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Analysis of Wine Components in Cynthiana and Syrah Wines

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Red wine is composed of a complex matrix of compounds that can interfere with analysis. A high-performance liquid chromatography (HPLC) procedure was developed to efficiently analyze organic acids, sugars, glycerol, and ethanol in Cynthiana (*Vitis aestivalis*) wine. Standard laboratory procedures (pH, titratable acidity, and color attributes) and HPLC were found reproducible for Cynthiana wine. HPLC recovery efficiency was determined by analysis of spiked and unspiked samples (model, Cynthiana, and Syrah (*Vitis vinifera*) wines). Although recovery of components was greater in the model wine, recovery in Cynthiana and Syrah wine was comparable. The HPLC procedure was further compared to commercial rapid enzyme analysis tests using model, Cynthiana, and Syrah wines. HPLC analyses were more accurate than enzymatic tests for determining components in the model, Cynthiana, and Syrah wines. Considering the complexity of the wines analyzed, reproducibility and recovery of the HPLC procedure was demonstrated and showed improvement and precision when compared to existing methods.

KEYWORDS: Cynthiana wine; Syrah wine; HPLC; rapid enzyme analysis; reproducibility

INTRODUCTION

Wines are composed of a complex mixture of water, phenolic compounds, organic acids, alcohols, and residual sugars responsible for sensory characteristics of wine. Because of interfering phenolic compounds, analysis of red wines, such as Cynthiana (*Vitis aestivalis*) and Syrah (*Vitis vinifera*), is more difficult than with white wines. Analysis of wine components can be time-consuming and difficult, but high-performance liquid chromatography (HPLC) has been used to simplify analysis. However, there are few HPLC procedures that encompass the broad span of compounds in wine in a reasonable amount of time per sample.

The red grape varieties, Cynthiana and Syrah, are both commercially produced. Syrah grapes are grown for commercial production in France, Australia, and the United States, whereas Cynthiana grapes are produced commercially only in limited quantities in the United States. Syrah produces a complex, full-bodied wine. Syrah wine can have high pH problems; however, high titratable acidity levels are uncommon. Cynthiana wine is deep-colored and can have both high pH (3.5−3.9) and high titratable acidity (8.5−12 g/L) caused by the presence of the weak acids (*1*).

Methods for wine analysis can include standard laboratory procedures, rapid enzymatic analysis tests, and HPLC. Standard laboratory analyses incorporate simple physical, chemical, and color measurements used for wine evaluation such as pH, titratable acidity, color, and phenolic levels measured by absorbance and transmittance (*2*, *3*). Numerous enzymatic determinations for wine analysis have been recognized and recommended by the Office Internationale du Vin in Paris and the American Association of Analytical Chemists (*4*). The Boehringer Mannheim Enzymatic Analysis and Food Analysis tests (R-Biopharm AG, Darmstadt, Germany) contain multi-tested reagents that allow simple, safe, and rapid analyses. Many European regulations and guidelines have been referenced by R-Biopharm in German, French, Swiss, and Austrian publications for determination of organic acids, sugars, and ethanol in wine (*4*).

The HPLC method most commonly used for separation of organic compounds in wines is an isocratic separation employing a single column packed with a strong cation-exchange resin in the hydrogen form with a dilute mineral acid as the eluant (*5*). HPLC analysis at 210 nm resulted in incomplete separation of organic acids because of coelution between some organic acids, phenolic compounds, and fructose (*6*, *7*). Other research showed that resin-based columns were unable to separate organic acids in juice and wine because of close elution of primary acids of wine; whereas, reversed phase chromatography allowed better separation (*8*, *9*). Ion exchange HPLC separated major carboxylic acids, sugars, glycerol, and ethanol in wine samples when used with a refractive index detector (*10*, *11*). Other HPLC procedures have also been developed for the analysis of organic acids, sugars, and ethanol in wines (*12*−*20*).

