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OBTAINING A STANDARDIZED DEHYDRATED PRODUCT FROM MURTILLA FRUIT (*Ugni molinae* TURCZ) AS A SOURCE OF NUTRACEUTICALS: EVALUATION OF ANTIOXIDANT ACTIVITY, POLYPHENOLICS COMPOUND PROFILE AND ENZYMATIC ACTIVITY.

TESIS PARA OPTAR AL GRADO DE DOCTOR EN CIENCIAS DE RECURSOS NATURALES

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"Obtaining a standardized dehydrated product from murtilla fruit (*Ugni molinae* TURCZ) as a source of nutraceuticals: evaluation of antioxidant activity, polyphenolic compounds profile and enzymatic activity"

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Abstract

'Murtilla' (*Ugni molinae* Turcz) is a native chilean species belongs to Myrtaceae. It produces a small globose fruit with pleasant flavor and recognized antioxidant activity. The antioxidant activity performed in murtilla fruits from different ecotypes is comparable to blueberry and lowers than maqui and calafate fruits. Various antioxidant components found in fruit show a close relationship between their dietary intake and potential health benefits. However several studies have found that these constituent may be affected by biotic and abiotic conditions, and drying processing methodologies affecting both polyphenolic compound content and antioxidant activity. For these reasons, the general objective of this work was to obtain a standardized dehydrated murtilla fruit with antioxidant activity comparable to fresh murtilla fruit. Specifically, it was evaluated the effect of the drying process on the: a) profile of polyphenols and anthocyanins, b) antioxidant activity, and c) peroxidase and polyphenol oxidase activity.

The profile of anthocyanins in fresh and dehydrated fruit murtilla was investigated, and high performance liquid chromatography (HPLC) with UV detector was used to separate and determine individual compounds found in metanolic extract of dehydrated murtilla fruit. The identification of each compound was based on retention time and UV spectra by comparison with pure commercial standards and it was corroborated by HPLC connected to a mass detector. Murtilla fruit prepared by freeze drying (FD), hot-air drying at 65ºC (HAD 65ºC), and a hot-air drying at 80ºC (HAD 80ºC) drying methods were evaluated for anthocyanin retention.

Polyphenols content, antioxidant activity and dry matter content in murtilla fruits significantly depended of the crop season. The best performances of the studied parameters were obtained in the 2007 harvest year, which presented the highest values of precipitation and number of frost (2006-2007 crop seasons). Canonical discriminant analysis of seasonal differences in Red Pearl-

INIA, South Pearl-INIA and 14-4 genotype resulted South Pearl-INIA in the best discrimination between cultivars. In this study was demonstrated the influence of genotype on total polyphenol content, DPPH antioxidant activity and dry matter in murtilla fruit.

The main polyphenolic compounds determined in fresh murtilla fruit were gallic acid, catechin, quercetin 3-β-D-glucoside, myricetin, quercetin and kaempferol. Moreover, the dehydration led to a loss of polyphenols with respect to the polyphenols found in fresh fruits. The main anthocyanin compounds determined in fresh and dried murtilla fruits were cyanidin-3-glucoside and peonidin-3-O-glucoside.

The comparative application of the convective hot-air drying at 65 ºC (HAD 65 ºC) and 80 ºC (HAD 80 ºC) and freeze drying (FD) on fresh murtilla fruits Red Pearl-INIA variety showed that FD had a positive effect by increasing the total polyphenols (TP) and total anthocyanins (TA). HAD 65 ºC, HAD 80 ºC and FD produced a variation in the composition of the individual polyphenolic and anthocyanin compounds respect to the fresh murtilla fruit and freeze drying showed higher contents of the total individual polyphenols and anthocyanins determined by HPLC comparing to HAD 65 ºC and HAD 80 ºC. The application of the HAD 65 ºC, HAD 80 ºC and FD showed a positive effect on antioxidant activity because these drying methods increased the TEDPPH and TEABTS of the dried murtilla fruits respect to the fresh fruit, which could be explained by variations in the composition of polyphenolic compounds like gallic acid and catechin. Finally, the freeze drying is the best method for retention of polyphenol compounds and antioxidant activity of the fresh murtilla fruit Red Pearl-INIA variety. This dried fruit could be considered as potential functional ingredient, opening interesting perspectives for the development of new products in the food and nutraceuticals industry.

Chapter 1

Introduction

General Introduction

'Murtilla', 'mutilla' or 'murta' (*Ugni molinae* Turcz) is a native Chilean species that produces a small berry fruit and polyphenolic compounds that has been associated to the health benefices reported for this native species. The potential use of leaves and fruits has stimulated the investigation of the processing on biological and chemical characteristics shown by the respective fresh sources. Previous researches have reported that this fruit have higher levels of flavonoids and phenolic compounds and higher antioxidant capacity found in leave and fruit metanolic extracts. Several reports have associated the antioxidant activity to murtilla fruits and leaves to the content of polyphenols compounds (Avello and Pastene, 2005; Rubilar *et al*., 2006; Shene et al., 2009; Ruiz et al., 2010; Rubilar et al., 2011). Flavonoids glycosides and triterpenoids have been isolated from the leaves, being associated with the beneficial effects observed (Delporte *et al*., 2007). Pentacyclic triterpenoids, such as asiatic, corosolic, alphitolic, betulinic, oleanolic and ursolic acid, have been identified in hexane, dichloromethane and ethyl acetate extracts of murtilla leaves) (Aguirre *et al*., 2006). Other works have reported the presence of flavonoids glycoside of quercetin, myricetin and kaempferol in leaves of *U. molinae* (Rubilar *et al*., 2006). As a result of the characterization of the extracts of murtilla leaves, polyphenolic compounds have been detected in aqueous, ethanol and methanol extracts in which gallic acid was identified in aqueous extracts; epicatechin derived and kaempferol have been detected in alcoholic extracts, and myricetin ramnoside, myricetin diramnoside, myricetin glucoside and quercetin glucoside were found in all the extracts, although in low quantity in the aqueous extract (Avello, 2005 Rubilar *et al*., 2006, Shene *et al.*,2009).

Ruiz *et al*. (2010) reported that the antioxidant activity of murtilla fruits was comparable to blueberry and lower than maqui and calafate fruits; also they reported the anthocyanins cyanidin-3-glucoside and peonidin-3-glucoside in murtilla fruits, and the flavan-3-ols identified in murtilla were catechin and epicatechin.

Nowadays, obtaining of processed foods and nutraceutical products from plants require a standardization associated to homogeneity characteristics of the: a) vegetal source, b) transformations process of the raw material, c) procedures for isolating active principles, and d) methodologies involve in characterization of the bioactive metabolites.

Moreover, the chemical characteristics of the plant depend strongly of the intrinsic factors (plant genetic) and extrinsic factors such as environmental conditions, collection methods, cultivation, harvest, post-harvest processing, transport, and storage practices (Kevers *et al*. 2011). On the other hand, several data such as climate conditions, season, geographical localization, environmental conditions, etc. must be observed and documented in order to elucidate future differences in bioactivity compared with other results found. For instance, the duration of sunlight, average rainfall, average temperature, including day-time and night-time temperature differences, also influence the physiological and biochemical activities of plants, and prior knowledge should be considered (van der Luis *et al*. 2001; Bolling *et al*. 2010; Howard *et al*. 2012). The soil should contain appropriate amounts of nutrients, organic matter, and other elements to ensure optimal plant growth and quality. Optimal soil conditions, including soil type, drainage, moisture retention, fertility, and pH, will be dictated by the selected plant species and/or plant part (Koca *et al*., 2009).

After harvest, bioactive compounds present in fresh fruits can change qualitative and/or quantitatively because the same factors above mentioned. Thus, several methods have been used to drying vegetal materials for being commercialized like a dehydrated fruit or for nutraceutical products. Classical drying methods include the dehydration in the open air, drying ovens/rooms, liophylization, microwave or infrared devices. However, regardless the methodology, temperature and moisture must be controlled to avoid damage to the active chemical constituents. The method and temperature used for drying may have a considerable impact on the quality of the resulting vegetal materials. Research is currently underway to determine how different processing methods may affect the levels of these bioactive agents and their biological activity in foods. Drying was shown to reduce antioxidant capacity in berries (Saskatoon berries [*Amelanchier alnifolia Nutt.*]) and raspberries (Kwok *et al*., 2004; Mejia-Meza *et al*., 2009).The mechanism how several drying methods affect the relative retention of antioxidant components has not been fully characterized. To our knowledge, only few studies has been conducted to assess the effects of different dehydration technologies on the retention of individual and total polyphenols in murtilla fruits, or the effect on their antioxidant capacity (Reyes *et al*., 2009 and 2010).

Drying is a traditional method used for fruit preservation. Conventional drying using convective hot-air has been applied for different berries. Lopez *et al*. (2010) reported a decreased on total polyphenol content (45 to 82%) and antioxidant activity in dried blueberry respect to fresh fruit for drying process using different air temperature (50-90 ºC). A lower decreased on blueberry total polyphenol content (28%) was determinate with convective drying at 90 °C for 10 hours. Also, losses on total anthocyanins (69%) and antioxidant activity (24%) were observed (Stojanovic and Silva, 2006). Convective hot-air drying at 75 and 76.6 ºC showed a decreasing of the 82.6 and 74.7% in the antioxidant capacity of Saskatoon berries (Amelanchier alnifolia Nutt.) and raspberries respectively (Kwok *et al*., 2004; Mejia-Meza *et al*., 2009). Fresh strawberry from cultivar Kent and Elsanta showed a decreasing in total polyphenols (35.8 and 35.9%, respectively), and anthocyanins (73.0 and 63.6%, respectively) when drying with convective hot-air at 70 °C and velocity of 1 m s^{-1} (Wojdyło *et al*., 2009).

Freezing drying (FD) or liophylization is considered as one of the best methods for the conservation of food (Ratti, 2001) and it is recommended for preserving polyphenols compounds in berries fruit (de Torres *et al*., 2010; Grace *et al*., 2012). de Torres et al. (2010) determinated that freeze drying method was less aggressive than oven-drying on phenolic compounds, anthocyanins and flavonols, identified in fresh grape skin (Carménère and Cabernet Sauvignon varieties) and dehydrated samples. Comparison of different drying methods applied for three strawberry cultivars showed that freeze drying reduced the content of soluble phenolic compounds to 51 at 56% of its initial values, while for convectively dried products (26-38%) and microwave dried strawberries (28-39%) a much lower level residual values were observed. Also, the antioxidant activity was favorable for freeze drying (Bôhm *et al*., 2006). Drying cranberry fruit using freeze drying (FD) resulted in greater retention of anthocyanins compared to air drying and preserved a higher level of cranberry antioxidant activity, but FD showed similar performance than vacuum-microwave drying. Difference in anthocyanins content and antioxidant activity was attributed to drying temperature, presence of oxygen during the drying process and drying time (Leusink *et al*., 2010). Grace *et al*. (2012) reported no significant differences in concentrations on each polyphenolic compound identified in fresh cranberries and after freeze drying. Reyes *et al*. (2009) evaluated the application of the atmospheric freeze drying for murtilla fruit as an alternative of the vacuum freeze drying; under optimum operation conditions (Drying

5 ºC, fast freezing and IR application), a large percentage of the antioxidant capacity founded in fresh fruit remains in the dry fruit.

No study has been performed to assess the effect of traditional hot air convection dehydration method and freeze drying on the retention of murtilla fruits polyphenols and their antioxidant capacity. These drying methods could be the ways to preserve the polyphenolic bioactive components reported in the literature for this fresh fruit and to collaborate for its commercialization as a dried product for its used as direct food or ingredient of the nutraceuticals product.

Phytochemical are active metabolites that necessarily require extraction and isolation from their natural sources which also contain many unwanted materials. This is necessary regardless the separation technique and analytical method used. The objective is the targeted isolation of new bioactive phytocompounds and this can mean either a full identification of a bioactive phytocompound after purification or partial identification to the level of a family of known compounds. The isolated compound is characterized by spectroscopic methods and rapid strategies for chemical characterizations of phytoconstituents of natural products as well as assessing the bioactivities of the natural products (Moronkola, 2012).

The use of polyphenols in food either as fortification (Pinelo *et al*., 2004a) or preservation is a common custom generally carried out to increase its nutritional value. Apple polyphenols, for instance, having a large amount of a catechin oligomer, were reported to be effective inhibitors of cholesterol oxidation in commercial meat products such as pork sausage, raw and roast ham, bacon, and hamburgers (Pinelo *et al*., 2004a). Similarly, other flavonoids (e.g., quercetin) are successfully employed to inhibit fish oil degradation (Pinelo *et al*., 2004a). Resveratrol is known to possess intense preservative and pharmacologic characteristics (Pinelo *et al*., 2004a). The ability of phenols to resist oxidative cleavage and polymerize, leading to an improvement in the overall antioxidant activity of plant foods, is highly associated with their structure (Denev *et al*., 2012). Further factors, such as pH, as well as the presence of oxygen and oxidative agents or enzymes, could play important roles (Pinelo *et al*., 2004b). Peroxidase (POD) and polyphenol oxidase (PPO) have been considered the principal enzymes responsible for quality deterioration

in most fruits and vegetable. The importance of controlling of PPO in fruits, largely determines the quality and economic value of the product harvested, stored and processed. POD is involved in enzymatic browning since diphenols may function as reducing substrate in this reaction (Chisari *et al*., 2007; Serrano-Martinez *et al*., 2008). Peroxidase is also intimately related to flavor loss and odor of stored food as well as to a great variety of biodegradation reactions (Rojas-Grau *et al*., 2008). The presence of POD and PPO in murtilla fruits have not been reported yet, then the study of these enzymes is key because they could be associate with the changes in polyphenols when the fruit is drying.

In summary, there are a few studies about the effect of drying technologies on polyphenolic compounds and antioxidant activity of murtilla fruit and no information about its enzymatic activity. The objective of this study was to determine and to compare the effect of convective hot-air drying at 65 ºC (HAD 65ºC), convective hot-air drying at 80 ºC (HAD 80ºC) and freeze drying (FD) on profile of polyphenol compounds, antioxidant activity and enzymatic activity on fresh murtilla (*Ugni molinae* Turcz) fruits and dehydrated products, with the purpose of standardizing a process for obtain a dehydrated product from murtilla fruits with antioxidant activity comparable to the fresh fruit.

Hypothesis

Based on these antecedents, the work hypothesis is:

The classic method for conventional hot-air drying with respect to freeze drying methodology affect the profile of polyphenol compounds and decrease the antioxidant activity in murtilla fruits.

Objectives

General goal

The objective of this study was to determine and to compare the effect of convective hot-air drying at 65 ºC (HAD 65ºC), convective hot-air drying at 80 ºC (HAD 80ºC) and freeze drying (FD) on profile of polyphenol compounds, antioxidant activity and enzymatic activity on fresh murtilla (*Ugni molinae* Turcz) fruits and dehydrated products, with the purpose of standardizing a process for obtain a dehydrated product from murtilla fruits with antioxidant activity comparable to the fresh fruit.

Specific goals

1. To establish initial condition in fresh murtilla fruit in relation to antioxidant activity, total concentration of polyphenols, anthocyanins and identify polyphenolic and anthocyanin compounds present in this fruit.
2. To evaluate the effect of HAD and FD, on the antioxidant activity of dehydrated murtilla fruits.
3. To evaluate the effect of HAD and FD, on the profile of polyphenols and anthocyanins in dehydrated murtilla fruits.
4. To evaluate the peroxidase and polyphenol oxidase activity of fresh murtilla fruits and dehydrated products.

References

Aguirre, M. C., Delporte, C., Backhouse, N., Erazo, S., Letelier, M. E., Cassels, B. K., Silva, X., Alegría, S., and Negrete, R. (2006). Topical anti-inflammatory activity of 2α-hydroxy pentacyclic triterpene acids from the leaves of *Ugni molinae*. Bioorganic and Medicinal Chemistry 14, 5673-5677.

Avello, M., Pastene, E. 2005. Actividad antioxidante de infusos de *Ugni molinae* Turcz ("Murtilla"). Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas 4, 33-39.

Böhm, V., Kühnert, S., Rohm, H. and. Scholze, G. (2006). Improving the nutritional quality of microwave-vacuum dried strawberries: A preliminary study Food Science and Technology International 12, 67- 75.

Bolling, B., Dolnikowski, G., Blumberg, J., Chen, C.Y. 2010. Polyphenol content and antioxidant activity of California almonds depend on cultivar and harvest year. Food Chemistry. 122, 819-825.

Chisari, M., Barbagallo, R. N., and Spagna, G. (2007) Characterization of polyphenol oxidase and peroxidase and influence on browning of cold stores strawberry fruit. Journal of Agriculture and Food Chemistry 55, 3469-3476.

Delporte C, Backhouse N, Inostroza V. (2007). Analgesic activity of *Ugni molinae* (murtilla) in mice models of acute pain. Journal of Ethno pharmacology 112, 162–165.

de Torres, C.; Díaz-Maroto, M.C.; Hermosín-Gutiérrez, I.; Pérez-Coello, M.S. (2010). Effect of freeze-drying and oven-drying on volatiles and phenolic compositions of grape skin. Analytica Chimica Acta 660, 177–182.

Grace, M.H., Massey, A.R., Mbeunkui, F., Yousef, G.G., Lila, M.A. (2012) Comparison of health-relevant flavonoids in commonly consumed cranberry products Journal of Food Science 77 H176-83.

Howard, L. R., Prior, R. L., Jackson O. L. **(2012)** Processing and storage effect on berry polyphenols: challenges and implications for bioactive properties. Journal of Agricultural and Food Chemistry 60,: 6678–6693.

Kevers, C., Pincemail, J., Tabart, J., Defraigne, J-O., Dommes, J. 2011. Influence of cultivar, harvest time, storage conditions and peeling on the antioxidant capacity and phenolic and ascorbic acid contents of apples and pears. Journal of Agricultural and Food Chemistry 59, 6165-6171.

Koca, I., Karadeniz, B. 2009. Antioxidant properties of blackberry and blueberry fruits grown in the Black Sea Region of Turkey. Scientia Horticulturae 121, 447-450.

Kwok, B.H.I., Durance, T., Kitts, D.D. (2004). Dehydration techniques affect phytochemical contents and free radical scavenging activities of Saskatoon berries (Amerlachier alnifolia Nutt). Journal of Food science 69, 122-6.

Leusink, G. J., Kitts, D., Yaghmaee, P., Durance, T. (2010). Retention of antioxidant capacity of vacuum microwave dried cranberry. Journal of Food Science 75, 311-316

López, J., Uribe, E., Vega-Gálvez, A, . Miranda, M., Vergara, J., González, E., and Di Scala, K. 2010. Effect of air temperature on drying kinetics, vitamin c, antioxidant activity, total phenolic content, non-enzymatic browning and firmness of blueberries variety O'Neil. Food and Bioprocess Technology 3, 772-777.

Mejia-Meza E.I., Yañez J.A., Remsberg C.M., Takemoto J.K., Davies N.M., Rasco B., and Clary C.(2010). Effect of dehydration on raspberries: polyphenol and anthocyanin retention, antioxidant capacity, and antiadipogenic activity. Journal of Food Science 75, 5-12.

Moronkola, D.O. (2012). Developments in phytochemistry, drug discovery research in pharmacognosy, Prof. Omboon Vallisuta (Ed.), ISBN: 978-953-51-0213-7.

Denev N. P., Kratchanov G.C., Ciz M., Lojek A., Kratchanova M. G. (2012). Bioavailability and Antioxidant Activity of Black Chokeberry (Aronia melanocarpa) Polyphenols: in vitro and in vivo Evidences and Possible Mechanisms of Action: A Review
Food Science and Food Safety 11 (5) 471–489.

Pinelo M., Manzocco L., Nuñez M., Nicoli M. C (2004a). Interaction among phenols in food fortification: negative synergism on antioxidant capacity. Journal of Agricultural and Food Chemistry 52,1177-80.

Pinelo M., Manzocco L., Nuñez M., Nicoli M. C (2004b). Solvent effect on quercetin antioxidant capacity. Food Chemistry 88, 201–207

Ratti, C. Hot air and freeze-drying of high-value foods: A review (2001) Journal of Food Engineering 49, 311-319.

Reyes, A., Bustos, R., Vásquez, M.B., Scheuermann, E. (2009). Optimization of "murtilla" berry drying in an atmospheric freeze dryer. Chemical Engineering Transactions 18, 183-188.

Reyes, A.; Bubnovich, V.; Bustos, R.; Vasquez, M.; Vega, R.; Scheuermann, E. (2010).Comparative study of different process conditions of freeze drying of 'murtilla' berry. Drying Technology 28, 1416–1425.

Rojas-Grau, M.A., Soliva-Fortuny, R. and Martin- Belloso, O. (2008) Effect of natural antibrowning agents on color and related enzymes in fresh-cut fuji apples as an alternative to the use of ascorbic acid. Journal of Food Science 73, 267-272

Rubilar, M., Pinelo, M., Ihl, M., Scheuermann, E., Sineiro, J., Nuñez, M.J. (2006) Murtilla leaves (Ugni molinae Turcz) as a source of antioxidant polyphenols. Journal Agriculture Food Chemistry 54, 59-64.

Rubilar, M., Jara, C., Poo, Y., Acevedo, F., Gutierrez, C., Sineiro, J., Shene, C. (2011). Extracts of Maqui (*Aristotelia chilensis*) and Murta (*Ugni molinae* Turcz): sources of antioxidant compounds and α-Glucosidase/α-Amylase inhibitors. Journal Agriculture Food Chemistry 59, 1630-7.

Ruiz, A., Hermosin-Gutierrez, I., Mardones, C., Vergara, C., Herlitz, E., Dorau, C., Winterhalter, P. and von Baer, D. (2010). Polyphenols and antioxidant activity of calafate (*Berberis microphylla*) fruits and other native berries from southern Chile. Journal Agriculture Food Chemistry 58, 6081–6089.

Serrano-Martinez, A., Fortea M.I., Del Amor F.M., Nuñez –Delicado E. (2008) Kinetic characterization and thermal inactivation study of partially purified red pepper (*Capsicum annuum* L.) peroxidase. Food Chemistry 107,193-1

Shene, C., Reyes, A., Villarroel, M., Sineiro, J., Pinelo, M., and Rubilar, M. (2009). Plant location and extraction procedure strongly alter the antimicrobial activity of murtilla extracts. European Food Research and Technology 228, 467-475.

Stojanovic, J. y J.L. Silva: (2007). Influence of osmotic concentration, continuous high frequency ultrasound and dehydration on antioxidants, colour and chemical properties of rabbiteye blueberries, Food Chemistry 101, 898-906

van der Sluis, A.A., Dekker, M., De Jager, A., Jongen, W.M.F. 2001. Activity and concentration of polyphenolic antioxidants in Apple: Effect of cultivar, harvest year, and storage conditions. Journal of Agricultural and Food Chemistry. 49, 3606-3613

Wojdyło, A., Figiel, A., Oszmianski, J (2009). Effect of drying methods with the application of vacuum microwaves on the bioactive compounds, color, and antioxidant activity of strawberry fruits Journal of Agricultural and Food Chemistry 57, 1337-1343.

Chapter 2

Influence of genotype and harvest year on polyphenols content and antioxidant activity in murtilla (*Ugni molinae* Turcz) fruits

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INFLUENCE OF GENOTYPE AND HARVEST YEAR ON POLYPHENOL CONTENT AND ANTIOXIDANT ACTIVITY IN MURTILLA (*UGNI MOLINAE* TURCZ) FRUIT

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Abstract

Polyphenol content and antioxidant activity in murtilla (*Ugni molinae* Turcz) fruit from three genotypes (the 14-4 genotype and the Red Pearl-INIA and South Pearl-INIA varieties) were studied over five growing seasons. Our results showed significant differences in total polyphenol content among yearly harvests. The lowest value (283 ± 72 mg GAE/100 g dw) was obtained for the 14-4 genotype in the 2008 harvest, and the highest value (2,152 ± 290 mg GAE/100 g dw) was observed for the variety South Pearl-INIA in 2007. The lowest value for antioxidant activity (2,234 ± 337 µmol TE/100 g dw) was obtained for the Red Pearl-INIA variety in 2008, and the highest value (4,073 ± 76 µmol TE/100 g dw) was observed for the 14-4 genotype in 2007. There was a significant effect of genotype and growing season on polyphenol content, antioxidant activity and dry matter content for the murtilla fruits evaluated in this study, but additional studies examining other abiotic and biotic factors are required to fully explain causality.

Keywords: DPPH, Trolox, rainfall, frost, climate conditions, native berry.

