Brassicasterol: a specific biomarker sterol in humidifier sediments in indoor pollution

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ABSTRACT
Brassicasterol [B(ol)] is a sterol that was found in marine invertebrates and in some vegetables of Brassica family and in a few genus of fungus. During a study concerning an HVAC building, analysis of tank humidifier sediments based on Ergosterol [E(ol)] assessment show the presence of a particular sterol: Brassicasterol. The mycological study of the tank humidifier sediments show a presence of fungus species: *Exophiala jeanselmei* var. *heteromorpha*, *Exophiala jeanselmei* var. *jeanselmei*, *Acremonium strictum*, *Phialophora malorum* and *Phialophora fastigiata*.

The aim of this study was to investigate the relationship between Brassicasterol and the occurrence of the genus *Exophiala*. From the aquatic fungus investigated in this study, Brassicasterol was detected only in the *Exophiala* and *phialophora* species related to *Exophiala*. The Brassicasterol mass percentage depended on the nature of the culture media. The ratio B(ol)/E(ol) was in linear correlation with biomass age on different culture media. Brassicasterol seems to be related specially to *Exophiala jeanselmei* species.

INDEX TERMS
Ergosterol; Brassicasterol; Biomarker; Spray humidifiers; *Exophiala jenselmei*; Sediments

INTRODUCTION

Microbial growth, wherever in an HVAC unit, leads to the dispersion of living microorganisms or cellular fragments into the working places. Their occurrence may be linked to different health problems among which are allergic disorders (asthma) or infectious diseases (rhinitis, pneumonitis, conjunctivitis) that are part of the so-called 'Sick Building Syndrome' or 'Building Related Illness' (Engelhart *et al.*, 2000). In the HVAC systems, air humidifiers are often the critical points as regards microbial contamination, especially with hydrophilic fungi like *Phialophora* sp., *Phoma* sp., *Fusarium sp.* (Austwick *et al.*, 1986) and *Verticillium* sp (Engelhart *et al.*, 2000).

Exophiala is a dematiaceous fungus widely distributed in soil, plants, water and decaying wood material. As well as being a saprophyte in nature, the *Exophiala* species are usually included among the fungi called 'black yeast'. They are the causative agent of various human infections. Some species of *Exophiala* are known to cause subcutaneous disease in humans and other vertebrates (Larone, 1995; Sutton *et al.*, 1998; De Hoog *et al*., 2000). Although not normally life threatening, these infections must be removed surgically or they may continue to grow for years. In handling these fungi, care must be taken not to accidentally inoculate oneself with contaminated instruments.

The use of ergosterol (EOH) as biological marker of fungi is interesting because it is the major and specific sterol in many fungi and mushrooms and it is found in living and dead cells. The use of brassicasterol as a specific biological marker of *Exophiala* species may contribute to better surround the humidifiers microbiology and therefore there sanitary state.

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METHODS

Sampling Procedure and Preparation

The sediments present in the water tank were drawn up into 1-l sterilized glass containers. The instrument used for suction consisted of unique sterilized stainless steel pipes of 60-cm length and 7-mm i.d. connected to a glass container by sterilized silicon tubing. Sediments' sampling was carried out by running a hand-operated vacuum pump (Merck Eurolab, hubi2103A). One litre of mixed water sediments was sampled and filtered through a polycarbonate isopore® membrane 0.8-µm filter (ATTP04700, Millipore). Depending on the total cake quantity, from 0.2 to 0.8 g of wet and homogenized sample was placed into a 17-ml Pyrex tube, sealed with a Teflon-lined screw cap. Whenever possible, triplicate EOH extractions were made. When enough matter was present, the dry weight of the deposit was determined by duplicate analyses after drying until constant weight in an oven at 110°C.

