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Determination of sterols and diterpenoids from brown algae (Cystoseiraceae)

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ABSTRACT

A high-performance liquid chromatographic method is described for the determination of secondary metabolites that belong to the brown algae of the Cystoseiraceae family. The study was focused on sterols, which are globally determined as fucosterol, and on the main structural families of diterpenes (mero- and linear diterpenes). An example is given of the analysis of three characteristic extracts from Atlantic and Mediterranean species.

INTRODUCTION

The lipidic extracts from the brown algae belonging to the Cystoseiraceae family have led to various studies on secondary metabolites [1–22]. They have permitted the identification of several sterols and diterpenes and the determination of the chemical structures of new compounds.

Gas chromatography and gas chromatography–mass spectrometry have often been used in order to identify sterols and determine their composition within the sterolic fraction [1,2,4,8]. The same holds for high-performance liquid chromatography (HPLC), which has been used to separate and purify the diterpenes [6,7,10–16,18–21]. However, we are not aware of any quantitative analysis that allows the precise evaluation of both the amount of sterols (the total amount of free sterols) and that of the main diterpenes from the studied algae. Therefore, such an investigation applied to all the seaweed species so far described could be useful for chemotaxonomic studies on the Cystoseiraceae family.

In this paper, we report a general method for the determination of secondary metabolites from brown seaweeds belonging to the Cystoseiraceae family using normal-phase HPLC. The study was focused on sterols, which are globally determined as fucosterol (**1**) and on the main structural families of diterpenes which have been isolated from Cystoseiraceae. These diterpenes include acyclic compounds with a geranylgeraniol skeleton (**2** and **3**) and meroditerpenes (diterpenes with mixed biogenesis) characterized by hydroquinonic methyl nucleus linked to a diterpenic chain. This chain might be either acyclic or cyclic (**4** and **6**) or even cyclically rearranged (**5A, B, C** and **D**).

EXPERIMENTAL

Instrumentation

Separations were carried out on an LDC liquid

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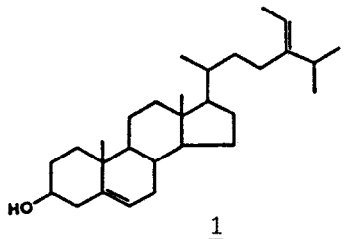

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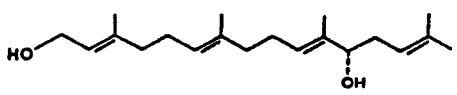

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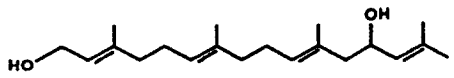

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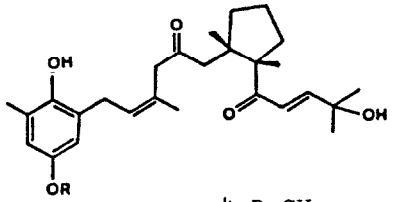
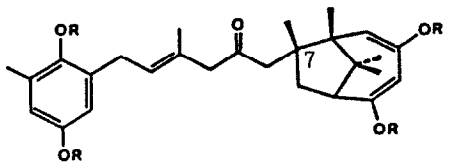
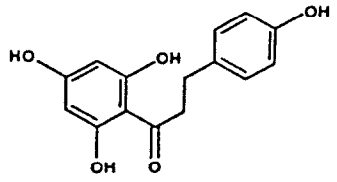


4 R=CH_3
6 R=H



5 [A R=H; B R=H, C_7(S*)]



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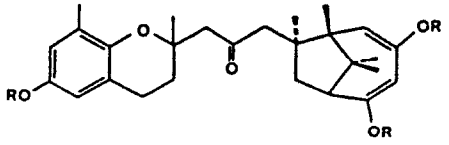
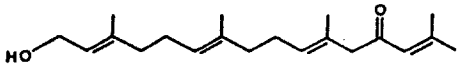


5 [C R=H; D R=H, C_7(R*)]



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chromatograph (Milton Roy, Riviera Beach, FL, USA) equipped with a Constametric 3000 solvent-delivery system. A Spectromonitor 3100 X variable-wavelength UV detector set at 289 nm, with a 15-μl flow cell, was used for the analysis of meroditerpenes, whereas a Waters Assoc. Model R401 differential refractometer was used for the detection of sterols and linear diterpenes. Retention times and peak areas were obtained with a Shimadzu Chromatopac C-R6A integrator. The column (stainless steel, 250 × 4 mm I.D.) was packed with 5-μm silica (Partisil 5, Whatman, Clifton, NJ, USA). The flow-rate was 1 ml/min.