1. Introduction

The native Chilean species *Ugni molinae* Turcz, commonly called "murtilla," "mutilla" or "murta," is a wild perennial shrub that grows in southern Chile. This plant produces a small globoid berry with an equatorial diameter of 0.71-1.31 cm (Seguel *et al*., 2000). Infusions of murtilla leaves are highly valued in Chilean indigenous mapuche folk medicine for the treatment of conditions such as diarrhea, dysentery, and urinary tract pain (Montenegro, 2002). Murtilla is most often consumed as a fresh fruit because of its organoleptic characteristics, but the fruit is also processed commercially to be sold canned or as jam, juice or liquor (Scheuermann *et al*., 2008).

The murtilla fruit is known for its typical and surrounding aroma, which is produced by 24 volatile compounds that have been identified in four ecotypes and range in concentration from 1.2 to 250.5 μg kg^{-1} fresh weight (Scheuermann *et al*., 2008). Additionally, murtilla fruit is considered a valuable source of high quality pectin that has a chemical composition similar to that of commercial citrus pectin (Taboada *et al*., 2010). Several reports show an association between the antioxidant activity of murtilla fruits and leaves and the levels of polyphenols (Avello and Pastene, 2005; Rubilar *et al*., 2006; Shene *et al*., 2009; Ruiz *et al*., 2010; Rubilar *et al*., 2011).

Antioxidant activity studies using different ecotypes of murtilla have shown that the levels of antioxidant activity in the fruit are comparable to those of blueberry and lower than those of maqui and calafate fruits (Ruiz *et al*., 2010; Arancibia-Avila *et al*., 2011). These results suggest that high levels of flavan-3-ols such as catechin and epicatechin, in addition to the anthocyanins cyanidin-3-glucoside and peonidin-3-glucoside, could be the responsible for the high antioxidant activity observed in murtilla fruits. The concentration of flavonols observed in murtilla fruit was two times higher than the concentrations observed in maqui and calafate fruits, with quercetin derivatives being the most abundant flavonol (Ruiz *et al*., 2010).

Polyphenols and many other natural antioxidants (vitamins, carotenoids and other endogenous constituents) may promote better health. These antioxidants are capable of fulfilling a number of

functional roles, acting as free radical scavengers, peroxide decomposers, singlet and triplet oxygen quenchers, enzyme inhibitors and synergists (Mandal *et al*., 2009). A close relationship has been shown between dietary intake of various antioxidant components and potential health benefits. However, several studies have found that these compounds may be affected by unaccounted factors, such as variety, growing environment, growing season, climate, temperature, light, soil type and other conditions (processing, post-harvest storage), which could affect both antioxidant content and antioxidant activity in the fruit (van der Sluis *et al*., 2001; Bolling *et al*., 2010).

The objective of this study was to determine potential seasonal differences in polyphenol content and antioxidant activity in murtilla fruits from three genotypes (the 14-4 genotype and the Red Pearl-INIA and South Pearl-INIA varieties) harvested in five different years.

2. Materials and methods

1.1. Sampling and experimental design

Fruits were obtained from three murtilla genotypes (the 14-4 genotype and the Red Pearl-INIA and South Pearl-INIA varieties) belonging to a germplasm collection developed by INIA-Chile through its murtilla research project. The plants were grown in an experimental field near Puerto Saavedra (38°45’ S in latitude, 73°21’ W in longitude). The genotypes used in this research were selected for their agronomical and organoleptic characteristics (Seguel *et al*., 2000; Scheuermann *et al*., 2008). The fruits were harvested in the same state and at the same stage in the plants’ life cycle in April of 2006, 2007, 2008, 2009 and 2011 and were transported immediately after harvest to the Food Science Laboratory at the Universidad de La Frontera.

Total polyphenol content, antioxidant activity and dry matter of the murtilla fruit were determined for the three genotypes and the five harvest years (2006, 2007, 2008, 2009 and 2011). However, lack of financial support made it impossible to adequately manage the crops. Thus, the data from the fruit harvested in 2010 were excluded from the study due to low reliability.

1.2. Extraction

A solvent extraction of the murtilla fruit was used to measure polyphenol levels and antioxidant activity according to the methodology described by Scheuermann (2009). Approximately 6 g of fresh fruit was weighed with a semi-analytical balance (+/- 0.001 g) and ground with a mortar. The crushed fruit was subsequently transferred to a 100 mL bottle and 20 mL of methanol (99.9%) that had been previously conditioned to a temperature e of 30 °C was added. The solvent fruit extraction was performed in an oven (GFL-3032, Germany) under agitation (170 rpm) for 20 min at 30 °C. After the extraction step, the fruit extract was separated from solid matter by vacuum filtration using Advantec 232 filter paper (Toyo, Japan). The extracts were stored in 100mL flasks protected with aluminum foil and were analyzed immediately.

1.3. Total polyphenol content

Total polyphenol content was determined according to the method described by Wong *et al.* (2006). Using this method, 3.16 mL of distilled water were added to 40 µL of the sample and mixed with 200 µL of Folin-Ciocalteau reagent. After 5 minutes, 600 µL of 20% Na_2CO_3 were added to the reaction mixture and the mixture was allowed to stand for 120 min. The absorbance of each sample was measured at 765 nm and expressed in mg of gallic acid equivalents (GAE) per 100 g of dry weight (dw) by calibrating the optical density of each sample to a standard curve that was previously established using varying concentrations of gallic acid.

1.4. DPPH antioxidant activity

The antioxidant capacity of the murtilla fruit extracts was determined by measuring the radical 2,2-diphenyl-1-picrylhydrazy (DPPH) scavenging of the antioxidant compounds present in the extracts. The degree of discoloration of the solution indicated the scavenging efficiency of the added substance. In its radical form, DPPH has an absorption band of 515 nm, which disappears following reduction by an antiradical compound (von Gadow *et al*., 1997; Atoui *et al*., 2005). The initial absorbance of the DPPH in methanol was measured at 515 nm and had a range of 0.630 to 0.640. An aliquot (50 µl) of the murtilla fruit extract was added to 1,950 µl of the metanolic DPPH solution. The change in absorbance at 515 nm was measured at 30 min. The antioxidant capacity, based on the DPPH free radical scavenging ability of the extract, was expressed in µmol Trolox equivalents (TE) per 100 g of dry weight (dw).

1.5. Dry matter

Dry matter was determined using fruit moisture content according to the Instituto de Salud Pública de Chile (1998). Five grams of halved murtilla fruits were oven-dried at 105 ºC for five hours and then weighed. This process was repeated until the difference between two successive weighings was less than 5 mg.

1.6. Climate condition data

Climate records for rainfall and number of frosts were obtained from the closest meteorological station, located in Tranapuente (38°41' S, 73°21' W). The first growing season (2006) ran from May 2005 to April 2006 when the fruit was harvested. The growing season was the same during subsequent years, up through the final harvest in April 2011.

1.7. Data analysis

For all experiments, three to five replicates were analyzed per treatment. The data were analyzed using a mixed-model ANOVA with genotype and season, followed by Tukey's Honestly Significant Difference tests as pos-hoc tests (Sokal and Rohlf, 1995). Alternatively, Student's t-tests were used to analyze pairs of independent samples (Zar, 1999). Prior to performing the statistical tests, the data for each variable were checked for normality and homogeneity of variance. A canonical discriminant analysis was performed to evaluate murtilla genotype performance using 95% confidence ellipses. Total polyphenol content, antioxidant activity and dry matter were correlated with rainfall and number of frosts (measured in the field) using the nonparametric Spearman's rank method. JMP v.8 software (SAS Institute Inc., Cary, NC) was used for all statistical analyses and differences were considered significant at $P \leq 0.05$.

3. Results and discussion

1.8. Total polyphenol content, DPPH antioxidant activity and dry matter of murtilla fruit from different growing seasons

The effect of harvest year on total polyphenol content, DPPH antioxidant activity and dry matter for the three genotypes studied was significant (Tukey's test, $P<0.05$) (Figure 1). Large differences in total polyphenol content were observed among harvest years, with polyphenol

content ranging from 283 ± 72 mg GAE/100 g dw for the 14-4 genotype in 2008 to 2,152 ± 290 mg GAE/100 g dw for the South Pearl-INIA variety in 2007. DPPH antioxidant activity ranged from 2,234 ± 337 µmol TE/100 g dw in the Red Pearl-INIA variety in 2008 to 4,073 ± 76 µmol TE/100 g dw for the 14-4 genotype in 2007.

<Insert Figure 1 here>

These differences could be explained by variation in weather conditions among the growing seasons, such as differences in rainfall and the number of frosts. The highest correlations were between total polyphenol content and rainfall, dry matter and rainfall, dry matter and number of frosts and DPPH antioxidant activity and rainfall (Table 1). These abiotic factors could also be responsible for the changes in dry matter. For instance, notable changes in fruit dry matter between the 2006 and 2009 harvest years were observed for the 14-4 genotype (30.9% decrease) and the Red Pearl-INIA variety (33.2% decrease) (Figure 1). This result is corroborated by the significant correlations between dry matter and rainfall (0.82) and dry matter and number of frosts (0.75).

<Insert Table 1 here>

Therefore, our results showed a trend in increasing total polyphenol content, DPPH antioxidant activity and dry matter with increasing rainfall and number of frosts. Several researchers attribute differences in the level of phenolic compounds and antioxidant activity in fruits and vegetables of the same genotype grown in different locations or different years to changes in environmental conditions, such temperature, water availability (drought or precipitation), light intensity, salinity and pollination (van der Sluis *et al*., 2001; Connor *et al*., 2002; Howard *et al*., 2003; Temime *et al*., 2006; Ksouri *et al*., 2008; Bolling *et al*., 2010; Kevers *et al*., 2011; Sun *et al*., 2011). However, the mechanism behind the effects of low temperature (number of frosts) and water availability (rainfall) on phenolic content and other secondary metabolites in plants is not clearly understood. Normally, the mechanism considered the biotic or abiotic stresses that result in the formation of reactive oxygen species (ROS) followed by activation of the enzyme and hormone systems of the plant, producing antioxidants that include enzymatic and non-enzymatic

components (Xin and Browse, 2000; Janda *et al*., 2003; Ksouri *et al*., 2008). The effects of water stress (*e.g.*, waterlogging or drought) and temperature (low v. high, constant v. fluctuating) on the medicinal herb *Hypericum brasiliense* included an increase in the levels of some phenolic compounds (quercetin, rutin, 1,5-dihydroxyxanthone and isouliginosin B). However, changes in phenolic content depended on the type of stress and the compound analyzed and were likely a response to the generation of ROS (de Abreu and Mazzafera, 2005).

Figure 2 shows the values for monthly total rainfall and minimum accumulated temperature used to correlate each growing season (Table 1) with total polyphenol content, DPPH antioxidant activity and dry matter for the three murtilla fruit genotypes. As shown in Figure 2A, the accumulated rainfall values for the month of August in the 2005-2006 and 2010-2011 growing seasons were similar to the total rainfall in the 2007-2008 and 2008-2009 growing seasons. There were differences in rainfall distribution and quantitative rainfall between April 2005 and April 2011, confirming that there was variance in this agronomic parameter over the course of the study period at the location of the experimental field is located (38°45' S latitude; 73°21'W longitude). A similar pattern occurred for minimum accumulated temperature (Figure 2B), with the 2008-2009 growing seasons showing the highest temperature (44 °C) and the lowest the number of frosts (Table 1) for the study period.

<Insert Figure 2 here>

Year-to-year variations in certain phenolic compounds that are important sources of secondary plant metabolites have been observed in other types of fruit (van der Sluis *et al*., 2001; Kevers *et al*., 2011). Connor *et al*. (2002) reported that the phenolic content of several highbush and interspecific hybrid blueberry cultivars grown at three locations varied considerably over two growing seasons. Howard *et al*. (2003) suggested that this effect was not surprising because abiotic and biotic factors such as temperature, irradiation, herbivory and pathogenic infection are known to induce the protective antioxidant mechanisms of plants. The correlation coefficients determined by Sun *et al*. (2011) showed that altitude and annual precipitation had a significant effect on sour jujube fruit (*Ziziphus jujuba* Mill.). Fruits grown in harsh, arid and high-altitude

areas can produce a larger amount of natural antioxidants and therefore may exhibit higher antioxidant activities than fruits grown in other areas.

1.9. Genotypic and seasonal effects on total polyphenol content, DPPH antioxidant activity and dry matter of murtilla fruit

The analysis of variance showed that the effects of genotype, season and genotype x season were significant for all variables, with the exception of genotype and genotype x season for DPPH antioxidant activity (Table 2). Howard *et al*. (2003) reported similar results in blueberries and attributed these results to genetic and environmental factors. The significant main effects of growing season, genotype and genotype x growing season on antioxidant activity measure by the Oxygen Radical Absorbance Capacity (ORAC) test, phenolic content and fruit weight demonstrated that environmental growing conditions can impact phenolic and ORAC levels in blueberry and that genotypes vary in their capacity to synthesize phenolic compounds under different growing conditions. In contrast, Connor *et al*. (2002) found that the antioxidant activity and phenolic content of blueberry cultivars were more strongly affected by genotype than by harvest year.

Correlation does not imply causation, but a correlation does indicate that changes in one variable are related to changes in another variable. New studies that include other abiotic and biotic factors are necessary to determinate all of the causes that could fully explain year-to-year changes in total polyphenol content, DPPH antioxidant activity and dry matter in the murtilla fruit varieties evaluated in this study. It is the first whose purpose is to relate some environmental and chemical variables for this fruit.

<Insert Table 2 here>

1.10. Importance of genotype

The multivariate discriminant analysis of murtilla genotypes resulted in two canonical variables accounting for 94.2% and 5.7% of the variation between genotypes. Reduced space plots of the two canonical variables distinguished among the genotypes based on 95% confidence ellipses (Figure 3). The analysis showed that the responses of the Red Pearl-INIA variety (RP-I) and the

14-4 genotype (14-4G) were indistinguishable from each other under the conditions of this study, while the South Pearl-INIA (SP-I) variety appeared to have a significantly different effect.

<Insert Figure 3 here>

In this study we demonstrated the effect of genotype and growing season on total polyphenol content, DPPH antioxidant activity and dry matter in murtilla fruit. There are reports showing that antioxidant activity in the fruits and leaves of blackberry, raspberry, and strawberry plants varies with cultivar and developmental stage (Wang and Lin, 2000). In one study, apple polyphenol content varied by at least 5.2-fold among 67 cultivars (Wojdylo *et al*., 2008). The effect of genotype on antioxidant activity has been shown in grapes (Lee and Talcott, 2004), plums (Gil *et al*., 2002), apples (Wolfe *et al*., 2003), citrus fruits (Bocco *et al*., 1998), guavas (Jiménez-Escrig *et al.,* 2001), nectarines and peaches (Gil *et al*., 2002). Bolling *et al*. (2010) found seasonal differences in total phenol and antioxidant constituents in almonds, reporting a 13% greater polyphenol content between different cultivars in 2005 compared to 2007. Because the qualitative and quantitative composition of phenolic compounds in fruits is unique to individual species and genotypes, it is possible to determine similarities and differences among standardized and confirmed genotypes and predict the response of new varieties to specific production and cultivation conditions (Sochor *et al*., 2010).

In this study, high correlations were found between climatic factors (rainfall and number of frosts) and the fruit characteristics evaluated (Table 1). Similar results were observed by Temime *et al*. (2006), who reported a positive linear relationship between precipitation and phenol content in Chétoui variety virgin olive oils. Moreover, the results of Janda *et al*. (2003) suggest that plants grown at low temperatures have higher levels of both antioxidants and antioxidant enzymes, and Xin and Browse (2000) suggest that low temperatures induce changes in plants, such as increased levels of antioxidants and reduced water content.

4. Conclusions

In summary, in this study, there was a statistically significant effect of growing season on polyphenol content, antioxidant activity and dry matter of murtilla fruits from three genotypes (the 14-4 genotype and the Red Pearl-INIA and South Pearl-INIA varieties). These characteristics trend to increase with higher rainfall and number of frosts. In addition, polyphenol content and dry matter were affected by genotype. A canonical discriminant analysis of seasonal differences in the Red Pearl-INIA, South Pearl-INIA and 14-4 genotypes showed that the South Pearl-INIA variety varied the most from the other genotypes. Additional studies that examine other biotic and abiotic factors that affect secondary metabolites are needed to fully explain the causes of year-to-year variation in polyphenolic compounds and antioxidant activity in murtilla fruit.

Acknowledgements

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References

Arancibia-Avila, P., Toledo, F., Werner, E., Suhaj, M., Leontowicz, H., Leontowicz, M., Martinez-Ayala, A., Paśko, P., Gorinstein, S. 2011. Partial characterization of a new kind of chilean murtilla-like berries. Food Research International 44, 2054-2062.

Atoui, A.K., Mansouri, A., Boskou, G., Kefalas, P. 2005. Tea and herbal infusions: their antioxidant activity and phenolic profile. Food Chemistry 89, 27-36.

Avello, M., Pastene, E. 2005. Actividad antioxidante de infusos de *Ugni molinae* Turcz ("Murtilla"). Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas 4, 33-39.

Bocco, A., Cuvelier,M-E., Richard, H., Berset, C. 1998. Antioxidant activity and phenolic composition of citrus peel and seed extracts. Journal of Agricultural and Food Chemistry 46, 2123-2129.

Bolling, B., Dolnikowski, G., Blumberg, J., Chen, C.Y. 2010. Polyphenol content and antioxidant activity of California almonds depend on cultivar and harvest year. Food Chemistry 122, 819-825.

Connor, A.M., Luby, J., Tong, C., Finn, C., Hancock, J. 2002. Genotype and environmental variation in antioxidant activity, total phenolic content and anthocyanins content among blueberry cultivars Journal of the American Society for Horticultural Science 127, 89-97.

de Abreu, I.N., Mazzafera, P. 2005. Effect of water and temperature stress on the content of active constituents of *Hypericum brasiliense* Choisy. Plant Physiology and Biochemistry 43, 241-248.

Gil, M.I., Tomás-Barberan, F.A., Hess-Pierce, B., Kader, A.A. 2002. Antioxidant capacities, phenolic compounds, carotenoids, and vitamin C content of nectarine, peach, and plum cultivars from California. Journal of Agricultural and Food Chemistry 50, 4976-4982.

Howard, L., Clark, J., Brownmiller, C. 2003. Antioxidant capacity and phenolic content in blueberries as affected by genotype and growing season. Journal of the Science of Food and Agriculture 83, 1238-1247.

Instituto de Salud Pública de Chile. 1998. Manual Métodos de Análisis Físico-Químicos de Alimentos, Aguas y Suelo. Andros Ltda., Santiago de Chile, Chile. 187p.

Janda, T., Szalai, G., Rios-Gonzalez, K., Veisz, O., Páldi, E. 2003. Comparative study of frost tolerance and antioxidant activity in cereals. Plant Science 164, 301-306.

Jiménez-Escrig, A., Rincón, M., Pulido, R., Saura-Calixto, F. 2001. Guava fruit (*Psidium guajava* L.) as a new source of antioxidant dietary fiber. Journal of Agricultural and Food Chemistry 49, 5489-5493.

Kevers, C., Pincemail, J., Tabart, J., Defraigne, J-O., Dommes, J. 2011. Influence of cultivar, harvest time, storage conditions and peeling on the antioxidant capacity and phenolic and ascorbic acid contents of apples and pears. Journal of Agricultural and Food Chemistry 59, 6165-6171.

Ksouri, R., Megdiche, W., Falleh, H., Trabelsi, N., Boulaaba, M., Smaoui, A., Abdelly, C. 2008. Influence of biological, environmental and technical factors on phenolic content and antioxidant activities of Tunisian halophytes Comptes Rendus Biologies 331, 865-873.

Lee, J-H., Talcott, S. 2004. Fruit maturity and juice extraction influences ellagic acid derivatives and other antioxidant polyphenolics in muscadine grapes. Journal of Agricultural and Food Chemistry 52, 361-366.

Mandal, S., Yadav, S., Yadav, S., Nema, R.K. 2009. Antioxidants: a review. Journal of Chemical and Pharmaceutical Research 1, 102-104.

Montenegro, G. 2002. Chile Nuestra Flora Útil. Universidad Católica de Chile, Chile. 267p.

Rubilar, M., Pinelo, M., Ihl, M., Scheuermann, E., Sineiro, J., Nuñez, M.J. 2006. Murta leaves (*Ugni molinae* Turcz) as a source of antioxidant polyphenols. Journal of Agricultural and Food Chemistry 54, 59-64.

Rubilar, M., Jara, C., Poo, Y., Acevedo, F., Gutierrez, C., Sineiro, J., Shene, C. 2011. Extracts of Maqui (*Aristotelia chilensis*) and Murta (*Ugni molinae* Turcz): sources of antioxidant compounds and α-Glucosidase/α-Amylase inhibitors. Journal Agriculture Food Chemistry 59, 1630-1637.

Ruiz, A., Hermosin-Gutierrez, I., Mardones,C., Vergara, C., Herlitz, E., Dorau, C., Winterhalter, P., von Baer, D. 2010. Polyphenols and antioxidant activity of calafate (*Berberis microphylla*) fruits and other native berries from southern Chile. Journal Agriculture Food Chemistry 58, 6081-6089.

Scheuermann, E. 2009. Estudio de componentes volátiles y capacidad antioxidante de frutos de murta (*Ugni molinae* Turcz) y evaluación de sus estabilidad en almacenamiento refrigerado, congelación y tratamiento térmico de pasteurización. Tesis para optar al grado académico de Doctor en Ciencias y Tecnología de Alimentos, Universidad de Santiago de Chile, Chile. 200p.

Scheuermann, E., Seguel, I., Montenegro, A., Bustos, R., Hormazábal, E., Quiroz, A. 2008. Evolution of aroma compounds of murtilla fruits (*Ugni molinae* Turcz) during storage. Journal of the Science of Food and Agriculture 88, 485-492.

Seguel, I., Peñaloza, E., Gaete, N., Montenegro, A., Torres, A. 2000. Colecta y caracterización molecular de germoplasma de murta (*Ugni molinae* Turcz.) en Chile. Agro Sur 28, 32-41.

Shene, C., Reyes, A., Villarroel, M., Sineiro, J., Pinelo, M., Rubilar, M. 2009. Plant location and extraction procedure strongly alter the antimicrobial activity of murta extracts. European Food Research and Technology 228, 467-475.

Sochor, J., Zitka, O., Skutkova, H., Pavlik, D., Babula, P., Krska, B., Horna, A., Adam, V., Provaznik, I., Kizek, R. 2010. Content of phenolic compounds and antioxidant capacity in fruits of apricot genotypes. Molecules 15, 6285-6305.

Sokal, R., Rohlf, J. 1995. Biometry. Third edition. W.H. Freeman and Co., New York, U.S.A. 887p.

Sun, Y-F., Liang, Z-S., Shan, C-J., Viernstein, H., Unger, F. 2011. Comprehensive evaluation of natural antioxidants and antioxidant potentials in *Ziziphus jujuba Mill. var. spinosa* (*Bunge*) *Hu ex H. F. Chou* fruits based on geographical origin by TOPSIS method. Food Chemistry 124, 1612-1619.

Taboada, E., Fisher, P., Jara, R., Zuñiga, E., Gidekel, M., Cabrera, J.C., Pereira, E., Gutierrez-Moraga, A., Villalonga, R., Cabrera, G. 2010. Isolation and characterization of pectic substances from murta (*Ugni molinae* Turcz) fruits. Food Chemistry 123, 669-678.

Temime, S.B., Wael, T., Bechir, B., Leila, A., Douja, D., Mokhtar, Z. 2006. Changes in olive oil quality of Chétoui variety according to origin of plantation. Journal of Food Lipids 13, 88-99.

van der Sluis, A.A., Dekker, M., De Jager, A., Jongen, W.M.F. 2001. Activity and concentration of polyphenolic antioxidants in Apple: Effect of cultivar, harvest year, and storage conditions. Journal of Agricultural and Food Chemistry 49, 3606-3613.

von Gadow, A., Jourbert, E., Hansmann, C.F. 1997. Comparison of the antioxidant activity of aspalathin with that of other plant phenols of rooibos tea (*Aspalathus linearis*). α-Tocopherol. BHT. and BHA. Journal of Agricultural and Food Chemistry 45, 632-638.

Wang, S.Y., Lin, H.S. 2000. Antioxidant activity in fruits and leaves of blackberry. raspberry. and strawberry varies with cultivar and developmental stage. Journal of Agricultural and Food Chemistry 48, 140-146.

Wolfe, K., Wu, X., Liu, R.H. 2003. Antioxidant activity of apples peels. Journal of Agricultural and Food Chemistry 51, 609-614.

