Headspace Solid-Phase Microextraction Analysis of Volatile Organic Sulfur Compounds in Black and White Truffle Aroma

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Headspace solid-phase microextraction (HS-SPME) combined with gas chromatography–ion trap mass spectrometry (GC–ITMS) has been shown to be a powerful technique for detection of volatile organic sulfur compounds (sulfur VOCs) in aromas of white truffles (*Tuber magnatum* Pico) and black Perigord truffles (*Tuber melanosporum*). For both species all of the compounds previously identified during several studies were detected in single analyses, and in the case of white truffles three new sulfur compounds were identified: dimethyl di- and trisulfide and 1,2,4-trithiolane. Comparison with traditional headspace Tenax adsorption/desorption GC–MS analyses of the aromas showed that the HS-SPME technique is less suited for quantitative analyses, especially because the polydimethylsiloxane fiber coating used in the SPME device strongly discriminates more polar and very volatile compounds. With the Tenax adsorption analysis two new sulfur compounds were identified in black truffle aroma: 1-(methylthio)propane and 1-(methylthio)-1-propene. The predominant sulfur compounds are dimethyl sulfide and bis(methylthio)methane in white truffle aroma and dimethyl sulfide in black truffle aroma. On evaporation of the sulfur compounds from cuttings of black truffle a distinct mushroom odor appeared that is ascribed to the considerable contents of 1-octen-3-ol and other C_8 compounds, characteristic for mushroom aroma, that are present in the black truffle aroma.

Keywords: *Aroma analysis; solid-phase microextraction; sulfur VOCs; truffles*

INTRODUCTION

Truffles are subterranean fungi of the order Tuberales. The white truffle (*Tuber magnatum* Pico) is highly appreciated for its unique and intense aroma and is found mainly in Italy (Giovanetti, 1984). The black Perigord truffle (*Tuber melanosporum* Vitt.) has a milder and less complex, but still highly appreciated, aroma and is found mainly in Italy and France.

The volatile constituents of black truffle have been analyzed by Ney and Freitag (1980), Claus *et al.* (1981), Talou *et al.* (1987, 1990a), and Flament *et al.* (1990). The list of identified volatiles includes alcohols, aldehydes, ketones, acids, esters, amines, aromatic ethers, hydrocarbons, sulfur compounds (see below), a few heterocyclic compounds, and even an androstenol (pig pheromone). White truffle aroma was investigated first by Fiecchi *et al.* (1967), who detected one of the most important odor constituents, namely bis(methylthio)-methane (Bianco *et al.*, 1988; Polesello *et al.*, 1989). The aroma of truffles, black as well as white, is characteristically sulfurous, and some sulfur-containing volatile organic compounds (sulfur VOCs) have been identified in both species. In black truffle these are dimethyl sulfide (Ney and Freitag, 1980; Talou *et al.*, 1987), dimethyl disulfide (Talou *et al.*, 1990a; Flament *et al.*, 1990), dimethyl trisulfide (Flament *et al.*, 1990), bis-(methylthio)methane (Flament *et al.*, 1990), and, in canned black truffle; 2-formylthiophene (Talou *et al.*, 1989). In white truffle aroma the identified sulfur VOCs comprise dimethyl sulfide (Bianco *et al.*, 1988), bis-(methylthio)methane (Fiecchi *et al.*, 1967; Bianco *et al.*, 1988; Polesello *et al.*, 1989), tris(methylthio)methane, and methyl (methylthio)methyl disulfide (Polesello *et al.*, 1989). The methods used in these studies were mainly designed for volatile organic compounds in general and not directed toward sulfur VOCs as target compounds. The extraction methods used included lyophilization followed by analysis of the condensate (Fiecchi *et al.*, 1967), vacuum distillation followed by clean-up on a polystyrene column (Polesello *et al.*, 1989), steam distillation (Ney and Freitag, 1980), and headspace purge and trap (Talou *et al.*, 1987; Flament *et al.*, 1990).

