Citrinin

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Citrinin (CIT) is a mycotoxin produced by different species of Aspergillus, Penicillium, and Monascus. CIT can contaminate a wide range of foods and feeds at any time during the pre-harvest, harvest, and post-harvest stages. CIT can be usually found in beans, fruits, fruit and vegetable juices, herbs and spices, and dairy products, as well as red mold rice. CIT exerts nephrotoxic and genotoxic effects in both humans and animals, thereby raising concerns regarding the consumption of CIT-contaminated food and feed.

Mycotoxins are poisonous secondary metabolites produced by filamentous fungi infesting crops and grain before harvest in the field or after harvest during storage. Improper storage conditions regarding moisture, temperature, and water activity play a significant role in the proliferation of storage fungi and the production of toxins. Citrinin (CIT) is a polyketide-derived mycotoxin most commonly occurring during storage. Hetherington and Raistrick isolated CIT for the first time from a culture of Penicillium citrinum in 1930s. Meanwhile, it was reported that the three fungal genera Penicillium (P. citrinum, P. verrucosum, and P. expansum), Aspergillus (A. carneus, A. niveus, and A. terreus), and Monascus (M. ruber) could produce CIT. Recently, CIT has also been found in food colorings traditionally made in Asia from rice fermented

with Monascus purpureus (“red mold rice”), conventionally used for meat preservation and food coloring.

Mycotoxins can contaminate the final food products and pose health concerns. However, recent advancements in food processing, such as hazard analysis of critical control points (HACCP) and good manufacturing practices (GMP), have aided in keeping final food products safe and healthy. Apart from this, several degradation methods can be applied for the partial or complete elimination of these toxins from food to ensure consumer food safety and avoid health concerns. Though CIT has shown antibacterial, anticancer, and neuroprotective properties, it is seldom used as a drug owing to its high nephrotoxicity and genotoxicity. Various in vitro and in vivo studies provided strong evidence of reproductive toxicity as well as the teratogenic and embryo toxic effects of CIT.

Major Source of Citrinin

The fungi of the genera Penicillium, Aspergillus, and Monascus are major producers of CIT. Penicillium spp. are of foremost importance and are reported to produce CIT worldwide during the drying and storage of cereal crops and other foodstuffs, among which Penicillium citrinum occurs most commonly in all kinds of food and feed, in almost all climatic conditions. Table 1 provides an overview of the current identity of microfungi Penicillium, Aspergillus, and Monascus species that can apparently produce CIT in foodstuffs.

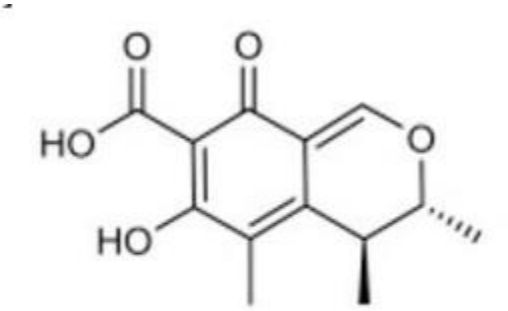
Table 1. Major citrinin producers among fungal species in foodstuffs

Genera	Subgenus	Series	Species
Penicillium	*Furcatum*	-	*P. citrinum* Thom
	Penicillium	Expansa	*P. expansum* Link
	Penicillium	Corymbifera	*P. radicicola* Overy & Frisvad
	Penicillium	Verrucosa	*P. verrucosum* Dierckx
	Penicillium	-	*P. viridicatum* Westling
	Penicillium	-	*P. camemberti* Sopp
Aspergillus	-	-	*A. carneus* Tiegh
	-	-	*A. niveus* Blochwitz
	-	-	*A. oryzae*
	Circumdati	-	*A. terreus* Thom
Monascus	-	-	*M. purpureus* Went
	-	-	*M. ruber* Tiegh

