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FTIR-determination of sterols from the red alga *Asparagopsis armata*: Comparative studies with HPLC

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Abstract

An analytical procedure was developed for the determination of the total amount of sterols in the red alga *Asparagopsis armata*, globally determined as cholesterol, which is the major sterol contained in red algae. Samples, previously saponified with KOH were preconcentrated on DSC-18 solid phase cartridges (SPE) and eluted with dichloromethane stabilized with β-amylene. Fourier transform infrared (FTIR) spectrometry was employed for selective detection at 1049 cm^{-1} with a baseline established between 1000 and 1079 cm^{-1}. The results were compared to those obtained by high performance liquid chromatography (HPLC). The concentration obtained in actual samples from alga was 3.37% (w/w) by FTIR and 3.30% (w/w) by HPLC, showing a good comparability between the two methods.

Keywords: Fourier transform infrared; Solid phase extraction; High performance liquid chromatography; Sterol determination; Red alga; *Asparagopsis armata*

1. Introduction

Many studies on the distribution of sterols in the various classes of algae had been described [1–5]. The composition of sterols provides interesting information for both chemotaxonomic and ecological purposes [2,6]. A cursory literature survey revealed that the major sterol of red algae is generally cholesterol and in some ones cholesterol was the only sterol present [1,6].

Currently, the typical sterolic isolation procedure from algae is based on extraction of the crude lipid fraction with a mixture of methylene chloride and methanol followed by saponification [7]. The unsaponifiable fraction is generally analyzed by chromatographic methods. Gas chromatography is very useful for the determination of the composition of sterols within the sterolic fraction [8–11]. Additionally, normal [12] and reversed-phase high performance liquid chromatography (HPLC) [13–15] have also been used to separate and purify the sterolic fraction from algae.

Recent efforts in the discovery of new sterols from algae have focused on the development of isolation procedures, purification techniques and on the use of different analytical methods [16–18]. However there are few precedents on the use of Fourier transform infrared spectrometry (FTIR), which has generally been used as complementary method for identification of the sterols from algae. Sekkal et al. used infrared spectrometry as a basic technique for the elucidation of the structure of a polysaccharide from red algae [19]. This is the single application of infrared spectrometry in this field.

The main objective of the present study has been the development of a new procedure for the determination of total sterols content in the red alga *Asparagopsis armata* by FTIR spectrometry after separation using solid phase extraction cartridges.

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As the cholesterol is the major sterol in the sterolic fraction of the liquid extract of this alga [20], sterols were globally determined as cholesterol. Additionally, the results obtained by FTIR spectrometry were carefully checked and compared with those obtained by HPLC.

2. Experimental

2.1. Apparatus and reagents

A Mattson (Madison, WI, USA) Model Genisis II FTIR spectrometer was employed to obtain FTIR spectra in the range 4000–400 cm^{-1}. The system was equipped with a temperature-stabilized detector with KBr beam splitter and precise digital signal processing (DSP). Spectra were obtained at 4 cm^{-1} nominal resolution and accumulating 15 scans per spectrum. All measurements were carried out using a micro-flow cell with CaF_2 windows and a path length of 0.5 mm. Connection tubes were PTFE with 0.8 mm internal diameter.

Separations were carried out on a high performance liquid chromatograph system consisting of a Constametric (Paris, France) 3500 solvent delivery system, a manual injection valve with a volume loop of 20 μl and a Waters (Paris, France) model R 10 differential refractometer for the detection. Retention times and peak areas were obtained with a Varian 4400 integrator. For normal phase analysis, the Purospher® Star Si (4.6 mm i.d. × 250 mm) analytical column was employed.

Cholesterol, cholesteryl acetate and 4-methoxyacetophenone standards were obtained from Merck (Darmstadt, Germany). Ethyl acetate (EtAcO) and 2,2,4-trimethylpentane (TMP) (isooctane) HPLC grade, and analytical reagent grade methanol and chloroform were obtained from Carlo Erba (Milano, Italy), analytical reagent grade dichloromethane, stabilized with amylene or with ethanol, from Panreac (Barcelona, Spain) and ethanol, from Prolabo (Fontenay-sous-Bois, France) were also employed.

