# UNIVERSIDAD DE INVESTIGACIÓN DE TECNOLOGÍA EXPERIMENTAL YACHAY

**Escuela de Ciencias Químicas e Ingeniería**

## TÍTULO: Design and development of an analytical method using HPLC for the determination of the chemical marker present in *Tropaeolum tuberosum*

Trabajo de integración curricular presentado como requisito para la obtención del título de Química


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# Dedicatoria

*"Dedico esta tesis a Dios y a las personas más importantes de mi vida: Mi madre, porque gracias a su apoyo, consejos y palabras de aliento, he podido llegar a este punto de mi vida profesional y sé que sin ella, nada de esto hubiera sido posible. Mi padre, que a pesar de que no está físicamente conmigo, siempre estuvo en mi mente y mi corazón, y estoy segura que estaría muy orgulloso de mí. Mi hermano, que siempre me ha apoyado y ha apoyado a mi madre incluso en mis momentos de ausencia. Mis abuelitos que siempre estuvieron orando por mí, pendientes de mí, apoyándome y deseándome siempre lo mejor. Por último, pero no menos importante, esta tesis está dedicada al ser que ocupa un gran espacio en mi corazón y que me acompañó día y noche mientras realizaba este escrito, mi perrita "Chía".*

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# Resumen

A pesar de la indudable creencia ancestral de que numerosas plantas herbales tienen poderes curativos, la complejidad química de las mismas ha dificultado el desarrollo de estudios científicos que avalen su actividad terapéutica. En los últimos años, la industria farmacéutica, a través de modernas técnicas de caracterización, se ha centrado en desarrollar métodos que permitan estudiar con más detalle estas plantas, con el fin de lanzar al mercado productos de alta calidad. La inexistencia de un método analítico centrado en el control de calidad del polvo de *Tropaeolum tuberosum* (mashua) como materia prima ha desarrollado el interés de la industria farmacéutica Ecuatoriana para investigar esta planta medicinal endémica de los Andes. De esta forma, mashua fue la planta medicinal objeto de estudio en este proyecto, que fue seleccionada por su reconocida actividad antiinflamatoria en la medicina popular. Esta investigación se centra en determinar un marcador químico presente en mashua, que será utilizado como patrón de referencia en el control de calidad de la materia prima (polvo de mashua). El marcador químico se obtendrá mediante el diseño y desarrollo de un método analítico basado en Cromatografía Líquida de Alta Resolución en Fase Reversa con detección UV-Vis (RP-HPLC-UV/Vis). La separación de estos compuestos en el equipo se logró utilizando tanto la columna THERMO L1 (C18) (150 mm x 4.6 mm y 5 $\mu$m) como un sistema de elución en gradiente. La fase móvil consta de una solución tampón pH 2.5 con agua (TEA 0.01 M, $H_3PO_4$) como Solvente A, Metanol como Solvente B y Acetonitrilo como Solvente C durante 25 minutos. El método de HPLC desarrollado en este trabajo demostró que a (+)-catequina y quercetina no son marcadores químicos apropiados para la caracterización y control de calidad de mashua porque estos dos compuestos no existen o existen en una cantidad residual en el extracto hecho a partir del polvo de esta planta medicinal andina. Así, es necesario adquirir otros estándares descritos en la literatura científica para mashua y disponibles en el mercado de forma a encontrar marcadores químicos apropiados para caracterizar y hacer en control de calidad de esta planta. Esta metodología es de extrema importancia para la industria farmacéutica ecuatoriana porque no existe actualmente ningún método de control de calidad para mashua, lo que impide su comercialización en el mercado nacional y internacional.

**Palabras Clave**: Compuestos fenólicos, mashua, medicina popular, TLC Preparativa, RP-HPLC, marcador químico, "screening" fitoquímico cualitativo preliminar, (+)-catequina.

# Abstract

Despite the unquestionable ancestral belief that numerous herbal plants have healing power, the chemical complexity has hampered scientific studies that support their therapeutic activity. In recent years, the pharmaceutical industry, through modern characterization techniques, has focused on developing methods that allow studying these plants in further detail, to launch high-quality products onto the market. The inexistence of an analytical method focused on quality control of *Tropaeolum tuberosum* (mashua) powder as a raw material has developed the Ecuadorian pharmaceutical industry's interest in researching this endemic Andean medicinal plant. In this way, mashua was the medicinal plant that is subject of study in this project, which was selected due to its well-known anti-inflammatory activity in popular medicine. This research is focused on determining a chemical marker present in mashua, which will be used as a reference pattern in quality control of raw material (mashua powder). The chemical marker was obtained through the design and development of an analytical method based on Reverse Phase High-Performance Liquid Chromatography with UV-Vis detection (RP-HPLC-UV/Vis). The separation of these compounds on the equipment was achieved using both the THERMO L1 (C18) column (150 mm x 4.6 mm and 5 $\mu$m) and a gradient elution system. The mobile phase consisted of buffer solution pH 2.5 with water ( TEA 0.01 M, $H_3PO_4$) as Solvent A, Methanol as Solvent B, and Acetonitrile as Solvent C during 25 minutes. The HPLC method developed in this work demonstrated that a (+)-catechin and quercetin are not appropriate chemical markers for the characterization and quality control of mashua because these two compounds do not exist or exist in a residual quantity in the extract made from the powder of this Andean medicinal plant. Thus, it is necessary to acquire other standards described in the scientific literature for mashua and available in the market in order to find appropriate chemical markers to characterize and do quality control of this plant. This methodology is extremely important to the Ecuadorian pharmaceutical industry because there is not currently any quality control method for mashua, which prevents its commercialization in the national and international market.

**Keywords**: Phenolic compounds, mashua, popular medicine, preparative TLC, RP-HPLC, chemical marker, preliminary qualitative phytochemical screening, (+)-catechin.

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# Abbreviations

UV: Ultraviolet

RP-HPLC: Reversed Phase-High Performance Liquid Chromatographic Method

HPLC: High Performance Liquid Chromatographic Method

DAD: Diode-Array Detection

UPLC: Ultra Performance Liquid Chromatography

UV-Vis: Ultraviolet–Visible

FA: Formic Acid

p/p: purple mashua

y/y: yellow mashua

p/y: mixture of purple and yellow mashua

AA: Acetic Acid

I: Isocratic

G: Gradient

AH: Acid hydrolysis

TEAP: Triethanolamine Phosphate

RP18: Reversed Phase 18

HSS T3: High Strength Silica with 1.8 $\mu$m particle size

C18: means that the molecules contain 18 carbon atoms

mm: milimeters

$\mu$m: micrometers

TEA: Triethylamine

$H_3PO_4$: Phosphoric acid

GC: Gas Chromatography

M: Molar

FW: Fresh weight

DM: Dry matter

AH: Acid Hydrolysis

ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

ORAC: Oxygen Radical Absorbance Capacity

TLC: Thin-Layer Chromatography

# 1. Introduction-Justification

## 1.1 Mashua: Chemical composition, therapeutic effects and ancestral belief

Mashua (*Tropaeolum tuberosum*), also known in the literature as "añu" or "isaño", belongs to the group of edible tubers from the cool-temperate Andes. This tuber's interest is related to chemical properties and hypotheses based on deep cultural beliefs that have attracted the curiosity in the scientific field. Traditional medicine has encouraged mashua tubers' use due to the therapeutic effects on the liver and kidneys alleviating prostate and urinary disorders. Among the most interesting therapeutic properties, we can find antibiotic, antifungal, insecticidal, nematicidal, and diuretic (which are related to secondary metabolites, glucosinolates, and isothiocyanates content) [1–3] .

Mashua is composed by aromatic glucosinolates (benzyl glucosinolate) that are considered one of the principal secondary metabolites in this tuber. These could experience enzyme hydrolysis (reaction related with the breakdown of a bond using water and the enzyme myrosinase, in this specific case) as we can see in Figure 1.1, this reaction results in the release of isothiocyanates (volatile compounds that produce the characteristic taste of mustard). So, these tubers used in traditional cuisine have better flavor with sun treatment because they have lower glucosinolate content [1, 4].

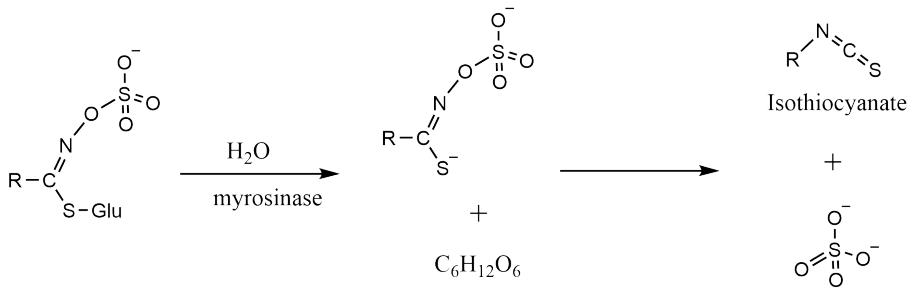


Figure 1.1: The reaction of enzymatic hydrolysis of glucosinolates and the product obtained: the release of isothiocyanates [4].

These tubers present undesirable substances such as isothiocyanates in mashua, oxalates in oca and astringent principles in mauka. These substances limit acceptability because they have harmful effects or generate some toxicity, which makes them unacceptable or inadequate for countless applications when consumed in large quantities [5]. Nevertheless, if the reaction produced when the roots of mashua are converted into powder is considered, the mayor's presence of isothiocyanates that could be toxic in the

body can be avoided [6].

Obtaining authentic information about medicinal plants is an arduous work due to both essential and non-essential constituents. Some of them have a pharmaceutical interest, and others even have specific toxicity to the human beings. A medicinal plant is considered a chemical factory due to its complex internal composition and the secondary metabolites are considered important constituents because many of these compounds can have medicinal and therapeutic effects [7]. Mashua presents in their composition different secondary metabolites such as flavonoids, phenols, tannins, anthocyanidins, quinones, saponins, lactones, coumarins, cardenolides, triterpenes, steroids, and alkaloids [8].

In our country, research should focus on the study of endemic plants such as mashua to demonstrate the different hypotheses about the positive health benefits of the medicinal Ecuadorian plants. However, despite the information behind this tuber, authors have not directly focused on determining the chemical composition of this tuber. According to the statements found within the literature, unfortunately, the available information regarding the identification of non-anthocyanin phenolic compounds in mashua tubers is scarce. In this way, we can realize that the characterization of other compounds (non-anthocyanin phenolic compounds) of mashua is undoubtedly an essential requirement to know which of them plays a critical role for the therapeutic activity of this tuber [9, 10].

Finally, we can highlight the lack of an analytical method focused on quality control of mashua powder as a raw material in the current scientific literature. Thus, the Ecuadorian pharmaceutical industry has high interest to research this endemic Andean medicinal plant and ensure the presence of this plant within a pharmaceutical formulation through the use of chemical markers.

## 1.2 Tropaeolaceae Family

Tropaeolaceae belongs to the herbaceous family and the most important and widely known genus is Tropaeolum, which contains 86 species [1]. The morphological diversity is directly related to the distinctive characteristics of their phenotype and genotype (color, shape, size, chemical composition, among others). In particular, the color of this type of tuber varies from cream or yellow to dark purple; generally, secondary metabolites are responsible for this properties (see Figure 1.2) [1, 11].


Figure 1.2: Some examples of Tropaeolaceae family: oca (*Oxalis tuberosa*), ulluco (*Ullucus tuberosus*) and mashua (*Tropaeolum tuberosum*) [1].

### 1.2.1 A tuber related to Mashua: *Oxalis tuberosa* (oca)

Oca belongs to the Tropaeolaceae family; thus, they have similar characteristics with Mashua including related compounds, antioxidant potential, appearance, among others (see Figure 1.2 and Table 1.3). Taking into account that mashua tubers have a sweet and pleasant taste is broadly consumed in the Andean region, even this tuber occupies the second position compared with potato in terms of nutritional value, antioxidant potential, and consumption. Despite its well-known use as food, oca tubers are renowned for their medicinal properties, which are used to treat skin ulcers, postpartum recovery, and urinary system diseases taking into account its anti-inflammatory properties. These medicinal properties are also associated with the antioxidant potential based on secondary metabolites' existence in their composition. So, the antioxidant capacity of these tubers is related to anthocyanin content in the case of purple (p/p) oca and flavonoids content in the case of yellow (y/y) oca [12].

Furthermore, considering that the antioxidant capacity is related to the total phenolics, it is essential to know that Total Phenolics in the same family have different values. So, oca tubers are in the range of 71–132 mg/100 g of Fresh Weigh (FW), the value for mashua tubers varies from 92–337 mg/100 g of FW, and in the case of potatoes, the range goes from 64–232 mg/100 of FW. These values differ in terms of their genotype [12].

## 1.3 Secondary Metabolites

Secondary metabolites, also known as phytochemicals, are an important source of active pharmaceuticals. During last decades, these molecules have been increasingly studied to the point of having a branch of science focused on the activity of these compounds, Phytochemistry. The most important classification is based on three principal groups: phenolic compounds, alkaloids, and terpenoids, and exists a subclassifi-

cation of these groups according to the complex chemical structure of the molecules that belong to them. In drug research and development, the importance of plants' individual components and particularly, second metabolites, lies in obtaining optimized extraction methods, pharmacokinetics, assurance quality methods, even isolate and characterize these compounds related to modern drug development. According to this statement, alkaloids are the most used secondary metabolites in the pharmaceutical industry due to its therapeutic relevance. Simultaneously, the wide variety of phenolic compounds has the utmost importance when it comes to drug quality control analysis [13, 14].

### 1.3.1 Phenolic Compounds

Phenolic compounds have as common feature that is the presence of a phenol group into their molecular structure. In this way, phenolic compounds could be classifed as a function of the number of phenol rings and the type of substituted groups that are linked to the skeleton of the molecule. It is widely known that antioxidant potential is attributed to these compounds due to their high reducing potentials; these compounds generally work as reducing agents (electron donors). Furthermore, phenolic compounds (secondary metabolites) plays an important role in the organoleptic, prevention and medicinal properties of plants [9, 12]. Table 1.1 shows the classification of the best known phenolic compounds.

Table 1.1: Classification of phenolic compounds.

| | |
|---|---|
| **Phenolic acids** | *o*-coumaric acid |
| | *p*-coumaric acid |
| | Protocatechiuc acid |
| | Ferulic acid |
| | Gallic acid |
| | Caffeic acid |
| | Chlorogenic acid |
| | *p*-hydroxybenzoic acid |
| **Flavonols** | Quercetin |
| | Rutin |
| | Myricetin |
| | Kaempherol |
| **Flavones** | Chrysin |
| | Luteolin |
| | Apigenin |
| **Flavanones** | Naringenin |
| **Flavan 3-ols and Procyanidins** | Cathechin |
| | Epicathechin |
| | Gallocathechin |
| | Epigallocatechin gallate |
| | Procyanidins (B1 and B2) |
| **Anthocyanidins** | Delphinidin |
| | Cyanidin |
| | Pelargonidin |
| | Malvidin |

#### 1.3.1.1 Flavonoids

Flavonoids (Latin "flavus" that means "yellow") are phenolic compounds found in nature in the form of glycosides (glucose is the most common sugar). Flavonoids are classified based on the structure of the C2-C4 carbon chain (see Figure 1.3) [15].

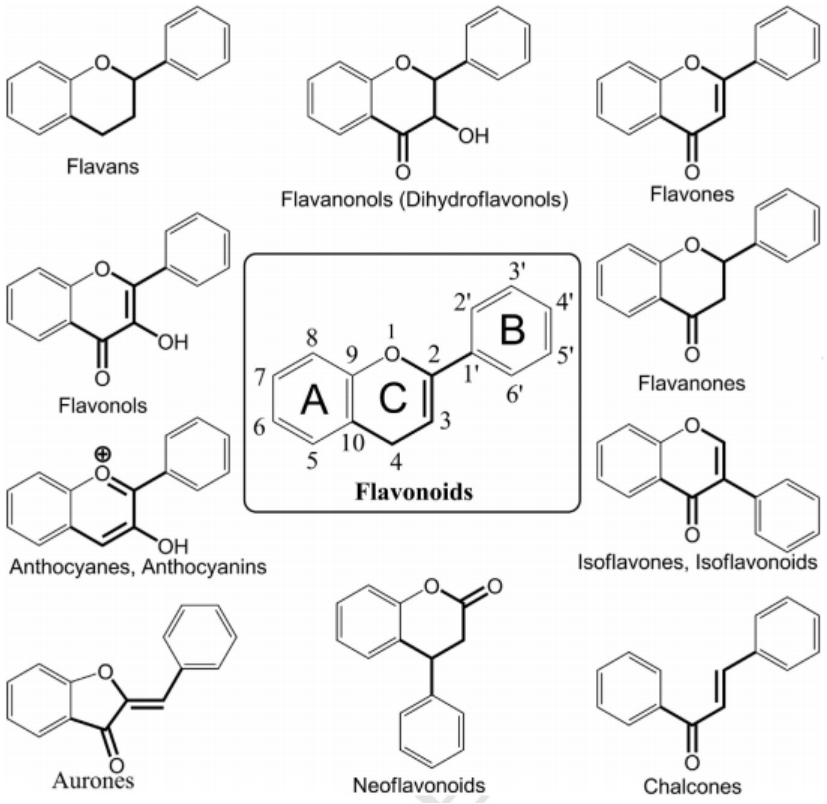


Figure 1.3: Classification of flavonoids based on the structure of the C2-C4 carbon chain (highlighted in bold) [15].

Also, according to his classification, each molecule presents a specific maximum wavelength at which they absorb UV radiation (see Table 1.2). In this way, flavonoids that contain hydroxyl groups tend to be more polar, so its extraction is performed with solvents such as methanol, ethanol, or water. In contrast, the flavonoids with methoxyl groups are less polar and they are extracted with solvents such as ether or chloroform [16]. The location of flavonoids in the plant is not specified; sometimes, these compounds are present in flowers, leaves, stems and fruits. In the specific case of mashua, we could found flavonoids in the roots; we can recognize them through its yellowish color (see Figure 1.2) [1, 17, 18].

Table 1.2: Maximal absorbance wavelengths of different flavonoid types.

| ***Flavonoid Type*** | **Range of Maximum Wavelength (nm)** |
|---|---|
| Flavones | 270-365 |
| Flavonols | 330-365 |
| Flavanones | 290 |
| Isoflavones | 236-260 |
| Chalcones | 340-360 |
| Dihydrochalcones | 280 |
| Anthocyanins | 502-520 |
| Cathechins | 210-280 |

**1.3.1.1.1 Glycosylation Reaction** The final product of glycosylation reaction is a glycoside, which is a molecule that contains a sugary portion (glycone) joined to a non-sugary portion (aglycone) through a glycosidic bond. The hydroxyl groups present in these compounds allow glycoside formation through glycosylation reaction which generate a complexity of molecules in the extract (see Figure 1.4) [19].

