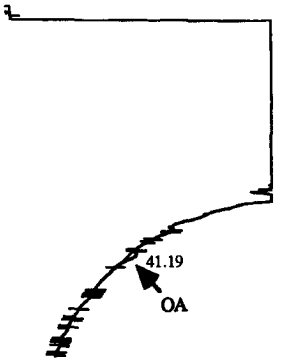

Fig. 8A: HPLC chromatogram (HPLC system A) of a not immunoaffinity column purified extract of a brew of commercial roasted coffee containing a calculated OA-amount of 1.0µg/kg ground, roasted coffee

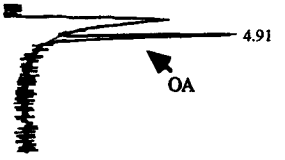

Fig. 8B: HPLC chromatogram (HPLC system B) of the same extract of a brew of commercial roasted coffee , with previous immunoaffinity column purification , containing a calculated OA-amount of 1.0µg/kg ground, roasted coffee

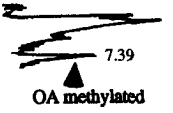

Fig. 8C: HPLC chromatogram (HPLC system B) of the methylated extract of Fig. 8B, containing a calculated OA-amount of 0.8µg/kg ground, roasted coffee

In addition, because of the low k-value (k =1.6) in the chromatograms of system B, one commercial roasted sample (2µg/kg) was also tested in system C which is similar to system A (used for the extracts not cleaned up by IAC). The relevant differences to the system A were the use of a more powerful fluorescence detector and the use of a narrowbore HPLC-column. This resulted in a 5 times lower detection limit for OA standard with system C than previously achieved with system A. The OA-identity was confirmed by the identical retention time (=18 min.; k=8) of the OA-standard peak, and the peak in the extracts without and with IAC-cleaning.
16 of the 40 analysed extracts of brews not cleaned by IAC contained detectable OA amounts (≤1µg OA/kg coffee) and tended to be higher than those in the ground coffee. This observation corresponds to the results described in Fig. 6. All the brew samples with amounts ≤ 2µg OA/kg ground coffee were confirmed by IAC cleaning including methylation. Also in two brew samples with amounts ≤1µg OA/kg in the first HPLC analysis small amounts of OA were detected after IAC cleaning.
In the cases where two or more aliquots of one sample were analysed, again a certain inhomogeneity of OA contamination could be seen.

Table 5: OA levels of 40 roasted coffee samples from the Swiss retail market (analysed without and with immunoaffinity column cleaning)

Type or Origin of coffee (as indicated on the package)	roasted [µg OA/kg ground coffee] NIPE	IPE	IPE, methylated	brew µg OA/kg [referred to ground coffee] NIPE	IPE	IPE, methylated
Arabica	< 1.0			< 1.0		
unknown	< 1.0			1.0	0.4	0.4
unknown	< 1.0			1.0	0.7	0.5
Arabica	< 1.0			< 1.0		
unknown	< 1.0			< 1.0		
unknown	< 1.0			1.0	1.7	1.9
Arabica	1.0; < 1.0	1.0; 0.3	2.0; 0.3	5.4; 3.6; 7.2		
Central-, Southamerica, Westafrica, India	< 1.0			< 1.0		
Arabica, Centralamerica, Eastafrica	1.5	1.1	0.8	< 1.0		
Arabica, Centralamerica	< 1.0			1.0	1.2	0.7
Arabica, Centralamerica, Eastafrica	1.0; < 1.0	1.0*; n.a.	0.6; n.a.	1.6	0.5	0.4
Arabica, Central-, Southamerica, Eastafrica, India	1.0	0.2	0.2	1.0	0.7	0.8
unknown	< 1.0			< 1.0		
70% Arabica, 30% Robusta	4.0; < 1.0			4.2; < 1.0		
Arabica, Robusta	4.2; 7.0			5.2; 7.9; 10.9; 7.2		
Southindia	< 1.0			< 1.0		
Kenia	< 1.0			2.0	1.1	1.4
Arabica	< 1.0			< 1.0		
Arabica	< 1.0			< 1.0		
unknown	< 1.0			< 1.0		
unknown	2.0; < 1.0	1.8*; 0.3	1.3; 0.3	6.7; 7.3; < 1.0		
unknown				< 1.0		
unknown				1.0	1.6	1.5
Arabica				1.0	0.9	0.5
Arabica				< 1.0		
Arabica				< 1.0		
Arabica				1.0	1.0	0.8
Arabica, Robusta				< 1.0		
Arabica, Robusta				< 1.0		
Arabica				< 1.0		
Arabica, Robusta				1.0	4.2	3.4
Arabica, Robusta				< 1.0		
Arabica				< 1.0		
Arabica				< 1.0		
Arabica, Robusta				< 1.0		
Arabica				< 1.0	0.4	0.3
unknown				< 1.0	0.7	0.5
unknown				< 1.0		
unknown				< 1.0		
Arabica, Robusta				1.0	2.0	2.3

n.a. : not analysed, NIPE: not immunopurified extracts, IPE: immunopurified extacts

* samples qualitatively confirmed by GC-MS

3.5. Discussion

The same analytical method was used in this study for the detection of OA in extracts of green as well as of roasted coffee beans whereas for coffee brew a faster extraction method was applied because of the low fat content (<1% dry weight of solid residues (Ratnayake *et al.*, 1993) of the brew compared to the green and roasted beans (lipid content between 9 and 20% (Smith, 1985)). Surprisingly, we detected significantly lower amounts of OA in relatively low contaminated samples of roasted beans than in the corresponding green beans or in the respective brew (samples no. 36, 37 and 38, Fig. 6). This is possibly due to incomplete extraction from the roasted beans due to matrix effects (e.g. a limited amount of relatively strong binding sites, which would influence extractions in the low level range only). Therefore, the extraction procedure used was not optimal for low contaminated roasted beans. Treatment with hot water prior to extraction, as it is done in coffee brewing, obviously improves the efficiency of OA analysis of low contaminated roasted coffee.

The lack of baseline separation and the small sample size of low contaminated roasted samples via HPLC system A only demanded a more reliable method. Therefore, to confirm the presence of OA in low contaminated commercial roasted samples and to get a more reliable quantification, an additional clean up of the extracts via an immunoaffinity column was applied. This step turned out to be very efficient as shown in Figure 8. The suspected low OA content could be convincingly confirmed after the additional cleaning step either as native compound or as methylated derivative. Also the indicated range of contamination after IAC-cleaning is reliable. But it must be underlined that even with inclusion of the immunoaffinity clean up the quantification of these results in the range of the detection limit (0.1-0.5 ppb) was only possible within a factor of about 2.

We thus strongly recommend to include a clean up step by IAC for analytical studies on low level OA contamination in coffee.

Supplementary qualitative confirmations of the OA-identity in the extracts were done via GC-MS and via a third HPLC-system (System C). All these analyses showed very clear and unequivocal evidence for the identity of OA in these immunoaffinity column cleaned extracts.

The presence of OA in commercial green coffee has been observed by other authors in a similar concentration range as reported here (Table 4). Cantafora *et al.* (1983) analysed 40 samples of which 9 were positive and contained between 0.5 and 23µg/kg, Tsubouchi *et al.* (1984) found 4 positive samples out of 22, containing between 9.9 and 46µg/kg and Micco *et al.* (1989) reported that 16 samples out of 29 were contaminated with OA levels between 0.2 and 15µg/kg.

Due to the inhomogeneity of OA contamination within the coffee samples it is difficult to determine destruction of OA during roasting when using samples containing only low amounts of OA (Table 3). Grinding and mixing of the beans could not completely eliminate this problem.