For preconcentration of the sterols, commercially available 6 ml DSC-18 octadecyl cartridges from Supelco (Bellefonte, USA), containing 1 g of solid phase with an average particle size of 50 μm and specific surface area of 480 $m^2\ g^{-1}$ were employed.

3. General procedure

3.1. Sample pretreatment

Samples of red alga (*A. armata*) were collected in June 2002 from the southern Mediterranean coast of Algeria, in Tipaza village. The alga was washed with water, spin dried, shade dried and all foreign materials were removed by hand.

Two hundred grams of algae were soaked with 2 l of methanol and chloroform 1:1 (v/v) mixture. The solution was filtered over celite, with an average porosity of 30 μm, under reduced pressure and evaporated to dryness. The procedure was repeated three times on the algal residue. The organic extract obtained was saponified before analysis by HPLC and FTIR spectrometry.

3.2. Saponification procedure

As sterol esters are sensitive to saponification with strongly basic reagents, a precise amount of organic extract was heated to reflux with 40 ml of 1 M ethanolic KOH at 90 °C for 1 h, then 40 ml of distilled water were added and the reaction mixture extracted by solid phase extraction (SPE) before its determination by FTIR or HPLC.

3.3. FTIR determination of sterols

One hundred milligrams of organic extract, previously saponified were loaded into 1 g of solid phase octadecyl silica DSC-18 extraction cartridges, preconditioned sequentially with 2 ml of methanol and 1 ml of water and eluted with four portions of 1 ml of dichloromethane stabilized with amylene. The residue was dried by using Na_2SO_4 and evaporated to dryness. The residue was diluted in 1 ml dichloromethane and the IR spectra were obtained at a 4 cm^{-1} resolution and accumulating 15 scans per spectrum, using a background of a dichloromethane blank obtained under the same experimental conditions.

The peak height of spectra was obtained by measuring the absorbance at 1049 cm^{-1} with a base line established between 1079 and 1000 cm^{-1}. Corrected absorbance values were interpolated in the corresponding calibration line obtained for mixture solutions containing 0.8–25 mg ml^{-1} of standards prepared and treated in the same way as the samples.

3.4. Solid phase extraction and HPLC determination of sterols

The chromatographic separation of organic extracts was carried out in the isocratic mode using a mixture of 2,2,4 trimethylpentane and ethyl acetate in 87:13 (v/v) proportion as the mobile phase and a flow rate of 1 ml min^{-1}. Twenty microliters of samples or standards were injected with 4-methoxyacetophenone as an internal standard. For standardization, 2.66–6 mg ml^{-1} solutions of cholesterol in EtAcO were used with 4-methoxyacetophenone 2 mg ml^{-1} in EtAcO as an internal standard. Under identical conditions, the extract of the alga was mixed with 20 μl of internal standard and diluted with EtAcO. This solution was injected directly into the HPLC system.

One hundred and ninety milligrams of organic extract, previously saponified was passed through a DSC-18 extraction cartridge, eluted with four portions of 1 ml of dichloromethane stabilized with amylene. The residue was dried with Na_2SO_4 and evaporated to dryness. 36.6 mg of the residue obtained were dissolved in EtAcO to a final volume of exactly 458 μl, by using a micropipette of 1000 μl

(±0.6). Twenty microliters of this solution were injected into the HPLC system.

4. Results and discussion

4.1. FTIR spectra of cholesterol and cholesteryl acetate

Fig. 1 shows the FTIR spectra of standard solutions of cholesterol (Fig. 1a), cholesteryl acetate (Fig. 1b) and that of a sample extract of alga (Fig. 1c) in CH_2Cl_2. As can be seen, in the region between 2935 and 2850 cm^{-1} all spectra show intense bands due the $–CH_2–$ and $–CH_3$ groups. Cholesterol present also an intense band at 1049 cm^{-1} that corresponds to the secondary C–OH. The –OH group of cholesterol and bands at 3602 and 3679 cm^{-1}. Cholesteryl acetate has an intense band at 1725 cm^{-1}, characteristic of the carbonyl group, and the acetate group provide a C–O stretching band at 1261 cm^{-1} and a second IR O–C band at 1032 cm^{-1} [21]. This specific band of cholesteryl acetate at 1032 cm^{-1} disappears after the saponification reaction to afford the characteristic bands of cholesterol at 1049, 3602 and 3679 cm^{-1}.

