UNIVERSIDAD DE INVESTIGACIÓN DE TECNOLOGÍA EXPERIMENTAL YACHAY

Escuela de Ciencias Químicas e Ingeniería

TÍTULO: Extraction and identification of saponins from *American agave* leaves, and their role as a potential source of phytonutrients with great economic value

Trabajo de titulación presentado como requisito para la obtención del título de Químico(a)

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Le dedico esta tesis a mis padres Juan Carlos y Durby, mi hermano Alan, mi pareja Eddy y mi pequeña mascota Charlotte porque es gracias a su apoyo que he podido superar los obstáculos que han surgido a lo largo de toda la carrera.

Polette Katerine Cevallos Gallegos

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Polette Katerine Cevallos Gallegos

RESUMEN

La planta de *Agave americana* más conocida como penco o maguey muestra una gran importancia en las tradiciones de grupos folklóricos presentes a lo largo del callejón interandino del Ecuador. Los productos más conocidos del *Agave americana* son las bebidas tradicionales como el Chaguarmishqui, la producción de fibras, como alimento y como fuente de saponinas esteroidales. Las saponinas son metabolitos secundarios presentes en el género agave, las cuales han sido investigados por su potencial terapéutico como antiinflamatorios, antitumorales, antifúngicos y antibacterianos, llamando la atención de fitoquímicos, biólogos y científicos para el descubrimiento de fármacos[1–3]. En función a esto, la presente tesis tiene como objetivo demostrar la presencia de saponinas esteroidales en las hojas de la subespecie ecuatoriana *Agave americana L.*, que son consideradas como producto de desecho durante la producción del Chaguarmishqui, Se secó y pulverizó la hoja de *Agave americana L* y prepararon extractos hidroalcohólicos aplicando ultrasonido como método de extracción, estos extractos crudos fueron particionados para obtener extractos de diferente polaridad a los cuales se les realizó el perfil fitoquímico y la identificación de saponinas utilizando técnicas cromatográficas y espectroscópicas. La caracterización fitoquímica resultó positiva para saponinas y otros metabolitos secundarios, tanto en el extracto crudo como en los extractos más polares. Se realizó una hidrólisis ácida directa para la obtención de las sapogeninas y el análisis fitoquímico resultó positivo para glicósidos y carbohidratos en la fracción polar, demostrando que la aglicona fue liberada encontrándose en la fracción no polar, demostrándose con los resultados obtenidos del análisis por cromatografía líquida de alta resolución, espectroscopia infrarroja y resonancia magnética nuclear. Con los resultados de este trabajo se logró determinar que si hay saponinas esteroidales en las hojas de *Agave americana L.* y que estás se encuentran en los extractos de mayor polaridad al particionar un extracto hidroalcohólico, y las sapogeninas se pudieron determinar en los extractos de menor polaridad al aplicar una hidrólisis ácida directa o indirectamente.

Palabras clave: *Agave americana*, saponinas, sapogeninas.

ABSTRACT

The *Agave americana* plant better known as penco or maguey shows a great importance in the traditions of folk groups present along the inter-Andean alley of Ecuador. The best-known products of *Agave americana* are traditional drinks such as Chaguarmishqui, the production of fibers, as food, and as a source of steroidal saponins. Saponins are secondary metabolites present in the genus agave, which have been investigated for their therapeutic potential as anti-inflammatory, antitumor, antifungal, and antibacterial drugs, drawing the attention of phytochemicals, biologists, and scientists for drug discovery[1–3]. Based on this, the present thesis aims to demonstrate the presence of steroidal saponins in the leaves of the Ecuadorian subspecies *Agave americana L.*, which are considered as a waste product during the production of Chaguarmishqui. The *Agave americana L.* leaf was dried and pulverized and prepared hydroalcoholic extracts applying ultrasound-Assisted Extraction (UAE) as an extraction method, these crude extracts were partitioned to obtain extracts of different polarity to which the phytochemical profile and the identification of saponins were carried out using chromatographic and spectroscopic techniques. The phytochemical characterization was positive for saponins and other secondary metabolites, both in the crude extract and in the more polar extracts. Direct acid hydrolysis was carried out to obtain the sapogenins and the phytochemical analysis was positive for glycosides and carbohydrates in the polar fraction, demonstrating that the aglycone was released being in the non-polar fraction, being demonstrated with the results obtained from the liquid chromatography analysis high resolution, infrared spectroscopy, and nuclear magnetic resonance. With the results of this work, it was possible to determine that if there are steroidal saponins in the leaves of *Agave americana L.* and that they are found in the extracts with the highest polarity when partitioning a hydroalcoholic extract, and the sapogenins could be determined in the extracts of lower polarity when applying acid hydrolysis directly or indirectly.

Keywords: *Agave americana*, saponins, sapogenins.

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ABREVIATIONS

PEF	Pulsed Electric extraction
UAE	Ultrasound-Assisted extraction
SFE	Supercritical Fluid Extraction
EAE	Enzyme Assisted Extraction
PLE	Pressurized Liquid Extraction
TLC	Thin-Layer Chromatography
HPLC	High Performance Liquid Chromatography
IR	Infrared Spectroscopy
NMR	Nuclear Magnetic Resonance
UV	Ultraviolet
nm	Nanometers
g	Grams
mg	Milligrams
mL	Milliliter

1. INTRODUCTION

The *Agave americana* is a perennial plant, up to 4 meters tall, with robust green or pale gray leaves, with a height of between 1 and 3 meters long[4,5].

This species is typical of Mexico, but it has been distributed throughout the world in countries such as Ecuador. In Ecuador, the subspecies *Agave americana L.* plant has been cultivated or found in the wild throughout the sierra region[6] and has been used commonly for the production of traditional drinks such as Chaguarmishqui, as food, for the manufacture of textiles and ropes. In the production of Chaguarmishqui, the core of the plant is used and the rest of its parts do not enter into this process.

The bibliography reports that a great variety of saponins are found in the leaves and rhizomes, where from the structural point of view of saponins they have a polar part corresponding to carbohydrates and a nonpolar part (aglycone or sapogenin) linked by a glycosidic bond[2]. Saponins are polar molecules, which are soluble in hydroalcoholic mixtures[7].

Saponins are secondary metabolites that can have a steroidal or triterpenoid framework. In *Agave americana L*, the presence of steroidal saponins of the spirostanol and furostanol type is reported[8]. From the point of view of their biological activity, they are characterized by being anti-inflammatory, antitumor, insecticidal, and antimicrobial. Being all these properties potential for the pharmaceutical industries.

Thus, the present research aims to determine the presence of steroidal saponins, using different extraction mechanisms applied to the *Agave americana L.* leaf provided by Uyamafarms S.A, which are not used in the production of Chaguarmishqui. In addition to carried out the phytochemical analysis, and identification with chromatographic and spectroscopic techniques to the extracts obtained. This makes it possible to take advantage of a residue with medicinal potential.

This work begins with the problem statement which provides guidance for the development of the research objectives, which demonstrates the importance of this study.

A background that is a compilation of general information about plant family, the *Agave americana* species, secondary metabolites present in the specie, kind of saponins and their classification, therapeutic properties and the methods studied for the extraction of saponins. Then the methodology is presented where the materials, reagents, and work methodology were performed, then the analysis of the samples. Later, the analysis and discussion of results obtained during the experimentation and their respective analyzes are reported, with their respective tables and figures. Finally, the conclusion of this work based on the results obtained, and the recommendations that can guide other future investigations.

2. PROBLEM STATEMENT

Through the years, the human being has been used plants for different activities such as rituals, for the manufacture of textiles, as food, for the production of beverages, and for medicinal uses. In countries like Ecuador, you can find great biodiversity of plants, which have been studied because their medicinal properties, however are many others properties that are waiting to be discovered and taken advantage of.

Plants such as *Agave americana*, a species that grows wild in the Sierra del Ecuador region, is used to make traditional drinks such as Chaguarmishqui, where only the core of the plant is used, and the leaves are not involved in the elaboration of this drink and could be considered as waste, or are used for the manufacture of textiles, fibers, and ropes.

Studies report that *Agave americana* leaves are a source of secondary metabolites, especially steroidal saponins such as hecogenin and tigogenin, the same ones that play a very important role within the pharmaceutical industry thanks to their therapeutic properties such as anti-inflammatory, antitumor, antibacterial and antifungal. Hecogenin and tigogenin are compounds of high economic value and high demand due to their low production, they could be obtained from the leaves of the *Agave americana* not used in the production of Chaguarmishqui.

Therefore, the present study analyzes the presence of steroidal saponins in the leaves of *Agave americana L.*, obtained by different extraction processes, taking advantage of the residues of the Chaguarmishqui production process.

3. OBJECTIVES

3.1. General objectives

To determine the presence of steroidal saponins in the extracts applying different extraction mechanisms in the leaves of *Agave americana L*. not used in the manufacture of Chaguarmishqui.

3.2. Specific objectives

- Evaluate different extraction mechanisms for the isolation of steroidal saponins present in the leaves of *Agave americana L.* obtaining different extracts.
- Analyze the qualitative phytochemical screening of the extracts obtained from the leaves of *Agave americana L.*
- Identify the presence of saponins in the fractions obtained from the leaves of *Agave americana L.* by means of chromatographic and spectroscopic techniques.

4. BACKGROUND

4.1. Agavaceae family

Agavaceae family is a perennial monocotyledonous family species [9]. Although they have some characteristics in common, they vary greatly. It includes around 8 genera: Agave L., Beschorneria Kunth, Furcraea Vent., Hesperaloe Engelm., Manfreda Salisb., Polianthes L., Prochnyanthes S. Watson, Yucca L, even Hesperoyucca (Engelm.) Baker, where 342 species are distributed[10]. This family originally grows in the tropical territory, highly cultivated in Costa Rica, but centered in Mexico and the Antilles, is distributed throughout the world in temperate and arid zones along all continents since they show a remarkable morphological diversity, and an adaptation in dry climates since it prevents the plant from losing water through perspiration[6,11].

The plants that compose the Agavaceae family are widely used ancestrally by folk groups around the world as food, fiber, for the production of fermented beverages and traditional medicine[12], and have been reported to have become a source of chemistry research showing a wide variety of secondary metabolites. The secondary metabolites produced in the Agavaceae family have some biological activity as a contraceptive activity, against inflammations and it is presumed that it may have properties against cancer. The main goal of the plant to produce secondary metabolites is to combat predators by using cytotoxic bioactive agents, and they also made use of these metabolisms to enhance their pollination[11,13].

4.2. *Agave americana*

Agave americana is a perennial plant of the Agavaceae family that belongs to the genus Agave (Table 1).

Table 1. Taxonomic classification of *Agave americana*.

Kingdom	**Plantae**
Division	Magnoliophyta
Class	Liliopside
Order	Asparagales
Family	Agavaceae
Genus	Agave
Specie	*Agave americana*
Scientific name	*Agave americana*
Common names	Century plant, maguey, American aloe, Penco[14]

Agave americana presents its modified stem provided with buds from which its large leaves come. They normally reproduce by seed or by suckers of the same plant. The *Agave americana* has a height of 2 m with 3 - 4 m in provided with an elongated stem with flowers on the top and robust leaves on the bottom, with approximately between 40 and 70 thick concave leaves of a height of 1 - 3 m in length and of a green or pale gray color (Figure 1). Flowering occurs in the months of June and August and fruiting occurs in the months of September to December [4,13].

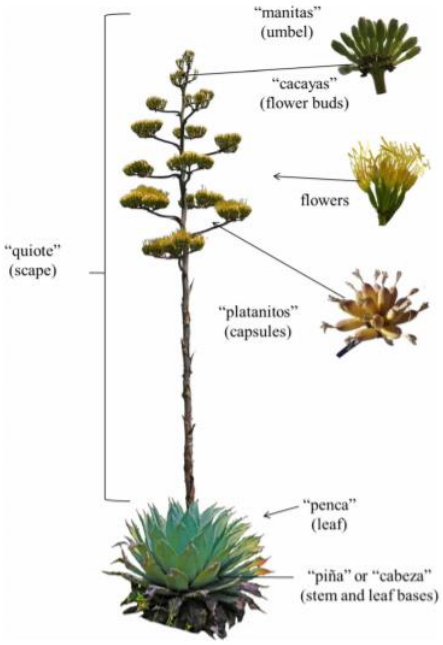


Figure 1. Parts of plant *Agave americana*[15].

Agave americana is native to Mexico, it can even be found in some parts of Europe, in the America continent, we can find it in countries such as Bolivia, Colombia, Chile, Ecuador, Panama, Peru, and Venezuela[16], because which over time has been introduced in different parts of the world with the aim of taking advantage of its properties in the production of sugars which in turn opened the production of alcoholic beverages such as tequila. Specifically , in Ecuador, we find subspecies such as Linnaeus, Marginata, Picta, and Furcraea Andina in plantations at an altitude of 1500 - 2500 meters above sea level [16,17] (Figure 2), along the entire inter-Andean alley in arid and semi-arid areas in the Sierra Region in a wild way, it can even be found in roads and parks. It is famous because it is used for the production of Chaguarmishqui traditional drink in Ecuador and for the production of tequila, a drink alcoholic in Mexican territory[18].

(a)  (b)  (c)  d) 

Figure 2. Subspecies of *Agave americana* a) *Agave americana Linnaeus*, b) *Agave americana Marginata*, c) *Agave americana Picta, d) Furcraea andina.*

Agave americana is a source of different secondary metabolites, among them saponins. The saponins are used for the manufacture of soaps, for therapeutic purposes, and even formerly the natives used the spines that are used to give relief to embroidery and as needles or nails. The leaves are used as fiber to make ropes, canvases, any type of textiles and other necessary utensils, the leaves are also used as room roofs. In addition, a study reported in 2019 shows the use of *Agave americana* leaves for the production of biomass[19].

Some applications are focused on industrial use, where this plant is massively cultivated for the production of tequila. Tequila is an alcoholic drink of Mexican

origin, produced in some parts of Ecuador. The process of obtaining tequila begins with the harvest when the plant has a maturity of 7-10 years to obtain the best sugars. To obtain this drink only use the heart as in the Chaguarmishqui, where an opening is made in the heart of the plant, it is covered and the juices are removed and it is carried out to fermentation, distillation, cleaning, and purification[20]. The leaves are cut and separated as a source for other uses or wastes so that they must be used due to their pharmaceutical properties with industrial and economic interest. The heart goes through a process of cooking, tearing, fermentation, distillation, and finally maturation[21].

The chemical content of the leaves have pharmaceutical potential and phytonutrients due to all the secondary metabolites that they present as steroidal saponins and saponins[2], hence the idea of taking advantage of this residue from the tequila industrial process and giving it use with great economic potential.

4.3. Secondary metabolites

Secondary metabolism of the plants allows the production and accumulation of compounds of a diverse chemical nature, called secondary metabolites. Secondary metabolites that are formed from precursors developed in primary metabolism. This occurs because plants allocate a significant amount of carbon and energy to the synthesis of a wide variety of organic molecules that are secondary metabolites. These metabolites are synthesized in small quantities and their production depends on the genus of the plant, the family, and species. Secondary metabolites are considered non-essential since they do not participate in the functions essential for the life of the plant, but are regulatory, since in the plant they have functioned as attractants or repellants for animals, providing color to flowers and fruits to ensure that pollinating insects ensure their reproduction, they can produce bitter flavors to combat predators or pathogens that can attack the plant[22,23]. While for human beings it is important and they take advantage of them because they are attributed an important amount of therapeutic, pharmacological, cosmetic, textile, ecological properties, among others.

The *Agave americana* in his chemical content presents a list of elements such as phosphorus, iron, sodium, potassium, magnesium, calcium, zinc, and copper. Also amino acids such as lysine, tryptophan, histidine, phenylalanine, leucine, tyrosine, methionine, valine, and riboflavin [13].

As an overview in the *Agave americana*, it has been reported their main antibacterial power, being a medicinal potential due to the secondary metabolites reported in the leaves as alkaloids, tannins, phenols, flavonoids, saponins, glycosides, and carbohydrates[24] and have be reported that the most abundant components are saponins and flavonoids [13]. The literature has been reported that the chemical content depends on the part of the plant where secondary metabolites were isolated. In roots carbohydrates, reducing sugar, steroids, cardiac glycosides, and saponin glycosides are present and which are attributed to their diuretic and antisyphilitic properties. In the leaves, rhizomes, flowers and callus cultures phenols, flavonoids, and saponins are present, and some biological activities as insecticidal has been evaluated. These secondary metabolites are considered a set of phytochemicals responsible for interfering with the physiology and behavior of pests[2,25,26].

4.3.1. Saponins

Saponins are bioactive compounds that can be found in many plants, however, certain animals such as marine animals and insects may have saponins present. We can find them as steroidal glycosides, steroidal alkaloids, and triterpenoids, within their structure they have 1 or more sugar molecules, however, they can also appear as aglycones that lack the sugar molecule, called sapogenin which is linked by a bond glycosidic gives it a hydrophobic and hydrophilic character (Figure 3). Its classification will depend on its aglycone, which can be steroidal or triterpenoid[22,23,27–29]. Saponins, whether steroidal or triterpenoid, are synthesized from the isoprenoid pathway, the biosynthesis of saponins is understood thanks to their characterization in proteins and genes of the biosynthetic pathway of terpenoids in plant cells[30].

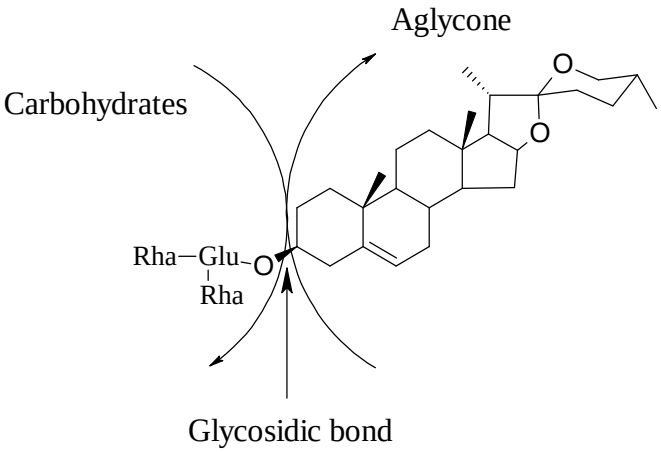


Figure 3. General structure of steroidal saponin.

As we mentioned earlier, the classification of saponins will depend on their steroid or triterpenoid aglycone. Content of steroidal saponins are prevalent in monocotyledonous plants, focusing on families Amaryllidaceae, Dioscoreaceae, and Liliaceae. Its structure is made up of 27 carbons, presenting a hexacyclic structure. These secondary metabolites are the protagonists in pharmaceutical productions such as cortisone, contraceptives, estrogens, among others.

Triterpenoid saponins have a structure made up of 30 carbons, we can find them in dicotyledonous plants, and they constitute the majority of saponins that are present in nature, among them they only differ in the number and type of sugar attached to their aglycone. They can be present in 3 different chemical structures (30-45 carbons) acyclic, tetracyclic, and pentacyclic[1,27,29,31].

Saponins are polar molecules so that they are soluble in hydroalcoholic solutions and insoluble in solvents of medium to low polarity. They are surfactants capable of forming foam and emulsions, hence their use and replacement of traditional soaps by these detergents. Saponins can also be toxic thanks to the formation of complex sterols, causing nausea, stomach pain, diarrhea, and dysfunctions in digestion, they can even break down red blood cells, releasing their content, however, despite this they also have a wide variety of therapeutic properties such as anti-tumor, anti-inflammatory, antioxidant, anti-pathogenic, anti-fever properties and can be stimulatory for the uterus. Saponins also have an effect on

animal products as it reduces the cholesterol present in meat, however, this topic requires further investigation[32]. Studies carried out in 2015[33] report the use of saponin extracts as an aid to eliminate Cr^{+6} by 16% and As^{+3} by 64.41% in contaminated waters. An important fact of hydrolyzed saponins (sapogenins) is that they can reduce methane production in living beings when the population of protozoa associated with methane-producing bacteria decreases. Furthermore, the literature states that saponins produce more propionate, resulting in less hydrogen production which is necessary to make more methane[29]. Sapogenin have a very important pharmaceutical use in the synthesis of hormones, as enhancers of the human immune system, for the manufacture of drugs for Alzheimer's, and even research on human vaccines[1,23,27,32,34]. Here their important role in the investigation field.

4.3.1.1. Saponins present in *Agave Americana*

The saponins of *Agave americana* are fundamentally steroidal. Steroidal saponins constitute a very large group of compounds with different properties and structures, which can be divided according to the structure of the spirostanol (27 carbons) or furostanol (26 carbon) type (Figure 4). The steroidal saponins that are reported in *Agave americana* are of the monoglycosides , diglycosides, triglycosides, hexaglycosides spirostanol and glycoside furostanol type, found in leaves, flowers, rhizomes (Table 2)[2,8].

