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Study of the Composition of Volatile Organic Compounds Emitted by the Filamentous Fungus *Fusarium Culmorum* by Gas Chromatography-Mass Spectrometry Combined with Solid Phase Microextraction

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Abstract—Gas chromatography-mass spectrometry combined with solid-phase microextraction was used to study the composition of volatile organic compounds emitted by the filamentous fungus *Fusarium culmorum*. β-phellandrene, α-phellandrene, α-terpinene, *p*-cymene, and limonene were identified as markers. Their presence in the equilibrium vapor over an object studied can be regarded as identification sign of the infection by this type of *Fusarium* fungus.

Keywords: gas chromatography–mass spectrometry, solid-phase microextraction, volatile organic compounds, *Fusarium*

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INTRODUCTION

It is known that at least a quarter of grain crops grown in the world is contaminated with mycotoxins produced by fungi of the genus Fusarium. Currently, grain fusariosis is widespread, posing a serious environmental problem [1]. Predominantly, winter crops are infected, which leads to a significant reduction in yield and deterioration of food quality. Even considerable grain infestation may occur without symptoms at all or at the background of their very weak manifestation. Therefore, fast detection of grain infestation by toxin-producing micromycetes in the early stage of the process in the absence of obvious signs of infection is the urgent problem [2].

Traditional micro-biological methods are labor- and time-consuming, whereas the chemical methods have a low detection limit and are not suitable for test objects with low mycotoxin concentrations. Importantly, the available methods are mainly focused on the identification of specific members of the mycotoxin family, of which more than 500 is known today. To detect the content of mycotoxins in food or feed by the existing methods, they are extracted with organic solvents, precleaned, and converted (if necessary) into a volatile, fluorescent or colored compound. At the final stage, they are analyzed by chromatographic methods [3–4]. For certain mycotoxins, the radioimmunoassay and enzyme-linked immunosorbent methods are applied [5].

Intermediate products of toxin biosynthesis are volatile organic compounds (VOCs), mainly from the sesquiterpene series. The study of a profile of VOCs emitted by toxin-producing micromycetes, the detection of volatile organic compound markers, and the development of methods for their rapid and high-sensitivity detection will make it possible to monitor, providing high reliability, the safety of raw materials and food and detect the species of toxin-producing fungi in the early stages of infestation of plant materials.

The fungus *Fusarium culmorum* is an ubiquitous pathogen of crops that can affect both the root system and the generative organs of plants. A growing fungus

produces trichothecene mycotoxins, which makes products and feed produced from infected grain unsuitable for human consumption. Owing to an extremely high prevalence and severity, *F. culmorum* is one of the most actively studied fungi of the genus *Fusarium*. Latest data on *F. culmorum*, including population diversity, pathogenesis and stability, mycotoxin biosynthesis, and infection diagnostics, as well as preliminary results of the genome decryption, are summarized in a review [6]. At the same time, the composition of the secondary metabolites of this fungus has scarcely been studied. For example, in [7] attempt is made to identify the VOC emissions from *F. culmorum*, another typical pathogen of barley, *Cochliobolus sativus*, and from barley roots in the presence and in the absence of these microorganisms. The goal of the study was to determine the mechanisms of the interaction of pathogens with the plants infected by them. Note, published data on the VOC emissions from the *Fusarium fungi* are controversial [8–10]. This is because in the determination of qualitative and quantitative composition of VOCs the authors used different substrates and cultivation times, and, which is no less important, the scenarios of the selection and concentration and the analytical procedures were different. Gas chromatography-mass spectrometry (GC–MS) method is used for the VOC detection, irrespective of the plan of experiments. In this method, mass spectra (MS) and chromatographic retention indices (RI) are the most important identification characteristics. In our previous study [11], "metabolome portraits" of VOCs emitted by different types of Fusarius fungi were detected by the GC–MS combined with solid phase microextraction (SPME). It was established that for all the types studied, including *F. cerealis, F. culmorum, F. graminearum, F. langsethiae, F. poae, and F. sporotrichioides*, the volatile organic compounds may by arranged in two groups with respect to the volatility: extremely volatile (RI 400–1000) and moderately volatile (RI 1400–1550). It is problematic, but desirable, to study both groups within the same procedure, because in a metabolic profile study the change in the composition of endogenous metabolites in a biological environment is commonly detected using a particular analytical method. Therefore, the "chromato-graphic," "electrophoretic" [12], and other profiles of biogenic compounds are the commonly used meta-bolomics terms. Compilation of fragments into a metabolic (chromatographic) profile is an independent task, which can lead to ambiguous results. The total amount of VOCs was determined in a 120-m chromatographic column within the same procedure. On the one hand, this ensured a good separation of light components with close RI values, but, on the other hand, led to inversion of the RI values of high-boiling point components, which were recalculated using poly-cyclic aromatic hydrocarbons as reference components.

