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Analysis of Phenolic Compounds in Portuguese Wild and Commercial Berries after Multienzyme Hydrolysis

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Supporting Information

ABSTRACT: Berry fruits are a good source of phenolic compounds and thus, potentially beneficial to health. Phenolic compounds are mainly present as a variety of conjugated forms, either with sugars via *O*-glycosidic bonds or with other polyols as esters. This chemodiversity makes characterization and identification highly demanding. Selected varieties of commercial blueberries, raspberries and blackberries and the two wild berries Portuguese crowberry and strawberry tree fruits were characterized for individual phenolic content by liquid chromatography−diode array detection and mass spectrometry (HPLC-DAD-MS) after hydrolysis by a novel combination of the fungal glycosidases hesperidinase and cellulase. This approach is shown to be a simple alternative to other existing methods for analysis of plant phenolic compound aglycones. The hydrolysis of glycosides and organic acid esters is efficient and less aggressive than acid and alkaline hydrolysis. This method is able to disclose new sources of dietary phenolic compounds, and the potential usefulness of Portuguese crowberry and strawberry tree fruit is herein demonstrated.

KEYWORDS: *hesperidinase, cellulase, Portuguese crowberry, strawberry tree fruit, polyphenols, bioaccessibility*

INTRODUCTION

Experimental and epidemiological evidence suggests that insufficient intake of fruits and vegetables may predispose the human body to a range of chronic health disorders. Consumption of fruits and vegetables has been associated with a decreased risk for certain degenerative diseases, such as coronary heart disease, stroke, cancer, diabetes mellitus, and osteoporosis.[1−6] These effects have been associated with the ingestion of polyphenols, a class of plant secondary metabolites, which although not being classically "essential", are described as having beneficial health effects such as anti-inflammatory, antioxidant, neuroprotective, and promotion of healthy aging.[7−14]

Berry fruits are particularly rich in a diverse range of polyphenols[1] from several different classes. The most abundant classes in berries are phenolic acids, tannins, and flavonoids, especially anthocyanins, responsible for the colored pigmentation.[10] Most phenolic compounds are present in plants as a variety of conjugated forms, either with sugars via *O*-glycosidic bonds or with other polyols as esters.[15] Numerous points of conjugation per aglycone can be possible (depending on class), leading to a diverse range of potential forms, differing in their bioavailabilities and bioactivities. This complexity also makes characterization and identification challenging. Following ingestion, although some polyphenols may be absorbed intact, typically absorption in the digestive tract requires hydrolysis of glycoside conjugates by small-intestinal lactase phlorizin hydrolase or cytosolic β-glucosidase.[16,17] Aglycones are then subjected to phase II metabolism and can be sulfated, methylated, and glucuronidated.[18] Compounds that escape from absorption in the small intestine can reach the colon and undergo biotransformation by the colonic microflora, resulting in simpler catabolites that may also be absorbed and metabolized.[19]

Commercial exploitation of a wider range of edible fruits could facilitate increased consumer intake, and characterization of polyphenols within these novel species may yield useful hints as to their potential bioactive capacity. Portuguese crowberry, *Corema album* L., is a low trailing shrub endemic to the Iberian peninsula, belonging to the Empetraceae family, and grows mainly in coastal habitats, particularly in sand dunes. It produces edible quasi-spherical drupes, approximately 1 cm in diameter, which are white when ripe and have a sugary and water-rich pulp.[20] The berries take months to ripen, and the peak of ripening happens in August and early September. Portuguese crowberries are edible, and, although they are not currently commercially exploited, are a potentially important source of nutrients and phytochemicals. Recently, they were described as a good source of phenolic acids and flavonols.[21]

The strawberry tree, *Arbutus unedo* L., belongs to the Ericaceae family and is a typical shrub of the Mediterranean

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region. It produces edible spherical red berries when ripe, which are mostly processed to manufacture alcoholic beverages, jams, and marmalades.[22] They are a good source of sugars, minerals, vitamins, carotenoids, organic acids, and phenolic compounds such as proanthocyanidins and flavonoids and phenolic acids, which as expected are mostly glycosylated or esterified with organic acids.[23−26]

In addition to these native berries, the specific climatic conditions in Portugal grant to producers the possibility of off-season production of high-quality berry fruits that are already commercially exploited, such as blueberries, raspberries, and blackberries. Several commercial varieties of these berries are being tested in the Experimental Field of Fataca (EFF) for perfecting production technologies and enhancing fruit quality and phytochemical value.

The aim of this study was to perform a novel combination of enzymes for hydrolysis of phenolic glycosides and quantification of aglycones by HPLC-DAD with confirmation by mass spectrometry. The usefulness of this method was tested in five different berries, Portuguese crowberry, strawberry tree fruits, blueberry, raspberry, and blackberry.

MATERIALS AND METHODS

Chemicals and Standards. Organic solvents were purchased from Fischer Scientific and were of HPLC grade. Formic acid was purchased from the same company. Phloroglucinaldehyde, *p*-coumaric acid, ferulic acid, caffeic acid, punicalagin, and avicularin were from Sigma-Aldrich (St. Louis, MO, USA). All other reagents unless otherwise stated were purchased from Sigma-Aldrich. Folin−Ciocalteu reagent and vanillic acid were from Fluka (Buchs, Switzerland). 5-Caffeoylquinic acid, 3-caffeoylquinic acid, and protocatechuic acid were from Acros Organics (Geel, Belgium). 3-Methylgallic acid was from Apin Chemicals Ltd. (Abingdon, UK) and syringic acid from Alfa Aesar (Haverhill, MA, USA). Gallic acid, daidzein, cyanidin-3-*O*-glucoside, (+)-catechin, (−)-epicatechin, ellagic acid, myricetin, quercetin, kaempferol, myricitrin, hyperoside, quercitrin, rutin, isoquercitrin, astragalin, and nicotiflorine were purchased from Extrasynthese (Lyon, France). Cellulase (EC 3.2.1.4) and hesperidinase (EC 3.2.1.40) from *Aspergillus niger* were purchased from Sigma-Aldrich.