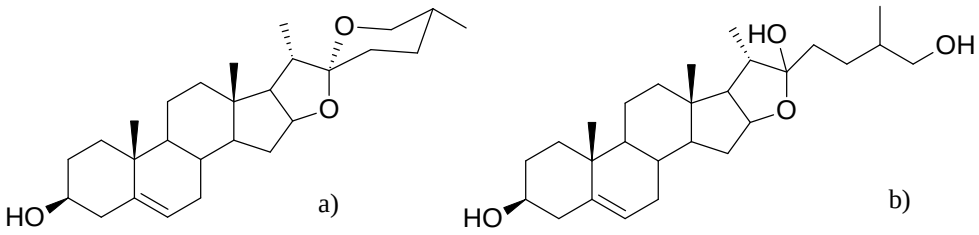


Figure 4. Steroidal saponins a) spirostanol, b) furostanol[35].

Table 2. Details of the steroidal sapogenins isolated from *Agave americana*[2].

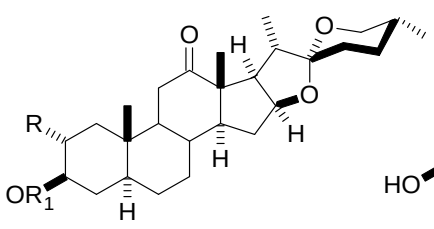
N°	Name	Source
Steroidal sapogenin (Figure 5)		
1	(25R)-5α-Spirostan-3β-ol-12-one (Hecogenin)	Leaves, fermented leaves
2	(25R)-5α-Spirostan-3β,6α,23α-triol (Hongguanggenin)	Fermented leaves
3	(25R)-5α-Spirostan-3β,6α-diol (Chlorogenin)	Flowers
4	(25R)-5α-Spirostan-3β,6α,11α,23α-tetraol (Agavegenin A)	Fermented leaves
5	(25S)-5α-Spirostan-3β,23α,27-triol (Agavegenin B)	Fermented leaves
6	(25S)-5α-spirostan-3β,6α,23α,24β-tetrol (Agavegenin C)	Leaves
7	(25S)-5α-Cholestane-3β,16β,22β,26-tetrol (Agavegenin D)	Leaves
Spirostanol monoglycosides (Figure 6)		
8	(25R)-5α-spirostan-3β-ol-12-one 3-O- β -D-galactopyranoside (hecogenin 3-O-β-D-galactopyranoside) (Agavoside A)	Fermented leaves
9	25R)-5α-Spirostan-3β,6 α,23α-triol 6-O-β-D-glucopyranoside (Hongguanggenin 6-O- β-D-glucopyranoside) (Agamenoside C)	Fermented leaves
10	(25S)-5α-Spirostan-3 β,23α,24-triol 24-O-β-D-glucopyranoside (Agamenoside I)	Leaves
Spirostanol diglycosides (Figure 7)		
11	(25R)-5α-Spirostan-3β,6α-diol 3,6-di-O-β-D-glucopyranoside (Chlorogenin 3,6-di-O-β-D-glucopyranoside)	Leaves
12	(25R)-5α-Spirostan-3β,6 α,23β-triol 3,6-di-O-β-D-glucopyranoside (Hainangenin 3,6-di-O-β-D-glucopyranoside)	Leaves
13	(25R)-5α-Spirostan-3β,6 α,23α-triol 3,6-di-O-β-D-glucopyranoside (Hongguanggenin 3,6-di-O-β-D-glucopyranoside) (Cantalasaponin-1	Leaves
14	(25R)-5α-Spirostan-3β,6α-diol-12-one 3,6-di-O-β-D-glucopyranoside	Leaves
15	(25S)-5α-Spirostan-3β,6α,23α,24β-tetrol 6,24-di-O-β-D-glucopyranoside (Agamenoside H)	Leaves
16	(25R)-5α-Spirostan-3β-ol-12-one 3-O-β-D-glucopyranosyl-(1→ 4)-O-β-D-galactopyranoside (Hecogenin 3-O-β-D-glucopyranosyl - (1→ 4)-O-β-D-galactopyranoside) (Agavoside B)	Fermented leaves

Table 2.
Continuation

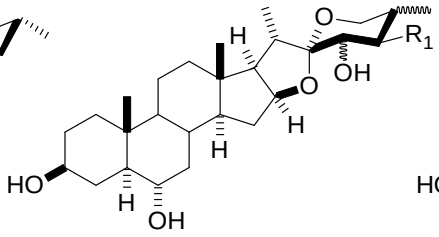
N°	Name	Source
Spirostanol triglycosides (Figure 8)		
17	(25R)-5α-Spirostan-3β-ol-12-one 3-O-β-D-glucopyranosyl-(1 → 2) -O -β-D-glucopyranosyl -(1→4)-O-β-D-galactopyranosid	Fermented leaves
Spirostanol tetraglycosides (Figure 9)		
18	(25R)-5 α -Spirostan-3β-ol-12-one 3-O- β -D-glucopyranosyl-(1→2)-O-[β -D-xylopyranosyl-(1 →3)]-O- β -D-glucopyranosyl-(1 → 4)-β-D-galactopyr	Leaves, fermented leaves
19	(25R)-5 α -Spirostan-3 β,6 α -diol 3-O- β -D-glucopyranosyl- (l→2)-O-[β -D-xylopyranosyl-(l→3)]-O- β -D-glucopyranosyl-(1 →4)-O- β -D-galactopyranoside (Agamenoside B)	Fermented leaves
20	(25R)-5 α -Spirostan-3 β -ol-12-one 3-O- α -L-rhamnopyranosyl-(1 → 3)-O-[β -D-glucopyranosyl-(1 → 2)]-O- β -D-glucopyranosyl-(1 → 4)-O- β -D-galactopyranoside (Agamenoside D)	Fermented leaves
Spirostanol pentaglycosides (Figure 10)		
21	(25R)-5 α -Spirostan-3 β -ol 3-O- β -D-xylopyranosyl-(1→3)-O- β -D-glucopyranosyl-(1 → 2)-O-[β -D-xylopyranosyl-(1 → 3)]-O- β -D-glucopyranosyl-(1 →4)-O- β -D-galactopyranoside	Rhizomes
22	(25R)-5 α -Spirostan-3 β -ol-12-one 3-O- α -L-rhamnopyranosyl-(1 → 3)-O- β -D-glucopyranosyl-(1 → 2)-O- [β -D-xylopyranosyl-(1→3)]-O- β -D-glucopyranosyl-(1 → 4)-O- β -D-galactopyranoside (Agamenoside E)	Fermented leaves
23	(25R)-5 α -Spirostan-3 β -ol-12-one 3-O- β -D-xylopyranosyl-(1 → 3)-O- β -D-glucopyranosyl-(1→2)-O-[β -D-glucopyranosy -(1→3)]-O- β -D-glucopyranosyl-(1→4) -O- β -D-galactopyranoside (Agamenoside F)	Fermented leaves

Table 2.
Continuation

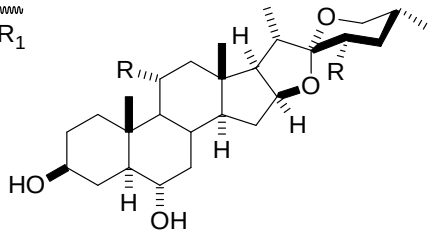
N°	Name	Source
24	(25R)-5 α -Spirostan-3 β -ol 3-O- α -L-rhamnopyranosyl-(1 →3)-O- β -D-glucopyranosyl-(1→2)-O-[β -D-xylopyranosyl-(1→3)]-O β -D-glucopyranosyl-(1→4)-O- β -D-galactopyranoside	Fermented leaves
25	(25R)-5 α -Spirostan-3 β,6 α,23 α -triol 3-O- α -L-rhamnopyranosyl-(1 → 3)-O- β -D-glucopyranosyl-(1→2)-O-[β -D-xylopyranosyl-(1→3)]-O- β -D-glucopyranosyl-(1→4)-O- β -D-galactopyranoside (Agamenoside A)	Fermented leaves
26	(25R)-5 α -Spirostan-3 β -ol 3-O- α -L-rhamnopyranosyl-(1→3)-O- β -D-glucopyranosyl-(1→2)-O-[β -D-glucopyranosyl -(1→3)]-O- β -D-glucopyranosyl-(1→4)-O- β -D-galactopyranoside (Agamenoside G)	Fermented leaves
Spirostanol hexaglycosides (Figure 11)		
27	(25R)-5 α -Spirostan-3 β -ol-12-one 3-O- α -L-rhamnopyranosyl-(1→4)-O- α -L-rhamnopyranosyl-(1→3)-O-[β -D-xylopyranosyl-(1→2)]-O- β -D-glucopyranosyl-(1→3)-O- β -D-glucopyranosyl-(1→4)-O- β -D-galactopyranoside (Agavesaponin E)	Leaves
Furostanol glycosides (Figure 12)		
28	(22S,23S,25R,26S)-23,26-Epoxy-5 α -furostan-3 β,22,26-triol 26- O- β -D-glucopyranoside (Agamenoside J)	Leaves
29	(25R)-26-O- β -D-Glucopyranosyl-5 α -furostan-3 β,22 α,26-triol-12-one 3-O- β -D-xylopyranosyl-(1→2)-O-[α -L-rhamnopyranosyl-(1→4)- O- α -L-rhamnopyranosyl-(1→3)]- O- β -D-glucopyranosyl-(1→4)-O- β -D-glucopyranosyl- (1→4)-O- β -D-galactopyranoside (Agavasaponin H)	Leaves



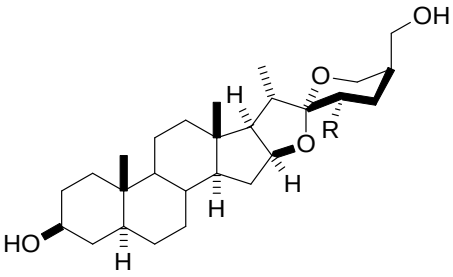
1: R = R_1 = H, 5α
1a: R = H, R_1 = CH_3CO, 5α
1b: R = H, R_1 = CF_3CO, 5α



2: 25R, 23α-OH, R_1 = H
6: 25S, 23α-OH, R_1 = β-OH



3: R = H
4: R = OH



5: R = H

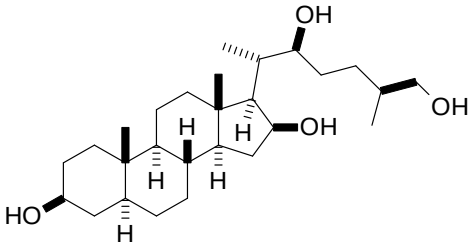


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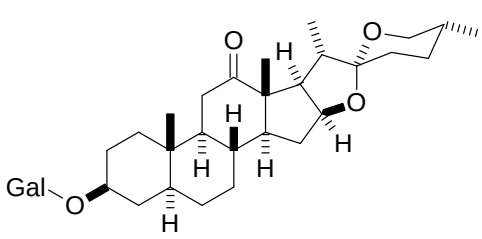
Figure 5. Chemical structures of the steroidal sapogenins reported from *Agave americana*.



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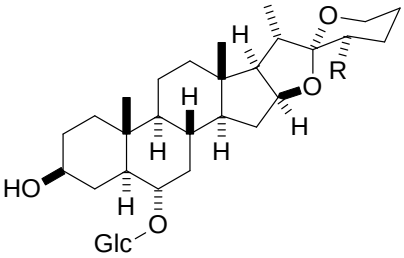

9: R = OH

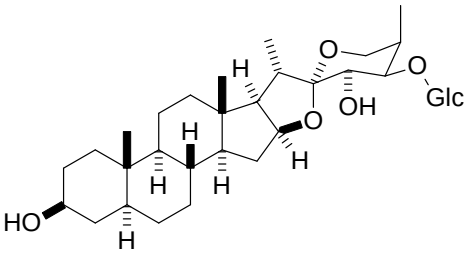

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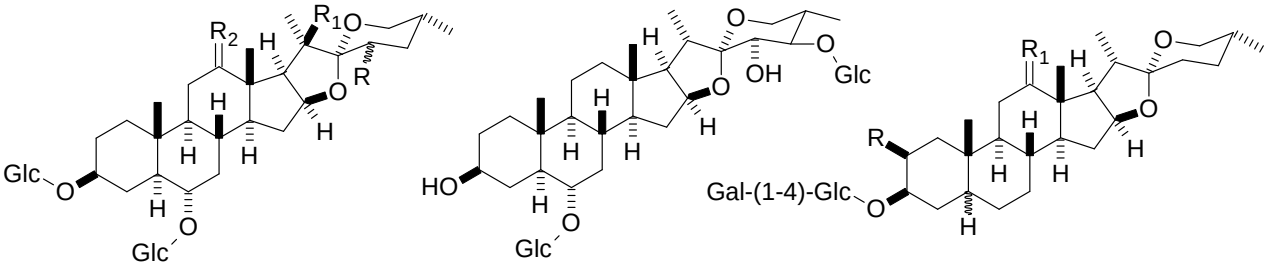
Figure 6. Spirostanol monolycosides of *Agave americana*.

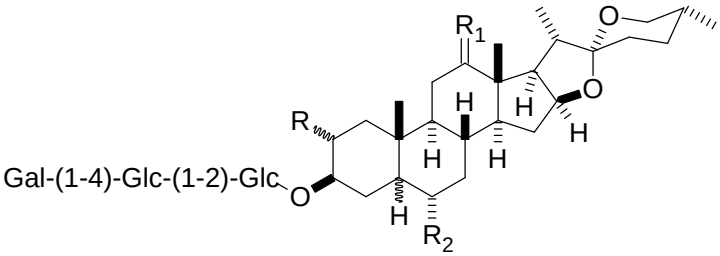


11: R = R_1 = H, R_2 = H,H
12: R = β-OH, R_1 = H, R_2 = H,H
13: R = α-OH, R_1 = H, R_2 = H,H
14: R = R_1 = H, R_2 = O

15

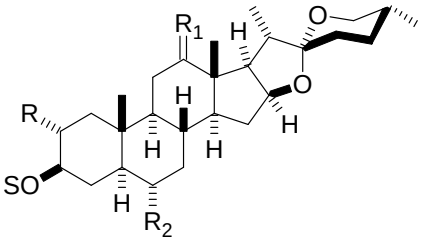
16: R = H, R_1 = O, 5α

Figure 7. Spirostanol diglycosides reported from *Agave americana.*



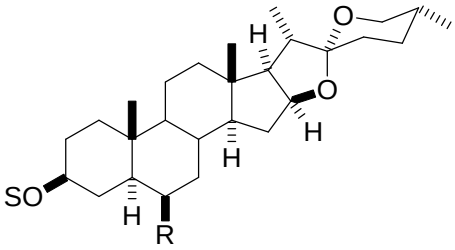
17: R = $R_{2=}$ H, R_1 = O, 5α

Figure 8. Spirostanol triglycosides reported from *Agave americana*.

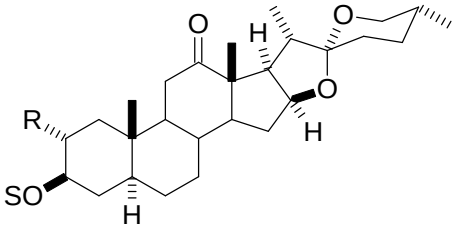


18: R = R_2 = H, R_1 = O, S = Glc-(1-2)-[Xyl-(1-3)]-Glc-(1-4)-Gal-
19: R = H, R_1 = H,H, $R_{2=}$ OH, S = Glc-(1-2)-[Xyl-(1-3)]-Glc-(1-4)-Gal-
20: R = R_2 = H, R_1 = O, S = Rha-(1-3)-[Glc-(1-2)]-Glc-(1-4)-Gal-

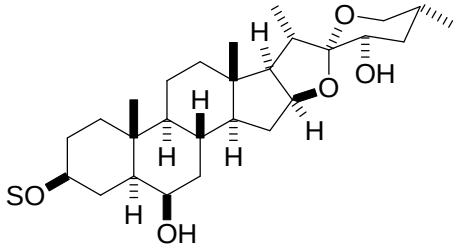
Figure 9. Spirostanol tetraglycosides reported from *Agave americana*.



21: R = H, S = Xyl-(1-3)-Glc-(1-2)-[Xyl-(1-3)]-Glc-(1-4)-Gal-
24: R = H, S = Rha-(1-3)-Glc-(1-2)-[Xyl-(1-3)]-Glc-(1-4)-Gal-
26: R = H, S = Rha-(1-3)-Glc-(1-2)-[Glc-(1-3)]-Glc-(1-4)-Gal-

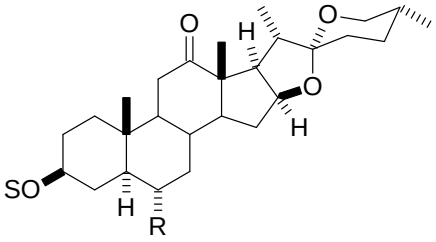


22: R = H, S = Rha-(1-3)-Glc-(1-2)-[Xyl-(1-3)]-Glc-(1-4)-Gal-
23: R = H, S = Xyl-(1-3)-Glc-(1-2)-[Glc-(1-3)]-Glc-(1-4)-Gal-



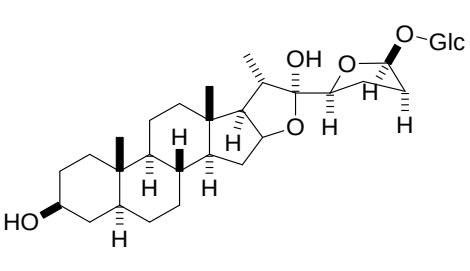
25: S = Rha-(1-3)-Glc-(1-2)-[Xyl-(1-3)]-Glc-(1-4)-Gal-

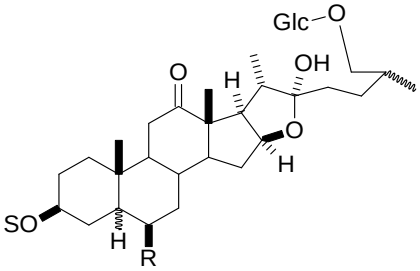
Figure 10. Spirostanol pentaglycosides reported from *Agave americana.*



27: R = H, S = Rha-(1-4)-Rha-(1-3)-[Xyl-(1-2)]-Glc-(1-3)-Glc-(1-4)-Gal-

Figure 11. Spirostanol hexaglycosides reported from *Agave americana*.





28 **29:** 25R, 5α, R = H, S = Xyl-(1-2)-[Rha-(1-4)-Rha-(1-3)]-Glc-(1-4)-Glc-(1-4)-Gal-

Figure 12. Structures of furostanol saponins of *Agave americana*.

4.3.1.2. Pharmacological activities steroidal saponins

Steroidal saponins are a group of natural antibiotics with great properties, when they are included in the diet, they can protect the body against diseases as serious as cancer since they attack tumor cells with their cytotoxic activity. Different studies have been tested against fungi and bacteria in saponins present in leaves to prevent the growth of these pathogens with great effectiveness in candida or dermatophytes. Saponins have been part of drugs known for their anti-inflammatory power, since they are responsible for not degrading corticosteroids, and acts as a barrier to the metabolism produced to cause inflammation. Even, among the great benefits that we can find in saponins, is their influence on cholesterol, heart problems, and in the treatment of gastric ulcers. It should be noted that the different pharmacological activities of saponins have only been studied in vitro[1–3].

4.3.1.3. Hecogenin and tigogenin

Hecogenin and tigogenin (Figure 13) are steroidal sapogenins and are present in different types of agave leaves, especially *Agave americana*, it is even possible to find them in the residues of the fermented juice of *Agave americana*[36]. The content of steroidal saponins depends on several factors such as cultivation, age, and geographical location. The concentrations of hecogenin and tigogenin in *Agave americana* depend a lot on the age of the plant, because as the plant grows the content of these glycosides varies, there is a greater amount if it is immature and less when the plant is mature[1]. Hecogenin, the compound found in the *Agave americana* in the highest proportion is tigogenin, the proportion between the two is very important, there must be a lower amount of tigogenin because, as we can see in Figure 13b, tigogenin lacks a group carbonyl, therefore, it does not allow the synthesis of new drugs such as hecogenin. Both come from the same mixture but at the time of their extraction and to separate them, acetylation of the hydroxyl group is required[37].

