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Citrinin*)

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Summary: Citrinin, a nephrotoxic mycotoxin, has been of growing importance also for the "International Agency for Research on Cancer", ever since its presumable role in the occurrence of Balcan endemic nephropathy (BEN) was discussed at the congress on "Mycotoxins, Endemic Nepthropathy and Urinary Tract Tumours" held in Lyon in June 1991 (12). In late 1991, citrinin was therefore also included in the list of toxins to be examined by the screening subcommittees on natural toxins of the International Live Science Institute, European Branch.

Zusammenfassung: Es wird eine Übersicht zu dem Mykotoxin Citrinin gegeben. Dabei werden die Citrininbildner und ihre Begleittoxine, Bildungsbedingungen, das natürliche Vorkommen, der Einfluß der Be- und Verarbeitung sowie die Kinetik und die Toxikologie berücksichtigt. 106 Literaturzitate.

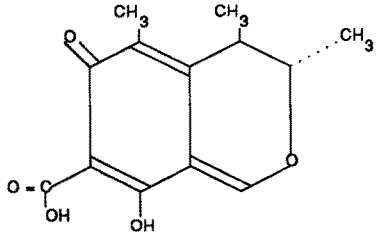
Key words: mycotoxins; citrinin; citrinin producers; biological effects; natural occurrence

Schlüsselwörter: Mykotoxine; Citrinin; Citrininbildner; biologische Wirkung; natürliches Vorkommen

Chemical data

IUPAC Systematic Name: (3R,4S)-4,4-dihydro-8-hydroxy-3,4,5-trimethyl-6-oxo-3H-2-benzopyran-7-carboxylic acid.

Structural formula:



Molecular formula: $C_{13}H_{14}O_5$ Mol.wt: 250.25
Description: yellow, odorless, crystalline solid.
Melting point: 170–173°C.
Solubility: practically insoluble in water; soluble in ethanol, dioxane, dilute alkali, acetone, benzene, and chloroform.
Spectroscopy data: see IUPAC (43).

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Citrinin producers and concomitant toxins

In searching for antibiotic natural substances Raistrick and Hetherington (74) isolated citrinin in 1931 from cultures of *Penicillium citrinum*. However, citrinin was never used as an antibiotic because of its toxic effects on vertebrates. Since then, many other *Penicillium* species and some *Aspergilli* have also been found to produce not only citrinin, but some other toxins as well, if conditions are favorable (see Table 1).

Table 1. Other mycotoxins, produced by citrinin-synthetizing molds

Species	Mycotoxin	Ref.
Aspergillus candidus	Kojic acid Xanthoascin	19, 80
A. carneus	?	80
A. egypticus	?	23
A. flavipes	?	8
A. niveus	?	80
A. terreus	Terreic acid Patulin 6-Hydroxymellein Geodin Geodoxin Acetylaranotin	19, 80
Penicillium canescens	Rugulosin Penicillic acid Penitrem A	80
P. citreoviride	Citreoviridin	80
P. citrinum	Kojic acid	80
P. claviforme	Patulin	80
P. cyclopium = *P. aurantiogriseum*	Ochratoxin A Cyclopiazonic acid Patulin Penicillic acid Viomellein Xanthomegnin Rugulosin	17, 28, 80
P. expansum	Curvularin Penicillic acid Patulin	56, 65, 80
P. fellutanum	Carlosic acid Spinolosin	80
P. implicatum	?	80
P. janthinellum	Penicillic acid Penitrem A	8, 80
P. jenseni	?	80
P. lividum	Penicillic acid	80
P. notatum	Roquefortine	80
P. palitans	Ochratoxin A 4-Hydroxy-ochratoxin A Penicillic acid Penitrem A, B, C	17, 28, 80

P. purpurrescens	Ochratoxin A Penitrem A	17, 28
P. roqueforti	Penicillic acid Patulin Fumigaclavin A Roquefortine PR-Toxin	8, 80
P. spinulosum	Penitrem A Spinulosin	80
P. steckii	Curvularin A	46, 80
P. velutinum	?	80
P. viridicatum	Ochratoxin A Penicillic acid Viomellein Xanthomegnin Oxalic acid Cyclopiazonic acid Penitrem A Griseofulvin	17, 19, 28, 51
Pythium ultimum	?	8

Taxonomy according to Raper and Fennell (75), and Raper and Thom (76), resp.

