PONTIFICIA UNIVERSIDAD CATOLICA DE CHILE
SCHOOL OF ENGINEERING

OPTIMAL HPL EXTRACTION AND ADSORPTION ISOTHERMS ON AGAROSE OF POLYPHENOLS OF MAQUI (*ARISTOTELIA CHILENSIS* [MOL.] STUNTZ) LEAVES

PAMELA RAQUEL RIVERA TOVAR

Thesis submitted to the Office of Graduate Studies in partial fulfillment of the requirements for the Degree of Doctor in Engineering Sciences

Advisors:
JOSÉ R. PÉREZ CORREA & MARÍA S. MARIOTTI CELIS

Santiago, Chile, August, 2021

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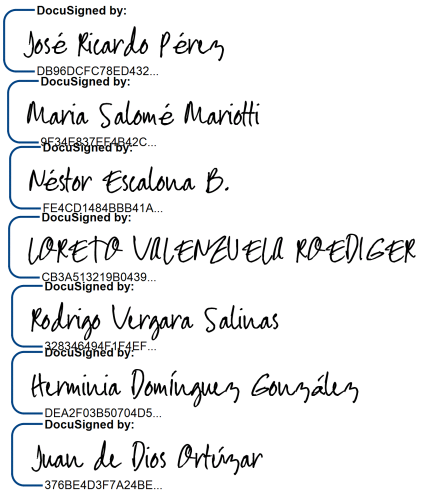

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To my parents, grandparents, sisters, and friends.

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Thesis submitted to the Office of Graduate Studies in partial fulfillment of the requirements for the Degree of Doctor in Engineering Sciences by

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ABSTRACT

In recent years, the production of processed maqui berry has increased dramatically, due to its attractive bioactive properties. Several studies have positioned maqui as one of the natural sources with the highest total polyphenols (highly antioxidant compounds) content. However, due to the complex distribution of the berries in the plant, the maqui industry extracts a significant amount of leaves during harvest, which are discarded. Maqui leaves are also a rich source of polyphenols, with even higher levels (~51%) than berries. Maqui leaves extracts have been shown *in vitro* assays to have antidiabetic and anti-hemolytic effects, and *in vivo* assays, anti-inflammatory, and analgesic effects. Despite these attractive results regarding maqui leaves extracts, only conventional low-yield extraction methods have been used and neither has the extraction process been optimized nor have purification processes been studied for the recovery of polyphenolic extracts from this agro-industrial waste. Therefore, the two hypotheses that guided this research were: (i) application of multi-response optimization to hot pressurized liquid extraction allows to determine the operating conditions that produce polyphenol extracts

with outstanding features from maqui leaves; and (ii) the detailed study of adsorption equilibrium of five maqui leaf polyphenols on agarose allows to characterize the system and generate relevant information for the isolation of these polyphenols by APLC. The methodology used in this thesis involves the following steps: (i) responses adjustment (total polyphenol content, antioxidant capacity and extract purity) to first and second order surfaces and subsequently the maximization of the defined global desirability functions, (ii) characterization of the three optimal extracts in terms of antioxidant capacity (through different reactions) and polyphenolic profile, (iii) evaluation of the effects of liquid phase composition and temperature on adsorption equilibrium of each polyphenol, and (iv) estimation of isothermal and thermodynamic equilibrium parameters for each evaluated scenario. The results of this thesis will allow the use of maqui leaves, which is currently an agro-industrial waste, to produce a functional ingredient with attractive polyphenolic characteristics for the food industry. In addition, the adsorption results will allow efficient and accurate design, optimization and scaling-up of adsorption preparative liquid chromatography for isolation of five polyphenols from natural extracts.

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PONTIFICIA UNIVERSIDAD CATÓLICA DE CHILE

ESCUELA DE INGENIERÍA

EXTRACCIÓN HPL OPTIMA E ISOTERMAS DE ADSORCIÓN EN AGAROSA DE POLIFENOLES DE HOJAS DE MAQUI (*ARISTOTELIA CHILENSIS* [MOL.] STUNTZ)

Tesis enviada a la Dirección de Postgrado en cumplimiento parcial de los requisitos para el grado de Doctor en Ciencias de la Ingeniería.

PAMELA RAQUEL RIVERA TOVAR

RESUMEN

En los últimos años, la producción de procesados de maqui ha aumentado drásticamente, debido a sus atractivas propiedades bioactivas. Varios estudios han posicionado el maqui entre las fuentes naturales con mayor contenido de polifenoles (compuestos altamente antioxidantes) totales. Sin embargo, debido a la compleja distribución de las bayas en la planta, la industria del maqui extrae una cantidad significativa de hojas durante la cosecha, las cuales son descartadas. Las hojas de maqui son también una rica fuente de polifenoles, incluso con mayores niveles (~51%) que las bayas. Los extractos de hoja de maqui han demostrado en estudios *in vitro* poseer efectos antidiabéticos y anti-hemolíticos, y en estudios *in vivo*, efectos antiinflamatorios y analgésicos. A pesar de estos resultados atractivos acerca de los extractos de hojas de maqui, solo se han empleado métodos convencionales de extracción de bajo rendimiento, tampoco se ha optimizado el proceso de extracción, ni se han estudiado procesos de purificación para la recuperación de extractos polifenólicos desde este descarte agroindustrial. Por lo tanto, las dos hipótesis que guiaron esta investigación fueron: (i) la aplicación de la optimización de respuesta

múltiple a la extracción con líquidos calientes presurizados permite determinar las condiciones de operación que producen extractos de polifenoles con características sobresalientes desde hojas de maqui, y (ii) el estudio detallado del equilibrio de adsorción de cinco polifenoles de hojas de maqui en agarosa permite caracterizar el sistema y generar información relevante para el aislamiento de estos polifenoles mediante la cromatografía líquida preparativa de adsorción. La metodología empleada en esta tesis implica los siguientes pasos: (i) el ajuste de las respuestas (contenido total de polifenoles, capacidad antioxidante y pureza polifenólica del extracto) a superficies de primer y segundo orden y posteriormente la maximización de funciones de deseabilidad global, (ii) la caracterización de tres extractos óptimos en términos de capacidad antioxidante (mediante diferentes reacciones) y perfil polifenólico, (iii) la evaluación de los efectos composición de la fase líquida y temperatura sobre el equilibrio de adsorción de cada polifenol, y (iv) la estimación de parámetros de equilibrio isotérmico y termodinámico para cada uno de los escenarios evaluados. Los resultados de esta tesis permitirán la valorización de las hojas de maqui, que actualmente son un descarte agroindustrial, para producir ingredientes funcionales con características polifenólicas atractivas para la industria alimentaria. Además, los resultados de adsorción permitirán el diseño, la optimización y el escalamiento eficientes y precisos de la cromatografía líquida preparativa de adsorción para el aislamiento de cinco polifenoles desde extractos naturales.

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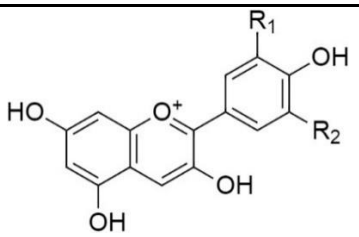
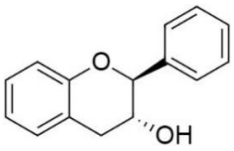
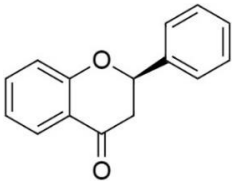
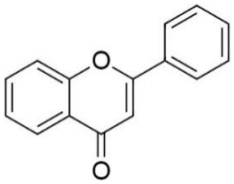
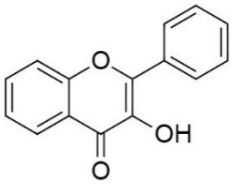
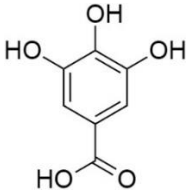
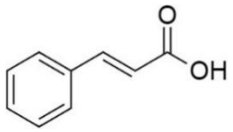
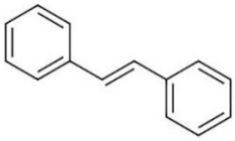
CHAPTER 1. INTRODUCTION

1.1. Polyphenols, natural antioxidant compounds: origin, molecular structure, and applications

Polyphenols are a prominent class of secondary metabolites in plants, which generate them in response to challenges from external factors such as UV radiation, herbivores, and microbial infections, and others (Beart et al., 1985; Mierziak et al., 2014). Since these compounds do not participate in primary metabolic functions such as photosynthesis, they are not present in uniform form and quantity in all plants. The polyphenols molecular structures contain one or more benzene rings with at least one hydroxyl group attached. This basic structure can be combined with mono and polysaccharides. There are more than 8,000 combinations that have been classified according to their molecular structure (Landete, 2012; Speisky et al., 2017). The most studied and most abundant groups and subgroups of polyphenols found in natural matrices are flavonoids (anthocyanins, flavanols, flavanones, flavones, and flavonols), phenolic acids (hydroxybenzoic and hydroxycinnamic acids), and stilbenes (Table 1-1) (Neveu et al., 2010).

These compounds have high antioxidant capacities; therefore, they can neutralize ROS (reactive species derived from oxygen) produced in excess by the human body when tobacco smoke, herbicides, pesticides, air pollution, high-fat diet, or others are present. For this reason, polyphenols are considered effective compounds for the prevention and slowing down of chronic, degenerative, and vascular diseases associated with oxidative stress (excess ROS) (Petti & Scully, 2009).

Table 1-1: Classification of polyphenols according to their molecular structure.

Group	Subgroup	Base molecular structure
Flavonoids	Anthocyanins	
	Flavanols	
	Flavanones	
	Flavones	
	Flavonols	
Phenolic acids	Hydroxybenzoic acids	
	Hydroxycinnamic acids	
Stilbenes	Stilbenes	

Polyphenols are incorporated into the human body generally through food intake, and then these undergo different transformations in different parts of the body. In the stomach only a small fraction of some low molecular weight polyphenols can be absorbed. In the small intestine, aglycones (species without associated non-phenolic compounds) and anthocyanins are absorbed directly, whereas species with non-phenolic bonds must be cleaved first. High molecular weight polyphenols (hydrolyzable tannins or oligomers) are not absorbed but can be partially depolymerized. The absorbed polyphenols reach the liver, where they are subjected to conjugations with one or more constituents to modify the hydrophilic/hydrophobic balance and thus facilitate their transport and excretion. Finally, those entering the bloodstream can exert their effect on different organs, either immediately or after their accumulation (Torres et al., 2017). Once in the active site, the main action mechanism is the oxidative reaction in which polyphenols are oxidized by ROS, thus maintaining the stability of essential biomolecules such as proteins and DNA. Some polyphenols can also act as chelating agents, that is, they can react with certain transition metals (such as copper and iron), preventing the formation of ROS (Del Rio et al., 2010; Petti & Scully, 2009).

Therefore, polyphenols are being investigated to develop new nutritional applications that aim to prevent diseases and improve skin health, specifically, the development of nutricosmetics from natural polyphenolic extracts (Khan et al., 2019). Studies on the potential of polyphenolic extracts in the inhibition of key enzymes in skin aging (tyrosinase and elastase), or as anti-inflammatory, anti-pyretic, antimicrobial, anti-viral, and analgesic agents justify and validate the use of these extracts for the formulation of new nutraceuticals or skin-care products (Khan et al., 2019; Royer et al., 2013). On the other hand, polyphenols have another application in the food preservation industry, as they can also scavenge free radicals that cause the degradation of food products during processing and storage (Khan et al., 2019).

1.2. Maqui leaves: an agroindustrial waste as a new and potential source of polyphenols

Maqui is a native evergreen shrub that belongs to the Elaeocarpaceae family and grows mainly in central and southern Chile. Maqui plant reaches 3 to 5 meters in height and produces small purple berries that can be eaten in one piece (Hoffmann et al., 1992; O. Muñoz, 2001). Maqui berry is considered a "super fruit" whose worldwide demand has been increasing in recent years due to its potent antioxidant properties. It was shown that this berry reached high values of total polyphenol content (TPC) and antioxidant capacity (AC); even higher than those of fruits known as potential polyphenols sources, such as blueberry, blackberry, olive, and grape (Figure 1-1) (DINTA Asistencia Técnica, 2018). This berry has also been reported to have multiple bioactive effects, including antibacterial, anti-inflammatory, cardioprotective, and anti-diabetic effects that are probably due to the presence of well studied bioactive polyphenols such as delphinidin, kaempferol, and quercetin (In Chapter 2, the bioactive properties of maqui are discussed in detail). The maqui berry is being marketed mainly as dried fruit, capsules, lyophilized powder, nectar, pulp, and juices. In 2015, Chile exported 190 tons of processed maqui berry (US$ 4.5 million) to Japan, South Korea, Italy, the United States, Germany, Australia, Denmark, and others. A year later, these figures increased to 433 tons and US$ 9.9 million. Today, these maqui berry-based products are found on Amazon or in gourmet stores and supermarkets in New York (Pontillo, 2018).

A limitation of maqui processing is the problematic collection of berries due to their complex distribution in the plant (Figure 1-2). Manual collection, a non-destructive but low yield method, requires 7,430 work hours to harvest a plantation of 1,905 trees (2.5 kg berries/tree). In turn, mechanically assistance collection (Pellenc Olivon T220-300) and fully mechanized collection (New Holland Braud 9090X Olivar) require 254 and 2 work hours, respectively, to harvest a similar plantation. Despite these two collection methods being much more efficient than manual collection, they generate high amounts of agro-

industrial waste because they remove berries and also a significant number of leaves and twigs (Gaete-Espinoza et al., 2020).

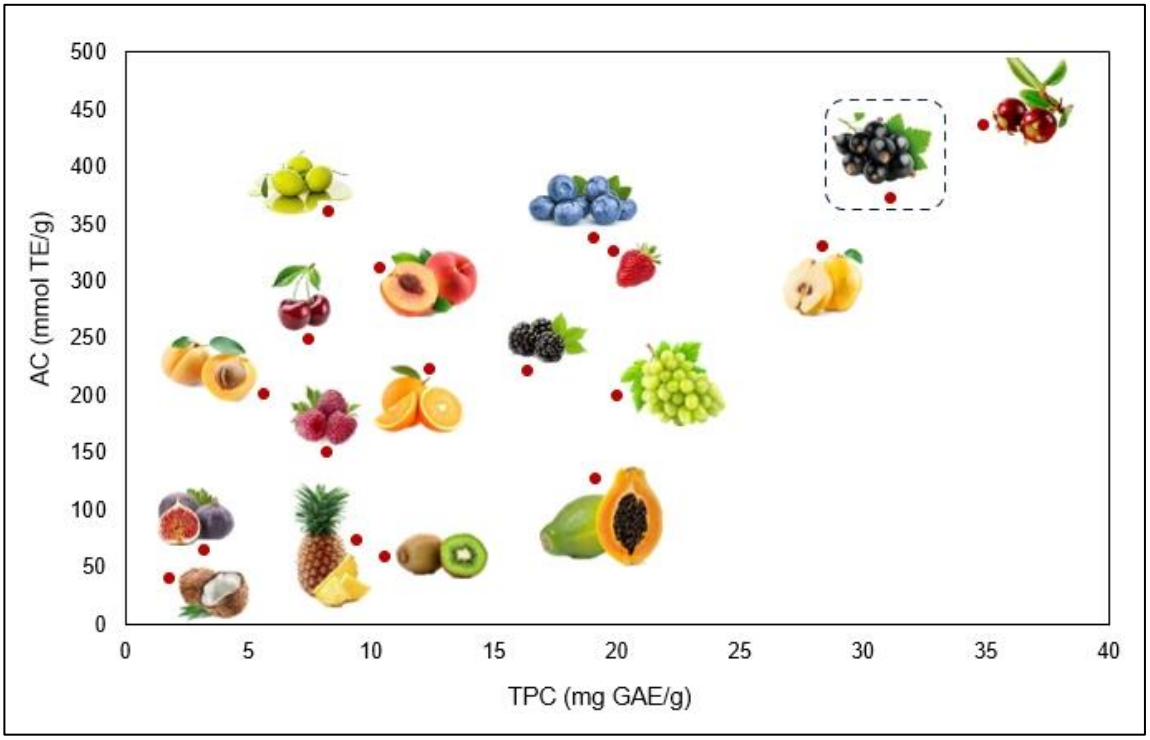


Figure 1-1: Total polyphenol content (TPC) and antioxidant capacity (AC) of different fruits. Data extracted from *DINTA Asistencia Técnica (2018)*

Maqui leaves are also a rich source of polyphenols, reaching even higher levels than maqui berries (51% and 98% in terms of TPC and AC, respectively) (Rubilar et al., 2011). Maqui leaves infusions have been used in traditional Mapuche medicine to treat various ailments such as diarrhea, throat infection and mouth ulcer (Zúñiga et al., 2017). Maqui leaves extracts have been shown *in vitro* assays to have antidiabetic and anti-hemolytic effects and *in vivo* assays anti-inflammatory and analgesic effects (Muñoz et al., 2011; Rubilar et al., 2011). Different polyphenols highlighted by their important beneficial effects for health were identified in extracts of maqui leaves such as catechin, resveratrol and pelargonidin, which demonstrated their neuroprotective, chemopreventive for skin cancer and photoprotective effects, respectively (Giampieri et al., 2012; Jang et al., 1997;

Mandel & Youdim, 2004; Vidal et al., 2013). The sale prices of these polyphenols are high which reflect the relevance of each of them (47,902; 1,752 and 120,768 US$/g, respectively, taken from *Merck website*). Valorizing this agro-industrial waste, which grows in proportion to the growth of the maqui berry industry, as a source of functional ingredients increases the maqui industry's benefits and reduces its environmental impact.

Figure 1-2: 4-year-old plants with defined sizes (left) and distribution of berries on the branches (right). Photographs taken from *"Manual técnico económico del maqui para cosecha mecanizada"*.

1.3. Main processes for the recovery of extractable polyphenols from maqui leaves

Extraction, pre-purification, and fractionation are necessary processes to obtain polyphenolic extracts and polyphenol fractions of high purity from maqui leaves and other solid natural matrices (Figure 1-3).

Extraction, the first fundamental process (Figure 1-3), moves compounds from natural matrix into the solvent. Generally, a solid matrix is previously prepared in steps that include drying, grinding and homogenizing. Yield extraction depends on solvent type, extraction time and temperature, sample-to-solvent ratio, chemical composition, and physical characteristics of th sample. Therefore, there is no universal extraction procedure applicable to all types of natural compounds (Dai & Mumper, 2010). Microwave assisted extraction (MAE), ultrasonic assisted extraction (UAE), hot pressurized liquid extraction

(HPLE), and supercritical fluid extraction (SFE) are widely applied methods in the extraction of polyphenols from natural matrices because they achieve high extraction yields. Extraction process is not necessarily selective, then the crude extract usually contains large amounts of other compounds (carbohydrates and/or lipoidal material) in addition to polyphenols. (Caballero-Valdés et al., 2017; Dai & Mumper, 2010).

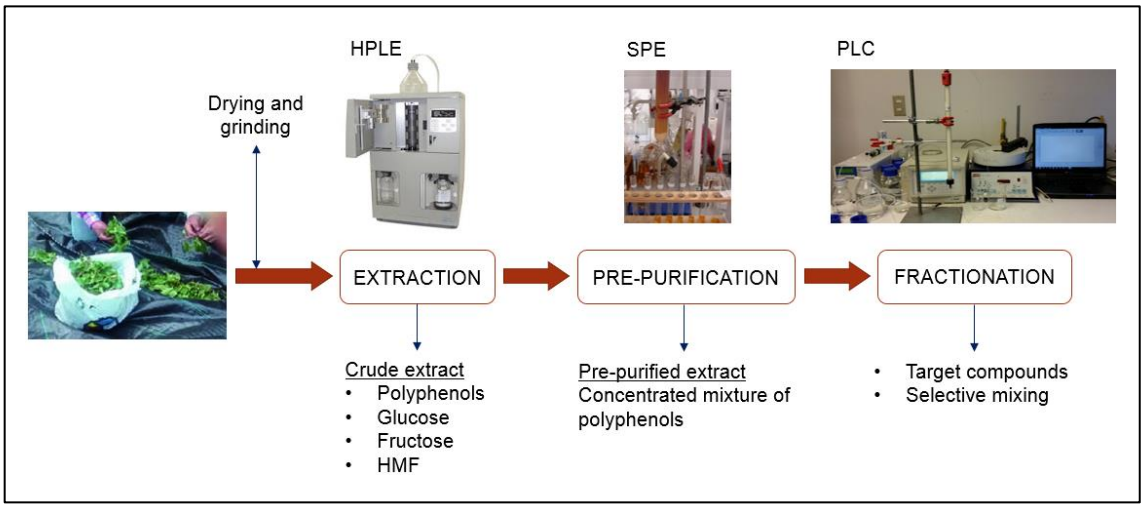


Figure 1-3: Process line for polyphenols recovery from natural matrices. HPLE: hot pressurized liquid extraction, SPE: solid phase extraction, PLC: preparative liquid chromatography, HMF: hydroxymethylfurfural.

Pre-purification is the next process (Figure 1-3) that has function of separating the non-polyphenolic compounds (difficult to handle in food and nutraceutical applications) from the crude extract to achieve a concentrated polyphenolic extract (Muzaffar et al., 2015). Solid phase extraction (SPE) method is usually carried out to remove sugars, organic acids, and other polar non-polyphenolic compounds. SPE involves adsorption and desorption with macroporous resins such as Amberlite, XAD-2, XAD-7, HP-20 that have been used successfully to concentrate polyphenolic extracts (Dai & Mumper, 2010; Huaman-Castilla et al., 2019).

Fractionation or isolation process is generally carried out by preparative liquid chromatography (PLC) that obtain specific polyphenol fractions or selective extracts. PLC

is an efficient separation method that was originally designed to obtain purified natural pigments from complex mixtures of plant origin. Today it has become an indispensable unit operation for fractionating high-value compounds on laboratory and industrial scales (M. Gu et al., 2006a). During process, a liquid fluid (mobile phase) is pumped through a bed of porous particles (stationary phase) while compounds dissolved in mobile phase interact to different degrees with stationary phase. This difference causes the compounds to move through the column at different speeds, which eventually leads to their separation. These compounds diffuse into and out of stationary phase particles, undergo thermodynamic interactions with stationary phase or form transient chemical bonds with it, until they finally leave the column (He et al., 2004).

Although the potential of maqui leaves as a natural source of polyphenols was demonstrated, until now no attempt has been made to optimize the recovery of polyphenols from this agro-industrial waste. Maqui leaves extracts have been obtained by maceration (Muñoz et al., 2011; Rubilar et al., 2011; Vidal et al., 2013), a conventional extraction technique that requires long extraction times and toxic organic solvents to achieve high yields. No purification process has been designed or optimized to isolate catechin, resveratrol, pelargonidin or other relevant polyphenols for the food, pharmaceutical or nutricosmetic industries.

1.4. Hot pressurized liquid extraction (HPLE): High yield green method in the extraction of polyphenols

HPLE method is based on the principles of green chemistry since aggressive organic solvents are not required, extraction times are reduced, quantities of processed samples are small and solvent used is low, which means reduced consumption of energy and resources, and guaranteed obtaining of safe and quality extracts (Mustafa & Turner, 2011). These situations make HPLE superior over conventional extraction methods (soxhlet extraction, sonication, blending and solid-liquid extraction) that use large amounts of solvent with negative environmental impact and inapplicable for food, process large amounts of sample and spend long times; moreover, they require post-extraction processes

that separate the extract from the solid residue, which concentrate and clean-up the extract (Camel, 2001; Ramos et al., 2002). In many cases, these operating conditions induce ionization, hydrolyzation or oxidation of those more sensitive and thermolabile polyphenols (Vergara-Salinas et al., 2017).

1.4.1. HPLE equipment and features

HPLE method is carried out at elevated temperatures (T < 200 °C) above the boiling point of solvent, which is kept in liquid phase thanks to the high operating pressure (generally set at 1500 psi). Extraction efficiency is improved when the extraction temperature is high because the surface tensions of solvent and matrix decrease, improving the matrix wetting with solvent; viscosity of solvent also decreases, improving its penetration into the matrix microstructure; and polyphenol diffusion in solvent is faster, reducing the amount of solvent used (Björklund et al., 2000; Möckel et al., 1987; Richter et al., 1996).

Accelerated solvent extraction equipment (ASE®) is an automated technique introduced by Dionex Corporation, that is used for HPLE on a laboratory scale mainly in static configuration, where during a predetermined time the extraction is carried out in one or more extraction cycles with substitution of solvent between cycles. At the end of the last extraction cycle and to avoid losses, sample cell is purged with inert gas to remove the solvent from sample cell and tube in the collection vial (Mustafa & Turner, 2011; Thermo Fisher Scientific Inc, 2011). ASE equipment can work in a wide range of temperature (T_{room} - 200 °C) in steps of 1 and 5 °C for ASE 150 and ASE 200, respectively. Previously, sample cell is filled with sample join with diatomaceous earth or some other inert particulate material which acts as a dispersing agent to avoid plugging due to caking of sample and/or dehydrating agent during extraction. This material is also used to reduce the volume of sample cell and therefore volume of solvent used (Mustafa & Turner, 2011; Saito et al., 2004).

Extraction temperature, static time (minutes the cell contents are kept at the set temperature), rinse volume (amount of solvent to rinse the cell after the static extraction), purge time (minutes the cell is purged with nitrogen) and static cycle (number of times the static extraction and rinse cycles are performed) are factors that can be manipulated and set by the ASE (Thermo Fisher Scientific Inc, 2011). While the amount of sample and type of solvent that can be a mixture of solvents, since it improves extraction yields by improving solubility and increasing interaction of the target compound with extraction solvent (Arapitsas et al., 2008; M. Mukhopadhyay & Panja, 2008), are manipulated variables defined outside the ASE.

1.4.2. HPLE optimization: influencing factors on polyphenols recovery

HPLE method was used to identify factors with the most significant effects on different response variables that measure both extract's purity and extraction yield. HPLE was also used to optimize the extraction of polyphenols from different natural matrices: Croatian olive leaves (Putnik et al., 2017), Brazilian pepper (Feuereisen et al., 2017), myrtle leaves (Díaz-de-Cerio et al., 2018), olive leaves (Xynos et al., 2014) and goji berry (Tripodo et al., 2018); using aqueous ethanol as solvent and ASE 200, ASE 300, and ASE 350 (Table 1-2). Ethanol has been presented as an affordable and non-toxic GRAS (generally recognized as safe by the Food and Drug Administration) extraction solvent for recovery of polyphenols and other bioactive compounds from natural matrices by HPLE (Herrero et al., 2011; Okiyama et al., 2018; Pazo-Cepeda et al., 2020; Taamalli et al., 2012; Tamkutė et al., 2019).

$$Polyphenol\ extraction\ yield = \frac{mg\ GAE}{g\ dry\ matrix} \tag{1.1}$$

$$Extract's\ polyphenol\ purity = \frac{mg\ GAE}{g\ dry\ extract} \tag{1.2}$$

where GAE is gallic acid equivalent. Yield and purity can also be expressed in terms of a specific polyphenol (oleuropein) or polyphenolic group (anthocyanins).

Table 1-2: Evaluated influencing factor ranges, fixed values for non-influencing factors, and optimal HPLE operating conditions.

Factors	Xynos et al. (2014)	Rodríguez-Pérez et al. (2016)	Putnik et al. (2017)	Feuereisen et al. (2017)	Díaz-de-Cerio et al. (2018)	Tripodo et al. (2018)
ASE system	300	200	350	350	350	200
Natural matrix	Olive leaves	Moringa leaves	Olive leaves	Brazilian pepper	Myrtle leaves	Goji berry
Pression (psi)	1500	1500	1500	1500	1015	1450
Sample (g)	7	1	1	0.5	1	1
Cell volume (mL)	33-100	11	34	10	22	11
Rinse vol. (%)	40-100	60	60	150	60	60
Purge time (sec)	60-180	60	90	60	100	60
Static time (min)	5-25	5	5-15	0-10	5-30	5
Number of cycles	1-3	1	1-2	1	1	1
Temperature (°C)	40-190	50-180	60-100	40-120	50-200	50-180
EtOH (% v/v)	0-100	0-100	50	0-100	50-100	0-100
Optimal conditions						
Static time (min)	n.c.	n.c.	5	10	19	n.c.
Number of cycles	3	n.c.	2	n.c.	n.c.	n.c.
Temperature (°C)	190	128	80	100	137	180
EtOH (% v/v)	100	35	n.c.	54.5	71	86
Max. response	46.64[a]	59[b]	53.15[b]	~51[c]	30.0[b]	65.98[b]

[a] Extraction yield (%), [b] total polyphenol content (mg gallic acid equivalent/g dried natural matrix), [c] total polyphenol content (mg caffeic acid equivalent/100 mL extract), and n.c.: not considered in optimization.

Rinse volume and purge time did not show significant effects on extraction yield and oleuropein content (Xynos et al., 2014). Generally, values in the ranges of 40%-150% and 60-180 seconds are established for these factors, respectively in the operation and/or optimization of HPLE (Díaz-de-Cerio et al., 2018; Feuereisen et al., 2017; Putnik et al., 2017; Tripodo et al., 2018) (Table 1-2). Static time effect on extraction yield was significant although it was the one with the least impact among the significant effects (Xynos et al., 2014). The lowest value for this factor (5 min) is usually set to avoid hydrolysis and oxidation reactions, which occur when exposure to high temperatures is prolonged, degrading thermally unstable polyphenols (Putnik et al., 2017; Tripodo et al., 2018; Xynos et al., 2014). For some natural matrices, the optimal static extraction time

was greater than 5 min (Díaz-de-Cerio et al., 2018; Feuereisen et al., 2017), it is possible that the majority polyphenols contained in these natural matrices are thermally resistant. Ethanol concentration, extraction temperature and number of cycles showed significant effects on HPLE process (in terms of TPC, AC, extraction yield and polyphenols' extraction yield), therefore, several HPLE optimizations were carried out considering the change impact of these three factors or two of them (Table 1-2).

According to the results of HPLE optimizations (Table 1-2): (i) extraction yield and TPC tend to increase with number of cycles, especially for samples that offer greater resistance to solvent penetration. With the addition of an extraction cycle, fresh solvent is provided to extraction matrix which maintains a favorable extraction equilibrium (Mottaleb & Sarker, 2012; Putnik et al., 2017); and (ii) optimal extraction temperature and ethanol concentration are a function of the chemical composition of each natural matrix and optimization objective; therefore, it is not possible to generalize a single trend. Temperatures above 100 °C significantly reduced anthocyanin recovery (Vergara-Salinas et al., 2013), while some phenolic acids (gallic, chlorogenic, vanillic, caffeic and ferulic), some flavanols (catechin and epicatechin), some flavonols (quercetin and kaempferol) and resveratrol stilbene were highly stable at 150°C and their recoveries increased significantly when temperature changed from 90 to 150 °C (Huaman-Castilla et al., 2019). Regarding ethanol concentration effect, some authors found optimum performance at high ethanol concentration (> 50%) (Díaz-de-Cerio et al., 2018; Tripodo et al., 2018), while others at low concentration (< 50%) (Rodríguez-Pérez et al., 2016). The recoveries of phenolic acids were higher at highest ethanol concentration (50%), on the other hand, flavanols and stilbenes recovered better at the intermediate ethanol concentration (32.5%), while higher recoveries of flavonols were achieved at the lowest ethanol concentration (15%) (Huaman-Castilla et al., 2019). Hence, when flavonols (quercetin and kaempferol acetyl glycoside isomers) are the majority compounds in the matrix (moringa oleifera leaves), polyphenol extraction has shown to be maximized at 35% of ethanol concentration (Rodríguez-Pérez et al., 2016), whereas a high ethanol concentration (71%)

would favor the extraction of matrixes such as myrtle leaves that have high gallic and ellagic acid derivatives (Díaz-de-Cerio et al., 2018).

1.5. Polyphenols isolation from natural extracts by adsorption preparative liquid chromatography (APLC)

The APLC method with Superose^{TM} 12 as stationary phase and different proportions of water (H_2O), ethanol (EtOH) and acetic acid (HAc) as mobile phase has been highly recommended to isolate corilagin, geraniin, protocatechuic aldehyde, gallic acid and salvianolic acid from extracts of Chinese medicinal plants (*Salvia miltiorrhiza* Bunge and *Geranium wilfordii* Maxim) due to these fractions reached high purities (87.2% - 99.4%) and high recoveries (76.8% - 88.1%) (M. Gu et al., 2008; Liu et al., 2011). These APLC phases were also used for the successful isolation of epigallocatechin gallate (Xu et al., 2006), benzoic acid, 4-hydroxybenzoic acid, gallic acid, fisetin, kaempferol, quercetin, myricetin, polydatin, and resveratrol (M. Gu et al., 2006a; Tan et al., 2010), puerarin (He et al., 2004), quercitrin, rutin, robinin, hesperidin, hesperetin, apigenin, and naringenin (M. Gu et al., 2006b).

Superose^{TM} 12 prep grade (Code No. 17-0536-01) is an agarose-based gel that offers a large specific surface (micropores), is an insoluble material, easily scalable to industrial size, mechanically and chemically stable to coupling, process and cleaning conditions (Cuatrecasas, 1970; Cuatrecasas & Anfinsen, 1971; M. Gu et al., 2008). The separation generated by this gel can be achieved in one stage and is based on the exclusion by size and the adsorption of the molecules of the mixture, canceling other types of interactions of the molecules with the phases of the system (Qi et al., 2007). Hence, the polyphenols that are more weakly adsorbed by agarose advance faster, while those that are more strongly adsorbed lag behind. This gel is resistant to temperature range 4-40 °C and to all solutions commonly used in gel filtration. It is stable for long periods in pH range 3-12 and for short periods in pH range 1-14. More characteristics in Table 1-3 (GE Healthcare -Superose ^{TM}, 2005).

Table 1-3: Some Superose 12 properties.