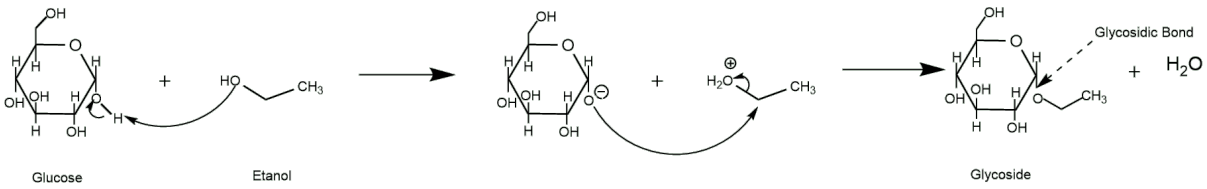


Figure 1.4: Mechanism of Glycosylation Reaction.

**1.3.1.1.2 Anthocyanins** Anthocyanins are water-soluble glycosides of anthocyanidins (aglycones). They are within the family of flavonoids, which belongs to the great family of phenolic compounds. The more substituted the anthocyanin, a bathochromic change (an increase of wavelength) occurs in a much more remarkable way, causing changes of color from violet to blue and even red; this phenomenon can be appreciated when analyzing roots, leaves, flowers, and fruits. Anthocyanins in the extract present photosensitive pigments, and for this reason, it is necessary to take into account storage conditions. These types of compounds are used in the treatment of inflammatory processes. The most used methods to perform separation and identification of anthocyanins are TLC and HPLC. The mobile phase used for this purpose consists of solvents such as formic acid, hydrochloric acid, and water (25:24:51); nevertheless, these proportions and solvents can vary according to the experimental conditions. The pH of the solvent used for the extraction should be low; this condition is crucial to the formation of the flavylium ion, which optimizes extraction times [20–22].

#### 1.3.1.2 Alkaloids

Alkaloids are secondary metabolites with basic nature (nitrogenous organic compounds). As a strict definition, it is not only necessary to have nitrogen in the structure to belong to this family, but also nitrogen must be within a heterocyclic ring (see Figure 1.5) [23]. The example of caffeine is taken because it is one of the only alkaloids that have been described in the literature for mashua tubers [24]. Alkaloids are associated with medicinal purposes (drugs); so, if a plant (root, leaf, flower, or fruit) presents alkaloids within its composition is synonymous of an undeniable pharmacological action. Thus, many of the synthetic drugs that have been developed for wellness and even recreation of humans have imitated therapeutic principles of medicinal plants that often contain alkaloids [12].

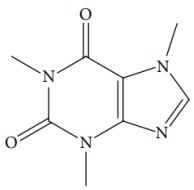


Figure 1.5: Chemical Structure of Caffeine [24].

## 1.4 Derivatives of phenolic compounds present in mashua

Previous studies have given information about the existence of a wide variety of not identified compounds, which generally are called "derivatives" of secondary metabolites (principally phenolic compounds); this, due to the complexity of the extract and lack of a significant number of standards required for characterization [9].

So, these derivative compounds present both similar structural and spectral (UV-Vis) features, which are compared experimentally or through literature. According to the different mashua tubers (yellow (y/y), mixture of yellow and purple (p/y) and purple (p/p)), some phenolic profiles could have greater complexity than others, depending on the associated compounds present within its composition. For example, the information found in the literature about yellow mashua, considered for this project, shows a smaller quantity of peaks compared to purple mashua, due to the inexistence of certain phenolic compounds such as anthocyanins in their composition. Common compounds present in the different types of mashua tubers are gallic acid, gallocatechin, epigallocatechin, and procyanidin B9. Supplementary bibliographic information about some derivatives of phenolic compounds present in this tubers are detailed in Table 1.3 [9].

Table 1.3: Derivatives of phenolic compounds present in Mashua.

| **Chemical Compound** | **Amount(mg/100 g mashua FW)** | **Ref.** | $\lambda_{max}$ (nm) | **Ref.** |
|---|---|---|---|---|
| Gallic acid | $0.02_{(p/y)}+0.03_{(p/p)}+0.01_{(y/y)}= 0.06$ | [9] | 260 - 272 | [25] |
| | $0.43_{(p/y)}+0.35_{(p/p)}+0.38_{(y/y)}= 1.16$ | [9] | | |
| | $0.46_{(y/y)}$ | [9] | | |
| | **Total**= 1.68 | [9] | | |
| Gallocatechin | $1.26_{(p/y)}+(-)_{(p/p)}+0.05_{(y/y)}= 1.31$ | [9] | 236, 270 | [9] |
| Epigallocatechin | $10.70_{(p/y)}+3.02_{(p/p)}+0.14_{(y/y)}= 13.86$ | [9] | 237, 270 | [9] |
| Procyanidin $B_2$ | $1.43_{(p/y)}+0.25_{(p/p)}+0.01_{(y/y)}= 1.69$ | [9] | 237, 280 | [9] |
| Malvidin | N/A | [12] | 272, 535 | [26] |
| | N/A | [12] | 510 | [27] |
| Quercetin(AH) | N/A | [9] | 258, 380 | [28] |
| Myricetin | N/A | [9] | 268 | [29] |
| Cyanidin(AH) | N/A | [9] | 506 | [27] |
| Delphinidin(AH) | N/A | [9] | 210, 275, 535 | [27] |
| Pelargonidin(AH) | N/A | [9] | 494 | [27] |
| Protocatechuic acid(AH) | N/A | [9] | 216, 257, 293 | [30] |
| Epicatechin(AH) | N/A | [9] | 210, 280 | [31] |
| Epicatechin derivatives | N/A | [9] | 236, 280 | [9] |
| Epigallocatechin derivatives | N/A | [9] | 237, 273 | [9] |
| Gallocatechin derivatives | N/A | [9] | 237, 270 | [9] |
| Cinnamic acid derivatives | N/A | [9] | 292 | [9] |
| Cinnamic acid derivatives(AH) | N/A | [12] | | |
| *o*-Coumaric acid derivatives | N/A | [9] | 274, 325 | [9] |
| *p*-Coumaric acid derivatives | N/A | [9] | 310 | [9] |
| Rutin derivatives | N/A | [9] | 260, 354 | [9] |
| *p*-Hydroxybenzoic acid derivatives | N/A | [9] | 267 | [9] |
| Myricetin derivatives | N/A | [9] | 253, 376 | [9] |
| Protocatechuic acid derivatives | N/A | [9] | 254, 273 | [9] |
| Vanillic acid derivatives | N/A | [9] | 254, 273 | [9] |
| Vanillic acid derivatives(AH) | N/A | [12] | | |
| Gallic acid derivatives | N/A | [9] | 274 | [9] |
| Caffeic acid derivative | N/A | [12] | 312.8 | [12] |
| Luteolin derivatives | N/A | [12] | 207, 260, 350 | [32] |
| Apigenin derivatives | N/A | [12] | 220, 325 | [32] |
| Naringenin derivatives | N/A | [12] | 288.7 | [33] |

AH: Sample subjected to an acid hydrolysis process to obtain the mentioned compound; p/p: purple mashua according to the genotype and phenotype; y/y: yellow mashua according to the genotype and phenotype; p/y: mixture of purple and yellow mashua according to the genotype and phenotype

This research is also based on a bibliographic analysis, which gives us general information about this tuber's related compounds. Table 1.3 shows synthetically this information, which is mostly related

to phenolic compounds. This table shows that most of these compounds are focused on a qualitative analysis instead of a quantitative one. Many of the compounds related to mashua tubers are described in the literature as derivatives of other compounds because there are no standards that corroborate the presence of specific compounds. They are called "derivatives" because, through chromatographic or spectrophotometric analysis, they have very similar UV-Vis spectra and retention times compared to the reference compound aglycone either through literature or a chemically identical compound (standard).

## 1.5 Pharmacological Activity of Secondary Metabolites in Mashua Tubers

### 1.5.1 Antioxidant Activity

Oxidation is a chemical reaction that involves the loss of electrons (generating free radicals), leading to a chain reaction that produces damage to cells. In this reaction, antioxidants as reducing agents play the role of donating electrons to the acceptor molecule. Furthermore, it is essential to know that these processes are closely related, so one cannot exist without the other [34].

The antioxidant activity is associated with aromatic rings and, more, especially due to the presence of phenol groups (phenolic compounds). In this way, phenol groups neutralize free radicals preventing the oxidation (creating non-free radicals in the body). So, this process prevents the appearance of cancer cells and has been found incredibly helpful in treating chronic diseases. Green tea is a clear example representing this assertion, so it is composed of catechins (phenolic compounds) with known antioxidant activity. Also, the antioxidant activity is related to electrochemical behavior, in such a way that, high reduction potential is directly proportional to high antioxidant activity [11, 35, 36].

Although the tubers discussed previously belong to the same family, the antioxidant activity and phenolic compounds content differs despite its similar appearance. In this way, some studies have shown that mashua tubers have the highest antioxidant activity indexes than other family members. Different studies have shown that the antioxidant activity of purple and yellow mashua is due to its anthocyanin content (principally delphinidin, cyanidin, and pelargonidin). The anthocyanin content varies according to the mashua type. Dark purple mashua tubers present a higher antioxidant activity than cream or yellow mashua tubers (5-7 times higher, generally attributed to anthocyanin content). Through this information, it is possible to think that beneficial health effects that provide yellow mashua tubers are linked to other types of compounds [9, 11].

Also, mashua tubers' nutritional value is compared even with potato (tuber consumed in large quantities in our country), turning it into one of the most important products that have a broad food potential in a few years. However, mashua tubers should be as recognized and appreciated due to their nutritional value as well as their medicinal activity: anti-inflammatory, diuretic, and antioxidant activity. Antioxidant activity has been the most studied parameter through different tests such as ABTS, ORAC, etc. These tests reveals equal values or even highest compared to products known precisely for this activity such as apples, grapes, cherries, broccoli, cabbages, among others [9].

## 1.6 Chirality of the Molecules

Chirality is related to forms spacially differents from the same molecule (enantiomers). So, the molecule has the same chemical structure but different mirror images (not superimposable). The concept of chirality is also related to daily life since many of the products we use are focus on chiral molecules. Thus, chiral bioactive compounds can be related to drugs, agrochemicals, flavors, and even odors. However, in some cases, such as Thalidomide Tragedy (a racemic mixture of a drug that caused severe malformations in children), they can become fatal due to the toxicity they generate. In less critical cases, the isomer of the molecule in question does not fulfill its function [37]. The characterization of chiral compounds found in medicinal plants' complexity is of great importance in analytical chemistry (separation and characterization of compounds) and pharmacology. The Chinese Pharmacopoeia has focused on studying medicinal plants, and many of the active substances have been shown to be chiral molecules [38].

### 1.6.1 Chirality of Catechins

Catechins are secondary metabolites present in different medicinal plants and foods, which belong to the group of polyphenolic flavan-3-ols (flavonoids) [39]. The catechin skeleton structure shows two chiral centers (one is on carbon two and the other on carbon three, which makes this molecule present four diastereoisomers, that is, two isomers of trans configuration called catechins and two isomers of cis configuration called epicatechins (see Figure 1.6)[40, 41].

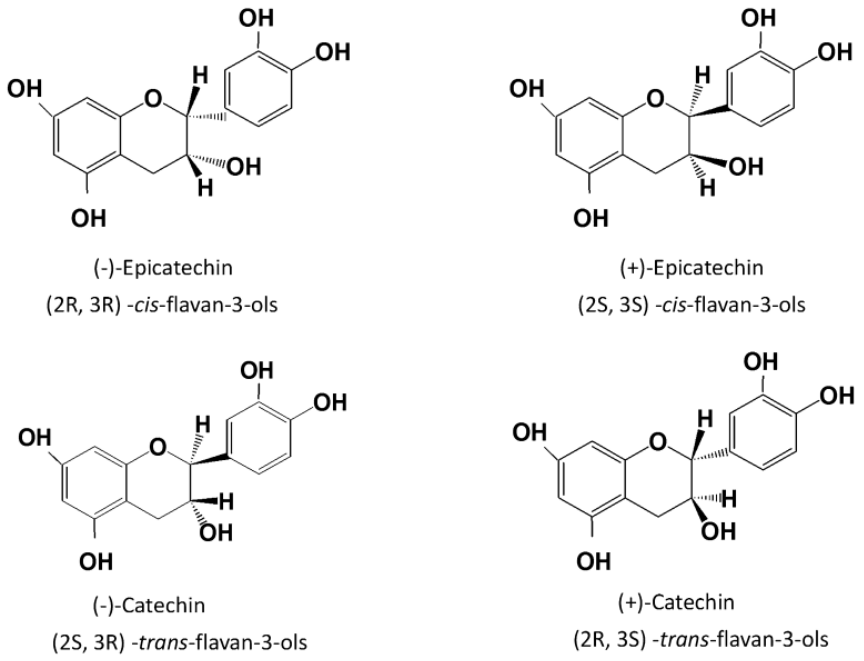


Figure 1.6: Chirality of Catechins [41].

The diastereoisomers (+)- catechin and (-)- epicatechin are the most studied, while (-)- catechin and (+)- epicatechin generally do not exist in nature. Diastereoisomers have the same adsorption properties; so, its separation by RP-HPLC with a regular column (achiral) becomes an impossible task [41]. In this way, if these types of diastereoisomers are required, they can be separated or recognized through a chiral chromatographic column for HPLC analysis [40]. Figure 1.7 shows an analysis by RP-HPLC with two types of columns: achiral (D) and chiral (E) [41]. The study with an achiral column has as fundamental requirement the use of standards to demonstrate diastereoisomers presence. The problem lies in the fact that it is difficult to find standards of these diastereoisomers due to their scarcity in nature.

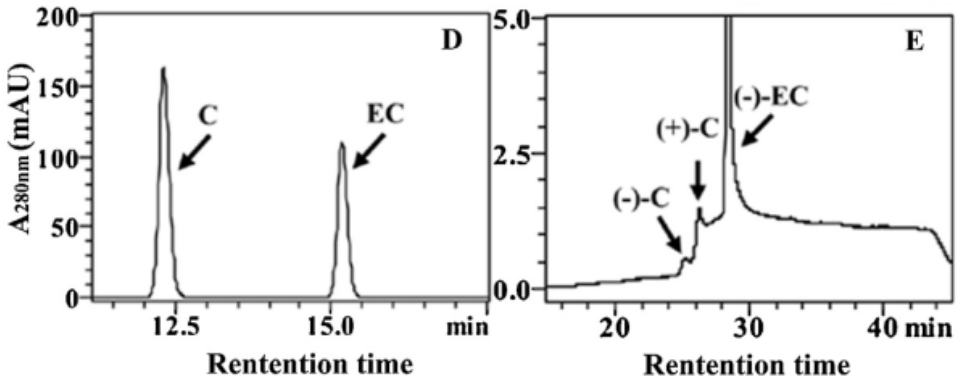


Figure 1.7: RP-HPLC analysis with two types of columns: achiral (D) and chiral (E) [41].

Thus, the use of a chiral column is necessary to separate the diastereoisomers present in a plant using reference retention times and specific chromatographic characteristics. So, analysis of catechins by HPLC using chiral columns or highly specific standards has demonstrated the variability of retention times in their different diastereoisomers (see Table 1.4). An indirect form to obtain diastereoisomers is through the manufacturing process. For example, products such as coffee contain (+)- catechin; however, due to treatment with high temperatures or alkalinity that they experience in the industrial process, could experience a change to (-)- catechin [42].

Table 1.4: Values of retention time between the diastereoisomers (+)-catechin and (-)-epicatechin.

| ***Diastereoisomers*** | ***Retention Time*** | ***Reference*** |
|---|---|---|
| (+)-catechin | 11.8 | [43] |
| (-)-epicatechin | 16.1 | [43] |

Catechins could have different activity according to its configuration cis or trans; in this way, for example, catechin (trans) is a more potent anti-atherogenic (prevent the formation of lipid deposits in the arterial wall) than epicatechin (cis) in hyperhomocysteinemia (a group of rare metabolic diseases characterized by an elevated level of the amino acid homocysteine in the blood plasma).

In the same way, catechins could have different activity according to its diastereoisomers; in this way, for example, (-)- catechin does not have medicinal activity, while (+)- catechin showed antibacterial activity. Also, many studies reported that (+)- catechin was more efficient than (-)- epicatechin in preventing deoxyribonucleic acid damage induced by heterocyclic amines [38].

Furthermore, it has been demonstrated through different studies the presence of catechins in the chemical composition of mashua tubers. Catechins have an antioxidant and anti-inflammatory effect that has been widely studied through in vitro and in vivo tests [42]. Generally, the antioxidant activity of purple mashua tubers is attributed to the high content of anthocyanidins. Considering that yellow mashua, the object of this research, through the literature shows a low content of these compounds, it is possible to deduce other compounds associated with this benefit for the body [44].

Considering that enantiomers have the same response in UV-Vis spectrophotometry, we can ensure that the signal and intensity will be the same regardless of its diastereoisomers. In this way, Figure 1.8 and 1.9 shows that epicatechin and catechin have the same spectrum, taking into account absorption maxima, peak shape, shoulders, and others [31, 43, 45].

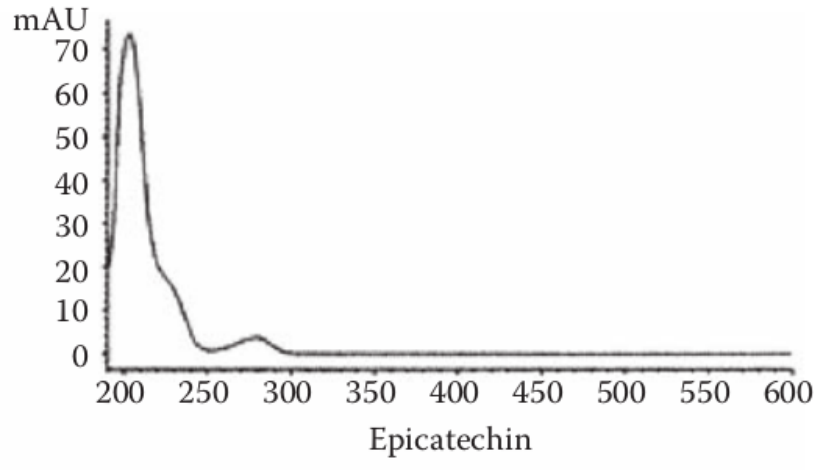


Figure 1.8: UV-VIS spectra for Epicatechin [31].

