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VOLATILE COMPOUNDS OF GREEN MICROALGAE GROWN ON REUSED WASTE WATER

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Key Word Index—*Scenedesmus* sp.; *Chlorella vulgaris*; microalgae; volatile products; reused waste water.

Abstract—The volatile compounds of two green algae, *Scenedesmus* sp. and *Chlorella vulgaris*, have been identified. These algae, which are dominant in biomass, have been harvested from a pond used for treatment of reused waste water. Volatile oils were obtained by steam distillation of the algae with a yield of 0.014–0.020% (w/w). The essential oil components were analysed by GC and GC-mass spectrometry. The major products in the oil of both species are palmitic acid in the acidic fraction (18–19%), phytol and 2-butyloctanol in the alcohol fraction (11–16%), methyl palmitate, methyl oleate, methyl linoleate, methyl stearate in the ester fraction (20–23%) and, heptadecane and 1-heptadecene in the hydrocarbon fraction (25–26%). Minor quantities of ketones and lactones were also detected.

INTRODUCTION

Studies of volatile oil components in algae grown in freshwater or in natural water are becoming of increasing interest [1–6] due to the increase in aquatic microorganisms with urbanization and industrialization [7]. Jüttner [1, 2] has reported volatile odorous excretion products of microalgae proliferating in natural aquatic environment or synthetic medium. Microalgae belonging to the Chrysophyceae, Chryptophyceae, Dinophyceae, Chlorophyceae, Bacillariophyceae and Cyanophyceae were reported to impart odours to water when blooms of these organisms occur. High concentration of β-cyclocitral has been correlated with the maximum development of *Microcystis wesenbergii*, a *Cyanobacterium* in eutrophic lakes and that of 1-heptadecene with the green algae, *Scenedesmus*, *Micractinium* and *Oocystis* [2]. The algal population can modify its environment by the production of volatile or excretion products [3, 4].

Pratt and Fong [8] were the first to isolate a substance called chlorellin which is a mixture of fatty acids from *Chlorella*. Collins and Bean [9] identified formaldehyde, acetaldehyde and butanone in the volatile oil obtained from the green microalgae, *Chlamydomonas globosa* (Chlorophyceae). Liersch [10, 11] found some monoterpenes, such as limonene, myrcene and proazulene, in the essential oils of some *Chlorella*, *Ankidostrodesmus* and *Scenedesmus* species. Velev *et al.* [12, 13] and Zolovitch and Velev [14] identified alcohols such as farnesol, 2-phenylethanol and some monoterpenes, like α- and β-pinene, ρ-cymene, limonene, δ-carene and β-phellandrene, in *S. acutus*.

Since then, many volatile products of low M_r have been found in some freshwater algae [1 and references therein, 5, 6] belonging to the Bacillariophyceae, Chlorophyceae, Cyanophyceae and Chrysophyceae. Sulphur compounds, such as dimethyl sulphide and dimethyl trisulphide have also been identified. Aldehydes and ketones have also been found in the Chryptophyceae and mono- and diterpenes in the Rodophyceae. New important groups of compounds have been detected (aldehydes, saturated and unsaturated hydrocarbons, alcohols and ketones) in algal media or in dominant species during algal blooms [1–4].

Alkenes and alkylbenzenes have been identified in *Tetraselmis* species [1]. In different strains of *Synechoccus* [2], 6-methyl hept-5-en-2-one, dihydroactinidiolide and tetrahydrogeranyl acetone were the major components of the volatile products [3]. Norcarotenoids, such as β-cyclocitral and β-ionone have also been reported in *Synura uvella*, *Microcystis* and *Aphanizomen*[1, 4].

Recently, long-chain fatty acids (n-hexadecanoic, n-octadecanoic), n-C_{26} and n-C_{28} carboxylic methyl esters, phytol and n-heptane were identified in thermal extracts of *C. fusca* and *S. obliquus* [15]. The growth of microalgae coupled to waste water treatment could therefore be a source of useful chemical products, including fatty acids, glycerol, lipids, enzymes, amines and essential oils [16]. Quite often *Chlorella* or *Scenedesmus* are found in waters

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rich in organic matter [7]. When the latter is abundant these waters can impart musty odours.

