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Use of headspace solid-phase microextraction and headspace sorptive extraction for the detection of the volatile metabolites produced by toxigenic *Fusarium* species

Jan C.R. Demyttenaere[a,*], Rosa M. Moriña[a,b], Norbert De Kimpe[a], Pat Sandra[b]

[a] *Department of Organic Chemistry, Faculty of Agricultural and Applied Biological Sciences, Ghent University, Coupure Links 653, Ghent B-9000, Belgium*
[b] *Department of Organic Chemistry, Faculty of Sciences, Ghent University, Krijgslaan 281 (S4), Ghent B-9000, Belgium*

Abstract

An efficient methodology was developed to determine the growth of toxigenic *Fusarium* spp., based on headspace solid-phase microextraction (SPME) and stir bar sorptive extraction of the fungal volatile metabolites produced. SPME and headspace sorptive extraction (HSSE) were used to monitor the de novo production of sesquiterpene hydrocarbons, such as trichodiene, a volatile marker and intermediate in the biosynthesis of trichothecenes. On growth media such as malt extract agar and potato dextrose agar, it was found that trichodiene was produced by toxigenic strains of *Fusarium sambucinum* and *Fusarium sporotrichioides*. It was the main volatile metabolite in the headspace extract of the cultures. On the other hand, deoxynivalenol producing *Fusarium graminearum* showed a completely different pattern of volatile sesquiterpenes and could easily be distinguished from a zearalenone producing strain of *F. graminearum* based on the headspace profile. Hence, it can be concluded that headspace analysis of volatile fungal metabolites by SPME and HSSE in combination with gas chromatography/mass spectrometry is a suitable monitoring technique to differentiate toxigenic strains of *Fusarium*.

Keywords: Headspace analysis; Extraction methods; *Fusarium* spp.; Mycotoxins; Trichothecenes; Deoxynivalenol; Trichodiene; Volatile organic compounds

1. Introduction

Fusarium fungal species are known to infect agricultural products such as grains, maize and wheat throughout the world [1–3]. *Fusarium* fungi are probably the most important mycotoxin producers infecting cereals in northern and temperate regions where they produce mycotoxins such as trichothecenes and zearalenone (ZEA). The most important trichothecenes are deoxynivalenol (DON or vomitoxin), nivalenol (NIV), T2 toxin and diacetoxyscirpenol (DAS), which are produced by various *Fusarium* spp., such as *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium poae*, *Fusarium sporotrichioides*, *Trichothecium* and others [4].

Another important producer of trichothecene mycotoxins is *Fusarium sambucinum*, a field fungus occurring in moderate climatic zones, distributed widely on vegetables and as a plant pathogen. It is the main species that causes dry rot of potato tubers in North America and Europe [5].

The biosynthesis of trichothecenes has been extensively studied and reviewed [6,7]. The sesquiterpene hydrocarbon trichodiene is a precursor in trichothecene biosynthesis and is presumably the last intermediate in the pathway prior to oxygenative reactions [8]. Trichodiene has also been shown to be a volatile marker for trichothecene biosynthesis [9,10]. The monitoring of fungal volatiles as markers for mycotoxin production and for early detection of fungal infection in grains and agricultural commodities has received increasing interest over the last years [11,12] and is subject of the current study. The correlation between infection of potato tubers and emission of fungal volatiles by *F. sambucinum* was also demonstrated more recently by a Canadian group [13].

Previously, we reported the use of solid-phase microextraction (SPME) and headspace sorptive extraction (HSSE) to monitor the production of the fungal volatile metabolite (+)-aristolochene by toxigenic strains of *Penicillium roque-*

* Corresponding author. Tel.: +32-9-264-59-64; fax: +32-9-264-62-43.
E-mail address: jan.demyttenaere@ugent.be (J.C.R. Demyttenaere).

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forti [14,15]. This paper describes the use of SPME and HSSE for the detection and identification of the volatile metabolites produced by toxigenic *Fusarium* species.

As model fungal cultures, two strains of *F. sambucinum* and one strain of *F. sporotrichioides* were selected, known for their production of trichothecenes and their emission of the volatile marker trichodiene [5,9]. The method was then applied to two commercially available strains of *F. graminearum*, one of which produces ZEA and the other one of which produces DON.

2. Experimental

2.1. Microorganisms and cultivation

Five *Fusarium* strains were used in this study: two trichothecene producing strains of *F. sambucinum* (marked KF-735 and KF-749) [5,9]; one T2 toxin producing strain of *F. sporotrichioides* (marked KF-196) [9], obtained from the Fungal Collection of the Institute of Food Technology, Agricultural University of Poznañ, Poland and kindly provided to us by Dr. Henryk Jeleñ of the same institute; and two toxigenic strains of *F. graminearum*, obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany), namely DSM 4527, producer of DON [16] and DSM 1095, producer of ZEA [17]. The fungi were cultivated in both Petri dishes and slants and conserved at 4 °C on malt extract agar (MEA; malt extract 2%, bacteriological peptone 0.1%, glucose 2% and agar 2%, pH 5.4 ± 0.2) and on potato dextrose agar (PDA; potato extract 0.4%, glucose 2% and agar 1.5%, pH 5.6 ± 0.2). All culture media were obtained from Fluka (Bornem, Belgium).