Sample Treatment

Microwave-assisted extraction (MAE) of EOH

The MAE technique was adapted from Young (1995). The samples were first saponified with a 2 M methanolic solution of NaOH for 30 s at 60% of the maximum output power (800 W) of a home microwave (Zanker, model MWV DC8205E). After adding 2 ml of a 2.0 µg/ml solution of cholesterol as internal standard, the reaction mixture was extracted three times with 2 ml *n*-hexane. The combined hexane extracts were transferred into a 10-ml conical flask and rotary evaporated to dryness at 35°C.

Derivatization of the sterols

Trimethylsilyl (TMS) derivatives of sterols were synthesized in 200 µl of equal amounts of anhydrous pyridine and BSTFA–TMCS (99:1) (Sigma Chemicals) at 90°C for 30 min. The residual reagent was then evaporated to dryness under a gentle stream of nitrogen, dissolved in 200 µl of *n*-hexane and finally transferred into 2-ml vial before GLC analysis.

Gas Chromatography

The determination of EOH was performed on a Hewlett Packard HP6890 series gas chromatograph. Manual injections were made in the split-less mode at 290°C. Helium was the carrier gas with a flow rate of 1.9 ml/min (linear velocity of 47.0 cm/s). The capillary column was Hewlett Packard HP-5 (5% phenylmethyl polysiloxane) with the following characteristics: 30 m × 0.25 mm i.d.; 1.00-µm film thickness). Temperature programme was from 200 to 290°C at 15°C/min with a final hold of 33 min. The FID was maintained at 300°C with a nitrogen make-up flow of 20.0 ml/min. The occurrence of EOH in a sample was confirmed by GC-MS on a Hewlett Packard HP5971 mass spectrometer coupled to a HP5890 Series II gas chromatograph in the following conditions: ion source 200°C; interface temperature 290°C; EI mode at 70 eV; mass range scanned from 50 to 550 amu. The MS operated in scan or SIM mode (*m*/*z*: 329 and 363) according to the quantity of EOH present in the sample.

The microbiological numbering was carried out according to Heinemann *et al.* (1994).

CULTURES

All the cultures were from the collection at the Scientific Institute of Public Health—Louis Pasteur, Brussels, Belgium. Fourteen-day incubations were carried out for the cultures on S10 liquid media. Biomass recovery was done by vacuum filtration of cultures on Stericup (0.2 µm) materiel (Millipore). For the solid cultures media, malt extract, mineral media S10

(sporulation favouring poor media) and potato dextrose agars (PDA, rich media) were used; 47 days of incubation time were necessary for culture.

RESULTS

Ergosterol and brassicasterol analysis were carried out on both water and sediments of the tank humidifiers. However, sample from water contains ergosterol but not brassicasterol.

The GC-MS analysis of the samples shows a peak between the internal standard (cholesterol) peak and the ergosterol peak (Figure 1). The spectral database search reveals the identity of this peak to be 94% similar to brassicasterol [(22E)-ergosta-5,22-dien-3β-ol].

