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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF PROFILES OF MYCOTOXINS AND OTHER SECONDARY METABOLITES

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SUMMARY

A reversed-phase high-performance liquid chromatographic determination of profiles of mycotoxins and other fungal secondary metabolites has been developed. *Penicillium*, *Aspergillus* and *Fusarium* polyketides, terpenes and alkaloids have been emphasized. In a gradient elution, using water–acetonitrile containing 0.05% trifluoroacetic acid, 134 secondary metabolites were eluted evenly with retention times from 1.08 to 34.48 min. Metabolites with the same retention time were usually not produced by the same species. As UV detection at 254 nm was used, some mycotoxins (type A trichothecenes, viridicatumtoxin, peptide-like compounds and xanthomegnin) could not be detected. The method appears to be valuable for chemotaxonomic studies of fungi. Unpurified concentrated chloroform–methanol extracts of petri dish cultures analysed by the proposed method presented gave species-specific characteristic profiles of known and unknown secondary metabolites and mycotoxins.

INTRODUCTION

High-performance liquid chromatography (HPLC) is being used increasingly for the analysis of individual and related mycotoxins in foods and fungal cultures[1–4]. These HPLC methods differ significantly in the choice of normal- or reversed-phase columns of different types, elution mixtures and gradients, detection methods and sample preparation and purification procedures[1,2]. A general chemotaxonomic determination of all secondary metabolites from a fungal strain is difficult (if not impossible) using a relatively simple HPLC method because secondary metabolites are chemically very diverse[5–7]. Further, some secondary metabolites are structurally related to primary metabolites[6,7], complicating the choice of culture media, extraction methods, purification and analysis. Many chemotaxonomic studies have therefore been centred around one or two chemosyndromes[8]. Other chemotaxonomic studies, based on thin-layer chromatography (TLC)[9,10] have shown that at least three chemosyndromes are necessary to identify a strain of *Penicillium* and other genera to species level. Three very different *Penicillium* species, *P. sclerotigenum*, *P. griseoful-*

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vum and *P. coprophilum*, are all able to produce the different chemosyndromes griseofulvin and roquefortines, for example[10].

The aim of this study was to develop a general method, based on HPLC, for the chemotaxonomic study of moulds, with special emphasis on the most important secondary metabolites in fungi (polyketides, alkaloids and terpenes). Other less common fungal secondary metabolites, such as cyclic peptides, sterols, fatty acids and pyrimidine- and purine-like compounds, were deliberately not included, as they require UV detection at wavelengths where primary metabolites would interfere, chemical derivatization, extraction with polar solvents or other compound-specific treatments.

EXPERIMENTAL

Fungi

Several *Penicillium* subgenus *Penicillium* isolates (about 160 isolates) were tested using the method described below. All strains were maintained and inoculated from Czapek yeast autolysate agar (CYA)[9]. Conidium suspensions made from a 1-week-old culture (25°C) of the latter medium[9] were used to inoculate six petri dishes (9 cm) containing 17 ml of CYA agar and six petri dishes containing yeast extract sucrose (YES) agar[9]. The plates were incubated for 12 (or 14) days at 25°C in the dark in an upright position.

The contents of six petri dishes were placed in Stomacher polyethylene bags and extracted with 100 ml of chloroform–methanol (2:1, v/v) in a Colworth Stomacher 400 for 2.5 min. After freezing at −30°C for at least 16 h, the contents of a stomacher bag was filtered through Whatman 1 PS phase separator filters and the organic phase was evaporated *in vacuo* at 39°C on a rotary evaporator. The residue was dissolved in 2 ml of chloroform and transferred into a 4-ml dram vial and stored in a freezer (−18°C) before analysis. The fungal extracts were filtered through glasswool in a pasteur pipette before injection (10 µl) into the chromatograph.

Fungal secondary metabolite standards

Standards of secondary metabolites were dissolved in chloroform (2 ml). If insoluble in chloroform, 1 ml of methanol was added to the standard. In a few instances acetone (1 ml) was also added. The sources of the secondary metabolites were as follows: K. Hult, Royal Technical University, Stockholm, Sweden (orsellinic acid, barnol, tenuazonic acid, sulochrin); Y. Ueno, Faculty of Pharmaceutical Sciences, Tokyo University of Science, Tokyo, Japan (emodin, rugulosin); Løvens Kemiske Fabrik, Copenhagen, Denmark (gliotoxin, mycophenolic acid, monorden, brefeldin A); L. Leistner, Bundesanstalt für Fleischforschung, Kulmbach, F.R.G. (cyclopiazonic acid, penitrem A, PR-toxin); R. T. Gallagher, Ruakura Agricultural Research Centre, Hamilton, New Zealand (janthitrem A, verruculogen); A.E. de Jesus, Council for Scientific and Industrial Research, Pretoria, South Africa (citreoviridin, viridamin, viridicatumtoxin); U. L. Diener, Auburn University, AL, U.S.A. (roquefortine c); P. M. Boll, Chemical Institute, Odense University, Denmark (carlosic acid, dehydrocarolic acid); A. Ciegler, Computer Sciences Corp., National Space Technology Lab., MS, U.S.A. (brevianamide A, xanthomegnin, viomellein); S. Ohmomo, Institute of Applied Biochemistry, University of Tsukuba, Ibaraki-Ken,