2.2. Headspace analysis of surface cultures by SPME and HSSE

The fungi were cultivated as small sporulated surface cultures in 22 ml SPME vials (Supelco, Bornem, Belgium) and the volatile metabolites were extracted by headspace SPME during 30 min at 25 °C with a 100 μm polydimethylsiloxane (PDMS) fiber as described previously [15] (Fig. 1). SPME fibers were obtained from Supelco. For HSSE extraction, a PDMS stir bar with 24 μl of stationary phase (Gerstel, Mülheim a/d Ruhr, Germany) was hung in the headspace of a 22 ml vial in which a fungal surface culture had developed, by attaching the magnetic stir bar against a paper clip by simple magnetic force (Fig. 1). The stir bar was then exposed to the headspace of the surface culture during 30 min at 25 °C [15].

2.3. Analysis of the headspace extracts by GC–MS

GC–MS analyses of the SPME extracts were performed with an Agilent 6890 GC Plus coupled to a quadrupole mass spectrometer 5973 MSD (Agilent), and equipped with a HP-5 MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness). Working conditions were: injector 250 °C, transfer line to MS system 250 °C, oven temperature—start 40 °C, hold 2 min, programmed from 40 to 200 °C at 10 °C min^{-1}, from 200 to 250 °C at 15 °C min^{-1}, hold 5 min; carrier gas (He) 1.0 ml min^{-1}; SPME desorption was carried out using a CIS-4 programmed temperature vaporizer PTV injector (Gerstel) in split mode (1/10); electron impact ionization 70 eV; acquisition parameters (scanned *m*/*z*): 40–200 (2–10 min), 40–300 (>10 min).

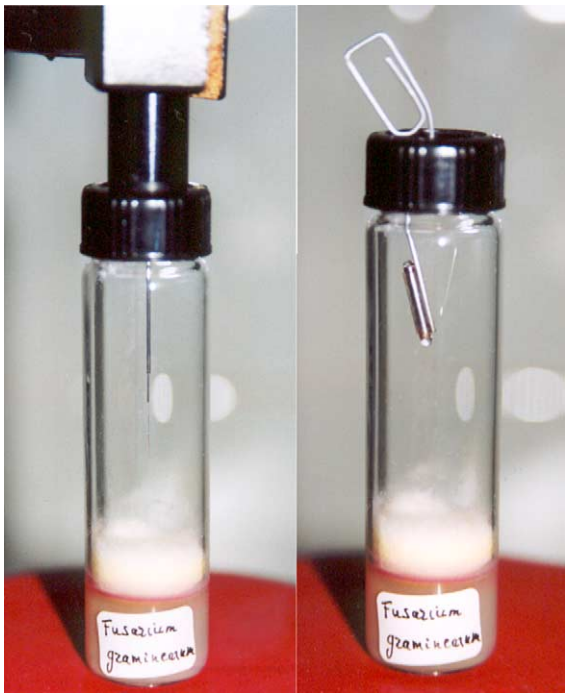

Fig. 1. Experimental design of SPME and HSSE of surface cultures of *F. graminearum* in SPME vials.

For the analysis of the HSSE extracts, the stir bar was placed into a glass tube (178 mm length, 6.0 mm o.d.; Gerstel) and then thermodesorption was carried out using a Gerstel Thermo Desorption System (TDS2; Gerstel), coupled to a CIS-4 PTV injector (Gerstel). The TDS2 oven was programmed from 20 to 250 °C at 60 °C min^{-1} (held 7 min at 250 °C) and the volatile analytes were thermally desorbed in splitless mode and cryo-focused in a CIS-4 PTV-injector (Gerstel) at −50 °C; injection of the analytes was done in split mode (1/10) by fast heating the injector from −50 to +250 °C at 12 °C s^{-1}. The analysis was carried out using the same analytical conditions as for the analysis of the SPME extracts described earlier.

The identification of the main fungal metabolite from the toxigenic cultures, trichodiene was based on comparison of its mass spectrum and retention index with literature data [5]. The Kováts retention index of trichodiene on the HP-5 MS stationary phase is 1541.

