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Use of solid phase microextraction (SPME) for profiling fungal volatile metabolites

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ABSTRACT

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Aims: The influence of isolation methods: solid phase microextraction (SPME) with different fibres and simultaneous distillation extraction (SDE) on the profile of isolated fungal volatile metabolites was investigated.

Methods and Results: Four SPME fibre types: Polydimethylsiloxane, Polyacrylate, Carboxen/PDMS and Carboxen/Divinylbenzene/PDMS were evaluated in terms of their efficiency in extracting volatile metabolites emitted by *Penicillium roqueforti* grown on wheat kernel medium. All fibres showed varied efficiency and selectivity in extracting volatile compounds. Sesquiterpene hydrocarbons were the predominant fraction of volatile compounds isolated by all fibres, and ranged from 55·4 to 93·7% of all volatiles depending on the type of fibre used. Alcohols and ketones ranged from 2·7 to 20·5%, esters from 1·2 to 12·8%, and monoterpene hydrocarbons from 1·2 to 5·4%. Profile of volatile compounds obtained by SDE differed from SPME and the oxygenated sesquiterpenes formed the predominant fraction of volatiles isolated using SDE.

Significance and Impact of the Study: The data in this study show that analysed profile of volatile compounds emitted by fungi is highly dependent on the extraction method.

Keywords: *Penicillium roqueforti*, solid phase microextraction, volatile metabolites.

INTRODUCTION

Research on fungal volatile metabolites is focused on few major fields: characterization of volatiles specific for fungal presence and growth, profiling volatiles in chemometrical comparison of strains or species and detection of off-odours caused by fungi (Börjesson *et al.* 1993; Larsen and Frisvad 1995a; Jeleń and Wąsowicz 1998).

For all these purposes solid phase microextraction (SPME) is a promising tool for extraction of volatile compounds. It has been demonstrated that SPME finds applications in quantification of different compounds both from solid and liquid matrices in concentrations ranging from low ppt to ppm (Pawliszyn 1997; Jeleń and Wąsowicz 2000). Apart from its advantages in quantitative analyses it can be a valuable method for qualitative determination of volatile compounds.

The aim of this research was to investigate the influence of sampling procedure especially extraction selectivity on the profile of volatile metabolites isolated from fungal culture using various SPME fibres, and comparing SPME to simultaneous distillation extraction (SDE) method. *Penicillium roqueforti* was selected as a model micro-organism for the experiments because of its ability to synthesise vast amounts of volatile metabolites of diversified character.

MATERIALS AND METHODS

Fungal incubation

Penicillium roqueforti IBT 16404 was obtained from Department of Biotechnology, Denmark Technical University at Copenhagen. Isolate was grown on potato dextrose agar (Chełkowski 1985) slants for 10 days, washed with sterilized

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water and combined spore suspension was used for inoculation of medium. Wheat grain moistened to 40% water content and autoclaved at 121°C for 20 min prior to inoculation was used as a medium for fungal growth. Incubation was performed in 100 ml bottles (Supelco, Inc., Bellefonte, PA, USA), each filled with 20 g of medium, in triplicates. To provide aerobic conditions for *P. roqueforti* growth bottles were plugged with sterile cotton stoppers, which were replaced by caps with teflon-lined silicon rubber membrane at the sampling time. Fungal cultures were incubated at 20°C for 14 days.

Extraction of volatile metabolites

Volatile compounds produced by *P. roqueforti* were isolated by SPME at 20 or 50°C, depending on the experiment, or by SDE method.

For the evaluation of different fibres for volatiles profiling the following ones were examined: Polydimethylsiloxane (PDMS) 100 μm, Polyacrylate (PA) 85 μm, Carboxen^{TM}/PDMS (C/P) 75 μm, and Carboxen^{TM}/Divinylbenzene/PDMS (C/D/P) 50/30 μm. All fibres were obtained from Supelco, and conditioned prior to analyses according to the manufacturer's recommendations. To avoid differences related to fungal growth and production of volatile metabolites in different fungal cultures a series of four extractions using different fibres were performed from the same bottle. The subsequent series of extraction was performed from next culture bottle in a random fibre order. All extractions were performed for 20 min.

For SDE samples (20 g) were transferred to 250 ml round bottom flasks, to which 100 ml of water was added, and distillation/extraction was performed for 2 h. As an extraction solvent a mixture of ethyl ether/pentane (1 : 1, v : v) was used (5 ml), which was concentrated to 0·5 ml before chromatographical analysis. SDE was performed in a Likens–Nickerson-type micro apparatus (Chrompack, Cat. No. 16050).