Properties	Superose 12 prep grade
Exclusion limit globular proteins	2×10^6
Matrix composition	Composite of cross-linked agarose
Average particle size (µm)	30 ± 10
Max back pressure (psi)	105 (0.7 MPa, 7 bar)
Recommended flow rate[a] (cm/h)	Up to 40
Yields and activity recovery (%)	80-100
Sample loading capacity[b]	0.5-4% of total column volume

[a] At room temperature in aqueous buffer, if the column is used at 4 °C half flow rate compare to room temperature. [b] For maximum resolution, apply as small a sample volume as possible, but not less than 0.5%.

Achieving efficient PLC and high purity fractions requires carefully defining several parameters that significantly influence the process to varying degrees, such as column dimensions (length and diameter), stationary phase properties (e.g., porosity and void fraction), volume and feed flow, flow and composition of the mobile phase and temperature. Optimal analytical-scale separation conditions, where mixtures are handled under very dilute conditions, are usually different from the optimum conditions for separating concentrated mixtures. Therefore, direct transfer of laboratory methods to the separation bench does not lead to optimal operation. Experimental trial-and-error methods can be applied to improve separating conditions, but that would need a significant investment of time and materials (T. Gu, 2015; Tarafder, 2013). Under time and material constraints, a better option for developing optimal separation conditions is model-based optimization, which is more efficient and accurate than experimental trial and error methods.

APLC models, regardless of the level of abstraction to model mass transfer, are composed of differential mass balances within the liquid and on the stationary phase surface, and an equation that represents the thermodynamic equilibrium which is the most important since this phenomenon determines the separation (elution time) of the mixture's components (Guiochon et al., 2006a; Tarafder, 2013). Hence an accurate estimation of

equilibrium parameters contributes to the success of theoretical mathematical models for design, optimization and scale-up of APLC (T. Gu, 2015). To quantify the thermodynamic interaction between polyphenols and the mobile and stationary phases, a mathematical formulation called the equilibrium isotherm is used (Tarafder, 2013). Regardless of the physical nature of the mobile phase-polyphenol-stationary phase interactions (ion exchange, size exclusion, reverse phase, etc.), the adsorption equilibrium isotherm attempts to quantify this relationship (T. Gu, 2015; Qi et al., 2007).

1.5.1. Polyphenol adsorption equilibrium on agarose gel

Adsorption isotherms of polyphenols in agarose with H_2O:EtOH:HAc are not available in the existing publications in the literature. Experimental measurement of these isotherms consists of isotherm data points are first measured experimentally (polyphenol concentrations in both liquid phase and adsorbent when equilibrium is reached, for different polyphenol initial concentrations) and then these data are fitted or correlated with theoretical isotherm models. In general, there are two experimental methods to obtain adsorption isotherm data:

- Column method consisting of frontal adsorption (also known as breakthrough analysis) on mini column to avoid excessive use of polyphenol. In this case, the equation to fit experimental data includes the agarose particle porosity and bed void volume fraction (T. Gu, 2015).
- Batch adsorption equilibrium method that consists in putting a solution with fixed polyphenol concentration in contact with a certain amount of agarose in a test tube, for a sufficient time until adsorption equilibrium is established (T. Gu, 2015).

Both adsorption equilibrium and thermodynamic behavior do not change with the process configuration, therefore the results of both methods should be the same. However, batch experiments are usually more simple, inexpensive, and less time-consuming than

column procedures, therefore, this technique is generally preferred to carry out adsorption studies (Loebenstein, 1962).

Mathematically, equilibrium isotherms express polyphenol concentration in adsorbent (q_e) as a function of liquid phase concentration (C_e). Various standard isotherms are discussed in the literature, describing a wide variety of elution profile shapes (Guiochon et al., 2006b). The two most used models for solid-liquid adsorption are Langmuir isotherm and Freundlich isotherm. Langmuir model represents ideal systems characterized by a monolayer adsorption (the thickness of the adsorbed layer is one molecule), fixed and identical number of adsorption sites, no lateral interaction between adsorbed molecules and homogeneous adsorption (all sites have equal enthalpies or affinities for the adsorbate) (Davis et al., 2003; Foo & Hameed, 2010).

$$q_e = q_{max} K_L C_e / (1 + K_L C_e) \tag{1.3}$$

where q_{max} (mmol/g) is the maximum adsorption capacity and K_L (L/mmol) is the adsorption equilibrium constant. Freundlich model is also a widely used model in the polyphenol adsorption onto macro and micro porous resins, and it represents more complex systems in which an infinite number of molecules can be adsorbed and where adsorption sites have a degree of heterogeneity (Allen et al., 2004; Foo & Hameed, 2010).

$$q_e = K_F C_e^{1/n} \tag{1.4}$$

where K_F (mmol/g)(L/mmol)$^{1/n}$ and n are model parameters associated with adsorption capacity of the adsorbent and adsorption intensity or degree of surface heterogeneity, respectively (Davis et al., 2003). According to the thermodynamically consistent theory of ideal adsorbed solution (IAS), these estimated parameters for each polyphenol can be used to determine the adsorption equilibrium of each polyphenol when it is contained in a multicomponent mixture, that is, considering that interactions of polyphenols with two phases (mobile and stationary) can be influenced by each other, as happens in APLC. Models of multicomponent isotherms such as the Competitive Langmuir isotherm and Freundlich-Langmuir isotherm are used (Guiochon et al., 2006b; Tarafder, 2013).

Numerical values of these parameters can also be used to accurately define the stationary and mobile phases for APLC system, as these values can provide relevant information about adsorption system. The n value can indicate whether the adsorption is irreversible ($10 < n$), very favorable ($2 < n < 10$), moderately favorable ($1 < n < 2$) and unfavorable ($n < 1$) (Hamdaoui, 2006; Tran et al., 2016). The K_F and q_{max} values provide information on the type of polyphenol-agarose-liquid phase interactions (Davis et al., 2003). Further understanding of the adsorption process can be attained through thermodynamic analysis. The enthalpy change (ΔH, kJ/mol) indicates whether the adsorption process is exothermic (negative value) or endothermic (positive value), in addition its absolute value ($|\Delta H|$) shows if the process is ruled by chemisorption (80-200 kJ/mol) or physisorption (2.1-20.9 kJ/mol) (Saha & Chowdhury, 2011). The isosteric adsorption enthalpy (ΔH_x, kJ/mol) is the enthalpy change at a constant amount of adsorbed adsorbate which corroborates or contrasts the information given by ΔH about the nature of adsorption ($|\Delta H_x| < 80$ kJ/mol physisorption or $80 < |\Delta H_x| < 400$ kJ/mol chemisorption), it also provides information about the degree of heterogeneity of agarose surface (Saha & Chowdhury, 2011; Ghosal & Gupta, 2015; Unnithan & Anirudhan, 2001). Gibbs free surface energy change (ΔG, kJ/mol) reveals the degree of spontaneity and thermodynamic feasibility of the adsorption process (Saha & Chowdhury, 2011). The entropy change (ΔS, kJ/mol K) suggests how the adsorbate molecules settle on the adsorbent surface during the adsorption process ($\Delta S < 0$ means less random and $\Delta S > 0$ means more random) (Li et al., 2005; Saha & Chowdhury, 2011).

Thermodynamic parameters can be determined by evaluating equilibrium conditions (Eq. 1.5), using the van't Hoff equation (Eq. 1.6) and the integrated Clausius-Clapeyron equation (Eq. 1.7) (Ghosal & Gupta, 2015; Tran et al., 2016; Wang et al., 2020).

$$\Delta G = -RT \ln K_{eq} \tag{1.5}$$

where T is the absolute temperature (K), R is the ideal gas constant (8.314 J/mol K), and K_{eq} is the thermodynamic equilibrium constant (dimensionless).

$$\ln K_{eq} = -\frac{\Delta H}{R}\frac{1}{T} + \frac{\Delta S}{R} \quad (1.6)$$

$$\ln C_e = \frac{\Delta H_x}{R}\frac{1}{T} + K \quad (1.7)$$

where K is integration constant. ΔH_x can be determined from the slope of the isosteres, plot of ln C_e versus 1/T. The different equilibrium concentrations (C_e) of the isosteres were obtained at a constant adsorbed amount (q) at three temperatures.

1.6. Statement of hypotheses and objectives of the thesis

This thesis was supported by two hypotheses, which are:

Application of the multi-response optimization to HPLE allows to determine the operating conditions that produce polyphenol extracts with outstanding features from maqui leaves.

Detailed study of the adsorption equilibrium of five maqui leaf polyphenols on agarose allows to characterize the system and generate relevant information for the isolation of these polyphenols by APLC.

To overall goal that leaded this thesis is:

Optimize the HPLE and evaluate the adsorption equilibrium of polyphenols of maqui leaves to produce balanced crude extracts with optimal polyphenolic features and to characterize APLC polyphenol isolation system.

The specific goals are:

- Review and identify the polyphenolic properties (antioxidant capacity, bioactive effects, main polyphenols identified) of maqui leaves.
- Determine the HPLE operating conditions that individually and simultaneously maximize extract's polyphenol purity and polyphenol extraction yield.
- Model adsorption equilibrium of five maqui leaf polyphenols on agarose gel, using theoretical models.

1.7. Summary of methodologies and approach of the thesis

Each following chapter of this doctoral thesis is associated with a journal manuscript that have been submitted (chapter 4) or already published (chapters 2 and 3), where the above specific goals were developed (Figure 1-4).

Chapter 2 presents the initial bibliographic review that allowed the definition of the maqui leaf as the natural matrix of study. This review discusses relevant information about the antioxidant potential of maqui and murta, focusing on the bioactivity of leaf and berry extracts, evaluated *in vitro* and *in vivo* assays. It shows maqui polyphenolic profiles determined by different extraction and analysis methods. It collects some details regarding the bioactivity of its most relevant polyphenols and a comparative analysis of their contents in maqui with those of other matrices. Finally, it presents the study of phenolic variability of extracts caused by external factors such as genotype, environment, stage of harvesting, storage, and processing.

Chapter 3 describes the optimization and assessment of the efficiency of HPLE in the recovery of low molecular weight extractable polyphenols from maqui leaves. The optimization of the process focused on maximizing the TPC measured by the Folin-Ciocalteau method, AC measured by the ABTS radical scavenging activity assay and polyphenols purity (g of gallic acid equivalent/100 g of dry extract, %) of the extracts generated in an ASE 200 device (5 mL extraction cell). To further characterize the optimal extracts, additional extractions were carried out under optimal conditions in an ASE 150 device (100 mL extraction cell). These extracts were evaluated in terms of TPC, purity, *in vitro* antioxidant capacity (DPPH and ORAC), and low molecular weight polyphenol profile.

Chapter 4 presents the measurement and characterization of the adsorption on agarose of five relevant low weight polyphenols identified in maqui leaves. Superose^{TM} 12 prep grade and between three and six solutions with different water compositions, ethanol, and acetic acid were used as adsorbent and liquid phases. The chosen adsorbent and liquid phases were relevant for designing an adsorption preparative liquid chromatography

(APLC) process to isolate these polyphenols. Adsorption isotherms were fitted to experimental data and then used to evaluate the effect of temperature and composition of the liquid phase on each of the five studied polyphenols' adsorption capacity. The adsorption process was further assessed through thermodynamic analysis, where accurate and significant thermodynamic equilibrium parameters were obtained.

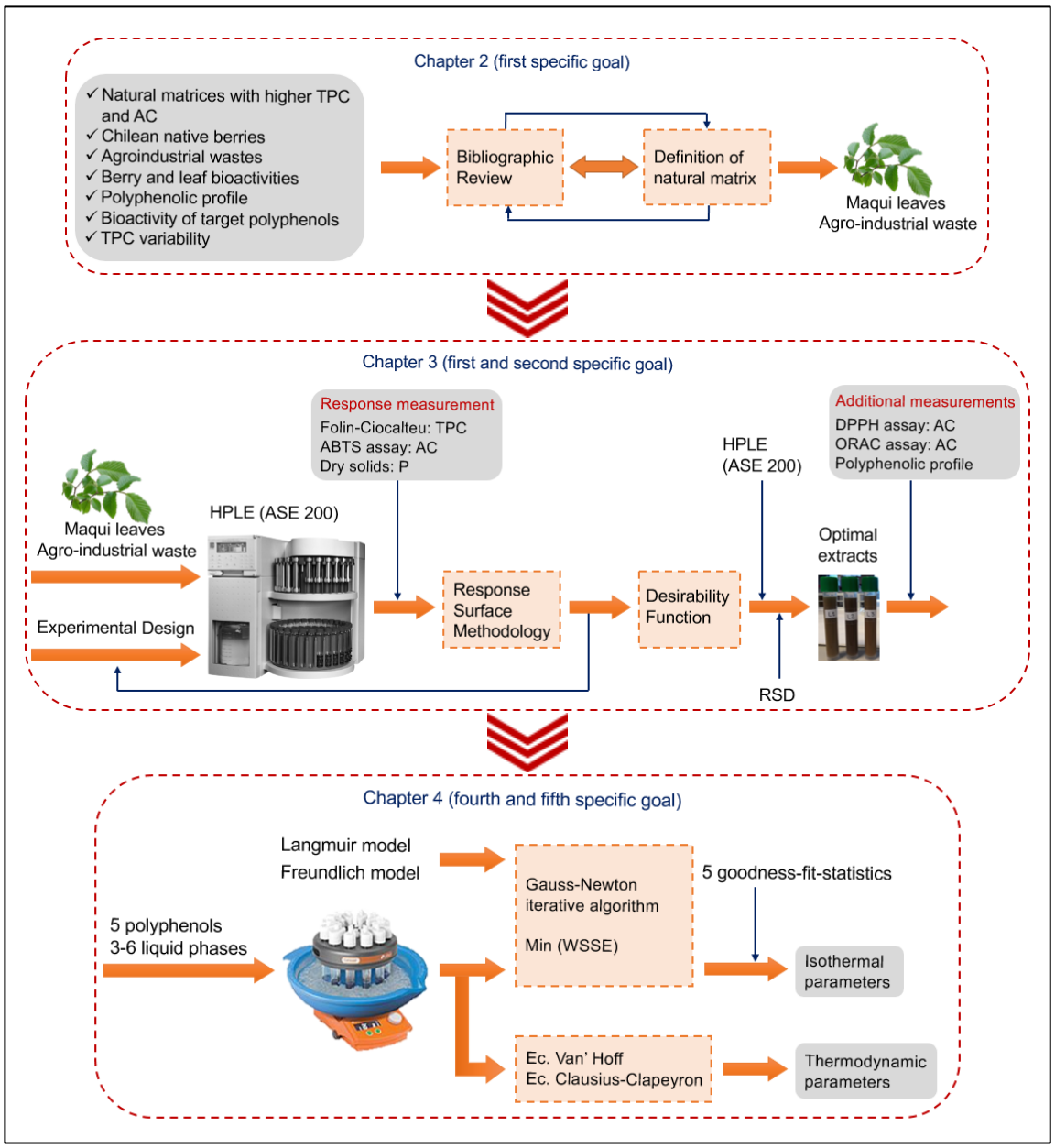


Figure 1-4: Thesis general overview.

CHAPTER 2. MAQUI (*ARISTOTELIA CHILENSIS* [MOL.] STUNTZ) AND MURTA (*UGNI MOLINAE* TURCZ): NATIVE CHILEAN SOURCES OF POLYPHENOL COMPOUNDS

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2.1. Introduction

Scientific and commercial interest in producing functional ingredients has encouraged the search for natural sources with high polyphenol content. Phenolic compounds may act as antioxidants through several different mechanisms, including free radical scavenging, metal chelation and protein binding (Maurya & Devasagayam, 2010). There is abundant evidence to suggest that polyphenols have beneficial effects on health, including the prevention of chronic diseases associated with oxidative processes (Puupponen-Pimiä et al., 2001; Tomás-Barberán, 2003). However, despite this clinical evidence, there is still no widely accepted Dietary Reference Intake for these compounds. In this sense, further research that considers the bioavailability of specific polyphenols and their combinations, as well as their safety evaluation, should be carried out in order to warrant polyphenol intake recommendations (Speisky et al., 2017).

Polyphenols can be classified chemically into (i) phenolic acids, (ii) flavonoids (flavonols, flavanols, isoflavones, anthocyanins and flavanones) and the less common (iii) stilbenes and (iv) lignans. The first group has a single ring of 6 carbon atoms, while the second group has two or three rings. The stilbenes have two rings connected together by a chain of three carbons and the lignans do not have a defined common structure (Scalbert & Williamson, 2000; Tsao, 2010). The main sources of dietary polyphenols are fruits, fresh vegetables, beverages (fruit juice, wine, tea, coffee, chocolate, and beer) and, to a lesser extent, dry legumes, and cereals (Scalbert & Williamson, 2000).

Berries (small fruits with little seeds and that can be eaten in one piece) usually present a higher total polyphenol content (35% - 55%) and antioxidant capacities (17% - 67%) than other natural sources (Fredes, 2009) (Figure 2-1). Chile ranks as the third largest world exporter of cultivated berries (blueberry, strawberry, raspberry, and blackberry) behind only Spain and the USA. However, the Chilean blueberry industry has risen to be the world's largest exporter. Last year, Chile exported ~104,472 tons of fresh blueberries, mainly to USA (64%) and United Kingdom (11%) (*Oficina de Estudios y Políticas Agrarias. Servicio Público Centralizado.*, 2017). Similarly, the exportation of some native wild berries is growing significantly. This is the case of maqui, which in January-September 2015 recorded exports of 188 tons (US$ 4.4 million), an increase of 63% compared to the same period of the previous year. The main destinations were Japan, Italy, USA, and Germany (Aguilar, 2015).

Maqui (*Aristotelia chilensis* (Mol.) Stuntz) is a native evergreen shrub that belongs to the Elaeocarpaceae family and mainly grows in central and southern Chile. Maqui plants can reach 4 - 5 m high and produce round and purple edible berries (about 5 mm) (Hoffmann et al., 1992; O. Muñoz, 2001). In Chile, the potential output of maqui is 37,400 tons of fresh fruit per year, considering the estimated 170,000 ha of maqui from the IV to XI region, with an average yield of 220 kg/ha (*Paquete Tecnológico Maqui Productos Forestales No Madereros En Chile*, 2015).

Murta (*Ugni molinae* Turcz) is a native evergreen shrub that belongs to the Myrtaceae family and grows from the VII to the XI region in Chile. This plant normally reaches 1-2 m high, and its fruits are small globular red berries (0.7 to 1.3 cm) with pleasant smell and taste (C. Muñoz, 1966; M. Muñoz et al., 1986). Data from 2011 show that the planted (cultivated) area in Chile is 200 ha and the wild area is 25,000 ha, with an estimated production of 37,500 tons (1,500 kg/ha) (ODEPA, 2013; Torralbo et al., 2011).

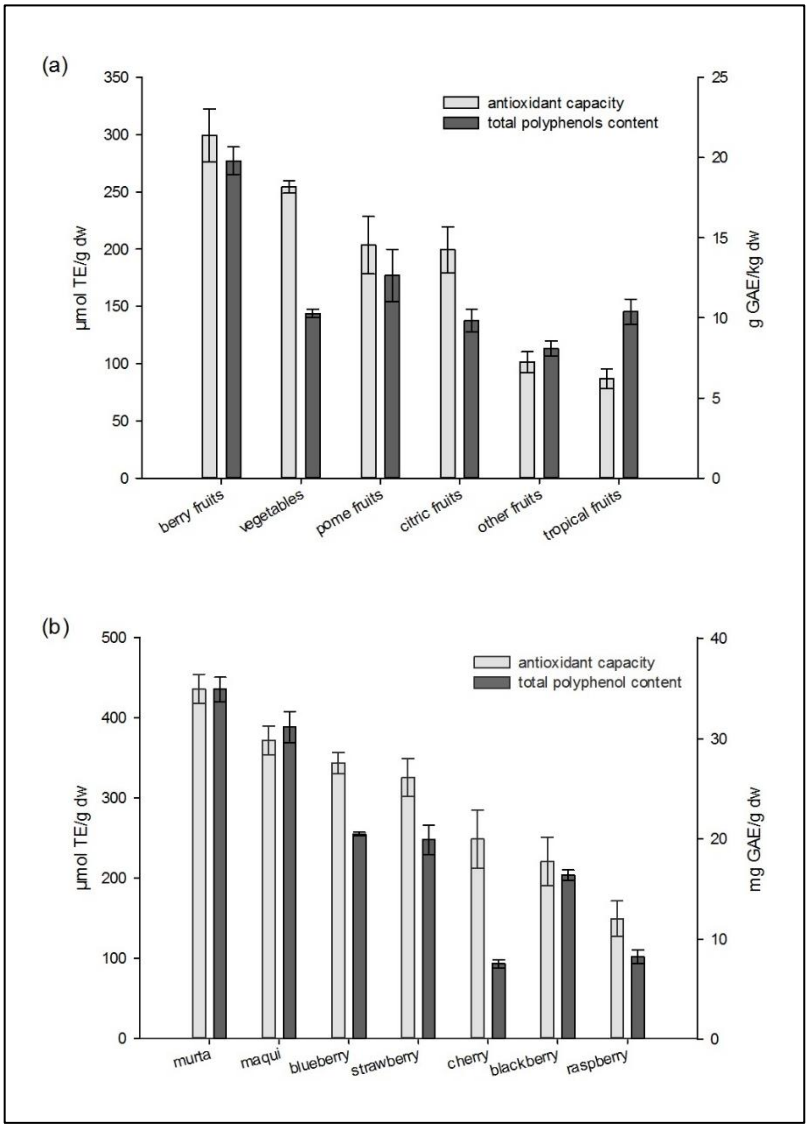


Figure 2-1: Antioxidant capacity and total polyphenol content of natural sources. (a) Different kind of fruits and vegetables, and (b) Some berries cultivated or grown in Chile. TE: Trolox equivalent, GAE: Gallic acid equivalent, dw: dry weight. (Calculated based on the online database: *Primer portal antioxidantes, alimentos y salud en el mundo de habla hispana*)

Maqui and murta berries are called superfruits that gave high total polyphenol content and antioxidant capacity. Total polyphenol contents oscillate between 14.2 to 51.6 g GAE/kg for maqui berry and 9.2 to 40.3 g GAE/kg for murta berry (Table 2-1). Meanwhile, the antioxidant capacities vary in intervals of 1.7 to 399.8 and 10.94 to 82.9

mg/L measured by DPPH test for maqui berry and murta berry, respectively. The large size of the AC ranges of both fruits shows the dependence of the concentration of the methanol DPPH solution (Table 2-2). Interestingly, extracts of maqui and murta leaves present higher TPC and AC than those of their respective berries (Table 2-1), suggesting that these agroindustrial wastes can be used as a potential source of natural antioxidants (López de Dicastillo et al., 2017; Rubilar et al., 2011).

Table 2-1: Total Polyphenol Contents of extract from maqui and murta obtained at atmospheric pressure.

Source	Operating conditions			TPC (g GAE/kg)	References
	Temperature (°C)	solid:liquid ratio	Solvent composition		
Maqui berry	Room	1:200	Ethanol 50%	45.7 ± 1.1[a]	Rubilar et al. (2011)
	Room	1:4	Methanol (0.1% HCl)	14.5 ± 2.4[b]	Fredes et al. (2014)
	Room	1:125	Methanol (0.1% HCl)	**51.6 ± 0.9[a]**	Brauch et al. (2016)
	Room	1:10	Methanol 80% (0.1% HCl)	49.7 ± 0.6[a]	Genskowsky et al. (2016)
	Room	1:4	Methanol (0.1% HCl)	14.2 ± 1.5[b]	Fredes et al. (2012)
Murta berry	Room	1:200	Ethanol 50%	10.1 ± 1.6[a]	Rubilar et al. (2011)
	Room	1:10	Methanol (0.1% HCl)	9.2 ± 0.3[a]	Brito et al. (2014)
	40	1:2	Ethanol 50%	34.5 ± 1.2[a]	López de Dicastillo et al. (2017)
	30	1:3.3	Methanol	21.5 ± 1.6[a]	Alfaro et al. (2013)
	30	1:30	Ethanol 50%	**40.3 ± 0.0[a]**	Augusto et al. (2015)
Maqui leaf	Room	1:200	Ethanol 50%	**69.0 ± 0.9[a]**	Rubilar et al. (2011)
Murta leaf	Room	1:200	Ethanol 50%	32.5 ± 3.1[a]	Rubilar et al. (2011)
	40	1:2	Ethanol 50%	127.9 ± 6.3[a]	López de Dicastillo et al. (2017)
	Room	n.d.	Ethanol n.d.	**260.6 ± 3.7[a]**	Peña-Cerda et al. (2017)

GAE: gallic acid equivalents, n.d.: not defined, [a]: dry weight, [b]: fresh weight.

Table 2-2: Antioxidant Capacities of maqui and murta extracts determined by different assays.

Source	Analysis method (unit)	MIN - MAX values	References
Maqui berry	DPPH (mg/L)	1.700[a] - 399.8[b]	Céspedes et al. (2008), Céspedes et al. (2010), Céspedes et al. (2017), Fredes et al. (2014), Rubilar et al. (2011).
	FRAP (mol Fe^{2+}/kg dw)	0.101 – 0.389	Brauch et al. (2016), Fredes et al. (2012), Fredes et al. (2014), Genskowsky et al. (2016).
	ORAC (g ET/kg dw)	74.83	Gironés-Vilaplana et al. (2014).
Murta berry	DPPH (mg/L)	10.94[a] – 82.90[b]	Brito et al. (2014), Rubilar et al. (2011).
	FRAP (g ET/kg dw)	20.29 – 74.50	Brito et al. (2014), López de Dicastillo et al. (2017).
	ORAC (g ET/kg dw)	78.44	López de Dicastillo et al. (2017).
Maqui leaf	DPPH (mg/L)	8.000[b] – 12.10[c]	O. Muñoz et al. (2011), Rubilar et al. (2011).
	FRAP (mol Fe^{2+}/kg dw)	n.r.	-
	ORAC (g ET/kg dw)	n.r.	-
Murta leaf	DPPH (mg/L)	21.60[b]	Rubilar et al. (2011).
	FRAP (g ET/kg dw)	188.5	López de Dicastillo et al. (2017).
	ORAC (g ET/kg dw)	281.9 - 4,981	López de Dicastillo et al. (2017), Peña-Cerda et al. (2017).

DPPH: bleaching rate of the radical stable 2,2-diphenyl-1-picrylhydrazyl (concentration of the sample required for the inhibition of DPPH radical by 50%), FRAP: ferric reducing antioxidant power, ORAC: oxygen radical absorption capacity, ET: Equivalent Trolox, dw: dry weight, n.r.: not reported, [a, b, c]: concentration of the methanol DPPH solution 100, 400, 50 µM, respectively.

The variation in the TPC and AC can be attributed to the interaction of several factors, some related to the extraction process such as temperature and solvent's polarity (Mariotti-Celis, Martínez-Cifuentes, Huamán-Castilla, Vargas-González, et al., 2018) as well as pre-processing factors like geographical conditions and harvest time (Fredes et al., 2014; Rodríguez et al., 2016). Additionally, the analysis method can also be an influential factor, especially in the determination of antioxidant capacity. Observed differences in these values can be attributed to intrinsic characteristics of the assay, such as steric impossibility and differences in the polyphenols profile of the extracts (Mariotti-Celis, Martínez-Cifuentes, Huamán-Castilla, Vargas-González, et al., 2018).

This review discusses relevant information about the antioxidant potential of maqui and murta, focusing on the bioactivity of the extracts of both leaves and fruits. We also

analyze the polyphenolic profiles of these superfruits as well as the main factors that can affect them. Finally, some details regarding the bioactivity and bioavailability of their most relevant polyphenols are given.

2.2. Bioactivity of maqui and murta extracts

2.2.1. Berry extracts

Maqui and murta are fruits increasingly consumed in Chile as food (freeze-dried powders and jams) and drinks (soda drinks, juices, and liquors) because of their high antioxidant capacity and health promoting bioactivities (Torralbo et al., 2011).

There are several studies showing important bioactivities of maqui berry (Table 2-3). For example, infusions of maqui are used to treat simple enteritis and dysentery (Bonometti, 2000). In addition, maqui berry showed significant protection against hydrogen peroxide-induced intracellular oxidative stress in human endothelial cell cultures (Miranda-Rottmann et al., 2002). Maqui is exceptionally effective in inhibiting α-glucosidase (IC_{50} = 0.33 ± 0.02), a key enzyme involved in the metabolism of carbohydrates, compared not only with acarbose control (IC_{50} = 3.89 ± 0.79) but also with other Latin American fruits like cape gooseberry (IC_{50} = 56.03 ± 0.32), noni (IC_{50} = 27.32 ± 2.79), acai (IC_{50} = 2.14 ± 0.18) and papaya (IC_{50} = 1.58 ± 0.26) (Gironés-Vilaplana et al., 2014). Moreover, like other berries, maqui presents antibacterial properties (Genskowsky et al., 2016; Khalifa et al., 2015), probably due to its high anthocyanins content. The maqui berry extract also showed an anti-inflammatory effect in macrophage cells (Céspedes et al., 2017), which was subsequently evaluated with *in vivo* assay (Céspedes et al., 2010).

In vivo assays with maqui berry, methanol extracts showed a cardioprotective effect on acute ischemia/reperfusion performed in rat hearts (Céspedes et al., 2008). Anti-diabetic effects of a standardized anthocyanin-rich formulation from maqui berry (ANC) were studied in mice. It was observed that oral administration of ANC improved fasting blood glucose levels and glucose tolerance in hyperglycemic obese mice fed with a high-

fat diet, suggesting that ANC might aid in preventing and treating type II diabetes by controlling the chronic hyperglycemia of diabetic patients (Rojo et al., 2012). Maqui berry has also been related to the prevention of retinal diseases because it protects the photoreceptor cells from their degeneration caused by sunlight (Tanaka et al., 2013).

Even though the antibacterial effect of murta fruits has been reported only on an *in vitro* scale up to now (López de Dicastillo et al., 2017), it is used in folk medicine as anti-inflammatory and analgesic for different kind of pains (Montenegro, 2000). Considering its similarities with related berries such as blackberry, blueberry, cranberry, red raspberry, and strawberry, it is expected that murta berries would be beneficial to treat several human health ailments. For instance, the inhibition of growth and the stimulation of the apoptosis of human cancer cells (Seeram et al., 2006) and the attenuation of induced gastric lesions in rats by the activation of antioxidant enzymes (Alvarez-Suarez et al., 2011).

2.2.2. Leaf extracts

Infusions of maqui and murta leaves have long been used in traditional and native medicine in Chile. These beneficial effects have been assessed in several *in vitro* and *in vivo* studies (Table 2-3).

Maqui leaves have been applied for the treatment of diarrhea, amygdalitis, pharyngitis, dysenteries, tonsillitis, and oral ulcers (O. Muñoz, 2001). Leaves also show an anti-diabetic effect that exceeds 87% of the maqui berry (Rubilar et al., 2011). *In vivo* assays in mice with extracts of maqui leaves, showed stronger anti-inflammatory effects than the reference drug "Nimesulide" (O. Muñoz et al., 2011).

Alcoholic beverages and infusions made of murta leaves are useful to attenuate urinary tract pains and as astringent, stimulant, and phytoestrogenic (Montenegro, 2000). In mice trials, methanol extracts of murta leaves showed a dose-dependent antinociceptive effect applying intraperitoneal, oral, and topical administrations. These results were close to those of the reference medicine "Ibuprofen" (Delporte et al., 2007). *In vitro* assays with aqueous extracts of murta leaves showed significant protection of human erythrocytes

exposed to the oxidative stress induced by an extremely toxic biological oxidant (HClO). A concentration as low as 10 µM GAE of murta aqueous extract neutralized the effect of a HClO concentration as high as 0.25 mM (Suwalsky et al., 2007). Moreover, aqueous extracts of murta leaves were used as an additive in the development of edible films made from tuna-fish gelatin. The transparent films enriched with this additive exhibited increased protection against UV light as well as stronger antioxidant capacity (Gómez-Guillén et al., 2007).

Finally, crude extracts of fruit, leaves and stems of murta and maqui were compared based on their capacity to inhibit α-glucosidase. Crude extracts of maqui stem and leaves were the two most active inhibitors, followed by crude extracts of murta leaves. The inhibition of this enzyme could control hyperglycemia and could be useful to develop functional foods for diabetes patients (Rubilar et al., 2011).

2.3. Polyphenolic profile

The polyphenolic profiles of fruit and leave extracts of maqui and murta, have been identified and quantified using High Performance Liquid Chromatography (HPLC) coupled to Diode Array Detector (DAD) and Mass Spectrometry (MS).

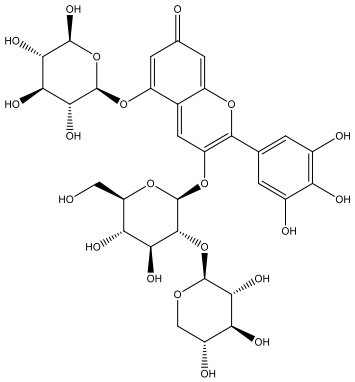
The analyses of these superfruits have shown that both maqui and murta contain mainly anthocyanins such as delphinidin 3-glucoside-5-sambubioside and pelargonidin 3-arabinoside; and flavonols such as quercetin 3-glucoside and 3-rutinoside. The chemical structures of the polyphenols identified in these natural extracts are shown in Figures 2-2, 2-3 and 2-4.

Table 2-3: Bioactivity of maqui and murta extracts.

