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The Influence of Temperature on the Growth of Mould *Aspergillus ochraceus* NRRL 3174 and on Ochratoxin A Biosynthesis in Pure and Mixed Culture

Utjecaj temperature na rast plijesni *Aspergillus ochraceus* NRRL 3174 i na biosintezu okratoksina A u čistoj i mješovitoj kulturi

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Summary

In the present study the growth of ochratoxigenic mould ***Aspergillus ochraceus*** *NRRL 3174 and ochratoxin A (OTA) synthesis were investigated during the cultivation of the mould on corn grains in a pure culture, as well as in a mixed culture with the non-ochratoxigenic mould* ***Trichothecium roseum*** *ZMPBF 1226.*

The amount of synthesised biomass was determined indirectly by measuring the chitin, while the quantification of OTA was performed fluorodensitometrically.

During the 35 days cultivation of the tested mould in a stationary culture at temperatures 15 °C, 20 °C, 25 °C and 30 °C, and the water content in the substrate of 38 %, the highest concentration of OTA was reached at 20 °C. At higher temperatures, 25 °C and 30 °C, the biomass synthesis was increased, but at the same time OTA synthesis decreased. OTA concentration was maximal after 14 days of cultivation.

A mixed culture of the tested moulds reduced OTA biosynthesis, and after 14 days the OTA concentration dropped to 50–60 % of the highest value obtained in the pure culture.

Sažetak

U ovom je radu istražen rast okratoksikotvorne plijesni ***Aspergillus ochraceus*** *NRRL 3174 i sinteza okratoksina A (OTA) na kukuruznom zrnu u čistoj i mješovitoj kulturi s neokratoksikotvornom plijesni* ***Trichothecium roseum*** *ZMPBF 1226.*

Rast je biomase posredno određivan mjerenjem količine hitina, a koncentracija OTA praćena fluorodenzitometrijski.

Istraživanjem plijesni ***A. ochraceus*** *NRRL 3174 provedenim tijekom 35 dana u stacionarnoj kulturi pri temperaturama od 15, 20, 25 i 30 °C, te pri udjelu vode u supstratu od 38 %, utvrđeno je da se najveća koncentracija OTA postiže pri temperaturi uzgoja od 20 °C. Više temperature uzgoja, 25 i 30 °C, povoljno djeluju na rast plijesni, ali istodobno utječu na smanjenje biosinteze OTA. Maksimalna koncentracija OTA postignuta je nakon 14 dana uzgoja.*

Mješovita kultura istraživanih plijesni snižuje koncentraciju OTA, a nakon 14. dana koncentracija OTA iznosila je 50–60 % od maksimalne vrijednosti dobivene tijekom procesa biosinteze u čistoj kulturi.

Introduction

Among numerous mycotoxins, as secondary metabolites of the moulds of *Aspergillus* and *Penicillium* species, there is a group of mycotoxic and cancerogenic compounds named ochratoxins *(1)*. The best known and

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certainly the most toxic metabolite produced by *Aspergillus ochraceus* is ochratoxin A (OTA), isolated for the first time in 1965 in South Africa *(2,3)*.

OTA is a naturally occurring mycotoxin with the pronounced nephrotoxic, hepatotoxic, haemorrhagic and teratogenic effects. It was isolated from several individual species of *Aspergillus ochraceus* group, and from *Penicillium* sp. *(4,5)* which are typical moulds found as contaminants in warehouses. The activity of these fungi depends on environmental conditions, mostly on the air temperature and the water content in a substrate.

Chemically, OTA is 7-carboxy-5-chloro-8-hydroxy--3,4-dihydro-3R-methyl iso-coumarin linked to β-L--phenylalanine through peptide bond.

Numerous authors referred to natural occurrence of OTA in food, feed and related products *(6,7)*. It was isolated from oats and barley *(8,9)*, flour and mouldy bread *(10)*, naturally moistly wheat *(11)*, commercial corn, rye, peanuts, coffebeans, soya, cocoa beans and from commercial food like pork meat, sausages *(12)* and smoked ham and meat *(13)*.

