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Determination of Polyphenolic Compounds by Ultra-Performance Liquid Chromatography Coupled to Tandem Mass Spectrometry and Antioxidant Capacity of Spanish Subtropical Fruits

Adelaida Esteban Muñoz[1,2], Montserrat Barea Álvarez[1], María-Jesús Oliveras-López[3], Rafael Giménez Martínez[1], José Ángel Rufián Henares[1], Manuel Olalla Herrera[1*]

[1]Department of Nutrition and Bromatology, School of Pharmacy, University of Granada, Granada, Spain
[2]Nutrition and Food Science, University of Granada, Granada, Spain
[3]Department of Molecular Biology and Biochemical Engineering, Area of Nutrition and Food Science, University Pablo de Olavide of Sevilla, Seville, Spain
Email: *olalla@ugr.es

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Abstract

In an analysis of the seven types of subtropical fruits most consumed and produced in southern Spain, UPLC-ESI-MS/MS was used to quantify 14 phenolic species: five hydroxycinnamic acids, seven hydroxybenzoic acids and two flavonoids (quercetin and naringenin). In each case, in addition, antioxidant capacity was determined by FRAP, ABTS and DPPH. Of these fruits, carambola (or starfruit) presented the highest levels of phenolic compounds, cherimoya (custard apple) and kiwi were the richest in non-flavonoid phenolic compounds and papaya had the highest levels of the flavonoids studied. Higher mean values were recorded in home-grown fruits than in imported varieties by ABTS and DPPH methods. Persimmon's antioxidant capacity was well above that of the other fruits, according to our analyses.

Keywords

Phenolic Compounds, Spanish Subtropical Fruits, Antioxidant Activity, UPLC-ESI-MS/MS, Q-TOF

1. Introduction

In recent years, there has been increasing interest in dietary phytochemicals or

bioactive compounds, in view of their potential protective action against cardiovascular disease and certain types of cancer. These compounds include phenolic compounds such as isothiocyanates, flavonoids, isoflavones and lignans, together with other compounds such as saponins and cumestrol [1] (AICR, 2015).

The antioxidant capacity of bioactive compounds in foods is interesting from two standpoints: food technology and nutrition. Phenols and phenolic compounds are secondary metabolites, of vegetable origin, that are among the main antioxidant components in food and, in addition, the most abundant antioxidant compounds in the human diet. Many of the beneficial effects associated with the consumption of foods of vegetable origin are attributed to these phenolic compounds [2] (Rinaldo *et al.*, 2010). Phenolic compounds also influence the sensory properties, such as flavour and colour, and contribute to the aroma and taste of many foodstuffs of vegetable origin. Consequently, they are of great importance in the food industry.

Spain is one of the main suppliers of tropical fruit to other European countries, and exported 115,129 tonnes in 2012 (latest available data), corresponding to 0.95% of all its fruit and vegetable exports [3] (MAPAMA, 2017). The Spanish Mediterranean coast presents environmental characteristics typical of a Mediterranean subtropical climate, which makes the coastal zones of Granada and Málaga provinces the only areas suitable in Andalusia for this type of crop [4] (CMAOT, 2015).

The present study was conducted to evaluate the bioactive compounds of Spanish-grown tropical fruits, especially their antioxidant capacity. The data obtained on these tropical fruits can be usefully incorporated into tables of nutritional composition.

In addition to the above study goals, ultra-high performance liquid chromatography (UPLC), coupled with mass spectrometry, was used to quantify individual phenolic compounds of nutritional importance in these home-grown subtropical fruits.

2. Materials and Methods

2.1. Samples

The following tropical fruits are the types most consumed in Spain and have been analysed in this study: carambola (starfruit) (*Averrhoa carambola*, n = 10) cherimoya (custard apple) (*Annona cherimolla*, Mill., n = 10), kiwi (*Actinidia deliciosa* cv Hayward, n = 10) (*Mangifera indica* L., n = 10), papaya (*Carica papaya* L., n = 10), persimmon (*Diospyros kaki* L.) and avocado (*Persea Americana* Mill., *Hass* and *Bacon* varieties). Samples of Spanish-grown and imported fruits were analysed (except for some phenolic species of which, due to limited seasonal availability, only Spanish-grown samples were analysed). The samples were obtained from retailers in the cities of Granada and Málaga and were immediately taken to the laboratory to be processed. All non-edible parts—skin, seeds and/or pits—were removed; the separated edible part was then triturated

in a blender and the juice extracted from each fruit. The processed samples were stored at 4˚C until needed for analysis, which was performed as soon as possible. Each sample consisted of two randomly-chosen pieces of fruit from the items contained in the sales unit, usually a basket or mesh bag. All analyses were conducted in triplicate.

2.2. Reagents and Standards

The following reagents were obtained from Panreac Química SL (Barcelona, Spain): gallic acid (GA), p-coumaric acid, caffeic acid, vanillic acid, ferulic acid, sinapic acid, chlorogenic acid, ellagic acid, p-hydroxybenzoic acid, protocatechuic acid, pyrogallic acid, 3,5-dimethoxybenzoic acid, quercetin, naringenin, diethyl ether, acetic acid (glacial), anhydrous sodium sulphate and methanol. Sigma-Aldrich (Steinheim, Germany) supplied gallic acid, Folin-Ciocalteu reagent, sodium carbonate, 2,4,6-tri (2-pyridyl)-s-triazine (TPTZ), ferric chloride hexahydrate, sodium acetate, 6-hydroxy-7,8-tetramethylchroman-2-car-carboxylic acid (Trolox), chlorogenic acid, hydrochloric acid, 2,20-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), sodium persulphate, sodium phosphate monobasic, sodium nitrite and aluminium chloride. All reagents were of analytical grade.

2.3. Phenolic Compounds

Individual phenolic compounds. The UPLC-ESI-MS/MS method was used to identify the different phenolic compounds [5] (Rueda *et al.*, 2016) in fruits with modifications.

Treatment of the sample. A liquid-liquid extraction with final concentration was performed. Then, 20 mL of diethyl ether was added and the resulting solution was frozen at −20˚C for 24 hours, after which it was centrifuged for 10 minutes at 9000 rpm. The supernatant was transferred to a separatory funnel and three extractions made with 20 mL diethyl ether. A spatula tip of anhydrous sodium sulphate was added to the organic extract; this was then filtered and the filtrate was passed to a heart-shaped flask for rotary evaporation at 30˚C to the smallest possible volume. The extracts were collected with 1 mL methanol/water mixture (1:1), filtered through a 0.20 μm membrane filter and passed to a chromatography vial for analysis.