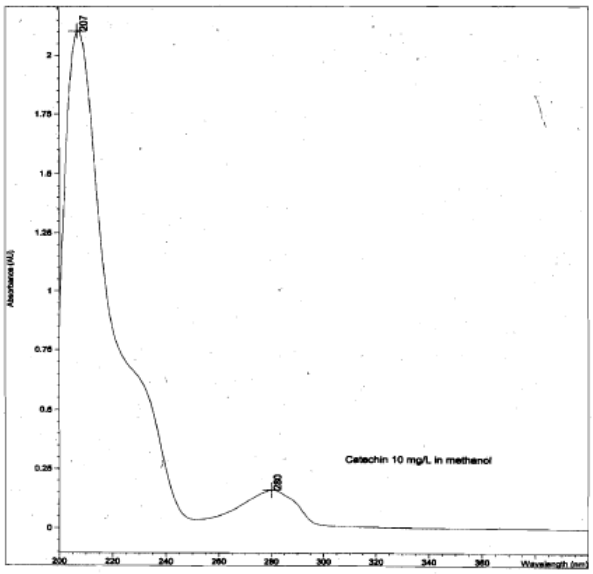


Figure 1.9: UV-VIS spectra for Catechin [43].

## 1.7 Chemical Markers

Chemical markers are characteristic molecules or specific constituents of plants, regardless of whether they possess any therapeutic activity. Generally, chemical markers are related to the medicinal constituents, but these often are dificult to obtain since the plant's complexity. Furthermore, chemical markers development presents other difficulties, for example, temperature, light, and solvents that often cause degradation and/or transformation of constituents [46].

Chemical markers are widely studied to optimize methods of extraction and purification, characterize

and elucidate structures, discover active substances present in medicinal plants, and even are related to the development of new drugs. The choice of an appropriate chemical marker is crucial for the quality control analysis of raw material in herbal medicines. The importance of quality control analysis of raw material in herbal medicines ensures their safety, traceability, and efficacy. Also, it allows to avoid adulterants or toxic substances. In this way, according to Li, *et al.*, 2018 [46], exist a chemical marker classification used in quality control analysis: general constituents coupled with 'fingerprints'. This classification has a fundamental requirement; it chooses general components (common and specific in the plant) to be used simultaneously with 'fingerprints' to differentiate from its substitutes and adulterants [46].

Chemical fingerprinting is the most powerful technique used for quality control analysis by using specialized analytic methods such as RP-HPLC and Gas Chromatography (GC) coupled to detectors such as mass spectrometer or spectrophotometer. A chemical fingerprint is a unique pattern with multiple chemical markers that contain the complete information of the sample [46].

## 1.8 Thin Layer Chromatography (TLC)

TLC is a type of Planar Chromatography, considered one of the simplest analytical methods used to perform a brief scan of a mixture of compounds. This technique has many advantages such as simultaneous processing of samples, saving time in each analysis, and allowing versatility in the choice of mobile phase (reducing the use of solvents, which is reflected in the economic factor). This type of research is usually qualitative and semi-quantitative [20].

In this method, sample (extract) in the form of a small dot, is placed in the baseline (reference of the starting point) drawn at one side of the silica ($SiO_2$) or alumina ($Al_2O_3$) plates (stationary phase with the polar character) (see Figure 1.10). It is essential to take into account that the stationary phase must be inert to not interact with the samples to be analyzed. The plate is then placed in a close chamber, which contains a solvent or mixture of miscible solvents with different polarities and proportions (mobile phase). The ideal mobile phase used during the development of the first analytical tests through TLC was found through the trial and error method. By capillarity, the components of the mixture migrate at different speeds through the stationary phase. This difference in speed is related to the affinity (polarity) of the components of the mixture with the stationary and mobile phase so that the polarity of the mobile phase is proportional to the elution order. So, solutes with more polar characters tend to be more retained, while those with non-polar character elute easily. During the adsorption process, the interactions carried out are hydrogen bonding, electron-pair donor/electron-pair acceptor (charge transfer), ion-ion, ion-dipole, and Van der Waals interactions. Finally, the silica plate is removed from the camera, and it is necessary

to limit the end of the path of the solvent (arrival line) to calculate retention factor (Rf). The plate is allowed to dry for a few minutes. It then proceeds to observe it through ultraviolet (UV) light, with or without applying a visualization reagent (generally by spraying). It is recommended to choose a solvent for that the compounds of interest have a RF in a range of 0.3 - 0.5. In other words, the compounds should be in the middle of the silica plate to obtain a value in this range [20].

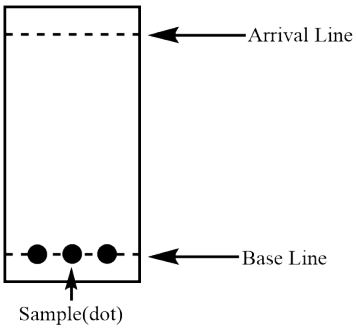


Figure 1.10: Execution of TLC experiment.

It is important to note that TLC analysis in a complex mixture (extract) will not solve all the doubts due to their complexity of the sample. Some compounds can interact with the mobile phase while others do not produce "candles" in the process. Nevertheless, TLC provides traces of information that will clear some specific doubts. In a TLC analysis, it is necessary to compare either in experimentally or theoretically way, against a reference standard or a known composition sample, in order to determine the degree of purity of the compound that will be analyzed or with the purpose of separate and identify compounds present in the mixture; this comparison is associated with the calculation of retention factors; generally, these values are not easily reproducible because it depends on the specific conditions of each Laboratory [20].

## 1.9 UV-VIS Spectrophotometry

The compounds present in the complexity of a plant extract are subjected to a pre-treatment and purification process in order to obtain their separation through basic chromatographic techniques such as TLC or column chromatography. Taking into account that UV/Vis Spectrophotometry is an analytical method based on the fact that each molecule absorbs a specific wavelength and generates a characteristic spectrum, it is used as a preliminary technique to high-resolution chromatographic analysis. In this way, it is possible to identify even between the same family compounds due to specific functional groups within each molecule. These identity properties allow comparing experimental results with theoretical data [47].

## 1.10 Reverse Phase-High Performance Liquid Chromatography (RP-HPLC)

RP-HPLC is a column chromatographic technique with a stationary phase and a liquid phase. Phenolic compounds are usually identified through this method due to its versatility, precision, high resolution, and relative cost-effectivity. The principal detectors of HPLC are UV–Vis and diode array detection (DAD). However, one drawback of this analytical technique is that in some cases, several compounds present similar retention times and spectrophotometric characteristics, making their identification a difficult task. So, the crude extracts analyzed by this method need a pre-purification to avoid noisy baseline in the chromatogram or a hydrolysis pre-treatment to simplify the chromatograms by decreasing the peaks correspondent to glycosidic forms of the compounds under study. In this manner, chromatograms with better resolutions are obtained [9, 22].

Many experiments demonstrated that chromatographic conditions used in HPLC experiments should be implemented concerning the type of compounds or family of compounds to be studied, using this information as a reference and considering the interactions of the molecule with the whole system should be balanced. So, the results of phytochemical screening of the extract, or literature should be considered as relevant information for this purpose [9, 22]. In the Table 1.5 we can see bibliographic information about the chromatographic conditions used in UPLC focused on phenolic compounds. UPLC is a much more sophisticated technique than HPLC in terms of equipment sensitivity and the need for a smaller sample amount. However, it has the same principle as HPLC, which helped us design the analytical method.

The nature of the compound (for example, hydrophilic or lipophilic nature) gives considerable information support to obtain an efficient separation during the experiment; this is due to separation by HPLC has as one of the fundamental principles, polarity of the samples and solvents. For example, a lipophilic molecule is generally retained in the C18 stationary phase. The peaks of the compounds should appear quickly in the chromatograms, taking into account that in terms of quality control analysis, the retention times do not should exceed 30 minutes [9, 22]. On the other hand, hydrophilic compounds have more affinity towards mobile phase (generally polar) and appear in the first few minutes of the chromatogram. Sometimes, this behavior is considered an advantage and it is an optimizacion of resources. Although the peaks of the compounds must have retention times greater than the uracil (base peak) [9, 22].

Table 1.5: Chromatographic conditions for UPLC handling in experiments related to different phenolic compounds.

| Ref. | Column | Dimensions | Mobile Phase | Gradient | Flow Rate | Temperature | Detection | Vol. |
|---|---|---|---|---|---|---|---|---|
| [48] UPLC | C-18 | 50 mm x 2.1 mm 1.7 um | Solvent A: 0.1% of FA Solvent B: MeOH | 0-80%B—-0-12min 80-100%B—-12-14min 100%B—-14-15min | 0.4 ml/min | 60°C | 200 nm to 400 nm | 10 ul |
| [49] UPLC | HSS T3 | 100 mm x 2.1 mm 1.8 um | Solvent A $H_2O$ with 0.1% FA Solvent B ACN with 0.1% FA | 3-8%B—-0-1min 8%B—-1-6min 8-20%B—-6-14min 20-24%B—-14-15min 24%B—-15-26min | 0.4 ml/min | column:25°C sample: 4°C | 280nm | 5 ul |
| [50] UPLC | C-18 | 150 x 2.1 mm i.d. 1.7 um | Solvent A 0.5% FA in water Solvent B 0.5% FA in ACN | 5%B——0min 25%B——20min 50%B——25min 90%B——30-32min 5%B——35-40min | 0.3 ml/min | 30°C | 280 nm Catechin 350 nm Flavonol | - |
| [51] UPLC | RP18 | 2.1 x 100 mm 1.7 um | Solvent A 0.1% AA in MeOH Solvent B 0.1% AA aqueous | (I) 5%A—-0.6min (G) 5-15%A—0.6-0.7min 15-42%A—0.7-3.2min 42-100%A—-3.2-3.3min 100%A—-3.3-8.9min 100-5%A—-8.9-9.0min 5%A—-9.0-10.0min | 0.44 ml/min | 60°C | - | 10ul |
| [52] UPLC | RF-C18-A | 150mm x 2 mm 3um | Solvent A 0.1% water : FA (1000 : 0.05 v/v) Solvent B: 0.1% MeOH | 10-80%B——0-70min Isocratic—-10min 10%B——90min Isocratic——10min | 0.2 ml/min | - | 280 nm 320 nm | 2ul |
| [53] UPLC | C-18 | 50 x 2.1 mm 1.8 um | Solvent A: TEAP (pH 2.5; 0.1 M) Solvent B TEAP/ACN (pH 2.5; 0.1 M) 60:40 (v/v) | 15-80%B—0-3.5min | 0.9 ml/min | 60°C | 230nm | 1 ul |
| [54] UPLC | C-18 | 100 x 2.1 mm, 2.2 um | Solvent A 0.1% AA in water Solvent B:(50:50 v/v) MeOH and ACN | 6%B——0-0.5min 6-24%B——0.5-6.5min 24-26%B——6.5-9min 26-6%B——9-10min | 0.4 ml/min | 30°C | - | 5ul |
| [55] UPLC | C-18 | 50 x 2.1 mm, 1.7 um | Solvent A Water with 50mM AA Solvent B ACN | 20-24%B——0-2min 24-26%B——2-4min 26-32%B——4-5min 32-35%B——5-12min 35-100%B——12-15min | 0.25 ml/min | 25°C | 270nm | 1ul |

# 2. Problem Statement

The widespread knowledge of the medicinal properties of this tuber, according to ancestral wisdom, has led to the launch of products that claim to contain mashua (raw material) in its formulation. Still, it isn't easy to prove this without analytical techniques that support it [3]. So, previous studies that involves chemical composition of mashua are not focused on analytic methods that allow knowing the chemical structure of active compounds or the search of chemical fingerprint for quality control analysis purposes. Even, despite the existence of studies focused on the antioxidant capacity of this tuber, most of them have not been interested in the chemistry behind this therapeutic property. In the same way, some authors do not emphasize or delve into the study of this tubers because most of them only see this tuber as food potential, not as a potential drug. The disinterest of studying a tuber lies in the ignorance about its chemical nature since, generally, only its nutritional value has been taken into consideration. In this way, this research lies an attempt to provide information about chemical composition of mashua tubers related to high-end analytic techniques [56]. Furthermore, the search of an chemical marker will solve an specific problem of Ecuadorian pharmaceutical industry related to quality control of this medicinal plant and make posible its comercialization in national and international market as a food supplement.

# 3. General and Specific Objectives

## 3.1 General Objective

- To design and develop an analytical method using RP-HPLC-UV/Vis for the determination of the chemical marker present in mashua powder which will be used as a reference pattern in quality control of this Andean medicinal plant in pharmaceutical industry of Ecuador.

## 3.2 Specific Objectives

- To analyze the preliminary qualitative phytochemical screening of the hexane, dichloromethane, chloroform, and ethanol extracts of mashua powder.
- To determine the advantages and disadvantages of the pre-treatment process of the extract through two techniques Simple Distillation and Rotary Evaporation.
- To analyze fractions obtained through a Preparative TLC using UV-Vis spectrophotometry and RP-HPLC coupled to UV-Vis spectrophotometer.
- To determine the method of extraction and treatment of the sample (mashua powder) to obtain high-resolution separation conditions through RP-HPLC coupled to UV-Vis spectrophotometer.
- To develop a method through RP-HPLC-UV/Vis in order to find a chemical marker.
- To study (+)-catechin and quercetin as chemical markers for mashua powder.

# 4. Methodology

## 4.1 Reactives, Standards and Equipment

### 4.1.1 Reactives

- Ferric Chloride Hexahydrate ($FeCl_3.6H_2O$) were purchased from Mallinckrodt Pharmaceuticals (United Kingdom)
- 1-butanol were purchased from Fisher Chemical (UK)
- Resublimated Iodine were purchased from Fisher Chemical (UK)
- Hydrochloric acid were purchased from Merck (Darmstadt, Germany)
- Dichloromethane were purchased from Fisher Chemical (UK)
- Ethanol were purchased from Merck (Darmstadt, Germany)
- Chloroform were purchased from Merck (Darmstadt, Germany)
- Hexane were purchased from Merck (Darmstadt, Germany)
- Sulfuric acid were purchased from PHARMCO (USA)
- Potassium Iodide were purchased from Merck (Darmstadt, Germany)
- Magnesium were purchased from Merck (Darmstadt, Germany)
- Picric Acid were purchased from Merck (Darmstadt, Germany)
- Sodium Hydroxide were purchased from Merck (Darmstadt, Germany)
- Potassium hydroxide were purchased from Merck (Darmstadt, Germany)
- Ammonia Solution 25% were purchased from Isolab Chemicals (USA)
- Phosphoric acid 85% were purchased from Merck (Darmstadt, Germany)
- Triethanolamine were purchased from Merck (Darmstadt, Germany)
- HPLC-grade methanol and acetonitrile were purchased from Merck (Darmstadt, Germany)
- Ethyl Acetate for analysis from Merck (Darmstadt, Germany)

- Formic Acid for analysis from Merck (Darmstadt, Germany)
- Glacial Acetic Acid for analysis from Merck (Darmstadt, Germany)
- Methanol for analysis from Merck (Darmstadt, Germany)
- Silica gel 60 F254 plates from Merck (Darmstadt, Germany) were used in the separation by thin layer chromatography of phenolic compounds (preparative method)
- Type II HPLC-Water

### 4.1.2 Standards

- (+)-Catechin hydrate from $\geq$ 98 % Sigma Aldrich (USA)
- Quercetin dihydrate $\geq$ 98 % from Sigma Aldrich (USA)

### 4.1.3 Equipment

- HPLC (High Performance Liquid Chromatography) Hitachi Chromaster VWR 1549043
- Vacuum pump Boeco R-300/AGTA047
- Karl Fischer automatic titrator METROHM 915/1915001017174
- Rotary Evaporator ISOLAB 605.01.001/XZ173AD0000092
- Ultrasonic Shaker VWR Scientific Products 75T
- UV Chamber CHROMATO CC-10
- Stove UMCO
- Glass thermometer ALLA FRANCE
- Chromatographic Column 150-4,6 THERMO SCIENTIFIC 10611115
- Chronometer MONTERO
- Electronic Automatic Pipette Brand HandyStep electronic/17ESS612
- Analytical balance Sartorius BP210S/70804614
- Precision balance Mettler Toledo ME 2002/B712853703
- Spectrophotometer Merck PHARO 300/1031120936
- pH meter Mettler Toledo Seven Easy S20 K/1231405242

- Extraction hood WEMA LABOR 62000/12468
- Moisture analyzer Mettler Toledo HB43-S/B205650467
- Conductimeter Mettler Toledo Seven Compact S230/B321347891

## 4.2 Powder Preparation

The yellow mashua tubers were provided by Central Market (Ambato, Ecuador) in August 2019. These tubers were subsequently submitted to the evaporation process in an industrial stove (reducing its size considerably). Then, they were turned into powder. This process was made to decrease considerably the amount of water that contains (79-94 %), avoid enzymatic degradation of compounds such as glycosides, and facilitate the extraction process [1]. The drying process is directly proportional to extraction times; thus, when the drying becomes optimal, the extraction times are optimal, requiring less solvent in each step. However, due to the small and limited amount of powder that could be obtained during this process and with the knowledge that prepared mashua powder exists on the market, it was necessary to get it to start with the execution of several essays. In mashua powder, several tests such as moisture percentage and water content (Karl Fisher titration) were made to monitor the status of the sample before the development of this study (see in Figures 4.1 and 4.2). The results of these tests were 6.95% and 6.63%, respectively The powder was stored at 22° C in a hermetic container [17, 18].


Figure 4.1: Analysis of Moisture in Mashua Powder.

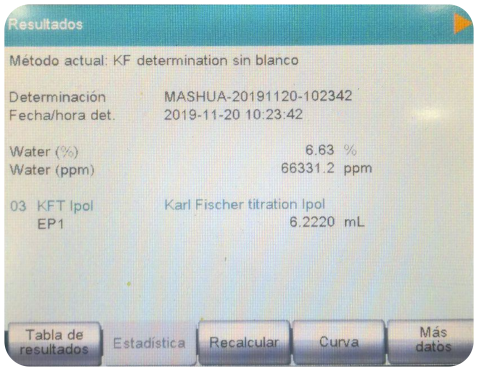


Figure 4.2: Analysis of Mashua Powder Water Content (Karl Fischer titration).

