Journal of Chromatography, 157 (1978) 345–355
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CHROM. 11,100

HIGH-PRESSURE LIQUID CHROMATOGRAPHIC ANALYSIS OF HYDROXYCINNAMIC ACID–TARTARIC ACID ESTERS AND THEIR GLUCOSE ESTERS IN *VITIS VINIFERA**

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(Received January 26th, 1978)

SUMMARY

A high-pressure liquid chromatographic technique has been developed to separate and quantify the amount of monocaffeoyl-, mono-*p*-coumaroyl-, and monoferuloyl-tartaric acid in grapes. The method features direct analysis of the grape juice with minimum sample preparation. The identity of the hydroxycinnamic acid–tartaric acid esters has been established through hydrolysis of the esters followed by gas–liquid chromatography and high-pressure liquid chromatography. The absence of chlorogenic and neochlorogenic acids often reported in grapes has been substantiated. Evidence will be presented for the occurrence of an ester of caffeic acid–glucose–tartaric acid.

INTRODUCTION

Esters of the hydroxycinnamic acids are widely distributed in the plant world, especially in fruits [1–3]. Numerous researchers[1,4–7] have identified chlorogenic acid (3-caffeoylquinic acid), its various positional isomers, and the analogous *p*-coumaroyl- and feruloylquinic acids[8] in *Vitis vinifera* grapes. Hydroxycinnamic acid–tartaric acid esters have been shown in grape skins[9], whole grapes[10], spinach chloroplasts[11–14], and the leaves of members of the Compositae[2,3,15–18].

As late as 1976 Singleton and Noble[19] noted that whether the esters of quinic acid, tartaric acid, or both are present in *V. vinifera* grapes is still unresolved. Recently, Singleton has shown conclusively the presence of hydroxycinnamic acid–tartaric acid esters in *V. vinifera*[20]. Glucose esters and glycosides of the hydroxycinnamic acids have been identified in numerous plants by Harborne and Corner[21], but have not been shown to be present in grapes.

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One of the major problems involved in separating and identifying hydroxycinnamic acid esters is their similarity in chemical characteristics. The quinic acid, tartaric acid, and glucose esters of a single hydroxycinnamic acid show similar ultraviolet (UV) absorption spectra with maxima for each ester typically varying by 3–5 nm from each other[22,23]. Many of the common paper chromatography solvents can not adequately separate the different esters of a single hydroxycinnamic acid[22].

High-pressure liquid chromatography (HPLC) using reversed-phase packings has been shown to completely separate the various hydroxycinnamic acids[24]. Court[25] separated the various positional isomers of chlorogenic acid on a reversed-phase packing. 3-Caffeoylquinic acid and 3-feruloylquinic acid are cleanly separated by HPLC and isochlorogenic acid, composed of three isomers of dicaffeoylquinic acid, yields three distinct peaks when analyzed by HPLC[16]. The sensitivity of HPLC for phenolic compounds is extremely good, easily detecting as little as 5 ng of the hydroxycinnamic acids.

In this study, we set out to establish the identity of the major hydroxycinnamic acid esters present in *V. vinifera* grapes, and to quantify the amounts present in various varieties.

EXPERIMENTAL

Apparatus

A Waters Assoc. (Milford, Mass., U.S.A.) high-pressure liquid chromatograph consisting of two Model 6000A chromatography pumps, a Model 660 solvent programmer, and a U6K injector was used. A Micromeritics Chromonitor 785 (Norcross, Ga., U.S.A.) was used to measure the optical density of the eluates. A Houston Instruments Omniscribe strip chart recorder (Austin, Texas, U.S.A.) provided a visual display while an Autolab Minigrator (Spectra-Physics, Santa Clara, Calif., U.S.A.) was used to integrate the peak areas. A 25 mm × 4.6 mm I.D. Zorbax ODS chromatography column (Du Pont Instruments, Wilmington, Del., U.S.A.) equipped with a 5 cm × 4.6 mm I.D. pre-column packed with 10-μm μBondaPak ODS (Waters Assoc.) was used for the chromatographic separations.