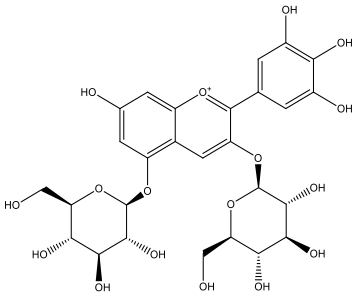
Source	State of source	Solvent's kind	Bioactivity	Assay's details	
				in vitro	*in vivo*
Maqui fruit	Freeze-dried	Aqueous methanol 80%	Antibacterial effect expressed in MIC and MBC.	Microbial strains, associated with microorganisms of decomposition [a]	
	Air-dried	Water, aqueous methanol 60% and acetone	Anti-inflammatory effect by inhibiting enzymes (iNOS and COX-2) or of their products involved in inflammatory response.	RAW 264.7 murine macrophage cells [b]	
	Air dried	Ethanol and acetone	Anti-inflammatory effect against 12-deoxyphorbol-13-decanoate (TPA).		Ear edema in mice [c]
	Air-dried	Water, aqueous methanol, and fractions	Cardioprotective effect on acute ischemia-reperfusion performed.		Male rats weighing 250 - 300 g [d]
	Freeze-dried	Aqueous methanol 70%.	Anti-diabetic effects.	Rat liver cells [e]	Per-os administration in hyperglycemic obese mice fed a high fat diet [e]
	Fresh	Aqueous methanol 70%.	Anti-diabetic effect by inhibition of lipase and **α**-glucosidase.	Enzymes involved in the metabolism of carbohydrates [f, g]	
	Fresh	Concentrated aqueous juice	Inhibitory effect of copper-induced LDL (low density lipoprotein) oxidation.	Human LDL prepared from plasma normolipidemic blood donors [h]	
	Fresh	Concentrated aqueous juice	Protects from hydrogen peroxide-induced intracellular oxidative stress.	Human umbilical vein cells [h]	
	Fresh	Aqueous ethanol	Protective effect against the death of photoreceptor cells induced by light.	661W murine cells [i]	
	D3G5G extract	Aqueous ethanol	Restores tear secretion in dry eye by decreasing the formation of ROS.		Female 8-week-old rats [j]
Maqui leaf	Dried	Aqueous ethanol 50% and fractions	Anti-diabetic effect by inhibition of **α**-glucosidase/**α**-amylase and anti-hemolytic activity.	Was performed according to the chromogenic method [f]	

	Air-dried	Aqueous and methanol	Anti-inflammatory effect against 12-deoxyphorbol-13-decanoate (TPA) and arachidonic acid.		Topical administration in mice [k]
	Air-dried	Aqueous and methanol	Analgesic effect by tail flick and tail formalin tests.		Per-os administration in guineapigs [k]
Murta fruit	Try dried	Water and aqueous ethanol 50%	Antibacterial effect expressed in MIC and MBC.	Escherichia coli and Listeria monocytogenes [l]	
Murta leaf	Air-dried	Water	Protective effect the human erythrocytes exposed to oxidative stress induced by an extremely toxic natural oxidant (HClO).	Red blood cells from healthy donors used to hemolysis assays and scanning electron [m]	
	Dried	Ethyl acetate and methanol	Antinociceptive effect (dose-dependent).		Intraperitoneal, oral, and topical administration in tail formalin test in mice [n]
	Dried	Aqueous ethanol 50% and fractions	Inhibiting effect on **α**-glucosidase/**α**-amylase and anti-hemolytic activity.	Was performed according to the chromogenic method [f]	
	Try dried	Water and aqueous ethanol 50%	Antibacterial effect expressed in MIC and MBC.	Escherichia coli and Listeria monocytogenes [l]	

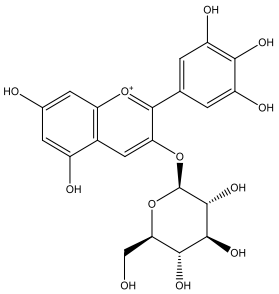
D3G5G: delphinidin 3, 5-diglucoside, MIC: minimum inhibition concentration and MBC: minimum bactericidal concentration. [a] (Genskowsky et al., 2016), [b] (Céspedes et al., 2017), [c] (Céspedes et al., 2010), [d] (Céspedes et al., 2008), [e] (Rojo et al., 2012), [f] (Rubilar et al., 2011), [g] (Gironés-Vilaplana et al., 2014), [h] (Miranda-Rottmann et al., 2002), [i] (Tanaka et al., 2013), [j] (Nakamura et al., 2014), [k] (O. Muñoz et al., 2011), [l] (López de Dicastillo et al., 2017), [m] (Suwalsky et al., 2007), [n] (Delporte et al., 2007).



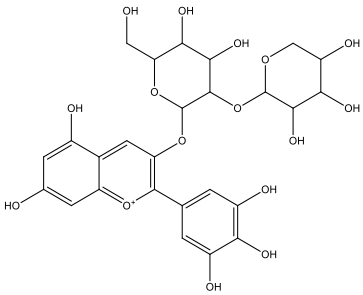
(a) Delphinidin 3-sambubioside 5-glucoside



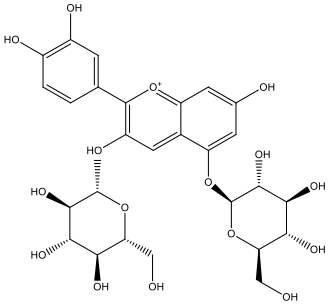
(b) Delphinidin 3,5-diglucoside



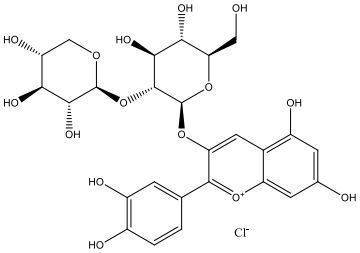
(c) Delphinidin 3-glucoside



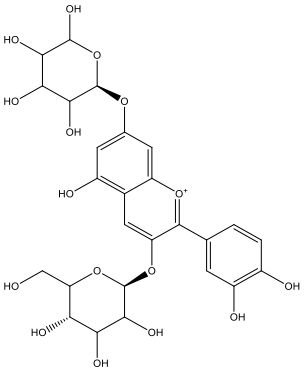
(d) Delphinidin 3-sambubioside



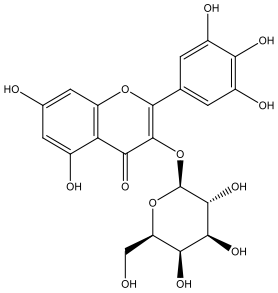
(e) Cyanidin 3,5-diglucoside



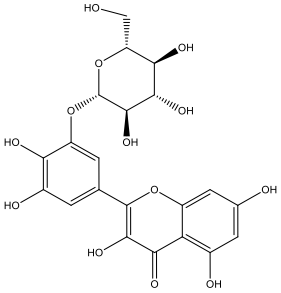
(f) Cyanidin 3-sambuioside



(g) Cyanidin 3-glucoside 7-rhamnoside



(h) Myricetin 3-galactoside



(i) Myricetin 3-glucoside

Figure 2-2 contd…

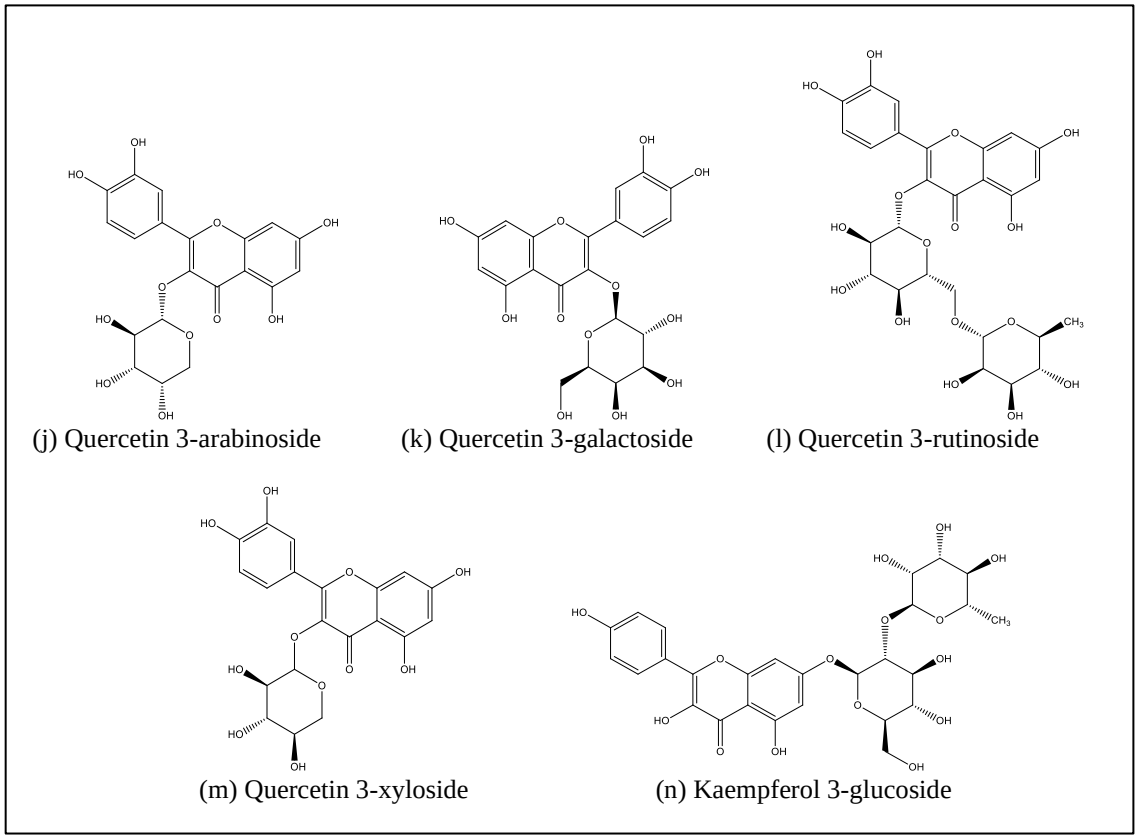


Figure 2-2: Chemical structures of maqui berry's polyphenols. (a-g): anthocyanins and (h-n): flavonols.

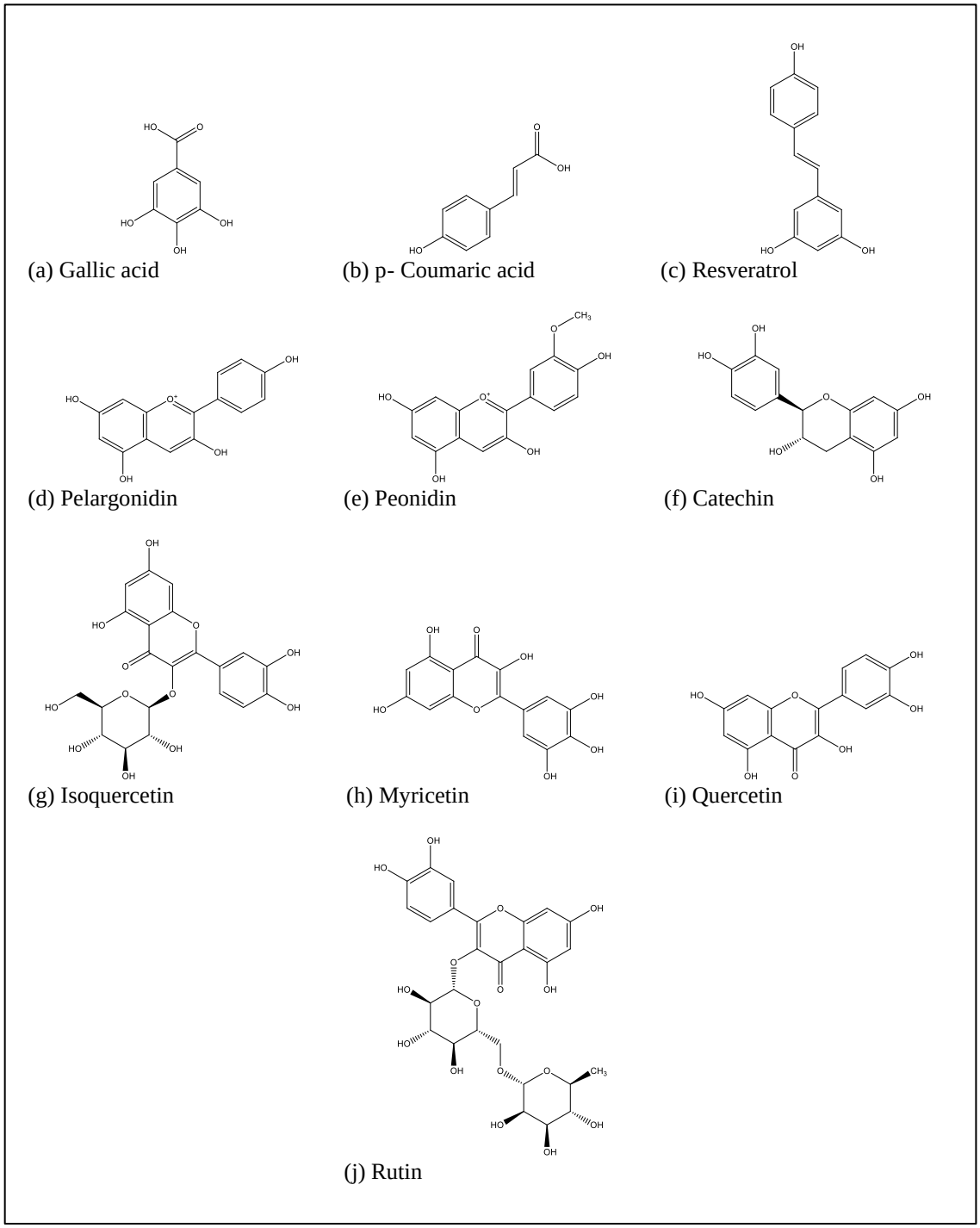
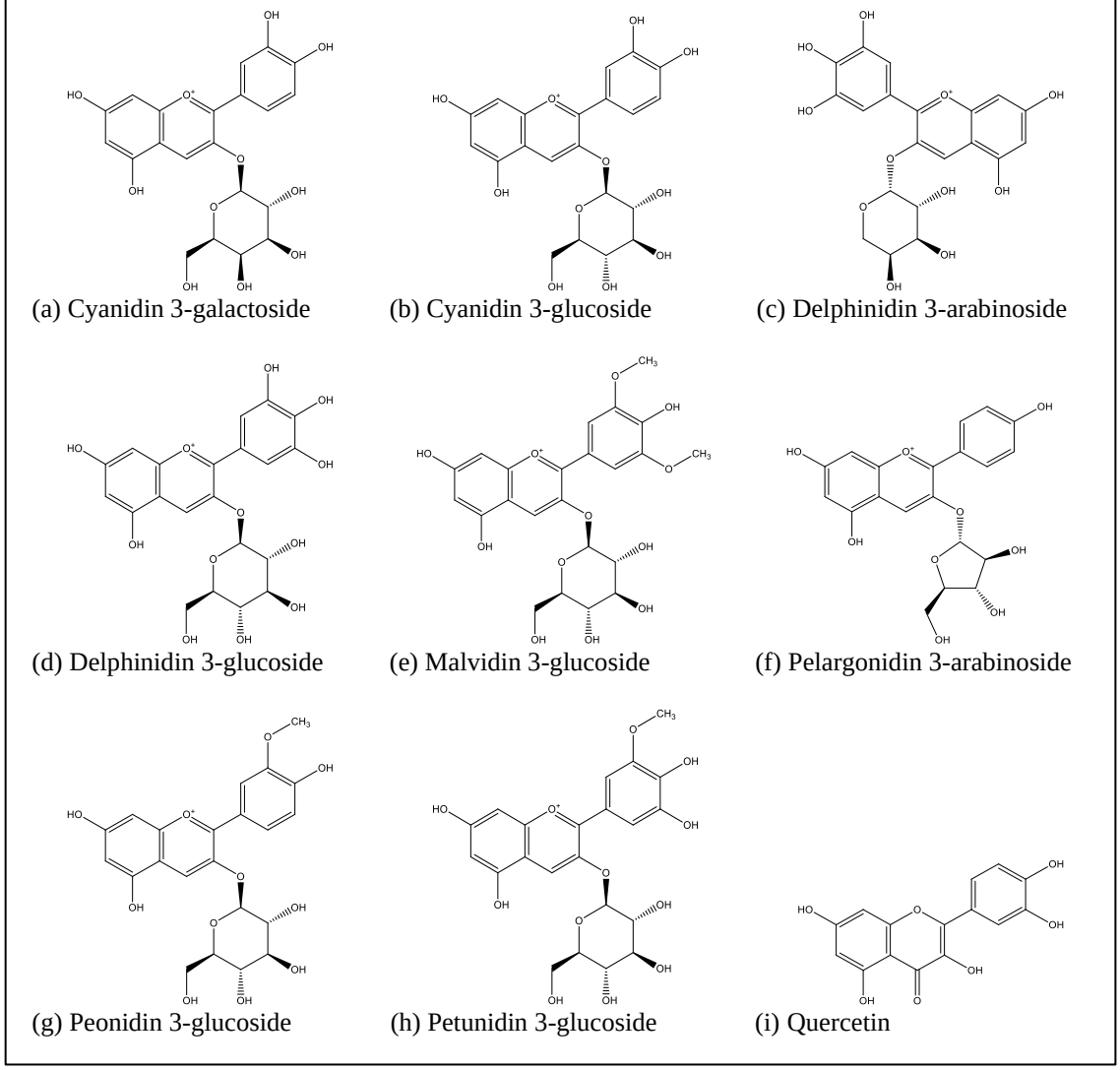


Figure 2-3: Chemical structures of maqui leaf's polyphenols. (a-b): phenolic acids, (c): stilbene, (d-e): anthocyanins, (f): flavanols and (g-j): flavonols.



(a) Cyanidin 3-galactoside (b) Cyanidin 3-glucoside (c) Delphinidin 3-arabinoside

(d) Delphinidin 3-glucoside (e) Malvidin 3-glucoside (f) Pelargonidin 3-arabinoside

(g) Peonidin 3-glucoside (h) Petunidin 3-glucoside (i) Quercetin

Figure 2-4 contd…

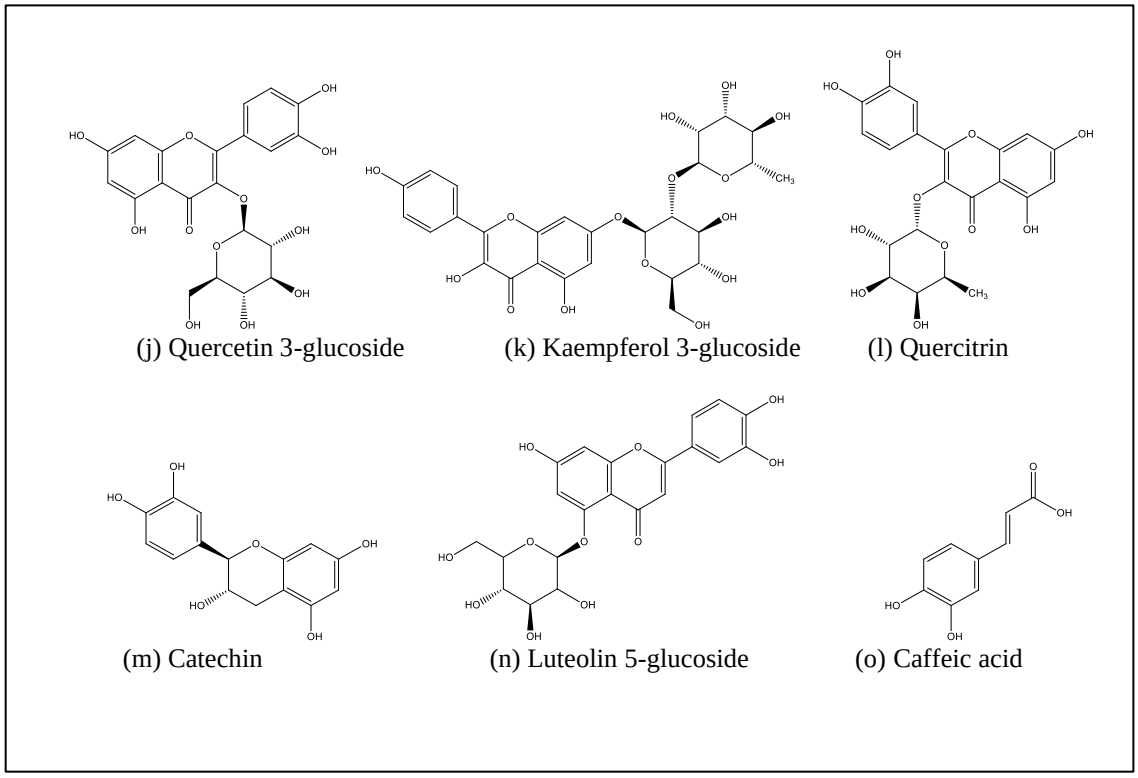


Figure 2-4: Chemical structures of murta berry's polyphenols. (a-h): anthocyanins, (i-l): flavonols, (m): flavanol, (n): flavone and (o): phenolic acid.

In extracts of maqui berry, anthocyanins were the major polyphenols identified. The most abundant anthocyanins were delphinidin derivatives. Céspedes et al. (2010), Escribano-Bailón et al. (2006) and Gironés-Vilaplana et al. (2014) found delphinidin-3-sambobioside-5-glucoside as the main anthocyanin in maqui berry, whereas Genskowsky et al. (2016) and Rojo et al. (2012) reported that delphinidin-3-glucoside was the most abundant. This variability can be attributed to several factors, like extraction solvent, type of drying, location of the plant, year, and time of harvest as well as the quantification method. Non-anthocyanins were found in lesser amounts, of which two were phenolic acid and 13 flavonols. Tables 2-4 and 2-5 show the detailed phenolic content of maqui extracts (Brauch et al., 2016; Céspedes et al., 2010; Escribano-Bailón et al., 2006; Genskowsky et al., 2016; Gironés-Vilaplana et al., 2014; Rojo et al., 2012). The most abundant maqui polyphenols (delphinidin derivatives) are also present in other sources

such as: black currant, grape wine, common bean, pomegranate juice and other berries. Figure 2-5 includes known natural sources that contain this kind of polyphenols. This data was taken from the database "Phenol-Explorer" (Neveu et al., 2010), selecting relatively recent reports that used similar analytical methods. Maqui is the only natural source of delphinidin 3-glucoside-5-sambubioside reported in this database, and the rest of the delphinidin derivatives are more abundant in maqui berry than in any other source in this database.

Table 2-4: Anthocyanins present in maqui fruit extracts.

Polyphenols	g/kg dw Escribano-Bailón et al. (2006)[a]	Céspedes, Valdez et al. (2010)[a]	Gironés-Vilaplana et al. (2014)[b]	Rojo et al. (2012)	Brauch et al. (2016)	Genskowsky et al. (2016)
Del 3-S-5-G	0.715 ± 0.002	1.011	2.503 ± 0.114	3.2	8.57 ± 0.95	4.36 ± 0.01
Del 3,5-diG	0.365 ± 0.003	0.498	2.404 ± 0.085	3.3	16.83 ± 1.48	7.23 ± 0.04
Del 3-S	0.219 ± 0.002	0.305	0.632 ± 0.004	8.8	1.26 ± 0.11	7.06 ± 0.15
Del 3-G	0.263 ± 0.003	0.325	2.109 ± 0.018	13.5	4.02 ± 0.32	9.48 ± 0.25
Cy 3-S-5-G	0.288 ± 0.003	0.207	1.347 ± 0.033	2.2	6.35 ± 0.53	6.89 ± 0.06
Cy 3,5-diG		0.187				5.36 ± 0.05
Cy 3-S	0.137 ± 0.000	0.174	0.822 ± 0.005	0.2	2.01 ± 0.11	0.73 ± 0.11
Cy 3-G	0.132 ± 0.002	0.172	n.d.	8.3		1.24 ± 0.02
Cy 3-G-5-R	n.d.	n.d.	0.025 ± 0.019	n.d.	n.d.	n.d.

Del: delphinidin, Cy: cyanidin, S: sambubioside, G: glucoside, R: rhamnoside, dw: dried weight, n.d.: not detected. [a]: g equivalents of delphinidin 3-glucoside, [b]: g equivalents cyanidin 3-glucoside.

Maqui leaves are rich in gallic acid (47.55%), catechin (21.75%), pelargonidin (14.45%) and resveratrol (3.55%) (Vidal et al., 2013). The last two are found in few other sources and in lesser amounts. For example, Figure 2-6 shows that maqui leaves contains at least 3 times more pelargonidin than strawberry (a well-known rich source of pelargonidin). Figure 2-6 was prepared following the same procedure used in Figure 2-5.

Ethanol extracts of murta fruit contain mainly three non-anthocyanins, caffeic acid 3-glucoside, quercetin-3-glucoside, and quercetin, as well as two main anthocyanins, pelargonidin-3-arabinose, and delphinidin-3-glucoside (Junqueira-Gonçalves et al.,

2015). On the other hand, methanolic extracts contain mainly two anthocyanins petunidin 3-rutinoside and peonidin 3-glucoside (Brito et al., 2014).

The following polyphenols were detected in murta leaf extracts: (i) flavonols: mainly branched to 3-glycoconjugates of myricetin and quercetin; (ii) flavanols: epicatechin, and (iii) phenolic acids: gallic acid (Rubilar et al., 2006).

The properties of the most relevant of these polyphenols and their derivatives are detailed below.

Table 2-5: Non- anthocyanins present in maqui fruit extracts.

Family	Polyphenol	g/kg dw	
		Gironés-Vilaplana et al. (2014)	Genskowsky et al. (2016)
Phenolic acids	Ellagic acid	0.0201[a] ± 0.0015	0.94 ± 0.01
	Granatin B	0.0053[a] ± 0.0011	n.d.
Flavonols	Quercetin 3-rutinoside	0.0513[b] ± 0.0087	n.d.
	Myricetin 3-galoyglucoside	0.0320[b] ± 0.0024	n.d.
	Myricetin 3-galactoside	0.0247[b] ± 0.0034	0.32 ± 0.01
	Quercetin 3-arabinoside	0.0224[b] ± 0.0009	n.d.
	Quercetin 3-galactoside	0.0217[b] ± 0.0060	0.17 ± 0.00
	Myricetin 3-glucoside	0.0192[b] ± 0.0038	0.62 ± 0.01
	Quercetin 3-xyloside	0.0155[b] ± 0.0009	n.d.
	Dimethoxy-quercetin	n.d.	0.28 ± 0.00
	Kaempferol 3-rutinoside	0.0074[b] ± 0.0029	n.d.
	Myricetin	n.d.	0.25 ± 0.01
	Rutin	n.d.	0.20 ± 0.01
	Quercetin 3-glucoside	n.d.	0.07 ± 0.00
	Quercetin	n.d.	0.06 ± 0.00

[a]: g ellagic acid-3-glucoside equivalent, [b]: g quercetin-3-glucoside equivalent, n.d.: not detected.

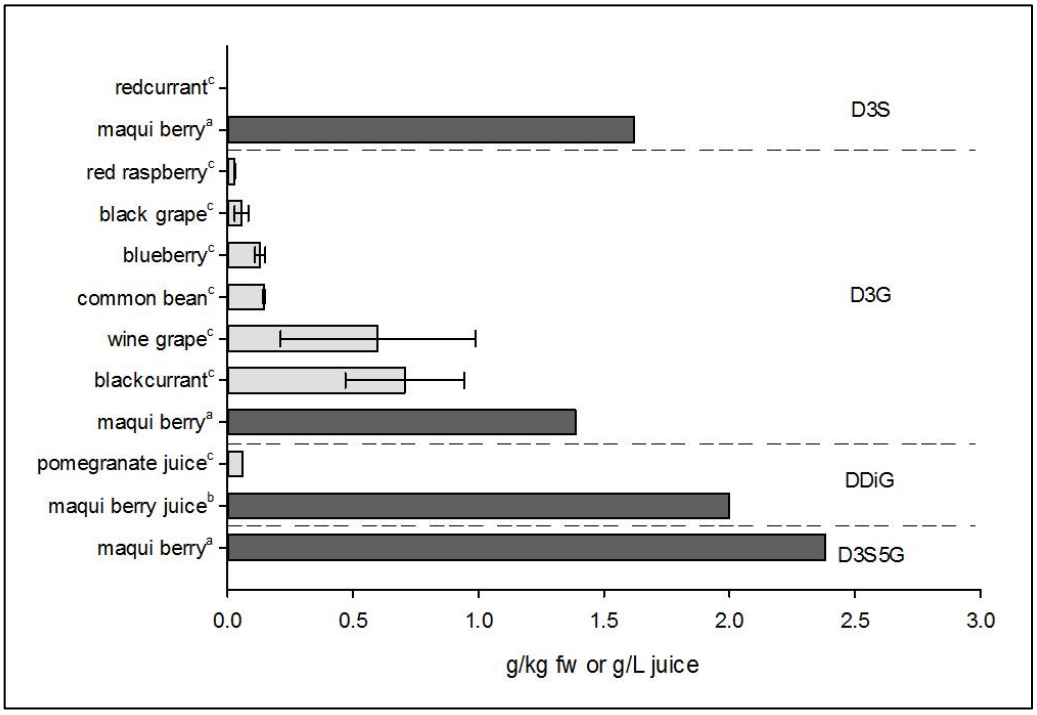


Figure 2-5: Contents of delphinidin derivatives in maqui berry and other natural sources. Where: D3S: delphinidin-3-sambubioside, D3G: delphinidin-3-glucoside, DDiG: delphinidin-3,5-diglucoside and D3S5G: delphinidin-3-sambubioside-5-glucoside, fw: fresh weight. [a]: mean of values obtained from (Brauch et al., 2016; Escribano-Bailón et al., 2006), [b]: value obtained from (Brauch et al., 2016), [c]: mean of different cultivars or varieties values obtained from (Neveu et al., 2010).

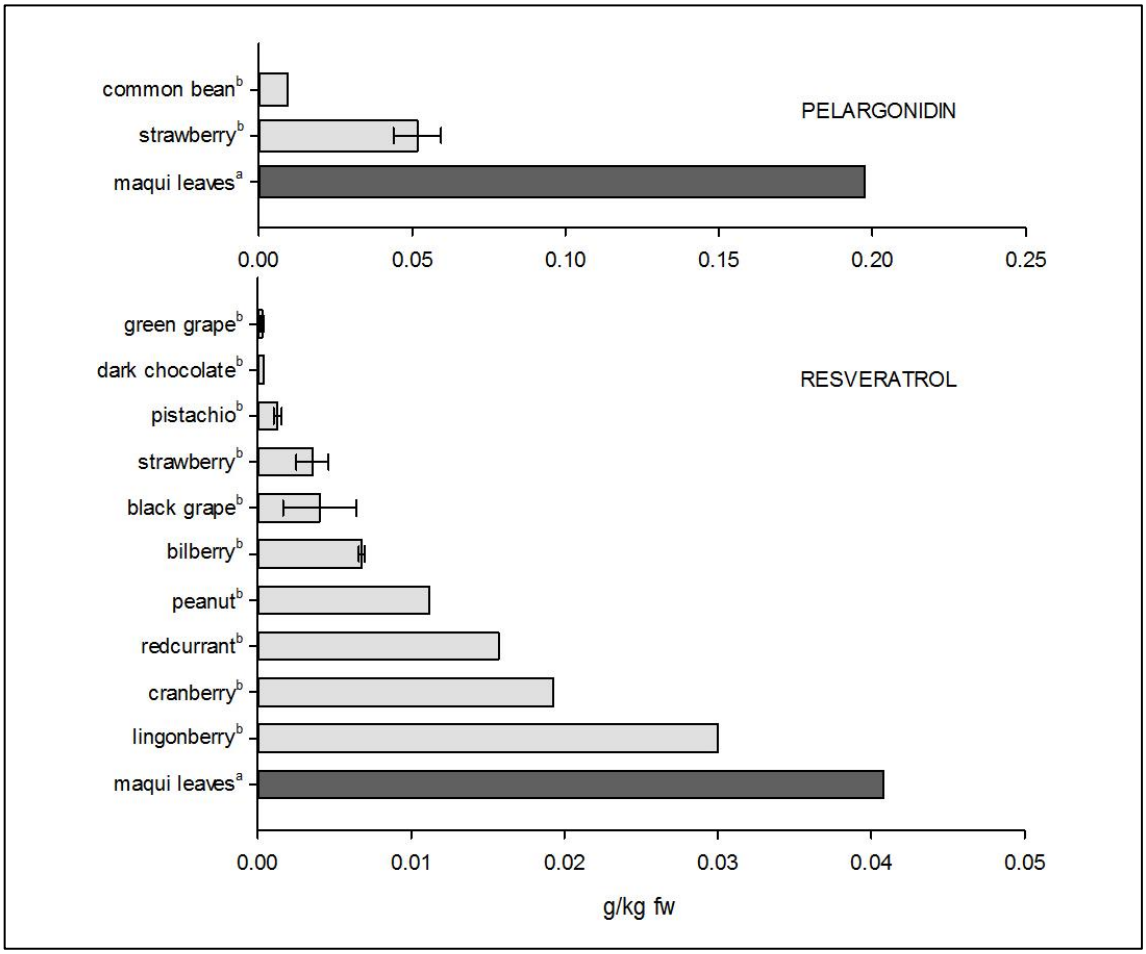


Figure 2-6: Pelargonidin and resveratrol contents in maqui leaves and other natural sources. Where fw: fresh weight, [a]: values adapted from (Vidal et al., 2013), [b]: mean of different cultivars or varieties values obtained from (Neveu et al., 2010).

2.3.1. Pelargonidin (Anthocyanin)

Anthocyanins such as delphinidin, cyanidin, peonidin and pelargonidin are a group of flavonoid compounds that are responsible for the attractive colors (orange, red and blue) of many flowers, vegetables, and fruits (Aguilera-Otíz et al., 2011; Junqueira-Gonçalves et al., 2015). Hence, in addition to their application as functional food ingredients, they can be used as natural colorants in foods and beverages.

Recent reports have shown multiple health benefits associated with consumption of fruits rich in pelargonidin such as strong protection against oxidative stress, reduction of ischemic brain damage, protection of neurons against stroke-induced damage, reversal of age-related changes in brain (Dreiseitel et al., 2008) and antidiabetic effects (Roy et al., 2008).

