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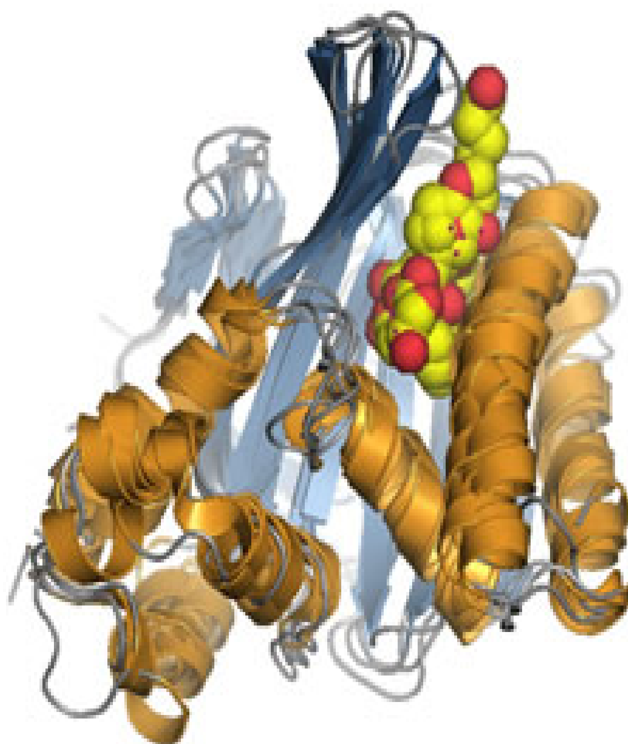

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Determination of free sterols in red and brown algae by FTIR spectroscopy

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Abstract. *A new analytical method was developed for the determination of free sterols in the extracts of the red alga Asparagopsis armata and the brown one Cystoseira sedoides by using FTIR spectrometry. Sterols samples were globally refered to cholesterol content because it is the major sterol contained in red algae [1] and to fucosterol because it is the principal one contained in brown ones [2]. The method involves the use of cholesterol as reference standard in the case of red alga and a correction factor of 1.259 ±0.003, which represents the ratio between the slopes of calibration lines obtained from fucosterol and cholesterol in the case of brown alga. To isolate the sterols, the lipidic extract of the algae were heated in methanol and diluted with water and then preconcentrated on DSC-18 solid phase cartridges and eluted with 1 mL dichloromethane stabilized with β-amylene. The determination of free sterols by Fourier transform infrared spectrometry was carried out by considering the specific band of cholesterol at 1047 cm^{-1} corrected with a baseline established between 1060 and 1014 cm^{-1}. The sterols content were obtained by the external calibration. The concentrations obtained were 1.9 ± 0.5 % and 1.5 ± 0.4 % in Asparagopsis armata and Cystoseira sedoides, respectively.*

Key Words: Sterol Determination, Cholesterol, Fucosterol, *Asparagopsis Armata, Cystoseira Sedoides, Fourier Transform Infrared*, Solid Phase Extraction

Introduction

It has been well documented that the primary sterols of red algae are cholesterol and other C-27 sterols. On the other hand, the major sterol in brown algae is fucosterol and cholesterol has been found at trace levels. However in the green algae there is no single major sterol.[1,7]

Sterols are commonly used in taxonomy, because they are constituents of the cell membranes and their composition depend on the taxonomic position of organism investigated, on the environment and seasonal variation.[8,9] The usual work up for sterol purification includes

digitonin precipitation, alkaline hydrolysis and silica gel chromatography, yielding the sterol fraction which was analyzed by gas chromatography (GC) or GC with mass spectrometry detection (GC/MS) or liquid chromatography (HPLC).[1,6,8-13]. Fourier transform infrared spectroscopy (FTIR) is a rapid and non destructive technique that can provide reliable qualitative information of biological samples and is a very useful tool for accurate quantitative analysis of many compounds without requiring complex sample preparation procedures.[14,15] Considering environmental friendliness of solid phase extraction (SPE) which is well established method for the concentration and extraction of sterol,[16-18]we have recently combined the infrared Fourier transform technique with the solid phase extraction to perform quantitative determination of total sterols in the extract of red alga *Asparagopsis armata* Harvey (globally determined as cholesterol which is the major sterol in this alga) and in four brown algae (globally determined as fucosterol which is the major sterol in the brown algae) [19,20]. In such a frame, the objective of this work has been to quantify free sterol in the red alga *Asparagopsis armata* Harvey and in the brown one *Cystoseira sedoides* by FTIR spectrometry coupled to SPE trying to obtain as greenest as possible analytical method.
Results obtained by FTIR spectrometry were carefully checked and compared with those obtained by HPLC.

