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Quantitative Analysis of Free Phytosterols by UV-Vis Spectrometry using Two Methods of Extraction in Marine Macroalgae

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Abstract

Objective: The present study was performed to evaluate the presence and concentration of free phytosterols by UV-Vis spectrometry using two extraction methods in marine macroalgae of the Caribbean.

Methods: The algae were collected, dried and homogenized. A portion of the material was extracted with a reflux system with dichloromethane, another portion was macerated with ethanol for three days and concentrated in vacuum. Both samples were centrifuged and phytosterols were quantified by spectrophotometric analysis with the Liebermann-Burchard method.

Results: Two Phaeophytas algae, *Spatoglossum schroederi* (5.35 μg/mg with CH_2Cl_2 and 3.85 μg/mg with ethanol) and *Sargassum* sp (3.35 μg/mg with CH_2Cl_2 and 2.98 μg/mg with ethanol) showed the results with the highest amounts of sterols. Values around

1 μg/mg were reported for *Gracilaria* sp, *Caulerpa racemosa*, *Ulva lactuca* and *Padina pavonica*. Rhodophyta algae *Galaxaura rugosa* reports the lowest presence of sterols by both extraction techniques (0.15 μg/mg with CH_2Cl_2 and 0.26 μg/mg with ethanol).

Conclusions: The results of the extraction with CH_2Cl_2 and ethanol showed to be statistically equivalent (t_{exp}=2.25, $p<0.05$), suggesting the possibility of replacing the use of a halogenated solvent with another with lower environmental impact for the recovery of sterols.

Keywords: Phytosterols, marine algae, UV-VIS spectrophotometry, Liebermann-Burchard.
JEL Code: N56

1. Introduction

Seaweeds are a promising source of natural products with interesting activities. They have been used as food in the diet for centuries, due to their content of carotenoids, fiber, proteins, unsaturated fatty acids, vitamins, and minerals (Makkar et al., 2016; Kumar & Pal, 2016; Gaillande et al., 2017). Many substances obtained from algae have been used in medicine and pharmacy, such as its phycocolloids, among multiple applications are mentioned for alginic acid, carrageenans and agar (Pawar & Edgar, 2012; Necas & Bartosikova, 2013; Zia et al., 2017; Capillo et al., 2017).

The nutraceutical potential of marine algae has recently been studied (Tanna et al., 2018) Phytosterols are important structural components in the cell membranes of algae, they have an important role during their development in metabolism. They have been associated with health benefits, especially in the reduction of cholesterol levels in the blood, related to a lower incidence of cardiovascular diseases (Kritchevsky & Chen, 2005; Chen et al., 2008; Kamal-Eldin & Moazzami, 2009; El Shoubaky & Salem, 2014; Muñoz & Ramos, 2016). They are also considered essential nutrients that promote functioning optimal defense mechanisms of the body and present anti-inflammatory effect (Kumar, 2017).

There are many analytical methods that have been reported for the quantification of phytosterols in vegetal material, mainly by HPLC (coupled to UV and/or MS detector) and GC (coupled to FID and/or MS detector) (Kamenarska et al., 2003; Hassan and Chin, 2006; Shah et al., 2010; Gachumi & El-Aneed, 2017; Lee et al., 2018; Chen et al., 2018); there are also reports of the use of nuclear magnetic resonance for its detection (Zhang et al., 2016). These methods have certain disadvantages related to the complexity in the preparation of samples and the high costs of the equipment associated. One of the analytical techniques that avoids these factors is UV-Visible spectrophotometry, widely used for decades in the quantification of secondary metabolites (Kritchevsky & Tepper, 1979; Swift, 1984; Nariya et al., 2014). It is still used in the estimation of metabolites in plants and marine organisms due to its simplicity and its low cost of implementation (Araújo et al., 2013; Marques et al., 2013; Park et al., 2016). The spectrophotometric test that has been most used for the determination of phytosterols is Liebermann-Burchard, which uses the combination of sulfuric acid and acetic acid for the oxidation of sterols, generating colored compounds that can be quantified at 650 nm (Liebermann, 1885; Xiong et al., 2007; Anasane & Chaturvedi, 2014).

Due to the attractive properties that phytosterols present in the marine flora, the objective of this investigation is to quantify free sterols by UV-Vis spectrometry using two methods of extraction (with halogenated and non-halogenated solvent) in seven marine Caribbean macroalgae: Rhodophyceae (*Galaxaura rugosa* and *Gracilaria* sp), Phaeophyceae (*Spatoglossum schroederi*, *Padina pavonica* and *Sargassum* sp) and Chlorophyceae (*Caulerpa racemosa* and *Ulva lactuca*).