Preparative column chromatography was done on a 50 cm × 2.4 cm glass column packed with Polyamid CC-6 (Brinkmann Instruments, Westbury, N.Y., U.S.A.). Fractions were collected on an automatic fraction collector (Instrumentation Specialties Company, Lincoln, Nebr., U.S.A.).

Gas–liquid chromatography (GLC) was performed on a Varian Aerograph Model 1740-1 gas chromatograph (Palo Alto, Calif., U.S.A.) equipped with dual flame ionization detectors. A 6 ft. × 2 mm I.D. stainless-steel column packed with 3% SE-30 on Chromosorb W (70–80 mesh) was used for the separation of silylated sugars and tartaric acid.

Spectrophotometric measurements were made with a Beckman DU modified with an optical density converter, light source stabilizer, and cuvette positioner (Gilford Instrument Labs, Oberlin, Ohio, U.S.A.). A Buchler rotary evaporator (Fort Lee, N.J., U.S.A.) was used for concentration of samples. All samples and HPLC solvents were filtered through a 0.45-μm Millipore cellulose acetate or PTFE membrane filter (Bedford, Mass., U.S.A.) prior to use.

Reagents

Reagent-grade methanol, acetonitrile, pyridine, phosphoric acid, glutamic acid, tartaric acid, chlorogenic acid, glucose, and xylose were obtained from J. T. Baker (Phillipsburg, N.J., U.S.A.). Trimethylchlorosilane (TMS), hexamethyldisilazane (HMDS), and N,O-bis(trimethylsilyl)-acetamide (BSA) were purchased from Pierce Chemical Company (Rockford, Ill., U.S.A.). Dr. Joseph Corse (Western Regional Research Lab., USDA, Albany, Calif., U.S.A.) kindly furnished samples of 3-caffeoylquinic acid (chlorogenic acid), 5-caffeoylquinic acid (neochlorogenic acid), and 3-feruloylquinic acid.

All organic solvents used for HPLC were re-distilled prior to use. All water was distilled and stored in glass.

Procedure for isolation and characterization

Grapes (*V. vinifera*) held frozen at $-40°$ were quickly defrosted in a microwave oven. Sulfur dioxide (1000 ppm), as sodium bisulfite, was added. The grapes were crushed and forcefully squeezed by hand through cheesecloth. The pulp and seeds were discarded and the juice (200–400 ml) was extracted three times using the ethanol–ammonium sulfate technique of Singleton[27] to remove sugars and organic acids and concentrate the phenolic compounds. Ethanol was removed from the combined extracts on a rotary vacuum evaporator. The extraction process was repeated. The combined extracts were evaporated to a volume of 2–10 ml and filtered through a 0.45-μm Millipore filter.

The unknowns were then separated from the extract by preparative HPLC methods using conditions similar to analytical conditions or by column chromatography on Polyamid CC-6. Column chromatography on Polyamid CC-6, due to its different selectivity from C_{18}, is preferred since it removes compounds which might otherwise interfere with HPLC separations.

A 50 cm $\times$ 2.4 cm glass column was packed with Polyamid CC-6 equilibrated to 2% formic acid. The extract was applied to the top of the column. Any sugars, salts, or organic acids present were eluted off the column with 250 ml of 2% formic acid. The column was then eluted with 1500 ml methanol–formic acid–water linear gradient progressing from 25:2:73 to 50:2:48 followed by an additional 500 ml of methanol–formic acid–water (50:2:48). Fractions of 10 ml were collected. The elution order of the esters from Polyamid CC-6 is the opposite of that for C_{18} reversed-phase packing. The fractions were analyzed by HPLC for the occurrence of the various esters. The appropriate fractions were pooled and evaporated to 2–5 ml on a rotary vacuum evaporator.

