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Artículo / Article

Linking the platelet antiaggregation effect of different strawberries species with antioxidants: Metabolomic and transcript profiling of polyphenols

[Asociando el efecto de antiagregación plaquetaria de distintas especies de frutillas con antioxidantes: perfil metabolómico y transcriptómico de polifenoles]

Carolina Parra-Palma[1], Eduardo Fuentes[2], Ivan Palomo[2], Carolina A. Torres[3,4], María A. Moya-León[1] & Patricio Ramos[1]

1Laboratorio de Fisiología Vegetal y Genética Molecular, Instituto de Ciencias Biológicas, Universidad de Talca, Talca, Chile
2 Departamento de Bioquímica Clínica e Inmunohematología, Facultad de Ciencias de la Salud, Programa de Investigación Interdisciplinaria de Excelencia en Envejecimiento Saludable (PIEI-ES), Universidad de Talca, Talca, Chile
3 Facultad de Ciencias Agrarias, Universidad de Talca, Talca, Chile
4 Centro de Pomaceas, Facultad de Ciencias Agrarias, Universidad de Talca, Talca, Chile
Contactos | Contacts: **Patricio RAMOS - *E-mail address:* pramos@utalca.cl**
Contactos | Contacts: **María A. MOYA-LEON - *E-mail address:* alemoya@utalca.cl**

Abstract: A comparative study of antioxidant properties, platelet antiaggregation activity and transcriptional analysis of flavonoid biosynthesis genes were performed in *Fragaria* x *ananassa*, *F. vesca* and *F. chiloensis* subsp *chiloensis* f. *chiloensis* and f. *patagonica*. Furthermore, differences in flavonoid content were found by UHPLC-MS. The highest free radical scavenging activity by DPPH assay was observed in *F. chiloensis* f. *chiloensis*, meanwhile, *F. vesca* presented the highest antioxidant capacity by FRAP. Biosynthetic flavonoids-related transcripts were higher abundant in *F.* x *ananassa* and lower in *F. vesca*. Additionally, all strawberry extracts showed antiaggregant effect (1 mg mL-1), but *F. vesca* and *F. chiloensis* subsp. *chiloensis* f. *patagonica* were still active at lower concentration. This study suggests that platelet antiaggregation effect of different strawberries could be due to isoflavones and flavonoids precursors in addition to anthocyanins. Results could usefully to take decisions in future breeding programs to improve the content of healthy compounds in strawberry fruits.

Keywords: Antioxidant capacity, flavonoid biosynthesis, gene expression, platelet antiaggregation, *Fragaria* spp.

Resumen: Se realizó un estudio comparativo de propiedades antioxidantes, actividad de antiagregacion plaquetaria, análisis transcripcional de genes de biosíntesis de flavonoides y contenido de estos en *Fragaria* x *ananassa*, *F. vesca* and *F. chiloensis* subsp *chiloensis* f. *chiloensis* and f. *patagonica*. La mayor actividad removedora de radicales libres por DPPH se observó en *F. chiloensis* f. *chiloensis*, mientras *F. vesca* presentó la mayor capacidad antioxidante mediante FRAP. Transcritos relacionados con biosíntesis de flavonoides fueron mas abundantes en *F.* x *ananassa* y menores en *F. vesca*. Adicionalmente, todos los extractos de frutillas mostraron efectos antiagregante (1 mg mL-1), pero *F. vesca* and *F. chiloensis* subsp. *chiloensis* f. *patagonica* fueron activos a concentraciones menores. Este estudio sugiere que efectos de antiagregación plaquetaria en distintas frutillas podría deberse a isoflavonas y precursores de flavonoides además de antocianinas. Los resultados podrían ser útiles en programas de mejoramiento genético para mejorar el contenido de compuestos saludables en frutilla.

Palabras clave: capacidad antioxidante, biosíntesis de flavonoides, expresión génica, antigregación plaquetaria, *Fragaria* spp.

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INTRODUCTION

During the last years, increasing consideration has been placed in plants and foods which can contain antioxidant properties, with special attention to berries due to their low calories intake and high content of potential bioactive compounds, such as polyphenols, fiber, minerals, and vitamins (Aaby *et al*., 2007; Alvares-Suarez *et al*., 2014). Specifically, strawberries are a rich source of polyphenolic compounds (Kosar *et al*., 2004) and one of the most widespread edible spring and summer fruit. Polyphenolics are secondary metabolites that differ in molecular size and structure, and consider molecules from very simple structures such as hydroxycinnamic acid to more complex ones, such as flavonoids and proanthocyanidins (large polymers of high molecular weight). These compounds contribute to fruit quality parameters such as appearance, taste and flavor (Tomás-Barberán *et al*., 2001). Among the polyphenolics, the most abundant and greatest observed health impact compounds are the flavonoids, representing close to two-third parts of plant dietary polyphenols (Robbins, 2003). Flavonoids are divided into six main classes: anthocyanins, flavan-3-ols, flavonols, flavanones, flavones, and isoflavones (Del Rio *et al*., 2013).

In strawberries, anthocyanins are quantitatively the most important type of polyphenols. Major anthocyanins have been identified as pelargonidin- and cyanidin- glycosides or acylated forms (Cheel *et al*., 2007; Simirgiotis *et al*., 2009; Salvatierra *et al*., 2010). Additionally, despite their low concentrations in strawberry, increasing interest has been placed on flavonols (quercetin and kaempferol derivatives) due to the high bioavailability and bioactivity of their main aglycones and glucosides derivatives (Sesink *et al*., 2003; Schmeda-Hirschmann *et al*., 2011).

Breeding programs of strawberry species have narrowed the genetic diversity (Gil-Ariza *et al*., 2009) reducing the variability of the polyphenols content, as it has been indicated by several studies performed on the characterization of phenolic composition of different *Fragaria* species (Kosar *et al*., 2004; Cheel *et al*., 2007; Simirgiotis *et al*., 2009; Muñoz *et al*., 2011). Additionally, the transcriptional level of genes involved in the first part of the phenylpropanoid metabolic pathway has been analyzed indicating that there are strong differences in expression among species which could explain differences in their phenolic composition (Muñoz *et al*., 2011).

On the other hand, cardiovascular diseases (CVD) are the world's major cause of death among non-transmittable diseases (Butler, 2011). To prevent CVD, a diet rich in vegetables and fruit has been proposed to play a crucial protective role (Bach-Faig *et al*., 2011). Platelets play a key role in CVD development as major component of thrombi (Vorchheimer & Becker, 2006). Studies with fruits (red grapes, strawberries, kiwis, and pineapples) and vegetables (garlic, onions, melons, and tomatoes) have shown *in vitro* anti-aggregant activity (Torres-Urrutia *et al*., 2011). Studies conducted in strawberry concluded that their main positive effects in the prevention of CVD might be summarized in antihypertensive, anti-atherosclerotic and antioxidant effects (Giampieri *et al*., 2015). Therefore, all efforts performed to identify the compounds responsible of those healthy effects and also antiaggregant activity, are still scarce.

Recently, a study conducted in humans indicated that the regular consumption of a strawberry-rich anthocyanin diet reduces platelet activation (Alvarez-Suarez *et al*., 2014). Therefore, the aim of this study was to evaluate the platelet antiaggregation activity of extracts prepared from different strawberry genotypes and to identify the expression profile of genes related to the biosynthesis of phenylpropanoids (Figure 1), as a way to associate the genetic variability to their health promoting effect.