In the investigations carried out with animals to which different doses of OTA were applied, consequences were ochratoxicosis in birds, diarrhoea and polyuria in rats *(14)*, skin fibrosarcome in rainbow trout *(15)* and other symptoms. Given during a longer period of time, OTA caused chronic damages of liver and kidney, like atrophy of the proximal tubules, necrosis of hepatocytes and fat liver infiltrations *(16–18)*.

After oral administration, a lethal dose determined for young rats was 20–22 mg kg^{-1} body weight *(19)*, and for male and female guinea pigs it was 9.1 and 8.1 mg g^{-1} body weight, respectively *(20)*.

Mycotoxic porcine nephropathy (MPN), an endemic disease described for Danish swine over 70 years ago *(21)*, was mainly associated with the fungal contamination of feed with OTA *(22, 23)*. A similar nephropathy has been described in several other European countries *(6,7)*. The incidence of nephropathy was often especially high following wet harvesting periods.

The fact that OTA is highly nephrotoxic for almost all laboratory and domestic animals was an important indicator of the possible involvement of OTA in the aetiology of Balkan Endemic Nephropathy (BEN) *(24, 25)*. BEN is a renal disease in humans, reported in some rural areas in northern Croatia, Bulgaria, Romania, Bosnia and Herzegovina and Serbia *(26–30)*. The disease is characterised by slow, progressive tubular and/or glomerular lesions, accompanied by a decrease in renal function. The mycotoxic hypothesis postulated in the aetiology of BEN seems even more probable having in mind that functional and structural changes in kidneys of nephropathes were comparable to damages obtained at ochratoxicoses in animals, especially in pigs *(31)*.

For that reason, human exposure to OTA has been followed based on detection of OTA in food, feed and blood of the inhabitants from the endemic nephropathic areas *(32–34)*.

Despite the fact that OTA mutagenecity was experimentally confirmed in rats *(35)*, but not in humans, because of its pronounced toxicity OTA is considered to be a potent carcinogen. Tolerable daily intakes for humans were estimated to be 0.2 to 4.2 ng kg^{-1} body weight per day *(7)*.

Besides being a great risk to human health, feeding animals with contaminated feed might produce significant damage in agriculture.

In view of the great importance of OTA biosynthesis and the complexity of problems rising during the fungal growth in mixed cultures, the aim of this study was to elucidate how particular parameters, similar to those found in warehouses, influence biomass synthesis and OTA production by *Aspergillus ochraceus* NRRL 3174 in pure culture, and in mixed culture with naturally occurring, non-ochratoxigenic mould, *Trichothecium roseum* ZMPBF 1226.

Materials and Methods

Microorganisms

The mould found as a usual contaminant on corn, which does not produce ochratoxin, was identified as *Trichothecium roseum* and is held in the Collection of Microorganisms of the Faculty of Food Science and Biotechnology of Zagreb (ZMPBF) under the serial No. 1226.

Aspergillus ochraceus NRRL 3174 strain, described as one of the strongest OTA producers, was obtained from the USDA Fermentation Laboratory of the Northern Regional Research Centre, Peoria, Il.

Biomass and OTA production were compared under different cultivation conditions:

a) *A. ochraceus* NRRL 3174 grown in a pure culture

b) *A. ochraceus* NRRL 3174 grown in a mixed culture with *T. roseum* ZMPBF 1226

Preparation of inoculum

The pure culture of the investigated mould was subcultured on potato-dextrose agar slants for sporulation. After 7 days' incubation at 28 °C, 5 cm^3 of sterile Triton X-100 solution (5 mg dm^{-3}) were added. Conidia, along with parts of mycelium were harvested with an inoculation loop, and homogenised in a Poter's homogeniser.

Inoculum was diluted to the concentration of $5 \cdot 10^7$ spores per cm^3.