Material obtained in this manner was then further purified by HPLC. An aliquot of sample to be purified was injected on to the Zorbax ODS column and eluted with 6–10% acetonitrile in distilled water adjusted to pH 2.6 with phosphoric acid. In all cases the distilled water was adjusted to the specific pH prior to the addition of the acetonitrile. The appropriate fractions were collected and pooled. Further purification was performed as necessary.

Characterization

Once the purified ester had been obtained, the UV absorption spectrum of the compound was determined and the components of the ester were identified through

hydrolysis and GLC and HPLC analyses of the hydrolysate. In order to obtain a UV spectrum of the purified material, it was first necessary to remove any traces of mineral acid introduced in the eluting solvent during purification of the ester. This was most easily done by injecting an aliquot of pure ester into the chromatograph and eluting with distilled water to which no acid had been added. Any acid present in the sample elutes in the solvent front followed by the ester which elutes as a broad peak, probably due to ionization of the tartaric acid. This material can then be evaporated to dryness and dissolved in 100% ethanol for spectral measurements. Failure to remove mineral acid prior to suspension in 100% ethanol results in major spectral changes.

The ester was then hydrolyzed for either 4 h in 2 *N* NaOH at room temperature or 6 h in 2 *N* HCl in a boiling water bath. A small aliquot of hydrolysate was adjusted to pH 2–4 and analyzed by HPLC using the Zorbax ODS column with 2.5 ml/min of 10–20% acetonitrile in distilled water, pH 2.6. This confirmed the hydrolysis of the ester and the identity of the hydroxycinnamic acid moiety. The retention time of the hydrolysate peak was compared to that of known standards. Alkaline hydrolysates were then treated by the addition of Dowex 50 (hydrogen form), filtered, and evaporated to dryness. Acid hydrolysates were directly evaporated to dryness.

Hydrolysates were prepared for paper chromatography by the addition of a small amount of methanol–water (1:1). Whatman No. 1 paper was used with butanol–acetic acid–water (5:1:4) ascending for tartaric acid analysis along with standards of quinic, citric, malic, tartaric, and caffeic acids. The chromatogram was first visualized under UV light to detect hydroxycinnamic acids and then sprayed with a 1% solution of sodium metavanadate in water. Ethyl acetate–pyridine–water (12:5:4) was used, ascending, for glucose analysis with standards of glucose, galactose, fructose, xylose, arabinose, and rhamnose. The chromatogram was sprayed with *p*-anisidine–phthalic acid[28] and heated for 2–5 min at 100°.

For GLC, the same hydrolysis procedure was used. The concentration of the ester solution to be hydrolyzed was first determined as will be outlined later. An internal standard, glutamic acid for tartaric acid esters and xylose for glucose esters, was then added to the hydrolysate in an amount equimolar to the hydroxycinnamic acid present. The ester plus internal standard was then hydrolyzed and dried as just discussed. The dry hydrolysate was then silylated with either 0.3 ml BSA or 0.3 ml HMDS–TMCS (2:1) in 0.2 ml of dry pyridine. Samples were heated for 10 min at 80° to complete the silylation reaction. The same procedure was followed for an equimolar standard of either tartaric acid or glucose and the appropriate internal standard.

An amount of 1–2 μl of sample was injected into the gas chromatograph using essentially the same conditions as used by Johnson and Nagel[29]. The retention times of peaks from the hydrolyzed ester sample were compared with those of the standards. The ratio of the peak height or area of the hydrolysate peaks to that of the internal standard was established. A similar ratio was established for the standard solution containing equimolar amounts of glucose or tartaric acid and internal standard. Since the internal standard had been added in an equimolar amount to the ester prior to hydrolysis, a ratio for the two peaks equal to that of the equimolar standard would indicate a 1:1 ratio of hydroxycinnamic acid to glucose or tartaric acid.

