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Prenyl Ethers: Novel Fungal Volatiles Formed by *Penicillium digitatum*

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Abstract: Prenyl ethyl ether (PEE) was previously described as the cause for a solvent-like off-note in ground hazelnuts, but its origin remained unclear. Investigations were carried out by analytical groups of Coop and Givaudan over four years to elucidate this phenomenon. From mouldy citrus fruits a strain of *Penicillium digitatum* was isolated and found to form PEE. Formation on citrus and other fruits was prominent and contributed to the particular smell of decayed fruits. Several strains of *P. digitatum* formed PEE, while other fungal species did not. In contrast to citrus fruit, prenyl methyl ether (PME) was formed as dominant prenyl ether on hazelnuts while only small amounts of PEE were found. PME has not been previously described as volatile metabolite of fungi or as a food-taint. Spiking experiments with deuterated ethanol showed that the ethyl group is likely incorporated into PEE *via* the aldehyde form. On hazelnuts strongly decayed by *P. digitatum* yet another prenyl ether was tentatively identified: Prenyl isopropyl ether. Prenyl ethers present a novel group of volatile metabolites of *P. digitatum*. They are likely typical for this species and have not been described before. Prenyl ethers seem to play a significant role in the smell of food decayed by *P. digitatum* and should be considered in cases of off-notes and taints.

Keywords: Citrus · Hazelnut · Prenyl ethyl ether · Prenyl isopropyl ether · Prenyl methyl ether · Volatile microbial metabolites

Introduction

Taints and off-flavours render foods unacceptable for human consumption and thereby they cause significant economic losses for the food industry. They can originate from chemical reactions within the food, migration from the environment into the food or they can be formed by microorganisms.[1] Volatile metabolites from fungi or bacteria comprise a very broad range of different compounds and many of them have been associated with spoilage of food and agricultural commodities.[2] Prenyl ethyl ether (PEE) was recently identified as the source for a solvent-like off-note in ground hazelnuts and cake prepared thereof.[3] Further studies confirmed the relevant contribution of PEE to the aroma to such hazelnuts. After infection of different fruits with moulds PEE was detected in decayed fruits reminiscent of the distinct smell of mouldy citrus fruits.[4]

In the present study we report the identification of *Penicillium digitatum* as a fungal species capable of forming PEE. First insights in the formation of this compound are presented, which seems to be specific for *P. digitatum*. In addition, the identification of other, newly described prenyl ethers is reported here.

Experimental

Chemicals were of analytical grade and obtained from Sigma-Aldrich (Buchs, Switzerland). Prenyl ethyl ether and prenyl methyl ether were provided by Givaudan (Dübendorf, Switzerland). Mass spectrum (EI^+) of PME *m/z*: 100 (M^+, 12), 85 (100), 41 (54), 55 (41), 45 (26), 39 (23), 53 (20), 69 (17), 67 (17), 68 (13). Deuterated methanol and ethanol were from CIL (Cambridge, UK). Fungal strains (Table 1) were purchased from DSMZ (Braunschweig, Germany) and cultivated on Sabouraud-agar plates or tubes (Biolife, Milan, Italy). Inoculation was made either with a defined solution of spores or with an undefined inoculum prepared by washing out spores from an agar tube with NaCl–peptone water (0.85% NaCl, 0.1% peptone; Oxoid, Pratteln, Switzerland). Defined solutions of spores were obtained by harvesting spores from Agar plates (sporulation induced by storage in the fridge at 5 °C), dispersion in NaCl–peptone water and quantitation in different dilutions on agar plates. Growth experiments on ground hazelnuts and homogenised citrus fruits (powder obtained by cryogenic milling) were performed with 30 g of material sterilised in 250 mL Duran flasks (Duran, Wertheim, Germany). Aerobic incubation of samples was at 25 °C for two weeks as a standard length of time. For fruits and malt extract (ME, 9 mL) the inoculum was present in 1 mL of liquid, while for hazelnut 5 mL were needed to allow for a significant growth. Quantitative experiments were performed in ME medium (malt extract 17 g/L + mycological peptone 3 g/L in water; Biolife) in glass tubes with aluminium caps allowing for air supply. SPME analysis was performed qualitatively and sampling was done in the headspace of the flasks for 30 min at room temperature and the fibre (50/30 µm DVB/CAR/PDMS, Stableflex 24 Ga; Sigma-Aldrich) was directly thermally desorbed in the GC injector. Quantitation of prenyl ethers in liquid ME samples was done in duplicates using decane as an internal standard (5 mg/L) and by extracting 2 mL of ME broth with 2 mL tert. butyl methyl ether (Sigma). 1 µL was injected split-less and analysed with GC-MS with detection in SIM mode for quantitation and in fullscan mode (*m/z* 20–300) for identification. Calibration levels were obtained by adding analytes and internal standard into pure ME broth followed by extraction like the samples. Identification of prenyl ethers was done with two different stationary phases: Optima delta-3 (30 m, i.d. 0.32 mm, film 0.25 µm; Macherey-Nagel, Oensingen, Switzerland) and ZB-