On comparing the spectra of standard solutions (Fig. 1a) with those of a real sample (Fig. 1c), it can be seen that the direct determination of cholesterol in red alga by FTIR spectrometry could be difficult without a previous isolation and preconcentration process (see Fig. 2a). Therefore, in this study where the sample is very complex, the absorbance measurements at 1049 cm^{-1} were obtained after saponification then preconcentration of the unsaponificable fraction. Absorbance values for both, samples and standards were corrected, with a base line established between 1079 and 1000 cm^{-1}.

4.2. Solid-phase extraction FTIR spectrometric determination of cholesterol

In order to made the determination of cholesterol levels in red alga by FTIR the analytical features of different calibration lines obtained in either CH_2Cl_2 or $CHCl_3$ stabilized with either β-amylene or ethanol were obtained. As see in Table 1, CH_2Cl_2 stabilized with amylene is an appropriate solvent, providing a low limit of detection (about 10 μg ml^{-1}), an extremely good variation coefficient of the absorbance signals of 1.2% and a good linearity of the calibration lines.

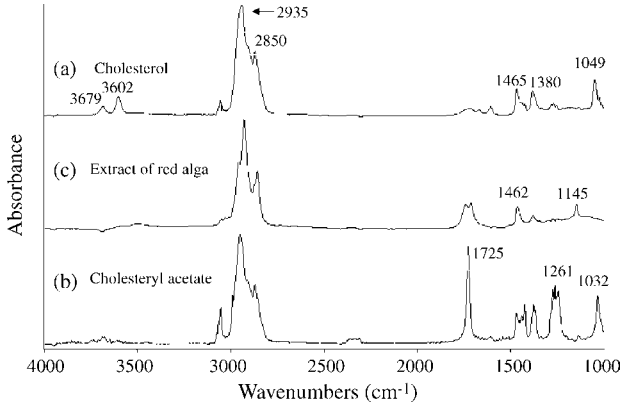

Fig. 1. FTIR absorbance spectra of: cholesterol 10 mg ml^{-1} (a); cholesteryl acetate 10 mg ml^{-1} (b), and an extract of red alga containing 100 mg ml^{-1} (c) dissolved in CH_2Cl_2 without any pre-treatment.

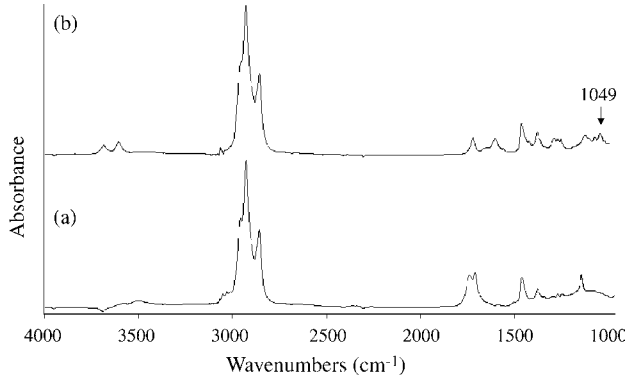

Fig. 2. FTIR spectra for 100 mg for a real sample of the extract of the red alga *A. armata*, dissolved in CH_2Cl_2 without any pre-treatment (a), and after saponification and preconcentration in a DSC-18 cartridge and elution with CH_2Cl_2 stabilized with amylene (b).