Material, equipment and operating conditions for UPLC-ESI-MS/M-QTOF Analysis: Chromatograph: Acquity UPLC System. Column: Waters ACQUITY UPLC™ HSS T3 2.1 × 100 mm, 1.8 μm. Column temperature: 400˚C. Injection volume: 10 μL. Mobile phase: Channel A: water with 0.5% acetic acid; Channel B: acetonitrile. Gradient: initial: 5% B, T15: 95% B, T15.1: 95% B. Analysis time: 18 min. Flow rate: 0.4 mL/min. Detector: SYNAPT G2 HDMS Q-TOF high resolution spectrometer. Waters. Ionisation source: electrospray ionisation. Ionisation mode: negative. Measurement range: 50 - 1200 uma.

Identification and quantification: Phenolic compounds were identified by

comparing the negative masses recorded in previous research, using MassLynx V4 software (Waters Laboratory Informatics, Waters, 2010). Individual phenolic compounds were quantified by obtaining a series of solutions, with a concentration of 5 - 40 moles, of standard phenolic compounds with different retention times. For each phenolic compounds selected, a calibration curve with R2 ≥ 9.997 was performed, to ensure the linearity of the method. The standards were analysed under the same working conditions as the samples. As the retention times and the spectrum areas were known, the phenolic compounds in the different samples could be identified and quantified.

Analytical validation. Table 1 and Table 2 show the main analytical validation parameters of the method used.

Figure 1 shows a chromatogram obtained for a sample of mango.

Table 1. Analytical parameters for the chromatographic determination of phenolic compounds.

Phenolic compound	Linear range (ppm)	Linear equation	R coefficient	R^2 coefficient	LOD (ppm)	LOQ (ppm)
Caffeic acid	0.10 - 20	y = 258.156x + 198.750	0.993	0.985	2.732	9.106
Ferulic acid	0.10 - 20	y = 211.196x + 70.825	0.993	0.987	1.730	5.766
Sinapic acid	0.10 - 20	y = 123.085x + 74.989	0.994	0.989	3.705	12.352
p-coumaric acid	0.10 - 20	y = 130.054x + 100.156	0.992	0.983	3.617	12.056
Chlorogenic acid	0.10 - 20	y = 274.309x – 44.400	0.999	0.999	0.459	1.529
Gallic acid	0.10 - 20	y = 121.185x + 72.692	0.994	0.988	2.261	7.538
Vanillic acid	0.10 - 20	y = 97.610x + 36.396	0.995	0.989	1.278	4.260
Ellagic acid	0.10 - 20	y = 32.644x + 22.078	0.995	0.990	1.736	5.787
p-hydroxybenzoic acid	0.10 - 20	y = 50.425x + 31.859	0.997	0.995	1.264	4.214
Protocatechuic acid	0.10 - 20	y = 92.879x + 70.475	0.993	0.987	2.412	8.038
Pyrogallic acid	0.10 - 20	y = 104.227x + 51.756	0.995	0.991	2.616	8.721
3,5-dimethoxybenzoic acid	0.10 - 20	y = 120.380x + 56.541	0.994	0.990	1.477	4.922
Quercetin	0.10 - 20	y = 993.424x + 551.770	0.994	0.988	1.343	4.476
Naringenin	0.10 - 20	y = 1440.661x + 754.607	0.992	0.984	1.253	4.177

R: correlation coefficient; LOD: Limit of detection; LOQ: Limit of quantification.

Table 2. Parameters to verify the accuracy and precision of the method used to determine individual phenolic compounds for carambola fruit.

Phenolic compound	Carambola			
	n	Mean area ± SD	S^2	CV (%)
Gallic acid	10	516.38 ± 67.266	0.46	1.3
Naringenin	10	97.16 ± 9.299	7.31	0.95
Hydroxybenzoic acid	10	171.92 ± 23.752	2.75	1.38
Caffeic acid	10	108.83 ± 7.213	0.51	0.66
p-coumaric acid	10	89.75 ± 2.367	0.73	0.26
Ferulic acid	10	65.544 ± 6.412	5.85	0.97

n: sample number; SD: Standard deviation; S^2: Variance; CV: Coefficient of variation. Limit of detection = 0.005 mg/L.