Highly contaminated green beans, where the inhomogeneity was less pronounced, were therefore used for the investigation of OA destruction during roasting. In the three spoiled samples (no. 6, 7 and 8) containing up to 1300µg/kg (Fig. 3) no statistically significant destruction of OA could be observed after roasting. However due to the inhomogeneity of OA content (especially samples no. 7 and 8) a loss of OA during roasting between 14% and maximally 62% for the samples 6,7,and 8 would not have been detected. In the two artificially contaminated samples (no. 43 and 44) OA was very homogeneously distributed, as indicated by the low standard error of the mean. From the results of sample no. 43 a statistically significant reduction of 2%-29% of the OA content during roasting was calculated. It is likely therefore that a reduction of the OA content during roasting of less than 30% takes place. This calculated small decrease is due partially to some epimerization at C(3), the loss of OA adsorbed on the silver skin, and a possible chemical destruction of OA by substances in the coffee bean. The results of the heat stability experiment with pure OA-solution demonstrating that OA is not detectably destroyed up to 6 minutes of exposure to 270°C which represents a maximum of temperature and double the time normally used for roasting support our findings of negligible destruction during roasting.

Several studies have been published regarding the heat stability of OA during the roasting process. Tsubouchi *et al.* (1987) reported a reduction of 0-12% OA after roasting artificially inoculated beans contaminated in the range of 200-140'000µg/kg, which corroborates our own findings. One can assume a relatively high homogeneity in artificially contaminated samples

as indicated by the results presented here, although Tsubouchi *et al.* (1987) did not report corresponding data .

In all the other studies (Cantafora *et al.*, 1983; Gallaz and Stadler, 1976; Levi *et al.*, 1974; Micco *et al.*, 1989) a destruction of OA during roasting in the range between 50 and 100% was reported. Levi *et al.* (1974), Gallaz and Stadler, (1976) and Micco *et al.* (1989) added pure OA directly to the beans in the range of 40-350µg/kg. It is very likely that with the loss of the silver skin from the coffee beans during roasting also the superficially added OA was removed. The almost complete destruction of OA in 6 mouldy samples containing <20-40µg OA/kg found by Gallaz *et al.* (1976), Cantafora *et al.* (1983) and Micco *et al.* (1989) is contradictory to our results. These authors did not take problems of inhomogeneity and incomplete extraction in aliquots of low contaminated samples into account. In addition they did not compare the content in green beans and in the corresponding brew as it was done in this study.

In Fig. 6 it is shown that OA is neither destroyed appreciably by the roasting process even in the low level range nor is it retained in the ground roasted coffee during brewing since the levels found in green coffee beans and in the corresponding brew remained almost the same and no statistically significant difference can be seen. However, the destruction that would have had to take place to detect significant differences between the green coffee and the corresponding brew would have had to be over 52%, 64% and 74% respectively in these experiments. Since the inhomogeneity in these low contaminated samples is enormous and only a small number of analyses was carried out, it can, based on these data, only be stated that more than 25-50% of the OA, detected in green coffee, is - after roasting- found in the brew. Indeed, Tsubouchi *et al.* (1987) reported that 100% of the OA detected in roasted coffee could be eluted into the brew, thus corroborating the assumption that a higher percentage (50% or more) of OA found in green coffee is transferred into the coffee brew.

Similar data as discussed earlier for samples 36, 37 and 38 (Fig. 6) concerning the OA level of roasted coffee and the brew were obtained from samples of roasted coffee and the corresponding brews from the Swiss retail market (Table 5). In 6 samples where OA was detected in the ground coffee and in the brew as well, the same or higher OA concentrations were found in the brew (Table 5). The fact that in one

sample no OA was detected in the brew, whereas a small amount was found in the ground coffee was probably due to inhomogeneity of OA contamination. This inhomogeneity was further illustrated with the samples where several aliquots of the same sample were analysed.

OA in commercially roasted beans was also found by Tsubouchi *et al.* (1988) in 5 of 68 samples purchased in Japan (=7% positive), containing OA in the range of 3.2 - 17µg/kg (detection limit: 2µg/kg). The higher percentage of positive samples in our study (45%) was due to the lower detection limit (0.1µg/kg) as only 10% of the samples in Table 5 had concentrations >2µg/kg.

The lower average concentration of OA in commercially available roasted coffee from the Swiss retail market (Table 5) in comparison to the green samples received from coffee companies (Table 4) is possibly due to the mixture of beans from different origins in the Swiss samples, whereas the green beans, on the other hand, were well defined, but surely not representative samples. Therefore the results of the two tables (Table 4 & 5) cannot be directly compared.

In order to achieve a more precise estimate of the degree of OA contamination in coffee, as well as to assess the overall contribution of coffee consumption to human OA intake, analyses of a large number of commercial coffee samples should be undertaken.

Based on the preliminary results presented here, a daily intake from coffee drinking of about 25ng OA per person corresponding to approximately 0.4ng/kg bw/day is easily possible (based on a daily use of 25g of ground roasted coffee (about 3-4 cups) and a contamination level of 1µg/kg). The daily intake of OA via food in general was previously estimated to be about 100ng (DFG, 1990), corresponding to 1.6ng/kg bw. This indicates that coffee consumption can contribute significantly to the OA intake of humans. The calculated total daily intake of about 2.0ng/kg bw (from the major sources known so far) is lower than the PTWI (Provisional Tolerable Weekly Intake of the Joint FAO/WHO Expert Committee on food additives (WHO, 1991) of 112ng/kg bw/week corresponding to 16ng/kg bw/day. However, this PTWI accounts for renal damage only and does not take carcinogenicity into consideration.

Regarding carcinogenicity data, Kuiper-Goodman and Scott (1989) calculated a tolerable daily intake of OA either with a NOEL/safety factor

approach or with a mathematical low-dose extrapolation. With an experimentally observed NOEL for tumours at 21µg/kg bw/day (Boorman, 1989) and a safety factor of 5000 they calculated an estimated tolerable intake of 4.2ng/kg bw/day for humans. According to the Delaney clause, which was introduced in the US legislation in 1958, compounds with carcinogenic properties shall not be present in food. This very strict requirement had obviously to be weakened during the past 30 years when many unavoidable compounds with carcinogenic properties were detected in food. It was therefore agreed in many countries that a theoretical lifetime cancer risk for individual compounds in food of $1:10^6$ ("virtually safe dose", VSD) had to be accepted. For OA the mathematical extrapolation for a life time tumour risk of $1:10^6$ resulted in a VSD of 0.2ng/kg bw/day. At present it is not possible to fully evaluate these theoretical estimates of the carcinogenic risk of human exposure to OA at low dose levels since the carcinogenic mechanism of OA is not known and no kinetic data for humans are available. If, however, the most conservative approach (as used for the calculation of the VSD) of a linear high to low dose extrapolation is applied, a cumulative theoretical life time risk for a kidney tumour of only 0.0012% can be calculated from the preliminary data of the total daily OA intake of ca. 2ng/kg bw. The cumulative life time kidney tumour incidence for men and women in Switzerland is 1.3% and 0.6%, respectively (IARC, 1992). This suggests that OA -if at all- plays only a minor role among the factors leading to kidney tumours in humans. In addition, one must underline that the linear dose-effect extrapolation for OA carcinogenicity, generally only used for genotoxic carcinogens, represents a worst case scenario and the calculated -already small- theoretical risk most likely overestimates the real risk. On the other hand, there are profound species differences in OA kinetics (Fuchs, 1988) and a longer persistence of OA in humans compared to rats is likely. As long as all these questions are not yet solved and in view of the many different and potent toxic properties of OA, the exposure to OA should be kept as low as possible.

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4. Intraindividual variation in plasma levels and kinetic parameters of ochratoxin A in humans

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4.1. Abstract

Ochratoxin A (OA) is a carcinogenic mycotoxin which is produced by ubiquitous fungal species (Aspergillus and Penicillium). OA is found in a variety of food items and as a consequence it is also most frequently detected in human plasma (average concentrations found: 0.1-1ng OA/ml plasma). In the present study the intraindividual variation of plasma levels was investigated in eight persons over a period of two months. The concentrations measured ranged from 0.2-0.9ng/ml. The plasma levels in some individuals remained nearly constant over the time, others varied considerably (e.g. increase of 0.4ng/ml within 3 days, decrease of 0.3ng/ml within 5 days) during the observation period.