These sapogenins have great potential and applicability in the industry, especially hecogenin for all its pharmaceutical properties, having a worldwide demand of 900 tons per year, but the production is only enough to cover 44%, the other 56% not covered has sparked interest in developing new and improved methodologies for obtaining hecogenin[38]. In addition to being a compound of pharmacological interest, the use of this raw material that is not used in the production of Chaguarmishqui is added.

According to Sigma Aldrich, these saponins are priced at up to $ 477.74 per 10 mg of hecogenin or tigogenin, so they are compounds of great economic value and one of the reasons why the use of waste from the agave is very important.

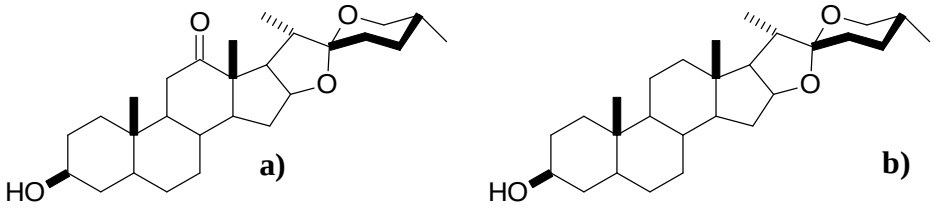


Figure 13. Steroidal sapogenins a) hecogenin, b) tigogenin.

Hecogenin is a compound present in the agave, it is a compound that has a carbonyl group as illustrated in Figure 13a, which allows the introduction of a double bond that allows the inclusion of fluorine for the manufacture of hormones, it has hemolytic properties and inclusive toxic for some animals such as fish, but one of the most representative values of hecogenin is its use in diseases such as cancer, one of the most destructive diseases of the body worldwide and one of the pathologies of greatest interest in the pharmaceutical industry. Currently, you can find commercial hecogenin with a purity of 95 and 99%[38].

Tigogenin participates in the production of steroids in the pharmaceutical industry, which is where the interest in its extraction comes from, in addition to having antibacterial properties[39].

4.4. Methods

4.4.1. Conventional extractions

4.4.1.1. Maceration extraction

This extraction is about putting the raw material, together with a solvent in a closed container or system for a long time, avoiding the entry of sunlight, avoiding possible reactions, at room temperature, for a specific time that can be days, weeks, or even months, with frequent movements during the maceration process in order to reduce the operating time. After the necessary time for the maceration, filtration is carried out, using the same solvent used in the maceration to wash the cake, in order to filter what has been retained in it[40,41].

There are several solvents used in the mash, such as hydroalcoholic solvents, water, or glycerin[40].

The disadvantages of using this technique for a long time and the amount of solvent required when using this technique is also possible that a complete extraction may not be achieved. However, it is a low-cost extraction as it does not require the use of sophisticated equipment. Maceration is a widely used method for the extraction of saponin with a good yield, however, as it is a very long process, the use of a stir bar is recommended[42].

4.4.1.2. Percolation extraction

This extraction has two stages, first an initial wetting stage, followed by an exhaustive extraction of the active substances of interest from the plant. This extraction presents a constant flow of solvent that can be water, alcohol, or another, which will pass through the raw material in a porous material, dragging all the active principles of interest. The success of the extraction will depend on the level of fineness of the raw material, the speed of expansion of the active substances from the raw material to the solvent, and the speed with which the solvent flows. This method is used mainly in the pharmaceutical industry for the production of trickling extracts[43,44].

The advantage of this technique is the extraction time compared to the time that is invested in the maceration. However, this technique maintains a high solvent consumption. The percolation method has low yields in saponins, this is due to the contact between the solvent and the matter, which is less in relation to other solvents, in addition to having a long extraction time[45,46].

4.4.1.3. Soxhlet and reflux extraction

This extraction is carried out in a Soxhlet device, which has 3 parts, one that acts as a refrigerant, another that is the extractor, and the last that is the balloon. The process begins with heating the balloon until the solvent boils, the vapors will rise to the refrigerant where they will condense and fall on the sample that was previously put in the extractor, macerating it until when in the extractor, when it fills falls through the side tube until it reaches the boiling balloon, this process is repeated continuously, so that the solvent is not lost, on the other hand, the solvent is recycled, and the active substances are concentrated in the balloon[43].

Soxhlet operation is similar to reflux extraction, the difference is that it only has two parts, one that is the refrigerant where the solvent will condense ensuring that it is recycled and the volume is not lost, the other part is the balloon where the raw material is put together with the solvent in heat and constant stirring for a certain time until the active principles are extracted.

Both extraction methods are a distillation process used in industrial laboratories for different purposes. The disadvantage of this technique is the time required for extraction, as a minimum of one hour is required for reflux and in Soxhlet, it can take 24 to 72 hours, in spite of that, it is a widely used method for the extraction of saponins but involving high risk in the decomposition of pharmaceutical substances due to the use of heat[42].

4.4.2. Unconventional extractions

4.4.2.1. Accelerated solvent extraction

The method started in 1995 thanks to Dionex Corporation, considered as ecological in the preparation of samples of plant material prior to chromatographic analysis. It involves a rapid, pressurized solvent extraction, with a small solvent consumption, but applying high pressures and temperatures (100 ° C, 1500 psi), with the aim of improving the solubility of the active principles in the solvent, controlling that the solvent does not reach the boiling point and extraction be effective. The estimated time it takes for extraction to be 15 to 25 minutes in samples of 1 to 30 g using 15 to 45 ml of solvent during several extraction cycles. This technique is preferably applied in environmental, polymer, pharmaceutical, and food fields. This technique for the extraction of saponins is not common, however, it has been shown that there are positive results in the extraction of saponins in cow cockle seeds compared to extraction with ultrasound[42,47].

4.4.2.2. Microwave-assisted extraction

Microwaves are electromagnetic waves of a high frequency that are found between the radio frequency and the far-infrared regions of the electromagnetic spectrum with a frequency range of 0.3 to 300 GHz. One of the great differences of this technique in relation to others is the shape heating of the raw material, normally when it is heated, the heat begins from the outside to the inside, on the other hand, with microwave-assisted extraction the heat begins at the center of the material, that is, from the inside to the outside. What happens is that the microwave energy alters the molecules by ionic conduction, where the ions will migrate when an electromagnetic field is applied, and the resistance of the solution to this movement of the ions will result in friction that will lead to heating. Now, another point to consider in this extraction is the correct selection of solvents for successful extraction, since we must take into account the microwave absorption properties of the

solvent, the interaction of the solvent with the matrix, and the solubility of the analyte in the solvent. The higher the dipole moment of the solvent, the faster the solvent will be heated in the presence of microwave irradiation[48]. This method has higher performance in the use of saponins compared to other techniques and this is due to simultaneous heating of the samples and the solvent, obtaining an improved yield[42,49].

4.4.2.3. Ultrasound-assisted extraction (UAE)

This form of extraction achieves an efficient, complete, and high-performance process by applying high-power, low-frequency ultrasound waves to raw material and a solvent, these waves pass through the liquid leading to changes in temperature and pressure, producing bubbles that they break on the surface of the solid, perforating the cell membrane and induce the extraction. This extraction has several benefits, including being effective in extraction, saves energy, solvent and temperatures are moderate, which is why it favors compounds that can be temperature sensitive, however, for a satisfactory extraction it must be taken into account the ultrasonic potential that is used, the frequency, temperature and the interaction between the sample and the solvent. Although in recent years this technique is an important procedure in food engineering, it is also considered one of the most efficient extractions for obtaining saponins. The interest in compounds such as saponins and other bioactive substances of pharmaceutical interest led to the finding of a method that would give us a greater quantity of these bioactive compounds in a shorter and cheaper time[50].

4.4.2.4. Pulsed Electric Field Extraction (PEF)

In recent years, due to the demand in the industry for the active principles of plants, researchers have been forced to look for a method that is useful and profitable, so that the pulsed electric field extraction technique is positioned as a method for the industry, as it provides better yields when it comes to the extraction of active compounds and shorter times. The pulsed electric field

acts on the cell membrane inducing power of electrical pulses increasing the conductivity and permeability, this increase allows a reversible or irreversible rupture of the cell membrane of plants[51]. For the extraction of saponins, this method is considered to have a better performance compared to traditional methods, and all this due to its speed of extraction[52].

4.4.2.5. Supercritical Fluid Extraction (SFE)

This technique uses, as its name says, supercritical fluids to carry out the extraction, since it can act as a liquid due to its solubility and can act as a gas due to its diffusivity, favoring the extraction of a great variety of natural products. The process consists of heating fluid and it is pressurized until it becomes a supercritical fluid, ensuring that it has gas and liquid properties, acting as a solvent to extract materials from a sample. For this technique, supercritical carbon dioxide is used due to its low critical temperature, selectivity, economics, and ensuring a correct extraction for labile compounds[46]. Its use in saponins has been reported, resulting in a successful yield due to its rapid and efficient separation, however, the yield does not exceed that obtained by microwave-assisted extraction[42,46].

4.4.2.6. Enzyme Assisted Extraction (EAE)

One of the limitations when extracting natural products is when there are compounds that are affected by temperature (thermolabile) so that enzyme-assisted extraction favors this procedure thanks to the hydrolytic action of enzymes on the cell membrane of our material releasing the active principles of interest, in addition to providing high yields, unlike other extraction techniques, with lower energy consumption and shorter times. Pectin or cellulase, among others, is generally used to carry out the extraction. This method is widely used for the extraction of polysaccharides[46].

4.4.2.7. Pressurized Liquid Extraction (PLE)

It is a method that came as an alternative to conventional extraction methods. This technique is similar to accelerated solvent extraction but improved. Its mechanism basically is to apply high pressures during extraction to keep the solvents in a liquid state above their boiling point, favoring their diffusion, ensuring better penetration into the material we want to extract, reducing the time to obtain the bioactive compounds of interest, with low solvent consumption and high performance. This method has been used for the extraction of saponins, flavonoids, and even essential oils, however, with the latter, there are opposite criteria as to whether it is a suitable method for thermolabile compounds[46,53].

4.4.3. Thin-layer chromatography

TLC is a suitable technique to monitor saponins during the extraction and fractionation process. In addition, if a standard is available, the purity of the sample could be analyzed, however, we take into account that in TLC its visualization and analysis will be easier if we have an adequate screening of the sample, since the polar nature and Its high molecular weight causes difficulty in visualization, if a better resolution is desired, techniques such as HPLC can be used[54].

The most commonly used solvents for the visualization of saponins are a mixture of polar solvents, and even low polarity solvents are used at varied proportions to improve elution. The reported solvents are chloroform, methanol, ethanol, and water, in addition, it can be helped by developers such as 10% sulfuric acid and p-anisaldehyde where yellow marks show the presence of spirostanol and furostanol, and green, blue, and violet marks show the presence of steroids[2,54]. The use of developers is due to the fact that saponins are not easy to visualize by UV due to the lack of chromophore groups in their structure[54].

5. MATERIALS AND METHODOLOGY

5.1. Materials

Filter paper, TLC Silica Gel 60 F_{254} and silica XG TLC Plates, w/UV254, aluminum backed, 200um, 20x20cm, 25pk

5.2. Biological material

Agave americana Linnaeus leaves were proportionated by UyamaFarms S.A located 5 km from Mira – Carchi, the taxonomic determination of this plant was developed according to the available literature.

5.3. Reactives and equipments

5.3.1. Reactives

The chemical reactives used in this work are of analytical grade reagents and were: ethanol (93 %), distilled water; n-hexane, ethyl acetate and methanol with certified ACS grade from Fisher Chemical; hydrochloric acid (35 %) from Fisher Chemical; acetone, p-anisaldehyde, iodine and potassium iodide and Sigma Aldrich Life Science; sodium carbonate, sodium citrate dehydrated and copper (II) sulphate pentahydrated with granular certified from Fisher Chemical; glacial acetic acid with certified ACS grade from J.T. Baker; chloroform (99.8%) from LOBA Chemie; potassium sodium tartrate tetrahydrate with certified ACS grade from AppliCHem Panreac ITW Companies; iron (III) chloride hexahydrated (≥97%) from ISOLAB chemicals; magnesium powder with particle size 0.06 – 0.3mm from Merk KGaA; sodium hydroxide and sulfuric acid (95-97 %) with certified ACS grade from Merk KGaA; formic acid, acetonitrile and methanol with certified HPLC grade, LiChrosolv® from Sigma Aldrich Life Science; Tetrachloroethylene (99% extra pure) from Acros Organics

5.3.2. Equipments

The following equipments were used in this work: balance Cobos precision HR-150A (COBOS precision, Spain), BUCHI Rotavapor R-210, HPLC (UltiMate

3000), C-18 column for HPLC Hypertensil GOLD™ (150 x 4.6 mm 5µ particle size), the Agilent Cary 630 FTIR spectrometer, NMR (Nanalysis Corp, NMReady60 PRO spectrometer), drying stove (Pol-Eko Aparatura SLN 115), UV light (366 nm, Vilber Lourmat), ultrasound bath (Selecta, frequency of 40 KHz), coffee Grinder Electric (Daewoo, DCG-362), micropipettes (Finetech S series, model MS-1000H and MS-10H)

5.4. Tissue preparation

The plant tissue was washed, after that, the water excess removes and the leaves were cut in a transverse way of approximately 7 cm small pieces, were dried in the oven at 40 ° C until constant weight. The dried material was milling in a Coffee Grinder Electric until a homogeneous reasonably fine powder was obtained, which was weighed and then proceeded with the extraction of the material (Figure 14).

Figure 14. Drying of plant tissue

The yield of dried plant material was calculated as follow:

$$Dried\ plant\ material\ \% = \frac{Dried\ plant\ material}{Plant\ material} \times 100 \quad (1)$$

5.5. Extraction methods

5.5.1. Extraction procedure 1

For the extraction, the powder material was the first place an Erlenmeyer flask and then 90% ethanol solvent was added with ratio tissue-solvent 1:5 (w:v), allowed to ultrasonic (40KHz frequency) extraction for 15 min at 21°C during three cycles.

Finally, after the extraction, the plant tissue was filtered with the help of vacuum and filter paper, and the extract **(E1)** sample was evaporated to dryness with a rotary evaporator and stored until the phytochemical analysis is performed (Figure 15).

The percentage of yield of the process was calculated as follow:

$$\%Yield = \frac{M_{Af}}{M_{Ai}} x100 \quad (2)$$

Where:

M_{Ai}= Initial *Agave americana L.* mass

M_{Af}= E1

5.5.1.1. Fractioned (E1) by solvent extraction using partition coefficient

The first extract **E1** was concentrated and dissolved in water, then partitioned in a separatory funnel with solvents of increasing polarities first using n-hexane, second using ethyl acetate (1:3 v/v) for 3 times, obtaining three fractions hexanoic fraction **(F1A)**, ethyl acetate fraction **(F1B)** and aqueous **(F1C)**, and all fractions **F1A**, **F1B, F1C,** samples were evaporated to dryness with a rotary evaporator and stored until the phytochemical analysis is performed.

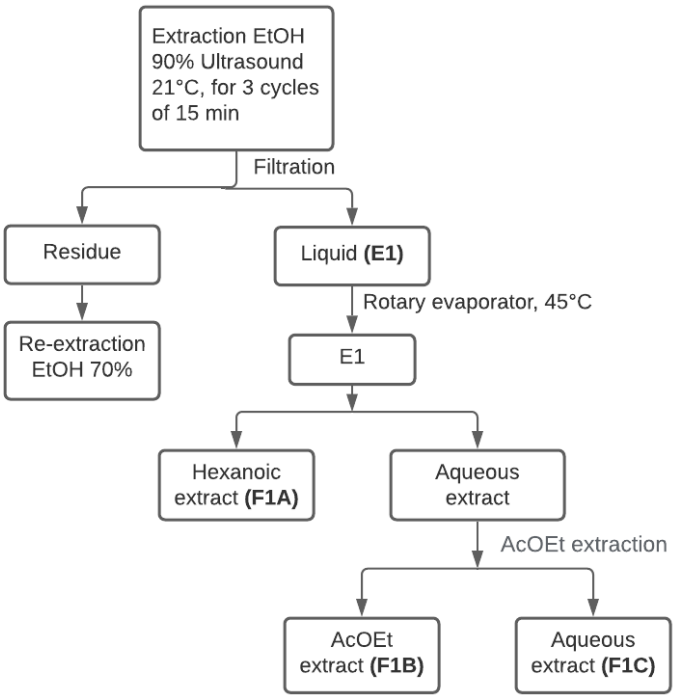


Figure 15. Overview of ultrasound extractions of *Agave americana L.*

5.5.2. Extraction procedure 2

To separate the polar part from the nonpolar of the saponin was carried out a second extraction first according to Extraction procedure 1, to get the extract **(E2)**. Then, this extract was hydrolyzed by reflux at 100°C for 3 hours with EtOH: H_2O: HCl 2N (20:65:15 v/v), after that was cooled and filtered with help of vacuum and filter paper, and obtained a solid residual **(E2B)** and liquid extract **(E2A)**. The liquid extract **E2A** was neutralized at pH 7, to get **(E2A-1).** Extract **E2A-1** was further extracted with n-hexane by reflux at 60°C for 1 hour, the mixture extraction obtained was separated in a separatory funnel to obtain a hexanoic extract fraction **(F2A)**. The aqueous layer was extracted in a separatory funnel with ethyl acetate twice to obtain ethyl acetate fraction **(F2B)** and aqueous fraction **(F2C),** samples were evaporated to dryness with a rotary evaporator and stored until the phytochemical analysis is performed (Figure 16).

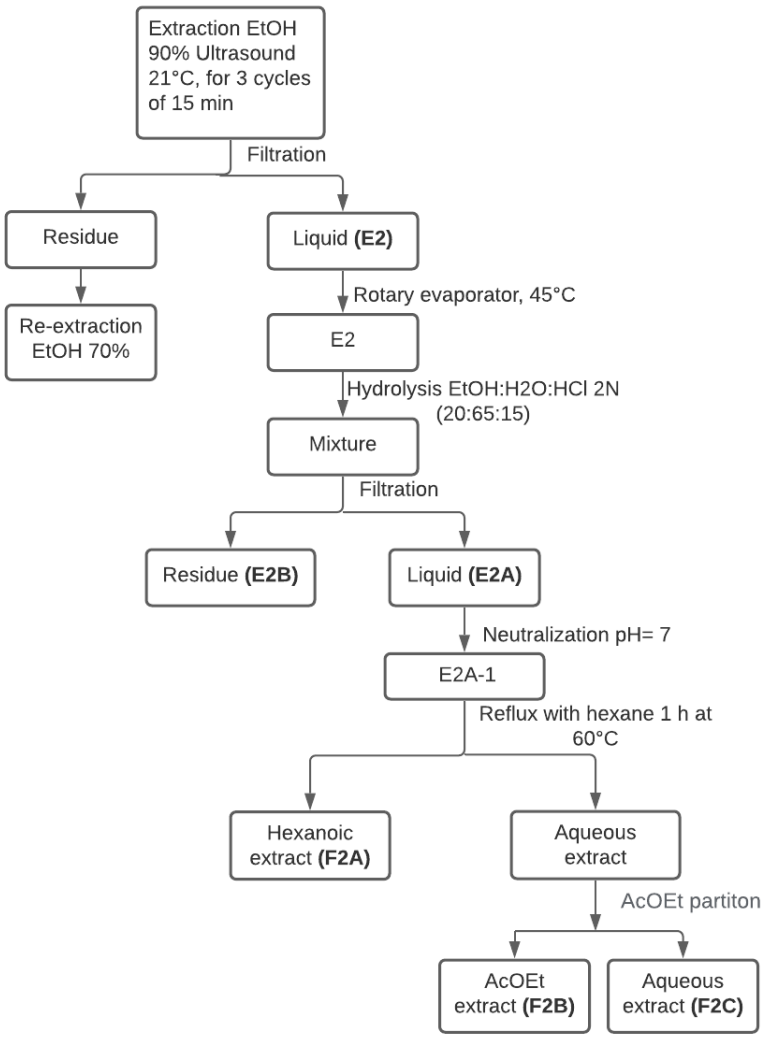


Figure 16. Overview of sapogenin obtention of *Agave americana L.* by hydrolysis of saponins.

5.5.3. Extraction procedure 3

To separate the polar from the nonpolar part of saponin a direct hydrolysis process was applied. The powder material was first placed in an Erlenmeyer flask and the EtOH: H_2O: HCl 2N (20:65:15 v/v) (1:4 w/v) solvent was added, allowed to ultrasonic (40KHz frequency) extraction for 45 min at 45°C. The mixture was neutralized at pH 7 **(E3)** and was filtered by vacuum and filter paper, to obtain a solid residual **(E3B)** and liquid extract **(E3A).** Extract **E3A** was extracted with n-hexane by reflux at 60°C for 1 hour, the mixture obtained was separated in a separatory funnel to obtain a hexanoic extract fraction **(F3A)**. The aqueous layer was extracted in a separatory funnel with ethyl acetate twice to obtain ethyl

acetate fraction **(F3B)** and aqueous fraction **(F3C).** These fractions were dried in a rotary evaporator until dried and stored until the phytochemical analysis (Figure 17).