The ratio of tartaric acid to monocaffeoyltartaric acid was also determined by hydrolyzing a known amount of ester and then performing a metavanadate test for tartaric acid[30]. The amount of tartaric acid found was then compared to the amount

of tartaric acid that would have been present if the hydroxycinnamic acid to tartaric acid ratio was 1:1. Similarly, the phenol–sulfuric acid assay[31] was used to quantify the amount of glucose in the ester containing caffeic acid, tartaric acid and glucose. Standards of caffeic acid and tartaric acid were also run to determine if either would interfere with the test.

Standard curves

The concentration of the various hydroxycinnamic acid–tartaric acid esters was determined from purified solutions of the esters in 100% ethanol. The optical density of each solution was measured at λ_{max} and compared to the molar absorptivity of the corresponding free hydroxycinnamic acid. For the quinic acid esters of the hydroxycinnamic acids, the molar absorptivity is essentially the same as that of the corresponding free acids[1,32,33] and the same is assumed to be true for esters of glucose and tartaric acid. Varying amounts of each standardized ester solution were then injected into the liquid chromatograph and eluted with 6–10% acetonitrile in water, pH 2.6. The area under the peak was measured by the integrator using a detection wavelength of 320 nm. Three injections were made for each quantity of ester injected and the results averaged. The area was plotted against the amount injected.

Grape sample analysis

Grapes (*V. vinifera*) were obtained from the Irrigated Agriculture Research and Experiment Station, Prosser, Wash., U.S.A. White Riesling grapes were sampled twice during the growing season while other varieties were harvested at a random date. The grapes were transported to Pullman, Wash. the same day and placed in a blast freezer at −40°. They were stored in glass jars until analysis.

For analysis, 1000 ppm of sulfur dioxide as sodium bisulfite was added to 100 g of de-stemmed grapes. The sample was quickly defrosted by microwave oven, immediately crushed, and forcefully squeezed through cheesecloth. The juice yield was recorded. The juice was centrifuged, filtered through a 0.45-μm Millipore cellulose acetate filter, and stored refrigerated under nitrogen prior to analysis.

An aliquot of sample, usually 50–100 μl, was injected into the chromatograph. For White Riesling grapes, the following solvent elution pattern was used: (1) 2.5 ml/min of 6% acetonitrile in distilled water, pH 2.6 for 15 min followed by (2) a linear gradient from 6% to 16% acetonitrile in water, pH 2.6, in 15 min at 2.5 ml/min, (3) column flush with 1.5 ml acetonitrile, and (4) equilibration for 5 min with the solvent used in (1). For other varieties, an isocratic elution using 6% acetonitrile in distilled water, pH 2.6, gave comparable results. The first system is useful when analyzing for caffeic acid and chlorogenic acid in addition to hydroxycinnamic acid–tartaric acid esters. Using isocratic elution excessive peak spreading occurs for any peak eluting after monoferuloyltartaric acid.

The area under each peak was determined at 320 nm by the integrator. The average of duplicate or triplicate analyses of each sample was determined.

In the case of White Riesling grapes, the elution times of the various peaks were compared to those for chlorogenic and neochlorogenic acid. Also, samples of these standards were simultaneously injected along with grape samples.

RESULTS

Fig. 1 shows a typical chromatogram of White Riesling grape juice using the 6–16% acetonitrile program detailed above. Peaks 1–5 correspond to esters that were purified as described previously. Peak 6 was identified by the use of a commercially available standard. The UV absorption maxima of peaks 1–5 in 100% ethanol are shown in Table I along with those of reference compounds. The esters which will be shown to be hydroxycinnamic acid–tartaric acid esters have, as a group, absorption maxima slightly higher in wavelength than their corresponding quinic acid esters which, in turn, are slightly higher than the corresponding free hydroxycinnamic acids.

