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ISOLATION AND IDENTIFICATION OF ODOUROUS COMPOUNDS FROM A LAKE SUBJECT TO CYANOBACTERIAL BLOOMS

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ABSTRACT

Buffalo Pound Lake in southern Saskatchewan, Canada, is subject to heavy blooms of blue-green algae, mainly Anabaena, Oscillatoria and Aphanizomenon. The occurrence of any of these species is usually followed by the appearance of pronounced tastes and odours. Water samples were extracted using a Likens- Nickerson or continuous liquid-liquid apparatus and capillary GC of the extracts showed that a large number of compounds are always present although the taste and odour vary in intensity. Many of these compounds have been identified using capillary GC-MS. Geosmin, which imparts a muddy flavour to water, was detected but methylisoborneol was absent. Other compounds present in the water included alkanes, alkylbenzenes, acetophenone, cyclohexanone, phenylacetonitrile, and α-pinene, some of which could affect the palatability of the water. Although some water samples had a musty odour extracts were frequently dominated by a pungent, smoky or tobacco flavour consistent with the presence of β-cyclocitral which was also indicated by GC-MS.

KEYWORDS

Blue-green algae, geosmin, alkanes, alkylbenzenes, ketones, β-cyclocitral, Likens-Nickerson extraction, liquid-liquid extraction.

INTRODUCTION

Buffalo Pound Lake is located in the southern portion of the province of Saskatchewan in Western Canada and is the source of the municipal water supply for two nearby cities. Blooms of blue-green algae typically occur during the summer months and are usually followed by the appearance of a characteristic "muddy" or "musty" taste and odour in the water. Although this taste and odour disappear an unpalatable flavour persists in the water into the winter. Off-flavours in water which coincide with the proliferation of microorganisms are generally attributed to geosmin and 2-methylisoborneol (MIB), metabolites of actinomycetes (Gerber, 1968, 1969) and blue-green algae (Tabachek and Yurkowski, 1976), although several other compounds are also known to affect the quality of water similarly (Montiel, 1979).

The analysis of water for taste- and odour-causing compounds is usually undertaken when the problem is at its worst. However, we were also interested in the compounds which might be responsible for the off-flavour which was still detectable in lake water well after the algae blooms had subsided. Since the water was being analyzed up to four months after a bloom had disappeared the quantity of organic compounds was expected to be low. Thus it was desirable to use methods that would allow processing of relatively large water samples (4.7 l), give good recovery, and avoid formation of artifacts. From previous experience (Blok and co-workers, 1983) it appeared that continuous liquid-liquid extraction, or Likens-Nickerson extraction (Likens-Nickerson, 1968), using dichloromethane as solvent, would be suitable.

EXPERIMENTAL

Water samples were collected from the intake to the Buffalo Pound Lake treatment plant, 50 mg/l of ascorbic acid added (Vogt and Leimer, 1977) and the samples stored at 4° in high density polyethylene containers. These were cleaned by flushing with hot water for 30 minutes and rinsing with methanol, and then with lake water on the day of collection.

Finished water samples were collected in glass containers, treated with sodium metabisulfite (5 mg/l) and ascorbic acid (50 mg/l) and stored at 4°. The bottles were previously cleaned with chromic acid, rinsed with distilled water and then flushed with water sample on the day of collection.

The dichloromethane used in the extraction of the water was purified by distillation through a vacuum-jacketed Vigreux column (70 cm) from a water bath at 45-47°, the same conditions used for initial concentration of the extracts. The distillate was distilled again and concentrated to the same level as required for the water extracts. The still residues from the first and second distillations, were examined by capillary GC (Fig. 1 a,b) and the residue from the second distillation concentrated further to determine the degree of concentration necessary to show the presence of impurities (Fig. 1c). Pentane was purified similarly by distilling from a bath at 40 - 42°.

The Likens-Nickerson extractions were carried out for 24 h on duplicate samples of water (4.7 l) using 60 ml solvent for each sample. The extracts were cooled in a freezer until excess water solidified and further dried with anhydrous $MgSO_4$ (2 g) and rapidly filtered into a flask with a drawn-out tip using a measured amount of solvent (15 ml) to rinse each flask and the filter funnel. The extracts were then concentrated as described for distillation of solvent to leave a residue of 1-2 ml. This was transferred to a graduated 4 ml threaded micro-volumetric flask (Wheaton Scientific, Millville, N.J., U.S.A.) and further concentrated to 0.5 ml through a 25 cm Vigreux column. The concentrated sample was sealed in the distillation flask using a screw cap and a Tuf-Bond Teflon-Silicone disc (Chromatographic Specialties, Brockville, Ontario, Canada).

