Original article

Wild *Arbutus unedo* L. and *Rubus ulmifolius* Schott fruits are underutilized sources of valuable bioactive compounds with antioxidant capacity

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Wild *Arbutus unedo* L. and *Rubus ulmifolius* Schott fruits are underutilized sources of valuable bioactive compounds with antioxidant capacity.

Abstract – Introduction. Several studies reveal the important role played by 'lesser-known' wild fruits since they contain nutritional and functional compounds which have biological properties. **Materials and methods**. Our work studied the presence of bioactive compounds such as vitamin C (ascorbic acid and dehydroascorbic acid), total phenolic content, phenolic acids, flavonols, anthocyanins and the antioxidant capacity (FRAP, ABTS$^{\bullet+}$ and DPPH$^{\bullet}$ *in vitro* tests) in wild fruits of *Arbutus unedo* L. and *Rubus ulmifolius* Schott of Spanish origin, including samples from different seasons and locations. **Results and discussion**. A wide variability was found in the composition of fruits of the same species, which substantiates the importance of analyzing several batches of wild fruits, to achieve representative results, taking into account the natural variability. *Arbustus unedo* fruits showed higher vitamin C, mainly in the ascorbic acid form, and phenolic content than *R. ulmifolius* [(172–419) mg ascorbic acid·100 g^{-1} fw *vs.* (5.99–26.83) mg ascorbic acid·100 g^{-1} fw, and (773–1621) mg total phenolic compounds·100 g^{-1} fw *vs.* (376–1326) mg total phenolic compounds·100 g^{-1} fw, respectively]; phenolic acids and anthocyanins are the major groups of phenolic compounds found in both species, with gallic acid and cyanidin 3-glucoside as the main compounds. *Arbustus unedo* fruits showed significantly higher Folin-Ciocalteu values than those of *R. ulmifolius*. These values are higher than those reported for the majority of berries. The significant correlations found among different antioxidant compounds ($r > 0.6300$, $P < 0.001$) may reveal a protective effect between ascorbic acid and phenolic acids or anthocyanins in the fruits. Therefore, *Arbutus unedo* and *R. ulmifolius* fruits should be considered as new important sources of safe antioxidants.

Spain / *Arbutus unedo* / *Rubus ulmifolius* / fruits / phenolic compounds / phenolic content / antioxidants

Les fruits sauvages d'*Arbutus unedo* L. et de *Rubus ulmifolius* Schott sont des sources peu employées de composés bioactifs intéressants présentant une capacité antioxydante.

Résumé – Introduction. Plusieurs études indiquent le rôle important joué par les fruits sauvages « peu connus » car ils contiennent des composés nutritionnels et fonctionnels avec des propriétés biologiques. **Matériel et méthodes**. Nos recherches ont étudié la présence de composés bioactifs comme la vitamine C (acide ascorbique et acide déhydroascorbique), la teneur totale en phénols, les acides phénoliques, les flavonols, les anthocyanes, et la capacité antioxydante (mesurée par des essais FRAP$^{\bullet+}$, ABTS$^{\bullet}$ et DPPH *in vitro*) dans des fruits sauvages de *A. unedo* L. et de *R. ulmifolius* Schott d'origine espagnole, les échantillons provenant de différentes saisons et différents sites. **Résultats et discussion**. Une large variabilité a été révélée dans la composition des fruits d'une même espèce ; cela a justifié l'importance d'analyser plusieurs séries de fruits sauvages, pour obtenir des résultats représentatifs, prenant en considération la variabilité naturelle. Les fruits d'*A. unedo* ont montré une plus haute teneur en vitamine C, principalement sous forme d'acide ascorbique, et en phénols que les fruits de *R. ulmifolius* [(172–419) mg acide ascorbique·100 g^{-1} pf *vs.* (5.99–26.83) mg acide ascorbique·100 g^{-1} pf, et (773–1621) mg de composés phénoliques totaux·100 g^{-1} pf *vs.* (376–1326) mg de composés phénoliques totaux·100 g^{-1} pf, respectivement] ; les acides phénoliques et les anthocyanes ont été les groupes principaux de composés phénoliques trouvés dans les deux espèces, l'acide gallique et la cyanidine 3-glucoside étant les composés principaux. Les fruits d'*A. unedo* ont présenté des valeurs sensiblement plus élevées avec le test de Folin-Ciocalteu que ceux de *R. ulmifolius*. Ces valeurs se sont révélées plus hautes que celles enregistrées pour la majorité de baies. Les corrélations significatives trouvées parmi les différents composés antioxydants ($r > 0{,}6300$, $P < 0{,}001$) pourraient indiquer un effet protecteur dans les fruits entre l'acide ascorbique et les acides phénoliques ou les anthocyanes. Pour cela, les fruits d'*A. unedo* et de *R. ulmifolius* devraient être considérés comme de nouvelles sources importantes d'antioxydants.

Espagne / *Arbutus unedo* / *Rubus ulmifolius* / fruits / composés phénoliques / teneur en phénols / antioxidant

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1. Introduction

Wild edible plants significantly contribute to the diet of rural Mediterranean regions; they are consumed throughout the year in fresh or processed forms [1]. Their nutritional role and health uses have been reported in many nutritional and ethnobotanical studies worldwide, as they often contain higher amount of nutrients and bioactive compounds than many cultivated species [2–4]. From wild plants, Mediterranean wild fruits could also be considered as interesting high-value sources of antioxidants for nutraceuticals, dietary supplements or functional foods [5], as is the case of unusual wild fruits, such as those of *Arbutus unedo* L. and *Rubus ulmifolius* Schott, which may have potential as a source of bioactive compounds with antioxidant activity.

Wild *Arbutus unedo* L. (strawberry tree) fruits have been traditionally eaten in Mediterranean areas, as raw fruits or in the production of digestive liqueurs and the development of preserves and jams. Also, some medicinal properties have been attributed to *A. unedo* fruits, leaves, roots or bark (*e.g.*, antiseptic, diuretic, antihypertensive) [6–8]. *Rubus ulmifolius* Schott (blackberry) fruits are eaten either alone or mixed with wine and sugar, or in jams.

