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Lipids metabolites and essential oil from the green alga *Chara vulgaris*

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دراسة مادة الأيض الذهنية والزيوت العطرية لطحلب المياه العذبة

تم تحليل الستيرول، الحمضيات الدهنية والزيوت العطرية لطحلب المياه العذبة (*Chara vulgaris*). الستيرول الأكثر وجودا هو 24 -éthylcholest ـ 5-èn-3-ol، أما الحمضيات الذهنية الأساسية فهي : C16:0، C18:0 و C18:1. تم تحليل الزيوت العطرية المحصل عليها بواسطة الكروماطوغرافيه و السبكترومتري. تحتوي المواد الطيارة على الإدروكربورات، الإستر، الألدييد و الكحول. أما المركبات الطيارة الموجودة بكثرة فهي الإبتاديكان العادي، الإبتاديسين-7، الفيتول، 6-10- 14 تريميتيلبانتاديكان-2- أون و الميتيلأنديكانوات. كما تم التعرف على اثنان من التيربينويد وهي 5,6- إبوكسي-ß- يونون و ß- يونون. يمكن استعمال النتائج المحصل عليها كمعيار كيموطاكسونومي.

الكلمات المفتاحية : طحلب المياه العذبة ـ الستيرول ـ الحمضيات الذهنية ـ الزيوت العطرية ـ *Chara vulgaris*

Métabolites lipidiques et huiles essentielles de l'algue d'eau douce *Chara vulgaris*

Les stérols, les acides gras et les huiles essentielles ont été analysés chez l'algue d'eau douce *Chara vulgaris*. Les stérols majeurs sont le 24-éthylcholest-5-èn-3-ol et l'isofucostérol alors que les principaux acides gras sont le C16:0, le C18:0 et le C18:1. L'huile essentielle obtenue est analysée par CPG-SM. Les produits volatiles contiennent des hydrocarbures, des esters, des aldéhydes et des alcools. Le n-heptadécane, le 7-heptadécène, le phytol, le 6,10,14-triméthylpentadécan-2-one et le méthylundécanoate sont les composés volatiles les plus abondants. Deux terpénoides ont été identifiés, la 5,6-époxy-ß-ionone et la ß- ionone. Les résultats obtenus peuvent être utilisés comme critères chemo-taxonomiques.

Mots clés : *Chara vulgaris* - Characeae - Algue d'eau douce - Stérols - Acides gras - Huiles essentielles - Chromatographie en phase gazeuse - Spectrométrie de masse

Lipids metabolites and essential oil from the green alga *Chara vulgaris*

Sterols, fatty acids and essential oils were analyzed in freshwater green algae *Chara vulgaris*. Two major sterols were identified as 24-ethylcholest-5-en-3-ol and isofucosterol, together with 9 fatty acids from lipids with C16:0, C18:0 and C18: 1, which were the principal abundant acids. GC-MS was used to analyze the essential oil obtained from *Chara vulgaris*. Identified compounds included hydrocarbons, esters, ketones, aldehydes and alcohols; they constituted the volatile oil derived from *Chara vulgaris*. The most abundant volatile compounds were n-heptadecane, 7-heptadecene, phytol, 6,10,14-trimethylpentadecan-2-one and methylundecanoate. Two terpenoids were identified as 5,6-epoxy-ß-ionone and ß -ionone . The results obtained can be used in chemosystematics.

Key words : *Chara vulgaris* - Characeae - Freshwater green alga - Sterols - Fatty acids - Essential oils - GC-MS

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INTRODUCTION

Studies of the components in marine and freshwater algae have recently prompted a wider interest among the scientific community, as they have shown important eco-chemical and bioactive roles. Most of them have some commercial importance related to foodstuffs, cosmetics, dye and biological activities (Flament & Ohloff, 1984; Kodama, 1986; Rzama, 1994).

The algae are widespread around the globe. The charale green algae are found in many habitats (rivers, ponds, ...) apparently in freshwater only and can reach 1 m length. *Chara vulgaris* belongs to the terrestrial order of the charales in the class of Charophyceae, a group generally considered to be the ancestor of plants (Langangen & Svirîdeniks, 1995). *Chara vulgaris* can grow in freshwater with high salt concentrations and has a pungent odor (Bourelly, 1966). When occurring in a pond, this species dominates the algal flora of the ecosystem.