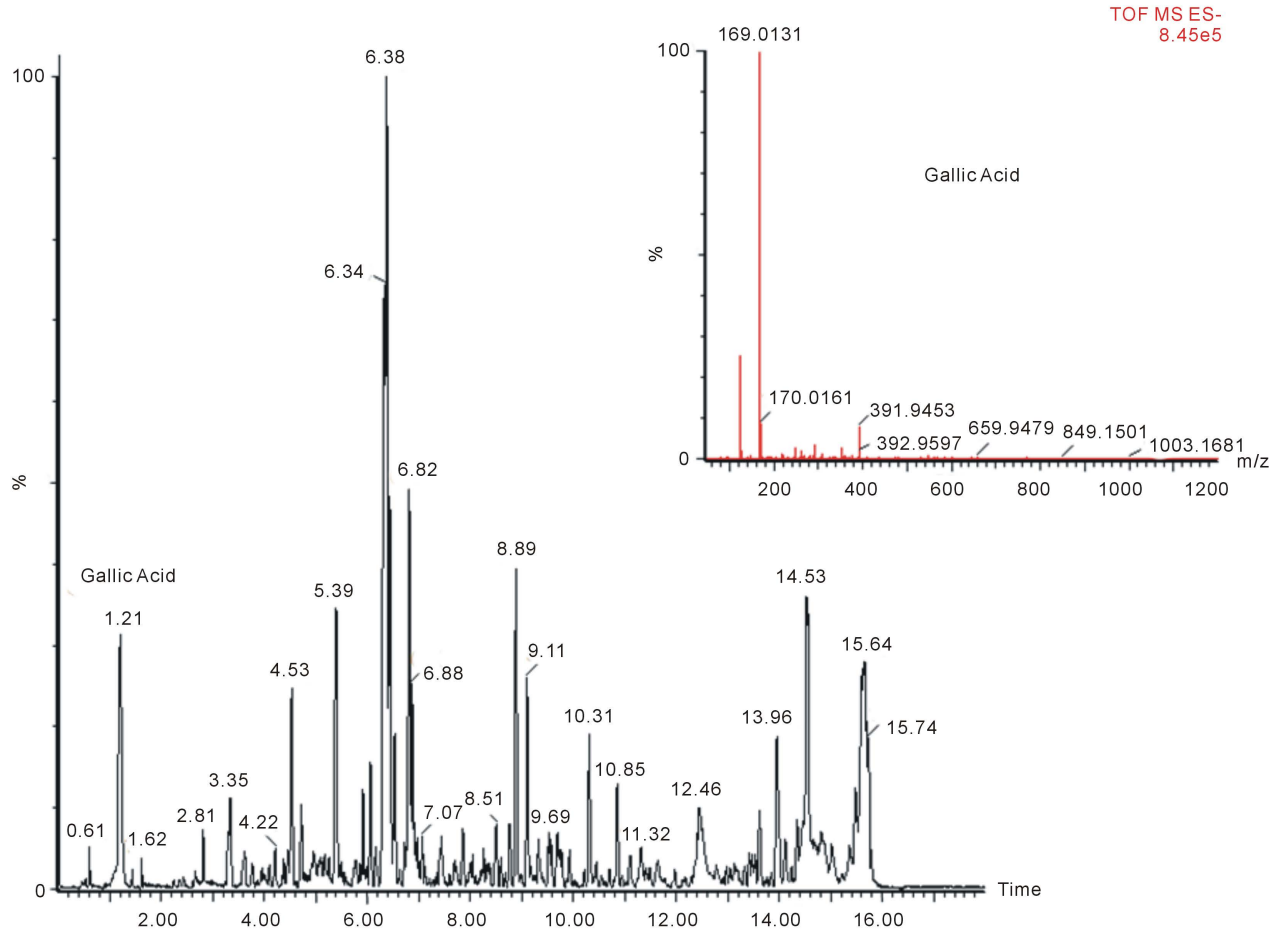

Figure 1. Chromatogram of a sample if Mango and mass spectrum of gallic acid.

2.4. Antioxidant Activity

2.4.1. FRAP Assay

The ferric-reducing ability of each sample solution was estimated using the procedure described previously [6] by Benzie and Strain (1996), adapted to a microplate reader as reported by Pastoriza *et al.* [7]. Calibration curves were performed using Trolox stock solutions. The results are expressed as mmol equivalents of Trolox per L of sample.

2.4.2. ABTS + Assay

The antioxidant capacity was estimated as the radical scavenging activity, following the procedure described previously [8] by Roberta *et al.* (1999) as adapted to a microplate reader by Pastoriza *et al.* [7]. Calibration was performed with a Trolox stock solution as described above. Results are expressed as mmol equivalents of Trolox per L of sample.

2.4.3. DPPH Assay

Radical scavenging activity was determined according to the method reported previously [9] by Julián-Loaeza *et al.* (2011) as adapted to a microplate reader by

Pastoriza *et al.* [7]. Calibration was performed with a Trolox stock solution as described above. Results are expressed as mmol equivalents of Trolox per L of sample.

2.5. Statistical Analyses

The statistical analyses were conducted using the Statgraphics Centurion XVI.II53 (2009) software package. The normality of the data set was confirmed by the Shapiro-Wilk test, and the homogeneity of the variances was confirmed by Levene's test, for a significance level of 5% ($p > 0.05$). One-way analysis of variance (ANOVA) was performed to compare more than two groups, and in cases where significant differences were obtained ($p < 0.05$), the multiple LSD test or Tukey's rank test was applied. If a non-parametric test was needed, the Kruskal-Wallis test was used. The degree of significance for all tests was $p \leq 0.05$.

3. Results and Discussion

3.1. Individual Phenolic Compounds

Our analysis quantified the 14 phenolic compounds present in the triturated pulp of seven tropical fruits Spanish grown cultivated in the Costa Tropical area of the Spanish provinces of Granada and Málaga (Andalusia, Spain). The phenolic species analysed by UPLC-ESI-MS/MS were five hydroxycinnamic acids (caffeic acid, ferulic acid, sinapic acid, p-coumaric acid and chlorogenic acid) and seven hydroxybenzoic acids (gallic acid, vanillic acid, ellagic acid, p-hydroxybenzoic acid, protocatechuic acid, pyrogallic acid and 3,5-dimethoxybenzoic acid). All of these phenolic species were non-flavonoid phenols; in addition, two flavonoid species were analysed: quercetin (a flavonol) and naringenin (a flavanone). **Table 3** shows in detail the quantity of each phenolic compounds in the fruit samples analysed.

3.1.1. Hydroxycinnamic Acids

In general, the values obtained from our samples for this group of phenols were lower than those reported previously [10] [2] Latocha *et al.* (2010); Rinaldo *et al.* (2010) ranging from 5 - 100 mg/100g of fresh fruit in a wide variety of fruits and vegetables [11] (Macheix and Fleuriet, 1990).

Among hydroxycinnamic acids, caffeic acid was determined at highest quantity. Except in pitaya (dragon fruit) and paraguayo (Saturn peach), this acid was present in all analysed samples, in quantities ranging from 8.53E−03 ± 0.001 mg/100g of papaya to 1.98E−01 ± 0.011 mg/100g of cherimoya.

The second most abundant hydroxycinnamic acid in fruit samples was ferulic acid, which was present in four of the seven fruits analysed, in quantities ranging from 5.48E−02 ± 0.002 mg/100g in mango to 2.84E−01 ± 0.022 mg/100g in carambola.

Ferulic acid is a natural antioxidant, abundant in cell walls of plants, and is commonly found in fruits and vegetables [12] (Santos *et al.*, 2006). Many

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Table 3. Individual phenolic compounds in Spanish-grown fruits (μg/100g of fresh fruit).

