PONTIFICIA UNIVERSIDAD CATOLICA DE CHILE
ESCUELA DE INGENIERIA

SEASONAL ANALYSIS OF SAPONIN CONTENT OF LEAVES OF YOUNG *QUILLAJA SAPONARIA* TREES FROM A PLANTATION

TRINIDAD SCHLOTTERBECK SUAREZ

Thesis submitted to the Office of Research and Graduate Studies in partial fulfillment of the requirements for the Degree of Master of Science in Engineering

Advisor:
RICARDO SAN MARTIN

Santiago de Chile, march, 2015

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To my family, for their unconditional support and company.

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RESUMEN

El árbol *Quillaja saponaria*, endémico de Chile, ha sido ampliamente explotado para la extracción de saponinas, las cuales son utilizadas en la minería, la agricultura y diversas industrias como la farmacéutica y la de alimentación. La sobreexplotación de los bosques nativos ha planteado la necesidad de establecer plantaciones. Hasta la fecha, la biomasa se ha obtenido de la madera y corteza de árboles adultos nativos, excluyendo el uso de las hojas debido a su baja contribución (respecto del árbol total) y también porque las líneas de producción industrial han sido diseñadas específicamente para madera y corteza, no para las hojas que contienen diferentes compuestos, como la clorofila.

En esta tesis, se propone el uso de árboles completos de plantaciones jóvenes, incluyendo las hojas. Se realizaron cuantificaciones de saponinas en hojas de 24 árboles de una plantación de tres años de edad durante las cuatro estaciones de un año. Nuestros resultados mostraron que las hojas representan el 27,8% del peso total de árboles jóvenes y que contienen en promedio 2,58% de saponinas. Teniendo en cuenta que esa cantidad de saponinas es similar a la encontrada en madera, las hojas podrían contribuir hasta en un tercio de las saponinas de los árboles jóvenes, siendo clave el procesamiento de estas para alcanzar buenos rendimientos.

A partir de los análisis realizados respecto del contenido de saponinas en las hojas, se observaron diferencias significativas en el contenido de saponinas durante las cuatro estaciones. En promedio, la concentración más baja se encontró durante el invierno (julio = 1,39% respecto a materia prima seca) y la mayor en otoño (abril = 4,49% respecto a materia prima seca), lo que sugiere que los factores abióticos podrían tener un efecto sobre la producción de saponinas.

Además, se observaron diferencias significativas en cuanto a las concentraciones de saponinas entre los 24 árboles. El valor mínimo en contenido de saponinas fue de 0,90 y el máximo de 4,71%, ambos respecto a materia prima seca. Esto plantea la necesidad de seleccionar árboles superiores en producción de saponinas.

Estos resultados contribuyen a la comprensión de una producción más sostenible de extractos de *Quillaja*, al mostrar el potencial de utilizar plantaciones de árboles jóvenes, incluyendo sus hojas, en lugar de sólo la madera y corteza ambos provenientes de bosques nativos. A su vez, permiten desarrollar nuevas líneas de investigación en torno a entender el comportamiento de plantaciones jóvenes y considerando las hojas de estos árboles como una posible nueva fuente de materia prima.

Palabras Claves: *Quillaja saponaria*, plantaciones, hojas, sólidos solubles, saponinas

ABSTRACT

Quillaja saponaria is an endemic tree in Chile. It has been extensively exploited for the extraction of saponins, which are used in mining, agriculture and in the pharmaceutical and food industries. The overexploitation of native adult *Quillaja* forests has brought the necessity to establish plantations. Until now, biomass has been obtained from wood and bark of native adult trees, excluding the use of leaves due to their low biomass contribution to the whole tree, and also because industrial production lines have been designed specifically for wood and bark and not for leaves that contain different compounds (e.g. chlorophyll).

In this thesis, we propose using complete trees from young plantations including the leaves. Quantification of saponins in leaves of 24 three-year-old *Quillaja* trees from a plantation was carried out during the four seasons of one year. Our results showed that leaves represent 27.8% of the weight of the whole young trees and that leaves contain on average 2.58% of saponins. Considering that this amount is similar to that found in wood, we argue that leaves could contribute up to one-third of the saponins of young trees, making its processing key to attain good yields.

Based on the analyses developed for saponin content in leaves, significant differences were observed in saponins contents during the four seasons. On average, the lowest concentration was found during winter (July = 1.39% with respect to dry weight) and

the highest in autumn (April = 4.49% with respect to dry weight), suggesting that abiotic factors may have an effect on saponins production.

A comparison between the 24 trees showed significant differences in saponins concentration among trees, supporting the need to select consistently superior saponins-producing trees. The lowest value for saponin concentration was 0.90 and the highest 4.71%, both with respect to dry weight.

These results contribute to the understanding of a more sustainable production of *Quillaja* extracts, showing a potential to use whole young trees from plantations rather than only the wood and bark from native *Quillaja* forests. Furthermore, these results allow the development of new research lines aiming to understand the behavior of young *Quillaja* plantations and the use of their leaves as a new possible source of raw material.

Keywords: *Quillaja saponaria,* plantations, leaves, soluble solids, saponins

1. INTRODUCTION

Quillaja saponaria Molina, commonly known as Quillay, is a native Chilean tree, which belongs to the sclerophyll forest in this country. It presents the characteristic of being an evergreen tree. It grows between the IV and IX Regions (Donoso et al., 2011), from the Andes Mountain to the Coastal Range (Sfeir, 1990).

The economic interest of this tree lies in the ability it has to produce saponins. *Quillaja* saponins are high molecular weight glycosides, containing a hydrophobic triterpenic nucleus and two hydrophilic sugar chains. *Quillaja* saponins have been used in various industries, mainly as biopesticide and plant growth stimulant in agriculture (Zúñiga et al., 2012, Chapagain et al., 2007, Fischer et al., 2011), emulsifier and foaming agent for animal and human food and beverage (Yang et al., 2013), wetting agent in film production and mining (San Martin et al., 2005b, San Martin et al., 2005a), and as adjuvant for human and non-human animals vaccines (Kensil et al., 1991, Kensil & Kammer, 1998, Barhate et al., 2013, Sun et al., 2009).

During 1996 a new method for extracting saponins was developed. Before this new method, only bark of native adult trees was used and the total of it was exported to the United States, Germany and Japan, countries that processed *Quillaja*. The new production process included the use of wood and bark of native trees. A reduction in the need of debarking the trees was achieved when using all the woody biomass, which decreased the ecologic damage (San Martin, 2000).

However, both processes use native trees as raw material. The ecologic impact of using native trees is significant (Honeyman, 2013). Moreover, it has been estimated that the sustainable limit of native forests will not cover the annual demand by the year 2020, considering that the industry is growing with mean annual rates of 19.4% (Instituto Forestal, 2012, San Martin, 2000, Honeyman, 2013). Plantations of young *Quillaja* trees could be the solution to meet up future demands. By including the leaves in the process that already considers wood and bark, the yields of young trees could be increased, making plantations interesting for the industry. It is noteworthy to point out that until now, leaves of adult native trees have not being used, due to their low contribution of biomass to the whole tree (4 to 6% of the total weight, Sfeir, 1990, Pulido, 2000). Also, the industrial production lines are designed exclusively for the extraction of the woody parts of trees and does not considers the need of removing the different compounds contained in leaves (e.g. chlorophyll).

Ongoing research highlights understanding the best plantation techniques to maximize the biomass obtainment per tree (Correa & Martínez 2013, Grandón et al. 2013). However, there is a lack of knowledge in regards to the production of saponins in young trees from plantations (one to seven years old) and in their leaves. Several factors might be implicated in the regulation of the saponin production in trees, such as seasonal changes, soil fertility and trees genetic, among others (Copaja et al., 2003, Szakiel et al., 2011, Kamstrup et al., 2000).

The aim of this work was to evaluate saponin content in leaves of young trees during one year, to obtain evidence that support the plantation of *Quillaja* trees and to consider the use of leaves of young trees as a new source of raw material for obtaining saponins. Also, evaluate the influence of the different seasons in the saponin production and the variation among trees. Thus, saponin contents of leaves of 24 three-year-old *Quillaja* trees from a plantation were studied during the four seasons of one year.

