Influence of Acid and Base Hydrolysis on the Phenolic Composition of Red Raspberry Juice†

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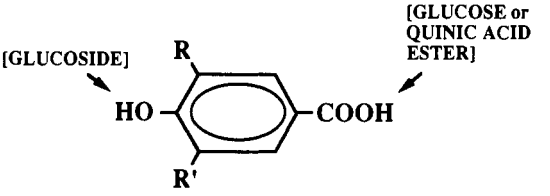
Acid and base hydrolysis simplified the complex HPLC chromatographic profile of red raspberry juice phenolics dramatically. In three acid-hydrolyzed juices, ellagic acid, two unidentified ellagic acid compounds, and gallic, *p*-hydroxybenzoic, protocatechuic, caffeic, *p*-coumaric, and ferulic acids, as well as (+)-catechin, (−)-epicatechin, quercetin, and kaempferol were separated and identified using HPLC diode array spectral techniques. The percentages of HPLC peak area for these phenolics were similar in the acid-hydrolyzed juices. In base-hydrolyzed samples the same phenolics, except the second ellagic acid compound, were present, and the first ellagic acid compound was present in smaller quantity. Sep-Pak C_{18} cartridges were used for sample preparation. Hydrolysis could become a very useful tool for rapidly screening raspberry juices for qualitative deviations from authentic HPLC profiles to determine adulteration by other fruit juices or phenolic mixes.

INTRODUCTION

It is of great importance to the food industry and regulatory agencies to guarantee the authenticity of red raspberry juices and concentrates, which are expensive and therefore targets for adulteration. Red raspberries and products made thereof are also of interest because of the anticarcinogenic effects of their phenolic constituents, particularly quercetin and ellagic acid. The health effects of these phenolics are reviewed in Rommel et al. (1992).

Secondary plant metabolites (e.g., flavonoids and other phenolics) are useful in authenticity investigations because they are present in plants within a finite range (Herrmann, 1976). Analysis of anthocyanin pigments, the major flavonoids in raspberries, can be effective for detecting the presence of juices and colorants derived from other anthocyanin-containing fruits (Spanos and Wrolstad, 1987). Analysis of other phenolics (flavonols, catechins, cinnamic acids, etc.) should be useful in authenticity investigations as an adjunct to anthocyanin analyses and for detecting non-anthocyanin-containing fruits such as white grape or pear. The flavonols (Henning, 1981; Rommel and Wrolstad, 1993a), ellagic acid derivatives (Daniel et al., 1989; Maas et al., 1991; Rommel and Wrolstad, 1993b; Wilson and Hagerman, 1990), and cinnamic and benzoic acids (Herrmann, 1989; Schuster and Herrmann, 1985) are present as glycosides or esters and provide a very complex profile. Acid or base hydrolysis transforms glycosylated and esterified phenolics into their aglycons (Hong and Wrolstad, 1986; Markham, 1982) and so reduces the number of raspberry juice phenolics significantly. A simplified HPLC phenolic profile of raspberry juice would be advantageous for screening of samples for authenticity.

The flavonol and ellagic acid compositions of red raspberries are reviewed in Rommel and Wrolstad (1993a,b). The most common benzoic acid compounds in plants are derivatives of the hydroxybenzoic acids: *p*-hydroxybenzoic, protocatechuic, vanillic, and gallic acids



R = R' = H	p-HYDROXYBENZOIC ACID
R = OH R' = H	PROTOCATECHUIC ACID
R = R' = OH	GALLIC ACID

Figure 1. Structures of hydroxybenzoic acids and derivatives.

