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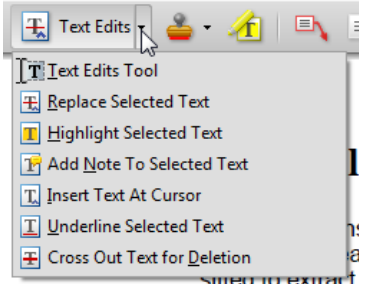

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Accumulation patterns of phenolic compounds during fruit growth and ripening of *Berberis buxifolia*, a native Patagonian species

Miriam E Arena[a], Pablo Postemsky[b] and Nestor R Curvetto[b]*

[a]*Laboratorio de Recursos Agronómicos, Centro Austral de Investigaciones Científicas, Ushuaia, Argentina;*
[b]*Laboratorio de Biotecnología de Hongos Comestibles y Medicinales, CERZOS, Bahía Blanca, Argentina*

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The accumulation patterns of phenolic compounds during fruit growth and ripening of *Berberis buxifolia* and their correlations were studied to determine the optimal time and conditions needed to obtain maximum phenolic content. Anthocyanin content increased from 1.7 to a maximum of 752.7 mg·100 g^{-1} FW at the end of ripening, while the flavonoid content was maximal in unripe fruits [604.0 mg (+)-catechin equivalents·100 g^{-1} FW]. Total phenolic compounds decreased from 968.1 to a minimum of 746.3 mg gallic acid equivalents·100 g^{-1} FW just as soluble solids started to accumulate; a maximum of 1522.9 mg gallic acid equivalents·100 g^{-1} FW was attained at the end of ripening. Both variables (1,1-diphenyl-2-picrylhydrazyl scavenging effect and reducing power) were maximum in unripe fruits, and decreased during the ripening period, although the 1,1-diphenyl-2-picrylhydrazyl scavenging effect increased again towards the end of this period. The accumulation patterns of phenols varied depending on the specific group of compounds considered, and could be correlated with fruit quality.

Keywords: Argentina; indigenous flora; Small fruits; black–blue berries; anthocyanins; flavonoids; antioxidant activity

Introduction

During recent decades, research on healthy practices has been particularly focused on the beneficial effects that can result from the adoption of a diet rich in fruits and vegetables. In fact, these foods can help reduce the risk of chronic, degenerative and oxidative-stress-mediated diseases, such as cancer and cardiovascular and neurodegenerative diseases (Roussos et al. 2009). As a result, attention is now also directed toward many small fruits that are considered to be a source of organic and inorganic nutrients and metabolic-regulating factors. In addition, these fruits are particularly regarded because of their nutraceutical properties as functional foods (Béliveau & Gingras 2005; Kuskoski et al. 2005). Indeed, small fruits of cultivated *Ribes*, *Rubus* and *Vaccinium* species are an excellent source of natural products with antioxidant properties (Deighton et al. 2002), which are mainly attributed to their high polyphenolic content, especially phenolic acids, flavonoids and anthocyanins, in addition to other important natural antioxidants like ascorbic acid (vitamin C) (Béliveau & Gingras 2005). The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers; they also have metal chelation potential (Kähkönen et al. 1999). The selection of any standardized method to evaluate antioxidant activity should meet some 'ideal' requirements (Prior et al. 2005). The 1,1-

*Corresponding author. Email: arena1@infovia.com.ar

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diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity (Shimada et al. 1992), the reductive ability for Fe^{3+}–Fe^{2+} transformation (Oyaizu 1986) in the presence of plant extracts and quantification of phenols through the Folin–Ciocalteu reagent have been shown to be simple, rapid, sensitive and precise techniques, while exhibiting a good reproducibility. The phenolic content and antioxidant activity show good correlation (Ferreyra et al. 2007; Turkoglu et al. 2007), highlighting the importance of the total phenolic content for the antioxidant activity found in several plant materials (Turkoglu et al. 2007).

Phenolics are much more than simple antioxidants and focus is currently turning toward other modes of action. It is important to understand how phenolics and their subgroups (e.g. flavonoids) change during fruit development, and how to measure their activity. Phenolics can be classified as flavonoid or non-flavonoid compounds. Flavonoids make up a significant portion of the phenolic compounds in small fruits, for example, grapes (Conde et al. 2007), and include several classes, such as proanthocyanidins (tannins), anthocyanins and flavan-3-ol monomers (i.e. catechins). Anthocyanins are the major phenolic components of soft berry fruits, and their antioxidant activity has been found to be closely related to total phenolic content (Deighton et al. 2002). These substances play a role in plant defence and they appear to possess beneficial effects against various diseases (Hou 2003). The biosynthesis of flavonoids is a tissue-specific process which is developmentally regulated. It is thought that these compounds play a variety of functions in plants, providing protection against UV radiation, participating in the defence against pathogens, providing a chemical guide to pollinating insects in flowers, being responsible for flower and fruit pigmentation, as well as playing an essential role in reproduction. In addition, flavonoids also contribute to the quality characteristics of fresh and processed food products including astringency, texture, taste and colour (Vvedenskaya & Vorsa 2004). Preharvest factors such as the plant genetic background, the environmental conditions during plant growth, the cultural practices employed and the stages of fruit ripening, all influence the synthesis of phenolic compounds (Kähkönen et al. 2001; Ferreyra et al. 2007) and their antioxidant capacity (Roussos et al. 2009), with ripening stages being even more relevant when immature berries are used, for example, cranberries in the juice industry (Çelik et al. 2008).

