JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Phenolic Composition and Antioxidant Activities in Flesh and Achenes of Strawberries (*Fragaria ananassa*)

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High performance liquid chromatography coupled with diode array and coulometric array detectors were used to characterize and quantify phenolic compounds in achenes and flesh of ripe strawberries (cv Totem and Puget Reliance). Total phenolics and total monomeric anthocyanins were measured and antioxidant activities were evaluated by the FRAP and the ORAC assays. Strawberries contained 1% achenes on a fresh weight basis; however, they contributed to about 11% of total phenolics and 14% of antioxidant activities in strawberries. Ellagic acid, ellagic acid glycosides, and ellagitannins were the main contributors to the antioxidant activities of achenes. The major anthocyanin in flesh was pelargonidin-3-glucoside, whereas achenes consisted of nearly equal amounts of cyanidin-3-glucoside and pelargonidin-3-glucoside. Phenolic content and antioxidant activity of strawberry achenes were reduced by industrial processing. However, the levels were still high and strawberry waste byproduct could thus be a possible source of nutraceuticals or natural antioxidants.

KEYWORDS: Strawberry; *Fragaria ananassa;* achenes; antioxidant activity; ORAC; FRAP; HPLC; anthocyanins; phenolic compounds; ellagic acid; ellagitannins

INTRODUCTION

Polyphenolic compounds are associated with prevention of diseases thought to be induced by oxidative stress, such as cardiovascular diseases, cancer, and inflammation (*1*−*4*). The possible protective effects reported are generally associated with the antioxidant activity of the polyphenolics. Strawberries have been shown to be a rich source of phenolic compounds with antioxidant and antiproliferative activities (*5*−*9*). It has further been shown that total antioxidant activity in serum from elderly women increased after consumption of strawberries (*10*). The antioxidant properties of strawberries have been demonstrated to be mainly due to high content of phenolic compounds rather than vitamin C (*7*, *8*).

The phenolic compounds responsible for the red color in strawberry flesh, the anthocyanins, have been widely investigated and identified as glycosides of pelargonidin and cyanidin, with pelargonidin-3-glucoside as the dominant compound (*11*−*15*). Glucosides and glucuronides of quercetin and kaempferol have been identified as the main flavonols (*14*−*17*). The primary cinnamic acid derivative is *p*-coumaroylglucoside, but also glucosides of ferulic and caffeic acid have been identified (*14*, *18*). Strawberries, together with raspberries and blackberries, are the main sources of ellagic acid in the human diet (*19*, *20*). Literature reports of ellagic acid content are inconsistent, with some workers reporting ellagic acid content as strictly ellagic acid and others combining it with ellagic acid glycosides and ellagitannins (*15*, *21*−*23*). Ellagitannins, which consist of a central core of glucose esterified with hexahydroxydiphenic acid(s) (HHDP), have been detected in strawberries (*15*). One of the ellagitannins is identified as casuarictin (*24*). In strawberry leaves the dimer sanguiin H-6 and its two constitutive monomers casuarictin and potentillin, as well as pedunculagin, are identified (*25*). Large differences in ellagic acid content are found among strawberry cultivars (*23*). The highest level of total ellagic acid was found in strawberry leaves followed by achenes and finally flesh (*23*). In strawberry pulp with achenes the total ellagic acid content for red fruit (cv Camarosa) was more than 6-fold higher compared to that of pulp without achenes (*26*). In a study on antifungal activity of strawberries, it was found that antifungal compounds included phenolics and the highest activity was found in achenes (*27*).

When strawberry fruits are processed to give juice and puree, substantial waste material that contains high levels of achenes is generated. This processing waste could be a potential source of nutraceuticals instead of being fed to livestock or sent to sanitary landfill. To be able to evaluate strawberry achenes as a source of natural antioxidants, it is important to know their phenolic composition and antioxidant properties. While the polyphenolic composition and antioxidant properties of strawberries has been the subject of several investigations, quantitative

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10.1021/jf048001o CCC: $30.25
Published on Web 04/20/2005

and qualitative comparisons of their distribution in the flesh and achenes are missing.

The aim of this study was to characterize and quantify the phenolic compounds and antioxidant activities in achenes and flesh of two strawberry cultivars, Totem and Puget Reliance. In addition, achenes generated from industrial production of seedless strawberry puree were analyzed to evaluate their potential as a source for nutraceuticals. Comparative extraction methods for recovery of polyphenolics from achenes were also investigated from the standpoint of finding those procedures that would be more suitable for industrial practice.

MATERIALS AND METHODS

Chemicals. Gallic acid, chlorogenic acid, (+)-catechin, (−)-epicatechin, ellagic acid, caffeic acid, *p*-coumaric acid, ferulic acid, quercetin-3-rutinoside (rutin), β-phycoerythrin (β-PE), and Folin and Ciocalteu's phenol reagent were purchased from Sigma Chemical Co. (St. Louis, MO). 2,4,6-Tripyridyl-*s*-triazine and 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox) were obtained from Fluka Chemie GmbH (Buchs, Switzerland). 2,2′-Azobis(2-amidinopropane)dihydrochloride (AAPH) was obtained from Polysciences Inc. (Warrington, PA). $FeCl_3 \cdot 6H_2O$, $FeSO_4 \cdot 7H_2O$, and sodium were obtained from Merck KGAa (Darmstadt, Germany). All solvents were of HPLC grade (EM Science, Gibbstown, NJ), and water was of Milli-Q quality (Millipore Corp., Bedford, MA).

Samples. Strawberries (cultivars Totem and Puget Reliance) were grown at the Oregon State University (OSU) Research and Extension Center, Aurora, OR. Fruit was harvested at commercial ripeness in June, 2002 and transported to the OSU Food Science and Technology pilot plant in cooled, insulated containers. They were immediate individually quick frozen in a blast freezer (−23 °C) and subsequently placed in glass jars and stored at −20 °C. The berries were stored for 6 months before use. The achenes were separated from the flesh in two ways. (1) The berries were partly thawed (2 h at room temperature) before blending in a food processor. The achenes were then separated from the flesh by pressing through a strainer. The collected achenes were rinsed several times with running, cold water and left to dry at room temperature. (2) Achenes were isolated manually from frozen freeze-dried berries with a pincer. Freeze-drying was performed in a Consol 4.5 freeze-drier (Virtis, The Virtis Company, Inc., Gardiner, NY). Dry weight of flesh and achenes were determined after freeze-drying. Percentage (weight/weight) of achenes was determined after separation from flesh, by either method 1 or 2.