(Figure 1); salicylic, syringic, and gentisic acids are less common (Goodwin and Mercer, 1983; Herrmann, 1989; Spanos, 1988). Most hydroxybenzoic acids are present in the form of glucosides and some as esters with glucose; however, gallic acid is mainly esterified to quinic acid or catechins and usually present in polymeric forms as soluble tannins, i.e., condensation products (Goodwin and Mercer, 1983; Herrmann, 1989). The hydroxybenzoic acid glucosides and esters that have been quantified in red raspberries (Herrmann, 1989; Schuster and Herrmann, 1985) are summarized in Table I; structures are shown in Figure 1. In raspberries the content of *p*-hydroxybenzoic acid β-D-glucoside is distinctly higher than in other rosaceaous fruits (Schuster and Herrmann, 1985). Swain et al. (1985) reported the presence of high concentrations of salicylic acid in raspberries; however, Herrmann (1989) considers trace amounts more realistic. Nonhydroxylated benzoic acid compounds, such as esters with glucose (e.g., 6-benzoylglucose), also occur in fruits in trace amounts (Herrmann, 1989). The presence of free hydroxybenzoic acids can result from hydrolysis of flavonoids (e.g., anthocyanins) and of hydroxybenzyl glucosinolates using alkali or nonspecific enzymes (Herrmann, 1989) during extraction from plants or juice processing.

Hydroxycinnamic acid compounds (Figure 2) are almost exclusively derived from the widespread *p*-coumaric, caffeic, and ferulic acids (Herrmann, 1989; Spanos, 1988). These acids may be bound to cell wall polymers (Herrmann, 1989); however, they occur in plants most frequently as simple esters with quinic acid (cyclitol) or glucose and also with carboxylic acids (e.g., malic, tartaric, galactaric;

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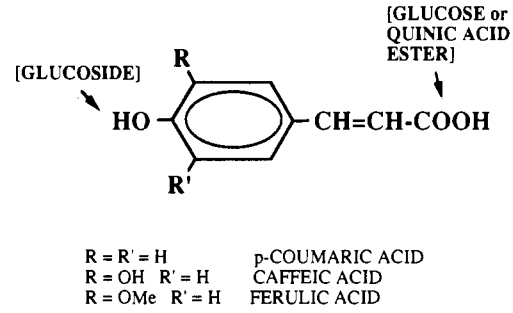

Figure 2. Structures of hydroxycinnamic acids and derivatives.

Table I. Concentrations of Hydroxybenzoic and Hydroxycinnamic Acids Present in Fresh Red Raspberries[a]

compound	concn, ppm[b]
hydroxybenzoic acid glucosides (cultivars: Glen Cova, Malling Exploit, Malling Promise)	
p-hydroxybenzoic acid β-D-glucoside	32–59
protocatechuic acid 4-β-D-glucoside	tr
gallic acid 4-β-D-glucoside	tr
hydroxybenzoic acid esters (Glen Cova and Malling Exploit)	
5-galloylquinic acid	tr
1-*O*-galloyl-β-D-glucopyranose	tr
salicylic acid	tr–1 (Mosel and Herrmann, 1974)
	39–51 (Swain et al., 1985)
hydroxycinnamic acid glucosides (Glen Cova, Malling Exploit, Malling Promise)	
p-coumaric acid β-D-glucoside	4–10
ferulic acid β-D-glucoside	tr–2
hydroxycinnamic acid ester (Glen Cova and Malling Exploit)	
1-*O*-coumaroyl-β-D-glucopyranose	6–14
1-*O*-feruloyl-β-D-glucopyranose	4–7
1-*O*-caffeoyl-β-D-glucopyranose	3–7
5-*p*-coumaroylquinic acid	1–2
5-caffeoylquinic acid (=chlorogenic acid)	tr–1
5-feruloylquinic acid	tr

[a] Herrmann (1989) and Schuster and Herrmann (1985). [b] Milligrams per kilogram of fresh fruit.

Herrmann, 1989; Schuster and Herrmann, 1985). The range of esters of the hydroxycinnamic acids is far greater than that of any other plant phenols (Goodwin and Mercer, 1983). The hydroxycinnamic acid glucosides and esters that have been quantified in red raspberries (Herrmann, 1989; Schuster and Herrmann, 1985) are given in Table I. Natural forms of cinnamic acids apparently have the trans-configuration but tend to isomerize to cis with the exposure to UV light during extraction from plants; oxidation in the O-position also occurs as an artifact (Herrmann, 1989; Spanos, 1988). Amides of hydroxycinnamic acids have been identified in the reproductive organs of raspberries but are absent from the green parts, petals, and sepals (Herrmann, 1989).