Large areas with indigenous flora which include the *Berberis* L. genus exist in Patagonia (Monge et al. 2000). At present, commercial *Berberis* orchards are being planted: this crop has economical potential, not only for flavour and tasteof the fruit, but also because of its putative high antioxidant properties. In fact, black–blue fruits are now consumed fresh, in marmalades and jams, in non-alcoholic beverages and in ice creams.

Berberis buxifolia is a spiny evergreen shrub with a large distribution in Patagonia and particularly in Tierra del Fuego (Moore 1983; Orsi 1984). The kinetic growth behaviour of the fruit and the evolution of chemical properties during the fruiting period and different growing seasons have been studied in this species (Arena & Curvetto 2008), and recent research on the *Berberis* genus has studied the phenol content in leaves (Koncic et al. 2010), roots (Surveswaran et al. 2007; Tomosaka et al. 2008) and fruits (Ruiz et al. 2010). The beneficial effect of *B. buxifolia* fruits on oxidative stress induced by chloramphenicol on human blood has also been reported (Albrecht et al. 2010).

However, there is a lack of information on the important role of preharvest factors on the evolution of secondary metabolism during fruit growth and ripening in *B. buxifolia*. Thus, the aim of this work was to study the patterns of accumulation of anthocyanin, flavonoid and phenol contents and antioxidant activity during growth and ripening of *B. buxifolia* fruits, and their correlations with

fruit quality, to understand the optimal time and conditions needed to obtain maximum phenolic content for use in the preparation of extracts for nutraceutical purposes.

Materials and methods

Chemicals and reagents

Sodium nitrite (Merck), aluminium chloride hexahydride (AppliChem), (+)-catechin (Sigma-Aldrich), sodium carbonate (Merck), Folin–Ciocalteau reagent (Sigma-Aldrich), gallic acid (Sigma-Aldrich), DPPH (Sigma-Aldrich), butylated hydroxyanisole (Sigma-Aldrich), α-tocopherol (Sigma-Aldrich), sodium phosphate buffer (pH 6.6, Sigma-Aldrich), potassium ferricyanide (Sigma-Aldrich), trichloroacetic acid (w/v, Anedra) and ferric chloride hexahydrate (Anedra) were purchased from Global Lab S.A. (Buenos Aires, Argentina). Ascorbic acid (Cicarelli) and all other chemicals and solvents were of analytical grade and obtained from Chemit Argentina S.R.L (Buenos Aires, Argentina).

Plant material and sampling

Berberis buxifolia plants ($n = 50$, with a mean height of 0.91 ± 0.15 m, with the full flower phase occurring at mid-spring for 1–2 weeks), were growing naturally in a representative area located near Ushuaia city, 54°48′S, 68°19′W (Tierra del Fuego, Argentina). Healthy and sun-exposed fruits (300 g) were collected manually every 14 days from 13 November 2007 (14 days after full flower phase; AFFP) to 4 March 2008 (126 days AFFP), and bulked as one sample for each date. The mean, maximum and minimum daily air temperatures from October to March during the 2007–2008 growing season were 8.6, 13.0 and 4.6 °C, respectively, these values being comparable with those described earlier by Arena & Curvetto (2008).

Fruit growth phase definition and characterization

Weight

Fresh and dry fruit weights were recorded the entire fruiting period (from days 14 to 126 AFFP) to define the fruit growth phases. In order to characterize the last fruit growth phases and ripening, the following variables were analysed from day 70 to 126 AFFP.

Firmness

Firmness was recorded using a digital penetrometer, Wagner Instruments Model FDI 2 (0.001 a 1 kgf), with tips of 1 mm diameter. AQ4

Soluble solids and total titratable acidity

Soluble solids were determined in fruit juice using an ATAGO N1-α refractometer with a 0–32°Brix measurement range with 0.2°Brix increments, and no temperature compensation. Total titratable acidity was measured by titration with 0.1 N NaOH solution. Total titratable acidity was expressed as malic acid, the most abundant organic acid in *B. buxifolia* fruits. The soluble solids/total titratable acidity ratio was also determined.

Fruit colour evolution and anthocyanin content

The percentage of fruit surface having a purple colour was recorded, while anthocyanin content was quantified using the pH differential method (Giusti & Wrolstad 2001). Samples (5 g) of initially frozen fruits were extracted for 24 h in 50 mL 0.1% HCl–MeOH solution at 4°C. Aliquots were then diluted from 1:5 to 1:80 to meet the Lambert–Beer's law, with either 0.025 M KCl (pH 1) or 0.4 M sodium acetate buffer (pH 4.5). Absorbance values at 510 and 700 nm were recorded with a Shimadzu 1203 UV–Vis spectrophotometer. Anthocyanin content was determined on the basis of a molar

extinction coefficient of 26,900 $M^{-1} \cdot cm^{-1}$ and a molecular mass of 449.2 g for cyanidin 3-glucoside. Anthocyanin content was determined as

$$(\text{mg} \cdot 100\,\text{g}^{-1}\text{fresh} - \text{frozen furit weight}) = (A \times \text{molecular mass} \times \text{dilution factor} \times \text{initial volume}/\varepsilon \times \text{sample weight}) \times 100,$$

where A (absorbance) $= (A_{510} - A_{700})_{\text{pH1.0}}$-$(A_{510} - A_{700})_{\text{pH4.5}}$. Values were expressed in mg anthocyanin·100 g^{-1} fresh weight (FW), mg anthocyanin·g^{-1} dry weight (DW) and mg anthocyanin per fruit.