MATERIALS AND METHODS

Plant material

Ripe fruits of *Fragaria chiloensis* subsp. *chiloensis* f. *chiloensis* (Rosaceae) were obtained from a commercial orchard in Purén, Bio-Bio Region, Chile. Ripe *Fragaria chiloensis* subsp. *chiloensis* f. *patagonica* (Rosaceae) fruits were obtained from collections growing in its native habitat in Termas de Chillán, Bío-Bío Region, Chile. Ripe fruits of *Fragaria* x *ananassa* (cv. Chandler) (Rosaceae) were obtained from the local market in Talca, Chile. Ripe fruits of *Fragaria vesca* L. (Rosaceae) were collected from plants growing in a greenhouse at the Instituto de Ciencias Biológicas of Universidad de Talca, Chile (Figure 2). Ripe fruits were harvested during 2014 and 2015 summer season. Strawberries were sorted to eliminate damaged or poor quality fruit in order to obtain a uniform sample in size and color. Pool samples of at least 20 fruits per specie were immediately frozen at -80° C until use.

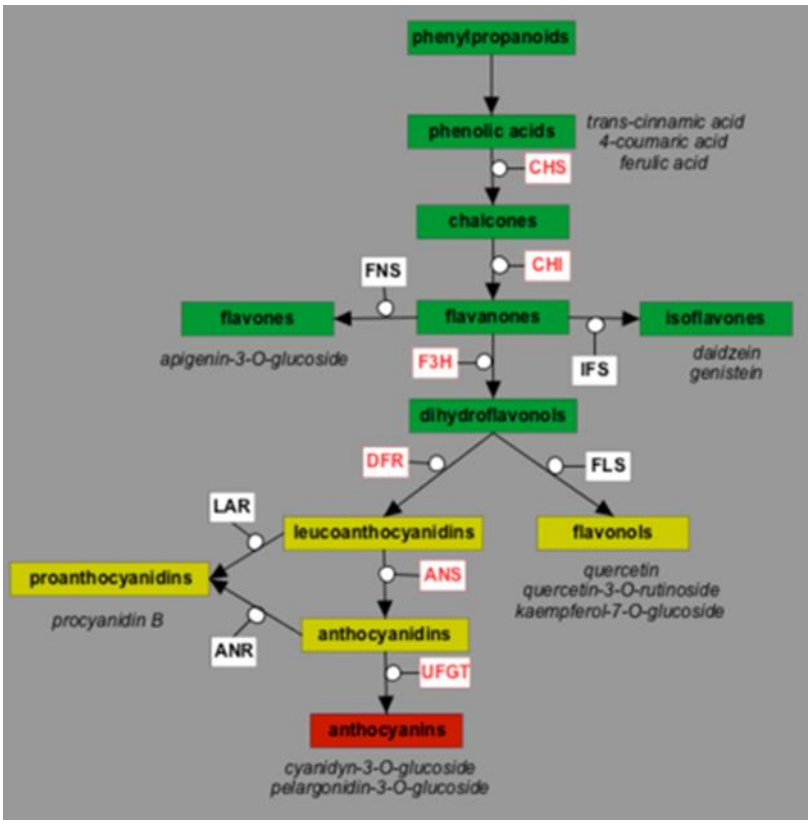

Figure 1
Simplified biosynthesis pathway of flavonoid. The gene encoding to enzymes investigated are displayed in the metabolic pathway. *CHS*, Chalcone synthase; CHI, Chalcone isomerase; *F3H*, Flavanone 3-hydroxylase; *DFR*, Dihydroflavonol reductase; ANS, Anthocyanidin synthase; UFGT, UDP glucose:flavonoid 3-O-glucosyl transferase.

RNA extraction and cDNA synthesis

Three independent total RNA samples were isolated from pools of fruits using the CTAB method with minor modifications (Chang et al. 1993). A treatment with DNAse (Invitrogen) was carried out to remove contaminant genomic DNA. The integrity of isolated RNA was checked on agarose gels stained with GelRed (Biotium Inc.), and their concentration determined in a ND-1000 UV spectrophotometer (Nanodrop Technologies, Montchanin, DE, USA). First strand cDNA synthesis was performed using First Strand cDNA Synthesis kit (Fermentas Life Science, Glen Burnie, MD, USA) following the manufacturer's instructions.

Transcriptional analysis by real time PCR (qPCR)

Primers for quantitative real-time PCR (qRT-PCR) of genes of the flavonoid biosynthesis pathway and the housekeeping (FcGAPDH, glyceraldehyde 3-phosphate dehydrogenase) based on Salvatierra *et al*. (2010) PCR amplification reactions were performed using Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Scientific) according to the manufacturer's instructions, in a Stratagene Mx3000P thermocycler (Agilent Technologies, Santa Clara CA, USA). The relative expression level of each gene corresponds to the mean of three biological and two technical replicates, normalized against the expression level of FcGAPDH1. Expression levels were calculated according to Pfaffl (2001) and expressed in arbitrary units ± standard error (SE).

