Antioxidants in Raspberry: On-Line Analysis Links Antioxidant Activity to a Diversity of Individual Metabolites

JULES BEEKWILDER,*,† HARRY JONKER,† PATRICK MEESTERS,‡ ROBERT D. HALL,†
INGRID M. VAN DER MEER,† AND C. H. RIC DE VOS†

¹Plant Research International, Postbus 16, 6700 AA Wageningen, The Netherlands, and
²PCF-Proeftuin Aardbeien en Houtig Kleinfruit, Sint-Truidersteenweg 321, 3700 Tongeren, Belgium

The presence of antioxidant compounds can be considered as a quality parameter for edible fruit. In this paper, we studied the antioxidant compounds in raspberry (*Rubus idaeus*) fruits by high-performance liquid chromatography (HPLC) coupled to an on-line postcolumn antioxidant detection system. Both developmental and genetic factors were assessed by comparing fruits from a single cultivar of different ripening stages and by comparing ripe fruits of 14 raspberry cultivars, respectively. The HPLC-separated antioxidant compounds were identified using HPLC-photodiode array coupled to mass spectrometry (quadrupole time-of-flight tandem mass spectrometry), using a reference lock mass for determining accurate masses. The dominant antioxidants could be classified as anthocyanins, ellagitannins, and proanthocyanidin-like tannins. During fruit ripening, some anthocyanins were newly produced, while others, like cyanidin-3-glucoside, were already present early in fruit development. The level of tannins, both ellagitannins and proanthocyanidin-like tannins, was reduced strongly during fruit ripening. Among the 14 cultivars, major differences (>20-fold) were observed in the levels of pelagonidin type anthocyanins and some proanthocyanidin type tannins. The content of ellagitannins varied approximately 3-fold. The findings presented here suggest that the content of individual health-promoting compounds varies significantly in raspberry, due to both developmental and genetic factors. This information will assist in the future development and identification of raspberry lines with enhanced health-promoting properties.

KEYWORDS: Raspberry; fruit; antioxidant; mass spectrometry; HPLC

INTRODUCTION

Fruits are well-known sources of health-promoting compounds. Such compounds comprise vitamins, minerals, and a range of different polyphenolic antioxidants, such as flavonoids and tannins. The intake of antioxidant flavonoids, and more precisely specific classes of flavonols such as quercetin, has been shown by epidemiological studies to reduce the risk of coronary heart disease (*1*). Presently, however, the mechanism by which these compounds exert their beneficial effect is not fully understood. Understanding the cellular effects of flavonoid metabolites is important for predicting which dietary flavonoids might be most beneficial in vivo (*2*). Flavonoid compounds are efficient oxygen free radical scavengers in vitro, and in relation to this, they have been demonstrated to inhibit a range of damaging effects induced by oxidative agents, such as lipid peroxidation, protein oxidation, topoisomerase activity, tumor growth, mutagenesis by carcinogenic chemicals, and other oxidation reactions that have long-term damaging effects on tissues (*3*). However, most of these experiments have been performed on in vitro systems or isolated tissues, and it is

unclear if dietary antioxidants have the same effect when taken up through the digestive system. Also, there is evidence that antioxidant activity per se does not sufficiently explain some of the observed health effects. For instance, one class of polyphenolic antioxidants, the ellagitannins, has been shown to have vasorelaxing effects, which may contribute to protection from coronary heart disease, while a different class of polyphenolic antioxidants, the anthocyanins, did not have such an effect in the same experiment (*4*). Vasorelaxation may therefore not be directly related to antioxidant activity, which indicates that our understanding of the mode of action of dietary antioxidant compounds is still very limited.

Raspberries (*Rubus idaeus*) are among the fruits containing the highest antioxidant levels. In addition to vitamin C, the antioxidant activity of raspberries is primarily constituted by two classes of compounds: anthocyanins and ellagitannins. Anthocyanins, which are red pigment polyphenols, are mainly found in berry fruits and grapes. They have been implicated in protection against coronary heart disease and certain types of cancer (*3*). Ellagitannins, which are complex derivatives of ellagic acid (*5*), have been identified in tea, many medicinal plants, and several fruits, including raspberries (*6*, *7*). In addition to their vasorelaxation properties (*4*), ellagitannins have been

* To whom correspondence should be addressed. Tel: +31-317-477164. Fax: +31-317-418094. E-mail: Jules.Beekwilder@wur.nl.
† Plant Research International.
‡ PCF-Proeftuin Aardbein en Houtig Kleinfruit.

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described to have general antioxidant effects (*8*). Raspberry could therefore be considered as a model fruit source for a variety of potentially healthy compounds.

In view of the potential health-related activities of polyphenolic antioxidants such as anthocyanins and tannins, these can be regarded as markers for fruit quality. We have used a high-performance liquid chromatography (HPLC)-based system to identify and monitor, on-line, the presence and antioxidant activity of individual compounds in plants (*9*). Such a monitoring system can be applied to assess the quality of fruits, to select for varieties that are rich in particular antioxidant compounds, and to monitor the uptake and efficacy of antioxidants, thereby contributing to the understanding of their health effects. Here, results are reported from the application of an on-line monitoring system, in combination with absorbance spectrometry and quadrupole time-of-flight high-resolution mass spectrometry (QTOF-MS), to identify the compounds concerned. To establish the use of antioxidants as markers for fruit quality, the fate of such compounds was assessed, both during raspberry fruit ripening and in a range of cultivars with diverse genetic backgrounds.

MATERIALS AND METHODS

Raspberry Material. Tulameen raspberry fruits at five different ripening stages were harvested at the PPO field station in Randwijk, The Netherlands, on June 27, 2001. Within 1 h after harvest, the berries were snap-frozen in liquid nitrogen and stored at −80 °C for further analysis. In addition, ripe fruits of seven cultivars of summer raspberries (Tulameen, Glen Ample, Encore, MA69-2, MEHQ69, Nova, and Prelude) and seven cultivars of autumn raspberries (Autumn Bliss, Autumn Britten, Carmen, Himbo Top, Polka, P93453, and P93563) were harvested in the same way on June 17, 2002, at the PCF-Proeftuin Aardbeien en Houtig Kleinfruit (Tongeren, Belgium).