A *Scenedesmus* species and *Chlorella vulgaris* were harvested from the High Rate Algal Pond (HRAP) which is a purification system for domestic waste water treatment based on the coupled action of algae and bacteria [17]. The degradation of organic matter in these waste waters by bacteria allows the growth of algae. During each season, the treatment of waste water in HRAP exhibits a succession of microalgae of the Chlorophyceae and the algal biomass is always dominated by one species [18]. During waste water treatment in HRAP, the bloom of this species may be correlated with the water purification process in the pond [19]. We report the volatile substances of *Scenedesmus* sp. and *Chlorella vulgaris* which are the dominant species in the algal biomass of the HRAP.

RESULTS AND DISCUSSION

The volatile oil was obtained by steam distillation of fresh algae; the diethyl ether extract of the distillate gave essential oil (0.014–0.017%, w/w) with a fishy odour.

The essential oil and the fractions obtained by column chromatography (to complete identification) were analysed by GC and GC-mass spectrometry. The compounds identified by GC and GC-MS are listed with their relative peak areas in Table 1. The assignment of volatile

Table 1. Composition of essential oil from *Scenedesmus* and *Chlorella vulgaris*

Compounds	Relative peak area (%) *Scenedesmus* sp.	*Chlorella vulgaris*
Acids	17.91	18.91
Dodecanoic	0.08	0.05
Tetradecanoic	0.10	0.14
Hexadecanoic	11.20	12.60
Octadecanoic	0.09	0.10
Octadec-9-enoic	2.10	1.90
Octadec-9,12-dienoic	1.30	1.60
Octadec-9,12,15-trienoic	3.04	2.52
Alcohols	15.94	11.39
Pentadecanol	0.58	–
Hexadecanol	0.25	0.20
Octadecanol	2.53	1.38
2-butyl octanol	5.50	–
Linalool	–	0.87
Geraniol	–	0.64
Phytol	7.08	8.30
Ketones	1.05	0.91
5-Ethyl furanone-2	0.04	0.08
2-Decanone	0.10	0.04
2-Undecanone	0.16	0.10
α-Ionone	0.08	0.14
Epoxy-β-ionone	0.14	0.10
β-Ionone	0.16	0.18
2-Tridecanone	0.17	0.12
6,10,14-Trimethylpentadecan-2-one	0.20	0.15
Lactone		
Dihydroactidiolide	0.17	0.22
Esters	23.12	20.43
Methyl benzenebutyrate	0.39	0.25
Ethyl benzenepropionate	+	0.14
Methyl tetradecanoate	0.08	0.15
Methyl pentadecanoate	0.20	0.18
Ethyl hexodecanoate	0.70	0.15
Methyl hexadecanoate	9.25	8.50
Methyl octadecanoate	3.60	2.40
Methyl hexadec-9-enoate	0.08	0.09
Ethyl octadec-9-enoate	0.22	0.02
Methyl octadec-9-enoate	4.52	3.54
Methyl octadec-9,12-dienoate	3.40	4.56
Diethyl phthalate	+	+
Dipropyl phthalate	0.20	0.09
Dibutyl phthalate	+	+
Di(2-ethylhexyl)phthalate	0.48	0.36

Table 1. *Continued*

Compounds	Relative peak area (%) *Scenedesmus* sp.	*Chlorella vulgaris*
Hydrocarbons	25.86	24.74
Tetradecane	0.56	0.02
1-Tetradecane	0.15	0.09
1-Pentadecene	0.16	0.15
4-Methylpentadecane*	0.20	0.12
Pentadecane	0.69	0.50
1-Hexadecane	0.06	0.07
Hexadecane	1.02	0.14
1-Heptadecene	6.40	5.30
6-Methylheptadecane*	+	+
Heptadecane	15.60	16.90
Octadecane	0.39	0.33
Icosane	0.35	0.05
Tetracosane	0.16	0.95
Pentacosane	0.05	0.04
Heptacosane	0.07	0.08
Miscellaneous	0.36	0.32
2-Methyl,5-methoxy pyridinol	0.03	0.01
1-Acetoacetic,ethyl-cyclohexylester	0.02	—
N,*N*-bis(2-hydroxylethyl)Dodecanamide	0.04	0.05
Benzenepropanoic acid	0.08	0.10
8-Amino,2-naphthenol	0.12	0.10
5-Dodecyldihydrofuran-2-one	0.07	0.06

+ Less than 0.01%; —compound absent; *tentative identification.

products to specific algae is much more difficult when they are grown in their natural environment than in laboratory cultures [1]. The products identified in *Scenedesmus* sp. and *C. vulgaris* are grouped in Table 1 by chemical class. The essential oil of both species was composed of acids (18–19% of volatile substances), alcohols (11–16%), esters (20–23%) and hydrocarbons (25–26%). In addition, we found ketones and lactones as minor constituents. In both algae, the relative amount of each class of components is about the same, except for alcohols, which appear in higher quantities in *Scenedesmus*.