Wojdylo, A., Oszmainski, J., Laskowski, P. 2008. Polyphenolic compounds and antioxidant activity of new and old apple varieties. Journal of Agricultural and Food Chemistry 56, 6520-6530.

Wong, S.P., Leong, L.P., William, J.H. 2006. Antioxidant activities of aqueous extracts of selected plants. Food Chemistry 99, 775-783.

Xin, Z., Browse, J. 2000. Cold Comfort farm: the acclimation of plants to freezing temperatures. Plant, Cell and Environment 23, 893-902.

Zar, J. 1999. Biostatistical Analysis. Fourth edition. Prentice Hall, U.S.A. 663p.

Table 1. Precipitation and number of frosts by growing season and their degree of correlation with the parameters measured in murtilla fruit.

	Growing Seasons							
	2005-2006	**2006-2007[a]**	**2007-2008**	**2008-2009**	**2010-2011**	**Total polyphenols [b]**	**DPPH AA [b]**	**Dry matter [b]**
Rainfall (mm)	1713	---	935	1014	1646	0.83 (39)	0.75 (36)	0.82 (36)
Number of frosts	9	---	8	2	7	0.62 (39)	0.56 (36)	0.75 (36)

[a] Missing data.

[b]All correlation coefficients (Spearman's rho) are significant at the $P<0.001$ level. Numbers in parentheses indicate sample size.

DPPH AA: DPPH antioxidant activity.

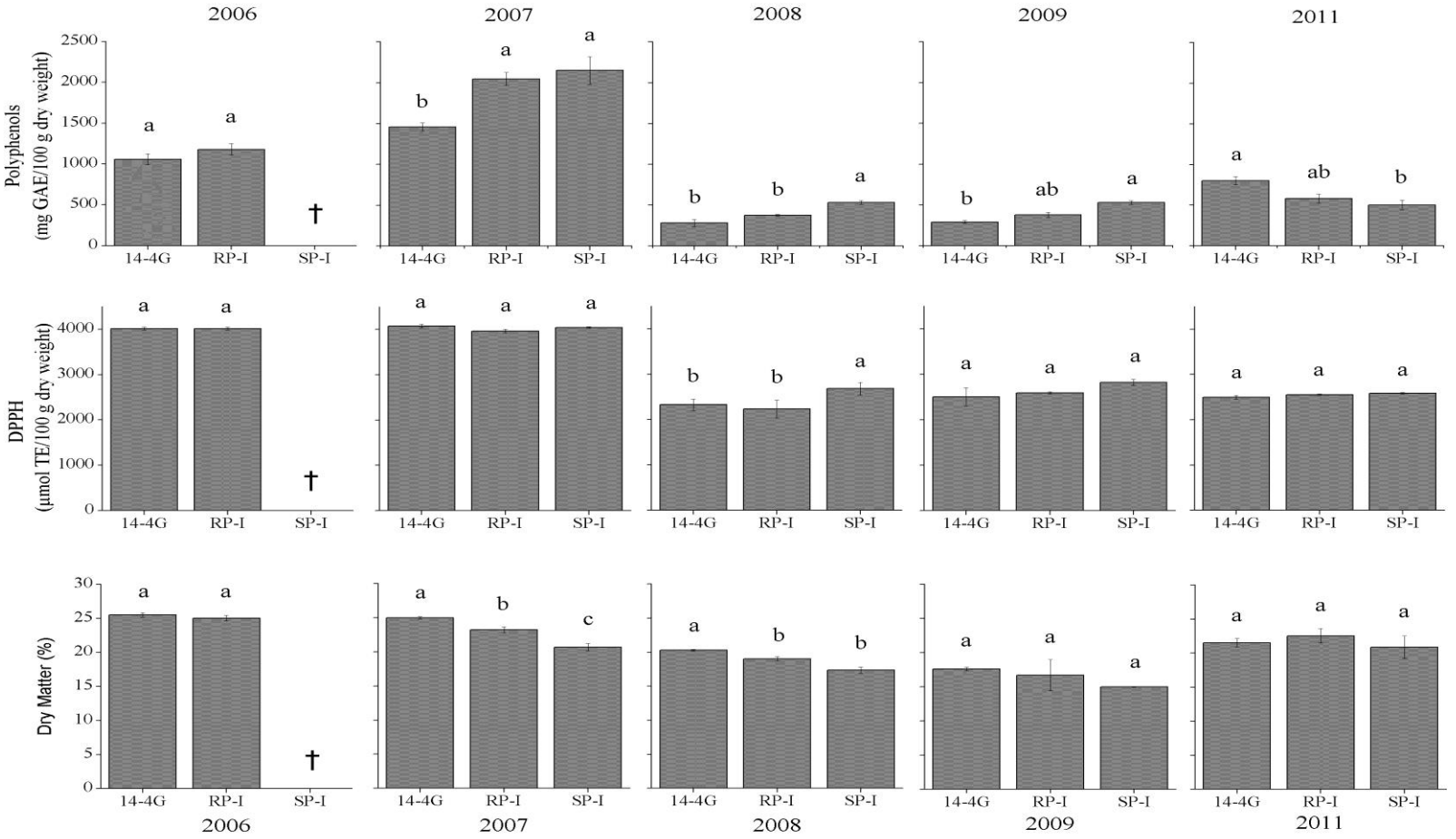
Table 2. *F*-values and significance of the main effects and interaction effect of the variables analyzed by multifactorial ANOVA.

	Genotype	Season	Genotype x Season
Total polyphenols content	11.17**	186.46**	7.83**
DPPH antioxidant activity	3.25ns	39.83**	0.67ns
Dry matter	10.77**	54.00**	2.31*

* $P<0.05$; ** $P<0.01$

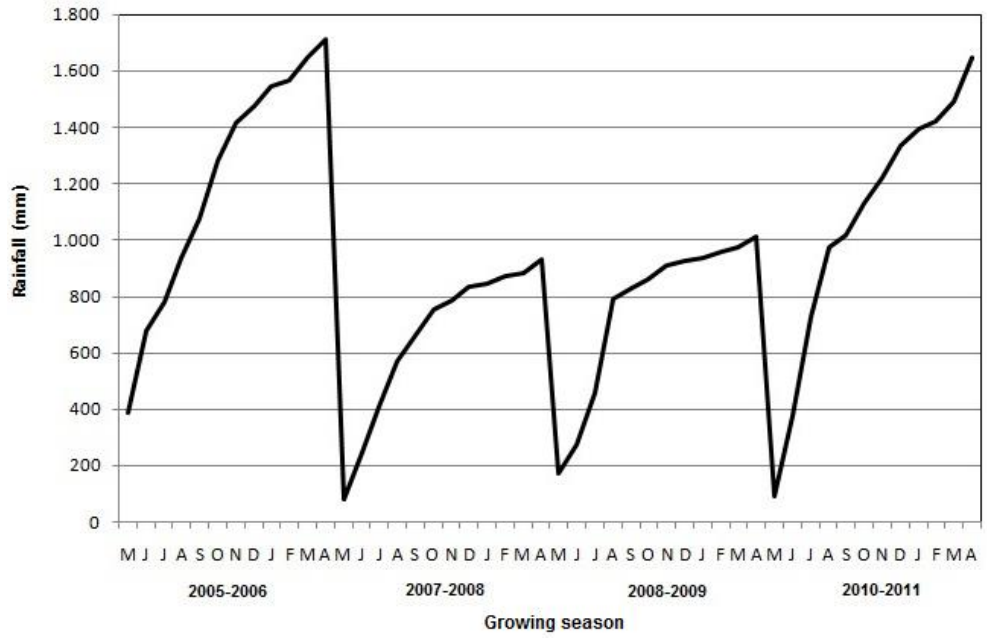
ns: non-significant difference.

Figure 1. Total polyphenol content (mg GAE/100 g dry weight), DPPH antioxidant capacity (μmolTE/100 g dry weight) and dry matter (%) in murtilla fruit for the genotype and the two varieties over the five study years.



14-4 G: Genotype 14-4; RP-I: Red Pearl-INIA variety; SP-I: South Pearl-INIA variety.
† Missed treatment.
Letters for each combination of parameter/year indicate significant differences according to the HSD-Tukey test (Student's t-test was used to compare treatments within the 2006 harvest year) (P<0.05).

(A)



(B)

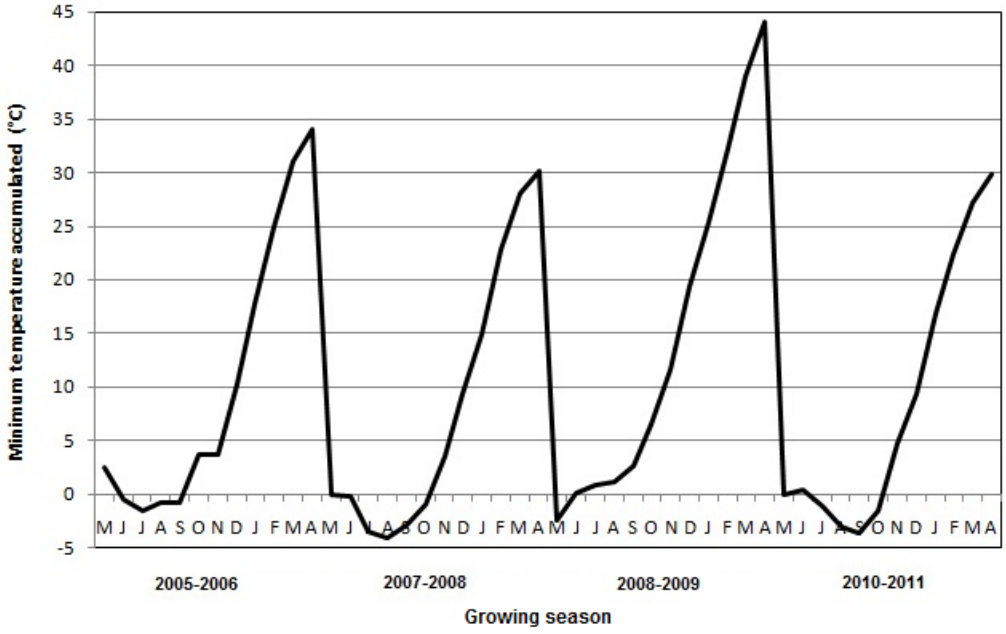


Figure 2. Changes in monthly total rainfall (A) and minimum accumulated temperature (B) for each growing season. Correlations with total polyphenol content, DPPH antioxidant activity and dry matter of the three murtilla fruit genotypes (the 14-4 genotype and the Red Pearl-INIA and South Pearl-INIA varieties) are shown in Table 1.

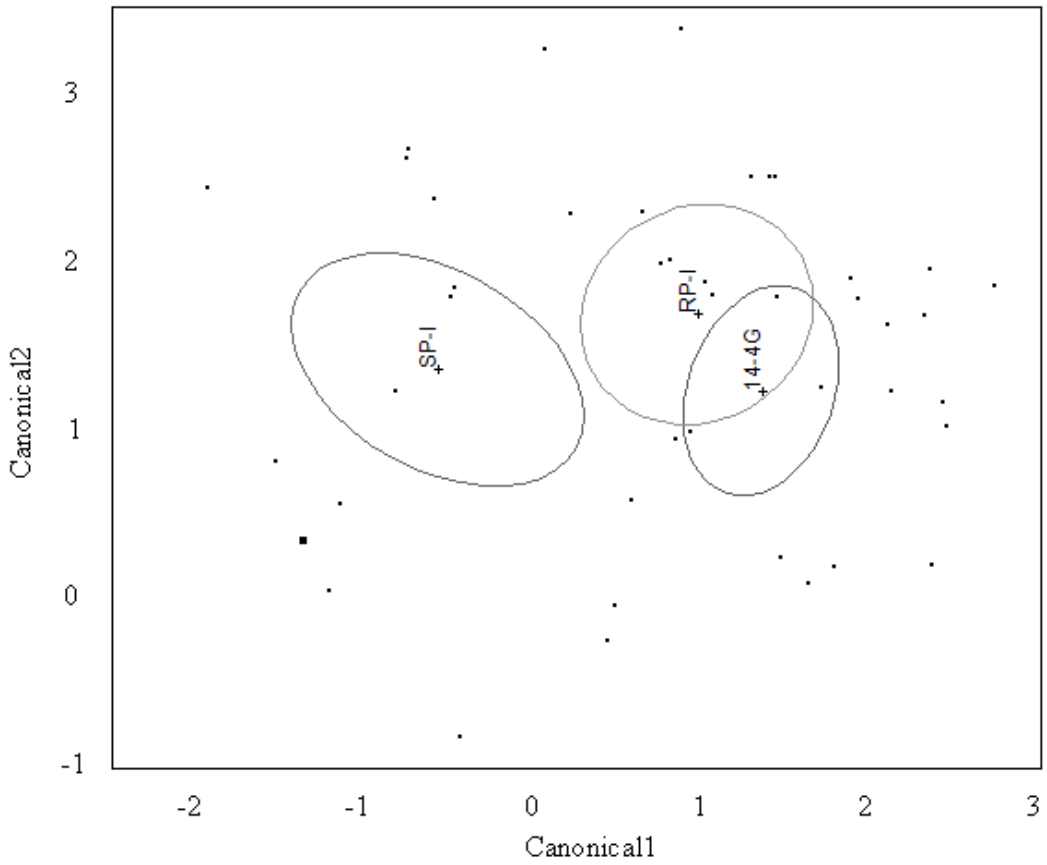


Figure 3. Canonical discriminant analysis of murtilla varieties based on polyphenol content, DPPH antioxidant activity and dry matter. Labels: SP-I: South Pearl-INIA variety, RP-I: Red Pearl-INIA variety, 14-4G: 14-4 genotype. Data represent the first and second canonical variables (Canonical 1 and Canonical 2) for the murtilla sample by variety (95% confidence ellipses).

Chapter 3

A comparative study of freeze drying and conventional drying techniques on the polyphenol compounds and antioxidant activity of the murtilla (Ugni molinae Turcz) fruits

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Author contributions: S A and ES designed experiments; S A, E S and I S. performed research; SA, AM and E S analyzed data; SA, AM, AQ and ES wrote the paper

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A comparative study of freeze drying and conventional drying techniques on the polyphenol compounds and antioxidant activity of the murtilla (*Ugni molinae* Turcz) fruits

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ABSTRACT

'Murtilla' (*Ugni molinae* Turcz) is a native chilean species belongs to Myrtaceae. It produces a small globose fruit with pleasant flavor and recognized antioxidant activity. Convective air and freeze drying are important methods for fruit preservation, but their effect on murtilla fruit antioxidant activity has not been studied simultaneously. The objective of this study was to determine and to compare the effect of convective hot-air drying at 65 ºC (HAD 65 ºC), at 80 ºC (HAD 80 ºC) and freeze drying (FD) on polyphenol compounds and antioxidant activity on fresh murtilla (*Ugni molinae* Turcz) fruits from Red Pearl-INIA variety. Total polyphenols, total anthocyanins, individual polyphenolic and anthocyanin compounds and antioxidant activity by DDPH (TE_{DPPH}) and ABTS (TE_{ABTS}) were analyzed for fresh and dried fruit. Freeze drying trends to retain higher contents of the total individual polyphenols and anthocyanins by HPLC than HAD 65 ºC and HAD 80 ºC. The application of all treatments showed a positive increased on TE_{DPPH} and TE_{ABTS} antioxidant activity of the dried murtilla respect to the fresh fruit. Freeze drying is considerate as the best method for retention of polyphenol compounds and antioxidant activity of the fresh murtilla fruit from Red Pearl-INIA variety.

Keywords: Dehydration methods, *Ugni molinae* Turcz, polyphenol and anthocyanin compounds, flavonoid, antioxidant capacity

INTRODUCTION

'Murtilla', 'mutilla' or 'murta' (*Ugni molinae* Turcz) is a native chilean species that produces a small berry fruit with recognized fruity, sweet and floral aroma and antioxidant capacity.[1, 2, 3, 4, 5] Polyphenolic compounds have been associated to antioxidant and antimicrobial activity for this native species.[2, 3, 4] Ruiz et al.[5] reported that the antioxidant activity of murtilla fruits was comparable with blueberry but it was lower than both maqui (*Aristotelia chilensis*) and calafate (*Berberis microphylla*) fruits; two anthocyanins, cyanidin-3-glucoside and peonidin-3-glucoside, and the flavan-3-ols catechin and epicatechin were identified in murtilla fruits by these authors. Whereas, Shene et al. [3] reported myricetin glucoside, quercetin glucoside, quercetin glucuronide and quercetin dirhamnoside in 50% water/ethanol extract from murtilla fruit.

Drying is a traditional method used for fruit preservation. Conventional drying using convective hot-air has been applied for different berries. López et al.[6] reported a decreased in the antioxidant activity and total polyphenol content range from 45 to 82% in dried blueberry respect to fresh fruit using different air temperature (50-90 ºC). Total polyphenol content, total anthocyanins and antioxidant activity of fresh blueberry decreased 28, 69 and 24% respectively when convective air drying at 90 °C for 10 hours was used.[7] Saskatoon berries (*Amelanchier alnifolia* Nutt.) and raspberries drying with convective hot-air at 75.0 and 76.6 ºC showed a decreasing in the antioxidant capacity from 82.6 to 74.7% respectively.[8, 9] Fresh strawberry from cultivar Kent and Elsanta showed a decreasing in total polyphenols (35.8 and 35.9%, respectively) and anthocyanins (73.0 and 63.6%, respectively) when drying with convective hot-air at 70 °C and velocity of 1 m s^{-1}.[10]

Freeze drying (FD) or liophylization is considered the best methods for the conservation of food[11] and is recommended for preserving polyphenols in berries fruit.[12, 13] de Torres et al.[12] determined that freeze drying method affected less than oven-drying the content of phenolic compounds, anthocyanins and flavonols, identified in fresh grape skin (Carménère and Cabernet Sauvignon varieties) and the respective dehydrated samples. Different drying methods applied on three strawberry cultivars showed that freeze drying maintained the highest content of soluble phenolic compounds (51-56%) compared with convective air (26-38%) and microwave (28-39%) drying. Also, the antioxidant activity was less affected when freeze drying was applied.[14] Drying cranberry fruit using freeze drying result in greater retention of anthocyanins than air drying, preserving high level of cranberry antioxidant activity. Difference in both anthocyanins content and antioxidant activity was attributed to drying temperature, presence of oxygen during the drying process and drying time.[15] Grace et al.[13] reported that the liophylization did not affect the concentrations of polyphenolic compounds identified in cranberry fruits. Reyes et al.[16] evaluated the application of the atmospheric freeze drying technique on murtilla fruit as an alternative of the vacuum freeze drying; under optimum operation conditions (5 ºC, fast freezing and infrared radiation - IR), a large percentage of the antioxidant capacity presented in fresh fruit remained in the dry fruit. Also, drying kinetics of halved murtilla fruit was determined using vacuum and atmospheric freeze drying with and without IR application, showing a considerably difference in drying rate between these methods when the moisture content was higher than X/Xo=0.1.[17]

No study has been performed to compare the effect of traditional convective hot-air drying method and freeze drying on the retention of murtilla fruits polyphenols and their

antioxidant capacity. These drying methods could be the ways to preserve polyphenolic bioactive components reported in the literature for fresh fruit and to contribute in the formulation of functional food for human diet. According to this, the objective of this study was to determine and to compare the effect of convective hot-air drying at 65 ºC (HAD 65 ºC), convective hot-air drying at 80 ºC (HAD 80 ºC) and freeze drying (FD) on polyphenol compounds and antioxidant activity of fresh murtilla (*Ugni molinae* Turcz) fruits from Red Pearl-INIA variety.

MATERIALS AND EXPERIMENTAL METHODS

Plant Material

Fresh murtilla (*Ugni molinae* Turcz) fruits collected from Red Pearl-INIA variety were provided by Agricultural Research Institute (INIA-Carillanca). Fresh harvested fruits were subjected to rapid freezing using liquid nitrogen (-196 ºC), and were subsequently stored at -20 ºC in a freezer (Electrolux FE-26). This fruit was considered as fresh fruit (Moisture of 79% weight base, w.b.), and was used in all the experiments.

Drying Methods

Murtilla fruits were dehydrated by: a) convective hot-air drying at 65 ºC (HAD 65 ºC) and 80 ºC (HAD 80 ºC), and b) by freeze drying (FD). The convective hot-air drying was carried out using an oven (Memmert model UFE 400) with convective flow air and temperature control. Murtilla fruit was drying at 65 °C (for 8 h) to a final moisture content of 17% (w.b.) and 80 ºC (for 4.5 h) to a final moisture content of 7% w.b. A Christ Alpha 1-2 Freeze at vacuum pressure of the 0.05 millibar equipped with a condenser temperature of the -56 ºC was used for freeze-drying. The drying time to

reduce moisture content to 5% w.b. was 48 h. The evaluation of the polyphenolic compounds and antioxidant activity was carried out with three replicates for each drying methods (HAD 65 ºC, HAD 80 ºC and FD).

Chemicals

Quercetin, quercetin 3-β-glucoside, kaempferol, myricetin, catechin, gallic acid, cyanidin-3-glucoside, peonidin-3-O-glucoside, methanol HPLC grade and sodium carbonate (Na_2CO_3) were purchased from Sigma-Aldrich Co. (St. Louis, USA). Acetonitrile HPLC grade, formic acid, 2, 2'azino-bis-(3 ethylbenzothiazoline-6-sulfonic acid) (ABTS), 6-hydroxy-2, 5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Folin-Ciocalteu reagent were purchased from Merck (Darmstadt, Germany).

Extraction Procedure

The extraction was carried out according to the methodology described by Scheuermann.[18] Fresh murtilla fruit (6 g) or dehydrated fruit (1 g) was ground in a mortar, transferred to a bottle and adding methanol (20 mL) previously conditioned at 30 ºC. The mixture was shaken in an incubator (GFL-3032, Germany) at 170 rpm at 30 °C for 20 min and filtrated (Whatman Nº 1) by vacuum. The methanolic murtilla fruit extract was protected from the light and used for determination of total polyphenols and anthocyanins content, and antioxidant activity. For anthocyanins determination by HPLC analysis, one portion of methanolic extract was centrifuged at 4,000 rpm for 10 min at room temperature, the supernatant was removed and freezing at -20 ºC. The individual polyphenols were determined directly from methanolic murtilla fruit extract.

However, before HPLC analysis the original extract or supernatant was concentrated to dryness using a rotary evaporator (Büchi R-210, Germany) at 30 ºC and 140 rpm and redissolved at 5 mL in methanol–formic acid (99:1, v/v).

Total Polyphenols Content

Total polyphenols content was determined using the Folin-Ciocalteau method according to Wong et al.[19] Methanolic murtilla fruit extract (40 µL) was mixed with distilled water (3.16 mL) and then added 200 µL of Folin-Ciocalteau reagent. After 5 min, 600 µL of 20% Na_2CO_3 were aggregated to the reaction mixture and maintained at 20 ºC for 120 min in darkness. The absorbance was measured at 765 nm, and the results were expressed as mg of gallic acid equivalent (GAE) per 100 gram of dry weight (d.w.).

Total Anthocyanins

The total anthocyanin content was determined by the pH difference method described by Giusty and Worlstad[20] and Sellappan et al.[21] In two test tubes, 1.8 mL of methanolic murtilla fruit extract was mixed with 0.2 mL of both potassium chloride buffer solution at pH 1.0 and sodium acetate buffer at pH 4.5. Then, absorbance at 510 and 700 nm respectively were measured for each extract-buffer mixtures using a spectrophotometer (Spectronic Genesys 5, Sweden). The total anthocyanin content was determined using the following equations:

$$\mathbf{A} = \left[(\mathbf{A_{510}} - \mathbf{A_{700}})_{\mathbf{pH=1.0}} - (\mathbf{A_{510}} - \mathbf{A_{700}})_{\mathbf{pH=4.5}}\right] \quad \text{Eq. 2.1}$$

$$\mathbf{C} = \frac{\mathbf{A \times PM \times FD}}{\boldsymbol{\varepsilon}} \times \mathbf{1000} \quad \text{Eq. 2.2}$$

Where: C = anthocyanin concentration (mg/L), A = anthocyanin absorbance; PM = molecular weight of the cyanidin-3-glucoside and ε = molar extinction coefficient of the cyanidin-3-glucoside. The result was expressed as mg of equivalent cyanidin-3-glucoside equivalent per 100 gram of dry weight (d.w.).