As a result of the study, an original research paper was submitted to a scientific journal, text that is included in this thesis (Chapter 3). An introduction chapter and hypotheses are included for wider analyses of *Quillaja* characteristics and the market developed around this natural Chilean resource. Finally, a last section is included for further discussions that were not included in the research paper.

1.1 General description of *Quillaja saponaria* Molina

Quillaja is one of the characteristic trees of the sclerophyll forest in Chile. The other typical species that grow in this kind of forests are: *Peumus boldus, Lithraea caustic, Cryptocarya alba, Maytenus boaria* and *Acacia caven* (Correa & Martínez, 2013).

Quillaja trees grow between Ovalle (IV Region) and Collipulli (IX Region), from the Andean Range to the Costal Mountains. These trees are found in locations 15 m above sea level and up to 1,600 m (Gotor, 2008). *Quillaja* trees resist broad ranges of temperatures and adverse conditions such as water deficit during the dry periods of summer, frosts in winter, strong winds, among others.

In terms of the size, adult native trees can reach diameters of 1.5 m approximately and heights of 20 to 30 m (Quintana, 2008). The natural rate of growth is characterized as slow, reaching a maximum growth of 0.6 cm of diameter at breast height and 30 cm of height per year (Correa & Martínez, 2013).

1.1.1 Characteristics of growth

a) Ground

Quillaja trees are able to grow in diverse type of grounds, including those with poor nutrients and harsh climate conditions (Correa & Martínez, 2013). The wide range of zones where these trees can grow supports its diversity, as it covers from the Andean to the Coastal mountain range. However, *Quillaja* does not grow well with excess of water and in grounds that have bad drainage. Specifically, trees do not grow in lacustrine organic and clay type grounds (Nuñez, 2006).

b) Climate

The optimal conditions for the growth of *Quillaja* trees are in Mediterranean climate, which presents average temperatures near 14 ˚C and precipitations of 150 to 1,500 mm annually (Correa & Martínez, 2013).

In terms of water requirements, the location of the trees depends on the amount of annual precipitations of the place. Where precipitations are over the 800 mm annually; trees are located principally in slopes facing north. Where annual rainfalls are near the 300 mm, trees are located in slopes facing south. Locations with precipitations below 250 mm, trees grow in flat lands (Nuñez, 2006).

c) Sunlight

With respect to sunlight, *Quillaja* trees are intolerant to locations and positions where there is mainly shadow. Moreover, in shadowy and humid locations *Quillaja* trees are displaced by other species of the sclerophyll forest like *Peumus boldus* and *Cryptocarya alba* (Álvarez, 2003, Nuñez, 2006).

d) Density

In 1999, a study established the national distribution of *Quillaja* trees. It was determined that 230,605 hectares had *Quillaja* as dominant specie (Cruz & Palma, 1999). Another study mentioned that by year 2000, *Quillaja* forests covered approximately 200,000 hectares (San Martin, 2000).

In terms of the density of this specie, reports indicated that sclerophyll forests have commonly 30 to 50 *Quillaja* trees per hectare. However, in the case of forests with high density of *Quillaja*, this specie can be present at approximately 100 individuals per hectare. (Donoso, 1981, Núñez, 2006).

e) Native forest availability

Regarding biomass productions of native forests, it has been estimated that every 15 years each hectare can produce 2 tons of biomass (San Martin, 2000). With approximately 200,000 hectares available mainly with *Quillaja* forests (San Martin, 2000), the annual sustainable limit of the native forests could de up to 27,000 tons of biomass per hectare. Probably this value is currently lower considering that the forest has been intensively harvested since 1999, many times without adequate techniques for the control of the specie (Álvarez, 2003) and possibly as a consequence of the effects of climate changes.

It is noteworthy that between 2009 and 2012 the industry of *Quillaja* has harvested 5,700 and 11,600 tons of biomass respectively, presenting a mean annual growth rate of 19.4% (Instituto Forestal, 2012, Natural Response S.A.). Considering those demands and growth rates, estimations indicate that approximately 40,000 to 48,000 tons of biomass will be needed by 2019 and 2020. Therefore, the sustainable limit of native forests will not cover the annual demand by 2020 (Honeyman, 2013).

Moreover, until 2012 only one company was producing *Quillaja* extracts (Natural Response S.A.). Due to the increasing demand for saponins, during the last two years two new companies have entered the Quillaja extraction industry (Chile Botanics S.A. and BASF).

1.1.2 Parts of the tree and their biomass contribution

The main aerial parts of *Quillaja* trees are trunk, branches, twigs, leaves and bark. Each of these components contributes in different percentages to the total weight, depending on the age of the tree and the zones where they grow.

Diverse values are found in the literature for the different components of adult native trees in terms of their weight contribution. The trunk represents between 55 to 77% of the aerial biomass and branches contribute between 5 to 37%. The lowest contributions are leaves and bark, with range values of 4 to 6% and 5 to 16%, respectively (Pardé, 1980, Sfeir, 1990, Pulido, 2000, Correa y Martínez, 2013).

Those values differ in the case of young trees. *Quillaja* trees within one to seven years old are much bushier than adults, as leaves have higher contribution in biomass due to the necessity of trees to do photosynthesis for growing (Quintana, 2008). Donoso et al. (2011) reported that leaves represented 52.9% of the total weight of two-year-old trees. Quintana (2008) in a plantation of seven-years-old *Quillaja* trees determined that the trunk represented 50.7% of the aerial weight, 31.9% were branches and 17.4% leaves. No further information can be found of young plantations besides the ones mentioned above.

1.1.3 Biomass functions

Due to the economic interest of *Quillaja* saponins, different biomass functions have been developed to have an estimation of the amount of wood and bark of adult native forests available for its exploitation. According to the location of trees, ages, variability in size and previous harvests, size and weight of trees can differ. Thus, different equations can be used to model these phenomena, depending on each scenario. Examples of different biomass equations are presented as follows.

Prado et al. (1986) weighted 32 adult native trees located in the north, center and south of Chile, in order to develop an equation to estimate biomass. They applied two type of equations: allometric and multiple linear. Both were fitted using mean squared error and calculated the coefficient of determination. The model that fitted better for the total tree was the multiple linear one, with values of 21.1% and 0.97 for mean squared error and coefficient of determination, respectively. The biomass equation developed for total trees was:

$$Y = - 599.0813 + 0.0281 (D^2 H) + 29.164 (B) \quad (1.1)$$

where Y is the estimating green weight of trees in kg, B is the basal stem diameter in cm, D is the diameter at breast height in cm and H is the total height of the tree in m.

Pulido (2000) also developed an equation for estimating *Quillaja* biomass. The function was done for vegetative regenerated trees. These were located in the interior zone of the VI region. The fitting of the model was also done using mean squared error and the coefficient of determination was calculated, which presented values of 45.86% and 0.98 for the selected equation. The function developed for dry weight was:

$$Y = 0.149 (B^{2.089}) \quad (1.2)$$

where Y is the estimating dry weight of trees in kg, B is the basal stem diameter in cm.

Durán (2002) analyzed 25 adult trees located in the VII region of Chile. On average, trees measured 11.06 m of height and 37.81 cm of diameter at breast height. The three types of models used were: no logarithmic transformation of the dependent variable, logarithmic transformation of the dependent variable and logarithmic transformation of both variables, dependent and independent. The best equation developed for dry total weight was using the first type, without any logarithmic transformation, with a mean squared error of 17% and a coefficient of determination of 0.84. The equation was:

$$Y = 103.289 + 0.017498\ (D^2\ H) \tag{1.3}$$

where Y is the estimating dry weight of trees in kg, D is the diameter at breast height in cm and H is the total tree height in m.

Quintana (2008) developed an equation for seven-year-old trees from a plantation located in the VI region of Chile. Trees presented on average a height of 1,507 mm and a diameter in the bottom of the main stem of 39 mm. Three different models were tried: lineal regression, allometric and potential model. The one that fitted best was the potential, which presented values of 72.1% for the mean squared error and 0.95 for the coefficient of determination. The equation developed for dry weight was:

$$Y = 0.000008253\ (B^2)^{0.61781}\ (B\ H)^{0.65135} \tag{1.4}$$

where Y is the estimating dry weight of trees in kg, B is the basal stem diameter in mm and H is the total height of the tree in mm.