Preparation of extracts from fruits and extraction yield

Ten grams of freeze-dried fruits were extracted with 100 mL of methanol at 25°C for 24 h with continuous stirring. The residue was later extracted twice with additional volumes of 100 mL methanol for 48 h, and the three extracts were combined. The extraction yield was determined gravimetrically from the methanolic extracts from two freeze-dried fruit portions (10 g) carried out in triplicate. The extracts were then used to determine the flavonoid and total phenolic contents, as well as the DPPH scavenging activity and reducing power, as described below.

Flavonoid content

Flavonoid content was determined according to Zhishen et al. (1999). Briefly, each methanolic extract (1.5 mg·250 μL^{-1} methanol solution) was mixed with 1.5 mL of distilled water and 75 µL of 50 g·L^{-1} sodium nitrite. After 5 min, 150 µL of 100 g·L^{-1} aluminium chloride hexahydride was added. After 6 min, 500 µL of 1 M sodium hydroxide and 275 µL of distilled water were added to the mixture. After vigorous mixing, the absorbances of the resulting solutions were measured against a blank at 510 nm using a Shimadzu 1203 UV–Vis spectrophotometer. The flavonoid content was obtained from the calibration curve prepared using (+)-catechin as standard and was expressed as mg (+)-catechin equivalents·100 g^{-1} FW, mg (+)-catechin equivalents·g^{-1} DW and mg (+)-catechin equivalents per fruit.

Phenol content

Phenol content was determined according to the Folin–Ciocalteau method (Taga et al. 1984), with some modifications. Briefly, 100 µL of each methanolic extract [20 mg·5 mL^{-1} of 1.3% HCl in methanol/water (60:40 v/v) solution] was extracted and 2 mL of 2% (w/v) aqueous sodium carbonate solution and 100 µL of 50% Folin–Ciocalteau reagent were added to the mixture. After standing for 30 min at 24°C, the absorbances of the resulting coloured solutions were measured against a blank at 750 nm using a Shimadzu 1203 UV–Vis spectrophotometer. The phenol content was obtained from the calibration curve prepared using gallic acid as standard, and expressed as mg gallic acid equivalents·100 g^{-1} FW, mg gallic acid equivalents·g^{-1} DW and mg gallic acid equivalents per fruit.

Scavenging activity on DPPH radicals

Each methanolic extract (0.0–1.0 mg·mL^{-1}) in methanol (2 mL) was mixed with 0.25 mL of a methanolic solution containing DPPH radicals, resulting in a final concentration of 0.1 mM DPPH. The mixture was shaken vigorously and allowed to stand in darkness for 30 min. The absorbance was then measured at 517 nm against a blank in a Shimadzu 1203 UV–Vis spectrophotometer (modified method of Shimada et al. 1992). A low absorbance value for the reaction mixture indicates high free radical scavenging activity. Ascorbic acid, butylated hydroxyanisole and α-tocopherol were used as antioxidant standards. The capability of

methanolic extracts to scavenge DPPH radicals was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = \left[\left(A_{\text{blank}} - A_{\text{sample}}/A_{\text{blank}}\right) \times 100\right]$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance of the reaction mixture for the compound scavenging effect. To avoid anthocyanin interference, A_{sample} was calculated by subtracting the absorbance of the fruit extracts in the absence of DPPH. The values for the DPPH scavenging effect were calculated for all methanolic extracts and antioxidant standard concentrations under study. The EC_{50} value ($mg \cdot mL^{-1}$) is the effective concentration of methanolic extract at which the antioxidant activity was 50%; it was obtained by interpolation from linear regression analysis between the sample concentration (0–0.5 $mg \cdot mL^{-1}$) and the DPPH scavenging effect.

Reducing power

The reducing power was determined following the method of Oyaizu (1986). Each methanolic extract (0.0–5.0 $mg \cdot mL^{-1}$) in methanol (2.5 mL) was mixed with 2.5 mL of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide, and the mixture was incubated at 50 °C for 20 min in darkness. Then, 2.5 mL of 10% trichloroacetic acid (w/v) was added and the mixture was centrifuged at 3000 rpm for 10 min. The upper layer (5 mL) was mixed with 5 mL of deionized water and 0.5 mL of 0.1% ferric chloride hexahydrate, and the absorbance was measured at 700 nm against a blank using a Shimadzu 1203 UV–Vis spectrophotometer. A high absorbance indicates a high reducing power. Ascorbic acid, butylated hydroxyanisole and α-tocopherol were used as antioxidant standards. The reducing power (RP) was calculated using the following equation:

$$(RP)(\%) = \left[\left(100 - A_{\text{maximum}} - A_{\text{sample}}/A_{\text{maximum}}\right) \times 100\right]$$

where A_{maximum} is the maximum absorbance value obtained with the colour solution developed by the reaction of ferricyanide salt with the compound under testing and A_{sample} is the absorbance value obtained with the colour solution developed by the reaction of ferricyanide salt with the compound under testing for each concentration. The values of reducing power (%) were calculated for all methanolic extracts and antioxidant standard concentrations under study. The EC_{50} value ($mg \cdot mL^{-1}$) is the effective concentration of methanolic extract at which the reducing power was 50%; it was obtained by interpolation from linear regression analysis between sample concentration (0–1 $mg \cdot mL^{-1}$) and reducing power.