In the study, the composition of volatile organic compounds emitted by strain of the fungus *F. culmorum* was determined by a combined method of gas chromatography-mass spectrometry and solid phase extraction.

EXPERIMENTAL

We studied MFG 102100 strain of microscopic fungus *F. culmorum* from a collection of the Laboratory of Mycology and Phytopathology of the All-Russian Institute of Plant Protection, which was isolated from oat grain grown in the Kirov region in 2007. The identification of fungal species was confirmed by evaluating its morphological and cultural features [13] and also with the help of species-specific molecular primers [14].

Fungal strain was grown in 20-mL glass chromatography vials on 2 mL of potato-sucrose agar medium (PSA) in the dark at 23–24°C for 7–10 days. During solidification, the vials with agarized medium were placed at an angle of 45 deg with respect to the horizontal direction to increase the surface occupied by the culture, and, consequently, the concentration of its VOC emissions in equilibrium vapor. The samples were withdrawn from the vials onto microfiber after 7, 9, and 10 days. The composition of the equilibrium vapor over the fungal cultures was determined by the gas chromatography-mass spectrometry (GC–MS) on a QP-2010 Shimadzu plus instrument (Japan). The analytes were prepared by solid-phase extraction from an equilibrium vapor phase onto a Carboxen/PDMS Stable Flex 85-μm microfiber. The microfiber was conditioned in the injector block of the chromatograph at 250°C for 30 min and then inserted into an equilibrium vapor over the sample at a temperature of 40°C through the pinhole in the lid of the vial. Sampling was performed during 60 min at 40°C. Thermal desorption of the analytes from the microfiber in the GC injector was carried out at 250°C.

Gas chromatography separation: evaporator temperature was 250°C, injection was splitless (0.5 min); DB-5 column (120 mm × 0.2 m × 0.33 m); carrier gas