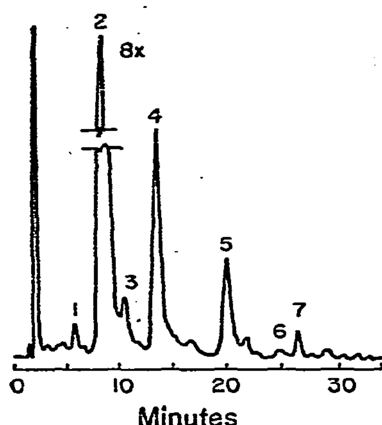

Fig. 1. Elution pattern of a typical White Riesling juice sample. Peaks: 1, caffeic acid–tartaric acid–glucose ester; 2, monocaffeoyltartaric acid; 3, *p*-coumaric acid–tartaric acid–glucose ester; 4, mono-*p*-coumaroyltartaric acid; 5, monoferuloyltartaric acid; 6, caffeic acid; 7, unknown.

TABLE I

ABSORPTION MAXIMA OF CINNAMIC ACID DERIVATIVES

Peak No.	*Compound*	*Absorption maximum (nm)*
1	Caffeic acid–tartaric acid–glucose	330
2	Monocaffeoyltartaric acid	333
3	*p*-Coumaric acid–tartaric acid–glucose	318
4	Mono-*p*-coumaroyltartaric acid	312
5	Monoferuloyltartaric acid	327
	Chlorogenic acid	328*
	p-Coumaroylquinic acid	315*
	Feruloylquinic acid	325*
	Caffeic acid	324
	p-Coumaric acid	310
	Ferulic acid	322

* From ref. 22.

Peak 2 yields caffeic acid upon alkaline hydrolysis as determined by both paper chromatography and HPLC of the hydrolysate. Paper chromatography of the hydrolysate followed by spraying with 1% metavanadate revealed a diffuse violet-orange spot. A tartaric acid standard yielded an identical color reaction while standards of malic, quinic, and citric acids yielded clear spots on the yellow background. Quantitative analysis of the hydrolysate by the metavanadate technique yielded an average of 0.87 tartaric acid residues per caffeic acid. Quantitative GLC yielded 0.91 (peak height analysis) and 0.69 (peak area calculated by integrator) tartaric acid residues per caffeic acid.

Peak 4 yielded *p*-coumaric acid upon alkaline hydrolysis as determined by HPLC. Silylation of the hydrolysate followed by GLC revealed 0.92 tartaric acid residues per *p*-coumaric acid based on peak height analysis.

Peak 5 yielded ferulic acid upon alkaline hydrolysis as determined by HPLC. Ratios of 0.73 and 0.88 tartaric acid residues per ferulic acid were found based on peak areas and peak height analysis, respectively. Based on the above results peaks 2, 4, and 5 are respectively assigned the identities of monocaffeoyl-, mono-*p*-coumaroyl-, and monoferuloyltartaric acids.

Peak 1 yielded, under both acid and alkaline hydrolysis conditions, caffeic acid as shown by HPLC. Paper chromatography of an alkaline hydrolysate indicated glucose, but was not definitive. Analysis of the same hydrolysate by GLC showed significant amounts of glucose. Using the phenol–sulfuric acid assay with glucose as a standard, the hydrolysate yielded 1.15 glucose residues per caffeic acid. GLC analysis of acid hydrolysates yielded 1.26 glucose and 1.77 tartaric acid per caffeic acid based on peak area.

Peak 3 yielded *p*-coumaric acid as determined by HPLC. Acid hydrolysates rendered an average of 0.78 glucose and 1.26 tartaric acid residues per *p*-coumaric acid based on the peak area of GLC analysis.

Based on the results it is postulated that peak 1 is composed of caffeic acid, glucose and tartaric acid and peak 3 is composed of *p*-coumaric acid, glucose and tartaric acid in equimolar amounts.

