METHODS FOR THE IDENTIFICATION OF TAINTING TERPENOIDS AND OTHER COMPOUNDS FROM ALGAE

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ABSTRACT

Off-flavour compounds produced by algae in freshwater ecosystems were studied for their structure using integrated sensory and spectroscopic methods: mass spectrometry (MS), nuclear magnetic resonance (NMR) and Fourier transform infrared (FTIR). Both solvent extraction and thermal desorption were used to isolate and to introduce the compounds into gas chromatography/mass spectrometry (GC/MS) and gas chromatography/Fourier transform infrared (GC/FTIR) systems. Ten ng of a terpenoid compound gave a readable IR spectrum. For $^1$H NMR studies the compounds were collected directly into NMR solvent by preparative gas chromatography. About 5 μg of a monoterpenoid compound was needed for a reliable $^1$H NMR spectrum. The mass spectral data indicated that the series of odorous substances detected by GC were terpenoid hydrocarbons and a later eluting series of compounds were sesquiterpene alcohols. In addition, one compound with an intense off-odour (aromatic, nitrogen containing compound) and some tainting carboxylic acids (derived from a starch factory) were detected.

KEYWORDS

Terpenoids; algae; off-flavour; thermal desorption; GC/MS; GC/FTIR; $^1$H NMR.

INTRODUCTION

The structure determination of off-flavour and related compounds is of great importance, because the configuration and conformation of those compounds primarily takes account for their sensory activity (Polak et al., 1978; Ohloff, 1981; Gerber, 1968, 1983).

Combination of the gas chromatography/mass spectrometry (GC/MS) with SNIFF and TASTE detection was used for the identification and determination of previously unknown tainting substances in water (Mallevalle and Suffet, 1987; Veijanen, 1990). Such identification was based on mass spectral and GC retention time comparison with model substances. However, model compounds are not always available and, in many cases, structural isomers give almost identical mass spectra. Therefore, many off-flavour substances remain incompletely identified, especially in the case of terpenoid compounds (Martin, 1987). IR and $^1$H NMR spectroscopies can be useful to distinguish those isomers and ring compounds which give similar mass spectra (Werkhoff et al., 1990).
To obtain the chemical structures of odorous compounds GC/FTIR/MS combined with sensory GC analysis and micro $^1H$ NMR spectroscopy have formed valuable techniques. The low sensitivity, the greatest disadvantage of NMR methods in comparison with IR and mass spectroscopy, can be partly overcome by suitable sample preparation and measuring techniques to achieve a detection level which is practical for fractions originated from natural samples (Werkhoff et al., 1990; Veijanen et al., 1988; Kolehmainen et al., 1989, 1990). Infrared spectroscopy reveals the functional groups (e.g., hydroxyl, carbonyl, nitrogen containing groups) which are not so easily seen by $^1H$ NMR (Herres, 1984; Schreier et al., 1984; Schomburg et al., 1984).

**MATERIALS AND METHODS**

Odorous water samples were taken from Lieksa River (Eastern Finland) in March 1988 and August 1988 and Ahtävää River (Western Finland) in August 1990. Algal samples were from Ahtävää River and also studied were blue green algae collected from different lakes in Finland.

Water samples (0.2-1 litre) were passed by suction through the adsorbent tubes (containing 200 mg of Tenax TA). In some cases, an open-stripping method was used (Deprez et al., 1986). The stripping gas was purified nitrogen 50 ml/min at the room temperature for 1-2 hours and the adsorbent tube was filled with Molecular Sieve (5Å). Algal samples were freeze dried and placed into a glass tube (8 x 0.5 cm). The small desorption injection technique (Veijanen et al., 1983; Veijanen, 1990) was used to introduce the samples into the gas chromatograph. Desorption temperature was 40-120°C (in most cases 100°C) and the flow rate (of the desorption gas = carrier gas He) 1 ml/min. The applied desorption times varied from 5 to 20 minutes.