Statistical analysis

Data were analysed statistically by one-way analysis of variance (ANOVA), and means were then separated using the Tukey multiple range test at $P < 0.05$. Linear coefficient correlations and regression analysis were performed between some pairs of variables.

Results and discussion

Fruit growth phase definition

The time course of fruit growth (fresh weight basis) from day 14 to day 126 AFFP shows a typical double sigmoid growth curve, as reported previously for this species (Arena & Curvetto 2008), with the highest fruit growth rate around days 42 and 56 AFFP (first phase of rapid increase), reaching a plateau (second lag phase) by day 70 AFFP (300.2 mg), which extended to day 84 AFFP (Figure 1A). During the third phase of rapid increase to day 112 AFFP (when the fruits also ripen) the highest

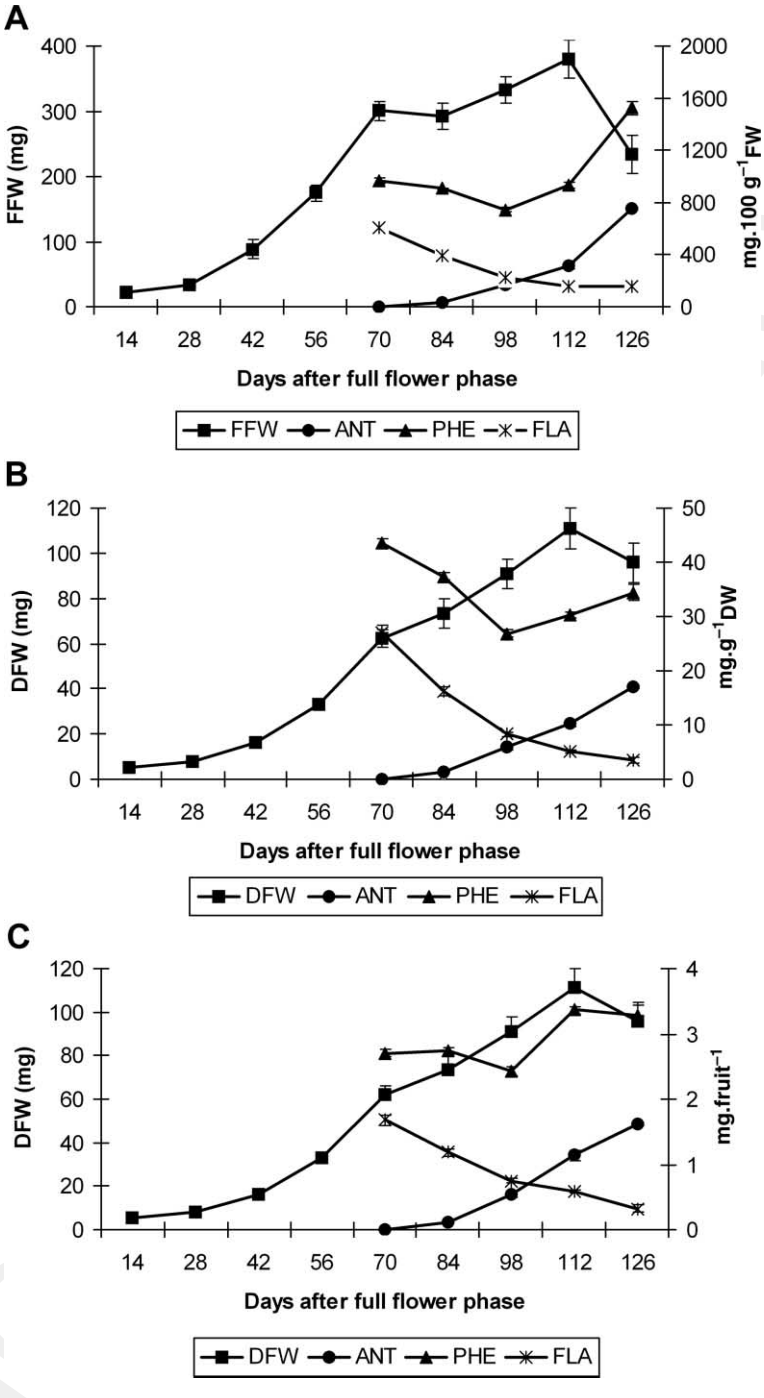

Figure 1. Phenolic compound evolution in the days after the full flower phase in *B. buxifolia* fruits. **A,** Expressed on the basis of fresh weight (mg·100 g^{-1} FW). **B,** Expressed on the basis of dry weight (mg·g^{-1} DW). **C,** Expressed on the basis of per unit fruit (mg·fruit^{-1}). ANT, anthocyanin content; PHE, phenol content; FLA, flavonoid content; FFW, fresh fruit weight; DFW, dry fruit weight ($n = 6$). Error bars represent $\pm$ 1 standard error of the mean.

biomass was obtained (379.8 mg), a decrease in fresh biomass then occurred towards the end of the growing season. The time course evolution on the basis of dry fruit weight also showed a double sigmoid curve with a less pronounced plateau, and increased slightly to reach an average biomass of 111.1 mg·fruit^{-1} at day 112 AFFP (Figure 1B). Fruit firmness changed significantly during the fruiting period (Table 1), with a maximum at days 70–84 AFFP, then decreasing towards the end of this period, with mean values of 0.15 and 0.19 kgf at days 112 and 126 AFFP, respectively.