		Carambola (*Averrhoa carambola*)	Cherimoya (*Annonacherimolla* Mill.)	Kiwi (*Actinidiachinensis*)	Mango (*Mangifera indica* L.)	Papaya (*Carica papaya* L.)	Pitaya (*Stenocereusqueretaroensis*)	Paraguayo (*Prunus pérsica* var. *Platycarpa*)	Persimmon (*Dyospiros kaki L.*)
PHENOLIC COMPOUND	FORMULA	***Mean ± SD***							
				Non-Flavonoids					
				Hydroxycinammicacids					
Caffeic acid	$C_9H_8O_4$	1.37E+02 ± 0.011	1.20E+02 ± 0.011	1.68E+02 ± 0.010	1.58E+01 ± 0.001	8.53E+00 ± 0.001	ND	ND	4.58E+01 ± 0.060
Ferulic acid	$C_{10}H_{10}O_4$	2.84E+02 ± 0.022	1.32E+02 ± 0.015	ND	5.48E+01 ± 0.002	ND	1.20E+02 ± 0.007	ND	1.69E+01 ± 0.008
Sinapic acid	$C_{11}H_{12}O_5$	ND	ND	3.49E+01 ± 0.002	ND	3.98E+01 ± 0.001	ND	ND	1.83E+00 ± 0.001
p-coumaric acid	$C_9H_8O_3$	4.00E+02 ± 0.020	2.69E+02 ± 0.031	ND	2.42E+01 ± 0.002	ND	ND	ND	1.12E+02 ± 0.055
Chlorogenic acid	$C_{16}H1_8O_9$	ND	ND	ND	ND	ND	ND	4.84E+01 ± 0.001	6.57E+00 ± 0.001
				Hydroxybenzoicacids					
Gallic acid	$C_7H_6O_5$	4.75E+03 ± 0.230	7.35E+02 ± 0.044	1.17E+04 ± 0.585	1.01E+03 ± 0.047	3.41E+02 ± 0.008	5.46E+02 ± 0.025	3.56E+03 ± 0.398	9.53E+02 ± 0.344
Vanillic acid	$C_8H_8O_4$	1.68E+02 ± 0.002	ND	ND	ND	ND	1.21E+02 ± 0.002	ND	5.55E+01 ± 0.013
Ellagic acid	$C_{14}H_6O_8$	7.43E+02 ± 0.067	ND	ND	ND	ND	1.48E+02 ± 0.001	1.50E+02 ± 0.241	3.27E+00 ± 0.173
p-hydroxybenzoic acid	$C_7H_6O_3$	1.59E+03 ± 0.090	ND	3.76E+02 ± 0.009	7.56E+01 ± 0.003	ND	ND	ND	1.08E+02 ± 0.039
Protocatechuic acid	$C_{13}H_{16}O_9$	1.21E+02 ± 0.010	ND	ND	ND	ND	ND	ND	1.30E+01 ± 0.010
3,5-dimethoxybenzoic acid	$C_{16}H_{18}O_9$	2.50E+02 ± 0.034	ND	8.80E+01 ± 0.001	ND	ND	ND	1.49E+02 ± 0.004	5.37E+01 ± 0.025
Pyrogallic acid	$C_6H_6O_3$	ND	ND	ND	5.65E+01 ± 0.002	ND	ND	ND	2.75E+01 ± 0.009
Flavonoids									
Flavonols									
Quercetin	$C_{15}H_{10}O_7$	1.52E+01 ± 0.001	ND	3.22E+00 ± 0.000	1.86E+00 ± 0.001	1.45E+01 ± 0.0012	2.86E+00 ± 0.001	4.94E+00 ± 0.008	6.89E+00 ± 0.006
Flavanones									
Naringenin	$C_{15}H_{12}O_5$	3.75E+00 ± 0.002	2.06E+00 ± 0.018	ND	1.86E+00 ± 0.002	ND	ND	4.20E+00 ± 0.012	ND

ND: Not detected..SD: Standard deviation.

physiological functions have been described, including anti-microbial, anti-inflammatory, anti-thrombosis and anti-cancer activities [13] (Merlin *et al.*, 2012).

Chlorogenic acid was only quantified in paraguayo (4.84E−02 ± 0.001 mg/100g fruit). This phenolic compound, one of the most abundant in the human diet, is formed by the esterification of caffeic acid and quinic acid. According to Santos *et al.* (2006) it presents anti-oedematogenic, anti-cancer and anti-nociceptive properties in rats, which is indicative of significant antioxidant activity [14] (Zare *et al.*, 2015).

Sinapic acid was identified in kiwi and papaya (3.49E−02 ± 0.002 and 3.98E−02 ± 0.001 mg/100g of fruit, respectively). In a recent study [14], by Zare *et al.* (2015), it was shown to have neuroprotective potential in rats presenting Parkinson's disease.

Our analysis identified **p-coumaric acid** in three fruits: carambola, cherimoya and mango (4.00E−01 ± 0.020, 3.54E−01 ± 0.031 and 2.42E−02 ± 0.002 mg/100g of fruit, respectively). This phenolic compound is used as a substrate to determine the activity of the cholesterol esterase enzyme [15]. (Planutis *et al.*, 1986).

The fruits richest in the phenolic compounds analysed were carambola and cherimoya, with 0.821 and 0.791 mg/100g of fresh fruit, respectively. According to Macheix *et al.* [11], 5 - 100 mg/100g of fresh fruit is the normal range, but in our analysis of these hydroxycinnamic acids, none of the fruit samples fell within this interval.

3.1.2. Hydrobenzoic Acids

As observed above, **gallic acid** was the only phenolic compound quantified in all the fruits analysed, with a presence ranging from 3.41E−01 ± 0.008 mg/100g of fruit in papaya to 7.99E + 00 ± 0.585 mg/100g of fruit in kiwi. This phenolic compound is found in a wide range of fruits and vegetables, at a concentration of 0.5 - 15 mg/100g of fresh fruit [11] (Macheix *et al.*, 1990), and therefore our samples, while heterogeneous, all contain a significant quantity within this interval.

Gallic acid is the main phenolic compound in mango [16]. (Kim *et al.* 2007). In our samples, the content was 4.86E−01 ± 0.047 mg/100g of fruit. Although this quantity only exceeded that found in papaya, gallic acid was the majority phenolic compound quantified in mango.

Several studies have been conducted to investigate the anti-cancer action of gallic acid. *In vitro* studies have demonstrated its high efficacy against human prostate cancer [17] (Ji *et al.*, 2009). Moreover, it exercises a selective effect in cancer cell death and strengthens the gastric mucosal barrier against gastro-intestinal cancer [18] (Singh *et al.*, 2004).

Protocatechuic acid was identified only in carambola (1.92E−01 ± 0.010 mg/100g of fruit). It has an anti-proliferative and apoptosis-inducing effect on HL-60 cells (used in the study of leukaemia) [19] (Tseng *et al.*, 2000).

Vanillic acid was identified in carambola and paraguayo (1.68E−01 ± 0.002 and 1.21E−01 ± 0.002 mg/100g of fruit, respectively). This common phenolic compound is found in herbs or roots used as medicine in China.