Frozen raspberries were ground to a fine powder in liquid nitrogen using a precooled coffee grinder. Compounds were extracted by sonicating exactly 0.5 g of frozen powder for 15 min in 2 mL of extraction solution, consisting of water with 62.5% methanol and 0.125% formic acid (FA). The mixture was centrifuged for 10 min at 2500 rpm, and the supernatant was filtered through a 0.45 μm Anotop 10 filter (Whatman) into 2 mL glass vials before injection into the HPLC.

HPLC Antioxidant Detection System. The HPLC antioxidant detection system was adapted from that described in ref *9*. The HPLC system comprised a Waters 600 controller, a Waters 996 photodiode array (PDA) detector, and a column incubator at 40 °C. The HPLC column used was a Synergi 4u max-RP80A 250 mm × 4.6 mm (Phenomenex, CA). The polar eluent was 0.5% FA in MQ water. Separation of compounds in the extracts was conducted in a 60 min run during which a linear acetonitrile gradient was applied from 5 to 30%. Compounds eluting from the column passed first through a PDA detector (set at an absorbance range of 240−600 nm) and were then allowed to react for 30 s with a buffered solution of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS; Roche) cation radicals on-line, before passing through a second detector, which monitors ABTS reduction. The ABTS radical solution was prepared by dissolving 55 g of ABTS in 50 mL of water, followed by the addition of potassium permanganate. After 16 h of incubation in the dark, the radical solution was diluted further in three volumes of 0.2 M Na-phosphate buffer, pH 8.0. The postcolumn reaction loop was a 3 m stainless steel tube (internal diameter 0.508 mm) at 40 °C. The decreased absorption by ABTS by reaction with antioxidants was monitored as it passed through a dual-wavelength UV−vis detector (Waters) at 412 and 650 nm. The absorption at 412 nm is the maximum absorption of the ABTS cation, while the 650 nm absorption was used when interference of compounds at 412 nm was observed. Modifications relative to ref *9* will be described elsewhere and comprise a higher ABTS concentration, temperature control of the postcolumn reaction, and pH stabilization.

HPLC/QTOF-MS. To identify the polyphenolic antioxidants in the extracts, samples were subjected to accurate mass LC-MS and MS/MS on a high-resolution time-of-flight mass spectrometer with lock mass correction (*10*), in combination with spectral analysis using a PDA detector on-line. Samples were injected and separated on a Synergi 250 mm × 4.6 mm column described above, using a Waters Alliance 2795 HT HPLC system providing a linear gradient from 5 to 30% acetonitrile (acidified with 0.5% FA) at a flow rate of 1 mL/min. Eluting compounds were first detected on-line at 240−600 nm using a Waters 2996 PDA, before entering a QTOF Ultima API mass spectrometer equipped with an electrospray ionization (ESI) source and a separate LockSpray. The eluent flow was split after PDA detection to obtain a flow of 0.2 mL/min into the MS. The following settings were applied during LC-MS runs: desolvation temperature of 300 °C with a nitrogen gas flow of 500 L/h, capillary spray at 3 (ESI$^+$) or 2.5 (ESI$^-$) kV, source temperature of 120 °C, and cone at 35 eV with 50 L/h nitrogen gas flow. Ions in the *m/z* range of 100−3000 were detected using a scan time of 0.9 s and an interscan delay of 0.1 s. Survey scans were performed at a collision energy of 5 (ESI$^+$) or 10 (ESI$^-$) eV. Tandem MS was performed on-line using three different collision energies (5, 10, and 50 eV) on up to eight masses detected per survey scan. The MS was calibrated with phosphoric acid in 50% acetonitrile. Leucine enkaphalin (Sigma), dissolved in 50% acetonitrile with 0.1% FA (ESI$^+$) or 20 μM ammoniumacetate (ESI$^-$), was used as a lock mass and was measured every 10 s. Masslynx software version 4.0 was used to control all instruments and to calculate accurate masses and corresponding elemental compositions of compounds.

RESULTS

On-Line Antioxidant Analysis. Conventional methods for identifying antioxidant compounds in complex mixtures imply time-consuming assay-guided fractionation procedures, followed by the identification of the purified compounds. The system described here is meant to screen for compounds with antioxidant activity in extracts more rapidly and directly. It is based on chromatographic separation of compounds in an extract. After HPLC and PDA detection, the eluent flow is mixed with a buffered free radical solution (ABTS cations) in a postcolumn reactor. The amount of ABTS quenched by antioxidants is subsequently measured by a second detector as a decrease in absorption at 412 nm. In this way, all separated compounds are individually monitored for both their PDA spectrum and their antioxidant activity simultaneously.

A similar system has been described previously (*9*). However, the system described in this paper is more dedicated to analyze fruit antioxidants and especially for the separation of polyphenolic components such as anthocyanins and tannins. Moreover, it is suitable for reproducible and automated screening of large sample numbers. The use of a column with a small pore size in combination with FA as acidifier resulted in good chromatographic resolution and thereby in resolution of antioxidant peaks, notwithstanding the unavoidable peak broadening in the postcolumn reactor. The pH settings of the system were a compromise between the demands of the column for optimal separation and the demands of the ABTS reaction for optimal reactivity. For reasons of chromatographic separation, 0.5% FA was used, as lower concentrations of FA did not produce sharp chromatographic peaks. At this FA concentration, the phosphate buffer in the antioxidant detector compensated the pH to 3.5, which is a suboptimal pH for the antioxidant activity of anthocyanins but still allows accurate measurements. Lowering the concentration of FA in the eluens (0.1%) provided a more physiological pH in the phosphate-buffered reaction mixture but resulted in poor chromatographic separation of the anthocyanins.