Recently, a novel headspace solid-phase microextraction (HS-SPME) technique has been introduced by Zhang and Pawliszyn (1993) in which volatile sample constituents are adsorbed on a thin, fused silica fiber, coated with a layer of an organic polymer placed in the headspace above the sample, and subsequently thermally desorbed inside an ordinary GC injection port. This new, solvent-free technique may replace the large and expensive equipment used in traditional headspace techniques and has the further advantage of avoiding injection of relatively large amounts of water into the GC and loss of analytes by adsorption onto large surfaces.

The most important physicochemical parameters for the sensitivity in HS-SPME analyses have been reported to be moderate volatility and high lipophilicity of the analyte (Nilsson *et al.*, 1995a). Since sulfur VOCs, typically being low molecular weight thiols and sulfides, are rather lipophilic, the HS-SPME technique combined with ITMS, capable of producing full-scan

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mass spectra at very low concentration levels, would be expected to be well suited for analysis of such compounds.

We report here the identification of the principal sulfur VOCs in white and black truffle aromas using HS-SPME with GC–ITMS. For comparison, traditional headspace Tenax adsorption analyses were performed.

MATERIALS AND METHODS

Truffles. Fresh truffles (*T. magnatum* Pico and *T. melanosporum* Vitt.) were collected in Alba, Italy, in October 1993 and 1994. The 1993 samples were kept for 7 days and the 1994 samples for approximately 1 month in darkness at 5 °C until analysis.

Sample Preparation. Immediately before analysis, the truffles were rinsed with tap water, brushed, and air-dried. For the first (1993) SPME analysis razor thin slices of truffle flesh were cut with a sharp knife and approximately 1 g (wet weight) was placed in a 10 mL glass vial closed with a Teflon-lined membrane cap. The second (1994) SPME analysis was performed with approximately 5 g of freshly cut dices of truffle flesh in a 25 mL glass vial closed with a Teflon-lined membrane cap. For the headspace Tenax adsorption thin strips of truffle flesh (1994), ~0.2 g, were used.

Headspace Solid-Phase Microextraction. The SPME device for this investigation (Supelco) uses a fine ($d = 110\ \mu m$) fused silica fiber coated with a short (10 mm), thin (95 μm) layer of polydimethylsiloxane. The fiber is housed in a stainless steel needle which allows for penetration of the membrane covering the sample vial and the septum in the GC injection port. Once inside the sample vial, the fiber was pushed out of the housing and exposed to the headspace above the truffle sample for 30 min at 80 °C and at room temperature in the first and the second analyses, respectively. At this point, the fiber was pulled into the housing and the SPME device was removed from the sample vial and inserted into the injection port of the GC–MS system.

Headspace Tenax Adsorption. A Spantech TD4 two-stage thermal desorber was used. The thin strips of sample material were placed between plugs of glass wool in an empty stainless steel tube which then was mounted in the desorber oven. The oven was heated to 60 °C, and approximately 20 mL of He gas was led through the tube to the liquid carbon dioxide cooled (−40 °C), Tenax-filled cold trap during 4 min. The trapped volatiles were desorbed by rapid electrical heating to 250 °C for 45 s and transferred to the GC column via a deactivated fused silica tube heated to 150 °C.

Gas Chromatography–Mass Spectrometry. The GC for the headspace SPME analyses was a Varian 3400 with a septum-equipped injector. For the first (1993) SPME analysis a 75 m × 0.53 mm DB-624 fused silica column (J&W scientific) with a 3 μm film thickness was installed in the GC oven and operated with the following temperature program: −20 °C for 2 min (liquid nitrogen cooling) followed by a temperature rise of 8 °C/min to 200 °C, which was held for 5 min. Helium was used as carrier gas at 25 mL/min. The GC was interfaced via a jet separator (0.3 Torr in the separator and 0.03 Torr in the analyzer) and a transfer line (220 °C) to a Varian ITS-40 ion trap mass spectrometer (ITMS). The ion trap was operated at 300 °C in the electron impact mode, scanning from m/z 35 to 260 in 1.5 s.

The second (1994) SPME analysis was carried out under identical conditions, except that a 30 m × 0.32 mm DB-624 fused silica column (J&W Scientific) with a 1.8 μm film thickness was used with helium at 2 mL/min as carrier gas and the GC was coupled directly to the ITMS.