Ellagic acid was identified in carambola, pitaya and paraguayo (7.43E−01 ± 0.067, 1.48E−01 ± 0.001 and 1.50E−01 ± 0.241 mg/100g of fruit, respectively). This phenolic compound is present in some fruits, nuts, seeds and berries, mainly strawberry and raspberry. Several studies have demonstrated its antioxidant, oestrogenic and/or anti-oestrogenic, anti-inflammatory, anti-microbial and prebiotic effects [20] (Landete, J. M., 2012).

p-hydroxybenzoic acid was identified in three fruits: carambola, kiwi and mango (8.17E−01 ± 0.090; 3.76E−01 ± 0.009; and 7.55E−03 ± 0.003 mg/100g of fruit, respectively).

Among the fruits analysed, kiwi had the highest amount of hydroxybenzoic acid (8.4561 mg/100g of fruit). With respect to the normal range of hydroxybenzoic acid in fruits and vegetables of 0.5 - 15 mg/100g of fruit, as proposed by Macheix *et al.* [11], all the fruits in our samples except papaya fell within this range.

3.1.3. Flavonols

Except in cherimoya, **quercetin** was quantified in all the fruits analysed., with a content ranging from 1.86E−03 ± 0.001 mg/100g of fruit in mango to 1.45E−02 ± 0.0012 mg/100g in papaya.

According to Macheix and Fleuriet [11], the usual range of flavonols is 0.5 - 25 mg/100g of fruit, and therefore all the fruits sampled are expected to include other flavonols in their chemical composition.

Studies have reported that among other phenolic compounds, quercetin is present in mango [10] (Latocha *et al.*, 2010), albeit at low levels, of around 0.15 mg/100g of fruit.

With respect to the flavonol content in papaya, other authors [21] (Lako *et al.*, 2007) measured 9 mg/100g of fruit. In comparison with other tropical fruits [2] (Rinaldo *et al.*, 2010), papaya is the richest in this group of phenolic compounds.

3.1.4. Flavanones

Naringenin was identified and quantified in four of the fruits analysed, with a content ranging from 2.01E−03 ± 0.002 mg/100g of fruit in mango, to 4.20E−03 ± 0.012 mg/100g in paraguayo. The other two fruits in which it was present were cherimoya and carambola (2.06E−03 ± 0.018 and 3.75E−03 ± 0.002 mg/100g of fruit, respectively).

Of the fourteen phenolic compounds and seven hydroxybenzoic acids analysed, gallic acid was found in all the fruits, and was present in the highest quantities. The mean values were significantly higher ($p < 0.01$) for kiwi and paraguayo than for the other fruits, with a content ranging from3.41E−01 ± 0.008 mg/100g of fruit in papaya to 7.99E+00 ± 0.585 mg/100g in kiwi. Gallic acid provides important health benefits through its anti-cancer and cardioprotective

(Priscila and Prince, 2009) potential [22].

Analysis of the variance among the phenolic compounds quantified in each of the fruits analysed revealed statistically significant differences ($p < 0.001$) for gallic acid, vanillic acid, p-coumaric acid and quercetin ($p < 0.005$).

With respect to the total quantity of phenolic compounds (as determined by liquid chromatography), the fruit with the highest quantity was kiwi, followed by paraguayo and carambola (kiwi > paraguayo > carambola > cherimoya > mango > papaya).

Carambola had the highest concentrations of six individual phenolic species: p-coumaric acid ($p = 0.0135$), vanillic acid ($p = 0.038$), ferulic acid, ellagic acid, pOH benzoic acid and 3,5 dimethoxybenzoic acid, and in another three – quercetin ($p = 0.0383$), caffeine and naringenin – it was the second-most abundant (difference non-significant). Papaya had the highest content of quercetin and sinapic acid. Kiwi had more gallic acid ($p < 0.01$) than any other fruit, and cherimoya had the highest caffeine content ($p < 0.01$).

3.2. Antioxidant Capacity

Due to the hydrophilic and lipophilic nature of the antioxidant components found in tropical fruits, diverse methods have been proposed to evaluate their antioxidant capacity. We performed three of these—ABTS, DDPH and FRAP.

3.2.1. ABTS Method

The results obtained for Spanish-grown and imported tropical fruits, expressed as mmol of Trolox equivalent antioxidant capacity (TEAC) per g of fruit, are shown in **Table 4**.

In our samples, the mean antioxidant activity ranged from 2.226 ± 0.761 mmol/g for avocado (var. *Bacon*) to 31.532 ± 16.801 mmol/g for persimmon.

Another fruit presenting high antioxidant capacity was cherimoya, with 22.094 ± 11.101 mmol/g. After persimmon and cherimoya, the next highest values were obtained for carambola, kiwi and papaya (**Table 4**) (8.734 ± 4.471, 5.406 ± 0.805 and 3.769 ± 0.834 mmol/g, respectively). Like persimmon, these fruits have abundant vitamin C; on the other hand, the levels of beta-carotene are lower than in persimmon, which may explain their lower antioxidant capacity [23] (Morillas-Ruíz and Delgado-Alarcón 2012).

Kiwi and avocado (var. *Hass*) had similar values in imported and Spanish-grown samples (5.65 ± 0.723 and 2.293 ± 0.592 mmol/g, respectively) but in papaya and carambola, the values in the imported fruits were lower than in the Spanish-grown ones (3.13 ± 0.414 and 7.18 ± 4.952 mmol/g, respectively).

Our values for the antioxidant capacity of carambola were lower than those obtained by previous studies [23] [24] [25] (Morillas-Ruíz and Delgado-Alarcón, 2012; Clerici and Carvalho-Silva, 2011; Muñoz *et al.*, 2007). In this respect, kiwi and papaya presented values of 5.160 ± 0.837 and 4.406 ± 0.633 mmol/g respectively, which were also lower than those reported elsewhere for kiwi [26] (Lim *et al.*, 2007) and papaya [27] [28] (Zuhair *et al.*, 2013; Morais *et al.*, 2015).

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Table 4. Antioxidant capacity of Spanish-grown and imported tropical fruits (mmol TEAC/g).

