

RESEARCH ARTICLE

Headspace Solid Phase Microextraction (HS-SPME) and Analysis of *Geotrichum fragrans* Volatiles[‡]

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Abstract

Geotrichum fragrans (syn. *Saprochaete suaveolens*) can produce fruity aromas (pine-apple like) such as esters and alcohols, when cultivated in glucose containing liquid media. Consumers' preferences for the use of natural products especially in foods and cosmetics have motivated the production of aroma chemicals by biotechnological means. In the present study, the volatile compounds of the *G. fragrans* during the 9 days cultivation in liquid media were accumulated by Headspace- Solid Phase Microextraction (HS-SPME) technique, and, analysed by gas chromatography/flame ionisation detector (GC/FID) and GC coupled to mass spectrometry (GC/MS), simultaneously. The main volatiles of the *G. fragrans* culture were determined as ethyl tiglate (8.5-74.6%), ethyl isovalerate (36.1-41.6%) and methyl isovalerate (0.1-10.9%) during the time course. Daily variation trends of the aroma volatiles were determined, where ethyl acetate was postulated as an intermediate metabolite in this bioconversion pathway.

Keywords: *Geotrichum fragrans*, bioconversion, HS-SPME, GC-FID, GC/MS, aroma volatiles

Introduction

Although a trend towards the use of natural ingredients is increasing, most of the aroma compounds are still produced by chemical synthesis. Consumers' preferences and demands for natural substances have also motivated the industry to produce aroma chemicals by biotechnological methods, which include enzymes, plant cell cultures and fermentation process by using microorganisms (Berger, 2007; De Oliveira et al., 2013). Today, approximately more than hundred bioflavours are on the market produced by enzymatic or microbial bioprocesses. Vanillin, γ -decalactone, 2-phenylethanol and raspberry ketone are considered as high value bioflavour compounds among others (Grondin et al., 2015).

Fungal fermentation is an important tool for the production of new natural flavour substances. It is well-known that some fungi have remarkable metabolic enzymes and pathways, which are able to synthesize *de novo* volatile compounds, which can be directly used in the aroma industry. Among them, *Geotrichum* is a genus of yeast like fungi mainly found in soil, water and air. Especially, *G. candidum* and *G. fragrans* (also known as *Saprochaete suaveolens* and *Oidium suaveolens*) produce fruity aromas (like pine-apple) such as esters and alcohols derivatives when cultivated in glucose and *l*-valine containing media (Goldberg & Williams, 1991; Neto, Pastore, & Macedo, 2006). Recent publications have outlined the importance and utilisation of *G. fragrans* for the production of aroma compounds.

[‡]This work was presented at 17th BiHAT Symposium, İzmir, Turkey, 2007.

Additionally, investigations on alternative wastes as a media and the influence of amino acids, glucose and fructose in the media for the feasible production of natural aroma compounds were of high interest (De Oliveira, 2013; Grondin, 2015; Neto, Pastore, & Macedo, 2006; Pinotti, 2006; Midaini, 2006; Damasceno, Cereda, Pastore, & Oliviera, 2003).

2-Phenylethanol and ethyl tiglate are aroma compounds widely used in food, cosmetics and fragrances industries, which they can also be produced by fungal fermentation processes. Synthetic ethyl tiglate is used as raspberry, strawberry, pineapple and rum flavouring additive for beverages, ice cream, candy and liquors. Ethyl isovalerate is colourless and oily liquid and used as pineapple flavouring additives for beverages, icecreams, candy, baked goods, chewing gum, gelatine desserts. Ethyl isovalerate is also used in perfumery or due to its apple-like odour (Berger, 1995 and 2007 Winter, 2009).

In the present study, *G. fragrans* (NRRL Y-17571) was cultivated in liquid glucose and peptone medium for 9 days. During the fermentation process volatiles were concentrated from the head space of the liquid media with polymer-coated SPME fiber which then injected into the heated injector of the GC/MS system. Daily variations of major bioflavours such as ethyl tiglate, ethyl and methyl isovalerate were evaluated.

Materials and Methods

Microorganism

The strain NRRL Y-17571 of *Geotrichum fragrans* (syn. *Saprochaete suaveolens*) used in this study (Figure 1). The fungi was stored at -85°C in sterile 15% glycerol solutions. Culture media were refreshed on Sabouraud glucose agar plate (Merck) at 26°C and inoculated in liquid media (containing glucose, peptone, yeast extract, Na₂HPO₄ and NaCl) and placed in an orbital shaker (New Brunswick Scientific, USA) operating at 200 rpm and 26°C for 10 days.

