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Analysis and Screening for Mycotoxins and Other Secondary Metabolites in Fungal Cultures by Thin-Layer Chromatography and High-Performance Liquid Chromatography

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Abstract. Methods for the screening of fungal cultures for toxic secondary metabolites are reviewed. Thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) are good general analytical methods for secondary metabolites in unpurified extracts. The combination of normal phase TLC using different chemical spray reagents with reversed phase HPLC, using alkylphenone retention indices and diode array detection, is a powerful technique for identifying the individual mycotoxins detected. The results of the screening methods are very dependent of the growth media and incubation conditions and a general method for the detection of *Penicillium, Aspergillus, Fusarium, Alternaria* and *Cladosporium* toxins is suggested.

The presence of mycotoxins in foods and feedstuffs is of great importance for development of disease in humans and animals (Krogh 1987). Therefore, accurate analytical methods is being developed for the detection of mycotoxins (Cole 1986; Betina 1984) in different commodities. A large number of mycotoxins is known, however, and it would be impossible to analyze for all these toxins in all commodities. It is therefore very important to determine which moulds are present in which foods (under specified environmental conditions) and determine the profile of mycotoxins produced by each species (Frisvad 1986) to reduce the number of mycotoxin analyses. The screening for many mycotoxins in a large number of isolates of moulds in each food sample is dependent on very simple methods or reliable identifications and established connections between fungal taxa and their profile of mycotoxins. While the screening of many fungal isolates is only practical using simple thin layer chromatography (TLC) techniques, the connection between fungal taxa and profiles of mycotoxins should be investigated with a combination of separating techniques and specific spectroscopic detection methods, such as high-performance liquid chromatography (HPLC) with diode-array detection (DAD), mass spectrometric detection (MSD) or Fourier transformation infrared detection (FTIRD) or gas chromatography (GC) using MS or FTIR detectors. TLC results can be confirmed by chemical reactions or full UV-VIS scanning on the TLC plates. Chang *et al.* (1984) have reported on direct analysis of TLC spots by fast atom bombardment mass spectrometry. GC methods are only applicable for a small number of mycotoxins since it is difficult to convert most mycotoxins to volatiles (Beaver 1986; Vesonder and Rohwedder 1986) and the use of MSD and FTIRD is only in the beginning phase as detectors in liquid chromatography. Even though the method requires expensive apparatus, mass spectrometry-mass spectrometry is a very promising method for mycotoxin analysis, especially in food samples (Plattner 1986). TLC and HPLC-DAD are the only techniques which will be discussed here, because they are especially suitable for screening of fungal extracts.

Screening for Mycotoxins in Fungi: Use of TLC

Scott *et al.* (1970) were the first to describe a good general method for detecting several mycotoxins in fungal cultures by a semimicro culture technique.

They suggested the use of yeast extract sucrose (YES) agar for production of the mycotoxins and acidic systems like toluene/ethylacetate/90% formic acid as a general eluent and an acidic anisaldehyde mixture as a spray reagent for mycotoxins. These methods are still very effective in the detection of most neutral and acidic mycotoxins. Some mycotoxins like cyclopiazonic acid, citrinin, and luteoskyrin streak in the TEF eluent, and an optimal detection of these toxins are obtained on silica gel plates impregnated with oxalic acid (Steyn 1969). The method of Scott *et al.* (1970) was modified by Bullerman (1974) to include the use of a 5% rice powder—4% cornsteep liquor medium in screening for mycotoxins in mould cultures. Barr and Dawney (1975) used a third medium, malt extract yeast extract glucose peptone (MYGP), and a multiple inoculation technique in their screening for mycotoxin production in fungal isolates. Lötzsch *et al.* (1974) proposed the use of a "stomacher" in mycotoxin analysis, because of the simplicity and low risk compared to a Waring blender and similar homogenizers. This technique was used by Leistner and Eckardt (1979) for analyzing the content of petri dishes with fungal agar cultures for mycotoxins. They used Merck malt extract agar containing soya peptone as a general mycotoxin producing medium. All the methods mentioned above included the use of chloroform (usually 55°C) as extraction liquid, sometimes after freezing the agar cultures for 24 hours. After filtering and drying over sodium sulphate the organic phase was evaporated and the residue redissolved in chloroform or other organic solvents. Gorst-Allman and Steyn (1979) proposed the eluent chloroform/acetone/isopropanol (CAP) for roquefortine C and cerium sulphate spray as a general spray reagent. A systematic analysis of mycotoxins, using several eluents and data on spot colours before and after treatment with anisaldehyde and ferrichloride sprays, was introduced for mycotoxins by Duracková *et al.* (1976).