Japan (cyclopenin, cyclopenol, roquefortine A, B and D, rugulovasine A); R. J. Cole, National Peanut Research Lab., Dawson, GA, U.S.A. (aflatrem, austdiol, *epi*-10-verruculotoxin, 3,5-dimethyl-8-hydroxy-6-methoxyisochroman, emodin, chaetoglobosin C, paspalinine, paxillin, verruculotoxin); H. Achenbach, Institut für Pharmazie and Lebensmittelchemie, Universität Erlangen-Nürnberg, Erlangen, F.R.G. (chromanol 1, 2 and 3); J. A. Quintanilla Compania de Industrias Agricolas, Valladolid, Spain (cyclopaldic acid); M. O. Moss, Department of Microbiology, University of Surrey, Guildford, U.K. (rubratoxin B); D. C. Aldridge, ICI Pharmaceutical Division, Macclesfield, Cheshire, U.K. (canadensolide, canescin, asterric acid, ethisolide, fulvic acid, mycelianamide, palitantin, phoenicin, purpurugenone, lapidosin, roseopurpurin, spinulosin, terrein, terrestric acid, viridicatin, wortmannin, xanthocillin X); R. R. Paterson, Commonwealth Mycological Institute, Kew, Surrey, U.K. (carolic acid, desacetylpebrolide, 3,5-dimethyl-6-hydroxyphthalide, epoxysuccinic acid, gentisyl alcohol, gladiolic acid, glauconic acid, griseophenone C, 5'-hydroxyasperentin, hydroxyisocanadensic acid, 6-methylsalicylic acid, lichexanthone, norlichexanthone, 2-pyruvoylbenzamide, secalonic acid D); G. W. van Eijk and H. J. Roeijmans, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands (ascochitine, asperthecine, byssochlamic acid, catenarin, cladofulvin, curvularin, cynodontin, chrysophanol, dehydrocurvularin, dermoglaucin, dothistromin, echinulin, erythroglaucin, flavoglaucin, frequentin, duclauxin, 2,7-dimethoxy-5-hydroxy-6-(1-acetoxyethyl)-1,4-naphthoquinone, ω-hydroxypachybasin, helminthosporin, iodinine, islandicin, isoemodin, 2,7-dimethoxy-6-juglone, lambertellin, macrosporin, mollicin, naphthalic anhydride (ref. 6, p. 151), nectriafurone, pachybasic acid, pachybasin, phomarin, questin, questinol, ravenelin, scytalone, "steckiin", soranjidiol, stipitatic acid, torreyol, 3,4,5-trihydroxy-7-methoxy-2-methylanthraquinone); L. M. Seitz, U.S. Grain Marketing Research Lab., Manhattan, KS, U.S.A. (alternariol, altenuene, altertoxin I); K. V. Rao, Department of Medical Chemistry, University of Florida, Gainesville, FL, U.S.A. (bostrycin, 4-deoxybostrycin); H. R. Burmeister, Northern Regional Research Center, Peoria, IL, U.S.A. (equisetin, moniliformin); R. A. Baker, Citrus and Subtropical Products Lab., Winter Haven, FL, U.S.A. (fusarubin, javanicin, norjavanicin, bostrycoidin, marticin, isomarticin); D. Parisot, Laboratoire de Cryptogamie, Orsay, France (fusarubin, isomarticin, javanicin, anhydrofusarubin); Y. Kimura, Faculty of Agriculture, Tottori University, Tottori, Japan (solaniol); T. Yoshizawa, Department of Food Science, Kagawa University, Kagawa, Japan (butenolide); R. Greenhalgh, CBRI Agriculture Canada, Ontario, Canada (culmorin, butenolide); Sigma, St. Louis, MO, U.S.A. (kojic acid, luteoskyrin, diacetoxyscripenol, HT-2 toxin, T-2 toxin, T-2 tetraol, T-2 triol, roridin A, verrucarin A, verrucarol, zearalenone, fusaric acid); Karl Roth, Karlsruhe, F.R.G. (aflatoxin B_1 and G_1, ochratoxin A, patulin, sterigmatocystin); Supelco, Bellefonte, PA, U.S.A. (citrinin); Aldrich-Europe, Beerse, Belgium (griseofulvin, penicillic acid); Wako Pure Chemicals, Dallas, TX, U.S.A. (deoxynivalenol, fusarenone-X, neosolaniol, nivalenol).

Reagents

HPLC-grade acetonitrile (Rathburn Chemicals, Walkerburn, U.K.), doubly glass-distilled water and trifluoroacetic acid (Ferak, Berlin, F.R.G.) were used as eluents.

Chromatographic equipment

A Hewlett-Packard HP 1084B high-performance liquid chromatograph with two pumps, a variable-wavelength UV detector, an HP 79850B terminal and a column oven held at 30°C was used. The solvent temperature was held at 50°C. The UV detector was set to monitor at 254 nm. Injections were made with a Hewlett-Packard autosampler injector system (10 μl). Analyses were performed on a 120 mm × 4.6 mm I.D. Nucleosil 5-μm C_{18} column (Macherey, Nagel & Co., Düren, F.R.G.).

HPLC analyses

A gradient solvent system (with solvent A = water and solvent B = 0.05% trifluoroacetic acid in acetonitrile) was developed after several experiments with different time programmes, solvents (water, acetonitrile and methanol) and column materials [Nucleosil, Spherisorb ODS (5 μm particles) and Nova-Pack C_{18} (5 μm particles)]. The final solvent programme was as follows: the initial percentage of solvent B was 10%, which was raised to 50% in 30 min, then to 90% in 4 min, held at 90% for 4 min and lowered to 10% again in 7 min at a flow-rate of 2.0 ml/min.

RESULTS AND DISCUSSION

Development of the method

Initial experiments on the HPLC of few mycotoxins showed that a reversed-phase column material was superior to a normal-phase material with regard to peak shape and reversible binding of secondary metabolites to the column, in agreement with other workers[1–3,11,12]. Different column materials (Nucleosil, Spherisorb and Nova-Pack) were tested on several mycotoxins and fungal extracts (*P. roqueforti*) and all were found to be excellent. As unpurified and lipid-containing fungal extracts were used, it was necessary to use short columns (< 13 cm); the use of 25-cm columns resulted in a gradual increase in column pressure after a few runs. Dissolving the fungal extracts in acetonitrile and defattening with light petroleum allowed the use of longer HPLC columns. Our columns did not show signs of degeneration after about 120 runs with unpurified chloroform extracts, even though one would expect a build-up of lipids and secondary metabolites binding irreversibly to the column (*e.g.*, xanthomegnin and viomellein)[11], owing to the introduction of chloroform into water–acetonitrile or water–methanol eluents. The columns were always flushed with methanol after not more than eight runs with fungal extracts.