Flavan-3-ols (or catechins) and polymeric proanthocyanins (procyanins or condensed tannins) are the most abundant phenolic compounds besides hydroxycinnamic acid esters in fruits found in cool to moderate climates; they are not found in citrus fruits or in vegetables (Herrmann, 1974). Both monomeric catechins and dimeric procyanidins (flavan-3-ol and flavan-3,4-ol dimers) are usually not glycosylated or esterified in plants (Herrmann, 1974). (−)-Epicatechin is the most important catechin in most fruits; however, (+)-gallocatechin is the primary catechin in peaches and some plums, strawberries, currants, and gooseberries (Herrmann, 1974). If at all present, (+)-gallocatechin and (−)-epigallocatechin do not occur until fruit ripeness. Blackberries, raspberries, and strawberries contain mainly (−)-epicatechin and (+)-catechin; occasionally (+)-gallocatechins are found (Mosel and Herrmann, 1974; Table II). Concentrations of these catechins peak during ripening of these berries (Mosel and Herrmann, 1974; Stoehr and Herrman, 1975). Dimeric procyanidins yield anthocyanidins and catechins during heating with acid (Hahlbrock, 1981; Herrmann, 1974; Spanos, 1988).

It was the objective of our study to create an expanded compositional database for red raspberry juice consisting of flavonoids and other phenolics, sugars, and organic acids. This paper is restricted to ellagic acid, non-anthocyanin flavonoids, and benzoic and cinnamic acids in hydrolyzed red raspberry juices. The objective was to separate and identify hydrolyzed phenolics in red raspberry juice so that they can be used (a) as supplementary authenticity indicators together with anthocyanins, sugars, and organic acids and (b) as a database for evaluating the effects of raspberry juice on health.

MATERIALS AND METHODS

Red raspberry juices (n = 4) made from Willamette (n = 3) and Meeker (n = 1) cultivars were analyzed. Juices were processed in the Food Science and Technology pilot plant at Oregon State University as described in Rommel and Wrolstad (1993a).

Juice Sample Preparation and Hydrolysis. The flavonoids and other phenolics of single-strength red raspberry juice (ca. 5 mL) were adsorbed onto activated Sep-Pak C_{18} cartridges (Waters Associates, Milford, MA) using cleanup procedure I of Hong and Wrolstad (1990); the eluate (ca. 15 mL) containing sugars and organic acids was discarded. C_{18} cartridges were activated with methanol. Flavonoids and phenolics were eluted from cartridges with acidified methanol (ca. 10 mL); this fraction was evaporated to dryness using a rotary evaporator and a 40 °C water bath. The dried phenolics were redissolved in a few milliliters of 4% phosphoric acid before hydrolysis.

Percent recovery was estimated by comparing the individual HPLC peak areas measured for solutions of standards before and after the sample preparation procedure.

Acid and Base Hydrolysis. Juice samples were hydrolyzed (a) in 2 N HCl for 30 min in boiling water as described by Hong and Wrolstad (1986) and (b) in 2 N NaOH for 2 h at room temperature in the dark in nitrogen atmosphere, as described by Markham (1982), followed by a 30-min hydrolysis in 2 N HCl in boiling water. Hydrolyzed samples were adjusted to pH greater than 2.0 with 2 N NaOH and readsorbed onto C_{18} cartridges and prepared as described above. Phenolics redissolved in 4% phosphoric acid were filtered with Millipore filters, type HA, 0.45-μm pore size (Millipore Corp., Bedford, MA).

Analysis by High-Performance Liquid Chromatography (HPLC). Descriptions of the HPLC instrumentation, diode array detection system, and column are given in Rommel and Wrolstad (1993b); solvent A = 100% acetonitrile, and solvent B = 1% acetic acid in deionized water. Flow rate was 0.6 mL/min, and injection volume was 50 μL. The following gradient elution program gave the best separation of the phenolics present in hydrolyzed juice samples: from 5 to 15% A in 35 min; to 25% A in 20 min; to 55% A in 18 min; to 100% A in 5 min; 5 min at 100% A; return to initial conditions in 7 min (total run time: 90 min). Ellagic and benzoic acids were detected at 260 nm, gallic acid and catechins at 280 nm, cinnamic acids at 320 nm and flavonols at 360 nm.