Figure 1. *Geotrichum fragrans* culture and the light microscope image (x400)



Headspace-Solid Phase Microextraction (HS-SPME)

2 x 50 mL liquid culture sample were transferred aseptically into 2 different flasks. Sampling and absorption process were performed same conditions and times as daily. 100 µm PDMS (Polydimethylsiloxane) coated SPME fibres inserted in to the head space of the culture flasks that covered with Parafilm for 15 min at 35-40°C. After the collection process SPME fibres needle (Figure 2.) directly injected into the injector of the GC-FID and GC/MS systems simultaneously.

Figure 2. Analysis of the volatiles by using HS-SPME/GCMS technique



Gas Chromatography and Gas Chromatography-Mass Spectrometry (GC/FID, GC/MS)

The GC analysis was carried out using an Agilent 6890N GC system. FID detector temperature was 300°C. In order to obtain same elution order with GC/MS, simultaneous injection was done by using same column and an appropriate operational condition. Relative percentage amounts of the separated compounds were calculated from FID chromatograms. The results of analysis are shown in Table 1. The GC/MS analysis was carried out with an Agilent 5975 GC-MSD system. Innowax FSC column (60m x 0.25mm, 0.25µm film thickness) was used with helium as carrier gas (0.8 ml/min). GC oven temperature was kept at 60°C for 10 min and programmed to 220°C at a rate of 4°C/min, and kept constant at 220°C for 10 min and then programmed to 240°C at a rate of 1°C/min. The injector temperature was at 250°C. MS were taken at 70 eV. Mass range was from m/z 35 to 450.

Compound Identification

Identification of the volatile components were carried out by comparison of their relative retention times with those of authentic samples or by comparison of their relative retention index (RRI) to series of n-alkanes. Computer matching against commercial (Wiley GC/MS Library, Adams Library, MassFinder 3 Library) (McLafferty & Stauffer, 1989; Koenig, Joulain, & Hochmuth, 2004) and in-house "Başer Library of Essential Oil Constituents" built up by genuine compounds and components of known oils, as well as MS literature data (Joulain & König, 1998; Adams, 2001) was also used for the identification.

Results and Discussion

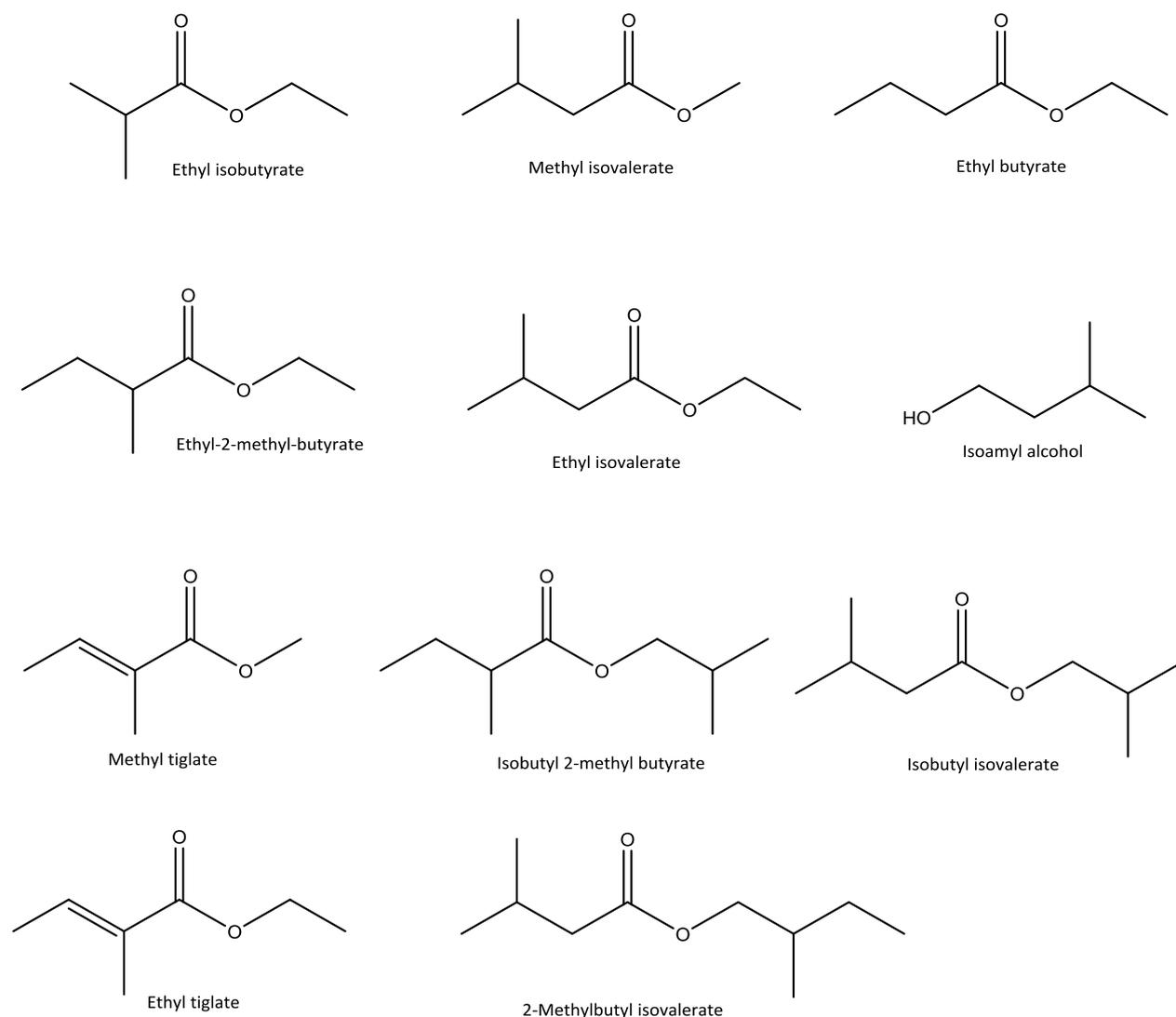
The volatile compounds of the *G. fragrans* were detected during 9 days culture period by using head space GC/FID and GC/MS systems simultaneously (Table 1.). The main volatiles were ethyl tiglate (8.5-76.4%), ethyl isovalerate (36.1-41.6%), methyl isovalerate (0.1-11%), 2-methylbutyl 3-methylbutyrate (0.4-10.2%) and Ethyl 2-methyl-butyrate (1-7.9%) (Figure 3.).