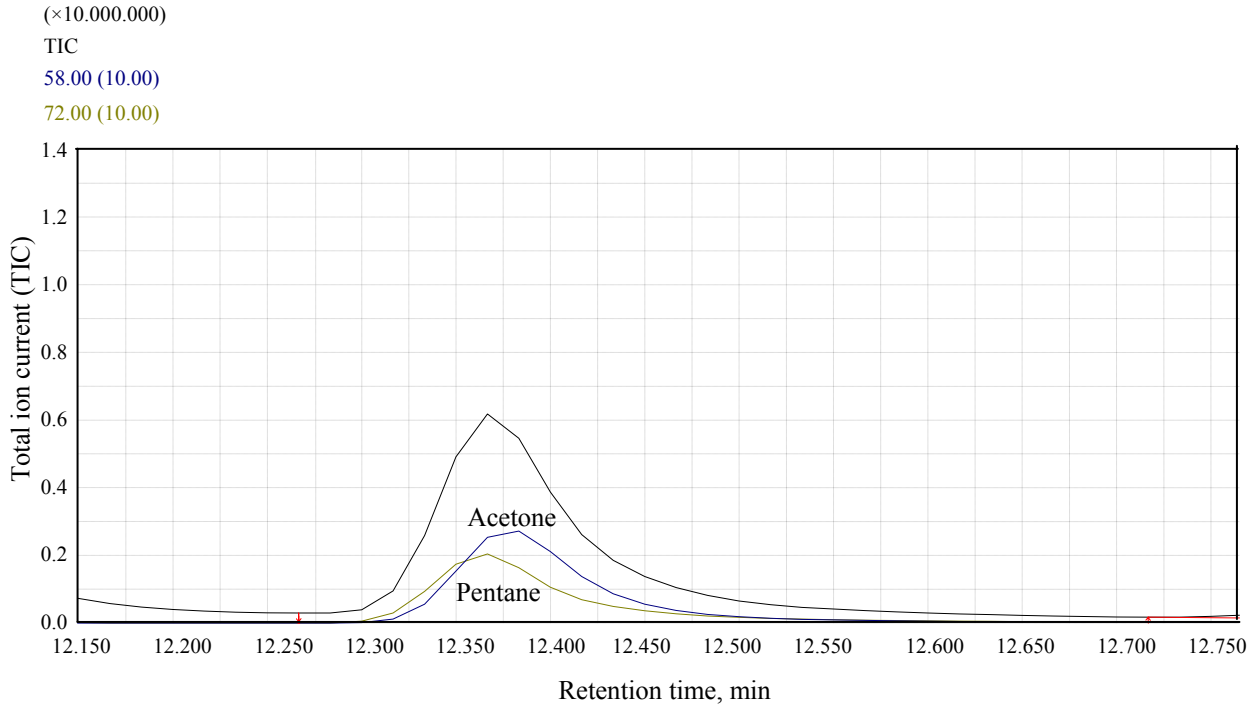

Fig. 1. A fragmentary total ion current chromatogram corresponding to VOC emissions from the culture 10 days of age. The unresolved peak is due to a pentane-acetone mixture.

was helium, the flow velocity of a carrier gas through the column was 1 mm^3/min. The temperature of the interface and the detector was 280°C. The chromatographic column was temperature-programmed in the range from 40 to 280°C with a step of 4°C/min.

Mass spectrometric analysis: the energy of electron ionization energy 70 eV, interface temperature 280°C, temperature of ionization source 200°C. Registration mode: TIC, mass range *m/z* 45–400.

To determine the chromatographic retention indices under the applied chromatographic conditions, we analyzed a mixture of normal alkanes C^6–C^{30}. For the identification of components we used the NIST 08 Mass Spectral Library with a computer search program and the Retention Index Database of organic compounds. Most of the reference RI values were taken from the original sources, because it is important to choose the values obtained for a 5% PDMS in the programmed temperature mode.

As reference we used a potatosucrose agar (PSA) culture medium without fungi. Under the conditions of our experiments (sampling temperature 40°C), no VOC emissions from the culture medium in the equilibrium vapor occurred. Background peaks in the chromatograms of the reference and analyte are mostly due to siloxanes of the column and microfiber.

The retention index of a compound was calculated relative to normal alkanes C^3–C^{18}. The retention indices ≥1200 were corrected using compounds with the known retention indices (polyaromatic hydrocarbons: acenaphthene, phenanthrene, and chrysene).

RESULTS AND DISCUSSION

Contrary to exhaustive extraction, SPME deals with equilibrium processes. On the one hand, this is unfavorable from the viewpoint of concentration. On the other hand, the study of the sample may be repeated after attaining the equilibrium in the system and, consequently, the changes in the metabolic profile with age of the culture can be determined.