Peak Characterization. Peaks separated by HPLC were characterized by their UV spectra and their retention times as compared with standards.

Standards Used for Peak Characterization: flavonols, kaempferol and quercetin (Sigma Chemical Co., St. Louis, MO); ellagic acid (Sigma); benzoic acids, gallic, protocatechuic, and *p*-hydroxybenzoic acid (Sigma); cinnamic acids, caffeic, *p*-coumaric, and ferulic acid (Sigma).

Table II. Flavan-3-ols Present in Fresh Raspberries, Blackberries, and Strawberries As Reported in the Literature

	total flavan-3-ols, ppm[a]	(+)-catechin, ppm	(−)-epicatechin, ppm	(+)-gallocatechin, ppm	ref
raspberries					
unknown variety	133	22	111		Mosel and Herrmann (1974)
Gevalo	46	1.1	45		Mosel and Herrmann (1974)
Malling Jewel	48	6.2	42		Mosel and Herrmann (1974)
Golden Queen	32	12	20		Mosel and Herrmann (1974)
Promiloy	49	14	35		Mosel and Herrmann (1974)
mean	62	11	51	tr[b]	
blackberries (6 varieties, mean)	131	19	112	tr[b]	Mosel and Herrmann (1974)
strawberries (16 varieties, mean)	47	33	6.9	7.4	Stoehr and Herrmann (1975)

[a] Milligrams per kilogram of fresh fruit. [b] Trace, in some samples.

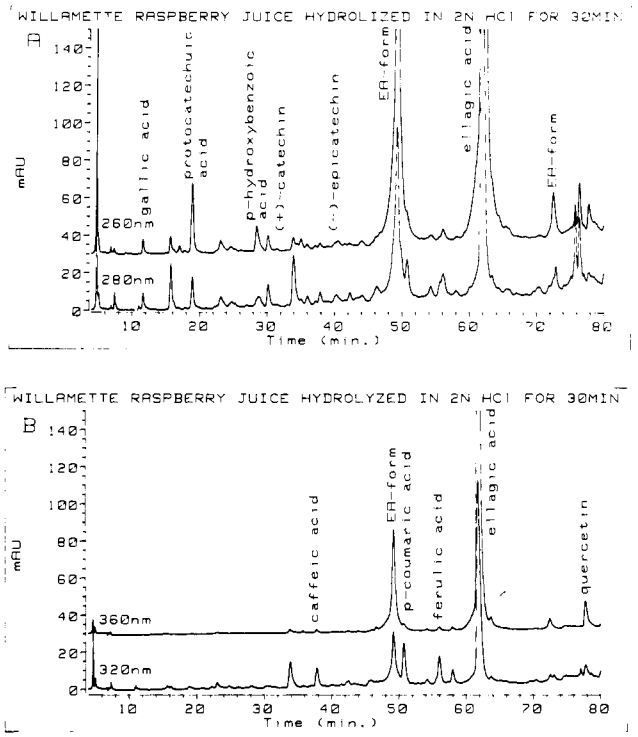

Figure 3. HPLC chromatograms of Willamette red raspberry juice hydrolyzed in 2 N hydrochloric acid for 30 min. (A) Detection at 260 (ellagic acid, ellagic acid derivatives, and benzoic acids) and 280 nm (gallic acid and catechins); (B) detection at 320 (cinnamic acids) and 360 nm (flavonols).

RESULTS AND DISCUSSION

The HPLC chromatograms of the hydrolyzed red raspberry juice samples analyzed were very similar for both Meeker and Willamette cultivars. An example of Willamette juice, hydrolyzed in 2 N HCl for 30 min, is shown in Figure 3. While recovery of flavanol aglycons, flavan-3-ols, and ellagic acid approached 100%, recovery of benzoic standards, particularly gallic and protocatechuic acids, from C_{18} cartridges was low (ca. 10%) and variable. Table III summarizes the results for the phenolic aglycons present in hydrolyzed red raspberry juices; peak areas were measured at the absorbance maximum for respective compound classes (260, 280, 320, or 360 nm). The three acid-hydrolyzed samples had similar percentages of HPLC area for the ellagic acid compounds, hydroxycinnamic acids, flavonol aglycons, and flavan-3-ols as evident by the standard deviations for their means (Table III).