Pelargonidin has been identified in in few sources such as beans (0.0095 g/kg fresh weight), strawberries (0.0517 g/kg fresh weight) and recently in maqui leaves (0.1974 mg/g fresh weight) as can be observed in Figure 2-6.

This compound is among the six flavonoids that have shown strongest *in vitro* and *in vivo* anti-inflammatory effects (Hämäläinen et al., 2007). Pelargonidin is also a better lipid peroxidation inhibitor than other anthocyanins such as cyanidin and delphinidin (Tsuda et al., 1996). Moreover, pelargonidin has the highest absorption rate among all anthocyanins (Felgines et al., 2003; X. Wu et al., 2004) which in general show poor absorption (Manach et al., 2004). Clinical trials have found that the absorption of pelargonidin can be improved by the effect of the food matrix, such as in strawberries with cream. In this case, the cream delays the passage through the gastrointestinal tract, enhancing the absorption of pelargonidin (Mullen et al., 2008). In addition, studies in rats showed that oral administration of pelargonidin (3-10 mg/kg body weight) may attenuate the hyperalgesia caused by diabetes mellitus in rats (Mirshekar et al., 2010; Roy et al., 2008). Finally, photoprotective effects and decreasing DNA damage on human skin cells against the adverse effects of UV-A radiation have been attributed to pelargonidin aglycon and its glucosides (Giampieri et al., 2012).

Pelargonidin shows complex synergistic and antagonistic interactions in polyphenol mixtures. It can reduce the antioxidant capacity of mixtures of p-coumaric acid and catechin, while it can increase the antioxidant capacity of mixtures of quercetin and cyanidin as well as mixtures of p-coumaric acid and quercetin (Reber et al., 2011).

2.3.2. Delphinidin (Anthocyanin)

Delphinidin, cyanidin and peonidin are the most abundant anthocyanins in berries. In these fruits, delphinidin is present as aglycone and bound to glucosides, arabinosides, galactosides, rutinosides, sambubiosides and xylosides (Neveu et al., 2010; X. Wu & Prior, 2005). The bioactive properties of only few bound delphinidins are known, showing similar health benefits than their aglycones. For example, delphinidin is the most potent inhibitor of osteoclastogenesis and *in vivo* studies have shown that it is effective to prevent the degradation of bones (Moriwaki et al., 2014).

Delphinidin 3-glucoside-5-sambubioside, a characteristic anthocyanin from maqui berry, seems to be partially responsible for the anti-diabetic effect of standardized anthocyanin-rich formulation of maqui berry. Specifically, this compound has shown anti-diabetic properties in rats, reducing both the glucose levels in the plasma and the glucose production in liver cells (Rojo et al., 2012). It also presented protective effects against death of photoreceptor cells *in vitro* assays with murine cells. This effect is associated with the prevention of night blindness and visual field constriction caused by sunlight (Tanaka et al., 2013).

Delphinidin-3,5-diglucoside also showed a therapeutic or preventive effect in ophthalmic disorder. It was demonstrated that the application of this polyphenol by oral route prevents dry eye, which has increased remarkably due to the radical expansion in the use of technological devices (Nakamura et al., 2014).

Delphinidin 3-glucoside is found mainly in berries, grape, wines, and beans (Neveu et al., 2010). *In vitro* tests, delphinidin 3-glucoside presented antiapoptotic effects in endothelial cells (Lamy et al., 2006; Martin et al., 2003) and fotoquimiopreventive effects against oxidative stress induced by UV-B (Yun et al., 2009). In prostate cancer, tumor growth inhibition and induction of apoptosis in malign cells has been shown (Bin Hafeez et al., 2008). Recent studies on bioavailability confirmed these potential therapeutic effects and established an absorption rate about 1% after oral administration (Martin et al., 2003).

Little is known about the bioactivity and bioavailability of delphinidin 3-sambubioside, a red pigment (Tsai et al., 2002) also found in redcurrant (Neveu et al., 2010) and in the *Hibiscus abdariffa L.* flower (Hou et al., 2005). A study proposes it is chemopreventive against leukemia (Hou et al., 2005).

Finally, Delfinol®, a standardized extract with ≥ 25% of delphinidin from maqui berry, was developed and evaluated in mice and rats as a blood glucose reducer. It was shown that a dosage of 20 mg/kg body weight significantly reduces postprandial blood glucose in subjects with moderate glucose intolerance (Hidalgo et al., 2014).

2.3.3. Resveratrol (Stilbene)

Resveratrol (3, 4, 5–trihydroxystilbene) is a phytoalexin produced by some plants in response to stress (Adrian & Jeandet, 2012). Foods containing resveratrol are limited to some berries like lingonberry and cranberry, grapes, red wine, peanuts, chocolate, and pistachio. It has also been reported in some non-edible leaves such as vine, eucalyptus and maqui (Figure 2-6) (Counet et al., 2006; Neveu et al., 2010; Vidal et al., 2013).

Resveratrol has received significant attention for its chemopreventive activity against cardiovascular diseases and various cancers (Jang et al., 1997). In addition, it mitigates the symptoms of neurodegenerative diseases (Singh et al., 2015; C. F. Wu et al., 2013). In nature, resveratrol can be found in different forms, such as the isomers trans- and cis-, the derivates piceid or piceatannol or the dimers viniferin, presenting different absorption and bioactivity properties. For example, it was shown that cis- and trans-resveratrol exhibited different ligand binding properties to human estrogen receptors. Moreover, the colonic-derived metabolite dihydro-resveratrol showed a biphasic effect, i.e., at high concentrations it inhibited the proliferation of tumor cells, while at low concentrations it favored their growth (Anisimova et al., 2011).

Several positive effects of resveratrol were demonstrated using *in vitro* models. For example, it reduces the proliferation of human colon cancer cells (Schneider et al., 2000) and removes excessive or inappropriate aggregations of platelets, decreasing the transient

ischemic risk, myocardial infarction, or stroke (Zini et al., 1999). In addition, it could be a candidate for retarding the progression of Alzheimer (Carpenter, 2017; Marambaud et al., 2005).

In vivo studies have revealed that resveratrol acts as a chemopreventive agent for skin cancer in mice models (Jang et al., 1997). In addition, it has been found responsible for reducing insulin secretion from pancreatic rats islets (Szkudelski, 2008), and for inhibiting angiogenesis and human breast cancer (Garvin et al., 2006). It also has shown to reduce injuries to the kidneys, spinal cord, liver, lungs, intestine, and colon (Baur & Sinclair, 2006).

Although *in vitro* and *in vivo* assays have been mostly conclusive, clinical studies are still unconvincing. There are several reasons explaining this discrepancy. One may be related to the metabolic fate of resveratrol since the intact compound exhibits a low bioavailability (Walle et al., 2004).

Nevertheless, further studies showed an extensive circulation and accumulation in several tissues of resveratrol-derived metabolites, some of them with reported biological effects. So other aspects, such as short-term exposures or the use of non-physiological metabolites or non-physiological concentrations have been suggested as potential reasons for the contradictions between *in vitro*, preclinical, and clinical results (Walle, 2011; Walle et al., 2004).

In addition, dietary intake of resveratrol from foods (dietary intake) is poor, *i.e.*, few milligrams per day, making relevant the development of strategies for increasing their intake such as the reutilization of murta and maqui leaves to produce concentrated extracts. It should be in mind though, that resveratrol presents a NOAEL (non-observed-adverse-effect-level) of 300 g/kg/day, and higher dosages can cause neurotoxicity, leukocytosis, anemia, and dehydration (Sáenz Chávez et al., 2014).

2.4. Phenolic variability

The antioxidant properties and phenolic composition of natural extracts are often not reproducible. This is due to genotype, environment, stage of harvesting, storage, and processing (Céspedes et al., 2013). Several studies have quantified the impact of some of these factors on the composition of maqui and murta extracts. The factors that cause larger variability in the TPC of maqui and murta berries are drying and extraction solvent; both linked to processing (Figure 2-7).

Changing drying temperature from 40 to 80°C can generate a significant loss of the original polyphenols found in the natural source. At the same time, neo-antioxidants due to the Maillard reaction can be generated depending on the temperature and drying time (Rodríguez et al., 2016). Freeze-drying is the most effective method since avoids polyphenols degradation and breaks the structure of the matrix, facilitating the subsequent extraction of the bioactive components by increasing the access of the solvent (Chan et al., 2009).

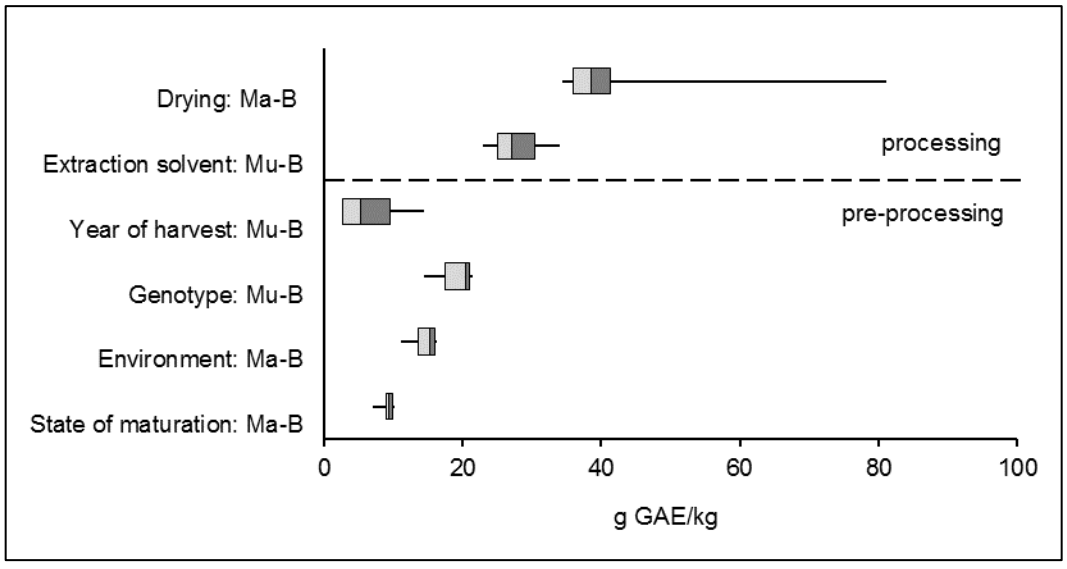


Figure 2-7: Variability of Total Polyphenol Content in maqui berries (Ma-B) and murta berries (Mu-B) due processing (López de Dicastillo et al., 2017; Rodríguez et al., 2016) and pre-processing factors (Alfaro et al., 2013; Fredes et al., 2012; Fredes et al., 2014).

Fruit and leaf polyphenols present different solubility for different solvents, explaining part of the variability found in the composition of maqui and murta extracts. For example, flavonol glycosides are more soluble in water when they have a high degree of glycosylation; contrarily aglycon flavanols (like epicatechin) and monoglycosides are more soluble in ethanol (López de Dicastillo et al., 2017; Shene et al., 2009). Other factors were assessed, such as the extraction system, the solid/liquid ratio, the particles size as well as the processing temperature and time, (Augusto et al., 2015; Rubilar et al., 2006). For example, Rubilar et al. (2006) observed in the extraction of murta leaves that total polyphenol content in the extract is favored by increasing the extraction time and temperature as well as the solvent-to-solid ratio. In addition, methanol was the best solvent followed by ethanol and water.

Although pre-processing factors generate less variability in extract composition, this is still significant and should be considered. In order of importance, these factors are: year of harvest > genotype > environment > maturation stage (Figure 2-7). These effects could be related to the stress these factors induce in plants. For example, for plants harvested in different years, the TPC trends to increase with higher precipitation and number of frosts in the growing period (Alfaro et al., 2013). To those plants that grew in different regions, the temperature and soil characteristics of the region have the greatest influence. For example, maqui berries collected in the mountain presented higher polyphenols content than those collected in the valley or coast (Fredes et al., 2014; Shene et al., 2009). Meanwhile fruits harvested at different stages of maturity presented variation in the contents and the type of polyphenols, with proanthocyanidins being predominant in immature fruits, while in mature fruits the anthocyanins predominated (Fredes et al., 2012). Regarding the genotype effect, a study carried out with 10 genotypes of murta leaves (cultivated with the same soil, climate, and agronomical management) showed significant differences in the composition of the extracts. The genotype ZF-18 (ZF: “zona fría” in Spanish) showed the highest phenolic content and antioxidant activity. Since the original zone of this genotype is cold, the plant rapidly accumulates phenolic compounds to defend against the Reactive Oxygen Species (ROS) induced by low temperatures (Peña-

Cerda et al., 2017). This confirms that the most extreme climatic conditions result in an increase in the polyphenols contained in the plants, as these are used as protective agents. In this sense, the research showed that crop's domestication can cause a decrease in the relative amounts of flavonols in plants. Leaves of wild murta showed a higher amount of flavonols than leaves of cultivated murta. This is because plant resources are allocated to produce higher yields rather than to protect against adverse conditions (Chacón-Fuentes et al., 2015).

It seems, that pre-processing factors may be more determinant of leaves polyphenols content than processing factors. For example, Peña-Cerda et al. (2017) reported a genotype variability in the TPC of murta leaves that is 5 times larger than the TPC variability due to extraction solvent (López de Dicastillo et al., 2017).

2.5. Conclusions

Maqui and murta are rich natural sources of polyphenols, showing higher contents than other plants of the same family (cranberry, strawberry, and others) and even higher contents than popular sources such as grapes, chocolate, and tea.

These berries present high amounts of health-promoting polyphenols such as resveratrol, pelargonidin, delphinidin, catechin and gallic acid. Leaves of these plants are the focus of recent attention since they are even a better source of phenolic compounds than the respective berries.

To generate consistent extracts for food, cosmetic and even pharmaceutical applications, it is necessary to study the effect of processing factors and cultivation conditions.

Further study of these increasingly consumed native Chilean berries is of great economic significance since it supports the commercial activities of gatherers, growers, micro-companies, and associated industries.

CHAPTER 3. MULTI-RESPONSE OPTIMAL HOT PRESSURIZED LIQUID RECOVERY OF EXTRACTABLE POLYPHENOLS FROM LEAVES OF MAQUI (*ARISTOTELIA CHILENSIS* [MOL.] STUNTZ)

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3.1. Introduction

Maqui (*Aristotelia chilensis* [Mol.] Stuntz) is a native evergreen shrub that mainly grows in central and southern Chile. Its fruits have been used in food, pharmaceutical, nutraceutical, and cosmeceutical products due to their potent antioxidant capacity demonstrated in several *in vitro* and *in vivo* studies. The total polyphenol content (TPC) values reported from maqui fruit are higher than those of other berries such as blueberry, strawberry, cherry, blackberry, and raspberry; the antioxidant capacity (AC) of maqui, as measured with the oxygen radical absorbance capacity (ORAC) method, was also higher than the berries mentioned above. Moreover, berries present higher TPC, and AC levels than other widely studied natural sources such as vegetables, pome fruits, citric fruits, and grapes (Rivera-Tovar et al., 2019). Maqui fruit extracts have shown bioactivities related to i) prevention of atherosclerosis, ii) promotion of hair growth, iii) anti-photoaging of the skin, iv) inhibition of low-density lipoprotein oxidation (Avello et al., 2009; Rivera-Tovar et al., 2019; Zúñiga et al., 2017), v) anti-hemolytic protection, vi) inhibition of α-glucosidase and α-amylase, vii) obesity control, viii) diabetes control and ix) cardioprotection. In addition, maqui extracts have shown anti-bacterial, anti-inflammatory, nematocidal, and antiviral activities (Rivera-Tovar et al., 2019; Zúñiga et al., 2017).

Maqui leaves, usually discarded in the agro-industrial production of fruits, could also be used to obtain bioactive ingredients since O. Muñoz et al. (2011) and Rubilar et al. (2011) showed that they have even higher total polyphenol content and antioxidant

capacity than maqui berries (Rivera-Tovar et al., 2019). Aqueous, ethanolic, and methanolic leaf extracts have anti-inflammatory and analgesic properties (O. Muñoz et al., 2011) and the potential to control diabetes by inhibiting αglucosidase and α-amylase (Rubilar et al., 2011). Several polyphenols have been identified in maqui leaves: two phenolic acids (gallic acid and coumaric acid), four flavonols (quercetin, isoquercetin, myricetin, and rutin), two anthocyanins (pelargonidin and peonidin), one flavanol (catechin), and one stilbene (resveratrol) (Vidal et al., 2013). Maqui leaves contain indole and quinoline alkaloids (aristoteline, serratoline, aristone, horbatine, horbatinol, protopine, aristoquinoline, 3fromylindole), as well as minerals, such as calcium, phosphorus, iron, and potassium (Zúñiga et al., 2017). Despite these appealing properties, no previous research has focused on optimizing the polyphenol's extraction process from maqui leaves, and few studies discussed their possible applications.

Rubilar et al. (2011) extracted maqui leaves by maceration with 50% water/ethanol at room temperature and a solvent-to-solid ratio of 5:1. The TPC of the obtained extracts was 69.0 ± 0.9 mg GAE/g dry weight, and the antiradical scavenging capacity against DPPH showed an IC_{50} = 8.0 ± 0.1 mg of extract/L. Hot pressurized liquids extraction (HPLE), also known as accelerated solvent extraction (ASE), can yield extracts with higher polyphenol content in less time and using lower amounts of solvent (Mustafa & Turner, 2011). This green extraction technique is efficient for the extraction of plant bioactives, where extraction temperature, solvent composition, and the number of cycles are the factors that have the most influence on TPC, AC, and the polyphenolic profile of the extracts (Díaz-de-Cerio et al., 2018; Putnik et al., 2017; Tripodo et al., 2018).

This work hypothesizes that by applying multi-response optimization to HPLE, it is possible to find operating conditions that yield several polyphenol extracts of maqui leaves with outstanding features. The optimization of the process was focused on maximizing the polyphenols' extraction yield. Three optimization objectives associated with the extracts obtained in an ASE 200 device (5 mL extraction cell) were considered: i) total polyphenol content (TPC) according to the Folin-Ciocalteu method; ii) antioxidant capacity (AC) as measured by ABTS radical scavenging activity assay; and iii) purity (g

of gallic acid equivalent/100 g dry extract, %). Additional extractions were carried out under optimal conditions in an ASE 150 device (100 mL extraction cell). These extracts were evaluated in terms of total polyphenol content of maqui leaves, the extract's purity, *in vitro* antioxidant capacity (DPPH and ORAC based on the dry mass of leaves and extracts), and the low molecular weight polyphenol profile.

3.2. Materials and methods

3.2.1. Plant materials

Adult maqui (*Aristotelia chilensis*) leaves (four-year-old) were obtained from the Región de La Araucanía, Chile on May 18 – 20, 2016. They were air-dried at 5 – 20 °C and relative humidity of 83% for seven days, ground in a meat mincer, and stored in sealed plastic bags in a dry, dark place at −18 °C before use. The main properties of the raw material were obtained using standard analytical procedures, among them: moisture (10.54 ± 0.09%), protein (15.15 ± 0.18 g/100 g leaves), and ash (0.07 ± 0.00 g/100 g leaves). Mineral content was measured after microwave-assisted acid digestion with nitric acid at 1600 W, 15 min, and 200 °C for 10 min (Marsxpress-CEM Corporation, USA). Sodium and potassium were determined by atomic emission spectrophotometry. Calcium, copper, magnesium, cadmium, iron, and zinc were determined by atomic absorption spectrophotometry in a 220 Fast Sequential Spectrophotometer (Varian, USA). The mineral content was potassium (10.50 ± 0.38 mg/g), calcium (21.23 ± 0.30 mg/g), magnesium (1.98 ± 0.01 mg/g), sodium (20.14 ± 0.99 mg/kg), iron (237.93 ± 4.46 mg/kg), zinc (12.33 ± 0.14 mg/kg), copper (< 7.00 mg/kg), cadmium (< 5.00 mg/kg) and lead (< 10.00 mg/kg).

3.2.2. Solvents and standards

Ethanol 96% (reagent grade Solvents, Scharlau) was used as a cosolvent to prepare the ethanol/water solvent mixtures (5, 15, 20, 25, 50, and 80% v/v). Methanol and acetone (≥99.9%) (HPLC, Sigma Aldrich) were used to prepare aqueous solvents for successive

extraction at ambient conditions (20 °C, 1 atm). For the analytical determinations, the following chemical reagents and standards were used: Folin-Ciocalteu reagent (D ≈ 1.24 g/mL); 2,2′-azino-di-(3ethylbenzthiazoline sulfonic acid) or ABTS (> 98%, Aldrich Chemistry); 2,2-diphenyl-1-picrylhydrazyl or DPPH (Sigma Aldrich); 2,2′–azobis(2-methylpropionamidine) dihydrochloride or AAPH (97%, Sigma Aldrich); sodium carbonate; sodium chloride; potassium chloride; potassium dihydrogen phosphate; disodium phosphate; potassium persulfate; dipotassium phosphate; fluorescein sodium salt; (±)-6hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid or Trolox reagent (97%, Aldrich Chemistry), gallic acid monohydrate (≥99%, Sigma Aldrich), quercetin-3-β-D glucoside (≥90%, Sigma Life Science), hydroxytyrosol (≥98%, Sigma Aldrich), oleuropein (≥98%, Sigma Aldrich). Catechin (≥98%), epigallocatechin (≥98%), epicatechin (≥98%), kaempferol (≥98%), resveratrol (≥98%), quercetin (≥97%), caffeic acid (≥99%), chlorogenic acid (≥98%), vanillic acid (≥99%), protocatechuic acid (≥99%) and ferulic acid (≥98%). These were purchased from Xi'an Haoxuan Bio-Tech Co., Ltd. (Baqiao, China). All solutions were prepared and stored cold and in the dark.

3.2.3. Aqueous-organic successive extraction (SE) at ambient conditions

Successive extractions of maqui leaves with aqueous methanol and aqueous acetone were performed according to Pérez-Jiménez et al. (2008) to obtain reference crude extracts (RCE). Samples of 0.5 g were placed in contact with 20 mL of an acidified (0.8% HCl 2 N) methanol/water solution (50% v/v, pH 2) and vigorously shaken for 1 h; the mixture was then centrifuged at 6000 rpm for 10 min. Subsequently, 20 mL of an acetone/water solution (70% v/v) was added to the remaining solids, and the mixture was then stirred and centrifuged. The methanolic and acetonic extracts were combined to determine the total polyphenol content of the mixture. It can be assumed that the combined extract (RCE1) contains close to 100% of the extractable polyphenols since the natural matrix first comes into contact with a polar and acidified solvent and then with a more non-polar solvent. Additionally, simple individual extractions with both solvents were performed,

which were used as reference extracts (RCE2, RCE3) to identify and quantify polyphenols.

3.2.4. Hot pressurized liquid extraction (HPLE) method

Extraction was performed in an Accelerated Solvent Extraction System ASE 200 (Dionex Corporation, Sunnyvale, CA, USA) using water/ethanol solutions as a solvent. Samples of dried leaves (1 g, dry weight) were mixed with 0.75 g of diatomaceous earth and placed into a 5 mL volume extraction cell. The fixed operating conditions were: 5 min static extraction time, 70% of flush volume, 1 min of purge, and 102.1 atm (1500 psi). The main factors influencing the measured responses (Xynos et al., 2014) were varied within a predefined range: temperature (80 – 200 °C), ethanol concentration (5% – 80% v/v), and the number of cycles (1 – 5). The obtained extracts were protected from light and stored at −20 °C until analysis. Additionally, extractions at the optimum conditions with the same solid-to-extract ratio (~1:45) were replicated in an ASE 150 (Dionex Corporation, Sunnyvale, CA, USA). AC with two other methods (DPPH and ORAC) and the low molecular weight polyphenol profile were determined to complete the characterization. A sample of 2 g (dry weight) was mixed with 1.8 g of neutral quartz sand (instead of diatomaceous earth) and placed into a 100 mL volume extraction cell previously filled with the sand to reduce the volume of the water/ethanol solution used for the extraction.

3.2.5. Experimental design and optimization

TPC and AC of a natural extract can be used to assess process yields (expressed in terms of mg per g of dry weight of the natural matrix) or to chemically characterize the extract obtained (expressed in terms of g per g of dry extract). Both global responses are complementary, providing a better description of a given natural matrix's antioxidant properties. Typically, the extraction process optimization goal is to maximize TPC and AC considering the process yield because the extracts are purified in subsequent steps (e.g., microporous resin purification). Consequently, we assessed the impact of the studied

factors (temperature (x_1), ethanol concentration (x_2), and the number of cycles (x_3)) on TPC (measured in mg GAE/g maqui leaves) and AC_{ABTS} (measured in mg TE/g maqui leaves). All experiments were carried out in a randomized order to minimize the effect of extraneous factors on measured responses.

Optimization was carried out in three sequential steps, each of them defining a specific experimental region (Figure 3-1). In the first two steps, the Box-Behnken experimental design (BBD) was applied to define the experimental points. The third step included a selection of the experimental points of the two previous steps. The response surface methodology (RSM) (Myers et al., 1989) was applied in each experimental region defined at each optimization step. The levels of the factors of the first optimization step were defined based on previous research that applied HPLE to different natural matrixes such as olive leaves (Putnik et al., 2017; Xynos et al., 2014), goji berry (Tripodo et al., 2018) and myrtle leaves (Díaz-de-Cerio et al., 2018). The second optimization step's levels resulted from moving the first experimental region towards the steepest ascent direction, although considering ASE 200 device constraints. The final step considered a selection of the experimental points of the two previous regions, discarding outliers. The latter were identified by looking at the residuals vs. order plots and applying the Minitab® Statistical Software v.19 criteria (standardized residuals with absolute values greater than two). Five goodness of fit statistics were calculated for the fitted models, with and without outliers, to determine their deleterious effect on the model performances. The standard error of the regression (*S*), in response variable units, represents the deviation of the measurements from the modeled response. The lower the value of *S*, the better the model;

$$S = \sqrt{\frac{\sum(y_i - \hat{y}_i)^2}{n - p - 1}} \tag{3.1}$$

y_i is the i^{th} value of the observed response, $\hat{y}_i$ is the i^{th} adjusted response, n is the number of observations, and p is the number of terms in the model.

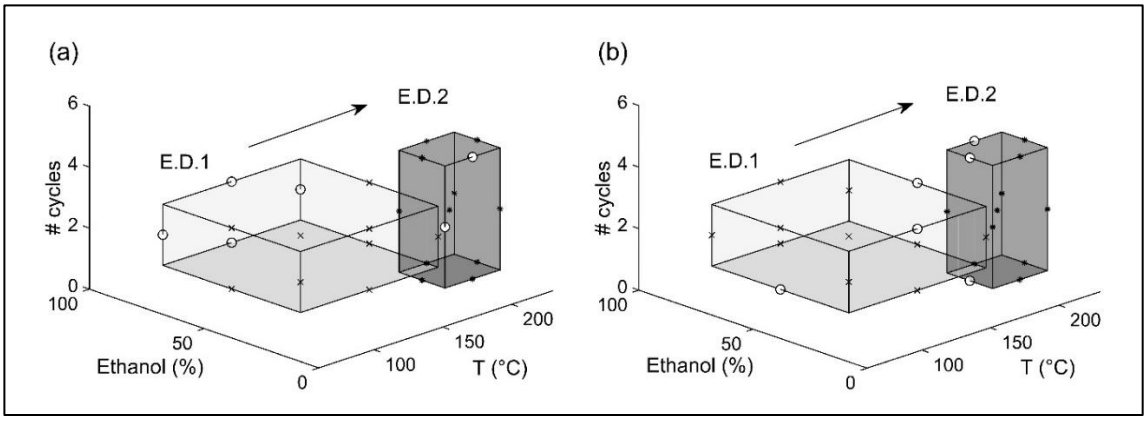

Figure 3-1: Graphical representation of the two preliminary experimental designs (BBD) and outliers (round markers in white) not considered on the final experimental region for (a) TPC and (b) AC_{ABTS}.

The determination coefficient (R^2) varies between 0 and 1; the higher the value of R^2, the better the model fit the experimental values (calibration set). R^2 is most useful when comparing models of the same size because its value increases when additional predictors are added to the model, even when there is no real improvement in the model's fit,

$$R^2 = 1 - \frac{\sum(y_i - \hat{y}_i)^2}{\sum(y_i - \bar{y})^2} \tag{3.2}$$

$\bar{y}$ is the mean response.

The adjusted R-squared (R^2_{adj}) is a modified version of the R^2 that incorporates the number of predictors in the model. The R^2_{adj} increases only if the new term improves the model more than would be expected by chance,

$$R^2{}_{adj} = 1 - \left[\frac{\sum(y_i - \hat{y}_i)^2}{\sum(y_i - \bar{y})^2}\right]\left(\frac{n-1}{n-p-1}\right) \tag{3.3}$$

The predicted R-squared (R^2_{pred}) is a form of leave-one-out cross-validation that is calculated by systematically removing one observation from the original data set and then estimating the regression equation and determining how well the model predicts the

removed observation. Models with an R^2_{pred} value substantially smaller than the corresponding R^2 value may indicate that the model is over-fitted.

$$R^2{}_{pred} = 1 - \frac{\sum(y_i - \hat{y}_{(i)})^2}{\sum(y_i - \bar{y})^2} \tag{3.4}$$

$\hat{y}_{(i)}$ represents the modeled response of the omitted observations.

Akaike's information criterion (*AIC*) describes the relationship between the accuracy and complexity of the model. If the number of parameters of a model increases, the model gains complexity, but at the same time, the mismatch between model and observations decreases. Therefore, the model with the lowest *AIC* value is expected to achieve a higher balance between reducing complexity (parsimony principle) and maintaining a minimum mismatch value,

$$AIC = n\, ln\, \sigma^2 + 2\,(p + 1) \tag{3.5}$$

σ^2 is the standard error between the model and the experimental values.

The responses of each design were initially adjusted to first-order models (plane) (Eq. 3.6) and when a curvature was detected, a second order model (Eq. 3.7) was fitted, which was expressed as a function of linear, interaction, and second-order terms.

$$y_i = \beta_0 + \beta_1 x_1(℃) + \beta_2 x_2(\%) + \beta_3 x_3 \tag{3.6}$$

$$y_i = \beta_0 + \beta_1 x_1(℃) + \beta_2 x_2(\%) + \beta_3 x_3 + \beta_4 {x_1}^2(℃)^2 + \beta_5 {x_2}^2(\%)^2 + \beta_6 {x_3}^2 + \beta_7 x_1 x_2(℃)(\%) + \beta_8 x_2 x_3(\%) + \beta_9 x_1 x_3(℃) \tag{3.7}$$

y_i are the dependent variables (simple or multi-response), β_i are the regression coefficients (fitted from experimental data), and x_i are the studied factors.

Pareto charts of the effects were used to determine which terms (effect) contribute the most to the response's variability. The *p*-value effect was compared with the significance level (α = 0.05) to determine whether the association between the response and each term in the model was statistically significant. Where $p \leq \alpha$ indicates that the

association is statistically significant. These procedures were performed with the Minitab® Statistical Software v.19.

The objective of the first two experimental designs was to maximize TPC and AC_{ABTS} independently. A multi-response optimization problem was formulated for the final design, where TPC, AC_{ABTS}, and extract purity (P) were simultaneously maximized. Purity was defined as the ratio between the total polyphenol content and the total soluble compounds content (SCC) in the extract, expressed as a percentage (Eq. 3.8). SCC is affected by temperature, ethanol concentration, and the number of cycles; therefore, including purity in the optimization allowed determining the conditions for the most selective extraction.

$$P\ (\%) = \frac{grams\ gallic\ acid\ equivalent}{grams\ dry\ extracts\ (soluble\ compounds)} 100 \tag{3.8}$$

where the grams of dry extracts were obtained by drying the liquid extracts (1 g) in an oven at 105 °C until constant weight (24–72 h).

Multi-response optimization was performed using the desirability function (DF) technique (Derringer & Suich, 1980). The method consists of converting each of the estimated response variables $\hat{y}_i\ (x)$ into desirable values $d_i(x)$ that can vary between 0 (the response value is "undesirable") and 1 ("completely desirable or ideal" response). The individual desirabilities of each estimated response are then combined using the geometric mean to obtain an overall or composite desirability (Eq. 3.9).

$$D(x_1, x_2, x_3) = \left(d_{TPC}{}^{w_{TPC}} \cdot d_{AC_{ABTS}}{}^{w_{AC_{ABTS}}} \cdot d_P{}^{w_P}\right)^{1/\left(w_{TPC}+w_{AC_{ABTS}}+w_P\right)} \tag{3.9}$$

w_i values vary between 0.1 and 10, which are arbitrarily assigned to define the priority of each response variable.

The transformation function for maximization is given by

$$d_i(x_1, x_2, x_3) = \begin{cases} 0 & \hat{y}_i(x_1, x_2, x_3) \leq L_i \\ \left[\dfrac{\hat{y}_i(x_1, x_2, x_3) - L_i}{T_i - L_i}\right]^s & L_i < \hat{y}_i(x_1, x_2, x_3) < T_i \\ 1 & \hat{y}_i(x_1, x_2, x_3) \geq T_i \end{cases}$$

L_i , an unacceptable value, is the lower specification bound, and T_i is a target value (a large enough response). Minitab software sets the lower bound and the target value to the minimum value and maximum value of the data, respectively. The exponent *s* defines how the desirability is distributed on the interval between the lower bound and the target value. The distribution can be convex ($s < 1$) whereby any response that falls within the $L_i - T_i$ interval is highly desirable, concave ($s > 1$) where only responses that fall close to the T_i value take high desirability values, or linear ($s = 1$) where the desirability increases linearly towards the T_i value. This last distribution was used in this study as it represents a neutral configuration that gives equal importance to the T_i and L_i values.

Three composite desirability functions were defined. OPT1 includes the maximization of TPC and AC_{ABTS} (equal priority was assigned to both responses), whereas OPT2 and OPT3 add P as a third response, but with different priorities. OPT2 assigned equal priority to the three objectives, while OPT3 assigned higher priority to P.