Most of the equations described above were developed for estimating the biomass for adult native trees, not for trees of plantations. Only Quintana (2008) developed equations for young plantations. In general terms, equations were fitted using mean squared error and the coefficient of determination was calculated to determine the correlation between the dependent and independent variables. By having those values as common parameters among the different equations, it is possible to compare between each other how models fitted empiric values.

1.2 *Quillaja* saponins

Saponins are glycosides present in more than 100 different families of plants (Güçlü-Üstündag & Mazza, 2007) such as quillaja, yucca, ginseng, among others. Specifically, *Quillaja* saponins consist of a triterpene with two sugars moieties attached at positions at carbon positions 3 and 28. (van Setten & van de Werken, 1996). Figure 1-1 shows the basic structure of *Quillaja* saponins. At least 50 different saponins have being identified from *Quillaja* extracts (van Setten & van de Werken, 1996, Nord & Kenne, 1999).

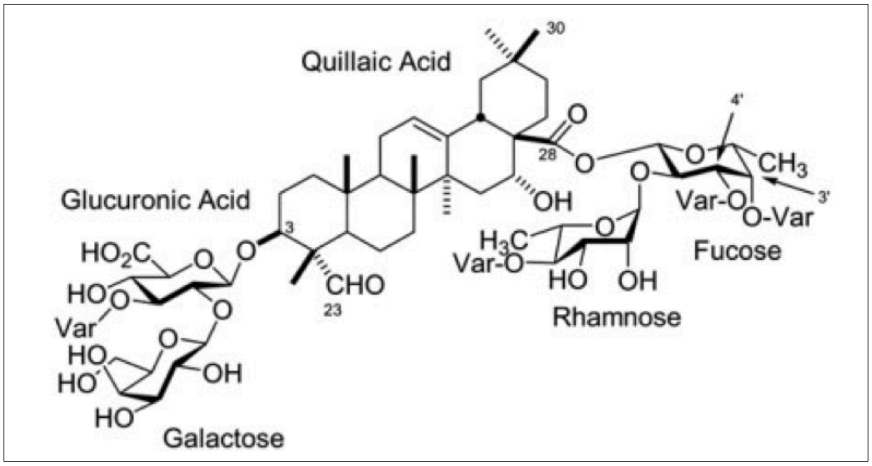

Figure 1-1 Basic structure of *Quillaja* saponins. The triterpene is usually a quillaic acid. It contains several sugars, like rhamnose, fucose, glucuronic acid and galactose (Nord & Kenne, 1999, Grandón et al., 2013).

It has been reported that *Quillaja* saponin content in adult trees varies within the genetic origin, the component of the plant used for the extraction and the environmental factors. With respect to the genetics of trees, Kamstrup *et al.* (2000) observed that trees from similar locations presented different saponin composition due to genetic factors, rather than a selective pressure from external factors (soil type, altitude or tree age). In terms of the component of plants, Grandón et al. (2013) found values of saponins between 1.2% and 2.2% in bark while San Martin (2000) reported 5% in bark and 1.2% in branches. Copaja et al. (2003) found 5.8 and 14.4% of saponins in twigs. Finally, Copaja et al. (2003) concluded that environmental factors do affect the production of saponins, which found that *Quillaja* trees submitted to poor agricultural conditions, such as low water content and poor soil quality had higher saponin content. All saponin contents mentioned above were determined using comparable HPLC methods.

1.2.1 Methods of extraction

Two different processes are used for extracting *Quillaja* saponins. One uses bark as raw material and the other utilizes the whole *Quillaja* wood (San Martin, 2000).

The one using bark consists on first peeling the bark from 30 to 40 years old trees, during the months of October to December (spring). This material is then dried for storing or direct exporting. Later, it is chipped for the extraction process, which can be carried out using water and/or alcohol. Additives are added for stabilization. Filtration is carried out with diatomaceous earth to remove unwanted extractives. Finally, evaporation is used for concentrating the products and it is hot-packed or spray dried (San Martin, 2000).

The process that considers the use of the whole wood, including the bark, is very similar to that described above; the difference lies in the initial raw material. By using both, wood and bark, the ecologic damage generated by the traditional production process is reduced, as there is no need for felling and debarking trees.

The process that includes all the wood impacted economically the *Quillaja* market. Figure 1-2 shows the decrease in the bark's exportation due to the development in Chile of the production process using wood and the increase in the exportation of elaborated products (*Quillaja* extracts) since 1996, respectively (Instituto Forestal, 2012). The decrease of bark exportations, which were sent mainly to the United States, Japan and Germany, was from approximately 1,000 tons to 200 tons. The increase in the exportations of elaborated *Quillaja* products was from cero to 1,000 tons, from 1999 up to 2012.

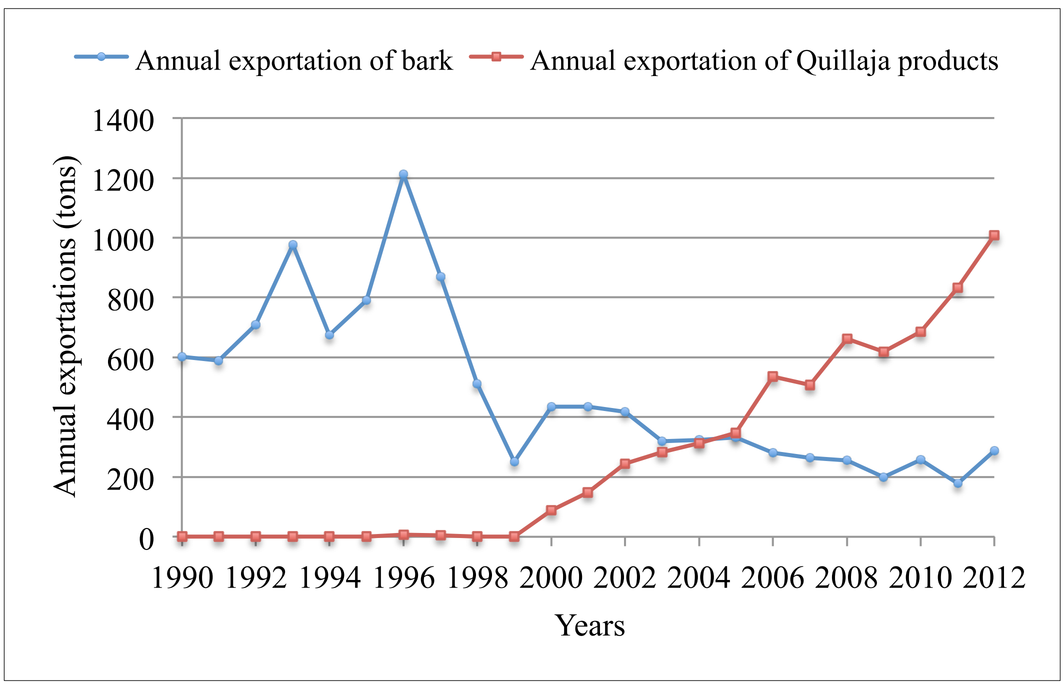

Figure 1-2 Annual exportations of *Quillaja* bark and products between 1990 and 2012 in tons, contemplating production process using bark and wood.

1.2.2 Uses and economic importance

Due to the chemical and biological properties that saponins have, such as tensoactive, emulsifier and surfactant property, antibacterial, insecticide, antioxidant, among others, saponins are widely used in various industries (Grandón et al. 2013). These industries include mining, agriculture, human food and pharmaceutical industries. Also, *Quillaja* saponins are registered as a GRAS product (Generally recognized as safe) and they are approved for human consumption in the USA and the European Union.

The main applications are (San Martin & Briones, 1999, van Setten & van de Werken, 1996):

1. Pharmaceutical: saponins are used as adjuvants for human (ie. Malaria) and animal (ie. Foot-and-mouth disease) vaccines and pharmaceutical applications as a suspension stabilizer.