Strawberry achenes used for evaluation of extraction conditions were the waste after the production of seedless puree at Kerr Concentrates Inc. (Salem, OR). The achenes were rinsed several times with running, cold water and left to dry at room temperature. Dried achenes were stored at −20 °C before extraction.

Extraction. Extraction was performed as earlier described (*28*) with some modifications. Liquid nitrogen-milled freeze-dried samples (0.5 g) were mixed with 10 mL of solvent (70% aqueous acetone) and sonicated for 10 min. After centrifugation, the supernatant was collected, and the insoluble plant material was re-extracted three times with solvent (6 mL). Pooled extracts were mixed with chloroform (1:1, v/v) and the aqueous and the lipophilic phase was partitioned after centrifugation (170 × *g* for 20 min). The aqueous phase was collected, and residual acetone was removed in a rotary evaporator at 40 °C under reduced pressure. The weight of the extract was accurately recorded.

Strawberry flesh from puree (5 g) was extracted in the same way, but with pure acetone as the solvent in the first extraction. In the extraction experiment, achenes were extracted whole (5 g) or liquid-nitrogen-milled (3 g), with water or 70% acetone as the extraction solvent. The extraction procedure, including volumes of solvents, was the same as given above.

All extractions were made in duplicate. Extracts were stored at −70 °C until analysis.

Purification of Samples. Solid-phase extraction using C_{18} Sep-Pak cartridges (Waters Assoc., Milford, MA) was used for removal of sugars and acids and to isolate anthocyanins from other phenolic compounds (*29*).

Hydrolysis. Alkaline and acid hydrolyses of extracts were performed as described earlier (*30*).

Total Phenolics. Total phenolics in extracts were determined according to the Folin-Ciocalteu procedure (*31*, *32*). Appropriately diluted extract (0.2 mL) was mixed with 1.0 mL of Folin-Ciocalteu reagent (1:10, v/v diluted with water) and incubated for 1 min before 0.8 mL sodium carbonate (7.5% w/v) was added. The mixture was incubated for 2 h at room temperature before absorption was measured at 765 nm (Shimadzu UV160U, UV−vis spectrophotometer, Kyoto, Japan). Total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per 100 g fresh weight of achenes or flesh (mg GAE/100 g fw). All extracts were analyzed in duplicate.

Total Monomeric Anthocyanins. Total monomeric anthocyanins (TMA) were determined according to the pH differential method (*33*). The extracts were appropriately diluted in two buffers, 0.025 M potassium chloride pH 1.0 and 0.4 M sodium acetate pH 4.5. After 15 min of incubation at room temperature, absorption was measured at 496 and 700 nm (Shimadzu UV160U, UV−vis spectrophotometer, Kyoto, Japan). TMA was calculated using molar absorptivity of 27 300 L cm^{-1} mol^{-1} and molecular weight of 433.2 g mol^{-1} for pelargonidin-3-glucoside (*33*) and expressed as milligrams per 100 g fresh weight of achenes or flesh (mg/100 g fw). All extracts were analyzed in duplicate.

FRAP Assay. The FRAP assay was carried out as described by Benzie and Strain (*34*) with some modifications according to the procedure described earlier (*35*). Briefly, 2.4 mL of freshly prepared FRAP reagent containing 10 mM TPTZ in 40 mM HCl, 20 mM $FeCl_3 \cdot 6H_2O$, and 300 mM acetate buffer, pH 3.6, in the ratio of 1:1:10 (v:v:v) was mixed with 80 μL of appropriately diluted sample. The mixture was allowed to stand for 60 min at room temperature before absorption was measured at 593 nm (Agilent 8453 Spectrophotometer, Agilent Technologies, Waldbronn, Germany). Aqueous solutions of Fe(II) ($FeSO_4 \cdot 6H_2O$) in the concentration range of 125−1000 μmol/L were used for calibration of the FRAP assay. Trolox was used as a standard. All extracts were diluted and analyzed in duplicate. FRAP values were expressed as μmol Trolox equivalents per gram sample (μmol TE/g fw). The ferric reducing ability of 1 μmol of Trolox was equivalent to that of 2.0 μmol of $FeSO_4 \cdot 6H_2O$.

ORAC Assay. The ORAC assay was performed as previously reported (*36*) with some modifications (*35*). The measurements were carried out on a Wallac 1420 Victor^2 96-well plate reader (EG & Wallac, Turku, Finland) with fluorescence filter (excitation 540 nm/8 nm, emission 570 nm/7 nm). β-PE (16.7 nM) was the fluorescence probe and target molecule for free radical attack from AAPH (4 nM) as the peroxyl radical generator. The reaction was conducted at 37 °C at pH 7.0 with Trolox (1 μM) as control standard and phosphate buffer as blank. The extracts were appropriately diluted with buffer prior to analysis. The β-PE fluorescence was recorded every 2 min after addition of AAPH. All measurements were expressed relative to the initial reading. Final results were calculated using the differences of areas under the β-PE decay curves between the blank and a sample and were expressed as μmol Trolox equivalents (TE) per gram sample (μmol TE/g fw).

HPLC Analysis. Anthocyanins and polyphenolics were separated using a Perkin-Elmer Series 400 liquid chromatograph (PerkinElmer Inc., Shelton, CT) equipped with a HP1040A photodiode array detector (Hewlett-Packard GmbH, Waldbronn, Germany) and a Beckman 501 autosampler (Beckman Instruments, Inc., San Ramon, CA).