## 4.3 Extraction Process

Despite the herbal plant's complexity, the choice of solvent chosen is related to the type of phenolic compounds extracted. The choice of this solvent is based on physicochemical properties such as polarity and solubility. In Figure 4.3, we can see some solvents used in the extraction of medicinal plant compounds; each solvent has on its right side the value of the dielectric constant, related to its polar or apolar character [8, 16, 17, 57–65]. Likewise, we can observe that polar solvents such as methanol extract polar compounds such as phenolic compounds. In contrast, non-polar solvents such as hexane will extract non-polar compounds such as fats. Annex 7.1 and 7.2 show additional information about polarity of the solvents.

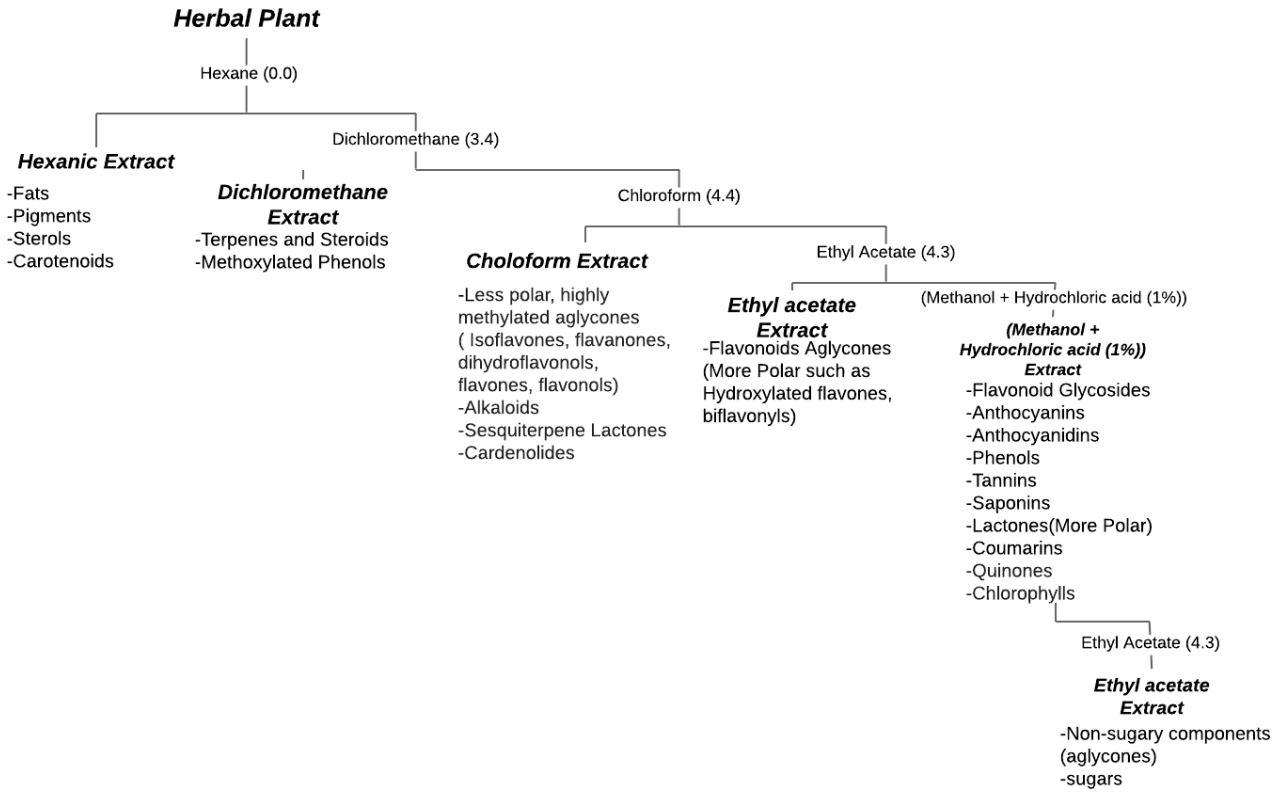


Figure 4.3: Solvent election diagram for extraction process of several natural compounds based on solvent polarity [8, 16, 17, 57–65].

Ethanolic extracts are commonly used in analytical research methods [45]. Due to their polarity, this solvent recovers a high amount of interest compounds such as alkaloids, flavonoids, and anthocyanidins. However, on many occasions, these compounds are present as glycosides, making their structural determination difficult with the available analytical methods. Hydrolysis is a process that simplifies a sample by separating the sugars from the compounds of interest (see Figure 4.4).

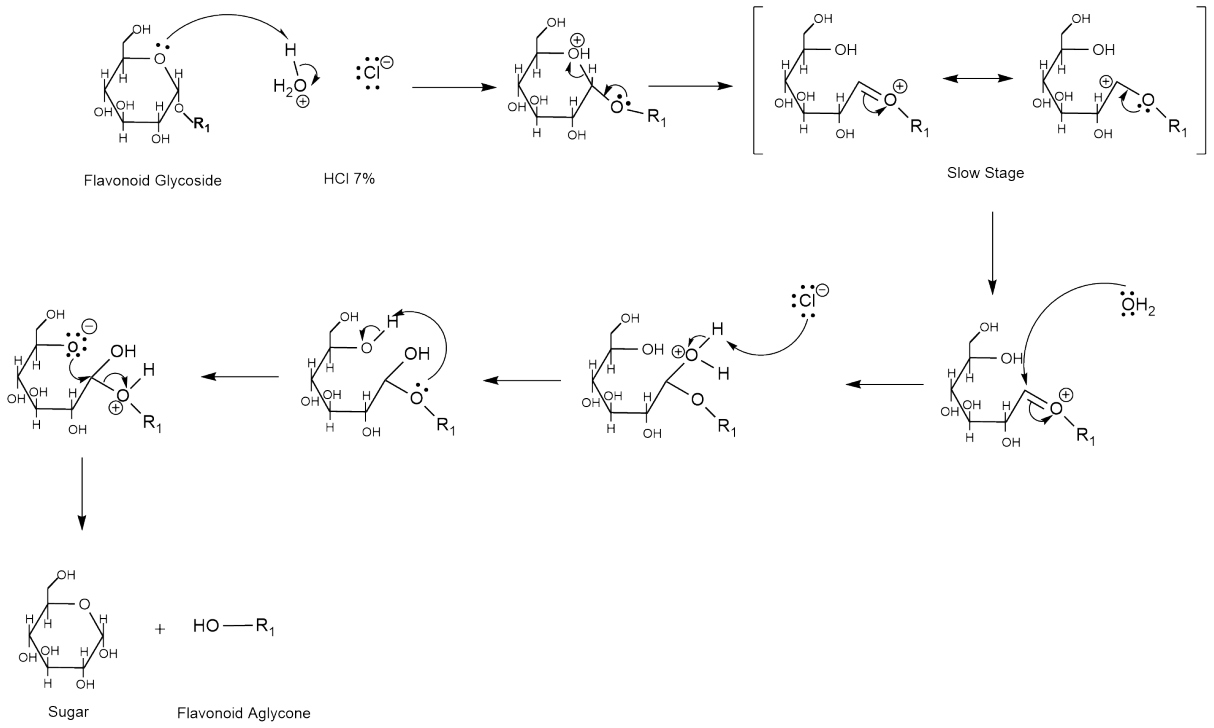


Figure 4.4: Mechanism of Acid Hydrolysis that occurs in the Flavonoid Glycosides.

## 4.4 Maceration

Maceration is the most basic process used by the ancestors to create herbal remedies. It is based on placing a large amount of plant material suspended in solvents, such as alcohol, oils, vinegar, honey, or water for long periods (weeks and even months). Maceration is considered a type of infusion because it can be done to any plant, but its main characteristic is that it does not contact heat. Some macerations have been used directly as cosmetics (tinctures, hair treatments), while others are used as medicines (antiseptics, antifungals) [66]. So, to obtain as much information as possible, it proceeds to realize six different extractions with 200 grams of mashua powder and 500 mL of each solvent (different solvents or concentration of the same solvents). For analytical purposes, approximately 200 g of mashua powder was placed in a 500 mL Erlenmeyer flask, 500 mL of the solvent was added (to macerate and realize a solid-liquid extraction) and stirred with a glass rod to homogenize the powder with the solvent. The flask was sealed with foil to avoid impurities and was placed in an ultrasonic shaker for 15 min. Then, it was kept in the dark. It is important to note that it is necessary to allow to macerate every procedure of extraction at least 24 hours. The supernatant was collected, filtered three times (cotton, sterile gauze, and filter paper), and placed in a 500 mL beaker. The graphycal procedure of the extraction is established in Annex 7.3.

So, extraction with Ethanol 70% and one with Hexane was carried out. After these extractions, was realized, a new extraction with a mixture of 150 mL of EtOH 96% and 1.5 mL HCl 7% in the same Erlenmeyer (to prove if it is possible to extract more compounds after the extractions mentioned above and likewise to see if an acidic environment affects the second extraction). These last extractions are called $2^{nd}$ Ethanolic 70% Extract and $2^{nd}$ Hexanic Extract.

## 4.5 Phytochemical Screening: Chemical Tests

Phytochemical screening of mashua powder is focused on realize the chemical tests mentioned in this section using different types of extracts.

### 4.5.1 Ferric Chloride (5%) Test (Polyphenols)

In a test tube, put 2 mL of ethanolic solution (extract) and add a few drops of Solution A. Then, perform the same experiment with Solution B. Blue, green, or violet colors suggest a positive test [19, 67]. Note: Solution A (Dissolve 2.5 g Ferric Chloride ($FeCl_3$) and dilute to the mark in a 50 mL volumetric ask with distilled water) and Solution B (Dissolve 2.5 g Ferric Chloride ($FeCl_3$) and dilute to the mark in a 50 mL volumetric ask with Ethanol).

### 4.5.2 Rosemhein Test (Anthocyanidins)

In a test tube, put 1 mL of alcoholic solution (extract), add 1 mL of concentrated HCl, and heat for 10 minutes in a water bath. Then, add 5 mL of water and 10 mL of 1-butanol, shake and allow to rest until cool. The test is positive when 2 phases appear with colors such as red to brown, orange, or red bluish [68].

### 4.5.3 Wagner Test (Alkaloids)

Wagner Reagent: Dissolve 1.27 g resublimated iodine ($I_2$) and 2 g potassium iodide (KI) and dilute to the mark in a 100 mL volumetric flask with distilled water [19, 67].
In acidic solutions of the extract, when adding a few drops of Wagner reagent, a brown precipitate suggests the presence of alkaloids [19, 67].

#### 4.5.3.1 Confirmation of Wagner test

The most common chemical tests used to demonstrate alkaloids within Ethanolic extract (70%) are Wagner, Dragendorff, and Mayer tests; however, due to some reagents' inexistence, only the Wagner test was performed, generating a positive test. In this way, to corroborate this, we proceed to discard a false-positive result since Wagner's test reacts to the presence of nitrogenous compounds. So, this result

could be related to proteins or fertilizers such as urea, generally used to treat this type of crop. For this purpose, approximately 100 g of mashua powder was placed in a 250 mL Erlenmeyer flask. It was then added about 150 mL of chloroform, stirring carefully with a glass rod to homogenize the powder with the solvent. Maceration requires approximately 96 hours. After that, with the help of a dropper, we added 15 drops of ammonia solution (25%) in the Erlenmeyer, let stand for 5 minutes in the ultrasonic shaker, and filter three times (gauze, cotton, and filter paper). After that, we put 5 mL of the content of the Vacuum flask in a 25 mL separation funnel, let to stand for 5 minutes, and two phases (aqueous and organic) were observed. If these phases will not clearly be observed, it was necessary add 2 mL of purified water in the separation funnel, shake and wait a few minutes; the chloroform fraction (bottom) was collected in a 25 mL beaker to perform the Wagner test. In this case, the Wagner test shows a positive test generating a dark brown precipitate at the bottom of the test tube (see Annex 7.4), verifying the previous result. Adding an ammonia solution (25%) in the chloroform extract, the acidic form of the raw material or extract (due to flavonoids) breaks and becomes a free base. In the case of a protein or urea, these compounds will move to the aqueous phase (solubility), while the organic phase will be free of these possible compounds to avoid any interference in the test.

### 4.5.4 Borntrager Test (Quinones)

In a test tube, put 1 mL of chloroform solution (extract) and 1 mL of potassium hydroxide (KOH (5%)), shake to mix the phases, and allow to rest for a few minutes. If the color of the alkaline phase (upper) turns pink or red, a positive test is suggested [19, 67].

### 4.5.5 Resin Test

In a test tube, put 2 mL of alcoholic solution (extract) and 10 mL of distilled water. The presence of a precipitate suggests a positive test [67].

### 4.5.6 Shinoda Test (Flavonoids)

In a test tube, put 2 mL of alcoholic solution (extract), add small magnesium pieces, and a few drops of concentrated hydrochloric acid (HCl). Allow that reaction take place (5 minutes); then, add 4 mL of amyl alcohol or chloroform, shake and allow to rest for a few minutes. In a positive test, the amyl alcohol phase or chloroform phase appears with colors such as orange, pink, red, or purple [19, 67].

### 4.5.7 Baljet Test (Sesquiterpene Lactones and Cardiac Glycosides)

In a test tube, put 2 mL of ethanolic solution (extract), add a few drops of Bajlet reagent. The test is positive when the color of the sample turns orange or red. Baljet reagent mix Solution A and B (1:1)

[19, 67]. Note: Solution A: Dissolve 1 g picric acid and dilute to the mark in a 100 mL volumetric flask with ethanol and Solution B: Dissolve 2.5 g sodium hydroxide (NaOH) and dilute to the mark in a 25 mL volumetric flask with ethanol.

### 4.5.8 Liebermann–Burchard Test (Sterols)

In a test tube, mix 1 mL of chloroform and 1 mL of anhydrous acetic acid, cool to 0°C and add one or two drops of concentrated sulfuric acid. Add cloroform extract and observe. In a positive test, the color turns blue, green, red, or orange [19, 67].

### 4.5.9 Foam Test (Saponins)

In a test tube, put 1 mL of alcoholic solution (extract) and 5 mL of distilled water, shake vigorously during 2-3 minutes and observe. The test suggests a positive test when foam persists for more than 2 minutes [67].

### 4.5.10 Salkowski Reaction (Sterols)

In a test tube, dissolve 2 mg of the sample (powder) in 1 mL of chloroform and add 1 mL of concentrated sulfuric acid, forming two phases. Red or yellow color indicates the presence of sterols and methylated sterols [19].

## 4.6 Mobile Phase of Preparative TLC, pre-treatment of the sample (Distillation process) and Spectra for purified Fraction

Each experiment performed to find the correct TLC mobile phase requires to know that ethanolic extract (70%) contain water in their composition. In this way, it was necessary to evaporate to dryness the extract to avoid "candles" (usually 2 mL of the ethanolic extract (70%)(test tube) in a water bath) before "sowing the sample" in the baseline of TLC.

Table 4.1: Mobile Phase-A17 for Preparative TLC.

| ***Dichloromethane*** | ***Ethyl acetate*** | ***Glacial Acetic Acid*** | ***Methanol*** |
|---|---|---|---|
| 75 mL | 10 mL | 5 mL | 10 mL |
| 18.75 mL | 2.5 mL | 1.25 mL | 2.5 mL |

For analytical purposes, approximately 100 g of mashua powder was placed in a 500 mL Erlenmeyer

flask (168.18 g), 500 mL of ethanol 70% was added (to perform a solid-liquid extraction) and stirred with a glass rod to homogenize the powder with the solvent. The flask was sealed with foil to avoid impurities and was placed in an ultrasonic shaker for 15 min; then, it was kept in the dark for 24 hours. The supernatant was collected, filtered three times (cotton, sterile gauze, and filter paper), and placed in a 500 mL balloon flask(295.10 g), obtaining 274.71 g (approximately 275 mL) of extract. The balloon flask was placed in simple distillation equipment, and after around 30 minutes, the distillation process started. The sample's alcohol content was distilled (this process takes about 3 hours), obtaining 81.93 g of the aqueous fraction. At the final of the process, some bubbles and several lumps could be appreciated (critical point). This phenomenon may be associated with water-insoluble compounds such as tannins. Furthermore, it is essential to take into account that lumps exhibited a dark brown color, which indicates that compounds may have been exposed to oxidation due to high temperatures in the distillation process. This parameter could not be controlled because it was necessary to reach the boiling point of alcohol (78.37°C) to start the process). In this way, the temperature reached was approximately 73.5°C. After that, we place the balloon flask contents in a 250 mL separation funnel, add 60 mL of ethyl acetate, mix vigorously, and let stand for 15 minutes until two phases(aqueous and organic) were separated. The organic phase (upper) was collected in a 400 mL beaker. A small TLC was performed with the A17 mobile phase (small chamber) to corroborate the separation of the components (initial test to proceed with the second distillation process). So, five washes with ethyl acetate were performed on the extract (20, 20, 20, 10, and 10 mL respectively), each one less intense than the previous one (see Annex 7.6); organic fractions were collected in the same 400 mL beaker (bulk). The organic phase (bulk-107.07 g) was placed in a 250 mL balloon flask (121.41 g) and put it in the simple distillation equipment (see Annex 7.7). After 1.5 hours, the extract has reached a critical point when present a caramelized aspect. The sample was reconstituted with 6-7 mL of ethyl acetate and place the contents of the glass ballon in a 25 mL beaker. Then, "sow in-band" the sample in the preparative TLC baseline with the help of a glass capillary and using the correct mobile phase (A17) in the chamber. After the separation (see Annex 7.7), we scrape the different fractions, and the silica powder is placed in a 25 mL beaker. It was reconstituted with 5 mL of methanol, and the beaker was placed in an ultrasonic shaker for 10 minutes. Then, the content was filtered two times with a syringe and a 0.45 um filter. Spectrophotometric scanning was performed with methanol as a blank.

## 4.7 Pre-Treatment of Extract (Rotary Evaporator), Preparative TLC and Spectra for purified Fractions

For analytical purposes, approximately 100 g (100.03 g) of mashua powder was placed in a 500 mL Erlenmeyer flask (168.19 g), 500 mL of ethanol 70% was added (to perform a solid-liquid extraction) and stirred carefully with a glass rod to homogenize the powder with the solvent. The flask was sealed

with foil to avoid impurities and was placed in an ultrasonic shaker for 15 minutes; then, it was kept in the dark for 1 hour. The supernatant was collected, filtered three times (cotton, sterile gauze, and filter paper), and placed in a 500 mL beaker obtaining approximately 275 mL of extract. The extract was placed in a rotary evaporator using the equipment's vacuum system to evaporate to dryness; so, the speed and temperature conditions (25 RPM and 50°C respectively) were adjusted to start the process. After 30 minutes, ethanol was gentle removal, leaving only an aqueous fraction inside the glass ballon. The glass balloon was moved away from the rotary evaporator with the water bath. The temperature was set to 20°C (the aqueous fraction do not evaporate); so, using the vacuum system of the rotary evaporator again, we add 20 mL of ethyl acetate inside the glass ballon to "wash" the aqueous fraction (the temperature was controlled so that ethyl acetate does not evaporate during this procedure). Then, add 20 mL of ethyl acetate and wash again for five minutes, maintaining the glass balloon in the vapor bath with lower temperature (25°C). It is necessary to maintain the same rotation speed to recover the sample of the ballon (this step was performed without pressure to do not evaporate the solvent). Then, we place the contents of the glass balloon(aqueous phase+ethyl acetate) in a 100 mL separation funnel, mix and let to stand until two phases were observed; so, the different phases were collected in separate 50 mL beakers. A new "wash" was then executed in the sample with 10 mL of ethyl acetate in the separation funnel, and the different phases collected. Organic phases (bulk) were placed back in the rotary evaporator to evaporate to dryness (see Annex 7.8). The sample was reconstituted with 7 mL of ethyl acetate (to wash the ballon) and put the contents in a 25 mL beaker.

