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FINAL DEGREE PROJECT IN MARINE SCIENCES

Natural products of cyanobacteria *Spirulina* spp. with deepening in *S. platensis* and its applicability in the pharmaceutical, cosmetic and nutraceutical sectors

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Abstract

Spirulina platensis is a cyanobacteria widely used as a dietary supplement due to its many beneficial applications for health and is of great interest in the pharmaceutical, food and cosmetic sectors. In the latter, the use of phycocyanin (a pigment present in this cyanobacteria) as a natural dye, which also has antioxidant, anti-cancer and anti-inflammatory properties, is very common.

The genus Spirulina is also of great interest because of the variety of macro and micronutrients it contains, some of which can not be synthesized by the human organism naturally.

The aim of this research is to deepen the chemical knowledge of the species S. platensis, using modern techniques of purification and structural analysis (chromatography, nuclear magnetic resonance (NMR) and mass spectroscopy (MS)).

The biomass of *S. platensis* was obtained by *Soxhlet* extraction with acetone, followed by filtration and concentration giving a dark product. By column chromatography, eluting with a combination of hexane/ acetone/ methanol, successive fractions of increasing polarity were obtained.

Pure metabolites were purified by preparative TLC and were identified by the spectroscopic methods (NMR and MS). Among the isolated metabolites are carotenoids, chlorophylls, fatty acids, xanthophylls, sulfoglycolipid (**II**) and a new compound, 4- [(2-methylheptyl) oxy] benzenesulfonic acid (platensic acid, **III**)

Keywords: *Spirulina platensis*, nutritional, metabolite.

1. Introduction

In modern therapy, natural products are important elements (molecules and mixtures made by living organisms). They are subdivided according to their function as antibiotics, vitamins, etc. or according to their chemical composition as polypeptides, polysaccharides, alkaloids, or terpenoids, for example. Natural products also represent a promising basis for the development of subtances with novel activities (Schwarzer *et al.*, 2003).

These products have been the source of active ingredients of drugs. Approximately 100 new products are in clinical development, particularly as antiinfectives and anticancer agents (Harvey, 2008).

Antimicrobial activity of certain fresh water microalgae from India was described (Prakash *et al.*, 2011). Also cyanobacteria is a source of structurally bioactive secondary metabolites that is receiving a lot of attention (Tan, 2007). It is also a very rich source of bioactive molecules with pharmaceutical importance (Dixit & Suseela, 2013). This research is based on the *Spirulina* genus. The term "Spirulina" has been widely used to refer to two species of cyanobacteria, *S. platensis* (*Arthrospira platensis*) *and S. maxima* (*Arthrospira maxima*), which are of great interest because they are grown to produce a large number of products that are attributed beneficial, nutritional properties and good for health (Ramírez-Moreno & Olvera-Ramírez, 2006). Thus, interesting glycolipids were isolated from *S. maxima* (Kataoka *et al.*, 1983).

Spirulina is of great interest in the food, pharmaceutic and cosmetic sectors, mainly for its protein content and also because of its pigments (Lupatini *et al.*, 2016).

Arthrospira platensis (blue-green algae) is commonly used as a dietary supplement. It is cultivated for consumption as food for humans and animals (Cheevadhanarak *et al.*, 2012). In addition, this cyanobacteria is an alternative for production of nutraceuticals and for treatment of certain diseases (Lupatini *et al*., 2016).

Phycocyanin, a pigment present in *A. plantensis*, is widely used in the cosmetic sector, as it is used as a natural dye, which also has anti-inflamatory, antioxidant and anticancer properties. This species is an excellent source of this pigment since his protein fraction contains about 200 g kg^{-1} phycocyanin (Lupatini *et al.*, 2016).

Acording to Lupatini et al. (2016), proteins from *A. platensis* can be hydrolyzed to bioactive peptides which can improve human health, besides the antioxidant, antiproliferative, anti-obesity properties, among others.

Spirulina spp. is of great interest because of the variety of macro and micronutrients it contains, some of which can not be synthesized naturally by the human organism, as well as being able to increase energy levels, reduced appetite and offer antioxidant protection, among others (Ramírez-Moreno & Olvera-Ramírez, 2006).

Spirulina lacks toxicity and has corrective properties against viral attacks, tumor growth, malnutrition and anemia (Thengodkar, & Sivakami, 2010). *A. platensis* contains a diversity of bioactive molecules which could improve human health (Winter *et al.*,

2014). In addition, numerous animal studies have described antioxidant (Wu *et al.*, 2016), antiviral (Pham & Durand-Chastel, 2003; Barron *et al.*, 2008), antibacterial (Elshouny *et al.*, 2017) and antiretroviral activity (Winter *et al.*, 2014). According to Bensehaila *et al.* (2015) and Juvekar and Nachankar (2005), *A. platensis* is also well known as a protein source of high biological value containing vital nutrients (mainly vitamin B_{12} and pro-vitamin A, minerals (especially iron) and α-linoleic acid (an essential fatty acid precursor for prostaglandins), reported to promote physical health, improve defense mechanisms of the human body and enhance longetivity of life. Also, *Spirulina maxima*¸ another species of the genus *Spirulina*, have been reported as insulin-containing photosynthetic organisms (Anwer *et al.*, 2011).

The purpose of this study is to find pure *A. platensis* products with special interest for use in pharmacy, cosmetics or nutraceuticals. For this, a rigorous laboratory work is required in which extraction, purification and structural elucidation of metabolites are carried out.

2. Experimental part

2.1. Chemicals

The solvents used such as hexane, dichloromethane, acetone, chloroform and methanol (Panreac brand) were technical grade and were purchased from Medina Cejudo (Las Palmas de Gran Canaria, Spain). The solvents for nuclear magnetic resonance spectroscopy were acquired from BioSigma (Santa Cruz de Tenerife, Spain). Pure water was obtained by reverse osmosis in a Milli-Q water system (Millipore, Bedford, MA, USA). The silica gel for column chromatography (*Scharlau*) was acquired from Melcan (Santa Cruz de Tenerife, Spain).

2.2. Isolation and identification of the cyanobacteria

The material, which was identified as *Arthrosgira platensis* [=*Spirulina platensis* (Gomont) Geitler, 1925 (Spirulinales, Spirulinaceae, *Spirulina*)], was obtained from GSN laboratories (General company of nutritional supplements), placed in Madrid, Spain. Samples of *A. platensis* were extracted by liquid solid extraction (*Soxhlet*). Photos of the species are provided in the *Figure 1*.

Figure 1.- Photos of *Spirulina platensis*

2.3. Obtaining crude Spirulina extract

The *Spirulina* powder is introduced into filter paper envelopes to be extracted by *Soxhlet* extraction. This consists of boiling a solvent (acetone) in a flask for 6-24 h until the natural products are separated from the solid sample. Several extractions were made. The solution is rotaevaporated (*BÜCHI Rotavapor R-114*) and carried almost to dryness. It is then left in the vacuum for 24 h. The extract is dissolved with chloroform and then silica gel is introduced to create the chromatographic head. Finally, it is rotaevaporated.

2.4. Apparatus and analytical methods

Analytical TLC was performed on silica gel plates (0.25 mm thickness) using a combination of hexane/ acetone at a ratio of 75:25 as the eluent. Spots were revealed by spraying with *Oleum* [sulfuric acid (4%) + acetic acid (80%) + water (16%)] and heated for 2-3 minutes.

Column chromatography was performed on silica gel (*Scharlau*) 0.06-0.02 mm particle sized and a 0.04-0.06 mm particle sized stationary phase. Hexane, acetone and metanol were used as the mobile phase and they were introduced using a motor (*Fluid Metering Inc.*). The motor was equipped with a controlling membrane to stabilize the pressure pulses.

Preparative TLC was performed on silica gel plates 20 x 20 cm (0.25 mm and 1 mm thickness). The sample is applied as a linear band across the width of the plate and analyzed by UV detection (254 and 365 nm). Finally, the plates were scraped to isolate the substance of interest. Both preparative TLC of the crude extract and the products obtained in the column chromatography were performed.

12.5 mg of crude extract per plate were applied on 8 silica gel plates and then eluted with hexane/ acetone in a ratio of 75:25 in a rectangular tank for 20 minutes. After eluting, the plates showed nine bands with different colours (carotenes, xanthophylls, chlorophylls, glycolipids, etc.) under natural light (*Figure 2*). They were analyzed by UV detection (254 and 365 nm) and the chromatographic parameters (Rf) were calculated. The nine bands were scraped with a spatula and extracted in Erlenmeyers flasks with acetone (x2), chloroform (x2) and methanol (x2). The bands which contained the same components were placed together.

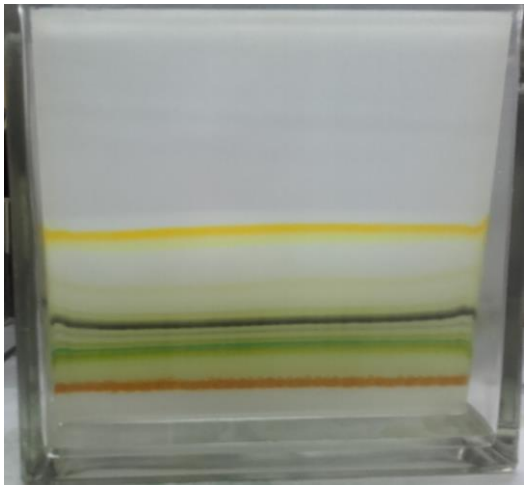


Figure 2.- Preparative TLC of crude *Spirulina* extract eluted with hexane/ acetone 75:25.

Preparative TLC of the products obtained from the chromatographic column were also performed. 50 mg of product were dissolved in 5 ml of chloroform and applied as a band across 4 silica plates (0.25 mm thickness) and other 100 mg of product were dissolved in 5 ml of chloroform and applied on 2 silica gel plates (1 mm thickness). Then, the procedure was the same as for the crude extract.

Nuclear magnetic resonance spectroscopy, most commonly known as NMR spectroscopy, is a research technique that exploits the magnetic properties of certain atomic nuclei. This type of spectroscopy determines the physical and chemical properties of the molecules in which they are contained. It relies on the phenomenon of nuclear magnetic resonance and can provide detailed information about the structure of molecules. The intramolecular magnetic field around an atom in a molecule changes the resonance frequency, thus giving access to details of the structure of a molecule and its individual functional groups.

Most frequently, NMR spectroscopy is used by chemists and biochemists to investigate the properties of organic molecules, although it is applicable to any kind of sample that contains nuclei possessing spin. NMR spectra are unique, well-resolved, analytically tractable and often highly predictable for small molecules. Thus, in organic chemistry practice, NMR analysis is used to confirm the identity of a substance. Different functional groups are obviously distinguishable, and identical functional groups with differing neighboring substituents still give distinguishable signals. A disadvantage is that a relatively large amount, 1–50 mg, of a purified substance is required, although it may be recovered through a workup. Preferably, the sample should be dissolved in a solvent, because NMR analysis of solids may not give equally well-resolved spectra. The timescale of NMR is relatively long, and thus it is not suitable for observing fast phenomena, producing only an averaged spectrum. NMR spectrometers are relatively expensive; universities usually have them, but they are less common in private companies. Modern NMR spectrometers have a very strong, large and expensive liquid helium-cooled superconducting magnet, because resolution directly depends on magnetic field strength. Less expensive machines using permanent magnets and lower resolution are also available, which still give sufficient performance for certain application such as quick checking of samples.

The vast majority of nuclei in a solution would belong to the solvent, and most regular solvents are hydrocarbons and would contain NMR-reactive protons. Thus, deuterium (hydrogen-2) is substituted (99+ %). The most used deuterated solvent is deuterochloroform ($CDCl_3$), although deuterium oxide (D_2O) and deuterated DMSO (DMSO-d_6) are used for hydrophilic analytes. The chemical shifts are slightly different in different solvents, depending on electronic solvation effects. NMR spectra are often calibrated against the known solvent residual proton peak or the added tetramethylsilane [$(CH_3)_4Si$].

3. Results and discussion

A thin layer chromatography (TLC) of the crude acetone extract of *A. platensis* is shown in *Figure 3*. Structural allocation was based on recently published data for *A. maxima* (Bravo de Laguna *et al.*, 2015).