Chromatographic separation was performed on a Prodigy ODS-3 column (250 mm × 4.6 mm i.d, 5 μm particle size) from Phenomenex (Torrance, CA) equipped with a 5 μm ODS guard column (4.0 mm × 4.6 mm i.d., Phenomenex, Torrance, CA). The mobile phase for separation of polyphenols other than anthocyanins consisted of acetonitrile (A) and 70 mM KH_2PO_4 adjusted to pH 2.4 with phosphoric acid (B). Initial mobile phase composition, 5% A and 95% B, was held in 3 min, followed by a linear gradient to 25% A in 27 min, then a linear increase to 55% A in 7 min, and finally isocratic conditions for 2 min. Column temperature was ambient. For separation of anthocyanins the mobile phase consisted of acetonitrile (A) and 1% phosphoric acid and 10% acetic acid (v/v) in water (B). The program

Table 1. Percentage of Achenes (on Total Weight Basis) and Dry Weight (%) of Achenes and Flesh of Red Ripe Strawberries

cultivar	percentage of achenes (%)	dry weight (%)	
		achenes	flesh
Totem	1.0	93.5	12.6
Puget Reliance	1.0	93.5	10.1
unknown (waste)		95.5	

followed a linear gradient from 2% to 20% A in 25 min and then from 20% to 40% A in 15 min. Column temperature was held at 40 °C.

In both separation systems the solvent flow rate was 1 mL/min and the column was allowed to equilibrate for 10 min between injections. Samples were filtered through a Millex HA 0.45 μm filter (Millipore, Ireland) before injection (20 μL). Spectra for all wavelengths between 220 and 600 nm were recorded by the photodiode array detector. The phenolic compounds were characterized by their UV spectra, retention times relative to external standards, and peak spiking. Anthocyanin composition was presented as percent (%) of total peak area at 520 nm. The other phenolic compounds were grouped into classes, quantified by external standards, and expressed as mg/100 g fw of achenes or flesh. Cinnamic acid derivatives were quantified as chlorogenic acid (320 nm), (+)-catechin and proanthocyanidins as (+)-catechin (280 nm), flavonols as rutin (370 nm), ellagic acid and ellagic acid glycosides as ellagic acid (260 nm), and ellagitannins as gallic acid (280 nm).

HPLC with Coulometric Array Detection. Chromatographic separation of phenolic compounds, other than anthocyanins, was performed on a Betasil C_{18}-column (250 mm × 4.6 mm i.d., 5 μm particle size) equipped with a 5 μm C_{18} (ODS) guard column (4.0 mm × 4.6 mm i.d.), both from Thermo Hypersil-Keystone (Bellefonte, PA). The mobile phase and elution program was the same as described above. Column temperature was 30 °C, and 50 μL of sample was injected.

The analyses were performed as described earlier (*35*), using an HP 1050 series HPLC (Hewlett-Packard GmbH, Waldbronn, Germany) interfaced to an ESA coulometric array detector (ESA Inc., Chelmsford, MA) with eight porous graphite working electrodes with associated palladium reference electrodes. The detector array was set from 100 to 800 mV in increments of 100 mV. The ESA CoulArray operating software (ESA Inc., Chelmsford, MA) was used to collect voltammetric data. Raw data was processed using Microsoft Excel. Peak areas for all compounds in a sample were summed. The results were calculated as peak areas and cumulative peak areas at the electrodes, expressed as coulomb per gram strawberry flesh or achenes (C/g fw). Cumulative responses at 800 mV had the highest correlation with antioxidant activities, and therefore those results were presented. For identification of individual compounds, the HDVs (cumulative response vs oxidation potential) were plotted (*35*).

ES-MS Method. Low-resolution mass spectroscopy was performed by electrospray ionization using a PE SCIEX API III Biomolecular Mass Analyzer (PerkinElmer Inc., Toronto, Canada) equipped with an ion spray source (ISV = 4000, orifice voltage = 60). The instrument was operated in the positive mode. Isolated anthocyanin fractions were introduced to the ES-MS by a 100 μL glass syringe at a flow rate of 12.55 μL/min. Only anthocyanin fractions isolated by solid-phase extraction from freeze-dried achenes of Totem and Puget Reliance and flesh from Totem were analyzed by ES-MS.

Statistical Analysis. Regression analysis to determine ORAC values was performed by Microsoft Excel. Linear regression between antioxidant activities was performed by Minitab Statistical Software (Release 13.30, Minitab Inc., State College, PA).

RESULTS AND DISCUSSION

Sample Characteristics. Percentage of achenes (on total weight basis) and dry weight (%) of achenes and flesh of the strawberries are given in **Table 1**.

Total Phenolics, Total Anthocyanins, Antioxidant Activities, and Voltammetric Response. Total phenolics in flesh of strawberries of Totem and Puget Reliance were in the range of 230−340 mg GAE/100 g fw (**Table 2**), which corresponds to 2000−2800 mg GAE/100 g dry weight. These levels are in agreement with previously reported values for strawberries (*9*, *37*−*39*). The TMA of the flesh of Totem and Puget Reliance, about 60 and 40 mg/100 g fw, respectively, was in the same range as previous reported for strawberries (*9*, *40*). The average ORAC value of strawberry flesh of about 13 μmol TE/g fw was similar to values reported in previous studies (*14*, *41*). The somewhat higher FRAP values obtained, calculated into the same units, i.e., about 4.3 mmol/100 g fw, compared with previous results, 2.2 and 3.3 mmol/100 g fw (*5*, *7*), could be due to longer reaction times in the assay in the present study (*35*). The electrochemical responses for the flesh samples coincided with the levels of total phenolics, TMA, and antioxidant capacities.

Strawberry achenes manually separated from freeze-dried berries contained high amounts of total phenolics, averaged 3600 mg GAE/100 g fw (**Table 2**). In achenes from the same berries but isolated from puree, content of total phenolics was less than half, about 1340 mg GAE/100 g fw. TMA of achenes separated from freeze-dried strawberries was about 59 mg/100 g fw, more