Liquid-liquid extraction with dichloromethane was done in duplicate in an apparatus which contained 1.5 l of water sample. The water was replaced with a fresh sample and extracted without changing solvent so that the total volume treated was the same as in the Likens-Nickerson extraction. Addition of solvent was necessary from time to time and the volume required was noted. The extract was processed as described previously for the Likens-Nickerson procedure.

Continuous liquid-liquid extractions with pentane (70 ml) were done in an apparatus containing the entire sample which was stirred magnetically during the extraction period (24 h.). The extract was treated as described previously.

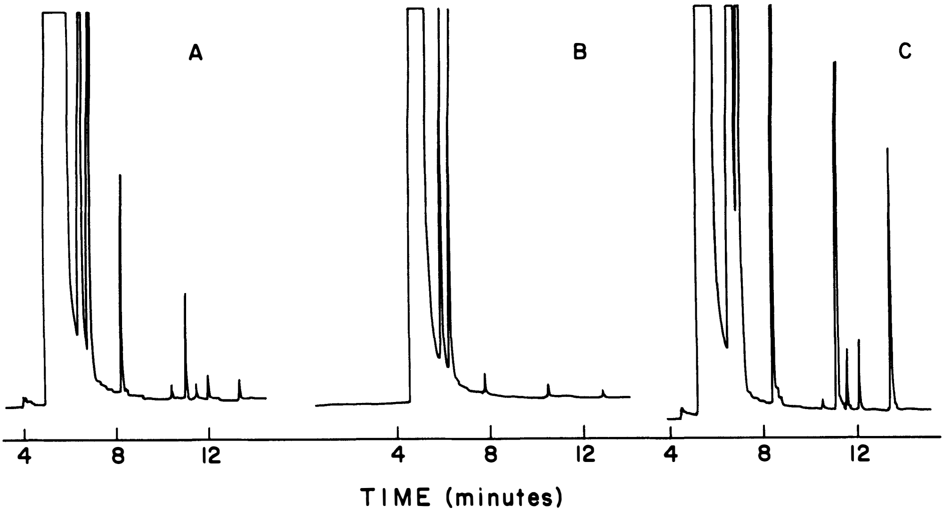

Fig. 1. Capillary gas chromatogram of: a) residue from first distillation of dichloromethane (conc. x200); b) residue from second distillation of dichloromethane (conc. x600); c) as in b but con. x 3800.

All glassware was cleaned in chromic acid and rinsed with distilled water before use and then rinsed with redistilled dichloromethane between experiments. The extractions and distillations were protected with a guard tube containing activated charcoal to minimize sorption of impurities from laboratory air.

Extracts were analyzed on a Hewlett Packard 5700 gas chromatograph fitted with a flame ionization detector and a capillary injection system (18740 A). The capillary column was 60 m x 0.32 mm fused silica Durabond DB-1 (J and W Scientific Inc., Rancho Cordova, Calif.) with helium as carrier gas (19.5 cm/sec at 100°). The injector was maintained at 200° and the detector at 250°. The column was programmed at 20°/8 min., 4°/min. to 230° and held 30 min. The splitless injection technique using 1 μl injections and a 30 sec. delay was used.

Mass spectra were obtained with a Finnigan 4000 quadrupole GC-MS equipped with a 60 m x 0.32 mm Durabond DB-5 fused silica column. The column was connected directly to the ionizer of the mass spectrometer and was programmed as above. The injector port was kept at 250° and the flow rate of carrier gas (helium) was 40 cm/sec at 150°. Injections were made in the splitless mode with a delay time of 0.8 min. The mass spectrometer was scanned from 41-500 a.m.u. every second and the data were processed by an Incos 2300 data system.

