# Thermal Decomposition and Detoxification of Citrinin under Various Moisture Conditions

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The decomposition and detoxification of citrinin by heating to 175 °C under three moisture conditions were examined. Citrinin was decomposed at 175 °C by dry heating. The decomposition products had different ultraviolet and fluorescence spectra from those of citrinin and were not cytotoxic toward HeLa cells. Under semimoist conditions, temperatures above 140 °C caused decomposition and detoxification; therefore, the detoxification temperature was decreased by 35 °C with slight moisture. Heating citrinin with water at around 140 °C yielded decomposition products as toxic as or more toxic than citrinin. Above 160 °C derived compounds were nontoxic.

## INTRODUCTION

Citrinin, a mycotoxin, is a toxic secondary metabolite of fungi. Several *Penicillium* and *Aspergillus* species produce citrinin, which has been implicated in nephropathy and cancer (Betina, 1989; Ueno and Kubota, 1976) and has been found as a natural contaminant of cereals, grains, and decaying fruits (Betina, 1989; Saito et al., 1971; Neely et al., 1972). The structures of citrinin and its biosynthetic intermediates are known (Colombo et al., 1981), and the degradation pathway of citrinin by *Penicillium citrinum* has been examined (Barber et al., 1988). The thermal decomposition and detoxification of citrinin during food processing or heat treatment have been rarely studied (Kawashiro et al., 1955).

Food that is contaminated by mycotoxins with strong acute toxicity can be surveyed before marketing. However, it is difficult to find and eliminate all food that is contaminated by mycotoxins with weak chronic toxicity, such as citrinin. Thermal food processing could be a more effective way to detoxify causal agent.

Citrinin becomes molten and decomposes at 175 °C (Betina, 1989). The heat stability of citrinin and the effects of pH under certain conditions have been reported (Kawashiro et al., 1955; Neely et al., 1972). These studies suggested that citrinin might be detoxified by heat.

It seems likely that the decomposition and detoxification of citrinin by heat depends on moisture as well as the temperature conditions. Various moisture conditions are used in food processing and cooking. The purpose of this study was to examine the decomposition and detoxification of citrinin under various temperature and moisture conditions: dry, semimoist, and moist. A cytotoxicity test with HeLa cells was used for both citrinin and decomposed citrinin, and the relationship between decomposition and detoxification was studied.

## MATERIALS AND METHODS

**Materials.** Citrinin was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Spectrophotometric grade $CHCl_3$ was from Nakalai Tesque, Inc. (Kyoto, Japan). Dulbecco's modified Eagle's medium (type Auto-Mod) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO). Trypsin, 2-propanol, and fetal bovine serum (FBS) were obtained from GIBCO/BRL Life Technologies, Inc. (Gaithersburg, MD), Wako, and Whittaker Bioproducts, Inc. (Walkersville, MD), respectively. Other reagents were purchased from Nakalai Tesque and Wako. The HeLa cell line was obtained from Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan).

**Heating of Citrinin.** Citrinin was dissolved in $CHCl_3$ (200 μg/mL) and stored at −20 °C until use. The concentration of citrinin was found by absorbance; molecular extinction was $1.61 \times 10^4$, at 332 nm using $CHCl_3$ (Neely et al., 1972). One milliliter (200 μg) of this stock solution, pipetted into a 1-mL glass vial (V-1A, Nichiden Rika Glass Co., Ltd., Tokyo), was dried under dry $N_2$ gas. Then 150 μL, 8 μL, or no distilled water was added to the dried citrinin and the vial sealed with an airtight aluminum cap. The citrinin was heated under three conditions: dry, semimoist, and moist. The vials were placed in an oil bath and heated at the rate of 3 °C/min. The vial internal temperature was checked with a thermoindicator (Thermolabel, Nichiyu Giken Co., Ltd., Tokyo). Samples were heated to 175 °C. When samples containing 150 μL of water were heated to >150 °C, a thermoregulated pressure vessel (type TEM-V, Taiatsu Scientific Glass Co., Ltd., Tokyo) was used. When the temperature reached a certain point, heating was stopped. After being cooled to room temperature, the sample was lyophilized to yield a powder. All steps were done in the dark or in UV-free conditions to protect the citrinin or its derivatives from decomposition.