HPLC Analysis

The compounds used in both identification and quantification of polyphenols and anthocyanins by HPLC was based on those previously identified by Rubilar et al.[2], Shene et al.[3] and Ruiz et al.[5] for murtilla fruit and leaves. HPLC system Merck Hitachi (LaChrom, Tokyo, Japan) equipment consisted of an L-7100 pump and L-4250 UV-VIS detector. The column used was C-18 RP Inertsil ODS-3 (GL Sciences Inc, Tokyo, Japan) of 250 mm x 4.60 mm i.d, 5μm maintained at 25 ºC. The extract sample was filtered through a 0.45 μm filter and 20 μL was injected for analysis of polyphenol and anthocyanin compounds. The identification was confirmed by: a) comparison of their retention time with pure standards, and b) co-injection.

Polyphenols

HPLC analysis were performed using a linear gradient solvent system consisting of 1% formic acid (A) and acetonitrile (B) as follow: 0-2 min, 100%A; 2-15 min, 80%A/20%B; 15-20 min, 70%A/30%B; 20-30 min, 40%A/60%B; 30-35 min, 100%A; at a flow rate of 1 mL/min. The polyphenolic compounds were monitored at 280 nm.

Anthocyanins

HPLC analysis were performed using a linear gradient solvent system consisting of 1% formic acid (A) and acetonitrile (B) as follow: 0-2 min, 100%A; 3-9 min, 90%A/10%B; 10-11 min, 80%A/20%B; 11-15 min, 75%A/25%B; 16-30 min, 70%A/30%B, 31-32 min, 60%A/40%B; 33-35 min, 100%A; at a flow rate of 1 mL/min. The anthocyanins were monitored at 525 nm.

Antioxidant Activity by DPPH Assay

Antioxidant activity was determined by free radical scavenging, measuring the discoloration degree of methanolic DPPH (2,2-diphenyl-1-picrylhydrazyl) solution.[2, 22, 23] The initial absorbance of the DPPH solution ranged from 0.630 to 0.640. An aliquot (50 µl) of methanolic murtilla fruit extract was added to 1,950 µl of methanolic DPPH solution. The change in absorbance at 515 nm was measured at 30 min. Trolox (6-hydroxy-2, 5,7,8-tetramethylchroman-2-carboxylic acid) and a calibration curve was used to evaluate the DPPH scavenging ability of the methanolic murtilla fruit extract. The results were expressed as µmol Trolox equivalent per 100 gram on dry weight (d.w.).

Antioxidant Activity by ABTS Assay

Antioxidant activity by the inhibition of the free radical ABTS (2, 2'azino-bis-(3 ethilbenzothiazoline-6-sulfonic acid) was assessed according to Re et al.[24] The $ABTS^+$ radical cation is formed during the reaction of ABTS and potassium. Then, the resulting blue-green colored $ABTS^+$ solution reacts directly with the antioxidant present in the sample. The reaction was carried out with 1 mL of the working solution of $ABTS^+$

inside of semi microcuvettes, and was read at 734 nm (Reagent blank, E_1). Methanolic murtilla fruit extract (20 µL) and ethanol (20 µL) were mixed with working solution of $ABTS^+$. After 6 min, the absorbance was measured (Sample value, E_2) at 734 nm. The difference among E_1-E_2 (ΔE) was graphed versus the corresponding concentration. For this, a calibration curve was prepared using 20 µL of ethanol and 20 µL of Trolox solution that were added to the working solution of $ABTS^+$. The results were expressed as µmol Trolox equivalent per 100 gram on dry weight (d.w.).

Statistical Analysis

The data were analyzed using a one-way analysis of variance (ANOVA). Values of $p < 0.05$ were considered significant. The differences between means were determined using the Tukey's multiple comparison tests and t-Student tests. Results were expressed as mean of measurements and their corresponding standard deviations. The SPSS 20.0 software (SPSS Inc., Chicago, IL) was used to analyze the data.

RESULTS AND DISCUSSION

Total Polyphenols and Total Anthocyanins Content in Fresh and Dried Murtilla Fruit

Total polyphenols (TP) and total anthocyanins (TA) in dried fruit obtained by convective hot-air drying at 65 ºC (HAD 65 ºC) and 80 ºC (HAD 80 ºC) and freeze drying (FD) showed significant differences respect to the TP and TA recorded in fresh murtilla fruit (Table 1).

Freeze drying had a positive effect by increasing the TP and TA content in dried fruit respect to fresh murtilla. FD was a best method for retention of the total polyphenols

and anthocyanins in murtilla fruit from Red Pearl-INIA variety in comparison with HAD 65 ºC and HAD 80 ºC. Several authors have reported that freeze drying either increases slightly or retains a higher content of TP or TA among others drying methods applied to berries and others fruits. According to Kwok et al.[8], freeze drying method maintained higher concentrations of TP and TA in the Saskatoon berries (*Amelanchier alnifolia* Nutt.) than others methods such as air-drying, vacuum microwave drying and combination method with both air and microwave vacuum drying. Similar result for TP content in strawberry was observed when fresh fruit was submitted to convective air, microwave vacuum and freeze drying methods. The last one showed a tendency to maintain the initial values obtained from the fresh fruit of three different strawberry varieties.[14] Michalczyk et al.[25] reported a slightly decreased or increased on TP and TA content when freeze drying was applied to bilberry, strawberry and raspberry, founding greater losses on TP and TA content when air drying method was applied. On the contrary, Mejia-Meza et al.[9] reported that total polyphenol glucoside and aglycones compounds were retained in higher concentration in blueberries dried by vacuum microwave and combinated method (hot-air drying + vacuum microwave) than those fruit submitted to freeze drying.

The unusual high increasing of TP and TA content showed by murtilla fruit submitted to FD could be due to changes in cell structure and chemical degradation associate with freezing and subsequent sublimation. Aguilera et al.[26] observed an important increment in the dihydroxybenzoic acid content in dried lentil with dehydration process. These authors considers that this increase might be originated from the disruption of cell walls during processing or the breakdown of insoluble phenolic compounds because it could have led to better extractability of these compounds.

Decreasing in total polyphenols (37.3%) and total anthocyanins (38.5%) content were observed in the dried fruit at HAD 65 °C compared to the fresh fruit, but for murtilla fruit treated with HAD 80 °C, TP content increases (21.4%) while TA decreases (66.7%). Böhm et al.[14] observed a decreasing in total polyphenols ranging from 23.6 to 33.1% depending on the strawberry variety when convective hot-air drying (~60 °C for 220 min) was used. Also a decrease was observed for TP (35.9%) and TA (73 and 63.5%) in strawberry cultivars Kent and Elsanta when convective hot-air (70 °C and air velocity of 1 m/s) was applied for drying fruit.[10] Total phenolic and anthocyanin glucosides and aglycones were reduced in approximately 79% when raspberries were drying with air at 76.6 ºC for 4.5 h (Final moisture content ~5%).[8] Saskatoon berries (*Amelanchier alnifolia* Nutt. cv. Thiessen and Smoky) drying using convective air at 75 ºC showed a decreasing of TP and TA ranging from 56.5 to 65.5% and 83.4 to 87.6%, respectively.[8] Our result are consistent with those reported at the literature when berries are drying using hot-air, except for the TP content which increased with HAD 80 °C. Particularly, Serratosa et al.[27] related the raise in the concentrations of phenol compounds with: a) water evaporation, b) improving of extraction from skins, c) compounds hydrolysis and/or biosynthesis, when red grapes were drying using hot-air at 40 ºC and relative humidity of *ca*. 20%.

Arancibia-Avila et al.[28] reported higher total polyphenol content for no ripe (121.3 mg GAE/g d.w.) and ripe (61.2 mg GAE/g d.w.) wild fresh murtilla fruit than our result. This divergent result could be attributed to different extraction conditions, such as the ratio fruit-solvent, and the time. However, Reyes et al.[16] obtained similar content on total polyphenols (1,460 mg GAE/100 g d.w.) for fresh murtilla fruit ecotype 14-4 INIA Carillanca Genetic Bank. Total anthocyanins founded in fresh murtilla fruit from Red

Pearl-INIA variety (Table 1) is lower than the reported in fresh blueberries.[7, 29] Ruiz et al.[5] reported that wild murtilla fruit presented a lower total anthocyanins concentrations than founded in maqui, calafate and blueberries.

In summary, the effect of the convective air drying on total polyphenol and anthocyanin of murtilla fruits from Red Pearl-INIA variety is consistent with the behavior observed for other berries, showing a greater decreasing for higher temperature and apparently anthocyanins were more affected. Decreasing in polyphenols and anthocyanins content by heat treatments is associated with compounds degradation which generates smaller molecules or in the case of anthocyanins producing molecular rupture ring.[30, 31] On the other hand, high temperatures can affect the anthocyanins monomers stability resulting polymerization.[32] These changes may affect the reactivity of the aromatic rings, which will be reflected when the polyphenols are measured in the presence of Folin-Ciocalteu reagent.[33]

Individual Polyphenols and Anthocyanins in Fresh and Dried Murtilla Fruit by HPLC Analysis

The polyphenolic compounds identified in murtilla fresh fruit Red Pearl-INIA variety (Table 2) were gallic acid, catechin, quercetin glucoside, myricetin, quercetin and kaempferol which have been also reported by Rubilar et al.[2], Shene et al.[3] and Ruiz et al.[5] for murtilla fruit and leaves. Significant differences in these individual polyphenolic compounds were observed between fresh and dried fruit. The concentration found for these polyphenolic compounds in fresh fruit ranged from 0.5 to 157 mg/100 g d.w. The most abundant was quercetin 3-β-D-glucoside which represented 75.4% of the identified compounds (208.2 mg/100 g d.w.). A strong decline

on this glucoside was observed when the fresh fruit was submitted to HAD 65 ºC, HAD 80 ºC and FD. Among the identified polyphenols, catechin is the compound that is present in higher content in murtilla fruit dried by any of methods evaluated (For HAD 65 ºC represent 67.4%, HAD 80 ºC 87.3% and FD 88.6%). Respect to the fresh fruit content (35.1 mg/100 g d.w.), catechin concentration showed a no significant increased for dried fruit with HAD 80 ºC and FD method and no significant decreased with HAD 65 ºC (Table 2). The gallic acid content trend to increase in the dried fruit by the three methods compared with the fresh fruit concentration (0.51 mg/100 g d.w.). Myricetin and kaempferol concentrations decrease significantly in dried fruit respect to the fresh, following the same behavior as quercetin 3-β-D-glucoside. Degradation of these compounds and others polyphenols no identified could be result in tendency to increase the gallic acid and catechin concentration in dried fruit by HAD 65 ºC, HAD 80 ºC and FD respect to their fresh fruit content. Wojdyło et al.[10] reported an increasing in the levels of (+)-catechin in dried strawberry after different drying processes, especially for the Elsanta cultivar, which was attributed to the depolymerized effect of proantocyanidin in strawberry and their conversion into elementary units. Only for quercetin was established a significant increasing of the concentration in the dried fruit obtained by HAD 80 ºC.

HAD 65 ºC, HAD 80 ºC and FD produced a decreasing in the total content of individual polyphenolic compounds determined by HPLC (Table 2) respect to the fresh murtilla fruit (208.2 mg/100 g d.w.), but the FD method showed the higher retention (79.0 mg/100 g d.w.) of these components. This result agrees with those reported by Wojdyło et al.[10] and de Torres et al. [12] who also reported that among different drying methods,

freeze drying produced the greater retention of total polyphenols and flavonols determined by HPLC in dried strawberry and Carménère grape skins, respectively.

The content of individual anthocyanin compounds in murtilla (Red Pearl-INIA variety) fresh and dried fruit are shown in Table 3. Cyanidin-3-glucoside and peonidin-3-glucoside had been reported by Ruiz et al.[5] in wild murtilla fruit. The concentration of individual anthocyanins in dried fruit decreased respect to fresh murtilla. Between both anthocyanin compounds, cyanidin-3-glucoside was the most stable compound and it was retained in highest proportion (38.7%) in murtilla fruit dried using freeze drying. Also FD was the method that retained the highest proportion (21.2%) of peonidin-3-glucoside respect to the initial concentration present in the fresh fruit.

Similar to total individual polyphenols (Table 2), drying methods evaluated in this research produced a decreasing in total individual anthocyanins content determined by HPLC (Table 3) respect to the fresh murtilla fruit (0.799 mg/100 g d.w.). The retention of HPLC total individual anthocyanins trends to be better for FD (23.5%). According to this, studies in dried strawberry[10] and Carménère grape skins[12] showed that freeze drying provided the greater retention of total anthocyanins evaluate by HPLC respect to others drying methods. Dried cranberry obtained by freeze drying showed a higher content for three anthocyanins than fruit dried by air and vacuum microwave drying.[15]

The contents of both individual polyphenols (Table 2) and individual anthocyanins (Table 3) determined using HPLC analysis considered only the compounds previously reported for wild murtilla fruit.[3, 5] This would explain the higher values determined for total polyphenol using Folin-Ciocalteu method and total anthocyanins by pH differential method informed for fresh and dried murtilla fruit Red Pearl-INIA variety (Table 1). Discrepancy in total anthocyanin content obtained by HPLC and pH differential method

has previously been reported for blueberry powdered extract, juice concentrate and puree. Chakraborty et al.[34] reported that HPLC method results were 1.4 to 2.1 times greater than the results obtained from the pH differential method. In summary, our results are consistent with those reported in the literature showing that freeze drying is better than HAD 65 ºC or HAD 80 ºC to preserve polyphenolic compounds in murtilla fruits from Red Pearl-INIA variety.

Antioxidant Activity in Fresh and Dried Murtilla Fruit by DPPH and ABTS Assay

Antioxidant activity measure by DPPH and ABTS assay in fresh and dried murtilla fruit Red Pearl-INIA variety drying by different methods is presented in Table 4. Antioxidant activity (TE_{DPPH} and TE_{ABTS}) was expressed as μmol Trolox equivalent (TE) per 100 g d.w. for both assays. An increasing in TE_{DPPH} and TE_{ABTS} of the dried fruit respect to fresh murtilla was observed for HAD 65 ºC, HAD 80 ºC and FD. The HAD 65 ºC and FD dried murtilla fruits showed similar antioxidant activity level measured by TE_{DPPH} or TE_{ABTS}. For FD dried murtilla fruits, the TE_{DPPH} was higher than TE_{ABTS}. This fact could possibly be attributed to variations in the polyphenols composition that benefits one of the reactions used for determinate the antioxidant activity. This increase in TE_{DPPH} and TE_{ABTS} is not a typical behavior reported for other berries. Different drying methods evaluated for Saskatoon berry (*Amelanchier alnifolia* Nutt.), strawberry, raspberry and cranberry have showed a decreasing in antioxidant activity.[8, 9, 10, 14, 15] DPPH antioxidant capacity of decreased in 9.4-41.3% and 73.9% with application of freeze drying and convective air drying, respectively.[8] Wojdyło et al.[10] reported a significant decrease in DPPH antioxidant activity when applied freeze drying (12.8%) and convective air drying (37.9%) to strawberry fruit.

The increase in TE_{DPPH} and TE_{ABTS} antioxidant activity of the murtilla fruit after drying by HAD 65 ºC, HAD 80 ºC and FD represent a favorable effect. Reyes et al.[16] also reported an increasing in DPPH antioxidant activity (EC_{50}) and decreasing in polyphenols when murtilla fruits from ecotype 14-4 INIA Carillanca Genetic Bank were evaluated by vacuum and atmospheric freeze drying. The application of the infrared radiation drying to blueberry puree, juice and extract at 65 to 90 °C for 5 to 9 minutes produced in some cases a slightly increase in the total antioxidant capacity ranging from 6.1 to 13.2%, but the difference respect to liquid products was no statistical significant.[34] Also, the antioxidant efficiency ($1/EC_{50}$) was not significantly affected in the dried blueberry fruit compared to fresh fruit by using of the vacuum or atmospheric freeze-drying.[35]

The increase in antioxidant activity would be related to changes in the polyphenols composition and possibly associated to the increase in the aglycones compounds as gallic acid and catechin (Table 2). Mrkic et al.[36] reported higher antioxidant activity of the air dried (50-100 °C, 25-90 min) broccoli respect to the fresh vegetable. The authors explained this result by an increase of compounds release from the matrix, hydrolytic phenomena during drying and polyphenol oxidation resulting in oligomers with higher antioxidant activity than native compounds. Serratosa et al.[27] also reported an increased on hydrophilic and lipophilic antioxidant activities in red grapes during air drying process (40 °C and relative humidity of ca. 20%) which was related to the increase in phenol compounds.

CONCLUSIONS

The comparative application of the convective hot-air drying at 65 ºC (HAD 65 ºC) and 80 ºC (HAD 80 ºC) and freeze drying (FD) on fresh murtilla (*Ugni molinae* Turcz) fruits Red Pearl-INIA variety showed that FD had a positive effect by increasing the total polyphenols (TP) and total anthocyanins (TA) and it was the best for TP and TA retention among the three drying condition evaluated. HAD 65 ºC, HAD 80 ºC and FD produced a variation in the composition of the individual polyphenolic and anthocyanin compounds respect to the fresh murtilla fruit and freeze drying showed higher contents of the total individual polyphenols and anthocyanins determined by HPLC comparing to HAD 65 ºC and HAD 80 ºC. The application of the HAD 65 ºC, HAD 80 ºC and FD showed a positive effect on antioxidant activity because these drying methods increased the TE_{DPPH} and TE_{ABTS} of the dried murtilla fruits respect to the fresh fruit, which could be explained by variations in the composition of polyphenolic compounds like gallic acid and catechin. Finally, the freeze drying is the best method for retention of polyphenol compounds and antioxidant activity of the fresh murtilla fruit Red Pearl-INIA variety. This dried fruit could be considered as potential functional ingredient and open interesting perspectives for the development of new products in the food and nutraceuticals industry.

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REFERENCES

1. Scheuermann, E.; Seguel, I.; Montenegro, A.; Bustos, R.; Hormazábal, E.; Quiroz, A. Evolution of aroma compounds of murtilla fruits (*Ugni molinae* Turcz) along storage. *Journal of the Science of Food and Agriculture* **2008**, 88(3), 485-492.
2. Rubilar, M.; Pinelo, M.; Ihl, M.; Scheuermann, E.; Sineiro, J.; Nuñez, M.J. Murtilla leaves (*Ugni molinae* Turcz) as a source of antioxidant polyphenols. *Journal Agriculture Food Chemistry* **2006**, 54(1), 59–64.
3. Shene, C.; Reyes, A.; Villarroel, M.; Sineiro, J.; Pinelo, M.; Rubilar, M. Plant location and extraction procedure strongly alter the antimicrobial activity of murtilla extracts. *European Food Research and Technology* **2009**, 228(3), 467–475.
4. Rubilar, M.; Jara, C.; Poo, Y.; Acevedo, F.; Gutierrez, C.; Sineiro, J.; Shene, C. Extracts of maqui (*Aristotelia chilensis*) and murta (*Ugni molinae* Turcz.): sources of antioxidant compounds and α-glucosidase/α-amylase inhibitors. *Journal of Agricultural and Food Chemistry* **2011**, 59(5), 1630–1637.
5. Ruiz, A.; Hermosin-Gutierrez, I.; Mardones, C.; Vergara, C.; Herlitz, E.; Dorau, C.; Winterhalter, P.; von Baer, D. Polyphenols and antioxidant activity of calafate (*Berberis microphylla*) fruits and other native berries from southern Chile. *Journal of Agriculture Food Chemistry* **2010**, 58(10), 6081–6089.
6. López, J.; Uribe, E.; Vega-Gálvez, A.; Miranda, M.; Vergara, J.; González, E.; Di Scala, K. Effect of air temperature on drying kinetics, vitamin c, antioxidant activity, total phenolic content, non-enzymatic browning and firmness of blueberries variety O´Neil. *Food and Bioprocess Technology* **2010**, 3(5), 772–777.

7. Stojanovic, J.; Silva, J.L. Influence of osmotic concentration, continuous high frequency ultrasound and dehydration on antioxidants, color and chemical properties of rabbiteye blueberries. *Food Chemistry* **2007**, 101(3), 898–906.

8. Kwok, B.H.I.; Durance, T.; Kitts, D.D. Dehydration techniques affect phytochemical contents and free radical scavenging activities of Saskatoon berries (*Amerlachier alnifolia* Nutt). *Journal of Food Science* **2004**, 69(3), SNQ122–SNQ126.

9. Mejia-Meza, E.I.; Yañez, J.A.; Remsberg, C.M.; Takemoto, J.K.; Davies, N.M.; Rasco, B.; Clary, C. Effect of dehydration on raspberries: polyphenol and anthocyanin retention, antioxidant capacity, and antiadipogenic activity. *Journal of Food Science* **2010**, 75(1), 5–12.

10. Wojdyło, A.; Figiel, A.; Oszmianski, J. Effect of drying methods with the application of vacuum microwaves on the bioactive compounds, color, and antioxidant activity of strawberry fruits. *Journal of Agricultural and Food Chemistry* **2009**, 57(4), 1337–1343.

11. Ratti, C. Hot air and freeze-drying of high-value foods: a review. *Journal of Food Engineering* **2001**, 49(4), 311–319.

12. De Torres, C.; Díaz-Maroto, M.C.; Hermosín-Gutiérrez, I.; Pérez-Coello, M.S. Effect of freeze-drying and oven-drying on volatiles and phenolic compositions of grape skin. *Analytica Chimica Acta* **2010**, 660(1-2), 177–182.

13. Grace, M.H.; Massey, A.R.; Mbeunkui, F.; Yousef, G.G.; Lila, M.A. Comparison of health-relevant flavonoids in commonly consumed cranberry products. *Journal of Food Science* **2012**, 77(8), H176–H183.

14. Böhm, V.; Kühnert, S.; Rohm, H.; Scholze, G. Improving the nutritional quality of microwave-vacuum dried strawberries: a preliminary study. *Food Science and Technology International* **2006**, 12(1), 67–75.

15. Leusink, G. J.; Kitts, D.; Yaghmaee, P.; Durance, T. Retention of antioxidant capacity of vacuum microwave dried cranberry. *Journal of Food Science* **2010**, 75(3), 311–316.

16. Reyes, A.; Bustos, R.; Vásquez, M.B.; Scheuermann, E. Optimization of "murtilla" berry drying in an atmospheric freeze dryer. *Chemical Engineering Transactions* **2009**, 18, 183–188.

17. Reyes, A.; Bubnovich, V.; Bustos, R.; Vasquez, M.; Vega, R.; Scheuermann, E. Comparative study of different process conditions of freeze drying of 'murtilla' berry. *Drying Technology* **2010**, 28(12), 1416–1425.

18. Scheuermann, E. Estudio de Componentes Volátiles y Capacidad Antioxidante de Frutos de Murta (*Ugni molinae* Turcz) y Evaluación de su Estabilidad en Almacenamiento Refrigerado, Congelación y Tratamiento Térmico de Pasteurización. D.S. Thesis, Unversidad de Santiago de Chile, Chile, 2009.

19. Wong, S.P.; Leong, L.P.; William Koh, J.H. Antioxidant activities of aqueous extracts of selected plants. *Food Chemistry* **2006**, 99(4), 775–783.

20. Giusti, M.M.; Wrolstad, R.E. Characterization and measurement of anthocyanins by UV-visible spectroscopy, Unit F1.2. In *Current Protocols in Food Analytical Chemistry*; Wrolstad, R.E., Schwartz, S.J., Eds.; John Wiley & Sons, Inc.: New York, 2001; F1.2.1–F1.2.13.

21. Sellappan, S.; Akoh, C.C.; Krewer, G. Phenolic compounds and antioxidant capacity of Georgia-grown blueberries and blackberries. *Journal Agriculture Food Chemistry* **2002**, 50(8), 2432–2438.

22. Von Gadow, A.; Jourbert, E.; Hansmann, C.F. Comparison of the antioxidant activity of aspalathin with that of other plant phenols of rooibos tea (*Aspalathus linearis*), α-tocopherol, BHT, and BHA. *Journal Agriculture Food Chemistry* **1997**, 45(3), 632–638.

23. Atoui, A.K.; Mansouri, A.; Boskou, G.; Kefalas, P. Tea and herbal infusions: their antioxidant activity and phenolic profile. *Food Chemistry* **2005**, 89(1), 27–36

24. Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology & Medicine* **1999**, 26(9-10), 1231–1237.

25. Michalczyk, M.; Macura, R.; Matuszak, I. The effect of air-drying, freeze-drying and storage on the quality and antioxidant activity of some selected berries. *Journal of Food Processing and Preservation* **2009**, 33(1), 11–21.