The toxicokinetic profile of OA was studied in one human volunteer after ingestion of 395ng radio labelled OA. A two compartment open model was found consisting of a fast elimination and distribution phase ($T_{1/2}$ = 22h) followed by a slow elimination phase with a calculated plasma half-life of 35.5 days. During the elimination phase it was found that most of the OA and its metabolites were excreted via urine. The renal clearance of the elimination phase was estimated to be around 0.048ml/min. Based on these data a daily uptake of OA of 85ng/person was estimated.

The ten fold higher elimination half-life of OA in humans compared to rats may influence considerably the evaluation of the rat carcinogenesis data with regard to human cancer risk extrapolation.

4.2. Introduction

The mycotoxin ochratoxin A is produced by ubiquitous Penicillium and Aspergillus species. Food and feed contamination by the toxin is found mainly in countries with temperate or continental climate. Data regarding the wide spread contamination of agricultural commodities and animal products by ochratoxin A (OA) have been published extensively over the past few years (for review see Kuiper-Goodman and Scott, 1989; IARC, 1993).

The main target organ of OA toxicity in several animal species is the kidney (Kuiper-Goodman and Scott, 1989) and OA is a strong kidney carcinogen in rats (Boorman, 1989). The International Agency for Research on Cancer (IARC) classified ochratoxin A as *possibly carcinogenic to humans* (IARC, 1993). Ochratoxin in human sera has first been looked for in the Balkan area (Hult et al., 1982; Petkova-Bocharova et al., 1988), because OA was thought to be a major factor in the induction of Balkan endemic nephropathy. Extensive studies were undertaken and a large number of samples of human blood from this region was analysed. Between 6 and 26% of the samples contained OA in the range of 1-35ng OA/ml blood (Petkova-Bocharova et al., 1988) and 1-40 ng OA/ml sera (Hult et al., 1982). In studies carried out in other countries OA was found in the most human blood samples (see Table 1) when sensitive analytical methods are used (detection limit: 0.05ng/ml).

The daily intake of OA in humans via food can be calculated based on a relatively good data base concerning OA concentration in food items (DFG, 1990; Rühl et al., 1992; Studer-Rohr et al., 1995). However, until now no data exist on the variation of OA-plasma levels over a prolonged time period in the same individual and kinetic parameters of OA in humans such as half-life and renal clearance are unknown. At present risk evaluation for humans based on animal data is difficult, as large species differences are known to exist: plasma half-lives determined in various species range from 0.68h in fish to 510h in monkeys (Hagelberg et al., 1989). One of the main factors influencing plasma half-lives is the capacity of OA to bind to plasma proteins, which accordingly appears to differ among species (Chang and Chu, 1977; Galtier et al., 1979; Hagelberg et

al., 1989). As a consequence also the values for clearance differ considerably (Hagelberg et al., 1989).
It was, therefore, the aim of the present study to look for intraindividual variations in OA plasma levels as well as to determine OA kinetic parameters in humans in order to have a better data base for the risk assessment of OA to humans.

Table 1. Occurrence of ochratoxin A in blood

Country	No. of samples	Positive samples	Detection limit	Content (ng/ml)	Reference
Canada	159	40%	no indication	0.27-35.33[a)]	(Frohlich et al., 1991)
Czechoslo-vakia	143	25%	0.1ng/ml	0.1-1.26[a)]	(Fukal et al., 1990)
Denmark	144	54%	0.1ng/ml	0.1-13.2[a)]	(Hald et al., 1991)
Germany	306	57%	0.1ng/ml	0.1-14.4[a)]	(Bauer et al., 1987)
Italy	133	100%	0.05ng/ml	0.05-14.0[a)]	Breitholz-Emanuelsson et al., 1994)
Poland	1065	7%	1.0ng/ml	0.27[a),b)]	(Golinski et al., 1991)
Sweden	297	13%	0.3ng/ml	0.3-6.7[a)]	(Breitholz et al., 1991)
Sweden	39	100%	0.04ng/ml	0.09-0.94[c)]	(Breitholz-Emanuelsson et al., 1993)
Switzerland	368	100%	0.01ng/ml	0.06-6.02[a)]	(BAG, 1994)

a) serum or plasma
b) mean of all samples measured
c) blood

4.3. Material and Methods

4.3.1. Chemicals

Ochratoxin A and phosphate buffered saline (PBS), pH 7.4, were purchased by Sigma LTD (St. Louis MO, USA), sodium chloride p.a. Art. 71380, chloroform p.a. Art. 25690 and ammonium hydroxide p.a., ≈25% in water Art. 9860 were purchased from Fluka Chemie AG (9470 Buchs, Switzerland). Acetic acid 100%, p.a. Art. 1.00063, ethanol p.a. Art. 1.00983 and ortho phosphoric acid min. 85%, p.a. Art. 573 were from Merck (Darmstadt, Germany), methanol 205 was from Romil Chemicals Limited (Loughborough, Leics UK) and the immunoaffinity columns were from Biocode Limited (York, UK). The ^{3}H-labelled OA was purchased from Moravek Biochemicals (Brea, California, USA). The scintillation cocktail for normal samples was Ready Gel from Beckman (USA) and the scintillation cocktail used for the oxidised samples was Monophase 40 from Packard.

4.3.2. Methods

4.3.2.1. Chemical analysis of plasma

The analyses were carried out according to Zimmerli and Dick (1995). Up to 2ml of human plasma were mixed with 10ml of 2M NaCl solution, containing 34ml of 85% H_3PO_4/litre. After addition of 5ml chloroform the mixture was shaken for 2 minutes and then centrifuged at 2500g for 20 minutes. The organic phase was transferred into a flask and the extraction with chloroform was repeated an additional three times. The combined organic phases were evaporated to dryness. The residue was dissolved in 4x 5ml phosphate buffered saline (pH 7.4, PBS) /15% methanol (v/v). This solution was applied to an immunoaffinity column (IAC) and pushed through the column using low pressure (1-2ml/min).

The column was washed with 10ml distilled water and air dried. The PBS/methanol and the water was discarded. OA, now bound in the IAC was then eluted from the column with 3ml of 100% methanol. The OA containing methanol was evaporated in a water bath (40°C) under a

nitrogen stream. The residue was taken up in 50-100µl of methanol and analysed by HPLC (HPLC conditions see Table 2).

Table 2: HPLC conditions

Instrument:	Kontron HPLC Systems 600
Column:	Spherisorb ODS 2 5µm, 250x4mm, Knauer Säulentechnik (Germany)
Precolumn:	Spherisorb ODS 2 5µm, 30x4mm, Knauer Säulentechnik (Germany)
Column-Temp.	50°C
Injection volume:	20-50µl
Eluent(isocratic):	65% Methanol, 35% Water/Acetic Acid (91:9 v/v)
Post-column alkalinisation:	25% Ammonium hydroxide, 0.2ml/Min
Flow:	1.0ml/Min
Detection:	Fluorescence Detector (Merck F 1000 Fluorescence Spectrophotometer)
Excitation Wavelength:	390nm
Emission Wavelength:	440nm
Detection limit for standards (S/N=2) : 4pg	

4.3.2.2. Testing the radiochemical purity of ^{3}H-Ochratoxin A

The radiochemical purity of the labelled OA was tested by HPLC and by immunoaffinity-column (IAC)/HPLC analyses.

For the HPLC analyses 25nCi of uniformly tritium labelled OA (specific activity: 3.9Ci/mmol) spiked with 13.4ng of unlabelled OA, was injected into the HPLC. The HPLC-eluate was collected at 1 minute intervals and the amount of radioactivity of each sample was determined in a liquid scintillation counter (LS 6000 LL Beckman, USA). The amount of cold OA was detected by fluorescence and determined by integrating the peak area. The HPLC-fraction with the highest amount of radioactivity was again injected into the HPLC and the same steps as in the first HPLC-run were repeated, in order to detect loss of ^{3}H-label.