Plant Material. Blueberry (*Vaccinum* spp.) fruits of six cultivars (Biloxi, Sharpblue, Misty, Jubilee, Star, Georgia Gem), raspberry (*Rubus idaeus*) fruits of three varieties (Glen Lyon, Polka, and Himbo Top), and blackberry (*Rubus* L. subgenus Rubus Watson) of five varieties (Arapaho, Loch Tay, Olallie, Oros, and Karaka Black) were grown at the Fataca experimental field in Odemira, Portugal. Wild Portuguese crowberry fruits (*Corema album* L.) were collected in the Comporta region, and wild strawberry tree fruits (*Arbutus unedo* L.) were collected from the Alentejo region of Portugal and grouped. Fruits were hand-harvested at a mature stage determined by their color and were stored until analysis at −20 °C for no more than 2 months.

Soluble Solids Content (SSc) and Total Titratable Acidity (TAc). Samples of the fruits were pooled to obtain a composite sample and analyzed for SSc using a hand refractometer Milwaukee model MR32ATC, with a scale range of Brix degrees (°Brix) from 0 to 32.0%.

TAc was determined by titrating a known volume of fruit juice with 0.1 N NaOH until the pH reached 7. The pH was measured with a Crison micro-pH 2002 pH-meter. The volume of NaOH required was used to calculate TAc, which is expressed as grams (tartaric acid) per liter.

Extraction of Phytochemicals. Fruits were blended using a domestic food processor until they were homogeneous, freeze-dried, and then stored at −80 °C. For total phenolics, anthocyanins, and oxygen radical absorbance capacity (ORAC) analysis, phytochemicals were extracted as previously described.[23] To each gram of lyophilized powder was added 12 mL of ethanol/water (1:1) solvent, and the mixture was shaken for 30 min at room temperature in the dark. The mixture was then centrifuged at 12400*g* during 10 min at room temperature. The supernatant was filtered through 0.20 μm cellulose acetate membrane filters. The resulting extracts were stored frozen at −80 °C.

Total Phenolic Measurement. Determination of total phenolic compounds was performed according to the Folin−Ciocalteau method[27] adapted for microplates as described elsewhere.[28] Gallic acid was used as standard, and the results were expressed in milligrams of gallic acid equivalents per gram of dry weight of plant material (mg GAE/g dw).

Anthocyanin Content. The total anthocyanin content of the fruit extracts was determined using a pH differential absorbance method as described elsewhere.[29] Absorbance readings were related to anthocyanin content using the molar extinction coefficient of 26900 L mol^{-1} cm^{-1} for cyanidin-3-*O*-glucoside.[30] Results were expressed as milligrams of cyanidin-3-*O*-glucoside equivalents per 100 g of dry weight of plant material (mg cy-3-gluc/g dw).

Peroxyl Radical Scavenging Capacity Assay. Peroxyl radical scavenging capacity was determined according to the ORAC method[31,32] adapted to microplate as described elsewhere.[28] The final results were calculated using the area differences under the fluorescence decay curves between the blank and the sample and were expressed as millimolar Trolox equivalents per 100 g of dry weight of plant material (mM TE/100 g dw).

Vitamin C Determination. To quantify the total amount of vitamin C (ascorbic acid plus dehydroascorbic acid), 50 g of fruits was homogenized with 50 mL of phosphoric acid. The homogenate was then centrifuged at 13000*g* for 20 min at 4 °C and the supernatant filtered using 0.20 μm cellulose acetate membrane filters. To 1 mL of the filtrate was added 0.2 mL of dithiothreitol (20 mg/mL) to reduce dehydroascorbic acid, and the mixture was allowed to stand in darkness for 2 h.[33] The solution was then passed through a Millipore 0.45 μm PTFE filter and injected into the HPLC system. Ascorbic acid quantification was conducted on a Hitachi HPLC instrument (VWR) equipped with EZChrom Elite software (Agilent), a model L-2130 pump system, a model L-2200 autosampler, a model L2300 column oven, and a model L2455 DAD system. A sample volume of 10 μL was injected, and separations were achieved on an Inertsil ODS-3 V column (250 × 4.6 mm, 5 μm), operated at 30 °C. The column was held at 30 °C, and ascorbic acid was eluted isocratically using a 0.7 mL/min flow of 0.01 M H_2SO_4 (pH 2.5). Solutions of L-ascorbic acid prepared in water with concentration values between 5 and 500 mg/L were used for the calibration curve, and detection was made at 262 nm.

Enzymatic Hydrolysis of Glycosides. To perform enzymatic hydrolysis of glycosides, phytochemicals were extracted by homogenizing 0.3 g of blended fruits with 2.7 mL of ethanol/water (1:1) containing 1 mM butylated hydroxytoluene (BHT) and 0.1 mM daidzein as internal standard. Samples were vortexed (10 min), sonicated (10 min), then vortexed again (10 min), and then centrifuged at 3000*g* for 20 min at 4 °C. After collection of the supernatant, the pellet was re-extracted according to the same procedure with 0.9 mL of the extraction solution. Supernatants were combined, centrifuged as described, and filtered through a 0.2 μm PTFE filter.

One part of the extract was used directly for HPLC analysis of total anthocyanins and caffeoylquinic acids. Another part of the extract was dried and reconstituted in water with 1 mM ascorbic acid, to protect samples from oxidation. Samples for aglycone analysis were adjusted to pH 3.8 with 0.2 M acetate buffer and incubated for 16 h at 40 °C with 0.02 U/mL hesperidinase [1 unit (U) corresponds to 333 mg of protein and is defined as the amount required to liberate 1.0 μmol of glucose from hesperidin per minute at pH 3.8 at 40 °C]. The pH was then increased to 5.0 with sodium acetate, and cellulase was added to a final concentration of 20 U/mL (1 U corresponds to 0.885 mg of protein and is defined as the amount required to liberate 1.0 μmol of glucose from cellulose in 1 h at pH 5.0 at 37 °C). Samples were incubated for a further 4 h at 37 °C and then extracted three times with ethyl acetate. The ethyl acetate phase (containing phenolic aglycones) was separated and evaporated, and samples were reconstituted with 2 mL of 0.1% (w/v) ascorbic acid solution in