After insertion into the GC injection port, the SPME fiber was pushed out and thermodesorbed for 5 min at 200 °C. In this way the analytes were transferred from the SPME coating to the top of the GC column, where they were cryofocused before being chromatographed. The total run time was 32 min.

The GC–ITMS data were acquired on a personal computer with Saturn I software (Varian). The mass spectral identifications of the sulfur VOCs were carried out by comparison to the NBS mass spectral library as well as search in MassLib (Chemical Concepts GmbH). Sulfur compounds were screened for by m/z 45 + 46 + 47 mass chromatograms. These masses, corresponding to the ion compositions CHS, CH_2S, and CH_3S (among others), are typical for compounds with saturated sulfide or mercaptan structure elements. Whether a constituent selected in this way in fact is a sulfur compound was determined by evaluation of its full spectrum.

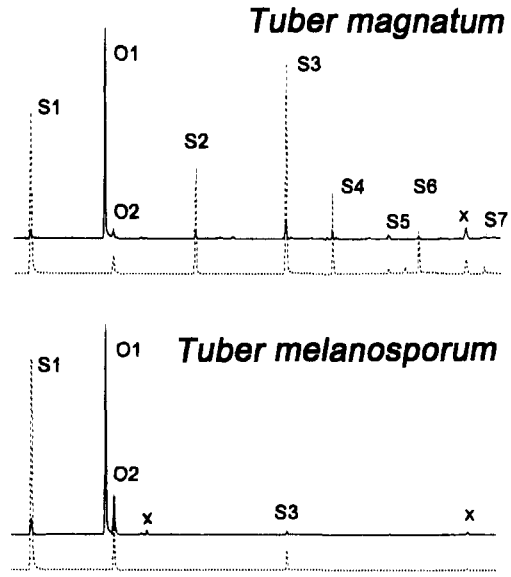

Figure 1. TIC (solid lines) and m/z 46 mass chromatograms (dotted lines) of HS-SPME GC–ITMS of white (upper) and black (lower) truffle aromas; X = unknown.

For the Tenax adsorption analyses a HP series 5890 GC was used. The column was a 30 m × 0.31 mm (i.d.) DB-5 fused silica column (J&W Scientific) with a film thickness of 1 μm. The carrier gas was helium with a flow rate of 2 mL/min. The inlet end of the column was connected to the transfer line from the Spantech desorber with a union inside the oven. The temperature program was 20 °C for 2 min (solid carbon dioxide cooling) followed by a rise of 10 °C/min to 250 °C. The column outlet end was coupled directly to the ion source of a VG Trio 2 quadropole mass spectrometer operated at 200 °C in the electron impact mode, scanning from m/z 20 to 350 in 1 s. Data were acquired on a personal computer with Labbase software (VG). The mass spectral identifications of the volatiles were supported by the NBS library.

RESULTS AND DISCUSSION

Total ion current (TIC) and m/z 46 mass chromatograms of the first (1993) HS-SPME GC–ITMS analyses of white and black truffle volatiles are shown in Figure 1. The sulfur and two major non-sulfur compounds identified from mass spectral data are listed in Table 1, together with their TIC area percentages.

Seven volatile sulfur compounds were found in the aroma of white truffles, S1–S7, including the four previously reported, S1, S3, S6, and S7, and three new compounds, S2, S4, and S5. In the latter group dimethyl di- and trisulfide have been identified in black truffle aroma, whereas 1,2,4-trithiolane (S5) has not been encountered in truffles. This compound, together with other cyclic polysulfides and the open-chain counterpart, methyl(methylthio)methyl disulfide (S6), has been found in the aroma of the edible mushroom *Lentinus edodes*, shiitake (Shieh and Sumimoto, 1992). The limited number of other occurrences known to us comprise the marine alga *Chondria californica* (Wratten and Faulkner, 1976), the freshwater alga *Ochromonas danica* (Jüttner *et al.*, 1982), North Sea fish oils (Chris-