Filtenborg and Frisvad (1980) introduced the very simple "agar plug method". A plug of agar from a petri dish culture is placed directly on the TLC plate, thus avoiding any extraction, filtering or purification steps. This method is only applicable for extracellular mycotoxins, so the method was extended to include intracellular mycotoxins by Filtenborg *et al.* (1983). In the latter method, a drop of effective extraction solvent (*i.e.*, dichlormethane/methanol, 2:1 or other mixtures) is placed on the mycelial side of a plug of a mould colony and after an extraction period of a few seconds the mycelial side of the agar plug is pressed against the application line on the TLC plate. In the latter extract, some extracellular mycotoxins may also be present, because they are often concentrated in exudate droplets on the fungal colony. This very time and resource saving method allows the screening of hundreds of fungal cultures within eight hr: 22 samples together with the external standard griseofulvin can be placed on the same TLC plate (20 × 20 cm) or even twice that number if the samples are run from each side (*i.e.*, 10 cm migration of eluent). Therefore, a higher number of different media and incubation conditions can be tested on the same fungal isolate. The sensitivity of the method is high: 8 petri dishes were extracted, using the stomacher method and redissolved in 5 ml methanol after evaporation of the extraction liquid gave a much weaker result that a single agar plug of the same isolate of *P. viridicatum* (Frisvad, unpublished). Problems with poor producers can usually be overcome by selecting an optimal mycotoxin production medium or by using several superimposed extracted agar plugs on the same application spot (Frisvad and Filtenborg 1983). The eluents and spray reagents used by Filtenborg *et al.* (1983) were selected from optimal systems employed by the workers mentioned in the former paragraph. Based on our previous papers and further experience, we recommend the TLC conditions listed in Table 1 for screening filamentous fungi for mycotoxins. Such a general system is, of course, not optimal for all mycotoxins. Good TLC systems for known mycotoxins are given in Cole and Cox (1981) and Betina (1984).

Most known mycotoxins can be efficiently analyzed in the eluent TEF followed by sulphuric acid and/or anisaldehyde spray treatments. There are three important exceptions (Table 1): 1) alkaloids are best analyzed by the eluent CAP followed by spraying with cerium sulphate (these compounds are generally intracellular and produced in greatest amounts on CYA). The alkaloids include the important mycotoxins penitrem A, roquefortine C and D, isofumigaclavine A and B, rugulovasine A and B, atramentins and oxaline. 2) Very acidic mycotoxins, such as citrinin, cyclopiazonic acid, viridicatumtoxin and some anthraquinones like rugulosin and luteoskyrin are best analyzed on oxalic acid-treated TLC plates. 3) Cyclic peptides, penicillins, and similar compounds should be analyzed in a eluent containing *n*-butanol and water, *n*-butanol/acetic acid/water (4:1:2) (Ghosh *et al.* 1978). Spray reagents for such compounds are rather insensitive and they include iodine or chlorine vapors followed by spraying with *o*-tolidine (Ghosh *et al.* 1978) or bromecresol green (Moskowitz and Cayle 1974). The same eluent (*n*-butanol/acetic acid/water) is also optimal for kojic acid and 3-nitropropionic

Table 1. Screening for different secondary metabolites in fungal extracts: Use of thin-layer chromatograhic methods