An example of such an antioxidant analysis of a raspberry extract is shown in **Figure 1**. **Figure 1A** shows the PDA
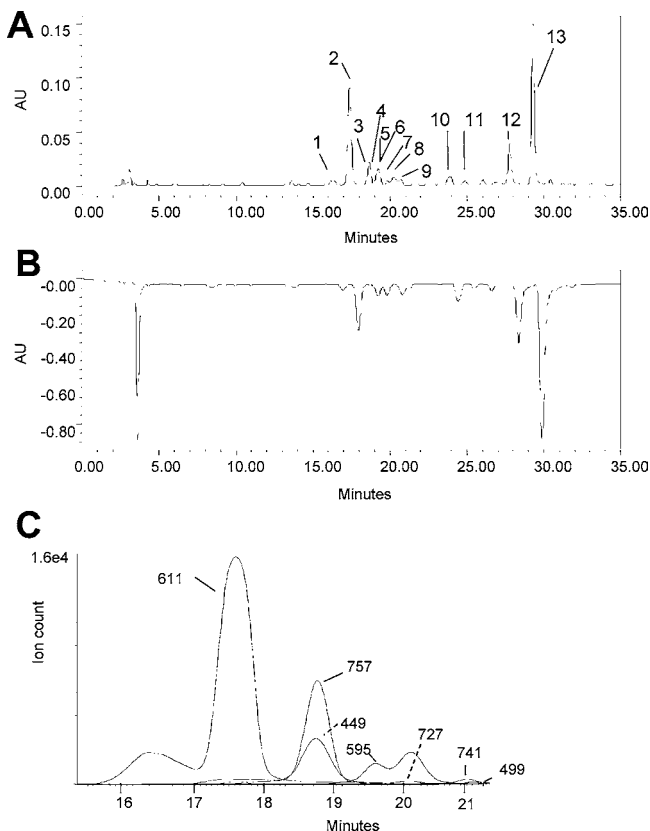
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Figure 1. HPLC analysis of antioxidant compounds of ripe Tulameen raspberries. (**A**) HPLC chromatogram recorded by PDA at 280 nm from a methanol extract of ripe Tulameen raspberry. Labels 1–13 on the peak indicate the assignments of the peaks as described in the text. (**B**) HPLC chromatogram recorded simultaneously at 412 nm after reaction with ABTS. Peaks are shifted 0.5 min relative to the upper panel. (**C**) HPLC chromatogram recorded at *m/z* 611, 757, 449, 595, 727, 741, and 433 by QTOF-MS. The time axis is not completely linear between panels **C** and **A**, due to different void volumes in the HPLC-QTOF and HPLC-PDA systems.

chromatogram as measured at 280 nm; **Figure 1B** shows the corresponding ABTS radical quenching activities of the same compounds. By integrating the areas of all of the (negative) peaks in the antioxidant detector chromatogram, the antioxidant activity of each separated compound can be calculated as a percentage of the total antioxidant activity of the extract. The delay in retention time observed between the corresponding PDA and ABTS peaks (30 s) is fixed and represents the eluent travel time through the postcolumn reaction tube between the two detectors and is equivalent to the antioxidant reaction time.

Contribution of Various Compounds to Antioxidant Activity of Raspberry Extracts. On the basis of the size of each antioxidant peak in the ABTS chromatograms (**Figure 1B**), the contribution of each antioxidant compound to the total antioxidant activity within an extract of ripe fruits of the raspberry variety Tulameen was calculated. Essentially, three main regions containing most of the antioxidant activity were observed. The first region consisted of a sharp peak early in the chromatogram, comprising compounds eluting in the flow-through of the column. This region represented roughly 20% of the total antioxidant activity in the extract. As was deduced from injecting pure compounds (data not shown), this region contained vitamin C (t_R = 2 min) and glutathione (t_R = 3 min) and probably also other highly polar but less abundant antioxidants such as cysteine. The second region comprised nine

antioxidant peaks eluting between 16 and 21 min after sample injection (peaks 1−9). The spectral analyses showed that these peaks all have an absorbance maximum at around 520 nm, which is indicative of anthocyanins (see also below). These nine anthocyanins together contributed about 25% to the total antioxidant activity (**Table 1**). The third region (retention times from 27 to 31 min) contained two major antioxidant peaks (numbered peaks 12 and 13), which were identified as ellagitannins (see below). The contribution of these two antioxidants to the total antioxidant activity in the extracts was about 12 (peak 12) and 40% (peak 13) (**Table 1**). Additionally, relatively minor antioxidant peaks were observed for proanthocyanidins and some other phenolic compounds. Together, these contributed around 5% to the total antioxidant activity in ripe Tulameen raspberries (**Table 1**).

Identification of Antioxidants by PDA and QTOF-MS/ MS. Compounds in the 13 peaks separated by HPLC were identified by running the same samples in the same column and the same gradient, while the column was connected to a QTOF high-resolution mass spectrometer, in sequence with a PDA detector. A standard reference compound (lock mass) injected on-line through a separate ionization source was used to correct for small fluctuations in mass accuracy during HPLC runs. After this lock mass correction, the accurate masses of eluting compounds could be calculated and the correspondingly most likely elemental composition deduced. With the instrumental calibration used and with (nonsaturating) mass peak intensities between 50 and 200 ions per scan in continuum mode, the lock mass-corrected accurate masses observed for standard compounds tested (i.e., chlorogenic acid, rutin, kaempferol-rutinoside, ellagic acid, and naringenin) were always within 5 ppm from the calculated masses. This minor deviation from the expected masses is sufficiently small to allow elemental composition calculation and, especially in combination with PDA spectra and MS/MS analyses, the identification of the antioxidant compound. Mass peak intensities lower than 50 or higher than 200 counts per scan usually gave rise to larger deviations from the calculated exact masses.

Both positive ions (HPLC peaks 1−11) and negative ions (HPLC peaks 12 and 13) and fragments obtained thereof were detected by analyzing samples in ESI-positive and -negative modes, respectively. In **Figure 1C**, superimposed specific mass traces of the MS peaks belonging to anthocyanins in part of the chromatogram are shown. Fragments of parent ions were generated in MS/MS mode at three different collision energies. **Table 1** lists the absorbance maxima and accurate mass measured for each peak, the calculated mass of expected compounds and the MS/MS fragments obtained from the parent mass, and their contribution to the total antioxidant activity.

All peaks, except for peaks 10 and 11, clearly match with previously published results (*4*, *11*), with respect to order of elution, absorption spectrum, mass, and MS/MS fragments. The absorbance maxima are mainly important for the identification of cyanidin (514 nm) and pelagonidin (500 nm) anthocyanins. The observed mass is mostly within 5 ppm from the calculated mass; where this is not the case (for instance, in the case of peak 1), this most likely results from the low intensity of these peaks. Peaks 3 and 4 partially overlap, as do peaks 6 and 7.