The algal population can modify its environment through production of volatile or excretion metabolites (Jüttner, 1983). Stom (1981) used Characeae algae as a biofilter absorbing and transforming the toxic pollutant products from effluents. The chemical constituents responsible for significant antimicrobial effects of 21 Minisotan aquatic plants and *Chara vulgaris* were isolated and identified (Lee *et al.*, 1972). El-Naggar (1995) analyzed *C. vulgaris* for the major constituents (ash, crude fiber, protein, lipids and carbohydrate). Hussain *et al.* (1996) studied six Chara species in an irrigation canal for their chemical products, ecology and seasonal succession. Beceiro-Gonzalez *et al.* (2000) also studied the interaction between arsenic and *C. vulgaris*.

Patterson (1972) identified clionasterol and 28-isofucosterol as the principal sterols of *C. vulgaris*. Small quantities of cholesterol and 24-methylene-cholesterol were also detected in this alga. Clionasterol was identified as a major sterol from two *Chara* species (Patterson *et al.*, 1991). Anthoni *et al.* (1980) reported the isolation and structure elucidation of two volatile compounds, partially responsible at least for inhibiting photosynthesis and for the peculiar odor of C *globularis*. Jacobsen *et al.* (1983) isolated two major volatile heterocyclic compounds (4-methylthio-1,2-dithiolane and 5-methylthio-1,2,3,-trithiane) from *Chara sp*. They found that these products had insecticide properties. Lipid composition was determined in *Chara sp.* (Dembitsky *et al.*, 1993).

This paper describe a new chemical composition of sterols and fatty acids from *Chara vulgaris*. Also, the essential oil has been identified for the first time in this specis.

MATERIALS & METHODS

1. Plant material

Chara vulgaris plant material was collected in July 1996 from ponds in "Jardin d'essais" near INRA (The National Agronomic Research Institute, Rabat, Morocco). This alga occurs in ponds with some aquatic plants. The species was identified microscopically in the Department of Algology (The Faculty of Sciences, Rabat).The alga was cleaned from the epiphytes and after manual sorting, it was dried and then ground to yield a fine powder.

2. Preparation of sterols and fatty acids

2.1. Sterols

A fine powder of alga (200 g) was extracted continuously with $CHCl_3$ - MeOH (2:1 ; v:v) in a soxhlet apparatus 4 h. The extract was evaporated and the residue was saponified as described by Creca & Monaco (1989) with alcoholic KOH 2N and concentrated. One equal volume of hot water was added to the residue and the unsaponifiable (0.4% of dry weight biomass) was recovered with diethyl-ether. This latter was washed with H_2O to a neutral pH, dried over Na_2SO_4 and the solvent evaporated. The free sterols were then acetylated using Ac_2O - pyridine (1:1 ; v:v) at room temperature overnight and then purified on silica gel using $CHCl_3$ - Et_2O (9:1 ; v:v) as solvent and washed and submitted to gaz chromatography (GC) analysis.

2.2. Fatty acids

The aqueous layer was acidified to pH = 1 with HCl (4N) and acids were extracted with diethyl-ether. The organic layer was washed to neutral pH, dried and the solvent evaporated. The fatty acids were esterified with MeOH saturated by HCl in heat to reflux 1 h. Analytical CCM in Hexane - Et_2O - AcOH (89:10:1 ; v:v) indicated no free acids. An

equal volume of hot water was added and esterified acids were extracted with diethyl-ether. The organic layers were washed to pH=7, dried over Na_2SO_4 and active charcoal was added to remove chlorophyll and pigments. Esterified fatty acids were obtained with 1.7% of dry weight biomass.

3. Preparation of essential oil

The essential oil was obtained from a 100 g fine powder homogenized in distilled water (250 ml) using a steam distillation apparatus for 4 hr. The distillate was saturated with NaCl and extracted four times with diethyl-ether. The extract was dried (Na_2SO_4) and concentrated under vacuum to yield yellow oil, which had a sulphuric odor (0.02% dry weight biomass).

4. Sterols analysis

The sterol acetates were analyzed on a fused silica capillary DB1 (30 m x 0.32 nm i.d.), with phase thickness 0.25 µm ; injector temperature 240°C and the interface temperature for GC-SM 250°C. The linear temperature programming conditions were from 180 to 300°C at a rate of 5°C/min. The total duration of analysis was 40 min per injection. The GC (Gilson) was coupled with a Spectrometer (Fisons). The ionization voltage was 70 eV. The identification of the compounds was accomplished using a databank Nist libraries of Spectrometer.

5. Fatty acids analysis

Esterified fatty acids were analyzed on a fused silica capillary OV17 (50 m x 0.32 nm i.d.), injector temperature 180°C, detector temperature 250°C, N_2 gas 15 ml/min from 160°C to 180°C, at a rate of 3°C/min. The total duration of a rate was 10 min. detector FID was used.