RESULTS AND DISCUSSIONS

When using extraction techniques it is essential to show that the solvent contains no impurities which might interfere with the analysis. The chromatogram of the residue from the first distillation of dichloromethane is shown in Fig. 1a and is equivalent to a 200-fold concentration. Redistillation of the solvent to represent concentration by a factor of 600 indicated only two relatively large peaks on the solvent tail (Fig. 1b). These two peaks were identified by GC-MS as butan-2-one and chloroform and do not interfere with the compounds extracted from the water. Further concentration (3800 times) of the residue from the second distillation of

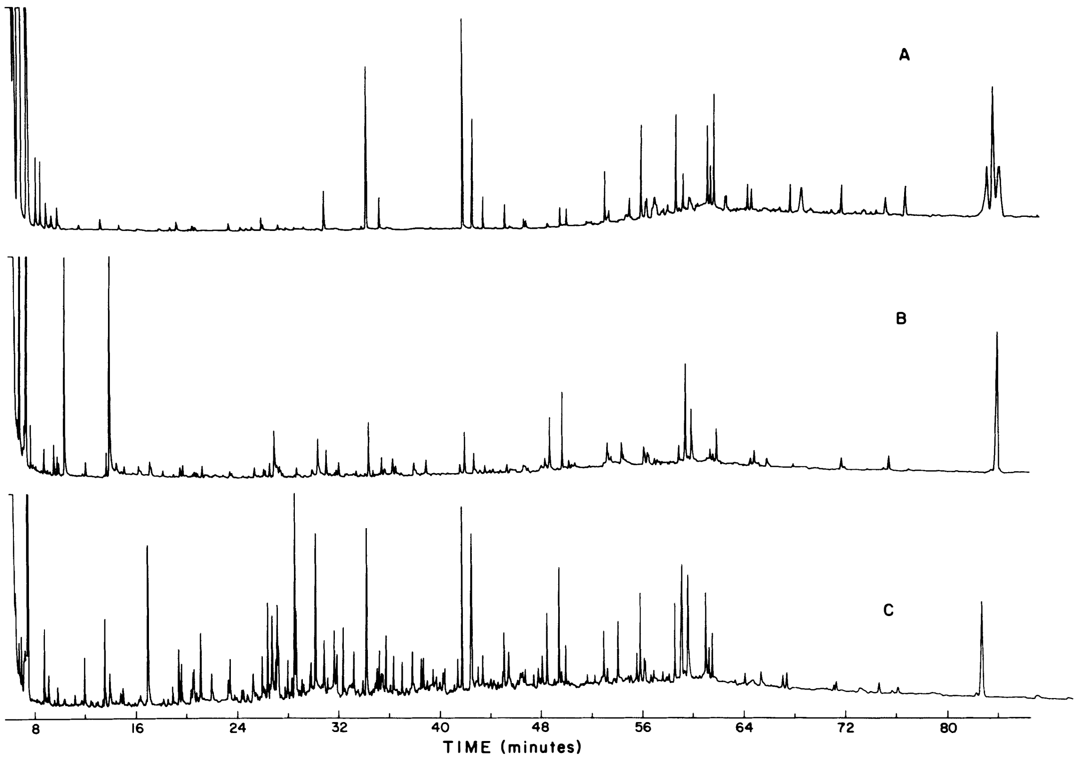

Fig. 2. Capillary gas chromatogram of: a) pentane extract (0.1 ml) of lake water Dec., 1981; b) subsequent dichloromethane extract (0.5 ml); c) extract with dichloromethane only (0.5 ml).

the solvent revealed that several impurities were still present (Fig. 1c), however the extracts were never concentrated more than 600x so that the only extraneous peaks were those shown in Fig. 1b. GC of double-distilled pentane also indicated that the only interfering impurities occurred on the solvent tail as indicated in Fig. 2a.

A water sample obtained in December, 1981, was continuously extracted with pentane and then with dichloromethane. The capillary gas chromatograms of the two extracts are shown in Fig. 2a,b and, apart from the two large peaks near the front of chromatogram 2b, contain peaks of similar retention times, but of different sizes. This indicates that both solvents remove essentially the same organic compounds from the water. However, the pentane extract (Fig. 2a) was concentrated to 0.1 ml while the dichloromethane extract was only reduced to 0.5 ml. Because of the sizes of the respective extractors the water sample could be extracted in a single batch when using pentane, but had to be processed in three batches with dichloromethane as solvent. Since all extracts were carried out for 24 h it could be argued that the water was extracted three times longer with dichloromethane. However, the pentane extract was concentrated to one fifth of the dichlormethane extract and taking these factors into consideration it is apparent from Fig. 2a,b that dichloromethane removes more material from the water. This is more clearly indicated by comparison with Fig. 2c which is the chromatogram of an extract (0.5 ml) obtained by continuous extraction of a second sample of the same water with dichloromethane only.