Table 1. Chromatography Parameters of Standard Compounds Used for Peak Identification

standard	retention time (min)	wavelength (nm)	LOD (mg/mL)	LOQ (mg/mL)	calibration range (mg/mL)
gallic acid	2.59	272	0.25	0.5	0.5−100
protocatechuic acid	5.26	272	0.09	0.2	0.5−100
3-methylgallic acid	6.28	272	0.09	0.2	0.5−100
3-caffeoylquinic acid	6.03	324	0.5	0.75	0.75−100
cyanidin-3-*O*-glucoside	8.07	520	0.75	2.5	2.5−100
5-caffeoylquinic acid	8.40	324	0.25	0.75	0.75−100
(+)-catechin	8.60	280	0.55	1	2.5−100
vanillic acid	9.41	272	0.09	0.2	0.5−100
caffeic acid	9.84	324	0.04	0.1	0.5−100
syringic acid	10.06	272	0.05	0.2	0.25−100
(−)-epicatechin	10.54	280	0.3	0.55	1−100
phloroglucinaldehyde	12.00	280	0.05	0.1	0.1−100
p-coumaric acid	12.11	302	0.04	0.09	0.1−100
ellagic acid	12.97	370	0.1	0.4	0.75−100
ferulic acid	13.16	324	0.05	0.15	0.25−100
myricetin	15.50	370	0.1	0.25	0.25−100
daidzein	17.20	302	0.1	0.25	0.25−100
quercetin	18.30	370	0.1	0.25	0.25−100
kaempferol	20.83	370	0.1	0.25	0.25−100

ethanol/water (1:1) and filtered through a 0.2 μm PTFE filter prior to analysis. The procedure was performed in triplicate for each fruit. Hydrolysis conditions were optimized by testing the hydrolysis efficiency of fruit extracts by testing individual enzymes incubated for 4 and 18 h and combined (hesperidinase for 18 h followed by cellulase for 4 h and cellulase for 18 h followed by hesperidinase for 4 h). The efficiency of hydrolysis was also tested by incubating different commercial standards of flavonol-glycosides (isoquercitin, hyperoside, rutin, quercitrin, avicularin, myricitrin, astragalin, and nicotiflorine), 5-caffeoylquinic acid, 3-caffeoylquinic acid, and punicalagin. Standards were dissolved to a concentration of 500 μM and were incubated with enzymes as described previously. The efficiency of hydrolysis was evaluated by substrate disappearance. Recoveries of the identified aglycones were calculated for each compound by spiking each fruit with the identified aglycones (Table 5). Recoveries were used to correct the quantification values of aglycones by dividing the determined value by the percentage of the recovery.

HPLC Analysis of Polyphenols. HPLC analysis was conducted on a series 1200 HPLC instrument (Agilent Technologies, Manchester, UK) equipped with ChemStation software (B.03.01), a model G1379B degasser, a model G1312B binary gradient pump, a model G1367C temperature-controlled autosampler, a model G1316B column oven, and a model G1315C diode array detection system (DAD). A sample volume of 5 μL was injected, and separations were achieved on a Zorbax Eclipse XDB-C18 (4.6 × 50 mm; 1.8 μm), operated at 30 °C, with a 0.2 μm stainless steel in-line filter. The method was performed as described by Pandino and co-workers[34] with the exception of the mobile phase consisting of 0.1% (v/v) formic acid in water (solvent A) and 0.1% (v/v) formic acid in acetonitrile (solvent B), used at a flow rate of 0.5 mL/min. The gradient started with 5% B to reach 10% B at 5 min, 40% B at 20 min, 90% B at 25 min, and 90% B at 29 min.

Chromatograms were recorded at 272, 280, 302, 324, 370, and 520 nm from diode array data collected between 200 and 600 nm. All standards were dissolved in ethanol or ethanol/water (1:1) and used as stock solutions. Calibration curves of peak area against concentration showed a good linear correlation with $r^2 > 0.99$. Limits of detection (LOD) and quantification (LOQ) as well as the wavelengths use to detect each compound are presented in Table 1.

Statistical Analysis. The results reported in this work are the averages of at least three independent experiments and are represented as the mean ± SD. Differences among treatments were detected by analysis of variance with Tukey's Honest Significant Difference (HSD) multiple-comparison test ($\alpha = 0.05$) using SigmaStat 3.10 (Systat).

RESULTS AND DISCUSSION

Portugal has good climatic conditions to facilitate production of different types of berries, and several different varieties have already been introduced in experimental fields. To select fruits to be further analyzed with a diverse chemical composition, an initial screening of chemical parameters for available commercial varieties and two wild species was performed.

Soluble Solid Content and Titratable Acidity. Fruit quality parameters, such as SSc and TAc, important as a quality indices for the consumer, were determined for the berries and their varieties (Table 2). Strawberry tree fruit showed the

Table 2. Analysis of Fruits for Soluble Solids Content (SSc) and Titratable Acidity (TAc)

fruit	variety	SSc[a] (°Brix)	TAc [g(tartaric acid)/L]	SSc/TAc
blueberry	Biloxi	12.2 ± 0.8a	3.5	3.4
	Sharp Blue	12.0 ± 1.3a	5.6	2.1
	Misty	11.3 ± 0.8a	5.5	2.1
	Jubilee	12.7 ± 2.3a	5.4	2.3
	Star	14.2 ± 2.1a	2.9	4.8
	Georgia Gem	15.3 ± 1.5	2.6	5.8
raspberry	Glen Lyon	9.2 ± 1.0a	14.6	0.6
	Polka	8.7 ± 1.5a	9.1	1.0
	Himbo Top	9.0 ± 0a	13.3	0.7
blackberry	Loch Tay	13.0 ± 2.1ab	5.1	2.5
	Olallie	7.4 ± 0.9c	4.5	1.7
	Arapaho	9.8 ± 1.0bc	7.1	1.4
	Oros	7.1 ± 0.9c	8.9	0.8
	Karaka Black	13.7 ± 2.0a	6.2	2.2
Portuguese crowberry		6.8 ± 0	8.7	0.8
strawberry tree fruit		27.8 ± 0.1	7.4	3.7

[a]Letters a−c represent the significance levels between varieties of the same fruit.