Table 1. Volatiles Identified in White and Black Truffle Aromas by HS-SPME GC–ITMS

peak no.	retention time (min)	chemical name	formula	area percentage	
				white truffle	black truffle
S1	10.2	dimethyl sulfide	C_2H_6S	3.6	6.5
O1	13.4	2-butanone	C_4H_8O	81	78
O2	14.1	2-butanol	$C_4H_{10}O$	3.8	14
S2	17.4	dimethyl disulfide	$C_2H_6S_2$	2.0	
S3	21.4	bis(methylthio)methane	$C_3H_8S_2$	5.9	0.9
S4	23.5	dimethyl trisulfide	$C_2H_6S_3$	2.7	
S5	26.7	1,2,4-trithiolane	$C_2H_4S_3$	0.1	
S6	27.4	methyl (methylthio)methyl disulfide	$C_3H_8S_3$	1.1	
S7	30.4	tris(methylthio)methane	$C_4H_{10}S_3$	0.3	

tensen *et al.*, 1981), plants (Whitfield *et al.*, 1981; Gmelin *et al.*, 1981), and egg aroma (Gil and MacLeod, 1981).

Only two sulfur VOCs were found in the black truffle aroma, dimethyl sulfide (S1) and bis(methylthio)-methane (S3). The other previously reported black truffle sulfur VOCs in fresh, black truffle aroma, dimethyl di- and trisulfide, were not detected.

The analyses were repeated 1 year later (1994) with white and black truffles from the new harvest. This time the truffles had been stored for a longer time (1 month) before analysis, and, as discussed below, the white truffle samples probably had become too old. To obtain higher sensitivity (Nilsson *et al.*, 1995a), and also to avoid possible production of artifacts, the SPME headspace samplings were performed at room temperature. In the first analyses a temperature of 80 °C was chosen interrupt metabolic activity in the samples. In the second analysis of white truffle aroma only four sulfur VOCs were detected; 1,2,4-trithiolane, methyl (methylthio)methyl disulfide, and tris(methylthio)-methane were missing. On the other hand, the second analysis of black truffle aroma showed all four sulfur VOCs that had been found previously in fresh, black truffle aroma.

It should be noted that factors other than storing time and headspace sampling conditions play a role for the aroma picture. The biological nature of these fungi makes them less well-defined as the growth state and growing conditions are determining for the evolution of volatiles. In the case of black truffles, Talou *et al.* (1990b) have found that dimethyl sulfide is the major volatile compound in the early growth state (October–December), whereas aldehydes, ketones, and alcohols become more prominent during the last growth stage (January–March). Further, continued production of volatiles through biochemical and maybe purely chemical processes during sampling might complicate the picture. Little is known, however, about the formation of volatile organosulfur compounds from organisms (Kjær, 1977; Bremner and Steele, 1978).

Regarding quantitative aspects, the SPME technique would not be expected to produce a true picture of the relative quantities of the aroma constituents. This is because of the fact that with the nonpolar polydimethylsiloxane fiber coating there is a strong discrimination against very volatile compounds (Nilsson *et al.*, 1995a) and polar compounds (Nilsson *et al.*, 1995b). Even with this in mind, it was surprising that only two non-sulfur VOCs could be identified in the aromas, especially considering that 120 non-sulfur VOCs have been identified in black truffle aroma (Flament *et al.*, 1990). However, our headspace Tenax sampling analysis of freshly cut black truffle material, discussed below, showed that these two non-sulfur VOCs, 2-butanone and 2-butanol, together constitute more than 80% of the VOCs and that, aside from very volatile C_2 and C_3 compounds which would not be expected to be detected with the SPME technique, other VOCs were present in trace amounts only, except for some C_8 compounds, with 1-octen-3-ol constituting about 4% of the total. The failure to detect these constituents with the SPME technique in the present study might be due to saturation of the fiber coating with the C_4 VOCs; no problems were encountered in previous analyses of C_8 compounds, including 1-octen-3-ol, from *Penicillium* fungi with HS-SPME (Nilsson *et al.*, 1995b). The success in detecting sulfur VOCs, on the other hand, might be due to such compounds being especially adsorptive. It may be noted that with the HS-SPME technique all of the previously reported sulfur compounds in the aromas of fresh white and black truffles, have been detected in single analyses. In the case of white truffle three new sulfur compounds were detected.

