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# PRODUCTION OF ANTIFUNGAL METABOLITES FROM VARIOUS ASPERGILLUS SPECIES: A STATE OF REVIEW

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## ABSTRACT

Filamentous fungi in the Aspergillus section Nigri (the black aspergilli) represent some of the most widespread food and feed contaminants known but they are also some of the most important workhorses used by the biotechnological industry. The Nigri section consists of six commonly found species from which currently 145 different secondary metabolites have been isolated and detected. From a human and animal safety point of view, the mycotoxins ochratoxin A and fumonisin B2 are currently the most problematic compounds. Especially in foods and feeds such as coffee, nuts, dried fruits, and grape-based products where fumonisin-producing fusaria are not a problem, fumonisins pose a risk. Bioactives were analyzed using gas chromatography-mass spectroscopy technique, then the in vitro antibacterial and antifungal activity of the methanolic extract was evaluated. 47 bioactive compounds were identified in the methanolic extract of Aspergillus terreus. Crude extract of Gramineae poaceae plant was very highly active. The results of antibacterial activity produced by Aspergillus terreus showed that the volatile compounds were highly effective to suppress the growth of Streptococcus pneumonia. Aspergillus terreus produce many important secondary metabolites with high biological activities. Based on the significance of employing bioactive compounds in pharmacy to produce drugs for the treatment of many diseases, the purification of compounds produced by Aspergillus terreus species can be useful.

**KEYWORDS:** Production of antifungal matabolites, Aspergillus.

## INTRODUCTION

The black aspergilli are some of the most important mycotoxigenic food and feed contaminants, especially in postharvest decay of fresh and dried fruits and certain vegetables, nuts, beans, and cereals.[1,2] This is due to their fast growth, pH tolerance, and high abundance in many environments. For the analytical chemist, issues such as fungal taxonomy and correct identification may seem of low relevance, but in fact biosystematics is a vital part of mycotoxin research and food safety. Since the profile of mycotoxins and other secondary metabolites is species specific[3–5], correct identification at the species level provides the key for planning the analytical determination of all relevant compounds.

The Aspergillus niger group (the black aspergilli, Aspergillus subgenus Circumdati section Nigri) comprises 18 species, of which Aspergillus niger, Aspergillus tubingensis, Aspergillus brasiliensis, Aspergillus acidus, Aspergillus carbonarius, and Aspergillus ibericus are common, whereas the remaining species are rare and found mainly in tropical regions (Table 1). A cladification of Aspergillus section Nigri using the β-tubulin and calmodulin genes showed that three clades could be distinguished[5]: the Aspergillus niger clade, a clade consisting of the two rare species Aspergillus homomorphus and Aspergillus ellipticus, and the clade of uniseriate black aspergilli (Aspergillus aculeatinus, Aspergillus aculeatus, Aspergillus japonicus, and Aspergillus uvarum), the members of which differ significantly from the remaining black aspergilli regarding their morphology, physiological behavior, and secondary metabolite production (e.g., producers of neoxaline, asperparalines, secalonic acids, asperamide, and aculeasins)[5], and this third clade has therefore not been included in this review. The identity and metabolite production of the uniseriate black aspergilli is usually not confused with the identity and metabolite production of Aspergillus niger and other biseriate black aspergilli. Aspergillus niger and Aspergillus tubingensis are probably the most common of the black aspergilli; however, in many studies describing secondary metabolites from these aspergilli, the producing organism has been identified as a black Aspergillus and then in many cases incorrectly named Aspergillus niger. A wrong identification may be further complicated by insufficient molecular identification based on sequencing of ribosomal DNA with low resolution.[5,6]

**Table 1: Various species of Aspergillus.**

| SPECIES | RARE |
|---|---|
| Aspergillus acidus | - |
| Aspergillus piperis | Yes |
| Aspergillus niger | - |
| Aspergillus ellipticus | Yes |
| Aspergillus vadensis | Yes |