We realize a preparative TLC using the correct mobile phase and put it in the chamber (which was conditioned for 1 hour). After the separation (see Annex 7.9), we scrape the different fractions; the silica powder was placed in a 25 mL beaker, it was reconstituted with 5 mL of methanol, and the beaker was allowed to stand in an ultrasonic shaker for 10 minutes. Then, the content was filtered two times with a 0.22 um filter. Spectrophotometric scanning was performed with the blank (methanol). The same sample analyzed on the spectrum was analyzed in HPLC with a UV-Vis detector to have more information on the extract. To the remaining aqueous phase collected previously, add 5 mL of HCl (7%) or HCl (3 M) and let to stand in an ultrasonic shaker for 15 minutes. Using the correct mobile phase, perform a preparative TLC as in the previous assay. This phase did not show a separation, possibly due to its water content(see Annex 7.10).

## 4.8 Pre-treatment of extract to RP-HPLC Analysis

For analytical purposes, approximately 100 g (100.03 g) of mashua powder was placed in a 500 mL Erlenmeyer flask (168.19 g), 500 mL of ethanol 70% was added (to perform a solid-liquid extraction) and stirred carefully with a glass rod to homogenize the powder with the solvent. The flask was sealed with

foil to avoid impurities and was placed in an ultrasonic shaker for 15 minutes; then, it was kept in the dark for 12-14 hours to macerate. The supernatant was collected, filtered three times (cotton, sterile gauze, and filter paper), and placed in a 500 mL beaker obtaining approximately 275 mL of extract. The extract was placed in a rotary evaporator using the equipment's vacuum system until the alcohol is distilled; so, the speed and temperature conditions (25 RPM and 40°C respectively) were adjusted to start the process. After 30 minutes, ethanol was gentle removal, leaving only an aqueous fraction inside the glass ballon. The glass balloon was moved away from the rotary evaporator with the water bath. The temperature was set to 20°C (the aqueous fraction do not evaporate). Using the vacuum system of the rotary evaporator again, we add 15 mL of ethyl acetate inside the glass ballon to "wash" the aqueous fraction (the temperature was controlled so that ethyl acetate does not evaporate during this procedure). Then, we place the contents of the glass balloon(aqueous phase+ethyl acetate) in a 100 mL separation funnel, mix and let to stand until two phases were observed; so, the organic phase was collected in a 50 mL beaker and placed in a glass ballon of the rotary evaporator. Then, a new "wash" was performed with 15 mL of ethyl acetate in the sample using the equipment's vacuum system, and in the same way, after 2 minutes, add 20 mL of HCl (2 N) to perform the hydrolysis of the extract during 20 minutes. Then, we place the glass balloon's contents in a 100 mL separation funnel, add 30 mL of ethyl acetate, mix, and let to stand until two phases were observed. The organic phase was collected and evaporated to dryness in a rotary evaporator and reconstituted with acetonitrile with 5 mL.

## 4.9 HPLC Conditions

### 4.9.1 HPLC Conditions analysis of Preparative TLC Fractions

A HPLC method was developed in this work to analize the preparative TLC fractions. The binary system involves an isocratic elution system with a mobile phase consisted of buffer solution pH 3.180 with acidified water (0.1 % $CH_3COOH$, v/v) as Solvent A and methanol as Solvent B in a relation (70/30, v/v) during 30 minutes. A Thermofisher RP18 (5 $\mu$m, 150 mm x 4.6 mm) column was used, and the temperature was set up to 40°C, with a flow rate of 1.2 mL/min and an injection volume of 30 uL (samples). This information is summarized in Table 4.2. Samples and mobile phases were filtered through a 0.22 $\mu$m Millipore filter before HPLC injection. The purified mashua extracts (preparative TLC fractions) were separated using a Hitachi equipment (RP Chromatography) equipped with an autoinjector, a UV-Vis detector, and Clarity software. Spectral data were recorded at 275 nm during the whole run.

Table 4.2: HPLC Conditions analysis of Preparative TLC Fractions

| | |
|---|---|
| Equipment (HPLC) | Hitachi Chromaster consisting of UV-VIS detector, autosampler, oven and Clarity software |
| Stationary Phase | Column THERMO L1 (C18) (150 mm x 4,6 mm and 5 $\mu$m (C18)) |
| Mobile Phase | Buffer solution pH 3.180 with acidified water (0.1 % $CH_3COOH$, v/v) as Solvent A<br>Methanol as Solvent B in a relation<br>(70/30, v/v) |
| Flow | 1.2 mL/min |
| Wavelength | 275 nm |
| Injection Volume | 10 uL (Uracil)<br>30 uL (Samples) |
| Temperature | 40°C |
| Time | 30 minutes |

### 4.9.2 HPLC Conditions for analysis of Hydrolized Mashua Extract and Standards

A HPLC method was developed in this work to analize the hydrolized mashua extract and standards. The system involves a gradient elution system with a mobile phase consisted of buffer solution pH 2.5 with water (0.01 M, $H_3PO_4$) as Solvent A, methanol as Solvent B, and Acetonitrile as Solvent C during 25 minutes. A Thermofisher RP18 (5 $\mu$m, 150mm x 4.6 mm) column was used and the temperature was set up to 30°C, with a gradient flow rate and an injection volume of 30 uL(samples) and 6uL (standard). This information is summarized in Table 4.3. Samples and mobile phases were filtered through a 0.22 $\mu$m Millipore filter prior to HPLC injection. The pretreatment mashua extract (hydrolyzed extract) was separated using a Hitachi equipment (RP Chromatography) equipped with an autoinjector, a UV-Vis detector, and Clarity software. Spectral data were recorded at 280 nm during the whole run.

Table 4.3: HPLC Conditions for analysis of Hydrolized Mashua Extract and Standards

| | |
|---|---|
| Equipment (HPLC) | Hitachi Chromaster consisting of UV-VIS detector, autosampler, oven and Clarity software |
| Stationary Phase | Column THERMO L1 (C18) (150 mm x 4,6 mm and 5 $\mu$m (C18)) |
| Mobile Phase | Buffer solution pH 2.5 with water (0.01 M, $H_3PO_4$)<br>85%Buffer,5%MeOH,10%ACN————0-7min<br>50%Buffer,22.5%MeOH,27.5%ACN——— 7.1-17min<br>85%Buffer,5%MeOH,10%ACN————17.1-25min |
| Flow | 0.8 mL/min————0-7min<br>1.2 mL/min————7.1-17min<br>0.8 mL/min————17.1-25min |
| Wavelength | 280 nm |
| Injection Volume | 10 uL (Uracil and Standard)<br>30 uL (Sample) |
| Temperature | 30°C |
| Time | 25 minutes |

# 5. Results, interpretation, and discussion

## 5.1 Phytochemical Screening Analysis

### 5.1.1 Preliminary results of qualitative phytochemical screening for Mashua Powder

The Chemical Tests suggested in Section 4.2.3 were applied to Ethanolic Extract (70%), Hexanic Extract, $2^{nd}$ Hexanic Extract and $2^{nd}$ Ethanol 70% Extract.

In the Test **A** (Ferric Chloride 5% Test (Polyphenols)), we can see three test tubes (first test tube: contain 2 mL of Ethanolic extract(70%), second test tube: contain 2 mL of Ethanol extract(70%) + 5 drops of Solution C, third test tube: contain 2 mL of Ethanol extract (70%) + 4 drops of Solution D), from left to right; so, second and third test tube presents a change of color that goes from reddish-brown to olive green (with some turbidity), which suggest a positive test (see Figure 5.1).

In the Test **B** (Rosemhein Test (Anthocyanidins)), we can see two test tubes (first test tube: contain 3 mL of Ethanolic extract (70%), second test tube: development of the test), from left to right; in the second test tube, we can see the presence of two phases with a change of color that goes from reddish-brown to brown, which suggest a positive test (see Figure 5.1).

In the Test **C** (Wagner Test (Alkaloids)), we can see three test tubes (first test tube: contain 2 mL of Ethanolic extract (70%), second test tube: evaporated Ethanolic extract (70%) + 2 mL of HCl (7%) + 8 drops of Wagner reagent, third test tube: evaporated Ethanolic extract (70%) + 2 mL of HCl (7%) + 15 drops of Wagner reagent, from left to right. In the second and third test tube, we can see brown precipitate at the bottom of the test tube, which suggests a positive test (see Figure 5.1).

In the Test **D** (Borntrager Test (Quinones)), we can see two test tubes (first test tube: evaporated Ethanolic extract (70%) + approximately 1 mL Chloroform (to reconstitute), second test tube: development of the test), from left to right; in the second test tube, we can see the presence of two phases but does not exist a change of color (pink or red), which suggests a negative test (see Figure 5.1).

In the Test **E** (Resin Test), we can see one test tube which contains 2 mL of Ethanolic extract (70%) + 10mL of distilled water; there is no presence of precipitate in the test tube, which suggests a negative test (see Figure 5.1).

In the Test **F** (Shinoda Test(Flavonoids)), we can see two test tubes (first test tube: contain 2 mL of Ethanolic extract (70%), second test tube: development of the test), from left to right. In the second test, the tube is observed the presence of two phases (the upper phase (reddish) and the bottom phase (orange)). So, the second test tube suggests a positive test (see Figure 5.1).

In the Test **G** (Baljet Test (Sesquiterpene Lactones and Cardiac Glycosides)), we can see two test tubes (first test tube: contain 2.5 mL of Ethanolic extract(70%), second test tube: development of test), from left to right. The second test tube experiment a change of color, which suggests a positive test (see Figure 5.1).

In the Test **H** (Liebermann–Burchard Test(Sterols)), we can see two test tubes (first test tube: evaporated Ethanolic extract (70%) + approximately 1.5 mL of chloroform, second test tube: development of the test), from left to right. In the second test tube, it is essential to note that when adding approximately three drops of sulfuric acid, the sample experiment changed color that goes from reddish-brown to brown (with some turbidity), as you can see at the bottom of the test tube. However, adding extra sulfuric acid, we can observe 2 phases due to the difference in their densities. So, the second test tube suggests a negative test (see Figure 5.1).

In the Test **I** (Foam Test (Saponins)), we can see two test tubes (first test tube: contain 1 mL of Ethanolic extract (70%) + 5 mL of distilled water, second test tube: development of the test), from left to right. The foam of the second test tube is kept after 2 minutes, which suggests a positive test (see Figure 5.1).

In the Test **J** (Salkowski Reaction (Sterols)), we can see a test tube with the development of the test; in this way, the presence of 2 phases is observed (the upper phase (transparent) and the bottom phase (red, but after a few minutes acquire a brown color); so, this suggest a positive test. It is essential to take into account that this chemical test is used as a confirmation of the Liebermann–Burchard Test (see Figure 5.1).

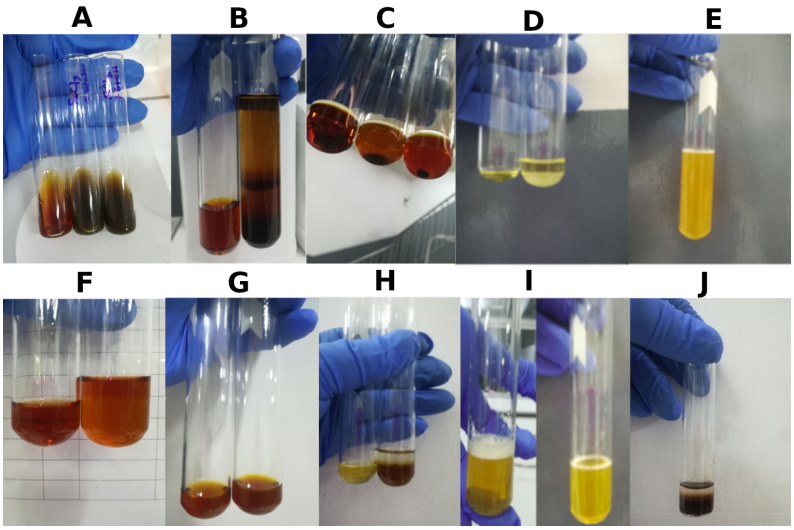


Figure 5.1: Qualitative Results of Chemical Test: Ethanol extract

(A): Ehanolic Extract ($1^{st}$ test tube), Ferric Chloride (5%) Test in water ($2^{nd}$ test tube) and Ferric Chloride (5%) Test in ethanol ($3^{rd}$ test tube); (B): Ehanolic Extract ($1^{st}$ test tube) and Rosemhein Test($2^{nd}$ test tube); (C): Ehanolic Extract ($1^{st}$ test tube) and Wagner Test ($2^{nd}$ and $3^{rd}$ test tubes); (D): Ehanolic Extract ($1^{st}$ test tube) and Borntrager Test ($2^{nd}$ test tube); (E): Ehanolic Extract ($1^{st}$ test tube) and Resin Test ($2^{nd}$ test tube); (F): Ehanolic Extract ($1^{st}$ test tube) and Shinoda Test ($2^{nd}$ test tube); (G): Ehanolic Extract ($1^{st}$ test tube) and Baljet Test ($2^{nd}$ test tube); (H): Evaporated Ethanolic extract (70%) + approximately 1.5 mL of chloroform ($1^{st}$ test tube) and Liebermann-Burchard Test ($2^{nd}$ test tube) ; (I): Ehanolic Extract ($1^{st}$ test tube) and Foam Test ($2^{nd}$ test tube); (J): Salkowski Reaction

The Chemical Tests suggested in Section 4.2.3 were applied to Hexanic Extract, $2^{nd}$ Hexanic Extract and $2^{nd}$ Ethanolic Extract 70%.

In the Test **A** (Ferric Chloride 5% Test (Polyphenols)), we can see three test tubes, each one contains 2 mL of each extract (Hexanic Extract, $2^{nd}$ Hexanic Extract and $2^{nd}$ Ethanolic Extract 70%, from left to right) + 3 drops of Solution C (it is possible to use solution D), from left to right. So, the first test-tube does not present a change of color, while the second and third test tubes present a significant change of color to olive green color but with less intensity than in the Ethanolic 70% Extract. In this way, the first test-tube suggest a negative test, while the second and third test tubes suggest a positive test (see Figure 5.2).

In the Test **B** (Rosemhein Test (Anthocyanidins)), we can see three test tubes, each one contains 2 mL of each extract (Hexanic Extract, $2^{nd}$ Hexanic Extract and $2^{nd}$ Ethanolic Extract 70%, from left to right). In each case, the test was developed; so, all of them showing the presence of two phases. The first test-tube does not present the color that suggests a positive test, while the second and third test tubes present a significant change of color to brown, which suggests a positive test (see Figure 5.2).

In the Test **C** (Wagner Test (Alkaloids)), we can see three test tubes; each one contains 2 mL of each extract (Hexanic Extract, $2^{nd}$ Hexanic Extract and $2^{nd}$ Ethanolic Extract 70%, from left to right). In each case, the test was developed. The first test-tube experience a change of color in one of its phases,

but this color does not suggest a positive test. Second and third test tube experience a brown precipitate after a few minutes visualized on the walls and at the bottom of the test tube, which suggest in both cases a positive test (see Figure 5.2).

In the Test **D** (Borntrager Test (Quinones)), we can see three test tubes. Each one contains 2 mL of each extract (Hexanic Extract, $2^{nd}$ Hexanic Extract and $2^{nd}$ Ethanolic Extract 70%, from left to right). In each case, the test was developed. So, all of them experience two phases; however, it does not exist a change of color(pink or red), which suggests a negative test (see Figure 5.2).

In the Test **E** (Resin Test), we can see three test tubes; each one contains 2 mL of each extract (Hexanic Extract, $2^{nd}$ Hexanic Extract and $2^{nd}$ Ethanolic Extract 70%, from left to right). In each case, the test was developed, so all of them do not experience the presence of precipitate, which suggests a negative test (see Figure 5.2).

In the Test **F** (Shinoda Test (Flavonoids)), we can see three test tubes; each one contains 2 mL of each extract (Hexanic Extract, $2^{nd}$ Hexanic Extract and $2^{nd}$ Ethanolic Extract 70%, from left to right). In each case, the test was developed. So, the first test-tube do not show any change, the second test, we can see two phases (the upper phase (reddish-orange) and the bottom phase (orange)), and in the third test tube, we can see two phases(the upper phase (red) and the bottom phase (orange)). The first test-tube suggests a negative test, while the second and third test tubes suggest a positive test (see Figure 5.2).

In the Test **G** (Baljet Test (Sesquiterpene Lactones and Cardiac Glycosides)), we can see three test tubes, each one contains 2 mL of each extract ( Hexanic Extract, $2^{nd}$ Hexanic Extract and $2^{nd}$ Ethanolic Extract 70%, from left to right). In each case, the test was developed. The first test-tube does not experience a change of color while the second and third present a change of color that goes from yellow to orange. The first test-tube suggests a negative test, while the second and third test tubes suggest a positive test (see Figure 5.2).

In the Test **H** (Liebermann–Burchard Test (Sterols)), we can see three test tubes, each one contains 2 mL of each extract (Hexanic Extract, $2^{nd}$ Hexanic Extract and $2^{nd}$ Ethanolic Extract 70%, from left to right). In each case, the test was developed (1-2 drops of sulfuric acid). The first test-tube experiences a change of color that goes from yellow to green; the second test tube experience a change of color that goes from light brown to greenish-brown; and the third test tube does not show a change of color. The first and second test tube suggests a positive test, while third test tubes suggest a negative test (see Figure 5.2).

In the Test **I** (Foam Test (Saponins)), we can see three test tubes, each one contains 2 mL of each

extract (Hexanic Extract, $2^{nd}$ Hexanic Extract and $2^{nd}$ Ethanolic Extract 70%, from left to right). In each case, the test was developed; so, in all of them, the foam is not kept after 2 minutes, which suggests a negative test (see Figure 5.2).