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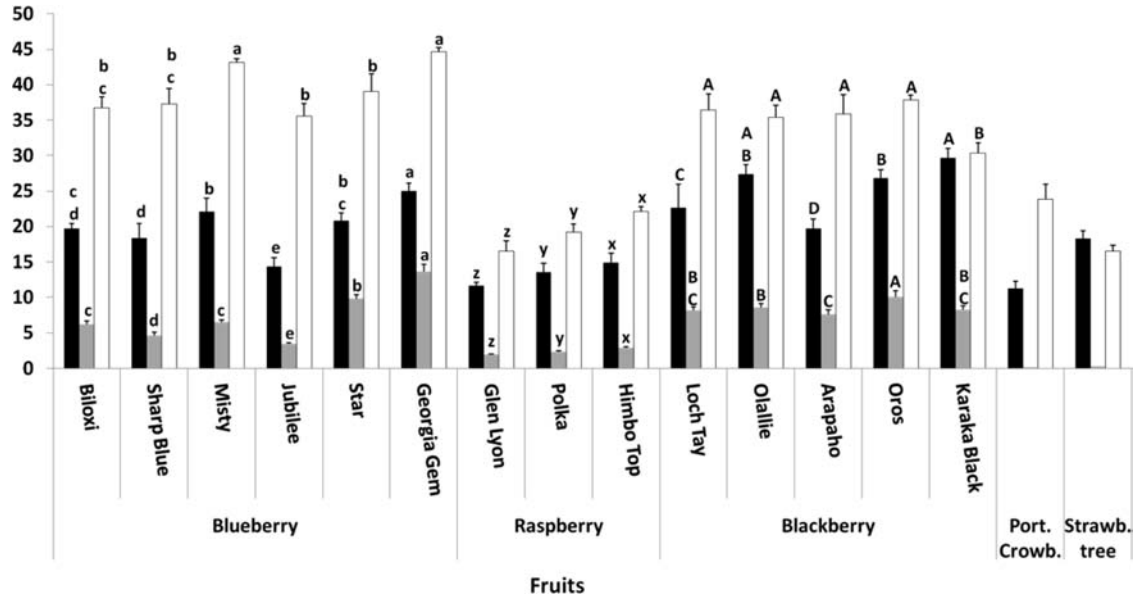

Figure 1. Phytochemical analysis of commercial varieties and wild fruits: measurement of total phenols (black bars, mg GAE/g dw), anthocyanins (gray bars, mg cy-3-glu/g dw), and antioxidant capacity (white bars, μmol TE/100 g dw) in fruit hydroethanolic extracts. Statistical comparisons are made between the varieties of each fruit. Letters a–e, x–z, and A–D represent the levels of significance between differences for each analyzed parameter independently.

Table 3. Quantification[a] of Total Anthocyanins, 5-Caffeoylquinic Acid, 3-Caffeoylquinic Acid, and Total Vitamin C in Fruits by HPLC-DAD

	fruits				
compound	blueberry var. Georgia Gem	raspberry var. Himbo Top	blackberry var. Karaka Black	Portuguese crowberry	strawberry tree
anthocyanins	433.2 ± 28.8	61 ± 1	160.6 ± 3.7	nd	9.72 ± 0.30
3-caffeoylquinic acid	nd	nd	nd	37.1 ± 3.7	nd
5-caffeoylquinic acid	47.10 ± 0.51	nd	nd	33.2 ± 2.6	nd
total vitamin C[b]	0.97 ± 0.12	12.52 ± 0.09	4.69 ± 0.08	5.43 ± 0.06	89.1 ± 1.7

[a]Contents are expressed as means of mg/100 g fw of fruits. Anthocyanins are quantified as means of cyanidin-3-*O*-glucoside equivalents. nd, not detected. [b]Total vitamin C was quantified as mg (L-ascorbc acid equivalents)/100 g fw of fruits.

highest SSc, far above the other analyzed berries, in contrast to Portuguese crowberry, which exhibited the lowest value. Some variability between the varieties of each commercial berry was observed, although in general blueberry varieties possessed the highest SSc and raspberries the highest TAc values, whereas blueberries were lowest. The ratio between SSc and TAc was particularly low for raspberry fruits, Portuguese crowberry, and blackberry variety Oros, suggesting that acidity would be obvious to consumers. The opposite was observed for blueberry varieties and strawberry tree fruit, for which a sweet flavor would be more prevalent. The high sugar content of strawberry tree fruit translates to a proclivity toward fermentation, making its chief utilization as a processed product rather than a fresh fruit. On the other hand, the high acidity of Portuguese crowberry fruits may contribute to the lack of demand by Portuguese consumers.

Phytochemical Analysis. Fruit extracts were quantified for total phenols, anthocyanins, and antioxidant capacity (Figure 1). These methods, although having some limitations and interferences, were selected as preliminary assays for quantification of phenolic compounds, in order to choose one variety of each for subsequent studies. Blackberries and blueberries were the fruits with higher content in total phenolic compounds and antioxidant capacity, which can be attributed to their high content of anthocyanins. The values of total phenolic compounds and antioxidant capacity of both wild berries were at a similar level to raspberries, although their content of anthocyanins was very low. Strawberry tree fruit has a red peel, but its anthocyanin level is low. In terms of varietal diversity, for blueberries, Georgia Gem contained the highest values for total phenolic compounds and anthocyanins. For raspberries, Himbo Top contained the highest values of total phenolic compounds, anthocyanins, and antioxidant capacity. All blackberry varieties possessed high values for anthocyanins and antioxidant capacity, and although Karaka Black contains slightly lower values for both parameters, it was the highest for total phenolic compounds content.

On the basis of these results, the blueberry variety Georgia Gem, the raspberry variety Himbo Top, and the blackberry variety Karaka Black were selected together with strawberry tree and Portuguese crowberry fruits for a more detailed analysis of phenolic compounds and free aglycones with the proposed method.

HPLC Analysis. Analysis of intact, nonhydrolyzed fruit extracts by HPLC-DAD revealed a great complexity and diversity of compounds, hampering individual identification of compounds. Only 3-caffeoylquinic and 5-caffeoylquinic acids were identified and quantified on the basis of their retention time and absorption spectra in comparison with standards (Table 3). Both caffeoylquinic acids were found to be the major

compounds in Portuguese crowberry fruits, and 5-caffeoylquinic acid was also abundant in blueberry. In a recent study, 5-caffeoylquinic acid was identified as the most abundant compound in Portuguese crowberry fruits, at higher levels than observed in our study, although reported levels of 3-caffeoylquinic acid were lower.[21] The values of 5-caffeoylquinic acid are slightly lower than described previously for blueberry (47 mg/100 g fw compared with published values of 64.6−208 mg/100 g fw[35,36]). In the other analyzed berries, if present, these compounds were too low to be detected or were obscured by other peaks. Anthocyanins were also analyzed by HPLC with detection at 520 nm and expressed as cyanidin-3-*O*-glucoside equivalents. Blueberry expressed the highest values, whereas anthocyanins were not detected in Portuguese crowberry fruits. Despite the fact that strawberry tree fruits have a red exocarp (when mature), they contained only 9.7 mg/100 g fw anthocyanins, but even this is higher than the value of 0.51 mg/100 g fw previously reported.[37] The anthocyanins present in strawberry tree fruits are expected to be cyanidin and delphinidin, both conjugated with galactose and glucose and the former also conjugated with arabinose.[24,37]

Total vitamin C was determined for the berries (Table 3). Strawberry tree fruit vitamin C content was far above that of the other fruits and exceeded values reported previously by Pallauf and co-workers,[24] but was in agreement with that of Alarcão-E-Silva and co-workers, who found a similarly high ascorbate content compared to other common fruits.[22] Values for vitamin C in Portuguese crowberries were similar to those in blackberry, whereas blueberries contained the smallest amount (Table 3).

