Phenolic Compounds in Ecuadorian Fruits

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Cover: Andean Blackberry, mortiño berry, physalis and tree tomato
(photos: Lucía Vasco)

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Abstract

A group of eighteen fruits cultivated in Ecuador were evaluated for their total soluble phenolic compounds and antioxidant activity and attempts were made to identify the group and content of phenolic compounds responsible for the antioxidant activity. In terms of total phenolic content, three groups (with <100, 200-500 and >1000 mg gallic acid equivalents/100 g FW) were clearly distinguishable. RP-HPLC-DAD and/or LC-MS/MS were used to study the phenolic compounds in four *Rosaceae* fruits (Andean blackberry, strawberry, plum and capulí cherry), three *Passifloraceae* and mortiño berry. Andean blackberry contained ellagitannins, ellagic acid derivatives and cyanidin glycosides as major compounds, plus considerable amounts of (-)-epicatechin, proanthocyanidins, quercetin derivatives, gallic acid and galloyl esters. Strawberry had ellagic acid derivatives as major components followed by pelargonidin glycosides, proanthocyanidins, quercetin derivatives and galloyl esters. Plum and capulí cherry both contained chlorogenic acids among the hydroxycinnamate derivatives, (-)-epicatechin among the flavan-3-ols and similar levels of proanthocyanidins and quercetin derivatives. In addition, capulí cherry had a high concentration of (+)-catechin.

Two fruits were studied for the first time: mortiño berry (*Vaccinium floribundum* Kunth, Family *Ericaceae)* and banana passion fruit or taxo (*Passiflora mollissima* H.B.K.). In mortiño, (-)-epicatechin and one trimer A were found. Of the flavonol glycosides, quercetin and myricetin were found as -3-*O*-hexosides, -3-*O*-pentosides and -3-*O*-deoxyhexosides. Chlorogenic and neochlorogenic acids together with caffeic/ferulic acid derivatives were found as predominant components among the hydroxycinnamic acids. Anthocyanins were the major class quantified in the berry. Banana passion fruit contained the monomers (E)GC, (+)-catechin, (-)-epicatechin and the rare (epi)afzelechin, and proanthocyanidin dimers and trimers (E)GC-(E)GC, (E)C-(E)GC, (E)Azf-(E)C, (E)C-(E)C-(E)C, (E)C-(E)GC-(E)C, and (E)GC-(E)GC-(E)C. The total content of proanthocyanidins was quantified as ~1955 mg/100 g FW, indicating that banana passion fruit is an excellent source of proanthocyanidins.

Keywords: fruits, Ecuador, phenolic compounds, antioxidants, flavonoids, mortiño, banana passion fruit, Andean blackberry

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Dedication

To Esperanza Carrillo Rueda, my dear Grandmother

Contents

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List of Publications

This thesis is based on the work contained in the following papers, which are referred to in the text by their Roman numerals:

I   Vasco, C., Ruales, J., Kamal-Eldin, A. (2008). Total phenolic compounds and antioxidant capacities of major fruits from Ecuador. *Food Chemistry* 111 (4), 816-823.

II Vasco, C., Àvila, J., Ruales, J., Svanberg, U., Kamal-Eldin, A. (2009). Physical and chemical characteristics of golden-yellow and purple-red varieties of tamarillo fruit (*Solanum betaceum* Cav.). *International Journal of Food Science & Nutrition*. DOI: 10.1080/09637480903099618.

III Vasco, C., Riihinen, K., Ruales, J., Kamal-Eldin, A. (2009). Phenolic compounds in *Rosaceae* fruits from Ecuador. *Journal of Agricultural and Food Chemistry* 57 (4), 1204-1212.

IV Vasco, C., Riihinen, K., Ruales, J., Kamal-Eldin, A. (2009). Chemical composition and phenolic compound profile of mortiño (*Vaccinium floribundum* Kunth). *Journal of Agricultural and Food Chemistry* (*in press*).

V   Vasco, C., Riihinen, K., Ryynänen, A., Ruales, J., Kamal-Eldin, A. Predominant flavan-3-ols and proanthocyanidins and other polyphenols in taxo (*Passiflora mollisima* H.B.K.) (*submitted*).

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The contribution of Catalina Vasco to the papers included in this thesis was as follows:

I Partial responsibility for planning the study and main responsibility for the analytical work and for preparation and revision of the article.

II Partial responsibility for the analytical work and main responsibility for preparation and revision of the article.

III Partial responsibility for planning the study and main responsibility for the analytical work and for preparation and revision of the article.

IV Partial responsibility for planning of the study and for the analytical work and main responsibility for preparation and revision of the article.

V Partial responsibility for planning the study and main responsibility for the analytical work and for preparation of the manuscript.

Abbreviations

A	Absorbance
ABTS	2,2'-azinobis-3-ethylbenzotiazoline-6-sulfonic acid
AC	Antioxidant capacity
B	Beauty (a variety of plum)
AH	Acid hydrolysis
C	Carbon, *i.e.* in numbering C-1
(C)	(+)-catechin
DAD	Diode array detector
DPPH	1,1-diphenyl-2-picrylhydrazyl
DW	Dry weight
(E)Afz	(Epi)afzelechin
EC	(-)-epicatechin
ECG	(-)-epicatechin gallate
EGC	(-)-epigallocatechin
EGCG	(-)-epigallocatechin gallate
ESI	Electrospray ionisation
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalents
GC	(+)-gallocatechin
FW	Fresh weight
HHDP	Hexahydroxydiphenic acid
HPLC	High performance liquid chromatography
LC	Liquid chromatography
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
P	Peel
PU	Pulp
ROS	Reactive oxygen species

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RP	Reversed phase
RS	Reference sample
SR	Santa Rosa (a variety of plum)
std	Standard
TEAC	Trolox equivalent antioxidant capacity
TPTZ	2,4,6-tripyridyl-s-triazine
PAs	Proanthocyanidins
UV	Ultraviolet
Vis	Visible

1 Literature background

Ecuador produces a wide range of delicious fruits from tropical, subtropical and Andean areas that are reaching international markets, as well as some that are not known outside the local markets and neighbouring counties, but only limited processing of different food products is carried out. Fruits are a very interesting plant material that can be eaten unprocessed or processed.
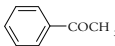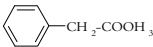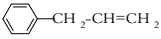
A diet rich in fruit and vegetables is associated with a reduced risk of developing chronic diseases such as cancer (Rajamanickam & Agarwal, 2008; Fresco *et al.*, 2006; Block *et al.*, 1992), cardiovascular disease (Hu, 2009; Bazzano *et al.*, 2003), and age-related neurodegenerative diseases (Shukitt-Hale *et al.*, 2008; Ramassamy, 2006). In fruits, the components involved in the prevention of degenerative diseases include soluble and insoluble dietary fibre, vitamin C, E, folate, carotenoids, selenium, and phenolic compounds (Szajdek & Borowska, 2008; Abdel-Aal & Akhtar, 2006; Feeney, 2004). Interest in phenolic compounds has increased dramatically over recent years since they are present in all plants and are therefore part of our diet (Bravo, 1998). Evaluating antioxidants in food is still challenging from an analytical point of view because they are always present as very complex mixtures. A complete extraction has not been achieved yet, their bioactivity cannot be attributed to one compound or group of compounds and the absorption, metabolism and physiological effects are different when they are ingested with food than when they are ingested as supplements (Bravo, 1998). Phenolic compounds are less potent than pharmaceutical drugs, but the advantage is that they are always present in our diet, giving a long-term physiological effect (Espín *et al.*, 2007).
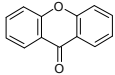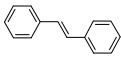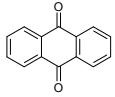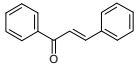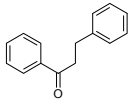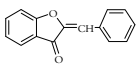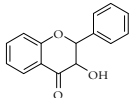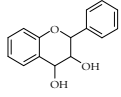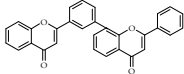
1.1 Phenolic compounds in plants

The four major secondary metabolites in plants are terpenoids, alkaloids, sulphur-containing compounds and phenolic compounds (Dillard & Bruce German, 2000). The phenolic compound family is huge and comprises a complex group of compounds (about 8000 identified compounds), varying from simple phenols to highly polymerised compounds such as tannins (Bravo, 1998). Phenolic compounds can be divided into several classes depending on the structure of the aglycone (Table 1).

The main phenolic subclasses in fruits are phenolic acids (hydroxybenzoic and hydroxycinnamic acids), coumarins, flavonoids and hydrolysable and condensed tannins (Hounsome *et al.*, 2008; Bravo, 1998; Macheix *et al.*, 1990). In this thesis, coumarins are not discussed and only flavonols, flavones, flavan-3-ols, and anthocyanins are included as flavonoids.

Table 1. *Classification of phenolic compounds*

Basic skeleton	Class	Basic structure	Examples
C_6	Simple phenols		Phenol, cresol, resorcinol
	Benzoquinones		Benzoquinone
C_6-C_1	Hydroxybenzoic acids	see Figure 1	Gallic acid, vanillic acid
	Condensed tannins	see Figure 1	Gallotannins, ellagitannins
C_6-C_2	Acetophenones		Annphenone
	Phenyl acetic acids		*p*-Hydroxyphenylacetic acid
C_6-C_3	Hydroxycinnamic acids	see Figure 1	Caffeic acid, ferulic acid
	Phenylpropenes		Eugenol, myristicin
	Coumarins, isocoumarins		Umbilliferone, scopoletin
	Chromones		Eugenin

C_6-C_4	Naphthoquinones		Juglone
$C_6-C_1-C_6$	Xanthones		Mangostin, mangiferin
$C_6-C_2-C_6$	Stilbenes		Resveratrol
	Anthraquinones		Emodin
$C_6-C_3-C_6$	Chalcones		Phloridzin, arbutin
	Dihydrochalcones		Phloretin
	Aurones		Sulferetol
	Flavones	see Figure 2	Apigenin, luteolin
	Flavonols	see Figure 2	Quercetin, myricetin
	Dihydroflavonol		Taxifolin
	Flavanones	see Figure 2	Hesperitin, naringenin
	Flavanol	see Figure 2	(epi)Catechin
	Flavandiol or leucoanthocyanidin		(+)-Leucocyanidin
	Anthocyanidins	see Figure 2	Cyanidin, pelargonidin
	Isoflavonoids	see Figure 2	Daidzein, genistein
$(C_6-C_3-C_6)_2$	Biflavonoids		Agathisflavone
$(C_6-C_3-C_6)_n$	Proanthocyanidins	see Figure 2	Procyanidins
$(C_6-C_3)_2$	Lignans, neolignans		Sesamin, secoisolariciresinol
$(C_6-C_3)_n$	Lignins		

Sources: Hijova (2006), Bravo (1998), Singh *et al.* (1997), Tomás-Barberán & Clifford (2000b), Villemin *et al.* (1998).

Phenolic acids in general are phenols with one carboxylic acid group. Hydroxybenzoic acids have the carboxylic acid group directly attached to the ring, while hydroxycinnamic acids have a three-carbon side chain. The different phenolic acids (Figure 1) differ in the number and position of the hydroxyl and methoxyl groups attached to the aromatic ring (Robbins, 2003; Macheix et al., 1990).