2. Cosmetic industry: surfactant and emulsifier in shampoos, exfoliating agents, lipsticks and other cosmetic products.

3. Mining: for water treatments and for controlling and reducing acid mist in electrowinning of metals, like copper and zinc.

4. Agriculture: saponins are used in the formulation of pesticides such as fungicides, nematicides and insecticides.

5. Human food industry: foaming agent, emulsifier, flavor enhancer and surfactants for beverage and food. Also, as an ingredient in low-cholesterol foods.

6. Animal food industry: additives for food to prevent pathogen infections, for reducing the emission of ammonium in feces and to reduce cholesterol in eggs.

1.2.3 Saponins content in aerial parts of the tree

The different parts of *Quillaja* trees contain distinct amounts of saponins. Also, differences are observed between the same parts of the trees. It has been reported that the differences in saponin contents could be associated to different factors, such as the method of extraction utilized, genetics of trees, seasonal changes, among others (Kamstrup et al., 2000, Copaja et al., 2003, Szakiel et al., 2011, Grandón et al., 2013).

In the case of bark, Grandón et al. (2013) found values of saponins between 1.2% and 2.2% of dry weight, with an extraction done using 2 g of dried bark and 50 mL of MeOH: H_2O (7/3, v/v) at room temperature for 24 hours. While San Martin (2000) reported 5% in bark, with the common water extraction method, at 50 – 60 ˚C for 3 hours. Copaja et al. (2003) determined values between 8.6 and 15.8% when the extraction was done with water in a soxhlet apparatus for 10 hours at a temperature of 100 ˚C and 6.5 to 8.5% when extracting with water by maceration at room temperature for 24 hours. All saponin contents mentioned above were determined using comparable HPLC methods.

For branches, San Martin (2000) reported values of 1.2% of dry weight, also using water extraction and HPLC for saponin detection. These results contrast with the ones found by Copaja et al. (2003) of 2.3 and 6.7% with water in a soxhlet apparatus and 2.3 to 3.9% with water extraction by maceration. Both saponin contents determined by Copaja et al. (2003) were using HPLC method.

There is a lack of reports for the case of leaves from adult trees, because until now the use of leaves of adult trees has been excluded. Probably the exclusion is related with the low contribution leaves have with respect to the weight of the whole tree, as leaves only represent 4 to 6% of the total weight of adult trees (Sfeir, 1990, Pulido, 2000). Furthermore, today leaves are not perceived to have an economic value. This is supported by the inexistence of registration in volumes of exportation neither economic gain due to the usage of leaves (Instituto Forestal, 2012).

It is important to point out that all values described above are for adult native trees. For the case of young ones, there is a lack of information in the literature, as the use of plantations is a new technique for obtaining *Quillaja* biomass.

The only report belongs to Sfeir (1990), who studied young *Quillaja* trees from the Experimental Center belonging to the Department of Agriculture and Forestry of Universidad de Chile, located in Talca Province (VII region). Trees were four-years-old when analyses were done. In leaves and stems, the annual average of saponin concentration was 4.28% and 10.86% with respect to dry initial matter, respectively (Sfeir, 1990). The method used to determine saponin contents was based on the one proposed by Campos (1970). It consisted of an alcoholic extraction with 10 g of chipped material and 250 mL of ethanol for 24 hours in a soxhlet apparatus. The alcohol was then evaporated and the remaining was mixed with 0.5 g of calcium oxide per 100 mL of solution. The solution was then filtered. To remove the excess of calcium oxide, the solution was exposed to a flow of carbon dioxide. Finally, the solution was filtered again and dried at low temperature.

2. HYPOTHESES

For the present research, the hypotheses are:

Hip1 Leaves of young *Quillaja* trees from plantations contain considerable amounts of saponins, making its processing key to attain good yields.

Hip2 Saponin production in leaves of young *Quillaja* trees suffers variations during the different seasons of the year.

Hip3 Saponin production in leaves is different within young *Quillaja* trees that have not been selected and domesticated.

3. THE USE OF LEAVES FROM YOUNG TREES OF *QUILLAJA SAPONARIA* (MOLINA) PLANTATIONS AS A NEW SOURCE OF SAPONINS

Trinidad Schlotterbeck, Mario Castillo-Ruiz, Hernán Cañon-Jones, Ricardo San Martin

ABSTRACT

Quillaja saponaria is an endemic tree in Chile. It has been extensively exploited for the extraction of saponins, which are used in mining, agriculture and in the pharmaceuticals and food industries. The overexploitation of native adult *Quillaja* forests has brought the necessity to establish plantations. Until now biomass has been obtained from wood and bark of native adult trees, excluding the use of leaves due to their low biomass contribution to the whole tree, and also because industrial production lines have been designed specifically for wood and bark and not for leaves that contain different compounds (e.g. chlorophyll).

In this study, we propose using complete trees from young plantations including the leaves. Quantification of saponins in leaves of 24 three-year-old *Quillaja* trees from a plantation was carried out during the four seasons of one year. Our results showed that leaves represent 27.8% of the weight of the whole young trees and that leaves contain on average 2.58% of saponins. Considering that this amount is similar to that found in

wood, we argue that leaves could contribute up to one-third of the saponins of young trees, making its processing key to attain good yields.

Significant differences were observed in saponin contents during the four seasons. On average, the lowest concentration was found during winter (July = 1.39% dry weight) and the highest in autumn (April = 4.49%), suggesting that abiotic factors may have an effect on saponin production. A comparison between the 24 trees showed significant differences in saponin concentration among trees (0.90 to 4.71%), supporting the need to select consistently superior saponin-producing trees.

3.1 Introduction

Quillaja saponaria Molina, commonly known as Quillay, is a native Chilean evergreen tree that belongs to the sclerophyll forest of Chile. It grows between the IV (30˚ S) and IX (38˚ S) regions (Donoso et al., 2011). The tree has been used extensively for extraction of saponins, which are high molecular weight glycosides containing a hydrophobic triterpenic nucleus and two hydrophilic sugar chains (San Martín & Briones, 2000, Nord & Kenne, 1999). *Quillaja* saponins have been traditionally used in various industries, such as biopesticides and plant growth stimulant in agriculture (Zúñiga et al., 2012, Chapagain et al., 2007, Fischer et al., 2011), emulsifier and foaming agent for animal and human food and beverage (Yang et al., 2013), wetting agent in film production and mining (San Martin et al., 2005b, San Martin et al., 2005a) and as vaccine adjuvants for human and non-human animals vaccines (Kensil et al., 1991, Kensil & Kammer, 1998, Barhate et al., 2013, Sun et al., 2009).

Due to the diverse uses of *Quillaja* saponins described above and a rise of demand for *Quillaja*-based products, exploitation of native trees has increased in the last few years. Between 2009 and 2012, harvests increased from 5,700 and 11,600 tons of *Quillaja* biomass, representing a mean annual growth rate of 19.4% (Instituto Forestal, 2012). With those demands, estimations indicate that approximately 40,000 and 48,000 tons of biomass could be needed by 2019 and 2020. Native forests could not cover those values, as the sustainable limit of native forests has been estimated in 27,000 tons per year. This, because approximately 200,000 hectares are available mainly with native *Quillaja* forests, and each hectare produces 2 tons of biomass every 15 years (San Martin, 2000). Therefore, the sustainable limit of native forests could not cover the annual demand by 2020 (Honeyman, 2013).

The historical source of saponins for extraction has only involved native trees (Correa & Martínez, 2013). Two different processes are developed for the extraction of *Quillaja* saponins. One is using bark as raw material and the other is utilizing whole *Quillaja* biomass, including wood and bark (San Martin, 2000). Until now, leaves of adult native trees have not been used, due to their low biomass contribution to the whole tree (4 to 6% of the total weight, Sfeir, 1990, Pulido, 2000). Also, the industrial production lines do not considers the removal of the different compounds contained in leaves (e.g. chlorophyll).

With the increased demand for *Quillaja* products, plantations are being studied as an opportunity for supplying the shortage of this native resource and preventing an unsustainable exploitation (Donoso et al. 2011). We argue that by including the leaves in the process that already considers wood and bark, the yields of young trees could be increased, making plantations interesting for the industry.