6. Essential oil analyses

Quantitative analyses were carried out by GC using fused-silica capillary column SE-54 (30 m x 0.32 i.d) with a film thickness of 0.25 µm. Helium was used as the carrier gas at flow rate of 1.1 ml/ min. The column temperature was kept at 40°C for the first 10 min, and then programmed at a rate of 4°C/min from 40 to 480°C. GC was coupled with an electronic integrator for determining the quantitative composition of the essential oil.

GC-MS analyses were carried out using a Spectrometer (Fisons Instruments). The GC chromatograph was equipped with a fused silica capillary column CP-Sil 5CB (50 m x 0.22 i.d) at 40°C for 10 min and then programmed from 40 to 280°C at 4°C/min. The detector ionization energy was 70 eV. It was coupled with a mass detector.

RESULTS AND DISCUSSION

The macroalgae living in clear pounds or in brackish waters were not exhaustively studied (Dembitsky *et al.*, 1993). In this work, the isolated fractions of sterols, fatty acids and volatile oils from *Chara vulgaris* were investigated by GC and mass spectroscopy. These compounds were identified by comparing with authentic samples and mass spectral bank (NIST library of Spectrometer) and the existing literature (Kodama, 1986 ; Lafferty & Stauffer, 1989 ; Adams, 1989 ; Patterson, 1992).

The obtained sterols composition is listed in table 1. Two main sterols (24-ethyl-cholesterol (30%) and isofucosterol (65%)) were found in agreement with the work of Patterson *et al.* (1991). 24-ethyl-cholest-5, 22-dien-3-ol (2%) and fucosterol or 24-methylene-cholesterol (1%) were discovered for

Table 1. Sterol composition of *Chara vulgaris* (% from the total sterols)

Sterol	*C. vulgaris*	*C. vulgaris**
Cholesterol	1	tr
24-methyl-cholest-5,22-dien-3-ol	-	-
24-methyl-cholest-5-en-3-ol	-	-
24-methylene-cholesterol (fucosterol)	1	tr
24-ethyl-cholest-5,22-dien-3-ol	2	-
24-ethyl-cholest-5-en-3-ol (clionasterol)	**30**	**39**
Isofucosterol	**65**	**54**

tr = trace <0. 01; - not detected ; * (Patterson, 1992)

the first time in *Chara vulgaris*. 24-ethyl-cholesterol is a major sterol in some freshwater algae (Rzama *et al.*, 1994a) and marine algae (Kajiwara *et al.*, 1990). Fucosterol and cholesterol were also detected but in significant amounts than in some *Chara* sp. analyzed by Patterson (1992). Fucosterol was the main sterol only in brown marine algae (Tsenka *et al.*, 1997).

Fatty acid (FA) composition of *C. vulgaris* is summarized in table 2. Palmitic (C16:0), stearic (C18:0), oleic (C18:1) and linoleic (C18:2) acids were predominant in the total fatty acid composition. Low amounts of C18:3, C20:0, C20:4, C20:5 and C22:0 were detected. Total saturated fatty acids (SAFA) are higher than polyunsaturated fatty acids (PUFA) : the percentage was 64.1% and 30.35%, respectively. Dembitsky *et al.* (1993) found that fatty acids contained C16:0 saturated, mono-unsaturated and C18 acids with 1-3 double bonds as predominant acids in *C. vulgaris* and two other macrophytes. Other freshwater species and marine algae seem to contain the same level of saturated and unsaturated fatty acids (Ahlgren *et al.*, 1992 ; Zhukova & Aizdaicher, 1995).

Table 2. Composition of fatty acid from *Chara vulgaris* (% from the total fatty acids)

Fatty acid	Concentration (%)
C14: 0	2.25
C16:0	**30.80**
C16: 1	3.80
C18: 0	**27.90**
C18:1	**10.90**
C18:2	**8.85**
C18:3	3.40
C20:0	1.80
C18:4 + C20:1	0.85
C20:4	1.30
C20:5	1.25
C22:0	1.35
Others	5.55
SAFA	64.10
PUFA	30.35

Abbreviations : Fatty acids C14:0, 14: number of atom carbon, 0: number of unsaturation ; SAFA: saturated fatty acids ; PUFA: polyunsaturated fatty acids.

Xu *et al.* (1998) analyzed fatty acid profiles of 12 green macroalgae. The major fatty acids were 16:0, 18:1, 18:3, 16:3, 18:2 and 20:4. Total PUFA contents ranged from 17.2 to 54.4%. Often, the percentage of PUFA was higher than SAFA in microalgae than macroalgae (Ibanez-Gonzalez *et al.*, 1998 ; Rebolloso-Fuentes *et al.*, 2001 ; Renaud *et al.*, 1999).

