Gas Chromatography-Mass Spectrometry Analysis of Volatile Organic Compounds Produced by Some Micromycetes

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Key Words

Gas chromatography-mass spectrometry
Fungi and yeasts
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Summary

The volatile organic compounds evolved from various fungi were analyzed by gas chromatography-mass spectrometry. All the fungi studied were selected for their relevance in human mycology. According to their behaviour in the production of volatile compounds the fungi were divided into three groups. Group I was composed of fungi characteristically yeasts producing very high levels of volatile compounds. Ethanol, ethyl acetate, ethyl propanoate, isoamyl acetate, isobutanol and 3-methyl-1-butanol were evolved by members of this group. Group II comprised fungi producing at least ethanol, 3-methyl-1-butanol and 2-methyl-1-butanol. This group included saprophytic or opportunistic filamentous fungi. Group III consisted mainly of the keratinolytic species of fungi which produce very few volatile compounds.

Introduction

Gas chromatography-mass spectrometry (GC-MS) techniques have been used for studying volatile organic compounds produced by microorganisms and in particular, by fungi [1–5]. However, as different methods have been used it is not possible to compare the reported data. In this study, we have described a technique used to determine volatiles evolved from about twenty fungi selected for their involvement in human mycology. Futhermore, an attempt was made to classify these fungi according to the volatiles they produced.

Experimental

Organisms and Culture Conditions

This study was based on the use of several strains of yeasts and filamentous fungi (listed in Table I) recently recovered from various clinical samples sent for analysis to our hospital laboratory. The identifications of yeasts were performed by physiological tests (germ tube formation in serum and chlamydosporulation) and standard auxanographic methods according to Kreger van Rij [6]. Filamentous fungi were identified on the basis of their macroscopical and microscopical morphology.

All isolates were cultivated on Sabouraud dextrose agar slants in 25-mL vials (5 mL of medium per vial). Stationary growth phase organisms were obtained by incubating the cultures at 37 °C for 48 h for yeasts, and at 25 °C for 4 to 7 days for filamentous fungi. In order to preserve volatiles each of the fungi was cultivated and stored until analysis in the same 25-mL glass vial which was sealed with a teflon-faced silicone rubber septum.

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

The volatile compounds were analyzed using a procedure previously described [7]. The volatiles were extracted from the gas phase in the vial by passing a stream of helium over the sample, and then concentrated in a refrigerated trap. The trap was a stainless steel tube (4 × 0.3 cm I.D.) containing 200 mg of a polymer adsorbant (Tenax®) and was cooled at − 50 °C with liquid nitrogen. After a purge time of 5 min, the trap was rapidly heated to 280 °C for 30 s and the adsorbed substances were flushed onto two fused silica capillary columns (RSL 160 Alltech, 25 m × 0.32 mm I.D., 5.0 µm polydimethylsiloxane film) in a gas chromatograph (Varian model 6000). One of the capillary columns was

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connected to a flame-ionization detector (FID) and the other to a mass spectrometer ion-trap detector (ITD 800 Finnigan) which was used for the identification of the volatile compounds (mass range = 45–200 m/z). After an initial step at 40 °C for 5 min, the column temperature was increased to 200 °C at a rate of 10 °C/min. The injector and the detector were maintained at 250 °C. Helium was used as the carrier gas at a flow rate of 1 mL/min.

Results

Chromatograms of the volatile compounds present in the medium or produced by the fungi are shown in Figure 1A and 1B. Most of the organic constituents were identified by comparing GC-MS data with those of reference compounds whilst some of the volatiles were identified using the NBS library of the ITD system. Both qualitative and quantitative differences could be observed. The production of ethanol was taken as the criterion for the classification of the fungi into the three categories as listed in Table I. Group I is composed of fungi producing very high levels of volatile compounds, especially ethanol which saturated the FID. Ethyl acetate, ethyl propanoate, isoamyl acetate, isobutanol, 3-methyl-1-butanol and 2-methyl-1-butanol are also present in large concentration. Group II consists of fungi also producing ethanol, 3-methyl-1-butanol and 2-methyl-1-butanol, but to a lesser extent than fungi of Group I. Other compounds were also detected for these two groups. The last group is formed by fungi producing very few colatile compounds and, in particular, no ethanol.