Fruit	ABTS Mean ± SD			DPPH Mean ± SD			FRAP Mean ± SD		
	Spanish	**Imported**	**Mean**	**Spanish**	**Imported**	**Mean**	**Spanish**	**Imported**	**Mean**
Cherimoya	2.59E+01 ± 13.892	1.82E+01 ± 5.680	2.20E+01 ± 11.101	3.89E+00 ± 1.427	0.96E+00 ± 0.391	2.43E+00 ± 1.814	14.549E+01 ± 6.018	18.135E+01 ± 7.393	16.342E+01 ± 6.842
Avocado									
var. *Hass*	2.27E+00 ± 0.502	2.29E+00 ± 0.592	2.28E+00 ± 0.537	3.82E+00 ± 0.827	3.56E+00 ± 0.581	3.69E+00 ± 0.711	2.28E+00 ± 1.198	2.59E+00 ± 1.289	2.44E+00 ± 1.227
var. *Bacon*	1.86E+00 ± 0.856	2.58E+00 ± 0.444	2.22E+00 ± 0.761	6.48E+00 ± 4.419	3.22E+00 ± 1.382	4.85E+00 ± 3.610	6.09E+00 ± 7.422	2.87E+00 ± 1.950	4.48E+00 ± 5.556
Kiwi	5.16E+00 ± 0.837	5.65E+00 ± 0.723	5.40E+00 ± 0.805	8.48E+00 ± 1.150	1.00E+01 ± 2.172	9.24E+00 ± 1.870	5.12E+00 ± 1.666	5.97E+00 ± 1.391	5.54E+00 ± 1.562
Mango	6.37E+00 ± 0.902	0.48E+00 ± 0.167	3.42E+00 ± 3.075	0.73E+00 ± 0.443	0.97E+00 ± 0.426	0.85E+00 ± 0.442	7.21E+00 ± 1.444	6.26E+00 ± 2.722	6.73E+00 ± 2.185
Papaya	4.40E+00 ± 0.633	3.13E+00 ± 0.414	3.76E+00 ± 0.834	1.41E+01 ± 2.510	1.06E+01 ± 1.788	1.24E+01 ± 2.788	6.70E+00 ± 1.551	4.38E+00 ± 1.465	5.54E+00 ± 1.562
Persimmon	4.09E+01 ± 18.487	2.21E+01 ± 7.544	3.15E+01 ± 16.801	2.28E+02 ± 119.521	1.54E+02 ± 99.870	1.91E+02 ± 114.168	3.49E+01 ± 35.890	4.41E+01 ± 48.661	3.95E+01 ± 42.076
Carambola	1,02E+01 ± 3.465	7.18E+00 ± 4.952	8.73E+00 ± 4.471	1.48E+01 ± 4.732	1.56E+01 ± 4.273	1.52E+01 ± 4.431	7.78E+00 ± 1.854	6.14E+00 ± 2.755	6.96E+00 ± 2.444
ALL	1.21E+01 ± 15.368	7.71E+00 ± 8.399	9.93E+00 ± 12.550	3.51E+01 ± 84.206	2.49E+01 ± 60.077	3.00E+01 ± 73.130	1.05E+01 ± 16.049	1.13E+01 ± 21.450	1.09E+01 ± 18.897

For persimmon, the values observed in our samples were lower than those reported by Fei *et al.* [29], for both Spanish-grown and imported fruit.

For mango and cherimoya, too, our values were somewhat lower than those obtained previously [30] (Vasco *et al.*, 2008), for Spanish-grown and imported fruit.

3.2.2. DPPH Method

The results obtained for the Spanish-grown fruit samples, expressed as mmol of TEAC per g of fruit, are shown in **Table 4**. The mean values ranged from 191.657 ± 114.168 mmol/g of fruit for persimmon to 0.859 ± 0.442 mmol/g for mango. As with the ABTS method, the values ranged widely among samples.

With the DPPH method (see **Table 4**), although persimmon continued to present the greatest antioxidant capacity, the values were lower, both overall (2.432 ± 1.814 mmol/g) and in Spanish-grown and imported fruit (3.898 ± 1.427 and 0.966 ± 0.391 mmol/g, respectively). The values for Spanish-grown carambola (14.828 ± 4.732 mmol/g) were the second highest of all the samples, but were followed very closely bypapaya (14.167 ± 2.510 mmol/g). The Spanish-grown kiwi samples presented values of 8.486 ± 1.150 mmol/g, while lower ones were obtained for avocado (var. *Hass*) (3.820 ± 0.827 mmol/g), cherimoya (3.898 ± 1.427 mmol/g) and mango, which had the lowest value of all the fruits sampled (0.738 ± 0.443 mmol/g).

In the imported fruits, as with the Spanish-grown samples, the highest antioxidant value was found in persimmon (154.611 ± 99.870 mmol/g) and the lowest one in mango (0.979 ± 0.426 mmol/g). Cherimoya also presented a very low value (0.966 ± 0.391 mmol/g), well below that found in Spanish-grown fruits. In avocado and papaya, too, the values observed were lower than those of the home-grown varieties (3.563 ± 0.581 and 10.65 ± 1.788 mmol/g, respectively).However, imported kiwi and carambola had higher values than the Spanish-grown equivalents (10.010 ± 2.172 and 15.690 ± 4.273 mmol/g, respectively).

Our values for avocado and papaya were lower than those reported previously for fruit pulp [23] [28], by Morais *et al.* (2015) and Morillas-Ruiz *et al.* (2012), but for papaya they were higher than those observed in a previous paper [27], by Zuhair *et al.* (2013). For persimmon, both Spanish-grown and imported, the values we obtained using the DPPH method were higher than those of Fei *et al.* [29].

The values for kiwi were lower than those reported before [31] [23] by Du *et al.* (2009) and Morillas-Ruiz *et al.* (2012) while for carambola, they were lower than those of Clerici *et al.* [24] but higher than those found by Morillas-Ruiz *et al.* [23]. The latter author [23] reported higher values than ours for mango, which is in line with the findings of Vasco *et al.* [30].

3.2.3. FRAP Method

Table 4 shows our results for Spanish-grown and imported fruit samples, expressed as mmol of TEAC per g of fruit. The antioxidant activity of the Span-

ish-grown fruit samples ranged from 34.970 ± 35.890 mmol/g for persimmon to 2.284 ± 1.198 mmol/g for avocado (var. *Hass*).

As with the methods described above, FRAP showed persimmon to have a greater antioxidant activity than the other fruits. Avocado had the lowest antioxidant capacity (as in the ABTS method). Among the varieties of avocado, the antioxidant capacity of var. *Bacon* (6.096 ± 7.422 mmol/g) was nearly three times stronger than that afforded by var. *Hass* (2.284 ± 1.198 mmol/g).