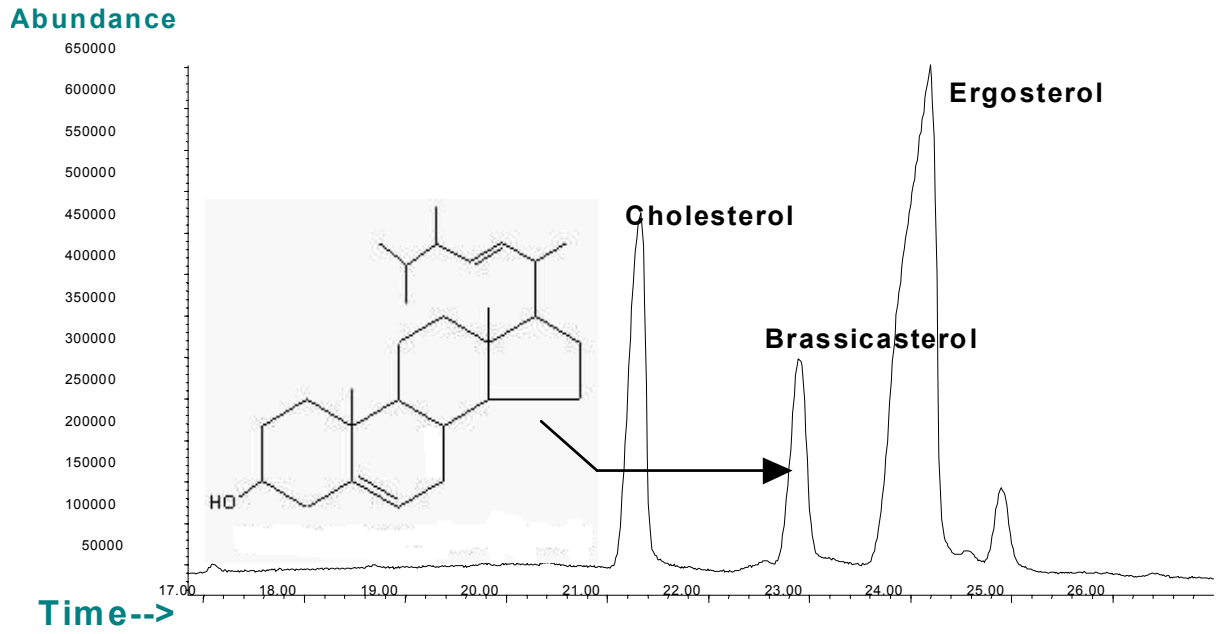

Figure 1 Total ion chromatogram of a sample from humidifier tank sediments.

Analysis results for the whole tank humidifier sediments experienced are shown in Figure 2.

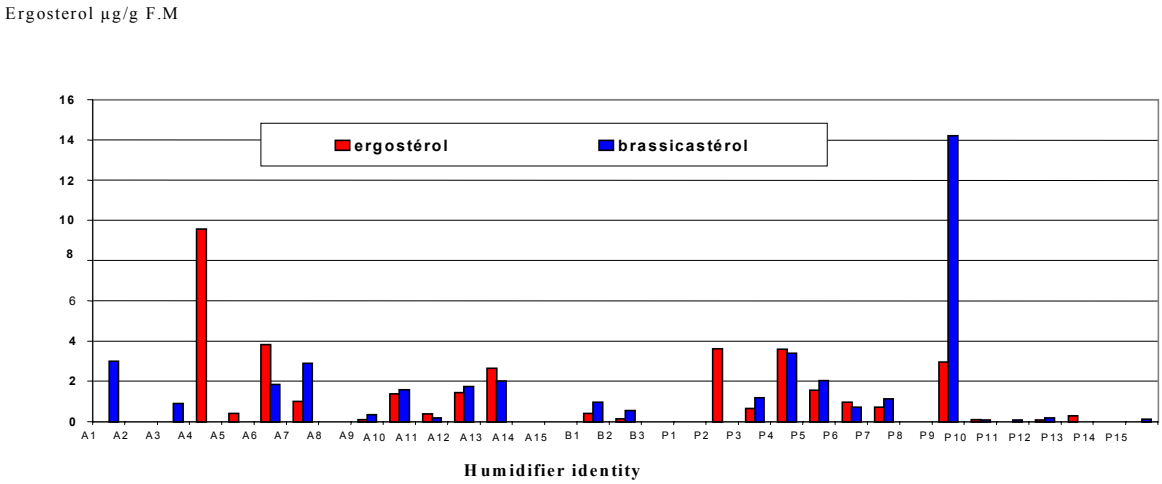

Figure 2 Ergosterol and brassicasterol ratio in sediments of 33 humidifiers. [Letters A, B and P mean the way of dampening: A (amazon), B (honeycombs), P (spray).]

From the 33 cases studied, only 17 cases were positives for brassicasterol. The ratio B(ol)/E(ol) was in the range of 0.5–4.8. Nevertheless, brassicasterol was found in four sediments but not ergosterol and vice versa for four other cases. This led us to suppose that brassicasterol will be considered as a specific biomarker for a specific humidifier fungus contamination. In order to get more information about the occurrence of this sterol in the sediments of tank humidifiers, we follow-up the evolution of both ergosterol and brassicasterol in one of the most contaminated humidifier from the 33 samples experienced.