Some freeze-dried algal samples (0.46 g) were also extracted in a Soxhlet device with 120 ml of a solvent mixture of hexane-acetone-diethyl ether-petroleum ether (9:5.5:2.5:1) for six hours. The solvent was concentrated by evaporation to 1 ml and the concentrate was cleaned up by liquid chromatography (LC) using a florisil column (1 g): The 60/100 mesh florisil PR (Fluka) was activated by heating at 160°C for 24 hours then deactivated with 1.25 % of water. The compounds were eluted out of the column with 4 ml of hexane, then with 10 ml of hexane-diethyl ether (9:1), with 10 ml of hexane-diethyl ether (5:5) and at last with 10 ml of diethyl ether. All eluates were concentrated with nitrogen stream to a low volume.

A gas chromatograph (HP 5890) fitted with a wide bore capillary column (HP-5, 25 metre long, 0.53 mm i.d. and 2.65 μm film thickness) was used preparatively to collect the compounds of interest for NMR identification. The gas chromatograph was equipped with a flame ionization detector (FID) and with an infrared detector (IRD, HP 5965A). In the GC/FTIR system, the column was split after IR-registration into two columns, one for FID and the other for sniffing or for fraction collection. Mass spectra were obtained either by the use of a mass selective detector (HP 5970) or a mass spectrometer (Varian MAT 212).

Samples for $^1H$ NMR were collected from the GC fractions directly into ampoules containing about 100 μl of deuterated chloroform (CDCl$_3$), which had previously been found to be a suitable NMR solvent for samples with terpenoid structures and without aromatic moieties. After sufficient fraction collection, about 5 μg, the samples were transferred to 5 mm o.d., 2 mm i.d. thick wall NMR sample tubes. Proton NMR spectra were measured on a JEOL GSX-270 spectrometer at 270 MHz. Solvent signals were used as references (Veijanen et al., 1988; Kolehmainen et al., 1989).

**RESULTS AND DISCUSSION**

The water in the Ahtävää River had an unpleasant earthy, manure odour. Geosmin was found (30-40 ng/l), but the presence of this compound could not account for...
the whole nature of the odour. Sensory GC analysis showed that the unpleasant odours were associated with some terpenoid hydrocarbons (five isomers, molecular weight 164, C_{10}H_{16}) which eluted before geosmin (molecular weight 182), and sesquiterpene alcohols (molecular weight 222) which eluted after geosmin. Alkyl substituted benzenes were also detected in the retention time area of these alcohols. In addition, a compound with an interesting mass spectrum with main peaks at m/z 133 and 151 was found. The spectrum is not yet interpreted; however, it might be an aromatic, nitrogen containing acidic compound. Its IR spectrum was not yet obtained. The same compound was recently found from groundwater with the same type of manure odour. Sulfur compounds were also found in small concentrations in this river. Studies are continuing in efforts to identify the exact structures of the hydrocarbons and alcohols responsible for the river odour. Preliminary, Gerber (1968) has suggested several bicyclic structures to the C_{10}H_{16} (argosmins) to the hydrocarbons formed by elimination of water from geosmin. The present hydrocarbons could be the same compounds, although odours of these compounds have not been reported earlier.

The rancid odour of the water was found to be caused by carboxylic acids that were present in abundant quantities. A factory was observed to leak starch into the river and as starch is known to decompose to carboxylic acids this was considered the source of the problem. The amount of hexanoic acid (unpleasant, manure odour) was especially significant. Van Gemert and Netterbreijer (1977) reported the Threshold Odour Concentration (TOC) for hexanoic acid in water to be 3 mg/kg.

Algal samples gave the same terpenoid compounds as waters; the amounts of these compounds were, however, greater than in the water samples. When the algae were extracted by solvent, many volatile terpenes of interest which elute before geosmin, were lost during the concentration/evaporation process. Cold hexane shake of algal samples, however, showed that the volatiles existed in the original algae.

The Lieksa River has odour problems, especially associated with sulfur containing compounds. There is a mill that manufactures cardboard. The samples for study were taken upstream from the mill, from the wastewater clarifier, and from wastewater that was run to the river. The stripping method was used to analyse for the sulfur compounds more precisely. Many sulfur compounds were found only in the wastewater samples. Dimethyl disulfide and methyl isothiocyanate were found in concentrations of 1-10 μg/l each. Other identified sulfur compounds were: hydrogen sulfide, carbon disulfide, dimethyl sulfide, thiophene, methylthiophenes, methylpropyl sulfide and dimethyl trisulfide.