In each case the tri-ester elutes just prior to its corresponding tartaric acid ester. This would imply that a ferulic acid–tartaric acid–glucose ester should elute just prior to the monoferuloyltartaric acid. A very small, poorly resolved peak has been observed in the chromatogram just prior to monoferuloyltartaric acid. Its response to changes in either the pH of the eluting solvent or the UV detection wave length parallel those of monoferuloyltartaric acid. While it is anticipated that this peak would correspond to an ester of ferulic acid, tartaric acid, and glucose, it is a minor peak requiring great concentration before meaningful amounts can be isolated.

The effect of pH of the eluting solvent on relative retention was examined. A pH shift in the eluting solvent from 2.3 to 3.4 drastically alters the retention time of the hydroxycinnamic acid–tartaric acid esters. At a flow-rate of 3.0 ml/min using 4% aqueous acetonitrile, monocaffeoyltartaric acid elutes in 735 sec at pH 2.3, 510 sec at pH 2.6, 380 sec at pH 2.7 and 210 sec at pH 3.4. The first pK_a of tartaric acid is 2.98 while the pK_a values of the hydroxycinnamic acids are in the range of 4.5. The shift in retention time attributable to the change in pH is most likely linked to the percent ionization of the tartaric acid. The higher pH yields a more polar compound which is retained less by the reversed-phase packing material. The opposite is true

at lower pH values. While this pH effect necessitates careful preparation of solvents it can also be used to optimize HPLC separations where two compounds can not be adequately resolved and only one is pH sensitive. By simply shifting the pH slightly the peaks may be resolved. This method was used to optimize the HPLC conditions used for grape analysis and has also been a useful tool in preparative HPLC.

On the evidence available it is possible to speculate on the type of linkages found in the tri-ester. The absorption maxima of the compounds are very close to that of the glucose, tartaric acid, and quinic acid esters of the hydroxycinnamic acids and not similar to the maxima quoted by either Harborne and Corner[21] or Steck[22] for the corresponding glycosides. Alkaline hydrolysis of peak 1 yields caffeic acid and glucose. The above results suggest the hydroxycinnamic acid is bound by an ester linkage. Since the compounds are pH sensitive and respond in an identical manner to the corresponding tartaric acid ester, at least one of the carboxylic acid groups on the tartaric acid must be free. It might be hypothesized that both carboxylic acid groups are free since the pH response is similar to that of the tartaric acid esters which have two ionizable carboxylic acid groups[10,23].

Fig. 2 shows a chromatogram of some standard compounds using the same eluting conditions as in Fig. 1. Both chlorogenic and neochlorogenic acids have been reported in grapes[1,4,6,8]. While it appears that neochlorogenic and chlorogenic acids could be peaks 3 and 7, respectively, simultaneous injection of neochlorogenic and chlorogenic acids along with juice samples into the liquid chromatograph showed neochlorogenic acid eluting between peaks 2 and 3 and chlorogenic acid eluting between peaks 6 and 7. There is no evidence for the occurrence of either chlorogenic or neochlorogenic acid in any *V.vinifera* grape analyzed. It is unlikely, based on the above, that 4-caffeoylquinic acid occurs in *V. vinifera*.

Peak 6, based on simultaneous injections with a standard, was caffeic acid. The relatively insignificant amount of free caffeic acid present confirms firstly that the

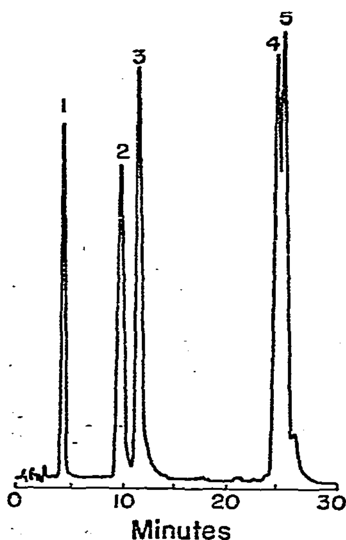

Fig. 2. Elution pattern of various standard phenolic acids. Peaks: 1, gallic acid; 2, neochlorogenic acid; 3, gentisic acid; 4, caffeic acid; 5, chlorogenic acid.

major amount of caffeic acid is found in bound forms, and secondly that very little, if any, hydrolysis of caffeic acid-containing compounds occurred during sample preparation and analysis. It should be noted that caffeic acid elutes prior to chlorogenic acid in our elution program. This is the opposite of other investigations[25,34] using C_{18} reversed-phase packings.