**Thin-Layer Chromatography.** Thin-layer chromatograms (TLC) were developed on TLC plates, silica gel 60 without a fluorescence indicator (Merck, Darmstadt, Germany). Fluorescence detection was at 365 nm. The solvent for development was a 5:5:1 mixture of ethyl acetate, acetone, and water. Citrinin and the heated products solubilized in $CHCl_3$, and 2.5 μg was spotted on the TLC plate.

**Ultraviolet and Fluorescence Spectra.** Ultraviolet (UV) absorption and fluorescence spectra were obtained on a Shimadzu UV–vis spectrophotometer UV-160A and a Hitachi fluorometer, respectively, in $CHCl_3$; 12 μg/mL citrinin solution was used for assay.

**Cytotoxicity.** The toxicity of citrinin and heated products were assayed with HeLa cells. The complete bioassay has been described (Trivedi et al., 1990). Briefly, citrinin or the products of heated citrinin were dissolved in fetal bovine serum, which was diluted with Dulbecco's modified Eagle's medium. Then 50 μL of the diluted solution containing 5 μg of citrinin or the products was put in a well of 96-well microplates (Nunc). To each well was added 50 μL of a HeLa cell suspension of $6 \times 10^4$ cells/mL. These followed incubation at 37 °C under 5% $CO_2$. Cell growth was measured by a colorimetric method with the use of MTT, which gave good correlation between the cell number and the color development from the reduction of MTT under suitable conditions (Trivedi et al., 1990). Developed color was measured with a microplate reader (Tosoh MPRA-4, Tokyo) at the test wavelength of 540 nm and the reference wavelength of 620 nm. The percentage of cytotoxicity of citrinin and heated products was calculated according to the following equation: % cytotoxicity = [1 − (absorbance of the treated well/absorbance

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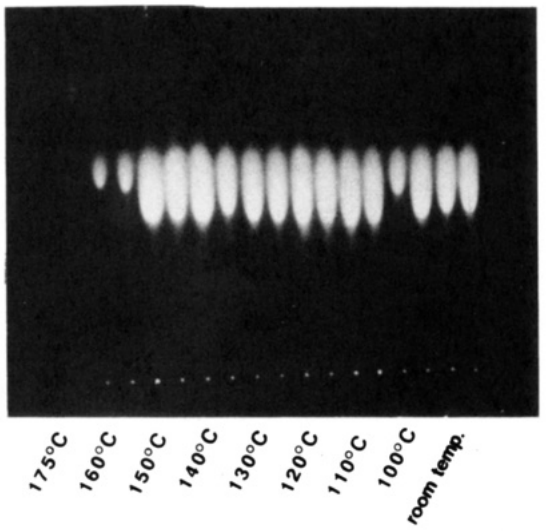


**Figure 1.** Thin-layer chromatogram of citrinin and citrinin heated at different temperatures under dry condition. Duplicates have been done for each temperature.

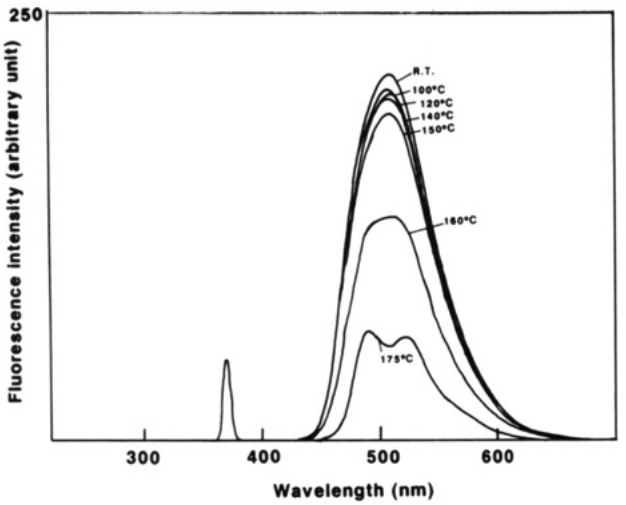


**Figure 2.** Fluorescence spectra of citrinin and citrinin heated at different temperatures under dry condition.

of the control well)] × 100. Absorbance of the control well is the absorbance of the well containing the medium and HeLa cells without citrinin or heated products.