Different isocratic eluent systems [acetonitrile–water (1:1), acetonitrile with 0.05% trifluoroacetic acid–water (1:1) or methanol–water (1:1)] were compared with gradient elution [from 10% B (with the organic solvents mentioned above in the B channel) to 50% B in 30 min and to 90% in 4 min] for fungal extracts containing known mycotoxins (*P. griseofulvum*, *P. vulpinum* and *P. roqueforti* chemotype I). The isocratic systems yielded chromatograms with poor separation of the first peaks and broadening of the last peaks (the general elution problem[13]), but the chromatograms from gradient elution with methanol, acetonitrile or acetonitrile containing trifluoroacetic acid (channel B) and water (channel A) were good (narrow, sharp, symmetrical peaks and an even distribution of the retention times of the secondary metabolites). The system with acetonitrile containing 0.05% trifluoroacetic acid as the organic eluent was the best with regard to the number of peaks in the chromatograms

of the fungi mentioned above. A flow-rate of 2.0 ml/min, was better than 1 ml/min with the gradient mentioned under Experimental with regard to the even distribution of metabolites. The time programme of the gradient (see Experimental) was designed for automatic runs (a smooth return to the initial %B) and to use relatively more time (30 min) on the majority of metabolites eluting before B reached 50% (112 standard secondary metabolites out of 134 tested).

Standard secondary metabolites

The retention times on one particular Nucleosil column for 134 mycotoxins and other fungal secondary metabolites using the final system mentioned under Experimental are listed in Table I. The retention times of the secondary metabolites were evenly distributed from 1.08 min (moniliformin) to 34.48 min (erythroglaucin). All peaks, except those of PR-toxin, terrestric acid and rugulovasine A, had excellent shapes. The asymmetric tailing of PR-toxin, the broad but sharp peak of rugulovasine A and the very broad, rounded peak of terrestric acid were consistent properties, however. Of the other peaks, representing unknown secondary metabolites from many different penicillia, very few were broad or asymmetric. Griseofulvin was used as an external standard at least before and after analyses on one day, but a retention index using alkylphenones might be necessary in order to standardize the retention times when using different batches of columns and for the tentative identification of mycotoxins in different laboratories[3]. Table II list the retention times of some metabolites on a different batch of Nucleosil columns to that represented in Table I, and it is clear that without effective standardization, retention times might differ substantially on different batches of columns. Table III lists the percentage differences between the retention times on two batches of Nucleosil columns based on actual retention times and retention times relative to griseofulvin. More than one retention index standard is clearly needed. The Nucleosil batch differences were not the only differences, however: the analyses on which Tables I and II are based were run in different years and differences in solvents and temperatures might also have affected the results.

The fixed detection wavelength used (254 nm) precluded the analysis of compounds absorbing weakly at that wavelength, such as Type A trichothecenes (T-2 toxin, T-2 tetraol, T-2 triol, diacetoxyscirpenol and HT-2 toxin), 3-nitropropionic acid, epoxysuccinic acid, hadacidin, gibberellic acid, cyclochlorotine, penicillin G, butenolide, culmorin, echinulin, torreyol and most sterols[5]. Other secondary metabolites, such as xanthomegnin, viomellein, viridicatumtoxin, luteoskyrin, dothistromin, iodinine, 5′-hydroxyasperentin, roridin A, verrucarin and verrucarol, may have been retained on the column. It has been shown that xanthomegnin and viomellein will only elute efficiently from silica gel (normal- or reversed-phase) columns if a detergent such as sodium dodecylsulphate is added to the mobile phase[11]. Optimal liquid chromatographic methods for some of the toxins mentioned above have been developed (see Table IX in ref. 3).

HPLC of fungal extracts

A large number of the secondary metabolites listed in Table I are known mycotoxins. The HPLC method described here may be used for multi-mycotoxin screening methods, but the presence of several secondary and primary metabolites in foods

TABLE I

RETENTION TIMES OF FUNGAL SECONDARY METABOLITES AND KNOWN PRODUCERS OF THESE METABOLITES* IN A REVERSED-PHASE HPLC SYSTEM

Secondary metabolite	*Retention time (min)*	*Known producers*
Moniliformin	1.08	*Fusarium avenaceum, F. chlamydosporum, F. oxysporum, F. pallidoroseum, F. verticillioides*
Kojic acid	1.14	*Aspergillus flavus, A. oryzae, A. oryzae* var. *effusus, A. parasiticus, A. tamarii, A. terricola, A. thomii, A. wentii, Penicillium lanosum* and other *Penicillium* spp.
Nivalenol	1.39	*F. avenaceum*?, *F. camptoceras*?, *F. culmorum, F. equiseti, F. graminearum, F. poae, F. sambucinum*?, *F. sporotrichioides, F. pallidoroseum, F. tricinctum*?
Carolic acid	1.47	*P. fellutanum, P.* cf. *spinolosum, Trichoderma* sp.
Patulin	1.98	*A. clavatus, A. giganteus, A. terreus, Byssochlamys fulva, B. nivea, Eupenicillium lapidosum, Paecilomyces variotii, P. expansum, P. glandicola, P. griseofulvum, P. melinii, P. novaezeelandiae, P. roqueforti* chemotype II, *P. vulpinum*
Terrein	1.98	*A. terreus, Neosartorya fischeri, P. raistrickii*
Dehydrocarolic acid	2.05	*P. fellutanum, P.* cf. *spinulosum*
Roquefortine B	2.08	*P. crustosum, P. hirsutum, P. roqueforti*
Roquefortine D	2.24	*P. crustosum, P. hirsutum, P. roqueforti*
Carlosic acid	2.29	*P. fellutanum*
Deoxynivalenol	2.43	See nivalenol
Stipitatic acid	2.94	*Talaromyces stipitatus*
epi-10-Verruculotoxin	3.15	*P. brasilianum*
Austdiol	3.93	*A. ustus*
Rugulovasine A	4.18	*P. commune, P. concavorugulosum, P. islandicum, P. purpurugenum*
Fusarenone X	4.28	See nivalenol
Spinulosin	4.32	*A. fumigatus, P.* cf. *spinulosum*
Fusaric acid	5.04	*F. lateritium, F. oxysporum, F. solani, F. verticillioides, Peziza atrovinosa*
Penicillic acid	5.11	*A. alutaceus, A. auricomus, A. fresenii, A. melleus, A. sclerotiorum, Eupenicillium baarnense, E. ehrlichii, Paecilomyces lilacinus, Penicillium aurantiogriseum, P. brasilianum, P. fennelliae, P.* cf. *hirsutum, P. hispanicum, P. janczewskii, P. lividum, P. matriti, P. raistrickii, P. roqueforti* chemotype II, *P. thomii, P. viridicatum, Petromyces alliaceus*
Phoenicin	5.16	*E. cinnamopurpureum*
Scytalone	5.24	*Ceratocystis minor, P. hirsutum, Phialophora lagerbergii, Scytalidium* sp., *Verticillium dahliae*
Ethisolide	6.13	*Micropera (Foveostroma) caespitosa, P. decumbens*
Tenuazonic acid	6.69	*Alternaria alternata, Alt. brassicae, Alt. brassicicola, Alt. cheiranti, Alt. citri, Alt. japonica, Alt. kikuchiana, Alt. longipes, Alt. porri* f. sp. *solani, Alt. raphani, Alt. tenuissima, Phoma sorghina, Pleospora infectoria, Pyricularia oryzae*
Orsellinic acid	7.02	Widespread
Terrestric acid**	7.27	*P. aurantiogriseum* chemotype I, *P. crustosum, P. hirsutum, P. hordei*
Roquefortine A	7.49	*P. crustosum, P. hirsutum, P. roqueforti*
Bostrycin	7.95	*Alt. eichorniae, Arthrinium phaeospermum, Bostriconema alpestre, Khuskia oryzae*