Hydroxybenzoic acid   **Hydroxycinnamic acid**

p-Hydroxybenzoic acid $R_1=R_2=H$

Protocatechuic acid $R_1=OH$, $R_2=H$

Vanillic acid $R_1=OCH_3$, $R_2=H$

Syringic acid $R_1=R_2=OCH_3$

Gallic acid, $R_1=R_2=OH$

p-Coumaric acid $R_1=R_2=H$

Caffeic acid $R_1=OH$, $R_2=H$

Ferulic acid $R_1=OCH_3$, $R_2=H$

Sinapic acid $R_1=R_2=OCH_3$

Gallic acid   **Ellagic acid**   **Ellagitannin**
(galloyl-bis-HHDP-glucose)
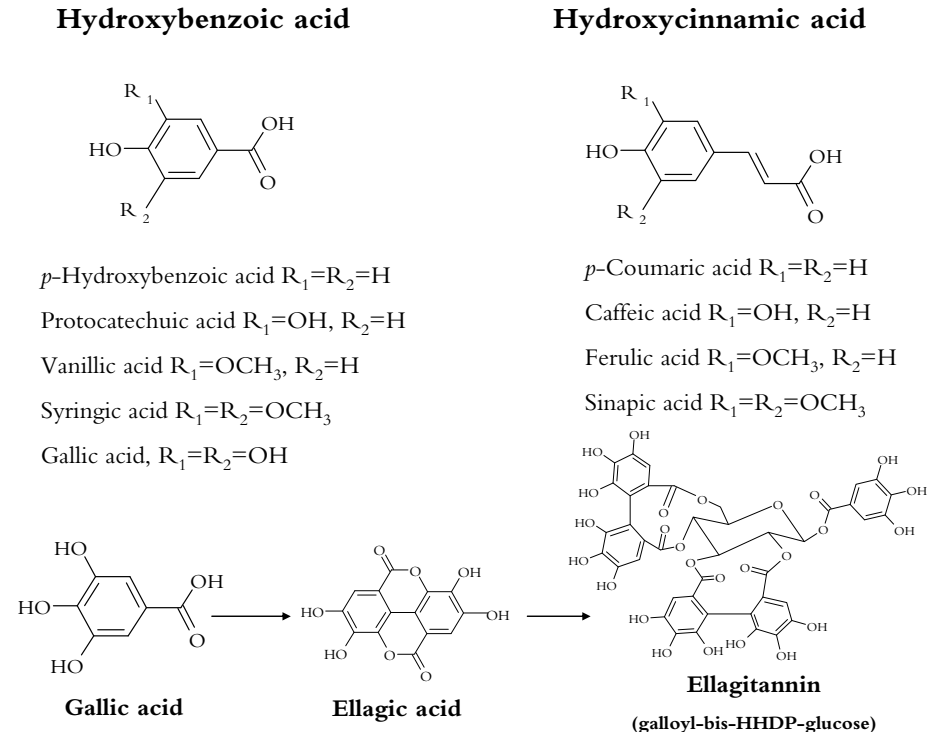
Figure 1. Chemical structure of hydroxybenzoic acids, hydroxycinnamic acids and ellagitannins.

Ellagic acid is the dimer of gallic acid (Figure 1) and both can condense with other galloyls to form gallotannins or with hexahydroxydiphenic acid (HHDP) to form ellagitannins. Free HHDP spontaneously lactonises to ellagic acid after acid hydrolysis of ellagitannins (Bakkalbasi et al., 2009; Haddock et al., 1982).

Flavonoids, the most studied phenolic compounds, are diphenylpropanes (C_6-C_3-C_6). Their basic skeleton and carbon numbering are presented in Figure 2 (Bravo, 1998). The structural subclasses of flavonoids depend on the modifications of the C-ring and are presented in Figure 3.

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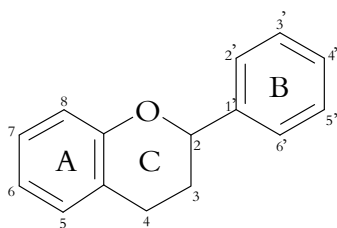
Figure 2. The flavan–nucleus.

Flavonols and flavones have a double bond between C-2 and C-3 but flavones lack the hydroxyl group at C-3. Of these, quercetin, myricetin, kaempferol, isorhamnetin, apigenin, luteolin and their glycosides are the most widespread compounds in plants.

Flavan-3-ols lack the oxygen group at C-4 and contain two centres of asymmetry at C-2 and C-3. The predominant forms are (+)-catechin (C), (-)-epicatechin (EC), (+)-gallocatechin (GC), (-)-epigallocatechin (EGC) and the gallic acid esters (-)-epicatechin gallate (ECG) and (-)-epigallocatechin gallate (EGCG) (Hollman & Arts, 2000; Macheix *et al.*, 1990). There are two additional monomers considered rare or absent in fruits, the monohydroxylated forms afzelechin and epiafzelechin (Macheix *et al.*, 1990).

Oligo- and polymeric forms of flavan-3-ols are known as proanthocyanidins (PAs), the name being based on their characteristic of yielding anthocyanidins when hydrolysed in acidic medium (Santos–Buelga & Scalbert, 2000). The diversity of PAs is based on their combination of monomers ((epi)catechin, procyanidin), (epi)gallocatechin, prodelphinidin) and (epi)afzelequin, propelargonidins), the different types of interflavonoid bonds (C-C or C-O-C bonds) and the length of the chains (degree of polymerisation) (Santos–Buelga & Scalbert, 2000; Bravo, 1998).

Anthocyanins are the glycosylated forms of the anthocyanidins. Cyanidin, delphinidin, peonidin, pelargonidin, petunidin, and malvidin are the most significant aglycones and differ in the number of hydroxyl and methoxyl groups in the B-ring (De Pascual-Teresa & Sanchez-Ballesta, 2008).
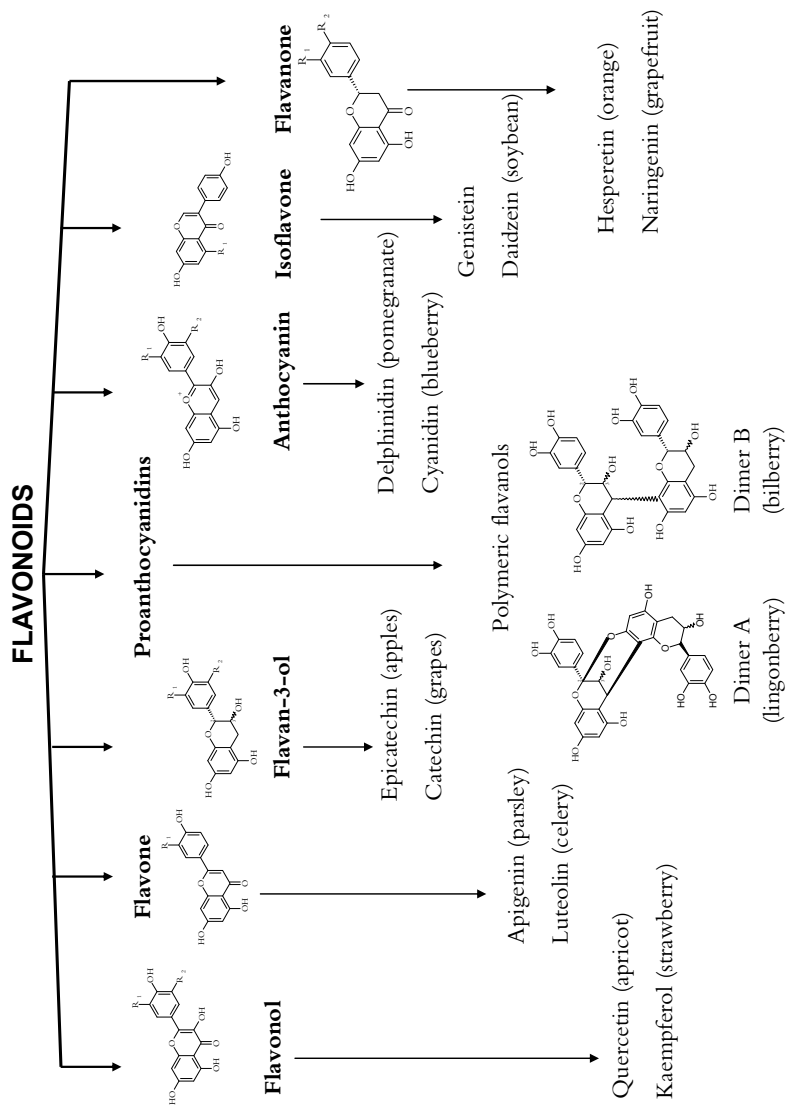
Figure 3. Major flavonoid classes with some examples of aglycones and their sources.

Phenolic acids are mainly found as conjugates with sugars, organic acids or bound to cell wall structures (Clifford, 2000b; Tomás-Barberán & Clifford, 2000a). The soluble forms usually occur as conjugates with the carboxyl group esterified with sugar, quinic, shikimic and other organic acids (Lafay & Gil-Izquierdo, 2008; Bravo, 1998). Glycosilation occurs mostly with glucose attached to the oxygen of the phenolic group (Herrmann, 1989). The esters of caffeic and quinic acids are known as neochlorogenic acid (3-caffeoylquinic acid), cryptochlorogenic acid (4-caffeoylquinic acid) and chlorogenic acid (5-caffeoylquinic acid) (Clifford, 2000b).

Flavonols and flavones are found as *O*-glycosides, usually with the sugar moiety in the 3-position, but *C*-glycosides have also been identified in plants (Bravo, 1998). The most common sugar residues are hexoses (glucose and galactose), pentoses (arabinose and xylose) and deoxyhexose (rhamnose) (Aherne & O'Brien, 2002). Flavonol glycosides sometimes are acylated with organic acids or phenolic acids (Bravo, 1998).

Flavan-3-ols are usually found in the free form, but they can polymerise as result of autoxidation or catalytic activity of polyphenol oxidase. Glycosides of these phenolic compounds are very rare (Hollman & Arts, 2000).

Among the anthocyanin derivatives, *O*-glycosides are mainly found, with hexoses and pentoses almost always attached at the C-3 position (Castañeda-Ovando *et al.*, 2009). Di- and triglycosides also occur, as well as acylated anthocyanins (Castañeda-Ovando *et al.*, 2009; Clifford, 2000a).

1.2 Functions and biosynthesis of phenolic compounds in plants

Phenolic compounds are important for plant physiology, being involved in their morphology (*i.e.* colour and mechanical support in the case of lignin), growth (phenolic acids have been related to nutrient uptake, protein synthesis, enzyme activity, photosynthesis, *etc.*), reproduction (attracting birds and insects that help with pollination) and in protection against attack by pathogens, herbivores and other stress factors such as UV radiation, where flavonols and flavones in particular act as a screen within the cuticle of the plant (Stalikas, 2007; Schijlen *et al.*, 2004; Heim *et al.*, 2002; Parr & Bolwell, 2000). In addition, phenolic compounds have been used for taxonomic purposes (Bravo, 1998).