Experimental

Cholesterol and fucosterol standards were obtained from Merck (Darmstadt, Germany). Analytical reagent-grade methanol and chloroform were purchased from Carlo Erba (Rodano, Italy). Analytical reagent-grade dichloromethane, stabilized with β amylene were obtained from Panreac (Barcelona, Spain).
The samples of *Asparagopsis armata* Harvey and those of Cystoseira sedoides were collected from the southern Mediterranean coast of Algeria in Tipaza village in May and july 2005, respectively . The algae were washed with water, spin dried, shade dried and all foreign materials were removed by hand.
For preconcentration of sterols, commercially available 3 mL solid phase extraction DSC-18 octadecyl cartridges from Supelco (Bellefonte, PA, USA), containing 500 mg of solid phase with an average particle size of 50 μm and specific surface area of 480 m^2g^{-1}, were employed.

A Thermo Nicolet FTIR spectrometer (model Nexus) was employed to obtain the FTIR spectra in the range of 4000-400 cm^{-1}. The system was equipped with a temperature stabilized detector with KBr beam splitter and precise digital signal processing. Spectra were obtained at 4 cm^{-1} nominal resolution and accumulating 20 scans per spectrum. All measurements were carried out using a micro-flow cell with CaF_2 windows and a pathlengh of 0.5 mm. Connection tubes were in PTFE with 0.8 mm internal diameter.

100 g of algae were soaked with 1 L of a mixture of methanol and chloroform 1:1 (v/v) for 24 H. The solution was filtered over celite under reduced pressure and evaporated to dryness. The procedure was replicated three times on the algal residue. The residue was dissolved in methanol and analyzed by FTIR spectrometry.
A precise amount (100 mg) of organic residue was heated with 10 mL of methanol until dissolution and 40 mL of distilled water were added. The mixture was loaded into 500 mg of solid phase octadecyl silica DSC-18 extraction cartridges (preconditioned sequentially with 2 mL of methanol and 1 mL water) and then eluted with 3 portions of 1 mL of dichloromethane stabilized with amylene. The residue was dried by using Na_2SO_4 and evaporated to dryness to remove traces of solvents. The residue was diluted in 1 mL of dichloromethane and the IR

spectra were obtained by using a background of a dicloromethane blank obtained in the same experimental conditions.

The peak height of spectra was obtained by measuring the absorbance at 1047 cm^{-1} with a base line established between 1060 and 1014 cm^{-1}. Corrected absorbance values were interpolated in the corresponding calibration line obtained for solutions containing from 0.7 to 10 mg mL^{-1} of a cholesterol standard which were prepared and treated in the same way than samples.

Results and discussion

The spectrum of the red alga extract residue dissolved directly in CH_2Cl_2 (Figure 1) shows that the direct determination of cholesterol from an extract of red algae is not possible and a pre-treatment is mandatory.

After dissolution of cholesterol in methanol and water, it was extracted and preconcentrated by using solid phase extraction cartridges with 500 mg of a DSC-18 phase, previously conditioned with methanol and water. Results obtained by "in batch" elution of loaded cartridges with three portions of 1 mL of CH_2Cl_2 stabilized with amylene demonstrated the total efficiency of the retention–elution process which corresponds to a 92 % recovery yield. These results were obtained by comparison of the calibration lines obtained with CH_2Cl_2 solutions of cholesterol and with aqueous solutions of a cholesterol previously diluted in methanol, preconcentrated on the cartridges and eluted off-line with CH_2Cl_2 (Table 1).