After persimmon, cherimoya presented the second highest value (14.549 ± 6.018 mmol/g), as in the ABTS method. The other fruits had similar values to those obtained by ABTS. The following results were obtained: kiwi (5.123 ± 1.666 mmol/g), papaya (6.703 ± 1.551 mmol/g), mango (7.211 ± 1.444 mmol/g) and carambola (7.784 ± 1.854 mmol/g).

Among the imported samples, persimmon still gave the highest value (44.127 ± 48.661 mmol/g), and this was higher than that obtained for the Spanish-grown equivalent. Avocado (2.595 ± 1.289 mmol/g), mango (2.872 ± 1.950 mmol/g), carambola (6.264 ± 2.722 mmol/g) and papaya (4.388 ± 1.465 mmol/g) all had lower values than those obtained for home-grown fruits. The value for kiwi was slightly above that found in Spain-grown fruit (5.970 ± 1.391 mmol/g).

The values obtained for avocado and papaya were lower than those reported for fruit pulp [28] by Morais *et al.* (2015). However, by the ABTS method, the value for our papaya samples was higher than that reported [27] by Zuhair *et al.* (2013).

In our study, carambola had a lower antioxidant capacity than that observed by other authors [23] [24] [25] (Morillas-Ruíz and Delgado-Alarcón, 2012; Clerici and Carvalho-Silva, 2011; Muñoz *et al.*, 2007).

In addition, for kiwi our results were lower than those obtained in previous studies [29] [31] (Fei *et al.*, 2013; Du *et al.*, 2009). This was also the case for persimmon [29] (Fei *et al.*, 2013), mango and cherimoya [31] (Vasco *et al.*, 2008), for both Spanish-grown and imported fruit.

One-way analysis of the variance for the antioxidant capacity data, according to the ABTS method (**Figure 2**), revealed significant differences ($p < 0.05$) between the values obtained for persimmon, carambola and cherimoya, on the one hand, and the other fruits analysed. Similar differences were observed between persimmon and the other fruits, according to the DPPH method (**Figure 2**).

ANOVA also revealed higher mean values in Spanish-grown fruits than in the imported ones, both by ABTS (12.152 versus 7.716 mmol TEAC/g) and by DPPH (35.141 versus 24.961 mmol TEAC/g, mean values) although the differences were only significant in the first case ($p = 0.0139$). By the FRAP method, the values were slightly higher in imported fruits (11.312 mm TEAC/g) than in the Spanish-grown ones (10.590 mm TEAC/g). The difference was not statistically significant ($p = 0.7919$).

In the ANOVA by type of fruit, regarding the antioxidant capacity determined by FRAP (**Figure 2**), eight pairs presented statistically significant differences at

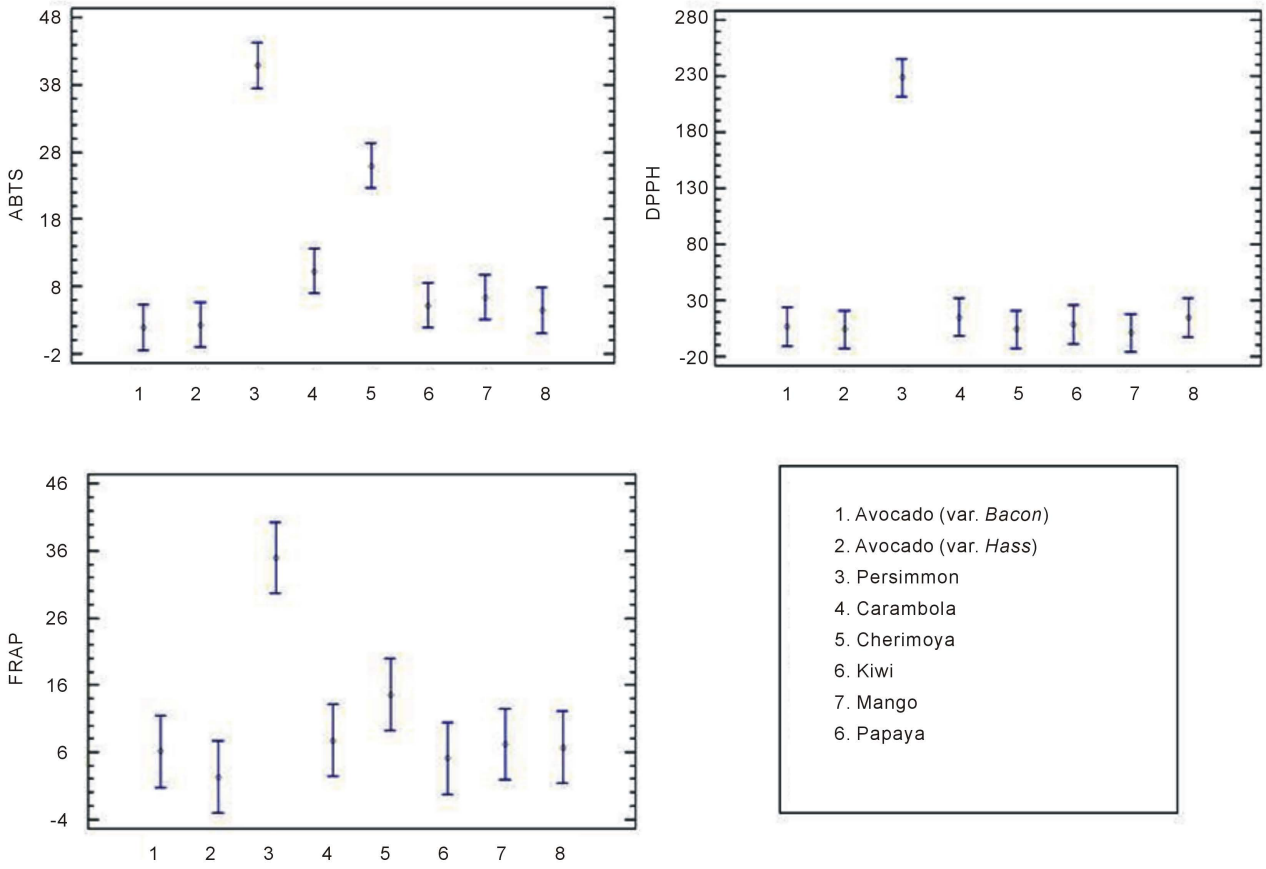

Figure 2. ANOVA of the data obtained by ABTS, DPPH and FRAP (mmol TEAC/G) for each fruit analyzed.

the 95% confidence level. The fruits that presented significant differences in this comparison were persimmon and cherimoya. Persimmon, in particular, was significantly different to all other fruits, with notably higher values. Cherimoya also had higher values than the other fruits, while avocado (var. *Hass*) presented the lowest values.

