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ORIGINAL PAPER

Guttation droplets of *Penicillium nordicum* and *Penicillium verrucosum* contain high concentrations of the mycotoxins ochratoxin A and B

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Abstract Eight of eleven ochratoxigenic isolates of *Penicillium nordicum* and *Penicillium verrucosum* produced guttation droplets when grown on Czapek yeast extract (CYA) agar for 10–14 days at 25°C. Parallel cultivation of one strain each of *P. nordicum* and *P. verrucosum* on malt extract agar demonstrated that higher volumes of exudate are produced on this agar. However, HPLC analyses revealed higher concentrations of ochratoxin A (OTA) and B (OTB) in droplets originating from cultures on CYA. For quantitative determination of the mycotoxin contents, triplicates of three isolates each of *P. nordicum* and *P. verrucosum* were grown as single spot cultures on CYA for up to 14 days at 25°C. Guttation droplets were carefully collected between day 11 and 14 with a microliter syringe from each culture. Extracts from exudates and corresponding mycelia as well as fungal free agar were analyzed by HPLC for the occurrence of ochratoxin A (OTA) and B (OTB). Mean concentrations ranging between 92.7–8667.0 ng OTA and 159.7–2943.3 ng OTB per ml were detected in the guttation fluids. Considerably lower toxin levels were found in corresponding samples of the underlying mycelia (9.0–819.3 ng OTA and 4.5–409.7 ng OTB/g) and fungal free agar (15.3–417.0 ng OTA and 12.7–151.3 ng OTB/g).This is the first report which shows that high amounts of mycotoxins could be excreted from toxigenic *Penicillium* isolates into guttation droplets.

Keywords Exudate · Fungal droplets · Guttation · Mycotoxin · Ochratoxin A · Ochratoxin B · *Penicillium* spp. · Secondary metabolites

Abbreviations

CYA	Czapek yeast extract agar
HPLC	High performance liquid chromatography
MEA	Malt extract agar
OTA	Ochratoxin A
OTB	Ochratoxin B

Introduction

Guttation, the phenomenon of active excretion of water and dissolved materials from leaves and other uninjured plant organs has been known for over 300 years [1]. The term guttation originates from the latin ‘‘gutta’’ = drop. The production of

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guttation droplets is also a common feature on the surface of fungal mycelia. However, reference to these exudates on various fungal structures is infrequent, and often these liquid droplets are only casually mentioned. Many penicillia and aspergilli exude drops and particularly in *Penicillium* spp. the droplet production has been used taxonomically [2–4]. About the mechanism and significance of droplet formation only less is known.

Jennings [5] suggested that droplets could serve as a reservoir of water allowing hyphae, not in direct contact with liquid medium, to continue growth when the feeding mycelium might be subjected to an unfavorable water potential.

Biochemical analyses of exudates from various fungi including *Claviceps purpurea*, *Fusarium culmorum*, *Penicillium vulpinum* (syn. *claviforme*), and *P. chrysogenum* (syn. *notatum*) indicated the presence of proteins in the liquid droplets, and enzyme activities were demonstrated in most.

McPhee & Colotelo [6] noted that exudate from *Fusarium culmorum* rapidly degrade plant tissues, as did the liquid from *Sclerotinia sclerotorium* [7].

These findings clearly point to ecological functions of guttation droplets. However, up to now no information is available about the occurrence of toxic secondary metabolites in exudates from *Penicillium* spp. although just these are well-known producers of guttation droplets [2–4, 8, 9].

The aim of this study was to examine various *Penicillium* strains for the occurrence of liquid droplets to determine whether mycotoxins are excreted into these exudates. For that purpose *Penicillium* strains known to be producers of the nephrotoxic and cancerogenic mycotoxin ochratoxin A were investigated.

Material and methods

Fungal cultures

A total of 11 *Penicillium* strains from the culture collections of the Institute of Microbiology and Toxicology of the Federal Center for Nutrtition and Food (SP strains) and from the BioCentrum-DTU, Technical University of Denmark (IBT strains) have been used: *Penicillium nordicum* (SP: 134, 1337, 1340, 1840; IBT: 13308, 14873, 19486) and *Penicillium verrucosum* (SP: 1002; IBT: 10039, 13077, 22123).