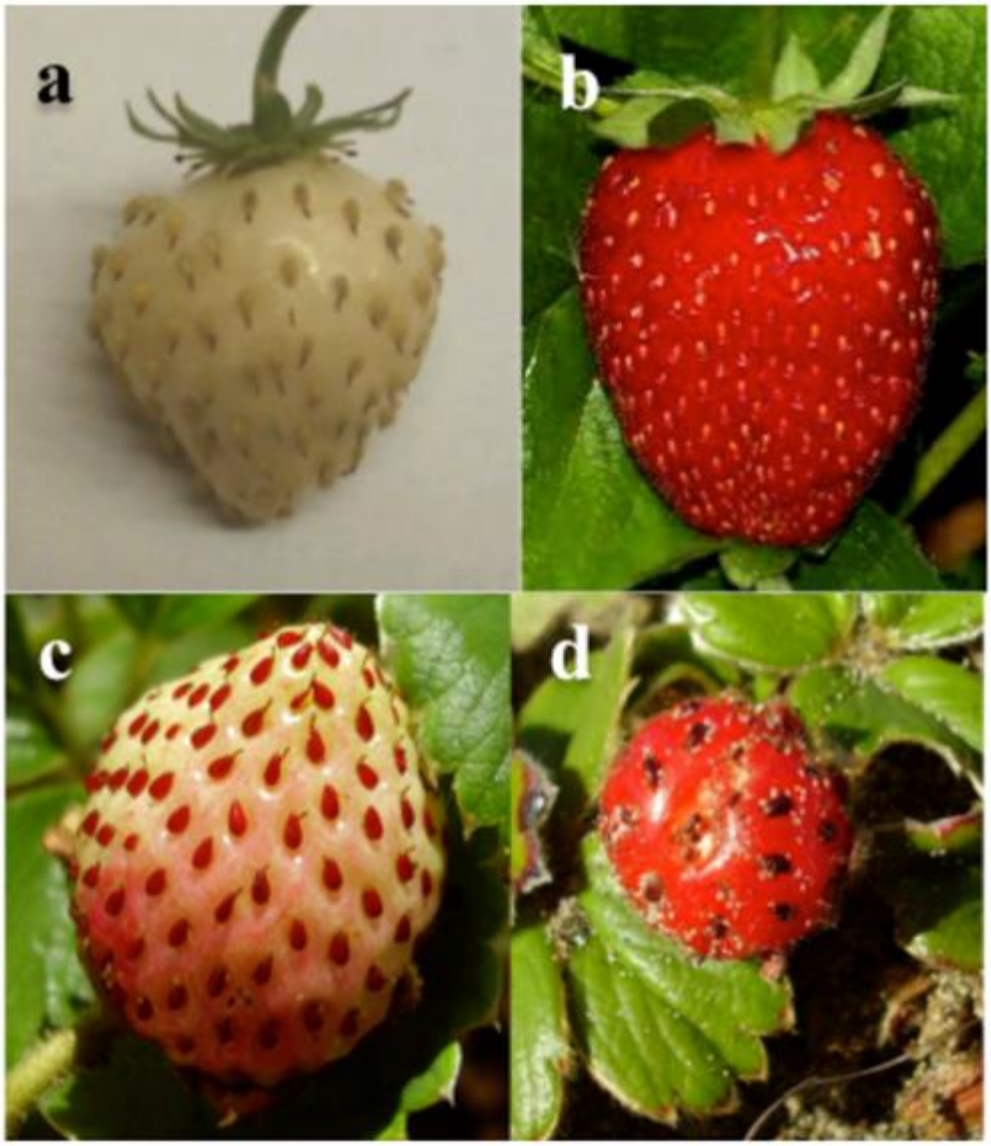

Figure 2
Strawberry fruit species analyzed. The different strawberry fruit species used in the chemical, transcriptional and biological assays were: a] *Fragaria vesca* ssp. *vesca* cv. Hawaii 4; b] *Fragaria* x *ananassa* cv. Chandler; c] *Fragaria chiloensis* ssp. *chiloensis* f. *chiloensis*; d] *Fragaria chiloensis* ssp. *chiloensis* f. *patagonica*

Total phenolics, flavonoids and anthocyanins content

Fruit tissue (receptacle and achenes) was homogenized in 1% HCl solution prepared in methanol (5 mL g^{-1} of fruit) with the help of an Ultraturrax (Labsynth, Brasil) for 5 min at 4000 rpm. The extracts were stirred for 1.5 h at room temperature and then centrifuged at 5000 rpm, recovering the supernatant. Three independent extractions were carried out for each strawberry species. The extracts obtained were used to determine total phenolics, anthocyanins and flavonoids content. The total phenolics content was determined using the method of Singleton et al. (1999) Briefly, an appropriate extract dilution was oxidized with the Folin–Ciocalteu reagent and then neutralized with sodium carbonate. The absorbance of the resulting blue color was measured at 700 nm after 30 min

using the Infinite® 200 PRO NanoQuant spectrophotometer (Tecan, Switzerland). Quantification was done on the basis of a standard curve of gallic acid. Results were expressed as mg gallic acid equivalents per 100 g of fresh weight (GAE 100 g^{-1} FW), corresponding to means ± SE of three biological replicates. The total flavonoid content of samples was determined by the aluminum chloride colorimetric method based on Chang *et al*. (2002). Quercetin was used as a reference for the calibration curve. Briefly, quercetin was dissolved in 80% ethanol and then diluted to 25, 50 and 100 µg/mL. The diluted solutions (0.5 mL) were mixed with 1.5 mL of 95% ethanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1M potassium acetate and 2.8 mL of distilled water each one and incubated during 30 min at room temperature. The absorbance of the reaction mix was measured at 415 nm. Strawberries methanol extracts (0,5 mL) were measured by this method. Results were expressed as mg quercetin equivalents per 100 g of fresh weight (QE 100 g^{-1} FW) and correspond to means ± SE of at least three biological replicates.

The anthocyanin concentration of the extract solution was determined by the pH differential method as described by Lee *et al*. (2008). Briefly, the sample (150 µL) was separately mixed with 750 µL of each of the following buffers: 0.025 M potassium chloride at pH 1.0 and 0.4 M sodium acetate buffer at pH 4.5. After incubation at room temperature for 50 min, absorbance at 524 and 700 nm were measured. The anthocyanin content was expressed as mg cyanidin 3-glucoside equivalents per 100 g fresh weight using the absorbance values of $A = (A524nm - A700nm)pH\ 1.0 - (A524nm - A700nm)pH\ 4.5$, considering a molar extinction coefficient of 26,900.

Total antioxidant capacity

Methanol extracts prepared above were tested for their radical scavenging ability based on 1,1-diphenyl-2-picrylhydrazyl (DPPH) discoloration assay (Cheel *et al*., 2007). An aliquot of 20 µL of methanol extract was mixed with DPPH solution (0.5 mM, 0.25 mL) and acetate buffer (100 mM, pH 5.5, 0.5 mL). The reaction mixtures, in triplicate, were incubated for 30 min at 25º C, and then the absorbance was measured at 517 nm in an Infinite® 200 PRO NanoQuant spectrophotometer (Tecan, Switzerland). Scavenging of DPPH radical was evaluated by comparison with a negative control group (DPPH with methanol). The absorbance (Abs_{sample}) of the resulting solution was converted into percentage of antioxidant activity (AA), using the following formula: $AA\% = 100 - ((Abs_{sample}/Abs_{control})\ x100)$. Methanol was used as negative control, and gallic acid as positive control.

Additionally, the ferric reducing antioxidant power (FRAP) analysis was performed according to Benzie & Strain (1996). For that, 100 µL of metanol extract was mixed with 3 mL FRAP reagent, left for 6 min at room temperature, and absorbance at 593 nm was measured in a NanoQuant spectrophotometer. FRAP reagent was prepared mixing 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tris[2-pyridyl]-s-triazine (TPTZ) in 40 mM HCl, and 20 mM $FeCl_3$ x 6 H_20 in a volume ratio of 10:1:1. A standard curve with $FeSO_4$ was performed and results were expressed as µmol Fe^{+2} per gram of fresh weight (µmol Fe^{+2} g^{-1} FW).

UHPLC-MS analysis of flavonoids and anthocyanins

UHPLC analyses were carried out with the methanolic extracts described above and using a Thermo Dionex ultimate 3000 (Thermo Scientific) equipped with a refrigerated autosampler. Samples (1 µL) were injected into a Hypersil Gold C18 column (50 x 2.1 mm x 1.9 µm, Thermo Fisher Scientific, Bremen). The following solvents were used: (A) 75% acetonitrile, 24.5% water and 0.5% formic acid (v/v), and (B) 5% acetonitrile, 94.5% water and 0.5% formic acid (v/v). Elution gradient was as follows: 0-1 min (0% B), 1-5 min (10% B), 5-10 min (30% B), 10-18 min (100% B) and 18-24 min (0% B). The flow rate was set at 300 µL min^{-1}.

The mass spectrometry (MS) experiments were performed with a Thermo Exactive Plus (Thermo Scientific) equipped with an electrospray interface (ESI) operating in the negative ionization mode. Each standard was infused into the electrospray ion source at 5 µg mL^{-1} in methanol using a syringe pump at a flow rate of 300 µL min^{-1}.

The ESI conditions were as follow: spray voltage, 2500 V; vaporizer temperature, 350° C; sheath gas pressure, 40 arbitrary units (au); auxiliary gas pressure, 10 au. The collision gas used was Nitrogen at a pressure of 1.5 mTorr. The data was processed using Xcalibur software 2.1 (Thermo Scientific).

Preparation of human platelet suspensions

The protocol was authorized by the ethic committee

of Universidad de Talca in accordance with the Declaration of Helsinki (approved by the 18th World Medical Assembly in Helsinki, Finland, 1964). Blood samples were taken from six young healthy volunteers in 3.2% citrate tubes (9:1, v/v) by phlebotomy with vacuum tube system (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA). Tubes were centrifuged (DCS-16 Centrifugal Presvac RV) at 200 g for 10 min to obtain platelet-rich plasma (PRP). Then the original tubes were centrifuged at 650 g for 10 min to obtain platelet-poor plasma (PPP). Finally PRP was adjusted to 200 x 10^9 platelets L^{-1} with PPP, and platelet counts were performed in a hematologic counter (Bayer Advia 60 Hematology System, Tarrytown, NY, USA).