A polyphasic approach where many different types of characters (microscopy, metabolite profiling, molecular methods) are used is recommended for the identification of these aspergilli. Certain molecular methods have proven quite successful, including restriction fragment length polymorphism and β-tubulin, or calmodulin sequencing. Until recently, the main mycotoxin from the black aspergilli was considered to be ochratoxin A (OTA), produced in variable amounts within certain species of the group. Aspergillus carbonarius consistently produces large amounts of OTA, whereas only 6–10% of members of the Aspergillus niger group produce OTA and in 10– 1000 lower amounts.[7–9] The third species producing OTA in section Nigri is Aspergillus sclerotioniger, but this species has only been found once in coffee. However, Aspergillus niger clearly has the widest distribution and has been reported to grow and damage a much larger number of crops and foods worldwide, including corn, peanuts, raisins, onions, mango, apples, and dried meat products.[2] This combined with the recent discovery that Aspergillus niger can also produce fumonisin B2 (FB2) and fumonisin B4 (FB4)[10,11] (Fig. 1) necessitates the addition of fumonisins in a number of food and feed screening programs. Aspergillus niger exhibits a remarkably versatile metabolism, which has made it one of the most important production organisms used for industrial fermentations.[12,13]

Since 1923, Aspergillus niger has been exploited commercially for its production of citric acid, mostly for use in food, cosmetics, and pharmaceutical preparations.[14] In addition, the fungus has been a rich source of industrial enzymes such as α-amylases, cellulases, and pectinases for use in the food industries since the 1960s.[12] Aspergillus niger possesses posttranslational mechanisms capable of correctly processing proteins that are difficult to express in traditional host organisms. As a result, it is widely used as a cell factory for heterologous expression of proteins.[14]

Aspergillus niger has been considered to be nontoxic under industrial conditions[12], and thus to be considered a safe production organism. As a result, quite a number of Aspergillus niger fermentations have been granted the generally regarded as safe (GRAS) status by the US Food and Drug Administration.[12] However, the potential presence of both OTA and fumonisins in Aspergillus niger emphasizes the need to adjust and/or reconsider the screening procedures for simultaneous targeting of multiple classes of mycotoxins.

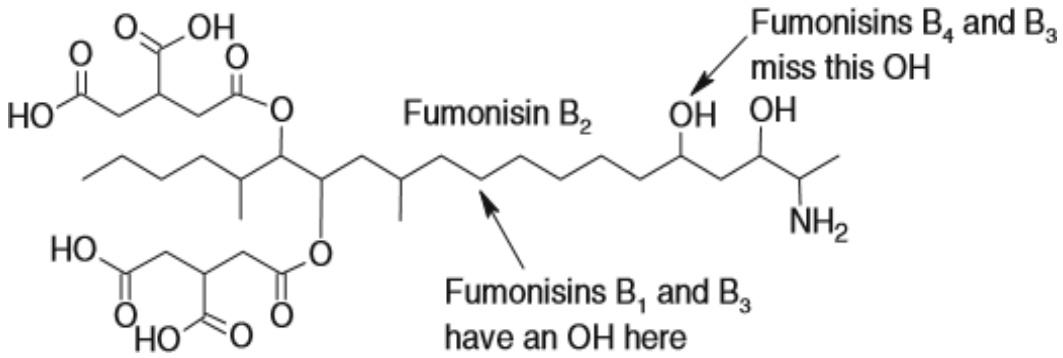



**Fig. 1: Structure of Fumonisin.**

The large extracts from the years 1983–1995 were analyzed by LC [acidic, 15–100% acetonitrile (CH3CN) gradient, 40 min, Nucleosil C18 column] with DAD (200–600 nm) as described by Frisvad and Thrane.[16] Data were available as printed reports with chromatographic traces (210 and 280 nm) and UV/vis spectra (200–600 nm). Microextracts from the years 1995–2003 were analyzed by LC-DAD with parallel fluorescence detection (FLD) under similar chromatographic conditions using a Nucleosil BDB C18 column[17] with the FLD set at 230→333 and 230→450 nm. Microextracts from the years 2003–2009 were analyzed by LC-DAD-FLD as mentioned above using a Luna C18 II column (15% CH3CN to 100% CH3CN in 20 min).[18] The analyses were performed using a LC system coupled to an orthogonal TOF mass spectrometer (Micromass LCT oaTOF) equipped with an electrospray source.[19] Two different gradients were used: (1) Luna II C18 15% CH3CN to 100% CH3CN in 20 min, 20 mM formic acid in both solvents[20]; (2) as described before but with the gradient starting at 30% CH3CN and going to 60% CH3CN in 5 min and then to 100% in 1 min. About 300 extracts were analyzed by LC-DAD–TOF mass spectrometry (TOFMS) in the years 2003–2009; of these, most have also been analyzed by LC-DAD-FLD.

