total aldehydes. Although ketones also react in the above method, they are present in negligible concentrations and do not contribute much to the flavor.

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Moniliformin, a Mycotoxin from *Fusarium fusarioides*

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Fusarium fusarioides was found to be highly toxic to ducklings and rats. The toxic metabolite, moniliformin (sodium or potassium salt of 1-hydroxycyclobut-1-ene-3,4-dione) was isolated as the sole toxin from *F. fusarioides*. Isolates obtained from peanuts, sorghum, millet, peaches, soil, and dried fish were all toxic to ducklings. The chronic toxicity of an isolate from millet was determined, and 10, 25, and 50% moldy meal incorporated into commercial rat mash all proved toxic. The effect of different incubation temperatures and periods was investigated. Material produced at three different temperatures was of approximately equal toxicity, although less of the material produced at higher temperature was consumed. Analysis showed that the material incubated at 31 °C contained the most moniliformin. Self-heating to a maximum of 6–8 °C took place at all incubation temperatures. The thin-layer chromatographic analytical method was applied to four randomly selected isolates, all of which produced moniliformin, ranging in quantities from 200–840 mg/kg. Methods are described for the analyses and isolation of moniliformin from yellow corn inoculated with cultures of *F. fusarioides*. The analytical methodology involves either thin-layer chromatography of the extracts or purification of these on Dowex-1 (Cl^-) resin; 0.2 M sodium chloride elutes the moniliformin which is subjected to quantitation by UV spectroscopy.

Toxin production is frequently encountered amongst many species and isolates of the genus *Fusarium*, the most common being zearalenone (Pathre and Mirocha, 1976; Mirocha and Christensen, 1974), moniliformin (Cole et al., 1973; Kriek et al., 1977), and the chemically related group of trichothecenes (Bamburg, 1976; Smalley and Strong, 1974; Saito and Ohtsubo, 1974). The associations of these toxins, and the species of *Fusarium* that produce them, with mycotoxicosis have been reviewed in detail (Joffe, 1974; Saito and Ohtsubo, 1974).

Samples of millet, *Pennisetum typhoides* (Burm.) Staph and Hubb., obtained from the households of patients in South West Africa suffering from the hemorrhagic disease Onyalai were mycologically investigated. Amongst others, *Fusarium fusarioides* (Frag. and Cif.) Booth could readily be isolated and proved to be toxic in feeding trials with ducklings (Rabie et al., 1975; Steyn and Rabie, 1976). Although the occurrence of *F. fusarioides* appeared to be less widespread than *Phoma sorghina* (Sacc.), it could nevertheless be isolated quite frequently from millet, which

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Table I. Toxicity of Various Isolates of *Fusarium fusarioides* to Ducklings

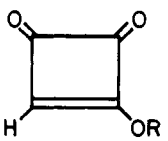
Isolate	Isolated from	Region	Number of deaths	Average time to death, days	Moniliformin production,[a] g/kg
M.R.C. 5	Peanuts	Mozambique	4/4	5	0.42
M.R.C. 6	Peanuts	Mozambique	4/4	5	*b*
M.R.C. 84	Peanuts	Mozambique	1/4	7	*b*
M.R.C. 79	Peanuts	Mozambique	4/4	5	*b*
M.R.C. 7	Dried fish	Mozambique	4/4	7	0.84
M.R.C. 85	Dried fish	Mozambique	1/4	9	*b*
M.R.C. 484	Soil	Pakistan	4/4	3	0.42
M.R.C. 485	Peach	Israel	3/4	5	*b*
M.R.C. 35	Millet	South West Africa	4/4	4	0.20
1998	Millet	South West Africa	4/4	3	0.84
1976	Millet	South West Africa	4/4	5	*b*
M.153	Sorghum	Mozambique	4/4	3	*b*
O.P.126	Beans	South Africa	4/4	7	*b*

[a] Determined by thin-layer chromatography. [b] Not determined.

is the single most important staple in the diet of most of the indigenous people. *F. fusarioides* has not to our knowledge been reported to be toxic.