Intake of both *A. unedo* L. and *R. ulmifolius* Schott fruits could be a good strategy to increase the quality of daily food for rural populations. They are valuable potential sources of safe antioxidants of natural origin, which require reconsideration of their role in traditional as well as contemporary diets, being sources of bioactive compounds. In fact, the potential health benefits of incorporating strawberry tree and blackberry fruits or their fruit extracts into yogurts, pie and pastry fillings, cereal, or meat products has already been described [9, 10].

The chemical composition and biological activity of *A. unedo* leaves has been generally studied, while those of its fruits have been less surveyed [11, 12]. To our knowledge, available scientific literature about anthocyanin and flavonol distribution in these wild fruits is scarce. Hence, it seems important to provide information on the bioactive compound content and their antioxidant capacity, taking into account natural variability, in order to promote and recover their consumption.

Our work focused on the evaluation of strawberry tree and blackberry wild fruits as potential sources of bioactive compounds (vitamin C as ascorbic acid and dehydroascorbic acid, total phenolic compounds, and the profile of families of phenolic compounds), as well as the evaluation of their antioxidant capacity measured by different *in vitro* methods.

2. Materials and methods

2.1. Plant material and sample preparation

The sampling procedures followed were according to the recommendations of Greenfield and Southgate to obtain composition data of wild fruit samples [13]. To take into account geographical and environmental variability, six individual samples of fruits of *A. unedo* and *R. ulmifolius* were collected from two different sites of Spain for each species, named Site 1 and Site 2 (*figure 1*), in 2007, 2008 and 2009. The species were clearly identified following the descriptions and keys of the two genera included in the work *Flora Iberica* [14, 15].

These four wild plant populations located in natural forests were the object of a wider study [16] and biometrical data about those of *A. unedo* can be found in previous papers [12, 17]. The fruits were gathered when fully ripe, *i.e.*, when they reach their characteristic mature color (red in the strawberry tree fruits, black in the blackberries) and enough sweetness and softness to make them more palatable. The fruits of *R. ulmifolius* were collected around the end of September, while those of *A. unedo*. were gathered from the end of October to the middle of December, depending on the site and the year (*figure 1*). They were harvested randomly inside the studied wild populations, from twenty-five trees in the case of *A. unedo*,

Arbutus unedo L.

	Site 1	Site 2
Municipality	S. Martín de Valdeiglesias	Salorino
Province	Madrid	Cáceres
Coordinates	4°19' W; 40°23' N	7°01' W; 39°25' N
Altitude	681 m	320 m
Collecting dates	24/10/2007, 12/11/2008, 30/11/2009	28/11/2007, 18/11/2008, 14/12/2009

Rubus ulmifolius Schott

Municipality	Tielmes	Madrid - Cantoblanco
Province	Madrid	Madrid
Coordinates	3°18' W; 40°14' N	3°41' W; 40°32' N
Altitude	594 m	690 m
Collecting dates	24/09/2007, 1/10/2008, 30/09/2009	19/09/2007, 24/09/2008, 29/09/2009

Figure 1.
Arbutus unedo L. and *Rubus ulmifolius* Schott fruits, and sampling details.

and from twenty-five 40 cm × 40 cm-quadrats in the case of *R. ulmifolius*.

All the selected wild fruit presented a healthy external appearance. Each sample was composed of at least 500 g of fruits, packed in plastic boxes and carried to the laboratories in a cooler-box cold system within the day. The stems and leaves were removed and fruits were freeze-dried (Lyophilizer Telstar-Cryodos equipment, Tarrasa, Spain) at −45 °C under vacuum, protected from light. The lyophilized product obtained was homogenized and stored in darkness, in sealed polyethylene bottles at −22 °C until analysis. Three replicates were extracted and measured for each analysis.

2.2. Dry matter determination

Dry matter (DM) was determined by desiccation to constant weight at (100 ± 2) °C following AOAC procedures [18].

2.3. Vitamin C analysis

Contents of ascorbic acid, dehydroascorbic acid and total vitamin C were quantified by high-performance liquid chromatography (HPLC), based on the method proposed by Sánchez-Mata *et al.* [19]. Briefly, freeze-dried fruits were extracted in 4.5% (w/v) meta-phosphoric acid, filtered by Albet 1242 paper and a 0.45-µm PVDF membrane filter, and 100-µL samples were injected into the HPLC system for ascorbic acid analysis. An aliquot of filtrate was subjected to reaction with 4% (w/v) L-cysteine, at pH 7, to convert dehydroascorbic acid into ascorbic acid, filtered and injected into the HPLC system to quantify total vitamin C content.

The instrument was a liquid chromatographer (Micron Analítica, Madrid, Spain) equipped with an isocratic pump (model PU II), an AS-1555 automatic injector (Jasco, Japan), a Sphereclone ODS (2) (250 mm × 4.60 mm, 5 µm) a Phenomenex column, a UV-visible detector (Thermo Separation Spectra Series UV100); and Cromanec XP software (Micronec, Spain). The mobile phase was 1.8 mM H_2SO_4 (pH 2.6), at a flow rate of 0.9 mL·min^{-1}, and UV detection at 245 nm was applied. Quantification was performed by the construction of a linear calibration curve of ascorbic acid (Merck, Darmstadt, Germany) in meta-phosphoric acid. Dehydroascorbic acid was determined by the difference between total vitamin C (measured as ascorbic acid in reduced extracts) and directly measured ascorbic acid contents.

2.4. Phenolic acid, flavonol and anthocyanin HPLC analysis

An aliquot of 0.5 g of freeze-dried fruits was extracted with 20 mL of acidic (0.01 M formic acid) methanol/water (50:50, v/v; pH 2). The extract was centrifuged (1935 *g*, 15 min) and the supernatant was recovered. Twenty mL of acetone/water (70:30, v/v) were added to the residue, and the tubes were shaken and centrifuged again. Methanol and acetone extracts were combined and used to determine phenolic compounds and antioxidant activity in the samples [20].