Peak 10 (t_R = 23.8 min), with λ_{max} = 240 nm, is most abundant in green raspberries. However, in the ripe Tulameen raspberry, it still corresponded to 3% of the total antioxidant activity. MS/MS analyses in ESI-positive mode revealed an accurate mass of 579.1572 $(M + H)^+$, which deviates 1.1 ppm from a $C_{30}H_{27}O_{12}$ compound (**Figure 2**). The 291.0884 fragment

Table 1. Properties of Antioxidant Compounds from Ripe Raspberries from Three Cultivars after HPLC, Detected by Absorbance and MS/MS; Peak Numbers Refer to **Figure 1**

peak no.	$R_T{}^a$	$\lambda_{max}{}^b$	ion mass^c	formula	calcd mass^d	Δppm^e	postulated structure^f	% of total antioxidant activity
1	16.74	514	611.1650	$C_{27}H_{31}O_{16}$	611.1612	6.3	cyanidin-3,5-diglucoside	
			287.0597	*$C_{15}H_{11}O_6$*	287.0556	1.4		1
2	17.42	514	611.1601	$C_{27}H_{31}O_{16}$	611.1612	−1.8	cyanidin-3-sophoroside	
			287.0572	*$C_{15}H_{11}O_6$*	287.0556	5.6		10
			449.1115	*$C_{21}H_{21}O_{11}$*	449.1084	6.9		
3	18.67	514	757.2147	$C_{33}H_{41}O_{20}$	757.2191	−5.8	cyanidin-3-(2^G-glucosylrutinoside)	
			287.0583	*$C_{15}H_{11}O_6$*	287.0556	9.4		2^g
			611.1608	*$C_{27}H_{31}O_{16}$*	611.1612	0.7		
4	18.73	510	449.1075	$C_{21}H_{21}O_{11}$	449.1084	−1.7	cyanidin-3-glucoside	
			287.0587	*$C_{15}H_{11}O_6$*	287.0556	9.5		g
5	19.01	499	595.1662	$C_{27}H_{31}O_{15}$	595.1663	−0.2	pelagonidin-3-sophoroside	
			271.0604	*$C_{15}H_{11}O_5$*	271.0606	−0.9		g
6	19.09	510	727.2144	$C_{32}H_{39}O_{19}$	727.2179	7.7	cyanidin-3-xylosylrutinoside	4^g
7	19.23	510	595.1655	$C_{27}H_{31}O_{15}$	595.1663	0.3	cyanidin-3-rutinoside	g
			287.0580	*$C_{15}H_{11}O_6$*	287.0556	8.5		
			449.1115	*$C_{21}H_{21}O_{11}$*	449.1084	6.9		
8	20.14	504	741.2279	$C_{33}H_{41}O_{19}$	741.2242	4.8	pelagonidin3-(2^G-glucosylrutinoside)	ND^h
			271.0615	*$C_{15}H_{11}O_5$*	271.0606	3.3		
9	20.70	495	433.1173	$C_{21}H_{21}O_{10}$	433.1135	−1.8	pelagonidin-3-glucoside	ND^h
			271.0598	*$C_{15}H_{11}O_5$*	271.0606	3.1		
10	23.89	250	579.1572	$C_{30}H_{27}O_{12}$	579.1503	1.1	pentahydroxyflavan dimer (proanthocyanidin)	3
			271.0583	*$C_{15}H_{11}O_5$*	271.0606	−8.7		
			289.0717	*$C_{15}H_{13}O_6$*	289.0712	1.7		
			291.0884	*$C_{15}H_{15}O_6$*	291.0869	5.3		
			427.1056	*$C_{22}H_{19}O_9$*	427.1029	6.3		
11	24.87	250	563.1552	$C_{30}H_{27}O_{11}$	563.1553	−0.1	tetrahydroxyflavan-pentahydroxy-flavan dimer (proanthocyanidin)	1
			273.0776	*$C_{15}H_{13}O_5$*	273.0763	4.8		
			291.0888	*$C_{15}H_{15}O_6$*	291.0869	6.7		
			411.1103	*$C_{22}H_{19}O_8$*	411.1080	5.6		
			427.1059	*$C_{22}H_{19}O_9$*	427.1029	7.0		
12	27.76	250	1401.1128	$C_{123}H_{78}O_{78}$	1401.1068	4.3	lambertianin C	14
			933.7486	$C_{123}H_{78}O_{78}$	933.7353	14.2		
			300.9980	*$C_{14}H_5O_8$*	300.9984	−1.5		
13	29.29	250	1869.1517	$C_{82}H_{53}O_{52}$	1869.1503	1.4	sanguiin H6	40
			934.0730	$C_{82}H_{53}O_{52}$	934.0712	0.8		

^{a} Retention time on HPLC in minutes. ^{b} Wavelength at which maximum absorption occurs. ^{c} Measured mass of the ions in the MS/MS spectrum. The parent mass is in normal font, while the MS/MS fragments are in italics. ^{d} Mass calculated based on the elemental composition of a known raspberry compound. ^{e} Distance between measured mass and calculated mass in ppm of the measured mass. ^{f} Postulated structure, based on information in ref *4*. ^{g} Peaks 3–5 and peaks 6 and 7 overlap in the antioxidant chromatogram. ^{h} The antioxidant activity of peaks 8 and 9 is below the limit of detection.
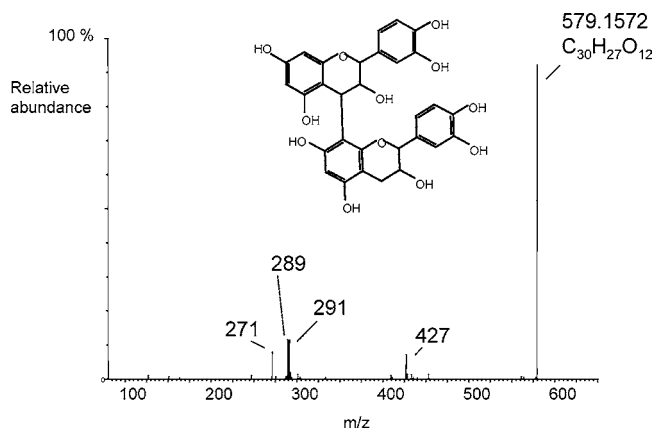
Figure 2. Product ion spectrum of peak 10 (*m/z* 579) on QTOF-MS/MS. The proposed proanthocyanidin structure is shown in the inset.