Mycotoxin type	Eluent[a]	Plug method[b] (medium)	Spray reagent
Basic mycotoxins: alkaloids: roquefortine C & D, atramentins, fumigaclavines, penitrems, paxilline, aflatrem etc.	CAP	I (CYA)	$Ce(SO_4)_2$ in 6 N H_2SO_4 (1%) occasionally followed by spraying with $AlCl_3$ spray and heating
Neutral and acidic mycotoxins	TEF	I (CYA + YES) E (YES)	0.5% anisaldehyde in methanol/acetic acid/sulphuric acid (17:2:1), and heating
Very acidic mycotoxins: Viridicatumtoxin, ochratoxin A, citrinin, rugulosin, luteoskyrin	TEF (OX)	I (CYA + YES) E (YES)/citrinin, ochratoxin A)	
Peptide like mycotoxins	BAW	Extraction of cultures	Chlorine or iodine vapours, followed by *o*-tolidine spray (cyclochlorotin, simatoxin) Fast blue B salt followed by NH_3 fumes (kojic acid, 3-nitro-propionic acid)
Special Cases[c]			
Xanthomegnin, viomellein, ochratoxin A, penicillic acid, mycophenolic acid	TEF	I (CYA + YES) E (YES)	NH_3 fumes (1 min.)
Patulin	TEF	E (PDA, MEA)	0.5% methylbenzothiazolonehydrochloride in water and heating
Verruculogen	TEF	I (CYA)	cold 6N sulphuric acid
Cylopenin, cyclopenol, viridicatin, viridicatol	TEF	I (CYA)	cold 6N sulphuric acid
Penitrem A, sterigmatocystin	TEF	I (CYA)	20% $AlCl_3$ in 65% ethanol, heating
PR-toxin	TEF	E (YES)	½ min UV radiation at 254 nm followed by examination at 366 nm
Tremorgens	TEF, HE	I (CYA)	20% $AlCl_3$ in 65% ethanol, heating
Cyclopiazonic acid	TEF (OX)	I (CYA)	Ehrlich spray: 1% p-dimethylbenzaldehyde in 96% ethanol, followed by HCl fumes or 6N sulphuric acid spray

[a] Eluents: TEF: toluene/ethylacetate/90% formic acid (5:4:1), CAP: chloroform/acetone/2-propanol (85:15:20), BAW: 1-butanol/acetic acid/water (4:2:1), HE: hexane/ethylacetate (6:4), OX: the TLC plate is dipped in a 8% methanolic solution of oxalic acid and dried
[b] I: The mycelial side of an agar plug from the culture is wetted with dichlormethane/methanol (2:1) and pressed against the TLC plate, E: the agar plug is placed directly on the TLC plate. CYA: Czapek yeast autolysate agar, YES: yeast extract (Difco)-sucrose agar
[c] These mycotoxins can be analyzed using the general methods, but the sensitivity is much better using the specified reagents and methods. For *Fusarium* mycotoxins see Thrane (1986). See also Cole and Cox (1981)

acid; fast blue salt B spraying followed by ammonia fumes is the most sensitive spray for these compounds (Harwig and Scott 1971). For some individual toxins, a certain combination of growth medium for the producing fungus and spray reagent is 10–100 times more sensitive than the general TEF eluent/anisaldehyde spraying system. Patulin is, f.ex., optimally produced on potato dextrose agar and optimally visualized by methylbenzothiazolone-hydrochloride (MBTH) spray (Scott and Kennedy 1973).

The simple TLC screening method of Filtenborg and coworkers was extended to include 182 mycotoxins and other fungal metabolites in the eluents TEF and CAP (Frisvad and Thrane 1987) and the eluent toluene/acetone/methanol (TAM) was added to the TEF and CAP eluents for optimal screening of *Fusarium* mycotoxins by Thrane (1986). In the latter two papers, all retardation factors (Rf) were listed relative to griseofulvin to reduce the risk of very variable Rf values in different laboratories. Paterson (1986) added data on colors of most of the secondary metabolites treated by Frisvad and Thrane (1987)—before and after anisaldehyde spray treatment—using the agar plug method developed by Filtenborg *et al.* (1983). Thus, the "agar plug method" is strongly recommended for screening mycelial fungi for mycotoxins.