Table 2. Total Phenolics, Total Monomeric Anthocyanins (TMA), ORAC and FRAP Values, and Voltammetric Response[a] in Flesh and Achenes of Red Ripe Strawberries

part of strawberries	cultivar/abbr[b]	total phenolics (mg GAE/100 g fw)	TMA (mg/100 g fw)	ORAC (μmol TE/g fw)	FRAP (μmol TE/g fw)	voltammetric response (C/g fw)
flesh						
freeze-dried[c]	Totem	341 ± 5	60.1 ± 1.3	16 ± 2	24.8 ± 0.2	1.7 ± 0.0
	Puget	230 ± 7	32.3 ± 1.0	12 ± 1	17.7 ± 0.3	1.2 ± 0.1
puree[d]	Totem	300 ± 18	58.9 ± 1.0	11 ± 1	21.7 ± 0.1	1.7 ± 0.2
	Puget	280 ± 17	45.4 ± 0.4	12 ± 1	21.3 ± 0.3	1.5 ± 0.0
achenes						
from freeze-dried[c]	Totem	3907 ± 544	67.5 ± 9.7	174 ± 32	474 ± 66	10.4 ± 1.2
	Puget	3314 ± 329	45.9 ± 2.0	172 ± 23	364 ± 3	7.4 ± 0.4
from puree[d]	Totem	1429 ± 46	11.3 ± 0.6	72 ± 1	154 ± 0	3.1 ± 0.2
	Puget	1256 ± 92	11.0 ± 0.2	72 ± 7	108 ± 7	3.1 ± 0.1
industrial waste product[e]	MA	584 ± 38	13.5 ± 0.8	39 ± 3	52 ± 4	1.9 ± 0.0
	WA	179 ± 5	11.1 ± 0.3	1.4 ± 0.1	9.9 ± 0.6	0.4 ± 0.0
	MW	120 ± 1	6.0 ± 0.1	12.4 ± 0.7	7.4 ± 0.0	1.6 ± 0.3
	WW	21 ± 3	3.1 ± 0.4	4.1 ± 0.3	1.5 ± 0.1	*f*

[a] Cumulative peak area at the end of the coulometric array, at 800 mV. [b] MA = milled, acetone extracted, WA = whole, acetone extracted, MW = milled, water extracted, WW = whole, water extracted. [c] Achenes were removed from freeze-dried berries using a pincer. [d] Achenes were separated from puree in a strainer. [e] The waste after the production of seedless puree. [f] Not measured.

Table 3. Tentative Identification of Anthocyanins Detected in Red Ripe Strawberries Based on Retention Times (t_R), Wavelength of Maximum Absorption (λ_{max}), Molecular Ion (M^+), and Response to Alkaline Hydrolysis

peak	t_R (min)	λ_{max} (nm)	response to alkaline hydrolysis	M^+ (*m*/*z*)	tentative identification
1	8.7	520	increased as peak 4 disappeared	449	cyanidin-3-glucoside
2	11.0	500	increased as peak 5 disappeared	433	pelargonidin-3-glucoside
3	12.2	502	not detected	579	pelargonidin-3-rutinoside
4	14.0	520	disappeared	535	cyanidin-3-glucoside-malonate
5	16.5	503	disappeared	519	pelargonidin-3-glucoside-malonate

Table 4. Anthocyanin Composition (Area %) of Anthocyanins in Flesh and Achenes of Red Ripe Strawberries

part of strawberries	cultivar/abbr[a]	cyanidin-3-glucoside	pelargonidin-3-glucoside	pelargonidin-3-rutinoside	cyanidin-3-glucoside-malonate	pelargonidin-3-glucoside-malonate
flesh						
freeze-dried[b]	Totem	0.9 ± 0.1	86.4 ± 0.2	2.5 ± 0.0	ND[c]	9.5 ± 0.1
	Puget	4.2 ± 0.3	91.3 ± 0.5	2.9 ± 0.5	ND	ND
puree[d]	Totem	1.1 ± 0.0	85.8 ± 0.1	2.1 ± 0.0	ND	10.1 ± 0.0
	Puget	3.8 ± 0.1	90.9 ± 0.0	3.5 ± 0.0	ND	ND
achenes						
from freeze-dried[b]	Totem	39.3 ± 0.1	36.1 ± 0.8	ND	18.6 ± 0.0	6.1 ± 0.2
	Puget	43.6 ± 0.0	34.8 ± 0.1	ND	21.6 ± 0.2	ND
from puree[d]	Totem	41.4 ± 0.4	37.3 ± 0.4	ND	13.7 ± 0.3	7.5 ± 0.4
	Puget	37.0 ± 1.9	51.7 ± 2.5	ND	11.3 ± 0.0	ND
industrial waste product[e]	MA	ND	90.2 ± 0.1	ND	ND	9.8 ± 0.1
	WA	ND	89.8 ± 0.2	ND	ND	10.2 ± 0.2
	MW	ND	85.9 ± 0.9	ND	ND	14.1 ± 0.9
	WW	ND	87.4 ± 0.2	ND	ND	12.6 ± 0.2

[a] MA = milled, acetone extracted, WA = whole, acetone extracted, MW = milled, water extracted, WW = whole, water extracted. [b] Achenes were removed from freeze-dried berries using a pincer. [c] Not detected, i.e., 0%. [d] Achenes were separated from puree in a strainer. [e] The waste after the production of seedless puree.

than four times the content of achenes separated from puree. The highest antioxidant activities were detected in achenes manually isolated from freeze-dried berries. The ORAC and FRAP values averaged 173 and 419 μmol TE/g fw, respectively. These antioxidant activities were more than twice the values of achenes separated from the puree. The antioxidant activities of achenes separated from puree were comparable to activities previously reported for blackberry seeds (*42*). Of the 117 dietary plants investigated in the Norwegian study (*5*), only dog rose had higher total antioxidant activity (FRAP) than strawberry achenes separated from puree. Among peel, pulp, and seed fractions of 28 common fruits, only white pomegranate peel and seeds from red rose grape had higher FRAP values, and only seeds from longan and lychee had comparable antioxidant activities (*7*). Voltammetric response was highest in achenes from freeze-dried Totem followed by achenes from freeze-dried Puget Reliance, whereas the values of achenes isolated from puree were about one-third.

Relationships between the Methods. The correlation between antioxidant activities of all samples ($n = 12$) measured in the FRAP and the ORAC assay was high ($r = 0.98$, $p < 0.001$). The FRAP values were about twice the ORAC values (**Table 2**). The antioxidant activities followed the same trend as the total phenolics, with positive correlations of $r > 0.99$ ($p < 0.001$). Between TMA and total phenolics or between TMA and antioxidant activities there were no significant correlations, mainly because achenes had high phenolic content but low anthocyanin concentrations. High correlations ($0.92 < r < 0.98$) were found between antioxidant activities and electrochemical responses at 300 mV and cumulative responses at 400 mV. However, the highest correlations were with cumulative responses at 800 mV for both the FRAP and the ORAC values, $r = 0.99$ and 0.96, respectively. In a previous study with standards of flavonoids and cinnamic acid derivatives, electrochemical responses at 300 mV and cumulative responses at medium oxidation potential (400 and 500 mV) had the highest correlation with FRAP values, whereas cumulative responses at 800 mV correlated best with ORAC values values (*35*).