As it can be seen in Fig. 2, the direct determination of cholesterol from an extract of red algae dissolved in CH_2Cl_2 is not possible due to the presence of cholesteryl acetate and other matrix components. The saponification of the extract transforms cholesteryl esters to free cholesterol

Table 1
Analytical features obtained for cholesterol determination by FTIR using different solvents

Solvent/stabilizer	Parameters	
CH_2Cl_2/amylene	Regression line	0.00234 + 0.01743*C*
	Correlation coefficient (*r*)	0.9999
	L.D. (μg ml^{-1})	10.5
	R.S.D.% (*n* = 10)	1.2
CH_2Cl_2/ethanol	Regression line	0.00228 + 0.01660*C*
	Correlation coefficient (*r*)	0.9999
	L.D. (μg ml^{-1})	21.9
	R.S.D.% (*n* = 10)	0.19
$CHCl_3$/amylene	Regression line	0.00225 + 0.01455*C*
	Correlation coefficient (*r*)	0.9998
	L.D. (μg ml^{-1})	18.2
	R.S.D.% (*n* = 10)	0.11
$CHCl_3$/ethanol	Regression line	0.00892 + 0.00886*C*
	Correlation coefficient (*r*)	0.9985
	L.D. (μg ml^{-1})	37.0
	R.S.D.% (*n* = 10)	2.3

C: concentration in mg ml^{-1}; R.S.D. (in %): relative standard deviation (*C* = 10.28 mg ml^{-1}). L.D.: limit of detection expressed in μg ml^{-1} and established for *k* = 3 (a probability level 99.6%). *Experimental conditions*: Measurements were carried out at a nominal resolution of 4 cm^{-1}, 15 scans were accumulated and 0.5 mm optical path length and CaF_2 were used. A band at 1049 cm^{-1} was selected and the baseline was established between 1079 and 1000 cm^{-1}.

Table 2
Comparison of calibration plots obtained for cholesterol in CH_2Cl_2 and for a mixture of cholesterol and cholesteryl acetate after saponification and SPE in BondElut C18 cartridge

Parameters	Cholesterol: direct CH_2Cl_2 solution	Mixture: after saponification and SPE
Regression line	0.00289 + 0.01772*C*	0.00111 + 0.01149 C
Correlation coefficient (*r*)	0.9999	0.9970
L.D. ($\mu g\,ml^{-1}$)	11.1	51.2
R.S.D.% ($n = 10$)	0.25	0.33
Concentration range ($mg\,ml^{-1}$)	0.7–25	

C: concentration in $mg\,ml^{-1}$; R.S.D. (in %): relative standard deviation ($C = 10.28\,mg\,ml^{-1}$ for cholesterol and $12\,mg\,ml^{-1}$ for the mixture); L.D.: limit of detection expressed in $\mu g\,ml^{-1}$ and established for $k = 3$ (a 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 a mixture of cholesterol and cholesteryl acetate in the proportion 9:1 previously saponified, preconcentrated on the cartridges and eluted off-line with CH_2Cl_2. *Experimental conditions*: Measurements were carried out at a nominal resolution of $4\,cm^{-1}$, 15 scans were accumulated and 0.5 mm optical path length and CaF_2 were used. A band at $1049\,cm^{-1}$ was selected and the baseline was established between 1079 and $1000\,cm^{-1}$.

but it produces a dilution of the analyte and the aqueous medium is not suitable for the direct measurement by FTIR. In this sense we have used the solid phase extraction and elution with CH_2Cl_2 as a separation and preconcetration technique for total cholesterol analysis after the previous saponification.

It has been reported in the literature high differences in the sterol composition of algae, which could be explained by seasonal or geographical changes [4,22]. So for the evaluation of the saponification process and the SPE and FTIR determination of cholesterol, aqueous solutions spiked with cholesterol and cholesteryl acetate in an arbitrary proportion 9:1 (w/w) were saponified and extracted through BondElut C18 cartridges. Results found for samples were interpolated in a calibration line obtained for standards of cholesterol directly prepared in CH_2Cl_2.