Among these concomitant toxins, which are exclusively nephrotoxins, penicillic acid has been found eight times, ochratoxin A four times, and viomellein and xanthomegnin twice (Table 1). These findings suggest the probability of additive and even superadditive effects of natural contaminations of foods and feedstuffs.

Aspergillus candidus, with a minimum a_W of 0.75, belongs to the xerotolerant species and hence is of some importance as a parasite on stored produce. In temperate and cool climates, *A. terreus*, *Penicillium cyclopium*, *P. expansum*, *P. roqueforti* and *P. viridicatum* are ubiquitous fungi on agricultural produce, but also on treated and processed food. Some of them even belong to the normal "refrigerator flora (81). All species listed in Table 1 have been found in soil; optimal conditions for growth on organic substrates and for mycotoxin production are air humidities of 70 % and more, while humidities from 65 % on allow retarded growth only.

However, as many authors report, the ability to produce the above-mentioned mycotoxins simultaneously or at certain quantities is not common to all isolates of the fungal species mentioned. This failure is due to the genetic lability of the asexually multiplying molds (*Deuteromycotina*) and to their ability to exchange nuclei with different genetic information through anastomoses among individuals of one species.

Conditions of citrinin production

Citrinin production is dependent upon the growth of one or several of the fungal species listed in Table 1. All these species are so-called storage fungi, infesting the produce superficially with conidia, while the fungal mycelium develops mainly under unfavorable storage conditions. In the initial phase after germination of conidia no mycelium grows. It is only in the so-called idiophase that secondary metabolic products form under favorable nutritional conditions provided by

stored food and feedstuffs, and in the presence of sufficient water and appropriate temperatures. However, the parameters humidity and temperature are controllable by storekeepers (see Table 2). The limits for fungal growth are usually wider than those for fungal mycotoxin production.

Table 2. Conditions of growth and citrinin production of some species (81)

Species	Temp. (°C)		Water activity (a_w*)
	Min.	Opt.	Min.
A. candidus	3–4	20–24	0.75
P. citrinum	5–7	26–30	0.80
P. cyclopium	–2	25–30	0.85
P. expansum	–3	25–26	0.82
P. viridicatum	–2	21–23	0.81

* a_w = equilibrium water content. Water content of a product which depends on the contents of fat, protein and carbohydrates, and which is in equilibrium with a certain relative humidity ($a_w \times 100$).

According to Takahashi et al. (93), who studied *Penicillium citrinum* in parboiled rice, appearance of fungal mycelium and mycotoxins after 14 days' incubation seems to be limited to the external layers of the grains.

It is interesting that the individual mycotoxins produced by a species may be controlled by different parameters. *P. viridicatum*, for example, has been found to grow well at 85 % relative humidity (r.h.), but to produce only ochratoxin A and penicillic acid, while at 90 % r.h. citrinin is produced in addition (66). In storage tests of durum wheat containing 15 % and 19 % water, citrinin production began after 24 weeks, reached its maximum of 80 mg/kg after 48 weeks, and decreased to 20 mg/kg by the end of the 60th week (3).

The presence of other fungal species may be of influence as well. Pravindra et al. (73) report that *P. citrinum* fails to produce citrinin in the presence of *A. niger* and/or *Trichoderma viride*, while normal citrinin biosynthesis is observed if other fungal species are present. These observations, although very interesting, are of little practical importance, as the fungal flora changes in its composition, and as temperature and humidity are subject to daily and seasonal fluctuations. But they help to explain the highly inhomogeneous distribution of mycotoxins in granular bulk material.

From the values listed in Table 2 those storage conditions may be derived which have to be observed in order to prevent fungal growth and mycotoxin production in stored produce.