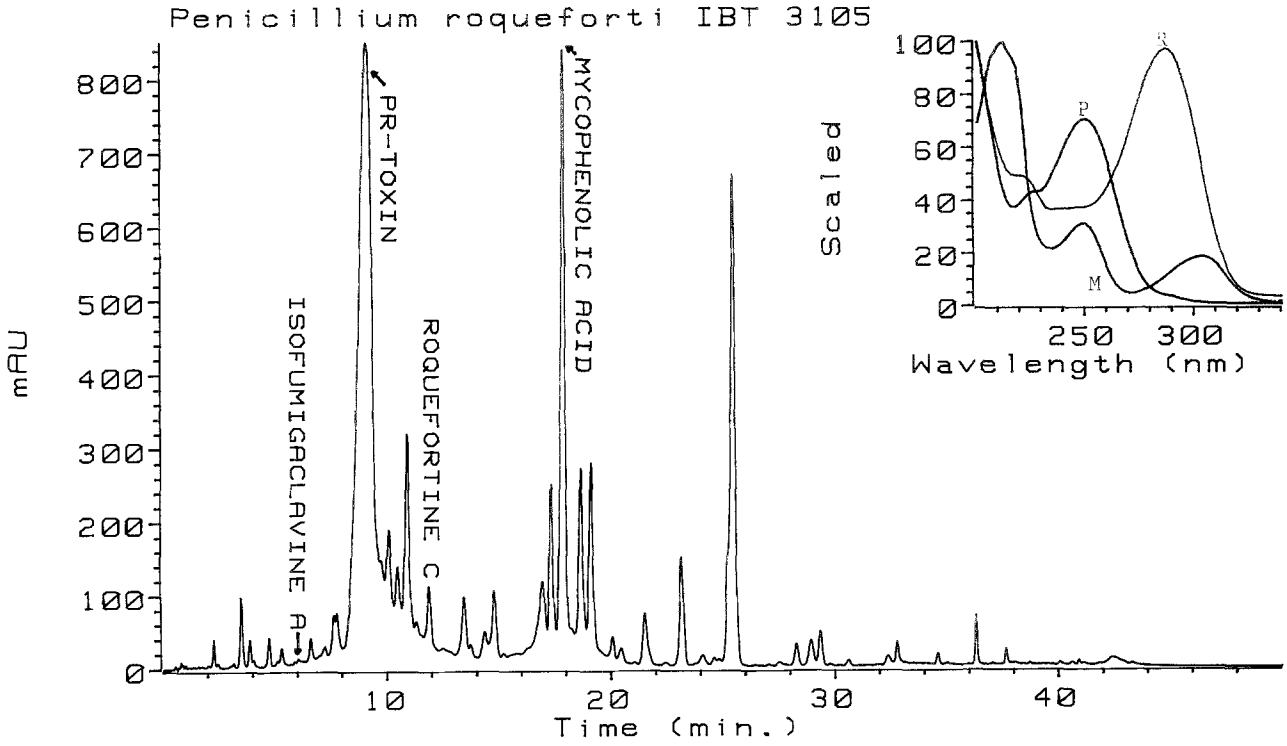

Fig. 1. HPLC trace of an extract of the contents of 8 YES agar petri dishes of an industrial strain of *Penicillium roqueforti*. The UV spectra of three mycotoxins, measured with the fast scanning diode array detector, are inserted in the upper right of the figure. The extract was monitored at 225 nm, but shown here at the signal B wavelength 254 nm. Many of the other peaks had UV spectra close to that of PR-toxin or mycophenolic acid