Realizing that the HS-SPME technique no doubt would give a distorted picture of the composition of the aromas, as discussed above, traditional headspace Tenax adsorption analyses combined with GC–quadropole MS were performed on the truffles collected in 1994. TIC chromatograms of white and black truffle aromas are shown in Figure 2, and the identified compounds are listed in Table 2 together with their TIC area percentages.

In black truffle aroma ethanol, acetone, 2-propanol, 2-butanone, 2-butanol, and C_8 ketones and alcohols are the predominant non-sulfur compounds; dimethyl sulfide is the only quantitatively important sulfur compound. The presence of dimethyl di- and trisulfide was confirmed, whereas bis(methylthio)methane was not detected with Tenax adsorption GC–quadropole MS. Traces of 1-(methylthio)propane and 1-(methylthio)-1-propene were detected (reproducibly) for the first time in truffle aroma. The finding of these compounds breaks the pattern of the sulfur compounds containing one carbon units only and complicates speculation on the origin of the sulfur compounds. None of the aromatic ethers previously reported by Flament *et al.* (1990) to give the truffle a nutty and earthy aroma were detected. Actually, our list of aroma constituents is more than 100 members shorter than that of these authors, whereas, qualitatively, it more resembles that of Talou *et al.* (1987), who, however, did not report the presence of the C_8 compounds which we believe play a role in the aroma, as discussed below. Quantitatively, very large differences are observed: The predominant constituents in the present study, 2-butanone and 2-butanol, were minor constituents, whereas acetaldehyde, acetone, ethanol, 2-methyl-1-propanol, and 2-methyl-1-butanol were the major constituents. Thus, it appears that truffle aroma is not a well-defined quantity.

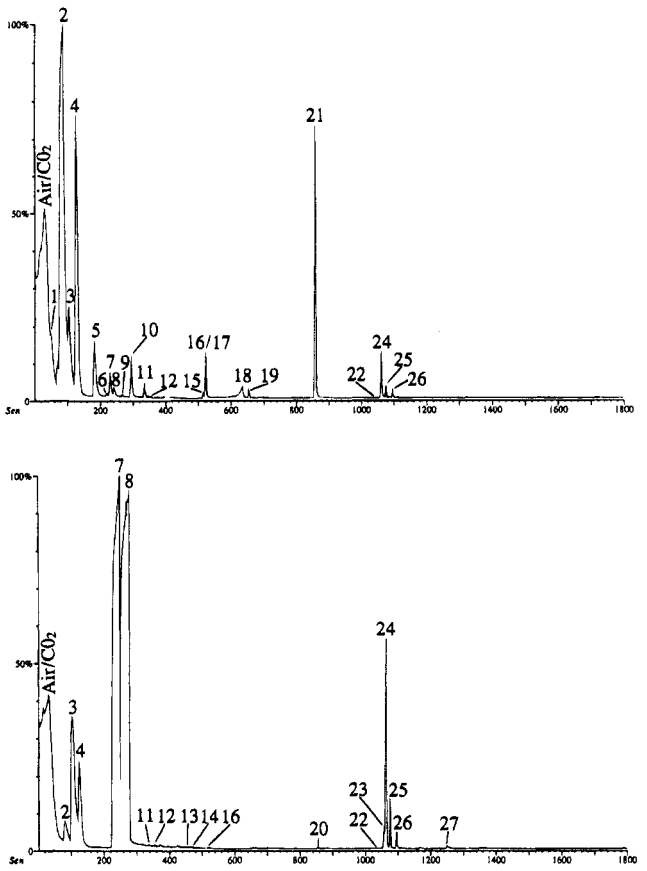

Figure 2. TIC chromatograms of headspace Tenax adsorption GC–quadropole MS of white (upper) and black (lower) truffle aromas.