In another way, the metabolite was analysed by LC–tandem mass spectrometry. This was performed using a Quattro Ultima triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source and operated in multiple reaction mode (MRM).[11] Separation was done on a Gemini C6phenyl column

(acidic, 20% CH3CN to 55% CH3CN in 6 min, then to 100% in 30 s). MRM transitions for OTA, fumonisin B1 (FB1), FB2, fumonisin B3 (FB3), and FB4 are described elsewhere.[11,21] Malformin A2 was detected using m/z 516→304 at 30 V and m/z 516→417 at 20 V; malformins C and A were detected using m/z 530→372 at 25 m/z 530→417 at 20 V; ochratoxin β was detected using m/z 223→103 at 36 V and m/z 223→159 at 36 V; ochratoxin α was detected using m/z 257→193 at 36 V and m/z 257→221 at 33 V; and ochratoxin B was detected using m/z 370→205 at 36 V and m/z 370→324 at 36 V. About 350 extracts were analyzed by LC–tandem mass spectrometry (MS/MS) in the years 2008–2009; many of these have also been analyzed by LC-TOFMS and LC-DAD-FLD. For the LC-DAD-FLD analyses, OTA, ochratoxin B (Sigma-Aldrich), ochratoxin α, kotanin, nigragillin, and malformins A, A2, and C were coanalyzed. For LC– mass spectrometry (MS), FB1, FB 2, and FB3 (Biopure, Tuln, Austria) and AAL toxins TA1 and TB1 (Sigma Aldrich) were also included. Tensidols A and B and aurasperones A, B, and E were purified and validated by NMR.[22]

LC-DAD and LC-DAD-FLD data were analyzed manually by comparing retention times, and UV spectra for the detected peaks as well as the retention times of the fluorescence peaks. These data were matched with LCDAD-TOFMS data for tentative identification of the peaks by searching Antibase (Wiley) for compounds with the same accurate mass (±0.01-Da tolerance) and similar UV properties and estimated retention time (based on theoretical log D values).[19] Compounds that we were able to tentatively identify in extracts from black aspergilli were aurasperones C–G, pyrophen, funalenone, fonsecinones B and C, fonsecin (TMC-256B1B), TMC-256A2, rubrofusarin B, fonsecin B, tensyuic acid A, asperazine, pyrano nigrins A– D, and FB4. The identity of FB4 was further validated by showing that its accurate mass, retention time and tandem spectra from 25–50 eV were identical to those of to the presumed FB4 in culture extracts from Fusarium verticillioides.

The black aspergilli can produce a diverse range of mainly polyketide-derived secondary metabolites along with non ribosomal peptides and a number of compounds of mixed biosynthetic origin. Annotation of the fully sequenced genome of Aspergillus niger showed the presence of an impressive 34 polyketide synthase (PKS)-encoding genes as well as 17 nonribosomal peptide synthase (NRPS) genes and seven PKS-NRPS hybrids, accentuating the biosynthetic potential and versatility of this species.[13] So far, a total of 145 metabolites have been isolated and their structures elucidated from the biseriate black aspergilli.

Historically, OTA and its precursors (ochratoxins B, β, and α) have been the most significant mycotoxins produced within the Aspergillus niger complex. In extracts from approximately 400 agar cultures, OTA was always detected in amounts at least 10 times higher than the precursors using LC-FLD, LC-TOF analysis, and LC-MS/MS, and in many cases only OTA was detected. It has been claimed that Aspergillus tubingensis can produce OTA[23,24], presumably owing to unspecific chemical analyses. It was not possible to confirm this from the same cultures in our laboratory by LC-FLD, LC-TOF analysis, nor LC-MS/MS in culture extracts from up to ten different media. Other researchers have also doubted OTA production by Aspergillus tubingensis.[3,25] Owing to the array of adverse toxicological effects on animals and humans, OTA is one of the most studied mycotoxins. The very low regulatory levels in food and feed mean that numerous highly sensitive analytical methods have been developed[26–28] and these have been reviewed several times.[29,30] Thus, OTA will not be in the major scope of this paper since many of the commodities where Aspergillus niger is a problem have already been covered.[29,31–35]

However, it must be emphasized that we have found that direct analysis of OTA from A. niger using LC-DAD is not possible in crude extracts owing to coeluting malformins and naptho-γ- pyrones (NGPs) (see later), of which the NGPs are present in more than 100 times higher quantities than OTA. Furthermore, with use of our LC- DADFLD method, four to five peaks of closely eluted substances are detected from many black Aspergillus extracts (230→ 333 nm, acidic conditions). This indicates a risk that some of these compounds may be coeluted with OTA even under other slightly different conditions. Thus, careful control of chromatographic conditions is necessary.