TABLE I (*continued*)

Secondary metabolite	*Retention time (min)*	*Known producers*
2-Pyruvoylaminobenzamide	8.45	*Colletotrichum lagenarium, P. chrysogenum*
Cyclopenol	9.05	*P. aurantiogriseum, P. aurantiogriseum* var. *neoechinulatum, P. commune* chemotype II, *P. crustosum, P. echinulatum, P. solitum, P. viridicatum*
Hydroxyisocanadensic acid	9.11	*P. arenicola*
Gladiolic acid	9.24	*P. gladioli*
Viridamin	9.68	*P. viridicatum*?
"Steckiin"***	10.51	*P. steckii*
6-Methylsalicylic acid	10.99	Widespread
Chromanol 3	12.63	*A. duricaulis*
Cyclopaldic acid	12.95	*A. duricaulis, A. puniceus, Neosartorya quadricincta, Penicillium commune* chemotype I, *Pestalotia palmarum*
Altenuene	13.12	*Alt. alternata*
Barnol	13.23	*Eupenicillium baarnense*
Desoxybostrycin	13.33	See bostrycin
Gliotoxin	13.39	*A. fumigatus, A. terreus, A. ustus, P. adametzii, P. bilaii, P. corylophilum, P.* cf. *spinulosum, P. vinaceum, Trichoderma hamatum*
Cyclopenin	14.40	See cyclopenol
Brevianamide A	14.43	*P. brevicompactum, P. viridicatum*
Aflatoxin G_1	14.59	*A. flavus, A. parasiticus*
Roseopurpurin	14.70	*P. roseopurpureum*
Questinol	14.77	*P. glabrum*
Palitantin	15.71	*Eupenicillium brefeldianum* (= *E. javanicum*), *Helicomyces roseus, P. commune* chemotype II, *P. spinulosum*
Asperthecin	15.95	*Emericella nidulans* and other *Emericella* species
Norjavanicin	16.19	*F. javanicum, F. solani*
Aflatoxin B_1	16.34	*A. flavus, A. parasiticus*
Altertoxin I	16.38	*Alt. alternata*
Canadensolide	16.42	*A. tamarii, Neosartorya stramenia, P. arenicola*
Roquefortine C	16.50	*P. chrysogenum, P. coprophilum, P. crustosum, P. expansum, P. glandicola, P. griseofulvum, P. hirsutum, P. hordei, P. oxalicum, P. roqueforti, P. sclerotigenum, P. vulpinum*
3,7-Dimethyl-8-hydroxy-6-methoxyisochroman	16.52	*P. steckii*
Fusarubin, dihydrofusarubin	16.54	*F. javanicum, F. solani*
Brefeldin A	16.58	*Curvularia subulata, Cur. lunata, Eupenicillium brefeldianum* (= *E. javanicum*, stat. anam. *P. simplicissimum*), *Nectria radicicola, P. decumbens, Phoma herbarum* var. *medicaginis*
Chromanol 2	16.63	*A. duricaulis*
Canescin	16.71	*P. canescens*?
Lapidosin	16.82	*Eupenicillium lapidosum*
Desacetylpebrolide	16.82	*P. brevicompactum*
Frequentin	16.99	*P. bilaii, P. commune* chemotype II, *P. spinulosum*
3,5-Dimethyl-6-hydroxy-phthalide	17.31	*P. gladioli*
Monorden	17.42	*Monocillium nordinii, Nectria radicicola, P. resedanum, Petriellidium boydii*?

(*Continued on p. 340*)

TABLE I (*continued*)