Screening for Mycotoxins: Use of HPLC

HPLC methods have been used in the analysis of many individual mycotoxins and this has been reviewed by Scott (1981), Gorst-Allman and Steyn (1984) and Shephard (1986). The only multi-mycotoxin screening method available is that developed by Frisvad (1987). This method is based on a gradient elution using water and acetonitrile (containing 0.005% trifluoracetic acid) on a reversed phase column. All available mycotoxins and other fungal secondary metabolites could be analyzed using that method. The value of the method was enhanced by the use of an alkylphenone retention time index system and diode array detection (Frisvad and Thrane 1987). In addition to the 166 fungal secondary metabolites detectable in the HPLC system of Frisvad and Thrane (1987), aspergillic acid, asteltoxin, averufin, cladosporin (=asperentin), dihydrosterigmatocystin, dihydroxyaflavinin, 4-hydroxymellein, mitorubrin, mitorubrinol, mitorubrinol-acetate, mitorubrinic acid, norsolorinic acid, parasiticol, trypacidin and vermiculin have been successfully analyzed by the method of Frisvad and Thrane (1987) (Frisvad, unpublished). Thus, even though the HPLC method mentioned above requires expensive apparatus, the results are effectively standardized, using alkylphenone retention time indices and diode array detection. Combined with the use of TLC and mycotoxin standards, this method allows characterization of mycotoxins from fungi close to a positive identification. Future development of FTIR and MS detectors for HPLC will permit an even more secure identification of small amounts of mycotoxins in fungal extracts. In most cases, however, the simple "agar plug method" is sufficient, at least for screening purposes, and this method requires inexpensive and generally available apparatus. TLC methods should, however, be used for identification with caution. Using TLC and external standards, Paterson *et al.* (1987) found ochratoxin A in *Penicillium expansum* and *P. brevicompactum* and rubratoxin B in *P. expansum*, *P. citrinum* and *P. brevicompactum*. HPLC analysis or TLC analysis using other eluents than TEF, using the same strains as Paterson *et al.* (1987), has shown that ochratoxin A and rubratoxin B are not produced by the species listed above (Frisvad, unpublished).

An example of the use of the HPLC method of Frisvad (1987) and Frisvad and Thrane (1987) is shown in Figure 1. An industrial strain of *P. roqueforti* was grown on eight 9 cm petri dishes of YES agar and extracted with chloroform followed by re-

dissolving in methanol and defattening with petroleum benzine. Several secondary metabolites could be characterized by the HPLC method and the production of PR-toxin, isofumigaclavine, mycophenolic acid and roquefortine C could be verified. The identity of the toxins was confirmed by TLC, external and internal standards and different chemical treatments and by identical alkylphenone indices as standard toxins and superimposable UV-VIS spectra (as determined by the diode array detector). Higher yields of the alkaloids isofumigaclavine A and roquefortine C were obtained on CYA agar however. Thus, the use of HPLC allows a quantitation of the different toxins and a good verification of identity.

References

Barr JG, Dawney GA (1975) A multiple inoculation technique for the screening of fungal isolates for the evaluation of growth and mycotoxin production on agar substrates. J Sci Fd Agric 26:1561–1566

Beaver RW (1986) Gas chromatography in mycotoxin analysis. See Cole RJ (1986), pp 265–292

Betina V (ed) (1984) Mycotoxins Production, Isolation, Separation and Purification. Developments in Food Science 8. Elsevier, Amsterdam, 528 pp

Bullerman LB (1974) A screening medium and method to detect several mycotoxins in mold cultures. J Milk Food Technol 37:1–3

Chang TT, Lay JO, Francel RJ (1984) Direct analysis of thin-layer chromatography spots by fast atom bombardment mass spectrometry. Anal Chem 56:109–111

Cole RJ (ed) (1986) Modern methods in the analysis and structural elucidation of mycotoxins. Academic Press, Orlando, 471 pp

Cole RJ, Cox RH (1981) Handbook of toxic fungal metabolites. Academic Press, New York, 874 pp

Duracková Z, Betina V, Nemec P (1976) Systematic analysis of mycotoxins by thin-layer chromatography. J Chromatogr 116:141–154