Further testing for radiochemical purity was done by employing an immunoaffinity column (IAC) for OA. 25nCi labelled OA spiked with 50ng of cold OA was mixed with 20ml PBS/15% methanol (v/v) and the solution was poured on to the column. The IAC was then rinsed with 10ml

of distilled water and the OA was eluted with 3ml of methanol. The PBS/methanol and water fractions were collected in 5ml samples and the amount of radioactivity was determined by scintillation counting. The amount of radioactivity in the eluate was determined by counting two aliquots of 20µl. The amount of cold and radioactive OA in the eluate was also determined by HPLC analysis after injection of two aliquots of 20µl. The HPLC-fraction during the OA-elution time was collected for determination of the amount of radioactivity. With the rest of the IAC eluate the whole procedure was repeated twice. The whole experiment including three IAC-runs was carried out three times.
Statistically significant differences at $p<0.05$ were calculated by applying a one sided t-Test.

4.3.2.3. Testing the radiochemical stability of ^{3}H-Ochratoxin A
2.5nCi of the uniformly labelled OA spiked with 3.4µg of cold OA was mixed either with 2.5ml phosphate buffered saline (PBS) pH 7.4 or with 2.5ml PBS pH 7.4 with 0.1% bovine serum albumin (BSA). These solutions were kept for 1, 4, 7 and 21 days at 37°C. At the end of the incubation time the solutions were acidified with 10µl of conc. HCl and OA was extracted twice with 1ml chloroform. The solutions were then centrifuged for 20 min. at 2000g to separate the phases. Aliquots of the chloroform-phase (100µl) and of the aqueous phase (1ml) were taken out and the amount of radioactivity was determined in a liquid scintillation counter. The percentage of radioactivity found in the aqueous phase was calculated on the base of radioactivity found in the PBS divided by the amount of radioactivity found in the chloroform phase. The cold OA was not analysed.

4.3.3. Experimental design of human studies

4.3.3.1. Intraindividual variation of OA plasma levels
OA plasma levels of 8 volunteers (4 females and 4 males; age 26 to 57) were determined over a period of 8 weeks. In the first week blood samples were taken every second day, and thereafter in weekly intervals. The blood

samples were centrifuged, plasma and erythrocytes separated and stored at -20°C until analysis.

4.3.3.2. Determination of kinetic parameters

3.8µCi ^{3}H-labelled ochratoxin A were dissolved in pure ethanol (corresponding to 395ng ochratoxin A) and ingested by a male volunteer (age: 57). Blood samples (9-18ml) were taken at 7, 13, 24, 36 and 58h and at 3, 4, 6, 9, 13, 20, 33, 54 and 75 days after ingestion. Complete daily urine samples were collected for the first 4 days. Thereafter, urine samples were collected at days when blood samples were taken, with the exception of day 13. The blood was centrifuged, the plasma was separated and the plasma and the erythrocytes were stored at -20°C until analysis. The urine volume was determined and also stored at -20°C until analysis.

0.2-1ml of each plasma sample and 1-2ml of each urine sample was counted in a liquid scintillation counter.

8 plasma samples (0.2-2.0ml) and 4 urine samples (2ml) were analysed by IAC/HPLC as described previously, however, allowing for the following changes: the samples were spiked with cold OA. After the extraction of the plasma with chloroform, 2.5ml of the aqueous phase were taken for analysis of radioactivity. All fractions from the IAC including the PBS/methanol and the water fractions were collected for analysis of radioactivity. From the methanol eluate (3ml) of the IAC 1ml was taken directly for liquid scintillation counting. The other 2ml were evaporated to dryness, dissolved in 100µl methanol and 50µl were injected into the HPLC. The HPLC eluate was collected in 2 minute-fractions and the radioactivity was determined by liquid scintillation counting. The recovery of the cold OA was calculated by integrating the peak area of the HPLC-analysis.

The amount of radioactivity in the erythrocytes was determined by liquid scintillation counting with previous oxidation (Packard Oxidiser 306) of 200-500µl of each sample.

4.3.4. Calculations

The elimination half-life of OA was fitted to the exponential expression

$$y = Ae^{-\alpha t} + Be^{-\beta t}$$

where y is the plasma concentration, A and B are the intercepts on the concentration axis of the residual line and the extrapolated curve of the β–phase (Figure 2), and α and β are the elimination constants of the α- and β–phase respectively. The residual line (Figure 2) resulted from the differences between the measured concentrations during the α-phase and the corresponding extrapolated concentrations of the β–phase. The corresponding half-lives were calculated by

$$T_{1/2\alpha} = \ln 2 / \alpha, \quad T_{1/2\beta} = \ln 2 / \beta$$

The elimination rate constant k_{el} (including the rate constant between the two compartment and the excretion) is described by the following equation:

$$k_{el} = \alpha \beta (A + B) / \alpha B + \beta A.$$

The renal clearance of radioactivity was calculated according the following expression:

$$Cl = (U \times V) / P$$

where Cl is the clearance of the radioactivity, U the amount of radioactivity in the urine, P the amount of radioactivity in the plasma and V the volume of urine per time.

The cumulative excretion of radioactivity in the urine after day 4 was calculated by extrapolation of the concentration of radioactivity found in the urine samples taken during the experiments.

Statistically significant differences of radioactivity counting were calculated by applying a one sided t-Test at $p<0.05$.

4.4. Results

4.4.1. Analytical methods

Levels of ochratoxin A as low as 0.05ng/ml in the plasma could be detected if the plasma extract was previously cleaned by an IAC before injection into the HPLC. The recovery of the IAC with the plasma extract was ≥80%. The IA-columns were regenerated with 20ml of PBS and were used repeatedly up to 10 times without a significant decrease of OA-affinity. The reproducibility within and between the columns was good, as indicated by a maximal standard deviation of 10% of the arithmetic mean of double analyses (range of OA levels measured: 0.1-1.0ng/ml). The detection limit was 0.05ng/ml (S/N=3).

4.4.2. Radiochemical purity of ^{3}H-Ochratoxin A

In the experiment using HPLC only, the peak of radioactivity corresponded with the elution time of the OA-peak. The recovery of the label collected during the OA elution time in the first HPLC-run was 88.1% (based on the amount of total radioactivity injected). 87.9% ± 1.7% (arithmetic mean of 3 injections ± 1SD) of the total radioactivity injected for the second HPLC-run was found after HPLC analysis in the area where OA is eluted. The ratio of radioactivity to unlabelled OA was determined by dividing the dpm collected during the OA elution time through the area under the curve of the OA peak. This ratio remained the same in the two consecutive HPLC-runs.

The results of the three consecutive IAC purification steps are shown in Table 3. In the first IAC purification step 1.7% ± 0.2% (arithmetic mean of the three columns ± SD) of the radioactivity appeared in the PBS/MeOH fraction, whereas in the following two runs only 0.15% ± 0.17% of the radioactivity was found in the corresponding fractions. The amount of radioactivity found in the following water-fractions of the three consecutive IAC-runs remained always the same and no additional purification effect could be seen. However the main part of radioactivity was found in the methanol eluate, where also the cold OA is eluted. To

determine whether or not the behaviour of the labelled OA was identical to that of cold OA in the IAC, the methanol eluate was injected into the HPLC and the ratio of radioactivity to unlabelled OA was determined as described above. Over the three consecutive IAC-purification steps followed by the HPLC analysis this ratio remained stable.