Mycotoxicological work with *Fusarium* species is complicated by the fact that different systems of classification are used, with a resultant confusion in nomenclature. The local isolates were identified as *Fusarium fusarioides* according to the system proposed by Booth (1971). This corresponds to *F. chlamydosporum* Wr. et Rkg. as proposed by Seemüller (1968), Wollenweber and Reinking (1935) and probably to *F. sporotrichioides Sherb.* var. *Chlamydosporum* (Wr. and Rkg.) Joffe according to the system proposed by Joffe (1973, 1974). The first reports of *F. chlamydosporum* from Southern Africa was its isolation from dead locusts in South West Africa (Wollenweber and Reinking, 1935) and subsequently from *Brachiaria pubifolia* Stapf. by Doidge (1938).

The mycotoxin moniliformin was recently isolated by Cole et al. (1973) from *Fusarium moniliforme* and by Kriek et al. (1977) from *Fusarium moniliforme* var. *subglutinans* from South African corn. The toxin has been isolated in both its sodium (1, R = Na) and potassium (1,



1, R = Na or K

R = K) forms (Springer et al., 1974). Its structure, synthesis (Springer et al., 1974), and spectroanalytical parameters (Lansden et al., 1974) have been reported. In rats, moniliformin causes progressive muscular weakness, respiratory distress, cyanosis, coma, and death (Kriek et al., 1977).

The present report describes the identification of the mycotoxin moniliformin in several strains of *F. fusarioides* and the isolation of this toxic metabolite from whole yellow corn inoculated with a culture of *F. fusarioides*.

Methods are described for the detection and quantitation of moniliformin. Investigations were carried out (a) to determine the frequency of occurrence of isolates of *F. fusarioides* in Southern Africa, (b) to determine whether toxigenicity is common to all isolates of *F. fusarioides*, (c) to evaluate its effect in laboratory animals, (d) to determine the optimum growth conditions for toxin production, and (e) to investigate the self-heating of *F. fusarioides* inoculated corn cultures.

RESULTS AND DISCUSSION

F. fusarioides was frequently isolated from millet and was sporadically found in other crops. The toxicity of all the isolates of *F. fusarioides* after incubation at 25 °C for 21 days was evaluated in ducklings (Table I). All the isolates were toxic to varying degrees. All further work was done with M.R.C. 35.

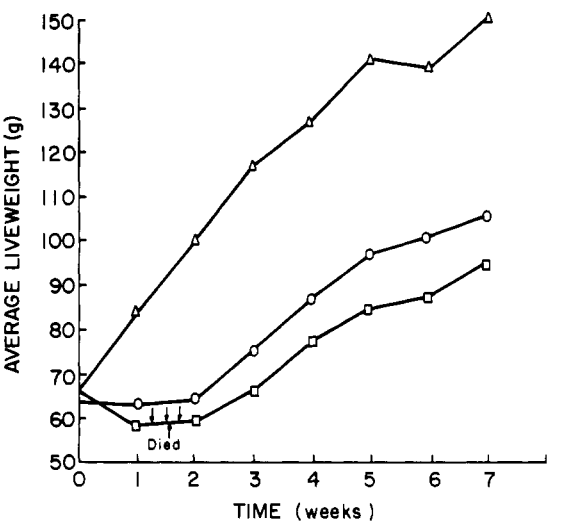


Figure 1. The influence of *Fusarium fusarioides* on the weight gains of rats, using 25% (○) and 50% (□) incorporation of moldy meal into the rat rations, compared with that of the control (△).

The toxicity of *F. fusarioides* to rats was determined using 25 and 50% incorporations of moldy meal into the ration. The results (Figure 1) showed that four out of eight rats which received the 50% moldy material died within 10 days, the survivors making very poor weight gains. None of the rats which received the 25% moldy meal ration died, but the weight gained was only 60% of that of the controls.

The long-term chronic effect of lower concentrations of the moldy meal was subsequently studied on rats. Commercial rat mash rations containing 5 and 10% moldy material were fed to rats over a period of 15 months. The results (Figure 2) showed that the 5% moldy material had a minimal effect on weight gains, but the 10% moldy diet resulted in an average of 25% reduction in weight gains as compared to the controls. None of the rats died.