When the strips of black truffle flesh used for the headspace Tenax adsorption analysis were left open to the air, their characteristic, strong sulfurous aroma disappeared in a short time. A reanalysis of the material (Figure 3) showed that the strong dimethyl sulfide peak had nearly vanished and that the other sulfur VOCs were not detectable. The pattern of non-sulfur VOCs was less changed, the major difference being a considerable increase in the amounts of the aldehydes 2- and 3-methylbutanal. Thus, the sulfur VOCs definitely are of basic importance for the truffle aroma. In the case of black truffles dimethyl sulfide probably is the only important sulfur VOC.

When the black truffle material had lost its characteristic sulfur aroma by evaporation, an intense mushroom odor was noticed. This prompted us to investigate the aroma of freshly cut, white, cultivated mushrooms (*Agaricus bisporus*) for comparison. TIC chromatograms are shown in Figure 3. The compounds responsible for the characteristic mushroom odor, 1-octen-3-ol, 3-octanone, and 3-octanol, are present in considerable amounts in the black truffle aroma, which also contains a small amount of 1-octen-3-one. This compound, not found in the aroma of the raw mushroom investigated, has a high aroma value (Fischer and Grosch, 1987). This explains the development of mushroom odor of black truffle on evaporation of the sulfur VOCs. It is interesting to observe the pronounced difference between the cap and the stalk of the mushroom, with 3-octanone and 1-octen-3-ol, respectively, being the most abundant compounds. Mushroom aroma has been the subject of a number of studies; for another recent reference see Buchbauer *et al.* (1993), but to our knowledge this difference has not been reported previously.

In white truffle aroma 2-butanone and 2-butanol are relatively weak; ethanol is the major non-sulfur VOC. Dimethyl sulfide and bis(methylthio)methane, characteristic for white truffle, are major compounds, constituting more than 25% of the VOCs. Dimethyl di- and trisulfide are present in small amounts, whereas methyl (methylthio)methyl disulfide, tris(methylthio)methane, and 1,2,4-trithiolane were not found, as was the case in the second HS-SPME analysis. The high contents of small alcohols, aldehydes, and ketones, and especially

Table 2. Volatiles Identified in White and Black Truffle Aromas by Headspace Tenax Adsorption GC–Quadropole MS

peak no.	retention time (s)	chemical name	formula	area percentage	
				white truffle	black truffle
1	49	acetaldehyde	C_2H_4O	6.5	
2	83	ethanol	C_2H_6O	45.0	1.1
3	103	acetone + 2-propanol	$C_3H_{6/8}O$	7.9	8.5
4	126	dimethyl sulfide	C_2H_6S	18.9	3.8
5	182	1-propanol	C_3H_8O	3.2	
6	220	2,3-butanedione	$C_4H_6O_2$	0.1	
7	229/249[a]	2-butanone	C_4H_8O	1.1	38.2[a]
8	242/278[a]	2-butanol	$C_4H_{10}O$	0.4	42.7[a]
9	267	ethyl acetate	$C_4H_8O_2$	0.1	
10	295	2-methyl-1-propanol	$C_4H_{10}O$	1.4	
11	335	3-methylbutanal	$C_5H_{10}O$	0.4	tr[b]
12	355	2-methylbutanal	$C_5H_{10}O$	tr	tr
13	454	1-(methylthio)propane	$C_4H_{10}S$		tr
14	475	1-(methylthio)-1-propene	C_4H_8S		tr
15	516	3-methyl-1-butanol	$C_5H_{12}O$	0.1	
16	522	dimethyl disulfide	$C_2H_6S_2$	0.1	tr
17	523	2-methyl-1-butanol	$C_5H_{12}O$	1.2	
18	635	3-hydro-2-butanone (acetoin)	$C_4H_8O_2$	0.7	
19	654	hexanal	$C_6H_{12}O$	0.2	
20	856	unknown, possibly artifact			0.1
21	860	bis(methylthio)methane	$C_3H_8S_2$	8.8	
22	1040	dimethyl trisulfide	$C_2H_6S_3$	tr	tr
23	1057	1-octen-3-one	$C_8H_{14}O$		0.3
24	1063	1-octen-3-ol	$C_8H_{16}O$	1.3	4.3
25	1076	3-octanone	$C_8H_{16}O$	0.3	0.7
26	1096	3-octanol	$C_8H_{18}O$	0.2	0.3
27	1248	2-octen-1-ol	$C_8H_{16}O$		tr

[a]Column overload and detector saturation. [b]Trace.