Biomass determination

Whole corn grains were used as a substrate. In the experiments with the pure culture, substrates (50 g of maize in 500 cm^3 Erlenmeyer flasks) were inoculated with 1 cm^3 suspension containing $5 \cdot 10^7$ spores of the mould *A. ochraceus* NRRL 3174, and in the experiments with mixed culture the inoculum contained $5 \cdot 10^7$ spores of each mould, *A. ochraceus* NRRL 3174 and *T. roseum* ZMPBF 1226.

The parameters maintained during the cultivation were as follows:

- initial water content in the substrate: 38 %;
- initial number of conidia: 10^6 per gram of substrate;
- incubation temperature: 15 °C, 20 °C, 25 °C and 30 °C;
- cultivation time: 35 days.

Prior to inoculation, the water content in the substrate was determined and then sterile water was added to the final water content of 38 %. Flasks were shaken on a laboratory shaker for 30 min to achieve equilibration between liquid and solid phase, and sterilisation was performed at 121 °C for 30 min. During the incubation, growth was followed by measuring the amount of biomass and OTA concentration in samples of two flasks taken in 7-day intervals. For the biomass amount determination a »chitin method« was applied, described earlier by Donald and Mirocha *(36)*.

OTA determination and measurement

OTA was isolated by extraction with chloroform (150 cm^3) on a laboratory shaker (100 cycles per min) for 30 min at room temperature. Crude extracts were separated from the culture medium by filtration under reduced pressure and evaporated to approximately 5 cm^3 on a rotary vacuum evaporator (50 °C, 0.66 kPa). The extracts were further purified by chromatography on a 40 × 400 mm column filled with silica gel *(37,38)*. OTA was identified by thin-layer chromatography (TLC) using precoated 20 × 20 cm silica gel F_{254} plates (Merck, Darmstadt, Germany). The plates were developed with benzene : acetic acid (9 : 1) *(39)* and after drying OTA was detected as a fluorescent spot made visible by UV light ($\lambda = 366$ nm). R_f values of the samples were comparable with the R_f value of the OTA standard chromatographed simultaneously. Fluorescence intensity, linearly proportional to the OTA concentration, was measured directly using a »Camag« fluorodensitometer, equipped with the 1100 W + W plotter *(40)*.

Results and Discussion

It is well known that the growth of fungi strongly depends on conditions maintained during cultivation, but considerably less is known about their influence on the production of secondary metabolites. According to the present knowledge, secondary metabolites often start to be produced after a decrease in growth rate *(41)*.

In the studies published so far, temperatures reported to be optimal for OTA production by fungi from *Aspergillus* species were 28 °C and 37 °C, respectively *(42, 43)*. Insufficient and miscellaneous results actuated the authors of this study to investigate the relationship between fungal growth (measured as glucosamine equivalents) and ochratoxin A production in a pure culture of *A. ochraceus* NRRL 3174, as well as in a mixed culture of *A. ochraceus* NRRL 3174 and *T. roseum* ZMPBF 1226 at different temperatures (Fig. 1).

Whole corn grains were used as a substrate, since naturally occurring contaminations were mostly found on cereals. One of the reasons for the lack of information about mould growth and OTA production on natural solid substrates might be due to difficulties in measuring fungal growth on solid substrates. The plate dilution method has often been used, but this method only reflects the number of live fungal elements and not the ac-