indicates the presence of a catechin group ($C_{15}H_{15}O_6$, +5.3 ppm). Another fragment, with *m/z* 427.1072, corresponds to one catechin unit plus 136 Da, which probably represents the A ring part of the second catechin unit. This MS/MS spectrum, recorded in the positive mode, matches the negative mode spectrum of a procyanidin catechin−catechin dimer of the B type (*12*). These authors, however, do not report on the presence of the other MS/MS fragment (*m/z* 271.0583; i.e., $C_{15}H_{11}O_5$) that we clearly

observe in our ESI-positive experiments. This fragment became more intense upon increasing the collision energy and may originate from *m/z* 289.0717 ($C_{15}H_{13}O_6$) upon loss of water. The parent mass 579 is apparently composed of two catechin units, probably interconnected by a (4 → 8) or (4 → 6) CC bond.

Peak 11 (t_R = 24.9 min, λ_{max} = 240 nm) resulted in 1% of antioxidant activity in red raspberries and was mainly detected in green, unripe raspberry fruits (see below). It had an accurate mass of 563.1544, corresponding to $C_{30}H_{27}O_{11}$ (−1.6 ppm; [M + H]$^+$). Fragments of *m/z* 291.0888 and 273.0776 were detected, matching catechin ($C_{15}H_{15}O_6$) and an afzelechin fragment ($C_{15}H_{13}O_5$). The 291 fragment, and also a fragment of *m/z* 427.1059, were also observed in peak 10. The catechin and afzelechin fragments suggest that peak 11 corresponds to a propelagonidin-like compound. The 427 fragment can be explained as a catechin group with the A ring part of the afzelechin group. Likewise, the observed fragment of *m/z* 411.1103 can be explained as the afzelechin group with part of the A ring of the catechin group. A propelagonidin consisting of an afzelechin and a catechin unit has been identified in raspberries before (*12*).

The antioxidant detection system clearly indicates that both ellagitannins make major contributions to the total antioxidant activity of the raspberry fruit. Unfortunately, some compounds
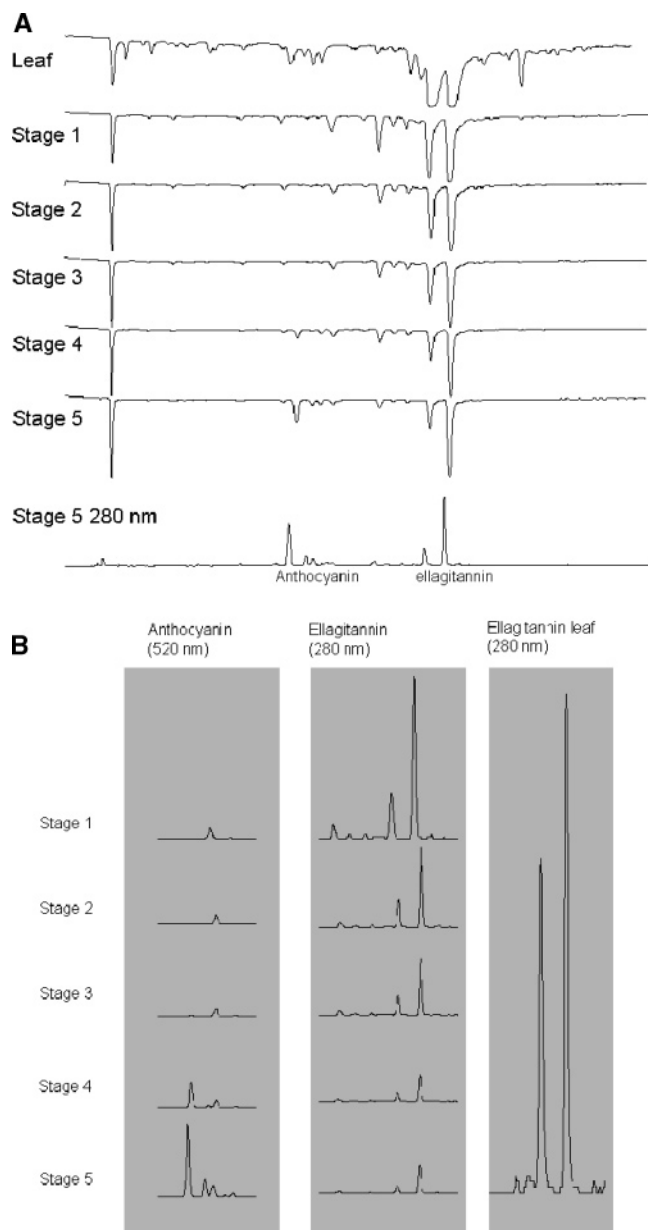
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Figure 3. Antioxidant compounds in different fruit stages. (**A**) HPLC chromatogram recorded at 412 nm after reaction with ABTS for a green leaf (leaf) and five different fruit stages of Tulameen raspberry (stages 1–5). For reference, the HPLC chromatogram recorded by PDA at 280 nm is shown at the bottom. (**B**) The HPLC chromatograms for five fruit stages recorded at 520 (left panel) and 280 (middle panel) nm. The panel on the right shows a chromatogram at 280 nm from a fresh leaf extract. The *y*-axis scales of the fruit stages and the leaf are identical.

separated by the HPLC column could not be separated properly by the antioxidant detection system, due to its slightly lower resolution resulting from peak broadening in the postcolumn reactor. This was the case for peaks 3 and 4 and peaks 6 and 7. As a result, the contribution of these peaks cannot be separately established. Standards of known antioxidant compounds such as quercetin-glycosides eluted after peak 13 (see also ref *4*), but in this study, hardly any visible antioxidant activity was observed in this region of the raspberry fruit extracts (**Figure 3B**). It was concluded that these flavonols do not contribute significantly to the total antioxidant activity of ripe raspberries.