Careful control of potential carryover between samples in the autosampler should be done if high- level OTA-producing Aspergillus carbonarius strains are analyzed in sequence. For OTA analysis, our LC-TOFMS was approximately two fold less sensitive than FLD. Here sensitivity could be enhanced tenfold by shifting to alkaline conditions[32]; however, other compounds would suffer from a pH change (e.g., by post column addition of $NH_4OH$ solution) and comparison with old data will not be possible. Our LC-MS/ MS instrument is about 20–50 times more sensitive than acidic FLD and has until now not yielded any interfering peaks on any of the two MRM transitions. It should be noted that the latest LC-TOFMS and Orbitrap instruments will, according to the manufactures, provide 10 times higher mass resolution and 10– 100 times better sensitivity than the LC-TOFMS instrument used here. ESI of OTA mainly yields the protonated molecular ion $[M+H]^+$ in positive mode and $[M-H]^-$ in negative mode, with approximately 10 times higher signal in positive mode.

With high in-source fragmentation settings, OTA yields several diagnostic ions for further confirmation. An unidentified fragment ion at m/z 358 can be observed along with the sodium adduct $[M+Na]^+$ and the 37Cl $[M+H]^+$ at m/z 406.[19] Care should be taken when using nominal mass LC-MS, since a very common

contaminant from plasticware (presumable a phthalate) with a molecular mass of 386 Da is eluted very close to OTA and makes at predominant $[M+NH4]^+$ ion at m/z 404. OTA can be differentiated from the contaminant on the basis of the chlorine isotope pattern.

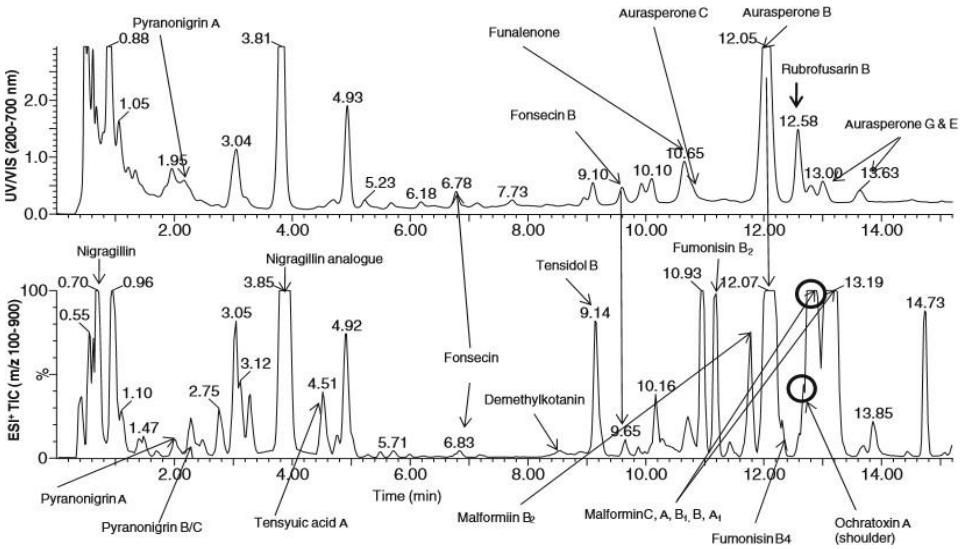



**Fig. 2: Aspergillus niger extract from 8-day culture on yeast extract sucrose agar analyzed by liquid chromatography – diode-array detection– time-of flight mass spectrometry.**

Endophytic fungi which thrive inside plant tissues as asymptomatic mutualists had been renowned as an important and novel source of bioactive compounds.[1] An array of secondary metabolites that were used as bio-control agents, immunosuppressive compounds, etc. was produced by these endophytes.[2] The metabolic interactions of endophytes with its host might favor the synthesis of biologically active secondary metabolites. The endophytes had metabolic impacts on host plants and possibly regulated the biochemical status of host plants.[3] Based on structure and biosynthetic pathway, fungal secondary metabolites were mainly classified as polyketides, non ribosomal peptides, alkaloids, or terpenes.[4,5]