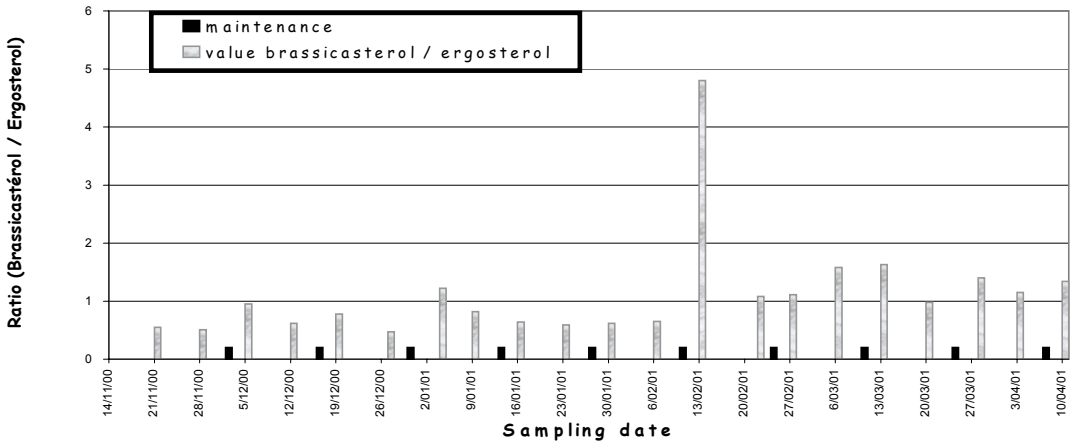

Figure 3 Evolution of the ratio B(ol)/E(ol) during different sampling periods.

The ratio B(ol)/E(ol) was in the range of 0.5–1.5. The maximum ratio observed was 4.8. To surround this particular sterol, more investigations concerning some humidifier aquatic fungus were realized. The fungus humidifier collection was from the Scientific Institute of Public Health—Louis Pasteur, Brussels, Belgium.

The stock experienced and the results related to this analysis are reported in Table 1.

Table 1 Values of ergosterol and brassicasterol in some fungus encountered in the humidifiers experienced

Experience; SSTC-003	No. IHEM	Ergosterol (% vs. DM)	Brassicasterol (% vs. DM)	Filtered biomass aspect
Acremonium strictum	993	0.56	Absent	Pinkish dough
Acremonium strictum	3937	1.01	Absent	Pinkish dough
Phialophora fastigiata	5319	0.74	Absent	Brownish dough
Phialophora malorum	5320	0.70	Absent	Brownish dough
Exophiala jeanselmei var. *heteromorpha*	4090	0.38	0.032	Individual black pellets
Exophiala jeanselmei var. *jeanselmei*	4779	0.48	0.12	Homogenous black dough

Brassicasterol was detected only in the *Exophiala* species from the six studied and that fact was observed also for the most brassicasterol positive cases. However, the ratio B(ol)/E(ol) for the two species of *Exophiala* after 14 days of culture (0.08 for stock 4090; and 0.25 for stock 4779) are much lower than the ratio calculated for the 33 humidifiers (range of 0.5–1.5; Figure 2) and the unique contaminated case (range of 0.5–4.8; Figure 3). All these observations lead us to reveal the relation between the ratio B(ol)/E(ol) and both the culture media composition and the age of fungus.

The two *Exophiala* species (Table 1) were cultured on three different solid media:

- malt extract (ME);
- mineral media S10 (poor media);
- potato dextrose agar (PDA; rich media).

After 47 days (incubation time) at 25°C, cultures were extracted as described above. The ratio B(ol)/E(ol) seemed to be dependant on the composition of the culture media. The value obtained for the stock *Exophiala* 4090 on the PDA media is twice the value obtained on ME media (Figure 4).