Secondary metabolite	*Retention time (min)*	*Known producers*
Sulochrin	17.72	*A. fumigatus, A. wentii, P. glabrum*
Chromanol 1	17.72	*A. duricaulis*
Curvularin	17.83	*Alt. cinerariae, Alt. zinniae, Curvularia lunata, Cur. subulata, Drechslera australiensis, P. restrictum, P. steckii, Pseudodiplodia obiones*
Isomarticin	18.02	*Fusarium* spp., *Neocosmospora* spp.
Citrinin	18.14	*A. carneus, A. terreus, Ceuthospora* sp., *Clavariopsis aquatica, Fennellia nivea, P. citreonigrum, P. citrinum, P. expansum, P. godlewskii* (= *P. westlingii*), *P. manginii, P. miczynskii, P. odoratum, P. verrucosum* chemotype II, *Pythium ultimum*?
Dehydrocurvularin	18.26	*Alt. aureofulgens, Alt. cinerariae, Alt. tomato, Alt. zinniae, Curvularia lunata, Cur. subulata, Cercospora scirpicola, P. roseopurpureum, P. steckii, Pseudodiplodia obiones*
Alternariol	18.55	*Alt. alternata, Alt. brassicicola, Alt. cheiranthi, Alt. cucumerina, Alt. dauci, Alt. kikuchiana, Alt. longipes, Alt. porri* f. sp. *solani, Alt. raphani, Alt. tenuissima, P. diversum, Pleospora infectoria*
Griseophenone C	18.66	*P. griseofulvum*
Wortmannin	18.70	*Myrothecium roridum, Talaromyces wortmannii*
Fulvic acid	18.90	*Cercospora beticola, Eupenicillium brefeldianum* (= *E. javanicum*), *P. glabrum, P. griseofulvum*
Marticin	19.10	*Fusarium* spp., *Neocosmospora* spp.
Nectriafurone	19.23	*Nectria haematococca*
Solaniol	19.41	*F. solani*
Glauconic acid	19.45	*P. purpurugenum*
PR-toxin[§]	19.57	*P. chrysogenum, P. roqueforti*
Javanicin	20.18	*F. javanicum, F. solani*
Lambertellin	20.38	*Lambertella corni-maris, Lam. hicoriae, Pseudospiropes simplex*
Asterric acid	20.47	*A. terreus, P. citrinum, P. glabrum, Ovadendron sulphureo-ochraceum, Scytalidium* sp.
Mycophenolic acid	20.60	*P. brevicompactum, P. raciborskii, P. roqueforti, Septoria nodorum, Verticicladiella abientina*
Viridicatin	20.90	See cyclopenol
ω-Hydroxypachybasic acid	20.96	*Penicillium* sp.
Griseofulvin	21.27	*A. sydowii, Khuskia oryzae, Khuskia sacchari, (Eupenicillium brefeldianum?), P. aethiopicum, P. coprophilum, P. griseofulvum, P. griseofulvum* var. *dipodomyicola, P. janczewskii, P. jensenii, P. lanosum, P. raistrickii, P. sclerotigenum, Trichoderma viride*?
Isoemodin	21.33	*Penicillium* sp.
Norlichexanthone	21.35	*Lecanora reuteri, P. griseofulvum, Pithomyces chartarum*
2,7-Dimethoxy-5-hydroxy-6-(1-acetoxyethyl)-1,4-naphthoquinone	22.33	*Hendersenula turuloidea*
Rubratoxin B	22.76 + 24.97	*P. purpurugenum*
Pachybasic acid	23.31	*Penicillium* sp.
Questin	23.33	*A. flavus, Dermocybe cinnamoneolutea, Eurotium cristatum, Monascus ruber, P. glabrum, A. terreus*

TABLE I (*continued*)

Secondary metabolite	*Retention time (min)*	*Known producers*
Citreoviridin	24.05	*A. terreus* var. *africanus*, *A. terreus* var. *aureus*, *Eupenicillium ochrosalmoneum*, *P. citreonigrum*, *P. manginii*, *P. miczynskii*, *P. smithii*
Bostrycoidin	24.12	*F. oxysporum*, *F. solani*
Zearalenone	25.13	*F. culmorum*, *F. equiseti*, *F. graminearum*, *F. oxysporum*?, *F. pallidoroseum*, *F. sambucinum*, *F. sporotrichioides*?, *F. verticillioides*
Ochratoxin A	25.59	*A. alutaceus*, *A. fresenii*, *A. melleus*, *A. ostianus*, *A. petrakii*, *A. sclerotiorum*, *P. purpurescens*?, *P. verrucosum*
Dermoglaucin	25.96	*Cortinarius sanguineus*, *Mycoblastus sanguineus*
Ascochitine	26.27	*Ascochyta fabae*, *Pseudodiplodia obiones*
Revenelin	26.57	*Drechslera holmii*, *Dre. monoceras*, *Dre. raveleni*, *Dre. turcica*
Phomarin	26.87	*Phoma foveata*
Sterigmatocystin	26.92	*A. egyptiacus*?, (*A. flavus*), *A. multicolor*, (*A. parasiticus*), *A. sydowii*?, *A. ustus*, *A. versicolor*, *Chaetomium thielavioideum*, *Cha. udagawae*, *Drechslera sorokiniana*, *Emericella aurantiobrunnea*?, *Eme. cleistominuta*, *Eme. corrugata*, *Eme. foveolata*, *Eme. fructiculosa*, *Eme. heterothallica*, *Eme. nidulans* and its varieties, *Eme. navahoensis*, *Eme. parvathecia*, *Eme. quadrilineata*, *Eme. rugulosa*, *Eme. spectabilis*, *Eme. striata*, *Eme. unguis*? *Eme. variecolor* var. *variecolor*, *Eurotium amstelodami*, *Eur. chevalieri*, *Eur. repens*, *Eur. rubrum*, *Farrowia* sp., *Talaromyces luteus*?
Xanthocillin X	26.94 (24.10) (29.75)	*A. clavatus*, *A. ustus*, *Eupenicillium egyptiacum*, *Eurotium chevalieri*, *P. chrysogenum*
Verruculogen	27.38	*A. caespitosus*, *A. fumigatus*, *Neosartorya fischeri*, *P. brasilianum*, *P.* cf. *paxilli*
Rugulosin	27.51	*Acroschyphus sphaerophoroides*, *Endothia fluens*, *End. gyrosa*, *End. japonica*, *End. macrospora*, *End. parasitica*, *End. viridistroma*, *P. brunneum*, *P. rugulosum*, *P. tardum*, *P. variabile*, *Sepedonium ampullosporum*, *Talaromyces wortmannii*
Soranjidiol	27.81	
Duclauxin	28.04	*P. duclauxii*, *Talaromyces stipitatus*
Emodin	28.31	*A. aculeatus*, *A. alutaceus*, *A. wentii*, *Acroschyphus sphaerophoroides*, *Caloplaca* spp., *Cetraria cullulata*, *Cortinarius sanguineus*, *Drechslera catenaria*, *Eurotium chevalieri*, *Eur. cristatum*, *Eur. echinulatum*, *Fulvia fulva*, *Nephroma laevigatum*, *Penicilliopsis clavariaeformis*, *Penicillium brunneum*, *P. islandicum*, *P. tardum*, *Hypocrea austo-grandis*, *Phoma foveata*, *Pyrenochaeta terrestris*, *Talaromyces avellaneus*, *T. stipitatus*, *Xanthoria fallax*
Macrosporin	28.48	*Alternaria cucumerina*, *Alt. porri*, *Alt. porri* f. sp. *solani*, *Dactylaria lutea*, *Phomopsis juniperovora*
Cyclopiazonic acid	28.49 + 28.53	*A. flavus*, *A. oryzae*, *A. tamarii*, *P. camemberti*, *P. commune*, *P. griseofulvum*
Janthitrem A	28.58	*E. brefeldianum* (= *E. javanicum*)
2,7-Dimethoxy-6-ethyljuglone	29.11	*Hendersenula toruloidea*
Mollicin	29.13	*Mollisia caesia*, *Mol. gallens*