Table 1. Relative percentages and daily variations of volatiles compounds of *G. fragrans* determined by GC/MS.

Compounds	Olfactory note	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9
Ethyl acetate	-	24,7	4,2	1,6	0,8	0,4	0,4	0,7	16,6	2,8
Ethanol	-	4,6	8,8	5,2	2,2	1,3	0,2	0,2	-	-
Ethyl propionate	Pineapple like	-	-	-	0,6	0,6	0,5	-	-	-
Ethyl isobutyrate	Fruity, Citrus like	1,4	2,2	2,5	2,1	1,7	1,1	tr	-	-
Methyl isovalerate	Fruity, estery, harsh	0,3	0,3	0,1	0,2	0,3	1,0	10,9	5,8	7,0
Ethyl butyrate	Pineapple	1,5	1,0	1,0	0,4	tr	-	-	-	-
Ethyl 2-methyl-butyrate	Green apple, raspberry	4,7	7,9	6,5	3,9	2,3	1,0	tr	-	-
Ethyl isovalerate	Banana, apple	37,6	41,6	40,1	40,6	39,0	36,1	tr	-	-
2-methylpropan-1-ol	-	-	2,7	0,1	0,1	tr	-	-	-	-
Isobutyl 2-methylbutyrate	Sweet fruity	-	1,0	0,6	0,4	0,3	0,2	-	-	-
Methyl tiglate	Ethereal	-	-	-	-	-	-	0,7	2,1	5,4
Isobutyl isovalerate	Apple-strawberry	0,2	3,2	3,1	3,4	4,1	4,5	1,0	-	-
1,4-cineole	Herbal, minty, camphor	2,5	tr	-	-	-	-	-	-	-
Isoamyl alcohol	Banana	0,8	5,2	2,5	1,5	0,9	0,4	-	-	-
1,8-cineole	Herbal, eucalyptus	2,4	0,4	0,1	0,1	tr	tr	-	-	-
Ethyl tiglate	Sweet Fruity, caramel	8,5	12,8	23,2	31,0	33,4	34,5	72,3	66,9	74,6
2-methylbutyl 3-methylbutyrate	Herbal fruity, green apple	0,4	3,3	4,1	5,1	7,3	10,2	2,8	0,6	-
Phenyl ethyl alcohol	Floral, Rose like	-	0,1	0,7	0,5	0,5	0,9	0,2	tr	-

Tr: < 0.1%; -: not detected

Ethyl tiglate production of the fungi was increased during nine days and reached maximum amount at 9th day (76.4%). First sampling day showed that ethyl isovalerate (37.6) and ethyl acetate (24.7%) were the main volatiles of the *G. fragrans* culture. Ethyl acetate and ethanol were decreased day by day. They seemed to be intermediate metabolites (Figure 4). After the 6th day Isobutyl 2-methyl butyrate, ethyl isovalerate, ethyl butyrate, 1,8-cineole and 2-methylpropan-1-ol were not detected in the head space of the liquid culture.