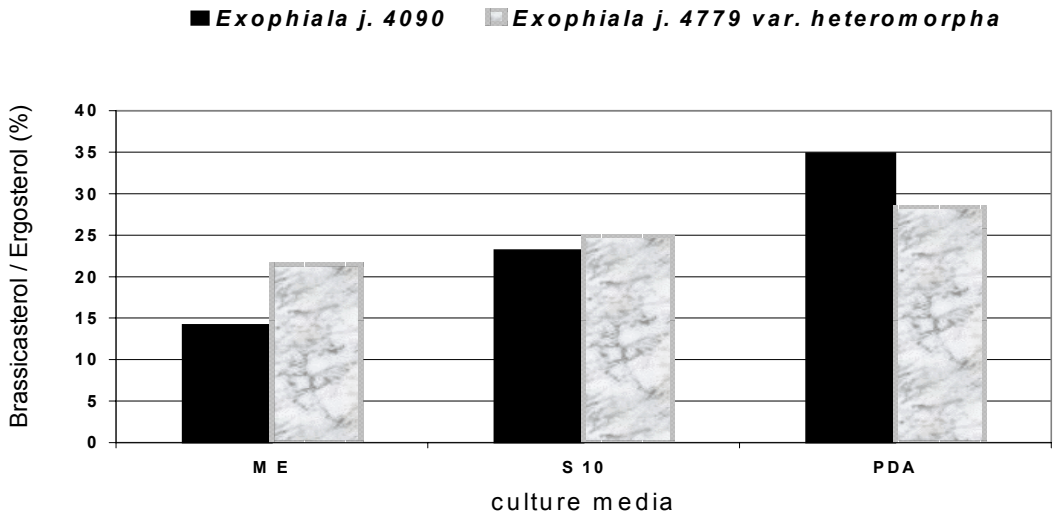

Figure 4 Ratio B(ol)/E(ol) depending on culture media for two *Exophiala* species.

The evolution of the ratio B(ol)/E(ol) depending on the age of the two species was studied by incubating six flasks of each species culture on the liquid S10 media at 25°C. Samples

were then checked at regular time ranges. The ratio B(ol)/E(ol) maintained a linear increase with time after 14 days of culture incubation time. The determination coefficients are about 0.987 and 0.971 for *Exophiala* 4090 and *Exophiala* 4779, respectively (Figure 5). This may signify that a higher ratio of B(ol)/E(ol) is probably a characteristic of old culture presenting an important mortality.