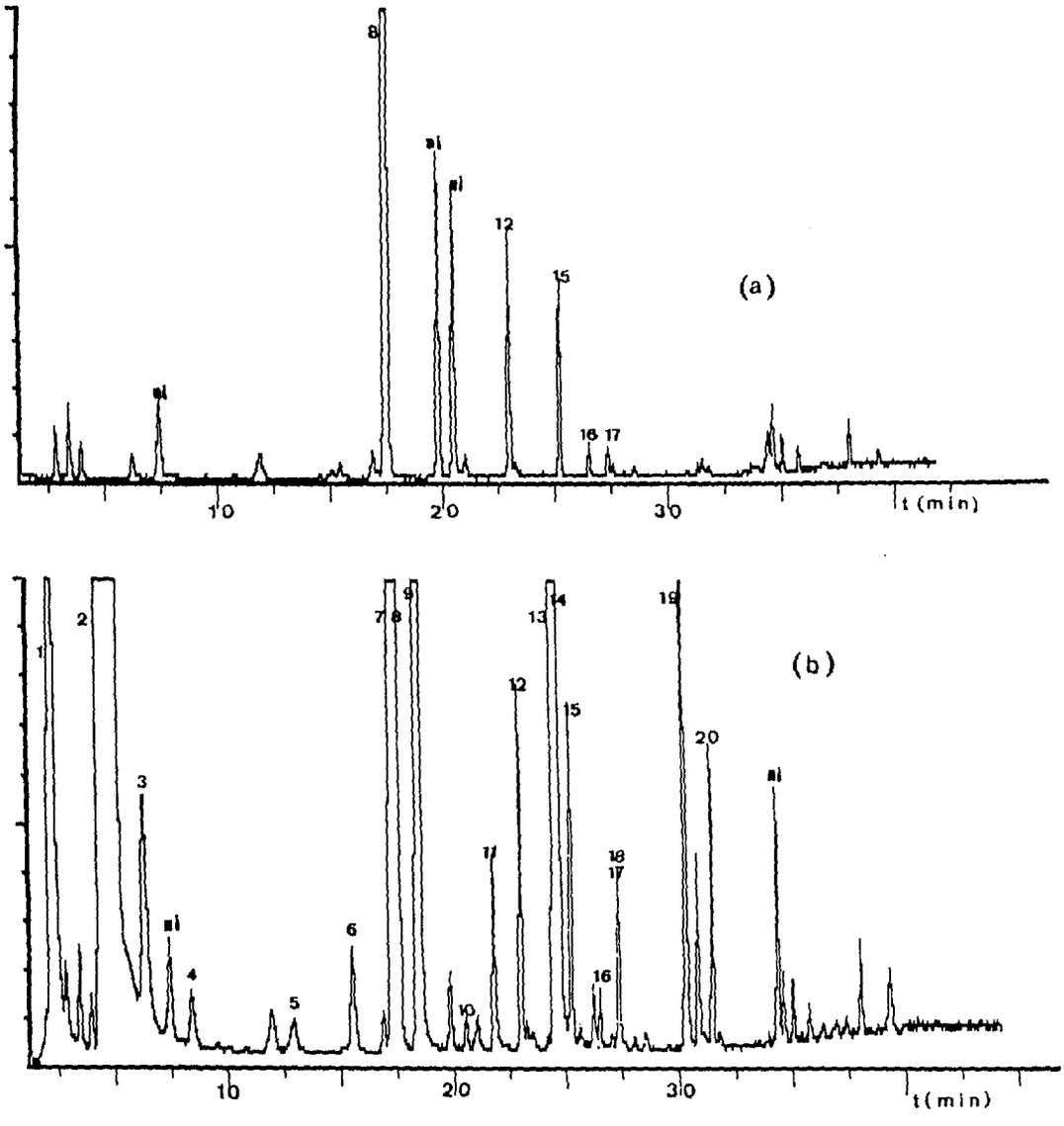

Figure 1A

Gas chromatograms of volatile organic compounds evolved from the culture medium (a) and from *Saccharomyces cerevisiae* (b). GC-MS techniques enabled us to identify the following compounds: **1** = acetaldehyde; **2** = ethanol; **3** = acetone; **4** = ethylformate; **5** = 1-propamol; **6** = 2-butanone; **7** = ethyl acetate; **8** = chloroform; **9** = isobutanol; **10** = benzene; **11** = 2-pentanone; **12** = bromodichloromethane; **13** = 3-methyl-1-butanol; **14** = 2-methyl-1-butanol; **15** = dimethyl disulfide*; **16** = toluene; **17** = dibromochloromethane; **18** = methyl butanoate; **19** = isoamyl acetate; **20** = bicyclo (2,4,0) octa-1,3,5-triene*; **21** = 2,5,4 nonadienal*.

*compounds identified only by comparison with NBS library. n.i. = compound not identified.

Although there is an overall similarity in the compounds isolated from fungi of the same group, a number of qualitative and quantitative differences could be observed in Groups I and II (Table II). For example, in Group I, *Candida inconspicua* and *C. kefyr* produced a large quantity of isoamyl alcohol with saturation of the FID, while *C. parapsilosis* produced only a small quantity of this alcohol. Likewise, production of 1-propanol was observed for all the yeasts tested except for *Candida inconspicua, C. krusei* and *C. parapsilosis*. In Group II, *Aspergillus niger* and *A. versicolor* failed to produce ethyl acetate in contrast to the other fungi of this group. These differences may be useful for the identification of a specific fungus.