Analysis of Aglycones after Enzyme Hydrolysis of Glycosides. To overcome the complexity of polyphenol analysis, the extracts were incubated with enzymes from *Aspergillus niger*: hesperidinase containing *α*-L-rhamnosidase and *β*-D-glucosidase activities and cellulase containing endo-1,4-*β*-D-glycosidic activity. Other enzymes from *A. niger* have previously been used to release phenolic acids from carbohydrates.[38−40]

Optimization of hydrolysis conditions and incubation time by using this novel combination of enzymes was performed. Fruit extracts and flavonol glycoside standards were used to test hydrolysis efficiency. Blueberry, due its phytochemical complexity, was selected for this optimization process, and the results were extrapolated to the other fruits. First, individual and a combination of enzymes were incubated with fruit extracts, and production of aglycones was compared. A combination of enzymes proved to be more efficient to obtain a higher degree of aglycones than individual enzymes. Hesperidinase hydrolysis was time-dependent, whereas cellulase had a similar efficiency in flavonol-glycoside hydrolysis for both 4 and 18 h incubation periods. On the basis of these results, incubation of fruit extracts with both enzymes, hesperidinase for 18 h and cellulase for 4 h, allowed complementary hydrolysis and optimized the reaction to obtain maximum yield of aglycones.

Further tests were performed to determine the hydrolytic efficiency of flavonol glycosides, by using standard compounds (Table 4). Incubation with the above enzyme combination enabled efficient hydrolysis of glucosides, rutinosides, and galactosides. The hydrolysis of arabinoside bonds was less efficient, as was hydrolysis of the rhamnoside bond in myricitrin and quercitrin. The enzymes also hydrolyzed the ester bond between caffeic and quinic acids of 3- and 5-caffeoylquinic acids, although hydrolysis of 3-caffeoylquinic acid was not complete (Table 4). The combination of enzymes was observed to hydrolyze punicalagin, an ellagitannin, although not completely, resulting in smaller compounds such as ellagic (Table 4). This additional hydrolysis (of nonglycosidic bonds) was probably due to the presence of other enzyme activities such as esterases in commercial enzymes, which are only partially purified.

Table 4. Hydrolysis Efficiency of Standard Compounds Incubated with Hesperidinase for 18 h, followed by Cellulose for 4 h

aglycone	conjugated sugar	compound name	efficiency of hydrolysis
quercetin	glucoside	isoquercitrin	complete
	galactoside	hyperoside	complete
	rutinoside	rutin	complete
	rhamnoside	quercitrin	incomplete (18%)
	arabinoside	avicularin	incomplete (56%)
myricetin	rhamnoside	myricitrin	incomplete (17%)
kaempferol	glucoside	astragalin	complete
	rutinoside	nicotiflorine	complete
		5-caffeoylquinic acid	complete
		3-caffeoylquinic acid	incomplete (61%)
		punicalagin	incomplete (73%)

For each fruit extract, recovery of aglycones was calculated after spiking with the reference standard and the values were used for correction of quantification (Table 5). Values for most compounds in all extracts were satisfactory, with the exception of gallic acid. However, the (low) efficiency of extraction of gallic acid was reproducible, implying that the limitation was in the incomplete partitioning and not due to instability.

As expected, HPLC-DAD chromatograms of fruit extracts were far less complex following hydrolysis, and the respective aglycones present in these berries were revealed (Figures 2−6). Identification of the aglycones in the extracts was performed by use of LC-MS data and confirmed with reference standards. LC-MS data are shown in the Supporting Information. Blueberry analysis revealed the presence of a high diversity of aglycones (Figure 2; Tables 5 and 6), with caffeic and gallic acids as the most abundant compounds. The majority of caffeic acid resulted from hydrolysis of 5-caffeoylquinic acid (as initially quantified), but a proportion is also likely derived from the hydrolysis of caffeic-4-*O*-glucoside, a minor compound previously reported in these berries.[36] The flavonols myricetin, quercetin, and kaempferol were also present in blueberries and are present in glycosylated form with values comparable to those reported here.[35,41] Protocatechuic, gallic, *p*-coumaric, and ferulic acids present in hydrolyzed extracts were previously found conjugated with glucose.[36] The same study also reported the presence of ferulic, *p*-coumaric, and caffeic acids esterified with quinic acid. Of the flavanols, only (−)-epicatechin was detected in blueberries. This compound was found in previous studies in concentrations between 0.7 and 1.1 mg/100 g fresh fruit.[15,42] However, in another study, no (−)-epicatechin was observed in four varieties of blueberries, whereas in three of these varieties, (+)-catechin and procyanidin B2 were identified, neither of which was found in our study.[43] This clearly reflects some chemical diversity of the minor compounds within blueberry varieties.