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Table 1. Detection of PEE after growth of different fungi in ME medium (incubation for 19 d), lemon and hazelnut (qualitative data, both 15 d).

Species and strain	ME medium	Lemon	Hazelnut
P. digitatum wt	PEE: 0.49 mg/L	PEE	PME
P. digitatum DSM 2732	PEE: 0.43 mg/L	nd	PME
P. digitatum DSM 62840	PEE: 0.32 mg/L	PEE, PME	PME
P. citrinum DSM 1179	nd	nd	nd
P. italicum DSM 2734	nd	nd	nd
P. chrysogenum DSM 895	nd	nd	nd
Eurotium rubrum DSM 62631	nd	nd	nd
Rhizopus stolonifer DSM 63011	nd	nd	nd
Aspergillus flavus DSM 818	nd	nd	nd
Fusarium lateritium DSM 62244	nd	nd	nd
Cladosporium cladosporioides DSM 62121	no experiment	nd	nd

nd: not detected, LOQ for ME medium: 0.1 mg/L.

5MS (30 m, i.d. 0.25 mm, film 0.25 µm; Brechbühler, Schlieren, Switzerland). Further analytical details can be found in preceding publications.[3,4]

Results

Since the odour of PEE is reminiscent of mouldy, decayed citrus fruits, different citrus fruits were analysed as fresh fruits (whole and cut) and left to decay in the lab. Clementines, oranges, lemons and grapefruits were used and qualitative analysis by SPME-GC-MS showed that PEE was not present in the fresh fruits. After spontaneous infection and significant growth of moulds for about two weeks the samples were analysed again and PEE was found in all samples with the strongest signal in clementines. Moulds were isolated from this clementine sample and pure colonies of three different moulds were obtained. They were sent to a specialised laboratory for identification based on analysis by MALDI-TOF, gene sequencing, and morphological criteria (Mabritec, Riehen, Switzerland). The fungi were identified as *Mucor circinelloides*, *Penicillium mali*, and *Penicillium digitatum* (wildtype, wt). Testing of these mould species on sterilised clementines revealed that only *P. digitatum* wt formed PEE while *M. circinelloides* and *P. mali* did not. The three moulds were again tested on sterilised, homogenised material of the four citrus fruits for confirmation. PEE was formed by *P. digitatum* wt only and found in all four matrices infected by this fungal species after about two weeks. Thus, *P. digitatum* wt was responsible for the presence of PEE on decayed citrus fruits. Their smell was typical and the contribution of PEE to it was notable and confirmed by GC olfactometry. After about 14 days the signal of PEE was equal or even higher than limonene as determined in qualitative analysis by SPME-GC-MS. Thus, PEE may be a compound typical for decayed citrus fruits and possibly specific for *P. digitatum.* Experiments with other fruits such as strawberries, apricots and nectarines showed that PEE is also found in these matrices after infection with *P. digitatum* wt.[4] Results of duplicates varied considerably, which is likely due to the non-standardised inoculum used. Ethanol and in particular ethyl acetate were other volatile metabolites which were regularly found along with PEE. These metabolites as well as other alcohols and esters have been previously described as volatiles from citrus fruits infected with *Penicillium*.[5–7] PEE has not been described before as volatile metabolite from *P. digitatum* or other fungi. In general, ethers present a chemical group of fungal volatiles, which are rarely reported, with a few exceptions such as methoxy benzenes.[2] Among many other compounds, hints for ether structures in monoterpenes were found by GC-FTIR during analysis of fungal volatiles but clear identification was not given.[8] However, during characterisation of volatile metabolites from 47 *Penicillium* taxa Larsen and Frisvad reported an unidentified compound with molecular mass 114 and fragment masses and RI value which match PEE very well.[7] They found it in *P. digitatum* and three other species of *Penicillium*. In addition, Ariza and coworkers reported an unknown volatile compound for oranges infected with *P. digitatum* with a molecular weight of 114 and causing the characteristic mouldy odour.[9] It is very likely that both research groups came across PEE, but were not able to identify the compound because its mass spectrum was only published in 2010.[3] This demonstrates that the relation of PEE and *P. digitatum* is not an exotic phenomenon, which is only present in our laboratories, but is likely a typical combination that has been detected but not identified by other, independent groups 19 and 12 years ago.