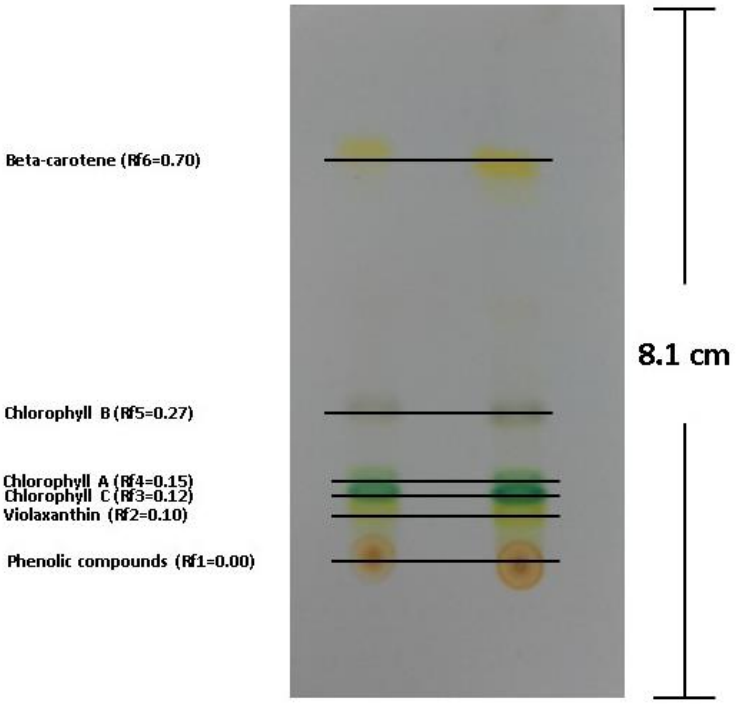


Figure 3.- TLC analysis of crude *A. platensis* extract. Plate of Silica gel (0.25 mm). Eluent: hexane-acetone (75:25).

TLC experiment (*Figure 3*) revealed six spots with six different Rf values. It was posible to identify: β-carotene (yellow spot, Rf_6=0.70), chlorophyll B (green spot, Rf_5=0.27), chlorophyll A (green spot, Rf_4=0.15), chlorophyll C (green spot, Rf_3=0.12), violaxanthin (yellow spot, Rf_2=0.10) and a mixture of phenolic compounds (red spot, Rf_1=0.00). These results were slightly different from those obtained by others investigators of our group for *A. maxima* (Bravo de Laguna *et al.*, 2015).

In order to confirm such structures by spectroscopic methods as well as to identify the minor components, column chromatography was performed through silicic acid, eluting with hexane and incremental amounts of acetone, up to 100% acetone, and then with acetone and incremental amounts of methanol, up to 100% methanol. This yielded 24 chromatographic fractions of progresive polarity, namely:

a) Nine products purified by preparative TLC from crude extract:
   Cree-S1 (C-1), Cree-S2 (C-2), ..., Cree-S9 (C-9)

b) Fifteen products purified by column chromatography:
   Sp-1, Sp-2, Sp-3, Sp-4, ….., Sp-15

Among these, the fractions Sp-1, Sp-3, Sp-4, Sp-5, Sp-6, Sp-7, Sp-8 and Sp-9 were selected for re-purification by column chromatography, of which fifty-two pure products came out (according to the analytical TLC):

Cree-Sp-1 (C-10),

Cree-Sp-3.1 (C-11), Cree-Sp-3.2 (C-12), Cree-Sp-3.3 (C-13), Cree-Sp-3.4 (C-14), Cree-Sp-3.5 (C-15), Cree-Sp-3.6 (C-16),

Cree-Sp-4.1 (C-23), Cree-Sp-4.2 (C-24), Cree-Sp-4.3 (C-25), Cree-Sp-4.4 (C-26), Cree-Sp-4.5 (C-27), Cree-Sp-4.6 (C-28),

Cree-Sp-5.1 (C-17), Cree-Sp-5.2 (C-18), Cree-Sp-5.3 (C-19), Cree-Sp-5.4 (C-20), Cree-Sp-5.5 (C-21), Cree-Sp-5.6 (C-22),

Cree-Sp-6.1 (C-29), Cree-Sp-6.2 (C-30), Cree-Sp-6.3 (C-31), Cree-Sp-6.4 (C-32), Cree-Sp-6.5 (C-33), Cree-Sp-6.6 (C-34),

Cree-Sp-7.1 (C-35), Cree-Sp-7.2 (C-36), Cree-Sp-7.3 (C-37), Cree-Sp-7.4 (C-38), Cree-Sp-7.5 (C-39), Cree-Sp-7.6 (C-40), Cree-Sp-7.7 (C-41), Cree-Sp-7.8 (C-42), Cree-Sp-7.9 (C-43),

Cree-Sp-8.1 (C-44), Cree-Sp-8.2 (C-45), Cree-Sp-8.3 (C-46), Cree-Sp-8.4 (C-47), Cree-Sp-8.5 (C-48), Cree-Sp-8.6 (C-49), Cree-Sp-8.7 (C-50),

Cree-Sp-9.1 (C-51), Cree-Sp-9.2 (C-52).

All these substances were sent to the Institute of Natural Products and Agrobiology (IPNA, Tenerife) of the Higher Council of Scientific Research (CSIC) for their spectroscopic analysis, obtaining, to date, the following results:

In *Figure 4* the ^{1}H-NMR spectrum of Cree-S1 (C-1) substance can be observed. Apart from the deuterated chloroform signal, at δ 7.26, signals corresponding to aromatic protons (δ 6.8-7.8), olefinic protons (δ 5.0-5.4), geminal to heteroatom protons (δ 3.4-4.3), and methylenes / methyls in the range δ 0.5-2.3 are observed. Since its colour is yellow-orange, it is understood that it is a β-carotene impurified with some aromatic substance. The product was discarded.

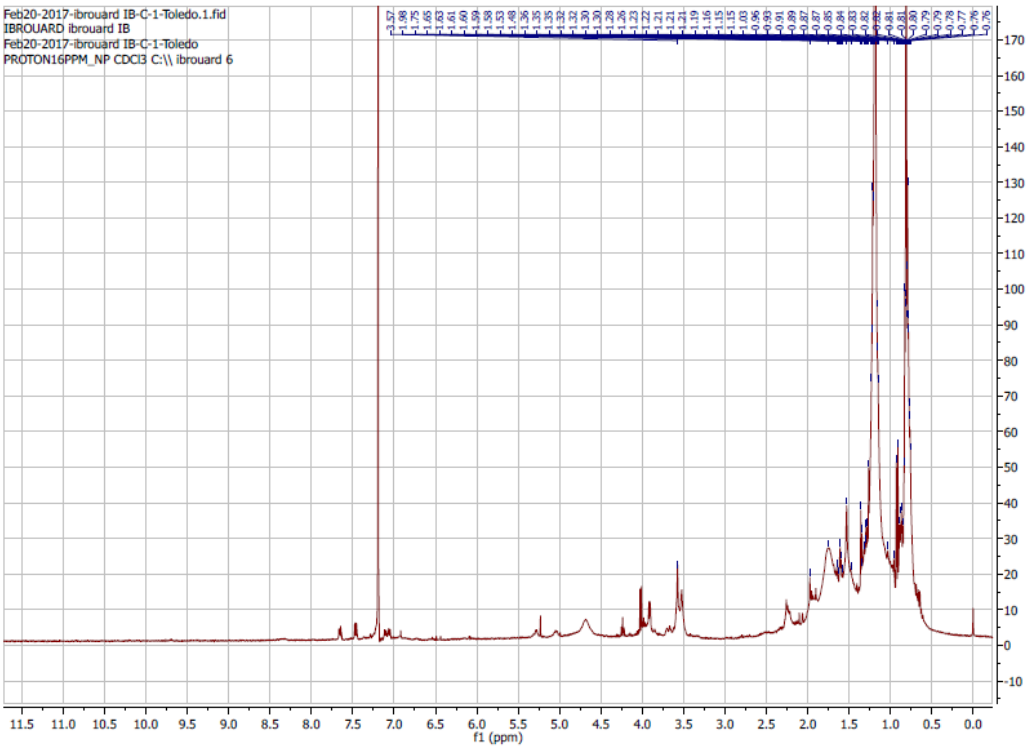


Figure 4.- ^{1}H-NMR spectrum of Cree-S1 (C-1) substance.

In *Figure 5*, the ^{1}H-NMR spectrum of Cree-S2 (C-2) substance can be observed. Apart from the deuterated chloroform signal, at δ 7.26, signals corresponding to aromatic protons (δ 6.8-7.8), geminal to heteroatom protons (δ 3.4-4.8), and methylenes / methyls in the range δ 0.5-2.5 are observed. Since its colour is yellow-orange, it is understood that it is a carotenoid impurified with some aromatic substance. The product was discarded.

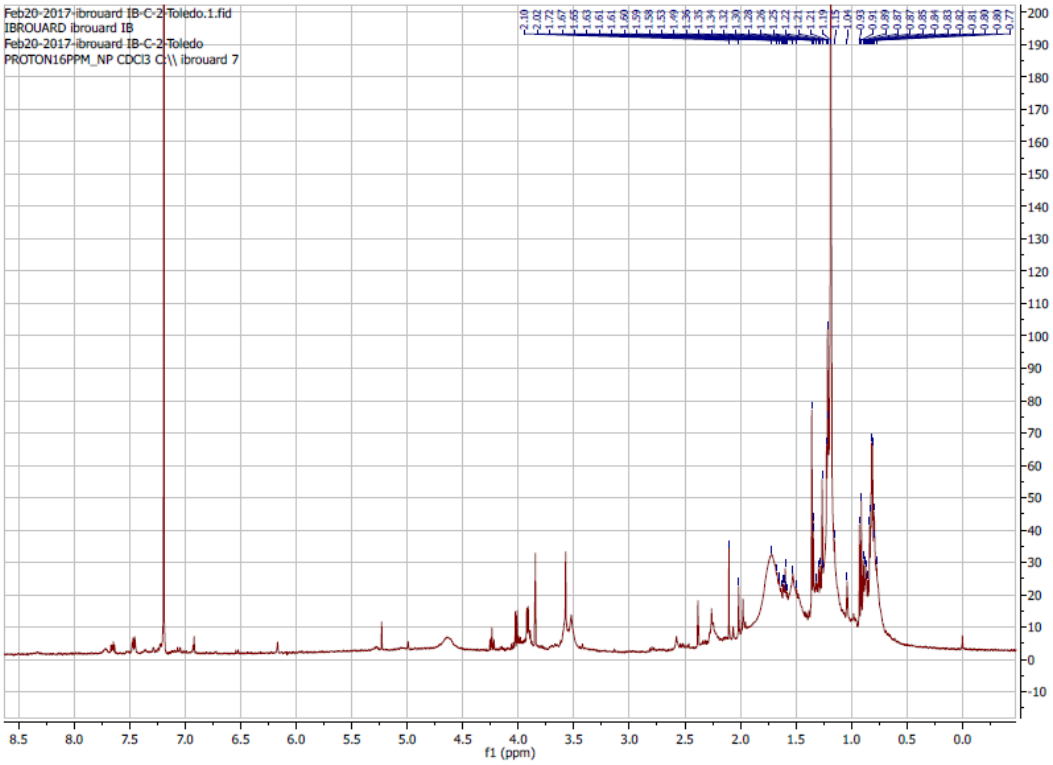
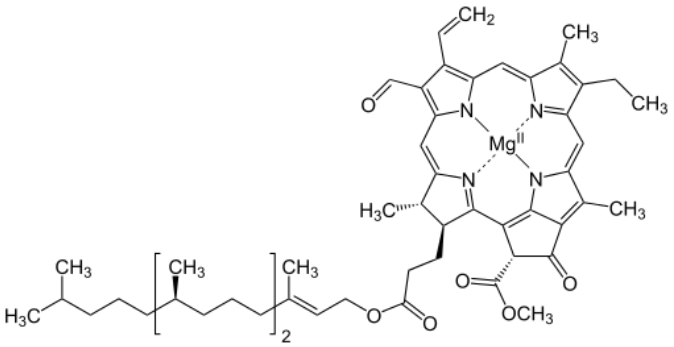


Figure 5.- ^{1}H-NMR spectrum of Cree-S2 (C-2) substance.