Platelet aggregation

For platelet aggregation assays, ripe fruits were homogenized in distilled water at a concentration of 1 mg mL^{-1} with the help of Ultraturrax (Labsynth, Brasil) for 5 min at 4000 rpm on an ice bath and filtered on 0.45 µm cellulose filter disc. Platelet aggregation was monitored according to Born & Cross (1963), using a lumi-aggregometer. Briefly, 480 µL of adjusted platelets (200 x 10^9 platelets L^{-1}) were pre-incubated with a dilution of strawberry aqueous extract (0.5, 0.75 and 1 mg mL^{-1}) for 5 min before starting the platelet aggregation by the addition of 20 µL of agonist (ADP 8 µmol L^{-1} or collagen 1.5 µg mL^{-1}). Platelet aggregation was measured at real time during a 6 min period at 37º C while stirring (150 g). All measurements were performed in six replicates and platelet aggregation (maximal amplitude (%)) was determined by the software AGGRO/LINK (Chrono-Log, Havertown, PA, USA). Inhibition of the maximal platelet aggregation by the extracts was expressed as: 100 – ((% AgX x 100) % AgC^{-1}), where % AgX is aggregation of the component under study; % AgC, relative aggregation of the control; negative control, 0.9% saline solution (Fuentes *et al.*, 2012). Adenosine 10 µM was used as positive control (100% of platelet inhibition).

Statistical analysis

All data was evaluated by one-way analysis of variance (ANOVA), in order to determine differences between genotypes for several parameters: antioxidant activity, phenolics, flavonoids and anthocyanins content, as well as the relative transcript abundance of each gene. The differences between means were determined by the Tukey's multiple comparison test; $p \leq 0.05$ values were considered significant. To determine the relationship between the antioxidant activity, the phenolic content and the relative expression level of each gene the Spearman correlation coefficient with 95% confidence was calculated.

RESULTS AND DISCUSSION

The total phenolics content of strawberry fruits with different phenotypes (Figure 2) was determined in ripe fruit using the Folin-Ciocalteu reagent based on the protocol reported by Singleton *et al*., (1999). Phenolic compounds are bioactive due, in part, to their ability to chelate metals, to inhibit lipoxygenase and to scavenge free radicals (Martínez-Valverde *et al*., 2000). Phenolic compounds are accumulated in fruits and plant young tissues (Häkkinen *et al*., 1999). Scalzo *et al*. (2005) determined the phenolic content of thirteen different fruit and vegetables, and reported high levels in red commercial strawberry. However, significant differences in phenolics content have been reported for the same strawberry varieties (Häkkinen *et al*., 1999; Scalzo *et al*., 2005).

In this study, we evaluate the phenolics content of different strawberry species (Figure 3A) with remarkable color differences as shown in Figure 2. Results showed that the highest phenolics content was determined in *F. chiloensis subsp. chiloensis* f. *patagonica*, with 83.7 ± 6.6 mg GAE 100 g^{-1} FW. *F. vesca* presented also a high phenolic content (64.6 ± 3.0 mg GAE 100 g^{-1} FW) but lower than the *F. chiloensis subsp. chiloensis* f. *patagonica*. Finally, the lowest phenolic content was observed in the *F. chiloensis subsp. chiloensis* f. *chiloensis* and *F. x ananassa*. Previously, Aaby *et al*. (2012) in a study involving 27 strawberries cultivars reported that the content of total phenolic compounds depend on the cultivar and also dependent on growing conditions, all different as in this study.

The total flavonoids content was also determined (Figure 3B). Values ranged from 224.2 mg QE 100 g^{-1} FW for the *F. chiloensis subsp. chiloensis* f. *patagonica* to 10.5 mg QE 100 g^{-1} FW for the white *F. chiloensis subsp. chiloensis* f. *chiloensis*. High flavonoids content was also determined in *F. x ananassa* (218.7 mg QE 100 g^{-1} FW). *F. vesca* has approximate 40% less flavonoids (135.1 mg QE 100 g^{-1} FW) than *F. x ananassa* or *F. chiloensis subsp. chiloensis* f. *patagonica*. The lowest flavonoids content was determined in *F. chiloensis*

subsp. chiloensis f. *chiloensis*. Buendía *et al*. (2010) also reported cultivar differences in the content of some flavonoids classes for strawberries grown in Spain, such as flavonols content (1.5 – 3.4 mg 100 g^{-1} FW) and proanthocyanidins (54 – 163 mg 100 g^{-1} FW).

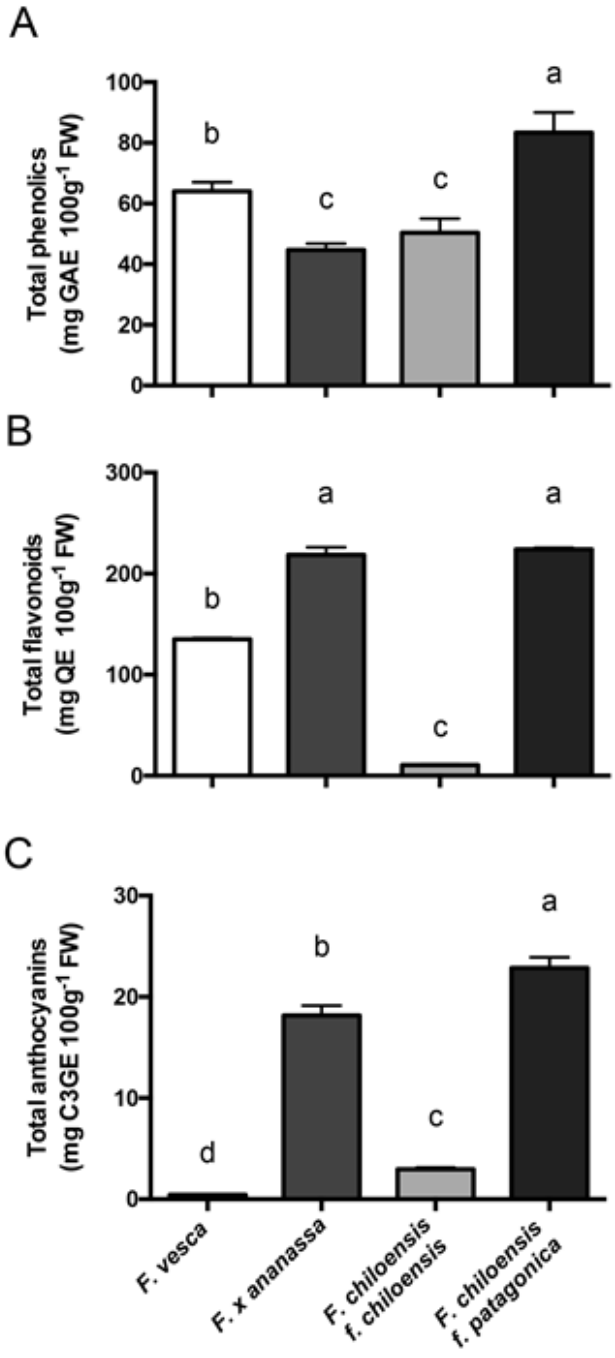

Figure 3
Chemical analyses of different strawberry species. Total phenolics [A], total flavonoids [B] and total anthocyanins content [C] were analyzed at the ripe fruit stage. Data correspond to mean ± SE of three biological replicates. Different letters indicate significant differences between samples [$p \leq 0.05$; ANOVA].