The major peaks present were ellagic acid and an unidentified ellagic acid compound (no. 1), which eluted before ellagic acid (Figure 3). Another unidentified ellagic acid compound (no. 2) eluted about 12 min later than ellagic acid; both ellagic acid compounds had absorbance spectra very similar to that of ellagic acid. In unhydrolyzed red raspberry juices ellagic acid and up to 16 additional ellagic acid compounds were present (Rommel and Wrolstad, 1993b). A reduction to only ellagic acid and two ellagic compounds during hydrolysis probably resulted from deglycosylation, demethylation, and demethoxylation of ellagic acid (Daniel et al., 1991; Maas et al., 1991) as well as from the removal of glucose and gallic acid molecules from ellagitannins through cleavage of ester bonds (Bate-Smith, 1959, 1972; Wilson and Hagerman, 1990). Hydrolysis may also have dissociated metal complexes of ellagic acid (Press and Hardcastle, 1969) and possibly open lactone forms of ellagic acid.

Three hydroxybenzoic acids were identified: gallic, protocatechuic, and *p*-hydroxybenzoic; protocatechuic acid had the greatest peak area. Also identified were three hydroxycinnamic acids: caffeic, *p*-coumaric, and ferulic acids, with *p*-coumaric having the greatest peak area. Herrmann (1989) and Schuster and Herrmann (1985) reported the presence of these hydroxybenzoic and hydroxycinnamic acids as glucosides and esters. We did not detect salicylic acid, perhaps because of low recovery in the sample preparation procedure. Reports of small concentrations of salicylic acid in red raspberries were made by Herrmann (1989) and Mosel and Herrmann (1974); Swain et al. (1985) reported quantities of 39–51 ppm of salicylic acid in red raspberries.

The flavan-3-ols (−)-epicatechin and (+)-catechin were detected. Their concentrations were lower, however, than would be anticipated from the quantities found in the unhydrolyzed samples (Rommel and Wrolstad, 1993b). While the flavonol aglycons quercetin and kaempferol were detected, the quantities were lower than would be anticipated from the amounts of quercetin and kaempferol glycosides in the unhydrolyzed samples (Rommel and Wrolstad, 1993b). It is evident that some loss of flavan-3-ols and flavonols occurs with hydrolysis. There are 13 quercetin and kaempferol glycosides and as many as 36 additional flavonols detected in trace quantities in unhydrolyzed samples (Rommel and Wrolstad, 1993b). Hydrolysis simplified the complex flavonol profile considerably. There are a number of peaks that remain unidentified, however.

A HPLC chromatogram of the same Willamette juice, hydrolyzed with 2 N NaOH for 2 h followed by 2 N HCl for 30 min, is shown in Figure 4. The phenolic pattern of this sample was very similar to that of the acid-hydrolyzed sample of the same juice, and the same compounds were present (Table III). However, ellagic acid compound 2 was absent, and the ratio of the percentages of peak area of ellagic acid and ellagic acid compound 1 was greater (5.7) than that for the acid-hydrolyzed sample (3.0). We speculate that both of these ellagic acid compounds were esters of ellagic acid (i.e., ellagitannins), which were hydrolyzed in basic but not in acidic conditions.

Table III. Percentages of HPLC Peak Area of Phenolics Present in Acid- and Base-Hydrolyzed Red Raspberry Juices