HPLC analysis of phenolic compounds was performed using a C18 Hypersil ODS stainless steel column (250 mm × 4.6 mm, 5 µm) (Teknokroma, Barcelona, Spain) thermostated at 30 °C. The equipment consisted of an Agilent 1100 Series System equipped with a quaternary pump, autosampler system and rapid scanning UV-visible photodiode array detector. The solvent system used was a gradient of acetonitrile (solvent A) and formic acid 2% (solvent B), as follows: 0 min, 4% of solvent A; 10 min, 10% of solvent A; 20 min, 20% of solvent A; 30 min, 40% of solvent A; 35 min, 40% of solvent A; 40 min, 60% of solvent A; 45 min, 60% of solvent A; 55 min, 4% of solvent A. The flow rate was 1 mL·min^{-1} and runs were monitored with the UV-visible photodiode array detector set at 280 nm (phenolic acids), 360 nm (flavonols) and 520 nm (anthocyanins). Data were processed by Agilent ChemStation software. Identification of the main phenolic compounds was carried out by comparing the retention times and UV-visible absorption spectrum of the compounds with those of the standards and by comparing with chromatographic data found in the literature. Phenolic acids (λ 280 nm) were quantified as mg of gallic acid equivalents (GAE)·100 g^{-1} of fresh weight, flavonols (λ 360 nm) were quantified as mg of rutin equivalents (RE)·100 g^{-1} of fresh weight, and anthocyanins (λ 520 nm) as mg of pelargonidin 3-glucoside equivalents (P3-GE)·100 g^{-1} of fresh weight. The quantification was made using external standard calibration curves (gallic acid, rutin and pelargonidin 3-glucoside) ranging between 50 µg·mL^{-1} and 300 µg·mL^{-1}. The total phenolic compounds were the sum of the three families of phenolic compounds.

2.5. Antioxidant activity determination

Previously obtained extracts (described in section 2.4) were used for all the *in vitro* antioxidant activity assays.

2.5.1. Folin-Ciocalteu method

Although the Folin-Ciocalteu assay has been traditionally used as a method to determine total phenol content in many plant foods, this reagent can also measure the total reducing capacity of a sample [21, 22]. This method, as well as the FRAP assay, is based on electron transfer reactions, and thus is used for antioxidant capacity determination [21], providing complementary information to phenolic compound content and other antioxidant assays. In our study, an aliquot of 0.5 mL of methanol per water extract was added to test tubes; 0.5 mL of Folin-Ciocalteu reagent and 10 mL of sodium carbonate (7.5%, w/v) were added and flasks were made up to 50 mL with distilled water [23]. After 60 min in the dark, absorbance was measured at 750 nm in a Lambda Ez 210 UV-visible spectrophotometer (Perkin Elmer, Massachusetts, USA). Results were compared with a standard curve prepared daily with different concentrations of gallic acid and results were expressed as mg of gallic acid equivalents (GAE)·100 g^{-1} of fresh weight.

2.5.2. Ferric reducing antioxidant power (FRAP) assay

In the FRAP assay, a potential antioxidant reduces ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) at low pH, with the formation of a blue complex (Fe^{2+}/TPTZ) [24]. The FRAP reagent was freshly prepared by mixing together 0.3 M acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tris-2,4,6-tripyridyl-2-triazine) in 40 mM HCl and 20 mM $FeCl_3$ in the proportion 10:1:1 (v/v/v), respectively. The assay was carried out in a 96-well microplate, by adding 10 µL of each extract and 290 µL of the FRAP reagent. After 20 min

of shaking in the dark at 37 °C, absorbance was measured at 593 nm. Results were compared with a standard curve prepared daily with different concentrations of Trolox and expressed as mmol of Trolox equivalents (TE)·100 g^{-1} of fresh weight.

2.5.3. 2,2´-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) scavenging capacity assay

The $ABTS^{\bullet+}$ assay is a decolorization assay applicable to both lipophilic and hydrophilic antioxidants. The pre-formed radical monocation of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) is generated by oxidation of ABTS with potassium persulfate and reduced in the presence of hydrogen-donating antioxidants, according to the method of Re *et al.* [25], with some modifications. ABTS radical cation ($ABTS^{\bullet+}$) was produced by the reaction of ABTS with 2.45 mM potassium persulfate ($K_2S_2O_8$) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The $ABTS^{\bullet+}$ solution (stable for two days) was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. Then, ten µL of each extract were incorporated in a 96-well microplate, and 290 µL of 7 mM $ABTS^{\bullet+}$ were added, mixed well and, after 20 min in the dark at 30 °C, absorbance was measured at 734 nm. Results were compared with a standard curve prepared daily with different concentrations of Trolox and expressed as mmol of Trolox equivalents (TE)·100 g^{-1} of fresh weight.

2.5.4. 2,2´-Diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) scavenging capacity assay

$DPPH^{\bullet}$ is a stable radical widely used to monitor the free radical scavenging abilities of various antioxidants, through the loss of absorbance at 515 nm as the pale yellow non-radical form is produced. The method proposed by Sánchez-Moreno *et al.* [26], with some modifications, was followed. Briefly, ten µL of each extract were mixed with 290 µL of 100 µM $DPPH^{\bullet}$ in methanol in a 96-well microplate and, after one hour of incubation in the dark, absorbance was measured at 515 nm in a microplate reader. Results were compared with a standard curve prepared daily with different concentrations of Trolox and expressed as mmol of Trolox equivalents (TE)·100 g^{-1} of fresh weight.

2.6. Statistical analysis

All the analyses were carried out in triplicate. Statgraphics Plus 5.1 (Statistical Graphics Corporation, Inc., Rockville, MD, USA) was used for statistical treatment of the analytical data. The multivariate ANOVA test and Fisher's Least Significant Difference (LSD) *post hoc* test were used to compare pairs of means and determine statistical significance at the $P < 0.05$ level. The correlations within variables were examined by Pearson correlation. Also, Principal Component Analysis (PCA) was performed among the variables.

3. Results and discussion

In agreement with previous studies about other fruits [27], geographical, seasonal and ripening status variations were expected to influence the chemical composition of the fruits as a result of differences in soil composition, sun exposition and climate. These variations justified the necessity of analyzing several batches of wild fruits, from different sites and years of collection, in order to take into account this natural variability in the final results.