The results derived from the different tests varied widely as regards total phenolic content and antioxidant capacity. Nevertheless, the fruits analysed can be grouped as having high, medium or low phenolic content and antioxidant capacity. In this sense, persimmon and cherimoya can be assigned to the high phenol and high antioxidant capacity group. Kiwi, papaya, carambola and mango belong to the medium group, in these two respects, and avocado would be classed as belonging to the group with low phenol content and low antioxidant capacity.

3.3. Nutritional Impact of Subtropical Fruits as Antioxidants in the Diet

In recent years, there has been increasing interest in dietary phytochemicals—including isothiocyanates, phenolic compounds, flavonoids, isoflavones, lignans, saponins and cumestrol—due to their possible protective action against cardio-

vascular disease and certain types of cancer [1] (AICR, 2015).

Phenolic compounds influence many sensory properties of foods, such as flavour and colour, and contribute to the aroma and taste of numerous food products of plant origin. Accordingly, they have long been of great importance in the food industry [32] (Ambigaipalan *et al.*, 2016).

The antioxidant capacity of bioactive compounds in foods is of interest in terms of nutrition because some foods have an important antioxidant activity and flavonoid content, which justifies the importance of their presence in the human diet, helping to reduce free radicals and reducing related diseases [33] (Pereira *et al.*, 2015).

Once the antioxidant capacity has been determined in the different fruits analysed, we can establish, taking its consumption into consideration, its total antioxidant contribution to diet. The antioxidant capacity and daily phenolic compound intake were calculated from the consumption patterns of each tropical fruit in Spain in 2014 [34] (MERCASA 2015), taking into account the normal portion sizes of each fruit [35] (Carvajal and Sánchez, 2003).

The contribution to antioxidant capacity is reported as mmol TEAC/day. For antioxidant intake, per edible portion, the results are expressed as mmol TEAC/portion. Due to the novelty of the fruits which have been studied in this paper (most of them have been introduced as *novel food* in Europe) there are no reliable published data with regard to daily recommended intakes as it does happening other fruits (e.g. citrus fruits). Nevertheless, an approximation of this contribution was made taking into account the data regarding the total antioxidant capacity in the Spanish diet reported by Saura-Calixto *et al.* [36].

The contribution made by the consumption of Spanish-grown tropical fruit to antioxidant capacity and phenolic compound intake is shown in **Table 5**. As observed above, the consumption of tropical fruits in Spain is low, ranging from 0.4 to 0.9 kg/year, except for that of kiwi, which rises to 3.0 kg/year. The daily consumption of one kiwi (Spanish grown) provides over 20% of total antioxidant needs, and so this tropical fruit, the most commonly consumed in Spain, contributes significantly not only to mineral intake but also to antioxidant capacity.

The contribution to real personal diets is best calculated using the edible portion size of each fruit, which ranges from 90 g for avocado to 130 g for kiwi. Persimmon makes the highest daily contribution in the Spanish diet to antioxidant capacity, exceeding 700% [37]. The consumption of a portion of Spanish-grown cherimoya or papaya contributes over 50% of antioxidant capacity needs. Mango is the tropical fruit that makes the lowest contribution to total antioxidant capacity (16.14%) in the Spanish diet.

In order to enhance antioxidant capacity and to increase the intake of phenolic compounds, the consumption of tropical fruit, cultivated in the coastal areas of Granada and Málaga provinces, could be included in nutritional recommendations.

Table 5. Daily values of antioxidant capacity obtained from the consumption of tropical fruits in Spanish diet (intake per portion of fresh fruit).

FRUIT	Daily intake (g/person/day)	ABTS mmol TROLOX	DPPH mmol TROLOX	FRAP mmol TROLOX	Edible portion (g)	Mean value from ABTS, DPPH and FRAP (mmol TROLOX)	% of intake of total dietary antioxidant capacity (%[1])
Cherimoya	2.36	52.142	5.739	38.567	120	1775.880	50.04
Avocado	2.42	5.455	10.338	8.378	90	289.485	8.16
Kiwi	8.44	45.627	78.061	46.810	130.5	816.408	23.00
Mango	1.42	4.889	1.220	9.566	120	572.880	16.14
Papaya	1.12	4.221	13.897	6.210	174	1796.898	50.63
Persimmon	1.01	31.847	193.573	39.943	174	26752.500	753.80
Carambola	0.26	2.271	3.967	1.811	108	1453.464	40.95

[1]Taking into account the total antioxidant capacity in the Spanish diet: 3.549 mmolTrolox/day (Saura-Calixto and Goñi, 2007).

The consumption of tropical fruits, cultivated in the coastal areas of Granada and Malaga provinces, could be included in nutritional recommendations, with the aim of enhancing antioxidant capacity and increasing the intake of polyphenols.

The presence of other nutrients in these foods (especially sugars, which have well-known implications for health) should also be taken into account. It is also important to consider the real bioavailability of phenolic compounds in these fruits.

4. Conclusions

According to our liquid-liquid extraction technique and UPLC-ESI-MS/MS analysis, carambola had the highest total content of the 14 individual phenolic compounds studied. Cherimoya and kiwi were the richest in non-flavonoid phenolic compounds, and papaya was richest in flavonoids. Gallic acid was the only phenolic compound quantified in all seven fruits.

The mean values found for antioxidant capacity, by ABTS, DPPH, were higher in Spanish-grown than in imported fruits. Persimmon show dean antioxidant capacity that was notably superior to that of the other fruits, which nevertheless also presented useful antioxidant capacity and, therefore, health benefits. Carambola and cherimoya were also found to be very rich in these bioactive compounds.

Finally, our study contributes local data that can be incorporated into tables of nutritional composition in reference to flavonoid and non-flavonoid phenolic species. We also supply data on antioxidant capacity obtained from the consumption of a portion of these fruits, and relate these values to the Spanish daily intake. In this respect, we note, in particular, that the consumption of a portion of persimmon or cherimoya supplies over 100% or 50%, respectively, of the daily antioxidant requirements of a healthy adult.

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Compliance with Ethical Standards

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent: Not applicable.

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