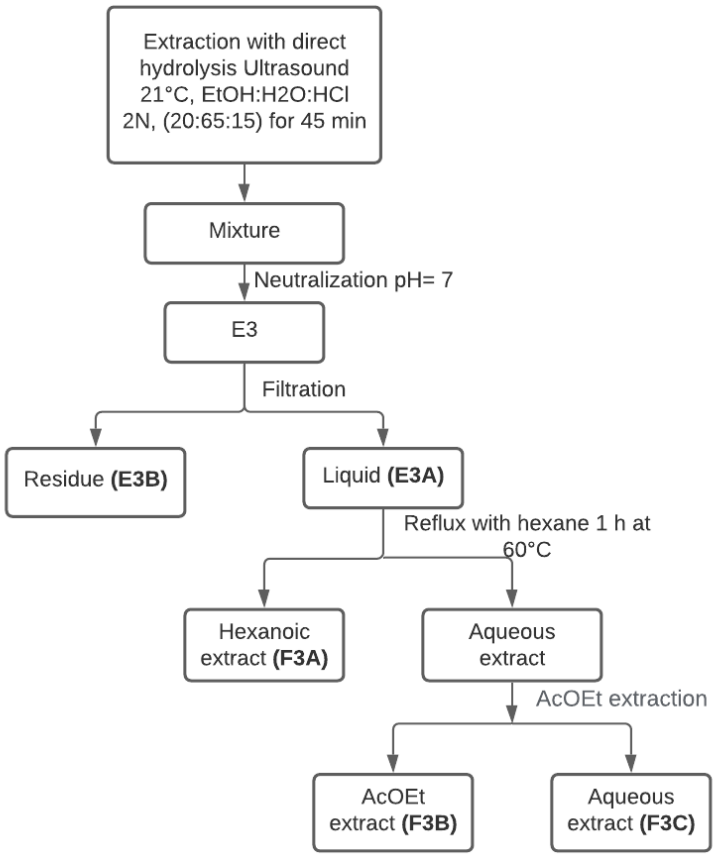


Figure 17. Overview of sapogenin obtention of *Agave americana L.* by direct hydrolysis.

5.5.4. Extraction procedure 4

To separate the polar part from the non-polar part of the saponins a direct hydrolysis process was applied, the treatment of this procedure was focused on the aglycones (non-polar part of saponins) that is soluble in nonpolar solvents. The powder material was first placing an Erlenmeyer flask and EtOH: H_2O: HCl 2N (20:65:15 v/v) (1:4 w/v) solvent was added, allowed to ultrasonic (40KHz frequency) extraction for 90 min at 45°C, the mixture obtained **(E4)** was filtered by vacuum and filter paper, to obtain a solid residual **(E4B)** and liquid extract **(E4A)**.

Liquid extract **E4A** was neutralized with NaOH 0.2M at pH 7 and filtered again by vacuum and filter paper, to obtained two fractions, a solid residual **(F4B)** and liquid extract **(F4A)**.

The solid residual **E4B** was extracted with n-hexane by reflux at 60°C for 3 hours, the mixture obtained **(F4C)** was filtered by vacuum and filter paper, to obtain a solid residual **(F4C1)** that was discarded as a waste and hexanoic extract **(F4C2)** that was purified as follow. The hexanoic extract **F4C2** was precipitated with acetone at 5°C, centrifugated and separate the supernatant **(F4D)** and a solid **(F4E)** and was stored for further analysis.

The supernatant **F4D** was analyzed by chromatography technics as TLC and HPLC, using different polarity eluents in order to found the best condition to separate the compounds and was purified by TLC in an 18x10 cm plate (silica XG TLC Plates, w / UV254, aluminum backed, 200um, 20x20cm, 25pk), applying as eluent a mixture ethanol: water: glacial acetic acid (90: 5: 5), the spots were visualized by UV at 254 nm, marked, scrape, extracted with acetone and filtered to obtain 5 fractions **F4D1, F4D2, F4D3, F4D4, F4D5**. These fractions were dried in **a** rotary evaporator until dried and stored at 5°C for further NMR analysis (Figure 18).

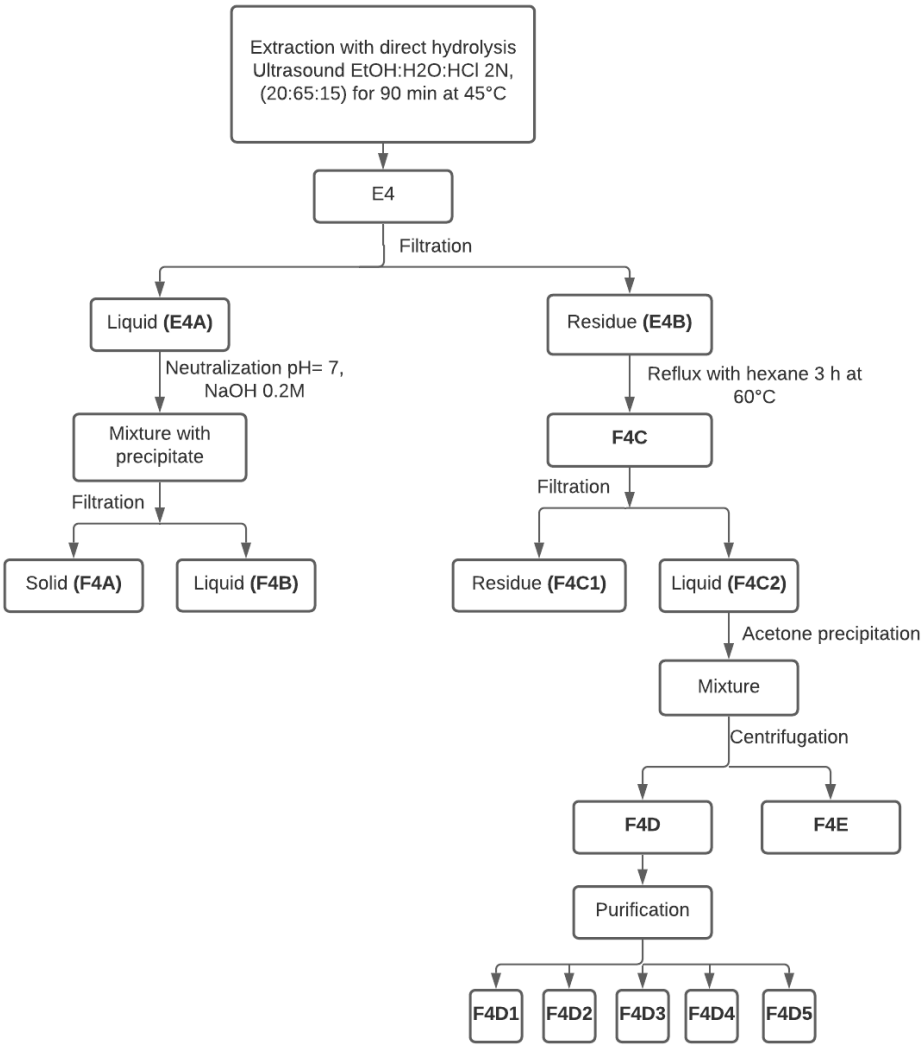


Figure 18. Overview of extraction and purification of *Agave americana L.* by direct hydrolysis.

5.5.5. Thin-layer chromatography analysis of the fractions obtained

TLC was applied with plates TLC Silica Gel 60 F_{254} and silica XG TLC Plates, w/UV254, aluminum backed, 200um, 20x20cm, 25pk, to identify the compounds present in the extracts, different solvents with different polarity such as: methanol, ethanol, distilled water, hexane, ethyl acetate, acetonitrile, glacial acetic acid, and chloroform were used.

The mobile phases used in TLC for each fraction are in Table 3. The plates were developed by 254 nm UV light and by p-anisaldehyde, except for the F4D1, F4D2, F4D3, F4D4, and F4D5 fractions, which were developed with iodine.

Table 3. Data of mobile phase used in different extractions.

Fraction	Mobile Phase
E1	Ethanol: water: glacial acetic acid (82:13:5) v/v
F1A	Ethyl acetate: chloroform (80:20) v/v
F1B	Ethyl acetate: chloroform (80:20) v/v
F1C	Ethyl acetate: chloroform (80:20) v/v
F2A	Ethyl acetate: chloroform (80:20) v/v
F2B	Ethyl acetate: chloroform (80:20) v/v
F2C	Ethyl acetate: chloroform (80:20) v/v
F3B	Ethyl acetate: chloroform (80:20) v/v
F3C	Chloroform: methanol: water (65:35:10) v/v
F4C	Hexane: EtOH (40:10) v/v, 0.5% glacial acetic acid
F4D	Ethanol: water: glacial acetic acid (90:5:5) v/v
F4E	Ethanol: water: glacial acetic acid (90:5:5) v/v
F4D1, F4D2, F4D3, F4D4, F4D5	1. Ethanol: water: glacial acetic acid (90:5:5) v/v 2. Hexane: Ethyl acetate (50:50) v/v

5.6. Phytochemical screening

The phytochemical screening of the extracts was performed applying qualitative tests in order to identify the different secondary metabolites. The qualitative tests were performed in test tubes, 1 mL of E1, F1A, F1B, F1C, F2C, F3C extracts was used following the next methods.

5.6.1. Test for alkaloids

Wagner test: 1.7% v / v of hydrochloric acid and a few drops of Wagner's reagent were added to acidify the 1.0 mL of extract of the fractions dissolved in EtOH

90%, then the formation of a brown precipitate or reddish indicates the presence of alkaloids.

Wagner's reagent: 0.3 g of iodine is dissolved in 1.5 g of potassium iodide (KI 2); add 30 ml of water to produce a solution[55].

5.6.2. Test for saponins

Foam test: We added 0.05g of extract of the fractions and added 5 mL of distilled water and heated for 2 minutes in a water bath at 70 ° C, vigorously shaken until we observed a stable foam that lasts more than 5 minutes[56].

5.6.3. Test for tannins and phenols

Ferric chloride test: 1 mL of 2% aqueous ferric chloride was mixed with 1.0 mL of extract of the fractions dissolved in EtOH 90%. A bluish-black or bluish-green color has formed indicating the presence of tannins and phenols[57].

5.6.4. Test for flavonoids

Shinoda's test: 1.0 mL of extract of the fractions dissolved in EtOH 90% was mixed with some fragments of magnesium tape and concentrated HCl was added dropwise. Within a few minutes, a scarlet pink or brown color appeared, indicating the presence of flavonoids[57].

Alkaline reagent test: 1.0 mL of extract of the fractions dissolved in EtOH 90% was mixed with 1 mL of a 2% solution of NaOH. A deep yellow color was formed which became colorless with the addition of a few drops of dilute acid indicating the presence of flavonoids[57].

5.6.5. Test for glycosides

Liebermann- Burchard test: 1.0 mL of extract of the fractions dissolved in EtOH 90% was each mixed with 1 mL of chloroform and 1 mL of acetic acid. The mixture was cooled on ice. Carefully concentrated H_2SO_4 was added. A color change from violet to blue to green indicated the presence of a steroid nucleus and dark pink to red indicated the presence of triterpenoids, that is, the aglycone portion of the glycoside[27,57].

Salkowski test: 1.0 mL of extract of the fractions dissolved in EtOH 90% was mixed with 1 mL of chloroform. Then 1 mL of concentrated H_2SO_4 was carefully added and stirred gently. A reddish-brown color indicated the presence of a steroid ring, that is, the aglycon portion of the glycoside[57].

Keller-Kilani test: 1.0 mL of extract of the fractions dissolved in EtOH 90% was mixed with 1 mL of glacial acetic acid containing 1-2 drops of a 2% solution of $FeCl_3$. The mixture was then poured into another test tube containing 1 mL of concentrated H_2SO_4. A brown ring at the interface indicated the presence of cardiac glycosides[57].

5.6.6. Test for carbohydrates

Fehling's test: The 1.0 mL of extract of the fractions dissolved in EtOH 90% was mixed with 1.0 mL of Fehling's solution. The mixed solutions were boiled for a few minutes. The formation of a red or brick red precipitate indicates the presence of reducing sugar[55].

Benedict's test: To the 1.0 mL of extract of the fractions dissolved in EtOH 90%, 1.0 mL of Benedict's reagent was added and boiled in a hot water bath for about 5 minutes. The appearance of a red, yellow, or green precipitate showed the presence of reducing sugar[55].

5.7. High performance liquid chromatography

To get a preliminary idea of the complexity of our extracts and for the purpose of getting an idea of the polarity of the following analyzed extracts, F1C, F1B, F2C, F3C, F4C, F4D and F4E, HPLC chromatograms were determined using an apparatus UltiMate 3000. Compounds were separated in a HPLC Hypersil GOLD™ C-18 (150 mm x 4.6 mm, 5µ particle size; Thermo Scientific). For data processing was used Chromeleon 7.1. Mobile phase flow was 1.0 mL/min. Mobile phase was composed by (A) 0.1 % formic acid in water and (B) Acetonitrile. The gradient was as follows: 95% solvent A from 0-10 min (isocratic), 95-70% solvent A from 10-15 min, 70-50% solvent A from 15-18 min and 50-5% solvent A from 18-20 min. Sample volume was 20 µL. The column

temperature and the detection wavelength were set at 30°C and 190, 260, 204 and 310 nm, respectively.

5.8. Infrared spectroscopy

To identify the possible functional groups, present in the fractions F1C, F2C, F3C, F4D, and F4E, IR spectra were determined using The Agilent Cary 630 FTIR spectrometer. The instrumental method used was the ATR sampling technique.

5.9. ^{1}H Nuclear magnetic resonance

In order to determine the molecules, present in fractions F4D1, F4D2, F4D3, and F4D5, we use NMR spectra using NANALYSIS NM60 PRO apparatus. The solvent was tetrachloroethylene (99% extra pure).

6. RESULT, INTERPRETATION AND DISCUSSION

6.1. Plant Material

In this work, the *Agave americana L.* plant provided of UyamaFarms S.A. 624.47 g wet leaves were used. The resulting weight of leaves after the drying process was 84.28 g. The moisture percentage resulted in 13.49%.

6.2. Extraction 1 analysis

The solid-liquid extraction was carried out taking into account the literature affirms that an ideal method for the extraction of saponins is the ultrasound. This method has the advantage of highly efficient extraction with good performance and high purity. In addition to allowing extraction to be carried out in less time, with less consumption of reagents, less complex, and does not use temperature, guaranteeing that no compound is degraded.

The saponins in their structure have a part nonpolar and polar[31], for this reason, is solubility in hydroalcoholic mixtures, and insoluble in low polarity solvents. In such a way that the E1 extract obtained with 90% EtOH, considered as an extremely complicated product, was fractionated for sieving, as a previous step to chromatographic and spectroscopic techniques, using solvents of increased polarity, starting with hexane (defatted), then with ethyl acetate and finally with water.

E1 extract after ultrasound extraction starting from 20 g of the dry material of *Agave americana L.* had a weight of 2.31 g. The yield of the E1 extract was calculated according to equation 2, resulting in a yield of 11.55%. The resulting weight of the fractionation with increased polarity was: 0.26 g (1.3%) for F1C, 0.13g (0.65%) for F1B, and 1.22 g (6.1%) for F1C.

Monitoring by TLC to E1, F1A, F1B, and F1C was carried out with different eluents, obtaining the results presented in table 4.

Table 4. Results of the TLC elaborated for E1, F1A, F1B and F1C.

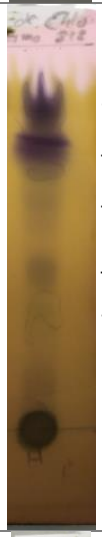
Sample and eluent mixture	Mark	Observations	Result
E1 EtOH: H2O: CH_3COOH (82:13:5) v/v	1	Yellow mark	 -1 -2
	2	Dark green mark	
F1A Ethyl acetate: chloroform (80:20) v/v	1	Dark blue mark	 -1 -2 -3 -4 -5 -6 -7
	2	Dark blue mark	
	3	Blue mark	
	4	Violet mark	
	5	UV light 254 nm	
	6	Violet mark	
	7	Dark green and UV light 254 nm mark	
F1B Ethyl acetate: chloroform (80:20) v/v	1	Blue mark	 -1 -2 -3 -4 -5
	2	Blue mark	
	3	Violet mark	
	4	Mark in UV light 254 nm	
	5	Dark green mark and UV light 254 nm	

Table 4.
Continuation

Sample and eluent mixture	Mark	Observations	Result
F1C Ethyl acetate: chloroform (80:20) v/v	1	Dark green mark	 -1

The TLC results indicate that our extract and fractions are very complex because it does not have a defined, and fine spots, demonstrating that we have more than a single compound. If we compare the three plates of F1A, F2B, and F3C, which were eluted with the same eluent (Ethyl acetate: chloroform (80:20v/v)), we can see how the marks in F1A and F1B have a similar profile with spots with similar polarity, however in F1C shows more polar compounds due to the spot is retained in the application mark, demonstrating that the sample is too polar in comparison with F1A and F1B.

The phytochemical screening results for E1, F1A, F1B and F1C can be seen in table 5.

Table 5. Phytochemical tests on ethanolic extract and fraction of the extraction 1.

Metabolites	Chemical Test	E1	F1A	F1B	F1C
Saponin	Foam test	+	-	-	+
Alkaloids	Wagner test	+	-	-	-
Tannins & Phenols	2% FeCl3 test	+	-	+	+
Flavonoids	Alkaline reagent test	+	+	-	+
	Shinoda test	+	(nd)	(nd)	(nd)
Glycosides	Keller Kiliani test	+	-	+	(nd)
	Salkowski test	+	-	+	+
	Liebermann-Burchard test	+	+	+	+
Carbohydrates	Fehling test	+	(nd)	(nd)	(nd)
	Benedict test	+	+	+	+

(+) indicates the presence of the evaluated secondary metabolite while (-) indicates its absence and (nd) not determine (Annex 1)

According to table 5, the ethanolic extract E1 of the leaves of *Agave americana L.* shows us the qualitative presence of some groups of secondary metabolites such as saponins, alkaloids, tannins, phenols, flavonoids, glycosides, and carbohydrates, as previously reported by Krishnaveni (2017)[58]. Based on our knowledge that saponins are polar compounds, and we expect to see them in F1C, and not in F1A and F1B, the qualitative responses turn out to be correct to the premise, since we obtained a positive response for saponins in the more polar fraction and also have a set of polar secondary metabolites such as phenols, qualitatively resulting in that fractionation with different polarity was developed correctly. Additionally, the phytochemical screening of the Liebermann-Burchard test showed us the presence of steroidal saponins, due to the blue and green color that presented.

Additionally, we performed the HPLC analysis on one of the moderately polar samples, to compare the peaks and differentiate between the two fractions. F1B and F1C according to phytochemical screening, these fractions were dissolved in water reaching a concentration of 1.1 mg / mL for F1B and 0.7 mg / mL for F1C.

According to the methodology, we set the acquisition at different wavelengths, in order to see the major number of peaks present in the samples considering as a compound.

Previous to each sample a blank sample was runner and the peaks were discarded from the chromatogram sample.

The reverse-phase HPLC analysis for the F1B sample indicates the presence of the most intense peaks between 14 – 16 minutes in the chromatogram (Figure 19) at a wavelength of 310 nm, show us that the less polar compounds are present in this fraction correlating the TLC profile. The peaks corresponding to the first retention time are due to the presence of phenols that are present, according to the phytochemical analysis carried out.