Figure 7 shows the ^{1}H-NMR spectrum of Cree-S5 (C-5) substance. Apart from the deuterated chloroform signal, at δ 7.26, signals corresponding to aromatic protons (δ 8.4-9.75), olefinic protons (δ 6.00-6.30), geminal to heteroatom protons (δ 4.00-3.0), and methylenes / methyls in the range δ 0.50-2.50 are observed. It is understood that it is an aromatic substance and, being dark green, could be contaminated by some chlorophyll. The chlorophylls a, b, c1, c2, d and f (*Figure 6*) was announced to be present in cyanobacteria and other oxygenic microorganisms that form stromatolites (Jabt, 2010; McAlpine, 2010)



I

Figure 6.- Structure of chlorophyll *f*.

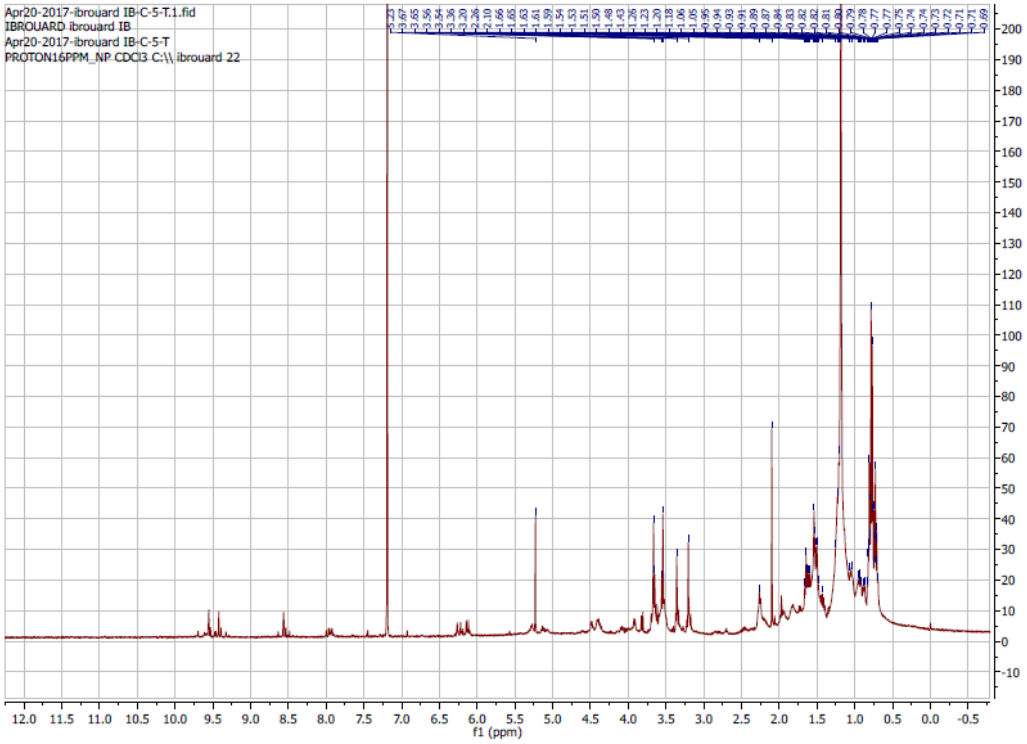
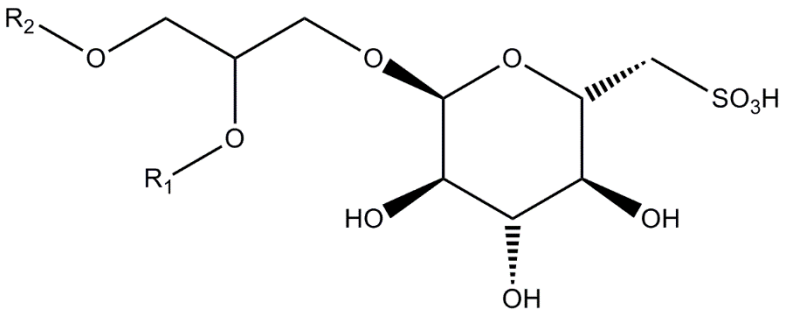


Figure 7.- ^{1}H-NMR spectrum of Cree-S5 (C-5) substance.

The ^{1}H-NMR spectrum of Cree-S7 (C-7) substance can be seen in *Figure 9*. Apart from the deuterated chloroform signal at δ 7.26 and the one for dichloromethane at δ 5.20, signals corresponding to geminal to heteroatom protons (δ 3.6-4.3), and methylenes / methyls in the range δ 0.5-2.5 are observed. It is understood that it is some lipid substance. Despite its yellow-green colouration, the substance looks quite pure, so it is being studied in more depth.

The ^{1}H-NMR of Cree-S9 (C-9) spectrum is observed in *Figure 10*. Apart from the deuterated chloroform signal at δ 7.26 and the one for dichloromethane at δ 5.20, signals corresponding to olefinic protons (δ 5.2-5.4), geminal to heteroatom protons (δ 3.8-4.5), and methines/ methylenes / methyls in the range δ 0.5-2.5, are observed. It is understood that it is some glycolipid substance. Despite its brownish colouration, the substance seems to be fairly pure, so it is being studied more deeply. For now, it is suspected that this substance has the structure of sulfoglycolipid (**II,** *Figure 8*) proposed in a worldwide patent (Pham & Durand-Chastel, 2003). As to the nature of the radicals R_1 and R_2, it is understood that they are polyunsaturated fatty acids, probably linoleic and linolenic, since ten peaks corresponding to other olefinic carbons are observed in the range of δ 127 - 131 in the spectrum of ^{13}C-NMR.



II

Figure 8.- Sulfoglycolipids of *Arthrospira* spp.

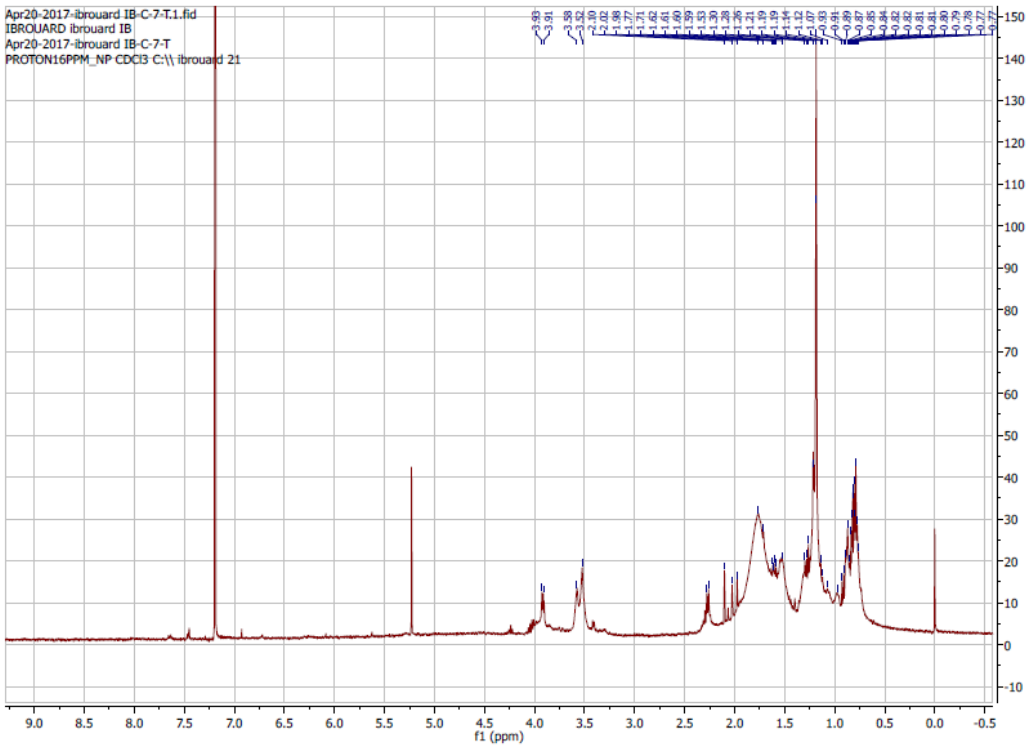


***Figure* 9.-** ^{1}H-NMR spectrum of Cree-S7 (C-7) subtance.

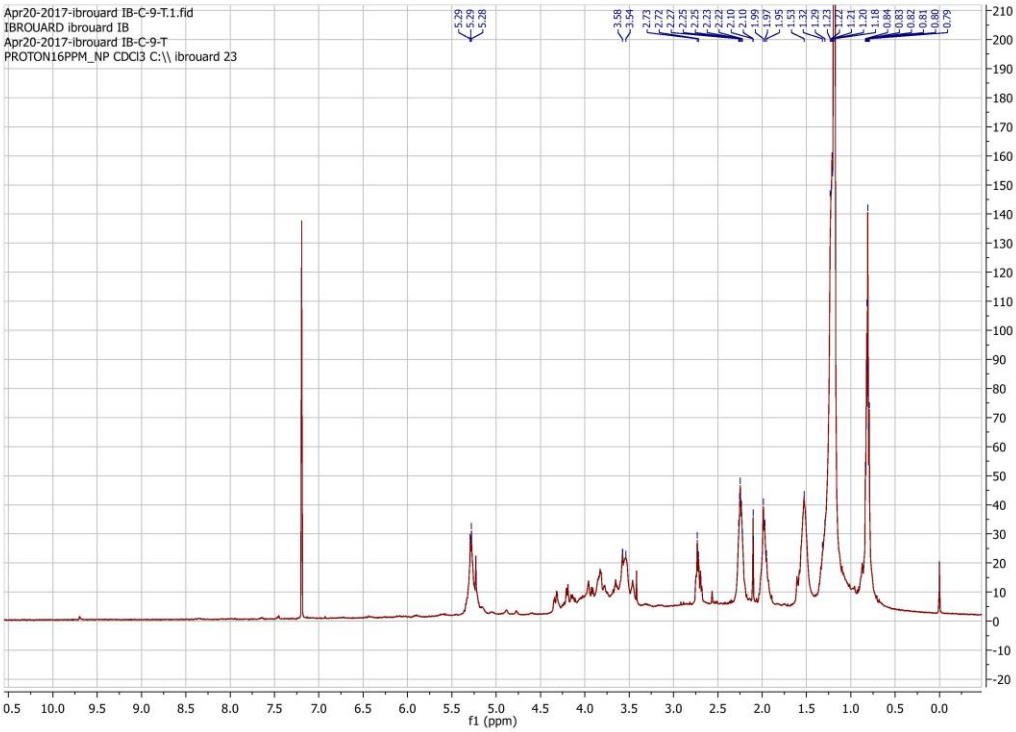


Figure 10.- ^{1}H-NMR spectrum of Cree-S9 (C-9) substance.