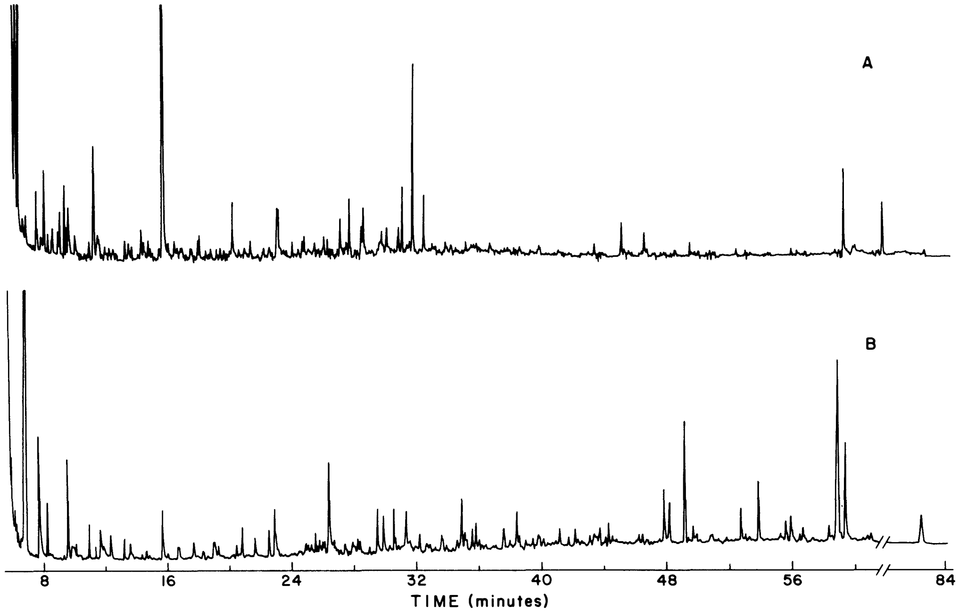

Fig. 3. Capillary gas chromatogram of Dec. 1981 lake water extracted by the a) Likens-Nickerson procedure and then by b) the liquid-liquid procedure.

The two large peaks on the solvent tail in Fig. 2b are the two impurities previously shown in Fig. 1b. The peaks at approximately 10 min and 14 min in Fig. 2b were identified as butan-2-ol and pentan-1-ol, respecitvely. These compounds have not been found in other extracts of this, or any other, lake water sample and are regarded as contaminants. In Fig. 2c only one solvent impurity (chloroform) is present because a different batch of dichloromethane was used which was free of butan-2-one. The large peaks immediately following the solvent in Fig. 2a are impurities, such as hexane which can not be removed completely from pentane by distillation, however these do not interfere with the compounds extracted from the water.

The compounds identified by GC-MS analysis of the extract obtained by continuous extraction with dichloromethane only are listed in Table 1 in order of elution. These were initially identified by comparison with the mass spectra in the computer library and then a series of mixtures containing the indicated compounds were examined using the same GC-MS conditions. Following this a sample of the extract was spiked with one of the indicated compounds (toluene) and reanalyzed by GC-MS. The increase in the size of the toluene peak indicated that this compound had been correctly identified and it was then possible to confirm the identities of many of the components based on retention times relative to toluene and corresponding mass spectra.

GC-MS analysis of extracts of the water sample obtained by continuous extraction first with pentane and then with dichloromethane indicated that each contained compounds previously isolated by extraction with dichloromethane alone (Table 1). However, in addition, octanal, tetradecane, hexadecanoic acid, heneicosane and docosane were detected in the second dichloromethane extract (Table 1). Fewer

TABLE 1 Compounds Recovered from Lake Water (Dec., 1981) by Continuous Liquid-Liquid Extraction

	Solvent		
Compounds	CH_2Cl_2 [1]	Pentane	CH_2Cl_2 [2]
Acetic Acid	+		
4-Methylpentan-2-one	+		
Toluene	+	+	+
Hexanal	+	+	
3-Methylheptan-2-one	+		
m-xylene	+	+	+
Cyclohexanone	+		
o-xylene	+	+	
Heptanal	+	+	
α-Pinene	+	+	
Benzaldehyde	+		
Alkylbenzene MW120	+	+	+
Octanal			+
1,3,5-Trimethylbenzene	+	+	
Decane	+		
1,4-Dichlorobenzene	+		+
1,2,3-Trimethylbenzene	+	+	
Limonene	+		
Acetophenone	+	+	+
2-Ethyl-1,3,dimethylbenzene	+		
Undecane	+		
Nonanal	+	+	+
1,2,4,5-Tetramethylbenzene	+		+
Naphthalene	+	+	+
Decanal	+	+	+
4-Chloroacetophenone	+		
Nonanoic Acid	+		
Biphenyl	+	+	+
Tetradecane			+
Diphenylether	+	+	+
2,6-Di-*t*-butyl-*p*-benzoquinone	+	+	+
Diethylphthalate	+	+	+
1-Hexadecene	+		+
Heptadecane	+	+	+
Tetradecanoic Acid	+		
Pristane	+		
Octadecane	+	+	+
Diisobutylphthalate	+		+
1-Hexadecanol	+		+
Nonadecane	+	+	+
Dibutylphthalate	+	+	+
Eicosane	+	+	
Phthalate Ester	+		+
Hexadecanoic Acid		+	+
Heneicosane			+
Docosane			+
Diisooctylphthalate	+	+	+