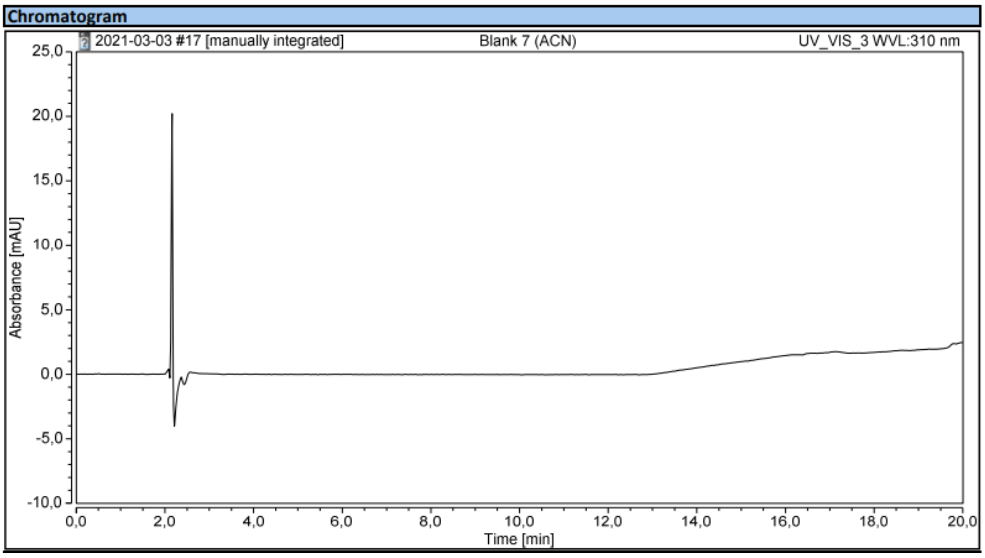


Figure 19a

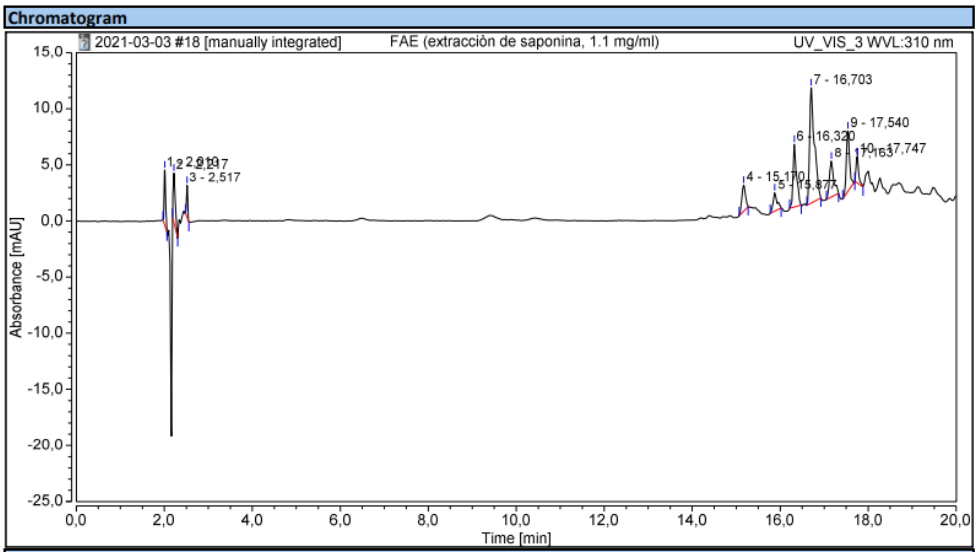


Figure 19b

Figure 19. HPLC chromatograms of blank (19a) and F1B fraction (19b).

The reverse-phase HPLC analysis, for F1C, showed the highest number of peaks at 260 nm (Figure 20), demonstrating to be a complex fraction even after the partition, wants to indicate that they are not pure fractions, it is necessary a more exhaustive process of purification. In addition, the chromatogram provides us with information about the polarity character of their structures according to the retention time. The peaks that are between 1.5 and 4 minutes, corresponding to polar compounds such as could be phenols compounds, and the peaks from 14 to 20 minutes, corresponding to less polar compounds as carbohydrates. Since saponins are less polar than phenols we can speculate that the first peaks correspond to the phenols, the peaks in the middle of the chromatogram, correspond to saponins and the final peaks correspond to the non-polar part of saponins. Lacking a reference, we cannot associate directly one of the peaks with saponins.

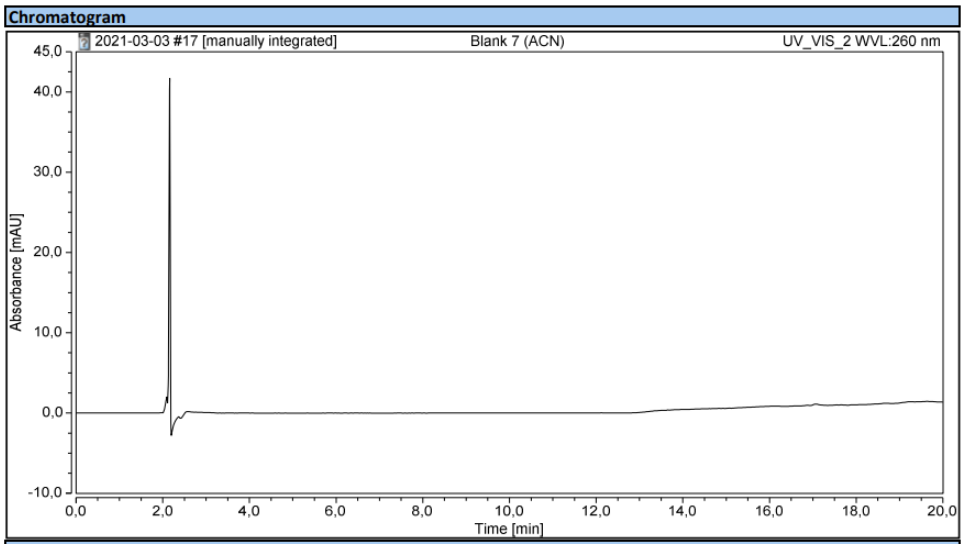


Figure 20a

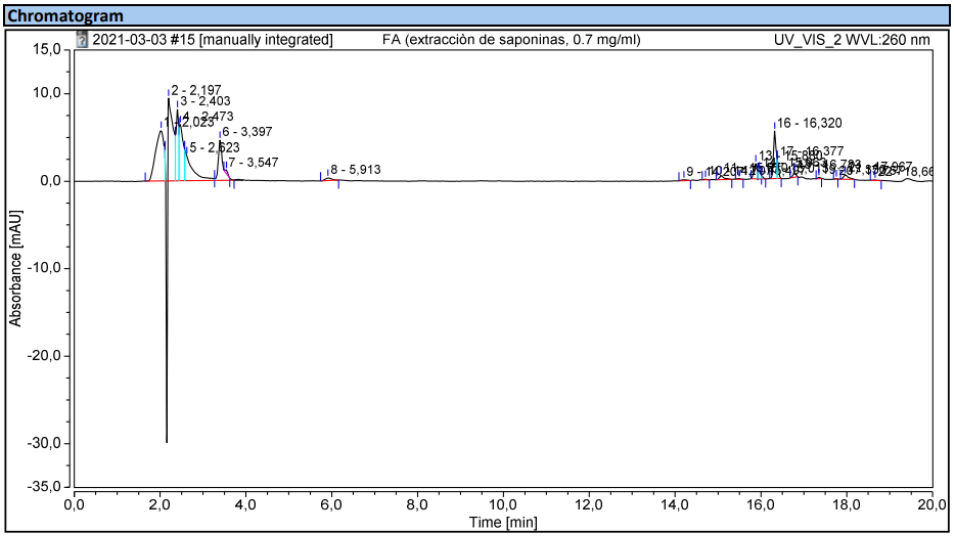


Figure 20b

Figure 20. HPLC chromatograms of blank (20a) and F1C fraction (20b).

This result is related due to the partition performed increasing polarities, where this fraction F1C has the polar compounds and F1B the moderately polar ones, the comparison is in annex 8.

Finally, taking into account the phytochemical screening, TLC and HPLC, the F1C sample was selected as the most suitable to continue with the infrared spectrum analysis, in order to analyze the characteristic functional groups present.

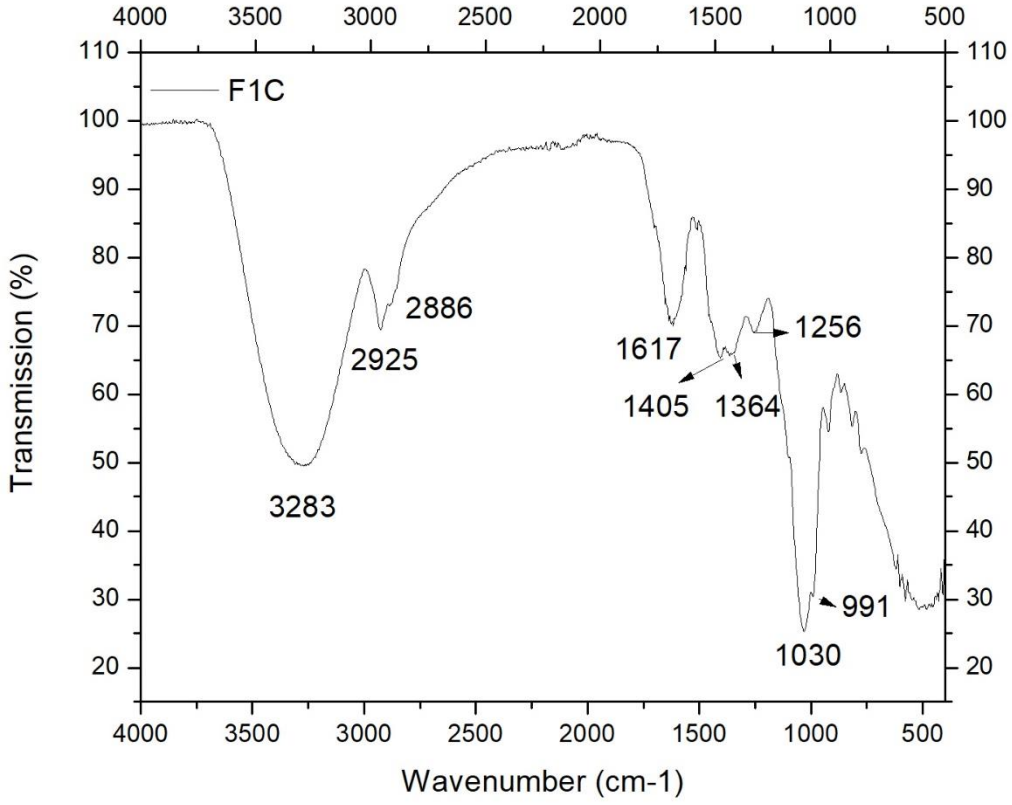


Figure 21. IR spectra of F1C.

Table 6. Interpretation of IR spectra of saponin F1C.

No.	Wavenumber (cm-1)	Absorption Intensity	Typical vibrations
1	3283 cm^{-1}	Medium, broad	O-H group
2	2928 cm^{-1}; 2886 cm^{-1}	Weak	C-H sp^3 group
3	1617 cm^{-1}	Weak	C=C group
4	1405 cm^{-1}; 1364 cm^{-1}; 1256 cm^{-1}	Weak	C-OH group
5	1030 cm^{-1}; 991 cm^{-1}	Strong	C-O-C group

The infrared spectrum shows the presence of typical functional groups as alcohol (O-H), double bond (C=C) and ether (C-O-C) present in a steroidal saponin structure. The presence of a C-O-C vibration indicates functional groups characteristic of saponins since phenols present lack this functional group.

6.3. Extraction 2 analysis

The extraction was carried out from an ethanolic extract, where we have the presence of saponins and other secondary metabolites, followed by hydrolysis, taking into account that the literature affirms that under acidic or basic conditions, the glycosidic bond present in the saponins is broken, separating the polar part that is the carbohydrate, from the nonpolar part that is the aglycone or sapogenin. Acid hydrolysis is generally a preferred method of obtaining complete glycosidic bond breaking from extracts without producing artifacts, while alkaline hydrolysis produces partial hydrolysis.

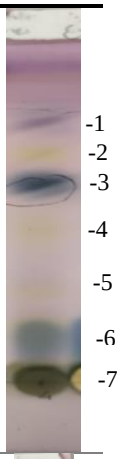
Sapogenins being hydrophobic, are soluble in nonpolar compounds so that when hydrolysis was carried out, the sapogenins remained insoluble. In order to analyze the polar part, indirectly showing the presence of sapogenins in the insoluble part, we performed a fractionation with increased polarities, to lead to a characterization that confirms the hypothesis.

The weight resulting from the fractionation with increased polarities to the ethanoic extract, from 4 g of dry tissue of *Agave americana L.* was 0.07 g for F2A, 0.82 g for F2B, and 2.21 for F2C. F2A, F2B, and F2C were monitored by TLC, obtaining the results presented in table 7.

Table 7. Results of the TLC elaborated for F2A, F2B and F2C.

Sample and eluent mixture	Mark	Observations	Result
F2A Ethyl acetate: chloroform (80:20) v/v	1	Blue mark	 -1 -2 -3
	2	Yellow mark	
	3	Violet and UV light 254 nm mark	

Table 7.
Continuation

Sample and eluent mixture	Mark	Observations	Result
F2B Ethyl acetate: chloroform (80:20) v/v	1	Violet mark	
	2	Yellow mark	
	3	Blue and UV light 254 nm mark	
	4	Yellow mark	
	5	Yellow mark	
	6	Blue mark	
	7	Dark green and UV light 254 nm mark	
F2C Ethyl acetate: chloroform (80:20) v/v	1	Blue mark	
	2	Green mark	

The marks resulting from TLC, unlike those obtained in extraction 1, indicate that our fractions are less complex because they have better-defined marks. In each figure, we can observe the polarity difference of each fraction, if we focus on the F2A plate, we can observe how the compounds present in this fraction elute in the presence of a low polarity mixture, in the same way in the F2B figure, we observe some marks that correspond to the marks of the F2A plate, however, we also have the presence of very polar marks in the lower part of the plate. Analyzing the F2C plate we can see how it is very difficult to elute this very polar fraction in the presence of a low polarity mixture. So, we can say that the fractionation at different polarities was reflected in the plates, where we visualized marks with different polarities.

The phytochemical screening performed on the most polar fraction F2C according to TLC can be seen in table 8.

Table 8. Phytochemical tests on F2C fraction of the extraction 2.

Metabolites	Chemical Test	F2C
Saponin	Foam test	-
Alkaloids	Wagner test	-
Tannins & Phenols	2% FeCl3 test	-
Flavonoids	Alkaline reagent test	-
Glycosides	Keller Kiliani test	+
	Liebermann-Burchard test	-
Carbohydrates	Benedict test	+

(+) indicates the presence of the evaluated secondary metabolite while (-) indicates its absence (Annex 5)

According to table 8, the analyzed fraction shows us the qualitative presence of glycosides and carbohydrates. Based on this, we can say that the most polar part of the saponins dissolved in this fraction, and the sapogenins are not present. The phytochemical screening of this sample also indicates that the hydrolysis process required more time and temperature to be more efficient because by analyzing the foam test in annex 5 we can see that the sample presented a foam but not stable, that it may be due to the fact that part of the sapogenins remained in this fraction. As sapogenin loses solubility in water when hydrolyzed, its foam productivity decreases and maybe the reason for the slight foam that occurred. The F2C fraction with 0.4 mg/mL of concentration was analyzed by HPLC at 260 nm when were observed the highest number of peaks, the retention times of the peaks in the chromatogram (Figure 22) demonstrate that the majority of the compounds are mostly polar.

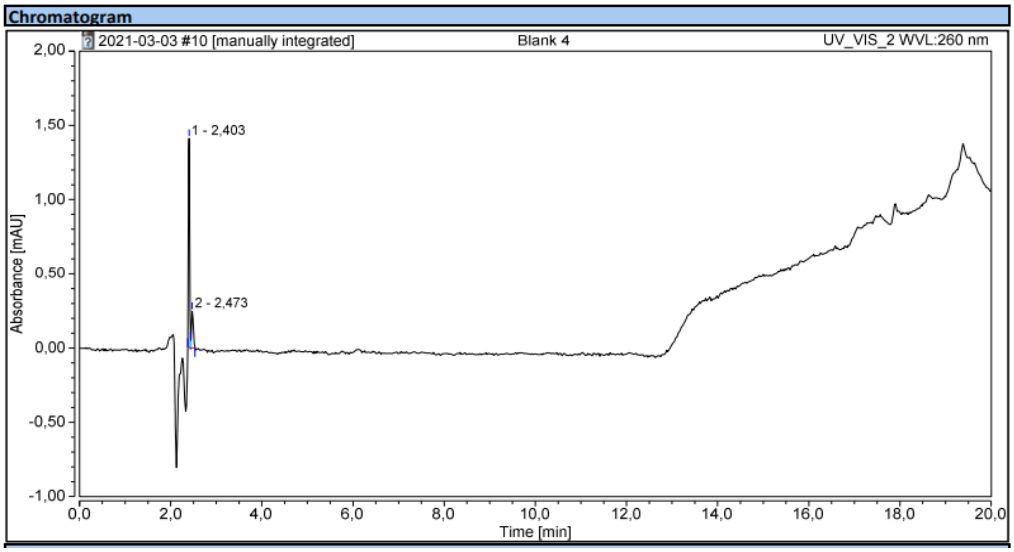


Figure 22a

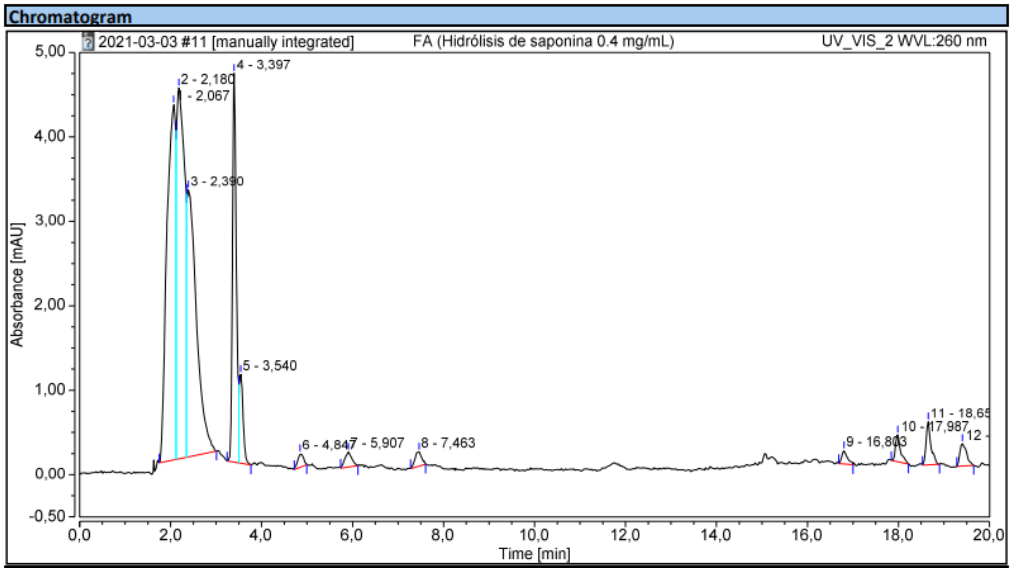


Figure 22b

Figure 22. HPLC chromatograms of blank (22a) and F2C fraction (22b).

The chromatogram for the F2C fraction demonstrates a very complex sample with at least 12 peaks, very intense peaks were visualized between 2 and 8 minutes, thus showing the presence of very polar compounds. However, we can still observe slight peaks on the right side of the chromatogram at from 16 to 20 minutes, corresponding to nonpolar compounds, indicating that possibly not

everything was hydrolyzed, as we could confirm it by foam test in phytochemical screening.

The identification for the functional groups presents in F2C fraction, the infrared spectra were obtained with the following result.

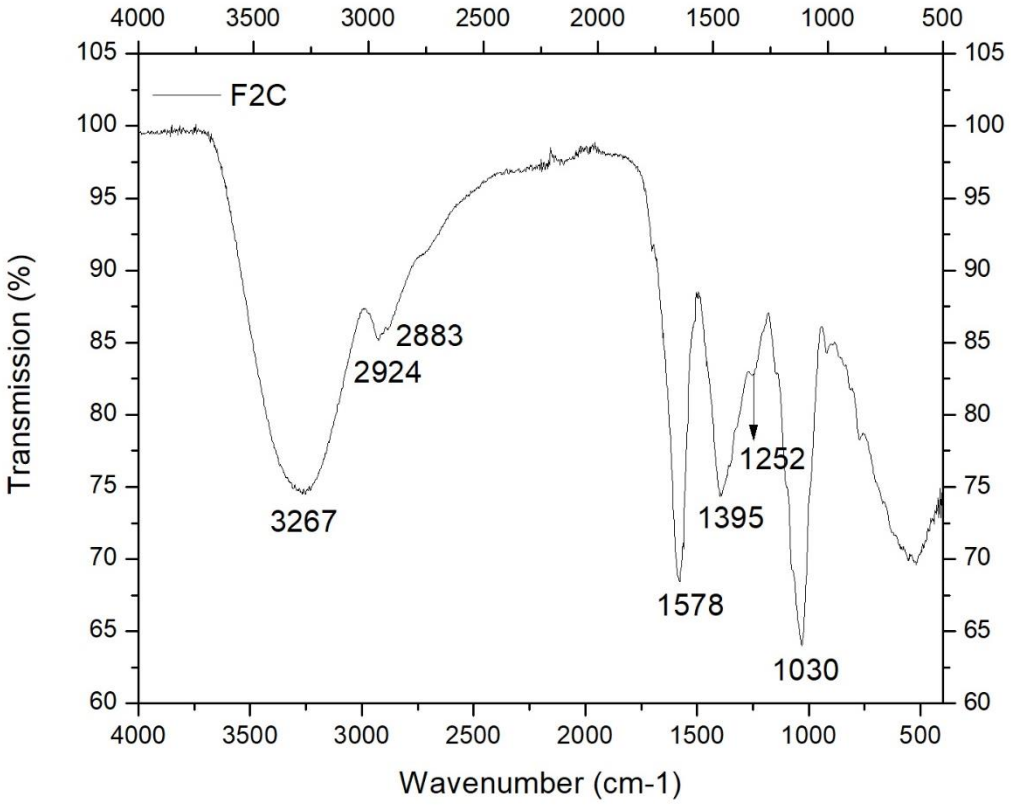


Figure 23. IR spectra of F2C.