The total anthocyanin content was also analyzed in ripe fruit samples (Figure 3C), and the highest value was determined in the *F. chiloensis subsp. chiloensis* f. *patagonica* followed by *F. x ananassa*. Results agreed with the color of fruit receptacle, as *F. chiloensis subsp. chiloensis* f. *patagonica* and *F. x ananassa* show a receptacle with an intense red color. The achenes of *F. chiloensis subsp. chiloensis* f. *patagonica* also display a deep red coloration, and this fact could explain its highest total anthocyanin content compared to *F. x ananassa* with yellow achenes. Additionally, *F. vesca* and *F. chiloensis subsp. chiloensis* f. *chiloensis* showed the lowest content of anthocyanins according to their receptacle and achenes coloration. *F. chiloensis subsp chiloensis* f. *chiloensis* have red achenes and pink pale receptacle, while in *F. vesca* the receptacle is white and the achenes are yellow. The data obtained is in agreement with Cheel *et al*. (2007) that reported levels of 2.3 mg cyanidin-3-glucoside 100 g^{-1} FW for the *F. chiloensis subsp. chiloensis* f. *chiloensis* and 30.6 mg cyanidin-3-glucoside 100 g^{-1} FW for *F. x ananassa* cv. Chandler.

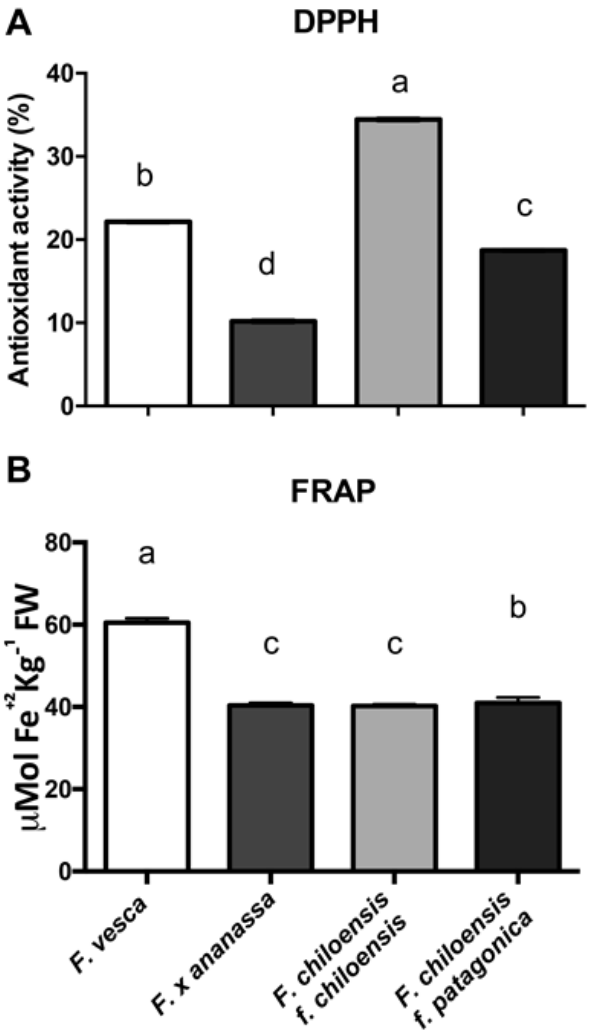

Figure 4
Antioxidant capacity of different strawberry species. Free radical scavenging activity by DPPH [A] and FRAP [B] were estimated in ripe fruit samples. Data correspond to mean ± SE of three biological replicates. Different letters indicate significant differences between samples [p≤ 0.05; ANOVA].

Our results showed a positive correlation between total flavonoid and total anthocyanin content ($r = 0.727$, $p \leq 0.05$). No correlation between total

The antioxidant capacity of ripe fruit extracts was assayed by DPPH and FRAP methodologies (Figure 4). The highest % of DPPH discoloration assay associated with high radical scavenging activity (Figure 4A) was observed in *F. chiloensis subsp chiloensis* f. *chiloensis* (34.4%), followed by *F. vesca* (22.1%) and *F. chiloensis subsp. chiloensis* f. *patagonica* (18.7%). The lowest % of DPPH discoloration was found in *F. x ananassa* cv. Chandler (10.2%). This method is limited due to the DPPH radicals can also interact with other radicals (Brand-Williams *et al*., 1995). Due to this and considering that different species could accumulate different metabolites, an additional method was assessed.

The antioxidant capacity was also evaluated by the ferric ion reducing antioxidant power (FRAP) (Fig. 4 B). *F. vesca* presented the highest FRAP capacity followed by the *F. chiloensis subsp. chiloensis* f. *patagonica*, *F. x ananassa* and *F. chiloensis subsp chiloensis* f. *chiloensis* with 77%, 68% and 65% of *F. vesca´*s FRAP capacity, respectively. The main problem with this approach is that is measuring the reducing capacity. Higher values of FRAP indicate a high reducing capacity, but not reflect the antioxidant activity because the assay not include an oxidisable substrate.

As described, both methodologies employed to determine the antioxidant capacity of fruit extracts (DPPH and FRAP) reached to different results. This apparent controversy is due to the different principle involved in each methodology. While one method evaluates the radical scavenging ability (DPPH), the other determines the capacity to reduce iron; both capacities are considered important to maintain the reductive state of biological systems. A positive correlation was observed between FRAP and total phenolics content ($r = 0.65$; $p \leq 0.1$), while a strong negative correlation was observed between DPPH values and flavonoids content ($r = -0.84$; $p \leq 0.01$) perhaps indicating that the high free radical scavenging activity (DPPH) in this species is not due to flavonoids, but other compounds generated previously to the biosynthesis of chalcone could be responsible of this antioxidant capacity evaluated by this methodology.

phenolics and total anthocyanins content was found, which is in agreement with other reports (Cordenunsi *et al*., 2002; Cheel *et al*., 2007).

The mRNA levels of six genes encoding for key enzymes of the flavonoid biosynthesis pathway (Figure 1) were quantified by qRT-PCR in selected strawberries species at the ripe stage: chalcone synthase (CHS), chalcone isomerase (CHI), flavonone 3-hydroxylase (F3H), anthocyanidin synthase (ANS), dihydroflavonol reductase (DFR) and UDP glucose:flavonoid 3-O-glucosyl transferase (UFGT). The transcript level of these genes showed a great variability among genotypes (Figure 5). A consistent high transcript level was determined in *F. x ananassa* for almost all genes analyzed, except for *UFGT*. On the contrary, *F. vesca* presented the lowest relative transcript levels for all the genes evaluated.

A strong negative correlation was found between FRAP assays and the expression levels of *CHI* ($r = -0.78$; $p \leq 0.01$) and *F3H* ($r = -0.78$; $p \leq 0.01$), and between the total phenolics content and the expression level of the same genes ($r = -0.76$; $p \leq 0.01$). On the other hand, positive correlations were found between the total flavonoids content and the expression levels of *CHS* ($r = 0.63$; $p \leq 0.05$), *DFR* ($r = 0.63$; $p \leq 0.05$) and *ANS* ($r = 0.63$; $p \leq 0.05$). Also strong positive correlations were found between the anthocyanins content and the transcript levels of *CHS* ($r = 0.78$; $p \leq 0.01$), *DFR* ($r = 0.78$; $p \leq 0.01$), *ANS* ($r = 0.78$; $p \leq 0.01$) and *UFGT* ($r = 0.78$; $p \leq 0.01$), all these genes drive the path to anthocyanin biosynthesis.