3.1. Compounds of *Arbutus unedo* L. fruits

According to our results, strawberry tree fruits are rich in vitamin C, with mean values from (172 to 419) mg ascorbic acid·100 g^{-1} fw, which means a wide variability, chiefly due to the different origin of the samples. The different moisture contents found depending on the geographical and seasonal conditions should be taken into account (*table I*). These vitamin C levels were similar to other wild fruits such as rose fruits, which are used as a source of vitamin C in teas and other products [28]. Previous studies of

Table I.
Vitamin C (ascorbic and dehydroascorbic acid), total phenols, phenolic acid, and flavonol and anthocyanin contents of *Arbutus unedo* L. and *Rubus ulmifolius* Schott fruits (fw = fresh weight).

Arbutus unedo L. (strawberry tree)

Year of the study	Location of the study	Moisture (%)	Total vitamin C (mg ascorbic acid·100 g^{-1} fw)	Ascorbic acid (mg ascorbic acid·100 g^{-1} fw)	Dehydroascorbic acid (mg ascorbic acid·100 g^{-1} fw)	Total phenolic compounds[1] (mg total phenolic compounds·100 g^{-1} fw)	Phenolic acids[2] (mg gallic acid Eq·100 g^{-1} fw)	Flavonols[3] (mg rutin Eq·100 g^{-1} fw)	Anthocyanins[4] (mg pelargonidin 3-glucoside Eq·100 g^{-1} fw)
2007	Site 1	53.22 ± 2.99 b	238.87 ± 4.64 b	208.63 ± 2.32 b	28.00 ± 2.59 bc	1618.16 ± 25.88 e	463.93 ± 2.88 a	25.72 ± 0.43 b	1154.23 ± 9.07 f
	Site 2	47.45 ± 3.86 a	411.72 ± 17.72 c	370.44 ± 1.28 d	31.12 ± 5.10 bc	1475.44 ± 18.43 d	856.91 ± 4.12 e	46.64 ± 0.22 d	618.53 ± 0.23 d
2008	Site 1	59.32 ± 1.53 c	171.70 ± 5.05 a	146.96 ± 2.55 a	26.59 ± 1.49 b	1077.81 ± 12.75 c	643.68 ± 5.43 d	47.26 ± 0.23 e	434.13 ± 2.58 c
	Site 2	46.82 ± 1.72 a	418.99 ± 14.32 c	382.13 ± 7.74 e	34.21 ± 2.48 c	1621.84 ± 43.87 f	944.81 ± 8.54 f	45.13 ± 2.32 c	677.03 ± 4.79 e
2009	Site 1	60.17 ± 0.39 c	238.60 ± 4.95 b	232.33 ± 2.66 c	4.58 ± 0.44 a	773.28 ± 16.54 a	513.29 ± 3.02 b	48.92 ± 1.34 f	260.01 ± 1.04 b
	Site 2	71.89 ± 0.70 d	245.59 ± 8.48 b	208.79 ± 4.89 b	34.15 ± 0.54 c	842.86 ± 23.64 b	585.03 ± 3.96 c	13.18 ± 0.15 a	257.83 ± 2.01 a
Average		56.27	287.58	258.21	26.44	1234.90	667.94	37.81	566.96

Rubus ulmifolius Schott (blackberry)

Year of the study	Location of the study	Moisture (%)	Total vitamin C (mg ascorbic acid·100 g^{-1} fw)	Ascorbic acid (mg ascorbic acid·100 g^{-1} fw)	Dehydroascorbic acid (mg ascorbic acid·100 g^{-1} fw)	Total phenolic compounds[1] (mg total phenolic compounds·100 g^{-1} fw)	Phenolic acids[2] (mg gallic acid Eq·100 g^{-1} fw)	Flavonols[3] (mg rutin Eq·100 g^{-1} fw)	Anthocyanins[4] (mg·100 g^{-1} fw)
2007	Site 1	38.41 ± 2.04 a	16.33 ± 1.14 c	11.85 ± 0.11 d	5.15 ± 0.30 b	1326.17 ± 18.84 f	942.38 ± 25.43 f	85.61 ± 0.64 e	298.18 ± 2.65 f
	Site 2	79.60 ± 0.26 e	25.41 ± 1.51 d	18.20 ± 0.18 f	7.21 ± 0.33 c	403.19 ± 5.25 b	240.80 ± 2.72 b	31.46 ± 0.16 c	130.93 ± 1.13 e
2008	Site 1	77.76 ± 0.39 d	5.99 ± 0.31 a	3.80 ± 0.29 a	2.94 ± 0.22 a	523.33 ± 8.15 d	400.77 ± 6.45 d	24.57 ± 0.23 b	98.00 ± 0.85 b
	Site 2	72.10 ± 0.38 b	26.83 ± 2.36 d	15.94 ± 0.12 e	8.58 ± 0.78 d	583.03 ± 6.35 e	434.47 ± 4.97 e	9.01 ± 0.08 a	94.56 ± 0.87 a
2009	Site 1	74.89 ± 0.50 c	12.68 ± 0.54 b	8.16 ± 0.73 b	4.52 ± 0.35 b	376.27 ± 3.24 a	197.72 ± 1.68 a	60.70 ± 0.48 d	117.85 ± 1.06 d
	Site 2	71.21 ± 0.30 b	16.61 ± 1.33 c	10.18 ± 0.02 c	5.10 ± 0.02 b	479.04 ± 3.97 c	269.91 ± 2.42 c	97.29 ± 0.79 f	111.84 ± 1.05 c
Average		70.77	17.25	10.59	5.32	607.67	414.34	51.44	141,89

Values expressed as mean ± standard deviation (SD), $n = 3$.
In each column, different letters indicate statistically significant differences ($P < 0.05$).
[1] Sum of all the components determined by HPLC; [2] HPLC (λ = 280 nm); [3] HPLC (λ = 360 nm); [4] HPLC (λ = 520 nm).