Discussion

The major part of the volatile compounds evolved from fungi consist of oxygen containing molecules: alcohols, aldehydes and esters. Most of them have been previously identified in microfungi [5]. However, the fungal species previously analyzed were different from those, specifically involved in human mycology, that we studied.

In contrast to other methods which require drying or steam-distillation [5], the analytical method described in this study is rapid. No manipulation of the fungi is necessary to transfer volatile components from the culture vials to the chromatograph. Moreover, as our method is non-destructive, it is likely that the volatile compounds are secreted from the growing fungi and do not result from thermal degradation of higher molecules.

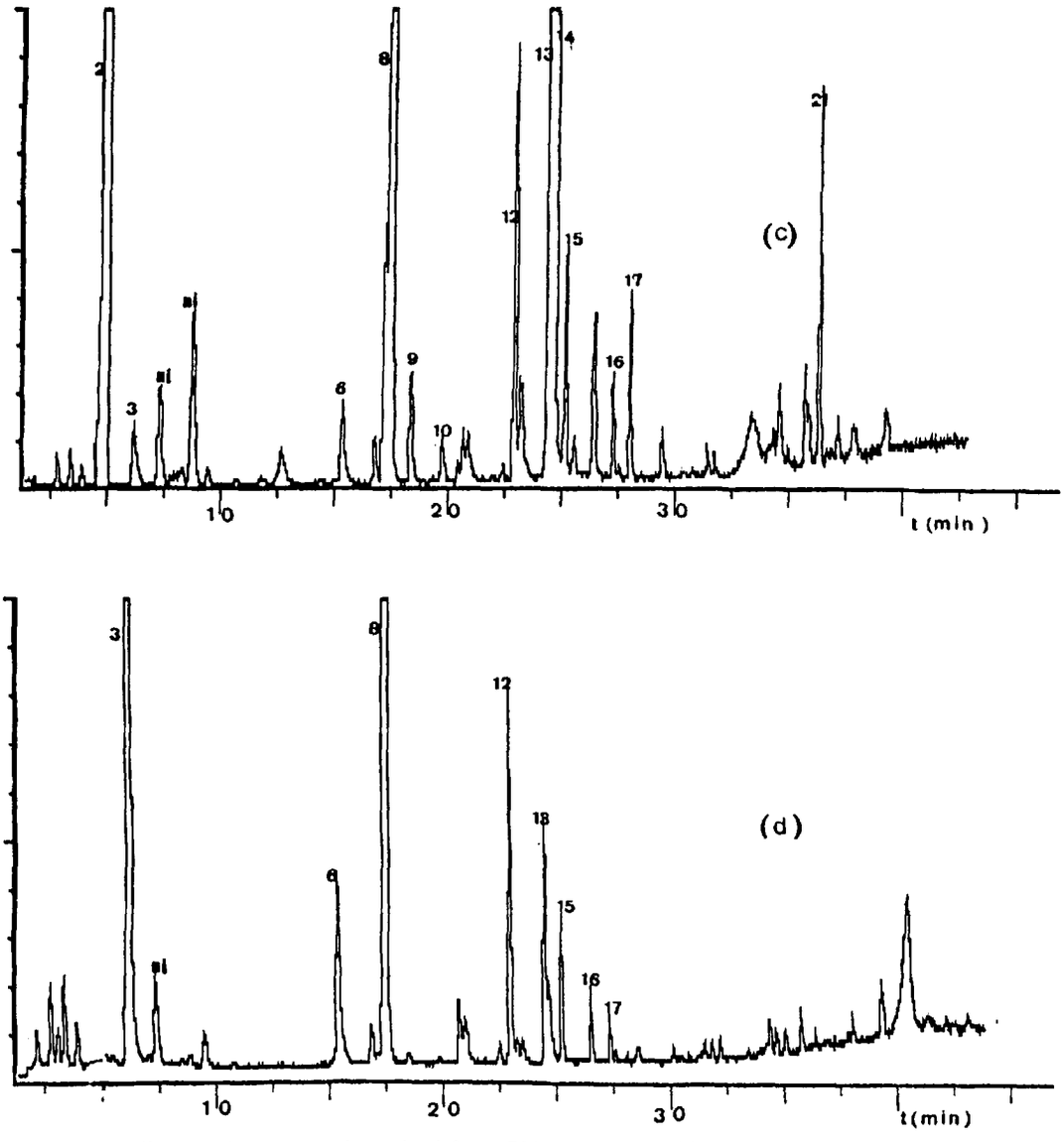

Figure 1B
Gas chromatograms of volatile organic compounds evolved from *Rhizopus oryzae* (c) and from *Trichophyton mentagrophytes* (d). For the identification of peaks, see Figure 1A.