Because PEE was identified as an off-note in ground hazelnuts, the main interest was to find out if *P. digitatum* wt was able to form PEE also on hazelnuts. Various experiments with different samples of ground, sterilised hazelnuts showed that *P. digitatum* wt formed only very small amounts of PEE and formation was clearly delayed as compared to citrus fruits, where PEE was already detected after one week. In contrast, another prominent peak appeared during qualitative analysis of these decayed hazelnuts: prenyl methyl ether (PME). The corresponding chromatogram, the structure of PME and its mass spectrum are shown in Fig. 1. Similar chromatograms were also obtained for the other two strains of *P. digitatum*. This compound was often the dominant signal in hazelnuts after incubation of about 2 weeks. The identity of PME was confirmed by analysis on two different stationary phases, comparison of the retention time (RI on DB-5: 751) and mass spectrum to the pure compound and by GC-olfactometry. Similar to PEE PME

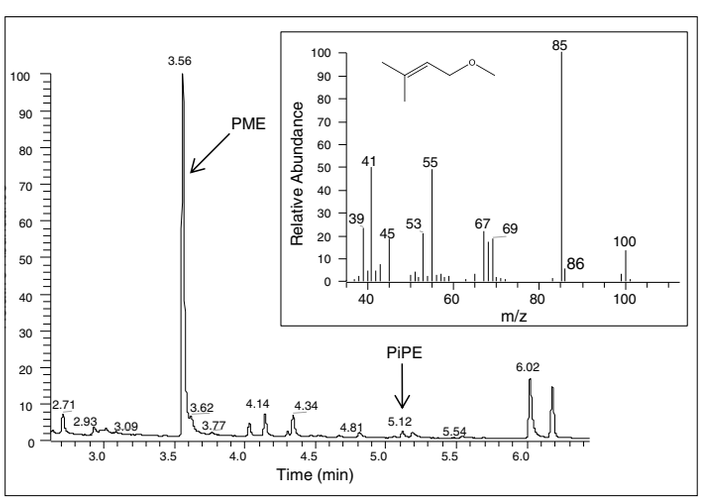

Fig. 1. SPME-GC-MS analysis of hazelnuts with *P. digitatum* DSM 62840: Chromatogram with prominent peak for PME at 3.57 min and small peak for PiPE at 5.12 min, box with mass spectrum and structure of PME.

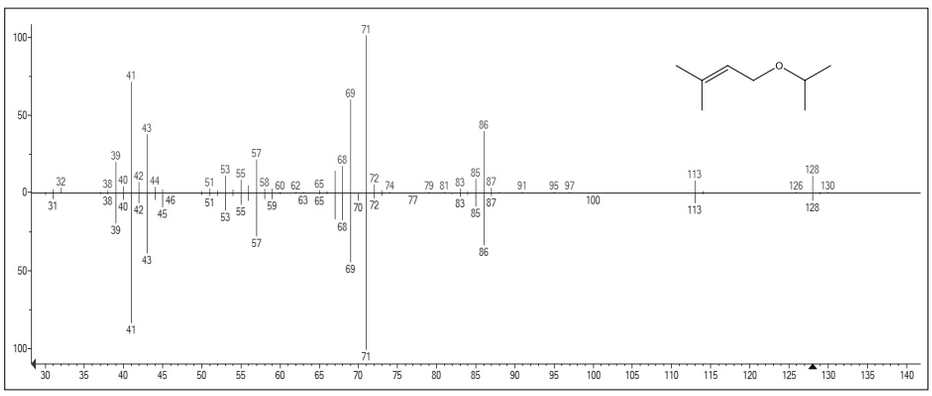

Fig. 2. Structure of PIPE and its mass spectrum found in hazelnuts with *P. digitatum* (top) and reference spectrum from library (bottom). Main masses *m*/z: 128 (M^+, 5), 71 (100), 41 (82), 69 (44), 43 (38), 86 (33), 57 (27), 68 (17), 67 (16), 53 (11), 113, (7).