26. Aguilera, Y.; Dueñas, M.; Estrella, I.; Hernández, T.; Benítez, V.; Esteban, R.M.; Martín-Cabrejas, M.A. Evaluation of phenolic profile and antioxidant properties of Pardina lentil as affected by industrial dehydration. *Journal of Agricultural and Food Chemistry* **2010**, 58(18), 10101–10108.

27. Serratosa M.; Marquez, A.; Lopez-Toledano, A.; Medina, M.; Merida J. Changes in hydrophilic and lipophilic antioxidant activity in relation to their phenolic composition during the chamber drying of red grapes at a controlled temperature). *Journal of Agricultural and Food Chemistry* **2011**, 59(5), 1882–1892.

28. Arancibia-Avila, P.; Toledo, F.; Werner, E.; Suhaj, M.; Leontowicz, H.; Leontowicz, M.; Martinez-Ayala, A.L.; Pasko, P.; Gorinstein, S. Partial characterization of a new kind of Chilean Murtilla-like berries. *Food Research International* **2011**, 44(7), 2054–2062.

29. Lohachoompol, V.; Srzednicki, G.; Craske, J. The change of total anthocyanins in blueberries and their antioxidant effect after drying and freezing. *Journal of Biomedicine and Biotechnology* **2004**, 2004(5), 248–252.

30. Furtado, P.; Figueiredo, P.; Chaves das Neves, H.; Pina, F. Photochemical and thermal degradation of anthocyanidins. *Journal of Photochemistry and Photobiology A: Chemistry* **1993**, 75(2), 113–118.

31. Gil-Izquierdo, A.; Gil, M.I.; Ferreres, F. Effect of processing techniques at industrial scale on orange juice antioxidant and beneficial health compounds. *Journal of. Agriculture and Food Chemistry* **2002**, 50(18), 5107–5114.

32. Markakis, P. Stability of anthocyanins in foods. In *Anthocyanins as Food Colors*; Markakis, P., Ed.; Academic Press: New York, 1982, 163–180.

33. Vijayakumari, K.; Siddhuraju, P.; Pugalenthi, M.; Janardhanan, K. Effect of soacking and heat processing on the levels of antinutrients and digestible proteins in seeds of *Vigna aconitifolia* and *Vigna sinensis*. *Food Chemistry* **1998**, 63(2), 259–264.

34. Chakraborty, M.; Savarese, M.; Harbertson, E.; Harbertson, J.; Ringer, K.L. Effect of the novel radiant zone drying method on anthocyanins and phenolic of three blueberry liquids. *Journal of Agricultural and Food Chemistry* **2010**, 58(1), 324–330.

35. Reyes, A.; Evseev, A.; Mahn, A.; Bubnovich, V.; Bustos, R.; Scheuermann, E. Effect of operating conditions in freeze-drying on the nutritional properties of blueberries. *International Journal of Food Sciences and Nutrition* **2011**, 62(3), 303–306.

36. Mrkic, V.; Cocci, E.; Dalla Rosa, M.; Sacchetti, G. Effect of drying conditions on bioactive compounds and antioxidant activity of broccoli (Brassica *Oleracea* L.). *Journal of the Science of Food and Agriculture* **2006**, 86(10), 1559–1566.

TABLE 1

Total polyphenol (TP) and total anthocyanins (TA) content in fresh and dried murtilla fruit Red Pearl-INIA variety

Treatments	**TP[1]**	**TA[2]**
Fresh	1,149.2^{c} ± 30.1	7.8^{b} ± 0.8
HAD 65 ºC	720.9^{d} ± 41.1	4.8b^{c} ± 0.7
HAD 80 ºC	1,462.5^{b} ± 113.5	2.6^{c} ± 1.1
FD	2,192.4^{a} ± 181.9	13.4^{a} ± 2.0

[1]Total polyphenol as mg gallic acid equivalent per 100 g d.w.

[2]Total anthocyanins as mg cyaniding-3-glucoside equivalent per 100 g d.w.

Data are expressed as means ± standard deviation of the three replicates. Values with different letters within columns differ significantly, tested by ANOVA and Tukey's HDS, $P \leq 0.05$. Abbreviations: hot-air drying at 65 ºC (HAD 65 ºC) and 80 ºC (HAD 80 ºC) and freeze drying (FD).

TABLE 2

Individual polyphenol compounds content (mg/100 g d.w.) in fresh and dried murtilla fruit Red Pearl-INIA variety

Polyphenol compound	**Fresh**	**HAD 65 ºC**	**HAD 80 ºC**	**FD**
Gallic acid	$0.51^{b} \pm 0.07$	$2.16^{ab} \pm 0.15$	$1.97^{ab} \pm 0.50$	$6.32^{a} \pm 3.37$
Catechin	$35.11^{ab} \pm 0.41$	$18.78^{b} \pm 3.24$	$43.57^{ab} \pm 9.65$	$69.91^{a} \pm 30.99$
Quercetin 3-β-D-glucoside	$157.17^{a} \pm 26.42$	$0.94^{b} \pm 0.19$	$1.73^{b} \pm 0.56$	$1.41^{b} \pm 0.52$
Myricetin	$14.07^{a} \pm 5.42$	$1.05^{b} \pm 0.12$	$1.11^{b} \pm 0.15$	$1.03^{b} \pm 0.21$
Quercetin	$0.66^{b} \pm 0.26$	$0.86^{ab} \pm 0.18$	$1.41^{a} \pm 0.44$	$0.24^{b} \pm 0.17$
Kaempferol	$0.84^{a} \pm 0.05$	$0.09^{bc} \pm 0.01$	$0.11^{b} \pm 0.03$	$0.02^{c} \pm 0.01$
Total individual polyphenols by HPLC	208.20 ± 27.90	23.97 ± 3.43	49.91 ± 10.39	79.02 ± 30.58

Data are expressed as means ± standard deviation of the three replicates. Values with different letters within rows differ significantly, tested by ANOVA and Tukey's HDS, $P \leq 0.05$. Abbreviations: hot-air drying at 65 ºC (HAD 65 ºC) and 80 ºC (HAD 80 ºC) and freeze drying (FD).

TABLE 3

Individual anthocyanin compounds content (mg /100 g d.w.) in fresh and dried murtilla fruit Red Pearl-INIA variety

Anthocyanin compound	**Fresh**	**HAD 65 ºC**	**HAD 80 ºC**	**FD**
Cyanidin-3-glucoside	$0.106^{a} \pm 0.006$	$0.012^{b} \pm 0.002$	$0.017^{b} \pm 0.003$	$0.041^{b} \pm 0.031$
Peonidin-3-glucoside	$0.692^{a} \pm 0.118$	$0.078^{b} \pm 0.035$	$0.071^{b} \pm 0.024$	$0.147^{b} \pm 0.095$
Total individual anthocyanins by HPLC	0.799 ± 0.115	0.091 ± 0.033	0.089 ± 0.027	0.188 ± 0.030

Data are expressed as means ± standard deviation of the three replicates. Values with different letters within rows differ significantly, tested by ANOVA and Tukey's HDS, $P \leq 0.05$. Abbreviations: hot-air drying at 65 ºC (HAD 65 ºC) and 80 ºC (HAD 80 ºC) and freeze drying (FD).

TABLE 4

Antioxidant activity (μmol TE/100 g d.w.) measure by DPPH and ABTS in fresh and dried murtilla fruit Red Pearl-INIA variety

Treatments	**TE_{DPPH}**	**TE_{ABTS}**
Fresh	$2{,}111.1^{cA}$ ± 200.6	$2{,}247.8^{cA}$ ± 7.8
HAD 65 ºC	$3{,}567.4^{aA}$ ± 47.0	$3{,}397.2^{aA}$ ± 20.1
HAD 80 ºC	$2{,}945.4^{bA}$ ± 249.1	$2{,}664.8^{bA}$ ± 293.5
FD	$3{,}677.6^{aA}$ ± 27.0	$3{,}162.9^{aB}$ ± 16.3

TE_{DPPH}: antioxidant activity measure by DPPH assay and expressed as μmol Trolox equivalent (TE) per 100 g d.w.

TE_{ABTS}: antioxidant activity measure by ABTS assay and expressed as μmol Trolox equivalent (TE) per 100 g d.w.

Data are expressed as means ± standard deviation of the three replicates. Values with different lowercase letters within columns differ significantly, tested by ANOVA and Tukey's HDS, $P \leq 0.05$ and values with different capital letters within rows differ significantly by t-Student test. Abbreviations: hot-air drying at 65 ºC (HAD 65 ºC) and 80 ºC (HAD 80 ºC) and freeze drying (FD).

Chapter 4

Determination of polyphenols and anthocyanins in fresh and dried murtilla (*Ugni molinae* Turcz) fruit Red Pearl cultivar by HPLC-ESI MS/MS

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Manuscript in preparation

Determination of polyphenols and anthocyanins in fresh and dried murtilla (*Ugni molinae* Turcz) fruit Red Pearl cultivar by HPLC-ESI MS/MS

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ABSTRACT

'Murtilla', 'mutilla' or 'murta' (*Ugni molinae* Turcz) is a native Chilean species that produces a small berry fruit, which polyphenolic content have been associated to the health benefits reported for this native species.

Qualitative analysis by high performance liquid chromatography (HPLC) coupled with ESI-MS/MS detection method was performance to separate and identified individual compounds in metanolic extract of fresh and dehydrated murtilla fruit Red Pearl variety prepared by freeze drying (FD), hot-air drying at 65ºC (HAD 65ºC), and hot-air drying at 80ºC (HAD 80ºC).

A good separation of different analytes was obtained and satisfactory peaks were achieved by gradient elution on a RP C18 column. Electrospray ionization mass spectrometry in the negative mode was used to identify individual phenolic compounds and in positive mode to identify anthocyanin compounds present in fresh and dried murtilla fruit (*Ugni molinae* Turcz) Red Pearl variety.

Various flavonols aglycones, flavan-3-ol and flavonols-glycosides were detected in fresh murtilla fruit and the method developed was useful for monitoring the changes of polyphenol profile in the processed fruit by three different dehydration techniques.

Keywords: Flavonoid, phenolic compounds, anthocyanins, dehydrated fruit, HPLC-ESI-MSMS.

INTRODUCTION

'Murtilla', 'mutilla' or 'murta' (*Ugni molinae* Turcz) is a native Chilean species that produces a small berry fruit, which both organoleptic and antioxidant properties have been recently investigated. Polyphenolic compounds have been associated to the health benefits reported for this native species (Aguirre 2006; Rubilar et al. 2006; Delporte et al., 2007; Shene et al., 2009; Rubilar et al., 2010). Previous research has reported that this berry has high levels of flavonoids and phenolic compounds in leaves and fruit metanolic extracts (Ruiz et al., 2010). The main types of metabolites found in murtilla leaves are represented by triterpenoids and flavonoids, and they have been reported as possibly responsible of the medicinal properties of murtilla leaves (Aguirre et al., 2006; Rubilar et al., 2006). Flavonoids glycosides and triterpenoids have been isolated from the leaves, being associated with the observed beneficial effects (Delporte et al., 2007). Other works have reported the presence of flavonoids glycoside of quercetin, myricetin and kaempferol in leaves of *U. molinae* (Rubilar et al., 2006). As a result of the characterization of the extracts of murtilla leaves, polyphenolic compounds have been detected in aqueous, ethanol and methanol extracts in which gallic acid was identified in aqueous extracts; epicatechin derivatives and kaempferol have been detected in alcoholic extracts (Rubilar et al., 2010). Myricetin ramnoside, myricetin diramnoside, myricetin glycoside and quercetin glycoside were found in all the extracts, although in low quantity in the aqueous extract (Rubilar et al., 2006; Shene et al., 2009). Ruiz et al. (2010) identified the anthocyanins cyanidin-3-glucoside and peonidin-3-glucoside and the flavan-3-ols catechin and epicatechin. in murtilla fruits

The free radical scavenging activity shown by the methanolic extract obtained from fresh fruit from this plant has been demonstrated in our previous study (Alfaro et al., 2013a), and a quantitative determination of the flavonoid content was performed for

corroborating the biological activity of these natural compounds with both anti-radical (antioxidative) activity (Alfaro et al., 2013b). Moreover, we have studied the effects of different dehydration technologies on the antioxidant activity and the concentration of the main polyphenols and anthocyanins components identified in murtilla fruits (Alfaro et al., 2013b).The results showed that the application of all the dried treatments showed a positive increased on the antioxidant activity of the dried murtilla respect to the fresh fruit. The comparative application of the convective hot-air drying at 65 ºC (HAD 65 ºC) and 80 ºC (HAD 80 ºC) and freeze drying (FD) on fresh murtilla showed that FD had a the best positive effect by increasing both total polyphenols (TP) and anthocyanins (TA) (Alfaro et al., 2013b). However, the concentration of both individual polyphenols and anthocyanins decreased when fruits were dried by means of the methodologies above mentioned (Alfaro et al., 2013b). These results suggested that the increase of the antioxidant activity could be associated to the presence of other polyphenolic and/or anthocyanins compounds in the methanolic extract, or the generation of metabolites produced by enzymatic degradation.

On the other hand, the quality control of standardized murtilla fruit extract is essential in regarding therapeutical properties, efficacy and applicability of extract. The obtaining of a standardized extract implicates the resolution and identification of the most of the components present in the compounds complex mixture responsible of the biological studied activity. In that sense, high-performance liquid chromatography (HPLC) combined with mass spectrometry detection (HPLC-ESI-MS/MS) is a valuable technique for the resolution of a complex mixture of compounds. For this reason, the main objective of this study was to characterize polyphenolic components in murtilla

fruits treated with freeze drying (FD), hot-air drying at 65ºC (HAD 65ºC)and hot-air drying at 80ºC (HAD 80ºC).

MATERIALS AND METHODS

Plant material

Fresh murtilla fruits *(Ugni molinae* Turcz) Red Pearl variety was provided by INIA Carillanca and they were used in all the experiments. Fresh harvested fruits were subjected to rapid freezing using liquid nitrogen (-196 ºC), and were subsequently stored at -20 ºC in an Electrolux FE-26 freezer. The dehydrated murtilla fruit were obtained by freeze drying (FD), hot-air drying at 65ºC (HAD 65ºC), and a hot-air drying at 80ºC (HAD 80ºC) techniques according to the methodology described by Mejias Meza et al. (2010) with some modifications.

Chemicals

Quercetin, quercetin 3-β-glucoside, kaempferol, myricetin, catechin, gallic acid, cyanidin-3-glucoside and peonidin-3-O-glucoside were purchased from Sigma Chemicals Co. USA and all solvent used for extraction were analytical grade. HPLC grade methanol was purchased from Sigma Aldrich, USA. HPLC grade acetonitrile and formic acid was purchased from Merck, Darmstadt, Germany.

Extraction procedure

The extraction was carried out according to the methodology described by Scheuermann (2009). Six g of fresh fruit or 1g of dehydrated murtilla fruit was ground in a mortar, and transferred to a bottle, adding 20 mL of methanol previously conditioned at 30 º C. Afterwards, the mixture was taken into an oven (GFL-3032, Germany), keeping it under agitation (170 rpm) for 20 minutes at 30 ºC. After this extraction stage, the mixture was

separated by vacuum filtration, using Whatman Nº 1 filter paper. The filtrates were stored, and protected from light and stored for determination of polyphenolic compounds. Then, a portion of the extract was centrifuged at 4000 rpm for 10 min at room temperature and kept under freezing at -20ºC for the determination of anthocyanins. The filtrates (20 ml) were concentrated to dryness using a rotary evaporator Buchi R-210 at 30ºC and 140 rpm and redissolved in MeOH–formic acid (99:1, v/v) and afforded at 5 ml.

HPLC MS/MS analysis

The chromatographic separation was carried out using a column RP-C18 Inertsil ODS-3 (2.1 x 150 mm, 3mm), injecting 10 µl at 0.2 mL/min and 35ºC. Standards and sample extracts were dissolved in MeOH–formic acid (99:1) and submitted to LC–MS. The chromatographic separation was performed using a linear gradient solvent system consisting of 0.1% formic acid (A) and Methanol (B). The initial composition of the mobile phase was 100 % A. The composition changed to 95 % A within 5 min, 5 % A within 20 min and maintained by 5 min, 95% A within 30 min. Each standard compound or separated peak was injected with an electro spray ionization (ESI) source into the mass spectrometer (LC-MS MS Shimadzu Prominence coupled at Mass spectrometer Applied Biosystems/MDS Sciex 3200 Qtrap, Columbia, USA). The capillary temperature was 450 ºC, and the capillary voltage was 3.5 kv. For anthocyanins determination data were collected as positive-ion spectra respectively. In a first step enhanced mass scan (EMS) and enhanced product ion (EPI) over an m/z 100-1500 range were used for obtaining a finger print of the compounds under study. Afterward, multiple reaction monitoring (MRM) method was applied in positive

polarity mode for sample analysis. The determination of polyphenolics compounds, such phenolic acids and flavonoids was similar to those carried out for anthocyanins, but the analysis performed in negative polarity. Table1 shows the transition analyzed by each compounds in negative polarity and the instrumental conditions for the MRM experiment. For the case of anthocyanins, two transitions for each compound analyzed. Table 2 shows the transitions analyzed by anthocyanin compound in positive polarity and the instrumental conditions for the MRM experiment. Parent and fragment ions of polyphenolic and anthocyanins compounds are shown in tables 3 and 4. A dwell time of 50 ms was used between each transition. Declustering potential (DP), entrance potential (EP), collision energy (CE) and collision exit potential (CXP) values are attached in the respective tables. As a positive identification parameter the retention times, UV spectra and transitions precursor ion/ion fragment were taken into account.

RESULTS AND DISCUSSION

In this study, polyphenolic compounds present in fresh fruit of murtilla (*U. molinae*) and the respective dehydrated samples obtained from three different methods were determined by HPLC–DAD and HPLC-MS/MS. All compounds were positively identified by comparison of retention time and UV spectrum with authentic standards. Afterwards, HPLC-MS/MS analysis was developed for ensuring the UV-identification and for exploring the presence of other phenolic compounds. Table 3 shows the spectral data and retention time of each polyphenol detected in fresh fruit and dehydrated fruit obtained from HPLC-MS analysis. Polyphenols identified in the different samples of murtilla investigated in this work can be classified as: a) hydrocynnamic acids and their derivatives, such as caffeic, *p*-coumaric, ferulic acids and chlorogenic acid; b) benzoic acids (gallic and vanillic acids); c) polyphenols, such as catechol; d) flavones (luteonin); e) flavonols and sugar conjugates, such as quercetin, kaempferol, myricetin, quercitrin and quercetin-3-β-D-glucoside; f) flavan-3-ols and sugar conjugates, such as epicatechin, epicatechin gallate and gallocatechin gallate; and g) anthocyanins: cyaniding-3-glucoside and peonidin-3-glucoside (Table 3 and 4). In addition of these 18 compounds, 39 derivative polyphenolic compounds were identified in at least one of the treatments (Table 3), and one derivative anthocyanin compound was identified in all the samples (Table 4). The assignments of derivative compound were based on the presence of the parent ion and the respective transitions, with different retention time related to the standard. For instance, the retention time of the gallic acid standard was 2.39 min, and it is characterized by its precursor ion m/z 169 (M-H) and the fragments ions (m/z 125 and 107), but HPLC-MS analysis revealed the presence of one chromatographic peak (28.40 min) with the parent and fragments ion. These derivate or sugar conjugates

were not considered in this analysis. Compounds were characterized by MS spectra showing their expected parent ion and MS/MS spectra showing one or two fragment ions. In the case of gallic acid $[M-H-125]^-$ is in agreement with a decarboxylation (Chua et al., 2011; Del Rio et al., 2004), the fragment ion m/z 107 could be explained by a deprotonation from the molecular ion, a subsequent decarboxylation and the loss of a hydroxyl group. Catechol is characterized for dehydration from the $(M-H)^-$ ion resulting in m/z 91, and the fragment m/z 65 is generated for a loss of acetylene from the fragment ion m/z 91. Epicatechin was identified on the basis of retention time and MS/MS fragmentation pattern [M-H]- at m/z 289 and the main MS2 fragment at m/z 245 (Del Rio et al., 2004), and the fragment m/z 123 could be explained as fission of the C-ring (Tsimogiannis et al., 2007). Peak at 23.3 min was assigned as chlorogenic acid because the $(M-H)^-$ parent ion (m/z 353) and the fragment ions m/z 191 (M-163) corresponding to the quinate moiety ([quinic acid-H^+]$^-$), and the m/z 161 ([caffeic acid-H_2O-H^+]$^-$) (Jaiswal and Kuhnert, 2010; Swatsitang et al., 2000). The MS spectra of ferulic acid (t.r. 23.6 min) exhibited the expected deprotonated pseudomolecular ion (negative ion) at m/z 193, and the m/z 134 produced by $(M-60)^-$ (Swatsitang et al., 2000).The fragment ion m/z 152 of the vanillic acid (t.r. 26.7 min) can be explained as a loss of the methyl group from the parent ion m/z 167 (Chua et al., 2011). The presence of m/z 167 is in accordance of a decarboxylation and a methyl loss (Chua et al., 2011; McLafferty and Stauffer, 1989). The identification of caffeic and *p*-coumaric acid was corroborated by the comparisons of the retention time and UV spectra of authentic standards. Moreover, a characteristic decarboxylation was observed for caffeic (t.r. 24.1 min) and *p*-coumaric (t.r. 26.9 min) acids, where MS^2, m/z 135 and 119 corresponded to CO_2 elimination respectively (Chua et al., 2011; Plazonic et al., 2009). Two

proanthocyanins, gallocatechin gallate (t.r. 24.8 min) and epicatechin gallate (t.r. 25.8 min) possess characteristic tandem MS spectra reported in the literature (Jaiswal et al., 2011). Gallocatechin gallate is characterized by the parent ion $[M-H]^-$ at m/z 457, and the main MS^2 ion at m/z 169, and epicatechin gallate was identified on the basis of retention time and MS-MS fragmentation pattern $[M-H]^-$ at m/z 441, and the main MS^2 fragment at m/z 289 and 169 with those of authentic standard (Jaiswal et al., 2011; Del Rio et al., 2004). A chromatographic signal at 28.4 min was assigned as quercetin-hexose conjugate on the basis of MS-MS fragmentation. This signal corresponded to a ($[M - H]^-$) at m/z 463, yielding a main fragmentation ion MS^2 of m/z 301, corresponding to hexose elimination. The injection of the respective glucoside conjugate, allowed assigning this signal as quercetin-3-β-glucoside (Del Rio et al., 2004). By comparing the chromatographic behavior with published data, its MS^n spectra of the molecular ion $[M-H]^-$ at m/z 317 where according to those of the authentic standard myricetin (Lin et al., 2012). As product of retro Diels-Alder fragmentation in the C-ring, fragment ions m/z 151 and 179 are produced (Ma et al., 1997). This fragmentation pattern is typical for flavon-3-ols where the ring A and B are di and tri-hydroxylated respectively (Lin et al., 2012). This pattern is quite characteristic for myricetin (Lin et al., 2012).The deprotonated molecule m/z 447 at retention time 30.8 min had a fragment ion peak at m/z 301 $(448-H-146)^-$ and 300 (448-H-147), this fragmentation is characteristic of quercitrin (Mämmelä et al., 2000). The ion m/z 301 found at 33.3min, presented one fragment at m/z 151 $(M-1-150)^-$, consistent with quercetin fragmentation pattern, and corresponding to a retro Diels Alder fragmentation in ring-C (Rodriguez-Medina et al., 2009). Luteolin was assigned to the peak at 39.7 min because presented the M-H molecular ion at m/z 285, and m/z 133 corresponding to the fission of B-ring reported

for flavones (Justesen, 2000). At 40.5 min an intense signal corresponding to m/z 285 is indicative of the kaempferol. This flavone does not produce significant fragmentation (Justensen, 2000).The MS spectra of three anthocyanins (19.5, 20.1 and 22.6 min) showed molar and fragmented ion masses (anthocyanidins agliconic parts of anthocyanins). Based on the agreement between scanned molecular masses using the MS detector and known anthocyanin molecular weights we confirmed peak at 19.5 min to be cyanidin-3-glucoside (449.0), and peak at 20.1 min corresponded to peonidin-3-glucoside (463), and peak at 22.6 would correspond to peonidin-conjugate (Mozetič and Trebše, 2004).