Soluble solids and total titratable acidity

Soluble solids increased significantly during the ripening phase, to reach a maximum (30.6 °Brix) at day 126 AFFP (Table 1). Whereas total titratable acidity decreased significantly during the ripening period (Table 1) from a maximum (6.3%) at day 70 AFFP. As expected, the ratio soluble solids/total titratable acidity increased significantly during the ripening phase, with the maximum (13.9) at day 126 AFFP (Table 1).

Fruit colour evolution and anthocyanin content

The percentage of the fruit surface having a purple colour changed significantly during the study period (Table 1), increasing from day 70 AFFP (20%) and attaining the highest values at days 112 (98.7%) and 126 (100%) AFFP, respectively. Anthocyanin content increased significantly during the study period in terms of fresh weight ($F = 792.7$, $P = 0.000$), dry weight ($F = 284.36$, $P = 0.000$) and on a per fruit basis ($F = 296.87$, $P = 0.000$) (Figure 1A–C), reaching a maximum at day 126 AFFP in terms of both fresh and dry weight (752.7 mg·100 g^{-1} FW and 16.9 mg·g DW^{-1}, respectively), and similarly on a per fruit basis (1.6 mg·fruit^{-1}). Thus, in *B. buxifolia*, biosynthesis of anthocyanin compounds starts after a pronounced lag phase from fruit setting (day 84 AFFP), increases markedly during the ripening period when fruit biomass is at maximum values, and continues to increase until the end of the fruiting period, even though fruit biomass decreases. The precise conditions that initiate anthocyanin synthesis are not well established, but it has been argued that sugar accumulation provides the substrate needed for anthocyanin synthesis (Jackson 2008). In *B. buxifolia* fruits, the remarkable positive and significant correlation between anthocyanin accumulation and colour, and soluble solids and soluble solids/total titratable acidity ratio is consistent with this view (Table 2). The anthocyanin content found in *B. buxifolia* fruits at maturity (753 mg·100 g^{-1} FW) was comparable with that obtained in earlier growing seasons (Arena & Curvetto 2008), and similar to that reported by Ruiz et al. (2010)

Table 1. Firmness (kgf), colour (%), soluble solids (°Brix), total titratable acidity (%) and soluble solids/total titratable acidity ratio in *Berberis buxifolia* fruits in the days after the full flower phase.

Days AFFP	F	C	SS	TTA	SS/TTA
70	0.24ab	20.00c	10.96e	6.31a	1.72e
84	0.27a	56.25b	13.36d	4.03b	3.32d
98	0.21ab	93.75a	14.86c	2.79c	5.33c
112	0.15b	98.75a	19.93b	2.45cd	8.10bc
126	0.19ab	100.0a	30.56a	2.19e	13.95a
F (P)	2.95 (0.029)	33.44 (0.000)	11856.24 (0.000)	417.43 (0.000)	400.81 (0.000)

Note: AFFP, after the full flower phase; F, firmness; C, colour; SS, soluble solids; TTA, total titratable acidity; SS/TTA, soluble solids/total titratable acidity ratio. ANOVA *F(P)*, *F* statistic and probability at $P = 0.05$. Values followed by different letters in each column are significantly different with the Tukey multiple range test at $P < 0.05$ ($n = 6$).

Table 2. Linear correlation coefficients (*r*) between pairs of variables.

	ANT FW	ANT DW	FLA FW	FLA DW	PHE FW	PHE DW	SE	RP
FFW	−0.315^{ns}		−0.182^{ns}		−0.488**		−0.347^{ns}	−0.202^{ns}
DFW		0.534**		−0.636**		−0.651**	−0.380**	−0.579**
F	−0.373^{ns}	−0.417^{ns}	0.357^{ns}	0.382^{ns}	−0.086^{ns}	0.456*	0.329^{ns}	0.405*
C	0.676**	0.770**	−0.901**	−0.907**	0.213^{ns}	−0.778**	−0.683**	−0.844**
SS	0.993**	0.975**	−0.724**	−0.761**	0.822**	−0.421**	−0.070^{ns}	−0.505^{ns}
TTA	−0.704*	−0.801**	0.959**	0.982**	−0.223^{ns}	0.894**	0.647*	0.921**
SS/TTA	0.984**	0.986**	−0.782**	−0.808**	0.742*	−0.531^{ns}	−0.163^{ns}	−0.562^{ns}
ANT FW	1.000		−0.719**		0.828**		−0.086^{ns}	−0.480*
ANT DW		1.000		−0.473*		−0.388^{ns}	−0.226^{ns}	−0.599**
FLA FW			1.000		−0.276^{ns}		0.653**	0.894**
FLA DW				1.000		0.843**	0.638**	0.896**
PHE FW					1.000		0.344^{ns}	0.005^{ns}
PHE DW						1.000	0.843**	0.910**
SE							1.000	0.862**
RP								1.000

Note: ANT, anthocyanin; FLA, flavonoid; PHE, phenol; FW, fresh weight; DW, dry weight; SE, scavenging effect on 1,1-diphenyl-2-picrylhydrazyl radicals; RP, reducing power; FFW, fresh fruit weight; DFW, dry fruit weight; F, firmness; C, colour; SS, soluble solids; TTA, total titratable acidity; SS/TTA, soluble solids/total titratable acidity ratio (SS/TTA). ^{ns}, nonsignificant; significant at $*P<0.05$; $**P<0.01$.

for *Berberis microphylla* from southern Chile. However, the anthocyanin content found in this work was higher than that of other reddish-purple berries, such as blackcurrants (350 mg·100 g^{-1} FW), raspberries (55-60 mg·100 g^{-1} FW), strawberries (40 mg·100 g^{-1} FW) (Lister et al. 2002), and blueberries (1.2 mg·g^{-1} FW) (Zheng & Wang 2003).