Table 9. Interpretation of IR spectra of sapogenin F2C.

No.	Wavenumber (cm-1)	Absorption Intensity	Typical vibrations
1	3267 cm^{-1}	Medium, broad	O-H group
2	2924 cm^{-1}; 2883 cm^{-1}	Weak	C-H sp^3 group
3	1578 cm^{-1}	Strong, sharp	C=C group
4	1395 cm^{-1}; 1252 cm^{-1}	Medium	C-OH group
5	1030 cm^{-1}	Strong, sharp	C-O-C group

In this spectrum, we have the presence of functional groups as alcohols (O-H) that correspond to carbohydrates and ether (C-O-C) signal corresponding to glycosides and carbohydrates, related to the phytochemical screening result, the

F2C sample was positive for carbohydrates being the polar part of the saponin and glycosides.

6.4. Extraction 3 analysis

Based on what was analyzed in the previous extraction, this new chemical process was carried out in order to reach the same conclusion as extraction 2, the difference in this process is that we did not obtain an ethanolic extract, but we carried out a process directly with the plant tissue and breaking the glycosidic bond in the same way to be able to separate the polar part from the nonpolar part in saponins in acid condition.

In this extraction we also fractionate with solvents of different polarities hoping to separate the polar part of the saponins, confirming that with this new chemical process we can also isolate the sapogenins. Unlike extraction 2 when we fractionated this extraction, we did not obtain a non-polar phase when we used n-hexane, which shows us that everything non-polar remained in the residue, including sapogenins.

The weight resulting from the fractionation with increased polarities starting from 5 g dry tissues of *Agave americana L.* was 0.09 g of F3B and 3.71 g of F3C. We monitor fractions F3B and F3C with TLC, obtaining the results presented in table 10.

Table 10. Results of the TLC elaborated for F3B and F3C

Sample and eluent mixture	Mark	Observations	Result
F3B Ethyl acetate: chloroform (80:20) v/v	1	Blue and UV light 254 nm mark	
	2	Yellow and UV light 254 nm mark	
	3	Dark green mark	
F3C $CHCl_3$: MeOH: H_2O v/v (65:35:10)	1	Blue mark	
	2	Yellow mark	
	3	Green mark	
	4	UV light 254 nm	
	5	Brown mark	

The marks resulting from TLC indicate that our fractions are less complex because they have better-defined marks. In these two plates, an elution mixture of different polarity was used, waiting to observe a more adequate separation. In the phase that corresponds to F3B we observed moderately polar compounds that could be eluted with the mixture; however, we have a mark at the point of origin, showing the presence of polar compounds that could not be eluted. Analyzing the F3C we can observe a better elution due to the increase in polarity of the eluent mixture, evidencing the presence of polar compounds.

The phytochemical screening performed on the most polar fraction (F3C) can be seen in table 11.

Table 11. Phytochemical tests on F3C fraction of extraction 3.

Metabolites	Chemical Test	F3C
Saponin	Foam test	-
Alkaloids	Wagner test	-
Tannins & Phenols	2% FeCl3 test	-
Flavonoids	Alkaline reagent test	-
Glycosides	Keller Kiliani test	+
	Liebermann-Burchard test	-
Carbohydrates	Benedict test	+

(+) indicates the presence of the evaluated secondary metabolite while (-) indicates its Absence (Annex 6)

According to table 11, the analyzed fraction shows us the qualitative presence of glycosides and carbohydrates, the same results obtained in extraction 2, which presented a different chemical process. Here we can also witness an unstable foam in foam test in annex 6, caused by the presence of hydrolyzed sapogenins that remained in that polar fraction.

The HPLC chromatogram profile (Figure 24) at 260 nm of F3C fraction shows the similar retention times of the peaks present in fraction F2C obtained in the extraction 2, in this case, the sample has 1.4 mg / mL of concentration, more concentrated.

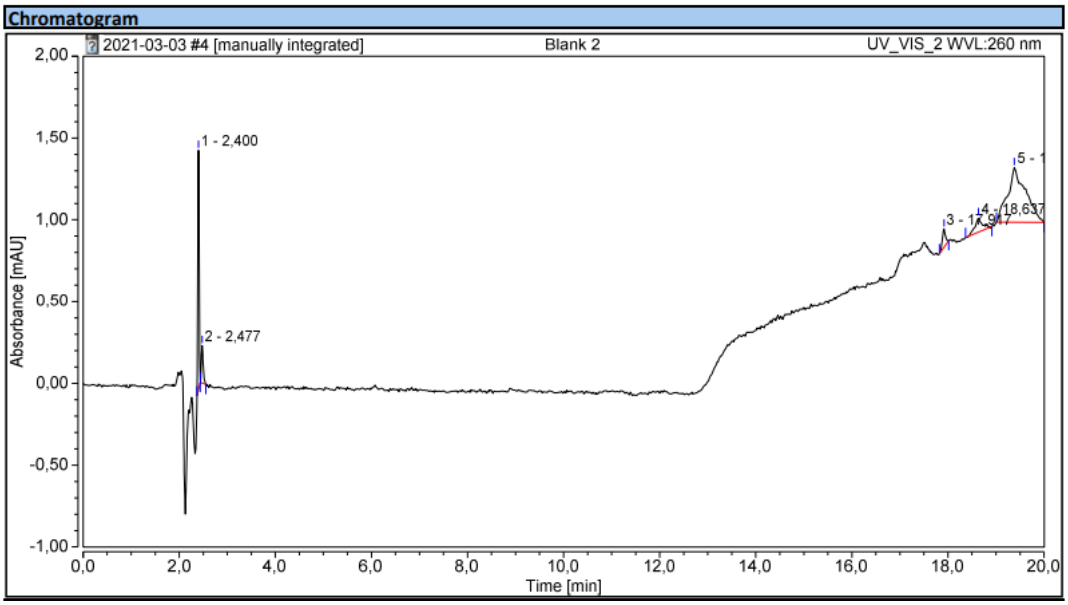


Figure 24a

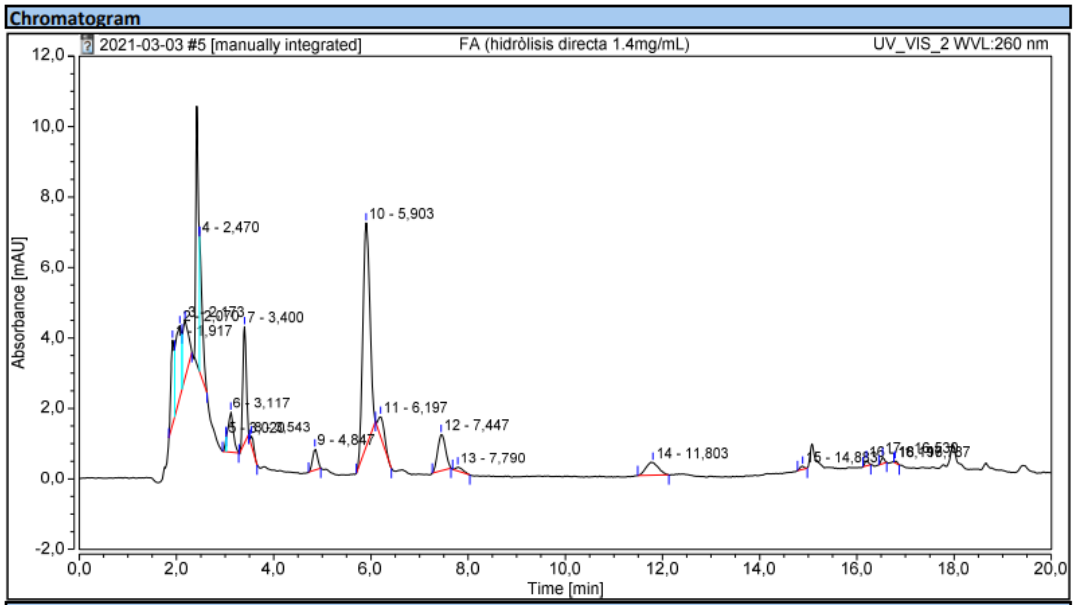


Figure 24b

Figure 24. HPLC chromatograms of blank (24a) and F3C fraction (24b).

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The chromatogram from F3C has more defined and separated 17 peaks in 260 nm. Several peaks present in the chromatogram profile of F3C correspond to the same signals in F2C, the main difference is that the peaks that emerge in the first minutes are more intense, this may be due to the fact that the concentration of the F3C sample is greater than F2C, but basically, we have a similar profile of polar compounds, which is what was expected (Annex 9).

The identification for the functional groups presents in F3C fraction, the infrared spectra was obtained with the following result.

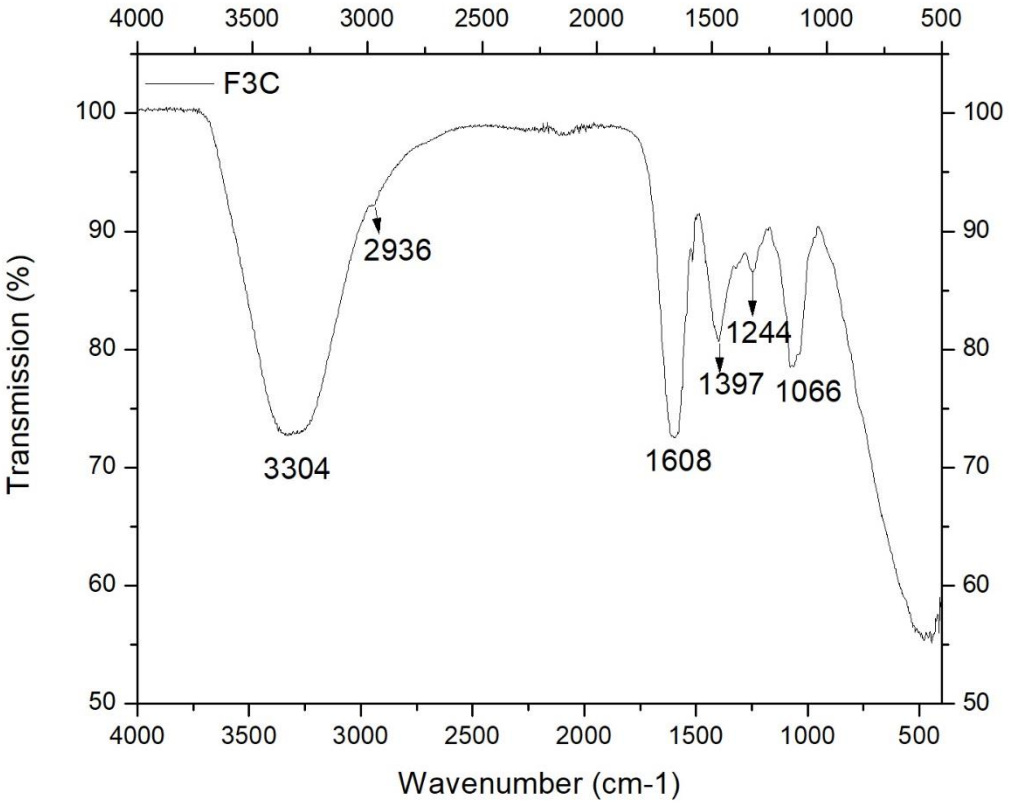

Figure 25. IR spectra of F3C.

Table 12. Interpretation of IR spectra of sapogenin F3C.

No.	Wavenumber (cm-1)	Absorption Intensity	Typical vibrations
1	3304 cm^{-1}	Medium, broad	O-H group
2	2936 cm^{-1}	Weak	C-H sp^3 group
3	1608 cm^{-1}	Medium	C=C group
4	1397 cm^{-1}; 1244 cm^{-1}	Weak	C-OH group
5	1066 cm^{-1}	Weak	C-O-C group

If we compare both the spectra of extraction 2 and extraction 3, it is basically the same profile, with the same functional groups as alcohol (O-H), and ether (C-O-C) corresponding to glycosides and carbohydrates. So that apart from having the polar part of the saponin, carrying out a different chemical process leads us to the same result.

6.5. Extraction 4 analysis

In the extraction processes described above, we analyze the polar part of the saponins, in this extraction and purification, we focus on the non-polar part where it is expected to have the sapogenins. To develop this extraction, we made direct hydrolysis to the *Agave americana L.* tissue, but instead of using ultrasound as in extraction 3, put heat and accelerate the hydrolysis process, ensuring the breakdown of the glycosidic bond that the saponins.

As sapogenins are hydrophobic compounds, it is expected that in the hydrolysis process they will remain insoluble in the acid solution. We worked with the residue, which was purified, to see if it is possible to find sapogenins such as hecogenin and tigogenin, according to the procedure reported by Cerqueira et al.[59] that way, we can suppose that are present in insoluble residue.

In this process, we use n-hexane to extract all the non-polar portion of the saponin (Aglycone) after the hydrolysis, in the F4C2 fraction. After F4C2 was precipitated with acetone, the F4D and F4E were obtaining, both are fractions were analyzed by TLC (Table 13), HPLC, and IR.

Table 13. Results of the TLC elaborated for F4C, F4D and F4E.

Sample and eluent mixture	Mark	Observations	Result
F4C Hexane: EtOH (40:10) v/v, 0.5% CH_3COOH	1	UV light 254 nm mark	 -1 -2 -3 -4 -5
	2	UV light 254 nm mark	
	3	Violet and UV light 254 nm mark	
	4	Yellow and UV light 254 nm mark	
	5	Violet and UV light 254 nm mark	
F4D EtOH: H2O: CH_3COOH (90:5:5) v/v	1	Yellow and UV light 254 nm mark	 -1 -2 -3
	2	Yellow and UV light 254 nm mark	
	3	Mark in UV light 254 nm	
F4E EtOH: H2O: CH_3COOH (90:5:5) v/v	1	Green and UV light 254 nm mark	 -1 -2
	2	Yellow and UV light 254 nm mark	

In the plate F4C, in the presence of a moderately polar eluent, we have the presence of nonpolar compounds present that are visualized with 254 nm UV light and more polar compounds in the lower part of the plate.

Observing the marks in F4D and F4E we have polar and nonpolar compounds, the first mark on the F4D plate corresponds to the first mark that is visualized in F4E. In addition, both plates have a mark that was not eluted at the origin.

In order to analyze their complexity and polarity, we took the F4C, F4D and F4E fractions to HPLC dissolving them in methanol obtaining the following concentrations, 0.8 mg / mL for F4C, 1.2 mg / mL for F4D and 1.6 mg / mL for F4E.

Before each measurement, a sample corresponding to the blank was inserted to discard peaks that are not part of the sample.

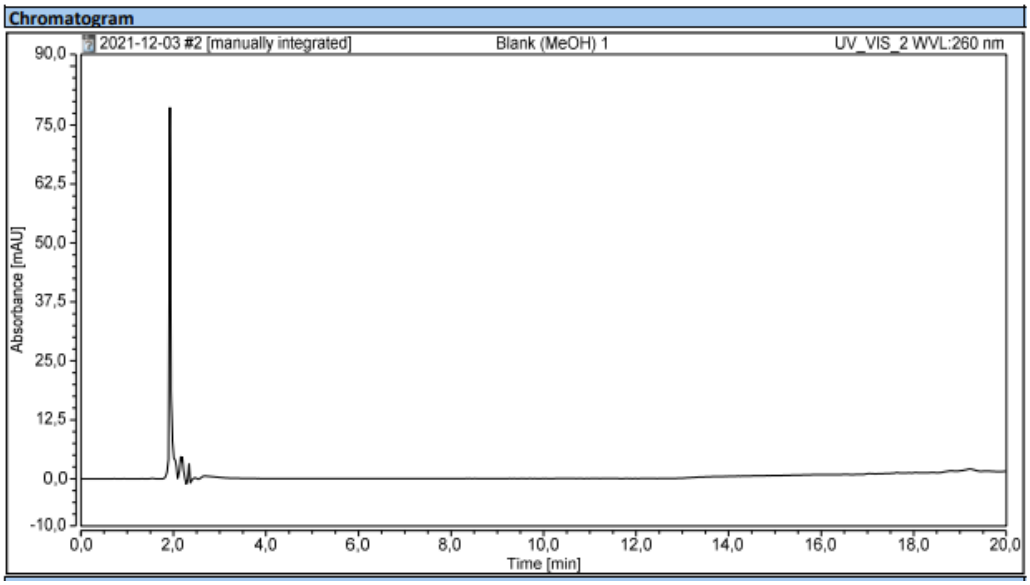

Figure 26a

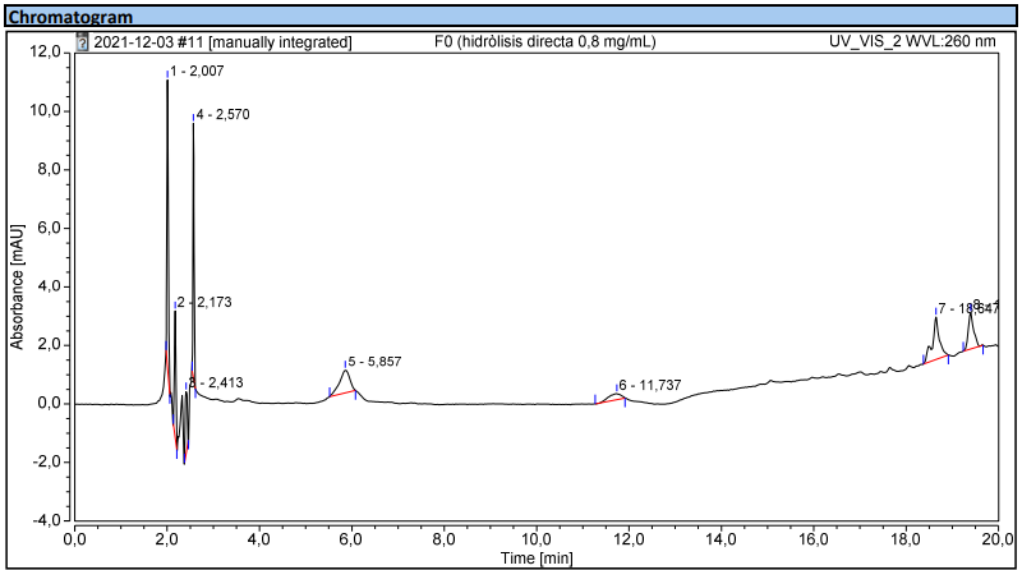

Figure 26b

Figure 26. HPLC chromatograms of blank (26a) and F4C fraction(26b).

In the F4C fraction where extraction was carried out with n-hexane to obtain nonpolar compounds, it shows us 8 peaks that do not correspond to the white, at a wavelength of 260 nm, with a clear presence of less polar compounds, however, there very polar peaks at 2 – 4 minutes, that could not be separated with the extraction.