In the hydrolyzed extracts, 3-methylgallic acid, syringic acid, vanillic acid, and phloroglucinaldeyde were also present, which

Table 5. Recovery Values[a] (Percent) for Each Compound Calculated by HPLC-DAD after Spiking of Fruit Extracts

		fruits				
peak	compound	blueberry var. Georgia Gem	raspberry var. Himbo Top	blackberry var. Karaka Black	Portuguese crowberry	strawberry tree
1	gallic acid	23.8 ± 1.0	47.3 ± 3.1	28.6 ± 1.6	34.2 ± 4.9	31.6 ± 3.7
2	protocatechuic acid	47.3 ± 4.0	86.6 ± 4.0	66.8 ± 5.1	74.6 ± 5.7	65.7 ± 2.8
3	3-methylgallic acid	46.8 ± 2.0	nd	nd	nd	nd
4	(+)-catechin	nd	nd	nd	nd	77.4 ± 7.4
5	vanillic acid	80.8 ± 1.5	nd	nd	82.4 ± 5.0	nd
6	caffeic acid	100.4 ± 10.5	93.7 ± 6.6	nd	89.6 ± 16.3	nd
7	syringic acid	59.5 ± 5.5	nd	nd	nd	nd
8	(−)-epicatechin	62.7 ± 4.9	74.7 ± 4.3	78.5 ± 4.2	69.2 ± 3.0	nd
9	phloroglucinaldeyde	78.8 ± 5.8	88.4 ± 4.5	74.4 ± 9.9	nd	71.9 ± 8.8
10	*p*-coumaric acid	99.0 ± 5.8	nd	nd	92.5 ± 6.7	nd
11	ellagic acid	96.9 ± 10.5	61.1 ± 16.4	95.9 ± 14.0	nd	50.0 ± 9.1
12	ferulic acid	97.1 ± 7.3	95.8 ± 6.8	96.7 ± 5.6	90.7 ± 4.2	nd
13	myricetin	78.9 ± 5.4	nd	nd	87.7 ± 7.4	85.0 ± 2.0
15	quercetin	85.2 ± 5.9	79.6 ± 5.7	79.9 ± 6.8	97.8 ± 8.5	80.2 ± 10.9
16	kaempferol	73.2 ± 3.9	nd	75.2 ± 2.0	92.7 ± 5.4	nd

[a]nd, not determined (because it was not detected after hydrolysis).

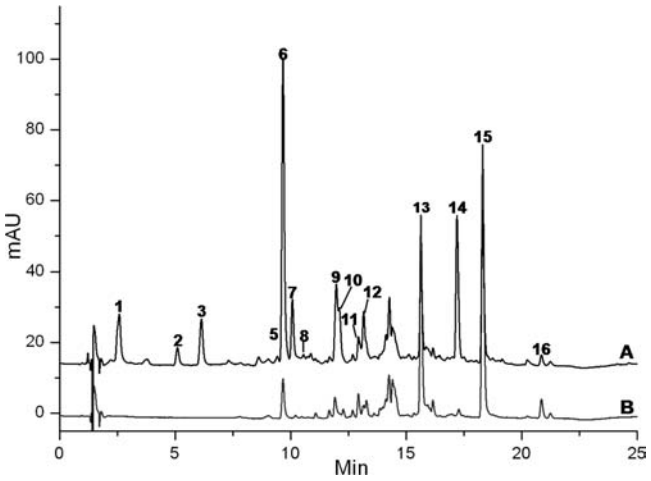

Figure 2. HPLC-DAD chromatograms of aglycones present in blueberry var. Georgia Gem extracts after enzyme hydrolysis: (A) recorded at 270 nm; (B) recorded at 370 nm. Peaks: 1, gallic acid; 2, protocatechuic acid; 3, 3-methylgallic acid; 5, vanillic acid; 6, caffeic acid; 7, syringic acid; 8, (−)-epicatechin; 9, phloroglucinaldehyde; 10, *p*-coumaric acid; 11, ellagic acid; 12, ferulic acid; 13, myricetin; 14, daidzein (IS); 15, quercetin; 16, kaempferol.

might be derived from hydrolysis or catabolism of compounds initially present in berries.

For raspberries (Figure 3; Table 5 and 6), the most abundant compound was ellagic acid. Although this compound has been previously quantified in the free form or attached to glycosides, it is mainly found esterified in ellagitanins such as sanguiin-H6 and lambertianin C.[44,45] Of the flavanols, only quercetin and (−)-epicatechin were detected. The presence of other flavanols such as (+)-catechin and procyanidin B2 is inconsistent between studies and appears to vary between varieties.[42,46,47] The same appears to happen for kaempferol, which was not detected in this work and also in some previous studies[46,47] but was found in other studies.[44,45] Caffeic acid was detected and may be derived from the 5-caffeoylquinic acid content previously reported in raspberries.[48] Mullen and co-workers reported a similar method for quantification of phenolics in raspberry, performing a hydrolysis of the extracts with β-glucosidase,[45] but of the phenolic acids, the presence of only *p*-coumaric, caffeic, and ellagic acids was reported. In this work, we also found gallic, protocatechuic, and ferulic acids and phloroglucinaldeyde in the hydrolyzed extracts (Figure 3; Table 6).

In blackberries (Figure 4; Table 5 and 6), the most abundant compounds were protocatechuic acid, phloroglucinaldeyde, and ellagic acid. Ellagic acid was previously found in the free form in blackberries but also esterified as ellagitannins[49,50] whereas protocatechuic acid was previously found in low amounts conjugated with glucose.[36] As in blueberry and raspberry, of flavanols, only (−)-epicatechin was detected in blackberries following hydrolysis. However, in previous studies, (+)-catechin was also found in low concentrations (6.6 mg/kg)[42,51] as well as other flavanols (0.06 and 0.83 mg/100g).[51] Therefore, the presence of some flavanols might be variety dependent, sometimes under the limit of detection. Quercetin was the most abundant flavonol in blackberries, with kaempferol in lower amounts in contrast with its absence in previous studies.[41,52] Gallic and ferulic acids were also found in hydrolyzed blackberry extracts, both found previously conjugated with glucose and ferulic acid with quinic acid.[36,49] To be noted is the absence of caffeic acid, which was previously described by Schuster in blackberries as conjugated with glucose and quinic acid.[36] Differences in the presence or absence of some compounds in comparison with previous studies might be the result of differences in cultivar between fruits and growing conditions.