Although many HPLC methods have been developed for the analysis of organic acids in white wines, red wines contain more phenolic compounds that prevent complete separation of peaks using current techniques. Because of limited information for Cynthiana wine, the objectives were to develop a HPLC procedure to efficiently analyze organic acids, sugars, and ethanol content and to establish reproducibility of HPLC and

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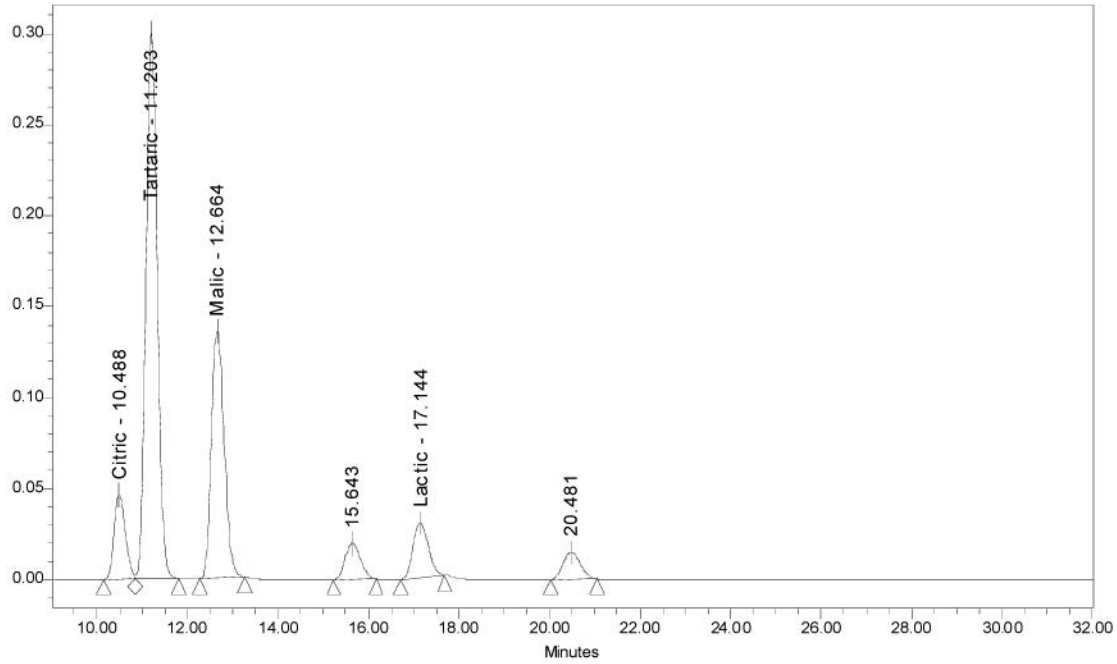

Figure 1. High-performance liquid chromatography chromatogram of compound separation and retention time in Cynthiana wine used for detection of citric and tartaric acids at 210 nm.

standard laboratory wine analyses in Cynthiana wine. The HPLC procedure was also evaluated for reproducibility and by comparison with commercial enzymatic analysis using a model wine, Cynthiana wine, and Syrah wine.

MATERIALS AND METHODS

Wine for Analysis. A model wine and red wines, Cynthiana and Syrah, produced at the Experimental Winery at the University of Arkansas Food Science Department were used. The model wine contained known amounts of monohydrate citric acid (1.0 g/L); L-(+) tartaric acid (2.5 g/L); L-(−) malic acid (1.25 g/L); succinic acid (0.6 g/L); L-(+) lactic acid (2.5 g/L); glacial acetic acid (0.5% v/v); D-(+) glucose (0.2 g/L); D-(−) fructose (0.2 g/L); glycerol (7.0 g/L) and ethanol (11% v/v) in a water matrix. The amounts in the model wine are representative of levels found in the varieties tested. The model wine was prepared for the HPLC recovery analysis and for use in a comparison of HPLC and rapid enzymatic analyses. Chemicals were purchased from Sigma Chemical Company (St. Louis, MO), Aldrich Chemical Co. (Milwaukee, WI), and Fisher Scientific (Fair Lawn, NJ).