hydrolysis method: cultivar: sample code:[d]	base + acid Willamette WI-OR-OR-HP-PP	acid Willamette WI-OR-OR-HP-PP	acid Willamette WI-BC-R-MH-DE	acid Meeker ME-OR-UR-HP-PP	acid, mean ± SD[a]
ellagic acid comopund					
ellagic acid	68.34	67.05	60.24	68.29	65.19 ± 4.33
ellagic acid form 1	11.95	22.36	20.69	21.11	21.39 ± 0.87
ellagic acid form 2	nd[b]	2.40	6.27	1.35	3.34 ± 2.59
hydroxybenzoic acids					
gallic	0.31	0.32	0.16	0.08	0.19 ± 0.12
protocatechuic	4.11	1.39	0.75	0.44	0.86 ± 0.48
p-hydroxybenzoic	1.60	0.74	0.46	1.03	0.74 ± 0.29
hydroxycinnamic acids					
caffeic	0.74	0.49	0.38	0.74	0.54 ± 0.18
p-coumaric	2.72	1.52	2.01	1.91	1.81 ± 0.26
ferulic	4.58	1.71	1.85	1.14	1.57 ± 0.38
flavonol aglycons					
quercetin	3.47	1.07	4.06	3.38	2.84 ± 1.57
kaempferol	0.10	0.28	2.57	tr[c]	1.43 ± 1.62
flavan-3-ols					
(+)-catechin	tr	0.11	tr	0.18	0.15 ± 0.05
(−)-epicatechin	2.08	0.57	0.58	0.37	0.51 ± 0.12

[a] Statistical summary applies to columns 2–4; standard deviation (alpha = 0.05). [b] Not detected. [c] Trace. [d] Sample codes defined in Rommel and Wrolstad (1993a).

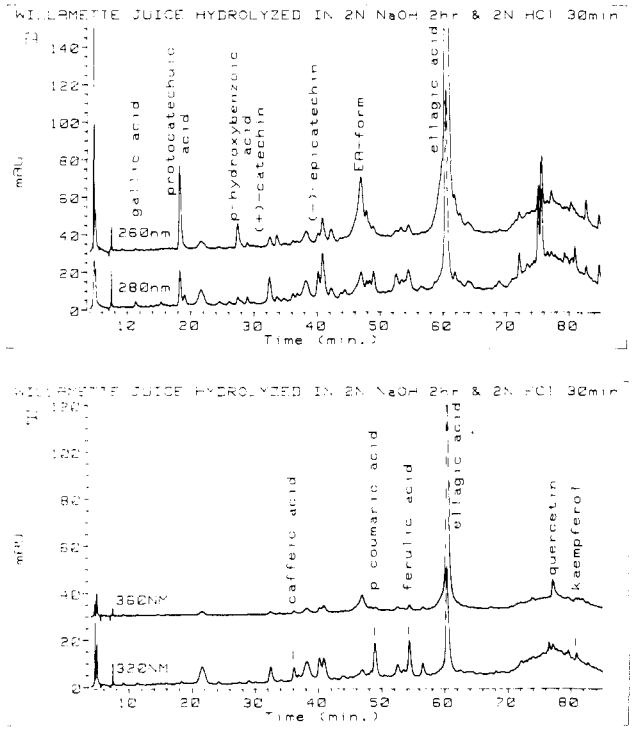

Figure 4. HPLC chromatograms of Willamette red raspberry juice hydrolyzed in 2 N sodium hydroxide for 2 h in 2 N hydrochloric acid for 30 min. (A) Detection at 260 (ellagic acid, ellagic acid derivatives, and benzoic acids) and 280 nm (gallic acid and catechins); (B) detection at 320 (cinnamic acids) and 360 nm (flavonols).

SUMMARY AND CONCLUSIONS

The complex HPLC chromatographic profile of red raspberry juice phenolics was simplified dramatically by both acid and base hydrolyses. In acid-hydrolyzed juices, ellagic acid and two unidentified ellagic acid compounds and also gallic, *p*-hydroxybenzoic, protocatechuic, caffeic, *p*-coumaric, and ferulic acids, as well as (+)-catechin, (−)-epicatechin, quercetin, and kaempferol were identified. In base-hydrolyzed samples the same phenolics, except for the second ellagic acid compound, were present; the first ellagic acid compound was detected in smaller quantity. The three acid-hydrolyzed juices had similar percentages of HPLC peak areas for the phenolics present, except for the benzoic acids. Hydrolysis provides a useful auxiliary method for raspberry adulteration investigations through examination of qualitative deviations from authentic HPLC profiles of ellagic acid compounds, hydroxycinnamic acids, flavan-3-ols, and flavonol aglycons. There are quantitative limitations as recovery is particularly low for benzoic acids, and there is some degradation of aglycons during hydrolysis.

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