Plants, known for ethnomedicinal properties and applications have been found to harbor novel endophytic microflora (Ravindra et al. 2016) which showed the importance of selection of plants for endophyte isolation. Several studies had revealed that different species of Morus had antioxidant, antiviral, anti-inflammatory, hypolipidemic, anti- hyperglycemic, neuroprotective[6], anti-HIV, anti- hypotensive and cytotoxic activities.[7] Antibiosis is considered as the most important type of antagonism in which the antagonists produce an array of secondary metabolites such as antibiotics and toxin, which contribute to the antagonistic activity of fungal biocontrol agents against plant pathogens.[8] Root rot, due to its epidemic nature and potential to kill the plants, is a dangerous disease of mulberry. Hence, this study highlighted the exploration and documentation of metabolite profile connected with mulberry endophytic fungi, Aspergillus terreus and evaluation of its bio-efficacy in controlling of charcoal rot pathogen, Macrophomina phaseolina.

The surface sterilization of collected plant samples was done according to the method described by Petrini[9] with modifications. The surface sterilized samples were macerated with 1 ml of Sterile Distilled Water (SDW) in a sterile mortar and pestle, and each sample was serially diluted in test tubes containing 9 ml of SDW. The dilutions of 10-4 and 10-5 were plated on Petri plates containing PDA medium supplemented with antibiotics. The plates were then incubated at 28±2°C for 10 days and observed for fungal growth. Each colony obtained was sub cultured on PDA slants and maintained for further use.[10] The isolated fungi were identified on the basis of morphological characteristics according to Domsch et al.[11] The charcoal rot pathogen, M. phaseolina was isolated from infected samples by root bit method[12] and identified based on the morphological characters. The isolated endophytes were screened for in vitro antagonistic activity against M. phaseolina by using dual culture technique.[13]

Based on the performance of isolates in the dual culture assay, potential isolate was further identified using molecular techniques. Genomic DNA was extracted from ground mycelium.[15] The DNA concentration and integrity were checked by electrophoresis on 0.8% agarose gel. PCR amplification of the DNA was performed using primer ITS1 (5' TCCGTAGGTGAACCTGCGG-3'), as described by Rhoden et al.[16], and primer ITS4 (5'TCCCCGCTTATTGATATGC-3'), as described by White et al.[17] Afterwards, samples were quantified again by electrophoresis on 1.2% agarose gel and documented. The PCR amplified products were sequenced. The metabolites were extracted according to the method followed by Choudhary et al.[22] with modifications. The fungi were inoculated in Potato

Dextrose Broth (PDB) and maintained at 28±2°C for 15 days. Liquid state fermentation method was followed for the production of metabolites. The culture broths were filtered and procedure using ethyl acetate as organic solvent. Equal volume of the filtrate and ethyl acetate was kept for overnight incubation in rotary shaker at 150 rpm. Then, the solvent phase was extracted using separating funnel and was dried in vacuum flask evaporator to yield the crude metabolite. The extract was scraped using HPLC grade methanol and stored for further analysis. Mycelia were separated. The broth containing metabolite was extracted by solvent extraction procedure using ethyl acetate as organic solvent. Equal volume of the filtrate and ethyl acetate was kept for overnight incubation in rotary shaker at 150 rpm. Then, the solvent phase was extracted using separating funnel and was dried in vacuum flask evaporator to yield the crude metabolite. The extract was scraped using HPLC grade methanol and stored for further analysis.

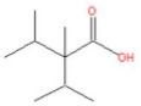
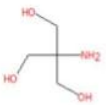
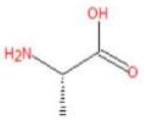
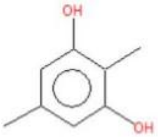
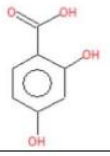
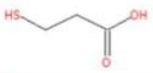
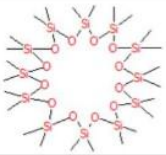
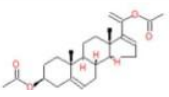
The purified extract was subjected to GC- MS analysis to identify the bioactive compounds. The sample was analysed in PERKIN ELMER CLARUS SQ8C gas chromatograph with DB-5 MS capillary standard non-polar column (Dimension: 30 Mts, ID: 0.25 mm, Film: 0.25 IM) was used. Helium was used as a carrier gas.