Fruit methanolic extract yield

The yield of methanolic extracts increased significantly during the studied fruiting period ($F=34.61$, $P=0.000$). Minimum values were obtained at days 70 and 84 AFFP (379.1 and 365.7 mg dry extract·g^{-1} DW, respectively), increasing by day 98 to 421.9 mg dry extract·g^{-1} DW and reaching the highest level from days 112 to 126 AFFP (453.3 and 443.2 mg dry extract·g^{-1} DW, respectively). The yield of methanolic extracts was positive and significantly correlated with the anthocyanin content ($r=0.779$; $P=0.007$).

Flavonoid content

Flavonoid content decreased significantly during the fruiting period in terms of fresh weight ($F=85.6$, $P=0.000$), dry weight ($F=147.5$, $P=0.000$) and on a per fruit basis ($F=83.23$, $P=0.000$) (Figure 1A–C). The highest content was obtained at day 70 AFFP [604.0 mg (+)-catechin equivalents·100 g^{-1} FW, 27.1 mg (+)-catechin equivalents·g^{-1} DW and 1.7 mg·fruit^{-1}], whereas the lowest contents were measured at day 126 AFFP [152.3 mg (+)-catechin equivalents·100 g^{-1} FW and 3.4 mg (+)-catechin equivalents·g^{-1} DW], following the same behaviour on a per fruit basis (0.3 mg·fruit^{-1}). In the study by Cheel et al. (2007), flavonoid content ranged between 30.0 and 123.2 mg quercetin equivalents·100 g^{-1} FW for *Fragaria* sp. fruits.

The synthesis of flavonoids (detected at 510 nm, like quercetin when they form complexes with aluminium) tends to decrease and may cease during ripening, as found for grapes, and

with non-flavonoid phenolics (Jackson 2008). This pattern of flavonoid accumulation and subsequent decline during ripening suggests the degradation of flavonoids and their utilization in the biosynthesis of other compounds and/or association with other cellular compounds by stable covalent links. In *B. buxifolia* fruits, the flavonoid content was positively and significantly correlated with the total titratable acidity, whereas it was negatively and significantly correlated with colour, soluble solids, soluble solids/total titratable acidity ratio and anthocyanin content.

Phenolic content

The phenolic content varied significantly during the fruiting period in terms of fresh ($F = 99.3$, $P = 0.000$) and dry fruit weight ($F = 37.9$, $P = 0.000$), and on a per fruit basis ($F = 19.07$, $P = 0.000$) (Figure 1A–C). Peaks for phenolic content were different when expressed on a fresh weight, dry weight or per fruit basis. When considering phenolic content on the basis of fresh fruit weight, this decreased from day 70 AFFP to a minimum at day 98 AFFP and then increased to a maximum at day 126 AFFP (1522.9 mg gallic acid equivalents·100 g^{-1} FW). However, when considering phenolic content on the basis of dry fruit weight, the maximum (43.5 mg gallic acid equivalents·g^{-1} DW) was reached at day 70 AFFP, then decreased to a minimum at day 98 AFFP and then increased at day 126 AFFP. Peaks for phenolic content on a per fruit basis were attained at days 112 and 126 AFFP (3.4 and 3.3 mg gallic acid equivalents/fruit, respectively). Phenolic contents measured in ripe *B buxifolia* fruits in this study (27–34 mg·g^{-1} DW) were comparable with those cited for *Vaccinium myrtillus* (33–38 g·g^{-1} DW) (Kähkönen et al. 2001) and *Ribes nigrum* (1000 mg·100 g^{-1} FW) (Deighton et al. 2002), and higher than those of other reddish-purple berries, such as *Ribes rubrum* (14 mg·g^{-1} DW), *Fragaria ananassa* (16–24 mg·g^{-1} DW) (Kähkönen et al. 2001), *Rubus idaeus* (300 mg·100 g^{-1} FW) (Lister et al. 2002) and native South American *Fragaria* sp. (106–268 mg·100 g^{-1} FW) (Cheel et al. 2007).

In some berries, the synthesis of phenolics begins shortly after fruit development starts, such as in grape (Jackson 2008), where some anthocyanins can be synthesized earlier, but most production involves other flavonoids or non-flavonoid phenolics. The phenolic content in *B. buxifolia* decreased significantly from the fruit growth lag phase to day 98 AFFP, reaching a minimum just when soluble solids begin to accumulate, to then begin another steeper increase to reach a maximum at the end of the ripening period. The initial decrease could be, in part, due to the decrease in tannin content, as found in grape (Conde et al. 2007); these particular phenolics play an important role in the defence against predators (Raffo et al. 2004). At the time fruits began to ripen at day 98 AFFP and phenolic content reached its minimum value, anthocyanin content was still not at a high level and the flavonoid content began to show a marked decrease. The phenolic content on a fresh weight basis showed behaviour similar to soluble solids, soluble solids/total titratable acidity ratio and anthocyanin content (Table 2), whereas the phenolic content on a dry weight basis showed similar behaviour to the flavonoid content, as shown by the significant and positive correlations found between them (Table 2). The differences in timing observed in the presentation of the phenolic peaks at the beginning and end of the ripening period could be due to the differential dry fruit weight as a percentage of fresh weight accumulated; dry fruit weight as a percentage of fresh weight doubled at day 126 AFFP (39.8%), with respect to day 70 AFFP (20.6%). Also, phenolic compound metabolism varies greatly according to the physiological stage of the fruit, and their biochemical production rates rely on the participation of many key enzymes, which are also limited by the availability of precursors. Thus, it is expected that each class of phenolic compound shows a