All strains have been previously characterized as producers of the mycotoxin ochratoxin A.

Screening for exudate and mycotoxin production

In order to examine exudate production, all strains were cultivated as three point cultures on Czapek Yeast Extract agar (CYA; Merck, Darmstadt, Germany) up to 3 weeks at 25°C and examined visually every day.

Two exudate producing strains (*P. nordicum* SP: 1840 and *P. verrucosum* SP: 1002) were cultivated in parallel on malt extract agar (MEA; Merck). Droplets from single cultures were carefully collected with a 10-µl Hamilton syringe (Alltech, Munich), transferred to glass vials, the volumes then quantified and subsequently analyzed by HPLC for the presence of ochratoxins.

Quantitative determination

Triplicates of single spot cultures of six isolates (*P. nordicum*: SP: 1840; IBT: 13308, 14873; *P. verrucosum*: SP: 1002; IBT: 10039, 13077) were grown on CYA for 14 days at 25°C. Guttation droplets of each culture were collected as described above. The underlying mycelia were cut off with a sterile scalpel, weighed, and transferred into a 100 ml glass vial. The fungal free agar of every agar plate was completely removed, weighed, and also transferred into 100 ml glass vials.

Extraction of ochratoxins

To 20 µl of exudate 80 µl of methanol were added and the mixture was then vortexed for 30 s. About 10 µl of the exudate mixture, equivalent to 2 µl of exudate, were directly applicated onto the HPLC column.

The fungal mycelia and the remaining fungal free agar samples were extracted each with 20 ml $CHCl_3$ for 10 min. The mixtures were then filtered through Na_2SO_4 and the solvent evaporated. The

residues were dissolved in 2 × 3 ml methanol, filtered through a 0.45 μm Millex HV_4 microfilter and transferred to 10 ml glass vials. The organic phase was evaporated to dryness with a gentle stream of nitrogen at 40°C and then the remaining residue redissolved in 3 ml. About 10 μl of each sample, equivalent of 0.03 g mycelium and 0.13 g fungal free agar, were injected for HPLC-analysis.

HPLC analysis of ochratoxin A and B

Analysis was carried out with a HPLC method according to Curtui et al. [10]. The system was equipped with a Model 422 pump, a Model 465 autosampler, a Model SFM 25 fluorescent detector (all from Kontron, Neufahrn, Germany), and an Altima C18 5 μm column (Alltech Assoc. Inc., Unterhaching, Germany). The mobile phase was acetonitrile-water-acetic acid (57:41:2; v/v/v) at a flow rate of 1 ml/min and a column temperature of 25°C. Fluorescence excitation and emission wavelengths were 330 nm and 460 nm, respectively. A volume of 10 μl was injected for standards (Sigma) and samples which gave clear chromatograms and retention times of ~8.2 min for OTA and ~5.3 min for OTB. Confirmation of OTA and OTB was done as previously described by re-chromatography of methyl esters of both toxins [10, 11]. For that purpose 50 μl methanol containing 14% boron trifluoride (Sigma) were added to the evaporated sample, the mixture kept at 80°C for 15 min and then evaporated to dryness under a gentle stream of nitrogen. Under the chromatographic conditions described above, the retention times of the methyl esters of OTA and OTB were ~17.8 and ~10.5, respectively. The detection limits for OTA and OTB were 0.1 ng/g and 0.2 ng/g, respectively.

For quantification, peak areas of OTA and OTB in samples were compared with the standards and integrated by the program DS 450-MT2/DAD V1.32 (Kontron).

Results

Guttation droplets were produced by eight of the eleven *Penicillium* strains tested after growth for 10–14 days on CYA. The *P. nordicum* strains SP: 134, 1337, 1340 produced no exudate at all and the droplets observed on cultures of *P. nordicum* IBT: 19486 and *P. verrucosum* IBT: 22123 were too tiny to be picked up with the micro liter syringe.

Analyses of the exudates produced by the two *Penicillium* strains (Fig. 1) grown in parallel on CYA and MEA were found to be positive for OTA and OTB (Table 1). High concentrations of both mycotoxins were detected in droplets originating from CYA cultures although the total volume of droplets collected from a single culture was higher when strains were grown on MEA. The HPLC analyses gave very clear chromatograms and the identity of the ochratoxins could be confirmed by methyl ester formation and re-chromatography (Fig. 2).