The effect of different ripe strawberry aqueous extracts on platelet aggregation induced by ADP and collagen were evaluated (Figure 6). The human platelet aggregation induced by ADP was strongly inhibited ($p \leq 0.05$) in the presence of all strawberry extracts at 1 mg mL^{-1} (Figure 6A). A significant high platelet antiaggregant effect (59% of inhibition) was observed for *F. vesca* at 1 mg mL^{-1}. Human platelet aggregation induced by ADP was also inhibited by more diluted fruit extracts, with 46% and 30% platelet aggregation inhibition ($p \leq 0.05$) for *F. vesca* and *F. chiloensis subsp chiloensis* f. *patagonica* extracts, respectively, at a dilution of 0.75 mg mL^{-1} (Figure 6B). At a dilution of 0.5 mg mL^{-1} none of the strawberry extracts had inhibitory effect on ADP-induced human platelet aggregation

(data not shown). On the other hand, strawberry extracts did not have inhibitory effect on collagen-induced platelet aggregation at a concentration of 1 mg mL^{-1} (Figure 6C).

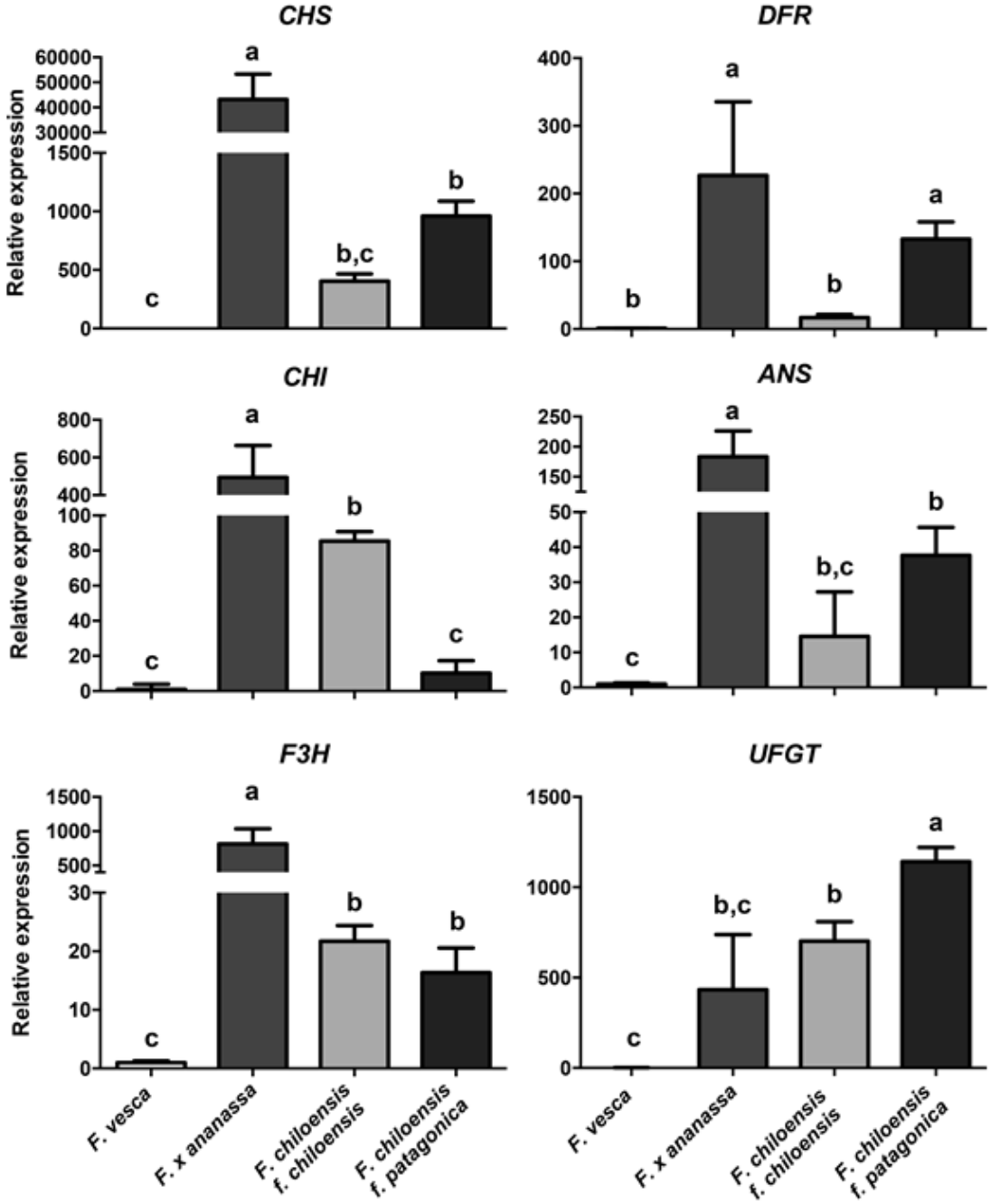

Figure 5
Analysis of the transcriptional profile of flavonoid biosynthetic pathway genes. The expression profile of genes involved in the flavonoid biosynthesis pathway was evaluated in ripe strawberry fruit from different species. The genes analysed were chalcone synthase [CHS], chalcone isomerase [CHI], flavonone 3-hydroxylase [F3H], anthocyanidin synthase [ANS], dihydroflavonol reductase [DFR] and UDP glucose:flavonoid 3-O-glucosyl transferase [UFGT]. Data correspond to mean ± SE of three biological replicates, using F. vesca's transcript abundance level as control with a nominal value of 1. Different letters indicate significant differences between samples [$p \leq 0.05$; ANOVA].