Table 1
Production of sesquiterpene hydrocarbons and relative contribution (%) of fungal metabolites in the headspace extracts of surface cultures of *F. sambucinum*, strains KF-749 and KF-735, grown on two media, PDA and MEA

Peak no.	t_R (min)	*I*			Compound	KF-749		KF-735	
		Exp.[a]	Lit.[b]	Lit.[c]		PDA	MEA	PDA	MEA
1a	14.62	1428	1503		α-Chamigrene	7.76	7.46	n.d.	n.d.
1b	14.65	1430			Unidentified sesquiterpene hydrocarbon	1.60	5.81	4.50	5.39
2	14.86	1447		1447[d]	Unidentified sesquiterpene (bp[e] 161)	**29.11**	**55.57**	n.d.	n.d.
3	14.90	1450	1434		Thujopsene	n.d.	n.d.	1.19	0.14
4	14.94	1453			Unidentified sesquiterpene	0.28	0.94	0.83	0.25
5	15.04	1461	1446	1462	(*E*)-β-Farnesene	**4.83**	**2.30**	**16.41**	**17.49**
6	15.20	1474	1460	1468	β-Santalene	1.14	0.82	0.00	0.16
7	15.31	1483	1464	1476	Acoradiene	0.77	4.73	0.57	0.65
8	15.40	1490	1474	1481	β-Chamigrene	**5.93**	**2.07**	**3.82**	**4.00**
9	15.43	1492	1473	1490	Aryl-curcumene	n.d.	n.d.	3.54	3.70
10	15.51	1498			Siloxane + β-chamigrene isomer	6.30	9.42	11.22	1.83
11	15.65	1510	1450		α-Himachalene or stereoisomer	0.00	0.00	2.62	2.12
12	15.74	1517	1503	1516	β-Bisabolene	**4.22**	**1.73**	**12.73**	**13.45**
13	15.78	1521	1418		α-Cedrene	0.65	1.12	5.34	3.17
14	15.81	1523	1498		Cuparene	0.42	0.00	0.24	0.68
15	15.86	1527	1500	1510	β-Himachalene	0.55	0.00	0.73	0.93
16	15.95	1535	1516		β-Sesquiphellandrene	0.39	0.00	0.94	1.51
17	16.03	1541		1533	Trichodiene	**35.11**	**8.03**	**30.85**	**41.00**
18	16.14	1550			Unknown compound	0.94	0.00	4.48	3.54

Sampling by HSSE; main compounds are displayed in bold; *I* = Kováts retention index.
[a] Exp. = experimental (HP-5 MS column).
[b] Lit. = literature (CpSil5 column) [19].
[c] Lit. = literature (DB-5 column) [5].
[d] It is postulated that compound no. 2 is the same one as isolated by Jeleñ et al. from *F. sambucinum* UM-N60B [5].
[e] bp = base peak in mass spectrum.

The other fungal metabolites were identified by comparison of their mass spectra and Kováts retention indices with literature data [5,18,19] and by comparison with the US National Institute of Standards and Technology (NIST) Mass Spectral Library (version 1.6d, 1998). Retention indices were measured by spiking the stir bar with 1 μl of a solution of *n*-alkanes (*n*-tetradecane–*n*-hexadecane) in Et_2O (0.01%, v/v) after absorption of the fungal volatiles and prior to thermal desorption and GC–MS analysis using the same analytical conditions as described earlier.

3. Results and discussion

3.1. Analysis of the volatiles produced by F. sambucinum

In the first experiment, the production of volatile compounds by two toxigenic strains of *F. sambucinum* was monitored by HSSE. The results with the relative contribution of the fungal metabolites in the headspace extracts are depicted in Table 1.

From these results, it can be seen that the two different strains of *F. sambucinum* can clearly be distinguished by HSSE. The strain KF-749 produces significant amounts of a sesquiterpene hydrocarbon which is the main constituent (55.6%) in the headspace when grown on MEA. It is assumed that it is a sesquiterpene hydrocarbon, based on the presence of a molecular ion at *m*/*z* 204 in the mass spectrum (see inset in Fig. 2). It has a very characteristic base peak at *m*/*z* 161. Based on its retention index and its mass spectrum, this compound seems to be the same as isolated as main metabolite by Jeleñ et al. from *F. sambucinum* UM-N60B [5]. No traces of this compound could be detected in the headspace extract of strain KF-735, demonstrating that it is characteristic for strain KF-749 and not for the other strains tested in the current study, whereas the main volatile constituent in the headspace extract of strain KF-735 was trichodiene, marker for trichothecene biosynthesis. Other important sesquiterpene hydrocarbons that were detected in the headspace extract of both cultures were (*E*)-β-farnesene, β-chamigrene and β-bisabolene. Also the presence of aryl-curcumene, the only aromatic sesquiterpene (molecular weight 202) detected in the headspace extracts of the fungal cultures and which is characteristic for strain KF-735 is interesting in this respect. Tentative identification of α- and β-himachalene and α-cedrene was based on mass spectrometry alone, since their mass spectra were very similar with both literature data [18,19] and the NIST mass spectral Dbase. The other compounds could positively be identified based on both mass spectrometry (NIST and literature) and retention index [5,18,19]. Therefore, it is assumed that compounds 11, 13 and 15 might be stereoisomers of the proposed structures. Whereas the relative composition of the headspace extracts was quantitatively dependent on the type of medium in the case of strain KF-749, the volatiles profile from strain KF-735 was similar for both