In different periods of the cultivation (7–10 days) of the strain of the fungus *F. culmorum* the largest variability in composition was observed for volatile compounds. In the equilibrium vapor over the fungal culture 7 days of age the content of ethanol was twice lesser, whereas the contents of isobutanol, 2- and 3-methylbutanoic aldehydes, and alcohols were twice larger than for a 9–10-day culture. For the culture 7 days of age, the non-separated peak of a pentane-

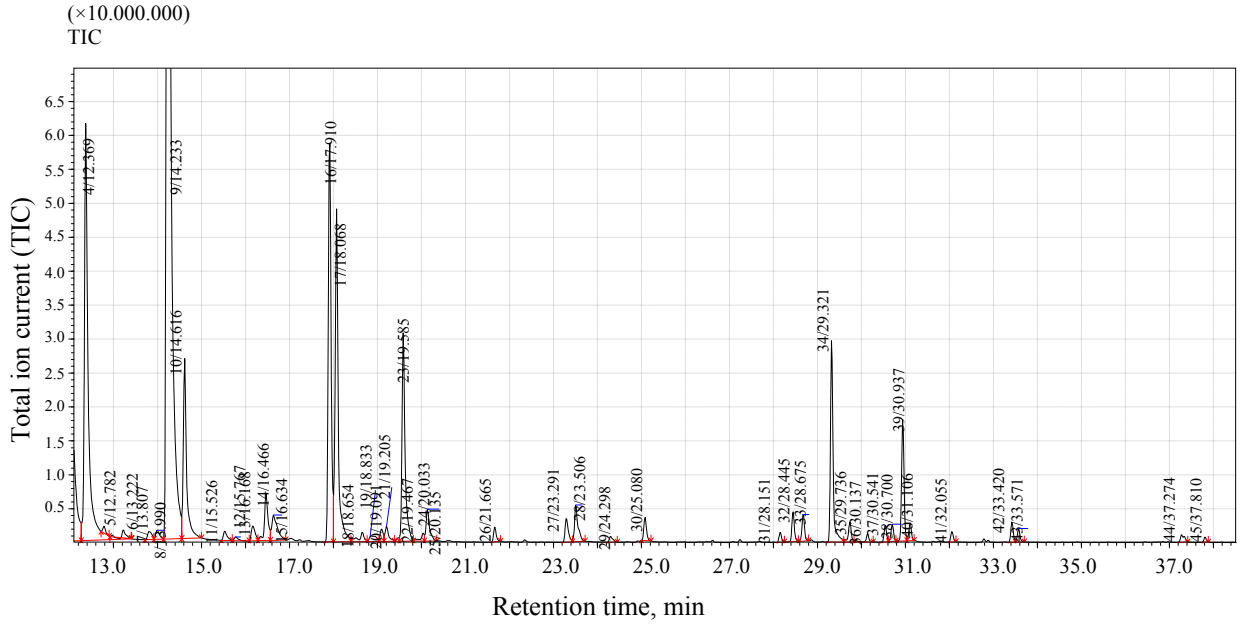

Fig. 2. The total ion current chromatogram obtained after sampling the equilibrium vapor over the *F. Culmorum culture* 10 days of age.

acetone mixture dominates with acetone, whereas for a 9–10-day culture, with pentane.

Under the conditions of our experiment, the pentane and acetone peaks are non-separable. A reconstruction of the mass fragmentograms of acetone (*m/z* 58) and pentane (*m/z* 72) allows a conclusion to be made that the culture in the age of 7 days emits pentane only hardly and the peak (RI 495) is almost completely corresponds to acetone, while for a 10-day culture, fraction of pentane in the acetone–pentane mixture is already significant (Fig. 1).

The remaining components of a seven-day culture are less pronounced. Therefore, the table data are given for 9- and 10-day cultures. Chromatographic profile of VOCs for a 10-day culture is demonstrated in Fig. 2. The most significant peaks correspond to ethanol (15%), a mixture of pentane and acetone (7%), ethyl acetate (22%), isobutanol (3%), 3-methyl 1-butanol (5.2%), 2-methyl 1-butanol (4.6%), 4-methyl-1,3-heptadiene (3%), hexyl acetate (2.4%), and β-phellandrene (1.4%).