Laboratory Analyses. Standard laboratory and color and phenolic analyses were performed (*2*, *3*). Sample pH was measured with a Beckman 250 model pH meter (Beckman Coulter, Inc., Fullerton, CA). Titratable acidity (tartaric acid in g/L) was measured by placing 5 mL of wine sample into 125 mL of deionized water and titrating with 0.1 N sodium hydroxide to an endpoint of pH 8.2. Color measurements were made using a Colorgard system/05 (BYK Gardner, Columbia, MD) and a Unicam Helios Beta UV−vis spectrophotometer (Unicam, Cambridge, UK). The Colorgard system was standardized with deionized water to CIE Lab transmission values of $L = 100$, $a = 0$, and $b = 0$. Darkness (L value) of the samples was evaluated. Absorbance measurements were made at 280, 420, and 520 nm for total phenolics, concentration of yellow-brown pigments, and concentration of red-colored anthocyanins, respectively (*2*). Total red pigment color (OD^{HCl}_{520}), color density ($OD_{520} + OD_{420}$), color hue (OD_{420}/OD_{520}), and total phenolics (OD_{280}) were measured.

HPLC Procedure. The HPLC procedure developed and utilized was adapted from Frayne (*5*). Organic acids, sugars, and ethanol content were determined using HPLC. HPLC was equipped with a Bio-Rad HPLC Organic Acid Analysis Aminex HPX-87H ion exclusion column (300 × 7.8 mm) and a Bio-Rad HPLC column for fermentation monitoring (150 × 7.8 mm) in series. A Bio-Rad Micro-Guard Cation-H refill cartridge (30 × 4.5 mm) was used for a guard column. Columns were maintained at 65 ± 0.1 °C by a temperature control unit. Mobile phase consisted of a pH 2.28 solution of sulfuric acid and water with a resistivity of 18 M obtained from a Millipore Milli-Q reagent water system. The sulfuric acid solution was used as the solvent with 0.65 mL/min flow rate. The solvent delivery system was a Waters 515 HPLC pump equipped with a Waters 717 plus autosampler. Injection volumes were 10 μL for all wines, and run time for completion was 32 min.

A Waters 410 differential refractometer to measure refractive index connected in series with a Waters 996 photodiode array detector monitored the eluting compounds. Citric and tartaric acids were detected by photodiode array at 210 nm and malic, lactic, succinic and acetic acids, glucose, fructose, glycerol, and ethanol were detected by a differential refractometer (**Figures 1** and **2**). The peaks were quantified using external standard calibration based on peak height estimation with baseline integration.

Commercial standards were used for identification. The standard solutions consisted of organic acids, sugars, and ethanol in various concentrations typical of the range found in Cynthiana and Syrah wines. Components were identified by a comparison of their retention times with those of external standards. Standard solutions were prepared with a range of different levels of standards. A 10 μL amount of standard solution was injected into the HPLC to determine the linearity of response and to develop standard curves for the HPLC method. The standard solution contained known amounts of monohydrate citric acid, L-(+) tartaric acid, L-(−) malic acid, succinic acid, L-(+) lactic acid, glacial acetic acid, D-(+) glucose, D-(−) fructose, glycerol and ethanol in a water matrix. Only analytical reagent grade components were used and were prepared in a pH 2.28 sulfuric acid solution. Waters Millennium[32] Chromatography Manager software was used for collecting and processing HPLC data.

Commercial Enzymatic Analysis. Boehringer Mannheim enzymatic tests (R-Biopharm AG, Darmstadt, Germany) were purchased to determine the level of acetic acid, citric acid, D-glucose, D-fructose, ethanol, glycerol, L-lactic acid, L-malic acid, and succinic acid in wine and to compare those values with HPLC. A Unicam Helios Beta UV−vis Spectrophotometer was used to determine enzymatic generation of reduced nicotinamide−adenine dinucleotide measured by the increase in light absorbance at 340 nm. Manufacturer recommendations were followed on each enzyme test.