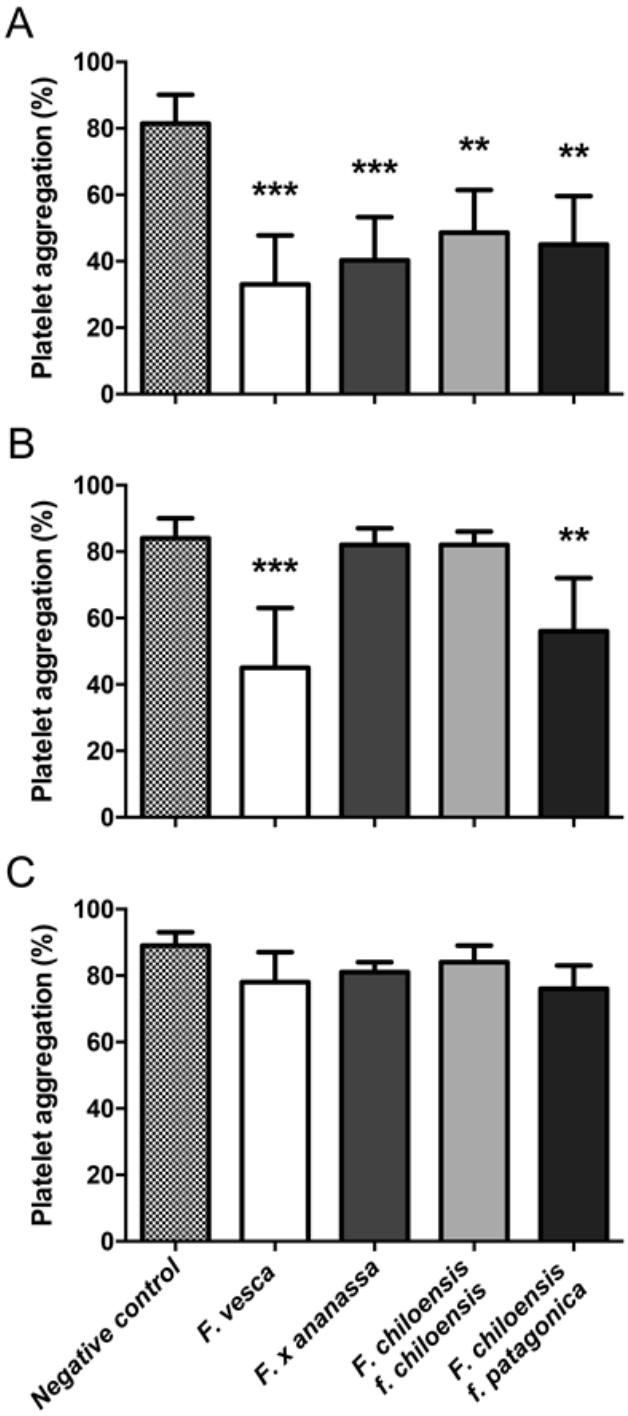

Figure 6
Effect of strawberry extracts on ADP [A, B] and collagen [C] induced platelet aggregation. Several fruit extract dilutions were prepared from each genotype: 1 mg mL-1 [A, C] and 0.75 mg mL-1 [B]. Platelet aggregation was induced by the addition of 20 µL of agonist, either 8 µmol L-1 ADP or 1.5 µg mL-1 collagen. Results are expressed as % platelet aggregation [mean ± SE, n=6]. Asterisk denotes statistically significant differences when compared with negative control [saline solution 0.9%], analyzed by ANOVA and Tukey's post-hoc test [* $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$].

The primary function of platelets is to contribute to hemostasis: the process of stopping blood loss after tissue damage (Golebiewska & Poole, 2015). Thus, when the endothelium is injured, the subendothelial collagen is exposed to the bloodstream and on contact with collagen, platelets become activated, with platelet adhesion, secretion of platelet contents, and platelet aggregation at the site of injury (Nieswandt *et al*., 2001). However, the barrier between hemostasis and pathological thrombosis is very narrow, and it has been increasingly recognized that platelets aggregation are at least partially liable for the pathological development of thrombosis (Freedman, 2005). Therefore, the results of this study suggest that strawberry extracts do not affect physiological hemostasis, however fruit extracts prevent the pathological thrombosis effect interfering with ADP receptors (P2Y1/P2Y12).

Platelet activation is crucial for development of CVD (Fitzgerald *et al*., 1986; Palomo *et al*., 2008), therefore compounds that inhibit platelet activation could have human health impact. The high content of phenolics, flavonoids and anthocyanins of fruit has been associated to their platelet aggregation inhibitory effect (Kim *et al.*, 2008; Alvarez-Suarez *et al.*, 2014; Santhakumar *et al.*, 2015), but it has not been performed so far a comparative analysis between strawberry species in order to identify metabolites to be considered in future breeding programs. *F. chiloensis* subsp. *chiloensis* f. *patagonica* has a promisory platelet antiaggregation effect, as well as *F. vesca*. The high content of total phenolics and flavonoids of *F. chiloensis* subsp. *chiloensis* f. *patagonica* could explain its high platelet antiaggregation activity. In the case of *F. vesca* as the level of total phenolics, total flavonoids and total anthocyanins is lower than *F. chiloensis* subsp. *chiloensis* f. *patagonica*, its antiaggregation activity could be due to the presence of other bioactive phenolic compounds that are biosynthesized previous to flavonoids pathway as indicated by the lower transcript abundance of *CHS*. In fact, we have tested that main representative phenolic compounds related to the flavonoid route (kaempferol and quercetin, alone or in combination). Results indicate that they do not have inhibitory effect on platelet aggregation induced by ADP (data not shown), on contrary to the reported by Bojić *et al.* (2011). This result could be due to the differences in concentration of ADP used to induce the platelet aggregation, which was higher in our evaluation.

The relative composition of anthocyanins in strawberry was reported for some cultivated and native species (Cheel *et al*., 2007). Also anthocyanins and flavonoids content were analyzed in whole fruit of native and commercial strawberry (Simirgiotis *et al*., 2009). In here, a comparative analysis of flavonoids content, and pelargonidin and cyanidin anthocyanins in extracts prepared from ripe fruits of four strawberry species was conducted to correlate their content with the transcript level of flavonoid biosynthesis genes. The list of flavonoids determined by UHPLC-MS is presented in Table 1. Pelargonidin-3-*O*-glucoside and cyanidin-3-*O*-glucoside are the most abundant anthocyanins found in native and commercial strawberry, according to a previous report (Simirgiotis *et al*., 2009). In *F. vesca*, pelargonidin was not detected and cyanidin was found in low quantity compared to the other species analyzed, which is in agreement with the transcript abundance of biosynthetic flavonoid pathway genes. The most bioactive fruit extract regarding to antioxidant capacity and inhibition of human platelet aggregation activity was *F. vesca*. The transcript profile of flavonoid biosynthetic genes is in accordance with the abundance of metabolites observed in this species, because transcripts of the first committed enzyme of the flavonoid pathway (*CHS*) and those downstream are low compared to the other species under study (Figure 1). In agreement with this, a precursor metabolite such as 4-coumaric acid is more abundant in *F. vesca* than in the other fruit species, as well as trans-cinnamic acid which is only detected in *F. vesca*. All these suggest that in *F. vesca* CHS is limiting the formation of flavonoids and therefore other phenolics are accumulated instead which could explain its high antioxidant capacity. Additionally, genistein and daidzein were identified as differentially accumulated in the four strawberry species (table 1). These compounds are accumulated in several fruits and nuts, in which are include strawberry fruit (Liggins *et al*., 2000). Nakashima *et al*. (1991) investigated the mechanism of genistein and daidzein in the inhibition of human platelets aggregation by these isoflavones, concluding that they act as protein tyrosine kinase inhibitors. These results are also according to the analysis of antiaggregatory activity of several flavonoid aglycones (Bojić *et al*., 2011). According

to the previous results of antithrombotic activity of strawberry extract in murine model (Alarcon *et al*., 2015), it is possible to estimate that the amount of strawberry extract necessary in humans for proven antiplatelet effects is about 70 mg/kg. However, additional studies are needed to confirm the strawberry extract dose to observe acute and chronic antiplatelet effects in humans.