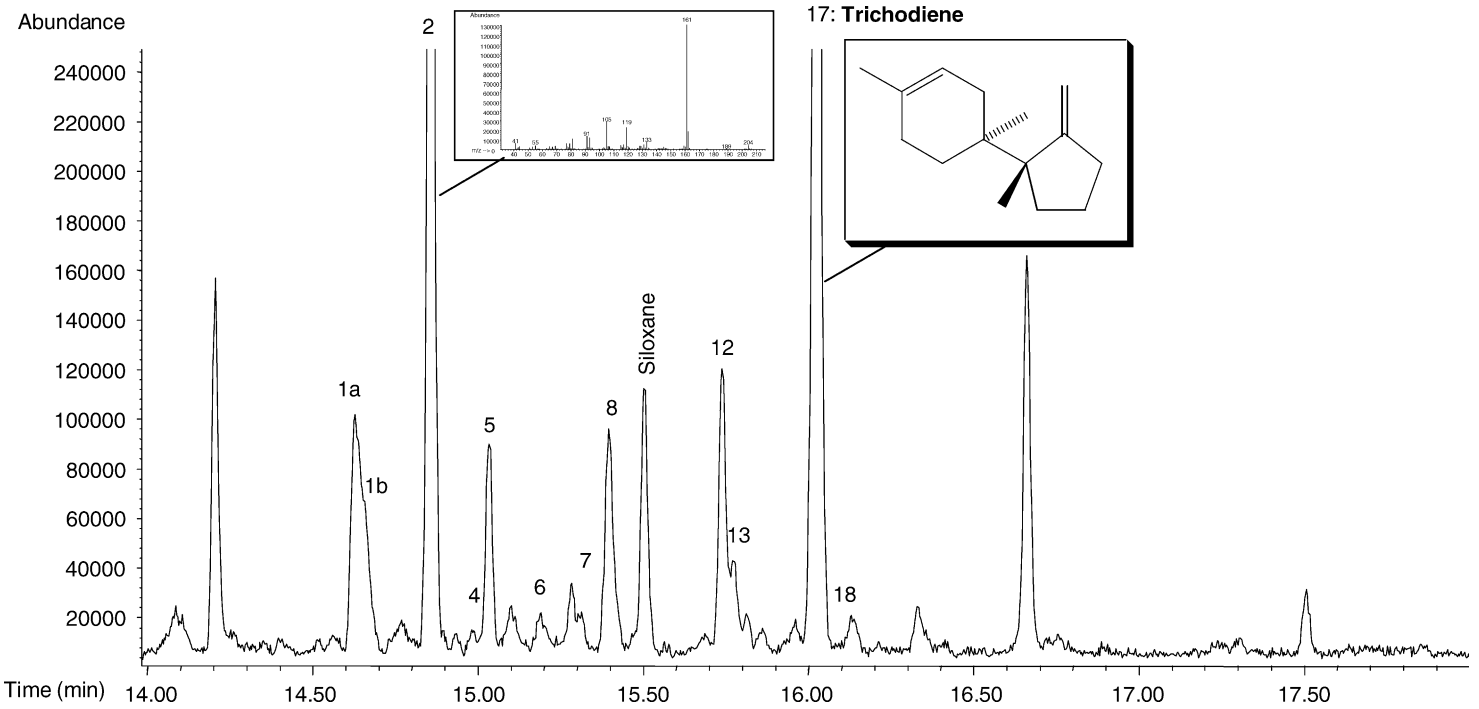

Fig. 2. Gas chromatogram of the HSSE extract of sporulated surface culture of *F. sambucinum* KF-749 culture grown on PDA medium. Peak numbers refer to the compounds listed in Table 1.