Consequently, for quantitative determination of the mycotoxins contents in exudates, the underlying mycelia and the fungal free agar medium, triplicates of three strains each of *P. nordicum* and *P. verrucosum* were cultivated as single spot cultures on CYA for 14 days.

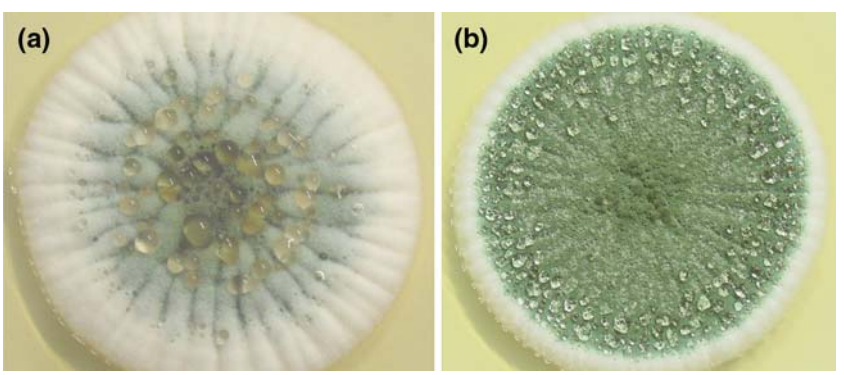

Fig. 1 Production of guttation droplets by (**a**) *Penicillium nordicum* SP: 1840 and (**b**) *Penicillium verrucosum* SP: 1002 cultivated on Czapek yeast agar for 14 days at 25°C

Table 1 Production of exudate and ochratoxin A and B levels by two *Penicillium* strains cultivated for 14 days on Czapek yeast agar and malt extract agar

Isolate	Growth on	Volume of exudate of one single culture	Toxin concentration
P. nordicum SP: 1840	CYA	55.7 µl	688 ng OTA/ml 414 ng OTB/ml
	MEA	70.7 µl	14 ng OTA/ml OTB negative
P. verrucosum SP: 1002	CYA	97.3 µl	4750 ng OTA/ml 3563 ng OTB/ml
	MEA	211.5 µl	630 ng OTA/ml 1661 ng OTB/ml

CYA: Czapek yeast agar

MEA: Malt extract agar

OTA: ochratoxin A

OTB: ochratoxin B

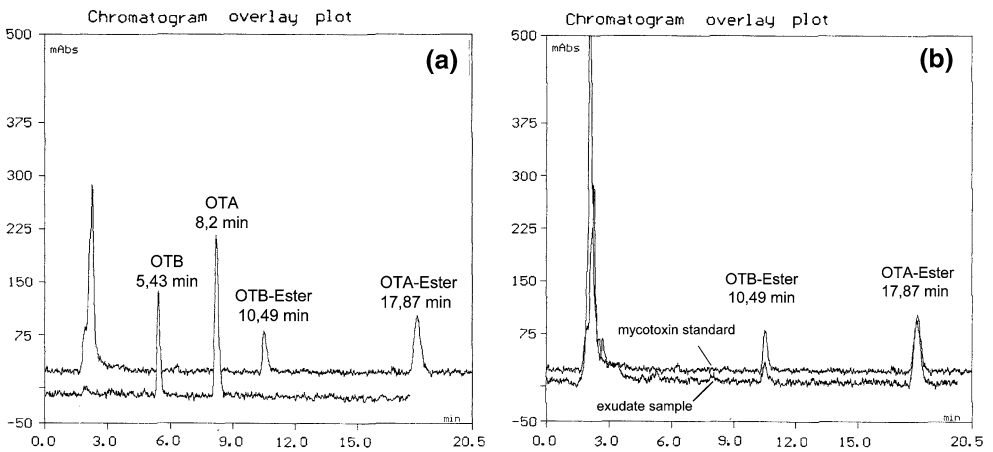

Fig. 2 HPLC analysis of ochratoxin A (OTA) and B (OTB). (**a**) Chromatogram overlay of non-derivatized toxin standards and corresponding methyl esters (**b**) Chromatogram overlay of derivatized toxin standards and an exudate extract from *P. nordicum* SP: 1840

The guttation droplets were big enough between 11 and 14 days of cultivation to be collected with the microliter syringe.