A total of 30 endophytic fungal isolates were obtained from healthy tissues of M. indica. Amongst them, 13 isolates namely ENF1, ENF5, ENF8, ENF12, ENF15, ENF16, ENF18, ENF22, ENF24, ENF26, ENF28, ENF29 and ENF30 were recovered from mulberry leaf. Based on the in vitro antifungal efficacy against M. phaseolina, the best performed isolate ENF12 was selected for further studies. The morphological and microscopic characteristic of ENF12 isolate was observed. The results revealed that the young mycelium was white in color later it turned into yellowish brown color. The yellow pigmentation was observed over the media. The hyphae were septate and hyaline. The conidia were small in size, smooth and oval in shape. The aleurioconidia, asexual spores produced directly on the hyphae were noticed (Figure 1). The sequential steps in the formation and wall structure of A. terreus conidia were studied by Transmission Electron Microscope. Solitary or botryose globose lateral cells 'aleurioconidia' occurred on the vegetative mycelium.[24]

The result of this study was collaborated with earlier findings. Pang et al.[25] isolated the endophytic fungus Aspergillus sp. CPCC 400735 from the plant Kadsura longipedunculata. Similarly, Aletaha et al.[26] isolated endophytic A. terreus from Suaeda aegyptica. The pathogen was isolated from the root rot infected plant and identified as M. phaseolina based on morphological characters and sclerotial structures. The ENF12 isolate was screened against mulberry charcoal rot pathogen, M. phaseolina by dual culture technique. The ENF12 isolate inhibited the growth of pathogen by 77.80% over control with an inhibition zone of 7.50 mm. The interaction between ENF12 isolate and pathogen was suggested that the endophyte could antagonize the growth of pathogen by antibiosis mechanism with presence of reactive zone between endophyte and pathogen (Figure 2). The outcome of in vitro study was accordance with the findings of Hamdi et al.[27] who evaluated the antifungal potential of A. terreus against soil borne fungi (Fusarium oxysporum f. sp. melonis and M. phaseolina) of watermelon and inhibited the mycelial growth by 36.14% and 46.63% respectively.

The metabolic profiles of living organisms reflect features of their life activities. The metabolites were identified using GC-MS analysis showed the relative abundance of various compounds. A Total of 91 metabolites were identified from A. terreus. The metabolites were classified according to their nature of the compounds such as amino acids, sugars, alcohol, fatty acids, organic acids, ketone, vitamins, sterols, terpenoids, alkaloids, carboxylic esters, hydrocarbons and others (Figure 4). Among the secondary metabolites, fatty acids were significantly high followed by amino acids, terpenoids, alcohols and hydrocarbons. The metabolite array of Aspergillus species were supported by Pang et al.[25] who extracted thirty- three metabolites including five phenalenone derivatives (1−5), seven cytochalasins (6−12), thirteen butenolides (13−25) and eight phenyl derivatives (26−33) from endophytic Aspergillus sp. CPCC 400735 isolated from rice.

**Table 2: Compounds identified from Aspergillus terreus.**

| S. no. | Retention time (minutes) | Peak area percentage | Compound name | Molecular weight | Molecular formula | Molecular structure | Biological function |
|---|---|---|---|---|---|---|---|
| 1. | 7.345 | 0.926 | Acetylthiooctanoic acid | 232 | $C_{11}H_{20}O_3S$ |  | Antifungal Antimicrobial Antibacterial |
| 2. | 7.440 | 1.487 | Butanoic acid | 158 | $C_9H_{18}O_2$ |  | Antimicrobial |
| 3. | 8.345 | 7.283 | Tromethamine | 121 | $C_4H_{11}NO_3$ |  | Antibacterial Anticancer agent |
| 4. | 8.561 | 0.517 | L-Alanine | 89 | $C_3H_7NO_2$ |  | Antibacterial |
| 5. | 9.796 | 7.916 | 1,3-Benzenediol | 138 | $C_8H_{10}O_2$ |  | Anti-oxidant Antibacterial |
| 6. | 15.008 | 2.226 | Dodecyl acrylate | 240 | $C_{15}H_{28}O_2$ |  | Antimicrobial |
| 7. | 18.129 | 3.963 | Benzoic acid | 154 | $C_7H_6O_4$ |  | Antibacterial |
| 8. | 20.430 | 1.106 | n-Hexadecanoic acid | 256 | $C_{16}H_{32}O_2$ |  | Anti-inflammatory Antioxidant Nematicidal Pesticidal Antimicrobial |
| 9. | 21.456 | 0.830 | Propanoic acid | 106 | $C_3H_6O_2S$ |  | Antifungal Antibacterial |
| 10. | 24.752 | 0.643 | Octadecanoic acid | 310 | $C_{20}H_{38}O_2$ |  | Antifungal, Antitumor activity, Antibacterial |
| 11. | 28.463 | 7.774 | Tetracosamethyl-cyclododecasiloxane | 888 | $C_{24}H_{72}O_{12}Si_{12}$ |  | Anti-oxidant Antimicrobial |
| 12. | 29.299 | 6.020 | 5,16,20-Pregnatriene | 398 | $C_{25}H_{34}O_4$ |  | Antifungal |