Analysis of volatiles

Volatile compounds were analysed on a gas chromatograph coupled to quadrupole mass spectrometer (Hewlett-Packard HP 5890 II/5971) working in EI mode and resolved on a MDN-5 column (30 m × 0·25 mm × 0·25 μm) (Supelco). Compounds were desorbed at 260°C, temperature equal for all fibres in a split/splitless injection port, equiped with a 0·75 mm SPME liner and working in a splitless mode. Analysis was performed in programmed temperature: 40°C for 3 min, then 8°C min^{-1} to 280°C. Gas chromatography/mass spectrometry (GC/MS) interface temperature was set to 280°C. Peak areas (of total ion current) were used for comparison of volatile compound fractions, fibre coatings and extraction methods. Compounds were identified using NBS 75K library of mass spectra or by comparison of retention times and spectra with those of authentic standards.

RESULTS

Four evaluated fibres showed different ability to extract volatile compounds. The highest amount of isolated volatiles expressed as total peak areas was observed for fibres based on Carboxene (Carboxene/PDMS 2476 ± 343, and Carboxene/PDMS/DVB 1999 ± 8·4 mln area counts), the lowest for polyacrylate (333 ± 6·1). Total peak area for PDMS fibre was 1064 ± 22·2 mln area counts. Influence of temperature on the amount of isolated compounds was also investigated. The increase of extraction temperature from 20 to 50°C significantly improved extraction efficiency – amount of total volatile compounds doubled at 50°C compared with 20°C (1258 ± 100 compared with 530 ± 43 mln area counts). Increase in sesquiterpene hydrocarbon extraction efficiency was even higher (1000 ± 123 compared with 294 ± 39 mln area counts). However, when monitoring production of volatiles especially during fungal growth, higher temperatures can influence fungal metabolism or even destroy them, therefore the possibility to extract volatile compounds 'on line' at ambient temperature is a valuable advantage of SPME. Small fibre phase volume, relatively large amount of inoculated medium (20 g), its large volume (two-thirds of incubation bottle) and the ability of *Penicillium* to produce volatile metabolites in large amounts allowed multiple extractions (up to eight times) from each bottle without significant decline in amounts of extracted compounds.

The use of different fibres for extraction influenced not only the amounts of isolated compounds, expressed as peak areas, but also their profile (Fig. 1). Although for all examined fibres, the dominating fraction was sesquiterpene hydrocarbons eluting from 18 to 22 min, the biggest differences were observed for remaining compounds. To facilitate the comparison between different fibres a list of volatile compounds extracted by four examined fibres is presented in Table 1. When PDMS fibre was used for extraction sesquiterpene hydrocarbons formed 93·7% of volatile compounds, whereas for PA fibre it was 78·1%, for Carboxen/DVB/PDMS 77·2%, and for Carboxen/PDMS only 55·4% of total volatiles. Alcohols and ketones represented 2·7–20·5% of volatile compounds depending on the type of fibre used. Esters were extracted in the range of 1·2–12·8%, whereas monoterpene hydrocarbons were extracted in the range of 1·2–5·4%.

Samples of *P. roqueforti* grown for 17 days were subjected to volatile compound isolation using SPME and SDE. A majority of identified compounds was the same in case of SPME and SDE; however, the profile of extracted

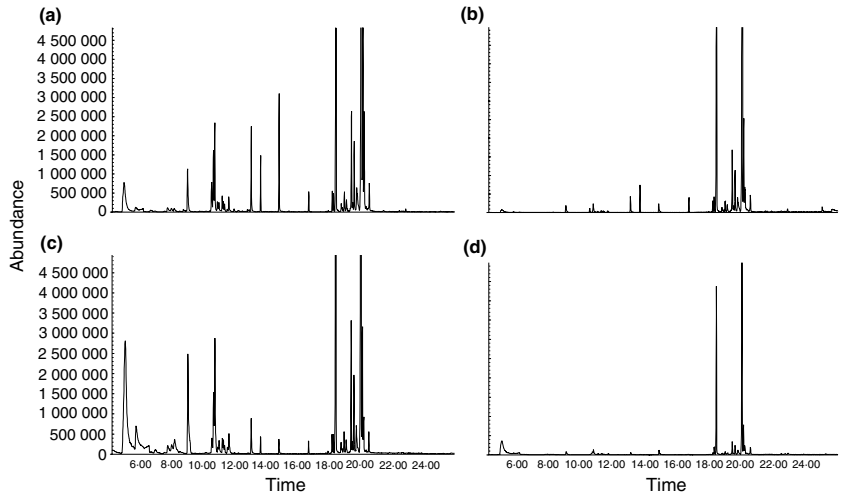

Fig. 1 Chromatograms of volatile compounds isolated from *Penicillium roqueforti* strain IBT 16404 after 14-day incubation on wheat kernel medium using solid phase microextraction fibres with various coatings: (a) Carboxen/PDMS/DVB; (b) Polydimethylsiloxane (PDMS); (c) Carboxen/PDMS; (d) Polyacrylate (PA). Column MDN-5 (30 m × 0·25 mm × 0·25 μm; Supelco)