Anthocyanin Composition. Anthocyanins were tentatively identified from chromatographic behavior, UV−vis spectra, and response to alkaline hydrolysis (**Table 3**). Ester bonds, but not glycosidic bonds, are susceptible to mild, alkaline hydrolysis (*12*, *30*), and the presence of acylated anthocyanins was confirmed by the disappearance of peaks after alkaline hydrolysis and simultaneous increase of corresponding non-acylated compounds. No UV absorbance maxima in the 310−320 nm range were detected, indicating no acylation of anthocyanins with aromatic acids, rather acylation with aliphatic acids such as acetic, succinic, or malonic acid (*12*). ES-MS and comparison of relative intensity of MS- and DAD signals was used to confirm peak identification. The main ion in the MS spectra was *m*/*z* 433, corresponding to the molecular weight of pelargonidin-3-glucoside. In strawberry flesh the ion with *m*/*z* 579, the molecular weight of pelargonidin-3-rutinoside, was detected. In spectra of achenes, a peak with *m*/*z* 449, matching cyanidin-3-glucoside, had relative intensity comparable to that at *m*/*z* 433. Peaks with *m*/*z* 535 were detected in the spectra of achenes, whereas both *m*/*z* 519 and 535 were detected in flesh and achenes of the cultivar Totem. The molecular weights of 519 and 535 correspond to the masses of pelargonidin-3-glucoside and cyanidin-3-glucoside plus *m*/*z* 86, respectively. Molecular weight of 86 equals malonic acid minus water (*43*). This leads to the conclusion that the acylated anthocyanins were pelargonidin-3-glucoside-malonate (*m*/*z* 519) and cyanidin-3-glucoside-malonate (*m*/*z* 535).

The relative composition of anthocyanins of flesh from Totem and Puget Reliance (**Table 4**) was as previously reported for strawberries (*11*−*14*). Both cultivars contained pelargonidin-3-glucoside as the main constituent, with minor amounts of cyanidin-3-glucoside and pelargonidin-3-rutinoside. Totem con-

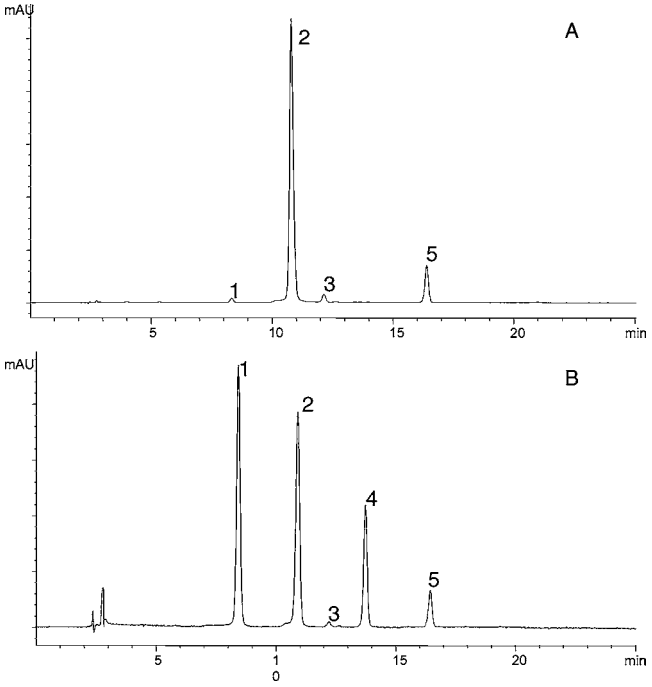

Figure 1. HPLC chromatograms (recorded at 520 nm) of anthocyanins of red ripe strawberries (cv Totem): (A) flesh and (B) achenes separated manually from freeze-dried berries. **1** = cyanidin-3-glucoside; **2** = pelargonidin-3-glucoside; **3** = pelargonidin-3-rutinoside; **4** = cyanidin-3-glucoside-malonate; **5** = pelargonidin-3-glucoside-malonate.

tained about 10% pelargonidin-3-glucoside-malonate, whereas no acylated anthocyanin was detected in flesh from Puget Reliance. Also Määttä-Riihinen et al. (*15*) reported pelargonidin-3-glucoside acylated with malonic acid in strawberries (cv Jonsok). However, cyanidin-3-glucoside and pelargonidin-3-glucoside acylated with succinic acid have been reported for several strawberry genotypes (*13*, *14*), and also pelargonidin-3-glucoside acylated with acetic acid has been identified in strawberries (cv Camarosa) (*44*).

The relative anthocyanin composition of achenes was quite different from that of flesh (**Figure 1** and **Table 4**). In achenes the relative amounts of cyanidin- and pelargonidin-3-glucoside were about equal and accounted for approximately 80% of the anthocyanins (**Table 4**). The third major anthocyanin in achenes was cyanidin-3-glucoside-malonate. As in flesh, achenes from Totem, in contrast to Puget Reliance, contained pelargonidin-3-glucoside-malonate. To our knowledge anthocyanin composition of strawberry achenes has not previously been reported.

The content of anthocyanins (TMA) in strawberry achenes separated manually from freeze-dried berries was more than four times the content in achenes separated from puree (**Table 2**). An explanation of this could be that achenes separated from freeze-dried berries contained remnants of flesh. However, anthocyanin profiles of achenes and flesh were different, and anthocyanin composition of achenes, whether separated from freeze-dried or puree, was similar (**Table 4**). It is thus likely that the anthocyanins were endogenous to the achenes and leached out during sample preparation.