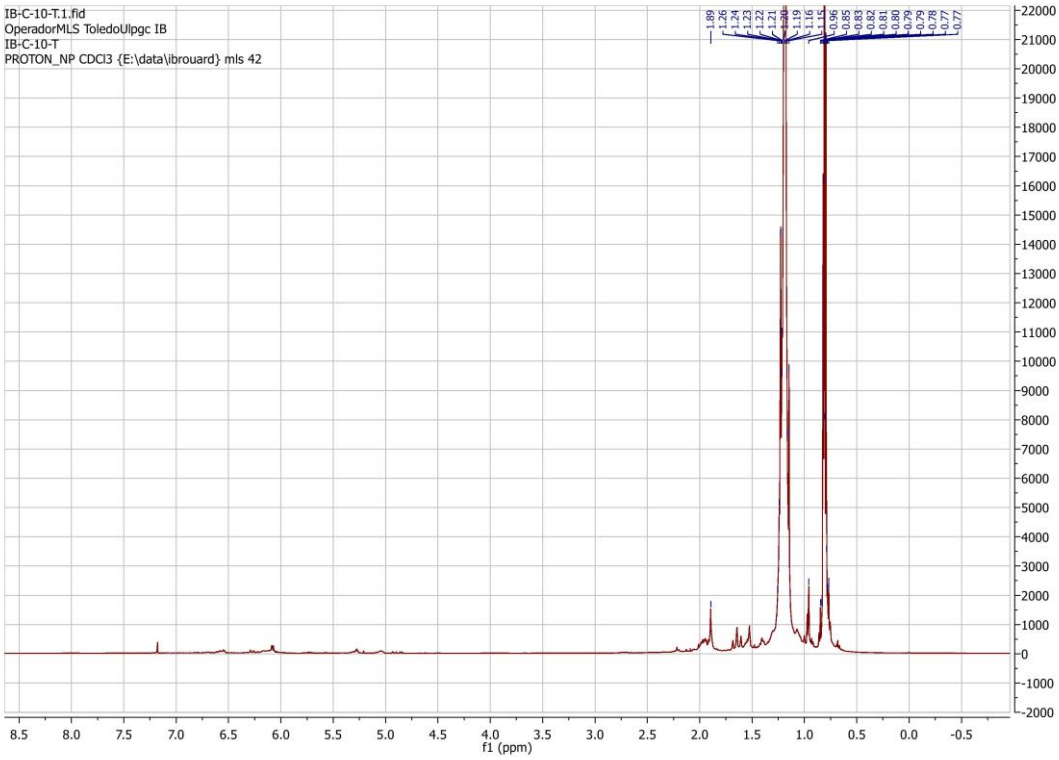


Figure 11.- ^{1}H-NMR spectrum of Cree-Sp-1 (C-10) substance.

Figure 11 shows the ^{1}H-NMR spectrum of Cree-Sp-1 (C-10) substance. Signals corresponding to protons of alkanes at δ 0.7-2.0 are observed, so it is understood that it is a wax.

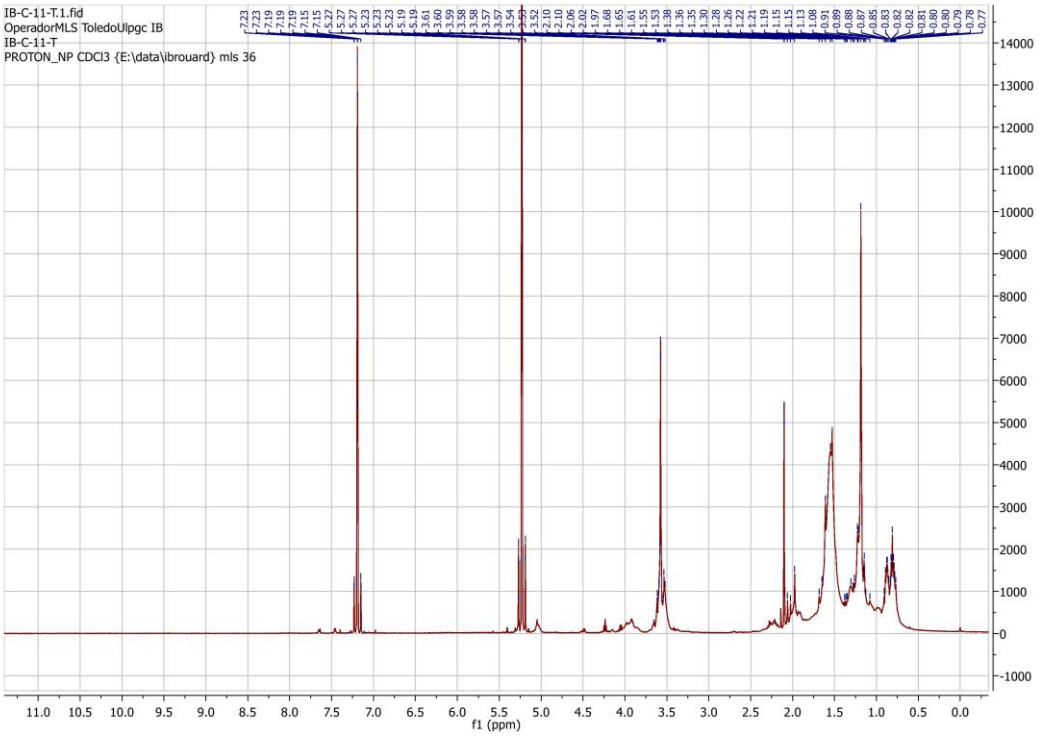


Figure 12.- ^{1}H-NMR spectrum of Cree-Sp-3.1 (C-11) substance.

The ^{1}H-NMR spectrum of Cree-Sp-3.1 (C-11) substance is shown in *Figure 12*. Signals corresponding to the solvents used (Cl_3CD at δ 7.26, CH_2Cl_2 at δ 5.25, and acetone at δ 2.2), geminal to heteroatom protons at δ 3.4-4.3 and methines / methylenes / methyls of alkyl groups at δ 0.6-2.4 are observed. This indicates that it could be a glycolipid. Since the substance has an orange colour, it is believed that it could be impurified with some polar xanthophyll.

In *Figure 13*, the ^{1}H-NMR spectrum of Cree-Sp-3.2 (C-12) substance can be observed. Signals corresponding to the solvents used (Cl_3CD at δ 7.26, CH_2Cl_2 at δ 5.23, and acetone at δ 2.2), geminal to heteroatom protons at δ 3.4-4.3 and methines / methylenes / methyls of alkyl groups at δ 0.6-2.3 are observed. This indicates that it could be another glycolipid. Since it has a yellow colour, it is understood that it could be contaminated by a carotenoid or a xanthophyll.

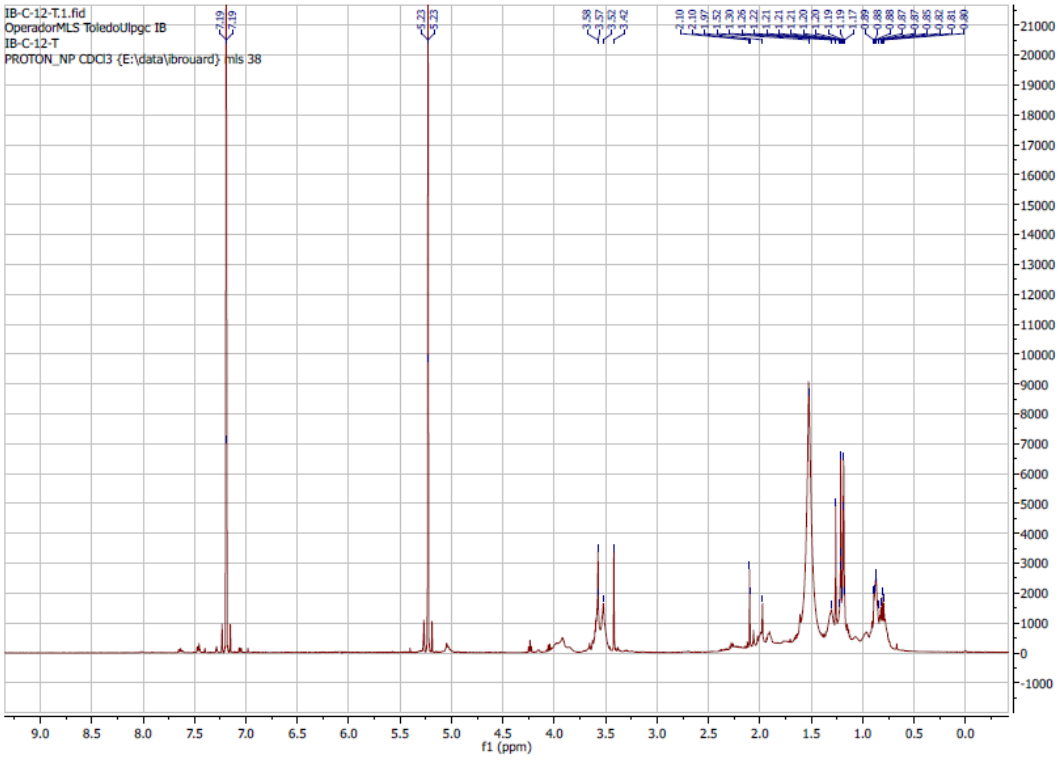
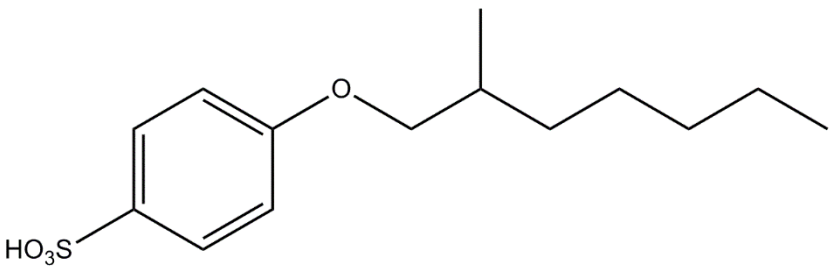


Figure 13.- ^{1}H-NMR spectrum of Cree-Sp-3.2 (C-12) substance.

Figure 15 and *Figure 16* show the ^{1}H-NMR and ^{13}C-NMR spectra of Cree-Sp-3.3 (C-13) substance. Signals corresponding to the solvents used (Cl_3CD at δ 7.26, CH_2Cl_2 at δ 5.23, and acetone at δ 2.2), aromatic protons at δ 7.4-7.6 (AB-type spin coupling system), geminal to heteroatom protons at δ 3.3-4.6 and methines / methylenes / methyls of alkyl groups at δ 0.6-1.7 are observed in Figure *15*. This indicates that it could be a *para*-disubstituted benzene compound. This is verified by the ^{13}C-NMR spectrum, where six aromatic carbons at δ 128.81-167.76, a geminal to oxygen carbon at δ 68.18 and seven aliphatic carbons at δ 38.76, 30.39, 28.95, 23.78, 23.00, 14.06 and 10.98 are observed. That is why it is considered that this compound could be some *para*-substituted aryl-alkyl eter as 4-[(2-methylheptyl)-oxi]-benzenesulfonic acid (platensic acid, **III**) (*Figure 14).*



III

Figure 14.- Structure of compounds C-13 and C-14 (platensic acid).

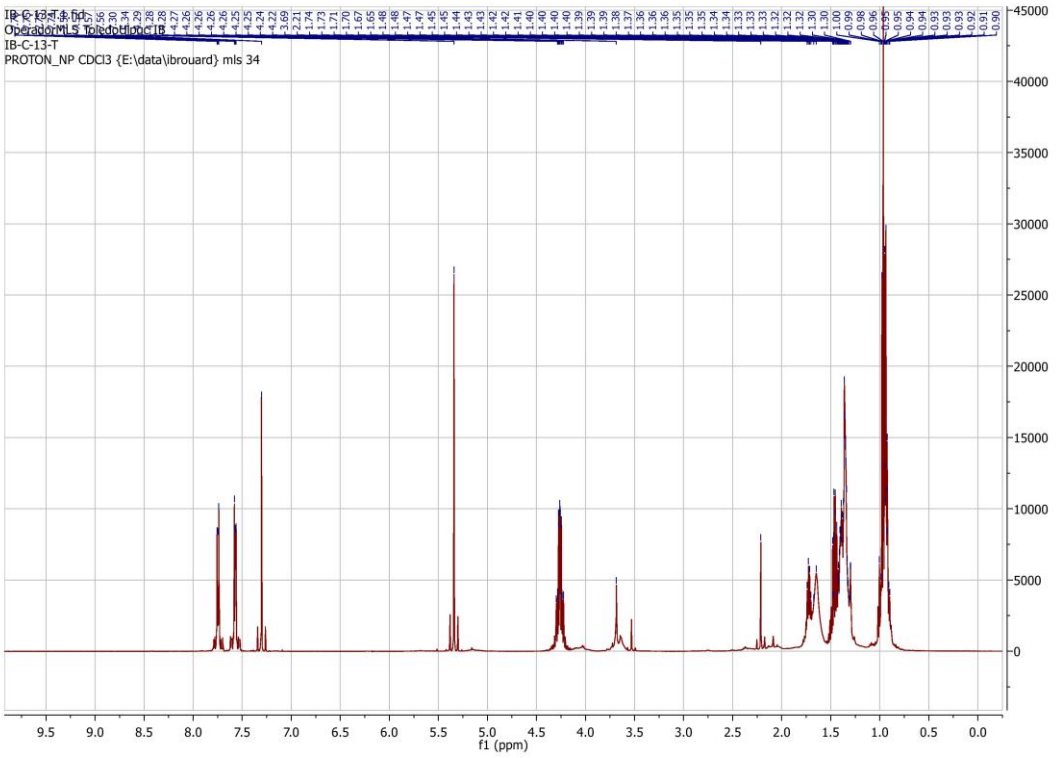


Figure 15.- ^{1}H-NMR spectrum of Cree-Sp-3.3 (C-13) substance.