quite different content evolution during ripening, which is seen in peaks at different harvesting dates (Raffo et al. 2004), and explains the differences found in the yield of methanolic extracts during ripening. Phenolic compounds are usually higher in young than in mature fruits with the exception of anthocyanins (Kähkönen et al. 2001). Typically, evolution of the phenol content in other fruit crops may show a decrease during growth. In some cases, the level of phenols continues to decrease steadily, as occurs in some species and white-coloured fruits (varieties of white grapes, mango and banana), or it may increase with maturation, as occurs in red fruits (apples) in which anthocyanins or other flavonoids accumulate (Häkkinen et al. 2000). Phenols decrease in both blackberries and strawberries as the fruit matures (Wang & Lin 2000).

Scavenging activity on DPPH radicals

The DPPH scavenging effect varied during the fruiting period and with the methanolic extract concentration (Figure 2). The DPPH scavenging effect at days 70 and 126 AFFP was effective at a lower concentration (0.25 $mg \cdot mL^{-1}$) than at days 84, 98 and 112 AFFP (0.50 $mg \cdot mL^{-1}$), which could be explained through the similar pattern of accumulation of phenol content and their significant and positive correlations (Table 2). The DPPH scavenging effect for standard antioxidants was effective at lower concentrations (0.10 $mg \cdot mL^{-1}$) than for fruit methanolic extracts (0.25 and 0.50 $mg \cdot mL^{-1}$) (data not shown). The effective concentration at which the DPPH radicals were scavenged by 50% (EC_{50}) was significantly different among methanolic extracts from different days after full flower phase (Table 3). The lowest values were found at days 70 and 126 AFFP (0.13 and 0.15 $mg \cdot mL^{-1}$, respectively), whereas the highest value (0.23 $mg \cdot mL^{-1}$) was obtained at day 98 AFFP. The DPPH scavenging effect was positive and significantly correlated with total titratable acidity, with flavonoid ($R^2 = 40.8$; $y = 30.6893 + 0.8693x$) and phenol content ($R^2 = 71.1$; $y = -15.6222 + 1.6468x$) in terms of dry fruit weight (Table 2), as found in *Diospyros kaki* L. cv. Mopan (Chen et al. 2008), although it was not correlated with anthocyanin content as found for strawberries (Cheel et al. 2007). The DPPH scavenging effect observed in *B. buxifolia* was comparable with that reported for *B. vulgaris* fruits (Motalleb et al. 2005) and for *B. koreana* bark (Qadir et al. 2009).

Reducing power

The reducing power varied during the fruiting period and with the methanolic extract concentration (Figure 3). Reducing power at day 70 AFFP was effective at a lower concentration (0.75 $mg \cdot mL^{-1}$) than at days 84, 98, 112 and 126 AFFP (2.50–5.00 $mg \cdot mL^{-1}$), which might be explained by the similar pattern of flavonoid content evolution. In addition, the reducing power of standard antioxidants was effective at lower concentrations (0.10–0.75 $mg \cdot mL^{-1}$) than for fruit methanolic extracts (0.75–5.00 $g \cdot mL^{-1}$) (data not shown). The effective concentration at which the reducing power was 50% (EC_{50}) was significantly different among the methanolic extracts obtained at different days after full flower phase (Table 3). The lowest values were found at 70 AFFP (0.36

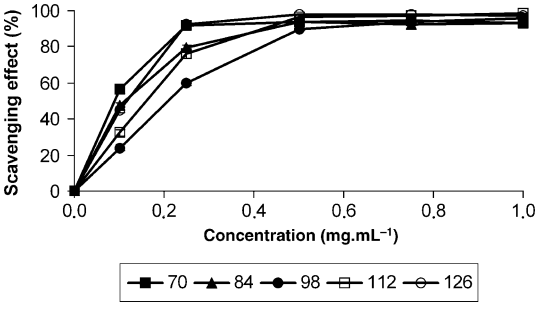

Figure 2. Scavenging effect on 1,1-diphenyl-2-picrylhydrazyl radicals (%) on *B. buxifolia* fruit extracts from day 70 to day 126 after full flower phase and at different methanolic extract concentrations (0.0–1.0 $mg \cdot mL^{-1}$) ($n = 6$). Error bars represent ± 1 standard error of the mean.