The yield of the diethyl-ether extract was 0.02% of essential oil. The compounds of essential oil were identified by GC and GC-MS (Table 3). The percentage given for each component is the average for three samples. Some of these volatile products have been described in the literature (Flament & Ohloff, 1984 ; Lafferty & Stauffer, 1989).

The main hydrocarbons were n-heptadecane (17.4% of total volatile oil) and 7-heptadecene (10.5%). The similar result was previously reported for the volatile oils of freshwater green algae (Jüttner, 1983, 1984 ; Rzama *et al.*, 1994b) and marine green algae (Kajiwara *et al.*, 1990). 7-heptadecene is known as the characteristic compound of edible kelp flavors (Kodama, 1986). Methyl-undecanoate was represented by a high amount (10.2%) and methyl-hexadecanoate with an appreciable percentage (2.5%). Significant levels of C:18 saturated methyl-esters and mono-, di- and tri-unsaturated methyl-esters were identified. The methyl-ester of arachidonic acid has also been detected.

Phytol (14.7%) was a predominant alcohol, it is known as a degradation product of chlorophyll (Jüttner, 1979). The 6,10,14-trimethyl-pentadecan-2-one (10.5%) was the major ketone, which has been shown to be formed from phytol (Kodama, 1986). Two terpenoids, 5, 6-epoxy-ß-ionone and ß-ionone have been identified, these products are reported to originate from carotenoids degradation (Kodama, 1986). These carotenoids are also reported in volatile oils of freshwater algae (Jüttner, 1979 ; Walsh *et al.*, 1998) and in marine algae (Kajiwara *et al.*, 1990).

Tetradecanal (2.82%) and hexadecanal (0.45%), volatile aldehydes were found in low amounts. Diethyl-phthalate (0.13%) was detected as a minor product. Phthalate products have been found in relatively low concentration in freshwater green algae (Rzama *et al.*, 1994 b ; Walsh *et al.*, 1998), in marine green algae (Koshimizen & Iwamura, 1986) and in drinking water (MacCarthy & Klusman, 1993).

Dimethyl-sulphide and octacycloatomic sulphur were detected in low amounts, the latter was found in some freshwater algae or in their environment (Jüttner, 1983 ; Kodama, 1986).

Table 3. Volatile components of *Chara vulgaris*

Component	RT (mn)	Relative peak area (%)
Methanetiol	1:10	tr
Dimethyl-sulphide	1:45	0.28
Pinene	2:05	0.43
Pinene	2:36	tr
p-cymene	3:29	0.23
Eucalyptol ou Cineol	3:37	0.67
Limonene	3:41	0.86
p-Menth-1-en-4-ol*	8:30	0.35
Methyl-undecanoate	17:15	**10.20**
Geranyl-aceton	18:06	0.04
Methyl-dodecanoate	20:39	0.55
1-Pentadecene*	20:19	0.24
Diethyl-phthalate	21:42	0.13
Tetradecanal	22:57	2.82
Pentadecane	23:13	3.62
7-Heptadecene	25:14	**10.50**
1-Heptadecene	25:32	1.52
Heptadecane	25:59	**17.40**
Methyl-tetradecanoate	26:15	2.21
5,6-Epoxy-ß-ionone	27:30	2.34
ß-Ionone	27:42	0.45
Octadecane	28:33	0.50
6,10,14-Timethyl-pentadecan-2-one	29:18	**10.50**
3, 7, 11,15-tetramethyl-hexadec-2-ene	29:40	0.51
Cyclooctasulfur	30:18	0.38
Hexadecanal	30:24	0.45
Farnesyl acetone	30:44	0.45
Methyl-hexadecanoate	30:19	2.50
Phytol	31:57	**14.70**
Furanone*	32:34	0.22
Methyl-octadecanoate	32:48	2.66
Methyl-oleate	33:54	1.52
Methyl-linoleate	34:10	0.25
Methyl-arachidonate	34:59	1.52
Heptacosane	39:00	0.50

tr = trace < 0.01%; * tentative identification

CONCLUSION

This paper focused on the chemical composition of sterols, fatty acids and the essential oil of freshwater green alga *Chara vulgaris*. Two sterols 24-ethyl-cholesta-5, 22-dien-3-ol and fucosterol or 24-methylene-cholesterol were discovered for the first time in *Chara vulgaris*. A new distribution of fatty acids was discussed and the essential oil was described for the first time. The terpenoids, sulphur and sterol compounds could play a significant role in the taxonomy of *Chara vulgaris* and the place of this alga in Charophyta.

Further research needs to be conducted to explain the relationship existing between the volatile excretion products in the ecosystem from *Chara vulgaris* and the species predominance in the pond.

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