Chemistry and Biosynthesis of Citrinin

CIT (Figure 1) is a polyketide-containing mycotoxin (C13H14O5, IUPAC: (3R, 4S)-4,6- dihydro-8-hydroxy3,4,5-trimethyl-6-oxo-3H-2-benzopyran-7-carboxylic acid). It is a solid poison with the appearance of lemon-yellow needles at pH 4.6. The color changes to cherry red at pH 9.9. It melts at temperatures between 175 and 178.5 ◦C. In practice, it is insoluble in cold water but somewhat soluble in hot water, and it is soluble in aqueous sodium hydroxide, sodium carbonate, or sodium acetate, as well as in polar organic solvents such as ethanol, methanol, and acetonitrile. It has a UV-light absorption maximum ranging from 250 to 321 nm depending on the solvent. CIT decomposes at temperatures exceeding 175 ◦C in dry conditions and over 100 ◦C in wet ones. CIT can

be destroyed by acidic or alkaline liquids, as well as by heating. CIT is a quinone with two intramolecular hydrogen bonds.



Citrinin

Genes Responsible for Citrinin Production

Most polyketide metabolite pathway genes are grouped together. Six of the genes have comparable sequences to Monascus purpureus BCRC33325 citrinin biosynthesis pathway genes. PksCT, ctnA, orf1, orf3, orf4, and orf5 are all implicated. They successively encode a polyketide synthetase, a regulator, an aldehyde dehydrogenase, an oxygenase, an oxidoreductase, and a membrane transporter . The capacity to generate citrinin was lost in the pksCT disruptant. The pksCT mutant was not genetically stable, and citrinin production was recovered following repeated culture. Similarly, in Monascus aurantiacus, loss of pksCT resulted in a significant reduction in citrinin synthesis. Surprisingly, this mutant was able to generate more red and yellow pigments. Furthermore, transcriptional regulation of fungal secondary metabolite pathways is tightly controlled. In M. purpureus, ctnA is a key transcriptional activator of citrinin production. The deletion of ctnA

dramatically reduced the production of the pksCT transcript, which resulted in lower citrinin production. The phenotype of citrinin production is lost when pksCT is disturbed. The ctnA gene encodes a Zn(II)2Cys6 binuclear DNA binding protein that is a significant inducer of citrinin synthesis. As a result, the ctnA-deficient strain of M. purpureus produces so little CIT that it is barely detectable

Occurrence in Food and Feed

CIT is found mainly in foodstuffs of vegetable origin. In addition, its presence is detected in various cereals (maize, wheat, rye, rice, corn, barley, oat) and cereal-based products, pomaceous fruits and fruit juices, roasted nuts (almonds, peanuts, hazelnuts, pistachio nuts), oilseeds (e.g., sunflower), and spices (e.g., turmeric, coriander, fennel, black pepper, cardamom and cumin). Cheese is also contaminated by CIT where toxigenic strains directly grow in the cheese mass. CIT production is most likely when grains are not properly dried, retaining higher moisture content (>16%). The favorable temperature range for growth of CIT is between 12 and 37 ◦C, with an optimum temperature at 30 ◦C.

Effects on Agricultural Food and Feed

Agricultural products can be contaminated during pre-harvest, harvest, and postharvest conditions. CIT contaminates harvested grains, dairy products, spices, juices from fruits and vegetables, herbs, and citrus fruits. Wang et al observed the significant presence of toxigenic strains of

CIT in cheese. According to the EFSA, CIT is mostly found in a few agricultural products, fruits, biological fluids, animal feed, and dairy products. Irradiation (ultraviolet, UV) of fruits containing 280–400 µg/kg of CIT resulted in destruction, with no presence of CIT found in the fruits. Following the report by the EFSA , CIT can be associated with ochratoxin A and aflatoxin B in grain products and cereals as well as with patulin in the case of apple juices and apple jams. CIT production is also regulated by nutritive elements such as oxygen availability, fatty acids, nitrogen, and carbon sources, besides environmental factors such as water activity, temperature, commodity preservation, and storage conditions. There is a regular occurrence of CIT in food and feed with the potential chance of consumer exposure to the toxin; still, no legal limits have been set.