The single cultures produced on average between 55 and 110 µl of exudate (Table 2). Some strains started immediately after collection of the droplets at day 11 with an additional production of exudate. The amount of these droplets collected 2 days later was found to be about 50% of the volume of the first collection.

HPLC analyses of exudates, underlying mycelia and fungal free agar media showed the presence of the mycotoxins OTA and OTB in all samples (Tables 3 and 4).

However, in comparison with the mycelium and agar extremly high concentrations of the toxins were found in the guttation droplets.

The *P. nordicum* isolates produced on average between 1656.7 and 6179.0 ng OTA/ml of exudate per culture. In the corresponding samples of mycelium and agar the levels were only in the range of 531.0–819.0 ng/g and 35.3–417.0 ng/g, respectively (Table 3). Among the *P. verrucosum* strains the mean concentrations in the guttation fluids reached from 92.7 ng OTA/ml by the weak ochratoxin producer SP: 1002 to 8667.0 ng OTA/ml by isolate IBT: 10039, which produced the highest amount of

Table 2 Production of guttation droplets by six *Penicillium* strains grown on Czapek yeast agar

Isolate	Volume of exudate collected (µl)			
	Plate 1	Plate 2	Plate 3	Mean
P. nordicum SP: 1840*	110	105	116	110
P. nordicum IBT: 13308	45[a]	60[a]	60[a]	55[a]
	26[b]	31[b]	23[b]	27[b]
P. nordicum IBT: 14873	70[a]	50[a]	50[a]	55[a]
	32[b]	20[b]	Trace[b]	26[b]
P. verrucosum SP: 1002*	85	106	82	91
P. verrucosum IBT: 10039	90[a]	103[a]	79[a]	91[a]
	60[b]	32[b]	35[b]	42[b]
P. verrucosum IBT: 13077	86[a]	75[a]	94[a]	85[a]
	27[b]	50[b]	55[b]	44[b]

[a] First collection on day 11 after inoculation

[b] Second collection on day 13 after inoculation

* Collected on day 14 after inoculation

Table 3 Concentration of ochratoxin A in exudates, mycelium, and fungal free agar

Isolate	Mean concentration (ng/ml or g) [SD]		
	Exudate $n = 3$	Mycelium $n = 3$	Agar $n = 3$
P. nordicum SP: 1840*	2045.0 [99.9]	819.3 [108.0]	37.3 [4.6]
P. nordicum IBT: 13308	6179.0 [2170.0][a]	711.7 [547.2]	35.3 [47.4]
	3533.3 [1672.0][b]		
P. nordicum IBT: 14873	1656.7 [499.6][a]	531.0 [113.8]	417.0 [269.0]
	1350.0 [733.2][b]		
P. verrucosum SP: 1002*	92.7 [23.4]	9.0 [3.0]	65.3 [39.4]
P. verrucosum IBT: 10039	8667.0 [1002.1][a]	783.7 [240.0]	248.3 [86.6]
	5823.3 [945.1][b]		
P. verrucosum IBT: 13077	1526.7 [370.7][a]	226.7 [34.2]	15.3 [3.2]
	1067.0 [187.1][b]		

SD: standard deviation

[a] Exudate collected on day 11 after inoculation

[b] Exudate collected on day 13 after inoculation

* Collected on day 14 after inoculation

toxins detected in this study. In the samples of mycelia and agar clearly lower levels of OTA were found (Fig. 3).

Analyses of the exudate droplets collected from the isolates IBT: 13308, 14873, 10039, and 13077 two days after the first collection revealed still very high mean toxin concentrations up to 5823.3 ng OTA/ml (Table 3).

OTB was also detected in high concentrations in the guttation droplets of all strains investigated (Table 4). Mean exudate levels ranged between 159.7 (*P. verrucosum* SP: 1002) to 2943.3 ng OTB/ml (*P. nordicum* IBT: 13308). Again comparably lower concentrations were found in the corresponding samples of mycelium and fungal free agar (4.5–409.7 OTB/g and 17.3–151.3 OTB/g, respectively) (Fig. 4).

These results clearly indicate that both ochratoxins are concentrated to a great degree in the liquid fluids of the toxigenic fungi.