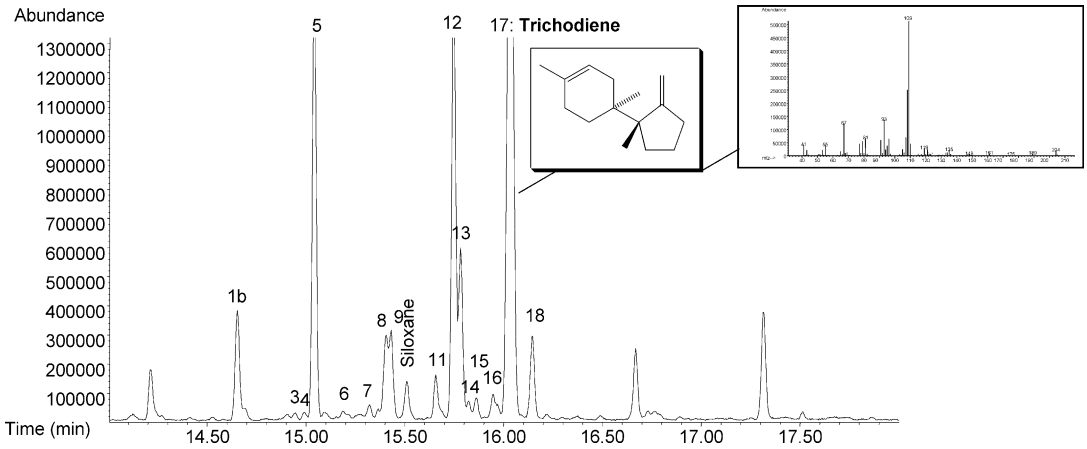

Fig. 3. Gas chromatogram of the HSSE extract of sporulated surface culture of *F. sambucinum* KF-735 culture grown on MEA medium. Peak numbers refer to the compounds listed in Table 1.

culture media. It is important to stress that the culture medium had no influence on the type of volatile metabolites produced, since the qualitative composition of the headspace extracts of the cultures, grown on both media was similar.

The chromatograms obtained after HSSE extraction of both *F. sambucinum* cultures are depicted in Figs. 2 and 3, respectively. From these chromatograms, the qualitative difference in the headspace profiles of both strains can clearly be seen from the presence of the large peak no. 2 in the extract of strain KF-749.

3.2. Analysis of the volatiles produced by F. sporotrichioides

In the next experiment, the headspace profile of *F. sporotrichioides* (KF-196) was investigated using both SPME and HSSE, as described in Section 2.

The results with the relative composition of the headspace extracts of all cultures (average of two cultures for each test) are depicted in Table 2. It can be concluded that the qualitative pattern of fungal volatiles is not affected by the type of medium. Moreover, there is also no significant difference in percentage composition of the main volatiles when the two growth media are compared. Only the relative contribution of trichodiene is slightly higher when the fungus is grown on PDA than when it is grown on MEA.

When the results obtained after HSSE are compared with those obtained after sampling by SPME, it can be concluded that the same qualitative pattern in the headspace profile is obtained. However, the relative contribution of each volatile metabolite is slightly different, and a higher recovery of trichodiene is obtained after SPME analysis than with HSSE analysis.

Table 2
Production of sesquiterpene hydrocarbons and relative contribution (%) of fungal metabolites in the headspace extracts of surface cultures of *F. sporotrichioides*

Peak no.	t_R (min)	*I*	Compound	Medium/sampling method		
				PDA/HSSE	MEA/HSSE	PDA/SPME
1	14.65	1430	Unidentified sesquiterpene hydrocarbon	**7.35 ± 1.23**[a]	**8.23 ± 0.52**	**6.39 ± 1.57**
3	14.90	1450	Thujopsene	0.47	0.23	0.38
4	14.94	1453	Unidentified sesquiterpene	0.49	0.52	0.41
5	15.04	1461	(*E*)-β-Farnesene	**11.11 ± 0.64**	**12.33 ± 1.59**	**7.85 ± 1.54**
6	15.20	1474	β-Santalene	0.37	0.87	0.35
7	15.31	1483	Acoradiene	0.95	1.13	0.98
8	15.4	1490	β-Chamigrene	**9.56 ± 0.67**	**10.41 ± 0.43**	**8.39 ± 1.19**
10	15.51	1498	Siloxane + β-chamigrene isomer	2.84	3.82	0.88
11	15.65	1510	α-Himachalene or stereoisomer	0.53	0.47	0.37
12	15.74	1517	β-Bisabolene	**12.66 ± 5.66**	**12.75 ± 0.41**	**10.38 ± 1.50**
13	15.78	1521	α-Cedrene	1.28	4.72	0.99
14	15.81	1523	Cuparene	0.64	0.21	0.24
15	15.86	1527	β-Himachalene	0.62	0.80	0.38
16	15.95	1535	β-Sesquiphellandrene	1.34	0.68	0.68
17	16.03	1541	Trichodiene	**47.58 ± 2.06**	**40.54 ± 0.72**	**58.32 ± 0.78**
18	16.14	1550	Unknown compound	2.11	2.29	2.42

Sampling by SPME and HSSE; main compounds are displayed in bold; *I* = Kováts retention index.
[a] Data are averages of two cultures grown on the same medium; standard deviation values are given for the main metabolites (in bold).