Mechanism of Toxicity and Health Effects of Citrinin

1. Mechanism of Toxicity

The two basic mechanisms of CIT-mediated harmful effects in biological systems are assumed to be the effects of oxidative stress and altered enzymatic antioxidative responses (e.g., epithelial glutathione and transhydrogenase). In the respiratory chain, CIT has been discovered to promote the creation of reactive oxygen species (ROS) and boost the synthesis of superoxide anions. These bioactivities could explain lipid peroxidation and cell death associated with mitochondrial malfunction [90]. The activation of caspases-3, -6, -7, and -9 has been linked to CIT

triggered apoptosis in kidney PK15 cells and human promyelocytic leukemia (HL-60) cells. CIT (108, 324, and 970 ppm) has been shown in several studies to cause harmful consequences in varieties of yeast cells by inducing oxidative stress and upregulating genes from oxidative stress response such as AADs, OYE3, FLR1, GRE2, and MET17.

CIT has previously been shown to accumulate in the budding yeast mitochondria, and exposure with CIT causes malfunction of respiratory system as well as mitochondrial complex I inhibition. Dysfunction in mitochondria caused by suppression of mitochondrial complex I resulted in superoxide anion (O2 −) production. Similarly, exposure with CIT for 60 min increased the amount of ROS in hepatocarcinoma HepG2 cells (10–30 μM) . As a result, it seems that CIT-induced ROS generation is required for initiation in apoptosis and that antioxidant system activation and adaptive responses mediated through ROS-sensitive transcription factors are activated. CIT treatment (1000 μM) of cells (107 mL−1) for 60 min at pH 4.5 resulted in a considerable rise in peroxides and total ROS as well as a 3-fold increase in glutathione concentration, with no change in superoxide or hydroxyl radical levels . CIT treatment raised ROS levels in hepatocarcinoma HepG2 cells (10–30 μM) for 60 min [95] and in single cells from the murine skin suspensions at 50 μM for 12–72 h.

2.Health Effects of Citrinin

CIT has been shown to be nephrotoxic and hepatotoxic to humans. The kidney is the major target organ of CIT. CIT is commonly found along with ochratoxin, and an additive or synergic effect has been shown to increase the toxicity, causing kidney disease in humans. Other than the kidney, the target organs of CIT include the liver, mitochondrial respiratory chain, and bone marrow. This nephrotoxin is also considered as one possible reason for porcine nephropathy. In the absence of adequate exposure data, the risk of CIT as a food contaminant was assessed based on an estimate of the critical CIT concentrations in grains and grain-based products that would result in nephrotoxicity.

Effects of Processing on Citrinin

The toxigenic potential of the fungi and thereby the yield of toxins can be affected by conditions during harvest, storage, and processing operations. CIT levels decreased in the products after processing due to its sensitivity to heat. CIT is decomposed into two other complexes, namely CIT-H1 and CIT-H2, after the heat treatment generally above 175 ◦C under dry conditions and above 100 ◦C in the presence of water. CITH2 has lower toxicity than CIT, while CIT-H1 is more toxic [101,113]. Dicitrinin A is another decomposition product of CIT reported recently together with other degradation products. Besides temperature, the addition of compounds such as flavonoids can also affect the level of CIT.