Natural occurrence of citrinin

Sterile food inoculated with citrinin producers, but also artificial media contained citrinin after some time. This shows that this mycotoxin may, in principle, occur in all foods or feedstuffs infested with the corresponding fungi. However, the number of investigations dealing with natural occurrence of citrinin is small as compared to studies concerning other mycotoxins, and available data are far from being systematic.

Food of vegetable origin

Reports on citrinin in cereals and cereal products are available from Denmark (52), Canada (89), Tunesia (6), India (68), and Switzerland (18). The latter only includes information about quantities: 11 out of 21 flour samples tested contained 0.2–1 μg/kg citrinin (mean value: 0,6 μg/kg); two out of four samples of durum wheat contained 0.3 and 0.7 μg/kg, resp.; one of two samples of pasta contained 0.5 μg/kg; five samples of wheat bran, and two each of maize semolina, oats, rice, and barley were found negative (limit of detection: 0.1 μg/kg). In Germany, citrinin was detected in various kinds of bread in the lower ppb range (81).

In Japan, Takahashi et al. (93) found citrinin in rice and rice flour, and Nishijima (67), who investigated 53 samples of commercial cereal products withdrawn in and around Tokyo, detected 27 μg/kg citrinin in one sample of maize flour. Fourteen positive samples containing up to 1390 μg/kg were found among 23 samples of imported maize kernels and maize flour withdrawn at wholesale establishments. Tu et al. (96) reported on citrinin in China in soy sauce which had been prepared using uncontrolled starter cultures. Citrinin together with aflatoxin was found also in peanuts, and in coconut products from India (90, 55).

Cerutti et al. (13) did not find citrinin in 23 samples of imported beer; this may be explained by the fact that during the first 5 days of barley germination eventually present mycotoxins are destroyed (22, 82).

Harwig et al. (37, 38) detected citrinin, besides patulin, in apples with brown rot (*P. expansum*), but not in apple juice and other apple products; this may be due to the instability of citrinin.

At the 10th AOAC Congress in 1987 on the worldwide occurrence of mycotoxins in food and feeds, Jelinek et al. (45) reported that citrinin, as concomitant toxin of ochratoxin A, was frequently found in many products, but that data about quantities were not available.

Food of animal origin

Mycotoxins may get into meat, offals, blood, milk, and into products made of these in two different ways. The products start molding and the toxins diffuse into the products. The second way, called carry-over, is by mycotoxin-containing feed; the mycotoxins are stored in the animal organism for varying lengths of time until they degrade or are excreted with feces and/or urine, partly also with milk and, in the case of poultry, with eggs.

Citrinin producing fungi were frequently isolated from meat products and sausages. Ciegler et al. (14) inoculated (raw) sausages with five citrinin-positive isolates, but failed to detect the toxin in the product after the usual maturing time of 10 weeks. In a similar experiment with country cured ham, however, Wu et al. (104) found small citrinin quantities at 15°C and considerable ones at 25–30°C, while the five strains of *P. viridicatum* which the investigators used failed to grow on this substrate at 10°C. Leistner and Eckardt (57) therefore recommend temperatures of 15°C and less for storage and maturation of products which are susceptible to molding. The authors emphasize, however, that citrinin formation is possible on every meat and should be regarded as serious.

In England, Jarvis (44) investigated 19 moldy commercial cheeses; 15 of them contained citrinin up to 50 μg/kg as well as ochratoxin A. Due to diffusion the concentrations were higher in the cheese than on its surface.

Contamination by carry-over was studied in hens by Abdelhamid and Dorra (2), who fed poultry feed containing 100 μg/kg citrinin over 6 weeks. After this time they detected 10 μg/kg in muscles and egg yolk and 6 μg/kg in the albumen. It should be underlined, however, that in practice such concentrations are extremely rare and certainly not fed over weeks. Comparable experiments in mammals, as far as we know, have not been published.

Feeds

Feeds, usually less carefully stored than food, are less protected against uptake of water from the atmosphere. This applies primarily to feed produced at the farm, but also to commercial feed stored by the farmer. Inadequate storage facilitates the infestation by fungi listed in Table 1 and contamination by mycotoxins. Citrinin in feedstuffs is of secondary importance, however; contamination of poultry meat and eggs is limited to extremely rare cases (see the preceding section).