Table 1: Comparison of calibration plots obtained for cholesterol in CH_2Cl_2 and cholesterol after solubilization and SPE in BondElut C18 cartridge

Parameters	*Cholesterol: direct CH_2Cl_2 solution*	*Cholesterol: after solubilization in methanol and SPE*
Regression line	-0.0005 +0.01666 **C**	0.0020 + 0.01454 C
Correlation coefficient	0.9999	0.9939
L.D / µg mL^{-1}	1.5	1.3
R.S.D. % (n=10)	0.4	0.8
Extraction yield / %		92
Concentration range /mg mL^{-1}	0.7-10	

C: concentration/ mg mL^{-1}, **R.S.D** en %: relative standard deviation. (C =5 mg mL^{-1} for cholesterol).
L.D: limit of detection / µg mL^{-1} and established for k=3 for probability level 99.6 %. These results were obtained by the comparison of the calibration lines obtained for cholesterol in CH_2Cl_2 solutions and for aqueous solutions of cholesterol previously solubilized in methanol, preconcentrated on the cartridges and eluted off-line with CH_2Cl_2.
Experimental Conditions: measurements were carried out at a nominal resolution of 4 cm^{-1}, 20 scans were accumulated and 0.5 mm optical , pathlength and CaF_2 were used. A specific band at 1047 cm^{-1} was selected and the baseline was established between 1060 and 1014 cm^{-1}.

Figure 2 shows the spectra found for a series of four standards prepared in the same way as samples in order to assure a quantitative recovery of cholesterol.
100 mg of the organic extract of the algae, previously prepared as indicated in the experimental part, were analyzed by FTIR using the stopped-flow mode. The FTIR spectra of a natural samples obtained after treatment were shown in Figures 3 and 4.. In order to assure a quantitative recovery of fucosterol in the brown alga, a coefficient of 1.26 was considered. This coefficient represent the ratio between the two slopes of calibration lines of fucosterol and cholesterol, directly prepared in dichloromethane stabilized with β amylene.

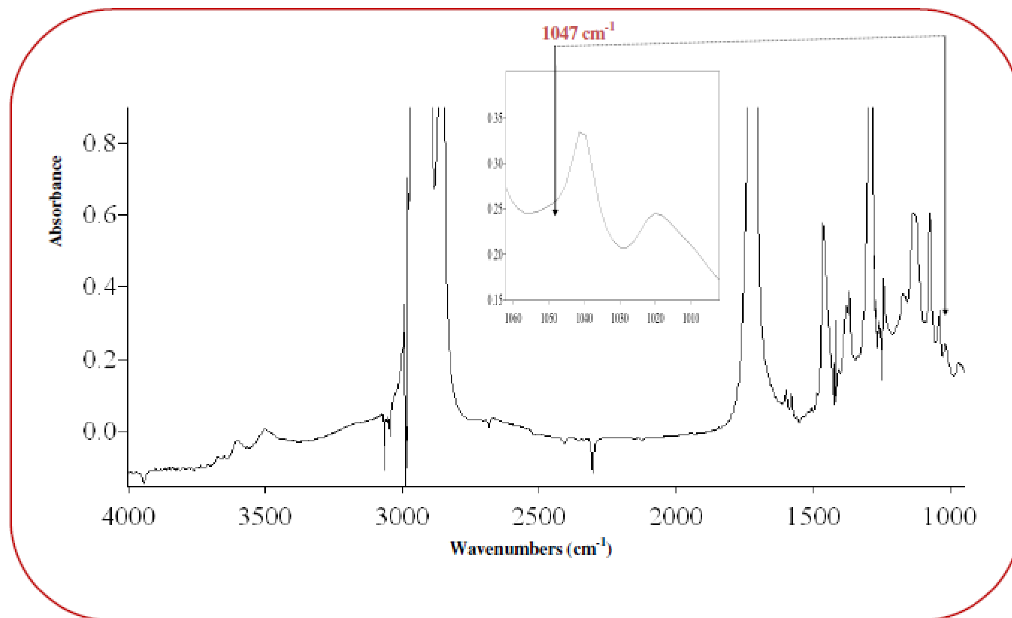

Figure 1: FTIR spectrum of 100 mg mL^{-1} crude algae extract dissolved directly in CH_2Cl_2