Phenylalanine

↓

Cinnamic acid

↓

Coumarins ← **Hydroxycinnamic acids** → Sugar derivatives

↓

Benzoic acids ← | Hydroxycinnamoyl CoA / *p*-Coumaroyl CoA | → Esters with organic acids / Hydroxycinnamoyl amides / Lignins

↓

Chalcones

↓

Flavanones

Flavones   **Flavonols**   **Anthocyanins**   **Flavan-3-ols**

↓

Proanthocyanidins
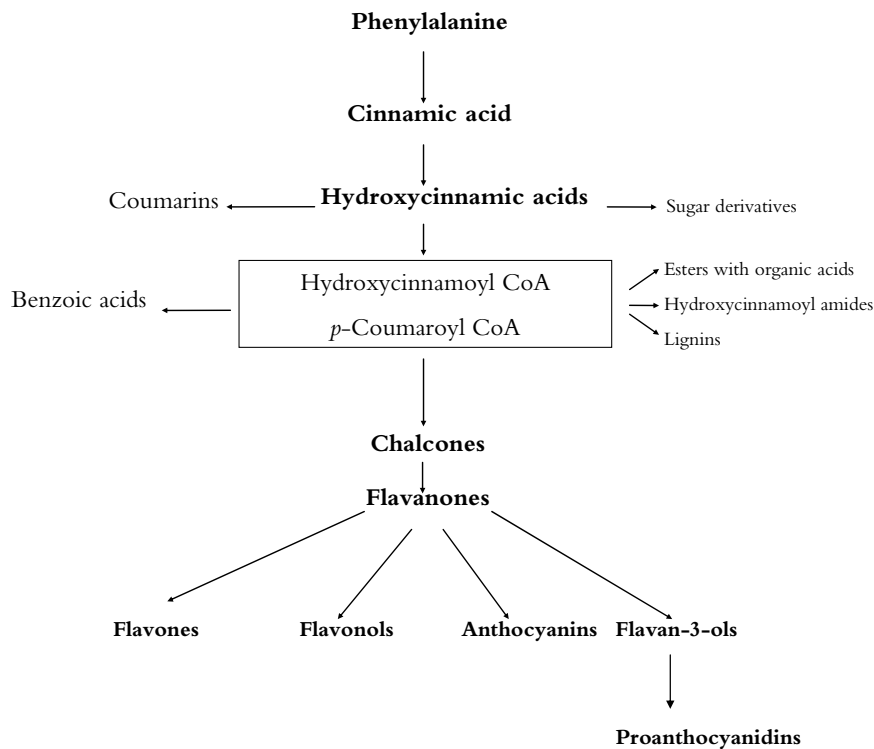
Figure 4. Phenolic compounds biosynthesis (Macheix *et al.*, 1990).

Phenolic compounds are generally synthesised *via* the shikimate pathway, which starts with the formation of phenylalanine and tyrosine. In Figure 4, phenylalanine is deaminated to form cinnamic acid in a reaction catalysed by phenylalanine ammonia-lyase (PAL), the key enzyme in phenolic biosynthesis (Chen *et al.*, 2009; Tomás-Barberán & Espín, 2001). Hydroxycinnamoyl and *p*-coumaroyl coenzyme A esters are the common structural precursors of various phenolic classes. The cleavage of acetate leads to hydroxybenzoic acids, hydroxylation and methoxylation to hydroxycinnamic acids (Macheix *et al.*, 1990). The final products of the flavonoid branch are anthocyanins, starting with the formation of chalcones and with the rest of flavonoids being formed from intermediates of anthocyanin biosynthesis (Schijlen *et al.*, 2004).

1.3   Phenolic compounds in foods

Phenolic compounds are found in almost every plant-derived food. The main sources are fruits, vegetables, cereals, legumes, and nuts (Tura &

Robards, 2002; Bravo, 1998). Table 2 presents a compilation of the main sources of the different phenolic classes. However, the best dietary source also depends on cultivation practices and on the precise dietary composition (Tura & Robards, 2002).

Table 2. *Main sources of the most significant dietary classes of phenolic compounds*

Phenolic class	Food source
Hydroxybenzoic acids	Berries, grapes, oranges, kiwi, apples, peaches, grapefruit, pears, cherries, potatoes, cereals, olives, vegetables, wine, beer, propolis, herbs, spices
Hydroxycinnamic acids	Apples, pears, cherries, plums, peaches, apricots, blueberries, white grapes, kiwi, tomatoes, coffee, white wine, cider, citrus juices, potatoes, olives, vegetables, cereal brans
Flavonols	Berries, apples, grapes, some fruit skin, grapefruit, onions, olives, vegetables, black tea, red wine
Flavones	Sweet red pepper, celery, parsley, red wine, citrus fruits
Flavanones	Citrus fruits
Flavan–3-ols	Apples, apricots, peaches, plums, sweet cherries, green and black tea, red wine
Proanthocyanidins (Condense tannins)	Grapes, cherries, apples, pears, blueberries, raspberries, blackberries, cider, red wine, beer, cocoa bean, cereals
Tannin-like compounds	Tea and wine
Anthocyanins	Berries, red grapes, red-coloured fruits and peel, blood oranges, plums, rhubarb, cabbage, eggplant, onion, red wine
Ellagic acid and ellagitannins	Raspberries, strawberries, blackberries, nuts

Sources: Robbins (2003), Heim *et al.* (2002), Clifford (2000a), Clifford (2000b), Clifford & Scalbert (2000), Hollman & Arts (2000), Santos-Buelga & Scalbert (2000), Tomás-Barberán & Clifford (2000a).

1.4 Phenolic compounds and human health

In general, a diet rich in fruit and vegetables has been linked to various beneficial effects on human health, such as reducing the risk of developing coronary heart disease (Dauchet *et al.*, 2006), cancer (Zhang *et al.*, 2009), hypertension (Mignone *et al.*, 2009), diabetes, and inflammatory processes (Zafra-Stone *et al.*, 2007). The constituents responsible for these protective effects include some vitamins (*e.g.* A, C, E, folate), minerals (*e.g.* potassium,

zinc, selenium), carotenes, dietary fibre and phenolic compounds (Anderson *et al.*, 2009; Mignone *et al.*, 2009; Muhammad *et al.*, 2006; Dillard & Bruce German, 2000). Phenolic compounds are considered to be antioxidants, scavengers of free radicals, metal chelators, antimutagens, and signalling agents (Dillard & Bruce German, 2000; Bravo, 1998). There is a wealth of epidemiological evidence on the impact of diet and lifestyle on the risk of developing chronic diseases, but in the case of fruit and vegetable consumption the results are sometimes inconsistent (Huxley *et al.*, 2009).

Although negative effects have not been reported, some phenolic compounds can be harmful when consumed in large amounts. The best-known negative properties attributed to phenolic compounds are the capacity to precipitate proteins, form complexes with polysaccharides, affect lipid metabolism and interfere with the bioavailability of metal ions (Bravo, 1998).

1.5   Determination of phenolic compounds in fruits

1.5.1 Antioxidant capacity assays

An 'antioxidant' is defined as a substance that inhibits free radicals and reactions promoted by oxygen (Bravo, 1998). For example, in medicine the antioxidants should prevent or delay damage in animal tissues. In food science, the application is broader and ranges from the protection of food products from oxidation processes (*i.e.* protection against rancidity) to dietary antioxidants that protect humans from negative effects of reactive species that cause degenerative diseases (Huang *et al.*, 2005). Total antioxidant power (content and effectiveness) is measured by a wide range of assays (Moon & Shibamoto, 2009; Stratil *et al.*, 2006; Huang *et al.*, 2005), some of which are described below and used in this thesis.

Folin-Ciocalteu assay

This is very popular, convenient, simple and reproducible, and is commonly known as the total phenolic compounds assay (Huang *et al.*, 2005). A redox reaction occurs between the phenolic compounds and the Folin-Ciocalteu reagent under basic conditions (pH ~10) obtained using sodium carbonate. The reaction is monitored by the change in colour, which is proportional to the concentration of phenolic compounds. The reagent is non-specific, as it can also be reduced by other non-phenolic species (Stratil *et al.*, 2006; Prior *et al.*, 2005; Folin & Ciocalteu, 1927). However, the test has become a

routine assay in studying phenolic compounds in plant materials and considerable amounts of data have been generated using the method (Huang *et al.*, 2005; Moyer *et al.*, 2002). Gallic acid equivalents (GAE) are used as reference in most cases (Magalhães *et al.*, 2006; Vinson *et al.*, 2001).

1, 1-diphenyl-2-picrylhydrazyl radical scavenging capacity assay (DPPH• assay)

DPPH• is a stable organic nitrogen radical that gives a deep-purple colour in methanol. The colour fades upon reaction with phenolic compounds in the test solution (Huang *et al.*, 2005). The reaction is monitored at 515 nm until it reaches the plateau. The percentage of DPPH• remaining in the solution is calculated and the reduction is proportional to the concentration and strength of antioxidants (Sánchez–Moreno *et al.*, 1998; Brand-Williams *et al.*, 1995). The concentration of antioxidants that decreases the initial DPPH• concentration by 50% is defined as EC_{50} and the time needed to reach the plateau at that concentration as T_{EC50} (Prior *et al.*, 2005). Sánchez–Moreno *et al.* (1998) defined the 'antiradical efficiency' (AE) as $AE = 1/EC_{50} T_{EC50}$. The drawbacks are mainly due to the reactivity of the DPPH• radical, which may react slowly or not at all with some antioxidants, and possible interference with compounds that present UV–Vis absorption maxima around 515 nm, leading to underestimations (Huang *et al.*, 2005).

Ferric reducing antioxidant power assay (FRAP)

This test uses a ferric complex $Fe(III)(TPTZ)_2Cl_3$ as an oxidant that is reduced to the ferrous Fe(II) form in contact with the antioxidant solution. The reaction is monitored for 4 to 6 min at 593 nm and the change in absorbance ($\Delta A = A_t - A_{0\,min}$) related to ΔA of a Fe(II) standard solution, which is linearly proportional to the concentration of the antioxidant (Benzie & Strain, 1996). The drawbacks are associated with the specificity of the reaction because any compound with a redox potential lower than 0.77 V will reduce the ferric complex (Pérez-Jiménez *et al.*, 2008; Huang *et al.*, 2005).

2,2'-azinobis-3-ethylbenzotiazoline-6-sulfonic acid radical cation decolourisation assay (ABTS•+)

The blue/green ABTS•+ is pre-generated through reaction between ABTS and potassium persulphate and diluted in ethanol to an absorbance of 0.7 $\pm$ 0.02 at 734 nm. The reduction of the radical when mixed with antioxidants is monitored by spectrophotometric readings after 1 min and 6 min and results are expressed as Trolox equivalents (Re *et al.*, 1999).

In biological systems, there are multiple free radicals and oxidant sources and antioxidants may respond in very different ways to them through single or multiple mechanisms (Prior et al., 2005). The antioxidant capacity measured in vitro cannot be taken as direct evidence of in vivo effects (Espín et al., 2007). The assays are useful as a first step in the evaluation of interactions between food items, extracts or supplements and reactive oxygen species (ROS) that can be potentially harmful and in the search for possible sources of antioxidants for further applications in food products or health (Pérez-Jiménez et al., 2008; Espín et al., 2007; Prior et al., 2005).

1.5.2 Extraction

There is no universal extraction method for all type of samples and compounds. Complete extraction should be the goal but this is sometimes compromised by the ability of the solvent to extract all compounds completely, as well as by chemical changes and destruction of the compounds. Therefore, the realistic aim with the extraction is to get extracts with sufficient analyte concentration and no interferences for the analysis (Tura & Robards, 2002). However, there is usually a need for additional steps, such as serial extraction, purification and fractionation to remove unwanted substances (Naczk & Shahidi, 2004; Tura & Robards, 2002). The most frequently selected solvents to extract phenolic compounds are: methanol, ethanol, acetone, water, ethyl acetate, and their combinations (Naczk & Shahidi, 2004). Anthocyanins are usually extracted as the flavylium cations with acidified methanol (Naczk & Shahidi, 2004) and aqueous acetone in general gives the best yields in the extraction of proanthocyanidins (Tura & Robards, 2002).

1.5.3 Hydrolysis

Phenolic compounds are usually found as conjugates (glycosides and esters) or attached to the plant cell wall. Chemical and enzymatic hydrolysis is commonly used to break these bonds (Stalikas, 2007). Enzymatic hydrolysis cannot be used when a wide range of conjugates are studied because their action is specific to certain compounds (Chuankhayan et al., 2007). Acid hydrolysis has been the conventional aid as part of structural analysis of conjugates to confirm the identity of flavonol and flavone aglycones when appropriate standards are not available (Tura & Robards, 2002).

Hydrolysis can also be considered essential for the analysis of condensed and hydrolysable tannins. Heating in acidic medium is used to analyse proanthocyanidins based on depolymerisation to the corresponding

anthocyanins for further colorimetric measurements (Santos–Buelga & Scalbert, 2000). Gallotannins and ellagitannis are hydrolysed to depolymerise to gallic and ellagic acid, respectively. After acid hydrolysis in methanol (ellagic acid is poorly soluble in water), ellagitannins release HHDP, which lactonises spontaneously to ellagic acid, a stable compound under strong hydrolysis conditions (Bakkalbasi *et al.*, 2009; Lei *et al.*, 2001; Clifford & Scalbert, 2000).