(*Continued on p. 342*)

TABLE I (*continued*)

Secondary metabolite	*Retention time (min)*	*Known producers*
Chaetoglobosin C	29.22	*Chaetomium cochlioides, Cha. globosum, Cha. mollipileum, Cha. rectum, Cha. subaffine, P. echinulatum* var. *discolor*
3,4,5-Trihydroxy-7-methoxy-2-methylanthraquinone	29.44	
Secalonic acid D	29.61	*P. oxalicum*
Anhydrofusarubin	30.03	*F. solani*
Purpurugenone	30.14	*P. purpurugenum*
Mycelianamide	30.24	*P. griseofulvum*
Catenarin	31.21	*Eurotium* spp., *Talaromyces stipitatus*
Byssochlamic acid	31.50	*Byssochlamys fulva, Bys. nivea*
Cladofulvin	31.97	*Fulvia fulva*
Pachybasin	32.23	*Aschochyta pisi, Phoma foveata, Trichoderma viride*
Chrysophanol	32.29	*Ascochyta pisi, Drechslera catenaria, Hypocrea austo-grandis, P. islandicum, Phoma foveata, Pseudospiropes simplex, Sepedonium ampullosporum*
Paxillin	32.40	*"P. paxilli"*
Penitrem A	32.77	*P. aurantiogriseum* (traces), *P. clavigerum, P. crustosum, P. glandicola, P. janczewskii*
Paspalinin	32.85	*A. flavus, Claviceps paspali*
Skyrin	32.99	*Acroschyphus sphaerophoroides, Endothia fluens, End. gyrosa, End. havanaensis, End. japonica, End. longirostris, End. macrospora, End. parasitica, End. radicalis, End. singularis, End. tropicalis, Hypomyces lactifluorum, Hyp. trichothecioides, Penicilliopsis clavariaeformis, P. brunneum, P. islandicum, P. rugulosum, P. tardum, Physica obscure* var. *endococcina, Pyxine endochrysina, Sepedonium ampullosporum, Trypetheliopsis boninensis*
Helminthosporin	33.36	*Drechslera* spp.
Physicon	33.52	*Achaetomium crystalliferum, Alternaria porri, A. flavus, A. wentii, Caloplaca murorum, Cetraria cullulata, Dermocybe cinnabarina, Eurotium chevalieri, Eur. cristatum, Eur. echinulatum, Eur. repens, Xanthoria fallax, Xan. mandschurica*
Equisetin	33.60	*F. equiseti*
Flavoglaucin	33.68	*A. flavus, Eurotium amstelodami, Eur. chevalieri, Eur. echinulatum, Eur. herbariorum, Eur. heterocaryoticum, Eur. repens, P. fellutanum, P. islandicum*
Islandicin	33.75	*P. islandicum*
Lichexanthone	33.82	Several lichens
Cynodontin	34.04	*Cercospora cari, Curvularia lunata, Cur. pallascens, Drechslera* spp., *Phoma* spp., *Pyrenochaeta terrestris, Spilocaea olieaginea*
Naphthalic anhydride	34.21	*A. silvaticus, Fusicoccum putrefaciens, P. herquei, Roesleria pallida*
Aflatrem	34.29	*A. flavus*
Erythroglaucin	34.48	*Alternaria porri, Dermocybe cinnabarina, Eurotium chevalieri, Eur. cristatum, Eur. herbariorum, Eur. repens, Talaromyces stipitatus, Xanthoria fallax, Xan. mandschurica*

* Producers of the secondary metabolites were taken from Turner[6], Turner and Aldridge[7], and revised according to Carmichael *et al.*[14], Hawksworth *et al.* (1983)[15], Samson and Pitt (1985)[16] and Frisvad (1986)[10]. Incorrectly identified fungi have not been included in this list. As an example, *P. cyaneum* has been cited as a producer of brefeldin A[17]. The original producer strain (V. Betina S-11 = CCM F-376) was a *P. simplicissimum*, however. Paterson[18], based on the system of Frisvad and Filtenborg[9], provides TLC data for many of the secondary metabolites treated in this paper.

** Terrestric acid is seen as a very broad peak.

*** "Steckiin" is an unknown compound ($C_{22}H_{26}O_9$) from *P. steckii* isolated by G. J. van Eijk.

§ PR-toxin shows characteristic tailing.

TABLE II

RETENTION TIMES FOR SOME OF THE MYCOTOXINS AND OTHER SECONDARY METABOLITES LISTED IN TABLE I, BUT ON ANOTHER BATCH OF NUCLEOSIL COLUMN MATERIAL (HPLC)

Secondary metabolite	*Retention time* of standards (min)*	*Mean and standard deviation** based on fungal extracts*
Kojic acid	1.22	
Carlosic acid	1.57	
Dehydrocarolic acid	1.63	
Terrein	2.25	
Patulin	2.27	2.26 ± 0.03 (8)***
Roquefortine B	2.67	
Roquefortine D	2.76	
Deoxynivalenol	2.87	
Austdiol	4.44	
Spinulosin	5.08	
Fusarenone X	5.26	
Rugulovasine A	5.31	
Penicillic acid	6.09	6.09 ± 0.01 (11)
Neosolaniol	7.59	
Terrestric acid	7.76	7.75 ± 0.27 (11)
Orsellinic acid	8.47	
Roquefortine A	9.16	
Cyclopenol	10.17	10.15 ± 0.06 (18)
Viridamin	10.61	
Chromanol 3	13.39	
Gliotoxin	14.22	
Brevianamide A	14.70	
Cyclopenin	15.46	15.44 ± 0.10 (22)
Palitantin	16.66	
Roquefortine C	17.22	17.22 ± 0.36 (27)
Brefeldin A	17.26	
Chromanol 2	17.80	
Monorden	19.12	
Alternariol	19.90	
Citrinin	20.01	
PR-toxin	21.27	21.25 ± 0.02 (4)
Viridicatin	21.98	21.97 ± 0.10 (13)
Mycophenolic acid	22.05	21.86 ± 0.27 (7)
Griseofulvin	22.21	22.16 ± 0.13 (29)
Rubratoxin B	23.84, 26.33	
Citreoviridin	24.45	
Zearalenone	26.93	
Ochratoxin A	27.27	
Xanthocillin X	28.22 (25.66; 28.81)	
Sterigmatocystin	28.55	
Verruculogen	28.93	
Rugulosin	29.43	
Janthitrem B	30.02	
Cyclopiazonic acid	30.25§	30.29 ± 0.07 (13)
Paxillin	33.08	
Penitrem A	33.48	33.49 ± 0.03 (10)
Paspalinin	33.51	
Equisetin	34.01	
Aflatrem	34.80	

* Average of three values determined on three different days.