Polyphenolic Composition. Polyphenolics other than anthocyanins were tentatively identified from chromatographic behavior, UV spectra, HDV, and response to alkaline and acidic hydrolysis. Identification of ellagitannins were based on UV spectra showing maxima only below 280 nm (*15*, *42*, *45*) (**Figure 2a**) and disappearance of peaks after alkaline hydrolysis, followed by increase of free ellagic acid. Ellagic acid glycosides, mainly 4-glycosides of ellagic acid (*46*, *47*), were characterized by UV spectra similar to that of ellagic acid (maxima at 253 and 362 nm) (**Figure 2b**) and disappearance of peaks after acidic but not after alkaline hydrolysis. The identification of the polyphenols based on UV spectra was supported by the HDVs. Ellagic acid and its glycosides had dominant potential at 300 mV with no further oxidation at higher potentials, whereas the ellagitannins started to oxidize at 100 mV and had dominant potential at the end of the coulometric array (at 800 mV) (**Figure 3**). Catechin and proanthocyanidins were classified on the basis of the characteristic UV spectra of flavan-3-ols and similar HDVs as previously reported for catechin (*35*). Identification of catechin and ellagic acid were confirmed by coelution with authentic standards in two different HPLC separation systems. Appearance of *p*-coumaric acid after alkaline hydrolysis, as previously detected cinnamic acid derivatives disappeared, indicated the presence of esters of *p*-coumaric acid, which have previously been reported as the main cinnamic acid derivatives in strawberries (*14*, *15*, *18*, *48*, *49*). Two late eluting compounds were recognized as flavonol glycosides from their spectra, HDVs, and responses to alkaline hydrolysis.

The content of free ellagic acid in strawberry flesh (**Table 5**) was lower than the values previously reported for whole strawberries (*14*, *15*, *21*); however, in those investigations the

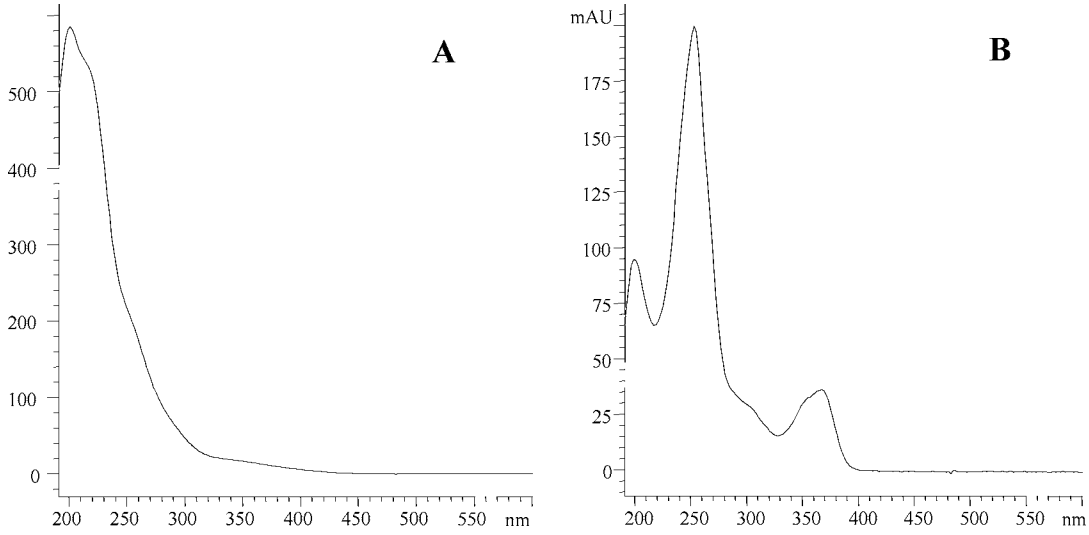

Figure 2. On-line UV spectra of characteristic compounds in strawberry achenes: (A) an ellagitannin and (B) ellagic acid.

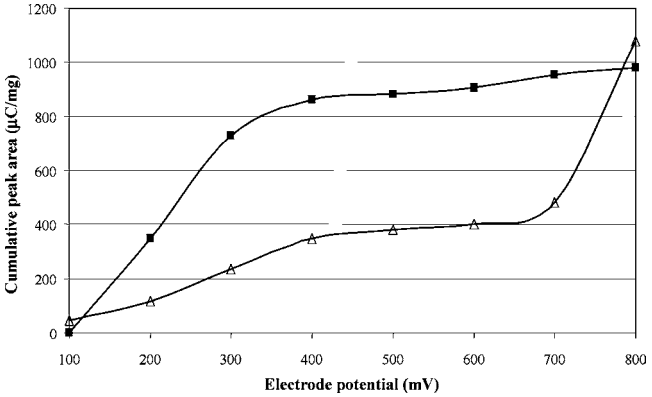

Figure 3. Hydrodynamic voltammograms (HDV) of an ellagitannin (Δ) and ellagic acid (■).

achenes were included. Levels of ellagic acid glycosides detected were of the same order of magnitude as the free ellagic acid. Ellagitannins were the main ellagic acid form and occurred at the same order of magnitude as reported earlier (*15*, *38*). Content of catechin and proanthocyanidins in strawberry flesh were relatively high and averaged 8 and 16 mg/100 g fw, respectively. Previously about 18 mg/100 g (*37*) and 9 mg/100 g (*38*) of total flavan-3-ols have been detected in strawberries. Two cinnamic acid derivatives were detected in strawberry flesh. The most abundant, probably a *p*-coumaroyl ester, contributed about 90% of the total content. Flavonol contents, averaging 4.9 mg/100 g fw, were of the same order of magnitude as previously reported (*16*, *37*). Wang et al. (*14*, *48*) reported lower quantities.

The composition of phenolic compounds in strawberry achenes was very different from that of flesh. HPLC chromatograms of flesh and achenes of Totem recorded at 260 nm are given in **Figure 4**. The majority of the phenolic compounds in strawberry achenes showed a UV max at 260 nm, rather than 280, 320, and 360 nm, and thus consisted of ellagic acid and its derivatives (**Figure 4b** and **Table 5**). Achenes of Totem contained the highest concentration of phenolic compounds, the concentration of ellagitannins in freeze-dried achenes being especially high (833 mg/100 g fw). The amount detected in achenes of Puget Reliance was nearly half. Also the content of flavonols in achenes was relatively high. Strawberry achenes contained a low amount of proanthocyanidins, particularly as compared to grape seed, but the content was also lower than in blackberry seeds (*42*).