Comparison of the qualitative composition of VOC emissions of *F. culmorum* with the data of [15] shows that these components are typical for fungi in general. Obviously, the quantitative ratio of the com-ponents does not coincide. Among the coinciding brutto components are isoamyl alcohol, 2-methyl-1-butanol, and 3-octanone. Ethyl acetate and ethanol, prevailing quantitatively in fusaric fungi, in fruit body *Calvatia gigantea* are present only in trace amounts. The linear retention indices of coinciding components obtained with a DB-5 column are taken from [15].

The authors of [16] studied the composition of VOC emissions from fungi *Aspergillus fumigatus, Aspergillus nidulans, F. solani,* and *Penicillium paneum*, cultured on agar medium. As markers of the microbial contamination of plants and soils served 2-ethyl-1-hexanol and 2-undecanone, which become prominent in the early stages of culturing and do not occur, in the authors's opinion, in the environment in the absence of microbial contamination. In the authors's opinion, other identified alcohols, aldehydes, and ketones, are common to all organisms. The intensity of their isolation by the microorganisms increases as the cultivation period increases from 2 to 10 days.

2-ethyl-1-hexanol, 3-octanone, and 2-undecanone, typical emissions of all the microorganisms studied, have no specificity. It was established that only fungi of the genus *Fusarium* emit 3-octanol, 2-phenyl-ethanol, 2-phenylisopropanol, hexanal aldehyde, 2-pentadekanone, and 2,2-phenylethanol. The number of spores correlates with the concentration of 3-octanone in the equilibrium vapor. Contrary to other micro-organisms, strains of all the *F. solani* studied emit no sesquiterpenes.

Volatile organic compounds identified in the equilibrium vapor over the *F. culmorum* culture 9–10 days of age[a]

Peak number	*RT*, min	Identified component	*m/z*	RI exp./ref.	% of total VOCs	
					9 days	10 days
1	12.010	Etanol[a,b]	45	427	17	15
2	12.367	Pentane +Acetone[a] (unresolved)	42, **43**, 57, 58 (acetone), 72 (pentane)	495	8	7
3	13.850	Acetic acid[b]	42, **43**, 45, 60	588	0.7	0.2
4	13.980	2-Butanone[b]	**43**, 57, 72	592	–	0.2
5	14.247	Ethyl acetate[b]	43, 70	605	11	22
6	14.631	Isobutanol[b]	43,74	620	4	3
7	15.518	3-Methyl butanal[b] (isovaleraldehyde)	**43**, 58, 71, 86	648	0.7	–
8	15.533	3-Methyl-2-butanone	**43**, 58, 71, 86	678	–	00.2
9	15.776	2-Methylbutanal[b]	57,86	656	–	0.06
10	16.467	Heptane[b]	100	700	0.7	0.8
11	16.634	3-Pentanone	**57**, 86	702	1	0.6
12	17.919/	3-Methyl-1-butanol[b] (isoamyl alcohol)	55,70	740	7	5
13	18.074	2-Methyl-1-butanol[b]	41,57,70	742	6	5
14	18.650	2- Methylpentanal	**43,** 58**,** 71, 100	755	1	0.2
15	18.930	Pyridine	52**, 79**	765/750	0.8	0.2
16	19.091	1-Pentanol[b]	**42**, 55, 70, 79	771/770	–	0.2
17	19.200	Isobutylacetate	**43**, 56, 73, 86	775/774	–	0.2
18	19.583	4-Methyl-1,3-Heptadiene	67, **95**,110	790/792	5	3
19	20.133	Octane[b]	114	800/800	0.6	0.5
20	20.430	1-Hexanal	**44,** 56, 95, 110	809/806	0.3	–
21	21.667	1-Hydroxy-3-methyl-2-butanone	**43**, 69, 71, 84	836/833	0.2	0.2
22	23.317	1-Hexanol[6]	43,**56**, 59	875/860-884	0.4	0.4
23	23.506	Isoamyl acetate[b]	**43**, 55, 70, 87	880/855-881	0.4	0.6
24	24.300	2-Heptanone[b]	**43**, 58, 71	898/890-892	0.1	0.1
25	25.080	Amyl acetate	**43**, 55, 70	914/884	0.4	0.3
26	28.150	1-Octen-3-ol[b]	43, **57,** 72, 85	987/986	0.2	0.1
27	28.433	3-Octanone[b]	**43,** 57, 71, 72, 99, 128	994/990	0.6	0.3
28	28.675	2-Pentylfuran[b]	53**, 81,** 138	1000/993	0.4	0.3
29	29.300	Hexyl acetate	**43,** 56, 61, 69, 84	1015/984	2	2.4
30	29.736	α-Phellandrene[b,c]	77**, 93,** 136	1025/1007	0.3	0.3