Wang et al. investigated the effect of is flavone and genistein on CIT production by Monascus aurantiacus Li AS3.4384 (MAL) during liquidstate fermentation containing rice powder as a carbon source and 2.0 g/L genistein. The results showed a significant reduction in CIT levels (approximately 80%) and an increase in biomass. Other flavonoids such as quercetin, kaempferol, myricetin, and genistin were also tested for their effectiveness; however, the maximum reduction was observed in the case of genistein. Further research by Ouyang et al. showed that the reduction was due to changes occurring at the transcription level. When transcriptome analysis of groups treated with genistein and control was performed, several genes that were significantly downregulated with genistein addition, thereby demonstrating their involvement in CIT production. A similar study by Huang et al. investigated the effect of the addition of rutin and its derivatives, α-glucosylrutin and troxerutin, in fermentation media on Monascus aurantiacus Li AS3.4384 CIT production. The results showed that inhibition by rutin derivatives was significantly higher (>50%) than rutin (around 20%) when added at the same concentration. In addition, the media composition also affected the reduction in CIT yield. The highest reduction of about 90% was observed after 14 days of fermentation when 15.0 g/L of troxerutin was added to low-starch peptone containing liquid media. In addition, several novel technologies have been incorporated in the food industry, such as high hydrostatic pressure (HHP), ultrasonication, and cold atmospheric

pressure plasma (CAPP). Application of HHP on the infected olives successfully reduced the microbial population by 90–100% and degraded CIT up to 100%. Moreover, HHP also enhanced phenolic compounds and antioxidant activity. The addition of 6–9% NaCl significantly reduced CIT production during olive storage. Similarly, ultrasonication of red yeast rice degraded up to 87.7% of CIT produced by Monascus purpureus during fermentation. On the other hand, CAPP degraded up to 50% of CIT developed by Penicillium sp. collected from wheat, oat, corn, and rice, without affecting the nutritional quality of grains.

Effects of Environmental Factors on Citrinin Production

Environmental factors such as temperature, pH, and light (especially during storage) affect the growth of fungus and the production of mycotoxin. Regulation of such environmental conditions, therefore, would help control the growth of fungus and thereby the release of toxins. Wawrzyniak and Wa´skiewicz investigated the effect of temperature (10, 20, and 30 ◦C) and different cereal substrates (wheat, triticale, rye, barley, maize, rice) on the growth of P. verrucosum and the production of CIT while maintaining the moisture content. For the experiment, the cereals were moistened, autoclaved, and then inoculated with the fungal spores. The inoculated cereals were then kept under different storage temperatures for 40 days. To determine growth, ergosterol (ERG) was used as a biomarker and the mycotoxin content was determined using HPLC. The results showed maximum ERG at 30 ◦C, although growth was

observed at all temperatures. Mycotoxin (CIT) was observed to be accumulated more at 20 ◦C in rice. The study suggested that irrespective of the temperature and cereal substrate, there is no strong correlation between the production of ERG and mycotoxin. This is reflected by the experiment since the optimal conditions for growth (30 ◦C) and CIT production (20 ◦C) do not coincide.

Detection Techniques

1. Sample Preparation

Sample preparation for the detection of CIT involves a process of extraction and clean-up that plays an important role in analyzing CIT with improved sensitivity, precision, accuracy, and specificity. Extraction is mainly carried out using solvents like acetonitrile and methanol in combination with other salts such as sodium chloride, potassium chloride and citric acid, and solvents like formic acid, acetic acid, and water. Some extraction processes even involve acidification using undiluted hydrochloric acid, sulfuric acid, or phosphoric acid to improve recovery and reproducibility. Further, various clean-up methods have been reported for purification of samples containing CIT, for example liquid–liquid/solid extraction (LLE/LSE), dispersive liquid–liquid microextraction (DLLME), solid-phase extraction (SPE), immunoaffinity columns (IAC), and the quick, easy, cheap, effective, rugged, and safe method (QuEChERS). The LLE/LSE method for sample preparation is

performed using polar organic solvents like ethanol, methanol, acetonitrile, and polyethylene glycol. However, with this method, it is difficult to extract all analytes of interest with good recoveries, as they comprise of an extensive range of physicochemical properties. This method involves co-extraction of polar matrix components from the organic solvents used in the extraction process, thereby entailing a clean-up step. Thus, making the method unsafe due to the use of large amounts of toxic organic solvents, apart from making it time consuming and labor-intensive.