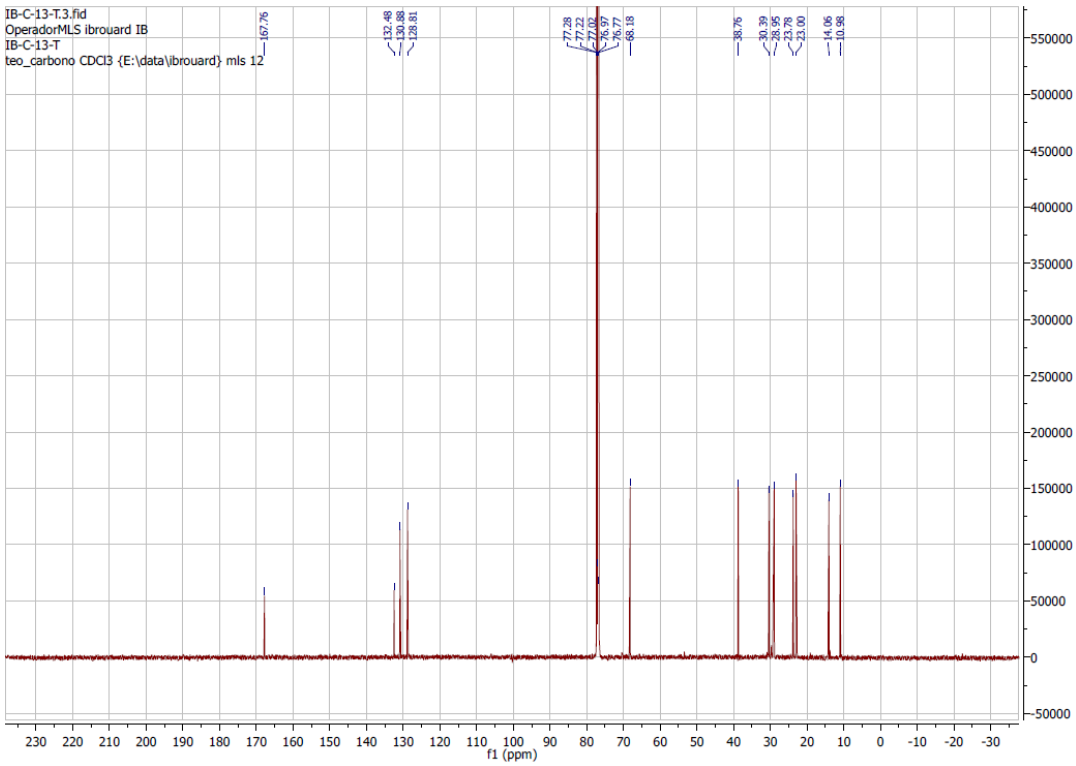


Figure 16.- ^{13}C-NMR spectrum of Cree-Sp-3.3 (C-13) substance.

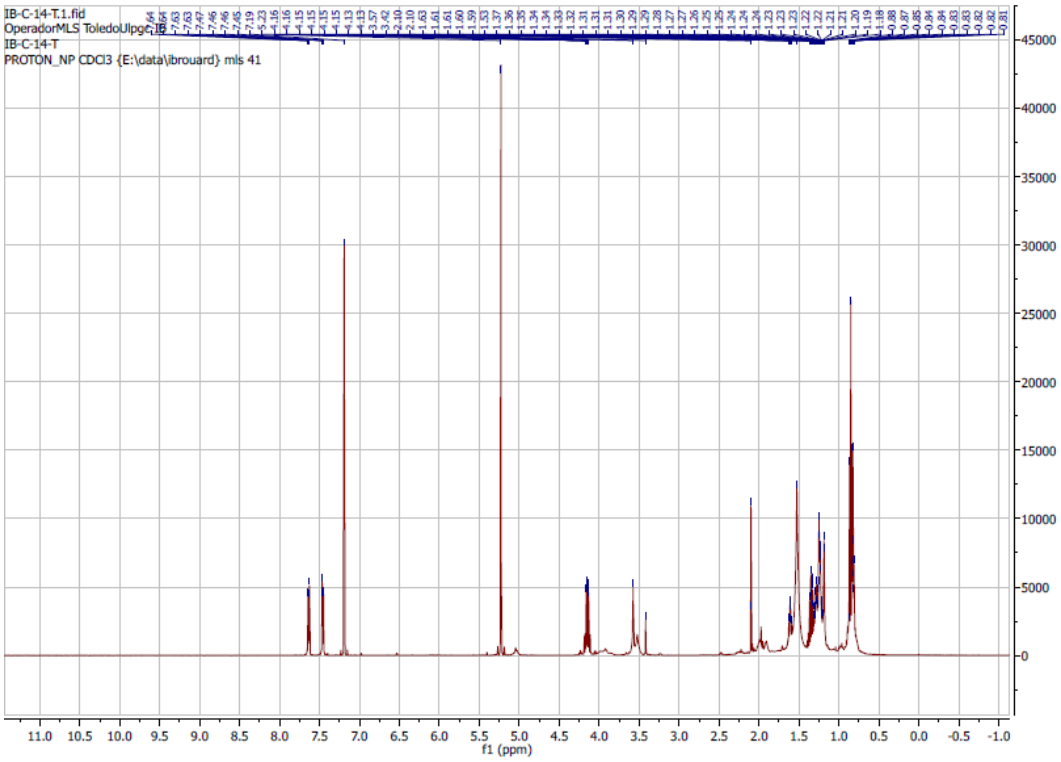


Figure 17.- [1]H-NMR spectrum of Cree-Sp-3.4 (C-14) substance.

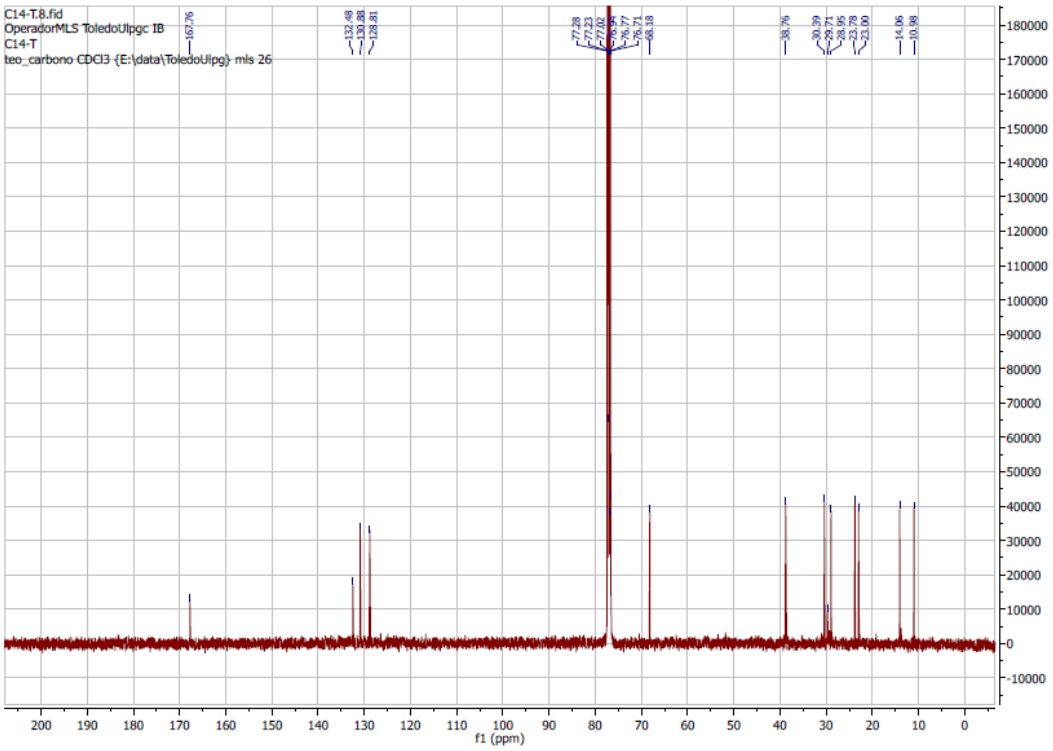


Figure 18.- *[13]C-NMR spectrum of Cree-Sp-3.4 (C-14) substance.*

Figure 17 and *Figure 18* show the ^{1}H-NMR and ^{13}C-NMR spectra of Cree-Sp-3.4 (C-14) substance. Again, signals corresponding to the solvents used (Cl_3CD at δ 7.26, CH_2Cl_2 at δ 5.23, and acetone at δ 2.20), aromatic protons at δ 7.4-7.6 (AB-type spin coupling system), geminal to heteroatom protons at δ 3.3-4.6 and methines / methylenes / methyls of alkyl groups at δ 0.6-1.7 are observed in *Figure 17*. This indicates that it could be a *para*-disubstituted benzene compound. This is verified by the ^{13}C-NMR spectrum (*Figure 18*), identical to the ^{13}C-NMR spectrum of Cree-Sp-3.3 (C-13) substance (*Figure 16*). From the DEPT, COSY, HMBC, and HSQC nuclear magnetic resonance spectra, it is confirmed the structure of 4-[(2-methylheptyl)-oxi]-benzenesulfonic acid (platensic acid, **III**) (*Figure 14*) for this metabolite.

The ^{1}H-NMR spectrum of Cree-Sp-3.5 (C-15) substance is shown in *Figure 19*. Again, signals corresponding to the solvents used (Cl_3CD at δ 7.26, CH_2Cl_2 at δ 5.23, and acetone at δ 2.2), olefinic protons at δ 5.0, geminal to heteroatom protons at δ 3.3-4.5 and methines / methylenes / methyls of alkyl groups at δ 0.6-2.5, are observed. This indicates that it could be a glycolipid with unsaturated fatty acids.

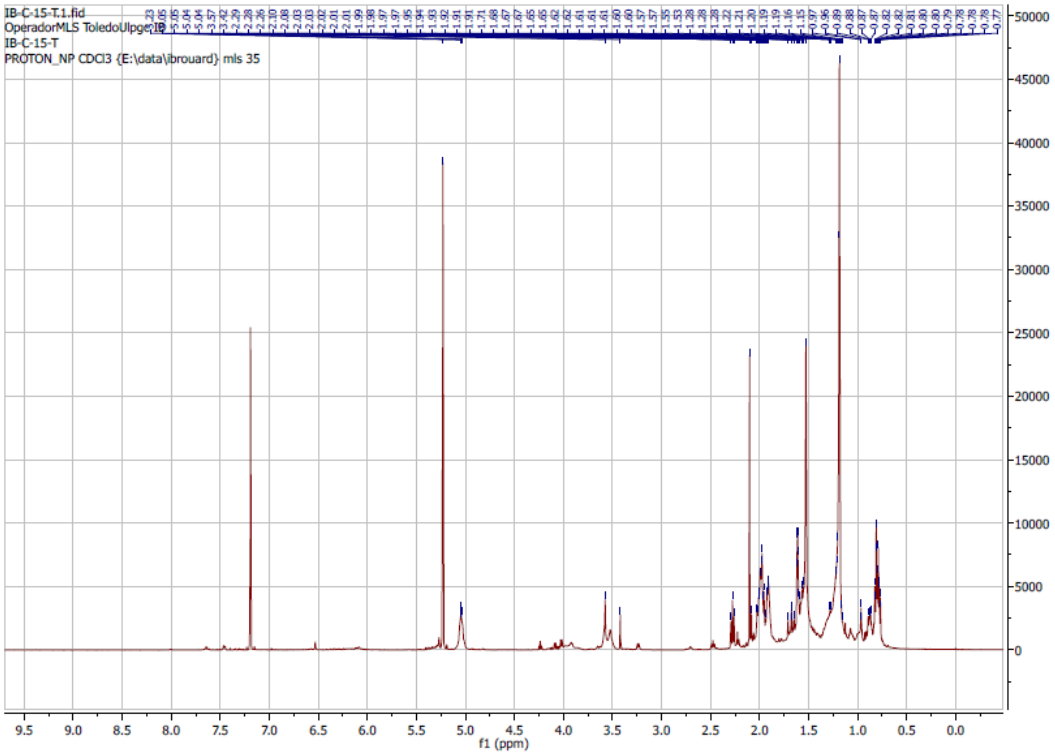


Figure 19.- ^{1}H-NMR spectrum of Cree-Sp-3.5 (C-15) substance.