compounds was different. When SPME was used as a method of isolation 1,3-octadiene was detected, absent in samples subjected to SDE. However, compounds absent in SPME samples were detectable in SDE. Of the identified compounds two fatty acids, hexadecanoic, and 9,12-octadecadienoic were present and also 2-ethoxy-butane, 1,1-diethoxy ethane, benzeneacetaldehyde, 2,4-decadienal and tetrahydro-6-propyl-2H-pyran-2-one were detected. However, the largest group of compounds found in SDE samples, that was absent in SPME samples, were sesquiterpene oxygenated compounds. They had a molecular weight of 216, 218 and 220 Da (Fig. 2).

DISCUSSION

Selection of fibre providing the highest extraction efficiency is an important issue in qualitative but mainly in quantitative analyses, where limits of detection are related to amount of adsorbed compound on phase covering the fibre. For qualitative purposes fibre selectivity is a more important issue, as this parameter will determine the profile of isolated metabolites. Nilsson *et al.* (1996), in their study on *Penicillium* volatile isolation by SPME, noted that for polar compounds polyacrylate-coated fibre was more efficient than PDMS fibre providing better detector responses. Moreover, an increasing response was observed with decreasing volatility of compounds. Larsen (1997) used PDMS fibre to extract terpenes produced by various fungi used in cheese making and distinguish between investigated species based on the profile of these metabolites.

The different profiles obtained with the examined fibres show the SPME selectivity of extraction. As PA fibre is often recommended for extraction of polar analytes, a large percentage of extracted alcohols and ketones confirms that. PDMS fibre with non-polar coating enables extraction of non-polar analytes, which is reflected in the highest percentage of absorbed sesquiterpene hydrocarbons of all examined fibres. Fibres in which isolation of volatile compounds relies on their adsorption on active sites of polymers used as a coating are advantageous in isolation of low molecular volatiles. This feature was observed for metabolites of *P. roqueforti* in the present study: the percentage of isolated alcohols, ketones and esters for Carboxen/PDMS fibre was over 33%, whereas for Carboxen/DVB/PDMS it was over 13% of total volatiles, mainly because of the large amount of extracted 3-methyl-1-butanol.

For isolation of volatiles produced by various *Penicillium* strains different methods have been used, based on purging volatile compounds with a stream of gas with subsequent adsorption on polymer traps, headspace diffusive sampling or simultaneous steam distillation/extraction (Börjesson *et al.* 1993; Larsen and Frisvad 1995b). Nilsson *et al.* (1996) observed striking similarities in profile and amounts of volatile compounds isolated using non-equilibrium SPME extraction and adsorption on Tenax traps. SDE is a method used for extraction of volatiles, especially when high boiling compounds need to be extracted. However, the main drawback is the possible formation of artefacts due to the long-term influence of high temperature. A comparison of SPME with SDE in this study confirms this phenomenon: the high abundance of oxygenated sesquiterpenes in SDE samples can be caused by long-term temperature influence and oxygenation of highly unsaturated sesquiterpene hydrocarbons. Additionally, polarity and high molecular weight of oxygenated sesquiterpenes make them difficult to migrate to gaseous phase, from which they can be extracted by SPME fibre. Similarly, fatty acids observed in SDE extracts, a product of lypolytic activity of fungi, can be

Table 1 Volatile compounds extracted from *Penicillium roqueforti* strain IBT 16404 using various solid phase microectraction fibres. Percentage of isolated compounds shown in columns 4–7