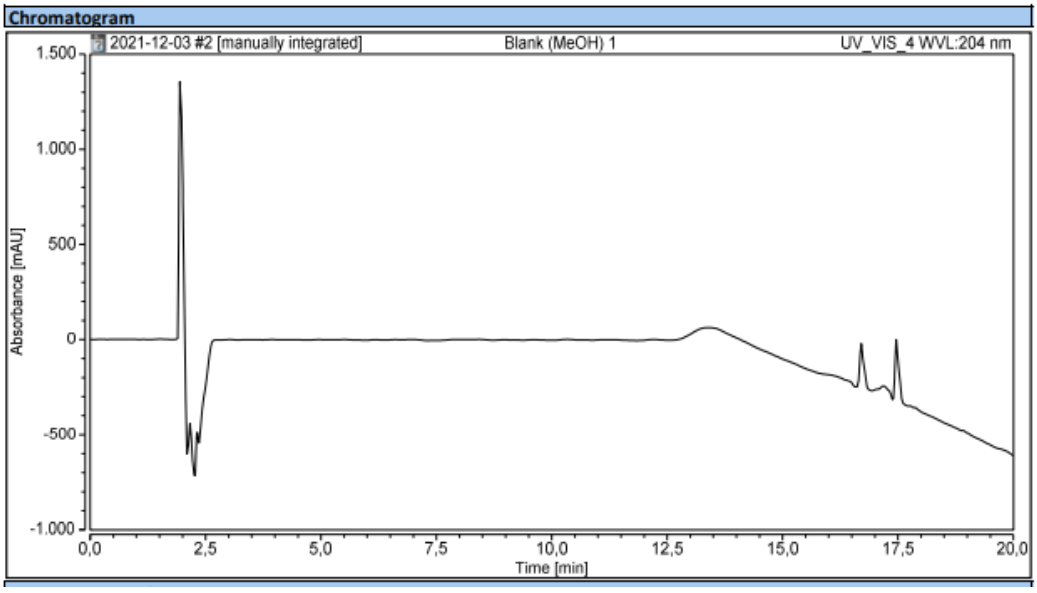

Figure 27a

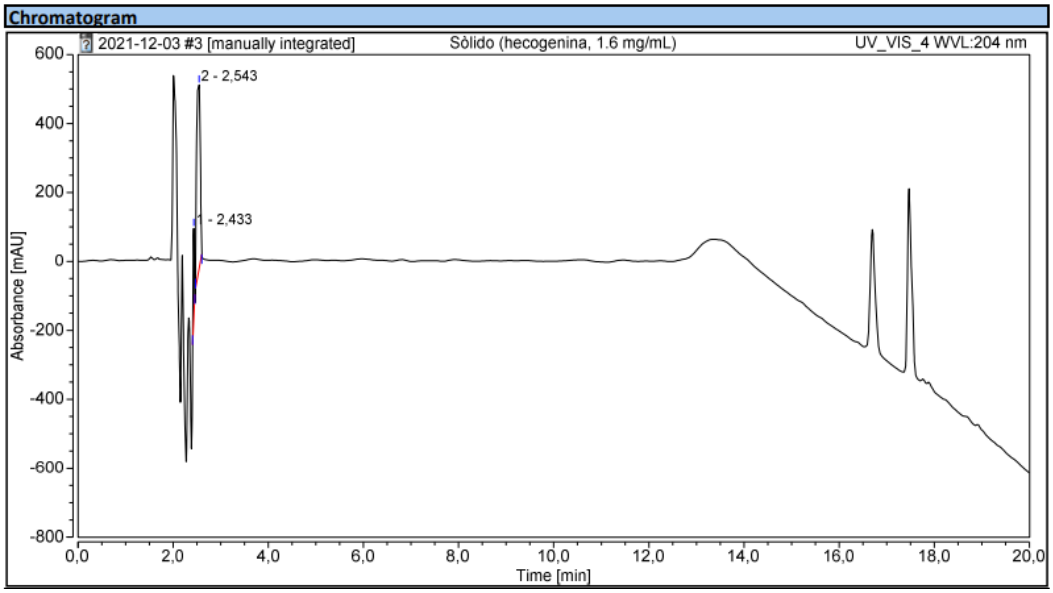

Figure 27b

Figure 27. HPLC chromatograms of blank (27a) and F4E fraction (27b).

In the following chromatogram belonging to F4E, we can observe the presence of 2 very polar peaks at 204 nm at 1.5 -3 minutes, that does not correspond to the blank, in this fraction, it was expected to observe nonpolar compounds, however, we can think that nonpolar compounds were separated in the centrifugation process and stayed in the phase mentioned below.

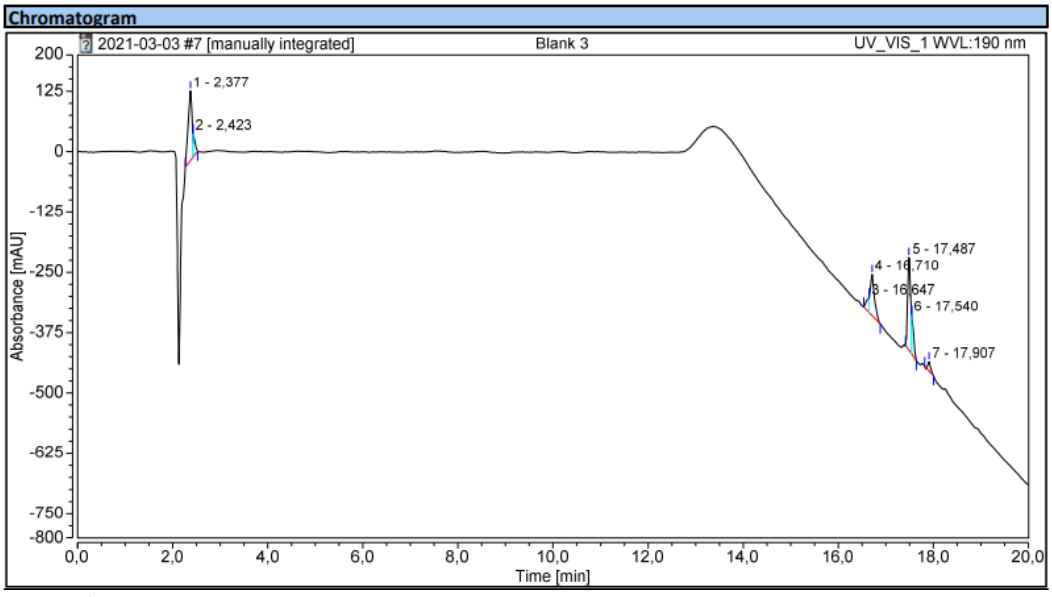

Figure 28a

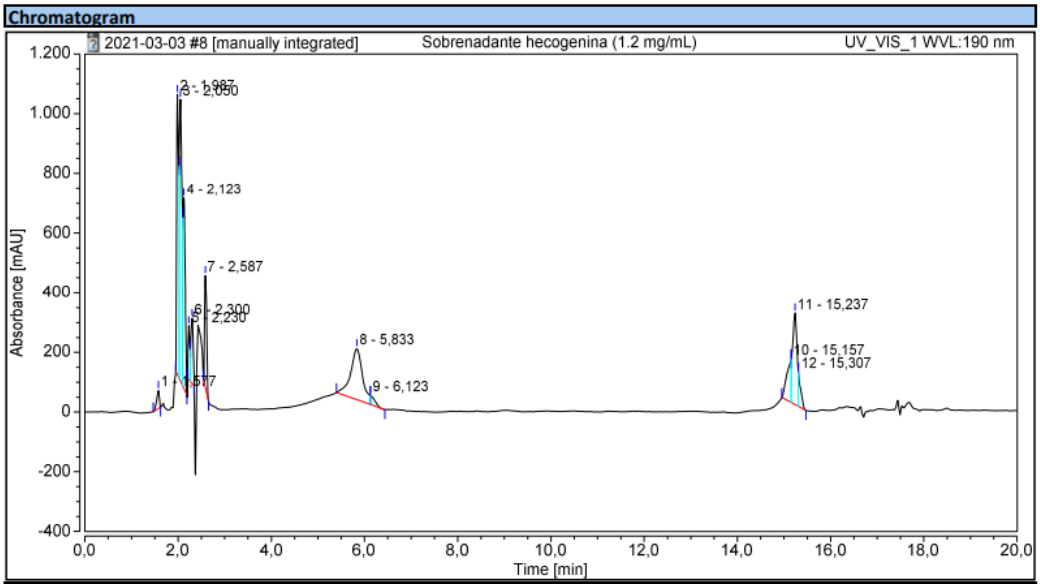

Figure 28b

Figure 28. HPLC chromatograms of blank (28a) and F4D fraction (28b).

The wavelength in which we can observe the greatest number of peaks in F4D corresponds to 190 nm where we have the presence of 12 peaks, where we see both polar compounds at 1.5 – 7 minutes and nonpolar compounds at 14 – 16 minutes so that it is possible that we do not have completely isolated the sapogenin, since only the presence of peaks is expected in larger retention times and in the chromatogram, we only see one peak, and the highest number of peaks is found in the first minutes, corresponding to polar compounds. However, unlike the previous chromatogram here, we see nonpolar peaks that the previous one lacked.

IR analysis was carried out in the F4D, and F4E fractions corresponding to the extraction 4, producing the following images spectral.

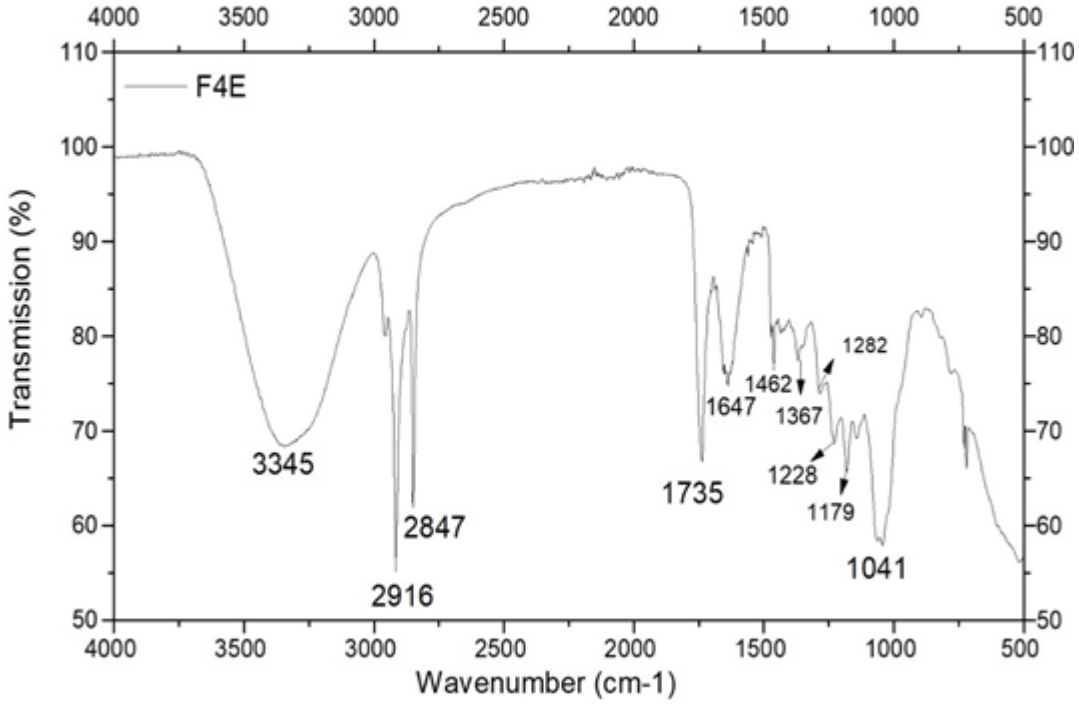

Figure 29. IR spectra of F4E.

Table 14. Interpretation of IR spectra of F4E.

No.	Wavenumber (cm-1)	Absorption Intensity	Typical vibrations
1	3345 cm^{-1}	Medium, broad	O-H group
2	2916 cm^{-1}; 2847 cm^{-1}	Strong, sharp	C-H sp^3 group
3	1647 cm^{-1}	Medium	C=C group
4	1462 cm^{-1}; 1367 cm^{-1}; 1282 cm^{-1}	Weak	C-OH group
5	1179 cm^{-1}; 1041 cm^{-1}	Medium	C-O-C group
6	1735 cm^{-1}	Strong, sharp	C=O group

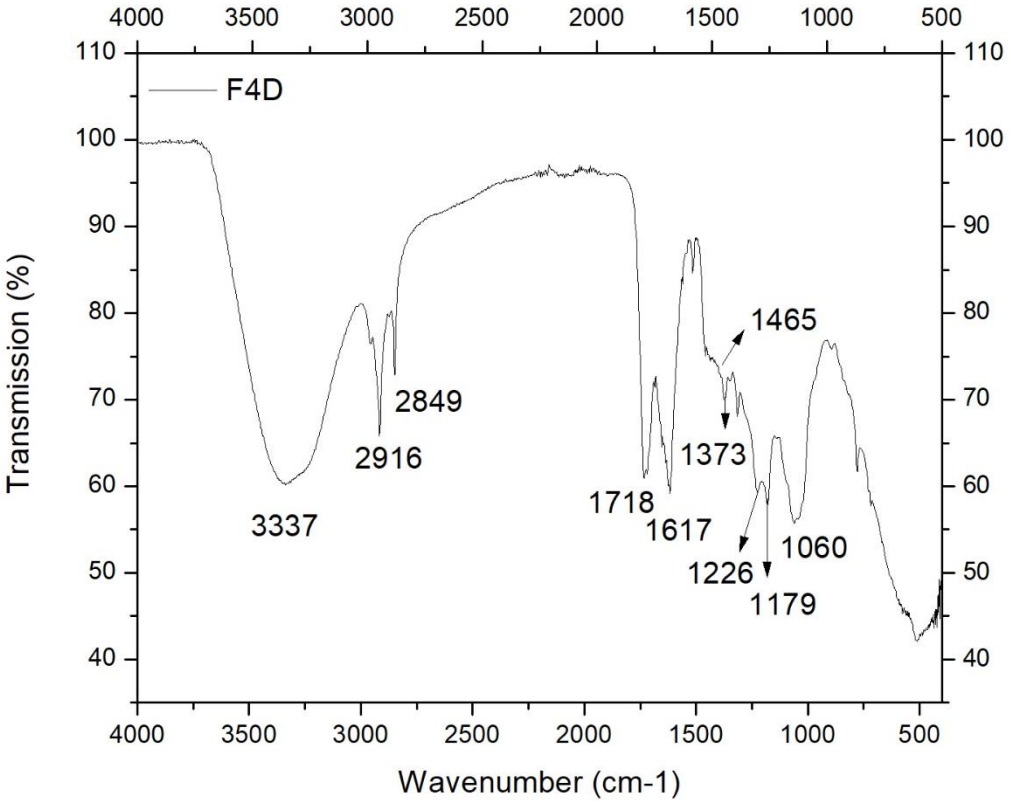

Figure 30. IR spectra of F4D.

Table 15. Interpretation of IR spectra of F4D.

No.	Wavenumber (cm-1)	Absorption Intensity	Typical vibrations
1	3337 cm^{-1}	Medium, broad	O-H group
2	2916 cm^{-1}; 2849 cm^{-1}	Medium, sharp	C-H sp^3 group
3	1617 cm^{-1}	Medium, sharp	C=C group
4	1373 cm^{-1}	Weak	CH_3 group
5	1465 cm^{-1}	Weak	CH_3 group CH_2 group
6	1226 cm^{-1}; 1179 cm^{-1}; 1060 cm^{-1}	Medium	C-O-C group C-OH group
7	1718 cm^{-1}	Medium, sharp	C=O group

Next, two spectra corresponding to the fraction F4D and F4E were observed, where identical profiles with the same functional groups such as alcohols and cyclic ether, which are characteristic of saponins, are observed, and additionally, it is noted in F4D spectra the carbonyl group that is present in the structure of the hecogenin, however, let us remember that we cannot confirm without having

analyzes that allow us to correctly elucidate the structure. In F4E we also have the presence of a carbonyl group corresponding to an ester, as we do not have an ester within the reported structures of the saponins in the *Agave americana*, we can think of two things, the first that the sample was contaminated, and second that we have a new compound not reported, so it is advisable to repeat the analysis to analyze the result.

According to the results of TLC from fraction F4D, where a more complex sample is observed than F4E, it was decided to purify, hoping to have a better screening of the sample, making use of thin-layer chromatography, the five spots present in the sample were extracting as preparative thin layer chromatography procedure to obtain F4D1, F4D2, F4D3, F4D4 and F4D5 marks that are distributed as follows (Figure 31).

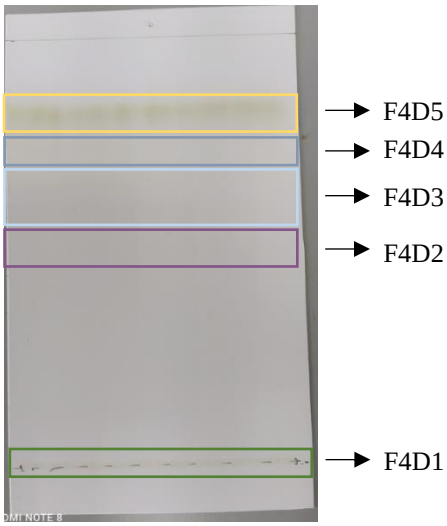

Figure 31. Result of purification by TLC with eluent mixture ethanol: water: glacial acetic acid (90: 5: 5) v/v.

Fractions F4D1, F4D2, F4D3, F4D4, and F4D5 were monitored by TLC. (Table 16)

Table 16. Results of the TLC elaborated for F4D1, F4D2, F4D3, F4D4 and F4D5.

Sample and eluent mixture	Mark	Observations	Result
F4D1 Hexane: EtOH (50:50) v/v	1	UV light 254 nm mark	
F4D2 EtOH: H2O: CH_3COOH (90:5:5) v/v	1 2	UV light 254 nm mark	
F4D3 (with two eluent)	/	/	
F4D4 EtOH: H2O: CH_3COOH (90:5:5) v/v	1	Yellow and UV light 254 nm mark	
F4D4 Hexane: EtOH (40:10) v/v	1	UV light 254 nm mark	
	2	UV light 254 nm mark	
F4D5 (with two eluent)	/	/	

(/) no mark

In the plates F4D1, F4D2, F4D3, F4D4, and F4D5, we can see how we obtain responses from F4D1 in the presence of a polar eluent, we have a nonpolar compound. F4D2 in the presence of a more polar eluent, a polar compound is shown, and finally F4D4 shows us polar and nonpolar compounds.

Next, we will see the series of four NMR spectra of the different fractions F4D1, F4D2, F4D3, F4D4, and F4D5. Within these spectra we will see signals for which the different types of hydrogen are responsible, more specifically the nucleus of the atoms of hydrogen are those who produce absorption of electromagnetic radiation; that is captured by the equipment to give these signals, each signal will correspond to a different type of hydrogen present.

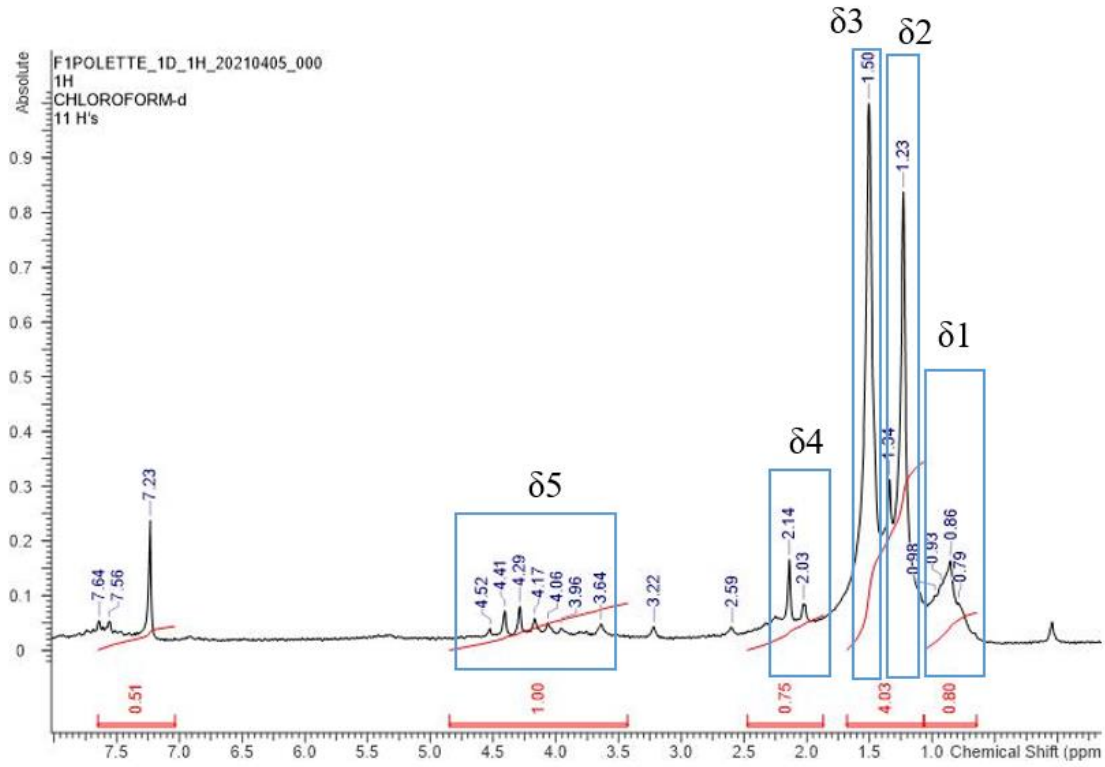

Figure 32. ^{1}H NMR spectra of F4D1 obtained by TLC.