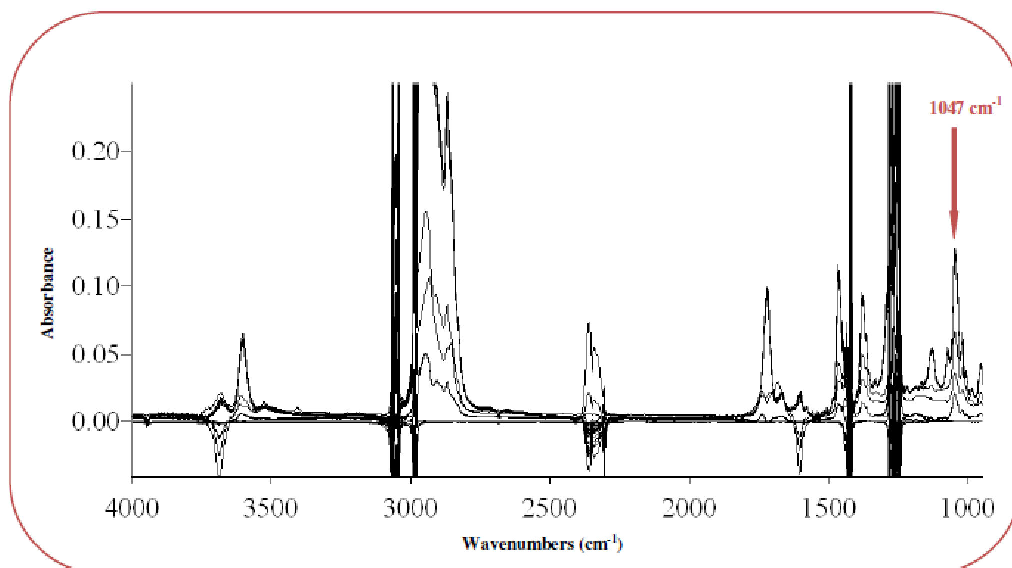

Figure 2: FTIR spectra of cholesterol standards obtained after preconcentration in DSC-18 cartridges and eluted with CH_2Cl_2 in the concentration range of 0.7-10 mg mL^{-1}

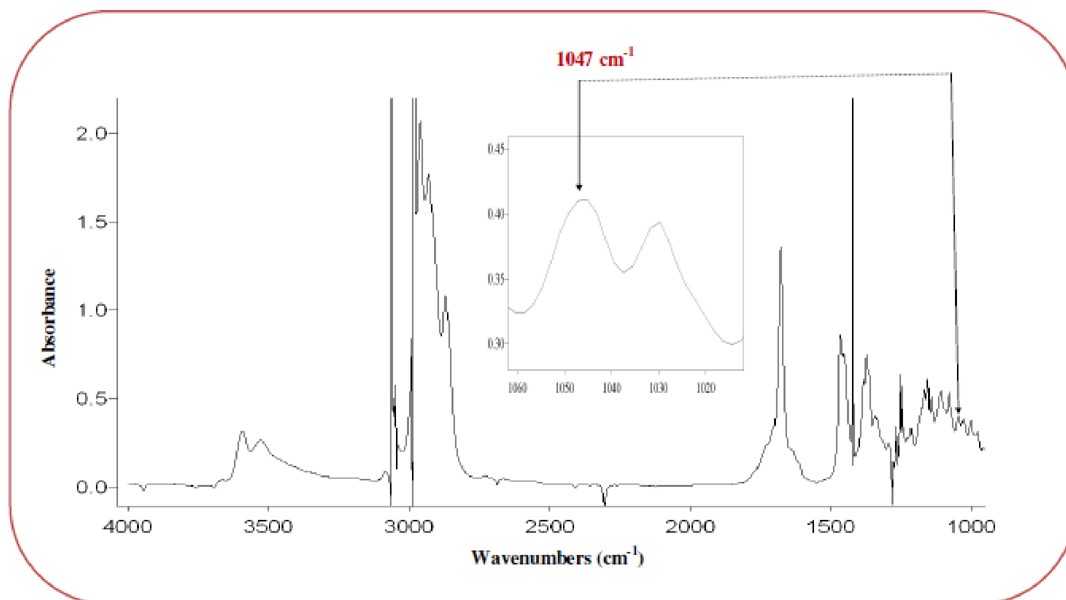

Figure 3: FTIR spectrum of 100 mg mL^{-1} of the *Asparagopsis armata* Harvey extract after treatment and EPS extraction and elution with 1 mL of CH_2Cl_2