2. Materials and Methods

2.1. Material Collection and Identification

The algae were collected in the intertidal zone of the Caribbean coast during low tide. The specimens were carefully removed from the coral substrate, washed with seawater, placed in polypropylene containers, and finally transported to the laboratory at an average temperature of 4 °C. The material was cooled during transportation to prevent decomposition and possible decomposition of metabolites (Haüssermann, 2004; Sánchez-Rodríguez et al., 2006; Borbón et al., 2016). A sample of each species was preserved in 10% formaldehyde for its identification with Taylor's taxonomic guide (Taylor, 1979) and the report by Soto and Ballantine (1986).

2.2. Material Preparation and Extraction

Two extraction methods were carried out for the sterols: extraction by reflux with CH_2Cl_2, a method adapted from Araújo et al. (2013); and extraction by ethanolic medium proposed by students of the Biochemistry Laboratory, National University (UNA). Table 1 shows the quantities that were measured for each species by each of the methods.

Table 1: Sample mass for each extraction method

Specie	CH_2Cl_2 Reflux (±0.1 g)	Ethanolic Extract (±0.1 g)
Galaxaura rugosa	72.9	72.9
Gracilaria sp	39.2	42.9
Spatoglossum schroederi	11.3	11.2
Padina pavonica	17.6	26.9
Sargassum sp	27.4	27.8
Caulerpa racemosa	26.3	30.7
Ulva lactuca	31.4	31.4

The algae material was dried at room temperature, cut, and milled, then placed with 100 - 150 mL of CH_2Cl_2. They were extracted with reflux for 1 hour at 50 °C, then cooled to room temperature (25 °C) and the extract obtained was collected. A second extraction was carried out and both were filtered with cotton to remove any solid residue present. This filtered was concentrated under reduced pressure on a rotary evaporator at 40 °C, the residue was centrifuged at 8000 g for 15 min to obtain a clarified supernatant, which was transferred to a 50 mL volumetric flask, completing the volume with CH_2Cl_2 (Araújo et al, 2013).

For the ethanolic extraction, between 100 and 150 mL of ethanol was added to the samples, and they were macerated for three days. The ethanolic extract obtained was filtered to remove solid residues, the filtrate was concentrated under reduced pressure on a rotary evaporator at 50 °C. The residue was resuspended in 30 mL of ethanol that was subsequently centrifuged at 8000 g for 15 min to obtain a clarified supernatant. This was transferred to a 50 mL volumetric flask, adjusting its volume with ethanol.

2.3. UV-Vis Spectrophotometric Analysis

For the calibration curve, cholesterol standards were prepared in glacial acetic acid, with concentrations between 0 and 10 mg/mL. Liebermann-Burchard reagent was added (6 mL), they were incubated for 10 min in a water bath at 37 °C. The absorbance was recorded in UV-Vis equipment Thermo Scientific® at 650 nm, in a time range no longer than 10 min after the end of the incubation. Samples were prepared in 16 x 100 mm test tubes, taking 5 mL aliquot for each extract. A previous heating allowed the evaporation of the CH_2Cl_2 and the ethanol from each tube, then 100 µL of glacial acetic acid was added and stirred, to finally 6 mL of the Liebermann-Burchard reagent was added and

homogenized in a Vortex. The samples were incubated for a period of 10 min in a water bath at 37 ° C and after cooling, their absorbances were recorded at 650 nm (Burke et al., 1974).

3. Results and Discussion

The results of total concentration of phytosterols in marine algae are reported in Table 2, obtained from the Liebermann-Burchard curve (y = 0.039x – 0.009; R^2 = 0.9970). The alga Phaeophyta *Spatoglossum schroederi* presented the highest content of sterols (5.35 and 3.85 µg/mL), followed by *Sargassum* sp (3.35 and 2.98 µg/mg). The presence of high concentrations of sterols in *Sargassum* has been reported by other types of techniques such as ^{1}H-NMR (Zhang et al., 2016). The Rhodophyta *Gracilaria* sp algae and the Chlorophyta *Caulerpa racemosa* and *Ulva lactuca* presented values close to 1 µg/mg, as well the alga Phaeophyta *Padina pavonica*. These are normal values reported for other marine organisms studied (Swift, 1984; Kanazawa, 2001; Bataglion et al., 2016). *Galaxaura rugosa* (Rhodophyceae) showed the lowest presence of sterols by both extraction techniques (≤ 0.3 µg/mg).