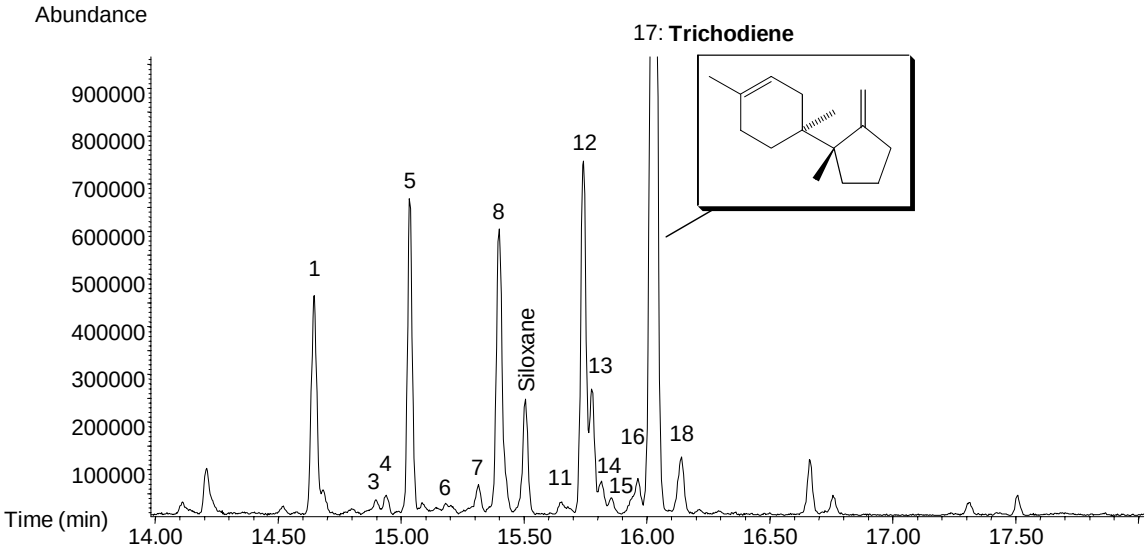

Fig. 4. Gas chromatogram of the HSSE extract of *F. sporotrichioides* culture grown on PDA medium. Peak numbers refer to the compounds listed in Table 2.

The chromatograms obtained after HSSE extraction and SPME extraction of the fungal cultures of *F. sporotrichioides* are depicted in Figs. 4 and 5, respectively. Both chromatograms look very similar. One drawback of sampling by HSSE is the presence of a siloxane peak in the chromatogram, probably due to bleeding of the PDMS stationary phase of the stir bar during thermal desorption. This siloxane peak was not detected in the chromatograms obtained after SPME. The siloxane peak overlaps with a β-chamigrene isomer, which makes the correct identification, detection and quantification of the latter compound difficult in the case of HSSE.

When the chromatograms of the headspace extracts of *F. sporotrichioides* and *F. sambucinum* are compared, it can be seen that the unidentified compound (no. 2) is characteristic for *F. sambucinum* KF-749 whereas aryl-curcumene is characteristic for *F. sambucinum* KF-735. No traces of the latter two compounds were detected in the headspace extracts of *F. sporotrichioides*. A scheme with an overview of the tentatively identified structures of the volatile fungal metabolites produced by *F. sambucinum* and *F. sporotrichioides* is depicted in Fig. 6. The exact stereochemical configuration of the metabolites was not determined as this was beyond the scope of the current study.

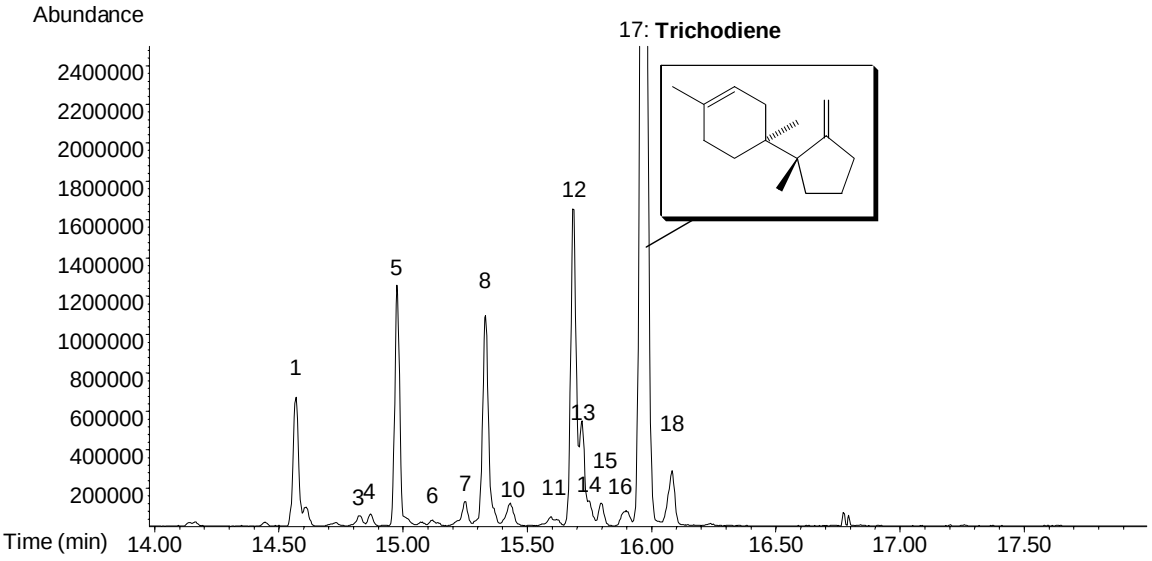

Fig. 5. Gas chromatogram of the SPME extract of *F. sporotrichioides* culture grown on PDA medium. Peak numbers refer to the compounds listed in Table 2.