Ongoing research highlights understanding the best plantation techniques to maximize the biomass obtainment per tree (Copaja et al. 2003, Correa & Martínez 2013, Grandón et al. 2013). However, there is a lack of knowledge in regards to the production of saponins of young plantations (one to seven years old). Several factors might be implicated in the up or down regulation of the saponin production in trees, such as seasonal changes, soil fertility, availability of light and water (Copaja et al. 2003, Szakiel et al. 2011).

Therefore the aim of this study was to evaluate the saponin content in leaves of young trees as a potentially new source of raw material. Also, we researched the influence of seasons in saponin production and the variation among trees.

3.2 Materials and methods

3.2.1 Sample collection

Samples were collected from a three-year-old plantation located at La Candelaria, Colchagua Province, Chile (35º36´40,09´´S; 71º29´57,04´´O). No selection of any kind was done to the seeds used for the plantation. Seeds came from trees of the same region where the plantation is located (VI Region). During the first 8 months trees were grown in a vivarium, until they reached a height of approximately 30 cm and then were planted during October 2010. Irrigation at the plantation was controlled using a drip line and supplying water during November to April, each tree receiving 16 L/month. No artificial application of water was given to trees between May and September, as they received water from rainfall. Mean annual precipitations were 459.62+/-125.40 mm during the past five years (2009 to 2013). Most of the rain period was during May and September with 83.58%+/-13.96% of the total annual precipitations (Dirección Meteorológica de Chile, 2014).

A total of 24 individual trees were used for the extraction and analysis during the four seasons of the year. Samples of 30 grams of leaves with their respective twigs were collected. Sample collections were done on November 2013 (Spring), February 2014 (Summer), April 2014 (Autumn) and July 2014 (Winter). All trees were analyzed to determine the soluble solids and saponin concentrations.

3.2.2 Determination of total tree biomass

The same 24 selected trees were cut down after the one-year period of analysis. Leaves, branches and trunk were separated and weighted to determine their contribution to the total tree biomass. The 30 grams of leaves taken during the four periods of the year were added to the final weight of each tree.

3.2.3 Extraction and quantification of soluble solids from leaves

The extractions were done on the same trees during each season of the year. Samples were grinded using a mixer for 20 s to obtain a maximum particle size of 5 mm. Two grams of chopped leaves were weighted using an analytical scale and then air-dried for 18 h at 70 ˚C to determine dried weight of each raw material (W_i).

For soluble solids and saponins extraction, 10 g of recently grinded material were mixed with 30 mL of water and incubated for 2 h at 50˚C. The extracted material was filtered using a Whatman filter paper N˚2.

To quantify soluble solids from samples, a 2 mL aliquot of the filtered liquid was weighted and dried for 18 h at 70 ºC using the oven until constant weight was achieved (W_f). Extractable soluble solids were expressed as g of soluble solids/100 g of dried plant and calculated as $\frac{W_f}{W_i} \cdot 100$. All the quantifications were carried out in triplicates.

3.2.4 Quantification and analysis of saponins in leaves

Estimation of saponin content was determined by RP-UPLC based on the procedure described by San Martín and Briones (2000) for RP-HPLC. Briefly, an aliquot from extracts of trees was filtered (0.22 μm) and analyzed by reverse phase chromatography on a C_{18} column, using a gradient of 34-45% ultrapure acetonitrile in ultrapure water with 0.15% v/v formic acid. The wavelength for determination of saponin content in the aliquot was carried out at 210 nm (Kamstrup et al., 2000). The total concentration of saponins (C_s) in samples was determined using the chromatographic area of all saponin peaks. A highly purified standard (> 90% total saponins purity, Supersap®, Natural Response) was used as a control for saponins profiling, quantification and to produce a calibration curve to quantify each saponin previously described. Saponin content was calculated as g of saponins in 100 g dried initial material $\frac{C_s}{W_i/V_i} \cdot 100$.

3.2.5 Nutritional analysis of soil

Soils samples were taken at 20 cm height, 20 cm width and 5 cm depth from each tree (within the area covered by the treetop) were absorbing roots are located. The edaphic level was carried out for electric conductivity (CE), nitrogen content (N), organic matter content (OM), phosphorus content (P), potassium content (K) and pH. All procedures were done according to the methods for soil and sludge (Zagal & Sadzawka, 2007).

Electric conductivity and pH analyses were carried out by mixing 20 g of dried soil with 50 mL of water at 20-25 ˚C to dissolve salts and then filtered. Nitrogen content was first extracted in a solution of 2 mol/L KCl, then NH_3 was distillated and finally, titration was used for determining nitrogen. Organic matter content was obtained by oxidation with dichromate in an acid medium and determined using colorimetric method (Zagal and Sadzawka, 2007). Olsen's method was used to determine phosphorus (Grandón et al., 2013). Briefly, phosphorus was extracted in a solution of 0.5 mol/L $NaHCO_3$ at pH 8.5 and detected by colorimetric technique. Potassium was extracted in a solution of 1 mol/L $NH_4C_2H_3O_2$ at pH 7.0 and detection was carried out using spectrophotometry emission.

3.2.6 Statistical analysis

Descriptive statistical analysis and Kruskal – Wallis tests were carried out to determine any differences occurring in soluble solids and saponin contents between the 24 trees and during the four seasons of the year. Paired Tukey tests were performed whenever differences existed. Pearson, Kendall and Spearman tests were done to determine the existence of correlation between saponin content and the different edaphic levels of soils. Differences were considered at $p < 0.05$. All statistical analysis were carried out using the free statistical software R (R Development Core Team, 2014).

3.3 Results and discussion

The contents of soluble solids and saponins during the four seasons of the year can be observed in Figure 3-1 to 3-4. Figure 3-1 shows annual soluble solids contents in leaves, which varied between 27.49+/-3.07 and 37.19+/-5.62% amongst the 24 trees. The analysis in each season, considering all trees, showed that soluble solids contents are in a range of 27.75+/-2.72 (winter) and 35.07+/-2.36% (autumn) as shown in Figure 3-2. In the case of saponin contents, Figure 3-3 shows the annual values in leaves, which varied between 0.90+/-0.34 and 4.71+/-1.99% amongst the 24 trees. As shown in Figure 3-4, during the different seasons the lowest concentration was in winter (July = 1.39+/-0.81%) and the highest in autumn (April = 4.49+/-1.84%), taking into account all trees.