Extraction of fungal cultures of *F. fusarioides* with chloroform yielded nontoxic extracts. Subsequent extraction with 80% methanol removed all the toxicity from the moldy meal. The toxic material was isolated and identified as moniliformin by comparison of its thin-layer chromatographic (TLC) behavior and IR and UV spectra with those of moniliformin (obtained from *F. moniliforme*). Thick-layer chromatography (system B) of a portion of the aqueous methanol extract gave moniliformin as a mixture

Table II. Influence of Temperature on Moniliformin Production and Toxicity of F. fusarioides to Ducklings

Incubation temperature, °C	Incubation period, days	Number of deaths	Average time to death, days	Total mass feed consumed, g	Moniliformin production,[a] g/kg
20	7	3/4	7	213	0.32
20	14	4/4	8	98	0.24
20	21	4/4	6	94	0.16
25	7	4/4	8	100	0.40
25	14	4/4	6	54	0.40
25	21	4/4	7	22	0.20
31	7	4/4	6	14	0.80
31	14	4/4	4	3	0.60
31	21	4/4	7	7	0.30
Control		0/4		390	0.00

[a] Determined by thin-layer chromatography.

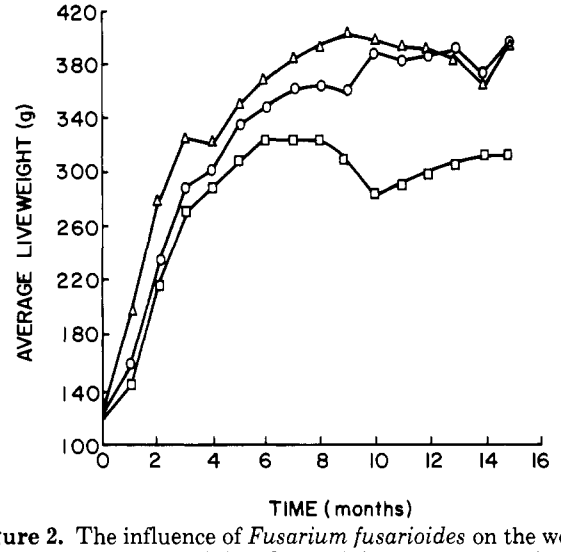


Figure 2. The influence of *Fusarium fusarioides* on the weight gains of rats, using 5% (○) and 10% (□) incorporation of moldy meal into the rat rations, compared with that of the control (△).

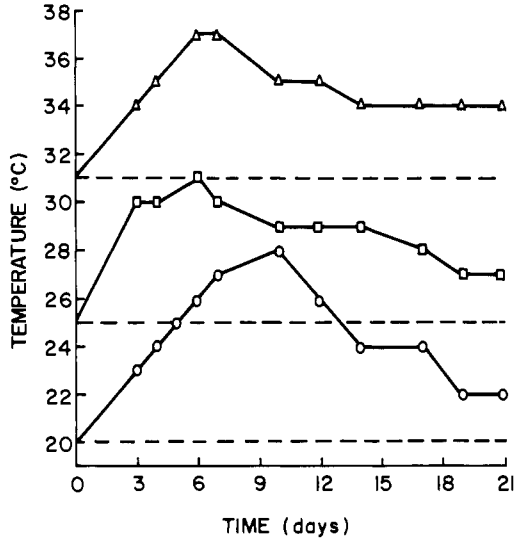


Figure 3. Self-heating of *Fusarium fusarioides* cultures at temperatures (---) of 20 °C (○), 25 °C (□), and 31 °C (△).

of sodium and potassium salts 150:1, respectively. The UV spectrum of moniliformin showed λ_{max}^{MeOH} at 229 (ϵ 18000) and 260 nm (ϵ 5000).

The effect of incubation temperature and period of incubation on toxin production and toxicity to ducklings was investigated. Cultures were incubated at constant temperatures of 20, 25, and 31 °C for periods of 7, 14, and 21 days. The results (Table II) show that all the cultures were toxic to ducklings.

Temperature measurements inside the jars showed that appreciable self-heating of the cultures occurred at all three incubation temperatures (Figure 3). When the actual culture temperatures recorded in the flasks are taken into consideration, it is clear that maximum toxin production occurred at a temperature of at least 34 °C, possibly at 36–37 °C. Relatively few fungi are capable of good growth at this temperature. *F. fusarioides* is a field fungus, and it is doubtful if temperatures above 30 °C are frequently encountered during its natural growth and development.

The amounts of moniliformin in each of the cultures which were incubated for various periods at three temperatures were estimated by comparing the absorption intensities of known standards against those of the extracts of TLC (systems A and B) (Table II). The highest yield was obtained at 31 °C after incubation for 7 days. This method of estimating moniliformin by quantitative TLC was also applied to five isolates of *F. fusarioides*, obtained from different geographical locations as seen in Table I. Attempts to determine moniliformin quantitatively by UV spectroscopy of the aqueous methanol extracts failed due to the interfering absorption of other metabolites at both 230 and 260 nm.