Results summarized in Table 2 show that the IR determination of cholesterol in CH_2Cl_2, using absorbance peak height values at $1049\,cm^{-1}$ indicates an incomplete extraction of this compound from the unsaponifiable phase of treated standards. In this context, it must be noted that the equation of aqueous solutions of a mixture was compared with that obtained for cholesterol standard dissolved in CH_2Cl_2 in order to obtain the total amount of both sterols, free and linked. In fact the recovery of cholesterol could be estimated on 65% from the comparison of the two obtained slope values of the calibration lines.

4.3. Selection of conditions for solid phase extraction of cholesterol and cholesteryl acetate

A series of spiked samples, containing $10.0\,mg\,ml^{-1}$ cholesterol or $11.1\,mg\,ml^{-1}$of a mixture of cholesterol and cholesteryl acetate were analyzed using BondElut C18 as solid phase extraction and different solvents for the elution process. The average yields obtained from the solid phase extraction-elution process by using CH_2Cl_2 stabilized with β-amylene as solvent of elution were 70 ± 6% for cholesterol and 66 ± 5% for the mixture of cholesterol and acetate. CH_2Cl_2 stabilized with ethanol give 74 ± 7% for cholesterol and 61 ± 4% for the mixture. Data found by using $CHCl_3$ stabilized with β-amylene and $CHCl_3$ were 54 ± 6 and 51 ± 4% for cholesterol and mixtures with cholesteryl acetate and these found for $CHCl_3$ with ethanol were 55 ± 3 and 46 ± 7%, respectively, thus, it indicates that dichloromethane is a suitable solvent for both FTIR determination and SPE of cholesterol and its ester.

To select the most appropriate solid phase for extraction of the total sterolic fraction from unsaponifiable phase, different commercial C18 (Octadecyl) SPE cartridges were tested: BondElut C18, DSC-18 and C18, being found extraction yields of 72 ± 4, 92 ± 7 and 68 ± 4% respectively. Thus it can be see that only DSC-18 cartridges retain quantitatively the total sterolic fraction from the unsaponifiable phase, being also found by those cartridges an average extraction field of 92 ± 8% on considering mixtures of cholesterol and cholesteryl acetate.

4.4. FTIR determination using preconcentration by SPE

After saponification reaction of a mixture of cholesterol and cholesteryl acetate in 9:1 proportion, as described in Section 2, the cholesterol was extracted and preconcentrated by using solid phase extraction cartridges with 1 g of a DSC-18 phase, previously conditioned with methanol and water. Results obtained by "in batch" elution of loaded cartridges with four portions of 1 ml of CH_2Cl_2 stabilized with amylene demonstrated that the total efficiency of the retention-elution process corresponded to 86%. These results were obtained by comparison of the calibration lines obtained with CH_2Cl_2 solutions of cholesterol and with aqueous solutions of a mixture previously saponified, preconcentrated on the cartridges and eluted off-line with CH_2Cl_2. Results obtained evidence the need to prepare the standards in the same way than samples in order to assure a quantitative determination of cholesterol from the esterified fraction.

4.5. Determination of total sterols in real samples of red alga by FTIR

One hundred milligrams of the extract of the red alga, *A. armata*, previously filtered and prepared as described in Section 2 were analyzed by FTIR using the stopped-flow mode. The FTIR spectrum of a real sample obtained after treatment is shown in Fig. 2b. The real samples of extract submitted to the same treatment as the standard solutions containing cholesterol and cholesteryl acetate 9:1 (w/w) provided a concentration of sterol in the extract of red alga of 3.37 ± 0.01%, which shows a very good precision.

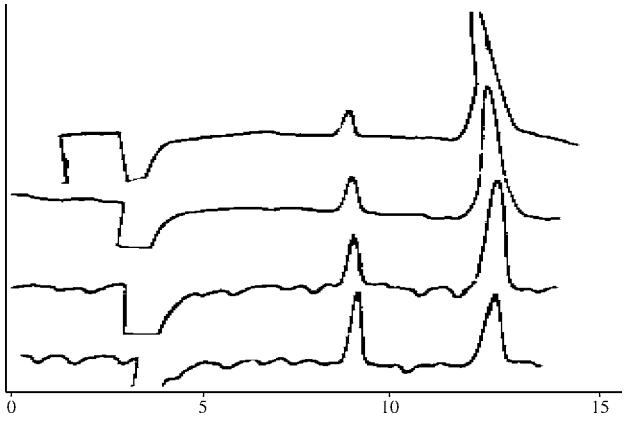

Fig. 3. Chromatograms of a series of four standards containing cholesterol and internal standard (4-methoxy acetophenone). A 20 μl volume was injected into a purospher® Star Si (4.6 mm i.d. × 250 mm) analytical column. A flow of 1 ml min^{-1} of TMP:EtOAc 87:13 (v/v) was used for separation and differential refractometer was used as detector.