3.2.6. Determination of responses in the extracts

a) Total polyphenol content (TPC)

The total polyphenol content was spectrophotometrically determined by the Folin-Ciocalteu method (Singleton et al., 1999). Aliquots of samples (250 µL) were mixed with Folin–Ciocalteu reagent (125 µL), 10% w/v aqueous sodium carbonate solution Na_2CO_3 (250 µL) and 1875 µL of distilled water, shaken and allowed to react for 1 h at room temperature (20 °C) in darkness; then the absorbance was measured, reading at 765 nm. TPC was calculated from a calibration curve using gallic acid (0.1 g/L maximum concentration), so the results were expressed as mg of gallic acid equivalents (GAE) per g of maqui leaves, dry weight.

b) Radical scavenging activity: ABTS at a fixed time (AC_{ABTS})

We applied the procedure described in Re et al. (1999). Radical cation $ABTS^{\bullet+}$ was produced by reacting a 7 mM ABTS solution with potassium persulfate (final concentration 2.45 mM). The $ABTS^{\bullet+}$ solution was diluted with phosphate buffer saline (PBS) (pH 7.4) to an absorbance of 0.70 at 734 nm and equilibrated at 30 °C. Then 1 mL of this diluted solution was added to 10 µL of extract or Trolox, and the absorbance was read after 6 min. The percentage inhibition of absorbance was referred to the concentration of extracts and Trolox. AC_{ABTS} was calculated from a calibration curve using Trolox (2.4 mM maximum concentration) and expressed as mg of Trolox equivalents (TE) per g of maqui leaves, dry weight.

3.2.7. Statistical analysis

All the extractions and chemical analyses were performed in triplicate. The experimental results obtained were expressed as means ± SD. Statistical analysis was carried out using Minitab® Statistical Software v.19. Analysis of variance (ANOVA) at 95% confidence level ($p < 0.05$) was applied to compare all the obtained responses.

3.2.8. Additional analytical determinations in the optimal extracts (ASE 150 extracts only)

a) DPPH radical-scavenging activity

The samples' ability to capture free radicals was measured with the DPPH method (Moure et al., 2005). 50 µL of extract dilutions or absolute methanol (as control) was added to 2 mL of methanolic DPPH (0.06 mM). Soon after vortexing the reaction mixture for 1 min, the tubes were placed in the dark for 16 min, and absorbance was measured at 515 nm. The EC_{50} parameter, which reflects the quantity of antioxidant needed to reduce the initial DPPH concentration by 50%, has been expressed as g maqui leaves in dry weight/g DPPH. This way of expressing DPPH values better reflects the antioxidant

capacity because it includes the concentration of the DPPH methanol solution and the sample concentration, which usually vary in each study.

b) ORAC

The ORAC assay was performed as described in Brescia (2012) with some modifications; 250 mL of phosphate buffer PBS pH 7.4 (75 nM) was prepared with 25 mL K_2HPO_4 solution (581.17 mM) and 25 mL KH_2PO_4 solution (168.72 mM) and distilled water. Briefly, 0.2034 g of AAPH was dissolved in 10 mL of PBS to the final concentration of 75 mM and made fresh daily. A fluorescein stock solution (1 mM) was made in PBS and stored in the dark at 4 °C. The stock solution was diluted sequentially, 1:500 and then 1:250, with PBS. The sample (25 µL), either of Trolox solution or PBS (blank), were added and mixed with 150 µL of sodium fluorescein to a 96-well plate, and they were incubated for 30 min at 37 °C. The reaction was initiated by the addition of 25 µL of AAPH solution. The fluorescence was measured every minute using the Synergy HTX Operators (BioTek Instruments, Inc.). Excitation was performed at 485/20 nm, and emission was measured at 528/20 nm. The reference calibration curve was performed with Trolox solutions between 4 and 48 µM. The results were expressed as mg of Trolox equivalents (TE) per g of maqui leaves, dry weight.

c) Identification and quantification of polyphenols

Eighteen polyphenols (8 phenolic acids, 3 flavanols, 3 flavonols, and 4 other polyphenols) were identified and quantified by applying the procedure described in Huaman-Castilla et al. (2019); 5 µL of extract diluted with distilled water (1:10) and filtrated (0.22 mm membrane) was injected (in triplicate) into an ultra-performance liquid chromatography-mass spectrometry (UPLC–MS, Dionex Ultimate 3000 with Detector MS Orbitrap Exactive plus, Thermofisher, Massachusetts, USA) equipped with a reverse-phase Acquity UPLC BEH C18 column (1.7 µm × 2.1 × 100 mm). Gradient elution was conducted at 35 °C with acetonitrile/0.1% formic acid (mobile phase A) and water/0.1% formic acid (mobile phase B) at a constant flow rate of 0.2 mL/min. The gradient elution

steps were as follows: the first 6 min, 80% A – 20% B; the next 18 min, 15% A – 85% B; and finally, the last 30 min, 80% A – 20% B. Polyphenol contents were calculated from calibration curves using standards for each compound, and the results were expressed as mg of specific polyphenols per g of maqui leaves (dry weight). Table 3-1S shows the linearity range, the regression equation, the determination coefficient, the limit of detection (LOD), and the limit of quantification (LOQ) for each standard calibration curve.

3.3. Results and discussion

3.3.1. Modelling extraction results of exploratory experimental designs

The TPC (y_1) and ACABTS (y_2) responses of each exploratory experimental design (Table 3-2S) were fitted to first or second-order models according to the presence or absence of curvature (see Table 3-3S). The TPC values of both experimental designs (E.D.1 and E.D.2) fit well to linear models, where the three main effects (β_1, β_2, and β_3) were statistically significant (Table 3-4S). The AC_{ABTS} values of the E.D.1 fit well to a linear model, where the temperature effect (β_1) was the only statistically significant. On the other hand, a second-order model fit well to the AC_{ABTS} values in the E.D.2, where two linear effects (β_2 and β_3), one quadratic effect (β_6) and one interaction effect (β_8) were statistically significant (Table 3-5S). The determination coefficients, R^2, of these four fitted surfaces ranged between 0.817 and 0.971. The significant effects were identified by analyzing their Pareto charts and their p-values at a significance level of $\alpha = 0.05$.

Figure 3-2 shows the relationships between TPC and AC_{ABTS} responses with the three factors studied in E.D.1 and E.D.2. Temperature is the most important factor (steepest slope) and is directly proportional (positive slope) to the two responses for both exploratory regions, except for AC_{ABTS} in E.D.2, where this effect presents a slight curvature. Ethanol concentration is the most important factor affecting AC_{ABTS} in the E.D.2 region. Both responses are inversely proportional to ethanol in E.D.1 and directly proportional in E.D.2. The number of cycles shows a potent effect in TPC and AC_{ABTS} in

the E.D.2 region. TPC is directly proportional to the number of cycles in both regions, while AC_{ABTS} is directly proportional in E.D.1 and presents a strong curvature in E.D.2.

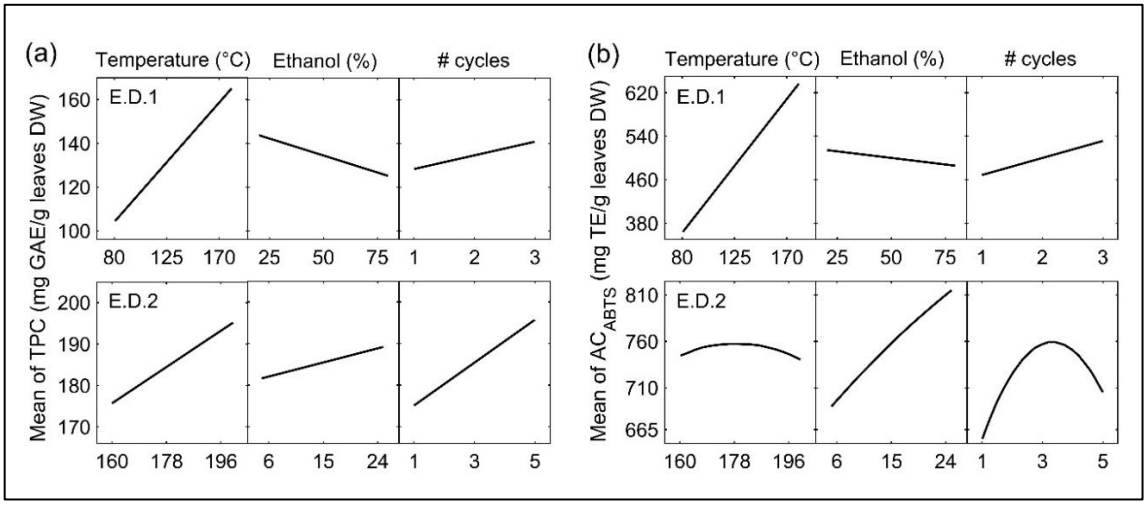

Figure 3-2: Main effects plot for (a) TPC and (b) AC_{ABTS} in regions E.D.1 (top row) and E.D.2 (bottom row). DW means dry weight.

According to the previous analysis, several changes in the trend of the two original regions' responses were detected. Hence, we decided to define a new experimental region using both exploratory regions. When all 30 observations of the original regions were considered, fitted models with unusual points (outliers) for the TPC and AC_{ABTS} responses were obtained. Therefore, a sequential elimination of outliers was applied until reliable TPC and AC_{ABTS} models were obtained. The goodness of fit statistics calculated with the 30 original points and with the outliers removed are shown in Table 3-6S. The elimination of 8 outliers for each response yielded second-order models that considerably improved all the statistical indexes considered. These second-order models were used in the optimization. The outliers removed did not present optimal responses, and most of them were in the descent zone of the response surfaces, away from the optimal extraction conditions. In addition, some repetitions of the central points of the experimental designs were identified as outliers (Table 3-2S).

3.3.2. Modelling extraction results of the final experimental design

The second-order models fit well to TPC and AC_{ABTS} values of the final experimental design (Table 3-7S), where the three factors (temperature, ethanol concentration, and the number of cycles) showed significant influence on the variation of both responses. Temperature (β_1) was the most statistically significant effect on TPC, followed by the quadratic effect of the ethanol concentration (β_5) and the number of cycles (β_3). AC_{ABTS} was significantly influenced by 7 effects (in decreasing order of statistical significance): temperature (β_1), the quadratic effect of the number of cycles (β_6), the quadratic effect of ethanol concentration (β_5), the interaction effect of temperature-number of cycles (β_9), the number of cycles (β_3), the interaction effect of temperature-ethanol concentration (β_7) and quadratic effect of temperature (β_4) (Table 3-8S).

Purity, the additional response considered in the final design, was only affected by two factors: temperature and ethanol concentration. A second-order model fit well with these data (S = 1.16, R^2 = 0.943, R^2_{adj} = 0.904, R^2_{pred} = 0.845), where four effects were relevant (in order of statistical significance): temperature (β_1), the quadratic effect of ethanol concentration (β_5), ethanol concentration (β_2) and the quadratic effect of temperature (β_4) (Table 3-8S).

Response surface plots for the models are displayed in Figure 3-3. Individual maximum values of TPC (208.94 mg GAE/g dry leaves) and AC_{ABTS} (818.02 mg TE/g dry leaves) were achieved at the maximum temperature of the experimental region (200 °C), 20% - 26% ethanol and 3 – 5 cycles. Instead, maximum P (40.07%) was obtained at the lowest temperature (80 °C), 80% ethanol, and the minimum number of cycles (one extraction).

Like in previous HPLE optimization studies, with Croatian olive leaves and Goji berry, we found that temperature is the most significant factor affecting TPC and AC values. High temperatures enhance both mass transfer and polyphenols solubility, as well as reduce solvent viscosity (Putnik et al., 2017; Tripodo et al., 2018). The impact of ethanol concentration is difficult to generalize. Some authors found optimum performance

(higher yield of mg GAE/g dry natural matrix) at high ethanol concentrations (71% v/v for myrtle leaves (Díaz-de-Cerio et al., 2018), 86% v/v for Goji berry (Tripodo et al., 2018)), while others at low concentrations (35% v/v for *Moringa oleifera* leaves (Rodríguez-Pérez et al., 2016)). This dissimilar behavior could be attributed to the particular matrix phenolic composition since each polyphenol family responds differently to ethanol as a co-solvent (Huaman-Castilla et al., 2019). It has been reported that the number of cycles tends to increase the extraction efficiency. Despite recovering 87% of the polyphenols in the first cycle of HPLE of black cohosh, the second and third cycles extracted 9% and 4.2%, respectively (S. Mukhopadhyay et al., 2006). In the optimizations of HPLE of olive leaves, the highest polyphenol content was reached by extracting with 2 and 3 cycles (Putnik et al., 2017; Xynos et al., 2014).

Purity showed a different behavior compared to the other two responses (Figure 3-3). The extracts with the highest TPC values reached low P values; this suggests that under these conditions, the soluble compounds content (SCC) increased mainly due to the extraction of nonphenolic compounds (those not reducing the Folin Ciocalteu reagent). Hydroxymethylfurfural (an unwanted compound), generated by the Maillard reaction, was reported as non-interfering in the determination of TPC by the F-C assay (Bastola et al., 2017) and was identified in extracts obtained at temperatures above 130 °C (from *Carménère* grape pomace) (Mariotti-Celis, Martínez-Cifuentes, Huamán-Castilla, Pedreschi, et al., 2018). Similarly, ethanol-soluble compounds such as alkaloids and glucose (identified in maqui leaves (Zúñiga et al., 2017)) were reported as noninterfering in the determination of TPC (Bastola et al., 2017). On the other hand, the extracted non-phenolic compounds (purity determinants) have a particular behavior for the studied factors, which generates differences in their respective TPC and P ratios.

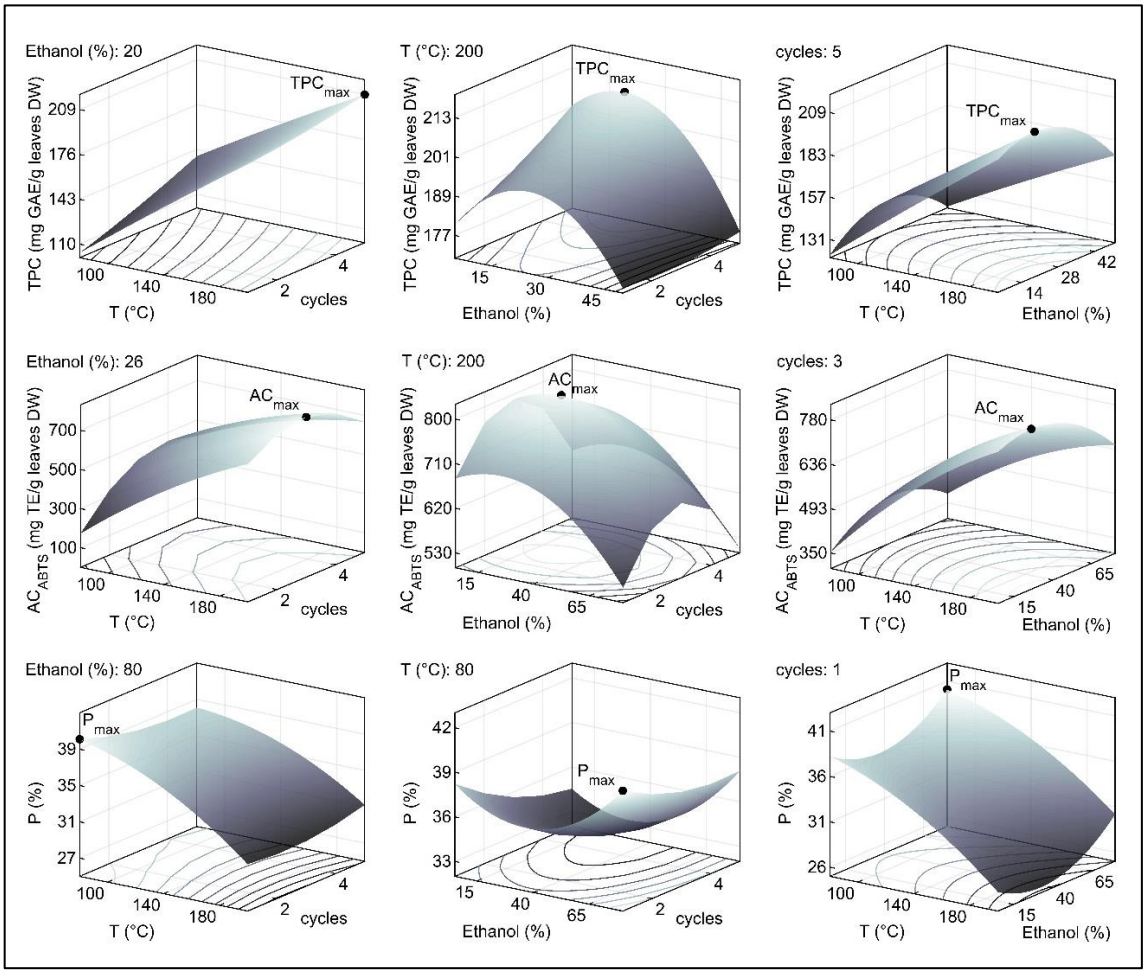

Figure 3-3: Response surface plots of the total polyphenol content (first row), antioxidant capacity measured by ABTS method (second row) and extract purity (third row) as a function of the studied factors. Where DW is dry weight.

3.3.3. Obtaining and evaluating optimal extracts

a) Multi-response optimization and model validation

Three composite desirability functions were applied to identify the combination of factor values that maximizes the set of responses, assigning different priorities to each response (w in Eq. 3.9) according to three scenarios (Table 3-1). Scenario 1 (OPT1) maximized TPC and AC_{ABTS} with equal priority, but neglected purity P. In this case, the

composite desirability (0.952) was close to 1, the ideal case, indicating an outstanding overall performance; since purity was not considered in the optimization, it presented a low value (24.91%). The second (OPT2) and third (OPT3) scenarios included the maximization of P but with different priorities. According to Harrington's rating system, these two scenarios showed low composite desirabilities, indicating that they achieved an acceptable but poor overall optimization. This result was expected as TPC and AC_{ABTS} objectives conflict with the purity objective. OPT3 (higher priority to P) seems to be a better option than OPT2 (all responses with the same priority) since it showed higher composite desirability than OPT2.

Table 3-1: Optimization of the 3 established objective functions.

Extract	Optimization function			Adjusted factors			Estimated responses		
	$w_{TPC=AC}$	w_P	D	T (°C)	EtOH (%)	# Cycles	TPC[a]	AC_{ABTS}[a]	P[a]
OPT1	1	0	0.952	200	23	3	200.71	815.35	24.91
OPT2	1	1	0.524	143	22	3	167.68	664.24	30.83
OPT3	1	10	0.550	122	5	3	126.08	541.52	35.94

[a] TPC: mg GAE/g maqui leaves, AC_{ABTS}: mg TE/g maqui leaves and P: %.

Extractions at the 3 optimum conditions were performed (in triplicate) on an ASE 200 device to verify them experimentally and assess the predictive ability of the fitting models. The optimal conditions of the first and second scenarios were slightly changed, as shown in Table 3-2, since the ASE 200 device operates in conjunction with a Solvent Controller product that mixes solvents in a range of 5 to 100%, in 5% increments for the ethanol concentration. The relative standard deviations (RSD) between the estimated and experimental responses were low (< 8%), which means that the experimental values were in good agreement with those estimated theoretically. Additionally, extractions were performed under the original optimal conditions (Table 3-1) in the ASE 150 equipment, which has no constraints on ethanol concentration. The second-order models obtained with ASE 200 were accurate to predict the experimental responses obtained in ASE 150 extractions since the experimental values of TPC and P were in good agreement with those estimated (RSD < 6%) (Table 3-2).

Table 3-2: Predicted and experimental values of the responses measured in the optimal extracts of maqui leaves, processed by ASE 200 and ASE 150 equipment.

	Extract	Optimal conditions	Response	Predicted	Experimental	*SD*	*RSD*
ASE 200	OPT1	200 °C 25% ethanol[a] 3 cycles	TPC (mg GAE/g[b]) AC_{ABTS} (mg TE/g[b]) P (%)	199.87 817.03 24.94	188.73 ± 3.02 825.43 ± 51.19 28.03 ± 0.85	7.90 5.64 2.18	4.07 0.69 8.04
	OPT2	145 °C 20% ethanol[a] 3 cycles	TPC (mg GAE/g[b]) AC_{ABTS} (mg TE/g[b]) P (%)	164.03 668.21 30.69	162.86 ± 1.50 710.65 ± 21.94 31.41 ± 0.63	0.80 30.06 0.51	0.49 4.39 1.64
	OPT3	122 °C 5% ethanol 3 cycles	TPC (mg GAE/g[b]) AC_{ABTS} (mg TE/g[b]) P (%)	126.08 541.52 35.87	129.57 ± 0.49 593.37 ± 8.53 36.34 ± 0.47	2.49 36.40 0.33	1.95 6.42 0.92
ASE 150	OPT1	200 °C 23% ethanol 3 cycles	TPC (mg GAE/g[b]) P (%)	200.71 24.51	205.14 ± 1.64 26.92 ± 0.21	3.10 1.42	1.53 5.48
	OPT2	143 °C 22% ethanol 3 cycles	TPC (mg GAE/g[b]) P (%)	167.68 30.83	170.74 ± 1.33 33.06 ± 0.27	2.14 1.58	1.26 4.94
	OPT3	122 °C 5% ethanol 3 cycles	TPC (mg GAE/g[b]) P (%)	126.08 35.94	115.79 ± 1.01 36.29 ± 0.62	7.27 0.25	6.01 0.79

[a] Extraction conditions adjusted to the operating range of the ASE 200 Solvent Controller. [b] Grams of maqui leaves in dry weight.

b) Characterization of optimal extracts in terms of TPC and AC

As expected, successive extraction (SE) with acidified methanol/water (50% v/v, pH 2) and acetone/water (70% v/v) yielded an extract (RCE1) with the highest TPC values (264.53 ± 8.21 mg GAE/g maqui leaves). Therefore, SE can be considered a reference extraction method that achieved complete recovery of extractable polyphenols (Pérez-Jiménez et al., 2008).

OPT1 conditions achieved the highest TPC, whereas OPT3 conditions reached the highest purity; OPT2 conditions were in a middle ground (ASE 150, Table 3-2). OPT1 achieved a high recovery of TPC compared to RCE1 (78%). OPT3 extract presented a lower polyphenol extraction yield than RCE1 (44%) but a significantly higher purity

(35%) than the OPT1 extract. The intermediate extract (OPT2) achieved 65% of the TPC of RCE1 and 22% more purity than the OPT1 extract. The three optimal extracts yielded higher TPC values (ASE 150, Table 3-2) than the hydroalcoholic extracts (50% of ethanol) of maqui leaves obtained by maceration in a previous study (Rubilar et al., 2011), which reached only 26% of the TPC of RCE1. At the same time, the best extract (OPT1 extract) presented a TPC ~6 times higher than the average TPC of those reported for maqui fruit (35.14 ± 1.30 mg GAE/g maqui fruit) (Rivera-Tovar et al., 2019).

The three optimum HPLE extractions reached yield values (g dry extract/100 g dry leaves, %) of 72%, 52%, and 34%, respectively. Similar yields were reported in previous work with HPLE of other natural matrices: 75% (Goji berry, 50% ethanol – 180 °C) (Tripodo et al., 2018), 56% (*Moringa leaves*, 35% ethanol – 128 °C) (Rodríguez-Pérez et al., 2016) and 54% (olive leaves, 50% ethanol – 190 °C) (Xynos et al., 2014).

The AC of maqui leaves in the optimum extracts were determined by three methods that measure a sample's free radical scavenging capacity (ABTS, DPPH, and ORAC) to provide comprehensive information that considers the different mechanisms of actions of the different antioxidants contained in the natural matrix (Table 3-9S). The one-way analysis of variance (ANOVA) with Tukey's multiple comparison method showed that the three optimum conditions (OPT1, OPT2, and OPT3) yielded significantly different AC values (ABTS and ORAC) of the MLEs. Whereas using the DPPH method, only OPT2 extracts showed significantly different AC values, lower than OPT1 and OPT3 extracts.

OPT1 extracts presented the highest AC (ABTS, ORAC), between 14% and 39% more than OPT2 and OPT3 (Table 3-9S). AC_{DPPH} behavior differed from that of AC_{ABTS} and AC_{ORAC}; this could be due to the characteristics of the compounds extracted under optimum conditions. It was previously reported that compounds such as carotenoids and anthocyanins, present in maqui leaves (Vidal et al., 2013; Zúñiga et al., 2017), absorbs at 515 nm (λ_{max} to DPPH radical absorption), which generates an overestimated measurement (Boligon, 2014). In contrast, the ABTS method is more effective for

analyzing plant extracts because the measurement at 734 nm eliminates possible interferers, and also, the radical can interact with a broader range of antioxidants (Mareček et al., 2017).

The antioxidant capacities of the optimum extracts (based on the grams of dry extract) reached values in the ranges of 1225.92 – 1668.20 mg TE/g dry extract (ABTS method), 1204.13 – 2142.50 mg TE/g dry extract (ORAC method), and 0.17 – 0.37 g dry extract/g DPPH (EC_{50}, DPPH method); the MLE with the highest AC was OPT3 followed by OPT2 (Table 3-10S). Two previous studies on the recovery of polyphenols from maqui leaves determined the antioxidant capacity with the DPPH method using different concentrations of the DPPH methanol solution (400 and 200 µM) (O. Muñoz et al., 2011; Rubilar et al., 2011). Therefore, to compare adequately our AC results with the literature values, it was necessary to express all DPPH values in the same units (g dry extract/g DPPH). Our best extract in terms of antioxidant capacity and P (OPT3 extract, ASE 150) needed only 0.17 g dry extract/g DPPH for 50% depletion of the free radical, ~3 times lower than the results of previous studies, which showed AC_{DPPH} values of 0.55 g dry extract/g DPPH (Rubilar et al., 2011) and 0.46 g dry extract/g DPPH (O. Muñoz et al., 2011). This comparison should be made with caution, AC results depend strongly on extraction methods and conditions, solvent, particle size, and pre-treatment (Pérez-Jiménez et al., 2008), as well as on factors related to the plant (genotype, environment, stage of harvesting and storage) (Rivera-Tovar et al., 2019). Nevertheless, our extracts' high AC_{DPPH} values confirm the extraction method's efficiency and the optimization procedure's adequacy.

A correlation between TPC and AC_{ABTS} (R^2 = 0.996) as well as between TPC and AC_{ORAC} (R^2 = 0.991) was observed, although the correlation was only statistically significant for the first case (*p-value* = 0.04 and 0.062, respectively).

Hydroxymethylfurfural (HMF) could be present in OPT1 and OPT2 extracts because they were generated at temperatures above 130 °C (Huaman-Castilla et al., 2019; Mariotti-Celis, Martínez-Cifuentes, Huamán-Castilla, Pedreschi, et al., 2018). However,

these two extracts were focused on optimizing the polyphenol's yield; in a subsequent process, these extracts can be purified to eliminate or reduce HMF concentration (an undesirable extractable compound) to levels below those that generate carcinogenic effects. Applying solid-phase extraction with HP-20 macroporous resin (Huaman-Castilla et al., 2019), the HMF concentration in grape pomace crude extracts (23.61 mg HMF/g dry pomace) was reduced almost entirely (~95%). In our case, maqui leaves extracts probably contain HMF concentrations similar or lower than those of grape pomace and notably lower than those of natural matrices such as ground coffee or bakery products, which already formed HMF during high-temperature processes before extraction.

3.3.4. Low molecular weight phenolic compounds

The three optimum ASE 150 (OPT1, OPT2, OPT3) MLEs and two reference extracts (RCE2 = 70% acetone and RCE3 = 50% methanol) were characterized in terms of their low molecular weight polyphenols; 11 phenolics of the 18 analyzed were quantified (Table 3-3). OPT1, OPT2, and OPT3 extracts contained 54%, 44%, and 58% of the total quantified polyphenols in RCE2, as well as 57%, 46%, and 61% of the total of quantified polyphenols in RCE3, respectively (Figure 3-1S a). All extracts, except OPT1, contained more flavonoids than non-flavonoids (Figure 3-1S a). RCE2 contained almost two times more flavonoids than non-flavonoids, while OPT1 contained equal amounts of flavonoid and non-flavonoids. OPT3 showed the highest recovery of quantified flavonoids in HPLE, probably due to the low ethanol content (5% v/v) in the extraction solvent, which has been shown to favor their recovery (Rodríguez-Pérez et al., 2016).

All extracts presented a similar distribution of flavonols (41% – 48%), phenolic acid (35% – 45%), flavanols (4% – 22%), and others (1% – 7%) (Figure 3-1S b). However, only for phenolic acids, the effect of extraction temperature showed a clear trend. OPT3 (122 °C, 5% ethanol) was particularly efficient to recover gallic and cinnamic acids (Table 3-3). High temperatures (200 °C) have been shown to accelerate these phenolic acids' degradation (Khuwijitjaru et al., 2014). The recovery of gallic acid is significantly enhanced at moderately high temperatures (≤150 °C) compared with extractions at lower

temperatures (90 °C) (Huaman-Castilla et al., 2019). In our case, its recovery at 200 °C (OPT1) was severely affected; hence, we estimated that maximum recovery of gallic acid could be achieved in the range of 120 – 150 °C. Previous studies with other natural matrices have shown that gallic acid can be extracted better with higher ethanol concentrations (15% – 50%) (Dhanani et al., 2017; Huaman-Castilla et al., 2019). The optimum ethanol concentration to extract gallic acid may depend on interactions between this acid and specific polyphenols of the given matrix.

OPT1 contained higher amounts of chlorogenic acid (~2 times) and coumaric acid (~4 times) than OPT3. These phenolic acids are resistant to thermal degradation, and probably high temperatures are required to weaken their bonds with the matrix (Khuwijitjaru et al., 2014). Also, moderate ethanol concentration (15%) in the extraction solvent favored chlorogenic acid recovery (Huaman-Castilla et al., 2019).

Both temperature and ethanol content did not clearly show an effect on other polyphenols' recovery (Table 3-3). The catechin and quercetin glucoside recovery showed a slight improvement with OPT2 conditions (143 °C – 22% ethanol). At 200 °C, quercetin 3-glucoside and catechin may have suffered significant degradation, as observed in microwave-assisted extraction at temperatures higher than 125 °C for compounds with many hydroxyl groups (Liazid et al., 2007). It was previously reported that maximum recoveries of catechin were achieved at 150 °C (evaluated in the range 90 – 150 °C) from *Carménère* wine pomace (Huaman-Castilla et al., 2019), and at 130 °C (evaluated in the range 100 – 200 °C) from tea leaves and grape seeds (Piñeiro et al., 2004); explaining the good performance of OPT2 conditions.

Table 3-3: Low molecular weight phenolic compounds quantified in optimum HPLE and references maqui leaves' extracts (ASE 150).

Compound	mg/g leaves (dry weight)				
	OPT1 (200 °C - 23% - 3 cycles)	OPT2 (143 °C - 22% - 3 cycles)	OPT3 (122 °C - 5% - 3 cycles)	RCE3 (20 °C - 50%)	RCE2 (20 °C - 70%)
Phenolic acids					
Gallic acid[a]	0.07 ± 0.00^{D}	0.216 ± 0.028^{C}	0.36 ± 0.01^{B}	0.23 ± 0.01^{C}	0.64 ± 0.02^{A}
Chlorogenic acid	1.10 ± 0.00^{B}	0.762 ± 0.006^{C}	0.65 ± 0.02^{C}	1.47 ± 0.01^{A}	1.44 ± 0.02^{A}
Vanillic acid	n.d.	n.d.	n.d.	n.d.	n.d.
Caffeic acid	n.d.	n.d.	n.d.	n.d.	n.d.
Ferulic acid	n.d.	n.d.	n.d.	n.d.	n.d.
Protocatechuic acid	1.06 ± 0.03^{A}	0.925 ± 0.049^{A}	1.13 ± 0.07^{A}	1.57 ± 0.02^{A}	0.93 ± 0.01^{A}
Coumaric acid[a]	0.10 ± 0.03^{A}	0.027 ± 0.002^{B}	0.03 ± 0.00^{B}	0.03 ± 0.00^{B}	0.03 ± 0.00^{B}
Cinnamic acid[a]	0.06 ± 0.00^{C}	0.084 ± 0.006^{C}	0.31 ± 0.02^{B}	0.54 ± 0.02^{A}	0.53 ± 0.01^{A}
Other polyphenols (Stilbenes, tyrosols, dihydrochalcones)					
Resveratrol[b]	n.d.	0.076 ± 0.013^{B}	0.13 ± 0.02^{A}	n.d.	n.d.
Hydroxytyrosol	0.38 ± 0.00^{A}	0.120 ± 0.007^{B}	0.17 ± 0.02^{B}	0.18 ± 0.01^{B}	0.07 ± 0.01^{B}
Oleuropein	n.d.	n.d.	n.d.	n.d.	n.d.
Phloridzin	n.d.	n.d.	n.d.	n.d.	n.d.
Flavanols					
Catechin	0.21 ± 0.01^{D}	0.507 ± 0.011^{C}	0.43 ± 0.00^{C}	1.67 ± 0.02^{B}	2.25 ± 0.03^{A}
Epicatechin	n.d.	n.d.	n.d.	n.d.	n.d.
Epigallocatechin	n.d.	n.d.	n.d.	n.d.	n.d.
Flavonols					
Quercetin - 3 glucoside	0.56 ± 0.01^{C}	1.078 ± 0.017^{B}	1.04 ± 0.02^{B}	2.01 ± 0.03^{A}	2.09 ± 0.03^{A}
Quercetin	1.53 ± 0.03^{A}	0.668 ± 0.024^{B}	1.52 ± 0.05^{A}	1.31 ± 0.02^{A}	1.35 ± 0.03^{A}
Kaempferol	0.44 ± 0.01^{C}	0.098 ± 0.007^{E}	0.29 ± 0.01^{D}	0.67 ± 0.02^{B}	0.90 ± 0.01^{A}
∑ Polyphenols identified	5.50 ± 0.13	4.485 ± 0.157	5.93 ± 0.22	9.68 ± 0.15	10.23 ± 0.15

[a] Expressed as ferulic acid, [b] mg/100 g maqui leaves and n.d. means not detected. [(A - E)] Values that do not share a letter are significantly different.