Solvents

The solvents used were ethyl acetate–isooctane (2,2,4-trimethylpentane) (2:3, v/v). They were freshly distilled and then filtered and degassed *in vacuo* through a sintered-glass filter.

Standards

Reference samples for the standardization were (1) Commercial fucosterol (**1**) (Sigma, St. Louis, MO, USA) for the analysis of the sterols; (2) bifurcadiol (**2**), eleganediol (**3**), methoxybifurcarenone (**4**) and a mixture of mediterraneols and bifurcarenone[a] (**5** and **6**) for the analysis of the diterpenes (compounds **2**, **3**, **4** and mixture **5–6** were previously isolated and identified from natural sources and purified by semi-preparative HPLC); and (3) commercial phloretin (**7**) (Sigma) and 2-methyl-2-butanol (**8**) (Carlo Erba, Milan, Italy), which can be used separately as an internal standard depending on the extract studied.

Sample preparation for HPLC analysis

Reference mixtures were obtained from stock solutions of each standard: 0.4–0.8 g/l in ethyl acetate for meroditerpenes (UV detection) and ten times more concentrated for sterols and linear diterpenes [refractive index (RI) detection]. Calibration was achieved using phloretin (**7**) as internal standard (stock solution at 0.5 g/l for UV detection and 2 g/l for RI detection) or using 2-methyl-2-butanol (**8**) as internal standard (stock solution at 5 g/l, only for RI detection). For the preparation of reference mixtures, 0.3 ml of internal standard solution was added to given volumes of each standard solution and diluted to 10 ml (UV detection) or 0.6 ml (RI detection) with ethyl acetate. Under identical conditions, a diethyl ether extract of the studied species (stock solution at 4 g/l for UV detection and 8 or 18 g/l for RI detection) was mixed with 0.3 ml of internal standard solution and diluted with ethyl acetate. This solution was injected directly into the HPLC apparatus.

[a] Compounds **5** and **6** are minor constituents of *Cystoseira stricta* which were isolated in very small amounts in order to effect their identification. Therefore, the mixture **5–6**, obtained from the extract studied, could be used for standardization purposes.

Standardization

Calibration graphs $w_i/w_{is} = f(A_i/A_{is})$, where w_i/w_{is} = sample weight per unit internal standard weight and A_i/A_{is} = sample peak area per unit internal standard peak area, were straight lines (regression lines were obtained from four points). Equations and correlation coefficients (r) are given in Table I for fucosterol and linear diterpene standards [RI detection; internal standard phloretin (**7**) or 2-methyl-2-butanol (**8**)] and in Table II for meroditerpene standards [UV detection at 289 nm; internal standard phloretin (**7**)].

RESULTS AND DISCUSSION

Compounds **1** to **6** were determined by normal-phase HPLC with isocratic elution with ethyl acetate–isooctane (2:3, v/v). Their retention times are given in Tables I and II and typical chromatograms of standard mixtures are shown in Figs. 1, 2.

Under these conditions, sterols are eluted together and globally determined as fucosterol (**1**), which is the main component of the sterolic fraction from the brown algae. The linear diterpenes (**2** and **3**) can only be detected by means of a differential refractometer. UV detection, which is more sensitive, could only be used for the linear diterpenes of the eleganolone (**9**) type, for which the conjugated ketone part of the molecule shows an absorption at 254 nm. For the meroditerpenes (**4–6**), which show a maximum wavelength at 289 nm, UV detection is more sensitive.

Ethyl acetate–isooctane has often been used as an eluent in thin-layer chromatography (TLC) or semi-preparative HPLC in order to separate the diterpenes from brown algae [6,7,16,18,19,21]. Isooctane is preferable to hexane because it is far less volatile and nearly as viscous (0.50 cP) as ethyl acetate. After several preliminary experiments, including gradient elution in combination with UV detection, we selected ethyl acetate–isooctane (2:3, v/v), which allows an optimum separation of the analyte compounds.