## RESULTS

**Heating under Dry Condition.** Up to 150 °C, the appearance of the citrinin did not change. By heating at and above 160 °C citrinin became brown from yellow. The TLC patterns of the heated citrinin at various temperatures are shown in Figure 1. Citrinin heated up to 150 °C gave only one yellowish green fluorescent spot with the same $R_f$ (0.61) as unheated citrinin. The fluorescence of the citrinin heated to 160 °C was weaker than that of the original citrinin. At 175 °C, the spot corresponding to citrinin had completely disappeared, and new fluorescent spots with $R_f$ values (0.78–0.91) higher than that of citrinin were observed. This change in the TLC pattern was confirmed by the fluorescence spectra (Figure 2). An emission peak at 510 nm (excitation, 254 nm) found with the original citrinin was reduced in the sample heated to 160 °C and had disappeared in the sample heated to 175 °C. Two peaks at 495 and 525 nm appeared when the peak at 510 nm disappeared. Changes were similar in the UV spectra (Figure 3). The absorbance at 332 nm, which is the absorption maximum of citrinin, gradually decreased

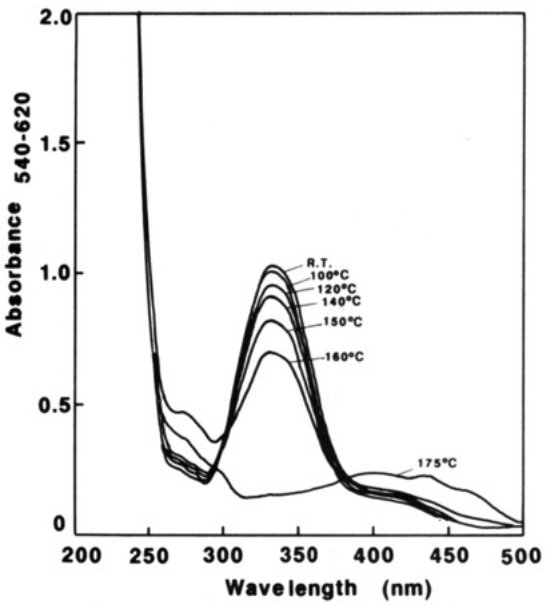


**Figure 3.** Ultraviolet spectra of citrinin and citrinin heated at different temperatures under dry condition.

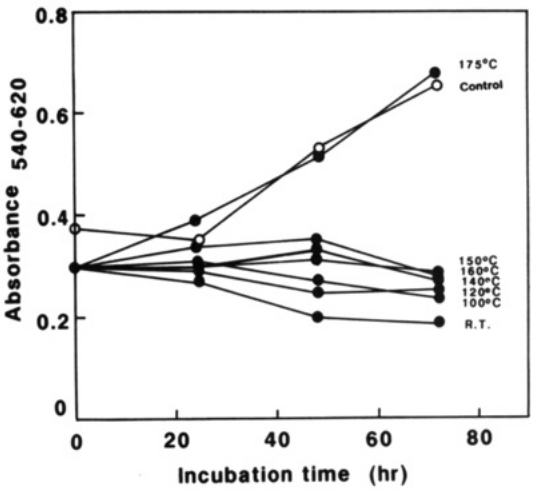


**Figure 4.** Cytotoxicity of citrinin and citrinin heated at different temperatures under dry condition. The control experiment, without citrinin, is shown by an open circle.

as the temperature increased, and at 175 °C the peak at 332 nm disappeared completely. From these results, we concluded that in dry heating the decomposition of citrinin started at 160 °C and that at 175 °C the compound was completely decomposed. Next, the cytotoxicity of the samples heated under dry conditions at various temperatures was examined. The cells proliferated well in the absence of citrinin, and with increasing incubation time, the absorbance gradually increased (Figure 4), indicating an increased cell number. With citrinin, the cells did not grow, and the number of viable cells decreased; that is, citrinin was toxic to HeLa cells. Heated citrinin had weaker toxicity than the original citrinin. The toxicity of citrinin decreased when treated at higher temperatures, but the citrinin heated to 160 °C was still toxic. Heating at 175 °C gave complete detoxification. This result means that the decomposed citrinin shown by fluorescent spots in the TLC with a higher $R_f$ value (0.78–0.91) than that of citrinin was not cytotoxic.