Resveratrol recovery was favored at low extraction temperatures and ethanol contents; hence, OPT3 conditions yielded the highest recoveries. Hydroxytyrosol and kaempferol contents were higher in the OPT1 extracts. These polyphenols require high temperatures (180 °C and 150 °C, respectively) and low concentrations of ethanol (< 50% and 15%, respectively) to optimize their extraction from natural matrices (Cea Pavez et al., 2019; Huaman-Castilla et al., 2019). Resveratrol, hydroxytyrosol, and coumaric acid were extracted better in our optimal HPLE conditions than in the reference extracts, probably because room temperature (20 °C) was not effective.

Quercetin and protocatechuic acid contents did not present significant differences (according to one-way ANOVA, Tukey Pairwise comparisons) between optimum extracts (OPT1, OPT3) and reference extracts (RCE2, RCE3). Only OPT2 extract showed a significantly lower quercetin content, which suggests that these polyphenols were stable in the entire operating range explored in this research.

The observed variability in the recovery of some polyphenols with temperature and ethanol could be caused by some specific interactions between polyphenols and other compounds present in the natural matrix as well as between polyphenols and the extraction solvent. A deep chemical characterization that considers a computational calculation would improve the understanding of these interactions (Huaman-Castilla et al., 2019).

Some of the identified polyphenols present in our MLEs are potentially beneficial for human health, according to *in vitro* and *in vivo* assays and clinical trials (Heleno et al., 2015; Pohl & Lin, 2018).

The HPLE of maqui leaves has become a new promising alternative for the sustainable extraction of polyphenols (Figure 3-4). Compared with other natural matrices and extraction processes (mechanical shaking, sonication, ultrasound-assisted, stirring, and supercritical fluid), our optimal extracts place HPLE of maqui leaves as among the best natural source and extraction technique to obtain protocatechuic acid (1.13 mg protocatechuic acid/g maqui leaves), quercetin (1.53 mg quercetin/g maqui leaves) and catechin (0.51 mg catechin/g maqui leaves) (Figure 3-4 a, c, and e). Chlorogenic acid and

kaempferol contents of our MLEs (1.10 and 0.44 mg/g maqui leaves, respectively) were equivalent to those obtained from other natural matrices using non-eco-friendly techniques and were the best among the natural matrices processed by HPLE (Figure 3-4 b and d). Resveratrol, kaempferol, cinnamic acid, and hydroxytyrosol have been identified only in ~14, ~12, ~8, and ~5 solid natural matrices, respectively (Neveu et al., 2010). Therefore, maqui leaves that have been discarded in the maqui fruit industry become an attractive new potential source of these polyphenols.

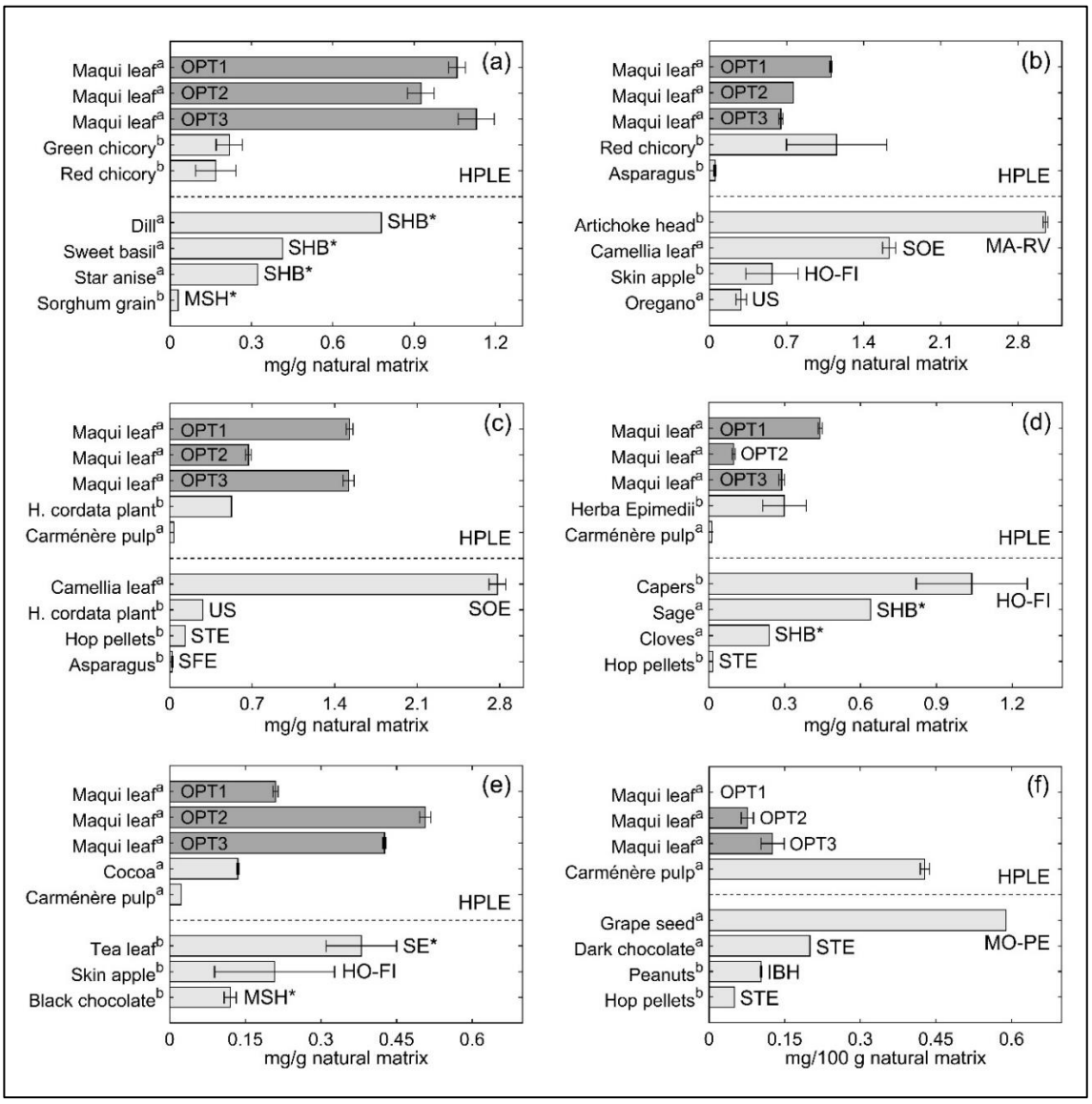

Figure 3-4: Comparison of the content of target polyphenols in maqui leaves with those of different natural matrices that were obtained by HPLE-ethanol (above) and by other extraction technologies-ethanol (down). (a) protocatechuic acid, (b) chlorogenic acid, (c) quercetin, (d) kaempferol, (e) catechin, and (f) resveratrol. [a]: dry weight, [b]: fresh weight, *: methanol instead of ethanol, SHB: shaking bath, STE: Stirring extraction, MSH: mechanical shaking, US: ultrasound-assisted, HO-FI: homogenization and vacuum filtration, SOE: extraction with sonication, MA-RV: maceration and rotary evaporator, SFE: supercritical fluid extraction, SE: static extraction, IBH: ice bath homogenizer and MO-PE: mortar and pestle. (Callemien et al., 2005; Chen et al., 2008; Haghi & Hatami, 2010; Huaman-Castilla et al., 2019; Neveu et al., 2010; Okiyama et al., 2018; Piñeiro et al., 2004; Shrikanta et al., 2015; Solana et al., 2015; Y. Zhang et al., 2008).

3.4. Conclusions

HPLE, an eco-friendly technique, was used to recover polyphenols from maqui leaves currently discarded in the maqui berry industry. RSM and DF were applied for HPLE multi-response optimization. For the first time, HPLE optimal extracts were obtained considering the simultaneous maximization of TPC, AC, and polyphenol purity. Optimal HPLE conditions (OPT3: 122 °C, 5% EtOH and 3 cycles) that prioritized polyphenol purity (P) more than TPC and AC achieved extracts 35% more pure than the other optimum MLEs. In turn, optimal extraction conditions (OPT1: 200 °C, 23% EtOH and 3 cycles) that prioritized TPC, and AC equally achieved extracts with the highest AC and a TPC that was 78% of the TPC of the reference extract (RCE1), that supposedly recovered 100% of the polyphenols contained in the original matrix. Eleven polyphenols were identified and quantified by UPLC-MS in the optimum extracts of maqui leaves. Chlorogenic and coumaric acids, hydroxytyrosol, and kaempferol were better recovered at OPT1 conditions. Gallic acid, cinnamic acid, and resveratrol were better recovered at OPT3 conditions since they experience thermal degradation. Many of the polyphenols

quantified in this study showed higher contents in our MLE than in other natural sources processed with HPLE or with other extraction technology.

3.5. Appendix A. Supplementary materials

Table 3-1S: Analytical and sensitivity data of the calibration curves of the 18 polyphenols quantified in the extracts using ultra-performance liquid chromatography-mass spectrometry (UPLC–MS).

Polyphenol	linearity range (mg/L)	Regression equation	R^2	*LOD* (mg/L)	*LOQ* (mg/L)
Chlorogenic acid	0.04-3.80	$Y = 1.14227 \cdot 10^8 X$	0.998	0.01	0.04
Vanillic acid	0.04-4.08	$Y = 1.51206 \cdot 10^6 X$	0.995	0.05	0.16
Caffeic acid	0.03-3.32	$Y = 2.85586 \cdot 10^8 X$	0.994	0.01	0.03
Ferulic acid[a]	0.02-1.96	$Y = 3.86029 \cdot 10^7 X$	1.000	0.01	0.02
Protocatechuic acid	0.06-5.80	$Y = 1.08551 \cdot 10^8 X$	0.997	0.02	0.06
Resveratrol	0.02-1.96	$Y = 6.18861 \cdot 10^7 X$	1.000	0.01	0.02
Hydroxytyrosol	0.04-3.76	$Y = 9.06219 \cdot 10^6 X$	1.000	0.01	0.04
Oleuropein	0.01-1.04	$Y = 2.74689 \cdot 10^8 X$	0.999	0.00	0.01
Phloridzin	0.01-1.03	$Y = 1.56960 \cdot 10^8 X$	1.000	0.00	0.01
Catechin	0.03-2.72	$Y = 1.71436 \cdot 10^8 X$	0.992	0.01	0.03
Epicatechin	0.04-3.48	$Y = 1.85467 \cdot 10^8 X$	0.996	0.01	0.04
Epigallocatechin	0.05-4.64	$Y = 1.46855 \cdot 10^8 X$	0.997	0.01	0.05
Quercetin-3 glucoside	0.01-0.40	$Y = 1.74576 \cdot 10^8 X$	0.999	0.00	0.01
Quercetin	0.04-3.76	$Y = 1.12383 \cdot 10^8 X$	0.998	0.01	0.04
Kaempferol	0.07-6.60	$Y = 1.94372 \cdot 10^8 X$	0.990	0.01	0.02

[a] Gallic, coumaric and cinnamic acids were expressed as ferulic acid.

Table 3-2S: Levels (coded values in brackets) of factors for the Box-Behnken design and responses (TPC, AC and P) of both exploratory experimental designs and final experimental design (both exploratory regions less the outliers).

	Run	x_1	x_2	x_3	TPC (mg GAE/g leaves)	AC_{ABTS} (mg TE/g leaves)	P (%)
E.D. 1	3	80 (-1)	20 (-1)	2 (0)	109.18 ± 2.37	316.05 ± 7.73	35.32 ± 0.76
	2	80 (-1)	80 (1)	2 (0)	**94.93 ± 6.66**	287.62 ± 15.84	39.18 ± 2.75
	15	180 (1)	20 (-1)	2 (0)	181.00 ± 3.87	702.71 ± 16.89	27.03 ± 0.58
	13	180 (1)	80 (1)	2 (0)	**154.27 ± 3.17**	593.01 ± 23.85	32.43 ± 0.67
	11	80 (-1)	50 (0)	1 (-1)	102.92 ± 4.42	**393.22 ± 23.19**	36.67 ± 1.58
	7	80 (-1)	50 (0)	3 (1)	108.26 ± 3.71	449.17 ± 42.22	34.21 ± 1.17
	4	180 (1)	50 (0)	1 (-1)	157.78 ± 6.52	629.76 ± 24.66	28.80 ± 1.19
	14	180 (1)	50 (0)	3 (1)	165.71 ± 1.89	**618.97 ± 42.40**	27.27 ± 0.31
	10	130 (0)	20 (-1)	1 (-1)	137.01 ± 6.93	415.99 ± 25.56	**34.25 ± 1.81**
	9	130 (0)	20 (-1)	3 (1)	154.28 ± 13.58	**418.78 ± 7.22**	36.34 ± 3.20
	12	130 (0)	80 (1)	1 (-1)	**77.00 ± 2.94**	308.39 ± 40.44	**33.31 ± 1.27**
	5	130 (0)	80 (1)	3 (1)	**138.20 ± 6.89**	529.41 ± 32.21	36.03 ± 1.80
	6	130 (0)	50 (0)	2 (0)	129.53 ± 3.16	**490.67 ± 15.29**	33.75 ± 0.82
	1	130 (0)	50 (0)	2 (0)	**81.78 ± 6.21**	**371.92 ± 14.07**	**31.02 ± 2.36**
	8	130 (0)	50 (0)	2 (0)	129.32 ± 6.72	568.19 ± 30.68	34.43 ± 1.79
E.D. 2	4	160 (-1)	5 (-1)	3 (0)	**180.28 ± 11.81**	674.35 ± 50.37	32.09 ± 2.10
	10	160 (-1)	25 (1)	3 (0)	178.04 ± 6.54	729.72 ± 10.22	**34.60 ± 1.27**
	13	200 (1)	5 (-1)	3 (0)	187.30 ± 9.50	826.92 ± 26.99	26.33 ± 1.34
	14	200 (1)	25 (1)	3 (0)	204.85 ± 11.33	799.74 ± 27.69	**29.53 ± 1.63**
	3	160 (-1)	15 (0)	1 (-1)	162.89 ± 8.68	**624.43 ± 23.19**	31.36 ± 0.20
	12	160 (-1)	15 (0)	5 (1)	190.42 ±1.13	**710.25 ± 14.68**	29.76 ± 0.18
	1	200 (1)	15 (0)	1 (-1)	189.06 ± 2.19	660.98 ± 43.53	27.61 ± 0.32
	11	200 (1)	15 (0)	5 (1)	208.44 ± 2.03	659.62 ± 30.62	**30.98 ± 0.30**
	5	180 (0)	5 (-1)	1 (-1)	164.26 ± 5.26	619.04 ± 35.86	28.81 ± 0.92
	2	180 (0)	5 (-1)	5 (1)	**172.30 ± 4.75**	604.79 ± 18.46	27.31 ± 0.75
	8	180 (0)	25 (1)	1 (-1)	181.52 ± 7.95	670.74 ± 30.13	27.59 ± 1.21
	15	180 (0)	25 (1)	5 (1)	189.66 ± 2.13	**796.41 ± 10.13**	28.87 ± 0.32
	6	180 (0)	15 (0)	3 (0)	**208.10 ± 12.59**	759.40 ± 13.59	**31.68 ± 1.92**
	7	180 (0)	15 (0)	3 (0)	187.64 ± 6.04	784.89 ± 19.30	27.26 ± 1.45
	9	180 (0)	15 (0)	3 (0)	178.99 ± 0.37	728.83 ± 25.95	27.35 ± 0.06

$x_{1\text{-}3}$: temperature (°C), ethanol concentration (% v/v), and number of cycles. Outliers removed in bold.

Table 3-3S: Analysis of Variance test for models fitted to both exploratory experimental designs.

	Source	Degree of freedom	Sum of squares	Mean square	*F-value*	*p-value*
E.D. 1			TPC (R^2 = 0.971)			
	Model	3	8169.0	2723.0	101.64	0.000
	Residual	9	241.1	26.8		
	Lack of fit	8	241.1	30.1	1420.37	0.021
	Pure error	1	0.0	0.0		
	Total	12	8410.1			
			AC_{ABTS} (R^2 = 0.838)			
	Model	3	156126	52042	13.79	0.002
	Residual	8	30191	3774		
	Lack of fit	7	27187	3884	1.29	0.592
	Pure error	1	3005	3005		
	Total	11	186317			
E.D. 2			TPC (R^2 = 0.818)			
	Model	3	1685.1	561.7	13.43	0.001
	Residual	9	376.4	41.8		
	Lack of fit	8	339.0	42.4	1.13	0.625
	Pure error	1	37.3	37.3		
	Total	12	2061.4			
			AC_{ABTS} (R^2 = 0.970)			
	Model	8	55985	6998	16.33	0.008
	Residual	4	1715	429		
	Lack of fit	2	139	70	0.09	0.919
	Pure error	2	1576	788		
	Total	12	57699			

Table 3-4S: Regression coefficients and significance for the models fitted to the TPC response (y_1) of to both exploratory experimental designs. (Level of significance of 0.05).

Regression coefficients	E.D. 1 Coded value	*p-value*	E.D. 2 Coded value	*p-value*
β_o	134.36	0.000	185.38	0.000
Linear				
β_1 (T)	30.43	0.000	9.75	0.002
β_2 (E)	-9.42	0.001	3.79	0.049
β_3 (C)	6.26	0.012	10.34	0.002

T, *E* and *C*: temperature, ethanol concentration and number of cycles, respectively.

Table 3-5S: Regression coefficients and significance for the models fitted to the AC_{ABTS} response (y_2) of to both exploratory experimental designs. (Level of significance of 0.05).

Regression coefficients	E.D. 1 Coded value	*p-value*	E.D. 2 Coded value	*p-value*
β_o	499.6	0.000	757.7	0.000
Linear				
β_1 (T)	137.3	0.000	-2.42	0.800
β_2 (E)	-14.5	0.599	62.93	0.002
β_3 (C)	31.3	0.273	24.99	0.027
			Quadratic	
β_4 (T^2)			-15.3	0.270
β_5 (E^2)			-5.4	0.677
β_6 (C^2)			-78.6	0.003
			Interaction	
β_8 (E*C)			36.0	0.025
β_9 (T*C)			-21.8	0.103

In brackets the factors to which the coefficients correspond. Where *T*, *E* and *C* are temperature, ethanol concentration and number of cycles, respectively.

Table 3-6S: Selection of experimental region for final analysis according to five goodness of fit criteria.

# Obs.	model	*S* (mg/g)	R^2	R^2_{adj}	R^2_{pred}	*AIC*
Response 1: Total Polyphenol Content (TPC)						
30	linear	15.74	0.846	0.828	0.800	106.87
25	linear	7.41	0.956	0.950	0.924	103.78
22[a]	**2nd order**	**5.38**	**0.983**	**0.971**	**0.941**	**80.67**
Response 2: Antioxidant Capacity (AC_{ABTS})						
30	linear	79.61	0.775	0.749	0.696	267.31
25	linear	51.83	0.899	0.884	0.850	201.04
22[a]	**2nd order**	**25.83**	**0.985**	**0.974**	**0.963**	**149.73**

[a] One of the three repetitions at the central points of each experimental design was an outlier. The other six outliers of each response are show in Figure 3-1.

Table 3-7S: Analysis of Variance test for the three second order models fitted to final experimental design.

Source	Degree of freedom	Sum of squares	Mean square	*F-value*	*p-value*
TPC (R^2 = 0.983)					
Model	9	20526.1	2280.9	79.22	0.000
Residual	12	345.5	28.8		
Lack of fit	10	308.5	30.9	1.67	0.432
Pure error	2	37.0	18.5		
Total	21	20871.6			
AC_{ABTS} (R^2 = 0.985)					
Model	9	531274	59030	88.49	0.000
Residual	12	8005	667		
Lack of fit	10	6430	643	0.82	0.666
Pure error	2	1575	788		
Total	21	539280			
P (R^2 = 0.943)					
Model	9	289.5	32.2	23.96	0.000
Residual	13	17.5	1.3		
Lack of fit	10	10.9	1.1	0.50	0.820
Pure error	3	6.5	2.2		
Total	22	307.0			

Table 3-8S: Regression coefficients and significance for the second order models fitted to the three responses of the final experimental design. (Level of significance of 0.05).

Regression coefficients	TPC		AC_{ABTS}		P	
	Coded value	*p-value*	Coded value	*p-value*	Coded value	*p-value*
β_o	161.96	0.000	664.6	0.000	31.15	0.000
Linear						
β_1 (*T*)	38.09	0.000	176.6	0.000	-4.50	0.000
β_2 (*E*)	-2.42	0.468	-28.0	0.089	1.66	0.020
β_3 (*C*)	10.75	0.003	54.9	0.024	-0.67	0.397
Quadratic						
β_4 (T^2)	-0.97	0.787	-38.6	0.039	-1.46	0.042
β_5 (E^2)	-18.84	0.000	-82.1	0.001	2.82	0.002
β_6 (C^2)	1.47	0.605	-131.7	0.000	0.73	0.266
Interaction						
β_7 (*T*E*)	-6.78	0.074	-41.4	0.028	0.31	0.679
β_8 (*E*C*)	-4.77	0.262	5.4	0.822	0.43	0.708
β_9 (*T*C*)	-4.96	0.274	-71.4	0.024	0.75	0.501

In brackets the factors to which the coefficients correspond. Where *T*, *E* and *C* are temperature, ethanol concentration and number of cycles, respectively.

Table 3-9S: Antioxidant capacities of maqui leaves determined in the optimum extracts by three *in vitro* methods.

Extract	ASE 200		ASE 150	
	ABTS (mg TE/g[a])	DPPH EC_{50} (g[a]/g DPPH)	ORAC (mg TE/g[a])	DPPH EC_{50} (g[a]/g DPPH)
OPT1	825.43 ± 51.19	0.53 ± 0.01	917.59 ± 69.81	0.49 ± 0.01
OPT2	710.65 ± 21.94	0.46 ± 0.02	807.79 ± 44.57	0.36 ± 0.06
OPT3	593.37 ± 8.53	0.54 ± 0.01	683.70 ± 98.26	0.53 ± 0.04

[a] Grams of maqui leaves in dry weight.

Table 3-10S: Antioxidant capacities of three optimum extracts from maqui leaves determined by three different *in vitro* methods.

Extract	ASE 200		ASE 150	
	ABTS (mg TE/g[a])	DPPH EC_{50} (g[a]/g DPPH)	ORAC (mg TE/g[a])	DPPH EC_{50} (g[a]/g DPPH)
OPT1	1225.92 ± 95.65	0.36 ± 0.01	1204.13 ± 117.03	0.37 ± 0.02
OPT2	1370.22 ± 57.29	0.24 ± 0.02	1564.11 ± 105.19	0.18 ± 0.01
OPT3	1668.02 ± 38.11	0.19 ± 0.01	2142.50 ± 325.58	0.17 ± 0.00

[a] Grams of dry extract.

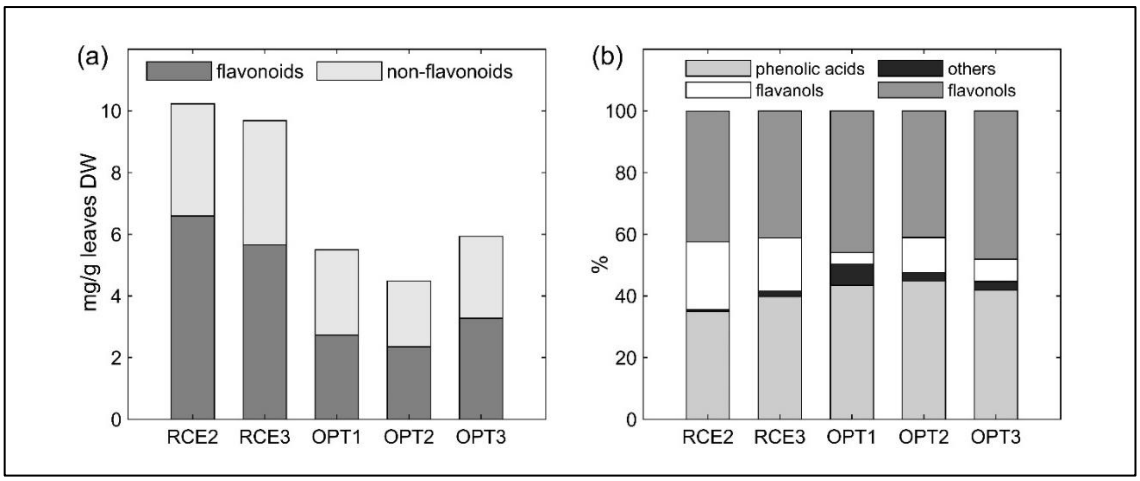

Figure 3-1S: Polyphenol contents quantified in the three optimal and the two reference extracts. (a) Total content of quantified polyphenols (grouped by family) and (b) Contribution of each subfamily to the total quantified (in percentage).

CHAPTER 4. ADSORPTION OF LOW MOLECULAR WEIGHT FOOD RELEVANT POLYPHENOLS ON CROSS-LINKED AGAROSE GEL

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4.1. Introduction

Polyphenols (or phenolic compounds) have received particular attention as functional food ingredients and nutraceuticals due to their health-related bioactivities, including protective effects against arteriosclerosis, coronary heart disease, cancer, and many neurodegenerative diseases (Chiva-Blanch et al., 2013; Kanno et al., 2015; Sławińska-Brych et al., 2016; Suganuma et al., 2016; Yang et al., 2014). These relevant human health benefits have been ascribed to ferulic acid (FA), protocatechuic acid (PCA), gallic acid (GA), kaempferol (KAE), catechin (CAT), and resveratrol (RSV) (Figure 4-1), according to many *in vitro* and *in vivo* assays (Garvin et al., 2006; Harini & Pugalendi, 2010; Heleno et al., 2015; Jang et al., 1997; Mandel & Youdim, 2004; Zhu et al., 2017). Interestingly, these bioactive compounds can be recovered from agroindustrial wastes such as *Aristotelia chilensis* leaves (Rivera-Tovar et al., 2021), *Carménère* wine pomace (Huaman-Castilla et al., 2019), spent coffee grounds (Mariotti-Celis et al., 2018) and brewery waste streams (Barbosa-Pereira et al., 2014), among other sources. The content of many of these polyphenols is higher in these matrices than in other traditional polyphenol sources such as grape seeds, apple peel, dark chocolate, and tea leaves (Neveu et al., 2010; Rivera-Tovar et al., 2021). Regarding this, the recovery of food ingredients from agroindustrial wastes has attracted many researchers' attention because it reduces the environmental impact of this industry and increases the availability of food-relevant micronutrients such as polyphenols (Mirabella et al., 2014).

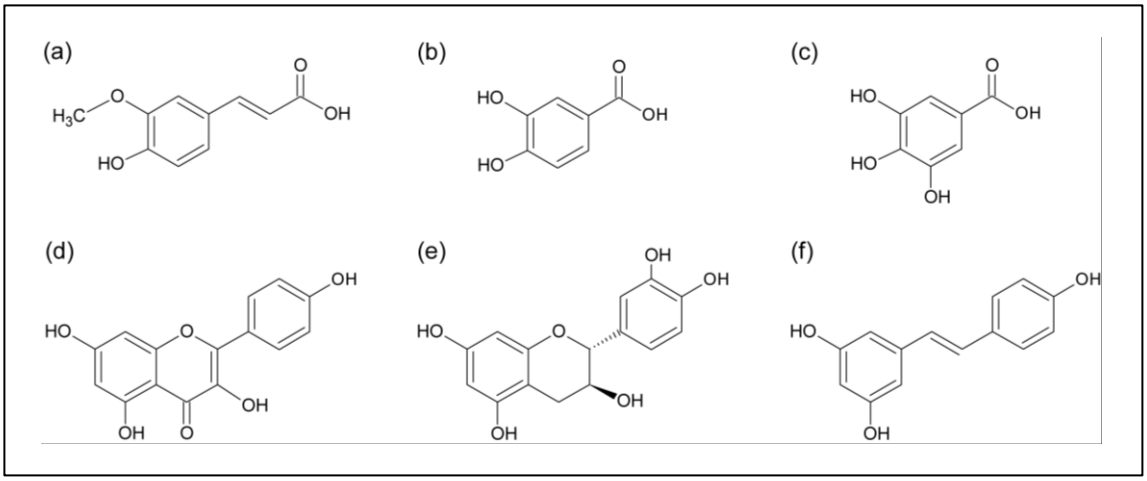

Figure 4-1. Molecular structures of (a) ferulic acid, (b) protocatechuic acid, (c) gallic acid, (d) kaempferol, (e) catechin, and (f) resveratrol.

Polyphenols consumption through the diet has been associated with several health benefits, attributed mainly to reducing free radicals' activity. Each polyphenol presents specific properties such as antioxidant capacity, bioavailability, and solubility, as well as specific bioactivities. The desired bioactivity of a given polyphenolic natural extract can be enhanced by separating those polyphenol(s) that reduce the total antioxidant capacity of the extract (antagonistic property of some polyphenols in the mixture) (Reber et al., 2011). Therefore, polyphenols purification is required to obtain selective natural extracts with the desired bioactive strength.

Techniques developed to isolate polyphenols generally include variations of preparative liquid chromatography, although these methods have been described as tedious, time and solvent-consuming, as well as difficult to scale up (M. Gu et al., 2006a; Valls et al., 2009). Adsorption preparative liquid chromatography (APLC) with cross-linked 12% agarose gel (Superose^{TM} 12 prep grade) has been highly recommended for polyphenols' isolation because it can be achieved in one step, and it is easily scalable to industrial size. Agarose is also stable to harsh chemical cleaning procedures (M. Gu et al., 2006b, 2008; T. Gu, 2015; Liu et al., 2011; Xu et al., 2007). High purities (87.2% - 99.4%) and recoveries (76.8% - 90.7%) can be obtained in step isocratic elution using mobile

phases containing different proportions of water, ethanol (EtOH), and acetic acid (HAc) (M. Gu et al., 2008; Liu et al., 2011).

Despite the promising results obtained for polyphenol's isolation using Superose^{TM} 12 prep grade adsorption chromatography, to our knowledge, there is no research regarding the adsorption equilibrium of polyphenols on this matrix considering mixtures distilled water, ethanol, and acetic acid as mobile phase. This research generates preliminary information for APLC experimental optimization and scaling-up, resulting in a more efficient experimental exploration. Specifically, the estimated adsorption isotherm parameters for each polyphenol in liquid phases of different H_2O:EtOH:HAc compositions, which represent the mobile phases for polyphenols elution in APLC, are essential input data to develop predictive mathematical models of isocratic and especially gradient APLCs; these models are useful for process optimization and scaling-up (T. Gu, 2015; Guiochon et al., 2006b). These models typically require system parameters (e.g., porosity), mass transfer parameters (e.g., axial dispersion), and adsorption equilibrium parameters (isotherm constants), which define the elution time and consequently the polyphenols' separation (Guiochon et al., 2006a; Tarafder, 2013). Thus, detailed adsorption equilibrium studies are necessary to develop reliable predictive APLC models.

Langmuir and Freundlich models are widely used to represent the solid-liquid adsorption equilibrium, i.e., the relationship between the concentration of adsorbate (polyphenols for this work) in the liquid phase (H_2O:EtOH:HAc) and on the adsorbent (agarose), after reaching equilibrium at a constant temperature (Freundlich, 1907; Langmuir, 1918). Gauss-Newton, an iterative numerical method, allows fitting experimental data to nonlinear functions, such as theoretical Langmuir and Freundlich isotherms, minimizing the weighted sum of squared errors (Motulsky & Christopoulos, 2004). The models and parameters fitted to these isotherms by these methods are usually adequate, accurate, and statistically significant. Additionally, from isothermal equilibrium parameters, it is possible to infer the characteristics of the adsorbent surface and information regarding each adsorbate's interaction with both phases (liquid phase and adsorbent). The thermodynamic analysis, through the determination of enthalpy change,

Gibbs energy change, and entropy change, allows completing the process description since these values reveal valuable information about the process thermal nature, adsorbate-adsorbent bonding mechanism, degree of process spontaneity, and uniformity of adsorbate organization on the adsorbent surface (Gao et al., 2013; Tran et al., 2016).

This study aimed to explore and characterize the adsorption behavior of six highly bioactive polyphenols representatives of different subclasses (FA a hydroxycinnamic acid, PCA a hydroxybenzoic acid, GA a hydroxybenzoic acid, KAE a flavonol, CAT a flavanol, and RSV a stilbene) on highly cross-linked agarose. Adsorption isotherms were fitted to experimental data and then used to evaluate the effect of temperature and composition of the liquid phase (H_2O:EtOH:HAc) on each of the six studied polyphenols' adsorption capacity. The adsorption process was further assessed through thermodynamic analysis, where accurate and significant thermodynamic equilibrium parameters were obtained. The estimated isothermal equilibrium parameters are helpful to develop APLC models for process design, optimization, and scaling-up.

4.2. Materials and methods

4.2.1. Solvents and polyphenols standards

Ethanol (gradient grade for liquid chromatography LiChrosolv®, Merck S.A.) and glacial acetic acid (anhydrous for analysis EMPARTA® ACS, Merck S.A.) were used to prepare liquid phases where the polyphenols were dissolved. Superose^{TM} 12 prep grade (GE Healthcare Lifesciences), a highly cross-linked agarose with an average particle size of 30 ± 10 µm, was used as an adsorbent. The evaluated adsorbates, ferulic acid, protocatechuic acid, gallic acid, kaempferol, catechin, and resveratrol, were purchased from Xi'an Haoxuan Bio-Tech Co., Ltd. (Baqiao, China); additional information on the chemical samples is shown in Table 4-1.

Table 4-1: Chemical sample description.