For the determination of these substances we used as an internal standard either phloretin (**7**) or the 2-methyl-2-butanol (**8**), depending on the extract studied. Phloretin (**7**), with its phenolic structure, is suitable for the determination of the meroditerpenes. It is eluted immediately after phlorogluci-

TABLE I

RETENTION TIMES AND EQUATIONS OF CALIBRATION GRAPHS FOR FUCOSTEROL AND LINEAR DITERPENE STANDARDS

Normal-phase column (silica, 5 μm) eluted with ethyl acetate–isooctane (2:3, v/v) at a flow-rate of 1 ml/min; RI detection; internal standard, (a) phloretin (7) or (b) 2-methyl-2-butanol.

No.	Compound	Retention time (min)	Equation	Correlation coefficient (r)	R.S.D.
1	Fucosterol	5.8	(a) $y = 2.12x - 0.02$	0.998	0.026
			(b) $y = 0.35x - 0.01$	0.999	0.003
2	Bifurcadiol	10.1	(a) $y = 1.71x + 0.005$	0.999	0.005
			(b) $y = 0.28x + 0.01$	0.999	0.012
3	Eleganediol	11.2	(a) $y = 1.77x + 0.02$	0.999	0.016
			(b) $y = 0.30x - 0.03$	0.999	0.022
7	Phloretin (I.S. 1)	15.8	–	–	–
8	2-Methyl-2-butanol (I.S. 2)	6.2	–	–	–

nol. Moreover, it allows the determination of sterols and linear diterpenes but not of eleganediol (**3**). By normal-phase HPLC this compound often leads to the formation of a small amount of an artifact which shows a retention time similar to that of **7**. In this instance, it is better to use the 2-methyl-2-butanol (**8**), which elutes between the sterols and the diterpenes. When a peak free of interferences is obtained, **8** is suitable as an internal standard for the determination of the sterols. Tables I and II give equations and correlation coefficients for the calibration graphs obtained with fucosterol and diterpene standards. The relative standard deviation (R.S.D.) of residues from the regression line of each standard is also given (calculated from four points).

The accuracy of the method is estimated, in the analysis range of each compound, by the difference ($d = w_i - \bar{w}_i$) between a known weight of the studied sample and the mean of the calculated value from the calibration graph (Table III). In this table, the value of the experimental coefficient ($t_{exp.}$) is lower than the corresponding Fischer coefficient (t) evaluated for a confidence level of 95%. On this basis, the method is not burdened with a systematic error.

The resuls obtained using this HPLC method are shown in Fig. 3 and Table IV. Three characteristic

TABLE II

RETENTION TIMES AND EQUATIONS OF CALIBRATION GRAPHS FOR MERODITERPENE STANDARDS

Normal-phase column (silica, 5 μm) eluted with ethyl acetate–isooctane (2:3, v/v) at a flow-rate of 1 ml/min; UV detection at 289 nm; internal standard, phloretin (7).

No.	Compound	Retention time (min)	Equation	Correlation coefficient (r)	R.S.D.
4	Methoxybifurcarenone	9.9	$y = 3.80x + 0.08$	0.999	0.024
5	Mediterraneols	12.7	$y = 20.50x + 0.73$	0.998	0.335
6	Bifurcarenone	14.0			
7	Phloretin (I.S)	15.8	–	–	–

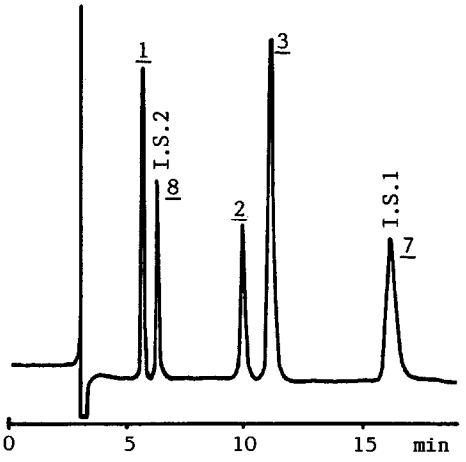

Fig. 1. Separation of standard mixtures of fucosterol and linear diterpenes. Experimental conditions as given in Table I. Fucosterol (**1**) (23.8 μg), bifurcadiol (**2**) (12 μg), eleganediol (**3**) (36 μg), phloretin (**7**) (20 μg) as internal standard 1,2-methyl-2-butanol (**8**) (49 μg) as internal standard 2.