A. unedo fruits have shown very variable amounts of vitamin C, from 5.50 mg ascorbic acid·100 g^{-1} fw [11, 29] to 264 mg ascorbic acid·100 g^{-1} dw [12]. Those differences may be due to the sample origin, moisture content, analytical methodologies, and also the method of extraction. The values found by these authors were similar to the mean values (142 mg ascorbic acid·100 g^{-1} fw) obtained with the fresh fruits by our group in a previous work [12]. However, the mean values of ascorbic acid obtained in the present work with freeze-dried samples were even higher (258 mg ascorbic acid·100 g^{-1} fw). This fact probably reveals that for these kinds of fruits the extraction may be more efficient when it is performed using recently powdered freeze-dried material, better than fresh samples, where the homogenization during extraction is more difficult. These results also showed that vitamin C contents in the fruits were more influenced by the location than by the year of harvest. Data obtained during the three years confirm the presence of ascorbic acid as a major form, being almost always higher than 90% of total vitamin C content (*figure 2a*).

Total phenolic compounds in the *A. unedo* fruits analyzed by HPLC ranged between (773 and 1622) mg·100 g^{-1} fw (*table I*), which is a very high level, in the range of rose fruits [29]; it is higher than many fruits considered rich in phenols, such as blueberries, with 670 mg GAE·100 g^{-1} fw [30]. Values of total phenolic compounds in 2009 were significantly lower than those obtained in 2007 and 2008 ($P < 0.05$) (*table I*, *figure 2b*). Major fractions of phenolic compounds in *A. unedo* fruits were phenolic acids and anthocyanins (*figure 2b*). The amounts of phenolic acids in *A. unedo* fruits were between (464 and 945) mg GAE·100 g^{-1} fw and the anthocyanin fraction was between (258 and 11548) mg P3-GE·100 g fw. Finally, flavonol content ranged from (13.18 to 48.92) mg RE·100 g fw (*table I*). Alarcão-E-Silva *et al.* showed much lower values of anthocyanins (101 mg·100 g^{-1} dw) [9], but the determination was made by a mathematical calculation from the direct measurement of absorbance at 535 nm, less specific than the method used in our study.

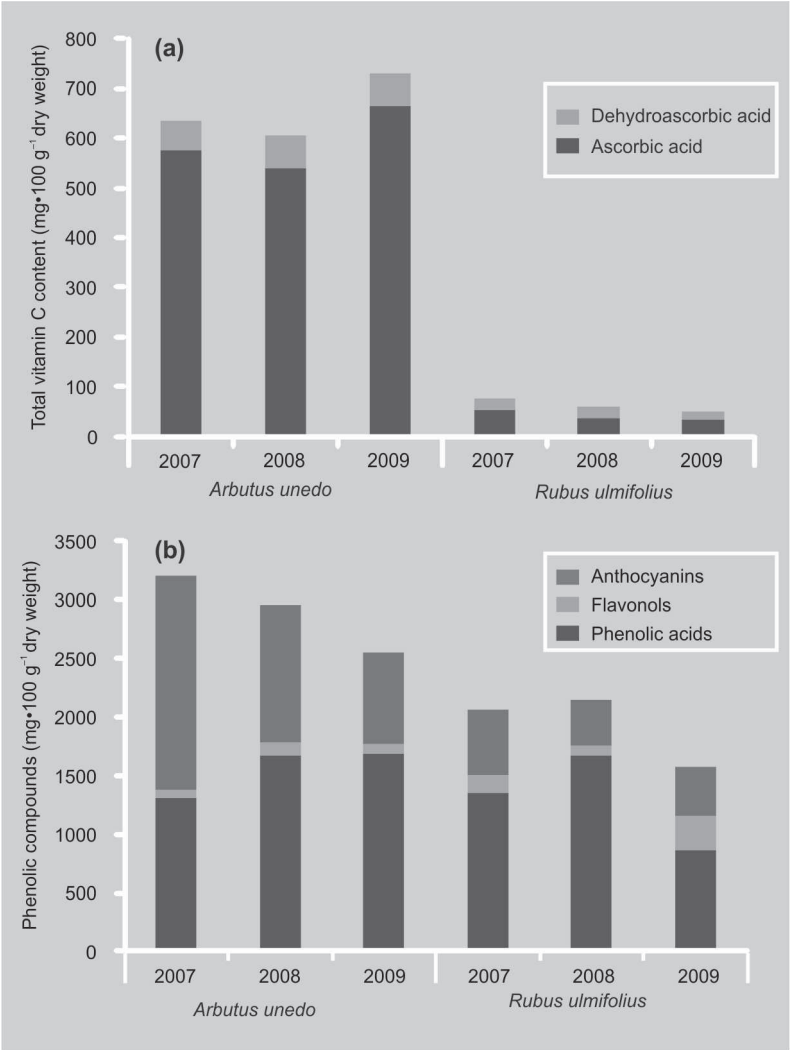

Figure 2.
Distribution of different antioxidant compounds in *Arbutus unedo* and *Rubus ulmifolius* fruits (average of each year of harvest expressed as dry weight). a) Ascorbic acid (ascorbic acid) and dehydroascorbic acid (DHA); b) phenolic compounds.

Identification of the three main groups of phenolic compounds identified (phenolic acids, flavonols and anthocyanins) was tested in *A. unedo* and *R. ulmifolius* fruits (*table II*). The identification of phenolic acids (280 nm), flavonols (360 nm) and anthocyanins (520 nm) in fruit extracts was performed by comparing their chromatographic and spectrophotometric behavior with those of authentic standards and with data found in the literature for these compounds present in similar fruits. Thus, the HPLC chromatogram of *A. unedo* extract at 280 nm presented two main compounds

Table II.
Chromatographic and spectroscopic characteristics and tentative identification of the three main groups of phenolic compounds identified (phenolic acids, flavonols and anthocyanins) in *Arbutus unedo* L. and *Rubus ulmifolius* Schott fruits.