1.5.4 Separation, detection and identification

High performance liquid chromatography (HPLC) is the most popular analytical technique used to separate phenolic compounds (Stalikas, 2007). Reversed–phase columns (RP C$_{18}$) are widely used with isocratic or gradient elution using an aqueous acidified (acetic, formic, phosphoric) solvent and an organic modifier (acetonitrile or methanol) (Stalikas, 2007; Robbins & Bean, 2004).

Identification of phenolic compounds is based on different detection techniques such as UV–Vis, fluorescence, electrochemical, mass spectrometry, and nuclear magnetic resonance (NMR), to name the most frequently used (Stalikas, 2007).

Phenolic compounds absorb in the UV region because they have at least one aromatic ring in their structure (Stalikas, 2007). Two absorption bands are characteristic for flavonoids: band I with a maximum between 300-550 nm, coming from the B-ring, and band II with a maximum between 240-285 nm, coming from the A-ring (Merken & Beecher, 2000). The different subclasses of phenolic acids and flavonoids show characteristic UV–Vis spectra that can be used for primary classification (Sakakibara *et al.*, 2003). The absorption maxima of the aglycones can experience shifts to higher wavelengths (bathochromic shift) as shown in Figure 5 due to sugar esters, or to lower wavelengths (hypsochromic shifts) due to –O-glycosidic bonds (Määttä *et al.*, 2003). However, a single wavelength is not enough to study mixtures of various phenolic compounds. In general, 280 nm is used for simultaneous screening but for identification and quantification purposes, data on hydroxybenzoic acids, flavan–3–ols and proanthocyanidins are collected at 280 nm, hydroxycinnamic acids at 320 nm, flavonols at 360 nm, flavones at 340 nm, and anthocyanins at 520 nm (Stalikas, 2007; Merken & Beecher, 2000).
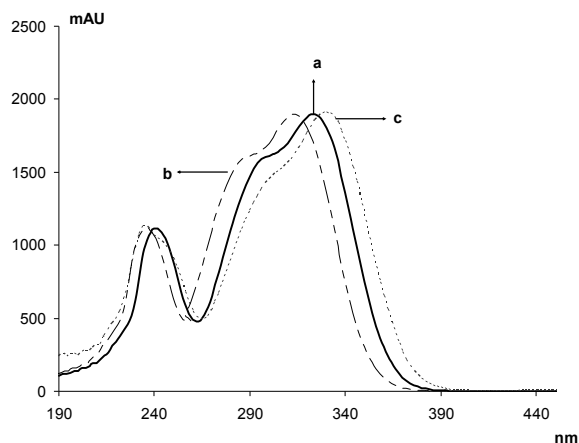
Figure 5. Shifts in the UV-Vis spectra of caffeic acid: (a) aglycone, (b) caffeic acid sugar ester and (c) caffeic acid-4-O-glucoside.

Even though the UV-Vis spectrum is a valuable analytical tool, its use is limited. Mass spectrometry can provide additional structural information and solve coeluting compounds in complex mixtures (Wu & Prior, 2005). Electrospray ionisation (ESI) mass spectrometry provides the molecular masses as a soft ionisation technique after chromatographic separation, while tandem mass spectrometry (MS/MS) provides extra information on the distribution of the substituents on the phenolic rings, useful for tentative identification but only rarely providing sufficient data for full structural analysis (Cuyckens & Claeys, 2004; Ryan *et al.*, 1999).

Ionisation can be performed in the positive (protonation, M+H) and/or negative (deprotonation, M-H) mode. Phenolic acids easily deprotonate in the negative mode (Pérez-Magariño *et al.*, 1999) and form adducts with the cations in the sample or mobile phase in the positive mode (Määttä *et al.*, 2003). However, hydroxybenzoic acid derivatives, hydroxycinnamates and monomeric flavan-3-ols such as catechin and epicatechin have been shown to have difficulties in ionising in the negative mode under certain conditions (Mertz *et al.*, 2007; Määttä-Riihinen *et al.*, 2004b; Määttä *et al.*, 2003; Tomás-Barberán *et al.*, 2001). Flavan-3-ols, oligomeric proanthocyanidins, flavonol glycosides, and anthocyanins respond in both ion modes (de Souza *et al.*, 2008; Ferreres *et al.*, 2007; Li & Deinzer, 2006; Seeram *et al.*, 2006; Cho *et al.*, 2005; Wu & Prior, 2005).

Phenolic compounds in plant foods are known to have several implications in the sensory and nutritional value of these foods. Some phenolic

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compounds are excellent indicators of the physiological development of the fruits (Macheix *et al.*, 1990). They also contribute to organoleptic properties such as colour, astringency, bitterness, and aroma (Drewnowski & Gomez-Carneros, 2000). In addition, scientists are interested in these substances since they have been reported to have multiple biological properties, while some phenolic compounds are interesting for the food industry for potential uses as natural pigments and as additives for their activity as antioxidants (Bravo, 1998).

2 Objectives

The main objective of this thesis was to evaluate a selected group of fruits cultivated in Ecuador for their total soluble phenolic compound content and their antioxidant activity and to identify the group of phenolic compounds that may be responsible for this activity in fruits with high activity.

Specific objectives for the individual studies were:

1. To study the total soluble phenolic compound content and antioxidant capacity of the eighteen Ecuadorian fruits using different antioxidant activity methods (Paper **I**).

2. To study antioxidant properties of Ecuadorian golden-yellow and purple-red tamarillo fruits (Paper **II**).

3. To identify and quantify the main phenolic compounds in Andean blackberry, strawberry, capulí cherry and plum from the *Rosaceae* family (Paper **III**), mortiño from the *Ericaceae* family (Paper **IV**) and three *Passifloraceae* fruits (Paper **V**).

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3 Materials and methods

3.1 Fruit samples

Eighteen different fruits from Ecuador were studied. These fruits are presented in Table 3 and their growing locations are marked in Figure 6. The fruits (pictured in Figure 7) were purchased at full ripeness from three main markets in Quito on three occasions. Fruits were cleaned, peeled if necessary, freed from seeds, chopped, frozen and freeze-dried. The samples were grinded and stored at -20 °C until analysed.

Figure 6. Growing location of the fruits by province. Fruit numbers are shown in Table 3.

Table 3. *Fruit samples collected in Ecuador for the studies*

Latin name	Common name (*name in Ecuador*)	Sample	Paper
Anacardiaceae			
Mangifera indica L.	Mango *(mango)* (1)	Pulp	**I**
Spondias mombin L.	Red mombin *(hobo)* (2)	Whole	**I**
Annonaceae			
Annona cherimolia Mill.	Cherimoya *(chirimoya)* (3)	Pulp	**I**
Bombacaceae			
Calocarpum zapota	Zapote (*zapote*) (4)	Pulp	**I**
Ericaceae			
Vaccinium floribundum Kunth	Blueberry *(mortiño)* (5)	Whole	**IV**
Myrtaceae			
Psidium guajava L.	Guava (*guayaba*) (6)	Whole	**I**
Passifloraceae			
Passiflora edulis var. flavicarpa	Passion fruit (*maracuyá*) (7)	Pulp	**I, V**
Passiflora ligularis L.	Sweet granadilla (*granadilla*) (8)	Pulp	**I, V**
Passiflora mollissima L.	Banana passion fruit (*taxo*) (9)	Pulp	**I, V**
Rosaceae			
Fragaria ananasa Duch.	Strawberry (*frutilla*) (10)	Whole	**I, III**
Prunus salicina Lindl[a]	Plum (*reina-claudia*) (11)	Whole	**I, III**
Prunus serotina var. Capuli[b]	Capuli (*capuli*) (12)	Whole	**I, III**
Rubus glaucus Benth	Andean blackberry (*mora*) (13)	Whole	**I, III**
Solanaceae			
Cyphomandra betacea Sendt[c]	Tamarillo (*tomate de árbol*) (14)	Pulp	**I, II**
Lycopersicon esculentum Mill.	Tomato (*tomate*) (15)	Whole	**I**
Physalis peruviana L.	Physalis (*uvilla*) (16)	Whole	**I**
Solanum muricatum Ait	Sweet pepino (*pepino*) (17)	Whole	**I**
Solanum quitoense Lam	Naranjilla (*naranjilla*) (18)	Pulp	**I**

[a]Two varieties were collected: Santa Rosa and Beauty; [b]Peel and pulp were analysed separately; [c]Two varieties were collected: golden–yellow and purple–red. Numbers in brackets correspond to Figure 7.

(1)   (2)   (3)   (4)

(5)   (6)   (7)   (8)

(9)   (10)   (11)

(12)   (13)   (14)

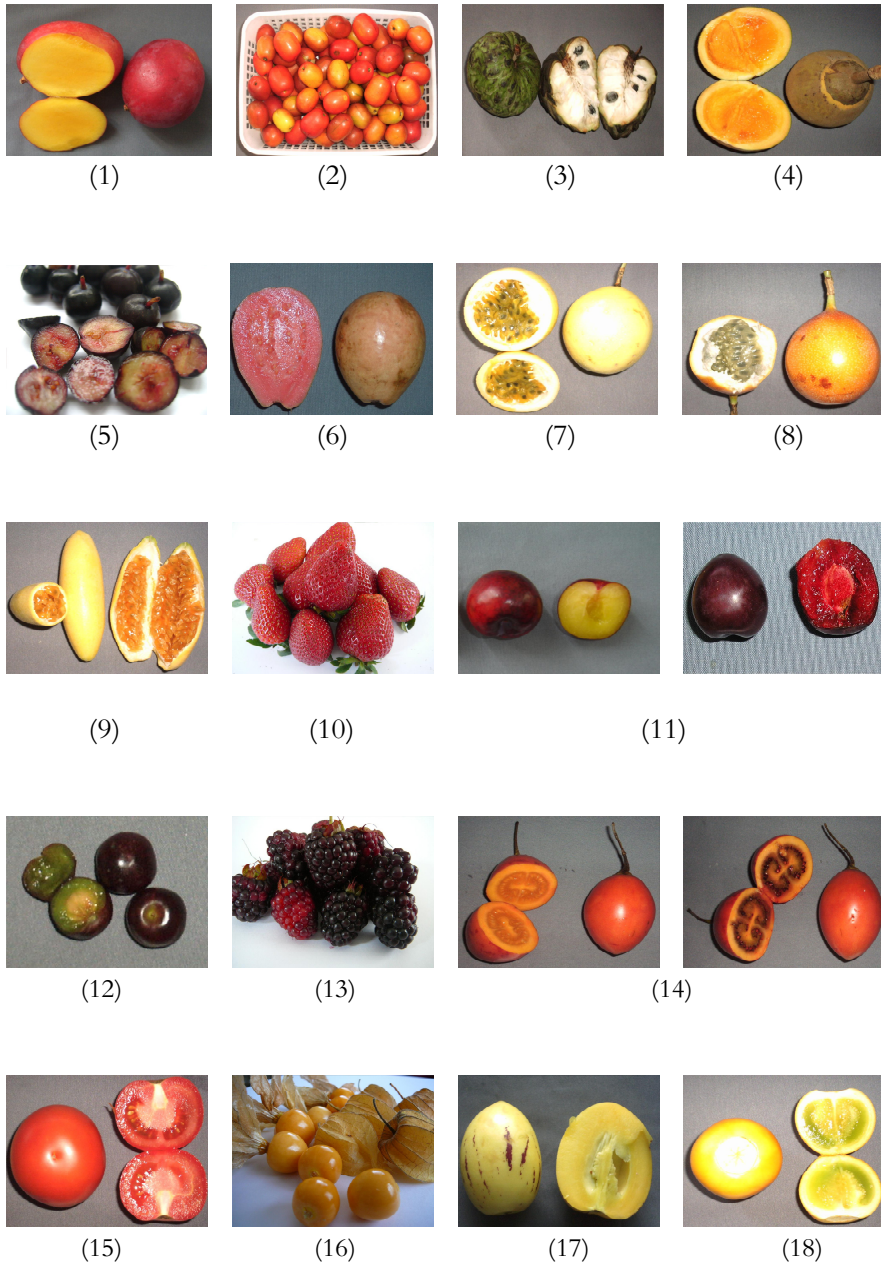(15)   (16)   (17)   (18)

Figure 7. Fruits and berries in the different studies (Pictures: C. Vasco and L. Vasco). Picture number refers to Table 3.