Table 17. Interpretation of NMR spectra of F4D1.

δ	Chemical Shifts ppm	Type of proton
1	0.79-0.98	R−C**H**$_3$
2	1.23-1.34	R−C**H**$_2$-R
3	1.50	R−C**H**(R)−R
4	2.03-2.14	R-**H**C(R)−C=O
5	3.64-4.52	—C(**H**)=C— ; **H**−C(−O—)

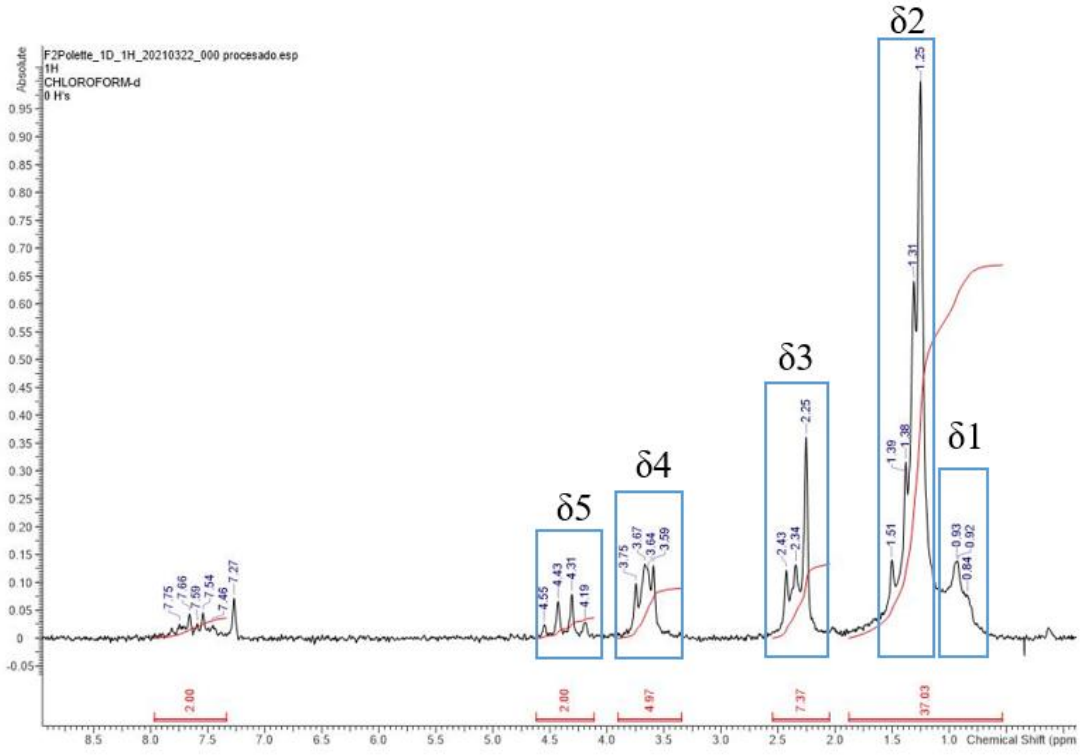

Figure 33. [1]H NMR spectra of F4D2 obtained by TLC.

Table 18. Interpretation of NMR spectra of F4D2.

δ	Chemical Shifts ppm	Type of proton
1	0.84-0.93	R−C**H**$_3$
2	1.25-1.51	R−C**H**$_2$·R
3	2.25-2.43	R-**H**C(R)−C(=O)−R
4	3.59-3.75	**H**$_3$C−O— R−O−C**H**$_2$-
5	4.19-4.55	—C(**H**)=C— **H**−C(−O—)

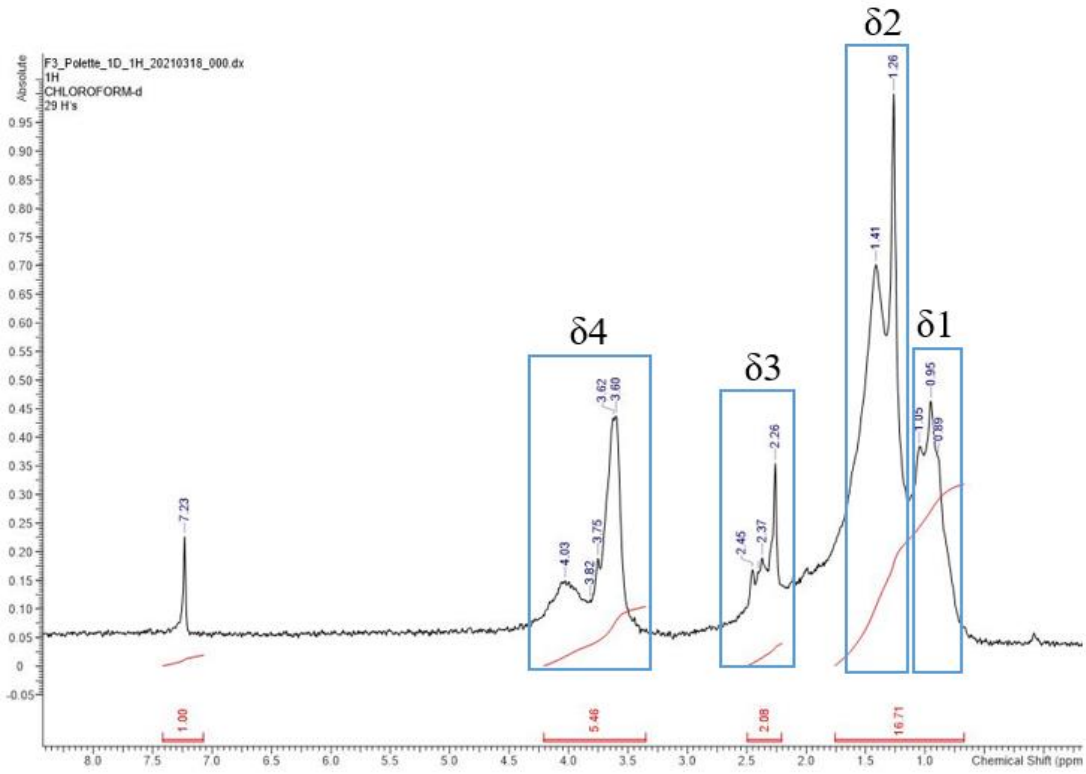

Figure 34. ^{1}H NMR spectra of F4D3 obtained by TLC.

Table 19. Interpretation of NMR spectra of F4D3.

δ	Chemical Shifts ppm	Type of proton
1	0.95-1.05	R−C**H**$_3$
2	1.29-1.41	R−C**H**$_2$-R
3	2.26-2.45	R R-**H**C−C=O R
4	3.60-4.03	**H**$_3$C−O— R−O−C**H**$_2$-

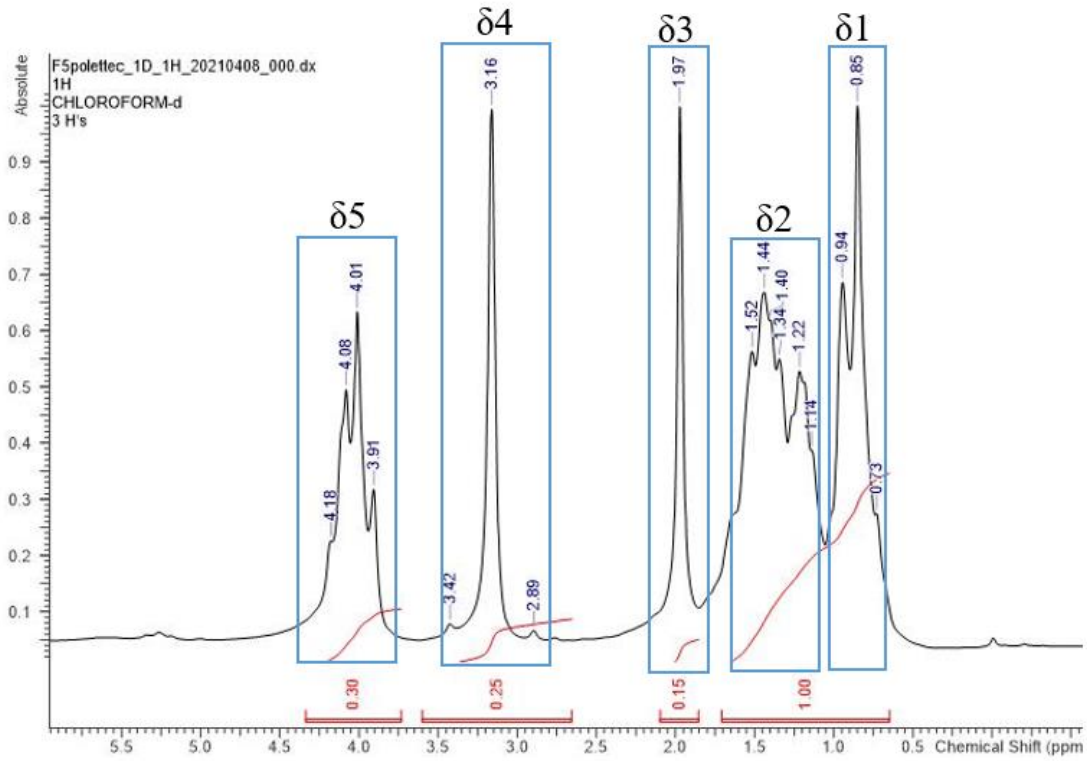

Figure 35. ^{1}H NMR spectra of F4D5 obtained by TLC.

Table 20. Interpretation of NMR spectra of F4D5.

δ	Chemical Shifts ppm	Type of proton
1	0.73-0.94	R−C**H**$_3$
2	1.14-1.52	R−C**H**$_2$-R
3	1.97	R−C=C−C−**H**
4	2.89-3.42	**H**$_3$C−O— R−O−C**H**$_2$-
5	3.91-4.18	**H**−C−O— R−O−C**H**$_2$-

After observing the types of hydrogens present in the NMR spectra, we can think that we have saponins present in the samples, however, we have hydrogens

attached to functional groups as aromatic groups in 7.5 -8 ppm, that do not correspond to the functional groups obtained by IR of F4D from where this series of fractions were obtained. So that we can say that the NMR gave us limited information due to the complexity of the structure and that we have low-resolution equipment, so this technique did not provide us with information that allows us to elucidate the structure. With this in mind, having very complex samples and low-resolution equipment, it is difficult to observe one of the important points in these spectra, such as the multiplicity and the integral.

In most of the spectra, we have a sign at 7 -7.5 ppm that corresponds to the signals of the solvent used for the analysis of the samples.

7. CONCLUSIONS

- Extraction procedure 1 directly demonstrates in the phytochemical and IR analyzes the presence of saponins in the ethanolic extract (E1) and the more polar fraction (F1C), which indicates that the leaves of *Agave americana L.* are a source of saponins, and they can be used to obtain economically and pharmacologically important secondary metabolites.
- Extraction procedures 2 and 3 demonstrated the majority presence of the polar part of the saponins in the polar fractions, based on the results of the phytochemical screening that indicated the presence of glycosides and carbohydrates.
- When comparing the phytochemical analysis of extractions 2 and 3, it is concluded that despite using a different chemical extraction process, in both processes we obtained the polar part of the saponins in the polar extracts.
- The extraction procedure 4, allows suspecting the presence of sapogenins such as hecogenin, however, it is necessary to carry out additional analyzes and have a standard to confirm it.

8. RECOMMENDATION

- With interest in increasing the yield of saponins to obtain a greater quantity for a better characterization, it is necessary to explore other conventional and unconventional methods.
- Develop more purification processes, in order to characterize less complex samples.
- Qualitative and quantitative identification of the hecogenin and tigogenin present in the extracts, with the help of HPLC quality standards.
- Characterize the fractions with more powerful NMR equipment, in order to allow the structure to be elucidated.

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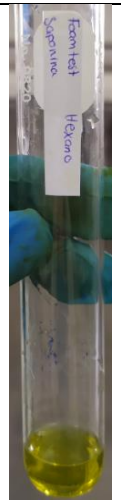
10. ANNEX

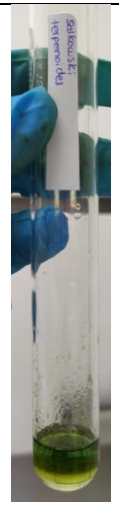
Annex 1. Results of the phytochemical screening of E1 Agave americana.

E1 ETHANOLIC EXTRACT OF LEAVES									
Saponin	Alkaloids	Tannins & Phenols	Flavonoids		Glycosides			Carbohydrates	
Foam test	Wagner test	2% FeCl3 test	Alkaline reagent test	Shinoda test	Keller Kiliani test	Salkowski test	Liebermann-Burchard test	Fehling test	Benedict test
									
Stable foam	Brown precipitate	Bluish-green color.	Presence of intense yellow color, with the presence of acid it clarified.	Presence of a brown color.	Formation of a brown ring at the interface.	Formation of a reddish ring at the interface.	Presence of a blue color that turned green.	Presence of a dark red precipitate.	Presence of a dark green precipitate.

Annex 2. Results of the phytochemical screening of F1A Agave americana.

F1A FRACTION OF **E1**							
Saponin	Alkaloids	Tannins & Phenols	Flavonoids	Glycosides			Carbohydrates
Foam test	Wagner test	2% FeCl3 test	Alkaline reagent test	Keller Kiliani test	Salkowski test	Liebermann-Burchard test	Benedict test
							
No foam	No precipitate	Precipitate formation	With the acid it clarified	No ring	No ring	Blue color that turned green	Reddish and green precipitate

Annex 3. Results of the phytochemical screening of F1B Agave americana.

F1B FRACTION OF **E1**							
Saponin	Alkaloids	Tannins & Phenols	Flavonoids	Glycosides			Carbohydrates
Foam test	Wagner test	2% FeCl3 test	Alkaline reagent test	Keller Kiliani test	Salkowski test	Liebermann-Burchard test	Benedict test
							
No foam	No precipitate	Bluish-green color	No color change	Slight brown ring on the interface	Reddish ring at the interface	Blue color that turned green	Green precipitate

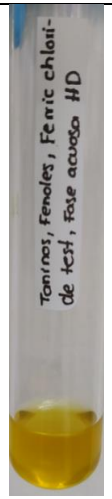
Annex 4. Results of the phytochemical screening of F1C Agave americana.

F1C FRACTION OF **E1**						
Saponin	Alkaloids	Tannins & Phenols	Flavonoids	Glycosides		Carbohydrates
Foam test	Wagner test	2% FeCl3 test	Alkaline reagent test	Salkowski test	Liebermann-Burchard test	Benedict test
						
Stable foam	No precipitate	Bluish-green color	With the acid it clarified	Reddish ring at the interface	Blue color that turned green	Reddish and green precipitate

Annex 5. Results of the phytochemical screening of F2C Agave americana.

F2C FRACTION OF EXTRACTION 2							
Saponin	Alkaloids	Tannins & Phenols	Flavonoids	Glycosides			Carbohydrates
Foam test	Wagner test	2% FeCl3 test	Alkaline reagent test	Keller Kiliani test	Salkowski test	Liebermann-Burchard test	Benedict test
							
Unstable foam	No precipitate	No color change	No color change	Slight brown ring on the interface	Slight reddish ring on the interface	No color change	Slight red precipitate

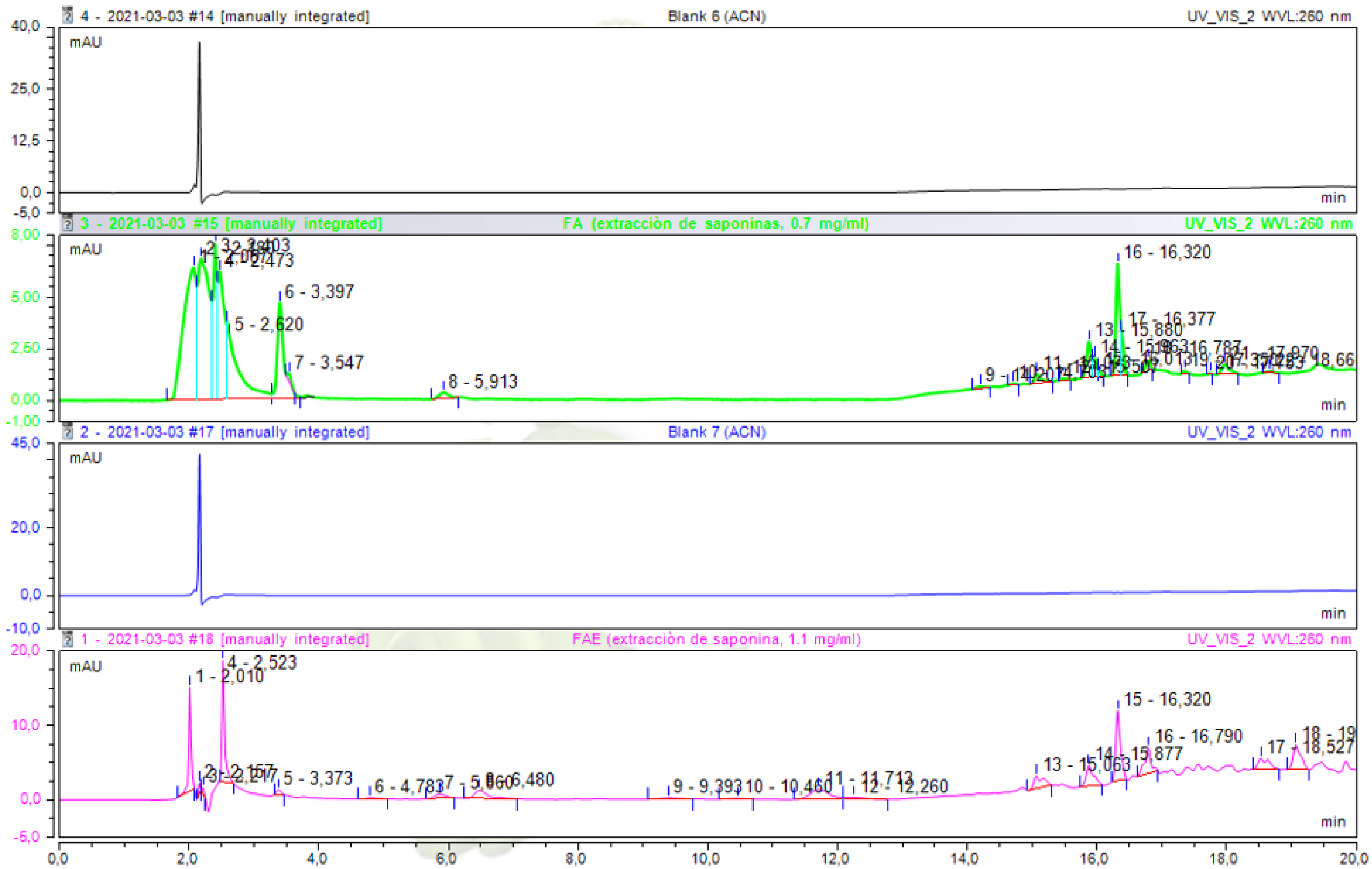
Annex 6. Results of the phytochemical screening of F3C Agave americana.

F3C FRACTION OF EXTRACTION 3							
Saponin	Alkaloids	Tannins & Phenols	Flavonoids	Glycosides			Carbohydrates
Foam test	Wagner test	2% FeCl3 test	Alkaline reagent test	Keller Kiliani test	Salkowski test	Liebermann-Burchard test	Benedict test
							
Unstable foam	No precipitate	No color change	No color change	Slight brown ring on the interface	Slight reddish ring on the interface	No color change	Slight red precipitate

Annex 7. Chromatogram list.

Fraction	Chromatogram name
F1B	FAE (extracción de saponina 1.1 mg/ml)
F1C	FA (extracción de saponina 0.7 mg/ml)
F2C	FA (Hidrólisis de saponina 0.4 mg/ml)
F3C	FA (Hidrólisis directa 1.4 mg/ml)
F4C	F0 (Hidrólisis directa 0.8 mg/ml)
F4D	Sobrenadante hecogenina (1.2 mg/ml)
F4E	Sólido (hecogenina, 1.6 mg/ml)

Annex 8. Comparison of F1C (green line), with F1B (pink line).



Annex 9. Overlays of F2C (pink line) with F3C (black line).