With regard to the origin of isobutanol and of some other alcohols, they are known to be intermediates of amino acid metabolism [4]. Some alk-1-en-3-ols, which were not found in the present study, have been described as resulting from the metabolism of fatty acids [5]. Thus, probably the majority of the volatile compounds are linked to the metabolism of the studied microorganism. As the fungi differ in their cell-wall structure and in their growth, it is no surprise that they differ in their metabolism and in their production of volatiles.

In this study, the classification of the tested fungi into three groups was based only on the chromatographic data corresponding to the production of volatile compounds. It is interesting to note that the three groups consisted of yeasts, saprophytic or opportunistic filamentous fungi and keratinolytic species, respectively. All yeasts were characterized by an important production of various metabolites in which ethanol, ethyl acetate, ethyl propanoate, 2-methyl-1-butanol and 3-methyl-1-butanol predominated. In contrast, very few metabolites were detected in the tested keratinolytic fungi, i.e. various dermatophytes of the genus *Microsporum* or *Trichophyton* and non-dermatophyte keratinolytic fungi, *Anixiopsis stercoraria* and *Scopulariopsis brevicaulis*. Different saprophytic or opportunistic fungi, particularly some aspergilli and zygomycetes, constituted the intermediate group which was characterized by an important production of ethanol and lesser quantities of 2-methyl-1-butanol and 3-methyl-1-butanol.

Interestingly, isoamyl acetate was detected exclusively in yeasts and its production seemed to be a common feature of these fungi since all the tested yeast species produced this compound. Further studies are needed to clarify whether the production of this volatile component could be considered as a specific marker of yeasts, and so could be used for taxonomic differentiation. However, in other studies reviewed by Vanhaelen et al.

Table I List of the fungi tested and attempted classification.

Group I:	*Candida inconspicua*
	Candida kefyr
	Candida krusei
	Candida pelliculosa
	Candida albicans
	Candida famata
	Candida glabrata
	Candida tropicalis
	Candida parapsilosis
	Clavispora lusitaniae
	Saccharomyces cerevisiae
Group II:	*Absidia corymbifera*
	Aspergillus niger
	Aspergillus fumigatus
	Aspergillus versicolor
	Rhizopus oryzae
Group III:	*Microsporum persicolor*
	Microsporum canis
	Scedosporium apiospermum
	Trichophyton rubrum
	Trichophyton mentagrophytes
	Trichophyton terrestre
	Scopulariopsis brevicaulis
	Anixiopsis stercoraria

Table II Qualitative and quantitative differences in the production of volatile organic compounds from fungi of Groups I and II.

Fungi	Volatile organic compounds											
	acetal-dehyde	ethanol	1-pro-panol	ethyl acetate	isobutanol	ethyl propanoate	n.i.	3-methyl-1-butanol	2-methyl-1-butanol	ethyl butyrate	n.i.	isoamyl acetate
Group I:												
Candida inconspicua	+	sat		sat	++	+++	++	++	++	+	+++	sat
Candida kefyr	++	sat	+	sat	++	+++	++	+++	++	++	++	sat
Candida parapsilosis	+	sat		sat	++	+		++	++	+		+
Candida krusei	++	sat		sat	+++	+++		+++	++	++	++	++
Candida pelliculosa	+	sat	+	sat	++	+++		+++	++	+	++	++
Candida albicans		sat	+	++	++	+++		+++	++	+	+	++
Candida famata	++	sat	+	++	++	+		+++	++			++
Candida glabrata	++	sat	+	++	++	++		+++	++	+		++
Candida tropicalis		sat	+	++	++	+		+++	++	+		++
Clavispora lusitaniae	++	sat	+	sat	++	+++		++	++	+		+
Saccharomyces cerevisiae	++	sat	+	++	++	+		++	++			++
Group II:												
Absidia corymbifera		+++	+	++	++	+		+++		+		
Aspergillus niger	+	+++	+		++	+			++	+		
Aspergillus fumigatus	+	++	+	+	+		+	++				
Aspergillus versicolor		++					+	++				
Rhizopus oryzae		++		+				++				

Note: n.i., not identified; sat, saturation of the FID; +++, ++, +, indicate the relative abundance of the volatile compounds.

[5], production of isoamyl acetate was also detected with *Dipodascus aggregatus* which is related to ascosporous yeasts [8] and with *Ceratocystis moniliformis*, a filamentous ascomycete [9].

In conclusion, our study indicates that analysis of the volatile compounds evolved from fungi can be useful for classifying different species of microfungi. As this analysis also gives an insight into their metabolism it is possible that this method could be useful in research into antifungal drugs.

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