3.2 Chemicals and reference compounds

Specific reagents and standards are listed in Table 4. Bog whortleberry, blackcurrant, rowanberry, strawberry, raspberry previously studied by Määttä *et al.* (2004a; 2004b; 2003) were used as reference samples in Paper **III**. Bilberry previously studied by Riihinen *et al.* (2008) was used as reference sample for anthocyanins in Paper **IV** and lingonberry-derived proanthocyanidin mixture purified and characterized previously by Määttä-Riihinen *et al.* (2005) was used in Paper **V**.

Table 4. *Special reagents and standards used in the studies*

Name	Supplier	Paper
ABTS	Sigma–Aldrich St. Louis, USA	**I, IV**
Caffeic acid	Sigma–Aldrich St. Louis, USA	**I-V**
(+)-Catechin	Sigma–Aldrich St. Louis, USA	**II-V**
Chlorogenic acid	Sigma–Aldrich St. Louis, USA	**II-V**
p-Coumaric acid	Sigma–Aldrich St. Louis, USA	**II-V**
Cyanidin-3-*O*-glucoside chloride	Extrasynthese Geney, France	**II-IV**
DPPH	Sigma–Aldrich St. Louis, USA	**I-II**
Ellagic acid	Sigma–Aldrich St. Louis, USA	**III**
(-)-Epicatechin	Sigma–Aldrich St. Louis, USA	**II-V**
$FeCl_3. 6H_2O$	Sigma–Aldrich St. Louis, USA	**I**
Ferulic acid	Sigma–Aldrich St. Louis, USA	**I-V**
$FeSO_4. 7H_2O$	Sigma–Aldrich St. Louis, USA	**I**
Folin–Ciocalteu reagent (2.0 N)	Sigma–Aldrich St. Louis, USA	**I-II, IV**
Gallic acid	Sigma–Aldrich St. Louis, USA	**I-V**
p-Hydroxybenzoic acid	Sigma–Aldrich St. Louis, USA	**II-V**
Kaempferol	Sigma–Aldrich St. Louis, USA	**II-V**
Myricetin	Fluka Buchs, Switzerland	**II-V**
Pelargonidin-3-*O*-glucoside chloride	Extrasynthese Geney, France	**III**
Potassium persulphate	Sigma–Aldrich St. Louis, USA	**I, IV**
Quercetin	Sigma–Aldrich St. Louis, USA	**I-V**
Rutin	Sigma–Aldrich St. Louis, USA	**I, III**
TPTZ	Sigma–Aldrich St. Louis, USA	**I**
Trolox	Sigma–Aldrich St. Louis, USA	**I-II, IV**
Tungstosilicic acid hydrate	Sigma–Aldrich St. Louis, USA	**II**
Vanillic acid	Sigma–Aldrich St. Louis, USA	**II-V**

3.3   Antioxidant activity methods

The experimental conditions of the methods used to measure total soluble phenolic compound content and antioxidant activity are summarised in Table 5. More details can be found in Paper **I**.

In Papers **I**, **II** and **IV**, freeze-dried samples were extracted twice to analyse total soluble phenolic compounds and antioxidant activity. First, the samples (0.5 g) were extracted with 20 mL of a mixture of methanol:water (50:50, v/v) with continuous stirring for one hour, centrifuged (4000 rpm, 15 min) and the supernatant placed in a volumetric flask. The residue was then re-extracted with 20 mL of a mixture of acetone:water (70:30, v/v) for one hour, centrifuged and the supernatants pooled in the volumetric flask. The total volume was made up to 50 mL with water.

Table 5. *Experimental conditions for total soluble phenolic compounds and antioxidant activity*

Method and reference	Reaction mixture	Final Volume	Reaction time	λ	Standard
Folin & Ciocalteu (Folin & Ciocalteu, 1927)	0.5 mL sample extract + 0.5 mL Folin & Ciocalteu reagent + 10 mL sodium carbonate (75g/L)	25 mL	1 h	750 nm	Gallic acid
DPPH[•] (Sánchez-Moreno et al., 1998)	0.1 mL sample extract + 3.9 mL DPPH methanolic solution (0.025 g/L)	4 mL	Until plateau	515 nm	Trolox
ABTS[•+] (Re et al., 1999)	10μL sample extract + 1mL ABTS[•+] ethanolic solution	1.010 mL	6 min	734 nm	Trolox
FRAP (Benzie & Strain, 1996)	100 μL sample extract + 900 μL FRAP reagent	1 mL	Until plateau	593 nm	$FeSO_4.7H_2O$ and Trolox

Analyses were performed in triplicates and results presented as mean $\pm$ SD; n=9 except for red mombin (n=8), cherimoya (n=7), plum (n=5) and purple tree tomato (n=2) in Paper **I**. For mortiño, pooled samples were prepared for each market, representing three subsamples from the different purchases in Paper **IV**.

3.4 Extraction methods for the identification and quantification of phenolic compounds

The extraction procedures for the identification and quantification of phenolic compounds in fruits are described in Figure 8. In Papers **III–V**, the two-step extraction method described by Määttä *et al.* (2001) was used. In brief, 0.5 - 3 g of freeze-dried sample were extracted with ethyl acetate using intermittent mixing and centrifugation. The solid residue was acidified and extracted with methanol. In every case, extracts were combined, evaporated, dissolved in methanol and injected into the HPLC. More details on the other extractions can be found in Papers **III** and **V**.
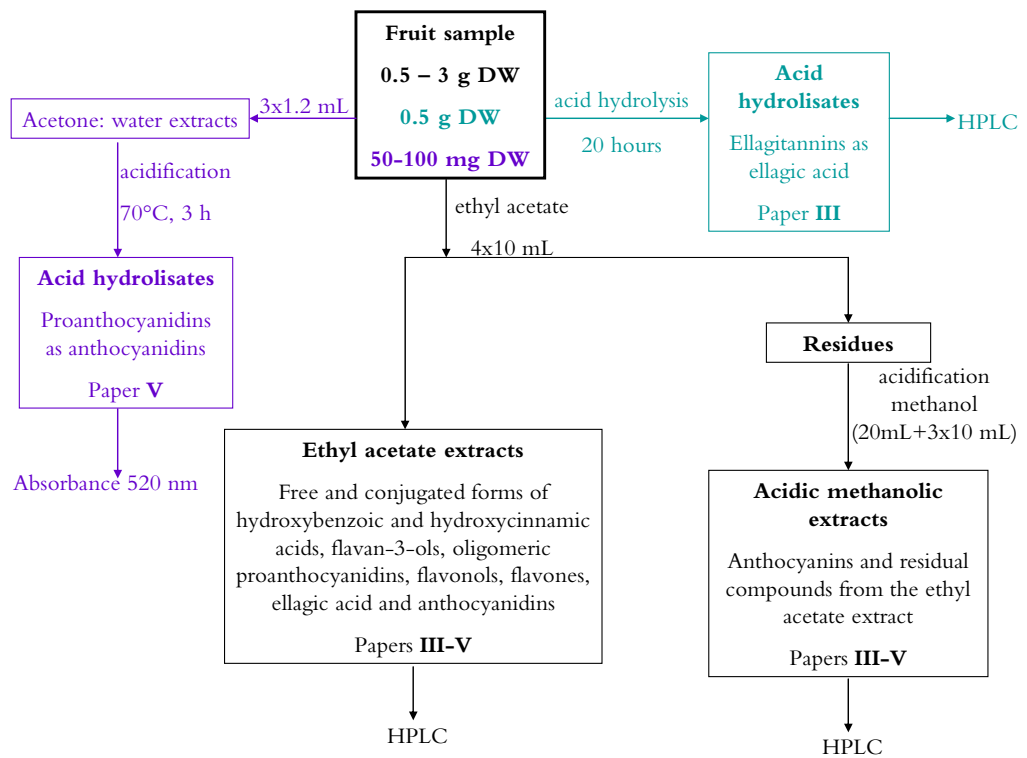

Figure 8. Extraction steps for Papers **III-V**.

3.5 Identification and quantification of phenolic compounds

Ethyl acetate and methanol extracts were analysed simultaneously for free and conjugated forms of hydroxybenzoic acids (280 nm), hydroxycinnamic acids (320 nm), flavan-3-ols (280 nm), oligomeric proanthocyanidins (280 nm), flavonols and flavones (360 nm), ellagic acid and ellagitannins (250 nm), and anthocyanins (520 nm). Columns and chromatographic conditions for the separation, identification and further quantification of the different phenolic compounds in the fruit extracts are summarised in Table 6. More details can be found in Papers **III–V**.

Table 6. *Chromatographic conditions used in the different studies*

Extract	Column	Mobile phase	Gradient	λ (nm)	Paper
Ethyl acetate and methanolic extracts	LiChroCART Purospher RP-18e (125 x 3 mm i.d., 5 μm)	1% formic acid (A), acetonitrile (B)	20 min: 5–30% B, 0.5 mL/min	280 320 360	**III**
	Gemini C$_{18}$ (150 x 3 mm i.d., 5μm)	1% formic acid (A), acetonitrile:methanol (85:15, v/v) (B)	25 min: 5–30% B, 1 mL/min	280 320 360	**III-V**
Methanolic extract	LiChroCART Purospher RP-18e (125 x 3 mm i.d., 5 μm)	5% formic acid (A), acetonitrile (B)	5-10% B (0-5 min), 10% B (5-10 min), 10-40% B (10-25 min), 0.5 mL/min.	520	**III**
	Gemini C$_{18}$ (150 x 3mm i.d., 5μm)	8.5% formic acid (A), acetonitrile:methanol (85:15, v/v) (B)	4-10% B (0-2 min), 10% B (2-20 min), 10-15% B (20-35 min), 15-35% B (35-40 min), 35-80% B (40-50 min), 80%B (50-52 min), 1 mL/min.	520	**IV**
Acid hydrolysate	LiChroCART Purospher RP-18e (125 x 3 mm i.d., 5 μm)	1% formic acid (A), acetonitrile (B)	20 min: 5–30% B, 0.5 mL/min	250 360	**III**

Analyses were performed in triplicates and results presented as mean ± SD; n=9 except for Andean blackberry, strawberry and capulí cherry in Paper **III** (n=3), pooled samples were prepared for each market, representing three subsamples from the different purchases.

A Finnigan LTQ linear ion trap mass spectrometer (Thermo, San Jose, CA) was used. Electrospray ionisation (ESI)-MS-MS was carried out in positive mode using a capillary voltage of 3.8 kV, temperature of 275 °C, and collision energy of 35%. Data were collected in full scan mode over a mass range of m/z 170-1000 on the ethyl acetate and methanol extracts using the 25 min linear gradient with 1% formic acid (solvent A) and acetonitrile:methanol (85:15, v/v, solvent B).

Peak identification was based on retention times and UV-Vis spectra of the standards listed in Table 4. Some reference berry samples, an on-line library of berry phenolic compounds published in the study of Scandinavian berry species by Määttä-Riihinen *et al.* (2004a) and MS/MS were used. The identity of flavonol glycosides was confirmed by acid hydrolysis of the ethyl acetate extract to release the aglycones. Quantification was based on peak area at the characteristic maximum absorption wavelength for every class.