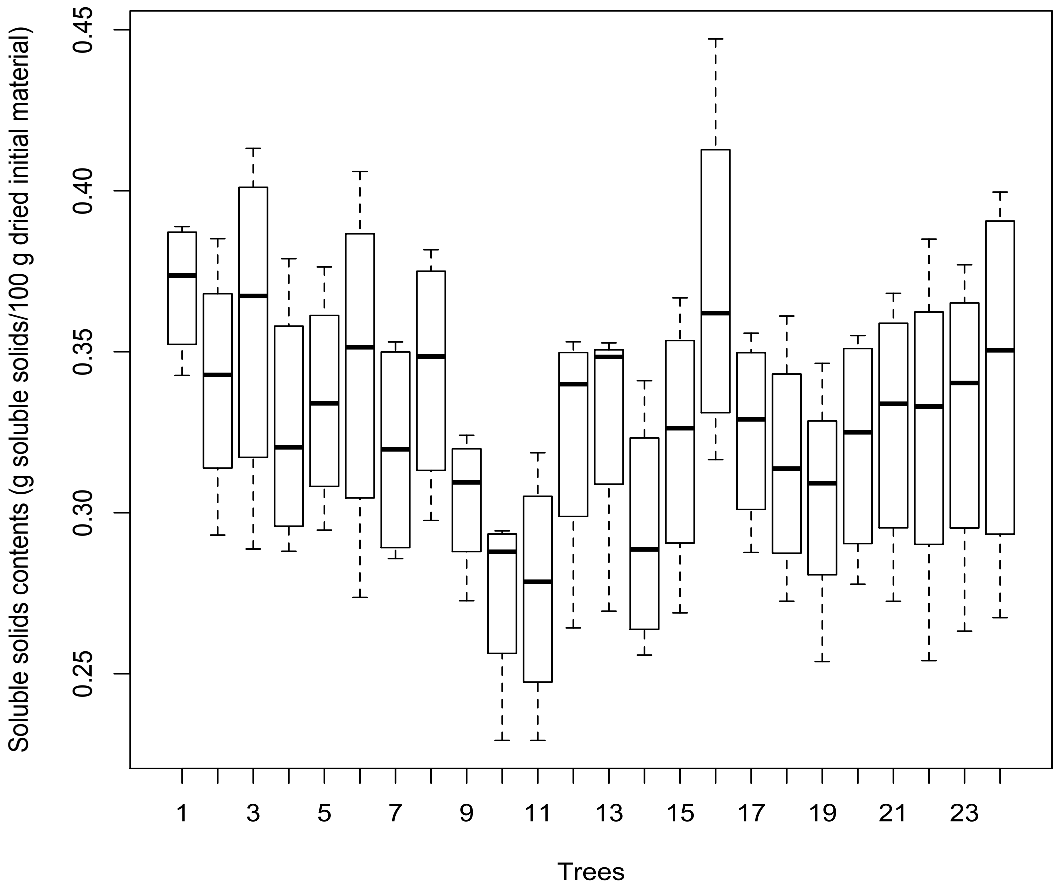

Figure 3-1 Annual distributions of soluble solids content in the leaves of the 24 trees (N=4, X^2 = 26.52, df = 23, p = 0.2769).

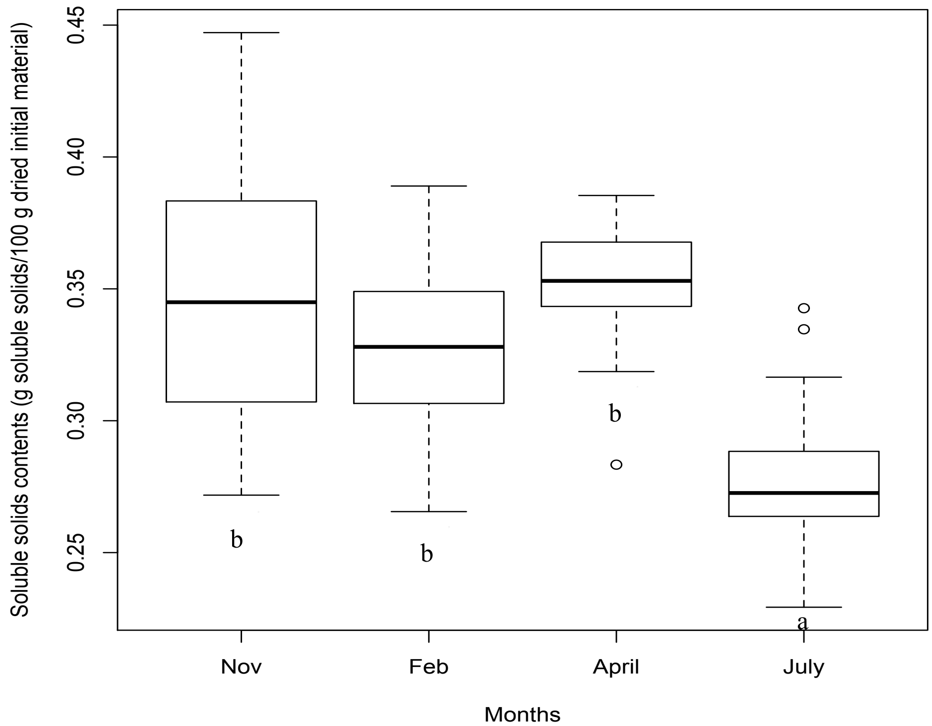

Figure 3-2 Soluble solids contents of leaves in the 24 trees according to the four sampling months (N=24, X^2 = 43.63, df = 3, p < 0.001).

These results represent the first evidence of the contribution of saponins in leaves of young *Quillaja* trees from plantations. Saponin contents were in accordance to that found for *Quillaja* bark, trunks and branches. Grandón et al. (2013) found values of saponins between 1.2% and 2.2% in bark. While San Martin (2000) reported 5% in bark and 1.2% in branches. However, our results contrast the ones found by Copaja et al. (2003) of 5.8 and 14.4% of saponins in twigs. These differences may be attributed to different extraction procedures, as Grandón et al.

(2013) used an alcoholic extraction with 2 g of dried bark in 50 mL MeOH: H_2O (7/3, v/v) at room temperature for 24 hours. San Martin (2000) described the common water extractive method done at 50-60 ˚C for 3 hours. Copaja et al. (2003) used two different procedures, one done in a soxhlet apparatus with 10 to 20 g of dried material with 300 mL of water at 100 ˚C for 10 hours and the other by maceration in 100 mL of water at room temperature for 24 hours. All saponin contents described above were quantified using reverse phase HPLC.

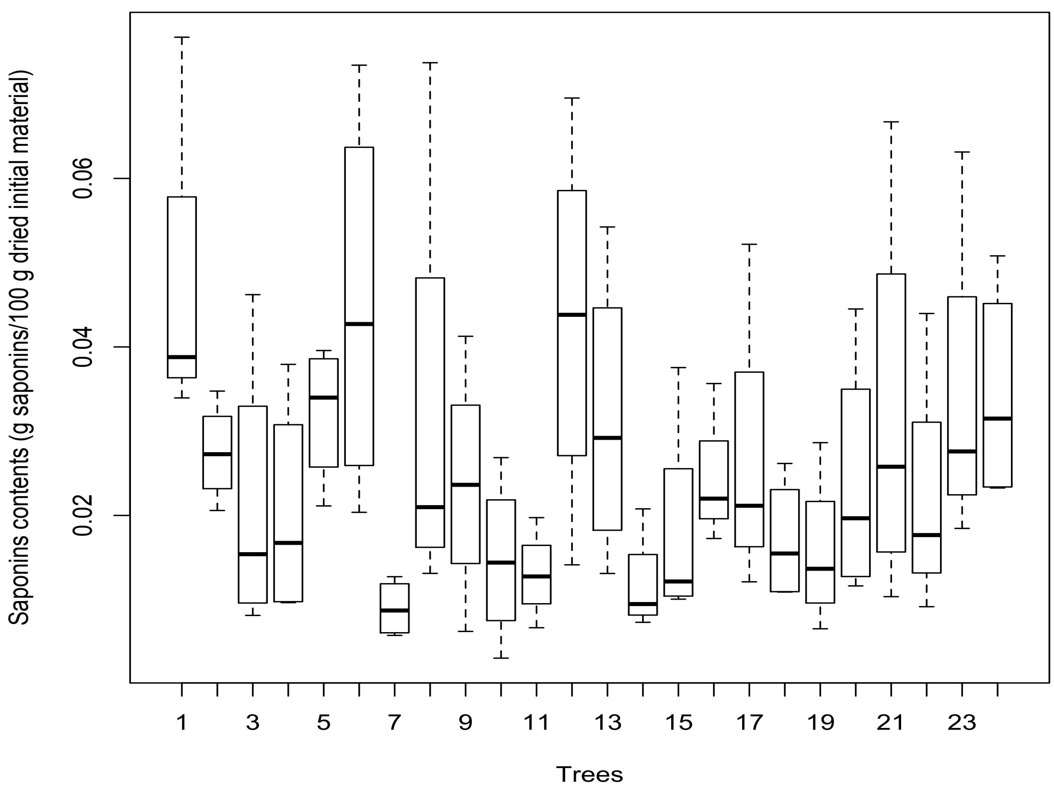

Figure 3-3 Annual distributions of saponin content in the leaves of the

24 trees (N=4, X^2 = 39.56, df = 23, p = 0.017)