Experimental Design and Analysis. Data were analyzed by analysis of variance using the Statistical Analysis System PROC GLM procedure

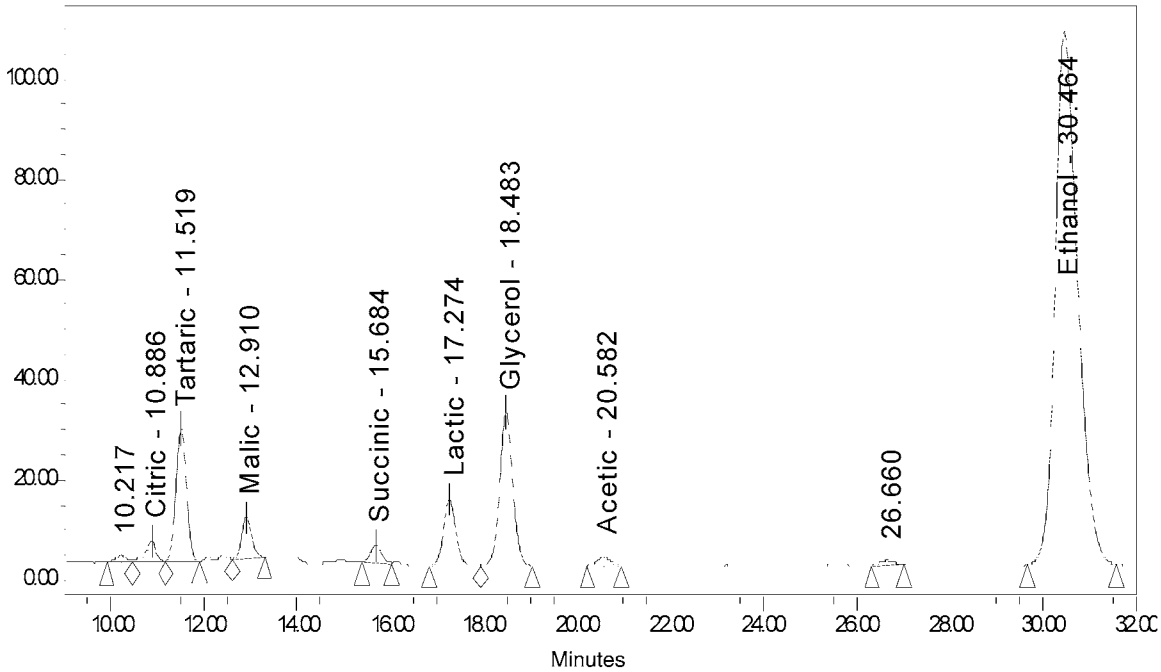

Figure 2. High-performance liquid chromatography chromatogram of compound separation and retention time in Cynthiana wine used for detection of malic, succinic, lactic and acetic acids, glycerol, and ethanol by refractive index. (Although glucose and fructose were not present in this sample, glucose elutes at 12.415 min, and fructose elutes at 13.424 min.)

(*21*). Treatment means were separated by Duncan's Multiple Range Test ($p \leq 0.05$). Mean, standard deviation, and coefficient of variation (CV) values were calculated using JMP_{IN} 4.0.2 (*22*).

Reproducibility. A reproducibility study was designed to compare day-to-day means and the amount of variation between samples of the same Cynthiana wine. HPLC and standard laboratory analyses were conducted on nine wine samples per day for a period of three consecutive days (27 total bottles). HPLC wine samples were each injected in triplicate (81 total injections).