4 Results and discussion

4.1 Total soluble phenolic compound content and antioxidant capacity

The total soluble phenolic compound content and antioxidant capacity measured for all samples are presented in Figure 9.

In Paper **I**, the samples were classified according to their total soluble phenolic compound content into three groups (Figure 9). The first group included Andean blackberry (2167 mg GAE/100 g FW), capulí cherry peel (1494 mg GAE/100 g FW) and banana passion fruit (1010 mg GAE/100 g FW). The second group included samples ranging in total soluble phenolic compound content from 462 to 238 mg GAE/100 g FW and the third group included samples with contents ranging from 91 to 26 mg GAE/100 g FW. Total antioxidant capacity was measured by three different methods (DPPH$^{\bullet}$, FRAP and ABTS$^{\bullet+}$) but Figure 9 shows the TEAC values in order to include mortiño berry from Paper **IV**.

The results obtained by the three assays also described three groups, despite some differences in the order within each group. The three groups for total antioxidant capacity were named as high (Andean blackberry, 52 μmol Trolox/g sample FW; capulí cherry peel, 66 μmol Trolox/g sample FW; and banana passion fruit, 102 μmol Trolox/g sample FW), intermediate, including guava, plum, capulí cherry pulp, cherimoya, zapote, red mombin, and strawberry (42 to 22 μmol Trolox/g sample FW) and low, including sweet granadilla, naranjilla, physalis, tree tomato, passion fruit, mango, sweet pepino, and tomato (4.4 to 1.8 μmol Trolox/g sample FW). Mortiño berry (48 μmol Trolox/g sample FW) was also located in the high capacity group.
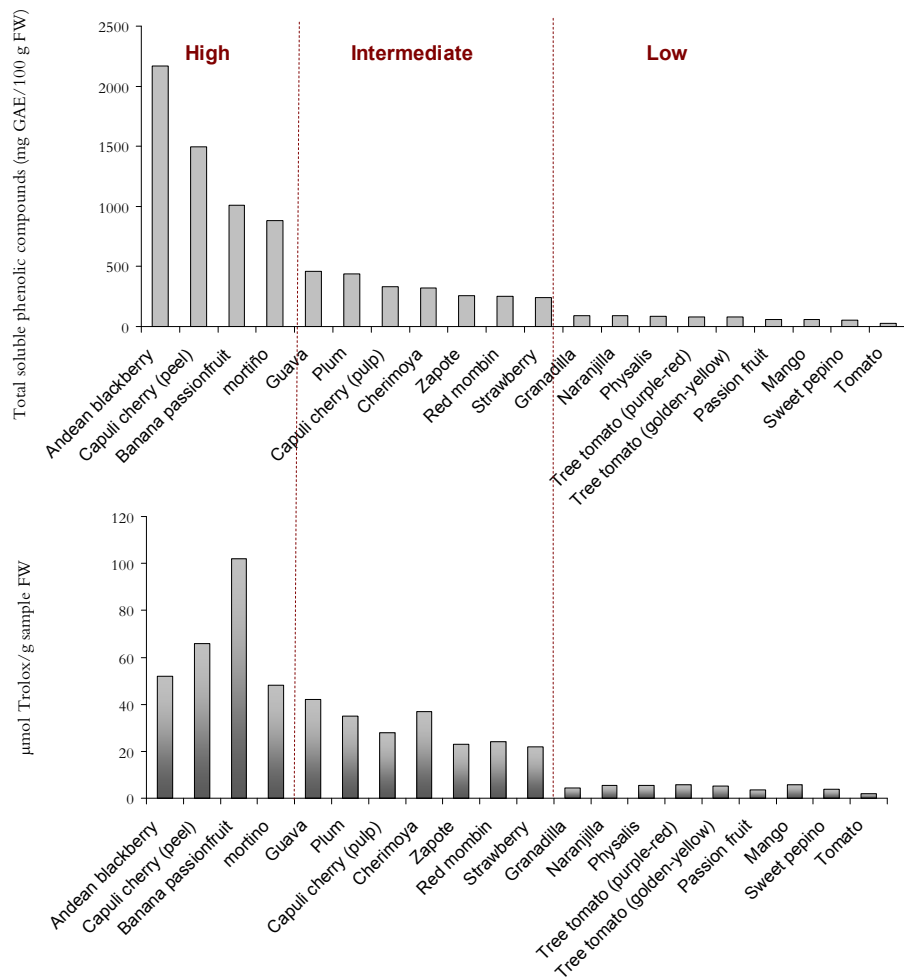
Figure 9. Total soluble phenolic compound content and TEAC values for all samples.

4.2 Identification of phenolic compounds in fruits with high antioxidant capacity

The fruits classified as having high antioxidant capacity were further studied together with other members of the same botanical family for their composition in terms of different phenolic compound classes. Andean blackberry and capulí cherry from the *Rosaceae* were studied together with strawberry and plum (Paper **III**), and mortiño (Paper **IV**) and banana passion

fruit from the *Passifloraceae* together with sweet granadilla and yellow passion fruit (Paper **V**). A summary of the compounds identified in Papers **III**, **IV** and **V** is presented in Table 7. The tools used for identification were standard compounds, some reference samples, UV-Vis spectral characteristics, and the molecular ion and MS/MS fragments.

4.2.1 Hydroxybenzoic acids

Free gallic acid was only identified in Andean blackberry, while galloyl esters were identified using a Finnish strawberry as reference sample and by the bathochromic shifts in the absorption maximum of the gallic acid spectrum (Figure 10). The presence of gallic acid in blackberry might be due to natural or forced degradation of the hydrolysable tannins that are major constituents of the berry (Bakkalbasi *et al.*, 2009). Strawberry has been cited as one of the major sources of gallic acid and derivatives among the berries in the *Rosaceae* family (Tomás-Barberán & Clifford, 2000a). The occurrence of four galloyl esters has been reported for strawberry (Määttä-Riihinen *et al.*, 2004b) but the Ecuadorian strawberry analysed here contained only two esters (Paper **III**).

gallic acid λmax =232, 272 nm; galloyl esters λmax = 232-234, 282-288 nm

p-hydroxybenzoic acid λmax =256 nm; derivatives λmax =262 nm

vanillic acid λmax =262, 292 nm; derivatives λmax =260-268, 296 nm

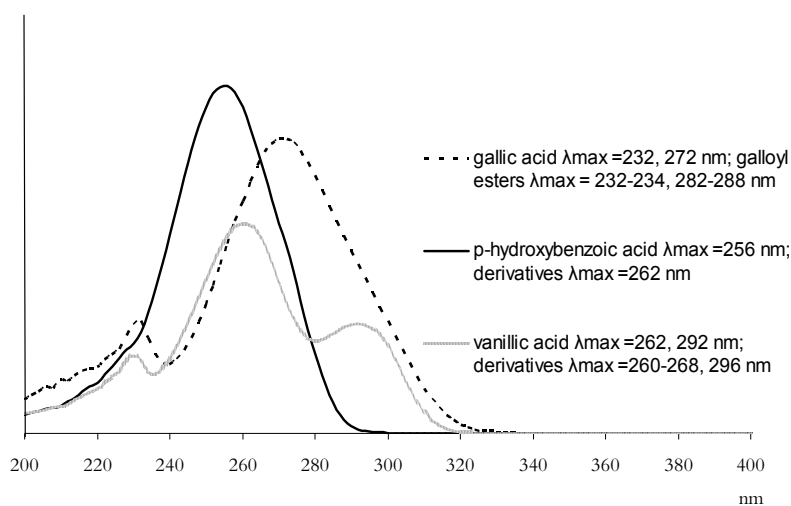*Figure 10.* Characteristic spectra for gallic, *p*-hydroxybenzoic and vanillic acids and the corresponding bathochromic shift of the derivatives.

p-Hydroxybenzoic acid derivative was found in mortiño together with two vanillic acid derivatives (Paper **IV**). These compounds were tentatively identified based on their characteristic UV-Vis spectra since they did not ionise under the MS conditions.

4.2.2 Hydroxycinnamic acid and derivatives

Free forms of hydroxycinnamic acids are rarely found in fruits (Määttä–Riihinen *et al.*, 2004b). Hydroxycinnamic acid derivatives were mainly found in plum, capulí cherry and mortiño (Papers **III** and **IV**). The isomers, neochlorogenic acid and chlorogenic acid, were identified in the *Prunus* species and mortiño, while several unidentified caffeic/ferulic acid derivatives (Figure 11) were found in the plum Beauty and in mortiño. Esters of caffeic acid *m/z* 367 and 369 were found together with caffeoylshikimic acid and a caffeoyl hexoside in mortiño. *p*-Coumaric acid derivatives (Figure 11) were mainly detected in the plum Beauty but were also present in Andean blackberry, strawberry and mortiño. Hydroxycinnamic acid derivatives were also found in tree tomato peel and pulp in Paper **II**.
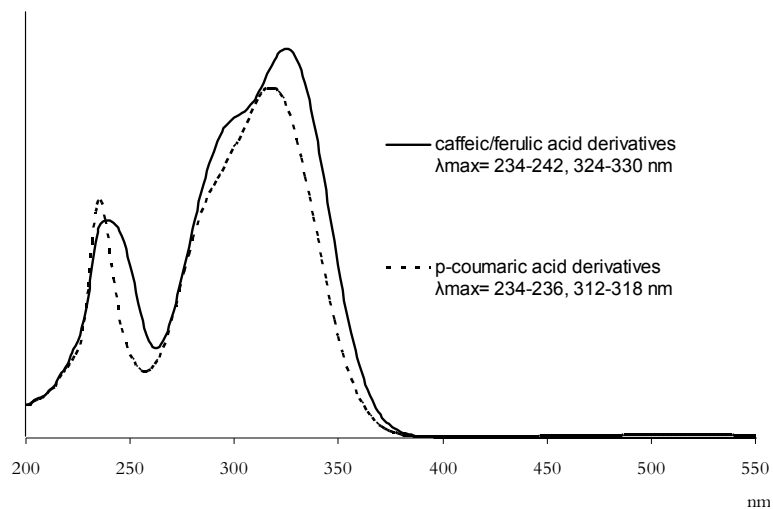
Figure 11. Examples of the typical spectra of chlorogenic acid (caffeic acid derivative) and *p*-coumaric acid derivatives.

4.2.3 Flavan-3-ols and proanthocyanidins

Flavan–3–ols are important constituents of fruits since they are the monomeric forms in the structure of proanthocyanidins (condensed tannins) (Macheix *et al.*, 1990). We determined the four main flavan-3-ols found in fruits: (+)-catechin (Papers **III** and **V**), (–)-epicatechin (Papers **III**, **IV** and V) and (epi)gallocatechin (Paper **V**) but we also identified a fifth monomer, (epi)afzelechin (Paper **V**), which is considered rare in fruits (Macheix *et al.*, 1990). Various dimeric and trimeric proanthocyanidins (types A and B) were

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identified in capulí cherry, plum and mortiño; some could only be classified according to the UV–Vis spectra and/or the molecular ion (Papers **III** and **IV**). In Paper **V**, proanthocyanidins were major compounds in banana passion fruit. The dimers (E)C–(E)C, (E)C–(E)GC and (E)GC–(E)GC and the trimer (E)GC–(E)GC–(E)C were found, as well as dimeric forms containing (epi)afzelechin. More complex molecules could not be identified under our chromatographic conditions and polymeric forms were only quantified in the fruit.