In the United States, Stahr et al. (92) investigated pig feed as cause of ochratoxin A intoxication. They found citrinin nearly as often as ochratoxin. Gedek (28) reports on studies in Denmark where ochratoxin A was found in 58 % of barley samples (mean value: 3 mg/kg) and citrinin in 9 % (mean value: 1 mg/kg). In Egypt, Abdelhamid (1) detected citrinin in 15 % of 52 feed samples (mean value: 26 μg/kg), with maximum citrinin contents found in fish meal (40–70 μg/kg).

However, all these concentrations are far below those experimental values which induced nephropathy in pigs (200 μg/kg) (27, 52).

Occurrence in humans

Reports on the occurrence of citrinin in the human organism are not available.

Influence of treatment and processing of the content of citrinin

Citrinin is more susceptible to heat than other mycotoxins (85). Its effectiveness, in terms of antibiotic activity, decreased after 15 min at 100°C and pH 2–9.5, with degradation beginning at 60–70°C. Also, UV light has been found to reduce its activity to some extent (81). In terms of its pharmacological effect on young hens, heat treatment of 100°C for 8 min was little effective, while no diuretic effect was observed any more if citrinin had been exposed to 105°C for 16 min (50).

Citrinin in stored crops is not stable. Its degradation rate has been found to increase with increasing water content. Harwig et al. (37) have shown that in barley, maize, and wheat, at a_W of 0.70 and 25°C, the half-life values were 7.8, 15.5, and 11.9 days, resp., at a_W of 0.90, 1.8, 10.4, and 3.0 days only, however. The authors assume this to be due to reactions with SH groups, similar to patulin or penicillic acid. This assumption was refuted by Ciegler et al. (14). Glucosidation in living vegetable tissue, as in the case of zearalenone, would be another explanation; zearalenone has been found to escape analytical detection by glucosidation, but does not lose its toxicity.

During malt production (1 mg/kg citrinin in barley) and brewing of beer citrinin and ochratoxin A have been found to degrade (53).

Citrinin kinetics and metabolism

Thirty minutes after i.v. administration of 3 mg/kg body weigth in rats, 15 % of the citrinin dose was found in the liver and 6 % in the kindeys; after 6 h, these

levels decreased to 8 % and 5 %, resp. 74 % was excreted with the urine during the first 24 h. Four percent and 11 % were found after 24 and 48 h, resp., in the feces (71). According to Reddy et al. (77), citrinin is excreted in two phases of different rates, one resulting in a biological half-life value of 1.95 h, the other of 39.7 h. After i.v., i.m., or s.c. application to rats, citrinin was still detected in the blood as late as after 24 h. After oral application, maximum serum levels were observed after 3 h which subsequently decreased quickly. About 20 % are bound to serum albumin (16, 84).

Of a single oral dose of 30 mg citrinin/kg body weight administered to rats, 15.4 % was metabolized into dihydrocitrinone, while 42.5 % was found unchanged in urine and blood plasma (21). However, *Penicillium viridicatum* was found to produce the same metabolite after extended growth in culture broth, which was subsequently converted by the fungus into ochratoxin A (69).

Reddy et al. (77) found three metabolites of higher polarity than that of citrinin in pregnant rats; these metabolites, in contrast to the mother compound, were not detected in the fetuses.

Toxicology of citrinin

Citrinin has been classified as toxic to moderately toxic. LD_{50} after oral application to rats is 50 mg/kg body weight, to mice 112, golden hamsters 67, and guinea pigs 43 mg/kg (8, 42, 80).

The citrinin action in the cell is characterized by accumulation in the mitochondria and interference with the electron transport system (4, 35, 36). The process, which depends on the pH and does not affect the permeability of the cell membrane, leads to an inhibition of the synthesis of DNA and, subsequently, of protein and RNA as well (15, 63, 105, 106).

Nephrotoxicity

Citrinin has nephrotoxic effects. Response of the animals investigated was similar as far as the renal sites of citrinin action and susceptibility of the different cell kinds are concerned (105, 106). Damage to the renal tubuli in the dose range of 20–50 mg/kg body weight were observed in mice, rats, guinea pigs, rabbits, dogs, and pigs (11).