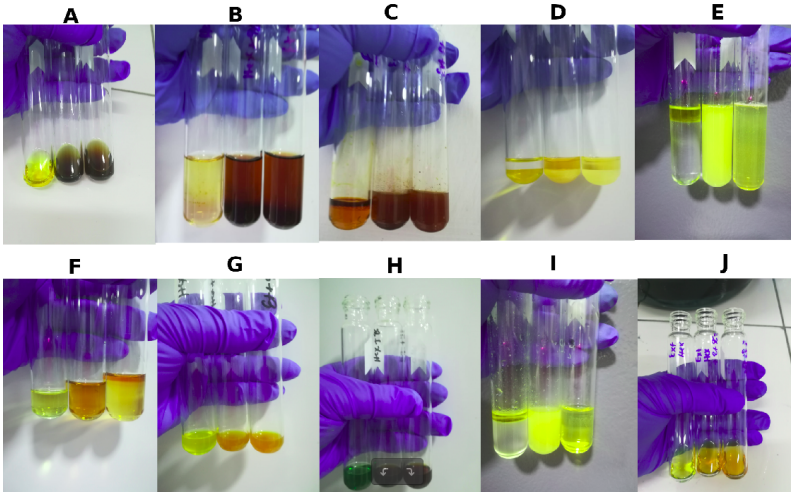


Figure 5.2: Results of different Chemical Tests in Hexanic Extract ($1^{st}$ test tube), $2^{nd}$ Hexanic Extract ($2^{nd}$ test tube) and $2^{nd}$ Ethanolic Extract 70% ($3^{rd}$ test tube)

(A): Ferric Chloride (5%) Test in water; (B): Rosemhein Test; (C): Wagner Test; (D): Borntrager Test; (E): Resin Test; (F): Shinoda Test; (G): Baljet Test; (H): Liebermann-Burchard Test; (I): Foam Test;(J): Comparison Extracts

The results displayed in Figures 5.1 and 5.2 are summarized in Table 5.1. So, ten tests were performed and symbolized by the letters A-J. In this way, taking into account the conditions explained in Section 4.2.3 to consider a positive test (change of color, presence of precipitate or visualization of 2 phases), it proceeds to establish a qualitative result: Abundant(+++), Moderate(++), Slight(+), Null(-).

Table 5.1: Qualitative Results of different Chemical Tests in Ethanolic Extract 70%, Hexanic Extract, $2^{nd}$ Hexanic Extract and $2^{nd}$ Ethanolic Extract 70%

| ***Chemical Test*** | ***Ethanol Extract 70%*** | ***Hexanic Extract*** | $2^{nd}$ ***Hexanic Extract*** | $2^{nd}$ ***Ethanol 70% Extract*** |
|---|---|---|---|---|
| A.-Ferric Chloride(5%) | +++ | - | ++ | ++ |
| B.-Rosemhein | +++ | - | ++ | ++ |
| C.-Wagner | +++ | - | + | + |
| D.-Borntrager | - | - | - | - |
| E.-Resin | - | - | - | - |
| F.-Shinoda | +++ | - | + | + |
| G.-Baljet | - | - | ++ | ++ |
| H.-Liebermann–Burchard | - | +++ | - | - |
| I.-Foam | + | - | - | - |
| J.-Salkowski | + | N/A | N/A | N/A |

Ethanol 70% Extract. (Ethanolic 70% Extract), Hexane Extract (Hexanic extract), $2^{nd}$ Hexane Extract (Hexanic Extract (evaporated) + EtOH 96% (reconstituted)), $2^{nd}$ Ethanol 70% Extract (EtOH 70% Extract (evaporated) + Ethanol 96% (reconstituted))

The Chemical Tests suggested in Section 4.2.3 were applied to Choloroform and Dichloromethane Extract.

In the Test **A** (Ferric Chloride 5% Test (Polyphenols)), we can see two test tubes (first test tube: contain 2 mL of a chloroform extract (evaporated) + 0.75 mL of alcohol + 3-5 drops of Solution C, second test tube: contain 2 mL of dichloromethane extract (evaporated) + 0.75 mL of alcohol + 3-7 drops of Solution C), from left to the right; so, both test tubes do not present a characteristic change of color that goes from reddish-brown to olive green, which suggests a negative test (see Figure 5.3).

In the Test **B** (Rosemhein Test (Anthocyanidins)), we can see two test tubes where the experiment was developed; none show the presence of two phases with a change of color that goes from reddish-brown to brown, which suggest a negative test (see Figure 5.3).

In the Test **C** (Wagner Test (Alkaloids)), we can see two test tubes (first test tube: evaporated chloroform extract(70%) + 1.5 mL of HCl (7%) + 12 drops of Wagner reagent, second test tube: evaporated dichloromethane extract(70%) + 1.5 mL of HCl (7%) + 12 drops of Wagner reagent, from left to right; none present a brown precipitate at the bottom of the test tube, which suggests a negative test (see Figure 5.3).

In the Test **D** (Borntrager Test (Quinones)), we can see two test tubes where the execution of the test was developed, each one showing the presence of two phases, but does not exist a change of color (pink or red); so, both cases suggest a negative test (see Figure 5.3).

In the Test **E** (Resin Test), we can see two test tubes where the execution of the test was developed; each one does not show the presence of precipitate, which suggests a negative test (see Figure 5.3).

In the Test **F** (Shinoda Test (Flavonoids)), we can see two test tubes where the execution of the test was developed; each one does not show any change in color in the phases; in this way, we can see two phases with similar colors(yellow). So, both cases suggest a negative test (see Figure 5.3).

In the Test **G** (Baljet Test (Sesquiterpene Lactones and Cardiac Glycosides)), we can see two test tubes, where the execution of the test was developed. In both cases, we can see that the sample does not experience a change of color that goes from yellow to orange. So, both test tubes suggest a negative test (see Figure 5.3).

In the Test **H** (Liebermann–Burchard Test (Sterols)), we can see two test tubes, where the execution

of the test was developed using 1-2 drops of sulfuric acid. In both cases, we could see a change of color that goes from yellow to green. So, this suggests a positive test (see Figure 5.3).

In the Test **I** (Foam Test (Saponins)), we can see two test tubes, where the execution of the test was developed. The experiment shows that foam does not keep after 2 minutes, suggesting a negative test in both cases. Figure **J** shows two test tubes that contain 1.5 mL of chloroform extract and 1.5 mL of dichloromethane extract as a reference of color for the different tests (see Figure 5.3).

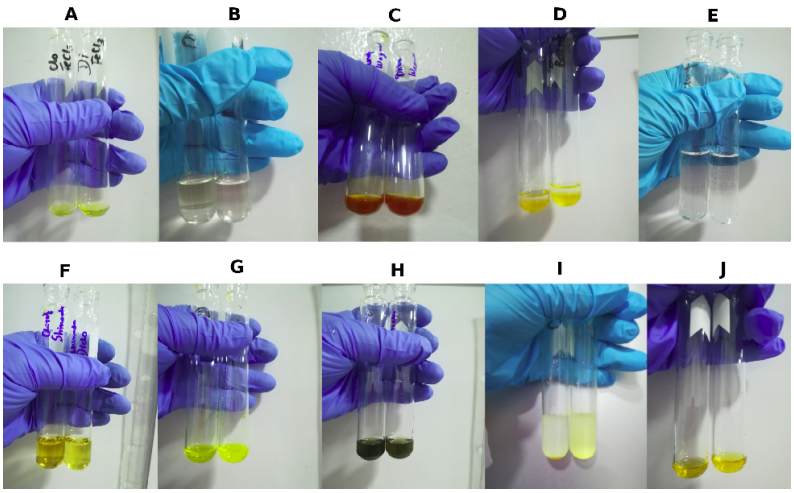


Figure 5.3: Results of different Chemical Tests in Chloroform Extract ($1^{st}$ test tube) and Dichloromethane Extract ($2^{nd}$ test tube).

(A): Ferric Chloride (5%) Test in water; (B): Rosemhein Test; (C): Wagner Test; (D): Borntrager Test; (E): Resin Test; (F): Shinoda Test; (G): Baljet Test; (H): Liebermann-Burchard Test; (I): Foam Test;(J): Comparison Extracts

The results displayed in Figure 5.3 are summarized in Table 5.2. Finally, the Chemical Tests suggested in Section 4.2.3 were applied to Chloroform Extract and Dichloromethane Extract.

Table 5.2: Qualitative Results of Chemical Test: Chloroform Extract and Dichloromethane Extract.

| ***Chemical Test*** | ***Chloroform Extract*** | ***Dichloromethane Extract*** |
|---|---|---|
| A.-Ferric Chloride(5%) | - | - |
| B.-Rosemhein | - | - |
| C.-Wagner | - | - |
| D.-Borntrager | - | - |
| E.-Resin | - | - |
| F.-Shinoda | - | - |
| G.-Baljet | - | - |
| H.-Liebermann–Burchard | ++ | ++ |
| I.-Foam | - | - |
| J.-Comparison Extracts | N/A | N/A |

Considering the information presented in Table 5.1 and 5.2, phytochemical screening results show that recovery of phenolic content could be affected by the type of solvent used in the maceration process. In this way, (70%) and (96%) ethanol solution extracted the highest amount of phenolic compounds, whereas the solvents like hexane, chloroform, and dichloromethane only extract the fats of the sample (washing-up)[69]. Regarding the results of ethanolic extract, we can stand out the results related to picture A and C (see Figure 5.2). Picture A shows a positive result to Ferric Chloride 5% Test, which demonstrates phenolic compounds in the extract. Picture C shows a positive result for alkaloids, which could prove pharmacological activity in the ethanolic extract of mashua. Figure 5.3 exhibits another of the highlighted results. So, picture H in the hexanic extract shows a positive result for sterols, which could prove anti-inflammatory activity due to this type of compound in the extract. After obtaining the results of the phytochemical screening, we proceed to carry out the maceration using ethanol (70%) as a solvent; so, a pre-treatment of the extract is carried out through filtering, distillation, and washing with ethyl acetate. In this stage, the organic phase was used to perform a TLC and try to separate the compounds of the extract. In order to achieve this purpose, it is necessary to find the correct mobile phase, which will be one of the fundamental requirements for analysis by HPLC.

### 5.1.2 Confirmation of Wagner test

According to the development of this analysis, alkaloids, which are related to pharmacological activity, exists in the extract and, therefore, corroborated their existance in mashua tubers.

## 5.2 Pre-treatment of the Sample using Simple Distillation and Rotary Evaporator

The identification of compounds could be affected by the degradation of phenolic compounds; this can happen during the distillation process due to the high temperature used. Total degradation or losses of phenolic compounds may be related to high extract exposure temperatures, maceration time, hydrolysis, or degradation of the sample when any of these factors are not controled[9].

The distillation process is not the best option to concentrate an extract containing phenolic compounds since these compounds can degrade at temperatures higher than 40°C, which could interfere with the chromatogram of HPLC. In this way, a similar procedure was carried out but associated with a rotary evaporator, the advantage of this equipment is that function at vacuum pressure. It requires lower temperatures to evaporate the solvent and thus ensure that there is no degradation of compounds. Also, the concentration-time of the sample can be controlled, since, with the use of vacuum in this equip-

ment, the time decreases considerably. To obtain the aqueous fraction of the ethanolic extract 70%, the procedure of elimination of alcohol was performed with both methods: simple distillation and rotary evaporator to compare the advantages and disadvantages of each one during the same procedure. Thus, the experiment confirmed that using a rotary evaporator is the most efficient option in terms of time since obtaining the desired fraction lasted about 30 minutes while the distillation process lasted about 3 hours. Another essential advantage of the rotary evaporator is the temperature control (the temperature used in the process was 40°C, which remains constant during the experiment). In simple distillation, the reference temperature is the boiling point of ethanol (78.37°C) so, the temperature reached in the distillation process was 73.5°C, which was monitored continuously. Temperature is a fundamental aspect of the degradation of compounds since phenolic compounds are susceptible to degradation in high temperatures. Another related problem refers to glycosylated forms of the phenolics compounds (due to their nature), making the impossible separation of the compounds with available chromatography techniques. The method used to resolve this problem is the hydrolysis process.

## 5.3 Spectral Analysis

Since the mobile phase A17, detailed in Table 4.1 showed an evident separation of compounds (see Annex 7.5), we continued with the pre-treatment of the ethanolic extract of the mashua by evaporating the ethyl acetate, that is, concentrating the sample through of the distillation method until obtaining a caramel mass, which was "sow in a band" in a preparative TLC. We scraped the fluorescent fraction of the preparative TLC ( see Annex 7.7) to read in the spectrophotometer through a scan that goes from 200 to 400 nm. Figure 5.4 shows the spectra of the preparative TLC fraction that resulted in a maximum wavelenght of 285 nm, which corresponds to the ultraviolet absorption for some of the derivatives of catechins.

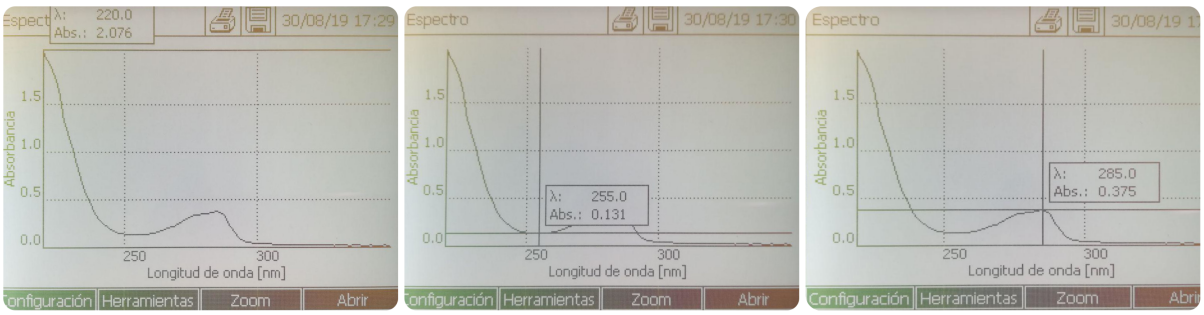


Figure 5.4: Experimental Analysis of the Fraction present in Preparative TLC (Distillation Process) through UV-Vis Spectrophotometry.

Also, these results ruled out the presence of anthocyanins (see Table 1.2), or at least it was discarded as one of the main components of the extract. In this way, anthocyanins will not be the point of reference for this investigation. Among the interest compounds related to y/y mashua type, we have proanthocyanidin content, phenolic acids (*o*-coumaric, protocatechuic, and gallic acid), flavan-3-ols monomers (epicatechin), flavonols (rutine) [9]. So, it is essential to consider that the result of this spectrum has a similar shape and maximum wavelength to the spectrum for epicatechin and catechin (see Figure 1.8 and 1.9). In this way, considering that catechin and epicatechin have maximum wavelengths of 210 and 280 nm, it is possible that the out of range result, mentioned above, may be due to some degradation of the compounds of the extract. This degradation could be the consequence of using high temperature during the distillation process.

So, the extraction of the compounds through preparative TLC using the rotary evaporator to concentrate the sample is completely necessary and generates two very notorious fractions in this experiment, analyzed through spectrophotometry. The spectrum correspondent to fraction 2 (upper fraction) of the preparative TLC (see Annex 7.9) can be seen in 5.5. This spectrum is very similar to the spectra of epicatechin and catechin (see Figure 1.8 and 1.9) with maximum wavelengths of 210 and 275 nm.

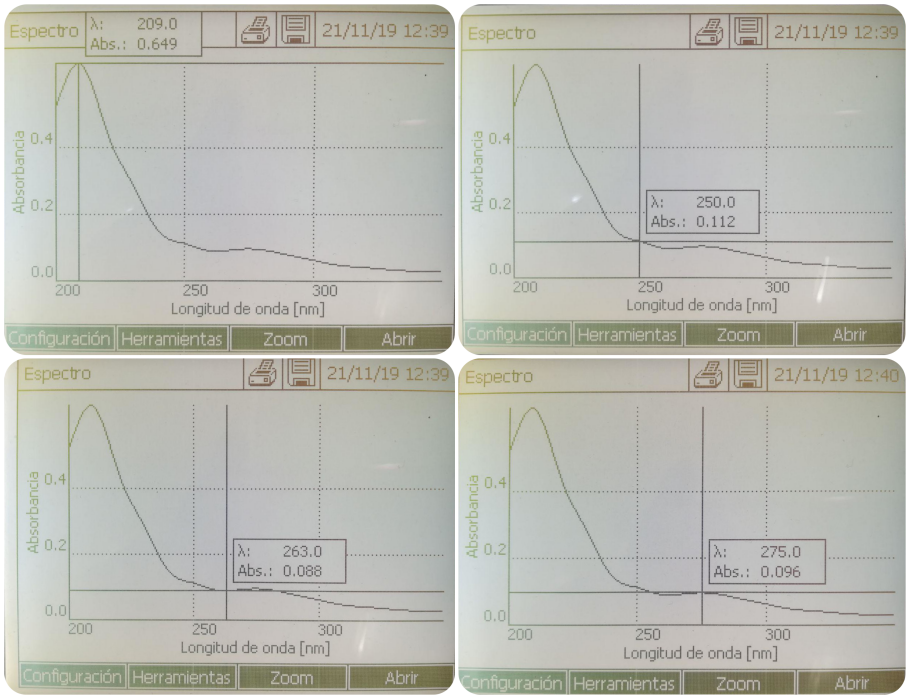


Figure 5.5: Experimental Analysis of the Fraction 2 present in Preparative TLC (Rotary Evaporator) through UV-Vis Spectrophotometry.

In contrast, Figure 5.6 shows the scan of fraction 1 (lower fraction) of the preparative TLC, which can be related to other compounds of the extract of mashua.