Table. (Contd.)

Peak number	*RT*, min	Identified component	*m/z*	RI exp./ref.	% of total VOCs	
					9 days	10 days
31	30.137	α-Terpinene[c]	77, 79, **93,** 105, **121,** 136	1035/1023 1019[2]	0.1	0.1
32	30.533	*p*-Cymene[b,c]	91**, 119,** 134	1044/1042 1026[2]	0.3	0.2
33	30.700	Limonene[b]	**68**, 93**,** 136	1048/ 1032[2]	0.2	0.2
34	30.937	β-phellandrene[b,c]	77, **93**, 136	1054/1034[2]	1.4	1.4
35	31.106	2-Nonanone	**43**, 58, 84	1058/1052	–	0.2
36	32.055	3-Octen-1-ol	**55,** 67, 81	1081/1056	0.2	0.1
37	33.420	Heptyl acetate	**43,** 56, 70, 98	1114/1111-1118	0.4	0.2
38	33.567	2-Heptenyl acetate	**43,** 54, 81, 96, 114	1118/1091	–	0.2
39	37.274	Octyl acetate	**43,** 56, 70, 83, 84, 112	1214/1201-1220	0.1	0.1
40	37.810	Piperitol (menth-1-en-3-ol)	**84**, 93, 139	1229/1208	–	0.05
41	45.133	Sativene	79, 91, **108,** 119, 133, 161, 189, 204	1409/1387-1404	0.2	0.2
42	49.617	Trichodiene	67, **109**	**1543**/(1533-1541)	< 0.05	< 0.05

[a] (*RT*) is the retention time of a component; (Identified component) is the substance responsible for a peak with a given number; (*m/z*) are mass numbers of main signals of a given component in the mass spectrum; (RI exp/ref) are the experimental and reference values of the chromatographic retention indices; retention indices for the well-known compounds are not included; (% of the total amount of VOCs) is fraction of the total amount of VOCs (%). [b] VOCs identified in fruit body of the fungus *Calvatia gigantean* [15]. [c] VOCs typical of *F. culmorum* and absent in the substrate (barley roots) [7].

Of all the VOCs listed in [16], we found only 3-octanone. During the observation period (7–10 days) its content in the equilibrium vapor above culture *F. culmorum* changed only slightly. Among sesquiterpenes, typical fusaric fungi [17], only 0.2% sativene was detected. Trichodiene and the sesquiterpene hydrocarbons with a molecular weight of 204 in amounts exceeding 0.1% of the total amount of VOCs were not detected. It was shown that trichodiene is the most abundant volatile metabolite of the sesquiterpene series in trichothecene-producing fungal species [17, 18].

Differences in the spectra of sesquiterpenes suggest that they can be used for differentiation of toxin-producing fungi. Although the relation between the concentration of trichodiene and the determined amounts of the synthesized trichothecene mycotoxins does not always exist, presence of trichothecene can be a clear indicator of the contamination of substrate with trichothecene mycotoxins [19].

Interestingly, trichodiene in strain of *F. culmorum* was not also identified in [7]. This compound is regarded as a marker of trichothecene mycotoxins, which are a pathogenicity factor [20]. As a consequence, the authors of [13] suggested that other VOCs formed by this fungus, rather than trichothecene metabolites, are responsible for the pathogenicity of strain for barley.