Arbutus unedo L. (strawberry tree)

Detection λ (nm)	Retention time (min)	Compound	% peak area	λ_{max} (nm)	Average contents (mg·100 g^{-1})[1]
280	4.97	Gallic acid	68.58	270	383.89 ± 114.90
	6.88	Gallic acid derivative	26.19	275	150.01 ± 58.48
360	18.32	Myricetin 3-xyloside	11.22	345	12.65 ± 10.73
	22.04	Quercetin 3-xyloside	10.78	360	2.71 ± 1.98
	25.67	Quercetin 3-rutinoside	29.20	355	13.40 ± 0.32
	27.46	Quercetin 3-rhamnoside	18.77	355	7.25 ± 4.85
520	24.91	Delphinidin 3-galactoside	8.63	280, 535	88.95 ± 43.16
	27.07	Cyanidin 3-glucoside	80.19	275, 520	355.98 ± 144.22
	29.59	Cyanidin 3-arabinoside	11.18	275, 520	72.47 ± 50.96

Rubus ulmifolius schott (blackberry)

Detection λ (nm)	Retention time (min)	Compound	% peak area	λ_{max} (nm)	Average contents (mg·100 g^{-1})[1]
280	5.08	Gallic acid	68.73	265	268.72 ± 183.35
360	10.68	Quercetin 3-galactoside	8.55	325	5.44 ± 2.56
	18.30	Quercetin 3-glucoside	19.56	340	18.18 ± 8.77
	25.66	Quercetin 3-rutinoside	9.72	355	6.45 ± 4.33
520	27.46	Cyanidin 3-glucoside	78.91	280, 520	86.73 ± 10.34
	29.55	Pelargonidin 3-rutinoside	2.51	280, 510	4.23 ± 2.41
	31.04	Cyanidin 3-glycoside	18.57	287, 520	19.49 ± 2.37

[1] Average amount obtained for each compound at each collection site and three different years.

and one of them was identified as gallic acid (retention time of 4.97 min) by comparison with the authentic standard (*table II*). The other compound, eluting at 6.88 min, showed a UV spectrum similar to gallic acid and has been identified by different authors as a gallic acid derivative such as theogallin (3-*O*-galloylquinic acid) by HPLC-MS analysis. At 360 nm, four major compounds were identified as myricetin 3-xyloside, quercetin 3-xyloside, quercetin 3-rutinoside and quercetin 3-rhamnoside, according to their retention times and absorption maxima in the UV-Vis spectra. Ellagic acid derivative was not detected due to the type of extraction applied. At 520 nm, delphinidin 3-galactoside, cyanidin 3-glucoside and cyanidin 3-arabinoside were identified by comparing this anthocyanin profile and the absorption maxima in the UV-Vis spectrum (*table II*) with those found in the literature for this fruit [11, 31]. Gallic acid was the most abundant phenolic compound (384 mg GAE·100 g^{-1} fw) in the strawberry tree fruit, followed by anthocyanins, where the most abundant compound was cyanidin 3-glucoside (356 mg P3-GE·100 g^{-1} fw) (*table II*). These values are higher than those reported by Pallauf et al. and Pawlowska *et al.* in *Arbutus unedo* L. fruits [11, 31]. Also, quercetin derivatives were the most abundant flavonols in these fruits [(2.71 to 13.40) mg RE·100 g^{-1} fw].

3.2. Compounds of *Rubus ulmifolius* Schott fruits

Blackberry fruits showed much lower vitamin C levels than the strawberry tree fruits

(*figure 2a*) although ascorbic acid was also the major form, accounting for more than 60% of the total vitamin C. There are only a few studies reporting individual values of dehydroascorbic acid and ascorbic acid, the main compounds responsible for antioxidant activity in fresh fruits [9, 12]. A wide variability was found in ascorbic acid, dehydroascorbic acid and total vitamin C, with coefficients of variation up to 46.07%. Wide natural variations in vitamin C and many other nutrients' content can be found in edible fruits, as can be seen from data in the scientific literature as well as food nutrient databases [3, 12, 27, 32–35]. Different factors may be involved in vitamin C content in the fruits, particularly in wild species, very influenced by climate conditions, due to its high instability at high temperatures or during light exposure. The higher moisture content in 2008 and 2009 samples could also explain their lower vitamin C content by a dilution mechanism, in the same way as it happens to other nutritional and phytochemical components.

The amount of total phenolic compounds in *R. ulmifolius* was significantly lower than in *A. unedo* fruits, ranging from (376 to 1326) mg·100 g^{-1} fw (*table I*). However, these values of both species are in the highest range or even above those reported for the majority of berries [(192 to 929) mg·100 g^{-1} fw] [36, 37][1].

The major families of phenolic compounds in both *R. ulmifolius* and *A. unedo* fruits were phenolic acids and anthocyanins; however, phenolic acids showed a higher predominance in *R. ulmifolius*, with an average contribution of 68.19% to total phenolic compounds, while in A. unedo fruits both families contributed in similar proportions (*figure 2b*). Phenolic acids in *R. ulmifolius* ranged from (198 to 942) mg GAE·100 g^{-1} fw, and anthocyanins from (95 to 298) mg P3-GE·100 g^{-1} fw, while the total amount of flavonols, as the minor family in *R. ulmifolius*, was between (9.01 and 97.29) mg RE·100 g^{-1} fw (*table I*). Previous studies have reported lower values in many cultivated and wild berries: total anthocyanins ranging from (12.70 to 262) mg cyanidin 3-glucoside·100 g^{-1} fw [36–38]. Also, lower values of flavonols and anthocyanins in *R. ulmifolius* wild fruits have been reported (7.60 mg RE·100 g^{-1} fw and 100 mg cyanidin 3-glucoside·100 g^{-1} fw, respectively) [10].

Identification and peak assignment was performed in blackberry fruits as mentioned before in strawberry tree fruit; the major compound identified at 280 nm was gallic acid (*table II*). Also, at 360 nm, the HPLC profile showed three main compounds which chromatographic and spectral data matched with quercetin 3-galactoside, quercetin 3-glucoside and quercetin 3-rutinoside. Finally, the HPLC chromatogram at 520 nm showed four major compounds identified as different glycosides of cyanidin and pelargonidin 3-rutinoside, but delphinidin derivatives were not detected, unlike in *A. unedo* (*table II*)[1] [39]. The same as in *A. unedo* fruits, gallic acid was the major phenolic acid (269 mg GAE·100 g^{-1} fw) in *R. ulmifolius*, followed by cyanidin 3-glucoside (86.73 mg P3-GE·100 g^{-1} fw). Quercetin glycosides are the most commonly identified flavonols in berries [39] and our study observed their content in the range of (5.44 to 18.18) mg RE·100 g^{-1} fw. Blackberry fruits showed a variable distribution of anthocyanins from (4.23 to 86.73) mg P3-GE·100 g^{-1} fw. This wide range has been previously reported for phenolics in other fruits [27], and may be influenced by genotype and environment; factors such as climate or soil conditions might influence anthocyanin content of wild fruits.