In summary, the identity of all the compounds was confirmed with authentic standards and comparing UV spectra and MS fragmentation patterns reported in the literature. These results are consistent with those from by Ruiz et al. (2010) who reported that fresh fruits of murtilla showed antioxidant activity and the presence of anthocyanins, flavonols and flavan-3-ols. The presence of cyaniding-3-glucoside and peonidin-3-glucoside is corroborated by Ruiz et al. (2010) and Alfaro et al., (2013b) who founded these anthocyanins in murtilla fruits. This is the first report identifying, by HPLC-ESI-MS, the presence of gallic acid, catechol, epicatechin, vanilic acid, caffeic acid, epicatechin gallate, *p*-coumaric acid, quercetin-3-β-glucoside, myricetin, quercitrin, quercetin, luteonin, kaempferol in both fresh fruits. A comparative qualitative analysis of the polyphenols identified in fresh and dehydrated fruit showed that any dehydration methodology studied in this work produced changes in the polyphenol profile (Table 3). Chlorogenic acid is an important intermediate in lignin biosynthesis (Boerjan et al., 2003), and it was present in fresh and freeze drying samples. FD was the treatment retaining the less number of components in comparison with fresh fruit (60%), and hot-

air drying at 65 ^{o}C retained the most of the components in relation to the fresh fruit (80%). However, all the flavonols and sugar conjugates were present in FD fruits. These results do not allow explain the bigger antioxidant activity shown by FD fruits than fresh fruits (Alfaro et al. 2013b). A possible explanation would be the presence of a significant number of unknown polyphenol conjugates detected in this study. Preliminary analysis suggests that the flavonoids identified in this work could be conjugate with other hexoses than glucose. Further studies of HPLC-ESI-MSMS will be necessary for dilucidating the structure of these unknown conjugates.

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REFERENCES

Aguirre, M. C., Delporte, C., Backhouse, N., Erazo, S., Letelier, M. E., Cassels, B. K., Silva, X., Alegría, S., and Negrete, R. (2006). Topical anti-inflammatory activity of 2α-hydroxypentacyclictriterpene acids from the leaves of *Ugni molinae*. Bioorganic and Medicinal Chemistry 14. 5673-5677.

Alfaro, S. Mutis, A. Palma, R. Quiroz, A. Seguel, I. Scheuermann E. (2013a). Influence of genotype and harvest year on polyphenol content and antioxidant activity in murtilla (*Ugni molinae* Turcz) fruit. Journal of Soil Science and Plant Nutrition 13: 67-78.

Alfaro, S., Mutis, A. Palma, R. Quiroz, A. Seguel, I. Scheuermann E. (2013). Drying Methods Effect on Polyphenol Compounds and Antioxidant Activity of the Murtilla (Ugni molinae Turcz) Fruits Red Pearl-INIA Variety (Manuscript submitted to International Journal of Food Science and Technology)

Boerjan, W., Ralph, J. and Baucher, M. (2003). Lignin biosynthesis. Annu. Rev. Plant Biol. 54: 519–46. doi:10.1146/annurev.arplant.54.031902.134938. PMID 14503002.

Chua, L.S., Latiff, N.A., Lee, S.Y., Lee, C.T., Sarmidi, M.R. and Aziz, R.A. (2011). Flavonoids and phenolic acids from *Labisia pumila* (Kacip Fatimah). Food Chemistry 127: 1186-1192.

Delporte C, Backhouse N, Inostroza V. (2007). Analgesic activity of *Ugni molinae* (murtilla) in mice models of acute pain. Journal of Ethnopharmacology 112:162-165.

Del Rio, D., Stewart, A.J., Mullen, W., Burns, J., Lean, M.E.J., Brighenti, F. and Crozier, Al. (2004). HPLC-MS^n Analysis of phenolic compounds and purine alkaloids in green and black tea. Journal of Agricultural Food 52: 2807-2815.

Jaiswal, R., Jayasinghe, L. and Kuhnerta, N. (2011). Identification and characterization of proanthocyanidins of 16 members of the *Rhododendron* genus (Ericaceae) by tandem LC–MS. Journal of Mass Spectrometry 47: 502-515.

Jaiswal, R. and Kuhnert, N. (2010). How to identify and discriminate between the methyl quinates of chlorogenic acids by liquid chromatography–tandem mass spectrometry. Journal of Mass Spectrometry 46: 269-281.

Justesen, U. (2000). Negative atmospheric pressure chemical ionisation low-energy collision activation mass spectrometry for the characterisation of flavonoids in extracts of fresh herbs. Journal of Chromatography A, 902: 369-379.

Lin, Y., Wu, B., Li, Z., Hong, T., Chen, M., Tan, Y., Jiang, J. and Huang, C. (2012). Metabolite identification of myricetin in rats using HPLC coupled with ESI-MS. Chromatographia 75: 655-660.

Ma, Y.L., Li, Q.M., VandenHeuvel, H. and Claeys, M. (1997). Characterization of flavone and flavonol aglycones by collision-induced dissociation tandem mass spectrometry. Rapid. Communication Mass Spectrometry 11: 1357-1364.

Mämmelä, P.,Savolainen, H., Lindroos, L., Kangas, J. and Vartiainen, T. (2000). Analysis of oak tannins by liquid chromatography-electrosprayionisation mass spectrometry. Journal of Chromatography A, 891: 75-83.

Markakis, P. (1974). Anthocyanins and their stability in food. Critical Review in Food Technologies 4: 341-437.

Mejia-Meza, E.I., Yañez, J.A., Remsberg, C.M., Takemoto, J.K., Davies, N.M., Rasco B., and Clary, C. (2010). Effect of dehydration on raspberries: Polyphenol and anthocyanin retention, antioxidant capacity, and antiadipogenic Activity. Journal of Food Science 75:H5-12.

McLafferty, F. W. and Stauffer, D.B. (1989).The Wiley/NBS Registry of Mass Spectra Data, John Wiley & Sons, New York.

Plazonić, A., Bucar, F., Maleš, Ž.,Mornar, A., Nigović, B. and Kujundžić, N. (2009). Identification and quantification of flavonoids and phenolic acids in burr parsley (*Caucalisplaty carpos* L.), using high-performance liquid chromatography with diode array detection and electrospray ionization mass spectrometry. Molecules 14: 2466-2490.

Mozetič, B. and Trebše, P. (2004). Identification of sweet cherry anthocyanins and hydroxycinnamic acids using HPLC coupled with DAD and MS detector. Acta Chimica Slovenica 51: 151−158.

Rodríguez-Medina, I.C., Segura-Carretero, A. and Fernández-Gutiérrez, A. (2009). Use of high-performance liquid chromatography with diode array detection coupled to electrospray-Qq-time-of-flight mass spectrometry for the direct characterization of the phenolic fraction in organic commercial juices. Journal of Chromatography A, 1216: 4736–4744.

Rubilar, M., Pinelo, M., Ihl, M., Scheuermann, E., Sineiro, J., Nuñez, M.J. **(**2006). Murtilla leaves (*Ugni molinae*Turcz) as a source of antioxidant polyphenols. Journal Agriculture Food Chemistry 54: 59-64.

Rubilar, M., Jara, C., Poo, Y., Acevedo, F., Gutierrez, C., Sineiro, J. and Shene, C. (2010). Extracts of Maqui (*Aristotelia chilensis*) and Murta (*Ugni molinae*

Turcz.): Sources of antioxidant compounds and α-glucosidase/α-amylase inhibitors. Journal Agriculture Food Chemistry 59: 1630-1637.

Ruiz, A., Hermosin-Gutierrez, I., Mardones, C., Vergara, C., Herlitz, E., Dorau, C., Winterhalter, P. and von Baer, D. (2010). Polyphenols and antioxidant activity of calafate (*Berberis microphylla*) fruits and other native berries from southern Chile. Journal Agriculture Food Chemistry 58: 6081-6089.

Scheuermann, E. 2009. Estudio de componentes volátiles y capacidad antioxidante de frutos de murta (*Ugni molinae* Turcz) y evaluación de sus estabilidad en almacenamiento refrigerado, congelación y tratamiento térmico de pasteurización. Tesis para optar al grado académico de Doctor en Ciencias y Tecnología de Alimentos, Universidad de Santiago de Chile, Chile. 200p.

Shene, C., Reyes, A., Villarroel, M., Sineiro, J., Pinelo, M., and Rubilar, M. (2009). Plant location and extraction procedure strongly alter the antimicrobial activity of murtilla extracts. European Food Research and Technology 228: 467-475.

Swatsitang, P., Tucker, G., Robards, K. and Jardine, D. (2000). Isolation and identification of phenolic compounds in *Citrus sinensis*. Analytica Chimica Acta 417: 231-240.

Tsimogiannis, D., Samiotaki, M., Panayotou, G. and Oreopoulou, V. (2007). Characterization of flavonoid subgroups and hydroxy substitution by HPLC-MS/MS. Molecules 12: 593-606.

Table 1: Experimental conditions for MRM experiment for the HPLC MSMS analysis of polyphenol compounds.

ID	Q1 Mass (Da)	Q3 Mass (Da)	Time (ms)	DP (volts)	EP (Volts)	CE (Volts)	CXP (Volts)
Vanillic acid	167	152	200	-42.72	-8	-13.620	-0.71
		108	200	-42.72	-8	-23.940	-1.00
Ferulic acid	193	134	200	-53.70	-10	-22.030	-1.00
		178	200	-53.70	-10	-10.580	-4,68
Chlorogenic acid	353	191	200	-313.57	-8.34	-26.130	-1.00
		161	200	-312.57	-8.34	-36.01	-1.99
Gallocatechin gallate	457	169	200	-239.72	-11	-26.300	-3.17
		125	200	-239.72	-11	-55.980	-1.00
Epicatechin gallate	441	169	200	-130.00	-9	-28.780	-2.66
		289	200	-130.00	-9	-27.610	-2.84
Quercetin	301	151	200	-21195	-10	-28.350	-0.68
		121	200	-211.95	-10	-33.940	-2.93
Kaempferol	285	117	200	-155,00	-12	-60.220	-1.68
		143	200	-155.00	-12	-44.900	-1.00
Luteolin	285	133	200	-111.94	-12	-46.380	-0.83
		151	200	-111.94	-12	-36.830	-1.00
p-Coumaric	163	119	200	-47.80	-10	-19.000	-1.21
		104	200	-47.80	-10	-37.000	-0.81
(-)-Epicatequin	289	123	200	-54.00	-12	-40.000	-0.90
		245	200	-54.00	-12	-23.000	-1.88
Gallic acid	169	125	200	-269.30	-12	-17.110	-0.81
		107	200	-269.30	-12	-27.220	-1.02
Caffeic acid	179	135	200	-308.00	-12	-19.990	-2.56
		107	200	-308.00	-12	-32.160	-1.21
Myricetin	317	151	200	-167.97	-10	-33.690	-1.00
		137	200	-167.97	-10	-36.190	-2.00
Quercitrin	447	300	200	-167.15	-12	-34.860	-2.47
		271	200	-167.15	-12	-53.580	-2,03
Catechol	109	91	200	-72.57	-10	-25.640	-1.02
		65	200	-72.57	-10	-35.420	-1.00
Quercetin-3-β-D-glucoside	463	300	50	-97.69	-10	-36.130	-2.78
		271	50	-97.69	-10	-56.740	-1.87

Q1 (precursor ions) and Q3 (fragment ions), DP (Declustering potential), EP (entrance potential), CE (Collision Energy) and CXP (Collision exit potential).

Table 2. Experimental conditions for MRN experiment by anthocyanins analysis

ID	Q1 Mass (Da)	Q3 Mass (Da)	Time (ms)	DP (volts)	EP (Volts)	CE (Volts)	CXP (Volts)
Cianidin-3-glucoside	449	287	50	46	3.31	40	8.320
Peonidin-3--glucoside	463	301	50	62	10.35	37.45	2.610
		286	50	62	10.35	59.4	2.750

Q1 (precursor ions) and Q3 (fragment ions), DP (Declustering potential), EP (Entrance potential), CE (Collision Energy) and CXP (Collision exit potential).

Table 3. Polyphenols identified in murtilla fruits

Compounds	Retención time (min)	Parent Ion (M-H) m/z	MS^2 base peak m/z	Sample			
				1	2	3	4
Hydrocinnamic acids							
Chlorogenic	23.3	353	191 161	x			**x**
Caffeic	24.1	179	135	x	x	x	**x**
p-Coumaric	26.9	163	119 104	x	x	x	x
Benzoic acids							
Gallic acid	8.7	169	125	x	x	x	x
Vanillic acid	23.7	167	152 108	x	x	x	
Polyphenols							
Catechol	16.9	109	91 65	x	x		
Flavones							
Luteolin	32.7	285	133	x		x	
Flavonols and sugar conjugates							
Quercetin-3-β-D-glucoside	28.4	463	301	x	x	x	x
Myricetin	30.5	317	151 179	x	x	x	x
Quercitrin	30.8	447	301 300	x	x	x	x
Quercetin	32.3	301	151	x	x	x	x
Kaempferol	40.5	285	117 143	x	x	x	x
Flavan-3-ols							
Epicatechin	21.7	289	245	x	x	x	x
Proanthocyanins							
Gallocatechin gallate	24.8	457	169	x			
Epicatechin gallate	25.8	441	169 289	x			

1: fresh fruit; 2: hot-air drying at 65°C; 3: hot-air drying at 80°C; 4: freeze drying

Table 4: Anthocyanins identified in murtilla fruits

Retention time (min)	Q^1 Precursor Ions	Q^3 Fragment Ions	Compound	Samples
19.5	449	287	Cianidin-3-glucoside	1 2 3 4
20.1	463	303 286	Peonidin-3-glucoside	1 2 3 4

Code samples: 1: fresh fruit; 2: hot-air drying 65ºC (HAD 65ºC); 3: hot-air drying 80ºC (HAD 80ºC); 4: freeze drying (FD).

Chapter 5

PARTIAL CHARACTERIZATION OF ENZYMATIC ACTIVITY OF POLYPHENOLOXIDASE (PPO) AND PEROXIDASE (POD) IN FRESH AND DRIED MURTILLA FRUITS (*UGNI MOLINAE* TURCZ) RED PEARL-INIA VARIETY

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ABSTRACT

The activities of polyphenol oxidase (PPO) and peroxidase (POD) in murtilla fruit (*Ugni molinae* Turcz) were determinate using spectrophotometric method. The polyphenol oxidase enzyme and peroxidase enzyme were extracted with sodium phosphate buffer solution at pH 7.5; PPO and POD activities were determined using catechol and *p*-phenylenediamine as substrate respectively.

The enzymatic activity was study in fresh and dehydrated murtilla fruit and was expressed as the change in absorbance per minute per ml of enzymatic extract and as the change in absorbance per minute per milligram of protein extracted (specific activity).

Enzymatic activities of work of POD and PPO were investigated in murtilla fruits because the optimum conditions for determination of both enzyme activities and the relation with the changes in polyphenols content have not been investigated for this fruit.

The highest activity of PPO and POD was obtained at 25º C. at values of pH between 6.5 at 7.0. The PPO enzyme was kinetically characterized with catechol as substrate and K_m and V_{max} values were 2,967.88 ± 238.02 mM and 0,1474 ±0.02 μM/min, by fresh murtilla fruit.

Keywords: Flavonoids, phenolic acid, total polyphenols, antioxidant capacity, dehydrated fruit, HPLC**,** PPO, POD, enzymatic activity, phenolic oxidation.

1. INTRODUCTION

The murtilla or murta (*Ugni molinae* Turcz.), is a native plant to Chile, which belongs to the family of Myrtaceae. It's kind of bushy growth, produces fruits globose, small, pleasant flavor and aroma. In the wild the species develops into plants of varying height between 0.3 m and 1.70 m, and exceptionally, can reach a height greater than 3 m (Seguel and Torralbo, 2004).

The potential use of leaves and fruits as a source of polyphenolic compounds with antioxidant activity has stimulated the study of different methods of processing fresh fruit for ensuring their availability around the year and do not alter its chemical and biological properties. The importance of controlling of polyphenol oxidase (PPO) in fruits, largely determines the quality and economic value of the product harvested, stored and processed.

Polyphenol oxidase (PPO) is a cooper-containing enzyme present in all plants. It is widely distributed enzyme involved in the biosynthesis of melanin in animals and in the browning of plants (Arnnok et al., 2010). The enzyme required cooper and oxygen; it is an oxide- reductase class of enzyme with oxygen functioning as hydrogen acceptor. Catalyzes the oxidation of phenolic compounds to form corresponding quinone intermediated which polymerize to form undesirable pigment, by two types of oxidative reactions the hydroxylation of monophenols to *o*-diphenols and the oxidation *o*-diphenols to *o*-quinones, which lead to formation of black or brown pigments (Lee et al., 2007; Gawlik et al.,2007).

Peroxidase (POD) is an oxidoreductase that is directly involved in many plant functions such as hormone regulation, defense mechanism, indolacetic degradations and lignin biosynthesis (Serrano, Martinez *et al.,* 2008). It catalyzes reactions in which hydrogen

peroxidase acts as the donor of hydrogen atom (Arnnok *et al*.,2010). POD is involved in enzymatic browning since diphenols may function as reducing substrate in this reaction (Chisari et al., 2007; Serrano-Martinez *et al*., 2008). Peroxidase is also intimately related to flavor loss and odor of stored food as well as to a great variety of biodegradation reactions (Rojas-Grau *et al.*, 2008).

Peroxidase (POD) and Polyphenol oxidase (PPO) have been considered the principal enzymes responsible for quality deterioration in most fruits and vegetable. POD can contribute to adverse changes in the flavor, color, texture or nutrient value of raw and processed fruits (Gonzalez et al., 2000). PPO activity may be responsible for the loss of the red color of some fruits through the degradation of anthocyanin pigments (Markakis, 1974).

The objective of this study was to quantify the effect of freeze drying (FD), hot-air drying at 65ºC (HAD 65ºC), and a hot-air drying at 80ºC (HAD 80ºC) techniques on these properties in fresh and dried murtilla fruits and to evaluate the effect of peroxidase and polyphenol oxidase activity of fresh fruits and dehydrated products.

2. MATERIALS AND METHOD

2.1 Plant material

Fresh fruits of murtilla (*Ugni molinae* Turcz) obtained from a selected plant (Red Pearl variety) were provided by INIA Carillanca and they were used in all the experiments. Fresh harvested fruits were subjected to rapid freezing using liquid nitrogen (-196 º C) and were subsequently stored at -20 ºC in an Electrolux FE-26 freezer. The dehydrated murtilla fruit were obtaining by freeze drying (FD), hot-air drying at 65ºC (HAD 65ºC), and a hot-air drying at 80ºC (HAD 80ºC) techniques.

2.2. Enzyme extraction

The extraction procedure was using the method described by Fortea *et al.* (2009) with some modifications. Initially all steps of enzyme extraction were carried out at 4ºC. Subsequent, the activities of polyphenol oxidase (PPO) and peroxidase (POD) in murtilla fruit (*Ugni molinae* Turcz) were extracted from 10 g of pulverized liquid nitrogen frozen murtilla fruit, and it was homogenized and mixed for 1 min with 15 ml of extraction buffer solution (0.2 M sodium phosphate buffer pH 7.0) and 100 µl of Protease Inhibitor Cocktail. The homogenate was filtered through eight layers of gauze and centrifuged at 4,000 g for 15 min. The supernatant was discarded and the precipitate was extracted with 20 ml of 4% (w/v) Triton X-114 in 100 mM sodium phosphate buffer (pH 7.0). The mixture was subjected to temperature-induced phase partitioning and kept at 4ºC for 15 min and then warmed to 37ºC for 15 min. This turbid solution was centrifuged at 10,000 g 10 min at 25ºC. The detergent rich phase was discarded and the clear detergent-poor supernatant was used as enzyme source and was stored at -20ºC.

2.3. Chemicals*:*

Reagents, Triton X-114, catechol, and p-phenylenediamine were obtained from Sigma Aldrich Co Germany. The hydrogen peroxide solutions were freshly prepared every day.

2.4. Determination of PPO activity

PPO activity measurement: Polyphenol oxidase activity was determined at 25ºC by using spectrophotometric method (Fortea *et al.,* 2009) an initial rate of increase in absorbance at 400 nm by adding 3 ml of reaction mixture, consisting of 2.7 ml of 0.5 M catechol in 0.1 M sodium phosphate buffer, pH 7.0 and 300 µL of extract to a 4.5 ml quartz cuvette path length. The change in the absorbance at 400 nm was recorded every 5 s up to 3 min from the time the enzyme extract using ultraviolet-visible spectrophotometer, (Spectronic Genesis 10 S). One unit of PPO activity was defined as a change in absorbance of 0.001 per minute and ml of enzymatic extract immediately after extract addition and as the change in absorbance per minute per milligram of protein extracted (specific activity). All determinations were performed in triplicate.

2.5. Determination of POD activity

POD activity measurement: Peroxidase activity was determined at 25 º C measuring the increase in absorbance at 485 nm, according to method of Rojas Grau *et al*., (2000), with some modifications. The reaction was started by adding 0,9 ml of Sodium phosphate buffer (0.05 M at pH 6,5), 0.67 ml of *p*-phenylenediamine 1% (w/v), 0.33 ml of hydrogen peroxide 1.5% (w/v), freshly prepared and 0,67 tropolone 1% (w/v) to discard any contributions of polyphenol oxidase at the progress of reactions, in a final

volume of 1ml and 0.1 ml of extract to a 4.5 ml quartz cuvette of 1 cm path length. The changes in the absorbance at 485 nm were measured using ultraviolet-visible spectrophotometer, (Spectronic Genesis 10 S). One unit of POD activity was defined as a change in absorbance of 0,001 per minute and ml of enzymatic extract immediately after extract addition and as the change in absorbance per minute per milligram of protein extracted (specific activity). All determinations were performed in triplicate.

2.6. Effect of pH

A study was made on the effect of pH on the catechol oxidation by murtilla PPO and apple PPO as a positive control. Enzyme activity was determined in 0.2 M sodium phosphate buffer at different pH values ranging from 4.0 to 9.0.

2.7. Effect of temperature

A study was made on the effect of temperature on catechol oxidation by murtilla PPO and apple PPO as a positive control. Enzyme activity was determined at different temperatures ranging from 25ºC to 80ºC. The enzyme solution was incubated in a heating bath at different temperatures (25, 37, 45, 55, 65, and 80º C). After heating, samples were cooler in ice and assayed immediately at 25ºC.

2.7. Protein determination

Protein concentration of the extracts was measured according to the Bradford (1976) method. The absorbance was measured at 595 nm with bovine serum albumin (BSA) as a standard. Analyses were made in triplicate for each sample.

2.8 Effect of substrate concentration

Solutions of catechol concentrations ranging from 0.05 M to 0.5 M were employed to study the effect of substrate concentration on PPO activity of fresh and dehydrated murtilla fruit. In a glass cuvette, 0.3 ml of enzyme solution was mixed with 2.7 ml of catechol at different concentrations in 0.2 M sodium phosphate buffer (pH 7.0) Michaelis constant ($K_{m)}$ and maximum velocities (V_{max}) values for the PPO were calculated from a plot of 1/activity v/s 1/ substrate concentrations by the Lineweaver-Burk plots for fresh and dehydrated murtilla fruit respectively, using catechol as substrate.

2.9. Statistical analysis

The data collected were analyzed using a one-way analysis of variance (ANOVA). Values of $p < 0.05$ were considered significantly. The differences between means were determined using the Tukey's multiple comparison tests. Results were expressed as mean of measurements and their corresponding standard deviations. The SPSS software system version 17.0 for Windows was used to analyze the data.

3. RESULT AND DISCUSION

3.1. Selection of conditions for enzymatic activity analysis

The optimal pH values obtained from this study were utilized for preparing the murtilla fruit samples (Table 4.1). In this work the entire enzyme assays were conducted at pH 7.0 for PPO and POD enzyme extractions. In this work, use of PVPP is was not possible to utilize in murtilla assay because no good result with this methodology and, consequently, extracted PPO and POD activities were very low. The use of PVPP has been reported to produce goods results in PPO extraction from other plant tissues, such raspberry, banana, strawberry and papaya (Gonzalez *et al* 1999). However, the use of a detergent (Triton X-114) in buffer showed that method increased the enzymatic activity extracted. This two phase step involved full advantage in the removal of phenols and hydrophobic proteins) and to prevent browning yielding a PPO and POD in a clear solution, (Sanchez–Ferrer *et al*.1993, Sojo *et al* 1998).This method has been used for the purification of purple skinned grapes PPO (Fortea *et al* 2009, Nuñez Delicado *et al*. 2003), red pepper POD (Serrano-Martinez *et al*. 2008), blackberry PPO and POD (Gonzalez *et al*.,2000), raspberry PPO (Gonzalez *et al* 1999), and banana PPO (Sojo et al., 1998).

The reaction medium at 25ºC a 0.2 M sodium phosphate buffer pH 7.0, contained 100 µl protease inhibitor cocktail, 300 µl enzyme extract and 0.1M catechol for obtaining a good reproducibility and measure of the PPO enzyme activity.