Portuguese crowberry extracts also contained a great diversity of phenolic aglycones after enzyme hydrolysis (Figure 5; Table 5 and 6). The most abundant compound was caffeic acid, derived mainly from the hydrolysis of 3-caffeoylquinic and 5-caffeoylquinic acids, which were abundant in the non-hydrolyzed extracts (Table 3). Other hydroxycinnamates such as ferulic and *p*-coumaric acids were found at lower levels. The flavonols quercetin, kaempferol, and myricetin were present in the hydrolyzed extracts. The same compounds were also detected conjugated with glycosides in a previous study by HPLC-DAD-MS/MS.[21] (−)-Epicatechin, however, was not found in the same study. Gallic, protocatechuic, and vanillic acids were also found in Portuguese crowberry fruits (Table 6). These compounds had been found previously by GC-MS but

Table 6. Quantification[a] of Phenolics in Fruit Extracts after Enzyme Hydrolysis Using HPLC-DAD

		fruits				
peak	compound	blueberry var. Georgia Gem	raspberry var. Himbo Top	blackberry var. Karaka Black	Portuguese crowberry	strawberry tree
1	gallic acid	16.32 ± 3.10	1.44 ± 0.06	6.08 ± 0.25	1.12 ± 0.15	117.2 ± 2.4
2	protocatechuic acid	1.95 ± 0.03	3.88 ± 0.30	12.98 ± 0.97	1.32 ± 0.05	2.27 ± 0.12
3	3-methylgallic acid	5.28 ± 0.10	ud	ud	ud	ud
4	(+)-catechin	ud	ud	ud	ud	25.46 ± 0.02
5	vanillic acid	1.03 ± 0.05	ud	ud	0.63 ± 0.03	ud
6	caffeic acid	31.94 ± 0.54	4.08 ± 0.23	ud	39.42 ± 2.15	ud
7	syringic acid	5.08 ± 0.12	ud	ud	ud	ud
8	(−)-epicatechin	5.24 ± 0.43	3.31 ± 0.19	4.18 ± 0.06	1.83 ± 0.33	ud
9	phloroglucinaldeyde	1.21 ± 0.17	3.63 ± 0.12	12.19 ± 0.40	ud	0.41 ± 0.03
10	*p*-coumaric acid	1.89 ± 0.16	ud	ud	0.89 ± 0.02	ud
11	ellagic acid	2.9 ± 0.05	12.45 ± 0.77	8.62 ± 0.58	ud	7.43 ± 0.12
12	ferulic acid	3.06 ± 0.07	1.98 ± 0.09	0.62 ± 0.02	0.56 ± 0	ud
13	myricetin	9.20 ± 0.13	ud	ud	1.39 ± 0	1.13 ± 0.05
15	quercetin	11.30 ± 0.33	2.14 ± 0.14	3.04 ± 0.05	3.92 ± 0.18	3.16 ± 0.10
16	kaempferol	1.01 ± 0.01	ud	0.36 ± 0.01	0.27 ± 0	ud

[a]Contents are expressed as means of mg/100 g dw of fruits. ud, under the limit of detection.

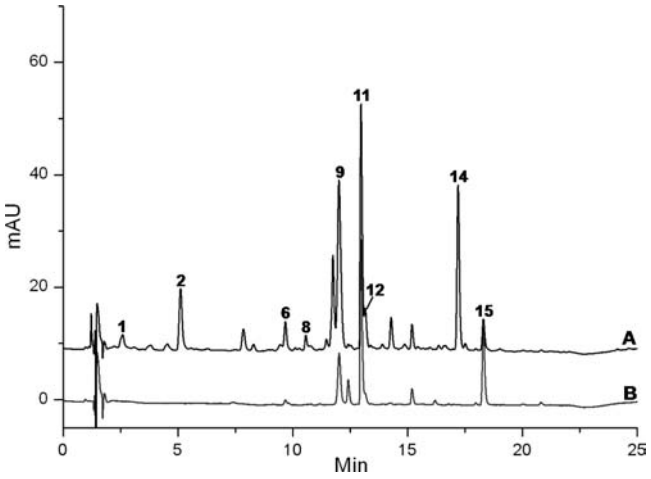

Figure 3. HPLC-DAD chromatograms of aglycones present in raspberry var. Himbo Top extracts after enzyme hydrolysis: (A) recorded at 270 nm; (B) recorded at 370 nm. Peaks: 1, gallic acid; 2, protocatechuic acid; 6, caffeic acid; 8, (−)-epicatechin; 9, phloroglucinaldehyde; 11, ellagic acid; 12, ferulic acid; 14, daidzein (IS); 15, quercetin.

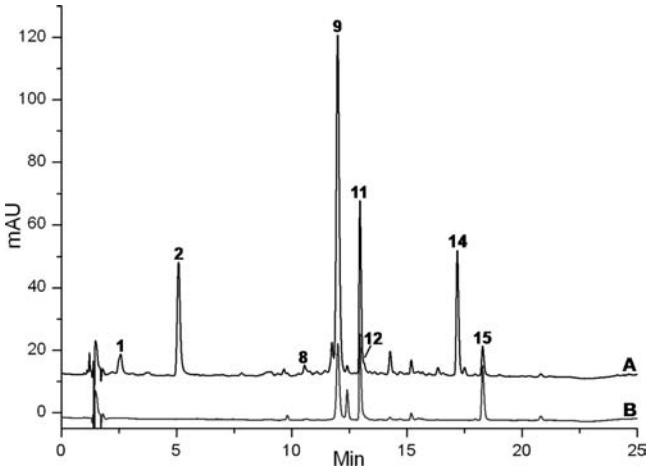

Figure 4. HPLC-DAD chromatograms of aglycones present in blackberry var. Karaka Black extracts after enzyme hydrolysis: (A) recorded at 270 nm; (B) recorded at 370 nm. Peaks: 1, gallic acid; 2, protocatechuic acid; 8, (−)-epicatechin; 9, phloroglucinaldehyde; 11, ellagic acid; 12, ferulic acid; 14, daidzein (IS); 15, quercetin; 16, kaempferol.

not by HPLC,[21] demonstrating the utility of the proposed hydrolysis method.