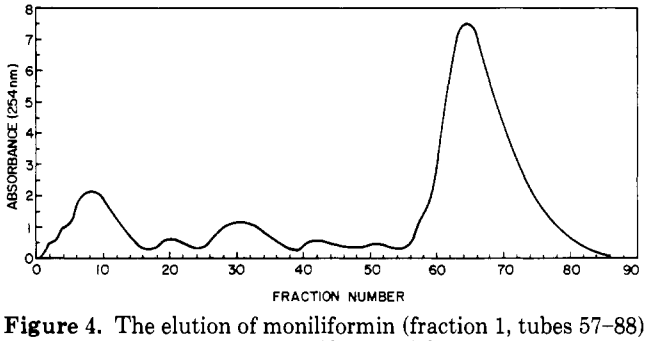


Figure 4. The elution of moniliformin (fraction 1, tubes 57–88) from a column of Dowex 1X8 (Cl^- form) by the successive use of 0.1 M sodium chloride (tubes 1–38), 0.15 M sodium chloride (tubes 39–48), and 0.2 M sodium chloride (tubes 49–88). The eluate was monitored by its absorption at 254 nm.

Moniliformin was isolated from a sample of maize meal inoculated with a culture of *F. fusarioides* and incubated at 31 °C and 7 days. A portion of this material was extracted with chloroform, followed by extraction with 80% methanol. An aqueous solution of the methanolic extract was absorbed on to a small amount of Dowex-1 (Cl^-) resin, applied to a column of the same resin, and eluted successively with 0.1, 0.15, and 0.2 M sodium chloride. The eluate was monitored by its UV absorption (254 nm) (Figure 4). Moniliformin was eluted by 0.2 M sodium chloride (tubes 57–88), the UV spectrum of this eluate

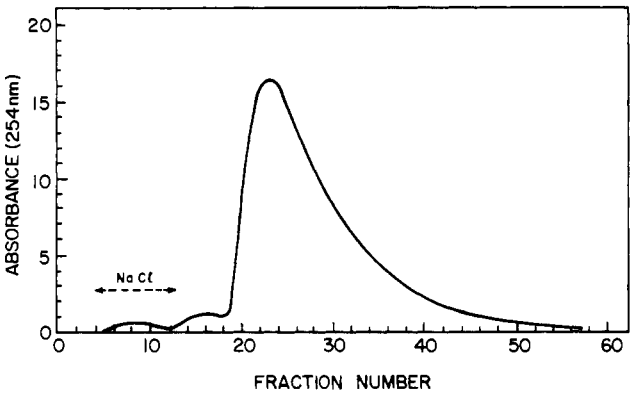


Figure 5. The elution of moniliformin (fraction 2, tubes 20–50) from a charcoal column with water. Sodium chloride was eluted in tubes 4–13. The eluate was monitored by its absorption at 254 nm.

showed a ratio for A_{260}/A_{229} of 0.295.

An aqueous solution of the foregoing fraction was applied to a charcoal column (French, 1955) and eluted with water, the eluate being monitored by its UV absorption (Figure 5). After the initial elution of sodium chloride, moniliformin (1, R = Na) was eluted (fraction 2), identical with an authenic sample of moniliformin.

The amount of moniliformin in the culture incubated at 31 °C for 7 days was determined from the extinction coefficient values (at 229 and 260 nm) of the saline solution obtained from the foregoing ion-exchange chromatography of the aqueous methanol extract. The value obtained by this UV spectrophotometric method (0.68 g/kg) is in close agreement with the concentration as determined by quantitative TLC of the same methanol extract (0.8 g/kg, Table II).

In another experiment the effect of time on the production of moniliformin by M.R.C. 35 at 31 °C was quantified by the above ion-exchange chromatography and UV spectrophotometric method. Corn samples were taken every third day after the start of the fermentation over a period of 30 days. Moniliformin yields indicated that production started at day three (~25 mg/kg) and reached a maximum after 12 to 18 days (950 mg/kg). The yields fluctuated after this period. The reason for the later production of maximum yield in this experiment is unknown, but probably due to factors such as differences in inoculum size or differences in water content of sterilized corn which may slightly differ from one batch to another.