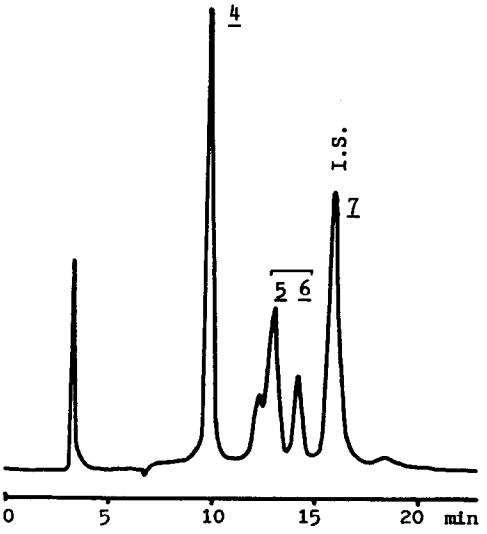

Fig. 2. Separation of a standard mixture of meroditerpenes. Experimental conditions as given in Table II. Methoxybifurcarenone (**4**) (1.2 μg), mediterraneols–bifurcarenone mixture (**5–6**) (5.9 μg), phloretin (**7**) (0.3 μg) as internal standard.

diethyl ether extracts from Atlantic and Mediterranean species were studied: *Bifurcaria bifurcata* from two different sources, (a) Atlantic coast, Cap Blanc, Morocco and (b) Atlantic coast, Casablanca, Morocco, and *Cystoseira stricta*, (c) from the Mediterranean coast, Saint Aygulf, Var, France. In Table IV, results are given in mg/g of dried seaweed. RI detection was used for sterols [globally determined as fucosterol (**1**)] and linear diterpenes **2** and **3**; UV detection was used for meroditerpenes **4** and **5–6**. Phloretin (**7**) was the internal standard in Fig. 3a and c′ and 2-methyl-2-butanol (**8**) in Fig. 3b and c.

The precision of the results is given as a percentage for a confidence level of 95% (Table IV). It was obtained from four measurements on the same sample.

TABLE III

COMPARISON OF A KNOWN WEIGHT OF STANDARDS WITH CALCULATED VALUE

$\bar{w}_i$ = Mean weight of sample calculated from the calibration graph; s = standard deviation from three measurements (n); w_i = known weight of sample; $t_{exp.} = d/s/\sqrt{n}$; t = corresponding Fischer coefficient for a confidence level of 95%.

No.	Compound	$\bar{w}_i$ (mg)	s (mg)	$d = w_i - \bar{w}_i$	$t_{exp.}$	t
1	Fucosterol	15.83	0.88	0.07	0.14	4.30
2	Bifurcadiol	24.03	1.46	0.03	0.04	4.30
3	Eleganediol	35.09	0.95	0.91	1.67	4.30
4	Methoxybifurcarenone[a]	0.374	0.008	0.007	1.62	4.30
5	Mediterraneols[a]	0.256	0.001	0.011	1.83	4.30
6	Bifurcarenone[a]					

[a] UV detection at 289 nm.