4.2.4 Flavonol and flavone derivatives

In fruits, mainly glycosides of myricetin, quercetin, kaempferol, and isorhamnetin are found (Figure 12) (Macheix *et al.*, 1990). Quercetin glycosides were found in all the fruits in Papers **II**, **III**, and **IV** and in banana passion fruit in Paper **V**. Most fruits usually have a combination of two aglycones (Macheix *et al.*, 1990) and kaempferol was present as glycosides in strawberry, plum and capulí cherry in Paper **III**, myricetin glycosides in mortiño (Paper **IV**), tamarillo peel (Paper **II**) and banana passion fruit (Paper **V**). Banana passion fruit in Paper **V** contained trace amounts of laricitrin hexoside, isorhamnetin hexosides and *C*-hexosides of the flavones apigenin and luteolin.
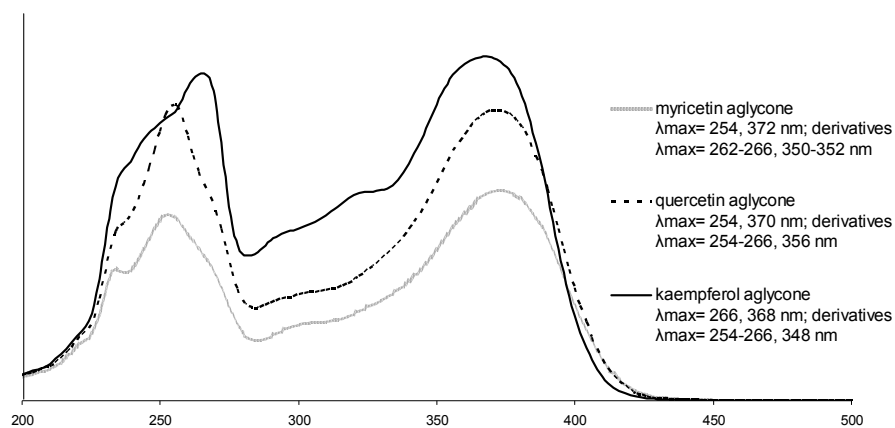

Figure 12. Spectra of three common flavonol aglycones found in fruits.

4.2.5 Anthocyanins

Cyanidin is the most common anthocyanidin found in red fruits (~90%) (Macheix *et al.*, 1990), with very few red-coloured fruits not containing cyanidin (*e.g.* tomato). In all the fruits studied here, cyanidin glycosides (Figure 13) were the major pigments found as hexosides (glucoside and/or galactoside), pentosides (xyloside and/or arabinoside) and rutinoside (Papers **III** and **IV**). Pelargonidin-3-*O*-glucoside and pelargonidin-3-*O*-rutinoside (Figure 13) were the major anthocyanins in strawberry and 3-*O*-rutinoside a minor one in Andean blackberry (Paper **III**). Two delphinidin glycosides (hexoside and pentoside, Figure 13) were identified in mortiño (Paper **IV**). The berries of *Ericaceae* (*Vaccinium* spp.) are an example of a great complexity in the anthocyanin profile (12 to 16 glycosides or more) but mortiño appeared to be an exception (Macheix *et al.*, 1990). Tree tomato (Paper **II**) also contained anthocyanins in the peel and seed-jelly. The individual compounds were not identified but Hurtado *et al.* (2009) reported cyanidin, pelargonidin and delphinidin glycosides.

The anthocyanin content of fruits is affected by different factors such as light and temperature, but genetic factors are critical for the quality and quantity of pigments in fruits (Macheix *et al.*, 1990).
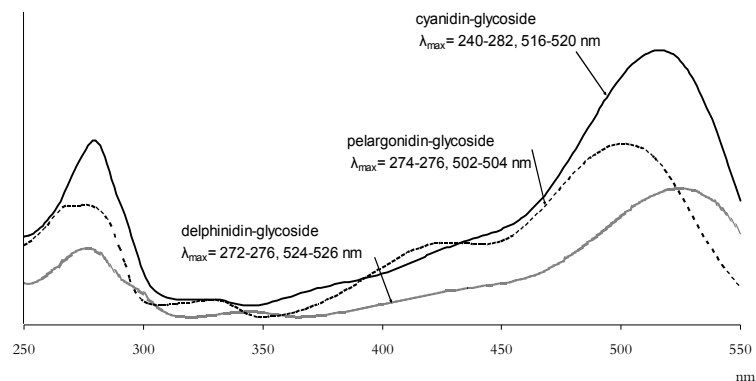

Figure 13. Spectra of three anthocyanins found in fruits.

Table 7. *Summary of the phenolic compounds identified in Papers III, IV and V*

Phenolic compound[a]	DAD characteristic absorption maxima (nm)	m/z	Fruit[b]	Paper
Gallic acid (280 nm), ellagitannins and ellagic acid derivatives (250 nm)				
Gallic acid (std)	234, 272	–	Andean blackberry	III
Galloyl esters (RS)	232–234, 282–288	–	Strawberry, Plum (B, SR)	III
Gallic acid derivative (UV–Vis)	232, 288	–	Mortiño	IV
Ellagitannins (UV–Vis)	232–233, 253–256	–	Andean blackberry	III
Ellagic acid derivatives (RS)	254, 362–370	–	Andean blackberry, strawberry	III
Ellagic acid (std, RS)	254, 368	–	Andean blackberry, strawberry	III
Hydroxybenzoic acids and derivatives (280 nm)				
Vanillic acid derivatives (UV–Vis)	260–268, 296	–	Mortiño	IV
p–Hydroxybenzoic acid derivative (UV–Vis)	262	–	Mortiño	IV
Hydroxycinnamic acid derivatives (320 nm)				
Neochlorogenic acid (UV–Vis, MS)	240–242, 324–325	355	Plum (B, SR), mortiño	III, IV
Caffeic/ferulic acid derivative (UV–Vis)	234–242, 324–330	–	Plum (B, SR), mortiño, tamarillo	II, III, IV
p–Coumaric acid derivatives (UV–Vis)	234–236, 312–314	–	Strawberry, plum (B), mortiño	III, IV
p–Coumaroyl sugar ester (RS)	234, 314	–	Andean blackberry, strawberry	III
Chlorogenic acid (std, MS)	238–240, 325–326	355	Plum (B, SR), capulí (P, PU), mortiño	III, IV
Caffeoylshikimic acid (MS)	240, 326	337	Mortiño	IV
Caffeic acid esters (MS)	240, 326	369	Mortiño	IV
Caffeic acid esters (MS)	242, 328	367	Mortiño	IV
Caffeoyl hexoside (MS)	242, 326	325	Mortiño	IV

Mix of hydroxycinnamic acids (UV–Vis)	236, 318	-	Plum (B)	III
Flavan-3-ols and proanthocyanidins (280 nm)				
Flavan-3-ols/proanthocyanidins (UV–Vis)	238, 278	-	Andean blackberry, strawberry, plum (B, SR)	III
(E)GC–(E)GC (MS)	234, 272	611	Banana passion fruit	V
(E)GC (MS)	236, 272	307	Banana passion fruit	V
(E)C–(E)GC (MS)	236, 276	595	Banana passion fruit	V
(E)GC–(E)GC–(E)C (MS)	236, 278	899	Banana passion fruit	V
(E)C–(E)C (MS)	236, 278	579	Banana passion fruit	V
(+)–Catechin (std, MS, RS)	236–238, 278	291	Strawberry, capulí (P, PU), plum (SR), banana passion fruit	III, V
(E)Afz–(E)C (MS)	236, 280	563	Banana passion fruit	V
(-)-Epicatechin (std, MS)	234–238, 278	291	Andean blackberry, capulí (P, PU), plum (B, SR), mortiño, banana passion fruit	III, IV, V
(epi)Afzelechin	232, 280	275	Banana passion fruit	V
Trimer A (MS)	236, 280	865	Mortiño	IV
Procyanidin trimers B (MS)	234–236, 278	867	Capulí (P, PU), plum (SR, B)	III
Procyanidin dimer B (MS)	236, 280	579	Capulí (P, PU)	III
Proanthocyanidins (UV–Vis)	238, 278	-	Plum (B, SR)	III
Proanthocyanidin (MS)	236, 278	593	Capulí (PU), plum (B)	III
Flavonols (360 nm)				
Myricetin hexosides (MS)	262–266, 352	481	Banana passion fruit	V
Myricetin deoxyhexoside (MS)	-	465	Banana passion fruit	V
Myricetin pentosides (MS)	262, 350	451	Mortiño	IV

Compound	UV	m/z	Source	Group
Laricitrin hexoside (MS)	264, 348	495	Banana passion fruit	V
Apigenin–C-hexoside (MS)	-	433	Banana passion fruit	V
Luteolin–C-hexoside (MS)	-	449	Banana passion fruit	V
Quercetin glycoside (rutin) (std, MS)	254, 352	611	Capulí (P), Andean blackberry	III
Quercetin hexosides (MS)	254–256, 352–355	465	Capulí (P), plum (B), banana passion fruit	III, V
Quercetin-3-O-glucuronide (RS, AH)	256, 355	-	Andean blackberry, strawberry	III
Quercetin pentosides (MS)	254–258, 352–356	435	Capulí (P), plum (B, SR), mortiño	III, IV
Quercetin dipentoside (MS)	256, 352	567	Capulí (P), plum (B, SR)	III
Kaempferol hexoside (MS)	264–266, 348	449	Capulí (P)	III
Quercetin deoxyhexoside (MS)	256, 350–352	449	Mortiño, banana passion fruit	IV, V
Kaempferol-3-O-glucuronide (RS, AH)	254, 348	-	Andean blackberry, strawberry, plum (SR,B)	III
Quercetin glycosides (UV-Vis, AH)	256, 354–356	-	Andean blackberry, plum (B, SR), tamarillo	II, III
Kaempferol-O,C-dipentoside (MS)	264, 348	551	Capulí (P)	III
Kaempferol pentoside (MS)	264, 348	419	Capulí (P)	III
Kaempferol glycoside (UV-Vis, AH)	254, 348	-	Plum (B, SR)	III
Isorhamnetin hexosides (MS)	254, 354–360	479	Banana passion fruit	V
Quercetin derivative (MS)	256, 348	593	Mortiño	IV
Quercetin (std, MS)	254–256, 370–372	303	Capulí (P), mortiño	III, IV
Anthocyanins (520 nm)				
Delphinidin hexoside (MS)	276, 526	465	Mortiño	IV
Delphinidin pentoside (MS)	276, 526	435	Mortiño	IV
Cyanidin hexosides (std, MS)	240–280, 516–518	449	Andean blackberry, strawberry, capulí	III, IV

Compound	λmax (nm)	m/z	Source	
Cyanidin–3–O–rutinoside (MS)	280, 518	595	(P), plum (B, SR), mortiño Andean blackberry, capulí (P), plum (B, SR)	**III**
Pelargonidin-3-O-glucoside (std, RS)	276, 502	-	Strawberry	**III**
Pelargonidin-3-O-rutinoside (RS)	276, 504	-	Andean blackberry, strawberry	**III**
Cyanidin glycosides (UV-Vis)	278–282, 516–520	-	Andean blackberry, plum (B)	**III**
Cyanidin pentoside (MS)	280, 518	419	Mortiño	**IV**
Delphinidin (MS)	272, 530	303	Mortiño	**IV**
Cyanidin (std, MS)	274–278, 524–526	287	Andean blackberry, plum (B), capulí (P), mortiño	**III, IV**

[a]Identification based on (std) = standard retention time and UV–Vis spectra, (UV–Vis)= characteristic absorption maxima, (RS) = reference sample, (AH) = aglycone confirmed by acid hydrolysis, (MS) = mass spectrometry. [b]P= peel; PU = pulp; B = plum variety Beauty; SR = plum variety Santa Rosa.

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4.3 Quantification of phenolic compounds

It is important to bear in mind that accurate and precise quantification of dietary phenolic compounds is often questionable in simultaneous measurement of prominent compounds and quantities given should be considered semi-quantitative due to uncertainties related to extraction efficacy and limitations in the availability of suitable reference compounds for quantification (Naczk & Shahidi, 2004; Tura & Robards, 2002).