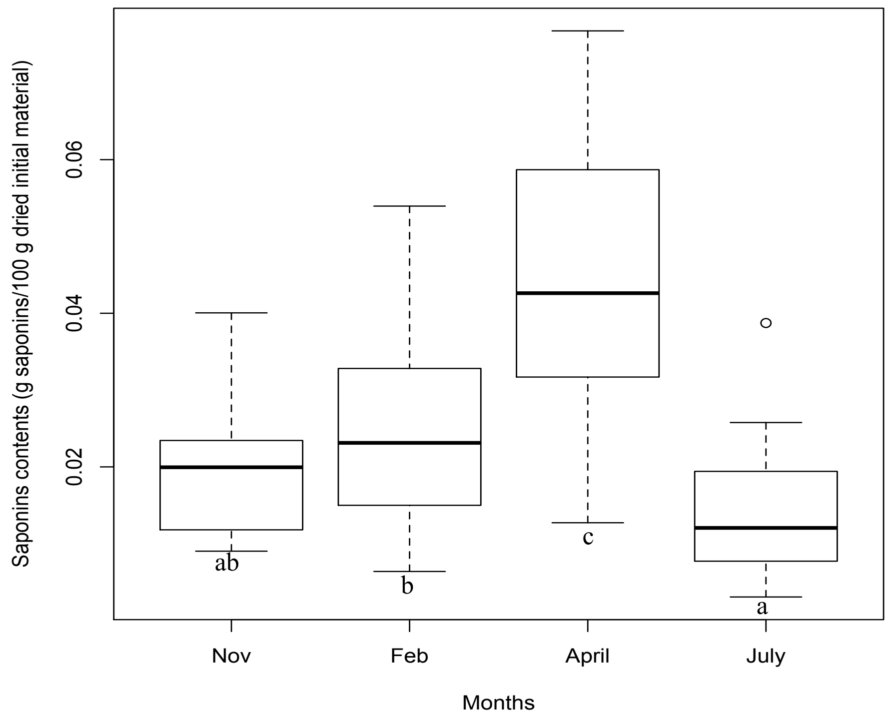

Figure 3-4 Saponin contents of leaves in the 24 trees according to the four sampling months (N=24, $X^2 = 42.02$, df = 3, $p < 0.001$).

To corroborate the contribution in biomass of the leaves as raw material in *Quillaja* trees, we weighted leaves, branches and trunk separately at the end of the study, as shown in Table 3-1. Our results showed that leaves represented 27.8+/-5.7% of the weight of the whole three-year-old tree. This result was lower to previously reported studies (Donoso et al., 2011), which showed that 52.9% of the weight of two-years-old trees was from leaves. The differences in biomass contribution may be attributed to different cultivation techniques, soil composition, sampling season and that two- years-old trees could be bushier than older ones.

Table 3-1 Contribution of leaves, branches and trunk to the total weight of the sampled *Quillaja* trees

Tree	Total weight (kg)	Contribution of leaves, branches and trunk to whole tree (kg)		
		Leaves	Branches	Trunk
1	6.7	2.4	2.5	1.8
2	3.6	1.2	1.3	1.1
3	1.4	0.4	0.3	0.7
4	8.4	2.2	4.2	2
5	1.2	0.4	0.5	0.3
6	0.7	0.3	0.1	0.3
7	5.1	1.3	3.2	0.6
8	4.5	1.1	2.4	1
9	5.4	1.5	2.5	1.4
10	9.8	2.6	5.7	1.5
11	4.6	1	2.8	0.8
12	4.1	1.1	2	1
13	21.8	6	12.8	3
14	5.1	1.5	2.5	1.1
15	2.6	0.6	1.1	0.9
16	2.6	1	0.8	0.8
17	9.5	2.7	5.1	1.7
18	8	1.6	5.1	1.3
19	9.2	2.2	4.9	2.1
20	3	0.9	1.6	0.5
21	6.7	1.3	3.6	1.8
22	4.7	1.2	2.7	0.8
23	4.4	1	2	1.4
24	12.4	3.1	7.1	2.2

From a productive point of view, the use of leaves of young trees as an additional raw material could increase up to one-third the current total yield per tree. This, since the amount of saponins in leaves was on average 2.58%, similar to that found in woody parts of native trees (Grandón et al., 2013, San Martin, 2000, Copaja et al., 2003) and leaves represented almost 30% of the weight of the whole tree. This could make processing of leaves of young trees key to attain good yields per hectare.

These results suggest a new way of obtaining *Quillaja* raw material, by including leaves of young trees as a source for saponins, as well as the currently used (wood and bark). Using whole young trees could denote a significant ecological impact by reducing the use of native trees, preserving natural forest and increasing the plantations of young *Quillaja* trees. Therefore, future studies could quantify the ecologic impact of including leaves as a new raw material. These, by evaluating how many less trees are needed if leaves are also used as raw material, including them to the current ones (wood and bark). Also, evaluate the economic opportunity of developing new *Quillaja* plantations as new businesses (Honeyman, 2014). Probably no importance was given to leaves when using adult native trees, because they only represent between 4 to 6% of the biomass of adult trees (Sfeir, 1990, Pulido, 2000).

Our results showed a statistically significant difference in the saponin content between the 24 trees, independently of the sampling months (Figure 3-3, X^2 = 39.56, df = 23, p = 0.017). For example, trees 1, 6 and 12 showed higher amounts of saponins during the whole study. On the other hand, trees 7, 11 and 14 were consistently low saponins producers during the same four periods. These results suggest the implementation of selection and domestication of *Quillaja* trees as an agricultural method to farm trees superior in the production of saponins. Therefore, plantations could contain only trees that produce high amount of saponins, as it is the molecule of interest.

It is important to point out that no significant difference was observed in the production of soluble solids between the 24 trees (Figure 3-1, X^2 = 26.52, df = 23, p = 0.2769). This suggests that the production of saponins is mediated by a specific response, because variability between trees exists for saponins, but not for soluble solids. For this reason, soluble solids contents cannot be used as indicator of a hyper saponin producer tree. Future research should be aimed to understand the underlying mechanism of saponin production, irrespective of soluble solid content.

Also, the 24 trees of our study produced saponin contents differently between each season of the year (Figure 3-4, X^2 = 42.02, df = 3, $p < 0.001$). During spring (November), summer (February), autumn (April) and winter (July) saponin contents were 1.96%, 2.50%, 4.49% and 1.39%, respectively. Our results showed that during summer and autumn trees accumulated more saponins. As it has been reported, this could be related to abiotic factors such as water deficit during dry periods of summer, frosts during winter, different annual amounts of sunlight received by the plants, position in the field or others (Copaja et al., 2003, Correa & Martínez, 2013, Szakiel et al. 2011). Also, it may be due to the fact that saponins are considered to be part of the defensive system of plants (Grandón et al. 2013), so probably the stress caused during the months of summer generates an extra production of saponins in leaves (Copaja et al. 2003). Taken together, our results suggest that leaves should be harvested in autumn and air-dried for storage, because of the higher saponin contents in that period. In this study, we controlled water delivery during summer and autumn, therefore future studies should be conducted to determine other variables such as wind exposure or evaporation rate that may be influencing our results.

Soluble solids contents did not show statistical significant differences (Tukey Contrasts, $p > 0.1$) between spring (November = 34.67%), summer (February = 32.98%) and autumn (April = 35.07%). The only statistical significantly lower value was during winter (Figure 3-2, $X^2 = 43.63$, df = 3, $p < 0.001$) with 27.75% of soluble solids with respect to dried matter. This finding could be explained by the physiological phase of dormancy of trees (Szakiel et al., 2011). These results contribute to explain that abiotic factors may influence the production of specific metabolites, like saponins, and in turn, do not affect significantly soluble solids contents.

Soil sample from trees did not show significant differences for EC, K, N, OM, P and pH (Kruskal – Wallis tests, $p > 0.1$). Electric conductivities were 0.03 +/- 0.01 mS/cm, potassium 97 +/- 26 mg/kg, nitrogen had values of 15 +/- 7 mg/kg, organic matter 2.31 +/- 0.61%, phosphorus of 8 +/- 4 mg/kg and pH 5.71 +/- 0.34. Nitrogen levels were within the range for the coastal and central zones of Chile reported by Grandón et al. (13.9 +/- 10 and 14 +/- 9 mg/kg, respectively) P, K and OM content were approximately 60% lower than those reported for the coastal zone of Chile (Grandón et al., 2013). No correlation was observed between the differences in saponin contents in the 24 trees and quality of their soils (Pearson, Kendall & Spearman Correlation tests, $p > 0.1$). Therefore, the edaphic characterization of soils of each tree cannot explain the differences in the contents of the metabolite of interest.