Comparison of Phenolic Content and Antioxidant Activities in Different Parts of Strawberries. The achenes isolated from puree contained substantially lower levels of phenolic compounds than achenes separated manually from freeze-dried berries. The reduced amount of phenolic compounds in achenes separated from strawberry puree was most likely due to leaching of water-soluble compounds during water rinsing of seeds on the strainer. The level of the highly polar water-soluble ellagitannins was dramatically reduced in achenes separated from puree (**Figure 4** and **Table 5**). In a separate experiment, it was shown that achenes separated from puree followed by freeze-drying had the same phenolic content as the same achenes not subjected to freeze-drying (results not shown). Thus the higher amount of phenolic compounds in freeze-dried achenes was not due to easier extraction of these compounds from freeze-dried material.

Because of the low water content of achenes (**Table 1**) the amount of phenolic compounds per fresh weight in achenes was high (**Table 2** and **Table 5**). However, if expressed on a dry matter basis, the concentrations of phenolic compounds and antioxidant activities of achenes and flesh were in many instances comparable. The composition of phenolic compounds in achenes isolated manually from freeze-dried berrries, in contrast to achenes separated from puree, indicates the "true" content of the achenes and compares as follows to the phenolic content of flesh. Whereas strawberries consisted of about 1% achenes on a fresh fruit basis, the achenes contributed about 11% of total phenolics and 14% of antioxidant activities in strawberries. The main contribution was from ellagic acid and its derivatives (**Table 5**). In accordance with earlier findings (*23*, *26*), the level of these compounds was substantially higher in achenes than in flesh. Ellagitannins, and especially sanguiin H-6, have previously been shown to be major contributors to the antioxidant capacity of raspberries (*50*). Sanguiin H-6 has been detected in strawberry leaves (*25*) and is most likely also present in strawberry fruits (*15*). A high free radical scavenging activity has been reported for ellagic acid (similar to that of flavan-3-ols and gallic acid), whereas the activities of the 4-glycosides of ellagic acid detected in raspberries were lower (*46*). Content of catechin and flavonols in achenes, except the flavonol content in achenes from freeze-dried berries, were about 4-fold higher compared to flesh. The proanthocyanidins and the cinnamic acid derivatives were in the same order of magnitude in flesh and achenes on a fresh weight basis.

Table 5. Concentration of Phenolic Compounds (mg/100 g fw) in Flesh and Achenes of Red Ripe Strawberries

part of strawberries	cultivar/abbr[a]	ellagic acid (as ellagic acid)	ellagic acid glycosides (as ellagic acid)	ellagitannins (as gallic acid)	catechin (as catechin)	procyanidins (as catechin)	cinnamic acid deriv (as chlorogenic acid)	flavonols (as rutin)
flesh								
freeze-dried[b]	Totem	0.3 ± 0.0	0.2 ± 0.0	9.6 ± 0.6	8.0 ± 0.1	16.5 ± 0.3	7.8 ± 0.4	6.5 ± 0.3
	Puget	0.2 ± 0.0	0.5 ± 0.0	7.6 ± 0.2	8.1 ± 0.0	13.7 ± 0.1	7.9 ± 0.0	4.9 ± 0.1
puree[c]	Totem	0.3 ± 0.1	0.2 ± 0.0	11.3 ± 0.3	6.2 ± 0.2	17.9 ± 0.8	8.1 ± 0.1	4.0 ± 0.0
	Puget	0.2 ± 0.0	0.6 ± 0.1	8.7 ± 0.0	9.0 ± 0.1	15.5 ± 1.2	10.2 ± 0.2	4.2 ± 0.0
achenes								
from freeze-dried[b]	Totem	87.3 ± 14.6	52.3 ± 12.4	833.0 ± 162.9	26.7 ± 7.2	32.0 ± 5.6	19.3 ± 3.6	93.7 ± 6.5
	Puget	34.4 ± 3.7	57.0 ± 4.8	440.6 ± 47.7	59.8 ± 3.2	20.5 ± 1.2	13.4 ± 2.6	49.5 ± 1.5
from puree[c]	Totem	37.0 ± 2.4	17.5 ± 0.8	35.8 ± 6.0	22.0 ± 0.6	20.0 ± 4.2	12.3 ± 2.9	26.7 ± 1.5
	Puget	13.5 ±1.8	13.2 ± 2.0	8.8 ± 0.4	35.0 ± 2.9	10.5 ± 0.4	10.0 ± 1.1	15.7 ± 0.8
industrial waste	MA	3.4 ± 0.4	2.8 ± 1.0	83.2 ± 10.8	13.3 ± 1.5	38.7 ± 5.4	22.5 ± 2.1	9.5 ± 0.9
product[d]	WA	1.9 ± 0.2	1.0 ± 0.1	5.2 ± 0.1	4.2 ± 0.0	6.4 ± 0.3	8.5 ± 1.0	6.0 ± 0.2
	MW	0.2 ± 0.0	0.5 ± 0.0	8.2 ± 0.1	5.4 ± 1.0	11.8 ± 2.3	20.9 ± 0.3	0.4 ± 0.3
	WW	0.6 ± 0.0	0.1 ± 0.0	2.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.5 ± 0.1	0.4 ± 0.0

[a] MA = milled, acetone extracted, WA = whole, acetone extracted, MW = milled, water extracted, WW = whole, water extracted. [b] Achenes were removed from freeze-dried berries using a pincer. [c] Achenes were separated from puree in a strainer. [d] The waste after the production of seedless puree.