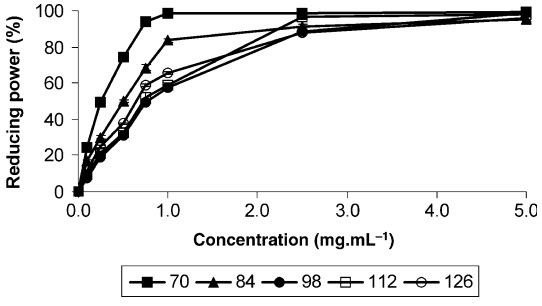

Figure 3. Reducing power (%) on *B. buxifolia* fruit extracts from day 70 to day 126 after full flower phase and at different methanolic extract concentrations (0.0–5.0 mg·mL^{-1}) ($n = 6$). Error bars represent ± 1 standard error of the mean.

mg·mL^{-1}), and the highest values at day 98 AFFP (0.80 mg·mL^{-1}), decreasing to 0.65 mg·mL^{-1} at day 126 AFFP. Reducing power was positively and significantly correlated with total titratable acidity, flavonoid ($R^2 = 80.4$; $y = 6.2790 + 0.6229x$) and phenol content ($R^2 = 82.8$; $y = 17.5062 + 0.9074x$) in terms of dry fruit weight, as found for *B. vulgaris* and *Berberis croatica* (Koncic et al. 2010), whereas it was negatively and significantly correlated with anthocyanin content (Table 2). A positive and significant correlation was found between phenol content and antioxidant activity measured through the TEAC assay in *B. microphylla* fruits (Ruiz et al. 2010). The described relationships might explain the maximum DPPH scavenging effect and reducing power values obtained at day 70 AFFP, as well as the minimum values for DPPH scavenging effect at day 98 AFFP, and the contribution of the flavonoid and phenolic content, which are responsible at least in part for the demonstrated antioxidant activity of *B. buxifolia* methanolic extracts. Although the DPPH scavenging effect of *B. buxifolia* extracts was more effective at lower concentrations (0.50 mg·mL^{-1}) than it was for reducing power (0.75–5 mg·mL^{-1}), there was a strong relationship between both methods with regard to the antioxidant activity, as shown by the significant linear correlation found between both parameters (Table 2). It is noteworthy that the DPPH scavenging effect and reducing power of *B. buxifolia* fruit extracts showed maximum values at concentrations scarcely higher than the standard antioxidant.

The antioxidant activity of the fruits and their phenolic composition involve complex relationships, and thus are very difficult to describe using statistical tools (Kähkönen et al. 2001). The antioxidant activities attributed to these substances show divergences within subgroups of compounds, hence antioxidant properties of a single compound within a group can vary remarkably. Therefore, equal levels of one specific compound do not necessarily mean an equal antioxidant response as a whole. Synergism, i.e. the ability of an antioxidant compound to improve when summed with the activity of another, is another reason for the divergences. Differences may also arise from the methodology used to assay the antioxidant activity, for example, whereas the ORAC method has positive correlations with anthocyanins in blueberry extracts (Prior et al. 1998), the DPPH method does not show any correlation with anthocyanins in strawberries (Cheel et al. 2007; Ferreyra et al. 2007), thus explaining the complex relationship between the antioxidant activity of the fruits and their phenolic composition (Kähkönen et al. 2001).

Conclusions

The accumulation patterns of phenolic compounds during fruit growth and ripening in *B. buxifolia* varied depending on the specific group considered, i.e. anthocyanins, flavonoids or phenols, and can be correlated with fruit quality characteristics including fruit weight, firmness and colour, soluble solids, total titratable acidity and their ratios, which are responsible for astringency, texture, taste and colour. This, together with the variation in antioxidant activity during fruiting and the definition of adequate terms of reference, i.e. biomass of secondary metabolites on the basis of fresh or dry fruit weight, or on a per fruit unit basis, are

Table 3. Concentration of methanolic extracts ($mg \cdot mL^{-1}$) at which the 1,1-diphenyl-2-picrylhydrazyl radicals were scavenged by 50% ($mg \cdot mL^{-1}$) and the reducing power ($mg \cdot mL^{-1}$) was 50% in *Berberis buxifolia* fruits in the days after the full flower phase.

Days AFFP	SE	RP
70	0.13b	0.36c
84	0.16b	0.51bc
98	0.23a	0.80a
112	0.20a	0.77a
126	0.15b	0.65ab
F(P)	35.27 (0.000)	37.87 (0.000)

Note: AFFP, days after the full flower phase; SE, scavenging effect on 1,1-diphenyl-2-picrylhydrazyl radicals; RP, reducing power. The EC_{50} value was obtained by interpolation from linear regression analysis between the sample concentration ($mg \cdot mL^{-1}$) and SE and RP. ANOVA F(*P*), *F* statistic and probability at $P = 0.05$. Values followed by different letters in each column are significantly different with the Tukey multiple range test at $P < 0.05$ ($n = 6$).

of considerable importance when compared with other species, or from a practical point of view when the nutraceutical value of a fruit has to be examined. Spectrophotometric methods to quantify phenolic contents are simple, fast and quite accurate, although they do not characterize a specific component. Although this study was carried out during a single, but representative, growing season, knowledge of the accumulation patterns of these secondary metabolites during *B. buxifolia* fruit growth and ripening will allow us to understand the effect of preharvest factors such as light and fertilization on this period, and contribute to selecting the most appropriate practices and assigning the best alternative uses (beginning of ripening for fresh market, ending of ripening for industrial processing, etc.). Irrespective of differences due to the methodology used to study the antioxidant responses of the methanolic fruit extracts, and taking into consideration the antioxidant responses by comparison with widely used standard antioxidant substances and with other small fruit species, *B. buxifolia* fruit appeared to possess excellent antioxidant activity during the second and third fruit growth phases (lag phase and the time when fruit ripening occurs, respectively), which may contribute to its functional value, acquiring relevance and applicability for nutraceutical purposes.

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