It is noteworthy that K, N and P soil contents were lower than the recommended ranges for applying fertilizers for growth, which are between 100 and 150 mg/kg for potassium, 50 and 150 mg/kg for nitrogen and 60 to 100 mg/kg for phosphorus (Quiroz et al., 2009). In accordance to Copaja *et al*. (2003) who concluded that *Quillaja* trees exposed to poor soil quality had higher saponin content, these trees could be benefited in terms of saponin production due to the poor soil.

Extraction methods are an important part for the recovery of saponins within tree tissues. Our study and most of the cited herein utilizes chipping, water extraction and temperature to release saponins from tree tissues (Kensil et al., 1991, Kamstrup et al., 2000, San Martin & Briones, 2000, San Martin, 2000, Copaja et al., 2003). However, recent studies have used other techniques such as ultrasound, microwave, accelerated solvent, pulse electric field and enzyme-assisted extractions to obtain saponins and other bioactive compounds in plant species (Azmir et al., 2013, Cheok et al., 2014, Gaete-Garreton et al., 2011). For example, Cares et al (2010) showed that using ultrasound enhances the extraction from *Quillaja* stems and bark and reduced from 3 hours to 20 minutes and temperature from 60˚C to 20˚C to obtain the same amount of extracted solids. Therefore, future studies should aim to optimize the extraction process of saponins from leaves, based on the results that indicated leaves had similar amounts of saponins that wood and bark. This, to evaluate techniques that could enhance the extraction of *Quillaja* leaves.

3.4 Conclusions

Our results indicate that in a three-years-old plantation, leaves represented on average 27.8% of the weight of the whole tree. Saponin contents in the leaves were on average 2.58% of dry weight, amount that is similar to that found in wood. Leaves of young trees should be used as a new source of raw material to obtain saponins commercially, as the total content of these glycosides is significant and gives economic value to them. Moreover, if leaves of young trees are considered in the production, these can contribute up to one third in the amount of saponins per young tree. This makes the processing of leaves key to attain good yields from trees of young plantations.

In all, our results open the opportunity to improve the quality of raw material by the selection and domestication of superior saponin producing trees under optimal conditions, with the consequent increase in the sustainability of the *Quillaja* native forest in Chile. The disparity of saponin contents between trees during the four seasons of the year (0.90 to 4.71%) suggested that differences exist in terms of the production of saponins. This did not happen in the case of the soluble solids production, as no significant differences were observed.

The follow up of each tree during the four seasons of the year showed a significant difference in the saponin contents, being 1.39% the lowest amount during winter (July) and 4.49% the highest in autumn (April). These results suggested the impact of abiotic factors in the formation of saponins in the trees. Also, that leaves should be collected during autumn, period were the production of saponins was the highest.

Finally, research efforts should also focus in relevant areas such as agronomic/horticultural sciences, as well as the evaluation of the economic and social impact of the use of *Quillaja* trees in order to obtain good and sustainable quality products.

3.5 Acknowledgements

The authors would like to thanks Mr. Hugo Vera and Mr. Patricio Guzmán from Las Palmas *Quillaja* farm for their constant support and collaboration during the sampling of the trees and their innovation in starting the first industrial *Quillaja* domestication. Also, the authors are grateful to Dr. Leandro Padilla of Natural Response S.A. for facilitating the saponin standard, UHPLC analysis and his kind comments and revision of the manuscript.

4. FURTHER DISCUSSION

The measurement of biomass performed to the 24 trees used for the saponin analysis opened the possibility to evaluate the existing equations for estimating biomass using height and diameter. Therefore, the empiric values obtained for this research were used to evaluate the existing equations for estimating *Quillaja* biomass.

This comparison between the real weight of the 24 trees and the ones obtained by using the equations demonstrated the necessity of establishing new equations for young trees from plantations. First, the majority of the existing equations use the diameter at breast height as a variable for biomass estimation. In the case of young trees, that variable cannot be measured always, because some are smaller than 1.3 m (height were the diameter is measured).

On the other hand, the two equations that do not include diameter at breast height (Pulido, 2000 & Quintana, 2008) as a variable, do not estimate precisely the biomass of three-year-old trees from plantations. Table 5-1 has the values of the weight of the 24 trees and the values estimated using the equations of total trees developed by Pulido (2000) and Quintana (2008). Pulido (2000) developed an equation for vegetative regenerated trees located in the interior zone of the VI region. Quintana (2008) developed an equation for seven-year-old trees from a plantation located in the VI region of Chile. Pulido (2000) had values for the mean squared error and the coefficient of determination of 45.86% and 0.98, while Quintana (20008) has values of 72.1% and 0.95, respectively. The coefficient of differentiation for the weight of the 24 trees was of -0.14 using Pulido's equation (2000) and 0.23 for Quintana (2008). As Quintana (2008) mentioned, the coefficient of determination should be higher than 0.83 to achieve a good correlation between the dependent and independent variables. Prado et al (1986) mentioned that values of mean squared error lower than 20% should be considered acceptable for estimating *Quillaja* biomass. The values of mean squared error for the 24 trees and the equations of Quintana (2008) and Pulido (2000) were 449% and 15971%, respectively. Neither equations presented values near the acceptable one.

This opens the opportunity of elaborating equations to estimate biomass in young trees. Specific equations are not only needed for three-years-old plantations, like the one studied within this thesis, also growing equations that could allow having precise data of trees from one to seven years old.

Table 5-1 Comparison between real weight of trees and equations done by Quintana (2008) and Pulido (2000)

Trees	Real dry weight (kg)	Quintana (2008)	Pulido (2000)
1	3.7	2.12	4.30
2	2.0	1.52	3.45
3	0.8	0.85	2.42
4	4.6	2.44	3.95
5	0.7	0.62	1.48
6	0.4	0.57	1.28
7	2.8	2.11	4.30
8	2.5	1.97	4.86
9	3.0	1.54	2.70
10	5.4	2.42	4.48
11	2.5	0.93	1.80
12	2.3	1.12	2.42
13	12.0	4.36	8.17
14	2.8	1.98	4.30
15	1.4	1.38	2.29
16	1.4	1.67	3.61
17	5.2	4.60	10.03
18	4.4	3.59	6.29
19	5.1	2.91	4.86
20	1.7	1.10	3.14
21	3.7	5.61	12.39
22	2.6	2.74	5.86
23	2.4	2.29	4.30
24	6.8	2.79	5.45

The comparison between the existing equations and the empiric weight of trees measured within this thesis, are just one example of the need for new research that allows understanding the behavior of young *Quillaja* trees from plantations. Also, the lack of reports indicating the amount of saponins present in young trees is an opening opportunity for finding further information. Therefore, research efforts should also focus on areas related to young plantations, for understanding the agronomic/horticultural sciences of it and the saponin production capacity. As well as the evaluation of the economic and social impact of using young *Quillaja* trees for achieving a sustainable industry.

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A P P E N D I X

5. APPENDIX A: IMAGES OF TREES

Figure 5-1 Tree n˚ 1

Figure 5-2 Tree n˚ 2

Figure 5-3 Tree n˚ 3

Figure 5-4 Tree n˚ 4

Figure 5-5 Tree n˚ 5

Figure 5-6 Tree n˚ 6

Figure 5-7 Tree n˚ 7

Figure 5-8 Tree n˚ 8

Figure 5-9 Tree n˚ 9

Figure 5-10 Tree n˚ 10

Figure 5-11 Tree n˚ 11

Figure 5-12 Tree n˚ 12

Figure 5-13 Tree n˚ 13

Figure 5-14 Tree n˚ 14

Figure 5-15 Tree n˚ 15

Figure 5-16 Tree n˚ 16

Figure 5-17 Tree n˚ 17

Figure 5-18 Tree n˚ 18

Figure 5-19 Tree n˚ 19

Figure 5-20 Tree n˚ 20

Figure 5-21 Tree n˚ 21

Figure 5-22 Tree n˚ 22

Figure 5-23 Tree n˚ 23

Figure 5-24 Tree n˚ 24