The acidic fraction obtained by column chromatography, converted into methyl esters, was composed of saturated and unsaturated monocarboxylic acids with chain lengths ranging from C_{12} to C_{18}. In this fraction, palmitic acid was dominant. Appreciable amounts of linolenic, linoleic and oleic acids were also detected. The fatty acid composition of *S. obliquus* and *C. vulgaris* [20] also showed the predominance of palmitic and linolenic acid. Volatile carboxylic acids were identified in green freshwater microalgae [21].

The alcohol fraction (11–16%) was considered to possess the characteristic flavour of microalgae freshwater [1]. Three main components, phytol, 2-butyloctanol and octadecanol were identified. Zolovitch and Velev [14] have reported saturated aliphatic alcohols ranging from C_{10} to C_{24} with a high proportion of heptadecanol and octadecanol in extracts of *S. acutus*. 2-Butyloctanol with a M_r of 226 is identified for the first time in *Scenedesmus*. This compound was also found in the volatile products from a synthetic meat flavour system [22]. Linalool (0.87%) and geraniol (0.64%) which were reported earlier in marine green algae [23] and more recently in the red algae, *Porphyra tenera* [24], were detected only in *C. vulgaris*, while 2-butyloctanol (5.50%) was found only in *Scenedesmus*. Geraniol was also detected in small amounts in *Synechoccus* and *Anacystis nidulans* [3]. The terpene compounds in the cells of microalgae are probably intermediates in the formation of higher terpene compounds [14].

Ketones (1%), were found as minor components in the essential oil of the two algal species. It has been reported that there was excretion of low M_r products, such as aldehydes, alcohols, and ketones (β-ionone, 2-undecanone, epoxy-β-ionone) in *Synura uvella* [1], a brown alga, which dominates during algal blooms in natural waters. It was suggested that these compounds originated from the degradation of unsaturated fatty acids and carotenoids. Carotenoid degradation products have been reported as tobacco constituents [25] and have frequently been observed in natural waters [26]. We have identified α- and β-ionone (0.2%) and also 6,10,14-trimethyl-decan-2-one (0.15–0.20%), which has been shown to be formed from phytol, a derivative of chlorophyll [27]. These ketones have been reported in some marine green algae [28–30]. Epoxy-β-ionone (*ca* 0.10%) has also been reported as a component of the products

from a *Cyanobacterium* [3]. The lactone, dihydroactinidiolide (*ca* 0.20%), has been identified in *Scenedesmus* and *Chlorella*, the dominant species of HRAP, and found in marine green algae [29]. This compound was also reported in excretion products of different strains of *Synechoccus* [3], a blue-green freshwater alga.

The ester fraction was characterized by esterified fatty acids ranging from C_{12} to C_{18}, with methyl palmitate as a major constituent, and by significant amounts of C_{18} saturated methyl esters, mono- and triunsaturated methyl esters. The methyl esters of hexadecanoic and 9-octadecenoic acid have already been reported as major products in the blue-green alga, *Oscillatoria* [31], which is characterized by a strong odour. Ethyl palmitate and oleate were also found in *Scenedesmus* and *Chlorella* in our study.

Phthalate products are regarded as toxic pollutants in industrial waste water [32]. The relative amount of these components is 0.6% below that of essential oil (0.014% of the total biomass). Di(ethylhexyl)phthalate and dipropylphthalate were detected in volatile organics of waste water influents and effluents [33]. Dibutylphthalate was identified in some blue-green algae [5], such as *Oscillatoria*, *Phormidium*, *Aphanisomenon* and *Anabaena*, and in the green alga, *Enteromorpha* [30]. In our studies of organics products in waste water (unpublished results) we have found diethyl- and dibutyl phthalate as the principal constituents. Lubomir and Vera [34] have detected di(2-ethylhexyl)phthalate in drinkable water. In the ester fraction, methyl benzylbutyrate and ethyl benzylpropionate were also identified. Benzene derivative have been detected in freshwater microalgae [21, 26, 35]. Tants [35] has isolated phenylcarboxylic acid and esters from *Chlorella* species grown in axenic culture. Alkylbenzenes were reported in a *Tetraselmis* species during a bloom in natural water [26].