Discussion

Observation of guttation droplets is part of everyday experience of practising mycologists. The reason for this exudation activity by fungi has

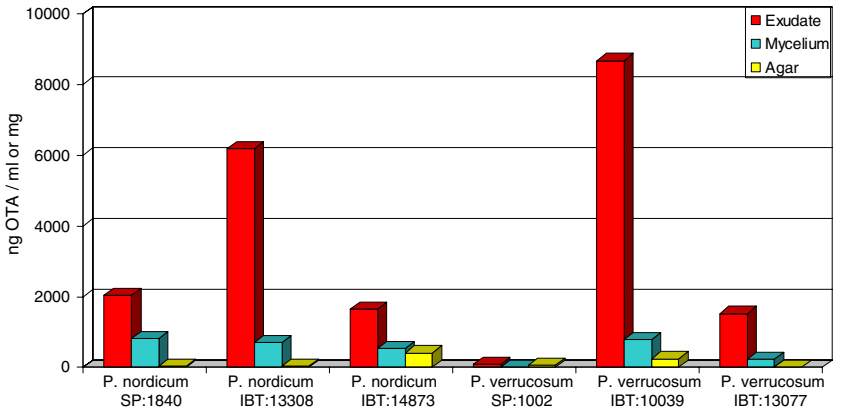

Fig. 3 Comparison of mean ochratoxin A (OTA) levels in exudate, mycelium, and fungal free agar from six *Penicillium* isolates

Table 4 Concentration of ochratoxin B in exudate, mycelium, and fungal free agar

Isolate	Mean concentration (ng/ml or g) [SD]		
	Exudate $n = 3$	Mycelium $n = 3$	Agar $n = 3$
P. nordicum SP: 1840*	1953.3 [177.2]	409.7 [199.1]	22.0 [2.0]
P. nordicum IBT: 13308	2943.3 [756.9][a] 1843.3 [713.0][b]	222.3 [194.7]	25.3 [19.5]
P. nordicum IBT: 14873	2046.7 [657.7][a] 1420.3 [762.8][b]	334.7 [198.9]	151.3 [150.6]
P. verrucosum SP: 1002*	159.7 [105.38]	4.5 [3.8]	17.3 [10.7]
P. verrucosum IBT: 10039	1100.0 [181.9][a] ND[b]	119.3 [39.7]	42.7 [9.7]
P. verrucosum IBT: 13077	1710.0 [285.1][a] 726.7 [257.4][b]	36.0 [2.6]	12.7 [3.8]

SD: standard deviation

[a] Exudate collected on day 11 after inoculation

[b] Exudate collected on day 13 after inoculation

* Collected on day 14 after inoculation

ND: not detected

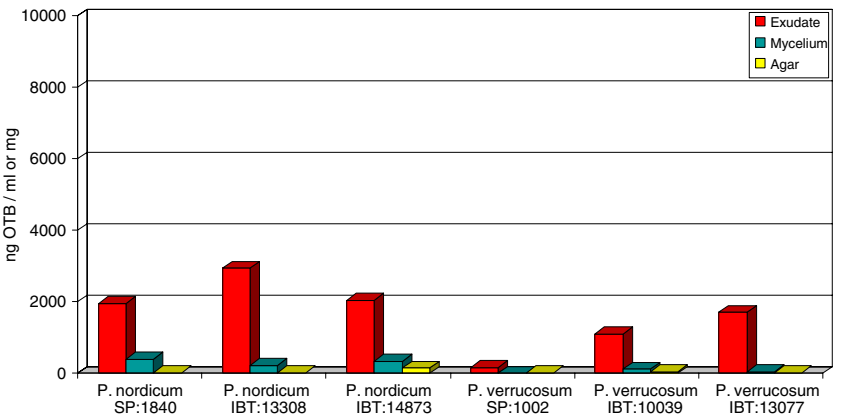

Fig. 4 Comparison of mean ochratoxin B (OTB) levels in exudate, mycelium, and fungal free agar from six *Penicillium* isolates

been attributed with no importance [12] or has been controversly discussed [5,7,13]. While Jennings [5] believed that the role of the droplets is just in helping to maintain a constant growth rate of aerial hyphae, some authors discussed the function of droplets as reservoir for enzymes and

a variety of solutes [7, 13–15]. McPhee and Colotelo [13] have found a definite pattern in the distribution of droplets produced by *Fusarium culmorum* and argued that the hyphal tips are a very active metabolic region and devoid of vacuoles, use the droplet system as a reservoir for secondary metabolites, by-products, or metabolite reserves, which could be readily available when needed. Grovel et al. [16] reported about the excretion of the cytotoxic and immunosuppressive gliotoxin in an exudate of a strain of *Aspergillus fumigatus*, which has been isolated from sediments of a mussel bed. They also found an accumulation of this mycotoxin in the meat of mussels.