After a single i.p. application of 50 mg/kg body weight to rats, mainly cytoplasmic vacuolization of epithelial cells of proximal convoluted tubuli was observed after 24 h, followed by multifocal areas of necrotic proximal convoluted tubuli after 48 h. After 72 h, regeneration of the damaged cells began and was nearly completed after 96 h (60). Similar results have been reported by Jordan et al. (47), and Phillips et al. (72). Urinary excretion increased with decreasing osmolality (7), a reaction observed in hens as well (29).

Phillips and Hayes (70) report an increase in kidney weight of mice during the first 2 days after i.p. application of 35 mg/kg body weight, which was accompanied by decrease of DNA, protein, and later, also RNA. Similar results were obtained by Jordan et al. (48) in golden hamsters, by Thacker et al. (94) in guinea pigs, and by Hanika et al. (33, 34) in rabbits.

In young hens (24–28 days old) citrinin added to the feed caused a dose-dependent increase of water uptake and urinary excretion at doses from 3.86 mg on during 4 h (50). After renal portal infusion of 0.2 ml of citrinin solution con-

taining 200 and 800 mg/kg citrinin, Hnatow and Wideman (40) did not find histopathological changes, but a dose-dependent increase of urinary excretion and simultaneous decrease of osmolality. The effects of citrinin were reversible.

The nephrotoxic effects of citrinin are characterized by kidney enlargement, degeneration of the tubuli with subsequent cortical fibrosis, and functional decrease of tubular activity (42).

Genotoxicity and other short-term tests

In the rec assay in *Bacillus subtilis* citrinin still produced a positive reaction following 20 μg/disc (97), while Manaba et al. (61) did not find any response to 10 and 100 μg/disc. The Ames test in *Salmonella typhimurium* was negative up to 400 μg/plate (24, 54, 98, 102). Thust and Kneist (95) describe citrinin as a potent inductor of chromosome aberrations after metabolic activation of liver microsomes of humans and rats; sister chromatid exchanges were not found, however. Martin et al. (63) report single- and double-strand breaks in the DNA of intact cells of *E. coli*. Base substitution mutations have also been found (9).

Carcinogenicity

In a study in groups of 20 male DDD mice each which were given 0, 100, and 200 mg/kg citrinin added to the feed, no tumors were found in the surviving animals after 70 weeks (49). Renal tumors were also not observed in male Sprague-Dawley rats after 48 weeks fed a diet containing 200 and 500 mg citrinin/kg body weight (91).

Long-term feeding tests (80 weeks) in male F344 rats which were fed a diet containing 1 g citrinin/kg body weight resulted in 35 out of 48 animals (which were sacrificed after 40, 60, and 80 weeks) developing histopathologically verified benign clear-cell adenomas of the kidneys (5).

Embryotoxicity and teratogenicity

In rats which received a single s.c. dose of 35 mg citrinin/kg body weight on one of the days 3–15 of gestation no skeletal malformations of the fetuses have been found, but enlarged kidneys, internal hydrocephalus and cleft palate were found. Thirty – 50 % of the mother animals died; the resorption rate of fetuses in the treated group was higher than in controls (78). Comparable results were obtained by Hayes et al. (39) and Hood et al. (41) in mice, and by Mayura et al. (64) after a single i.p. application of 30 or 40 mg citrinin/kg body weight to rats.

Effect on the immune system

Immune-suppressive effects, as have been found with ochratoxin A, were not observed after application of citrinin to mice. According to Reddy et al. (79), even a slight stimulation of the immune system appears after i.p. doses of up to 3 mg/kg body weight.

Combination effect with other toxins

After simultaneous application of citrinin and ochratoxin A in vitro Creppy et al. (15) observed in hepatome cells an immediate inhibition of RNA and protein synthesis and, after a short time, blocking of the DNA synthesis.

Mice: After combined oral application of 25 mg ochratoxin A/kg body weight and 200 mg citrinin/kg body weight Kanisawa (49) found a clear increase of renal cell tumors after 70 weeks.