3.3. Antioxidant capacity of *A. unedo* and *R. ulmifolius* fruits

A great variability was found in the antioxidant capacity value of strawberry tree and blackberry fruits, depending on the site of collection and the harvest year (*table III*). In our study, *Arbutus unedo* analyzed by the Folin-Ciocalteu method gave results ranging from (952 to 1974) mg GAE·100 g^{-1} fw. The *R. ulmifolius* fruits analyzed showed values of (449–1337) mg GAE·100 g^{-1} fw, higher

[1] Phenol-Explorer, Database on Polyphenol content in Foods, http://www.phenol-explorer.eu/contents/food/70. October 2013.

Table III.
Antioxidant capacity of *Arbutus unedo* L. and *Rubus ulmifolius* Schott fruits (fw = fresh weight).

Arbutus unedo L. (strawberry tree)

Year of the study	Location of the study	Folin-Ciocalteu (mg gallic acid Eq·100 g^{-1} fw)	FRAP	$ABTS^{\bullet+}$	$DPPH^{\bullet}$
			(mmol trolox Eq·100 g^{-1} fw)		
2007	Site 1	1973.01 ± 151.51 d	9.86 ± 0.49 b	5.29 ± 0.52 c	3.70 ± 0.17 b
	Site 2	1973.68 ± 122.63 d	8.45 ± 0.45 a	1.22 ± 0.09 a	4.38 ± 0.07 c
2008	Site 1	1736.50 ± 80.43 c	8.40 ± 0.12 a	5.14 ± 0.21 c	3.51 ± 0.27 b
	Site 2	1954.62 ± 198.46 cd	17.72 ± 0.27 c	10.65 ± 0.28 d	6.54 ± 0.24 d
2009	Site 1	1351.29 ± 123.32 b	10.12 ± 0.18 b	1.77 ± 0.40 ab	3.27 ± 0.28 b
	Site 2	951.72 ± 49.00 a	8.00 ± 0.17 a	2.42 ± 0.07 b	2.78 ± 0.10 a

Rubus ulmifolius Schott (blackberry)

Year of the study	Location of the study	Folin-Ciocalteu (mg gallic acid Eq·100 g^{-1} fw)	FRAP	$ABTS^{\bullet+}$	$DPPH^{\bullet}$
			(mmol trolox Eq·100 g^{-1} fw)		
2007	Site 1	1337.15 ± 121.89 c	14.16 ± 0.40 d	8.89 ± 0.88 d	9.35 ± 0.22 d
	Site 2	449.39 ± 3.85 a	4.51 ± 0.12 a	2.42 ± 0.13 a	2.63 ± 0.12 a
2008	Site 1	541.32 ± 3.80 ab	4.90 ± 0.09 a	3.74 ± 0.33 b	3.41 ± 0.16 b
	Site 2	587.02 ± 41.31 b	4.45 ± 0.09 a	2.28 ± 0.05 a	3.12 ± 0.06 b
2009	Site 1	604.94 ± 50.99 b	9.22 ± 0.41 c	5.76 ± 0.57 c	4.41 ± 0.11 c
	Site 2	599.95 ± 34.34 b	8.50 ± 0.46 b	4.08 ± 0.32 b	4.46 ± 0.15 c

Values expressed as mean ± standard deviation (SD), $n = 3$.
In each column, different letters indicate statistically significant differences ($P < 0.05$).

values than that reported by Egea *et al.* [40] (297 mg GAE·100 g^{-1} fw). Considering the mean values of the antioxidant capacity of the two fruits obtained for each method used in the two locations and the three different years, we found that *A. unedo* showed significantly higher FRAP values than *R. ulmifolius* [(10.43 ± 3.46) mmol TE·100 g^{-1} fw *vs.* (7.62 ± 3.61) mmol TE·100 g^{-1} fw]. However, no statistically significant differences were found between *A. unedo* and *R. ulmifolius* in $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ values.

Although $ABTS^{\bullet+}$, $DPPH^{\bullet}$ and FRAP values were significantly correlated with each other, only FRAP showed significant correlations with all the antioxidant compounds analyzed. As could be expected, several correlations were found between the different antioxidant compounds in the fruits (*table IV*). Relevant positive correlations were found between ascorbic acid and all the antioxidant compounds analyzed except for flavonol content, which was the minor fraction of phenolic compounds in these fruits. Therefore, total phenolic content was correlated with the major constituents: phenolic acid and especially anthocyanin content ($r = 0.8741$, $P < 0.05$), but not with flavonols.

The significant correlations found between ascorbic acid and phenolic acids in the fruits (*table IV*) could reveal some reciprocal protective effect between ascorbic acid and other bioactive compounds analyzed. This correlation could mean that the presence of high levels of some antioxidants in fruits could also preserve the presence of other antioxidants, and as a result, a higher total antioxidant capacity may be found.

3.4. Characterization of *A. unedo* and *R. ulmifolius* fruits according to their bioactive compounds

In our study, we analyzed and included a great number of variables and significant

Table IV.
Correlation analysis of antioxidant activity values with total vitamin C, ascorbic acid, dehydroascorbic acid, total phenols and the three main groups of phenolic compounds identified (phenolic acids, flavonols and anthocyanins).