			(Peak area%)/Fibre type			
	Compound	Rt#	PA	PDMS	C/P	C/D/P
1	Heptane*	4·04	0	0	0·12	0
2	3-methyl-1-butanol*	4·86	14·02	0·42	16·96	4·73
3	Toluene*	5·36	0·35	0	0·59	0·12
4	Acetic acid, 2-methylpropyl ester	5·55	1·03	0	4·45	0·55
5	2-methyl propanoic acid	6·00	1·91	0	1·79	0·69
6	1,3-octadiene	6·78	0	0·20	0·38	1·00
7	Xylene*	7·84	0	0	0·77	0·32
8	Isoamyl acetate*	8·03	0	0	1·59	0·33
9	Styrene*	8·36	0	0	0·18	0·06
10	Propanoic acid 2-methyl-2-methylpropyl ester	8·88	0·99	0·82	5·13	2·14
11	1-octene-3-ol*	10·42	0·25	0·29	0·65	1·50
12	3-octanone*	10·53	0·62	2·00	2·22	2·30
13	β-myrcene*	10·62	1·46	0·84	4·30	3·06
14	3-octanol*	10·80	0	0	0·28	0·45
15	Butanoic acid, 2-methyl-2-methylpropyl ester	10·88	0	0·11	0·58	0·38
16	Butanoic acid 3-methylbutyl ester	11·10	0·30	0·22	0·89	0·87
17	Propanoic acid, 2-methyl-3-methylbutyl ester	11·17	0	0·08	0·19	0·21
18	(+)-2-carene	11·24	0	0·16	0·26	0·32
19	1-methyl-4-(1-methylethyl) benzene	11·43	0	0	0·26	0·11
20	Limonene*	11·52	0·24	0·19	0·84	0·60
21	3-carene*	11·85	0	0	0·02	0·13
22	α-phellandrene*	12·13	0	0	0	0·07
23	Undecane	12·95	0·50	1·07	1·07	2·26
24	(1,1-dimethylethyl)-2-methylphenol	14·73	1·09	0·73	0·42	3·17
25	β-patchoulene	18·14	0·33	0·67	0·49	0·64
26	β-elemene-isomer	18·23	1·23	0·95	0·55	0·59
27	β-elemene	18·39	23·61	24·55	16·46	14·98
28	Diepi-α-cedrene	18·72	0·27	0·39	0·32	0·29
29	β-gurjunene	19·30	0	0·15	0·05	0·12
30	β-patchoulene-isomer	19·37	2·23	3·73	3·15	3·07
31	Aristolochene	20·02	35·26	44·06	21·83	35·86
32	Valencene	20·10	5·65	6·06	3·77	6·63
33	α-selinene	20·18	2·32	2·61	1·39	3·65
34	β-himachalene	20·24	0	1·01	0·51	0·48
35	α-chamigrene	20·33	0	0·18	0·18	0·18
36	β-bisabolene	20·41	0·01	0·25	0·11	0·21
37	α-panasinsene	20·51	1·31	1·27	0·58	1·05
	Unidentified		0·24	0·10	1·31	0·56
	Unidentified sesquiterpenes		4·78	6·89	5·36	6·32

*Compounds identified based on the comparison of retention time and maas spectra with standard analysed under the same conditions.
Rt#, retention time (min).

released in this method and are more difficult to extract by SPME without derivatization. Larsen and Frisvad (1995b) observed differences in profiles of volatile compounds obtained by SDE and headspace, but no destruction or rearrangements in sesquiterpene fraction were observed after 20 min SDE.

One of the advantages of SPME is the relatively short time required for extraction, mainly because of a small volume of phase covering the fibre (Pawliszyn 1997). The limiting step in shortening total analysis time in this study was GC/MS run. SPME extraction time could be shortened without a significant decrease in amount of

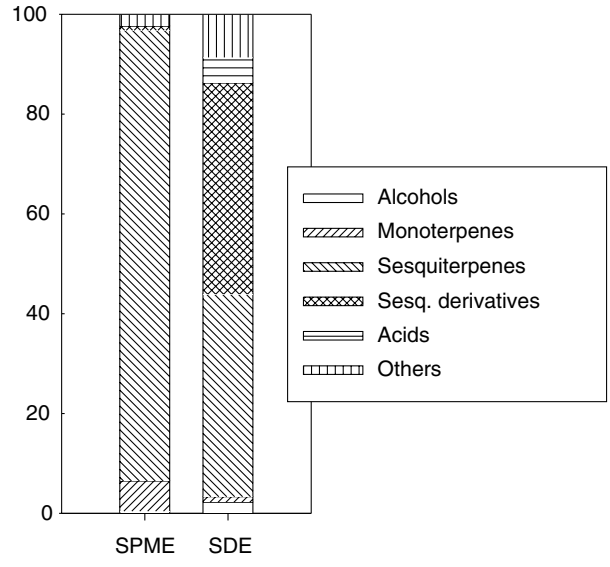

Fig. 2 Main fractions of volatile compounds (percentage of total peak areas) isolated from *Penicilium roqueforti* using SPME (PDMS fibre) and SDE methods

extracted compounds. Therefore, SPME, especially with subsequent fast GC separation, can be the fastest screening method for volatile compounds emitted by microorganisms.

There are also some drawbacks of SPME. One must be aware that drawing conclusions on the percentage content of particular compounds in the matrix (medium) based on the area percentage of corresponding peak obtained by SPME extraction may be misleading. The peak areas of compounds extracted by SPME are influenced by various factors, such as distribution coefficients between phases, compound affinity towards polymer fibre coatings, type and composition of matrix, extraction temperature, time, pH and ionic strength of liquid medium to name out a few. Therefore, for all quantitative purposes calibration for specific analysed compounds is required. It should be remembered that SPME remains a valuable tool both in qualitative and quantitative analysis of volatile fungal metabolites.

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