A summary of the main components quantified in Andean blackberry, strawberry, capulí cherry, plum, mortiño, and banana passion fruit is given in Table 8. Andean blackberry was rich in ellagic acid derivatives and ellagitannins (3926 mg/kg FW) and cyanidin glycosides (508 mg/kg FW). Ellagic acid derivatives and ellagitannins were also the major compounds in strawberry (278 mg/kg FW), while proanthocyanidins made up 78 mg/kg FW and anthocyanins (pelargonidin derivatives) 65 mg/kg FW. The major phenolic compounds in capulí cherry were the flavan-3-ols (–)-epicatechin (972 mg/kg FW), (+)-catechin (454 mg/kg FW) and proanthocyanidins (658 mg/kg FW). The two varieties of plum were similar in their composition but differed in the amounts of certain classes. Santa Rosa (710 mg/kg FW) had 1.5-fold the amount of proanthocyanidins found in Beauty (469 mg/kg FW), while the (–)-epicatechin content in Beauty (415 mg/kg FW) was 2.4-fold higher than in Santa Rosa. The quercetin glycoside contents were similar, 168 and 144 mg/kg FW for Santa Rosa and Beauty, respectively. The difference between the plum varieties in the content of anthocyanins was related to the fact that Santa Rosa (76 mg/kg FW) contained anthocyanins only in the peel, while Beauty (250 mg/kg FW) had coloured peel and pulp. Mortiño, like other blueberries, was very rich in anthocyanins (2030 mg/kg FW) but also in proanthocyanidins (400 mg/kg FW), quercetin glycosides (350 mg/kg FW) and hydroxycinnamic acids (295 mg/kg FW of caffeic/ferulic acid derivatives and chlorogenic acid). Finally, the phenolic composition of banana passion fruit was predominantly flavan-3-ols (955 mg/kg FW of (E)GC, (+)-catechin, (–)-epicatechin and (epi)azfelechin) and dimeric and trimeric proanthocyanidins (2767 mg/kg FW).

In five of the six dark coloured fruits studied, cyanidin derivatives were major constituents (76-1807 mg/100 g FW), delphinidin was the second most abundant pigment (223 mg/100 g FW) and pelargonidin the third (65 mg/100 g FW in strawberry), in agreement with several studies of anthocyanins in fruits summarised by Macheix et al. (1990).

Table 8. *Summary of the contents (mg/kg FW) of the major compounds and classes estimated in fruits*

	Andean blackberry	Capuli	Strawberry	Plum Santa Rosa	Plum Beauty	Banana passion fruit	Mortiño
Gallic acid and galloyl esters	49	-	46	5	-	-	31
Ellagic acid derivatives and ellagitannins	3926	-	278	-	-	-	-
Hydroxybenzoic acids and derivatives	-	-	-	-	-	-	71
Neochlorogenic acid	-	-	-	76	92	-	25
Chlorogenic acid	-	188	-	40	-	-	145
Caffeic/ferulic acid derivatives	12	8	-	11	45	-	150
p-Coumaric acid derivatives	4	-	18	7	85	-	17
Epigallocatechin	-	-	-	-	-	173	-
(+)-Catechin	-	454	-	-	-	488	-
(-)-Epicatechin	68	972	-	173	415	201	80
(epi)Afzelechin	-	-	-	-	-	93	-
Proanthocyanidins	58	658	78	710	469	2767	100
Quercetin glycosides	72	72	36	168	144	-	350
Myricetin glycosides	-	-	-	-	-	-	26
Kaempferol glycosides	4.0	3.0	5.0	14	5.0	-	-
Cyanidin glycosides	508	89	6.0	76	250	-	1807
Delphinidin glycosides	-	-	-	-	-	-	223
Pelargonidin glycosides	2.4	-	65	-	-	-	-
Total	**4703**	**2443**	**532**	**1280**	**1502**	**3722**	**3025**

5   General discussion

Total phenolic compound content in the fruits studied ranged from high (2167-882 mg GAE/100 g FW) to intermediate (462-238 mg GAE/100 g FW) and low (91-26 mg GAE/100 g FW) (Paper **I**). The first group contained very high levels of antioxidants in comparison with commonly consumed fruits such as citrus fruits (reported content 31-760 mg GAE/100 g FW) (Abeysinghe *et al.*, 2007; Sun *et al.*, 2002). Apples, pears and red grapes contain 270, 54 and 182 mg GAE/100 g FW of total phenolic compounds, respectively, according to Sun *et al.* (2002).

Szajdek & Borowska (2008) summarised some results for different berries such as bilberry (*V. myrtillus*, 525 mg/100 g FW), blackberry (*R. fruticosus*, 361-555 mg/100 g FW), blackcurrant (*R. nigrum*, 318-1342 mg /100 g FW), blueberry (*V. corymbosum*, 181-473 mg/100 g FW), chokeberry (*Aronia melanocarpa*, 663-690 mg/100g FW), raspberry (*R. idaeus*, 114-517 mg/100 g FW) and strawberry (*Fragaria x ananassa*, 102-443 mg/100 g FW). Some TEAC values have also been reported by García-Alonso *et al.* (2004) for blackberry and blueberry (19.2 and 18.7 μmol Trolox/g FW, respectively) and by Scalzo *et al.* (2005) for strawberry (10-16 μmol Trolox/g FW). Comparisons of the results are difficult because of differences in extraction, analytical techniques and standards used in the different studies.

In our studies, total phenolic content and antioxidant capacity showed a positive correlation except in Andean blackberry, capulí peel and banana passion fruit (Paper **I**). The negative correlation obtained for these three fruits might be explained by the type of compounds involved in the activity and not only by the concentration. Among the three fruits, banana passion

fruit had the lowest total phenolic content but the highest activity (Paper **I**), while the phenolic profile (Paper **V**) showed that it has high amounts of flavan-3-ol monomers and is very rich in dimeric and trimeric proanthocyanidins (2767 mg/100 g FW estimated by HPLC). Moreover, the quantification of proanthocyanidins using the lingonberry-derived proanthocyanidin mixture in Paper **V** gave a total of 19550 mg/kg FW, which is very high. In banana passion fruit, the relationship between the activity and the compounds is more evident since they are predominant in the fruit, but it is not known specifically whether the activity is due to the monomers, oligomers, polymers or whether it is a synergic effect. However, it has been reported that flavan-3-ols and galloyl groups easily release electrons to free radical species, the number and position of hydroxyl groups in their structure enhance the free radical scavenging activity (Aron & Kennedy, 2008). Tea and wine are good examples of these forms of phenolic compounds and molecules containing (epi)afzelechin have been found in hops (*Humulus lupulus*), which is an important herb in the beer brewing industry (Li & Deinzer, 2006). Flavan-3-ols and proanthocyanidins are involved in the astringency sensation (Dillard & Bruce German, 2000) that is very characteristic of this fruit flavour.

For capulí cherry (Paper **III)**, it proved difficult to correlate the content of compounds with the antioxidant activity because the measurements were made separately for peel and pulp but the peel, which showed high activity, was also rich in (+)-catechin and (-)-epicatechin and proanthocyanins (B type dimer and trimers) and had high concentrations of flavonol glycosides (mainly quercetin derivatives) and anthocyanins which were not present in the pulp. The high content of (+)-catechin and (-)-epicatechin and the non-detectable level of ascorbic acid in capulí cherry may be linked to the rapid and intense browning of the berry when peeled. Some of the proanthocyanidins detected in the berry might be products of autoxidation or the action of polyphenol oxidase.

The rest of the fruits studied showed profiles with different distributions of the phenolic compound classes and in very variable amounts. Andean blackberry and strawberry in Paper **III** might then owe their antioxidant capacity to the ellagic acid derivatives and ellagitannins that comprise 83.5% and 52.3% of the phenolic compounds quantified in those berries, respectively, and blackberry might also owe its activity to cyanidin glycosides (11%). Unlike banana passion fruit, mortiño (Paper **IV**) had a complex profile with many of the subclasses of flavonoids studied present in

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the berry, but anthocyanins were the major class (67%). However, we can only give a very general evaluation of the subclass of compounds involved in the antioxidant activity because even if the amounts were similar, we do not have enough information on *e.g.* the hydroxylation, glycosilation or substitution in the structures, factors that can modify or even suppress the activity of the compounds in the extracts (Heim *et al.*, 2002). Furthermore, our focus was on the fruits as a food item and their potential contribution to the diet and on evaluating the fruits as possible sources of different phenolic compounds.

The interest in 'exotic' fruits is rising but consumer acceptance is still predominantly related to the sensory properties (*e.g.* taste, colour and aroma), the nutritional value and potential health benefits (Sabbe *et al.*, 2009). In addition, phenolic compounds are responsible for the bitter and astringent taste of many foods and beverages (Drewnowski & Gomez-Carneros, 2000). Low molecular weight phenolic compounds tend to be bitter, *e.g.* naringin and tangeretin in citrus fruits and quercetin and (epi)catechin in wine and tea. On the other hand, high molecular weight phenolic compounds (*e.g.* tannins) are astringent as a result of the interaction between the compounds and the proline-rich proteins in saliva (Haslam, 2007; Drewnowski & Gomez-Carneros, 2000). Colours imparted by phenolic compounds make food attractive and appetising. Anthocyanins are responsible for the different shades in the colour of fruits and berries, with the characteristic colours depending on the anthocyanidins present. For example, the red colour of ripe strawberry is given by pelargonidin glycosides being present as major anthocyanins. The qualitative phenolic compound composition is very important because it can significantly affect the colour of the food or food products. Colour changes can be perceptible to the human eye, *e.g.* flavan-3-ols change the colour of wine due to co-pigmentation reactions (Asen *et al.*, 1972).

The health-promoting properties of foods are very popular nowadays among health-conscious consumers, but the development of functional foods or nutraceuticals needs much study and the key issues are still the bioavailability, metabolism, dose/response and possible toxicity. However, there is strong evidence that food has a direct impact on health (Espín *et al.*, 2007). Identification and quantification of phenolic composition in different fruits will help to reveal the impacts of different compounds/compound classes on the sensory and/or nutritional properties of these fruits, as well as their roles in plant protection and development.

6 Main findings

❖ The total soluble phenolic compound content divided the fruits into three groups: High content (Andean blackberry > banana passion fruit > mortiño berry), intermediate (guava > plum > capulí cherry > cherimoya > zapote > red mombin > strawberry) and low (granadilla > naranjilla > physalis > tree tomato > passion fruit > mango > sweet pepino > tomato). The fruits with high antioxidant capacity were banana passion fruit > Andean blackberry > mortiño (Papers **I, IV**).

❖ Quercetin and myricetin glycosides were identified in tree tomato peel and hydroxycinnamic acid derivatives in the edible part of the fruits (Paper **II**).

❖ The phenolic compound profile of Andean blackberry was dominated by ellagitannins, ellagic acid derivatives and cyanidin glycosides. Strawberry had ellagitannins, pelargonidin glycosides and proanthocyanidins as major compounds. The profiles of the two plum varieties differed in their contents of anthocyanins. Capulí, a special Andean cherry, contained mainly (+)-catechin and (-)-epicatechin, cyanidin glycosides, proanthocyanidins and quercetin glycosides (Paper **III**).

❖ Mortiño berry, a new *Vaccinium* species, was characterised and the phenolic profile was found to be rich in quercetin glycosides, hydroxycinnamic acids and anthocyanins. The anthocyanin profile only contained cyanidin and delphinidin glycosides, unlike other blueberry species which contain petunidin, peonidin and malvidin glycosides and might also contain acetylated anthocyanin glycosides (Paper **IV**).

❖ Banana passion fruit was found to be a rich source of flavan-3-ols and proanthocyanidins and to contain the rare (epi)afzelechin and its dimer (Paper **V**).

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