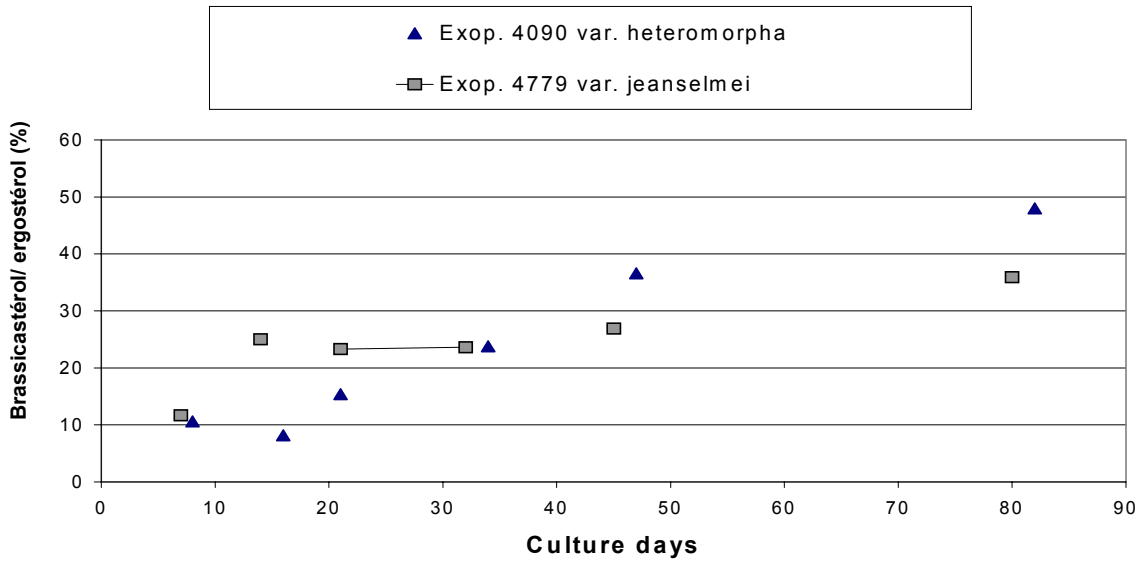

Figure 5 Evolution of the ratio B(ol)/E(ol) depending on time (number of culture days).

The high value of the ratio B(ol)/E(ol) recorded was about 0.48 (*Exophiala* 4090) and 0.36 (*Exophiala* 4779). These values are too low compared to those recorded in tank humidifier sediments (values in the range 0.5–1.5 with a peak value at 4.8).

In order to confirm the relation of brassicasterol and the occurrence of the *Exophiala* species, sterol profiles of some aquatic species were studied. The results illustrated in Table 2 show that all the species containing brassicasterol (positive species) were from the *Exophiala* and *phialophora* species related to *Exophiala*. Nevertheless, some *Exophiala* and *phialophora* species were not positive. These results lead us to suppose that brassicasterol seems to be specially related to *Exophiala jeanselmei* species in the tank humidifier. More investigations have to be done to clarify this point.

Table 2 Sterols from some aquatic species (cultures were done on the liquid S10 media for 14 days at a temperature of 25°C)

Fungus	Origin	N° IHEM	Ergosterol mg/g D.M	Brassicasterol mg/g D.M	Ratio B-ol/E-OL
Acremonium strictum	HVAC	993	5,6	ND	
Acremonium strictum	HVAC	3937	10,1	ND	
Phialophora fastigiata	HVAC	5319	7,4	ND	
Phialophora malorum	HVAC	5320	7	ND	
Phialophora radiciola	swimming pool	5341	10,2	ND	
Phialophora olivacea	swimming pool	5347	4,9	0,56	0,11
Phialophora oxyspora	swimming pool	9316	7,3	0,98	0,13
Phialophora mustea	fruit juice	4115	6,1	ND	
Phialophora japonica	rotted wood	5641	2,2	ND	
Exophiala spinifera	mycosis	3804	8,7	ND	
Exophiala pisciphila	swimming pool	5366	3,3	ND	
Exophiala pisciphila	HVAC	4036	6,4	ND	
Exophiala pisciphila	skin	7912	4,8	ND	
Exophiala J. var. jeans.	contact lences	4736	6	ND	
Exophiala J. var. jeans.	tropical swimming pool	5375	4,6	0,68	0,15
Exophiala J. var. jeans.	HVAC	4779	4,8	1,2	0,25
Exophiala J. var. jeans.	swimming pool	5335	4,9	ND	
Exophiala J. var. hetero	tropical swimming pool	5379	6,8	1,2	0,18
Exophiala J. var. hetero	HVAC	4090	3,8	0,3	0,08

CONCLUSION

This work was completed from the point of view of revealing the relation between brassicasterol and the occurrence of *Exophiala* species in the tank humidifier sediments. We found that a high value of brassicasterol mass percentage [high ratio B(ol)/E(ol)] characterizes an old biomass present in the sediment. Other analysis shows the ratio to depend on the culture media nature.

In the case of humidifier tank, brassicasterols seem to be specific to *Exophiala jeanselmei* species. Ergosterol and brassicasterol assessment in the humidifiers may be complementary for gathering sanitary information. The analysis is relatively short (1 day) compared to the mycological ones (25–30 days).

More investigations on brassicasterol and the *Exophiala* species have to be done. The confirmation of the brassicasterol structure is in progress and complementary studies on *Exophiala* will be carried out. The final goal is to draw up target values in parallel with those of ergosterol.

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