[1] Liquid-Liquid extract with this solvent only.

[2] Liquid-liquid extract of water previously extracted with pentane.

TABLE 2 Compounds Recovered from Lake Water (Dec, 1981) by Likens-Nickerson Extraction and then by Continuous Liquid-Liquid Extraction with Dichloromethane

Compounds	Likens-Nickerson	Liquid-Liquid
Acetic acid		+
Benzene		+
Toluene	+	
Hexanal		+
m-Xylene		+
Cyclohexanone		+
Heptanal		+
Benzaldehyde	+	+
Alkylbenzene MW120		+
Decane		+
Octanal		+
1,4-Dichlorobenzene	+	+
Acetophenone	+	+
Undecane		+
Nonanal		+
Decanal	+	+
2,6-Di-t-butyl-p-benzoquinone	+	+
Nonanoic acid		+
Tetradecane		+
Diethylphthalate	+	+
Heptadecane		+
Tetradecanoic acid	+	+
Octadecane		+
Diisobutylphthalate		+
1-Hexadecanol		+
Nonadecane		+
Dibutlyphthalate	+	+
Eicosane		+
Heneicosane		+
Docosane		+
Phthalate Ester		+
Diisooctylphthalate	+	+

compounds were identified when the water was extracted consecutively with pentane and dichloromethane compared to extraction with dichloromethane alone (Table 1). This may be due to the smaller amounts, or even absence, of several components in the consecutive extracts as indicated by capillary GC (Fig. 2).

Several of the compounds isolated from the water are commonly found in plastics, for example, the phthalates and 2,6-di-t-butyl-p-benzoquinone. These could have been leached from the plastic containers used to store the water samples although phthalates are found in most water bodies presumably due to their widespread use. The 2,6-di-t- butyl-p-benzoquinone is an antioxidant and was found in very small amounts but has also been detected in finished water stored in glass (Table 4). The aldehydes hexanal to decanal, and benzaldehyde, and the ketones, 4-methyl-pentan-2-one, 3-methylheptan- 2-one, cyclohexanone, acetophenone and chloro-acetophenone have not been reported as algal products. Neither have the terpenes, α-pinene or limonene, although other terpenoid compounds were identified in *Cyanidium caldarium* cultures by Juttner (1976, 1979) and Juttner and Wurster (1979). These authors identified 1,4-dichlorobenzene in *C. caldarium* as well as some alkylbenzenes, however Buffalo Pound Lake water contains several alkylbenzenes