Parameters studied	Folin-Ciocalteu		ABTS$^{\bullet+}$		DPPH$^{\bullet}$		FRAP	
	Pearson correlation coefficient	*p*-value	Pearson correlation coefficient	*p*-value	Pearson correlation coefficient	*p*-value	Pearson correlation coefficient	*p*-value
Total vitamin C	0.8119	0.0000	0.0753	0.6625	0.0277	0.8727	0.4960	0.0021
• Ascorbic acid	0.8127	0.0000	0.0767	0.6565	0.0345	0.8417	0.5079	0.0016
• Dehydroascorbic acid	0.7223	0.0000	0.1376	0.4234	–0.0381	0.8255	0.3505	0.0361
Total phenolic compounds	0.9340	0.0000	0.4664	0.0041	0.4857	0.0027	0.6859	0.0000
• Phenolic acids	0.5787	0.0002	0.3940	0.0174	0.5668	0.0003	0.5068	0.0016
• Flavonols	0.0355	0.8372	0.3919	0.0181	0.6260	0.0000	0.4487	0.0061
• Anthocyanins	0.8537	0.0000	0.2960	0.0796	0.1487	0.3868	0.4605	0.0047

variability was observed in all of them; therefore, multivariate analysis was applied in order to characterize and classify the fruits studied according to their bioactive compounds. A principal component analysis (PCA) was performed, reducing the multidimensional structure of the data and providing a two-dimensional map for explaining the variance observed. The first two components of the PCA explained 81.57% of the total variance (55.55% first and 26.02% second) (*figure 3*). The first component is highly positively correlated with moisture and negatively correlated with total phenols and phenolic acid variables, vitamin C and its fractions (ascorbic acid), and the Folin-Ciocalteu and FRAP assays. The second principal component separates the samples according to flavonols and the DPPH$^{\bullet}$ assay (positive correlation), and it is negatively correlated with dehydroascorbic acid and vitamin C. All the samples were plotted on the reduced space of the two principal components generally; *A unedo* fruits were negatively characterized by the first and second principal components (higher vitamin C, phenolic acids and antioxidant activity measured by the Folin-Ciocalteu and FRAP assays, and lower moisture) and *R. ulmifolius* positively correlated with both components, which means lower anthocyanins and vitamin C (ascorbic acid) and higher moisture and flavonol content, which statistically confirmed the observations in the data presented.

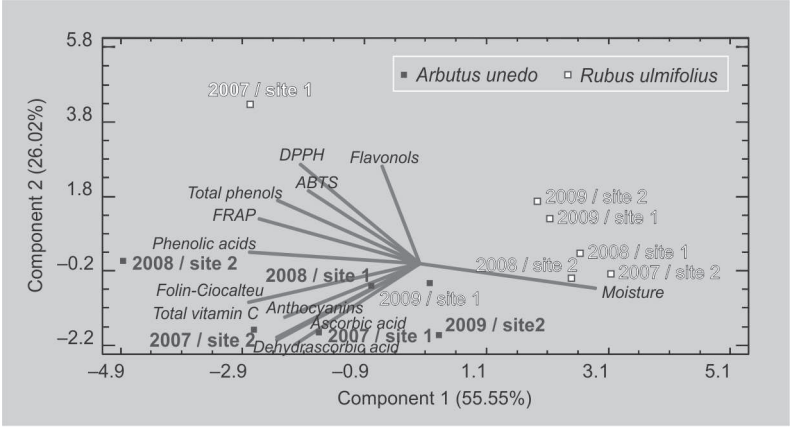

Figure 3.
Principal Component Analysis (PCA) of bioactive compounds of *Arbutus unedo* L. and *Rubus ulmifolius* Schott fruits.

4. Conclusion

The wild fruits of *Arbutus unedo* L. and *Rubus ulmifolius* Schott are valuable sources of bioactive compounds with antioxidant activity. *Arbustus unedo* fruits showed higher vitamin C and phenolic content than *R. ulmifolius*. In both species the major groups of phenolic compounds found were phenolic acids (gallic acid as the major one) and anthocyanins (cyanidin 3-glucoside as the main compound). *Arbustus*

unedo showed significantly higher Folin-Ciocalteu values than *R. ulmifolius*. The significant correlations found among different antioxidant compounds ($r > 0.6300$, $P < 0.001$) may reveal a protective effect between ascorbic acid and phenolic acids or anthocyanins in the fruits. These correlations should be further studied in the context of the complex synergistic and antagonistic actions of the different bioactive compounds involved in the antioxidant metabolism of plants.

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Los frutos silvestres de *Arbutus unedo* L. y *Rubus ulmifolius* Schott son valiosas fuentes de compuestos bioactivos con capacidad antioxidante.

Resumen - Introducción. Según diversos estudios previos, algunos frutos silvestres "menos conocidos" pueden desempeñar un importante papel en la dieta, ya que contienen compuestos nutricionales y funcionales que tienen propiedades biológicas. **Materiales y métodos**. Este trabajo estudia la presencia de compuestos bioactivos como la vitamina C (ácido ascórbico y ácido deshidroascórbico), el contenido de compuestos fenólicos totales, ácidos fenólicos, flavonoides, antocianinas, y la capacidad antioxidante a través de ensayos *in vitro* (FRAP, $ABTS^{\bullet+}$ y $DPPH^{\bullet}$), en frutos silvestres de *Arbutus unedo* L. y *Rubus ulmifolius* Schott de origen español, incluyendo muestras recolectadas en diferentes años y localidades. **Resultados y discusión.** Se ha encontrado una amplia variabilidad en la composición de los frutos de la misma especie, que permiten sustentar la importancia de analizar varios lotes de frutos para lograr resultados representativos, que incluyan la posible variabilidad natural. Los frutos de *A. unedo* presentaron mayor contenido de vitamina C (especialmente la forma de ácido ascórbico) y contenido fenólico que los frutos de *R. ulmifolius* {[(172 *vs* 419) *vs* (5.99 a 26.83)] mg·100 g^{-1} de peso fresco y de [(773 a 1.622) *vs* (376–1.326)] mg·100 g^{-1} de peso fresco, respectivamente}, y fueron los ácidos fenólicos y las antocianinas los principales grupos de compuestos fenólicos encontrados en ambas especies, siendo el ácido gálico y la cianidina 3-glucósido los más abundantes. Los frutos de *A. unedo* mostraron valores significativamente más altos en el ensayo de Folin-Ciocalteu que los de *R. ulmifolius*. Estos valores son más altos que los indicados para la mayoría de los frutos silvestres. Las correlaciones significativas encontradas entre diferentes compuestos antioxidantes ($r > 0{,}6300$, $P < 0{,}001$) pueden revelar un efecto protector entre ácido ascórbico, y ácidos fenólicos o antocianinas en los frutos. Por todo ello, los frutos de *Arbutus unedo* y los de *R. ulmifolius* deben ser considerados en la actualidad como importantes fuentes de antioxidantes.

España / *Arbutus unedo* / *Rubus ulmifolius* / fruits / compuestos fenólicos / contenido fenólico / antioxidantes