Recovery Efficiency. A recovery study was designed to evaluate the accuracy of the analyses and to determine the level of interferences for the compounds identified. Recovery efficiency was performed by spiking a model wine, Cynthiana wine, and Syrah wine. The spiking solution contained known amounts of monohydrate citric acid (1.0 g/L); L-(+) tartaric acid (2.0 g/L); L-(−) malic acid (2.0 g/L); succinic acid (1.0 g/L); L-(+) lactic acid (2.0 g/L); and glacial acetic acid (1.0% v/v); D-(+) glucose (1.0 g/L); D-(−) fructose (1.0 g/L); glycerol (2.0 g/L) and ethanol (5% v/v) in a water matrix. Spiked samples were prepared by using 1 mL of the spiking solution and 24 mL of the model wine, Cynthiana wine, or Syrah wine. HPLC analysis was conducted on the spiked and nonspiked samples.

Comparison of HPLC with Commercial Enzymatic Analysis. A study was designed to compare variation between HPLC and rapid enzyme tests. HPLC and enzymatic tests were conducted on nine samples from a model wine, Cynthiana wine, and Syrah wine. Analyses for each wine were conducted on the same day.

RESULTS AND DISCUSSION

Reproducibility. Standard laboratory wine analyses were completed on Cynthiana wine. The analysis of Cynthiana wine resulted in reproducibility with p values of $p = 0.3699$, 0.5022, 0.6295, 0.4772, 0.1245, and 0.1301 for titratable acidity, darkness, color density, color hue, total phenolics, and total red pigment color, respectively. Since no differences from a day-to-day analysis were found, a comparison of means and standard deviations of laboratory analyses was completed to explain sample variation. The CV ranged from 0.13 to 7.46% for components evaluated by laboratory analyses (**Table 1**). For most components, the CV value was small (less than 2%) indicating a high degree of reproducibility for the laboratory method.

Table 1. Reproducibility of Laboratory and High-Performance Liquid Chromatography (HPLC) Analyses of Cynthiana Wine

analysis methods and components	mean	standard deviation	coeff. of variation
Laboratory components ($n = 27$)			
titratable acidity (g/L tartaric acid)	0.56	0.01	0.13
darkness (CIE '*L*')	15.74	1.04	6.59
total phenolics (OD_{280})	11.83	0.88	7.46
total red pigment color ($OD^{HCL}{}_{520}$)	24.12	0.29	1.20
color density ($OD_{520} + OD_{420}$)	52.67	0.68	1.29
color hue (OD_{420}/OD_{520})	0.86	0.01	1.40
HPLC compounds ($n = 81$)			
tartaric acid (g/L)	1.89	0.01	0.59
malic acid (g/L)	2.04	0.01	0.45
succinic acid (g/L)	0.49	0.01	1.86
lactic acid (g/L)	4.05	0.01	0.34
glycerol (g/L)	6.20	0.02	0.36
acetic acid (%)	0.07	0.01	2.07
ethanol (% v/v)	9.91	0.04	0.45

Reproducibility of the HPLC procedure was evaluated. HPLC analysis of Cynthiana wine resulted in reproducibility with p values of $p = 0.1391$, 0.4387, 0.9208, 0.3927, 0.3067, 0.6826, and 0.4456 for tartaric, malic, succinic, lactic, glycerol, acetic, and ethanol content, respectively. Citric acid was not present in the wine. Since no differences existed from day-to-day analysis, a comparison of means and standard deviations of HPLC analyses was completed to explain sample variation. The CV ranged from 0.34 to 2.07% for compounds evaluated by HPLC (**Table 1**). Since all but one of the CV values were $<2.1\%$ and standard deviations were small, a high degree of reproducibility was achieved by the HPLC method.

Recovery Efficiency. A recovery analysis was performed on the model wine, Cynthiana wine, and Syrah wine. These wines were spiked with a known amount of each component. Spiked wines were analyzed and compared to the nonspiked wines.