exhibits a solvent-like, ethery and fresh odour. PME was also found in apricots and clementines after advanced decay of the sample and the signal in these matrices was clearly smaller compared to PEE. To the best of our knowledge PME has not been described as a fungal metabolite so far and this prenyl ether is likely another volatile compound typically formed by *P. digitatum*. PME was not detected in blank samples.

Yet another prenyl ether was tentatively identified by a very good match of its mass spectrum: Prenyl isopropyl ether (PiPE). The signal of PiPE was considerably smaller as compared to PME (Fig. 1) but increased after very long decay (no data shown). Its mass spectrum and the reference spectrum are shown in Fig. 2. The identification of PiPE could not be fully confirmed, because a reference compound was not available and presently no description of its odour can be given. Further experiments are needed to confirm the presence of PiPE on decayed nuts and fruits. In addition to prenyl ethers methanol, methyl acetate, different ketones (*e.g.* acetone) were found in mouldy hazelnuts. The signals at 6.02 and 6.18 min (Fig. 1) were identified as 3-methyl-4-heptanone and α-pinene, respectively, which were also detected in blank hazelnuts. Thus, they are not related to *P. digitatum*. The differences in the pattern of prenyl ethers in fruits and nuts are likely due to the different composition of the substrate. Similar effects of substrate on the formation of volatile metabolites have been described for many moulds.[2] The fact that mainly PME was found on hazelnuts affected by *P. digitatum* while only minor amounts of PEE were detected indicates that PEE, causing an off-note in ground hazelnuts,[3] may have been transferred from the environment to the hazelnuts rather than being formed on the nuts. If it had been formed by *P. digitatum* on the nuts, a strong signal for PME would have been found, but this was not the case.

Since the prenyl ethers were accompanied by their corresponding alcohols and acetate esters, we asked ourselves whether the formation of PEE was dependent on the presence of ethanol. Therefore, sterilised hazelnuts were spiked with 0.5% ethanol inoculated with *P. digitatum* wt. After 2 weeks a massive signal of PEE was found in the sample. PME, methanol, methyl acetate and ethyl acetate were also detected (data not shown). The addition of ethanol greatly enhanced the formation of PEE on hazelnuts. It can be assumed that *P. digitatum* wt needs ethanol for the formation of PEE and either the substrate provides it or allows the fungus to form it. To check if added ethanol is incorporated into PEE 0.5% D6-ethanol was added to a sterilised lemon matrix inoculated with *P. digitatum* wt. After about 2 weeks a PEE peak with a fronting shoulder and mass spectra were found with shifts of +4 for ions containing still the ethyl residue (M^+ at *m/z* 118, $(M\text{-}CH_3)^+$ at *m/z* 103) as compared to unlabelled PEE (Fig. 3). These signals are likely to originate from D4-PEE showing that added deuterated ethanol was incorporated into PEE. The fact that only four deuterium atoms were incorporated suggests acetaldehyde as an intermediate in the formation mechanism. There were also minor signals observed which could be assigned to D5-PEE, *e.g.* M^+ at *m/z* 119, $(M\text{-}CH_3)^+$ at *m/z* 104 (not shown in Fig. 3). These signals minimally preceded the ones assigned to D4-PEE. A minor formation of D5-PEE would imply that incorporation of ethanol into PEE may also proceed *via* the alcohol.