In POD enzymatic activity case, using the optimized activity conditions a 0.05 M sodium phosphate buffer pH 6.5. The reaction medium contained 67 µl Tropolone 1% w/v, 33 µl enzyme extract, 33 µl peroxide and 67 µl p-phenylenediamine 1% w/v.

Tropolone was the most effective inhibitor, achieving almost completely inhibiting enzyme PPO of a banana pulp, according to Sojo *et al.*, 1998.

PH optimizations for extractions and activity: The pH stability was measured by extraction the PPO enzymes from Read Pearl-INIA variety murtilla fruit and Fuji apple cultivar as a positive control (figure not shown), in buffers ranging from pH 4.0 to 9.0 (Figure 4.1 and Figure 4.2). The optimal pH values obtained from this study were utilized in the murtilla fruit samples. There were two maximum PPO values, at pH levels of 6, 0 and 7.0. Did not obtaining enzymatic activity at extractions pH values <5.0 in both assay. In this work the entire enzyme assays were conducted at pH 7.0 for PPO and at pH 6.5 for POD enzymes activity.

In general, most plant showed the optimum pH for any enzymes at or near neutral pH values. The strawberry PPO had a maximum activity at pH 4.0 (Gonzalez *et al*.1999), potato PPO was fully active at pH 4.5 and pH 6.5 (Sanchez Ferrer *et al*. 1993), hot pepper PPO and POD the optimum pH was 7.0 (Arnnok *et al*., 2010).

Temperature optimizations for extractions and activity:

Enzymatic inactivation is the result of a process strongly-temperature dependent of protein denaturation and loss of some functional group were involucres. (Fortea *et al.*, 2009).Generally, the reaction rate decreased because of thermal denaturation wen the temperature is increased. Temperature dependence in the enzyme activities is showed in Figure 4.3 and 4.4 for PPO and POD respectively.

The thermal stability of PPO enzymes from Read Pearl variety murtilla fruit and Fuji apple cultivar as a positive control (figure not shown) was studied. At temperatures > 50ºC the Fuji apple PPO enzyme was strongly inactivated and not shown activity, and so at lower temperatures this effect is less significant, finding that process of activation

of the enzyme was strongly dependent of temperature. In the case of Red Pearl murtilla fruit at range of temperature ≥ 60ºC it shown enzymatic activity, which would indicate that Read Pearl murtilla PPO is highly thermostable. The range of temperatures required for the inactivation of murtilla fruit Red Pearl INIA variety was determined for this assay, at temperatures ≥ 65ºC.

The POD activity showed the highest activity was in the range of 25 at 30 ºC, and then decreased at temperature above 37°C. In case of POD, the enzyme was highly active up to 37º C and lost its activity at higher temperatures in a range of 45 at 80º C. PPO showed the highest activity at 25º C and its activity decreased slightly between 37 at 55ºC, and then decreased strongly from 65 at 80º C.

From previous studied, PPO and POD of hot pepper activity decreased at temperatures above 40º C, (Arnnok et al., 2010), the range of temperature required for the inactivation of red pepper POD was at temperatures >40º C (Serrano- Martinez *et al*,.2008). The optimum temperature of activity for strawberry PPO was in a range of 50º at 65 ºC and PPO retained this activity over the range of 40 at 65º C and the POD activity was maximal at 25º C. Temperatures higher than 30º C caused a progressive decrease of activity until inactivation at 70ºC (Chisari et al., 2007).

3.2. Effect of dehydration process on Red Pearl murtilla fruit PPO and POD activity

The enzymatic activity from murtilla fruit Red Pearl-INIA variety was shown trend to decrease when it submitted to dehydrating process. The drying process leads to decrease the PPO activity in the dehydrated murtilla fruit. Read Pearl PPO activity being affected by drying at 80 °C which produced a 83 % decrease of enzyme activity, while drying it

at 65 °C by 79,6 % compared with freeze-drying, which showed no significant difference with the enzyme activity in the fruit.(Table 4.2). In the case of POD activity the drying process that use high temperature decreased in a 97% the enzymatic activity and not shown significant difference with respect to the fruit, when the dehydration is carried out by freeze-drying method (Table 4.3). The effect of the dehydration process is identical on both enzymes but in the case of PPO, this enzyme is thermostable to a temperature range higher than the POD.

Respect to results related to our work on dehydrated fruit, Fortea et al 2009 performed a thermal inactivation study of PPO and POD from table grape. These results showed that inactivation occur in this case at temperatures > at 70º C and this process is strongly-temperature dependent and this author suggested that this thermostability may be taking into account when thermal treatments were used to obtain processed products.

Zhu *et al.* 2010 indicated that the best processing parameters for the intermittent heating mode are a surface temperature of 75º C, 5 mm slice, and 7.5 min processing time, which lead to final product with 10% residual POD, less than 1% residual PPO, and this method has a significant effect on product quality and processing characteristics, as faster inactivation of enzymes.

3.3. Effect of substrate concentrations on PPO activity in dehydrated murtilla fruit

The enzymatic activity is shown in Table 4.4. The results show that high concentration of catechol produce an increase of the PPO activity. However, at low concentrations it is possible to observe a decrease of the enzyme activity with respect to fresh fruit. These results are consistent with those reported by others authors (Arnnok et al., 2010,

Gonzalez *et al*., 2000), in which a catechol oxidation was found to be dependent to an increase in the substrate concentration.

3.4. Determination of K_m and V_{max} on PPO enzyme.

In a first approximation, K_m and V_{max} values for PPO activity with catechol as substrate were 2,967.88 ± 238.02 mM and 0.1474 ±0.02 μM/min, by fresh murtilla fruit; obtaining the highest value of K_m for dried fruit by HAD 65 °C and HAD 80º C drying methods (Table 4.4). The V_{max} obtained from these methods were 1.2579 ±0.10 μM/min, for dehydrated fruit by HAD 65º C and 1.4050 ±0.21 μM/min by HAD 80ºC. The lowest values of K_m and V_{max} were obtained for dehydrated fruit by freeze drying and for fresh murtilla fruits. This could be indicating that the activity of PPO in murtilla fruits is more affected by thermal methods of dehydration, which used higher temperatures in the drying process. The K_m and V_{max} values for PPO of fresh murtilla fruit were unknown until this study was conducted.

4. CONCLUSIONS

The stability of the polyphenol components in the fruits murtilla of variety Red Pearl-INIA by action of the enzyme polyphenol oxidase is favored by drying using hot air convection**.**

The advances of the results in relation to the PPO activities allow suggesting that optimum value of pH is 7.0 and the enzyme is thermo stable up to 60°C**.** In the case of POD the optimum value of pH is 6.5 and the enzyme is less thermostable and decreased at temperature above 30°C.

With respect to the influence of dehydrating process over the enzymatic activity these results showed that inactivation occur in this case at temperatures up to 65º C for PPO activity and above 37º C for POD activity. This process is strongly-temperature dependent in the dehydrating process that used high temperature. Freeze drying shows no significant differences in relation to the fresh fruit.

The use of optimal experimental conditions for determination of PPO activity allowed obtaining a partial characterization of this enzyme, determining K_m and V_{max} values, which have not been reported previously.

Study the specific affinity of these enzymes with different phenolic substrates present in fresh and dried murtilla fruit, and to understand the behavior of these naturally occurring enzymes when as acting in enzymatic browning processes as well as during postharvest processing and storage of this fruit.

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REFERENCES

Armonk, P., Ruangviriyachai, C., Mahachai, R., Techawongstien, S., and Chantihai,S. (2010) Optimization and determination of polyphenol oxidase and peroxidase activities in hot pepper (Capsicum annuum L.) pericarb. International Food Research Journal 17. 385-392.

Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding" Analytical Biochemistry. 72:248-254

Chisari, M., Barbagallo, R. N., and Spagna, G. (2007) Characterization of polyphenol oxidase and peroxidase and influence on browning of cold stores strawberry fruit. Journal of Agriculture and Food Chemistry 55.(9). 3469-3476.

Fortea, M.I., López-Miranda, S., Serrano-Martínez, A., Carreño, j., Núñez-Delicado, E. **(2009)** Kinetic characterization and thermal inactivation study of polyphenol oxidase and peroxidase from table grape (Crimson Seedless). Food Chemistry 113.1008-1014.

Gawlik-Dziki, U., Złotek, U. and Świeca, M. (2007) Characterization of polyphenol oxidase from butter lettuce (Luctuca sativa var. capitata L.). Food Chemistry 107: 129-135.

González, E.M., De Ancos, B. and Cano, M.P. (1999) Partial Characterization of Polyphenol Oxidase Activity in Raspberry Fruits. Journal Agriculture Food Chemistry 47: 4068-4072.

González, E.M., De Ancos, B. and Cano, M.P. (2000) Partial characterization of Peroxidase and Polyphenol oxidase activities in Blackberry Fruits. Journal Agriculture Food Chemistry 48. 5459-5464.

Lee, M-K. and Park, I. (2007) Studies on inhibition of enzymatic browning in some foods by Du-Zhong (Eucommia uimoides Oliver) leaf extract. Food Chemistry 114: 154-163.

Markakis, P. (1974) Anthocyanins and their stability in food. Critical Review in Food Technologies 4. 341-437.

Mejia-Meza E.I., Yañez J.A., Remsberg C.M., Takemoto J.K., Davies N.M., Rasco B., and Clary C.(2010) Effect of Dehydration on Raspberries: Polyphenol and Anthocyanin Retention, Antioxidant Capacity, and Antiadipogenic Activity. Journal of Food Science 75(1).5-12.

Núñez-Delicado, E., Sojo, M., García-Carmona, F., Sánchez-Ferrer, A., (2003) Partial purification of latent persimmon fruit polyphenol oxidase. Journal of Agriculture and Food Chemistry. 51: 2058-2063.

Rojas-Grau, M.A., Soliva-Fortuny, R. and Martin- Belloso, O.(2008) Effect of Natural Antibrowning Agents on Color and Related Enzymes in Fresh-Cut Fuji Apples as an Alternative to the Use of Ascorbic Acid. Journal of Food Science. Vol. 73 (6):267-272.

Seguel, I.; Torralbo, L. (2004). Murtilla: el berry nativo del Sur de Chile. Revista Tierra Adentro, 57(4):20-25.

Serrano-Martinez, A., Fortea M.I., Del Amor F.M., Nuñez –Delicado E. (2008) Kinetic characterization and thermal inactivation study of partially purified red pepper (Capsicum annuum L.) peroxidase. Food Chemistry 107:193-1

Sojo, M., Nuñez-Delicado, E., García-Carmona, F., Sánchez-Ferrer, A. (1998) Partial purification of a banana polyphenol oxidase using Triton X-114 and PEG 8000 for removal of polyphenols. Journal Agriculture Food Chemistry 46: 4924-4930

Zhu, Y., Pan, Z., McHugh, T., Barrett, D. (2010) Processing and quality characteristics of apple slices processed under simultaneous infrared dry-blanching and dehydration with intermittent heating. Journal of Food Engineering 97: 8–16.

Table 4.1: Influence of extraction buffer composition on Red Pearl murtilla fruit PPO activity

Composition buffer	Red Pearl murtilla fruit PPO activity (ΔOD/min/mg protein extract.)	Red Pearl murtilla fruit POD activity (ΔOD/min/mg protein extract)
0,2M sodium phosphate buffer pH 7.0 + 4.0 % (w/v) Triton X-114 + 0.100 ml of Protease Inhibitor Cocktail + 0.2 mM tropolone (POD assay)	3.86 ± 0,28	3.08 ±0.37

Activity values are the average of three independent determination ± standard deviation

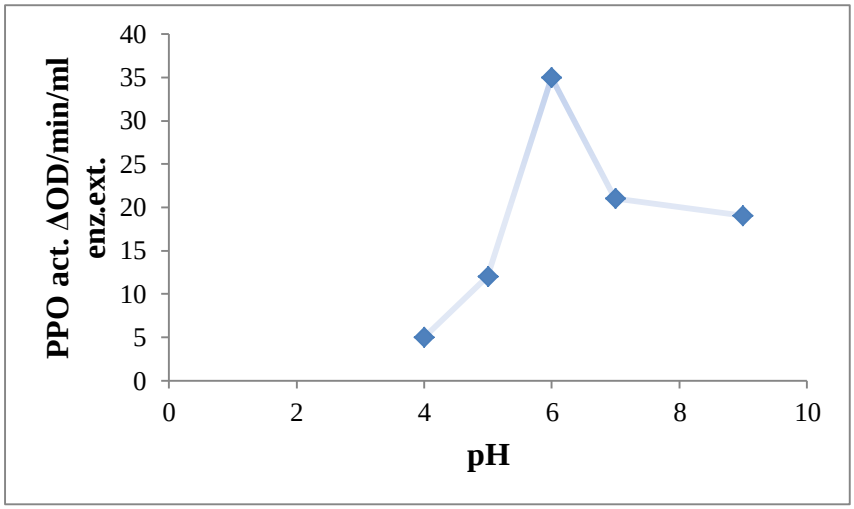

Fig.4.1. Effect of pH on Red Pearl murtilla fruit PPO activity with a McIlvaine buffer solution (1:1) at range of pH = 3.0 at 6.5, 0.2M sodium phosphate buffer at range of 7.0 at 9.0.The reaction medium at 25ºC contained 100 µl protease inhibitor cocktail, 300 µl enzyme extract and 0,1 M catechol

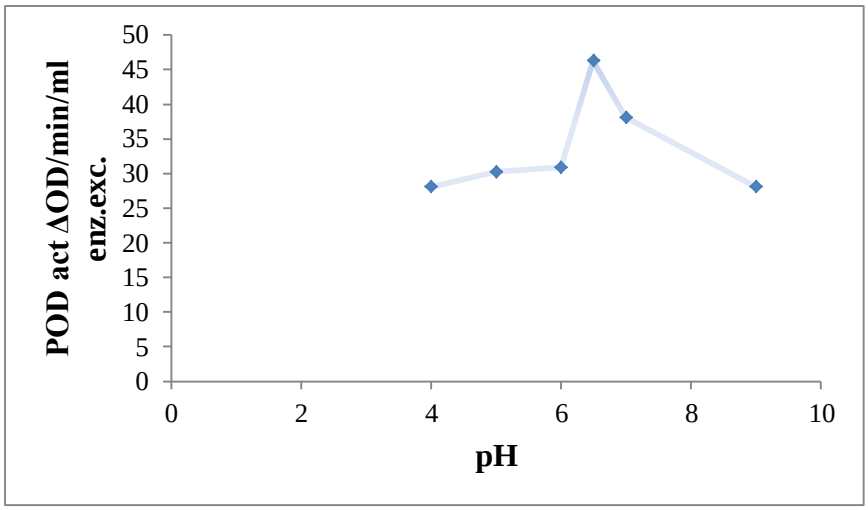

Fig.4.2. Effect of pH on Red Pearl murtilla fruit POD activity with a McIlvaine buffer solution (1:1) at range of pH = 3.0 at 6.5, 0.2M sodium phosphate buffer at range of 7.0 at 9.0.The reaction medium at 25ºC contained 67 µl Tropolone 1% w/v, 33 µl enzyme extract, 33 µl peroxide and 67 µl p-phenylenediamine 1% w/v.

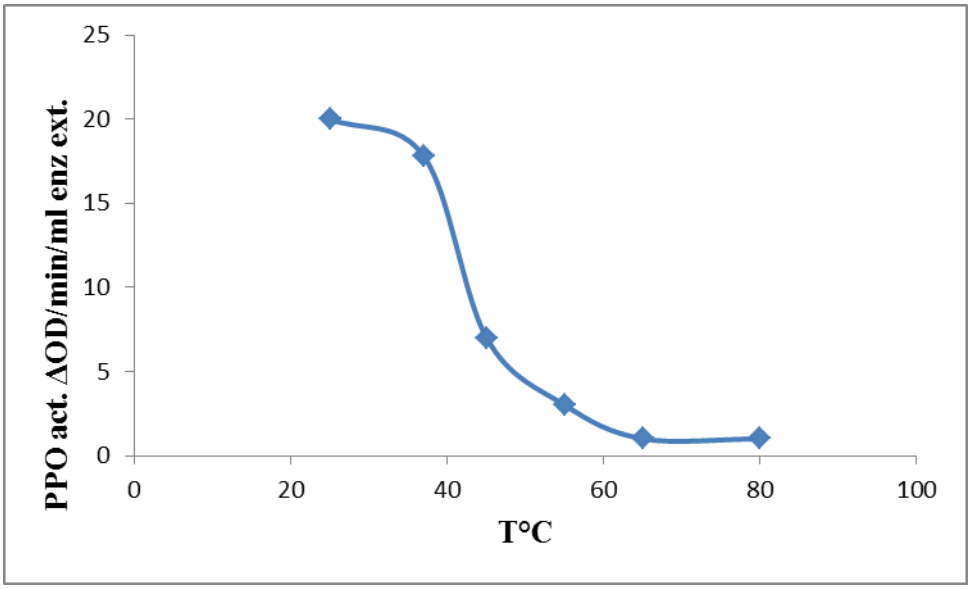

Fig.4.3. Effect of Tº on Red Pearl murtilla fruit PPO activity with a Phosphate buffer solution (1:1) at range of pH = 7.0 containing Triton X 114 (4%). The reaction medium at 25ºC contained 100 µl protease inhibitor cocktail, 300 µl enzyme extract and 0.1 M catechol.

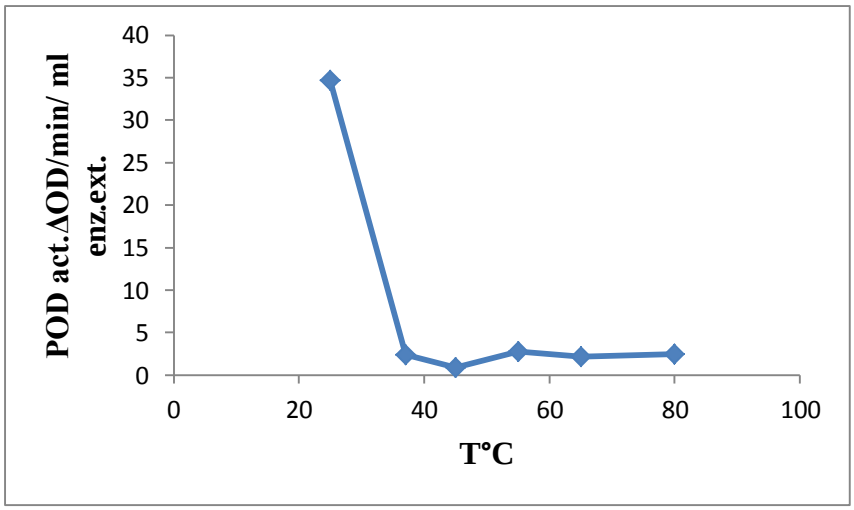

Fig.4.4. Effect of Tº on Red Pearl murtilla fruit POD activity with a Phosphate buffer solution (1:1) at range of pH = 6.5. The reaction medium contained 67 µl Tropolone 1% w/v, 33 µl enzyme extract, 33 µl peroxide and 67 µl p-phenylenediamine 1% w/v.

Table 4.2 Effect of dehydration treatments on enzymatic activity (PPO) of murtilla fruits.

TREATMENTS	PPO ACTIVITY (ΔOD/min/mL extract)
FRESH	19.7 ± 4.2 [a]
FD	24.0 ± 4.3 [a]
HAD 65°C	4.0 ± 1.0 [b]
HAD 80°C	3.3 ± 0.6 [b]

Values expressed as mean ± SD. Different letters indicate statistical differences according to the Tukey test, $p < 0.05$, $n = 3$. HAD 65 ° C, HAD 80 ° C, hot air drying at 65 ° C and 80 ° C respectively.

Table 4.3 Effect of dehydration treatments on enzymatic activity (POD) of murtilla fruits.

TREATMENTS	POD ACTIVITY (ΔOD/min/mL extract)
FRESH	102.1 ±36,9[a]
FD	112.0±5.48[a]
HAD 65°C	3.3±1.3[b]
HAD 80°C	2.4±0.51[b]

Values expressed as mean ± SD. Different letters indicate statistical differences according to the Tukey test, $p < 0.05$, $n = 3$. HAD 65 ° C, HAD 80 ° C, hot air drying at 65 ° C and 80 ° C respectively.

Table 4.4 Effect of substrate concentrations on PPO activity in dehydrated murtilla fruit

		PPO activity (ΔOD/min/g dw)		
Catechol (mM)	FRESH	HAD 65ºC	HAD 80ºC	FD
0.50	0.011±0.004	0.028±0.005	0.030±0.003	0.045±0.005
0.10	0.008±0.046	0.028±0.010	0.027±0.001	0.032±0.000
0.05	0.003±0.001	0.007±0.003	0.006±0.002	0.010±0.001

Activity values are average of three independent determinations ± standard deviations

Table 4.5 Determination of *Km* and *Vmax* from Lineweaver-Burk plots for dehydrated murtilla fruit, using catechol as substrate at different range of concentration.

Sample	K_m (mM)	$V_{max.}$ (μM/min)
FRESH	2,967.88 ± 238.02	0.1474 ± 0.02
HAD 65ºC	7,322.33 ± 4,027.22	1.2579 ± 0.10
HAD 80ºC	12,745.68 ± 2,729.46	1.4050 ± 0.21
FD	1,779.21 ± 55.16	0.3757 ± 0.02

Activity values are average of three independent determinations ± standard deviations

Chapter 6

General Discussion

General Discussion

Quality is an important parameter that can affect the efficacy and beneficial properties of natural products. Extrinsic and intrinsic factors can affect the quality of the final product.. Intrinsic factors such as species differences or genotypes variations can affect the qualitative and quantitative accumulation of bioactive chemical constituents in the plant material. Extrinsically, environmental factors, collection methods, cultivation, harvest, post-harvest (transport, appropriated storage, and industrial processes), and diverse causes such as inadvertent contamination, or intentional adulteration are contributing factors to the quality of the final product.

In this context, to relate some environmental and chemical variables such as genotype, harvest year and polyphenol content, and its effect on the antioxidant activity in murtilla fruits is very relevant for determining similarities and differences among standardized and confirmed genotypes and for predicting the response of new varieties to specific production and cultivation conditions (Sochor *et al.,* 2010).

Standardization is a key step in the production of natural products. From a quality-control point of view, it allows to the manufacturers to ensure consistency of their products from batch to batch. It is essential to determine the effect of the processing on the antioxidant activity and other health-promoting properties of murtilla fruits, for allowing define that drying method is more favorable for the obtaining a specific type of dehydrated products. It is very important to define those relevant variables for the dehydration process, such as temperature, time, drying velocity, relative moisture, etc. depending of method utilized. The optimizations of these variables will allow finding the best condition affecting less the chemicals characteristics of the vegetal material dehydrated. In this study the focus was to find the optimal conditions for keeping the

chemical and bioactive characteristics of the original fruit after a dehydration process. Different drying methods and temperatures were evaluated in this study and the murtilla fruit dried was compared to polyphenol and antioxidant characteristics of the raw fruit.

This research showed changes in the concentration and profile of polyphenols and antioxidant activity depending of the drying method employed. It is relevant to mentioned that many foods which contain anthocyanins are thermally processed prior to consumption and this process can greatly influence anthocyanin content in the final product (Giusti & Wrolstad, 2003). On the other hand, the group of enzymes, collectively called "phenolase" is responsible for browning of some fruits and vegetables. When the tissue is bruised, cut, peeled, diseases, or exposed to any number of abnormal conditions, the colour of the fruits or vegetables is changed. This enzyme group includes such diverse enzymes as phenoloxidase, cresolase, dopa oxidase, catecholase, tyrosinase, polyphenoloxidase, potato oxidase, sweet potato oxidase, and phenolase complex. The study of the activity of these enzymes is very important when is necessary to define the type of drying for using because two main reasons: (1) the undesirable development of off-color and (2) the formation of off-flavors. The control of enzymatic browning involves drying methods by heat applications. It is essential to control the heating time at high temperatures, so that the enzymes can be inactivated avoiding significant changes in flavor and texture. A balance should be worked out in terms of each particular raw material and desired food product. For this reason is essential standardizing in this level. At this respect, the temperature of the drying method used was studied in relation to the quality of the final product. Drying temperature affects both the products properties and the production costs. Generally, a low drying temperature is preferred during the production to conserve the properties of

the active ingredients. However, the practical drying temperature should also be at the level where complete drying of the initial drying could be achieved to obtain a reasonable production yield in a short period. Moreover the standardized of drying method is necessary for optimization and preserver the essential components, while heating techniques cause a loss of phenolic compounds (Patras et al. 2012, Mejias-Meza et al. 2010, Thakur et al.,2012). In this study, the dried murtilla fruit obtained by two methods showed significant differences in quantitative and qualitative polyphenols and different antioxidant capacities. Fruit dried obtained by freeze drying showed higher concentration of polyphenols compared with that obtained using the other method assayed (convective air drying method). Then, the effect of the hot air drying on total polyphenols and anthocyanins was different respect to freeze drying, but the two methods exhibits a favorable effect antioxidant activity on dried fruit respect to fresh. In some case is important to consider other standardization requeriment as pretreatments and others process parameters for dehydration (Mohanta *et. al*. 2011) or botanical standardizing of herbal extract (Nilfani *et al*. 2009).