Table 3: Results (recoveries) of the three consecutive IAC-runs for testing the radiochemical purity of the labelled ochratoxin A

	Column 1	Column 2	Column 3
1.Run:			
PBS/MeOH-Fraction	1.7%	1.9%	1.6%
Water-Fraction	7.8%	12.0%	21.4%
Methanol-Eluate	90.6%±2.8	86.0%±1.5	77.10%
HPLC-Analysis (Labelled OA)	69.4%±2.3	70.2%±3.0	69.10%
HPLC-Analysis (cold OA)	86.2%±1.1	92.1%±1.5	82.80%
2. Run			
PBS/MeOH-Fraction	0.1%	0.1%	0.5%
Water-Fraction	8.9%	13.4%	20.7%
Methanol-Eluate	73.7%±0.9	79.4%±3.3	58.0%
HPLC-Analysis (Labelled OA)	57.4%±2.6	53.7%±2.5	47.0%
HPLC-Analysis (cold OA)	80.0%±0.2	72.2%±0.2	56.8%
3. Run			
PBS/MeOH-Fraction	<0.1%	0.1%	<0.1%
Water-Fraction	8.5%	13.7%	17.1%
Methanol-Eluate	68.3%±2.6	55.2%±1.5	38.4%
HPLC-Analysis (Labelled OA)	49.0%±2.8	43.6%±2.4	31.6%
HPLC-Analysis (cold OA)	65.7%±7.0	58.2%±5.8	45.9%

All percentage values are recoveries calculated on the base of the amount of radioactivity and cold ochratoxin A added before the first run of each column was started

4.4.3. Radiochemical stability of ^{3}H-Ochratoxin A

The results of the radiochemical stability tests are shown in Table 4. Each figure represents a single experiment. The amount of radioactivity found in the PBS was between 1 and 3%. Without BSA there was a significant increase of radioactivity in the water found after 7 days (compared to the amount after 1 day) and after 21 days (compared to all other incubation periods). In the solutions with BSA there was no significant increase during the 21 days of incubation. Comparing the solutions with and without BSA at the same incubation time significant differences were seen following an incubation time of 1 and 21 days. The solutions without BSA showed a lower amount of radioactivity after the incubation time of 1 day, whereas after 21 days in the solutions with BSA the amount of radioactivity was lower.

Table 4: Stability of 3H-labelled OA at 37°C and pH 7.4: percentage of radioactivity found in the aqueous phase

time	without BSA	with 0.1% BSA
1 day	1.1%	1.5%
	0.9%	1.6%
4 days	0.8%.	1.8%
	1.7%	1.5%
	1.6%	1.3%
7 days	1.8%	2.2%
	1.8%	1.3%
	1.3%	1.5%
21 days	2.7%	1.7%
	3.0%	2.2%

for significant differences at $p < 0.05$ see text

4.4.4. Intraindividual variation of OA-plasma levels

The results of all 8 persons are shown in Figure 1a and 1b. The levels measured ranged between 0.20ng/ml and 0.88ng/ml. Over all, significant differences between males and females were not observed. The time course of OA in plasma showed an individual pattern in each volunteer. Female 4 and male 1 showed rather constant plasma levels, while in the other males a high plasma OA-variability was found.
In the HPLC chromatograms mainly of female 1, but also in some samples of female 3 and 4 and of male 2, 3 and 4 a second peak was observed. This peak was identified with cochromatography as a diastereomer of ochratoxin A which was already found in coffee (Studer-Rohr et al., 1995). The amount of the diastereomer in the plasma was between 10 and 20% of the measured concentration of ochratoxin A. The calculation of the diastereomer concentration was identical as for OA. For final plasma level determination this amount was added to the calculated OA amount.

Figure 1a: Individual OA plasma levels of the 4 female volunteers

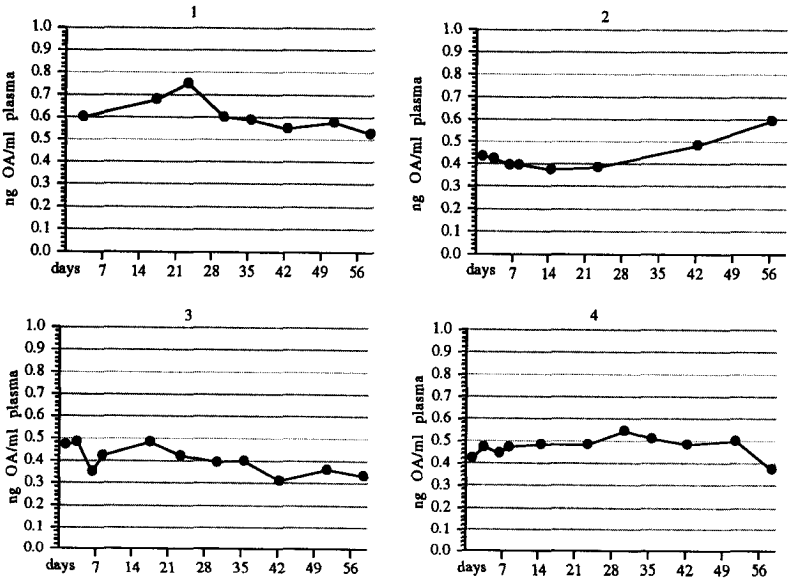
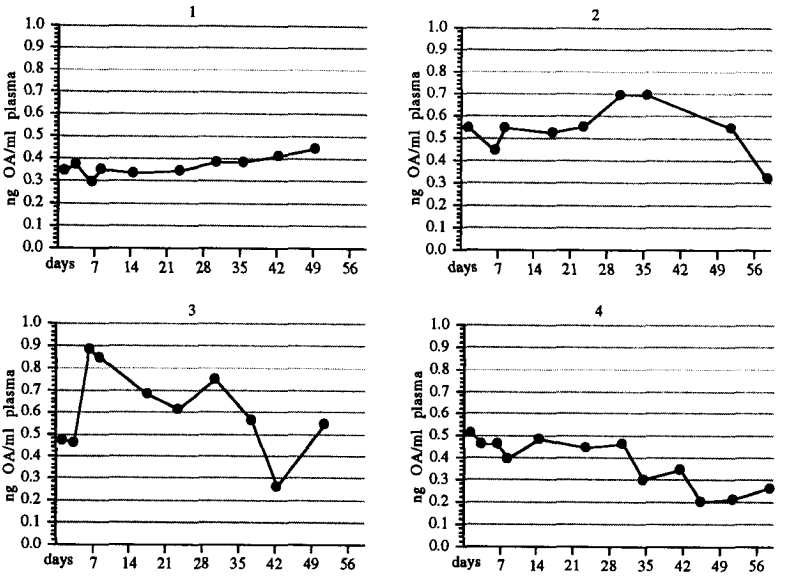


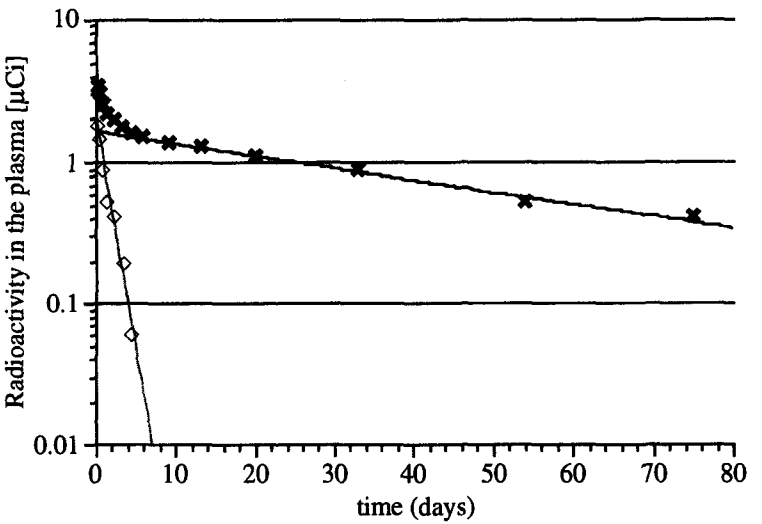
Figure 1b: Individual OA plasma levels of the 4 male volunteers



4.4.5. Determination of kinetic parameters

The change of radioactivity in the plasma after ingestion of 3.8µCi radio labelled OA (=395ng OA) is shown in Figure 2. Already 7 hours after ingestion a maximum level of 90% (=3.44µCi) of the total administered dose was found in the plasma. A fast decrease of the total plasma level from 3.44 to 1.48µCi was seen during the first 6 days. Thereafter only a slow decrease of the plasma level was observed.

Figure 2: Semilogarithmic plot of radioactivity determined in the total plasma (✖) after ingestion of 3.8µCi (=395ng ochratoxin A) suggesting a two compartment, open system. The dashed line (◇) is obtained by the method of residuals (see text)



Based on these observations a two compartment model was postulated. The plasma half-life during the first 6 days (α-phase, Figure 2) was calculated to be around 22 hours. The elimination half-life of the radioactivity during the β-phase (from day 6 on) from the plasma was calculated to be 35.5 days.