4.6. Comparison of the concentration of sterols in red alga obtained by FTIR and HPLC

To confirm the accuracy of the FTIR procedure high performance liquid chromatography was applied to the determination of sterols in the red alga, following the procedures given in Section 3.4. Fig. 3 shows the chromatograms obtained for a series of four standards containing cholesterol, which was eluted at 12.2 min, and 4-methoxy acetophenone, as internal standard, which was eluted at 8.8 min.

The regression equation, between the ratio of peak area values found for cholesterol and internal standard can be described by $C = 1.29A + 0.211$, with a regression coefficient of 0.9987 in the concentration range of cholesterol from 2.66 to 6 mg ml^{-1}.

Fig. 4a shows the chromatogram obtained for a real sample of extract of red alga dissolved directly in EtAcO and a chromatogram of the extract prepared for the determination of total sterols after saponification and SPE (Fig. 4b). From these, data concentration of 1.5 ± 0.4 and 3.3 ± 0.2% were found for free cholesterol and total cholesterol, respectively.

The concentration obtained by HPLC and FTIR in red alga were 3.3 ± 0.2 and 3.37 ± 0.01%, respectively. These are in excellent agreement.

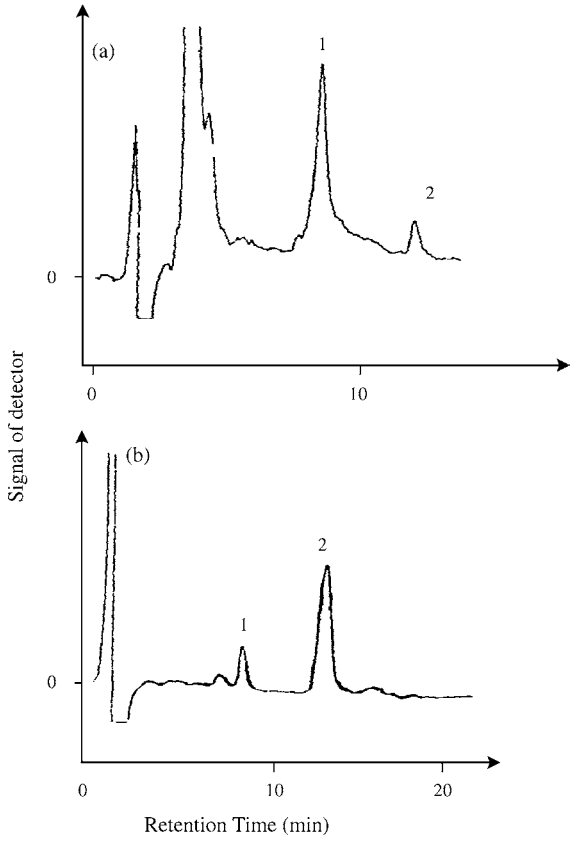

Fig. 4. Chromatograms for real samples of the extract of red algae *A. armata*: without any pretreatment (a) and after saponification and SPE in a DSC-18 cartridge (b). (1) is the peak of the internal standard, and (2) the peak of the sterol.

5. Conclusion

The studies carried out have shown that total sterols in extract of the red alga *A. armata* can be determined by FTIR after saponification and solid phase extraction through DSC-18 cartridges. The determination can be carried out at the absorbance of 1049 cm^{-1} corrected with a baseline established between 1079 and 1000 cm^{-1}.

The method proposed in this paper is easy, rapid, reproducible and provides similar values to those obtained by HPLC.

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