As was noted above, the mass chromatograms in our study were determined by monitoring total ion current and no peak assignable to trichodiene was

detected at cultivation times 7–10 days. On mass chromatograms reconstructed by the characteristic numbers of a trichodiene ion (*m/z* 109, 67), peaks in the region of its retention time are also barely visible. Trichodiene produces no contribution to the VOC pool of *F. culmorum* and can be determined in targeted rather than non-targeted analysis.

In [7], attempt is made to identify VOCs emitted from barley root damaged by *F. Culmorum*. For chromatographic separation, the authors used a column with a stationary phase VF-WAX. Therefore, the retention indices of the identified compounds could not be used as reference in our study. Nevertheless, it is interesting to compare the final results of the identification. Among the VOCs, released by barley roots only in the case of infection with *F. culmorum*, the following compounds are listed: 1,3-octadiene, heptan-4-one, α-phellandrene, α -terpinene, β-phellandrene, 1-ethyl-3-methylbenzene, γ-terpinene, *p*-cymene, 4-heptanol, 1-octen-3-one, 5-methyl-5-hepten-3-one, 3-octanol, α-acoradiene, longifolene, *epi*-bicyclosesquiphellandrene, beta-acoradiene, *cis*-piperitol, and 3-methyl-6-propan-2-yl cyclohex-2-ene-1-one. In relative amount, beta-phellandrene is predominant (6.5% of the total amount of VOCs). By our data, the latter compound may be also classed with moderate-volatility macrocomponents (1.4% of the total amount of VOCs). Another three components, α-phellandrene, α-terpinene, and *p*-cymene, coincide with the results of [7]. In two cases, we can say about relative compounds: 2-heptanone (in our study) and 4-heptanone (in [7]), 4-methyl-1,3-heptadiene (in our study) and octa-1,3-diene (in [7]). Errors in the identification of structural isomers within standardless analysis are certainly possible, but the given structural variants are easily distinguishable by the retention indices. Metabolic (chromatographic) VOC profiles are mostly presented without comprehensive description of analytical procedures used for their preparation. Therefore, data available from the literature on the composition of VOCs emitted by the microorganisms are contradictory. Interlaboratory reproducibility of the metabolic profiles can be achieved when the standard experimental conditions will be accepted by the community of laboratories specializing in this field [21]. Thus, volatile organic compounds released by the microscopic fungus *F. culmorum* contain alcohols (ethyl, isobutyl, 2- and 3-methylbutanoic, pentyl, hexyl, and 3-octene) as the main component. Among volatile acids, only acetic acid is determined. Esters are found in the form of alcohol acetates. The contribution of aliphatic hydrocarbons (pentane, heptane, and octane) to the total amount of VOCs is significant. Terpenes (α-phellandrene, α-terpinene, *p*-cymene, limonene, and β-phellandrene), in addition to aldehydes (2- and 3-methylbutanoic, 2-methylpentanal, and hexanoic), ketones (acetone, 2-butanal, 3-methyl-2-butanal, and 3-octanone) and pyridine can presumably be considered as the most organoleptically active components responsible for the signaling functions in interaction of fungi with the insects ensuring their propagation. No other nitrogen-containing VOCs, with the exception of pyridine, as well as sulfur-containing VOCs were detected. Among sesquiterpenes, only sativene was determined. Our results and the discrepant data published in the literature on the qualitative and quantitative composition of VOC emissions from the microscopic fungus *F. Culmorum* are insufficient to call VOC markers, which, as identified in the equilibrium vapor, unambiguously indicate that test object is infected just by this fungus. At the same time, a group of terpene hydrocarbons, including β-phellandrene, α-phellandrene, α-terpinene, *p*-cymene, and limonene, as a whole, can be regarded as sufficiently stable species-specific identification feature. Importantly, the determination of VOCs is performed in the equilibrium vapor. Therefore, no correction for the type of the matrix studied is required in the GC–MS analysis.

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