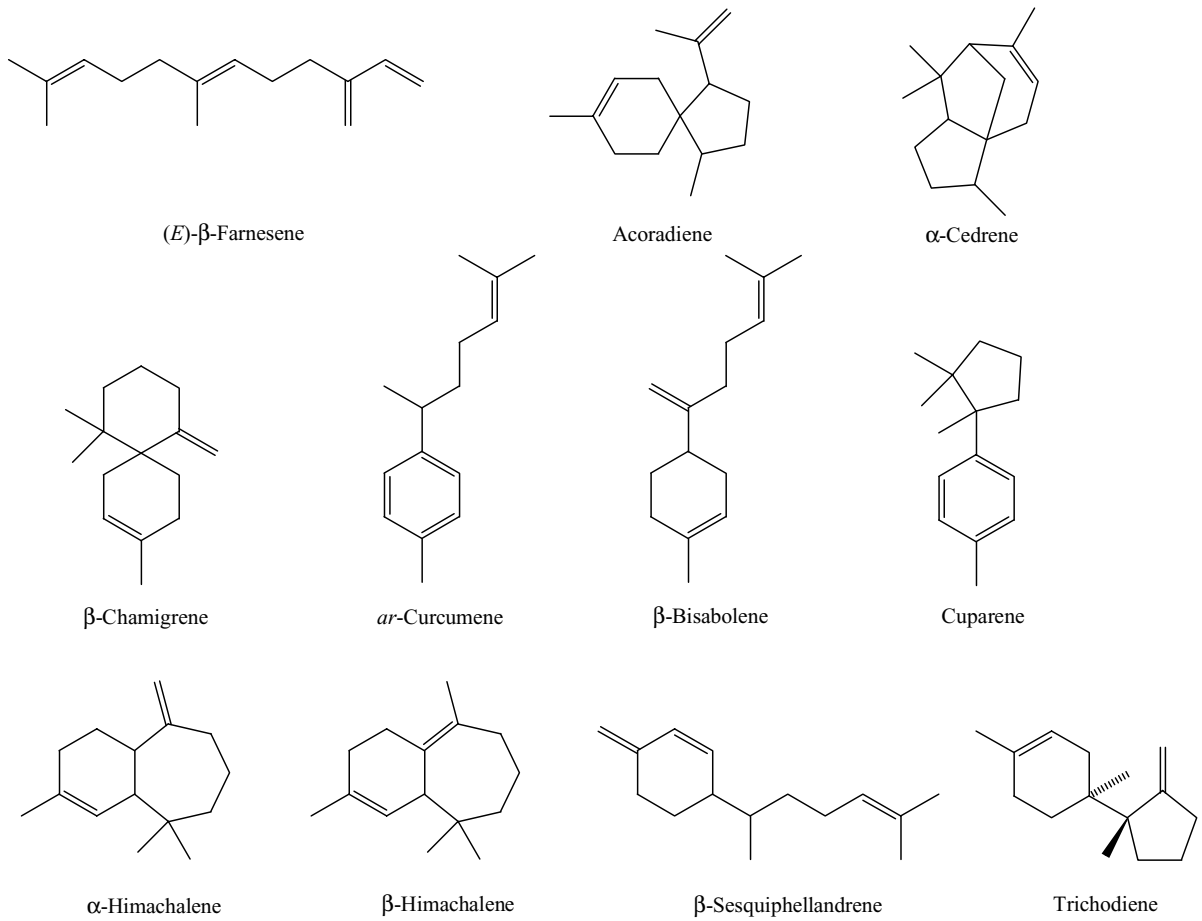

Fig. 6. Structures of volatile fungal metabolites produced by toxigenic *Fusarium* spp. (exact stereochemistry not determined).

3.3. Analysis of the volatiles produced by F. graminearum

In the final experiment, both sampling methods, SPME and HSSE, were applied to two strains of *F. graminearum*, one of which produces DON and the other one of which produces ZEA. The aim of this experiment was to distinguish two strains of the same species, each producing another class of mycotoxins, based on the headspace profile.