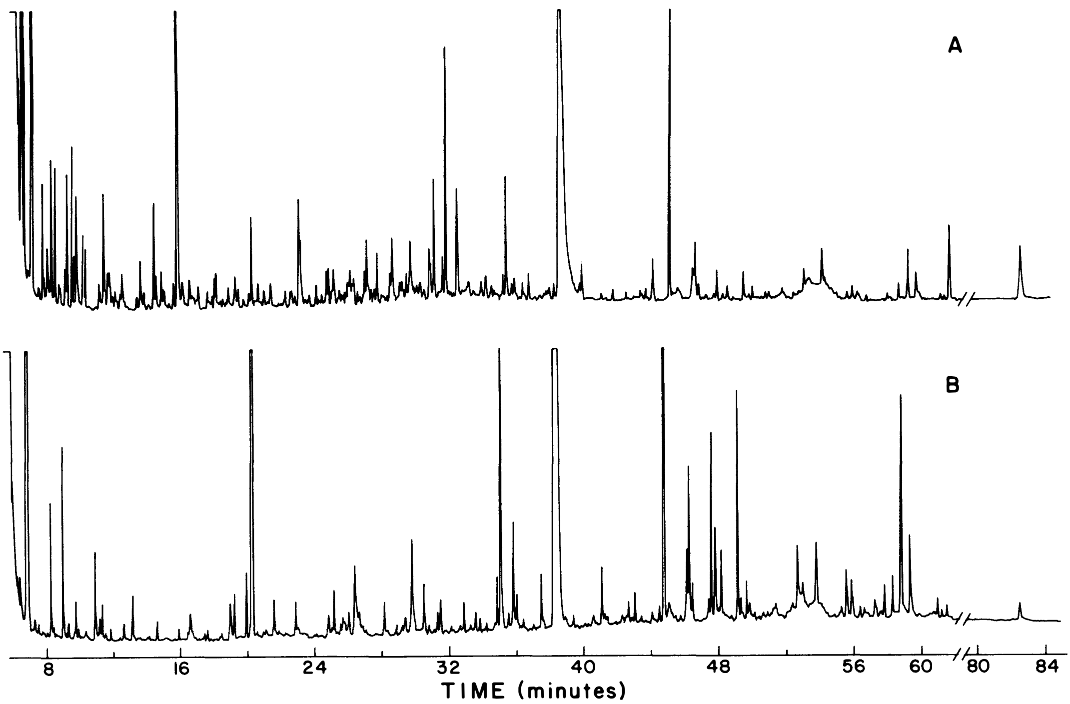

Fig. 4. Capillary gas chromatogram of Aug. 1981 lake water extracted by: a) Likens-Nickerson method; b) liquid-liquid extraction.

(Table 1) not reported by Jüttner and Wurster (1979). The only branched hydrocarbon isolated from lake water was pristane (2,6,10,14-tetramethylpentadecane) which has been identified as a minor constituent of only one blue-green alga, Anacystis nidulans (Oro and coworkers, 1967), whereas 7-, 8- and 4- methylheptadecanes have been obtained from several blue-green algae (Han and coworkers, 1968; Juttner, 1976). The majority of the other hydrocarbons found in the water have previously been obtained from blue-green algae (Oro and coworkers, 1967; Han and coworkers 1967; Juttner, 1976; Juttner and Wurster, 1979). Two other compounds isolated from lake water are biphenyl and diphenyl ether (Table 1). These have not been detected in finished water stored in glass containers and would appear to be leached from the plastic containers used to store the lake water samples.

In the Likens-Nickerson method only those compounds which are volatile in steam would be expected to accumulate in the solvent and hopefully yield an extract less complex than that obtained by continuous liquid-liquid extraction and hence be easier to identify by capillary GC-MS. Comparison of the chromatograms of extracts of the December water sample obtained by the Likens-Nickerson method (Fig. 3a) and the continuous liquid-liquid methods (Fig. 2c) indicates little similarity between the early portions of these chromatograms where the most volatile compounds would be expected. When the water sample previously extracted by the Likens-Nickerson method was reextracted continuously with dichloromethane the extract (0.5 ml) gave the chromatogram in Fig. 3b. It was expected that compounds which had not been