Summarizing the analytical observations, it appears that validated analytical methods only exist for OTA and the fumonisins. For the remaining compounds produced by the black aspergilli, the existing papers are mainly descriptions of the isolation procedure and structural elucidation, and no reference standards are commercially available.

However, it should be possible to include them in an analytical scheme on a nonquantitative scale by using A. niger extracts for tuning MRM transitions when using triple quadrupole instruments, as, for example, is done for F. avenaceum metabolites in infected apples.[41]

Profiles of culture extracts can also be compared with those of crude extracts of infected food and feed samples obtained using newer LC-TOFMS and Orbitrap instruments.[41] These are getting increasingly more and more sensitive, and are a more interesting alternative to MRM analysis as they allow reprocessing of data files for more compounds.[42] With this approach, it is our recommendation to simultaneously monitor compounds

within a series, for example, both FB2 and FB4, several of the malformins (A–C), or several NGPs, as compounds within a class are usually always coproduced. This will help pinpoint suspicious samples, where, for example, only one malformin is detected. Given that these compounds will be produced in infected crops, monitoring of NGP s, tensidols, fumonisins, malformins, bicoumarins (kotanins), and asperazines should enable the detection of growth of black aspergilli to species level.

Even though LC combined with DAD and accurate mass determination is a strong tool for identifying mycotoxins and other fungal metabolites, a correct elementary composition, characteristic UV spectra, and the same elution profile[47,49] are not sufficient for unambiguous identification of positional isomers of, for example, the NGPs or malformins. Detection in the original strain will provide a very strong tentative identification; however, for absolute identification NMR data are required. Improvements in the area of LC-NMR have made it an option even for the analytical chemist, as it is now possible to obtain data in the nanomole range.[44] Combining accurate mass determination with a few NMR recognizable features will make it possible to quickly identify positional isomers of known compounds.[44,48] In addition, it should be possible to quantify compounds in a fraction by NMR, since signals are proportional the number of moles in the tube. Subsequently one can calibrate against other (standardized) tubes containing accurate amounts of other compounds.[45,48,50]

## CONCLUSION

In conclusion, species within Aspergillus section Nigri are excellent producers of a large number of diverse secondary metabolites. Several new metabolites and maybe even new biosynthetic pathways are expected to be discovered in the near future now that the full genome of Aspergillus niger has been sequenced and soon also the full genomes of other black aspergilli will be sequenced. Currently LC-DAD with accurate mass determination provides the easiest and most efficient strategy for tentative mapping of secondary metabolites in Aspergillus niger and its close relatives, especially if compared with already published chromatographic profiles, MS/MS data, and UV whole spectra. If absolute identification and positional isomer identification is needed, LC- NMR will be necessary. For determination in food and feed, direct analysis of diluted crude extracts using LC-MS/MS analysis or LChigh-resolution mass-spectrometric detection is suggested. If sample pretreatment is needed, care should be taken when using anion exchange since A. niger can produce extremely high amounts of organic acids which can outsalt acidic target metabolites.

The endophytic fungi A. terreus isolated from mulberry leaf had potential antifungal efficacy against M. phaseolina. The crude ethyl acetate extracts of A. terreus showed strong inhibitory action against charcoal rot pathogen. The metabolite profile of A. terreus was studied and the identified metabolites involved pathways were analyzed. The metabolic pathway analysis revealed that the major metabolites identified from A. terreus were also reported in mulberry plants thus showed the mutualistic interaction between the host and endophyte.

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