Changes in Antioxidant Compounds during Fruit Ripening. To determine the effect of ripening stage on the antioxidant

profile in raspberries, we investigated which of the identified (phenolic) antioxidant compounds accumulate or diminish during fruit ripening. Analyses were performed with extracts from five successive ripening stages of fruits (**Figure 3A**) and, for comparison, on a mature leaf from plants of the cultivar Tulameen. The ripening stages are defined as follows: stage 1, small (immature) green fruits; stage 2, full size (mature) green fruits; stage 3, pink fruits; stage 4, red fruits, still attached to the crown; and stage 5, ripe red fruits, easily detached from the crown. Antioxidant profiles of these fruits are shown in **Figure 3B**. The HPLC chromatogram at 280 and 520 nm is shown in **Figure 3C**.

The pattern of antioxidant activity shows two major trends during fruit ripening. First, anthocyanins accumulated only in stages 4 and 5, coinciding with red fruit color, while only traces were observed in the pink fruits from stage 3. Second, the tannins, which dominated the antioxidant profiles especially in leaf and stage 1 fruits, gradually diminished during fruit ripening. Polar antioxidants including vitamin C (in first region of the chromatograms) only increased slightly upon ripening, especially between stages 1 and 2.

To study the relative changes in more detail, the peak area of the MS parent ion (ESI$^+$) of each of the anthocyanins was recorded for all five developmental stages (**Table 2**). Stages 1 and 2 show hardly any differences with regard to anthocyanins: the main anthocyanin peak was cyanidin-3-glucoside (peak 3), with some traces of cyanidin rutinoside (peak 7). The third stage showed the onset of ripening-related accumulation of anthocyanins. At this stage, small amounts of cyanidin sophoroside and cyanidin glucosylrutinoside were detectable, which probably contribute to the pink color of the fruit. The fourth stage showed a sharp increase in these two compounds and, in addition, the onset of pelagonidin-glycosides accumulation. In the last ripening stage of the fruits (stage 5), the compounds that appeared during the third and fourth stages of development increased even further. Thus, most anthocyanins observed in red fruits are associated with fruit ripening, while cyanidin-3-glucoside occurs in both unripe and ripe fruit. This compound was also observed in old purple leaves of raspberry but not in fresh green leaves (data not shown). Despite a five times increase during ripening, the contribution of this compound to fruit pigmentation was minor as compared to some other components, such as cyanidin sophoroside (peak 2) and cyanidin glucosylrutinoside (peak 4), which were present at much higher levels in ripe fruits.

Tannins markedly decrease during ripening. Data in **Figure 3C** and **Table 2** show that both ellagitannins (peaks 12 and 13) and proanthocyanidin-like tannins (peaks 10 and 11) follow a similar pattern. However, some differences can be observed. Peak 13 did not decrease significantly during the transition from the fourth to the fifth stage, while both proanthocyanidin-like compounds (peak 10 and 11) were strongly reduced in this phase. During the first stages (1−3), differences were observed in the patterns of decrease in the proanthocyanidin-like compounds: Peak 11 is much less affected by the early fruit development than is peak 10. Notably, the content of ellagitannins in leaf material was much (4−5 times) higher in leaf, as compared to the green stages (**Figure 3C**).

Antioxidants in Different Raspberry Cultivars. To assess the effect of genetic background on variation in the antioxidant compounds, the composition of anthocyanins and ellagitannins was assessed for 14 different cultivars. Cultivars were grown at one location, and ripe red fruits were harvested and pooled per cultivar. Aqueous methanol extracts were prepared and

Table 2. Content of Antioxidant Compounds during Development of Tulameen Raspberries and in Ripe Prelude and Autumn Britten Raspberries[a]

peak no. (specific ion)[b]	peak identity	Tulameen					Prelude	Autumn Britten
		stage 1	stage 2	stage 3	stage 4	stage 5	stage 5	stage 5
1 (m/z 611)	cyanidin-diglucoside	ND[c]	ND	ND	1630	2110	1199	685
2 (m/z 611)	cyanidin-sophoroside	ND	ND	55	3924	10078	6098	5304
3 (m/z 757)	cyanidin-glucosylrutinoside	ND	ND	ND	217	3497	6852	7262
4 (m/z 449)	cyanidin glucoside	443	359	454	1002	2007	947	2260
5 (m/z 595)	pelagonidin-sophoroside	ND	ND	ND	93	667	2156	145
6 (m/z 741)	cyanidin-xylosylrutinoside	ND	ND	ND	5	54	113	184
7 (m/z 595)	cyanidin-rutinoside	ND	ND	ND	169	1216	3023	5832
8 (m/z 741)	pelagonidin-glucosylrutinoside	ND	ND	ND	7	145	3118	890
9 (m/z 433)	pelagonidin-glucoside	ND	ND	ND	35	74	284	233
10 (m/z 579)	proanthocyanidin	4103	2622	2141	1406	531	841	891
11 (m/z 563)	proanthocyanidin	3034	2062	2074	1257	654	48	723
12 (240 nm)[d]	lembertianin C	2.8×10^6	2.0×10^6	1.1×10^6	8.0×10^5	5.8×10^5	1.6×10^5	8.0×10^5
13 (240 nm)[d]	sanguiin H6	7.5×10^6	4.4×10^6	2.8×10^6	1.8×10^6	1.9×10^6	1.1×10^6	3.0×10^6