** Based on peaks in chromatograms of fungal extracts (confirmed by external and internal standards and TLC in different systems).

*** Numbers in parentheses are numbers of different fungal chromatograms. For patulin the values were taken from chromatograms of *P. expansum, P. vulpinum, P. griseofulvum, P. glandicola* and *P. roqueforti* chemotype II, for example.

§ Cyclopiazonic acid was often seen as two peaks (the two tautomers).

TABLE III

PERCENTAGE DIFFERENCES IN RETENTION TIMES AND RETENTION TIMES RELATIVE TO GRISEOFULVIN ON TWO DIFFERENT BATCHES OF NUCLEOSIL

Secondary metabolite	*Percentage difference*	
	Retention time	*Relative retention time*
Kojic acid	7.0	1.3
Patulin	14.6	8.5
Terrein	13.6	7.5
Roquefortine B	28.4	21.5
Roquefortine D	23.2	17.1
Deoxynivalenol	18.1	12.3
Austdiol	13.0	7.0
Rugulovasine A	27.0	19.8
Fusarenone X	22.9	16.4
Spinulosin	17.6	11.3
Penicillic acid	23.1	16.7
Orsellinic acid	20.7	14.2
Terrestric acid	12.5	6.4
Roquefortine A	22.3	15.9
Cyclopenol	13.5	7.5
Viridamin	9.6	3.7
Chromanol 3	6.0	0.3
Cyclopaldic acid	5.5	−0.2
Gliotoxin	6.2	0.5
Cyclopenin	8.1	2.2
Brevianamide A	2.2	−3.2
Palitantin	6.0	0.3
Roquefortine C	4.4	4.3
Fusarubin	5.5	−0.1
Brefeldin A	4.1	−1.5
Chromanol 2	7.0	1.3
Monorden	9.8	3.9
Chromanol 1	6.8	1.1
Alternariol	7.3	1.6
PR-toxin	9.7	3.8
Mycophenolic acid	7.9	2.1
Viridicatin	5.2	−0.5
Griseofulvin	5.6	0
Rubratoxin B	4.7, 5.4	−0.8, −0.1
Citreoviridin	1.7	−3.8
Zearalenone	7.2	1.4
Ochratoxin A	7.0	0.9
Sterigmatocystin	6.1	0.4
Xanthocillin X	4.8	−0.9
Verruculogen	5.7	0
Rugulosin	7.0	1.3
Cyclopiazonic acid	6.2	0.6
Janthitrem A	5.0	−0.6
Chaetoglobosin C	6.3	0.7
Paxillin	2.1	−3.3
Penitrem A	2.2	−3.3
Paspalinin	2.0	−3.4
Equisetin	1.1	−4.4
Aflatrem	1.5	−3.9

and feedstuffs presents the analytical chemist with several challenges. The presence of unknown metabolites in the growth substrate of the fungi examined did not cause problems in the HPLC analysis of unpurified chloroform–methanol extracts: extracts of uninoculated YES or CYA agar media showed no peaks in the chromatograms except for two small peaks that eluted before the first fungal secondary metabolite detected (moniliformin). As no peaks, except for the two peaks mentioned above, were common to all fungal chromatograms, it appears that all peaks detected were secondary metabolites. This is probably due to the poor absorbtion of most primary metabolites (amino acids, carbohydrates and fatty acids) at 254 nm and the polarity of many primary metabolites, leaving them undissolved in the chloroform extracts. The notable exception is most fungal lipids, which are present in the chloroform extracts; however, fatty acids absorb very poorly at 254 nm and this appear to apply also to other lipids, such as most fungal sterols.

HPLC traces of chloroform–methanol extracts of different species of terverticillate penicillia showed species-specific profiles of known and unknown secondary metabolites. Fig. 1 and 2 show chromatograms of *Penicillium griseofulvum* (two isolates) and *P. vulpinum* (formerly *P. claviforme*) (two isolates), respectively. The production of patulin, roquefortine C, griseofulvin and cyclopiazonic acid is indicated for *P. griseofulvum* and the production of patulin and roquefortine C is indicated for *P. vulpinum*. The production of these toxins was confirmed by the use of internal standards and TLC of the same fungal extracts using internal standards and different chemical treatments[9]. In addition to these known toxins, many other metabolites contribute to the specific profile of secondary metabolites. Even though these unknown

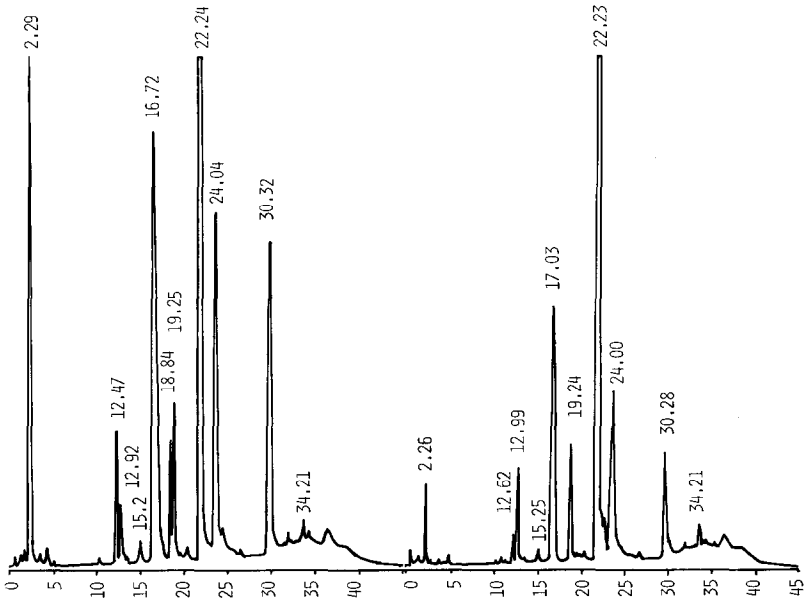

Fig. 1. HPLC traces of two isolates of *P. griseofulvum* (left, IMI 285525, right, KBA 34b). Note the many peaks at the same retention time and the peaks representing patulin (2.29 and 2.26 min), roquefortine C (16.72 and 17.03 min), griseofulvin (22.24 and 22.23 min) and cyclopiazonic acid (30.32 and 30.28 min). The extracts were made from six petri dishes of Czapek yeast extract agar (CYA).