Sub-classes	Chemical name	CAS no.	Formula	Molar mass (g/mol)	Purity[a]
Hydroxycinnamic acid	Ferulic acid	1135-24-6	$C_{10}H_{10}O_4$	194.19	≥0.980
Hydroxybenzoic acid	Protocatechuic acid	99-50-3	$C_7H_6O_4$	154.12	≥0.990
Hydroxybenzoic acid	Gallic acid	149-91-7	$C_7H_6O_5$	170.12	≥0.990
Flavonol	Kaempferol	520-18-3	$C_{15}H_{10}O_6$	286.24	≥0.980
Flavanol	Catechin	154-23-4	$C_{15}H_{14}O_6$	290.26	≥0.980
Stilbene	Resveratrol	501-36-0	$C_{14}H_{12}O_3$	228.25	≥0.980
	Ethanol	64-17-5	C_2H_6O	46.07	≥0.999
	Acetic acid	64-19-7	$C_2H_4O_2$	60.05	≥0.997

[a] Informed by the corresponding chemical suppliers.

4.2.2. Batch adsorption system and experimental procedure

Adsorption isotherms were obtained using the Carousel 12 Plus™ Reaction Station (Radleys, Saffron Walden, UK) described in (Cuevas-Valenzuela et al., 2015). Adsorption experiments were performed for each polyphenol using liquids phases of different compositions (H_2O:EtOH:HAc) (Table 4-2). Polyphenolic solutions were prepared in the concentration ranges constrained by each compound's water solubilities (Table 4-2). The liquid phases used for the experiments differ between the polyphenols due to the significant differences in their solubilities.

In the adsorption experiments, 0.005 g dry weight of cross-linked 12% agarose was weighed (previously washed with distilled water to displace the storage ethanol, moisture: 86.1%) in an analytical balance with a resolution of 0.0001 g and then added to the glass tubes inside the Carrousel. Once the Carrousel system reached a temperature of 20 °C, 0.005 L of the polyphenolic solutions (at 20 °C) were poured into the corresponding flask. All glass tubes were carefully capped to avoid evaporation. The solution-adsorbent mixture was kept under agitation (500 rpm) at 20 °C for 60 min. The adsorption time was defined based on GA adsorption evaluation over time for 6 hours, where the change in GA concentration in liquid phase occurred in the first minutes (~20 min) and then remained practically invariant (Figure 4-1S). A similar procedure was carried out to assess

the effect of temperature in polyphenols adsorption from a liquid phase (W70, Table 4-2). In this case, the experiments were performed at 10 °C and 30 °C, keeping the rest of the operating conditions invariable. Isotherm curves were set up using 5 or 6 different initial concentrations of each polyphenol.

Once the adsorption equilibrium was reached, the liquid and solid phases were quickly separated using a polytetrafluoroethylene (PTFE) syringe filter with a pore size of 0.45 μm and a diameter of 13 mm (Bonna-Agela Technologies, Delaware, USA). The filtrates were diluted, so polyphenol concentrations could be measured using an ultraviolet-visible spectrophotometer (Reyleigh UV-1601, Beijing Beifen-Ruili Analytical Instrument Co. Ltd., Beijing, China). Each polyphenol's absorbance was measured within the range of 200-600 nm to establish the maximum wavelengths for spectrophotometric measurements (Table 4-2).

Table 4-2: Some specifications for adsorption experiments: water solubility, concentration range of polyphenolic solutions, liquid phase composition, and absorbance reading.

Compound	Water solubility[a] (mg/L water) at 20 to 25 °C	Polyphenol concentration range (mmol/L liquid phase)	Liquid phase composition (H_2O:EtOH:HAc v/v)	Maximum wavelength (nm)
FA	524.8 (Haq et al., 2017) 527.0 (Shakeel et al., 2017)	0.515-2.575 (100-500)[b]	90:5:5 (W90) 70:15:15 (W70) 50:25:25 (W50) 30:35:35 (W30)	325
PCA	18521 (Noubigh et al., 2007) 29400 (Srinivas et al., 2010)	6.488-38.931 (1000-6000)[b]	100:0:0 (W100) 70:15:15 (W70) 50:25:25 (W50) 30:35:35 (W30)	260
GA	9583 (Lu & Lu, 2007) 14940 (Dabir et al., 2018)	7.054-35.269 (1200-6000)[b]	100:0:0 (W100) 94:3:3 (W94) 80:10:10 (W80) 70:15:15 (W70) 30:35:35 (W30)	260 (water) 270
KAE	1.25 (Telang et al., 2016) 1.34 (K. Zhang et al., 2015)	0.028-0.168 (8-48)[b]	70:15:15 (W70) 50:25:25 (W50) 30:35:35 (W30)	265
CAT	4544 (Cuevas-Valenzuela et al., 2015) 7620 (Takanori et al., 2014)	2.412-14.470 (700-4200)[b]	100:0:0 (W100) 94:3:3 (W94) 80:10:10 (W80) 70:15:15 (W70) 50:25:25 (W50) 30:35:35 (W30)	275
RSV	24.15 (Ha et al., 2019) 50.00 (Robinson et al., 2015)	0.035-0.210 (8-48)[b]	100:0:0 (W100) 94:3:3 (W94) 80:10:10 (W80) 70:15:15 (W70) 30:35:35 (W30)	305

[a] values used as a reference in the definition of maximum concentrations of each polyphenol. [b] equivalent values in mg/L.

Calibration curves (R^2 > 0.997) for each polyphenol in each liquid phase were prepared to correlate absorbance and concentration. Increases in concentration during absorbance determination due to evaporation were prevented by using cell caps. After the concentration of the polyphenolic solutions was calculated, C_e (mmol/L), the equilibrium adsorption capacity, q_e (mmol/g), was calculated using a mass balance,

$$q_e = (C_o - C_e) \cdot V / m_A \quad (4.1)$$

Where V is the volume of the polyphenolic solution (L), m_A is the dry weight of agarose (g), and C_0 is the concentration of the solution before the adsorption process (mmol/L).

4.2.3. Fitting of adsorption isotherm models

The adsorption isotherms describe the relationship between the amount of polyphenol adsorbed on agarose (q_e, mmol/g) and the polyphenol diluted in the liquid phase (C_e, mmol/mL) at equilibrium. The most commonly used isotherm models, Langmuir and Freundlich, were applied in our study to find which one represents better the adsorption process of the studied system (polyphenol-agarose-liquid phase). The Langmuir model assumes monolayer adsorption and a fixed number of adsorption sites. It also assumes that all adsorption sites are equal, and there is no interaction between adsorbed molecules (Davis et al., 2003). This model can be described as:

$$q_e = q_{\max} K_L C_e / (1 + K_L C_e) \quad (4.2)$$

where q_{max} (mmol/g) is the maximum adsorption capacity and K_L (L/mmol) is the adsorption equilibrium constant.

The Freundlich model is often used to represent non-ideal adsorption. It is characterized by multilayer formation, heterogeneous surface, and irregular heat adsorption distributions (Foo & Hameed, 2010). The equation that describes the Freundlich isotherm is:

$$q_e = K_F C_e^{1/n} \quad (4.3)$$

where K_F (mmol/g)(L/mmol)$^{1/n}$ and n are model parameters associated with adsorption capacity of the adsorbent and adsorption intensity or degree of surface heterogeneity, respectively (Davis et al., 2003).

Model parameters were estimated through nonlinear weighted regression where the weighted sum of the squares of the distances of the data points to the modeled curve (*WSSE*, Eq. 4.4) was minimized by Minitab® Statistical Software v.19 using a Gauss-Newton iterative algorithm (maximum number of iterations: 200 and convergence tolerance: 0.00001). Initial values of the parameters that allowed convergence to the minimum values were: [0.1 0.1] and [1 1] for Langmuir and Freundlich parameters, respectively.

$$WSSE = \sum_{i=1}^{N} w_i \left(q_{calc} - q_{exp}\right)_i^2 \tag{4.4}$$

where q_{calc} is the absorbed polyphenol value calculated by the model, q_{exp} is the experimental measurement of the adsorbed polyphenol, N represents the number of data points, and w_i is the weight assigned to each observed point. The reciprocal of the coefficient of variation (*CV*) of each observation's replications is generally considered an appropriate weight because observations with small experimental errors weigh more, and observations with large experimental errors weigh less; this compensates for the heteroscedasticity of the residuals.

Discrimination between Langmuir and Freundlich fitted models for each case was carried out according to several criteria. Three of them referred to the residuals: the standard regression error (*S*, Eq. 4.5), the coefficient of determination (R^2, Eq. 4.6) and residual plots; and three other criteria referred to the estimated parameters: correlation matrix (*C*, Eq. 4.7), confidence intervals (*CI*, Eq. 4.8) and confidence coefficient (*CC*, Eq. 4.9). Although these criteria were defined for linear functions, which underestimate nonlinear equation's true uncertainty, they can be useful and accepted as a valid approximation if they are interpreted correctly.

S is measured in the units of the response variable and represents how far the data values fall from the fitted values,

$$S = \sqrt{WSSE/(N-p)} \tag{4.5}$$

p is the number of model parameters.

R^2 represents the ratio between the explained variance and the total variance,

$$R^2 = 1 - \frac{WSSE}{SST} = 1 - \frac{\sum_{i=1}^{N} w_i\left(q_{calc} - q_{exp}\right)^2}{\sum_{i=1}^{N}\left(q_{exp} - \bar{q}_{exp}\right)^2} \quad (4.6)$$

$\bar{q}_{exp}$ is the mean of the experimental values of the adsorbed polyphenol.

The correlation matrix of the parameter estimates (C) was used to identify those parameters that were strongly correlated ($|C_{pq}| > 0.99$). Matrix C was calculated by Minitab® Statistical Software v.19 based on the approximate variance-covariance matrix of the parameter estimates:

$$C = S^2(R'R)^{-1} = R^{-1}(R^{-1})' \quad (4.7)$$

The approximate correlation between the estimates of θ_p and θ_q is:

$$\left(C_{pq}\right)/\left(\sqrt{C_{pp}C_{qq}}\right)$$

R is the (upper triangular) matrix from the QR decomposition of the Jacobian evaluated at θ^i (parameter estimate after iteration i) for the final iteration. θ_p and θ_q represent the two estimated parameters of each model.

CI defines the range of values that are likely to contain the true value of the model parameter (95% confidence). The function nlparci of MATLAB v. R2019a was used to determine the confidence intervals based on the variability observed in the sample, the sample size, and the confidence level.

$$CI = \theta \pm \sigma \cdot t_{\alpha/2} \quad (4.8)$$

σ is the standard deviation of the estimated parameter (variance-covariance matrix function), and $t_{\alpha/2}$ is the upper point of the Student's t distribution with N-p degrees of freedom.

CC was calculated according to (Sánchez et al., 2014). A parameter was considered statistically significant (different from zero) when the following criterion is fulfilled:

$$CC = \Delta(CI)/\theta < 2 \quad (4.9)$$

ΔCI is the width of the confidence intervals.

4.2.4. Thermodynamic analysis

Further understanding of the adsorption process can be attained through thermodynamic analysis. The enthalpy change (ΔH, kJ/mol), Gibbs free surface energy change (ΔG, kJ/mol), and the entropy change (ΔS, kJ/mol K) can provide information related to the energy changes that occurred on agarose after adsorption and the mechanisms involved in this process (Gao et al., 2013). The spontaneity of the system was determined by evaluating ΔG at equilibrium conditions, as was done for the solid-liquid adsorption of polyphenols from *Eucommia ulmoides* oliv. leaves on macroporous resin (Wang et al., 2020), sulforaphane on macroporous resin (Yuanfeng et al., 2016), polyphenols on eucalyptus bark powders (Parada & Fernández, 2017) and cadmium on orange peel (Tran et al., 2016):

$$\Delta G = -RT \ln K_{eq} \quad (4.10)$$

where T is the absolute temperature (K), R is the ideal gas constant (8.314 J/mol K), and K_{eq} is the thermodynamic equilibrium constant (dimensionless), which was calculated from the distribution coefficient, K_d (L/g), by plotting $\ln(q_e/C_e)$ versus C_e and extrapolating to zero (Tran et al., 2016). To achieve a dimensionless constant, K_d was multiplied by $\rho(T)$ of the liquid phase (H_2O:EtOH:HAc), as proposed by Milonjić (Milonjić, 2007). The ΔH and ΔS values were determined by plotting $\ln K_{eq}$ against $1/T$ (the van't Hoff equation, Eq. 4.11) and by multiplying the slope and the intercept by R.

$$\ln K_{eq} = -\frac{\Delta H}{R}\frac{1}{T} + \frac{\Delta S}{R} \quad (4.11)$$

This well-known equation is obtained by substituting Eq. 4.10 into the Gibbs Helmholz (Eq. 4.12) equation and the fundamental relation between ΔG, ΔH, and ΔS (Eq. 4.13).

$$\left(\frac{\partial \frac{\Delta G}{T}}{\partial T}\right)_P = -\frac{\Delta H}{T^2} \tag{4.12}$$

$$\Delta G = \Delta H - T\Delta S \tag{4.13}$$

The enthalpy change of adsorption at a constant amount of adsorbed adsorbate is defined as the isosteric adsorption enthalpy change (ΔH_x, kJ/mol). This important thermodynamic parameter is an indicator of the performance of adsorption and surface energy heterogeneity (Ghosal & Gupta, 2015). The isosteric adsorption enthalpy change with a constant surface coverage is obtained from the integrated Clausius-Clapeyron equation and assuming that ΔH_x is independent of temperature, as follows:

$$\ln C_e = \frac{\Delta H_x}{R}\frac{1}{T} + K \tag{4.14}$$

where K is integration constant. ΔH_x can be determined from the slope of the isosteres, plot of ln C_e versus 1/T. The different equilibrium concentrations (C_e) of the isosteres were obtained at a constant adsorbed amount (q) at three temperatures.

Each parameter is presented with the corresponding combined standard uncertainty (U) calculated as described in (Farrance & Frenkel, 2012).

4.2.5. Statistical analysis

All the adsorptions and chemical analyses were performed in triplicate (in some cases, additional repetitions were performed). Experimental values obtained were presented as means ± SD, and estimated parameters were presented with uncertainties. Statistical analysis was carried out using Minitab® Statistical Software v.19 and MATLAB v. R2019a.

4.3. Results and discussion

4.3.1. Experimental adsorption isotherms

Adsorption isotherms of six polyphenols (FA, PCA, GA, KAE, CAT, RSV) on agarose considering liquid phases with different compositions (H_2O:EtOH:HAc) at 20 °C were studied; the experimental curves are presented in Figure 4-2. According to the isotherm classification system defined by Giles & Smith (Giles et al., 1960) observing the form of the initial slope, all our curves followed the L-type shape, indicating that as more sites on agarose are filled in, it becomes much more difficult for a polyphenol molecule to find a vacant site. Also, polyphenols are either horizontally adsorbed or show no strong competition from the liquid phase.

The obtained isotherms show different shapes past the origin (a subgroup of the Giles classification). The FA curves exhibit subgroup L1 shapes due to the absence of an inflection point. This absence means that these curves are incomplete, probably because FA initial concentrations in the liquid phases were not high enough. Nevertheless, these high concentrations are not relevant for the current study since they have not been found in natural sources. Whereas GA, KAE, CAT, and RSV curves show subgroup L2 shapes, having an inflection point, an apparent plateau (e.g., GA - W30), a slight change in slope post the inflection point (e.g., CAT - W94), or a continually rising curve (e.g., KAE - W50). Finally, PCA curves present L4 shapes that are characterized by a second rise and a second plateau. This behavior could have appeared due to a re-orientation of the adsorbed PCA molecules (horizontal orientation at the first plateau and vertical orientation at the second) or because a second layer has been formed (Giles et al., 1960). PCA curves did not agree with any of the two theoretical isotherm models evaluated in this study. Consequently, this polyphenol was not considered in the subsequent analyzes. PCA isotherms could be analyzed by increasing the number of observations in the first rise and considering only this region; the second rise contains exceptionally high concentrations not found in natural matrices.

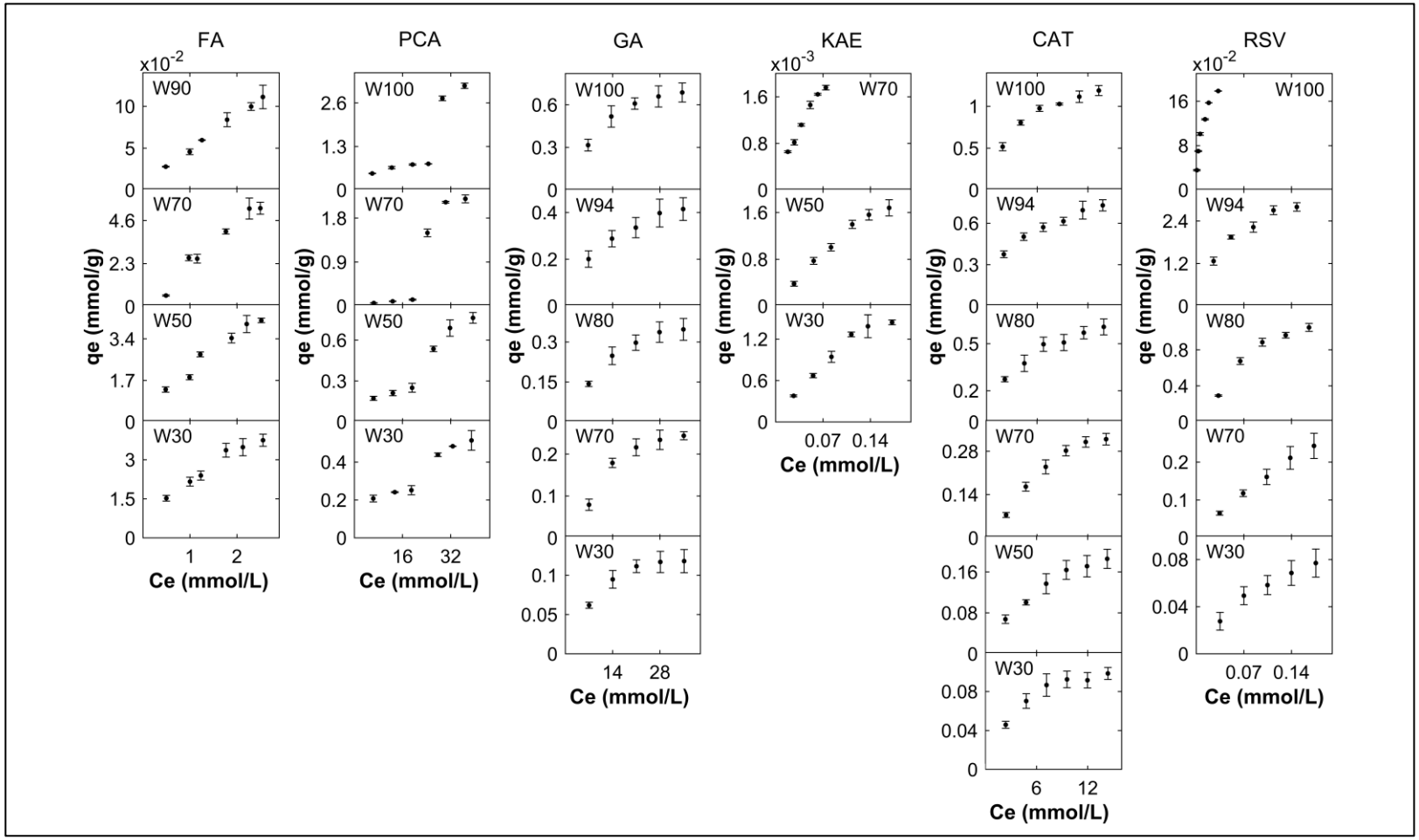

Figure 4-2. Equilibrium experimental data of the six polyphenols evaluated in 3 - 6 liquid phases with different compositions (W100, W94, W90, W80, W70, W50, and W30) at 20 °C. Each column corresponds to a given polyphenol: FA (ferulic acid), PCA (protocatechuic acid), GA (gallic acid), KAE (kaempferol), CAT (catechin), and RSV (resveratrol).

The effect of the liquid phase composition on the adsorption capacity was the same for all the polyphenols analyzed (Figure 4-3). At high water concentrations (W100 for GA, CAT, and RSV; W90 for FA; and W70 for KAE), the highest adsorption capacities were observed, which decreased (in different proportions for each polyphenol) with decreasing concentrations of water. RSV's mean maximum adsorption reached 93.6%, which was the highest of the polyphenols evaluated here, followed by CAT (13.3%), FA (4.5%), GA (3.1%), and finally KAE (2.8%). These results show the high affinity of RSV with agarose, which in chromatography translates into a problematic elution that must necessarily be accelerated with the decrease in water concentration in the mobile phase (Bai et al., 2014). On the other hand, GA or KAE, showing the lowest affinities to agarose, would elute more efficiently, even with a liquid phase with high water concentrations. This behavior was observed by Tan et al. (2010) who found that GA eluted at ~170 min with a mobile phase of 5% EtOH and 5% HAc (W90) while RSV elution required a gradient in the mobile phase with 30% EtOH and 30% HAc (W40) as the final mobile at ~321 minutes.

The average adsorptions of GA, CAT, and RSV with the W70 liquid phase were 0.8%, 2.6%, and 1.4%, respectively. The change from W100 to W70 generated a more significant impact on RSV adsorption (reduced ~59 times) followed by CAT adsorption (reduced ~5 times) and GA adsorption (reduced ~3 times). Similarly, the change from W70 to W30 generated more similar reductions on the adsorptions of five polyphenols (~2-3 times less). Whit W30 liquid phase, most polyphenols (safe FA with 2.8%) only reached an average adsorption of ~0.9%. Due to these low adsorptions, W30 (or solutions with even less water) is an excellent mobile phase to elute these polyphenols in APLC. The adsorption capacities of FA were similar with W30, W50, and W70; these liquid phases reduced ~2 times the average maximum adsorption (4.5%).

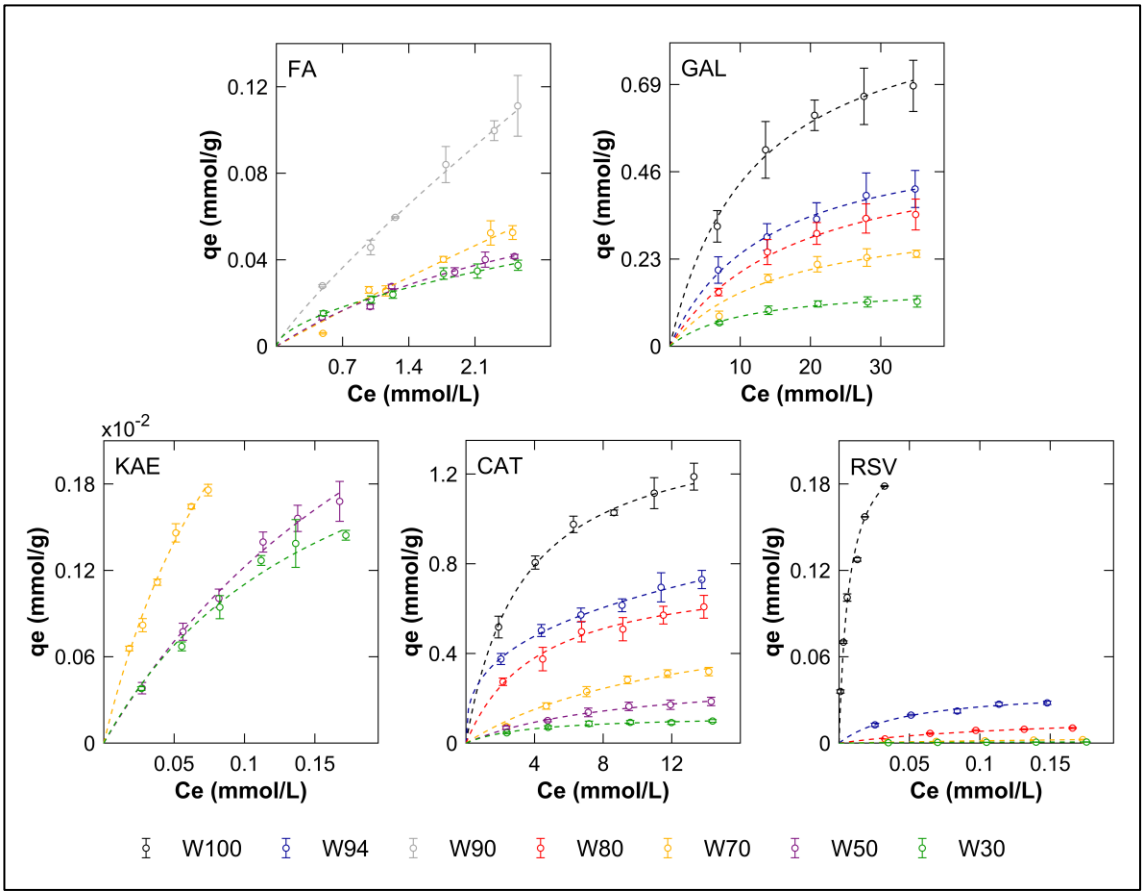

Figure 4-3. Effect of liquid phase composition (W100, W94, W90, W80, W70, W50, W30, and W90; see Table 4-2) on polyphenol adsorption for the five studied polyphenols: FA (ferulic acid), GA (gallic acid), KAE (kaempferol), CAT (catechin) and RSV (resveratrol). This effect for first plateau of PCA is shown in Figure 4-2S.

The adsorption behavior against changes in liquid phase composition (decrease in water) can be attributed to polyphenols solvophobicity. A greater polyphenol solvophobicity in a specific liquid phase means a stronger tendency of liquid phase molecules to push polyphenol molecules towards agarose. Silva et al. (2007) observed high polyphenol solvophobicities in liquid phases with high dielectric constants (water, ε_{H2O} = 78.5) and low solvophobicities in liquid phases with low dielectric constants (ethanol and acetic acid, ε_{EtOH} = 24.3 and ε_{HAc} = 6.15). High acetic acid concentrations in the liquid phase may also be responsible for adsorption reduction since it reduces the

available sites for polyphenols adsorption. Interaction of acetate ions and delocalized electrons from the agarose surface could also influence the process (M. Gu et al., 2006a).

4.3.2. Models fitting

The estimated isothermal equilibrium parameters and the goodness of fit statistics obtained by nonlinear weighted regression of Langmuir and Freundlich models for each isotherm (33 total) are presented in Table 4-3.

R^2 values ranged between 0.9828-1.0000 for Langmuir and between 0.9747-0.9999 for Freundlich. *S* values for both models were low; they represented less than 10.0% of the mean of the endogenous variable (q_e). Together with residual plots, these results indicate that both isotherm models fitted correctly to all experimental curves and were adequate models because their residuals were independent and normally distributed. In most cases, except for FA-W90, FA-W30, and CAT-W94, Langmuir fitted models achieved a slightly higher R^2 and a slightly lower *S* than Freundlich fitted models. However, these statistics were not discriminant enough to choose Langmuir as the best model, given the minor differences in their values ($\Delta R^2_{max} = 0.0081$ and $\Delta S_{max} = 3.2\%$).

Statistical criteria referring to estimated parameters (*CI*, *CC*, and $|C|$) provided additional information to choose the best-fit model. The fitted parameters of both models for the studied polyphenols were statistically significant ($CC < 2$), except those of FA-W70-20°C of the Langmuir model, whose *CC*s were high (8.87 for q_{max} and 9.63 for K_L) respectively. For FA and KAE isotherms, Freundlich parameters were more accurate than Langmuir's since *CI*s of Freundlich parameters were significantly smaller. For CAT and RSV, model parameters showed the same trend, but *CI*s of Freundlich model parameters were only slightly smaller in these cases. For GA isotherms, parameter *CI*s of both models were almost the same size. The parameter correlation matrix ($|C|$) is helpful to identify non-determinable model parameters, i.e., those that are correlated. Most FA and KAE Langmuir isotherms (except two, see Table 4-3) presented highly correlated parameters ($|C| \geq 0.9934$); therefore, this supports that the Freundlich model represents better the

adsorption of these two polyphenols on agarose. Similarly, Freundlich represents the RSV isotherms better since Langmuir contains highly correlated parameters in two isotherms, and the $|C|$ Langmuir values were larger than Freundlich in five isotherms. Langmuir is the best-fit model for GA and CAT since all isotherm's parameter correlations were lower than Freundlich, which showed highly correlated parameters in three GA isotherms (W100, W94, and W70 at 20°C).

It is worth mentioning that the overall difference between the two models was not evident with our data; hence, both can be used to represent the isothermal adsorption of the studied polyphenols on agarose adequately. Previous studies have also shown good agreement between specific equilibrium data and the two models (Huang et al., 2007; Qiu et al., 2007; Ribeiro et al., 2002).

The n parameter values in the Freundlich model can indicate whether the adsorption is irreversible ($10 < n$), very favorable ($2 < n < 10$), moderately favorable ($1 < n < 2$) and unfavorable ($n < 1$) (Hamdaoui, 2006; Tran et al., 2016). In most cases, the five polyphenols had moderately favorable adsorptions. A few cases showed very favorable adsorption (n higher than 2 with $n_{max} = 2.979$). Moderate absorptions are adequate for chromatography, where analytes should be retained momentarily to achieve differentiated elutions. In addition, n inverse value in range 0-1 is a measure of absorbent surface heterogeneity, being more heterogeneous as n inverse value gets closer to zero (Foo & Hameed, 2010; Haghseresht & Lu, 1998). Therefore, the agarose surface exhibited a slight degree of heterogeneity in all cases due to n inverse values were far from zero ($1/n > 0.5$ for most cases and $1/n > 0.35$ only for 9 of 33 cases). The adsorption capacities (K_F) and the maximum adsorption capacities (q_{max}) of all the well-fitted Langmuir models decreased with ethanol and acetic acid, verifying that adsorption is higher when the liquid phase has greater water proportion, as previously discussed. These isothermal equilibrium parameters provide information on the type of polyphenol-agarose-liquid phase interactions. They can also be used to develop first-principles models to optimize and scale-up APLC systems.

Table 4-3: Estimated parameters and the goodness-of-fit of Langmuir and Freundlich models.