The main components of the hydrocarbon fraction were *n*-heptadecane and 1-heptadecene as previously reported in the unsaponifiable fraction of *S. quadricauda*, *Chlorella* species [36] and *C. pyrenoidosa* [37], as well as in the volatile oil of freshwater blue-green algae [38] and in marine algae [39]. In microalgae, of the Chlorophyceae, pentadecane and heptadecane have been reported as originating from decarboxylation of palmitic and stearic acid, respectively [37]. Although the hydrocarbon fraction was represented exclusively by straight chain compounds ranging from C_{14} to C_{20}, we have tentatively identified two branched-chain compounds, viz. 4-methylpentadecane and 6-methylheptadecane. Methylalkanes, such as 7- and 8-methylheptadecane, were also found in some blue-green algae [5, 37, 38] and in *S. quadricauda* [40]. In our species, in which *n*-heptadecane was the major component, 4-methylheptadecane and 6-methylheptadecane were minor components. The presence of branched hydrocarbons is thought to be characteristic of these prolific species in HRAP. Minor amounts of C_{17}, C_{24} and C_{25} n-alkanes, like those in *S. quadricauda* and *Chlorella* species, [36] were detected.

In our study, most of the compounds identified have low odour thresholds. Esters and alcohols, which are responsible for the unpleasant odours emanating from algae [25, 26] represent *ca* 40% of the volatile oil. On comparing the oil composition of the two algal species we have found, as a main trend, the same major components in each chemical class for both algae, except for the alcohols. High concentrations of 2-butyloctanol are found only in *Scenedesmus*, while only trace amounts of linalool and geraniol were detected in *Chlorella*. This latter point of difference between the two algal species may be correlated respectively to the maximum growth of each species and could serve as a taxonomic marker for HRAP biomass.

EXPERIMENTAL

Harvest of algae. Algae were harvested by centrifugation (3000 rpm) of the effluent of the High Rate Algal Ponds (HRAP) in the IAV Hassan II Institut (Rabat, Morocco). Centrifugation was performed with a creaming centrifuge. Green algae were grown in a pond of 24 m^2 area and 0.5 m depth. Algal species were identified microscopically. In the September 1990 harvest, *Scenedesmus* represented 88% of the total biomass. Some minor species, such as *S. quadricauda* (5%) and *Oocystis* (7%) were also found. In April 1991, the harvest yielded *C. vulgaris* (85%), *S. obliquus* (5%), *S. quadricauda* (6%) and *Micractinium pucillum* (4%).

Preparation of essential oil. The algal biomass of dominant species (90 g fr. wt) was homogenized in distilled H_2O (300 ml). The homogenate was distilled twice for 2 hr, the distillates combined, satd with NaCl and extracted $\times 4$ with Et_2O. The Et_2O extract was dried (Na_2SO_4) and concd to yield a brown oil which possessed a fishy odour (0.014% dry wt biomass). The essential oil was stored at 4° under a N_2 atmosphere.

Fractionation of essential oil. According to the method already described [41], a portion of the volatile oil was placed on a column (10 × 1 cm) of silica gel (Merck 60, 230–400 mesh). The first fr. which was eluted with pentane, yielded hydrocarbons. The second fr. which was eluted with Et_2O and Et_2O–MeOH (9:1) contained polar and oxygenated compounds. The third fr. eluted with Et_2O–MeOH–HOAc (45:4:1) gave the acidic fr.

Analysis of volatile organic compounds. Total volatile substances and the three frs obtained by CC were analysed by GC and GC-MS. Identification of the components was based on a mass spectral data bank (NIST library of the spectrometer), and comparison with ref. substances and lit. [42–44].

GC-MS. The capillary column was OV-1 (50 m × 0.32 mm i.d.) and the column temp. was held at 50° for 5 min and prog. to increase at 2° min^{-1} from 50° to 300°. The inj. and det. temps were 220° and 200°, respectively. Helium was the carrier gas at a flow rate of 25 ml min^{-1}. The electron impact was 70 eV. The sample was dissolved in Et_2O and the inj. vol. was 1 ml.

GC analysis. A fused silica capillary column CPSil5 CB (50 m × 0.22 mm i.d.) was used. The column temp. was held at 40° for 10 mm and prog. to increase at 4° min^{-1} from 40° to 280°. The inj. temp. was 220°, that of the det. 280°. The quantitative composition of the essential oil was determined by GC (FID) coupled to an electronic integrator.

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