The quantitation of moniliformin is readily accomplished by the UV spectrophotometric method or alternatively by thin-layer chromatography. The combination of ion-exchange and charcoal chromatography allows moniliformin to be isolated by a simple and rapid procedure.

EXPERIMENTAL SECTION

General Instrumentation. Ultraviolet (UV) spectra were determined (for solutions in methanol) with a Unicam SP 800 spectrometer. Infrared (IR) spectra were recorded with a Perkin-Elmer 237 spectrophotometer for 4% dispersions in potassium bromide. Atomic absorption spectrometry was carried out on a Perkin-Elmer 303 Spectrometer.

Materials. Fifty seed samples of millet were obtained from different geographical areas in Ovamboland, South West Africa, as well as a number of samples from Swaziland and the Transvaal Province of South Africa. Samples of various agricultural products such as peanuts, corn, casava, beans, sorghum, dried fish, etc. were obtained from Mozambique and microbiologically examined for the presence of *F. fusarioides*. The seeds were surface sterilized, macerated in a Waring blender, and plated out on potato dextrose agar containing albamycin. Plates were incubated at 25 °C and examined at regular intervals for the presence of *F. fusarioides*, which was subsequently isolated in pure culture. Eight of the resultant isolates of *F. fusarioides* were selected for evaluation of toxicity. Two isolates (M.R.C. 484 and M.R.C. 485) were obtained from Dr. C. Booth, Commonwealth Mycological Institute, and one (O.P. 126) from Dr. W.F.O. Marasas, South African Medical Research Council. Two additional isolates were obtained from dried fish collected in Mozambique. The origin and toxicity of the various isolates used in the present study are shown in Table I.

Culture Techniques. Inocula were prepared by growing the fungi on 30 mL of potato dextrose agar in 250-mL Erlenmeyer flasks after incubation at 25 °C for 10 days. Spore suspensions were used to inoculate whole yellow corn in 2-L fruit jars. The corn (400 g of corn and 400 mL of H_2O) was previously autoclaved from 1 h on two consecutive days at 121 °C and, after inoculation, incubated at 25 °C for 21 days. The material was dried in a forced-draught oven at 50 °C for 24 h, milled in a Wiley mill to a fine meal, and stored at 5 °C until used. Control meal was produced in the same way, except that it was not inoculated.

Toxicity Tests. (*a*) *Ducklings.* The moldy meal was incorporated into a commercial chicken mash on a 50% weight basis. Control feed consisted of yellow corn meal (autoclaved for 1 h on two consecutive days) mixed (50% by weight) with commercial chicken mash. One-day-old Pekin ducklings were used and were fed ad libitum for 14 days. Weights of survivors were recorded.

(*b*) *Rats.* Rats were BD IX males, used shortly after weaning. The moldy meal was incorporated into the commercial rat ration on a weight basis at the levels indicated in the various experiments. Control feed consisted of commercial rat mash to which had been added the same amounts of control meal. Six rats were used for each treatment.

Temperature Measurements. Temperature measurements of corn cultures were made by means of a Y.S.I. tele-thermometer. Glass tubes sealed at one end were inserted through the centers of the cotton plugs of the culture jars, within 3–4 cm of the bottom. Thermistor leads were passed down the tubes after autoclaving and positioned in the center of the culture mass. Temperature readings were recorded daily. Cultures were incubated in incubation rooms at constant temperatures of 20, 25, and 31 °C.

Extraction Procedure. Samples (50 g) of the moldy meal from *F. fusarioides* were extracted with chloroform (500 mL, 48 h) in Soxhlet extractors, followed by extraction with aqueous methanol (80%, 500 mL, 48 h). Toxicity tests showed that only the methanol extracts contained the toxic component. The methanol extracts were concentrated (ca. 90 mL), water added to 100 mL, and extracted with *n*-hexane. The aqueous methanol layers (100 mL) were analyzed for the presence of moniliformin by quantitative thin-layer chromatography.