Simultaneous injection also showed that the relatively broad peak eluting after monoferuloyltartaric acid was a mixture of *d,l*-catechin. This peak is not observed in Fig. 1 because the low molar absorptivity of catechin yields a relatively small peak, and at 320 nm the absorptivity of catechin is only 7% of that at maximum. Thus, the catechin simply does not show. The elution order of catechin relative to monoferuloyltartaric acid can be changed by adjusting the solvent pH as discussed.

One of the major aspects of this analytical technique is the minimum amount of sample preparation necessary. Because this is a direct sampling technique, there is no need for internal standards or standard curves related to sample preparation. Standard curves related to chromatographic parameters were all linear and passed through the origin. The percent error on repeated injections of the same sample typically runs 1% for caffeoyltartaric acid, 2% for *p*-coumaroyltartaric acid, and as high as 5% for feruloyltartaric acid. The decrease in reproducibility of the monoferuloyltartaric acid is attributable to the small amount of this compound present in the grape juice, slight increases in peak spreading, and some variation in the resolution of minor peaks in this area of the program. In the latter case, the integrator used to analyze the peak areas readily detects these small changes in resolution.

Table II shows the range of variation for the three tartaric acid esters among a few *V. vinifera* varieties. Two sets of values are also given for mature and immature White Riesling grapes. There is considerable variation in the quantity of hydroxycinnamic acid–tartaric acid ester both on a varietal basis and during the course of maturation.

TABLE II

CONCENTRATION OF THE TARTRATE ESTERS IN THE JUICE OF DIFFERENT GRAPE VARIETIES

Variety	*°Brix*	*Caffeoyl-tartaric (ppm)*	*p-Coumaroyl-tartaric (ppm)*	*Feruloyl-tartaric (ppm)*
Semillon	23.3	74.8	8.3	2.9
Chardonnay	22.5	99.4	19.8	3.1
White Riesling (immature)	7.2	392.8	63.4	30.8
White Riesling	21.8	198.2	20.9	15.9
Cabernet Sauvignon	21.7	70.9	16.7	2.4
Pinot Noir	22.8	211.4	27.5	6.3

Additional detail and more varieties of *V. vinifera* along with detailed data regarding the change in hydroxycinnamic acid–tartaric acid ester content of White Riesling grapes during maturution will be published elsewhere[35].

Sufficient monocaffeoyltartaric acid was available for a taste evaluation. At 80 ppm in distilled water a bitter, astringent, drying taste was present. Since all grapes

sampled showed monocaffeoyltartaric acid concentrations at or considerably above this amount, the compound must contribute to the astringency of grapes and wine.

CONCLUSION

HPLC techniques have been developed for analysis of the hydroxycinnamic acid–tartaric acid esters that is quantitative and requires a minimum of sample preparation. The presence of monocaffeoyl-, mono-*p*-coumaroyl-, and monoferuloyltartaric acid in the juice of *V. vinifera* has been substantiated. There is evidence for esters of caffeic acid, tartaric acid and glucose, and *p*-coumaric acid, tartaric acid and glucose. Neither chlorogenic nor neochlorogenic acid occurs in *V. vinifera*.

ACKNOWLEDGEMENTS

The authors would like to thank Mr. Robert Fay, Irrigated Agriculture Research and Extension Center, Prosser, Wash. for assistance in gathering grape samples. The assistance of Mr. Larry Wulf with technical HPLC problems is gratefully acknowledged. This research was supported in part by Project No. 10790052 from the Pacific Northwest Regional Commission.

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