Figure 20 shows the ^{1}H-NMR spectrum of Cree-Sp-3.6 (C-16) substance. Signals corresponding to the solvents used (Cl_3CD at δ 7.26, CH_2Cl_2 at δ 5.23, and

acetone at δ 2.2), olefinic protons at δ 5.3, and methines / methylenes / methyls of alkyl groups at δ 0.6-2.5 are again observed. This indicates that it could be a mixture of unsaturated fatty acids.

The ^{1}H-NMR spectrum of Cree-Sp-4.1 (C-17) substance is represented in *Figure 21*. Again, signals corresponding to the solvents used (Cl_3CD at δ 7.26, CH_2Cl_2 at δ 5.23, and acetone at δ 2.20), geminal to heteroatom protons at δ 3.3-4.3, and methines / methylenes / methyls of alkyl groups at δ 0.6 -2.6 are observed, indicating that it could be another glycolipid, but now, with saturated fatty acids.

In *Figure 22*, the ^{1}H-NMR spectrum of Cree-Sp-4.2 (C-18) is observed. Signals corresponding to the solvents used (Cl_3CD at δ 7.26, CH_2Cl_2 at δ 5.23, and acetone at δ 2.2), geminal to heteroatom protons at δ 3.6–4.3 and methines / methylenes / methyls of alkyl groups at δ 0.7 -2.5 are, again, observed. This indicates that it could be another glycolipid with saturated fatty acids.

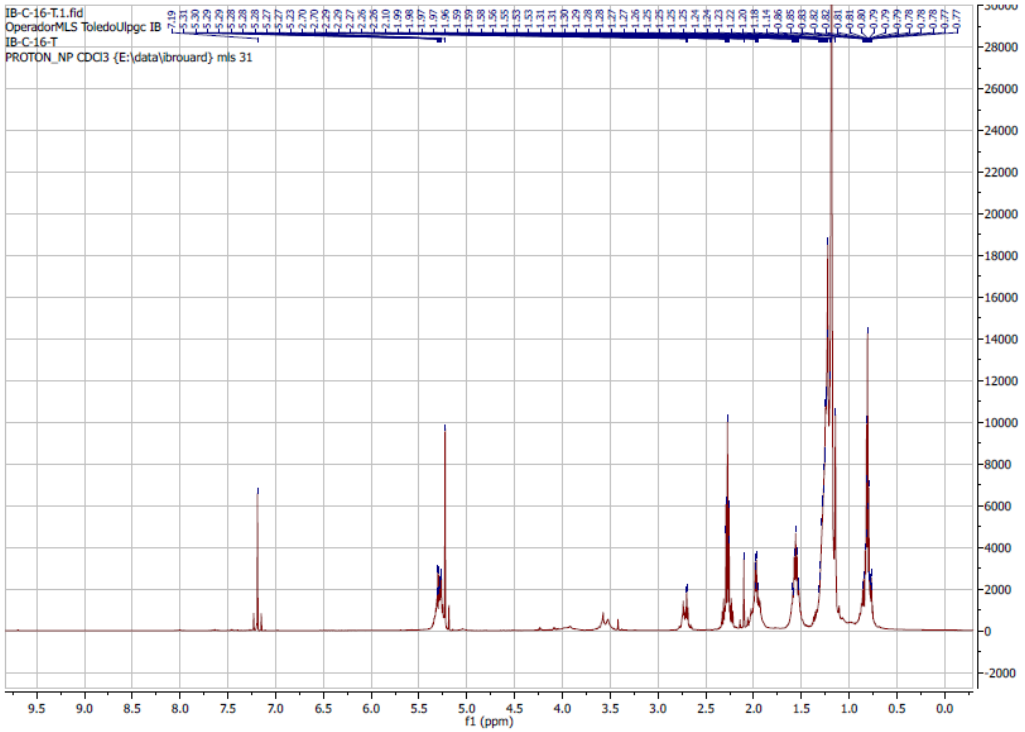


Figure 20.- ^{1}H-NMR spectrum of Cree-Sp-3.6 (C-16) substance.

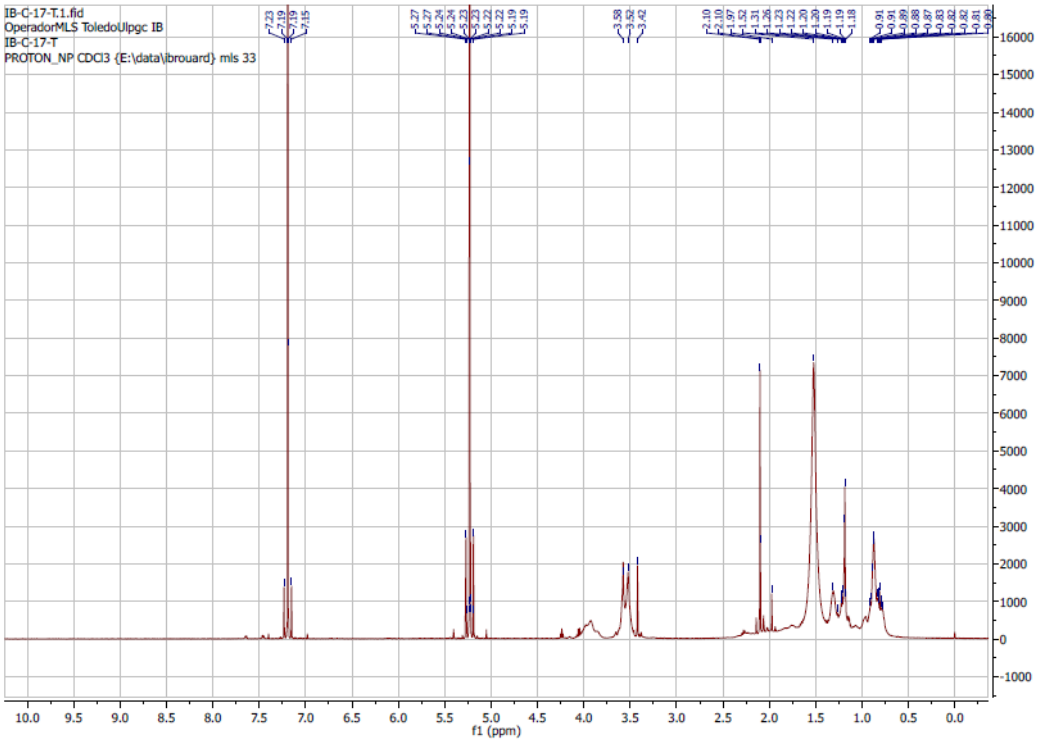


Figure 21.- ^{1}H-NMR spectrum of Cree-Sp-4.1 (C-17) substance.

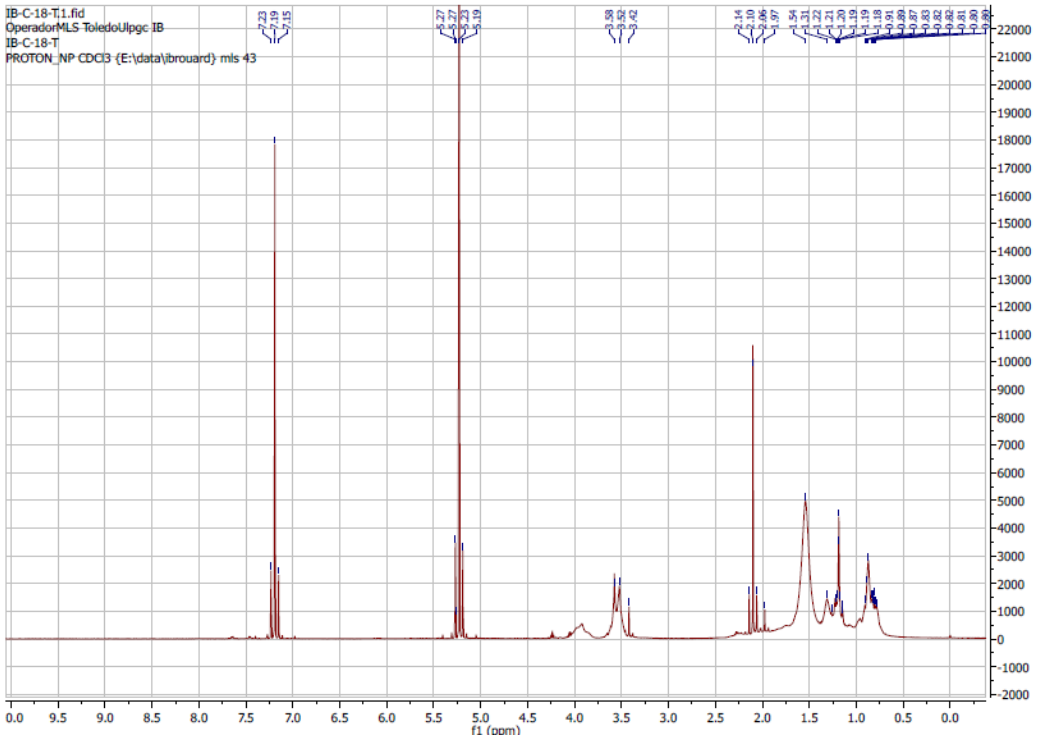


Figure 22.- ^{1}H-NMR spectrum of Cree-Sp-4.2 (C-18) substance.

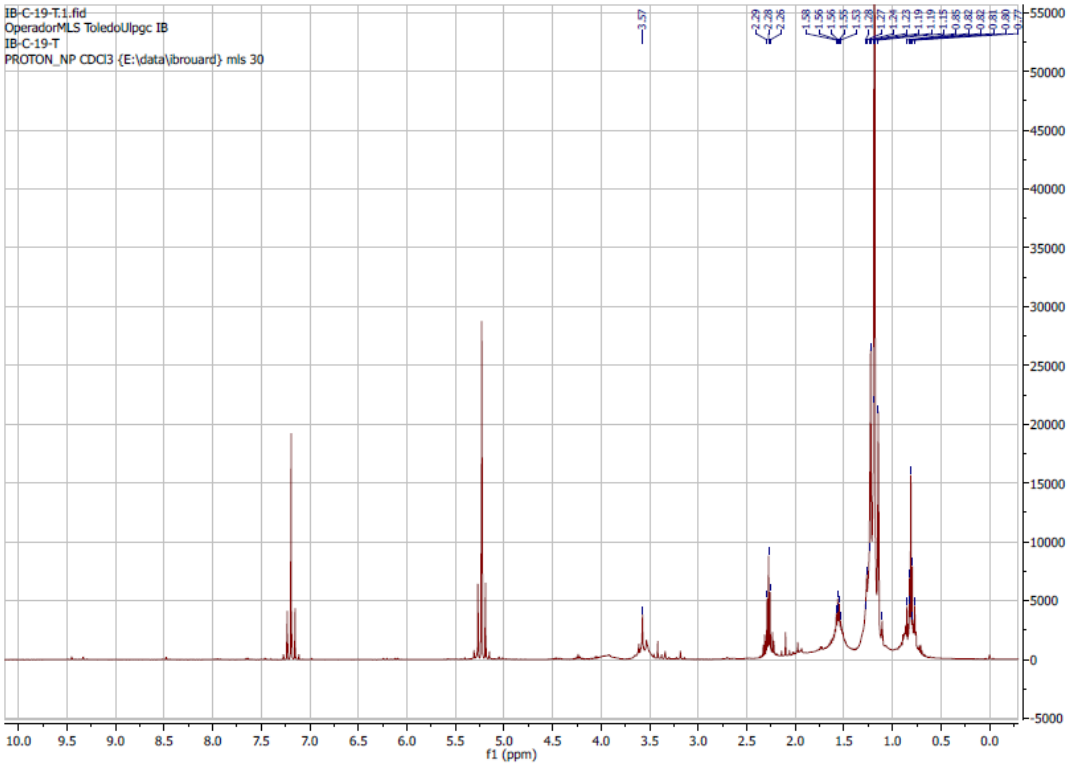


Figure 23.- ^{1}H-NMR spectrum of Cree-Sp-5.3 (C-19) substance.