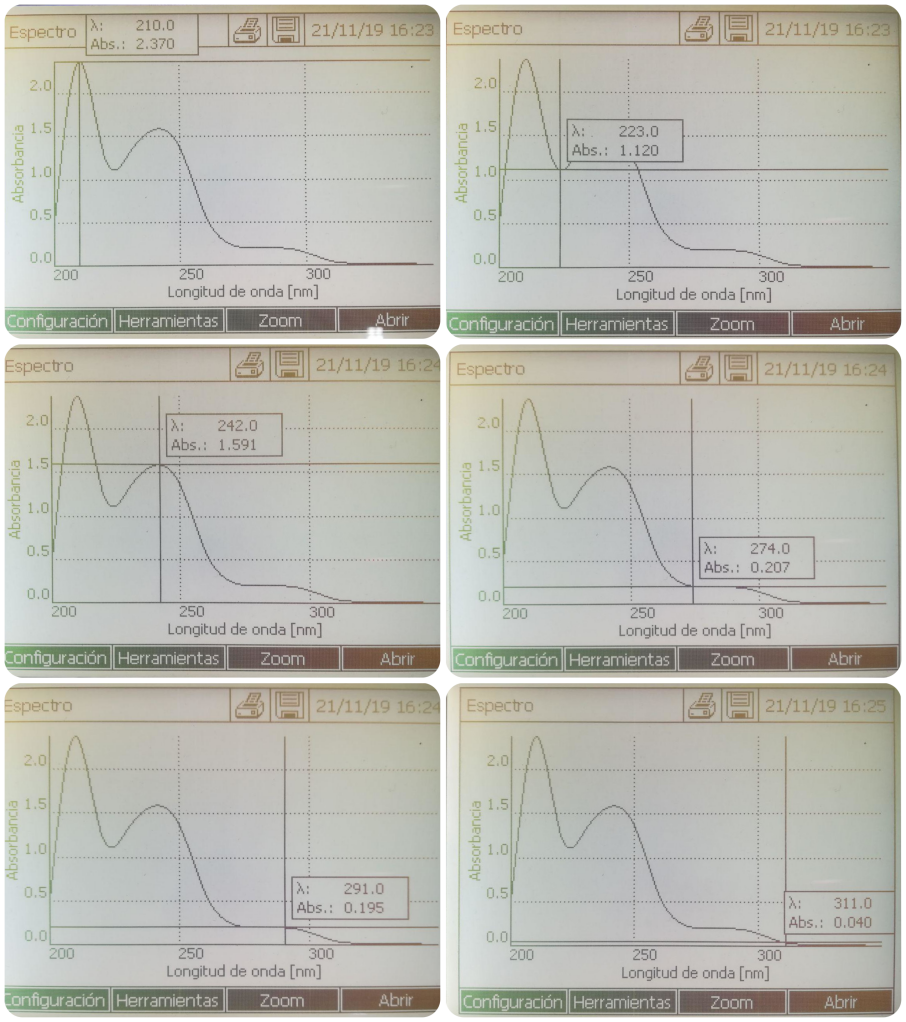


Figure 5.6: Experimental Analysis of the Fraction 1 present in Preparative TLC (Rotary Evaporator) through UV-Vis Spectrophotometry.

## 5.4 Chromatographic Analysis

Qualitative or quantitative analysis of the extract requires many standards due to the complexity of herbal plants. This task is further complicated due to the chirality of the molecule of interest because, in many cases, the same molecule can have different chiral forms. An example that demonstrates this type of behavior is the presence of catechin in the mashua tubers. The bibliographic analysis states that this compound could be found in different chiral forms (diastereoisomers): (-)- catechin, (+)- catechin, (+)- epicatechin or (-)- epicatechin. Furthermore, small variations related to the same compound can also be found. For example, gallocatechin or epigallocatechin, making the identification process even more difficult. The standard used in this research was (+)-catechin, which is a specific type of catechin that, in turn, has a defined retention time, which could differ in a certain way with the retention times of other

catechin derivatives.

The chromatograms obtained for uracil and different fractions of the yellow mashua type are shown in Figures 5.7, 5.8 and 5.9, and the superposition of them are displayed in the Figure 5.10. Separation through preparative TLC originated two well-defined fractions (fraction 1 and fraction 2) analyzed by HPLC. During a preliminary analysis, fraction 1 showed two peaks with poor resolution, while fraction 2 had a well-defined peak. The peaks had retention times lower than uracil (base peak). The fact that uracil had a longer retention time was not optimal, but this point was improved through new chromatographic conditions. Thus, in the subsequent analysis, the mobile phase and other conditions were changed in order to improve the resolution and retention times. In this case, identification was executed at 275 nm, a wavelength at which the majority of phenolic compounds are detected.

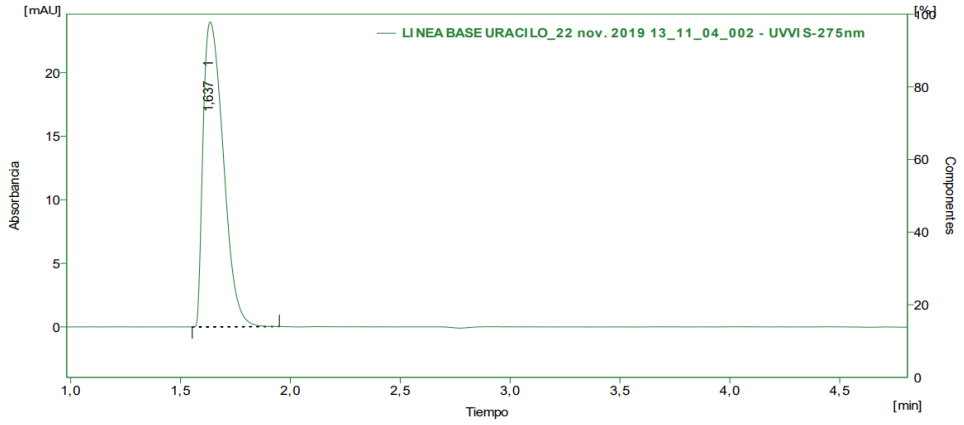


Figure 5.7: Uracil related to the analysis of Preparative TLC Fractions.

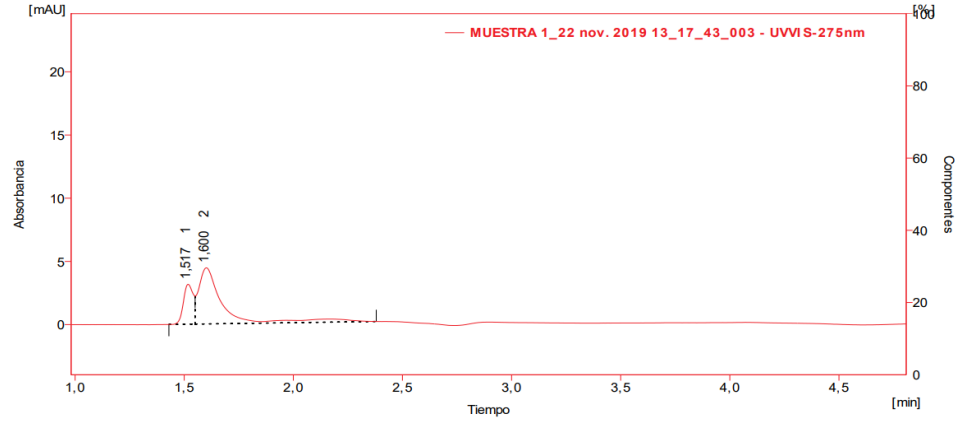


Figure 5.8: Fraction 1 of the Preparative TLC analyzed by RP-HPLC.

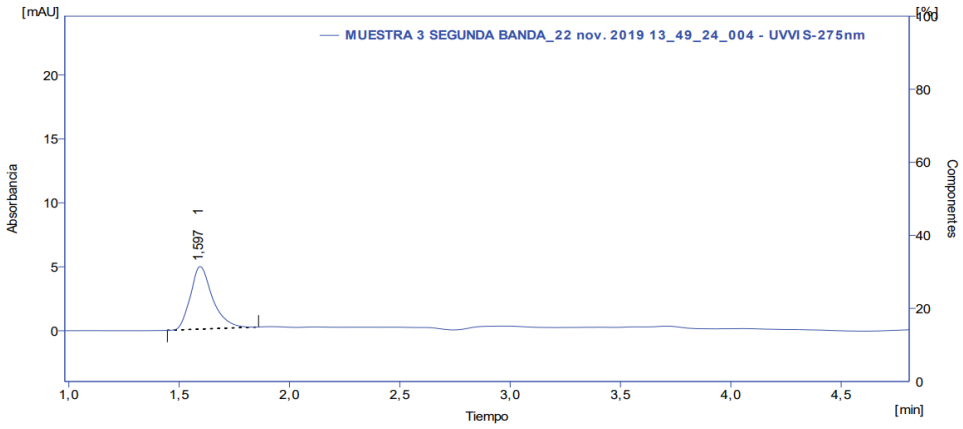


Figure 5.9: Fraction 2 of the Preparative TLC analyzed by RP-HPLC.

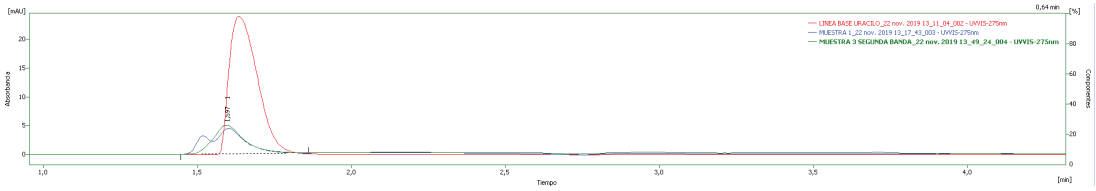

Figure 5.10: Superposition of the chromatograms of Fraction 1 and 2 (Preparative TLC).

After the HPLC analysis of the purified fractions, an analysis of the hydrolyzed extract of mashua was performed. This extract follows the same pre-treatment pattern used previously, with slight differences. This chromatogram can be seen in Figure 5.11, which shows a well-defined chromatogram and a clear separation of the compounds through the presence of tall and thin peaks of the different compounds in this extract. The number of peaks is related to the complexity of the mashua extract and the different related compounds known through the bibliographic analysis.

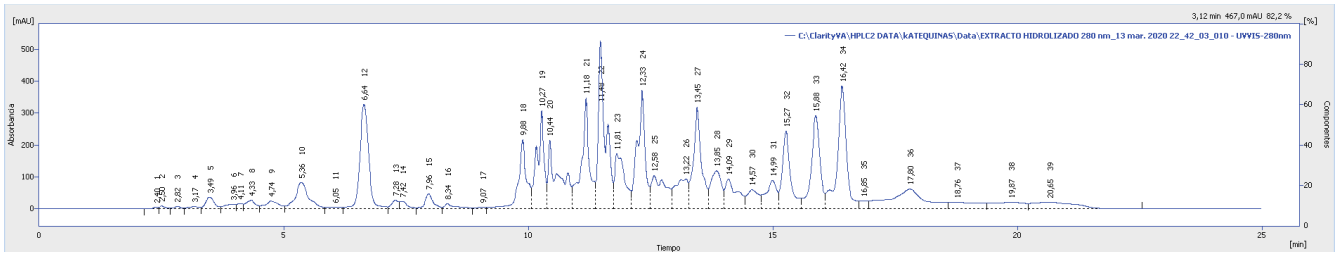


Figure 5.11: Chromatographic Profile of Hydrolyzed Mashua Extract.

In Figures 5.12 and 5.13 , it can be seen comparing the preparative TLC fractions with the hydrolyzed extract of mashua. In this way, it is observed that these purified fractions were found in tiny amounts concerning the extract as such.

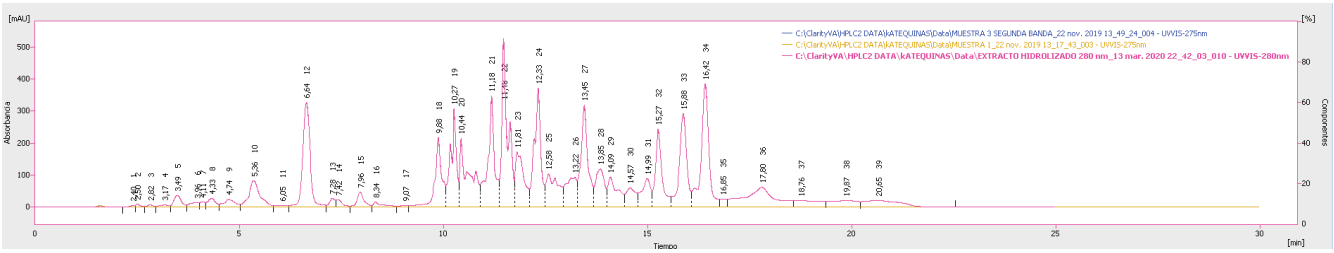


Figure 5.12: Comparison of Fractions 1 and 2 (Preparative TLC) with Hydrolyzed Mashua Extract.

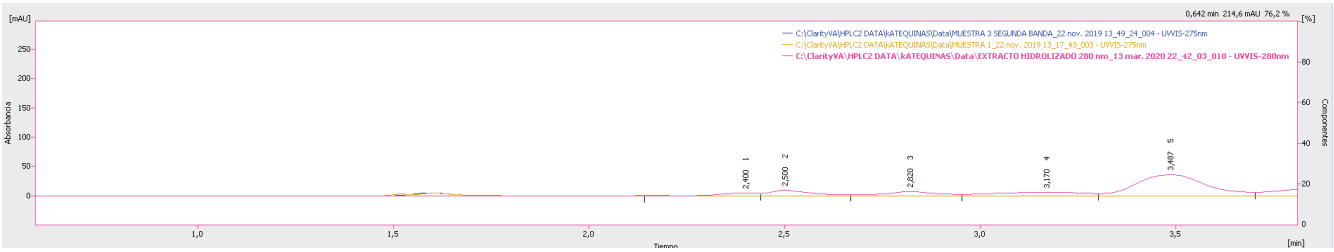


Figure 5.13: Zoom of Comparison of Fractions 1 and 2 with the Hydrolyzed Mashua Extract.

Figure 5.14 show the (+)-catechin standard (blue peak) injected in a posterior experiment that pretended to have the same conditions. Still, due to Buffer pH or external factors, the same retention time result could not be obtained between the standards. The solvent used for disolve the pre-treatment in this case was Buffer Solution. So, this solvent generate a better resolution of the standard peak.

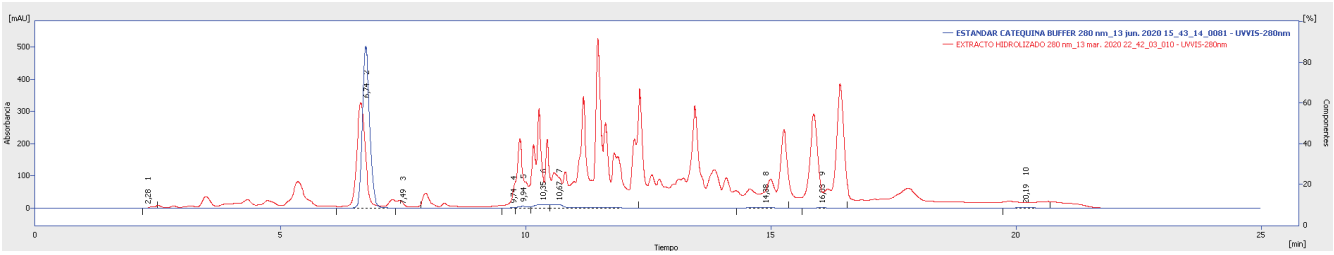


Figure 5.14: Comparison of (+)-catechin standard injected for the second time with Hydrolyzed Mashua Extract.

Also, Figure 5.15 shows the signals that the catechin standard shows when it is diluted with different solvents. This was done to show that the type of solvent with which the sample is diluted does not have a radical influence on the compound's retention time. However, the resolution is improved by diluting the standard with Buffer or Methanol.

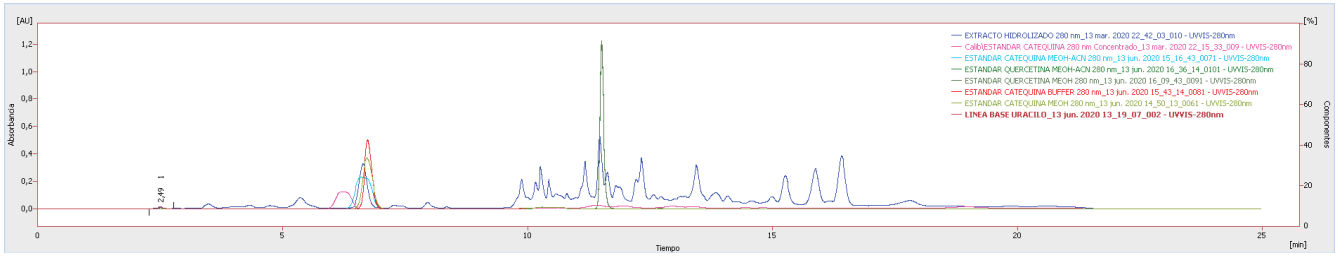


Figure 5.15: Comparison of (+)-catechin Standard diluted with different solvents.

Figures 5.16 and 5.17 shows the comparison of the (+)-catechin standard with the hydrolyzed extract of mashua. This chromatogram indicates that the standard's retention time is 6.20, while the retention time for the nearest peak in the hydrolyzed extract is 6.64. This demonstrates a variation that may be associated with the chirality of the catechin compound or one of its derivatives. However, it is definitively assured that this specific compound is not related to the extract. Taking as a reference the Table 1.4 associated with the retention times of phenolic compounds, we can propose epicatechin as the possible compound related to the extract. In comparison with catechin, epicatechin has a longer retention time.

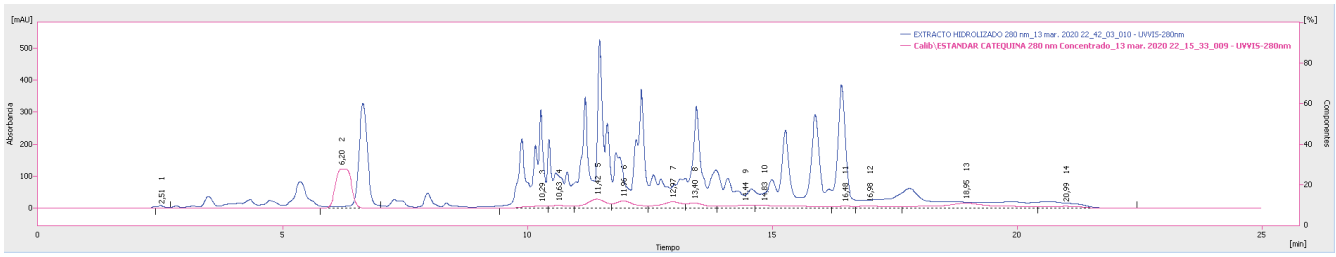


Figure 5.16: Comparison of (+)-catechin Standard and Hydrolyzed Mashua Extract.