To verify, whether or not the measured radioactivity in the plasma consisted of unchanged OA and/or some radioactive metabolites, some plasma samples were analysed by IAC/HPLC. The results are shown in Table 5. No significantly elevated radioactivity levels were found in the aqueous phase after the extraction with chloroform nor could higher radioactivity levels be measured in the PBS/MeOH fraction of the IAC. Between 8.8% and 16.3% of the radioactivity were found in the water fraction. Similar amounts in the water fraction were also found with the standard (Table 3). In the methanol fraction, between 77.6% and 101.1% of the radioactivity was eluted from the IAC. These recoveries were slightly, but not significantly lower compared to the recoveries with standard. After HPLC-analyses between 51.3% and 64.8% of the initial radioactivity was recovered in the fraction, where also the OA is eluted (4'-6'). These values were around 17% lower compared to the results with the standard. However, no elevated levels of radioactivity were found in the other fractions (0'-2, 2'-4', 6'-8', 8'-10') of the HPLC. The OA amounts found in the corresponding fractions of the different plasma samples remained relatively stable over the whole observation period.

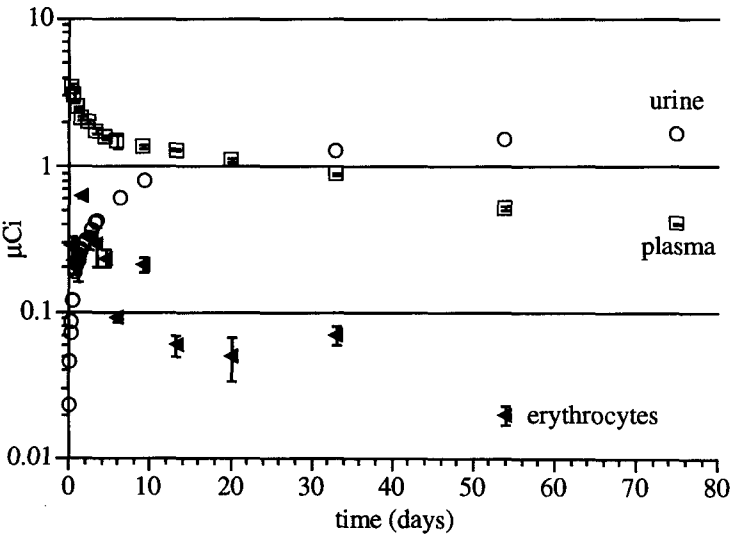
Table 5: Radioactivity measured in plasma samples during IAC and HPLC analyses (bold values indicate the fraction where the OA is eluted)

Time after ingestion	13 hours	3 days	6 days	9 days	20 days	33 days	54 days	75 days
Aqueous phase (after extraction)	<5.0%	<5.0%	<5.0%	<5.0%	<5.0%	<5.0%	<5.0%	<5.0%
IAC:								
PBS/MeOH-Fraction	<5.0%	<5.0%	<5.0%	<5.0%	<5.0%	<5.0%	<5.0%	<5.0%
Water-Fraction	8.8%	11.3%	10.1%	16.3%	10.6%	11.8%	11.0%	9.1%
Methanol-Eluate	**86.1%**	**101.0%**	**82.1%**	**75.4%**	**87.7%**	**77.6%**	**88.3%**	**87.6%**
HPLC (time intervall):								
0'-2'	<5.0%	<5.0%	<5.0%	<5.0%	<5.0%	<5.0%	<5.0%	<5.0%
2'-4'	<5.0%	<5.0%	<5.0%	<5.0%	<5.0%	<5.0%	<5.0%	<5.0%
4'-6'	**64.8%**	**52.8%**	**55.6%**	**51.3%**	**58.6%**	**52.1%**	**61.4%**	**59.9%**
6'-8'	<5.0%	<5.0%	<5.0%	<5.0%	<5.0%	<5.0%	<5.0%	<5.0%
8'-10'	<5.0%	<5.0%	<5.0%	<5.0%	<5.0%	<5.0%	<5.0%	<5.0%
OA-Recovery (cold OA)	130.0%	109.0%	90.0%	81.5%	89.5%	76.5%	94.0%	93.0%

All percentage values are calculated on the base of the initial value

The amount of radioactivity measured in the erythrocytes (after oxidation, recovery from the oxidiser: ≥95%) is shown in Figure 3. In the first 4 days of the experiment the values varied from 0.22-0.63µCi with the highest concentration after 36h. Despite of some fluctuations of the concentrations, the curve form showed a similar shape as the one of the plasma. Compared to the concentration of radioactivity measured in the plasma, the amount found in the erythrocytes was between 4 and 30%. If the peak values are not taken into account, the percentage ranged from 4-10%.

Figure 3: Semilogarithmic plot of the radioactivity determined in the total plasma volume (□), the total volume of erythrocytes (◀) and the extrapolated cumulative excretion of radioactivity via urine (O)



Of plasma and erythrocytes the arithemetic mean of 3 samples ±1SD is shown.

The cumulative excretion of radioactivity in the urine is shown in Figure 3. During the first 6 days of the experiment 0.61µCi were excreted in the urine, whereas in the plasma in the same time span the amount of

radioactivity decreased by 1.96µCi. In the following days (6-75) 1.06µCi were excreted via urine and in the plasma the level decreased from 1.48 to 0.41µCi (=1.07µCi) during the same time. Renal clearance levels of the radioactivity varied in the first nine days between 0.12 and 0.09ml/min. A renal clearance of 0.054ng/ml was calculated at day 33, and at day 53 and 75 it was 0.042 and 0.047ng/ml, respectively.

As with plasma samples, four urine samples were analysed via IAC and HPLC. However, the urine samples appeared to be difficult to analyse with IAC columns: the predominant portion of radioactivity of urine samples was found in the PBS/MeOH fraction, in contrast to plasma samples where most of the radioactivity was found in the methanol fraction. In addition the recovery of cold OA in the methanol fraction was low. Therefore it was tested, whether the radioactivity found in the PBS/MeOH fraction was OA or a metabolite. It was shown that most of radioactivity found in the PBS/MeOH fraction was unchanged OA indicating that the IAC column was not functioning properly four urine sample analysis. The amount of unchanged OA in the urine was, therefore, determined without the use of the IAC. The residue of the chloroform phase was directly taken up in 200µl of methanol and 50µl were injected into the HPLC. The results of two extractions of each urine samples and the HPLC-fractions are shown in Table 6. Clear differences compared to the results with plasma can be seen. Already in the aqueous phase between 33% and 38% of the radioactivity were found. To test whether the radioactivity in the aqueous phase was tritiated water or a metabolite, 2ml urine of two samples were lyophilised and the radioactivity of the residue as well as of the collected water was measured. The radioactivity found in the residue was 93 and 87% respectively, whereas the amount of radioactivity in the water was 2.2% and <4.5%.

In the HPLC between 42% and 54% of the radioactivity was eluted with the OA. In contrast to the findings in plasma where no elevated levels of radioactivity were found in other fractions than the OA-fraction, in the time interval before the OA (2'-4') between 14% and 20% of the radioactivity was detected. The amounts found in the corresponding fractions of the analyses of the different urine samples remained relatively constant over the 9 days analysed.

Table 6: Radioactivity measured in urine samples after extraction and HPLC analyses (bold values indicate the fraction where OA is eluted)

Time after ingestion	1 day	2 days	6 days	9 days
Aqueous phase (after extraction)	37.8%	34.8%	32.5%	36.3%
HPLC (time intervall):				
0'-2'	<7.0%	<7.0%	<7.0%	<7.0%
2'-4'	19.5%	20.4%	14.7%	13.8%
4'-6'	**42.3%**	**44.5%**	**53.7%**	**49.9%**
6'-8'	<7.0%	<7.0%	<7.0%	<7.0%
8'-10'	<7.0%	<7.0%	<7.0%	<7.0%

All percentage values are calculated on the base of the initial value

4.5. Discussion

4.5.1. Analytical methods

The detection of OA via IAC/HPLC in plasma samples is a relatively simple, and very sensitive method. However, for urine samples it could not be used, as recoveries varied widely and unsystematically. This might be explained that some components of urine may block the antibodies of the column. Biological samples with varying compositions, which have to be analysed for OA via IAC should, therefore, first be tested with spiked samples.