After these qualitative experiments the aim was to obtain quantitative data in a standardised and reproducible way and to check if addition of selected compounds affects the formation of PEE. This was done by inoculation of a liquid ME medium with a defined solution of spores and incubation under aerobic conditions at 25 °C. Preliminary experiments showed that formation of prenyl ethers was dependent on the presence of oxygen from air. A first experiment with *P. digitatum* wt (9200 spores per mL) was carried out in 2012 and showed that PEE was found only after one week. In 2014 the strains *P. digitatum* wt (8000 spores per mL, experiment 2), *P. digitatum* DSM 2732 (7000 spores per mL) and *P. digitatum* DSM 62840 (4000 spores per mL) were compared in parallel. Fig. 4 shows the course of PEE formation by *P. digitatum*. The formation of PEE by *P. digitatum* wt was somewhat delayed in 2014 as compared to the first experiment. In general, formation of PEE started after about 7 to 10 days and concentration reached about 0.3 mg/L after 2 weeks, and 0.5 mg/L after 3 weeks. The variation of

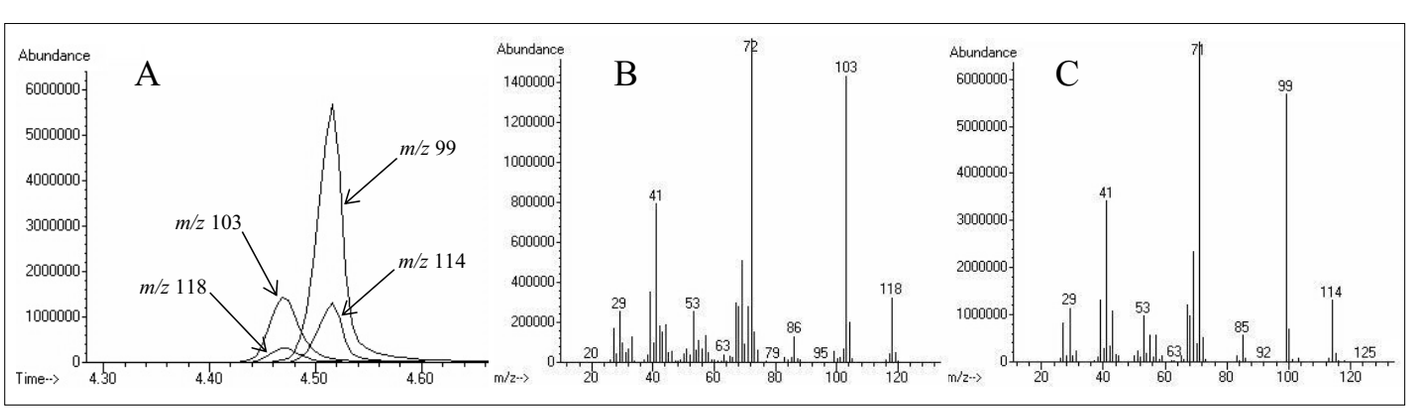

Fig. 3. Extracted ion chromatogram (A) and mass spectra of D4-PEE (B) and PEE (C) obtained for lemon with 0.5% D6-ethanol added and inoculated with *P. digitatum* wt for 2 weeks.

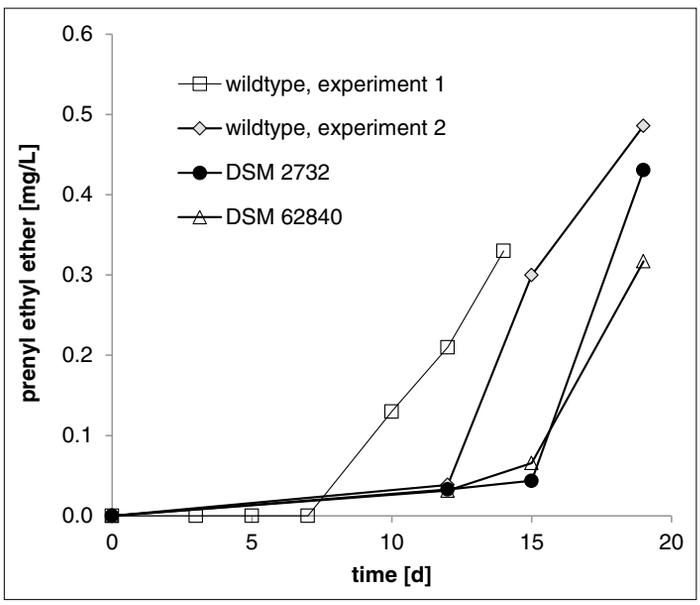

Fig. 4. Course of PEE formation by different *P. digitatum* strains in liquid ME medium. Two independent experiments are shown for *P. digitatum* wild-type.