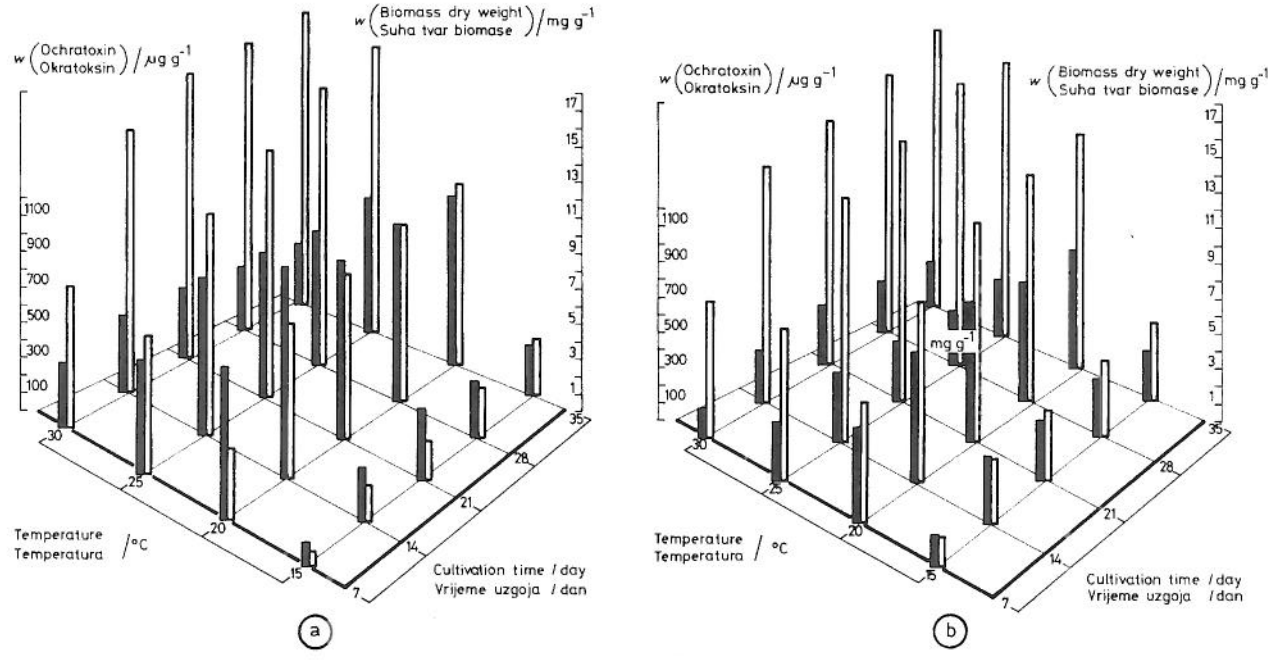

Fig. 1. Comparison of mould biomass and synthesized ochratoxin A
a) pure culture of *Aspergillus ochraceus* NRRL 3174,
b) mixed culture of *A. ochraceus* NRRL 3174 and *Trichothecium roseum* ZMPBF 1226 on the whole maize grain with 38 % water content, with respect to cultivation time and temperature (OTA ■, biomass □)
Slika 1. Usporedba biomase i sintetiziranog okratoksina A
a) čista kultura plijesni *Aspergillus ochraceus* NRRL 3174,
b) mješovita kultura *A. ochraceus* NRRL 3174 i *Trichothecium roseum* ZMPBF 1226 na cijelom zrnu kukuruza s udjelom vode od 38 %, ovisno o vremenu i temperaturi uzgoja (OTA ■, biomasa □)

Table 1. Decrease in OTA mass fraction with respect to temperature during the cultivation of *A. ochraceus* NRRL 3174 grown in pure culture and *A. ochraceus* NRRL 3174 grown in mixed culture with *T. roseum* ZMPBF 1226 on corn grain

Tablica 1. Snizivanje masenog udjela OTA u ovisnosti o temperaturi tijekom rasta čiste kulture plijesni *A. ochraceus* NRRL 3174 i mješovite kulture plijesni *A. ochraceus* NRRL 3174 i *T. roseum* ZMPBF 1226 na zrnu kukuruza

Mould	*A. ochraceus* NRRL 3174				*A. ochraceus* NRRL 3174 *T. roseum* ZMPBF 1226			
Initial water fraction / %	38				38			
Incubation temperature / °C	15	20	25	30	15	20	25	30
Decrease in OTA mass fraction / %	30	21	15	20	31	17	19	25

*Data express the relationship between OTA mass fraction obtained after 35 days incubation and maximal fraction reached after 14 days during the same incubation

*Rezultati izražavaju odnos udjela OTA nakon 35. dana uzgoja i maksimalnog udjela dobivenog nakon 14. dana istog uzgoja

cumulated level of fungal mycellium that may have contributed to toxin production earlier during the incubation. Herewith applied »chitin« method is based on alcalic hydrolysis of chitin to glucosamine, which is then determined spectrophotometricaly. Using this method, Duraković *(44)* has shown that the mass fraction of chitin in healthy maize corns was 105–120 μg g^{-1}, while *A. ochraceus* NRRL 3174 mycelium contained 228 mg g^{-1} dry weight biomass.