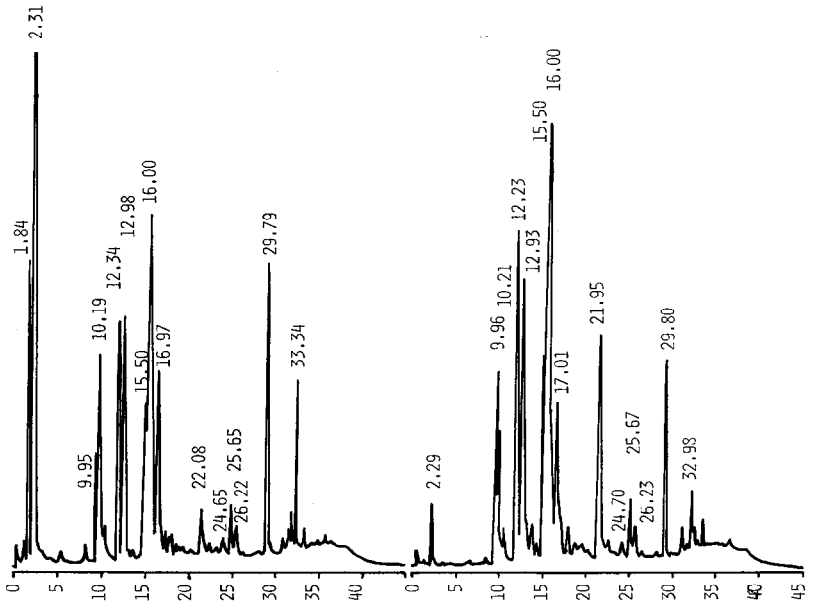

Fig. 2. HPLC traces of two isolates of *P. vulpinum* [left, our subculture of NRRL 1002; right, ATCC 58612 (from LKF ZL16)]. Note the many peaks at the same retention time (most of them different from those of *P. griseofulvum*) and the mycotoxins patulin (2.31 and 2.29 min) and roquefortine C (17.03 and 17.01 min). The cultures were grown on CYA for 12 days at 25°C.

secondary metabolites appear in a specific time sequence and often in specific correlated amounts, a retention time index and partial peak identification or characterization (*i.e.*, by post-column derivatization, mass spectrometry or diode array UV–VIS detection), seem to be necessary for the general identification of the terverticillate penicillia based on HPLC of secondary metabolites in different laboratories. We are currently working on such a chemotaxonomic system based on HPLC and diode array detection.

Some secondary metabolites had nearly the same retention times in the chromatographic system, but these metabolites were not produced by the same species, the exception being patulin and terrein, both of which are produced by *Aspergillus terreus*. For each of the four important food-borne genera in foods, *Penicillium*, *Aspergillus*, *Fusarium* and *Alternaria*, the most important secondary metabolites produced by species in these genera are evenly distributed along the time axis in the chromatograms (Table I). The many other genera producing the secondary metabolites listed in Table I and the many types of secondary metabolites detectable by liquid chromatography (polyketides, terpenes and alkaloids) indicate that the HPLC method presented here may be applied in chemotaxonomic studies of most fungal taxa.

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REFERENCES

1 R. D. Cooker, in J. Gilbert (Editor), *Analysis of Food Contaminants*, Elsevier, Amsterdam, London and New York, 1984, p. 207.
2 P. M. Scott, in J. F. Lawrence (Editor), *Trace Analysis*, Academic Press, New York, 1981, p. 193.
3 D. W. Hill, T. H. Kelley, K. J. Langner and K. W. Miller, *Anal. Chem.*, 56 (1984) 2576.
4 M. E. di Menna, D. R. Lawrence and P. A. Wyatt, *Appl. Environ. Microbiol.*, 51 (1986) 821.
5 R. J. Cole and R. H. Cox, *Handbook of Toxic Fungal Metabolites*, Academic Press, New York and London, 1981.
6 W. B. Turner, *Fungal Metabolites*, Academic Press, London and New York, 1971.
7 W. B. Turner and D. C. Aldridge, *Fungal Metabolites II*, Academic Press, London and New York, 1983.
8 C. C. Culberson and W. L. Culberson, *Syst. Bot.*, 1 (1976) 325.
9 J. C. Frisvad and O. Filtenborg, *Appl. Environ. Microbiol.*, 46 (1983) 1301.
10 J. C. Frisvad, in R. J. Cole (Editor), *Modern Methods in the Analysis and Structural Elucidation of Mycotoxins*, Academic Press, New York and London, 1986, p. 415.
11 J. H. Wall and E. B. Lillehøj, *J. Chromatogr.*, 28 (1983) 461.
12 J. A. Lansden, *J. Assoc. Off. Anal. Chem.*, 67 (1984) 728.
13 V. Meyer, *Praxis der Hochleistungs-Flüssigchromatographie*, 3. Auflage, Sauerländer, Aarau, 1984.
14 J. W. Carmichael, W. B. Kendrick, W. B. Conners and L. Sigler, *Genera of Hyphomycetes*, University of Alberta Press, Edmonton, 1980.
15 D. L. Hawksworth, B. C. Sutton and G. C. Ainsworth, *Ainsworth and Bisby's Dictionary of the Fungi*, 7th ed., Commonwealth Mycological Institute, Kew, 1983.
16 R. A. Samson and J. I. Pitt (Editors), *Advances in Penicillium and Aspergillus Systematics*, Plenum Press, New York and London, 1985.
17 J. Fuska and I. Kuhr, *Folia Microbiol.*, 10 (1965) 357.
18 R. R. M. Paterson, *J. Chromatogr.*, 368 (1986) 249.