Table 2. Recovery Efficiency (%) of Components in a Model Wine Solution, Cynthiana Wine, and Syrah Wine Measured by High-Performance Liquid Chromatography

component	Recovery efficiency (%) model wine	Cynthiana wine	Syrah wine
citric acid, monohydrate (g/L)	98	98	**97**
L-(+) tartaric acid (g/L)	92	91	**98**
L-(–) malic acid (g/L)	95	75	**87**
succinic acid (g/L)	94	82	**89**
L-(+) lactic acid (g/L)	100	85	**101**
acetic acid, glacial (% v/v)	100	99	**99**
D-(+) glucose (g/L)	101	109	**110**
D-(–) fructose (g/L)	103	117	**79**
glycerol (g/L)	100	78	**82**
ethanol (% v/v)	99	99	**99**
total average of components	98	93	**94**

Percent recovery of the compounds added as the spike is shown in **Table 2**. The lowest recoveries for compounds were ≥92, 75, and 79% for the model wine, Cynthiana wine, and Syrah wine, respectively. A higher percent recovery in the model wine solution than the Cynthiana and Syrah wines indicated the presence of interfering compounds. There was a range of 92−103% for components measured in the model wine, 75−117% for Cynthiana wine, and 79−110% for Syrah wine. The average of all components measured was 98, 93, and 94% for the model wine, Cynthiana wine, and Syrah wine, indicating an overall affective method of analysis.

Table 3. Comparison of the Percent (%) Differences of High-Performance Liquid Chromatography (HPLC) and Rapid Enzyme Analysis Tests (Enzyme) on Components in a Model Wine Solution as Compared to Known Levels

component	level in model wine solution	Analysis comparison HPLC vs known	enzyme vs known
citric acid, monohydrate (g/L)	1.00	–10.3	–8.9
L-(+) tartaric acid (g/L)	2.50	–6.8	*a*
L-(–) malic acid (g/L)	1.25	–10.3	–24.4
succinic acid (g/L)	0.60	–24.3	–90.3
L-(+) lactic acid (g/L)	2.50	–13.9	–48.6
acetic acid, glacial (% v/v)	0.50	–11.2	–13.8
D-(+) glucose (g/L)	0.20	–3.7	–3.0
D-(–) fructose (g/L)	0.20	–3.6	–18.0
glycerol (g/L)	7.00	–18.0	–23.9
ethanol (% v/v)	11.00	–0.9	–9.2

[a] Enzyme analysis test is unavailable % for tartaric acid.

Comparison of HPLC with Commercial Enzymatic Analysis. The HPLC procedure was evaluated by verification of levels of components with commercial enzymatic analysis and the model wine. The model wine was analyzed by HPLC and rapid enzyme analysis. Results of the means were compared to the known levels of components in the standard solutions (**Table**

Table 4. Comparison of the Means, Standard Deviations, and Coefficients of Variation of High-Performance Liquid Chromatography (HPLC) and Rapid Enzyme Analysis Tests (Enzyme) on Wine Components