Water content in a substrate necessary for OTA production by fungi *A. ochraceus* NRRL 3174 is rather high (more than 20 %), so in these experiments 38 % was used. This value corresponded to $a_w = 1$, so that water activity did not represent a limiting factor for the fungal growth. Besides, such high humidity may be found in maize in our fields during rainy and moist harvest seasons.

For the cultivation, the following temperatures were chosen: 15 °C, 20 °C, 25 °C and 30 °C. Experiments with the pure, as well as with the mixed culture have shown that for OTA biosynthesis a temperature higher than 15 °C is required. The temperature optimal for OTA biosynthesis was found to be 20 °C.

The greatest mass fraction of biomass that was synthesised after 35 days during the growth of the tested mould in the pure culture at 20 °C was 10.30 mg g^{-1}. In the mixed culture 13.20 mg of biomass per g of substrate was found.

The mould tested produced considerable amounts of OTA both in pure and in mixed culture (Fig. 1). The highest mass fraction of OTA obtained in a pure culture was 1225 μg g^{-1} biomass dry weight, and was measured after 14 days of incubation at 20 °C. In the mixed culture the maximal OTA fraction was lower, and after 21 days of incubation reached the level of 790 μg g^{-1} biomass dry weight (Fig. 1.).

During the incubation at 25 °C the growth of the moulds was intensified, but simultaneously OTA synthesis was suppressed. After 35 days of cultivation the yields of biomass obtained in pure and mixed culture were 16.20 mg g^{-1} of substrate, and 15.90 mg g^{-1} of substrate, respectively. The maximal level for OTA found in pure culture grown at 25 °C was 895 μg g^{-1} biomass dry weight, while in mixed culture only 395 μg g^{-1} biomass dry weight was found after 14 days of cultivation. Comparing these results with those obtained at 20 °C it can be calculated that despite the increased biomass growth, mass fraction of OTA was reduced by 25 % in pure, and even by 50 % in mixed culture (Fig. 1.).

The maximal values of the mass fraction of biomass synthesized during the growth of *A. ochraceus* NRRL 3174 at 30 °C, both in pure and mixed culture, did not differ significantly from the value obtained at 25 °C, and were found after 35 days cultivation to be 16.50 mg g^{-1} substrate, and 15.70 mg g^{-1} substrate, respectively. Under the same conditions, the highest fraction of OTA produced by *A. ochraceus* NRRL 3174 in pure culture was detected after 14 days and was 440 μg g^{-1} biomass dry weight. In the mixed culture, where *A. ochraceus* NRRL 3174 and *T. roseum* ZMPBF 1226 were grown together, the maximal OTA mass fraction was reached after 21 days with the value of 345 μg g^{-1} biomass dry weight.

Table 1. summarises the results obtained for OTA production by fungi *A. ochraceus* NRRL 3174 grown on maize with 38 % water content at different temperatures in pure culture, as well as in mixed culture with fungi *T. roseum* ZMPBF 1226. The values were calculated on the basis of the OTA mass fraction after 35 days incubation against the maximal OTA fraction reached after 14 days during the same cultivation. As can be seen, in all the cases after reaching the maximal value within 14–21 days, OTA fraction decreased with time. Mixed culture has shown a greater ability to suppress OTA fraction, except, at 20 °C, the temperature found to be optimal for OTA synthesis.