**Heating under Semimoist Condition.** The TLC pattern in Figure 5 shows that a yellowish green fluorescent spot, corresponding to citrinin, decreased with the increase in temperatures, and greenish fluorescent spots ($R_f$ 0.78–

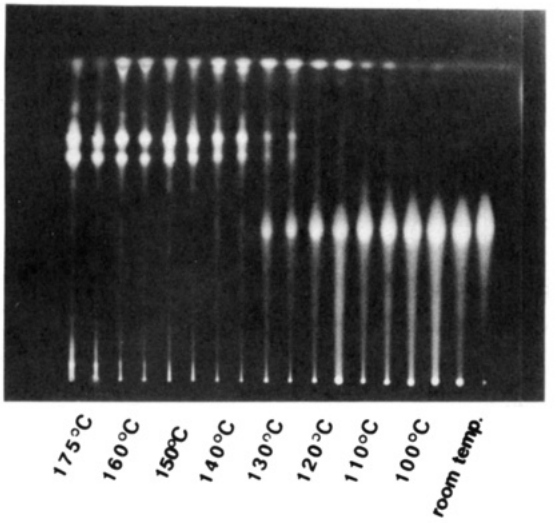


**Figure 5.** Thin-layer chromatogram of citrinin and citrinin heated at different temperatures under semimoist condition. Duplicates have been done for each temperature.

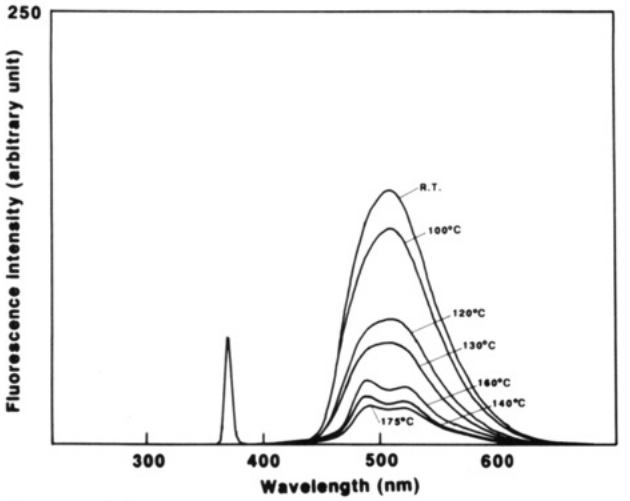


**Figure 6.** Fluorescence spectra of citrinin and citrinin heated at different temperatures under semimoist condition.

0.91) and other fluorescent spots (greenish and brownish) with high $R_f$ values (0.95–1.00) appeared upon heating at 140 °C and higher temperatures. Figure 6 confirmed these results. With the increase in temperature, the fluorescence intensity at 510 nm decreased, and fluorescence disappeared at 140 °C. Above 140 °C, the spectrum pattern changed. UV absorption spectra gave similar results (Figure 7). With the increase in temperature, the absorption at 332 nm reduced, and at 140 °C this peak disappeared. Heating to >140 °C gave a slight increase in absorbance at 420–440 nm; this corresponded to the occurrence of the spots found in TLC.

The cytotoxicity of the samples is shown in Figure 8. As the temperature increased, the cytotoxicity of the sample decreased, and heating up to 140 °C completely detoxified citrinin. Under semimoist condition, the decomposition and detoxification temperatures were both reduced by about 35 °C compared with those under dry heat.