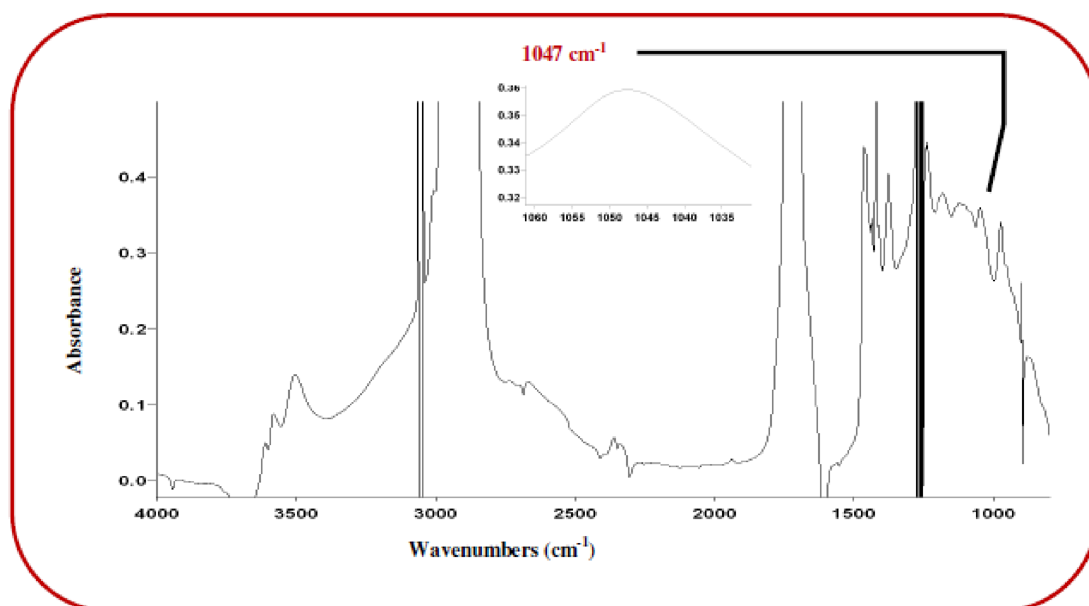

Figure 4: FTIR spectrum of 100 mg mL^{-1} of the *cystoseira sedoides* extract after treatment and EPS extraction and elution with 1 mL of CH_2Cl_2

Table 2 show that for the pair fucosterol and cholesterol respectively, the coefficient was stable and about 1,26.
Sample extracts submitted to the treatment, described in the experimental section, provided a concentrations of free sterol in the extracts of *Asparagopsis armata* and *Cystoseira sedoides,* of 1.9 ± 0.5 % and 1.5 ± 0.4 %, respectively. These results were obtained by making three independent analysis.

The developed FTIR- SPE method is suitable for the quantification of free sterols in red and brown algae and contributes to the broad fields of green analytical chemistry by reducing the amount of reagents and organic solvents and provides a relatively short time treatment and low cost with minimum of energy consumption. However, it is clear that due to their high transparency, chlorinated solvents are required. However, all employed solutions were distilled at the end of their use to recover the CH_2Cl_2 and $CHCl_3$. Additionally, the use of stopped-flow strategy for sample and standards measurements offers an excellent way for the on-line process of wastes as suggested in previous studies [21, 22].

Table 2: Evaluation of the correction coefficient obtained to determine fucosterol using cholesterol calibration lines

Regression line of fucosterol	*Regression line of cholesterol*	*Correction coefficient*	
0.000148 +0.018708C	0.000587 + 0.014882C	1.2571	1.259 ± 0.003
0.000357 +0.018754C	0.000729 + 0.014890C	1.2595	
0.000405 +0.018552C	0.000620 + 0.014807C	1.2529	
0.000040 +0.018647C	0.000691 + 0.014782C	1.2615	
0.000307 +0.018755C	0.000688 + 0.014764C	1.2599	
0.000488 +0.018758C	0.000540 + 0.014879C	1.2607	

Conclusions

On the basis of our present results, as well as the previous ones[19, 20], The predominant free sterols were cholesterol in red seaweed (56 % of total sterols content) and fucosterol in brown brown seaweeds (96 % of total sterols content). The developed procedure provides a sensitivity enough methodology to quantify the total concentration of free sterols in algae which offers an alternative to the classical chromatographic approaches for screening purposes thus being a fast screening methodology.

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