[a] Values represent mass counts at this specific ion and are an average of two independent measurements of two independent samples. [b] The specific ion (ESI+) that was used for quantification is indicated. [c] ND, not detected. [d] Values for lambertianin C and sanguiin H6 are peak surface areas measured at 240 nm in the HPLC chromatogram.
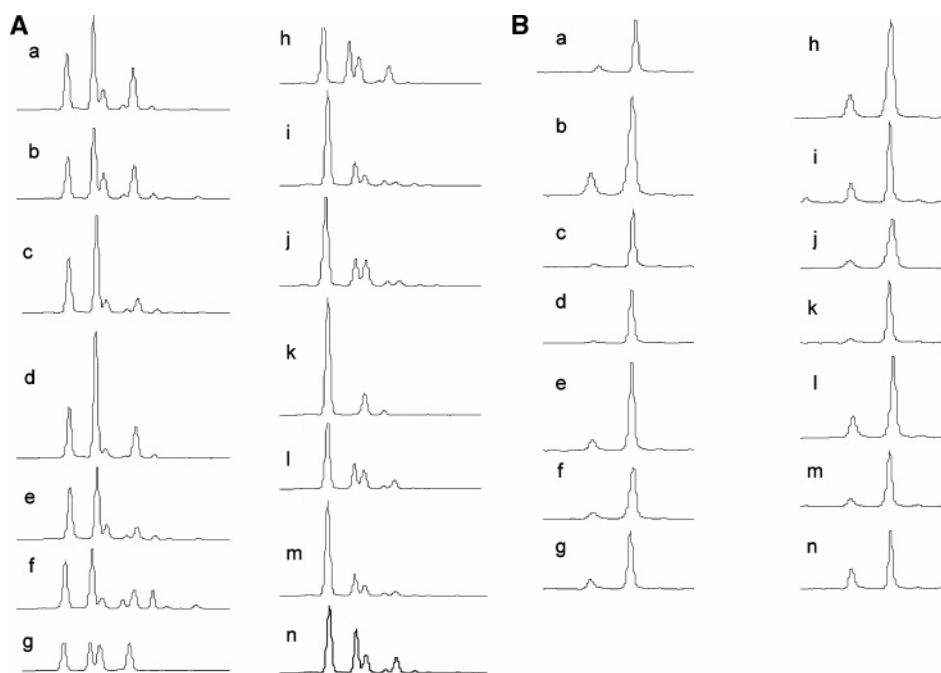

Figure 4. HPLC analysis of anthocyanins (**A**) and ellagitannins (**B**) in different raspberry cultivars. Shown are identical time frames from the chromatograms at 520 (15–22 min, panel **A**) and 280 (25–32 min, panel **B**) nm. The *y*-axis scales are identical for each cultivar. Cultivars are Autumn Bliss (a), Autumn Britten (b), Carmen (c), Nova (d), Himbo Top (e), Prelude (f), P93453 (g), MA69-2 (h), MEHQ69 (i), P93563 (j), Polka (k), Encore (l), Tulameen (m), and Glen Ample (n).

analyzed by HPLC/PDA followed by an on-line antioxidant detector. In most cultivars, all nine anthocyanin peaks (absorbing at 520 nm) were observed. However, large differences existed between the cultivars with respect to the relative amounts of each of the components (**Figure 4A**). Three groups of cultivars can be distinguished based on their anthocyanin patterns. For the first group (Autumn Bliss, Autumn Britten, Carmen, Himbo Top, and Nova), the major anthocyanin was peak 3 [cyanidin-3-(2G-glucosylrutinoside)], contributing about 40% of total absorbance at 520 nm. For the second group (Tulameen, MEHQ-69, Polka, P93453, MA69-2, Encore, Glen Ample, and P93563), peak 2 (cyanidin-3-sophoroside) is the dominant anthocyanin (about 50% of total absorbance at 520 nm). In the third group (Prelude and P93453), both peaks 2 and 3, as well as several other anthocyanin peaks, were dominant. For ripe fruits of representative cultivars of each group (i.e., Autumn Britten, Tulameen, and Prelude), the specific mass counts of anthocya-

nins and tannins in the MS analysis are shown in **Table 2** (last three columns).

Pelagonidins (peaks 5, 8, and 9) vary stronger than cyanidins between the cultivars. Pelagonidins were relatively low abundant in the group of cultivars represented by Tulameen, while they were readily observed in Prelude. Differences from 5 (peak 5)- to 20 (peak 8)-fold were observed. In Autumn Britten, pelagonidin sophoroside (peak 5) was hardly found, but other pelagonidins were well-detected in this cultivar. The proanthocyanidin-like peak 11, on the other hand, was about 20-fold lower in Prelude than in the other cultivars.

The ellagitannins were the dominant antioxidants in all cultivars, contributing to 30−60% of total antioxidant activity. In most of the 14 cultivars, both Lambertianin C (peak 12) and Sanguiin H6 (peak 13) were observed (**Figure 4B**). However, when there was a relatively low abundance of ellagitannins, like in cultivar Nova, lambertianin C seems to be hardly detectable.

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There is no obvious correlation between the abundance of ellagitannins and the abundance or distribution of anthocyanins.

DISCUSSION

The aims of this study were to monitor and identify individual antioxidant compounds in raspberry fruits, to determine their contribution to the total antioxidant activity in aqueous methanol extracts, and to compare differences associated with developmental stages and cultivars. Clearly, the advantage of on-line antioxidant measurement lies in its ability to distinguish the antioxidant activity of compounds that can hardly be separated by fractionation but still elute as separate peaks. The relative importance of these compounds for antioxidant activity can be observed and measured directly, without time-consuming fractionation. Thus, an independent measurement of the functional quantity of compounds is performed, independent of their molar absorption constant or wavelength optimum. It provides a way of functional quantification for different classes of compounds, relative to each other. This is particularly useful when no standards are available, as is, for instance, the case for many tannins and anthocyanin-glycosides.

To identify the compounds with apparent antioxidant activity, the extracts were also analyzed by a HPLC-PDA-QTOF-MS/MS system using the same chromatographic conditions and the same column. Peaks on both systems could be interrelated based on the retention time and PDA spectrum of the separated compounds. Consequently, although the mass spectrometry analyses were performed on a separate HPLC system, it was possible to elucidate the identity of compounds that had been identified as significantly antioxidant, through exact mass measurements and MS/MS fragmentation patterns, and comparison to available literature data on compounds present in raspberry (*4*, *12*). The use of a lock-mass reference spray for mass correction (*10*) allowed for highly accurate mass determinations. For most parent ion masses, the measured mass was within 5 ppm of the expected mass, which was calculated from the elemental composition of compounds known to be present in raspberry. From an analytical–chemical point of view, such a tight match confirms the elemental composition of the detected compound (*13*).