**Heating under Moist Condition.** Figure 9 shows the TLC pattern of citrinin heated under moist condition to different temperatures. As the temperature increased, the citrinin spot became smaller, disappearing at 160 °C. However, the TLC pattern was different from that obtained under semimoist condition. Fluorescent spots

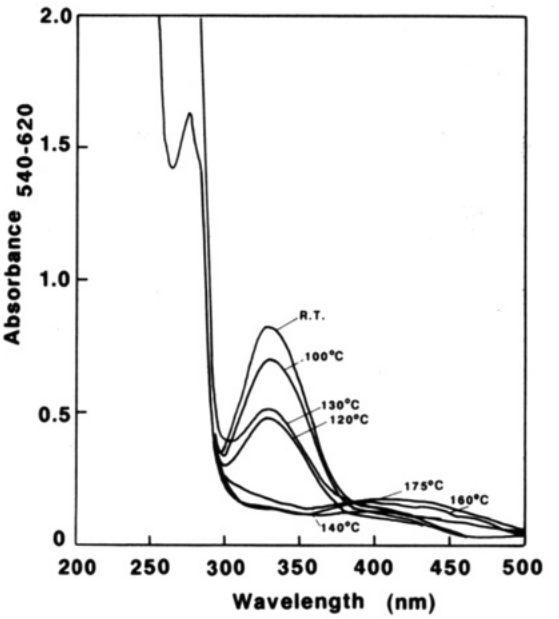


**Figure 7.** Ultraviolet spectra of citrinin and citrinin heated at different temperatures under semimoist condition.

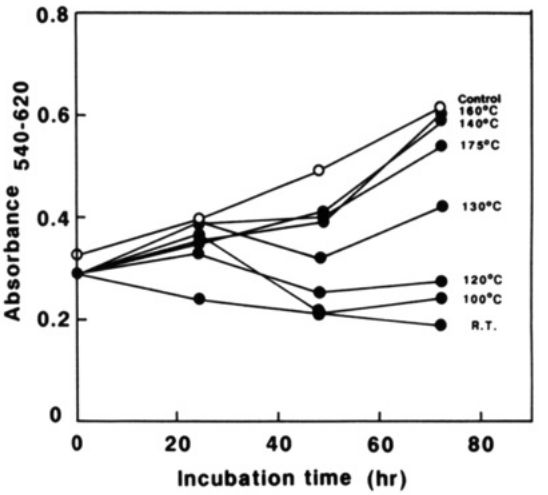


**Figure 8.** Cytotoxicity of citrinin and citrinin heated at different temperatures under semimoist condition. The control experiment, without citrinin, is shown by an open circle.

($R_f$ 0.95–1.00) appeared at >140 °C and other fluorescent spots ($R_f$ 0.78–0.91) appeared when heating was above 160 °C.

Fluorescence spectra (Figure 10) showed that the fluorescence intensity at 510 nm was reduced by heat and that the peak at 510 nm became the minimum at 140 °C. The emission fluorescence increased again as the temperature increased. Two peaks similar to those obtained under dry and semimoist conditions appeared. The UV spectra are shown in Figure 11. The peak at 332 nm decreased with heat, and the lowest value was obtained at 160 °C.

The cytotoxicity of each sample treated at various temperatures is shown in Figure 12. Cytotoxicity decreased as the temperature increased from 100 to 130 °C; however, at 140 °C toxicity increased. The samples heated to 140 and 150 °C have toxicity similar to that of the original citrinin. When heated at 160 °C and above, toxicity decreased again and disappeared.

Some toxic compounds seem to occur by heating to 140 and 150 °C. These compounds may have little absorbance at 332 nm and give spots with $R_f$ values of 0.95–1.00.

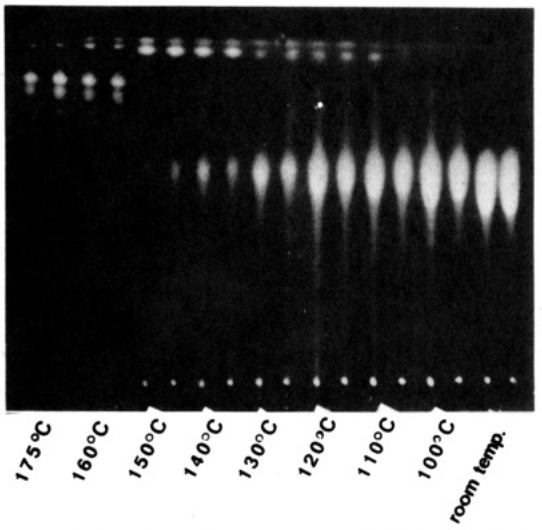


**Figure 9.** Thin-layer chromatogram of citrinin and citrinin heated at different temperatures under moist condition. Duplicates have been done for each temperature.