Gupta and Chatterjee (31), who applied methaqualone following i.p. doses of 10–30 mg citrinin/kg body weight to mice report longer sleeping times of the animals. Gupta and Sasmal (32) found the same effect after paraldehyde and citrinin, and paraldehyde and ochratoxin A, and attribute the potentiating effect to a decrease of the acetylcholine level in the brain.

Rats: Citrinin was found to significantly increase the carcinogenicity of N-nitrosodimethylamine (25) and N-(3.5-dichlorophenyl) succinimide (91).

Mayura et al. (64) applied 30 mg citrinin/kg body weight and 1.0 mg ochratoxin A/kg body weight to rats in a single s.c. dose on 1 day of days 5–14 of gestation. Twenty-two – 40 % of the mother animals died, and malformations of skeleton and tissue were more frequent than in the controls, while these effects were completely absent when single doses of either citrinin or ochratoxin were administered.

Hens: Manning et al. (62) and Brown et al. (10), who fed a diet containing 300 mg/kg citrinin and 3 mg/kg ochratoxin A to 3-week-old chickens did not observe any additive or synergistic effect. In adult hens, Glahn et al. (30) did not find any additive effect on renal function, manure moisture, or diuresis.

Vesela et al. (101), however, who treated chicken embryos with 4 μg citrinin and 0.03–0.5 μg ochratoxin A report clear additive effects in terms of retarded growth and various malformations.

Insects: Dowd (20) fed naturally occurring concentrations of citrinin, ochratoxin A, and penicillic acid (2.5 mg/kg), and combinations of these, to freshly hatched larvae of corn earworms (*Heliotis zea*) and fall armyworms (*Spodoptera frugiperda*). Ochratoxin and citrinin both had a synergistic effect on *Spodoptera*, while the combination of penicillic acid and citrinin had the same effect as the individual toxins. The latter combination was additive in *Heliotis*, however. All remaining combinations had neither synergistic nor additive effects.

Some other combination tests with ochratoxin A in dogs, rats, and mice (17) were conducted with such high concentrations as are never reached in foods and only exceptionally reached in feedstuffs.

Long-term tests using sub-acute doses, also in combination with other agents, have not been reported.

Analysis

TLC and HPLC have been found useful to detect citrinin. Various methods for extraction and purification depending on the matrix have been available. With TLC the limit of detection is 1–20 μg/kg, with HPLC 0.1–0.01 μg/kg. Fluorescence measurement is more sensitive than UV absorption (18, 100). Analytical methods were published by Betina and by Scott (8, 86–88).

Unspecific bioassays, according to Lenz et al. (59) and Lenz and Süssmuth (58), are growth inhibition of *Bacillus thuringensis*, inhibition of pigment synthesis in *Serratia marcescens* or prevention of swarming of *Azospirillum brasiliense* and of *Proteus mirabilis*.

Discussion

It is not possible at this time to estimate the risk caused by citrinin for consumers to any reliable extent. Our knowledge of combination effects with concomitant mycotoxins and other toxic compounds present in food is insufficient. Data about the average and/or maximal daily uptake by humans and animals have not been available. In animals, the question of the carry-over of the compound itself and its metabolites has not yet been fully explored. Furthermore, the effects of long-term exposure to low doses have not been investigated.

Tremendous efforts in research will be necessary to clarify open questions, as not a single toxin, but interactions of several toxins are involved. What seems to be quite sure is that citrinin is no genotoxic carcinogen. It has, therefore, been classified by IARC as a substance of limited evidence for carcinogenicity (42).

However, it is a well-known fact that mycotoxins of so-called storage fungi, to which all citrinin producers belong, are preferentially found at places where products for human and animal nutrition are stored under inappropriate conditions, i.e., where stored food and feed are not kept dry (26). Preventive measures included improvement of post-harvest techniques, strict control of the relative humidity in stores, and additional use of protective agents. In view of the relatively high instability of citrinin, even at sufficiently low a_W values, this way seems not only promising, but certainly also helpful in minimizing concomitant mycotoxins as well.

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