	Model wine solution		Cynthiana wine		Syrah wine	
	HPLC	enzyme	HPLC	enzyme	HPLC	enzyme
citric acid (g/L)						
mean	0.90	0.91	0.64	0.06	0.63	0.04
standard deviation	0.01	0.01	0.01	0.02	0.01	0.01
coefficient of variation (%)	0.61	1.02	1.13	26.97	1.00	18.33
tartaric acid (g/L)						
mean	2.33	*a*	3.40	*a*	1.68	*a*
standard deviation	0.01		0.03		0.01	
coefficient of variation (%)	0.46		0.77		0.54	
malic acid (g/L)						
mean	1.12	0.95	1.57	0.47	1.09	0.35
standard deviation	0.01	0.17	0.01	0.00	0.01	0.03
coefficient of variation (%)	1.03	18.04	0.56	0.00	0.69	7.83
succinic acid (g/L)						
mean	0.45	0.06	0.66	0.13	0.63	0.35
standard deviation	0.02	0.11	0.01	0.09	0.01	0.00
coefficient of variation (%)	4.25	200.29	0.70	65.22	1.57	0.00
lactic acid (g/L)						
mean	2.15	1.29	3.14	22.15	2.03	23.95
standard deviation	0.03	0.22	0.01	0.35	0.01	0.21
coefficient of variation (%)	1.43	17.48	0.45	1.56	0.70	0.86
acetic acid (%)						
mean	0.44	4.31	nd[b]	0.56	nd	0.26
standard deviation	0.01	0.02	0.00	0.01	0.00	0.02
coefficient of variation (%)	1.37	0.50	0.00	1.42	0.00	7.91
glucose (g/L)						
mean	nd	0.19	nd	0.07	nd	0.09
standard deviation	0.00	0.01	0.00	0.04	0.00	0.01
coefficient of variation (%)	0.00	2.36	0.00	61.19	0.00	2.41
fructose (g/L)						
mean	nd	0.16	nd	0.02	0.63	0.02
standard deviation	0.00	0.02	0.00	0.01	0.01	0.01
coefficient of variation (%)	0.00	9.41	0.00	26.99	1.07	13.53
glycerol (g/L)						
mean	5.74	5.33	0.62	5.42	0.79	7.02
standard deviation	0.04	0.44	0.01	0.05	0.01	0.33
coefficient of variation (%)	0.64	8.18	0.48	0.95	0.41	4.75
ethanol (% v/v)						
mean	10.91	9.98	10.87	9.30	10.98	9.26
standard deviation	0.03	0.82	0.03	0.11	0.04	0.28
coefficient of variation (%)	0.32	8.20	0.27	1.19	0.36	2.98

[a] Enzyme analysis test is unavailable for tartaric acid. [b] Not detected (nd).

3). HPLC analysis resulted in ≤13.9% lower than the known level of citric, tartaric, malic, lactic and acetic acids, sugars, and ethanol. Analysis of succinic acid and glycerol by HPLC was 24.3 and 18.0% lower than known levels, respectively. Analyses of glucose, citric acid, and ethanol by enzyme tests were 3, 8.9, and 9.2% lower than known levels, respectively. Enzyme tests showed excessively lower than known levels of all other compounds. Zoecklein et al. (*3*) reported that enzymatic procedures using test kits are difficult to carry out successfully because of interferences in the wine and small volumes of samples and reagents required for analysis. Overall recoveries by the enzyme tests were not as accurate as HPLC analyses.

A comparison of means, standard deviations, and coefficient of variation of wine components in a model, Cynthiana wine, and Syrah wine measured by HPLC and enzyme tests was completed (**Table 4**). Except in the samples with the means equal to zero, standard deviations were generally higher for the measurement of compounds using the enzyme tests than the HPLC. The small CV values also demonstrated a higher level of reproducibility by the HPLC than the enzymatic tests. Because of color compounds present in Cynthiana and Syrah wine, the HPLC is believed to be better at determining organic acids, sugars, and ethanol than the rapid enzymatic analyses since rapid tests use light absorbance from a spectrophotometer to determine the compounds. Larger standard deviations and CV values indicated less repeatability of the replications. Generally, the standard deviations and CV values were greater for the Cynthiana and Syrah wine than the model solution, possibly indicating interference of compounds in the wines.

CONCLUSIONS

The standard laboratory analyses utilized and HPLC method developed were acceptable and reproducible procedures for determining components in Cynthiana wine. Although recovery efficiencies for HPLC analysis were greater for the model wine, recovery in Cynthiana and Syrah wine were comparable. HPLC analyses were more accurate than enzymatic tests for determining organic acids, sugars, and ethanol content in model, Cynthiana, and Syrah wines. The analysis of red wine in this study is representative of complex material for analysis, indicating that this HPLC procedure could be easily and more accurately used on lighter colored wine and juice products. For wine and juice companies where analysis of components is routine, this HPLC procedure could be used to reduce the total time for analysis and cost per sample.

ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; CV, coefficient of variation.

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