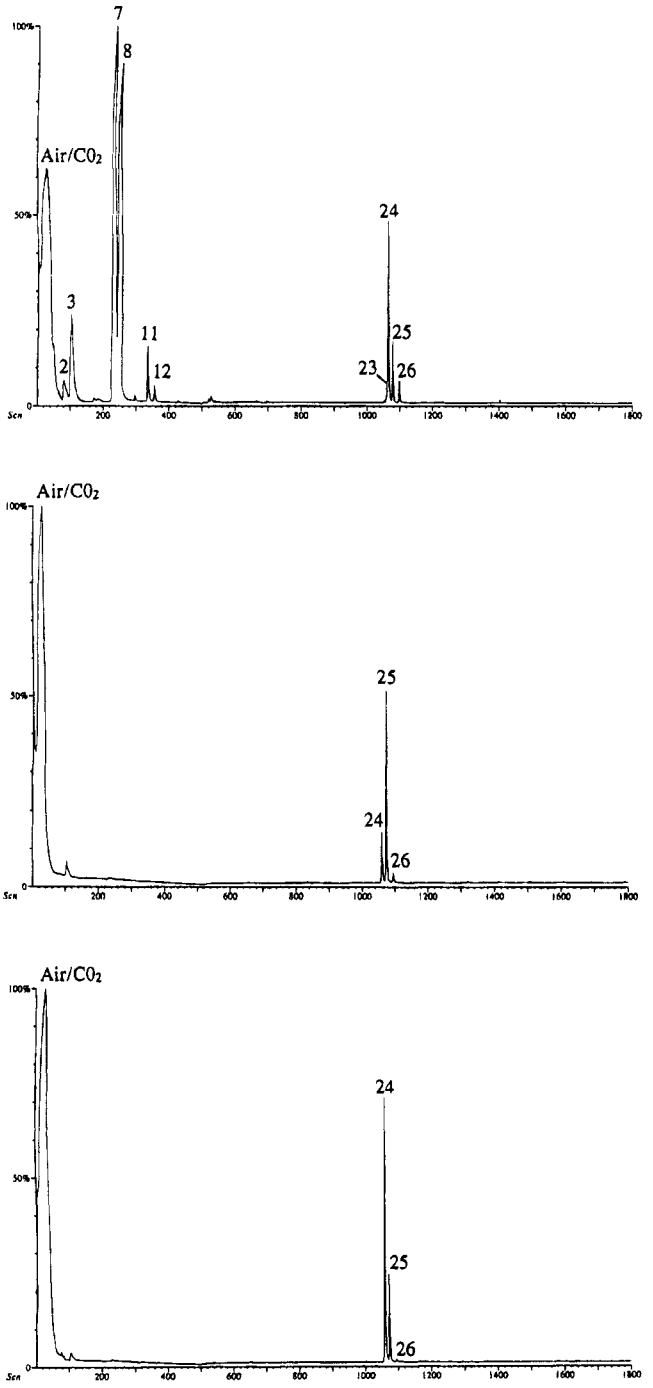

Figure 3. TIC chromatograms of headspace Tenax adsorption GC–quadropole MS of black truffle after evaporation of the sulfur VOCs (upper), mushroom cap (middle), and mushroom stalk (lower).

of acetoin, suggest microbiological growth, meaning that the time from collection until analysis might have been too long for the white truffle. It is evident, however, that dimethyl sulfide and bis(methylthio)methane are the compounds responsible for the unique aroma of that truffle species.

The results obtained by HS-SPME GC–ITMS are in good accordance with those obtained by headspace Tenax adsorption GC–quadropole MS for the sulfur VOCs, while the expected discrimination of the polar or very volatile compounds by HS-SPME was confirmed.

CONCLUSION

Headspace solid-phase microextraction combined with GC–MS detection is a powerful technique for analysis of volatile organic sulfur compounds in truffle aromas, but since HS-SPME strongly discriminates more polar and very volatile compounds, it is less suited for the quantitative analysis.

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