4.5.2. Radiochemical purity of ^{3}H-Ochratoxin A

In two consecutive HPLC runs the ratio of labelled to unlabelled OA remained stable. Therefore, it was concluded that the labelled OA was, when tested with this HPLC-method, radiochemically pure.

The results of the three consecutive IAC runs were interpreted as followed: the 1.7% of radioactivity that appeared only in the PBS/methanol fraction of the first IAC run but not in the two following runs, indicated that a maximum of 2% of the radioactivity did not consist of labelled OA. The amount of radioactivity lost with the water on the IAC remained the same over three consecutive IAC purification steps and was not considered to be an impurity but an incomplete chromatographic separation. This was confirmed by the fact that the ratio of radio labelled to unlabelled OA remained the same in the three consecutive IAC runs followed by HPLC analysis. Thus, the radiochemical purity of OA can be demonstrated also with the IAC/HPLC method. It was therefore concluded, that with the methods available no significant radiochemical impurities could be detected. The labelled OA was considered radiochemically pure enough to be used for the kinetic study without previous purification steps.

4.5.3. Radiochemical stability of ^{3}H-Ochratoxin A

The observed amount of radioactivity in the aqueous phase was small and the loss of radioactive OA during the 21 days was considered as insignificant for the experiment presented. The addition of BSA to the PBS did not seem to have an influence on the stability of OA up to 7 days. The lower amount of radioactivity found after one day of incubation in the PBS without BSA was probably due to incomplete separation of the PBS and the chloroform phase of the PBS/BSA solution, as the separation of this mixture turned out to be difficult. Only after 21 days a significantly higher stability of the labelled OA was found in the PBS solutions with BSA compared to those without BSA. This could be due to the fact, that the proteins stabilise the OA molecule, which otherwise degrades in alkaline medium and in the presence of water (personal communications: T.J. Jansen, Vicam, USA; R. Dick, BAG, Switzerland). As in human plasma OA is also bound to proteins, the radiochemical stability of the labelled OA was found to be sufficient for our purposes.

4.5.4. Human experiments

4.5.4.1. Intraindividual variation of OA-plasma levels

The levels and frequencies of OA found in the plasma of the eight test persons are in line with the data found by Zimmerli et al. (BAG, 1994) and by Breitholz-Emanuelsson et al. (1993 and 1994) (Table 1). In these three studies all of the samples contained OA. The higher percentage of OA positive samples found in these three studies, compared to the other studies listed in Table 1, was most probably due to improved analytical methods with lower detection limits.

An interesting finding was the high variability of OA-plasma levels in some of the volunteers during the 8 week observation period (Figure 1a and 1b). Thus for determination of representative individual OA-plasma levels a single analysis is not sufficient. Peak OA-plasma concentrations may be induced by small quantities of food items containing high levels of OA. For example in male 3, the steep increase of 0.44ng OA/ml plasma between day 4 and 7 was probably due to the consummation of dried fruits. These are known to contain punctually high concentrations of OA (Baumann and Zimmerli, 1988; Steiner et al., 1993; Zohri and Abdel-Gawad, 1993). Other reasons for a steep increase or for differences between individuals in OA plasma levels can not be given as the 8 volunteers did not keep a diary about their nutrition. Relatively steep drops in OA plasma levels as shown by male 2, 3 and 4 and by female 3 and 4 can be explained by distribution (as seen in the α-phase, see below) of OA into the organs after ingestion of OA.

A diastereomer of OA was detected and identified by Rásonyi et al. (1995b). The detection of this diastereomer of ochratoxin A in food items was first described by (Studer-Rohr et al., 1995). It was found in roasted but not in green coffee beans. As the diastereomer was also found after heating pure OA, the conclusion was drawn, that the diastereomer is formed under the influence of heat. It can, therefore, theoretically be present not only in roasted coffee but also in other food items treated with heat before consumption. The detection of the diastereomer also in human plasma is described in this paper for the first time. As the diastereomer was also detected in plasma of volunteers which do not (or only rarely) drink coffee, other dietary sources than coffee must be present. However the

person with the highest levels of diastereomer (female 1) is a strong coffee consumer.

4.5.4.2. Determination of kinetic parameters

98% of the administered radioactivity was found in the plasma and erythrocytes 7 hours after ingestion. This high absorption level could be explained by the administration form: the rapid and complete absorption of pure ethanol. It was therefore not possible to estimate - by this experiment- the percentage of OA which is absorbed when OA is ingested as food contaminant. Hagelberg et al. (1989) measured the bioavailability in several species. Considerable species differences were seen. The relative bioavailability for mouse, rat and monkey was 97%, 44% and 57% respectively when the OA was administered orally versus. i.v. dissolved in 51mM $NaHCO_3$.

The curve form of the measured radioactivity in the plasma indicated a two compartment model. In the α-phase, which lasted about 6 days, the plasma level decreased rapidly for 1.96μCi, whereas in the same time span only 0.61μCi where excreted via urine. The amount of OA excreted via faeces for this time span is unknown. However, it is assumed that not all the OA was excreted but that an unknown amount was distributed within the body. In the β-phase (69 days) the plasma level decreased for 1.07μCi. The elimination half-life (β-phase) was calculated to be 35.5 days. This is in line with the findings of (Hagelberg et al., 1989) in monkeys. They found an elimination half-life of 21.3 days upon oral administration and 35 days for i.v. administration. The long elimination half live can be explained by a high affinity of OA to plasma proteins (Galtier et al., 1980; Hult and Fuchs, 1986).

A high percentage of radioactivity that was measured in the plasma could be identified as ochratoxin A. No significant decrease of the recovery of radioactivity was found after the IAC when compared to the results with the standard. A significant decrease of the recovery of radioactivity after the HPLC analyses (compared to the analyses with the standard) of 17% was calculated. It is therefore possible, that low concentrations of metabolites were not detected. Nevertheless it can be concluded, that over 80% of the radioactivity measured in the plasma was ochratoxin A and that

the concentration of metabolites was low. These values remained unchanged over the whole observation period (day 1-75).
The concentrations of radioactivity found in the erythrocytes was only around 5-10% of the radioactivity found in the plasma. This illustrates, that over 90% of the OA in the blood is found in the plasma.
Whereas in the plasma no metabolites were detected and over 80% of the radioactivity could be identified as OA, in the urine only between 42 and 54% of the radioactivity was recognised as unchanged OA. Around 33% of the radioactivity was found in the aqueous phase after extraction and around 14-20% in the HPLC fraction before the OA was eluted (2'-4'). In the four urine samples analysed (at day 1, 2, 6 and 9) these values remained relatively constant and no big differences between the samples of the different time points were seen. Urine samples from later time points could not be analysed via HPLC, as the amount of radioactivity was too low. The metabolites were not further identified, but it was shown, that the radioactivity found in the aqueous phase was not tritiated water. The fact, that no metabolites were detected in the plasma, whereas around 50% of the radioactivity in the urine was not unchanged OA, could be due to either concentration of the metabolites in the urine because of renal filtration of the blood or/and due to metabolisation of OA in the kidney. The metabolisation of OA to 4-hydroxy-OA was shown to occur at least in rat kidneys via cytochrome P-450 (Stein et al., 1985; IARC, 1993).
The renal clearance of radioactivity during the elimination phase was very low and was calculated to be about 0.048ml/min. The renal clearance of OA was calculated to be, depending on the amount of unchanged excreted OA, between 0.014-0.024ml/min. Hagelberg et al. (1989) postulated a renal clearance in humans of 0.033ml/min. This value was estimated based on the rate of filtration in the glomeruli for inulin and the free fraction of OA measured in human plasma.
The finding, that in the β-phase, the plasma level decreased by the same amount as radioactivity was excreted via urine, indicated, that most of the OA and its metabolites were excreted via urine and that the biliary excretion was negligible. In addition Hagelberg et al. (1989) found that in monkeys the excretion via urine corresponded to the total clearance. This result stands in contrast to other species. For example in mice and rats, which show half-lives of 40 and 120h respectively, it was found that only

about 10% of the OA was excreted through the urine (Hagelberg et al., 1989).