As expected, the headspace profile of the DON producing strain (DSMZ 4527) was completely different from the one of *F. sambucinum* and *F. sporotrichioides* and that of the ZEA producing strain (chromatogram not shown). The emission pattern was characterized by one main metabolite (77% in the HSSE extract and 84% in the SPME headspace extract). The exact structure of this compound could not be elucidated, but based on the mass spectrum and the retention index (1498) two structures can be proposed, namely germacrene D and bicyclosesquiphellandrene. Other minor metabolites were β-elemene (2.6–3.4%), β-farnesene (1.5–2.6%), γ-cadinene (1.0–2.1%) and two unidentified sesquiterpene hydrocarbons. No trichodiene was detected in the headspace of the DON producing *F. graminearum* strain. Since ZEA is not a trichothecene and its metabolic pathway is completely different, no sesquiterpenes were detected in the headspace profile of the ZEA producing *F. graminearum* strain (chromatogram not shown). This means that infection of maize by zearalenone producing *F. graminearum* cannot be detected by monitoring of the emission of the fungal metabolites. Hence, the proposed method is restricted to trichothecene producing fungi.

4. Conclusion

It can be concluded that headspace analysis of volatile fungal metabolites by solid-phase microextraction and headspace sorptive extraction in combination with gas chromatography/mass spectrometry is a suitable monitoring technique for the fast detection of trichothecene producing fungi. Trichodiene could be used as a volatile marker for trichothecene producing *F. sambucinum* and *F. sporotrichioides* fungi, and also DON producing *F. graminearum* could be easily distinguished based on its very characteristic profile of volatile metabolites.

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nañ, Poznañ, Poland) for offering the toxigenic *F. sambucinum* and *F. sporotrichioides* strains. Part of this research was supported by grant BOF/GOA 1205898. The authors are indebted to the Fund for Scientific Research, Flanders (F.W.O.-Vlaanderen, Belgium) for a post-doctoral fellowship.

References

[1] D.R. Lauren, M.P. Agnew, J. Agric. Food Chem. 39 (1991) 502.
[2] A.E. Desjardins, G. Manandhar, R.D. Plattner, C.M. Maragos, K. Shrestha, S.P. McCormick, J. Agric. Food Chem. 48 (2000) 1377.
[3] J.M. Cooney, D.R. Lauren, M.E. di Menna, J. Agric. Food Chem. 49 (2001) 522.
[4] D. Bhatnagar, J. Yu, K.C. Ehrlich, in: M. Breitenbach, R. Crameri, S.B. Lehrer, (Eds.), Fungal Allergy and Pathogenicity, vol. 81, Chemical Immunology, Karger, Basel, 2002, p. 167.
[5] H.H. Jeleñ, C.J. Mirocha, E. Wasowicz, E. Kamiñski, Appl. Environ. Microbiol. 61 (1995) 3815.
[6] A.R. Hesketh, L. Gledhill, D.C. Marsh, B.W. Bycroft, P.M. Dewick, J. Gilbert, Phytochemistry 30 (1991) 2237.
[7] D.W. Brown, S.P. McCormick, N.J. Alexander, R.H. Proctor, A.E. Desjardins, Fungal Genet. Biol. 32 (2001) 121.
[8] A.R. Hesketh, L. Gledhill, B.W. Bycroft, P.M. Dewick, J. Gilbert, Phytochemistry 32 (1993) 93.
[9] H. Jeleñ, D. Latus-Zietkiewicz, E. Wasowicz, E. Kamiñski, J. Microbiol. Methods 31 (1997) 45.
[10] K. Wilkins, K.F. Nielsen, S.U. Din, Environ. Sci. Pollut. Res. 9 (2002) 1.
[11] T. Börjesson, U. Stöllman, J. Schnürer, Appl. Environ. Microbiol. 58 (1992) 2599.
[12] H. Jeleñ, E. Wasowicz, Food Rev. Int. 14 (1998) 391.
[13] A.C. Kushalappa, L.H. Lui, C.R. Chen, B. Lee, Plant Dis. 86 (2002) 131.
[14] J.C.R. Demyttenaere, A. Adams, K. Van Belleghem, N. De Kimpe, W.A. König, A.V. Tkachev, Phytochemistry 59 (2002) 597.
[15] J.C.R. Demyttenaere, R.M. Moriña, P. Sandra, J. Chromatogr. A 985 (2003) 127.
[16] G. Engelhardt, M. Schuster, J. Lepschy, P.R. Wallnöfer, Z. Lebensm. Unters. Forsch. 182 (1986) 123.
[17] C.W. Bacon, J.D. Robbins, J.K. Porter, Appl. Environ. Microbiol. 33 (1977) 445.
[18] R.P. Adams, Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry, Allured Publishing Corporation, Carol Stream, IL, 1995.
[19] D. Joulain, W.A. König, The Atlas of Spectral Data of Sesquiterpene Hydrocarbons, E.B.-Verlag, Hamburg, 1998, p. 99.