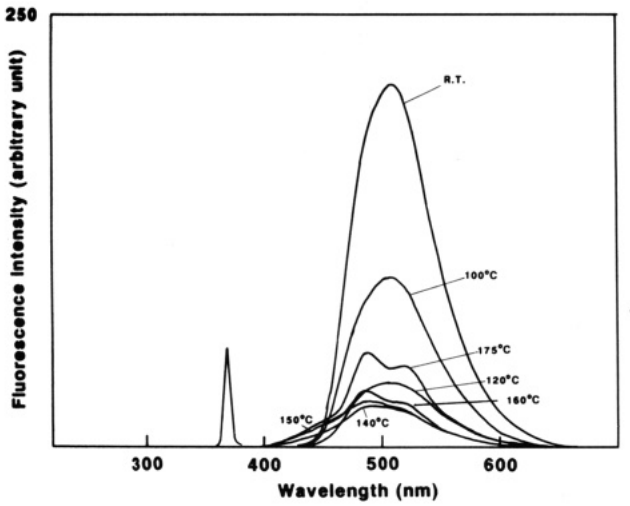


**Figure 10.** Fluorescence spectra of citrinin and citrinin heated at different temperatures under moist condition.

## DISCUSSION

Bioassays done to detect mycotoxins can be classified as microbial, cytological, immunological, invertebrate, and vertebrate; each method has advantages and disadvantages (Betina, 1989). The use of a cell culture to help predict mycotoxicity makes it fairly easy to demonstrate and measure toxic effects and, additionally, to define their specificity and mechanism (Robbana-Barnat et al. 1989). Natori et al. (1970) have used HeLa cells to evaluate the toxicity of ochratoxins and penicillic acid. Cell counts or the incorporation of [$^3$H]thymidine may be used as an indicator of toxicity. The MTT method introduced by Mosmann (1983) and Green et al. (1984) is useful to evaluate the cell number by the amount of color that develops because of the reduction of MTT. By use of an immunoreader, many samples can be treated in a short time; however, when the MTT method is used, suitable conditions must be selected in advance.

The HeLa cell is a popular cell line and can be obtained easily. The bioassay system using the combination of the HeLa cell line and MTT was developed by Trivedi et al. (1990). This bioassay system is convenient to do a screening test of the sample containing toxin and to examine the toxicity of the derivatives of toxic compounds including mycotoxin. In this study, the decomposition of