Based on the experimentally determined half-life and the estimated body burden, the daily intake can be calculated using the equation:

$$\text{uptake} = (k_{el} \times \text{body burden}) / f \quad \text{(Klaassen, 1986).}$$

This equation shows the relationship between continuous intake (ng/day) which equals the daily excretion if the body is in a steady state. Besides the elimination constant (k_{el} = 0.0426) and the fraction absorbed (f) also the body burden has to be taken into account. The body burden in humans was estimated based on OA concentration relationships found in the different organs of the swine thus assuming a similar organ distribution of OA in humans and pigs. The following relative OA organ concentrations expressed as % of the serum value were found in pigs (Kühn, 1993): 7.3% in kidney, 6.9% in liver, 4.7% in fat and 3.8% in muscle. If one calculates with an average plasma level of 0.4ng/ml in humans, the resulting body burden is approximately 2µg. Assuming that 100% of the ingested OA is absorbed the estimated daily intake is 85ng/person or 1.2ng/kg bw. This daily intake calculated based on body burden and the elimination constant is very well in line with the daily intake calculated on the basis of the contamination found in food items, which was estimated to be between 80-160ng (DFG, 1990; Studer-Rohr et al., 1995).

The half-life of OA in rats was between 56 and 120h (Galtier et al., 1979; Ballinger et al., 1986; Hagelberg et al., 1989). The half-life determined in humans is therefore 6-13 fold longer than in rats. If rats are fed 35µg OA/kg bw for 4 weeks, plasma levels between 1.06µg/ml for females and 1.37µg/ml for males are found (Rásonyi et al., 1995a). When these plasma levels are extrapolated linearly to the average plasma levels found in humans (≈0.4ng/ml) a daily intake of 12ng OA/kg bw would result. This is 10 times higher than it is calculated for humans. Considering, however, that the plasma half-life of humans is 10 fold longer than that in rats, a good correlation results.

This fact has to be considered, when risk extrapolations from rats to humans are made. It signifies, that when the data of Boorman (1989) for kidney tumors in rats are extrapolated linearly, the estimated tumor risk has

to be multiplied by a factor 10. Therefore, a daily intake of 1.2ng/kg bw for humans, as calculated above, would then result in a life time kidney tumor incidence of $75/10^6$, thus, clearly exceeding the general accepted risk of 1 additional tumor $/10^6$ lives. For the linear extrapolation as used here, the same tumor induction mechanisms and the same sensitivity of cells to OA in rats and humans are assumed. In light of the fact, that species and sex differences regarding tumor induction in rats and mice vary by a factor of 20-100, the latter assumption is highly questionable.
However, as long as the mechanism(s) of tumorgenesis are still unknown, it can not be evaluated how high the risk of kidney tumor for humans induced by OA really is. The exposure of humans to OA should therefore be kept as low as possible.

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5. General conclusions

This work was initiated with the aim to increase the knowledge on OA in order to provide a better data base for human risk assessment.

In the first part it was shown, that OA is frequently found in food items also in Switzerland. A main source for human exposure are cereals and cereal products. The daily intake of OA via food items was previously calculated to be around 80ng/person based on a large data base on OA contamination in cereals and meat (DFG, 1990; Rühl et al., 1992). The investigations on coffee revealed that OA is very heat stable and that coffee consumption may contribute significantly to human OA exposure. For regular coffee drinkers the daily intake is therefore probably increased by approximately 25ng, depending on the daily quantity consumed. However, the daily intake can vary considerably due to the inhomogenous distribution of OA contamination.

An other approach to estimate the daily intake was using human plasma levels. The ubiquitous occurrence of OA in food items was demonstrated by the fact, that OA could be detected in most of the human blood samples in European countries when sensitive analytical methods are used. The mean concentrations found in blood samples were around 0.2-0.6ng OA/ml. The half-life of OA was determined to be 35.5 days. This is in line with the long half-life that was found in monkeys (Hagelberg et al., 1989) but it demonstrates that, compared to rats, the half-life is ten times longer and, therefore, the systemic exposure in humans may be significantly higher. In conjunction with an estimated body burden of about 2µg (based on a mean plasma concentration of 0.4ng/ml) a daily intake of 85ng/person was calculated.

The daily intake estimated by two independent approaches showed a very good agreement. It can therefore be concluded that the main sources of OA for human exposure are presently known.

In order to set the daily intake of 1-2ng/kg bw into perspective with the doses known to cause cancer in rodents a risk extrapolation for humans was carried out.

The IARC (International Agency for Research on Cancer) classified OA as possibly carcinogenic to humans (Group 2B). Although there is no clear evidence that OA is carcinogenic for humans, Kuiper-Goodman and Scott

(1989) calculated an acceptable daily intake and a virtually safe dose based on the data of the 2 year carcinogenicity study with rats (Boorman, 1989). Applying a safety factor of 5000 to the no observed effect level (NOEL) an acceptable daily intake (ADI) of 4.2ng/kg bw was calculated. If, on the contrary to the latter model, the lowest dose level where tumors were found, was extrapolated linearly down to the virtually safe dose (VSD: 1 additional tumor / 10^6 lifes) a value of 0.2ng/kg bw/day was obtained.
The ten times longer half-life of OA in humans compared to rats and the probably ten times higher plasma levels in humans compared to rats at the same dose levels (Rásonyi et al.) is an additional point, that has not yet been taken into account for human cancer risk assessment. It is important to consider this fact in light of the exposure of OA to the target organ. Risk extrapolations are usually based on the oral intake. In case of large species differences, however, the levels measured in the plasma are more valid. Hence based on the kinetic data in humans the systemic dose can be determined, thus indicating a more precise dose measurement than the oral intake would give. It signifies, therefore, that the VSD would decrease by a factor of ten to 0.02ng/kg bw/day. For the NOEL/safety factor model it has no consequences as this approach does not consider any dose-effect relationship nor any kinetic data.
The NOEL/safety factor approach is usually considered to be more adequate when the carcinogen is known to cause no irreversible damages to the DNA at low doses and a NOEL can therefore be established. But this approach is only valid when a non-linear dose effect curve exists and based on the known mechanism a functional threshold (NOEL) can be determined.
The VSD approach is used for carcinogens which react with DNA. For such compounds theoretically also at very low doses a biological effect can not be dismissed. As the mechanism(s) of tumorgenesis for OA are presently not known it seems to be questionable if the NOEL/safety factor approach can be used at the moment. The large species and sex differences however indicate, that a direct genotoxic mechanism is very unlikely. On the other hand low numbers of DNA adducts were found by ^{32}P-postlabelling in mice and humans (Pfohl-Leszkowicz et al., 1991; Pfohl-Leszkowicz et al., 1993a; Pfohl-Leszkowicz et al., 1993b). It seems,

therefore, to be crucial to know more about the mechanism of action of OA.

Our results could contribute to elucidate the systemic exposure of OA to humans. But, as long as the mechanism is unknown, it remains difficult to predict more precisely the carcinogenic risk of human OA exposure.

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Curriculum vitae

1966	Born in Lucerne on the 7th of April, as youngest daughter of 4 children of Madeleine and Klaus Rohr-Rast
1972-78	Primarschule (Elementary school) in Lucerne
1978-85	Kantonsschule Luzern, completed with the Matura, typus B
1985-91	Studies in Food Engineering at the Swiss Federal Institute of Technology at Zürich Diploma Thesis on "Replacement of the Mouse Bioassay for the Analysis of PSP-Toxins (Saxitoxin) in Mussels and Preserved Mussels by a Physicochemical Method" at the Institute of Toxicology in Schwerzenbach/Zürich supervised by Prof. Ch. Schlatter
1987	Practical studies at the Milchhof Emmen
1987	Practical studies at the Kantonales Laboratorium der Urkantone
1990	Practical studies at the Latinreco Nestlé SA, Quito, Ecuador
1992-95	Carried out the work presented in this Doctoral Thesis at the Institute of Toxicology
1992	Marriage with Bernhard Studer