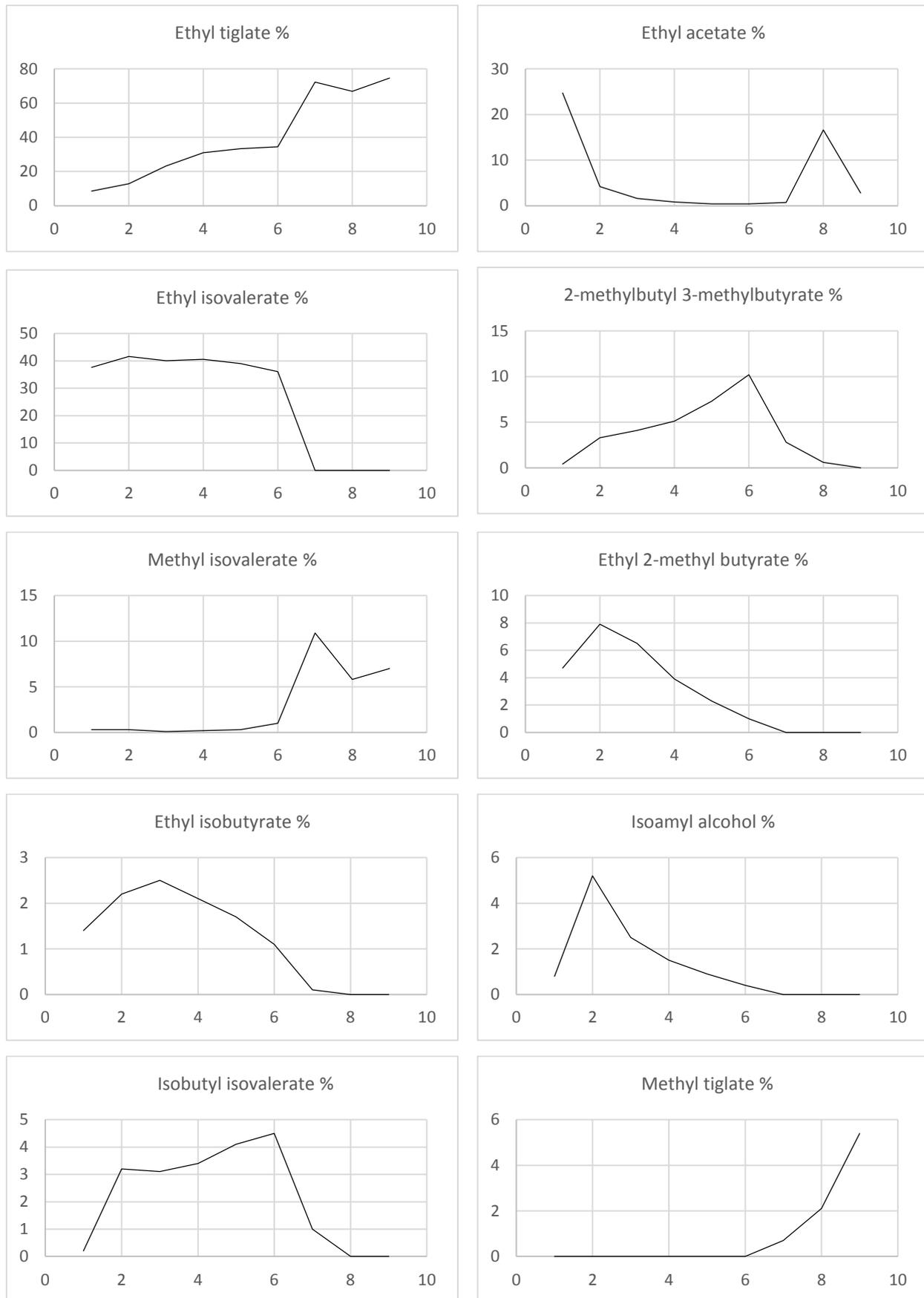
Figure 3. Volatile compounds produced by *G. fragrans*

Furthermore, in this present work sterilized molasses, a by-product of the sugar beet industrial refining process, was also used as fungal liquid culture medium. According to our ongoing studies, molasses can be utilized as a source of raw material for fermentative media for the production of valuable volatiles, which showed a similar profile to our glucose-peptone liquid medium results.

G. fragrans is a special fungi strain which produces ester, alcohol and acid-like fruit aromas (Damasceno, Cereda, Pastore, & Oliviera, 2003). Detected molecules from the culture head-space are well known flavouring compounds using in food and cosmetic industries. Ethyl isovalerate and especially ethyl tiglate are widely used in beverages, ice creams, baked goods and desserts as flavouring agents. They are also used as perfuming agents and precursors for the synthesis of more valuable aroma chemicals.

The use of biotechnology and fungal fermentation processes for the production of natural flavouring substances is an economic and easy way instead of the chemical synthesis or extraction and purification from the plants.

Figure 4. Daily variations (%) of volatile compounds of *G. fragrans*



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