Thin-Layer Chromatography (TLC). TLC was performed on precoated silica gel-60 F-254 plates (0.25 mm; Merck). The following solvent systems were used: (A) 1-butanol–acetic acid–water (4:1:5, upper layer; 16 h at 5 °C); (B) chloroform–methanol (3:2; 3 h at 20 °C). Moniliformin was detected by (a) observing the developed plates under UV light (254 nm) and (b) spraying the plates

with 2,4-dinitrophenylhydrazine (2,4-DNP) spray reagent (2 g of 2,4-DNP and 4 mL of sulfuric acid in 100 mL of methanol, or 1 g of 2,4-DNP in 500 mL of 1 N hydrochloric acid) and heating at 140 °C for 20 min. Moniliformin appeared as a brown spot (method b) at R_f 0.40 (system A) and 0.53 (system B). The minimum amounts of pure moniliformin detectable on a silica gel TLC plate by this method were found to be 0.15 and 0.75 μg for methods a and b, respectively. The estimated amounts of moniliformin in each of the methanol extracts were determined by quantitative TLC. Solvent system A was found to be preferred for TLC estimation of moniliformin. In these analyses, known quantities of moniliformin (5.0, 7.5, and 10.0 μg) were applied to the same plate as the samples under investigation. The results are listed in Table II. This method was also applied to the analysis of moniliformin in five isolates of *F. fusarioides*, obtained from different geographical locations (Table I). The lowest concentration of moniliformin found in a corn culture was 80 mg/kg of corn.

Thick-Layer Chromatography. Thick-layer chromatography was carried out on precoated silica gel-60 plates (2 mm; Merck). A portion of the aqueous methanol extract obtained from the material which was incubated at 31 °C for 7 days was applied to six plates (20 × 20 cm). The plates were developed twice (solvent system B) and dried. The moniliformin (detected under UV at 254 nm) was scraped off, eluted with methanol, and crystallized from aqueous methanol. An aqueous solution of moniliformin was analyzed for sodium and potassium by atomic absorption using the 5890 Å (Na^+) and 7665 Å (K^+) lines. Sodium and potassium were found to be present in a ratio of 150:1.

Isolation of Moniliformin. (*a*) *Extraction.* A sample (50 g) of moldy meal from *F. fusarioides* (incubation at 31 °C for 7 days) was extracted as described above.

(*b*) *Ion-Exchange Chromatography.* The aqueous methanol extract (3.4 g) was dissolved in water (30 mL) and Dowex 1X8 (Cl^- form, 200–400 mesh, 5 g) was added. The mixture was stirred for 30 min and filtered. A UV spectrum of the filtrate showed that all the moniliformin had been removed from the solution. The resin (on the filter) was washed with water until no UV-absorbing material was eluted and applied to a column of Dowex 1X8 (Cl^- form, 200–400 mesh, 25 × 1 cm). The column was successively eluted with 0.1 M sodium chloride (760 mL), 0.15 M sodium chloride (200 mL), and 0.2 M sodium chloride (800 mL) at a flow rate of 40 mL/h (Buchler Polystaltic Pump), 20-mL fractions being collected. The eluate was monitored by a Uvicord at 254 nm. The elution curve is given in Figure 4. Moniliformin (1, R = Na) was eluted in the 0.2 M sodium chloride (tubes 57–88). The amount of moniliformin in the eluate was estimated by UV spectroscopy to be 34 mg, using extinction coefficient value of 18000 (229 nm) and 5000 (260 nm). Fractions 61 to 76 (fraction 1, containing 28 mg of moniliformin) were combined and freeze-dried.

(*c*) *Charcoal Column Chromatography.* A mixture (3:2, by weight) of (A) Nuchar C-190 decolorizing carbon (Eastman Organic Chemicals, practical) and (B) activated cocoanut charcoal (50–200 mesh, Fisher Scientific Company) was successively washed with ethanol (5 volumes), saturated sodium chloride solution (5 volumes), and water (20 volumes) in a sintered-glass funnel. Fraction 1 was dissolved in water (10 mL), applied to a column (15 × 1 cm) of the charcoal mixture, and eluted with water at a flow rate of 20 mL/h. Initially, 3-mL fractions were collected. The eluate was monitored by a Uvicord at 254 nm. Fractions 1–15 were in addition monitored for sodium chloride by means of silver nitrate solution. The elution curve is shown in Figure 5. The fraction size was increased to 10 mL after tube 25. Sodium chloride was eluted in tubes 4–13 and moniliformin from tube 20 onwards. Fractions 21–35 were combined and freeze-dried (fraction 2, 25 mg). The UV spectrum of fraction 2 had λ_{max}^{MeOH} 229 nm (ε 18000) and 260 nm (ε 5000). The IR spectrum (λ_{max}^{KBr} 1780, 1705, 1680, 1615, 1110, and 840 cm^{-1}) and R_f values (systems A and B) of fraction 2 were identical with those of pure moniliformin.

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