Michalzyk et al. (2009) considered that air-drying caused different percentage losses in the analyzed components of the various fruits. The relatively large decrease in the polyphenol and anthocyanin content in bilberries may have been due to the relatively long drying period (72 h), causing a naturally occurring waxy layer on the fruits and the degradation of anthocyanins depends, among other factors, on polyphenol oxidase activity, organic acid content, sugar concentration, pH and the content of more reactive anthocyanins, e.g., cyaniding-3-glucoside (de Ancos et al., 2000). Changes in the polyphenols composition caused by hot air drying treatments showed increase in the aglycones compound such as gallic acid and catechin respect to fresh murtilla fruit

suggest that polyphenols are sensitive to heat and prolonged time treatment affecting their antioxidant activity (Mejia-Meza et al., 2008). In freeze drying method the increase in the total antioxidant and higher retention of total anthocyanins and polyphenols in the glycoside form is caused by the use of vacuum pressure and low temperature (Mejia-Meza et al., 2008). Lopez et al. (2010) considered that decreasing caused by hot air drying (50-90 ºC) in total phenolic content and antioxidant activity respect to fresh fruit is probably due to the generation of different antioxidant compounds which have a varying degree of antioxidant activity. Although, Fiol et al. (2013) observed that in kale (Brassica oleracea var. sabellica), the thermal processing of 2 and 4 h cooking showed tendency to generate higher antioxidant activity (increase of 12% and 20% for 'Winterbor' respectively 8% and 6% for 'Altmärker Braunkohl' cultivars). However, these authors showed that cooking affected flavonoids profile and distribution, due to the simultaneously loss of antioxidants, alteration of existing antioxidants as well as the formation of neo-formed antioxidant compounds, whereas the latter are able to compensate the loss. Determination of the antioxidant activity of isolated compounds from the HPLC was allowed to observe that the neo-formed compounds and reaction products exhibited an antioxidant capacity which was able to compensate the loss of the antioxidant capacity of the raw material. In this study, catechin is the compound that is present in higher content in murtilla fruit dried by any of methods evaluated. Respect to the fresh fruit, catechin concentration showed a no significant increased for dried fruit with convective hot-air drying at 80 ºC (HAD 80ºC) and freeze drying (FD) methods, and no significant decreased with hot-air drying at 65 ºC (HAD 65ºC). Gallic acid content trend to increase in the dried fruit by the three methods compared with the fresh fruit concentrations. Myricetin and kaempferol

concentrations decreased significantly in dried fruit respect to the fresh fruit, following the same behavior as quercetin 3-β-D-glucoside. Degradation of these compounds and others polyphenols no identified could result in increasing both gallic acid and catechin concentration in dried fruit obtained by HAD 65 ºC, HAD 80 ºC and FD respect to their fresh fruit. HAD 65 ºC, HAD 80 ºC and FD produced a decreasing in the total content of individual polyphenolic compounds determined by HPLC respect to the fresh murtilla fruit (208.2 mg/100 g d.w.), but the FD method showed the most higher retention of these components. The concentration of individual anthocyanins in dried fruit decreased respect to fresh murtilla and cyanidin-3-glucoside was the most stable compound and it was high retained in murtilla fruit dried by freeze drying. This method retained the highest proportion (21.2%) of the peonidin-3-glucoside respect to the concentration present in the fresh fruit, and the best retention, considering the sum of the individual anthocyanins, was obtained applying FD (23.5%) method. An increasing in both TEDPPH and TEABTS was observed when fresh fruit was dried by means of HAD 65 ºC, HAD 80 ºC and FD. The HAD 65 ºC and FD dried murtilla fruits showed similar antioxidant activity level measured by TEDPPH or TEABTS.

In this respect, and in accordance with the results obtained in this study, is possible to propose that standardization should consider the type of product dehydrated. For instance, if the target product will be develop for the nutraceuticals industry, the drying method must consider the conservation or increasing of the components that have more beneficial properties on a given health problem. In this context, Popovich et al. (2005) reported that the retention of the specific gingenosides changed depending of the different drying methods used; influencing the bioactivity of the final product.

In the case of food industry, the standardization of the drying method must consider the organoleptic or nutritional qualities to be highlighted in the final dried product and the restrictions on the operating conditions and final cost. At this respect, drying time, temperature and water activity influence the quality of the final product. Low temperatures generally have a positive influence on the quality of biological materials, but require longer processing times, which may have a detrimental effect and a higher cost (Raghavan et al., 2007). Determining the appropriate drying conditions is very important for achieving desirable concentration and profile of polyphenolic in the dehydrated murtilla fruit. For instance, drying temperature has influence on stability of polyphenol compounds found in murtilla fruit, but this parameter affect also the polyphenol oxidase and peroxidase activity positively affecting the compositions and bioactivity of the compounds in the dehydrated fruit. The chemicals modifications can occurs during the HAD and FD process affecting the polyphenolic profile due to chemicals changes induced by increments of temperature. Sadilova et al. (2006) observed that elderberry anthocyanin contents were very sensitive to thermal treatment. Patras et al. (2009) demonstrated that anthocyanins (cyanindin-3-glucoside and pelargonidin-3-glucoside) in blackberry and strawberry puree were significantly affected by thermal process treatments of 70 ºC during holding times of 2 minutes. Anthocyanins, in concordance with other polyphenols, are enzymatically degraded in the presence of polyphenol oxidase. Thermal degradation of anthocyanins results in the formation of polyphenolic degradation products; it is not clear if the formation of these components results in an overall reduction in antioxidant activity and the polyphenolic components formed may also possess antioxidant properties (Patras *et al*. 2010).

Polyphenol oxidases are mainly responsible for browning. However, because of both the high affinity of PPO for their natural substrates and the low levels of hydrogen peroxide in fruit, the extent of the involvement of POD in enzymatic browning has remained doubtful (Nicolas et al., 1994). In studies carried out in apple drying, higher degradation was observed for catechin and epicatechin with respect to chlorogenic acid in model solution and in apple juice (Lavelli *et al*., 2010). This can be explained by the fact that catechin and epicatechin are oxidized by the coupled oxidation mechanism with chlorogenic acid quinones and are also involved in the formation of addition products with quinones. Heavy losses of anthocyanins and chlorogenic acid occurred with milling and depectinization, which is believed to have been aggravated by native PPO. The anthocyanin profile changed drastically because of varying stability of individual pigments (Skrede *et al*., 2000). Freeze drying at low temperature and vacuum condition causes a low damage on the texture of the dried products and the degradation of vitamins and loss of aroma are significantly limited (Rahimi *et al*., 2013).

Rate of enzymatic browning reactions depends on temperature of drying, pH, moisture content of the product, time of heat treatment, the concentration and nature of the reactants. Rate increases with increasing temperature, and the increase is faster in systems with high sugar content. The PPO enzyme needs sufficient water to be active. Drying process inhibit the enzyme activity. Water activity influences non-enzymatic browning, lipid oxidation, degradation of vitamins, enzymatic reactions, and protein denaturation. Freeze drying retains original characteristics of the product and decrease water activity, and offers highest quality in a dry product compared to other drying methods (Rahimi et al. 2103). In relation to the results obtained in this study for the enzymatic activity is possible to confirm the evidence of the influence of temperature on

the decrease of the enzymatic activity of both enzymes in the processes of heat drying up to 65 ºC, unlike what happens when subjecting the fruit to the process drying by freeze drying. However, in the case of freeze-drying, although there is not significant decrease in enzyme activity with respect to fresh fruit, the enzymatic browning no occurs due to low water activity that is obtained in this process.

Conclusions

The information generated in this study gave directions about selection of genotypes and agricultural practices to enhance the polyphenolic compounds and antioxidant quality of murtilla (*Ugni molinae* Turcz) fruit.

The application of the hot-air drying at 65 ºC and 80 ºC and freeze drying showed a positive effect on antioxidant activity because these drying methods increased the TE_{DPPH} and TE_{ABTS} of the dried murtilla fruits respect to the fresh fruit, which could be explained by variations in the composition of polyphenolic compounds like gallic acid and catechin.

Freeze drying is the best method for retention of polyphenol compounds and antioxidant activity of the fresh murtilla fruit Red Pearl-INIA variety.

The combination of HPLC UV-VIS and HPLC-ESI-MS coupled to electro spray ionization (ESI) has proved to be an accurate method for the analysis of murtilla fruit Red Pearl INIA variety phenolic composition. Presence of epicatechin, quercetin 3-β-D glucoside, quercitrin and quercetin was corroborated in fresh murtilla fruits and the main anthocyanin compounds found in murtilla fruit were cyanidin-3-glucoside and peonidin-3-O-glucoside, the latter being more labile compounds to the action of high temperatures.

Freeze drying is the best method for retention of polyphenol compounds and antioxidant activity of the fresh murtilla fruit Red Pearl-INIA variety. Finally, this work can be useful for achieved defined steps and processes in the standardization of dehydrated products from murtilla fruit. In this context the recommendation that give is to prefer

freeze drying if the formulation is thinking a pharmaceutical product because this method is associated whit quality because low temperatures protect the active material during processing and is approved by regulatory authorities. When thinking in nutraceuticals or functional food, alternative drying process was possible to use , because in this type of product is necessary to evaluate to other aspects of interest concerned with the organoleptic, physical, and chemical changes that take place in the dried fruit.

The activity of the enzyme polyphenol oxidase and peroxidase from murtilla fruits variety Red Pearl INIA can be decreasing using a convective hot air drying at temperature of the 65 ºC or higher.

Finally, this work can be useful for achieve and define steps and processes in the standardization of dehydrated products from murtilla fruit; having in mind properties of compounds analyzed in conjunction with procedures utilized.

Perspectives

This work has contributed with important information about unknown aspects about the chemical composition and bioactivity of the compounds found in the murtilla (*Ugni molinae* Turcz) fruit. However, it is necessary to further investigation about the potential therapeutic value of these identified compounds. Also a relevant aspect is the scaling of drying methods from the laboratory to industry conditions for preserve the quality to this fruit and the polyphenol antioxidant activity normally associated with health benefits.

From the viewpoint of the stability of the compounds and enzymes identified in this work which affect the quality of the fruit, it would be interesting to assess future type of substrates that are generated by direct action of enzymes present in the fruit and during drying of the fruit.

Finally, is necessary propose new investigations in this fruit as potential functional ingredient and for the development of products for the food, nutraceuticals and cosmetics industry, keeping in view technological aspects related to the development of new products that incorporate murtilla fruit as active ingredient in pharmaceutical specialties.

REFERENCES

1. Aguilera, Y.; Dueñas, M.; Estrella, I.; Hernández, T.; Benítez, V.; Esteban, R.M.; Martín-Cabrejas, M.A. 2010 Evaluation of phenolic profile and antioxidant properties of Pardina lentil as affected by industrial dehydration. Journal of Agricultural and Food Chemistry, 58(18), 10101–10108.

2. Alfaro, S. Mutis, A. Palma, R. Quiroz, A. Seguel, I. Scheuermann E. 2013 " A comparative study of freeze drying and conventional drying techniques on the polyphenol compounds and antioxidant activity of the murtilla (*Ugni molinae* Turcz) fruits" (Manuscript submitted to International Journal of Food Science and Technology).

3. Armonk, P., Ruangviriyachai, C., Mahachai, R., Techawongstien, S., and Chantihai, S. 2010 Optimization and determination of polyphenol oxidase and peroxidase activities in hot pepper (Capsicum annuum L.) pericarb. International Food Research Journal 17. 385-392.

4. Böhm, V.; Kühnert, S.; Rohm, H.; Scholze, G. 2006. Improving the nutritional quality of microwave-vacuum dried strawberries: a preliminary study. Food Science and Technology International, 12(1), 67–75

5. Connor, A.M., Luby, J., Tong, C., Finn, C., Hancock, J. 2002. Genotype and environmental variation in antioxidant activity, total phenolic content and anthocyanins

content among blueberry cultivars Journal of the American Society for Horticultural Science 127, 89-97.

6. Chisari, M., Barbagallo, R. N., and Spagna, G. 2007. Characterization of polyphenol oxidase and peroxidase and influence on browning of cold stores strawberry fruit. Journal of Agriculture and Food Chemistry 55.(9). 3469-3476.

7. Fiol M. , Weckmüller A., Neugart s., Schreiner M., Rohn S., Krumbein A., W. Kroh L. 2013. Thermal-induced changes of kale's antioxidant activity analyzed by HPLC–UV/Vis-online-TEAC detection. Food Chemistry 138. 857–865.

8. Fortea, M.I., López-Miranda, S., Serrano-Martínez, A., Carreño, j., Núñez-Delicado, E. 2009. Kinetic characterization and thermal inactivation study of polyphenol oxidase and peroxidase from table grape (Crimson Seedless). Food Chemistry 113.1008-1014

9. Furtado, P.; Figueiredo, P.; Chaves das Neves, H.; Pina, F.**1993**. Photochemical and thermal degradation of anthocyanidins. Journal of Photochemistry and Photobiology A: Chemistry, 75(2), 113–118.

10. Gil-Izquierdo, A.; Gil, M.I.; Ferreres, F. 2002 Effect of processing techniques at industrial scale on orange juice antioxidant and beneficial health compounds. Journal of. Agriculture and Food Chemistry, 50(18), 5107–5114

11. González, E.M., De Ancos, B. and Cano, M.P. 1999. Partial Characterization of Polyphenol Oxidase Activity in Raspberry Fruits. Journal Agriculture Food Chemistry 47: 4068-4072.

12. González, E.M., De Ancos, B. and Cano, M.P. 2000 Partial characterization of Peroxidase and Polyphenol oxidase activities in Blackberry Fruits. Journal Agriculture Food Chemistry 48. 5459-5464

13. Howard, L., Clark, J., Brownmiller, C. 2003. Antioxidant capacity and phenolic content in blueberries as affected by genotype and growing season. Journal of the Science of Food and Agriculture 83, 1238-1247

14. Koca, I., Karadeniz, B. 2009. Antioxidant properties of blackberry and blueberry fruits grown in the Black Sea Region of Turkey. Scientia Horticulturae 121, 447-450.

15. Kwok, B.H.I.; Durance, T.; Kitts, D.D. 2004. Dehydration techniques affect phytochemical contents and free radical scavenging activities of Saskatoon berries (*Amerlachier alnifolia* Nutt). Journal of Food Science, 69(3), SNQ122–SNQ126.

16. **Lavelli V., Caronni P. 2010**. Polyphenol oxidase activity and implications on the quality of intermediate moisture and dried apples. European Food Research and Technology. 231 (1).93-100

17. Leusink, G. J.; Kitts, D.; Yaghmaee, P.; Durance, T. **2010**. Retention of antioxidant capacity of vacuum microwave dried cranberry. Journal of Food Science 75(3), 311–316.

18. López, J., Uribe, E., Vega-Gálvez, A., Miranda, M., Vergara, J., & Di Scala, K. 2010 . Effect of air temperature on drying kinetics, vitamin C, antioxidant activity, total phenolic content, non- enzymatic browning and firmness of blueberries variety O'Neil. Food and Bioprocess Technology, 3(5), 772–777.

19. Markakis, P. Stability of anthocyanins in foods. In *Anthocyanins as Food Colors*; Markakis, P., Ed.; 1982. Academic Press: New York, , 163–180.

20. Mejia-Meza, E.I.; Yañez, J.A.; Remsberg, C.M.; Takemoto, J.K.; Davies, N.M.; Rasco, B.; Clary, C. 2010 Effect of dehydration on raspberries: polyphenol and anthocyanin retention, antioxidant capacity, and antiadipogenic activity. Journal of Food Science, 75 (1), 5–12

21. Núñez-Delicado, E., Sojo, M., García-Carmona, F., Sánchez-Ferrer, A., (2003) Partial purification of latent persimmon fruit polyphenol oxidase. Journal of Agriculture and Food Chemistry. 51: 2058-2063.

22. Patras A., Brunton N. P., Da Pieve S., Butler F. 2009. Impact of high pressure processing on total antioxidant activity, phenolic, ascorbic acid, anthocyanin content and colour of strawberry and blackberry purées. Innovative Food Science and Emerging Technologies 10: 308–313

23. Patras A., Brunton N. P., O'Donnell C., Tiwari B.K. 2010. Effect of thermal processing on anthocyanin stability in foods; mechanisms and kinetics of degradation.Review. Trends in Food Science & Technology 21: 3-11.

24. Popovich DG, Hu C, Durance TD, Kitts DD. 2005: Retention of ginsenosides in dried ginseng root: Comparison of dying methods. Journal Food Science.; 70(6): S355–S358.

25. Rahimi J., Singh A., Adewale P. O., Adedeji A. A.,. Ngadi M. O., Raghavan V. 2013. Effect of Carboxylmethyl Cellulose Coating and Osmotic Dehydration on Freeze Drying Kinetics of Apple Slices. Food 2: 170-182.

26. Raghavan G. S. V, Orsat V. 2007 Recent advances in drying of biomaterials for superior quality bioproducts. Asia-Pacific Journal of Chemical Engineering 2: 20–29

27. Ruiz, A.; Hermosin-Gutierrez, I.; Mardones, C.; Vergara, C.; Herlitz, E.; Dorau, C.; Winterhalter, P.; von Baer, D. 2010. Polyphenols and antioxidant activity of calafate (*Berberis microphylla*) fruits and other native berries from southern Chile. Journal of Agriculture Food Chemistry, 58(10), 6081–6089

28. Sadilova E., Stintzing F. C., Carle R. 2006. Thermal degradation of acylated and nonacylated anthocyanins. Food Chemistry and Toxicology. 71 (8) 504-512.

29. Serrano-Martinez, A., Fortea M.I., Del Amor F.M., Nuñez –Delicado E. (2008) Kinetic characterization and thermal inactivation study of partially purified red pepper (Capsicum annuum L.) peroxidase. Food Chemistry 107:193-199.

30. Shene, C.; Reyes, A.; Villarroel, M.; Sineiro, J.; Pinelo, M.; Rubilar, M. **2009**. Plant location and extraction procedure strongly alter the antimicrobial activity of murtilla extracts. *European Food Research and Technology*, 228(3), 467–475

31. Skrede G, Wrolstad RE, Durst RW. 2000. Changes in anthocyanins and polyphenolics during juice pro- cessing of highbush blueberries (Vaccinium corym-bosum L.). Journal of Food Science 65(2):357-364.

32. Sochor, J., Zitka, O., Skutkova, H., Pavlik, D., Babula, P., Krska, B., Horna, A., Adam, V., Provaznik, I., Kizek, R. 2010. Content of phenolic compounds and antioxidant capacity in fruits of apricot genotypes. Molecules 15, 6285-6305

33. Vijayakumari, K.; Siddhuraju, P.; Pugalenthi, M.; Janardhanan, K. **1998** Effect of soacking and heat processing on the levels of antinutrients and digestible proteins in seeds of *Vigna aconitifolia* and *Vigna sinensis*. Food Chemistry, 63(2), 259–264.

34. Wojdyło, A.; Figiel, A.; Oszmianski, J. 2009. Effect of drying methods with the application of vacuum microwaves on the bioactive compounds, color, and antioxidant

activity of strawberry fruits. Journal of Agricultural and Food Chemistry, 57(4), 1337–1343.

35. Zhu, Y., Pan, Z., McHugh, T., Barrett, D. (2010) Processing and quality characteristics of apple slices processed under simultaneous infrared dry-blanching and dehydration with intermittent heating. Journal of Food Engineering 97: 8–16.

ANNEXE

SCIENTIFIC PRODUCTIVITY

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Publications:

1. “Effects of packaging and preservation treatments on the shelf life of murtilla fruit (Ugni molinae Turcz) in cold storage” Erick Scheuermann, Mónica Ihl, Lisette Beraud, Andrés Quiroz, Sonia Salvo, **Susana Alfaro**, Rubén O. Bustos and Ivette Seguel. Packaging Technology and Science, DOI:10.1002/pts.2014.

2. “Study of polyphenols content and antioxidant activity in murtilla (Ugni molinae Turcz) fruits in function of genotype and harvest season” **Alfaro, S**. Mutis, A. Palma, R. Quiroz, A. Seguel, I. Scheuermann E. (Publishing in Journal of Soil Science and Plant Nutrition, 13(1), 67-78, 2013).

3.”A comparative study of freeze drying and conventional drying techniques on the polyphenol compounds and antioxidant activity of the murtilla (*Ugni molinae* Turcz) fruits” **Susana Alfaro**, Ana Mutis, Andrés Quiroz, Ivette Seguel, and Erick Scheuermann (Manuscript submitted to International Journal of Food Science and Technology).

4.”HPLC-ESI-MS/MS for analysis of polyphenol and anthocyanin compounds in fresh and dried murtilla fruit red pearl variety” (Manuscript in preparation)

5. “Caracterización parcial de la actividad enzimática de polifenoloxidasa (PPO) en frutos frescos y deshidratados de murtilla (Ugni molinae Turcz) de la variedad Red Pearl-INIA” **Alfaro, S**., Mutis, A., Quiroz, A., Scheuermann, E. Revista de Farmacología de Chile 2012 (5) 3: 70.

6. “Partial characterization of enzymatic activity of polyphenol oxidase (PPO) and peroxidase (POD) in fresh and dried murtilla fruits (*Ugni molinae* TURCZ) Red Pearl-

INIA variety" Alfaro, S., Mutis, A., Quiroz, A., Scheuermann, E. (Manuscript in preparation).

Congresses

1. **Alfaro, S.,** Scheuermann, E., Quiróz, A Seguel, I. y Montenegro A "Estudio del contenido de polifenoles y actividad antioxidante de frutos de murtilla (Ugni molinae Turcz) en función del genotipo y año de cosecha". VII Simposio de recursos genéticos para América Latina y el Caribe. 28 ,29 y 30 de Octubre 2009, Pucón Chile.

2. **Alfaro, S.,** Scheuermann, E., Quiróz, A Seguel, I. y Montenegro A. "Efecto de la congelación y tratamiento térmico en el contenido de polifenoles y actividad antioxidante de frutos de murtilla (Ugni molinae Turcz).XVII Congreso Nacional de Ciencias y Tecnología de los Alimentos "Chile en la Era de los Alimentos Funcionales: Regulación y Proyecciones" y III Congreso del Caribe y I latinoamericano "Higiene y Calidad en la Agricultura y la Alimentación" 4, 5 y 6 de Noviembre 2009, Iquique, Chile.

3. **Alfaro, S.,** Scheuermann, E., Quiróz, A. "Métodos para determinación de capacidad antioxidante en plantas nativas chilenas" 1º Workshop y 4º Curso internacional avances en ciencias y tecnologías de recursos naturales 23, 24 y 25 de Noviembre, 2009 Pucón, Chile

4. Velazquez R., Mutis A., **Alfaro S**., Scheuermann E., Quiroz A., Hormazabal E. "Characterization of antioxidant activity of leaves and flowers of Bougainvillea sp." 2º Workshop y 5º Curso internacional avances en ciencias y tecnologías de Recursos Naturales 23, 24 y 25 de Octubre2010, Pucón, Chile.

5. 2º Workshop Internacional “Compuestos Bioactivos y Recubrimientos Comestibles” Universidad De La Frontera 11 y 12 de Noviembre de 2010.

6. **Alfaro, S**., Scheuermann, E., Mutis, A., Quiroz, A "Polyphenols compound profile determined in a fresh and dehydrated Red Pearl variety of murtilla fruit".3nd International Workshop Advances in science and Technology of Bioresources. November 2-3-4 2011 Universidad De La Frontera, Pucón Chile.

7 **Alfaro, S**., Mutis, A., Quiroz, A., Scheuermann, E. "Partial characterization of enzymatic activity of Polyphenol oxidase (PPO) in fresh and dehydrated murtilla fruit (Ugni molinae Turcz) Red Pearl-INIA variety" XXXIV Congreso Anual de la Sociedad de Farmacología de Chile ,21 - 24 Noviembre 2012, Pucón Chile.