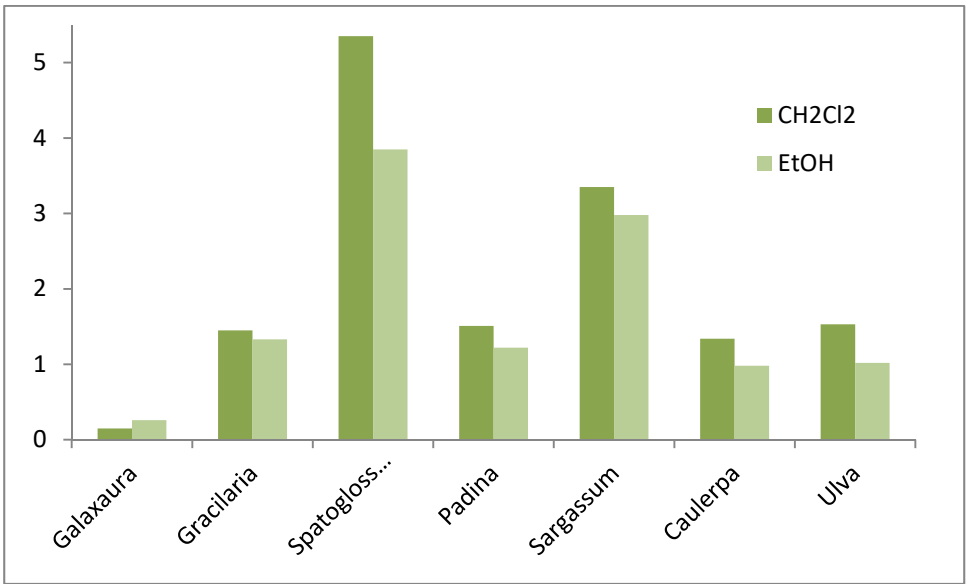
Table 2: Absorbance values and total concentration of phytosterols in algae by Liebermann-Burchard test using two extraction techniques

Especie	Abs $_1$	Abs $_2$	TCP $_1$	TCP $_2$
Galaxaura rugosa	0.034	0.068	0.15	0.26
Gracilaria sp	0.218	0.218	1.45	1.33
Spatoglossum schroederi	0.232	0.163	5.35	3.85
Padina pavonica	0.097	0.122	1.51	1.22
Sargassum sp	0.357	0.321	3.35	2.98
Caulerpa racemosa	0.131	0.111	1.34	0.98
Ulva lactuca	0.183	0.119	1.53	1.02

Abs = Absorbance (1 = CH_2Cl_2 extraction, 2 = ethanolic extraction)
TCP = Total Concentration of Phytosterols (µg/mg) (1 = CH_2Cl_2 extraction, 2 = ethanolic extraction)

Figure 1 shows a comparative graph of the phytosterols concentrations of each seaweed by the two extraction methods. A *t*-test for paired data did not show significant differences at 95% confidence (t_{exp} = 2.25), suggesting that both extractions generate equivalent results. Many studies have used extraction solvents for phytosterols that are highly toxic to the environment (Hsu et al, 2017; Uddin et al., 2018). Currently the trend is to use methods and solvents with less impact on the environment; in this way, the use of supercritical fluids such as CO_2 to extract sterols has been successfully tested (Hrabovski et al., 2012; Uddin et al, 2015). The efficiency of other less toxic solvents has also been reported, such as the use of ethanol, which has shown in some cases an equal or better extraction of sterols than hexane, petroleum ether, ethyl ether, ethyl acetate, chloroform and acetone (Dunford et al., 2009; Hussain & Mohamad, 2015).

Figure 1: Total concentration of sterols (µg/mg) of seven marine algae from the Caribbean obtained by two extraction methods.



A study published by Chen et al. (2014) reported phytosterols found in *Sargassum* sp, such as fucosterol and saringosterol, with a potential regulator of cholesterol metabolism in the body through the activation of receptors in the liver. These observations show the importance of investigating more exhaustively the marine biodiversity, to evaluate its potential use in beneficial applications for humans (Ito et al., 2017).

The results obtained in this study allow to establish a new alternative for the extraction of phytosterols in algal material with a solvent with low impact on the environment, in order to preliminarily know the content in the algae before subsequent identification. Marine organisms studied on the Caribbean coast have shown several interesting activities with promising biotechnological potential (Borbón et al., 2012; Rojas-Brenes et al., 2012; Borbón et al., 2017; Córdoba-Cháves et al., 2017), suggesting further exploration of their resources.

Summary and Concluding Remarks

A higher concentration of free sterols is reported in Phaeophyceae class over Rhodophyceae and Chlorophyceae classes. However, it must be taken into account that it is a preliminary study of free phytosterols, in order to identify and quantify each of the sterols present, more specific analytical techniques must be applied, such as HPLC-MS or GC-MS. The results show the possibility of using ethanol as an extraction solvent instead of CH_2Cl_2, avoiding the use and generation of waste of a halogenated solvent in teaching-research laboratories.

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Conflict of Interest Statement

We declare that we have no conflict of interest.

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