The ^{1}H-NMR spectrum of Cree-Sp-4.3 (C-19) substance is shown in *Figure 23*. Again, signals corresponding to the solvents used (Cl_3CD at δ 7.26, CH_2Cl_2 at δ 5.23, and acetone at δ 2.2), geminal to heteroatom protons at δ 4.6-3.2, and methines / methylenes / methyls of alkyl groups at δ 0.6- 2.5 are observed, indicating that it could be another glycolipid with saturated fatty acids.

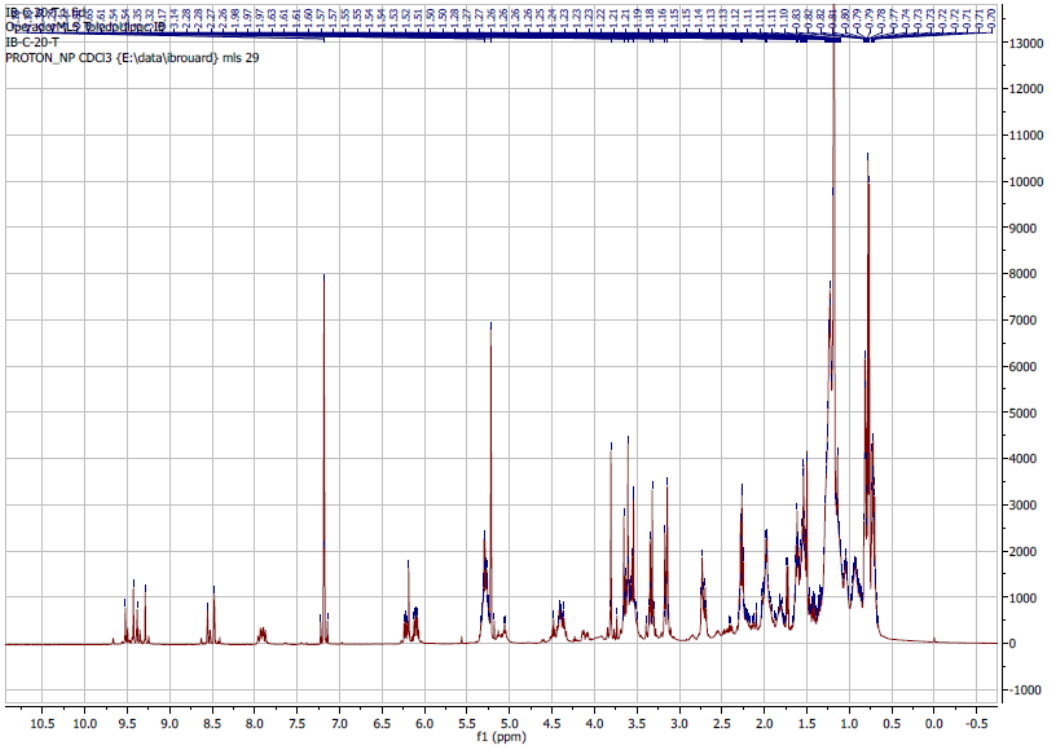


Figure 24.- ^{1}H-NMR spectrum of Cree-Sp-5.4 (C-20) substance.

In *Figure 24*, ^{1}H-NMR spectrum of Cree-Sp-4.4 (C-20) substance is showed. Again, signals corresponding to the solvents used (Cl_3CD at δ 7.26, CH_2Cl_2 at δ 5.23, and acetone at δ 2.2), aromatic protons at δ 9.75–9.20, 8.75-8.40 and 8.0-7.8, olefinic protons at δ 6.30-6.00, and 5.35-5.00, germinal to heteroatom protons at δ 3.60-3.50, a methoxy group at δ 3.80, geminal to heteroatom protons at δ 4.60-4.26, unidentified signals at δ 3.35, 3.32, 3.17 and 3.14, and methines / methylenes / methyls of alkyl groups at δ 2.75-0.50, are observed. This indicates that it could be a mixture of aromatic compounds. At this moment, we are working on its possible identification.

The ^{1}H-NMR spectrum of Cree-Sp-4.5 (C-21) substance is shown in *Figure 25*. Signals corresponding to the solvents used (Cl_3CD at δ 7.26, CH_2Cl_2 at δ 5.23, and acetone at δ 2.2), aromatic protons at δ 9.75–8.50, olefinic protons at δ 6.10-6.30, geminal to heteroatom protons at δ 4.60-3.00, which include a methoxy group at δ 3.80, geminal to heteroatom protons at δ 3.60-3.50, and methines / methylenes / methyls of alkyl groups at δ 2.50-0.50 are, again observed. This indicates that it could be a new aromatic product. At the moment it is deepening in its structural elucidation.

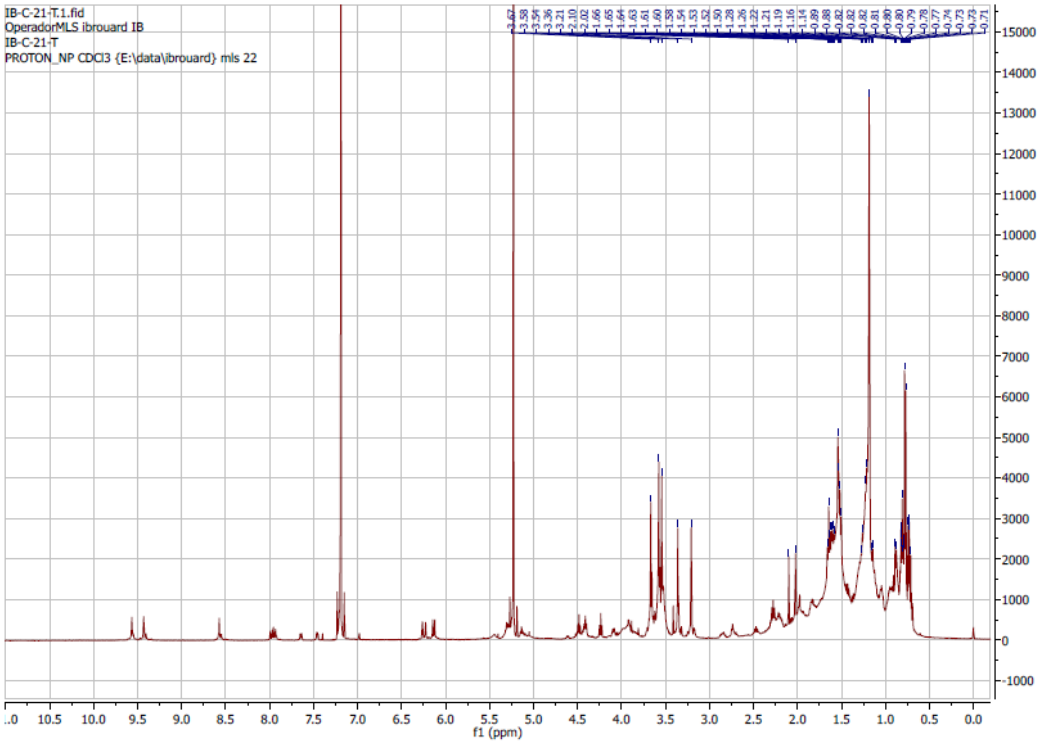


Figure 25.- ^{1}H-NMR spectrum of Cree-Sp-4.5 (C-21) substance.

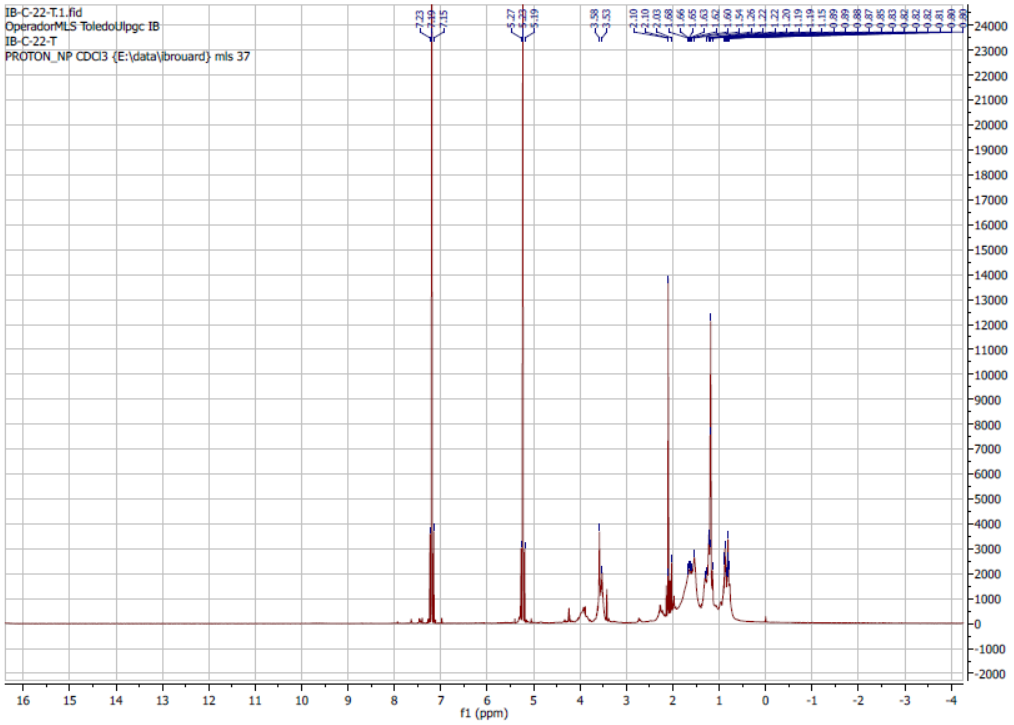


Figure 26.- ^{1}H-NMR spectrum of Cree-Sp-4.6 (C-22) substance.

Figure 26 shows the ^{1}H-NMR spectrum of Cree-Sp-5.6 (C-22) substance. Again, signals corresponding to the solvents used (Cl_3CD at δ 7.26, CH_2Cl_2 at δ 5.23, and

acetone at δ 2.2), geminal to heteroatom protons at δ 4.30-3.30 and δ 3.60-3.50, and methines / methylenes / methyls of alkyl groups at δ 2.30-0.50, are observed, indicating that it could be a glycolipid with saturated fatty acids.

4. Conclusion

It is concluded, from the bibliographical study, that the cyanobateria *A. platensis* presents a multitude of beneficial properties for the human being. Thus, it has recently been found that this cianobacteria has anti-inflamatory, antioxidant and anticancer properties, which are, in principle, attributed to the high amount of phycocyanin they contain. It should also be mentioned that some studies claim that *A. platensis*, used as food, increases the life expectancy of citizens, probably because it contains proteins of high biological value.

On the other hand, many marine organisms are a rich source of structurally novel and biologically active secondary metabolites. These compounds are of interest in the pharmaceutical industry. The cells of *Arthrospira* (=*Spirulina*) *platensis* contain a relatively high proportion of lipids. Thus, the products Cree-S7 (C-7), Cree-S9 (C-9), Cree-Sp-3.1 (C-11), Cree-Sp-3.2 (C-12), Cree-Sp-3.5 (C-15), Cree-Sp-4.1 (C-17), Cree-Sp-4.2 (C-18), Cree-Sp-4.3 (C-19), and Cree-Sp-4.6 (C-22), isolated in this work, were shown as lipid substances thanks to their spectroscopy study (^{1}H-NMR and ^{13}C-NMR).