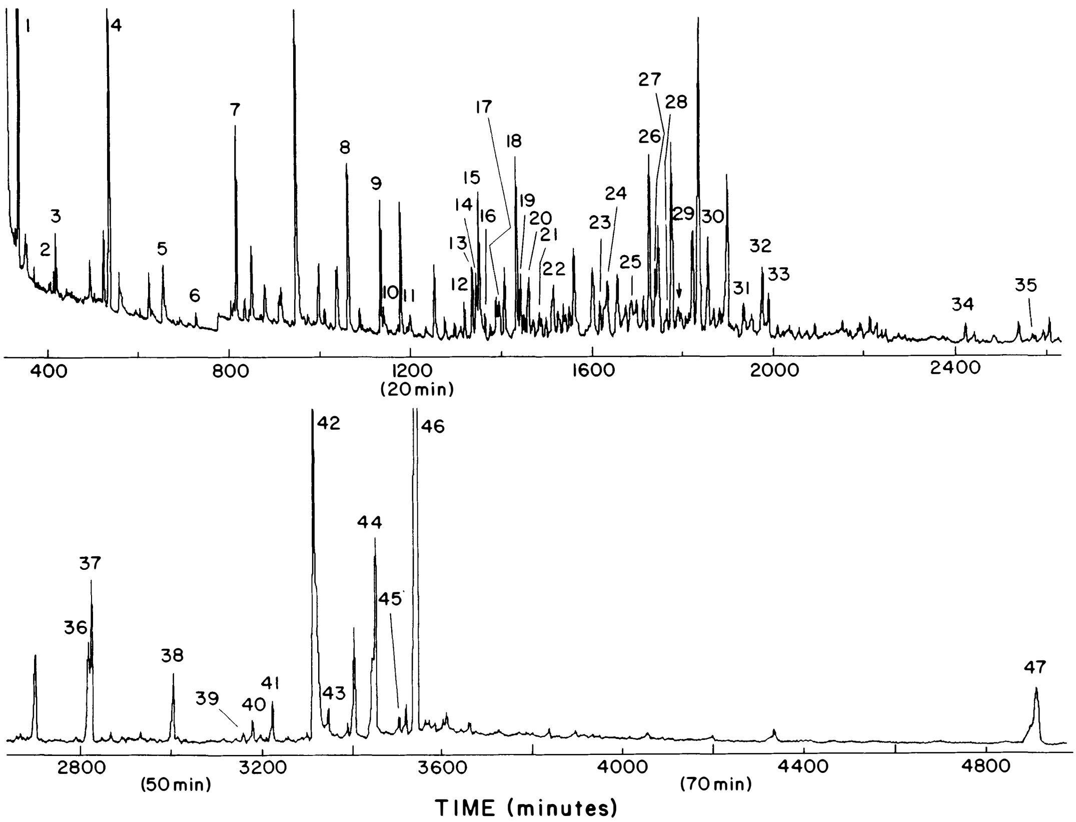

Fig. 5. GC-MS analysis of Likens-Nickerson extract of finished water (Aug., 1981).

removed by the Likens-Nickerson method would be recovered in the liquid-liquid extract, however, comparison of the chromatograms in Fig. 3 and Fig. 2c shows that this did not occur.

The compounds identified in the Likens-Nickerson and subsequent liquid-liquid extracts are listed in Table 2 in order of elution from the GC column. Only ten of the compounds in the former extract could be identified in comparison to thirty in the latter. The capillary gas chromatograms of these extracts (Fig. 3a,b) show that a larger number of early-eluting components are present in the Likens-Nickerson extract. Inadequate chromatographic resolution of these components may account for the failure to identify many of the compounds by GC-MS. The Likens-Nickerson extract also contains relatively small amounts of the later-eluting components which may not have been sufficient to give good mass spectra. Comparison of the results (Table 1, 2) indicates that of the methods tested, continuous liquid-liquid extraction with dichloromethane gives the best over-all recovery and identification of compounds in the water sample.

Extracts of a lake water sample from Aug. 31, 1981 (three weeks after appearance of a bloom) obtained by Likens-Nickerson and continuous liquid-liquid extraction using

TABLE 3 Compounds Recovered from Lake Water (Aug 31, 1981) by Continuous Extraction with Dichloromethane

Benzene	2,6-Di-t-Butylphenol
Toluene	1-Hexadecene
Hexan-2-one	Diethylphthalate
m-Xylene	1-Tetradecanol
Cyclohexanone	Heptadecane
Heptanal	Tetradecanoic acid
1,1,2,2-Tetrachloroethane	Octadecane
Benzaldehyde	Diisobutylphthalate
Octanal	1-Hexadecanol
1,4-Dichlorobenzene	Nonadecane
Acetophenone	Dibutlyphthalate
Undecane	Eicosane
Nonanal	Heneicosane
Decanal	Docosane
4-t-Butylphenol	Phthalate ester
Tetradecane	Diisooctylphthalate
2,6-Di-t-butyl-p-benzoquinone	

dichloromethane gave the chromatograms in Fig. 4. These show many peaks with similar retention times although there are variations in corresponding peak sizes. Comparison of the two chromatograms confirms that the Likens-Nickerson method recovers more of the low-boiling material and less of the high-boiling compounds than the liquid-liquid method. GC-MS analysis of the liquid-liquid extract indicated that essentially the same compounds were present in the August 31 sample (Table 3) as in the December sample (Table 1,2). The main differences are that the August water sample contained two relative large peaks eluting at approximately 20 min and 38 min (Fig. 4). These were identified as 1,1,2,2-tetrachloroethane and 4-t-butylphenol, respectively. The latter is an anti-oxidant and was probably leached from the plastic storage container as it was not found in the Likens-Nickerson extract of finished water (stored in glass) from the same period (Table 4). Tetrachloroethane was present as a minor component in the finished water extract (Table 4) and its presence as a large component of the raw water again must be attributed to leaching from the plastic container. The Likens-Nickerson extract evaporated while stored in the refrigerator and some of the more volatile compounds were lost. Consequently, only a few compounds could be identified and these were the same as found previously (Table 2).