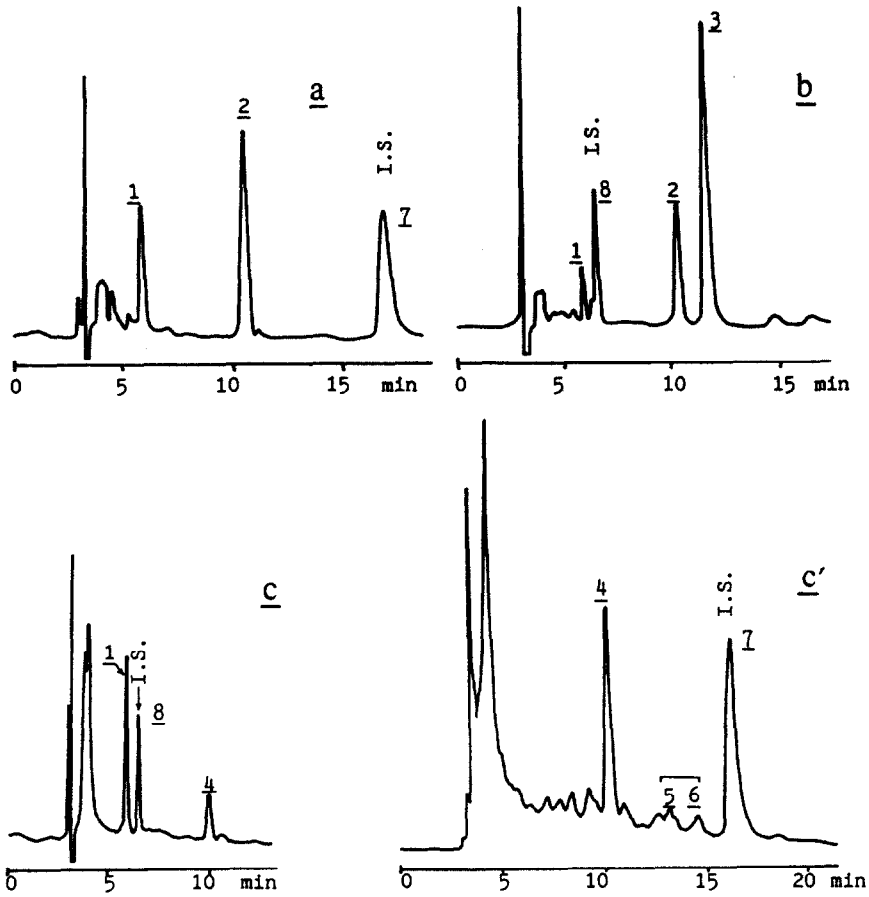

Fig. 3. Examples of HPLC analyses of diethyl ether extracts of (a) *Bifurcaria bifurcata* (Atlantic coast, Cap Blanc, Morocco) [experimental conditions as given in Table I (a)]; (b) *Bifurcaria bifurcata* (Atlantic coast, Casablanca, Morroco) [experimental conditions as given in Table I (b)]; (c) *Cystoseira stricta* (Mediterranean coast, Saint Aygulf, Var, France) [experimental conditions as given in Table I (b)]; (c') the same as (c), but experimental conditions as given in Table II.

The limit of detection was evaluated per gram of dried seaweed for each compound. The values were 0.20 mg/g (**7** as I.S.) and 0.01 mg/g (**8** as I.S.) for sterols; 0.24 mg/g (**7** as I.S.) and 0.57 mg/g (**8** as I.S.) for bifurcadiol (**2**); 0.44 mg/g (**8** as I.S.) for eleganediol (**3**); 0.015 mg/g (**7** as I.S.) for methoxybifurcarenone (**4**); and 0.22 mg/g (**7** as I.S.) for the mixture of mediterrancols and bifurcarenone (**5**–**6**).

CONCLUSIONS

This work, which was undertaken from a chemotaxonomic point of view, satisfies a double purpose: first, a rigorous phytochemical comparison between the species studied, and second, the evaluation of the geographic and seasonal evolution of their chemical components. This was achieved by means of a rapid, easy, reproducible method which could be applied to the determination of other secondary metabolites from different classes of seaweeds: (a) the sterols from red and green algae and (b) the diterpenoids eluted between sterols and phloretin (**7**) when using normal-phase HPLC for the determination.

TABLE IV

COMPOSITION OF EXTRACTS ORIGINATING FROM BROWN ALGAE BELONGING TO THE CYSTOSEIRACEAE FAMILY

(a) *Bifurcaria bifurcata* (Ross) (Atlantic coast, Cap Blanc, Morocco); (b) *Bifurcaria bifurcata* (Ross) (Atlantic coast, Casablanca, Morocco); (c) *Cystoseira stricta* (Mont.) Sauv. (Mediterranean coast, Saint Aygulf, Var, France). Results are given in mg/g of dried seaweed. Analyses were carried out in quadruplicate on the same sample.

No.	Compound	Amount (mg/g)					
		(a)	Precision (%)[a]	(b)	Precision (%)[a]	(c)	Precision (%)[a]
1	Fucosterol[b]	1.34	7.1	0.70	6.8	1.40	3.4
2	Bifurcadiol	4.41	2.5	3.97	10.8	–	–
3	Eleganediol	–	–	10.47	2.3	–	–
4	Methoxybifurcarenone[c]	–	–	–	–	0.29	5.5
5	Mediterraneols[c]	–	–	–	–	0.63	2.5
6	Bifurcarenone[c]						

[a] Precision for a confidence level of 95%.
[b] Total amount of free sterols.
[c] UV detection at 289 nm.

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