The concrete results obtained in this work, the structural field inform us of unsaturated fatty acids, possible unsaturated sulfoglycolipids with antiviral activity (structure **II**, *Figure 8*) and *para*-substituted benzene rings, as platensic acid (structure **III**, *Figure 14*). These last two compounds would be biosynthesized by *A. platensis* to acquire adaptive advantage in the environment and, therefore, they would be authentic alomones.

Finally, in conclusion it is necessary to continue to deepen the knowledge of these substances, particularly, in their purification, structural elucidation and biological activity.

5. Acknowledgements

Thanks to Ignacio Brouard (Instituto de Productos Naturales y Agrobiología, CSIC, Tenerife) for his contribution in making the NMR spectra showed in this paper and to the teachers Francisco Javier Toledo Marante y Elsa María Rodríguez Pérez for tutoring this project. Thanks also to the University of Palmas de Gran Canaria for giving me the opportunity to do this work

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7. Attachments

Ecología Química: alomonas de la cianobacteria *Arthrospira (=Spirulina) platensis*

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RESUMEN

A. platensis (familia Cyanophyceae, orden Cyanophyceae), es una cianobacteria comercial con aplicaciones en nutracéutica. Unas 530 publicaciones científicas describen el conocimiento que tenemos sobre los metabolitos de este microorganismo. Dentro de nuestra línea de investigación focalizada hacia el descubrimiento de alomonas, hemos observado que se describen para esta especie diversas actividades biológicas, entre las que sobresalen la actividad antiviral[1,2] y antioxidante.[3] Mientras que la actividad antiviral se achaca a metabolitos con supuesta estructura de sulfoglicolípido, la actividad antioxidante se ha relacionado con una amplia diversidad de estructuras químicas, como filohidroquinonas hidroxiladas, tocoferoles, carotenos, clorofilas, xantofilas, ácidos grasos poliinsaturados y fenoles. Ante dicha diversidad química, y dado que los mencionados compuestos fueron identificados mediante métodos analíticos nada rigurosos (normalmente, solo HPLC), nos hemos propuesto aquí profundizar en el conocimiento químico de la especie *A .platensis*, mediante modernas técnicas de purificación y análisis estructural (cromatografía, resonancia magnética nuclear y espectrometría de masas).

Por extracción con un aparato *Soxhlet* de la biomasa de *A. platensis* con acetona, seguida de filtración y concentración, se obtuvo un extracto bruto de la misma como un jarabe oscuro. Por cromatografía en columna, eluyendo con mezclas de hexano/ acetona/ metanol, se obtuvieron sucesivas fracciones de polaridad creciente. Los metabolitos puros se purificaron por TLC preparativa y fueron identificados por los mencionados métodos espectroscópicos (RMN y EM). Entre los metabolitos aislados figuran carotenos, xantofilas, clorofilas, ácidos grasos, el sulfoglicolípido (**II**) y un nuevo compuesto, el ácido 4-[(2-metilheptil)-oxi]-bencensulfónico (ácido platénsico, **III**), cuya estereoquímica, aún está por determinar.

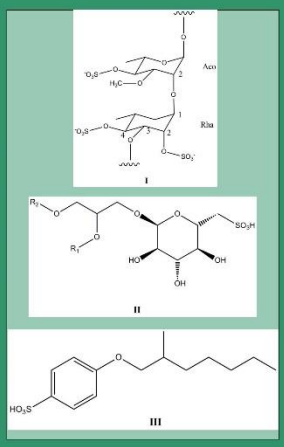

Figura 1: *Arthrospira platensis* (imagen tomada de internet)

LAS ALOMONAS DE *A. platensis*

La cianobacteria *A. platensis* posee tanto sustancias antivirales como antibacterianas, antifúngicas y antiparásito.[2] En la actualidad se atribuye la actividad antiviral de esta especie tanto a sus polisacáridos sulfatados aniónicos (*Calcium spirulan*, Ca-SP), como a sus sulfoglicolípidos.[2] Los sulfoglicolípidos de *A. platensis* también inhiben a la transcriptasa inversa, esto es, el enzima que cataliza la retrotranscripción.[2] Por otra parte, las unidades sulfatadas de O-ramnosil-acofriosa [→3)-α-L-Rha-(1 → 2)-α-L-Aco-(1 →] (**I**) parecen ser esenciales para la actividad del Ca–SP,[2] sin embargo, la estructura de los sulfoglicolípidos de *A. platensis* aún está sin caracterizar espectroscópicamente. En esta comunicación aportamos un avance en la solución de dicho problema. En concreto, tras aislarlo y hacerle espectroscopía, confirmamos que podría tener la estructura propuesta por Pham & Durant-Chastel (**II**).[1] En cuanto a la naturaleza de los ácidos grasos R_1 y R_2, según se deduce del espectro de ^{13}C-NMR, se trata de dos ácidos grasos polinsaturados, probablemente, el linoleico y el linolénico, dado que se observan diez carbonos olefínicos en el intervalo de δ 127 – 131 del espectro de ^{13}C-NMR.

EXPERIMENTAL

Extracción y cromatografía: Tras extraer la biomasa comercial de *A. platensis* (1500 g) con acetona se obtuvieron 34 g de un extracto bruto, Éste se sometió a cromatografía en columna de gel de sílice eluyendo con mezclas de hexano y acetona seguido de mezclas de acetona y metanol. Se obtuvieron así, tras su monitoreo por TLC analítica, 14 fracciones, las cuales fueron mezclas diversas de carotenos, xantofilas, clorofilas, ácidos grasos libres (saturados e insaturados), y otros compuestos aún no identificados.

Análisis por TLC: Se preparó aproximadamente 2 mL de una disolución del extracto bruto/ fracción cromatográfica/ metabolito puro (20 mg x mL^{-1}) de *A. platensis*. Dicha disolución (3 µL) se aplicó a una placa de TLC analítica (MACHEREY-NAGEL, 10 x 5 cm) y se eluyó con hexano: acetona (75:25), según se recomienda en la bibliografía.[4] Los compuestos separados se identificaron visualizando las placas de TLC bajo luz blanca, bajo radiación UV (240 o 370 nm) o por medio de la aplicación de un *spray* de *oleum* seguido de calentamiento a 120 ºC durante 20 minutos.

Análisis por NMR: La espectroscopía de resonancia magnética nuclear (^{1}H-NMR, ^{13}C-NMR, DEPT, COSY, NOESY, HSQC, HMBC) se realizó en el Instituto de Productos Naturales y Agrobiología del CSIC (Tenerife).

Figura 2: *Arthrospira (=Spirulina) platensis* comercializada por los laboratorios Solaray

AGRADECIMIENTOS

Los autores agradecen al Ministerio de Economía y Competitividad la subvención del proyecto: CTM2016-75457.

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XXI Simposio de Botánica Criptogámica
Aranjuez – Madrid, España, 20-24 de Junio, 2017

ATTACHED I. Poster presented to the XXI Symposium on Cryptogamic Botany (Aranjuez, Madrid, June 20-24, 2017).

Durante las seis primeras semanas de prácticas externas en el ámbito de la investigación, se realizó un trabajo de búsqueda bibliográfica relacionada con el tema a tratar: "Productos naturales de la cianobacteria *Spirulina* spp., con profundización en *S. platensis* y su aplicabilidad en los sectores de la farmacéutica, cosmética y nutracéutica" (véase *Ilustración 1*).

A medida que se realizaban las prácticas externas se iban seleccionando aquellos artículos más relacionados con las actividades realizadas en prácticas que ayudaban en el entendimiento de éstas. La introducción del TFG se pudo realizar gracias a la lectura de numerosos artículos científicos que hablaban de productos naturales para los campos ya mencionados anteriormente, y sobre todo, de la aplicabilidad de la *Spirulina* spp. en ellos. La parte de metodología se realizó prácticamente en base al trabajo de laboratorio y gracias a la ayuda de algunos libros que hablaban de algunas técnicas de laboratorio empleadas en las prácticas externas. Gracias al Instituto de Productos Naturales y Agrobiología del CSIC (Tenerife), en particular a su investigador D. Ignacio Brouard Martin por su estudio en espectroscopía de resonancia magnética nuclear (RMN) y gracias a la Universidad Las Palmas de Gran Canaria (ULPGC) por su estudio cromatográfico se pudo obtener los resultados necesarios para la realización y entendimiento del TFG. Los resultados obtenidos se compararon con los obtenidos por otros autores con el fin de observar similitudes y diferencias y, también, corroborar nuestros propios resultados. Finalmente, la parte de discusión y conclusión fueron una de las más complicadas debido a la escasez de artículos científicos que hablaran del tema a tratar, ya que la mayoría hablaba de biología y pocos de química. No se pudo llegar a una conclusión clara, por lo que se pide más investigación en este campo.

GRADO EN CIENCIAS DEL MAR. ASIGNATURA: 40632 – Trabajo Fin de Título

DOCUMENTO 1: DEFINICIÓN DE ACTIVIDADES Y TEMPORALIZACIÓN

Datos del TFT
Título: Productos naturales del alga Spirulina spp, con profundización en S. platensis y su aplicabilidad en los sectores de la farmacéutica, cosmética y nutracéutica
Periodo de duración. Desde (dd/mm): 1/2/2017 hasta (dd/mm): 30/6/2017

Datos alumno:	
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Beca:	☐ Si, Indicar: Movilidad X No

Datos tutor/es:	
Grupo/Centro/empresa: Tecnología Química y Desarrollo Sostenible	
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Grupo/Centro/empresa: Tecnología Química y Desarrollo Sostenible	
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I. Actividades propuestas.

Las actividades que se proponen para que realice el alumno durante **el desarrollo de su TFT serán las siguientes:**
1 Revisión Bibliográfica del tema propuesto
2 Experimentación relacionada con el tema propuesto
3
4
5
6

II. Cronograma (ver nota aclaratoria):

	Semana																
Actividad	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	HP	HNP
1	X	X	X	X	X	X											80	70
2							X	X	X	X	X	X	X	X			100	200
																Total	180	270

En Las Palmas de Gran Canaria , a 17 de Enero de 2016

Firma alumno Firma: tutor del TFT

Nota:

- Las semanas se cuentan desde la primera semana del segundo semestre hasta la última.
- Sombrear las celdas de las semanas en las que se vaya a trabajar la actividad indicada, que pueden ser o no consecutivas.
- En las últimas columnas indicar el nº de horas presenciales (HP) y no presenciales (HNP) por actividad. La suma total debe ser la indicada.
- El alumno debe enviar este documento al tutor de TFT tras la primera reunión de planificación.

Illustration 1. Scheduling of the activities carried out during the execution of the TFG

La formación recibida en cursos anteriores ha sido muy útiles a la hora de realizar el TFG, si bien es verdad que no se han realizado muchos artículos científicos a lo largo de la carrera, por lo que me he encontrado con algunos problemas a la hora de escribir el TFG.

Un aspecto bastante positivo a la hora de realizar el TFG fue la implicación de mi tutor de prácticas a la hora de resolver dudas y ayudar con el TFG. Otra cosa a nombrar, es la facilidad que tiene el alumno o alumna para obtener artículos científicos totalmente gratis, gracias a la plataforma El Faro o Scopus.

La longitud del trabajo de fin de grado es muy razonable, ya que te recomiendan no pasarte de x páginas, por lo tanto, no te exigen un máximo de 20 páginas, por ejemplo, lo que desde mi punto de vista, es muy poco para un trabajo de investigación.

Un aspecto muy negativo a la hora de realizar el TFG fue que muchos alumnos y alumnas tuvimos problemas a la hora de realizar el trabajo, ya que en la carrera no nos ha enseñado lo suficiente sobre la realización de artículos científicos, exceptuando una asignatura en la que tuvimos que realizar uno.

Personalmente, el TFG me ha ayudado bastante, ya que he aprendido a realizar correctamente un trabajo riguroso de investigación y, por ende, un trabajo científico. He aprendido, también, a buscar trabajos de investigación que me han servido bastante a la hora de realizar el TFG.