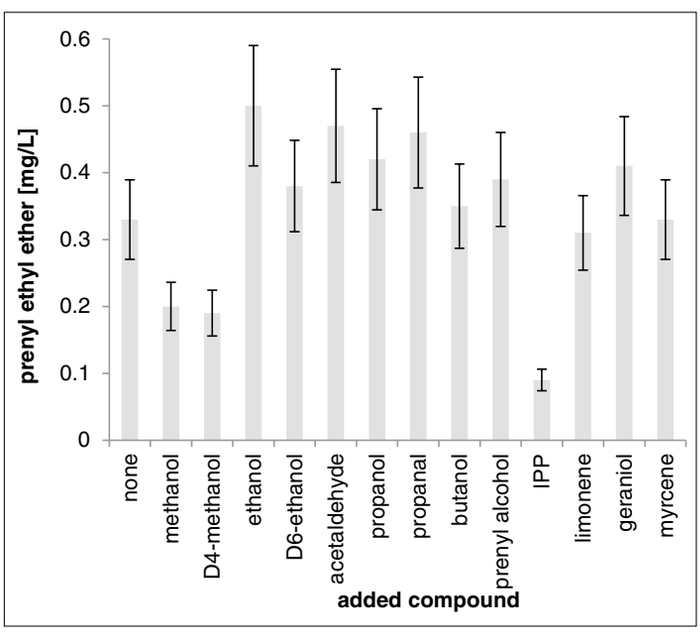

Fig. 5. Effect of addition of selected compounds to ME medium (5 mmol/L, terpenes and IPP: 0.15 mmol/L) on the formation of prenyl ethyl ether by *P. digitatum* wt (n = 2). Error bars represent the double standard deviation (18%).

PEE formation in experiment 1 was rather low, as determined in six tubes after 13 days: On average 0.2 mg/L were found with a CV of 9%. This data shows that all strains of *P. digitatum* form PEE in similar amounts and that the system with ME medium is reproducible. The differences in the course of PEE formation may be due to the different amount of spores used for inoculation. The total amount of PEE formed would even be larger because only the fraction present in the liquid was determined. Similarly to ME medium, PEE formation on citrus fruits and hazelnuts started only after about 10 days (data not shown).

In a further experiment different compounds were added to ME medium to check if they have an effect on PEE formation by *P. digitatum* wt (Fig. 5). Addition of ethanol and acetaldehyde led to an increase of PEE as compared to the control, but also n-propanol and its aldehyde had a similar effect. The effect of ethanol is in line with the formation of PEE on hazelnuts after addition of ethanol. Data analysis for samples with addition of n-propanol, propanal and butanol gave no signals which could be assigned to prenyl n-propyl ether or prenyl butyl ether, respectively. In the experiment with D6-ethanol small signals just before the PEE peak were found that could originate from D4-PEE which is in line with results shown in Fig. 3. Methanol seemed to have an inhibitory effect, which was also observed with D4-methanol and no signals for PME or D3-PME were found. No clear effect was found for prenol and the three terpenes tested, while with added IPP (γ,γ-dimethylallyl pyrophosphate) which is a precursor for terpene biosynthesis[10] a lower amount of PEE was found. This effect may be due to methanol which was the solvent for IPP. This data show that the prenyl residue is rather formed by *P. digitatum* itself and that procurement of a prenyl function from the medium is not a prerequisite.

To see whether the formation of PEE and PME is specific for *P. digitatum* a selection of some moulds typically found on hazelnuts[11] or citrus fruits[12] were added to sterilised lemon and hazelnuts as well as to liquid ME medium. Data in Table 1 show that all three strains of *P. digitatum* formed prenyl ethers and that on hazelnut mainly the methyl ether was found while on lemon and in ME medium the ethyl ether was found. Surprisingly, *P. digitatum* DSM 2732 did not form PEE on lemons while it formed it in ME medium. Further experiments are needed to clarify if this particular strain is indeed incapable of forming PEE on citrus or whether this was just an outlying experiment. In addition, other strains of *P. digitatum* and species of *Penicillium* should be checked for formation of prenyl ethers on different media to understand whether these ethers are formed only by certain strains/species or whether they are specific compounds formed by all strains of *P. digitatum*. If the latter turns out to be true then prenyl ethers may be used as an analytical target to specifically detect *P. digitatum* on mouldy foods and as a new compound for chemotaxonomy.[13]

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