When comparing all the results obtained in this study it was concluded that the optimal temperature for OTA production by fungi *A. ochraceus* NRRL 3174 under applied conditions is 20 °C. Having in mind that this is the mean temperature in the continental parts of the country during summer, it becomes obvious what risk to the health, both human and animal, can arise from mouldy corn and other food grains. Higher temperatures did, however, intensify biomass synthesis, but the rate of OTA production was reduced. During the growth of *A. ochraceus* NRRL 3174 in mixed culture with the nonochratoxigenic fungi *T. roseum* ZMPBF 1226, a decrease in OTA synthesis was even more pronounced. As shown in Figure 1, the amount of OTA produced by this biomass is much less than that produced in pure culture. Since *T. roseum* ZMPBF 1226 was originally isolated as a naturally occurring contaminant on corn, it is reasonable

to expect that such mixed mycoflora should be found in fields and warehouses. Although this mould under conditions applied in this study did not produce either OTA or chromatographically similar compounds, little is known about other possible metabolites that might cause a decrease in OTA production. Such a metabolite could inhibit either *A. ochraceus* biomass synthesis or directly OTA pathway. On the other hand, one of rate limiting factors for fungal growth on a solid substrate is the available substrate surface. In mixed cultures the species with higher specific growth rate will overgrow the substrate, thus preventing the growth of other species. Finally, we must not forget the possibility that OTA is being metabolised by other mould(s) in the mixed culture. Which of the mentioned possibilities is the right explanation for what really happens, still remains to be answered.

Conclusions

The highest concentration of ochratoxin A produced by fungi *A. ochraceus* NRRL 3174 grown on corn grains with 38 % water content was achieved at 20 °C after 14 days of incubation, that is during the intensive sporulation. Under these conditions, a mass fraction of 1225 $\mu g\ g^{-1}$ biomass dry weight was measured in pure, and 790 $\mu g\ g^{-1}$ biomass dry weight in mixed culture with the mould *T. roseum* ZMPBF 1226, respectively.

Raising the temperature from 20 °C to 25 °C caused the increment of 50 % in biomass synthesis, but meanwhile OTA production was reduced by 15–20 %. During the growth at 30 °C this effect was even more pronounced.