Filtenborg O, Frisvad JC (1980) A simple screening method for toxigenic moulds in pure cultures. Lebensm Wiss Technol 13:128–130

Filtenborg O, Frisvad JC, Svendsen JA (1983) Simple screening method for molds producing intracellular mycotoxins in pure cultures. Appl Environ Microbiol 45:581–585

Frisvad JC (1986) Taxonomic approaches to mycotoxin identification. See Cole RJ (1986), pp 415–457

——— (1987) High-performance liquid chromatographic determination of profiles of mycotoxins and other secondary metabolites. J Chromatogr 392:333–347

Frisvad JC, Filtenborg O (1983) Classification of terverticillate penicillia based on profiles of mycotoxins and other secondary metabolites. Appl Environ Microbiol 46:1301–1310

Frisvad JC, Thrane U (1987) Standardized high-performance liquid chromatography of 182 mycotoxins and other fungal metabolites based on alkylphenone retention indices and UV-VIS spectra (diode array detection). J Chromatogr 404:195–214

Ghosh AC, Manmade A, Bousquet JM, Townsend JM, Demain AL (1978) A convenient thin-layer chromatographic method for the detection of cyclochlorotine and simatoxin, toxins from *Penicillium islandicum* Sopp. Experientia 34:819–820

Gorst-Allman CP, Steyn PS (1979) Screening methods for the detection of thirteen common mycotoxins. J Chromatogr 175:325–331

——— (1984) Applications of chromatographic techniques in the separation, purification and characterization of mycotoxins. See Betina (1984), pp 59–85

Harwig J, Scott PM (1971) Brine shrimp (*Artemia salina* L.) larvae as a screening system for fungal toxins. Appl Microbiol 21:1101–1106

Krogh P (ed) (1987) Mycotoxins in food. Academic Press, New York, 280 pp

Leistner L, Eckardt C (1979) Vorkommen toxinogener Penicillien bei Fleisch Erzeugnisse. Fleischwirtschaft 59:1892–1896

Lötzsch R, Tauchmann F, Meyer W (1974) Einsatz des "Stomacher" in der Mykotoxin-Analytik. Fleischwirtschaft 54:943–945

Moskowitz GJ, Cayle T (1974) a method for the detection of 3-nitropropionic acid in crude biological extracts. Cereal Chem 51:96–105

Paterson RRM (1986) Standardized one- and two-dimensional thin-layer chromatographic methods for the identification of secondary metabolites in *Penicillium* and other fungi. J Chromatogr 368:249–264

Paterson RRM, Simmonds MSJ, Blaney WM (1987) Mycopesticidal effects of characterized extracts of *Penicillium* isolates and purified secondary metabolites (including mycotoxins) on *Drosophila melanogaster* and *Spodoptora littoralis*. J Invert Pathol 50:124–133

Plattner RD (1986) Mass spectrometry-mass spectrometry as a tool for mycotoxin analysis. See Cole (1986), pp 393–414

Scott PM (1981) High performance liquid chromatographic determination of mycotoxins. In: Lawrence JF (ed) Trace analysis, Academic Press, New York, pp 193–266

Scott PM, Kennedy BPC (1973) Improved method for the thin-layer chromatographic determination of patulin in apple juice. J Assoc Offic Anal Chem 56:813–816

Scott PM, Lawrence JW, Walbeek W van (1970) Detection of mycotoxins by thin-layer chromatography: Application to screening of fungal extracts. Appl Microbiol 20:839–842

Shepherd MJ (1986) High performance liquid chromatography and its application to the analysis of mycotoxins. See Cole (1986), pp 293–333

Steyn PS (1969) The separation and detection of several mycotoxins by thin-layer chromatography. J Chromatogr 45:473–475

Thrane U (1986) Detection of toxigenic *Fusarium* isolates by thin-layer chromatography. Lett Appl Microbiol 3:93–96

Vesonder RF, Rohwedder WK (1986) Gas chromatographic-mass spectrometric analysis of mycotoxins. See Cole (1986), pp 335–351

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