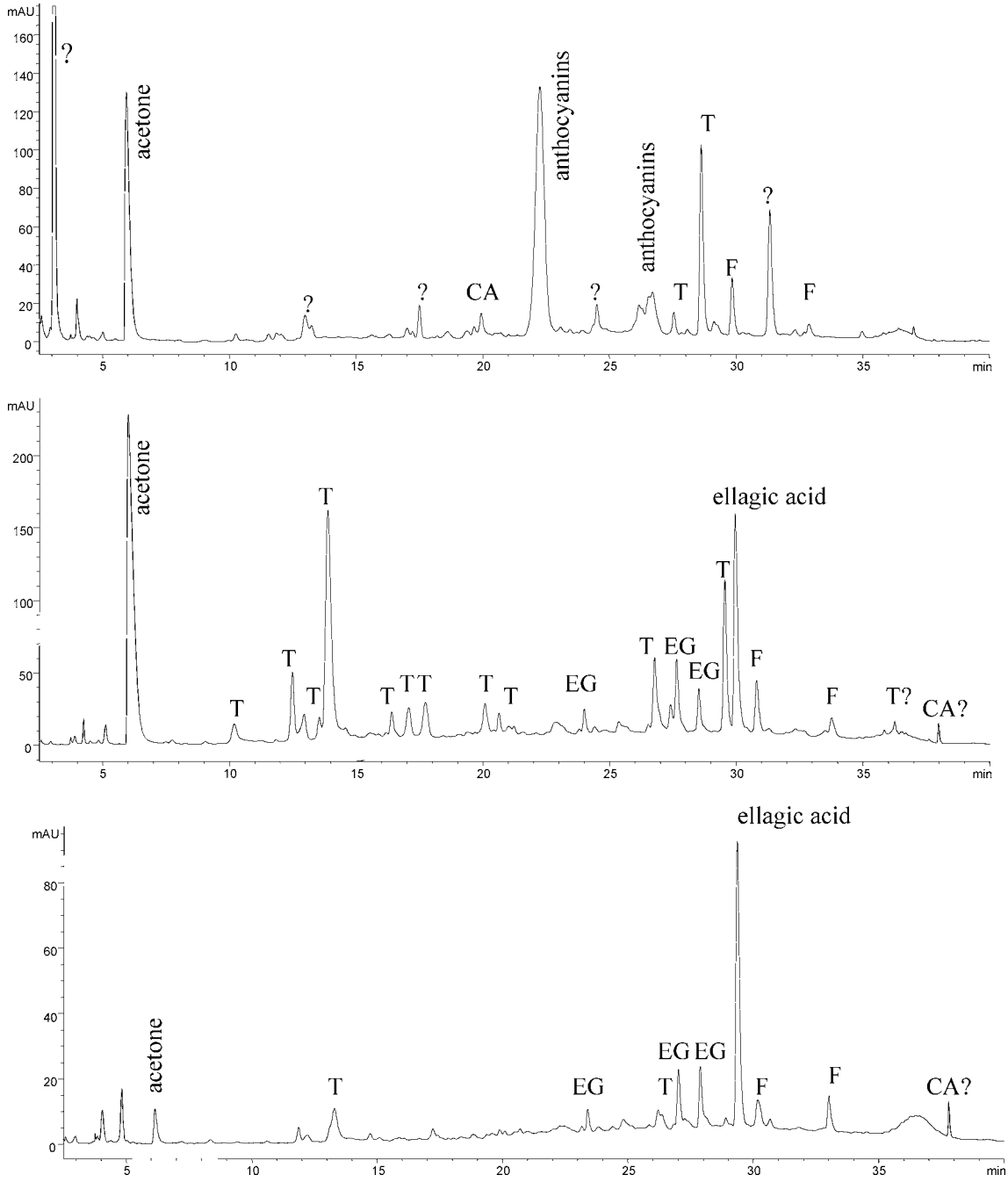

Figure 4. HPLC chromatograms (recorded at 260 nm) of phenolic compounds of red ripe strawberries (cv Totem): (A) flesh, (B) achenes separated from freeze-dried berries, and (C) achenes separated from puree. **T** = ellagitannin; **EG** = ellagic acid glycoside; **F** = flavonol; **CA** = cinnamic acid derivative; **?** = not identified.

Industrial Waste Product Achenes. Waste product achenes contained about 90% pelargonidin-3-glucoside and 10% pelargonidin-3-glucoside-malonate (**Table 4**). Cyanidin-3-glucoside and cyanidin-3-glucoside-malonate were not detected in the industrial waste product achenes.

Industrial waste product achenes extracted with 70% acetone after liquid-nitrogen-milling (MA) contained higher amounts of phenolic compounds and had higher antioxidant activities than strawberry flesh (**Table 2**). However, the values were about half compared to achenes from Totem and Puget Reliance isolated from puree. Industrial waste product achenes also contained lower amounts of ellagic acid and ellagic acid glycosides (**Table 5**). While achenes of Totem and Puget Reliance had quite similar phenolic profiles, the waste product achenes were quite different. The major compound was an ellagitannin eluting just prior to ellagic acid.

As expected, the most efficient extraction method of phenolic compounds from strawberry achenes was the one normally utilized in the laboratory, liquid nitrogen-milled samples extracted with 70% acetone (MA). Total phenolics of achenes extracted with water (MW) were 21% of the value obtained for achenes extracted with acetone, while ORAC and FRAP values were 32% and 14% of those obtained with acetone extraction (**Table 2**). Extraction of whole achenes instead of milled reduced total phenolics to 31% and 18% for acetone- and water-extracted achenes, respectively, and antioxidant activities were reduced to averages of 11% and 27%, respectively.

Processing of strawberry juice concentrate and seedless puree generates about 10% and 4% waste, respectively (personal communication, Sam Grubb, Kerr Concentrates, OR). Thus the achenes from these waste streams could be a source for phenolics to be used as nutraceuticals or natural antioxidants. While the phenolic content and antioxidant activities of strawberry achenes from the industrial source were substantially lower than that of achenes isolated from freeze-dried strawberries, the levels were still high.

ABBREVATIONS USED

AAPH, 2,2′-azobis(2-amidinopropane)dihydrochloride; β-PE, β-phycoerythrin; DAD, diode array detector; DP, dominant potential; ES-MS, electrospray mass spectroscopy; FRAP, ferric reducing activity power; fw, fresh weight; GAE, gallic acid equivalents; HDV, hydrodynamic voltammogram; HHDP, hexahydroxydiphenic acid(s); HPLC, high-performance liquid chromatography; MA, milled acetone extracted; MW, milled water extracted; MS, mass spectroscopy; ORAC, oxygen radical absorbance capacity; Trolox, 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid; TE, Trolox equivalents; TMA, total monomeric anthocyanins; WA, whole acetone extracted; WW, whole water extracted.

ACKNOWLEDGMENT

Robert W. Durst, Department of Food Science and Technology, OSU, is acknowledged for valuable discussions regarding HPLC and ES-MS analysis. We thank Sam Grubb, Kerr Concentrates Inc. (Salem, OR) for providing waste product achenes after production of seedless strawberry puree.

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Received for review November 30, 2004. Revised manuscript received March 14, 2005. Accepted March 22, 2005.

JF048001O