References

1. P. Krogh, S. Nesheim: Selected methods of analysis. In: *Environmental Carcinogens*, Vol. 5, H. Egan, L. Stoloff, P. Scott, M. Castegnaro, I. K. O' Neil, H. Bartsh, W. Davis (Eds.), IARC Publications, No. 44, International Agency for Research on Cancer, Lyon (1982) pp. 247–282.
2. K. J. Van der Merwe, P. C. Steyn, L. Fourie, D. B. Scott, *Nature, 205* (1965) 1112.
3. M. R. Adams, M. O. Moss: *Food Microbiology*, The Royal Society of Chemistry, Cambridge (1995) pp. 228–244.
4. S. M. Madhyastha, R. R. Marquardt, A. A. Frohlich, G. Platford, D. Abramson, *J. Agric. Food Chem. 38* (1990) 1506.
5. J. I. Pitt, *Appl. Environ. Microbiol. 53* (1987) 266.
6. J. Harwig, T. Kuiper-Goodman, P. M. Scott: *Handbook of Foodborne Diseases of Biological Origin*, M. Rechcigl, Jr. (Ed.), CRC Press, Boca Raton, Florida (1983) pp. 193–238.
7. T. Kuiper-Goodman, P. M. Scott, *Biomed. Environ. Sci. 2* (1989) 179.
8. B. G. Osborne, *Food Cosmet. Toxicol. 18* (1980) 615.
9. R. G. Rotter, R. R. Marquardt, A. A. Frohlich, D. Abramson, *J. Sci. Food Agric. 50* (1990) 155.
10. O. L. Shotwell, C. W. Hesseltine, M. L. Goulden, *Appl. Microbiol. 17* (1969) 765.
11. J. Harwig, Y. K. Chen, *Can. J. Plant. Sci. 54* (1974) 17.
12. M. Škrinjar, T. Horvat-Skenderović, *Tehnol. mesa, 30* (1989) 53.
13. F. E. Escher, P. E. Koehler, J. C. Ayres, *Appl. Microbiol. 26* (1973) 27.
14. M. H. Brown, G. M. Szczech, B. P. Purmalis, *Toxicol. Appl. Pharmacol. 37* (1976) 331.
15. R. C. Doster, R. O. Sinnhuber, J. H. Walks, D. J. Lee, *Fed. Proc. 30* (1971) 578.
16. F. S. Chu, C. C. Chang, S. H. Asboor, N. Prentice, *Appl. Microbiol. 29* (1975) 313.
17. L. Rutqist, K. Hult, J. Grabarkiewiz-Szcesna, J. Chelkowski, K. Szebiotiko, *Appl. Environ. Microbiol. 49* (1985) 1014.
18. T. Holmberg, A. B. Emanuelsson, P. Häggblom, O, Schwan, K. Hult, *Mycopathologia, 116* (1991) 169.
19. P. Krogh: *Endemic nephropathy*, A. Puchlev, V. Diner, D. Doichinov (Eds.), Sofia, Bulgarian Academy of Sciences (1974) pp. 266–270.
20. I. F. H. Purchase, J. J. Theron, *Food Cosmet. Toxicol. 6* (1968) 479.
21. S. Larsen, *Maanedsskr. Dyrl. 40* (1928) 289.
22. P. Krogh, *Acta Pathol. Microbiol. Scand.* Sect. A, Suppl. *269* (1978) 1.
23. F. C. Stormer, O. Storen, C. E. Jansen, J. J. Pedersen, A. J. Aasen, *Appl. Environ. Microbiol. 45* (1983) 1183.
24. J. M. Barnes: *The Balcan Nephrophaty*, Wolstenholme GEW, Knight, London: Churchill (1967) p. 110.
25. L. Rutqist, N. E. Björklund, K. Hult, S. Gatenbeck, *Zbl. Vet. Med. A. 24* (1977) 402.
26. R. L. Buchanan, L. A. Ocker, H. G. Stahl, *Arch. Microbiol. 142* (1985) 200.
27. A. A. Fronlich, R. R. Marquardt, A. J. Bernatsky, *J. Assoc. Off. Anal. Chem. 71* (1988) 949.
28. D. H. Monroe, C. J. Holeski, D. L. Eaton, *Food Chem. Toxicol. 24* (1986) 1273.
29. D. Dochev, *Münch. Med. Wschr. 115* (1973) 537.
30. B. Hald, P. Krogh, *J. Assoc. Off. Anal. Chem. 56* (1973) 1441.
31. D. Dimitrov, *Münch. Med. Wschr.* (1960) 201.
32. M. Pavlović, R. Pleština, P. Krogh, *Acta Pathol. Microbiol. Scand.*, Sect. B, *87* (1979) 243.
33. B. Radić, R. Fuchs, M. Peraica, B. Šoštarić, S. Čeović, *III naučni sastanak o mikotoksinima*, Sarajevo (1988).
34. K. Hult, R. Fuchs, M. Peraica, R. Pleština, *J. Appl. Toxicol. 4* (6) (1986).
35. G. A. Boorman: *Toxicology and carcinogenesis studies of ochratoxin A in F 344/N rats, NTP TR 358*, National Toxicology Program, Research Triangle Park, North Carolina (1989) p. 142.
36. W. W. Donald, C. J. Mirocha, *J. Cereal Chem. 54* (3) (1977) 466.
37. R. M. Epply, *J. Assoc. Off. Anal. Chem. 51* (1966) 473.
38. C. W. Hesseltine, *Biotechnol. Bioeng. 14* (1972) 24.
39. *Book of Methods*, 8th Ed. AACC, St. Paul, Minnesota (1983).
40. *Book of Methods*, 14th Ed. AOAC, Washington, D. C. (1984).
41. M. Luckner, L. Nover, H. Bohm: *Secondary Metabolism and Cell Differentiation*, Springer Verlag (1977) pp. 37–102.
42. L. Hugh, E. Mary, Fun Sun Chu, *Appl. Microbiol. 6* (1971) 1032.
43. A. Boćarov, *Arhiv biol. nauka, 35* (1983) 119.
44. S. Duraković, *Disertacija*, Sveučilište u Zagrebu (1981).