Interestingly, for *Penicillium* spp., which has a distinctive ability to produce guttation droplets, nothing is known about the function or content of these exudates. However, penicillin has been found in droplets in approximately the same concentration as in the underlying culture broth of a penicillin producing *Penicillium* isolate (K.B. Raper, personal communication cited in [7]).

More than 30 *Penicillium* species are described to produce exudates [4, 8], among them *P. nordicum* and *P. verrucosum* which are also potent producers of ochratoxins [17]. Ochratoxin A has been found predominantly in cereals and cereal products but also in a variety of other food commodities [18–21] and even airborne dust [22]. It is a potent nephrotoxin and renal carcinogen in rodents, causes mycotoxicosis in animals, particularly in swine, and is associated with human kidney disease in certain countries [20, 21, 23]. Ochratoxin B is the dechlorinated analog of OTA and is considered less toxic than the parent compound [24]

Results of our study demonstrate that the strains investigated are able to produce up to 110 µl guttation droplets per culture within 10–14 days of cultivation on CYA. Although not all droplets could be collected with the microliter syringe and therefore many of the tiny droplets remained on the mycelium, HPLC analyses demonstrated very high levels of OTA and OTB in the exudate as compared with the underlying mycelium. The concentration of OTA in the droplets reached more than 8 ppm for *P. verrucosum* IBT: 10039 and clearly exceed the levels detected in the mycelium and agar. Looking at all results obtained, the concentrations of OTA in guttation droplets were up to 11 times and 176 times higher than in the mycelium and agar, respectively. In case of OTB, the levels in the liquid fluid were found to be up to 47.5× and 132× higher than in the corresponding samples of mycelium and agar.

These data clearly indicate for the first time that high amounts of mycotoxins could be excreted from toxigenic *Penicillium* isolates into guttation droplets.

The reasons for this are not clear. It could be hypothesised that the fungus regulates by this way the concentration of the mycotoxins produced and, at certain levels, gets rid of these compounds. As the function of ochratoxins for the producing fungus and a natural target organism, if there is any, is not known, the interpretation of the findings remain speculative. An insecticidal effect of OTA containing sclerotia of *Aspergillus carbonarius* against larvae of the detritivorous beetle *Carpophilus hemipterus* and corn ear worm *Helicoverpa tea* have been reported by Wicklow et al. [9, 25]. Based on these results, the action of OTA could be directed to insects competing with the fungus for the substrate.

Several strains of the basidiomycete *Pleurotus* are known to produce droplets containing a toxin that killed *Rhabditis*, a nematode, on contact with the nematodes head [26]. The same function, i.e., the defense of mycophagic mites and other organisms could be true for the role of mycotoxin containing droplets produced by *Penicillium* spp.. However, a previous study has shown that *Tyrophageus casei*, a mycophagic mite, obviously is not negatively affected by ochratoxins [27]. After grazing the mycelia of an ochratoxigenic *P. verrucosum* strain grown on sterilized barley, living mites contained, in contrast to the substrate, high levels of OTA and OTB. Besides a possible mechanism to defense mycophagic organisms and substrate competitors, the attraction of organisms in order to spread fungal spores could be attributed to guttation droplets. The high ochratoxin concentrations found in the liquid exudates clearly point to an ecological function. However, more studies on the role and significance of guttation droplets are needed to fully understand

the physiological and ecological importance. If the toxic droplets should occur under natural conditions, it must be questioned if these exudates can play a role as vectors contaminating the environment.

Independent of these discussion points, the exudate droplets of highly toxigenic strains such as *P. verrucosum* IBT: 10039 could be used for laboratory purposes, i.e., getting ochratoxin A and B reference standard in a very simple way and without extensive cleanup procedure.

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