A sample of finished water from August 31, 1981, was extracted by the Likens-Nickerson method and the GC-MS analysis of the extract indicated a large number of components (Fig. 5). Of the many compounds identified (Table 4) several corresponded to those previously isolated from December and August raw water samples (Table 1 and 3, respectively) by continuous liquid-liquid extraction. The main differences were the presence of bromodichloromethane and dibromochloromethane in the finished water which are formed during the disinfection process. [The presence of chloroform in the extract (Table 4) is probably due to a combination of impurity in the solvent and formation during the chlorination of water]. Geosmin was also identified in this extract (Fig. 5, no. 34) as well as several new ketones, viz., 3-methylbutan-2-one, 4-methylpentan-2-one, and 3-methylcyclopent-2-en-1-one. Phenylacetonitrile was detected in this extract and the lower homologue benzonitrile was also indicated, however, an authentic sample was not available to confirm the latter compound.

The Likens-Nickerson extract of the finished water sample from August had a

TABLE 4 Compounds Recovered from Finished Water (August 31, 1981) by Likens-Nickerson Extraction with Dichloromethane.[1]

1.	Chloroform	25.	Alkylbenzene MW134
2.	Benzene	26.	Undecane
3.	3-Methylbutan-2-one	27.	Nonanal
4.	Bromodichloromethane	28.	1,2,4,5-Tetramethylbenzene
5.	4-Methylpentan-2-one	29.	Phenylacetonitrile
6.	Toluene	30.	Alkylbenzene MW134
7.	Dibromochloromethane	31.	Naphthalene
8.	m-xylene	32.	Dodecane
9.	o-Xylene	33.	Decanal
10.	Heptan-2-one	34.	Geosmin
11.	1,1,2,2-Tetrachloroethane	35.	2,6-Di-t-butyl-p-benzoquinone
12.	n-Propylbenzene	36.	Dibutylphthalate
13.	Benzaldehyde	37.	1-Hexadecene
14.	Alkylbenzene MW120	38.	Heptadecane
15.	3-Methylcyclo-pent-2-en-1-one	39.	Anthracene
16.	1,3,5-Trimethylbenzene	40.	Tetradecanoic Acid
17.	Alkylbenzene MW120	41.	Octadecane
18.	1,2,4-Trimethylbenzene	42.	1-Hexadecanol
19.	Decane	43.	Nonadecane
20.	Octanol	44.	Dibutylphthalate
21.	1,4-Dichlorobenzene	45.	Eicosane
22.	1,2,3-Trimethylbenzene	46.	Hexadecanoic Acid
23.	Alkylbenzene MW134	47.	Diisooctylphthalate
24.	Acetophenone		

[1] Numbers refer to peaks in Fig. 5..

pungent, smoky odour although the water itself had the musty aroma associated with geosmin. GC-MS analysis of the extract indicated a small peak at scan number 1793 (Fig. 5) containing all of the ions characteristic of β-cyclocitral. However, an authentic sample was not available to confirm the identity of this component.

The absence of geosmin in the August lake water sample may be due to microbial action during the storage period (3 months at 4°) since loss of geosmin can be prevented by addition of mercuric chloride (McGuire and coworkers, 1982). Very small amounts (50 pg) of geosmin can be detected by monitoring for the base peak, m/z 112 (Yasuhara and Fuwa, 1979). This was acheived when geosmin was the only compound present. In our case several of the compounds extracted from the water had ions at m/z 112 in their mass spectra so that scanning for a single ion would not have given uneqivocal identification.

CONCLUSION

Small amounts of organic compounds can be recovered from water samples more efficiently by continuous liquid-liquid extraction using dichloromethane rather than pentane as solvent. Likens-Nickerson extraction with dichloromethane removed more of the low-boiling components and less of the high-boiling compounds compared to liquid-liquid extraction. The preferential removal of the more volatile compounds by the Likens-Nickerson method is not necessarily advantageous since GC-MS analysis of Likens-Nickerson extracts of raw water gave poorer identification of organics than corresponding liquid-liquid extracts. However, GC-MS analysis of a Likens-Nickerson extract of a finished water sample also led to identification of a considerable number of compounds.

Water samples obtained in August and December contained many of the same compounds but none were identified that might be responsible for the taste of the water in winter time.

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