The system described was used to identify antioxidant compounds in raspberry fruit. Most compounds detected, such as the cyanidin anthocyanins, and ellagitannins, such as lambertianin C and sanguiin H6, have already been described. In addition to these known compounds, we were able to identify other proanthocyanidin-like compounds in raspberry fruit, based on accurate mass measurements and MS/MS fragmentation patterns, and could demonstrate directly that these proanthocyanins also have antioxidant activity. Having identified these antioxidant compounds, we then studied how they change during fruit ripening and to what extent differences occur between different cultivars. Total anthocyanin content is of course known to increase during fruit ripening, reflected in the color change of the fruit (*14*). However, our findings indicate that some anthocyanins, like cyanidin-3-glucoside, are already present in unripe (green) fruit and that these are also those detected in stressed leaves. Other anthocyanins, like cyanidin sophoroside, cyanidin glucosylrutinoside, and pelagonidin glucosylrutinoside, occur only when the red color of the fruit develops. On the other hand, other polyphenols such as ellagitannins are initially present at high levels but decrease strongly during fruit ripening. Nevertheless, these compounds still contribute a major portion to the total antioxidant activity of the ripe fruits. Proanthocyanidins were also found to contribute significantly to the

antioxidant activity but only in unripe fruits; they are present in only low amounts in most ripe fruits. What this investigation has clearly shown is that the antioxidant content of raspberry fruits is determined by a diverse range of components, which provide unequal contributions to the total antioxidant activity. Furthermore, during development and ripening, significant shifts occur in the relative contributions of the different classes of compounds, which cannot be detected by a general determination of total antioxidant activity. Consequently, only the application of an on-line system as described here will reveal the fundamental, biochemical basis of the antioxidant activity as a quality trait.

Fruit consumption is increasingly associated with a healthy lifestyle. Many consumers are interested in natural health-promoting compounds, such as antioxidants in food. For this reason, the content of antioxidants in fruit can, like firmness, taste, and processing qualities, be considered as quality traits. Consequently, if antioxidants are to be used as markers for fruit quality, more knowledge is required of the effect of fruit development and the genetic background on the presence of antioxidant compounds. The present study was aimed to collect such knowledge.

For grapes, detailed knowledge has already been established, and anthocyanin fingerprints are being used to differentiate grape cultivars. Several groups have studied the accumulation of anthocyanins during grape ripening (*15*, *16*). Esteban et al. studied the accumulation of seven different anthocyanins in the skin of two black grape cultivars by HPLC. The pattern of accumulation during ripening was found to differ only modestly. In raspberry, however, the present study indicates that a more pronounced variation in accumulation among the anthocyanins occurs. Some raspberry anthocyanins are present at all phases of fruit development, while others only occur in ripe raspberry fruits. The key difference between raspberry and grape anthocyanins is that the dominant raspberry anthocyanins are derived from cyanidin and pelagonidin and differ only in their sugar moieties, while in grape, cyanidin-, petunidin-, malvidin-, delphinidin-, and peonidin-based anthocyanins have been found and all of them in the monoglucoside, acetylglucoside, and coumarylglucoside forms (*15*, *16*). It is therefore possible that, in grape, the basic monoglucoside structures are synthesized simultaneously, while the additional glycoside side chains, which are found in raspberry, are differentially induced during ripening, probably due to differences in enzymatic activity of glycosyl transferases. Analysis of expression of anthocyanin pathway genes in grape berries indicates that glucosyl transferases are upregulated at the end of ripening and are independent from the other anthocyanin biosynthesis genes (*17*). Furthermore, inhibition of glucosyl transferase expression was shown to be detrimental to anthocyanin accumulation in grape, stressing the regulatory importance of these enzymes (*18*).

The patterns of tannin accumulation during fruit ripening are poorly understood. Proanthocyanindin-type of tannins of dimeric nature, like the molecules having m/z values 563 and 579, which were observed in the unripe raspberries, are known to cause astringency in wine. During maturation of wine, these compounds condense into polymers, their solubility decreases, and thus astringency is reduced (*19*). The astringency of raspberries is also reduced during fruit ripening. However, while Haslam and Lilley (*19*) suggest that tannins hardly decrease during raspberry fruit ripening and that the increase in the content of other compounds, such as polysaccharides, during fruit ripening disrupt the astringency effect, our data strongly suggest that both proanthocyanidin-like tannins and ellagitannins decrease by a

factor of 3−8 during raspberry fruit ripening. The procyanidins are even significantly reduced during the last ripening step (**Table 2**), while the ellagitannins stay more or less constant. When we tasted raspberry fruits of ripening stages 4 (unripe) and 5 (ripe), a strong difference in astringency between these stages was observed (results not shown), suggesting that especially the dimeric proanthocyanidins contribute significantly to the astringent taste of unripe raspberries.

Breeding for raspberry cultivars with higher levels of specific antioxidants could imply the introduction of germplasm from wild raspberries. The described on-line antioxidant analysis system provides a useful tool to select for variation among novel breeding lines. The profile of antioxidant compounds in the raspberry cultivars studied here seems, qualitatively, to be rather conserved, in the sense that most compounds occurred in all cultivars. However, a per compound comparison did reveal remarkable genetic differences. For instance, Prelude raspberries are unusual in that they contain very little of the procyanidin-like compound (563 *m/z*) and have relatively high peaks of pelagonidin sophoroside and pelagonidin glucosylrutinoside. This relatively high level of pelagonidins as compared to cyanidins suggests that hydroxylation of flavonoids may occur less efficiently in the variety Prelude, due, for example, to a lower activity of the enzyme flavonol-3′-hydroxylase. Another example of a contrasting anthocyanin profile is that for Polka raspberries. These lack detectable glucosyl rutinoside peaks for both cyanidin and pelagonidin, while glucosides (peak 2, 3, 5, and 9) were present (**Figure 4**), suggesting a lower activity of flavonoid-3-glucose rhamnosyl transferase (*20*). Clearly, genetic differences in content of individual antioxidant compounds exist between raspberry cultivars.

In conclusion, this paper describes the application of an on-line HPLC-based antioxidant detection system for the analysis of complex metabolic mixtures in raspberry extracts. This system is able to pinpoint which compounds present in a plant extract exert antioxidant activity. When used in combination with QTOF-MS/MS, it readily enables the detection and identification of the antioxidant compounds. The availability of such a system allows for critical assessment of harvesting and processing conditions, with regard to their effect on individual antioxidant compounds, and thus to effects on potentially health-promoting properties in foodstuffs.

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