	m/z	*RT (min)*	*F. vesca*		*F. x ananassa*		*F. chiloensis f. chiloensis*		*F. chiloensis f. patagonica*	
Cyanidin-3-*O*-glucoside	286.27	19.06	0.5330	± 0.1140 ^{b}	0.8252	± 0.0560 ^{b}	1.4177	± 0.1359 ^{a}	1.2752	± 0.1512 ^{a}
Pelargonidin-3-*O*-glucoside	270.30	18.03	ND		11.4544	± 0.3701 ^{b}	ND		20.4300	± 0.5767 ^{a}
Quercetin	303.03	6.63	1.2490	± 0.9650 ^{b}	2.0497	± 0.6540 ^{a}	1.6901	± 0.1184 ^{a}	1.8318	± 0.0544 ^{a}
Quercetin-3-*O*-rutinoside	449.08	4.73	0.0057	± 0.0001 ^{a}	0.0042	± 0.0021 ^{a}	0.0056	± 0.0024 ^{a}	0.0072	± 0.0022 ^{a}
Kaempferol-7-*O*-glucoside	449.08	4.64	0.0156	± 0.0056 ^{a}	0.0130	± 0.0026 ^{a}	0.0113	± 0.0036 ^{a}	0.0055	± 0.0034 ^{a}
Procyanidin B	578.52	0.90	1.1091	± 0.3167 ^{b}	0.8228	± 0.4287 ^{b}	1.1460	± 0.0716 ^{b}	1.5603	± 0.1029 ^{a}
4-Coumaric acid	177.04	2.91	4.9811	± 0.5233 ^{a}	1.8912	± 0.0433 ^{d}	3.3484	± 0.0801 ^{b}	2.2816	± 0.0192 ^{c}
Ferulic acid	194.14	7.70	ND		ND		ND		0.1412	± 0.0022 ^{a}
Apigenin-3-*O*-glucoside	433.09	5.20	0.0020	± 0.0006 ^{a}	0.0014	± 0.0004 ^{a}	ND		0.0021	± 0.0005 ^{a}
Daidzein	255.05	7.11	4.9702	± 0.1009 ^{b}	0.0115	± 0.0001 ^{c}	0.0046	± 0.0063 ^{c}	6.4391	± 0.2951 ^{a}
Trans-cinnamic acid	131.04	8.26	0.0006	± 0.0001	ND		ND		ND	
Genistein	271.04	9.33	0.1867	± 0.0044 ^{a}	0.1014	± 0.0025 ^{c}	0.1418	± 0.0051 ^{b}	0.0973	± 0.0097 ^{d}

Table 1
Concentration of the main anthocyanins and flavonoids (mg kg^{-1} Fresh Weight) determined in ripe fruits of four strawberry species through UHPLC-MS.
Standard calibration curves: pelargonidin-3-*O*-glucoside ($y = 3e^{-6}x$-34.95, R^2= 0.99); cyanidin-3-*O*-glucoside ($y = 2e^{-7}x$ + 153.83, R^2= 0.91); quercetin ($y = 0.0016202x + 0.571185$, R^2= 0.98); Quercetin-3-*O*-rutinoside ($y = 3e^{-7}x$ + 8,3477, R^2= 0.99); Kaempferol-7-*O*-glucoside ($y = 3e^{-7}x$-22.597, R^2= 0.99); Procyanidin B2 ($y = 0.000136891x + 1.66868$, R^2= 0.99); 4-Coumaric acid ($y = 0.00184371x$+1.55866, R^2= 0.99); Ferulic acid ($y = 5e^{-6}x$-55.532, R^2= 0.91); Apigenin-3-*O*-glucoside ($y = 3e^{-7}x + 1.5596$, R^2= 0.97); Daidzein ($y = 3e^{-6}x$-1.6126, R^2 = 0.96); Trans-cinnamic acid ($y = 1e^{-7}x$-0.2127, R^2 = 0.94); Genistein ($y = 7e^{-6}x$-26.057, R^2 = 0.91). Values in a row with different letters are statistically different ($p < 0.05$) as determined by Tukey's comparison test. ND = not detected.

Cinnamic acid derivatives is well known that are present in *F. chiloensis subsp chiloensis* f. *chiloensis* (Cheel *et al.,* 2005) and as well as ellagic and *p*-coumaric acid derivatives comprises a high percentage of the total phenolics composition of strawberry fruit (Häkkinen *et al.,* 1999), which agreed with the observed results (Table 1), but ellagic acid was not quantified. *F. vesca* and *F. chiloensis subsp. chiloensis* f. *patagonica* show greater phenolic content, displaying metabolites such as *p*-coumaric and ferulic acid, whose content is lower or was not detected neither in *F. x ananassa* nor in *F. chiloensis subp. chiloensis* f. *chiloensis*. According to the correlation analysis, the results of the FRAP method correlate directly with the phenolic content of the fruits, indicating that these metabolites (mainly phenolic acids) are responsible for the greater antioxidant activity presented by these two species. Piazzon *et al.* (2012) showed that some phenolic acids, including ferulic acid, although they are extensively metabolized after absorption, retain a strong antioxidant activity and can still exert a significant antioxidant action in vivo. In addition, the antioxidant effect of ferulic acid has been verified against several chronic and acute pathologies, such as cancer (Serafim *et al.,* 2011, Rocha *et al.,* 2012), cardiovascular (Ohsaki *et al.*, 2008), diabetes (Jung *et al.,* 2007), among others. In addition, its activity of free radical scavenging has been evaluated in several neurodegenerative pathologies, particularly in Alzheimer's disease (Sgarbossa *et al.*, 2015).

The most abundant phenolic acid found in

this work was 4-coumaric acid (or *p*-coumaric acid), mainly in *F. vesca*. Clifford (2000) showed that *p*-coumaric acid was able to reduce significantly the platelet aggregation induced by AA (arachidonic acid) and ADP after 2 weeks of treatment, in doses comparable to the daily consumption of cinnamates. This function has been related to its antioxidant activity (Salvemini & Botting, 1993; Neiva *et al.*, 1999). Furthermore, Luceri *et al.* (2007) showed that the administration of *p*-coumaric acid (5 mg/kg) significantly increased the antioxidant activity of the plasma after one week of treatment.

The presence of proanthocyanidins has been also reported in *Fragaria x ananassa* (Carbone *et al.*, 2009). Benefits are attributed to proanthocyanidins due to their antioxidant capacity, and reports from several *in vitro* assays have shown potentially interactions of proanthocyanidins with biological systems, such as antiviral, antibacterial and radical scavenging systems (De Bruyne *et al.*, 1999). Based on this, we understand that many phenolic compounds as proanthocyanidins, tannins and some derivatives which could be play an important role in anti-platelet action, are not fully extracted by aqueous solution, but this could be part of future determinations.

CONCLUSIONS

In this work, we have analyzed the antioxidant activity of four wild and commercial strawberry genotypes, correlating the expression level of genes involved in the flavonoids biosynthesis pathway with their metabolite abundance. Additionally, the platelet antiaggregation effect of wild strawberry species suggests their potential as human-protectors. As recently described, the dietary supplementation of rats with a white Chilean aqueous extract favors the normalization of oxidative and inflammatory responses after a liver injury induced by LPS (Molinett *et al*., 2015). Based on this and considering fresh fruits as the most common form of consumption of strawberry, we decided to evaluate the antiplatelet effect of aqueous extracts. The results obtained indicate that the protective effect of fruit extracts towards platelet aggregation was higher in *F. vesca* and *F. chiloensis* subsp. *chiloensis* f. *patagonica*. Transcripts and metabolite analysis revealed that anthocyanins are not the unique responsible of the antioxidant capacity in strawberry, as *F. vesca* shows the lowest flavonoids and anthocyanins content, according with the lowest mRNA transcript abundance. Results suggest that some flavonoid precursors and some derivatives, i.e isoflavones such as genistein and daidzein, could be important for human health due to high antioxidant activity and inhibition of human platelet aggregation showed by *F. vesca*. Composition analysis of phenolics, flavonoids, anthocyanins and antioxidant potential was not evaluated in aqueous extracts; based on a comparative analysis between methanolic, hexanic and aqueous extracts from leaves in which not strong differences were observed (Ahmed et al. 2015).

Strawberry fruit extracts can be considered as functional food due to its platelet antiaggregant effect, however as described, its capacity depends on the strawberry species. Therefore breeding programs directed to improve functional food capacity needs to take this information into consideration.

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