In strawberry tree fruit extracts (Figure 6; Table 5 and 6), gallic acid appears as the most abundant compound, at a much higher level than the other berries. The abundance of gallic acid is supported by previous studies in which gallic acid is in the free form or conjugated with either quinic acid, shikimic acid, or glucose.[23,37,53] The second most abundant compound was (+)-catechin, a compound previously reported in this fruit.[24] However, the (+)-catechin content of strawberry tree fruits reported here is similar to the total content in flavanols reported previously.[24] It is possible that this higher content in catechin comes from the breakdown of proanthocyanidins during enzyme incubation. Ellagic acid was the third most abundant compound and had been previously found conjugated in this fruit with glycosides.[23,24] Myricetin, quercetin, and ellagic acid were also detected in hydrolyzed extracts (Table 6) and were previously found in these berries conjugated with glycosides.[24] Protocatechuic acid and phloroglucinaldehyde were also detected and are believed to come mainly from hydrolysis and thermal degradation of anthocyanins, even at acidic pH.[54] Anthocyanin degradation is the probable source of 3-methylgallic acid observed in hydrolyzed blueberry extracts.[19] Kay and co-workers have detected absorption and phase II metabolites of protocatechuic acid and phloroglucinaldehyde in a Caco-2 cell model after spontaneous degradation of anthocyanins and suggest that these catabolites may be at least partially responsible for the biological activity of anthocyanins in vivo.[55]

The use of this method in five different fruit matrices reveals its versatility. Moreover, the method is very efficient for hydrolysis of glucosides, galactosides, and rutinosides, although rhamnosides are less efficiently hydrolyzed. Overall, it is a simple and rapid method for the identification and quantification of phenolic aglycones. The identification and quantification of phenolics in plant tissues can be sometimes difficult due to a high number and diversity of compounds.

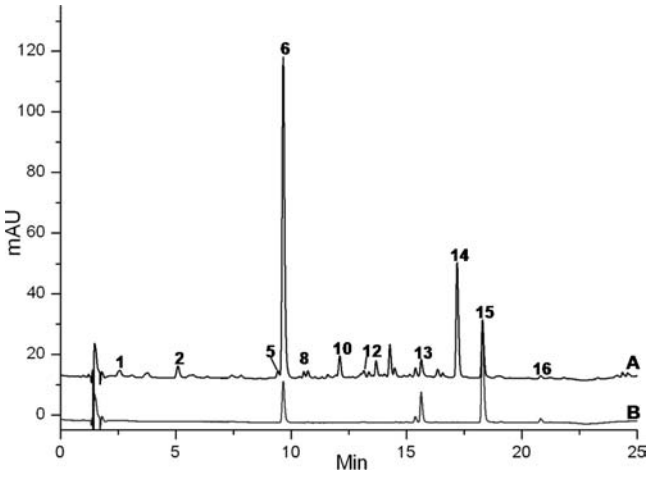

Figure 5. HPLC-DAD chromatograms of aglycones present in Portuguese crowberry fruit extracts after enzyme hydrolysis: (A) recorded at 270 nm; (B) recorded at 370 nm. Peaks: 1, gallic acid; 2, protocatechuic acid; 5, vanillic acid; 6, caffeic acid; 8, (−)-epicatechin; 10, *p*-coumaric acid; 12, ferulic acid; 13, myricetin; 14, daidzein (IS); 15, quercetin; 16, kaempferol.

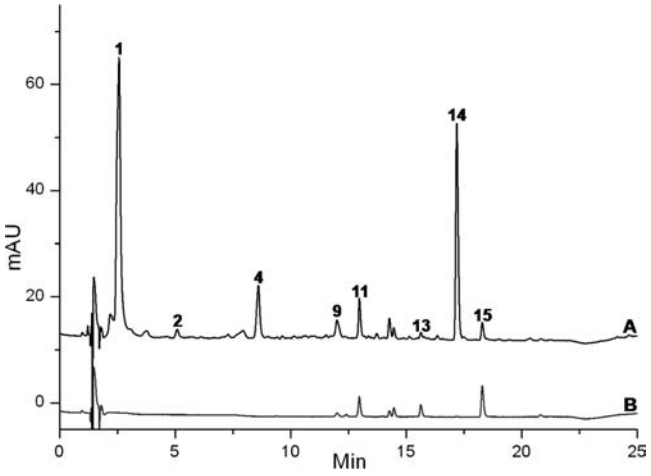

Figure 6. HPLC-DAD chromatograms of aglycones present in strawberry tree fruit extracts after enzyme hydrolysis: (A) recorded at 270 nm; (B) recorded at 370 nm. Peaks: 1, gallic acid; 2, protocatechuic acid; 4, (+)-catechin; 9, phloroglucinaldehyde; 11, ellagic acid; 13, myricetin; 14, daidzein (IS); 15, quercetin.

Therefore, hydrolysis of glycosides and analysis of the resulting aglycones is a usual methodology to overcome this problem. Traditional methods based on acid hydrolysis of glycoside bonds require relatively high concentrations (1−2 M) of mineral acids at temperatures close to 100 °C.[56,57] However, this methodology can be too harsh for some compounds, which may undergo partial degradation, as observed for catechins and flavonols such as myricetin, kaempferol, and quercetin.[58] It was observed that it is hard to reach a compromise as an increase in incubation time can increase degradation, but a reduced time can result in underestimation of the phenolic content.[56,58] Alkaline hydrolysis can also lead to significant losses in hydroxycinnamic acid derivatives as reported by Krygier and co-workers.[59]

Methods employing commercial enzymes to release and identify phenolic aglycones have also been previously used and have been reported to be adequate for phenolic acid quantification in cereals.[60−62] Hesperidinase has been used for hydrolysis of flavonol-glycosides and analysis of aglycones.[63] Fungal glycosidases can be very useful either for analytical or for other purposes. Previously, rhamnosidases were used to produce functional beverages by converting flavonoid-rhamnosides to glucosides, thus increasing bioavailability.[64,65]

Here, we report for the first time the combined use of hesperidinase and cellulase as an efficient method for releasing flavonols and phenolic acids aglycones from glycosidic bonds for analytical purposes. Other methods have been used for this purpose, but each has advantages and limitations and so should be chosen according to the aim of the determination. This method is a simple alternative to analyze complex samples, and its usefulness has been demonstrated by application to two noncommercial fruits. Blueberry, raspberry, and blackberry fruits were also analyzed according to this method, and results, with minor exceptions, are generally in agreement with published data.

The results highlight wild berries, Portuguese crowberry and strawberry tree, as good sources of phenolic compounds. Strawberry tree fruit is mostly characterized by the presence of gallic acid derivatives, whereas Portuguese crowberry fruit contains high amounts of caffeic acid derivatives. Therefore, they constitute a good alternative to the other berry fruits traditionally consumed to increase and diversify the ingestion of phenolic compounds in our diet.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary Tables 1−5. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

HPLC, hig-performance liquid chromatography; DAD, diode array detection; SSc, soluble solids content; TAc, titratable acidity; ORAC, oxygen radical absorbance capacity; GAE, gallic acid equivalents; TE, trolox equivalents; PTFE, polytetrafluoroethylene; IS, internal standard

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