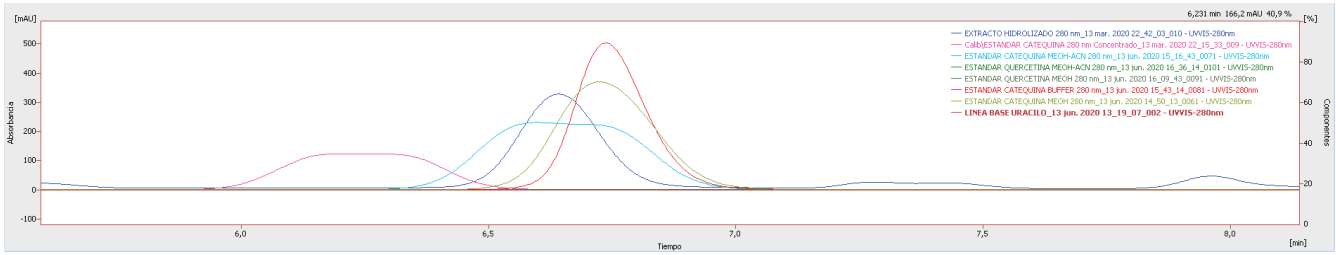


Figure 5.17: Zoom of Comparison of (+)-catechin Standard diluted with different solvents.

Furthermore, Figure 5.18 observe the well-defined peak of the quercetin standard, but since it was injected in posterior experiment, we cannot be sure of the peak's location about the hydrolyzed extract. We can say that it could be associated with one of the peaks to the right of it. But this can only be verified when the extract and the standards are injected at the same time. However, quercetin or a derivative could also be one of the main components of the mashua extract, which would demonstrate

the existence of another component with anti-inflammatory properties within the chemical composition of mashua.

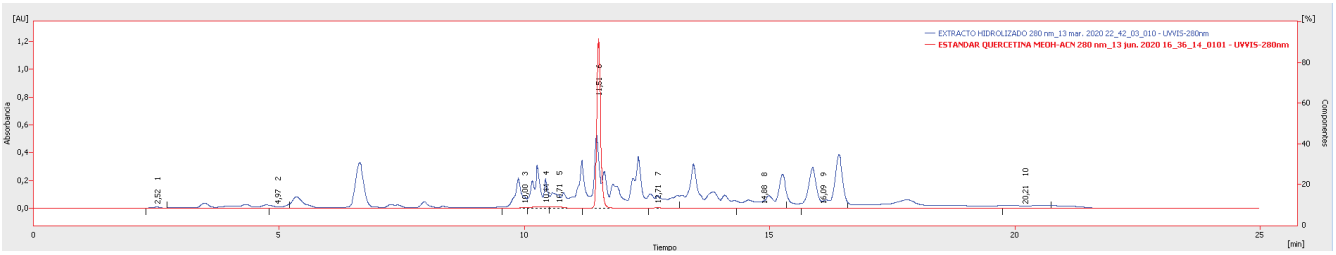


Figure 5.18: Comparison of quercetin standard and Hydrolyzed Mashua Extract.

# 6. Conclusions and recommendations

## 6.1 Conclusions

The scientific literature showed several types of phenolic compounds for mashua tubers associated with its therapeutical properties. Still, only a few of them have been identified and quantified through analytical techniques. In this way, only a few previous studies about this topic focus on finding a chemical marker for mashua.

Solvents with different polarities were used for the extraction process (ethanol, hexane, chloroform, and dichloromethane) in the phytochemical screening. In this way, ethanol (70 %) will be used for routine quality control due to the extraction of the highest number of compounds generating positive results in the different chemical tests and interesting results during Phytochemical Screening. In this way, it is essential to highlight that ethanolic (70%) shows a positive result to the Ferric Chloride test, which demonstrates phenolic compounds in the extract. The positive result for the Wagner test (alkaloids) is also important because it could prove pharmacological activity in the ethanolic extract of mashua. Hexanic extract shows a positive result for sterols, which could prove an anti-inflammatory activity due to this compound type in the extract.

The spectrophotometric analysis concludes the presence of catechins in the mashua extract. However, RP-HPLC analysis discards the presence of +(-) catechin. The chromatogram results may be related to another of the chiral compounds of catechin.

Despite that many compounds are described in literature about the chemical composition of mashua tubers, they are not enough at the moment of identifying the compounds. This is due to a lack of standards in the laboratory that confirm our literature information.

The distillation process procedure is not the best option to concentrate an extract that contains phenolic compounds since these compounds can degrade at high temperatures. In this way, a similar procedure using a rotary evaporator due to a vacuum pressure system requires lower temperatures to evaporate the solvent and ensure no degradation of compounds.

Considering the results, the method designed and developed herein is currently successful for the determination of a chemical fingerprint and subsequently an appropriate chemical marker for mashua. It

was verified that (+)-catechin and quercetin were not appropriate chemical markers for mashua because they did not exist in the extract of mashua powder in high concentrations. Therefore, we need acquire other chemical standards previously described in the scientific literature for this medicinal plant to find an appropriate chemical marker.

## 6.2 Recommendations

- Before analysis by HPLC related to the determination of a chemical marker of a plant, it is necessary to perform a thorough study of its physicochemical characteristics, since when this is not executed, resources are usually wasted, the columns are damaged and cannot be achieved an efficient separation in the chromatograms.
- After its pretreatment, the mashua extract should be also analyzed by HPLC/DAD and UPLC/MS. Due to the pandemic these analyzes could not be performed. These techniques are excellent for identifying potential chemical markers and facilitating their purchase on the market.
- The standards must be injected simultaneously with hydrolyzed extract in the same HPLC analysis so that there are no differences in retention times.
- The use of a chiral column is necessary to characterize a molecule with at least one chiral center in order to relate this compound to the chemical marker of the hydrolyzed extract of the mashua powder.
- In order to make possible to find an appropriated chemical marker for this medicinal plant, it is necessary buy other chemical standards available in the market (gallic acid, gallocatechin, epigallocatechin, procyanidin $B_2$, myricetin, coumaric acid, rutin, caffeic acid, apigenin, naringenin, luteolin, cinnamic acid, cyanidin) and already described in the scientific literature for this medicinal plant, as discussed previously in this work.

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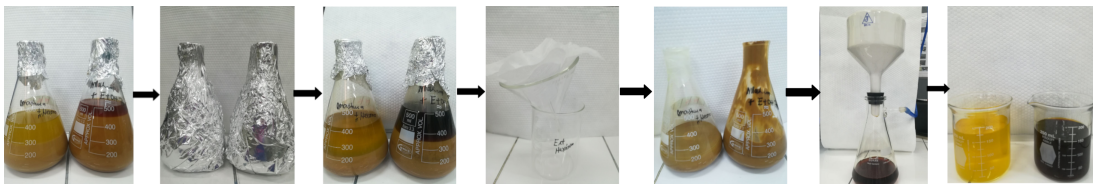
# 7. Annexes

Annex 7.1: Physical and Chemical properties of Solvents used in Phytochemical Screening.

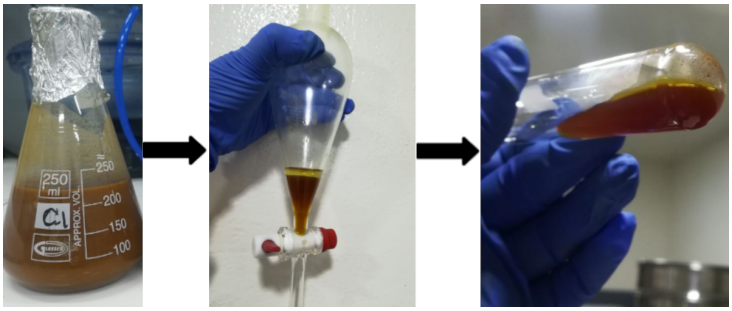
| ***Solvents*** | **Density(g/cm$^3$)** | **Molar Mass (g/mol)** | ***Polarity Index*** | ***Boiling Point*** *(25° C)* |
|---|---|---|---|---|
| Hexane | 0.6548 | 86.18 | 0.0 | 68.00 |
| Triethylamine | 0.7260 | 101.19 | 1.8 | 89.00 |
| Isopropyl Ether | 0.725 | 102.18 | 2.2 | 69.00 |
| Toluene | 0.8669 | 92.14 | 2.3 | 110.60 |
| Benzene | 0.8786 | 78.11 | 3.0 | 80.1 |
| Dichloromethane | 1.3300 | 84.93 | 3.4 | 39.60 |
| Tetrahydrofuran | 0.8900 | 72.11 | 4.2 | 66.00 |
| Ethyl Acetate | 0.9000 | 88.11 | 4.3 | 77.10 |
| 2-propanol | 0.7863 | 60.10 | 4.3 | 82.50 |
| 1-propanol | 0.7900 | 60.10 | 4.3 | 97.00 |
| Chloroform | 1.4830 | 119.38 | 4.4 | 61.20 |
| Ethanol | 0.7890 | 46.07 | 5.2 | 78.37 |
| Acetic acid | 1.0490 | 60.05 | 6.2 | 118.10 |
| Acetonitrile | 0.7860 | 41.05 | 6.2 | 82.00 |
| Methanol | 0.7918 | 32.04 | 6.6 | 64.70 |
| Water | 0.9970 | 18.01 | 9.0 | 100.00 |

Annex 7.2: Dielectrical Constants of Solvents used to extraction.

| ***Solvents*** | ***Dielectrical constant*** *25° C* |
|---|---|
| Hexane | 1.89 |
| Benzene | 2.30 |
| Toluene | 2.57 |
| Acetonitrile | 36.64 |
| Ethyl ether | 4.20 |
| Chloroform | 4.80 |
| Ethyl Acetate | 6.02 |
| Acetic acid | 6.15 |
| Tetrahydrofuran | 7.58 |
| Dichloromethane | 9.00 |
| 2-butanol | 17.26 |
| 1-butanol | 17.8 |
| 2-propanol | 18.3 |
| 1-propanol | 20.1 |
| Acetone | 20.7 |
| Ethanol | 24.55 |
| Methanol | 32.65 |
| Formic Acid | 57.9 |
| Water | 78.3 |

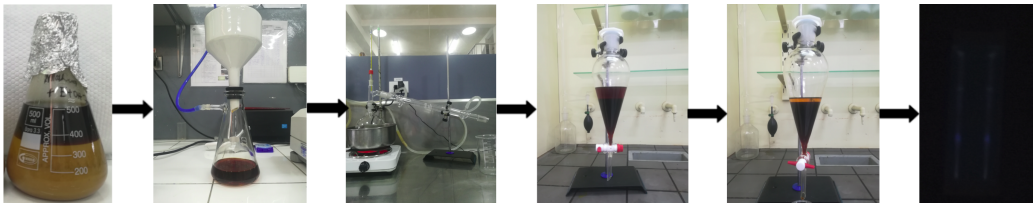


Annex 7.3: Steps of Solid-liquid extraction described in section 4.2.1.

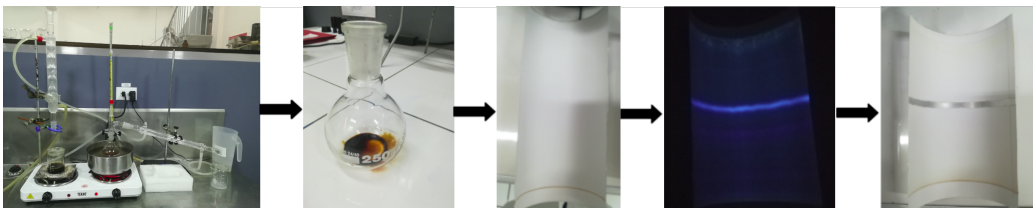



Annex 7.4: Confirmation of Wagner test in mashua extract (false-positive or false negative result).

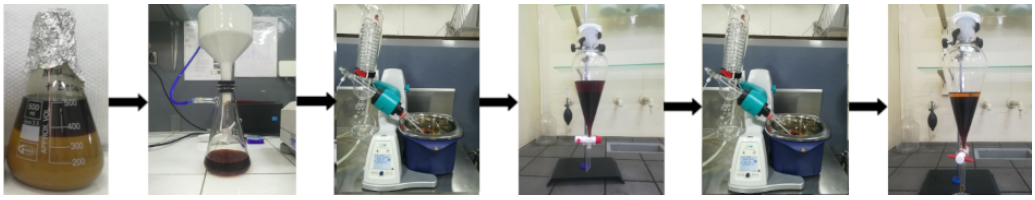
Annex 7.5: TLC of Mashua Ethanolic Extract 70% using A17-Mobile Phase.



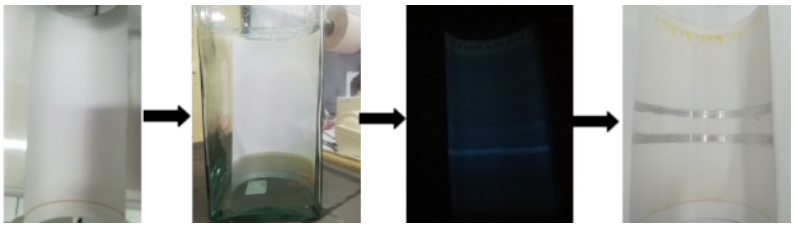
Annex 7.6: Asembly for Distillation Process of Mashua Ethanolic Extract 70% and execution of TLC.



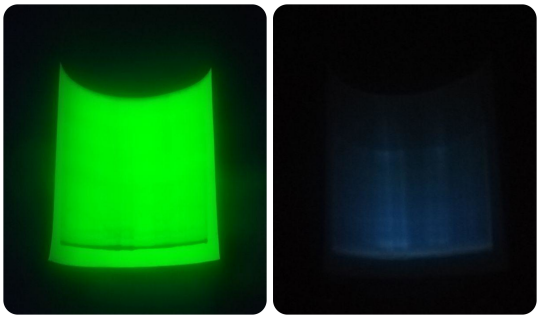
Annex 7.7: Assembly for Distillation Process of Pre-treatment Mashua Ethanolic Extract (Organic Phase) and execution of Preparative TLC.



Annex 7.8: Treatment Process of Mashua Ethanolic Extract 70% using a Rotary Evaporator.



Annex 7.9: Execution of Preparative TLC (Mashua Ethanolic Extract 70%).



Annex 7.10: Preparative TLC of the hydrolyzed aqueous phase.

Annex 7.11: Certificate of Analysis of (+)-catechin.

SIGMA-ALDRICH®

sigma-aldrich.com

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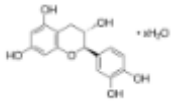
Email USA: techserv@sial.com
Outside USA: eurtechserv@sial.com

# Certificate of Analysis

Product Name:
(+)-Catechin hydrate – ≥98% (HPLC), powder



| | |
|---|---|
| **Product Number:** | **C1251** |
| **Batch Number:** | **WXBD0758V** |
| Brand: | SIGMA |
| CAS Number: | 225937-10-0 |
| Formula: | C15H14O6 · xH2O |
| Formula Weight: | 290,27 g/mol |
| Storage Temperature: | Store at 2 - 8 °C |
| Quality Release Date: | 29 AUG 2019 |
| Recommended Retest Date: | JUL 2023 |

| Test | Specification | Result |
|---|---|---|
| Appearance (Colour) | White to Light Beige to Light Brown | Light Brown |
| Appearance (Form) | Powder | Powder |
| Solubility (Colour) | Yellow to Very Dark Yellow and Light Brown-Yellow to Very Dark Brown-Yellow | Very Dark Brown-Yellow |
| Solubility (Turbidity) 50 mg/mL in Ethanol | Clear to Slightly Hazy | Very Slightly Hazy |
| Proton NMR spectrum | Conforms to Structure | Conforms |
| Water Content by NMR Report Result | | 6.56 % |
| Specific Rotation Report Result | | 24.7 ° |
| Purity (HPLC) | ≥ 98 % | 99 % |

Steven Chen, Manager
Quality Control
Wuxi, China CN

Annex 7.12: Certificate of Analysis of quercetin.

# Certificate of Analysis

SIGMA-ALDRICH

| | |
|---|---|
| **Product Name** | Quercetin dihydrate, ≥98% (HPLC), powder |
| **Product Number** | Q0125 |
| **Product Brand** | SIGMA |
| **CAS Number** | 6151-25-3 |
| **Molecular Formula** | $C_{15}H_{10}O_7 \cdot 2H_2O$ |
| **Molecular Weight** | 338.27 |

| TEST | SPECIFICATION | LOT 087K0744 RESULTS |
|---|---|---|
| **APPEARANCE** | YELLOW TO YELLOW WITH A GREEN TO BROWN CAST POWDER | YELLOW POWDER |
| **SOLUBILITY** | DARK RED SOLUTION AT 200 MG PLUS 4 ML OF 1 M SODIUM HYDROXIDE | CONFORMS |
| **PURITY BY HPLC** | NOT LESS THAN 98% | 99% |
| **QC RELEASE DATE** | | SEPTEMBER 2007 |
| **PRODUCT CROSS REFERENCE INFORMATION** | | REPLACEMENT FOR ALDRICH #171964 |

Rodney Burbach, Manager
Quality Control
St. Louis, Missouri USA

INTERVIEW

Transcript for Interview:

1. ¿Porqué razón las personas buscan la Mashua?

   **Nancy Sailema**: La mashua la buscan para la próstata, porque es desinflamatorio, es bueno para cuando a los hombres les duele mucho lo que es la próstata; por esa razón ocupan más la mashua.

   **Marcia Cañar**: Como desinflamante, para desinflamar los riñones, el hígado, la próstata.

2. ¿Quién la recomienda?

   **Nancy Sailema**: Aquí mismo en nuestra seción de Plantas medicinales la recomendamos y la gente nativa(las personas del campo), ellos recomiendan la mashua para la próstata.

   **Marcia Cañar**: Médicos, los doctores de los Centros Naturistas y nosotros también.

3. ¿Cuál es el método de preparación más común de la Mashua para fines terapéuticos?

   **Nancy Sailema**: La mashua se cocina normalmente en 2 Litros de agua, como es pequeña la mashua al menos 8 tubérculos se cocinan(si se desea se puede cocinar con la cashamarucha o sino solo la mashua); se cocina bien y se licua y se toma ese jugo en ayunas; además el agua de mashua se toma entre el día.

   **Marcia Cañar**: Se le prepara cocinando la mashua en agua, luego se cierne y los tubérculos se le licúa con la agua de la cashamarucha o la cola de caballo.

4. ¿Con qué regularidad se debe usar la Mashua?

   **Nancy Sailema**: El jugo se debe tomar de una a tres veces al día, mientras que en el transcurso del día se deben tomar 2 Litros de agua de mashua.

   **Marcia Cañar**: 3 veces al día por 9 días el jugo licuado.

5. ¿Qué enfermedades se pueden tratar a base de este tubérculo?

   **Nancy Sailema**: Inflamación de los riñones, hígado.

   **Marcia Cañar**: Para bajar la fiebre, para la inflamación del útero, vías urinarias, páncreas, intestino grueso y también se conoce que es antidiabético.