Compound, liquid phase/ T (°C)		Langmuir model					Freundlich model				
		q_{max} (CC)	K_L (CC)	S ($S\%$)	R^2	$\|C\|$	K_F (CC)	n (CC)	S ($S\%$)	R^2	$\|C\|$
FA	W90/20	4.98E-01 (1.22)	1.09E-01 (1.42)	1.23E-03 (2.0)	0.9992	0.9988[b]	4.93E-02 (0.06)	1.17E+00 (0.14)	9.86E-04 (1.6)	0.9996	0.7291
	W70/20	5.59E-01 (8.87)[a]	4.32E-02 (9.63)[a]	1.33E-03 (4.6)	0.9967	0.9998[b]	2.27E-02 (0.43)	1.03E+00 (0.61)	1.52E-03 (5.3)	0.9965	0.8990
	W50/20	1.02E-01 (1.10)	2.77E-01 (1.71)	8.79E-04 (3.5)	0.9972	0.9941[b]	2.20E-02 (0.28)	1.43E+00 (0.51)	1.02E-03 (4.1)	0.9970	0.8976
	W30/20	6.63E-02 (0.61)	5.17E-01 (1.15)	5.49E-04 (2.3)	0.9986	0.9837	2.23E-02 (0.17)	1.73E+00 (0.42)	5.75E-04 (2.4)	0.9987	0.8081
	W70/10	2.01E-01 (0.40)	2.64E-01 (0.62)	9.03E-04 (1.9)	0.9992	0.9934[b]	4.09E-02 (0.10)	1.36E+00 (0.17)	1.10E-03 (2.3)	0.9991	0.8711
	W70/30	7.24E-02 (1.58)	2.10E-01 (2.04)	1.35E-03 (9.1)	0.9828	0.9947[b]	1.22E-02 (0.36)	1.26E+00 (0.59)	1.83E-03 (10.1)	0.9747	0.8378
GA	W100/20	9.44E-01 (0.26)	8.35E-02 (0.73)	5.13E-03 (1.1)	0.9997	0.9642	1.67E-01 (1.10)	2.42E+00 (0.84)	1.26E-02 (2.7)	0.9986	0.9909[b]
	W94/20	5.77E-01 (0.25)	7.30E-02 (0.65)	2.45E-03 (0.9)	0.9998	0.9652	9.02E-02 (0.48)	2.30E+00 (0.35)	2.87E-03 (1.1)	0.9998	0.9903[b]
	W80/20	5.41E-01 (0.36)	5.62E-02 (0.79)	3.08E-03 (1.3)	0.9996	0.9689	5.83E-02 (1.11)	1.93E+00 (0.67)	6.78E-03 (3.0)	0.9985	0.9881
	W70/20	3.58E-01 (0.63)	6.47E-02 (1.64)	4.82E-03 (3.0)	0.9981	0.9707	5.15E-02 (1.76)	2.24E+00 (1.20)	7.79E-03 (4.9)	0.9962	0.9926[b]
	W30/20	1.60E-01 (0.31)	9.62-E02 (0.88)	1.32E-03 (1.6)	0.9993	0.9479	3.13E-02 (1.09)	2.53E+00 (0.90)	2.77E-03 (3.3)	0.9978	0.9860
	W70/10	6.56E-01 (0.30)	3.98E-02 (0.55)	2.95E-03 (1.3)	0.9997	0.9791	4.71E-02 (0.90)	1.68E+00 (0.48)	7.93E-03 (3.4)	0.9982	0.9878
	W70/30	2.30E-01 (0.40)	3.78E-02 (0.76)	9.00E-04 (1.1)	0.9997	0.9847	1.71E-02 (0.98)	1.73E+00 (0.52)	1.81E-03 (2.3)	0.9992	0.9922[b]

KAE	W70/20	4.54E-03 (0.66)	8.87E+00 (0.99)	2.76E-05 (2.6)	0.9984	0.9958[b]	1.20E-02 (0.65)	1.38E+00 (0.31)	3.63E-05 (3.4)	0.9977	0.9941[b]
	W50/20	4.61E-03 (0.88)	3.62E+00 (1.27)	1.84E-05 (1.9)	0.9993	0.9959[b]	6.79E-03 (0.67)	1.33E+00 (0.42)	2.75E-05 (2.8)	0.9987	0.9871
	W30/20	2.90E-03 (0.77)	6.12E+00 (1.36)	3.29E-05 (3.8)	0.9970	0.9871	4.81E-03 (0.85)	1.53E+00 (0.62)	5.15E-05 (5.9)	0.9941	0.9793
	W70/10	5.69E-03 (0.64)	7.06E+00 (0.90)	3.74E-05 (3.2)	0.9977	0.9968[b]	1.34E-02 (0.53)	1.36E+00 (0.25)	4.58E-05 (3.9)	0.9972	0.9944[b]
	W70/30	5.25E-03 (1.32)	6.15E+00 (1.72)	5.39E-05 (5.7)	0.9931	0.9967[b]	1.31E-02 (1.06)	1.26E+00 (0.44)	7.31E-05 (7.7)	0.9898	0.9926[b]
CAT	W100/20	1.44E+00 (0.17)	3.05E-01 (0.57)	1.20E-02 (1.5)	0.9993	0.9645	4.83E-01 (0.39)	2.83E+00 (0.52)	2.07E-02 (2.6)	0.9984	0.9839
	W94/20	8.52E-01 (0.25)	3.37E-01 (0.87)	1.05E-02 (2.1)	0.9985	0.9350	2.98E-01 (0.16)	2.94E+00 (0.22)	5.02E-03 (1.0)	0.9997	0.9718
	W80/20	7.77E-01 (0.22)	2.39E-01 (0.65)	5.88E-03 (1.5)	0.9994	0.9427	2.06E-01 (0.36)	2.40E+00 (0.39)	7.47E-03 (1.8)	0.9992	0.9725
	W70/20	6.38E-01 (0.76)	7.71E-02 (1.38)	5.08E-03 (2.6)	0.9986	0.9897	6.21E-02 (1.06)	1.56E+00 (0.69)	8.30E-03 (4.2)	0.9970	0.9885
	W50/20	3.08E-01 (0.35)	1.08E-01 (0.68)	1.57E-03 (1.3)	0.9995	0.9772	4.37E-02 (0.47)	1.80E+00 (0.37)	2.30E-03 (2.0)	0.9992	0.9784
	W30/20	1.25E-01 (0.21)	2.63E-01 (0.66)	9.67E-04 (1.4)	0.9994	0.9371	3.75E-02 (0.61)	2.68E+00 (0.70)	2.00E-03 (2.9)	0.9979	0.9755
	W70/10	7.26E-01 (0.47)	1.15E-01 (0.90)	5.51E-03 (2.0)	0.9990	0.9802	1.05E-01 (0.70)	1.78E+00 (0.58)	9.68E-03 (3.5)	0.9976	0.9772
	W70/30	4.02E-01 (0.63)	8.63E-02 (1.12)	2.64E-03 (2.0)	0.9991	0.9834	4.18E-02 (0.84)	1.56E+00 (0.58)	4.59E-03 (3.4)	0.9978	0.9770
RSV	W100/20	2.17E-01 (0.19)	1.40E+02 (0.72)	7.00E-03 (7.3)	0.9902	0.9609	5.83E-01 (0.88)	2.97E+00 (0.68)	8.58E-03 (9.0)	0.9883	0.9816
	W94/20	3.74E-02 (0.28)	2.05E+01 (0.77)	3.54E-03 (1.9)	0.9991	0.9556	6.14E-02 (0.56)	2.52E+00 (0.58)	5.54E-04 (3.0)	0.9983	0.9799
	W80/20	2.15E-02 (1.24)	6.18E+00 (2.03)	3.18E-04 (4.9)	0.9953	0.9874	3.75E-02 (1.31)	1.48E+00 (0.89)	4.86E-04 (7.5)	0.9918	0.9801

	W70/20	8.32E-03 (0.58)	2.39E+00 (0.75)	9.20E-06 (0.7)	0.9999	0.9967[b]	1.02E-02 (0.26)	1.23E+00 (0.15)	1.13E-05 (0.9)	0.9999	0.9807
	W30/20	1.29E-03 (0.21)	8.26E+00 (0.69)	3.70E-06 (0.8)	0.9999	0.9842	2.05E-03 (0.49)	1.80E+00 (0.41)	6.10E-06 (1.4)	0.9997	0.9836
	W70/10	7.47E-03 (0.21)	4.40E+00 (0.32)	9.70E-06 (0.5)	0.9999	0.9911[b]	1.18E-02 (0.45)	1.38E+00 (0.27)	3.65E-05 (2.0)	0.9994	0.9782
	W70/30	3.02E-03 (0.14)	5.43E+00 (0.22)	2.60E-06 (0.3)	1.0000	0.9891	4.79E-03 (0.40)	1.51E+00 (0.27)	1.14E-05 (1.3)	0.9997	0.9813

[a] statistically non-significant parameter, [b] highly correlated parameters.

4.3.3. Thermodynamic analysis

Adsorption experiments of the five polyphenols on agarose using the W70 liquid phase (70:15:15 v/v, H_2O:EtOH:HAc) were performed at three different temperatures: 10, 20, and 30 °C (Figure 4-4). For all the evaluated polyphenols, the adsorption was reduced with increasing temperature, confirming the exothermic characteristic of the adsorption process. This behavior was expected since almost any adsorption process is exothermic, where the total energy released at the adsorbent-adsorbate junction is larger than the total energy absorbed by bond breakage (Saha & Chowdhury, 2011). An increase in 10 °C (from 10 to 20 °C) reduces the average percentual adsorptions of the five polyphenols. FA was the most affected with a reduction of 34.8%, followed by GA, RSV, and CAT with similar adsorption reductions equal to 29.6%, 28.8%, 28.6%, respectively; KAE the least affected (7.0%). Similarly, an increment of 20 °C (10 to 30 °C) reduced the average adsorptions by 67.1% for FA > 65.5% for GA > 57.7% for RSV > 51.6% for CAT > 17.9% for KAE.

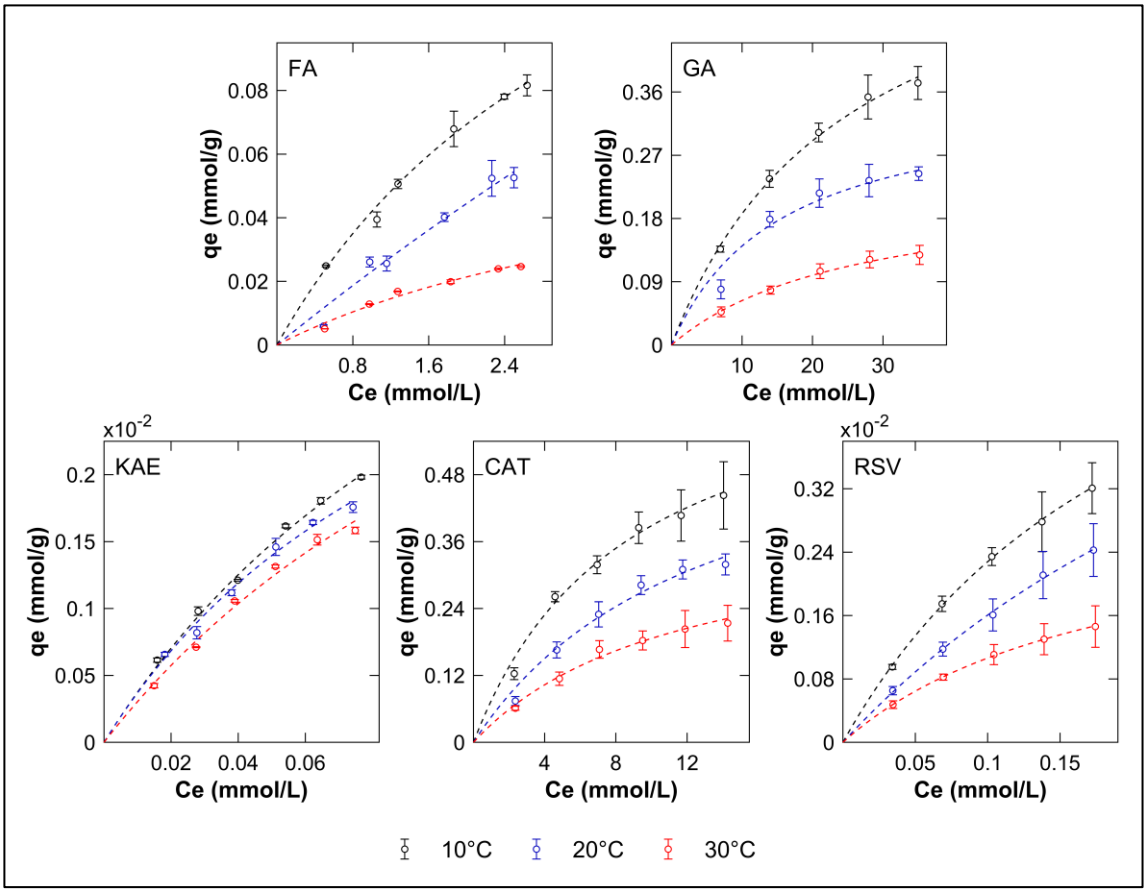

Figure 4-4. Temperature effect on polyphenols adsorption on agarose from W70 liquid phase for FA (ferulic acid), GA (gallic acid), KAE (kaempferol), CAT (catechin), and RSV (resveratrol). This effect for first plateau of PCA is shown in Figure 4-2S.

The enthalpy (ΔH) and entropy (ΔS) of adsorption were determined from Figure 4-5, whose values are summarized together with the Gibbs energy (ΔG) values (calculated from Eq. 4.10) in Table 4-4. The isosteric enthalpy (ΔH_x) of adsorption was computed from Figure 4-6 and is shown in Table 4-5. All these thermodynamic parameters appear with their respective combined standard uncertainty. For all cases, the negative values of ΔH suggest that the adsorption processes were exothermic and that an increase in temperature hindered the adsorption process. The $|\Delta H|$ value also indicates if the process is ruled by chemisorption (80-200 kJ/mol) or physisorption (2.1-20.9 kJ/mol) (Saha & Chowdhury, 2011). KAE adsorption was undoubtedly the only process ruled by

physisorption, which means that KAE-agarose binding interactions are relatively weak. Following what has been discussed above, KAE was the polyphenol with less affinity towards agarose among the five studied polyphenols. Contrarily, considering that the enthalpy of adsorption of FA, GA, CAT, and RSV was higher than the values established for physisorption and lower than those for chemisorption, these adsorption processes were presumed to be between both mechanism or could be a mixture of them. The $|\Delta H|$ values were closer to the upper limit of physisorption than to the lower limit of chemisorption; therefore, these adsorption processes are primarily physical. This idea is supported by the fact that hydrogen bonding energy (physisorption) is usually in the range of 8 – 50 kJ/mol (Huang et al., 2007) and cross-linked 12% agarose provides a large number of hydrogen bond acceptor sites for the polyphenol's hydroxyl groups. Hence, hydrogen bonding has been established as the dominant adsorption factor in polyphenol-agarose systems (Tan et al., 2010; Xu et al., 2006). Hence, no structural changes occurred on agarose, and no desorption limitations were involved (Gao et al., 2013). In the adsorption of FA and GA (both with the highest absolute values of enthalpy, see Table 4-4), multiple hydrogen bonding FA-agarose and GA-agarose could have been involved (Huang et al., 2007). Furthermore, since hydrogen bonding energy strongly depends on the distance of the atoms involved (Wendler et al., 2010), it may also be possible that the hydrogen bonds formed by FA and GA with agarose are much closer to the surface than the ones formed by KAE, CAT, or RSV. The $|\Delta H_x|$ value (Table 4-5) corroborated that FA, GA, CAT and RSV adsorptions were not process ruled by chemisorption, since $|\Delta H_x|$ values lower than 80 kJ/mol were established for physisorption and $|\Delta H_x|$ values between 80 and 400 kJ/mol indicate the possible presence of chemisorption (Ghosal & Gupta, 2015). In addition, according to the variation of $|\Delta H_x|$ with the surface coverage (adsorption capacity) (Table 4-5), the degree of heterogeneity of the adsorbent surface can be confirmed, while a constant value of $|\Delta H_x|$ would indicate a homogeneous surface (Saha & Chowdhury, 2011; Unnithan & Anirudhan, 2001). In this study, a slight degree of heterogeneity of the agarose surface can be attributed because the variation of $|\Delta H_x|$ was moderate in all cases, which agreed with that indicated by the *n* isothermal parameter.

Negative values of ΔG (Table 4-4) indicate that the adsorption process was spontaneous and thermodynamically feasible for all the studied cases (Saha & Chowdhury, 2011). Polyphenol's adsorption was more spontaneous and more favorable energetically at lower temperatures. Negative values of ΔS, for all cases studied, indicate that polyphenol molecules were organized less randomly (more ordered) at the polyphenol-agarose interface during the adsorption process ($\Delta S < 0$ means less random and $\Delta S > 0$ means more random (Li et al., 2005; Saha & Chowdhury, 2011)). $|\Delta S|$ value of KAE adsorption was lower than those of CAT and RSV adsorption and much lower than those of phenolic acids adsorption (FA and GA). It could be speculated that the lower $|\Delta S|$ of KAE is related to the higher -OH moieties of KAE, in which more spatial configurations are available for the adsorption of this compound on the surface. Hence, the order of the system does not change as much as when only one or two spatial configurations are available (as in the case of the phenolic acids).

Table 4-4: Thermodynamic parameters for the adsorption of five polyphenols on agarose.

Compound	T (°C)	$K_{eq} \pm U$	$\Delta G \pm U$ (kJ/mol)	$\Delta H \pm U$ (kJ/mol)	$\Delta S \pm U$ (J/mol K)	R^2
FA	10	49.0 ± 2.7	-9.16 ± 0.13	-49.3 ± 4.4	-141 ± 15	0.9947
	20	26.2 ± 2.8	-7.96 ± 0.26			
	30	12.3 ± 1.6	-6.32 ± 0.34			
GA	10	22.59 ± 0.20	-7.339 ± 0.021	-39.6 ± 2.9	-114 ± 10	0.9945
	20	13.7 ± 1.2	-6.37 ± 0.21			
	30	7.44 ± 0.16	-5.058 ± 0.055			
KAE	10	41.87 ± 0.42	-8.792 ± 0.024	-11.2 ± 1.1	-8.7 ± 3.6	0.9913
	20	34.7 ± 1.2	-8.643 ± 0.088			
	30	30.6 ± 1.6	-8.62 ± 0.13			
CAT	10	64.6 ± 3.9	-9.81 ± 0.14	-28.2 ± 1.1	-64.8 ± 3.7	0.9985
	20	44.1 ± 1.8	-9.23 ± 0.10			
	30	29.3 ± 1.3	-8.51 ± 0.12			
RSV	10	30.54 ± 0.35	-8.049 ± 0.027	-25.7 ± 3.4	-63 ± 12	0.9826
	20	19.37 ± 0.56	-7.224 ± 0.070			
	30	14.87 ± 0.18	-6.804 ± 0.030			

R^2 is the coefficient of determination of the van't Hoff plot, and U is the combined standard uncertainly.

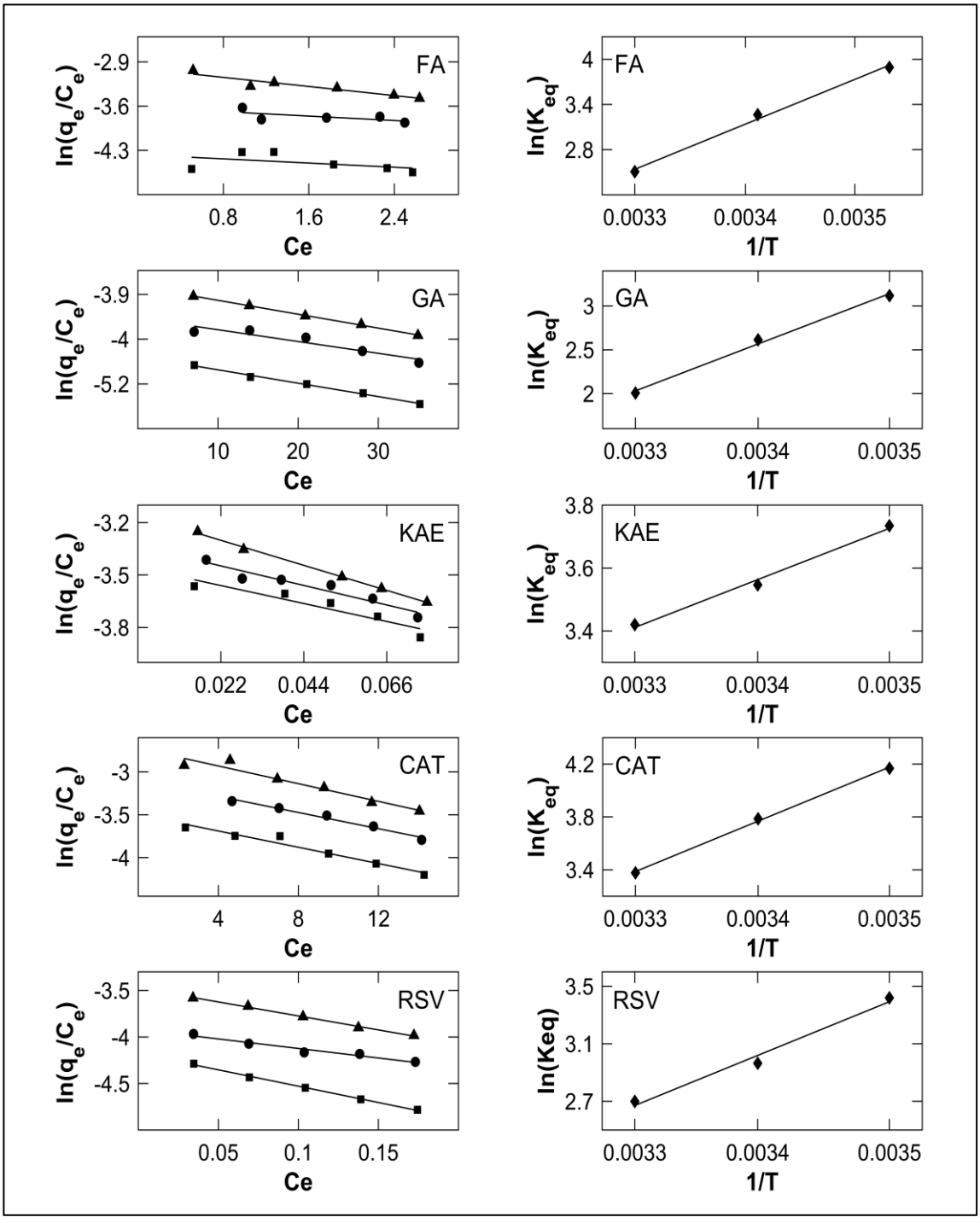

Figure 4-5. Plots of ln(q_e/C_e) versus q_e to calculated K_{eq} (left column) and van't Hoff plots (right column) for the five polyphenols (FA, GA, KAE, CAT, and RSV). ▲: 10 °C, ●: 20 °C, and ■: 30 °C.

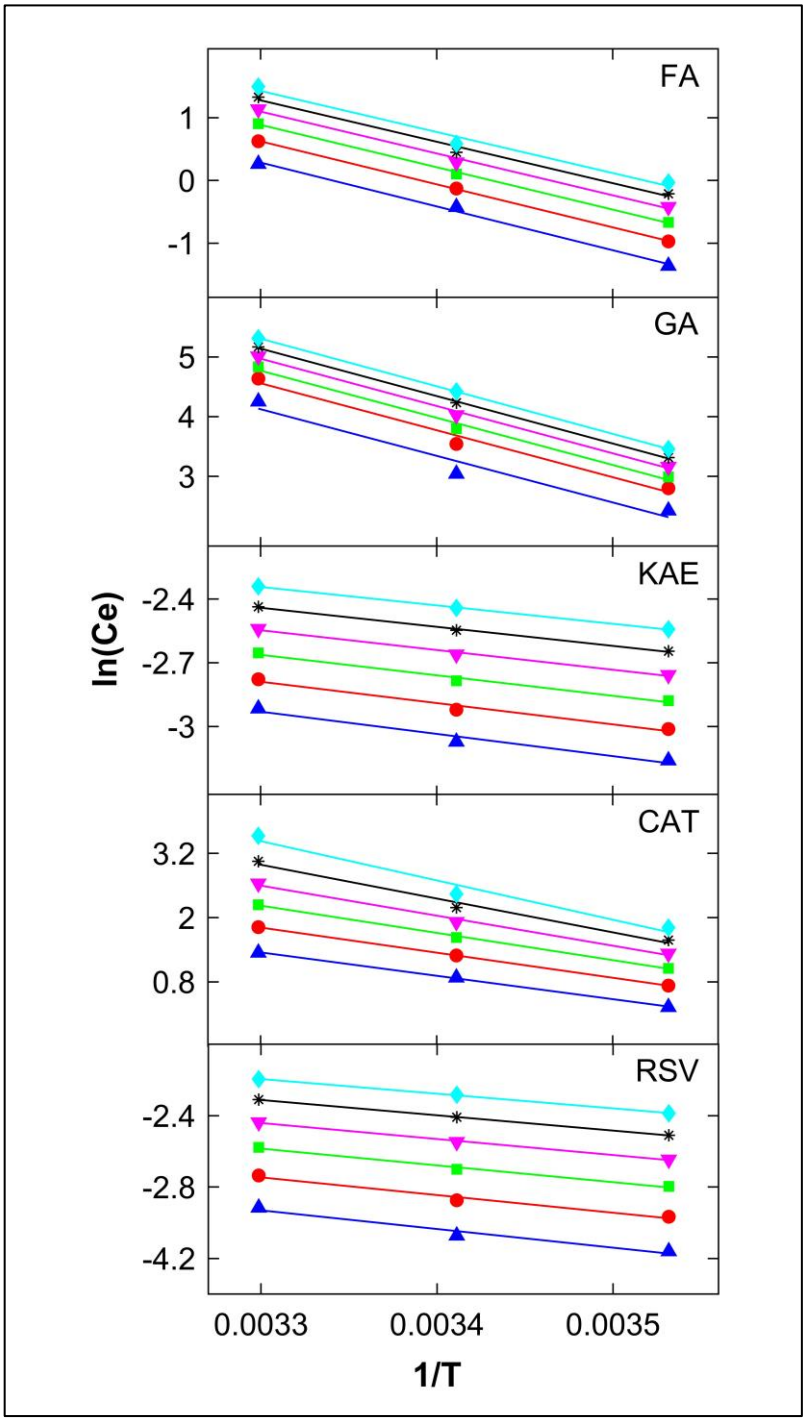

Figure 4-6. Plots of ln(C_e) versus 1/T for adsorptions of FA, GA, KAE, CAT, and RSV on agarose. ▲, ●, ■, ▼, *, and ♦ represent the six constant adsorbed amounts (q) defined in the concentration range of each polyphenol (Table 4-5).

Table 4-5: The isosteric adsorption enthalpy change of five polyphenols on agarose.

Compound	q (mmol/g)	$\Delta H_x \pm U$ (kJ/mol)	R^2
FA	1.5E-02	-58.06 ± 4.01	0.9953
	2.0E-02	-56.98 ± 0.73	0.9998
	2.5E-02	-56.1 ± 1.8	0.9990
	3.0E-02	-55.5 ± 3.9	0.9951
	3.5E-02	-54.9 ± 5.6	0.9895
	4.0E-02	-54.4 ± 7.2	0.9829
GA	2.0E-01	-64.9 ± 9.5	0.9585
	2.5E-01	-65.4 ± 8.5	0.9832
	2.8E-01	-65.6 ± 6.1	0.9917
	3.1E-01	-65.9 ± 3.8	0.9968
	3.4E-01	-66.1 ± 1.7	0.9993
	3.7E-01	-66.25 ± 0.18	1.0000
KAE	1.3E-03	-8.7 ± 1.6	0.9680
	1.5E-03	-8.3 ± 1.2	0.9785
	1.6E-03	-8.02 ± 0.93	0.9869
	1.8E-03	-7.73 ± 0.64	0.9931
	1.9E-03	-7.46 ± 0.38	0.9974
	2.1E-03	-7.21 ± 0.14	0.9996
CAT	1.0E-01	-36.3 ± 1.1	0.9991
	1.4E-01	-38.96 ± 0.11	1.0000
	1.8E-01	-42.3 ± 1.6	0.9986
	2.2E-01	-46.6 ± 3.5	0.9943
	2.6E-01	-52.5 ± 6.3	0.9857
	3.0E-01	-61.0 ± 9.8	0.9711
RSV	1.3E-03	-8.7 ± 1.6	0.9680
	1.5E-03	-8.2 ± 1.1	0.9815
	1.7E-03	-7.82 ± 0.73	0.9913
	1.9E-03	-7.46 ± 0.38	0.9974
	2.1E-03	-7.13 ± 0.11	0.9999
	2.3E-03	-6.84 ± 0.22	0.9990

4.4. Conclusions

Adsorption of five relevant low molecular weight polyphenols on Superose^{TM} 12 prep grade from liquid phases with different compositions (H_2O:EtOH:HAc) was explored and characterized. Between three and six liquid phases were evaluated for each polyphenol to simulate the different mobile phases used in isocratic and gradient APLCs.

Experimental values were fitted to standard isotherm models (Langmuir and Freundlich) by weighted least squares to compensate for the heteroscedasticity of the residuals.

Isotherms' form (L-type) indicated that probably all these polyphenols are horizontally adsorbed, there is no strong competition with the liquid phase, and that as the sites in agarose are filled, adsorption becomes more difficult. Different affinities of the polyphenols with agarose were observed. RSV showed the highest affinity towards agarose, and average adsorption was higher (93.6%) with pure water. The maximum average adsorption of the other polyphenols was 13.3% (CAT), 4.5% (FA), 3.1% (GA), and 2.8% (KAE) with water-rich liquid phases. Lowering the water proportion or increasing the EtOH:HAc proportion in the liquid phase reduced the studied polyphenols' adsorption. This reduction was different for each polyphenol, where the magnitude of this effect was: RSV > CAT > GA for the change from W100 to W70. Meanwhile, W30 liquid phase reduces the adsorptions of four of the polyphenols (except FA) up to ~0.9%, which indicates that a mobile phase of this composition could accelerate the elutions in APLC system. In the evaluated range, this factor did not have a significant effect on FA adsorption. This information is helpful to design efficient elution policies in gradient APLC.

The goodness of fit statistics (R^2 and S) indicated that both isotherm models were adequate and fitted correctly to all the experimental curves. However, the statistics referring to the parameters (CC, CI, C), especially C, indicated that the Freundlich model represented better FA, KAE, and RSV adsorptions, while the Langmuir model was better for GA and CAT. It should be mentioned that the differences between models were not significant in some cases.

The decrease in adsorption with temperature and the negative values of ΔH indicate that the adsorption processes studied were exothermic. KAE adsorption was governed only by physisorption, while FA, GA, CAT, and RSV were primarily physical. All the adsorption processes studied were spontaneous and thermodynamically feasible ($\Delta G < 0$).

In addition, the polyphenol molecules were less randomly organized (more ordered) at the polyphenol-agarose interface during the adsorption process ($\Delta S < 0$).

4.5. Appendix B. Supplementary materials

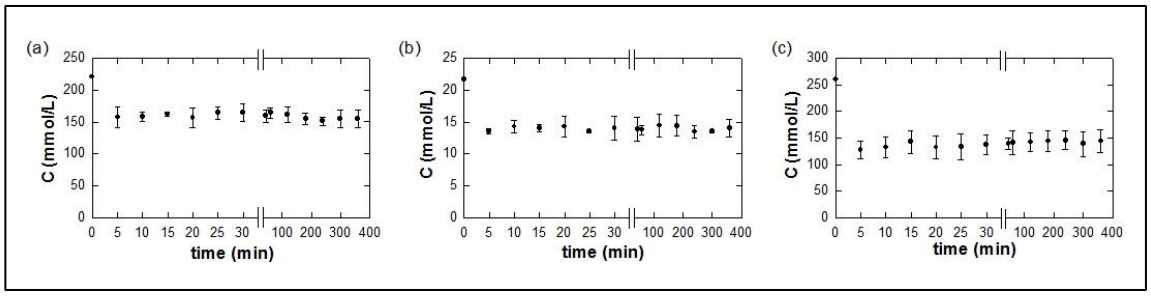

Figure 4-1S. GA adsorption (on agarose) evaluation over time from liquid phases: (a) W50, (b) W100 and (c) without water.

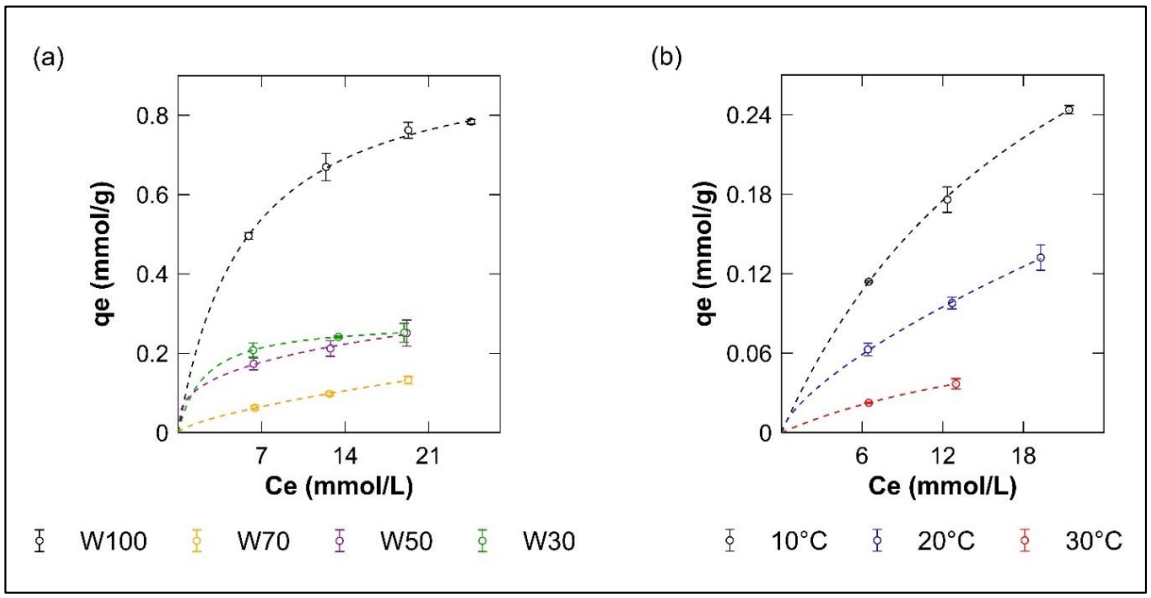

Figure 4-2S. (a) Effect of liquid phase composition and (b) effect of temperature on polyphenols adsorption on agarose at 20 °C and W70 liquid phase, respectively, for first plateau of PCA (protocatechuic acid).

GENERAL CONCLUSIONS

In this study, the multi-response optimization method was applied to HPLE for recovering polyphenolic extracts with outstanding characteristics from maqui leaves. In addition, the adsorption on agarose of five highly bioactive maqui leaves polyphenols were fully characterized for designing an efficient APLC isolation process.

An extensive literature search revealed that maqui is a natural source richer in polyphenols than other plants of the same family (blueberry, strawberry, and others). The leaves of these plants are the focus of recent attention as they are even a better source of phenolic compounds than the respective berries. It is necessary to study the effect of processing factors and growing conditions to generate consistent extracts for foods, cosmetics, and pharmaceuticals.

This doctoral research applied for the first time multi-response optimization (desirability function) and the response surface method (RSM) to design a maqui leaves HPLE process. RSM accurately predicted (RSD < 8%) total polyphenol content (TPC), antioxidant capacity (AC), and total polyphenol purity of maqui leaf extracts for two extraction scales (5 and 100 mL). The optimal HPLE conditions that prioritized both TPC and AC equally recovered ~3 times more TPC from maqui leaves than maceration, while the prioritization of purity allowed obtaining extracts with a purity of 36.29% and AC ~3 times better than the reported values. Maqui leaves and HPLE are among the best natural sources and extraction methods, respectively, to recover protocatechuic acid, quercetin, and catechin.

From the study of the adsorption equilibrium, it was possible to determine the different affinities for agarose that the studied polyphenols presented (in descending order of average adsorption): resveratrol (93.6%), catechin (13.3%), ferulic acid (4.5%), gallic acid (3.1%), and kaemferol (2.8%). The decrease in the proportion of water in the liquid phase (water:ethanol:acetic acid) reduced the adsorption of these polyphenols, except in ferulic acid. The adsorption processes studied were exothermic, spontaneous, and

thermodynamically feasible. Significant and uncorrelated isotherm parameters were estimated for 33 different scenarios; this information is critical for model-based design of the APLC process.

FUTURE PERSPECTIVES

The optimal HPLE conditions developed in this thesis are helpful to generate polyphenolic extracts from maqui leaves as functional ingredients. Currently, these leaves are a discard of the maqui processing industry. High recoveries and purities were achieved with the proposed method; therefore, the obtained extracts are suitable inputs for the following pre-purification and fractionation stages. The production of these extracts can support the commercial activities of gatherers, growers, micro-companies, and associated maqui industries in Chile.

The adsorption data collected in this thesis is critical for developing an APLC model, which in turn is required for model-based optimal design of a polyphenol's isolation process. The highly bioactive polyphenols considered in this study can reach extremely high prices depending on their purity. For example, Sigma Aldrich offer catechin (≥98%), resveratrol (≥99%), kaempferol (≥98%), and protocatechuic acid (≥99%) at 47,902 US$, 1,752 US$, 7,509 US$ and 5,331 US$ per gram, respectively. Hence, the potential of this agroindustrial discard can be further exploited to produce pharmaceuticals.

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