2. Detection and Quantification Method

2.1. Thin-Layer Chromatography (TLC)

TLC uses visual or fluorodensitometry procedures with a recovery limit of 0.01 ppm for quantitative as well as qualitative detection of mycotoxins, including the assessment of purity, separation, and the identification of organic compound heating. In TLC, CIT separation is completed using various solvents, and the chemical validation of CIT is performed using two types of treatment approaches. The first involves saturation of TLC plate with acid-organic solution followed by exposing the TLC plate (with developed chromatogram) to the vapors of acetic anhydride/pyridine. The second treatment involves direct immersion of the TLC plate in an aluminum chloride reagent. These treatments

transformed CIT into a new fluorescent compound that is detected under 365 nm light.

2.2. Colorimetric Technique of Detection

Under visual detection, the colorimetric technique is one of the most common methods for CIT detection. The conjugated planar structure imparts a natural fluorescence to CIT, that can be evaluated qualitatively and quantitively using a fluorometer. This natural fluorescence can be further intensified in acidic environments. Apart from this, other ultrasensitive methods for visual detection of CIT in the nano molar range.

2.3. High-Performance Liquid Chromatography (HPLC)

HPLC is one of the most common instrument-based techniques used to detect CIT in food and feed samples. It is usually combined with fluorescence, ultraviolet, and amperometric detection for high selectivity to achieve even low detection levels of CIT.

2.4. Liquid Chromatography-Mass Spectroscopy (LC-MS)

LC-MS is a high throughput analytical method for CIT detection with reduced costs, labor, and time. LC-MS/MS systems with a triple quadrupole analyzer (QqQ) is the most widely reported system used for the determination of CIT in food and feed samples. Apart from this, recently a high-resolution LC-MS/MS system coupled with Qtrap mass analyzer was reported for the detection of CIT due to its full-scan mode

operation that provides high specificity irrespective of the number of other mycotoxins detected.

2.5. Liquid Chromatography Fluorescence Detection (LC-FLD)

The natural fluorescence of CIT can be an effective alternative for its detection. Since only a few mycotoxins possess this property, this method cannot be used for multimycotoxin analysis. Typical excitation and emission wavelengths for CIT fluorescence detection have been reported to be 330–335 nm and 497–500 nm, respectively. However, there have been instances where CIT in foods like red yeast rice and coffee samples could not be detected using this method, implying lower sensitivity and selectivity for CIT detection.

2.6. Liquid Chromatography UV/Visible Detection (LC-UV/Vis)

HPLC-UV/Vis is the least-reported method for CIT detection owing to its reduced selectivity and sensitivity than even fluorescence detection. However, since it is a simple and economical method, efforts can have made to use this method in combination with a few pre-treatments for the estimation of CIT and other mycotoxins in food and feed.

2.7. Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA kits represent a portable and easy-to-apply in CIT detection technique that is commonly being used owing to its lower costs and fast analysis. In an ELISA assay, a complex is formed due to a competitive

assay that exists between the analyte and a specific primary antibody or a conjugate of an enzyme. This complex then interacts with the chromogenic substrate to determine the amount of analyte present.

2.8. Immunochromatographic Assay (ICA)

The properties of ICA make it a user-friendly method for CIT detection due to its suitability, simplicity, high speeds, and lower costs. ICA assisted by molecularly imprinted biopolymers allows biological detection of multiple mycotoxins in contaminated agricultural products.

2.9. Capillary Zone Electrophoresis (CZE)

The CZE method for detection of mycotoxins was developed in response to the drawbacks that were observed for the instrument-based (HPLC and GC) and biological detection methods (TLC, ELISA, and paper-based colloidal gold testing). Drawbacks of instrument-based methods include high costs, complex pre-treatments, time-intensiveness, and the usage of large amounts of organic solvents, while the drawbacks of biological methods are possible false-positive results, difficult reproducibility, and sensitivity.