Many terpenoid compounds were also found in the Lieksa River. The same compounds occurred in both wastewaters, but the concentrations were greater in the wastewater running to the river than in the clarifier wastewater. Only the concentration of methyl isothiocyanate decreased in the pool after the clarifier. In general, the concentrations of off-flavours were greater in August than in March (Veijanen, 1990). Aeration tests showed that even a short period of aeration decreased the amounts of odorous substances (Mononen, 1989).

In the IR studies, optimization of the instrument for sensitivity (below 50 ng) was critical. Column combinations (the wide bore column was too thick for the flow cell transfer lines), and gas flows (make up and split systems must all fit together) are critical factors. Thermal desorption from freeze dried algae and normal injection from extracted algal samples, however, all gave good IR spectra at a level of about 10 ng for terpenoid compounds. Many of these IR spectra have not yet been interpreted. Figure 1 shows the FTIR spectrum of geosmin, obtained from an Oscillatoria species by thermal desorption.

The FTIR spectrum of geosmin (Fig. 1) was quite similar to the optical IR spectra of geosmin recorded from AgBr disk and from CCl₄ solution (Gerber, 1979).
NMR studies were made mainly using model compounds [camphor, 2'-methylisoborneol (MIB) and 2,6-dimethylcyclohexanol isomers] in searching for the detection limits of the method. Geosmin was collected from algae by preparative gas chromatography for the NMR studies.

When the concentration of the substrate was very low (= or < 5 μg/100 μl), the solvent impurity signals in some cases interfered seriously. This problem was reduced by using small sample sizes (capillary tube) and/or by purifying the solvent by preparative GC. Reliable proton NMR spectra were obtained from 5 μg of terpenoid material. 1H NMR spectrum of MIB and camphor clearly showed signals indicative of methyl groups and some of the proton signals originating from the bicyclic skeleton were also well separated. The 1H NMR spectrum of geosmin (11 μg) was not as well defined as that of some other model compounds.

In the 1H NMR spectrum of commercial 2,6-dimethylcyclohexanol, the protons epimeric to the hydroxyl groups of the cis,cis-, cis,trans- and trans,trans-isomers all gave well separable patterns. Thus, these signals are very characteristic of structural isomerism (Eliel et al., 1962). The cis,cis-isomer importantly has an earthy odour resembling that of geosmin (Polak et al., 1978).

Gas-phase 1H NMR spectroscopy offers a new promising approach in this field of research. Harris and Rao (1983) e.g. have shown that there exist considerable differences in the NMR measuring techniques between the gas and liquid phases. In order to develop the gas-phase methodology for the off-flavour analyses, some experiments with model compounds were conducted. Camphor and MIB were collected, as GC fractions, directly into empty NMR tubes cooled by liquid nitrogen. A problem encountered was that camphor tended to sublimate at the top of the NMR tube during measurements at elevated temperatures, and the spectrum got weaker with time and eventually disappeared. To limit the sample space to the measuring area, a tight Teflon plug was used. This improvement slowed down the sublimation of camphor, but did not prevent it totally (Kolehmainen et al., 1990). Special micro cuvettes may be worth testing.
CONCLUSIONS

The techniques of combined GC/FTIR/MS, together with sensory GC analysis and micro NMR spectroscopy, have formed versatile and informative methods for the structural analysis of off-flavour compounds in water supplies. The sensitivity of both IR and NMR spectroscopy can be increased by the application of improved instrumental technique.

The GC fractions collected directly into the NMR solvent gave promising results. The mixture of 2,6-dimethylcyclohexanol isomers gave resolved signals which suggested that the fractionation of structural isomers may not always be necessary. Gas-phase $^1$H NMR spectroscopy forms an interesting alternative in the study of off-flavour compounds. Unfortunately, there exist only very few previous results from the application of this technique.

ACKNOWLEDGEMENTS

We are grateful to P. J. Salovaara for designing and constructing the equipment and to Kaarina Sivonen and Raija Luukkainen (University of Helsinki) for providing the algae samples. Ilpo Korhonen helped in the interpretation of mass spectra. We are grateful to Wolfgang Korth from CSIRO, Australia, for valuable advice. The Water and Environmental District of North Karelia and the City of Pietarsaari are acknowledged for financial support.

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