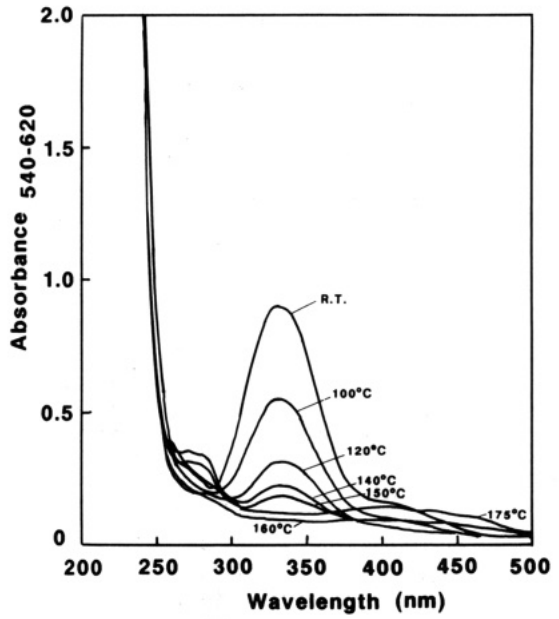


**Figure 11.** Ultraviolet spectra of citrinin and citrinin heated at different temperatures under moist condition.

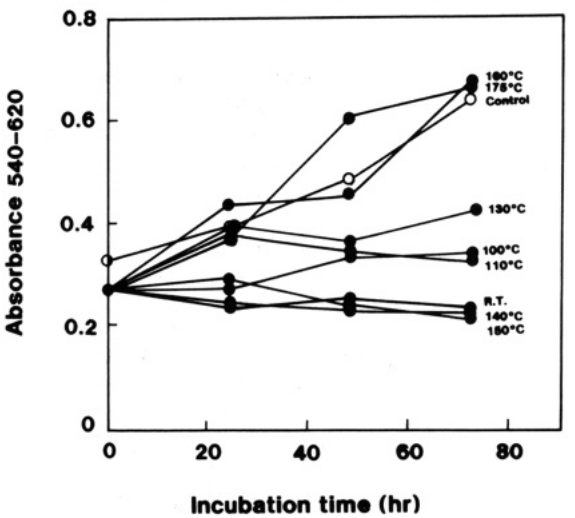


**Figure 12.** Cytotoxicity of citrinin and citrinin heated at different temperatures under moist condition. The control experiment, without citrinin, is shown by an open circle.

citrinin detected by TLC and by the UV or fluorescence spectra almost corresponded to the disappearance of cytotoxicity toward HeLa cells, indicating that the cytotoxicity assay used here was sensitive to evaluate the detoxification of citrinin.

The decomposition of citrinin by heating under dry condition was studied by Kawashiro et al. (1955), who reported a change in the fluorescence of citrinin. Its decomposition with wet heat has not been reported. In food processing and cooking, the moisture range used is broad. The heating conditions used here are typical for food cooking (frying and baking) and food processing (retort cooking and extrusion cooking). The decomposition temperature is a function of moisture conditions. The presence of small amounts of water decreased the decomposition temperature of citrinin from 175 to 140 °C, and the decomposition pattern also changed. A more important result is that the compound(s) with toxicity close to that of the original citrinin (or with higher toxicity) should occur by heating at 140 °C under moist condition. Above 175 °C under dry condition, above 140 °C under semimoist condition, and at about 160 °C under moist

**Table I. Decomposition[a] and Detoxification[b] of Citrinin by Heating**

| | dry | | semimoist | | moist | |
|---|---|---|---|---|---|---|
| | abs[a] | tox[b] | abs[a] | tox[b] | abs[a] | tox[b] |
| control[c] | 100 | 100 | 100 | 100 | 100 | 88 |
| 100 °C | 89 | 88 | 85 | 90 | 61 | 64 |
| 110 °C | 100 | ND[d] | 94 | 80 | 43 | 67 |
| 120 °C | 97 | 88 | 58 | 80 | 35 | 48 |
| 130 °C | 92 | 84 | 62 | 46 | 34 | 44 |
| 140 °C | 93 | 82 | 17 | 9 | 25 | 91 |
| 150 °C | 79 | 79 | ND[d] | ND[d] | 20 | 91 |
| 160 °C | 68 | 79 | 17 | 6 | 13 | −13 |
| 175 °C | 15 | −7 | 22 | 21 | 13 | −10 |

[a] Absorbance at 332 nm is shown by percent of that of the citrinin nonheated, based on the data in Figures 3, 7, and 11. [b] Toxicity is shown by percent cytotoxicity. Citrinin or heated products were incubated with HeLa cells for 72 h. The data for calculation were from Figures 4, 8, and 12. Details were described in the text. [c] Nonheated citrinin. [d] ND, not determined.

condition, a group of decomposed and apparently nontoxic compounds of citrinin ($R_f$ 0.78–0.91) was found. At about 140 °C, under moist condition, another group of decomposed compounds of citrinin ($R_f$ 0.95–1.00) was clearly found that were different from citrinin. The heated product containing these decomposed compounds was still toxic. These findings are summed up in Table I, where the decomposition and detoxification of citrinin by heating were shown by the change in absorbance at 332 nm (specific absorption peak of citrinin) and the percent cytotoxicity, respectively. Under dry and semimoist conditions the detoxification corresponds to the decomposition of citrinin, while under moist condition toxicity increased with decomposition at 140 and 150 °C.

From the structure of citrinin and the study of the degradation of citrinin by *P. citrinum* (Barber et al., 1988), decarboxylation from citrinin molecule might occur as the first step of decomposition and the decomposed compound may be a starting substrate for next reactions. The purification and identification of the decomposed products are in progress.

The present study showed that decomposition of citrinin by heating did not always result in detoxification but that citrinin can be detoxified if suitable temperature and moisture conditions are chosen.

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